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Detection of 4-hydroxycoumarin anticoagulants and their metabolites in urine as part of a systematic toxicological analysis procedure for acidic drugs and poisons by gas chromatography–mass spectrometry after extractive methylation[1,2]

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Abstract

A gas chromatography–mass spectrometry (GC–MS) procedure was developed for the detection of 4-hydroxycoumarin anticoagulants and their metabolites in urine as part of a systematic toxicological analysis procedure for acidic drugs and poisons after extractive methylation. The part of the phase-transfer catalyst remaining in the organic phase was removed by solid-phase extraction on a diol phase. The compounds were separated by capillary GC and identified by computerized MS in the full scan mode. Using mass chromatography with the ions *m/z* 291, 294, 295, 309, 313, 322, 324, 336, 343 and 354, the possible presence of 4-hydroxycoumarin anticoagulants and/or their metabolites could be indicated. The identity of positive signals in such mass chromatograms was confirmed by comparison of the peaks underlying full mass spectra with the reference spectra recorded during this study. This method allowed the detection of therapeutic concentrations of phenprocoumon and warfarin in human urine samples. In absence of human urine, acenocoumarol, coumachlor, coumatetrayl, pyranocoumarin (cyclocumarol) could be detected only in rat urine.

Keywords: Extractive methylation; 4-Hydroxycoumarin anticoagulants; Acidic drugs

1. Introduction

In clinical toxicology, the diagnosis or the definite exclusion of an acute or chronic intoxication is an important task. Before quantification of the drugs or poisons, a comprehensive screening is necessary to find out which compounds were taken. Urine is the sample of choice for such a screening, because the concentrations of analytes are higher than in plasma. A systematic toxicological analysis procedure (STA) was previously developed for simultaneous detection of most of the basic and neutral drugs in urine using GC–MS after acid hydrolysis, extraction and acetylation [3]. For the detection of acidic drugs like anticoagulants, ACE inhibitors, calcium antagonists,

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[1]Part of these results was reported at the 34th International TIAFT Meeting, Interlaken, August 11–15, 1996 [1]. They are part of the PhD thesis of J.W. Arlt [2].

[2]Dedicated to Prof. Dr. Karl-Artur Kovar, Tübingen (Germany), at the occasion of his 60th birthday.

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diuretics or non-steroidal anti-inflammatory drugs, a further GC–MS procedure has been developed using extractive methylation [1,4–6]. Extractive alkylation has proved to be a versatile method for sample preparation from biomatrices [4–12].

In the following, a GC–MS procedure is described for the detection of the 4-hydroxycoumarin anticoagulants and their metabolites. These compounds are used as therapeutics or as rodenticides of the so-called first generation. Such a method is necessary for the differential diagnosis of unclear coagulopathies, which may occur after ingestion of such coumarins. If necessary for toxicological interpretation, the identified anticoagulants can be quantified in plasma by HPLC [13,14].

2. Experimental

2.1. Chemicals and reagents

The following reference drug samples were kindly supplied by the manufacturers: acenocoumarol (Ciba–Geigy, Wehr, Germany), coumatetralyl (Bayer, Leverkusen, Germany), phenprocoumon (Hoffmann–La Roche, Grenzach-Whylen, Germany), warfarin (Boehringer Ingelheim, GB). Coumachlor and pyranocoumarin (cyclocumarol) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Diethyl ether was obtained from Asid Bonz (Boeblingen, Germany), ethyl acetate, methanol, methyl iodide and toluene were obtained from Merck (Darmstadt, Germany) and tetrahexylammonium hydrogensulfate from Fluka (Neu-Ulm, Germany). All these chemicals were of analytical grade, with the exception of methyl iodide (for synthesis). The solid-phase extraction (SPE) columns Isolute Diol, non end-capped, 500 mg/10 ml were obtained from ICT (Bad Homburg, Germany).

2.2. Urine samples

Authentic human urine samples were only available from patients treated with phenprocoumon or warfarin. Urine from rats was used for the studies on the medicament acenocoumarol or the rodenticides, coumachlor, coumatetralyl and pyranocoumarin (cyclocumarol). The rats were pretreated with vitamin K_1 to prevent coagulopathy. For recording of the reference data of the metabolites, 10–20 mg kg^{-1} body mass of the corresponding anticoagulant were administered in aqueous suspension by gastric intubation to female wistar rats (Ch. River, Sulzfleck, Germany). Urine was collected separated from the faeces. All urine samples were stored at −20°C before analysis.

2.3. Apparatus

The samples were incubated under agitation with a multifix shaker S 300 (Schwinherr, Schwäbisch Gmünd, Germany) in a water bath. A Vac-Master V-10 (ICT) was used for manual SPE.

The extracts were analysed using a Hewlett–Packard (HP, Waldbronn, Germany) Series 5890 gas chromatograph combined with a HP MSD Series 5970 mass spectrometer and a HP Chemstation Series G1034C version C03.00. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m×0.2 mm I.D., cross-linked methyl silicone, 0.33 μm film thickness); column temperature, programmed from 100°C to 310°C at 30°C min^{-1}, initial time 3 min, final time 8 min; injection port temperature, 280°C; carrier gas, helium; flow-rate, 1 ml min^{-1}. The MS conditions were as follows: full scan mode; ionisation energy, 70 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C.

2.4. Extractive methylation procedure

A 2 ml portion of urine was mixed in a centrifuge tube with 2 ml of the phase-transfer reagent consisting of 0.02 *M* tetrahexylammonium (THA) hydrogensulfate in 1 *M* sodium phosphate buffer adjusted to pH 12 with sodium hydroxide. After addition of 6 ml of 1 *M* methyl iodide in toluene, the closed tube was shaken in a 50°C water bath for 30 min. After phase separation by centrifugation at 1500×*g* for 3 min, the organic phase, containing the analytes and

THA salts, was transfered to the SPE column, conditioned as follows: 10 ml of methanol at a flow-rate of 10 ml min^{-1}, drying the column under vacuum for 15 s, 10 ml of toluene at a flow-rate of 10 ml min^{-1}. The organic phase was rinsed through the sorbent at a flow-rate of 3 ml min^{-1} to adsorb the THA salts. The part of the analytes also adsorbed on the sorbent was selectively eluted with 5 ml of diethyl ether–ethyl acetate (92.5:7.5, v/v) at a flow-rate of 3 ml min^{-1}. The combined eluates were carefully evaporated to dryness at 60°C (vacuum 300–500 mbar). The residue was dissolved in 50 μl of ethyl acetate and an 1 μl aliquot of this extract was injected into the GC–MS.

