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STUDY ON METABOLISM OF SCUTELLARIN IN RATS BY HPLC-MS AND HPLC-NMR

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Scutellarin is the major active constituent of *Scutellaria barbata D.* The metabolism of scutellarin has been investigated in rats. The solid-phase extraction and HPLC-DAD methods were established to separate and analyse metabolites. Five metabolites (M1–M5) were identified by enzymatic hydrolysis, HPLC-DAD, HPLC-MS and HPLC-NMR. M1 and M3 were conjugates of scutellarin with two sulfate groups, which have not been reported in natural plants. M2 was scutellarin; M4 was 6-methyl-scutellarin; and M5 was 6-methyl-scutellarein. The metabolic pathway was proposed.

Keywords: Scutellarin; Metabolism; HPLC-MS

INTRODUCTION

Scutellarin is the major active constituent of *Scutellaria barbata D.* The crude drug is widely used for bacteriostasis, anti-thrombosis, anti-tumor and immunoregulation [1]. Scutellarin shows wide pharmacological effects. The soluble sodium and calcium salts of scutellarin inhibit platelet thromboxane B2 production without alteration of HETE [2]. It also inhibits 6-hetoprostaglandin F1α production by endothelial cells [3]. For leukocytes it does not affect thromboxane B2 production, but it potentiates the effect of calcimycin in stimulating LTB4 formation. The calcium salt of scutellarin is also a stimulant to fibrinolysis and an anticoagulant of endothelial cells.

Flavonoids are the main constituents of *Scutellaria barbata D.* The total amounts of flavonoids and the content of scutellarin are used for the quality control of herbs of *Scutellaria barbata D.* in the year 2000 edition of *Chinese Pharmacopoeia* (Vol. I) [4]. To elucidate the metabolism of scutellarin in humans and provide scientific data for clinical use we studied the metabolism of scutellarin in rats.

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RESULTS AND DISCUSSION

HPLC-DAD Analysis of Scutellarin and its Metabolites

Urine, feces and bile were collected during 0–12 h and 12–24 h after oral administration of scutellarin to rats. The prepared samples from urine, feces and bile by ODS were analysed by an established HPLC-DAD method. The HPLC-DAD chromatograms are shown in Figs. 1 and 2. Five metabolites were detected in urine and bile by comparison with blank

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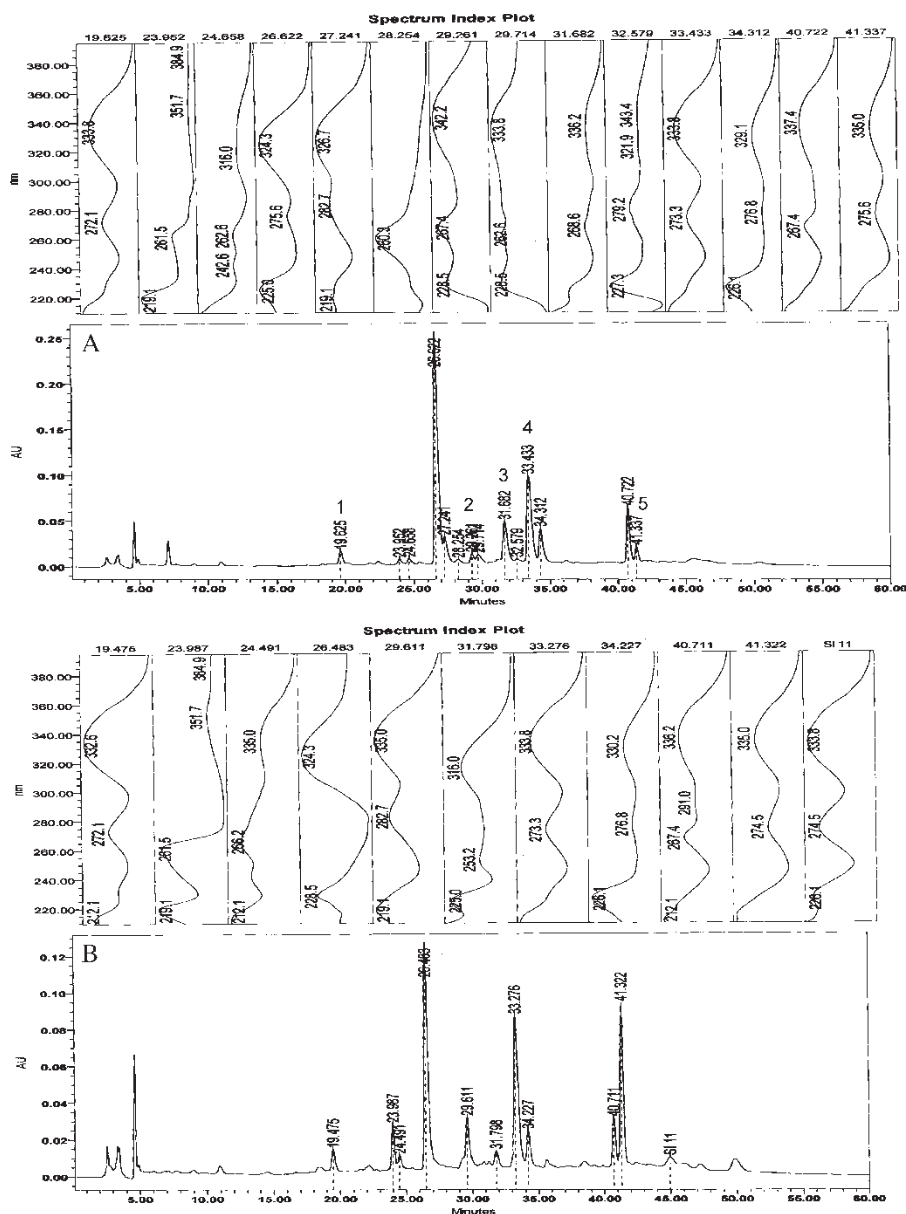

