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Quantification of perphenazine in Eurasian otter (*Lutra lutra lutra*) urine samples by gas chromatography–mass spectrometry☆

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Abstract

Perphenazine enanthate has been used in wild animals as a tranquilizer during the period of adaptation to new environments to reduce stress, mortalities and injuries. A gas chromatographic procedure for the quantitative measurement of perphenazine in otter urine has been developed and validated. The method involved an enzymatic hydrolysis with β-glucuronidase-arylsulfatase from *Helix pomatia*, followed by a solid-phase extraction with Bond Elut Certify cartridges. The resulting organic phase was evaporated, and the dry extract was derivatised with MSTFA to form the *O*-TMS derivative. The derivatised extracts were analysed by gas chromatography–mass spectrometry using SIM acquisition mode, measuring three diagnostic ions (*m*/*z* 246, 372 and 475). Another phenothiazine derivative, fluphenazine, was used as the internal standard (I.S.). Extraction recoveries for perphenazine and I.S. were 87.6±8.2% (n=4) and 106.7±13.4% (n=4), respectively. The calibration curves were linear in the range from 4 to 100 ng/ml (r^2=0.99). The limits of detection and quantification were estimated as 1.2 and 3.5 ng/ml, respectively. Precision and accuracy obtained in intra-assay studies were in the ranges of 1.3–8.7 and 1.7–19.5%, respectively, using control samples containing 6, 16 and 60 ng/ml of perphenazine. In inter-assay experiments, precision ranged from 4.3 to 14.9% and accuracy from 3.1 to 11.8%. Examples of the application of the perphenazine quantification method in otter urines after administration of perphenazine enanthate are presented.

Keywords: *Lutra lutra lutra*; Perphenazine

1. Introduction

Perphenazine is a potent neuroleptic, belonging to piperazinyl phenothiazine derivatives (Fig. 1). It has been used in therapeutics as an antipsychotic agent in order to decrease restlessness, aggressiveness and impulsive behaviour [1]. Perphenazine enanthate has been also successfully used in wild animals as a

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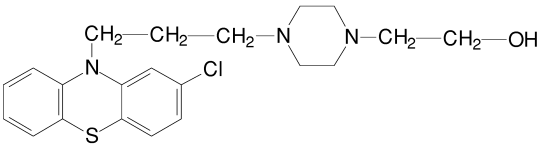
PERPHENAZINE



FLUPHENAZINE

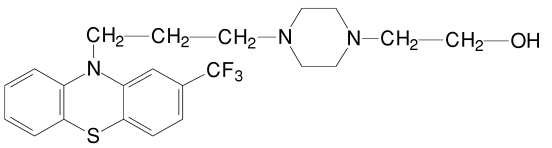


Fig. 1. Chemical structures of perphenazine and fluphenazine (internal standard).

tranquilizer during the period of adaptation to new environments to reduce stress, mortalities and injuries [2]. It is considered a long-acting tranquilizer with sedative effects noted from 10 to 16 h after injection, and lasts from 7 to 10 days. Their effectiveness varies among different species and among individual animals of the same species and the duration of effects is dose dependent [2]. Perphenazine enanthate has been recently used in wild Eurasian otters at the moment of capture to reduce stress during temporary confinement after capture [3].

Metabolic pathways described in humans are sulfoxidation, glucuronic conjugation of phenolic hydroxyl group, *N*-dealkylation and cleavage of piperazine ring [1,4]. Only 1% of daily oral dose is eliminated unchanged in humans, while glucuronide and sulfoxide metabolites are 30 and 12% of 24 h urine, respectively [1,4].

Several quantitative methods have been described for the determination of perphenazine in plasma, serum, whole blood and in tablet formulations based on gas chromatography with electron-capture or nitrogen–phosphorus detection (GC–ECD or GC–NPD, respectively) [5–7] or liquid chromatographic (LC) methods [8–19]. A few early articles are reported for the determination of perphenazine in human and rat urine samples, based on thin-layer chromatography [20,21] and using large volumes of urine with poor detection limits.

