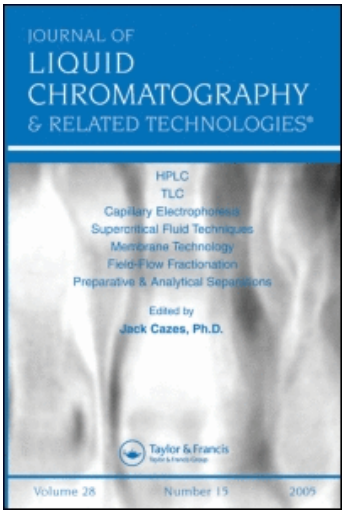

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Determination of Coumarin, 7-Hydroxy-Coumarin, 7-Hydroxycoumarin-Glucuronide, and 3-Hydroxycoumarin by High-Performance Liquid Chromatography

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DETERMINATION OF COUMARIN, 7-HYDROXY-COUMARIN, 7-HYDROXYCOUMARIN-GLUCURONIDE, AND 3-HYDROXYCOUMARIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A selective and sensitive method for the determination of coumarin and its main metabolites 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 3-hydroxycoumarin in human plasma and/or urine is described. Coumarin and 7-hydroxycoumarin were extracted from plasma with n-hexane/chloroform and with chloroform. After evaporation under vacuum, the residue was redissolved in methanol/water and injected onto the HPLC column (LiChroCART 250-4, RP 8e 5 µm; Merck, Darmstadt, Germany). The mobile phase consisted of methanol/water/tetrahydrofuran/acetic acid (45:40:10:5). 7-Hydroxycoumarin-glucuronide in plasma was enzymatically converted to 7-hydroxycoumarin by incubation with a mixture of β-glucuronidase and sulphatase in citrate-hydrochloric acid-buffer. Analysis of 7-hydroxycoumarin and its glucuronide in

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urine was performed without extraction using a linear gradient system of methanol/water/tetrahydrofuran/acetic acid as mobile phase. 3-Hydroxycoumarin was determined in urine by extraction and HPLC separation procedures as described for coumarin or 7-hydroxycoumarin in plasma samples. The assay was linear (mean: r=0.9993) in the concentration ranges studied; coumarin: 500 - 25,000 nmol/l, 7-hydroxycoumarin: 500 - 25,000 nmol/l; (plasma), 250 - 10,000 nmol/l (urine), 7-hydroxycoumarin-glucuronide: 1000 - 50,000 nmol/l (plasma), 250 - 10,000 nmol/l (urine) and 3-hydroxycoumarin: 500 - 25,000 nmol/l); with a mean accuracy of 4.6%. The lower limits of detection ranged from 60 nmol/l (7-hydroxy-metabolites in plasma) up to 300 nmol/l (coumarin). The interday precision data were determined using spiked plasma samples (range 4000-8500 nmol/l); with a mean coefficient of variation of 4.3%. The assay was used for the determination of coumarin and its 7- or 3-hydroxy-metabolites in healthy volunteers; examples of chromatograms and concentration vs. time curves are shown.

INTRODUCTION

In addition to antiphlogistic effects (1-3) and protective properties for endothelial cells (2), coumarin (5,6-benzo-α-pyrone) has been postulated to exhibit anticancer activities e.g. by inhibition of enzymes involved in carcinogenesis, like DNA-specific poly(ADP ribose)-polymerase (4). Therefore, it has been used in oral chemotherapy of metastatic renal cell carcinoma (5) and malignant melanoma (6,7). In view of the well established first-pass effect of coumarin (8), it is evident that therapeutic results observed after oral administration are unlikely related to coumarin itself but to one or more of its metabolites. Coumarin is known to exhibit hepatotoxic effects within rats and dogs, but not within mice and baboons (9). Since 7-hydroxylation is the major metabolic pathway in baboons, DBA-/2J mice and in man (10), whereas 3-hydroxylation in rats and rabbits (11), it has been suggested that hepatotoxic effects might be due to 3-hydroxycoumarin and/or its metabolites o-hydroxyphenylpyruvic-, o-hydroxyphenyllactic- and o-hydroxyphenylacetic acid (9).

