Journal of Chromatography, 582 (1992) 137–143
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6539

Rapid and sensitive determination of coumarin and 7-hydroxycoumarin and its glucuronide conjugate in urine and plasma by high-performance liquid chromatography

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(First received February 25th, 1992; revised manuscript received August 11th, 1992)

ABSTRACT

A rapid and sensitive high-performance liquid chromatographic method was developed for the analysis of coumarin, 7-hydroxycoumarin and its glucuronide conjugate in urine and plasma. This method was used to monitor the urinary excretion of these compounds following a single oral dose of coumarin (100 mg). This new method gives excellent chromatographic separation and includes an internal standard. The method was validated and shown to be both accurate and precise in the range 0.5–100 μg/ml.

INTRODUCTION

Coumarin is a naturally occurring constituent of many plants. It has been used in the treatment of a diverse range of diseases, such as cancer, burns, brucellosis and rheumatic disease. Coumarin has a wide variety of uses in industry, mainly due to its strong fragrant odour [1].

Coumarin is metabolised initially by a specific cytochrome P-450 system [2], resulting in hydroxylation prior to phase II conjugation. The most common routes of hydroxylation are at positions 7 and 3, to yield 7-hydroxycoumarin (7-OHC) and 3-OHC, respectively. In primates, such as man and baboons, the major metabolite formed is 7-OHC [1]. The major phase II metabolite is a glucuronide conjugate (7-OHCG). Moran *et al.* [3] showed that on average 63% of a total dose of 200 mg of coumarin was recovered as 7-OHC in the urine of volunteers over a 24-h period, with most of this recovered within the first 10 h.

A number of techniques have been applied to analyse coumarin. These include paper and gas chromatography [4], spectrophotofluorimetry [5] and high-performance liquid chromatography (HPLC) [3,6]. Moran *et al.* [3] developed a HPLC method with a satisfactory separation and short retention time. They used a μBondapak C_{18} radial compression type column, a fixed detection wavelength of 280 nm and a mobile phase of methanol–water–acetic acid (200:300:1, v/v/v). This method was used routinely to monitor coumarin metabolism. However, it requires substantial sample preparation (approximately 24 h) prior to chromatographic analysis. It was shown previously by Tan *et al.* [5], that diethyl ether could successfully be used to extract coumarin and 7-OHC from biological samples.

The new method which we have developed and validated is a significant improvement on the

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0378-4347/92/$05.00

Moran method [3], since the assay time is reduced (to approximately 2 h), the amount of enzyme and incubation time required to hydrolyse 7-OHCG is reduced, and an internal standard is used which is well resolved from both coumarin and 7-OHC.

EXPERIMENTAL

Chemicals

Coumarin was kindly provided by Schaper and Brummer (Salzgitter, Germany). 7-OHC was purchased from Sigma (St. Louis, MO, USA). 7-Amino-4-methyl-coumarin was purchased from Aldrich (Dorset, UK) and used as an internal standard (I.S.). A stock solution (1 mg/ml) of coumarin and 7-OHC was prepared daily in 10 ml of methanol (HPLC grade, Lab-Scan, Dublin, Ireland), and diluted to 100 ml with ultrapure water. Working solutions (in the range 5–1000 μg/ml) were prepared by appropriate dilution of the stock solution with ultrapure water. A 1 mg/ml working solution of I.S. was prepared in 100% methanol.

Sample preparation

The urine samples were obtained from human volunteers who had received a single oral dose of coumarin (100 mg). Urine samples were collected prior to administration of coumarin, and at 5, 10, 15 and 24 h, and the urinary volumes were recorded.

A 0.8-ml aliquot of control urine was transferred to a 12-ml glass tube (Medlabs, Dublin, Ireland), spiked with 0.1 ml of coumarin and 0.1 ml of 7-OHC standard to give final concentrations of 0.5–100 μg/ml. A 50-μl aliquot of I.S. was added and vortex-mixed.

A 1-ml aliquot of patient urine or standard was extracted using 3 ml of diethyl ether (HPLC grade, Lab-Scan) and mixed by inversion for 10 min. The mixture was centrifuged at 600 *g* for 10 min. A 1.8-ml volume of the organic layer was removed, evaporated to dryness under nitrogen and reconstituted with 200 μl of methanol. The samples were then centrifuged at 600 *g* for 5 min, and 120 μl of the supernatant were transferred to a clean glass tube. Aliquots (20 μl) of this supernatant were injected for HPLC analysis.

