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Short Communication

Rapid extraction and detection of mazindol in horse urine

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Introduction

Mazindol, 5-(*p*-chlorophenyl)-5-hydroxy-2,3-dihydro-5H-imidazo (2,1-a) isoindole, is used clinically to produce an anorectic effect, which occurs as a result of stimulation of the central nervous system (CNS). However, this action on the CNS gives mazindol the potential for abuse, particularly as a pre-race stimulant for dogs and horses. The metabolism of mazindol in rats and dogs, as well as in man, has been previously studied and the drug shown to undergo extensive biotransformation, producing some 15 metabolites in very low concentrations [1, 2]. Differences were apparent in the metabolites produced by different species.

Because of this extensive metabolism, the concentration of parent drug present in urine following an administration will consequently be in the low ng ml^{-1} range. As the metabolism of mazindol in the horse is unclear, and to avoid any subsequent legal arguement, it is important to identify the parent drug in the urine of racing animals. The approach to this analytical problem described here was, therefore, to develop a highly efficient extraction procedure.

Chromatographic methods for the analysis for mazindol in biological fluids are limited. There are no published liquid chromatographic methods for the determination of mazindol, and serious difficulties in the analysis for mazindol using gas chromatography have been reported [3]. The standard compounds tend to breakdown during injection into the gas chromatograph, and attempts to derivatize the primary amine group with trifluoroacetic acid have been unsuccessful. It would appear that the best approach for the gas chromatographic determination of mazindol is by the incorporation of a mass selective detector (MSD), operated in selective ion monitoring mode, into the system [3, 4]. The detection limit for this drug, using gas chromatography–mass spectrometry (GC–MS), was reported to be 5–10 ng ml^{-1} depending on sample background.

However, using this method [3, 4] mazindol could not be detected in the urine of racehorses following the oral administration of 50 mg of the drug. This lack of sensitivity may be due to the laborious and inefficient extraction procedure described, which further complicates the analysis. The extraction methods described by Timmings *et al.* [3], using either solvent extraction or extraction with XAD resin, resulted in only 50% recovery of mazindol from spiked urine.

Urine samples from horses which had been dosed with mazindol, found to be positive by immunoassay screen (ELISA), could not be confirmed in these laboratories by GC–MS using the above extraction procedures.

No literature is available concerning the

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selective solid-phase extraction or determination of parent mazindol in racehorse urine.

This paper presents a rapid, efficient and sensitive extraction method and determination procedure, capable of detecting parent mazindol in horse urine up to 8 h after the single oral administration of an effective dose.

Experimental

Materials

Mazindol (Sanorex) was obtained from Sandoz Pharmaceuticals (NJ). Spiked horse urine standards were prepared by adding aliquots of a standard solution of the drug (1 mg ml^{-1}) in methanol to 1 ml of sample. Solutions containing 10 ng to 10 μg ml^{-1} of drug were prepared in this manner, mixed by vortexing and kept at 4°C until required for analysis.

Three horses were each given an oral dose of 50 mg of mazindol, and urine samples were taken at time intervals of 0, 0–1, 1–2, 2–4, 4–6, 6–8, 28 and 52 h after administration. This was repeated following an oral dose of 5 mg of the drug. These samples were also stored at 4°C prior to analysis.

Extraction procedure

Bond-Elut columns (Analytichem International, Harbor City, CA) containing benzenesulphonic acid packing material (strong cation exchange) and with a capacity of 3 ml, were positioned in a Vac-Elut sytem. Vacuum pressure was adjusted to 15–20 mmHg and each column was activated by washing with 2 × 3 ml methanol followed by 2 × 3 ml of distilled water and 3 ml of dilute phosphoric acid (7 mmol l^{-1}) at pH 3.5. Without allowing the column to dry, 10 ml of urine was adjusted to pH 3.5 with phosphoric acid (5 ml), applied to the column and drawn through under vacuum. The sample was left to dry on the column for 30 s before being washed, in sequence, with 3 ml of dilute phosphoric acid (pH 3.5), 1 ml of 1 M acetic acid and 1 ml of methanol. The adsorbed drug was then eluted with 1 ml of 1% ammonium hydroxide in methanol (pH 10).

The extract was either injected directly into the chromatograph or evaporated to dryness and reconstituted in chloroform (25 μl), containing propranolol 10 ng ml^{-1} as internal standard. The latter was used for GC–MS determination. Liquid chromatography (LC) was used to determine the efficiency of the extraction procedure.

Horse urine was spiked with mazindol at concentrations of 0.1, 1, 5 and 10 μg ml^{-1}. Each sample was extracted and analysed for mazindol by LC and peak areas of the extracts were compared with the peak area of a similar concentration of the pure drug. Percentage recovery was expressed as the average of three determinations. Confirmation of the purity of the extracts and detection of low levels of mazindol was then performed using GC–MS.

