ELSEVIER

Journal of Chromatography B, 746 (2000) 209–218

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Quantification of phenytoin and its metabolites in equine plasma and urine using high-performance liquid chromatography

Fuyu Guan[a,1], Cornelius E. Uboh[b,*], Lawrence R. Soma[a], Eric K. Birks[a], Donna Teleis[a], Jeffrey A. Rudy[b], Anthony O. Watson[b], Deborah S. Tsang[a]

[a]*University of Pennsylvania School of Veterinary Medicine Department of Clinical Studies, New Bolton Center Campus, Kennett Square, PA 19348, USA*

[b]*PA Equine Toxicology and Research Laboratory, Department of Chemistry, West Chester University, West Chester, PA 19382, USA*

Received 17 January 2000; received in revised form 11 May 2000; accepted 31 May 2000

Abstract

A reliable and sensitive method for the extraction and quantification of phenytoin (5,5′-diphenylhydantoin), its major metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) and minor metabolite, 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) in horse urine and plasma is described. The method involves the use of solid-phase extraction (SPE), liquid–liquid extraction (LLE), enzyme hydrolysis (EH) and high-performance liquid chromatography (HPLC). The minor metabolite, 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) was not present in a reliably quantifiable concentration in all samples. The new method described was successfully applied in the pharmacokinetic studies and elimination profile of phenytoin and *p*-HPPH following oral or intravenous administration in the horse.

Keywords: Phenytoin

1. Introduction

Phenytoin (diphenylhydantoin) is an anticonvulsant agent that is widely used in racehorses for the management of chronic intermittent rhabdomyolysis (CIR) or ‘post-exercise tying up’. The drawback in the use of phenytoin in the management of CIR is that there is limited knowledge of the pharmacokinetics of phenytoin in horses [1]. The lack of information on withdrawal time for phenytoin treatment prior to race day has led to numerous post-race positive test results in racehorse urine samples in many racing jurisdictions in North America. More information on the pharmacokinetics of phenytoin will lead to establishment of a withdrawal time and allow medication of racehorses without violating medication rules in racing. Violations in the medication policy may result in penalties ranging from loss of purse to suspension. One possible way to address this problem is to establish an acceptable withdrawal time following treatment with phenytoin. Such a withdrawal time from treatment prior to race time is not likely to result in a post race positive test for the drug. A withdrawal time cannot be established

*Corresponding author. Tel.: +1-610-436-3501; fax: +1-610-436-3504.

E-mail address: ubohcorn@vet.upenn.edu (C.E. Uboh).

[1]On leave from Beijing Institute of Pharmacology and Toxicology, Beijing, China.

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00330-3

without pharmacokinetic and urinary excretion parameters for phenytoin and its metabolites in the horse. To achieve this, a reliable analytical method was developed for the quantification of phenytoin and its metabolites in equine plasma and urine. The method described in this study has been successfully applied to a pharmacokinetic and urinary excretion study of phenytoin in the horse [2]

Previous studies described methods for the determination of phenytoin in human plasma or whole blood [3–9] and the major metabolites in human urine [10–13], and for the identification of phenytoin metabolites in mice [14]. Such a method had not before been described for equine samples. Analytical techniques used in the previous studies for the determination of phenytoin included high-performance liquid chromatography (HPLC) with ultraviolet (UV) or electrochemical detection, gas chromatography coupled with mass spectrometry, and immunoassay. Since equine urine is more complex than human urine in consistency and appearance, the previously reported methods for other species could not be easily adapted to the analysis of these analytes in equine urine samples. The purpose of this study was to develop an HPLC method suitable for the quantification of phenytoin and its major metabolites in equine urine and plasma samples.

2. Experimental

2.1. Chemicals

Phenytoin (5,5′-diphenylhydantoin), 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH, *para*-hydroxyl metabolite) and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH, *meta*-hydroxyl metabolite) were commercially obtained (Alltech Applied Science, State College, PA, USA) and cyheptamide (internal standard, I.S.) from Sigma (Sigma, St. Louis, MO, USA). Acetonitrile (HPLC grade), methanol (HPLC grade), ethyl ether (pesticide grade) and water (HPLC grade) were obtained from Fisher (Fisher Scientific, Fair Lawn, NJ, USA) and Bond Elute Certify SPE column (3 cc/130 mg) from Varian (Harbor City, CA, USA). Other chemicals used were of ACS grade.