FIGURE 1 HPLC-DAD chromatograms of urine after oral administration of scutellarin, (A) during 0–12 h; (B) during 12–24 h 1. M1, 2. M2, 3. M3, 4. M4 and 5. M5.

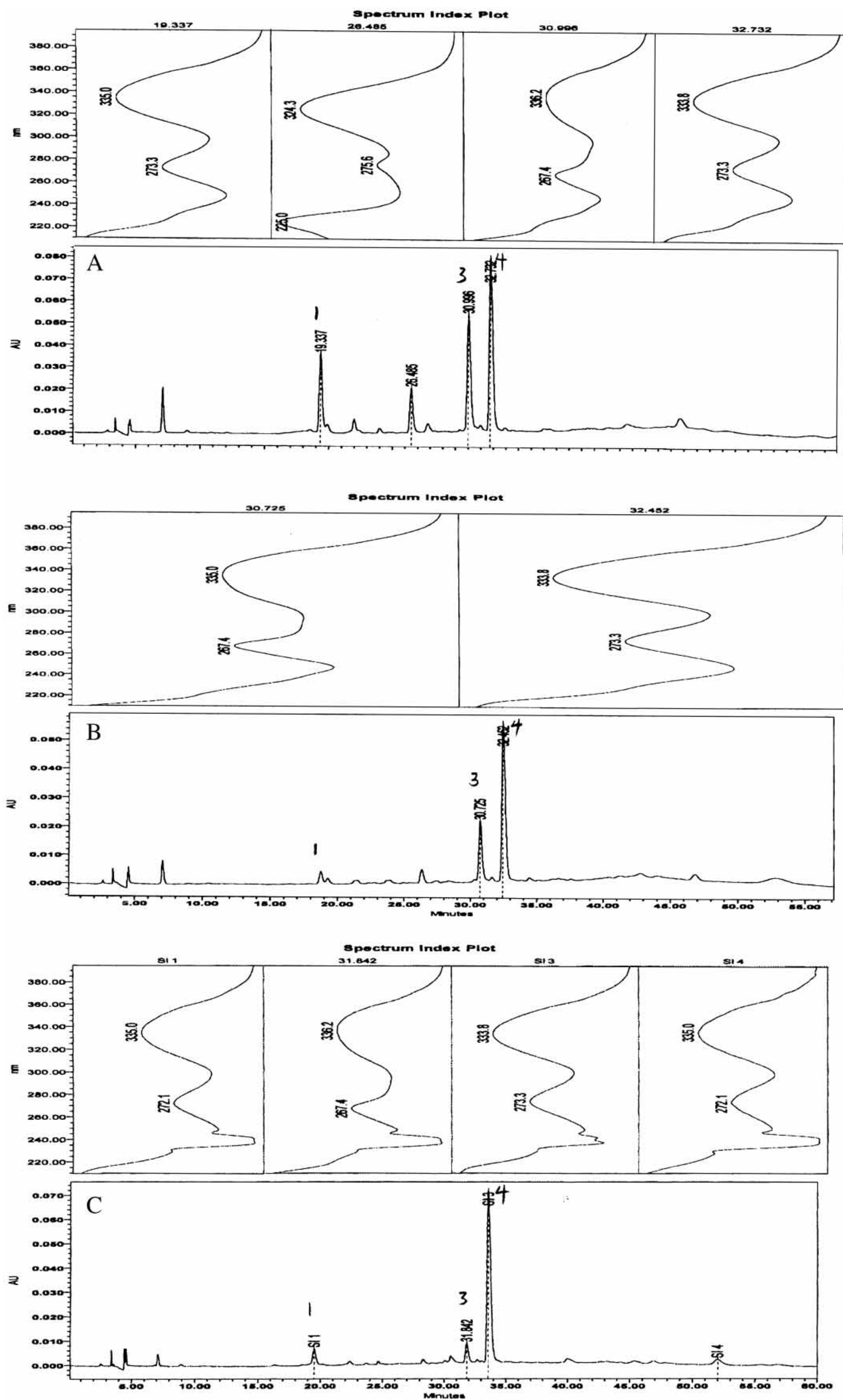

FIGURE 2 HPLC-DAD chromatograms of bile after oral administration of scutellarin, (A) during 0–12 h; (B) during 12–24 h; (C): During 24–36 h. 1. M1, 2. M2, 3. M3, 4. M4 and 5. M5.

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samples and UV spectrum of the parent drug. In the UV spectra of five metabolites maximal absorptions were at 270–280 nm and 325–335 nm, which are characteristic of the flavone skeleton. The five metabolites were designated as M1, M2, M3, M4 and M5 in the order of decreasing polarity. The results showed that scutellarin was metabolized in rats at a high rate and mainly excreted in urine and bile in the form of metabolites. Scutellarin and its metabolites were hardly found in feces.

Enzymatic Hydrolysis

Sulfatase can specifically hydrolyse sulfate conjugates, but β-glucuronidase is not specific, it can hydrolyse both glucuronic conjugate and sulfate conjugates. Urine and bile 0–12 h after oral administration were hydrolysed by sulfatase and β-glucuronidase at 37°C for 12 h. HPLC profiles of hydrolysed samples and unhydrolysed samples were compared, and the M1, M3 and M4 peaks were found to disappear or obviously decrease, while M5 increased after hydrolysis by glucuronidase. Only M1, M3 disappeared after hydrolysis by sulfatase. Thus, we concluded that M1 and M3 were sulfate conjugates and M4 was a glucuronic conjugate. Metabolite in bile 24–36 h after oral administration was mainly M4. When the bile after 24–36 h was hydrolysed by glucuronidase, M5 was the main product. We thus inferred that M4 was a glucoside of M5.

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Analysis of Scutellarin and its Metabolites by HPLC-MS

