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ELISA screening with GC–MS confirmation of the tranquilizer chlorprothixene administered in sub-therapeutic doses to horses

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Abstract: A commercially available generic promazine ELISA kit is available which shows cross-reactivity for the tranquilizer chlorprothixene (CPT). The ELISA test readily detects the presence of CPT or its metabolites in equine urine for up to 24 h after the i.v. and i.m. administration of sub-therapeutic doses (4.5 mg) to three horses. Maximum concentrations (CPT equivalents) are obtained 2 h after i.v. dosing. No distinct concentration peak values are observed after i.m. administration. Following solid-phase extraction, confirmation of CPT and its metabolites by electron impact mass spectrometry after sub-therapeutic administration is not successful. The use of chemical ionization mass spectrometry however revealed the presence of at least four metabolites including; chlorprothixene sulphoxide, hydroxylated chlorprothixene and hydroxylated chlorprothixene sulphoxide.

Keywords: *Chlorprothixene; doping; horse; metabolism; urine.*

Introduction

Phenothiazine tranquilizers have been used to enhance illegally the performance of both show and racing horses. A very small dose can help smooth a nervous jumper's approach and performance over the obstacles in jumping events. In other show horse events, a tranquilizer can assist a horse by rendering it less sensitive to disturbing influences. In the past the phenothiazine derivatives particularly acepromazine, promazine and propionylpromazine have been successfully misused in this respect. However, the introduction of a sensitive ELISA screening test [1, 2] capable of detecting amounts as low as 0.03 ng ml^{-1} of acepromazine resulted in the decrease of the illegal use of the drug. Moreover, the currently commercially available ELISA kit showed cross-reactivity for other phenothiazine tranquilizers and probably forced people to the illegal use of other tranquilizers including CPT. The present study relates to the ELISA screening for CPT.

Screening techniques, such as ELISA, require the support of confirmatory analytical methods for the unequivocal identification of a particular drug and its metabolites. The very high sensitivity of immunoassays implies the need for sensitive confirmatory methods. This requirement is met in the present study by isolation of the CPT metabolites by solid-phase extraction and analysis of the extracts by GC–MS in the chemical ionization mode.

Materials and Methods

Reagents

The reference sample of chlorprothixene was obtained through Roche (Brussels, Belgium) whereas the standard promazine was kindly donated by Wyeth (Brussels, Belgium). Certify-1 SPE cartridges were obtained from Varian (Walnut Creek, CA) and Sep-Pak C18 cartridges from Millipore (Milford, MA). The enzyme preparation Suc *Helix pomatia* (SHP) containing 100,000 Fishman units/ml β-glucuronidase and 1,000,000 Roy units/ml arylsulphatase was obtained from IBF (Villeneuve, France). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey–Nagel (Düren, Germany).

Experimental animals

Three standard-bred mares weighing respectively 540, 480 and 670 kg had food

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withheld from the afternoon preceding the day of the experiment. Chlorprothixene (Seda-Kel®, Wolfs Veterinaria, St Niklaas, Belgium) was administered at a reputedly used subtherapeutic dose of 4.5 mg intravenously via the right jugular vein. Two weeks later the same amount was given intramuscularly to the same horses.

A balloon-tipped catheter was placed in the bladder of each mare. The total urine in the bladder was collected prior to administration and post-administration at times 1, 2, 3, 4, 6, 9 and 12 h. An aliquot was also collected after 24 h. Urinary pH and volume were measured.

Equipment

The generic promazine ELISA kit was purchased from WTT Inc. (Lexington, KY). This kit contained a 96 well microtitre plate, washing and EIA buffers, generic promazine enzyme conjugate, K-blue substrate for horseradish peroxidase based ELISA and a negative and positive control urine samples.

EI mass spectra (70 eV) were recorded by means of a Hewlett–Packard (Palo Alto, CA) mass-selective detector. The gas chromatograph was equipped with a HP-Ultra 1 fused-silica column (25 m × 0.2 mm i.d., film thickness 0.11 μm). Injections were made in the splitless mode. GC conditions were: injector temperature: 250°C, oven temperature program from 60 to 210°C at a rate of 70°C min^{-1}, followed by a rise to 300°C (5°C min^{-1}).

