David Sérgio de Moura Ribeiro

Automatic analytical microflow systems for toxicological and pharmaceutical control

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Abstract

Considering the scope of this dissertation, automatic methodologies of flow analysis were developed for the chemical control of pharmaceutical formulations, as well as, for the detection and quantification of drugs involved in the drug facilitated crimes phenomenon, in beverages intentionally adulterated. To this end, flow microsystems based on the multipumping concept were used, in which its main features and analytical potentialities were explored and assessed, namely, the nature of the pulsed flow, high compactness and portability, reduced sample and reagent consumptions with consequent low production of residues, thus allowing the implementation of analytical systems very simplified, with low energy consumption and with high potential of automation and control. The high versatility associated to multipumping flow systems enabled performing several approaches of sample manipulation and also the implementation of different detection methods, such as, spectrophotometry, fluorometry and chemiluminescence.

The drugs indapamide, diazepam, glibenclamide and epinephrine were selected for the development of the research work because of its pharmacological secondary effects and due to the higher facility of commercial acquisition.

Taking advantage of the high operational versatility afforded by multipumping, it was implemented an analytical procedure for the spectrophotometric determination of indapamide in pharmaceutical formulations, based on the oxidative coupling reaction of the drug with 3-methylbenzothiazolin-2-one hydrazone and cerium (IV), in acidic medium. It was verified that the reaction can originate distinct reaction products depending on the sequence of reagents addition. The automation of this reaction scheme by means of a multipumping flow system easily allowed the implementation and control of the two different sequences of reagents addition, considering the individual and time programmed control of the insertion of reagent solutions. Additionally, the nature of the pulsed flow originated optimized conditions for the inline mixing of solutions, thus facilitating the reaction development.

An automatic methodology was developed for the analytical determination of diazepam, which consisted on the fluorometric monitoring of the products resultant from the photodegradation of the drug in alkaline medium. The developed methodology enabled to assess the performance of the coupling of the multipumping system with an ultraviolet photodegradation unit, in which the optimal conditions were created for its application in the detection and quantification of diazepam in spiked alcoholic drinks and also, in the chemical control of pharmaceutical formulations.

As part of the research work, a flow analysis microsystem with a very simplified configuration was also developed for the determination of glibenclamide in spiked alcoholic drinks and in pharmaceutical formulations. The analytical methodology consisted in the fluorometric monitoring of glibenclamide in acidic medium, in the presence of an anionic surfactant, promoting an organized micellar medium to enhance the sensitivity of the fluorometric measurements.

In another work and, in order to increase the sensitivity and selectivity of the methodology previously mentioned, a pre-separation unit was implemented into the microsystem. This coupling was facilitated due to the high degree of automation, operational simplicity and versatility of the multipumping systems. Given the complexity of the developed methodology, the microsystem included several solenoid valves in order to direct the flow by different analytical paths. This enabled an effective and simplified control of all the steps involved in the analytical procedure, namely, washing and conditioning of the separation column, sample insertion, separation, and by inverting the flow, the elution of the drug towards detection. The separation of glibenclamide from the samples was achieved through the adsorption of the drug into activated charcoal packed within a mini column followed by elution with a solution developed and optimized during this work, and composed by ethanol, hydrochloric acid and cetyltrimethylammonium bromide. The methodology was applied in the toxicological control of glibenclamide in spiked teas.

The potentiality of quantum dots nanocrystals (QD) to generate reactive oxygen species (ROS) in aqueous solution upon exposure to visible radiation was evaluated and applied in the chemical control of epinephrine in injectables. To this end, a multipumping flow system was coupled with a lab-made photocatalytic unit, constituted by two high power LED (Light Emitting Diode) lamps. In this system was implemented a methodology based on the quenching effect of epinephrine on the oxidation of luminol by the ROS species generated by the QD nanoparticles irradiation.

The proposed automatic methodologies of flow analysis furnished results statistically in agreement with those obtained by reference methods and also allowed to obtain recovery percentages that attested the quality of the results.

Keywords: multipumping, automation, pharmaceutical analysis, spiked drinks, drug facilitated crimes.

Resumo

No âmbito do tema desta dissertação foram desenvolvidas metodologias automáticas de análise em fluxo, para o controlo químico de fármacos em formulações farmacêuticas, assim como, para a deteção e quantificação de substâncias envolvidas no fenómeno de crimes facilitados por drogas em bebidas intencionalmente adulteradas. Para tal, recorreu-se a microssistemas de fluxo baseados no conceito da multi-impulsão tendo sido exploradas e avaliadas as suas principais características e potencialidades analíticas, tais como, a natureza do fluxo pulsado, elevada compacidade e portabilidade, reduzidos consumos de amostra e reagentes com consequente baixa produção de resíduos, permitindo assim a implementação de sistemas analíticos simples, com baixo consumo energético e com uma elevada facilidade de automação e controlo. A elevada versatilidade associada aos sistemas de fluxo por multi-impulsão possibilitou variadas formas de manipulação da amostra, e também a implementação de diferentes tipos de deteção, nomeadamente, espectrofotométrica, fluorométrica e quimioluminométrica. Considerando os efeitos farmacológicos secundários que acarretam e a maior facilidade de aquisição comercial, os fármacos indapamida, diazepam, glibenclamida e epinefrina foram selecionados para o desenvolvimento do trabalho de investigação.

Rentabilizando a elevada versatilidade operacional proporcionada pela multi-impulsão, foi implementado um procedimento analítico para a determinação espectrofotométrica da indapamida em formulações farmacêuticas, baseado na reação de acoplamento oxidativo do fármaco com os reagentes 3-metilbenzotiazolin-2-ona hidrazona e cério (IV), em meio ácido. Verificou-se que esta reação podia originar dois produtos de reação distintos dependendo da sequência de adição dos reagentes. A automação deste esquema reacional por intermédio de um sistema de fluxo por multi-impulsão permitiu facilmente a implementação e controlo das duas diferentes sequências de adição dos reagentes, dado o controlo individual e programado da inserção das soluções de reagentes. Adicionalmente, a natureza do fluxo pulsado originou condições otimizadas de mistura das soluções facilitando assim o desenvolvimento da reação.

Foi também desenvolvida uma metodologia automática para a determinação analítica de diazepam, baseada na deteção fluorométrica dos produtos formados por fotodegradação deste fármaco em meio alcalino. A metodologia desenvolvida permitiu avaliar o desempenho do acoplamento de um sistema multi-impulsão com uma unidade de fotodegradação com luz ultravioleta, tendo sido criadas as condições ótimas para a sua aplicação na deteção e quantificação de diazepam em bebidas alcoólicas adulteradas e também no controlo químico de formulações farmacêuticas.

No âmbito do trabalho desenvolvido foi adicionalmente concebido um microssistema de análise em fluxo com uma configuração muito simplificada para a determinação de glibenclamida em bebidas alcoólicas adulteradas e em formulações farmacêuticas. A metodologia analítica consistiu na monitorização fluorométrica de glibenclamida em meio ácido na presença de um tensioativo aniónico, o qual promoveu um meio micelar organizado para aumentar a sensibilidade das medições fluorométricas.

Noutro trabalho, e com o objetivo de aumentar a sensibilidade e seletividade da metodologia anteriormente mencionada, foi implementada uma unidade de pré-separação do fármaco glibenclamida no microssistema. Tal acoplamento foi facilitado dado o elevado grau de automação, simplicidade operacional e versatilidade dos sistemas por multi-impulsão. Em virtude da complexidade da metodologia desenvolvida, o microssistema incluiu várias válvulas solenoides para direcionar o fluxo por diversos percursos analíticos. Isto permitiu um controlo efetivo e simplificado de todas etapas envolvidas no procedimento analítico, nomeadamente, lavagem e acondicionamento da coluna de separação, introdução de amostra, separação, e por inversão do fluxo, a eluição do fármaco em direção à deteção. A separação de glibenclamida das amostras foi conseguida através da adsorção do fármaco em carvão ativado empacotado numa mini-coluna seguida pela eluição com uma solução desenvolvida e otimizada durante este trabalho e composta por etanol, ácido clorídrico e brometo de hexadeciltrimetilamónio. A metodologia foi aplicada no controlo toxicológico de glibenclamida em chás adulterados.

A potencialidade de nanocristais *quantum dots* (QD) gerarem espécies reativas de oxigénio (ROS) em solução aquosa após exposição a radiação visível foi avaliada e aplicada no controlo químico de epinefrina em injetáveis. Para tal, um sistema multi-impulsão foi acoplado com uma unidade fotocatalítica construída no laboratório, constituída por duas lâmpadas LED (*Light Emitting Diode*) de elevada potência, no qual foi implementado uma metodologia analítica que se baseou no efeito sequestrante da epinefrina sobre os ROS gerados pela fotoativação dos QD, limitando assim a reação de oxidação de luminol pelas espécies reativas. Deste modo, neste trabalho foi monitorizada a inibição do sinal de quimioluminescência gerado pela oxidação de luminol por ROS.

As metodologias automáticas de análise em fluxo propostas forneceram resultados estatisticamente concordantes com os obtidos pelos métodos de referência e também permitiram obter percentagens de recuperação que aferiram a qualidade dos resultados obtidos na validação dos sistemas analíticos desenvolvidos.

Palavras-chave: multi-impulsão, automação, análise farmacêutica, bebidas adulteradas, crimes facilitados por drogas.

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CHAPTER 1. Introduction

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List of acronyms and respective names

Acronym	Name
ALP	Alprazolam
API	Active pharmaceutical ingredients
BZD	Benzodiazepines
CE	Capillary electrophoresis
CHL	Chloral hydrate
CL	Chemiluminescence
CLO	Clonazepam
cmc	Critical micelle concentration
CPB	Cetylpyridinium bromide
CTAB	Cetyltrimethylammonium bromide
CZE	Capillary zone electrophoresis
DAD	Diode array detector
DED	Dual electrode detection
DEP	Direct electrospray probe
DESI	Desorption electrospray ionization
DFC	Drug facilitated crime
DIA	Diazepam
ECD	Electron capture detector
EMIT	Enzyme multiplied immunoassay
ESI	Electrospray ionization
FD	Fluorescence detector
FIA	Flow injection analysis
FID	Flame ionization detector

Acronym	Name
FLU	Flunitrazepam
FPIA	Fluorescence polarisation immunoassay
GBL	γ-butyrolactone
GC	Gas chromatography
GHB	Gamma-hydroxybutyric acid
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
IUPAC	International Union of Pure and Applied Chemistry
KET	Ketamine
LC	Liquid chromatography
LED	Light emitting diode
LIF	Laser induced fluorescence
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MBTH	3-methylbenzothiazolin-2-one hydrazine
MC	Methylcellulose
MCFIA	Multicommutated flow injection analysis
MDMA	3,4-methylenedioxymethamphetamine
MEKC	Micellar electrokinetic chromatography
MPA	3-mercaptopropionic acid
MPFS	Multipumping flow system
MS	Mass spectrophotometry
MSFIA	Multisyringe flow injection analysis
NIT	Nitrazepam

Acronym	Name
NMR	Nuclear magnetic resonance
NPD	Nitrogen phosphorous detector
PEG-ELS	Polyethylene glycol-electrolyte
PTFE	Polytetrafluoroethylene
PURGE	Presaturation utilizing relaxation gradients and echoes
QD	Quantum dots
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high-performance liquid chromatography
SDS	Sodium dodecyl sulphate
SIA	Sequential injection analysis
SIFA	Single interface flow analysis
SOFT	Society of Forensic Toxicologists
SPME	Solid phase microextraction
TMS	Trimethylsilane
UV	Ultraviolet
WHO	World Health Organisation
α-CD	α-cyclodextrin
β-CD	β-cyclodextrin
γ-CD	γ-cyclodextrin

CHAPTER 1

Introduction

1.1. Quality control of pharmaceutical formulations

The competent health authorities require the pharmaceutical industry to maintain high standards regarding the quality, safety and efficacy of the whole process of research, development, manufacture and control of medicines. Any person working in the pharmaceutical industry is well aware that a good part of its daily work tasks requires compliance with quality standards imposed by various regulatory agencies [1].

The development of a new medicine, as well as, the validation of a manufacturing process or the implementation of routine quality control involves the analysis of a significant amount of samples aiming to guarantee compliance of the formulations to the limits established by competent authorities.

Before any direct demonstration of the specifications of therapeutic efficacy and safety in patients by using clinical assays, it is important to ensure that the medicines have fulfilled all quality requirements, such as, identity, purity, content and stability. In this context, pharmaceutical analysis is essential to assure drug quality. The pharmaceutical analysis involve the analytical investigation of drug products (the formulated dosage form), active pharmaceutical ingredients (API, also mentioned as drug substance or bulk pharmaceutical chemical), raw materials, intermediates used in their synthesis, impurities and degradation products of drugs, or even the monitoring of the drug and its metabolites in biological samples, aiming to provide information that can contribute to the maximal efficacy and safety of drug therapy and also to the maximal economy of the production of pharmaceuticals products [2]. In fact, the information obtained from the analytical investigations is used to identify potential sources of safety problems, to meet the requirements of regulatory agencies, and as a basis for establishing quality control procedures and specifications for the product.

Alongside the pharmacological and toxicological tests essential to evaluate the efficacy and safety of medicines, the pharmaceutical industry resorts to chemical, physical-chemical and physical analysis to assess agreement with quality specifications.

Quality control includes analytical tests over all steps of drug development process, from the discovery of a new molecule with therapeutic activity, to its final clinical use. Usually, quality control tests involving the analysis of various samples comprise: (i) assay of final products; (ii) dissolution profiles during production and validation of manufacturing processes; (iii) Dosage uniformity tests; (iv) in-process blending uniformity for solid and semi-solid formulations [3].

These quality control tests have as main objective to contribute to the safety of drug therapy by guaranteeing that (i) the pharmaceutical formulation contains the labeled amount of the active ingredient (API); (ii) the drug substance (API) and formulated dosage form contain limited amounts of impurities (which is related with organic impurities and degradation products, inorganic impurities and residual solvents, etc.); (iii) the bioavailability of the active pharmaceutical ingredient in the formulation is well determined [4].

One of the most common analyses in pharmaceutical manufacturing control is the quantitative measurement of the active ingredient (API) in the pharmaceutical formulation. The importance of analytical control of this parameter arises from the possibility of a chemical alteration of the active ingredient during storage, the occurrence of a manufacturing error by the addition of different amounts than those prescribed for the final product, etc.

Due to the accelerated progress in the field of pharmaceutical analysis and quality control of medicines, there is a great demand for fast analytical procedures or methods with good performance characteristics [5]. The analytical methods used for the quantitative analysis of the active ingredient must be selective, precise and accurate. Moreover, in pharmaceutical analysis, wherein the sample matrix is not usually very complex and has reasonable high levels of analyte concentration when compared to biological samples, the main aim is to develop fast, simple, versatile, and reliable methods that can be readily adapted for routine analysis at relatively low cost.

The recommended methodologies for pharmaceutical analysis are encountered in official monographs and involve usually ultraviolet, visible or infrared spectrophotometry, titrimetric techniques and also chromatographic methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC). The chromatographic techniques are the instrumental methods of analysis that prevail in numerous monographs presented in the most recent editions of pharmacopoeias and also in the choice of many researchers for the development of new methodologies to the analysis of pharmaceutical formulations.

HPLC is the chromatographic technique most commonly used in the quantitative analyses of pharmaceutical formulation, being frequently coupled to a UV spectrophotometer as detection system. More recently, diode array detector systems have been increasingly used. Undoubtedly, the established procedures based on HPLC system afford good specificity and excellent precision and accuracy, however these procedures require time-consuming and laborious sample preparation, rigorous intervention of operator, lengthy analysis times, expensive instrumentation and specialized human operators. Therefore, its implementation for the routine analytical control in a large-scale may not be adequate

because of the impossibility of performing expeditious analyses at low cost, which could be very useful on a real time monitoring during the manufacturing process.

The spectroscopic techniques, particularly, UV/visible spectrophotometry are still often used in the various monographs, as well as, in several recently published scientific papers. These classic methods require a rigorous intervention of human operator which can lead to a higher exposure to hazardous substances and also an increased possibility of occurrence of errors.

In this context, there is a demand for new analytical methods that meet the necessary requisites for application in the chemical control of drugs in the pharmaceutical industry, thereby circumventing the above mentioned drawbacks of the spectroscopic and chromatographic techniques recommended by official monographs.

Automation in relation to analytical flow systems can significantly contribute to the development of new methodologies for chemical control of pharmaceutical formulations. In fact, taking into account their potentialities and features, all the necessary requirements for the implementation of analytical methods for routine control of drugs in a large-scale can be fulfilled. Thus, considering their potential importance in the pharmaceutical industry and as part of the research work presented in this dissertation, the concepts and characteristics of automation and flow analysis will be discussed in more detail in a subsequent section.

The detection or identification of counterfeit drugs is a growing challenge in preventing these products of entering health systems with serious consequences for consumers, drug industries and governments. In fact, recent years have seen an increase in the prevalence of counterfeit and substandard drugs on the market. The World Health Organisation (WHO) has defined counterfeit drugs as those with the correct active ingredients but fake packaging, with insufficient or without active ingredients or with incorrect ingredients. The substandard drugs are defined as those produced with little or no attention to good manufacturing practices. Illegal circuits seem inclined to manufacture either copies with the appearance of known trademark drugs or substandard or inadequate pharmaceuticals, including generic drugs. The poor quality of drugs, impairing their pharmacological effectiveness and safety, may be accidental with no intention to deceive, but oversights in manufacturing or neglected controls, particularly in terms of dosage, can have tragic consequences when considering treatment for acute and some chronic diseases, as example, becoming those drugs as potential toxic substances.

Although drugs are developed with therapeutics intentions, sometimes some substances are used for purposes other than those the pharmacologist had in mind. The word “drug” also involves psychoactive substances that can be used for medicinal or non-medicinal

purposes in which it is included substances that are legal or illegal. The use of drugs for intentions other than their therapeutic purposes has become a growing public concern making their study very important from the toxicological standpoint.

1.2. Toxicology: concepts and applications

Toxicology is a branch of science that deals with the study of the adverse effects of foreign compounds (xenobiotics, chemical, physical or biological agents) on living organisms and the ecosystem. Indeed, toxicology can be seen as the study of the detection, occurrence, properties, effects, and regulation of toxic substances. The study of toxicology is very important in society in several ways, not only as a tool to protect humans and environment from the harmful effects of toxicants but also to assist on the development of more selective and secure toxicants such as pesticides, anticancer and other clinical drugs. Undoubtedly, toxicology is essentially an applied science that contributes to the enhancement of the quality of life [6]. Toxicology is a complex and interdisciplinary subject, which comprises several areas of science such as, chemistry, biology, pharmacology, medicine, genetics, economics, and law. Thus, there are different application fields of toxicology which mostly are correlated, such as, environmental, occupational, clinical, regulatory, veterinary, forensic and analytical toxicology [7].

The environmental toxicology is concerned with the study of the deleterious effects resulting from the interaction between the toxicants present in the environment (chemical or physical agents), and several biological systems. Therefore, environmental toxicology studies the harmful effects caused by chemicals substances present in the environment (air, water, soil and food).

The occupational toxicology is the discipline which identifies chemical, physical or biological hazards, encountered in the working environment. Additionally, this field of toxicology recognizes adverse health effects that arise out of workers' exposures to these toxicants, establishing control measures to prevent or minimize exposures.

Clinical toxicology deals with the effects of toxins (typically in the form of drugs) on human patients. This includes the diagnosis and treatment of human poisoning.

Regulatory toxicology is intended to the formulation of laws, and regulations authorized by laws, with the aim to minimize the effect of toxic chemicals on human health and the environment.

Veterinary toxicology is the diagnosis and treatment of poisoning in animals, such as, livestock, companion animals and also feral species. Moreover, the veterinary toxicology is concerned with the possible transmission of toxins to the human population in meat, fish, milk, and other foodstuffs and also the care and ethical treatment of experimental animals.

Analytical and forensic toxicology will be discussed in more detail in the next section, and covers the fields of toxicology involved in the scope of the research work presented in this thesis.

1.2.1. Analytical and forensic toxicology

Analytical toxicology is the area of knowledge that deals with the detection, identification and quantification of toxic substances or poisons in several kind of samples such as, biological and related specimens (urine, blood, etc), environmental (soil, water and air) and also food samples (liquid and solid). In this regard, the continuous development and validation of analytical methodologies has been pursued in order to obtain increasingly accurate, precise and reliable results in the toxicological analysis.

Analytical toxicology plays an important role in the diagnosis, treatment, prognosis and, in some cases, the prevention of poisoning, since it is the only means by which the objective evidence of the nature and magnitude of exposure to a specific toxic compound can be obtained [8]. Toxicological analyses are requested in several cases, such as, (i) emergency and general hospital toxicology, including “poisons screening” and (ii) more specialized categories namely forensic toxicology and screening for drugs of abuse [9]. In fact, the study of the analytical methods used in toxicological analysis of different samples cannot be dissociated from the study of other application field of toxicology, essentially the clinical and forensic toxicology.

Forensic toxicology is concerned with the detection and quantification of toxic agents possibly present in criminal situations and therefore it is an important tool to aid medical or legal investigations of death, poisoning and drug abuse. Normally, attempted suicide and accidental self-poisoning cases are the responsibility of the clinical and analytical toxicologist. However, when an allegation of malicious poisoning occurs or the patient dies and a coroner’s inquest is ordered, the responsibility of investigation is assigned to the forensic toxicologist. The objective of the forensic toxicologist is not the legal outcome of the toxicological investigation namely, to determine who administered the poison or to

confirm the cause of death. Indeed, the main objective of the forensic toxicologist is to find the answers to the questions that arise during the criminal investigation, such as: (i) “Has this person been poisoned?”; (ii) “What is the identity of the poison?”; (iii) “How was it administered?”; (iv) “What are its effects?”; and (v) “Was it a dangerous or lethal amount?” [10]. To this end, the use of chemical analyses play an important role to detect the presence of the poison, quantify its concentration and relate this information to its known toxicity or effects on the organism.

Thus, forensic toxicology demands a continuous development of sensitive and specific analytical techniques and procedures to establish scientific facts relevant to specific cases.

The forensic toxicology can be divided into three different areas such as, death investigation toxicology (post-mortem forensic toxicology), human-performance forensic toxicology and forensic workplace drug testing [11]. These three distinct disciplines overlap significantly although each one was developed to support the judicial system in different ways, as briefly described next.

Post-mortem toxicology is the area of forensic toxicology where the forensic toxicologist aids in establishing the role of alcohol, drugs and poisons in the causation of death. The toxicologist identifies and quantifies the presence of drugs and chemicals in various fluids and tissues samples obtained during autopsy. To this end, it is required chemical and biomedical instrumentation capable to detect small amounts of toxic substances, positively identifying them, and accurately measuring how much is present. The accurately determination of the cause and manner of death has serious implications for public health and public safety.

Human-performance toxicology deals with the evaluation of the role of alcohol and drugs in the modification of human performance and behavior. The toxicologist is responsible to determine the absence or presence of ethanol and other drugs in appropriate specimens. This area of forensic toxicology is usually applied to investigations of impaired driving, vehicular assault and homicide, drug facilitated crimes and motor vehicle collision.

Forensic Workplace Drug Testing determines the absence or presence of prohibited drugs and their metabolites through the analysis of urine to demonstrate prior use or abuse. In this case the obtained results from these urine tests are usually applied to the workplace setting, where the use of drugs by employees can have noteworthy safety and economic implications.

Considering that the drug facilitated crime phenomenon has led to a growing public concern, this branch of forensic toxicology has gathered the interest of the scientific community. For this reason and also as part of the scope of the research work of this

thesis, the drug facilitated crime concept will be discussed in more detail in the next section.

1.2.2. Drug facilitated crime

Drug facilitated crime phenomenon (DFC) is related with the use of a drug to modify a human's behavior and performance for criminal gain, namely, sexual assault, robberies and homicides. A typical scenario of DFC may involve the surreptitious adulteration of beverages (so-called "drink spiking") or foodstuffs of a person with psychotropic drugs in order to render the victim passive, submissive, and unable to resist to the attacker. In cases of DFC, most of victims usually report loss of memory of what exactly has happened during and after the occurrence. Indeed, in the mind of the aggressor, an ideal drug should be one that is readily available, easy to administer, act rapidly in the loss of consciousness and cause the victim to have anterograde amnesia for events that occur under the influence of the intoxicating substance. Furthermore, it should also produce disinhibition, relaxation of voluntary muscles and loss of control [12].

The biggest challenge faced in the investigation of a DFC case is the difficulty in finding out what kind of drug was used to commit this crime. In fact, according to the Society of Forensic Toxicologists (SOFT) there are most of 50 drugs recognized or suspected to have been used to commit DFC [13]. The most commonly encountered drugs in alleged DFC (Table 1) are well-known recreational drugs of abuse, prescription medications, over-the-counter pharmaceuticals and ethanol. Among the pharmaceuticals usually involved in these cases, it can be highlighted the benzodiazepines (flunitrazepam, diazepam etc.), non-benzodiazepines (zopiclone, zolpidem, etc.), barbiturates, antihistamines and also less often the sulphonylurea antidiabetic glibenclamide. Most of the above mentioned pharmaceuticals have anxiolytic, sedative or hypnotic properties and they are fast-acting central nervous system depressants. Their effects can be dangerously potentiated if these drugs are ingested with alcohol [14].

Table 1 – List of drugs recognized or suspected to have been used to commit DFC. Adapted from [13].

Ethanol	**Non-benzodiazepines:**
GHB and analogs:	Zolpidem
Gamma-hydroxybutyrate	Zopiclone
1, 4-Butanediol	Zaleplon
Gamma-butyrolactone	**Antihistamines:**
Benzodiazepines:	Brompheniramine
Alprazolam	Chlorpheniramine
Clonazepam	Diphenhydramine
Chlordiazepoxide	Doxylamine
Diazepam	**Barbiturates:**
Flunitrazepam	Amobarbital
Lorazepam	Butalbital
Oxazepam	Pentobarbital
Triazolam	Phenobarbital
Stimulants:	Secobarbital
Amphetamine	**Miscellaneous Drugs:**
Cocaine	Ketamine
Methamphetamine	Scopolamine
MDMA	Valproic acid

MDMA, 3,4-methylenedioxymethamphetamine; GHB, gamma-hydroxybutyric acid

In DFC cases, the detection of the drug used to commit the crime (evidence) is extremely important to establish a complaint against the perpetrator, since the drug used is the "weapon" of the crime. The assessment of the pharmacological effects in victims is of little help when trying to trace a toxic profile in a specific DFC. In fact, the central nervous system depressant effects of most of the compounds involved in the DFC are very similar which makes it very difficult to determine the specific drug used in the DFC case only by the evaluation of the victims symptoms [15]. Moreover, the pharmacological effects of DFC drugs are themselves characteristic of ethanol intoxication, leading many law

enforcement agents to assume that the victim was drunk rather than drugged and consequently, a fully investigation to determine what really happened it is not conducted [16]. Therefore, it is imperative to resort to thorough and sensitive toxicological analysis of the best available evidence (sample) by using high-end laboratory analytical methodologies in order to increase the chance of detecting the DFC drug. In fact, one of the most important issues in a DFC investigation is the sensitivity of both screening and confirmatory methods, since some of the compounds, e.g. benzodiazepines, are frequently used in a single low dose. Thus, in the scientific literature some screening and confirmatory methods in different sample matrices have been proposed over the last years [17].

Preliminary screening methods for DFC drugs include enzyme multiplied immunoassay (EMIT), fluorescence polarisation immunoassay (FPIA), and Abuscreen OnTrak and OnLine imunoassays (Roche Diagnostics) [18].

For general DFC drug identification/confirmation some separation techniques have been proposed, namely, gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (including micellar electrokinetic chromatography (MEKC) and capillary zone electrophoresis (CZE)) which can be coupled with several detection methods, such as, mass spectrophotometry (MS), ultra-violet and diode array (DAD), fluorescence (FD) flame ionization (FID), electron capture (ECD) and nitrogen phosphorous (NPD) [17].

The selection of the proper sample for the toxicological analysis is another important issue that needs to be considered in a DFC investigation. The more usual matrices sampled for toxicological analysis are biological specimens, namely urine and blood-related matrices, including whole blood, plasma and serum. However, the results of the toxicological analysis in these kinds of samples can provide a negative result when the drug had in fact been ingested. This situation occurs because those biological specimens impose special sampling procedures. One of the most evident problems is the delay in reporting the crime, because of the anterograde amnesia suffered by the victim or doubts about what may have happened and possibly other psychological reasons, including, embarrassment, fear, and denial. Thus, enough time may have elapsed for the drug has been metabolized and eliminated from the biological samples (urine and blood) and consequently, the intoxicant substance can reach concentration levels below to the detection limit of most analytical methods usually applied. Indeed, most of the DFC drugs have very short half-life and therefore are rapidly biotransformated into several inactive metabolites that may be conjugated and then eliminated. As consequence of these problems, it is important for the analyst to ensure that the biological specimens are obtained quickly, nevertheless this is not always possible. The selection of blood specimens for toxicological analysis is

useful but only when the DFC event has occurred within 24 hours of sample collection. Instead, urine analyses have some advantages because these samples present larger windows of detection of drugs frequently used in DFC and their metabolites. However, it is recommended that a urine sample be collected up to 96 hours after the suspected drugging of the victim [19]. Exceeded the deadline for collecting samples of urine and blood, the toxicological analysis in these matrices to detect the DFC drug is of little interest, increasing the risk of not prove that in fact the victim was drugged.

In the last decade, the use of other matrices for complementary toxicological analysis has becoming more important in DFC investigation. The most commonly used samples for complementary toxicological analysis are hair and oral fluid (saliva) [20]. In addition, the drug-related evidence can also be found in drinks or drink containers at the crime scene.

Hair has been suggested as a valuable specimen to perform toxicological analysis. Indeed, hair analysis allow to increase the window of drug detection (weeks to months, depending on the length of the hair shaft) and also distinguish a single exposure from chronic use of a drug, which is of most importance. Additionally, the stability of the drug within the hair matrix is high for long periods of time, providing that specimens can be stored light-and moisture-protected. On the other hand, in the hair analyses the sample collection procedure is non-invasive and easy to perform. However, some drawbacks have been identified to the use of hair samples for toxicological analysis. An important problem mentioned is the possibility to obtain false positive results due to environmental contamination of the hair. Furthermore, taking into account that hair is a complex matrix, it is required a laborious sample pre-treatment to avoid the possibility of interferences in toxicological analysis by the hair constituents. Finally, it is important to refer that a long-term information of an individual's drug use is accessible through hair analysis, however for short-term information it is not recommendable to rely only on hair analysis since in these situations blood and/or urine are better specimens [20].

The saliva specimen was already used in DFC investigations presenting some advantages. One of the advantages is that sample collection can be performed under direct supervision without requiring intrusive techniques (blood sample collection) or loss of privacy (urine sample collection). Nevertheless, these samples have some disadvantages among which, it can be highlighted the insufficient amount of material collected from the victim. In fact, there are some drugs that can inhibit saliva secretion and cause dry mouth. Another disadvantage is the short detection window of illicit drug since saliva analysis can only be performed up to 72 hours after drug ingestion [20].

Beyond toxicological analyses performed on biological samples of the victim (blood, urine, saliva or hair) in order to detect the drug used in the crime, the competent authorities have

been advised by DFC investigators to search for any other items of possible relevance, such as the drink or drink residues suspected to have been adulterated (spiked drink). In fact, succeeding an accusation of suspected ‘‘spiked drink’’, as part of the investigation and if available, the concrete beverage can be analyzed to determine the presence of drugs. The alleged spiked drink left at the scene crime can be collected by the authorities and transported to the laboratory or, alternatively, if the authorities have sufficient means, on-site analysis can be performed. As it was already mentioned, the main problems confronted when conducting toxicological analysis in biological samples for DFC investigation are related with the necessary detection limits, sampling of specimens and time delay between drug ingestion and samples analysis. Thus, the combination of these factors indicates that analysis of spiked drink (physical evidence) in these crimes is very important since they could be the only way to circumvent some problems inherent to the analysis of biological samples collected from the victim. Indeed, the concentration levels of drugs detected in spiked drinks are much higher than those encountered on biological specimens. This is because of the higher stability of the drug in these samples (beverages), since the biometabolism of the drugs does not occur. Taking into account the increase of public concern surrounding the issue of drink spiking, a number of kits claiming to detect the presence of drugs in drinks have been commercialized in the United Kingdom. However, a study performed by Beynon *et al.* [21] demonstrated that two well-known commercially available test kits to detect some DFC drugs in beverages have a significant lack of selectivity and sensitivity. In fact, the use of such kits can provide false negative results for drug detection increasing the concern regarding the true magnitude of drink spiking within the United Kingdom.

The fast screening and toxicological analysis of drugs in beverages intentionally adulterated presents a challenge to forensic laboratories and a need for the development of new analytical techniques. Unlike the several published methods for analysis of DFC drugs from biological specimens, there are only a few published methods for analysis of spiked beverages especially as confirmatory methods. Indeed, in a review study focused between the years 2000 and 2010, Brown and Melton [17] revealed that the majority of analysis techniques for flunitrazepam, γ-hydroxybutyrate (GHB) and ketamine involve detection in biological matrices while only 4, 5 and 2 % of the analysis techniques, respectively, were carried out in beverages.

Table 2 – Methods for determination and quantification of DFC drugs in beverages.

Drug	Matrix	Sample preparation	Separation and detection	Recovery %	LOD µg mL^{-1}	LOQ µg mL^{-1}	Reference
DIA	Cold drink (soft drink, fruit juice)	LLE with chloroform and diethyl ether	HPTLC – UV	74 – 84	-	-	[22]
BZDs	Alcoholic beverages, teas and juice	LLE with chloroform: isopropanol	DEP – MS	-	-	-	[23]
DIA, ALP and CHL	Alcoholic beverages (Toddy)	-	RP-HPLC – DAD	-	0.4 – 4.5	-	[24]
GHB and BZDs	Soft drinks, water, alcoholic beverages, juices	LLE with ethyl acetate – BZDs and dilution – GHB	MECK	-	31.8 GHB 0.722 – 4.37 BZDs	-	[25]
BZDs and other sedatives	Water, soft drink and beer	-	LC – ESI – MS	-	-	-	[26]
GHB and GBL	Alcoholic beverages, juices and water	LLE with chloroform and TMS derivatization	GC – FID GC – MS	-	3 GHB 5 GBL	-	[27]
GHB	Water, beer and soft drinks	SPME on fiber derivatization	GC – MS	-	-	1.5	[28]
GHB	beer	-	NMR	-	-	-	[29]

Table 2 – continued.

Drug	Matrix	Sample preparation	Separation and detection	Recovery %	LOD $\mu g\ mL^{-1}$	LOQ $\mu g\ mL^{-1}$	Reference
CLO, FLU and NIT	beverages	LLE with ethyl acetate	MECK – LIF	79 – 88	13	-	[30]
BZDs	Soft drink, alcoholic beverage and juice	-	CZE – DAD	49.8 – 135.8	2.7 – 41.5	9.0 – 138.2	[31]
FLU and NIT	Soft drink	LLE with dichloromethane	LC – DED	78 – 95.5	0.02	-	[32]
BZDs and KET	Juice and beer	LLE with chloroform: isopropanol	GC – MS	73 – 112.6	1.3 – 34.2	3.9 – 103.8	[33]
GHB and GBL	Alcoholic beverage, water soft drink and juice	-	NMR – PURGE	-	0.03 – 0.1	1.1 – 9.8	[34]
FLU	Alcoholic beverage, juice and soft drink	-	DESI – MS	-	-	3	[35]

DIA, diazepam; BZDs, benzodiazepines; ALP, alprazolam; CHL, chloral hydrate; GHB, γ-hydroxybutyric acid; GBL, γ-butyrolactone; CLO, clonazepam; FLU, flunitrazepam; NIT, nitrazepam; KET, ketamine; TMS, trimethylsilane; LLE, liquid-liquid extraction; SPME, solid phase microextraction; HPTLC, high-performance thin-layer chromatography; DEP, direct electrospray probe; RP-HPLC, reversed-phase high-performance liquid chromatography; MECK, micellar electrokinetic chromatography; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; GC, gas chromatography; FID, flame ionization detection; NMR, nuclear magnetic resonance; LIF, laser induced fluorescence; CZE, capillary zone electrophoresis; DAD, diode-array detection; DED, dual electrode detection; PURGE, presaturation utilizing relaxation gradients and echoes; DESI, desorption electrospray ionization; LOD, limit of detection; LOQ limit of quantification

A literature survey allowed demonstrating the few scientific papers that have been published on the development of new analytical methodologies for the identification and/or quantification of DFC drugs in beverages, which are presented in the table 2 and that will be briefly described next.

In 1998, Sarin *et al.* [22] developed a high-performance thin layer chromatography method for the detection and determination of diazepam in cold drinks, involving one-step liquid-liquid extraction with chloroform and diethyl ether for sample pretreatment.

Then, Chen and Hu [23] applied a direct electrospray probe/mass spectrometry method for the qualitative analysis of nine benzodiazepines from several beverages. Also in this study, samples were treated with a chloroform liquid–liquid extraction prior to analysis. In the authors' opinion, the method provides an efficient mean for identifying drugs from aqueous leftovers found at the crime scene.

Later, in 2004, Rao *et al.* [24] proposed a reversed-phase HPLC method with a photo-diode array detector for simultaneous separation, identification and determination of chloral hydrate, diazepam and alprazolam in fermented alcoholic beverages. In this work, the only sample pretreatment required was filtration through 0.45 mm nylon membranes.

In the same year, Bishop *et al.* [25] described a micellar electrokinetic chromatographic screening method for the separation and detection of benzodiazepines and γ-hydroxybutyric acid (GHB) in a variety of beverages wherein an ethyl acetate liquid–liquid extraction was employed for beverages containing benzodiazepines. An anionic surfactant, sodium dodecyl sulfate, was used in the micellar mode to separate several DFC drugs.

In 2005, Olsen *et al.* [26] employed a liquid chromatography with electrospray ionization-mass spectrometry detection for the determination of sedating drugs in different beverages. They also assessed whether nine common sedating medicinal drugs added to different beverages could cause impairment or incapacitation and whether the drug tablets caused changing of the appearance and taste of the beverages. They concluded that if added in sufficient amounts to a beverage, the nine drugs could cause impairment or incapacitation. Additionally, the tested drugs caused altered appearance of the beverages and most of the drugs changed the taste of the original beverage.

Also in 2005, Elliott *et al.* [27] analyzed γ-hydroxybutyric acid and γ-butyrolactone in 50 beverages. In this work the samples were firstly analyzed using gas chromatography with flame ionization detection and then gas chromatography with mass spectrometry was used for confirmation of positive GC-FID samples. The authors extracted the drugs from several beverages using chloroform.

That same year, Meyers *et al.* [28] developed a method for the analysis of GHB in several alcoholic and non-alcoholic beverages. The developed method consisted initially on the extraction of GHB from aqueous samples by solid phase microextraction (SPME) followed by on-fiber derivatization and then analysis by GC-MS.

In 2006, Grootveld *et al.* [29] explored high resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy to investigate the detection and quantification of GHB in both human saliva and a non-alcoholic beer.

Bishop *et al.* [30], in 2007, developed a microfluidic approach which provided a fast and disposable procedure for the detection of nitrated benzodiazepines in spiked beverages. In this work, micellar electrokinetic chromatography was used with a cyanine dye (Cy5) for indirect laser-induced fluorescence detection. An ethyl acetate liquid-liquid extraction was used to concentrate samples.

Still in 2007, Webb *et al.* [31] reported a capillary zone electrophoresis (CZE) coupled with a diode array detector (DAD) for the separation and determination of 6 benzodiazepines in a variety of drinks frequently consumed at bars and parties. This method employed a double-coated capillary coated with poly(diallyldimethylammonium chloride) and then dextran sulfate. In this work, no sample pretreatment was required to quantify 5 benzodiazepines in the beverages tested, with the exception of wine, whose complexity of the sample matrix did not allow the accurate quantification of nitrazepam.

Honeychurch and Hart [32], in 2008, described a technique for determining flunitrazepam and nitrazepam in spiked soft drinks by liquid chromatography (LC) with dual electrode detection. The tested soft drinks needed a sample pre-treatment procedure prior to the analysis, involving a dichloromethane liquid-liquid extraction.

In 2009, Acikkol *et al.* [33] developed a gas chromatography with mass spectrometry method for the simultaneous determination of some benzodiazepines and ketamine from different drinks. The DFC drugs were extracted from spiked drinks by liquid-liquid extraction with chloroform: isopropanol 1:1 (v/v).

More recently, in 2011, Lesar *et al.* [34] analyzed common beverages adulterated with GHB and GBL using ^{1}H NMR with a water suppression method called presaturation utilizing relaxation gradients and echoes (PURGE). The PURGE method in combination with ^{1}H NMR technique permitted the direct identification and quantification of GHB and GBL in all beverages except wine in which the accurate quantification was not achieved due to small interferences of the sample matrix.

Also in 2011, D'Aloise and Chen [35] used a novel ambient ionization technique based on desorption electrospray ionization with mass spectrometry for the determination of

flunitrazepam in various beverages. The drug-containing drink was analyzed without any pretreatment.

The methodologies that have been developed for the toxicological analysis of DFC drugs in beverages are very precise and present high sensitivity, however most of the mentioned procedures requires expensive equipment, skilled operators and demands a laborious and specific sample treatment using environmental hazard reagents. Additionally, these techniques are only performed in very special laboratory conditions with rigorous intervention of specialized technicians. The methods above mentioned can be a valuable analytical tool especially as confirmatory methodologies. Nevertheless, the methodologies already developed for toxicological analysis of DFC drugs in beverages are completely inappropriate for fast screenings of the drinks at the crime scenes (*in situ*), since they imply large sized instruments and usually they are highly time consuming.

The development of new analytical methodologies for the fast *in situ* screening of DFC drugs in beverages is very important from a forensic and toxicological standpoint. Thus, for a fast *in situ* screening of DFC drugs, the main aim is to develop methodologies simple, fast, reliable and covering a wide range of other characteristics to enable the conduction of analysis out of the laboratory, such as automation, miniaturization, portability, versatility and easy of operation.

To this end, the recourse to automatic methods of analysis and more precisely methodologies based on flow analysis can assume an important role, which as part of the scope of the research work presented in this thesis, their concepts and importance will be described in more detail in the following sections.

1.3. Automatic methods of analysis

Over the last few decades, with the advances in science and technology has increased the need to develop and implement automatic methods of analysis in order to facilitate the analytical procedures, as well as, to perform a larger number of determinations in a reduced time, always keeping in mind the cost-effectiveness.

The automation of analytical procedures in chemical analyses enables to respond quickly and effectively to the request for analysis involving a large number of samples with diminishing human intervention thus allowing the reduction or elimination of several factors that impair the performance of the analytical procedures, as briefly described below.

In fact, the development of the automatic methods of analysis and its implementation in analytical laboratories has been justified by several reasons, among which are highlighted: (i) reduction or even replacement of human participation in routine or hazardous tasks (e.g. those involving toxic or explosive substances) resulting in an increased safety and avoidance of subjective errors; (ii) analytical performance is improved, especially regarding the precision of the results; (iii) more efficient use of the capacity of analytical instrumentation and better management of chemical reagents. Thus, reagent consumption and analytical costs are reduced and the environmental safety is improved, since there is a decreased production of hazardous wastes and a lower exposure of laboratory staff to toxic chemicals. Besides the need of minimizing the costs, there are reasons arising from social demands for increasing the standards of living, involving continuous controls of pollution, potable water, food and drink quality wherein it is usually necessary to resort to the analyses of a large number of samples [36].

Therefore, the automatic methods of analysis have been applied in varied fields, including clinical and toxicological analysis; industrial process control, from raw material to finished product; routine analysis of air, water and soil; and quality control of food, pharmaceutical and agricultural products [36].

The increasing implementation of such methodologies in many areas of application has led to apply new concepts and expressions, requiring a standardization effort by the IUPAC (International Union of Pure and Applied Chemistry) in order to standardize definitions. The IUPAC Commission for Analytical Nomenclature established a series of definitions which distinguish and specify the essential characteristics of automatic methods of analysis. Among the several concepts, the IUPAC recommended that a clear distinction should be made between automatic devices and automated devices [37].

Automatic devices are those that execute certain required actions to be performed at given points in an operation, without human intervention. They are systems without capacity to decide and therefore, they have no feedback system, performing the operations sequence always in the same way.

Automated devices are regulated or controlled by a feedback system which allows them to make decisions without human intervention. The sequence in which operations are executed depends on each situation, constantly adapted for each sample. These systems are self-monitoring and self-adjusting, possessing a higher independence of actuation when compared with automatic devices.

In the automatic methods of analysis, other terms are frequently used and therefore it is important to clarify the concepts associated with them in order to avoid ambiguousness.

Thus, IUPAC clearly distinguishes between mechanization, instrumentation and automation [37]:

(i) Mechanization is the use of mechanical devices to replace, refine, extend or supplement human effort.

(ii) Instrumentation is related to information production and transmission. An instrument is a device used to observe, measure or communicate a parameter, which replaces, refines or supplements human action.

(iii) Automation involves the use of instruments in which an element of non-human decision has been incorporated. More concretely, automation can be defined as the combined use of mechanical and instrumental devices to replace, improve, extend or supplement human effort and faculties in the performance of a process, in which at least one operation is controlled, without human intervention, through a feedback system. Finally, it should be mentioned that feedback system is an instrumental device combining sensing and commanding elements which can modify the performance of the process at different moments.

