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Simultaneous determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in human serum and plasma by high-performance liquid chromatography

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Abstract

A HPLC method was developed for the determination of the metabolites of coumarin and 7-hydroxycoumarin in plasma and serum. Separation was based on gradient elution of 7-hydroxycoumarin glucuronide, 7-hydroxycoumarin, coumarin and finally 4-hydroxycoumarin (which is used as an internal standard). Standards, prepared in plasma or serum, and samples were treated with trichloroacetic acid, mixed and centrifuged. The supernatant was removed and analyzed by reversed-phase high-performance liquid chromatography on a C_{18} column. The limit of detection was 50 ng/ml for 7-hydroxycoumarin and 200 ng/ml for coumarin and 7-hydroxycoumarin glucuronide. The linear range was 0.5–100 μg/ml for each of the analytes. The percentage relative standard deviation about the mean measured concentrations were all below 10%. There was no statistical difference between the standard curves prepared in plasma or serum. The method developed was applied to the determination of each of the three compounds in serum, after the administration of 7-hydroxycoumarin, and in plasma after the administration of coumarin. The concentrations of total 7-hydroxycoumarin in the serum samples were also determined by another HPLC method and the results were compared. There was no statistical difference between the results determined.

Keywords: Coumarin; 7-Hydroxycoumarin; 7-hydroxycoumarin glucuronide

1. Introduction

Coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin glucuronide and 4-hydroxycoumarin are all members of the benzo-pyrones. Coumarin and 7-hydroxycoumarin are found in nature and have both been used as trial drugs in cancer treatment [1,2] and in the treatment of oedemas [2–4]. In man 7-hydroxycoumarin is the principal phase I metabolite of coumarin in vivo [2,5] and it is then further metabolised to 7-hydroxycoumarin glucuronide, the principal phase II metabolite. Coumarin is metabolised by a specific cytochrome P450 in the liver (CytP450Coh or CytP4502A6) [5]. The coumarin 7-hydroxylation reaction requires the presence of nicotine adenine dinucleotide phosphate (NADPH). The identity of the coumarin 7-hydroxylase enzyme in other species has not been fully established [2,6]. 7-hydroxycoumarin is metabolised by uridine diphosphate glucuronyl transferase (UDPGT) to 7-hydroxycoumarin glucuronide by replacing the hydrogen in the hydroxy moiety with glucuronic acid from uridine diphosphate glucuronic acid at the 7-position.

After the administration of coumarin in man, it is

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quickly metabolised to 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide, and excreted principally in the urine, although free 7-hydroxycoumarin and the glucuronide form can be detected circulating in the plasma and serum [8–13]. The clinical activity observed after coumarin administration is generally attributed to its metabolite, 7-hydroxycoumarin [13]. Circulating serum levels of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide have been observed many days after coumarin administration [13]. It has been suggested that a cyclic deconjugating–conjugating system is in operation to enable the drug to act at a cellular level [14].

HPLC has been widely applied to the study of coumarin and its metabolites after both in vivo and in vitro metabolism [7,15]. It has also been used for the determination of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide simultaneously [16]. Capillary electrophoresis (CE) has also been used for the direct determination of 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin in urine [17]. CE was also used for the determination of the rate of glucuronidation of UDPGT in a crude liver enzymatic preparation [18]. The method developed below was also used for the determination of the rate of glucuronidation of 7-hydroxycoumarin in vitro and compared to the results from the CE method [19]. The majority of methods use β-glucuronidase to deconjugate 7-hydroxycoumarin from 7-hydroxycoumarin glucuronide and some solvent extraction step [9,15,20]. However, the method developed here does not require any deconjugation or solvent extraction step. The HPLC method developed was applied to the determination of 7-hydroxycoumarin, 7-hydroxycoumarin glucuronide and coumarin in plasma and serum from two studies with volunteers who were treated orally with coumarin or 7-hydroxycoumarin.

