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HPLC quantification of perphenazine in sheep plasma: Application to a pharmacokinetic study

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Abstract

A high performance liquid chromatographic (HPLC) method for the determination of perphenazine (PPZ) in sheep plasma was developed and validated. The separation was achieved using a 5 μm C18 column (125 mm × 4 mm) with a mobile phase composed of acetonitrile and an aqueous solution of H_3PO_4 and TBA (inverse gradient). The flow rate was 1.5 mL/min and the UV detection was performed at 258 nm. The method was validated with respect to linearity, intra and inter-day precision and accuracy, limit of quantification, limit of detection and storage stability. This method was used to perform a pilot pharmacokinetic study of PPZ after subcutaneous administration to one ewe.

Keywords: Perphenazine; HPLC; UV; Pharmacokinetic; Sheep

1. Introduction

Perphenazine belongs to the phenothiazine family and has a piperazine ethanol side chain [1]. It is considered a long-acting tranquillizer with sedative effects starting from 10 to 16 h after injection and lasting for 7 to 10 days. Its effectiveness varies among different species and among animals of the same species, and duration of its effects is dose dependent [2]. The use of long-acting tranquillizers, originally developed for the treatment of psychoses in non-compliant psychiatric patients, has markedly decreased mortality associated with the stress of translocation and confinement in wild ungulates [3]. This long action is achieved through esterification of the active compound, which is dissolved in vegetable oil, allowing delayed hydrolysis and slow absorption into the blood [4]. Due to this variation among individuals, it is important to have an easy and cheap method for perphenazine quantification in plasma in order to correlate the pharmacokinetic parameters with the tranquillizing effects.

Several analytical techniques have been reported in the literature involving gas chromatography [5], HPLC with coulometric detection [6] or HPLC with UV detection [6–8]. All these reported HPLC techniques involved, in the sample preparation, double or triple liquid–liquid extractions with organic solvent, followed by an evaporation [6,9] or solid phase extraction [8–11]. These sample preparation procedures require a lot of time, reagents and materials, and consequently they are quite expensive. Also, some of these reported techniques require electrochemical detectors, which are seldom available [9], or special chromatographic columns [7,8].

The aim of this study was to validate a rapid and easy HPLC method for perphenazine quantification using C18 columns and UV detection, in order to study PPZ pharmacokinetics in sheep plasma as a model for future studies in wild animal species.

2. Experimental

2.1. Materials

Perphenazine chemical standard was obtained from Sigma–Aldrich (part no. P6402, batch no. 064H0447). NaCl was purchased from Sharlau (part no. SO0225), ortho-phosphoric

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acid 85% from Riedel-de Haën (part no. 30417), tetrabutylammonium bisulphate 99% from Sigma–Aldrich (part no. T-7158), and acetonitrile ultra gradient HPLC grade from JT Baker (part no. 9017). Milli Q grade water was used for the mobile phase preparation. Drug free sheep plasma was obtained from the animals of the *Servei de Granges i Camps Experimentals* of the Veterinary School of the UAB, Barcelona, Spain.

For the sample preparation, 5 mL dispensable polypropylene tubes with caps and polypropylene eppendorf tubes of 1.5 mL and 2 mL were used.

2.2. Chromatographic system and conditions

The chromatographic system was composed of an on-line mobile phase degasser, a quaternary pump, an autoinjector with Peltier sample thermostat, a column oven and a diode array UV detector. All the modules were Agilent 1100 (Darmstadt, Germany). The chromatographic conditions and data analysis were performed using the Agilent Chem-Station (rev.B.01.03) software. The chromatographic analytical column was a 5 μm C18 column of 125 mm × 4 mm (Spherisorb, S2 ODS2, Waters, Milford, USA). The column oven temperature was set at 50 °C.

