VALIDATION OF A METHOD TO DETECT CENTRAL NERVOUS SYSTEM ACTING DRUGS IN BLOOD BY GC/MS. APPLICATION IN CASES OF DEATH CAUSED BY PHENOTHIAZINES IN THE NORTH OF CHILE

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ABSTRACT

A simple and rapid analytical method is developed and validated for the determination of antiepileptic and antipsychotic drugs in human whole blood using a solid-phase extraction and quantification by gas chromatography–mass spectrometry (GC/MS). Prazepam was used as internal standard (IS). The specificity, linearity, intra- and inter-assay precision and accuracy, and extraction recovery were fully evaluated. The limits of detection and quantification were in the range of 0.52 - 0.98 µg/mL and 1.56 - 2.97 µg/mL for antiepileptic drugs (pentobarbital, phenobarbital and carbamazepine) and 1.31 - 2.94 ng/mL and 3.98 - 8.89 ng/mL for antipsychotic drugs (chlorpromazine and thioridazine), respectively. The relative standard deviation (RSD) was less than 7.07%, while the intra-day accuracy was < 3.21% and the inter-day was < 8.80%, referred to RSD. The developed methodology was applied in forensic cases of death caused by phenothiazines in the North of Chile during 2010-2012.

Keywords: antiepileptic and antipsychotics drugs, gas chromatography, mass selective detector, solid-phase extraction

INTRODUCTION

Drugs acting in the Central Nervous System (CNS) like antipsychotic and antiepileptic drugs are currently used to treat diseases such as schizophrenia and epilepsy. This latter disease often coexists with psychiatric illness, including depression.^{1,2}

Many medications used in epilepsy have adverse effects, such as drowsiness, fatigue, dizziness, unsteadiness, blurred or double vision, difficulty concentrating, memory problems, irritability, and depression, which cause discomfort to the patient, and may jeopardize compliance.³ Regarding first- and second-generation antipsychotic drugs, large epidemiologic studies have reported that they are associated with increased risk of sudden cardiac death, but the absolute risk of death remains quite low.⁴

In this context, pharmacological treatment of the different forms of epilepsy and psychiatric disorders and the therapeutic drug monitoring is a well-established procedure, which helps to maximize the effectiveness of antiepileptic and antipsychotic therapy, increasing clinical efficacy while minimizing adverse effects.^{3,5,6} This fact has led to the development of reliable analytical methods for their analysis.⁷⁻¹⁰

One of the major goals of the Laboratorio Referencial Norte del Servicio Médico Legal, Iquique is to develop efficient and readily accessible chromatographic methods to detect drugs of abuse.¹¹⁻¹⁴ The aim of this study was, therefore, to establish a screening method for antiepileptic and antipsychotic drugs use. The target drugs include pentobarbital, phenobarbital and carbamazepine as antiepileptic drugs; and chlorpromazine and thioridazine as antipsychotic drugs (Figure 1). The present method was developed in human whole blood covering all therapeutic and toxic drug levels, and it was further applied to evaluate forensic cases of death caused by phenothiazine-like drugs as chlorpromazine and thioridazine in the North of Chile during 2010-2012.

MATERIALS AND METHODS

Chemicals and reagents

Pentobarbital (PTB), phenobarbital (PHB), carbamazepine (CBZ), chlorpromazine (CPZ), thioridazine (TDZ) and prazepam (PRZ), as internal standard (IS), were purchased from Cerilliant (TX, USA, > 99% purity grade). Ethyl acetate, acetonitrile, ammonia, and potassium hydroxide were purchased from Merck (Germany). Methanol (HPLC grade) was purchased from Sigma-Aldrich (USA). Potassium dihydrogen phosphate (analytical reagent grade) and hydrochloric acid were purchased from Riedel-de Haën (Germany).

Biological samples

Samples of whole blood from persons without story of drug consumption

and not receiving a pharmacological treatment were obtained from the blood bank of "Dr. Ernesto Torres Galdames" Iquique Hospital, and stored at -30 °C until analysis. According to the selected drugs, whole blood samples were further classified into two groups: the first one containing the antiepileptic drugs (pentobarbital, phenobarbital and carbamazepine) and the second one that contains the antipsychotic drugs (chlorpromazine and thioridazine).

