



2004

Development of a method for the detection and confirmation of the alpha-2 agonist amitraz and its major metabolite in horse urine

A. F. Lehner

University of Kentucky, alehner@uky.edu

C. G. Hughes

University of Kentucky

W. Karpiesiuk

University of Kentucky


J Daniel Harkins

University of Kentucky

L. Dirikolu

University of Kentucky

Follow this and additional works at: https://uknowledge.uky.edu/gerc_facpub

 *next page for additional authors*

Part of the [Large or Food Animal and Equine Medicine Commons](#)

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Repository Citation

Lehner, A. F.; Hughes, C. G.; Karpiesiuk, W.; Harkins, J Daniel; Dirikolu, L.; Bosken, Jeffrey M; Camargo, Fernanda; Boyles, J.; Troppmann, A.; Woods, W. E.; and Tobin, Thomas, "Development of a method for the detection and confirmation of the alpha-2 agonist amitraz and its major metabolite in horse urine" (2004). *Maxwell H. Gluck Equine Research Center Faculty Publications*. 80.

https://uknowledge.uky.edu/gerc_facpub/80

This Article is brought to you for free and open access by the Maxwell H. Gluck Equine Research Center at UKnowledge. It has been accepted for inclusion in Maxwell H. Gluck Equine Research Center Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

Development of a method for the detection and confirmation of the alpha-2 agonist amitraz and its major metabolite in horse urine

Digital Object Identifier (DOI)

<https://doi.org/10.1093/jat/28.7.553>

Notes/Citation Information

Lehner, A. F., Hughes, C. G., Karpiesiuk, W., Harkins, J. D., Dirikolu, L., Bosken, J., Camargo, F., Boyles, J., Troppmann, A., Woods, W. E., & Tobin, T. (2004). Development of a method for the detection and confirmation of the alpha-2 agonist amitraz and its major metabolite in horse urine. *Journal of Analytical Toxicology*, 28(7), 553-562. <https://doi.org/10.1093/jat/28.7.553>

Authors

A. F. Lehner, C. G. Hughes, W. Karpiesiuk, J Daniel Harkins, L. Dirikolu, Jeffrey M Bosken, Fernanda Camargo, J. Boyles, A. Troppmann, W. E. Woods, and Thomas Tobin

Development of a Method for the Detection and Confirmation of the alpha-2 Agonist Amitraz and Its Major Metabolite in Horse Urine*

A.F. Lehner^{1,†}, C.G. Hughes¹, W. Karpiesiuk¹, J.D. Harkins¹, L. Dirikolu², J. Bosken¹, F. Camargo¹, J. Boyles¹, A. Troppmann¹, W.E. Woods¹, and T. Tobin¹

¹Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky, Lexington, Kentucky and ²College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University, Tuskegee, Alabama

Abstract

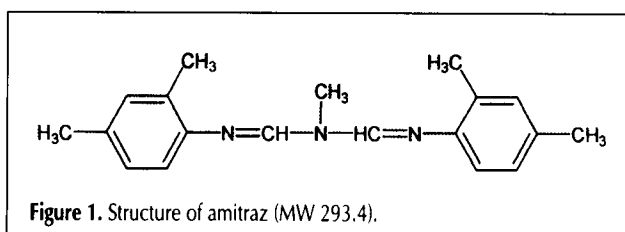
Amitraz (*N*'-(2,4-dimethylphenyl)-*N*'-[(2,4-dimethylphenyl)imino]methyl)-*N*-methyl-methanimidamide) is an alpha-2 adrenergic agonist used in veterinary medicine primarily as a scabicide- or acaricide-type insecticide. As an alpha-2 adrenergic agonist, it also has sedative/tranquilizing properties and is, therefore, listed as an Association of Racing Commissioners International Class 3 Foreign Substance, indicating its potential to influence the outcome of horse races. We identified the principal equine metabolite of amitraz as *N*-2,4-dimethylphenyl-*N*'-methylformamidine by electrospray ionization(+)-mass spectrometry and developed a gas chromatographic-mass spectrometric (GC-MS) method for its detection, quantitation, and confirmation in performance horse regulation. The GC-MS method involves derivatization with *t*-butyldimethylsilyl groups; selected ion monitoring (SIM) of *m/z* 205 (quantifier ion), 278, 261, and 219 (qualifier ions); and elaboration of a calibration curve based on ion area ratios involving simultaneous SIM acquisition of an internal standard *m/z* 208 quantifier ion based on an in-house synthesized *d*₆ deuterated metabolite. The limit of detection of the method is approximately 5 ng/mL in urine and is sufficiently sensitive to detect the peak urinary metabolite at 1 h post dose, following administration of amitraz at a 75-mg/horse intravenous dose.

Introduction

Amitraz (*N*'-(2,4-dimethylphenyl)-*N*'-[(2,4-dimethylphenyl)-

imino]methyl)-*N*-methyl-methanimidamide) is an Association of Racing Commissioners International (ARCI) Class 3 Foreign Substance (1) used widely in the field of veterinary medicine as a scabicide- or acaricide-type insecticide. Trade names include Mitaban (Pharmacia and Upjohn); Mitac (AgrEvo); and Aludex, Taktic, and Topline (Hoechst Roussel Vet.) (2). It is used, for example, in the treatment of demodicosis in dogs (3). Amitraz has a relatively low melting point of 86–87°C and is unstable to acidic pH (2) (Figure 1).

Toxicity of amitraz and other formamidine-type compounds to insects appear to involve activation of an octopamine-sensitive adenylate cyclase (4), and metabolism of the drug typically involves generation of the highly active metabolite *N*-2,4-dimethylphenyl-*N*'-methylformamidine, known also as BTS-27271. *Boophilus microplus* larvae, for example, absorb amitraz rapidly but do not demonstrate large internal concentrations, owing to rapid cleavage to BTS-27271; the expected complementary cleavage product 2,4-dimethylformanilide was not produced in equivalent quantity, but large amounts of polar metabolites and 2,4-dimethylaniline were produced instead. Of the identified compounds, only amitraz and BTS-27271 displayed toxicity to larvae (5). Amitraz applied to honeybee hives to prevent infestation with *Varroa jacobsoni* could be extracted with a liquid-liquid regimen, followed by high-performance liquid chromatography (HPLC) with diode-array detection (DAD) (6) or by gas chromatography with nitrogen phosphorous detection (GC-NPD) and mass spectrometric (MS) confirmation (7). Of seven acaricides detectable in honey and beeswax, amitraz was the least stable, degrading into BTS-



* Published as Article #322 from the Equine Pharmacology, Therapeutics and Toxicology Program at the Maxwell H. Gluck Equine Research Center and the Department of Veterinary Science, University of Kentucky. Published as Kentucky Agricultural Experiment Station Article # 03-14-040 with the approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experimental Station. Supported by a grant from The Kentucky Racing Commission, Lexington, KY.

[†] Author to whom correspondence should be addressed: Andreas F. Lehner, 108 Gluck Equine Research Center, Dept. of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099. E-mail: alehner@uky.edu.

27271 and 2,4-dimethylphenylformamide within 1 day in beeswax and within 10 days in honey (8).

