

**Analysis of a Neutral
“Dantrolene-Related Compound”
from Horse Urine**

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A Procedure Developed for
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Summary

Dantrolene, C₁₄H₁₀N₄O₅ (I), is a skeletal muscle relaxant and is commercially available as the sodium salt (Dantrium®). Detection of dantrolene and 5-hydroxydantrolene by HPLC was earlier reported (Stevenson, *et al.* 1997; Todi, *et al.* 1989) in horse urine and plasma. Although an immunoassay is commercially available for the detection of dantrolene (and/or its metabolites), no reproducible GC/MS method is available for confirmation. This method detects by GC/MS a neutral compound in the acid-neutral fraction from solid phase extracts of horse urine after oral administration of dantrolene sodium (1250 mg). This “dantrolene-related compound”, given the trivial name dantral, is assigned structure II based on H¹ and C¹³ NMR analyses and a partial synthesis from dantrolene. It differs in structure from the “degraded 5-hydroxydantrolene” reported previously (Stevenson *et al.* 1997). Dantral (II) was not detected when dantrolene supplemented samples were analyzed by SPE-GC/MS, which suggests that dantral (II) is a true metabolite of dantrolene.

Introduction

The skeletal muscle relaxant dantrolene (dantrolene sodium, Dantrium®, I, Fig. 1, Norwich Eaton) is indicated for the prevention and treatment of horse exertional rhabdomyolysis and other muscle disorders associated with exertion. Dantrolene is classified as a class IV substance by the Association of Racing Commissioners International (ARCI), indicating it may have some performance enhancing potential.

Dantrolene is reported to undergo extensive metabolism in humans, with only 5% of the parent drug excreted in urine, 5-hydroxydantrolene being the main metabolite. *p*-Actamidodantrolene (formed by reduction of the nitro group followed by acetylation) is a minor metabolite (Clark 1986). Hydroxydantrolene was also reported in horses (Court, *et al.* 1987) and dogs (Wuis, *et al.* 1982).

Although immunoassays are available for testing for dantrolene in horse urine, reliable and reproducible confirmatory techniques are limited. High performance liquid chromatographic (HPLC) techniques (Lerman, *et al.* 1989; Todi, *et al.*, 1989) generally lack specificity, while gas chromatographic/mass spectrometric (GC/MS) protocols (Stevenson, *et al.*, 1997) result in on-column degradation of dantrolene. These limitations prompted us to develop a reliable and sensitive GC/MS method for the detection of dantrolene and/or its metabolites in horse urine.

Scope

This SOP describes analysis of dantral (II, Fig.1) in horse urine. Screening for dantrolene-related products is accomplished by immunoassay (dantrolene ELISA, Neogen). Confirmation is based on the presence of dantral in either dichloromethene or acid treated methanol: dichloromethene (3:97) eluates from SPE columns.

Limitations

The sole effective means for detecting dantrolene-related compounds in horse urine is by immunoassay (ELISA) screening with an approximate limit of detection of 5-10 ng/ml (spiked with dantrolene). Since pure dantral and other metabolite standards are not commercially available, concentrations can only be estimated. In some instances, dantrolene can only be measured indirectly after acid treatment of urine extracts.

Safety Precautions

Protective clothing (safety goggles, lab coat, gloves) should be worn when handling organic solvents and concentrated acids and bases. Procedures should be performed in a fume hood or with otherwise adequate ventilation. Consult material safety data sheets for chemicals used in this protocol.

Materials and Methods

Animal dosing

Dantrolene sodium (1250 mg) was administered orally on two different occasions (3 months apart) to a horse. Urine was collected at 0, 1, 3, 5, 7, 10, 24, 48, and 72 hours after the first administration and at 0, 4 and 8 hours after the second.

Standards and chemicals

Five mg of dantrolene sodium (Sigma) was dissolved in water (3 ml), the pH was adjusted to 6.5 with 0.1M phosphate buffer, and dantrolene was extracted into dichloromethane (DCM, 5 ml). Chemicals and solvents used in this protocol were commercially available ACS grade.

Immunoassay kit

The dantrolene ELISA was available from Neogen and this assay was performed according to the manufacturer's instructions.

SPE columns

XTRACT (500 mg mixed bed) proprietary columns were purchased from United Chemical Technologies, Inc.

Reagents

1. 0 N Acetic Acid (HOAc) for SPE

Add 201 ml glacial acetic acid ($\text{CH}_3\text{CO}_2\text{H}$) to 3299 ml deionized water.

0.2 M Phosphate Buffer pH 6.5 for SPE

Dissolve 87.04 g potassium phosphate monobasic (KH_2PO_4) in 3200 ml deionized water. Mix well. Adjust pH to 6.5 with sodium hydroxide or potassium hydroxide pellets (approximately 11 g) or 5 – 10 N NaOH or KOH.

Synthesis of dantral (compound II)

Standard dantrolene or residues from extracts containing dantrolene and/or metabolites were dissolved in methanol: conc. hydrochloric acid (1:1,2 ml). After standing at room temperature for 2 hours, the solution was evaporated to dryness (N_2). The residue was extracted with DCM (2 x 250 μl), and this solution was dried by filtering through cotton.

After evaporation to dryness (N₂), the residue was reconstituted in 50 µl of DCM for GC/MS analysis.

