ELSEVIER

Journal of Chromatography B, 655 (1994) 63–71

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of the coumarin anticoagulant phenprocoumon and metabolites in human plasma, urine and breast milk by high-performance liquid chromatography after solid-phase extraction

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(First received October 27th, 1993; revised manuscript received January 24th, 1994)

Abstract

The coumarin anticoagulant phenprocoumon (PH) and metabolites (6-, 7- and 4′-hydroxyphenprocoumon) were analysed in plasma and urine samples from anticoagulated patients using solid-phase extraction and high-performance liquid chromatography with reversed-phase columns and ultraviolet and fluorescence detection; a simpler handling of samples, higher selectivity, precision, accuracy and analytical recovery were obtained compared to analysis using liquid–liquid extraction. Similarly, a method for the analysis of PH in human breast milk was developed to assess the passage of anticoagulants into breast milk in anticoagulated lactating women.

1. Introduction

The 4-hydroxycoumarin anticoagulant phenprocoumon (PH) (1) (Fig. 1) is used in middle Europe for the treatment and management of thromboembolic diseases in humans [1]. The therapeutic dose is adjusted by monitoring clotting times [2]; however, plasma concentration data of the drug and its metabolites, or their excretion rates in urine, are required for pharmacokinetic studies [3] or in suspected toxicological cases [4,5].

PH is partially oxidised to 6-hydroxyphenprocoumon (6-OH-PH; 2), 7-hydroxyphenprocoumon (7-OH-PH; 3) and 4′-hydroxyphenprocoumon (4′-OH-PH; 4); PH and metabolites are eliminated in bile and urine mainly as conjugates [6–10].

Several methods using high-performance liquid chromatography (HPLC) for the analysis of PH and metabolites were reported [6,7,12–14]; liquid–liquid extraction purification methods were used for plasma analysis. HPLC analysis of PH and metabolites in urine after liquid–liquid, or solid-phase extraction purification with C_{18} reversed-phase materials showed interfering peaks [6]; selective GC–MS methods were described for their assay [6–10,15,16]; these GC–MS methods require, however, further derivatisation steps after extraction, and equipment and trained staff which are not available in all laboratories. Lately, a solid-phase extraction method has been described for the HPLC analysis of

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0378-4347/94/$07.00
SSDI 0378-4347(94)00050-F

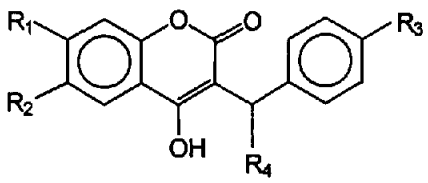

Fig. 1. Structure of anticoagulants and metabolites

Compound Number	Compound	R1	R2	R3	R4
1	Phenprocoumon (PH)	H	H	H	C_2H_5
2	6-Hydroxy-PH	H	OH	H	C_2H_5
3	7-Hydroxy-PH	OH	H	H	C_2H_5
4	4'-Hydroxy-PH	H	H	OH	C_2H_5
5	Warfarin (WA)	H	H	H	CH_2COCH_3
6	6-Hydroxy-WA	H	OH	H	CH_2COCH_3
7	7-Hydroxy-WA	OH	H	H	CH_2COCH_3
8	Warfarin alcohol 1	H	H	H	$CH_2CHOHCH_3$
9	Warfarin alcohol 2	H	H	H	$CH_2CHOHCH_3$
10	4'-Chloro-PH	H	H	Cl	C_2H_5
11	4'-Chloro-WA	H	H	Cl	CH_2COCH_3
12	Acenocoumarol (AC)	H	H	NO_2	CH_2COCH_3

conjugated and free PH and metabolites in urine using anion-exchange stationary phase [17].

The aim of this work was to develop simple assays to measure total PH and metabolite plasma and urine concentrations for studies on the disposition of these drugs in patients under PH anticoagulation [3], drug interactions [18] and suspected non-compliance cases [5].

In the present work plasma and urine samples were purified by solid-phase extraction, and total PH and metabolite concentrations were determined by reversed-phase HPLC with ultraviolet and fluorescence detection. An assay for PH in breast milk was also devised to investigate the possible passage of PH into breast milk of lactating women under PH therapy, and to evaluate the possible bleeding complications in lactants [19]; PH in human breast milk was analysed similarly with additional purification steps before the solid-phase extraction.