Figure 1. Structures of antiepileptic and antipsychotic drugs under study: pentobarbital (PTB), phenobarbital (PHB), carbamazepine (CBZ), chlorpromazine (CPZ) and thioridazine (TDZ).

Sample preparation

Human whole blood (1 mL) was shaken for 1 min and then homogenized thoroughly. To the homogenate, it was added to the antiepileptic drugs 100 µL of a 10 ng/mL solution of prazepam (IS), and 4 mL of bidistilled water; while to the antipsychotic drugs it was added 80 µL of a 5 ng/mL solution of prazepam. Then, 2 mL of 0.100 mol/L pH 6.0 phosphate buffer was added to the antiepileptic and antipsychotic drugs. The sample solutions were vortexed for 1 min, sonicated at room temperature for 30 min and centrifuged at 3500 rpm for 10 min and 4000 rpm for 15 min for antiepileptic and antipsychotic drugs respectively. The clean supernatants corresponding to samples were placed in the extraction column.

The concentration of the analytes was calculated using calibration curves from of spiked human whole blood sample. Linear regression lines were obtained by plotting the peak area ratios (the compound's peak area divided by that of the internal standard)

Solid-phase extraction (SPE)

The SPE was performed using an Oasis® HLB column. The SPE cartridges were preconditioned with 2 mL of methanol, 2 mL of bidistilled water and 2 mL of 0.100 mol/L pH 6.0 phosphate buffer, all under vacuum (less than 3 mm Hg). The prepared samples (containing either antiepileptic or antipsychotic drugs) were then applied and allowed to pass through the column at a rate of 1 to 1.5 mL/min. The sorbent was washed with 2 mL of methanol of a 5% in bidistilled water solution and 3 mL of methanol:water:ammonia (58:48:2) in the case of antiepileptic and antipsychotic drugs, respectively. To completely dry the column, the vacuum was maintained at 10 mm Hg for 10 min. Finally, the antiepileptic and antipsychotic drugs were eluted with 2 mL and 3 mL of methanol respectively. The solvent was evaporated under a gentle stream of nitrogen and the residue of antiepileptic drugs were reconstituted with 100 µL of ethyl acetate, while antipsychotic drugs were reconstituted with 100 µL of acetonitrile.

GC/MS Analysis

Chromatographic analysis was carried out on an Agilent 6890N series system (Agilent, USA - Weisser Analytical) equipped with a 7683 series Automatic Sampler linked with injector programmed temperature volatilization (PTV) and DB-5MS capillary columns (50 m x 0.22 mm, 0.33 µm film thickness). The injection volume was 5 µL in solvent vent mode. A selective mass detector together with a Chemstation software suite (Agilent, USA-Weisser Analytical) version A.09 was used for data processing and instrument control. The temperature of the PTV injector in solvent vent mode was set at 50 °C and the flow rate was kept at 1.2 mL/min using helium as carrier gas. The oven temperature was programmed as follows: the initial temperature was set at 50 °C, held for 3.5 min, and ramped at 36 °C/min to 175 °C, where it was held for 1 min and ramped at 18 °C/min to 260 °C and held for 1.0 min, and again ramped at 6 °C/min to 315 °C and held for 3.5 min. The GC-MS analysis for identification of antiepileptic and antipsychotic drugs were carried out in a gas chromatograph equipped with an Agilent 5975 mass selective detector operated in electron impact mode (Agilent USA- Weisser Analytical). The temperatures of the quadrupole, ion source, and mass selective detector interface were 150, 230 and 300 °C, respectively.

Validation of the method

The validation of the method was performed by calculating the following parameters: recovery value, linearity, intra- and inter-assay precision, limits of detection and quantification. The assessment of both intra- and inter-assay precisions were daily performed by using ten replicates.

RESULTS AND DISCUSSION

This work shows the development of a GC/MS analytical procedure for antiepileptic drugs (pentobarbital, phenobarbital and carbamazepine) and antipsychotic drugs (chlorpromazine and thioridazine) as a detection method in human whole blood. The optimization of this method was first carried out using prazepam as IS and then progressed to using a blank human whole blood sample spiked with each of the analytes. This experimental approach offers several advantages. One of the main advantages is the ability to maximize the information in cases with small sample availability as well as the ability to correlate the concentrations found in human whole blood with the clinical symptoms of the exposed individual. In addition, it allows the discrimination whether these concentrations are connected to the cause of death.