Amitraz has highly complex pharmacological and toxicological effects in mammals, and its use is specifically contraindicated in horses because of the not uncommon side-effect of severe colic when horses are exposed to this agent (9). Intestinal immobility and stasis have also been demonstrated for amitraz and BTS-27271 in sheep up to 4 h post dose, and reversal with yohimbine indicates alpha-2 adrenergic agonist properties as most likely responsible (10,11). Nevertheless, in tropical countries such as Brazil, amitraz continues to be used for reasons of effectiveness and economy (12). Behavioral and neurochemical studies of the effects of amitraz in rats showed that it decreased locomotion and increased immobility times in an open field, while increasing whole brain levels of noradrenaline and striatal levels of dopamine, possibly through inhibitory effects on monoamine oxidase (MAO) activity (13). Studies in horses similarly 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 here, as well, again indicating alpha-2 adrenergic agonist properties for this agent (14). Measurement of additional physiological parameters such as hoof withdrawal reflex and skin twitch reflex indicate that amitraz presents a marked, long-lasting, and powerful sedative effect in horses, whereas antinociceptive effects occur only at the highest amitraz doses (12).

In addition to its known effects on alpha-2 adrenergic receptors and on inhibition of MAO activity, amitraz has also been demonstrated to have a significant H1-histamine antagonist activity on isolated guinea-pig ileum. BTS-27271 had over threefold greater potency in this system, but the authors concluded that intestinal stasis was more likely due again to alpha-2 adrenergic agonist properties (10). Selective activation of a specific subset of alpha-2D adrenergic receptors was considered responsible for observed effects on inhibition of insulin secretion and increase of glucagon secretion by both amitraz and BTS-27271 in a concentration-dependent manner in perfused rat pancreas (15). Untoward effects of alpha-2 agonists such as xylazine include abortion, and Shin and Hsu (16) have demonstrated effects of amitraz and BTS-27271 on increasing motility of isolated porcine myometrium. The effect is mediated by an increase in extracellular calcium influx through voltage-dependent calcium channels. Yohimbine again reversed the effects, whereas the alpha-1 antagonist prazosin did not.

Bonsall and Turnbull (17) have suggested that depressor effects of amitraz may be countered by stimulatory effects, depending on the dose administered. In general, high doses may cause depression, whereas low doses may lead to excitatory symptoms such as hyperreactivity to external stimuli and possibly aggressiveness (16). Because of its known sedative/tranquilizing 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.

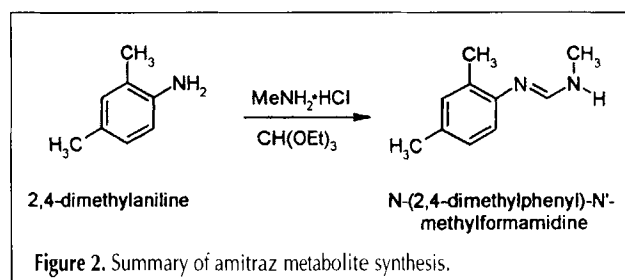
Materials and Methods

Standards

Amitraz was obtained as an analytical standard in free base form from Riedel-de Haën (Seelze, Germany) through Sigma-Aldrich. The *N*-2,4-dimethylphenyl-*N'*-methylformamide metabolite was obtained as a 10-ng/ μ L acetone solution from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The compound used for this solution was listed as having a 98.5% purity, based on HPLC with UV-DAD detection. The compound was also synthesized in free base form in-house, and its spectrometric properties agreed with those of the standard as follows: ESI(+)-MS scan, isotopic ratios expected for [M+H]⁺, +1, +2 (*m/z* 163, 164, 165): 100%, 12.1%, 0.54% (calculated for C₁₀H₁₅N₂ compound); 100%, 11.2%, 1.0% (Ehrenstorfer std); 100%, 11.1%, 0.63% (in-house prep); direct infusion-ESI(+)-MS-MS *m/z* 163 daughter ion scan: *m/z* 107 > 117 > 105 > 122 > 79 > 132 > 42 > 77 > 163 (same for both); EI-GC-MS of *t*BuDMS (*tert*-butyldimethylsilyl) derivative from reaction with MTBSTFA + 1% *t*BuDMCS (Pierce, Rockford, IL): *m/z* 205 > 219 > 73 > 59 > 156 > 162 > 88 > 276 > 261 (same for both; Ehrenstorfer 18.1 min, in-house 18.2 min retention times); EI-GC-MS of TMS (trimethylsilyl) derivative from reaction with BSTFA + 1% TMCS (Pierce): *m/z* 219 > 73 > 234 > 135 > 120 > 59 > 88 > 162 > 145 (same for both; Ehrenstorfer 10.9 min, in-house 10.8 min retention times). Underivatized compound observed under these conditions also demonstrated match (*m/z* 162 > 132 > 120 > 106 > 77 > 147 for both). EI-GC-MS revealed that our synthetic *N*-2,4-dimethylphenyl-*N'*-methylformamide metabolite was over 95% pure by total ion chromatogram total area comparisons, with contaminants including < 2% *N,N'*-bis(2,4-dimethylphenyl)-methanimidamide and < 3% amitraz, by library matching to the NIST98 EI-MS library.

Synthesis of *N*-(2,4-dimethylphenyl)-*N'*-methylformamide and its deuterated analogue

Synthesis of *N*-(2,4-dimethylphenyl)-*N'*-methylformamide is described only in German patent literature (18), but procedures for analogous formamidines (19) made it possible to elaborate a preparation of this metabolite. *N*-(2,4-Dimethylphenyl)-*N'*-methylformamide was synthesized from 2,4-dimethylaniline in a reaction with methylamine and triethyl orthoformate (Figure 2). Methylamine hydrochloride (1 g, 14.8mM) and triethyl orthoformate (2.19 g, 14.8mM) were first refluxed in 10 mL absolute ethanol for 15 min, at which point 2,4-dimethylaniline (1.79 g, 14.78mM) in 10 mL absolute ethanol was added dropwise. The addition was complete in 1 h. Following an additional reflux for 30 min, the reaction mixture



was cooled and concentrated in vacuo. The crude product was taken up in water and extracted with dichloromethane. 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_2SO_4), and concentrated to a crude crystalline material, which was purified by chromatography on silica gel (ethyl acetate/methanol, 95:5). Yield: 440 mg (18%) (Figure 2).

The deuterated standard of amitraz metabolite was synthesized similarly using deuterated 2,4-dimethyl- d_6 -aniline (20) instead of 2,4-dimethylaniline. Reaction of 2,4-dimethyl- d_6 -aniline (90 mg, 0.55mM) with methylamine hydrochloride (37.3 mg, 0.55mM) and ethyl orthoformate (81.8 mg, 0.55mM), followed by purification on silica gel column, produced 22 mg (24% yield) *N*-(2,4-dimethyl- d_6 -phenyl)-*N'*-methylformamidine.

Sample collection

Thoroughbred mares, weighing 450–550 kg, from our dedicated herd were used throughout. Horses were acclimated to their 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 dewormed quarterly with ivermectin (MSD Agvet, Rahway, NJ). A routine clinical examination was performed before each experiment to ensure 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 \times 60 in., Seamless, Ocala, FL). Urine samples were divided into aliquots, stored at -20°C until assayed, and then thawed immediately prior to analysis.

