International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2019): 7.583

A Review on LC-MS Method to Determine Antipsychotic Drugs in Biological Samples

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Abstract: ***Antipsychotic drugs, a class of psychiatric medication used to treat psychotic symptoms mainly in bipolar disorder, schizophrenia and other psycho-organic disorders. Antipsychotics are administered in oral doses of only few milligrams per day and are widely metabolized in the body, hence the concentration of these drugs in plasma is very low (pg-ng/ml levels), which complicate their detection using standard gas chromatography – mass spectrometry (GC-MS) procedures that cannot provide the required sensitivity. Due to the wide use of these drugs worldwide, there is a great need of analytical methods to analyze them in biological samples. Highly sensitive, selective and accurate bioanalytical methods are essential in order to conduct the pharmacology and toxicology studies and clinical TDM of antipsychotics. Recent advances in liquid chromatography – mass spectrometry (LC-MS) technique enables accurate detection and quantification of these drugs. This review provides LC-MS procedure for detection and quantification of antipsychotics with focus on sample preparation techniques.***

Keywords: Antipsychotic drugs, LC-MS, Sample preparation techniques

1. Introduction

Antipsychotic drugs are a class of psychiatric medication primarily used to manage psychotic symptoms mainly in schizophrenia, bipolar disorder and other psycho-organic disorders. The phenothiazine derivative chlorpromazine was the first drug introduced in the 1950s for the treatment of psychotic illnesses, replacing electroconvulsive therapy and psychosurgery. The main category of neuroleptic drugs is the phenothiazine derivatives, butyrophenones and thioxanthenes, known as 'typical' antipsychotics. While these drugs show significant improvement in the symptoms of psychotic illness, they are also associated with unwanted extra pyramidal side-effects resulting from their activity at dopamine receptors. Figure 1 demonstrates the general mechanism of action of anti-psychotics.

A new generation of antipsychotics introduced around 1995 largely overcame these side-effects via decreased activity at dopamine receptors compared with their traditional counterparts. These 'second generation' or 'atypical' antipsychotics now account for the vast majority of antipsychotic prescriptions. Studies in recent years have shown that atypical antipsychotics are not free from side effects. An increased risk of mortality in addition to cardiovascular complications has been reported in patients suffering from dementia when treated with atypical antipsychotics [1]. Furthermore, second-generation antipsychotics do not only increase the risk of diabetes [2] compared with typical agents, but also show a similar risk of sudden cardiac death to their typical counterparts. This drug class has rapidly gained importance in both a clinical and forensic setting, which makes the ability to reliably detect antipsychotics in human biological specimens a necessity. In a clinical environment, the analysis of antipsychotics in blood is necessary in order to monitor patient compliance and to maintain drug concentrations within the recommended therapeutic range of the respective drug. The absence of prescribed antipsychotics in a clinical case may also indicate non-compliance, a common issue among patients suffering from mental illness. In a forensic setting, the detection of antipsychotics is crucial in determining whether these drugs played a role in the cause of death. Based on World Health Organization, sixty four compounds are classified as antipsychotics and for about 70% of these, analytical methods have been developed to determine them in human matrices [3]. Figure 2 illustrates the chemical structures of some commonly used antipsychotics.

Due to the wide use of these drugs worldwide, there is a great need of analytical methods in order to analyze biological samples. The quantitative determination of antipsychotics in human matrices is of great interest both for therapeutic drug monitoring and for forensic toxicology. The modern trend in drug analysis is shifting from gas chromatography to liquid chromatography not only because of its good quantitative results, its high reproducibility, sensitivity and wide applicability, but also because most antipsychotic drugs are not volatile. MS in combination with LC now dominates the analytical field, providing a particularly convenient tool in the analysis of antipsychotics. The high sensitivity of LC-MS method often allows analysis times to be substantially reduced compared with traditional UV and EC methods, which is particularly useful for a large sample throughput or when fast turn-around times are required.

Conventional matrices used for this purpose are serum, plasma and whole blood. However, other alternative matrices like oral fluid and urine; which are easily collected, keratinized matrices namely hair and nails; which are stable and capable of providing information for long periods of time, dry blood spots (DBS) and cerebrospinal fluid, are widely used. Blood is the preferred specimen for antipsychotics analysis as it provides the most accurate representation of the relevant pharmacological effects. In a clinical setting, plasma and serum are matrices of choice for drug analysis, as they are the most common specimens used

in diagnostic medicine. Therapeutic drugs monitoring (TDM) methods are common and are more likely to focus on one or very few analytes. Whole blood is the most common specimen used in forensic cases since lysis is common in death investigations, and centrifugation shortly after collection is not always possible [4]. Urine is a useful specimen for general unknown screening (GUS) procedures, particularly when overdose is suspected and qualitative results are required. Antipsychotics are included in most published non-targeted screening procedures as part of big libraries. However, since these methods lack the ability to produce quantitative results, they are less relevant for the detection of antipsychotics and will not be discussed in this review. Hair has become an increasingly popular alternative specimen to blood, as drugs and their metabolites are likely to remain in hair samples long after the compounds have been eliminated from the body. Segmental hair analysis in particular can provide an indication of the long-term history of drug use in an individual. While hair analysis is frequently used as a tool in the analysis of drugs of abuse, only a limited number of methods targeting antipsychotics in hair using LC-MS technology have been published to date [5]. Oral fluid is used as an alternative to blood, which has increasingly gained importance due to the relatively short drug detection windows in addition to non-invasive collection of specimens. Antipsychotics are known to reduce salivary flow rate and may therefore not be ideal for detection in oral fluid. Cerebrospinal fluid (CSF) is commonly analyzed in order to help diagnose various diseases and conditions affecting the central nervous system (CNS), such as meningitis and encephalitis. It is also useful in diagnosing bleeding of the brain or tumors within the CNS. CSF is most commonly obtained by lumbar puncture, a complex and invasive procedure that requires specialized medical staff is complicated process of sample collection makes it a less favorable specimen in drug analysis. Table 1 shows the pharmacokinetic parameters of some antipsychotic drugs.

Due to the high complexity of biological materials, which often contain proteins, salts, organic compounds with similar properties to the analytes and other endogenous compounds that may deteriorate the performance of separation, a sample preparation procedure is required. An ideal sample preparation technique should be fast and comprise the minimum number of working steps, should be easy to learn and easy to use, should be economical and environmental friendly and should be compatible with many analytical instruments [6]. The four major sample preparation techniques used for those matrices are liquid–liquid extraction (LLE), solid-phase extraction (SPE), protein precipitation (PP) and direct injection. However, these conventional techniques tend to have many fundamental drawbacks because they include complicated, time-consuming steps and they require large amounts of sample and organic solvents, while there are many difficulties in automation. There is a big number of different microextraction techniques which are used for sample preparation of biological fluids and other biological matrices in order to enhance compatibility with modern analytical instrumentation, as well as to minimize the use of toxic chemicals and to decrease the size of biofluids or reagents demand. In this review we aim to present an overview of LC-MS procedure and microextraction techniques which are used in order to analyze biological fluids and to detect and quantify antipsychotics in conventional and alternative biological matrices.

