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Screening and quantification of antipsychotic drugs in human brain tissue by liquid chromatography–tandem mass spectrometry: Application to postmortem diagnostics of forensic interest

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ABSTRACT

A quantitative LC–MS/MS method has been developed for the simultaneous determination of 17 antipsychotic drugs in human postmortem brain tissue. Sample preparation was performed using Hybrid Solid Phase Extraction-Precipitation technology for the removal of endogenous protein and phospholipid interferences. The chromatographic separation was performed for 16 min on a C8 column, which used a gradient elution of formate ammonium and acetonitrile, and a flow rate gradient. Triple quadrupole mass spectrometry was employed to generate tandem mass spectrometric (MS/MS) data of the target analytes to select the ion *m/z* signals. Quantitation of the analytes was performed by operating in the dynamic multiple reaction monitoring (dMRM) mode using an electrospray ionization interface. Calibration curves prepared in the spiked brain tissue were linear in the range 20–8000 ng/g ($r^2 > 0.993$) for all drugs (except olanzapine). Within- and between-day coefficients of variation were lower than 25% for all drugs at the LOQ. The LOQ in the matrix ranged between 2 ng/g and 80 ng/g. The method was successfully applied to the unequivocal identification and accurate quantification of antipsychotic drugs in human postmortem brain tissues: therefore, this method can be used in forensic investigations.

1. Introduction

Schizophrenia is a severe psychiatric disorder affecting approximately 1.5% of the world's population [1]. The pharmacological treatment of schizophrenia is often performed with the simultaneous use of two or more antipsychotic agents to achieve the desired control of psychotic symptoms. Antipsychotic drugs are often associated with sudden death investigation. Detection of these drugs is necessary to establish their use and possible contribution to the cause of death. According to the World Health Organisation (WHO), a suicide occurs every 40 s, and it is the leading cause of violent deaths worldwide, ahead of deaths resulting from both war and homicides. The high rate of suicide in patients affected by psychiatric disorders has provoked clinical attention to this issue. A article published [2] on the suicide rate among schizophrenia patients treated at the same hospital in North Wales (UK) both before being treated with psychotropic drugs and after treatment (in and out of the hospital) with neuroleptics and/or atypical antipsychotics, found a 20-fold increase in the suicide rate since the use of psychotropic drugs.

The pharmacological and toxicological information obtained from the drug distribution in tissues and cells is important for understanding and predicting both drug reaction and toxicity [3,4]. Oral doses of antipsychotics for the treatment of schizophrenia patients are on the order of a few milligrams per day. Antipsychotics have been found to appear in plasma during short half-life but show persistence in the brain [5]. During legal medical autopsy human tissues are normally investigated to determine the cause of death, but the results from corresponding toxicology measurements often require further information. Using screening methods drugs were detected that had no connection to the cause of death. In these cases, the deceased had been undergoing a continuous therapeutic treatment, treatment during an operation, or an unsuccessful urgent therapy [6]. The concentration of these drugs in brain samples (ng/g levels) has resulted in the need to develop more sensitive, robust and precise analytical methods. There are several methods based on GC [7,8], and LC methods that have been published for the determination of some of these antipsychotics in brain samples using different detection systems, including chlorpromazine and thioridazine using an electrochemical detector [9];

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aripiprazole [10], haloperidol, olanzapine and clozapine [11] using ultraviolet detection; and olanzapine using coulometric detection [12].

Nevertheless, LC–MS has established itself as the clear leader in the quantitation of antipsychotics in biological samples. There are numerous reports of using LC–MS methods for the determination of some of these compounds in plasma [13,14], serum [15], blood [16,17], urine [18], cerebrospinal fluid [19], hair [20–22] and rat brain tissue [23]. However, to date, LC–MS/MS methods for the simultaneous screening and determination of the 17 most commonly used antipsychotic drugs, have not been developed.