2.5. GC–MS procedure

During the gas chromatographic separation, electron-impact full mass spectra was recorded and stored. To screen for the presence of the anticoagulants and their metabolites, mass chromatography was used with the ions m/z 291, 294, 295, 309, 313, 322, 324, 336, 343 and 354. These ions were selected from the mass spectra recorded during this study. Generation of the mass chromatograms could be started by clicking the corresponding pull down menu item (cf. Fig. 2) which executes the user defined macros [15] (the macros can be obtained from the authors: e-mail: pthmau@med-rz.uni-sb.de). The identity of the peaks in the mass chromatograms was confirmed by visual (Fig. 1) or computerized comparison [16] of the full mass spectra, underlying the peaks, with reference spectra recorded during this study (Fig. 1).

3. Results and discussion

3.1. Sample preparation

Authentic human urine samples were available only from patients treated with phenprocoumon and warfarin. For the studies on the medicament acenocoumarol and on the rodenticides human urine samples were not available. In these cases urine samples from rats were used.

As we have seen during our studies, cleavage of conjugates was not necessary for toxicological analysis, because the conjugation rate was relatively low. Derivatization, however, was essential for sensitive detection of these polar drugs (vinylogous carboxylic acids) and their metabolites (aniline/anilide derivatives or phenols). Only alcoholic hydroxy groups could not be methylated due to their lower nucleophilicity, but this fact did not markedly influence the sensitivity. The anticoagulants and their metabolites were isolated from urine and derivatized in one step by extractive methylation. The acidic compounds were extracted at pH 12 as ion pairs with the phase-transfer catalyst into the organic phase. Reaching the organic phase, the phase-transfer catalyst could easily be solvated due to its lipophilic hexyl groups. The poor solvation of the anionic analytes lead to a high reactivity against the methylation reagent methyl iodide. Part of the phase-transfer catalyst could also reach the organic phase as an ion pair with the iodide anion formed during the methylation reaction or with anions of the urine matrix. Part of these THA salts remained in the organic phase. Therefore, they had to be removed for maintaining the GC column's separation power and to exclude interactions with analytes in the GC injection port. We tested several SPE sorbents and different eluents for efficient separation of THA salts from the analytes. The diol sorbent yielded best reproducibility and recovery under the described conditions. Further advantages of such SPE columns were that they could easily be handled, that they were commercially available and that they had not to be manually prepared as described by Lisi et al. [12]. The overall recovery of the extractive methylation procedure for the different drug classes to be screened was tested for one typical compound of each class. For the class of the anticoagulants, the mostly used anticoagulant phenprocoumon was selected. Its recovery was 68±4% (n=5). The relatively low recovery can be explained by the moderate nucleophilicity of the vinylogous carboxylic acid. Since the recovery is reproducible and the detection limit is sufficient, this disadvantage can be accepted taking into consideration that this method is part of a comprehensive screening procedure for different acidic compounds.

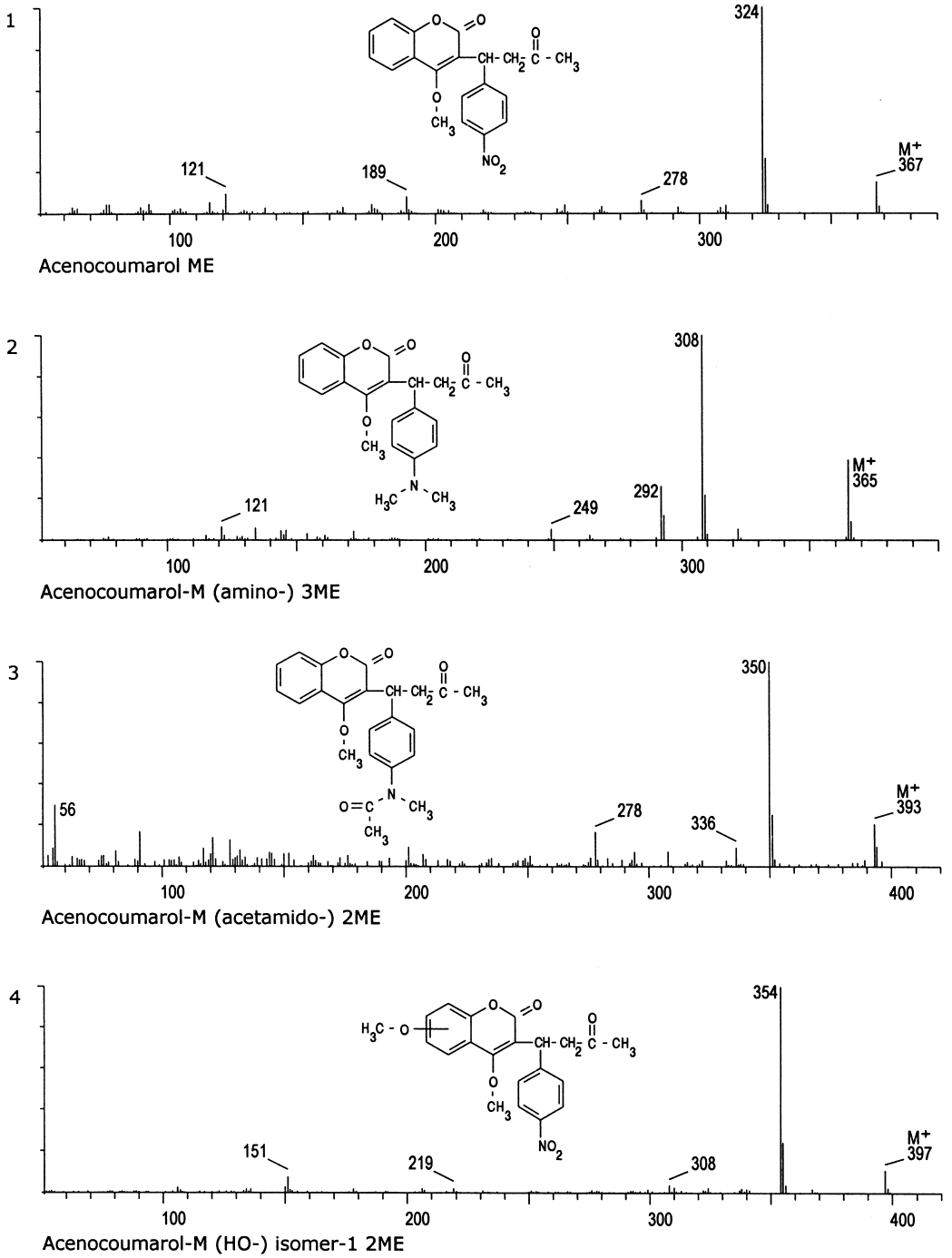


Fig. 1. Mass spectra and structures of the 4-hydroxycoumarins and their metabolites after methylation for precise identification (The abscissa represents the m/z value [u] and the ordinate the relative abundances of the fragment ions [%]).

