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Detection and identification of carprofen and its in vivo metabolites in greyhound urine by capillary gas chromatography–mass spectrometry

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Abstract

Rimadyl® (carprofen) was administered orally to the racing greyhound at a dose of 2.2 mg kg^{-1}. Following both alkaline and enzymatic hydrolysis, postadministration urine samples were extracted by mixed mode solid-phase extraction (SPE) cartridges to identify target analyte(s) for forensic screening and confirmatory analysis methods. The acidic isolates were derivatised as trimethylsilyl ethers (TMS) and analysed by gas chromatography–mass spectrometry (GC–MS). Carprofen and five phase I metabolites were identified. Positive ion electron ionisation (EI^+) mass spectra of the TMS derivatives of carprofen and its metabolites show a diagnostic base peak at $M^{+}\cdot - 117$ corresponding to the loss of $COO–Si–(CH_3)_3$ group as a radical. GC–MS with positive ion ammonia chemical ionisation (CI^+) of the compounds provided both derivatised molecular mass and some structural information. Deutromethylation-TMS derivatisation was used to distinguish between aromatic and aliphatic oxidations of carprofen. The drug is rapidly absorbed, extensively metabolised and excreted as phase II conjugates in urine. Carprofen, three aromatic hydroxy and a minor *N*-hydroxy metabolite were detected for up to 48 h. For samples collected between 2 and 8 h after administration, the concentration of total carprofen ranged between 200 and 490 ng ml^{-1}. The major metabolite, α-hydroxycarprofen was detected for over 72 h and therefore can also be used as a marker for the forensic screening of carprofen in greyhound urine.

Keywords: Carprofen

1. Introduction

Carprofen, a substituted carbazole [(±)-6-chloro-α-methyl-9*H*-carbazole-2-acetic acid or (±)-2-(6-chlorocarbazol-2-yl)propionic acid] is a non-narcotic, nonsteroidal anti-inflammatory drug (NSAID) with characteristic analgesic and anti-pyretic activity. In 1997, Pfizer Animal Health, USA marketed this medication as Rimadyl® (25, 75 and 100 mg caplets) for oral use in dogs for effective relief of pain and inflammation associated with osteoarthritis.

Pharmacokinetics and pharmacodynamics of (±)-carprofen, its ^{14}C-analogue and/or of the individual enantiomers have been studied in the man, dog, cat,

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sheep, cow, calves and horse [1–7]. The metabolic disposition of ^{14}C-labeled carprofen has been investigated in rats, dogs and humans. In all three species a small amount of the phase I metabolite, α-hydroxycarprofen, is formed and excreted unconjugated in urine [8]. The major metabolic pathways in dogs and rats were phase II conjugation to form the ester (acyl) glucuronide and phase I oxidations at C-7 and C-8 to form phenols which were subsequently conjugated to glucuronic acid. Also in both species, biliary secretion predominates and 70% of an intravenous dose of carprofen is eliminated in the feces while 15–20% is excreted in urine [8]. Enantioselective glucuronidation and subsequent biliary excretion of carprofen is reported to be species dependent [9–12]. In humans, direct glucuronide conjugation of carprofen represents the only significant pathway with 65–70% of the oral dose being excreted in urine, less than 5% as unconjugated carprofen [8]. Following intravenous administration of Zenecarp® (at 0.7 mg kg^{-1} per day for 5 days) to horses, >80% of carprofen is excreted in urine as the acyl glucuronide and, like in man, conjugation is the major metabolic route [Dumasia, unpublished data].

In common with the analysis of NSAIDs, most methods for the determination and quantification of carprofen in plasma and urine are based upon high-performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection [13–19]. However, other techniques such as thin-layer (planar) chromatography (TLC) and GC–MS have also been reported [1,8,20–22]. Carprofen is an acidic drug and the reported pretreatment of biological samples involved hydrolysis, liquid–liquid extraction (LLE), backextraction of the drug from acidified matrices (at pH 4) with organic solvent followed by either by HPLC or GC–MS analysis [13–22].

The current study was performed in order to develop screening and confirmatory methods for carprofen in postcompetition canine urine. The species-specific metabolic profile of carprofen in the greyhound was determined in order to define suitable analytes. For routine forensic screening using TLC and spray visualisation, the drug and its metabolites were extracted from conditioned base-hydrolysed canine urine by LLE. For studies related to confirmatory analysis, the drug and its metabolites were isolated from urine by a SPE method for acidic drugs using co-polymeric mixed mode cartridges [23], derivatised (TMS) and analysed by GC–MS in the EI$^+$ and ammonia CI$^+$ modes. For the identification of phenolic metabolites, selected samples were derivatised as deutromethyl-TMS and analysed by GC–EI$^+$MS. This paper describes the methods developed for the isolation, detection and identification of carprofen and its metabolites in greyhound urine.

