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Determination of free and total 7-hydroxycoumarin in urine and serum by capillary electrophoresis

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Abstract

A new method for the rapid determination of 7-hydroxycoumarin, the predominant metabolite of coumarin in humans, was developed for analysis in urine and serum, based on separation by capillary electrophoresis, with UV detection at 210 nm. The linear detection range for 7-hydroxycoumarin was 0–50 μg/ml while the limit of quantitation was 1 μg/ml. An internal standard, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, was utilised for the determination of free 7-hydroxycoumarin, but it was found not to be suitable in the analysis of total 7-hydroxycoumarin present. Urine from two volunteers, who had been administered coumarin, was analysed by both capillary electrophoresis and by HPLC. The results from the two methods were compared and contrasted. The CE method was found to decrease the analysis time in comparison to HPLC analysis, with results available after 1.5 min as compared to 12 min with HPLC. There was no statistical difference between the results determined by either method.

1. Introduction

Coumarin, a naturally occurring plant constituent, has been used in the treatment of cancer [1] and oedemas [2]. After administration, coumarin is quickly metabolised to 7-hydroxycoumarin by a specific cytochrome P-450, (P450Coh) in the liver [3]. Metabolism of 7-hydroxycoumarin to 7-hydroxycoumarin-glucuronide then occurs. Egan and O'Kennedy [4] and Rautio et al. [5] have shown that up to 95% of the coumarin administered is recovered within twenty four hours as 7-hydroxycoumarin or 7-hydroxycoumarin-glucuronide. The majority of coumarin and 7-hydroxycoumarin administered is excreted as 7-hydroxycoumarin-glucuronide. It has been suggested that 7-hydroxycoumarin is a prodrug for coumarin [6] and it is currently being investigated in clinical trials for its effectiveness in cancer treatment [7].

Capillary electrophoresis (CE) is a recent addition to the arsenal of techniques available to the analytical and research laboratory, and its range of applications is dramatically increasing, e.g. for the analysis of drugs, proteins, carbohydrates, and coumarin derivatives [8–12]. 7-Hy-

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droxycoumarin can be analysed by HPLC [4], spectrofluorimetry [13], and TLC [14]. The new method developed, based on CE, was validated for the analysis of free and total 7-hydroxycoumarin in urine and serum. The method was shown to be applicable for the determination of 7-hydroxycoumarin in the range 0–50 μg/ml. To improve the sensitivity of the method, comparable to that reported for TLC [14], various other detection methods could be utilised, e.g. laser induced fluorescence detection [15]. An internal standard, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, was utilised for the determination of free 7-hydroxycoumarin. Treating the sample with β-glucuronidase, to deconjugate the 7-hydroxycoumarin-glucuronide to 7-hydroxycoumarin, releases other endogenous species in the urine causing interference with the extraction of the internal standard and also at the migration time of the internal standard. Thus, it cannot be used during the analysis of total 7-hydroxycoumarin.

2. Experimental

2.1. Chemicals

7-Hydroxycoumarin and the internal standard, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, were purchased from Sigma (St. Louis, MO, USA). KH_2PO_4 and K_2HPO_4 were obtained from Riedel-de Haen (Hanover, Germany). 7-Hydroxycoumarin standards were prepared from a 1 mg/ml stock solution in methanol (HPLC grade, Labscan, Dublin, Ireland)–deionised water (10:90, v/v). Serial dilutions of the 7-hydroxycoumarin (10–500 μg/ml) were prepared in deionised water. A 1 mg/ml stock solution of the internal standard was prepared in methanol for the analysis of free 7-hydroxycoumarin. The electrolyte solution used was 0.025 *M* phosphate buffer (pH 7.5), which was prepared fresh daily by preparing 0.02 *M* K_2HPO_4 and 0.005 *M* KH_2PO_4 in deionised water.