Sample preparation for metabolite screens

Metabolite screening involved two methods for ESI(+)-MS direct infusion analysis. In the first, 5-mL urine aliquots were brought to 0.5% NH_4OH with 25 μL concentrated NH_4OH and extracted twice with 2 mL ethyl acetate (HPLC grade, Fisher, Fairlawn, NJ), the organic phases combined and acidified to 0.2% with formic acid (EM Science, Gibbstown, NJ), and immediately examined. In the second method, 1-mL urine aliquots were filtered through a 3000-MW cutoff Centricon-3 filter (Amicon, Inc., a division of Millipore, Beverly, MA) to remove high MW materials. The filters were centrifuged 60 min at $1000 \times g$ in a type AH-4 swinging bucket rotor in a Beckman (Palo Alto, CA) AccuSpinFR centrifuge. Filtrates were diluted 1:10 with a mixture of 50:50 acetonitrile/0.05% formic acid (aq.) (pH \sim 4) for analysis. In both methods, resultant mixtures were infused 1.2 mL/h via a Harvard (Holliston, MA) syringe pump equipped with a 500- μL Hamilton (Reno, NV) gas-tight syringe. Infusion was direct into the electrospray probe of the

Quattro II MS–MS (Micromass, Beverly, MA).

ESI-MS

Amitraz standards were prepared for direct infusion ESI (positive mode)-MS analysis by dissolution in 0.05% formic acid (aq)/acetonitrile (1:1) to 10 $\mu\text{g}/\text{mL}$. Infusion was carried out with a Harvard syringe pump equipped with a 500- μL Hamilton gastight syringe with infusion at 1.2 mL/h. The mass spectrometer was a Micromass Quattro II ESI-MS–MS, and typical ESI-MS voltage settings for detection and analysis of amitraz were as follows: capillary, 3.02 kV; HV lens, 0.54 kV; cone, 24 kV; skimmer lens, 2.1 kV; RF lens, 0.2 kV; source temperature, 120°C ; argon pressure for collisionally induced dissociation (CID) experiments, $3\text{--}4 \times 10^{-3}$ mbar; ionization energies: MS1, 1.0 and MS2, 3.9. Collision energy was set between 10 and 15. ESI-MS and MS–MS spectra were acquired as continuum data for a minimum of 1–2 min over the m/z 10–800 mass range, applying 1.8 s/scan duration. 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., Fairfield, CA).

HPLC methodology for ESI-MS

An Agilent (Atlanta, GA) 1050 HPLC was interfaced with the Quattro II MS–MS and equipped with a Luna phenyl-hexyl 1 \times 30-mm, 3- μ column (Phenomenex, Torrance, CA). The mobile phase was 52.5% acetonitrile/47.5% deionized water/0.05% formic acid run in isocratic mode at 0.15 mL/min. Multiple reaction monitoring was carried out for amitraz (m/z 294) daughter ions m/z 253, 163, 132, 122, and 117 with a 0.02 s dwell and for the *N*-2,4-dimethylphenyl-*N'*-methylformamidine metabolite (m/z 163) daughter ions m/z 122, 117, 107, 105, 79, and 77 with a 0.01 s dwell. Samples were resuspended in acetonitrile/water (1:1).

Extraction and derivatization 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 *N*-2,4-dimethyl- d_6 -phenyl-*N'*-methylformamidine as internal standard for quantitation. Then, 6 mL dichloromethane (EM Omnisolv) and 150 μL concentrated NH_4OH (\sim 30%, w/v) were added, the tube capped and mixed by vortex for 30 s, centrifuged 10 min at $1000 \times g$ 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. Twenty microliters *N,N*-dimethylformamide (HPLC grade, Aldrich, Milwaukee, WI) was 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 *N,N*-dimethylformamide remained. The residue was dissolved in 80 μL MTBSTFA-1% TBDMCS (Pierce Chemicals, Rockville, IL), vortex mixed 5 s, and immediately transferred to a microinjection vial. Derivatization occurred on injection of 1 μL into the GC–MS 250°C injector port.

GC–MS SIM quantitation

GC–MS SIM involved an Agilent 6890/5972 GC–MSD equipped with an HP-5 MS (Agilent) GC column (10 m \times 0.25

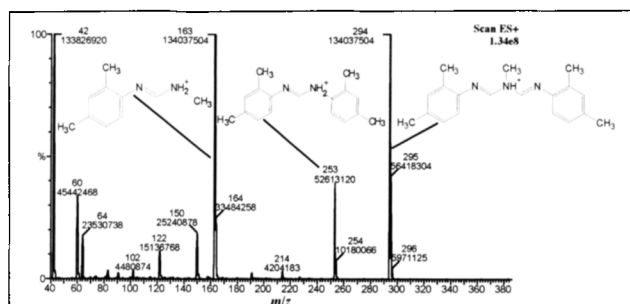


Figure 3. Amitraz study by direct infusion-ESI(+)-MS: 10 µg/mL amitraz standard in 0.05% formic acid/acetonitrile (1:1) was infused at 1.2 mL/h. Labels indicate peak m/z values, followed by intensity, with likely structures of three major components.

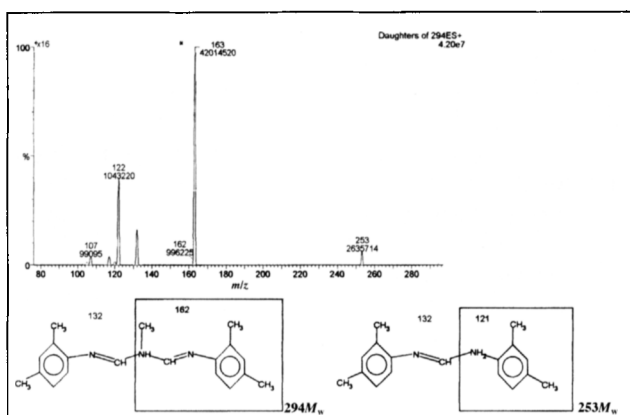


Figure 4. ESI(+)-MS for amitraz daughter ions at 10 µg/mL in 0.05% formic acid/ acetonitrile (1:1). Peak intensities between m/z 80 and 155 have been multiplied 16-fold. The structures below indicate the likely source of the m/z 163 ion by cleavage of a C-N bond with concomitant shift of a proton (left) and a rearranged amitraz (internal loss of CH_3CN) to provide a 253 MW analogue in turn responsible for generation of the m/z 122 fragment (right).

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 at 280°C, and the oven temperature was programmed as follows: 70°C for 2 min, then increased to 280°C at 20°C/min, and 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-formamidide derivative were m/z 278 (M^+), 261 (loss of CH_3), 219 (loss of *t*-butyl), and 205 (m/z 261 minus *t*-butyl), each at a 50 ms dwell; and for the *tert*-butyldimethylsilyl-*N*-2,4-dimethyl- d_6 -phenyl-*N'*-methyl-formamidide internal standard, m/z 225 (M^+ minus *t*-butyl) and 208 (m/z 264 loss of methyl minus *t*-butyl), again at 50 ms dwell. Preparation of the amitraz metabolite calibration curve was accomplished 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 500 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 coefficient of determination $r^2 > 0.99$. 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(+)-MS and found to give good response as an $[\text{M}+\text{H}]$ pseudomolecular ion of m/z 294 (Figure 3). The mass spectrum disclosed two significant peaks at m/z 253 and 163, for which structural interpretations suggest internal rearrangement with loss of CH_3CN and cleavage of a C-N bond, respectively, with likely structures included in the figure. These latter peaks are likely MS byproducts of this compound's relative instability.

