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Gas chromatographic–mass spectrometric analysis of veterinary tranquillizers in urine: evaluation of method performance

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Abstract

A method for analysis of veterinary tranquillizers in urine using gas chromatography–mass spectrometry (GC–MS) is described. Detection limits are 5 μg/l for ketamine, azaperone and the phenothiazines (chlor-, aceto- and propionylpromazine), 10 μg/l for haloperidol, 20 μg/l for xylazine and 50 μg/l for azaperol, recoveries for all analytes were higher than 70%. Method performance in terms of within-batch, between-days and between-analysts reproducibility was studied and found to be acceptable. Compliance with European Union criteria for confirmation of GC–MS ''positive'' results is evaluated and discussed.

Keywords: Ketamine; Azaperone; Phenothiazines; Haloperidol; Xylazine; Azaperol

1. Introduction

Veterinary tranquillizers are used to prevent mortality and loss of meat quality during transport to the slaughter house of animals that are sensitive to stress, especially pigs. The presence of residues of these drugs in edible tissues (e.g., kidney or liver) represents a potential consumer risk given their bioactivity. This fact has been recognised by the European Union (EU) and these compounds have been included among those that should be analysed in meat producing animals and their products (Group B-2-d in Council Directive 96/23/EEC [1]). Moreover, one of them (chlorpromazine) has been banned by the EU [2] and for another one (azaperone) there is a maximum residue limit [3].

The most common veterinary tranquillizers in our region are ketamine, xylazine, azaperone and the phenothiazines chlor-, aceto- and propionylpromazine. There are several published methods for analysis of these compounds based on high-performance liquid chromatography (HPLC) [4–7] and thin-layer chromatography (TLC) [8] but not for all of them, besides both techniques have some drawbacks. HPLC analysis of these drugs with the widely used reverse phase columns presents the problems that can be found with basic compounds, such as peak tailing and column batch-to-batch irreproducibility [9]. Besides full compound identification in HPLC is based on UV–visible spectra, which, even with modern diode-array detectors, pushes up the limit of unequivocal detection. TLC is a sensitive and relatively cheap technique that does

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not require extensive sample clean-up but positive results in EU countries require, at least, a further co-chromatography [10].

Gas chromatography–mass spectrometry (GC–MS) offers a good alternative in terms of sensitivity and ease of compound identification and it is the method of choice when full structural information and/or unequivocal identification is required (e.g., operated in SCAN mode). Two GC–MS methods have been published, although for tissue samples and not for all the compounds cited above [11,12].

Urine is a very convenient sample for drug monitoring since it can be easily obtained, it is readily available from both slaughter house and live animals (in fact it is almost the only feasible sample in this case) and can be used as a ''marker tissue''. Additionally, it is a somewhat cleaner sample than, for example, kidney or liver and does not require time consuming mincing, homogenising and extracting steps.

Our laboratory provides service to all veterinary officials in the region and has in consequence a high sample input, some of them being ''suspected'' samples that must be analysed as fast as possible, additionally samples from different sources are routinely received. Consequently, it was necessary to have a sensitive, reliable and high output method that could be used with urine samples from different species. The proposed method can be completed in 3 h for an up to 12-sample-batch and can be used for urine from bovines, sheeps and pigs.

2. Experimental

2.1. Chemicals and reagents

Ketamine, xylazine, chlorpromazine, acetopromazine, propionylpromazine and haloperidol were purchased from Sigma (St. Louis, MO, USA); azaperone and azaperol were obtained from Janssen Pharmaceutical (Beerse, Belgium). Stock solutions (1 mg/ml) in methanol of each standard were kept at −20°C in amber vials. Diluted mixed working standard was prepared from these stock solutions just before use.

Triethylamine (TEA) was purchased from Sigma. All additional chemicals used were analytical grade except ethyl acetate, which was residue analysis grade, all from Merck (Darmstadt, Germany). Purified water was produced with a Milli-Q system from Millipore (Bedford, MA, USA). For sample clean-up, 500 mg C_{18} columns from Varian (Harbor City, CA, USA) were used.

Sodium carbonate–sodium hydrogencarbonate buffer, pH 10 (carbonate–bicarbonate buffer) was prepared weekly dissolving together sodium carbonate and sodium hydrogencarbonate in purified water to obtain a concentration of 5% for both. The solution was kept at room temperature. All solutions containing TEA were prepared just prior to use and kept in a stoppered flask.

