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Determination of phenprocoumon, warfarin and their monohydroxylated metabolites in human plasma and urine by liquid chromatography–mass spectrometry after solid-phase extraction

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Abstract

A high-performance liquid chromatography–mass spectrometry (HPLC–MS) method for the quantification of phenprocoumon, warfarin, and their known monohydroxylated metabolites in human plasma and urine was developed using a simple, selective solid-phase extraction scheme. Chromatographic separation was achieved on a reversed-phase Luna C_{18} column and step gradient elution resulted in a total run time of about 13 min. Limits of quantification (LOQ) were ≤40 nM for the parent compounds and ≤25 nM for the metabolites and the limit of detection (LOD) was ≤2.5 nM for all analytes. Average recovery was 84% (±3.7) and 74% (±13.2) in plasma and urine, respectively. Intra- and inter-day coefficients of variation were ≤8.6 and ≤10.6% in plasma and urine, respectively. The method was successfully applied to the analysis of phenprocoumon samples from four healthy volunteers and should prove useful for future comparative studies of warfarin and phenprocoumon pharmacokinetics.
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Keywords: Phenprocoumon; Warfarin

1. Introduction

The 4-hydroxycoumarins phenprocoumon and warfarin are widely used as oral anticoagulants in the treatment and prevention of thromboembolic disorders [1]. They are characterised by a narrow therapeutic range and despite regular anticoagulant effect monitoring bleeding complications frequently occur. These are mainly attributed to pharmacokinetic variations due to genetic aspects or drug–drug interactions [1–3]. Previous methods for the determination of oral anticoagulants in biological fluids were mostly restricted to the quantification of the parent compounds [4–8]. However, it is often essential in the context of pharmacokinetic studies to determine the concentrations of both parent compounds and their metabolites, e.g. for phenotyping purposes by calculating metabolic ratios (parent compound/metabolite) that serve as a surrogate marker for the catalytic activity of hepatic cytochrome-P450 (CYP) enzymes [9].

In terms of warfarin, there have previously only two assays been published describing the simultaneous quantification of both parent drug and its monohydroxylated metabolites in rat plasma by high-performance liquid chromatography (HPLC) [10] or in human urine by thermospray HPLC–mass spectrometry (MS) [11]. None of these allow for the simultaneous analysis of warfarin and all five known monohydroxylated metabolites, namely 4′-, 6-, 7-, 8-, 10-hydroxywarfarin. In addition, a toxicological screening procedure for the detection of various 4-hydroxycoumarins and metabolites in urine by gas chromatography (GC)–MS has been described that, however, lacks any quantification of parent compounds or metabolites [12]. Other assays are restricted to warfarin and its two major metabolites hydroxylated in the 6- and 7-position utilizing HPLC [13–16] or thin-layer chromatography [17,18]. Finally, in vitro samples of warfarin and metabolites have been analyzed by LC–MS/MS [19] or GC–MS [20].

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Previous methods for the quantification of phenprocoumon and its major monohydroxylated metabolites, namely 4′-, 6-, 7-hydroxyphenprocoumon, in human plasma and urine by high-performance liquid chromatography (HPLC) after solid-phase extraction (SPE) were of relatively poor sensitivity [21]. Alternatively, they were complicated by interfering endogenous compounds requiring several cleaning steps and two separate assays for the determination of phenprocoumon and its three major monohydroxylated metabolites [22]. Previously described GC–MS methods required a time-consuming derivatisation procedure [23–25], were restricted to a semiquantitative analysis of phenprocoumon and metabolites in urine [24], or did not report reproducibility data [24,25].

We describe for the first time the determination of phenprocoumon, warfarin, and all of their known monohydroxylated metabolites in human plasma and urine by a single HPLC–MS assay. Compared to previous assays for the separate quantification of either phenprocoumon or warfarin and their metabolites, the assay performance has been further improved both with respect to sensitivity and simplicity of execution by developing a novel, selective solid-phase extraction scheme. Thus, the described method appears particularly attractive for pharmacokinetic studies of phenprocoumon and warfarin in vivo involving the quantification of both parent compounds and their known monohydroxylated metabolites.

2. Experimental

2.1. Chemicals and reagents

Racemic phenprocoumon (>99% chemical purity) was kindly provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Synthesised 4′-, 6-, 7-hydroxyphenprocoumon was a generous gift of Prof. W.F. Trager (Department of Medicinal Chemistry, University of Washington, Seattle, USA) [26]. Warfarin and its 4′-, 6-, 7-, 8-, and 10-hydroxylated metabolites as well as deuterium-labeled 7-hydroxywarfarin (>98% chemical purity) used as internal standard were purchased from Ultrafme Chemicals (Manchester, UK) (Fig. 1). Deuterium-labeled 7-hydroxywarfarin was dissolved in dimethylsulfoxide, whereas stock solutions of all other analytes were prepared in pure methanol. Internal standard and stock solutions were stored at −20 °C in the dark until use. Analyte stability in the stock solutions was confirmed over a period of 4 weeks. Acetonitrile, methanol, formic acid, dimethylsulfoxide, and ammonia as well as glacial acetic acid and sodium acetate for the preparation of acetate buffer were purchased from Merck (Darmstadt, Germany). Chemicals were of purest grade and solvents of HPLC grade. Sulphatase and β-glucuronidase were purchased from Sigma Chemicals (St. Louis, USA). Purified water was prepared using a Millipore Milli-Q purification system (Bedford, USA).

