METABOLISM OF THE α2-AGONIST AMITRAZ IN HORSES AND CONFIRMATION OF ITS ADMINISTRATION

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ABSTRACT

Metabolism and detection of the alpha-2 adrenergic agonist amitraz was investigated in the horse. This agent is useful in treating livestock as a result of its properties as an insecticide, but also has sedative properties as an adrenergic agonist. The Association of Racing Commissioners International (ARCI) lists amitraz as a Class 3 drug, indicating that it may have occasional recognised applications as a therapeutic agent, but nonetheless carries with it the potential to affect the outcome of horse races. The principal metabolite of amitraz in the horse has been identified by ESI$^+$ mass spectrometry as N-2,4-dimethylphenyl-N'-methylformamidine, and we have elaborated a GC/MS method for quantification of this metabolite in urine as a confirmation method for use by racing laboratories that face amitraz positive samples. The method involves extraction with NH$_4$OH-treated dichloromethane, derivatisation of extracted N-2,4-dimethylphenyl-N'-methylformamidine with *t*-butyldimethylsilyl groups, and selected ion monitoring of the derivative's m/z 278 (M$^+$), 261 (loss of CH$_3$), 219 (loss of *t*-butyl) and 205 (m/z 261 minus *t*-butyl) ions. Quantification based on m/z 205 provided a limit of detection of 5 ng/ml by GC/MS in comparison to the simultaneously acquired m/z 208 ion of a spiked synthetic d$_6$-deuterated metabolite introduced as an internal standard. The equine urinary metabolite peaked to only about 9 ng/ml 1 h after a 75 mg iv dose, and declined rapidly 4 h post dose. This indicates that the method's utility is probably restricted to samples taken within 2 h of dosing.

INTRODUCTION

Amitraz (N'-(2,4-dimethylphenyl)-N-[[(2,4-dimethylphenyl) imino]methyl]-N-methyl-meth-animidamide) is an ARCI Class 3 drug (Fig 1), used widely as an insecticide. It is used, for example, in the treatment of demodicosis in dogs (Medleau and Willemse 1995). Metabolism typically involves generation of N-2,4-dimethylphenyl-N'-methylformamidine (BTS-27271). For example *Boophililus microplus* larvae absorb amitraz rapidly but do not demonstrate large internal concentrations owing to cleavage to BTS-27271. Only amitraz and BTS-27271 display toxicity to larvae (Schuntner and Thompson 1978).

Amitraz has toxicological effects in mammals, and its use is contraindicated in horses owing to the side-effect of severe colic when horses are exposed to this agent (Smith 1994). Nevertheless, in tropical countries such as Brazil, amitraz continues to be used for reasons of effectiveness
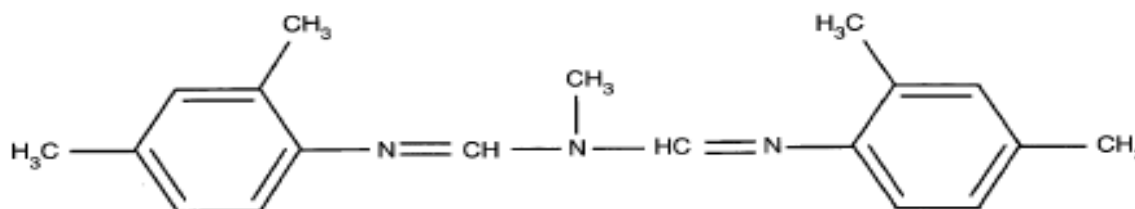

Fig 1: Chemical structure of amitraz.

and economy (Queroz-Neto *et al.* 1998). Studies in horses indicate a dose-dependent decrease in locomotor activity for amitraz, with effects lasting up to 3 h following a 0.15 mg/kg dose. Yohimbine immediately reversed the sedative effects of amitraz, indicating alpha-2 adrenergic agonist properties for this agent (Harkins *et al.* 1997). Hoof withdrawal reflex and skin twitch reflex measurements indicate that amitraz presents a powerful sedative effect in horses, with antinociceptive effects occurring only at the highest amitraz doses (Queroz-Neto *et al.* 1998).