2.2. Preparation of reagents

Phenytoin, *p*-HPPH, *m*-HPPH and I.S. stock solutions (1.0 mg/ml) were prepared in methanol, and each solution was stored at 4°C until analysis. A mixture (100 μg/ml or 10 μg/ml) of phenytoin, *p*-HPPH and *m*-HPPH was obtained by dilution. Phosphate buffer (0.10 or 0.08 *M*, pH 6.0) was prepared by dissolving 13.6 or 1.1 g of KH_2PO_4 in 900 ml of water, respectively, and adjusted pH to 6.0 using KOH (1 *M*) before bringing final volume to 1000 ml with de-ionized water. Phosphate buffer (0.080 *M*, as the mobile phase), was filtered using a 0.2-μm Nylon-66 filter (Rainin Instrument, Woburn, MA, USA). Saturated phosphate buffer (pH 4.5) was prepared by continuously adding KH_2PO4 to 1000 ml of de-ionized water while stirring until the solution was saturated. The saturated mixture was allowed to stand at room temperature for a minimum of 12–14 h before the clear solution was decanted into a fresh reagent bottle and labeled. β-Glucuronidase (Patella vulgata, Sigma), was diluted to 5000 AU/ml from concentrated stock vials, stored at 4°C and was discarded if not used within 6 days after preparation. Carbonate buffer (0.1 *M*, pH 10) was prepared by dissolving 5.3 g of sodium carbonate and 4.2 g of sodium bicarbonate in 900 ml of de-ionized water and q.s. to 1000 ml with de-ionized water.

2.3. Drug administration and sample collection

A dose of 8.8 mg/kg of phenytoin was administered to horses by either oral (p.o.) or intravenous (i.v.) route. Blood and urine samples were collected before drug administration and at various intervals post-drug administration. Blood samples were collected via a venous catheter into sodium oxalated tubes, centrifuged (2500–3000 rpm or 776–1318 *g*) for 15 min and the plasma was transferred into separate tubes and immediately stored at −20°C until analysis.

Urine samples were continually collected for a given period of time via an indwelling 24-F self-restraining catheter placed in the bladder and attached to a drainage bag. At each collection time, urine was aspirated from the bladder, and the total volume collected during each specified period was

measured. Urine samples were divided in 50-ml aliquots and stored at −20°C to avoid thawing and freezing cycles. Control plasma and urine samples used were analytically verified to be drug-free by immunoassay screening prior to use in this study.

2.4. Plasma sample preparation

2.4.1. Spike and drug administration samples

Spiked plasma samples used for method development were freshly prepared on the day of extraction. To 1.0 ml plasma, phenytoin, *p*-HPPH and *m*-HPPH (0.05–10 μg), and I.S. solution in methanol (20 μl) containing 2.0 μg cyheptamide were added. The sample was vortex-mixed for 5–10 s then 5.0 ml of phosphate buffer (0.1 *M*, pH 6.0) was added and similarly mixed. Plasma samples from drug administration groups (i.v. and p.o.) were similarly treated as described for spiked urine but analytes were not added.

2.4.2. Solid-phase extraction

Solid-phase extraction was manually conducted using a Vac Elut SPS 24 device (Varian, Habor City, CA, USA). Each Bond Elute Certify (3 cc/130 mg) SPE column was sequentially conditioned with 3.0 ml of methanol, 2.0 ml of water and 2.0 ml of phosphate (0.1 *M*, pH 6.0). Plasma sample was loaded onto the column and sequentially rinsed with 3.0 ml of water and 2.0 ml of 15% methanol and dried under vacuum for 10 min. Analytes were eluted with 5 ml of methanol. The eluent was evaporated to dryness at 70°C in a sample concentrator (Techne Dri-Block; Techne Cambridge, Cambridge, UK) under a stream of nitrogen, and ethyl ether (0.5–1.0 ml) was added to rinse the inner wall of the tube. The eluent was similarly dried at 45°C under a stream of nitrogen. The dried extracts were stored at 4°C until analysis.

2.5. Urine sample preparation

2.5.1. Spiked and drug administration samples

Spiked urine samples used were freshly prepared on the day of extraction. To 1.0 ml urine, phenytoin, *p*-HPPH and *m*-HPPH (0.1–20 μg) and 60 μl of I.S. solution in methanol containing 6.0 μg of 10,11-dihydrocarbamazepine were added. The sample was vortex-mixed for 5–10 s then 1.6 ml of de-ionized water and 0.40 ml of phosphate buffer (0.1 *M*, pH 6.0) were added and mixed. The urine samples (10 ml each) were centrifuged at 2500–3000 rpm (776–1318 *g*) for 20 min, and an aliquot (1.0 ml) of the supernatant was obtained by aspiration and similarly treated as described above for spiked urine but no analyte was added.

2.5.2. Liquid–liquid extraction (LLE)

Ethyl ether (5.0 ml) and isopropanol (0.20 ml) were added to each prepared urine sample (1.0 ml of urine–1.6 ml of distilled water–0.4 ml of 0.1 *M*, pH 6.0 phosphate buffer) and mixed by rotorack for 15 min. The extraction mixture was centrifuged at 2500–3000 rpm (776–1318 *g*) for 20 min, and the organic phase (top layer) was transferred into a fresh test tube. The extraction was repeated one more time. The extracts were combined and evaporated to dryness at 50°C in a sample concentrator under a stream of nitrogen, and ethyl ether (0.5–1.0 ml) was added to rinse the inner wall of the test tube and then dried as described above. The dried extracts were stored at 4°C until analysis.