To elucidate the role and disposition of coumarin and its metabolites in man, sensitive and selective methods are required for their quantification in urine or plasma. A sensitive method using fluoroscence detection after ether extraction and separation with glycine buffer has been published for the determination of coumarin and 7-hydroxycoumarin in whole blood (12). For quantification of coumarin and its hydroxy-metabolites in material obtained by ultracentrifugation of incubation mixtures containing washed rat liver microsomes, an assay using high-performance liquid chromatography (HPLC) without extraction procedures is described (13). The analysis of coumarin and its urinary metabolites by HPLC in urine samples has been demonstrated by Moran et al (14).

The aim of our study was to establish a sensitive and selective HPLC method for rapid quantitative analysis of coumarin, 7-hydroxycoumarin, its glucuronide, and 3-hydroxycoumarin in plasma or urine samples. This method should be useful for the estimation of pharmacokinetic data of coumarin and its 7- or 3-hydroxy-metabolites in man.

EXPERIMENTAL

Reagents and Chemicals

Coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin-glucuronide and 3-hydroxycoumarin were obtained from Schaper & Brümmer (Salzgitter, Germany). 7-Ethoxycoumarin, β-glucuronidase

(activity: 1,460 U/mg) from bovine liver (type B-1) and sulphatase (activity: 15.8 U/mg) from helix pomatia (type H-1) were purchased from Sigma (Deisenhofen, Germany). Chloroform and methanol were from J.T. Baker (B.V. Devener, Holland). All other chemicals were obtained from Merck (Darmstadt, Germany) and of analytical grade.

Apparatus

The HPLC system consisted of a 655 A-12 HPLC pump equipped with a 655 A-22 variable wavelength UV detector, a D-2000 chromato-integrator, and a 655 A-40 autosampler (all Merck/Hitachi, Darmstadt, Germany). As stationary phase, a LiChroCART 250-4, RP 8e 5 μm-column was used (Merck, Darmstadt, Germany). The composition of the mobile phase as well as the UV detector wavelength settings are described in the following.

Plasma and Urine Sample Preparation

Blood samples were withdrawn from the cubital vein and stored on ice in heparinized tubes for less than 60 min. After centrifuging (4000 g; 10 min), the plasma was set to pH 4.5 with 0.1 N hydrochloric acid. Four aliquots of urine collected within a period of 48 hours were set to pH 4.5 with 0.1 N hydrochloric acid. All samples were stored at -20°C until analysis.

Analysis of Coumarin and its 7-Hydroxy-metabolites in Plasma

For analysis of coumarin, 1 ml of plasma was spiked with 100 μl of the internal standard (1 mg of phenol dissolved in 20 ml of

water/methanol, 1:1, v/v) and extracted with 1.3 ml of n-hexane/chloroform, 3:10, v/v. After centrifuging (-20°C; 4000 g; 20 min) the upper (aqueous) layer was once again extracted with 1 ml of chloroform. The combined organic layers were evaporated to dryness under vacuum for about 40 min (in view of the volatility of coumarin, the extraction and evaporation must be done very carefully), and the residue was redissolved with 500 µl of water/methanol, 1:1, v/v. Fifty µl of this solution were injected onto the HPLC column. The mobile phase of the HPLC system consisted of methanol/water/tetrahydrofuran/acetic acid (45:40:10:5). The flow-rate was set to 1 ml/min and the temperature was ambient. The UV detector wavelength was set to 270 nm (coumarin) or 318 nm (7-hydroxy-metabolites).