The mobile phase was composed of acetonitrile (phase A) and a water solution (phase B) of H_3PO_4 (1 mL/L) and tetrabutylammonium bisulphate (TBA) (0.52 g/L) in a linear solvent gradient. The initial proportions of mobile phase were 72% of phase A and 28% of phase B. After injection of the samples these conditions were maintained for 1 min and after this time the proportions were linearly changed to 50% of phase A and 50% of phase B in 0.8 min, and maintained for 1.7 min. Then, the mobile phase returned to initial conditions in 0.1 min. The injection volume was 20 μL, and total run time was set at 6 min. The flow rate was set at 1.5 mL/min. The detection was performed at 258 nm (±5 nm band width) with a reference of 300 nm (±5 nm band width).

2.3. Preparation of the stock and spiking solutions

The PPZ stock solution (0.1 mg/mL) and spiking solutions (0.01, 0.001 and 0.0001 mg/mL) were prepared in HPLC grade acetonitrile. After preparation, the stock solution (100 mL) was divided into 2 mL aliquots and stored at −30 °C.

2.4. Calibration samples preparation

Five sheep plasma samples of 2 mL, free of drugs, were spiked, respectively, with 2, 5, 10, 50, 200 ng of PPZ to construct a calibration curve. With these spiking amounts the final PPZ plasma concentration was 1, 2.5, 5, 25 and 100 ng/mL, respectively. After spiking, the calibration samples were treated as the problem samples. Each day of analysis a complete calibration curve was prepared and analysed to calculate the problem sample concentration.

2.5. Sample preparation

After thawing the plasma samples, aliquots of 2 mL were placed in 5 mL polypropylene tubes. To precipitate plasma proteins, 2 mL of cold acetonitrile (−20 °C) were added to the samples and vortexed for 10 s after capping the tubes in a multivortex shaker (Vx-2500, VWR, USA). Then, 1 g of solid NaCl was added to the samples and vortexed for 5 min, again after capping. After shaking, the samples were centrifuged at 2700 × *g* for 5 min (Megafuge 1.0, Heraeus, Germany) to separate the organic phase from the aqueous phase. The complete upper organic phase was transferred to a 2 mL eppendorf tube and evaporated under N_2 stream, at 40 °C, in a thermostatic water bath. Finally, the samples were rediluted with 150 μL of mobile phase (72% phase A–28% phase B) for 5 min in the multivortex shaker and transferred to autosampler glass vials.

2.6. Method of validation

2.6.1. Selectivity

The selectivity of the method was assessed by analysing blank plasma samples obtained from non-treated sheep.

2.6.2. Linearity

The linearity of the calibration method was evaluated using a duplicate five level calibration curve. The calibration curves were constructed plotting the PPZ chromatographic peak area versus the spiked concentration of the calibration sample. A linear least square regression line was calculated. In order to assess the linearity, the back-calculated concentration was analysed for each calibration sample. A lack of fit test was performed and linearity was accepted when $p > 0.1$.

2.6.3. Intra-day and inter-day precision and accuracy

For the intra-day precision and accuracy assay, a calibration curve was prepared as specified in Sections 2.4 and 2.5 and analysed. Blank plasma samples (control samples) were spiked, in triplicate, with 1, 5 and 100 ng/mL of PPZ, respectively, and analysed using the calibration curve.

For the inter-day precision and accuracy assay, each day of analysis, three control samples were spiked with 1, 5 and 100 ng/mL of PPZ. They were prepared, processed and analysed with a complete calibration curve. The PPZ concentration of the control samples was calculated using the calibration curve.

The precision was calculated as the coefficient of variation (RSD %), in percentage, of the calculated concentration of the control samples ($n = 3$) at each concentration level. The accuracy was calculated as the percent error of the calculated concentration ($n = 3$) at each concentration level.

The acceptance criteria for the precision and accuracy results were as follows: for the precision the value obtained should not exceed the 15% of the coefficient of variation except for the LOQ where it should not exceed 20%; for the accuracy the mean value should be within the 15% of the actual value except at the LOQ where it should not deviate by more than 20%.

2.6.4. Limit of detection

The limit of detection (LOD) of the technique was determined by processing and analyzing five blank plasma samples. The chromatographic areas, in the PPZ retention time, of the blank samples were calculated. The limit of detection was calculated by multiplying the standard deviation of the areas obtained by 3.3 and dividing the result by the slope of the calibration curve obtained in the linearity study.