2. Experimental

2.1. Solvents and chemicals

Glass distilled grade organic solvents were purchased from Rathburn (Walkerburn, UK). Carprofen was obtained from Roche (Basel, Switzerland). Meclofenamic acid (2-[(2,6-dichloro-3-methylphenyl)amino]benzoic acid as sodium salt), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), β-glucuronidase from *Helix pomatia* (type HP2, a mixture of β-glucuronidase and aryl sulphatase enzymes), $^2H_3$-iodomethane and trimethylsulfonium hydroxide solution (Fluka, Switzerland) were obtained from Sigma (Poole, UK). Erlich's and Mandelins reagents were purchased from Militech (Washington, DC, USA). Worldwide Monitoring XTRACT® mixed-mode solid-phase extraction cartridges (XRDAH, 150 mg, 3 ml) were obtained from Anachem (Luton, UK). A 20-port SPE vacuum manifold for sample extraction was obtained from Whatman (Kent, UK).

2.2. Drug administration to animals, collection and storage of urine samples

Animal administrations were performed at the Veterinary Diagnostic Laboratory, Iowa State University, USA. Rimadyl® caplets (25 mg; Pfizer) were mixed with cat food and administered as a single oral dose (2.2 mg kg^{-1}) to two male greyhounds. Dog 11775 (34 kg) received three tablets (75 mg) and dog 4691 (43 kg) had four tablets (100 mg). Preadministration urine samples were obtained from each animal. Following administration, samples were collected at 2, 4, 8, 24, 48, 72 and 102 h postdose. The urine samples were stored at −20 °C and on completion of the collection period, transported

frozen to Horseracing Forensic Laboratory (UK) for analysis.

2.3. Routine forensic screening using thin-layer chromatography

A series of blank canine urine samples (2 ml), fortified with carprofen (at 1–5 μg ml^{-1}) were prepared. Postadministration urine aliquots (2 ml) were taken for analysis. The samples were base hydrolysed (2.5 *M* NaOH, 1 ml, 15 min at room temperature), potassium phosphate buffer (pH 3.3, 4 ml) was added, the pH adjusted to 3.3 with 5 *M* HCl and carprofen related analytes were isolated by LLE using dichloromethane–petroleum ether solvent mixture (10:1, v/v; 5 ml). The organic phase was reduced to dryness under oxygen-free nitrogen at 40 °C and the residue reconstituted in ethyl acetate (30 μl) for TLC with chemical over-spray visualisation.

TLC was performed on 0.25 mm precoated Kieselgel plates (silica gel 60 impregnated with F_{254} indicator, 20×20 cm; Merck, Darmstadt, Germany). Aliquots (10 μl) of the isolates were applied to each plate and the plates were developed in either of two chromatographic solvent systems (A) chloroform–ethanol (9:1, v/v) or (B) chloroform–cyclohexane–acetic acid (4:4:2, v/v). The developed plates were dried with gentle heat. For visualisation, the plates were sprayed with one of three different spray reagents (a) Erlich's reagent, (b) Mandelin's reagent and (c) silver nitrate reagent [$AgNO_3$ (1% aqueous), followed by mercuric chloride–diphenylcarbazone solution (2%:0.2%, in ethanol)] until just uniformly wet, then gently heated on a hot plate for colour formation.

2.4. Hydrolyses and extraction of total metabolites from urine

Aliquots of each urine sample (1 ml) were mixed with 10 *M* NaOH (20 μl) and left at room temperature for 30 min (pH≈10.5) to saponify the ester glucuronides. A 300-μl volume of 2 *M* HCl and 2.2 ml of 0.025 *M* ammonium acetate buffer, pH 5.5, were added to the base hydrolysed samples, mixed and the pH adjusted to 5.0. *Helix pomatia* mixed enzymes solution (20 μl) was added, mixed, the samples hydrolysed again at 50 °C for 2 h or overnight at 37 °C and centrifuged at 2000 *g* for 15 min to remove insoluble materials. XTRACT® columns were preconditioned for use by washing with 0.1 *M* phosphate buffer (pH 5.5)–methanol (9:1, v/v, 1 ml). The supernatant urine was passed through the columns (1 ml min^{-1}), rinsed with the 0.1 *M* phosphate buffer (pH 5.5)–methanol mixture (9:1, v/v, 0.5 ml), dried under full vacuum suction for 2 min, washed again with 1 *M* acetic acid (1 ml) and redried for a further 5 min. The acidic metabolites were recovered by elution with 2.5 ml ethyl acetate–hexane (3:1, v/v). The isolates were taken to dryness under N_2 at 40 °C. TMS derivatives were prepared by the addition of 50 μl MSTFA in toluene (1:1, v/v; 80 °C for 1 h) and analysed directly by GC–MS in the EI^+ and ammonia CI^+ modes.

2.5. Formation of deuteromethyl-trimethylsilyl (TMS) derivatives

The acid isolates were dissolved in dry acetone (200 μl) and $^2H_3$-iodomethane (25 μl) and potassium carbonate (~25 mg) were added. After mixing, the samples were heated at 60 °C for 45 min. The mixture was filtered through a cotton wool plug, the vial rinsed with acetone (2×500 μl) and the solvent reduced to dryness under nitrogen. The methylated residues were then silylated as described above and analysed directly by GC–EI^+-MS analysis.