For analysis of the 7-hydroxy-metabolites, 2 ml of plasma were spiked with 50 µl of the internal standard (3.79 mg of 7-ethoxycoumarin dissolved in 20 ml of water/methanol, 1:1, v/v). After 1:1 splitting, 10 µl of a mixture of β-glucuronidase (4000 U/ml) and sulphatase (40 U/ml) dissolved in citrate-hydrochloric acid-buffer (10 g citric acid-monohydrate dissolved in 100 ml of 1 N sodium hydroxide were set to pH 5.0 with 0.1 N hydrochloric acid) were added to one of the aliquots. Both aliquots were then incubated at 37°C for 24 hours. Thereafter, the samples were extracted and analyzed as described above. The concentration of the glucuronide was calculated as the concentration difference of 7-hydroxycoumarin from both aliquots.

Analysis of 7-Hydroxy-metabolites in Urine

Urine samples were diluted with water (1:500, v/v), and 250-µl-aliquots of the dilutions were mixed with 250 µl of

water/methanol (1:1, v/v). After addition of 50 µl of the internal standard (3.79 mg of 7-ethoxycoumarin dissolved in 40 ml of water/methanol, 1:1, v/v), 50-µl-aliquots were injected onto the HPLC column. A linear gradient system of methanol (0-2 min: 5%, 2-3 min: 5-40%, 3-15 min: 40%, 15-16 min: 40-5%, 16-23 min: 5%), water (0-2 min: 75%, 2-3 min: 75-40%, 3-15 min: 40%, 15-16 min: 40-75%, 16-23 min: 75%) and 20% of tetrahydrofuran/acetic acid (1:1; v/v) was used as mobile phase. The UV detector was set to 318 nm.

Analysis of 3-Hydroxycoumarin in Urine

Urine samples (1 ml) were spiked with 50 µl of internal standard (3.79 mg of 7-ethoxycoumarin dissolved in 20 ml of water/methanol, 1:1, v/v). The samples were extracted as described for coumarin analysis in plasma. The mobile phase (isocratic, 1 ml/min) consisted of methanol/water/tetrahydrofuran/acetic acid (45:40:10:5). The UV detector was set to 308 nm.

Calibration Curves, Precision and Recovery

For calibration, plasma and urine standards were spiked with fixed amounts of the internal standard and with variable concentrations of coumarin (range: 500 - 25,000 nmol/l), 7-hydroxycoumarin (range: 500 - 25,000 nmol/l; plasma, range: 250 - 10,000 nmol/l; urine), 7-hydroxycoumarin-glucuronide (range: 1000 - 50,000 nmol/l; plasma, range: 250 - 10,000 nmol/l; urine) and 3-hydroxycoumarin (range: 500 - 25,000 nmol/l). Calibration curves were plotted by correlating the peak-height ratio (analyte/standard) against the corresponding concentrations of the

analyte. The accuracy of the method was evaluated by assaying plasma samples spiked with known concentrations as described above and calculated according to (15). The interday precision of the assay was estimated by measuring plasma standards with known concentrations of the analyte on 30 days (3-hydroxycoumarin: 15 days). The extraction efficiency was determined by comparison of the detector signals obtained from HPLC separation of standard solutions and from spiked plasma samples analyzed as described above.

RESULTS

The extraction efficiencies as well as the lower limits of detection are listed in Table 1. The assay was linear in the concentration ranges studied; the least squares regression lines (y: analyte/standard peak height ratio, x: analyte concentration; nmol/l) and the corresponding coefficients of correlations are depicted in Table 2. The accuracy of the method is shown in Table 3; the theoretical concentrations (spiked conc.) agreed with the assayed concentrations with an accuracy mean value of 4.6%. For interday precision studies, the mean concentrations, standard deviations and coefficients of variation are shown in Table 4. The assay was used for the determination of coumarin and its metabolites in samples from healthy volunteers which had received different doses of coumarin either orally our intravenously (after written informed consent; the study has been approved by the ethics comittee of the medical faculty of the Georg-August-University, Göttingen, Germany). In Fig. 1 - 4, as examples,