2. Experimental

2.1. Chemicals and equipment

7-Hydroxycoumarin was purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid (Analar grade) and acetic acid were purchased from BDH (Poole, UK). 4-Hydroxycoumarin was purchased from Aldrich (Gillingham, UK). HPLC-grade methanol was purchased from Labscan (Dublin, Ireland). Screw cap (1.1 ml) vials were obtained from Labquip (Dublin, Ireland). Ultra-pure water was used for serial dilutions and for mobile-phase preparation.

The HPLC System Gold (Beckman Instruments, Fullerton, CA, USA) apparatus consisted of a solvent module 126, detector module 166, and autosampler module 507. All of the components were controlled by System Gold software. Separation was carried out on a Phenomenex (Macclesfield, UK) Bondclone 10 C_{18} column with gradient elution of the compounds of interest.

2.2. Preparation of standards and samples

Control human serum was obtained from St. James' Hospital (Dublin, Ireland), from people who had not been treated with coumarin or 7-hydroxycoumarin. Control human plasma was kindly donated from a healthy volunteer. Both control serum and plasma were stored at −20°C until required. A 1 mg/ml stock solution of 7-hydroxycoumarin and coumarin were both prepared in methanol–water (10:90). The 1 mg/ml stock solution of 7-hydroxycoumarin glucuronide was prepared in 100% water and the 1 mg/ml solution of 4-hydroxycoumarin was prepared in 100% methanol. A series of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide standards (0, 1, 5, 10, 20, 50, 80 and 100 μg/ml) were prepared in control plasma or serum to determine the precision and accuracy of the method.

Plasma samples: 250 mg of coumarin was administered to each of 3 volunteers and blood samples were taken at 0, 1 and 4 h, respectively. The blood was collected in NH_4-heparin coated tubes (Sarstedt, Wexford, Ireland) and the plasma was removed and stored at −20°C until required.

Serum samples: On day 1 and day 15, 4.0 g of 7-hydroxycoumarin were administered p.o. to 2 separate volunteers and 7.0 g to another. Blood samples were removed at specific time intervals (0, 5, 15, 30, 45, 60, 120, 240, 480 and 1440 min, respectively) and allowed to clot. The serum was removed and stored at −20°C until required.

Into 140 μl of control plasma or serum 20 μl of

each of the standards was added and vortex mixed – giving a final volume of 200 μl. To this 40 μl of a 20% (w/v) solution of trichloroacetic acid (TCA) was added and vortex mixed again. To 200 μl of an unknown sample of plasma or serum 40 μl of the 20% (w/v) solution of TCA was also added and vortex mixed. The samples were then centrifuged at 13 000 rpm for 10 min. The supernatant was removed into a 1.1 ml screw cap conical vial. To each vial 10 μl of the internal standard (1 mg/ml solution of 4-hydroxycoumarin) was added and the sample was vortex mixed. A 20-μl aliquot of the sample was then injected onto the HPLC column.

2.3. HPLC separation

Solvent A was a 50:950:2 (v/v) methanol–water–acetic acid mixture, and solvent B was 100% methanol. The solvents were prepared, mixed and degassed by sonication for 20 min. The eluent was monitored at 320 nm.

The 1 ml/min gradient was as follows: 0–14 min, 100% solvent A→solvent A (50%)–solvent B (50%); 14–22 min, solvent A (50%)–solvent B (50%); 22–23 min, solvent A (50%)–solvent B (50%)→solvent A (100%); 23–32 min, solvent A (100%).

3. Results and discussion

3.1. Development of the HPLC separation

A HPLC method was developed for the determination of coumarin, 7-hydroxycoumarin, and 7-hydroxycoumarin glucuronide and was applied to a range of biological fluids including urine, plasma and serum. It was found to be applicable to all three fluids. However, interference from endogenous species present in urine did not allow complete baseline separation of the analytes of interest (results from urine study not shown). Capillary electrophoresis was found to be more suitable for the direct determination of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in urine [17]. Samples were then spiked into each of the biological fluids and the method was adjusted until the analytes were successfully resolved from any endogenous species present in the matrix of interest (Fig. 1).