Figure 4 displays the ESI(+)-MS-MS daughter ion spectrum of the amitraz $\text{M}+\text{H}$ m/z 294 ion. Note the intense m/z 163 ion, ideal for implementation of a sensitive LC-MS method for its quantitation. Daughter ion analysis of the m/z 253 peak of Figure 3 provides ions m/z 132, 122 (bp), 120, 118, 107, and 105 (data not show) in very nearly the same ratios as obtained with amitraz in Figure 4, reinforcing the idea that m/z 253 in Figure 3 is directly related to amitraz by rearrangement and that this ion is likewise responsible for generation of these same ions in the amitraz daughter ion spectrum. We obtained approximately the same intensity of the m/z 163 peak [loss of $\text{CH}_2=\text{N}-\text{C}_6\text{H}_3(\text{CH}_3)_2$] whether amitraz was suspended in acid or base, which is valuable because the compound is considered acid unstable; however, infusing it in acetonitrile/water so as to avoid pH extremes reduced the m/z 163 intensity by approximately 50% (data not shown).

Table I lists urinary metabolites of amitraz identified in various species; phase II conjugates, should they form, would add 176 (glucuronide), 80 (sulfate), or 57 amu (glycine

Table I. Hypothetical Metabolites of Amitraz as Predicted on the Basis of Literature Reports in Animals and Plants

Metabolite	Formula	MW	Ring	Reference
			Hydroxyl (MW)	
<i>N</i> -(2,4-Dimethylphenyl)- <i>N'</i> -methylformamidide	$\text{C}_{10}\text{H}_{14}\text{N}_2$	162	178	5,8,10,11, 23,24,25
<i>N</i> -(2,4-Dimethylphenyl)-formamide	$\text{C}_9\text{H}_{11}\text{NO}$	149	165	8,16,23, 24,25
<i>N</i> -(4-Carboxy-2-methylphenyl)- <i>N'</i> -methylformamidide	$\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_2$	192	192	*
4-Amino-3-methyl-benzoic acid [†]	$\text{C}_8\text{H}_9\text{NO}_2$	151	167	16,23,24
4-(Formylamino)-3-methyl-benzoic acid	$\text{C}_9\text{H}_9\text{NO}_3$	179	195	23,24
4-(Acetamido)-3-methyl-benzoic acid	$\text{C}_{10}\text{H}_{11}\text{NO}_3$	193	209	*
<i>N,N'</i> -bis(2,4-Xylyl)-formamidide	$\text{C}_{17}\text{H}_{20}\text{N}_2$	252	268	*
2,4-Dimethylaniline	$\text{C}_8\text{H}_{11}\text{N}$	121	137	5,16,23, 24,25

* This information obtained from an International Program on Chemical Safety Monograph #944 (Amitraz-JMPR Evaluations 1998, Part II-Toxicological) available at the www.inchem.org.

[†] This metabolite is isomeric with 2-amino-5-methylbenzoic acid, also reported as a metabolite (18).

conjugates) with the assumptions that glucuronides form on hydroxyls or carboxylic acids, sulfates form on hydroxyls, and glycine conjugates form on carboxylic acids. This table provided a guide for further analyses. 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(+)-MS of 0 and 2 h post-dose samples. Figure 5 displays the results of such an analysis, and amidst the background ions one notes the appearance of a significant m/z 163 ion in the 2 h sample relative to the 0 h sample. The right portion of the figure displays the change in intensity of this ion over the time course of urine sample taking, with behavior

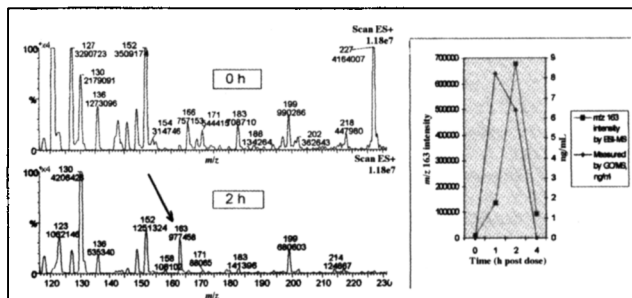


Figure 5. On the left is a comparison of 0- (top) and 2-h (bottom) post amitraz-dosage urine samples following ethyl acetate extraction and direct infusion ESI(+)-MS analysis. The principal change is the appearance of a significant m/z 163 peak in the 2 h sample. The right panel shows the variation in intensity of the m/z 163 metabolite with time, behavior suggestive of the metabolism of a typical oral pharmacological agent. The m/z 163 intensity curve compared favorably with the curve defined by GC-MS measured concentrations, derived according to the method discussed.

suggestive 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 (21); however, no candidate Phase II conjugates were identified by either approach. Table II summarizes potential metabolites screened in urine by these methods. The only other possible lead for an equine urinary metabolite was 4-amino-3-methyl-benzoic acid (MW 151, listed in Table I) and as a likely candidate at M+H m/z 152 in Table II, but this possibility remains to be pursued.

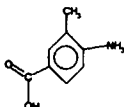
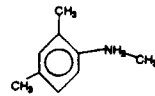
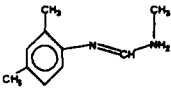
In Figure 6, we compare the ESI(+)-MS-MS daughter ion spectra of the m/z 163 metabolite discerned by the Figure 5 comparison of 0 and 2 h samples to a standard of *N*-2,4-dimethylphenyl-*N'*-methylformamide. Their identical appearance supports the proposed structure of the metabolite. The structure is shown as part of Figure 3 and is obtained directly from amitraz in the ESI source because the daughter ion spectrum of the amitraz m/z 163 byproduct (not shown) is also identical to both spectra in Figure 6. Intact amitraz as a m/z 294 ion was, however, not identified during analysis of urine ethyl acetate extracts.

The left-hand panel of Figure 7 presents an HPLC of amitraz with MRM of specific fragmentation events of Figure 4 daughter ion spectrum. The similarity of retention times demonstrates the simultaneity of elution of principle fragmentations. The center panel of Figure 7 compares 0- and 2-h post-dose ethyl acetate extracts run by gradient HPLC with MRM acquisition specific for the m/z 163 amitraz metabolite, disclosing its presence only following amitraz dosing. In the right-hand panel of Figure 7, the individual fragmentations coincide at the 3.97 RT

peak with area ratios corresponding to those of an *N*-2,4-dimethylphenyl-*N'*-methylformamide standard and agreeing with the intensities of the daughter ion spectra of Figure 6.

Demonstration of *N*-2,4-dimethylphenyl-*N'*-methylformamide 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. Figure 8 shows the ESI(+)-MS-MS daughter ion spectrum of a d_6 metabolite m/z 169 M+H ion, and Table III includes a comparative interpretation of the d_6 and d_0 metabolite fragment ions. However, quantitation of the m/z 163 ion was hampered by a severe reduction in sensitivity by the LC-MS-MS method. GC-MS was considered a viable alternative approach, and Figure 9 describes the results obtained with two methods of *N*-2,4-dimethylphenyl-*N'*-methylformamide silylation. The two methods gave similar results in terms of good chromatographic peak shape and reasonable mass spectra capable of providing characteristic high MW ions of good intensity for generation of SIM methods including quantitative and qualitative ions data acquisition. However,

Table II. Significant Examples of Potential Metabolites Screened from Equine Urine Following Amitraz Dosing

Ion(s) (m/z) Seen in 2 h Urine Absent in 0 h	Method of Extraction*	Possible Interpretation as an Amitraz Metabolite	Comment
83, 115, 136	CF	—	Probable sodium adducts
249, 308	CF	—	Probable potassium adducts
152	CF		Daughter ion spectrum matches structure
111, 171, 199	EA	—	Do not correspond to Table I predictions
136	EA		Daughter ion spectrum does not match structure
163	EA		Daughter ion spectrum matches structure

* CF = centrifugal filtration of urine and EA = ethyl acetate liquid-liquid extraction of urine.

