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Implementation in the clinical practice of the quantitative analysis of antidepressant drugs in patients with suspected psychotropic drug intoxication

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IMPLEMENTAÇÃO NA PRÁTICA CLÍNICA DA ANÁLISE QUANTITATIVA DE FÁRMACOS ANTIDEPRESSIVOS EM DOENTES COM SUSPEITA DE INTOXICAÇÃO POR PSICOFÁRMACOS

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Abstract

Antidepressant drugs (ATDs) contributed to the high usage of psychotropic drugs over the last decades, being the second cause of drug intoxications in Portugal. However, a transition of the prescriptions from highly toxic ATDs to newer ATDs, which are deemed safer, has been observed. Consequently, the mortality associated with ATDs intoxication is not, currently, as prominent. However, in the cases of polymedicated patients, patients with reduced hepatic and kidney function and patients with polymorphisms in the liver metabolism enzymes, the morbidity of intoxication may be significant. Therefore, the availability of qualitative and quantitative toxicological analyses techniques in healthcare facilities is of immense importance.

The main purpose of this study was to implement in the clinical practice an auxiliary method for the diagnosis and monitoring of patients suspected for ATDs intoxication or toxicity, which quantifies the extensive variety of ATDs available in the market.

First, the study was submitted for authorization of several departments and for approval of the hospital's board of directors. Next, the medical and nursing teams of the Urgent Care Centre and of the Emergency Room were briefed on the project procedures. Then, method verification was carried out to confirm the fitness of the proposed method for its intended use. Finally, quantification of the various ATDs and active metabolites was performed in patients' serum samples, using the verified commercial kits.

The method proved to be appropriate for the intended analysis, for most analytes, having adequate precision, accuracy and measuring range. However, in some analytes, the results did not meet the standards required and, for that reason, some parameters need to be further evaluated in the future. The method also showed to be valuable for the diagnosis and monitoring of patients suspected for ATDs intoxication or toxicity, since it allowed the detection of some ATDs, in patients samples, that are overlooked by the screening tests used in clinical routine.

Keywords: *Antidepressants, intoxication, quantitative analysis, LC-MS/MS, method verification.*

Resumo

Os antidepressivos contribuíram para o elevado consumo de psicofármacos nas últimas décadas, sendo os antidepressivos a segunda causa de intoxicações medicamentosas em Portugal. No entanto, tem se observado uma transição das prescrições de antidepressivos altamente tóxicos para antidepressivos mais recentes e mais seguros. Consequentemente, a mortalidade associada aos antidepressivos não é, atualmente, tão significativa. No entanto, nos casos de pacientes polimedicados, pacientes com função hepática e renal reduzida e pacientes com polimorfismos nas enzimas do metabolismo hepático, a morbidade da intoxicação pode ser significativa. Portanto, a disponibilidade de técnicas de análises toxicológicas qualitativas e quantitativas em estabelecimentos de saúde é de elevada importância.

O principal objetivo deste estudo foi implementar na prática clínica um método auxiliar para o diagnóstico e monitorização de pacientes com suspeita de intoxicação ou toxicidade por antidepressivos, que permita a quantificação da extensa variedade de antidepressivos disponíveis no mercado.

Em primeiro lugar, o estudo foi submetido para autorização de vários departamentos e para aprovação do conselho de administração do hospital. Em seguida, as equipas médicas e de enfermagem do serviço de urgência e da sala de emergência foram informadas sobre os procedimentos do projeto. Depois, foi realizada a verificação do método proposto para confirmar a adequação do mesmo para o uso pretendido. E, finalmente, a quantificação dos vários antidepressivos e respetivos metabolitos foi realizada em amostras de soro de pacientes usando kits comerciais verificados.

O método mostrou-se adequado para a análise pretendida, para a maioria dos analitos, apresentando precisão, exatidão e intervalo de medição adequados. No entanto, em alguns analitos, os resultados não foram de encontro aos padrões exigidos e, por esse motivo, alguns parâmetros terão de ser reavaliados no futuro. O método também se mostrou relevante para o diagnóstico e monitorização de pacientes com suspeita de intoxicação ou toxicidade por antidepressivos, pois permitiu a quantificação de alguns antidepressivos, em amostras de pacientes, que não são detetados pelos testes qualitativos utilizados na rotina clínica.

Palavras-Chave: *Antidepressivos, intoxicação, análise quantitativa, LC-MS/MS, verificação de método.*

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List of Abbreviations and Symbols

5-HT: 5-hydroxytryptamine or Serotonin

ATD: Antidepressant Drug

b (%): Relative Bias

CID: Collision-Induced Dissociation

CHUPorto: *Centro Hospitalar Universitário do Porto*. Porto's University Hospital Centre

CNS: Central Nervous System

CV (%): Coefficient of Variation

CYP: Cytochrome P450

DHD: Defined Daily Dose per 1000 Inhabitants per Day

ESI: Electrospray Ionization

HPLC: High Performance Liquid Chromatography

INEM: *Instituto Nacional de Emergência Médica*. Portuguese National Institute of Medical Emergency

ISTD: Internal Standard

LC: Liquid Chromatography

LOD: Limit of Detection

LLOQ: Lower Limit of Quantification

MAO: Monoamine Oxidase

MAOI: Monoamine Oxidase Inhibitor

MRM: Multiple Reaction Monitoring

MS: Mass Spectrometry

MS/MS: Tandem Mass Spectrometry

NE: Norepinephrine

NSAID: Non-Steroidal Anti-Inflammatory Drug

r (%): Relative or Apparent Recovery

R^2: Square of the Correlation Coefficient or Coefficient of Determination

SERT: Serotonin Transporter

SNRI: Serotonin and Norepinephrine Reuptake Inhibitor

SQC: *Serviço de Química Clínica*. Clinical Chemistry Service

SSRI: Selective Serotonin Reuptake Inhibitor

$S_{y/x}$: Standard Error of the Regression or Standard Error of the Estimate

TCA: Tricyclic Antidepressant

TDM: Therapeutic Drug Monitoring

UHPLC: Ultra-High Performance Liquid Chromatography

UV: Ultraviolet

1. Introduction

1.1. Intoxication by Antidepressants

An intoxication consists of a series of effects and symptoms that occur after exposure to a xenobiotic (chemical compounds foreign to an organism or biological system) in toxic concentrations for a short period of time (acute event) or in a continuous and repetitive manner (chronic event) (1).

An intoxication can result from several scenarios of intentional exposure: misuse of therapy, drug abuse or intentional overdose (e.g., suicidal attempt, murder attempt, to facilitate theft or sexual abuse) (2, 3). In children and the elderly, most intoxications are associated with accidental exposure (4, 5). Accidental intoxication can be iatrogenic; it can result from the practice of self-medication and can be caused by pharmacokinetic and pharmacodynamic drug interactions in polymedicated patients (3). This type of intoxication can also result from idiosyncratic reactions (inter-individual variability such as polymorphism) (2).

Drug intoxications, particularly with psychotropic drugs, is a serious global public health problem, due to their high morbidity and mortality (5). Patterns and causes of intoxication vary across countries and regions, which may reflect drug availability and prescribing practice in the population (4). Since the introduction of the first psychotropic drug, the number of cases of overdose with these drugs seems to accompany their sales (6).

Portugal, in particularly, has, in comparison with other European countries, the highest rates of consumption of psychotropic pharmaceuticals (7). Drugs that act on the central nervous system (CNS), have an evident economic weight in the Portuguese National Health Service and ATDs contributed particularly to the increase in the use of this type of drug over the last decades (**Figure 1**) (8).

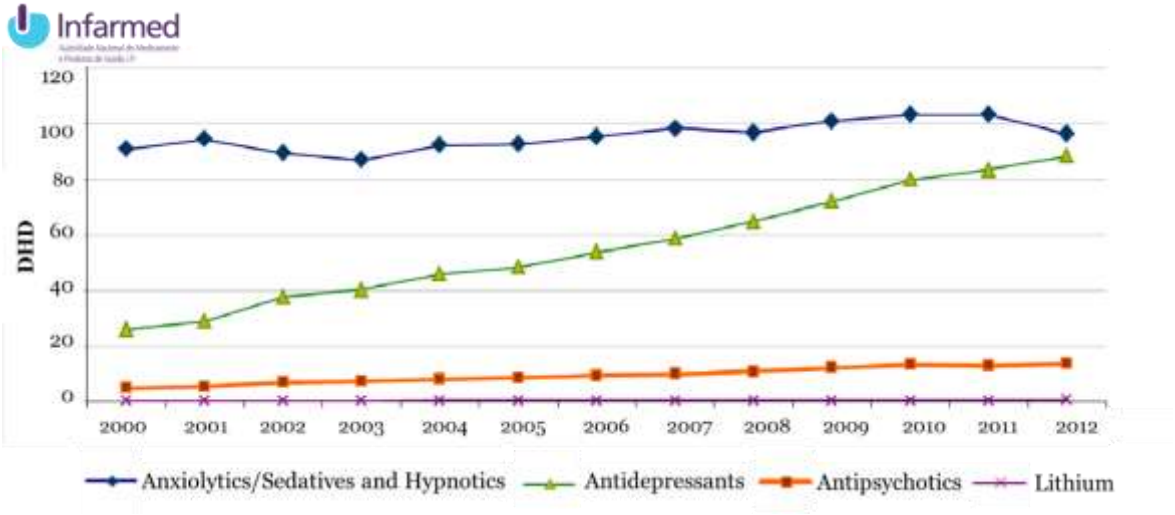


Figure 1 | *Evolution of psychotropic drug use expressed through the defined daily dose per 1000 inhabitants per day (DHD), by therapeutic subgroup between 2000 and 2012. Adapted from (8).*

This increase may reflect greater accessibility to medicines, longer use, the approval of new therapeutic indications and the deterioration of Portugal's population mental health (7).

The ATDs played a significant role in the increase in the prescription and use of psychotropic drugs in Portugal in the last 30 years. Thus, it is expected that intoxications with this group of psychotropic drugs follow the same trends. Data from the Portuguese National Institute of Medical Emergency (INEM) confirm this theory (**Figure 2**).

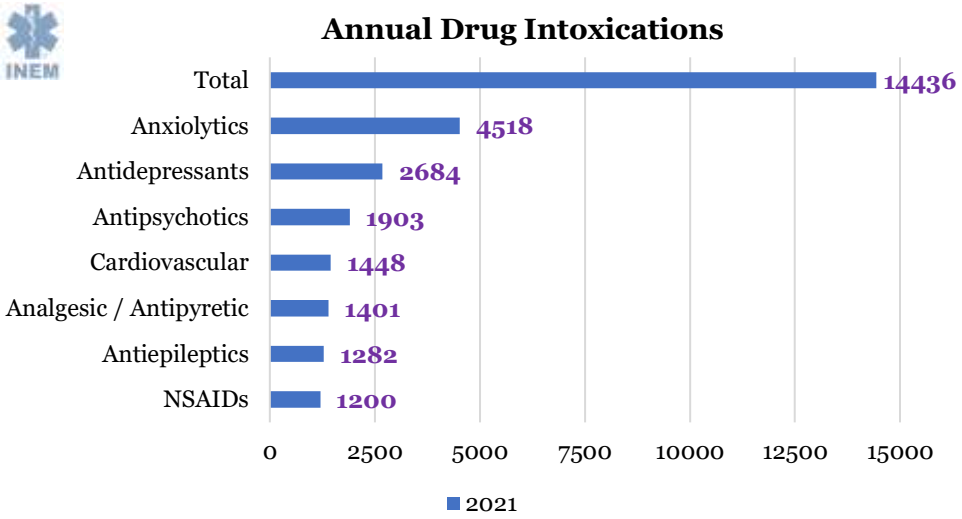


Figure 2 | *INEM's performance indicators: characterization of drug intoxications in 2021. Source: INEM.*

Given the trend of increasing prescriptions and, consequently, intoxication with psychotropic drugs, it becomes increasingly important to change prescribing patterns towards safer and equally effective drugs, to reduce the rates of fatal overdoses (6). Understanding country-specific patterns of drug use and intoxication is essential for

planning overdose prevention programs (4) and for the development and implementation of suitable toxicological analyses in health services.

In the case of ATDs, the change has already taken place: the prescriptions have changed from highly toxic tricyclic antidepressants (TCAs) to other ATDs, such as selective serotonin reuptake inhibitors (SSRIs), which are more selective and less toxic (**Figure 3**) (9).

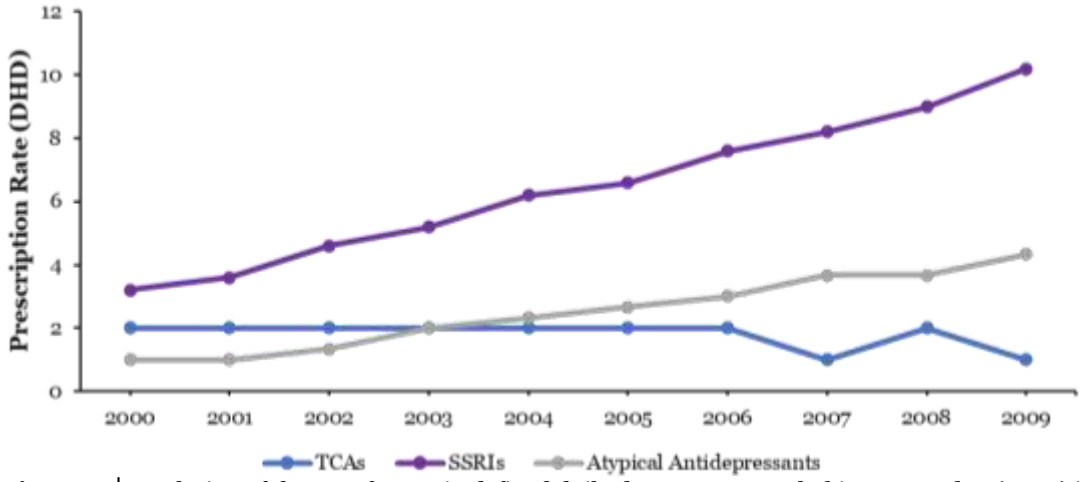


Figure 3 | *Evolution of the use of ATDs in defined daily dose per 1000 Inhabitants per day (DHD) in Portugal. Adapted from (10).*

Despite the increase in ATD overdose, primarily because of the increase in SSRI intake, fatalities have decreased, due to a better safety profile of these newer drugs (9).

So, the mortality associated with ATDs overdose is not, currently, as problematic as it was in the past (11). However, in the cases of polymedicated patients with possibility for drug interactions, risk groups with reduced hepatic and kidney function that compromise drug elimination, and patients with polymorphism in the enzymes involved in the pharmacokinetics of ATDs, the morbidity associated with the use of ATDs is significant and they may particularly benefit from the quantitative monitorization (12).

These facts reinforce the idea that the availability of the quantitative and qualitative toxicological analyses techniques to support clinical interventions in the intoxicated patient is of immense importance, both from a clinical and public health points of view.

1.2. Antidepressants Pharmacology

The ATDs are psychotropic drugs traditionally used in the treatment of depressive disorders, though, they have a broader therapeutic application (13). These psychotropic drugs aim to correct chemical imbalances of neurotransmitters in CNS, namely serotoninergic and noradrenergic neurotransmission, which are believed to be responsible for changes in mood and behaviour (14).

The ATDs are commonly divided into four major groups: SSRIs; atypical antidepressants, which include selective serotonin and norepinephrine reuptake inhibitors (SNRI); cyclic antidepressants including TCAs; and monoamine oxidase inhibitors (MAOI) (15).

The different classes of ATDs are very similar in terms of their therapeutic efficacy. Thus, knowing the toxicity profiles of these drugs is of particular importance to choose the therapy to be adopted (15).

1.2.1. Selective Serotonin Reuptake Inhibitors

The SSRIs emerged in the 1980s in the United States with the introduction of fluoxetine (Prozac®). After TCAs and MAOIs, the clinical introduction of SSRIs revolutionized the therapy for depression (16). SSRIs are effective, have a good tolerability profile and a wide therapeutic range (17). Therefore, they are widely prescribed, being currently used as the first-line treatment of depression (18).

The SSRIs are potent and selective inhibitors of serotonin reuptake at the presynaptic terminal by inhibiting serotonin transporters (SERT). This results in an increase in serotonin (5-HT) availability at serotonergic synapses (18). The pharmacological activity of SSRIs allows their use in major depression, minor depression and other psychiatric disorders thought to be associated with a dysfunctional state of the 5-HT sysyem (19).

Despite being considered safe, SSRIs may cause some adverse effects, which can arise at therapeutic doses. Gastrointestinal effects such as anorexia, nausea, vomiting and diarrhea are the most common. Sexual dysfunction, headaches, insomnia, fatigue, anxiety, and minor cardiovascular effects may also occur (18, 20).

The SSRIs undergo extensive oxidative metabolism catalysed by several hepatic enzymes including CYP, a family of more than 30 isoenzymes present in the endoplasmic reticulum of hepatocytes. At the same time, SSRIs can inhibit CYP isoenzymes, thus triggering pharmacokinetic interactions with other drugs or molecules that are metabolized by this set of isoenzymes. Co-administration may lead to a change in clearance or a change in the ratio

of parent compound to active metabolites, resulting in a subtherapeutic effect or toxicity (17, 21).

The SSRIs may also have pharmacodynamic interactions with other drugs through physiological mechanisms such as: direct competition at receptor binding sites; exacerbated increase in the same neurotransmitter pathway; or an effect on the physiological functioning of an organ or organ system (21). Co-administration with drugs that increase the bioavailability of 5-HT in the synaptic cleft precipitates serotonin syndrome. Serotonin syndrome symptoms include hypertension, mydriasis, diaphoresis, rigors, hyperthermia, agitation, tremors, delirium, convulsions, among others (22).

Signs of acute intoxication with SSRIs include nausea, emesis, dizziness, hyperopia, and less common symptoms such as CNS depression and sinus tachycardia. Thus, it is rare for an overdose of SSRIs to result in life-threatening effects. The treatment of intoxicated patients is fundamentally based on supportive care (20, 23).

