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Detection of diazepam in horse hair samples by mass spectrometric methods

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A method for the detection of diazepam in horse hair samples by low resolution gas chromatography–mass spectrometry (GC-MS) was developed. Two other techniques, gas chromatography–high-resolution mass spectrometry (GC-HRMS) and high-performance liquid chromatography–atmospheric pressure chemical-ionisation mass spectrometry (HPLC-APCI-MS-MS) were applied on some selected samples. Sample preparation was performed according to a technique previously described for human hair, involving incubation with Sorensen buffer and solvent extraction. Hair samples from different sites such as coat on the neck, coat on the back, mane and tail were collected from two thoroughbreds which had received several dosages of diazepam corresponding to a total dose of 750 mg and 200 mg of diazepam respectively. In the first experiment, by low resolution GC-MS using single ion monitoring, diazepam was detected in the mane for at least 85 d after the last administration. In the second one, using the same method, diazepam was detected in the coat on the neck up to 25 d following the last administration. Low resolution GC-MS data were confirmed by the two other techniques. Furthermore, GC-HRMS even made possible the detection of diazepam up to 38 d after the administration of 200 mg of diazepam.

Introduction

The 1,4-benzodiazepines are widely prescribed drugs, with diazepam being the most well-known. Their determination is important for pharmacological studies as well as in forensic science. Regarding mass spectrometry, 1,4-benzodiazepine compounds were successfully examined by electron impact, positive and negative chemical ionisation and electrospray.[1–4] More recently, Cirimele *et al.*[5] and McClean *et al.*[6] were interested in the detection of benzodiazepines in human hair. In the past decade, the analysis of hair for the detection and quantification of drugs has gained widespread interest in forensic science. A few papers deal with this subject in the horse field.[7] In order to investigate the detection of drugs in horse hair, we considered that diazepam was a compound of choice to carry out a pilot study. In the horse diazepam is mainly used for its tranquillising and muscle relaxant properties.[8] The goal of the present study is to look at diazepam and one of its main metabolites, nordiazepam,[9] in horse hair, *i.e.* coat on the neck and coat on the back, tail and mane after its administration to the horse. In order to evaluate the analytical performance, three techniques were used: (i) GC-MS on a quadrupole instrument in single-ion monitoring (SIM) and scan mode; (ii) gas chromatography–high-resolution mass spectrometry (GC-HRMS) in SIM mode on a magnetic sector instrument; and (iii) HPLC-atmospheric pressure chemical-ionization (APCI)-MS-MS on an ion trap instrument in scan mode. Low resolution GC-MS was applied to all samples, while the two other techniques were conducted on some selected samples only.

Experimental

Diazepam administration

Two bay thoroughbreds were selected for this study. Each administration corresponded to 50 mg of diazepam injected intramuscularly, alternately on each side of the lower part of the neck.

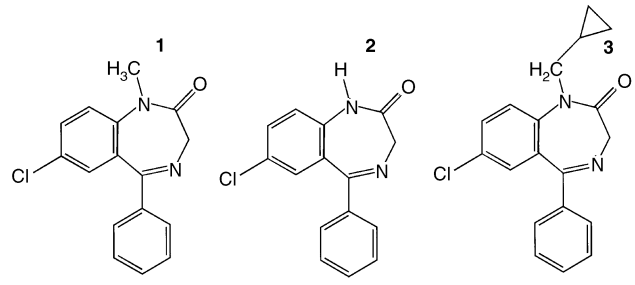
Experiment 1 was started in the autumn for the first horse which received 750 mg in total, administered in three sets of six, five and four dosages of diazepam, respectively. There was an interval of 11 d between the first two sequences and 37 d between the last ones.

Experiment 2 was started in spring for the second horse which received 200 mg of diazepam in total, corresponding to one sequence of four administrations within three days.

Samples of the coat, mane and tail were collected. Before starting the experiment, specific areas on the tail and the body were defined for sampling. The coat was taken on the left side of the neck in the upper part and on the back, behind the saddle position. These areas were clipped. Hair samples were collected periodically as shown in Tables 1 and 2.

Standards and reagents

Analytical grade solvents were obtained from Prolabo (Paris, France). Diazepam, nordiazepam and prazepam (Scheme 1) were purchased from Sigma (St. Louis, MO, USA). A stock



Scheme 1 Chemical structure of benzodiazepines of interest: 1, Diazepam (M_r 284.0716); 2, nordiazepam (M_r 270.0559); 3, prazepam (M_r 324.1029).

