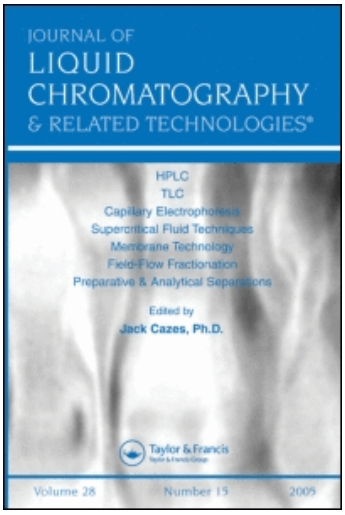

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Determination of Urinary Metabolites of Coumarin in Human Urine by HPLC

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ABSTRACT

Liquid chromatography with fluorometric detection has been developed for the determination of the metabolite of coumarin (7-hydroxycoumarin) in human urine. 7-Hydroxycoumarin and 5,7-dimethylcoumarin were separated simultaneously on a 25 cm × 4.6 mm i.d. Phenomenex CN 5 μm column with acetonitrile–water (40 : 60, v/v) as mobile phase at 1.0 mL/min, and detected at excitation 325 nm and emission 470 nm. The limit of detection was 0.28 ng/mL for 7-hydroxycoumarin. Analysis of human urine 1–6 days after ingestion of oral Chinese medicines, led to the conclusion that the concentration of 7-hydroxycoumarin was higher than for control urine.

Key Words: 7-Hydroxycoumarin; Human urine metabolite; Fluorescence detection.

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INTRODUCTION

Coumarin (1,2-benzopyrone) is common to plants of the Umbelliferae (including celery, parsnip, and parsley) and Rutaceae (citrus plants) family. Coumarin has also been examined as a therapeutic agent for the treatment of various cancers.[1,2] The metabolic pathways of coumarin in the human body leads to the intermediate 7-HC and, consequently, glucuronidation in the intestine and liver.[3–5] Methods for the determination of coumarin and 7-HC in biological fluids have been extensively published.[6–10] Analysis of the coumarin in plasma was performed on a C_8 column and was detected by measuring the UV absorbance at 275 nm.[6] Denise and Runkel et al. published a method for the quantification of 7-HC in urine and plasma by spectrofluorimetry.[7,8] Determination of 7-HC in urine and serum, based on separation by capillary electrophoresis with UV, was detected at 210 nm.[9,10] But, very few have dealt with its determination in human pharmacokinetics after oral administration of Chinese medicine. The aim of this work was to develop a sensitive HPLC method for the determination of urinary metabolites of 7-HC in humans after oral administration of Chinese medicines.

EXPERIMENTAL

Instrumentation

HPLC was performed with a Gasukuro Kogyo model 576 pump and model 7125 injector equipped with a 20-μL sample loop and a RF-10 A_{XL} spectrofluorometric detector. Chromatograms were acquired and peak areas calculated by means of an SISC chromatogram Data Integrator. Absorbance measurements were acquired with a Cary UV-visible spectrophotomer (Varian, Australia). Matched quartz cells, 1 cm path length, were used to hold all solutions for measurement.

Reagents and Materials

The coumarins tested were coumarin, 7-HC, and 5,7-DMC (citropten) from Aldrich.

Human Subjects

Five normal, healthy, men and women, aged from 22 to 25 years, were selected. Their average height was 165 ± 5 cm and average weight was

60 ± 10 kg. Each received 12 g umbelliferase Chinese herbal medicine per day for 1–5 days.

Sample Preparation

Urine Sample Collection

Urine samples were collected at specific time intervals (0,1, 4, 8, 16, 24, 48, 72, 96, and 120 hr, respectively) after medicinal intake. Time 0 values were used as background and subtracted from sample values. The remainder of the samples were kept in high density polyethylene containers and stored in a freezer (−20°C) for further determination of metabolites of 7-HC by HPLC.

