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Short Communication

Comparison of a novel thin-layer chromatographic–fluorescence detection method with a spectrofluorometric method for the determination of 7-hydroxycoumarin in human urine

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ABSTRACT

A novel method for the determination of 7-hydroxycoumarin in human urine which combines thin-layer chromatography (TLC) with fluorescence detection (FD) has been devised. The limit of detection (1 ng/ml) enables determination of 7-hydroxycoumarin after both administration of coumarin and environmental exposure to this fragrance material. When compared to a spectroflourometric method of analysis, the TLC–FD method proved to be more selective for the analysis of 7-hydroxycoumarin in human urine.

INTRODUCTION

Coumarin is a benzopyran which occurs naturally in many plants and essential oils [1,2]. Although no longer used as a food-flavouring agent, coumarin is used as a fragrance material in a wide range of household products including perfumes, toothpastes and tobacco products [1,3]. In man it is metabolised primarily by 7-hydroxylation to 7-hydroxycoumarin (umbelliferone) [4], a large proportion of which is subsequently conjugated with glucuronic acid and excreted in the urine [5].

Although separation techniques from paper

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chromatography [6] to high-performance liquid chromatography [5] have been employed to identify 7-hydroxycoumarin in biological samples, most detection methods take advantage of the fact that 7-hydroxycoumarin is a highly fluorescent compound.

Several spectrofluorometric methods have been used for the quantitative analysis of 7-hydroxycoumarin in metabolic studies *in vivo* and *in vitro* in man and animals [7,8] and also in *in vitro* studies where 7-hydroxycoumarin is the product of the O-deethylation of 7-ethoxycoumarin, a reaction widely used as a measure of mono-oxygenase activity [9]. Such methods rely on the fluorescence of an alkaline solution of 7-hydroxycoumarin and the measurement of the fluorophore at specific activation and emission wavelengths. In this paper a modified version of the spectrofluorometric method of Greenlee and Poland [9] was employed for the determination of 7-hydroxycoumarin in human urine after oral administration of coumarin. The same samples were then re-analysed using thin-layer chromatography (TLC) combined with fluorescence detection (FD), hereafter referred to as thin-layer chromatography–fluorescence densitometry (TLC–FD).

EXPERIMENTAL

Materials

Coumarin, umbelliferone (7-hydroxycoumarin) and β-glucuronidase (Type H-2 from *Helix pomatia*) were obtained from Sigma (Poole, UK). Organic solvents were HPLC grade and inorganic reagents were Analar grade from BDH (Poole, UK). Coumarin capsules (2 mg) were prepared by the Pharmacy, Royal Victoria Infirmary (Newcastle upon Tyne, UK).

Urine collection

Sixty-four healthy individuals (twenty female) consented to take part in this study which had been approved by the joint Health Authority–University Ethics Committee. Each subject was given a single oral dose of coumarin (2 mg) and all the urine produced in the subsequent 8-h period was collected, pooled and the total volume recorded. A pre-coumarin urine sample was also collected from twenty-four of the above volunteers. All urine samples were stored frozen (−80°C) until required for analysis.

Analysis of samples by spectrofluorometry

Unconjugated 7-hydroxycoumarin. Chloroform (4 ml) was added to either urine (2 ml) or standard solution (2 ml; 0.1–1.0 μg/ml). The mixture was vortex-mixed (0.5 min) and then mechanically shaken (20 min). Following centrifugation (2000 *g*; 5 min), the aqueous layer was removed and discarded and a portion (2 ml) of the organic phase was then extracted with a solution of 0.01 *M* NaOH–1 *M* NaCl (3 ml) by vortex-mixing, shaking and centrifugation as above. An aliquot (10 μl) of the alkaline phase was then added to 0.01 *M* NaOH–1 *M* NaCl (3 ml) and the concentration of 7-hydroxycoumarin determined fluorometrically (excitation maximum at 368 nm and emission maximum at 456 nm) with a Perkin-Elmer LS-2 fluorescence spectrometer (Beaconsfield, UK).

Conjugated 7-hydroxycoumarin. Hydrochloric acid (2 ml; 9 *M*) was added to either urine (1 ml) or standard solution (1 ml; 0.5–10.0 μg/ml) and the mixture was refluxed in a sealed screw-top vial (100°C; 5 h). After cooling, neutralization was achieved by the addition of NaOH (5 *M*; 3 ml). An aliquot (2 ml) of the resulting solution was extracted and fluorescence determined as above.