Owing to its known sedative/tranquilising properties, amitraz administration requires analytical monitoring to regulate its use in performance horses. The purposes of the current study are to investigate the metabolism of amitraz in the horse and develop chromatographic and mass spectrometric methods for its post administration detection in performance horses.

MATERIALS AND METHODS

Standards

Amitraz was obtained in free base form from Riedel-de Haën (Seelze, Germany) through Sigma-Aldrich. N-2,4-dimethylphenyl-N'-methylformamide was obtained as a 10 ng/μl acetone solution from Dr Ehrenstorfer GmbH (Germany). The compound was also synthesised in free base form in house, and its spectrometric properties agreed with those of the standard according to direct infusion-ESI+-MS scan, direct infusion-ESI+-MS/MS m/z 163 product ion scan, EI-GC/MS of tBuDMS (tert-butyl-dimethylsilyl) derivative from reaction with MTBSTFA + 1% tBuDMCS (Pierce), and EI-GC/MS of TMS (trimethylsilyl) derivative from reaction with BSTFA + 1% TMCS (Pierce).

N-(2,4-dimethylphenyl)-N'-methylformamidine synthesis

N-(2,4-Dimethylphenyl)-N'-methylformamidine was synthesised from 2,4-dimethylaniline in a reaction with methylamine and triethyl-orthoformate. 14.8 mMol methylamine hydrochloride and 14.8 mMol triethyl orthoformate were refluxed in 10 ml absolute ethanol 15 min, at which point 14.8 mMol 2,4-dimethylaniline in 10 ml of absolute ethanol was added drop-wise. Following an additional 30 min reflux, the reaction mixture was cooled and concentrated *in vacuo*. The crude product was taken up in water, extracted with dichloromethane, and a diluted aqueous solution

of NaOH was cautiously added to the aqueous layer until the mixture reached pH 8–9, after which it was extracted with chloroform. The chloroform layer was separated, washed with water, dried (Na$_2$SO$_4$) and concentrated to a crude crystalline material, which was purified by chromatography on silica gel (ethyl acetate – methanol 95:5). The yield was 440 mg (18%).

The deuterated standard of amitraz metabolite was synthesised similarly using deuterated 2,4-dimethyl-d$_6$-aniline (CDN 2001) instead of 2,4-dimethylaniline. Reaction of 0.55 mMol 2,4-dimethyl-d$_6$-aniline with 0.55 mMol methylamine hydrochloride and 0.55 mMol ethyl orthoformate followed by purification on silica gel column produced 22 mg (24% yield) of N-(2,4-dimethyl-d$_6$-phenyl)-N'-methylformamidine.

Sample collection

Thoroughbred mares, weighing 450–550 kg, were used throughout. Horses were placed in stalls for 24 h prior to drug administrations. All horses were fed twice a day with grass hay and feed (12%), which was a 50:50 mixture of oats and an alfalfa-based protein pellet, and 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 each animal was healthy and sound. During experimentation, each horse was provided water and hay *ad libitum*. Each mare served as its own control. Animals used in these experiments were managed according to the rules and regulations of the Institutional Animal Care Use Committee at the University of Kentucky, which also approved the experimental protocol. Amitraz (75 mg) was administered intravenously and urine samples were collected immediately before dose and at 1, 2, 4, 6, 8 and 24 h after administration using a Harris flush tube (24 Fr x 60 in; Seamless, Ocala). Urine samples were divided into aliquots stored at -20°C until assayed, then thawed immediately prior to analysis.

Sample preparation for metabolite screens

Metabolite screening involved 2 methods for ESI+-MS direct infusion analysis. In the first, 5 ml urine aliquots were brought to 0.5% NH$_4$OH with 25 μl concentrated NH$_4$OH, and extracted twice with 2 ml ethyl acetate (Fisher), the organic phases combined and acidified to 0.2% with formic acid (EM Science) and immediately examined. In the second method, 1 ml urine aliquots were filtered through a 3,000 mw cut-off centrifugal Centricon-3 filter (Millipore) to remove high mw materials.