To structurally identify the five metabolites in urine and bile, the prepared samples from urine and bile after oral administration were analysed by HPLC-MS (see Fig. 3) for structures). ESI in both negative and positive mode were tried. The results showed that ESI in positive mode was sensitive to scutellarin and its metabolites. Molecular ions $(M+1)^+$ of the five metabolites and scutellarin were collected using ESI in positive mode (Table I). The results showed that M2's molecular ion $(M+1)^+$ was 287, indicating the loss of glucuronic acid from scutellarin to form scutellarein. The molecular ions $(M+1)^+$ of M1 and M3 were 447, indicating that the conjugated compounds of scutellarein had two sulfate groups, in agreement with enzymatic hydrolysis. Two sulfate groups conjugated with either two of 4′,5,6,7-hydroxy groups of scutellarein at different positions resulted in a different elution order on the HPLC profile. M4's molecular ion $(M+1)^+$ was 477, revealing the addition of a $-CH_3$ group to scutellarin. M5's molecular ion $(M+1)^+$ was 300, indicating the loss of glucuronic acid from scutellarin and the addition of a $-CH_3$ group to scutellarin. On the basis of enzyme hydrolysis, the methyl position in M4 was the same as in M5. It may be that scutellarin was first methylated to produce M4, then M4 lost the glucuronic group and formed M5, or M2 was methylated to M5, then M5 was further conjugated with glucuronic acid and produced M4. The methylating enzyme in the cell liquid of liver specifically metabolized *o*-hydroxyphenyl so methylation may occur on the 5,6-hydroxy groups of scutellarin or on the 5,6,7-hydroxy groups of scutellarein. However, the 5-hydroxy group was chemically inert because of the formation of a hydrogen bond with the 4-carboxyl group; it could be supposed that the methylation most probably occurred on the 6-hydroxy group of scutellarin or the 6,7-hydroxy groups of scutellarein. In the metabolism of baicalein that had no 4′-hydroxy group compared with scutellarin in human urine, methylation only occurred on 6-hydroxy [5]. Consequently, we inferred that the methyl groups of M4 and M5 were mainly on the 6-hydroxy. To further confirm the position of methyl group of M4 and M5, M4 in bile during 24–36 h was analysed by HPLC-NMR. The structures of metabolites are given in Fig. 3.

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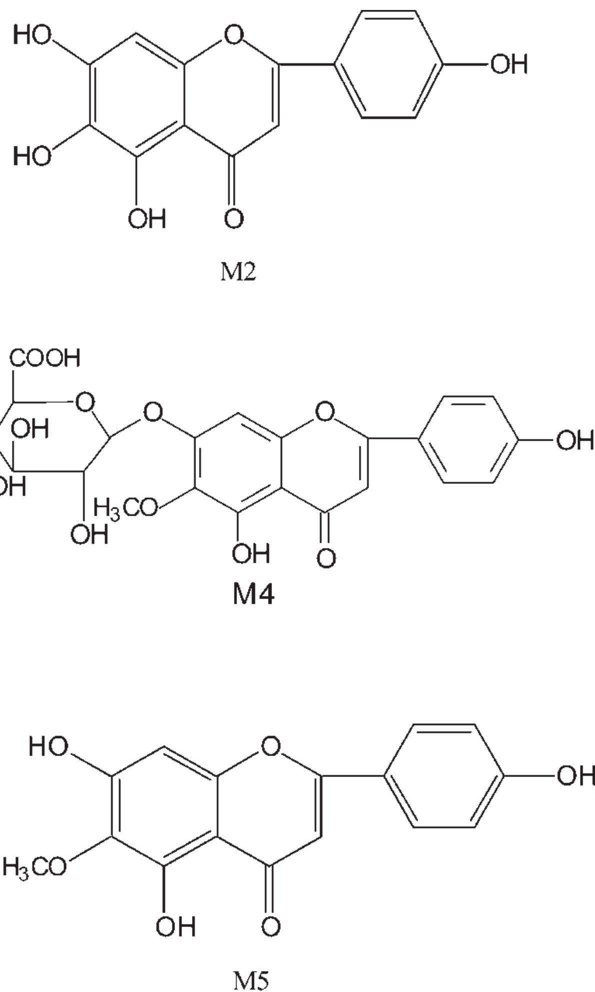

FIGURE 3 Structures of metabolites M2, M4 and M5.