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solution of each drug was prepared by dissolving 10 mg in 10 ml of methanol. The solutions were protected from light and stored at 4 °C. Appropriate dilutions were made to prepare the standard working solutions.

Sample preparation

The hair samples were washed using an aqueous solution of sodium dodecylsulfate at 1 mg ml^{-1}. The samples were then ground and treated with Sorensen buffer at pH 7.6, according to the method described by Cirimele *et al*.[5] Hair or mane (25 mg) were supplemented with 30 ng of prazepam (internal standard) in 1 ml of buffer and then incubated at 40 °C for 2 h. Solvent extraction was carried out using 1.5 ml of a mixture of ethyl ether–chloroform (8 + 2). Solvent was evaporated and the residue dissolved in 20 μl of chloroform. A 1.5 μl volume was injected. The residues which were analysed by HPLC-APCI-MS-MS were dissolved in 200 μl of mobile phase.

Instruments

GC-MS analyses were performed in the positive ion electron impact (EI) mode on a HP5971 (Hewlett-Packard, Palo Alto, CA, USA) and on a Finnigan-MAT (Bremen, Germany) 95 XL instrument.

The HP 5971 mass selective detector (MSD) was coupled with an HP 5890 gas chromatograph equipped with a bonded phase fused silica capillary column HP5 Trace (ref 19091M-433; 30 m × 0.25 mm id, 0.25 μm film thickness). Operating conditions were: time for the splitless injection was set at 1.0 min, helium inlet pressure was at 11.5 psi (1 psi = 6894 Pa), initial temperature was set at 60 °C, ramped at 20 °C min^{-1} up to 305 °C and held for 5 min. The transfer line temperature was set at 280 °C. Detection of diazepam and nordiazepam was achieved by monitoring ions at *m/z* 256 and *m/z* 283 for diazepam and at *m/z* 241 and *m/z* 270 for nordiazepam.

Quantification of diazepam was carried out using the ions mentioned above for diazepam and the ions at *m/z* 295 and *m/z* 324 for the internal standard (prazepam).

Table 1 Diazepam concentrations in horse hair samples from the first experiment obtained by low resolution GC-MS in SIM mode.

Time/d					
After the 2nd sequence of administrations	After the 3rd sequence of administrations	Diazepam concentration/pg mg^{-1}			
		Coat on the neck	Coat on the back	Mane	Tail
10	[a]	[b]	[b]	37	[b]
20	[a]	67	< 25	< 25	32
62	25	249	70	71	36
91	54	930	< 25	< 25	[b]
122	85	[b]	[b]	< 25	[b]
132	95	[b]	[b]	nd[c]	[b]

[a] Only two sequences of administrations took place. [b] Hair samples were not collected. [c] nd: not detected.

Table 2 Diazepam concentrations in horse hair samples from the second experiment obtained by low resolution GC-MS in SIM mode

Time after the last administration/d	Diazepam concentration/pg ml^{-1}	
	Coat on the neck	Mane
25	73	nd[a]
38	nd	nd
55	nd	nd

[a] nd: not detected.

GC-HRMS was carried out with a reversed geometry, double focussing mass spectrometer (Finnigan MAT 95) coupled to an HP 5890 gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a HP5 Trace column. GC conditions were: initial temperature was set up at 60 °C for 0.5 min, then ramped up to 20 °C min^{-1} to 300 °C. The ion source was held at 270 °C. High-resolution selected ion monitoring was performed by electric field scanning using a reference gas (perfluorophenanthrene) for mass locking and mass calibration.

At a mass resolution of 5000, the following ions were monitored at *m/z* 283.0638, *m/z* 256.0529 and *m/z* 241.0533 for diazepam, at *m/z* 269.0481 and *m/z* 241.0533 for nordiazepam and *m/z* 295.1002 and *m/z* 269.0481 for prazepam.

HPLC-APCI-MS-MS was performed on the LCQ mass spectrometer (Finnigan) which was linked to an HPLC pump P4000 and an autosampler AS3000 Spectrasystem from TSP. The HPLC column was a Colochrom Nucleosil C18 (Macherey Nagel, Düren, Germany), 100 Å, 3 μm (150 × 4.6 mm id) and the mobile phase was a mixture of methanol and water (8 + 2, v/v) flowing at 0.75 ml min^{-1}.