PH and its metabolites are chiral compounds; the enantiomer separation of the coumarin anticoagulants and metabolites was the subject of previous publications [13,14].

2. Experimental

2.1. Chemicals

Racemic warfarin (5) and 4'-chloro-warfarin (11) were supplied by Sigma Chemie (Deisenhofen, Germany); racemic phenprocoumon (1) and 4'-chloro-phenprocoumon (4'-Cl-PH) (10) were gifts from Hoffmann-La Roche (Basel, Switzerland); racemic hydroxyphenprocoumon metabolites (6-OH-PH, 7-OH-PH, and 4'-OH-PH) (2–4), racemic warfarin metabolites (6-OH-WA, 7-OH-WA) (6,7) and the racemic diastereomeric warfarin alcohols 1 and 2 (8,9) were obtained in our laboratories by chemical synthesis [11,13,20,21]. Racemic acenocoumarol (12) was a gift from Ciba-Geigy (Wehr, Germany); coumatetralyl, difenacoum and brodifacoum were gifts from Dr. N. Shearer, Laboratory of Hematology, Guy's Hospital, London; β-glucuronidase/arylsulfatase solution from *Helix pomatia* (40 U/ml and 20 U/ml respectively) was obtained from Merck (Darmstadt, Germany), tetramethylammonium hydroxide pentahydrate from Fluka Feinchemikalien (Neu-Ulm, Germany). Reagents and solvents for chromatography were analytical grade. Acetone, acetonitrile and methanol were spectroscopic grade (Merck). Solvents for HPLC were filtered and degassed with helium before chromatography.

2.2. Preparation of biological samples

Citrated plasma samples from patients under anticoagulant therapy or suspected non-compliance, were obtained after determination of clotting times by the Coagulation Laboratory of the Medical Clinic, University of Heidelberg (Heidelberg, Germany); heparinised or EDTA

plasma may also be used. Urine samples were collected over 24 h. The plasma and urine samples were kept frozen until analysis.

Breast milk samples from a premature child's mother under PH therapy were obtained by pumping and were kept frozen until analysis; the samples were collected by Dr. R. von Kries, Pediatric Clinic, University of Düsseldorf (Düsseldorf, Germany).

2.3. HPLC equipment

HPLC analysis was performed with a Model 1090 M liquid chromatograph equipped with a ternary solvent delivery system and autosampler; the UV-Vis photodiode array (DAD) and fluorescence detectors (Model M1046) were connected in series; a Model 79994A computer workstation was used for system control and data handling (Hewlett-Packard, Waldbronn, Germany). The DAD wavelengths were set at 280 and 312 nm and on-line spectra were recorded in the range 230–400 nm; fluorescence excitation and emission wavelengths were set at 292 and 380 nm respectively; both UV and fluorescence signals were recorded simultaneously.

2.4. Gas chromatographic–mass spectrometric equipment

Mass spectra were obtained using electron impact ionisation (EI) (ionization potential 70 eV) in the scanning or selected-ion monitoring mode, with a Model 5989A quadrupole mass spectrometry system interfaced to a Series 5890 Series II gas chromatograph (Hewlett-Packard). Gas chromatographic separations were performed with fused-silica columns (HP Ultra-1, 12 m × 0.23 mm I.D.) using a temperature program from 60°C to 250°C in 20 min. Samples (1 μl) (in isooctane) were injected in a cool-on column injector, and an HP-UNIX workstation was used for data acquisition and analysis.

2.5. Solid-phase extraction tube conditioning

Before sample purification the Bond Elut solid-phase extraction tubes were conditioned as follows: the C_{18} octadecyl phases (500 mg; reservoir volume 2.8 ml; ict, Frankfurt, Germany) were conditioned by filtering twice with 2 ml of methanol and twice with 2 ml 1% aqueous acetic acid. Bond Elut solid-phase tubes SAX (strong anion exchanger; same capacity and manufacturer as above) were washed twice with 2 ml methanol, twice with 10 ml tetramethyl ammonium hydroxide (0.1% aqueous solution) and 10 ml 1 *M* aqueous acetic acid. The solutions were allowed to filter by gravity.