Filtrates were diluted 1:10 with acetonitrile: 0.05% formic acid (aq), 1:1, pH~4, for analysis. Resultant mixtures were infused at 1.2 ml/h via a Harvard syringe pump equipped with a 500 µl Hamilton gas-tight syringe directly into the electrospray probe of the Quattro II MS/MS (Micromass, Massachusetts, USA).

Electrospray ionisation (ESI) mass spectrometry

Amitraz standards were dissolved to 10 µg/ml in 0.05% formic acid (aq): acetonitrile, 1:1 Infusion was carried out with a Harvard syringe pump equipped with a 500 µl Hamilton gastight syringe with infusion at 1.2 ml/h into a Micromass (Massachusetts, USA) Quattro II ESI-MS/MS, and typical ESI-MS settings for detection and analysis of amitraz were as follows: capillary, 3.02 kV; HV lens, 0.54 kV; cone, 24 V; skimmer lens, 2.1 V; RF lens, 0.2 V; source temperature, 120°C; argon pressure for collisionally-induced dissociation (CID) experiments, $3-4 \times 10^{-3}$ mbar; ionisation energies: MS1, 1.0 V; MS2, 3.9 V. Resultant data were background subtracted and smoothed with the Micromass MassLynx version 3.4 software. Spectra were deconvoluted with the assistance of Mass Spec Calculator Pro software, version 4.03 (Quadtech Associates, Inc. 1998).

Extraction and derivatisation for GC/MS

N-2,4-dimethylphenyl-N'-methylformamidine was extracted from 1 ml urine in a screw-cap test tube by first introducing 300 ng/ml d_6-N-2,4-dimethylphenyl-N'-methylformamidine as internal standard for quantification. Then 6 ml dichloromethane (EM Omnisolv) and 150 µl concentrated NH_4OH (~30% w/v) were added, the tube capped and mixed by vortex for 30 s, centrifuged 10 min at 1000 × g in a type AH-4 swinging bucket rotor in a Beckman AccuSpinFR centrifuge, the upper aqueous phase removed and discarded, and the organic phase transferred into a conical tube. 20 µl of N,N-dimethylformamide [DMF] (Aldrich, HPLC grade) were added, and the organic phase was evaporated to near dryness under a stream of N_2 in a 35–40°C water bath until just a drop of DMF remained. The residue was dissolved in 80 µl of MTBSTFA-1% TBDMCS (Pierce Chemicals, Illinois, USA), vortexed 5 s and immediately transferred to a micro-injection vial. Derivatisation occurred on injection of 1 µl into the GC/MS 250°C injector port.

GC/MS SIM quantification

GC/MS selected ion monitoring (SIM) involved an Agilent 6890/5972 GC/MSD equipped with a HP-5 MS (Agilent) GC column, 10 m × 0.25 mm × 0.25 µm film thickness operated in the splitless mode with 1 ml/min helium. The GC injector was at 250°C, the transfer line was at 280°C and the oven temperature was programmed at 70°C for 2 min, then increased to 280°C at 20°C/min, held at 280°C for 5 min. SIM data were collected as follows: ions monitored for the tert-butyldimethylsilyl-N-2,4-dimethylphenyl-N'-methyl-formamidine derivative were m/z 278 (M+), 261 (loss of CH_3), 219 (loss of t-butyl) and 205 (m/z 261 minus t-butyl), 50 ms dwell each; and for the tert-butyldimethylsilyl-N-2,4-dimethyl[d_6]phenyl-N'-methyl-formamidine internal standard, m/z 225 (M+ minus t-butyl), and 208 (m/z 264 loss of methyl minus t-butyl), 50 ms dwell each. The amitraz metabolite calibration curve was prepared by determining the internal standard (m/z 208) and amitraz (m/z 205) peak areas for a series of standards (0, 2, 5, 10, 50, 100, 300 and 50 ng/ml), calculating the ratio of standard area/internal standard area along the horizontal axis, and plotting expected concentrations as a function of this ratio. Standard curves prepared in this fashion provided correlation coefficient $R^2 > 0.99$. For GC/MS scanning experiments, the m/z 50–700 mass range was scanned at 1.19 scans/s.