2.6. Enzyme hydrolysis

2.6.1. Spiked and drug administration samples

Glucuronic acid conjugation occurs extensively in man and animals. It is a detoxification process to convert foreign compounds such as drugs in the body into a β-glucosiduronic acid that is a relatively strong organic acidic, more water-soluble, less toxic and therefore, more rapidly excreted from the body than its precursor. Glucuronide conjugates are not extractable by common extraction procedures. In order to de-conjugate the drug to make its extraction from urine possible, β-glucuronidase is used in the hydrolysis or cleavage process to release the drug or its metabolite for extraction. The optimum pH for enzyme hydrolysis using β-glucuronidase is 4.5–5.5.

To 0.20 ml urine, *p*-HPPH and *m*-HPPH (0.1–20 μg) were added. The sample was mixed by vortex for 5–10 s, then 0.80 ml of de-ionized water, 0.10 ml of saturated phosphate buffer (pH 4.5) and 0.50 ml of β-glucuronidase were added, mixed and hydrolyzed. Hydrolysis was conducted at 65°C in a water

bath for 3 h. The enzyme-hydrolyzed samples were cooled to room temperature, and stored at 4°C until extracted and analyzed. The drug administration urine samples were centrifuged at 2500–3000 rpm (776–1318 *g*) for 20 min. Following centrifugation, an aliquot (0.1 or 0.2 ml) of the supernatant was taken and similarly treated as described above for spiked urine but no analytes were added.

2.6.2. LLE and back extraction

Hydrolyzed urine contains much more impurities than the un-hydrolyzed sample. Many attempts to clean up the analytes from hydrolyzed urine samples were made through multiple extraction procedures. Phenytoin metabolites were initially extracted into ethyl ether, and back extracted into carbonate buffer (pH 10). After the pH of the buffer was adjusted to between 6 and 6.5, the analytes were again extracted into ethyl ether. The basis for this clean up procedure is the acidic phenolic group present in the chemical structure of phenytoin metabolites (*m*- and *p*-HPPH) that allows the metabolites to undergo the whole extraction procedure to the final step. For the same reason, the I.S. for the quantification of the metabolites should contain the same acidic phenolic group so that it is extractable by the multiple extraction procedures. Thus warfarin was chosen as the I.S. since it contains an acidic phenolic group in its molecule and its retention time was suitable under the HPLC conditions selected for this study.

A 60-μl solution containing 6.0 μg of warfarin as the I.S. with 2.4 ml of de-ionized water was added to each EH-urine sample and mixed. Ethyl ether (5.0 ml) was added, and the sample was mixed by rotorack for 15 min. The extraction mixture was centrifuged at 2500–3000 rpm (776–1318 *g*) for 20 min, and the organic phase (top layer) was transferred into a fresh test tube. The extraction was repeated and the two extracts were pooled and evaporated to dryness at 40°C in sample concentrator under a stream of nitrogen. To further clean up the samples, the dried extract was dissolved in 2.0 ml of ethyl ether, and back extracted into 4.0 ml of carbonate buffer (0.1 *M*, pH 10) by mixing for 15 min using a rotorack. The organic layer (top) was discarded. The aqueous layer (bottom) was adjusted to pH 6.0 using phosphoric acid (1 *M*) and then extracted twice with ethyl ether (2×5 ml). The two extracts were pooled and dried as described above. The dried extracts were stored at 4°C until analysis.

