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Thermospray and particle beam liquid chromatographic–mass spectrometric analysis of coumarin anticoagulants

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ABSTRACT

Positive ion mass spectra were obtained from several coumarin oral anticoagulants (phenprocoumon, warfarin, acenocoumarol and dicoumarol) and derivatives by liquid chromatography–thermospray mass spectrometry (LC–TSP-MS) and liquid chromatography–electron impact mass spectrometry (LC–EI-MS) to assess the use of LC–MS methods for the determination of these compounds in biological materials. LC–TSP mass spectra showed a single $[M+1]^+$ ion with no fragmentation; LC–EI mass spectra showed fragment ions which were similar in mass and relative intensities to those obtained by conventional EI-MS. These data should serve as a basis for the development of LC–MS methods for the qualitative and quantitative analysis of coumarin anticoagulants in biological samples. LC–TSP-MS was applied to the determination of phenprocoumon in a plasma extract from an anticoagulated patient.

INTRODUCTION

Coumarin-type anticoagulants are wideley used therapeutically for the management and prophylaxis of thromboembolic states and as rodenticides [1]. In Europe, phenprocoumon (PH) (Table I; **1**) is currently used clinically and its elimination and metabolism in humans have recently been elucidated [2–5]. Methods for the determination of phenprocoumon and its metabolites in plasma, urine and bile samples have been developed using high-performance liquid chromatography (HPLC) [2–5] and gas chromatography–mass spectrometry with electron impact (EI) ionization (GC–EI-MS) after methylation of the extracts [3–8].

PH metabolites (Table I; **4–7**) in urine cannot be determined by HPLC with UV or fluorimetric detection owing to interferences [3]. GC–EI-MS of the methylated derivatives has been used [3,6,8] and this method is sensitive and selective; however, it has several disadvantages: extracts have to be derivatized prior to GC–MS analysis [3,6–8]; the methylation yields a mixture of isomers

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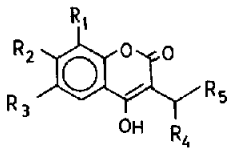
TABLE I

STRUCTURES OF COUMARIN ANTICOAGULANTS AND DERIVATIVES



No.	Compound	R_1	R_2	R_3	R_4	R_5
1	Phenprocoumon	H	H	H	C_2H_5	C_6H_5
2	Phenprocoumon-d_5	H	H	H	C_2H_5	$C_6^2H_5$
3	*p*-Chlorophenprocoumon	H	H	H	C_2H_5	C_6H_4Cl
4	6-Hydroxyphenprocoumon	H	H	OH	C_2H_5	C_6H_5
5	7-Hydroxyphenprocoumon	H	OH	H	C_2H_5	C_6H_5
6	8-Hydroxyphenprocoumon	OH	H	H	C_2H_5	C_6H_5
7	4'-Hydroxyphenprocoumon	H	H	H	C_2H_5	C_6H_4OH
8	6-Methoxyphenprocoumon	H	H	OCH_3	C_2H_5	C_6H_5
9	7-Methoxyphenprocoumon	H	OCH_3	H	C_2H_5	C_6H_5
10	8-Methoxyphenprocoumon	OCH_3	H	H	C_2H_5	C_6H_5
11	Warfarin	H	H	H	CH_2COCH_3	C_6H_5
12	*p*-Chlorowarfarin	H	H	H	CH_2COCH_3	C_6G_4Cl
13	Acenocoumarol	H	H	H	CH_2COCH_3	$C_6H_4NO_2$
14	Dicumarol	H	H	H	H	3-(4'-Hydroxycoumarin)

(with 2-O-methylchromone and 4-O-methylcoumarin structures) for each compound [3–8], hence this mixture must be well separated by the GC capillary column for quantification with selected ion monitoring (SIM); and the whole procedure of extraction, derivatization and GC–MS analysis is time consuming.

In recent years, several liquid chromatography–mass spectrometry (LC–MS) coupling systems and ionization methods have been described [9,10]. Thermospray (TSP) [11] ionization in particular has been applied to a variety of analytical applications in the biomedical field [10,12,13]. Lately, on-line LC–MS interfaces with EI ionization have become commercially available, using monodisperse aerosol generation (MAGIC) [14] and gas-phase diffusion separator interfaces [15]; these methods are also known as particle beam (PB) LC–MS methods and on-line LC–EI mass spectra can be obtained.

