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DETECTION AND QUANTITATION OF 3-HYDROXYBUPIVACAINE IN HORSE URINE

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ABSTRACT

The local anaesthetic bupivacaine is used in equine medicine and is classified as a Class 2 foreign substance by the Association of Racing Commissioners International (ARCI). Its identification in post race urine may cause the imposition of significant penalties. Therefore analytical procedures for screening and confirmation of this medication are being developed.

Gas chromatographic/mass spectrometric (GC/MS) screening of equine urine, after dosing with bupivacaine, revealed the major metabolite recovered as a hydroxylated bupivacaine. As a result, 3-hydroxybupivacaine was synthesised, purified and characterised and a quantitative mass spectrometric method was developed for the metabolite as recovered from horse urine. We confirmed hydroxylation at the 3-position by comparision with authentic 4-hydroxybupivacaine. Following subcutaneous injection of the highest no-effect dose (HNED) of bupivacaine (0.25 mg) the concentration of 3-hydroxybupivacaine recovered from horse urine reached a peak of 38 ng/ml at 4 h post administration, as measured by GC/MS. The analytical procedure involved β-glucuronidase hydrolysis, solid phase extraction with Clean Screen columns, derivatisation with N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) to give tBuDMS derivatives and automated injection on a H-P 6890/5972 GC/MS equipped with a 5%-diphenyl-95%-dimethylpolysiloxane capillary column. A standard curve was established with 3-hydroxylidocaine as internal standard.

Enzyme-linked immunosorbent assay (ELISA) of untreated urine was performed by means of an assay generated originally against the related drug mepivacaine and with which bupivacaine and its metabolites show high cross-reactivity. Results following subcutaneous injection of 0.25 mg bupivacaine generally mirrored those of GC/MS, with negligible (<10 ng/ml) measurements during the first hour, values of 20–40 ng/ml during the 2–6 h time span and negligible values at 8–24 h.

INTRODUCTION

Bupivacaine is a potent local anaesthetic (LA), capable of significant anaesthesia with doses as low as 0.5 mg/site (Harkins *et al.* 1996). It is considered to have a potency and toxicity 4 times that of the structurally related drug mepivacaine (Goehl *et al.* 1973). Bupivacaine produces conduction blockade in sensory neurons by retarding the influx of Na^+ ions and is listed as a Class 2 substance by the Association of Racing Commissioners International (ARCI). Identification of bupivacaine in post race urine samples can therefore result in substantial penalties. However, it is not often reported internationally. It is therefore important to determine dose and time response relationships for the local anaesthesia produced by this agent, as well as time periods for which bupivacaine metabolites remain detectable in plasma or urine after administration of therapeutic doses.

The Agriculture Canada Equine Drug Evaluation Center (1991) reports a 24 h detection time for bupivacaine following a 50 mg im dose. However, this report provided no further analytical details. Information on the nature of metabolites, their concentrations and means of detection, and biological effects are of importance for officials to assess the true nature of a positive bupivacaine report for a post race sample.

The objectives of this study were to: 1) identify the principal equine urinary metabolite of bupivacaine; 2) devise an analytical GC/MS method for its quantitation; 3) validate a sensitive ELISA test for bupivacaine in equine serum or urine; and 4) determine the urine concentration of bupivacaine and its metabolites after administration of the HNED to establish pharmacological relationships for bupivacaine in the horse. Because pharmacological effects and route of administration

are linked, the HNED must be defined with regard to a specific route of administration. It should be appreciated that this report is preliminary and further details, such as physiological determination of the HNED of 0.25 mg/site, will be published in the near future (Harkins *et al.* 1999b).

Materials and methods

Horses and sample collection

Animals used in these experiments were managed according to the rules and regulations of the University of Kentucky Institutional Animal Care Use Committee, which also approved the experimental protocol. Thoroughbred mares weighing 460–580 kg were maintained on grass hay and a twice daily dry feed (12% protein), consisting of a 50:50 mixture of oats and an alfalfa-based protein pellet. A routine clinical examination was performed before each experiment to ensure that the animals were healthy and sound. During experimental procedures, horses were provided with water and hay *ad libitum*.

The HNED for bupivacaine (0.25 mg) has been determined and will be reported elsewhere (Harkins *et al.* 1999b). Multiples of this HNED were injected subcutaneously in horses. During the first day, complete urine collection was accomplished with a Foley catheter (24 Fr; Rusch Inc., Georgia, USA) and attached plastic bag. Blank samples were collected during the 30 min period before administration and further test samples were then collected at 0–0.5, 0.5–1, 1–2, 2–4, 4–6 and 6–8 h after administration. Finally, 24 h after administration, a Harris flush tube (24 Fr x 60 in; Seamless, Florida, USA) was used to collect a maximum of 300 ml urine. All urine samples were divided into aliquots and stored at -20°C until assayed.

ELISA testing for bupivacaine

The one step mepivacaine ELISA used for this analysis has been described previously (Harkins *et al.* 1999a) and its cross-reactivity with related drugs has also been reported (Woods *et al.* 2000). Standard curves for the ELISA were generated with bupivacaine.