2.6. Gas chromatography–mass spectrometry

GC–EI^+-MS at 70 eV was performed on a Fisons MD800 bench top mass spectrometer interfaced to a Thermoquest Trace 2000 series GC. A SGE BPX5 column (~25 m×0.25 mm I.D., 0.25 μm film thickness) was used with helium as the carrier gas. The initial column temperature, 90 °C, was maintained for 1 min. The oven temperature was programmed at 15 °C min^{-1} to 320 °C and then maintained at 320 °C for 6 min. The injector, transfer line and the ion source temperatures were 250, 280 and 150 °C, respectively. Aliquots (1 μl) of the derivatised urine isolates were injected in the splitless mode (1 min) using an autosampler (CTC model A200S, Zwingen, Switzerland). Full scan EI+ mass

spectra were recorded from 8 to 20 min of run time by scanning from m/z 100 to 600 (2 scans/s). Authentic mixed carprofen and meclofenamic acid standards (5 ng μl^{-1} concentration) were prepared, derivatised and analysed with the samples. For semiquantitative analysis the same GC and MS conditions were used but analysis was carried out in selected ion monitoring (SIM) mode.

Full scan GC–CI^+-MS analysis was carried out using ammonia as the reactant gas. A Finnigan ThermoQuest Automass Multi quadrupole benchtop mass spectrometer interfaced with a ThermoQuest Trace 2000 series GC. A SGE BPX5 column (~25 m×0.22 mm I.D., 0.25 μm film thickness, SGE) was used, with helium as the carrier gas. The injector, transfer line and the ion source temperatures were 260, 250 and 150 °C, respectively. Aliquots (1 μl) of the derivatised urine isolates were injected in the splitless mode (1 min) using an autosampler (CTC Analytics model CombiPAL system). CI^+ mass spectra at 70 eV were recorded from 8 to 20 min by scanning from m/z 100 to 600 as described above.

2.7. Semiquantification of urinary carprofen

A calibration line (range 0–100 ng ml^{-1}) and quality control samples (25, 50 and 75 ng ml^{-1}) were prepared in duplicate using blank canine urine (1 ml) fortified with authentic carprofen as the analyte. Aliquots (1 ml) of all pre- and sequential postadministration urine samples from both experiments were analysed in duplicate. For both dogs, urine samples collected 4 and 8 h after administration were initially diluted by 1/5 and 1/10 with deionised water. Duplicate aliquots (1 ml) of each prepared sample were taken for analysis. The internal standard (I.S.), meclofenamic acid (Na salt) was added to all samples at a concentration of 50 ng ml^{-1} as the free acid. Samples were prepared, derivatised and analysed by SIM–GC–EI^+–MS acquiring the ions listed below:

Channel	Mass (m/z)	Name
1, 2 and 3	242.1, 277.1 and 367.2	Meclofenamic acid (I.S.) TMS
4, 5 and 6	265.1, 300.2 and 417.3	Carprofen TMS
7–10	353.2, 388.2, 462.3 and 505.3	Carprofen metabolites TMS

The SIM–GC–MS conditions were the same as those described above for full scan GC–EI^+-MS analysis. For quantification, the peak area response of the m/z 300.2 ion was used for derivatised carprofen, the m/z 388.2 ion was used for the derivatised metabolites 2 to 5 and the m/z 242.1 ion was used for derivatised meclofenamic acid (I.S.). The VOYAGER software was used to perform the semiquantification of these compounds from their respective ion responses (peak areas).

3. Results

3.1. Routine forensic TLC screening methods

TLC analysis with spray visualisation showed that carprofen gave a grey colour with Mandelin's reagent, a blue/grey colour with Erlich's reagent and a purple colour with the silver nitrate reagent. Only blank samples fortified at levels of 2000 and 5000 ng ml^{-1} were clearly identified. Samples spiked at levels of <2000 ng^{-1} and postadministration urine samples from both greyhound administrations yielded endogenous sample contributions, which partially or totally obscured the presence of carprofen or related components.

3.2. Qualitative analysis and metabolite identification

Following both alkaline and enzymatic hydrolysis, the aglycones were isolated by SPE and the residues co-injected in MSTFA–toluene (1:1, v/v) for GC–MS analysis. To distinguish between aromatic, heteroatom and aliphatic oxidation, deuteromethylation was performed to methylate the carboxylic acid and phenolic groups prior to trimethylsilylation and GC–MS analysis. The primary EI^+ fragmentation pathway for the TMS derivatives of carprofen and its metabolites is the initial loss of O=C–O–$Si(CH_3)_3$ group as a radical ($M^+\cdot - 117$) and the formation of the diagnostic charge-retained base ions at m/z 300, 316 and 388. In some cases, further loss of the Cl atom [$M^+\cdot - (117+35)$] resulted in the formation of the ions m/z 265, 281 and 353 (see Fig. 3).