Over the last years, along with the development of automatic methods of analysis, different methodologies and several strategies have emerged for the implementation of automation in analytical processes. According to the kind of sample processing, automatic methods of analysis can be divided into three categories, namely, discrete or batch methods, robotic methods and flow methods [36]:

(i) Discrete or batch methods: The samples are mechanically transported toward the detection unit, where each sample preserves its integrity in separated vessels. During the samples transport, the different analytical stages, such as dilution, reagent dispensing, mixing, heating, etc., can be performed in a sequential way.

(ii) Robotic methods involve the use of a high-precision mini robot controlled by a computer whose movements mimic the actions of a human operator in an analytical procedure. This mini robot usually consists of a moving arm fitted with a hand (grip) that affords the movements required to transfer the sample and the products resulting from the different stages of its processing to a series of apparatuses (dilutor, liquid dispensing units, extractors, centrifuges, heaters) and instruments (balance, photometer, chromatograph). A microprocessor controls the robot’s movements and the operation of the different apparatuses and instruments, from which it receives the corresponding signals to be treated in order to obtain the final results. These methods have the ability to perform tasks other than those programmed, based on "learning" and self-programming routines.

(iii) Flow methods: The sample solutions are successively introduced at the same point into a channel carrying a liquid or gas, commonly known as carrier stream, in which samples are propelled towards the detection. During the propulsion of samples from the insertion point to the detector, they can be subjected to one or more chemical reactions (or any other type of analytical operation) in order to condition the samples properly for the determination. Thus, the channels carrying the samples can merge or not with other channels carrying reagents, buffers, masking agents, etc. Upon reaching the detector, the analytical signal is continuously monitored over time and each sample or reaction product yields a transient signal in which its height or area is related with the parameter under evaluation.

According to the type of flow used, these flow methods can be classified as segmented flow methods and non-segmented flow methods:

(i) Segmented flow methods: In 1957, Skeggs [38] proposed a methodology based on segmented flow with continuous monitoring of flow parameters. For almost two decades, in the development of flow methodologies, it was necessary to resort to the use of segmentation in order to prevent sample dispersion and thus maintaining the identity of each sample, separating them from one another by air bubbles, to promote the continuous analysis of samples. The bubbles inserted into the system, in an intercalated way, are removed prior to reaching the detector in order to circumvent its interference in the obtained analytical signal, which when using an optical detection system can be due to the phase difference (liquid-air or air-liquid).

(ii) Non-segmented flow methods: In 1975, emerged the concept of non-segmented flow analysis, in which small sample volumes are inserted directly into the carrier stream. The sample zone is carried toward detection without the segmentation of the flow by air bubbles.

Ever since and to the present time, the non-segmented flow methods have been most studied by researchers and therefore they are subject to greater scientific interest. This widespread scientific interest has been verified mainly due to lower installation and operation costs, a higher flexibility based on a modular structure that allows its simplified reconfiguration to new analytical situations and also by a higher facility of operation and control. Additionally, these methods enable a reduced consumption of sample, reagents and analysis time. The importance of flow analysis, essentially non-segmented flow analysis in the overall perspective of automation in analytical laboratories justifies a more detailed approach of this subject, which is following presented.

1.4. Non-segmented flow methods

The non-segmented flow methods are undoubtedly the automatic methods of analysis on which more research work has been performed in the recent years. These methods have been subject to wide acceptance due to its high versatility and simplicity and also the required cost for its implementation in analytical laboratories that is very affordable. Moreover, they can provide excellent results regarding the rapidity, accuracy and precision. The operation mode can be directly modified by the operator in order to obtain the system optimization and the control of chemical and physical variables.

As previously mentioned, the non-segmented flow analysis is performed by the direct insertion of small sample volumes into a carrier stream without the presence of air bubbles. In these methods, the measurements are performed without the need to reach the chemical and physical equilibrium, since sample and standard solutions are subjected to identical dispersion levels and also they are processed always in the same way (high degree of reproducibility).

The fact that it is not necessary to resort to the insertion of air bubbles to avoid contamination between samples involves several advantages, such as, a higher determination rate, improved response times and higher reproducibility in detection.

The fundamental components of a non-segmented flow system are [37]:

i) Propelling system is the responsible device by the constant movement (either by aspiration or by propulsion) of the sample and reagents solutions inside the tubing. The propulsion of solutions throughout the flow system is usually conducted using a peristaltic pump or piston pump.

ii) Sample insertion system is intended to insert or inject, in a fully reproducible manner, an accurate volume of sample into a carrier solution, which it can be accomplished by means of a rotary valve, a proportional injector or a solenoid valve.

iii) Transport and reaction system serves to link the different parts of the flow system. The transport system normally consists of a number of polytetrafluoroethylene (PTFE) or polypropylene tubes, with several inner diameters. The reactor, a major component of the transport system, influences the residence time and the profile of the sample plug, and is designed to meet the particular needs of the system concerned. These reactors can be filled with a chemically active or inert material (packed reactors).

The flow system can also include mixing chambers or separating modules.

iv) Detection system is the responsible device for the transduction of the analytical signal. A variety of analytical detection techniques can be used such as optical (UV-visible spectrophotometry, fluorimetry and chemiluminescence) or electrochemical (voltammetry-amperometry and potentiometry).
v) Data acquisition and processing system is the device that consists in a chart recorder or a microcomputer which receives the analytical signals provided by the detector.
The growing technological evolution of these methods led to the emergence of several flow analysis techniques. The pioneer non-segmented flow system was the flow injection analysis (FIA) [39]. Ever since, other methodologies have emerged over the last decades, such as, sequential injection analysis (SIA) [40], multicommutated flow injection analysis (MCFIA) [41], multisyringe flow injection analysis (MSFIA) [42] and more recently, multipumping flow system (MPFS) [43] and single interface flow analysis (SIFA) [44]. After the appearance of FIA in 1975, it is important to mention that only from the 90's emerged other non-segmented flow methodologies. This period coincides with the great technological evolution of computing and electronic devices and also, with the significant decrease in the cost of these devices leading to its widespread use in the laboratory. On the other hand, the higher facility of development and use of programming languages, simplifies the conception, operation and control of automatic systems of analysis. Despite this progress, the FIA methodology remains the most widely used today and it is still the one that presents a higher number of scientific publications. This can be explained by the fact that this methodology does not require the use of computing and electronic devices, as well as, knowledge of programming.

1.4.1. Flow injection analysis

FIA methodology (Figure 1) was proposed by J. Ruzicka and E. H. Hansen [39], in 1975, as an alternative of segmented flow methods. The fundamental characteristic of this non-segmented flow method is the control of the dispersion of a sample plug intercalated into a carrier stream that can contain reagents while it is carried towards the detector.
Unlike to what happens in the segmented flow analysis, wherein samples are sequentially aspirated and transported between air bubbles, in FIA methodology a constant volume of sample is injected into a non-segmented carrier stream in which is dispersed in a certain extension during transport.

The control of the sample dispersion during the transport in the flow system is important because it allows controlling the sample dilution into the system, as well as, in a case of a chemical reaction, it promotes a higher sample and reagent mixture. The extension of sample dispersion into the carrier stream determines the profile of analytical signal. The sample dispersion should be well controlled in order to guarantee the same degree of sample dispersion during the transport in all determinations, thus ensuring the reproducibility of the analytical measurements.

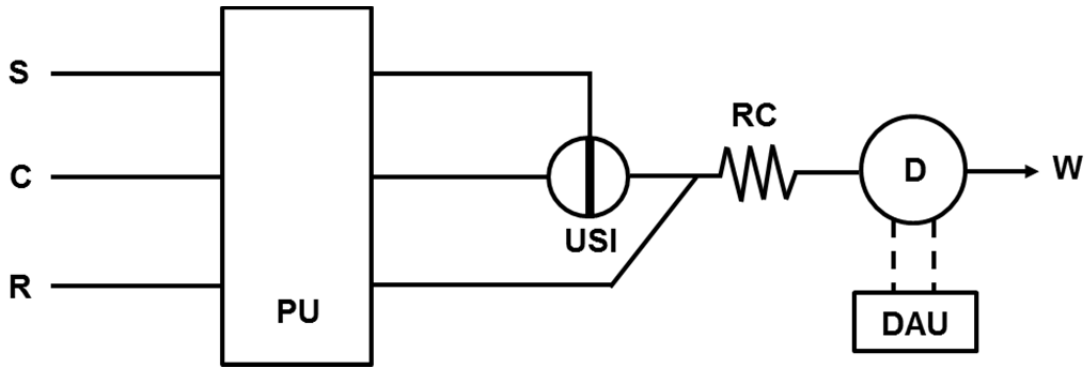

Figure 1 – Schematic diagram of a typical FIA manifold. S, sample; C, carrier; R, reagent; PU, propelling unit (e.g. peristaltic pump); USI, unit of sample insertion (e.g. rotary valve); RC, reactor; D, detector (e.g. spectrophotometer); DAU, data acquisition unit (e.g. chart recorder); W, waste.

The versatility of flow injection analysis is higher comparing with the segmented flow analysis, since it simplifies the implementation of several analytical approaches that were of high complexity to perform in a segmented flow system (with air bubbles between samples), namely, kinetic, enzymatic and stopped flow methods. Moreover, the versatility of FIA enables the implementation of non-chromatographic continuous separation techniques, such as, dialysis, liquid-liquid or solid-liquid extractions, ion exchange, etc., increasing the sensitivity or selectivity of the chemical determination. Another important advantage of FIA is its facility of conception and implementation, since it is relatively simple to develop a system using inexpensive equipment, such as, a peristaltic pump, injection valve, flow cell, Teflon® tubing, connectors, etc., and coupling this apparatus to detection instruments that are also common in laboratories (spectrophotometers, amperometric and potentiometric systems, etc.). Furthermore, this methodology does not require the specific use of computers and software. This facility of implementation associated with good analytical results is the reason why FIA systems have been

extraordinarily used in the research laboratories, justifying thus, the emergence of a large number of published scientific papers using this methodology, although in the last decade other non-segmented flow methodologies have also been the subject of research, development and improvement.

1.4.2. Sequential injection analysis

In 1990, J. Ruzicka and G. Marshall [40] proposed the sequential injection analysis (SIA) methodology as a progress of the control processes of the flow analysis. SIA is a non-segmented flow methodology based on the principle of controlled dispersion and reproducible manipulation, which mode of functioning is based on the programmable flow concept [45]. In other words, the programmable flow concept means that the SIA analysis protocols are automated and fully software-controllable.

One of the basic characteristics of SIA is the fact that the system is controlled by a computer which is responsible by the synchronized control of the propulsion device and a multi-position selection valve. The multi-position selection valve plays a fundamental role in SIA methodology (Figure 2), in which its functioning consists in the alternative connection of the central port to one of the side ports, and the number of possible positions depends on the number of side ports in the valve. The side ports of the valve have access to the sample and reagent solutions required for the application of the analytical method and also allow the connection to the detector. However, these side ports can be used for other purposes, such as, an outlet for waste, or can be linked to other devices, as for example, a mixing chamber, etc. A computer controls the alternative connection of the central port with the side ports of the valve and provide the instructions to the propulsion device in order to define the flow rate, volume and flow direction (direct or inverse) of the different solutions. At the same time, the computer can perform the data acquisition.

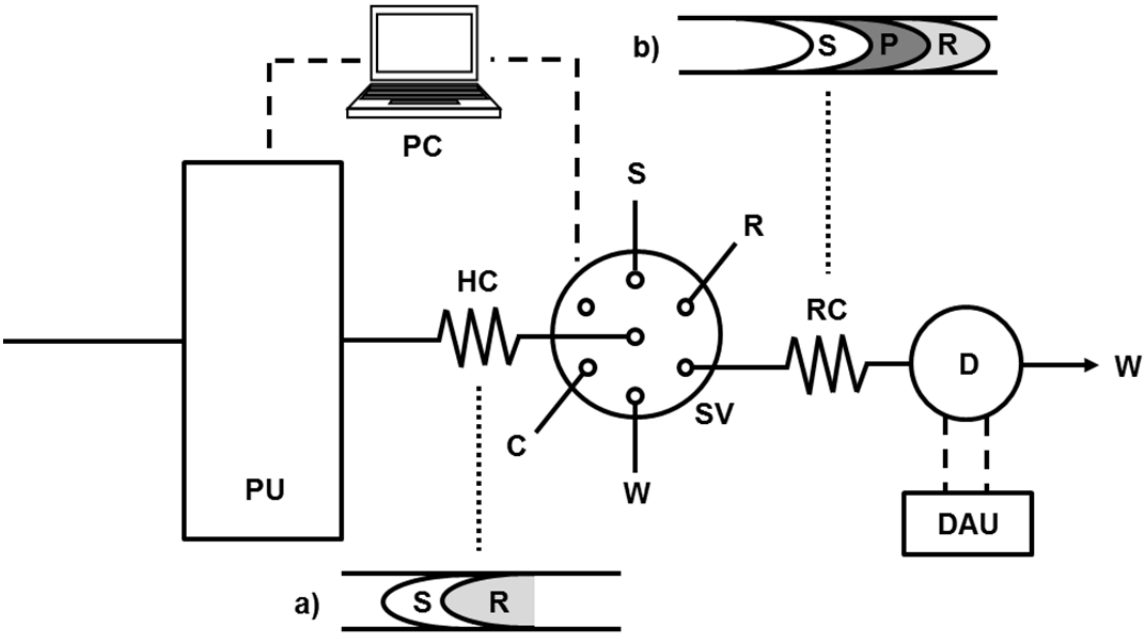

Figure 2 – Schematic diagram of a typical SIA manifold. S, sample; C, carrier; R, reagent; PU, propelling unit (e.g. peristaltic pump); PC, computer; HC, holding coil; SV, multi-position selection valve; RC, reactor; D, detector (e.g. spectrophotometer); DAU, data acquisition unit (e.g. chart recorder); W, waste; a), sequential aspiration of sample (S) and reagent (R) from VS; b), propulsion of reaction zone toward detection; P, reaction product.

In a usual analytical cycle, precise volumes of sample and reagents are sequentially aspirated to the holding coil (Figure 2, a)), and by inverting the direction of the flow, the formed reaction zone is transported towards detection (Figure 2, b)). During this procedure the mixture of sample and reagents occurs, not only due to the inversion of the flow direction but also to the radial and axial dispersion phenomenon that always occurs in the transport processes.

The main advantages of SIA systems relatively to FIA systems are a reduced consumption of sample and reagents and subsequent significant reduction in waste generation, since the solutions are used more efficiently being only aspirated a volume strictly necessary for the analysis. In addition, in SIA methodologies, by using a computer, it is possible to control the appropriate flow rate in a very reproducible way and also all the times used in each step of the analytical cycle, in which the experimental parameters can be easily changed without physical reconfigurations of the system. These features are the reason why SIA is regarded as a very versatile, simple and robust system with low maintenance requirements. When using SIA systems it is possible to implement more

complex chemical reactions involving a large number of reagents. The SIA versatility enables that the same manifold can be used in different analytical determinations.
However, some drawbacks are pointed to the SIA methodology, such as, the increased time required for the analytical determination not only because of the measurement and reaction time, but also due to the required time for the sequential aspiration of the different solutions. SIA systems usually involve higher costs in its implementation in analytical laboratories comparing with the FIA systems. Additionally, in SIA systems, the mixture between sample and reagents is more difficult because of its operation mode in which sample and reagents are aspirated one after the other, thereby hindering the interpenetration between the two zones.

1.4.3. Multicommutated flow injection analysis

Multicommutated flow injection (MCFIA) analysis was proposed in 1994 [41], by research groups of Center of Nuclear Energy in Agriculture of the University of São Paulo (CENA, Brazil) and the Faculty of Pharmacy of University of Porto (FFUP, Portugal), as a novel strategy to handle sample and reagents introduction in a non-segmented continuous flow system.
A multicommutation system (Figure 3) is defined as an analytical network that involves the actuation of n active devices (or “n” operations with a single device) on a single sample enabling the establishment of up to 2^n states. The required analytical steps for sample processing are defined by the control software, which can be modified at real-time [46].
The multicommutation is usually performed through the use of solenoid valves controlled by a computer. The fast switching between two states (ON/OFF) characteristic of this device provides significant advantages over others injection devices (such as, rotatory valve or multi-position selection valve) regarding the approach of insertion of sample and reagent volumes into the flow system. With a time-based control that can be easily altered or adjusted, small plugs of sample are intercalated with small plugs of reagent, enabling a faster sample/reagent homogenization and consequently, a higher reaction development. This novel concept of sample and reagent insertion is designated by binary sampling [41]. During the transport of the reaction zone towards detection, the sample and reagent mixture occurs by the mutual dispersion at the several liquid interfaces originated between sample and reagent aliquots. It is important to mention that for constant total volumes of sample and reagent, the mixture is enhanced by decreasing the aliquot volumes and

increasing the number of slugs. This means that the mixture between sample and reagent is more efficient with the increase of the number of reaction interfaces, which is easily achieved with a MCFIA system, thus constituting an important advantage in relation to the flow systems previously proposed.

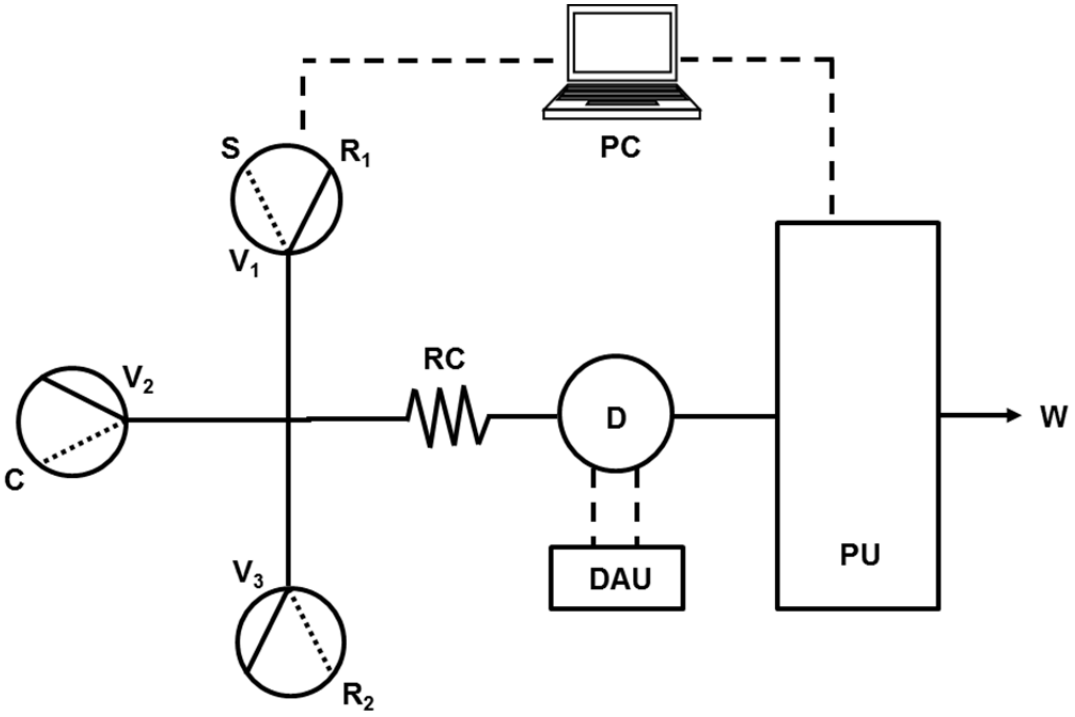

Figure 3 – Schematic diagram of a typical MCFIA manifold. S, sample; C, carrier; R_1 and R_2, reagents; PU, propelling unit (e.g. peristaltic pump); PC, computer; V_1 – V_3, solenoid valves; RC, reactor; D, detector (e.g. spectrophotometer); DAU, data acquisition unit (e.g. chart recorder); W, waste.

The multicommutation systems present high versatility since the topology of a MCFIA manifold can assume distinct configurations such as, linear, circular, etc., by means of a flow network that allows the flowing streams to be directed through different analytical pathways.

The combination of the advantageous operational features of MCFIA, such as, the simplicity of flow manifolds, high versatility, high degree of automation and the new concept of sample insertion by binary sampling, ensure the necessary means to achieve a rapid reaction development, low consumption of solutions, an efficient control of the analytical parameters and a wide variety of sample zone managements without requiring any physical reconfiguration of the flow manifold.

However, MCFIA systems have some drawbacks that are essentially related with the operational characteristics of the injection devices usually used in this methodology. Indeed, when the solenoid valves are used for a long period of time, it can be verified an inferior robustness comparing with the multi-position selection valve commonly used in SIA, since its excessive use can lead to a warming of the insertion devices increasing the probability of failure occurrence, which can affect the reproducibility of measurements.

1.4.4. Multisyringe flow injection analysis

In 1999, Cerdà and co-workers proposed multisyringe flow injection analysis (MSFIA) [42], as an alternative to the previous flow methodologies, particularly the multicommutated strategy, combining the multi-channel operation of FIA with the possibility of selecting the precise volume of sample and reagent required for analysis, as it happens in SIA [47].
MSFIA involves the use of a multisyringe piston pump which is employed as a multi-channel device (Figure 4). This device consists in an automatic burette containing up to four syringes, with different capacity (with volumes of 0.50, 1.00, 2.50, 5.00, 10.00, 25.00 mL), allowing to obtain a wide range of flow rates, which are driven by a single motor and controlled by a computer software. A solenoid valve is connected to the head of each syringe, allowing optional coupling to the flow system or to solution reservoir. Thus, it is possible the assembly of a flow network connected to the detector, wherein solutions from the different syringes can be carried to the flow system or returned to their reservoirs, according with convenience for the analytical determination.

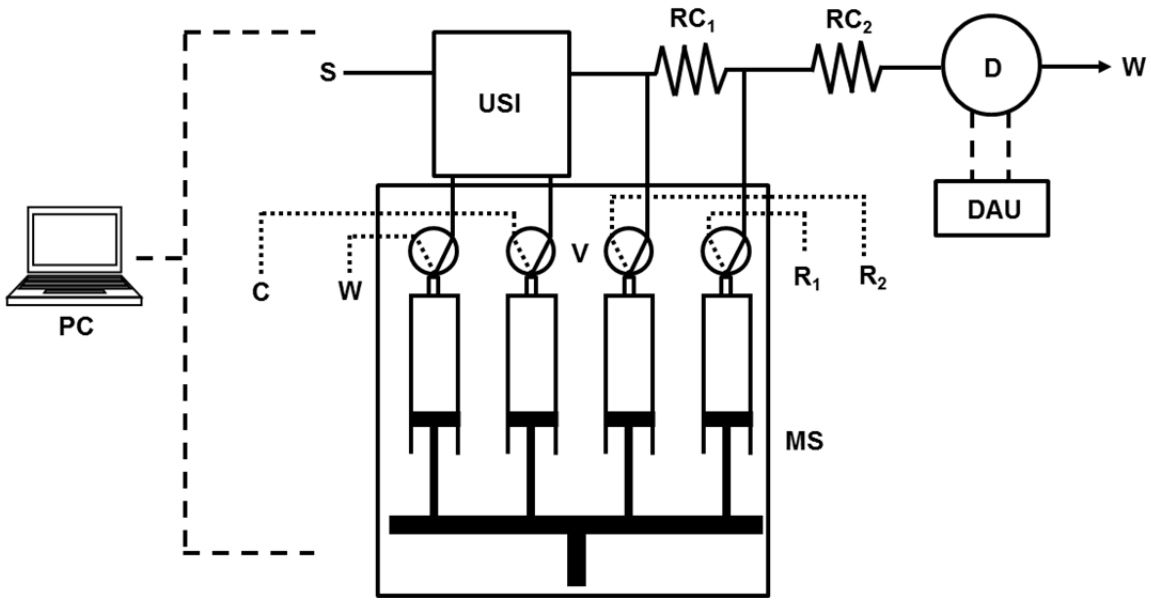

Figure 4 – Schematic diagram of a typical MSFIA manifold. PC, computer; S, sample; C, carrier; R_1 and R_2, reagents; MS, multisyringe piston pump; V, solenoid valve; RC_1 and RC_2, reactors; USI, unit of sample insertion; D, detector (e.g. spectrophotometer); DAU, data acquisition unit (e.g. chart recorder); W, waste.

This flow methodology has several advantages among which can be mentioned: (i) The possibility of avoiding excessive consumption of sample and reagents since there is the option for return of solutions to the reservoir when no longer needed. (ii) The use of multisyringe piston pump allows to circumvent one of the main drawback of peristaltic pump which is the use of propelling polymeric tubes that can be incompatible with strong acids and bases and organic solvents, or even in the case of being compatible, the tubes can have a short life time due to the systematic compression and decompression. iii) This propulsion device confers to MSFIA systems a higher robustness and precision in the transport of solutions comparing to the flow systems that use the peristaltic pumps.

Despite the advantages mentioned above, the functioning of multisyringe piston pump entails periodical refilling of syringes with the respective solutions that impairs the determination rate.

1.4.5. Multipumping flow system

The multipumping concept [43] appeared in 2002, which constituted a novel flow strategy with distinct hydrodynamic characteristics (pulsed flow) and with other important differences relatively to the previously proposed flow methodologies (FIA, SIA, MCFIA and MSFIA), namely, in terms of the conception and establishment of the flow manifold, since the successive stages of a typical procedure in flow analysis including the sample insertion, reagent addition and propulsion of the solutions are performed by the same component of the flow system and not by separate and individual active devices.
The concepts and features of this flow methodology will be described in more detail in a dedicated chapter (subchapter 1.5.), since the analytical methods developed in the scope of the research work explored the multipumping concept. Thus, in the next section (section 1.4.6.), the more recent flow analysis strategy of fluid management namely single interface flow analysis (SIFA) will be discussed.

1.4.6. Single interface flow analysis

In 2005, single interface flow analysis (SIFA) was proposed by Ribeiro *et al.* [44] and it represented a new concept involving the management of solutions and also the way of interaction between of sample and reagent zones. Indeed, in SIFA the mixing between sample and reagent zones occurs at a single reaction interface instead to the dual or multiple reaction interface concept, usually related to the previous flow techniques.
In a typical SIFA system (Figure 5), the sample and reagent insertion and propelling devices are usually symmetrically positioned around the detector placed at the core of the flow manifold, thus enabling repetitive flow reversals and consequently several interface manipulations, including multi-detection of the reaction interface.
Additionally, in SIFA systems the controlled dispersion and reaction zone formation do not depend of the sample and reagent volumes but only in the extension of the penetration of sample and reagent adjacent zones. The increase of penetration degree of the involved zones can be achieved resorting to multiple flow reversals and eventually with the use of pulsed flow (e.g. by using solenoid micro-pump).

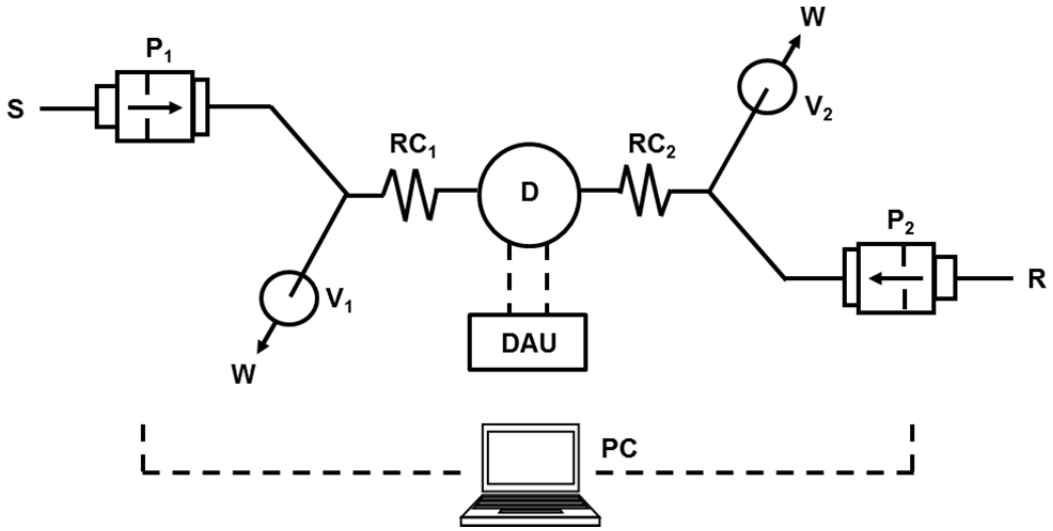

Figure 5 – Schematic diagram of a typical SIFA manifold. PC, computer; S, sample; R, reagent; P_1 and P_2, solutions insertion and propelling devices (solenoid micro-pumps); V_1 and V_2, solenoid valves; RC_1 and RC_2, reactors; D, detector (e.g. spectrophotometer); DAU, data acquisition unit (e.g. chart recorder); W, waste.

It is important to refer that the basic principles of functioning of SIFA system do not depend of multipumping concept and therefore, the design of these systems does not means the use of solenoid micro-pumps for propulsion and insertion of solutions.

In SIFA the way that sample and reagents solutions are mixed is similar to SIA systems, nevertheless SIFA presents some advantages. The use of a long holding coil is not required in SIFA systems to prevent penetration of sample zone into propelling device and also to avoid possible problems with cross-contamination, resulting in a simpler system configuration. Moreover, SIFA systems are more easily implemented and optimized, since the requirements of manipulating parameters such as coil length, sample volume and flow rate to achieve the optimal dispersion is reduced.

One of the most remarkable advantages of SIFA is the possibility of performing multiple reaction zone detections in a simple and efficient way, enabling thus to obtain accurate and precise results without loss of sensitivity.

1.5. Multipumping flow system

Multipumping flow system (MPFS) is a strategy of fluid management for the implementation of flow-based analytical procedures which were developed in 2002 by two research groups at Faculty of Pharmacy of University of Porto (FFUP, Portugal) and Center of Nuclear Energy in Agriculture of the University of Sao Paulo (CENA, Brazil) [43]. As mentioned in subchapter 1.4.5, MPFS differ from the previous proposed flow approaches in several aspects, including manifold components and configuration, operational mode and flow hydrodynamics characteristics.

1.5.1. Manifold components and configuration

The MPFS manifold (Figure 6), described for the first time by Lapa *et al.* [43] was based on the utilization of several solenoid micro-pumps which can be operated individually or in combination for the propulsion (or impulsion) of liquids while at the same time they can act as sample/reagent insertion devices and commutation units. Indeed, the fundamental operations involved in an automatic flow system, such as, sample insertion, reagent addition, solutions mixing and the transport of the reaction zone toward the detector are performed by the same manifold component (solenoid micro-pump), being the only active device. However, in the conception and establishment of the MPFS manifolds in more complex situations it can be necessary to use solenoid valves for re-directing the solutions through a flow network. The reduction of the number of manifold active components enables a very simplified configuration and control of the flow system while simultaneously minimizing the probability of occurrence of equipment failure, malfunctions or errors.

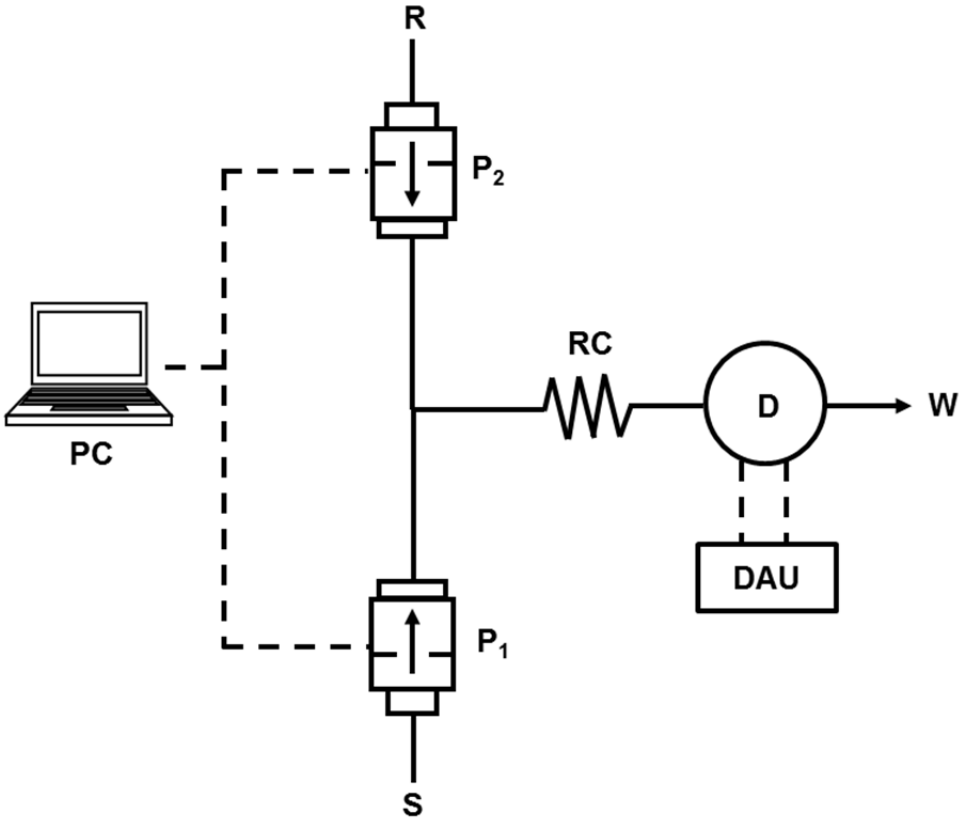

Figure 6 - Schematic diagram of a typical MPFS manifold. PC, computer; S, sample; R, reagent; P_1 and P_2, solenoid micro-pumps; RC, reactor; D, detector (e.g. spectrophotometer); DAU, data acquisition unit (e.g. chart recorder); W, waste.

The micro-pumps are solenoid operated devices designed to provide a precise, repeatable and discrete dispense of solution volumes. They exhibit operational characteristics that support a good analytical performance, as already demonstrated by the developed flow systems exploiting the multipumping concept and documented in the scientific literature. These solenoid micro-pumps are provided with a non-metallic path and chemically inert, and they are also compact, accurate, precise and robust. In fact, solenoid micro-pump is an important device that has been very useful in the assembly of flow systems exploring multipumping concept, which allowed the development of valuable methodologies applied to distinct analytical situation providing accurate, precise and reliable results. Considering its potential, the solenoid micro-pumps were initially used by Weeks and Johnson in the assembly of a flow manifold for the development of a methodology based on flow injection analysis [48]. However, in this research work the

micro-pumps were used only as propelling devices replacing the peristaltic pump in a typical two-channel FIA manifold.

1.5.2. Operational mode

In a multipumping flow system, the micro-pumps used in the flow manifold are controlled individually by computer assuring a high flexibility in terms of solutions insertion, including the possibility of real-time manipulation of the inserted volumes, flow rates and sampling strategy. The insertion of the sample and reagent solutions into the flow system are controlled by means of a time-based or a pulse-counting routine, providing a great versatility and precision in the selection or adjustment of the most adequate sample/reagent volume. In fact, considering the fixed stroke volume characteristic of the micro-pumps, the sample and reagent volume could be easily defined by the number of pulses inserted into the flow system through the repeated actuation of the respective micro-pump. Additionally, the flow rate is determined by the stroke volume and the frequency of the micro-pump actuation (pulse frequency). Another interesting feature of the operational mode of MPFS is the facility of monitoring the position of the sample zone in the interior of the flow system by means of pulse-counting or volume-measuring routines, which enables a more efficient control of the sample transport throughout the flow manifold, facilitating, for example, the synchronization of the reagents addition and the establishment of the reaction zone. Simultaneously, the effective and precise control of the position of sample zone also allows controlled stops of the flowing stream, which can be very important in the implementation of kinetic methods and stopped-flow strategies [49].

The multiple tasks performed by the micro-pumps combined with their individual control by means of a computer enables the utilisation of different sampling strategies including single sample volumes, binary sampling and merging zones. The single sample volumes approach consists in the insertion of a unique volume of sample solution between two identical plugs of reagent solution or into a flowing carrier stream, establishing only two reaction interfaces. The binary sampling strategy is the intercalation of small plugs of sample and reagent solutions, establishing multiple reaction interfaces, which enables a better homogenization of reaction zone comparatively to the single sample volumes approach (only two reaction interfaces). This is easily performed in a MPFS through the alternated actuation of sample and reagent micro-pumps for a certain number of

intercalation cycles and intercalation pulses, defining the reaction zone. The utilisation of the merging zones approach for sample insertion is carried out by simultaneous actuation of sample and reagent micro-pumps. The features above mentioned ensure a versatile manipulation of the sample/reagent mixing, sample dispersion and therefore, of reaction development.

1.5.3. Flow hydrodynamic characteristics

The multipumping flow systems are characterized by an important difference in the hydrodynamic properties of flowing stream regarding the more conventional flow-based procedures. Instead the typical laminar flow associated with continuous flow systems, MPFS is characterized by a pulsed flow that could be viewed as a chaotic movement of the solutions in all directions. Indeed, the actuation of solenoid micro-pumps based on the sudden displacement of the pump diaphragm produces a burst of solution at the pump outlet generating a pulsed flowing stream. The pulsed nature of the flow promotes a fast and efficient mixture between aliquots of sample and reagent solutions which can lead to an enhancement in the analytical sensitivity, especially when slow chemical reactions are involved or higher viscous solutions are used. As a result of the hydrodynamic characteristics of MPFS, a higher degree of sample/reagent mixture is achieved comparatively to the observed in the continuous flow methodologies with laminar flow regime, wherein the interpenetration between solutions only depends on the phenomena of diffusion and convection [49]. This assumption was already corroborated by some scientific works [43, 50, 51] in which the performance of a MPFS and a conventional continuous flow-based system with laminar flow regime were evaluated under the same circumstances by using similar manifolds configurations. Lapa *et al.* [43] in the determination of Cr(VI) exploiting the reaction with diphenylcarbazide concluded that the analytical signals provided by the multipumping system (pulsed flow) were three times higher than those obtained with the FIA system (laminar flow). Dias *et al.* [50] performed the comparison of the flow pattern inherent to multipumping and multicommutation concept, by exploiting the dye approach and by implementing a slow chemical reaction (molybdenum blue method for phosphate determination). The authors verified that the utilization of pulsed flows allowed better mixing conditions, lower reagent/sample consumption, improved the analytical sensitivity and reduced sample broadening when compared with laminar flow. Additionally, Fortes *et al.* [51] in their work emphasized that

by using MPFS, the height and width of the recorded peak, as well as, washing time were significantly improved, which means better sensitivity and sampling rate relatively to flow systems relying on laminar flow (Figure 7). All the referred scientific works highlighted the superior characteristics of pulsed flow which is the hallmark of the flow systems based on the multipumping concept.

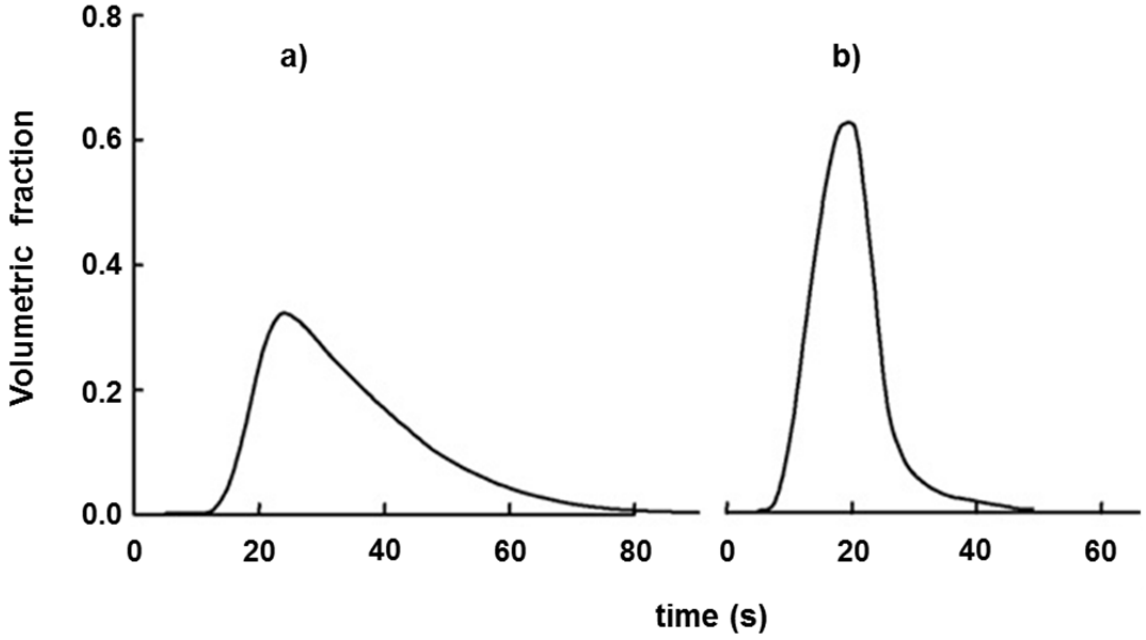

Figure 7 – Analytical signals obtained with a) multicommutated and b) multipumping flow systems. Bromocresol green solution inserted into the flow system (total flow rate = 2.0 mL min^{-1}, reactor length = 200 cm; sample inserted volume = 100 µL). Volumetric fraction (χ) concept in flow analysis was proposed by Zagatto *et al.* [52]. Figure adapted from [51].

The pulse volume (directly dependent on the stroke volume) is an important parameter regarding the efficiency of the reaction zone homogenization, since it determines the volume of segments that are mixed and/or intercalated. Thus, the utilization of higher stroke volume leads to more difficulties of sample/reagent mixing, as it was verified by Weeks *et al.* [48] that referred the irregularity and poor reproducibility of the analytical signals as major drawbacks of micro-pumps. However, by means of the utilization of micro-pumps with lower stroke volumes, a more reproducible pulsed flowing stream is produced allowing the enhancement of the mixing efficiency and enabling the attainment of improved analytical signals. Effectively, the pulse volume plays a fundamental role in the reaction development affecting also the analytical signal profile. The nature of the pulsed flow can be perceptible when analyzing the stair-like profile of the recorded

analytical signals (Figure 8). This profile is mainly dependent on the volume of the stroke, pulse frequency, length of the reactor and internal volume of the detector's flow cell.

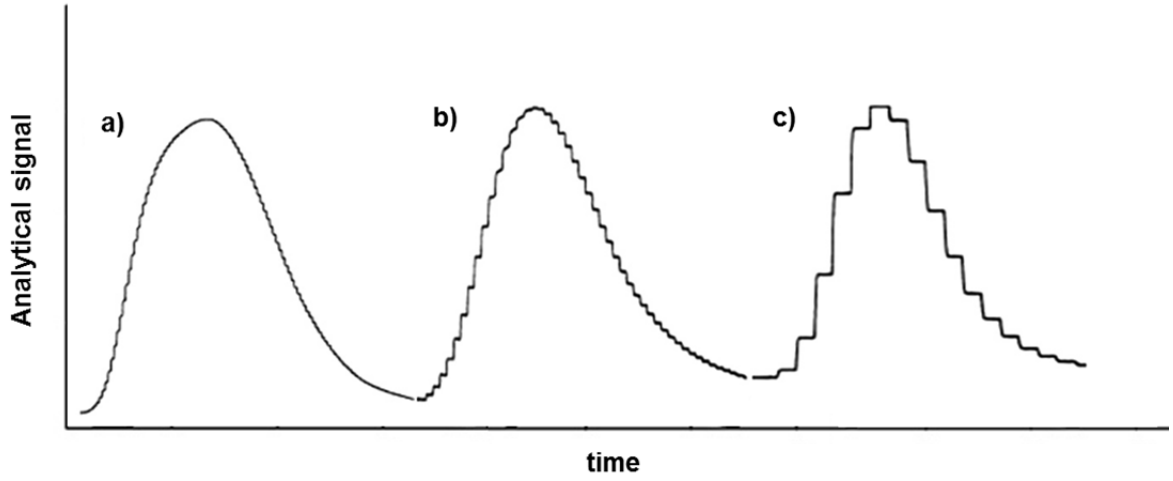

Figure 8 - Analytical signal profiles obtained by inserting into a MPFS, a Brilliant Green solution (95.0 mg L^{-1}) by means of a 3 µL (a), 8µL (b) and 25 µL (c) per stroke solenoid micro-pumps. Peaks recorded at 12 cm min^{-1}. Adapted from [43].

1.5.4. Potentialities of MPFS

The characteristics and potentialities of the flow systems based on the multipumping concept make this flow strategy very attractive for the implementation of automatic analytical procedures, which can be used with significant advantages relatively to the conventional strategies.

In fact, the nature of the pulsed flow in combination with the multiple tasks performed by the multi-pump and their individual control by means of a computer enable to implement more compact analytical flow systems integrating fewer components, being thus more easily assembled and controlled with a great operational simplicity. These important features provide to the multipumping strategy the necessary means to develop analytical systems characterized by a rapid reaction development with a significant reduction of sample and reagent consumption that leads to cost reduction and minimization of waste generation. The above mentioned features in combination with the high portability and low power demands exhibited by MPFS allow the miniaturization of automatic flow systems,

making possible the carrying out of analytical measurements outside the laboratory environment.