2.6. Extraction procedure for plasma

A 0.5-ml plasma sample was mixed with 5 μl 4'-Cl-PH solution (internal standard; 1 mg/ml); the mixture was transferred to a Bond Elut C_{18} preconditioned column, and washed successively twice with 2 ml 1% acetic acid and twice with 2 ml 20% aqueous methanol; the column was dried by air aspiration (Visiprep solid-phase vacuum manifold; Supelco, Bad Homburg, Germany) and eluted by rinsing the column twice with 0.5 ml acetone; the total acetone eluates were evaporated at 50°C under a stream of nitrogen. The extract was treated with 100 μl solvent [for elution system 1: 0.5% aqueous acetic acid–acetonitrile (40:60, v/v); for elution system 2: 0.1 *M* aqueous ammonium acetate pH 4.5–methanol (40:60, v/v)], vortex-mixed for 2 s and injected onto the HPLC system.

2.7. Extraction procedure for urine

A 5-ml urine sample was mixed with 15 μl β-glucuronidase/arylsulfatase and 0.25 ml acetate buffer (1 *M*, pH 4.5), and incubated overnight at 37°C; after the addition of 50 μl internal standard (see plasma extraction) the mixture was added to a Bond Elut SAX preconditioned column and washed twice with 2 ml water and twice with 2 ml potassium dihydrogen phosphate (0.1 *M*, pH 4.0); the column outlet was attached to a preconditioned Bond Elut C_{18} column, and washed six times with 2 ml of a hydrochloric acid–sodium chloride solution (0.04 *M*, 0.26 *M*, respectively). The C_{18} column was washed and processed as described above for plasma.

2.8. Extraction procedure for breast milk

A 1.0-ml milk sample was mixed with 100 μl 30% perchloric acid and 1.0 ml methanol and vortex-mixed for 2 min; to the sample 100 μl sodium hydroxide (5 *M*) was added; the sample was vortex-mixed and centrifuged at 900 *g* for 10 min. A 1.6 ml-aliquot of the supernatant was mixed with 100 μl glacial acetic acid, vortex-mixed and again centrifuged; a 1.3-ml aliquot was added to a preconditioned Bond Elut C_{18} column and further processed as described earlier for plasma samples.

2.9. HPLC plasma analysis

HPLC plasma analysis was carried out using a Nucleosil C_{18} 100-5 column (125 × 4 mm I.D., 5-μm spherical particles; Macherey Nagel, Düren, Germany) preceded by an ODS-Hypersil guard column (20 × 2.1 mm I.D., 5-μm spherical particles; Hewlett-Packard). The isocratic elution system 1 consisted of a mixture of a 0.5% aqueous acetic acid–acetonitrile (40:60, v/v); this system was used for the sole analysis of PH. Gradient elution system 2 was used for the analysis of PH and its metabolites; solvent A was an aqueous 0.1 *M* ammonium acetate pH 4.5 solution; solvent B was methanol; a linear gradient from 45–100% B in 50 min was programmed and the analysis was stopped at 20 min and the column reequilibrated during 10 min before the next injection. The total solvent flow-rate was 1 ml/min and the operating pressure and temperature were 10–12 MPa and 20–25°C, respectively. A 20-μl plasma extract was injected onto the HPLC system. Peak areas were integrated and the concentrations were calculated using the corresponding calibration factor for each compound analysed; PH was quantified by integrating the UV signal at 312 nm, and the metabolites by integrating the fluorescence signals. After an analytical series the columns were washed with water and methanol; the guard column was changed when the pressure in the system increased.

2.10. HPLC urine analysis

Chromatographic conditions: 20 μl of urine extract were injected onto the HPLC system as described for plasma; elution system 2 (see above) was used for analysis. Signals for quantification were the same as for plasma.

2.11. HPLC breast milk analysis

The same chromatographic conditions as described for plasma were used for the analysis of milk extracts; only PH was assayed using elution system 2 and fluorescence detection.

2.12. GC–MS analysis

PH and metabolites were analysed after treating the extracts with methyl iodide as described before [3], and were dissolved in isooctane (10 μl) before injection onto the GC–MS system; for quantitation in the selected-ion mode (SIM), the ions at *m/z* 294, 324 and 329 were monitored for the O-methyl derivatives of PH, metabolites and 4′-Cl-PH respectively.