In this study, a method for the quantitative measurement of pherphenazine in otter urine has been developed. The method consists of a solid-phase extraction, a derivatisation to form trimethylsilyl (TMS) derivatives and gas chromatographic analysis.

2. Experimental

2.1. Chemicals and reagents

Perphenazine and fluphenazine were supplied by Sigma (St. Louis, MO, USA). Methanol, 2-propanol, 25% ammonia, glacial acetic acid, sodium acetate trihydrate, potassium hydroxide and ammonium chloride were purchased from Merck (Darmstadt, Germany). Chloroform (HPLC grade) was supplied by Scharlau (Barcelona, Spain). Deionised water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

β-Glucuronidase-arylsulfatase from *Helix pomatia* type HP-2 used in enzymatic hydrolysis was provided by Sigma. *N*-Methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Düren, Germany). Bond Elut Certify solid-phase extraction columns (130 mg/10 ml) were provided by Varian International (Harbour City, CA, USA).

Ammonium chloride buffer, pH 9.5 was prepared by dissolving 28 g of ammonium chloride in 100 ml of deionised water, and adjusting the pH to 9.5 with concentrated ammonia solution. 1 *M* acetic acid was prepared by mixing 28.5 ml of glacial acetic acid with deionised water to a final volume of 500 ml. A 1.1 *M* acetate buffer, pH 5.2 was prepared by dissolution of 14.97 g of sodium acetate trihydrate and 2.2 ml of acetic acid in 100 ml deionised water. A 0.1 *M* acetate buffer, pH 4 was prepared by mixing 5.7 ml of glacial acetic acid in 800 ml of deionised water; the pH was verified and adjusted to 4, and the solution was taken to 1000 ml with deionised water. Organic extracts were evaporated to dryness with a Turbo-Vap LV evaporator from Zymark (Hopkinton, MA, USA).

2.2. Standard solutions

Stock standard solutions (1 mg/ml) of perphenazine and fluphenazine (internal standard, I.S.) were prepared using methanol as a solvent. Working standard solutions of 10 and 1 μg/ml were prepared by 1:100 and 1:1000 dilutions of the stock standard solutions with methanol.

2.3. Instrumental analysis

The instrumental analysis was performed in a 5890 gas chromatograph coupled to a 5970 mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA). The instrument was equipped with a cross-linked methyl siloxane fused-silica capillary column (17 m×0.2 mm I.D., 0.11 μm film thickness) from Agilent Technologies (USA). Injection was performed in splitless mode (2 min delay), and helium was used as the carrier gas (0.8 ml/min measured at 180 °C). Injection and transfer line temperatures were set at 280 °C. The sample volume injected was 2 μl. Initial oven temperature was set at 180 °C, increased at 20 °C/min to 290 °C and maintained for 5 min. The total run time was 10.50 min. Insert liners packed with silanised glass wool were used.

Analysis was performed in the selected-ion monitoring (SIM) acquisition mode, monitoring characteristic ions for perphenazine mono-TMS derivative (*m/z* 246, 372, 475), and for fluphenazine mono-TMS derivative (*m/z* 280).

2.4. Solid-phase extraction procedure

To 2 ml of urine samples, 20 μl of I.S. solution (fluphenazine, 1 μg/ml) was added. Then, urine samples were adjusted to pH 5.2 with 1 ml of 1.1 *M* acetate buffer, pH 5.2 and vortex mixed (5 s). For enzymatic hydrolysis, 50 μl of β-glucuronidase-arylsulfatase from *Helix pomatia* was added and after vortex mixing during 5 s, samples were incubated at 55 °C for 2 h. After cooling to room temperature for a few minutes, samples were centrifuged at 3500 rpm for 5 min. For solid-phase extraction, Bond Elut Certify columns previously conditioned with 2 ml of methanol and 2 ml of deionised water were used. Hydrolysed urines were applied in a time up to 2 min. Then, columns were washed with 2 ml of deionised water, 1 ml of 1 *M* acetic acid and 2 ml of methanol, and after drying for 2 min, two consecutive elutions of 2 ml were carried out with a mixture of chloroform–2-propanol (80:20, v/v) containing 2% ammonia. The organic extract was evaporated to dryness under a nitrogen stream in a water bath at 40 °C, kept in a desiccator containing diphosphorous pentoxide and potassium hydroxide pellets and maintained under vacuum for at least 1 h.