Another essential feature of MPFS is the high versatility which enables the implementation of a wide variety of assays, with assorted detection methods, at varied concentration values and by resorting to distinct sample manipulation, without requiring system reconfiguration. In the scientific papers published over the last decade, the versatility was well demonstrated, wherein several methodologies were developed in varied analytical situations exploring the multipumping concept which was coupled to different types of detection methods, namely, spectrophotometric, fluorometric and chemiluminometric detectors. In this particular the analytical potential of MPFS is interesting for the implementation of analytical determination involving chemiluminescence detection. Indeed, the chemiluminescence is usually yielded through very fast chemical reaction and for that reason efficient mixture in close proximity to the detector is fundamental to obtain high sensitivity measurements of the light emitted from the short-lived excited state intermediates produced in the chemiluminescent reaction.

Moreover, MPFS could be an analytical tool with a great potential for the implementation of extraction or separation processes because of the nature of pulsed flow that produce a chaotic movement of the particles, thus promoting a higher interaction between non-miscible fluids (liquid-liquid extraction) or between solid and solvents (solid-liquid extraction), which can facilitate the transfer processes between different phases of the analyte to be determined. Nevertheless, taking into account that few research works have been carried out in this field, there is a lot of work to be done in order to assess the real potential of MPFS systems in conducting analytical procedures involving extraction or separation processes.

Considering the scope of the research work presented in this dissertation, the analytical potential of MPFS was assessed in the development and implementation of methodologies for the chemical control of drugs in pharmaceutical formulation and also for toxicological analysis of DFC drugs in beverages intentionally adulterated. In order to fulfill the objectives of the research work, the potentialities of MPFS methodology were explored for the implementation of several analytical procedures, such as, photochemical degradation, chemical derivatization and adsorption/desorption at solid-liquid interface, that worked as innovative analytical strategies and could be applied to different analytical situations involving different types of detectors such as visible spectrophotometry, fluorometry and chemiluminescence.

Thus, the research works presented in this dissertation (from Chapter 3 to Chapter 8) are discussed in order to highlight the types of analytical strategies implemented, in which the

most particular aspects of each developed work are fully described, namely concerning to the development of flow manifold, the study of chemical and physical parameters, the analytical features of the developed method, the application to real samples and the comparison of the results obtained by the proposed methodology with those furnished by reference procedure. The developed works described from Chapter 3 to Chapter 8 are presented under the format of papers published or submitted in international peer reviewed scientific journals.

The first developed work (Chapter 3) involves the spectrophotometric determination of indapamide based on the oxidative coupling reaction between the drug and 3-methylbenzothiazolin-2-one hydrazine (MBTH) in the presence of cerium (IV). The automation of this reaction scheme in MPFS is emphasized, namely, the high versatility of the proposed flow system in terms of solutions manipulation in order to enable the exploitation of different addition sequence of reagents and also to carry out different strategies for sample insertion (binary sampling, merging zones and single sample volumes). This system was applied to the chemical control of indapamide in pharmaceutical formulation.

In Chapters 4 and 5 are discussed the photochemical reactions and its advantages as a valuable analytical strategy. This kind of reaction of reaction is implemented in a MPFS system for the fluorometric determination of diazepam in spiked drinks and pharmaceutical formulations (chapter 4 and 5, respectively). In these works is well demonstrated that the utilization of a photodegradation unit into an automatic flow system enable to obtain a higher efficiency of the photochemical reactions, improving the sensitivity of the methodology.

In Chapter 6 is referred the development of an automatic flow system with a very simplified configuration based on the multipumping concept for the fluorometric monitoring of glibenclamide in acidic medium in the presence of anionic surfactant (SDS), that promote an organized micellar medium to enhance the fluorometric measurements. In this work, the high portability and the easy control of MPFS are emphasized and it is also demonstrated the potential of the proposed methodology as a valuable analytical tool for the determination of glibenclamide in pharmaceutical formulation and in alcoholic beverages intentionally adulterated.

The performance of a separation process into a MPFS is discussed in Chapter 7, wherein a pre-separation unit based on a mini column packed with activated charcoal was incorporated into a miniaturized flow system to carry out the inline separation of glibenclamide from teas samples. The nature of the pulsed flow is assessed in the separation process, particularly, in the desorption step of the drug from activated charcoal.

This methodology was proposed for the toxicological control of glibenclamide in spiked teas.

Finally, in Chapter 8 the capacity of CdTe quantum dots to generate reactive oxygen species (ROS) when irradiated with visible light making possible the chemioluminometric chemical control of pharmaceutical formulations containing epinephrine is demonstrated. All reactional processes were implemented inline by using an automated MPFS in which a laboratory-made photocatalytic unit based on two high power visible light emitting diode (LED) lamps was incorporated. The main aim of the proposed methodology was to evaluate the use of a visible LED light to photoactivate QD and also to take advantage of the formed ROS to determine epinephrine that present antioxidant properties. Additionally, the hydrodynamic characteristics of MPFS are put in evidence since it allows improving the efficiency and sensitivity of the chemiluminescence measurements.

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CHAPTER 2

Experimental

2.1. Introduction

In this chapter, the experimental aspects concerning the several works performed and presented throughout this thesis are further discussed. These aspects include general procedures of the preparation of solutions and also the description of the devices used to assemble the flow systems highlighting their features, operation mode and control. Additionally, some aspects related to the optimization procedures of the proposed methodologies and the statistical treatment used to assess the quality of the obtained results are also described.

2.2. Reagents and solutions

The preparation of all solutions was carried out with water purified from a Milli-Q system (Millipore RG), equipped with mixed bed resins (QPack2 – CPMQ004D2) and a particle filter unit of 0.45 μm Millipack 40 Gamma gold. This purification system allows attaining water with resistivity superior to 18 MΩ.cm. All chemicals used were of analytical reagent grade without any treatment process or further purification.

Standard stock solutions were obtained by rigorous weighing of the respective solid reagent in a Mettler Toledo analytical balance (model AG 285), followed by dissolution in the appropriate solvent, into volumetric flasks (class A) of different capacities. The working standard solutions were obtained by rigorous dilution of standard stock solutions using glass pipettes or automatic LabMate HTL micropipettes of different capacities, the last ones with plastic disposable tips. The micropipettes models LM100, LM1000 and LM5000 with maximum capacities of 100, 1000 and 5000 μL, respectively, were regularly calibrated with deionized water. The preparations of standard and sample solutions in the several performed works are described in detail in the corresponding chapters.

2.3. Apparatus and instrumentation

2.3.1. Flow manifold based on the multipumping concept

In the following sections the fundamental components of the flow systems, based on the mutipumping concept, developed in the different analytical determinations presented in this thesis will be discussed in detail describing the devices and components used in the assemble of the flow systems.

2.3.1.1. Propelling and insertion devices

For the propelling and insertion of sample and reagent solutions, solenoid micro-pumps were used. The solenoid micro-pumps were purchased from Bio-Chem Valve Inc. (Boonton, EUA) of different stroke volumes namely 8, 10, 25 and 50 μL (models 090SP12–8, 120SP1210–4TE, 120SP12–25, 120SP12–50, respectively) which had a cylindrical configuration and reduced dimensions with a height between 5.0 – 6.4 cm and diameter between 1.8 – 2.5 cm (Figure 1).

The functioning of the micro-pump is based on the displacement of a flexible diaphragm operated by a solenoid. The diaphragm is maintained closed by means of an inner spring mechanism. When the solenoid is energized, the diaphragm is opened permitting the entry of fluid into the pump chamber through the inlet check valve, while simultaneously closes the outlet check valve. When the solenoid is de-energized the spring forces the diaphragm back to the closed status dispensing a determined volume of fluid through outlet check valve and simultaneously closing inlet check valve [1].

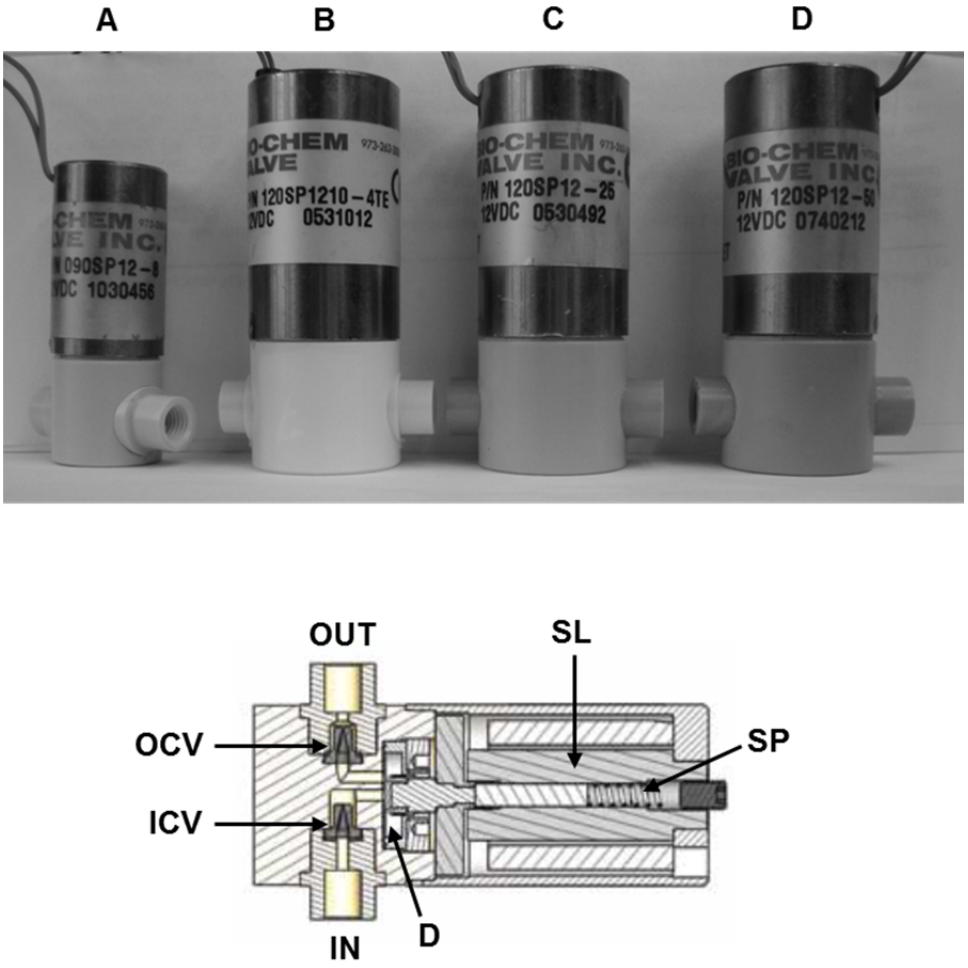

Figure 1 – Solenoid micro-pumps and schematic representation of its cross section (adapted from [1]). Micro pumps with a stroke volume of: A, 8 µL (090SP12 – 8); B, 10 µL (120SP1210 – 4TE); C, 25 µL (120SP12 – 25); D, 50 µL (120SP12 – 50). ICV, inlet check valve; OCV, outlet check valve; D, diaphragm; SL, solenoid; SP, spring.

For an ideal functioning, the micro-pumps require the application of a voltage of 12 V, over a period of at least 150 ms during which the solution is aspirated into the inner chamber of the micro-pump. This period of activation of the micro-pump is followed by a period of deactivation that is defined by the operator. The sum of activation and deactivation periods defines the pulse time. Thus, for each micro-pump, the pulse time in combination with the stroke volume of the micro-pump defines the flow rate, while the number of pulses defines the volume inserted into the flow system.

2.3.1.2. Commutation devices

The developed multipumping flow systems were basically constituted by solenoid micro-pumps as the unique active devices of the flow manifold, however in the assemble of the flow system described in Chapter 7, solenoid valves were used as commutation devices in order to direct the flow through different analytical paths. The 3-way solenoid valves, model 161 T031, were purchased from NResearch Inc. (West Caldwell, USA).

These devices had a cylindrical configuration and reduced dimensions with a height of 2.9 cm and a diameter of 1.9 cm (Figure 2). The functioning of solenoid valves was conditioned by the direction of pressure applied on two polytetrafluoroethylene (PTFE) membranes, which defines the path of solutions. Thus, one of the paths is established through the activation of solenoid thus applying a pressure on one of the membranes. When the solenoid was switched off, the coil spring displacement enabled the return of the membrane to its initial position establishing the alternative path. The solenoid valve acts as a switch, with two possible states, allowing at different moments, the selection between two alternate paths.

The solenoid valves are of low power consumption (2.4 Watts/12 Volts) and they have a reduced dead volume, reduced internal volume, short response time (5 to 20 ms for activation) and high chemical resistance.

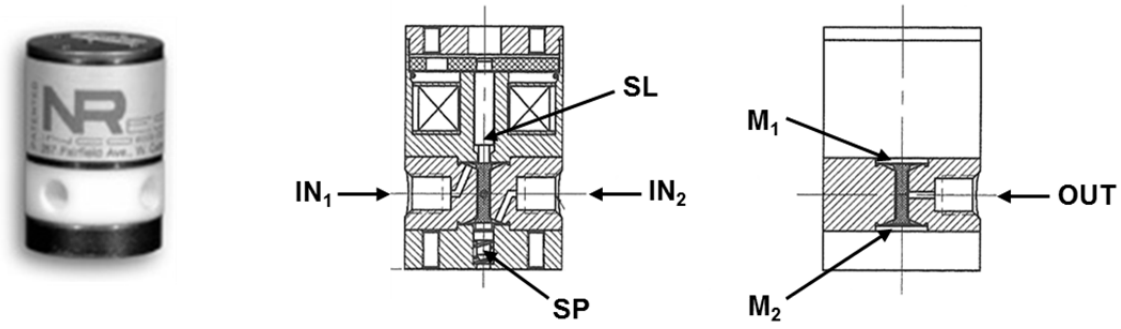

Figure 2 - Solenoid valve and schematic representation of its cross section. IN_1 and IN_2, inlet channels; OUT, outlet channel; M_1 and M_2, PTFE membranes; SL, solenoid; SP, spring. Adapted from [2].

2.3.1.3. Tubing and other manifold components

All the tubing connecting the different components of the multipumping flow systems was made of polytetrafluoroethylene (Omnifit), with 0.8 mm of internal diameter. End-fittings and connectors were also used. The reactors with different geometries were also made with PTFE tubes of 0.8 mm i.d., which were used in order to enhance radial transport and to reduce laminar transport throughout the analytical path [3]. Laboratory made acrylic (Perspex®) connectors were used as confluences in order to connect the tubes to one another and to other parts of the system. Confluences with different configurations [4] namely in triple (Y-shaped) or in quadruple (usually in the shape of an arrowhead) were used.

2.3.1.4. Photoirradiation units

In the work described in Chapter 4 and 5, an ultraviolet irradiation unit was used for the photodegradation of diazepam which consisted on a 2 m reactor coiled around of a 15 W Philips (model TUV 15W/G15T8) low pressure mercury lamp (Figure 3). These lamps are low-pressure mercury-vapour discharge lamps consisting of a tubular glass envelope, emitting a short-wave ultraviolet radiation with a radiation peak at 253.7 nm (UV-C).

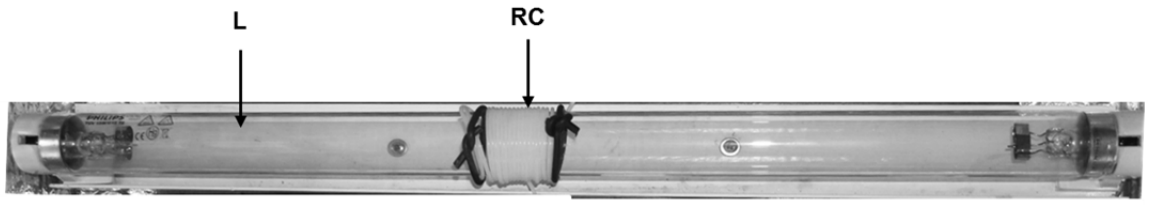

Figure 3 – Photograph of UV irradiation unit. RC, 2 m PTFE reactor; L, 15W Philips (model TUV 15W/G15T8) low pressure mercury lamp.

In the analytical methodology developed for the determination of epinephrine (Chapter 8), a photo-excitation unit was used for the photoactivation of CdTe quantum dots in aqueous solutions. This photo-excitation unit (Figure 4) consisted in a reaction coil made of PTFE

tubing (0.8 mm i.d.) placed between two high-power LED lamps (Parathom® R50 40 daylight).

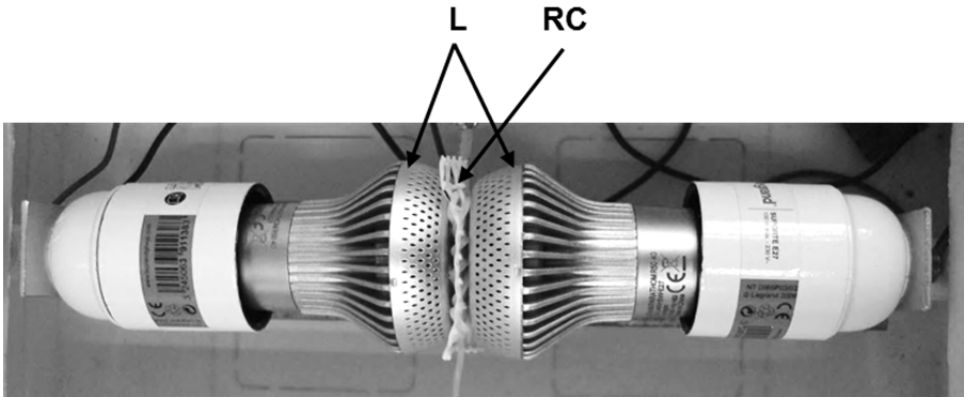

Figure 4 – Photograph of photo-excitation unit. RC, 50 cm reaction coil; L, high-power LED lamps.

The high-power LED lamps had low energy consumption, extremely long life (25000 h), efficient generation of white light and they had no UV and near-IR radiation in the light beam, as it can be seen in Figure 5.

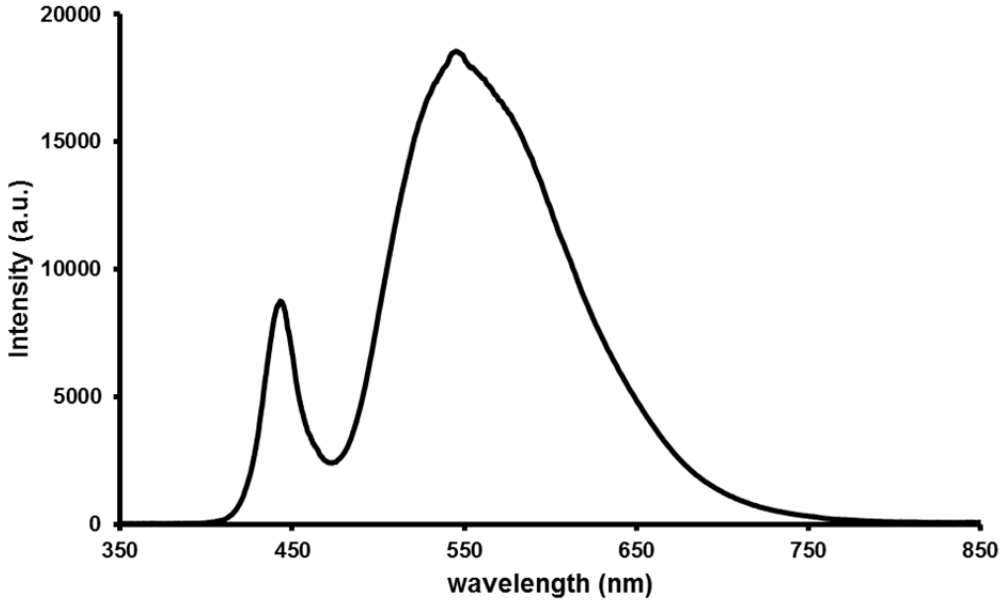

Figure 5 – Absorption spectrum of the visible light emitted from the high-power LED lamps.

2.3.1.5. Inline pre-separation unit

In the flow manifold described in Chapter 7, it was necessary to resort to an inline pre-separation unit to separate the drug glibenclamide from the sample matrix (teas). This pre-separation unit was based on a laboratory made acrylic (Perspex®) column filled with activated charcoal (Darco®, 20-40 mesh, in granular form, obtained from Sigma Aldrich®) in which two filters with 90 µm pore size (Mobicol "classic" M 1002) were placed at both ends of the column to entrap the activated charcoal. During the optimization of the flow system, 5 columns (A – E) with different lengths (L) and internal diameters (i.d.) were evaluated (Figure 6).

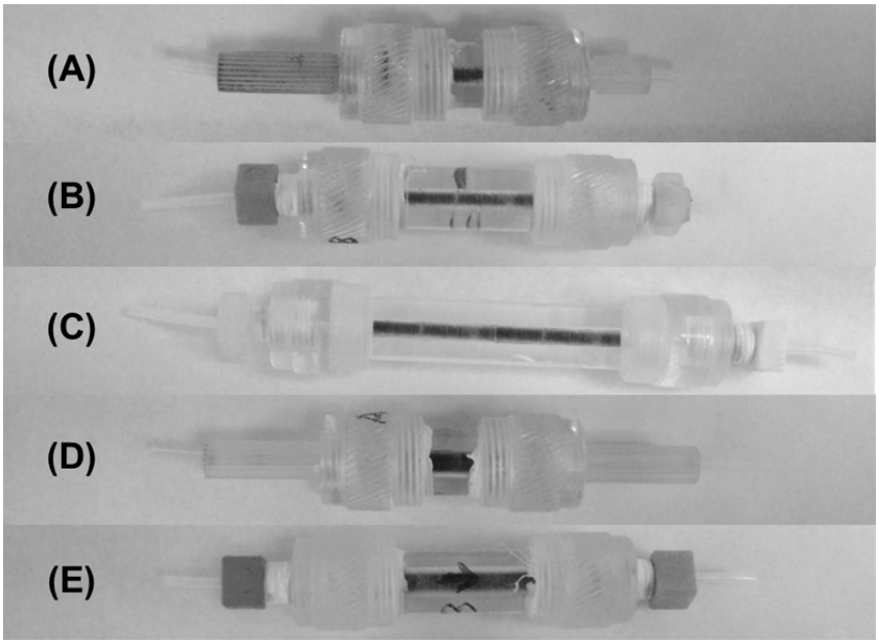

Figure 6 – Photograph of pre-separation unit. Acrylic (Perspex®) columns: A, L – 15 mm and 2 mm i.d.; B, L – 30 mm and 2 mm i.d.; C, L – 50 mm and 2 mm i.d.; D, L – 15 mm and 3 mm i.d.; E, L – 30 mm and 3 mm i.d..

2.3.1.6. Detection systems

For the spectrophotometric determination described in Chapter 3, an Ocean Optics S2000 spectrophotometer and a World Precision Instruments F-O-LITEH halogen lamp light source were used. The light source was connected to the CUV-UV Cuvette Holder (Ocean Optics) equipped with a Hellma flow-cell (30 µL inner volume, 10 mm optical path) via a single optical fiber with a 600 µm core diameter (Ocean Optics, Model: QP600-2-UV-BX), while the S2000 spectrophotometer was connected to the CUV-UV Cuvette Holder through a single optical fiber with a 200 µm core diameter (Ocean Optics, Model: QP200-2-UV-BX).

The flow manifolds described in Chapters 4, 5, 6 and 7 included a FP-2020 spectrofluorimeter (Jasco, Easton, MD, USA), equipped with a 16 µL internal volume flow cell. The FP-2020 spectrofluorimeter covers a wide wavelength range of both excitation and emission from 220 to 700 nm with proven stability. Versatile time-programming capabilities are provided for wavelength, response, sensitivity range and gain [5].

The flow manifold described in Chapter 8 used a FP-2020 Plus detector (Jasco, Easton, MD, USA) with a specially designed chemiluminescence module commercially available as option. This chemiluminescence attachment is based on a modular flow cell consisting of a spiralled PTFE tube with a 0.8 mm i.d. and an internal volume of 100 µL that was positioned in front of a highly sensitive photomultiplier.

2.3.1.7. Computer control and data acquisition

The developed analytical flow systems were fully controlled by means of a microcomputer, through lab-made software, allowing the selection and adjustment, in a simplified way, of all analytical parameters that conditioned the performance of the flow systems.

The automatic control of the active devices used in the different flow systems (solenoid micro-pumps and solenoid valves) was accomplished by an Intel Pentium® based microcomputer. For the actuation of micro-pumps a power drive was used, which is a crucial component of a MPFS because it provides the required electric current for the activation of solenoids. In the flow systems described in Chapters 3, 4, 5 and 6, a lab-made electronic interface using a CoolDrive™ power drive board (NResearch Inc., NJ, USA) was used to activate the micro-pumps. However, CoolDrive™ only enables the

control of 5 devices. Thus, for the flow systems described in Chapters 7 and 8, a home-made circuit based on a ULN2003 chip [6] was used as power drive in order to activate the solenoid of micro-pumps and valves. The connection between the microcomputer and the power drive was established through the LPT1 motherboard port.

Since the solenoid micro-pumps are the only active devices of multipumping flow systems, a simple software program was sufficed for setting up flow rate, sample and reagent volumes, sampling strategy, etc. The software program used for the control of the several parameters in the flow systems described in Chapter 3, 4, 5 and 6 was lab-made software written in Microsoft QuickBasic 4.5®. In the flow systems described in Chapter 7 and 8, lab-made software based on programming language Microsoft VisualBasic 6.0® was used as control program.

In the analytical determination described in Chapter 3, in which an ADC1000-USB External USB A/D Converter was used to interface S2000 spectrophotometer to the computer, the data acquisition was performed using the OOIBase32™ software version 2.0.6.5 of Ocean Optics.

The analytical signals obtained in the different determinations described in Chapters 4, 5, 6, 7 and 8 were registered by means of a model L250E Linseis chart recorder which was coupled to the detection system.

2.3.2. Additional Instrumentation

According to the required precision, all reagents were weighed in a Mettler Toledo AG 285 analytical balance (precision of 1×10^{-5} g) or in a Kern 440-35N balance (precision of 1×10^{-2} g).

The solutions were agitated by a Falc - F60 electromagnetical stirrer with option for heating. For degas the solutions, a VWR USC 100T5 ultrasonic bath was used.

When necessary, pH of solutions was measured using a combined glass pH electrode (Crison 52-02) and a Crison model GLP 22 milivoltimeter. The calibration of the combined electrode was performed with commercial standards of: pH = 4.00 (Riedel-de Haën, 33543), pH = 7.00 (Riedel-de Haën, 33546) and pH = 9.00 (Merck, 9889).

The preliminary batch assays to evaluate the reaction involved in the spectrophotometric determination of indapamide, described in Chapter 3, was performed in a Perkin Elmer Lambda 45 UV/VIS spectrophotometer by using quartz cells with 1 cm optical path (Hellma, ref. 6030-UV). The preliminary batch assays described in Chapters 4, 5, 6 and 7

were carried out in a Perkin Elmer LS 50 B spectrofluorimeter by using quartz cells with 1 cm optical path (Hellma, ref.104-QS).

The determinations of indapamide, glibenclamide and epinephrine in pharmaceutical formulations were also performed following the reference procedures described in British Pharmacopoeia [7], by using a Jasco LC-2000 Plus chromatograph system equipped with MD-2015 Diode Array detector and a PU-2080 analytical isocratic pump. LC-Net II/ADC was used as the hardware interface between the microcomputer and the HPLC system. ChromNAV data software was used to control all system and for data acquisition including all standard chromatography calculations.

In the determination of diazepam in beverages, described in Chapter 4, the comparison procedure that was adapted with modifications from the scientific literature, also involved the execution of a chromatographic method resorting to the HPLC system above mentioned.

The reference procedure recommended by British Pharmacopoeia [7] for the chemical control of pharmaceutical formulations containing diazepam (Chapter 5) was performed resorting to the Perkin Elmer Lambda 45 UV/VIS spectrophotometer above mentioned.

2.4. Development and optimization of the flow systems

In the development and optimization of flow systems, the analytical parameters such as sensitivity, determination rate, precision, accuracy and solutions consumption were taken into account. Thus, during the studies concerning the physical and chemical parameters in Chapters 4, 5, 6 and 8, a univariate method was used to the selection of the optimal values. The univariate method consisted on varying each parameter under study within a certain interval, while keeping the other parameters fixed. On the other hand, for the optimization studies performed in the works described in Chapters 3 and 7, a multivariate experimental design was used to optimize the parameters of the multipumping flow systems that influenced the analytical results. The multivariate experimental design is discussed in more detail in the corresponding Chapters, since each design was adapted for the analytical purpose.

The analytical determinations of the different samples were performed through the establishment of calibration curves. For each analytical determination the working concentration range was determined by inserting a series of standard solutions with different concentrations and determining the range where the analytical signal was linearly

related to the concentration. The drug concentrations of the different samples were obtained by interpolation in the established calibration curves.

Taking into account the recommendation of IUPAC [8], the limit of detection was defined as the concentration (C_{LD}) derived from the smallest measure (Y_{LD}) that can be detected with reasonable certainty for a given analytical procedure. Therefore, the value Y_{LD} was obtained from the equation: $Y_{LD} = b + 3S_{y/x}$, where b is the intercept value of the calibration curve and $S_{y/x}$ is the deviation (or uncertainty) of y values which was calculated by: $\sqrt{\frac{\sum(y-yi)^2}{(n-2)}}$. The limit of detection was calculated resorting to the equation: $C_{LD} = \frac{(Y_{LD}-b)}{a}$, in which a is the slope of the calibration curve [9].

The assessment of precision was performed through the calculation of the confidence interval for the determined concentrations of samples, by resorting to the equation: $t \times \frac{s}{\sqrt{n}}$, where s is the standard deviation of a series of repeated measures, t is obtained from Student's t- table taking into account the confidence level intended and the number of degrees of freedom (n - 1) and n is the number of determinations. The random uncertainty in the value for the measure, X, or the corresponding uncertainty in the estimate of concentration is represented by precision [8], being the final value of concentration, for a defined confidence level of 95%, presented as followed: $\bar{X} \pm t_{0.05} \times \frac{s}{\sqrt{n}}$, where $\bar{X}$ is the average value of the measurements.

The evaluation of accuracy, which relates the agreement between the measured concentration and the "true value", was performed through the calculation of the relative deviation (RD %) by the equation: $\frac{(C_{MPFS} - C_{ref})}{C_{ref}} \times 100$, where C_{MPFS} is the result obtained by the proposed MPFS methodology and C_{ref} is the result obtained by the reference or comparison methodology. In the situations where no reference methods exist in the scientific literature, and hence it was impossible to compare the obtained results by the proposed method with a reference or comparison procedure (Chapters 6 and 7), recovery assays were performed in order to assess the accuracy.

The determination rate expressed as the number of determinations per hour was estimated through the measuring, in millimeters, of the length of 5 peaks recorded in the paper equivalent to 5 repeated determinations, and subsequent conversion in the time required for its accomplishment from the paper speed (mm min^{-1}) of the chart recorder. Alternatively, the determination rate was calculated taking into account the time required for the accomplishment of all steps of the analytical cycle. This calculation was easy to perform since the flow systems were controlled by software and the flow parameters defined on a time and pulse number base.

2.5. Statistic assessment of results

In the analytical determinations described in Chapters 3, 4, 5, 6 and 7, the analytical signal, corresponding to the drug concentration under evaluation, was calculated as a function of the average of a set of measures obtained by consecutive insertions of the same sample. The final concentration was calculated by interpolation of the obtained analytical signal intensity in the calibration curve established for each methodology.

In Chapter 8, the reactive oxygen species (ROS) scavenging capacity of epinephrine was evaluated through the decrease in chemiluminescence (CL) emission signal. The percentage of CL inhibition ($(\%)\,\Delta CL$) was assessed using the formula $(\%)\,\Delta CL = \frac{(S_{blank} - S_{sample})}{S_{blank}} \times 100$, where S_{blank} and S_{sample} represent the analytical signal obtained in the absence and in the presence of scavenger compounds, respectively. In this case, a linear calibration curve was established by the percentage of CL inhibition as a function of the logarithm of epinephrine concentration.

In the scientific works where the results obtained from the developed methodologies and reference procedures were compared, a paired t-test was also applied for the assessment of accuracy. The t value was calculated from the formula $t = \frac{\bar{X}}{S}\sqrt{n}$, where $\bar{X}$ and S are the mean and standard deviation respectively of X, the difference between paired values. The number of degrees of freedom of t is $n - 1$. The t value calculated when compared to the reference t value (P = 0.05), for a significance level of 95 %, allowed to confirm the agreement between the two methods when the null hypothesis is verified [9].

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CHAPTER 3

Exploiting the oxidative coupling reaction of MBTH for indapamide determination

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Exploiting the oxidative coupling reaction of MBTH for indapamide determination

David S.M. Ribeiro, João A.V. Prior*, João L.M. Santos, João A. Lopes, José L.F.C. Lima

REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha no. 164, 4050-047 Porto, Portugal

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ABSTRACT

The oxidative coupling reaction between aromatic amines and 3-methylbenzothiazolin-2-one hydrazone (MBTH) in the presence of cerium(IV) has been extensively used with quantitative analytical purposes. Nevertheless, a literature survey reveals that different wavelengths (visible range) can be used to monitor the reaction products when using MBTH and Ce(IV) as colour developing reagents.

In the present work, the oxidative coupling reaction of indapamide (an oral antihypertensive diuretic drug) with MBTH in the presence of cerium(IV) was evaluated using distinct reaction approaches and was implemented in an automated multipumping flow system. The developed methodology was applied in the spectrophotometric control of the drug in pharmaceutical formulations. The optimization of the proposed multipumping flow system was performed by using an experimental design approach, namely by exploiting a Plackett–Burman factorial design and a central cubic faces design.

This work revealed the formation of products with different reaction kinetics, chemical stabilities and absorbance spectra, depending on the sequence of addition of the reagents. By exploiting a specific sequence in the addition of reagents, the proposed automatic system allowed the rapid quantification of indapamide in pharmaceutical formulations, with a determination rate of about 25 h^{-1}, and a relative deviation under 2.1% when comparing with the reference procedure. Detection limit was approximately 1 mg L^{-1}.

1. Introduction

3-Methylbenzothiazolin-2-one hydrazone (MBTH) was first introduced as a reagent in analytical chemistry for the detection and determination of aldehydes, indoles, aromatic amines, imino-hetero-aromatic compounds, arylalkylamines, carbazoles, phenols, etc. [1,2]. In the presence of oxidizing agents it forms strongly coloured products with several pharmaceutical compounds [3–11].

A literature survey focusing on the oxidative coupling reaction of MBTH and Ce(IV) [3–11] with several compounds reveals that without justification, very different wavelengths were used to monitor the formed products, probably indicating the formation of completely different reaction products.

This work was aimed at attaining an improved knowledge of the two-stage reaction involving MBTH and Ce(IV) through the spectrophotometric determination of indapamide. As no concise information is available in literature, extensive studies through mathematical modelling were carried out focusing the evaluation of the influence of several parameters on the formed reaction products. The analysis of the obtained mathematical models revealed that reaction kinetics, products stability and spectra were markedly affected by the experimental conditions.

With an analytical purpose, the reaction was subsequently implemented in a multipumping flow system. Automation of procedures for pharmaceutical analysis has been extensively exploited since it allows both a reduction in the consumption of reagents and generated wastes and a noteworthy increase in the sampling rate. In this context, flow-based techniques have proven to be valuable tools for the development of fast, simple, versatile and reliable methods that can be readily adapted for routine analysis at relatively low cost.

Multipumping pulsed flow system [12] is a low cost and simple approach to automate analytical determinations, and an advantageous alternative to the conventional flow injection analysis (FIA) [13], sequential injection analysis (SIA) [14], and multicommuted (MCFS) [15] systems relying on to the typical laminar flow regimen. MPFS rely exclusively on the utilization of multiple very small size solenoid actuated micropumps controlled by computer [16] that allow a very simplified configuration of the flow system as well as its automated control. By taking advantage of all operational characteristics provided by these flow systems, such as a high degree of mixture, high versatility in terms of both the sequence of solutions

* Corresponding author. Tel.: +351 222 078 994; fax: +351 222 078 961.
E-mail addresses: javprior@gmail.com, joaoavp@ff.up.pt (J.A.V. Prior).

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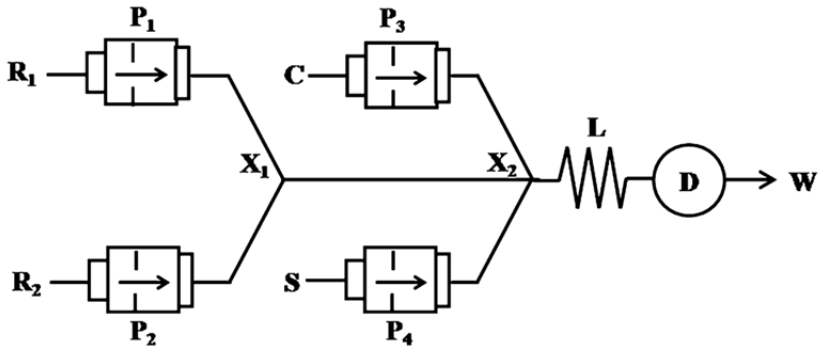

Fig. 1. Multipumping flow system. P_1–P_4: solenoid micropumps 8 μL stroke volume; X_1 and X_2, confluence points; *L*, reactor coil; *D*, detector (λ = 601 nm); *C*, carrier solution (0.1 mol L^{-1} H_2SO_4); R_1, MBTH (1.2×10^{-2} mol L^{-1} in 0.1 mol L^{-1} H_2SO_4); R_2, cerium(IV) (1.1×10^{-2} mol L^{-1} in 0.1 mol L^{-1} H_2SO_4); *S*, sample (in 5% ethanol and 0.1 mol L^{-1} H_2SO_4).

insertion and of the variability of inserted volumes, the oxidative coupling reaction was further exploited to the chemical control of indapamide formulations.

Indapamide, or 4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoyl-benzamide, is an antihypertensive and diuretic drug which belongs to the indolines class [17]. Several procedures for indapamide determination in pharmaceutical and biological samples have been employed. These procedures include high performance liquid chromatography [18–20], LC–MS [21,22], gas chromatography [23], chemiluminescence [24], capillary electrophoresis with amperometric [25] and UV [26] detection, colorimetry [27,28] and UV spectrophotometry [29]. Some flow methodologies have also been used in order to quantify indapamide in pharmaceutical formulations, such as, flow injection analysis with chemiluminescence [30] and spectrophotometric [31] detection.

Thus, this work aims at contributing to an improved knowledge of the oxidative coupling reaction of MBTH and Ce(IV), by establishing mathematical models focusing the relationships between several analytical parameters (chemical and physical) and the formation of different reaction products, despite using the same chemical reagents. Furthermore, a simple, low cost, and rapid flow analysis system, exploiting the multipumping concept for the spectrophotometric determination of indapamide in pharmaceutical formulations, was implemented.

2. Experimental

2.1. Apparatus

The flow system comprised four micropumps (Bio-Chem Valve Inc. Boonton, NJ, USA, ref. 090SP) of fixed displacement diaphragm type, being solenoid operated and dispensing 8 μL per stroke.

The manifold tubing was made from 0.8 mm i.d. PTFE, and the end-fittings, connectors and confluences were also made of the same material.

Automatic control of the analytical system was accomplished by means of a Intel Pentium based microcomputer and the software was developed using Microsoft Quick-Basic 4.5. A lab-made electronic interface using a CoolDrive™ power drive board (NResearch Inc., NJ, USA) was used to activate the micropumps through the LPT1 computer port.

The miniaturized optical detector involved a S2000 spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) and a World Precision Instruments F-O-LITEH halogen lamp light source. The light source was connected to the CUV-UV Cuvette Holder (Ocean Optics) equipped with a Hellma flow-cell (30 μL inner volume, 10 mm optical path) via a single optical fiber with a 600 μm core diameter (Ocean Optics, Model: QP600-2-UV-BX), while the S2000 spectrophotometer was connected to the CUV-UV Cuvette Holder through a single optical fiber with a 200 μm core diameter (Ocean Optics, Model: QP200-2-UV-BX). An ADC1000-USB External USB A/D Converter was used to interface S2000 spectrophotometer to an Intel Pentium III computer.

Data acquisition was performed using the OOIBase32™ software version 2.0.6.5 of Ocean Optics.

2.2. Samples, standards and reagents

All solutions were prepared with doubly deionized water (conductivity <0.1 μS cm^{-1}) and analytical grade chemicals were used.

A 400 mg L^{-1} indapamide stock solution was daily prepared by dissolving 40 mg of indapamide (Sigma) in 40 mL of ethanol (Panreac, 99.5%) and diluted to 100 mL with deionized water. Then a 50 mL aliquot of indapamide stock solution was transferred to a 200 mL volumetric flask and diluted to volume with deionized water. Thus, a 100 mg L^{-1} intermediate indapamide solution in 10% ethanol was obtained.

The working indapamide standards (5–50 mg L^{-1}) were prepared by appropriate dilution of the intermediate indapamide solution, using the following experimental procedure. Aliquots of intermediate indapamide solution were transferred into a series of 50 mL volumetric flaks. After that, appropriately aliquots of ethanol (Panreac, 99.5%) were added, with the aim to obtain the same concentration of ethanol (5%) in all the working indapamide standards. Finally, 2.5 mL of 2 mol L^{-1} H_2SO_4 solution was added and the volume was made up to the mark with deionized water.

A 1.2×10^{-2} mol L^{-1} MBTH solution was prepared by dissolving 258.84 mg of 3-methylbenzothiazolin-2-one hydrazone hydrochloride hydrate (Sigma–Aldrich®) in 100 mL of a 0.1 mol L^{-1} sulphuric acid solution.

A 1.1×10^{-2} mol L^{-1} Ce(IV) solution was prepared by dissolving 444.74 mg of cerium(IV) sulphate tetrahydrate (Riedel-de Haën®) in 100 mL of 0.1 mol L^{-1} H_2SO_4.

The sample solutions made with commercially available pharmaceutical formulations were prepared by weighing and powdering a representative number of tablets. Thereafter, an appropriate amount of sample was placed in a 50 mL flask and mechanically shaken with 2.5 mL ethanol (Panreac, 99.5%) and 2.5 mL of a 2 mol L^{-1} sulphuric acid for 25 min. The solution was filtered into a 50 mL volumetric flask. The contents of flask were diluted to 50 mL with deionized water. At last, the obtained solution was filtered with a syringe filter i.d. 0.20 μm (Corning®) before its insertion in the flow system.

2.3. Flow manifold

The analytical manifold (Fig. 1) comprised four solenoid micropumps (P_1 to P_4) used as solutions insertion and propelling devices.

Prior to sample insertion the simultaneous actuation of micropumps P_1 and P_2 allowed the combined insertion of MBTH and cerium(IV) solutions through X_1, by exploiting the merging zones approach. The number of solutions pulses was enough to guarantee that the resulting mixed solution filled the flow tubing placed between the confluence points X_1 and X_2 (about 20 cm). Next, the micropump P_4 was actuated and the sample solution was inserted in the flow system reaching the confluence point X_2. Finally, by actuating the micropump P_3, sulphuric acid solution was inserted into the system through X_2 allowing the rejection to waste of the exceeding volumes of the reagents solutions and establishment of the baseline.

The analytical cycle started by intercalating a unique volume of sample solution between two identical plugs of the MBTH/Ce(IV) solution. The sampling stage consisted firstly in the insertion of the MBTH/Ce(IV) solution for a pre-set number of pulses, through the simultaneous actuation of micropumps P_1 and P_2 (total volume of 16 μL per stroke), at a fixed pulse time of 1.2 s, corresponding to a pulse frequency of 50 min^{-1}, which defined the flow rate at 0.8 mL min^{-1}. Then, by inserting a pre-set number of pulses of micropump P_4, at a fixed pulse time of 0.6 s, a unique volume of sample solution was intercalated with another identical small plug of the MBTH/Ce(IV) solution. Thereafter, the reaction zone, fulfilling the second stage of the oxidative coupling reaction, was carried towards the detector through the repeated actuation of P_3 (8 μL per stroke), at a fixed pulse time of 0.6 s, corresponding to a pulse frequency of 100 min^{-1}, which defined the flow rate at 0.8 mL min^{-1}. The formed product was monitored at 601 nm.

2.4. Reference procedure

For validation of the results furnished by the developed methodology, the pharmaceutical formulations containing indapamide (tablets) were also analysed by reversed-phase liquid chromatography, according with the reference methodology [32]. The content of indapamide in the tablets was calculated from the chromatogram obtained and using the declared content of indapamide in standard solution. Each mg of indapamide in standard solution is equivalent to 1.0246 mg of the drug in tablets.

2.5. Experimental design

Different experimental strategies were adopted to optimize the values of physical and chemical parameters. For screening purposes full factorial and Plackett–Burman designs were adopted [33]. For optimization (or fine tuning) central cubic faces designs were selected [34]. The outcome of designed experiments was modelled with multivariate linear regression [35]. The structure of linear models depends on the selected experimental design. Each model structure was optimized by means of analysis of variance (ANOVA), namely by ensuring statistical significance of regression coefficients, regression and evaluating the lack-of-fit (ensured using replicates). Feedforward artificial neural networks were also used to model experiments when linear models were inadequate [36]. Three-layered networks with a non-linear hidden layer (hyperbolic tangent) and a linear output layer were selected. The training algorithm was pseudo-second-order Levenberg–Marquardt. Overfitting was avoided by an appropriate training-validation strategy (the data is split in calibration (70%) and validation (30%) subsets during network training). Models simulation was performed using data within the corresponding training range. Contour plots were produced whenever appropriate.