Methane chemical ionization spectra were obtained using a Finnigan MAT TSQ 70 interfaced to a Varian 3400 gas chromatograph. The column used was a 30 m × 0.32 mm i.d., SE30 (Alltech Econocap) (film thickness 0.2 μm). Injections were made in the splitless mode. GC conditions were: injector temperature 240°C, transfer line 280°C, the oven temperature program was 70°C for 1 min 70 to 320°C at 15°C min^{-1} and 320°C for 3 min. The ion source temperature was 150°C and the indicated source pressure was 8000 mtor.

ELISA screening

The one-step ELISA test was started by adding urine (20 μl) to each well previously washed with buffer. A solution (180 μl) containing a covalently linked promazine–HRP conjugate was then added to each sample on the microplate. During this step the presence of free drug or cross-reacting material competitively prevented the antibody from binding to the generic promazime–HRP complex. After a 60 min incubation (in the dark) the fluid was removed and the wells washed three times with buffer (300 μl). Peroxidase substrate solution (150 μl) was added to each well. After 60 min the colour was checked by eye. Negative samples turned blue, whilst positive urine samples were colourless. The negative and positive control urine sample containing acepromazine was treated similarly.

Semi-quantitative ELISA determination

Blank horse urine was spiked with CPT resulting in concentrations of 0, 1, 2.5, 5, 10, 25 and 50 ng ml^{-1}, respectively. The spiked urine samples (20 μl) were screened by ELISA as already described except that the absorbance was measured at 650 nm in a microplate reader. The linear portion of the S-curve was used to measure the CPT equivalent concentrations of the urine samples analysed concomitantly and in duplicate. Whenever appropriate, administration samples were diluted with blank horse urine.

Solid-phase extraction and derivatization

Equine urine (20 ml) was adjusted to pH 5.2 with 1 M hydrochloric acid and 1 M sodium acetate buffer (2 ml:pH 5.2) added. After the addition of SHP (100 μl) the urine was hydrolysed at 56°C for 2 h. After cooling, phosphate buffer (2 ml; 0.1 M, pH 6) was added and the urine was adjusted to pH 6 using 1 M NaOH. The hydrolysate was spiked with the internal marker solution (promazine 50 μg ml^{-1}; 50 μl) and centrifuged for 10 min. A solid-phase Sep-Pak C18 cartridge was primed with methanol (5 ml) followed by deionized water (5 ml). The centrifuged hydrolysate was passed through the cartridge and eluted with methanol (3 ml). The eluate was evaporated *in vacuo* and the residue reconstituted in methanol (100 μl). After the addition of deionized water (3 ml) and phosphate buffer pH 6 (2 ml), the sample was centrifuged (5 min). Certify-1 columns used with a vacuum manifold were preconditioned by washing with methanol (2 ml) followed by phosphate buffer (pH 6; 2 ml). The sample was slowly passed (2 ml min^{-1}) through the activated cartridge which was then rinsed with 1 M acetic acid (1 ml) dried under full vacuum for 5 min, washed with methanol (3 ml) and redried for a further 10 min. The CPT metabolites were recovered with ethyl acetate containing concentrated ammonium

hydroxide (2% v/v). The extracts were taken to dryness under N_2 at 40°C, the residue was dissolved in 10% MSTFA in toluene (30 μl) and 2 μl was injected for GC–MS analysis.

Unhydrolysed urine (20 ml) was also analysed by SPE by buffering with phosphate buffer pH 6 and the procedure described above. The final extract after elution with ethyl acetate containing ammonium hydroxide (2% v/v) was taken to dryness under N_2 at 40°C and the residue dissolved in ethyl acetate (50 μl) and 2 μl injected for EI GC–MS analysis.

