PHARMACOKINETIC STUDY OF RISPERIDONE. APPLICATION OF A HPLC METHOD WITH SOLID PHASE EXTRACTION

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ABSTRACT

A new, simplified solid phase extraction procedure for the determination of risperidone and 9-hydroxyrisperidone in human plasma has been developed. This method involves the use of an optimized extraction protocol developed in Waters OASIS® HLB 30mg 1cc extraction columns using 1 mL of human serum. Separation was performed by HPLC using a Waters XTerra RP-18 (5 µm, 150x4,6 mm) column with a mobile phase consisting in acetonitrile – potassium dihydrogen phosphate 50 mM pH 3.4 (27/73). UV detection at 278 nm was used to quantify analytes, encountering good linearity ($r^2 > 0.999$) in the 2-100 ng/mL concentration range. The mean recovery was 92.4 % and 92.8 % for risperidone and 9-hydroxyrisperidone respectively, with an intraday – interday precision below 7%, and accuracy below 115 %. The method has been successfully applied in pharmacokinetic studies that require a large sample number.

Keywords: Risperidone, 9-hydroxyrisperidone, solid phase extraction.

1.- INTRODUCTION

Risperidone (RSP, figure 1) is a benzoisoxazole derivative that is clinically used as an atypical antipsychotic. Since its introduction in the 1990s, it has been considered to be a first-rate choice in the treatment of schizophrenia and other mental disorders because of its pharmacodynamics¹ (selective blocking of 5-HT₂ and D₂ receptors in brain), providing effective management of positive, negative and cognitive symptoms of schizophrenia with a low profile of extrapyramidal and autonomic side effects².

Several methods have been proposed for therapeutic and toxicological monitoring of RSP and 9-hydroxyrisperidone (9-OH RSP, figure 1). These are more-or-less sensitive depending on the detection mode used. UV detection offers simplicity but higher limit of quantitation (LOQ=2 ng/ml) and presence of interferents if the extraction procedure is not selective^{3,4,5}. Electrochemical detection offers lower LOQ (< 0.5 ng/mL) but lacks selectivity and sample and standard preparation require high purity reagents^{6,7}. Ultimately, mass spectrometry detection offers high sensitivity and selectivity, but the cost is not accessible for most laboratories, especially in Latin America⁸⁻¹¹.

Figure 1: Chemical structures of Risperidone, 9-OH hydroxyrisperidone and Clozapine.

The extraction procedures described to isolate RSP and 9-OH RSP from biological matrices are predominantly based on liquid-liquid extraction^{3-5,8,9,11}. These are very effective and offer high recoveries (> 85 %) with good precision and accuracy, but sample handling requires the use of toxic solvents or multiple extraction steps in order to obtain clean extracts, and this factor is a drawback when the analysis demands to work with a large sample number. On the other hand, solid phase extraction (SPE) procedures offer an easier sample handling but the results are reliable only if the analyst avoids sorbent drying^{7,10,12-15}. In our laboratory, we tested the properties of the sorbent included in Waters OASIS® HLB cartridges because it offer more advantages compared to traditional SPE¹⁶. This result is explained by the chemical nature of the polymeric reversed-phase sorbent, which has the ability to remain moist with water, to retain a wider range of polar and non-polar compounds, and it does not lose efficiency if the sorbent dries. The final result is improved reproducibility with less time spent in sample clean-up.

Since it is necessary to concentrate the extracted sample prior to the HPLC analysis - given the relatively low sensitivity of RSP and 9-OH RSP at UV - we developed an optimized extraction methodology in OASIS HLB® 30 mg, 1 cc columns. The previously described characteristics of the sorbent allows the handling of a large number of samples in pharmacokinetic studies (e.g. bioavailability and bioequivalence studies) without using large amounts of organic solvents for washing and elution steps.

2.- EXPERIMENTAL

2.1.- Reagents

RSP (European Pharmacopoeia) was generously donated by Laboratorios Andrmaco (Santiago, Chile), 9-OH RSP was purchased from Toronto Fine Chemicals (Toronto, Canada), and the internal standard clozapine (CLZ, figure 1) was purchased from Sigma. Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid 100%, ammonia 27-30%, phosphoric acid 85% and potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). Water was purified and deionized by a Barnstead Nanopure Diamond system. Healthy human serum was generously donated from Diagnomed Clinical Laboratory and "Gustavo Torrejn" Clinical Laboratory (Concepcin, Chile).