The concentration of free and conjugated 7-OHC was determined by pre-treating a 1-ml aliquot of urine with 1 ml of β-glucuronidase (Sigma), at a concentration of 5000 U/ml in 1 *M* sodium acetate buffer, pH 5.0. The mixture was incubated for 30 min at 37°C and extracted as before. This liberated the conjugated 7-OHC and allowed the estimation of total 7–OHC. By subtracting the concentration of free from total, the concentration of 7-OHC excreted as 7-OHCG could be determined.

The optimum incubation time and β-glucuronidase concentration, in 1 *M* acetate buffer, pH 5.0, was determined using a patient sample with a high concentration of 7-OHCG.

To determine the intra-day precision and accuracy, five calibration sets of coumarin and 7-OHC were extracted from urine and plasma and injected on the same day. The inter-day variability for urine and plasma samples was determined by preparing a calibration set on five different days, with injection of each set on the day it was prepared.

The stability of coumarin and 7-OHC following extraction from urine was determined by storing one set of the inter-day calibration solutions at 4°C for 72 h prior to reinjection. The stability of unextracted standards was determined over a similar period following storage at room temperature.

HPLC conditions

The HPLC system used was a reversed-phase chromatograph, with a de-gassed mobile phase of methanol–water–acetic acid (300:700:2, v/v/v). The column used was a pre-packed Waters C_{18} μBondapak (300 mm × 3.9 mm I.D.) at ambient temperature, with a particle size of 10 μm. A pre-column (5 mm × 5 mm I.D.) of the same packing material was used for the analysis of urine samples. The flow-rate was 2 ml/min (Model 126 Beckman System Gold programmable solvent module) and the absorbance units full scale (a.u.f.s.) was 0.05, as measured at 324 nm (Model 166 Beckman System Gold programmable detec-

tion module). Quantitation of coumarin and 7-OHC was made by measuring peak-height ratio from a standard curve.

RESULTS AND DISCUSSION

We have developed a method for the assay of free and conjugated 7-OHC and coumarin in urine samples from human volunteers. This new method has also been applied to the analysis of coumarin and 7-OHC in plasma.

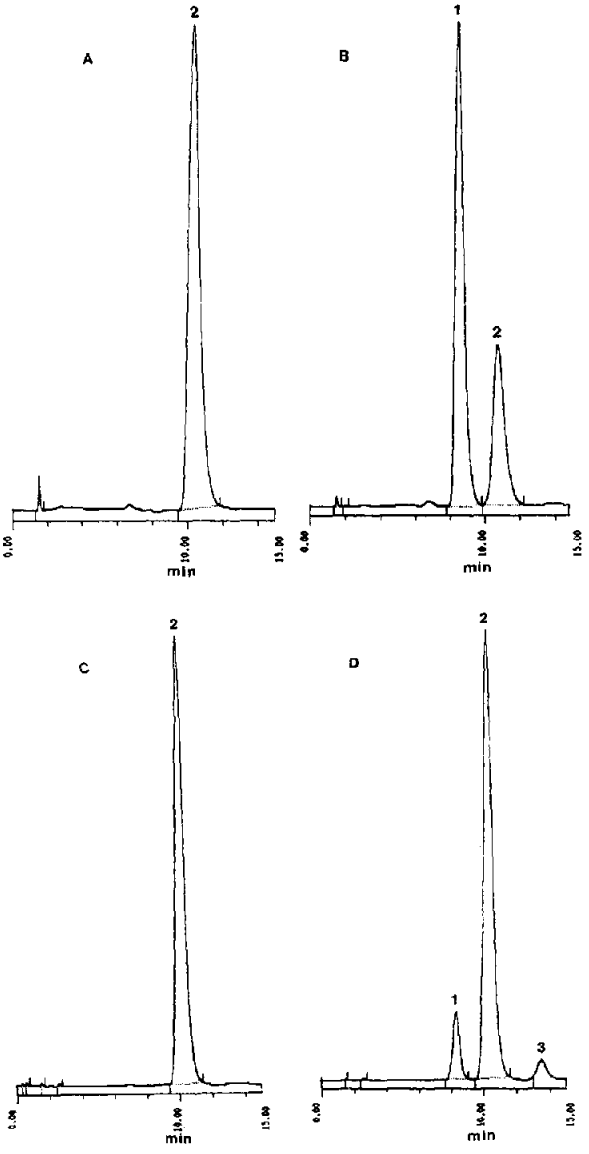

Fig. 1. Chromatograms of sample extracts. (A) Patient urine blank; (B) patient urine sample containing 7-OHC at a concentration of 85.7 μg/ml 10 h after drug administration; (C) blank plasma; (D) blank plasma spiked with coumarin and 7-OHC at a concentration of 5 μg/ml. Peaks: 1 = 7-OHC (8.3 min); 2 = I.S. (10.3 min); 3 = coumarin (13.5 min).