HPLC-NMR Analysis of M4 in Bile

M4 was the main metabolite in bile during 24–36 h after oral administration. The extracted sample from bile during 24–36 h was analysed by HPLC-NMR. The stop-flow detection mode was adopted at $t_R = 33.0$ min. After 1 h the ^{1}H NMR data of M4 were

TABLE I HPLC-MS data for parent drug and metabolites

Identified metabolites and parent drug	*Retention time (min)*	*UV*	*ESI$^+$ data*
M1	15.027	272.2, 335.0	447
Parent drug	16.493	282.7, 326.7	463
M2	23.81	282.7, 335.0	287
M3	22.693	268.8, 336.2	447
M4	25.860	273.3, 333.8	477
M5	37.293	274.5, 335.0	301

TABLE II ^{1}H NMR of parent drug and M4

	Compound	
Proton	*Parent drug*	*M4*
6-OCH_3		3.8s
3-H	6.82s	6.75s
5-OH	12.74s	–
6-OH	10.36s	–
8-H	6.99s	7.01s
2′,6′-H	7.94d (8.5)	7.96d (9.0)
3′,5′-H	6.95d (8.0)	7.00d (9.0)
4′-H	8.59s	–
7-O-GLU		
1″	5.22d (8.0)	5.20d (7.0)
2″, 3″, 4″, 5″	4.04**br**	–

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collected (see Table II). A methoxy proton signal appeared at δ 3.8. The 8-phenyl proton and anomeric proton of glucuronic acid were similar to those of the parent drug. Thus, we inferred that M4 was 6-methyl-7-glucuronic acid scutellarin and M5 was 6-methyl-scutellarin.

Pathway of Metabolism and Excretion

After oral administration of scutellarin to rats it was metabolized at a high rate. Five metabolites were detected and identified. There were two possible pathways of metabolism. In the first pathway scutellarin was absorbed into the blood and metabolized to scutellarein (M2) by glucuronic acid enzyme and to M4 by a methylating enzyme in the liver. M4 lost glucuronic acid and formed M5, and M2 with the methyl group added to the 6-hydroxy formed M5 (Fig. 4). M2 was conjugated with two sulfate groups on either two of 4′,5,6,7-hydroxy groups in different ways and produced M1 and M3.

The second pathway was that scutellarin was first biotransformed into M2 which was then absorbed into the blood and metabolized to M1, M3 and M5. Subsequently, M5 conjugated with glucuronic acid to produce M4.

EXPERIMENTAL

Instrumentation

HPLC analysis was carried out on a Waters 2690 pump and 996 PDA detector using a 5 μm Phenomenex RP-18 column (4.6 × 250 mm). The column was maintained at 25°C. Detection was carried out at 335 nm with peak scanning from 200 to 400 nm (2 nm step). The flow rate was 0.8 ml min^{-1}. HPLC-MS was performed with a Waters 2690 pump, 996 PDA detector and Micromass ZMD using a RP-18 3.5 μm Waters Symmetry column (2.1 × 150 mm) that was maintained at 25°C. Detection was carried out at 335 nm with peak scanning from 200 to 400 nm (2 nm step). Mass spectra were recorded using ESI in the positive mode, with a cone voltage of at 30 V for TIC and 10 V for SIR, source temperature 120°C, desolvent temperature 300°C and scanning from mass 200 to 800 nm. The flow rate was 0.3 ml min^{-1}. A LD5-2A centrifuge made in Beijing Medical Centrifuge Factory was used. HPLC-NMR analysis was performed on a Varian HPLC-NMR system using a 5 μm Phenomenex RP-18 column (4.6 × 250 mm).

