ELSEVIER

Journal of Chromatography B, 667 (1995) 95–103

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Detection of flunixin in equine urine using high-performance liquid chromatography with particle beam and atmospheric pressure ionization mass spectrometry after solid-phase extraction

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First received 26 October 1994; revised manuscript received 28 December 1994; accepted 28 December 1994

Abstract

A normal-phase HPLC method combined with particle-beam mass spectrometry (PB-MS) was developed for the analysis of non-steroidal anti-inflammatory drugs (NSAIDs). The forty one NSAIDs analysed responded in one or more (electron impact, positive and negative chemical ionisation) modes and highly characteristic spectra were produced. A mixed-mode solid-phase extraction (SPE) method for isolating acidic NSAIDs was developed using the Bond Elut Certify II cartridge. The average recovery was 88.5%. Flunixin, extracted by SPE from urine of a mare to which the meglumine salt had been administered was positively identified by HPLC–PB-MS and HPLC–atmospheric pressure ionization (API) MS methods.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are often detected during screening post-race samples from the thoroughbred racing jurisdiction under our control. These compounds reduce inflammation and have varying antipyretic and analgesic effects [1], thus allowing an otherwise lame horse to race. Due to their polar nature most NSAIDs are not suitable for GC unless derivatised. However, screening can easily be done by HPLC using UV [2–8] or tandem UV–differential refractive index [3] or tandem UV–fluorometric detection [9]. Suspected positives are confirmed by GC–MS of the derivatised extract [9–11]. Screening/confirmation by LC–MS is a less time-consuming option because no derivatisation step is required. This conservation of time and effort is especially important where more than one technique with MS detection is mandated for positive identification of the drug.

Several methods of introducing the analyte into the mass spectrometer after HPLC are available [12]. We elected to use particle-beam MS because of its ability to acquire spectra under classical EI as well as positive and negative chemical ionisation conditions. The speed of switching between ionization modes also increases efficiency and the spectra produced are probability match "library searchable" which makes identification simpler. Furthermore, the

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0378-4347/95/$09.50
SSDI 0378-4347(94)00603-2

limitation on sensitivity, which is the major drawback of this HPLC–MS method [13], is not as important with NSAIDs because the large dosage of these drugs that is required for efficacy translates into high concentrations in serum and urine. PB-MS has a range of operating conditions where optimal performance is achieved. It is better suited to introducing compounds separated by normal-phase rather than reversed-phase HPLC. This is because the efficiency of the analyte transfer through the PB is inversely proportional to the ΔH_{vap}, surface tension and the heat capacity of the mobile phase [14]. Many of the best suited solvents are only compatible with normal-phase chromatographic systems. Published normal-phase HPLC methods are not generally suited to PB-MS with negative chemical ionisation due to the use of chloroform or dichloromethane in the mobile phase (e.g. [15]). Therefore, a gradient HPLC (Hypersil SI) method with hexane–5% water in isopropanol to be used in conjunction with PB-MS was devised for separating NSAIDs. This normal-phase HPLC–PB-MS analytical method proved effective for 41 NSAIDs that were tested.

Solid-phase extraction (SPE) using a Bond Elut Certify II column was developed for isolating and concentrating flunixin and other acidic anti-inflammatory agents from urine. Extracts of samples collected after administration of flunixin meglumine to a thoroughbred mare were extracted by SPE and analysed by HPLC–PB-MS. The drug was detectable in all the acquired isolates using the electron-impact (EI), positive chemical ionisation (PCI) and negative chemical ionisation (NCI) modes. These samples were also analysed by reversed-phase (C_{18}) HPLC–atmospheric pressure ionisation (API) MS in the negative-ion mode and the analyte was detected.