Although many of the studied compounds have weakly actives metabolites [24–27], these were not included in the current study considering that their appearance in brain samples of suicide cases would be of little forensic relevance. In these cases, as discussed below, high concentrations of the parent drug are the determining factors when drawing conclusions. However, some of the compounds studied are matabolites: paliperidone, which is the main metabolite of risperidone and norclozapine, a metabolite of clozapine. The former was studied due to its prescription as an active substance and the latter due its ability to accumulate in the blood at levels similar to the parent drug, which has been extensively described by numerous authors [28].

Currently, rapid methods are needed for both the extraction and the sample clean-up to ensure accurate quantification techniques using LC–MS/MS. For the analysis of drugs in brain tissue, several column phases using SPE methodologies have been employed: C8 [29], silica [30], and C18 [31,32]. The proposed method used the Hybrid Solid Phase Extraction-Precipitation (Hybrid-SPE-PPT) technology. This method uses a simple and generic sample preparation, which is designed for the gross-level removal of endogenous protein and phospholipid interferences from biological samples prior to LC–MS or LC–MS/MS analysis. Excessive background noise from endogenous matrix components has always been a major analytical challenge in quantitative bioanalysis, and reducing the noise has become paramount along with decreasing the analytical run time. In bioanalytical mass spectrometry, the issue of excessive background noise contributes to the growing problem of ion-suppression. The pretreatment step avoids one of the major causes of ion-suppression in bioanalysis, which is the presence of phospholipids, during LC–MS or LC–MS/MS analysis in the positive ion electrospray mode (ESI+). The proposed LC–MS/MS method is able to simultaneously determine 17 antipsychotic drugs in human brain tissue at ng/g levels with a high level of accuracy. The drugs chosen for this study were the most commonly used antipsychotic according to data collected from the European Medicines Agency (EMEA). The identification of the analyte is performed using two *m/z* transitions in the dynamic MRM mode.

2. Material and methods

2.1. Reagents and chemicals

Standards of sulpiride, tiapride, amisulpride, paliperidone, risperidone, quetiapine, norclozapine, clozapine, levomepromazine, zuclopenthixol, chlorpromazine, and thioridazine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Haloperidol, haloperidol-D4 (Internal Standard), olanzapine, and ziprasidone were obtained from Cerilliant (Round Rock, TX, USA). Aripiprazole and sertindol tablets as a solid were donated by the Basque National Health Service (Osakidetza) and provided by Lundbeck (Barcelona, Spain).

The HPLC grade acetonitrile, 2-propanol and methanol were purchased from Scharlau (Barcelona, Spain). Ammonium formate, which was used to prepare a buffer solution (pH 8.2) in water, and formic acid (mass spectrometry grade), which was used in the pretreatment procedure, were purchased from Sigma–Aldrich (Steinheim, Germany). Deionized water was prepared by purifying demineralized water using a Milli-Q water filtration system (Millipore, Milford, MA, USA).

Individual stock solutions containing approximately 1000 mg/L of each analyte were prepared by dissolving solid standards in 2-propanol. The stock solutions were preserved at 4 °C. Mixed stock solutions of five by five analytes were prepared in 2-propanol at 1 mg/L. These solutions were used to obtain the mass spectra and qualitative information of standards.

A stock mixed standard solution of 10 mg/L was prepared monthly in water and preserved by refrigeration. Working solutions were prepared weekly at the required concentration by diluting the stock mixed standard in water.

2.2. Instrumentation

Chromatographic analysis was performed on an Agilent Technologies (Wilmington, DE, USA) 1200 Series HPLC system and 6410 Triple Quad mass spectrometer using MassHunter software and equipped with electrospray ionization (ESI) as the ion source operating in the positive mode. The column used was a ZORBAX (Agilent) Eclipse Plus C8 Narrow Bore (2.1 mm × 150 mm, 5 μm), and the guard column contained the same packing material.