Analysis

Liquid chromatography. The LC system consisted of a Perkin Elmer series 2 pump which was used to deliver solvent at a rate of 1.5 ml min^{-1}. The eluent was monitored at 254 nm with a Perkin Elmer LC-15B ultraviolet detector. The column was a μBondapak C18 (30 cm × 4.5 mm i.d. 5 μm) (Waters), fitted with a Rheodyne injection system incorporating a 20-μl loop. Separation was achieved with an eluent of 0.005 M pentane sulphonic acid–acetonitrile–85% H_3PO_4 (75:25:5, v/v/v) at pH 3.5, which gave a retention time for mazindol of 12 min. All solvents used were of HPLC grade. Peak areas were reported as the average of three determinations. The use of a fixed loop injection system together with triplicate analyses circumvented the need for an internal standard for quantitation of mazindol.

Gas chromatography–mass spectrometry. The GC–MS system used was a Hewlett Packard 5890 GC with a 5970 MSD and Chem Station Data System. The column was a DB-1 methyl silicone fused silica capillary (J + W) (15 m × 0.25 mm i.d. and 0.25-μm film thickness). The GC was operated in splitless injection mode for a 1 μl injection. Helium carrier gas was used at a flow rate of 1 ml min^{-1}. The oven was programmed from 50–280°C at 30°C min^{-1} after a 1-min initial delay, and was held at final temperature for 5 min.

The GC–MS was operated in selective ion monitoring mode (*m/z* 266 for mazindol and *m/z* 72 for the internal standard). Identification of mazindol in the urine extracts was achieved by examination of the full mass spectrum of the appropriate peak in the chromatogram.

Results and Discussion

The LC system described was suitable for

the examination of extracts of mazindol from horse urine. Since mazindol has a pK_a value of 8.6 [5], it would be poorly retained on a reversed-phase HPLC system at a neutral pH as a result of partial ionization. The possibility of ion-pair chromatography was then considered, with favourable results.

The extracts were clean and no interference from endogenous materials was observed. This was verified by LC analysis of an extract of "blank" urine (Fig. 1). Two unidentified peaks were apparent in the LC chromatogram and were presumed to be metabolites of mazindol. These compounds did not interfere with the mazindol peak and could be detected in the urine up to 12 h after oral administration of 50 mg of the drug. Mass spectral data also confirmed the identity and purity of the mazindol peak (Fig. 2). The "metabolites" were not seen in the GC–MS chromatograph. The standard curve was established by linear least-squares regression analysis of the peak area of mazindol as a function of concentration injected. The relationship was found to be linear for concentrations of mazindol between 0.1–10 μg ml^{-1}. The regression equation was found to be $y = 68.97x + 0.52$ at 0.02 AUFS, with a correlation coefficient of 0.9992. Standard curves constructed on three different days showed good reproducibility over the concentration range studied.

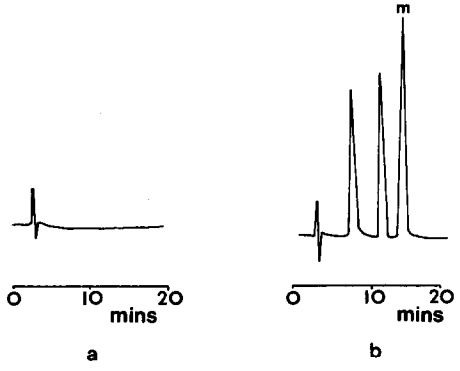

Figure 1
Chromatograms following solid-phase extraction of (a) blank horse urine, and (b) horse urine 4 h after administration of mazindol (m). Other peaks in the chromatogram are unidentified metabolites.

Recovery of mazindol from spiked urine using the outlined extraction procedure and LC analysis was found to be 100 ± 2.7% for urine between 0.1–10 μg ml^{-1}. No significant difference was seen in recovery over this concentration range. The minimum detectable level (signal to noise >2) of 25 ng ml^{-1} allowed the detection of mazindol up to 8 h after administration of a 50-mg oral dose (Table 1). A graph of urine concentration, against time is shown in Fig. 3, and is typical of the elimination profile seen with all three horses following a single oral administration of 50 mg. The reproducibility of the assay was better than 3.6%, which was determined from the RSD of triplicate assays of urine samples obtained after a 50-mg oral dose (Table 1). The data from this study are in close agreement with that previously described by Timmings *et al.* [3]. No parent mazindol was detected in samples taken after 28 h, or in urine collected from horses having received a 5-mg dose of the drug.