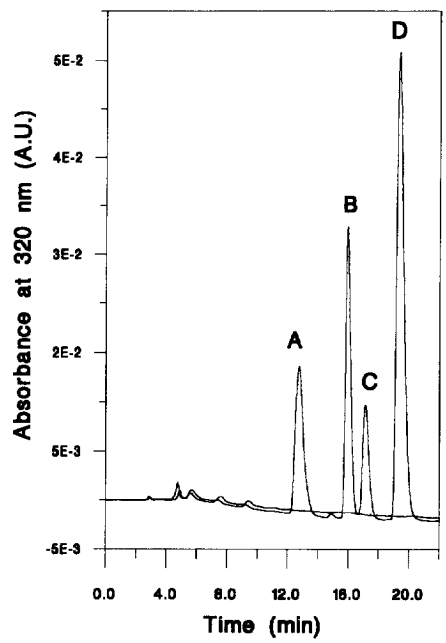

Fig. 1. Overlay of the chromatograms showing the separation of blank serum and a 20 μg/ml preparation of (A) 7-hydroxycoumarin glucuronide, (B) 7-hydroxycoumarin, (C) coumarin, and (D) the internal standard, 4-hydroxycoumarin, in serum.

The solvents used for the separation were selected because they had been successfully used previously by Egan and O'Kennedy [15] and Sharifi et al. [16]. However, when 7-hydroxycoumarin glucuronide was analyzed by the method of Egan and O'Kennedy [15] it eluted in the void volume, and, thus, a method that would retard it on the column was sought. This involved the use of a gradient elution based on their method [15]. If the percentage solvent of each changed more rapidly to 50:50 solvent A–solvent B, the samples were not fully resolved, and if the rate was slowed the time factor for separation became too long. After the gradient profile returned to 100% solvent A the absorbance was monitored until it returned to baseline zero. At this point (about 9 min later) the system was then set to inject the next sample. It is clearly seen that there is no interference from endogenous species present in the serum (Fig.

1). This is also seen for the analysis of blank plasma and standards in plasma (results not shown).

3.2. Limit of detection, linearity, precision and accuracy

In both plasma and serum the limit of detection was 50 ng/ml for 7-hydroxycoumarin and 200 ng/ml for coumarin and 7-hydroxycoumarin glucuronide. The linear detection range was from 0.5 μg/ml and 100 μg/ml for each of the analytes. The accuracy and precision was determined for standards between 1 μg/ml and 100 μg/ml. Correlation coefficients for any of the standard curves prepared were always better than 0.995. The absorbance ratio (absorbance of the analyte/absorbance of the internal standard) was plotted versus the concentration added to ascertain the precision of the method and the regression line. The typical equation for absorbance ratio for the intra assay for each of the analytes were: coumarin, $y=-0.01+0.013x$, $r^2=0.998$; 7-hydroxycoumarin, $y=-0.02+0.033x$, $r^2=0.998$; 7-hydroxycoumarin glucuronide, $y=-0.01+0.016x$, $r^2=0.999$. For the inter assay the mean standard curves were: coumarin, $y=-2e^{-3}+0.012x$, $r^2=0.999$; 7-hydroxycoumarin, $y=0.01+0.032x$, $r^2=0.999$; 7-hydroxycoumarin glucuronide, $y=2e^{-4}+0.015x$, $r^2=0.999$.

The results of the determination of the accuracy and precision of the method (Table 1) were calculated from these regression lines. Table 1 gives the mean measured concentration±standard deviation and the percentage relative standard deviations for some of the standards prepared for both intra- and inter-assay studies. It is evident that the method is both precise and accurate.