2. Experimental

2.1. Chemicals and reagents

Diclofenac was purchased from Adcock Ingram Pharmaceuticals (Johannesburg, South Africa). Ibuprofen and naproxen were purchased from Boots Pharmaceuticals (Nottingham, UK). Ketoprofen was purchased from Rhone Poulenc Rorer (Port Elizabeth, South Africa). Oxaprozin was a gift from Akromed (Johannesburg, South Africa). Piroxicam was purchased from Pfizer (Johannesburg, South Africa). Phenazone was purchased from Protea Chemicals (Johannesburg, South Africa). Phenylbutazone was purchased from Sigma (St. Louis, MO, USA). Tenoxicam was purchased from Roche (Johannesburg, South Africa). Alclofenac, aminoantipyrine, bumadizone, clonixin, diflunisal, dipyrone, famprofazone, fenbufen, fenoprofen, flufenamic acid, flunixin, flurbiprofen, ibufenac, indomethacin, indoprofen, isopyrin, isoxepac, ketorolac, meclofenamic acid, nabumetone, nefopam, niflumic acid, phenazone, phenazopyridine-HCl, propyphenazone, salicylamide, sulindac, suprofen, tiaprofenic acid, tolmetin, and zomepirac were gifts from the University of the Orange Free State (Bloemfontein, South Africa). Acetonitrile, hexane, isopropanol and methanol high purity solvents were from Burdick and Jackson (Muskegon, MI, USA). Acetic acid (>99.8% purity) was from Riedel-de Haën (Hannover, Germany). Orthophosphoric acid was from E. Merck (Darmstadt, Germany). Water was purified with a Milli-Q reagent water system from Millipore, Waters Chromatography division (Bedford, MA, USA).

2.2. Instrumentation and methods

HPLC with UV (diode array)-particle beam MS

HPLC with UV (diode array). A Hewlett-Packard Series II 1090 L liquid chromatograph with a diode-array UV detector (Hewlett-Packard, Palo Alto, CA, USA) was used. The monitoring frequency was 280 nm with a bandwidth of 40 nm. All mobile phases were filtered and sparged before use. The flow-rate was 0.4 ml/min and the oven temperature was 45°C. A Hypersil SI (100 × 2.0 mm I.D., 5 μm) column (Phenomenex, Torrance, CA, USA) was conditioned overnight with isopropanol and then stabilized (5 min) with the starting mixture of hexane–5% water in isopropanol (IPA) (98:2, v/v). From 0 to 8 min the composition was changed with a linear gradient to 70:30 and between 8 and 12

min the composition was altered to 0:100. This composition was maintained until 13 min. The column was stabilized (5 min) with hexane–5% water in IPA (98:2, v/v) before each run. Three microlitres of a 50:50 hexane–isopropanol solution containing 50 μg/ml NSAID and 20 μg/ml floctafenine internal standard were injected.

Particle-beam MS. The HPLC effluent was coupled to a Hewlett-Packard HP 59980B PB interface operated at 65°C with a helium pressure of 275 kPa. The PB was connected to a Hewlett-Packard HP5989A quadrupole mass spectrometer fitted with a 10-kV conversion high energy dynode (HED) (Hewlett-Packard). The viton O-ring of the PB gate valve was replaced with a nitrile ring of similar dimensions (ABES, Johannesburg, South Africa) in order to eliminate several prominent background ions found in the NCI mode. Methane gas (99.995% purity) at 333 Pa was used for chemical ionization. The following source temperatures: 150, 200, 250, 300°C, were evaluated for each standard in the EI, positive and negative CI modes. In addition 350°C was also evaluated for EI. The source temperature was 275°C for the analysis of the flunixin administration trial sample extracts.

Reversed-phase HPLC–atmospheric pressure ionization (API) MS

HPLC. A Thermo Separations Products membrane degasser and 3500MS/3200MS solvent delivery system was coupled to a AS3000 autosampler (Thermo Separations Products, Fremont, CA, USA). The Hypersil ODS (100 × 2.1 mm I.D., 5 μm) column (Hewlett-Packard) was stabilized (5 min) with the starting composition 1% aqueous acetic acid–acetonitrile (70:30, v/v). This was maintained up to 2 min. Thereafter the composition was changed with a linear gradient to 0:100 at 8 min. This composition was maintained until 10 min. The flow-rate was 0.4 ml/min.