Synthesis and characterisation of 3-hydroxybupivacaine

Synthesis of 3-hydroxybupivacaine from bupivacaine was analogous to the previously reported synthesis of 3-hydroxylidocaine (Harkins *et al.* 1998). Nitration of bupivacaine under classic conditions (concentrated sulphuric/nitric acid mixture) resulted in the 3-nitro compound which, when reduced with zinc dust in 50% acetic acid, produced 3-aminobupivacaine in high yield. After diazotisation of this amine with sodium nitrite in sulphuric acid and following hydrolysis in acidic conditions at 70°C, the desired 3-hydroxybupivacaine was obtained, which was then purified by column chromatography on silica gel and crystallised finally from acetone. The 4-hydroxybupivacaine was provided by Maxxam Analytics.

TABLE 1: Comparison of retention times for 3- and 4-hydroxybupivacaine mono-TMS derivatives with urine from a horse dosed with 250 mg bupivacaine

Compound	Retention time (min)	Average retention time (min)	Standard deviation
3-hydroxybupivacaine (synthetic)	1) 38.37 2) 38.37 3) 38.37 4) 38.42 5) 38.34 6) 38.36	38.372	0.024
4-hydroxybupivacaine (synthetic)	1) 38.61 2) 38.61 3) 38.60 4) 38.65 5) 38.56 6) 38.58	38.601	0.028
Urine from bupivacaine-dosed horse (hydroxybupivacaine of essentially one type only)	1) 38.36 2) 38.33 3) 38.32 4) 38.39 5) 38.47 6) 38.31	38.363	0.055

TABLE 2: Average concentration (ng/ml) of bupivacaine equivalents measured by ELISA after subcutaneous administration of different levels

Dose (mg)	Time post dosing (h)							
	0	0.5	1.0	2.0	4.0	6.0	8.0	24.0
0.25	7.0	23.7	20.9	23.3	18.2	15.3	15.1	9.7
0.75	45.6	51.1	40.8	26.1	12.9	15.6	27.4	2.6
2.25	18.3	22.0	69.2	110.9	52.8	24.5	13.1	69.6
2.50	0.0	0.0	13.2	21.8	15.2	47.9	14.9	15.6
6.75	13.6	15.7	55.9	68.3	45.4	73.3	36.0	2.3
20.0	0.0	101.5	432.6	275.4	341.8	334.9	203.2	16.0
25.0	5.0	0.0	66.8	25.2	94.1	66.8	71.7	16.9
60.0	24.2	123.9	1024	1343	707.3	1007	709.3	57.7
250.0	10.6	103.2	660.3	2418	3768	2135	2098	370.2

GC/MS quantitation of 3-hydroxybupivacaine

Urine samples from a bupivacaine-treated horse were quantitated for 3-hydroxybupivacaine by GC/MS. Samples (5 ml/sample) were hydrolysed with 1 ml β-glucuronidase (5,000 units/ml, Type L-II; Sigma) and 2 ml 1M sodium acetate, pH 5.0. Standard solutions of 3-hydroxybupivacaine with 3-hydroxylidocaine as internal standard were prepared in methanol and then added to blank hydrolysed urine over a range of 4–160 ng/ml. All samples were sonicated for 90 s and extracted with Clean Screen SPE columns. The dichloromethane eluent was evaporated under a stream of nitrogen and reacted with 40 µl MTBSTFA plus 1% TBDMCS (Regis). Further details on extraction and derivatisation are included elsewhere (Woods *et al.* 2000).

GC/MS methodology

GC/MS methods are essentially identical to those reported by Woods *et al.* (2000). For identification of 3-hydroxybupivacaine in horse urine, trimethylsily (TMS) derivatives were prepared by reaction of 50 µl BSTFA + 1% TMCS (Pierce) 30 min at 70°C with urine extracts described above. Full scan spectra were generated over the 50–550 m/z range. For quantitation, the MS was operated by selected ion monitoring (SIM) of ions 140.1 m/z and 86.0 m/z for 3-hydroxybupivacaine and 3-hydroxylidocaine, respectively.

RESULTS

Owing to nearly overlapping retention times of 3- and 4-hydroxybupivacaine TMS derivatives under standard GC conditions of 70°C (initial 2 min), 20°C/min oven increase, and holding at 280°C for 12 min, slower oven temperature increase (5°C/min) was attempted and found to separate the peaks adequately. Table 1 lists retention times for these compounds on repeated injection along with that for the β-glucuronidase-released metabolite from horse urine. The equine metabolite is statiscally indistinguishable from 3-hydroxybupivacaine. Mass spectra of the respective peaks support this conclusion (data not shown).

Cross-reactivity of bupivacaine, 3- and 4-hydroxybupivacaine and related LA with the mepivacaine ELISA have been summarised elsewhere (Woods *et al.* 2000). Standard curves for the ELISA test indicated that addition of 11.4 ng of bupivacaine/ml to the system produced 50% inhibition of the maximum ELISA response (I_{50}). Higher concentrations of bupivacaine increased the inhibition in a sigmoidal manner, with essentially complete inhibition of the ELISA test occurring at 100 ng of bupivacaine/ml. The I_{50}s for bupivacaine, 3-hydroxybupivacaine and 4-hydroxybupivacaine were 17, 145 and 450 ng/ml, respectively.