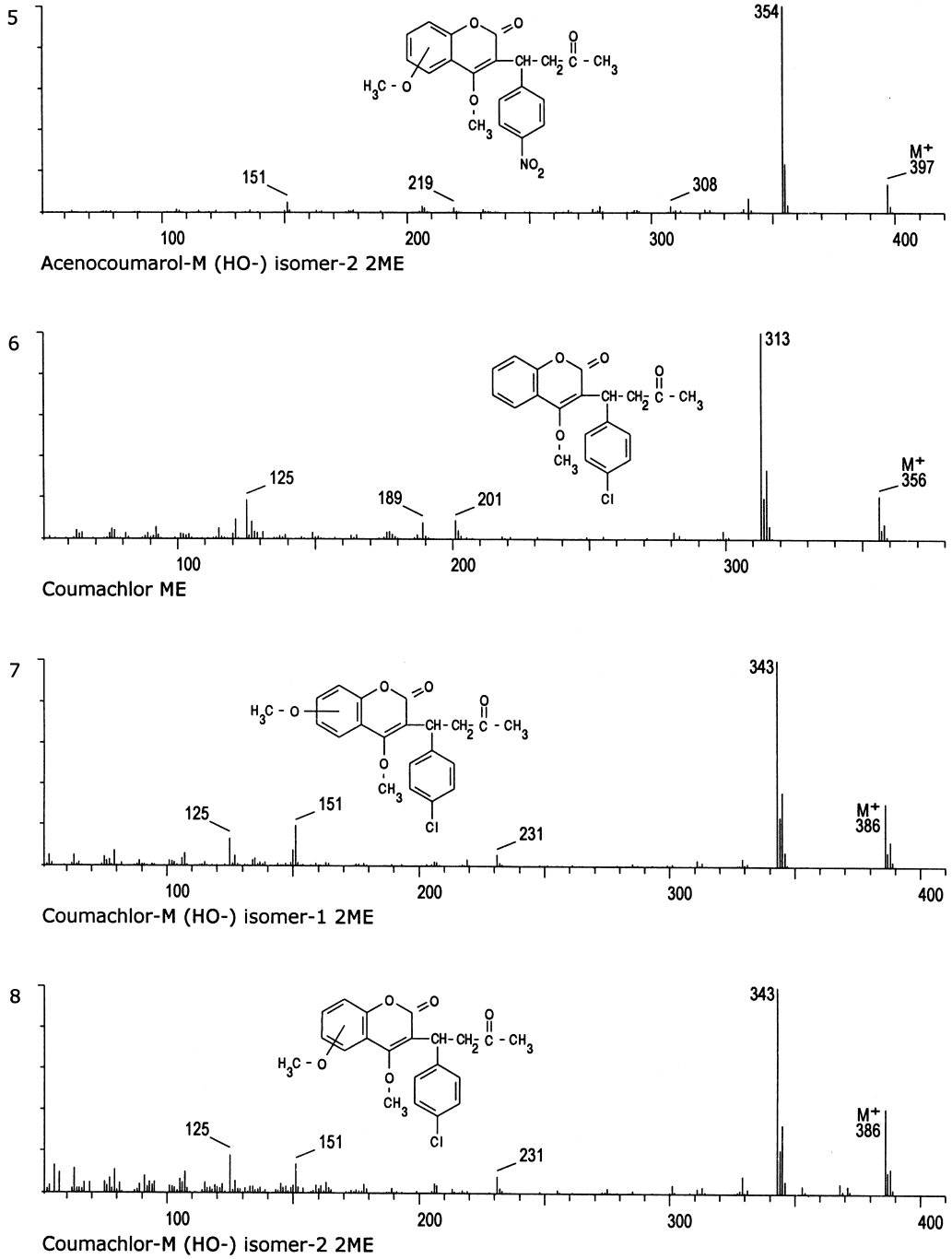
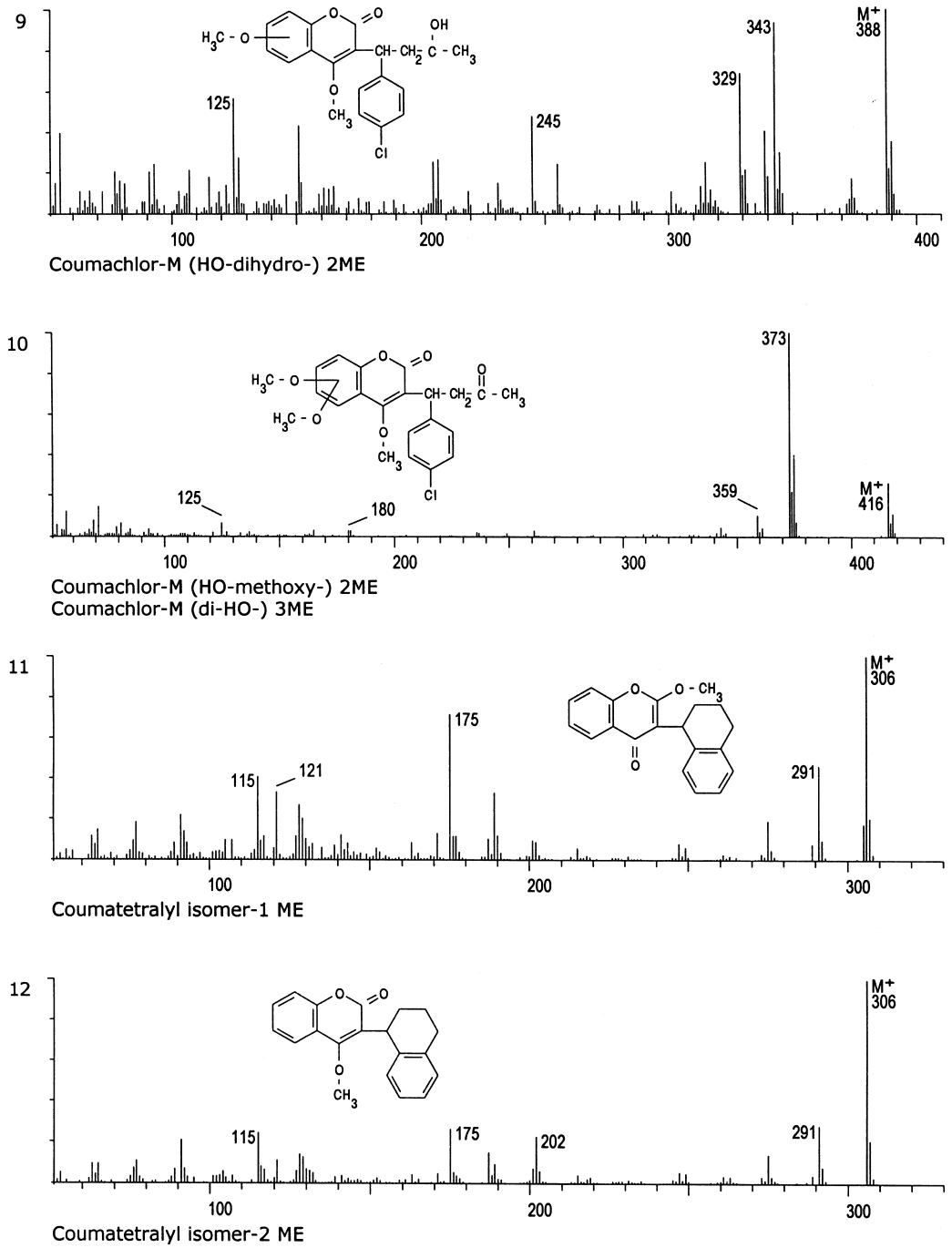