API-MS. The HPLC was coupled to a Finnigan M.A.T. SSQ 7000 MS (San Jose, CA, USA) via an electrospray ionization interface used in the negative mode. The nitrogen sheath gas pressure was 275 kPa and no auxiliary gas or liquid flow was used. The collision-induced dissociation voltage was set at 20 V. The electron multiplier voltage was at 1500 V with the collision dynode at 15 kV.

2.3. Flunixin equine administration

Finadyne (Fisons Pharmaceuticals, Chloorkop, South Africa) equivalent to 535 mg of flunixin was administered to an 482 kg thoroughbred mare. Urine samples were obtained using an urinary catheter and frozen to −20°C within 30 min of collection.

2.4. Solid-phase extraction (SPE) of flunixin from equine urine

A Bond Elut Certify II 3 ml cartridge (Varian, Harbour City, CA, USA) which contains a dual-chemistry (C_8 plus strong anion exchanger) solid phase [16], was primed with 3 ml of methanol followed by 3 ml of water. The pH of the urine sample was adjusted to 7 with 1 *M* NaOH or 1 *M* HCl and centrifuged (500 *g*, 15 min). A 3-ml volume of supernatant was drawn through the column at 0.2 ml/min using a vacuum manifold. The column was successively washed with 2 × 2.5 ml of water, 2 × 2 ml of methanol and dried for 20 min using the full vacuum. After washing with 2 × 2 ml of hexane the flunixin was eluted with 2 × 2 ml of 10% acetic acid in hexane. The naproxen internal standard (2.5 μg) was added to samples for reversed phase analysis and 2 μg ketoprofen to those analysed by normal-phase HPLC. The eluent was dried under oxygen-free nitrogen and reconstituted in either 100 μl of methanol (for reversed-phase HPLC) or 100 μl of 50:50 hexane–isopropanol (for normal-phase HPLC).

3. Results and discussion

The gradient normal-phase (Hypersil SI) HPLC separation with UV detection gave peaks (average base width = 0.239 min) with reproducible retention times for all the anti-inflammatory agents injected. These results, relative to the

internal standard floctafenine, are shown in Table 1.

Most of the 41 NSAIDs tested gave a detectable response by HPLC–PB-MS in at least two of the three ionisation modes used (Table 1). In fact only diflunisal and tolmetin could be detected with only one of the ionisation modes. The optimum conditions for each analyte are

Table 1
Normal-phase HPLC relative retention times and PB-MS responses in EI, PCI and NCI modes

