A LIQUID CHROMATOGRAPHIC-ELECTROSPRAY TANDEM MS/MS METHOD FOR QUANTITATION OF EQUINE COCAINE METABOLITES

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ABSTRACT

A highly sensitive and specific method has been developed for quantitation of cocaine (COC) and its principal metabolites, ecgonine methyl ester (EME), benzoylecgonine (BZE), and norcocaine (NOR). The method combines the sensitivity and low background of a high pressure liquid chromatograph-electrospray ionisation-tandem quadrupole mass spectrometer (LC/MS/MS) with the precision of low level extractions using Zymark robotic solid phase extraction (SPE) and the added precision of isotopic internal standards, in this case deuterated analogues EME-d_3, BZE-d_5, NOR-d_5 and COC-d_3.

Standards were dissolved in acetonitrile, diluted into blank plasma or urine (1 ml) to prepare standard curves, and automated SPE was performed on a Zymark RapidTrace system. Extraction efficiency for cocaine and its metabolites ranged from 70–85%. Equine samples were diluted 1:2 with pH6 phosphate buffer prior to extraction. SPE columns were washed with dichloromethane, methanol and buffer, loaded with sample then washed sequentially with water, 0.1 N HC1 and methanol prior to elution of the analytes with dichloromethane-isopropanol-NH_4OH (78:20:2). The eluent was collected in silanised test tubes and evaporated to dryness at <40°C under a stream of nitrogen. Each sample was then dissolved in 100 µl of mobile phase and transferred to a micro-injection vial.

Quantitative analysis was performed on a Hewlett Packard model 1050 HPLC interfaced with a Micromass Quattro-II MS/MS operated in ESI-positive mode. The LC system included a Phenomenex Luna phenyl/hexyl column (2 mm x 250 mm x 5µ) and utilised a water-0.05% formic acid-acetonitrile gradient with 50 µl sample injection. Quantitation was based on the following m/z transitions: COC 304–>182, NOR 290->168, BZE 290->168, and EME 200->182, with analogous ions for the deuterated internal standards. Our routine quantitative detection limit for 1 ml samples of plasma or urine was roughly 0.05 ng/ml (slightly higher for BZE). Standard curves were typically prepared over the range of 0.1–1,000 ng/ml and quantitation was accomplished with the Quattro MassLynx quantitation routine by fitting unknowns to a weighted second order quadratic regression curve (r^2 >0.999).

Additional metabolites of cocaine in equine urine, including meta- and para-hydroxy-benzoylecgonine and hydroxylated cocaine analogues were identified. Hydroxylated metabolites of cocaine are known to appear in human urine and have been identified in equine liver microsomal preparations (C. Uboh, personal communication). It is our expectation that the presence of para-hydroxy-BZE in equine urine will prove useful in the differentiation between actual equine cocaine metabolism and post collection contamination of samples.

INTRODUCTION

The plant alkaloid cocaine is a potent central nervous stimulant when administered to animals. Its mechanism of action involves increasing the effective epinephrine concentration at epinephrine receptors resulting in a general increase in brain stimulation (Tobin 1981). Cocaine also has significant local anaesthetic properties (Harkins *et al.* 1996) and may also increase glycogenolysis in

muscle tissue, thereby affecting athletic performance (Stanley *et al.* 1991).

The stimulatory action of cocaine in the horse results in increased respiratory rate, increased locomotor activity (Tobin *et al.* 1979) and stimulation of operant behavior in a variable-interval responding test (Shults *et al.* 1982). Typically, these effects follow a biphasic response pattern, producing stimulant effects at low doses and an inhibition of these responses after peak response has been reached.

Cocaine has long been used as a stimulant in racehorses, and its use was a major factor in the establishment of equine medication control and drug testing systems (Tobin 1981). Under stalled conditions, iv doses as low as 4 mg have been reported to stimulate a horse as measured by the operant behavior method (Shults *et al.* 1982). Cocaine appears to be readily absorbed from many routes of administration in horses including oral and topical. This ease of absorption of the drug, combined with its widespread availability through the illicit market, leads to cocaine being considered as a low level environmental contaminant in certain jurisdictions. It is listed as an Association of Racing Commissioners International (ARCI) class I agent; such agents are considered to have the greatest effect on altering the performance of racing horses.