2.6.5. Limit of quantification

The limit of quantification (LOQ) was considered as the lowest concentration level where the precision and accuracy results were within the acceptance range. This limit of quantification value was compared with the limit of detection, and was considered acceptable when its value was at least five-fold greater than the limit of detection.

2.6.6. Stability

The stability of the stock standard solution was tested at −20 °C. The stability of sample extracts kept in the autosampler at 10 °C for 48 h was assessed as well. The stability of PPZ in plasma under storing conditions (−20 °C) was also assayed.

2.7. Pharmacokinetic experiment

One *Lacaune* ewe, weighing 76.4 kg, was given an intramuscular administration of 1.5 mg/kg of perphenazine enanthate. Blood samples (10 mL) were taken from the jugular vein at 0, 0.5, 1, 2, 3, 4, 8, 12, 24, 36, 48, 60, 72, 84, and 96 h post-administration using heparinised vacuum tubes (Becton Dickinson, Vacutainer® LH 170 I.U). Blood samples were centrifuged at 3500 rpm during 10 min to obtain the plasma samples. Plasma samples were divided into two aliquots, placed in 5 mL polipropylene tubes, capped and stored at −20 °C until the day of analysis. On the day of the sample analysis two blank samples and a complete calibration curve were processed with the samples and the concentration of samples was obtained using this calibration curve.

This procedure was approved by the Institutional Animal Care and Use Committee of the UAB.

3. Results and discussion

3.1. Method development

The separation of lipophylic drugs by reverse phase usually involves the participation of an organic phase and an aqueous phase in the mobile phase, using a solvent gradient, where the proportion of the organic phase increases with time. In the present method there is an inverse solvent gradient where the organic solvent phase decreases from 72% of acetonitrile to 50%. This inverse solvent gradient was performed because, in the method development studies, we observed that the stationary phase retention of PPZ increased when the H_3PO_4 and TBA concentration of the aqueous phase decreased. On the other hand, the presence of the TBA in the mobile phase was to reduce the peak tailing effect of the PPZ. As a result of these observations, the final gradient began with a low percentage of aqueous phase and was maintained during 1 min. Under these conditions, the concentration of H_3PO_4 and TBA in the total mobile phase was not enough to elute the PPZ from the column; meanwhile, all the other substances from the plasma extract were submitted to a high percentage of acetonitrile and were not retained by the column. When all the possible interference substances eluted from the column, then, on increasing the aqueous phase, the amount of H_3PO_4 and TBA was enough to elute the PPZ. Under these conditions, the retention time of PPZ was 3.3 min (Fig. 1.). No interferences in the retention time of PPZ were observed after injection of a blank plasma sample (Fig. 1.) because the initial high percentage of acetonitrile eluted all the possible interferences. The base-line drift observed in the chromatograms shown in Fig. 1 was due to the solvent gradient response.

The extraction procedure was performed using 2 mL of plasma in order to increase the sensitivity of the technique as PPZ plasma concentration in the treated animals was very low, as described below. The extraction was a liquid–liquid partition between acetonitrile and water after saturation of the system with NaCl followed by acetonitrile evaporation under nitrogen stream. The mean recovery efficacy of the extraction was 87% (±5). This procedure was fast and cheap, as all the materials used are dispensable and of low cost. The time taken to process a sample is very low and depends on the capacity of the shakers, centrifuges and evaporator apparatus used.

3.2. Method validation

3.2.1. Selectivity

The chromatograms of a PPZ standard solution, a blank plasma sample and a plasma sample spiked with 1 and 25 ng/mL of PPZ are in Fig. 1. No interferences were detected at the retention time of PPZ.