NSAID	RRT[a]	EI[b]	PCI	NCI
Alclofenac	0.890	++^{150}	+++^{150}	–
Aminoantipyrine	1.289	+++$^{150-300}$	+++$^{150-200}$	+++$^{250-300}$
Bumadizone	1.346	++^{300}	+^{250}	+^{250}
Clonixin	1.030	++^{350}	++^{150}	++^{250}
Diclofenac	0.621	++^{350}	+^{300}	+^{300}
Diflunisal	2.134	–	++^{150}	–
Dipyrone	2.237	++^{350}	++^{300}	–
Famprofazone	0.359	+++$^{150-300}$	+++$^{150-300}$	++^{250}
Fenbufen	1.056	+++^{350}	++^{150}	–
Fenclofenac	0.626	+++^{150}	++$^{150-250}$	++$^{250-300}$
Fenoprofen	0.559	+++^{350}	+$^{150-200}$	–
Floctafenine	1.000	+++^{350}	++^{200}	+++$^{200-250}$
Flufenamic acid	0.680	+++^{350}	++$^{150-200}$	++^{250}
Flunixin	1.030	+++^{350}	++^{150}	++^{250}
Flurbiprofen	0.636	+++^{150}	+++^{150}	–
Ibufenac	0.656	+^{350}	++^{150}	–
Ibuprofen	0.463	++^{350}	++^{150}	–
Indomethacin	1.035	+++^{350}	+^{150}	++^{250}
Indoprofen	1.292	+++^{350}	+^{150}	–
Isopyrin	0.773	+++^{150}	+++$^{150-300}$	++^{250}
Isoxepac	1.239	+++^{350}	++^{150}	–
Ketoprofen	0.873	+++^{350}	+++$^{150-300}$	+++^{250}
Ketorolac	1.695	+++^{350}	++^{150}	++^{250}
Meclofenamic acid	0.468	+++^{350}	++$^{150-250}$	+++^{300}
Mefenamic acid	ND[c]	+++^{350}	+++$^{150-300}$	+^{250}
Naproxen	0.559	+++^{350}	+++$^{150\ 300}$	–
Nefopam	1.316	+++^{150}	+++$^{150-300}$	++^{300}
Niflumic acid	1.248	+++^{350}	+++^{150}	+++^{250}
Oxaprozin	1.164	+++^{350}	++^{150}	++^{250}
Phenazone	1.317	+++$^{300\ 350}$	+++$^{150-300}$	++^{250}
Phenazopyridine	0.741	+++^{350}	+++$^{150-250}$	+++$^{250-300}$
Phenylbutazone	0.251	+++$^{150\ 350}$	+++$^{150-300}$	+++^{250}
Piroxicam	1.433	+^{150}	+++$^{150-250}$	+++$^{250-300}$
Propyphenazone	0.452	+++$^{300\ 350}$	+++$^{150-250}$	–
Salicylamide	0.687	+^{350}	++$^{150-200}$	–
Sulindac	1.615	++^{350}	–	++$^{250-300}$
Suprofen	1.103	+++^{350}	+++^{150}	++^{300}
Tenoxicam	2.132	–	+^{250}	++^{300}
Tiaprofenic acid	1.451	+++^{350}	+++^{150}	++^{250}
Tolmetin	0.533	–	–	+^{250}
Zomepirac	1.770	+++^{350}	++$^{150-250}$	–

[a] Average ($n = 3$) retention times relative to floctafenine, monitoring frequency of 280 nm with a 40-nm bandwidth.
[b] + = Detectable response, ++ = moderate response, +++ = strong response. Superscript denotes temperature of the optimum base-peak response.
[c] Not determined.

indicated in Table 1 and the three major spectrum ions are listed in Table 2. No correlation could be found between the response for a particular compound in a mode of ionisation and its chemical class. This is probably due the large structural variations within the six classes tested. Nonetheless, in general, EI at 350°C gave the best response for the majority of NSAIDs. The

Table 2
Three characteristic ions in descending order of intensity for each NSAID in the EI, PCI and NCI modes of ionization