The heated capillary was maintained at 150 °C and the vaporizer heater at 450 °C. The corona discharge was set to 4.5 kV. The instrument was operated in MS-MS full scan mode on the ions at *m/z* 285 for diazepam, at *m/z* 271 for nordiazepam and *m/z* 325 for prazepam at 20.0, 20.0 and 19.0%, respectively, of collision.

Results and discussion

Low resolution GC-EIMS SIM mode

Under these chromatographic conditions, there was no interference with diazepam and the internal standard prazepam, by any extractable endogenous material present in horse hair. Validation parameters obtained from this method are summarised in Table 3. The limit of detection (calculated for a signal to noise ratio of 3) using a 25 mg sample size was in accordance with data on diazepam in human hair.[5] Nevertheless, due to an interfering compound, quantification of nordiazepam was not possible.

As shown in Tables 1 and 2, this method was applied to all the hair samples from the two horses. In Experiment 1, diazepam was detected in the coat of the back and in the coat of the neck 54 d after the third sequence of administration. Taking the entire length of the mane, diazepam was detected 85 d after the last administration, the diazepam concentration being less than 25 pg mg^{-1}.

In the tail, diazepam was detected 20 d after the second sequence of administration and 25 d after the third sequence of administration.

The collection of further samples was not possible, therefore the detection period remains unknown, especially for the hair on the neck. In comparison with urine, after an im administration of 10 mg of diazepam, the drug was detected for 30 h after injection.[8] In addition nordiazepam was detected in all the hair samples except the one in which diazepam was not.

Table 3 Validation data obtained by low resolution GC-MS in SIM mode

Parameters	Results
Extraction yield (spiked sample 75 pg mg^{-1})	80%
Calibration curve	$y = 0.0015x + 0.0038$ ($r = 0.998$)
Detection limit	2.5 pg mg^{-1}
Quantification limit	25 pg mg^{-1}
Accuracy (spiked sample 80 pg mg^{-1})	91%
Accuracy (spiked sample 120 pg mg^{-1})	86%