2.7. HPLC analysis of plasma and urine samples

Analysis was carried out with an HP-1090 High Performance Liquid Chromatograph (HPLC) equipped with autosampler and Chem-station software (Hewlett-Packard, Wilmington, DE, USA). An Eclipse XDB-C_8 column (150×4.6 mm I.D., 3.5 μm particle size) with an Eclipse XDB-C_8 guard column (12.5×4.6 mm I.D., 5 μm; MAC-MOD Analytical, Chadds Ford, PA, USA) was used for separation of phenytoin and its metabolites from urine and plasma samples. The dried plasma extracts were dissolved in 100-μl mixture of 32% acetonitrile and 68% phosphate (8 m*M*, pH 6.0), and the dried urine extracts were reconstituted in 100 μl of 40% methanol and 60% phosphate (8 m*M*, pH 6.0) solution. The reconstituted extracts were transferred into HPLC 2-ml glass auto-injection vials with 200-μl glass inserts using plastic disposable pipettes (Fisher Scientific, Fair Lawn, NJ, USA). HPLC analysis was performed at ambient temperature. An aliquot (20 μl) of each extract in solution was injected onto HPLC column. The flow-rate was 0.70 ml/min. The detection of phenytoin and its metabolite was conducted at a wavelength of 210 nm with a bandwidth of 4 nm, and the reference wavelength was set at 450 nm with a bandwidth of 100 nm. Three elution conditions were used. For analysis of plasma samples, the mobile phase gradient consisted of two pre-mixed components, A and B. Component A comprised 20% acetonitrile–80% phosphate buffer (8 m*M*, pH 6.0) and component B comprised 80% acetonitrile–20% phosphate buffer (0.008 *M*, pH 6.0). The mobile phase gradient was initially held at 80% A+20% B (0–1 min), increased to 55% A–45% B (1–7 min), and to 100% B (7–12 min), held at 100% B (12–13 min), and switched to the initial hold at 80% A–20% B (13.1–19 min). The elution order of the analytes was *p*-HPPH, *m*-HPPH, phenytoin and cyheptamide (I.S.), and HPLC run time was 19 min. For analysis of non-EH urine samples, the isocratic mobile phase comprised a pre-mixture of 48% methanol and 52% phosphate buffer (0.008 *M*, pH 6.0). The elution order of the analytes was *p*-HPPH, *m*-HPPH, phenytoin and 10, 11-

dihydrocarbamazepine (I.S.), and HPLC run time was 20 min. For the analysis of EH-urine samples, the isocratic mobile phase was a pre-mixture of 44% methanol and 56% phosphate buffer (0.008 *M*, pH 6.0). The elution order of the analytes was *p*-HPPH, *m*-HPPH and warfarin (I.S.), and HPLC run time was 20 min. All sample extracts were analyzed within 2–3 days following extraction.

3. Results and discussion

3.1. Phenytoin and its metabolites recovered from plasma and urine samples

Phenytoin and its two metabolites (*p*- and *m*-HPPH) were extracted from spiked plasma by Bond Elut Certify columns. Clean urine extracts were obtained from diluted urine using ethyl ether–isopropanol mixture for LLE. Ethyl ether extracts of phenytoin metabolites from EH-urine were further cleaned by back extraction into carbonate buffer and re-extracted from the buffer after pH was adjusted to 6.0. The recovery of phenytoin and its metabolites from these samples was determined using the extraction procedures for plasma, urine and hydrolyzed urine samples spiked with known concentrations of drug and metabolites (Table 1). The recovery of phenytoin and its metabolites from plasma by SPE was from 46 to 63%, and that for the analytes from urine by LLE was from 84 to 98%. The recovery of *p*-HPPH from hydrolyzed urine was 83 to 90%. It should be noted that *m*-HPPH was not detected in any of the plasma and non-EH urine samples from phenytoin administration samples. However, in EH-urine samples *m*-HPPH was detected but could not be reliably quantified.

3.2. HPLC mobile-phase systems for analysis of plasma, urine and hydrolyzed urine samples

The mobile phase gradient using pre-mixed acetonitrile and phosphate buffer for analysis of the analytes in plasma samples produced peaks with good resolution for phenytoin, *p*-HPPH and *m*-HPPH. However, this mobile phase gradient was not suitable for analysis of the analytes in the urine samples, since co-eluting substances in the urine samples interfered with quantification of phenytoin. To adjust the selectivity of the mobile phase for the analysis of the analytes in urine samples, acetonitrile

Table 1
Percent recovery of phenytoin and *p*-HPPH from plasma and urine[a]

Analytes	Samples					
	Plasma		Urine		Hydrolyzed urine	
	Conc. spiked (μg/ml)	Recovery (%) (n=6)	Conc. spiked (μg/ml)	Recovery (%) (n=6)	Conc. spiked (μg/ml)	Recovery (%) (n=6)
Phenytoin	0.40	60±2	0.4	92±5.0	–	–
	2.0	63±3	2.0	89±5.3	–	–
	10	61±4	8.0	96±1.7	–	–
p-Hydroxyl	0.40	56±2	0.4	89±5.0	4.0	83±5.2
metabolite	2.0	54±5	2.0	86±3.9	20.0	90±9.1
(*p*-HPPH)	10	46±5	8.0	96±1.7	–	–
m-Hydroxyl	0.40	56±3	0.4	85±7.1	–	–
metabolite	2.0	54±7	2.0	84±5.0	–	–
(*m*-HPPH)	10	46±6	8.0	98±1.7	–	–
I.S.[b]	2.0	70±3	6.0	91±3.5	6.0	47±7.1

[a] Recovery (%)=conc. detected×100 divided by conc. spiked. Results=mean±SD.

[b] Cyheptamide was used as I.S. for analyzing plasma samples, 10,11-dihydrocarbamazepine was I.S. for urine samples, and warfarin was I.S. for the analysis of EH urine samples.