Extraction of Urinary Metabolites of Coumarin

Since 7-HC is found predominantly in the 7-HC-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 50 μL of β-glucuronidase (Sigma) at 119,000 units/mL in 1 M sodium acetate buffer (pH 4.5). The mixture was gently mixed and incubated at 37°C for 30 min. The resulting solution was extracted with 10 mL of chloroform and the organic layer evaporated at 40°C under dry nitrogen. The dried extract was reconstituted with 0.5 mL of 50% (v/v) methanol–water and loaded onto a Sep-Pak® C_{18} Waters cartridge, which had been conditioned with 2 mL of methanol and 2 mL water prior to sample loading. An additional 0.5 mL of methanol was used to rinse the sample vial and was also loaded onto the C_{18} cartridge. The sample on the C_{18} cartridge was washed with 2.0 mL of water (eluant discarded), 2.0 mL of 22% acetonitrile–water solution (eluant discarded), 1.0 mL of 30% acetonitrile–water solution (eluant collected-contained 7-HC), 1.0 mL of 40% acetonitrile–water solution (eluant collected-contained 7-HC), and 1.0 mL of 55% acetonitrile–water solution (eluant collected-contained 7-HC). These three fractions were combined and dried under nitrogen at 45°C. The dry extract was reconstituted with 500 μL of pure methanol and filtered through 0.45 μm membrane filters before LC analysis.

RESULT AND DISCUSSION

Optimization of Chromatographic Conditions

In reverse-phase liquid chromatography, the retention of any solute depends on the proportion of the organic modifier in the aqueous eluant.

An organic-enriched composition results in a decreased retention time or capacity factor (k'). To illustrate this point, two different eluants, acetonitrile–methanol–water (80 : 10 : 10, 75 : 12.5 : 12.5, 60 : 20 : 20, and 50 : 25 : 25, v/v/v) and acetonitrile–water (30 : 70, 40 : 60, and 50 : 50, v/v) were prepared. The most substantial enhancement of selectivity was obtained when methanol was incorporated in the mobile phase. Figure 1 shows, with 50 : 25 : 25 (v/v/v) acetonitrile–methanol–water as mobile phase, the retention times of 7-HC and 5,7-DMC were found to be 3.2 and 3.5 min, respectively. However, this mobile phase was too polar for the rapid elution of 7-HC and 5,7-DMC. Figure 2 shows the chromatogram of urine that has an interfering peak, which was very close to the 7-HC peak and they may be combined to one peak. So, the 50 : 25 : 25 (v/v/v) acetonitrile–methanol–water as mobile phase was not suitable for quantitation of 7-HC in urine. The capacity factors and sensitivity were affected by the concentration

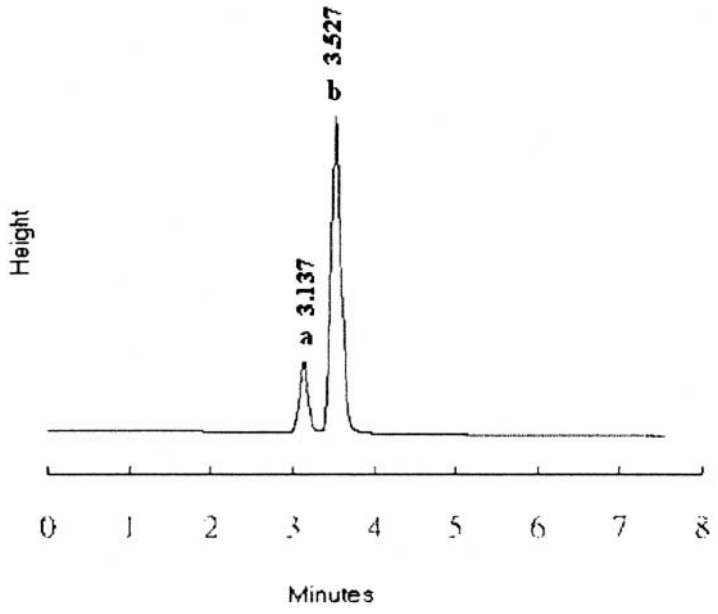

Figure 1. Chromatograms obtained by LC-FD from (a) 7-hydroxycoumarin, (b) limetin, 5,7-dimethylcoumarin, 20 ng/mL of 7-hydroxycoumarin, and limetin standard (100 ng/mL). Peak a is 7-hydroxycoumarin and peak b is limetin 5,7-dimethylcoumarin. Stationary phase, Phenomenex CN (5 μm, 250 × 4.60 mm i.d.); mobile phase, acetonitrile–methanol–water (2 : 1 : 1, v/v/v); flow rate, 1.0 mL/min; fluorescence detection, $\lambda_{exc} = 325$ nm; $\lambda_{em} = 470$ nm.