3.3. Clinical and pharmacokinetic studies

The method developed was applied to two separate studies:

3.3.1. Study 1. Plasma analysis

After the administration of coumarin to each of three volunteers plasma samples were taken from each at 0, 1 and 4 h; these were then analyzed by HPLC (Fig. 2). No free coumarin was observed in the plasma of any of the volunteers. There was a majority of 7-hydroxycoumarin glucuronide as compared to 7-hydroxycoumarin in the plasma samples. The highest levels were observed at 1 h, and after 4 h the level decreased significantly (Fig. 2). The highest concentration of 7-hydroxycoumarin glucuronide measured was 21.8 μg/ml with ca. 0.1 μg/ml of free 7-hydroxycoumarin present for volunteer 1 at 1 h. No free 7-hydroxycoumarin was detected in the plasma for any of the volunteers after 4 h. For volunteer 2, analysis of the excretion of free and conjugated 7-hydroxycoumarin in the urine indicated that approximately up to 43% of the 250 mg dose administered (ca. 98% as 7-hydroxycoumarin gluc-

Table 1
Intra-assay and inter-assay precision and accuracy for the determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in plasma and serum ($n=5$)

Concentration added (μg/ml)	Concentration measured[a] (mean±S.D.,$n=5$) (μg/ml)		
	7-Hydroxycoumarin	7-Hydroxycoumarin glucuronide	Coumarin
Intra-assay			
1.0	1.69±0.01 (0.5%)	1.39±0.04 (2.6%)	1.19±0.03 (2.8%)
10.0	9.60±0.05 (0.5%)	9.89±0.12 (1.2%)	9.70±0.07 (0.7%)
50.0	49.30±0.09 (0.2%)	49.16±0.11 (0.2%)	49.43±0.06 (0.2%)
100.0	102.5±0.59 (0.6%)	101.09±0.9 (0.9%)	102.4±0.63 (0.6%)
Inter-assay			
1.0	0.89±0.08 (8.8%)	0.97±0.04 (4.1%)	1.29±0.12 (9.6%)
10.0	10.16±1.04 (10.0%)	9.92±0.57 (5.8%)	9.93±0.67 (6.7%)
50.0	50.70±2.05 (4.0%)	51.13±1.86 (3.6%)	49.10±1.47 (3.0%)
100.0	100.10±6.09 (6.1%)	100.60±3.82 (3.8%)	100.08±6.45 (6.4%)

[a] Values in parentheses are R.S.D.s.

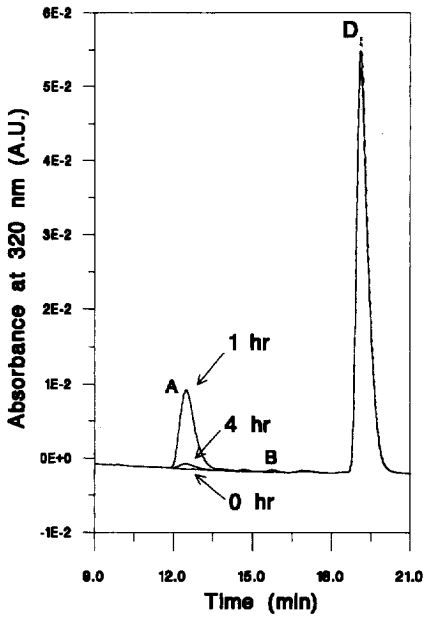

Fig. 2. Overlay of the chromatograms showing the separation of volunteer 2's plasma samples collected at 0, 1 and 4 h after coumarin administration. It shows the separation of (A) 7-hydroxycoumarin glucuronide, (B) 7-hydroxycoumarin and (D) 4-hydroxycoumarin. An absorbance ratio of 0.29 corresponds to 20 μg/ml of 7-hydroxycoumarin glucuronide.