Instrumentation

Mass spectra were obtained using a Hewlett-Packard 5972 MSD with a 5890 GC (15m HP5 MS column). NMR experiments were performed on a Bruker ARX-500 Fourier Transform Spectrometer. Samples were dissolved in 0.6 ml of CDCl₃ in 5 mm tubes. Proton NMR spectra were recorded at 500MHz and carbon spectra at 125MHz. Proton spectra utilized 640 scans while carbon required 30,000. A 30-degree pulse and 1 second delay were used in both instances.

Controls

Blank horse urine is used as a negative control. Either horse urine spiked at 2-3 ug of dantrolene per ml or urine collected at 4 to 8 hrs. after oral administration of dantrolene sodium (250 mg to 1250 mg) are recommended as positive controls.

Solid phase extraction (SPE) protocol

United Chemical Technologies' protocol was used with minor alterations. Five ml aliquots of urine at pH 5.5 – 6.5 were loaded onto pre-conditioned SPE columns (methanol, 5 ml; H₂O, 5 ml; phosphate buffer 0.2 M pH 6.5, 3 ml). The columns were then sequentially washed with phosphate buffer (0.2 M, pH 6.5, 5 ml), 1N acetic acid (2 ml), and hexane (5 ml). Prior to addition of hexane, the column was dried under strong vacuum (eg 12-15 in Hg) for ≈ 45-60 minutes (or until dry). Two fractions were collected: (1) DCM, 10 ml; and (2) DCM containing 3% methanol, 10 ml. Both extracts were evaporated to dryness under N₂. For GC/MS, residue (1) was dissolved in 50 µl DCM and analyzed for dantral. Metabolites of dantrolene in residue (2) were converted to dantral as described in this SOP.

Analytical Data

Immunoassay

Urine samples collected after the two administrations (through 72 hours for the first administration) contained dantrolene and/or its metabolites based on ELISA analysis (i.e., greater than 80% suppression as compared to zero hour controls).

Gas chromatography / mass spectrometry

Table (1) shows electron impact MS data for dantral (II) and dantrolene degradation product (produced after injecting parent dantrolene into the GC/MS system).

Table 1

Electron Impact Mass Spectral Data

Experimental conditions:

Injection port, 250° C; Initial oven temperature, 120° C (1 min.); Ramp rate, 20°/min; Final oven temperature, 300° C.

Dantral:

m/z (%), 217 (100, M⁺), 115 (51), 187 (36), 114 (31), 159 (15), 218 (13), 89 (13), and 88 (11)

Dantrolene (possible degradation product):

m/z (%), 214 (100), 140 (52), 184 (50), 156(38), 113 (34) and 89 (33)

Fraction (1) from urine collected from 4 to 8 hours in both administrations contained dantral. Small amounts of dantral and dantrolene degradation product (structure as yet undetermined) were seen in fraction (2) from these urine collections, but after conversion to dantral as described, this compound was readily detected in these fractions.

Dantrolene and/or its metabolites present in fraction two are difficult to analyze by GC/MS (Todi, *et al.* 1989; Stevenson, *et al.* 1997) without conversion to the aldehyde (dantral). In addition to dantrolene, related material in fraction two may include 5-hydroxydantralene and amino dantrolene. Only dantrolene and 5-hydroxydantralene are converted to dantral.

Nuclear Magnetic Resonance

Dantral was characterized as structure II (Fig. 1) on the basis of H¹ and C¹³ NMR data. The assignments of seven protons and eleven carbon atoms are demonstrated in Figure 2. The degradation product in fraction (2) (Table 1) differs from the reported degradation product of dantrolene/5-hydroxydantralene (Stevenson, *et al.* 1997) and remains uncharacterized.

Dantral as a Possible Metabolite

Whether dantral from urine extracts is a metabolite or extraction artifact was answered by supplementing urine with dantrolene (3 µg/ml) and analyzing by SPE and GC/MS. Dantral was not found in fractions 1 and 2, but was detected in fraction 2 after acid hydrolysis, suggesting that this compound is formed in vivo and not during the analytical protocol.

The imine linkage (as in dantrolene) is usually produced by the acid catalyzed reversible reaction of a primary amine and an aldehyde. The formation of dantral from an acid catalyzed cleavage of dantrolene is therefore expected. We have no direct evidence, however, that this reaction occurs as a metabolic pathway even though the acid analog was reported as a metabolite in plasma and urine (Wuis, *et al.* 1990).

Estimation of Dantrolene-Related Compounds

After qualitative GC/MS analysis of urine from different collection times, it was noted that the amount of dantrolene-related materials decreased rapidly within 8-10 hours after dosing. Confirmation was difficult after 24 hours.

Although one could estimate indirectly the amount of dantrolene-related materials in urine by measuring the concentration of dantral, this was not attempted due to the lack of a dantral reference standard.

Quality Assurance

This protocol was verified in other TIP laboratories demonstrating transferability.

Critical control points: The effectiveness of the conversion of dantrolene and related products to dantral is unknown. Preparation of standard solutions of dantrolene rely on extraction from acidic solution, making final concentrations presumptive.

Conclusions

- 1) Dantral is detectable by GC/MS in SPE fraction (1) from urine of horses dosed with dantrolene. Dantral is detectable in SPE fraction (2) (3% MEOH in DCM) after acid hydrolysis.
- 2) Dantral is not an extraction artifact and may be formed in vivo.
- 3) Dantrolene and related compounds are cleared relatively quickly from horse urine after a single oral dose of 1250 mg.
- 4) Dantral serves as a marker for the presence of dantrolene.

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