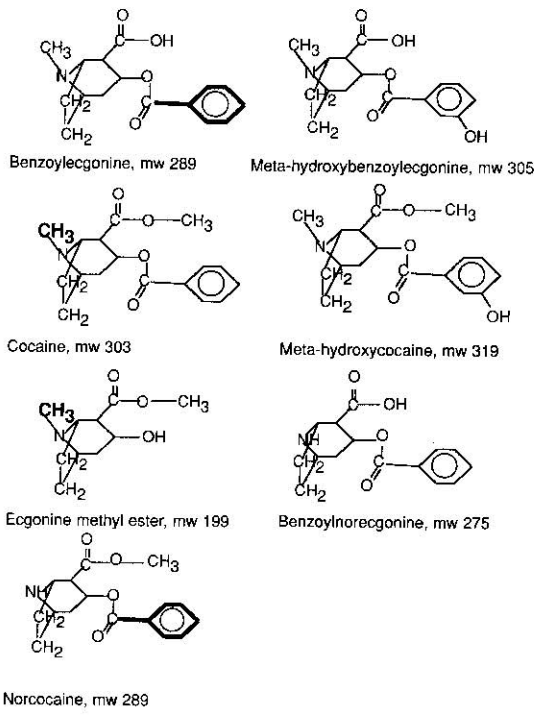

Fig 1: Structures of cocaine and its metabolites. Darkened lines and fonts indicate the site of deuterium labelling in cocaine, EME, BZE and NOR.

MATERIALS AND METHODS

Mature Thoroughbred mares weighing 500 kg were maintained on grass hay and feed (12% protein), ie a 50:50 mixture of oats and an alfalfa-based protein pellet given twice daily. The animals were vaccinated annually for tetanus and de-wormed quarterly with ivermectin (MSD Agvet, New Jersey, USA). A routine clinical examination was performed before each experiment to assure that the animals were healthy and sound. The horses were kept in a 20-acre field until placed in a box stall where they were provided water and hay *ad libitum*. All animals were managed according to the rules and regulations of the University of Kentucky's Institutional Animal Care Use Committee, which also approved the experimental protocol.

Cocaine was administered as a single iv dose at 1, 5, 25 or 100 mg total injection. Serum samples were collected by means of Vacutainer tubes (SST Gel and Clot Activator, Becton Dickinson, New Jersey, USA), before treatment started and then at 0.25, 0.5, 1, 2, 4 and 8 h after administration. During the first day, urine collection was accomplished with a Foley catheter (24 Fr, Rusch, Inc., Duluth, GA 30136) and attached plastic bag. Urine was collected for 30 min before treatment (0 h), and during the periods 0–1 h (1 h), 1–2 h (2 h), 2–4 h (4 h), 4–8 h (8 h). At 24 and 48 h after administration, a Harris flush tube (24 Fr x 152 cm; Seamless, Florida, USA) was used to collect a maximum of 300 ml urine. Urine was placed in aliquots and stored at –20°C. until assayed.

Standards of each compound analysed (cocaine, benzoylecgonine, norcocaine, and ecgonine methyl ester) were dissolved in acetonitrile and added to 1 ml blank plasma or urine at concentrations of 0.1 ng/ml to 1,000 ng/ml to prepare standard curves. Internal standards (10 µl of 10 µg/ml) consisting of deuterated analogues (Fig 1) were added to each sample prior to extraction.

Automated SPE was performed on a Zymark RapidTrace system equipped with Clean Screen SPE columns (Worldwide Monitoring, #CSDAU203, Bristol, PA), with 200 mg column matrix. Equine plasma or urine samples were diluted 1:1 with pH 6 phosphate buffer prior to extraction. SPE columns were washed with dichloromethane, methanol and buffer, loaded with sample and then washed sequentially with water, 0.1 N HCl and methanol prior to elution of the analytes with dichloromethane-isopropanol-NH_4OH (78:20:2). See Table 1 for extraction procedure summary.