Table 2
Calibration equation, quantification ranges and limit of detection for phenytoin and metabolites in spiked plasma and urine samples[a]

	Calibration equation ($y = mx + b$)		Concentration range (μg/ml)	Correlation coefficient (r^2)	n	Limit of detection (μg/ml)
	m±SD	b±SD				
Plasma						
Phenytoin	0.278±0.0024	0.00018±0.0149	0.1–10	0.999	12	0.1
p-HPPH	0.212±0.0033	0.0314±0.0209	0.2–10	0.997	11	0.1
m-OH-metabolite	0.265±0.0045	0.00792±0.0288	0.2–10	0.997	12	0.1
Urine						
Phenytoin	0.164±0.0030	−0.00688±0.0142	0.2–20	0.997	11	0.1
p-HPPH	0.160±0.0024	−0.00347±0.0109	0.1–15	0.997	12	0.1
m-OH-metabolite	0.203±0.0032	−0.00689±0.0145	0.1–15	0.997	12	0.1
EH-urine						
p-HPPH	0.124±0.0022	0.0368±0.0135	0.5–100	0.996	12	0.5

[a] y is the ratio of peak area of phenytoin or its metabolites to that of I.S., and x is the concentration (μg/ml) of phenytoin or its metabolites.

Table 3
Accuracy and precision (C.V.)[a] for quantification of phenytoin and metabolites in spiked plasma, urine and hydrolyzed urine samples (n=6)

Sample/ analytes	Analyte added (μg/ml)	Intra-day		Inter-day	
		Analyte detected (μg/ml)	C.V. (%)	Analyte detected (μg/ml)	C.V. (%)
Plasma					
	0.40	0.44±0.007	1.6	0.46±0.040	8.7
Phenytoin	2.0	2.1±0.10	4.9	2.1±0.13	6.0
	10	10±0.61	6.1	11±0.93	8.0
p-Hydroxyl	0.40	0.36±0.018	5.0	0.33±0.054	16
metabolite	2.0	2.2±0.23	10	2.0±0.10	5.1
(*p*-HPPH)	10	10±1.0	10	11±2.1	18
m-Hydroxyl	0.40	0.45±0.017	3.8	0.47±0.069	15
metabolite	2.0	2.3±0.29	13	2.1±0.067	3.1
(*m*-HPPH)	10	10±1.2	12	11±2.4	21
Urine					
	0.4	0.49±0.044	8.9	0.49±0.013	2.6
Phenytoin	2.0	2.0±0.038	1.9	1.9±0.086	4.6
	8.0	7.9±0.27	3.4	7.9±0.40	5.1
p-Hydroxyl	0.4	0.45±0.020	4.4	0.47±0.016	3.4
metabolite	2.0	2.0±0.074	3.7	1.9±0.086	4.0
(*p*-HPPH)	8.0	8.2±0.28	3.4	8.1±0.60	7.4
m-Hydroxyl	0.4	0.44±0.019	4.4	0.45±0.018	4.0
metabolite	2.0	1.9±0.031	1.6	1.9±0.12	6.5
(*m*-HPPH)	8.0	8.4±0.29	3.4	8.2±0.59	7.2
EH-urine					
p-Hydroxyl	4.0	3.4±0.48	14	3.8±0.46	12
metabolite	20	19.8±3.1	16	18.7±3.6	19
(*p*-HPPH)					

[a] Coefficient of variation (C.V.;%)=standard deviation of concentration detected/mean concentration detected×100. Results=mean±SD.

was replaced with methanol, and an isocratic mobile phase comprising 48% methanol and 52% phosphate buffer (0.008 *M*, pH 6.0) was used. The use of this new mobile phase resulted in well-resolved peaks for the analytes. With EH-urine extracted samples, co-eluting substances increased despite the introduction of additional clean-up steps. For better resolution of the peaks for the analytes from co-eluting substances in the EH-urine, slight adjustment in the mobile phase was made from 48 to 44% methanol and from 52 to 56% phosphate buffer (0.008 *M*, pH 6.0).

3.3. Calibration curves for phenytoin and its metabolites and limit of detection

Linearity of the methods for phenytoin and its metabolites was investigated over a concentration range of 0.1–20 μg/ml using spiked plasma and urine samples. Reliable detection limits for phenytoin and its metabolites in this study were defined as the concentration at which the analyte produced a chromatographic peak with a signal-to-noise ratio of >3. Table 2 shows the calibration curves and