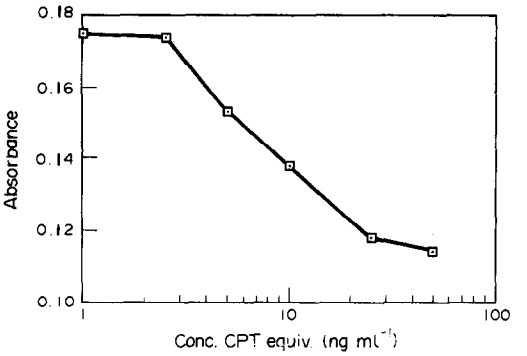

Figure 1
Urinary chlorprothixene calibration graph using the generic promazine ELISA kit (absorbance measured at 650 nm).

Results and Discussion

The ability of the anti-acepromazine antibody to detect several congeners has been evaluated *in vitro* by Kwiatkowski *et al.* [1]. From their results it appeared that this antibody showed strong cross-reactivity with other phenothiazine tranquilizers including promazine, chlorpromazine, propiopromazine and trifluprom azine. In the present study the ability of the ELISA test to detect CPT in equine urine was evaluated. Blank urine was spiked with different amounts of CPT. The calibration graph for the assay (Fig. 1) gives a sharp change in absorbance between 5 and 25 ng ml^{-1}. This corresponds to a distinct blue colour in the absence of CPT (absorbance >0.15) and no visually discernible colour in the presence of 10 ng ml^{-1} of CPT. In practice, the results of this ELISA test kit are read by eye. Hence the assay will visually detect the presence of CPT in urine for samples with concentrations exceeding 10 ng ml^{-1}. However, the cross-reactivity of the antibody with CPT metabolites or conjugates is currently unknown. Therefore a more realistic approach was the evaluation of the ELISA test using urine from CPT-dosed horses. In this way, the ELISA reaction readily detected CPT equivalents up to 24 h after the i.v. and i.m. administration of a sub-therapeutic dose to three horses. Inhibition of the ELISA reaction was essentially complete from 1 to 24 h (i.v.) and from 2 to 24 h (i.m.). Examples of concentration–time curves after the administration of 4.5 mg to two horses are given in Fig. 2.

In doping analysis the presence of a drug needs to be unequivocally confirmed by mass spectrometry. The detection limit for a given drug depends primarily on the sensitivity of the confirmation method. Obviously, the sensitivity and selectivity is determined by the signal to noise ratio and consequently by the 'cleanness' of the extract. A rapid technique for pretreatment of biological samples that met the

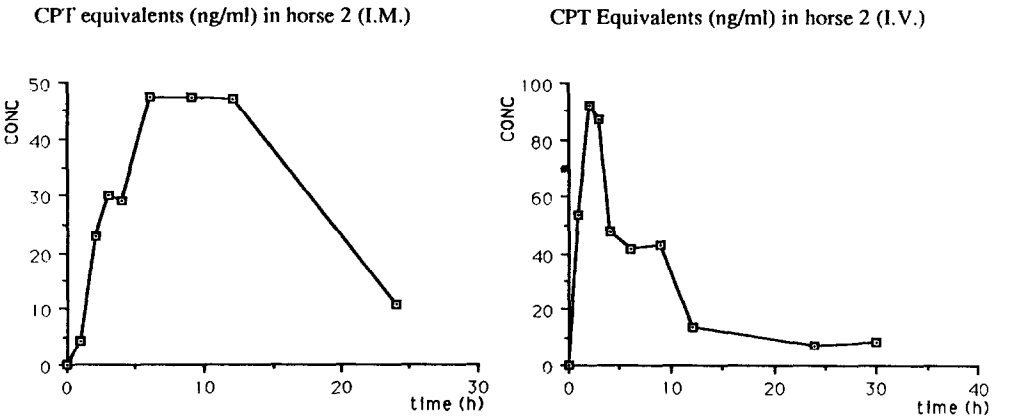

Figure 2
Chlorprothixene equivalent concentrations (ng ml^{-1}) in urine after the i.v. and i.m. administration of a sub-therapeutic dose (4.5 mg) to horse 2.