The eluent was collected in silanised test tubes and evaporated to dryness at <40°C under a gentle

TABLE 1: Sequential steps for solid phase extraction of cocaine and its metabolites on the Zymark RapidTrace

Automated solid phase extraction procedure			
Step	Solution	Volume	Rate
Condition	CH_2Cl_2/NH_4OH	6 ml	5 ml/min
Condition	methanol	2	20
Condition	PO_4 buffer	2	20
Load	Sample	2	1.5
Rinse	H_2O	6	20
Rinse	HCl	3	20
Dry 5 min	N_2		
Rinse	methanol	4.5	20
Rinse	methanol	4.5	20
Dry 2 min	N_2		
Elute	CH_2Cl_2/NH_4OH	2	1.5

stream of nitrogen. Each sample was then dissolved in 100 µl of mobile phase and transferred to a micro-injection vial. Extraction recoveries ranged from 70–85%.

Quantitative analysis was performed on a Hewlett Packard model 1050 HPLC interfaced with a Micromass Quattro-II MS/MS operated in ESI-positive mode. The LC system included a Phenomenex Luna phenyl/hexyl column (2 mm x 250 mm x 5µ); the mobile phase was initially 75% Solvent A (10 mM ammonium formate, pH 8.5, 5% w/v methanol), 25% Solvent C (0.05% w/v formic acid in water + 5% v/v acetonitrile), flowing at 0.45 ml/min and which varied along a linear gradient to Solvent B (0.05% w/v formic acid in acetonitrile) as follows: 0 min, 75% A, 25% C; 1 min, 75% A, 25% C; 5 min, 10% A, 90% B; 8 min, 10% A and 90% B; 8.5 min, 75% A, 25% C; 12.5 min, 75% A, 25% C. Sample injection volume was 25 µl. Quantitation was based on the following m/z transitions: COC 304→182, NOR 290→168, BZE 290→168, and EME 200→182, with analogous ions for the deuterated internal standards.

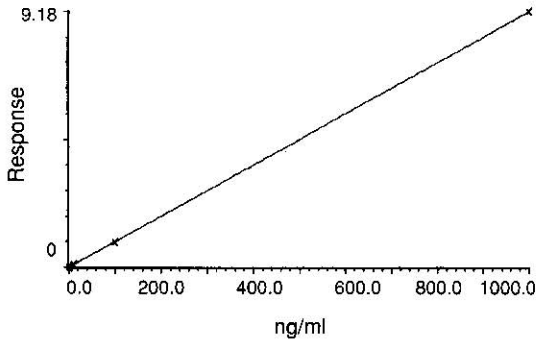

Fig 2: Typical calibration curve generated by Micromass MassLynx software for cocaine data acquired by ESI-MS/MS. Cocaine was acquired as an m/z 304→182 transition, and cocaine-d3 as internal standard (I.S.) as an m/z 307→184 transition. The data were fitted to a quadratic equation, in this case of the type 4.58020 x $10^{-8}x^2$ + 0.00913369x + 0.00155131. The origin was excluded, and the curve was weighted 1/x. Response was plotted as area x (I.S. concentration/I.S. area). The coefficient of determination, r^2, was >0.999.

Quantitation was accomplished with the Quattro II MassLynx quantitation software (Micromass, Massachusetts, USA) by fitting unknowns to a weighted second order quadratic regression curve (r^2 >0.999). A typical calibration curve is depicted in Figure 2. The routine quantitative detection limit in 1 ml samples of plasma or urine was roughly 0.05 ng/ml (slightly higher for BZE), determined by repeat analysis.