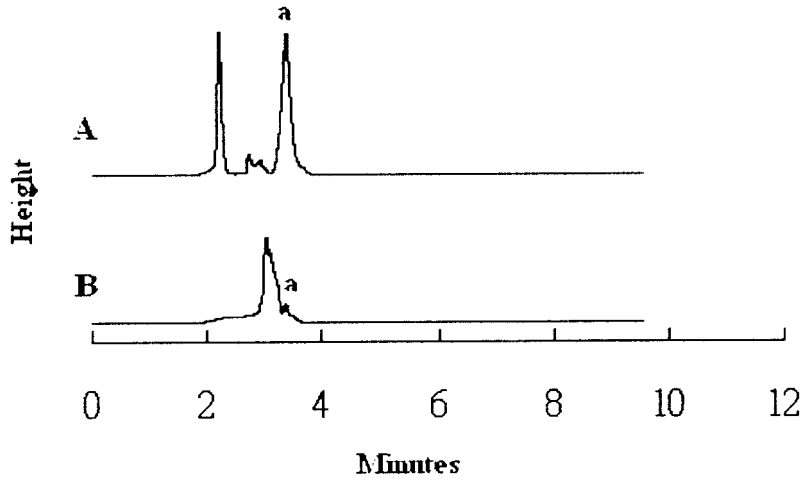

Figure 2. Chromatograms obtained by LC-FD from the human urine (a) subject F1 after 16 hr; (b) subject M1 after 8 hr of oral administration Umbelliferae Chinese herbs. Peak a is 7-hydroxycoumarin; Phenomenex CN (5 μm, 250 × 4.60 mm^2 i.d.); mobile phase, acetonitrile–methanol–water (2 : 1 : 1, v/v/v); flow rate, 1.0 mL/min; fluorescence detection, $\lambda_{exc} = 325$ nm; $\lambda_{em} = 470$ nm.

of acetonitrile in the mobile phase. The retention time of 7-HC decreases and sensitivity increases as the proportion of acetonitrile in the mobile phase is increased. The optimum composition of the mobile phase was acetonitrile–water (40 : 60, v/v) and the retention times of 7-HC and 5,7-DMC were found to be 4.3 and 8.0 min, respectively. When the commonly used settings for excitation (361 nm) and emission (490 nm) were applied, both coumarin and 7-HC were detected at similar levels of sensitivity as previously reported.[11] However, we were interested in developing a method with increased sensitivity for 7-HC, and found that the maximum response occurred at 325 nm excitation and 470 nm emission.

Linearity, Recovery, and Limit of Quantification of LC-FD Assay

Since the analyte (7-HC) and internal standard (5,7-DMC) in any sample or standard receive the sample treatment, the ratio of their signals will be unaffected by any lack of reproducibility in the procedure. A calibration curve of peak height of 7-HC/5,7-DMC vs. concentration of 7-HC

is linear (5–1000 ng/mL) with a slope of 1.5 ($r = 0.9990$). The limit of quantification was 5.6 pg for 7-HC. A 500 μL aliquot of the 7-HC and 5,7-DMC were added to 0.5 mL of diluted urine samples and to urine samples that contained known amounts of endogenous 7-HC, and extraction was carried out as described above. To calculate percentage recovery, the amount of endogenous 7-HC was subtracted from the measured total amount, divided by the added amount, and multiplied by 100. Table 1 shows the LC-FD traces obtained for a volunteer urine sample spiked with 7-HC. Recoveries and precision were observed (recoveries ranging from 95% ± 0.5% to 105% ± 0.7%).

Application to Human Urine

The proposed LC-FD method was applied to the determination of 7-HC in human urine. Representative LC-FD chromatograms of metabolites of 7-HC in volunteers urine, extract treated with and without β-glucuronidase after intake of medicine, were shown in Fig. 3(A) and (B), respectively. Figure 3(A) and (B) compare the chromatogram of pure standard [Fig. 3(C)]. Sample constituents with retention characteristics identical to those of 7-HC and 5,7-DMC were identified and measured. When coumarin

Table 1. Recovery of 7-hydroxycoumarin fortified human urine for LC-FD.

	7-Hydroxycoumarin		
Subjects	Added (ng/mL)	Found (ng/mL)	Recovery (%)[a]
1	50	49	97 (0.55)[b]
	100	95	95 (0.56)
	200	202	101 (0.34)
2	100	101	101 (1.8)
	200	198	99 (2.0)
	400	400	100 (0.64)
3	50	52	104 (0.15)
	100	104	104 (0.75)
	200	210	105 (0.67)

[a]($n = 3$).
[b]RSD, relative standard deviation.