2.13. Preparation of standards

Plasma

A standard stock solution containing 5 mg of PH in 5 ml phosphate buffer (0.1 *M*, pH 7.4) was prepared; stock solutions of the internal standard 10 and PH metabolites 2–4 were prepared dissolving each compound separately in methanol (1 mg/ml for 10, and 1 mg/10 ml for 2–4); solutions were maintained for one week at 2–8°C in the dark. PH stock solutions were diluted with buffer, and the metabolites and internal standard with methanol. Increasing amounts of the diluted solutions were added to blank plasma to obtain final plasma concentrations of 0.2, 0.5, 1.0, 2.0 and 5.0 μg/ml for PH, and 0.1, 0.2, 0.5 and 1.0 μg/ml for each metabolite; internal standard (10, 1.0 μg/ml) was also added to each plasma sample; each sample was purified as described above.

Urine

The same stock solutions used for plasma were used to obtain the urine standards; increasing amounts of the stock solutions were added to blank urine to obtain final concentrations of 0.1, 0.2, 0.5, 1.0 μg/ml for PH and 0.02, 0.05, 0.1, 0.2 μg/ml for each metabolite; each urine sample was incubated with β-glucuronidase/arylsulfatase, internal standard (10, 1.0 μg/ml) was added to each urine sample and the samples were purified as described above.

Breast milk

To 1-ml pooled breast milk aliquots, known amounts of PH were added (0.02, 0.05, 0.075, 0.1 μg/ml), and internal standard (0.1 μg/ml). The samples were processed as described above.

2.14. Calibration curves

Calibration curves for plasma and urine analysis were obtained by processing plasma and urine with known amounts of PH metabolites and internal standards as mentioned above. Least-squares linear regression of the ratio of PH (or metabolite) peak area/internal standard peak area *vs.* added concentrations was used to calculate the calibration factors for each compound. For breast milk calibration (blank breast milk contained already PH), the method of standard addition was used [22] and a calibration factor was calculated as described above.

2.15. Analytical recoveries

The analytical recoveries for each compound were determined by comparing the peak areas of extracts from standard plasma and urine samples with added known concentrations, to standard solutions (dissolved in buffer or methanol) of the compounds with the same concentration.

2.16. Interfering substances

The coumarin anticoagulants warfarin and metabolites (5–9) acenocoumarol (12), coumatetralyl, difenacoum and brodifacoum, as well as other drugs [9] were analysed by HPLC under the conditions used for the plasma and urine assays.

3. Results and discussion

The coumarin anticoagulant phenprocoumon (PH) and metabolites were analysed in plasma and urine samples from anticoagulated patients using solid-phase extraction and high-performance liquid chromatography with reversed-phase columns and ultraviolet and fluorescence detection. This method was optimized in order to use smaller samples, to simplify the sample purification steps and to obtain adequate selectivities, precision, accuracy and analytical recoveries in comparison to previous procedures using liquid–liquid extraction [6,12]. Calibration factors were calculated using internal standards; however, accuracy and precision were comparable when using external standard calibration. The use of shorter analytical columns with reversed-phase spherical particles combined with a guard column allowed the efficient separation of the main drug and its metabolites while saving HPLC solvent and analysis time; the analytical column was stable and gave reproducible results over long periods; more than one thousand analyses can be performed before column deterioration occurs; k' values for PH and metabolites, and other anticoagulants are tabulated in Table 1; in elution system 2 all peaks were baseline separated; other anticoagulants did not interfere and may be identified by their retention times and UV spectra; acenocoumarol is not fluorescent and may be clearly differentiated from warfarin (Table 1). Similarly, a method for the analysis of PH in human breast milk was developed to assess the passage of anticoagulants into breast milk in anticoagulated lactating women.

The same methodology was extended to the analysis of warfarin (WA) and its metabolites (5–9) in plasma and urine, and will be the subject of a separate communication.

Table 1
Column capacity values of phenprocoumon and its metabolites, warfarin and acenocoumarol

Compound No.	Compound	k'_1	k'_2
1	Phenprocoumon (PH)	3.25	7.20
2	6-Hydroxyphenprocoumon	1.25	4.87
3	7-Hydroxyphenprocoumon	1.38	6.27
4	4'-Hydroxyphenprocoumon	1.13	4.00
5	Warfarin (WA)	2.50	8.80
10	4'-Chlorophenprocoumon	4.75	10.00
12	Acenocoumarol	2.38	7.07

k'_1 Values correspond to elution system 1 and k'_2 values to elution system 2 (see Experimental).