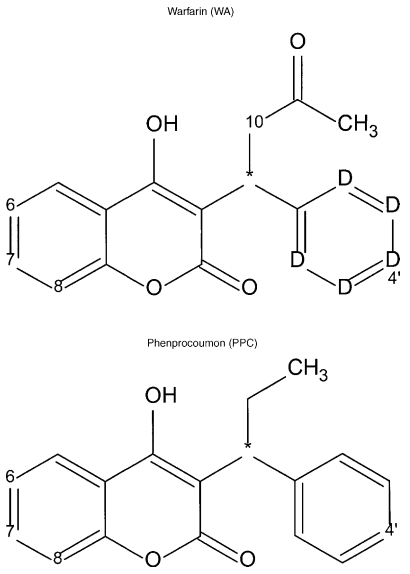

Fig. 1. Chemical structures of warfarin and phenprocoumon. The hydroxylation and deuterium-labeled positions are indicated by respective numbers and letters. The chiral center is marked by an asterisk (*).

2.2. Sample preparation

For assay validation blank plasma and urine was spiked with appropriate amounts of phenprocoumon, warfarin, and their metabolites as well as deuterium-labeled 7-hydroxywarfarin (0.5 μM). To 1 ml of spiked sample volume, 50 μl (for urine analyses) or 100 μl (for plasma analyses) of formic acid (1 M) was added to give a final pH of 3.0–4.0.

Plasma and urine samples from four healthy volunteers after intake of a single, oral dose of 12 mg racemic phenprocoumon were also analysed [27]. After thawing, 1 ml of these samples containing deuterium-labeled 7-hydroxywarfarin (0.5 μM) was adjusted to about pH 5.0 by addition of 100 μl acetate buffer (0.5 M) and incubated for 14 h at 37 °C in the presence of 25 μl sulphatase (37.0 U/ml) and 50 μl β-glucuronidase (9000 U/ml). Incubation conditions were selected based on a previous assay [22] and further validated by initial experiments using different incubation times (0, 10, 14, 18 h) and enzyme volumns (sulphatase: 0, 10, 25, 50 μl); β-glucuronidase: 0, 25, 50, 100 μl). Prior to SPE samples were adjusted to pH 3.0–4.0 by addition of 100 μl formic acid (1 M).

2.3. HPLC–MS conditions

The HPLC–MS system was equipped with Chemstation software for data registration and calibration and consisted of a reversed-phase Luna C_{18} 100 mm × 2.0 mm i.d. column with 3 μm particle size (Phenomenex, Torrance, USA), a vacuum degasser, a binary pump, an autoinjector, a column thermostat, and a single-quadrupole mass

spectrometer (Hewlett Packard 1100, Agilent Technologies, Avondale, USA). This was equipped with an atmospheric pressure-electrospray ionisation (AP-ESI) source used in the negative ionisation mode with the following spray chamber settings: nebuliser pressure: 20 psi, capillary voltage: 2000 V, fragmentor voltage: 70 V (for 4′-, 6-, 7-hydroxyphenprocoumon) or 100 V (for all other analytes), drying gas temperature: 350 °C, drying gas flow rate: 10 l/min. Selected ion monitoring (SIM) was chosen and each deprotonated molecular ion was monitored with a dwell time of 114 ms at *m/z* 279 and 295 for phenprocoumon and its metabolites, *m/z* 307 and 323 for warfarin and its metabolites, and *m/z* 328 for deuterium-labeled 7-hydroxywarfarin, respectively.

Chromatographic separation was achieved at a column temperature of 40 °C with an eluent A (2% acetonitrile in 25 mM formic acid) and B (70% acetonitrile in 25 mM formic acid). A step gradient was run at a flow rate of 0.3 ml/min with 55% eluent B for the first 5 min. Then the column was washed with 100% eluent B for 2 min followed by another 4 min equilibration to the initial conditions with 55% eluent B. The gradient mixer in the pump was bypassed to minimise gradient delay time. Including a subsequent needle wash this resulted in a total run time of about 13 min.

2.4. Sample purification

Sample purification was achieved by SPE using Sep Pak C_{18} light cartridges (130 mg, 1 ml) supplied by Waters (Eschborn, Germany). Spiked and volunteer samples were centrifuged at 17,500 × *g* for 10 min. After conditioning (1 ml methanol) and equilibration (2 ml purified water) 1000 μl of the supernatant was passed through the cartridges with gentle positive-pressure at a flow rate of about 1.5 ml/min. After washing with 500 μl of acetonitrile/water 20/80% (for plasma analyses) or 30/70% (for urine analyses) in 25 mM formic acid (pH 3.0) the samples were eluted under basic conditions with 1000 μl of acetonitrile/water 40/60% in 25 mM ammonia (pH 10.0). The eluate was subsequently vacuum centrifuged for 30 min to a final volume of 500–600 μl and 25 μl of the remainder were injected into the chromatographic system.

2.5. Standard curves

Standard curves were developed by spiking blank plasma and urine with appropriate amounts of phenprocoumon, warfarin, and their metabolites using eight different concentrations in the range of 0.04–10 μM (parent compounds) and 2.5–500 nM (metabolites), respectively. The peak height ratios of phenprocoumon, warfarin and metabolites over internal standard were plotted against the known concentrations, since peak height ratios showed better reproducibility and greater linearity of the calibration curves than using peak area ratios. Correlation coefficients were calculated by least-square regression analysis.