work directed towards defining instrument linearity quickly disclosed that the TMS derivative (Figure 9A and 9B) was inferior to the *t*BuDMS derivative (Figure 9C and 9D) in terms of sensitivity and linearity, primarily due to matrix interference with TMS derivative-specific ions; matrix components may interfere to some extent with formation of the TMS derivative but interfere in a more serious manner by chromatographic coelution. When it became clear that the *t*BuDMS derivative provided significant advantages, this silylation method was applied to the d_6 internal standard, with results as described in Figure 10. As with the d_0 compound in Figure 9C, extracted ion chromatography here also disclosed appropriate ions for quantitative and qualitative purposes (m/z 208, 225, and 282) (Figure 10A). The mass spectrum in Figure 10B shows displacement of the d_0 analogue m/z 219 ion (Figure 9D) by 6 amu to m/z 225 and of the m/z 205 (Figure 9D) by 3 amu to m/z 208, thus restricting the chances for isotopic interference by the internal standard with measurement of the metabolite. Figure 10C illustrates chromatography of the d_6 metabolite as an internal standard in conjunction with the d_0 metabolite; larger ions (m/z 208 and 225) are not shown for clarity but overlap m/z 264 and 282. Note the slight but reproducible difference in retention times of 0.02 min (7.86 minus 7.84 min). LC-MS-MS with MRM acquisition for the underivatized d_6 analogue *N*-(2,4-dimethyl- d_6 -phenyl)-*N'*-methylformamide m/z 169 daughter ion (138, 128, 120, and 110) transitions simultaneously showed no corresponding fragments related to the d_0 compound [m/z 163 daughter ion (122, 117, 107, 105, 79, 77)] (data not shown). However, application of the GC-MS method, followed by ion chromatography of m/z 282 and 276 (d_6 and d_0 metabolite M+

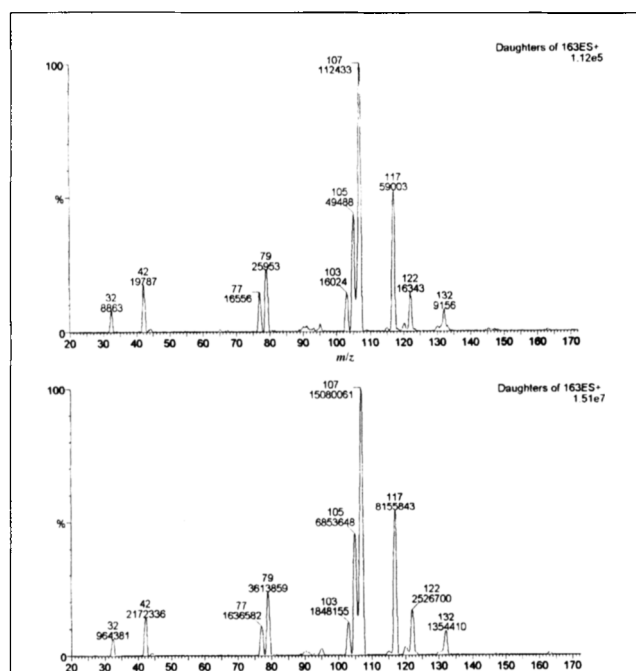


Figure 6. ES(+) daughter ion spectra for the m/z 163 species observed in ethyl acetate extracts of amitraz post-dose urine (top) (compare Figure 5) and for the standard of *N*-2,4-dimethylphenyl-*N'*-methylformamide (10 ng/ μ L) diluted 1:10 in 0.05% formic acid/acetonitrile (1:1) (bottom). The high collision energy of 26 eV enabled the relatively large diagnostic m/z 107 ion.

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 as presented in Figure 11.

Figure 12A illustrates ion chromatograms acquired from the extract of a 1-mL urine sample 1 h post dose and specifically indicating the location of coeluting quantifier (m/z 205) and qual-

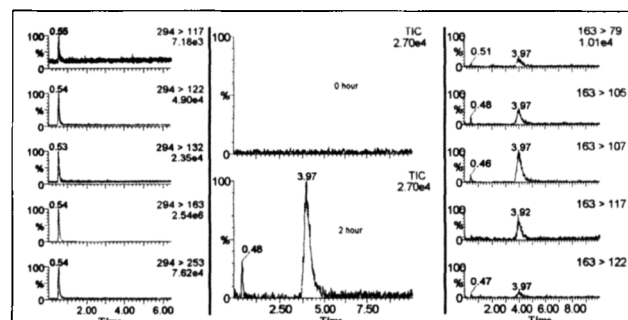


Figure 7. LC-MS-MS of amitraz (left panel) displaying early elution on a 30×1 -mm Luna phenyl-hexyl column (3- μ m particle size) with acetonitrile/0.05% formic acid (aq) (1:1) elution. Qualifier ions are shown, with the m/z 294 \rightarrow 163 being the largest transition. The amitraz 10 ng/ μ L standard was run at 0.150 mL/min with collection of the following ES(+) data by MRM (dwell time in ms in parentheses): m/z 294.00 > 253.00 (20); 294.00 > 163.00 (50); 294.00 > 132.00 (20); 294.00 > 122.00 (20); and 294.00 > 117.00 (20). The center panel shows amitraz-dosed urine extracted with ethyl acetate 0 (top) and 2 h (bottom) post dose and subjected to gradient HPLC, with ES(+) MS detection and MRM data acquisition and displaying the TIC for 0 and 2 h post-dose, with the amitraz metabolite at 3.97 min RT. The right panel shows the individual fragmentations for the 2-h sample. This confirmed *N*-2,4-dimethylphenyl-*N'*-methylformamide in the 2-h post-dose urine extract run at 0.150 mL/min with collection of the following ES(+) data by MRM (dwell time in ms in parentheses): m/z 163.00 > 122.00 (10); 163.00 > 117.00 (10); 163.00 > 107.00 (10); 163.00 > 105.00 (10); 163.00 > 79.00 (10); and 163.00 > 77.00 (10). Intensities of the individual ions in the right panel indicate the greater intensity of the 163 > 107 ion, in agreement with the compound's ES(+) MS-MS spectrum (Figure 6).

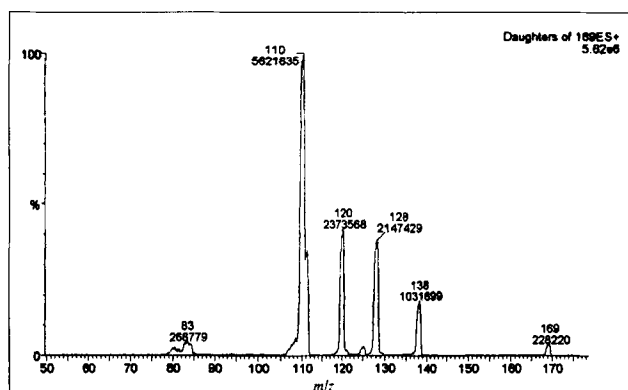


Figure 8. Development of a deuterated internal standard for quantitation of *N*-2,4-dimethylphenyl-*N'*-methylformamide in urine: the d_6 analogue ES(+) m/z 169 daughter ion mass spectrum. Note that ions m/z 163, 132, and 122 of Figure 6 are here represented by +6 amu variants, whereas m/z 117, 107, and 77, among others, are represented here by +3 amu variants.