2.2. Apparatus

Solvent evaporation by nitrogen sparging was carried out in a Turbovap II (Zymark, Hopkinton MA, USA).

GC was performed in a Hewlett-Packard GC 6890 (Palo Alto, CA, USA) fitted with a Hewlett-Packard 6890 Series autosampler. Instrument control, data acquisition and data processing were carried out with G1701AA Chemstation software, also from Hewlett-Packard. The column used was a DB 5MS (30 m×0.25 mm I.D., 0.25 μm film thickness) from J&W Scientific (Folsom, CA, USA) fitted with a 5 m×0.25 mm I.D. deactivated fused-silica tube from Supelco (Bellafonte, PA, USA). A Hewlett-Packard 6890 Series quadrupole mass-selective detector was used for detection.

2.3. Sample treatment

A 2.5-ml volume of 3‰ TEA carbonate–bicarbonate buffer was added to 5 ml urine in a stoppered plastic tube, mixed by tumbling and vortexing and centrifuged at 28 000 *g* for 5 min. The resulting supernatant was percolated through a C_{18} column which had previously been activated with 5 ml of 1‰ TEA in methanol and washed with 5 ml of 1‰ TEA in water. After sample percolation the column was washed once with 1 ml of 1‰ TEA carbonate–bicarbonate buffer and twice with 2 ml of 1‰ TEA in water. Final elution was carried out with 10 ml of

1‰ TEA in *n*-hexane–2-propanol (9:1); the eluent was collected in a 50-ml Turbovap tube and evaporated at 40°C to 0.5 ml final volume under nitrogen sparging in a Turbovap II. After this evaporation step, 1 ml carbonate–bicarbonate buffer was added mixed by vortexing and extracted twice with *tert*.-butylmethyl ether. Ether extracts were pooled and evaporated to dryness at room temperature under nitrogen sparging. Dried extracts were reconstituted with 50 μl ethyl acetate and injected into the GC–MS system.

Control samples were analysed for every sample batch and were prepared spiking at detection level bovine urine previously found to be absent of all analytes. Spiked extracted blank samples (SEB samples) were obtained spiking extracted urine samples just before the last evaporation step (no analyte loss was detected in this step). In both cases the drug concentrations were 5 μg/l urine for ketamine, azaperone and chlor-, aceto- and propionylpromazine, 10 μg/l for haloperidol, 20 μg/l for xylazine and 50 μg/l for azaperol. Working standard preparation and sample spiking was always performed by the same analyst to reduce variability from this source.

2.4. Gas chromatography–mass spectrometry

GC conditions were as follows: initial temperature, 90°C for 2 min; raised at 30°C/min to 160°C and final rate, 10°C/min to 275°C, this temperature was held for 15 min. A 1-μl aliquot was injected into the GC–MS system in splitless mode. The injector port temperature was 260°C, the transfer line from GC system to the mass-selective detector was held at 275°C. Helium was used as the carrier gas at 1.5 ml/min initial flow. The mass selective detector was operated in electronic impact mode, using single ion monitoring (SIM) with different acquisition groups for each analyte except azaperone and acetopromazine, which due to their very close elution were included in the same acquisition group. Dwell time in all cases was 50 ms. Full mass scan confirmation was carried out injecting 2.5 μl into the GC system and scanning eluting peaks between 50 and 450 u.

3. Results and discussion

3.1. Chromatographic separation and selection of diagnostic ions

Fig. 1 shows a representative chromatogram of a control sample, separation of all compounds was possible with the exception of acetopromazine and azaperone, which could not be achieved using different temperature ramps, isothermal elution or combinations of both. Nevertheless, this partial separation combined with selected ion monitoring made possible the simultaneous analysis of these two compounds (see Fig. 1B).