3.1. Plasma analysis

Plasma HPLC analysis of PH and metabolites was preceded by solid-phase extraction with a C_{18} phase. When only PH is to be determined in routine analysis of plasma, isocratic elution is simpler and quicker (HPLC run time 10 min); PH metabolites do not contribute to the anticoagulant effect [23], and do not always have to be assayed. The simultaneous determination of the parent drug and its metabolites required a gradient elution program (Table 1). In plasma the concentrations of the metabolites were lower than that of the main drug; therefore, PH was quantitated by integrating the UV absorbance signal and the metabolites by detection of the fluorescence, since the latter is 5–20 times more sensitive (depending on the fluorimeter settings). HPLC analysis of a control plasma extract is shown in Fig. 2A, that of a control plasma after addition of 2 μg/ml PH and 0.25 μg/ml of the PH metabolites in Fig. 2B, and the plasma extract of a patient under PH anticoagulation is shown in Fig. 2C. The method was validated checking the peak purities by recording the UV spectra with the DAD during the HPLC run, and by treatment of the extracts with methyl iodide and GC–MS analysis using selected-ion monitoring as described previously [3,4]. This method determines the total plasma (free plus plasma protein bound) PH and metabolites concentrations. The PH and its metabolites calibration curves were measured in a range of 0.2–5 μg/ml and 0.05–1 μg/ml respectively; calibration curves were linear ($r = 0.996$–0.999); precision and accuracy for different plasma concentrations are shown in Table 2; limits of detection were

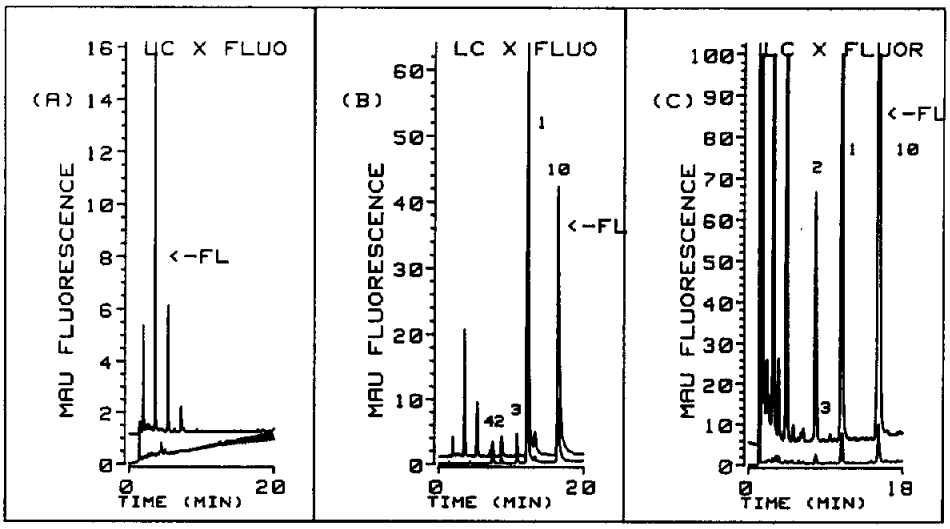

Fig. 2. HPLC analysis of plasma extracts: (A) control plasma; (B) control plasma after addition of 2 μg/ml phenprocoumon (PH) and 0.25 μg/ml of each metabolite; (C) plasma sample from a patient under PH therapy; PH concentration 0.67 μg/ml. HPLC system with gradient elution; UV (MAU = milli absorbance units) and fluorescence chromatograms (upper trace, FL) are overlayed; abscissa units is the fluorescence intensity. Numbers on the chromatographic peaks are those of Fig. 1.