The experimental design tables and linear models estimation was made using Modde version 6.0 (Umetrics, Umea, Sweden). Artificial neural networks and contour plots were made using Matlab version 6.2 (Mathworks, Natick, USA).

3. Results and discussion

3.1. Influence of reagents addition sequence

Some preliminary batch assays to evaluate the reaction involved in the spectrophotometric determination of indapamide revealed that the sequence of reagents addition is very important for the reaction development. This fact become evident when was conducted an experiment in which was monitored the absorbance of formed products by two different reagent addition approaches: MBTH mixed first with Ce(IV) and then with indapamide (1st addition sequence); or, MBTH mixed with indapamide and subsequently with Ce(IV) (2nd addition sequence). For a final volume of 15 mL the reagent volumes used were: 5 mL of a 8×10^{-3} mol L^{-1} MBTH solution, 5 mL of a 1.00×10^{-2} mol L^{-1} Ce(IV) solution and 5 mL of a 25 mg L^{-1} of indapamide. The obtained results (Fig. 2) showed that if MBTH and Ce(IV) solutions were mixed prior to the addition of indapamide a blue-coloured compound was formed with maximum absorbance wavelength at 601 nm; however, when MBTH was initially mixed with indapamide and then with the oxidizing agent, a red-coloured compound was produced with maximum absorbance in the visible range at 510 nm.

In the 1st addition sequence, the results comply with others mentioned in the literature affirming that the mechanism of reaction occurs in two stages [28]. Firstly, MBTH loses two electrons and one proton due to oxidation with Ce(IV), forming an electrophilic intermediate, which is the active coupling species. In the next stage, the electrophilic intermediate and the analyte undergoes electrophilic reaction with the formation of a coloured product and the elimination of one molecule of water.

In the 2nd addition sequence, in which the products formed exhibit an absorbance maximum at 510 nm, the mechanism of reaction is most likely different, probably involving the formation of a coupled intermediate between MBTH and the analyte, that is spontaneously oxidised after the addition of Ce(IV), originating different coloured species with different formation kinetics than the ones formed in 1st addition sequence.

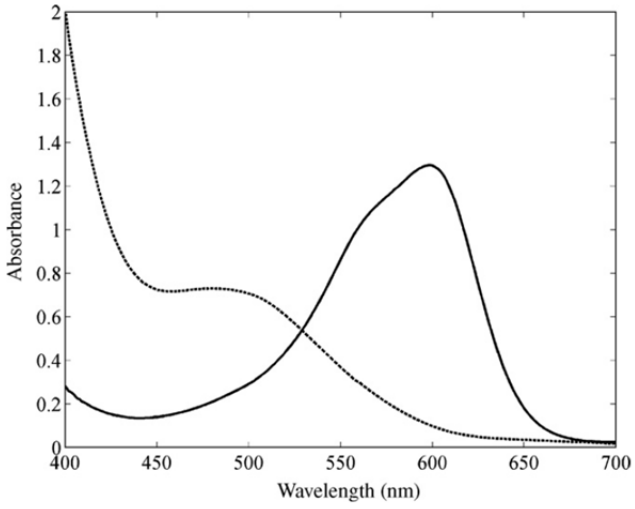

Fig. 2. Absorption spectra of indapamide (—, 1st addition sequence) and (––, 2nd addition sequence).

Table 1
Summary of regression models of chemical variables obtained for the screening and optimization stages.

Model	Design type	Design objective	Model equation (fitted using multivariate linear regression)	Analysis of variance		Q^2
				Regression	Lack-of-fit	
I	Full factorial	Screening	$A = (0.22 \pm 0.013) + (0.076 \pm 0.0071)\,[MBTH] + (0.11 \pm 0.015)\,[Ce] - (0.052 \pm 0.015)\,[H_2SO_4] + (0.046 \pm 0.0075)\,[MBTH]\,[Ce] + (0.041 \pm 0.0071)\,[MBTH]\,[H_2SO_4] - (0.080 \pm 0.015)\,[H_2SO_4]\,[Ce]$	$p < 0.0001$	$p = 0.864$	0.983
II	Central cubic faces	Optimization	$A = (0.55 \pm 0.036) + (0.012 \pm 0.031)\,[MBTH] + (0.18 \pm 0.040)\,[Ce] + (0.029 \pm 0.026)\,[H_2SO_4] + (0.51 \pm 0.11)\,[MBTH]^2 - (0.43 \pm 0.083)\,[Ce]^2 - (0.17 \pm 0.057)\,[H_2SO_4]^2$	$p < 0.001$	$p = 0.096$	0.911

With the aim to study the stability of the formed products through 2nd addition sequence, a study involving a programmed spectrophotometric monitoring during 120 min was performed, following the same experimental conditions as above. An absorbance spectrum (400–700 nm) was recorded at 5, 15, 30, 60, 90 and 120 min after the mixture of solutions and the obtained results are depicted in Fig. 3. The results revealed that initially, the maximum absorbance occurred at 510 nm, and that it decreases as the reaction time increases, in opposition to absorbance at 601 nm that increases at least till 60 min. This way, the red-coloured compound monitored at 510 nm is not stable, giving place to a complex with maximum absorbance at 601 nm. Moreover, at 90 min it was observed the formation of a blue precipitate, impairing spectrophotometric monitoring.

Similar stability and kinetic studies were conducted for the complexes formed by the 1st addition sequence, revealing a negligible variation in absorbance at 601 nm as reaction time increased, indicating a good complex stability. Comparing these results with the ones previously discussed for the 2nd addition sequence, it can be concluded that the formation of the complex detected at 601 nm is faster when applying 1st addition sequence approach. Additionally, no significant absorbance at 510 nm was observed.

These results justify the reason by which Youssef [28] by applying the 2nd addition sequence monitored the same reaction for indapamide determination at 601 nm. This determination methodology had to involve long waiting periods for the reaction development, because for the 2nd addition sequence the complex formation is slower than for the 1st addition sequence.

The influence on analytical signal of the waiting time before addition of the third reagent, for 1st and 2nd addition sequences, was also assessed, because it could originate some differences on the stability and formation kinetics of complexes. The analysis of the results comprising waiting times of 1, 5 and 10 min, showed that there was no observed influence of the waiting time on analytical signal before addition of the third reagent (indapamide, for the 1st addition sequence, and Ce(IV) for the 2nd addition sequence), not changing the conclusions previously stated.

One can then conclude that for indapamide determination through reaction with MBTH and Ce(IV), the 1st addition sequence approach is preferable, since it allowed a faster formation of a more stable complex, than the one formed by performing the 2nd addition sequence.

Taking into account the previous results, it was developed a multipumping flow system to implement the determination of indapamide (Fig. 1), in which the MBTH reagent was mixed with Ce(IV) solution creating a first reaction zone that was then mixed with the sample solution creating a second reaction zone.

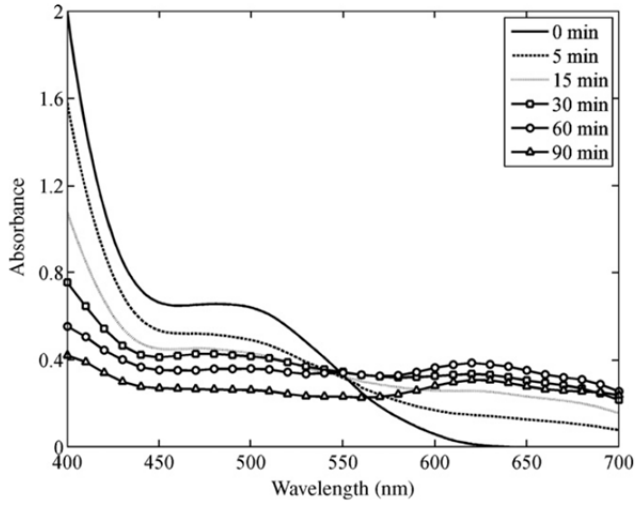

Fig. 3. Effect of development time on intensity of the coloured products of oxidative coupling reaction using the 2nd reagents addition sequence (MBTH and indapamide were mixed first and then with the Ce(IV) solution).

3.2. Optimization of the MPFS

Multivariate experimental design was used to optimize the parameters of the multipumping flow system that affected the analytical results. In order to evaluate the importance of all the influencing variables on the determination, a screening experimental design method was applied. The influence of the chemical variables on the analytical signal was first evaluated. It involved the analysis of the influence on absorbance of the concentrations of MBTH, cerium(IV) ammonium sulphate and sulphuric acid solutions. This study was performed using a full factorial design with 3 central points and 24 randomised runs, comprising the following ranges: 0.01–0.001, 0.1–1 and 0.01–0.001 mol L^{-1}, for MBTH, sulphuric acid and cerium(IV) ammonium sulphate, respectively. In this screening study it was verified that maximum absorbance values were obtained for lower concentrations of sulphuric acid and higher concentrations of MBTH and Ce(IV) solutions. The obtained model was described in Table 1 (model I). According to this model, a new range for each chemical parameter was defined for optimization purposes.

Next, a central cubic faces design was applied in order to fine tune the range of concentrations of the previously variables. This type of design is often adopted for optimization purposes since it is suitable for the estimation of cubic order models. In this optimization, the concentrations ranges assessed were 0.005–0.015 mol L^{-1} for MBTH and Ce(IV), and 0.08–0.1 mol L^{-1} for sulphuric acid solutions. The results revealed that the highest analytical signal was obtained for a combination of the following concentrations: 0.012, 0.011 and 0.1 mol L^{-1} for MBTH, Ce(IV) and sulphuric acid solutions, respectively. Results are compiled in Table 1 (model II).

The physical variables of the automatic flow system affecting the analytical signal were also studied using a Plackett–Burman factorial design with 12 randomised runs and 3 central points. The studied variables and their lower and upper levels were as followed: reactor length (10–100 cm), micropumps pulse time (0.2–1.0 s) and number of inserted sample pulses (5–15). As the Plackett–Burman design only provides the tendencies of variables to optimum values,

results revealed that the absorbance values increased with pulse time and number of sample pulses. However, when approaching the higher values of sample pulses and pulse time studied in the experimental design, it was verified the appearance of double peaks with different shapes, being this fact related with the length of the reactor used, indicating that the sample volume inserted into the system was too high and was not totally reacting. According with the results, and in order to surpass the difficulty related to double peaks that make determinations unfeasible, it could be implemented a reactor with a length superior to 55 cm, but that would impair the analysis time and increase sample dispersion.

So, three studies for which were fixed a different reactor length (10, 30 and 55 cm) were conducted, and the influence on analytical signal of the pulse time and number of sample pulses were assessed in the following ranges: 0.6–1.4 s and 8–16, respectively. A full factorial design with four levels for pulse time and number of pulses was adopted for each reactor length. According to this design, a total of 48 experiments were conducted. The obtained absorbances for each reactor length were modelled with an artificial neural network (one non-linear hidden layer with three nodes and a linear output layer with one node) since a linear model was found not to be appropriate. Details regarding the calibration are available in Section 2.5. Contour plots were produced for each reactor length by simulating the calibrated artificial neural network within the two physical parameters range. It was concluded that a number of pulses higher than 13 was not adequate for the determination, as double peaks were obtained. The obtained results were treated in order to exclude the ones corresponding to double peaks, being these graphically marked as filled circles (see Fig. 4A containing the contour plot for the 55 cm-length reactor). Subsequently, for each reactor was selected the optimum combination of pulse time/number of pulses, aiming to the higher absorbance value, and comparing at the end the selected results between the three assays. That comparison revealed that the highest absorbance value was obtained using a 55 cm reactor length, 1.1 s of pulse time and 11 pulses of sample insertion.

However, it was verified that the selected values provided a determination rate of 15 h^{-1}, approximately. Aiming to increase the determination rate without impairing analytical response, all the physical variables were further optimized (with exception for reactor length already fixed at 55 cm), with the objective of obtaining a maximum value for the ratio between absorbance signal and the time needed for one determination (in this work named "period"), and by attributing the same percentage importance (50%) to both factors. The period was the difference in time for maximum absorbance values between two consecutive determinations. For each assay, five determinations were conducted, and so, the calculated period corresponded to a four values average.

Thus, the results depicted in Fig. 4B revealed that the maximum value of absorbance/period for indapamide determination were obtained when using 11 pulses of sample solution, a pulse time of 0.6 s, for a reactor with 55 cm length.

Another flow parameter of great importance was the strategy used for sample introduction in the flow system, since it can influence the degree of mixture, and hence, reaction development. Under the optimized conditions, the effect of flow sampling strategy on the absorbance signal was studied. Some assays were carried out by exploiting different sampling methods at confluence point X_2 (Fig. 1), such as merging zones, binary sampling and single sample volume, and at the same time varying the number of pulses of the inserted sample solution within 6 and 14 (corresponding to sample solution volumes between 48 and 84 μL). Two different approaches for binary sampling were evaluated, designated in this work, by binary sampling A and B. Binary sampling A and B consisted in the insertion of aliquots of sample solution with pre-mixed aliquots of MBTH and Ce(IV) solutions, that is, plugs of sample were intercalated with plugs of MBTH/Ce(IV) solution. The binary sampling approaches A and B differed in the number of sample plugs intercalated: for the type A, one pulse of sample (8 μL) was intercalated with one pulse of MBTH/Ce(IV) (16 μL) solution, while, for approach B, two pulses of sample solution (16 μL) were intercalated with one pulse of MBTH/Ce(IV) (16 μL) solution.

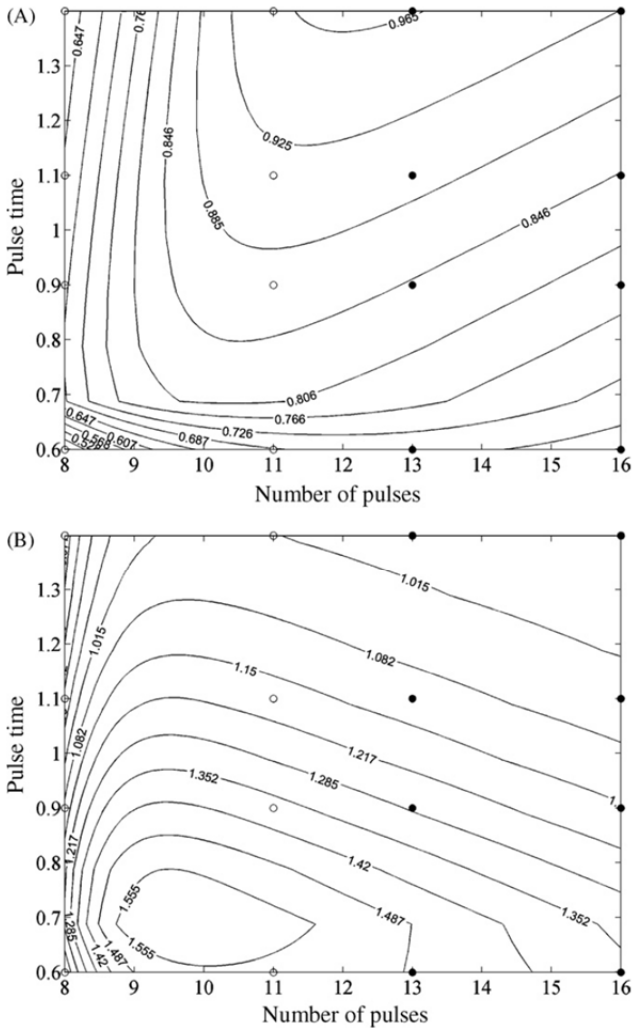

Fig. 4. (A) Effect of pulse time and number of pulses on absorbance signal using a reactor length of 55 cm (filled circles, double peak; empty circles, no double peak). (B) Effect of pulse time and number of pulses on absorbance signal per period using a reactor length of 55 cm (filled circles, double peak; empty circles, no double peak).

Performance of the merging zones process was evaluated by the simultaneous insertion of pre-mixed solution MBTH/Ce(IV) and sample solution. On the other hand, the single sample volumes approach was accomplish by inserting a unique volume of sample solution between two identical plugs of pre-mixed MBTH and Ce(IV) solution.

When programming these assays, the pulse time was modified and adjusted for all sampling strategies, in order to attain a similar flow rate throughout the system during sample insertion and transport phases. This was necessary since, in a multipumping flow system each individual solution flow rate is defined by the stroke volume and pulse time of the corresponding micropump, and when micropumps are activated simultaneously, the overall flow rate corresponds to the sum of the flow rates of all propelled solutions. This way, according to the sampling approach used, different flow rates are obtained. At the same time, the influence of the volume of sam-

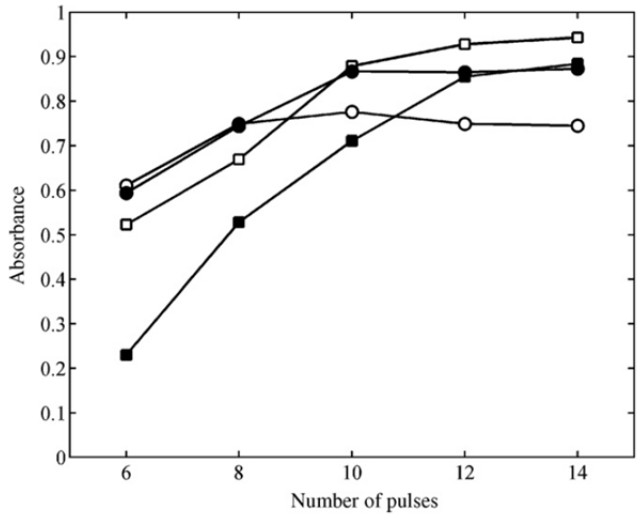

Fig. 5. Influence in the analytical signal of the number of sample pulses for distinct sampling strategies (empty squares, binary sampling A; filled squares, binary sampling B; empty circles, merging zones; filled circles, single volumes).

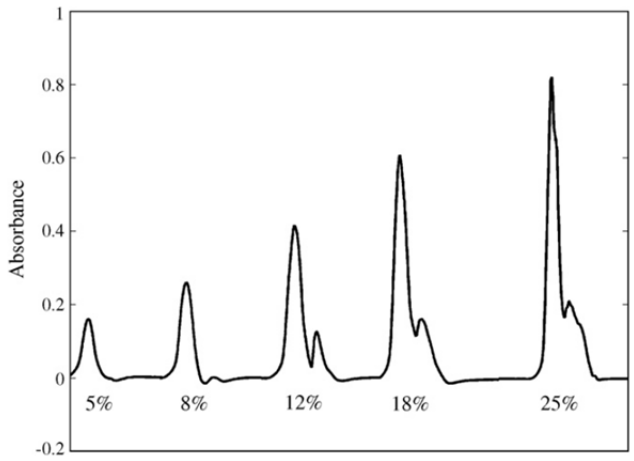

Fig. 6. Influence of ethanol concentration (%) in the analytical signal.

ple solution to be inserted into the flow system was evaluated for each sampling strategy, by analysing the effect on the analytical signal of the number of pulses of the micropump responsible for the sample insertion.

For the single sample volumes and binary sampling A approaches the obtained results revealed a more pronounced increase in the analytical signal up to approximately 10 pulses of sample solution (80 μL of sample), tending to stabilize for a higher number of pulses. However, for the merging zones approach an increase on analytical signal was verified up to 8 sample pulses (64 μL of sample), while that for binary sampling B the analytical signal increased up to 12 sample pulses (96 μL of sample).

After observing the obtained results (Fig. 5), it was concluded that both single sample volumes and binary sampling A approaches provided the highest analytical signals, and as a compromise between determination rate and sensitivity, 10 sample pulses (80 μL of sample solution) were selected for further optimizations.

Following last results, it was studied the performance of the single sample volumes and binary sampling A strategies by establishment of calibration curves with indapamide concentrations up to 50 mg L^{-1}. The results were evaluated resorting to a comparison between the slopes of calibration curves: linearity of the curves was represented by Abs = 0.0341 (±0.0005) × $C_{indapamide}$ (mg L^{-1}) + 0.24 (±0.01) and Abs = 0.0292 (±0.0004) × $C_{indapamide}$ (mg L^{-1}) + 0.20 (±0.01), for single sample volumes and binary sampling A, respectively. It was verified a sensitivity increase of about 14% when the single sample volumes approach was performed. This way, the sampling strategy applied for indapamide determinations was single sample volume.

As already mentioned in subchapter "sample, standards and reagents", since indapamide is practically insoluble in water, a stock solution of 400 mg L^{-1} was prepared in a 40% ethanol solution. The indapamide standard solutions were prepared from dilution of the stock solution with acid sulphuric solution. So, for indapamide concentrations between 5 and 50 mg L^{-1}, the ethanol concentration was between 0.5 and 5%. Nevertheless, the concentration of ethanol had a strong influence in absorbance signal, due to Schlieren effect. In order to evaluate this influence, a calibration curve with a concentration range of ethanol up to 25% was established. It was observed that the absorbance increased with the concentration of ethanol and for that reason its concentration was normalized between all standards with the objective to obtain the same influence of ethanol in analytical signal. On the other hand, it is important to mention that the utilization of ethanol concentrations higher than 5% caused intense refractive index gradients (Schlieren effect) in the sample zone, as it can be observed through the appearance of double peaks represented in Fig. 6. In order to circumvent the Schlieren effect problem, all indapamide standards were prepared in a 5% ethanol solution.

3.3. Analysis of pharmaceutical formulations

In order to apply the developed methodology to the determination of indapamide in pharmaceutical formulations, the influence of some compounds commonly used as excipients was assessed. A sample solution containing a fixed amount of indapamide (20 mg L^{-1}) and different concentrations of the excipients under evaluation were analysed by the developed method. A compound was considered as non-interfering if the analytical signal variation was ±4% compared to the analytical signal obtained in the absence of the referred compound. The results revealed that the excipients (starch, lactose, magnesium stearate, anhydrous colloidal silica, hypromellose, polyvinylpyrrolidone (povidone), stearic acid, and cellulose) on a 100-fold mass ratio regarding indapamide did not interfere.

Under all the optimal conditions previously established, a linear working response range for indapamide concentration up to 50 mg L^{-1} was obtained. The calibration curve was represented by Abs = 0.0325 (±0.0002) × $C_{indapamide}$ (mg L^{-1}) + 0.187 (±0.007) with a correlation coefficient of 0.9999. The detection limit calculated from the equation of the calibration curve according to Miller and Miller [37] was about 1 mg L^{-1}.

The precision of the proposed methodology was evaluated through the calculation of the confidence interval of a set of 10 repeated measures for each sample. From the results in Table 2, it can be confirmed that the developed methodology presents a good repeatability, taking into account the calculated concentration ranges for a confidence level of 95%.

For accuracy assessment, the results obtained for determination of indapamide in seven commercial pharmaceutical dosage forms by the proposed flow procedure, were compared with the ones obtained through the reference procedure of the British Pharmacopoeia. The results, summarised in Table 2, revealed a good agreement between both methods, with relative deviations comprised between −1.5 and 2.1%. Additionally, this agreement was confirmed using a paired *t*-test [37], in which the *t*-value estimated (0.127) was lower than the tabulated one (2.447) illustrating the

Table 2
Comparison of analytical results obtained in the determination of indapamide in pharmaceutical formulation by the proposed and the reference method [32].

Sample	Dosage (mg)/formulation	Amount found (mg)[a]		R.D. (%)[b]
		MPFS Methodology	Reference method	
Generis 2.5	2.5	2.76 ± 0.09	2.75 ± 0.18	0.2
Alter 2.5	2.5	2.56 ± 0.08	2.58 ± 0.12	−0.8
Fluidema 2.5	2.5	2.58 ± 0.08	2.59 ± 0.05	−0.3
GP 2.5	2.5	2.51 ± 0.06	2.54 ± 0.04	−1.5
Fludex LP 1.5	1.5	1.50 ± 0.03	1.46 ± 0.06	2.1
Tandix 1.5	1.5	1.52 ± 0.05	1.51 ± 0.05	0.7
Ind Generis 1.5	1.5	1.51 ± 0.04	1.49 ± 0.04	0.8

[a] Mean ± $t0.05$ (Student's t-test) × (S.D./$\sqrt{n}$).
[b] Relative deviation of the development method regarding the reference procedure.

Table 3
Analytical figures of merit of the proposed multipumping flow system (MPFS) and batch methodology [28].

Parameter	MPFS	Batch
Linear dynamic range (mg L^{-1})	Up to 50	1.2–9.6
Equation of linear calibration	$A = 0.0325 \times C_{indapamide} + 0.187$	$A = 0.1179 \times C_{indapamide} + 0.0086$
Detection limit (mg L^{-1})	1.00	0.14
Sampling rate (h^{-1})	25	Not mentioned
MBTH (mg)/determination	0.207	6.00
Ce(IV) (mg)/determination	0.356	10.00
H_2SO_4 (mg)/determination	12.55	49.04

absence of any statistical differences for a confidence level of 95% ($n = 7$).

The time required to complete an analytical cycle was approximately 147 s and consequently the determination rate equated to about 25 determinations per hour. For that reason, the developed automatic flow methodology represents a significant improvement in determination rate when compared to the reference method [32], in which, almost 12 min were required to complete a chromatographic run (determination rate of about 5 h^{-1}), meaning an improvement of about 80%.

3.4. Comparison of the proposed MPFS with other methodologies

Additionally, the MPFS and chromatographic methodologies were compared in terms of production of residues per determination. The HPLC procedure produced approximately 14.4 mL of residues per determination (containing: SDS 4.21 mg, glacial acetic acid 2.62 mg, triethylamine 100.91 mg, butan-2-ol 224.62 mg and acetonitrile 3.39 g), while the MPFS only produced 1.44 mL per determination (containing: MBTH 0.207 mg, Ce(IV) 0.356 mg and sulphuric acid 12.55 mg). This means that the proposed MPFS allowed a significant reduction in the production of residues in the order of 90%.

The developed MPFS was also compared with another spectrophotometric methodology carried out in batch for indapamide determination based on the same reaction [28]. In Table 3 are compiled some analytical figures of merit of both methodologies and also the reagents consumption per determination. According to Table 3, the obtained linear working range with MPFS was increased to up 50 mg L^{-1}. In batch methodology the determination rate is not mentioned, but since it involves waiting periods for reaction development (15 min total waiting time, according with authors), it can be predicted that the determination rate is significantly lower than that obtained by the proposed MPFS. Moreover, the comparison between the two methods demonstrated that the proposed MPFS allowed a significant reduction in the consumption of reagents in the order of 97, 96 and 74%, for MBTH, Ce(IV) and sulphuric acid, respectively. A lesser wastefulness of reagents was accomplished due to sulphuric acid solution that was the only reagent in carrier solution, allowing the very low consumption of MBTH or Ce(IV).

4. Conclusions

This work contributed to an improved knowledge of the oxidative coupling reaction involving the reagents MBTH and Ce(IV), often used in the pharmaceutical chemical monitoring.

The results obtained in this work showed that the oxidative coupling reaction involving MBTH and Ce(IV) can led to distinct reaction products depending on the experimental conditions, namely on the sequence of reagents addition. This assumption was confirmed when this reaction was applied in the spectrophotometric determination of indapamide by resorting to an automated flow-based system. Effectively, different reaction products with different kinetics, stabilities and absorbance spectra were obtained simply by varying the sequence of reagents insertion. If the reagents addition sequence involves mixing first MBTH and indapamide, and only then Ce(IV), a product with fast formation kinetics, relatively unstable and with a maximum absorbance wavelength at 510 nm is formed. But, by mixing first MBTH and Ce(IV), adding latter the indapamide solution, then the formed product is much more stable, with maximum absorbance wavelength at 601 nm.

This variability can be exploited as an advantageous versatile feature whenever the presence of given specie interfere with the determination, a situation that could be overcome by using a specific addition sequence that favoured the formation of a product with either distinct kinetics or a different maximum absorbance wavelength. For example, when applying this methodology for the determination of a substance in several matrices, and if interfering specie absorbing at one of the monitored wavelengths are formed, then the same reaction can still be used by exploiting one specific addition sequence of reagents, avoiding the detection at the wavelength where the interference is noticed.

The automation of a reactional scheme involving MBTH and Ce(IV) by means of a flow-based analytical system requires immediately high versatility in terms of solutions manipulation in order to enable the exploitation of these possibilities. In this regard, the multipumping flow system implemented for indapamide determination allowed the individual insertion, commutation and propelling of all solutions in a pre-determined and programmed sequence, making very easy the implementation of the two different addition sequences of reagents by using only one analytical

system. At the same time, it generated a pulsed flowing stream with improved mixing conditions that facilitate reaction development.

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CHAPTER 4

Automated determination of diazepam in spiked alcoholic beverages associated with drug-facilitated crimes

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Automated determination of diazepam in spiked alcoholic beverages associated with drug-facilitated crimes

David S.M. Ribeiro, João A.V. Prior*, João L.M. Santos, José L.F.C. Lima

REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, No. 164, 4050-047 Porto, Portugal

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ABSTRACT

In this work, a multipumping flow system (MPFS) coupled to a photodegradation unit was developed, for the first time, for the determination of diazepam (a benzodiazepine) in spiked alcoholic beverages by fluorimetry. The main features of MPFS such as, high portability, versatility and straightforward automation and control combined with the efficiency and simplicity of photodegradation and the selectivity and sensitivity of fluorimetric detection makes the developed analytical methodology an attractive tool and a valuable contribution for the prevention of drug-facilitated crimes (DFC). Drug-facilitated crimes involve the unauthorized administration of strong central nervous system depressant drugs, which have the capability of preventing victims from resist to the action of the perpetrator or fighting off. Most often, the drugs identified as being used in DFC are surreptitiously placed in drinks served to potential victims in entertainment places, like night clubs.

Five commercial alcoholic beverages (Eristoff®, Smirnoff®, Bacardi®, Dry Gin® and Brazilian Cachaça 51®) spiked with diazepam were analyzed by the proposed methodology, and the results revealed good agreement with those obtained through a HPLC comparison procedure. Relative deviations comprised between −1.97 and 2.05% were achieved, and additionally, the application of a paired *t*-test, revealed the absence of any statistical difference for a confidence level of 95% ($n = 5$). The detection limit was approximately 2.02 mg L^{-1}.

1. Introduction

Benzodiazepines are widely used in the clinical treatment of anxiety, insomnia and panic disorder [1]. Although their specific medical indications, the benzodiazepines can be used illegally in drug-facilitated crimes (DFC) in which victims are incapacitated and unable to resist to assaults or rapes [2,3]. The pharmacological effects, such as, anxiolytic, anticonvulsant and CNS depressant, makes diazepam classified as a DFC drug. The drugs included in this group are characterized by being easily placed surreptitiously in drinks consumed at entertainment places, such as nightclubs. Additionally, the combination of this kind of drugs with alcohol, normally contained in the beverages, can be extremely dangerous because of the potentiation of the pharmacological effects. The distance between moderate CNS depressant effects and coma, or even death, becomes very narrow. The intoxication effects can include loss of consciousness, vomiting, poor coordination and balance, respiratory difficulties and loss of control.

In recent years, drug-facilitated crimes have become a growing public concern and have highlighted the potential abuse of benzodiazepines, making their fast detection a priority from a forensic and toxicological standpoint [4]. In the cases of drug-facilitated crimes, the detection of the responsible benzodiazepine drug in the biological fluids of the victim is of paramount importance in establishing an effective prosecution. However, the results of the toxicological analysis in these cases are usually negative because, for example, the delay in reporting the criminal offense, the administration of a single dose and the short half-life of some of the drugs [5]. Thus, the screening and toxicological analysis of drugs in beverages intentionally spiked is becoming more requested, as it happens more frequently in the cases of DFC victims. When there is a delay between reporting the incident and collecting the appropriate biological specimen, the drug-related evidence may be found in drinks or drink containers at the crime scene, since in these samples the biometabolism of the drugs do not occur.

In literature several published methods for analysis of benzodiazepines in beverages can be found based either on Gas Chromatography/Mass Spectrometry (GC–MS) [6], Capillary Zone Electrophoresis/Diode Array (CZE–DAD) [4], Direct Electrospray Probe/Mass Spectrometry (DEP/MS) [7], High Performance Liquid Chromatography/Diode Array (HPLC–DAD) [8,9], High Performance Thin-Layer Chromatography (HPTLC) [10], etc.

* Corresponding author at: REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia da Universidade do Porto, Rua Aníbal Cunha, No. 164, 4050-047 Porto, Portugal. Tel.: +351 222 078 994; fax: +351 222 078 961.
E-mail address: joaoavp@ff.up.pt (J.A.V. Prior).

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The methods above mentioned are very precise and present high sensitivity, however they are only performed in very special laboratory conditions, and they are laborious and require skilled operators. Additionally, these methods are completely inappropriate for fast screenings of the drinks at the crime scene, since they involve the utilization of large equipments.

In this context, the development of portable miniaturized flow systems for in situ and real-time analysis, assume high importance and promising applicability. The exploitation of multipumping concept [11] allows implementing very compact and portable analytical systems, with high simplicity in automation and control, bringing together all the advantages associated to miniaturization, as for example, the reduction in the solutions consumption, high portability and reduced power requirements as well as minimized waste production. These features make the MPFS attractive for measurements out of laboratory. Furthermore, another essential advantage of MPFS is versatility because these flow-based methods permit distinct sample manipulations, without requiring system reconfiguration, or at least, by introducing only minor modifications, being this way able to accommodate a wide variety of assays, with assorted detection methods [12].

This work aims at contributing to the prevention of drug-facilitated crimes, by developing an automatic, portable and miniaturized analytical flow system, exploiting the multipumping concept, for fast in situ analysis, in the screening of benzodiazepines in surreptitiously spiked alcoholic drinks. The present paper describes, for the first time, the development of a flow-based system in which an automatic multipumping flow system was applied to detect drink spiking with diazepam. The determination of diazepam on commercial beverages, previously spiked, was implemented through photodegradation of diazepam and detection of degradation products by spectrofluorimetry.

2. Experimental

2.1. Samples, standards and reagents

All chemicals were of analytical reagent grade and doubly deionised water (specific conductivity <0.1 μS cm^{-1}) was used throughout.

The diazepam drug was supplied by "Laboratórios Bial" (Porto, Portugal). A 200 mg L^{-1} diazepam stock solution was prepared by dissolving 20 mg of diazepam in 40 mL of absolute ethanol (Panreac) and diluted to 100 mL with deionised water. This stock solution was stored under refrigeration and protected from the light.

A 2 mol L^{-1} NaOH solution was prepared by dissolving 40 g in 500 mL of deionised water.

A 0.1 mol L^{-1} sodium dodecyl sulphate (SDS) solution was prepared by dissolving 7.21 g of SDS (Fluka) in a 250 mL volumetric flask with deionised water.

Working diazepam standards (5–40 mg L^{-1}) were daily prepared by appropriate dilution of the stock solution using the following experimental procedure: aliquots of diazepam stock solution (0.5–5.0 mL) were transferred into a series of 20 mL volumetric flasks. Thereafter, 2 mL of 2 mol L^{-1} NaOH solution and 6 mL of 0.1 mol L^{-1} SDS solution were added. Finally, appropriate aliquots of absolute ethanol (Panreac) were added, aiming at obtaining the same ethanol concentration (20%, v/v) for all working diazepam standards. The volume was subsequently made up to the mark with deionised water.

Five highly consumed alcoholic beverages, including very popular brand names such as Eristoff®, Smirnoff®, Bacardi®, Dry Gin® and Cachaça 51®, were selected for analysis. The beverages were spiked aiming at a diazepam concentration of 25 mg L^{-1}, by adding 1.25 mL of a 500 mg L^{-1} diazepam stock solution into a 25 mL volumetric flask and the final volume was completed with the respective alcoholic beverage under analysis. Then, aliquots of the spiked drinks were transferred into a series of 25 mL volumetric flasks and subsequently 2 mL of 2 mol L^{-1} NaOH solution and 6 mL of 0.1 mol L^{-1} SDS solution were added, and the volume was made up to the mark with deionised water.

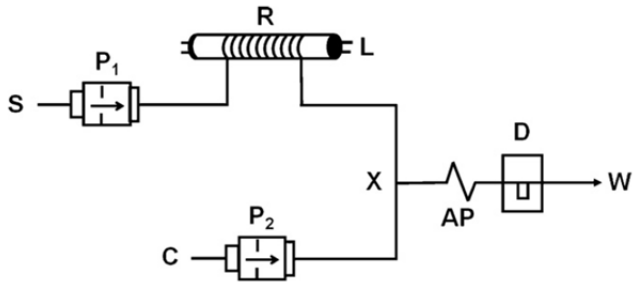

Fig. 1. Multipumping flow system (MPFS): P_1, P_2: solenoid micropumps (internal volume 10 μL); *X*: confluence point; *R*: 2 m reactor; *D*: fluorimeter detector (λ_{ex} = 272 and λ_{em} = 450 nm); *L*: Philips UV lamp; AP: 10 cm analytical path; *S*: sample; *C*: carrier (0.2 mol L^{-1} NaOH); *W*: waste.

2.2. Apparatus

A model FP-2020/2025 spectrofluorimeter (Jasco, Easton, MD, USA), equipped with a 16 μL internal volume flow cell was used for monitoring fluorescence intensity (λ_{ex} = 272, λ_{em} = 450 nm).

The designed flow manifold comprised two 120SP (Bio-Chem Valve Inc. Boonton, NJ, USA) solenoid actuated micro-pumps, which were of the fixed displacement diaphragm type, dispensing 10 μL per stroke. Flow lines made of 0.8 mm i.d. PTFE tubing, homemade end-fittings, connectors and confluences points were also used.

Automatic control of the analytical system was accomplished by means of a Pentium based microcomputer with software developed using Microsoft Quick-Basic 4.5. A CoolDrive™ power drive (NResearch, Caldwell, NJ, USA) was used to actuate the micro-pumps through the LPT1 computer port.

Irradiation of the solutions with UV light was performed using a 15 W Philips TUV 15W/G15T8 low pressure mercury lamp at 253.7 nm. The analytical signals were registered through a model L250E Linseis chart recorder.

2.3. Flow system operation

The configuration of the developed analytical flow manifold (Fig. 1) was designed taking in special attention the photoreaction of diazepam in alkaline medium, by exposure of UV light. Aiming at implementing a flow manifold that would allow a higher versatility in the control of the parameters that influenced the extension of diazepam photodegradation, particularly the time of exposure, UV lamp was assembled in line with sample insertion tubing and a micro-pump responsible for samples insertion and transport was placed prior to the UV light source. Since the UV lamp was placed in a sealed box to prevent any hazardous exposure its connection to the remaining flow system (at confluence point *X*) required a 36 cm tubing (with an internal volume of 180 μL) that contained sample solution not subject to irradiation, which was flushed to waste prior to the fluorescence measurements.

Thus, the analytical manifold employed two solenoid micropumps which were responsible for the individual handling of sample and carrier solutions. The micro-pump P_1 (Fig. 1) was responsible for inserting the diazepam solution, while micro-pump P_2 was used for inserting and propelling the 0.2 mol L^{-1} NaOH solution, which was used as carrier.

The analytical cycle started by actuating P_1, for inserting the sample solution in the analytical manifold filling the 2 m reactor tube (R) which was coiled around the UV lamp (L). The used number of pulses was enough to ensure that the reactor coil was completely filled with the sample solution, which will allow subsequently, and after sample irradiation, the carrying out of five consecutive determinations. Thereafter, P_1 was switched off and the UV lamp was turned on, starting the sample irradiation stage for a period of 15 min.

Following completion of the irradiation time, the lamp was turned off, P_1 was reactivated and the above referred 180 μL of non-irradiate sample solution were carried towards waste. Subsequently, P_1 was switched off and by actuation of P_2 the NaOH carrier solution was inserted and propelled through confluence point (X) and through the detector (D) for cleanup and baseline establishment.

The analytical cycle proceeded with the insertion of 100 μL of irradiated solution (10 pulses) in the analytical path by switching on P_1. Subsequently, by repeated actuation of micro-pump P_2, the reaction zone was carried toward the detector generating an analytical signal whose magnitude was proportional to the diazepam concentration. This process was repeated five times allowing the accomplishment of five consecutive determinations.

Micro-pump P_1 in the sample insertion stage and micro-pump P_2 in the transport stage were operated with a pulse time of 0.2 s corresponding to a pulse frequency of 171 min^{-1}, establishing a flow rate of 1.71 mL min^{-1}.

2.4. Reference procedure

Since no reference methodology was available for the determination of the drug in spirits, the results obtained by the proposed flow-based methodology were validated by comparison with those furnished by a HPLC reference methodology. This reference methodology was the one recommended by the United States Pharmacopoeia (USP) [13] for the determination of diazepam pure drug. However, this procedure was modified in conformity with the work of Rao et al. [9] due to the scarce worldwide availability of acetonitrile.

The comparison methodology involved the preparation of four diazepam standard solutions in methanol, for establishment of a calibration curve. Samples and standards solutions were filtered with 0.45 μm filters and 10 μL of the filtrate of each solution was injected into the chromatograph. The chromatographic system (Jasco, model MD-2015 Plus) incorporated a Kromasil C-18 column (250 mm × 4.6 mm), and the mobile phase was a mixture of methanol, water and acetic acid (650:350:0.1). The analysis was carried out under isocratic conditions at a flow rate of 1 mL min^{-1}. Chromatograms were recorded at 254 nm.

3. Results and discussion

Some preliminary assays revealed that diazepam solutions prepared in acidic medium and exposed to natural light for several days, underwent decomposition, that is, the concentration of diazepam gradually decreased. The decomposition of the drug was verified when different solutions containing 200 mg L^{-1} of diazepam prepared in 2 mol L^{-1} H_2SO_4 were exposed to natural light for 1, 10, 15 and 25 days. With the aim to demonstrate the acid hydrolysis of the compound in conditions of exposure to natural light, a solution of 200 mg L^{-1} diazepam in 2 mol L^{-1} H_2SO_4 was heated for 1 h at 100 °C, assuring this way the complete degradation of diazepam [14,15]. Afterwards, all the solutions above mentioned were diluted 10 times, and for each obtained solution the fluorescence excitation and emission spectra was recorded, at a wavelength range of 240–420 and 300–600 nm, respectively. The results (Fig. 2) demonstrated that one of the compounds resulting from the acid hydrolysis, either favored by exposure to natural light or by means of heating, was fluorescent with a wavelength of maximum excitation and emission at 262 and 463 nm, respectively. For the analysis of the results it was taken into consideration that an overall degradation of the drug was achieved when the solution of diazepam in acidic medium was heated to 100 °C during 1 h. Consequently, by comparing the obtained results for diazepam solutions exposed to natural light with those observed with the diazepam solution heated, it was verified that after 1, 10, 15, and 25 days of exposure, the degradation rate of the drug was approximately 1.5, 18.0, 28.0 and 41.0%, respectively. These results revealed that with the increase of exposure time to natural light the analytical signal was higher, and for 1 day of exposure the fluorescence intensity was not significant. This allows the assumption that the process of diazepam hydrolysis in acidic medium by exposure to natural light is slow, and may take several days to accomplish.

Therefore, aiming at increasing the diazepam hydrolysis by means of exposure to light, a more powerful light source would have to be used, and thus, several batch assays were performed involving the direct exposure to light from a UV lamp of glass beakers containing a 200 mg L^{-1} diazepam solution prepared in 2 mol L^{-1} sulphuric acid for 10, 25 and 45 min. After analysis of the recorded excitation and emission spectra it was verified that the effect of UV radiation for the short exposure period assayed in the batch procedure was negligible since the formation of fluorescence compounds was not perceptible.

A possibility to increase the efficiency of UV radiation and to accelerate the process of hydrolysis would be to coil a tubing around the UV lamp, which would be used to hold the diazepam solution during irradiation, in a flow-based configuration. In fact, the implementation of photodegradation in a flow system has proven to increase the surface area of exposure to UV radiation, to decrease the distance between the solution and the light source, to enable the exposure of reduced sample volumes and to assure an automatic control of the whole process. Accordingly, a multipumping flow system was developed and, a 50 mg L^{-1} diazepam in 2 mol L^{-1} H_2SO_4 solution was inserted in a reaction tube coiled around the tubular UV lamp in order to maximize the proximity to the radiation source. The inserted diazepam solution was exposed to UV radiation for 10 min through the stopped-flow technique, after which it was carried toward the fluorescence detector, for measuring the emission intensity at 463 nm (excitation at 262 nm). The obtained results revealed the formation of fluorescent compounds, confirming that the UV radiation had effectively promoted the hydrolysis of diazepam within tubing, and confirming as well, that a miniaturized flow system could be a valuable tool for carrying out the photodegradation of diazepam with improved efficiency, thereby increasing the reaction extension, and consequently, the intensity of fluorescence of the irradiated solution. According to a previously published work [16], acid-catalyzed hydrolysis produces a benzophenone compound, whilst the hydrolysis in alkaline media originates different compounds formed by fission of the amide linkage.

3.1. Optimization of chemical parameters

Sample pH, as well as, the presence of some substances, e.g. Fe (III), Cu (II) and Ce (IV) and substances that provide an organized media (including anionic, cationic or neutral surfactants), can strongly influence the photodegradation process. Therefore, some experiments to optimize the acid concentration, and also studies involving different reaction media were performed in order to provide a higher fluorescence intensity of the formed product.

