Certified reference standards and stable isotope internal standards for equine therapeutic medication regulation

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ABSTRACT

Regulators of equine events now recognize the need for defined regulatory "cut-offs" or "thresholds" for trace residues of therapeutic medications in blood or urine, thereby requiring accurate forensic quantification of residues of these medications. This creates a need for certified reference standards and stable isotope internal standards for use in medication analysis. In the United States, the Racing Medication Testing Consortium (RMTC) has identified approximately 50 equine therapeutic medications, dietary and environmental substances for regulation. Where appropriate, we are synthesizing and making available to the racing industry the appropriate certified reference standards and stable isotope internal standards for use in the analysis of these compounds. To date we have synthesized about 40 reference standards and are providing at least 20 unique certified reference standards and/or their appropriate stable isotope internal standards to the racing industry. Unique equine drug/metabolite standards/stable isotope internal standards which have been synthesized include Hydroxyethylpromazine (HEP) and Hydroxyethylpromazine sulfoxide (HEPS), HEP-*d*4 and HEPS-*d*4, and most recently, 4-Hydroxyxylazine and 4-Hydroxyxylazine-*d*6. Other reference standards synthesized and available include Carboxydetomidine, Hydroxydetomidine, Carboxydetomidine-*d*4, Hydroxydetomidine-*d*4, Furosemide-*d*5, Clenbuterol-*d*9, Flunixin-*d*3, Guaifenesin-*d*4, Ketoprofen-*d*4, 3-Hydroxylidocaine, 3-Hydroxylidocaine-*d*3, 4-Hydroxylidocaine, 3-Hydroxymepivacaine, 3-Hydroxymepivacaine-*d*3, 4-Hydroxymepivacaine-*d*3, Methocarbamol-*d*4, Phenylbutazone-*d*9, Procaine-*d*10, 7-Hydroxyfluphenazine, 3-Hydroxypromazine, Butorphanol-*d*6, Pyrilamine-*d*3, O-Desmethylpyrilamine, O-Desmethylpyrilamine-*d*3, Tetrahydrogestrinone-*d*4, Tramadol-*d*3, O-Desmethyltramadol, O-Desmethyltramadol-*d*4, Boldenone-*d*3 and others.

Keywords: Equine forensic chemistry, medication regulation, quantitative analysis, regulatory analytes, certified reference standards, stable isotope internal standards, chemical synthesis.

INTRODUCTION

Proper veterinary care of horses requires that horses in training have access to modern therapeutic medications. However, the sensitivity of equine drug testing now allows ready detection of pharmacologically insignificant concentrations, or insignificant "trace levels", of many therapeutic medications. In 1995, the Association of Racing Commissioners International (ARCI) resolved that members "address trace level detection so as not to lead to disciplinary action based on *pharmacologically insignificant traces of these substances* (our emphasis) (Tobin et al. 1999). More recently, the Racing Medication and Testing Consortium (RMTC) has identified approximately 50 therapeutic medications for which it is committed to developing regulatory thresholds (Spencer et al. 2008), as set forth in Table 1.

The "substances" identified and quantified in post-race samples may be the actual parent medications themselves, usually quantified in plasma, or, in a number of cases, fragments of metabolically transformed forms of the medication usually recovered from urine samples. Since these are the "analytes" on

which regulatory control of the medication depends, these substances may best be designated as "regulatory analytes" (Tobin et al. 1999). To enable their accurate quantification, certified reference standards of the regulatory analytes are required (Tobin et al. 1999) as well as corresponding stable isotope labeled analogs of the regulatory analytes to serve as internal standards (Karpiesiuk et al. 2000, 2002, 2004).

The University of Kentucky, in cooperation with Frontier Biopharm (Richmond, Kentucky) and Neogen Corp. (Lexington, Kentucky), is working to synthesize and provide to the equine forensic chemistry community certified reference standards for equine therapeutic medications prepared under US GMP standards that will satisfy ISO-17025 requirements for quantitative measurements, and ISO-34 requirements for reference materials. We are also synthesizing the corresponding stable isotope internal standards, which are also required for quantitative equine forensic testing. These reference standards will meet industry requirements for chemical identity, spectroscopic and chemical purity, including levels of residual volatile solvents, water and inorganics, in accordance with accepted ISO specifications for analytical standards.