1.2.2. Atypical Antidepressants

Atypical antidepressants are a set of pharmaceuticals with different mechanisms that expand the pharmacological properties of SSRIs (20). Atypical antidepressants, along with SSRIs, constitute the current standard treatment of depression (20). Included in this group are the SNRIs; serotonin reuptake antagonists and inhibitors; selective norepinephrine reuptake inhibitors; norepinephrine and dopamine reuptake inhibitors; and partial agonists and serotonin reuptake inhibitors.

SNRIs, commonly called “dual-action” agents, combine robust inhibition of SERT with variable degrees norepinephrine transporter inhibition (12). The addition of the norepinephrine transporter inhibition, theoretically, has therapeutic advantage by increasing the reach of these ATDs to monoamine neurotransmitter systems in more regions of the brain (24). Venlafaxine is an example of an SNRI.

Selective norepinephrine reuptake inhibitors selectively inhibit norepinephrin (NE) reuptake, avoiding the additional undesirable properties of other ATDs (e.g., TCAs) (24). The first selective noradrenergic reuptake inhibitor marketed in Europe was reboxetine (24).

An example of a norepinephrine and dopamine reuptake inhibitor is bupropion (24). The mechanisms of action of bupropion and its main active metabolite, hydroxybupropion, include inhibition of dopamine and, to a lesser extent, NE reuptake (20).

Serotonin antagonists and reuptake inhibitors combine 5-HT receptors inhibition and SERT inhibition. Trazodone is a 5-HT receptor antagonist and a weak serotonin reuptake inhibitor. Additionally, trazodone has α-adrenergic antagonist activity and weak histamine H1 receptor antagonism (15, 20).

Partial agonists and serotonin reuptake inhibitors are, essentially, SSRIs with additional 5-HT_{1A} receptor agonism. The combination of serotonin reuptake inhibition with partial 5-HT_{1A} agonism is known to enhance antidepressant properties and to enhance SSRI/SNRI tolerability in some patients (24). Vortioxetine is a partial agonists and serotonin reuptake inhibitor.

Mirtazapine is structurally classified as tetracyclic antidepressant (**Chapter 1.2.3.1**.). However, due to its unique mechanism of action, mirtazapine is considered an atypical antidepressant: central presynaptic inhibition of α_2-adrenergic receptors, which triggers an increase in the neuronal release of NE and 5-HT; inhibition of some 5-HT receptor subtypes; and blockade of histamine receptors (20).

1.2.3. Other Antidepressants

The emergence of first-generation antidepressants in the 1950's, such as MAOIs and TCAs, revolutionized psychopharmacology of depression and made fundamental contributions to the development of psychiatry. However, the establishment of safer ATDs (e.g., SSRIs) led to a decrease in their general use. Although the cases of intoxication and overdose with TCAs and MAOIs have been decreasing, it is still important to know their pharmacology and toxicology bacause they still have clinical use.

1.2.3.1. Cyclic Antidepressants

Cyclic antidepressants include the traditional TCAs, as well as other cyclic compounds, such as tetracyclic antidepressants. Cyclic antidepressants have at least three condensed rings in their chemical structure (20).

The pharmacological effect of TCAs include the inhibition of the reuptake of 5-HT and NE, which increases the amount of these neurotransmitters in the CNS receptors (14). Unfortunately, TCAs have unwanted pharmacological effects such as anticholinergic (muscarinic) action, antihistamine action and blockade of α-adrenergic receptors (20, 25). Consequently they have a narrow therapeutic range and may be associated with significant neurotoxicity and cardiotoxicity (26). During the administration of TCAs with other drugs, pharmacodynamic interactions may occur. The combination of TCAs with other drugs that

influence the 5-HT neurotransmission (e.g., MAOIs) may result in the development of serotonin syndrome (27).

As TCAs can cause serious and fatal side effects, they are not currently used as first-line treatment of depressive disorders.

1.2.3.2. Monoamine Oxidase Inhibitors

The monoamine oxidases (MAO) are a family of enzymes that catalyse the inactivation of monoamines, such as NE, 5-HT, and dopamine. They also metabolize exogenous molecules like tyramine. There are two types of MAO: MAO-A preferentially metabolizes NE and 5-HT and is concentrated in the intestine and liver; MAO-B is more concentrated in the brain (20). MAOIs inhibit the activity of these enzymes, increasing the availability of biogenic amines such as NE and 5-HT, decreassing the symptoms of depression, namely TCAs-resistant depression, and atypical depression (24, 28).

Patients taking MAOIs are vulnerable to severe hypertensive reactions: MAOIs indirectly cause an increase in catecholamine storage enhancing the action of sympathomimetics (that cause the release of those neurotransmitters); MAOIs decrease the protection that MAO-A offers, at the intestinal and hepatic level, against sympathomimetics consumed orally through food (e.g. tyramine present in fermented foods) (15). The emergence of selective reversible inhibitors of MAO-A (e.g., moclobemide) helped solve this problem because they can block sufficient MAO-A in the CNS to obtain an antidepressant effect, while dietary tyramine is effectively metabolized by the peripheral MAO-A by displacing the inhibitor (29).

1.3. Toxicological Analyses of Antidepressants

Analytical toxicology is the application of analytical chemistry tools in the detection, identification, and measurement of xenobiotics and their metabolites in biological samples, to aid in the diagnosis, treatment, prognosis and prevention of intoxication (30). Xenobiotics can be pharmaceutical agents, illicit drugs, gases, solvents, pesticides, toxic metals, toxins, and a multitude of other industrial and environmental toxicants (31). Analytical toxicology has several applications, namely in the clinical field. Clinical toxicology involves therapeutic drug monitoring (TDM) and urgent toxicological analyses that aim, respectively, to optimize pharmacological therapy and identify the intoxication agent (3).

Acute intoxication is a common reason for hospital admission, and most intoxication patients make a full recovery without specific treatment. However, for some common intoxication agents, analytical toxicology data can be important to establish and confirm a diagnosis and guide the choice of treatment and therapy (30).

Knowing the concentration of a xenobiotic is especially useful for two reasons: to establish a prognosis and treatment in the early stages of intoxication when there is minor toxicity; and to make decisions regarding the use of antidotes or interventions to accelerate drug elimination (20). When quantification is not feasible, a qualitative result is still of considerable value if symptoms are consistent with the detected compound (31).

1.3.1. Biological Samples

The biological samples most frequently collected at the hospital for clinical chemistry are urine and whole blood, which can be processed into serum (without anticoagulant) or into plasma (with anticoagulant) (3).

In toxicology, blood is the most important biological sample. The main advantages of using blood are: it is a widely accepted matrix; it has better pharmacokinetic and clinical correlation for therapeutic and toxic levels; it is a difficult sample to adulterate; and it has extensively studied reference values (3). Although blood can be collected from various anatomical sites, blood is usually collected from peripheral venous accesses (e.g., cephalic vein) (32).

Most quantitative assays are performed on serum or plasma (33). The use of serum has the advantage of not having a potential interference caused by additives, however its obtainment becomes time consuming, since the coagulation is slow, which becomes problematic in the analysis of unstable compounds (31).

1.3.2. Analytical Methods

The analytical methods used in clinical toxicology must meet certain requirements that make them applicable in a hospital context. They must be fast, analyte-specific, accurate, and sensitive for determining clinically useful concentrations in a small volume of sample.

The main methods used in toxicological analyses include spectrophotometric tests, immunoassays and chromatographic separation techniques including gas chromatography (GC) and liquid chromatography (LC) coupled to different detectors (20).

Chromatography is a separation method through which a complex mixture is physically separated into its individual components. The fundamental principle of the methodology is the differential partitioning of a mixture between stationary and mobile phases (20). LC and GC are column separation techniques that require more technical knowledge, but which, when associated with detectors, allow both the identification and quantification of a wide variety of xenobiotics with low detection limits (20, 34).

The LC separation is based on the differential distribution of solutes between a liquid mobile phase and a stationary phase. When small-diameter particles are used to support the stationary phase, the technique is known as high performance liquid chromatography, (HPLC) (34). Ultra-high performance liquid chromatography (UHPLC) operates at higher pressures than HPLC and allows for smaller particle sizes in columns, increasing sensitivity (35). In the clinical laboratory, HPLC is the most widely used form of LC (36). The type of stationary phase in the column is the main determinant of the selectivity and resolution of LC separations (34). Many different types of detectors have been used in LC including spectrophotometric detectors (e.g., UV-Vis), fluorometric detectors, electrochemical detectors, mass spectrometry (MS) and tandem mass spectrometry (MS/MS) detectors (20, 34).

1.3.2.1. Mass Spectrometry

The MS is an analytical method for studying the masses of molecules (or fragments of molecules in MS/MS) present in a sample (36, 37). MS ionizes the sample components, separates the resulting ions in vacuum based on their mass-to-charge ratios (m/z) and measures the intensity of each ion. This information indicates the concentration level of ions that have a given mass and is extremely valuable for quantitative and qualitative analysis (38).

Analysis by MS begins with the ionization of analyte molecules into gas-phase ionic species, that may break into characteristic fragments (36). Electrospray ionization (ESI), a type of atmospheric pressure ionization, is one of the softest ionization method available, which means fragmentation is minimal and it can be used for highly polar, least volatile, or thermally unstable compounds. ESI can generate positive or negative ions depending on the charge of the voltage applied. Since most of the compounds result in protonated (or deprotonated) molecular ions and adduct ions, without generating complicated fragment ions, determination of the molecular mass of compounds is very simple (38).

Because ionization in ESI and mass analysis happen at different pressures, there is a need for an interface that introduces the ions generated by the ionization probe unit into a

vacuum. After that transition, the ions generated are guided through a lens system that focuses them and introduces them into the mass analyser section (**Figure 4**) (38).

There are a variety of mass analysers including single MS and tandem/hybrid arrangements known as MS/MS systems. The most common form of MS/MS is the triple quadrupole (31). In a triple-quadrupole instrument, there are three sets of quadrupole filters, although only the first and third function as mass analysers. The first quadrupole (Q1), acting as a mass filter, transmits and accelerates a selected ion towards the second quadrupole (Q2), which is called a collision cell. The pressure in Q2 is higher, and the ions collide with a neutral gas (e.g., Argon). The result is fragmentation by collision-induced dissociation (CID). The fragments are then accelerated into the third quadrupole (Q3), another scanning mass filter, which sorts them before they enter a detector (39).

There are different data acquisition modes. When working with quadruple mass analyser for single MS, scan mode and selected ion monitoring mode can be used: in the first mode, a scan is performed in the defined mass range; In the second mode, only ions with target mass are selectively detected. With triple quadrupole, these modes can be mixed and matched in Q1 and Q3. In the specific case of multiple reaction monitoring (MRM) mode, both Q1 and Q3 are operated in selected-ion monitoring mode (39).

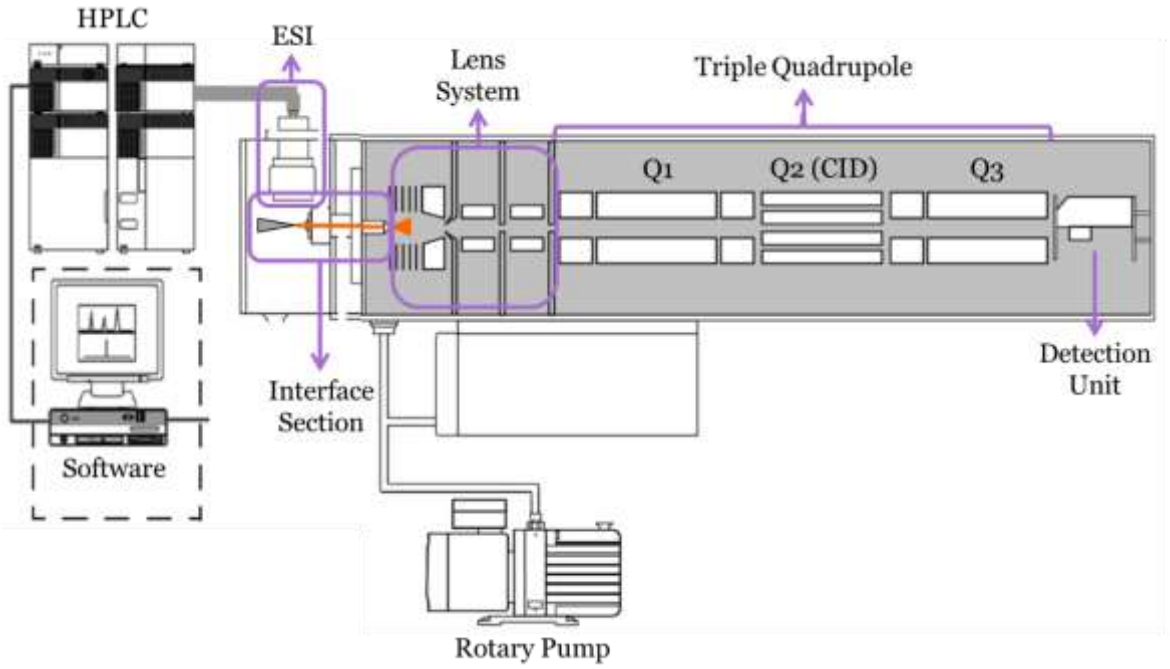


Figure 4 | *Components of an ESI-MS/MS. Source: Shimadzu.*

1.3.3. Method Validation and Method Verification

Method validation is the process of confirming, by examination of objective evidence, that the method under consideration fulfils the specific requirements for a intended use or application (40). In the context of toxicological analyses, validated procedures must be used to perform the appropriate analyses to the required degree of accuracy/reliability in an clinically relevant time scale (30).

For quantitative bioanalytical procedures, it established that at least the following validation parameters should be assessed: selectivity, linearity of calibration, stability, accuracy, precision, and lowest limit of quantification (LLOQ). Additional parameters which may be relevant include limit of detection (LOD), recovery, reproducibility, and robustness (41).

According to the Australian National Association of Testing Authorities, when a laboratory uses a commercial test kit and applies the methodology and reagents according to the manufacturer's instructions, the kit does not need to be revalidated in the testing laboratory (42). In this case, method verification is required.

Method verification is performed when basic validation work of the method has already been carried out, but the laboratory still needs to confirm its ability to apply the method (40). It is recommended to verify that the operators using their equipment in their laboratory environment can apply the method obtaining the same outcomes as in the validation data provided in the commercial kit (42). Verification is also required when there is an important change such as a new but similar instrument, relocation of equipment, among others (40).

Having this in mind, some parameters that are evaluated in method validation must be verified during method verification procedures. These include intraday and interday precision and accuracy, linearity of calibration, measuring range, LLOQ and LOD.

The precision of an analytical procedure expresses the degree of agreement between a series of measurements of replicates from the same sample and may be considered at three levels: repeatability (intra-assay), intermediate precision (inter-assay), and reproducibility (41). Precision is usually expressed numerically by measures of imprecision, such as relative standard deviation or coefficient of variation (42).

The accuracy of an analytical procedure expresses the proximity between a result and its true nominal value (42). The accuracy of a method is affected by both systematic error and random error (i.e. total error), but it is usually wrongly used to describe only the systematic error component (41).

The linearity of calibration is the ability to induce a direct proportional correlation between the analyte concentration and the magnitude of the signal produced (response), within a specified range (measuring range) (37). The standard error of the regression ($S_{y/x}$) is a measure of the linearity. The square of the correlation coefficient (R^2) derived from regression analysis is frequently used of as a test for linearity, but it may be misleading (42). It is important to note that, although linear models are preferable, non-linear models (e.g., second-order) can be used in the calibration (41).

The LOD is the smallest amount of analyte that can be distinguished from the blank, i.e., it can be detected, but not necessarily quantified as an exact value. The LLOQ is the smallest amount of analyte that can be measured with adequate precision and accuracy (41).

2. Research Plan

The main purpose of this work is to implement in the clinical practice of the *Centro Hospitalar Universitário do Porto* (CHUPorto) an auxiliary method for the diagnosis and monitoring of patients suspected for ATDs intoxication or toxicity, which allows the quantification of a wide range of ATDs in clinical use.

This research study is institutional, longitudinal, prospective, clinical and laboratory. The number of estimated samples was based on the number of patients with an altered state of consciousness, with a request for toxicology screening and with the suspicion of "intoxication", which was an average of 100 requests per six months in 2020.

To fulfil the purpose of this research study, many steps have been considered (**Figure 5**).

A prospective clinical investigation involving the healthcare provision requires the analysis and authorization of several departments that together meet the conditions for approval by the hospital. So, this study has been formally submitted to the Education, Training and Research Department of CHUPorto, to the Clinical Investigation Service, to the Ethics Committee of CHUPorto, to the Person Responsible for Access to Information and to the Data Protection Officer, to obtain institutional authorization by CHUPorto's board of directors.

Additionally, the medical and nursing teams of the Urgent Care Centre and of the Emergency Room, which are responsible for the care of the patients mentioned above and request for diagnostic tests, were briefed on the project procedure. Likewise, procedures were agreed with the head of CoreLab to establish a flow of samples from the collection site (Urgent Care Centre and of the Emergency Room) to the analysis site [Clinical Chemistry Service (SQC)].

A method verification exercise was carried out to confirm the suitability of the proposed method for its purpose and that the same outcomes, as defined in the validation data provided in the commercial method, are obtained.

The method was put to the test and the quantification of the various ATDs and active metabolites was performed in serum samples from patients with suspected intoxication, using the verified LC-MS/MS commercial kits.