TABLE 1

Extraction Efficiencies and Lower Limits of Detection

	Extraction efficiency (%)	Lower limit of detection (nmol/l; signal-to-noise ratio)
C(*)	95	300; 5:1
7HCP	83	60; 7:1
7HCU	90	200; 5:1
7HCGP	77(**)	60; 7:1
7HCGU	90	200; 10:1
3HC	95	120; 3:1

*C: coumarin; 7HCP: 7-hydroxycoumarin in plasma; 7HCU: 7-hydroxycoumarin in urine; 7HCGP: 7-hydroxycoumarin-glucuronide in plasma; 7HCGU: 7-hydroxycoumarin-glucuronide in urine; 3HC: 3-hydroxycoumarin.

**about 93% of the 7-hydroxycoumarin-glucuronide are enzymatically converted to 7-hydroxycoumarin.

TABLE 2

Regression Analysis of the Calibration Curves

y = mx + b; y: analyte/standard peak height ratio
x: analyte concentration; nmol/l

r = coefficient of correlation
n = number of calibration curves from which the data were calculated

	m (* 0.0001)	b (* 0.01)	r	n
C(*)	1.136	1.779	0.9996	15
7HCP	4.988	0.560	0.9997	15
7HCU	3.168	- 1.878	0.9998	15
7HCGP	3.773	8.498	0.9994	15
7HCGU	1.838	- 0.472	0.9996	15
3HC	0.440	- 3.023	0.9975	7

*see TABLE 1 Mean: 0.9993

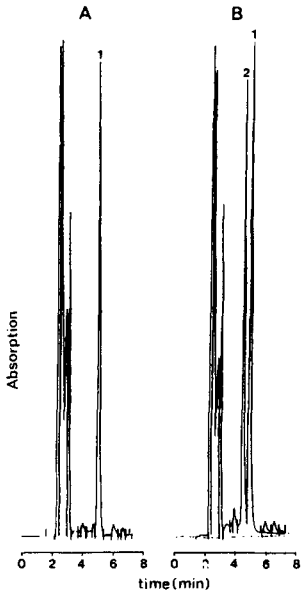

FIGURE 1

Chromatograms of peripheral venous plasma samples before (A) and 90 min after (B) i.v. application of 250 mg of coumarin (peak 1: internal standard, peak 2: coumarin). The coumarin concentration calculated from chromatogram (B) was 8000 nmol/l.

chromatograms of peripheral venous plasma or urine samples containing cumarin or its metabolites are depicted. Fig. 5 shows plasma concentration vs. time curves of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in a subject after i.v.-injection of 250 mg of coumarin.

DISCUSSION

It was the aim of our study to develop a method suitable for fast and easy quantification of coumarin and its major metabolites

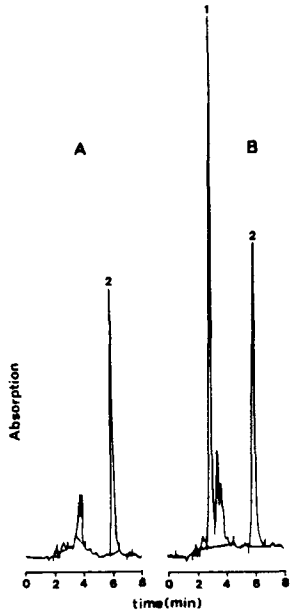

FIGURE 2

Determination of 7-hydroxycoumarin in human plasma. Chromatograms of peripheral venous plasma samples before (A) and 90 min after (B) i.v. application of 250 mg of coumarin (peak 1: 7-hydroxycoumarin, peak 2: internal standard). The 7-hydroxycoumarin concentration calculated from chromatogram (B) was 5400 nmol/l.