Initially, we evaluated the solid-phase extraction method, and confirmed the usefulness of the Oasis® HLB columns for extraction of antiepileptic and antipsychotic drugs from human whole blood.

Linearity data, as well as limit of detection (LOD) and limit of quantitation (LOQ) values, are reported in Table 1 for antiepileptic and antipsychotic drugs. The calibration curves showed good linear response ($r^2 > 0.988$) for human whole blood for all analytes. The ranges of linearity of the studied drugs were satisfactory with regard to the therapeutic range for forensic and clinical purposes.

The LOD values were calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve(s) at levels approximating the LOD according to the formula: $LOD = 3.3(SD/S)$. The LOD for antiepileptic drugs varied between 0.52 and 0.98 µg/mL and for

chlorpromazine and thioridazine drugs the values were between 1.31 and 2.94 ng/mL, respectively.

Table 1. Linearity, relative standard deviation (RSD), limit of detection (LOD), limit of quantitation (LOQ), and SPE efficiency in human whole blood using GC/MS.

Drugs	Tested range (µg/mL)	(r^2) ^a	RSD (%)	LOD (µg/mL)	LOQ (µg/mL)
Pentobarbital	1 - 20	0.9889	2.21	0.52	1.56
Phenobarbital	1 - 20	0.9902	2.56	0.98	2.97
Carbamazepine	1- 16	0.9966	3.43	0.52	1.58
Chlorpromazine	0.03 -0.8	0.9969	2.16	0.00131	0.00398
Thioridazine	0.1 - 5	0.9960	7.07	0.00294	0.00889

^a r^2 = Square of correlation coefficient with a weighting factor of 1/ concentration.

The limit of quantitation (LOQ) is the lowest concentration that can be measured on the standard curves with acceptable reproducibility. The LOQ values determined for the five drugs tested allowed the measurement of both the subtherapeutic as well as the toxic concentrations of these drugs.

The SPE were determined in triplicate at three concentrations (low, medium and high) of each drug in human whole blood. Table 1 shows the solid-phase extraction efficiency of the antiepileptic and antipsychotic drugs which was in the range of 1.0 – 20 µg/mL and 0.030 – 5.0 µg/mL, respectively. The average recoveries were 90.6% for antiepileptic drugs and 76.5% for antipsychotic drugs in human whole blood as shown in Table 2. The sensitivity showed by our method using GC/MS was similar to that previously reported by using chromatographic methods.¹⁵⁻¹⁶

Table 2. SPE efficiency in human whole blood.

Drugs	Whole Blood SPE efficiency (%)			
	Low*	Medium*	High*	RSD
Pentobarbital	82.1	77.4	72.1	6.5
Phenobarbital	102.1	94.2	99.6	4.1
Carbamazepine	94.5	101.3	92.4	4.9
Chlorpromazine	82.7	76.5	73.8	5.8
Thioridazine	80.2	74.8	71.2	6.0

RSD = relative standard deviation
*n=3

Table 3 shows the results obtained for intra-assay and inter-assay precision calculations and selected ions used for qualification and quantification of all the analytes. Inter-day and intra-day precision were < 9.0% and <3.5% for all analytes. It should be underlined that the precision of the analytes under investigation at reported concentrations met satisfactorily the internationally established acceptance criteria.¹⁷

Table 3. Intra- and inter-day precision and selected ions for pentobarbital, phenobarbital, carbamazepine, chlorpromazine and thioridazine.

Drugs	Inter-day precision*	Intra-day precision*	Selected ions (m/z)
	RSD (%)	RSD (%)	
Pentobarbital	2.48	2.24	<u>156</u> , 141, 197
Phenobarbital	5.80	3.19	<u>204</u> , 232, 117
Carbamazepine	2.19	1.35	<u>193</u> , 236, 165
Chlorpromazine	8.80	3.21	<u>318</u> , 58
Thioridazine	4.17	1.83	<u>245</u> , 198, 230

*n=10

The underlined ions were used for quantitation.