Table 1
Mean recovery of phenprocoumon, warfarin, and their metabolites in human plasma

	1 μM	4 μM	10 μM
Phenprocoumon	74	77	75
	25 nM	100 nM	250 nM
4′-Hydroxyhenprocoumon	71	76	73
6-Hydroxyhenprocoumon	71	74	72
7-Hydroxyhenprocoumon	69	71	69
	1 μM	4 μM	10 μM
Warfarin	77	77	76
	25 nM	100 nM	250 nM
4′-Hydroxywarfarin	66	75	75
6-Hydroxywarfarin	68	72	71
7-Hydroxywarfarin	70	78	76
8-Hydroxywarfarin	78	80	75
10-Hydroxywarfarin	81	78	73

Samples were spiked with analytes at three different concentrations and recovery data is provided as mean of triplicate experiments.

2.6. Assay validation

2.6.1. Sensitivity

The limit of detection (LOD) was defined as the concentration that could be determined with a signal to noise ratio of 3:1, whereas the lower limit of quantification (LOQ) corresponded to coefficients of variation of ≤10% (for precision data) and ≤20% (for accuracy data), respectively [28].

2.6.2. Recovery

Recovery was determined in triplicate at three different concentrations by comparing spiked plasma or urine samples with blank samples that were purified according to the described SPE procedure and subsequently spiked with appropriate amounts of the analytes to serve as 100% control (Tables 1 and 2).

2.6.3. Intra- and inter-day accuracy and precision

Spiked samples of analytes in plasma and urine were prepared at three different concentrations (Tables 3–6). To determine intra-day accuracy and precision, 6 replicate sample analyses were performed on the same day. Inter-day accuracy and precision was determined over a period of 3 days with three replicates per day ($n = 9$). Accuracy was calculated from the percentage ratio of measured over nominal sample concentration (mean of measured/nominal × 100). Precision was expressed as the percentage coefficient of variation.

2.6.4. Stability

Stability of phenprocoumon (1 μM), warfarin (1 μM), and their metabolites (25 nM) in human plasma and urine was

Table 2
Mean recovery of phenprocoumon, warfarin, and their metabolites in human urine

	1 μM	4 μM	10 μM
Phenprocoumon	109	93	85
	25 nM	100 nM	250 nM
4′-Hydroxyhenprocoumon	72	80	83
6-Hydroxyhenprocoumon	103	87	81
7-Hydroxyhenprocoumon	93	85	79
	1 μM	4 μM	10 μM
Warfarin	103	99	87
	25 nM	100 nM	250 nM
4′-Hydroxywarfarin	64	71	85
6-Hydroxywarfarin	49	60	80
7-Hydroxywarfarin	99	93	94
8-Hydroxywarfarin	76	75	88
10-Hydroxywarfarin	74	84	90

Samples were spiked with analytes at three different concentrations and recovery data is expressed as mean of triplicate experiments.

studied at −20 °C (7 days), +4 °C (4 days), as well as +18 and +37 °C (14 h). In addition, post-extraction stability of each analyte in the eluent was also assessed at +18 °C for 12 h.

2.6.5. Assay interference

Spiked plasma and urine samples of phenprocoumon (1 μM), warfarin (1 μM), and metabolites (25 nM) were analysed in the presence of naproxen, ibuprofen, and salicylic acid. The determined peak heights were compared to spiked samples alone.

2.6.6. Matrix effects

Spiked samples of phenprocoumon (1 μM), warfarin (1 μM), and metabolites (25 nM) were prepared in six different lots of plasma or urine and the determined peak heights were compared between each other.

Table 3
Intra- and inter-day accuracy and precision data for phenprocoumon and its metabolites in human plasma

	Intra-day ($n = 6$)			Inter-day ($n = 9$)		
	1 μM	4 μM	10 μM	1 μM	4 μM	10 μM
Phenprocoumon	4.8 (97)	3.7 (102)	2.9 (98)	2.1 (96)	2.3 (103)	1.3 (97)
	25 nM	100 nM	250 nM	25 nM	100 nM	250 nM
4′-Hydroxyhenprocoumon	6.6 (101)	7.4 (107)	4.9 (99)	1.9 (97)	2.1 (104)	4.8 (99)
6-Hydroxyhenprocoumon	7.5 (94)	8.6 (104)	5.1 (100)	0.7 (95)	0.6 (102)	3.2 (100)
7-Hydroxyhenprocoumon	6.3 (84)	7.9 (111)	5.5 (99)	4.2 (91)	2.2 (100)	4.9 (100)

Precision data is expressed as the coefficient of variation in % and accuracy data as mean % of the known spiked concentration.

Table 4
Intra- and inter-day accuracy and precision data for phenprocoumon and its metabolites in human urine

	Intra-day ($n = 6$)			Inter-day ($n = 9$)		
	1 μM	4 μM	10 μM	1 μM	4 μM	10 μM
Phenprocoumon	5.8 (103)	4.9 (101)	6.2 (99)	9.7 (104)	7.8 (103)	9.4 (99)
	25 nM	100 nM	250 nM	25 nM	100 nM	250 nM
4′-Hydroxyhenprocoumon	5.7 (110)	4.8 (110)	5.6 (99)	9.3 (117)	5.5 (112)	5.2 (98)
6-Hydroxyhenprocoumon	5.9 (120)	4.7 (114)	6.1 (98)	4.2 (123)	0.7 (114)	1.5 (98)
7-Hydroxyhenprocoumon	7.8 (112)	4.0 (105)	6.1 (100)	1.9 (120)	4.9 (109)	2.9 (99)

Precision data is expressed as the coefficient of variation in % and accuracy data as mean % of the known spiked concentration.