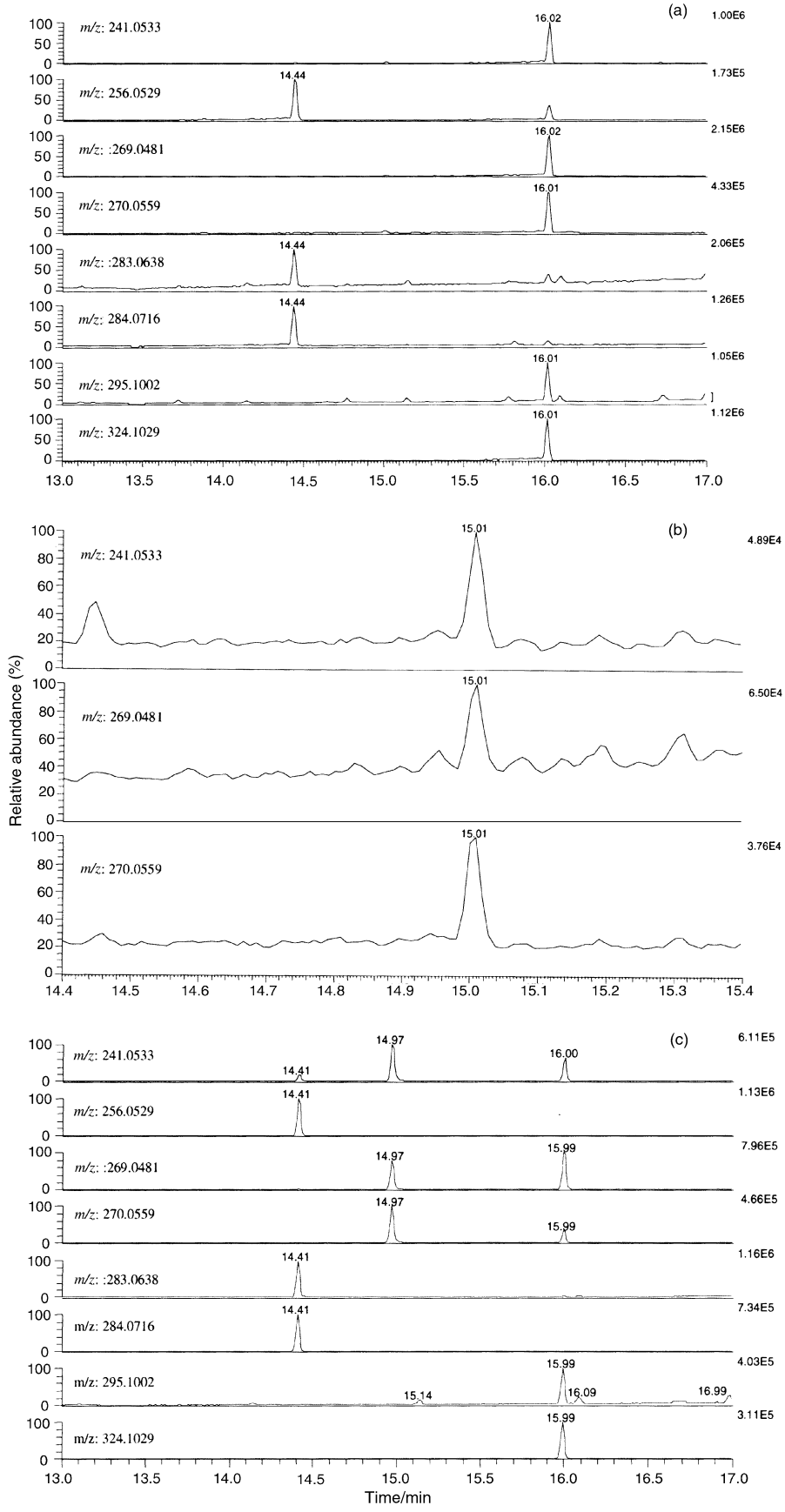

Fig. 1 Selected mass chromatograms (a and b) obtained from a horse hair sample (coat on the neck) collected 25 d after the third sequence of administrations (experiment 1); (c) a standard sample (0.2 ng μl^{-1}). The acquisition was made on the diazepam ions (14.44 min) at *m/z* 256.0529 $[M - CH_2N]^+$ and *m/z* 283.0638 $[M - H]^+$, the nordiazepam ions at *m/z* 241.0533 $[M - H–CO]^+$ and *m/z* 269.0481 $[M - H]^+$ and the prazepam ions (16.01 min) *m/z* 295.1002 $[M - H–CO]^+$ and *m/z* 324.1029 M^+.

In Experiment 2, diazepam was detected in the coat on the neck up to 25 d after the last 50 mg administration (total dose of 200 mg). In the urine, diazepam was detected up to 4 d after administration and hydroxydiazepam up to 6 d.

Low resolution GC-EIMS scan mode

As shown in Table 1, the highest concentration of 930 pg mg^{-1} was found in the hair sample (coat on the neck) collected at 54 d after the last administration. Diazepam in this sample was identified using the scan mode. This high concentration was unexpected although diazepam was injected in one part of the neck at a reasonable distance from the sampling area. Because of the lower diazepam concentration in the other samples, low resolution GC-EIMS scan mode was not applicable to the other samples.

High resolution GC-EIMS SIM mode

Diazepam was found in the hair sample (hair on the neck coat) taken 25 d after the third sequence of administrations (Fig. 1a). These data confirm the previous one obtained by low resolution GC-MS SIM mode with a higher degree of confidence. Furthermore (Fig. 1b), nordiazepam was also detected, the retention time being in accordance with the reference standard samples (Fig.1c). This method may be helpful to establish a correlation between the parent drug and nordiazepam.

Regarding the second experiment corresponding to a total dosage of 200 mg, diazepam was detected in the hair sample (hair on the neck coat) taken 38 d after the last administration. Therefore taking into account the HRMS data, the detection period has been significantly improved. As previously shown[10] for clenbuterol in hair, HRMS significantly improves the signal-to-noise ratio.

HPLC-APCI-MS-MS scan mode

Diazepam was found in the two samples which were submitted to this analysis. These samples corresponded to hair on the neck coat and mane collected at 25 d after the last sequence of administrations. Nordiazepam was not found in these samples. By HPLC-APCI-MS-MS scan mode, (Figs. 2a, b, c, d), the mass spectra (Figs. 2b and 2d) confirmed the presence of diazepam in the samples. Due to the full scan MS-MS spectra, the identification of the compound is more certain. To avoid the presence of interferents, additional work is needed to improve the HPLC method and determine the detection limit.