in human plasma and urine by only one extraction and HPLC separation procedure. The different chromatographic properties of the analytes, and interfering plasma and urine peaks, however, required special sample pretreatment procedures and HPLC conditions. In contrast to urine, the determination of 7-hydroxycoumarin-glucuronide was not successfull in plasma samples, since, in view of its high polarity, separation from plasma components could not be achieved. Therefore, enzymatic cleavage was performed by using a mixture of β-glucuronidase and

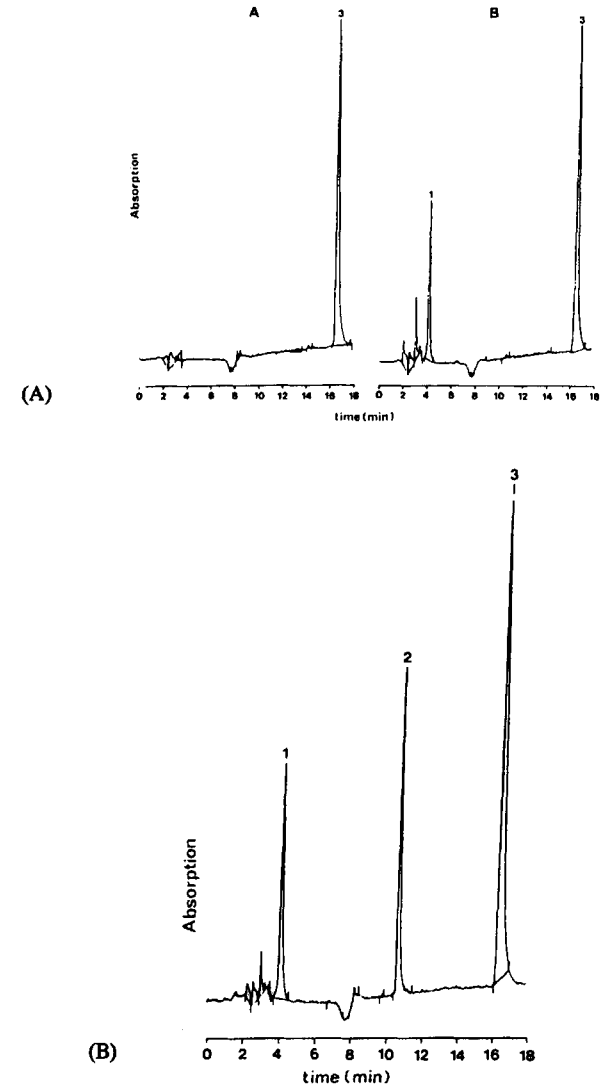

FIGURE 3

Determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine. Figure 3a shows chromatograms of urine samples before (A) and after (B) i.v. application of 250 mg of coumarin (peak 1: 7-hydroxycoumarin-glucuronide). The concentration of 7-hydroxycoumarin-glucuronide calculated from chromatogram (B) was 2800 nmol/l. Since 7-hydroxycoumarin was not dectectable in urine after this dose, a chromatogram of urine spiked with 7-hydroxycoumarin (peak 2) as well as its glucuronide (peak 1) is shown (Figure 3b). Peak 3: internal standard.

TABLE 3

Accuracy of the Assay

Accuracy (%) = 100 • |s - f|/s (*)

s: spiked concentration
f: found concentration (data not shown)

Spiked conc. (nmol/l)	Accuracy (%)					
	C(**)	7HCP	7HCU	7HCGP	7HCGU	3HC
250			4.0		2.0	
500	17.6	11.0	4.6		1.0	8.4
750			3.9		8.0	
1000	12.1	6.8	3.2	7.5	9.9	4.3
1250			1.6		6.7	
1500			0.4		1.3	
2000	3.5	4.7	3.0	5.9	1.9	2.9
2500			7.6		0.6	
4000				6.6		
5000	3.1	5.6	2.0		6.2	5.5
7500			1.2		2.0	
10000	6.3	3.0	0.7	7.1	0.1	3.7
12500						2.3
20000	0.9	1.6		5.4		4.8
25000	7.5	6.1				4.1
40000				1.5		
50000				3.3		