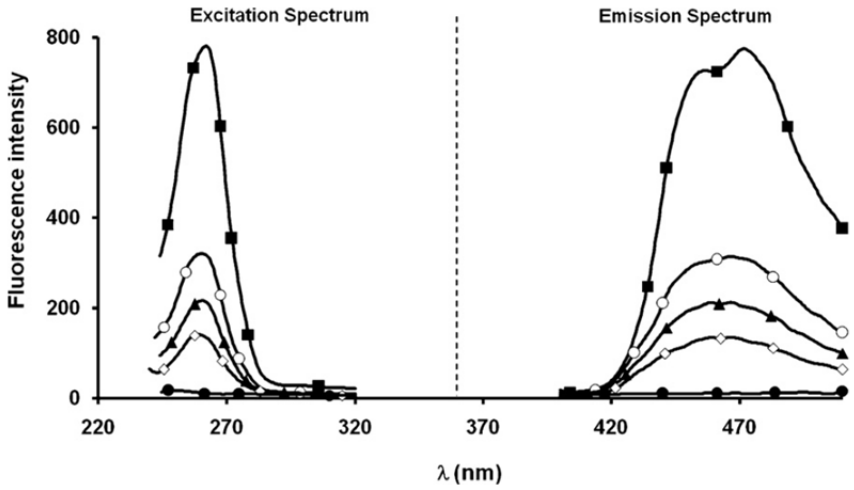

Fig. 2. Fluorescence emission and excitation spectra of hydrolyzed solutions of diazepam in acidic medium: (■) diazepam solution hydrolyzed by heating. The remaining results correspond to diazepam solutions hydrolyzed upon exposure to natural light for increasing number of days: (●) 1 day; (◊) 10 days; (▲) 15 days; (○) 25 days.

The influence of sulphuric acid on the analytical signal was assessed over a concentration range from 0.0125 to 4.0 mol L^{-1}, by inserting in the system 100 μL (10 pulses) of a 50 mg L^{-1} diazepam standard solution and exposing it to UV radiation for 10 min. The highest analytical signal was obtained using a sulphuric acid solution with a concentration of 0.050 mol L^{-1}.

Furthermore, with the goal of increasing the fluorescence intensity of the irradiated solutions in acidic medium, the influence on analytical signal of the presence of different substances in the photoreaction process was studied, namely, (i) metal ions, such as Cu (II), Fe (III), Ce (IV), (ii) non-ionic (methylcellulose, MC), cationic (cetyltrimethylammonium bromide, CTAB) and anionic surfactants (sodium dodecyl sulphate, SDS). The performed studies involved the preparation of seven solutions containing 50 mg L^{-1} of diazepam in 0.050 mol L^{-1} H_2SO_4. One of the solutions was used as reference solution and to each one of the remaining six was added a given amount of the different substances under evaluation. The final concentration of metal ions (Cu (II), Fe (III) and Ce (IV)) was 5×10^{-4} mol L^{-1} while for CTAB, SDS and MC surfactants was 0.01 mol L^{-1} and 0.01%, respectively, being these values higher than the respective critical micelle concentrations (cmc). The obtained results revealed a decrease of fluorescence intensity when using the metal ions, SDS and CTAB surfactants, whilst for MC no difference in analytical signal was notice, when comparing with the reference solution The photodegradation of diazepam in alkaline medium, instead of acid medium, was also studied by replacing the 0.050 mol L^{-1} H_2SO_4 by an equimolar NaOH solution. The assays were carried out using the multipumping flow system previously referred. The excitation and emission spectra of a 50 mg L^{-1} diazepam in 0.050 mol L^{-1} NaOH solution, exposed to UV radiation for 10 min within the flow system were recorded. The results revealed two wavelengths of maximum excitation and emission, namely: (a) λ_{ex} = 272, λ_{em} = 450 nm; (b) λ_{ex} = 381, λ_{em} = 463 nm. Nevertheless, a higher analytical response was obtained when using excitation and emission wavelengths of λ_{ex} = 272, λ_{em} = 450 nm. As it happened with the assays for acid hydrolysis, a study of the influence of the reaction conditions in the photodegradation extension under alkaline conditions was also carried out. In this evaluation were used the same metal ions and surfactants (with the exception of CTAB) at the same concentrations used in the above mentioned assays.

The obtained results (Fig. 3) revealed a pronounced fluorescence intensity increment with Fe (III), MC and SDS and a decrease with Cu (II) and Co (II). The highest increment value (110%) was obtained with SDS. These results led to the conclusion that the fluorescence detection of the diazepam photodegradation products in alkaline medium can be increased if the reaction occurs in micellar medium, as it results in an organized microenvironment that promotes the photodegradation reaction and/or the fluorescence emission. Therefore, in order to clarify the role of SDS in the proposed methodology, the determination of diazepam by photodegradation was carried out using two distinct experimental approaches. In the first case SDS was added to the sample before the irradiation step, and in the second case the anionic surfactant was only added after the irradiation step. The assay involved the utilisation of two 50 mg L^{-1} diazepam standard solutions: one prepared both in 0.05 mol L^{-1} NaOH and 0.01 mol L^{-1} SDS, and a second one prepared only in 0.05 mol L^{-1} NaOH. In the flow manifold (Fig. 1), a third micro-pump was added on the confluence point *X*, which allowed the insertion and propulsion of a 0.01 mol L^{-1} SDS solution used in the second approach. The assay involved the comparison of the analytical signals by using three different experimental procedures. The first procedure, used as reference, involved the irradiation for 10 min of the 50 mg L^{-1} diazepam in 0.05 mol L^{-1} NaOH solution, followed by the insertion, using the merging zones approach, of 10 pulses of the irradiated solution and 10 pulses of

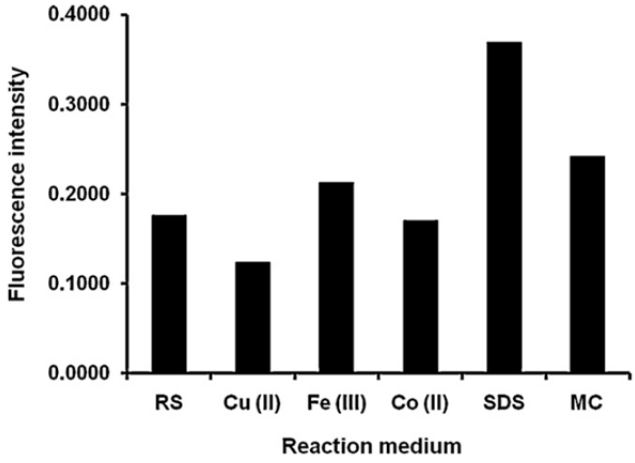

Fig. 3. Influence of the reaction medium on the alkaline photodegradation of diazepam: RS, reference solution; MC, methylcellulose; SDS, sodium dodecyl sulphate.

0.05 mol L^{-1} NaOH solution, and transport toward the detector with the NaOH solution. In the second procedure, 50 mg L^{-1} of diazepam in 0.05 mol L^{-1} NaOH solution was irradiated for 10 min followed by the insertion, using the merging zones approach, of 10 pulses of the irradiated solution and 10 pulses of 0.01 mol L^{-1} SDS solution, and afterwards the propulsion with the NaOH solution of the reaction zone to the detector. Finally, the third procedure involved the irradiation for 10 min of the 50 mg L^{-1} diazepam in 0.05 mol L^{-1} of NaOH and 0.01 mol L^{-1} SDS solution. After that, 10 pulses of irradiated solution and 10 pulses of 0.05 mol L^{-1} NaOH solution were inserted by merging zones followed by the propulsion of the reaction zone to the detector with NaOH solution.

Relative fluorescence intensities of 0.280 (±0.007), 0.256 (±0.004) and 0.580 (±0.02) were obtained for the first, second and third procedures, respectively. These results indicated that the use of SDS surfactant promoted the photoreaction development, because an increase of 109% in fluorescence intensity was verified when the diazepam solution was irradiated in the presence of the surfactant. On the other hand, when SDS was added to the diazepam solution after irradiation step, no significant variation in the analytical signal was observed regarding the one obtained with the first procedure. In fact, some surfactants, namely SDS, are known to originate an organized micellar system in the surrounding medium for concentrations higher than its cmc, increasing the analyte's concentration on the micellar surface, causing changes in the photochemical properties of analytes. More concretely, the increase of the analyte's concentration on the micellar surface improves its susceptibility to the exposition to UV radiation and the formation of fluorescence products, optimizing the photoinduced degradation of diazepam [17,18]. Consequently, the studies continued by preparation of the diazepam standards in a solution containing SDS and NaOH.

The study of the influence of sodium hydroxide concentration on the analytical signal was performed using several sample solutions, containing 50 mg L^{-1} of diazepam, 0.01 mol L^{-1} sodium dodecyl sulfate (SDS) and different NaOH concentrations under evaluation (0.01–0.50 mol L^{-1}). The volume of sample solutions inserted in the flow system was 100 μL (10 pulses), and the irradiation time was 10 min. In this study an increase of fluorescence intensity was verified for sodium hydroxide concentrations up to 0.20 mol L^{-1}, whereas, for higher concentrations the signal approached stabilization. Therefore, for the posterior optimization assays a concentration of 0.20 mol L^{-1} sodium hydroxide was selected.

Likewise the sodium hydroxide assays, in the assessment of the influence of the SDS concentration on the fluorescence intensity, an irradiation period of 10 min of a diazepam solution with concentration of 50 mg L^{-1} was used. A volume of 100 μL (10 pulses) of irradiated solution was inserted in the flow system, and subsequently the sample zone was carried towards the detector using as carrier the 0.20 mol L^{-1} NaOH solution. The obtained results demonstrated a pronounced increase in fluorescence intensity for SDS concentrations of up to 0.030 mol L^{-1} and stabilization for higher values. A 0.030 mol L^{-1} SDS concentration was selected for the following optimizations.

As it was previously mentioned the diazepam standard solutions were prepared from dilution of the stock solution of 200 mg L^{-1} containing 40% ethanol. Taking into account this fact, and considering that the proposed methodology is aimed at the determination of diazepam in commercial beverages served at night clubs, that contain very different alcoholic contents, the effect of the alcohol concentration in the analytical signal was assessed over a concentration range of up to 20% (v/v). Additionally, according with Caponetti et al. [19], the alcohol–water interaction has a pronounced effect on the micellisation of sodium dodecyl sulphate, causing a shift of the cmc of SDS when using samples with different

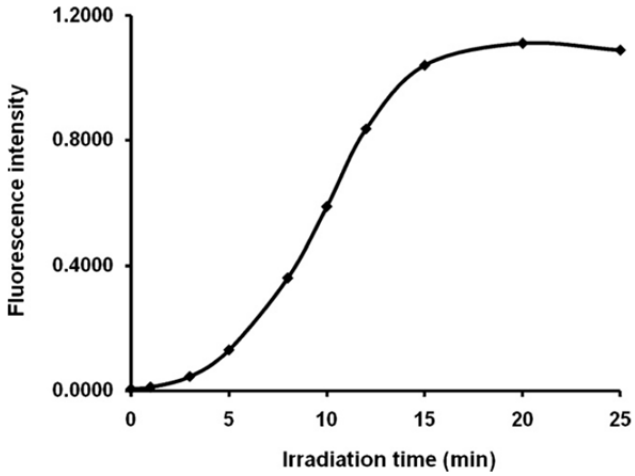

Fig. 4. Influence of irradiation time on the analytical signal.

water–alcohol proportions. Thus, it was important for the proposed work to use a concentration of SDS superior to its cmc, guaranteeing this way the micellar organization of the system. The results revealed no significant differences between the analytical signals for different ethanol concentrations, indicating also, that the use of a 0.03 mol L^{-1} SDS concentration guaranteed the formation of a micellar medium.

The influence of irradiation time on the analytical signal was also studied. This parameter was very important since it determined the extension of diazepam photodegradation, and therefore, the fluorescence intensity of the irradiated solution. This study involved the insertion in the analytical flow system of a 50 mg L^{-1} diazepam solution prepared in 0.20 mol L^{-1} NaOH and 0.030 mol L^{-1} SDS. After insertion the flowing stream was stopped under the UV light for increasing time periods, namely, 0, 1, 3, 5, 8, 10, 12, 15, 20 and 25 min, which corresponded to the irradiation interval. Following completion of the irradiation time, the volume of irradiated solution of diazepam equivalent to 10 pulses (100 μL) was carried to the detector, using as carrier the NaOH solution. The results (Fig. 4) revealed that increasing the irradiation time up to approximately 5 min, a small increase of the analytical signal was attained. However, with the increase of irradiation time between 5 and 15 min a markedly increase of fluorescence intensity signals was observed, tending to stabilization for irradiation times higher than 15 min. Accordingly, an irradiation time of 15 min allowing the maximization of the analytical signal was selected to perform the photodegradation of diazepam.

3.2. Optimization of physical parameters

The dispersion undergone by the reaction zone depended of the operational parameters of the flow manifold, and affected the extension of the reagents mixture and its dispersion within the flow system. Among the various physical parameters of the analytical flow system that could be studied and optimized, in order to yield an adequate dispersion level and to maximize the analytical signal, focus was set on the length of the analytical path (AP in Fig. 1) between the confluence point *X* and detector, the inserted sample volume and flow rate. The study of the influence of the sample volume and the length of the analytical path (AP) was simultaneously performed. The analytical response was evaluated relatively to the inserted number of pulses and for AP lengths of 10, 30, 60 and 85 cm. For each AP length, the number of pulses was varied between 2 and 18 (corresponding to sample volumes between 20 and 180 μL) of a 50 mg L^{-1} solution of diazepam containing

Table 1
Comparison of analytical results obtained in the determination of diazepam in spiked beverages by the proposed and the reference method.

Spiked drink	Concentration added (mg L^{-1})	Concentration found (mg L^{-1})[a]		% Recovery		R.D. %[b]
		MPFS methodology	Reference method	MPFS methodology	Reference method	
Eristoff®	25.05	25.7 ± 0.3	25.6 ± 0.8	102.4	102.3	0.12
Smirnoff®	25.05	25.7 ± 0.2	25.2 ± 0.2	102.5	100.4	2.05
Bacardi®	25.05	25.3 ± 0.2	25.8 ± 0.3	100.8	102.8	−1.97
Dry Gin®	25.05	26.0 ± 0.2	25.9 ± 0.2	103.6	103.2	0.40
Cachaça®	25.05	24.8 ± 0.2	25.2 ± 0.2	99.0	100.7	−1.65

[a] Mean ± t0.05 (Student's t-test) × ($S/\sqrt{n}$).
[b] Relative deviation of the development method regarding the reference procedure.

0.20 mol L^{-1} NaOH and 0.030 mol L^{-1} SDS, previously irradiated for 15 min. The pulse time used was of 0.2 s, which corresponded to a flow rate of about 1.71 mL min^{-1}. After observing the obtained results, it was concluded that an AP length of 10 cm allowed the attainment of the highest fluorescence intensity signal (Fig. 5). These results were in agreement with what would be expected, since the main purpose of this phase was to carry out the photoreaction products toward the detector, with a minimized dispersion of the reaction zone. In this case, for AP lengths higher than 10 cm, the residence time increased and consequently the dispersion undergone by the formed products was higher, thus diminishing the magnitude of the analytical signal. Taking into account these results, an AP of 10 cm was selected for further optimization assays.

The study of the influence on the analytical signal of flow rate and inserted sample volume was also performed simultaneously. In these assays, for pulse times of 0.1, 0.2, 0.3 and 0.4 s (corresponding to flow rates of 2.40, 1.71, 1.33, 1.09 mL min^{-1}), the number of inserted sample pulses was also varied between 2 and 18 (corresponding to volumes between 20 and 180 μL) of a solution containing 50 mg L^{-1} diazepam in 0.20 mol L^{-1} NaOH and 0.030 mol L^{-1} SDS, previously irradiated for 15 min. After analysis of the results, it was verified that the flow rate had no visible influence in the analytical signal intensity, which is clearly explained by the short length of the reaction coil used. Therefore, as a compromise between sampling rate and precision, a pulse time of 0.2 s (flow rate of 1.71 mL min^{-1}) was chosen.

A parameter that demonstrated a pronounced influence on analytical signal was sample volume, which was evaluated in terms of number of sample pulses (of a unitary value of 10 μL) inserted in the flow system. The obtained results (Fig. 6) revealed an accentuated increase on the analytical signal up to approximately 10 pulses (100 μL) and a subsequent stabilization for higher values. As a compromise between determination rate and sensitivity, this sample volume was selected for the analysis.

3.3. Analysis of commercial alcoholic beverages

In order to evaluate the performance of the developed methodology, and thus demonstrate its potential for routine laboratory procedures in forensic and toxicological analysis, the described multipumping flow system was applied to the determination of diazepam in alcoholic beverages (Eristoff®, Smirnoff®, Bacardi®, Dry Gin®, Cachaça®), previously spiked with the drug. Under the optimal analytical conditions formerly established, a linear working response range for diazepam concentration of up to 40 mg L^{-1} was obtained. The calibration curve was represented by the equation:

$$FI = 0.050(\pm 0.001) \times C - 0.046(\pm 0.022)$$

in which FI was the fluorescence intensity and C was diazepam concentration, in mg mL^{-1}. A correlation coefficient of 0.9992 was verified. The detection limit calculated from the equation of the calibration curve according to Miller and Miller [20] was about 2.02 mg L^{-1}.

Despite the fact that the linear working response range for diazepam concentration was up to 40 mg L^{-1}, the developed flow system can still be applicable in the detection of higher concentrations of diazepam and thus, provide a method for the identification of adulterated beverages. Aiming at validating the proposed miniaturized automatic methodology, the results obtained in the determination of diazepam in five spiked beverages by the proposed flow procedure, were compared with those furnished by the reference procedure. The results, summarized in Table 1, revealed a good agreement between both methods, with relative deviations between −1.97 and 2.05%. Additionally, the application of a

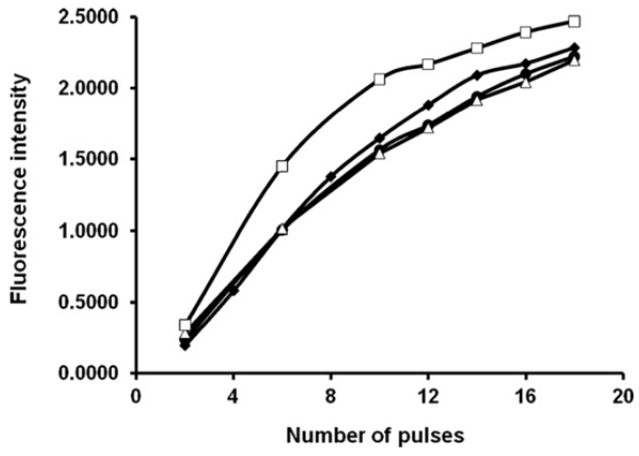

Fig. 5. Influence on analytical signal of the number of pulses using different AP lengths: (□) 10 cm; (♦) 30 cm; (●) 60 cm; (△) 85 cm.

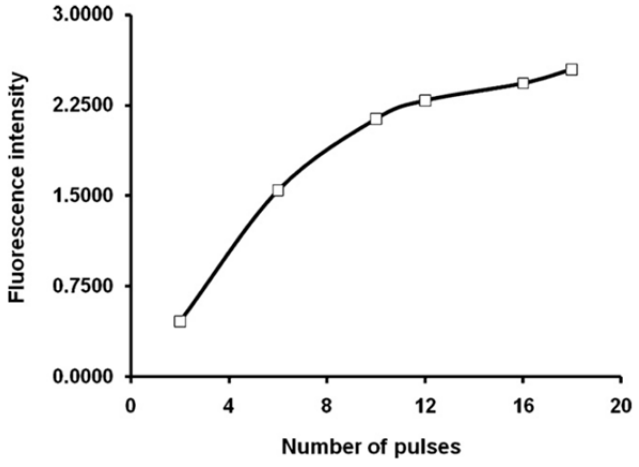

Fig. 6. Influence on analytical signal of the number of pulses using a flow rate of 1.71 mL min^{-1} (pulse time of 0.2 s).

paired Student's t-test [20] confirmed that there is no statistical differences ($t_{estimated} = 0.292$, $t_{tabulated} = 2.776$) between the results obtained by both procedures, confirming that the different alcohol contents of the beverages did not interfere in the analytical signal of the proposed methodology.

The results attained in the evaluation of the developed methodology precision, by repeated analysis (four consecutive determinations) of the spiked sample solutions, revealed that, taking into account the calculated concentration ranges determined in the different samples (Table 1), for a confidence level of 95%, a good repeatability was verified.

Due to the need of a 15 min sample exposure to UV radiation and taking into account the length of the reactor coiled around the UV lamp which limited the sample volume subject to radiation, a sample throughput of 3 h^{-1}, with five consecutive determinations per sample.

4. Conclusions

This work constitutes a noteworthy contribution to the prevention of drug-facilitated crimes based on the use of the drug diazepam, since the developed automatic miniaturized flow system successfully enabled the detection and determination of diazepam in spiked alcoholic beverages. The proposed methodology for the detection of diazepam in surreptitiously spiked beverages does not imply any complex sample preparation before analysis. The developed multipumping flow system additionally offers a high portability, low reagents and power consumption, allowing a promising application in in situ analysis.

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CHAPTER 5

Diazepam fluorimetric monitoring upon photo-degradation in an automatic miniaturized flow system

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ORIGINAL PAPER

Diazepam Fluorimetric Monitoring Upon Photo-Degradation in an Automatic Miniaturized Flow System

David S. M. Ribeiro · João A. V. Prior · João L. M. Santos · José L. F. C. Lima

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Abstract The present work describes the fully integration in line of a photo-degradation unit, comprising a low pressure UV lamp, in a Multipumping Flow System (MPFS), for the fluorimetric chemical control of commercially available pharmaceutical formulations containing diazepam. The utilization of an organized micellar medium provided enhanced fluorescence emission. The results allowed to obtain a linear working range for diazepam concentrations of up to 40 mg L^{-1} (r=0.9998) and the detection limit was about 0.97 mg L^{-1}. The results obtained by the miniaturized and automatic flow system were in agreement with those furnished by the reference procedure, with relative deviations comprised between −2.09% and 2.13%.

Keywords Miniaturized · Flow analysis · Multipumping · Diazepam · Photo-degradation · Spectrofluorimetry

Introduction

Diazepam is a benzodiazepine that is used for the management of anxiety disorders or for the short-term relief of related symptoms. Diazepam may also be used to relieve agitation, shakiness, and hallucinations during alcohol withdrawal and to relieve certain types of muscle spasms. It may also be used to treat seizures, insomnia and other conditions.

D. S. M. Ribeiro · J. A. V. Prior (✉) · J. L. M. Santos · J. L. F. C. Lima
REQUIMTE, Department of Physical-Chemistry,
Faculty of Pharmacy, University of Porto,
Rua Aníbal Cunha 164,
4050-047 Porto, Portugal
e-mail: joaoavp@ff.up.pt

Nowadays, worldwide benzodiazepines consumption has reached very high values, mostly in developed countries, becoming a serious health problem as it frequently reached abuse levels. Either under prescription or by auto-medication, benzodiazepines abuse is frequently a consequence of their widespread availability and of their toxic effects causing dependence and habituation. Although death and serious illness problems resulting from benzodiazepine abuse are relatively rare, their combined administration with either alcohol or other medications could have fatal consequences. Being one of the most commonly used benzodiazepines, diazepam related health problems are a major public concern not only for its potential toxicity (an overdose of diazepam can be fatal) or as a consequence of abuse practices, but also due to inappropriate dose administrations, which requires the development of improved methods for the chemical control of diazepam in pharmaceutical formulations. Several methodologies have already been proposed for the monitoring of diazepam in pharmaceuticals such as thin-layer chromatography—densitometry technique [1], capillary electrophoresis and reversed phase-high performance liquid chromatography (RP-HPLC) [2], potentiometry using solid contact ion-selective electrodes [3], fluorometry [4], extractive-spectrophotometric method [5] and colorimetry [6, 7].

Distinct continuous flow methodologies resorting to a variety of detection techniques were also developed, such as, flow injection analysis combined with fluorimetry [8, 9] and UV spectrophotometry [10] and sequential injection lab-on-valve with potentiometric [11] detection.

Nevertheless, a fast, simple, low cost and miniaturized analytical system for diazepam determination would allow a more expeditious and simplified chemical control of the formulations, and most probably, the encouragement of the use of manipulated formulations with adapted drug dosages

for every clinical situation. Ideally, the use of adapted dosages should always be applied whenever treating a clinical illness.

In pharmaceutical chemical control, the main aim is to develop fast, simple, versatile and reliable methods, which can be readily adapted for routine analysis at relatively low cost. In this context, multipumping flow analysis can play a prominent role since, due to the specific nature of components that comprised the flow system, they enable the development of miniaturized flow systems allowing a higher degree of simplicity and improved operational versatility. Therefore, its application in the chemical control of pharmaceutical formulations represents an attractive perspective, as an advantageous alternative to the reference methodologies that usually involve chromatographic techniques, being these highly time consuming, expensive and often requiring the use of environmental hazard reagents. Besides, applying separation chromatographic techniques for the analysis of samples that exhibit usually an uncomplicated composition, and not requiring low detection limits, as is the case of pharmaceutical formulations, could be considered somehow excessive.

The multipumping flow analysis methodology [12] is based on the use of very small size solenoid actuated micropumps controlled by computer, that make possible a very simplified configuration of the flow system as well as its automatic control, since the basic operations in a chemical flow determination, namely, sample insertion, reagents addition, strategy for solutions mixing and transport of the reaction zone towards the detector unit are carried out by a single type component, being the solenoid micro-pumps the only manifold active elements. The fully automatic control of these devices, under time-based and pulse-counting routines, makes MPFS an attractive methodology for implementation of reliable and versatile analytical alternatives for determination of pharmaceutical compounds, with the additional advantage of permitting a runtime access to important analytical techniques and parameters, such as solutions manipulation (namely, stopped flow and reaction zone formation), flow rate and sample insertion.

The proposed miniaturized flow system enabled the determination of diazepam in commercially available formulations by spectrofluorimetric monitoring after the photo-degradation of the drug, which was accomplished by using of a low pressure UV radiation source coupled in line with the flow system. The proposed methodology was very simple, since it just involved a hydrolysis reaction catalyzed by UV radiation, which was only dependant on the solutions medium used. The use of a low pressure mercury lamp, instead of a high pressure arc lamp, avoided the cumbersome excessive heating of the solutions exposed to the radiation, thus preventing air bubbles formation that impair detection.

Materials and methods

Samples, standards and reagents

All solutions were prepared with doubly deionised water and analytical grade chemicals were used.

A solution containing 0.1 mol L^{-1} sodium dodecyl sulphate (SDS) was prepared by dissolving 7.21 g of SDS (Fluka®) in a 250 mL volumetric flask, using deionised water as solvent. A 2 mol L^{-1} NaOH solution was prepared by dissolving 40 g in 500 mL of deionised water.

The diazepam solutions were prepared from the pure drug supplied by "Laboratórios Bial" (Porto, Portugal). A 200 mg L^{-1} diazepam stock solution was prepared by dissolving 20 mg of diazepam in 40 mL of absolute ethanol (Panreac®, 99.5%) and diluted to 100 mL with deionised water. This stock solution was protected from the light and stored under refrigeration.

The working diazepam standards (5–40 mg L^{-1}) were prepared, on a daily basis, by appropriate dilution of the stock solution: aliquots of diazepam stock solution (0.5–5.0 mL) were transferred into a series of 20 mL volumetric flasks and 2 mL of 2 mol L^{-1} NaOH solution and 6 mL of 0.1 mol L^{-1} SDS solution, were added. In order to obtain the same ethanol concentration (20%, v/v) for all working diazepam standards, appropriate aliquots of absolute ethanol (Panreac®) were also added. The volume was subsequently made up to the mark with deionised water.

Ten commercially available pharmaceutical formulations containing diazepam were used to prepare sample solutions, by weighing and powdering a representative number of tablets. Afterwards, an appropriate amount of sample, corresponding to 2.5 g of diazepam, was dissolved in absolute ethanol (Panreac®, 99.5%) by stirring for 25 min. The resulting solutions were filtered, transferred to 50 mL volumetric flasks and diluted with deionised water. Finally, appropriate volumes of the obtained solutions, with diazepam concentrations of approximately 50 mg L^{-1}, were transferred to 20 mL volumetric flasks and 2 mL of a 2 mol L^{-1} NaOH solution and 6 mL of a 0.1 mol L^{-1} SDS solution were added. The final volume was subsequently made up with deionised water.

Apparatus

The detector used to monitor the fluorescence intensity (λ_{ex}=272 nm, λ_{em}=450 nm) was a spectrofluorimeter Jasco (Easton, MD, USA), model FP-2020/2025, equipped with a 16 µL internal volume flow cell.

The developed flow manifold comprised two 120SP solenoid actuated micro-pumps (Bio-Chem Valve Inc. Boonton, NJ, USA), of fixed displacement diaphragm type, dispensing 10 µL per stroke. All flow lines were made of

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0.8 mm i.d. PTFE tubing. Homemade end-fittings, connectors and confluences were also used.

The irradiation of the solutions was carried out by using a 15W Philips TUV 15W/G15T8 low pressure mercury lamp, emitting short-wave ultraviolet radiation with a maximum radiation peak at 253.7 nm.

Automatic control of the analytical system was accomplished by means of a Pentium based microcomputer and software developed using Microsoft Quick-Basic 4.5. A CoolDrive™ power drive board (NResearch Inc., West Caldwell, NJ, USA) was used to activate the solenoid of micro-pumps through the LPT1 computer port.

Operation of the flow manifold

The developed analytical flow system, depicted in Fig. 1, employed two solenoid micro-pumps which were responsible for the individual handling of sample and carrier solutions.

The micro-pump P_1 (Fig. 1) was responsible for inserting the diazepam solution, while micro-pump P_2 was used for inserting and propelling the 0.2 mol L^{-1} NaOH solution, which was used as carrier. This flow scheme allowed a higher versatility in the control of the parameters that influenced the extension of diazepam photo-degradation, particularly the time of exposure to UV radiation, as the placement of micro-pump P_1 responsible for samples insertion and transport prior to the UV light source, assembled in line with sample insertion tubing, easily enabled halting the flow for the time interval necessary to promote diazepam degradation.

The analytical cycle started by actuating P_1 for inserting the sample solution into the analytical manifold, filling the 2 m reactor tube (R) that was helically coiled around the UV lamp (L). The number of pulses was enough as to ensure that the reactor coil was completely filled with the sample solution, allowing, after sample irradiation, the carrying out of five consecutive measurements. Thereafter, P_1 was switched off and the UV lamp was turned on, starting the sample irradiation stage for a period of 15 min.

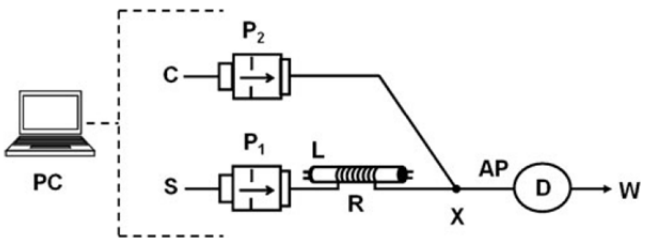

Fig. 1 Multipumping flow system (MPFS). *PC*—computer; *P1*, *P2*—solenoid micro-pumps (internal volume 10 µL); *X*—confluence point; *R*—2 m reactor; *D*—fluorimeter detector (λ_{ex}=272 nm; λ_{em}=450 nm); *L*—Philips UV lamp; *AP*—10 cm analytical path; *S*—sample; *C*—carrier (0.2 mol L^{-1} NaOH); *W*—waste

Following completion of the irradiation time, the lamp was turned off, and P_1 was reactivated, allowing the transport to waste of approximately 180 µL of non-irradiated sample solution. This non-irradiated volume was contained within the terminal 36 cm of the reaction tubing required to connect the UV sealed box (used to prevent any hazardous exposure) to confluence point X. This way, only the solution contained in the tubing coiled around the lamp was subjected to fluorimetric measurements. Subsequently, P_1 was switched off and by actuation of P_2 the NaOH carrier solution was inserted and propelled through confluence point (X) and through the detector (D) for cleanup and baseline establishment.

The analytical cycle proceeded with the activation of P_1, for the insertion of 100 µL of irradiated solution (ten pulses) in the analytical path. Then, by repeated actuation of micro-pump P_2, the reaction zone was carried toward the detector generating an analytical signal whose magnitude was proportional to the diazepam concentration. This process was repeated five times allowing the accomplishment of five consecutive measurements.

In all analytical procedures, the micro-pumps were operated at a pulse time of 0.2 s, establishing a flow rate of 1.71 mL min^{-1}.

Reference procedure

For assessing the accuracy of the results furnished by the developed procedure, commercially available pharmaceutical formulations containing diazepam were analyzed according to the reference procedure recommended by the British Pharmacopoeia [13], which rely on UV spectrophotometry. All pharmaceutical samples were weighed, powdered, and a determined amount was dissolved in a methanolic sulphuric acid solution, followed by dilution and filtration. The absorbance of the final solutions was determined by UV spectrophotometry at a wavelength of 284 nm.

Results and discussion

High pressure mercury lamps capable to emit radiation in the UV range have been used to carry out the photo-degradation of several drugs. Nevertheless, it is well know that these lamps produce a significant temperature increase in the solutions that could lead to undesirable chemical processes, parallel to those induced by the UV radiation. Moreover, in flow analysis increased temperatures result in oscillations in flow-rate and in the formation of air bubbles, leading to reduced reproducibility and sometimes even impaired detection. The use of high pressure arc UV lamps usually requires a cooling system, placed after irradiation,

to eliminate air bubbles formed at high temperatures [8]. To prevent this situation, and since preliminary results have shown that diazepam was adequately susceptible to UV irradiation, a low pressure mercury lamp was used to promote the photo-degradation of this compound. In order to evaluate the advantages in implementing the photo-degradation process in a miniaturized flow system by exploiting the multipumping concept, a comparison study of the results obtained upon diazepam exposure to UV radiation in glass beakers and inside a flow tube coiled around the UV lamp, was carried out. Therefore, batch assays were performed involving the exposure to UV radiation of several 200 mg L^{-1} diazepam solutions in 2 mol L^{-1} sulphuric acid contained in glass beakers, for 10 min, 25 min and 45 min. For the proposed flow-based procedure, a multipumping flow system comprising a UV lamp was implemented. A solution containing 50 mg L^{-1} diazepam in 2 mol L^{-1} sulphuric acid was inserted in the flow tubing coiled around the UV lamp, and exposed to the radiation by exploiting the stopped-flow technique, maximizing this way the radiations effects.

The results of the batch assays, involving the analysis of the recorded excitation and emission spectra, revealed that the effect of UV radiation for the assayed exposure intervals was insignificant and the formation of fluorescent compounds was negligible. On the other hand, when observing the results obtained from the flow-based procedure, it was concluded that even by exposing the solutions to UV radiation for a short time period of 10 min, a noteworthy production of fluorescent products (λ_{ex}=262 nm; λ_{em}= 463 nm) was accomplished, even taking into account that the parameters controlling the flow system were not optimized. These results confirmed that the fully integration of a UV light source in a miniaturized flow system, by coiling a flow tube around a UV lamp, exhibits several advantages, namely, the reduction of the distance between the solutions and the light source, the increase of the exposed area of solutions to radiation, the high reduction in the solutions volume used, and finally, the accomplishment of a fully automation of the whole analysis procedure. Therefore, it was concluded that the implementation of the photo-degradation of diazepam in a miniaturized flow system effectively promotes the hydrolysis of diazepam with improved efficiency, by means of increasing the reaction extension and the intensity of fluorescent signal.

In photochemical systems, the source intensity, irradiation time and medium composition, influence the nature and concentration of the degradation products formed in the process, which are frequently obtained via a multitude of possible pathways. Aiming at a high photo-degradation yield, some studies were conducted for optimization of chemical and physical parameters.

Optimization of chemical parameters

Since the pH of sample solutions can influence the extension of the photo-degradation process several studies involving the photo-degradation of diazepam in different acid and alkaline mediums were carried out. Simultaneously, several substances, namely, metal ions and surfactants, were tested for their capacity to increase the extension of the diazepam photo-degradation in acid and alkaline mediums. These studies were aimed at obtaining the higher fluorescence intensity for the degradation products. Several solutions were prepared and irradiated with UV light by making use of the developed flow system. Two groups of sample solutions were prepared: one containing 50 mg L^{-1} of diazepam in 0.050 mol L^{-1} sulphuric acid and a second one containing 50 mg L^{-1} of diazepam in an equimolar concentration of sodium hydroxide. Each of these solution groups was constituted by different solutions containing different substances that one would expect to increase the fluorescence intensities of the analyzed samples. The substances used were (i) Cu(II), Fe(III) and Ce(IV), in a concentration of 5×10^{-4} mol L^{-1}; (ii) non-ionic, cationic and anionic surfactants, respectively, methylcellulose (MC), cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS), in concentrations of 0.01% for MC and 0.01 mol L^{-1} for SDS and CTAB. The selected concentration values for the surfactants were higher than the respective critical micelle concentrations (cmc). For comparison purposes two reference solutions (RS) that contained only diazepam in the acid or alkaline medium (0.050 mol L^{-1} of sulphuric acid and sodium hydroxide, respectively) were used. All solutions were irradiated for 10 min.

A compilation of the assays and the obtained results is represented in Table 1.

The obtained results show a decrease of fluorescence intensity for the acid solutions containing the metal ions, SDS and CTAB surfactants, when comparing with the acid reference solution (Acid-RS). In the case of MC surfactant the differences of analytical signal comparatively to the reference solution were not significant. On the other hand, the assays in alkaline medium revealed a pronounced increase in fluorescence intensity when using Fe (III), MC and SDS, in relation to the alkaline reference solution (Alk-RS). Also, when using Cu (II) and Co (II) in alkaline medium a decrease in fluorescence intensity was observed. The highest increment value (110% regarding the reference solution) was obtained with SDS.

These results clearly demonstrate that enhanced fluorescence intensity is obtained under alkaline conditions in the presence of SDS. The formed micellar medium results in an organized microenvironment that promotes the photo-degradation reaction and/or the fluorescence emission. In

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Table 1 Evaluation of the influence of pH and different substances in the photo-degradation process of diazepam

	[Diazepam]	[Acid] or [Base]	[Tested substance]	λ_{ex} λ_{em}	Fluorescence intensity
Acid-RS			–		0.2871
Acid-Cu (II)			Cu (II)		0.2470
Acid-Fe (III)			Fe (III)	λ_{ex}=262 nm	0.0779
Acid-Ce (IV)	50 mg L^{-1}	$[H_2SO_4]$ = 0.050 mol L^{-1}	Ce (IV)	λ_{em}=463 nm	0.1889
Acid-CTAB			CTAB		0.1971
Acid-SDS			SDS		0.2592
Acid-MC			MC		0.2961
Alk-RS			–		0.1758
Alk-Cu (II)			Cu (II)		0.1233
Alk-Fe (III)	50 mg L^{-1}	[NaOH]=0.050 mol L^{-1}	Fe (III)	λ_{ex}=272 nm	0.2123
Alk-Ce (IV)			Ce (IV)	λ_{em}=450 nm	0.1697
Alk-SDS			SDS		0.3690
Alk-MC			MC		0.2420

order to discriminate between these two potential effects of SDS, some studies were performed involving the addition of the SDS reagent either before or after the irradiation stage. These studies were easily implemented in the flow system, due to its modular structure that allowed the inclusion of a third micro-pump, which was responsible for the insertion of SDS after the irradiation stage. Two different solutions of diazepam were prepared: one containing 50 mg L^{-1} of diazepam in 0.05 mol L^{-1} of NaOH, and a second one containing the same concentration of diazepam in 0.05 mol L^{-1} NaOH and 0.01 mol L^{-1} SDS. In the experiments, three different determinations using the flow system with three micro-pumps were conducted: (i) the first procedure, used as reference, involved the irradiation for 10 min of the 50 mg L^{-1} diazepam in 0.05 mol L^{-1} NaOH solution, followed by the insertion, using the merging zones approach, of ten pulses of the irradiated solution and ten pulses of 0.05 mol L^{-1} NaOH solution; (ii) in the second procedure, the 50 mg L^{-1} of diazepam in 0.05 mol L^{-1} NaOH solution was irradiated for 10 min followed by the insertion, using the merging zones approach, of ten pulses of the irradiated solution and ten pulses of 0.01 mol L^{-1} SDS solution; (iii) finally, the third procedure involved the irradiation for 10 min of the 50 mg L^{-1} diazepam in 0.05 mol L^{-1} de NaOH and 0.01 mol L^{-1} SDS solution, followed by insertion of ten pulses of irradiated solution and ten pulses of 0.05 mol L^{-1} NaOH solution by merging zones. For each of the described approaches, the 0.05 mol L^{-1} NaOH solution was used as carrier for the transport of the reaction zones towards the fluorescence detector.

The results revealed fluorescence intensities of 0.280 (±0.007), 0.256 (±0.004) and 0.580 (±0.02) for the (i), (ii) and (iii) procedures, respectively. Accordingly, it could be concluded that the use of SDS surfactant promoted the photoreaction development, since it was verified an increase of 109% in the fluorescence intensity when the diazepam solution was irradiated in the presence of SDS. For the subsequent assays, the solutions of diazepam were always prepared in NaOH and SDS solution.

Aiming at increasing the fluorescence intensities in the proposed methodology, several concentrations of sodium hydroxide (0.01–0.50 mol L^{-1}) were tested in solutions containing 50 mg L^{-1} diazepam and 0.01 mol L^{-1} SDS. After the UV irradiation stage for 10 min, by exploiting the stopped-flow method, 100 μL of sample (ten pulses) were transported towards the detector by using as carrier the corresponding solution of NaOH at the concentration under evaluation. The results showed that the fluorescence intensity increased at approximately up to a concentration of 0.20 mol L^{-1} of NaOH and, that for higher concentration values, the analytical signal tended to stabilization. For the subsequent assays a concentration of 0.20 mol L^{-1} of NaOH was selected.

The influence of the SDS concentration on fluorescence intensities was evaluated by analyzing several diazepam solutions containing different concentrations of SDS (0.001–0.05 mol L^{-1}). Several solutions containing 50 mg L^{-1} of diazepam, 0.20 mol L^{-1} NaOH and different concentrations of SDS, were inserted in the multipumping flow system and subjected to UV radiation for a period of 10 min. Afterwards, ten pulses of sample (100 μL) were transported to the fluorescence detector, using as carrier a 0.20 mol L^{-1} NaOH solution.

The obtained results demonstrated that the fluorescence intensity increased pronouncedly until a SDS concentration value of 0.030 mol L^{-1} and that for higher concentrations it approached stabilization. Thus, for the

remaining assays it was selected a SDS concentration of 0.030 mol L^{-1}.

The ethanol concentration used to prepare the stock solution of diazepam (200 mg L^{-1} in 40% ethanol), from which the diazepam standard solutions were prepared, could also affect the obtained fluorescence intensity. This way, the effect of ethanol in the analytical signal was assessed up to concentration value of 20% (v/v) but no significant differences in the fluorescence intensities were observed for all ethanol concentrations assayed.

Optimization of physical parameters

Since the fluorescence intensity could be influenced by the time of irradiation that determined the extension of diazepam photo-degradation, and by other parameters, namely the analytical path length (AP, Fig. 1), flow rate and sample volume inserted that could affect not only the residence time but also the extension of the reagents mixture and its dispersion inside the flow system, some studies were conducted aiming at the optimization of these physical parameters.

Different periods of irradiation with UV light, comprising 0 min, 1 min, 3 min, 5 min, 8 min, 10 min, 12 min, 15 min, 20 min and 25 min, were applied to solutions containing 50 mg L^{-1} diazepam in 0.20 mol L^{-1} NaOH and 0.030 mol L^{-1} SDS, by the stopped-flow technique. After the irradiation stages, 100 μL of irradiated sample was inserted into the system and transported toward the fluorescence detector for monitoring. The results revealed a small increase in fluorescence intensity when increasing the irradiation time up to approximately 5 min, but a pronounced increase of the fluorescence intensity signals between 5 min and 15 min. For irradiation periods higher than 15 min the fluorescence intensity tended to stabilization. Considering the results, an irradiation time of 15 min was selected for posterior assays, enabling this way the maximization of the photo-degradation process.

The optimization of the analytical path length (AP, Fig. 1) and inserted sample volume was performed simultaneously. This study involved the irradiation of solutions containing 50 mg L^{-1} diazepam in 0.20 mol L^{-1} NaOH and 0.030 mol L^{-1} SDS, for periods of 15 min. Next, by using a fixed pulse time of 0.2 s (1.71 mL min^{-1}), different sample pulses (two to 18 pulses, corresponding to 20–180 μL of sample) were inserted in the flow system and transported towards the detector for fluorescence intensity monitoring. This experiment was repeated for each of the AP lengths tested, namely, 10 cm, 30 cm, 60 cm and 85 cm. The obtained results, represented in Fig. 2, were in agreement with what would be expected, confirming that an AP length of 10 cm allowed obtaining the highest fluorescence intensity in all the experimental conditions. In

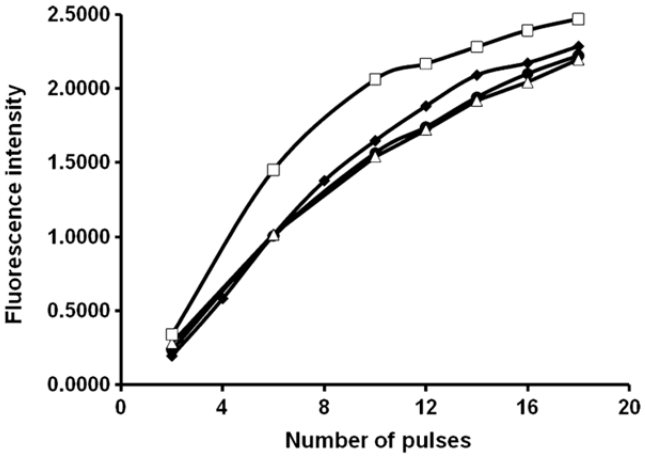

Fig. 2 Influence on analytical signal of the number of pulses using different AP lengths. □—10 cm; ♦—30 cm; ●—60 cm; △—85 cm

fact, a small AP length causes a minimized dispersion of the reaction zone and therefore an analytical signal maximization. Additionally, from Fig. 2 it can be observed an accentuated increase on the analytical signal up to approximately ten pulses (100 μL) and a subsequent stabilization for higher values. As a compromise between determination rate and sensitivity, this sample volume was selected for the analysis.