2.2. Sample preparation

Control urine was obtained from a volunteer who had not been treated with coumarin or 7-hydroxycoumarin. Urine from two volunteers, who had been treated with 100 mg of coumarin, was obtained at specific time intervals (0, 2, 6, 10, 14 and 24 h). The urinary volumes were recorded. Control serum was obtained from St. James' Hospital, Dublin, Ireland. It was not possible to analyse 7-hydroxycoumarin in serum or urine without extraction due to the endogenous species present in the matrix which absorb at 210 nm and interfere with the detection of the 7-hydroxycoumarin present.

A 0.1-ml volume of 7-hydroxycoumarin standard and 0.9 ml of control urine or serum was added into a 10-ml sterile blood tube (Medlabs, Dublin, Ireland), giving a series of calibration standards from 0 to 50 μg/ml. A 1-ml volume of a urine sample from a volunteer was also added into a 10-ml tube for analysis. The internal standard (0.05 ml) was added to both standards and unknowns and vortex-mixed for 20 s. Diethyl ether (3.5 ml) was added to each tube to extract the 7-hydroxycoumarin and internal standard. The tubes were mixed by rotation for 10 min and centrifuged at 600 *g* for 10 min. A 1.8-ml volume of the diethyl ether layer was removed into a 75 × 12 mm glass tube (M.S.C., Dublin, Ireland) and evaporated to dryness at 60°C. The sample was reconstituted into 0.1 ml of 0.025 *M* phosphate buffer (pH 7.5). Samples were not filtered before injection. Since 7-hydroxycoumarin is found predominantly in the 7-hydroxycoumarin-glucuronide form in urine, it must be treated with β-glucuronidase to liberate it to the free (non-conjugated) form for analysis. Therefore, the urine was treated with 1 ml of β-glucuronidase (Sigma) at 5000 units/ml in 1 *M* sodium acetate (Sigma) buffer, pH 5.0. The mixture was gently mixed and incubated at 37°C for 30 min. There was no internal standard used in the determination of total 7-hydroxycoumarin. The sample was then analysed on a Beckman capillary electrophoresis instrument (P/ACE System 2050).

2.3. Capillary electrophoresis separation

The capillary used was a 27 cm × 50 μm I.D. fused-silica column (Beckman Instruments), with a capillary-to-detector distance of 19.3 cm. The

preparation step for priming of the capillary was a 1-min rinse with 0.1 *M* sodium hydroxide, and then a 1-min rinse with electrolyte solution (0.025 *M* phosphate buffer, pH 7.5). The sample was applied to the capillary by a 3-s pressurized injection (0.5 p.s.i.) and separation achieved with an applied voltage of 20 kV (rise time 0.2 min) at 25°C. Typical running current was 100 μA. The resultant electropherogram was monitored at 210 nm with a fixed-wavelength detector using Beckman System Gold software. Migration times for all components varied within ± 0.1 min due to slight differences in operating conditions, e.g. capillary conditioning. The precision of migration time values could perhaps be improved by longer conditioning of the capillary with electrolyte solution. Concentrations for total and free 7-hydroxycoumarin in the volunteer's urine was calculated from the respective standard curves prepared. For the analysis of free 7-hydroxycoumarin, absorbance ratio versus concentration (μg/ml) was plotted. Absorbance ratios for the analysis of free 7-hydroxycoumarin were calculated from the equation: (absorbance of 7-hydroxycoumarin)/(absorbance of internal standard), and for total 7-hydroxycoumarin the concentration was calculated from a plot of 7-hydroxycoumarin absorbance versus standard concentration (μg/ml). Inter-assay and intra-assay variation in absorbances, and absorbance ratios were assessed [$n = 5$ (free), $n = 6$ (total)] by analysing calibration standards and calculating the mean absorbances and absorbance ratios and their related standard deviations. Percentage relative standard deviations were also calculated.

3. Results and discussion

3.1. Development of the CE separation

A new method has been developed for the separation of 7-hydroxycoumarin from coumarin and its quantitation in urine and serum using capillary electrophoresis. Various buffers were investigated in an attempt to separate the compounds. Although several of the buffers showed some separation, phosphate buffer was chosen, as it gave rise to the best resolution between coumarin and 7-hydroxycoumarin. The pH was then optimized, and the best separation achieved at pH 7.5. The ionic strength of the buffer was also assessed. At 0.1 *M*, a very high running current was exhibited, but use of 0.025 *M* phosphate buffer concentration was found to decrease the current to 100 μA. In the analysis of free 7-hydroxycoumarin, an internal standard, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, was utilised. However, the internal standard was not used in the determination of total 7-hydroxycoumarin.