Selection of diagnostic ions was carried out after acquisition of complete mass spectra for all analytes, both alone and in the working standard, with the same chromatographic conditions used to analyse samples. Ions chosen were those of both highest mass and intensity, the ions used in quantitation (e.g., quantifier ions) were the molecular ions when their intensity was not too low (as was the case of ketamine). Azaperol and haloperidol suffered high fragmentation in the ion source and only three high mass ions were compatible with a reasonable detection limit. Molecular ions were used for quantitation when possible with the exception of azaperone; in this case the molecular ion was sufficiently intense for quantitation but the extracted ion chromatogram at 327 u presented a peak for azaperone (M^+) and a very close peak for acetopromazine (isotopic of M^+ at 326 u) which complicated the reliable integration of the azaperone peak. Retention times of compounds and ions monitored are listed in Table 1. Analysis of known blank urines showed that no interfering peaks were present in the extracted ion chromatograms for each analyte.

3.2. Sample treatment

The veterinary tranquillizers to be analysed are basic compounds so that an alkaline environment during C_{18} clean-up was chosen to minimise their dissociation. Additionally, a high salt concentration was used to further increase their affinity to the C_{18} column. Preliminary studies led to very irreproducible recoveries for all analytes, even complete loss

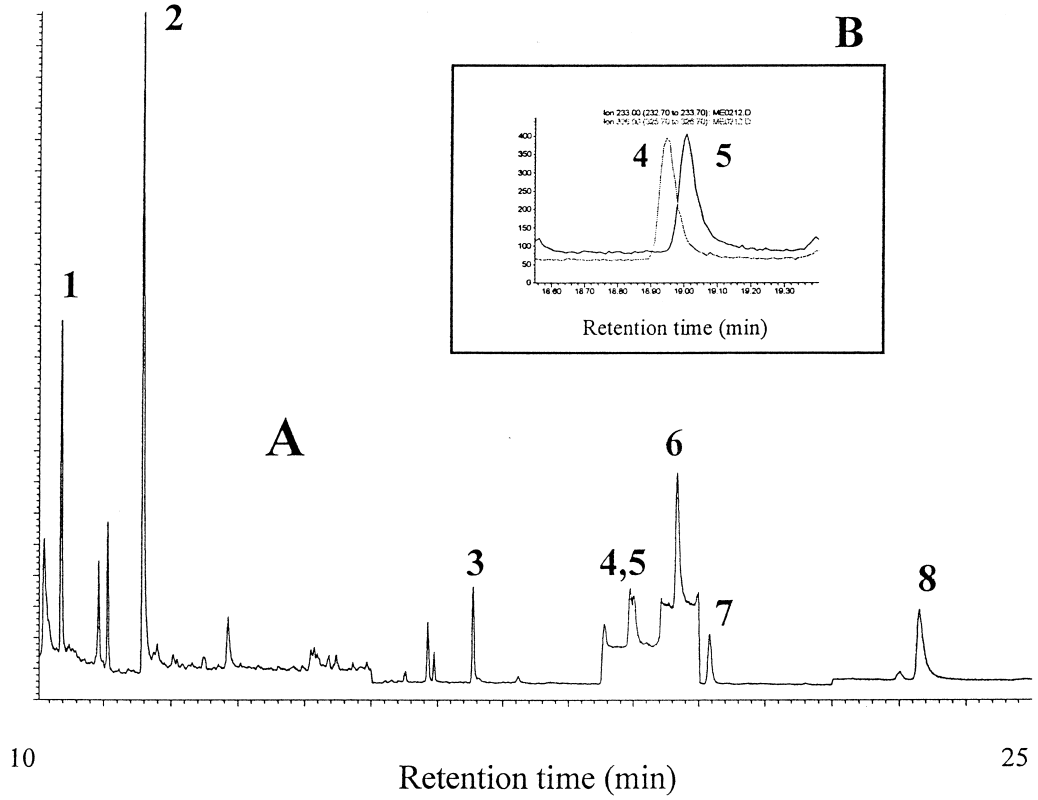

Fig. 1. (A) Total ion chromatogram of control sample. Numbers indicate individual compounds: 1=ketamine, 2=xylazine, 3=chlorpromazine, 4=acetopromazine, 5=azaperone, 6=azaperol, 7=propionylpromazine and 8=haloperidol. (B) Extracted ion chromatograms for acetopromazine and azaperone; 326 u and 233 u, respectively.

for some, within the same sample batch. After study of possible causes, the variability source was found to be the C_{18} column, very probably due to uncontrolled interactions of analytes with free silanols on the silica surface, a very common circumstance in the HPLC analysis of polar drugs [9]. 1‰ TEA was used to control this effect; this TEA concentration was present during activation, sample percolation,