Table III. Principal ESI(+)-MS-MS Daughter Ions of *N*-(2,4-dimethylphenyl)-*N*'-Methylformamide and Their Interpretation in Comparison to Its d_6 Analogue (Major Ions in Bold Type)

d_0 Amitraz Metabolite Peak (m/z)	Corresponding d_6 Amitraz Metabolite Peak (m/z)	$d_6 - d_0$ Difference (amu)	Likely Interpretation
163	169	6	M+H
132	138	6	[M+H] - CH ₃ NH ₂
122	128	6	[M+H] side chain rearrangement to NH ₂ (loss of CH ₃ - N = CH)
117	120	3	m/z 132 (d_0)/138 (d_6) - phenyl ring methyl
107	110	3	m/z 122 (d_0)/128 (d_6) - phenyl ring methyl
105	111	6	[M+H] - CH ₃ NH ₂ CH = N
103	109	6	unassigned
95	98	3	m/z 122 - phenyl ring C-CH ₃
90	94	4	m/z 105 (d_0)/111 (d_6) - phenyl ring methyl group
79	83	4	m/z 132 (d_0)/138 (d_6) - phenyl ring HC=CH-C-methyl
77	80	3	unassigned

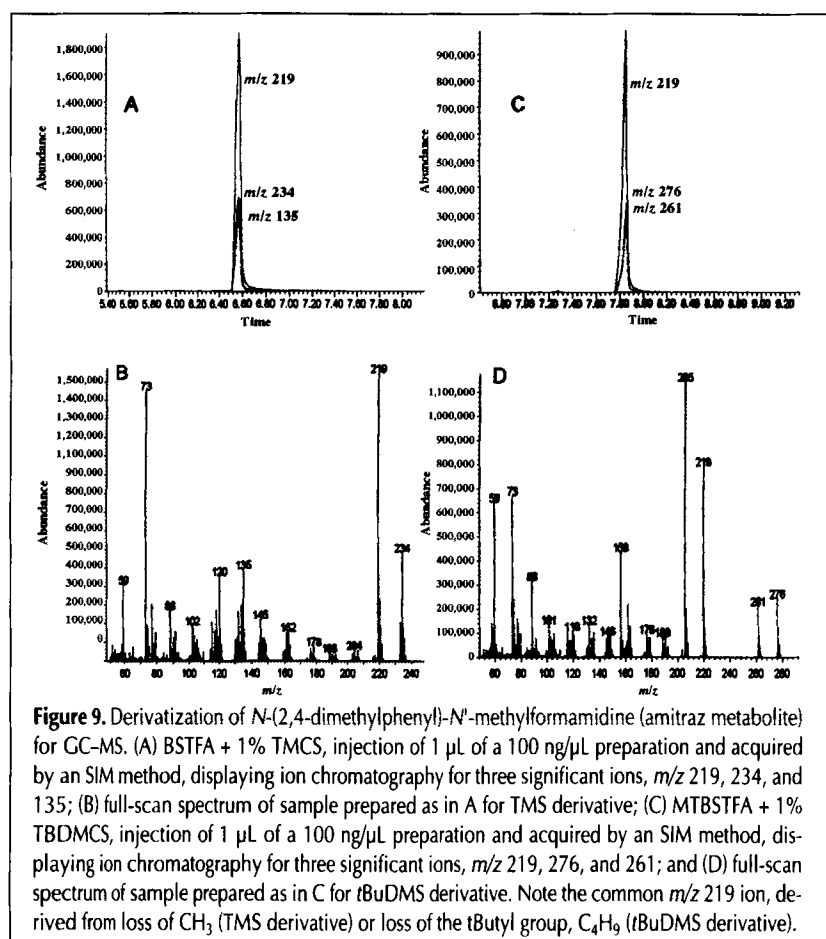


Figure 9. Derivatization of *N*-(2,4-dimethylphenyl)-*N*'-methylformamide (amitraz metabolite) for GC-MS. (A) BSTFA + 1% TMCS, injection of 1 μ L of a 100 ng/ μ L preparation and acquired by an SIM method, displaying ion chromatography for three significant ions, m/z 219, 234, and 135; (B) full-scan spectrum of sample prepared as in A for TMS derivative; (C) MTBSTFA + 1% TBDMCS, injection of 1 μ L of a 100 ng/ μ L preparation and acquired by an SIM method, displaying ion chromatography for three significant ions, m/z 219, 276, and 261; and (D) full-scan spectrum of sample prepared as in C for tBuDMS derivative. Note the common m/z 219 ion, derived from loss of CH₃ (TMS derivative) or loss of the tButyl group, C₄H₉ (tBuDMS derivative).

ifier ions (m/z 219, 261, and 276). The urine concentration of metabolite based on m/z 205 was 8.2 ng/mL. Figure 12B demonstrates measurements taken to determine peak height and noise height for m/z 219 for assurance that the S/N ratio had not fallen below the limit of 3. Finally, Figure 12C shows the time course of changes in urinary concentrations of *N*-2,4-dimethylphenyl-*N*'-methylformamide in urine following dosage with 75 mg amitraz (i.v.). Only two points exceeded the LOD of the assay, indicating the rapidity with which elimination of this particular metabolite occurs in the horse.

Validation information for the amitraz metabolite GC-MS confirmatory method is as follows. Standard curves showed a linear response between 2 and 300 ng/mL with a coefficient of determination $r^2 > 0.99$. Extraction efficiency for the SPE method ranged between 75–83% for the analyte spiked into urine. For the instrument lower LOD, S/N ratios were recorded for decreasing amounts of *tert*-butyldimethylsilyl-*N*-2,4-dimethylphenyl-*N*'-methyl-formamide, and the ratio fell below a limit of 3 at 50 pg (measured as underivatized metabolite) on column for two of the four principal SIM ions (m/z 261 and 276), corresponding roughly to a 5 ng/mL sample. The lower LOD in urine was determined by following the ion ratios to decreasing levels and determining whether ratios were maintained within Association of Official Racing Chemists minimum criteria (Proposed guidelines released 2001), namely 5% absolute or 30% relative for low resolution SIM. Measurements exceeded these criteria at a 50 pg injection, corresponding to a 5 ng/mL sample. Recovery of *N*-2,4-dimethylphenyl-*N*'-methyl-formamide during assays performed over three runs on different days provided an average measurement of 20.03 ng/mL with an average cv of 9.2% for a 20 ng/mL low standard and 291.7 ng/mL with an average CV of 7.4% for a 300-ng/mL high standard.

Specificity of the assay was tested by GC-MS confirmation analysis of unextracted standards of the structurally related α_2 -agonists guanabenz, guanfacine, guanethidine, and guanadrel; the structurally related antiulcer medication cimetidine; and furosemide and phenylbutazone, two therapeutic medications widely used in equine medicine (W. Carter, personal communication). None of the *t*BuDMS-derivatives coeluted with that of *N*-2,4-dimethylphenyl-*N*'-methyl formamide, with retention times relative to *tert*-butyldimethylsilyl-*N*-2,4-dimethylphenyl-*N*'-

methyl-formamidine as follows: guanethidine, four peaks at -1.99 , -1.69 , -1.37 , and $+0.07$ min; guanabenz, four peaks at -0.89 , -0.80 , $+0.36$, and $+2.87$ min; guanfacine, three peaks at $+0.29$, $+1.47$, and $+2.88$ min; guanadrel, two peaks at $+0.25$ and $+2.39$ min; cimetidine, three peaks at $+2.60$, $+3.03$, and $+4.46$ min; phenylbutazone-*t*BuDMS, $+4.86$ min; and furosemide, two peaks at $+7.05$ and $+9.64$ min. When these drugs were present at the equivalent of 100 ng/mL, no effect was observed on quantitation of the amitraz metabolite at concentrations of 50 or 500 ng/mL or even at the LOD of 5 ng/mL. Ion ratios m/z $276/205$ and $219/205$ for the amitraz- d_6 compound and $282/208$ and $225/208$ for the d_6 internal standard were unaffected, and there was no effect on their respective retention times. When the compounds were added to blank serum at concentrations of 500 ng/mL, extracted, and analyzed, no chromatographic interference was observed (i.e., no coeluting interference was observed in specific ion chromatograms for m/z 208 , 205 , 276 , or 219).