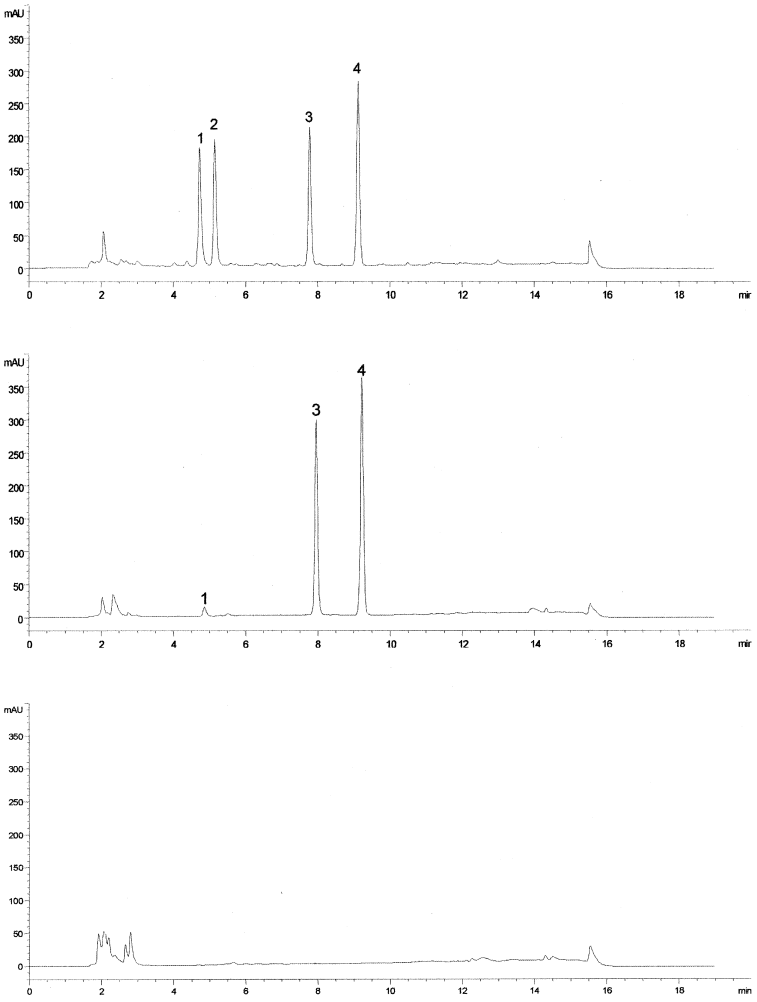

Fig. 1. HPLC chromatograms for plasma samples. The top panel is the chromatogram for a control plasma sample (1.0 ml) spiked with phenytoin, its two metabolites and I.S. (2.0 μg each). In the middle panel is the chromatogram for a plasma sample (1.0 ml) collected 8 h post oral administration of 8.8 mg/kg of phenytoin, and the bottom panel is the chromatogram for a blank plasma sample (1.0 ml) collected before the administration of phenytoin. Peak identification in order of elution: (1) *p*-HPPH; (2) *m*-HPPH; (3) phenytoin; (4) cyheptamide (I.S.).

detection limits for phenytoin and its metabolites in plasma, urine and EH-urine. The calibration curves were linear (r^2>0.99). The limit of detection for phenytoin, *p*-HPPH and *m*-HPPH was 0.1 μg/ml in plasma or urine and 0.5 μg/ml for *p*-HPPH in EH-urine.

3.4. Precision and accuracy of the method

Precision and accuracy for quantification of phenytoin and its metabolite were determined. Control plasma and urine samples were spiked with different concentrations of the analytes from which intra- and inter-day assays were performed. The results are summarized in Tables 3. The coefficient of variation (C.V.) for intra- and inter-day assays for phenytoin in plasma and urine was less than 9% (1.6–8.7%). For *p*-HPPH in plasma, urine and EH-urine, intra-day and inter-day C.V. was between 3.4 and 19%. The accuracy (concentration detected× 100/concentration spiked) for intra- and inter-day assays for phenytoin and its metabolites in plasma,