Acepromazine, hydroxyethylpromazine, and hydroxyethylpromazine sulfoxide

Acepromazine is a phenothiazine tranquilizer widely used in the control and handling of horses (Ballard et al. 1982). The effective dose is small and it is rapidly converted to its major urinary metabolites Hydroxyethylpromazine (HEP) and Hydroxyethylpromazine sulfoxide (HEPS) (Figure 1). Regulatory control of acepromazine currently depends on the recovery and quantification of either or both of these substances in post-competition urine samples (Dewey et al. 1981). We have synthesized these compounds and their deuterated analogs (Figure 1) and they are now available as certified reference standards for racing chemists.

Detomidine

Detomidine (4-((2,3-dimethylphenyl)methyl)-3H-imidazole) is a potent and relatively short-acting alpha2 adrenergic agonist tranquilizer widely used in equine medicine. Detomidine is administered by rapid intravenous injection and produces a profound but relatively short-lived tranquilization. Its actions are terminated by redistribution, with plasma concentrations of detomidine rapidly becoming undetectable (Grimsrud et al. 2009). Optimal regulatory control of its use is therefore dependent on detection and quantification of urinary metabolites or metabolite fragments. The principal metabolite fragments of detomidine found in equine urine are carboxydetomidine and hydroxydetomidine (Grimsrud et al. 2009). We have synthesized both of

Table 1. Rmtc therapeutic medications routinely used and identified as necessary by the veterinary advisory committee.

First Priority Group (Currently in Research)	Second Priority Group	Third Priority Group	Fourth Priority Group	Fifth Priority Group	Research Already Underway	Already in Body of Model Rules
Acepromazine	Boldenone	Albuterol	Atropine	Diazepam	Aminocaproic Acid	Cimetidine
Butorphanol	Stanozolol	Betamethasone	Beclomethasone	Dipyrone	Carbazochrome	DMSO
Detomidine	Testosterone	Diclofenac	Buscopan	Fluorprednisolone	Clenbuterol	Flunixin
Glycopyrrolate	Dantrolene	Methylprednisolone	Cromolyn	Guaifenesin	Procaine Penicillin	Furosemide
Lidocaine	Dexamethasone	Reserpine	Isoxsuprine	Isoflupredone		Ketoprofen
Mepivacaine	Fluphenazine	Triamcinolone	Pentoxyfylline	Prednisone		Omeprazole
Methocarbamol	Hydroxyzine	Trichlormethiazide	Phenytoin			Phenylbutazone
Pyrilamine	Nandrolone	Xylazine	Prednisolone			Ranitidine

This table is reproduced courtesy of Dr Scot Waterman and the Racing Medication and Testing Consortium. For each of these therapeutic medications the RMTC is developing appropriate regulatory thresholds in plasma or urine, and also associated withdrawal time guidelines (January, 2008).

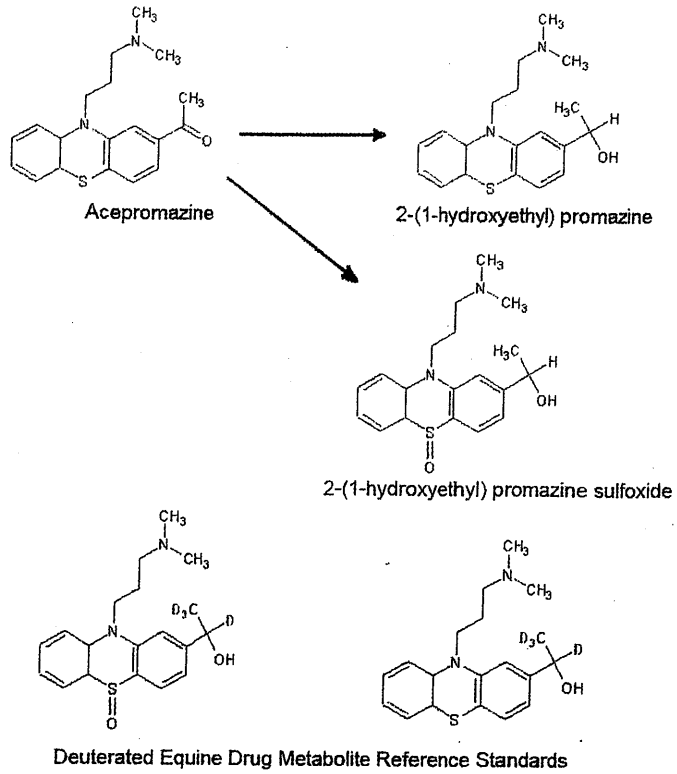


Figure 1: Top: Equine metabolic products of Acepromazine, HEP and HEPS. Bottom: Corresponding deuterated internal standards.