Full scan EI^+ mass spectra of derivatised authen-

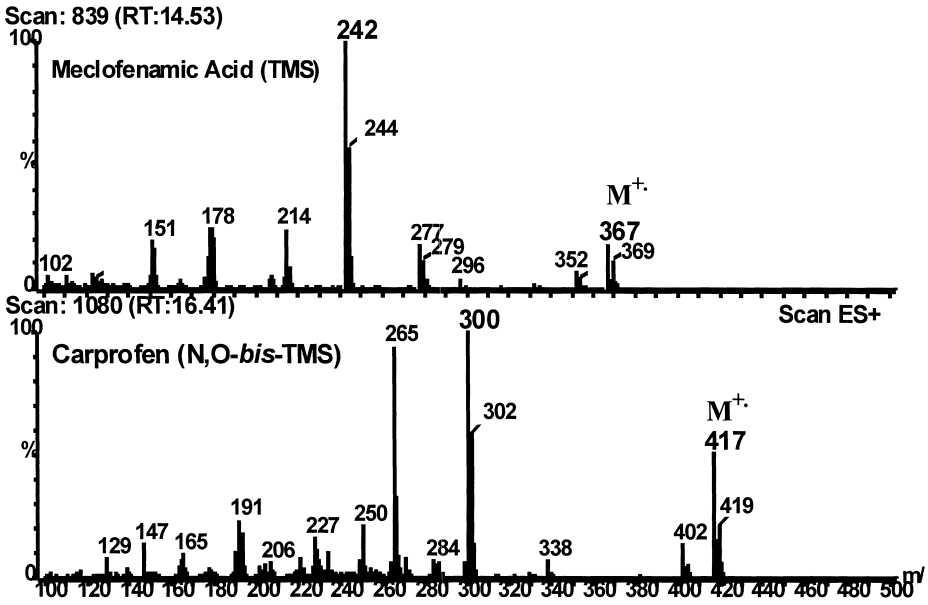

Fig. 1. Full scan EI^+ mass spectra of the TMS derivatives of authentic standards of carprofen and meclofenamic acid.

tic carprofen (as *N*,*O*-bis-TMS) and meclofenamic acid (as *O*-TMS) are shown in Fig. 1. The total ion chromatogram (TIC), mass chromatograms of the ions *m*/*z* 300, 388 and 505 and the full scan EI^+ mass spectra of the TMS derivatives of carprofen and five substrate-related compounds (**1**–**6**) obtained from analysis of a postadministration urine (dog 4691, U2, 4 h) are shown in Figs. 2 and 3. The full scan ammonia CI^+ mass spectra of the TMS derivatives of carprofen and its metabolites for the same sample are illustrated in Fig. 4 (spectra 1–6). Similar TIC, EI^+ and CI^+ data (not illustrated) were obtained for the second dog.

The parent drug (spectrum 1; Fig. 3) was identified by comparison of its retention time and EI^+ mass spectrum with those of the authentic standard (Fig. 1) and confirmed from its CI^+ mass spectral data (spectrum 1; Fig. 4). The EI^+ and CI^+ mass spectra of carprofen (1) and five substrate-related compounds (2–6) shown in Figs. 3 and 4, respectively, are tabulated in Table 1. On the basis of these data, the compounds were identified as *N*-hydroxycarprofen (peak 2; Fig. 2 and metabolite **1**, Fig. 9), α-hydroxycarprofen (peak 4; Fig. 2 and metabolite **2**, Fig. 9) and three regioisomers of aromatic monohydroxycarprofen (peaks 3, 5 and 6; Fig. 2 and metabolites **3**–**5**, Fig. 9).

The primary EI^+ fragmentation pathway for the deuteromethyl-TMS derivatives of carprofen and its metabolites again show the major loss 62u corresponding to the loss of $O{=}C{-}O{-}C^2H_3$ group as a radical ($M^+\cdot - 62$) and the formation of the diagnostic charge-retained base peaks at *m*/*z* 300, 333 and 388 (Fig. 5). The EI^+ mass spectrum of the O-deuteromethyl-*N*-TMS derivative of carprofen is shown in Fig. 5 (spectrum 1). It shows a molecular ion $M^{+\cdot}$, *m*/*z* 362 with a fragment ion at *m*/*z* 300 corresponding to the loss of 62 atomic mass units. For the regioisomeric monohydroxy metabolite 5 (spectrum 6; Fig. 5) aromatic oxidation was confirmed by deuteromethylation followed by silylation, resulting in the formation of O-,COO-bis-deuteromethyl-mono-*N*-TMS derivative showing a molecular ion $M^{+\cdot}$, *m*/*z* 395 and a base peak at *m*/*z* 333 ($M^+\cdot - 62$). The site of oxidation [on either of the two carbocycles of the carbazole nucleus] for any of the three phenolic metabolites could not be determined by mass spectrometry without the availability of authentic reference compounds. The EI^+ mass spectrum of the major regioisomeric monohy-