TABLE 2: Major daughter ions for cocaine and its principal metabolites. M+H+ is the molecular ion charged to plus one state with an acidic proton

Compound	M+H+, m/z (% relative abundance)	Product ion, m/z (% relative abundance)
COC	304(10)	182(100), 150(5), 119(3), 105(9), 85(15)
COC-d3	307(11)	185(100), 153(5), 119(3), 105(7), 85(14)
BZE	290(15)	168(100), 150(5), 105(25), 82(11)
BZE-d5	295(17)	168(100), 150(6), 110(11), 82(10)
EME	200(59)	182(100), 150(6), 119(7), 108(8), 93(8), 82(60)
EME-d3	203(58)	185(100), 153(5), 119(6), 111(8), 93(7), 85(60)
NOR	290(4)	168(100), 136(58), 105(10), 68(8)
NOR-d5	295(5)	168(100), 136(60), 110(8), 68(7)
m-OH-BZE	306(14)	168(100), 150(5), 121(13), 124(3), 82(10)
m-OH-COC	320(14)	182(100), 150(5), 121(4), 165(9)

Relative abundances are shown parenthetically.
COC = cocaine; COC-d3 = trideuterated COC; BZE = benzoylecgonine; BZE-d5 = pentadeuterated BZE; EME = ecgonine methyl ester; EME-d3 = trideuterated EME; NOR = norcocaine; NOR-d5 = pentadeuterated NOR; m-OH-BZE = meta-hydroxyBZE; m-OH-COC = meta-hydroxyCOC. m-OH-BZE and m-OH-COC are included as examples of hydroxylated metabolites discovered during our experimentation

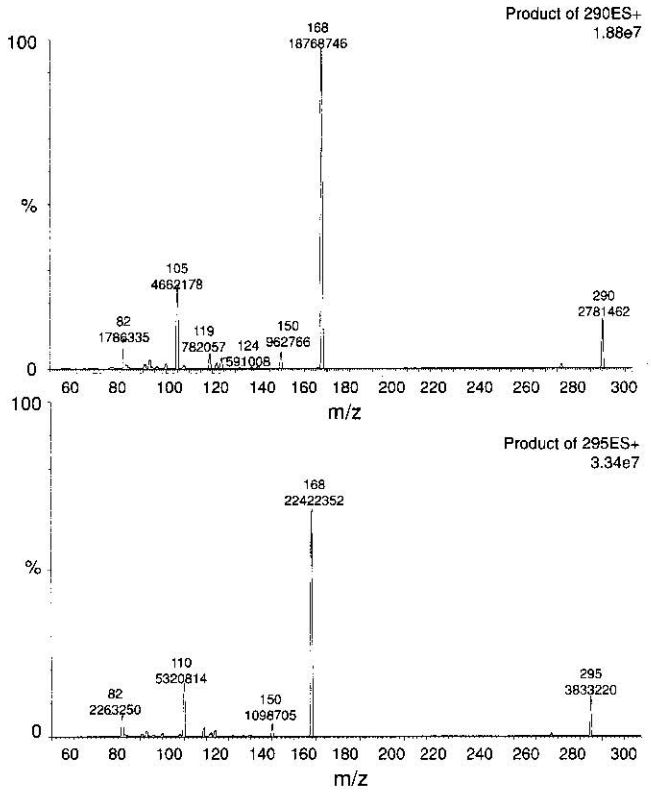

Fig 3: Product ion mass spectra of benzoylecgonine m/z 290 molecular ion [top] and d5-benzoylecgonine m/z 295 molecular ion [bottom]. Spectra were derived by direct infusion of standards at 10 ug/ml in acetonitrile:0.05% formic acid (aq), 1:1, with data acquisition by ESI(+)-MS/MS.

RESULTS

Direct infusion of cocaine metabolite standards in acetonitrile:0.05% formic acid (aqueous), 1:1, enabled generation of product ion spectra for each of the compounds of interest, whose structures are shown in Figure 1 with indication of the location of deuterium atoms in internal standards. Figure 3 shows such spectra for benzoylecgonine and its deuterated analogue as examples, and Table 2 summarises important product ions for each of the compounds shown in Figure 1. Tuning of the mass spectrometer depended on optimisation of detection of the cocaine m/z 304→182 transition (loss of benzoic acid). Such tuning resulted in concurrent optimisation of equivalent transitions for BZE and NOR. Although EME and d3-EME lack benzoyl groups and cannot undergo equivalent transitions, their principal molecular fragmentations involved loss of water (-18 amu) coincidentally at the equivalent carbon atom of the 8-azabicyclo[3.2.1]octane nucleus; these transitions were also optimised by focusing on tuning of the cocaine m/z 304→182 transition. Quantitation exploited the dependability of the optimised transitions, and standard curves for COC, BZE, EME and NOR were fitted to a second order quadratic regression curve with fitness R values estimated at >0.999.