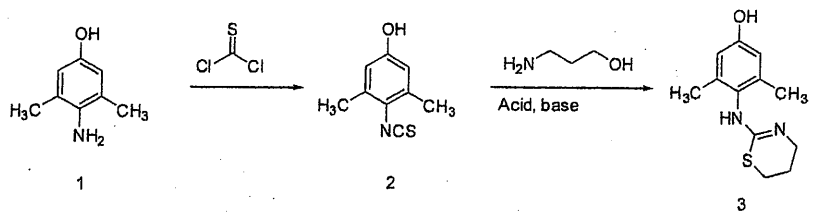


Figure 2: Synthesis of 4-hydroxyxylazine. Synthesis of 4-hydroxyxylazine commenced with substance 1, followed by conversion to isothiocyanate [2], followed by addition of 3-amino-1-propanol to alkylate the isothiocyanate group under basic and acidic conditions to yield the desired product, 4-hydroxyxylazine [3].

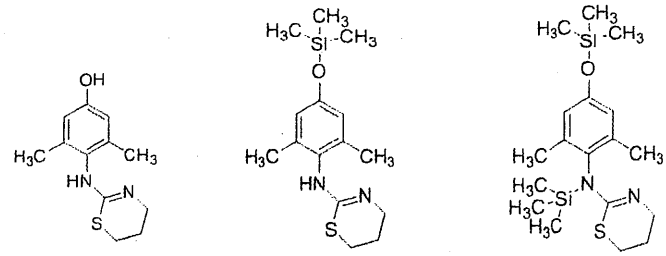
these compounds and their deuterated analogs and they are now available as certified reference standards.

Xylazine

Xylazine (N-(2,6-dimethylphenyl)-5,6-dihydro-4H-1,3-thiazin-2-amine) is a relatively short-acting alpha-2 adrenergic agonist tranquilizer widely used in equine medicine. Xylazine is administered by rapid intravenous injection and produces a profound but short-lived tranquilization. Its actions are terminated by redistribution, with plasma concentrations of xylazine rapidly becoming undetectable (Mama et al. 2005). Optimal regulatory control of its use is therefore dependent on detection and quantification of urinary metabolites or metabolite fragments (Mutlib et al. 1992). The principal metabolite fragment recovered from equine urine is 4-hydroxyxylazine which we have synthesized following the procedure outlined in the Figure 2.

Mass spectrometry of 4-hydroxyxylazine

Analysis of 4-hydroxyxylazine as its silyl derivative by GC-MS revealed two chromatographic peaks (Figure 4-a) from compounds resulting from the derivatization of the hydroxyl group (Figure 4-b, ret time 10.4) plus partial derivatization of the compound at the NH



4- hydroxyxylazine mono-TMS derivative bis-TMS derivative

Figure 3: 4-hydroxyxylazine and its silyl derivatives, 4-hydroxyxylazine-monoTMS, 4-hydroxyxylazine-bisTMS.

site (Figure 4-c, ret time 10.1) (structures in Figure 3). Consistent with previous experience, reducing the concentration of BSTFA derivatizing reagent (in this case from 100% to 30%) in dichloromethane resulted in a single chromatographic peak consisting of the mono-TMS derivative (mw 308).

NMR characterization

The 400 MHz Varian INOVA 400 MHz NMR located in the Chemistry Department at the University of Kentucky was used to obtain 1H, 13C, and Gradient-selected Correlation SpectroscopY (gCOSY) NMR spectra to confirm the structure of our

4-hydroxyxylazine product. The 1H, 13C, and gCOSY NMR spectra are consistent with a high-purity product. The spectra for 1H show the distribution of proton chemical shifts are associated with the functional group on the molecule, with the proton chemical shift ranges in good accord with their various functional groups and in good agreement with literature reports. Based on this information we can correlate the NMR spectral characteristics (Table 2) of our 4-Hydroxyxylazine as we will now detail. First, 4-Hydroxyxylazine contains two aromatic protons in the benzene ring, H2 and H6, which are expected to be in the 6.1 to 8.5 ppm range. However, because these peaks are mirror images of each other, our proton NMR shows them at 6.36 ppm as one peak.