A CE separation of a mixture of coumarin, 7-hydroxycoumarin and the internal standard is shown in Fig. 1. From this it can be seen that the

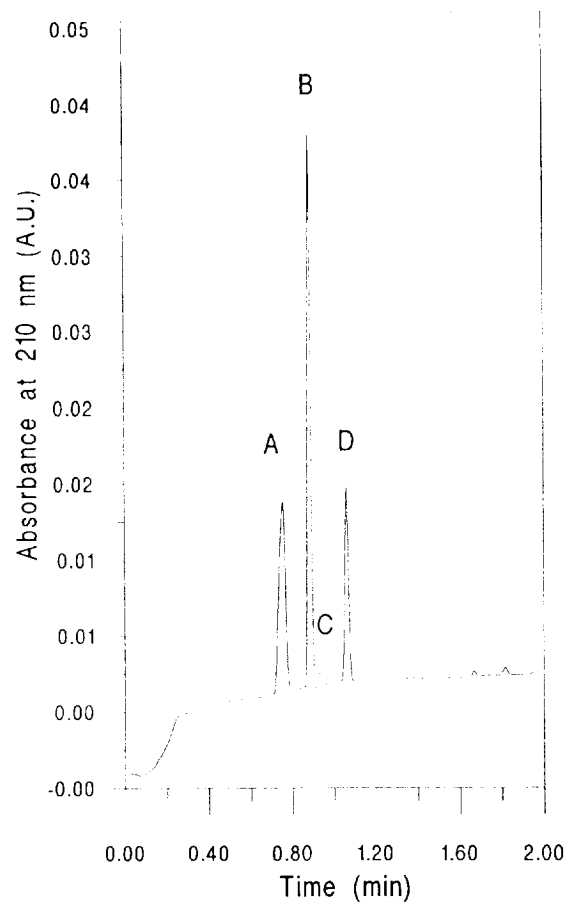

Fig. 1. Capillary electrophoresis separation of (A) coumarin, (B) 7-hydroxycoumarin, (C) contaminant of 7-hydroxycoumarin standard, and (D) internal standard. They were prepared in 0.025 *M* phosphate buffer, pH 7.5, and analysis was carried out as outlined under Experimental.

three compounds are well separated with baseline resolution within 1.2 min. Coumarin has a net zero charge at pH 7.5 and it migrates with the solvent front, i.e. with the same migration time as a neutral marker, benzamide. This means that the method described above cannot be used for the quantitation of coumarin in biological fluids. At the pH utilised one achieves the separation of coumarin from 7-hydroxycoumarin by virtue of the charge difference between the two compounds (7-hydroxycoumarin is negatively charged at pH 7.5). The separation was then applied to the analysis of urine and serum samples as outlined under the Experimental section. The separation of the compounds in the urine extract is shown in Fig. 2, where it can be seen that there is only minimal interferences with the analysis of 7-hydroxycoumarin from co-extracted endogenous species in the urine. The CE analysis of total 7-hydroxycoumarin in a urine sample taken from a volunteer 6 h after administration of coumarin is shown in Fig. 3. It indicates that most of the interferents have longer migration times compared with those of the drug and its metabolite. This figure also shows that the internal standard cannot be used in the analysis of total 7-hydroxycoumarin owing to the greater number of interferents occurring at its migration time. Also, irreproducible extraction of the internal standard was found after the treatment of the urine sample with β-glucuronidase. Efforts to find an alternative internal standard which would elute later in the electropherogram and extract reproducibly, proved fruitless. The CE analysis of a serum extract is shown in Fig. 4. From this it can be seen that there is good separation between the metabolite, the internal standard and the majority of the co-extracted interfering substances. There was no interferences from other glucuronides with 7-hydroxycoumarin after treatment of the serum with β-glucuronidase.