Fig. 1. (*continued*)



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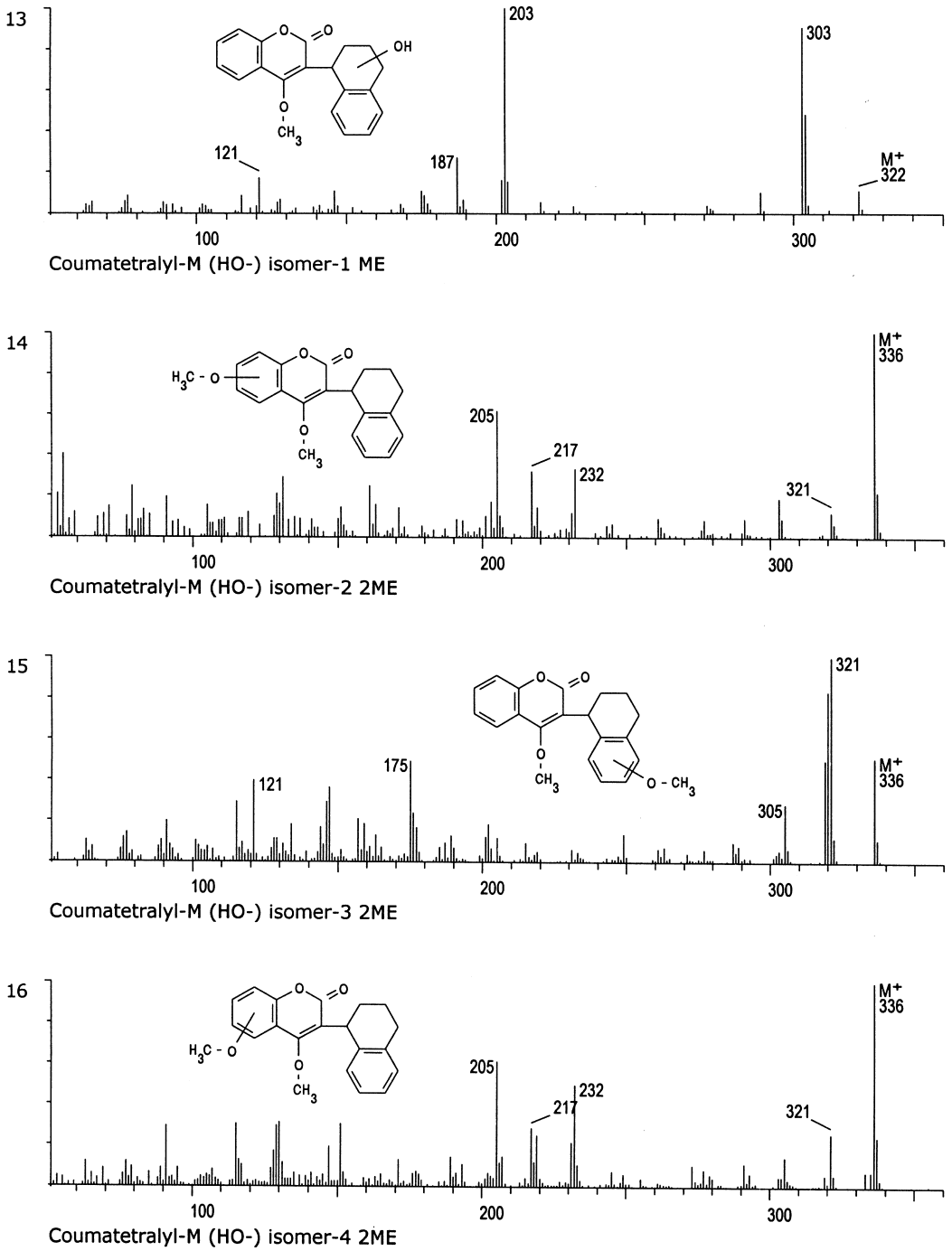