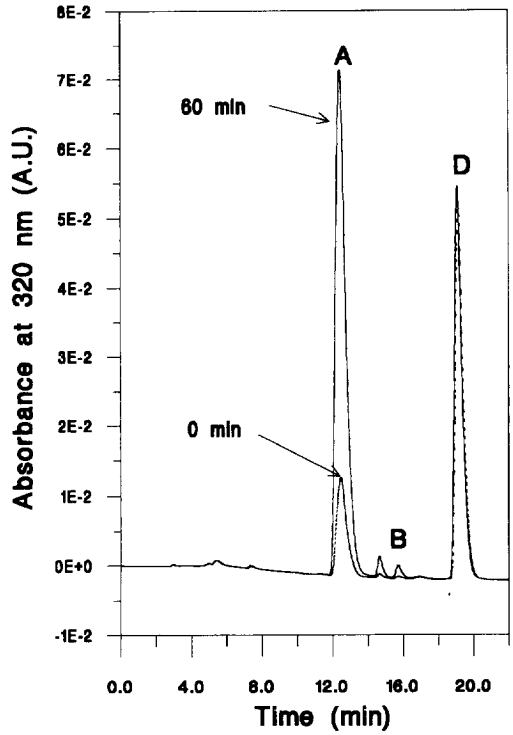

Fig. 3. Overlay of the chromatograms showing the separation of volunteer 3's serum samples (on the second day of the study, i.e. day 15) after the administration of 4 g of 7-hydroxycoumarin p.o. It shows the separation of (A) 7-hydroxycoumarin glucuronide, (B) 7-hydroxycoumarin, and (D) 4-hydroxycoumarin, at 0 min and at 60 min.

uronide and ca. 2% as 7-hydroxycoumarin) had been excreted [17] within the first 6 h and 65% after 24 h. The levels of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in the plasma were also in the same ratio, i.e. ca. 2:98%.

3.3.2. Study 2. Serum analysis

At any time approximately 95–99% of the 7-hydroxycoumarin detected in the serum is in the glucuronide form with the remainder circulating as free 7-hydroxycoumarin. Other metabolites were not detected. From the analysis of the serum 2 weeks after the initial dosage of the drug, 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin were detected in the serum of each of the volunteers (Fig. 3). On day 1 no 7-hydroxycoumarin, free or conjugated, was observed (results not shown) before the administration of the drug. The drug concentration–time profile for the third volunteer after the administration of 4.0 g of 7-hydroxycoumarin on day 15 is shown in Fig. 4. The levels of 7-hydroxycoumarin (free or conjugated) determined, the drug level time profile, or the amount remaining in circulation observed at day 15 were different for each of the volunteers studied. The concentrations of free 7-hydroxycoumarin were at a maximum at 4 h after the administration of 7-hydroxycoumarin. The highest 7-hydroxycoumarin concentration observed was 1.3 μg/ml. Fig. 5 shows a plot of the 7-hydroxycoumarin glucuronide concentrations (μg/ml) versus time (min) for each of the other two volunteers. It is shown that there is no inter-day difference for each of the volunteer's levels of conjugated drug in the serum.

The serum samples were also analyzed by the method of Egan and O'Kennedy [15]. In their method the total 7-hydroxycoumarin concentration was determined. There was no statistical difference observed between the results obtained by either

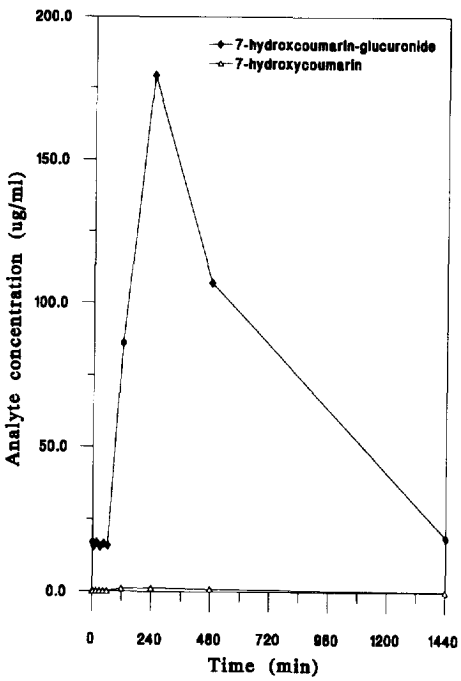

Fig. 4. Time profile of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide concentration versus time for a volunteer who had been administered 4 g of 7-hydroxycoumarin.