*calculated according to ref. (15)

**see TABLE 1

Mean: 4.6

sulphatase, which yielded the best recoveries. In urine, separation of 7-hydroxycoumarin as well as its glucuronide could easily be achieved without extraction and cleavage. This is an advantage compared to the method of Moran et al. (14) which requires enzymatic cleavage of the 7-hydroxycoumarin-glucuronide in urine samples. In comparison to the method using fluorescence detection (12), our assay, on the one hand, offers the possibilty to determine coumarin and 7-hydroxycoumarin by a selective HPLC

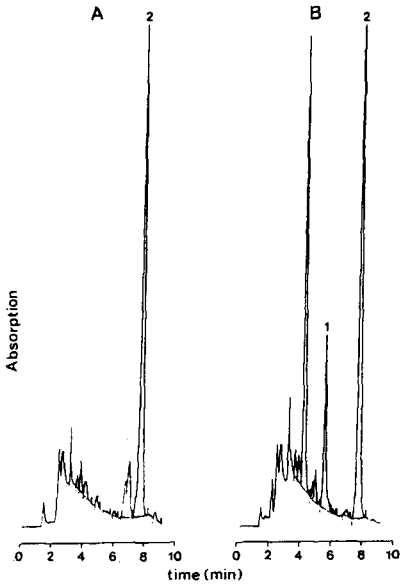

FIGURE 4

Determination of 3-hydroxycoumarin in urine. Chromatograms of urine plasma samples before (A) and 6 hours after (B) p.o. application of 1000 mg of coumarin (peak 1: 3-hydroxycoumarin, peak 2: internal standard). The 3-hydroxycoumarin concentration calculated from chromatogram (B) was 9600 nmol/l.

procedure, and on the other hand, it allows quantification of 7-hydroxycoumarin-glucuronide in plasma. A method is reported for the determination of 3-hydroxycoumarin in biological samples from cell incubations after ultracentrifugation without extraction procedures (13). In comparison, our method allows sensitive determination of 3-hydroxycoumarin in urine, however, extraction is required.

In conclusion, though it has not been possible to establish a "standard procedure" which allows quantification of coumarin and

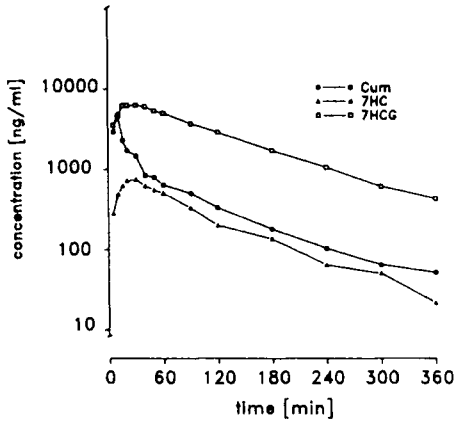

FIGURE 5

Plasma concentration vs. time curves of coumarin, 7-hydroxy-coumarin and 7-hydroxycoumarin-glucuronide after i.v.-injection of 250 mg of coumarin.

TABLE 4

Interday Precision

	Spiked concentration (nmol/l)	Found concentration (mean ± S.D.) (nmol/l)	Coeff. of Variation (%)	n (days)
C(*)	4000	4357 ± 189	4.3	30
7HCP	4000	3963 ± 187	4.7	30
7HCU	3750	3552 ± 139	3.9	30
7HCGP	8000	8050 ± 230	2.9	30
7HCGU	3750	3720 ± 204	5.4	30
3HC	8500	8736 ± 383	4.4	15
*see TABLE 1		Mean:	4.3	

its main metabolites by only one extraction and separation step independend from the kind of sample matrix, a sensitive and selective assay has been developed which already has been proven as a useful routine method for the estimation of pharmacokinetic parameters of coumarin and its main metabolites in man.

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