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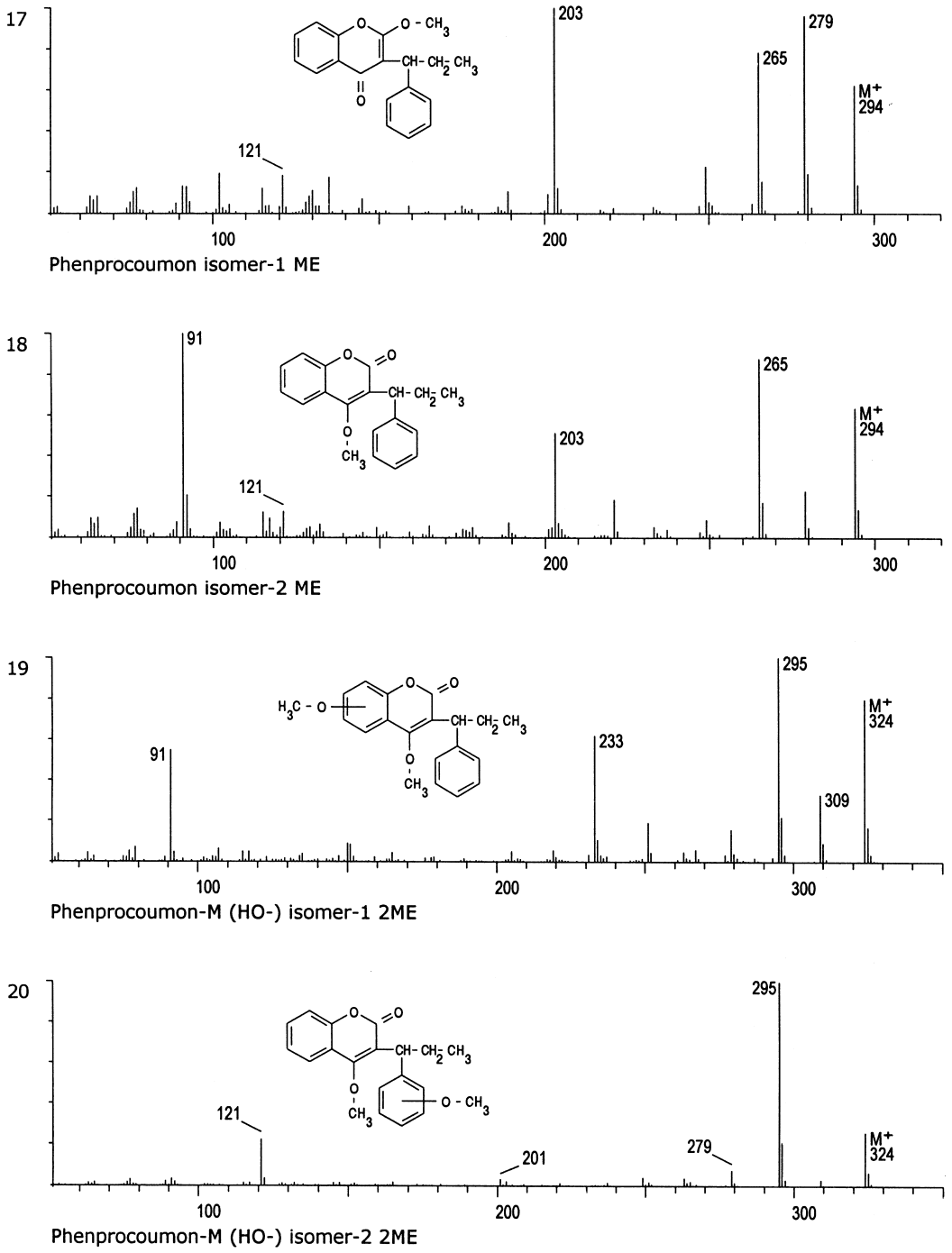

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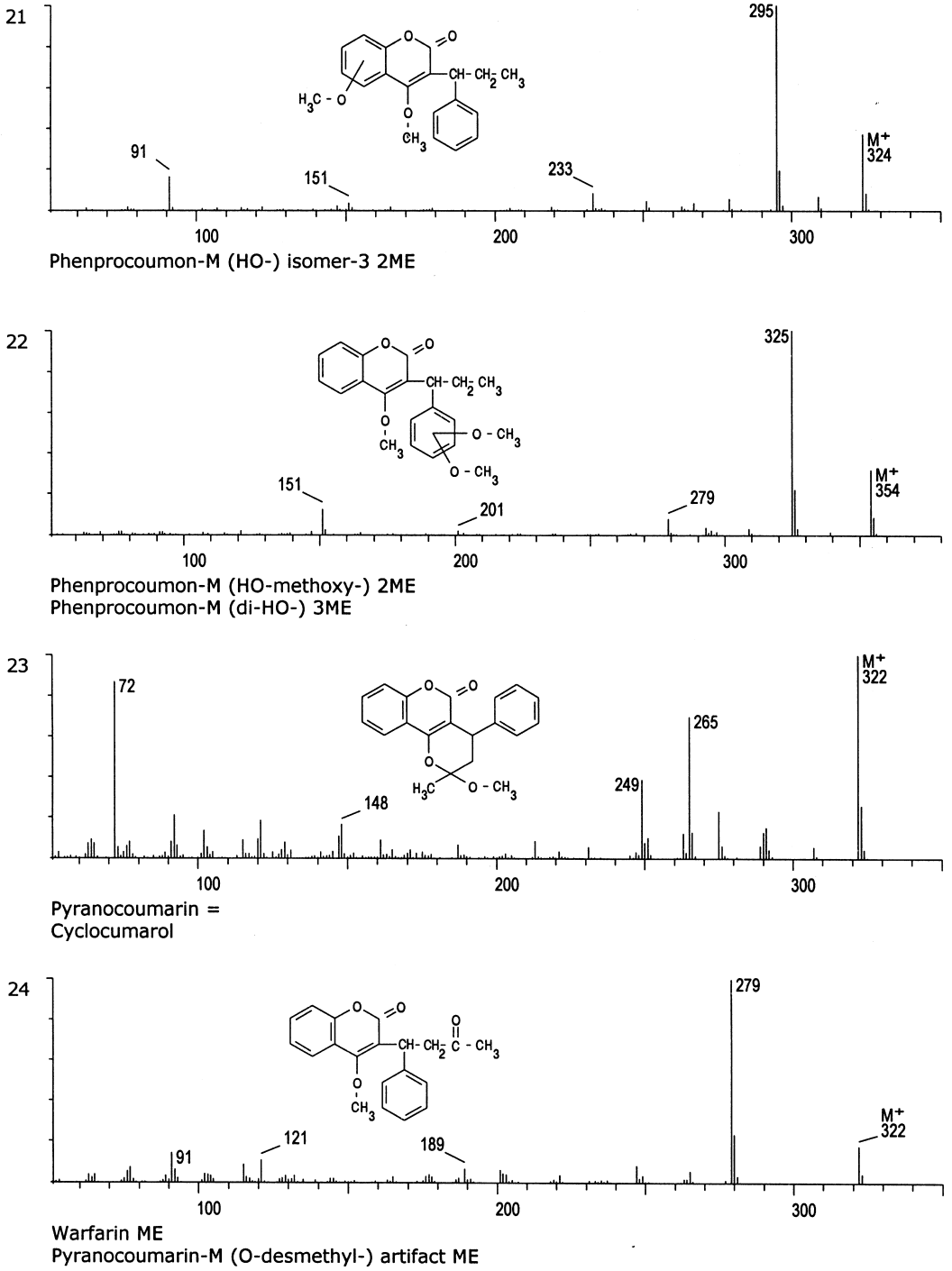
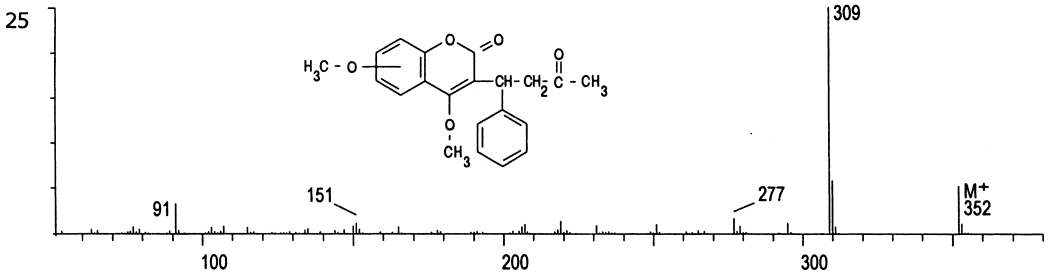
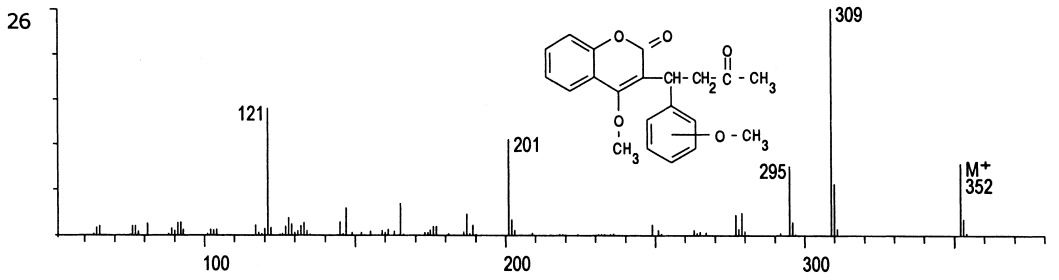