1.1 What is LC-MS?

Separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). Coupled chromatography - MS systems are popular in chemical analysis because the individual capabilities of each technique are enhanced synergistically. While liquid chromatography separates mixtures with multiple components, mass spectrometry provides structural identity of the individual components with high molecular specificity and detection sensitivity. In addition to the liquid chromatography and mass spectrometry devices, an LC-MS system contains an interface that efficiently transfers the separated components from the LC column into the MS ion source [9]. The interface is necessary because the LC and MS devices are fundamentally incompatible. While the mobile phase in a LC system is a pressurized liquid, the MS analyzers commonly operate under high vacuum (around 10^{-6} torr / 10^{-7} Hg). Thus, it is not possible to directly pump the eluate from the LC column into the MS source. Overall, the interface is a mechanically simple part of the LC-MS system that transfers the maximum amount of analyte, removes a significant portion of the mobile phase used in LC and preserves the chemical identity of the chromatography products (chemically inert). As a requirement, the interface should not interfere with the ionizing efficiency and vacuum conditions of the MS system [10, 11]. Nowadays, most extensively applied LC-MS interfaces are based on atmospheric pressure ionization (API) strategies like electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo-ionization (APPI). Figure 3 demonstrates the instrumentation diagram of LC-MS.

2. Sample Preparation

Due to the high specificity of LC-MS methods, it was initially thought that the sample preparation step may not be as crucial as with other analytical methods, particularly for MS/MS methods since transitions greatly reduce the risk of interference from other drugs. While endogenous components might no longer be detected using LC-MS methods, they can still significantly interfere with the quantification of a drug. Therefore, liquid-liquid extraction (LLE) [12] and solid-phase extraction (SPE) [13] are still most commonly used as a sample treatment prior to injection into the LC-MS system, as they provide the most thorough sample clean-up. The four major sample preparation techniques used for those matrices are liquid–liquid extraction (LLE), solid-phase extraction (SPE), protein precipitation (PP) and direct injection. However, these conventional techniques tend to have many fundamental drawbacks because they include complicated, time-consuming steps and they require large amounts of sample and organic solvents, while there are many difficulties in automation. Therefore, there is a great need of developing novel, relatively simple, fast and solvent-free microextraction procedures which use smaller volumes of

samples and solvents (micro liter range or even smaller) and can be widely used to analyze these samples. To date, there is a big number of different microextraction techniques which are used for sample preparation of biological fluids and other biological matrices in order to enhance compatibility with modern analytical instrumentation, as well as to minimize the use of toxic chemicals and to decrease the size of biofluids or reagents' demand.

1) Solid Phase Microextraction (SPME)

It is an efficient solvent-free sample preparation method which was first introduced in the early 1990s by Pawliszyn and co-workers. It enables automation, miniaturization and high-throughput performance. This technique uses fibers and capillary tubes coated by stationary phases and it can be applied to samples in any state of matter gaseous, liquid and solid. The technique is based on partitioning of the analytes between the sample matrix and the extraction phase which is immobilized on a fused-silica SPME fiber coated with polymers, until the equilibrium is reached and subsequent thermal desorption of the extracts into a gas chromatograph, reconstitution in the mobile phase used for a separation with a liquid chromatography, or direct injection to an HPLC injection port using suitable interface [14]. The type of the polymers which are used depends on the properties of the analyte. For drug analysis of biological matrices the most common coatings are the polydimethylsiloxane (PDMS) and polyacrylate (PA) while other polymers such as polypyrrole coatings, coatings based on restricted access materials, and those based on mixtures of biocompatible polymers with sorbents used for SPE have also been developed and used. The instrument used for SPME is given in figure 4.

The most widely used technique is fiber-SPME. In this technique the analyte is directly extracted onto the coating of the fiber which is usually is inside a needle in a device with an assembly holder. For the procedure of SPME the sample is placed in a capped vial with a septum which is pierced by the needle of the device followed by the extension of the fiber either to the vapor above the sample (Head-Space SPME) for volatile analytes or directly to the sample (Direct Immersion-SPME) for the extraction of non-volatile analytes until equilibrium is reached. An alternative microextraction technique is in-tube SPME that uses a fused-silica capillary column. The extraction of the analytes takes place either onto the inner coating of the fiber or onto a sorbent bed. Compared to fiber SPME, in-tube SPME is more mechanically stable and can be used with on line coupling with HPLC or LC/MS instruments [15]. Various SPME methods have been developed for the analysis of antipsychotic drugs in biological matrices prior to liquid chromatography analysis. Theodoridis et al. [16] developed a method for the determination of a typical antipsychotic; haloperidol, together with other four drugs: quinine, naproxen, ciprofloxacin and paclitaxel in urine. Each analyte was studied independently. Haloperidol was determined using an Analyticals Erbasil Symmetry C18 column, with a mixture of 0.05 M aqueous ammonium acetate and acetonitrile (35:65 v/v) as a mobile phase, while the detection was accomplished with a UV detector at 210 nm. For the SPME procedure, a PDMS 100 μm fiber was conditioned for 30 min in a GC injector operating at 250 ^{0}C. Then, 4 mL of a solution of each pharmaceutical in buffer (20 μg/mL in 0.9% NaCl, pH 9) was transferred in a glass vial containing a magnetic stirring bar, which was then capped and the sample was agitated at 700 rpm. At the end of the extraction, the analyte was desorbed in 200 μL of methanol and an aliquot of 80 μL was injected to the HPLC. Kumazawa et al. [17] have successfully developed an HPLC-MS/MS method to determine eleven phenothiazine derivatives (clospirazine, fluphenazine, perazine, thiethylperazine, thioridazine, flupentixol, thioproperazine, trifluoperazine, perphenazine, prochlorperazine and propericiazine) in human whole blood and urine using solid-phase microextraction (SPME) with a polyacrylate-coated fiber. The pH of the samples was adjusted to about 8 with KOH solution and the vial was sealed with a silicone-rubber septum cap. The syringe needle of the SPME device was passed through the septum and the polyacrylate fiber was pushed out from the needle and immersed directly in the sample solution in the vial at 40 ◦C and the extraction lasted 60 min at continuous stirring at 250 rpm. The fiber was then injected into the desorption chamber of the SPME-HPLC interface. This was filled with distilled water containing 10 mM ammonium acetate plus 0.1% formic acid—100% acetonitrile (70:30, v/v).The desorption time is 10 min. Subsequently the entire contents of the desorption chamber were flushed directly on to the HPLC column by means of the mobile phase flow at 0.2 mL/min. This method was effectively applied to real samples after oral administration and it can be recommended for use in both therapeutic monitoring and clinical or forensic toxicology.