The method was then compared to the previously developed HPLC method [4] for the quantitation of 7-hydroxycoumarin in the urine of volunteers who had been administered coumarin. Tables 1 and 2 compare the results obtained with both methods. When, however, one compares the run times for the samples, one immediately realises the decrease in the time needed for the determination step (at least eight fold) between the two techniques. The method also has a fast regeneration step to recondition the capillary column between analyses. There are no carryover problems, less solvents are used, and there is minimal organic solvent waste as compared with the reversed-phase HPLC method. To improve the sensitivity of the method, 324 nm could be used as the detection

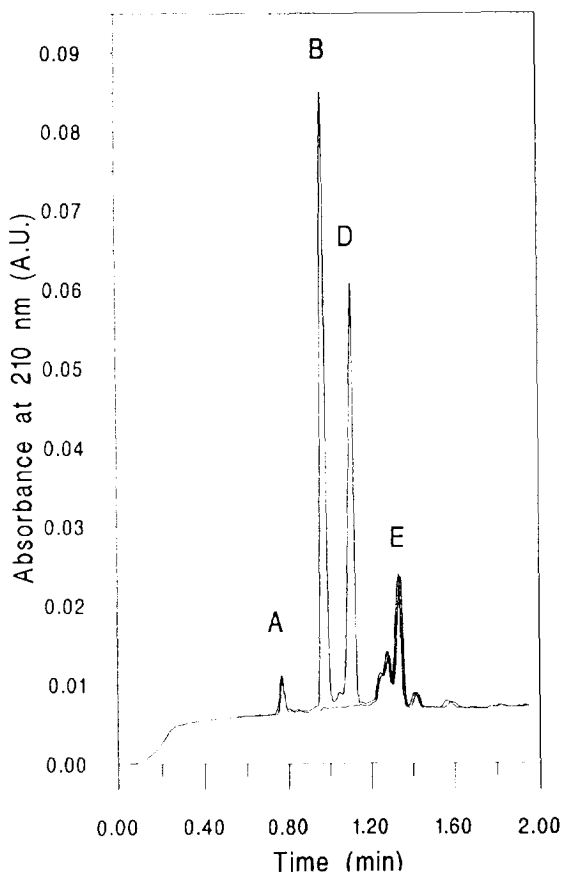

Fig. 2. Electropherograms of blank urine and 50 μg/ml 7-hydroxycoumarin standard and internal standard prepared in urine. (A) Solvent front, (B) 7-hydroxycoumarin, (D) internal standard and (E) co-extracted endogenous species. The samples were prepared and analysed as outlined in Experimental. The figure shows an overlay of two electropherograms.

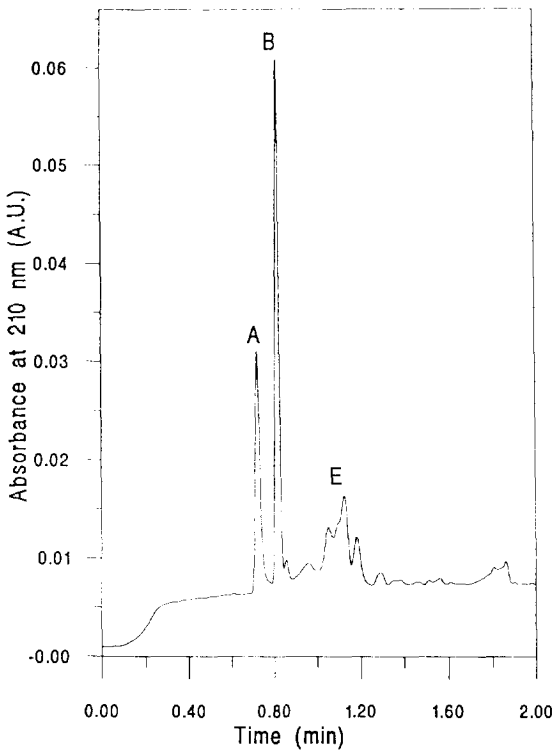

Fig. 3. CE analysis of total 7-hydroxycoumarin in a urine sample from volunteer 2, 6 h after administration of coumarin. (A) Solvent front containing any compounds which would be liberated by the addition of β-glucuronidase thus explaining the increase in absorbance at the solvent front as compared to untreated urine. (B) 7-hydroxycoumarin and (E) co-extracted endogenous species in urine.