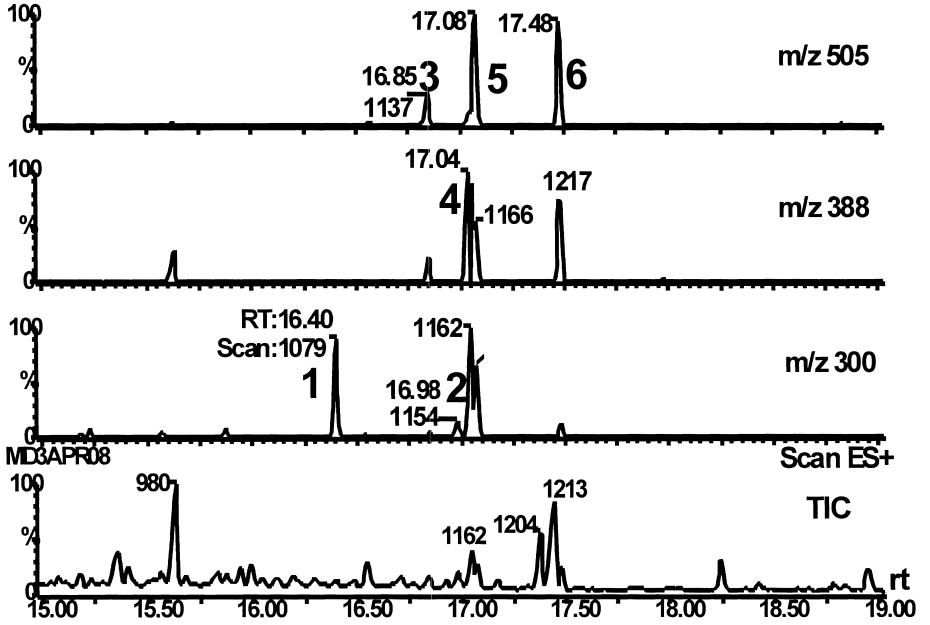

Fig. 2. TIC (EI^+) and mass chromatograms of m/z 300, 388 and 505 ions for the detection of the TMS derivatives of carprofen and its metabolites obtained from the isolate of a hydrolysed postadministration urine sample (dog 4691, U2, 4 h). Peaks: 1 = carprofen (as bis-TMS), 2–6 = various monohydroxycarprofen regioisomers (as bis- and tris-TMS).

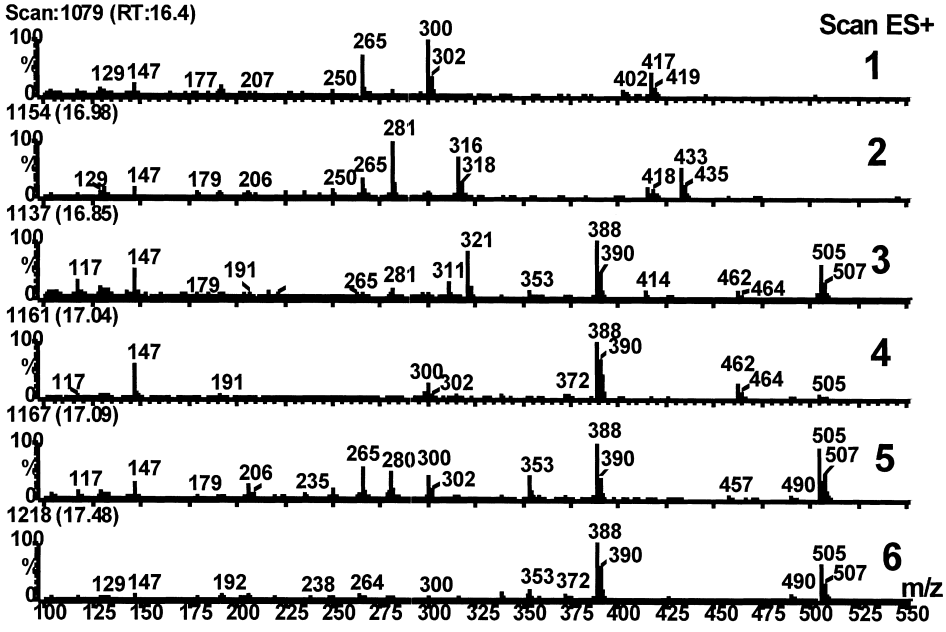

Fig. 3. Full scan EI^+ mass spectra of carprofen and its metabolites obtained from the derivatised isolate of a hydrolysed postadministration urine sample from dog 4691 (U2, 4 h). 1 = Carprofen (*N*,*O*-bis-TMS); 2 = *N*-hydroxycarprofen (*O*-bis-TMS; oxidation at the *N*-hereroatom of the fused pyrrole ring); 4 = α-hydroxycarprofen as tris-TMS (oxidation of the α-carbon on the 2-propionic acid moiety; 3, 5 and 6 = aromatic monohydroxycarprofen regioisomers as tris-TMS (possible sites of oxidation are C-7, C-8 and C-3 on the carbocyclic rings).