Discussion

Amitraz is known to possess characteristic sedative and tranquilizing properties in the horse and other species, principally due to its α -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. In this communication, 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 SIM of m/z 278 , 261 , 219 , and 205 fragments. The concentrations of this metabolite were quantitated in post-administration equine urines using an in-house synthesized d_6 internal standard derivatized to yield a *tert*-butyldimethylsilyl-*N*-2,4-dimethyl- d_6 -phenyl-*N'*-methyl-formamidine structure 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.

As seen in Figure 3, 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), 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. By TIC area count, these were present to the extent of 16% *tert*-butyldimethylsilyl-*N*-(2,4-dimethylphenyl)-formamide ($M + 263 = 149 + 114$), 11% *tert*-butyldimethylsilyl-*N*-(2,4-dimethylphenyl)-*N'*-methylformamidine ($M + 276 = 162 + 114$), 15% *tert*-butyldimethylsilyl-*N,N'*-bis(2,4-xylyl)-formamidine ($M + 366 = 252 + 114$), with the rest underivatized amitraz ($M + 293$).

Parent amitraz did not derivatize with MTBSTFA and chromatographed cleanly, yielding a 10.75 min peak (data not shown). 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 (data not shown), 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 in sheep and adult Shetland-cross ponies (11). 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 i.v. doses; elimination half-lives were significantly greater at 39.4 and 44.2 min, respectively. These doses were significantly greater than

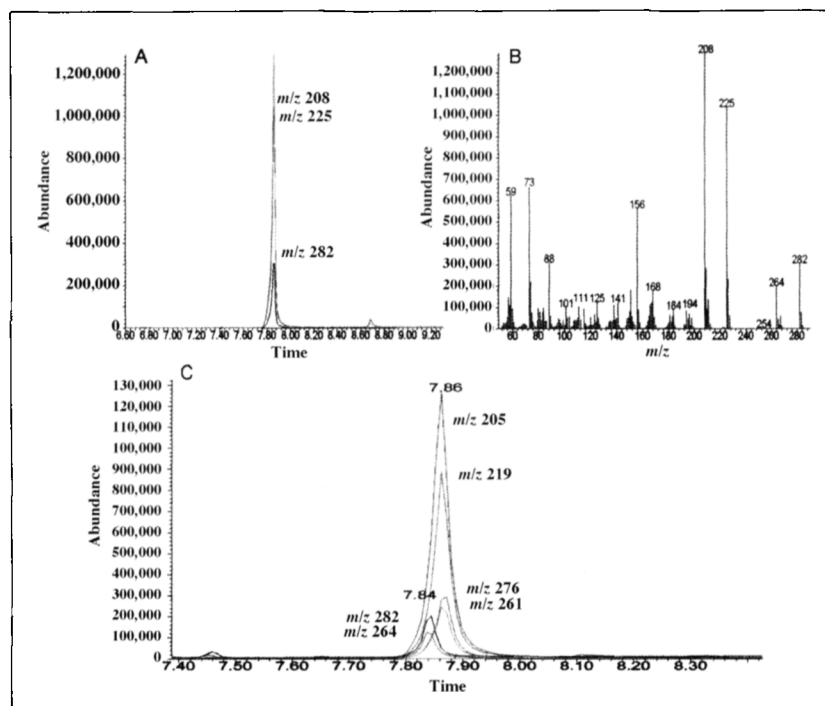


Figure 10. GC-MS chromatographic properties of the *N*-(2,4-dimethylphenyl- d_6)-*N'*-methylformamidine internal standard for urine concentration measurement. (A) MTBSTFA + 1% TBDMCS, injection of 1 μ L of a 100 ng/ μ L preparation and acquired by an SIM method, displaying extracted ion chromatography for three significant ions, m/z 208 , 225 , and 282 ; (B) full-scan spectrum of sample prepared as in A for *t*BuDMS derivative; and (C) ion chromatography for an extracted standard displaying ions m/z 282 and 264 specific to the 0.3 ng/ μ L internal standard and ions m/z 205 , 219 , 276 , and 261 specific to the 0.5 -ng/ μ L nondeuterated amitraz metabolite.

those utilized in the study reported here and resulted in a slow onset of sedation, which reached a maximum effect 10–15 min following injection. Although our purpose was not to measure pharmacological effect but metabolism, the 75-mg i.v. dose (roughly 0.15 mg/kg) nonetheless produced sedative effects in our horses (see also reference 14).

The pharmacokinetic parameters for amitraz and BTS 27271 measured by Pass and Mogg (11) were comparable to those of

the alpha-2 agonist xylazine (0.6 mg/kg i.v.), with a measured distribution half-life of 5.9 min and elimination half-life of 50 min in the horse (22). Such pharmacokinetic parameters are typically measured within 120 min following i.v. dose (11,22), because of the rapid decline in blood levels of these agents. Whereas it is our intention to guide laboratories in the development of methodologies for the confirmation of amitraz, 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'*-methylformamide metabolite as described herein, then the urine samples must optimally be taken within 2 h of the time of administration of this agent.

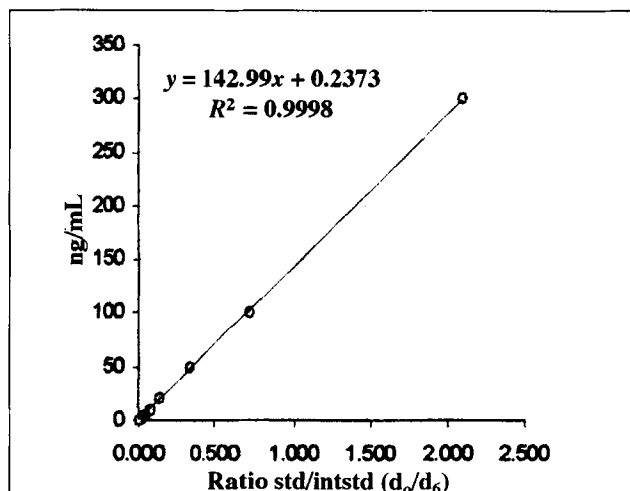


Figure 11. Typical standard curve generated for the amitraz metabolite *N*-(2,4-dimethylphenyl)-*N'*-methylformamide in equine urine spiked with the deuterated analogue. Note that concentration is displayed as a function of the ratio of metabolite standard/internal standard in order to enable generation of the linear equation as labelled.