The aim of this work was to assess the use of LC–MS methods for the determination of phenprocoumon, its metabolites and other anticoagulants in biological samples. The LC–mass spectra of the pure compounds (Table I; **1–14**) were measured using TSP and EI ionization techniques. Phenprocoumon was determined in a plasma extract obtained from an anticoagulated patient by LC–TSP–MS.

EXPERIMENTAL

Chemicals

Phenprocoumon and derivatives (Table I; **1–10**) were synthesized according to Heimark *et al.* [16]. Warfarin and *p*-chlorowarfarin were obtained from Sigma (Deisenhofen, F.R.G.) and dicoumarol from Aldrich (Steinheim, F.R.G.); acenocoumarol was a gift from Ciba-Geigy (Wehr, F.R.G.) and *p*-chlorophenprocoumon was obtained from Hoffman-LaRoche (Grenzach, F.R.G.). The purity of the coumarin anticoagulants was established by HPLC. Methanol, acetonitrile and water were of HPLC quality. All other reagents were of analytical-reagent grade.

LC–TSP-MS

Positive ion mass spectra were obtained using (a) a Hewlett-Packard (Bad Nauheim, F.R.G.) Model 5986A quadrupole mass spectrometer equipped with a Vestec Model 721A Thermospray LC–MS interface (Interion, Manchester, U.K.), with data acquisition and editing using a Model 7994A workstation (Hewlett-Packard), and (b) a Vestec Model 201A dedicated LC–MS system, with data acquisition and editing using a Univent Vector 1 data system (Teknivent, Maryland Heights, MO, U.S.A.).

TSP mass spectra were obtained using pure thermospray and discharge-aided ionization. Typical LC–MS parameters were as follows: temperatures, block 230°C, tip heater 250°C and vaporizer tip 218°C; electron multiplier, operated at 1800 – 2400 eV; mass spectral data obtained by scanning between m/z 200 and 450.

LC–EI-MS

Spectra were obtained with a Vestec Model 201A LC–MS dedicated system coupled to an advanced particle beam interface and a combined EI–thermospray source. Particle beam interface temperatures were, vapour tip 130°C, spray chamber 70°C, membrane separator 40°C and momentum separator 120°C. EI temperatures were, source 230°C and lens 120°C. The filament was operated at 70 eV and 300 μA and the electron multiplier at 2400 eV. Positive ion mass spectra were obtained by scanning between m/z 90 and 450.

Direct insertion EI-MS

Spectra with a direct introduction probe were obtained as described previously [7].

Liquid chromatography

The Hewlett-Packard Vestec interface TSP-MS system used a Model 510 HPLC pump (Millipore–Waters, Eschborn, F.R.G.) attached to a Nucleosil C_{18} (5 μm) HPLC column (125 mm × 4 mm I.D.) (Macherey, Nagel & Co., Düren,

F.R.G.) and a Rheodyne Model 7125 injector. A three-way valve (Model EQ-90; Valco Instruments, Houston, TX, U.S.A.) and an on-line filter were used before the capillary leading to the TSP inlet. The Vestec dedicated LC–MS system used a Philips Model 4100 HPLC pump and a Philips Model C6W injector.

Mixtures of acetonitrile (or methanol) and 0.1 *M* ammonium acetate (1:1) were used as the eluent (1 ml/min) and the samples (in methanol; 100 ng–1 μg) were analysed by flow injection via the loop injector.

Determination of phenprocoumon in human plasma

Plasma samples (0.5 ml) from a patient suspected of phenprocoumon overdose were extracted, evaporated, dissolved in methanol (100 μl) and analysed by reversed-phase HPLC with photometric detection as described previously [2,3]. *p*-Chlorophenprocoumon (**3**) was used as an internal standard; aliquots of the extract (20 μl) were injected into the LC–TSP-MS interface as described before with the same LC parameters, (see above). Selected ions were monitored at *m/z* 281 and 315. Urine samples after enzymatic hydrolysis [2] were similarly extracted and analysed by LC–MS. Quantification was achieved by comparing the ratio of the integrated areas of the *m/z* 281 and 315 signals with those of calibration samples.