Using the outlined extraction procedure

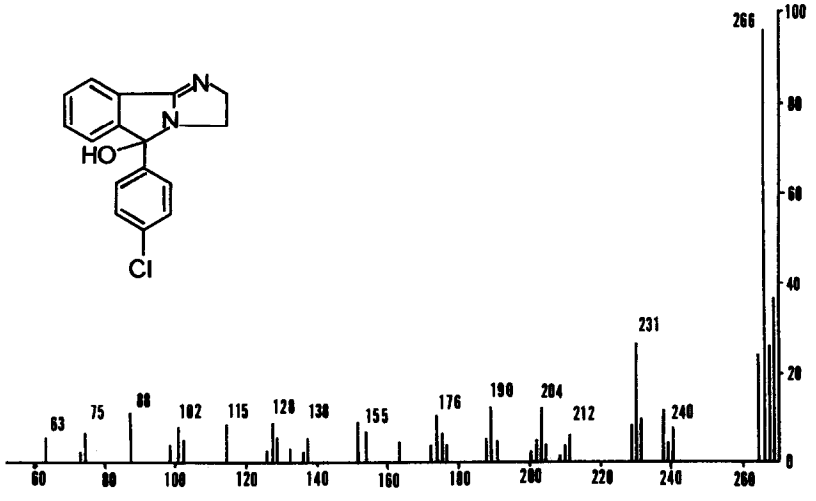

Figure 2
Mass spectrum of mazindol.

Table 1
Concentration of mazindol in horse urine following a single oral dose (50 mg), following triplicate analysis of each sample

Time (h)	Concentration (ng ml^{-1}) 1	2	3	Mean concentration (ng ml^{-1})	RSD (%)
1	41.6	44.5	45.3	43.8	3.6
2	160.4	166.2	166.6	164.4	1.7
4	211.5	218.7	220.2	216.8	1.8
6	310.2	313.8	315.6	313.2	0.7
8	184.2	182.5	183.0	183.2	0.3
28	ND	ND	ND	—	—
52	ND	ND	ND	—	—

ND = Not detected.

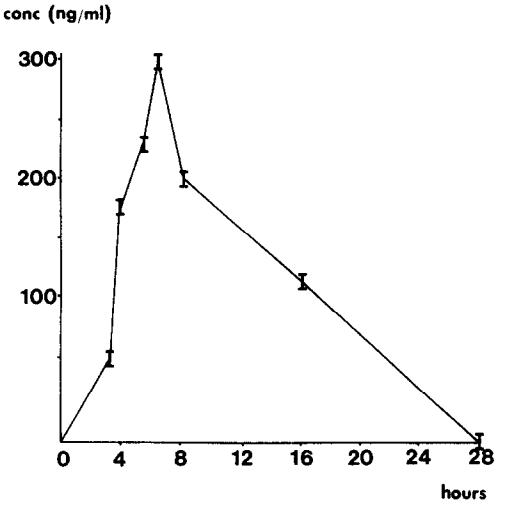

Figure 3
Mean excretion pattern (n = 3) of unchanged mazindol in the horse after a single 50-mg oral dose.

together with GC–MS analysis the minimum detectable level of mazindol was improved to 0.8 ng ml^{-1}. However even with this limit of detection, no mazindol could be seen in the urine of animals dosed with 5 mg of the drug.

Whereas a number of laboratories are now using solid-phase columns for drug extraction, the majority of reported methods involve the use of non-polar columns (C8, C18) or XAD resins. Non-polar columns are capable of giving excellent recoveries of drugs from body fluids, but each method tends to be specific for the extraction of a particular drug or group of drugs. Conversely XAD resins can be used for the extraction of a range of compounds but the extraction procedure often involves a number of stages with a subsequent loss of efficiency.

The use of ion exchange columns for drug extraction, as described in this paper, resulted in a highly efficient extraction procedure which should be applicable to any basic drug in which ionization can be induced. This was a simple and rapid technique which allows 100% recovery of low levels of drug from biological fluids. The extract was sufficiently clean to enable direct injection into an LC or GC–MS instrument. The advantages of this method of extraction were particularly apparent in the analysis of mazindol, where very little of the parent drug was present in the biological fluid.

A level of 800 pg of mazindol can be detected using this method and was detected, which should be sufficient to detect the drug following administration of pharmacologically relevant doses.

There is some debate as to the effective dose required to excite a horse. Even with doses of 100 mg, given intravenously, veterinarians at the Illinois Racing Board have observed (unpublished data) only a slight increase in irritability and respiratory rate and no change in pulse rate [6]. Therefore, even though we were unable to detect mazindol following a 5-mg intravenous administration, it is unlikely that doses as low as this would improve the racing performance of a horse.

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