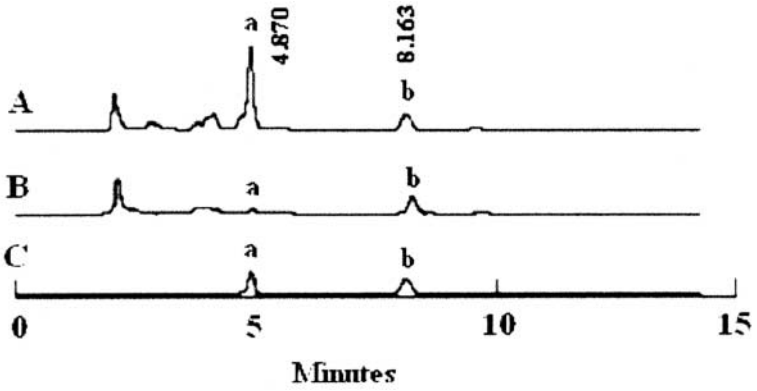

Figure 3. Chromatograms obtained by LC-FD from the human urine (A) with (B) without β-glucuronidase (C) a mixture of standards, after oral administration Umbelliferae Chinese herbs. The peaks are as follows: (a) 7-hydroxycoumarin (50 ng/mL), (b) 5,7-dimethylcoumarin, internal standard (100 ng/mL). Stationary phase, Phenomenex CN (5 μm, 250 × 4.60 mm i.d.); mobile phase, acetonitrile–water (40 : 60, v/v), flow rate, 1.0 mL/min; fluorescence detection, $\lambda_{exc} = 325$ nm; $\lambda_{em} = 470$ nm.

is dissolved in alkali (tetrabutylammonium hydroxide or potassium hydroxide), cleavage of the pyrone ring occurs when the anion of *cis*-coumaric acid is formed. Upon irradiation with long-wavelength UV light (365 nm), the cis-form is converted to the trans-isomer. Apparently, in the trans-isomer, the hydrogen atom of the phenol group form a chelate with a sterically unhindered, unsaturated carbon atom, giving rise to a six-membered ring which is the fluorophore.[11,12] Since the fluorescence intensity of coumarin depends upon the concentration of potassium hydroxide, that was not possible for analysis by LC-FD. Therefore, the coumarin and its major metabolite (7-HC) could not be simultaneously detected by the LC-FD method. However, the advantage of the fluorescence detection is of particular interest for 7-HC because there is no interference by other metabolites absorbing in the FD.

The 7-HC concentration–time profile for the two subjects after the administration of 12.0 g of Chinese medicine on days 1 and 4 were shown in Fig. 4. When the levels of 7-HC were determined, the medicine level time-profile, or the amount remaining in circulation, observed at day 4 was different for each of the subjects studied. The concentrations of free 7-HC were at a maximum at 4–8 hr after the administration of Chinese medicine. The highest 7-HC concentration observed was 137.3 ng/mL. It was shown

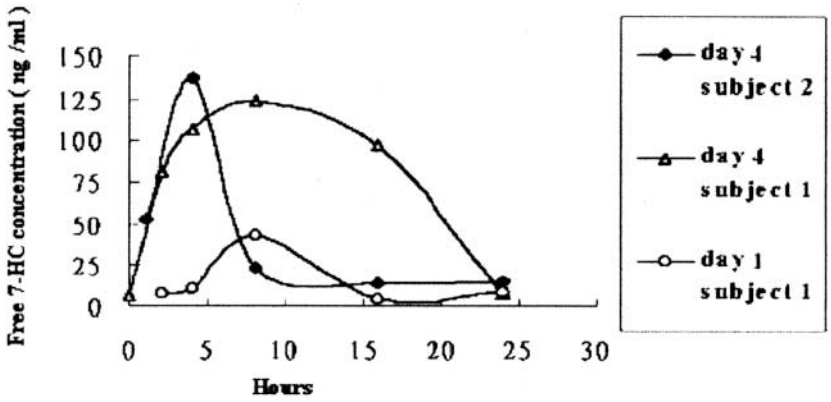

Figure 4. Time profile of urinary unconjugated (free) 7-hydroxycoumairn concentration vs. time for subjects 1 and 2 after intake of Chinese herbal medicine 12 g/day.