RESULTS

LC–TSP-MS

Analyses of compounds **1–14** (Table I) were performed with LC–TSP-MS systems using flow injection. TSP and TSP discharge-aided ionization with positive ion detection were used with ammonium acetate as modifier in the mobile phase. An intense $[M + 1]^+$ ion was detected for each compound with scarcely any fragmentation in the range *m/z* 200–450 (Table II) under the conditions described (see Experimental). The anticoagulants phenprocoumon (**1**), warfarin (**11**), acenocoumarol (**13**) and dicumarol (**14**) showed base peaks at *m/z* 280, 309, 354 and 337 respectively. The different monohydroxy- (**4–7**) and methoxyphenprocoumon isomers (**8–10**) ionized giving the same base peaks of *m/z* 297 and 311, respectively (Table II). Discharge-assisted TSP spectra showed higher ion intensities than TSP alone. Some compounds showed a further low-intensity ion (less than 1% of the base peak) at *m/z* $[M + 18]^+$, probably resulting from addition of an ammonium ion to the molecule.

LC–EI-MS

Compounds **1, 3–11** and **13** were analysed with positive EI ionization after separation of the mobile phase with an advanced particle beam interface using a combined EI–TSP source [15]. The spectra showed characteristic fragments for the compounds investigated (Table II). Fig. 1 shows the LC–EI-MS of (a) phenprocoumon, (b) 7-hydroxyphenprocoumon, (c) warfarin and (d) acenocoumarol.

TABLE II

LC–MS FRAGMENTS OF COUMARIN ANTICOAGULANTS AND DERIVATIVES

No.	Compound	Molecular weight	*m/z* (*I*)[a]	
			TSP	EI
1	Phenprocoumon	280	281(100)	280(30),265(16),251(100),223(12), 189(30),121(52),91(26)
2	Phenprocoumon-d_5	285	286(100)	
3	*p*-Chlorophenprocoumon	314[b]	315(100),317(42)	314[b](34),299[b](12),285[b](100), 257[b](11),189(45),125[b](88),121(84)
4	6-Hydroxyphenprocoumon	296	297(100)	296(15),281(13),267(69),239(7), 174(5),137(100)
5	7-Hydroxyphenprocoumon	296	297(100)	296(6),281(3),267(41),239(7), 174(19),137(100)
6	8-Hydroxyphenprocoumon	296	297(100)	296(15),281(10),267(59),239(7), 205(30),174(3),137(100)
7	4′-Hydroxyphenprocoumon	296	297(100)	296(16),281(2),267(100),189(6), 121(68),107(48)
8	6-Methoxyphenprocoumon	310	311(100)	310(36),295(19),281(100),219(18) 175(20),150(32)
9	7-Methoxyphenprocoumon	310	311(100)	310(11),295(3),281(69.5),182(39), 151(100)
10	8-Methoxyphenprocoumon	310	311(100)	310(7),295(6),281(30),219(18), 192(26),150(58),122(100)
11	Warfarin	308	309(100)	308(10),265(100),251(8),249(8), 187(24),121(3)
12	*p*-Chlorowarfarin	342[b]	343(100),345(25)	
13	Acenocoumarol	353	354(100)	353(3),310(50),295(2),281(15), 176(46),162(42),121(100)
14	Dicumarol	336	337(100)	

[a] *I* = Ion intensity (%) (base peak = 100), in parentheses.
[b] Molecular weight of lower mass isotope.

The mass spectra of the different monohydroxy- and methoxyphenprocoumons isomers were characteristic for each isomer (Table II).

Determination of phenprocoumon in human plasma and urine

Fig. 2a shows the mass chromatograms of the extract from a pretreatment plasma sample after addition of 1 μg/ml of PH (**1**) and *p*-chloro-PH (**3**). The ions monitored at *m/z* 281 and 315 correspond to PH and *p*-chloro-PH, respectively. Fig. 2b shows the corresponding recordings obtained from a patient suspected of PH overdose. Urine samples were also analysed by LC–MS (not shown). Concentration data from the LC–MS analysis were comparable to those obtained by conventional HPLC with photometric detection [2,3]. The precision (relative