Table 5
Intra- and inter-day accuracy and precision data for warfarin and its metabolites in human plasma

	Intra-day ($n = 6$)			Inter-day ($n = 9$)		
	1 μM	4 μM	10 μM	1 μM	4 μM	10 μM
Warfarin	4.3 (99)	2.6 (104)	2.2 (96)	0.7 (94)	0.6 (101)	3.2 (100)
	25 nM	100 nM	250 nM	25 nM	100 nM	250 nM
4′-Hydroxywarfarin	2.9 (97)	6.2 (106)	5.1 (99)	1.3 (95)	2.0 (101)	4.1 (100)
6-Hydroxywarfarin	5.0 (99)	7.0 (107)	3.3 (99)	3.1 (95)	1.4 (103)	3.3 (100)
7-Hydroxywarfarin	7.0 (106)	7.5 (106)	3.0 (99)	1.1 (101)	0.9 (104)	2.9 (99)
8-Hydroxywarfarin	6.8 (102)	6.3 (114)	3.2 (98)	2.9 (100)	3.4 (107)	2.3 (99)
10-Hydroxywarfarin	6.3 (99)	5.9 (108)	3.0 (99)	2.9 (93)	2.4 (102)	2.9 (100)

Precision data is expressed as the coefficient of variation in % and accuracy data as mean % of the known spiked concentration.

Table 6
Intra- and inter-day accuracy and precision data for warfarin and its metabolites in human urine

	Intra-day ($n = 6$)			Inter-day ($n = 9$)		
	1 μM	4 μM	10 μM	1 μM	4 μM	10 μM
Warfarin	5.8 (103)	4.9 (106)	6.2 (102)	10.6 (105)	9.1 (106)	7.3 (101)
	25 nM	100 nM	250 nM	25 nM	100 nM	250 nM
4′-Hydroxywarfarin	6.2 (101)	6.4 (105)	2.5 (99)	5.1 (101)	5.7 (110)	5.9 (99)
6-Hydroxywarfarin	3.4 (92)	6.6 (107)	4.5 (99)	0.6 (109)	1.5 (109)	3.6 (99)
7-Hydroxywarfarin	4.5 (110)	4.9 (114)	2.7 (98)	2.8 (97)	1.9 (109)	1.1 (99)
8-Hydroxywarfarin	4.6 (112)	5.2 (114)	3.4 (98)	7.5 (97)	5.9 (111)	4.7 (98)
10-Hydroxywarfarin	4.6 (94)	6.3 (108)	4.9 (99)	1.0 (110)	0.5 (108)	2.7 (99)

Precision data is expressed as the coefficient of variation in % and accuracy data as mean % of the known spiked concentration.

2.6.7. Application of the assay

The pharmacokinetics of phenprocoumon and its metabolites was studied in plasma from four healthy volunteers after administration of a single, oral dose of 12 mg phenprocoumon over a period of 144 h (approved by the ethics committees of Charité University Hospital, Berlin, Germany and Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden) [27]. Urine samples collected over 12 h from the same individuals were also measured. Each sample analysis was done in triplicate.

3. Results and discussion

In order to allow for comparative pharmacokinetic studies of phenprocoumon and warfarin, our initial aim was to develop a sensitive and simple methodology for the simultaneous analysis of phenprocoumon, warfarin, and their known monohydroxylated metabolites in human plasma and urine. The determination of both parent compounds and metabolites is essential for a comprehensive analysis of phenprocoumon and warfarin metabolism, e.g. for an assessment of metabolic ratios (parent compound/metabolite) and thereby of activities of hepatic CYP enzymes that are known catalysts of either anticoagulant drug. Each of the few previous assays described for the quantification of either phenprocoumon or warfarin and their metabolites in biological fluids incorporated some major limitations, such as relatively poor sensitivity [21], lack of reproducibility data [10,11,24,25], or a time-consuming sample purification [10,22] or derivatisation [23–25]. Moreover, an assay for the determination of phenprocoumon, warfarin, and all of their known monohydroxylated metabolites using the same sample purification and analytical procedure has not yet been described.

Typical chromatograms of spiked human plasma and urine samples at the lower limit of quantification show well-shaped and separated peaks (Fig. 2a and b). The analytes were identified on the basis of their retention times and mass spectra compared to standard solutions containing individual compounds. The determined retention times were the same in plasma and urine (Fig. 2a and b). As parent compounds and metabolites differ considerably with respect to their polarity, a step gradient was required to elute all compounds within a reasonable total run time. Thus, a change in the mobile phase composition from initial 55–100% eluent B was programmed from 5 to 7 min that actually occured with a lag time of about 2 min. Thereby all metabolites were eluted within the first 7 min followed by the parent drugs at 7.5 and 8.1 min, respectively. Since each monohydroxylated metabolite of phenprocoumon or warfarin possesses identical mass to charge ratios, good chromatographic resolution is a prerequisite and was achieved with a reversed-phase C_{18} column. Our thorough sample purification allowed for analysis of at least 500 samples with the same column without adversely affecting the chromatographic separation.

The optimal fragmentor voltage was selected on the basis of a maximum signal response. As all analytes are weak acids, the negative ionisation mode was chosen. MS conditions were further optimised using a one-variable-at-a-time approach.

It has previously been shown that phenprocoumon, warfarin, and their monohydroxylated metabolites are mainly excreted as conjugates. Thus, each urine sample was hydrolysed enzymatically by sulphatase and β-glucuronidase in order to determine the sum of free and conjugated analytes. At the selected conditions of 14 h incubation and 25 μl sulphatase (37.0 U/ml) and 50 μl β-glucuronidase (9000 U/ml) maximum analyte concentrations were obtained indicating complete deconjugation. These conditions have also previously been optimised by others on the basis of enzymatic hydrolytic activity towards a glucuronidated or sulphated coumarin analogue, namely 4-methylumbelliferone [22]. Thus, the existence of any remaining conjugated analytes in the matrix appears unlikely despite lack of the glucuronidated and sulphated standards thereby preventing an ultimate verification in this regard.