3.2.2. Linearity

The relationship between the PPZ peak areas and the plasma concentration was linear over the tested range of concentrations (1–100 ng/mL). The typical equation of the calibration curve ($n = 2$) was: $y = 0.5359x - 0.3651$ ($r^2 = 0.9998$). The results of the calibration curve linearity assay are summarised in Table 1

Table 1
Results of the linearity assay

Lack of fit test $p = 0.595$			
Concentration added (ng/mL)	PPZ peak area	Back calculated concentration (ng/mL)	Percent error
1	0.27	1.16	15.5
1	0.24	1.10	9.6
2.5	1.04	2.60	3.9
2.5	1.04	2.60	4.0
5	2.53	5.38	7.5
5	2.48	5.28	5.7
25	13.07	25.03	0.1
25	12.13	23.54	−5.8
100	53.52	99.83	0.5
100	53.13	100.49	−0.2

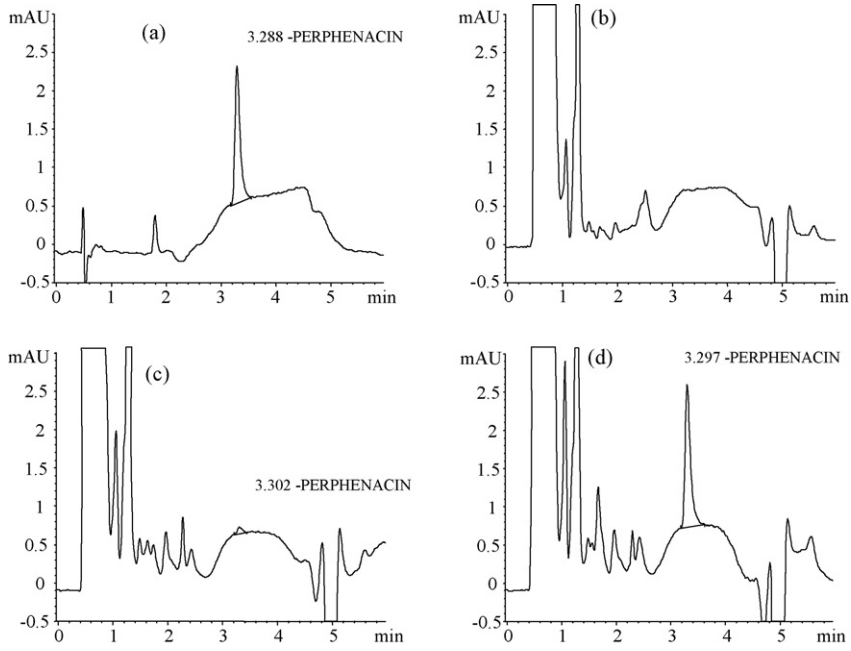

Fig. 1. Typical chromatograms of (a) PPF standard solution, (b) blank plasma sample, (c) blank plasma sample spiked with PPF at 1 ng/mL concentration level, and (d) blank plasma sample spiked with PPF at 25 ng/mL concentration level.

where the back calculated concentration and the accuracy of each sample are expressed. The correlation coefficient of all calibration curves was higher than 0.9998. The lack of fit test showed $p = 0.595$. All these tests indicated that the PPZ peak areas showed a linear relationship with plasma concentration.

3.2.3. Inter-day and intra-day precision and accuracy

Table 2 shows the intra-day and inter-day validation results. The accuracy and precision parameters obtained in both assays were within the acceptance criteria for the three PPZ plasma concentrations assayed.

3.2.4. Limit of detection and limit of quantification

The results of the limit of detection assay appear in Table 3. After extraction and analysis of five blank samples the mean peak area, corresponding to the peak interferences that appeared in the PPZ retention time, was 0.059 mAU s and the calculated LOD was 0.0365 ng/mL. The limit of quantification was 1 ng/mL, which is the lowest concentration level of the calibration curve. At this concentration level, the values of accuracy and precision were within the acceptance limits. The ratio between LOQ and LOD was 27.4, indicating that LOQ was, at least, five-fold the value of LOD.

Table 2
Intra-day and inter-day precision and accuracy results

	n	Spiked concentration (ng/mL)		
		1	5	100
Intra-day precision (RSD, %)	3	6.4	12.9	4.7
Intra-day accuracy (%)	3	15.9	−4.2	−1.8
Inter-day precision (RSD, %)	3	8.2	8	0.9
Inter-day accuracy (%)	3	6.2	5.5	−0.4

Table 3
Limit of detection assay

Sample	PPZ peak area
BK1	0.0156
BK2	0.0038
BK3	0.0033
BK4	0.0000
BK5	0.0067
Mean	0.0059
SD	0.0059
LOD	0.0365

3.2.5. Stability assay

The stock standard solution under the storing conditions was stable for at least 2 months. The sample extracts maintained in the autosampler for 48 h at 10 °C were stable as shown in Table 4. The long-term stability of PPZ in the plasma was assessed during a 50 day storage period at −20 °C and a concentration level of 5 ng/mL. After 50 days the PPZ area was 99.8% of the PPZ area obtained at the moment of spiking the plasma.