Table 1
Retention time and diagnostic ions monitored for GC–MS analysis of veterinary tranquillizers; ions are listed in relative intensity order, lowest to highest (* denotes ion used in quantitation)

Compound	Retention time (min)	Diagnostic ions (m/z)				Molecular ion (m/z)
Ketamine	10.38	102	152	209	180*	238
Xylazine	11.58	187	177	220*	205	220
Chlorpromazine	16.55	247	232	272	318*	318
Acetopromazine	18.95	197	241	280	326*	326
Azaperone	19.01	309	327	208	233*	327
Azaperol	19.67	176	222	235*		235
Propionylpromazine	20.17	269	255	294	340*	340
Haloperidol	23.30	206	237*	224		237

washing and final elution of the C_{18} columns. Use of TEA is essential to obtain reproducible results.

After elimination of water carry-over, the final eluate could be evaporated to dryness, reconstituted in ethyl acetate and injected into the GC–MS system. However, it was found better to resuspend the residue in carbonate–bicarbonate buffer and to extract the analytes with an organic solvent; *tert*.-butylmethyl ether was chosen since high recoveries from aqueous solutions have been reported for these analytes using this solvent [11] and it is readily evaporated at room temperature.

3.3. Detection limit

To estimate the limit of detection (LOD), spiked samples at different concentrations and from different sources were analysed. The LOD for each compound was fixed at the concentration in which it was possible to detect all ions with a signal-to-noise better than 10. This criteria results in LODs of 5 μg/l for ketamine, azaperone and the phenothiazines (chlor-, aceto- and propionylpromazine), 10 μg/l for haloperidol, 20 μg/l for xylazine and 50 μg/l for azaperol. Fig. 2 shows results obtained with two real samples, one of which contained azaperol as confirmed by mass spectra of the suspected peak.

3.4. Method performance

LODs are dictated by the least abundant ions, since the quantifier ions are more intense, it results in a high signal-to-noise ratio for quantitation, which implies reliable integration of the extracted chromatograms for the quantifier ion. This fact allows for easy control of analytical output. It should be noted, nevertheless, that the method was not used for quantification purposes of ''positive'' samples, since there is not an EU maximum residue limit for these compounds in urine [3].

To obtain data on within batch repeatability, both control and SEB samples were processed by the same operator. This approach was used to avoid ''matrix-effects'' on quantitation (see below). Recovery was calculated comparing abundance of quantifier ion in SEB samples with that of the control samples; results are shown in Table 2. Recoveries for all analytes were higher than 70% and with acceptable variability for a multiresidue method at trace levels.

Control of recovery through SEB samples is not an attractive alternative since it implies the need of processing an additional sample; on the other hand, to use standards to the same end makes it necessary to ascertain the presence of ''matrix-effects'', e.g., changes in ionization patterns in the ion source due to the presence of coeluting compounds; these effects are common for certain analytes/samples (e.g., pesticides in fat). To test for this effect, SEB samples and working standards of the same concentration were injected into the GC–MS system. Abundance of quantifier ion was determined and a ''matrix-effect factor'' was calculated as response in SEB samples divided by response in standards. To test whether the matrix-effect factor obtained reflected a real difference (e.g., matrix effects), response in SEB samples and standards were compared using a two-tailed Student *t*-test. Results summarised in Table 3 indicate statistically significant matrix effects for ketamine, xylazine, azaperone and haloperidol.

Method repeatability over time was estimated with data from all control samples processed by one operator in a several months period. Matrix-effect factors were used for ketamine, xylazine, azaperone and haloperidol. Results are shown in Table 2, again both recoveries and repeatability were acceptable.

The method described has been used in a routine basis for some months in our laboratory for monitoring of these drugs (over 300 samples processed) so that different operators have processed control samples; this fact gives an opportunity to study method reproducibility. The reproducibility data listed in Table 2 include both within-batch and between-days variability for operators 2 and 3 since each operator analysed one control sample each day. Lower recoveries were obtained by operator 3, probably due to his not performing the method on a regular basis and/or his somewhat lesser experience.