The study of the influence of flow rate on the fluorescence intensity signal was also performed. In this assay, different pulse times of 0.1 s, 0.2 s, 0.3 s and 0.4 s (corresponding to flow rates of 2.40 mL min^{-1}, 1.71 mL min^{-1}, 1.33 mL min^{-1}, 1.09 mL min^{-1}) were used to transport to the fluorescence detector the reaction zone, constituted by ten pulses (100 μL) of a previously irradiated solution (15 min) containing 50 mg L^{-1} diazepam in 0.20 mol L^{-1} NaOH and 0.030 mol L^{-1} SDS. After analysis of the results (Fig. 3), it was verified that the flow rate had a very small influence in the analytical signal intensity, for pulse times comprised between 0.2 s and 0.4 s, which is clearly explained by the short length of the reaction coil used. For 0.1 s of pulse time, the working frequency of the micro-pumps is high, and therefore the chaotic movement of the solutions inside tubing due to the characteristic pulsed flow nature of the MPFS originates a slightly increase of the sample dispersion, causing thus, a decrease in the fluorescence intensity. As a compromise between sampling rate and precision, a pulse time of 0.2 s (flow rate of 1.71 mL min^{-1}) was chosen.

Interferences

In order to apply the developed methodology to the determination of diazepam in pharmaceutical formulations, the influence of some compounds commonly used as excipients was assessed. Different sample diazepam sol-

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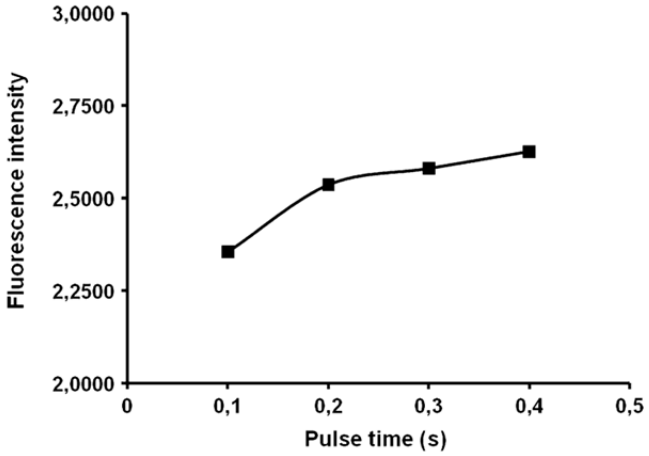

Fig. 3 Influence on analytical signal of different pulse times: 0.1 s, 0.2 s, 0.3 s and 0.4 s (corresponding to flow rates of 2.40 mL min^{-1}, 1.71 mL min^{-1}, 1.33 mL min^{-1}, 1.09 mL min^{-1})

utions containing a fixed amount of diazepam (20 mg L^{-1}) and different quantities of the excipients under evaluation were analyzed by the developed methodology. A compound was considered as non-interfering if the analytical signal variation was ±4% when compared to the analytical signal obtained in the absence of the referred compound. The results revealed that the excipients hypromellose and cellulose up to a 100-fold mass ratio, starch, povidone and colloidal silica up to a 50-fold mass ratio, and lactose up to a 25-fold mass ratio, did not interfere.

Chemical control of pharmaceutical formulations

By using the previously referred optimized experimental conditions a linear response range for diazepam concentrations of up to 40 mg L^{-1} was obtained. The calibration curve was represented by $FI = 0.0451\ (\pm\ 0.0004) \times C - 0.0083\ (\pm\ 0.0080)$, in which FI was the fluorescence intensity and C was diazepam concentration, in mg mL^{-1}. A correlation coefficient of 0.9998 was verified. The detection limit calculated from the equation of the calibration curve according to Miller and Miller [14] was about 0.97 mg L^{-1}.

In order to validate the proposed methodology, based in a miniaturized multipumping flow system, the results obtained in the determination of diazepam in ten commercial pharmaceutical formulations were compared with those furnished by a reference procedure of the British Pharmacopoeia [13]. The results, summarized in Table 2, showed a good agreement between both methods, with relative deviations between −2.09% and 2.13%. A paired Student's t-test [14] confirmed that there were no statistical differences ($t_{estimated}$=0.474, $t_{tabulated}$= 2.260) between the results obtained by both procedures, for a confidence level of 95% (n=10).

The results attained in the evaluation of the developed methodology precision, by repeated analysis (four consecutive determinations) of each commercial pharmaceutical formulation revealed a good repeatability (Table 2), at a confidence level of 95%.

The developed methodology allowed a determination rate of 14 h^{-1}.

Conclusions

The implementation of an automated multipumping flow system with a photo-degradation unit for diazepam determination, based on the susceptibility of this benzodiazepine to UV irradiation and the subsequent generation of fluorescent products further reinforced by a micellar medium, proved to be a valuable alternative for the chemical control of this drug, since the developed flow system was very simple to operate and control and exhibited high versatility and low reagents consumption, when in comparison with traditional methods, like for example, chromatographic procedures.

Table 2 Comparison of analytical results obtained in the determination of diazepam in pharmaceutical formulations by the proposed and the reference method

Pharmaceutical sample	Declared dosage mg/formulation	Amount found (mg/formulation)[a]		R. D. %[b]
		MPFS methodology	Reference method	
Valium roche 10	10	9.75±0.03	9.9±0.4	−1.40
Valium roche 5	5	4.93±0.04	4.89±0.05	0.92
Diazepam labesfal 10	10	10.1±0.1	10.02±0.01	1.16
Diazepam labesfal 5	5	4.87±0.04	4.81±0.09	1.29
Bialzepam 3	3	2.88±0.07	2.86±0.01	0.69
Bialzepam 6	6	5.68±0.01	1.51±0.05	0.67
Diazepam ratiopharm 10	10	10.0±0.2	10.10±0.02	−0.06
Diazepam ratiopharm 5	5	4.90±0.06	4.80±0.02	2.13
Metamidol winthrop 10	10	10.06±0.08	9.95±0.08	1.14
Metamidol winthrop 5	5	4.82±0.05	4.9±0.3	−2.09

[a] Mean ± *t0.05* (Student's t test) × $(S/\sqrt{n})$

[b] Relative deviation of the development method regarding the reference procedure

The developed MPFS can be further applied in the monitoring of natural fluorescence substances, or of their degradation compounds, obtained by photo-degradation through exposure to UV radiation, assuming that they exhibit fluorescence properties. The UV lamp coupled to the developed MPFS can be easily turned on or off, emphasizing the versatility of the analytical system. Additionally, by implementing a UV lamp in an automatic flow system the life-time of the lamp is increased, since the system control enables the use of the lamp only when it is strictly necessary. Moreover, it contributes to a lower power consumption and a lower and more stable operational temperature within the flow analytical system that present several advantages such as to avoid the formation of air bubbles and to prevent fluctuations in flow rate and interferences in the analytical signal reproducibility and magnitude.

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CHAPTER 6

Automatic miniaturized fluorometric flow system for chemical and toxicological control of glibenclamide

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Automatic miniaturized fluorometric flow system for chemical and toxicological control of glibenclamide☆

David S.M. Ribeiro, João A.V. Prior*, Christian J.M. Taveira, José M.A.F.S. Mendes, João L.M. Santos

Requimte, Department of Chemical Sciences, Laboratory of Analytical Chemistry and Physical-Chemistry, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha n° 164, 4099-030, Porto, Portugal

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ABSTRACT

In this work, and for the first time, it was developed an automatic and fast screening miniaturized flow system for the toxicological control of glibenclamide in beverages, with application in forensic laboratory investigations, and also, for the chemical control of commercially available pharmaceutical formulations. The automatic system exploited the multipumping flow (MPFS) concept and allowed the implementation of a new glibenclamide determination method based on the fluorometric monitoring of the drug in acidic medium (λ_{ex} = 301 nm; λ_{em} = 404 nm), in the presence of an anionic surfactant (SDS), promoting an organized micellar medium to enhance the fluorometric measurements.

The developed approach assured good recoveries in the analysis of five spiked alcoholic beverages. Additionally, a good agreement was verified when comparing the results obtained in the determination of glibenclamide in five commercial pharmaceutical formulations by the proposed method and by the pharmacopoeia reference procedure.

1. Introduction

Glibenclamide, is a potent, second generation antidiabetic drug in a class of medications known as sulfonylureas widely used to lower glucose levels in patients with type II non-insulin-dependent diabetes mellitus.

Alongside its therapeutic use, a literature survey reveals that glibenclamide has been subject to an increasing attention among the drugs that are used to incapacitate victims, mainly youngsters or elderly, with a criminal purpose that include sexual abuse or robbery. On the other hand, some reports have emphasized the danger of hypoglycemia with glibenclamide, even at low dose, especially among the elderly due to classic overdosage, leading to the use of this drug to commit suicides or even homicides [1,2]. Sedation induced by glibenclamide can be observed as overdosage presents lethargy. This can be a cause of incapacitation of a victim. Diagnosis can be difficult to achieve when aiming to the discrimination between insulinoma and antidiabetic drug-induced hypoglycemia. In order to differentiate between these two causes of hypoglycemia, antidiabetics must be screened and their concentrations evaluated.

Being glibenclamide one of the most commonly used sulfonylureas for type II diabetes treatment, related health problems are a major public concern not only for its potential toxicity (an overdose of glibenclamide can be fatal) or as a consequence of abuse practices (suicides or homicides), but also due to inappropriate dose administrations (very common among old people), putting in evidence the need for simple and expeditious methods for its chemical control. If one analyses the broad application of glibenclamide in our days, as stated before, it is emphasized the need to develop straightforward and versatile automatic procedures to carry out its screening in drug formulations that can be easily manipulated nowadays, as well as, in beverages that might be the "vehicles" for its surreptitious administration.

Several methodologies were already proposed for the determination of glibenclamide in pharmaceutical formulations involving chromatographic [3–12], potentiometric [13] and fluorometric [14] procedures. These methods are highly time consuming, expensive and often require the use of environmental hazard reagents. Besides, it could be considered somehow excessive applying chromatographic techniques for the analysis of samples that exhibit usually an uncomplicated composition, and not requiring low detection limits, as is the case of pharmaceutical formulations. Therefore, automatic flow analysis can play a prominent role in the chemical control of pharmaceutical formulations since it enables the development of miniaturized flow systems allowing a higher degree of simplicity and improved operational versatility.

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* Corresponding author at: Requimte, Department of Chemical Sciences, Laboratory of Analytical Chemistry and Physical-Chemistry, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha n° 164, 4099-030, Porto, Portugal.
Tel.: +351 222 078 994; fax: +351 222 078 961.
E-mail address: joaoavp@ff.up.pt (J.A.V. Prior).

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More important, when considering the toxicological screening of beverages for the presence of glibenclamide, not a single scientific work can be found in the literature. Normally, in criminal situations where exists the suspicion of drug abuse with glibenclamide, only biological testing is performed (e.g. hair through LC–MS/MS [15]), which involves laborious assays in a laboratory environment, even considering the risk of not obtaining any validated result due to the short half-lives of most drugs in biological fluids. Glibenclamide has been reported to be cleared within 5 h in human plasma.

This work was the first attempt to develop an automatic and fast screening miniaturized flow system for the chemical control of commercially available pharmaceutical formulations containing glibenclamide, and also, for the toxicological control of some beverages possibly used in hypothetic criminal practices, and thus, it may contributes to build strong legal evidences in a drug-facilitated crime. The implementation in a flow system of an originally developed glibenclamide detection procedure capable of rapidly quantify the drug in some alcoholic beverages and pharmaceutical formulations, documents the importance of versatile and miniaturized detection methods in forensic situations requiring the analysis of medications and food samples collected in crime scenes.

The exploitation of the multipumping flow concept constitutes a major contribution in the development of compact and portable analytical flow systems allowing a higher degree of automation, operational simplicity and improved versatility, bringing together all the advantages associated to miniaturization of flow analysis procedures.

In this work, the originally developed quantification method was based on the fluorometric monitoring of glibenclamide in acidic medium (λ_{ex} = 301 nm; λ_{em} = 404 nm), in the presence of an anionic surfactant (SDS), promoting an organized micellar medium to enhance the fluorometric measurements. The analytical potential of this novel chemical approach for glibenclamide determination was further enhanced through its implementation in a fully automated multipumping flow system [16].

2. Experimental

2.1. Apparatus

The developed flow manifold comprised three 120SP solenoid actuated micro-pumps (Bio-Chem Valve Inc., Boonton, NJ, USA), of fixed displacement diaphragm type, dispensing 10 μL per stroke. All flow lines were made of 0.8 mm i.d. PTFE tubing. Homemade end-fittings, connectors and confluences were also used.

The detector used to monitor the fluorescence intensity (λ_{ex} = 301 nm, λ_{em} = 404 nm) was a fluorometer Jasco (Easton, MD, USA), model FP-2020/2025, equipped with a 16 μL internal volume flow cell.

Automatic control of the analytical system was accomplished by means of a Pentium based microcomputer and software developed using Microsoft Quick-Basic 4.5. A CoolDrive™ power drive board (NResearch Inc., West Caldwell, NJ, USA) was used to activate the solenoid of micro-pumps through the LPT1 computer port.

2.2. Samples, standards and reagents

All solutions were prepared with doubly deionised water and analytical grade chemicals were used.

A 200 mg L^{-1} glibenclamide stock solution was prepared by dissolving 50 mg of the bulk drug (Sigma®, ≥99%) in 250 mL of absolute ethanol (Panreac®, 99.5%). This stock solution was protected from the light and stored under refrigeration.

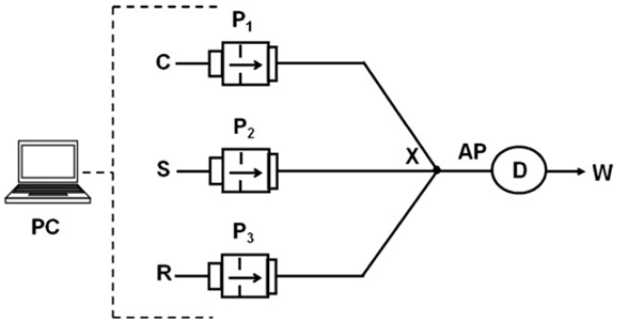

Fig. 1. Multipumping flow system (MPFS). PC, microcomputer; P_1–P_3, solenoid micro-pumps (internal volume 10 μL); X, confluence point; D, fluorometer detector (λ_{ex} = 301 nm and λ_{em} = 404 nm); AP, 10 cm analytical path; S, sample: glibenclamide in 50% ethanol/0.2 mol L^{-1} H_2SO_4; C, carrier: 0.2 mol L^{-1} H_2SO_4; R, 0.03 mol L^{-1} SDS; W, waste.

A solution containing 0.1 mol L^{-1} sodium dodecyl sulphate (SDS) was prepared by dissolving 7.21 g of SDS (Fluka®) in a 250 mL volumetric flask, using deionised water as solvent.

The working glibenclamide standards (5–75 mg L^{-1}) were prepared, on a daily basis, by appropriate dilution of the stock solution: aliquots of glibenclamide stock solution (0.5–7.5 mL) were transferred into a series of 20 mL volumetric flasks and 2 mL of 2 mol L^{-1} H_2SO_4 solution was added. In order to obtain the same ethanol concentration (50%, v/v) for all working glibenclamide standards, appropriate aliquots of absolute ethanol (Panreac®) were also added. The volume was subsequently made up to the mark with deionised water.

Five commercially available pharmaceutical formulations containing glibenclamide were used to prepare sample solutions, by weighing and powdering a representative number of tablets. Afterwards, an appropriate amount of sample, corresponding to 3.0 g of glibenclamide, was dissolved in absolute ethanol (Panreac®, 99.5%) by stirring for 25 min. The resulting solutions were filtered, transferred to 50 mL volumetric flasks and diluted with deionised water. Finally, appropriate volumes of the obtained solutions, with glibenclamide concentrations of approximately 60 mg L^{-1}, were transferred to 20 mL volumetric flasks and 2 mL of a 2 mol L^{-1} H_2SO_4 solution was added. The final volume was subsequently made up with deionised water. At last, the obtained solution was filtered with a syringe filter i.d. 0.20 μm (Corning®) before its insertion in the flow system.

Five commercial alcoholic beverages were selected for analysis. The drinks were spiked aiming at a glibenclamide concentration of 50 mg L^{-1}, by adding 6.25 mL of the 200 mg L^{-1} glibenclamide stock solution into a 25 mL volumetric flask and the final volume completed with the respective alcoholic beverage under analysis. Then, 2 mL of 2 mol L^{-1} H_2SO_4 solution were transferred into a series of 20 mL volumetric flasks, and the volume was made up to the mark with the respective alcoholic beverage previously spiked.

2.3. Flow manifold

The developed flow system exploiting the MPFS approach for the fluorometric determination of glibenclamide is depicted in Fig. 1. The manifold employed three solenoid micro-pumps (P_1, P_2, P_3), which were responsible for the individually handling of three different solutions.

The micro-pump P_1 was responsible for insertion and propelling of the 0.2 mol L^{-1} sulphuric acid solution, which was used as carrier; P_2 and P_3 were used for inserting the sample and 0.03 mol L^{-1} SDS solutions, respectively.

Preceding the analytical cycle, all flow tubing had to be filled with the corresponding solution. Then by actuating P_1, the sul-

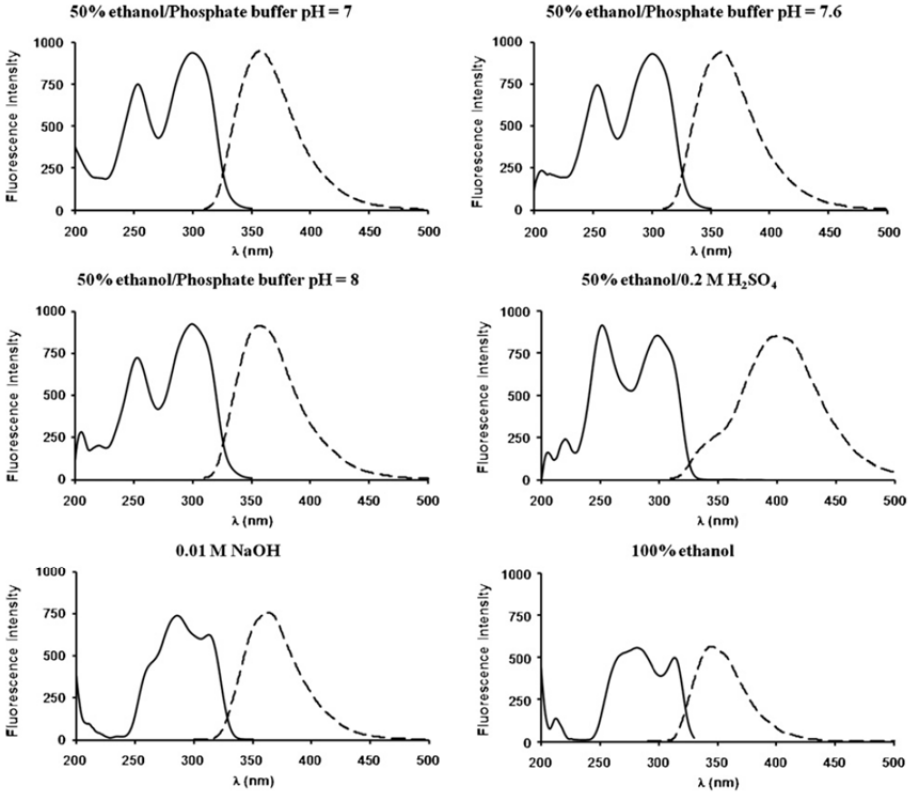

Fig. 2. Fluorescence excitation and emission spectra of glibenclamide solutions prepared in different reaction media.

phuric acid solution was inserted and carried out towards detection in order to establish baseline.

The analytical cycle started by intercalating a unique volume of sample solution between two identical plugs of the SDS solution. The sampling stage consisted in the insertion of the SDS solution for a pre-set number of pulses, through the actuation of micro-pumps P_3 (total volume of 10 μL per stroke), at a fixed pulse time of 0.2 s, corresponding to a pulse frequency of 171 min^{-1}, which defined the flow rate at 1.71 mL min^{-1}. Then, by inserting a pre-set number of pulses of micro-pump P_2, at a fixed pulse time of 0.2 s, a unique volume of sample solution was intercalated with another identical small plug of the SDS solution.

Thereafter, the established reaction zone was carried out towards the detector through the repeated actuation of P_1 (10 μL per stroke), at a fixed pulse time of 0.2 s, corresponding to a pulse frequency of 171 min^{-1}, which defined the flow rate at 1.71 mL min^{-1}. The fluorescence emission of the compound was monitored at 404 nm (λ_{ex} = 301 nm).

2.4. Reference procedure

For validation of the results furnished by the developed methodology, the pharmaceutical formulations containing glibenclamide (tablets) were also analyzed by reversed-phase liquid chromatography, according with the reference methodology [17].

3. Results and discussion

3.1. Preliminary assays

Some preliminary batch assays, aiming at evaluating the fluorescence properties of glibenclamide in different reaction media, were performed by carrying out the fluorescence excitation and emission spectra of six different glibenclamide standard solutions containing 50 mg L^{-1} of the drug. From this set of solutions, three were prepared in 50% ethanol and phosphate buffer with different pH values of 7, 7.6 and 8, whilst of the three remaining solutions one was prepared in 50% ethanol and 0.2 mol L^{-1} of H_2SO_4, another in alkaline medium, containing 0.01 mol L^{-1} NaOH and the last one in 100% ethanol. The selection of the composition of these solutions used to evaluate the glibenclamide fluorescence properties was based and dependent on the characteristic dissolution profile of the drug [18].

The analysis of the recorded glibenclamide excitation and emission spectra of the previous solutions (Fig. 2) demonstrated that the solutions prepared in 50% ethanol/phosphate buffer with a pH of 7, 7.6 and 8 exhibited maximums λ_{ex} = 254 and λ_{ex} = 300 nm and a maximum λ_{em} = 357 nm. Additionally, the results revealed no significant differences on the fluorescence excitation and emission spectra between the different fixed values of pH with phosphate buffer. A glibenclamide solution prepared in 50% ethanol/0.2 mol L^{-1} of H_2SO_4 exhibited λ_{ex} = 252 nm and λ_{ex} = 301 nm and λ_{em} = 404 nm. When the glibenclamide was dissolved in an alkaline solution of 0.01 mol L^{-1} NaOH it revealed fluorescence properties with maximum λ_{ex} = 284 nm and λ_{em} = 363 nm. For the solution of glibenclamide in 100% ethanol, the recorded spectra demonstrated that when the drug was excited at a wavelength 282 nm and/or 315 nm emitted a weak fluorescence at λ_{em} = 345 nm. Other compositions for glibenclamide solutions were assayed in order to evaluate fluorescence properties involving lanthanides, namely, europium and terbium, but they revealed that no detectable increment in fluorescence was measured.

Subsequently, after obtaining the maximum λ_{ex} and λ_{em} for each different solution of glibenclamide previously prepared, the fluorometric detection procedure was implemented in a miniaturized and automated analytical flow system based in the

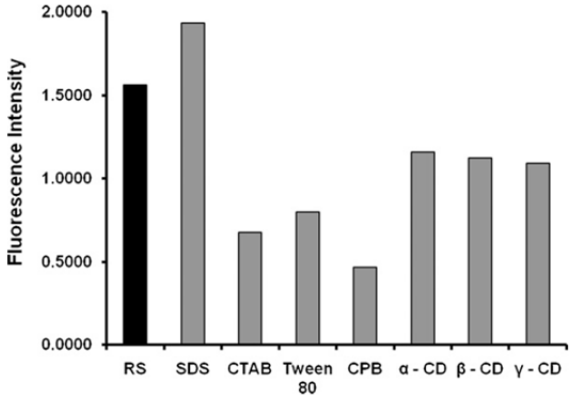

Fig. 3. Influence of surfactants and cyclodextrins on analytical signal. RS – reference sample; SDS – sodium dodecyl sulphate; CTAB – cetyl trimethylammonium bromide; CPB – cetylpyridinium bromide; α-CD – α-cyclodextrin; β-CD – β-cyclodextrin; γ-CD – γ-cyclodextrin.

multipumping concept, with the aim of confirming which one of the solutions media under evaluation allowed achieving the maximization of the analytical signal. The solutions containing 50 mg L^{-1} of glibenclamide dissolved in the different studied reaction media were inserted into the developed miniaturized multipumping flow system and the intensity of fluorescence for each of the solutions was recorded. For this, a MPFS with only two micro-pumps was used, in which, one micro-pump was responsible for the sample insertion and the other was used to propel the carrier solution (solution media under evaluation). The results showed that the glibenclamide solution prepared in a mixture of 50% ethanol and 0.2 mol L^{-1} sulphuric acid exhibited a higher fluorescence intensity (FI), approximately 165% when compared with the ones obtained with the others studied reactions media. Thus, a solution of glibenclamide prepared in 50% ethanol/0.2 mol L^{-1} sulphuric acid (λ_{ex} = 301 nm and λ_{em} = 404 nm) was selected to perform the optimization of the flow system.

3.2. Optimization of chemical parameters

It is documented in the scientific literature that the presence of reagents such as surfactants and sensitizers can have a strong effect on the fluorescence signal. In fact, some surfactants are known to originate an organized micellar system in the surrounding medium for concentrations higher than its CMC, increasing the analyte's concentration on the micellar surface, causing changes in the photophysical properties of analytes by improving its susceptibility to photoradiation and enhancing fluorescence emission.

Therefore, some experiments, with the goal of increasing the fluorescence intensity, were performed by studying the influence on analytical signal of the presence of different substances. Surfactants such as sodium dodecyl sulphate (SDS), cetyl trimethylammonium bromide (CTAB), Tween 80 and cetylpyridinium bromide (CPB) were used at concentrations above their critical micelle concentration (cmc). With the aim of assuring that all the surfactants used in the assay were at concentrations higher than their CMC, a concentration of 0.02 mol L^{-1} was selected for the surfactants SDS, CTAB and CPB, whilst for Tween 80 was 0.01%. Also, some cyclodextrins, namely, α, β and γ-cyclodextrin were used at a concentration of 5×10^{-3} mol L^{-1}. The assays were performed in the MPFS previously used, but comprising a third micro-pump that was responsible for the insertion of the studied solutions. The latter was merged with the stream of the glibenclamide sample solution by using the merging zones approach. A carrier solution of sulphuric acid 0.2 mol L^{-1} was used. Fig. 3 represents a compilation of the obtained results and a comparison with the FI originated by a 50 mg L^{-1} glibenclamide standard solution (reference sample). The analysis of the results demonstrated a decrease in FI for the glibenclamide solutions merged with α, β and γ-cyclodextrins, CTAB, Tween 80 and CPB surfactant solutions. However, by merging a solution of an anionic surfactant such as SDS to the glibenclamide solution, an increase of approximately 24% on the fluorescence signal was observed relatively to the reference sample. These results indicate that the use of SDS improves the sensitivity for the glibenclamide determination, most probably because the organized microenvironment promoted by this surfactant improved energy transfer and fluorescence efficiencies [19].

Subsequently, the influence of sulphuric acid (used as carrier) and SDS concentration on the analytical signal was assessed over a concentration range from 0.005 to 2 mol L^{-1} and 0.001 to 0.075 mol L^{-1}, respectively. The study of the influence of sulphuric acid concentration on the analytical signal was performed using several sample solutions all containing 50 mg L^{-1} of glibenclamide, 50% ethanol and different H_2SO_4 concentrations under evaluation. For these assays, a volume of 100 μL (10 pulses) of sample solution was inserted in the developed MPFS (Fig. 1) and merged with the same volume of 0.02 mol L^{-1} SDS solution. Next, the reaction zone was carried towards the detection with the sulphuric acid solution under evaluation. In this study, a significant increase of FI was observed for sulphuric acid concentrations up to 0.20 mol L^{-1}, whilst for higher concentrations stabilization of FI occurred. Thus, a concentration of 0.20 mol L^{-1} sulphuric acid was selected for further optimization assays.

In the assessment of the influence of SDS concentration on the analytical signal, a volume of 100 μL (10 pulses) of sample solution (50 mg L^{-1} of glibenclamide in 50% ethanol and 0.20 mol L^{-1} H_2SO_4) was merged with the same volume of solution containing different SDS concentrations under evaluation (0.001–0.075 mol L^{-1}), and subsequently the reaction zone was carried towards the detector with the 0.20 mol L^{-1} H_2SO_4 carrier solution. The obtained results showed an increase in FI for a SDS concentration of up to 0.01 mol L^{-1}, which was the value selected for the subsequent assays.

3.3. Optimization of physical parameters

Since the analytical signal could be influenced by physical parameters such as the flow rate, analytical path length (Fig. 1, AP), sample volume and sampling strategy that could affect the extension of the reagents mixture and also its dispersion inside the flow system, some optimization studies involving these parameters were carried out aiming at the maximization of the analytical signal.

The optimization of the analytical path length and the sample volume inserted in the flow system were assessed simultaneously. In this study, for each AP length evaluated, namely 10, 20, 40, 65 and 110 cm, was varied the number of pulses between 2 and 16 (corresponding to sample volumes between 20 and 160 μL) of sample solution (50 mg L^{-1} of glibenclamide in 50% ethanol/0.20 mol L^{-1} H_2SO_4) merged with the same number of pulses of a 0.01 mol L^{-1} SDS solution. The carrier was a solution 0.20 mol L^{-1} in H_2SO_4. The reaction zone was carried towards detection by actuating the micro-pump responsible for carrier insertion with a pulse time of 0.2 s, which corresponded to a flow rate of about 1.71 mL min^{-1}. The obtained results (Fig. 4) showed that for an AP length of 10 cm the highest analytical signal was achieved in all the experimental conditions, and that FI increased until a sample volume of approximately 10 pulses. The smallest AP length ensures the minimization of dispersion phenomena of the reaction zone and, consequently, enabled the maximization of FI.

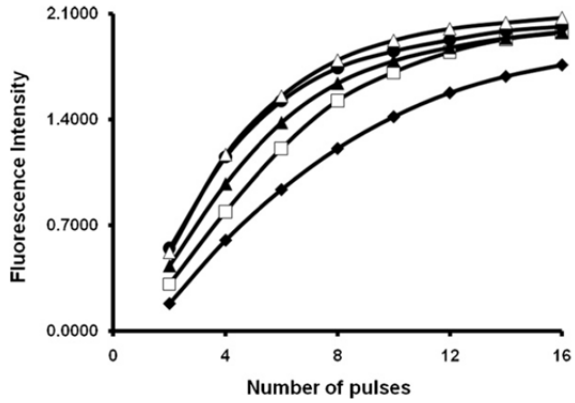

Fig. 4. Influence on the analytical signal of different path lengths. (♦) 110 cm; (□) 65 cm; (▲) 40 cm; (●) 20 cm; (△) 10 cm.

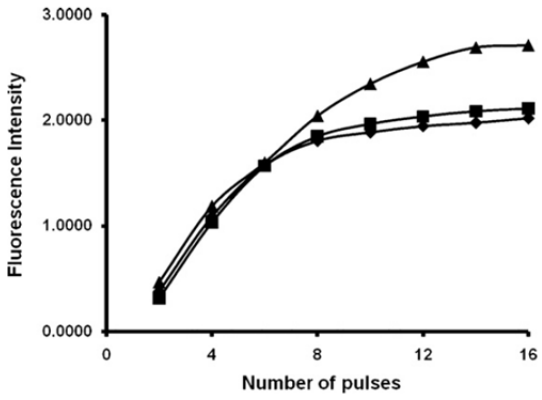

Fig. 6. Influence on the analytical signal of sampling strategies. (▲) Unique volume; (■) merging zones; (♦) binary sampling.

The study of the influence on the analytical signal of flow rate was also performed simultaneously with the inserted sample volume. In these assays, for each different pulse time of 0.1, 0.15, 0.2, 0.3 and 0.4 s (corresponding to flow rates of 2.40, 2.00, 1.71, 1.33, 1.09 mL min^{-1}), the number of inserted sample pulses was also varied between 2 and 16 (corresponding to volumes between 20 and 160 μL) of a solution containing 50 mg L^{-1} glibenclamide in 50% ethanol/0.20 mol L^{-1} H_2SO_4. In this study, the parameter that demonstrated a more pronounced influence on analytical signal was sample volume, which revealed an accentuated increase on the FI up to approximately 10 pulses (100 μL), tending towards stabilization (Fig. 5, graphic A) for higher values. On the other hand, it was verified that the pulse time (flow rate) had a very small influence in analytical signal, which could be explained by the small length of the analytical path (10 cm). However, by fixing the sample volume to 10 pulses and by analysing the variation of the FI with the pulse time, it was verified an increase of the analytical signal up to 0.2 s and a subsequent decrease in fluorescence intensity for higher values (Fig. 5, graphic B). Therefore, as a compromise between determination rate and sensitivity, a pulse time of 0.2 s (corresponding to a flow rate of 1.71 mL min^{-1}) and 10 pulses of sample (corresponding to a sample volume of 100 μL) was chosen.

Another flow parameter of great importance was the sampling strategy, since it could influence the degree of reagents mixture, and hence, the supramolecular organization in the reaction zone, being this fact determinant in the FI measured. Thus, some assays were carried out by exploiting different sampling approaches, such as merging zones, binary sampling and unique sample volume, and

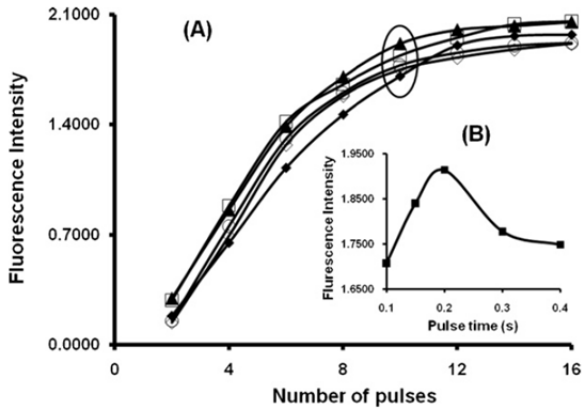

Fig. 5. Influence on the analytical signal of the pulse time (flow rate) and number of sample pulses (sample volume). (A) and (B) explained in the text. (♦) 0.1 s; (□) 0.15 s; (▲) 0.2 s; (○) 0.3 s; (◊) 0.4 s.

at the same time varying the number of pulses of the inserted sample solution within 2 and 16. The study of sampling strategy with number of sample pulses was important because, this way, a fine optimization of the number of pulses could be performed, taking into account that, the two mentioned parameters are very dependent one on another. For the accomplishment of these assays, the pulse time was modified and adjusted for each evaluated sampling strategy, in order to achieve a similar flow rate throughout the system during sample insertion and transport stages. This was important because when micro-pumps are activated simultaneously, theoretically the overall flow rate corresponds to the sum of the flow rates of all propelled solutions.

The obtained results (Fig. 6) demonstrated that there were no significant differences in the analytical signal for the three sampling strategies when inserting up to 6 pulses of sample solution in the flow system. However, for values higher than 6 sample pulses, a pronounced increase in analytical signal was observed when using the unique volume strategy comparatively with the others sampling strategies.

Accordingly with the principle of sampling strategy associated with binary sampling or merging zones, it should be expected from the results a higher efficiency in the mixture of sample and SDS solutions relatively to the unique volumes strategy. Nevertheless, the obtained results showed a higher FI when using the unique volumes strategy, indicating that a higher degree of mixture actually caused an increase in the dilution of the reaction zone, impairing the analytical signal. In this case, the influence on analytical signal of the degree of dilution markedly compromised the supramolecular organization created by SDS, in the enhancement of the fluorescence signal. Taking into account this data, and in order to obtain a better comprehension on the influence of SDS surfactant in combination with a unique volumes strategy (low degree of mixture), it was performed an assay involving again the study of the influence of the concentration of SDS on analytical signal when using the unique volumes strategy. As expected, and with the aim of assuring a micellar organization of the reaction zone to enhance the FI, a 0.03 mol L^{-1} SDS concentration was chosen for the determination of glibenclamide in samples.

3.4. Analysis of pharmaceutical formulations

In order to apply the developed methodology to the determination of glibenclamide in pharmaceutical formulations, the influence of some compounds commonly used as excipients was assessed. Different sample solutions containing a fixed amount of glibenclamide (30 mg L^{-1}) and different quantities of the excipients under evaluation were analyzed by the developed methodology. A com-

Table 1
Comparison of analytical results obtained in the determination of glibenclamide in pharmaceutical formulations by the proposed and the reference method.

Sample	Declared dosage (mg/formulation)	Amount found (mg/formulation)[a]		R. D. (%)[b]
		MPFS methodology	Reference method	
Pharmaceutical sample 1	2.5	2.52 ± 0.02	2.54 ± 0.08	0.82
Pharmaceutical sample 2	5	4.97 ± 0.05	4.976 ± 0.004	0.09
Pharmaceutical sample 3	2.5	2.47 ± 0.01	2.39 ± 0.02	−3.53
Pharmaceutical sample 4	5	4.86 ± 0.02	4.829 ± 0.001	−0.73
Pharmaceutical sample 5	2.5	2.43 ± 0.04	2.45 ± 0.07	0.74

[a] Mean ± $t0.05$ (Student's t-test) × $(S/\sqrt{n})$.
[b] Relative deviation of the developed method regarding the reference procedure.

Table 2
Results obtained in the toxicological control of glibenclamide in spiked alcoholic beverages.

Spiked sample	Concentration added (mg L^{-1})	Concentration found (mg L^{-1})[a]	Recovery (%)
Sample brand 1	50.0	49.3 ± 0.9	98.6
Sample brand 2	50.0	50 ± 2	99.2
Sample brand 3	50.0	49.8 ± 0.8	99.6
Sample brand 4	50.0	49 ± 1	98.7
Sample brand 5	50.0	49 ± 3	98.7

[a] Mean ± $t0.05$ (Student's t-test) × $(S/\sqrt{n})$.

pound was considered as non-interfering if the analytical signal variation was ±4% when compared to the analytical signal obtained in the absence of the referred compound. The results revealed that the excipients hypromellose, lactose, cellulose starch, povidone, magnesium stearate and colloidal silica up to a 100-fold mass ratio, did not interfere.

The linear working response range was assessed by inserting in the MPFS, several standard solutions containing different concentrations of glibenclamide and by using the previously referred optimized physical and chemical parameters. A linear response range for glibenclamide concentrations of up to 75 mg L^{-1} was obtained and the calibration curve was represented by FI = 0.0512 (±0.0007) × C + 0.09 (±0.03), R = 0.9992, in which FI was the fluorescence intensity and C was glibenclamide concentration, in mg L^{-1}. The detection limit calculated from the equation of the calibration curve according to Miller and Miller [20] was about 2.75 mg L^{-1}.

The validation of the proposed automatic methodology, based in a miniaturized multipumping flow system, was accomplished by comparison of the results obtained in the determination of glibenclamide in 5 commercial pharmaceutical formulations with those furnished by the reference procedure of the British Pharmacopoeia [17]. The results, summarized in Table 1, showed a good agreement between both methods, with relative deviations between −3.53 and 0.82%. Additionally, a paired Student's t-test [20] confirmed that there were no statistical differences ($t_{estimated}$ = 0.763, $t_{tabulated}$ = 2.776) between the results obtained by both procedures, for a confidence level of 95% (n = 5).

An evaluation of the precision of the proposed MPFS for glibenclamide determination in pharmaceutical formulations, through the repeated analysis of each commercial pharmaceutical formulation (5 consecutive determinations for each sample) revealed a very good repeatability (Table 1), at a confidence level of 95%.

The developed methodology allowed a determination rate of 39 h^{-1}.

3.5. Toxicological control in beverages

The configuration simplicity, versatility in controlling analytical parameters, portability, low power consumption of the proposed automatic flow system and the proved analytical performance of the novel chemical approach for the quantification of glibenclamide implemented in the MPFS, makes the analytical methodology described in this work a promising analytical tool with relevant applicability in the forensic or toxicological analysis of samples collected in crime scenes. Besides that, glibenclamide has been reported to be cleared quickly within 5 h in human plasma, emphasizing the need for new fast screening methodologies for glibenclamide detection in the possible sources for drug ingestion. Taking into account the history of glibenclamide abuse found in literature [15], the developed flow system was applied to the determination of glibenclamide in five alcoholic beverages previously spiked with the drug. However, additional tests have shown that some components of soft drinks and red wine interfered in the analytical response and make in general glibenclamide detection uncertain in these samples.

The samples were prepared accordingly with the procedure mentioned in Section 2.2. Only recovery studies were performed since in the scientific literature it cannot be found an alternative method for glibenclamide (or similar) determination in beverages. Hence, this work scientific novelty.

The obtained results (Table 2) revealed good recoveries comprised between 98.6% and 99.6% of glibenclamide from the spiked samples.

4. Conclusions

This work describes the development of the first automatic miniaturized flow system for the fast screening of glibenclamide in pharmaceutical formulations and alcoholic beverages, involving the implementation of a novel reactional scheme. The drug glibenclamide was directly monitored by fluorescence without any sample pre-treatment, like for example, pre-concentration or photodegradation of the analyte, and the analytical signal was enhanced by performing the determinations in an organized microsystem obtained with sodium dodecyl sulphate and sulphuric acid.

The developed analytical approach was characterized by high simplicity and versatility. The results obtained in the fluorometric determination of glibenclamide in commercial pharmaceutical formulations proved that the developed methodology can be a valuable and promising tool for pharmaceutical chemical control, being this way an advantageous alternative to the reference procedure, only requiring low quantities of reagents and producing reduced volumes of residues. The standard procedures for glibenclamide monitoring involve chromatographic techniques

that require high volumes of reagents often dangerous for environment, expensive equipment and are time-consuming in sample and solutions preparation.

Additionally, the developed automatic system demonstrated its potential for carrying out routine laboratory procedures in forensic and toxicological analysis, being an important analytical tool for the detection and quantification of glibenclamide in alcoholic beverages that can be used to perpetrate suicides, homicides and/or robberies. Rapid glibenclamide screening in beverages through this automatic flow system should be used to complement conventional blood and urine analysis as it helps to rapidly identify drug-induced hypoglycemia and permits to take proper clinical and legal actions, all in the good health and safety of the victim. Considering the high portability and the easy control of these systems, one could expect its application for *in situ* analysis performed by police technicians, being one advantage of the proposed work over standard chromatographic techniques. Nevertheless, the proposed system lacks of sensitivity and selectivity for samples with higher matrix complexity. However, the real potentiality of the developed system is still to be achieved through its application for the analysis of samples with different matrices after implementation in-line of pre-concentration procedures, relatively easily to perform due to the modular structure of MPFS.

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CHAPTER 7

Exploiting adsorption and desorption at solid-liquid interface for the fluorometric monitoring of glibenclamide in adulterated drinks

EXPLOITING ADSORPTION AND DESORPTION AT SOLID-LIQUID INTERFACE FOR THE FLUOROMETRIC MONITORING OF GLIBENCLAMIDE IN ADULTERATED DRINKS

David S. M. Ribeiro, João A. Lopes, João L. M. Santos, João A.V. Prior*

Requimte, Department of Chemical Sciences, Laboratory of Analytical Chemistry and Physical-Chemistry, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha nº 164, 4099-030, Porto, Portugal

* Author for correspondence

E-mail: joaoavp@ff.up.pt

Phone: 0351 222 078 994

Fax: 0351 222 078 961

Abstract

Nowadays, the use of a drug to modify a person's behavior with criminal intentions has become a growing public concern. In fact, stealthy drink spiking with certain drugs can cause the incapacitation of a potential victim of assault and in extreme cases can even lead to death. Belonging to the group of drugs used to commit drug-facilitated crimes is glibenclamide, which not only exhibits high sedation secondary effects but when subject to an overdose intake can lead to intense hypoglycemic episodes that could end with death. Suicide attempts and homicides through overdose with glibenclamide have already been reported.

In this work and for the first time, it was developed a new methodology for detection of glibenclamide in spiked liquid samples (teas) by fluorometry (λ_{ex}=300 nm; λ_{em}=404 nm). The novel methodology was also implemented in a miniaturized and portable automatic flow system based in the concept of multipumping with an in-line pre-separation unit. The separation of the drug from the liquid samples was achieved through adsorption of the drug into activated charcoal packed within a mini column followed by elution with a solution composed by ethanol, hydrochloric acid and the surfactant CTAB (70 %, 1.0 mol L^{-1}, 0.01 mol L^{-1}, respectively). The results allowed to obtain a linear working range for glibenclamide concentrations of up to 50 mg L^{-1} (r = 0.9999) and the detection limit was about 0.81 mg L^{-1} of glibenclamide.