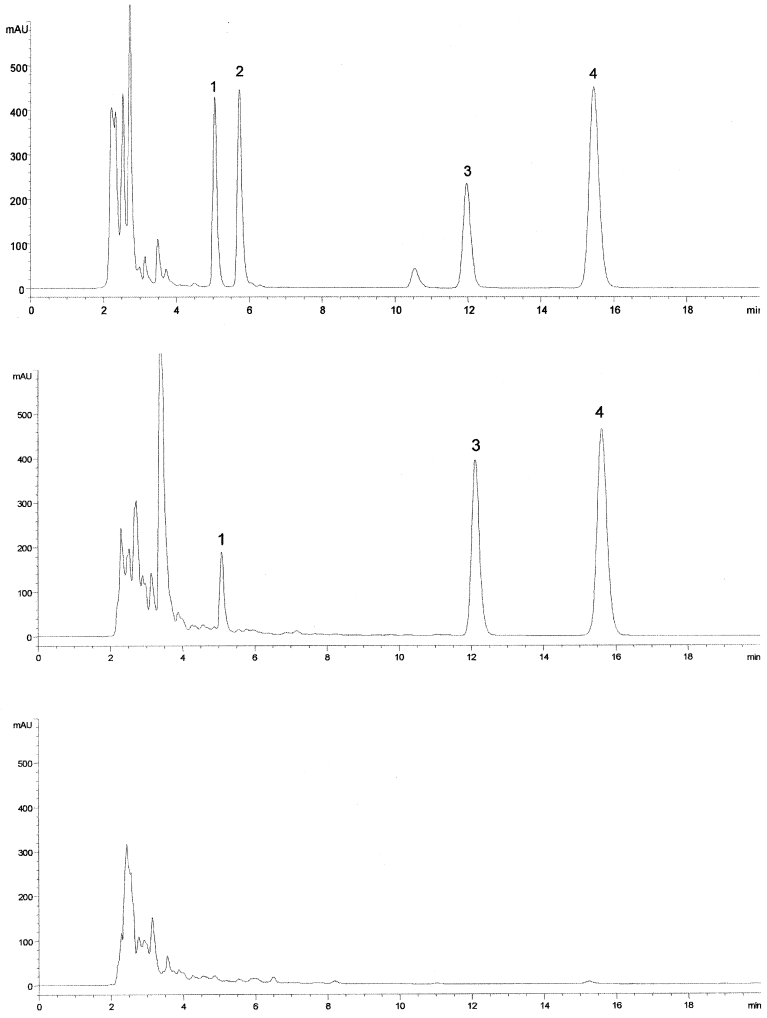

Fig. 2. HPLC chromatograms for urine samples. The top panel is the chromatogram for a control urine sample (1.0 ml) spiked with phenytoin, its two metabolites (4.0 μg each) and I.S. (6.0 μg). The middle panel is the chromatogram for urine sample (1.0 ml) collected 8 h post i.v. administration of phenytoin (8.8 mg/kg). The bottom panel is the chromatogram for a blank urine sample (1.0 ml) collected before the administration of phenytoin. Peak identification in order of elution: (1) *p*-HPPH; (2) *m*-HPPH; (3) phenytoin; (4) 10,11-dihydrocarbamazepine (I.S.).

urine and EH-urine samples ranged from 85 to 115%.

3.5. Determination of phenytoin and its metabolites in administration samples

Using the method described above, the chromatograms for phenytoin and its metabolites in plasma, urine, EH-urine and control samples were obtained as shown in Figs. 1–3. In Fig. 1, phenytoin and a very low concentration of *p*-HPPH were detected in plasma sample collected 8 h after oral administration of phenytoin (8.8 mg/kg). Fig. 2 (middle panel) shows the presence of phenytoin and its major metabolite, *p*-HPPH, in its unconjugated (free) form in urine samples collected 8 h post intravenous administration of phenytoin (8.8 mg/kg). Fig. 3 indicates that *p*-HPPH was detected and quantified in an EH-urine sample collected 10 h post intravenous administration of phenytoin (8.8 mg/kg). In the non-EH-urine sample extract, *p*-HPPH in its free or unconjugated form was present at very low concentrations (≈1.0 μg/ml). It should be noted that, although we detected what was considered *m*-HPPH