Fig. 1. (*continued*)



Warfarin-M (HO-) isomer-1 2ME
Pyranocoumarin-M (O-desmethyl-HO-) isomer-1 artifact 2ME



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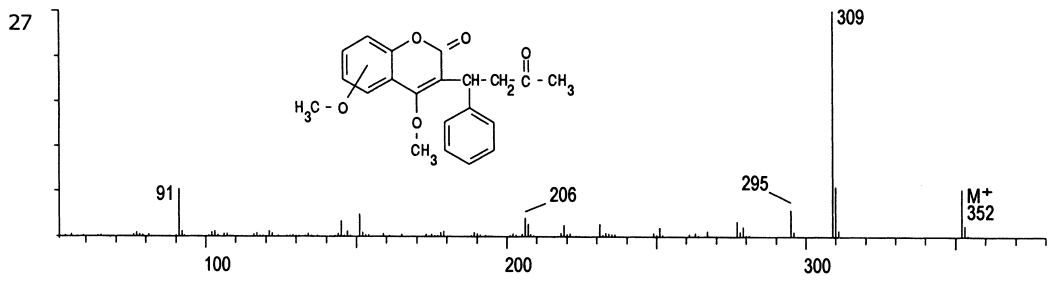
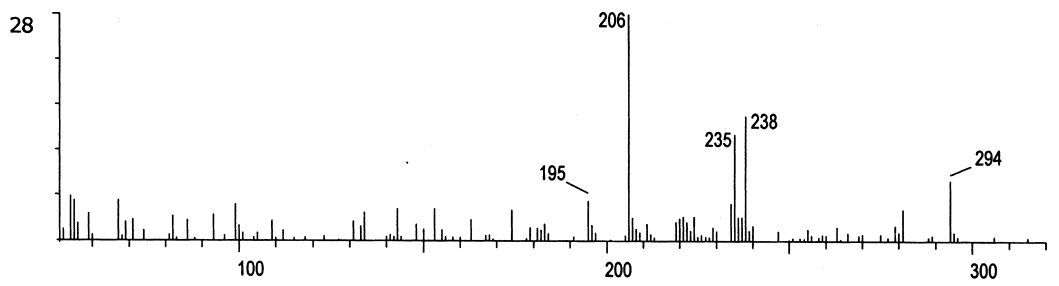
Pyranocoumarin-M (O-desmethyl-HO-) isomer-2 artifact 2ME



Warfarin-M (HO-) isomer-3 2ME
Pyranocoumarin-M (O-desmethyl-HO-) isomer-3 artifact 2ME



Endogenous biomolecule

Fig. 1. (*continued*)