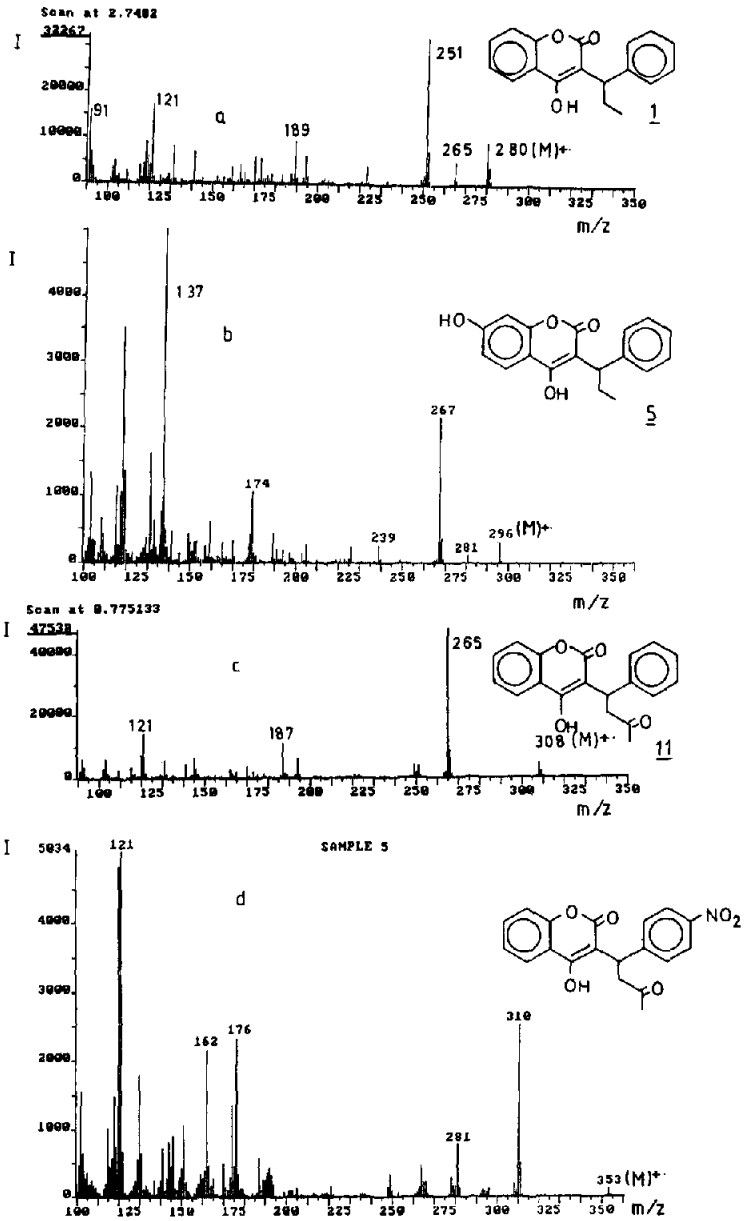


Fig. 1. LC–EI mass spectra of (a) phenprocoumon, (b) 7-hydroxyphenprocoumon, (c) warfarin and (d) acenocoumarin.

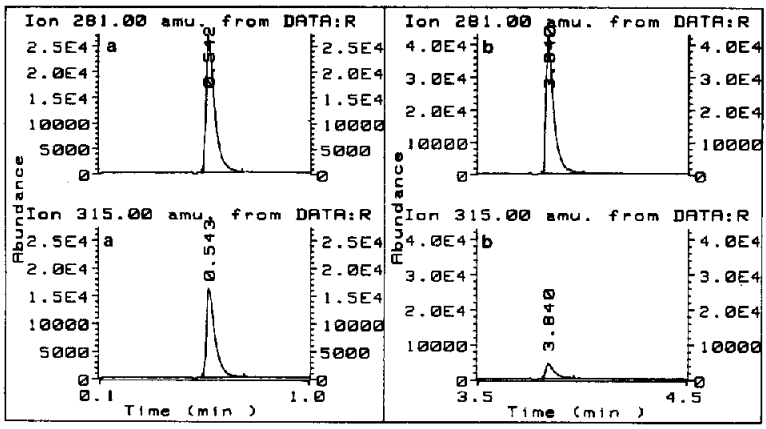


Fig. 2. LC–MS determination with selected ion monitoring of phenprocoumon (PH) and *p*-chlorophenprocoumon (p-Cl-PH) in plasma extracts. (a) Pretreatment plasma after addition of 1 μg/ml of pure compounds; (b) patients's plasma, PH concentration 4.8 μg/ml. The extracts were analysed by flow injection. The ions at *m/z* 281 and 315 correspond to PH and *p*-Cl-PH, respectively.

standard deviation) was 1.9% ($n = 5$), the accuracy was 3% at a PH plasma concentration of 50 ng/ml and the limit of detection of PH was 5 ng/ml; the calibration graph was linear in the range 0–5 μg/ml.