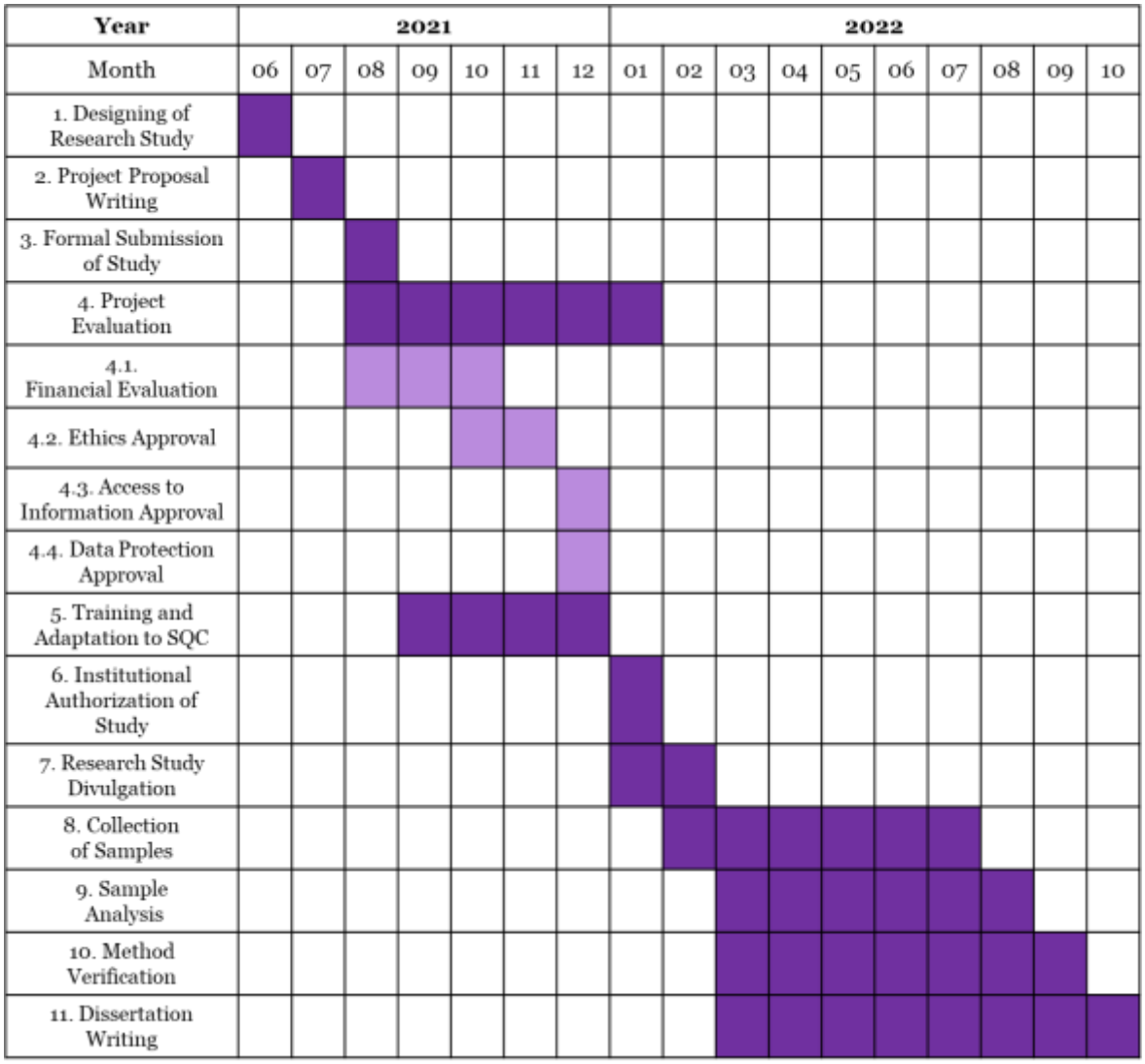


Figure 5 | *Workflow timeline.*

3. Research Methods

3.1. Pre-Analytical Procedures

As this study was elapsed in an hospital context using real patient's samples, several procedures had to take place before the actual analytical procedure. In this chapter, this will be explored.

3.1.1. Submission of the Research Study

As mentioned before, the formal submission of an academic research study was performed to obtain institutional authorization from CHUPorto. For that purpose, several essential documents and information were prepared, organised, and submitted. These included the research study cover pages (**Appendix 1**); the budget and respective funding; the appeal for institutional authorization (to the board of directors, the chair of the ethics committee and the education/training/research department); a statement of responsibility (from student and advisors); the local authorizations (from emergency department director, SQC director, pathology department director, emergency room officer); the student curriculum vitae; the academic research proposal; a letter requesting waiver of informed consent; the request for reuse of clinical records; the data protection form; and a conflict of interest disclosure form for research activities.

3.1.2. Clinical Toxicological Analyses in SQC

During the adaptation to the SQC, some training took place to better understand the clinical routine of a hospital laboratory. During that time, the quantitative methods used in the SQC within the scope of clinical toxicology were shown and explained.

The most used techniques for sample preparation were solid phase extraction, protein precipitation and acid hydrolysis. The biological specimens used for the analyses were whole blood, serum, plasma, and urine. The quantitative analytical methods were based in HPLC and UHPLC associated with different detectors: electrochemical detector, UV, MS, and MS/MS.

In the SQC, LC is used for TDM of immunosuppressants (e.g., mycophenolic acid), third generation antiepileptic drugs (e.g., lamotrigine, levetiracetam), antiarrhythmics (e.g., amiodarone), neuroleptics, benzodiazepines and thiopental. Chronic alcohol abuse evaluation is performed through the quantification of carbohydrate-deficient transferrin

(e.g., asialotransferrin, disialotransferrin, pentasialotransferrin, tetrasialotransferrin, trisialotransferrin). Lastly, to evaluate the impact of genetic polymorphisms in the metabolization of pharmaceuticals, particularly in the metabolism of thiopurines, the assessment of the activity of thiopurine methyltransferase is also performed.

The LC is also used in SQC for numerous other biological molecules: biogenic amines and active metabolites (dopamine, epinephrine, norepinephrine; normetanephrine, metanephrine, 3-methoxytyramine, 5-hydroxyindoleacetic acid, homovanillic acid, vanillylmandelic acid); porphyrins (uroporphyrin, heptacarboxyporphyrin, hexacarboxyporphyrin pentacarboxyporphyrin, coproporphyrin); vitamins [vitamin A, vitamin B1, vitamin B2, vitamin B6, vitamin E; methylmalonic acid (vitamin B12 cofactor)]; and steroids (aldosterone, testosterone, 17-OH-progesterone, dehydroepiandrosterone sulphate, androstenedione).

3.1.3. Samples Circuit

The patients to include in this study were recruited in the Emergency Room and at the Urgency Care Centre, upon the request of the analysis by the physicians of the specialties involved in the treatment of these patients (requisition exemplified in **Appendix 2**).

Blood samples were collected from patients aged 18 years and over with suspected drug intoxication with altered state of consciousness; and to patients aged 18 years and over chronically medicated with ATDs who had acute changes in consciousness. Pregnant women and children were excluded from the study.

The harvest method consisted of an immediate collection of blood sample into a tube without additives (454092 VACUETTE® TUBE 4 mL).

The blood samples were then transported to the central hospital laboratory (CoreLab), the serum was separated from the blood clot by centrifugation at 1,000–2,000 x g for 10 minutes and stored at 4º C for maximum of 24 hours. Afterwards, the serum samples were transported to the SQC and stored at -40º C protected from light, until the analysis.

3.1.4. Anonymization of Samples and Clinical Data

To obtain a waiver of informed consent and to proceed ethically with the study, the patient´s blood samples and clinical data were anonymized.

To accomplish the anonymization of the samples, a code consisting of the letter “S” followed by a sequential number corresponding to the chronological order of the collection was

attributed to each sample. Additionally, upon entry of the samples in SQC, a random sample analysis number generated by the laboratory information system was attributed by the supervisor of the study at the service to each sample.

To accomplish the anonymization of the clinical data, a couple steps were required: a list with the above-mentioned code numbers of each sample and the correspondent clinical record number was created by the supervisor of the study at the SQC and delivered to the supervisor in the emergency room; Then, the supervisor at the emergency room filled out the clinical data and deleted the clinical record number of each sample; the file with the anonymized clinical data was delivered to the student.

3.2. Analytical Method

3.2.1. Method Verification

The method used is extensively validated for 38 analytes, including ATDs and active metabolites. Therefore, method verification was performed to ensure that the same results as specified in the validation data provided by the manufacturer were attained in the SQC laboratory.

Precision and accuracy testing was performed by running plasma control solutions at two concentration levels (**Appendix 3-6**) in quadruplicates in a day (n=4) for a single day analysis (intraday) and by running triplicates for over 3 days (n=9) for analysis over different days (interday). Precision was expressed as the percentage of the coefficient of variation [CV (%)] (**Equation 1**) and a CV (%) of less than 15% was considered acceptable (42). Accuracy was reported as a percent difference between experimental and nominal values, relative bias [b (%)] (**Equation 2**), and a % difference of within 20% was accepted (40).

$$\text{CV (\%)} = \frac{\text{Standard Deviation of Replicates}}{\text{Mean of Replicates}} \times 100$$

Equation 1

$$\text{b (\%)} = \frac{\text{Mean of Experimental Values - Nominal Value}}{\text{Nominal Value}} \times 100$$

Equation 2

Recovery testing was performed by diluting the highest calibrator of each set with blank serum (Lyphochek® Drug Free Serum, Bio-Rad, United States of America) to two different concentration levels: toxic and therapeutic. The dilution was done in duplicates. The relative recovery or apparent recovery [r (%)] was calculated (**Equation 3**) and values within 80% to 120% were accepted (40).

$$r\ (\%) = \frac{\text{Mean of Experimental Values}}{\text{Nominal Value}} \times 100$$

Equation 3

Linearity testing was performed by analysing calibrators (**Appendices 7-10**) over 2 days and by analysing duplicates of each calibrator in one day. Linearity was reported as R^2 and as $S_{y/x}$, which were determined using the excel regression analysis output. It was considered acceptable if the R^2 value for each curve was greater than 0.9850 and the $S_{x/y}$ value was close to zero (42).

The LLOQ was determined in two different ways: 10 independent serum blanks were analysed (n=10) and the LLOQ was calculated as the mean concentration of the blank plus 10 times the standard deviation of the concentration of the blank (**Equation 4)**; 10 independent sample blanks fortified at the lowest clinically relevant concentration (**Appendix 11**) were analysed (n=10) and the LLOQ was calculated as 50% above the mean of measured concentration of the fortified blanks (**Equation 5)** (42).

$$LLOQ' = \text{Mean of 10 Blanks} + (10 \times \text{Standard Deviation of 10 Blanks})$$

Equation 4

$$LLOQ'' = 1.5 \times \text{Mean of 10 Fortified Blanks}$$

Equation 5

LOD was determined as the mean concentration of the blank plus 3 times the standard deviation of the concentration of the blank (**Equation 6**) (42).

$$LOD = \text{Mean of 10 Blanks} + (3 \times \text{Standard Deviation of 10 Blanks})$$

Equation 6

3.2.2. Sample Preparation

First, the vials of Internal Standard Mix, which consist of stable isotopically labelled (deuterated) and co-eluting ISTDs, were reconstituted with 1.0 mL of Reconstitution Buffer. The vials were then left at atmospheric temperature for 5 min, while mixing gently periodically until the solution was homogeneous. A volume of 800 µL of the reconstituted Internal Standard Mix solution was added to 12 mL of Precipitation Reagent to form Mixture A**.**

Vials of lyophilized plasma controls at two concentration levels [Control I and Control II (**Appendix 3-6**)] and of lyophilized plasma calibrators [Blank, Calibrator 1, Calibrator 2, Calibrator 3 (**Appendix 7-10**)] were reconstituted by pipetting 1.0 mL of distilled water directly into the glass vial.

Sample preparation was based on a protein precipitation step. For that, 50 µL of each serum sample were pipetted into a 1.5 mL transparent reaction vial. Next, 25 µL of Extraction Buffer were added to the reaction vial, mixed briefly with a vortex, and incubated for 2 min. Afterwards, 250 µL of Mixture A were added and mixed for a minimum of 30 s with a vortex and centrifuged at 15000 x g for 5 min. Finally, 50 µL of supernatant separated from the pellet were diluted with 150 µL of Dilution Buffer 1 (proportion of 1:4).

The same steps were followed for the calibrator solutions (i.e., Calibrator 1, Calibrator 2, Calibrator 3, Blank) and control solutions (i.e., Control I and Control II), mentioned above.

The Precipitation Reagent, the Extraction Buffer and the Dilution Buffer 1 used are components of MassTox® TDM Series A set from Chromsystems Instruments & Chemicals GmbH (Munich, Germany) and their composition is not disclosed by the manufacturer.

The Reconstitution Buffer, the Internal Standard Mix, the 3PLUS1® Multilevel Plasma Calibrators and the MassCheck® Plasma Controls used are components of the Tricyclic Antidepressants TCA 1, Tricyclic Antidepressants TCA 2, Antidepressants 1/EXTENDED, Antidepressants 2/EXTENDED sets from Chromsystems Instruments & Chemicals GmbH (Munich, Germany). The composition of the Reconstitution Buffer and of the Internal Standard Mix is not disclosed by the manufacturer. The respective concentration of the MassCheck® plasma controls and the 3PLUS1® multilevel calibrators is presented in **Appendices 3-10**.

3.2.3. LC-MS Instrumentation and Conditions

A volume of 200 µL of the diluted supernatants from the serum protein precipitation step was pipetted into a 300 µL insert of a 1.5 mL glass vials with screw caps and with pre-slit septa.

A volume of 5 µL of each sample was injected into a LCMS-8050 (Shimadzu, Kyoto, Japan) consisting of a LC-40D XS Solvent delivery module, a SCL-40 System controller, a DGU-405 Degassing Unit, a SIL-40C XS Autosampler, a CTO-40C Column Oven, a SPD-M40 Photo Diode Array Detector, a FCV-S Flow Channel Selection Valve, and equipped with the MassTox® TDM MasterColumn® A (Chromsystems Instruments & Chemicals GmbH, Munich, Germany). In **Table 1**, the chromatographic conditions for each ATD set are presented.

Table 1 | *LC conditions for each set of ATDs.*

TCA 1					
Mobile Phase 1	**Mobile Phase 2**	**Gradient**	**Total Analysis Time**	**Flow rate**	**Oven Temperature**
35%	65%	Isocratic	2.5 min	0.6 mL/min	28 ºC
TCA 2					
Mobile Phase 1	**Mobile Phase 2**	**Gradient**	**Total Analysis Time**	**Flow rate**	**Oven Temperature**
35%	65%	Isocratic	3.5 min	0.6 mL/min	28 ºC
ATD 1					
Mobile Phase 1	**Mobile Phase 2**	**Gradient Time**	**Total Analysis Time**	**Flow rate**	**Oven Temperature**
100%	0%	0.00 min	3 min	0.6 mL/min	30 ºC
100%	0%	0.20 min			
50%	50%	0.21 min			
50%	50%	0.70 min			
20%	80%	0.71 min			
20%	80%	2.40 min			
100%	0%	2.41 min			
100%	0%	3.00 min			
ATD 2					
Mobile Phase 1	**Mobile Phase 2**	**Gradient Time**	**Total Analysis Time**	**Flow rate**	**Oven Temperature**
70%	30%	0 min	2.8 min	0.6 mL/min	28 ºC
0%	100%	1 min			
0%	100%	2.10 min			
70%	30%	2.11 min			
70%	30%	2.80 min			

The Mobile Phase 1, the Mobile Phase 2 and the Rinsing Solution are components of the MassTox® TDM Basic Kit A from Chromsystems Instruments & Chemicals GmbH (Munich, Germany) and their composition is not disclosed by the manufacturer.

The LCMS-8050 (Shimadzu, Kyoto, Japan) is coupled to a mass spectrometer with an ESI unit ASSY (nebulizer and drying gas: N_2, heating gas: dry air), a desolvation line, a UF-Lens™ system, a triple quadrupole (Q2 collision gas: Ar), a dynode secondary-electron multiplier detector (pulse counting mode) and a E2M28 rotary vacuum pump; controlled by LabSolutions Version 5.97 SP 1 (Shimadzu, Kyoto, Japan) data system. In **Table 2**, the MS/MS conditions for each ATD set are presented.

Table 2 | *MS/MS conditions for each set of ATDs.*

Parameter	TCA 1	TCA 2	ATD 1	ATD 2
Nebulizing Gas Flow	3.0 L/min			
Drying Gas Flow	10 L/min			
Heating Gas Flow	10 L/min			
Ionization Mode	Positive			
ESI Voltage	0.8 kV	1.5 kV	1.0 kV	0.8 kV
ESI Temperature	300 °C			
Desolvation Temperature	526 °C			
DL Temperature	250 °C			
Heat Block Temperature	400 °C			
Conversion Dynode Voltage	10.0 kV			
Detector Voltage	1.74 kV			
CID Gas	270 kPa			
Analysis Mode	MRM			

The MRM parameters used are presented in **Appendices 12-15**.

4. Results and Discussion

4.1. Verification of Performance Parameters

The method of the commercial kit used is widely validated. Therefore, verification of performance parameters was executed. For that purpose, the linearity, the precision intra-day and inter-day, the accuracy intra-day and inter-day, the recovery, the LLOQ and the LOD were evaluated.

The calibration curves were obtained by drawing the graph in which the ratio of the ATD drug peak area and the correspondent ISTD peak area of each calibrator is a function of the respective concentration of the ATD (see concentrations in **Appendices 7-10**). In **Figure 6**, are presented as a representative example the calibration curves for sertraline.

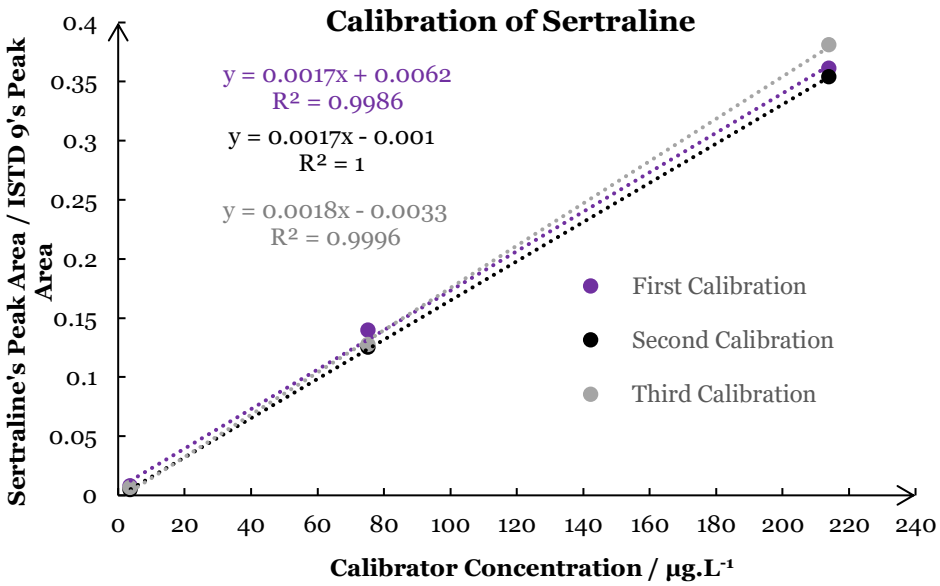


Figure 6 | *Independent calibration curves for sertraline performed in different days.*

Calibration curves for all ATDs analysed are presented in **Appendix 20**.