This preliminary study demonstrates that the concentrations found in hair samples are often low and therefore the use of different methods is preferable. Low resolution GC-MS by SIM mode on two ions made it possible to detect and quantify

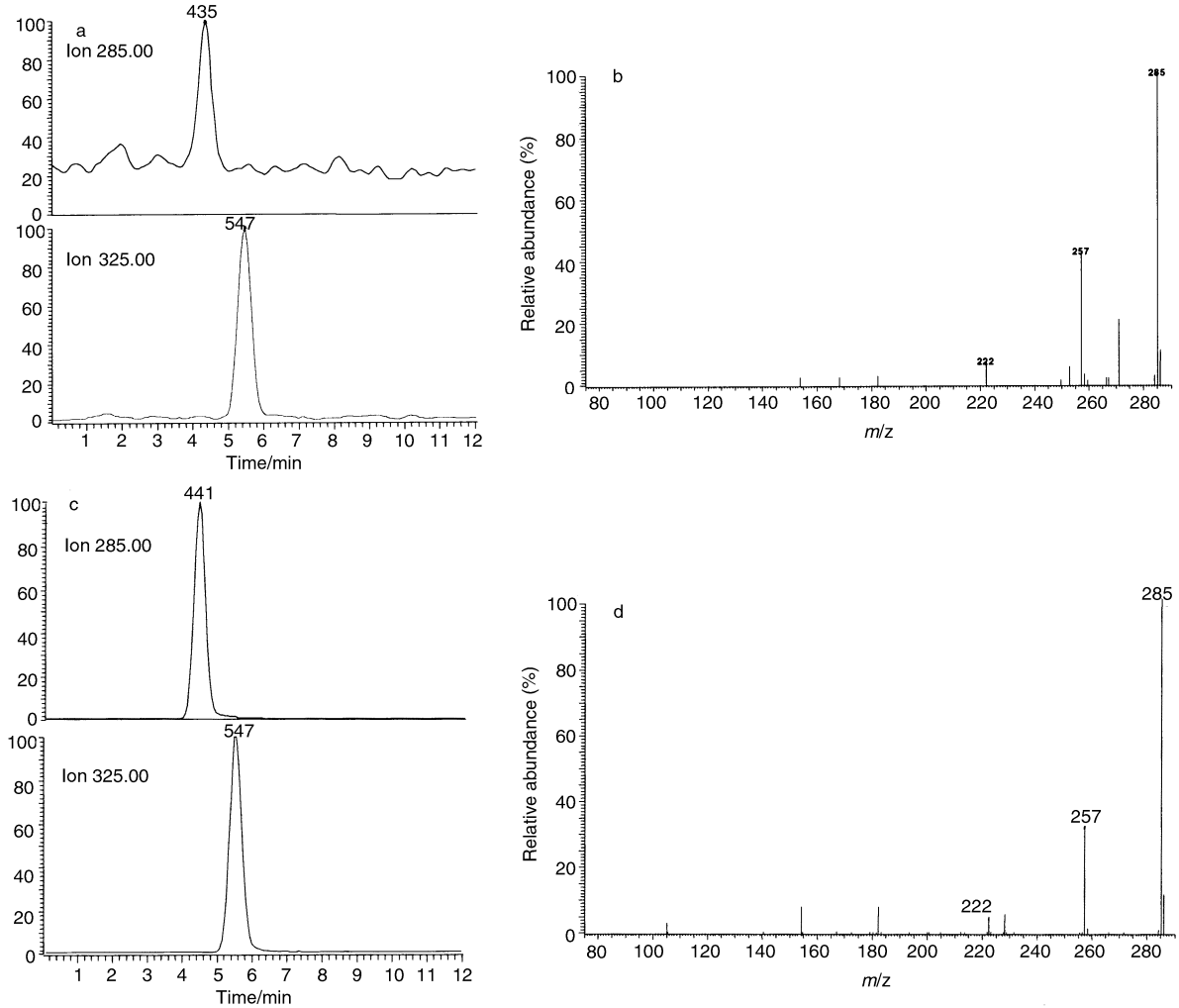

Fig. 2 Chromatogram (a) and full MS-MS spectrum of diazepam (b) obtained from a horse hair sample (coat on the neck) (experiment 1) collected 25 d after the third sequence of administrations; chromatogram (c) and full MS-MS spectrum (d) obtained from a standard. The two ions at *m/z* 285 and *m/z* 325 are respectively $[M + H]^+$ of diazepam and the internal standard prazepam.

diazepam for up to 85 d in the mane. A sensitivity improvement was observed by HRMS leading to a longer detection time for diazepam. In most cases it was impossible to obtain a full mass spectrum by low resolution GC-MS, therefore HRMS and LC-MS-MS were necessary to improve the data specificity. Further investigations are needed to examine fully the significance of drug testing in horse hair. Nevertheless, hair analysis should be limited to special applications of horseracing control, and might be proposed as an additional medium in the future.

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