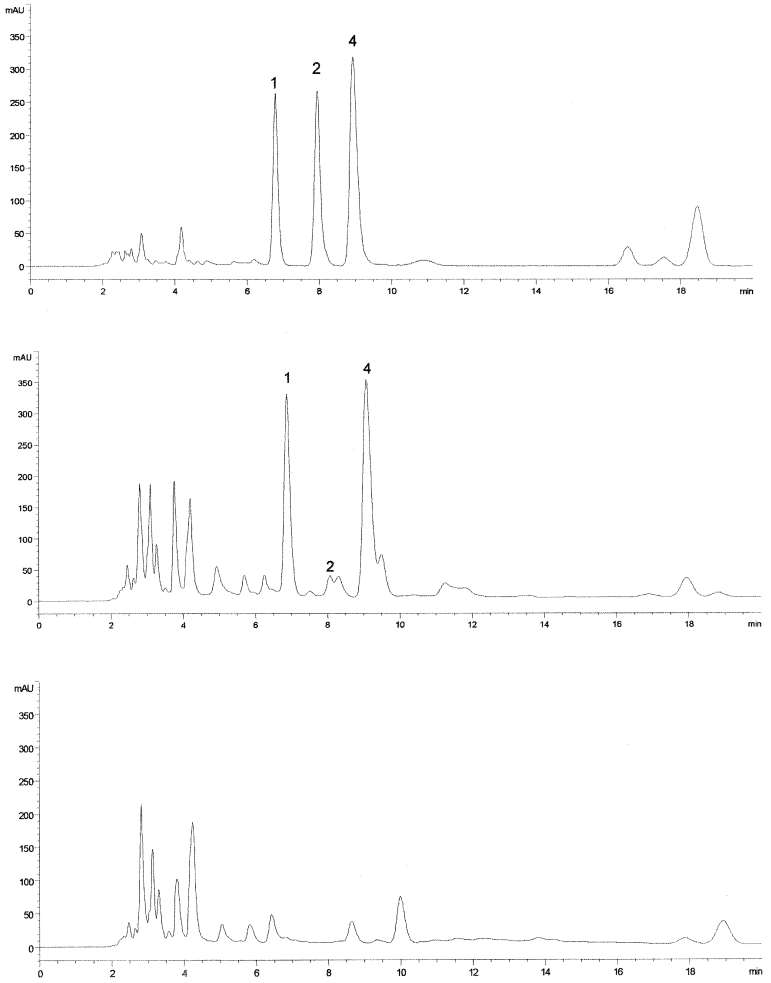

Fig. 3. HPLC chromatograms for EH-urine samples. The top panel represents the chromatogram for control urine sample (0.2 ml) spiked with *p*-HPPH, *m*-HPPH (4.0 μg each) and warfarin (I.S., 6.0 μg). In the middle panel is the chromatogram for urine sample (0.1 ml) collected 10 h post i.v. administration of phenytoin (8.8 mg/kg). The bottom panel is the chromatogram for control urine sample (0.2 ml) collected before the administration of phenytoin. Peak identification in order of elution: (1) *p*-HPPH; (2) *m*-HPPH; (4) warfarin (I.S.).

by its retention time in most of the samples at various times post phenytoin administration, the peak overlapped with a co-eluting substance that made any quantification from difficult to unreliable. From these observations, it appears that the production of *m*-HPPH in the horse is small and thus, it is a minor metabolite of phenytoin in the horse.

Hundreds of plasma, urine and EH-urine samples from 18 horses, to which phenytoin (8.8 mg/kg, i.v. or p.o.) was administered, were analyzed using the method we have described in this paper. By this method, we have successfully extracted, detected, and quantified phenytoin and *p*-HPPH in plasma and urine samples collected following its administration to horses. A low concentration ($\approx$1.0 μg/ml) of an unconjugated *p*-HPPH was detected and quantified in some of the non-EH-urine samples. Following enzyme hydrolysis for glucuronide de-conjugation of analytes in urine samples, a higher concentration of *p*-HPPH was recovered and quantified, suggesting that *p*-HPPH is excreted in urine as a glucuronide conjugate.

Acknowledgements

This study was funded by the Pennsylvania Horse and Harness Racing Commissions for which we are very thankful. The authors also express their appreciation to The Horsemen Association at Pocono Downs, Thoroughbred Horse Association at Philadelphia Park, The HBPA at Penn National and The MSOA at Meadows for their cooperation, continuing support and encouragement.

References

[1] D.F. Kowalczyk, J. Beech, J. Vet. Pharmacol. Ther. 6 (1983) 133–140.
[2] L.R. Soma, C.E. Uboh, F. Guan, E.K. Birks, D. Teleis, J.A. Rudy, A.O. Watson, Am. J. Vet. Res., accepted for publication (2000).
[3] R. Shimoyama, T. Ohkubo, K. Sugawara, T. Ogasawara, T. Ozaki, A. Kagiya, J. Pharm. Biomed. Anal. 17 (1998) 863.
[4] M.M. Bhatti, G.D. Hanson, L. Schultz, J. Pharm. Biomed. Anal. 16 (1998) 1233.
[5] M.J. Cwik, M. Liang, K. Deyo, C. Andrews, J. Fischer, J. Chromatogr. B 693 (1997) 407.
[6] G.L. Lensmeyer, B.E. Gidal, D.A. Wiebe, Ther. Drug Monit. 19 (1997) 292.
[7] L.A. Romanyshyn, J.K. Wichmann, N. Kucharczyk, R.C. Shumaker, D. Ward, R.D. Sofia, Ther. Drug Monit. 16 (1994) 90.
[8] A.T. Andresen, K.E. Rasmussen, H.E. Rugstad, J. Chromatogr. 621 (1993) 189.
[9] M.T. Maya, A.R. Farinha, A.M. Lucas, J.A. Morais, J. Pharmacol. Biomed. Anal. 10 (1992) 1001.
[10] S. Othman, W.A. al-Turk, A.S. Awidi, T.K. Daradkeh, O. Shaheen, Drug Design Deliv. 2 (1987) 41.
[11] J.H. Maguire, Epilepsia 29 (1998) 753.
[12] J. Hermansson, T. Iversen, U. Lindquist, Acta Pharm. Suec. 19 (1982) 199.
[13] C.E. Costello, J.E. Biller, K. Biemann, T.R. Browne, Clin. Chim. Acta 115 (1981) 263.
[14] S.A. Chow, L.J. Fischer, Drug Metab. Dispos. 10 (1982) 156.