Chromatographic separation

Five coumarin derivatives were tested for suitability as internal standards. These were 4-hydroxycoumarin, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, 3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (hydrochloride), coumarin-3-carboxylic acid and 7-amino-4-methylcoumarin. The latter was the only compound that was extracted and was well resolved from both coumarin and 7-OHC. The excellent separation and lack of possible interfering peaks can be seen in Fig. 1. The development of a method with an internal standard marks a significant improvement on the method by Moran *et al.* [3].

TABLE I

RECOVERY OF COUMARIN AND 7-OHC FROM URINE AND PLASMA (n = 3)

Concentration (μg/ml)	Urine		Plasma	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Coumarin				
5.0	101.6 ± 1.4	1.3	75.6 ± 1.2	1.6
10.0	89.3 ± 1.9	2.1	80.9 ± 1.4	1.7
20.0	96.9 ± 3.2	3.3	97.4 ± 1.4	1.4
40.0	97.9 ± 2.0	2.1	98.2 ± 1.3	1.3
60.0	100.7 ± 1.2	1.2	99.3 ± 1.3	1.3
80.0	97.2 ± 4.3	4.4	97.4 ± 1.2	1.3
100.0	103.4 ± 3.9	3.8	103.2 ± 1.6	1.5
7-OHC				
0.5	92.8 ± 1.4	1.5	90.2 ± 2.0	2.2
1.0	109.1 ± 4.3	3.9	96.4 ± 0.9	0.9
5.0	97.8 ± 3.8	3.9	97.4 ± 0.9	1.0
10.0	91.6 ± 2.3	2.5	99.1 ± 0.9	0.9
20.0	99.4 ± 4.5	4.5	99.4 ± 0.7	0.7
40.0	103.4 ± 0.9	0.9	102.2 ± 1.0	1.0
60.0	102.1 ± 2.4	2.4	100.2 ± 0.7	0.7
80.0	97.5 ± 0.9	0.9	99.4 ± 1.6	1.6
100.0	103.7 ± 1.6	1.5	102.0 ± 1.4	1.3

TABLE II

INTRA-ASSAY PRECISION AND ACCURACY FOR COUMARIN AND 7-OHC FOLLOWING EXTRACTION FROM URINE AND PLASMA (n = 5)

Concentration added (μg/ml)	Coumarin		7-OHC	
	Found (mean ± S.D.) (μg/ml)	R.S.D. (%)	Found (mean ± S.D.) (μg/ml)	R.S.D. (%)
Urine				
0.5	N.D.[a]		0.5 ± 0.0	5.4
1.0	N.D.		1.1 ± 0.1	5.5
5.0	5.3 ± 0.4	7.3	4.9 ± 0.2	3.4
10.0	8.9 ± 0.2	2.3	9.1 ± 0.2	2.0
20.0	19.1 ± 0.6	3.1	19.4 ± 1.0	5.0
40.0	38.2 ± 2.1	5.6	41.3 ± 0.4	1.1
60.0	61.7 ± 1.8	2.9	61.7 ± 1.3	2.1
80.0	78.7 ± 2.8	3.5	78.2 ± 0.7	0.8
100.0	100.9 ± 4.5	4.4	102.2 ± 3.1	3.1
Plasma				
0.5	N.D.		0.4 ± 0.0	2.5
1.0	N.D.		0.9 ± 0.1	8.4
5.0	5.0 ± 0.0	0.8	5.2 ± 0.2	3.2
10.0	9.8 ± 0.2	2.5	11.0 ± 0.2	2.0
20.0	20.7 ± 0.8	3.7	21.3 ± 0.5	2.4
40.0	39.6 ± 1.5	3.8	41.2 ± 0.3	0.8
60.0	61.0 ± 0.8	1.3	58.4 ± 1.2	2.1
80.0	80.7 ± 0.3	0.4	81.1 ± 1.5	1.9
100.0	95.5 ± 1.0	1.1	96.8 ± 0.7	0.7

[a] N.D. = not determined.