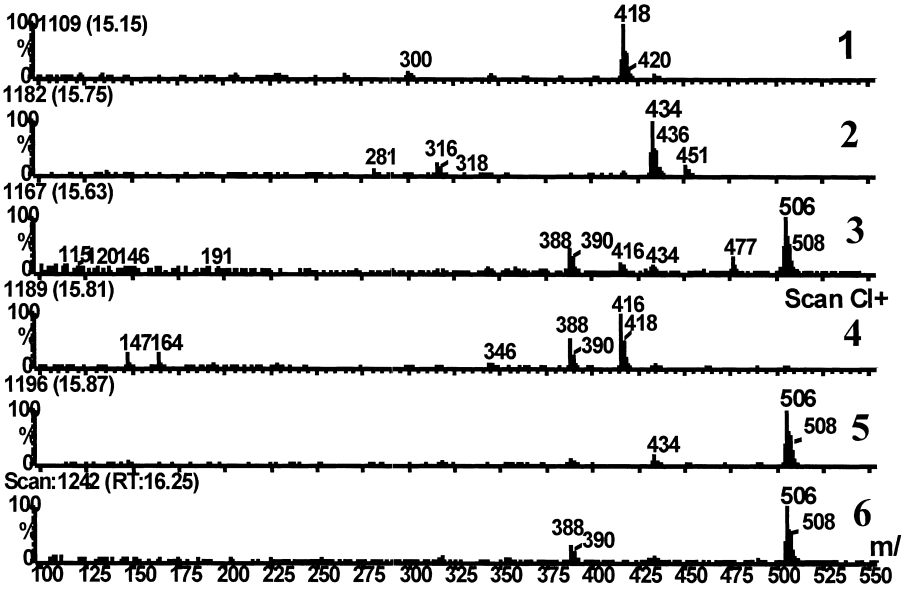

Fig. 4. Full scan ammonia CI^+ mass spectra of carprofen and its metabolites obtained from the TMS-derivatised isolate of the hydrolysed 4 h postadministration urine sample from Dog 4691. (1) carprofen (*N,O*-bis-TMS), (2) *N*-hydroxycarprofen (*O*-bis-TMS), (4) α-hydroxycarprofen (tris-TMS) and (3, 5 and 6) aromatic monohydroxycarprofen regioisomers (tris-TMS).

droxy metabolite (spectrum 4; Fig. 5) showed a molecular ion $M^{+}\cdot$, *m/z* 450 with fragment ions at *m/z* 435 ($M^{+}\cdot - 15$) and a base peak at *m/z* 388 again corresponding to the loss of 62 u. The molecular mass and the EI^+ fragmentation pattern of this metabolite therefore confirm the formation of the mono-deuteromethyl-bis-TMS derivative with deutromethylation of the COOH and silylation of both, an aliphatic hydroxy function and the *N*-heteroatom of the fused pyrrole ring of the carbazole nucleus. The site of oxidation for this compound was therefore assigned to the α-carbon on the 2-propionic acid moiety. This compound was therefore identified as α-hydroxycarprofen (metabolite **2**, Fig. 9).

3.2.1. Semiquantification of carprofen by GC–SIM-MS

Calibration standards, QC, pre- and postadministration samples were prepared, extracted, derivatised and analysed by GC–SIM-MS. The calibration line

Table 1
EI^+ and ammonia CI^+ mass spectral fragmentation data for the TMS derivatives of carprofen and its metabolites

Compound	EI^+ mass spectral data		CI^+ mass spectral data	
	Base peak (*m/z*)	Fragment ions and intensity (%)	Base peak (*m/z*)	Fragment ions and intensity (%)
Carprofen-*N,O*-bis-TMS (parent)	300/302	417/19 [$(M)^+$; 50], 402 (15), 265 (90)	418/420 $[M+H]^+$	300/302 (3)
N-Hydroxycarprofen-*O,O*-bis -TMS (metabolite 1)	281	433/435 [$(M)^+$; 40], 418/20 (12), 316/318 (65)	434/436$[M+H]^+$	316/318 (10)
α-Hydroxycarprofen-*N,O,O*-tris-TMS (metabolite 2)	388/90	505/07 [$(M)^+$; 5], 490/92 (8), 462/64 (25), 300/302 (3)	416/418 $[(M+H)-90]^+$	388/90 (31)
Hydroxycarprofen-*N,O,O* -tris-TMS (regioisomeric metabolites 3–5)	388/90	505/07 [$(M)^+$; 50], 490/92 (5), 353 (25)	506/508 $[M+H]^+$	388/90 (15–35)

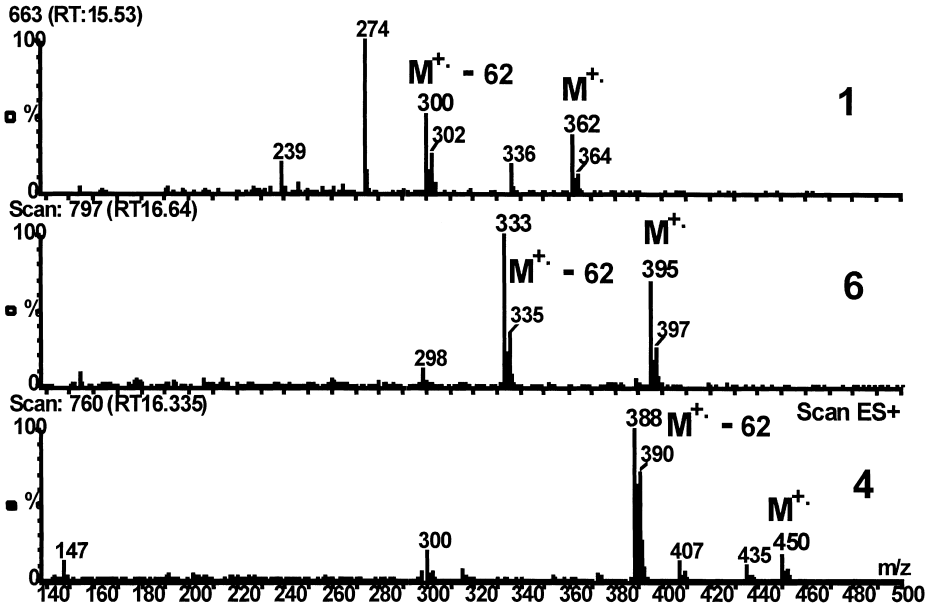

Fig. 5. Full scan EI^+ mass spectra of the deuteromethyl-TMS derivatives of (1) carprofen (6) an isomer of aromatic monohydroxycarprofen and (4) α-hydroxycarprofen.