3.5. Ion ratios and EU criteria

Confirmation of GC–MS positive results in EU countries needs to fulfil certain criteria [10], the most restrictive (although not mandatory) of which concerns ratios of diagnostic ions. These ratios are defined as abundance of each ion divided by abun-

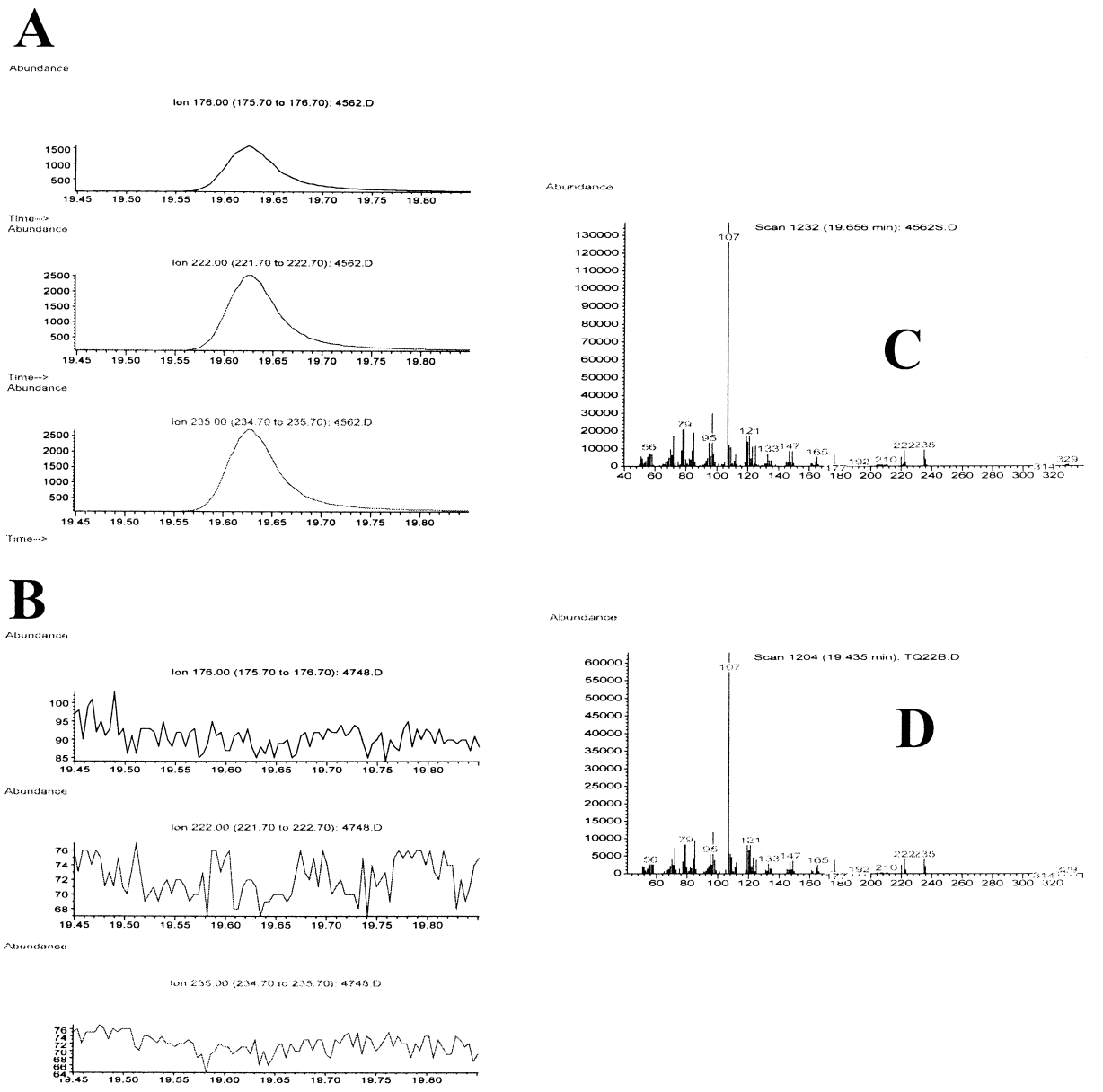

Fig. 2. Extracted ion chromatograms for azaperol peak in ''positive'' real sample (A) and in ''negative'' real sample (B). (C) Mass spectra of peak in (A). (D) Mass spectra of azaperol peak in working standard.

dance of the most abundant ion. EU criteria demands that for a sample to be considered positive the difference between each ion ratio of a sample and the same ion ratio of a standard does not exceed 10% the value of the standard. To evaluate compliance with the EU criterion, for each batch, the absolute difference between the control sample and the standard was divided by the corresponding ion ratio of the