2) Liquid Phase Microextraction (LPME)

It is a miniaturized form of liquid-liquid extraction, which was firstly introduced at 1990s, when Dasgupta [18] and Jeannot and Cantwell [19] suggested almost at the same time the use of extraction solvents in the low microliter range. It is considered as a simple, rapid and cheap sample preparation technique, which requires only several microliters of organic solvents in contrast to traditional LLE, which requires several hundred of milliliters. Based on hydrodynamic features, this technique can be classified into static LPME and dynamic LPME. In the static LPME, a solvent is used as an extractant and it is suspended in the sample. As a result transference of the target compounds to the extractant is carried out. On the other hand, in the dynamic mode, the exractant solvent forms a microfilm inside of an extraction unit, such as a microsyringe and the mass transfer of the analytes takes place between the sample and the microfilm. Figure 5 demonstrates the instrument for LPME.

The main forms of LPME are (1) single drop microextraction; the oldest form of LPME, which is less frequently used today compared to more recently developed techniques, because it was based on a droplet of solvent hanging at a needle of a syringe and it was not considered very robust, (2) Dispersive LPME (DLLME) and (3) Hollow fiber LPME (HF-LPME). Hollow fiber LPME is a recently developed technique, which is based on immobilized organic solvents inside pores of hollow fibers. This technique partly consists of (1) a donor phase, which is the aqueous sample containing the target compounds, (2) the porous fiber with the organic solvent trapped inside and (3) a receptor phase inside the hollow fiber lumen. Prior to

analysis, the organic solvent is immobilized in the fiber's pores by dipping the hollow fiber in a vial containing the solvent in order to form a layer. Next, the lumen is filled with the acceptor phase, which could be an organic, an acidic or a basic solution. For the analysis, the fiber is inserted in the sample, which is the donor phase containing the analytes and extraction takes place into the immobilized organic solvent. Depending on the acceptor phase that is used HF-LPME can be classified into (1) two phase HF-LPME, in which the organic solvent, which is immobilized in the hollow fiber and the receptor phase, which is inside the lumen of the fiber are the same solvent. In this case the solution that is used as a receptor phase can be directly injected to the gas chromatography system, whereas, for liquid chromatography and capillary electroapothesis, evaporation of the solvent and reconstitution in an aqueous solution are mandatory, so that the sample is compatible with the analytical apparatus. Another form of HF-LPME is the three-phase HF-LPME, in which the acceptor phase is an acidic or basic aqueous solution. In this case, extraction of the analytes takes place from the aqueous sample primarily into the immobilized organic solvent. After that, back extraction from the organic solvent takes place in the final receptor solution, which is the aqueous solution placed into the lumen of the hollow fiber. This extraction mode is limited to basic or acidic analytes that can be ionized. Hollow fiber-LPME can also be classified as static HF-LPME, which includes magnetic stirring of the solution and dynamic HF-LPME, in which small volumes of the sample are repeatedly pulled in and out of the fiber in order to increase the extraction speed. During the development of an ideal HF-LPME process, many parameters should be optimized in order to achieve the best results. These parameters are the material of the fiber, the type of organic solvent, the pH of the sample and the acceptor phase, the volume of sample and of solvent, the time, the temperature, the ionic strength and the stirring speed. Dispersive LLPME is a recent novel approach of liquid-phase microextraction, introduced by Assadi and their co-workers in 2006 [20]. This technique is based on a ternary solvent system consisting of an extraction solvent, a disperser solvent and an aqueous sample. A mixture consisting of the organic and the disperser solvent is rapidly and vigorously injected in the aqueous sample, which contains the target analytes. For this purpose a syringe is used. As a result, a cloudy solution is formed, which is supposed to be stable for a specific time. As a next step, phase separation takes place by gently shaking and centrifuging the mixture. If the density of the organic solvent is higher than this of water, the solvent goes to the bottom of the tube and it can be removed by a microsyringe, after discarding the aqueous solution. The crucial parameters in this procedure are the type and the volume of extraction and disperser solvents, the extraction time after the formation of the cloudy solution, the pH of the sample and its ionic strength. As for the extraction solvent, it should be miscible with the disperser solvent and it should be able to extract the target analytes. Moreover, its high density and low solubility in water assist the centrifugation step. As for the disperser solvent, it has to be soluble in the organic solvent and miscible in water in order to enable the organic solvent to be dispersed in the sample and to form a cloudy solution. The most common disperser solutions are acetone, methanol and acetonitrile.

2.1 DLPME (Dynamic Liquid Phase Microextraction)

Several LPME methods have been developed for the determination of antipsychotic drugs in biological matrices prior to liquid chromatography analysis using either HF-LPME or DLLME. Cruz-Vera et al. [21] published an almost solvent-less HPLC-UV method for the determination of seven phenothiazine derivatives in urine using the dynamic liquid-phase microextraction (dLPME) procedure. The whole process took place under dynamic conditions in an automatic flow system. The extraction unit was consisted of a syringe pump and a 1 mL syringe connected to a Pasteur pipette. For the microextraction, 100 µL of a mixture of ionic liquid 1-butyl-3-methyl imidazolium hexafluorophosphate and acetonitrile (50:50, v/v) were picked up in the pipette, which was then inserted into a vial containing the sample, the pH of which was primarily fixed at 8. A volume of 10 mL was drawn with a flow rate of 0.5 mL·min−1 . When the extraction was completed, 50 µL of the ionic liquid were drawn out at a flow rate of 0.05 mL·min−1 and recovered in a vial containing 50 µL of acetonitrile. Finally, 20 µL of the mixtures were injected into tandem LiChrosorb C8 (4.6 mm × 150 mm)–LiChrosorb C18 (4.6 mm × 150 mm) cartridge columns and determined with a mobile phase consisting of acetonitrile/water/acetic acid/trimethylamine 40/40/20/2 (v/v/v/v). A new pipette was used for each extraction so there is no carry-over effect. The recovery values was between 72% and 98%, the limits of detection were between 21 ng/mL and 60 ng/mL and the repeatability expressed as RSD varied between 2.2% and 3.9% and the method was successfully validated. Xiong et al. [22] developed a HPLC-UV method for the separation and quantitative determination of three psychotropic drugs (amitryptiline, clomipramine and thioridazine) in urine, using DLLME as a sample preparation technique. In 2011, Chen et al. [23] developed a DLLME-HPLC-UV method for the determination of two antipsychotic drugs; clozapine and chlorpromazine in urine. For the DLLME procedure 10 mL of the sample was placed in a test tube after adjusting the pH to 10 with NaOH and 200 µL of ethanol (as a disperser solvent) containing 40 µL CCl4 (as an extraction solvent) was fast and vigorously injected, in order to form a cloudy solution, which was then shaken and centrifuged for 2 min at 4000 rpm to achieve phase separation. After that, the precipitate was dissolved by 0.5 mL methanol after careful removal of the supernatant solution, the extract was filtered and injected into the HPLC. For the separation, a Symmetry® C18 column packed with 5.0 µm particle size of dimethyloctylsilyl bounded amorphous silica was used with a mixture of CH3COONH4 (0.03 g/mL, pH 5.5)-CH3CN (60:40, v/v) as a mobile phase. With these conditions, the limits of detection were lower than 6 ng/mL and the limits of quantification lower than 39 ng/mL. The absolute extraction efficiencies were higher than 97%. The method was successfully applied to the analysis of real samples obtained from patients.