DISCUSSION

Of the several available on-line LC–MS methods, TSP and EI (also referred as particle beam or MAGIC) ionization have been applied to the analysis of a wide variety of compounds [9–15]. LC–TSP positive ion mass spectra of the oral anticoagulants and derivatives analysed (Table I; **1–14**) were obtained after flow injection and TSP or TSP discharge-aided ionization. In all instances they showed a single $[M + 1]^+$ parent ion with no fragmentation (Table II). The anticoagulant drugs phenprocoumon, warfarin, acenocoumarol and dicumarol can be identified by their molecular ions $[M + 1]^+$ as none of them have the same molecular weight (Table II). The monohydroxy-PH isomers (Table I; **4–7**) showed the same $[M + 1]^+$ ion at *m/z* 297 (but the methoxy isomers (**8–10**) showed molecular ions at *m/z* 311) and cannot be differentiated by their TSP mass spectra alone. HPLC separation would be necessary for their characterization as they have different retention times [3].

The LC–EI positive ion mass spectra of several anticoagulants showed characteristic fragmentations which are similar to those obtained with conventional direct introduction probe EI–MS. Phenprocoumon, for example, shows the same molecular ion at *m/z* 280 ($M^{+\bullet}$) and fragments at *m/z* 265, 251, 121 and 91 and also similar relative ion intensities (Table II, Fig. 1) as previously reported for

direct inlet EI–MS [7]. Warfarin, acenocoumarol and other coumarin derivatives (Table II, Fig. 1) showed similar LC–EI-MS fragments to those in reported EI mass spectra [17,18] giving library-searchable spectra. It is probable that these compounds fragment following the same pathways as demonstrated for direct inlet EI–MS [7,17].

The determination of PH in plasma and urine samples from a patient suspected of PH overdose (Fig. 3) was carried out by LC–TSP-MS. Preliminary results showed that this method is an alternative to HPLC with photometric detection for the rapid, sensitive and selective assay of PH in biological extracts.

These data may serve as a basis for the development of assays for the determination of PH, its metabolites and other anticoagulants in biological samples.

REFERENCES

1 J. X. de Vries, R. Zimmermann and J. Harenberg, in K. Breddin, D. Gross and H. Rieger (Editors), *Angiologie und Hämostasiologie. 18.–20. Kitzbühler Symposien,* Fischer, Stuttgart, New York, 1988, p. 272.
2 J. X. de Vries, J. Harenberg, E. Walter, R. Zimmermann and M. Simon, *J. Chromatogr.*, 231 (1982) 83.
3 J. X. de Vries, M. Simon, R. Zimmermann and J. Harenberg, *J. Chromatogr.*, 338 (1985) 325.
4 J. X. de Vries, R. Zimmermann and J. Harenberg, *Eur. J. Clin. Pharmacol.*, 29 (1986) 591.
5 J. X. de Vries, R. Raedsch, U. Völker, I. Walter-Sack and E. Weber, *Eur. J. Clin. Pharmacol.*, 35 (1988) 433.
6 S. Toon, L. D. Heimark, W. F. Trager and R. A. O'Reilly, *J. Pharm. Sci.*, 74 (1985) 1037.
7 J. X. de Vries and D. Krauss, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 224.
8 L. D. Heimark and W. F. Trager, *Biomed. Mass Spectrom.*, 12 (1985) 67.
9 D. E. Games, *Adv. Chromatogr.*, 21 (1981) 1.
10 K. B. Tomer and C. E. Parker, *J. Chromatogr.*, 492 (1989) 189.
11 M. L. Vestal, *Science*, 226 (1984) 275.
12 I. G. Beattie and T. J. A. Blake, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 872.
13 W. M. Draper, F. R. Brown, R. Bethem and M. J. Mille, *Biomed. Environ. Mass Spectrom.*, 18 (1989) 767.
14 P. C. Winkler, D. D. Perkins, W. K. Williams and R. F. Browner, *Anal. Chem.*, 60 (1988) 489.
15 M. I. Vestal, D. Winn, C. H. Vestal and J. G. Wilkes, presented at the *37th ASMS Conference on Mass Spectrometry and Allied Topics, Miami Beach, FL, May 21–26, 1989.*
16 L. D. Heimark, S. Toon, L. K. Low, D. C. Swinney and W. F. Trager, *J. Labelled Compd. Radiopharm.*, 23 (1986) 137.
17 W. F. Trager, R. J. Lewis and W. A. Garland, *J. Med. Chem.*, 13 (1970) 1196.
18 J. X. de Vries, unpublished results, 1990.