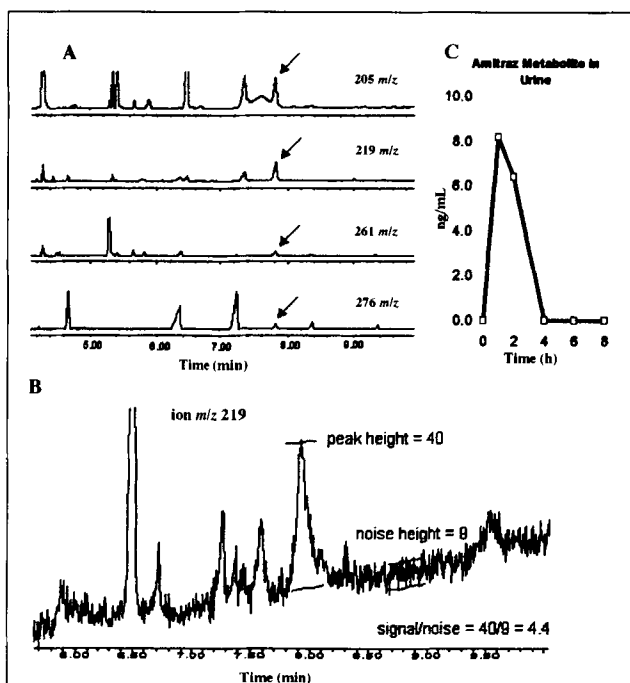


Figure 12. GC-MS confirmation of amitraz use on a urine sample 1 h following a 75-mg i.v. dose. Extracted ion chromatography for ions *m/z* 205, 219, 261, and 276 showing co-eluting peaks at 7.8 min retention (A); illustration of S/N ratio determination for qualifier ion *m/z* 219 for assurance that S/N exceeds 3.0 (B); and time course for amitraz metabolite between 0 and 8 h post administration (C).

References

1. Association of Racing Commissioners International. Uniform Classification Guidelines for Foreign Substances. <http://www.arci.com/drugtesting.html> (accessed January 2003).
2. Merck and Co., Inc. *The Merck Index on CD-ROM*, version 12:3. Chapman & Hall, CRC, Whitehouse Station, NJ, 2000.
3. L. Medleau and T. Willmense. Efficacy of daily amitraz therapy for refractory, generalized demodicosis in dogs: two independent studies. *J. Am. Anim. Hosp. Assoc.* **3**: 246–249 (1995).
4. L.C. Costa, G. Olibet, and S.D. Murphy. Alpha 2-adrenoceptors as a target for formamide pesticides: in vitro and in vivo studies in mice. *Toxicol. Appl. Pharmacol.* **93**: 319–328 (1988).
5. C.A. Schuntner and P.G. Thompson. Metabolism of [¹⁴C]-amitraz in larvae of *Boophilus microplus*. *Aust. J. Biol. Sci.* **31**: 141–148 (1978).
6. A.C. Martel and S. Zeggane. Determination of acaricides in honey by high-performance liquid chromatography with photodiode array detection. *J. Chromatogr.* **954**: 173–180 (2002).
7. C. Sanchez-Brunete, B. Albero, E. Miguel, and J.L. Tadeo. Determination of insecticides in honey by matrix solid-phase dispersion and gas chromatography with nitrogen-phosphorus detection and mass spectrometric confirmation. *J. AOAC Int.* **85**: 128–133 (2002).
8. E. Korta, L.A. Bakkali, B. Gallo, F. Vincente, V. Kilchenmann, and S. Bogdanov. Study of acaricide stability in honey. Characterization of amitraz degradation products in honey and beeswax. *J. Agric. Food Chem.* **49**: 5835–5842 (2001).
9. S.E. Smith. Treatment of mange in horses. *Vet. Rec.* **134**: 508–509 (1994).
10. M.A. Pass and T.D. Mogg. Effect of amitraz and its metabolites on intestinal motility. *Comp. Biochem. Physiol.* **C99**: 169–172 (1991).
11. M.A. Pass and T.D. Mogg. Pharmacokinetics and metabolism of amitraz in ponies and sheep. *J. Vet. Pharmacol. Therap.* **18**: 210–215 (1995).
12. A. Queiroz-Neto, G. Zamur, S.C. Goncalves, A.B. Carregaro, M.I. Mataqueiro, J.D. Harkins, and T. Tobin. Characterization of the antinociceptive and sedative effect of amitraz in horses. *J. Vet. Pharmacol. Therap.* **21**: 400–405 (1998).
13. J.C. Florio, M. Sakate, and J. Palermo-Neto. Effects of amitraz on motor function. *Pharmacol. Toxicol.* **73**: 109–114 (1993).
14. J.D. Harkins, A. Queiroz-Neto, G.D. Mundy, D. West, and T. Tobin. Development and characterization of an equine behavior chamber and the effects of amitraz and detomidine on spontaneous locomotor activity. *J. Vet. Pharmacol. Therap.* **20**: 396–401 (1997).
15. E.A. Abu-Basha, S. Yibchok-Anun, D.L. Hopper, and W.H. Hsu. Effects of the pesticide amitraz and its metabolite BTS 27271 on insulin and glucagon secretion from the perfused rat pancreas: involvement of alpha-2D-adrenergic receptors. *Metabolism* **48**: 1461–1469 (1999).
16. D.H. Shin and W.H. Hsu. Influence of the formamide pesticide amitraz and its metabolites on porcine myometrial contractility:

- involvement of alpha 2-adrenoceptors and Ca²⁺ channels. *Toxicol. Appl. Pharmacol.* **128**: 45–49 (2003).
17. S.L. Bonsall and G.J. Turnbull. Extrapolation from safety data to management of poisoning with reference to amitraz (a formamidine pesticide) and xylene. *Hum. Toxicol.* **2**: 587–592 (1983).
 18. I.R. Harrison, J.F. McCarthy, B.H. Palmer, and A. Kozlik. Pesticide Verbindungen, verfahren zu ihrer Herstellung und diese Verbindungen enthaltende Zubereitungen. *Chem. Abstr.* **75**: 63,443r (1971).
 19. M. Gall, M.M. McCall, R.E. TenBrink, P.F. VonVoigtlander, and J.S. Mohrland. Arylformamidines with antinociceptive properties. *J. Med. Chem.* **31**: 1816–1820 (1988).
 20. CDN Isotopes, Inc. 2,4-Dimethyl-d6-aniline. CDN Isotopes, Inc., Catalog 4A. <http://www.cdniso.com/englishversion/> (accessed April 2004).
 21. J.M. Bosken, A.F. Lehner, A. Hunsucker, J.D. Harkins, W.E. Woods, W. Karpiesiuk, W.G. Carter, J. Boyles, M. Fisher, and T. Tobin. Direct MS–MS identification of isoxsuprine-glucuronide in post-administration equine urine. *Can. J. Vet. Res.* **64**: 112–116 (2000).
 22. R. Garcia-Villar, P.L. Toutain, M. Alvinerie, and Y. Ruckebusch. The pharmacokinetics of xyazine hydrochloride: an interspecific study. *J. Vet. Pharmacol. Therap.* **4**: 87–92 (1981).
 23. C.O. Knowles and A.K. Gayen. Penetration, metabolism, and elimination of amitraz and N¹-(2,4-dimethylphenyl)-N-methylformamidine in southwestern corn borer larvae (Lepidoptera: Pyralidae). *J. Econ. Entomol.* **76**: 410–413 (1983).
 24. C.O. Knowles and H.J. Benezet. Excretion balance, metabolic fate and tissue residues following treatment of rats with amitraz and N¹-(2,4-dimethylphenyl)-N-methylformamidine. *J. Environ. Sci. Health, Part B* **16**: 547–555 (1981).
 25. Z. Naikai, D. Xiuqing, X. Xijuan, W. Shuqin, and A. Fengchun. Metabolic conversion of N¹-(2,4-dimethylphenyl)-N-methylformamidine pesticide and the analysis of the metabolites. *Bull. Environ. Contam. Toxicol.* **65**: 22–27 (2000).

Manuscript received March 18, 2003;
revision received September 9, 2003.