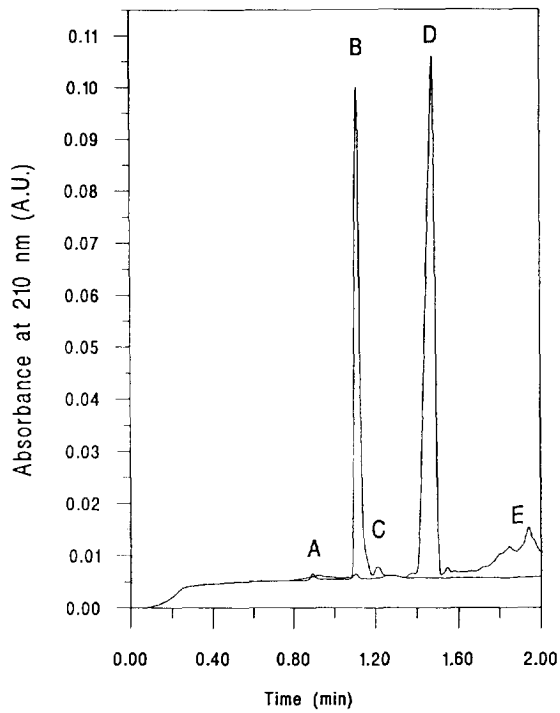

Fig. 4. Electropherograms of blank serum and 50 μg/ml 7-hydroxycoumarin standard and internal standard in serum. (A) Solvent front, (B) 7-hydroxycoumarin, (C) contaminant of standard, (D) internal standard, and (E) co-extracted endogenous species in serum.

wavelength, as in the HPLC [4] method. However, as with the HPLC method, a clean-up procedure is needed for the biological samples prior to analysis.

3.2. Limit of quantitation and linearity

In both urine and serum the limit of quantitation of 7-hydroxycoumarin was found to be 1 μg/ml and the linear detection range for the drug was 0–50 μg/ml.

The mean equation of the line for total 7-hydroxycoumarin is: $y = [1.39E-2 \pm 6.6E-4] + [1.43E-3 \pm 9.3E-5]x$ and for free 7-hydroxycoumarin is $y = [2.16E-2 \pm 8.9E-3] + [2.41E \pm 2E-3]x$, respectively. Correlation coefficients were always better than 0.990.

3.3. Accuracy and precision

Inter- and intra-assays for the determination of 7-hydroxycoumarin were carried out and the respective mean absorbances and mean absorbance ratios, and standard deviations calculated (Tables 3, and 4). The percentage relative standard deviation was found to be within 0.8% to 7.3%. The inter-assay accuracy and precision for free 7-hydroxycoumarin was determined over a five-day period. The intra-assay accuracy and precision for free drug was determined over five calibration sets on one specific day. The inter-assay analysis for total drug was determined over six days. The use of the internal standard was

Table 1
Comparison of HPLC and CE analysis of urinary concentrations of 7-hydroxycoumarin excreted over 24 h following oral administration of 100 mg of coumarin

Time (h)	7-Hydroxycoumarin concentration (μg/ml)					
	Volunteer 1			Volunteer 2		
	Free CE	Total	Total (HPLC)	Free CE	Total	Total (HPLC)
0	1.4	0	0	0	1.0	1.2
2	5.9	429.0	479.0	9.0	735.0	759.0
6	0	77.2	73.3	0	42.7	35.6
10	1.0	25.8	23.6	0	3.8	4.0
14	1.7	11.3	9.3	0	0	0
24	0	0	1.0	0	0	1.0

not of significant benefit for the determination of free 7-hydroxycoumarin.