Preparation of samples for TLC

Analysis for unconjugated 7-hydroxycoumarin. Chloroform (3.5 ml) was added to urine (2 ml) or standard solution (2 ml; 5–100 ng/ml). The mixture was vortex-mixed (0.5 min) and then mechanically shaken (20 min). Following centrifugation (2000 *g*; 5 min) the aqueous layer was removed and discarded and a portion of the organic layer (2 ml) was evaporated to dryness under vacuum at room temperature using a Gyrovap evaporating centrifuge (V. A. Howe, Banbury, UK).

Analysis for conjugated 7-hydroxycoumarin. Urine (0.5 ml) or standard solution (0.5 ml; 10–

600 ng/ml or 0.5–4.0 μg/ml) was incubated with β-glucuronidase (4000 U) for 16 h at 37°C. An aliquot (100–200 μl) of the mixture was removed and made up to 2 ml with distilled water. Chloroform (3.5 ml) was added and the extraction procedure continued as described above.

TLC procedures

Dry residues from the above procedure were reconstituted in chloroform (25 μl) and a portion (10 μl) was applied to TLC plates [precoated high-performance thin-layer chromatographic (HPLTC) glass-backed silica gel 60 F_{254}, 10 × 20 cm; E. Merck, Darmstadt, Germany] using a Linomat IV automatic sample applicator (Camag, Muttenz, Switzerland). Samples were applied to both sides of the plates in 6-mm bands with a lane separation of 4 mm. Chromatography was carried out in a Camag horizontal developing chamber in the saturation configuration using the lower phase of chloroform–water–ethyl acetate–acetic acid (24:12:6:0.5, v/v) as mobile phase. When separation was complete the plates were removed and allowed to dry at room temperature.

Densitometric analysis

Densitometric quantitation of 7-hydroxycoumarin was carried out using a Camag TLC scanner II connected to a Camag SP4290 TLC integrator with CATS evaluation software. The chromatographic lanes on the plates were scanned at a wavelength of 313 nm using the mercury lamp in the reflection mode in the presence of a K400 secondary filter. Peak heights were determined and using solutions containing known amounts of authentic standard taken through the above procedures, the concentration of 7-hydroxycoumarin in urine could be calculated by comparison of unknowns with calibration curves.

RESULTS

The spectrofluorometric and TLC methods described above were employed for the determination of 7-hydroxycoumarin in the urine of 64 individuals after the administration of a single oral dose of coumarin (2 mg). Pre-dose urine samples from 24 of the volunteers were analysed by TLC–FD only.

Results obtained by spectrofluorometry

The range of concentrations measured using this assay were 5–10 000 ng/ml with a limit of quantitation of 2 ng/ml. Calibration curves constructed up to 10 μg/ml were linear. Intra-assay variation was calculated by multiple analyses of a single-spiked urine sample in a single assay and gave a coefficient of variation (C.V.) of 3.7% (n = 8). Inter-assay variation was calculated by repeated analysis of a single spiked urine sample on

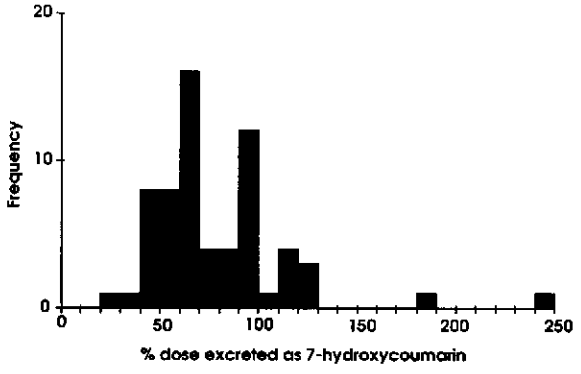

Fig. 1. Frequency distribution profile for percentage of the dose of coumarin excreted as 7-hydroxycoumarin (free and conjugated) in 0–8 h urine samples analysed by spectrofluorometry.

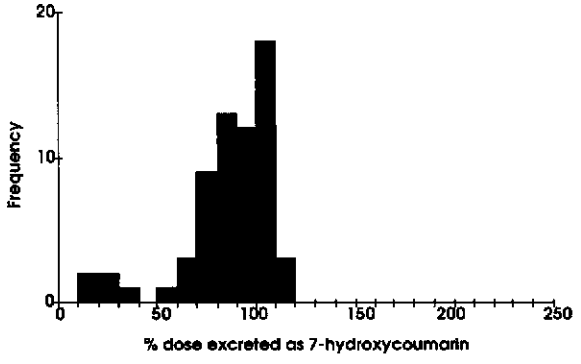

Fig. 2. Frequency distribution profile for percentage of the dose of coumarin excreted as 7-hydroxycoumarin (free and conjugated) in 0–8 h urine samples analysed by TLC–FD.