Alternative solid-phase extraction at pH 3 or pH 9.5 was also tested in order to optimise extraction procedure. Samples were adjusted to pH 3 with 1 ml of 1 *M* acetic acid before applying solid-phase extraction as previously described. Adjustment to pH 9.5 was done applying 100 μl of ammonium chloride buffer and in solid-phase extraction, columns were washed with 2 ml of deionised water, 1 ml of 0.1 *M* acetate buffer, pH 4 and 2 ml of methanol, consecutively; the elution was performed as described previously.

2.5. Derivatisation

The dried extracts were derivatised with 30 μl of MSTFA, vortex mixed during 10 s and kept at 80 °C for 30 min. After cooling to room temperature for 5 min, they were transferred with Pasteur pipettes to injection vials.

2.6. Assay validation

The method was validated by determining the selectivity and specificity, recovery of perphenazine and fluphenazine, linearity, limits of detection (LODs) and quantification (LOQs), and intra- and inter-assay precision and accuracy. Due to the difficulty in obtaining high volumes of blank urine samples from otters, calibration curves prepared with human blank urine were used. To demonstrate the ability of the calibration curve to predict values in otter urine, three control samples of 6, 16 and 60 ng/ml prepared in drug-free otter urine, were used in triplicate.

Selectivity and specificity were studied by the analysis of urine samples collected from otters not having received the drug and urine samples collected before the administration of the drug. The presence

of any interfering substance at the retention time of perphenazine and fluphenazine was verified.

Extraction recoveries of perphenazine and fluphenazine were calculated by comparison of peak areas of the compounds obtained after the analysis of samples spiked with 10 ng/ml of each compound ($n=4$), with the mean value of those obtained when the standards were added to extracted blank urines and subjected to the derivatisation process (representing 100% of extraction recovery).

Linearity was studied using a calibration curve with samples at the following concentrations: 4, 10, 40, 70, and 100 ng/ml. Calibration samples were prepared daily by adding appropriate volumes of stock solutions to 2-ml aliquots of drug-free human urine. In the first assay of validation, the calibration curves were prepared in quadruplicate. The ratio of the peak area of perphenazine (m/z 246) to peak area of the I.S. (m/z 280) was plotted against the concentration. Dixon's test ($\alpha=5\%$) was applied to detect outliers in the replicates at each concentration level. The behaviour of the variance over the calibration range (homoscedasticity/heteroscedasticity of the procedure) was evaluated by applying the Levene test ($\alpha=5\%$). In the rest of validation assays, the calibration curve was prepared in duplicate. To demonstrate the goodness of fit using the linear model, and F test ($\alpha=5\%$) was applied to compare the variance attributable to lack of fit with that due to random error.

The LOD and LOQ were defined as 3.3 and 10 times the value of noise, respectively. The standard deviation of the estimated concentration values for the lowest calibration point at 4 ng/ml ($n=4$) was used as a measure of the noise.

Intra-assay precisions and accuracy were determined by the analysis of control urine samples (prepared in drug-free otter urine) with concentrations of 6, 16 and 60 ng/ml of perphenazine at the same day ($n=3$ replicates of each). Inter-assay precision and accuracy were calculated by the analysis of the control samples in three different assays ($n=9$). Precision was expressed as the relative standard deviation (RSD) of the control samples concentration calculated using the calibration curve, and accuracy was expressed as the relative error (%) of the estimated concentration.

Suitability of sample dilution was verified by analysis of a control sample of 60 ng/ml diluted with blank urine to a final concentration of 15 ng/ml. The suitability of sample dilution was verified by comparison of the concentration obtained for this sample taking into account the dilution factor with the concentration obtained with a control sample of 60 ng/ml not subjected to dilution.