Table 2
Method repeatability and method reproducibility with control samples processed by three different operators[a]

Compound	Operator			
	1		2	3
	Within batch (n=9)	Between days (n=21)	Between days (n=13)	Between days (n=6)
Ketamine	84.6±4.2	80.6±6.4	88.0±3.0	84.0±3.4
Xylazine	98.2±6.0	85.9±8.5	95.5±7.1	66.0±15.6
Chlorpromazine	78.0±10.5	70.1±6.5	78.5±9.4	55.1±6.5
Acetopromazine	88.2±8.7	99.2±8.4	108.9±6.6	66.8±8.8
Azaperone	85.9±6.8	101.5±8.7	109.2±5.6	94.6±5.0
Azaperol	77.2±15.4	98.8±10.3	110.2±8.9	61.9±14.4
Propionylpromazine	85.1±9.9	78.6±7.4	90.2±9.2	57.7±6.4
Haloperidol	85.4±14.3	88.4±13.9	96.3±11.6	63.0±22.7

[a] The results shown are mean recoveries (%)±S.E.M., figures in parentheses are number of samples. Drug concentrations were 5 μg/l urine for ketamine, azaperone and chlor-, aceto- and propionylpromazine, 10 μg/l for haloperidol, 20 μg/l for xylazine and 50 μg/l for azaperol.

Table 3
Matrix effects for veterinary tranquillizers[a]

Compound	Matrix-effect factor
Ketamine	1.64*±0.08
Xylazine	0.64*±0.08
Chlorpromazine	1.05±0.07
Acetopromazine	0.81±0.08
Azaperone	0.58*±0.10
Azaperol	1.25±0.16
Propionylpromazine	1.06±0.09
Haloperidol	1.82*±0.25

[a] Matrix-effect factor is defined as response in SEB divided by response in standard (n=13). The values shown are mean±standard error of the mean (S.E.M.). * Denotes statistically significant difference (P<0.05) between both responses (two-tailed Student t-test).

standard and expressed as a percentage (RDR in Table 4). The 327 u fragment of azaperone was not used in the calculation due to the very close eluting peak of acetopromazine, see Section 3.1. The criterion demands that RDR for each ratio does not exceed 10%. The results in Table 4 indicate that the confidence intervals of means for ketamine, xylazine and chlorpromazine are outside the required values for, at least, one ion ratio, probably due to background from matrix compounds. The minimum number of replicates (both of standards and samples) for compliance are two for propionylpromazine, three for azaperone and four for acetopromazine; azaperol and haloperidol comply with a single analysis.

Table 4
Evaluation of compliance with EU criteria for ion ratios[a]

Compound	RDR (ratio 1)	RDR (ratio 2)	RDR (ratio 3)
Ketamine	3.7±10.4	93.6±27.0	7.6±1.4
Xylazine	6.3±1.0	20.3±7.4	2.7±0.9
Chlorpromazine	1.9±0.5	10.2±1.4	1.6±0.3
Acetopromazine	7.1±1.2	6.1±1.0	5.5±0.9
Azaperone	6.3±1.2	7.1±1.0	
Azaperol	3.9±0.9	1.8±0.4	
Propionylpromazine	6.3±0.8	4.8±0.9	3.2±0.3
Haloperidol	4.6±0.7	2.4±0.8	

[a] Ion ratios defined as abundance of each ion divided by abundance of the most abundant ion (see Table 1) were calculated for control samples and standards. For each ion ratio and sample batch, the relative difference in ratios (RDR) was calculated: RDR=100×|[ion ratio (spiked sample)−ion ratio (standard)]|/ion ratio (standard). The results shown are means±S.E.M. (n=21).

4. Conclusions

The method reported allows sensitive, high output analysis of veterinary tranquillizers in urine from different species. Analysis is performed by GC–MS so that direct structural information is obtained, which reduces the risk of false positives.

Detection limits are 5 μg/l for ketamine, azaperone and the phenothiazines (chlor-, aceto- and propionylpromazine); 10 μg/l for haloperidol, 20 μg/l for xylazine and 50 μg/l for azaperol. Both recovery for all compounds and reproducibility between experienced operators are good.

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