Prior to SPE samples were adjusted to pH 3.0–4.0 to reduce the polarity of the analytes and firmly attach them to the non-polar, stationary silica-phase of the cartridge. Thereby samples could be purified under these acidic conditions using rather high acetonitrile concentrations. Due to this highly selective SPE scheme interference by endogenous compounds was only marginal, but appeared more

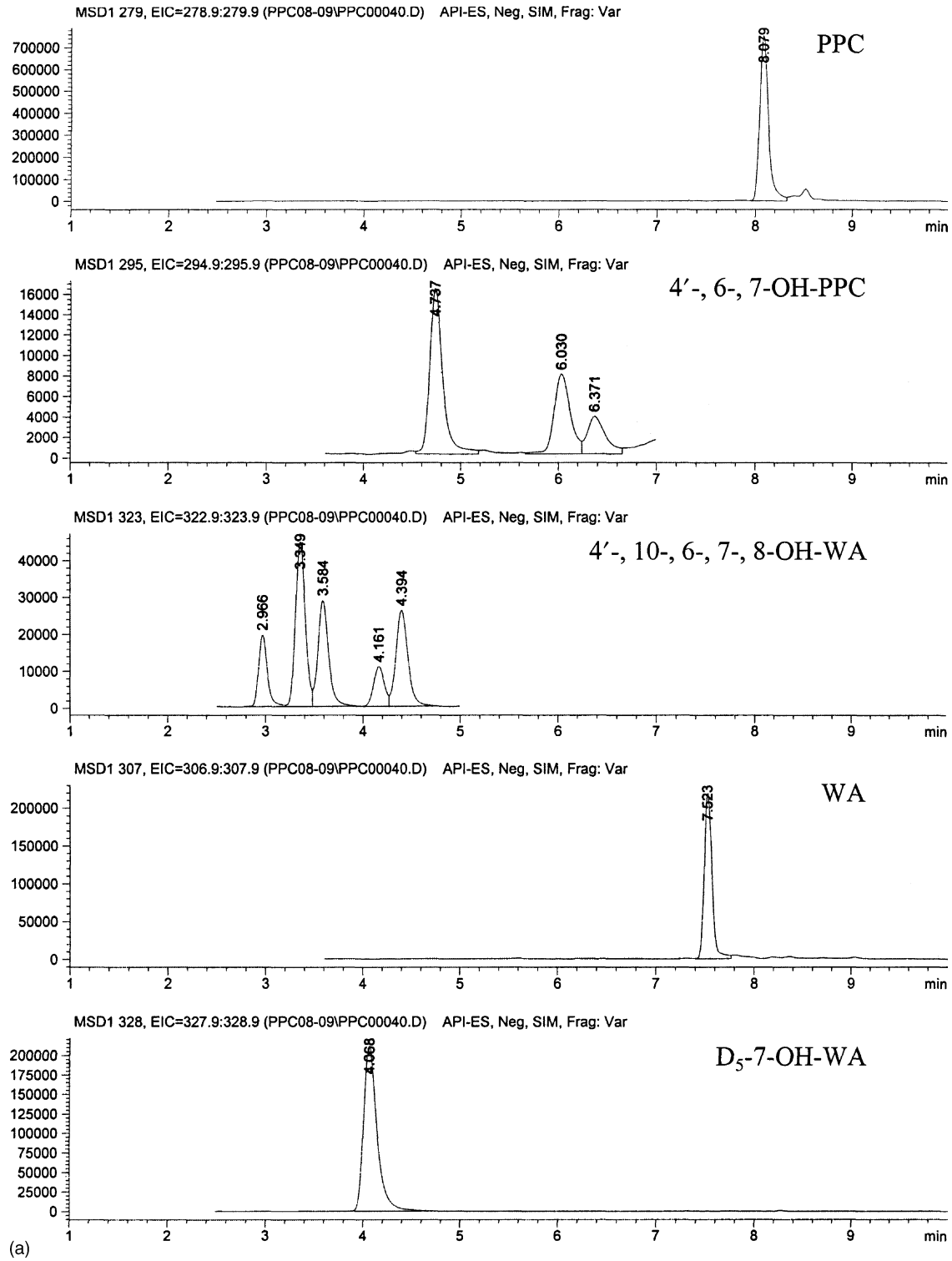

Fig. 2. Typical HPLC–MS chromatograms of human plasma (a) and urine (b) samples spiked with phenprocoumon (1 μM), warfarin (1 μM), and monohydroxylated metabolites (25 nM). Deuterium-labeled 7-hydroxywarfarin (0.5 μM) was used as internal standard. Analytes are mentioned in their elution order in each of the MSD windows.