2.2.- Equipment

The chromatographic equipment consisted in a Merck-Hitachi LaChrom Elite system (Japan) with an L-2130 quaternary pump (interface on board), an autosampler model L-2200, an UV detector model L-2400, and an oven model L-2300. The EZ Chrom Elite data acquisition software (Scientific Software, U.S.A.) was installed in a personal computer.

2.3.- Chromatographic conditions

The analyses were performed in a Waters XTerra column (5 µm, 150x4.6

mm) with an XTerra guard column (5 μ m 20mm). The mobile phase was prepared with acetonitrile and potassium dihydrogen phosphate 50 mM pH 3.4 (27-73), filtered with a 0.22 μ m nylon filter, and degassed with ultrasound, which was delivered at a flow of 1 mL/min. The temperature of the oven was adjusted at 32°C. Detection was set at 278 nm.

2.4.- Standard preparation

Stock solutions of RSP, 9-OH RSP and CLZ were prepared at a concentration of 1 mg/mL in methanol, and the working standards were diluted daily at a concentration of 5000 ng/mL in mobile phase. The stock solutions were stable for at least 6 months at -20°C.

2.5.- Sample preparation and extraction procedure

1 mL of serum was loaded with known amounts of RSP, 9-OH RSP and 10 μ L of the internal standard (Final CLZ concentration = 50 ng/mL). The OASIS® HLB cartridges were placed in a 12 gauge manifold (Novagen, U.S.A.) before their activation with the addition of 1 mL of methanol and conditioned with 1 mL of water. Then, the sample was added and washed with 3 mL of methanol/ammonia (50/2). Before washing, the cartridges were dried for 1 minute at 10 inches Hg of vacuum, and then finally, the analytes were eluted with 1 mL of methanol/acetic acid (55/2). The extracts were evaporated to dryness using N₂ at 65°C, and the residue was re-dissolved in 200 μ L of mobile phase, injecting 95 μ L into the chromatograph.

2.6.- Validation: Linearity, recovery, precision and accuracy

2.6.1.- Linearity

The method was validated in human serum through the concentration range of 5-100 ng/mL. Six points of 2, 5, 10, 20, 50 and 100 ng/mL (n=3) were analyzed twice in one day and once on another day. The slope was calculated using the ratio between RSP/CLZ and 9-OH RSP/CLZ areas. A linear regression analysis was performed to evaluate the linearity of the calibration curves. The limit of detection (LOD) was calculated measuring the signal/noise ratio of 3 with the lowest concentration of the calibration curve. The limit of quantification (LOQ) was expressed by the lowest concentration that gives an accuracy and precision below 20% in variation.

2.6.2.- Recovery

Recovery was determined analyzing samples (5, 50 and 100 ng/mL) spiked in serum (n=5). The results, expressed in area, were compared with reference standards prepared in mobile phase.

2.6.3.- Precision and accuracy

The method precision and accuracy were determined by analyzing samples (5, 50 and 100 ng/mL) spiked in serum. Intraday variation was evaluated by the injection of ten injections of extracts at the concentrations described, and interday variation was assessed injecting five samples of each concentration on three different days. The results were expressed as relative standard deviation (%RSD) in the case of precision and % difference from the theoretical concentration for accuracy.

2.7.- Application to a bioavailability study

Blood samples were obtained from a pharmacokinetic study previously approved by the University of Concepción ethics committee, in which a group of 13 healthy fasted volunteers (age between 18 – 40 years) ingested a 3 mg dose of Risperdal® (Trademark of Risperidone, Janssen Pharmaceuticals, Belgium) in the morning. Blood sampling was carried at 0, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, 34 hours. After extraction, samples were immediately centrifuged (3000 r.p.m. x 10 min) and the separated serum was stored at -54°C prior to analysis.

3.- RESULTS AND DISCUSSION

3.1.- Linearity

The calibration curves showed good linearity in the range of 2-100 ng/mL ($r^2 = 0.9998$) and were expressed by the linearity equations: $y = 0.020 (\pm 0.00107)x - 0.005 (\pm 0.01)$ for RSP and $0.0177 (\pm 0.0007)x - 0.0035 (\pm 0.0031)$ for 9-OH RSP, where x is the analyte concentration and y is the analyte/internal standard area ratio. The LOD for both analytes was 1 ng/mL. The LOQ was 2 ng/mL with a 10.4 % and 12.5 % variation in precision for RSP and 9-OH RSP, respectively.