4-Hydroxyxylazine also contains two methyl (CH_3) groups in the aromatic benzene. The usual saturated alkane proton range is from 0.5 to 1.5 ppm, but since our methyl groups are attached to the benzene, they may be expected to be slightly de-shielded due to the benzene ring; consistent with this interpretation, the chemical shift was found at 1.92 ppm. 4-Hydroxyxylazine also contains three different methylene (CH_2) groups, namely 4, 5, and 6', as set forth in Table 2. The methylene 5 has an expected chemical shift around 1.84 ppm, because it is surrounded by two saturated alkanes. The methylene 4 is connected to a sulfur that contains lone pairs of electrons, which makes the methylene more de-shielded; therefore, it is as expected at 2.84 ppm. The third methylene 6' is connected to a nitrogen that contains a lone pair of electrons and at the same time to a double bond; as a consequence, its chemical shift is expected to be more de-shielded than methylene 4. The proton NMR showed its chemical shift at 3.19 ppm, which is as expected. Finally, the proposed H-H correlation between methylene 4-5-6' was supported by the corresponding gCOSY spectrum.

Table 2: NMR spectral characterization table for 4-Hydroxyxylazine.

#	H	Chemical shift (ppm)
2, 6	H	6.36
3	CH_3	1.92
4	CH_2	2.84
5	CH_2	1.84
6'	CH_2	3.19

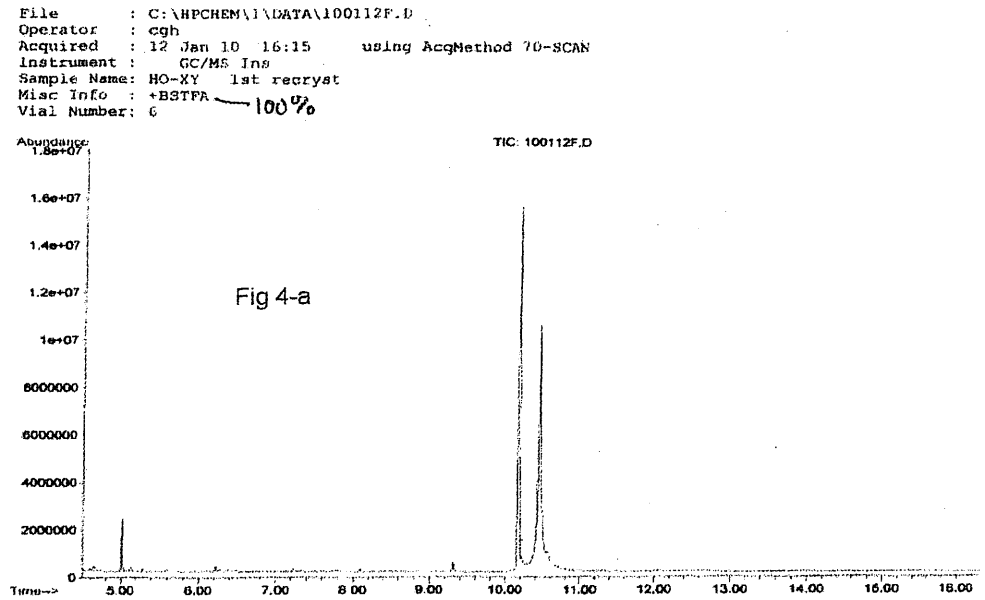
Note: Peak at 2.5 = DMSO-d_6 (deuterated solvent), and Peak at 4.10 = D_2O (deuterated solvent)