Table 2
Within-run precision and accuracy of phenprocoumon and metabolites determinations in plasma and urine

Compound No.	Compound	Plasma			Urine		
		Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration added (μg/ml)	Concntration found (mean ± S.D.) (μg/ml)	C.V. (%)
1	Phenprocoumon (PH)	0.20	0.201 ± 0.007	3.3	0.20	0.201 ± 0.014	6.8
		1.00	1.02 ± 0.02	2.3	1.00	0.96 ± 0.07	6.8
		5.00	5.14 ± 0.18	3.5			
2	6-Hydroxy-PH	0.10	0.093 ± 0.006	6.7	0.05	0.057 ± 0.002	3.9
		0.25	0.246 ± 0.014	5.5	0.20	0.0189 ± 0.011	6.0
3	7-Hydroxy-PH	0.10	0.098 ± 0.007	7.1	0.02	0.026 ± 0.001	3.0
		0.25	0.260 ± 0.016	6.3	0.10	0.094 ± 0.004	4.7
4	4'-Hydroxy-PH	0.10	0.105 ± 0.013	12.5	0.02	0.017 ± 0.002	9.1
		0.25	0.251 ± 0.021	8.3	0.10	0.101 ± 0.003	3.2

S.D. = standard deviation, C.V. = coefficient of variation ($n = 5$).

0.1 μg/ml and 0.02 μg/ml for PH and the metabolites; analytical recoveries for PH and the metabolites at different concentrations averaged 98 ± 2%, which is higher than obtained with liquid–liquid extractions (84%). Other anticoagulants and drugs (see Experimental) did not interfere with the assay.

Plasma total PH concentrations in anticoagulated patients were in the therapeutic range of 1–4 μg/ml; the therapeutic PH dose and concentration varies widely between different subjects, as the dose is adjusted according to the clotting times (Quick value). PH metabolites (2–4) were present in low concentrations (less than 0.3 μg/ml); the pattern of the metabolite concentrations was subject to high inter-individual variability.

3.2. Urine analysis

PH and its metabolites are excreted in urine mainly as conjugated compounds; previous to extraction, urine samples were enzymatically hydrolysed. A two-step purification procedure was used for urine samples: first, a solid-phase extraction with a strong anion-exchange phase, followed by filtration of the eluate through a C_{18} column. The interfering HPLC peaks which were detected with the previously used extraction method [3], were eliminated by using the described purifications (Fig. 3A); a simple purification procedure as described by Edelbroek *et al.* [13] showed interfering peaks. A chromatogram from a control urine after addition of PH and the metabolites is shown in Fig. 3B, and that of a patient urine after hydrolysis and extraction in Fig. 3C. The PH and metabolites calibration curves were measured in a range of 0.1–1 μg/ml and 0.02–0.2 μg/ml respectively; calibration curves were linear ($r = 0.996$–0.999); precision and accuracy at different concentrations are shown in Table 2; limits of detection were 0.05 μg/ml and 0.01 μg/ml for PH and the metabolites, respectively. Analytical recoveries for PH and the metabolites at the different concentrations averaged 85%, comparable to the values obtained with liquid–liquid extractions (84%), but with higher selectivity.

The total amount of PH and metabolites excreted in urine represented an average of 60% of the dose [6,10].

3.3. Breast milk analysis

No direct experimental evidence on the passage of PH into human milk in lactating mothers