3.2.- Recovery

The experimental values of recovery with their precision are shown in

Table 1.

Table 1: Recoveries of RSP, 9-OH RSP and CLZ in serum.

Analyte	[C] (ng/mL)	Recovery (%)	RSD (%) (n =5)
RSP	5	89.03	3.21
	50	94.39	1.84
	100	93.76	1.08
9-OH RSP	5	92.40	1.46
	50	92.74	3.68
	100	93.29	1.48
CLZ	50	86.20	2.36

Using the extraction procedure in serum samples spiked with the analytes, the average recovery for RSP was 92,39%, 92,81 % for 9-OH RSP and 86,20 % for CLZ. Because RSP, 9-OH RSP and CLZ are weak bases, it is necessary to reduce ionization in order to increase the affinity with the sorbent of OASIS® HLB cartridges, using an alkaline solution for washing. With 50 % of methanol in the washing solution, a clean extract was obtained without a significant decrease in the extraction yield. For elution, the acidic solution reduces the affinity for the sorbent by ionization of the basic nitrogen present in RSP, 9-OH RSP and CLZ, leading to a reduction in the amount of organic solvent needed for obtain the maximum recovery. An amount of methanol higher than 55 % led to elution of endogenous compounds present in the matrix.

Compared with other extraction procedures, like the developed by Price *et al.*, which involves the use of Varian Bond-Elut Certify cartridges, our method offers relevant advantages. These extraction cartridges contain a mixed bed sorbent (C₈ and anion exchange) which offers more selectivity, but the volume of Methanol required for conditioning and washing (12 mL per sample) appears to be a contradiction when the main objective of solid phase extraction is to reduce the amount of organic solvents used. On the other hand, Raggi *et al.*, developed a simplified method with Varian Bond-Elut C₈ extraction cartridges, and although the RSP and 9-OH RSP recoveries were high, this method does not appear to be suitable for the handling of larger sample number because it requires more attention in order to avoid sorbent drying, especially when is necessary the use of 24-gauge manifolds¹³. Furthermore, the analytical method was validated for toxicological purposes, and sample concentration and injection volume does not meet the LOD and LOQ requirements (using UV detection) for therapeutic drug monitoring and pharmacokinetic analyses. Nagasaki *et al.* and Mercolini *et al.* proposed the use of a cyano (CN) based sorbent for solid phase extraction of plasma samples obtained from patients¹⁴. The selection of this sorbent was justified by the fact of analysis costs, and the possibility of co-extract other antipsychotics for simultaneous analysis in the case of Mercolini *et al.* work, but the use of CN sorbents addresses the same issues presented by C8 or C18 sorbents. There are no comments or discussions about the suitability and reliability of the previously described methods on pharmacokinetic studies. Recently, Saracino *et al.* and Mandrioli *et al.*, have developed a clean-up procedure using a microextraction by packed sorbent technique, which supposedly has more advantages, like reduced time of analysis, less use of organic solvents and sample volume to process¹⁷⁻¹⁸. However, again, the use of reversed phase sorbents limits its suitability when is necessary to analyze a larges number of samples. An exception was found in the work of Locatelli *et al.*, because they used Phenomenex Strata X-CW solid phase extraction cartridges, which have a sorbent that shares some of the properties exhibited by the OASIS HLB sorbent¹⁹. It does not lose selectivity when the sorbent is dried, and this factor can be a drawback for the novelty of our work. However, the extraction recovery data obtained from the Strata SPE cartridges, showed a lack of consistence in the extraction yields of RSP and 9-OH RSP. At high RSP concentrations, the extraction yield of this compound decreased with an increase of the 9-OH RSP recovery, suggesting a probable sorbent saturation. The authors did not include a discussion that explains this phenomenon. With the OASIS HLB cartridges, the extraction yields of each analyte showed less variation through the work concentration range, and the sorbent amount required to perform the clean-up was half (60 mg of Strata X-CW sorbent vs. 30 mg of OASIS HLB) of the used on Strata X-CW SPE cartridges in order to achieve comparable results.

3.3.- Precision and accuracy

Table 2 presents the precision and accuracy results.

Table 2: Precision and accuracy for RSP and 9-OH RSP in spiked serum.