Table 4
Sample extracts stability assay

Spiked concentration (ng/mL)	Area at time 0 h	Area at 48 h	Stability percentage
1	0.268	0.263	98.1
2.5	1.041	1.000	96.0
5	2.53	2.447	96.7
25	13.067	12.872	98.5
100	53.518	53.795	100.5

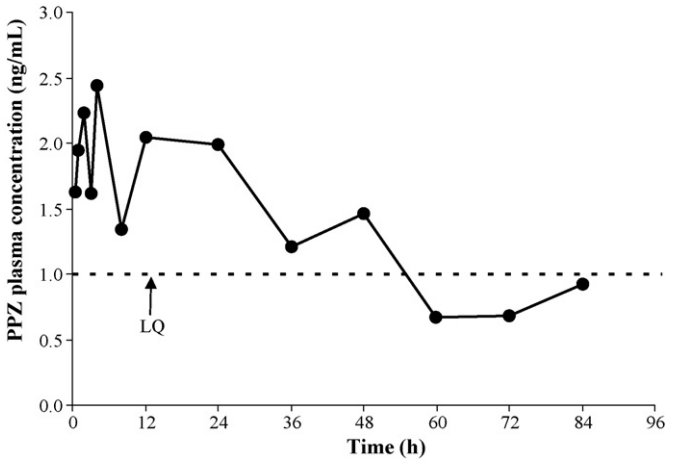

Fig. 2. Plasma concentration–time profile of PPZ in the ewe after administration of a 1.5 mg/kg intramuscular dose.

3.3. Pharmacokinetic analysis

This technique can add general knowledge on pharmacokinetics of perphenazine enantate on animals, which has only been addressed in the article published on Eurasian otter (*Lutra lutra lutra*) by Ventura et al. [12], to further relate this marked variation in plasma levels of the drug to behavioural changes.

Several quantitative methods have been described for the determination of perphenazine in plasma, serum and whole blood [9] and in urine [12]. However, most of these methods are expensive, with complicated procedures, and require a lot of time for less number of samples.

As far as we know, most of the information on the use and doses of neuroleptics in animals (wild and domestic) is based on empirical experiences. The availability of easy and cheap quantitative analytical methods, applicable to wild and domestic animals, is the main limiting factor for the development of pharmacokinetic studies in animals, especially in wild species.

Fig. 2 shows the plasma concentration–time profile obtained in the pilot pharmacokinetic study. The PPZ plasma concentration in the ewe after administration of a therapeutic dose was very low with a peak plasma concentration of 2.44 ng/mL obtained 4 h after administration. The PPZ plasma concentrations were below the LOQ of the technique at 60 h post-administration. The concentration values, calculated by extrapolation, at 60, 72 and 84 h post-administration were 0.67, 0.68 and 0.92 ng/mL, respectively. A relatively fast absorption was observed, followed by a slow elimination phase. In the elimination phase, a variable PPZ concentration decline suggested a possible slow pulse release of the PPZ from the injection site or accumulation of the drug in the lipidic tissues. Studies with more animals have to be performed to obtain sound information about the PPZ pharmacokinetic profile in this species.

4. Conclusion

A rapid and low-cost analytical method for the quantification of PPZ in sheep plasma is described. The sensitivity of the technique is high enough to study the pharmacokinetic profile of PPZ as this drug shows very low plasma concentrations. This methodology allows the analyst to prepare and analyse, by a simple HPLC method, at least 100 samples a day. All the reagents, materials and apparatus are of common use and dispensable tubes can be used. The method was validated in the concentration range of 1–100 ng/mL, which is enough for its use in pharmacokinetic studies.

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