requirements of selectivity is solid-phase extraction. The number of examples of the use of SPE in equine doping analysis is increasing. Formerly, XAD-resins [3, 4] and Extrelut [5] were used, but more recently copolymeric bonded-phase silica columns such as Certify cartridges which have both lipophilic and ion exchange properties [6–8] have provided a simple, rapid, selective and efficient method for the extraction of a broad range of basic drugs and their metabolites. The capacity of these columns however is limited. Moreover, due to the 'dirty' and viscous character of horse urine, methods that have been developed for human urine cannot always be applied in equine doping analysis. However, pretreatment by Sep-Pak extraction, followed by SPE using Certify-1 columns appeared to be a

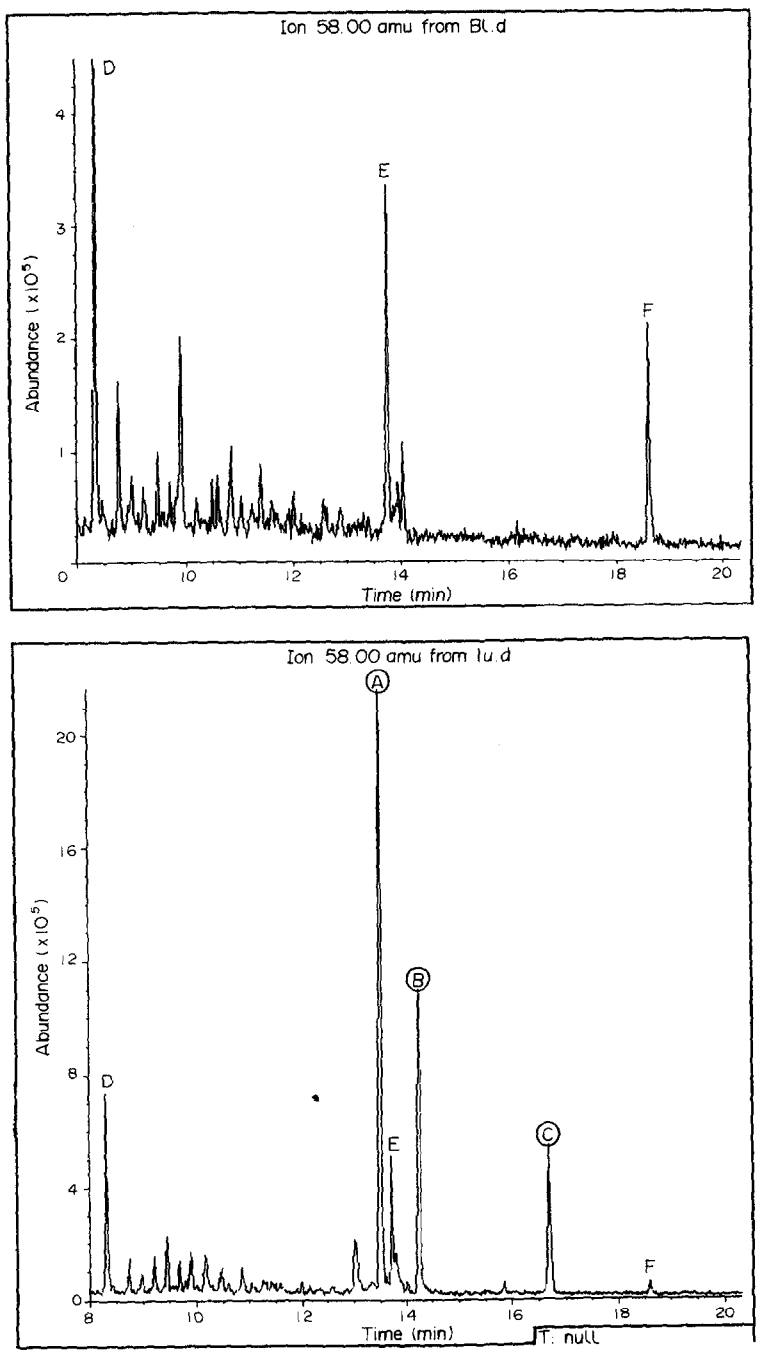

Figure 3
Ion chromatogram (m/z = 58) after EI GC–MS of a trimethylsilylated extract (upper:blank urine, lower:1 h after administration of 4.5 mg CPT to horse 3).

successful combination. This technique was used in this work to confirm the presence of CPT and/or metabolites in equine urine by GC–MS. A similar aprpoach has been used for the isolation of stanozolol metabolites from equine urine [9].