[C] spiked ng/mL RSP	Intraday (n=10)			Interday (n=5)		
	[C] measured ng/mL	Accuracy %	RSD %	[C] measured ng/mL	Accuracy %	RSD %
5	5.58 ± 0.3	111.6	5.38	5.22 ± 0.35	104.4	6.7
50	52.11 ± 1.62	104.2	3.1	51.8 ± 1.02	103.6	1.97
100	103.59 ± 0.84	103.6	0.81	102.51 ± 1.89	102.5	1.8
9-OH RSP						
5	5.55 ± 0.33	111	5.94	5.46 ± 0.25	109.2	4.6
50	51.7± 0.95	103.4	1.83	55.01 ± 1.57	110	2.8
100	104.33 ± 0.77	104.3	0.74	108.65 ± 3.38	108.7	3.1

3.4.- Sample analysis

Using the validated method, we were able to analyze a large number of samples obtained from the pharmacokinetic study previously described. Figure 2 presents the chromatograms of blood samples extracted from a volunteer at time 0, 1.25 and 4 hours. The concentrations of 9-OH RSP and RSP at these time intervals were respectively: 11.80 and 15.70 ng/mL (1.25 hours), 15.82 and 7.48 ng/mL (4 hours). The choice of analytical column was an important aspect to consider because the Waters XTerra® column presented a significantly longer life (expressed as number of samples injections) and higher efficiency (good resolution and less tailing) than other columns (LiChroCart, Chromolith Performance) tested by us for RSP and 9-OH RSP analysis²⁰. The selection of internal standard was based on clozapine chromatographic behavior (less retention time than other antipsychotics tested as internal standard) and the clinical fact that certain pharmacokinetic studies require healthy volunteers, and consequently there is a low probability that the volunteers use psychotropic drugs like anxiolytics, antipsychotics or antidepressants. Furthermore, clozapine is a drug that has a very specific use and is prescribed only if other therapeutic schemes involving atypical or classical antipsychotics (in association or not) fails. No interferences were found in any of the samples.

Figures 2A, 2B and 2C: Chromatograms of serum samples obtained at time (A) zero, (B) 1.25 hours, (C) 4 hours.

3.5.- Pharmacokinetic profile

Table 3 summarizes the results of the pharmacokinetic analysis, and figure 3 presents the mean (with standard deviation) pharmacokinetic profile of RSP and 9-OH RSP.

Table 3: Mean pharmacokinetic parameters determined from volunteers data with standard deviation (n = 13).

Parameter	RSP		9-OH RSP	
	Mean value ± SD	RSD % (n=13)	Mean value ± SD	RSD % (n=13)
AUC _{0-t}	78.17 ± 71.81	91.86	380.50 ± 111.01	29.18
AUC _{t-∞}	10.06 ± 5.68	56.44	80.53 ± 62.49	77.79
C _{max}	25.32 ± 15.25	60.23	23.33 ± 5.71	24.49
t _{max}	0.90 ± 0.30	33.00	2.79 ± 2.32	83.29
t _{1/2}	1.96 ± 0.80	40.94	12.67 ± 2.63	20.73
K _e	0.40 ± 0.13	33.16	0.057 ± 0.011	20.15

Figure 3: Mean pharmacokinetic profile with standard deviation of RSP (A) and 9-OH RSP (B).

4.- CONCLUSION

In bioavailability and bioequivalence studies, is necessary to have at least 12 volunteers in order to perform a reliable statistical analysis of the data. Additionally, sampling points at time intervals generally are higher to assure the correct calculation of the area under the curve (AUC). , not less of 500 samples must be analyzed and is very inefficient - and dangerous for the analyst - to use liquid-liquid extraction procedures, such as the described in other articles, since they require the handling of toxic (and inflammable) solvents and the execution of multiple steps for sample clean-up. The method described in this article overcomes these issues present in traditional solid phase extraction procedures (described previously) with reverse phase, CN or polymeric sorbents. It was demonstrated that is possible to perform a high throughput analysis of samples with reliable results.

Furthermore, the time required to perform extraction of RSP and 9-OH with the OASIS HLB sorbent can be reduced using 96-well plates and automatic sample processing systems (less time and solvent consumption), or with the use of more sensitive detection modes, such as mass spectrometry, which requires less sample for analysis, smaller injection volumes and provides higher sensitivity than UV detection⁸⁻¹¹.

In conclusion, the developed method is simple, fast, and can be applied to pharmacokinetic studies.

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