Keywords: multipumping; adsorption; charcoal; glibenclamide; drug-facilitated crimes; drink spiking.

1. Introduction

Nowadays, the stealthy drink spiking with hypnotic or psychotropic drugs, in entertainment places, turns the consumers into easy victims of assaults. The unconscious ingestion of drugs that modify the normal biological processes entails the need for the development of new analytical methodologies for the detection of such drugs in the places of utilization, in a way that should facilitate the prevention of its toxic action. Included in this group of drugs is glibenclamide. Glibenclamide is a potent, second generation oral sulfonylurea antidiabetic (hypoglycemic) agent widely used to lower glucose levels in patients with type II non-insulin-dependent diabetes mellitus with high sedation effects [1].

Although poisoning due to oral hypoglycemic agents is relatively uncommon, several occurrences of deaths and cerebral damage have been reported. Overdoses of this drug can lead to intense hypoglycemic episodes that end with death. A literature survey reveals the death of 6 persons in a total of 16 that had deliberately ingested toxic doses of sulfonylureas [2]. Other scientific reports have emphasized the danger of hypoglycemia with glibenclamide, even at low dose, especially among the elderly [3]. Suicides attempts and homicides through overdose with glibenclamide have also been reported [4]. Additionally, glibenclamide can be used as a drug to facilitate crimes due to its high sedation secondary effects that turn the victims unable to resist [5].

In the field of clinical and forensic toxicology, the analytical procedures not only have to be highly sensitive and selective but must also provide the results promptly, which can be achieved by in situ analysis. In the case of early sampling for toxicological analysis blood and urine have high interest, but food analyses are also of high importance when drugging related offences are suspected. The fast and in situ screening of drugs in food samples intentionally adulterated is imperative, as it happens more frequently in the cases of Drug-Facilitated Crime (DFC) victims. Nevertheless, and considering only

food samples as potential matrices, as this is the scope of this work, in scientific literature it can be only found a single work for glibenclamide determination in alcoholic beverages [6].

This work describes the development of an automatic analytical micro-system based in the concept of multipumping, compact and easy to transport for in situ analysis, applied in the toxicological control of teas surreptitiously spiked with glibenclamide. Taking into account that tea is a complex matrix, this work goes further in detecting glibenclamide in food samples when comparing with its detection in alcoholic samples that are a simpler matrix [6]. As glibenclamide is a common drug among the elderly being easily obtained in a drug store and since tea is also a common beverage for them, the use of tea spiked with abnormal quantities of glibenclamide might constitute a potential weapon for persons with criminal intentions.

The capacity of activated charcoal to adsorb chemicals has been recognized for centuries, and in many countries charcoal is used in medical emergencies being commonly used as an antidote of large spectrum in poisonings caused by sulfonylureas in general. The gastrointestinal absorption of many sulfonylureas is slow, which is partially due to their poor solubility at the gastric pH. Therefore, their absorption in acute intoxication may be delayed for several hours so that they may be adsorbed into charcoal given even several hours later. In fact, the study of the capacity of activated charcoal to adsorb different sulfonylurea drugs in vitro, including glibenclamide, was already reported in the scientific literature [7], but no work can be found involving the retention and elution (desorption) of the sulfonylurea drugs from the activated charcoal in order to perform its quantitative determination. In this work and for the first time, adsorption of sulfonylureas into charcoal followed by its desorption was developed and implemented in an automated procedure. Through a process of adsorption/desorption of drugs from activated charcoal the quantitative determination of those drugs in unknown's teas samples was feasible.

The multipumping flow system (MPFS), due to its versatility, operational simplicity and modular structure, allowed the easy implementation of a fluorometric detection approach for glibenclamide based on retention of the drug into activated charcoal followed by its desorption. Thus, a miniaturized flow system was developed in which a mini column packed with activated charcoal was incorporated to perform the in line separation of the drug from samples. Additionally, the use of activated charcoal makes the developed MPFS for glibenclamide determination an environmental friendly analytical methodology, since the charcoal is biological and environmentally innocuous.

2. Experimental

2.1 Apparatus

The multipumping flow system comprised three solenoid micro-pumps (Bio-Chem Valve Inc. Boonton, NJ, USA), two of which with 10-μL and one with 50-μL stroke volumes (series 120SP) and a set of four three-way solenoid valves (161T031, NResearch). The manifold was constructed with 0.8 mm i.d. PTFE tubing, homemade end-fittings, connectors and confluence points.

The detector was a fluorometer Jasco (Easton, MD, USA), model FP-2020/2025, equipped with a 16 μL internal volume flow cell. Analytical signals were recorded on a strip chart recorder, model Linseis L 250 E.

A perspex column of 50 mm length and 2 mm internal diameter was filled with activated charcoal to separate the analyte from the sample matrix. Two filters with 90 μm pore size (Mobicol "classic" M 1002) were placed at both ends of the column to entrap the activated charcoal.

Automatic control of the flow system was accomplished by means of an Intel Pentium based microcomputer with software developed using Microsoft VisualBasic 6.0. A homemade powerdrive was used to actuate the micro-pumps and solenoid valves through the LPT1 computer port.

2.2 Samples, standards and reagents

All solutions were prepared with doubly deionized water and chemicals of analytical grade were used.

A 500 mg L^{-1} glibenclamide stock solution was prepared by dissolving 125 mg of the drug in 250 mL of a 0.01 mol L^{-1} NaOH solution. This stock solution was stored under refrigeration.

The working glibenclamide standards (5–50 mg L^{-1}) were prepared, on a daily basis, by appropriate dilution of the stock solution: aliquots of glibenclamide stock solution (0.2–2.0 mL) were transferred into a series of 20.00 mL volumetric flasks and the volume was subsequently made up to the mark with 0.01 mol L^{-1} NaOH solution.

A solution containing 0.05 mol L^{-1} cetyltrimethylammonium bromide (CTAB) was prepared by dissolving 4.555 g of CTAB (Riedel-de Haën®) in a 250.0 mL volumetric flask, using deionized water as solvent.

The eluent mixture was prepared by adding 350 mL of absolute ethanol (Panreac®, 99.5%) plus 50 mL of 32 % hydrochloric acid (Panreac®) and 100 mL of a 0.05 mol L^{-1} CTAB solution into a 500.0 mL volumetric flask. Then, the eluent mixture was sonicated for 15 min, using an ultrasonic bath.

The activated charcoal was Darco®, 20-40 mesh, in granular form, obtained from Sigma Aldrich®. Approximately 46 mg of activated charcoal was used to fill the separating column.

Six commercial teas, including camomile, linden, lemon balm (Melissa), verbena, lemon and apple cinnamon, were selected for analysis. The different teas were spiked

aiming at a glibenclamide concentration of 25 mg L^{-1}, by adding 1.25 mL of the 500 mg L^{-1} glibenclamide stock solution into a 25.00 mL volumetric flask and the final volume completed with the respective tea sample under analysis. Then, the different spiked teas were filtered with a syringe filter i.d. 0.20 μm (Corning®) before the insertion in the flow system.

2.3 Flow manifold

The developed analytical flow system, depicted in figure 1, employed three solenoid micro-pumps (P_1 – P_3) which were responsible for the individual handling of the sample, conditioning and eluent solutions, and four solenoid valves (V_1 – V_4) which had the purpose of directing the different solutions towards the separating column, detection unit or waste.

In the developed MPFS the micro-pump P_1 was used for inserting the sample, P_2 was employed for the handling of the washing and conditioning solution of the column and, P_3 was responsible for insertion and propelling of the eluent solution.

Preceding the analytical cycle, all the flow tubing had to be filled with the corresponding solution. Thus, by simultaneously actuating the micro-pumps P_1 and P_2 and with the valves V_1 and V_4 in position ON (dashed lines, Figure 1), the sample and conditioning solutions were inserted and propelled directly to the waste. Then, the micro-pumps P_1, P_2 and valves V_1, V_4 were de-actuated and by actuating micro-pump P_3 with the valves V_2 and V_3 in position ON, the eluent solution was inserted, passing the separating column (CL) containing activated charcoal and carried towards the detection in order to establish baseline. Finally, by actuation of P_2 and with all solenoid valves in position off, NaOH conditioning solution was inserted and propelled through the column for cleanup and conditioning.

The analytical cycle was started by actuating the micro-pump P_1 to insert the sample into the column, thus performing the in-line separation of the drug through adsorption

into activated charcoal (Table 1, step 1). After introduction of the pre-determined sample volume, micro-pump P_1 was switched off and P_2 was actuated for the washing step (Table 1, step 2) directing the NaOH solution towards the activated charcoal column removing to waste the residual sample volume, thus reducing and minimizing matrix interferences. In the two previous steps, all the solenoid valves were switched off, allowing the direction of solutions through the column towards the waste. Thereafter, P_3 was actuated and simultaneously, solenoid valves V_2 and V_3 were switched ON to direct the eluent solution through the activated charcoal column towards the detection unit, being thus accomplished the elution step (Table 1, step 3). The fluorescence of the eluted solution was monitored at a wavelength of maximum excitation and emission of 300 and 404 nm, respectively. After the elution step, the column was washed and reconditioned (Table 1, step 4) for the next sample assay by actuating the micro-pump P_2. The analytical cycle was repeated five times allowing five consecutive determinations. The micro-pump P_1 (10 µL per stroke) in the separation step (Table 1, step 1) was operated with a pulse time of 0.4 s corresponding to a pulse frequency of 109 min^{-1}, establishing a flow rate at about 1.09 mL min^{-1}, whereas the micro-pump P_2 (10 µL per stroke) in the wash and reconditioning step (Table 1, step 2 and 4) was operated with a pulse time of 0.2 s corresponding to a pulse frequency of 171 min^{-1}, establishing a flow rate of 1.71 mL min^{-1}. In the elution step (Table 1, step 3), the micro-pump P_3 (50 µL per stroke) was operated with a pulse time of 0.5 s corresponding to a pulse frequency of 92 min^{-1}, establishing a flow rate of 4.62 mL min^{-1}.

3. Results and Discussion

3.1 Chemical conditions for adsorption and desorption of glibenclamide

Aiming at implementing the pre-separation of glibenclamide through adsorption into activated charcoal followed by its desorption and fluorometric monitoring, some preliminary assays for selection of the chemical conditions that favored the adsorption and desorption of a 20 mg L^{-1} glibenclamide standard solution from activated charcoal were conducted using an initial manifold (Figure 1 in supplementary material). For these assays, some reaction media involving combinations of different reagents used for the washing/conditioning of the column and the elution of the drug from the charcoal were studied. The nature of the tested chemical reagents that could influence the adsorption and desorption of glibenclamide from activated charcoal were chosen taking into account the characteristic dissolution profile of glibenclamide [8], the results of previous works namely those of Ribeiro et al. [6] and Neeman et al. [9], which study co-solvents for glibenclamide and, as well, the work of Atta-Politou et al. [10] that used a polyethylene glycol-electrolyte (PEG-ELS) solution to promote the desorption of a drug (fluoxetine) from activated charcoal. The characteristic properties of activated charcoal as a natural ion exchanger [11,12] were also considered. Thus, different combinations of acid (HCl), base (NaOH), alcohol (ethanol) and one surfactant (PEG-ELS) were tested.

Glibenclamide monitoring was conducted through the recording of the fluorescence excitation and emission spectra of the eluted solutions since different reaction media could affect the maximum wavelengths where excitation and fluorescence emission of glibenclamide would occur. Table 2 summarizes the assayed conditions and obtained results.

The performed assays revealed a positive detection of glibenclamide when using for the conditioning of the column a solution of 0.01 mol L^{-1} NaOH (also used for washing and as carrier) and as eluent a solution constituted by a mixture of 50 % ethanol and 0.7 mol L^{-1} HCl with corresponding maximum wavelengths of excitation and emission at 300 and 404 nm, respectively.

Nevertheless, during the carrying of the above assays, some problems in the flow system performance were detected, namely the clogging of the filters placed at the ends of the column. The gradual clogging of the filters caused variations in the flow rate that was gradually decreasing during repeated analytical determinations and impaired the elution of glibenclamide from the activated charcoal, thus originating a progressively reduction of the analytical signal.

In order to circumvent these drawbacks, the configuration of the multipumping flow manifold was modified so that the elution step (Table 1, step 3) was carried out with inversion of the direction of the flow relatively to the direction of flow during the pre-separation, washing and conditioning steps (Table 1, steps 1 and 2). The modified flow manifold is depicted in figure 1. Additionally, with the aim of further preventing the clogging of the column filters with particles of charcoal with dimensions inferior to the filters size pore (90 μm), the granule's size of activated charcoal used to fill the pre-separation column was standardized between 355 and 500 μm using tamises with corresponding mesh values.

Upon the modification of the flow system, the previous assays were repeated in order to evaluate the system performance in the pre-separation and detection of glibenclamide. It was observed an increased peak broadening (analytical signal) with concomitant failure returning to baseline, thus indicating a high residence time of the sample zone with consequently a higher sample dispersion phenomenon before drug detection. These results apparently indicated that the stroke volumes of the micro-pumps (10 μL) and hence the flow rate during the elution step was insufficient to elute completely the adsorbed drug on the activated charcoal, taking into account the

physical dimensions of the flow manifold and more concretely the internal volume of the pre-separating column. In order to improve the drug desorption, some assays were conducted to assess the most appropriate stroke volume of the micro-pump responsible for the propelling of the eluent solution (Figure 1, P_3), thus influencing directly the elution efficiency and aiming at higher peak's height.

Therefore, micro-pumps with stroke volumes of 10, 25 and 50 μL actuated at a pulse time of 0.5 s (0.9, 2.3 and 4.6 mL min^{-1}, respectively), were tested in the transport of the eluent. Considering the results, by choosing a micro-pump with 50 μL stroke volume it was obtained a higher chaotic movement of the eluent solution inside the separating column improving the desorption process, avoiding at the same time excessive sample dispersion and allowed to obtain the prompt return of the analytical signal to baseline after detection.

In the following assay the composition of the eluent solution (ethanol and acid) used for the determination of glibenclamide in the multi-pumping manifold was further studied to obtain a higher efficiency of elution and therefore the maximization of the analytical signal. The chemical nature of the acid, as well as, the presence of some anionic, cationic or neutral surfactants were studied which could strongly influence the efficiency of elution. Therefore, eluent solutions with 50 % ethanol and 0.72 mol L^{-1} of hydrochloric, sulphuric or nitric acids were evaluated for the determination of a 20 mg L^{-1} glibenclamide solution. It was observed that the fluorescence signal was enhanced rather with hydrochloric acid than with sulphuric or nitric acids.

Furthermore, with the goal of increasing the efficiency of elution of glibenclamide from the activated charcoal, it was studied the influence on the analytical signal of the presence of different substances namely, polyethylene glycol – electrolyte (PEG-ELS, non-ionic surfactant), cetyltrimethylammonium bromide (CTAB, cationic surfactant) and sodium dodecyl sulphate (SDS, anionic surfactant). The performed studies involved the preparation of three different eluents containing 50 % ethanol and 0.72 mol L^{-1} of HCl and to each solution was added a given amount of the different substances under

evaluation. The final concentration of PEG, CTAB and SDS was 20 mg L^{-1}, 0.01 and 0.02 mol L^{-1}, respectively. The three different solutions for elution were evaluated in the determination of two standard solutions of glibenclamide with concentrations of 25 and 50 mg L^{-1}. The obtained results (Figure 2) revealed a significant increase in the analytical signal, between 145 % and 205 %, when using CTAB in the eluent composition comparatively to the use of SDS or PEG-ELS.

Taking into account the obtained results, the optimization of the chemical and physical parameters of the MPFS was performed using an eluent solution with 50 % ethanol, 0.72 mol L^{-1} HCl and 0.01 mol L^{-1} CTAB.

3.2 Optimization of the MPFS

The dimensions of the separating column containing activated charcoal was expected to be of most importance in the determination of glibenclamide since it could determine the maximum adsorption capacity of the system for glibenclamide in the samples, influencing the mass quantity of adsorbed drug and the time necessary for the adsorption process to occur. Additionally, the dimensions of the column could also impair the flow rate considering the limited stroke volume of the micro-pumps and originate back pressure values that could be a cumbersome for the analytical determination through the developed multi-pumping flow system. These miniaturized systems lack of flow pressure when comparing with other more robust flow methodologies that use automatic syringes or peristaltic pumps as propelling units.

Thus, an optimization study was performed to evaluate the influence in analytical signal of 5 columns (A – E) with different lengths (L) and internal diameters (i.d.), discriminated in table 3, by establishing calibration curves with different glibenclamide standards (1 – 20 mg L^{-1}). According to the slopes of the calibration curves, it was verified that when using column C a higher sensitivity for the glibenclamide

determination was attained as a consequence of the enhancement in the adsorption efficiency, related with the length of the column, and the lower dispersion of the sample zone obtained when using a column with a small internal diameter in comparison with others with higher i.d.. This way, the column C was selected for further optimization assays.

The following assays aimed at evaluating the influence on the analytical signal of some physical and chemical parameters involved in the determination of glibenclamide through the developed MPFS, namely, sample volume, time for adsorption to occur, flow rate during elution and concentrations of the NaOH, ethanol, HCl and CTAB solutions. The flow rate during the washing step of the column (Table 1, step 2) had no influence on analytical signal because its only purpose was the removal of the sample matrix residues after adsorption of glibenclamide on the activated charcoal. The pulse time during step 2 of the automatic procedure (Table 1) was fixed in 0.2 s corresponding to a flow rate of about 1.7 mL min^{-1} thus not impairing the determination rate.

A multivariate experimental design approach was used to optimize the remaining physical and chemical parameters influencing the analytical signal. The influence on the maximum value of the analytical signal by the physical variables, comprising sample volume (SV), time for adsorption (TFA) and flow rate during elution (FRE) was first evaluated. During this optimization process, the concentrations of glibenclamide, HCl, ethanol and CTAB were fixed at 20 mg mL^{-1}, 0.72 mol L^{-1}, 50 % and 0.01 mol L^{-1}, respectively. The SV was evaluated by varying the number of sample pulses (5 – 20 pulses) inserted in the flow system by micro-pump P_1. The TFA to occur was assessed by using the stopped flow approach upon insertion of the sample solution for times between 120 and 480 s and the FRE was evaluated by varying the pulse time of the micro-pump P_3 between 0.5 – 1.25 s.

The set of experiments was created using a D-optimal design [13] with 20 runs conducted randomly. Each parameter was varied in 4 levels. After conducting the 20

runs, the maximum of each fluorescence signal was stored and modelled against the three factors (SV, TFA and FRE) using multiple linear regression [14]. Simple models and interaction models were attempted, but the former yielded better results. Non-significant factors (at a 95 % confidence level) were excluded from the final model. The obtained results (Table 4) revealed as expected that the most significant factor for the analytical signal was the SV, wherein for higher values the fluorescence intensity increased.

The TFA (time for adsorption) was also statistically significant and showed an inverse effect. These results are consistent with the fact of the adsorption of glibenclamide into activated charcoal is a dynamic process. Therefore, the optimization of the adsorption process was performed in a continuous flow mode. With this purpose, the influence on the analytical signal of flow rate during sample insertion and passage through the column containing activated charcoal, in alternative to the stopped flow approach used to evaluate TFA, was evaluated by varying the pulse time of micro-pump P_1, responsible for the sample insertion, between 0.2 and 1.0 s (1.7 and 0.5 mL min^{-1}, respectively). According to the obtained results and aiming at reaching a compromise between sampling rate and sensitivity, a pulse time for micro-pump P_1 of 0.4 s (flow rate of about 1.1 mL min^{-1}) during sample insertion was selected.

In contrast, the FRE (flow rate during elution) was found to be statistically non-significant at a confidence level 95 %. Considering that the FRE was statistically non-significant, a pulse time of 0.5 s (flow rate of about 4.6 mL min^{-1}) was chosen to conduct the determination of glibenclamide through the developed flow system, for not impairing the final determination rate.

Following the last results the influence on analytical signal of the SV (sample volume) was assessed by varying the number of sample pulses from 10 to 65 pulses (100 to 650 µL). This study showed that the analytical signal increased proportionally with the number of sample pulses up to 60 and for higher values tended to stabilization. However, a number of sample pulses of 20 (200 µL sample volume) was selected

because it allowed a better compromise between determination rate, sensitivity and analytical repeatability.

The study of the chemical parameters influencing the fluorescence intensity involved the analysis of the concentrations of NaOH, ethanol, HCl and CTAB solutions, while the configuration of the physical parameters during analysis was as compiled in table 1. This study was performed using a D-optimal design [13] with 28 runs conducted randomly, comprising the following concentration ranges: 0.01 – 0.1 mol L^{-1}, 10 – 70 %, 0.25 – 1.0 and 0.0025 – 0.015 mol L^{-1}, for NaOH, ethanol, HCl and CTAB, respectively. Four levels for each parameter were considered in the design. A multiple linear regression model was applied as previously described for the physical parameters optimization. The analytical results (Table 4) revealed that the fluorescence signal was significantly affected by the ethanol concentration, reaching the maximum fluorescence intensity values when using the highest concentrations of ethanol solution. Less important was the influence on the analytical signal of the concentration of the HCl solution, nevertheless a significant positive influence was observed. The variation of the CTAB concentration under evaluation was non-significant at the 95 % confidence level (P=0.118) although a positive correlation with the maximum of the fluorescence signal is suggested. Nevertheless, it is important to remember the importance of the presence of CTAB accordingly with the assays depicted in figure 3. The concentration of NaOH was found to be equally non-significant at the 95 % confidence level (P=0.328).

Considering these results it was concluded that the highest concentrations of ethanol and HCl yielded the highest fluorescence intensity, when using a CTAB solution with a concentration of approximately 0.01 mol L^{-1}. Since the NaOH concentration was found to be non-significant, for practical reasons, the lowest concentration level was selected (0.01 mol L^{-1}). Considering that the eluent solution consisted of a mixture of ethanol, HCl and CTAB, and by using a CTAB concentration fixed at 0.01 mol L^{-1}, the maximum concentration values of ethanol and HCl that could be used in the composition of the

eluent were 70 % and 1.0 mol L^{-1}, respectively. Therefore, the determination of glibenclamide on liquid samples through the developed multipumping flow system was performed using a 0.01 mol L^{-1} NaOH solution for conditioning and washing of the separation column and an eluent solution composed by 70 % ethanol, 1.0 mol L^{-1} HCl and 0.01 mol L^{-1} CTAB.

3.3 Analysis of commercial teas spiked with glibenclamide

The performance of the developed methodology, including inline separation of glibenclamide from the samples through adsorption into activated charcoal was evaluated in the determination of glibenclamide in commercial teas (camomile, linden, melissa, verbena, lemon and apple cinnamon), previously spiked with the drug.

Under the optimal analytical conditions formerly established, a linear working response range for glibenclamide concentration of up to 50 mg L^{-1} was obtained and the calibration curve was represented by FI = 0.0161 (±0.0001) × C + 0.015 (± 0.003), in which FI was the fluorescence intensity and C was glibenclamide concentration, in mg L^{-1}. A correlation coefficient of 0.9999 was verified. The detection limit calculated from the equation of the calibration curve according to Miller and Miller [15] was about 0.81 mg L^{-1}.

The precision of the proposed methodology was estimated by calculating the confidence interval of a set of 4 repeated measures of the spiked tea samples. The obtained results (Table 5) revealed a good repeatability taking into account the calculated concentration ranges for a confidence level of 95 %.

The time required to fully complete an analytical cycle was approximately 246 s and consequently the determination rate was about 15 determinations per hour.

For accuracy assessment, only recovery studies were performed since in the scientific literature it cannot be found to the present an alternative method for glibenclamide (or

similar) determination in teas or any other drinks. The obtained results (Table 5) revealed good recovery values comprised between 98.0 and 103.7 % of glibenclamide from the spiked samples.

4. Conclusions

In this work, it was successfully used activated charcoal, an environmental friendly material, to adsorb the drug glibenclamide from spiked teas, and for the first time it was developed an eluent mixture to promote the desorption of the drug from the charcoal. A solution mixture containing NaOH for the in-line reconditioning of the activated charcoal was also developed, but its efficiency was strongly dependent on the sample matrix analyzed since the activated charcoal is not selective for glibenclamide. For example, in this work, the detection of glibenclamide on black tea was unachievable due to the complexity in constituents of that sample.

It was demonstrated that the novel analytical approach for the inline pre-separation and quantification of glibenclamide implemented in an automatic miniaturized flow system constitutes a potential tool for routine laboratory procedures in forensic and toxicological analysis, allowing the rapid screening of glibenclamide in teas. The developed methodology can be used to complement LC-MS/MS glibenclamide analysis in blood or urine samples, when these samples are collected early enough taking into account the half-live of the drug in the organism, as it helps to rapidly detect glibenclamide in teas when exists a suspicion of drugging related offences through contaminated teas. Since the developed system was automatic, miniaturized and portable, the in-situ analysis of specific samples can be easily achievable by non-specialized technicians.

This work further proved that the activated charcoal effectively adsorbs different sulfonylureas, namely glibenclamide and thus, the activated charcoal can be used to possibly prevent their gastrointestinal absorption.

The developed MPFS can also be used to establish the correct dose of charcoal to be given to the victims of intoxication by performing surface adsorption assays into activated charcoal allowing the establishment of the adsorption isotherms and thus determining the adsorption ratio of drug-charcoal.

Additionally, it can be of great importance in the dissolution rate studies of glibenclamide or slightly soluble related substances, where different co-solvents and interactions with surfactants are evaluated aiming at improving its solubility. In these tests are often used chromatographic techniques that require high volumes of reagents dangerous for the environment, expensive equipment and are time-consuming in sample and reagents preparation.

The developed miniaturized automatic flow methodology represents a significant contribution for the fast screening of glibenclamide in spiked teas, assuring at the same time a high throughput and low consumption of both sample and reagents. Nevertheless, to achieve better selectivity different materials for performing solid-phase extraction should be tested.

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Figures:

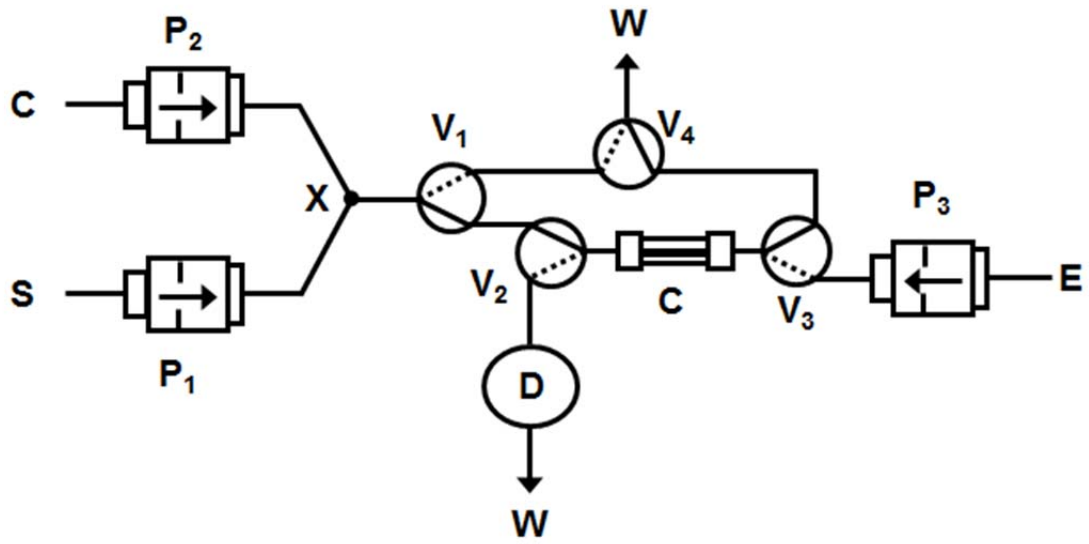


Figure 1 – Multipumping flow system (MPFS). P_1 – P_3: micro-pumps; X, confluence point; V_1 – V_4: solenoid valves (filled lines – solenoid OFF; dashed lines – solenoid ON); D, fluorometer detector (λ_{ex} = 300 nm and λ_{em} = 404 nm); CL, separation column containing activated charcoal (5.0 cm and i.d. 0.2 cm); S, sample: glibenclamide in 0.01 mol L^{-1} NaOH; C, conditioning and washing solution: 0.01 mol L^{-1} NaOH; E, eluent solution: 0.01 mol L^{-1} CTAB, 70 % ethanol and 1.0 mol L^{-1} HCl; W, waste.

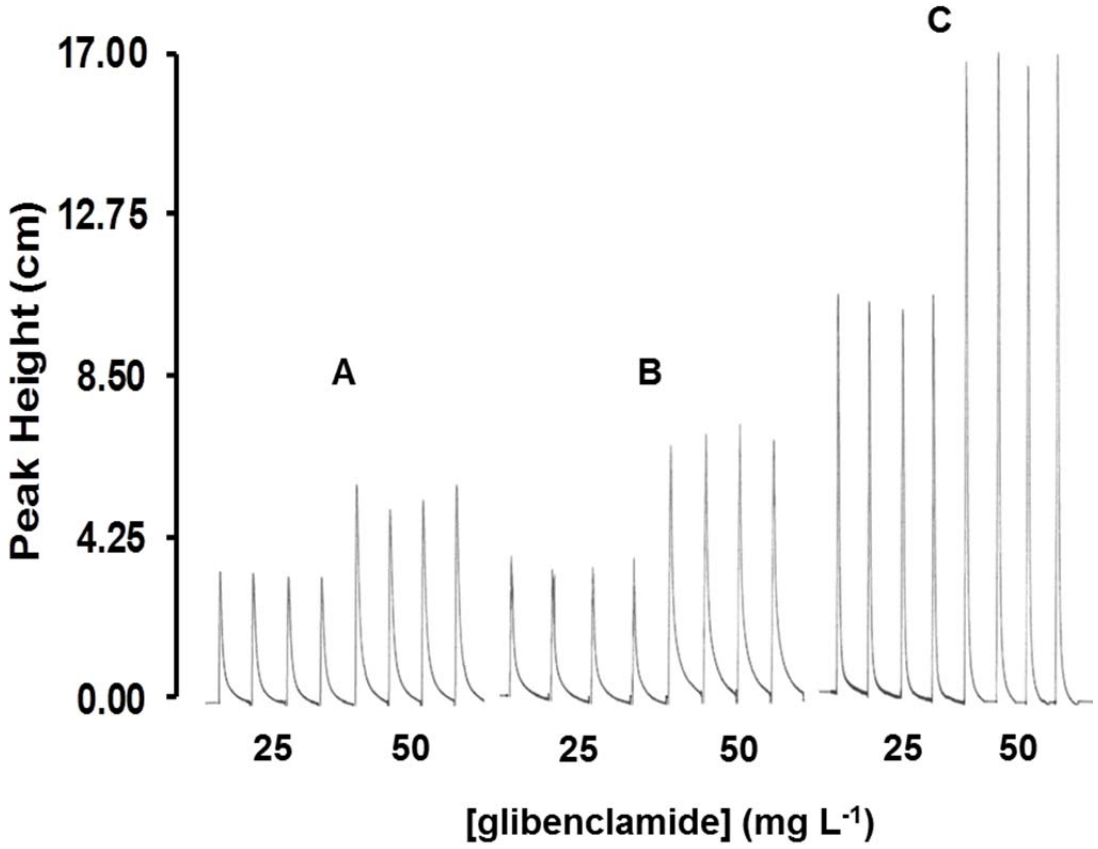


Figure 2 – Fiagram obtained in the study of the influence on analytical signal of three different eluent solutions (A, B and C). A, 50 % ethanol + 0.72 mol L^{-1} HCl + 20 mg L^{-1} PEG-ELS; B, 50 % ethanol + 0.72 mol L^{-1} HCl + 0.02 mol L^{-1} SDS; C, 50 % ethanol + 0.72 mol L^{-1} HCl + 0.01 mol L^{-1} CTAB.

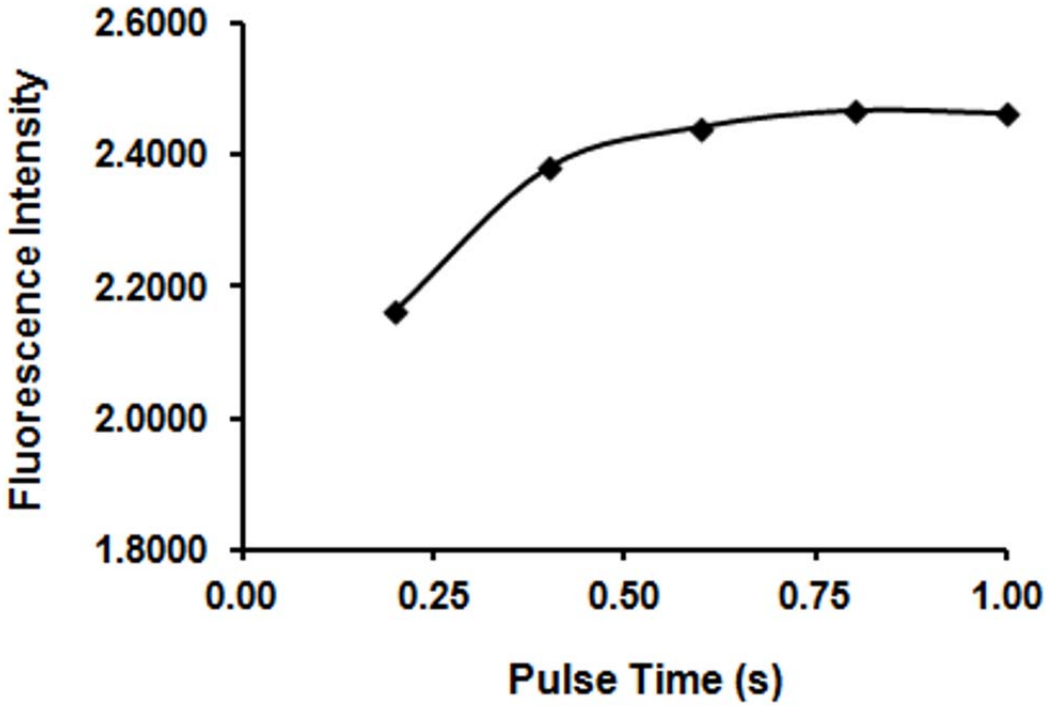
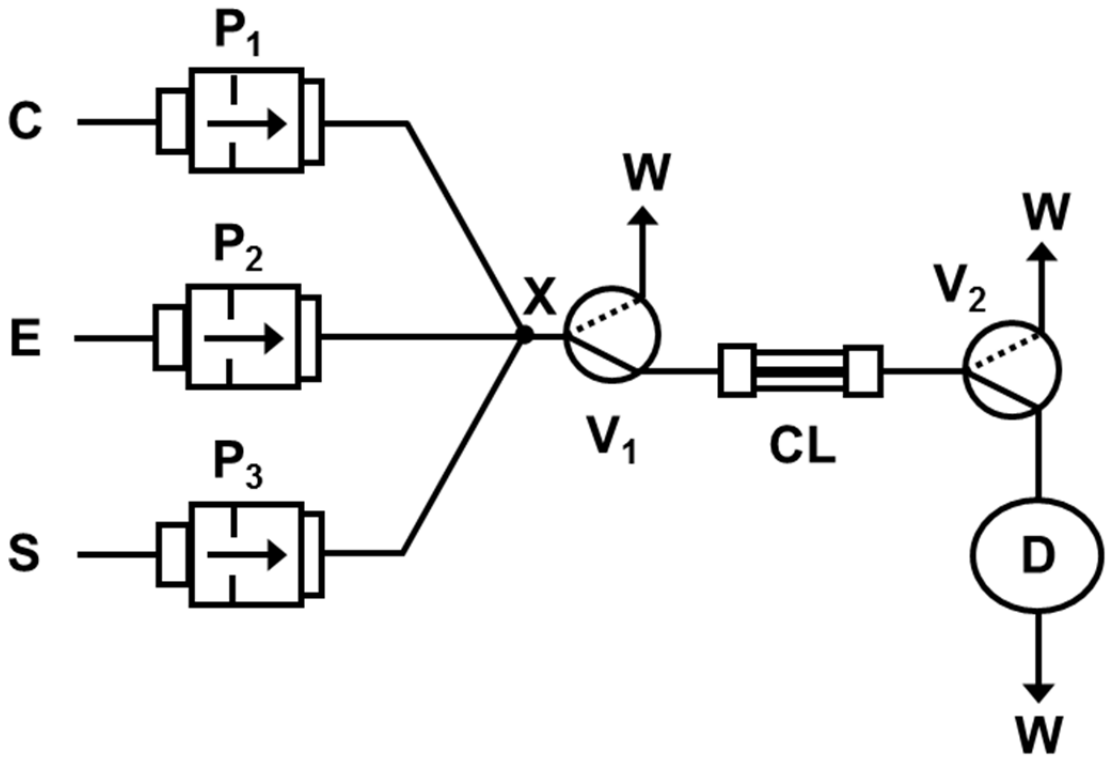


Figure 3 – Influence on analytical signal of different pulse times during sample insertion.

Supplementary material:



Supplementary material, Figure 1 – Preliminary multipumping flow system. P_1 – P_3: micro-pumps (10 µL stroke volumes); X, confluence point; V_1 – V_2: solenoid valves (filled lines – solenoid OFF; dashed lines – solenoid ON); D, fluorometer detector (λ_{ex} = 300 nm and λ_{em} = 404 nm); CL, separation column (1.5 cm and i.d. 0.3 cm); S, sample; C, conditioning and washing solution; E, eluent solution; W, waste.

Table 1 – Operation of the multipumping flow system for determination of glibenclamide.

Step	Operation	Micro-pump	Number of pulses	Pulse time (s)	Solenoid valves status	
					ON	OFF
1	Sample insertion	P_1	20	0.4		V_1, V_2, V_3, V_4
2	Washing of the column	P_2	220	0.2		V_1, V_2, V_3, V_4
3	Elution and detection	P_3	120	0.5	V_2, V_3	V_1, V_4
4	Washing/conditioning of the column	P_2	220	0.2		V_1, V_2, V_3, V_4

Table 2 - Studied chemical conditions for adsorption/desorption of the drug. PEG-ELS, polyethylene glycol-electrolyte solution; (+), positive detection of glibenclamide; (-), negative detection of glibenclamide.

Reaction media	Washing/Conditioning solution	Elution solution	Results
1	HCl + Ethanol	NaOH + Ethanol	(-)
2	HCl + Ethanol	Ethanol + PEG-ELS	(-)
3	NaOH	Ethanol	(-)
4	NaOH	PEG-ELS	(-)
5	NaOH	Ethanol + PEG-ELS	(-)
6	NaOH	Ethanol + HCl	(+)

Table 3 – Optimization of the pre-separation column dimensions. i.d., internal diameter; FI, fluorescence intensity; C, glibenclamide concentration in mg L^{-1}; R, correlation coefficient.

Column	Length (mm)	i.d. (mm)	Calibration equation	R
A	15	2	FI = 0.097 (±0.009) × C + 0.41 (±0.09)	0.9916
B	30	2	FI = 0.100 (±0.005) × C + 0.33 (±0.06)	0.9969
C	50	2	FI = 0.125 (±0.004) × C + 0.20 (±0.05)	0.9989
D	15	3	FI = 0.086 (±0.003) × C + 0.27 (±0.03)	0.9989
E	30	3	FI = 0.084 (±0.004) × C + 0.29 (±0.04)	0.9982

Table 4 – Summary of regression models aiming at the maximum of the fluorescence signal, estimated considering physical and chemical parameters experimental designs. Coefficients are scaled and centered.

	Parameter	Coefficients			Model (ANOVA)		
		Value	Conf.int. (±)[a]	P	P	RSD	Q^2
Physical parameters optimization model	Offset	1.595	0.0594	$<1\times10^{-6}$	$<1\times10^{-4}$	0.125	0.964
	SV	0.817	0.0694	$<1\times10^{-6}$			
	TFA	-0.222	0.0714	$<1\times10^{-6}$			
	FRE[b]	---	---	(0.295)[c]			
Chemical parameters optimization model	Offset	1.627	0.1423	$<1\times10^{-6}$	$<1\times10^{-4}$	0.346	0.895
	EtOH	1.486	0.2221	$<1\times10^{-6}$			
	HCl	0.392	0.1580	2.8×10^{-5}			
	CTAB[b]	---	---	(0.118)[c]			
	NaOH[b]	---	---	(0.328)[c]			

[a] Confidence interval for a 95 % confidence level.
[b] Statistically non-significant factor for a 95 % confidence level.
[c] P-value considering the inclusion of that factor in the model.

Table 5 – Results obtained for the determination of glibenclamide on spiked tea samples through the developed multipumping flow system.

Spiked sample	Concentration added (mg L^{-1})	Concentration found (mg L^{-1})[a]	Recovery (%)
Camomile	25.0	24.5 ± 0.1	98.0
Linden	25.0	25.7 ± 0.7	102.7
Melissa	25.0	25.5 ± 0.4	101.9
Verbena	25.0	25.9 ± 0.1	103.7
Lemon	25.0	24.8 ± 0.8	99.3
Apple cinnamon	25.0	25.6 ± 0.5	102.2

[a] Mean ± $t_{0.05}$ (Student's *t*-test) × (S.D./$\sqrt{n}$).

CHAPTER 8

Photoactivation by visible light of CdTe quantum dots for inline generation of reactive oxygen species in an automated multipumping flow system

Photoactivation by visible light of CdTe quantum dots for inline generation of reactive oxygen species in an automated multipumping flow system

David S. M. Ribeiro, Christian Frigerio, João L. M. Santos, João A.V. Prior*

Requimte, Department of Chemical Sciences, Laboratory of Analytical Chemistry and Physical-Chemistry, Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha nº 164, 4099-030, Porto, Portugal

* Author for correspondence
E-mail: joaoavp@ff.up.pt
Phone: 00351 222 078 994
Fax: 00351 222 078 961

Abstract

Quantum-dots (QD) are semiconductor nanocrystals able to generate free radical species upon exposure to an electromagnetic radiation, usually in the ultraviolet wavelength range.

In this work, CdTe QD were used as reactive oxygen species (ROS) generators for the chemiluminometric chemical control of pharmaceutical formulations containing epinephrine. Due to the relatively low energy band-gap of this chalcogenide a high power visible light emitting diode (LED) lamp was used as photoirradiation element and assembled in a laboratory-made photocatalytic unit. Owing to the very short lifetime of ROS and to ensure both reproducible generation and time-controlled reaction implementation and development, all reactional processes were implemented inline by using an automated multipumping micro-flow system.

The developed approach took advantage of the highly reactivity of ROS, obtained upon CdTe QD photoirradiation in aqueous solution, to oxidize luminol producing a strong chemiluminescence emission which was quenched in the presence of epinephrine.

A linear working range for epinephrine concentration of up to 2.28×10^{-6} mol L^{-1} ($r = 0.9953$; $n = 5$) was verified and the determination rate was about 79 determinations per hour.

The results obtained in the analysis of epinephrine pharmaceutical formulations by using the proposed methodology were in good agreement with those furnished by the reference procedure, with relative deviations lower than 4.80 %.

Keywords: Quantum dots; visible light photoirradiation; reactive oxygen species; chemiluminescence; multipumping flow system; epinephrine.

1. Introduction

Epinephrine or adrenaline [1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanol] is an hormone produced by suprarenal glands that belongs to the catecholamines group and plays an important role as neurotransmitter. This drug is widely used in the treatment of allergic emergencies, status asthmaticus, bronchial asthma, ventricular bradycardia, cardiac arrest, glaucoma and as styptic [1]. This drug has also been reported as being used in drug abuse situations to obtain stimulant effects through intravenous injection [2]. Due to its relevant therapeutical importance, several methodologies have been developed for epinephrine quantification in pharmaceutical formulations, such as, spectrophotometry [3, 4], fluorometry [5, 6], electrochemiluminescence [7], capillary electrophoresis with ultraviolet detection [8] and electrochemical [9-16] methods. Additionally, some methods based on distinct flow analysis techniques were also developed, mostly resorting to flow injection analysis with spectrophotometric [17, 18], spectrofluorimetric [19], amperometric [20] and electrochemiluminometric [21] detection and sequential injection analysis with potentiometric detection [22].

In this work, and for the first time, the advantageous features resulting from simultaneously exploiting the high catalytic activity of semiconductor nanoparticles and the antioxidant capacity of epinephrine [23] are put in evidence.

Colloidal semiconductor nanocrystals, often referred to as quantum dots (QD), can be defined as monodispersed nanoparticles made of a core of semiconductor material, surrounded by a capping organic layer or passivating molecule, in a diameter typically in the range 1–10 nm. These nanostructured materials comprise elements from the periodic groups IIB – VIB (e.g. CdSe, CdTe, CdS, ZnSe), IIIB-VB (e.g. InP, InAs) or IVB-VIB (e.g. PbSe) [24]. QD nanoparticles have important intrinsic properties, such as high photostability, high quantum yield, size-tunable, narrow and symmetric band emission and high absorption coefficient across a wide spectral range [25]. These photophysical properties of quantum dots have made them attractive materials in diverse fields of application, including their utilization as fluorescence probes and biomarkers in nanomedicine and their exploitation as chemosensors in analytical chemistry.