that there was no inter-day difference for each of the subject's level of the 7-HC in urine. Urine specimens from five different subjects were used in metabolism studies. Table 2 shows urine concentrations of metabolite in five subjects who continuously take Chinese medicines for 1, 2, 3, 4, and 5 days. The concentrations of metabolite (7-HC) with β-glucuronidase were higher than without. Figure 5 shows a plot of concentration of 7-HC vs. time for each of the five metabolism studied. It shows that each of the urine specimens has a different coumarin metabolism profile. The metabolite of coumarin (Fig. 5) resulted in a rise in urine levels, achieving a peak concentration 3–4 days after administration, before declining rapidly. There was no significant difference for this 7-HC in the urine circulation of the individual subjects. The assay described above was used to elucidate the pharmacokinetics of coumarin in human after a single and a continuous dose of Chinese herbal medicines.

CONCLUSION

The results of this study support the notion that 7-HC is the major metabolite of coumarin in man, and that it is largely excreted in the urine as the glucuronide conjugate. Very small amounts of the coumarin have been reported to be metabolized to and excreted as *o*-hydroxyphenylacetic acid.[13,14] However, our studies did not detect any of this metabolite.

Table 2. The concentration of coumarin metabolite (7-hydroxycoumarin) in human urine without and with β-glucuronidase after oral administration of Umbelliferae Chinese herbs.

		Days (ng/mL)[a]					
Subjects	β-Glucuronidase	1	2	3	4	5	6
F1	Without	24.6 (5.8%)[b]	33.4 (2.2%)	49.7 (0.9%)	52.9 (1.2%)	37.8 (2.6%)	24.6 (5.8%)[b]
	With	720 (3.5%)	64.0 (1.5%)	903 (0.9%)	1152 (1.4%)	851 (0.7%)	754 (3.5%)
F2	Without	52.3 (2.9%)	65.4 (3.5%)	61.2 (1.9%)	111 (3.8%)	—[c]	—[c]
	With	615 (3.0%)	919 (2.3%)	2032 (1.5%)	634 (0.8%)	850 (4.3%)	351 (2.9%)
F3	Without	67.6 (3.6%)	—[c]	33.2 (0.3%)	9.64 (0.6%)	295 (0.4%)	—[c]
	With	588 (0.7%)	491 (2.3%)	1041 (1.6%)	113 (2.4%)	700 (1.6%)	308 (5.0%)
F4	Without	50.9 (1.453%)	4.45 (7.6%)	36.6 (1.3%)	30.5 (2.2%)	60.7 (1.0%)	—[c]
	With	607 (2.2%)	55.5 (1.2%)	545 (0.6%)	860 (0.9%)	975 (3.6%)	152 (2.7%)
F5	Without	39.5 (0.2%)	31.5 (2.3%)	56.31 (5.8%)	11.28 (3.6%)	36.2 (0.8%)	7.70 (2.9)
	With	553 (1.5%)	1002 (1.0%)	1851 (1.3%)	519 (1.6%)	812 (6.3%)	272 (3.2%)

[a]($n = 3$).
[b]RSD, relative standard deviation.
[c]Not determined.

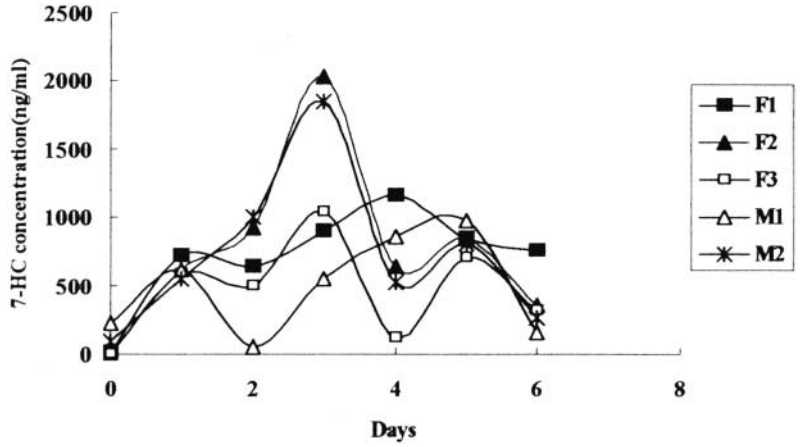

Figure 5. Day course of gastrointestinal absorption of coumarin metabolite after intake of Chinese herbal medicines 12 g/day. Column, Phenomenex CN (5 μm, 250 × 4.60 mm^2 i.d.); mobile phase, acetonitrile–water (40 : 60, v/v), flow rate 1.0 mL/min; fluorescence detection, $\lambda_{exc} = 325$ nm; $\lambda_{em} = 470$ nm.