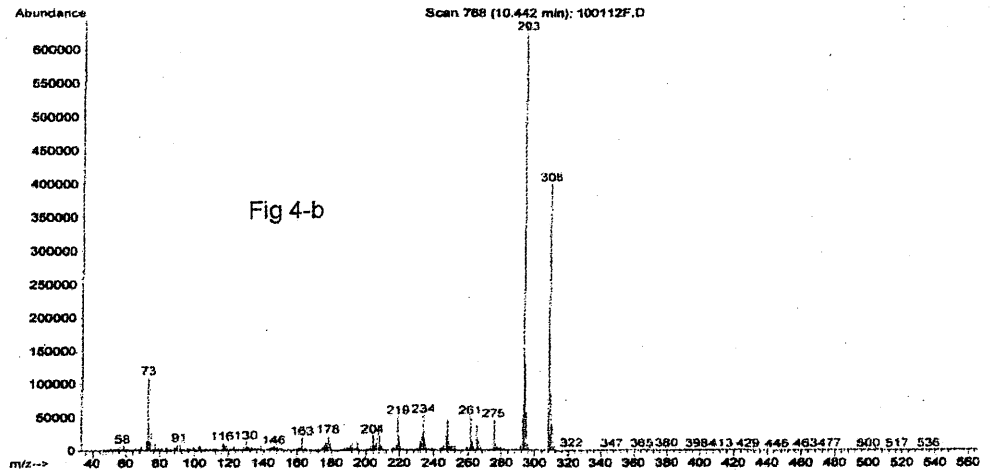
Other deuterated standards

Phenylbutazone-d_9, Flunixin-d_3, Ketoprofen-d_3, Methocarbamol-d_4, Guaifenesin-d_4, Clenbuterol-d_9, Furosemide-d_5, Procaine-d_{10}, and others.

We have previously synthesized and made available Phenylbutazone-d_9, Flunixin-d_3 and Ketoprofen-d_3 (Tobin et al. 2006), nonsteroidal anti-inflammatory medications approved by the RMTC and widely used in horses in training. These agents remain detectable in plasma for at least 24 h after the last administration; the regulatory analyte is the parent medication itself, and the appropriate internal standard is the stable isotope labeled medication, and these are being made available to racing chemists worldwide.

Methocarbamol is a neuromuscular relaxant widely used in horses in training and we have synthesized the d_4 analog of Methocarbamol. Guaifenesin is a structurally related neuromuscular relaxant and we have synthesized the d_4 analog of Guaifenesin. Additionally,





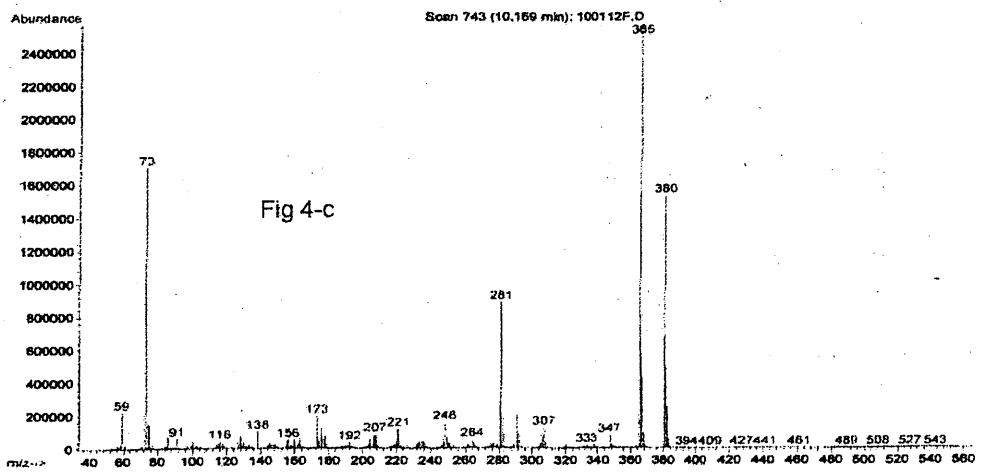


Figure 4: Chromatogram and mass spectra of 4-hydroxyxylazine-monoTMS (mw 308) and 4-hydroxyxylazine-bisTMS (mw 380).

as previously described, we have synthesized the d_4 analog of Clenbuterol, a widely used bronchodilator, and the d_5 analog of Furosemide, a diuretic widely used in the prophylaxis of Exercise-Induced Pulmonary Hemorrhage (EIPH) in racing horses. Procaine is a local anesthetic and a central nervous system stimulant and because its use in racehorses is regulated, we have synthesized Procaine-d_{10}.

CONCLUSION

Each of these regulatory analytes will be available as a 0.1 mg per mL certified reference standard, and also the corresponding stable isotope reference standard (www.frontierbiopharm.com). All analytes will be greater than 98% chemical and isotopic purity, absence of residual solvents is being established by thermal gravimetric analysis or static headspace analysis, all masses are determined using balances calibrated with NIST traceable balances, all concentration values are determined from an independent calibration curve and the shelf-lives of these reference standards in solution are at least 12 months unless otherwise stated.

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