2.7. Otter urine samples

Eurasian otters (*Lutra lutra lutra*) were live-trapped and transported to Barcelona Zoo, in the period between 1995 and 2000, in a translocation program to strengthen the eastern populations with animals from the western part of Spain. Trapped animals were chemically immobilized by manual injection of ketamine and medetomidine intramuscularly, following procedures described elsewhere [22,23]. At the moment of capture, and once they were chemically immobilized, a group were injected intramuscularly 20 mg of perphenazine enanthate. The rest of the otters remained untreated (non-treated group). In the Barcelona Zoo, they were individually housed indoors in wire-mesh cages (2.44 m long$\times$ 1.22 m wide$\times$1.22 m high), with attached wooden nest boxes (0.91 m long$\times$0.61 m wide$\times$0.51 m high) and suspended above the ground.

Urine samples from eight different otters (five females and three males), treated with a single dose of perphenazine enanthate were collected daily, during a period from 6 to 21 days after administration of the drug by using a plastic dish located on the floor under the cages [3]. For one of the female otters, two urine samples were collected 2 and 3 days before the administration of the drug.

Urines from three otters (one male and two females) belonging to the non-treated group were also collected at 1, 3, 4, 8, 11, 14, 16, or 18 days after arrival at the Barcelona Zoo.

3. Results and discussion

3.1. Derivatisation and chromatographic analysis

Perphenazine is a lipophilic molecule containing an alcohol and tertiary amine function in its chemical structure (Fig. 1). Fluphenazine was chosen as I.S.

due to its structural similarity to perphenazine. Analysis of perphenazine by GC–ECD in plasma samples was reported using TMS derivatives formed after reaction with *N,O*-bis-(trimethylsilyl)acetamide [5,6]. MSTFA is also widely reported for derivatisation of alcohol groups [24] and it has been used in this study. Stable TMS derivatives were formed for perphenazine and fluphenazine resulting from the derivatisation of the hydroxyl group in the lateral chain. Electron impact mass spectra of TMS derivatives of both compounds and proposed fragmentation profiles are presented in Fig. 2. As can be seen, mass spectra with adequate selectivity were obtained for perphenazine and fluphenazine TMS derivatives.

Common fragmentation pathways can be observed for the TMS derivatives obtained. The most important ions correspond to the phenothiazine ring and to the breakdown of the lateral chain at both sides of the first methylene group (m/z 232 and 246 for perphenazine-*O*-TMS, and m/z 266 and 280 for fluphenazine-*O*-TMS). Molecular ions are present in both cases with relative abundances between 10 and 20%. Ions resulting after the lost of methyl, –OTMS or $-CH_2-$OTMS groups from the molecular ion are also observed in both cases (m/z 460, 386 and 372, for perphenazine-*O*-TMS; and m/z 494, 420 and 406, for fluphenazine-*O*-TMS, respectively).

For perphenazine mono-TMS derivative, the diagnostic ions used were m/z 246, 372 and 475. These ions were also checked to evaluate the selectivity/specificity of the procedure. For quantitative purposes, the base ions for both perphenazine and fluphenazine derivatives were used (m/z 246 and 280, respectively).

Under the conditions studied, good chromatographic separation was obtained in a total run time of 10.5 min. The retention times of perphenazine and fluphenazine derivatives were 7.8 ± 0.1 and 6.2 ± 0.1 min, respectively. Chromatograms obtained after analysis of a blank urine, a calibration urine (40 ng/ml) and an otter urine obtained after administration of perphenazine enanthate are shown in Fig. 3.

3.2. Hydrolysis and solid-phase extraction procedures

Metabolism and urinary excretion of perphenazine in otter urine has not been previously described. However, conjugated perphenazine metabolites have been described in humans [1,4]. For this reason, enzymatic hydrolysis was applied before solid-phase extraction in this study. Extraction recovery was tested using Bond Elut Certify columns, due to its combined phase involving both lipophilic and ion-exchange properties. Interaction between the analytes and the sorbent can be modified at different pH conditions, so three different pH values were tested in this assay: pH 3, pH 5.2 and pH 9.5. Finally, pH 5.2 was chosen because of a higher selectivity, recovery and reproducibility compared to the other extraction pH values. These results can be explained taking into account the pK_a values of perphenazine and fluphenazine, which are 7.8 and 8.1, respectively [1,4]. At pH 5.2, both, perphenazine and fluphenazine are protonated, so interactions between the compounds and the sorbent were favoured; additionally, extraction recoveries at pH 5.2 were higher than using pH 3. Selectivity of sample extraction procedure is shown in Fig. 3.