The R^2 for all the curves using the linear regression model was higher than 0.9850, with a range of 0.9858-1.000. The curve with the lowest R^2 coefficient was that of duloxetine. The $S_{y/x}$ were lower than 0.4000, with a range of 0.0007-0.3819. The curve with the lowest standard error was that of N-desmethyldosulepin. The curve with the highest standard error was for reboxetine.

Table 3 lists the slope, intercept, R^2 and $S_{y/x}$ values obtained for all the analytes.

Table 3 | *Slope, intercept, R^2, $S_{y/x}$ values for every ATDs set.*

	Analyte	Slope	Intercept	R^2	$S_{y/x}$
TCA 1	Amitriptyline	0.0023	-0.0080	0.9994	0.0142
		0.0015	-0.0053	0.9987	0.0135
		0.0019	-0.0019	0.9999	0.0038
	Nortriptyline	0.0030	-0.0202	0.9983	0.0306
		0.0024	-0.0171	0.9977	0.0282
		0.0026	-0.0103	0.9997	0.0114
	Doxepin	0.0047	-0.0230	0.9974	0.0500
		0.0039	-0.0180	0.9977	0.0393
		0.0042	-0.0094	0.9995	0.0204
	Nordoxepin	0.0050	-0.0203	0.9975	0.0530
		0.0044	-0.0170	0.9980	0.0414
		0.0047	-0.0083	0.9996	0.0194
	Imipramine	0.0030	-0.0189	0.9979	0.0330
		0.0026	-0.0212	0.9960	0.0390
		0.0028	-0.0133	0.9990	0.0213
	Desipramine	0.0029	-0.0453	0.9991	0.0209
		0.0025	-0.0157	0.9968	0.0344
		0.0026	-0.0089	0.9992	0.0177
TCA 2	Clomipramine	0.0031	-0.0217	0.9958	0.0481
		0.0022	-0.0168	0.9958	0.0337
		0.0026	-0.0213	0.9930	0.0515
	Norclomipramine	0.0031	-0.0190	0.9972	0.0379
		0.0024	-0.0099	0.9986	0.0213
		0.0029	-0.0255	0.9927	0.0579
	Maprotiline	0.0025	-0.0326	0.9928	0.0461
		0.0025	-0.0226	0.9987	0.0193
		0.0027	-0.0375	0.9910	0.0563
	Normaprotiline	0.0015	-0.0495	0.9975	0.0150
		0.0014	-0.0402	0.9993	0.0071
		0.0017	-0.0517	0.9889	0.0354
	Protriptyline	0.0023	-0.0191	0.9965	0.0321
		0.0020	-0.0133	0.9986	0.0175
		0.0023	-0.0229	0.9920	0.0476
	Trimipramine	0.0076	-0.0630	0.9949	0.1421
		0.0051	-0.0439	0.9944	0.1001
		0.0059	-0.0598	0.9919	0.1402
	Nortrimipramine	0.0061	-0.0481	0.9954	0.1053
		0.0042	-0.0249	0.9973	0.0548
		0.0047	-0.0426	0.9932	0.0995
ATD 1	Citalopram	0.0014	0.0078	0.9963	0.0162
		0.0014	-0.0042	0.9999	0.0024
		0.0016	-0.0103	0.9975	0.0152

	Analyte	Slope	Intercept	R^2	$S_{y/x}$
ATD 1	N-Desmethylcitalopram	0.0016	0.0047	0.9991	0.0096
		0.0015	-0.0053	0.9999	0.0031
		0.0016	-0.0100	0.9981	0.0138
	Fluoxetine	0.0036	0.0008	0.9962	0.1000
		0.0037	-0.0566	0.9991	0.0509
		0.0042	-0.0588	1.0000	0.0030
	Desmethylfluoxetine	0.0038	0.0270	0.9867	0.2018
		0.0044	-0.2749	0.9984	0.0803
		0.0043	-0.1683	0.9986	0.0724
	Duloxetine	0.0141	0.0059	1.0000	0.0077
		0.0117	-0.0126	0.9993	0.0315
		0.0006	0.0079	0.9858	0.0070
	Fluvoxamine	0.0061	-0.0023	0.9905	0.1418
		0.0083	-0.1096	0.9986	0.0732
		0.0100	-0.1762	0.9944	0.1791
	Mirtazapine	0.0016	0.0035	0.9998	0.0059
		0.0017	-0.0066	0.9995	0.0114
		0.0018	-0.0061	0.9993	0.0127
	N-Desmethylmirtazapine	0.0039	0.0133	0.9995	0.0243
		0.0039	-0.0144	0.9996	0.0217
		0.0041	-0.0305	0.9979	0.0550
	Paroxetine	0.0122	-0.0065	0.9997	0.0184
		0.0117	-0.0240	0.9974	0.0519
		0.0028	-0.0093	0.9997	0.0041
	Sertraline	0.0017	0.0062	0.9986	0.0093
		0.0017	-0.0010	1.0000	0.0011
		0.0018	-0.0033	0.9996	0.0052
	N-Desmethylsertraline	0.0137	-0.0878	0.9966	0.189
		0.0091	0.0214	0.9969	0.122
		0.0114	-0.0332	0.9967	0.1555
	Venlafaxine	0.0026	0.0101	0.9953	0.0306
		0.0024	-0.0090	0.9997	0.0070
		0.0026	-0.0186	0.9971	0.0238
	O-Desmethylvenlafaxine	0.0028	-0.0093	0.9989	0.0427
		0.0031	-0.0585	0.9996	0.0289
		0.0031	-0.0804	0.9962	0.0860
ATD 2	Dosulepin	0.0073	-0.0674	0.9953	0.1320
		0.0071	-0.0106	0.9997	0.0326
		0.0069	0.0156	0.9999	0.0137
	N-Desmethyldosulepin	0.0118	-0.2080	0.9989	0.0456
		0.0099	-0.0386	1.0000	0.0007
		0.0094	0.0354	0.9976	0.0533

	Analyte	Slope	Intercept	R^2	$S_{y/x}$
ATD 2	Mianserin	0.0100	-0.0332	0.9994	0.0325
		0.0084	0.0305	0.9942	0.0815
		0.0079	0.0184	0.9981	0.0433
	Milnacipran	0.0045	-0.0208	0.9993	0.0300
		0.0045	-0.0042	1.0000	0.0053
		0.0044	0.0007	1.0000	0.0034
	Moclobemide	0.0011	-0.0435	0.9981	0.0766
		0.0011	-0.0040	0.9999	0.0133
		0.0011	0.0068	1.0000	0.0068
	Opipramol	0.0032	-0.0474	0.9974	0.0654
		0.0032	-0.0019	0.9998	0.0191
		0.0034	-0.0217	1.0000	0.0016
	Reboxetine	0.0147	-0.0321	0.9994	0.0985
		0.0127	0.1433	0.9949	0.2581
		0.0124	0.1383	0.9981	0.1518
	Tianeptine	0.0127	-0.0501	0.9999	0.0320
		0.0134	-0.0418	0.9998	0.0444
		0.0128	0.0438	0.9983	0.1392
	Tranylcypromine	0.0058	-0.0665	0.9972	0.1485
		0.0058	-0.0001	1.0000	0.0029
		0.0060	-0.0198	0.9998	0.0416
	Trazodone	0.0014	-0.1182	0.9997	0.0498
		0.0017	-0.0787	0.9997	0.0562
		0.0016	-0.0027	0.9986	0.1159
	Vilazodone	0.0165	-0.0780	0.9948	0.0651
		0.0163	-0.0223	0.9997	0.0148
		0.0153	0.0008	0.9996	0.0171
	Vortioxetine	0.0305	-0.0472	0.9979	0.1067
		0.0325	-0.0614	0.9991	0.0767
		0.0332	-0.0694	0.9994	0.0643

Most b (%) values for intra-day and inter-day accuracy evaluation purposes fell in the ±20% interval. The analyte with highest intra-day bias was vortioxetine at 83.30 µg/L with a b (%) of -18.88%. The analyte with highest inter-day bias was duloxetine at 15.00 µg/L with a b (%) of 19.05%.

Most CV (%) values for intra-day and inter-day precision evaluation fell under 15%. The analyte with the highest intra-day imprecision was fluvoxamine at 187.90 µg/L with a CV (%) of 14.38%. The analyte with the highest inter-day imprecision was duloxetine at 15.00 µg/L with a CV (%) of 15.35%.

The intra-day and inter-day accuracy and precision results are presented in **Table 4.**

Table 4 | *Accuracy and precision values for every ATDs set.*

	Analyte	Concentration /µg.L^{-1}	Accuracy [b (%)]		Precision [CV (%)]	
			Intra-Day (n=4)	Inter-Day (n=9)	Intra-Day (n=4)	Inter-Day (n=9)
TCA 1	Amitriptyline	59.10	1.79	-0.01	1.40	3.19
		265.00	8.63	6.39	0.44	2.14
	Nortriptyline	63.50	-2.89	-5.03	0.67	3.02
		279.00	-0.86	-2.26	0.47	1.54
	Doxepin	34.00	3.25	2.06	0.75	4.29
		224.00	-0.65	-1.99	1.10	1.57
	Nordoxepin	34.10	3.05	0.53	1.09	4.70
		223.00	0.09	-2.08	2.45	1.90
	Imipramine	70.60	-0.84	-3.29	1.14	2.78
		271.00	1.84	0.87	1.16	2.04
	Desipramine	48.80	-0.91	2.48	1.09	10.79
		270.00	-2.16	-3.30	1.54	2.08
TCA 2	Clomipramine	43.60	-5.50	-4.16	5.43	6.94
		255.00	10.40	2.16	10.55	12.72
	Norclomipramine	46.70	5.85	11.06	6.68	7.83
		255.00	16.66	-3.02	9.11	15.46
	Maprotiline	74.10	5.02	8.96	8.53	2.06
		264.00	12.01	6.12	7.60	5.05
	Normaprotiline	91.70	7.07	1.34	5.32	13.02
		294.00	-2.67	-3.45	7.92	7.68
	Protriptyline	72.50	2.51	7.14	6.95	2.55
		262.00	12.48	4.51	9.06	4.78
	Trimipramine	35.70	-5.99	-3.16	4.64	2.44
		242.00	11.52	4.98	11.52	4.37
	Nortrimipramine	35.90	-3.74	11.13	5.91	2.60
		264.00	-2.85	6.20	10.46	4.49
ATD 1	Citalopram	70.90	-1.54	-13.65	1.98	9.85
		134.00	-3.07	-5.91	0.81	8.73
	N-Desmethylcitalopram	72.90	-1.48	-2.14	1.74	5.71
		144.00	-6.45	4.64	0.61	9.27
	Fluoxetine	218.00	4.98	0.75	6.30	6.48
		365.00	-5.58	5.80	6.50	8.37
	Desmethylfluoxetine	251.00	1.03	1.42	5.66	6.35
		394.00	-5.75	3.89	8.05	9.94
	Duloxetine	15.00	-0.57	-19.05	2.31	15.35
		112.00	-6.48	-2.16	3.04	6.33
	Fluvoxamine	113.00	-5.15	-2.65	8.40	7.79
		187.00	-11.74	1.51	14.38	11.69

	Analyte	Concentration /µg.L^{-1}	Accuracy [b (%)]		Precision [CV (%)]	
			Intra-Day (n=4)	Inter-Day (n=9)	Intra-Day (n=4)	Inter-Day (n=9)
ATD 1	Mirtazapine	89.80	-3.64	-10.48	5.83	3.73
		202.00	-10.32	1.72	1.52	10.76
	N-Desmethylmirtazapine	103.00	-3.78	-5.11	1.37	3.82
		203.00	-7.41	2.05	1.28	12.11
	Paroxetine	53.00	0.11	-7.73	2.53	5.77
		75.00	-4.99	4.48	2.68	10.15
	Sertraline	51.30	-0.51	-1.02	1.24	3.52
		99.30	-2.38	8.73	1.51	10.70
	N-Desmethylsertraline	81.10	-10.95	-16.00	0.81	3.15
		157.00	1.46	17.23	0.54	13.27
	Venlafaxine	72.90	-4.58	-6.27	0.85	4.11
		132.00	-4.54	4.11	2.03	10.33
	O-Desmethylvenlafaxine	207.00	17.04	2.84	0.59	4.83
		358.00	18.09	12.11	0.37	9.31
ATD 2	Dosulepin	46.00	-9.15	0.17	3.49	7.61
		319.00	-12.69	-8.67	3.25	6.61
	N-Desmethyldosulepin	86.60	-2.75	-0.90	5.61	5.02
		267.00	-8.10	-11.68	2.50	9.55
	Mianserin	21.40	8.29	-13.62	8.43	6.04
		120.00	-17.44	-14.27	6.17	14.67
	Milnacipran	55.60	-10.62	-6.88	1.14	3.50
		339.00	-11.90	-6.98	2.35	4.35
	Moclobemide	381.00	-9.65	-2.98	0.30	1.32
		2237.00	-7.47	-3.62	2.26	4.61
	Opipramol	91.80	-17.60	-7.32	2.99	4.75
		656.00	-8.84	-5.36	2.92	1.91
	Reboxetine	57.70	-12.05	-7.82	3.87	13.02
		296.00	-7.31	-6.83	5.24	12.35
	Tianeptine	57.60	-13.71	-9.66	6.55	4.02
		325.00	-16.25	-11.94	3.57	4.73
	Tranylcypromine	75.10	-12.95	-2.22	1.37	3.97
		325.00	-13.69	-8.60	3.32	4.93
	Trazodone	516.00	10.82	-4.93	2.64	7.55
		2240.00	-0.65	-10.07	2.54	2.11
	Vilazodone	27.40	-14.44	-3.22	2.19	1.81
		77.00	-13.67	-5.02	1.41	6.57
	Vortioxetine	17.40	-11.52	-4.22	5.93	5.91
		83.30	-18.88	-9.24	3.53	5.24

Most r (%) values fell within the 80% to 120% interval and therefore were considered acceptable. The analyte with the worst recovery was duloxetine, with a r (%) of 355.70% and 344.74% at 60 µg/L and 120 µg/L, respectively, and the analyte with the best recovery was dosulepin, with a r (%) of 99.715% at 308.00 µg/L.

The recovery values for all analytes are presented in **Table 5**.

Table 5 | *Recovery values for every ATDs set.*

	Analyte	Concentration /µg.L^{-1}	Recovery [r (%)]
TCA 1	Amitriptyline	150.80	96.79
		301.60	95.98
	Nortriptyline	149.60	98.59
		299.20	96.07
	Doxepin	121.60	101.32
		243.20	97.12
	Nordoxepin	122.80	103.49
		245.60	99.30
	Imipramine	146.80	96.66
		293.60	95.70
	Desipramine	143.60	101.30
		287.20	98.38
TCA 2	Clomipramine	140.80	106.65
		281.60	105.01
	Norclomipramine	138.80	105.61
		277.60	107.28
	Maprotiline	136.80	108.50
		273.60	108.97
	Normaprotiline	139.20	110.35
		278.40	109.20
	Protriptyline	148.40	103.97
		296.80	107.41
	Trimipramine	151.60	105.49
		303.20	110.66
	Nortrimipramine	146.80	107.87
		293.60	112.43
ATD 1	Citalopram	111.20	113.54
		222.40	100.54
	N-Desmethylcitalopram	118.00	108.37
		236.00	95.35
	Fluoxetine	284.40	111.51
		568.80	101.60
	Desmethylfluoxetine	299.20	113.17
		598.40	105.74

	Analyte	Concentration /µg.L^{-1}	Recovery [r (%)]
ATD 1	Duloxetine	60.00	355.70
		120.00	344.74
	Fluvoxamine	148.40	101.24
		296.80	92.09
	Mirtazapine	158.00	109.00
		316.00	100.63
	N-Desmethylmirtazapine	164.80	114.41
		329.60	102.48
	Paroxetine	56.80	119.29
		113.60	101.28
	Sertraline	85.60	104.66
		171.20	91.94
	N-Desmethylsertraline	145.60	120.65
		291.20	113.82
	Venlafaxine	103.20	119.65
		206.40	104.93
	O-Desmethylvenlafaxine	274.40	120.86
		548.80	104.28
ATD 2	Dosulepin	154.00	102.95
		308.00	99.71
	N-Desmethyldosulepin	94.40	114.51
		188.80	111.30
	Mianserin	74.80	108.45
		149.60	95.54
	Milnacipran	150.80	109.76
		301.60	95.71
	Moclobemide	952.80	110.09
		1905.60	101.35
	Opipramol	235.20	110.06
		470.40	93.54
	Reboxetine	168.40	95.91
		336.80	105.87
	Tianeptine	158.00	113.21
		316.00	95.17
	Tranylcypromine	274.00	107.27
		548.00	97.13
	Trazodone	1182.80	98.88
		2365.60	110.51
	Vilazodone	36.92	109.72
		73.84	94.04
	Vortioxetine	46.40	101.27
		92.80	98.68

Overall, the LLOQ values obtained using the two different methods were much higher than the LLOQ values provided by the manufacturer ($LLOQ_m$). It is important to note that the method used to calculate these $LLOQ_m$ values is not detailed by the manufacturer. Despite this, most LLOQ values obtained in this method verification have clinical validity. When the results obtained in this study were compared, it was concluded that, although the LLOQ values were overall higher, fortification of blanks (LLOQ") gives a better approximation to analytical routine conditions and allows to have a greater certainty in the results obtained near the lower limit of the therapeutic range.