Although the detection limit for EI GC–MS in the full scan acquisition mode using 20 ml urine and SPE is 25 ng ml^{-1} no CPT could be detected in the urine of the dosed horses. As the ELISA screening method had a detection limit of 10 ng ml^{-1} CPT and semi-quantitative analysis indicated the presence of CPT equivalents far above 25 ng ml^{-1} (Fig. 2) cross-reactivity of the generic promazine antibody with CPT metabolites seemed to be responsible for the positive screening results.

As hydroxylation of the benzene ring followed by conjugation is a common pathway in equine drug metabolism, the urine after CPT administration was enzymatically hydrolysed and subjected to GC–MS analysis following SPE. Using EI GC–MS, derivatization with MSTFA and monitoring the ion m/z = 58 (Fig. 3) three supplemental peaks (A, B and C) were observed in the urine extracts after CPT administration. However the EI mass spectra did not enable unambiguous identification of the chemical structure of these compounds.

The urinary extracts therefore were derivatized and analysed by GC–MS in the CI mode. Injection of reference standards of chlorprothixene gave satisfactory MS data in this mode at the level of 500 pg injected. The mass chromatograms, m/z 58 confirmed the presence of four metabolites (Fig. 4). On the basis of molecular weight of the derivatized material peak 1 (Fig. 4) was identified as the sulphoxide metabolite (M^+ + H, m/z 332), peaks 2 and 3 (Fig. 4) as hydroxylated metabolites (M^+ + H, m/z 404) and peak 4 (Fig. 4) as a hydroxylated sulphoxide metabolite (M^+ + H, m/z 420). The mass spectra of the four compounds all showed chlorine isotope patterns and molecular weights were confirmed by the presence of methane adduct ions (M^+ + 29 and M^+ + 41).

The methane CI spectra of the TMS derivatives of the major hydroxylated metabolite (peak 2, Fig. 4) and the hydroxylated sulphoxide metabolite are shown in Figs 5 and 6. In each case the protonated molecular ion was the base peak in the spectrum and the spectra showed a prominent ion at m/z 58 due to α cleavage of the dimethylaminopropyl side chain. The generation of selected ion chromatograms for m/z 58 and the protonated molecular ions of other possible metabolites, the *N*-oxide (m/z 332), the *N*-desmethyl sulphoxide (m/z 318) the *N*,*N*-desmethyl (m/z 288), the *N*-oxide sulphoxide (m/z 348) demonstrated the major processes for CPT metabolism in the horse were hydroxylation and sulphoxide formation.

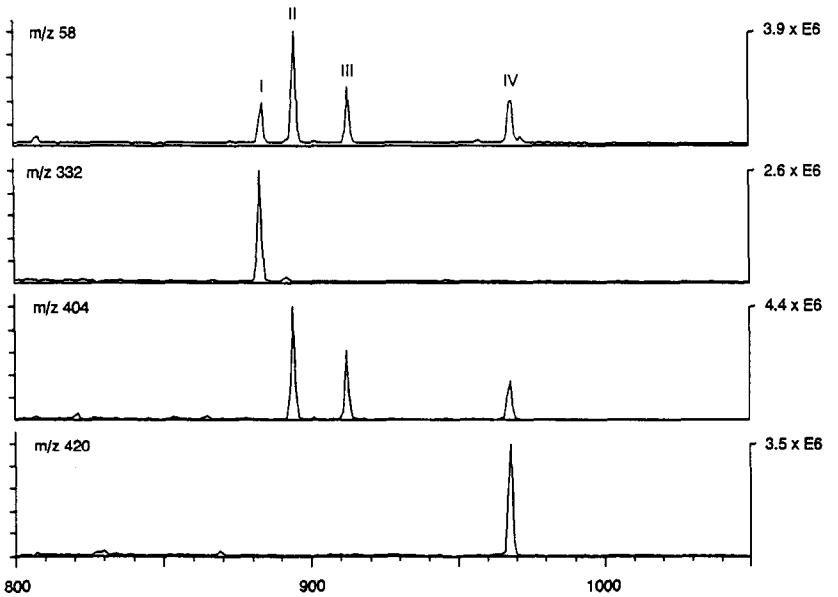

Figure 4
Mass chromatogram (m/z = 58) after CI GC–MS of a trimethylsilylated extract 3 h after i.v. administration of 4.5 mg CPT to horse 3.