RESULTS

Amitraz was examined by direct infusion-ESI+ mass spectrometry and found to give good response as an [M+H] pseudomolecular ion of m/z 294 (data not shown). The mass spectrum disclosed significant peaks at m/z 253 and 163 and structural interpretations suggest internal rearrangement with loss of CH_3CN, and cleavage of a C-N bond respectively, indicative perhaps of the compound's relative instability.

The ESI+-MS/MS product ion spectrum of the amitraz M+H m/z 294 ion included m/z 163 (base peak) > 263 > 122 > 132 > 107> 118 > 105. Product ion analysis of the m/z 253 peak of provided ions m/z 132, 122 (bp), 120, 118, 107 and 105, in very nearly the same ratios as obtained with amitraz, reinforcing the concept that m/z 253 is directly related to amitraz by rearrangement, and that this ion is responsible for generation of these same ions in the amitraz product ion spectrum. The m/z 163 peak (loss of $CH_2=N-C_6H_3[CH_3]_2$) occurred whether amitraz was suspended in acid or base, which is valuable because the compound is considered acid-unstable.

Urinary metabolites of amitraz have been identified in various species, and include N-(2,4-dimethylphenyl)-N'-methylformamidine, N-(2,4-
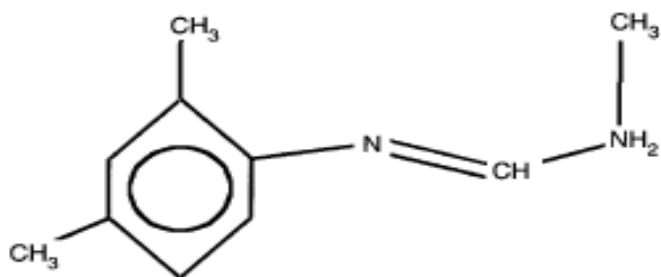
Fig 2: The likely structure of the amitraz m/z 163 metabolite.

dimethylphenyl)-formamide, N-(4-carboxy-2-methylphenyl)-N'-methylformamidine, 4-amino-3-methyl-benzoic acid, 4-(formylamino)-3-methyl-benzoic acid, 4-(acetamido)-3-methyl-benzoic acid, N,N'-bis(2,4-xylyl)-formamidine, and 2,4-dimethylaniline (Schuntner and Thompson 1978; Knowles and Benezet 1981; Knowles and Gayen 1983; Pass and Mogg 1991, 1995; Naikai *et al.* 2000; Korta *et al.* 2001; Shin and Hsu 2003). Urinary metabolites were investigated by noting the solubility of amitraz in ethyl acetate, performing a simple ethyl acetate liquid-liquid extraction of post amitraz dosage equine urine under high pH conditions, and comparing the ESI$^+$ mass spectra of 0 and 2 h post dose samples. Comparison of these samples' ESI$^+$ mass spectra disclosed a significant m/z 163 ion in the 2 h sample relative to the 0 h sample. M/z 163 examined in this way was found to change in intensity over the time course of urine sample taking, with behaviour indicative of that of a metabolite. A second approach to potential identification of urinary metabolites involved centrifugal filtration of the urine sample and direct examination of an acidified 1:10 dilution, a method that has previously been successful in identification of glucuronide metabolites (Bosken *et al.* 2000); however no candidate Phase II conjugates such as glucuronides or sulphates were identified by either approach. The likely structure of the amitraz m/z 163 metabolite is shown in Figure 2.

We compared the ESI$^+$-MS/MS product ion spectra of the m/z 163 metabolite to a standard of N-2,4-dimethylphenyl-N'-methylformamidine. Their identical appearance supports the proposed structure of the metabolite. Intact amitraz as a m/z 294 ion was not, however, identified during analysis of urine ethyl acetate extracts.