The LLOQ for desipramine, trimipramine, nortrimipramine, citalopram, desmethylfluoxetine, fluvoxamine, mirtazapine, N-desmethylmirtazapine, venlafaxine, milnacipran, tianeptine and tranylcypromine were higher than Calibrator 1 level. These values, although valid for clinical decisions, must be verified with more samples in the near future.

The LLOQ and LOD values for N-desmethylfluoxetine, duloxetine, paroxetine, N-desmethylsertraline, reboxetine, and trazodone were not satisfactory having in mind the therapeutic range (**Appendices 16-19**), so further evaluation is needed. The remaining LLOQ were considered clinically acceptable and useful for interpretation of patient samples included in this study.

The LLOQ and the LOD are presented in **Table 6**.

Table 6 | *LLOQ and LOD values for every ATDs set.*

	Analyte	**LLOQ"/µg.L^{-1}**	LLOQ'/µg.L^{-1}	**LOD /µg.L^{-1}**	$LLOQ_m$ /µg.L^{-1}
TCA 1	Amitriptyline	13.01	2.81	2.12	1.00
	Nortriptyline	15.39	4.00	3.67	1.00
	Doxepin	7.21	3.01	2.74	2.00
	Nordoxepin	6.57	2.96	2.58	1.00
	Imipramine	18.07	3.03	3.03	1.00
	Desipramine	19.67	8.98	8.62	1.00
TCA 2	Clomipramine	9.23	4.20	3.84	2.00
	Norclomipramine	8.75	4.00	3.61	2.00
	Maprotiline	29.72	10.35	10.35	3.00
	Normaprotiline	42.46	14.56	14.27	5.00
	Protriptyline	16.13	3.95	3.95	1.50
	Trimipramine	10.21	4.49	4.49	1.50
	Nortrimipramine	11.52	4.64	4.37	1.00

	Analyte	**LLOQ"/µg.L^{-1}**	LLOQ'/µg.L^{-1}	**LOD /µg.L^{-1}**	LLOQ$_m$ /µg.L^{-1}
ATD 1	Citalopram	11.75	5.52	4.40	2.00
	N-Desmethylcitalopram	7.81	4.73	3.73	2.50
	Fluoxetine	51.53	35.21	21.82	11.00
	Desmethylfluoxetine	155.14	52.91	34.07	30
	Duloxetine	---	---	---	0.5
	Fluvoxamine	41.15	24.61	16.10	11.00
	Mirtazapine	15.97	9.21	7.30	4.00
	N-Desmethylmirtazapine	23.66	14.44	9.45	2.50
	Paroxetine	---	---	---	2
	Sertraline	3.24	3.28	2.32	0.75
	N-Desmethylsertraline	---	---	---	6.00
	Venlafaxine	19.67	12.53	6.88	7.00
	O-Desmethylvenlafaxine	41.9	19.23	12.21	14.00
ATD 2	Dosulepin	11.35	8.34	2.32	3.56
	N-Desmethyldosulepin	72.67	1.63	0.70	8.40
	Mianserin	7.81	2.41	1.74	2.23
	Milnacipran	19.65	4.67	4.18	5.53
	Moclobemide	142.49	14.55	13.13	41.40
	Opipramol	27.66	7.02	4.12	9.96
	Reboxetine	14.52	---	---	5.81
	Tianeptine	22.42	8.28	5.04	11.30
	Tranylcypromine	11.44	4.56	4.24	5.11
	Trazodone	---	---	---	115.00
	Vilazodone	12.81	3.17	2.07	7.41
	Vortioxetine	7.95	7.99	4.46	4.11

LLOQ": LLOQ calculated using fortified blanks (see Equation 5);
LLOQ': LLOQ calculated using blanks (see Equation 4); LLOQ$_m$: LLOQ provided by the manufacturer.

The overall measuring ranges, which consist of the interval between the LLOQ and the highest calibration level, are presented in **Appendix 21**.

4.2. Quantification of Antidepressants in Patient's Samples

Prior to the study, it was predicted, based on the number of patients with altered consciousness, with "intoxication" at the initial diagnosis and a request for toxicology screening, during 6 months in 2020, that 100 patients would be included. Nevertheless, during the study, only 20 real patient samples were included and analysed. This was probably not related with a lower number of admissions to the CHUPorto of patients with ATDs intoxication or toxicity. Specific and thorough divulgation was performed in the emergency department, that still did not guarantee this "extra" request in the various medical teams. This resulted in a lower number of samples than it was estimated.

In the 20 cases, the patient's age average was 58.42 (standard deviation of 14.01) and the number of cases of the female gender was 14 (70%). The requests were made in two types of critical patients: confirmation of voluntary drug intoxication and suspected accidental overdose or iatrogenic reaction in patients on chronic medication.

The most suspected ATDs were trazodone with 7 cases, sertraline with 7 cases, mirtazapine with 3 cases, fluoxetine with 3 cases, venlafaxine with 3 cases. The top 5 suspected ATDs were either SSRIs or atypical antidepressants, which could not be detected or quantified using the previous screening methods established in CHUPorto.

The quantification set used for each sample was chosen based on the information given in the requisition filled out by the physicians of the Urgent Care Room and Emergency Room (**Appendix 2**). In cases where there was no information about the suspected ATD, a broad screening was performed, using all sets.

As explained in **Chapter 4.1,** the calibration curves were attained using the ratio of the ATD drug peak area and the ISTD peak area of each calibrator and the respective concentration of the ATD drug. The same calibration curves used in the method verification were used with the patients' samples: the slope and the intercept of those calibration curves (**Table 3**) were used to calculate the concentration of ATDs in each sample (**Equation 7**).

$$\frac{\text{ATD's peak Area}}{\text{ISTD's peak Area}} = \mathbf{Slope} \times \text{ATD's Concentration} + \mathbf{Intercept} \Leftrightarrow$$

$$\Leftrightarrow \text{ATD's Concentration} = \frac{\text{ATD's Peak Area}}{\text{ISTD's Peak Area}} \times \frac{1}{\mathbf{Slope}} - \mathbf{Intercept}$$

Equation 7

The ATDs concentrations calculated were then compared with the LLOQ (**Table 6**), with the correspondent metabolite and with the therapeutic ranges (**Appendices 16-19**). **Tables 7-10** present the ATDs concentrations in each patient's sample.

Table 7 | *TCAs concentration (µg/L) in selected patient's samples.*

TCAs	Samples											
Analyte	**S1**	**S4**	**S5**	**S6**	**S7**	**S9**	**S12**	**S14**	**S15**	**S17**	**S18**	**S19**
Amitriptyline	-	<LLOQ	-	-	-	<LLOQ	33.69	-	n.d.	-	-	-
Nortriptyline	-	<LLOQ	-	-	-	<LLOQ	19.11	-	<LLOQ	-	-	-
Clomipramine	53.09	n.d.	-	-	-	n.d.	-	-	<LLOQ	-	-	-
Norclomipramine	24.98	<LLOQ	-	-	-	n.d.	-	-	<LLOQ	-	-	-
Dosulepin	-	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	-	n.d.	n.d.	n.d.	n.d.	n.d.
N-Desmethyldosulepin	-	n.d.	n.d.	n.d.	n.d.	n.d.	-	n.d.	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Doxepin	-	<LLOQ	-	-	-	<LLOQ	n.d.	-	<LLOQ	-	-	-
Nordoxepin	-	<LLOQ	-	-	-	<LLOQ	n.d.	-	n.d.	-	-	-
Imipramine	-	<LLOQ	-	-	-	<LLOQ	n.d.	-	n.d.	-	-	-
Desipramine	-	n.d.	-	-	-	n.d.	n.d.	-	n.d.	-	-	-
Maprotiline+	47.68	n.d.	-	-	-	n.d.	-	-	n.d.	-	-	-
Normaprotiline	<LLOQ	n.d.	-	-	-	<LLOQ	-	-	n.d.	-	-	-
Protriptyline	<LLOQ	<LLOQ	-	-	-	<LLOQ	-	-	n.d.	-	-	-
Trimipramine	n.d.	n.d.	-	-	-	n.d.	-	-	n.d.	-	-	-
Nortrimipramine	n.d.	<LLOQ	-	-	-	n.d.	-	-	n.d.	-	-	-

- = not quantified; n.d. = below LOD; <LLOQ = below LLOQ.

+Is a tetracyclic antidepressant but has a similar pharmacological mechanism to TCAs.

Table 8 | *SSRIs concentration (µg/L) in selected patient's samples.*

SSRIs	Samples															
Analyte	**S2**	**S3**	**S4**	**S5**	**S7**	**S8**	**S9**	**S10**	**S11**	**S13**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**
Citalopram	n.d.	n.d.	n.d.	n.d.	n.d.	**464.67**	n.d.	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	n.d.	<LLOQ	<LLOQ	<LLOQ
N-Desmethylcitalopram	n.d.	n.d.	n.d.	n.d.	n.d.	**50.02**	n.d.	<LLOQ	<LLOQ	<LLOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluoxetine	154.68	156.31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LLOQ	<LLOQ	<LLOQ	n.d.	<LLOQ	<LLOQ
Desmethylfluoxetine	295.47	235.83	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluvoxamine	n.d.	n.d.	n.d.	n.d.	69.15	n.d.	n.d.	<LLOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Paroxetine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Sertraline	74.29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LLOQ	15.23	9.24	n.d.	n.d.	n.d.	49.96	7.19	n.d.
N-Desmethylsertraline	-	-	-	-	-	-	-	<LLOQ	25.08	70.83	<LLOQ	<LLOQ	<LLOQ	38.57	28.53	<LLOQ
- = not quantified; n.d. = below LOD; <LLOQ = below LLOQ; toxic concentrations in bold.																

Table 9 | *Atypical ATDs concentration (µg/L) in selected patient's samples.*

Atypical ATDs	Samples								
Analyte	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
Duloxetine	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.
Mirtazapine	n.d.	n.d.	n.d.	<LLOQ	-	n.d.	n.d.	n.d.	33.61
N-Desmethylmirtazapine	n.d.	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	<LLOQ
Venlafaxine	n.d.	n.d.	n.d.	n.d.	-	n.d.	**3621.50**	n.d.	<LLOQ
O-Desmethylvenlafaxine	n.d.	n.d.	n.d.	n.d.	-	n.d.	**390.00**	n.d.	<LLOQ
Mianserin	-	-	<LLOQ	53.24	<LLOQ	<LLOQ	-	<LLOQ	-
Milnacipran	-	-	<LLOQ	<LLOQ	<LLOQ	<LLOQ	-	<LLOQ	-
Opipramol+	-	-	<LLOQ	<LLOQ	<LLOQ	<LLOQ	-	<LLOQ	-
Reboxetine	-	-	<LLOQ	<LLOQ	<LLOQ	<LLOQ	-	<LLOQ	-
Tianeptine+	-	-	n.d.	n.d.	n.d.	n.d.	-	n.d.	-
Trazodone	-	-	<LLOQ	<LLOQ	**11582.87**	**5171.93**	-	<LLOQ	-
Vilazodone	-	-	<LLOQ	<LLOQ	n.d.	<LLOQ	-	<LLOQ	-
Vortioxetine	-	-	n.d.	n.d.	n.d.	n.d.	-	n.d.	-
Analyte	**S11**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**
Duloxetine	n.d.	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mirtazapine	n.d.	n.d.	-	n.d.	n.d.	38.69	n.d.	n.d.	8.74
N-Desmethylmirtazapine	n.d.	n.d.	-	n.d.	n.d.	<LLOQ	n.d.	n.d.	8.01
Venlafaxine	<LLOQ	<LLOQ	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
O-Desmethylvenlafaxine	<LLOQ	<LLOQ	-	<LLOQ	<LLOQ	n.d.	<LLOQ	<LLOQ	n.d.
Mianserin	-	-	n.d.	<LLOQ	-	<LLOQ	<LLOQ	<LLOQ	-
Milnacipran	-	-	n.d.	<LLOQ	-	<LLOQ	<LLOQ	<LLOQ	-
Opipramol*	-	-	<LLOQ	n.d.	-	n.d.	n.d.	n.d.	-
Reboxetine	-	-	n.d.	n.d.	-	n.d.	n.d.	n.d.	-
Tianeptine*	-	-	n.d.	n.d.	-	n.d.	n.d.	n.d.	-
Trazodone	-	-	165.93	<LLOQ	-	294.83	<LLOQ	**1108.58**	-
Vilazodone	-	-	n.d.	<LLOQ	-	<LLOQ	n.d.	<LLOQ	-
Vortioxetine	-	-	27.73	n.d.	-	n.d.	n.d.	n.d.	-

- = not quantified; n.d. = below LOD; <LLOQ = below LLOQ; toxic concentrations in bold.

+Structurally are TCAs but have different pharmacological mechanisms.

Table 10 | *MAOIs concentration (µg/L) in selected patient's samples.*

MAOIs	Samples									
Analyte	**S4**	**S5**	**S6**	**S7**	**S9**	**S14**	**S15**	**S17**	**S18**	**S19**
Moclobemide	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Tranylcypromine	<LLOQ	n.d.	n.d.	n.d.	n.d.	n.d.	<LLOQ	<LLOQ	<LLOQ	<LLOQ
n.d. = below LOD; <LLOQ = below LLOQ.										

Overall, the number of patients with toxic concentrations was 4 (namely, S6, S7, S8 and S19) for a total of 20 cases.

Afterwards, the patients' anonymised clinical data such as renal function clinical data (e.g., creatinine renal clearance), liver function clinical data (e.g., serum's alanine transaminase and aspartate transaminase concentration), history of intoxication, state of consciousness, and urine and serum screens test for psychotropic drugs, were evaluated in assemble with the obtained concentrations.

A high level of creatinine might indicate a reduced kidney function and, consequently, a reduced clearance of the ATDs that are eliminated in the urine. A high level of alanine transaminase and aspartate transaminase might indicate a liver disease, which may compromise the hepatic metabolism of the ATD. It should be mentioned that both alanine transaminase and aspartate transaminase are elevated in hepatic diseases, but alanine transaminase is more liver specific. Therefore, it is important to evaluate both hepatic function and renal function since it can affect the pharmacokinetics of ATDs. However, after inspecting the clinical data, it was concluded that none of patients with toxic concentrations (namely, S6, S7, S8 and S19) had altered renal or hepatic function.

Because the brain is the organ most affected by acute intoxication, any signs of disturbed behaviour, altered level of consciousness, or disturbed neuropsychiatric baseline, should prompt concerns about toxicity.

For the twenty adult patients included in this study, ten patients had intentional drug overdose history and an altered mental status. On samples S6, S7, S8, as mentioned before, toxic levels were found for suspected ATDs and active metabolites, namely trazodone, citalopram, and venlafaxine. Screen tests results for these patients were negative since screen methods only include TCAs. In five patients (namely, S2, S3, S10, S13 and S14), despite the intentional intoxication suspicion, the ATDs suspected were at therapeutic or under therapeutic levels. In this group, for S4 and S15 all sets of ATDs were under LLOQ. It is important to mention, that the time that passed between the ATD ingestion and blood sample collection was not taken in consideration for the interpretation of the results. Consequently, we cannot affirm with certainty that the patients that had therapeutic or under therapeutic levels, did not had toxic concentrations prior to the sample collection.

Ten patients with depressed mental status had no specific toxicologic history. Nevertheless, it was important to exclude accidental intoxication caused by adverse iatrogenic drug reaction as these patients were chronically medicated with several psychotropic pharmaceuticals. On sample S19 from a patient medicated with trazodone and sertraline, a toxic level for trazodone was quantified however sertraline was not detected. On sample S9

from a patient with psychiatric illness, medication list referred only antipsychotic drugs and all ATDs tested under the LLOQ. On sample S16 from patient medicated with sertraline, the ATD was also under LLOQ. The ATDs levels from seven patient samples (namely, S1, S5, S11, S12, S17, S18 and S20) were within therapeutic range and the depressed mental status could not be associated with them. Amongst this group of ten patients, four (namely S9, S16, S17 and S20) were also medicated with antipsychotics and benzodiazepines.

So, among 20 patient specimens, suspected ATDs were found in 16 (80%) with toxic level in 4 (20%). ATDs were not found in 4 specimens (20%) and not suspected ATDs were found in 4 (20%).

In **Figure 7** are presented the chromatograms of the patient's samples with toxic concentrations.