2.2. HF-LPME (Hollow Fiber Liquid Phase Microextraction)

For trace amounts of chlorpromazine in biological fluids, a hollow fiber liquid phase microextraction HF-LPME-HPLC-UV method was also developed. The drug was extracted

from 11 mL of sample into an organic phase which was n-dodecane trapped in the pores of the fiber followed by the back-extraction into a receiving aqueous solution consisting of 0.01 M phosphate buffer (pH 2.0), located inside the lumen of the hollow fiber. For the extraction 11 mL of the aqueous sample solution was placed into a glass vial with a stirring bar and the vials were put on a magnetic stirrer. The stirring speed was 1000 rpm. Then, 20 µL of the receiving phase were injected into the polypropylene fiber, which was placed into the organic solution for 5 s and then into water for 5 s to remove the extra organic solution from its surface. After that, the fiber was bent and placed into the sample for 60 min and at the end of the extraction the fiber was removed, the receiving phase was withdrawn into the syringe and 10 µL of the receiving phase was injected into the HPLC. The whole procedure was carried out in absence of salt. The detection limit for chlorpromazine was 0.5 µg/L and intra-day and inter-day assay (RSD %) were lower than 10.3%.The method was successfully applied to drug level monitoring in biological fluids (urine and serum) of patients and gave satisfactory results [24].

3) Microextraction using packed sorbent (MEPS)

Another microextraction technique developed in the last decade is microextraction by packed sorbent (MEPS). This novel technique is based on the same general principle of solid phase extraction (SPE), but with MEPS the packing is integrated directly into the syringe, in a very small barrel (BIN) which sets up the needle assembly of an HPLC syringe and not in a separate SPE cartridge. Figure 6 demonstrates the instrument of MEPS.

The sorbents that are used in MEPS are usually the same as conventional SPE columns. Most of the applied sorbents include silica-based sorbents (C2, C8 and C18). When the biological sample passes through the solid support of the syringe, the analytes are adsorbed onto the sorbent which is packed in the BIN. Because MEPS and SPE build on the same principles there is the option of transferring a method from conventional SPE to MEPS relatively straight forward. MEPS holds the high selectivity, the good sample purifying efficiency and extraction yields of SPE. Compared to traditional sample preparation techniques like LLE and SPE, MEPS procedure is faster, simpler, cheaper, more feasible, more environmental friendly, more user-friendly and uses both small amounts of biological sample (10 µL of plasma, urine or water) and large volumes (1000 µL). In general, MEPS can reduce sample volume and time necessary for the analysis. Moreover it can be fully automated and it can be connected to liquid chromatography (LC), gas chromatography (GC) or capillary electrochromatography (CEC). The most important factors in MEPS performance, which should be optimized before the sample analysis are conditioning, loading, washing and eluting solvents, sample flow rate, washing solution and the type and volume of the elution, which should be suitable for injection into LC or GC systems. Also, the volume of the sample should be optimized leading to the best equilibrium between a good analytical performance and a good extraction methodology. The optimum conditions will be contingent to the nature of the matrix being used and the retention capacity and specificity of the sorbent in order to obtain the highest recovery of the analytes. To date, there are several MEPS methods that have been developed for the determination of antipsychotic drugs in biological matrices prior to liquid chromatography analysis. In 2010 Saracino et al. [25] aimed to develop an analytical method for the determination of an atypical antipschycotic drug; risperidone and its main active metabolite 9-hydroxyrisperidone in human plasma and saliva based on HPLC with coulometric detection and an innovative MEPS procedure. Those two analytes were also studied with a SPME procedure prior to HPLC-MS/MS analysis. The microextraction procedure was carried out using a BIN containing 4 mg of solid-phase material silica-C8, after being activated with 100 µL of methanol for three times and conditioning with 100 µL of water for another three times, at a flow rate of 20 µL/s. For the extraction of the analytes, the samples were drawn up and down through the syringe 15 times (at a flow rate of 5 µL/s) without discarding and a washing step once with water (100 µL) and once with a mixture of water and methanol (95:5, v/v) took place, in order to remove biological interference from the samples. Then, the analytes were eluted with 250 µL of methanol and they were subsequently separated on a reversed phase C18 column, using a mobile phase composed of acetonitrile (26%) and a pH 6.5 phosphate buffer (74%).After extraction, the sorbent was cleaned similarly to the activating and conditioning step in order to decrease memory effects and to condition for the next extraction. The same sorbent was used for about 50 extractions. The limit of quantitation for the two compounds was 0.5 ng/mL, while the limit of detection was 0.17 ng/mL. Extraction yields were higher than 90.1% and intra-day and inter-day precision results were good. As a result, this method was successfully applied to real saliva and blood samples from patients. For the determination of the same analytes in plasma, urine and saliva, there is also a more recent MEPS-HPLC-UV method. The column which was used was a Chromsep C8 reversed-phase (150 × 4.6 mm i.d., 5 µm) and the mobile phase consisted of a mixture of acetonitrile (27%, v/v) and a pH 3.0, 30 mM phosphate buffer containing 0.23% (v/v) triethylamine (73%, v/v) with a gradient elution program. The UV detector was set at 238 nm and diphenhydramine was used as the internal standard. The C8 MEPS cartridges were activated and conditioned with 300 µL of methanol and then with 300 µL of water. For the loading step the samples were drawn into the syringe and discharged back 10 times. The cartridge was then washed with 200 µL of water and then with 200 µL of a water/methanol mixture. For the elution step, 500 µL of methanol were used. The eluate was dried and redissolved in mobile phase and 50 µL of the solution was injected into the HPLC system. For the MEPS procedure, extraction efficiencies were higher than 90%, while relative standard deviation (RSD) for precision was always lower than 7.9% for the two compounds. In the biological samples limits of quantification were lower than 4 ng/mL for risperidone and lower than 6 ng/mL for 9-hydroxyrisperidone. Finally, the developed method was successfully applied to the analysis of biological samples from patients and seems suitable for therapeutic drug monitoring. In 2014, Mercolini et al. [26] developed an HPLC method for the determination of a recent atypical antipsychotic; ziprasidone in plasma samples, using MEPS procedure. The analytes were separated on a RP C18 column, with a mobile phase which was a mixture of acetonitrile (30%, v/v) and a pH 2.5, 50 mM phosphate