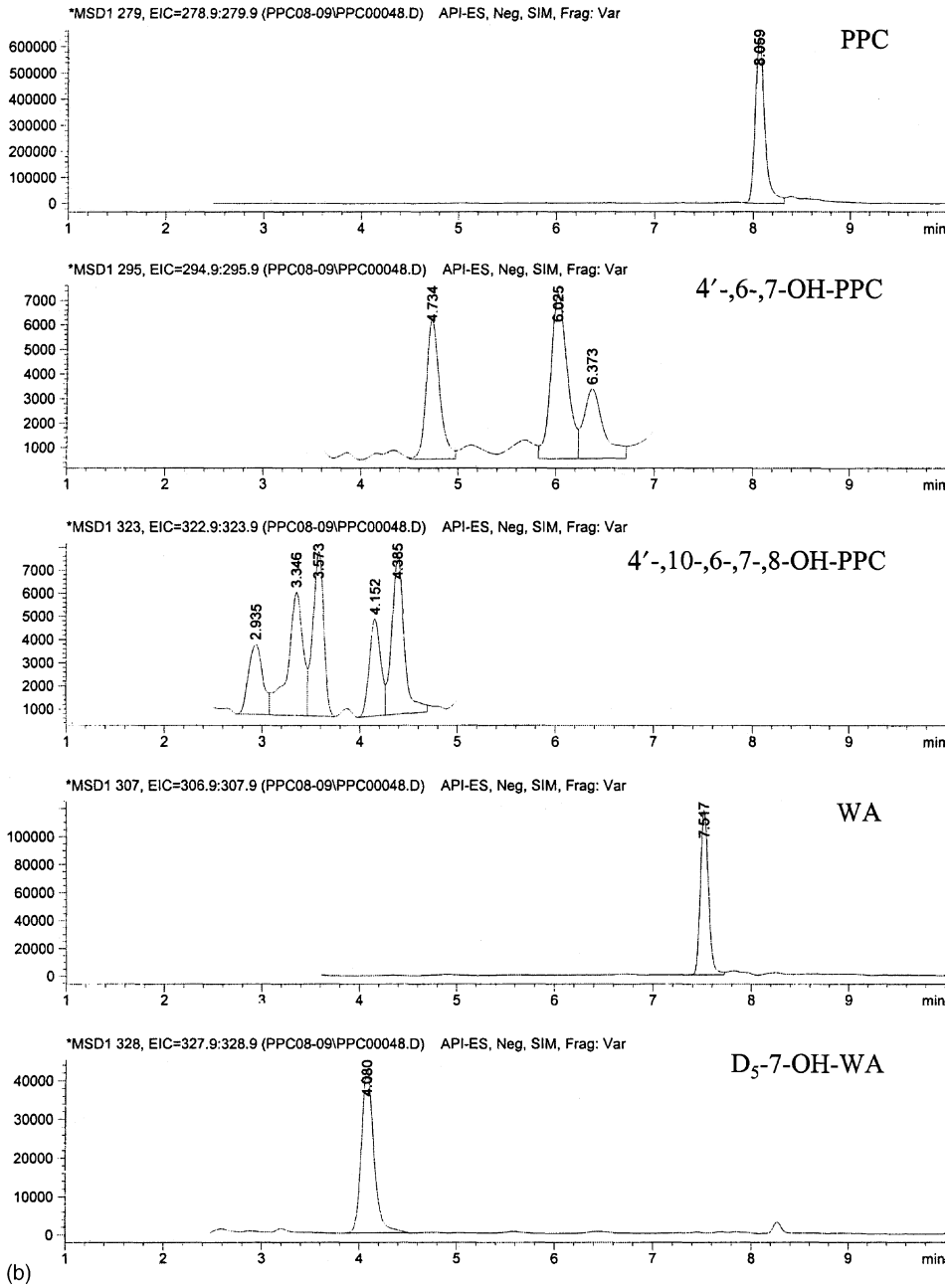

Fig. 2. (*Continued*).

pronounced in urine than in plasma. Consequently, the chosen acetonitrile concentration for the purification step was higher for urine (30%) than for plasma (20%) analyses. When comparing the peak height ratios of spiked samples prepared in six different lots of human plasma or urine, no interference by endogenous compounds in the matrix of blank drug-free plasma or urine samples was observed. Moreover, we have assessed the signal response of spiked samples in the presence of other exogenous anti-inflammatory drugs (naproxen, ibuprofen, and salicylic acid) that are also weak acids and commonly co-administered with either phenprocoumon or warfarin. No differences of peak height ratios compared to spiked samples alone were observed. Previously, it has also been shown by others that several drugs commonly co-administered to patients throughout therapeutic anticoagulation do not interfere with the quantification of phenprocoumon in plasma by HPLC analysis [5,29].

Despite the high solvent concentration used in the purification step, elution under strongly basic conditions (pH 10) resulted in high extraction efficiencies averaging to 84% (±3.7) and 74% (±13.2) in plasma and urine, respectively (Tables 1 and 2). With an eluent composition of 40% acetonitrile in 25 mM ammonia a subsequent evaporation step was

required to reduce the proportion of organic solvent and ensure sufficient retaining of the analytes on the HPLC column.

Precision and accuracy data confirmed a good reproducibility of the described method (Tables 3–6). A major concern with this analytical strategy requiring great pH variations certainly is the column performance and even more importantly the stability of the compounds. The latter issue was addressed by a comparison of the signal response from standard solutions prepared in purified water that were adjusted to pH 3, 5, 7, and 10 by addition of formic acid or ammonia, respectively. The observed differences in peak height for all analytes were less than 10%.

The current assay allows for the quantification of phenprocoumon, warfarin, and their metabolites over a wide concentration range. The standard curves of all metabolites were linear with r^2-values of $\geq$0.998 at concentrations ranging from 2.5 to 500 nM in human plasma and urine. Data of the parent compounds needed to be transformed based on a quadratic equation ($y = \sqrt{ax}$) to achieve linear calibration curves in a concentration range from 0.04 to 10 μM. The average r^2-values in plasma and urine amounted to 0.996 and 0.997 for phenprocoumon and warfarin, respectively. As the standards gave both in the presence or absence of sulphatase or glucuronidase the same results, each calibration curve was generated without addition of enzymes. It also indicates a lack of interference or ion suppression caused by any unknown, deconjugated endogenous compounds in the urine.