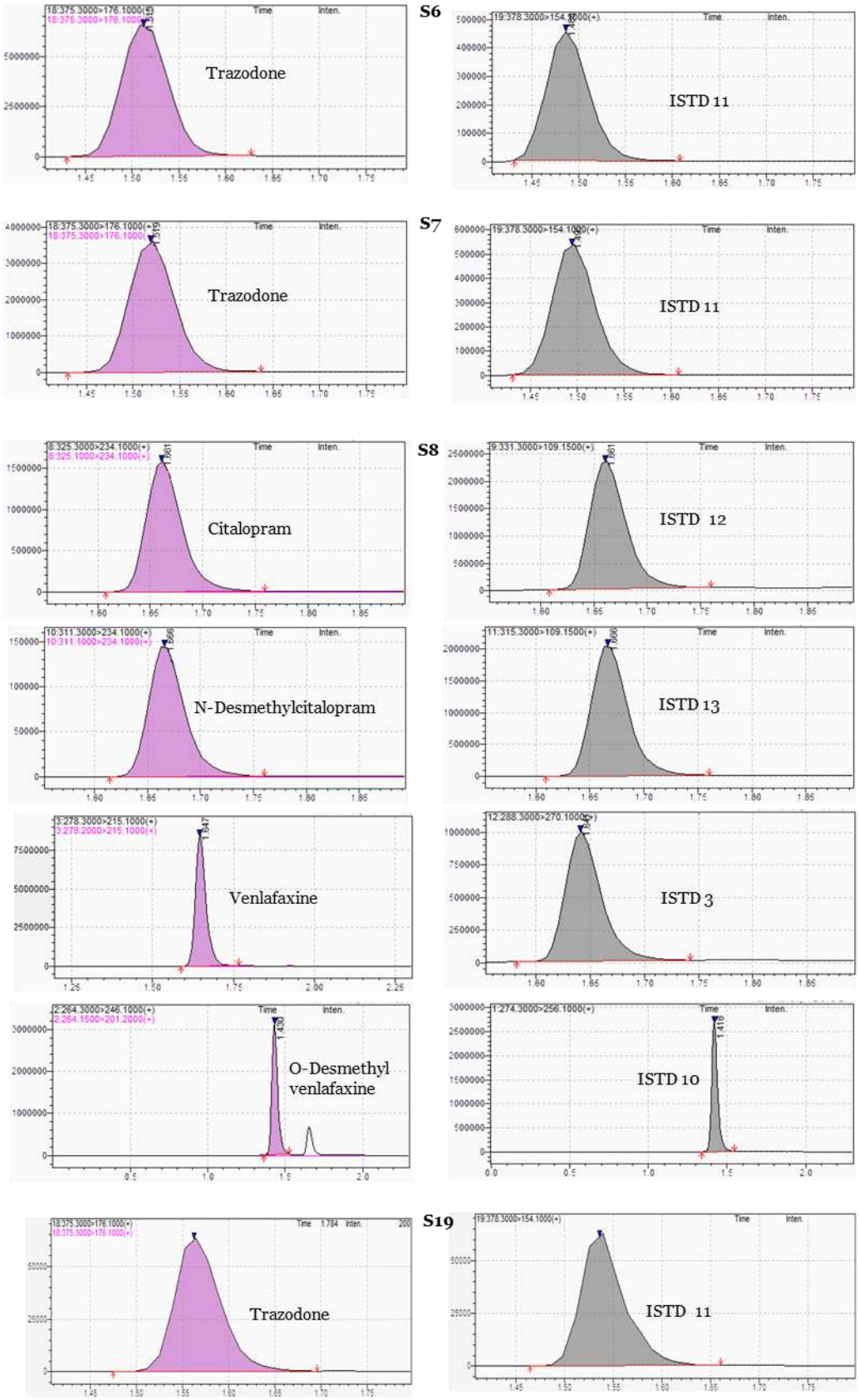


Figure 7 *| Chromatogram of ATD, metabolite and correspondent ISTDs for the patients with toxic concentrations.*

Rapid tests, such as immunoassays, are valuable for the qualitative and semi-quantitative determination of drugs in human urine and serum. However, positive and negative results are only preliminary, especially in critical patients with polydrug overdose, confusing mixed syndromes or worsening of clinical status, without known aetiology. In these cases, comprehensive quantitative analytical methods for specific drugs have better correlation with symptoms and are more useful for diagnosis, therapeutic decisions and monitoring.

According to Consensus Guidelines for TDM in Neuropsychopharmacology, chromatographic techniques, such as LC-MS/MS, are preferred when it comes to the monitoring of psychotropic drugs since they can be adapted to the analysis of almost every psychotropic drug, they can be applied with minimal sample preparation such as protein precipitation and dilution and many compounds can be analysed simultaneously. In the particular case of suspected intoxications, the use of LC-MS/MS is advantageous due to the high selectivity of MS for identification (12).

Likewise, the LC-MS/MS method used in this study proved to be useful as a complementary analysis for the diagnosis and monitoring of patients suspected for ATDs intoxication or toxicity, since it allowed the simultaneous quantification, in patients serum samples, of ATDs that went unnoticed by the screening tests used in routine practice, which only allow the detection of some TCAs.

5. Conclusion

The main aim of this work was to implement in the clinical practice of CHUPorto an auxiliary method which allows the quantification of the wide range of ATDs currently in clinical use.

To achieve this, the method's performance parameters were verified, and subsequently, real patient samples were analysed.

Having in mind the method verification performed, the method proved to be suitable for the intended analysis, for most analytes, having adequate precision, accuracy and measuring range for its clinical context. However, in some analytes, the results did not meet the standards required and, for that reason, some additional steps would need to be performed, such as the optimization of LC-MS/MS conditions for duloxetine, N-desmethylsertraline and respective ISTD; and further evaluation of the LLOQ and the LOD for duloxetine, paroxetine, N-desmethylsertraline, reboxetine, trazodone. This was not possible during the course of this study due to a limit of time and resources.

Considering the number of toxic concentrations of ATDs found using the LC-MS/MS analysis and knowing they were overlooked by the rapid screening tests, it is possible to conclude that the method verified in this study allows a complete, thorough screening and a more accurate serum quantification of a broad spectrum of ATDs.

In this study, LC-MS/MS for ATDs serum quantification was utilized in patients with suspected intoxication and toxicity. However, in the future, the same method may be used as tool for TDM, especially on older polymedicated patients, risk groups with reduced hepatic and kidney function, and patients with suspected polymorphism that affect the ATDs pharmacokinetics.

6. References

1. Klaassen CD. Casarett & Doull's Toxicology: The Basic Science of Poisons, 9th Edition: McGraw-Hill Education; 2018.

2. Gummin DD, Mowry JB, Beuhler MC, Spyker DA, Brooks DE, Dibert KW, et al. 2019 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 37th Annual Report. Clinical Toxicology. 2020;58(12):1360-541.

3. Moffat AC. Clarke's Analysis of Drugs and Poisons, 4th Edition: Pharmaceutical Press; 2011.

4. Okumura Y, Sakata N, Takahashi K, Nishi D, Tachimori H. Epidemiology of overdose episodes from the period prior to hospitalization for drug poisoning until discharge in Japan: An exploratory descriptive study using a nationwide claims database. Journal of Epidemiology. 2017;27(8):373-80.

5. Mathias TL, Guidoni CM, Girotto E. Tendências de eventos toxicológicos relacionados a medicamentos atendidos por um Centro de Informações Toxicológicas Revista Brasileira de Epidemiologia. 2019;22.

6. White N, Litovitz T, Clancy C. Suicidal antidepressant overdoses: a comparative analysis by antidepressant type. J Med Toxicol. 2008;4(4):238-50.

7. Silva M, Antunes A, Azeredo-Lopes S, Cardoso G, Xavier M, Saraceno B, et al. How did the use of psychotropic drugs change during the Great Recession in Portugal? A follow-up to the National Mental Health Survey. BMC Psychiatry. 2020;20(1):215.

8. Furtado C. Psicofármacos: Evolução do consumo em Portugal Continental (2000 – 2012). Infarmed-Autoridade Nacional do Medicamento e Produtos de Saúde, I.P.; 2013.

9. Stoner SC, Marken PA, Watson WA, Switzer JL, Barber MF, Meyer VL, et al. Antidepressant overdoses and resultant emergency department services: the impact of SSRIs. Psychopharmacology bulletin. 1997;33(4):667-70.

10. Furtado CR, M.; Gaspar, M. Análise da Evolução da Utilização de Psicofármacos em Portugal Continental entre 2000 e 2009. INFARMED; 2010.

11. McKenzie MS, McFarland BH. Trends in antidepressant overdoses. Pharmacoepidemiology and Drug Safety. 2007;16(5):513-23.

12. Hiemke C, Bergemann N, Clement HW, Conca A, Deckert J, Domschke K, et al. Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology: Update 2017. Pharmacopsychiatry. 2018;51(1-02):9-62.

13. Benkert O, Szegedi A, Müller MJ. Antidepressant Drugs. In: Smelser NJ, Baltes PB, editors. International Encyclopedia of the Social & Behavioral Sciences. Oxford: Pergamon; 2001. p. 529-35.

14. Hilal-Dandan R, Brunton L, Goodman LS. Goodman and Gilman Manual of Pharmacology and Therapeutics, Second Edition: McGraw-Hill Education; 2013.

15. Brown MJ, Sharma P, Bennett PN. Clinical Pharmacology: Elsevier Health Sciences; 2012.

16. López-Muñoz F, Alamo C. Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. Current pharmaceutical design. 2009;15(14):1563-86.

17. Preskorn SH. Clinically Relevant Pharmacology of Selective Serotonin Reuptake Inhibitors. Clinical pharmacokinetics. 1997;32(1):1-21.

18. Beck KM, Bhutani T, Koo JYM. Psychotropic Agents. In: Wolverton SE, editor. Comprehensive Dermatologic Drug Therapy (Fourth Edition): Elsevier; 2021. p. 382-96.e2.

19. Hiemke C, Härtter S. Pharmacokinetics of selective serotonin reuptake inhibitors. Pharmacology & therapeutics. 2000;85(1):11-28.

20. Nelson LS, Lewin NA, Howland MA, Hoffman RS, Goldfrank LR, Flomenbaum NE. Goldfrank's Toxicologic Emergencies, Ninth Edition: McGraw-Hill Education; 2010.

21. Taylor DM, Paton C, Kapur S. The Maudsley Prescribing Guidelines in Psychiatry: Wiley; 2015.

22. Volpi-Abadie J, Kaye AM, Kaye AD. Serotonin syndrome. Ochsner J. 2013;13(4):533-40.

23. Nelson LS, Erdman AR, Booze LL, Cobaugh DJ, Chyka PA, Woolf AD, et al. Selective serotonin reuptake inhibitor poisoning: An evidence-based consensus guideline for out-of-hospital management. Clinical toxicology (Philadelphia, Pa). 2007;45(4):315-32.

24. Stahl SM, Muntner N. Stahl's Essential Psychopharmacology: Neuroscientific Basis and Practical Applications: Cambridge University Press; 2013.

25. Abdollahi M, Mostafalou S. Tricyclic Antidepressants. In: Wexler P, editor. Encyclopedia of Toxicology (Third Edition). Oxford: Academic Press; 2014. p. 838-45.

26. Kompoliti K, Horn SS. Drug-Induced and Iatrogenic Neurological Disorders. In: Goetz CG, editor. Textbook of Clinical Neurology (Third Edition). Philadelphia: W.B. Saunders; 2007. p. 1285-318.

27. Gillman PK. Tricyclic antidepressant pharmacology and therapeutic drug interactions updated. Br J Pharmacol. 2007;151(6):737-48.

28. Livingston MG, Livingston HM. Monoamine Oxidase Inhibitors: An Update on Drug Interactions. Drug Safety. 1996;14(4):219-27.

29. Youdim MBH, Edmondson D, Tipton KF. The therapeutic potential of monoamine oxidase inhibitors. Nature Reviews Neuroscience. 2006;7(4):295-309.

30. Flanagan RJ. Developing an analytical toxicology service: principles and guidance. Toxicological reviews. 2004;23(4):251-63.

31. Cooper G, Negrusz A. Clarke's Analytical Forensic Toxicology: Pharmaceutical Press; 2013.

32. Dinis-Oliveira RJ, Vieira DN, Magalhães T. Guidelines for Collection of Biological Samples for Clinical and Forensic Toxicological Analysis. Forensic Sci Res. 2017;1(1):42-51.

33. Rosado T, Gonçalves A, Martinho A, Alves G, Duarte AP, Domingues F, et al. Simultaneous Quantification of Antidepressants and Metabolites in Urine and Plasma Samples by GC–MS for Therapeutic Drug Monitoring. Chromatographia. 2017;80(2):301-28.

34. Burtis CA, Bruns DE. Tietz Fundamentals of Clinical Chemistry and Molecular Diagnostics - E-Book: Elsevier Health Sciences; 2014.

35. Craige Trenerry V, Rochfort SJ. Natural Products Research and Metabolomics. In: Liu H-W, Mander L, editors. Comprehensive Natural Products II. Oxford: Elsevier; 2010. p. 595-628.

36. Rouessac F, Rouessac A. Chemical Analysis: Modern Instrumentation Methods and Techniques: Wiley; 2013.

37. Harris DC. Quantitative Chemical Analysis: W. H. Freeman; 2010.

38. Shimadzu. Basics of Liquid Chromatograph-Mass Spectrometry 2022 [Available from: https://www.shimadzu.com/an/service-support/technical-support/liquid-chromatograph-mass-spectrometry/index.html.

39. Dass C. Fundamentals of Contemporary Mass Spectrometry: Wiley; 2007.

40. Eurachem. The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics 2014 [

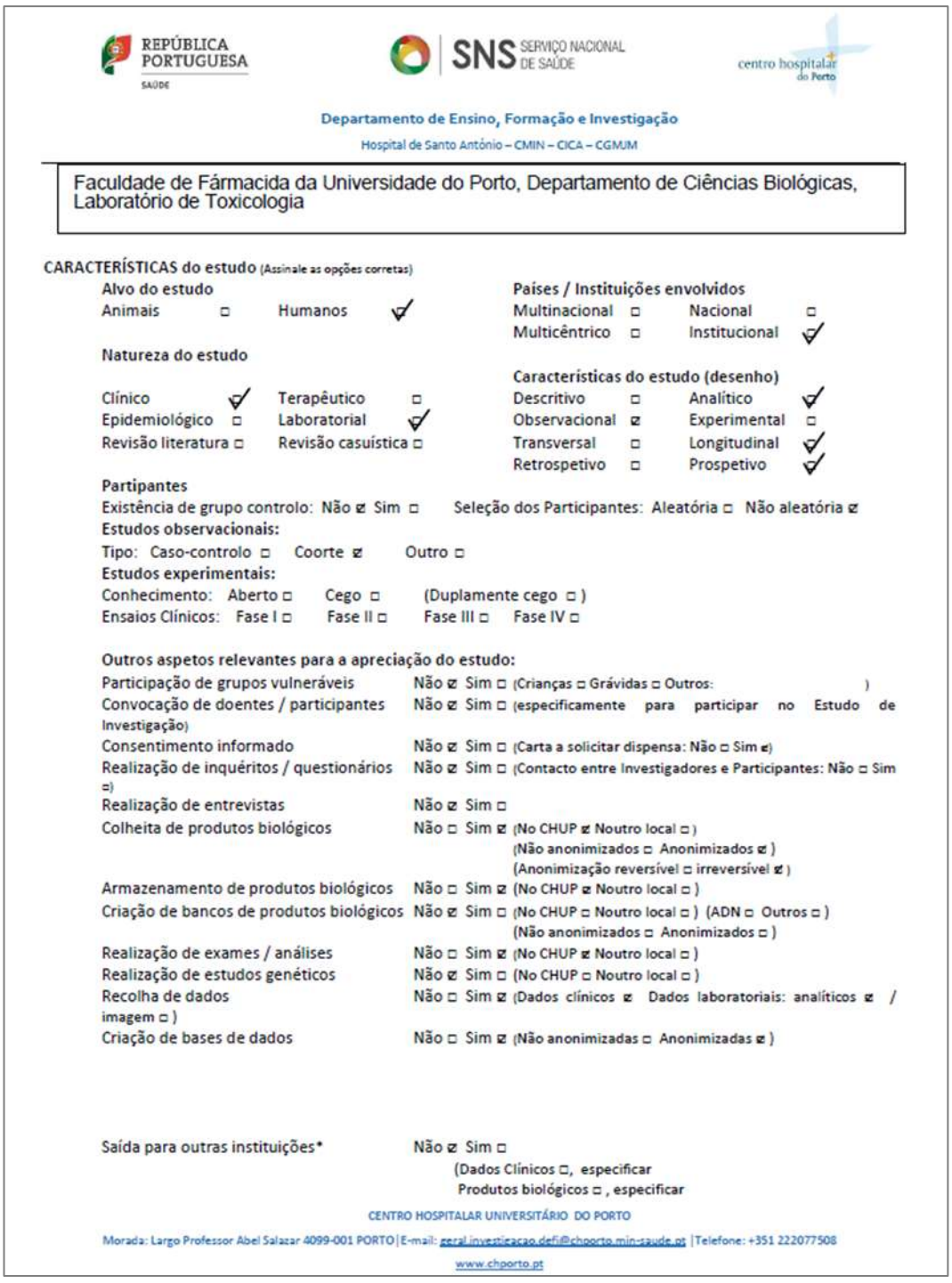
41. Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Science International. 2007;165(2):216-24.

42. NATA. Guidelines for the validation and verification of chemical test methods. In: NATA, editor. 2013.

43. TIAFT. Reference Blood Level List of Therapeutic and Toxic Substances. 2004.

44. Schulz M, Schmoldt A, Andresen-Streichert H, Iwersen-Bergmann S. Revisited: Therapeutic and toxic blood concentrations of more than 1100 drugs and other xenobiotics. Critical care (London, England). 2020;24(1):195.