Demonstration of N-2,4-dimethylphenyl-N'-methylformamidine as the structure of the principal amitraz equine urinary metabolite following oral administration led us to consideration of a deuterated analogue for elaboration of a quantitative confirmatory method. ESI$^+$-MS/MS examination of the d_6-metabolite provided a m/z 169 M+H ion and its major product ions included m/z 169, 138, 128, 120, 111 and 110, which correspond directly to d0-N-2,4-dimethylphenyl-N'-methylformamidine m/z 163, 132, 122, 117, 105 and 107 ions, respectively. GC/MS was considered as a viable alternative to a potential LC/ESI$^+$-MS/MS approach and 2 different methods of silyl derivatisation were compared: BSTFA + 1% TMCS (Pierce), with injection of 1 μl of a 100 ng/μl preparation and acquisition by a single ion monitoring (SIM) method, with chromatography for 3 significant ions, m/z 219, 234 and 135; and MTBSTFA + 1% TBDMCS (Pierce), with injection of 1 μl of a 100 ng/μl preparation and acquisition by SIM, with chromatography for 3 significant ions, m/z 219, 276 and 261. The 2 methods gave similar results in terms of good chromatographic peak shape and reasonable mass spectra with characteristic high mw ions of good intensity for SIM methods including quantitative and qualitative ions data acquisition. However, examination of instrument linearity soon disclosed that the TMS-derivative was inferior to the tBuDMS-derivative in terms of sensitivity and linearity, primarily due to matrix interference with TMS-derivative-specific ions. The tBuDMS-silylation method was then applied to the d_6-internal standard, and ion chromatography for 3 significant ions, m/z 208, 225 and 282 gave adequate yield and sensitivity. Mass spectra for the d_6 compound indicated displacement of the d0-analogue m/z 219 ion by 6 AMU to m/z 225, and of the m/z 205 by 3 AMU to m/z 208, thus restricting the chances for isotopic interference by the internal standard with measurement of the metabolite. LC/MS/MS with MRM acquisition for the underivatised d_6 analogue N-(2,4-dimethyl-d_6-phenyl)-N'-methylformamidine m/z 169→(138, 128, 120 and 110) transitions simultaneously showed no corresponding fragments related to the d0 compound (m/z 163→[122, 117, 107, 105, 79, 77]). However, application of the GC/MS method followed by ion chromatography of m/z 282 and 276 (d_6- and d_0-metabolite M$^+$ ions, respectively) of an extracted 0 ng/ml standard showed a measurable but slight amount of the d_0-compound present in the d_6-internal standard, to the extent of 0.3%. However, this did not interfere with generation of the calibration curve, and extraction from spiked horse urine allowed generation of a linear calibration curve.

Ion chromatograms acquired from the extracts of a 1 ml urine sample 1 to 4 h post dose

TABLE 1: Validation information for the GC/MS method for confirmation of the amitraz metabolite in equine urine

Parameter	Result
Standard curves	linear response between 2 and 300 ng/ml; correlation coefficient $r^2 > 0.99$
Extraction efficiency	75–83% from urine
Instrument lower limit of detection	5 ng/ml
Lower limit of detection in urine	5 ng/ml
Interassay reproducibility, low standard	average cv of 9.2% for a 20 ng/ml spike
Interassay reproducibility, high standard	average cv of 7.4% for a 300 ng/ml spike

specifically indicated co-elution of quantifier (m/z 205) and qualifier ions (m/z 219, 261 and 276). The urine concentration of metabolite based on m/z 205 was 8.2 ng/ml. The time course of changes in urinary concentrations of N-2,4-dimethylphenyl-N'-methylformamidine in urine following dosage with 75 mg amitraz iv showed that only 2 points exceeded the limit of detection of the assay, indicating the rapidity with which elimination of this particular metabolite occurs in the horse, with a peak at 1 h post dose.