2.3. Sample collection

The study was developed in compliance with policies of research and ethical review boards for postmortem brain studies. Human brain samples (n = 18), from subjects who had died from sudden and violent causes were obtained during autopsy and the deaths were associated with suicide cases certified by the Basque Institute of Legal Medicine (Bilbao, Spain). Samples from the prefrontal cortex (Brodmann's area 9) were dissected at the time of autopsy and immediately stored at −70 °C in the Brain Bank of the UPV/EHU until assay.

2.4. Calibration and real samples preparation

Pooled homogenized blank brain samples (n = 6) were used for the development and validation of the procedure. These samples were obtained from subjects who had died suddenly, usually in car accidents, who did no have a history of neuropathological or psychiatric disorders. The calibration curve samples were prepared by spiking 20 μL of the IS (1 mg/L) and 40 μL of the appropriate standard working solution to obtain antipsychotic drugs with final concentrations in brain of 2–8000 ng/g. Quality control samples (QCs) were prepared by spiking brain samples containing 20, 200 and 5000 ng/g as the final concentration of all analytes. A QC diluted 1:5 (eluate of 5000 ng/g was diluted using mobile phase) was prepared to ensure that a dilution from concentrations above the limits of the calibration curve did not affect sample integrity.

Prior to clean-up, human brain samples were thawed at 4 °C overnight and homogenized using an ultrasonic 450 Digital Sonifier Branson (Danbury, Connecticut, USA). Then, 100 mg of homogenized brain samples were deproteinized by the addition of 600 μL of formic acid in acetonitrile as a protein precipitating agent. The samples were shaken for 5 min and centrifuged at 10,000 rpm and 4 °C for 5 min in a Beckman Coulter Allegra™ X-22R Cryo-Centrifuge (Brea, CA, USA). The supernatants were transferred to 1 mL HybridSPE-Precipitation cartridges (HybridSPE-PPT) from Supelco (Sigma–Aldrich, St. Louis, MO, USA). The eluates were evaporated to dryness under a nitrogen stream at room temperature using a Techne Dri-Block 3D Evaporation System (Staffordshire, UK). The residues were reconstituted in 400 μL of the mobile phase and transferred into an autosampler microvial. Five microliters of the reconstituted eluates were injected into the LC–MS/MS system.

The spiked brain homogenate samples (standards and quality controls) were treated by HybridSPE-PPT for each analytical batch along with postmortem human brain tissue of psychiatric patients. All brain samples were processed in the same manner.

2.5. Chromatographic conditions

The mobile phase used for the chromatographic separation was composed of acetonitrile and aqueous ammonium formate adjusted to pH 8.2 with NH_4OH. Prior to use, the mobile phase was filtered (0.2 μm pore size) and degassed. The flow rate and gradient program were as follows: 30% acetonitrile (eluent B) at a flow rate of 0.4 mL/min; 0.0–9.0 min gradient increase to 50% eluent B and flow rate increase to 0.5 mL/min; 9.0–12.5 min gradient increase to 75% eluent B; 12.5–15 min gradient increase to 90% eluent B; 15–25 min flow rate decrease to 0.4 mL/min; 25–30 min gradient returns to the initial conditions to allow for equilibration. Sample analysis was performed at 30 °C. The autosampler was set to inject 5 μL of the sample aliquots, and the needle was rinsed for 90 s using a flushport with 2-propanol as the eluent.

2.6. Mass spectrometer conditions

Antipsychotics were monitored using a triple quadrupole mass spectrometer equipped with an electrospray ionization interface. For detection and quantification, the following ESI source conditions were applied: nitrogen was the nebulizer and desolvation gas, nebulizer gas flow: 8 L/min; gas temperature: 350 °C, nebulizer pressure: 30 psi and capillary voltage 4000 V. The electrospray ionization was in the positive mode, and the collision gas used was nitrogen.

The fragmentor values were optimized to maximize the intensity of the protonated molecular species $[M+H]^+$ and the collision energy was fixed to maximize the product ions characteristics of each antipsychotic. The mass