Appendices



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SNS SERVIÇO NACIONAL DE SAÚDE

centro hospitalar do Porto

Departamento de Ensino, Formação e Investigação

Hospital de Santo António – CMIN – CICA – CGMUM

Faculdade de Fármacida da Universidade do Porto, Departamento de Ciências Biológicas, Laboratório de Toxicologia

CARACTERÍSTICAS do estudo (Assinale as opções corretas)

Alvo do estudo
Animais ☐ Humanos ☑

Natureza do estudo
Clínico ☑ Terapêutico ☐
Epidemiológico ☐ Laboratorial ☑
Revisão literatura ☐ Revisão casuística ☐

Países / Instituições envolvidos
Multinacional ☐ Nacional ☐
Multicêntrico ☐ Institucional ☑

Características do estudo (desenho)
Descritivo ☐ Analítico ☑
Observacional ☒ Experimental ☐
Transversal ☐ Longitudinal ☑
Retrospetivo ☐ Prospetivo ☑

Partipantes
Existência de grupo controlo: Não ☒ Sim ☐ Seleção dos Participantes: Aleatória ☐ Não aleatória ☒
Estudos observacionais:
Tipo: Caso-controlo ☐ Coorte ☒ Outro ☐
Estudos experimentais:
Conhecimento: Aberto ☐ Cego ☐ (Duplamente cego ☐)
Ensaios Clínicos: Fase I ☐ Fase II ☐ Fase III ☐ Fase IV ☐

Outros aspetos relevantes para a apreciação do estudo:

Participação de grupos vulneráveis	Não ☒ Sim ☐ (Crianças ☐ Grávidas ☐ Outros:)
Convocação de doentes / participantes	Não ☒ Sim ☐ (especificamente para participar no Estudo de Investigação)
Consentimento informado	Não ☒ Sim ☐ (Carta a solicitar dispensa: Não ☐ Sim ☒)
Realização de inquéritos / questionários	Não ☒ Sim ☐ (Contacto entre Investigadores e Participantes: Não ☐ Sim ☐)
Realização de entrevistas	Não ☒ Sim ☐
Colheita de produtos biológicos	Não ☐ Sim ☒ (No CHUP ☒ Noutro local ☐) (Não anonimizados ☐ Anonimizados ☒) (Anonimização reversível ☐ irreversível ☒)
Armazenamento de produtos biológicos	Não ☐ Sim ☒ (No CHUP ☒ Noutro local ☐)
Criação de bancos de produtos biológicos	Não ☒ Sim ☐ (No CHUP ☐ Noutro local ☐) (ADN ☐ Outros ☐) (Não anonimizados ☐ Anonimizados ☐)
Realização de exames / análises	Não ☐ Sim ☒ (No CHUP ☒ Noutro local ☐)
Realização de estudos genéticos	Não ☒ Sim ☐ (No CHUP ☐ Noutro local ☐)
Recolha de dados	Não ☐ Sim ☒ (Dados clínicos ☒ Dados laboratoriais: analíticos ☒ / imagem ☐)
Criação de bases de dados	Não ☐ Sim ☒ (Não anonimizadas ☐ Anonimizadas ☒)
Saída para outras instituições*	Não ☒ Sim ☐ (Dados Clínicos ☐, especificar Produtos biológicos ☐ , especificar

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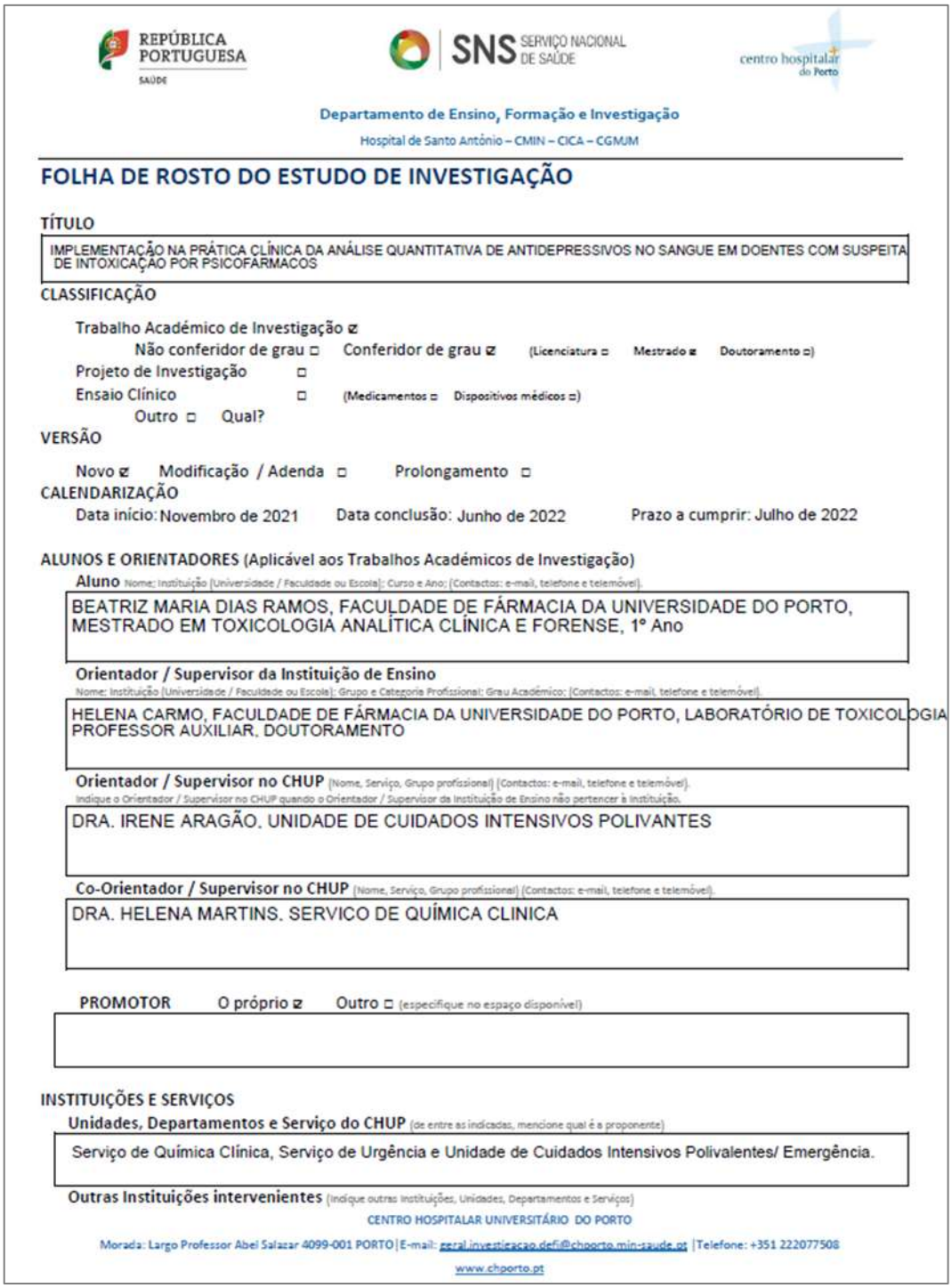
Appendix 1 | *Research study cover page.*



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Departamento de Ensino, Formação e Investigação

Hospital de Santo António – CMIN – CICA – CGMJM

FOLHA DE ROSTO DO ESTUDO DE INVESTIGAÇÃO

TÍTULO

IMPLEMENTAÇÃO NA PRÁTICA CLÍNICA DA ANÁLISE QUANTITATIVA DE ANTIDEPRESSIVOS NO SANGUE EM DOENTES COM SUSPEITA DE INTOXICAÇÃO POR PSICOFÁRMACOS

CLASSIFICAÇÃO

Trabalho Académico de Investigação ☑
Não conferidor de grau ☐ Conferidor de grau ☑ (Licenciatura ☐ Mestrado ☑ Doutoramento ☐)
Projeto de Investigação ☐
Ensaio Clínico ☐ (Medicamentos ☐ Dispositivos médicos ☐)
Outro ☐ Qual?

VERSÃO

Novo ☑ Modificação / Adenda ☐ Prolongamento ☐

CALENDARIZAÇÃO

Data início: Novembro de 2021 Data conclusão: Junho de 2022 Prazo a cumprir: Julho de 2022

ALUNOS E ORIENTADORES (Aplicável aos Trabalhos Académicos de Investigação)

Aluno Nome; Instituição (Universidade / Faculdade ou Escola); Curso e Ano; (Contactos: e-mail, telefone e telemóvel).

BEATRIZ MARIA DIAS RAMOS, FACULDADE DE FÁRMACIA DA UNIVERSIDADE DO PORTO, MESTRADO EM TOXICOLOGIA ANALÍTICA CLÍNICA E FORENSE, 1º Ano

Orientador / Supervisor da Instituição de Ensino

Nome; Instituição (Universidade / Faculdade ou Escola); Grupo e Categoria Profissional; Grau Académico; (Contactos: e-mail, telefone e telemóvel).

HELENA CARMO, FACULDADE DE FÁRMACIA DA UNIVERSIDADE DO PORTO, LABORATÓRIO DE TOXICOLOGIA PROFESSOR AUXILIAR, DOUTORAMENTO

Orientador / Supervisor no CHUP (Nome, Serviço, Grupo profissional) (Contactos: e-mail, telefone e telemóvel).

Indique o Orientador / Supervisor no CHUP quando o Orientador / Supervisor da Instituição de Ensino não pertencer à Instituição.

DRA. IRENE ARAGÃO, UNIDADE DE CUIDADOS INTENSIVOS POLIVANTES

Co-Orientador / Supervisor no CHUP (Nome, Serviço, Grupo profissional) (Contactos: e-mail, telefone e telemóvel).

DRA. HELENA MARTINS, SERVICO DE QUÍMICA CLINICA

PROMOTOR O próprio ☑ Outro ☐ (especifique no espaço disponível)

INSTITUIÇÕES E SERVIÇOS

Unidades, Departamentos e Serviço do CHUP (de entre as indicadas, mencione qual é a proponente)

Serviço de Química Clínica, Serviço de Urgência e Unidade de Cuidados Intensivos Polivalentes/ Emergência.

Outras Instituições intervenientes (Indique outras Instituições, Unidades, Departamentos e Serviços)

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Appendix 1 | *Continued.*

Centro Hospitalar Universitário do Porto

DOSEAMENTO DE ANTIDEPRESSIVOS NO SANGUE (*)

(EM DOENTES COM SUSPEITA DE INTOXICAÇÃO POR PSICOFÁRMACOS)

Colante do doente

Informação Clínica Relevante:

Medicação habitual e fármacos suspeitos de sobredosagem ou intoxicação:

Assinale os (s) fármacos suspeitos (s) de intoxicação.

	ANTIDEPRESSIVOS NÃO TRICÍCLICOS		ANTIDEPRESSIVOS TRICÍCLICOS
	Atomoxetina		Amitriptilina
	Bupropiom		Clomipramina
	Citalopram		Dosulepina (Dotiepina)
	Duloxetina		Imipramina
	Fluoxetina		Maprotilina
	Fluvoxamina		Nortriptilina
	Metilfenidato		Trimipramina
	Mianserina		
	Milnaciprano		Outros:
	Mirtazapina		
	Moclobemida		
	Paroxetina		
	Reboxetina		
	Sertralina		
	Tianeptina		
	Trazodona		
	Venlafaxina		
	Vortioxetina		

Estão indicados apenas os fármacos autorizados em Portugal

Colheita de sangue em tubo sem aditivos (tampa vermelha), Ref. nº 454092.

Identificar a amostra com o colante do doente.

Entregar a amostra no CoreLab, juntamente com a requisição em papel e mais um colante do doente.

Pedido por: _____ nº mec: _____Data: _____ Hora: _____

Colhido por: _____nº mec: _____Data: _____ Hora: _____

(*) Fase de implementação da metodologia
Projeto 2021.189 (156-DEFI/162-CE)

Appendix 2 | *Requisition for dosing of ATDs filled out by the physicians at the Urgency Care Centre and Emergency Room.*

Appendix 3 | *Concentration of MassCheck® TCA 1 Plasma Controls.*

Plasma Controls	Concentration / µg.L^{-1}	
Substance	Level I	Level II
Amitriptyline	59.10	265.00
Nortriptyline	63.50	279.00
Doxepin	34.00	224.00
Nordoxepin	34.10	223.00
Imipramine	70.60	271.00
Desipramine	48.80	270.00

Appendix 4 | *Concentration of MassCheck® TCA 2 Plasma Controls.*

Plasma Controls	Concentration / µg.L^{-1}	
Substance	Level I	Level II
Clomipramine	43.60	255.00
Norclomipramine	46.70	255.00
Maprotiline	74.10	264.00
Normaprotiline	91.70	294.00
Protriptyline	72.50	262.00
Trimipramine	35.70	252.00
Nortrimipramine	35.90	264.00

Appendix 5 | *Concentration of MassCheck® ATD 1/EXTENDED Plasma Controls.*

Plasma Controls	Concentration / µg.L^{-1}	
Substance	Level I	Level II
Citalopram	70.90	134.00
N-Desmethylcitalopram	72.90	144.00
Fluoxetine	218.00	365.00
Desmethylfluoxetine	251.00	394.00
Duloxetine	15.00	112.00
Fluvoxamine	113.00	187.00
Mirtazapine	89.80	202.00
N-Desmethylmirtazapine	103.00	203.00
Paroxetine	53.00	75.00
Sertraline	51.30	99.30
N-Desmethylsertraline	81.10	157.00
Venlafaxine	72.90	132.00
O-Desmethylvenlafaxine	207.00	358.00

Appendix 6 | *Concentration of MassCheck® ATD 2/EXTENDED Plasma Controls.*

Plasma Controls	Concentration / µg.L^{-1}	
Substance	Level I	Level II
Dosulepin	46.00	319.00
N-Desmethyldosulepin	86.60	267.00
Mianserin	21.40	120.00
Milnacipran	55.60	339.00
Moclobemide	381.00	2237.00
Opipramol	91.80	656.00
Reboxetine	57.70	296.00
Tianeptine	57.60	325.00
Tranylcypromine	75.10	615.00
Trazodone	516.00	2240.00
Vilazodone	27.40	77.00
Vortioxetine	17.40	83.30

Appendix 7 | *Concentration of 3PLUS1® TCA 1 Plasma Calibrators.*

Plasma Calibrators	Calibrator / µg.L^{-1}			
Substance	**1**	**2**	**3**	**Blank**
Amitriptyline	26.10	202.00	377.00	n.d.
Nortriptyline	26.80	202.00	374.00	n.d.
Doxepin	7.69	161.00	304.00	n.d.
Nordoxepin	6.78	162.00	307.00	n.d.
Imipramine	33.80	205.00	367.00	n.d.
Desipramine	14.30	192.00	359.00	n.d.
n.d. = not detected				

Appendix 8 | *Concentration of 3PLUS1® TCA 2 Plasma Calibrators.*

Plasma Calibrators	Calibrator / µg.L^{-1}			
Substance	**1**	**2**	**3**	**Blank**
Clomipramine	14.30	184.00	352.00	n.d.
Norclomipramine	14.60	182.00	347.00	n.d.
Maprotiline	38.10	193.00	342.00	n.d.
Normaprotiline	69.30	210.00	348.00	n.d.
Protriptyline	39.60	207.00	371.00	n.d.
Trimipramine	7.590	195.00	379.00	n.d.
Nortrimipramine	7.50	190.00	367.00	n.d.
n.d. = not detected				

Appendix 9 | *Concentration of 3PLUS1® ATD 1/EXTENDED Plasma Calibrators.*

Plasma Calibrators	Calibrator / µg.L^{-1}			
Substance	**1**	**2**	**3**	**Blank**
Citalopram	8.03	103.00	278.00	n.d.
N-Desmethylcitalopram	8.47	109.00	295.00	n.d.
Fluoxetine	77.30	290.00	711.00	n.d.
Desmethylfluoxetine	117.00	328.00	748.00	n.d.
Duloxetine	1.83	77.80	150.00	n.d.
Fluvoxamine	39.10	149.00	371.00	n.d.
Mirtazapine	6.41	155.00	395.00	n.d.
N-Desmethylmirtazapine	6.87	153.00	412.00	n.d.
Paroxetine	22.50	61.70	142.00	n.d.
Sertraline	3.75	75.40	214.00	n.d.
N-Desmethylsertraline	10.40	119.00	340.00	n.d.
Venlafaxine	22.00	104.00	258.00	n.d.
O-Desmethylvenlafaxine	52.50	286.00	686.00	n.d.
n.d. = not detected				

Appendix 10 | *Concentration of 3PLUS1® ATD 2/EXTENDED Plasma Calibrators.*

Plasma Calibrators	Calibrator / µg.L^{-1}			
Substance	**1**	**2**	**3**	**Blank**
Dosulepin	13.20	196.00	385.00	n.d.
N-Desmethyldosulepin	71.90	153.00	236.00	n.d.
Mianserin	7.12	95.50	187.00	n.d.
Milnacipran	17.90	201.00	377.00	n.d.
Moclobemide	148.00	1306.00	2382.00	n.d.
Opipramol	33.10	312.00	588.00	n.d.
Reboxetine	18.50	217.00	421.00	n.d.
Tianeptine	20.70	194.00	395.00	n.d.
Tranylcypromine	7.56	345.00	685.00	n.d.
Trazodone	242.00	1611.00	2957.00	n.d.
Vilazodone	15.00	579.20	92.30	n.d.
Vortioxetine	7.70	61.40	116.00	n.d.
n.d. = not detected				

Appendix 11 | *Concentration of all analytes in the fortified blank samples.*

	Analyte	Fortified Blank Concentration /µg.L-1
TCA 1	Amitriptyline	8.56
	Nortriptyline	8.79
	Doxepin	2.52
	Nordoxepin	2.22
	Imipramine	11.09
	Desipramine	4.69
TCA 2	Clomipramine	4.69
	Norclomipramine	4.79
	Maprotiline	12.50
	Normaprotiline	22.73
	Protriptyline	4.63
	Trimipramine	2.49
	Nortrimipramine	2.46
ATD 1	Citalopram	5.02
	N-Desmethylcitalopram	5.29
	Fluoxetine	48.31
	Desmethylfluoxetine	73.13
	Duloxetine	1.14
	Fluvoxamine	24.44
	Mirtazapine	4.01
	N-Desmethylmirtazapine	4.29
	Paroxetine	14.06
	Sertraline	2.34
	N-Desmethylsertraline	6.50
	Venlafaxine	13.75
	O-Desmethylvenlafaxine	32.81
ATD 2	Dosulepin	8.98
	N-Desmethyldosulepin	48.89
	Mianserin	4.84
	Milnacipran	12.17
	Moclobemide	100.64
	Opipramol	22.51
	Reboxetine	12.58
	Tianeptine	14.08
	Tranylcypromine	5.14
	Trazodone	164.56
	Vilazodone	10.20
	Vortioxetine	5.24

Appendix 12 | *Selected ions (m/z) in MRM analysis for the analytes and respective ISTD of TCA 1 set.*

Analyte Name	Precursor Ion	Ion Product 1	Ion Product 2
Amitriptyline	278	233	91
Nortriptyline	264	233	91
Doxepin	280	107	235
Nordoxepin	266	107	235
Imipramine	281	86	58
Desipramine	267	72	193
ISTD 1	283	107	235
ISTD 2	287	92	64
ISTD 3	267	233	91

Appendix 13 | *Selected ions (m/z) in MRM analysis for the analytes and respective ISTD of TCA 2 set.*

Analyte Name	Precursor Ion	Ion Product 1	Ion Product 2
Clomipramine	315	86	58
Norclomipramine	301	72	270
Maprotiline	278	250	191
Normaprotiline	264	169	117
Protriptyline	264	233	191
Trimipramine	295	100	58
Nortrimipramine	281	86	208
ISTD 2	287	92	64
ISTD 3	267	233	91
ISTD 4	318	89	61

Appendix 14 | *Selected ions (m/z) in MRM analysis for the analytes and respective ISTD of ATD 1/EXTENDED set.*