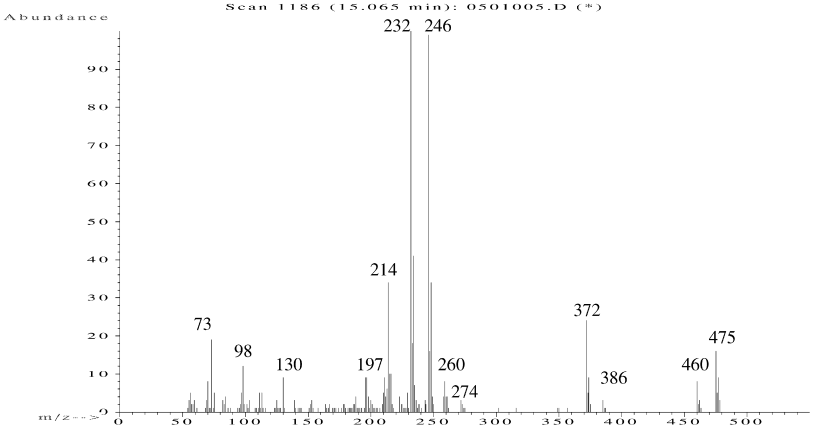
3.3. Assay validation

No matrix interferences were observed after analysis of urine samples collected from not treated otters ($n=9$) and in samples collected before the administration of the drug ($n=2$). Treatments with medetomidine and ketamine, used for the immobilization of the otters, did not show interferences in the analysis. Other phenotiazine tranquilizers, as chlorpromazine and trifluoperazine, did not interfere in the analysis of perphenazine, as the retention time of their TMS derivatives was lower than 6 min.

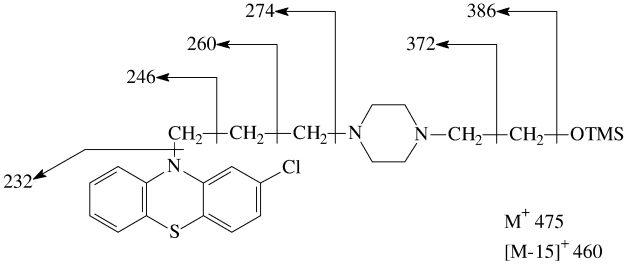
Extraction recoveries for perphenazine and the I.S. were 87.6 ± 8.2 and $106.7\pm13.4\%$, respectively, which improve most of previously reported perphenazine recoveries in human and rabbit plasma [6,8,17,18].

The procedure was found to be heteroscedastic, so peak area ratios between perphenazine and the I.S. were subjected to a proportional weighted least-square regression analysis. The method was linear in the range from 4 to 100 ng/ml ($r^2=0.99$). The test of comparison of variances was not significant, indicating the goodness of fit of the linear model. The LOD and LOQ were estimated as 1.2 and 3.5 ng/ml, respectively.

(a)



PERPHENAZINE



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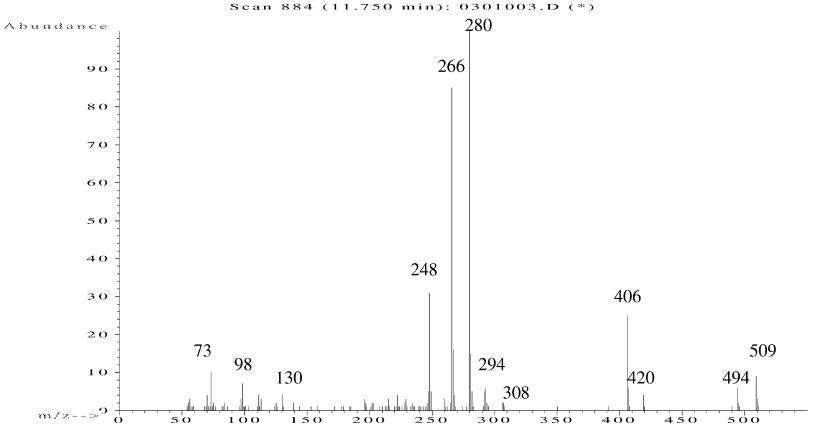