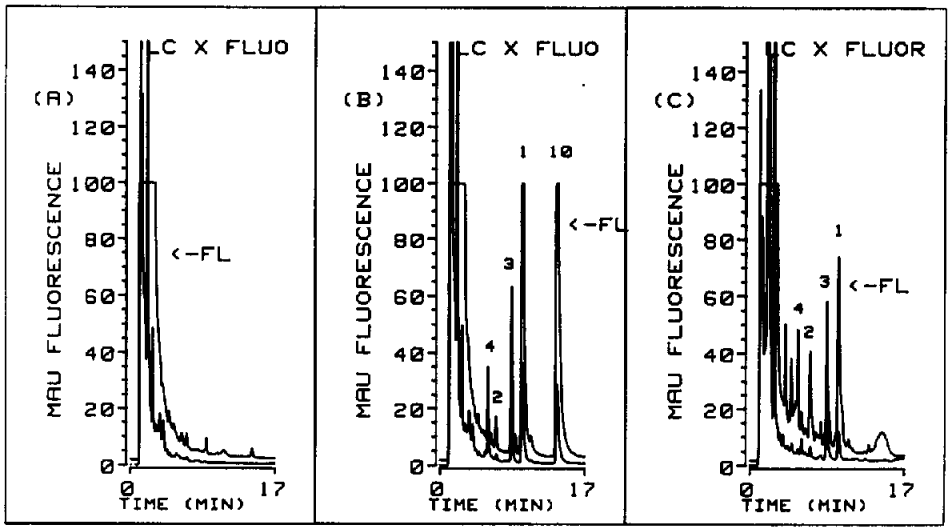

Fig. 3. HPLC analysis of urine extracts: (A) control urine; (B) control urine after addition of 0.5 μg/ml PH and metabolites: 6-OH-PH 20 ng/ml, 7-OH-PH 200 ng/ml and 4'-OH-PH 50 ng/ml; (C) urine sample from a patient under PH therapy (PH concentration 0.15 μg/ml). HPLC system with gradient elution; UV (MAU = milli absorbance units) and fluorescence chromatograms (upper trace, FL) are overlayed; abscissa units is the fluorescence intensity. Numbers on the chromatographic peaks are those of Fig. 1.

under coumarin anticoagulation has been published, prior to our studies [19].

An assay for the analysis of PH in human milk was developed; milk was processed with a series of precipitating agents in order to remove most of the matrix compounds, before submitting the sample to the solid-phase extraction as described for plasma. A high selectivity and sensitivity (limit of detection 5 ng/ml with fluorescence detection) was obtained with this method; results

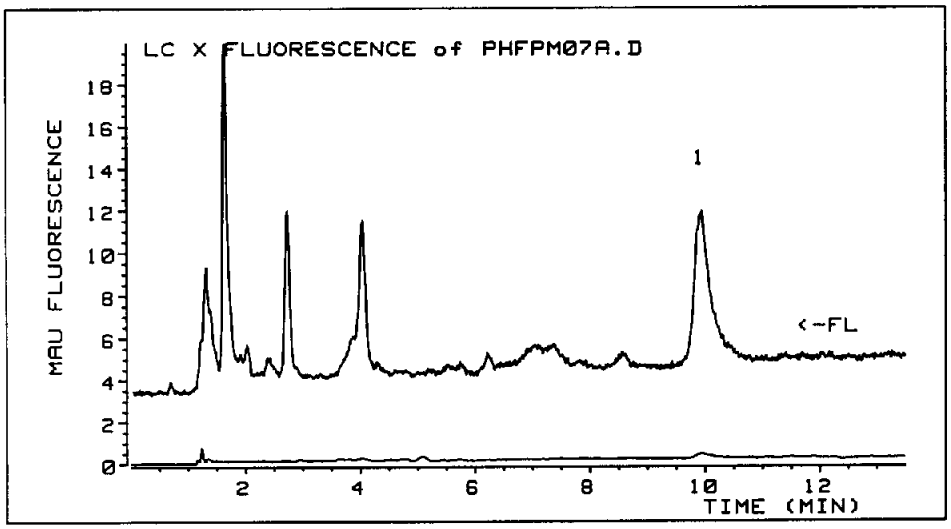

Fig. 4. HPLC of a breast milk extract; the sample was from a patient under phenprocoumon therapy (PH concentration 0.033 μg/ml). HPLC system with gradient elution; UV (MAU = milli absorbance units) and fluorescence chromatograms (upper trace, FL) are overlayed; abscissa units is the fluorescence intensity. Numbers on the chromatographic peaks are those of Fig. 1.

were validated by analysing the extracts, after methylation, with GC–MS. Fig. 4 shows the chromatogram from a breast milk extract. The method of standard additions was used for calibrations since no human control breast milk was available. The concentration of PH in the breast milk analysed varied from 26 ng/ml to 76 ng/ml; the amounts ingested with the milk will not present a high risk for the lactant when accompanied by supplementation with Vitamin K1 and clotting time controls [19].

The advantages of the present method consist in the simplicity of the solid-phase purification step, the use of smaller sample volumes for extraction, the elimination of chlorinated extraction solvents, and in the absence of interferences from endogenous compounds, other anticoagulants and drugs; also higher analytical recoveries, accuracy, calibration curve linearities and limits of detection were obtained compared with those obtained with liquid extractions.

4. Acknowledgements

We thank the Gesellschaft für Thrombose und Hämostaseforschung e.V. (Bad Nauheim, Germany) for support.

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