Analyte Name	Precursor Ion	Ion Product 1	Ion Product 2
Citalopram	325	109	234
N-Desmethylcitalopram	311	109	234
Fluoxetine	310	148	-
Desmethylfluoxetine	296	134	-
Duloxetine	298	154	188
Fluvoxamine	319	258	226
Mirtazapine	266	195	209
N-Desmetilmirtazapine	252	195	209
Paroxetine	330	192	123
Sertraline	306	159	129
N-Desmethylsertraline	292	159	129
Venlafaxine	278	260	215
O-Desmethylvenlafaxine	264	246	201
ISTD 2	270	195	211
ISTD 3	288	270	224
ISTD 6	305	154	195
ISTD 8	322	261	226
ISTD 9	309	159	129
ISTD 10	274	256	210
ISTD 11	256	195	211
ISTD 12	331	109	234
ISTD 13	315	109	234
ISTD 14	334	196	123
ISTD 15	315	153	-
ISTD 16	301	139	-
ISTD 17	296	160	133

Appendix 15 | *Selected ions (m/z) in MRM analysis for the analytes and respective ISTD of ATD 2/EXTENDED set.*

Analyte Name	Precursor Ion	Ion Product 1	Ion Product 2
Dosulepin	296	251	223
N-Desmethyldosulepin	282	251	218
Mianserin	265	208	193
Milnacipran	247	100	127
Moclobemide	270	183	140
Opipramol	364	206	234
Reboxetine	314	91	131
Tianeptine	437	228	246
Tranylcypromine	134	115	117
Trazodone	375	148	176
Vilazodone	442	197	397
Vortioxetine	299	256	120
ISTD 1	230	93	61
ISTD 2	244	93	61
ISTD 3	259	151	47
ISTD 4	139	119	122
ISTD 5	277	182	139
ISTD 6	257	110	127
ISTD 7	450	197	405
ISTD 8	319	91	176
ISTD 9	268	208	193
ISTD 10	299	251	223
ISTD 11	378	154	182
ISTD 12	368	206	234
ISTD 13	449	228	-
ISTD 14	307	260	124

Appendix 16 | *Therapeutic reference ranges for TCAs. Adapted from (12, 43, 44).*

TCAs	Therapeutic Range /µg. L^{-1}		
Drug	(12)	(44)	(43)
Amitriptyline	80-200*2	50-300	50-300
Nortriptyline	70-170	50-150	75-250
Clomipramine	230-450	90-250	90-250
Norclomipramine		230-450*1	150-550*1
Dosulepin	45-100	45-100	20-400
N-Desmethyldosulepin	-	-	100-200
Doxepin	50-150	30-100	20-150
Nordoxepin		50-150*1	200-350*1
Imipramine	175-300*2	50-350	45-150
Desipramine	100-300	10-500	75-250
Maprotiline+	75-130	75-130	75-250
Normaprotiline	-	-	100-400*1
Protriptyline	-	50-300	70-170
Trimipramine	150-300	150-300	10-300

*1- With parent compound. *2- With metabolite.

+Is a tetracyclic antidepressant but has a similar pharmacological mechanism to TCAs.

Appendix 17 | *Therapeutic reference ranges for SSRIs. Adapted from (12, 43, 44).*

SSRIs	Therapeutic Range /µg. L^{-1}		
Drug	(12)	(44)	(43)
Citalopram	50-110	50-110	20-200
Fluoxetine	120-500	120-500	15-500
Desmethylfluoxetine			100-500
Fluvoxamine	60-230	60-230	50-250
Paroxetine	20-65	2-65	10-75
Sertraline	10-150	10-150	50-250

Appendix 18 | *Therapeutic reference ranges for MAOIs. Adapted from (12, 43, 44).*

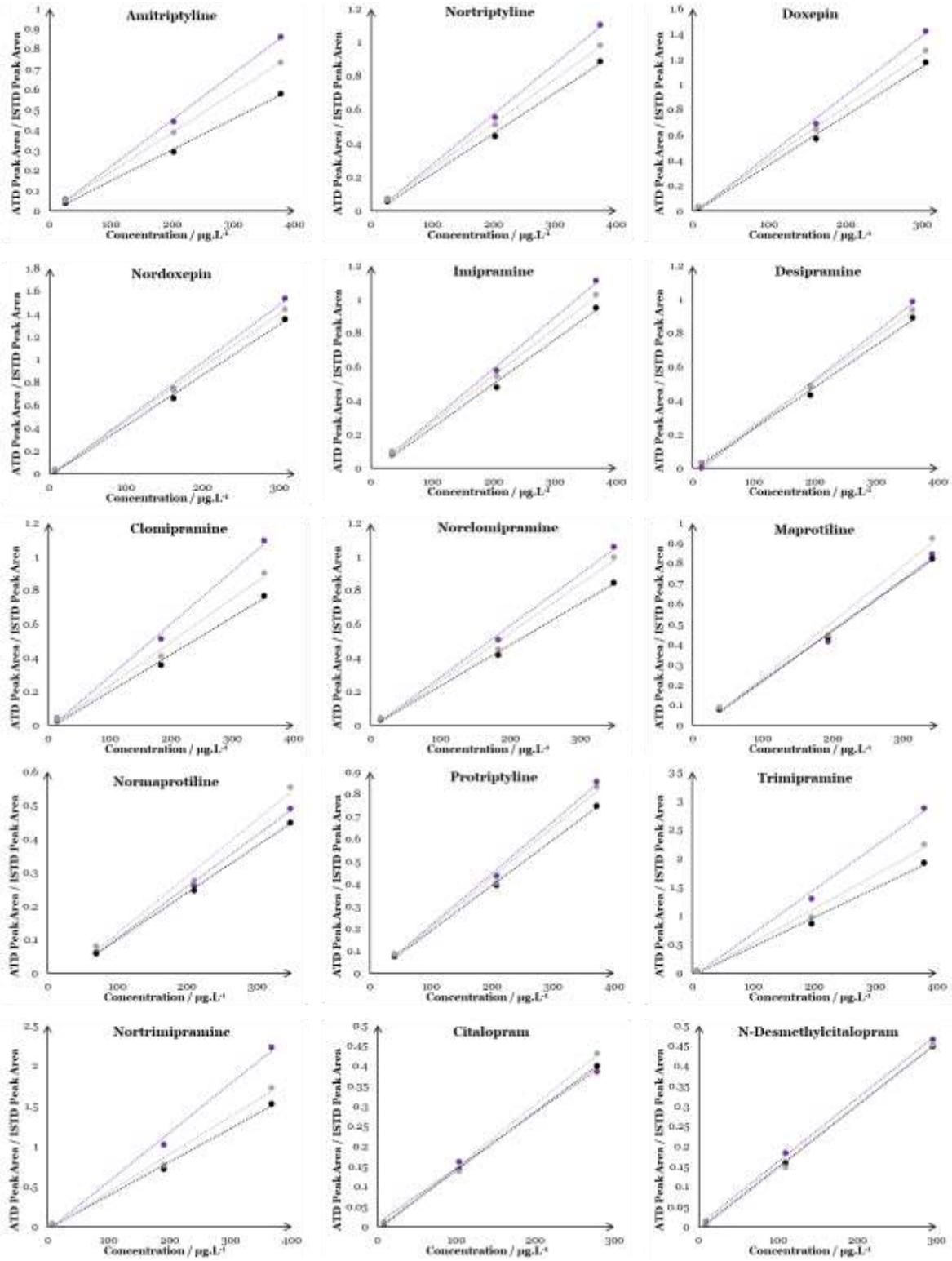
MAOIs	Therapeutic Range /µg. L^{-1}		
Drug	(12)	(44)	(43)
Moclobemide	300-1000	300-1000	400-1000
Tranylcypromine	≤50	≤50	50

Appendix 19 | *Therapeutic reference ranges for Atypical Antidepressants. Adapted from (12, 43, 44).*

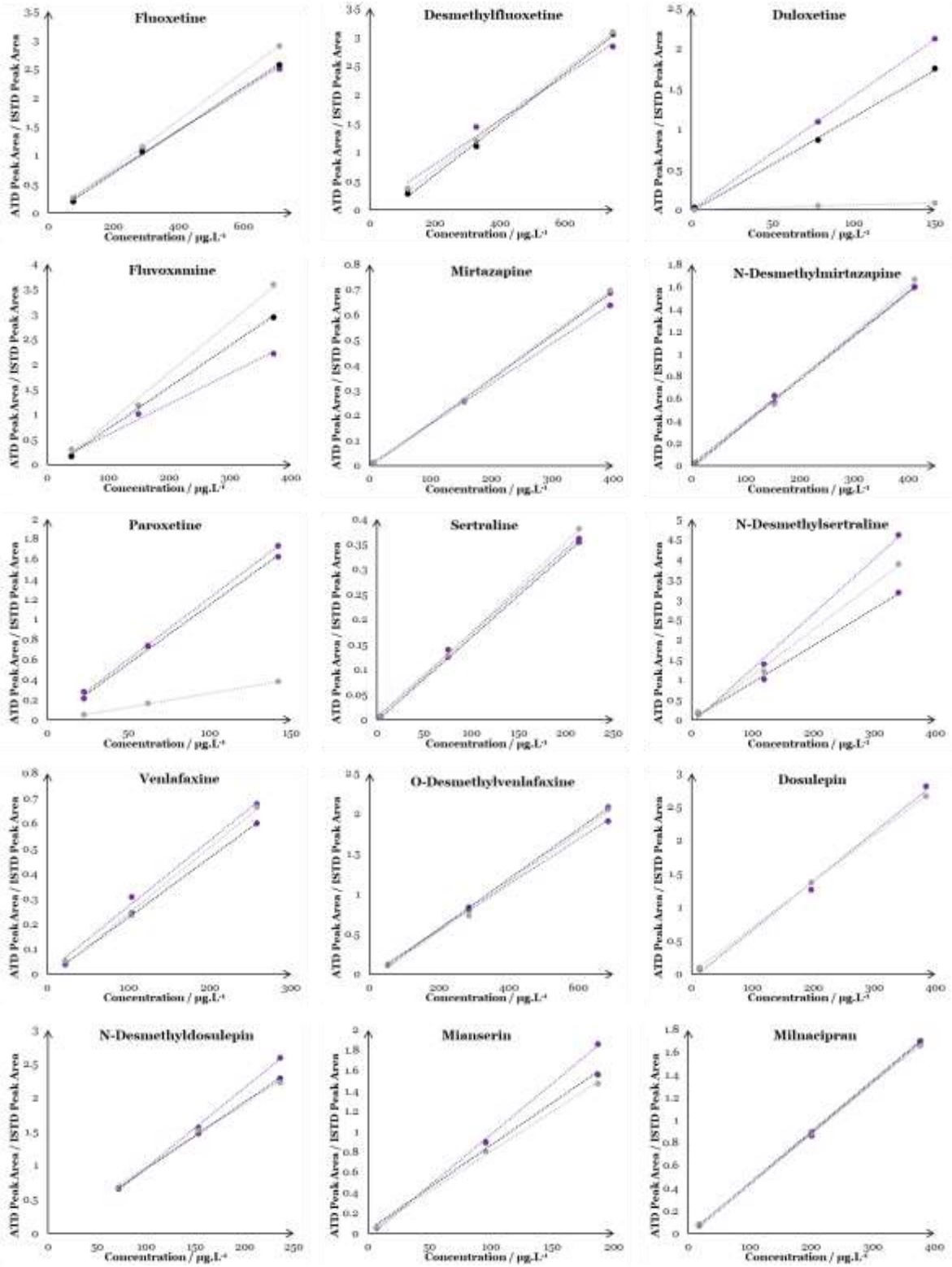
Atypical Antidepressants	**Therapeutic Range /µg. L^{-1}**		
Drug	(12)	(44)	(43)
Duloxetine	30-120	30-120	-
Mirtazapine	30-80	30-80	20-100
N-Desmethylmirtazapine	-	-	50-300*1
Venlafaxine	100-400	100-400	250-750
O-Desmethylvenlafaxine			
Mianserin	15-70	15-70	15-70
Milnacipran	100-150	100-150	-
Opipramol+	50-500	50-500	50-200
Reboxetine	60-350	60-350	-
Tianeptine+	30-80	30-80	-
Trazodone	700-1000	700-1000	300-1500
Vilazodone	30-70	30-70	-
Vortioxetine	10-40	6-40	-

*1- With parent compound.

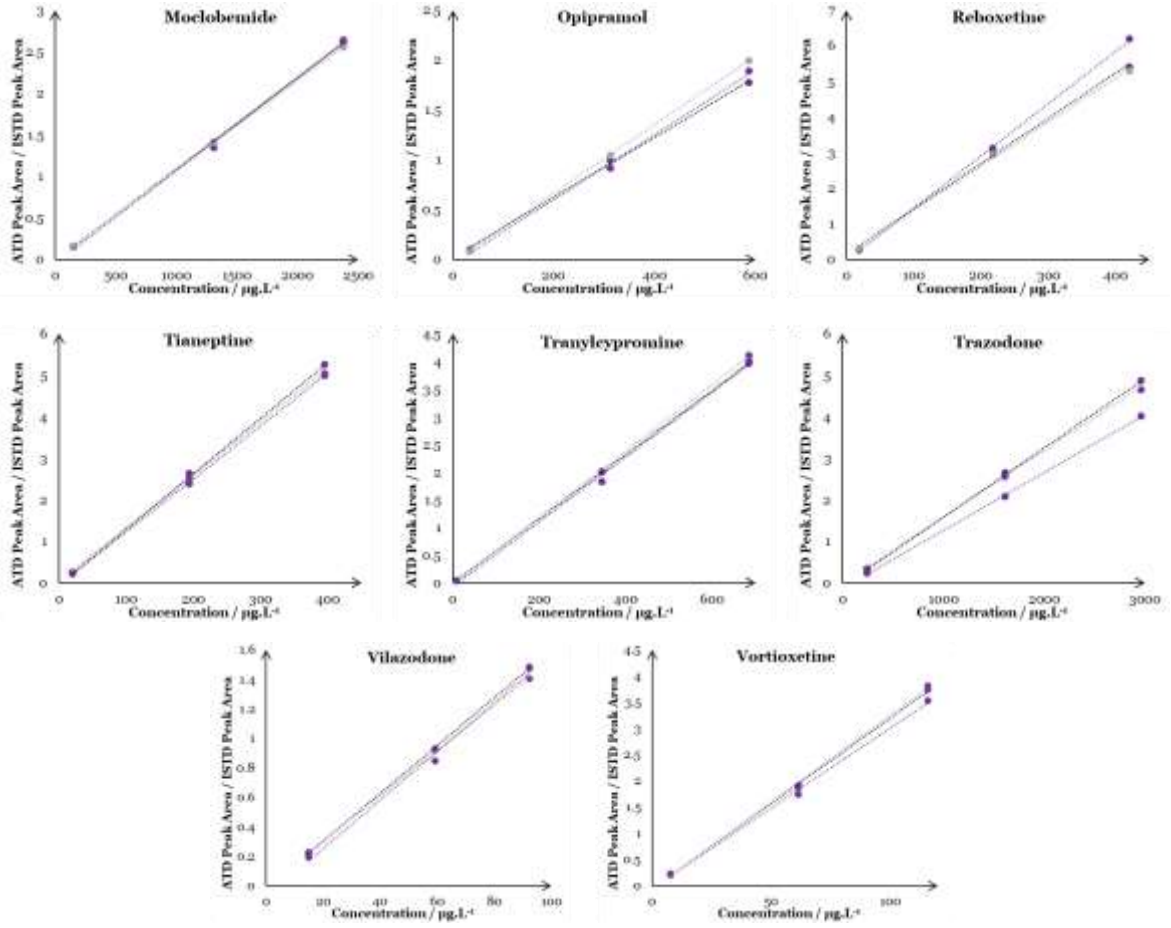
+ Structurally are TCAs but have different pharmacological mechanisms.



Appendix 20 | *Independent calibration curves performed in different days for all ATDs.*



Appendix 20 | *Continued.*



Appendix 20 | *Continued.*

Appendix 21 | *Measuring range for every ATDs sets.*

	Analyte	Measuring Range / µg.L^{-1}
TCA 1	Amitriptyline	[13.01 ; 377.00]
	Nortriptyline	[15.39 ; 374.00]
	Doxepin	[7.21 ; 304.00]
	Nordoxepin	[6.57 ; 307.00]
	Imipramine	[18.07 ; 357.00]
	Desipramine	[19.67 ; 359.00]
TCA 2	Clomipramine	[9.23 ; 352.00]
	Norclomipramine	[8.75 ; 347.00]
	Maprotiline	[29.72 ; 342.00]
	Normaprotiline	[42.46 ; 348.00]
	Protriptyline	[16.13 ; 371.00]
	Trimipramine	[10.21 ; 379.00]
	Nortrimipramine	[11.52 ; 367.00]
ATD 1	Citalopram	[11.75 ; 278.00]
	N-Desmethylcitalopram	[7.81 ; 295.00]
	Fluoxetine	[51.53 ; 711.00]
	Desmethylfluoxetine	[155.14 ; 748.00]
	Duloxetine	[1.83 ; 150.00]*
	Fluvoxamine	[41.15 ; 371.00]
	Mirtazapine	[15.97 ; 395.00]
	N-Desmethylmirtazapine	[23.66 ; 412.00]
	Paroxetine	[22.5 ; 142.00]*
	Sertraline	[3.24 ; 214.00]
	N-Desmethylsertraline	[11.6 ; 340.00]*
	Venlafaxine	[19.67 ; 258.00]
	O-Desmethylvenlafaxine	[41.9 ; 686.00]
ATD 2	Dosulepin	[11.35 ; 385.00]
	N-Desmethyldosulepin	[72.67 ; 236.00]
	Mianserin	[7.81 ; 187.00]
	Milnacipran	[19.65 ; 377.00]
	Moclobemide	[142.49 ; 2382.00]
	Opipramol	[27.66 ; 588.00]
	Reboxetine	[14.52 ; 421.00]
	Tianeptine	[22.42 ; 395.00]
	Tranylcypromine	[11.44 ; 685.00]
	Trazodone	[242.00 ; 2957.00]*
	Vilazodone	[12.81; 92.30]
	Vortioxetine	[7.95 ; 116.00]

*When the LLOQ determination was not possible, the lowest calibration level is considered.