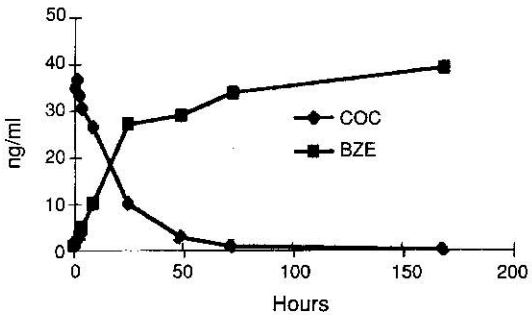

Fig 4: Cocaine degradation in urine at room temperature. Equine urine was spiked with 40 ng/ml cocaine and allowed to degrade over a week-long period; aliquots were measured for BZE, EME and NOR at various time intervals. EME and NOR responses were negative throughout.

In an effort to improve our handling of cocaine-related samples, we chose to measure cocaine stability in urine at room temperature by simply adding 40 ng/ml cocaine to fresh urine. As seen in Figure 4, cocaine rapidly hydrolysed to benzoylecgonine; this spontaneous reaction was over 90% complete within the first 48 h. Similar studies are ongoing with refrigerated and frozen urine and serum or plasma matrices dosed with

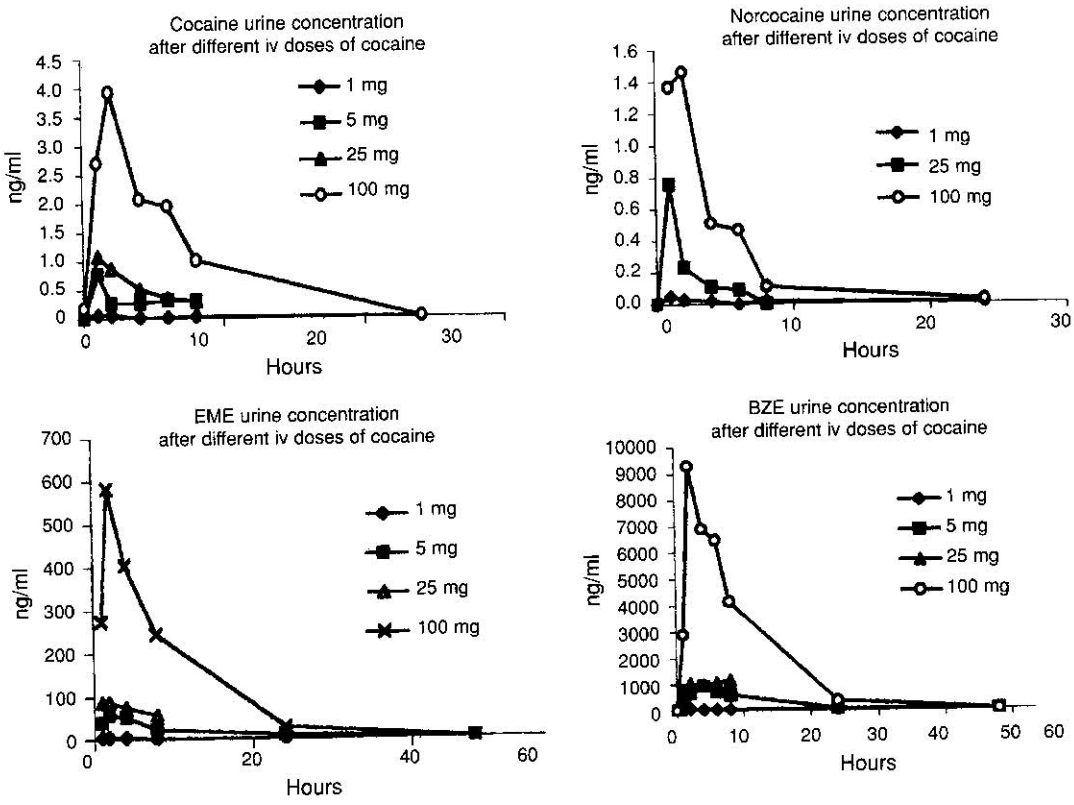

Fig 5: LC-MS/MS quantitation of cocaine and its principal metabolites in urine after differing iv doses (1, 5, 25, and 100 mg).