buffer containing 0.2% (v/v) diethylamine (70%, v/v) that was delivered isocratically and the detection was performed at 320 nm. For the microextration, the C2 sorbent which was chosen was conditioned with 200 µL of methanol and equilibrated with 200 µL of water. The sample was loaded and discarded back 10 times. Washing of the sorbent took place with 100 µL of water and 100 µL of a water/methanol mixture (90/10, v/v). Finally, the elution was done by drawing and discharging 500 µL of methanol. The eluate was dried under vacuum, redissolved in 100 µL of mobile4 phase and injected in the HPLC-UV system. Extraction yields were higher than 90% while limit of quantitation was 1 ng/mL. The sensitivity and the selectivity of the method was also good. The developed method was compared to a SPE procedure, using C2 cartridges and the results were satisfactory. As a result, this procedure was successfully applied to real plasma samples from patients who were using ziprasidone and can be used for therapeutic drug monitoring of patients undergoing treatment with ziprasidone. In 2015, Souza et al. [27] synthesized hybrid silica monoliths which were functionalized with aminopropyl- or cyanopropyl-groups by sol-gel process and used the mass selective stationary phase for MEPS to determine five antipsychotics, namely: olanzapine, quetiapine, clozapine, haloperidol and chlorpromazine) simultaneously with seven antidepressants, two anti-convulsants and two anxiolytics in plasma using UPLC-MS/MS. Due to the higher selectivity of the cyanopropyl hybrid silica for most of the drugs and its good mechanical strength, it was finally selected as the stationary MEPS phase. For the MEPS procedure, the stationary phase was conditioned with 4 × 200 µL of a methanol and acetonitrile mixture (50:50 v/v) and 4 × 200 µL of water. Then, 4 × 100 µL of plasma samples diluted with ammonium acetate solution (pH 10) was manually drawn. Then, the sorbent was washed with 150 µL of water and desorption took place using 100 µL of a 50:50 (v/v) mixture of methanol and acetonitrile. The extract was dried, and reconstituted with 50 µL of the mobile phase, which consisted of ammonium acetate solution 5 mmol/L (with 0.1% formic acid) and acetonitrile and then injected into a XSelects CSH C18 (2.5 µm, 2.1 × 100 mm) column for analysis with liquid chromatography. The linearity of the method ranged from 0.05 to 1.00ng/mL (limit of quantification) to 40–10,500 ng/mL. The absolute recoveries, the precision and the accuracy were good, so the developed method can be applied to the therapeutic drug monitoring of patients. Clozapine and its metabolites were also determined in dried blood spots on filter paper with a HPLC method coupled with a coulometric detection, after being extracted with phosphate buffer and cleaned-up with MEPS procedure. The use of this matrix has many advantages because it eliminates the blood withdrawal, it has low cost and low biohazard risk and it is easy to use and to store. For the microextraction procedure, the sorbent which was 4 mg of solid phase silica-C8 material, inserted into a syringe was activated using 3 × 100 µL of methanol and subsequently conditioned with 3 × 100 µL of water. For the clean-up 10 × 150 µL of the extract from DBS was drawn up and down, followed by a washing step first with 100 µL of water and second with a mixture of water and methanol (95:5, v/v). The elution step took place using 150 µL of the mobile phase and the liquid was injected into the HPLC system. For the HPLC analysis a reversed phase C18 column was used with a mobile phase composed of methanol, acetonitrile and phosphate buffer. All MEPS steps namely: activation, loading, washing and elution were carried out in manual mode. The extraction yields were higher than 90%, the method validation gave satisfactory results for accuracy, precision, sensitivity and selectivity. The developed method was successfully applied to real samples obtained from patients. Therefore, this developed method is suitable for therapeutic drug monitoring for patients undergoing treatment with clozapine.

2.2 LC-MS Method

1) LC separation

All antipsychotics possess hydrophobic properties and as such, all currently published methods for the detection and quantification of antipsychotics in biological matrices have employed reversed phase (RP) stationary phases, with mostly silica-based packings containing C8 and C18 chains. Cabovska et al. [28] and de Meulder et al. [29] used chiral columns in order to separate the (+) and (-) enantiomers of 9OH RIS. 9OH RIS is the main metabolite of the atypical antipsychotic RIS and has shown to be almost equipotent to risperidone in animal studies. Due to its efficacy, racemic 9OH RIS (paliperidone) is also marketed as a drug in its own right. The separation of the two enantiomers is useful for kinetic studies, as the formation of the (+)-form appears to be catalyzed by CYP2D6, whereas CYP3A4 and CYP3A5 are essential for the formation of the (+) - form [30]. The separation of these enantiomers is usually not essential in routine drug analysis. Columns packed with particle size were less significant than initially proposed. The column particle size appeared to make only a modest difference in the peak height, peak width, or resolution, with the difference for each parameter being less than a factor of 2. Higher flow rates distinctively increased peak height by 6–7 folds and the peak width decreased by about 3 fold when using the faster flow rate. In a post-mortem environment, larger particle sizes (3–5 mm) have proven to be favourable due to the higher robustness which is required for more complex matrices such as whole blood. The presented methods show a wide range of isocratic and gradient elutions, including various aqueous and organic elution solvents.

2) MS detection

Ionization of compounds in LC-MS technology is usually achieved with either electrospray ionization (ESI) or atmospheric-pressure chemical ionization (APCI). The reason ESI is used in the majority of presented methods for the detection of antipsychotics is likely to be associated with the higher sensitivity achieved by ESI. Bhatt et al. compared ESI with APCI, prior to development of their method for the detection of RIS and 9OH RIS in plasma. They found APCI to be less favourable when compared with ESI [31]. The higher sensitivity achieved by ESI, however, was at the expense of lower selectivity. Many authors have found matrix effects to be more prominent when applying ESI [32]. Ionization efficient neutral compounds including matrix particles, co-eluting compounds, or additives such as salts in biological samples, can compete with analytes during the evaporation process. This is likely to lower the ionization rate of the compounds of interest. It is further suggested that