NSAID	EI ions[a]			PCI ions			NCI ions		
	1	2	3	1	2	3	1	2	3
Alclofenac	226	181	167	227	229	181		NR[b]	
Aminoantipyrine	203	119	–	204	232	407	203	–	–
Bumadizone	184	162	133	283	192	311	234	342	189
Clonixin	247	262	181	163	277	391	262	246	264
Diclofenac	214	242	277	278	244	308	258	260	213
Diflunisal		NR		251	263	296		NR	
Dipyrone	230	217	123	218	246	230		NR	
Famprofazone	287	299	214	378	406	286	376	229	201
Fenbufen	181	152	254	296	255	237	254	216	210
Fenclofenac	296	298	217	299	251	279	278	180	244
Fenoprofen	242	197	104	243	391	197		NR	
Floctafenine	286	333	406	407	387	435	331	316	346
Flufenamic acid	281	263	235	282	262	243	281	263	216
Flunixin	281	263	296	297	282	277	296	278	262
Flurbiprofen	244	199	178	245	199	273		NR	
Ibufenac	192	105	–	193	245	147		NR	
Ibuprofen	163	206	199	207	161	193		NR	
Indomethacin	139	141	357	242	207	297	357	218	299
Indoprofen	236	281	218	282	245	297		NR	
Isopyrin	245	137	230	246	274	286	245	205	–
Isoxepac	268	223	165	269	297	325		NR	
Ketoprofen	254	209	177	254	210	225	254	210	225
Ketorolac	255	210	–	255	283	297	210	254	–
Meclofenamic acid	242	297	214	298	255	278	258	223	–
Mefenamic acid	241	223	208	242	224	–	223	210	240
Naproxen	185	230	170	231	185	259		NR	
Nefopam	225	179	165	254	231	282	252	195	–
Niflumic acid	282	237	263	282	311	263	282	254	258
Oxaprozin	293	248	165	294	322	334		NR	
Phenazone	188	–	–	189	217	229	266	187	–
Phenazopyridine	213	136	184	214	242	294	213	214	212
Phenylbutazone	308	184	252	309	337	349	308	189	252
Piroxicam	173	117	262	332	360	372	267	211	331
Propyphenazone	215	230	–	231	259	271		NR	
Salicylamide	137	120	–	138	166	178		NR	
Sulindac	297	233	312		NR		297	312	356
Suprofen	111	215	260	261	289	301	260	215	244
Tenoxicam		NR		121	338	261	273	255	337
Tiaprofenic acid	216	139	201	261	289	301	260	216	242
Tolmetin		NR			NR		162	102	–
Zomepirac	208	263	243	262	226	290		NR	

[a] Spectra obtained at optimum temperature as described in Table 1.
[b] No response.

Table 3
Flunixin recovered from spiked equine urine by solid-phase extraction

Flunixin concentration (μg/ml)	Recovery (%)	C.V. ($n = 6$) (%)
0.100	83.0	5.2
1.00	93.7	5.6
10.0	88.8	5.8

Average recovery = 88.5%

PCI (150°C) mode also gave good responses for a large number of these compounds. In most cases both the mode of ionisation and temperature of the source had a notable effect on the results obtained. There were a few exceptions, for example, phenylbutazone which gave a strong response using any of the ionisation modes and at all temperatures (Fig. 1) except for 150 and 200°C in NCI. For each mode of ionization the analytes produced several characteristic spectral peaks in addition to the molecular or pseudo-molecular ion. Phenylbutazone (Fig. 1), for example, produces a molecular ion in EI and pseudo-molecular ions in NCI $[M\text{-}H]^-$ and PCI $[M + H]^+$. The PCI spectrum, while lacking in fragments from the breakdown of the pseudo-molecular ion has prominent $[M + C_2H_5]^+$ and $[M + C_3H_5]^+$ adduct ions. Both the EI and NCI spectra show fragments at m/z 252 as well as unique fragments at m/z 183 and 265 for EI and m/z 189 for NCI. Therefore the different ionisation modes provide complementary data which can be used for confirming a 'positive' sample. This is especially important in our racing jurisdiction where we rely on more than one procedure using MS detection to confirm the presence of a drug. The normal-phase HPLC–PB-MS, because of its multiple ionisation