For subcutaneous doses of 250 mg (n=2), 60 mg (n=1), 25 mg (n=2), 20 mg (n=1), 6.75 mg (n=1), 2.5 mg (n=2), 2.25 mg (n=1), 0.75 mg (n=1) and 0.25 mg (n=13), the concentrations of apparent bupivacaine in urine reached peaks of 3768, 1343, 94, 432, 73, 48, 110, 51 and 24 ng/ml, respectively, between 0.5 and 6 h post dose. Data are shown in Table 2. The peak 'apparent bupivacaine' concentrations were directly related to the dose administered (dose=15.2 x peak concentration + 41.1; r^2=0.977).

The base peak of the mass spectra of bupivacaine and its 3- and 4-hydroxy metabolites, as either TMS or tBuDMS derivatives, is 140.1 m/z. Mass spectral quantitation of urine samples from a horse treated with the HNED of 0.25 mg for bupivacaine was performed by exploitation of the intensity of the 140.1 m/z peak in tBuDMS derivatives, and the data are presented in Table 3. 3-hydroxybupivacaine reached a peak of 38.6 ng/ml at 4 h post dose. ELISA data, derived from

TABLE 3: Bupivacaine quantitation in horse urine by GC/MS and ELISA following 0.25 mg subcutaneously

Time post dose (h)	Concentration (ng/ml)	
	GC/MS*	ELISA†
0	1.4	0.0
0.5	1.8	0.0
1	12.9	0.0
2	31.5	34.4
4	38.6	22.6
6	32.5	36.8
8	30.8	11.0
24	3.5	10.1

*As 3-hydroxybupivacaine
†As bupivacaine equivalents

samples analysed in a urine matrix with a bupivacaine standard curve generated with the mepivacaine ELISA, are also listed in Table 3 and are in general agreement, with significant bupivacaine equivalents occurring between 2 and 6 h post dose.

Discussion

Screening of urine from performance horses for potent medications like bupivacaine is largely dependent on ELISA testing. A previously described mepivacaine ELISA test (Harkins *et al.* 1998a) was used in this study, and its cross-reactivity with parent bupivacaine, mepivacaine, 4-hydroxybupivacaine and the principal urinary metabolite in the horse, 3-hydroxybupivacaine, have been summarised elsewhere (Woods *et al.* 2000). This ELISA test is significantly more sensitive for bupivacaine (I_{50}=17 ng/ml), mepivacaine (I_{50}=16 ng/ml), and 3-hydroxybupivacaine (I_{50}=145 ng/ml) than for 4-hydroxybupivacaine (I_{50}=450 ng/ml). With appropriate dilution of post race samples to reduce endogenous background, this screening test is able to detect the presence of apparent bupivacaine. Table 2 shows that incremental doses of bupivacaine became increasingly detectable by this ELISA test.

The apparent bupivacaine detected by the ELISA test is presumably composed of bupivacaine and structurally related metabolites of bupivacaine excreted in urine. Bupivacaine itself has not been found in significant concentrations in these samples and is not found commonly in post race urine samples. TMS-derivatisation of urine extracts from a horse dosed with 250 mg of bupivacaine disclosed that the principal urinary metabolite was a hydroxylated species of bupivacaine. Synthesis of 3-hydroxybupivacaine and procurement of 4-hydroxybupivacaine enabled us to attempt their chromatographic separation by slow oven ramping. By careful retention time comparisons, it can be seen from Table 1 that the principal hydroxylated species in equine urine is most likely to be the 3-hydroxy metabolite. Therefore, 3-hydroxybupivacaine was selected as the forensically important metabolite detected in post race samples.

With regard to the quantitation of 3-hydroxybupivacaine, MTBSTFA + 1% TBDMCS derivatised 3-hydroxybupivacaine to yield mono- and bis-derivatives, with the mono-form generally predominating. This contrasts with 3-hydroxylidocaine (Harkins *et al.* 1998) where the bis-derivative predominates or is the exclusive product. The second derivatisation occurs at the internal amide, since amides are generally much more difficult to silylate than phenolic groups (Pierce 1982) with reaction taking place at either the N or the O atom. Despite the propensity of the amide group to silylate during the derivatisation reaction, this approach is remarkably reliable and reproducible, consistently yielding linear standard curves with r values greater than 0.99 upon addition of areas of both mono- and bis-tBuDMS derivatives.

The analytical data show that the HNED of bupivacaine yielded concentrations of apparent bupivacaine in urine samples that were detectable by ELISA screening and were also detectable and recoverable as 3-hydroxybupivacaine by GC/MS. Although this is a preliminary report, at this point the simplest interpretation within the context of this experimental model is that the detection of a urinary concentration of 3-hydroxybupivacaine of less than ~40 ng/ml is unlikely to be associated with a recent pharmacological effect (Harkins *et al.* 1999b).

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