cocaine and will be reported in a future paper. Samples necessary for cocaine-related studies were kept refrigerated or on ice in order to obviate any degradation as an issue of concern in reporting metabolism results. Figure 5 displays LC-MS/MS quantitation of cocaine and its principal metabolites in urine after differing iv doses. Note that all of the metabolites peaked 2 h after iv administration, in the following order of prevalence: BZE > EME > COC > NOR. Peak concentrations after the 100 mg dose were respectively 9,200 ng/ml BZE, 580 ng/ml EME, 4.5 ng/ml COC, and 1.4 ng/ml NOR.

In our quest to extend mass spectral confirmatory procedures to plasma and serum samples, we made similar measurements of cocaine metabolites in plasma from the 100 mg iv dose. As expected, cocaine dropped precipitously during the course of the first hour, declining by over 90%. Benzoylecgonine rose rapidly in concurrent fashion to over 70 ng/ml by 30 min, with EME and NOR peaking similarly at 5 and 0.3 ng/ml respectively (data shown in Fig 6).

Figure 6 reports an additional principal equine metabolite of cocaine seen in plasma, para-OH-BZE which was specifically identified and quantitated using a Sigma (Missouri, USA) standard, and BZE-d5 as its internal standard. Other metabolites of cocaine identified in equine urine included meta- and para-hydroxy-benzoylecgonine and hydroxylated cocaine analogues, found in full scan acquisitions from the 100 mg iv dosage for cocaine. Ion chromatography of cocaine and benzoylecgonine masses + 16 amu for addition of an oxygen atom revealed appropriate candidates and acquired product ion spectra were interpretable by Mass Spec Calculator Pro software (Quadtech Associates, Inc., 1998). Figure 7 discloses that the hydroxylated metabolites are formed with the same general profile over time as the other metabolites, adding significant support to our contention that the spectra reported in Table 2 as m-OH-BZE and m-OH-COC are in fact equine metabolites. Their identities were confirmed by comparison to authentic cocaine metabolites available from Sigma.

Discussion

Our previous mass spectrometric methodologies for cocaine have depended solely upon GC/MS (Woods *et al.* 1996; Booze *et al.* 1997). However,

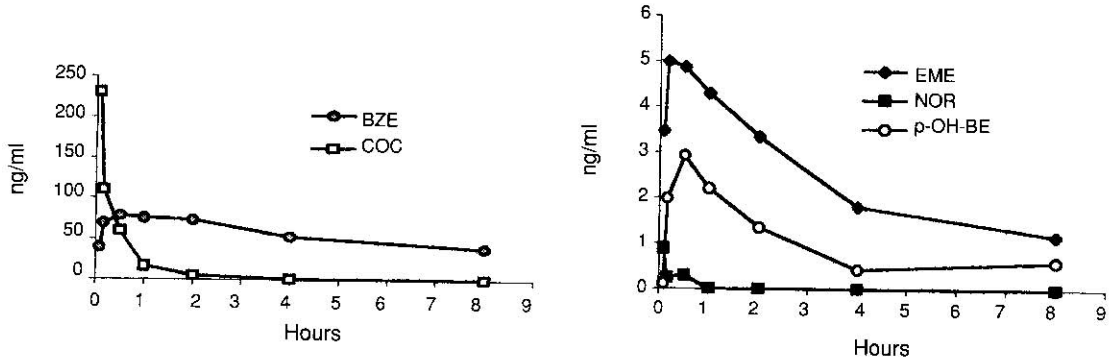

Fig 6: LC-MS/MS quantitation of cocaine and its principal metabolites in plasma after a 100 mg iv dose.