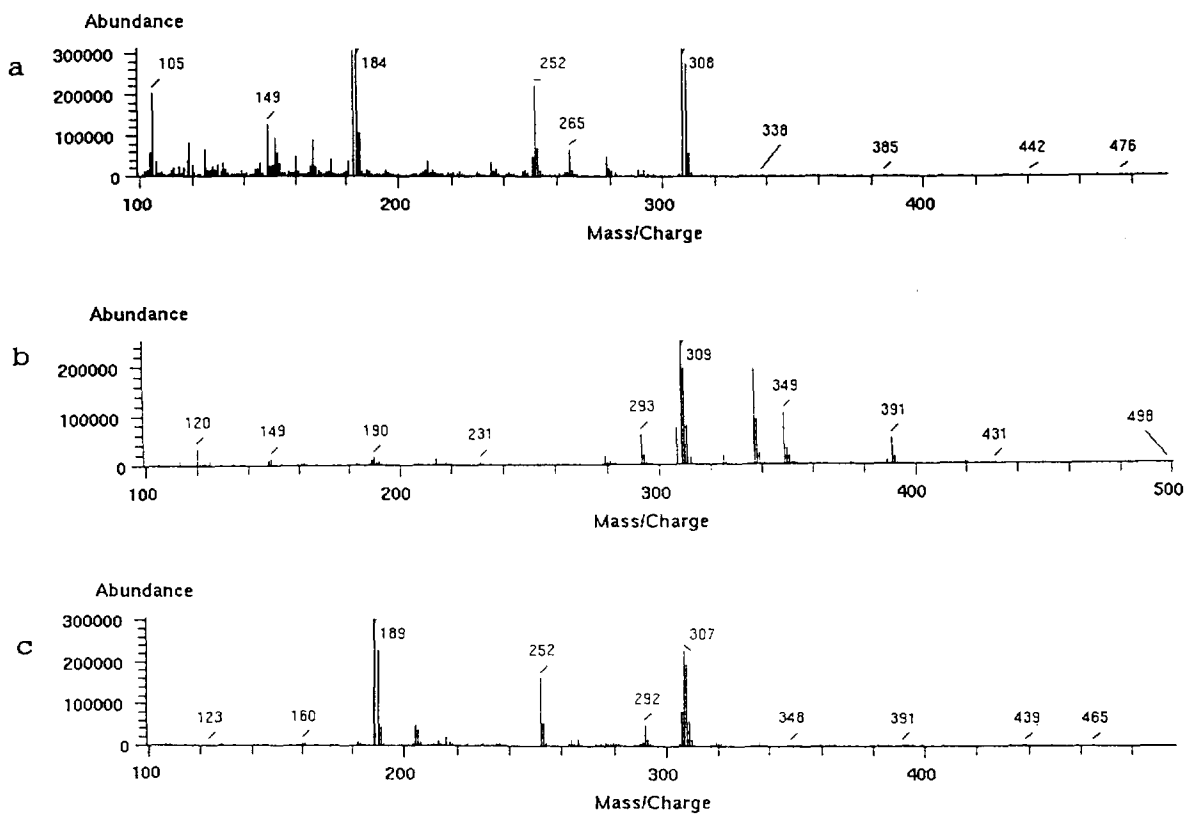

Fig. 1. Particle-beam mass spectra of phenylbutazone (a) EI, (b) PCI, (c) NCI.