Linearity and limit of quantification

A calibration graph of peak-height ratio for coumarin was found to be linear from 5 to 100 μg/ml, whilst for 7-OHC it was 0.5 to 100 μg/ml, following extraction from both urine and plasma. Therefore, the limit of quantification for coumarin extracted from urine and plasma was 5.0 μg/ml, whilst for 7-OHC it was 0.5 μg/ml.

Percentage recovery

The recoveries of coumarin and 7-OHC from urine and plasma were determined across the linear range, by dividing the peak height for the extracted solution by the peak height obtained by injecting equivalent amounts of an unextracted standard solution. As Table I shows, recovery rates were *ca.* 100%.

Accuracy and precision

To determine the intra-day accuracy and precision following extraction from urine and plasma, blank samples (n = 5) were spiked with both coumarin and 7-OHC at concentrations across the linear range. The extracts were injected five times, and the concentration determined from a calibration set. The percentage relative standard deviation (R.S.D.) for both compounds are shown in Table II.

The inter-day accuracy and precision of the method was also determined for coumarin and 7-OHC over a five-day period. Blank urine was spiked with coumarin and 7-OHC across the linear range. The concentrations were determined from a calibration set prepared daily and are shown in Table III.

TABLE III

INTER-ASSAY PRECISION AND ACCURACY FOR COUMARIN AND 7-OHC FOLLOWING EXTRACTION FROM URINE AND PLASMA (n = 5)

Concentration added (μg/ml)	Coumarin		7-OHC	
	Found (mean ± S.D.) (μg/ml)	R.S.D. (%)	Found (mean ± S.D.) (μg/ml)	R.S.D. (%)
Urine				
0.5	N.D.[a]		0.5 ± 0.0	6.3
1.0	N.D.		1.0 ± 0.1	6.3
5.0	5.4 ± 0.4	7.9	5.0 ± 0.1	2.7
10.0	10.0 ± 0.4	4.0	9.9 ± 0.4	4.3
20.0	19.7 ± 0.9	4.7	20.8 ± 1.0	4.9
40.0	38.5 ± 0.6	1.5	41.8 ± 1.7	4.0
60.0	59.3 ± 1.1	1.8	62.0 ± 2.1	3.4
80.0	80.1 ± 1.4	1.7	80.3 ± 2.7	3.3
100.0	101.9 + 1.1	1.1	99.5 ± 2.8	2.8
Plasma				
0.5	N.D.		0.3 ± 0.0	2.5
1.0	N.D.		1.0 ± 0.0	1.0
5.0	5.0 ± 0.1	1.8	5.1 ± 0.1	2.5
10.0	9.7 ± 0.5	5.0	10.0 ± 0.4	3.9
20.0	20.3 ± 0.9	4.6	20.9 ± 0.5	2.6
40.0	39.4 ± 0.7	1.7	40.7 ± 1.2	3.0
60.0	61.0 ± 1.1	1.8	58.6 ± 1.0	1.7
80.0	80.7 ± 0.5	0.6	82.4 ± 1.9	2.3
100.0	96.7 ± 1.8	1.9	97.8 ± 1.6	1.6

[a] N.D. = not determined.

Stability

The stability of a range of coumarin and 7-OHC standards following extraction from control urine was determined after storage at 4°C over a 72-h period. The results are shown in Table IV. The percentage accuracy indicates that both coumarin and 7-OHC remain stable. However, unextracted coumarin and particularly 7-OHC appear to become unstable following storage at room temperature for 72 h as indicated in Table IV. There is a significant loss in linearity which leads to erroneous results. This would, therefore, imply that fresh 7-OHC standards must be prepared daily.