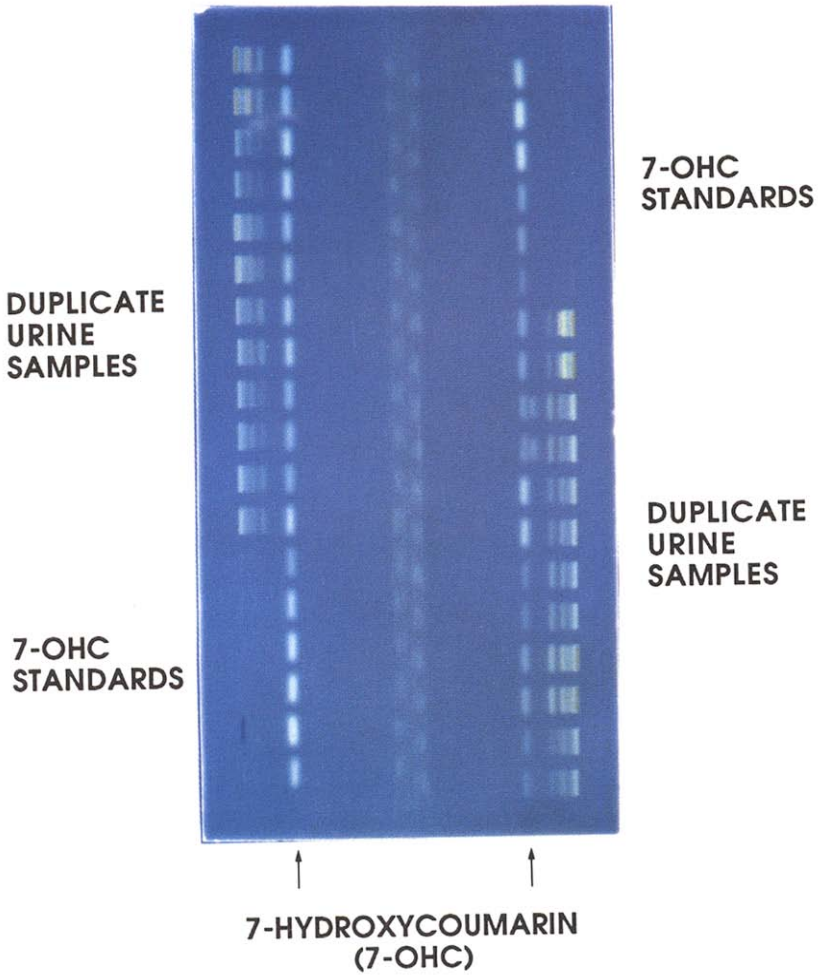

Fig. 3. Photograph of an HPTLC plate under UV illumination. On each side of the plate tracks 1–12 contain extracts of 0–8 h urine samples applied in duplicate and tracks 13–18 contain 7-hydroxycoumarin standards.

sequential days and produced a C.V. of 5.7% (n = 6). From Fig. 1 it can be seen that the percentage of the dose of coumarin excreted as total (both free and conjugated) 7-hydroxycoumarin by the 64 volunteers studied ranged from 27 to 241 with a mean ± standard error of 79 ± 4. Two individuals apparently excreted 188 and 241% of the dose as 7-hydroxycoumarin in the 8-h period following coumarin administration.

Results obtained by TLC with fluorescence densitometry

The range of concentrations using this assay were 5–4000 ng/ml with a limit of quantitation of 1 ng/ml. Calibration curves constructed up to 4 μg/ml were linear. The coefficient of variability within the assay was 4.8% (n = 16) and that between assays was 6.4% (n = 6). From Fig. 2 it can be seen that the percentage of the dose of coumarin excreted as total 7-hydroxycoumarin by the 64 volunteers studied ranged from 10 to 116 with a mean ± standard error of 86 ± 3. The two "outliers" as determined by the spectrofluorometric method of analysis excreted 91 and 100% of the dose as 7-hydroxycoumarin in the 8-h period following coumarin administration. Five of the subjects studied excreted less than 50% of the dose as 7-hydroxycoumarin, how-