during the evaporation process, the analyte of interest may precipitate from solution by itself or as a co-precipitate with non-volatile sample components. This highlights the need for thorough sample clean-up prior to MS analysis and the assessment of matrix-effects as a crucial part of method validation. Due to the predominantly basic properties of antipsychotics, ionization takes place in the positive mode. The vast majority of published methods apply selected reaction monitoring (SRM) as an easy way for the detection and quantification of antipsychotics. International guidelines [33] require a minimum of two SRM transitions for reliable identification of an analyte – unfortunately a large component of SRM methods do not comply with this rule. The best example of possible misidentification of a compound due to monitoring a single SRM transition is the structurally similar Odesmethyl metabolite of the antidepressant venlafaxine and the synthetic opioid tramadol. Due to their almost identical chemical structure, they do not only elute at the same time but also share the most abundant transition (m/z 264.2:58.2) [34]. Less common examples in the field of antipsychotics include the structural isomers promazine and promethazine. These drugs share the most abundant transition (m/z 285:86), representing the cleavage of the side chain and also elute at the same time. The isobaric compounds pipamperone and haloperidol share the two most abundant transitions (m/z 376.2:123 and m/z 376.0:165) [35]. If sensitivity can still be maintained, it is recommended to pick a transition with a smaller abundance for one of the two analytes or, alternatively, add a third transition in order to guarantee reliable differentiation. While MS in the SRM mode certainly provides an efficient tool for compound identification, these examples highlight the need to critically evaluate parameters (such as most abundant transitions) provided by the instrument during compound optimization.

Internal standard

A variety of internal standards (IS) have been used in the reviewed methods. Preferred internal standards are deuterated compounds of the drug class of interest, such as clozapine-d3, haloperidol-d4, olanzapine-d3, quetiapine-d8, and ziprasidone-d8. If these IS are unavailable to a laboratory, it is recommended to use a deuterated IS from a different drug-class rather than an antipsychotic that is in therapeutic use. To the contrary, it has been suggested that high concentrations of a drug can influence the peak areas of their coinjected deuterated analogues when using APCI mode with isotope peaks (M + 1 to M + 3) of analytes contributing to the peak area of the IS. This can lead to miscalculation of the IS concentration and subsequently underestimation of the drugs of interest. However, for masses (M + 5) and higher, no isotopic contribution was observed. As co-medication and therapeutic use of a compound can never be fully excluded, overestimation of an IS is likely to result in underestimation of a drug concentration. Swart et al. [36] did not achieve good results in their detection method for fluspirilene in human plasma when using dimethothiazine as an IS. Their decision not to use an IS at all defies the guidelines of acceptable analytical practice. Particularly in cases where only few analytes are included in a method, a suitable deuterated IS is preferred in all instances. Unfortunately, this is not an isolated event. A large number of analytical methods still use therapeutic drugs as IS.

Selectivity

In order to guarantee selectivity of an analytical method, it would be ideal that all possible interferences arising from matrix compounds, other drugs, and IS, are excluded. As this is impractical, the analysis of six blank specimens from different sources is widely considered acceptable and is applied by most authors. The testing of 10 blank specimens, however, has been employed by some authors and is encouraged for improved selectivity. Josefsson et al. [37] performed method validation in accordance with international guidelines in their method for the detection of OLZ and N-desmethyl OLZ in CSF; however, selectivity of the method was not investigated. This is surprising, as despite the more invasive nature of sample collection compared with taking blood, the authors obtained drug-free CSF samples from six different patients. Several authors do not state clearly how many different sources of blank specimens were tested for interferences. Klose Nielsen et al. [38] examined the interferences from other possible drugs in forensic samples by spiking blank blood samples with 66 common drugs such as benzodiazepines, analgesics, antidepressants, antipsychotics, b-blockers, narcotics and stimulants. Two ‘zero’ samples (blank sample containing IS) should be included in validation experiments in order to exclude possible interferences of the IS on the selectivity of the method.

Calibration

Linearity is an important part of method validation whenever quantification of analytes via a standard curve is carried out, which is the case in the vast majority of all published methods. An alternative is presented by Rittner et al. [39] in their method for the detection of 70 psychoactive drugs, where they semi-quantify several analytes using the method of standard addition. Peters et al. [40] comprehensively summarized the requirements for an adequate calibration model in their review (which is beyond the scope of this paper). The calibration range should cover at least the therapeutic range of the drug of interest; however, as long as linearity can be assured, a greater range can be included. Arinobu et al. [41] include 14 calibrators in order to cover the wide calibration range of 1 ng/ml–800 ng/ml for the detection of haloperidol and its metabolites in plasma and urine, measuring 10 replicates per calibrator. Moody et al. [42] could not guarantee linearity of calibration curves in their method targeting RIS and 9OH RIS when using ESI. As the calibration curves started to plateau above 10 ng/ml when using ESI, APCI was used to continue the method validation. The plateau could be caused by saturation of the detector. This is, however, unlikely as the concentrations injected are not very high with the highest calibrator at 25 ng/ml. Furthermore, the problem of the plateau does not exist in APCI mode, confirming that detector saturation is not the reason. A more likely cause is a saturation of the droplets during the ionization process; a problem not occurring in APCI mode as the ionization of compounds takes place in the gas-phase.

2.3 Stability

a) Processed sample stability

Prior to progressing to further validation experiments, the stability of the drugs of interest in processed samples must be verified. Extracted samples should not be stored longer than the stability in processed samples has been tested and assured; 24 hr is the most commonly investigated timeframe as runtimes are unlikely to exceed one day. Nevertheless, it can be useful to obtain stability information for a longer period of time in cases where instrument issues may cause samples to be re-run on the next day. There are three ways the result can be reported. Either as a percentage loss over a defined timeframe (given as the mean with SD); a comparison between the initial drug concentration and the concentration after storage using a paired t-test; [40] or as more frequent injections over the investigated timeframe, a curve is generated and (after regression analysis) a negative slope significantly different from zero ($p < 0.05$) indicates instability. Kratzsch et al.[43] accurately plotted absolute peak areas as opposed to relative peak areas against the time of injection, in order to prevent the IS from correcting for eventual losses. Some authors followed the recommendations of testing two concentrations (one low and one high of the calibration range), whereas others improved on this by including an additional concentration. Josefsson et al. investigated processed sample stability and found sample extracts to be unstable over 24 h, with significant losses for both OLZ and N-desmethyl OLZ. This outcome is not surprising as significant stability issues in processed samples containing OLZ have been reported in other matrices such as whole blood. If processed sample stability is not guaranteed over 24 h, it is recommended that analysis is completed prior to degradation of OLZ taking place.

b) Freeze-thaw stability

Assuring that multiple cycles of freezing and thawing do not compromise the integrity of tested samples is crucial in routine toxicological analysis. A blood sample is likely to be tested for different groups of analytes and therefore be thawed and frozen again several times. Experimental factors should be selected based on the conditions that are intended to be used on real cases, i.e. the temperature at which routine samples are being stored should be the temperature applied in the freeze-thaw (F/T) experiments. Shah et al. recommended the testing of at least three F/T cycles and two concentration levels in triplicate. [6,8] While there are variations in the number of concentration levels and F/T cycles tested by some authors, it is most concerning that there is still a large number of methods where no F/T stability experiments were conducted at all [44].