Representative chromatograms obtained by GC/MS following the extraction of pentobarbital, phenobarbital, carbamazepine, chlorpromazine, thioridazine and the IS prazepam are shown in Fig. 2. Using this method, all of the analytes of interest were resolved in 17 min for antiepileptic drugs and in 22.5 min for antipsychotic drugs. The authenticated standard samples of pentobarbital, phenobarbital, carbamazepine, chlorpromazine, thioridazine and prazepam were eluted at retention times of 14.7, 16.7, 17.11, 22.0, 22.4 and 23.5 min, respectively. The total ion chromatogram (TIC) in human whole blood showed good separation for these drugs.

Since the procedure proved to be sensitive, selective and reproducible, the method developed was applied to the fatal case presented in the Table 4. Antipsychotic drugs as thioridazine and chlorpromazine were detected in all the analyzed forensic cases. Toxicological results revealed that thioridazine and chlorpromazine toxic concentrations were present in the cases 1 and 4, respectively; while a venlafaxine lethal toxic concentration was only present in the cases 2 and 3. It is important to note that the all reported cases were 100%

female.

Acute chlorpromazine and thioridazine intoxication occurs as a consequence of intentional or accidental overdose. A publication reports that oral ingestion of 2.0 g of chlorpromazine and 0.90 g of thioridazine is causing seizures, fainting, hypotension, cardiac arrhythmia and coma.¹⁸ Fatal overdose is rare and only a few suicidal cases are available in the published literature.¹⁹⁻²¹

Sertraline, diazepam, nordiazepam and venlafaxine were also present but the literature is scarce about this point and evidence of a potential drug-drug interaction of these drugs with chlorpromazine and thioridazine is still lacking. Although chlorpromazine and thioridazine are usually well tolerated, several authors^{4,6,21} refer that their combined use with other psychotropic compounds may trigger an elevated risk of adverse reactions.

Taking into account the autopsy and toxicological findings, along with the circumstantial evidence, the cause of death was attributed to a nonspecific acute intoxication (case 1, 2 and 3) and to an acute pulmonary edema (case 4).

Figure 2. Chromatographic spectrum of pentobarbital (PTB), phenobarbital (PHB), carbamazepine (CBZ), chlorpromazine (CPZ), thioridazine (TDZ) and prazepam (PRZ) in SIM mode.

Table 4. Forensic cases of death caused by phenothiazines in the North of Chile during 2010-2012.

Case	Gender	Age	Cause of death	Concentration of phenothiazines	Other drugs detected	City	Year
1	Female	68	Non-specific acute intoxication	Thioridazine 2.5 µg/mL	Sertraline (traces) Diazepam (traces) Nordiazepam (traces)	Ovalle	2010
2	Female	41	Non-specific acute intoxication	Thioridazine traces	Venlafaxine (27 µg/mL) Diazepam (traces) Nordiazepam (traces)	Arica	2010
3	Female	49	Non-specific acute intoxication	Chlorpromazine 0.15 µg/mL	Venlafaxine (27.4 µg/mL) Diazepam (traces) Nordiazepam (traces)	Vallenar	2011
4	Female	41	Acute pulmonary edema	Chlorpromazine 0.45 µg/mL	----	Iquique	2012

CONCLUSION

The set up and the validation of a GC/MS method for the analysis of antiepileptic and antipsychotic drugs in whole blood samples has been reported. The use of Oasis® HLB column resulted in numerous advantages including reproducible recoveries, cleaner extracts, sensitivity and reduced solvent consumption for the detection of drugs acting on the Central Nervous System. For both class of drugs we calculated the limits of detection (LOD) and quantification (LOQ). For antiepileptic drugs like pentobarbital, phenobarbital and carbamazepine, they were in the range of 0.52 - 0.98 µg/mL and 1.56 - 2.97 µg/mL, respectively. With regard to antipsychotic drugs such as chlorpromazine and thioridazine, that values were in the range of 1.31 - 2.94 ng/mL and 3.98 - 8.89 ng/mL, respectively. Interestingly, the calculated LOQ values allowed the measurement of both sub-therapeutic as well as toxic levels of these drugs. The main advantage of this method lies on its selectivity and sensitivity for clinical and postmortem toxicological analysis. The current method provides a useful technique to be routinely used in Toxicology Laboratories.

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