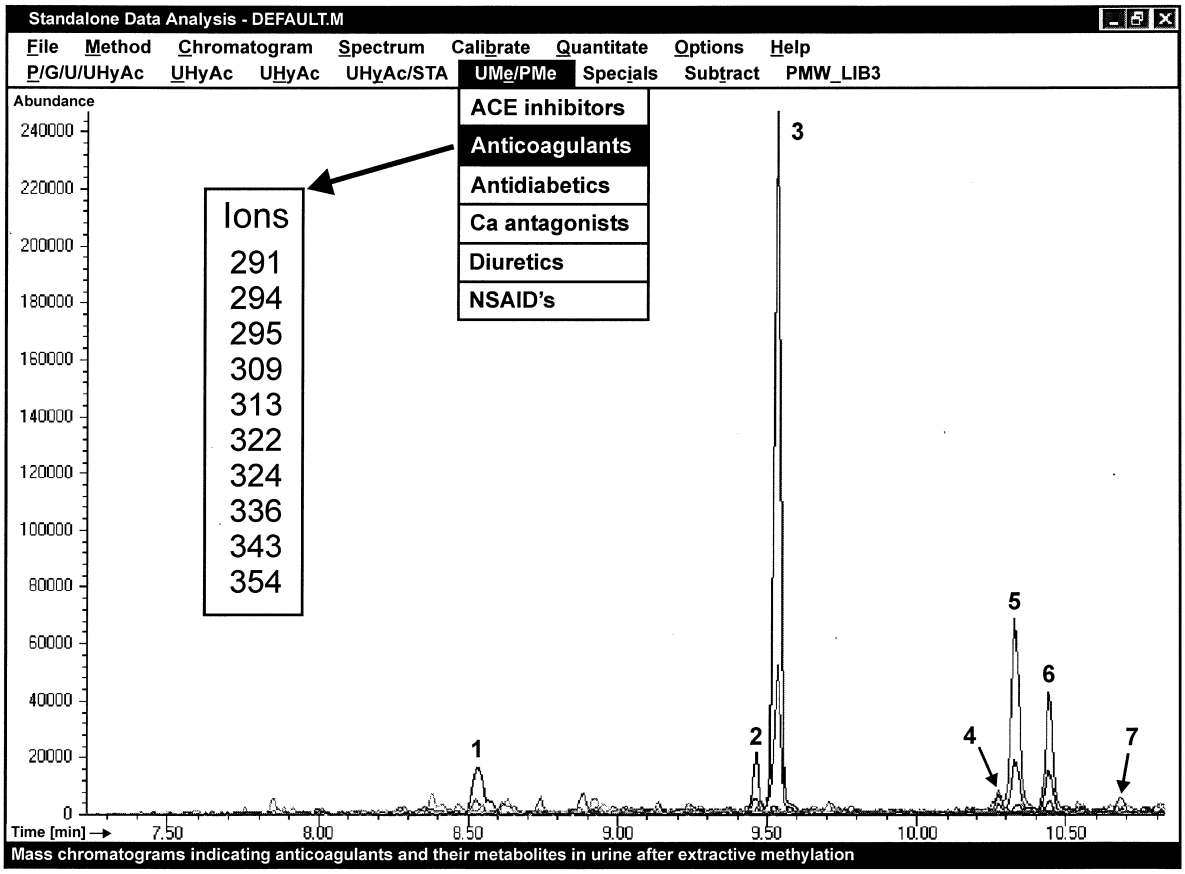


Fig. 2. Typical mass chromatograms with the ions *m*/*z* 291, 294, 295, 309, 313, 322, 324, 336, 343 and 354. They indicate the presence of phenprocoumon and its metabolites in a methylated extract of a urine sample taken from a patient after ingestion of a therapeutic dose (3 mg/day) of phenprocoumon. Peak 1 indicates an endogenous biomolecule (mass spectrum no. 28 in Fig. 1). Peaks 2–7 indicate the presence of phenprocoumon and its metabolites (mass spectra nos. 17–22 in Fig. 1). The merged chromatograms can be differentiated by their colours on a color screen.

3.2. Identification of metabolites

The urinary metabolites of the anticoagulants were identified by electron-impact (EI) MS after GC separation. The EI mass spectra of the metabolites were interpreted in correlation to that of the parent compounds according to the rules described by McLafferty and Turecek [17].

The EI mass spectra and the proposed structures of the anticoagulants and their metabolites after methylation are shown in Fig. 1 for precise identification via visual comparison. The formation of the shown isomers can result from metabolism (e.g. *p*- vs. *m*-hydroxylation or aliphatic vs. aromatic hydroxylation) or from derivatization due to tautomery. The isomers were numbered according to their retention time.

After methylation, the dihydroxy metabolites cannot be differentiated from the hydroxy methoxy metabolites. The *O*-demethyl metabolites of pyranocoumarin (cyclocumarol) were transformed to warfarin and its metabolites probably by cleavage of the cyclic semiketal. The GC and MS data of further metabolites detectable only in trace amounts will be included in the new edition of our handbook and library [16,18].

Table 1
List of the 4-hydroxycoumarins and their metabolites after methylation (ME) indicated by the given diagnostic ions

MS no. in Fig. 2	Compound	*m/z*										M^+	RI
		291	294	295	309	313	322	324	336	343	354		
1	Acenocoumarol ME							100				367	3035
2	M (amino-) 3ME				25		10					365	2985
3	M (acetamido-) 2ME								10			393	3265
4	M (HO-) isomer-1 2ME										100	397	3350
5	M (HO-) isomer-2 2ME										100	397	3500
6	Coumachlor ME					100						356	2770
7	M (HO-) isomer-1 2ME									100		386	2990
8	M (HO-) isomer-2 2ME									100		386	3035
9	M (HO-dihydro-) 2ME									90		388	3095
10	M (HO-methoxy-) 2ME									10		416	3195
11	Coumatetralyl isomer-1 ME	45										306	2655
12	Coumatetralyl isomer-2 ME	35										306	2690
13	M (HO-) isomer-1 ME						15					322	2910
14	M (HO-) isomer-2 2ME								100			336	2925
15	M (HO-) isomer-3 2ME								50			336	2935
16	M (HO-) isomer-4 2ME								100			336	2990
17	Phenprocoumon isomer-1 ME		75	15								294	2375
18	Phenprocoumon isomer-2 ME		75	15								294	2395
19	M (HO-) isomer-1 2ME			100	35			75				324	2655
20	M (HO-) isomer-2 2ME			100	5			25				324	2675
21	M (HO-) isomer-3 2ME			100	10			45				324	2705
22	M (HO-methoxy-) 2ME										30	354	2770
23	Pyranocoumarin (Cyclocumarol)						100					322	2670
24	M (O-desmethyl) artifact ME						20					322	2580
25	M (O-desmethyl-HO-) isomer-1 artifact 2ME			10	100							352	2810
26	M (O-desmethyl-HO-) isomer-2 artifact 2ME			30	100							352	2830
27	M (O-desmethyl-HO-) isomer-3 artifact 2ME			10	100							352	2870
24	Warfarin ME						20					322	2580
25	M (HO-) isomer-1 2ME			10	100							352	2810
26	M (HO-) isomer-2 2ME			30	100							352	2830
27	M (HO-) isomer-3 2ME			10	100							352	2870
28	Endogenous biomolecule		30	5									2140

Their relative abundances [%] are shown as well as the mass spectrum numbers (MS no.) in Fig. 2, the molecular masses (M^+) and the gas chromatographic retention indices (RI).

3.3. GC–MS analysis

For the detection of the anticoagulants and their metabolites, the full mass spectra recorded during temperature-programmed GC were evaluated using mass chromatography with the diagnostic ions *m/z* 291, 294, 295, 309, 313, 322, 324, 336, 343 and 354. In Table 1, the compounds which can be detected by these ions are listed. The metabolites and/or the isomers are listed in order of their retention indices. The relative abundances of the diagnostic ions in the corresponding mass spectrum are given as well as the molecular masses (M^+) and the gas chromatographic retention indices (RI). The RIs were recorded during GC–MS and calculated in correlation with the Kovats' indices [19] of the compounds of a standard solution of typical drugs which is measured daily for testing the GC–MS performance [20,21]. The reproducibility of retention indices measured on capillary columns was better using a mixture of drugs than that of the homologous hydrocarbons recommended by Kovats [19].