The sensitivity of the present method is very high compared with previous assays for the separate determination of either phenprocoumon and metabolites [21,22] or warfarin and metabolites in plasma and urine [10,11]. The lower LOD of all analytes was $\leq$1 nM in plasma and $\leq$2.5 nM in urine, respectively. The lower LOQ in plasma and urine was determined $\leq$40 nM for the parent compounds and $\leq$25 nM for all metabolites, respectively. The large injection volume of 25 μl was chosen in an attempt to maximise assay sensitivity and did not cause any significant peak distortion compared to lower injection volumes of 5, 10, 15, or 20 μl.

Stability of the analytes as influenced by different storage conditions was also evaluated. At room temperature of +18 °C, analytes were stable over a period of 12 h in the eluent. This allows for reliable overnight sample runs.

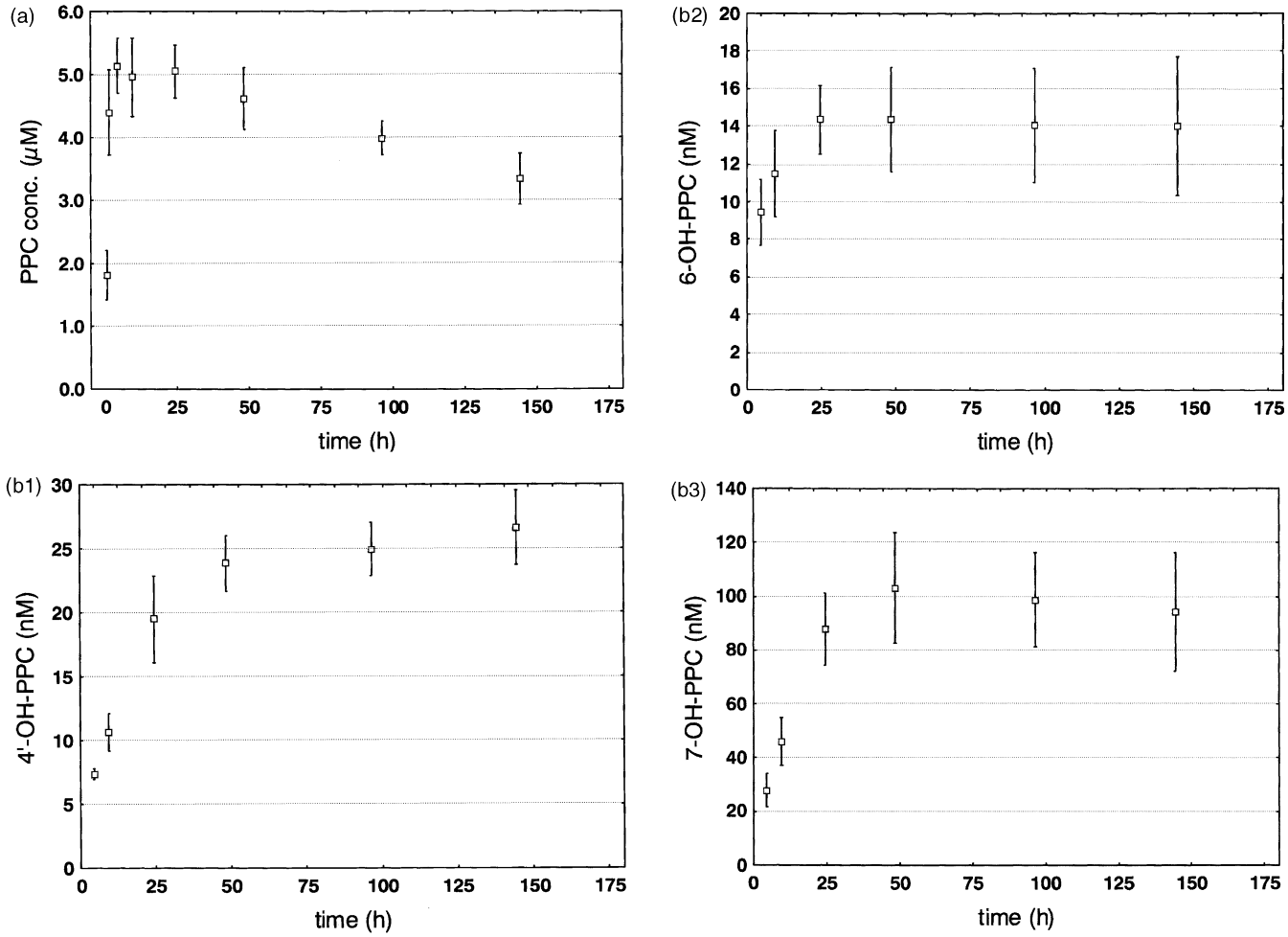

Fig. 3. Plasma-concentration–time curve of phenprocoumon (a) and its monohydroxylated metabolites (b) determined from four volunteer samples after administration of a single, oral dose of 12 mg racemic phenprocoumon. Data is expressed as mean ± 95% confidence interval.