3. Conclusion

Currently, there are more than 35 different antipsychotics available worldwide for the treatment of a range of psychotic illnesses. Over the past 15 years, recent advances in LC-MS technology has enabled the detection and quantification of these drugs in exceptionally low concentrations; the newer generation antipsychotics in particular. This has led to the development of numerous LC-MS methods for the analysis of antipsychotics in human biological specimens. Even in cases where simple biological matrices are involved, sample pretreatment cannot be avoided. Based on their useful benefits, microextraction techniques in the extraction and pre-concentration of various antipsychotic drugs in different biological matrices are growing. With the use of novel procedures, the main disadvantages of traditional sample preparation techniques such as LLE, SPE and protein precipitation can be overcome. At the same time, microextraction techniques are compatible with green chemistry, which is nowadays a trend in analytical chemistry. It also agrees with simplification and miniaturization which are trends gaining more and more interest day by day. Thus, more innovative methods can be developed, for the determination of a greater variety of typical and atypical antipsychotics in different biological samples. A requirement for the success of such detection methods is that they are suitably sensitive to cover the low therapeutic range in which antipsychotics are usually present. However, the quality of published methods with regard to validation criteria is not always consistent. The most significant issues relate to the evaluation of selectivity, linearity, and stability. Addressing these issues in future analytical studies is mandatory to accurately detect antipsychotics in biological specimens and, consequently, to better understand this increasingly prevalent class of drugs.

4. Acknowledgements

The authors are thankful to Dr. L. Rathaiah, Chairman, Vignan group of Institutions for providing the facilities to carry the review work.

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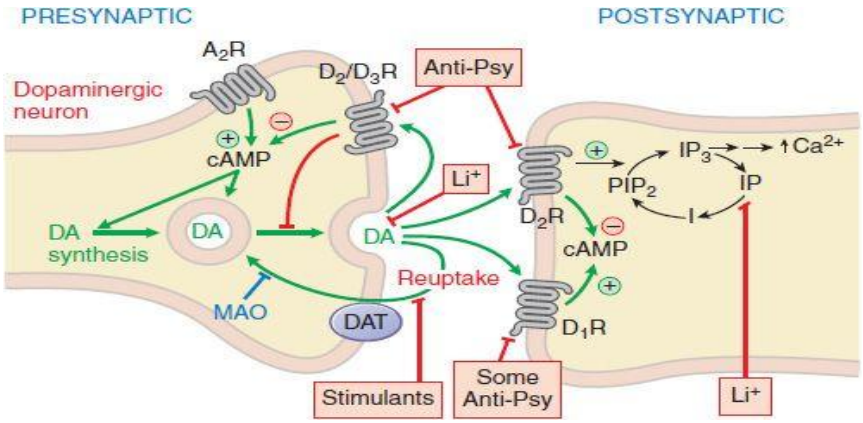