was plotted for the peak area ratio (m/z 300.2/242.1) against the amount (ng ml^{-1}) of fortified carprofen. With respect to the I.S., the calibration plot showed linearity in the range between 0 and 100 ng ml^{-1} and a correlation coefficient of 0.997 using the nonweighted linear regression (Fig. 6). Intra-day precision and accuracy data for the method is shown in Table 2 and demonstrate that reliable results for the concentration of total carprofen in canine urine can be obtained using the developed method. The concentrations of urinary carprofen (Table 3) were plotted for both administrations (Fig. 7). As no authentic metabolites were available, the peak area ratio of the metabolite response to the internal standard response (ions m/z 388.2/242.1) was determined. The excretion profile of the major metabolite (α-hydroxycarprofen, **2**) was plotted as the peak area ratio against time after administration (Table 3 and Fig. 8). Similar peak area ratio profiles were obtained for the two prominent carbocyclic (aromatic) hydroxy metabolites **4** and **5** (data not shown).

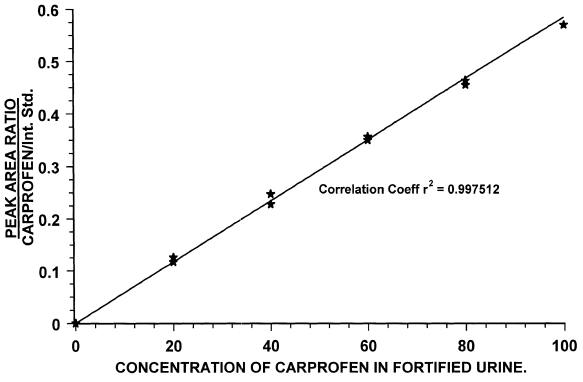

Fig. 6. Calibration line for semiquantification of carprofen in canine urine.

4. Discussion

Although TLC has previously been successfully used to detect carprofen and its metabolites [1,8,22], in this study it was found to be unsuitable for use as a high-throughput forensic screening method. This is mostly due to the lack of sensitivity and the masking of the developed colours by endogenous acidic co-extracted materials from canine urine.

Following the administration of a single oral therapeutic dose of Rimadyl, the in vivo biotransformation of the drug in the racing greyhound was

Table 2
Recovery, precision and accuracy of estimation for the quantification of carprofen in canine urine

Spiked level (ng ml^{-1})	Detected concentration (ng ml^{-1})			Precision RSD (%)	Accuracy RE (%)
		Mean	(±) SD		
25	24.57 26.80 27.50	26.29	1.53	5.82	5.16
50	47.67 52.04 55.10	51.6	3.74	7.25	3.2
75	72.73 67.98 68.36	69.7	2.63	3.8	7.1

Table 3
Amount of carprofen (ng ml^{-1}) and the peak area ratios of the major metabolite, α-hydroxycarprofen in canine urine, for both administrations

Urine	Time (h)	Carprofen (ng ml^{-1})		Metabolite **2** (peak area ratio)	
		Dog 4691	Dog 11775	Dog 4691	Dog 11775
U0	0	0.0	0.0	0.0	0.0
U1	2	13.0	54.6	0.37	1.7
U2	4	490.68	231.1	11.23	6.4
U3	8	358.75	195.53	12.86	8.41
U4	24	53.66	32.2	3.82	2.33
U5	48	6.85	4.9	0.57	0.43
U6	72	0.87	0.4	0.1	0.083
U7	102	0.1	0.04	0.054	0.03

investigated in order to determine a major analyte for use as a biomarker in post-race urine. Alkaline hydrolysis was used to cleave the β-1-*O*-acyl glucuronide conjugates including any positional isomers/anomers that may have been formed by internal acyl migration [24]. Enzyme hydrolysis was employed to

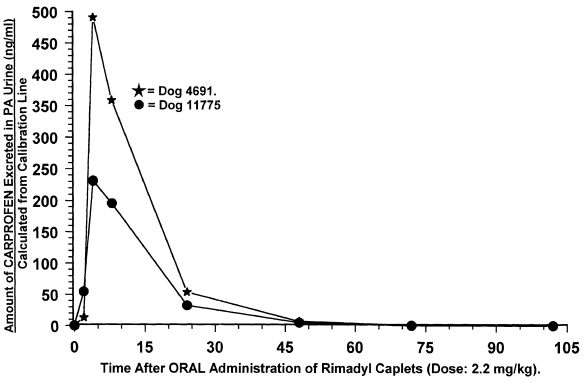

Fig. 7. Urinary excretion profile of total carprofen after oral administration of Rimadyl at 2.2 mg kg^{-1} to greyhounds.