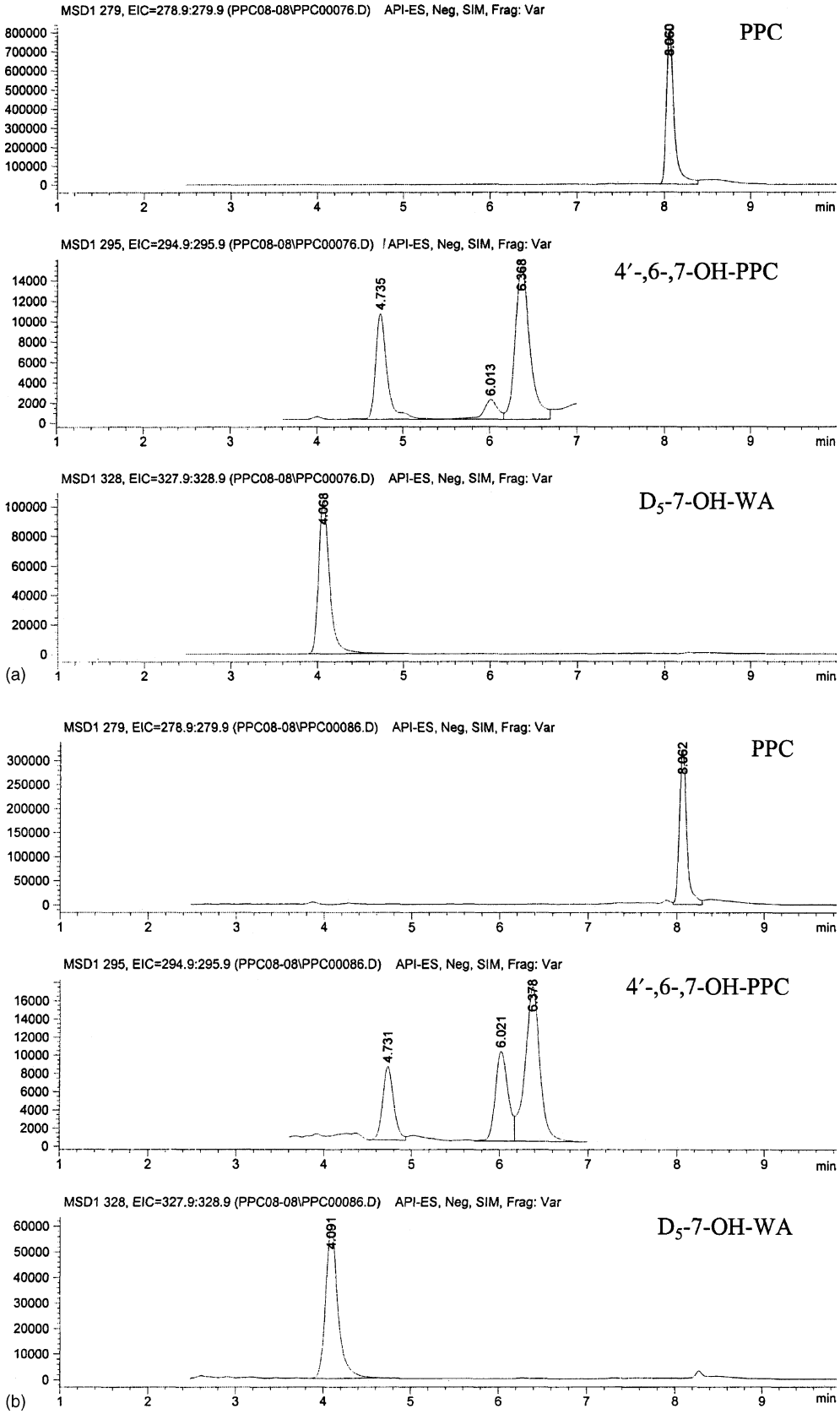

Fig. 4. Typical HPLC–MS chromatograms of human plasma (a) ($t = 36$ h) and 12 h collected urine (b) samples from a healthy volunteer after a single, oral-dose administration of 12 mg racemic phenprocoumon.

Moreover, analyte stability was also confirmed in the matrix as described under *Experimental*. The presence or absence of sulphatase or β-glucuronidase had no impact on the signal response obtained with the spiked standards. Finally, the stability of phenprocoumon and warfarin for several weeks in biological fluids has also previously been verified by others [5,30]. The applicability of the described method was confirmed by assessing the pharmacokmetics of phenprocoumon and its three major monohydroxylated metabolites in four healthy volunteers after administration of a single, oral dose of 12 mg racemic phenprocoumon [27]. Unfortunately, we did not obtain plasma or urine samples from volunteers after oral, single-dose administration of warfarin. Fig. 3a and b shows the plasma-concentration–time curves of phenprocoumon and its metabolites. A chromatogram from a plasma sample at $t = 36$ h (a) as well as from a urine sample collected over a period of 12 h (b) is also illustrated (Fig. 4a and b). The determined plasma-elimination half-life of 213 h (±43) is comparable to previously reported data [5,31]. In line with previous in vivo [32,33] and in vitro studies [34], 7-hydroxyphenprocoumon was clearly identified as quantitatively most important metabolite both in plasma and urine. Strikingly, each metabolite showed a very slow formation with t_{max} values of about 50 h (6- and 7-hydroxyphenprocoumon) and more than 144 h (4′-hydroxyphenprocoumon) after administration of a single, oral dose of 12 mg racemic phenprocoumon.

4. Conclusions

In conclusion, a sensitive, rapid, accurate, and precise HPLC–MS methodology has been developed for the determination of phenprocoumon, warfarin, and all of their known monohydroxylated metabolites in human plasma and urine. A simple and selective SPE scheme allowed for thorough sample purification and guaranteed good recovery of all analytes. The high sensitivity achieved with a single quadrupole appears to be sufficient for most applications and makes this assay particularly attractive for in vivo studies of phenprocoumon and warfarin metabolism.

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