Figure 1: Mechanism of action of anti-psychotics

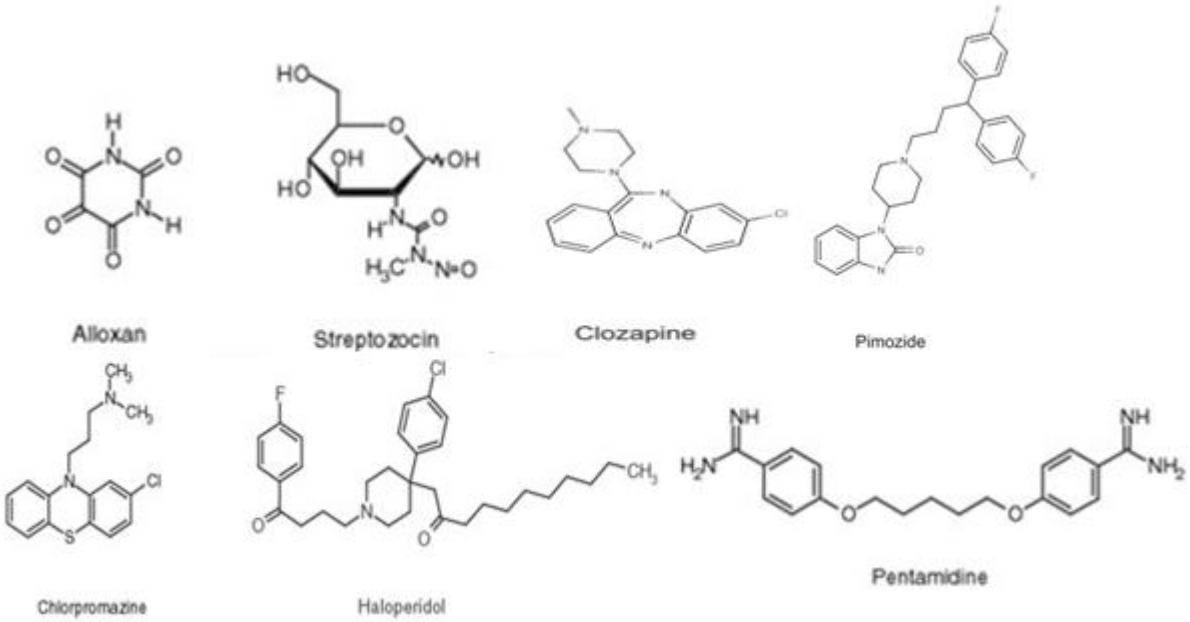

Figure 2: Chemical structures of common antipsychotic drugs

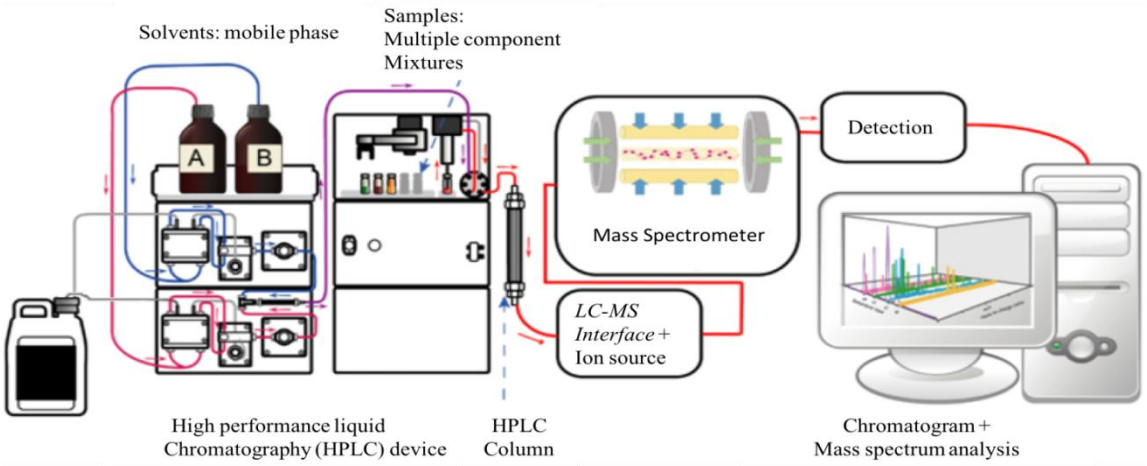

Figure 3: Instrumentation Diagram of LC-MS

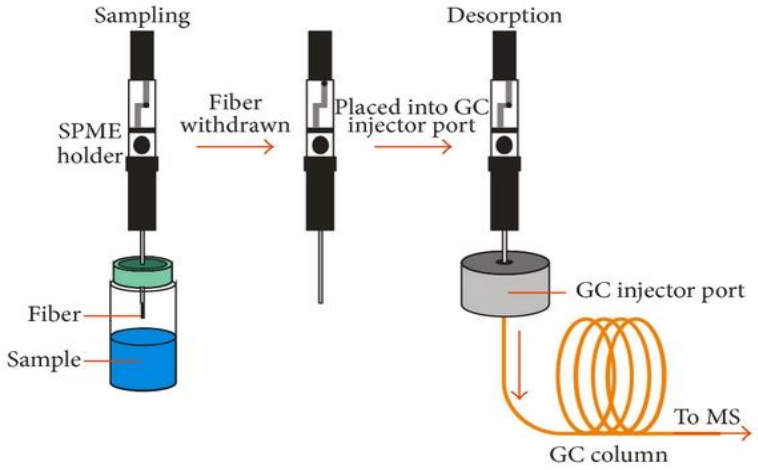

Figure 4: SPME

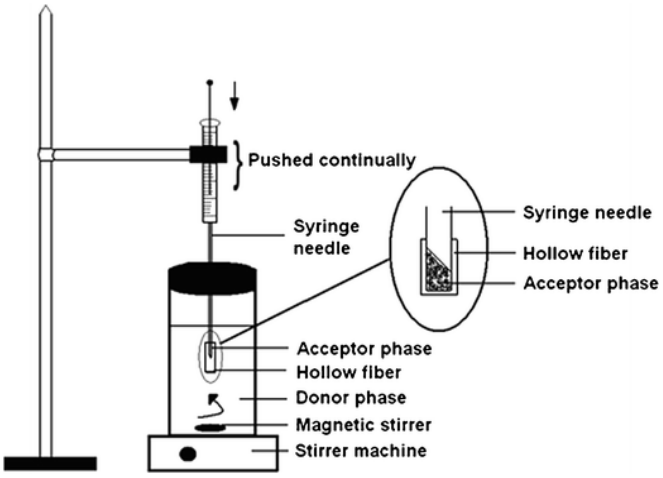

Figure 5: LPME

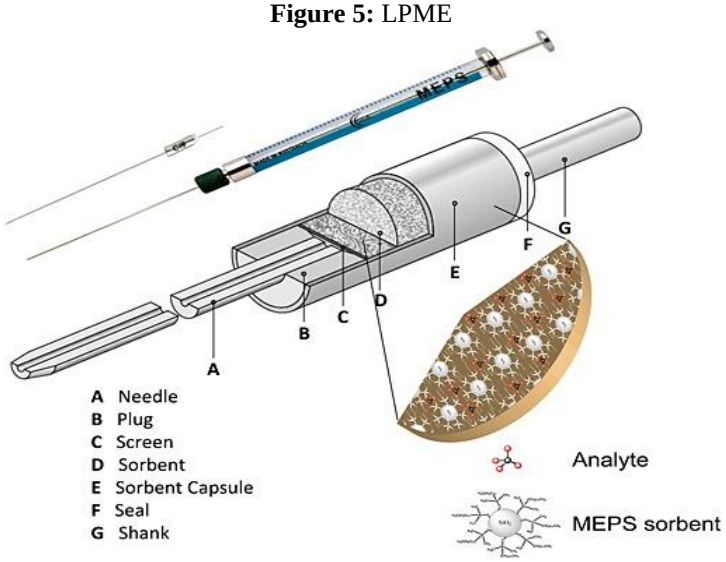

Figure 6: MEPS

Table 1: Pharmacokinetic parameters of common antipsychotic drugs

Drug	Common daily oral dose range in adults (mg)	Blood conc expected following therapeutic use (ng/ml)	$t_{1/2}$ (h)	V_D(L/Kg
9OH-Risperidone	3-12	10-10	23	N/A
Amisulpride	400-1200	50-400	11-27	13-16
Aripiprazole	10-30	50-350	60-90	4.9
Bromperidol	1-15	1-20	15-35	N/A
Buspirone	20-30	1-10	3-12	5-6
Chlorpromazine	200-600	30-300	7-119	10-35
Chlorprothixene	40-80	20-200	8-12	11-23
Clozapine	300-450	200-800	6-17	2-7
Flupentixol	3-6	1-15	19-39	14.1
Fluphenazine	1-5	2-20	13-58	220
Fluspirilene	2-5 (i.m)	N/A	21 days	N/A
Haloperidol	1-15	5-50	18	18-30
Levomepromazine	25-50	15-60	15-30	30
Loxapine	20-100	10-100	3-4	N/A
Melperone	100-400	5-40	2-4	7-10
Mesoridazine	100-400	15-100	2-9	3-6
Molindone	50-100	~500	1.2-2.8	3-6
Olanzapine	5-20	10-100	21-54	10-20
Penfluridol	20-60 (once per week)	4-25	70	N/A
Perazine	50-600	100-230	8-15	N/A
Perphenazine	12-24	0.6-2.4	8-12	10-35
Pimozide	7-10	15-20	28-214	11-62
Pipamperone	80-120	100-400	12-30	N/A
Prochlorperazine	15-40	10-500	14-27	13-32
Promazine	200-800	10-400	7-17	27-42
Quetiapine	300-4	70-170	6-7	8-12

Risperidone	2-6	10-100	3-20	0.7-2.1
Sulpiride	4-600	50-400	4-11	2.7
Thioridazine	150-300	200-2000	26-36	18
Thiothixene	6-30	N/A	12-36	N/A
Trifluoperazine	15-20	1-50	7-18	N/A
Trifluprom azine	165-37	30-100	N/A	N/A
Ziprasidone	40-160	50-120	2-8	1.5-2.3
Zotepine	75-300	5-300	12-30	50-168
Zuclopenthixol	20-50	5-100	12-28	15-20