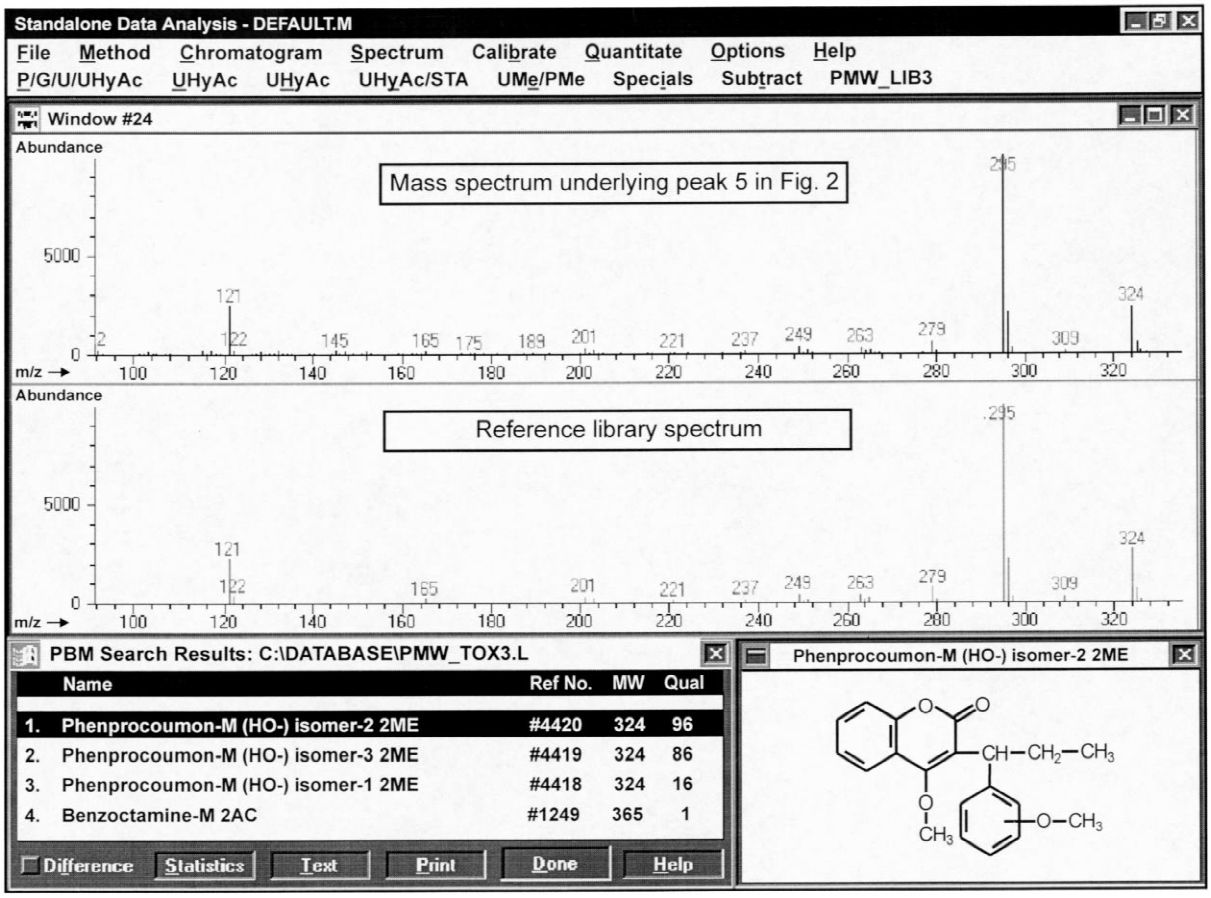


Fig. 3. Mass spectrum underlying the peak 5 in Fig. 2, the reference spectrum, the structure and the hit list found by computer library search.

Generation of the mass chromatograms could be started by clicking the corresponding pull down menu item which executes the user defined macros. Since further xenobiotics as well as endogenous biomolecules may also contain the diagnostic ions, the identity of an analyte must be confirmed by visual or computerized comparison of the peak underlying mass spectrum with the reference mass spectrum recorded during this study (Fig. 1 or [16,18]).

In Figs. 2 and 3 this procedure is illustrated for an authentic urine sample of a patient treated with a therapeutic dose of 3 mg/day of phenprocoumon. Fig. 2 shows the reconstructed mass chromatograms. Peak 1 indicates an endogenous biomolecule (mass spectrum no. 28 in Fig. 1). Peaks 2–7 indicate the presence of phenprocoumon and its metabolites (mass spectra nos. 17–22 in Fig. 1). Fig. 3 shows, as an example, the confirmation of the identity of peak no. 5 in Fig. 2 by computerized comparison of the underlying full mass spectrum (upper spectrum) with the reference spectrum (lower spectrum).

The drugs and their metabolites were sufficiently separated by GC. Most of the compounds could be differentiated by their mass spectra. However, some of the isomeric metabolites could only be differentiated via their retention indices.

Differentiation between pyranocoumarin (cyclocumarol) and warfarin was only possible by detecting the parent compound pyranocoumarin. As

already discussed in 3.2, the *O*-demethyl metabolites of pyranocoumarin were transformed to warfarin and its metabolites probably by cleavage of the cyclic semiketal. Interferences with other drugs or endogenous biomolecules indicated in the reconstructed mass chromatograms are improbable since in our experience these compounds have different gas chromatographic and/or mass spectral properties [16,18].

The sensitivity of the screening procedure was exemplified for phenprocoumon. The limit of detection was 25 ng ml^{-1} at a signal-to-noise ratio of 3 (S/N 3) empirically determined in the full scan mode. However, for urine analysis in clinical toxicology it is more important to know, whether the intake of a therapeutic dose of a drug can be detected. If this is possible, the intake of a toxic dose can be monitored all the more.

4. Conclusions

The presented procedure allowed the identification and differentiation of 4-hydroxycoumarin anticoagulants used as therapeutics or as rodenticides of the so-called first generation. The mostly used phenprocoumon and warfarin and their metabolites could be detected in urine of patients treated with therapeutic doses of these drugs. Acenocoumarol and the rodenticides of the first generation (coumachlor, coumatetralyl, pyranocoumarin) were detected in rat urine, since human urine samples were not available. In our experience, this method should be suitable to detect them also in urine of patients at least in the case of rodenticide intoxication. The low-dosed 4-hydroxycoumarin rodenticides of the second generation, the so-called 'superwarfarins', could not be detected by this general screening procedure. They can be identified and quantified by special HPLC procedures [13,14,22].

Furthermore, our method has proved to be suitable also for simultaneous detection of other acidic drugs and/or their acidic metabolites in urine [2], like ACE inhibitors and angiotensin II receptor antagonists [6], calcium channel blockers of the dihydropyridine type [5], diuretics, NSAID barbiturates, and oral antidiabetics [4]. The procedures for the detection of these drugs will be published elsewhere.

Acknowledgements

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