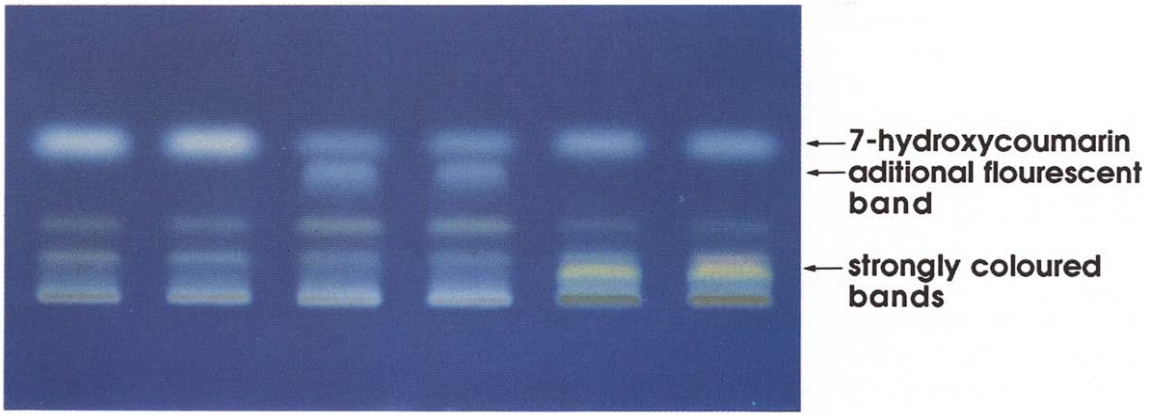

Fig. 4. Photograph of an area of an HPTLC plate under UV illumination to illustrate the presence of 7-hydroxycoumarin, an additional fluorescent band and strongly coloured bands in three 0–8 h urine samples.

ever, on repeat administration of coumarin, all excreted greater than 50% of the dose as this metabolite.

7-Hydroxycoumarin was absent in all unhydrolysed pre-coumarin urine samples and ranged from <1 to 1145 ng/ml with a mean ± standard error of 232 ± 58 in hydrolysed pre-coumarin urine samples.

Fig. 3 is a photograph of an HPTLC plate under UV illumination to which urine samples in duplicate and standards were applied and the plate developed as described above. It can be seen that the application of a 7-hydroxycoumarin standard results in the visualization of a single fluorescent band with an R_F value of 0.51. The application of post-coumarin urinary extracts prepared as described above gives rise to several bands including one which is fluorescent and has the same R_F value as 7-hydroxycoumarin. Fig. 4 is typical of the analysis of many of the urine samples collected in the 8-h period after coumarin administration which gave coloured bands with R_F values of less than that for 7-hydroxycoumarin. Fluorescent bands additional to 7-hydroxycoumarin were also seen in several urine samples including those from the two "outliers" and also in pre-coumarin samples.

DISCUSSION

The novel TLC–FD method presented in this paper allows more accurate measurement of 7-hydroxycoumarin in human urine than does spectrofluorometry. Although, for many of the urine samples, very similar results were obtained by both methods of analysis, certain samples appeared to contain more 7-hydroxycoumarin when analysed by spectrofluorometry than when analysed by TLC–FD and others less. Such findings may be explained by interference from other fluorescent compounds in some samples and a "quenching" of the fluorescence of 7-hydroxycoumarin by strongly coloured compounds in others. No attempt has been made to characterise either the additional fluorescent or the strongly coloured compounds present in certain urine samples. It is unlikely that the fluorescent compounds are ring-hydroxylated metabolites of coumarin since there is no evidence in the literature that any phase 1 metabolites other than 7-hydroxycoumarin and 2-hydroxyphenylacetic acid are produced in man [4]. Furthermore, similar fluorescent bands were detected in pre-dose urine samples from some of those individuals who demonstrated extra bands after coumarin administration. This suggests that the diet may be the source of additional fluorescent material.

Another possible, although less likely explanation, of the different results obtained by the two assays is the method of hydrolysis used. When analysed by spectrofluorometry, acid hydrolysis was employed. This was not possible for analysis

by TLC–FD since it resulted in the formation of a residue after the samples were evaporated to dryness which prevented their application to the TLC plate. Thus the degree of conjugation of 7-hydroxycoumarin was determined by TLC–FD after enzymic hydrolysis.

Trace amounts of 7-hydroxycoumarin have previously been detected in human urine presumably without prior administration of coumarin [10]. In the present study concentrations of 7-hydroxycoumarin in pre-coumarin urine samples were highly variable and this probably reflects inter-individual differences in exposure to coumarin-containing products.

The results of this study strongly support the notion that 7-hydroxycoumarin is the major metabolite of coumarin in man and that it is largely excreted in the urine as the glucuronide conjugate. The novel TLC–FD method outlined in this paper provides a selective method for the specific quantitative analysis of 7-hydroxycoumarin in human urine with or without prior administration of coumarin.

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