FLUPHENAZINE (ISTD)

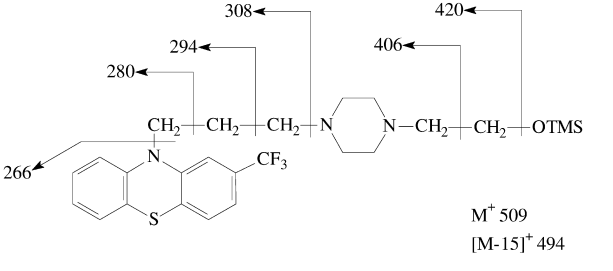

Fig. 2. Electron impact mass spectra of (a) perphenazine and (b) fluphenazine (internal standard) TMS derivatives and proposed fragmentation profiles.

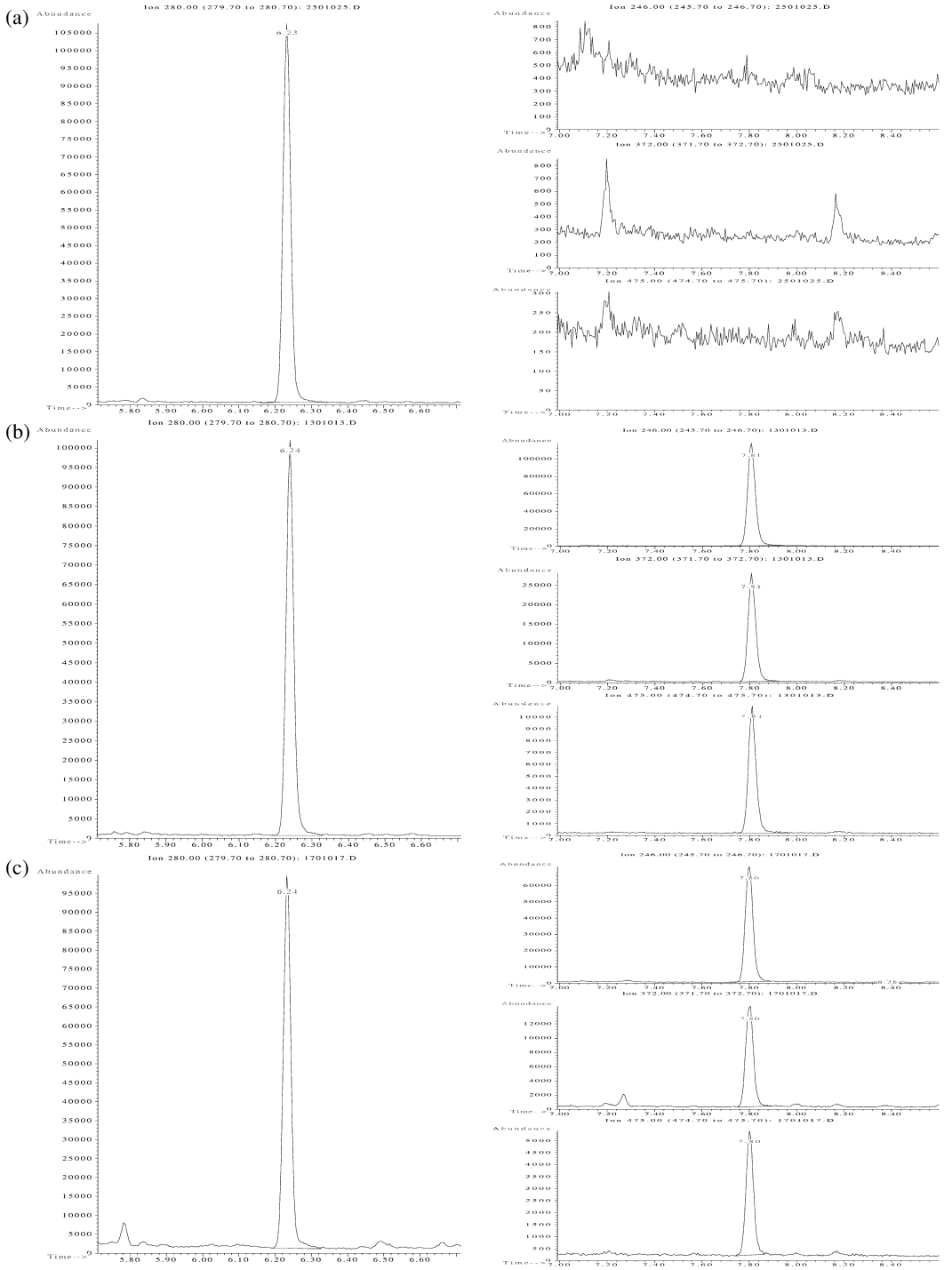

Fig. 3. Chromatograms obtained after analysis of (a) a blank urine, (b) a calibration urine spiked with 40 ng/ml of perphenazine and (c) an otter urine sample calculated concentration of 24.2 ng/ml.

Table 1
Intra-assay precisions and accuracies obtained in the quantification of perphenazine added to drug-free otter urine samples on 3 different days

Concentration (ng/ml)	Assay	*n*	Estimated concentration (mean±SD) (ng/ml)	Precision (RSD, %)	Accuracy (relative error, %)
6	1	3	4.8±0.2	5.3	19.5
	2	3	6±0.4	7.9	5.5
	3	3	6.6±0.5	8.6	10.5
16	1	3	15.8±0.3	2.6	1.7
	2	3	16.6±0.5	3.5	4.1
	3	3	16.6±0.7	5.2	3.6
60	1	3	65.5±1.7	3.3	9.1
	2	3	64.3±4.6	8.7	7.4
	3	3	65.6±0.7	1.3	9.4

Table 2
Inter-assay precision and accuracy obtained in the quantification of perphenazine added to drug-free otter urine samples

Concentration (ng/ml)	*n*	Estimated concentration (mean±SD) (ng/ml)	Precision (RSD, %)	Accuracy (relative error, %)
6	9	5.8±0.82	14.9	11.8
16	9	16.32±0.71	4.3	3.1
60	9	65.13±3.10	4.8	8.6

Intra- and inter-assay precisions and accuracies are presented in Tables 1 and 2, respectively. As can be observed, intra-assay precision and accuracy was from 1.3 to 8.7% and from 1.7 to 19.5%, respectively. Inter-assay precision and accuracy were in the ranges of 4.3–14.9 and 3.1–11.8%, respectively (Table 2). No significant differences were found between the concentrations of the control sample analysed with and without dilutions, so suitability of sample dilution was good.

3.4. Analysis of otter urine samples

The method was successful in determination of perphenazine in all studied samples, so detection of perphenazine was possible for a long time after administration (10–21 days depending on the otter). In Fig. 3, a chromatogram of an otter urine sample with perphenazine at a concentration of 24.2 ng/ml is shown.

Estimated concentrations were in the range of 4.1 ng/ml to 4.3 μg/ml. Maximum concentrations varied from 83.4 ng/ml to 4.3 μg/ml. No interferences were detected in otter urine samples, indicating the good selectivity of the procedure. In several urine otter samples a concentration higher than 100 ng/ml was obtained, so in these cases, suitable sample dilutions were prepared in order to obtain concentrations in the range of linearity.

In summary, a new procedure for determination of perphenazine in urine samples based on GC–MS has been developed. The method has proven to be sensitive, selective, accurate and precise. The method has been applied to the analysis of urines obtained from wild otters treated with perphenazine enanthate. Validation of the method has been performed using calibration curves prepared with human blank urine, and so, its applicability to the quantitation of perphenazine in human urine samples has also been demonstrated.

Acknowledgements

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