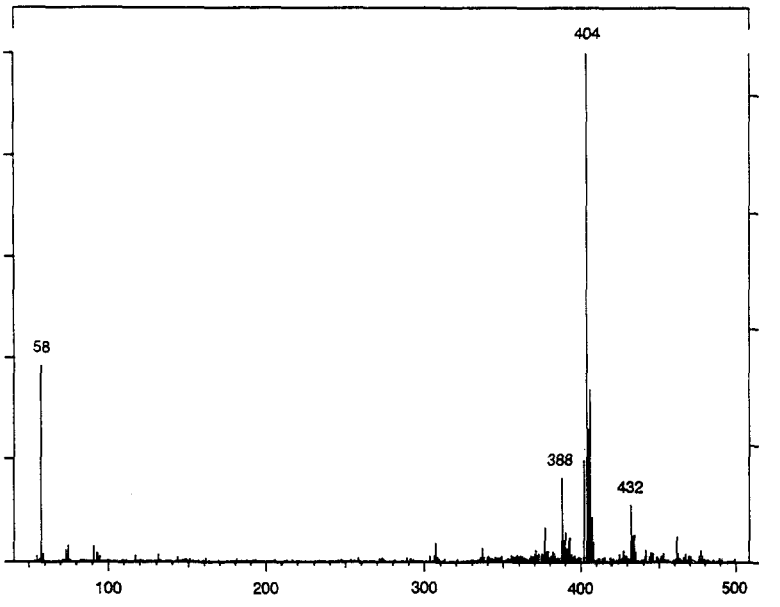

Figure 5
Methane CI spectrum of the TMS derivative of the hydroxylated CPT metabolite (peak II, Fig. 4).

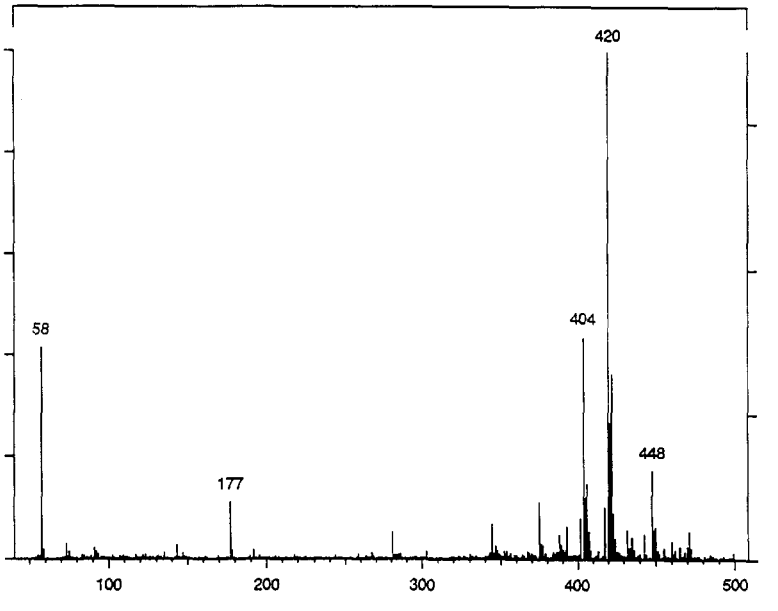

Figure 6
Methane CI spectrum of the TMS derivative of the hydroxylated CPT sulphoxide metabolite (peak IV, Fig. 4).

Similar metabolic profiles have been obtained for promazine and acetylpromazine in the horse [10]. The primary site for hydroxylation of promazine in the horse is the 3-position and for acetylpromazine, the 7-position. The formation of *N*-oxides and sulphoxides also was observed.

As the present study was limited to analysis of the samples by mass spectrometry in the EI and CI modes and no reference standards were available it was not possible to unequivocally determine the sites of hydroxylation.

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