Hydrolysis of 7-OHCG using β-glucuronidase

Previously, Moran *et al.* [3] hydrolysed 7-OHCG by incubating the urine sample with the enzyme β-glucuronidase at a concentration of 20 000 U/ml for 16 h in 1 *M* sodium acetate buffer, pH 3.8. However, it is shown in Table V that the enzyme is effective in completely hydrolysing 7-OHCG to form free 7-OHC, if incubated at 5000 U/ml in 1 *M* sodium acetate buffer, pH 5.0, for 30 min. The calculated 7-OHC concentrations using 5000 U/ml with an incubation time of 30 min were almost equivalent to those following incubation with 20 000 U/ml for 16 h (Table V). Sodium acetate buffer (1 *M*), pH 3.8, was found to be as equally effective as pH 5.0, in the com-

TABLE IV

STABILITY OF COUMARIN AND 7-OHC STANDARDS, BOTH BEFORE AND AFTER EXTRACTION FROM URINE, FOLLOWING STORAGE AT ROOM TEMPERATURE AND 4°C, RESPECTIVELY, FOR 72 h (n = 4)

Concentration added (μg/ml)	Unextracted		Extracted	
	Found (mean ± S.D.) (μg/ml)	Accuracy[a] (%)	Found (mean ± S.D.) (μg/ml)	Accuracy[a] (%)
Coumarin				
0.5	N.D.[b]		N.D.	
1.0	N.D.		N.D.	
5.0	5.5 ± 0.4	110	5.7 ± 0.4	114
10.0	9.3 ± 0.4	93	10.1 ± 0.3	101
20.0	16.6 ± 0.7	83	18.8 ± 0.6	94
40.0	35.2 ± 0.9	88	36.3 ± 1.0	91
60.0	56.4 ± 1.0	94	60.0 ± 1.2	95
80.0	84.8 ± 1.1	105	85.0 ± 1.3	106
100.0	100.8 ± 1.2	101	99.5 ± 1.5	99
7-OHC				
0.5	N.D.		0.5 ± 0.0	92
1.0	N.D.		0.9 ± 0.1	85
5.0	N.D.		4.1 ± 0.1	83
10.0	9.5 ± 0.9	95	9.2 ± 0.4	91
20.0	28.6 ± 1.0	143	20.8 ± 1.0	104
40.0	62.4 ± 1.1	156	42.9 ± 1.0	107
60.0	86.1 ± 1.2	144	66.0 ± 1.1	110
80.0	77.3 ± 1.2	97	83.0 ± 1.6	103
100.0	76.2 ± 1.1	76	92.1 ± 1.3	92

[a] Accuracy = (found/added) · 100%.
[b] N.D. = not determined.

TABLE V

HYDROLYSIS OF 7-OHCG WITH β-GLUCURONIDASE IN 1 M SODIUM ACETATE BUFFER, pH 5.0, AT 37°C TO YIELD FREE 7-OHC (n = 3)

Incubation time (h)	Activity (U/ml)	7-OHC found (mean ± S.D.) (μg/ml)	R.S.D. (%)
16	20 000	73.6 ± 0.03	0.04
16	10 000	79.0 ± 0.36	0.46
16	5 000	77.2 ± 0.67	0.87
0.5	20 000	76.1 ± 0.50	0.62
0.5	10 000	76.1 ± 0.16	0.21
0.5	5 000	78.4 ± 0.29	0.37

TABLE VI

URINARY CONCENTRATIONS OF FREE, TOTAL AND CONJUGATED 7-OHC EXCRETED OVER A 24-h PERIOD FOLLOWING ORAL ADMINISTRATION OF COUMARIN (100 mg)

Time after administration (h)	7-OHC concentration (μg/ml)		
	Free	Total	Conjugated
0.0	0.0	0.0	0.0
5.0	3.7	367.0	363.3
10.0	1.3	85.7	84.4
15.0	0.0	12.6	12.6
24.0	0.0	0.0	0.0

plete hydrolysis of 7-OHCG at 5000 U/ml and 30 min at 37°C (results not included).

Clinical and pharmacokinetic applications

The method proposed here has proven itself to be suitable for clinical use or pharmacokinetic studies. Analysis of urine samples, before and after drug administration in one patient on long-term coumarin therapy, was successfully carried out. The concentration of free and total 7-OHC excreted was determined at regular intervals following administration. The concentrations (μg/ml) are shown in Table VI. No free coumarin was detected in the urine samples analysed. This was similar to the findings of Moran *et al.* [3]. The results show that 96% of the total dose administered was recovered over a 24-h period.

CONCLUSION

In conclusion, the proposed method is rapid, simple, sensitive and reliable, thus enabling its use in routine clinical applications.

ACKNOWLEDGEMENTS

We would like to thank Schaper and Brummer, Germany, The Coumarin Research Fund and The Research and Postgraduate Studies Committee, Dublin City University, Ireland, for their financial support for this work.

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