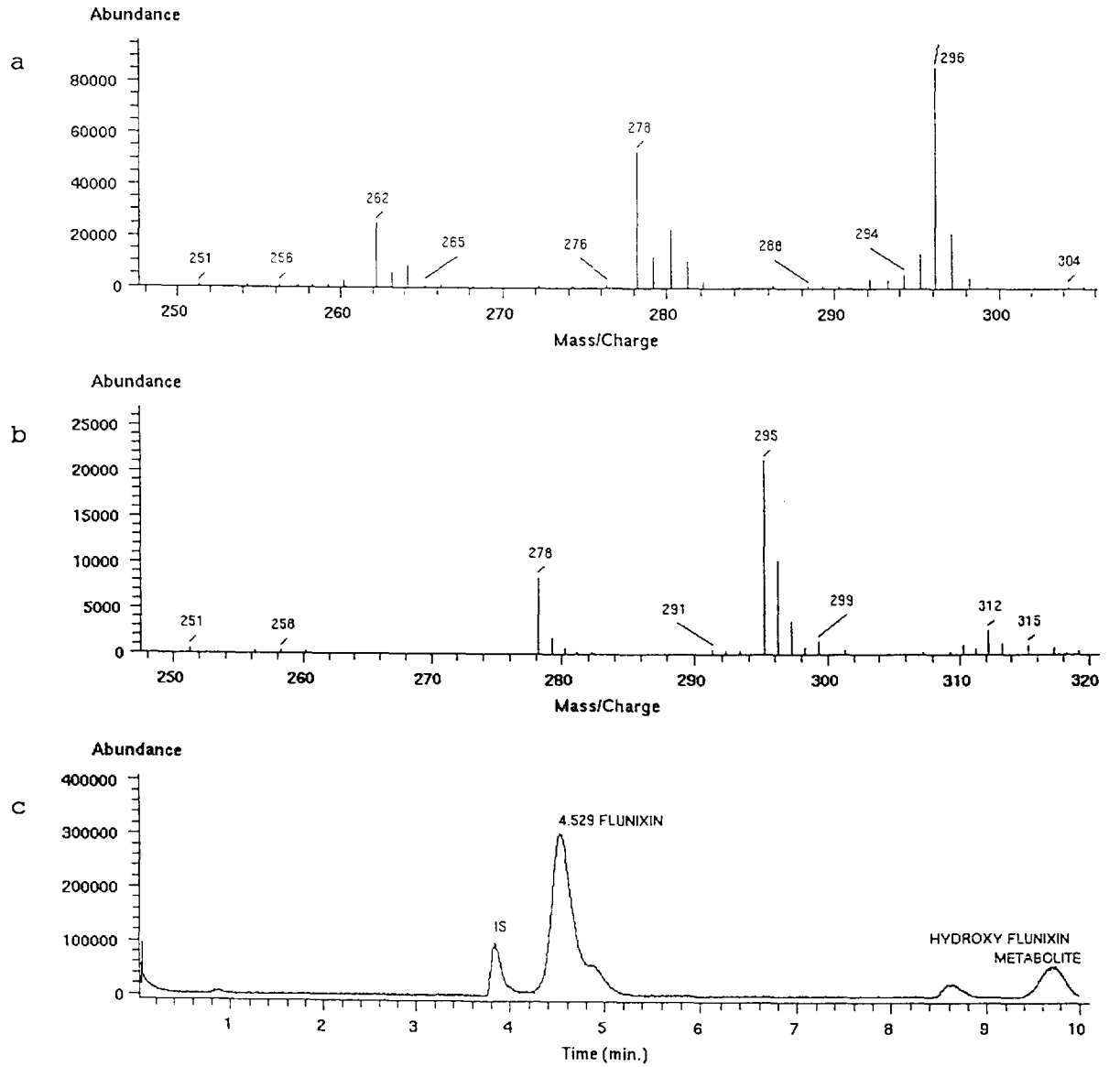

Fig. 2. Particle-beam mass spectral data (NCI) from the 8-h post-administration urine extract. (a) Mass spectrum of flunixin at 4.529 min; (b) mass spectra of hydroxyflunixin metabolite at 9.710 min; (c) total-ion chromatogram. IS = internal standard.

modes and abundant spectral information, provides a convenient way of achieving this.

Flunixin was extracted from spiked equine urine at an average recovery of 88.5% (Table 3) by SPE with a Bond Elut (mixed mode) Certify II cartridge. The dual chemistry of the packing material allows more rigorous washing steps and therefore the isolate contains fewer extraneous co-extractants than would be possible with a single-mode cartridge. This method was applied to urine samples obtained from a thoroughbred mare to which flunixin meglumine had been given by intravenous injection. Prior to analysis these samples were quantitated by HPLC-UV (diode array) (Table 4). Flunixin could be detected in the urine extracts by HPLC–PB-MS in