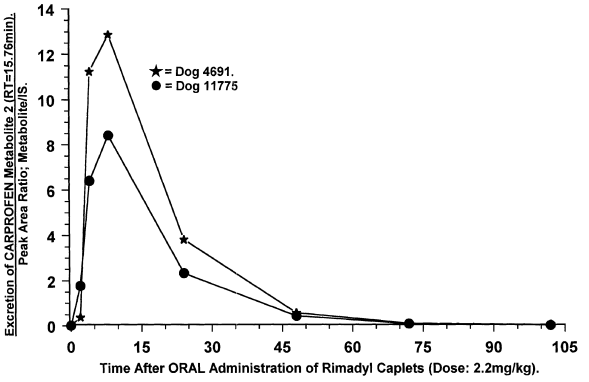

Fig. 8. Urinary excretion profile of α-hydroxycarprofen (metabolite **2**) expressed as peak area ratio against time after oral administration of Rimadyl to greyhounds.

liberate any ether glucuronides and sulfoconjugates of the phase I functionalised metabolites. Due to the unavailability of authentic reference compounds or any previously published data on the metabolites of carprofen in other species, structure elucidation of the putative metabolites is based primarily on the interpretation of the EI^+ and CI^+ mass spectral data of the derivatised compounds.

In most species, both carprofen and its metabolites are excreted as phase II conjugates in urine within the first 24 h of administration. It has been reported that following oral administration of carprofen to dogs, elimination by the fecal rather than renal route is predominant and two monohydroxy and the α-hydroxy metabolites were identified in dog urine [8]. In this study, carprofen and five isobaric monohydroxy metabolites (**1**–**5**, Fig. 9) were detected in the racing greyhound. These were formed in vivo by oxidations on the heteroatom, alicyclic and carbocyclic substructures of the carprofen molecule,

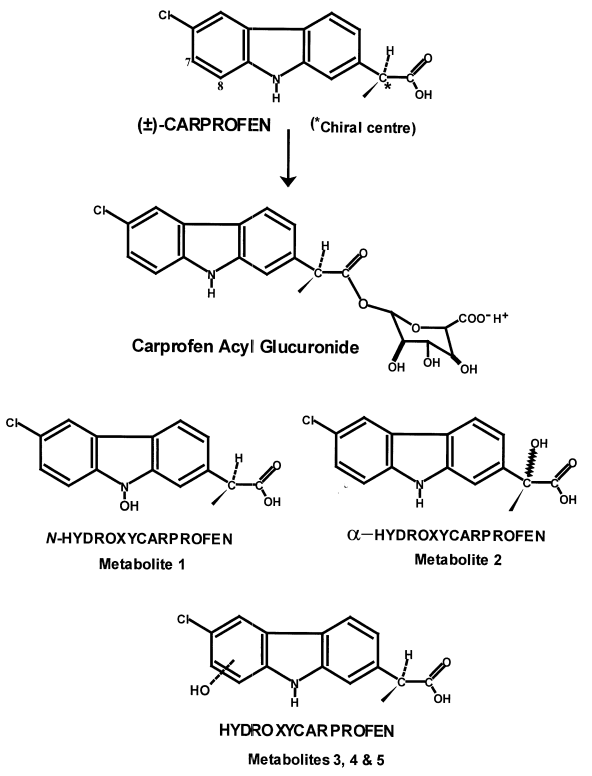

Fig. 9. Structures of carprofen and its in vivo metabolites detected and identified in greyhound urine.

resulting in the formation regioisomeric *N*-hydroxy, α-hydroxy and three phenolic metabolites. The formation and urinary excretion of *N*-hydroxcarprofen has not been reported previously in any species.

There is no requirement for the quantitative determination of thresholds or reporting levels for drugs that are banned in canine sports. Using meclofenamic acid as the I.S. a simple, rapid, GC–SIM-MS method was developed for the semiquantification of carprofen in canine urine. The detector response was linear over the range 0–100 ng ml^{-1}. The validation criteria described are satisfactory for the determination of excretion profiles or detection periods of drugs that are not listed as permitted medications. Further validation studies may be required depending on other laboratory's or sport authority's objectives and requirements. In this study, the quantitative data were only used for a graphical representation of the urinary excretion profile of carprofen and its major metabolite.

Using full scan GC–MS, carprofen was detected in postadministration greyhound urine samples for up to 48 h and quantified by SIM for up to 72 h. The major metabolite (α-hydroxycarprofen, **2**) was detected in urine for >72 h. The other metabolites can be detected at low levels for between 24 to 48 h. Inter-animal difference in the amount of carprofen and metabolites excreted in urine was observed in this study.

5. Conclusion

After oral administration, alkaline hydrolysis and solvent extraction followed by routine forensic TLC screening, cannot detect carprofen or related compounds at the concentrations present in greyhound urine. Carprofen and five regioisomeric monohydroxy metabolites were identified by GC–MS. A maximum level of 500 ng ml^{-1} of total carprofen was excreted in urine. *N*-Hydroxycarprofen has been tentatively identified for the first time as a urinary metabolite of carprofen in any species. The major metabolite α-hydroxycarprofen can be used as an appropriate biomarker for carprofen in postcompetition greyhound urine samples. The work presented in this study provides viable, rapid and specific methods for the detection of carprofen and its phase I

functionalised metabolites by GC–MS. The methods are also appropriate for the confirmatory analysis of the drug in postrace urine samples.

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