The novel LC-FD method outlined in this paper provides a selective method for the specific quantitative analysis of 7-HC in human urine with or without treatment of β-glucuronidase after oral administration of Chinese herbal medicines.

ABBREVIATIONS

7-HC	7-Hydroxycoumarin
5,7-DMC	5,7-Dimethylcoumarin
HPLC-FD	High-performance liquid chromatography with fluorescence detection

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REFERENCES

1. Lake, B.G.; Evans, J.G.; Lewis, D.F.V.; Price, R.J. Comparison of the hepatic effects of coumarin, 3,4-dimethylcoumarin, dihydrocoumarin and 6-methylcoumarin in the rat. Food Chem. Toxicol. **1994**, *32*, 743–751.
2. Weber, U.S.; Steffen, B.; Siegers, C.P. Antitumor-activities of coumarin, 7-hydrocoumarin and its glucuronide in several human tumor cell lines. Res. Commun. Mol. Pathol. Pharmacol. **1998**, *99*, 193–206.
3. Steensma, A.; Beamand, J.A.; Walters, D.G.; Price, R.J.; Lake, B.G. Metabolism of coumarin and 7-ethoxcoumarin by rat, mouse, guinea pig, cynomolgus monkey and human precision-cut liver slices. Xenobiotica **1994**, *24*, 893–907.
4. Deasy, B.; Bogan, D.P.; Symth, M.R.; O'Kennedy, R.; Fuhr, U. Study of coumarin metabolism by human liver microsomes using capillary electrophoresis. Anal. Chim. Acta **1995**, *310*, 101–107.
5. Beckley-Kartey, S.A.J.; Hotchkiss, S.A.M.; Capel, M. Comparative in vitro skin absorption and metabolism of coumarin in human, rat and mouse. Toxicol. Appl. Pharmacol. **1997**, *145*, 34–42.
6. Lamiable, D.; Vistelle, R.; Trenque, T.; Fay, R.; Millart, H.; Choisy, H. Sensitive high-performance liquid chromatographic method for the determination of coumarin in plasma. J. Chromatogr. **1993**, *620*, 273–277.
7. Egan, D.A.; O'Kennedy, R. Spectrofluorimetric method for the quantification of 7-hydroxycoumarin in urine and plasma using both extracted and unextracted samples. Analyst **1993**, *118*, 201–203.
8. Rankel, M.; Tegtmeier, M.; Legrum, W. Metabolic and analytical interactions of grapefruit juice and coumarin in man. Eur. J. Clin. Pharmacol. **1996**, *50*, 225–230.
9. Bogan, D.P.; Deasy, B.; O'Kennedy, R.; Smyth, M.R.; Fuhr, U. J. Chromatogr. **1995**, *663*, 371–378.
10. Bogan, D.P.; Thornes, R.D.; Tegtmeier, M.; Schafer, E.A.; O'Kennedy, R. Direct determination of 7-hydroxycoumarin and 7-hydroxycoumarin-glucuronide in urine by using capillary electrophoresis. Analyst **1996**, *121*, 243–247.
11. Tan, H.S.I.; Ritschel, W.A.; Sanders, P.R. Determination of coumarin and umbelliferone mixtures in whole blood by spectrophotofluorometry. J. Pharm. Sci. **1976**, *65*, 30–32.
12. Ichinose, N.; Schwedt, G.; Schnepel, M.; Adachi, K. *Fluorometric Analysis in Biomedical Chemistry Trends and Techniques in Including HPLC Applications*; John Wiley & Son: New York, 1979; 79.

13. Moran, E.; O'Kennedy, R.; Thornes, R.D. Analysis of coumarin and its urinary metabolites by high-performance liquid chromatography. J. Chromatogr. **1987**, *416*, 165–169.
14. Weber, U.S.; Steffen, B.; Siegers, C.P. Antitumor-activities of coumarin, 7-hydrocoumarin and its glucouronidae in several human tumor cell lines. Res. Commun. Mol. Pathol. Pharmacol. **1998**, *99*, 193–200.

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