Table 4
Concentration of flunixin found in post administration equine urine

Time (h)	Flunixin (μg/ml)
0.5	160.0
1	215.3
2	98.4
4	251.9
6	29.5
8	12.3
12	1.9

NCI (Fig. 2), EI and PCI modes. The retention time (4.5 min) of the compound correlated well with that of the standard and the spectra gave a probability match (PBM) [17] of >90% with the library entry. The limit of detection for flunixin was 10 ng/ml with a (peak-to-peak) signal-to-noise ratio of 10:1. The hydroxy metabolite [18] was also isolated by the SPE method and could be detected by PB-MS (Fig. 2) in all acquired urine extracts from 2-h post-administration. The characteristic retention time and spectrum of flunixin and its metabolite when analysed by HPLC–PB-MS allows the absolute identification of both these substances.

Flunixin and its metabolite were also detectable in these extracts following reversed phase (C_{18}) HPLC-API (electrospray ionisation) in the negative mode. However, because API is a "soft" ionisation mode, fragmentation was only achieved by collision-induced dissociation (CID) of flunixin in the octapole region of the SSQ 7000 (Fig. 3). This produced a spectrum with several ions in addition to the single pseudo-molecular anion observed with the CID off. The analyte was identified on the basis of a comparison between the retention time and spectral characteristics of the standard. These results were comparable with those produced by PB-MS. Although API can be operated in either the positive or negative mode only one of these will usually produce suitable results for a particular NSAID, unlike PB-MS which offers a choice of up to three different ionisation types. However, HPLC–PB-MS is not as sensitive as HPLC–API-MS. Nevertheless, despite the limitations of each HPLC–MS method, we believe that they are

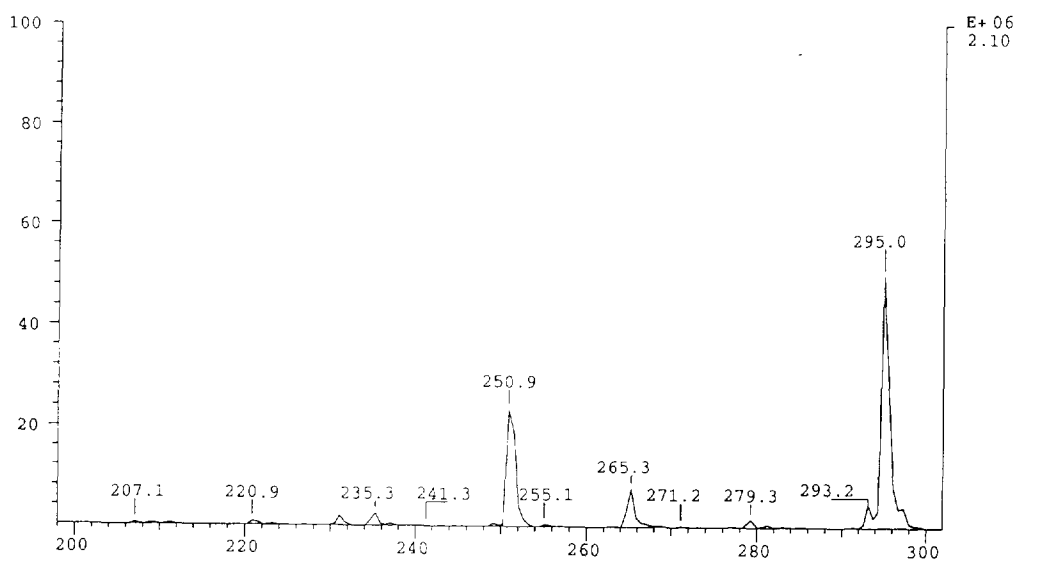

Fig. 3. Mass spectrum of flunixin obtained by reversed-phase HPLC–negative mode electrospray (CID) of the 2-h post-administration urine extract.

both well suited to the detection and confirmation of NSAIDs extracted from equine urine.

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