Validation information for the amitraz metabolite GC/MS confirmatory method are listed in Table 1. Specificity of the assay was tested by GC/MS confirmation analysis of unextracted standards of the structurally related alpha2-agonists guanabenz, guanfacine, guanethidine and guanadrel, the structurally related anti-ulcer medication cimetidine, and 2 widely used therapeutics, furosemide and phenylbutazone. None of the tBuDMS-derivatives co-eluted with that of N-2,4-dimethylphenyl-N'-methyl formamide. When these drugs were present at the equivalent of 100 ng/ml, no effect was observed on quantification of the amitraz metabolite at concentrations between 5 and 500 ng/ml. Ion ratios m/z 276/205 and 219/205 for the amitraz-d_0 compound, and 282/208 and 225/208 for the d_6 internal standard were unaffected, nor was there any effect on their respective retention times. When the compounds were added to blank serum at concentrations of 500 ng/ml, extracted and analysed, no chromatographic interference was observed, ie no co-eluting interference was observed in specific ion chromatograms for m/z 208, 205, 276 or 219.

DISCUSSION

Amitraz possesses characteristic sedative and tranquilising properties in the horse and other species, due to its alpha-2 adrenergic agonist characteristics. As such, regulatory control of the use of amitraz in performance horses requires the

availability of analytical monitoring techniques to prevent its unregulated use in equine performance events. We have demonstrated that an important metabolite of amitraz in the horse is N-2,4-dimethylphenyl-N'-methylformamidine, that this metabolite can be readily recovered from urine with a liquid-liquid extraction procedure, and that its presence can be monitored by GC/MS as its tert-butyldimethylsilyl-N-2,4-dimethylphenyl-N'-methyl-formamidine derivative followed by selected ion monitoring of m/z 278, 261, 219 and 205 fragments. The concentrations of this metabolite were quantified in post administration equine urine by using an in-house synthesised d_6-internal standard and monitored by SIM for m/z 208, followed by elaboration of a standard curve based on acquired area ratios of the m/z 208 to m/z 205 ions at the approximately 7.8 min retention time on GC/MS.

Amitraz standard examined by ESI$^+$-MS disclosed several components in addition to amitraz, in particular N-(2,4-dimethylphenyl)-N'-methylformamidine (m/z 163), N,N'-bis(2,4-xylyl)-formamidine (m/z 253) and most likely N-(2,4-dimethylphenyl)-formamide (m/z 150). The m/z 163 and 150 components are likely related to amitraz as simultaneous hydrolysis products, as mw 162 + 149 = 311, equivalent to amitraz 293 mw + H_2O (18 AMU), whereas the m/z 253 component results from a more complex rearrangement. Reaction of amitraz with MTBSTFA + 1% TMCS confirmed that these are present in the standard and are not electrospray source-derived breakdown products.

Parent amitraz did not derivatise with MTBSTFA, and chromatographed cleanly yielding a 10.75' GC peak. Elaboration of an SIM method for its principal peaks (m/z 293, 162, 147, 132 and 121) confirmed the absence of detectable parent amitraz in extracts of post administration serum and urine, in agreement with observations made by ESI$^+$-MS. The very rapid decline in urinary concentrations of the major amitraz metabolite, N-2,4-dimethylphenyl-N'-methyl formamide is in

good agreement with pharmacokinetic parameters established by Pass and Mogg (1991) in sheep and adult Shetland-cross ponies. These investigators reported distribution half-lives of 1.98 min for amitraz and 2.17 min for BTS 27271 (derived from amitraz) in ponies following 1 mg/kg iv doses; elimination half-lives were significantly greater at 39.4 and 44.2 min, respectively.

It is our intention to guide laboratories in the development of methodologies for the confirmation of amitraz, but the agent's rapid turnover suggests that if control of this agent is to be regulated by examination of the N-2,4-dimethylphenyl-N'-methylformamidine metabolite as described herein, then the urine samples must optimally be taken within 2 h of the time of administration of this agent.

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