In recent years, several works have been developed involving the use of QD in fluorescence or chemiluminescence based assays for the analytical determination of several compounds [26-35] and other chemical species, such as, heavy metals [36-39]. In addition, some other works have theoretically examined another highly promising

feature of QD, which is their capacity to generate reactive oxygen species (ROS) in aqueous solution [40-42] upon exposure to an electromagnetic radiation. Nevertheless, from a practical point of view, the QD potential to generate oxidizing species was only exploited by Silvestre *et al.* [43] in a work that aimed at the determination of the chemical oxygen demand of wastewaters.

In this work a novel approach involving the use of a visible light irradiation unit based on a LED lamp was develop aiming at the photoactivation of aqueous CdTe QD to generate ROS. The energy of the visible electromagnetic was more than enough to trigger the process of radicals generation, thus dispensing making use of a higher energy and more harmful UV lamp employed in the previously mentioned studies [40-43]. The use of a high-power lamp based on LED technology, instead of a UV low pressure mercury lamp, avoids the dangerous exposition of the analyst to UV radiation, as well as the cumbersome excessive heating of the solutions exposed to the radiation and also reduces significantly the energy consumption (up to 90 % energy savings). Aiming at implementing an automatic control of the QD photoactivation and also to reduce analytical reagents consumption, the novel analytical methodology was implemented in a micro-flow system that exploited the multipumping flow concept (MPFS).

The main characteristics of multipumping [44], such as, low reagents consumption, straightforward automation and control, high portability, versatility due to a modular structure, allowed in this work the easy implementation of a chemiluminometric approach for epinephrine determination in pharmaceutical samples. This analytical application was implemented because the main goal of the developed work was to evaluate the use of a visible LED light to photoactivate QD and also to take advantage of the formed ROS to determine substances that present antioxidant properties. Additionally, the hydrodynamic characteristics of MPFS, arising from the pulsed flow produced by the actuation of the micro-pumps promoted an efficient, reproducible and high sample/reagent intermixing in front of the detector, which is particularly important due to the nature of the short-lived species involved in the reactional scheme, allowing to further improve the efficiency and sensitivity of the chemiluminescence measurements in which light is usually generated by very fast reactions. The proposed methodology was based on the quenching effect of epinephrine on the oxidation of luminol by the ROS species generated by the QD nanoparticles irradiation.

2. Experimental

2.1 Apparatus

The developed flow manifold comprised four solenoid micro-pumps (model 120SP, Bio-ChemValve Inc. Boonton, NJ, USA), which were of the fixed displacement diaphragm type, delivering 10 µL per stroke. Automatic control of the micro-pumps was accomplished with an Intel Pentium® based microcomputer using software developed in Microsoft Visual Basic 6.0®. The solenoid devices were activated by a homemade power drive controlled through communication by the computer parallel port. Flow lines made of 0.8 mm i.d. PTFE tubing, homemade end-fittings, connectors and acrylic confluence points were also used.

The photo-excitation unit (LED-PEU) consisted in a 50 cm reactor coil made of PTFE tubing (0.8 mm i.d.) placed between two high-power LED lamps (Parathom® R50 40 daylight) emitting white light of high efficiency.

The detector used to monitor the chemiluminescence signal was a FP-2020 Plus model (Jasco, Easton, MD, USA) equipped with a modular flow cell consisting of a helical 0.8 mm i.d. PTFE tube with an internal volume of 100 µL that was positioned in front of a highly sensitive photomultiplier. Analytical signals were recorded on a strip chart recorder, model Linseis L 250 E.

2.2 Samples and standards

All solutions were prepared with doubly deionized water and chemicals of analytical grade were used.

For this work, three different samples for intramuscular injection were obtained. These pharmaceutical formulations had in their composition a high content of sodium metabisulfite, which is added as formulation preservative. A sample pre-treatment for the elimination of sodium metabisulfite was performed by applying a procedure adapted from Amorim *et al.* [22] with some improvements. Briefly, a first intermediate sample solution was prepared by adding, in a 20 mL volumetric flask, an appropriate volume of the injectable drug and 150 µL of 32 % HCl (Panreac®) and then the volume was made up to the mark with deionized water. Afterwards, this solution was bubbled with nitrogen for 25 minutes to release the dissolved sulfur dioxide originated by acidification of the medium containing metabisulfite. A second intermediate sample solution was prepared by appropriate dilution of the previous sample solution in a 25

mL volumetric flask and the pH was adjusted to approximately 5.7, with diluted sodium hydroxide solution. Finally, a dilution with water was carried out in order to obtain an epinephrine content included in the analytical range of the method.

Taking into account the technical demands on sample preparation, a similar approach was conducted for the standard solutions. A 2.28×10^{-3} mol L^{-1} epinephrine stock solution was prepared by dissolving 12.5 mg of the drug in 25 mL of water. A first intermediate epinephrine solution with a concentration of 6.83×10^{-5} mol L^{-1} was prepared by appropriate dilution of the stock solution and by adding 187.5 µL of 32 % HCl and then the volume was made up to 25 mL with water. A second intermediate epinephrine solution with a concentration of 2.28×10^{-5} mol L^{-1} was prepared by appropriate dilution in a 50.00 mL volumetric flask of the first intermediate epinephrine solution and the pH was adjusted to the pH of doubly deionized water, which was approximately 5.7, with a 0.2 mol L^{-1} sodium hydroxide solution. Finally, the volume was completed to the mark with water.

The working epinephrine standards (1.14×10^{-7} – 2.28×10^{-6} mol L^{-1}) were prepared by appropriate dilution of the second intermediate solution by transferring aliquots (0.125 – 2.50 mL) into a series of 25.00 mL volumetric flasks and the volume was subsequently made up to the mark with water.

A 1.0×10^{-2} mol L^{-1} luminol stock solution was prepared by dissolving 177.1 mg of 5-amino-2,3-dihydro-1,4-phthalazinedione (Sigma-Aldrich®) in 100 mL of 1.75×10^{-2} mol L^{-1} NaOH solution, which was used as solvent of luminol. This stock solution was stored under refrigeration. A working luminol solution of 1.5×10^{-3} mol L^{-1} was daily prepared by dilution of the stock solution in a 100.0 mL volumetric flask and the concentration of NaOH adjusted to 1.0×10^{-2} mol L^{-1}. All luminol solutions were always protected from the light.

2.3 Reagents and synthesis of CdTe quantum dots

In the synthesis of CdTe QD several reagents were used with the following amount: 1.6×10^{-3} mol of sodium borohydride (Sigma-Aldrich®), 0.4×10^{-3} mol of tellurium powder, 200 mesh (Sigma-Aldrich®), 4.0×10^{-3} mol of cadmium chloride (Sigma-Aldrich®) and 1.7×10^{-3} mol of 3-mercaptopropionic acid (Fluka®). Absolute ethanol (Panreac®) was also used in the synthesis process.

For the synthesis of CdTe QD capped with 3-mercaptopropionic acid (MPA) the procedure developed by Silvestre *et al.* [43] was executed, which in turn was based in the procedure described by Zou L. et al. [45].

For the assays, a solution containing 1.00 μmol L^{-1} of CdTe QD was prepared by dissolving 25.55 mg of the synthesized and purified CdTe QD, with a size of 3.00 nm, in 50 mL of water.

2.4 Flow manifold

The developed flow system exploiting the MPFS approach for the epinephrine determination is depicted in figure 1. The analytical manifold comprised four solenoid micro-pumps (P_1 to P_4) used for insertion and propulsion of the solutions of reagents. Preceding the analytical cycle, all flow tubing was filled with the corresponding solution by actuating the corresponding micro-pump. The analytical signal baseline was established by insertion of H_2O through actuation of P_3.

The analytical cycle started with the combined insertion of a pre-set number of sample pulses and CdTe QD solutions in confluence point X_1, by the simultaneous actuation of micro-pumps P_1 and P_2 at a fixed pulse time of 1000 ms. Subsequently, the micro-pumps P_1 and P_2 were switched off and by actuating micro-pump P_3 the carrier (H_2O) was inserted at a fixed pulse time of 1000 ms, corresponding to a pulse frequency of 52 min^{-1}, which fixed the flow rate during transport to detection at 0.52 mL min^{-1}. The inserted number of pulses of carrier solution was enough to guarantee the transport of the reaction zone (pre-mixed sample/QD solution) through the photo-excitation unit (LED-PEU) to the point of confluence X_2. Following, by the alternated actuation of the micro-pumps P_3 and P_4 a pre-set number of plugs of luminol solution were intercalated with plugs of the pre-mixed sample/QD solution previously irradiated and, immediately next, the reaction zone was transported through the repeated actuation of micro-pump P_3. To assure that from this moment the established reaction zone was rapidly carried to the detector unit, the pulse time of the actuated micro-pumps was fixed at 125 ms, corresponding to a flow rate of 2.18 mL min^{-1}.

3. Results and Discussion

3.1 Mechanism of reaction

The potential of QD for the formation of reactive oxygen species (ROS) through photoactivation was only recently recognized, by Ipe *et al.* [40], where it is stated that the absorption of photons with energy higher or equal to the quantum dot band gap energy promotes the formation of an exciton (electron–hole pair). This means that the

semiconductor QD under exposure to an ultraviolet or visible electromagnetic radiation can promote the delocalization of an electron (e^-) from the valence band (v_b) to the conduction band (c_b). This formed electron-hole pair ($e_{cb}^- + h_{vb}^+$) has redox properties that are dependent from the valence band and conduction band energies and principally from the flat-bands potentials. Charge transfer between QD and the nearby molecules will compete with radiative and non-radiative decay and energy transfer.

In this work, the potentiality of semiconductor quantum dots to produce free radicals upon electromagnetic irradiation in aqueous solution was exploited by studying the influence of some important characteristics of the synthesized QD on originating ROS. The methodology was tested by applying it in the determination of epinephrine in pharmaceutical formulations. The determination was based on the quenching effect of epinephrine on the chemiluminescence emission of luminol upon its oxidation by the reactive oxygen species generated through the photoactivation of aqueous CdTe QD nanoparticles.

One of the innovations of this work was the photoactivation of QD with a homemade visible light photocatalytic unit based on LEDs, which presents some advantages comparatively to the use of UV light.

The use of white LEDs, that are cold lamps, does not produce significant variations of temperature that often occurs with incandescent and gas-discharge lamps. This way, there are no variations of temperature that could influence the analytical determination or originate the formation of air bubbles, when using flow systems. Also, by using LEDs emitting in the visible wavelength range the photodegradation of epinephrine does not occur, since the radiation is insufficiently energetic for causing molecular degradation despite being sufficient for the QD photo-excitation. The homemade photo-excitation unit was built with lamps equipped with high power LEDs, with efficient generation of white light and without UV or near-IR radiation in the light beam. They were of very low energy consumption (up to 90 % energy savings) and long-life, filling the required standards for environmental friendliness.

The epinephrine determination method was accomplished through 3 distinct steps: (i) formation of radicals, (ii) oxidation of luminol and (iii) epinephrine quenching.

In the first step, QD were irradiated with visible light originating the formation of the exciton ($e_{cb}^- + h_{vb}^+$) (Equation 1). The excitons undergo redox reactions with oxidizing and oxidable species present in the irradiated medium, such as O_2 and hydroxide ion (OH^-) respectively. The conduction band potential (e_{cb}^-) is sufficient to reduce O_2 to superoxide radicals (Equation 2) and the valence band potential (h_{vb}^+) is enough to oxidize hydroxide ions to generate hydroxyl radicals (Equation 3).

1st step: reactive oxygen species (ROS) generation from photo-excitation of QD

$$\mathrm{CdTe} + h\upsilon \longrightarrow \mathrm{CdTe}\,(e^-_{cb} + h^+_{vb}) \qquad \text{(Equation 1)}$$

$$\mathrm{CdTe}\,\left(e^-_{cb}\right) + O_2 \longrightarrow O_2^{\cdot-} \qquad \text{(Equation 2)}$$

$$\mathrm{CdTe}\,\left(h^+_{vb}\right) + OH^- \longrightarrow \cdot OH \qquad \text{(Equation 3)}$$

In the second step, the free radicals ·OH and $O_2^{\cdot-}$, generated by photoactivation of QD in aqueous solution, oxidize luminol in alkaline medium to the aminophthalate ion which is produced in an electronically excited state and emits light (chemiluminescence signal, CL) on dropping to the ground state (Equation 4). In the absence of epinephrine, the obtained chemiluminescence signal was maximum (blank signal) since all the generated radicals were available for the oxidation of luminol.

2nd step: oxidation of luminol by ROS and emission of the photons (CL signal)

$$\text{luminol} + \begin{cases} O_2^{\cdot-} \\ \cdot OH \end{cases} \xrightarrow{OH^-} \text{3-aminophtlate} + h\upsilon \qquad \text{(Equation 4)}$$

In the last step, epinephrine acted as a free radical scavenger producing adrenochrome (Equation 5). The scavenging capacity of epinephrine for free radicals results in a decrease of the luminol oxidation rate producing a pronounced inhibition of the CL signal. The obtained CL quenching was used to quantify the epinephrine contents in pharmaceutical formulations.

3rd step: ROS scavenging capacity of epinephrine (quenching of CL signal)

$$\text{epinephrine} + \begin{cases} O_2^{\cdot-} \\ \cdot OH \end{cases} \longrightarrow \text{adrenochrome} \qquad \text{(Equation 5)}$$

3.2 Optimization of the MPFS

Taking into account some scientific works [46-48], the size of QD nanoparticles was expected to have a strong influence on the photochemical and electrical properties of the QD, determining thus its reactivity and the magnitude of the analytical signal.

Supported by the literature [49], the nanoparticle size was determined by the first absorption maximum resorting to the following formula:

$$D = (9.8127 \times 10^{-7})\lambda^3 - (1.7147 \times 10^{-3})\lambda^2 + (1.0064)\lambda - 194.84$$

in which D is the diameter of QD (nm) and λ is the wavelength of the maximum absorbance.

The molar concentration of the nanocrystals in solution can be also easily determined by simply taking an absorption spectrum of an aqueous CdTe QD solution with a known mass concentration and establishing the extinction coefficient (ε) using the following formula [35]:

$$\varepsilon = 3450\ \Delta E\ (D)^{2.4}$$

where ΔE is the transition energy corresponding to the first absorption peak expressed in eV. By knowing ε and the absorbance of CdTe QD solution, the molar concentration was calculated through the application of the Lambert-Beer's law.

For this work, were obtained by synthesis 3 different nanoparticle sizes of CdTe QD namely 1.87, 3.00 and 3.71 nm, which maximum absorption wavelengths were observed at 485, 531 and 606 nm, respectively.

An optimization study was performed in order to evaluate the influence in analytical signal of the 3 different sized QD above mentioned and at same time different QD concentration was assessed. For the QD with the smaller size (1.87 nm) a concentration range from 2.5 to 10 μmol L^{-1} was evaluated, while for the QD with higher size (3.00 and 3.71 nm) the concentration range studied was from 0.25 to 1 μmol L^{-1}.

The influence in the analytical signal of the different QD sizes and molar concentrations was assessed by establishing calibration curves with different epinephrine standards (2.28×10^{-7} – 2.28×10^{-6} mol L^{-1}). The results were analyzed resorting to a comparison between the slopes of the calibration curves. The results revealed that by increasing the size of the nanoparticles QD the production of ROS also increased, since for the nanoparticles of smaller size and for each tested concentration of QD (2.5 – 10 μmol L^{-1}) the scavenger activity of ROS by epinephrine was observed, while for QD of higher size and for the same range of QD concentrations (2.5 – 10 μmol L^{-1}) it was not observed any scavenging activity, that is, there was no difference between the recorded analytical signal in the presence or absence of epinephrine. In this last situation, the range of concentrations tested for epinephrine was not sufficient to

scavenge the elevated quantity of free radicals formed by photoactivation of the QD. Considering these last results, the concentrations of the solutions of QD of size 3.00 and 3.71 nm were 10 times reduced (0.25 e 1 μmol L^{-1}) and the assays were repeated. So, one can conclude that the use of QD of higher dimensions allows the use of smaller amounts of the nanoparticles when aiming for the photo-generation of ROS, making the proposed methodology environmental friendly by promoting the reduction in the reagents consumption. The results obtained in these assays revealed that the sensitivity of the proposed methodology increased with the concentration of QD of higher size (3.00 e 3.71 nm). A more thorough comparison of the results obtained with the QD of size 3.00 and 3.71 nm indicated that the highest sensitivity was obtained with the QD of 3.00 nm despite the QD of 3.71 nm in size originate a higher production of free ROS (highest blank signal).

The results demonstrated that a higher generation of free radicals does not implies higher sensitivity, since if a high quantity of ROS is generated than it is necessary to increase the working concentrations of epinephrine to be able to obtain a more accentuated diminishing of the analytical signal (CL quenching) and as a consequence the detection limit increases. This way, considering the obtained results and aiming at the best compromise between the parameters reagents consumption, sensitivity and detection limit, the QD of size 3.00 nm and with a concentration of 1 μmol L^{-1} in solution were selected for further optimization assays.

The following assays were conducted aiming the evaluation of the influence on the analytical signal of some chemical and physical parameters involved in the determination of epinephrine, through the proposed MPFS coupled with a photocatalytic unit emitting visible light, such as concentration of luminol and NaOH, sample volume, flow rate during the irradiation of QD and also the flow rate during the transport of the reaction zone to the detector. The optimization of the parameters under evaluation was made with the objective of better agreement between sensitivity, sample and reagent consumption and determination rate.

The influence of luminol and NaOH concentration on the analytical signal was assessed over a concentration range from 0.5×10^{-3} to 2.5×10^{-3} mol L^{-1} and 2.75×10^{-3} to 5.0×10^{-2} mol L^{-1}, respectively. The study of the influence of luminol concentration on the analytical signal was performed using a set of four epinephrine standard solutions (2.28×10^{-7} – 2.28×10^{-6} mol L^{-1}) and fixing the concentration of NaOH at 0.01 mol L^{-1}. For each concentration of luminol tested, calibration curves were established for evaluation of the sensitivity of the methodology through the analysis of the obtained slopes. The results demonstrated a more pronounced increase of sensitivity for concentrations of luminol from 0.5×10^{-3} up to approximately 1.5×10^{-3}mol L^{-1}, whereas

for higher concentrations the sensitivity tended towards stabilization. Therefore, for posterior assays the concentration of luminol was fixed at 1.5×10^{-3} mol L^{-1}.

In the same way, the study of the influence of NaOH concentration on the analytical signal was executed using a set of four epinephrine standard solutions (2.28×10^{-7} – 2.28×10^{-6} mol L^{-1}) and by using a solution of luminol 1.5×10^{-3} mol L^{-1}. The results (Figure 2) revealed that the sensitivity increased markedly with the concentration of NaOH up to 1.0×10^{-2} mol L^{-1} and for higher concentrations the increase was less pronounced. Taking into account the obtained results, the concentration of NaOH selected for further assays was 1.0×10^{-2} mol L^{-1}.

The influence of irradiation time on the analytical signal was also studied by varying the flow rate during the irradiation of the solution containing QD. In the multipumping flow concept the flow rate is directly determined by the internal volume of the micro-pumps (in the proposed MPFS was 10 µL) and the pulses frequency (often defined by the pulse time). The irradiation time was very important because it determined the extension of the generation of ROS and therefore, the amplitude of the chemiluminescence signal originated by the oxidation of luminol by the ROS. This optimization assay involved the establishment of calibration curves for each pulse time tested from 500 ms to 2500 ms (corresponding to flow rates from 0.92 mL min^{-1} to 0.23 mL min^{-1}). The optimization of the flow rate (pulse time) during the irradiation of QD was performed taking into account the sensitivity (slope of calibration curves) and the determination rate. According to the obtained results, the sensitivity of the methodology increased approximately 10 % by varying the pulse time from 500 to 2000 ms (corresponding to a decrease of flow rate from 0.92 up to 0.28 mL min^{-1}) and then, tended for stabilization. However, the determination rate decreased up to approximately 40 % with the increase of the pulse time. In order to obtain a better compromise between sensitivity and determination rate, a pulse time of 1000 ms (corresponding to a flow rate of 0.52 mL min^{-1}) was selected. In fact, for this pulse time the sensitivity increased about 5 % in a maximum of 10%, while the determination rate decreased approximately 17 % in a maximum of 40%.

The study of the influence of the flow rate during the transport of the reaction zone to the detector, immediately upon photoactivation of QD, was a relevant parameter also evaluated because the CL signal is originated by very fast reactions and is a transient signal. The flow rate during transport to detection determined the residence time of the reaction zone in the flow system, conditioning the light intensity measured. Indeed, depending on reaction kinetics, flow rates excessively low or high could result in a CL emission outside the detector's flow cell. Thus, some assays were carried in order to optimize the CL emission according to the flow rate during transport to detection. In

these assays, for different pulse times of 125, 250, 400, 500 and 750 ms (corresponding to flow rates of 2.18, 1.50, 1.09, 0.92, 0.67 mL min^{-1}), a calibration curve was performed for epinephrine concentrations up to 2.28 × 10^{-6} mol L^{-1}. The results (Figure 3) demonstrated a fast reaction kinetics since the sensitivity (slope of calibration curves) markedly increased with the flow rate (lower pulse times) up to 1.50 mL min^{-1}, approximately. For higher flow rates it was observed that the gain in sensitivity was not so pronounced. Considering that higher flow rates resulted in increased sensitivity and at the same time, determination rate, a flow rate of 2.18 mL min^{-1} (corresponding a pulse time of 125 ms) was selected for further optimization assays.

The influence of sample volume, defined by the pump stroke volume (10 µL) and number of pulses during sample insertion, was studied by varying the number of sample pulses from 4 to 12, corresponding to solution volumes comprised between 40 and 120 µL. The obtained results (Figure 4) showed that the sensitivity markedly increased with the number of sample pulses up to 8 and for higher values slightly decreased.

Another parameter of great importance was the flow strategy used for sample zone and luminol introduction in the flow system at the confluence point X_2 (Figure 1), since it could influence the degree of mixture of the solutions, and hence, determine the reaction development between free ROS and luminol reagent. Thus, some assays were carried out by exploiting two different flow sampling approaches, more specifically merging zones and binary sampling. The optimization method used for these assays was the same as above, aiming at the higher sensitivity of the proposed methodology. The slopes of the calibration curves obtained for each sampling strategy revealed a sensitivity approximately 4 % higher for binary sampling relatively to the merging zones approach. So, the flow sampling strategy selected for the chemical control of epinephrine formulations was binary sampling.

The results obtained in the optimization of the flow parameters are compiled on table 1.

3.3 Interferents

The high content of sodium metabisulfite (preservative) added to the composition of the pharmaceutical samples with the aim to prevent the oxidation of epinephrine interfered with the proposed methodology. Thus, a sample pre-treatment was required as already described in the subchapter “Samples, standards and reagents”. In this procedure, the nitrogen bubbling time was evaluated for a period of time between 5 and 30 min. This study showed that a time of 25 minutes was required to reduce the interference of

sodium metabisulfite in the analysis of the injectable samples, indicating its successful removal.

3.4 Analysis of commercial pharmaceutical formulations

After optimization of the physical and chemical parameters of the flow system, it was possible to achieve an analytical linear response range between 1.14×10^{-7} and 2.28×10^{-6} mol L^{-1} of epinephrine. The calibration curve was represented by the equation $\Delta CL = 65(\pm4) \times \text{Log } C + 464(\pm23)$ (R=0.9953, n=5), in which ΔCL was the chemiluminescence signal quenched, expressed in percentage and C was the epinephrine concentration in mol L^{-1}.

In order to evaluate the accuracy of the developed methodology, and consequently demonstrate its potential for routine laboratory procedures, the described miniaturized and automatic flow system was used for the determination of epinephrine in three formulations of different pharmaceutical laboratories, being all of them injectables, and the obtained results were compared with those found by conducting a reference procedure [50]. The results, summarized in Table 2, showed a good agreement between both methods, with relative deviations between 2.53 % and 4.80 %. A paired Student's t-test [51] confirmed that there were no statistical differences ($t_{estimated}$ = 2.255, $t_{tabulated}$ = 4.303) between the results obtained by both procedures, for a confidence level of 95% (n=3).

The evaluation of the precision of the proposed methodology for epinephrine determination in pharmaceutical formulations was performed through the repeated analysis of each injectable sample (4 consecutive determinations for each sample). The obtained results revealed a good repeatability taking into account the calculated concentration ranges for a confidence level of 95 % (Table 2).

The proposed MPFS allowed a determination rate of about 79 h^{-1}.

4. Conclusions

The main features of MPFS such as high portability, versatility, straightforward automation and control combined with the efficiency and simplicity of the LED photo-excitation unit and, additionally, the high sensitivity of chemiluminometric detection makes the developed analytical methodology an attractive tool for easily implementing chemical reaction schemes involving photoactivation of CdTe QD nanoparticles with visible radiation. The photoactivation of QD to generate ROS demands a high control of

the parameters that can influence the process, such as, time of irradiation and volume of solution containing the QD nanoparticles. The automatic control of those parameters, among others, that a MPFS coupled to a photocatalytic unit offers demonstrated its importance to conduct further scientific studies involving QD photoactivation and reaction mechanisms.

For the first time, a visible light module based on LEDs for the photoactivation of aqueous CdTe QD nanoparticles was implemented in an automatic analytical micro-system exploring the multipumping concept. The LED photo-excitation unit implemented in the MPFS allowed performing the QD photo-excitation without influence of temperature in the assays since the two LED lamps employed were cold-lamps. So, the developed LED-PEU can be safely applied in analysis that involve thermo labile substances or in assays that suffer influence from temperature variations. Additionally, being a non-hazard radiation the use in this work of visible radiation to photoactivate QD is expected to inspire researchers to use automatic flow systems coupled with a LED-PEU unit to conduct assays with QD involving its irradiation.

CdTe-MPA capped semiconductor colloidal nanocrystals can effectively participate in redox photocatalytic processes with solution surrounding species. The extension of radical generation is noticeably affected by nanoparticles size being more pronounced for bigger QD. There are many justifications for this size-radical generation dependence including variations on the surface-to-volume ratio and band-bap energy, occurrence of surface traps (less pronounced on bigger QD), etc. Further research is being carried out to explain these results.

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Figures:

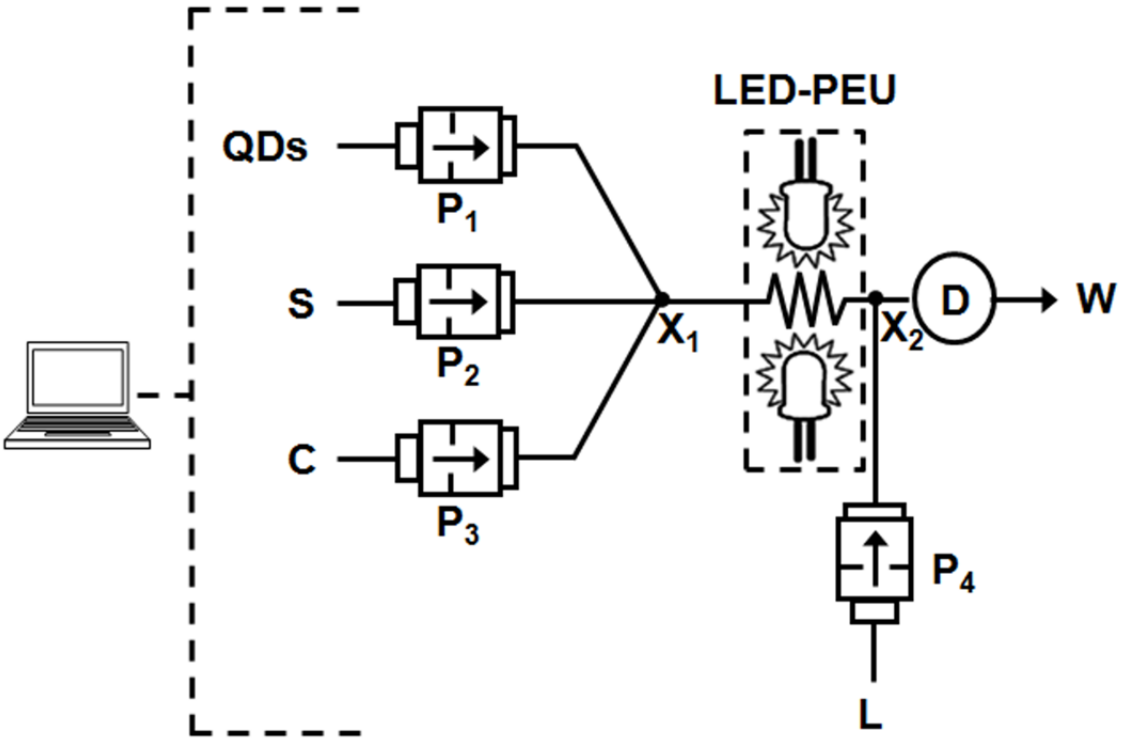

Figure 1 – Multipumping flow system (MPFS). P_1 – P_4, solenoids micro-pumps; X_1 and X_2, confluence points; LED-PEU, photo-excitation unit; D, chemiluminescence detector; S, sample: epinephrine; C, carrier: H_2O; QD, quantum dots: size particle of 3.00 nm with a concentration of 1 µmol L^{-1} in water; L, luminol: 1.5 mmol L^{-1} in 0.01 mol L^{-1} NaOH; W, waste.

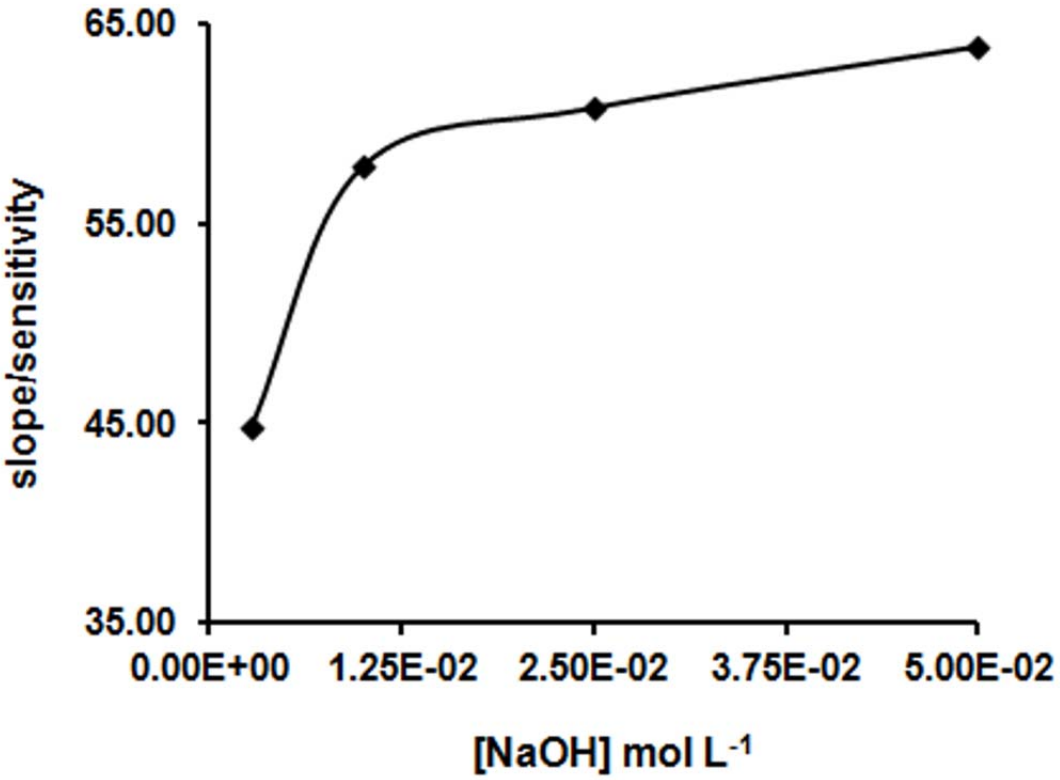

Figure 2 – Influence of sodium hydroxide concentration in the sensitivity of the methodology.

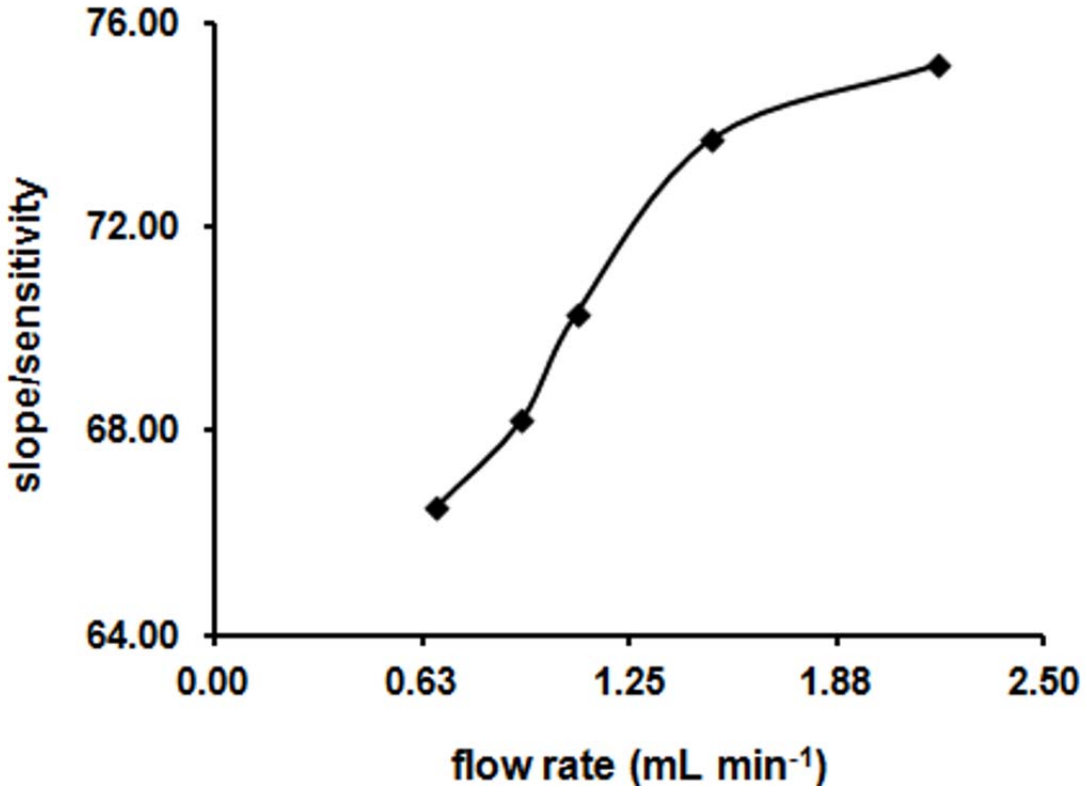

Figure 3 – Influence of the flow rate during the transport of the reaction zone to the detector on the sensitivity of the methodology.

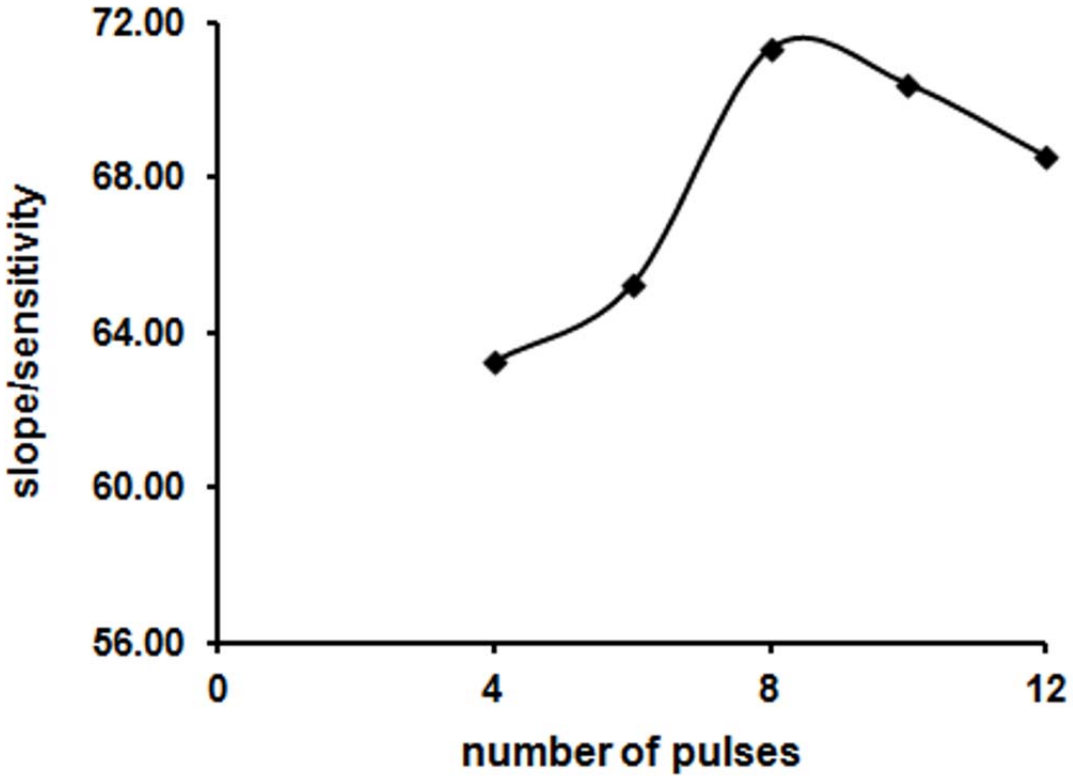

Figure 4 – Influence of number of pulses on the sensitivity of the methodology.

Table 1 - Compilation of the optimization results comprising the physical and chemical parameters.

Parameter	Studied values	Optimized values
QD nanoparticle size (nm)	1.87 – 3.71	3.00
QD concentration (μmol L^{-1})	0.25 – 10.00	1.00
Luminol concentration (mmol L^{-1})	0.50 – 2.50	1.50
NaOH concentration (mol L^{-1})	0.00275 – 0.05	0.01
Number of pulses	4 – 12	8
Sample volume (μL)	40 – 120	80
Pulse time during irradiation (ms)	500 – 2500	1000
Flow rate during irradiation (mL min^{-1})	0.92 – 0.23	0.52
Pulse time during detection (ms)	125 – 750	125
Flow rate during detection (mL min^{-1})	2.18 – 0.67	2.18

Table 2 - Comparison of analytical results obtained in the determination of epinephrine in pharmaceutical formulations by the proposed and the reference method.

Pharmaceutical sample	Declared dosage mg/formulation	Amount found (mg/formulation) (i)		R. D. % (ii)
		MPFS methodology	Reference method	
Pharmaceutical sample 1	0.3 mg/0.3 mL	0.313 ± 0.005	0.31 ± 0.03	2.53
Pharmaceutical sample 2	1 mg/1 mL	0.97 ± 0.02	0.95 ± 0.03	2.45
Pharmaceutical sample 3	1 mg/1 mL	1.04 ± 0.06	0.99 ± 0.09	4.80

(i) Mean ± *t0.05* (Student`s t test) × (S/$\sqrt{n}$).

(ii) Relative deviation of the development method regarding the reference procedure.

CHAPTER 9

Final Conclusions

All potentialities and characteristics of the multipumping flow concept, highlighted in Chapter 1, were explored and proved to be very useful in the different analytical applications described in this thesis. Indeed, the developed methodologies presented in this thesis allowed proving the high versatility associated to MPFS, since it was possible the implementation of several automated analytical procedures aiming the chemical and toxicological control of drugs in different samples, namely, pharmaceutical formulations, alcoholic and non-alcoholic beverages, with assorted detection methods, ranging varied concentration values by resorting to distinct sample manipulation approaches. In addition, due to the modular structure of MPFS, different devices were easily incorporated to perform analytical procedures for derivatization and pretreatment of sample within the system, which led to a significant increase of the analytical potentialities of the flow assemblies.

The insertion of a photodegradation unit allowed the carrying of photochemical reactions in the flow system offering a powerful mean for obtaining substances with improved fluorophoric properties and thus, higher sensitivity. Its implementation in a MPFS for determination of diazepam allowed combining all advantages of automated procedures, namely, high reproducibility due to the strict control of the experimental parameters, with the high analytical potential of the photodegradation unit. In fact, light has properties that make it an ideal reagent for analytical determinations since it allows reduction of the number of chemicals used or even its complete suppression. All these features combined with high sensitivity and selectivity of the fluorometric detection make the developed methodology an important and environmentally friendly analytical method, for chemical control of diazepam in pharmaceutical formulations and also for toxicological analysis of the drug in spiked drinks.

Additionally, the exploitation of multipumping concept made possible combining a photocatalysis process with quantum dots nanotechnology. Indeed, the automation of the chemical reaction scheme involving photoactivation of CdTe QD nanoparticles with visible radiation for ROS generation was successfully achieved because of the combination of the main characteristics of MPFS and the efficiency and safety of the LED photo-excitation unit, thus enabling the development of a powerful automated analytical tool for the photoactivation of CdTe QD. The developed approach was applied for the analytical determination of epinephrine.

The hydrodynamic characteristics of pulsed flow enabled a fast and efficient mixture between sample and reagent solutions, even under limited dispersion conditions improving the reaction development and consequently the sensitivity of the method. This

important advantage inherent to MPFS was demonstrated in the implementation of oxidative coupling reaction between indapamide and MBTH in the presence of cerium (IV) for the spectrophotometric determination of the drug. The high mixing capacity of MPFS was also evident in the results obtained in the chemiluminometric determination of epinephrine, where an efficient, reproducible and high sample/reagent intermixing in close proximity of the detector was achieved, which allowed improving the efficiency and sensitivity of the measurements, considering the reduced time of the light emitted from the short-lived excited state intermediates produced in the chemiluminescent reaction. Moreover, the nature of the pulsed flow demonstrated to be very useful in the developed methodology for fluorometric determination of glibenclamide wherein an inline pre-separation unit was incorporated into a MPFS for the separation of glibenclamide from tea samples. It was observed that a micro-pump with a higher stroke volume (50 µL) allowed to obtain an improved desorption process of glibenclamide from activated charcoal, which enabled an enhancement of the obtained analytical signal. In this flow manifold the combination of solenoid micro-pumps with solenoid valves facilitated the automation and control of the analytical procedure since the synergy between propulsion and commutation capacities of micro-pumps and the facility of directing the solutions afforded by solenoid valves allowed a higher versatility of management of solutions without impairing the determination rate.

In general, flow manifolds with a very simplified configuration and control were obtained because the use of micro-pumps to perform several tasks, namely, sample and reagents insertion, propulsion and commutation of solutions allowed a significant reduction of the number of manifold active components. This fact enabled the development of very compact flow systems with great operational simplicity and consequently, a reduction of cost and power consumption was also achieved.

The multiple tasks performed by micro-pumps in combination with their automatic and individual control enabled the utilization of different sampling strategies including single sample volumes, binary sampling and merging zones without implying physical reconfigurations of the system. The individual and independent control of solenoid micro-pumps by means of a microcomputer ensured a great flexibility in terms of manipulation of solutions. This was evidenced, for example, in the automation of a reaction scheme involving MBTH and Ce (IV), in which, in the flow-based analytical system proposed for indapamide determination, two different addition sequences of reagents could be easily implemented yielding different reaction products with different kinetics, stabilities and absorbance spectra. Also, the multitasking versatility of the micro-pumps was evidenced in the developed methodology for glibenclamide determination based on

adsorption/desorption of the drug into activated charcoal, where inclusively an approach of flow reversal was easily accomplished.

All the analytical methodologies developed in this work for the chemical control of drugs in pharmaceutical formulations demonstrated to be advantageous alternatives to the reference procedures recommended by the British Pharmacopoeia. These reference procedures, despite providing good specificity with excellent precision and accuracy, required time-consuming and laborious sample preparation, rigorous intervention of operator, lengthy analysis times, expensive instrumentation, consumption of large volumes of solutions and in some cases the handling of hazardous reagents. On the other hand, the proposed methodologies were fast, simple, versatile and reliable, apart from that the obtained results were statistically consistent with those furnished by the reference procedures. In comparison to the reference procedures recommended by the pharmacopeia, the proposed automatic methods allowed a significant reduction in reagents consumption with consequent minimization of waste generation while allowing a higher determination rate. Taking into account its main features, the proposed flow methods could be considered as valuable analytical tools that can be easily adapted for routine pharmaceutical analysis in the industry at a relatively low cost.

The automatic methodologies developed for the toxicological control of beverages constituted a noteworthy contribution for the fast screening of drugs surreptitiously spiked in alcoholic and non-alcoholic drinks used in DFC, which can provide a valuable contribution in preventing, and possibly assist in the establishment of legal responsibility for these crimes. Considering the unique features of several aspects of MPFS, including manifold components and configuration, operational mode and flow hydrodynamics characteristics, the proposed flow systems offered also a high portability and low power consumption, enabling a promising application in *in situ* analysis. The methods proposed for the fast and *in situ* screening of drugs in spiked beverages can be used, not as alternative but, as complement to conventional toxicological analysis of biological samples, namely, blood and urine analysis. The automated methods allow to rapidly identify and quantify the responsible drug used in DFC cases when spiked beverages are involved, as chemical control of these samples usually do not require quantification methods with low detection limits.

In the future, the development of more miniaturized flow-based analytical systems, increasingly aiming at low power consumption and reduced physical size, should be further exploited in order to obtain valuable analytical tools able to perform *in situ* analysis providing fast and real-time information related to the drug screening in beverages at the places of adulteration or usage.