recent development of a highly sensitive LC-MS/MS method for serum clenbuterol quantitation (Lehner *et al.* 2001a,b) suggested application of similar principles in development of an improved method for cocaine and its major metabolites. Impetus was also gained from an atmospheric pressure chemical ionisation (ApCI) method reported by Singh *et al.* (1999). Our electrospray LC-MS/MS procedure has been successful in providing high sensitivity and accuracy, arising from a combination of several advantageous elements (Lehner 2001): improved precision of SPE by positive-displacement Zymark RapidTrace units; availability of deuterated internal standards; elimination of the requirement for TMS derivatisation; and extremely low backgrounds available to MS/MS instrumentation with samples introduced via electrospray ionisation, ESI(+).

The previously used GC/MS procedure for cocaine and its metabolites had distinct problems, in particular: difficulties in chromatographic separation of BZE and NOR; matrix effects on measurements of early-eluting EME; occasional inadequacy of NOR derivatisation; insensitivity below a 10 ng/ml limit of detection for cocaine or any metabolite, particularly with small sample volumes such as 100 µl plasma; and occasional loss of BZE owing to presumed degradation (A. F. Lehner, unpublished results). The current LC-MS/MS method substantially reduces or eliminates all of these difficulties, in spite of the overlapping chromatography of NOR and COC. Equine metabolites arising from iv administration were detected readily in plasma and urine as displayed in Figures 5 and 6, respectively, and could be seen to peak in urine in the order of prevalence BZE > EME > COC > NOR at 2 h post administration. Plasma metabolites peaked roughly 30 min after administration in the order of prevalence BZE > EME > p-OH-BZE >> NC.

The LC-MS/MS procedure also provided sufficient sensitivity to visualise other equine metabolites of cocaine in urine and plasma, specifically a group of hydroxylated cocaine and benzoylecgonine metabolites differing from one another by site of hydroxylation on the phenyl ring (see Fig 1). To our knowledge this is the first report

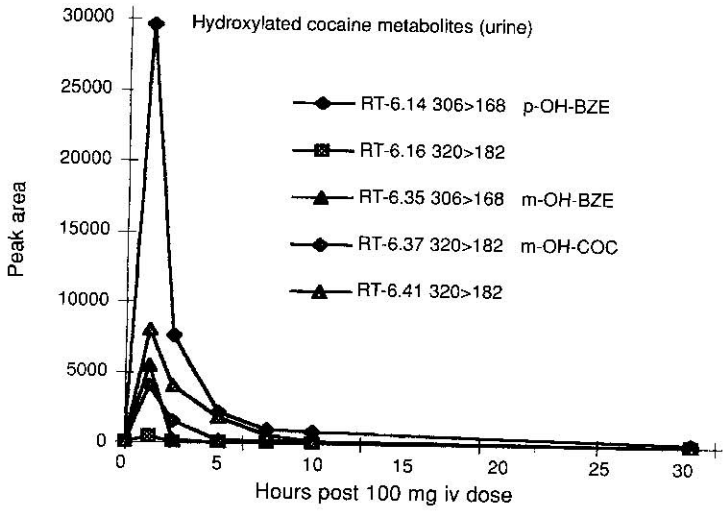

Fig 7: Peak area counts for hydroxylated metabolites of cocaine and benzoylecgonine observed in equine urine. The compounds p-OH-BZE (para-hydroxybenzoylecgonine), m-OH-BZE (meta-hydroxybenzoylecgonine) and m-OH-COC (meta-hydroxycocaine) were identified by comparison to Sigma drug metabolite standards; m-OH-BZE and m-OH-COC are shown structurally in Figure 1. The remaining m/z 320 →182 transitions occurring at 6.16 and 6.41 min retention times are presumably ortho and para-hydroxycocaine metabolites, respectively, but their definitive assignment requires additional work.

of such hydroxylated metabolites of cocaine in the horse, although they are known to appear in human urine (Zhang and Foltz 1990; Steele *et al.* 1993; ElSohly *et al.* 1998) and have in fact been found to arise in equine liver microsomal preparations incubated with cocaine (C. Uboh, personal communication). We specifically note that para-OH-BZE was the hydroxylated metabolite present to the highest extent, and it is therefore our expectation that its measurement in equine urine will prove useful in the differentiation between actual equine cocaine metabolism and post collection contamination of samples. This is especially true with regard to hydrolytic cleavages such as to BZE which, as seen in Figure 4, can arise from cocaine spontaneously when spiked into equine urine.