3.4. Clinical and pharmacokinetic studies

The method developed above was applied to urine samples obtained from two volunteers who had been administered coumarin. Their urine was analysed for free and total 7-hydroxycoumarin. During the analysis no free or conjugated coumarin was observed in the volunteer's urine (as determined from HPLC analysis). All of the coumarin appears in either of the 7-hydroxylated forms. The concentrations of free and total 7-hydroxycoumarin present were calculated from standard curves prepared on the day of analysis and the results obtained were related to the urinary volumes excreted giving the total amount of 7-hydroxycoumarin excreted in milligrams. Percentage of administered coumarin, excreted as 7-hydroxycoumarin, was then calculated from molecular mass ratios (Table 2).

Table 2
Comparison of HPLC and CE analysis of 7-hydroxycoumarin excreted over 24 h (milligrams), and percentage of administered coumarin excreted after 24 h

Time (h)	7-Hydroxycoumarin excreted (mg)			
	Volunteer 1		Volunteer 2	
	CE	HPLC	CE	HPLC
0	0	0	0.50	6.0
2	64.35	71.85	73.50	75.90
6	23.16	21.99	25.62	21.36
10	3.61	3.30	2.09	2.20
14	1.13	0.93	0	0
24	0	0.30	0	0.40
Total	92.85	98.37	101.71	100.46
Coumarin excreted[a] (%)	83.4	88.7	92.0	90.5

[a] % Coumarin excreted calculated from molecular mass ratios (0.9012), i.e. (mol.wt. coumarin)/(mol.wt. 7-OHC).

The results showed that up to 70% of the coumarin is excreted in the first two hours and that approximately 90% is excreted within 24 h. These results are in accordance with those of Egan and O'Kennedy [4] and Rautio et al. [5]. In the comparison of the results from CE and HPLC analysis of the volunteer's urine samples there was no statistical difference (Tables 1 and 2).

4. Conclusions

The use of capillary electrophoresis for the determination of 7-hydroxycoumarin in urine

Table 3
Inter-assay precision and accuracy for free and total 7-hydroxycoumarin analysis following extraction from urine (free $n = 5$, total $n = 6$)

Concentration (μg/ml)	Free Absorbance ratio (mean ± S.D.)	R.S.D. (%)	Total Absorbance (mean ± S.D.)	R.S.D. (%)
0	0	0	0	0
1	4.0E − 2 ± 2.8E − 3	6.9	2.35E − 3 ± 1.59E − 4	6.8
5	0.1541 ± 1.13E − 2	7.3	8.58E − 3 ± 5.30E − 4	6.0
10	0.2951 ± 9.90E − 3	3.3	1.70E − 2 ± 9.25E − 4	5.4
20	0.5812 ± 2.20E − 2	3.8	3.09E − 2 ± 1.68E − 3	5.4
50	1.2780 ± 3.63E − 2	2.8	7.13E − 2 ± 3.25E − 3	4.6

and serum was straight forward and reliable and, in comparison to more established methods (e.g. HPLC), the separation step was much more rapid. To determine concentrations of 7-hydroxycoumarin, in a pharmacokinetic study or clinical trial [16], where the coumarin dosage is from 100 mg upwards, the use of CE would be ideal due to the availability of autosamplers and the speed at which the data is obtained. Thus, the analysis of multiple samples can be performed faster. The total preparation time of the samples does not differ from that of the other methods available [4], but the analysis time is largely decreased. To analyse 7-hydroxycoumarin at lower concentrations, other detection methods could be utilised [14,15]. The availability of photodiode-array detectors, computer controlled systems, the minimal solvent waste involved, and the ease of method development make CE a very welcomed addition to the analytical chemist, and the researcher.

Table 4
Intra-assay precision and accuracy for free 7-hydroxycoumarin following extraction from urine ($n = 5$)

Concentration (μg/ml)	Absorbance ratio (mean ± S.D.)	R.S.D. (%)
0	0	0
1	3.52E − 2 ± 1.90E − 3	5.4
5	0.1509 ± 7.20E − 3	4.7
10	0.2809 ± 6.36E − 3	2.3
20	0.5221 ± 2.35E − 2	4.5
50	1.1750 ± 8.90E − 3	0.8

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