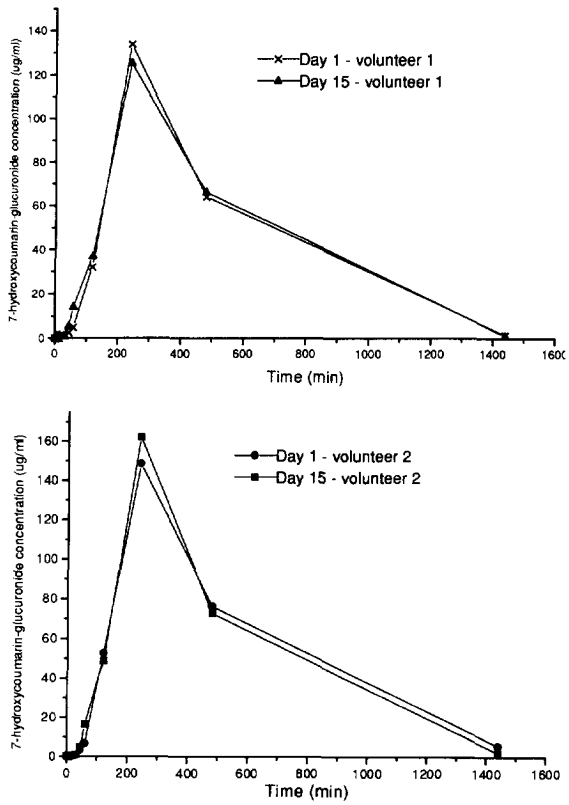

Fig. 5. Time profile of 7-hydroxycoumarin glucuronide concentrations (μg/ml) versus time (min) for volunteers 1 and 2 on each of the two days that the study was carried out.

method (Table 2). It was assumed that 99% of the 7-hydroxycoumarin in the serum was present as the glucuronide conjugate, i.e. the total 7-hydroxycoumarin (free and conjugated) content in the serum was assumed to approximately equal that of the total 7-hydroxycoumarin glucuronide content. It was, thus, possible to contrast the results determined from the two HPLC methods by comparing the concentrations (μM) of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in the serum calculated from either method. There is an average difference of up to $\pm 10\%$ between the results.

4. Conclusion

The method developed was suitable for the determination of coumarin, 7-hydroxycoumarin, and 7-hydroxycoumarin glucuronide in plasma and serum, after the in vivo metabolism of coumarin or 7-hydroxycoumarin. Previous methods required long incubation steps, extraction procedures, and required the use of enzyme solutions, i.e. β-glucuronidase. The analysis time is slightly longer than some other methods available; however, the method developed reduces the sample preparation time considerably. The method was shown to be applicable to the determination of the levels of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in clinical samples and for pharmacokinetic studies of coumarin metabolism.

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Table 2
Comparison of results for 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide concentrations (μg/ml) in serum for two volunteers who had been administered 7-hydroxycoumarin p.o.

Time (min)	Volunteer 1 (day 1)		Volunteer 3 (day 15)	
	7-OHC[1] concentration (μM)	7-OHCG[2] concentration (μM)	7-OHC[1] concentration (μM)	7-OHCG[2] concentration (μM)
0	0	0	5.62	5.12
5	0	0	5.25	4.66
15	0	0	5.55	5.02
30	6.79	3.30	4.94	4.58
45	11.11	7.30	4.81	4.87
60	24.10	14.30	5.99	4.68
120	106.80	93.90	316.60	254.20
240	380.20	392.00	575.30	530.40
480	177.80	187.70	396.90	316.50
1440	7.4	4.20	5.74	5.55

The levels of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in the serum were determined by two different HPLC methods. The serum samples were analyzed by the method as outlined under Experimental[1] and by the method of Egan and O'Kennedy [15][2]. The serum samples analysed were obtained from two volunteers who had both been administered 4.0 g of 7-hydroxycoumarin p.o.

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