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A HIGHLY SENSITIVE LC/MS/MS DETECTION METHOD FOR CLENBUTEROL IN EQUINE SERUM

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Abstract

A highly sensitive LC/MS/MS-based method for detection of the broncholdilator clenbuterol (4-amino-α-[(tert-butyl-d9-amino) methyl]-3,5-dichlorobenzyl alcohol) in equine serum samples has been developed. The method evolved from application of several recent developments in the authors' laboratory. First, deuterated clenbuterol (clenbuterol-d9) was synthesised as an internal standard. Secondly, solid phase extraction was run on a Zymark RapidTrace automated system, which afforded improved precision during extraction of low concentration samples. And thirdly, quantitative analyses were performed on a Hewlett-Packard 1050 HPLC equipped with a Micromass Quattro-II MS/MS set in ESI positive mode. The mobile phase was initially 0.05% formic acid + 5% acetonitrile in water, programmed along a linear gradient to 0.05% formic acid in acetonitrile. Quantitation of clenbuterol was based on the 277 m/z to 203 m/z transition corrected by the internal standard (clenbuterol-d9) 286 m/z to 204 m/z transition. The LC/MS/MS detector was optimised for detection of clenbuterol by adjusting argon levels in the intermediate gas cell for maximum production of the clenbuterol 203 m/z product ion. The standard curves in blank serum (0.01–1 ng/ml) were highly reproducible (r=0.9999) when fit to a secondary quadratic function, with an estimated limit of detection <10 pg/ml, and assay recovery was about 70%.

Five horses were administered oral clenbuterol (0.8 µg/kg, bid) for 10 days, and serum was collected for 14 days thereafter. Serum clenbuterol showed mean trough concentrations of ~150 pg/ml. After the last dose on Day 10, serum clenbuterol reached a peak of ~500 pg/ml and then declined with a half-life of roughly 7 h. Compared with previous results obtained from urine measurement of clenbuterol (Harkins *et al.* 2001), the serum-based approach proved to be more reliable and therefore more satisfactory for regulation of the use of clenbuterol.

Clenbuterol (90 µg) was also administered intra-tracheally to 5 horses. Peak serum concentrations of ~230 pg/ml were detected 10 min after administration, dropping to ~50 pg/ml within 30 min and declining much more slowly thereafter. These observations suggest the LC/MS/MS-based serum method can detect intra-tracheal clenbuterol when administered shortly before race time.

Introduction

Clenbuterol, a bronchodilator approved by the American Association of Equine Practitioners for use in horses, has the potential to alter athletic performance of racehorses, particularly if the horse has bronchospasm. This drug is classified by the Association of Racing Commissioners International (ARCI) as a Class 3 agent, meaning that its detection in post race samples may lead to significant penalties against trainers.

Previous work from the authors' laboratory has shown that clenbuterol can be detected readily in post administration urine samples at concentrations of 1–20 ng/ml using trimethylsilyl (TMS) derivatisation, and at concentrations of 1 ng/ml or less using methaneboronic acid (MBA) derivatisation methods with GC/MS (Harkins *et al.* 2001). The latter method for derivatisation is not preferred, however, because MBA is highly damaging to the GC column and the MS detector.

The application of urinary measurements for clenbuterol regulation raises concerns among regulators, horsemen and veterinarians for important reasons. First, clenbuterol persists in urine for at least 4 weeks after a prolonged therapeutic regimen. Secondly, recent work has shown that urine samples became negative (defined as <250 pg/ml) 5 days after dosing, but