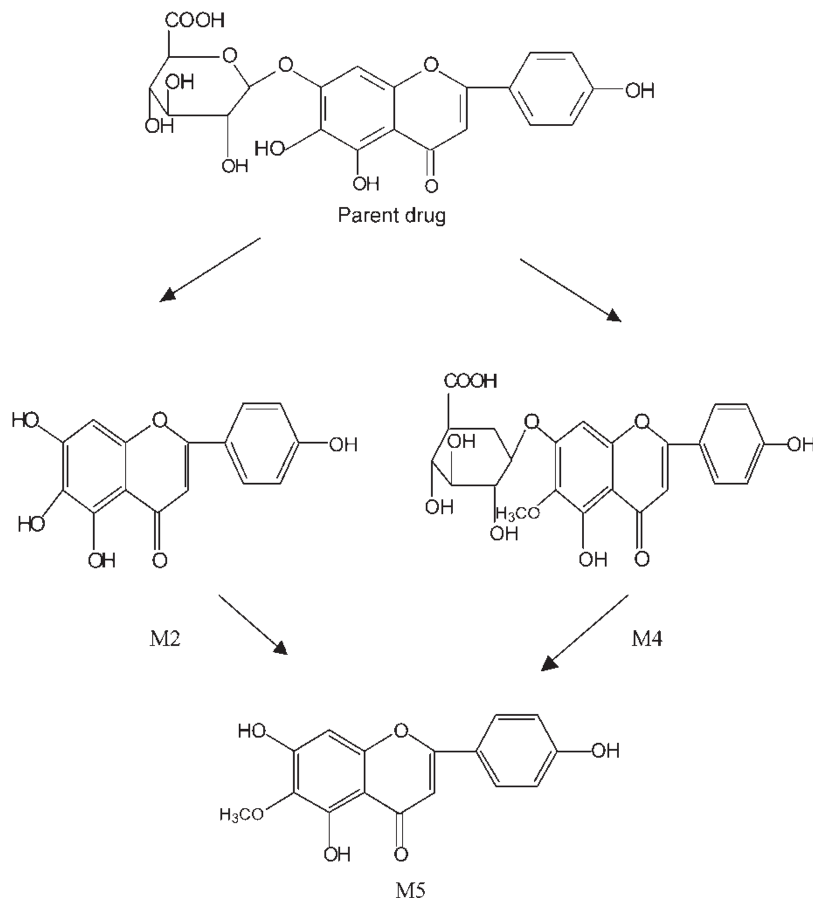

FIGURE 4 Metabolic pathway of scutellarin in rats.

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Chemicals

Acetonitrile was of HPLC grade from Fisher. Methanol was of GR grade from Peking Chemical Factory. Acetic acid was of analytical grade from Peking Chemical Factory. *N*,*N*-Dimethylformamide (DMF) was of analytical grade from Fixing Chemical Reagent Factory in China. Glucuronidase and sulfatase were purchased from Sigma. Physiological saline was purchased from Shandong Lingzi Pharmaceutical Company.

Scutellarin (>95% purity), provided by Professor Che Qingming, was dissolved in physiological saline to a concentration of 50 mg ml^{-1}; a little DMF helped dissolution.

Animal and Biological Samples Collection

Ten male Wistar rats (200 ± 20 g body weight) were provided by the Animal Center of Institute of Materia Medica. Before oral administration of scutellarin, ten rats were fasted in a metabolic cage for 12 h and maintained with physiological saline. In the meantime blank urine and feces were collected. Five rats were orally administered 200 mg kg^{-1} bodyweight of scutellarin, and urine and feces were collected during 0–12 h and 12–24 h periods. Five rats were anaesthetized using acetic ester and a polyethylene tube was inserted into the rat

biliary duct. After blank bile was collected for 4 h, the rats were orally administered 200 mg kg^{-1} body weight of scutellarin, and bile was collected during 0–12 h and 12–24 h periods.

Preparation of Biological Samples

Rats urine or bile was subjected to ODS (50 mg ODS per ml urine or bile) with H_2O and MeOH as eluent. MeOH fractions containing metabolites were evaporated to dryness under reduced pressure at 40°C and stored frozen until analysed by HPLC as described below. Feces were ground completely in 10 times the volume of water and the mixture was centrifuged. The upper clear solution was subjected to ODS as described above.

Enzymatic Hydrolysis

Urine or bile was incubated in 0.1 M NaAc buffer (pH 5.1) with β-glucuronidase 1500 U per ml urine or bile and in 0.5 M NaAc buffer (pH 7.1) with sulfatase 1 U per ml urine or bile for 12 h at 37°C. The reaction was terminated by removing the reaction mixture from the incubator and subjected to ODS as described in the Preparation of Biological Samples.

Analysis of Metabolites by HPLC-DAD and HPLC-MS

The residue of each sample was dissolved in MeOH, then centrifuged at 3500 rpm. The upper clear solution was analysed by HPLC-DAD, HPLC-MS or HPLC-NMR with 60 min gradient elution. Solvent A was 0.1% aqueous acetic acid and solvent B was acetonitrile. The gradient system was A/B (v/v) = 82/18 (0–10 min) → A/B (v/v) = 60/40 (40 min) → A/B (v/v) = 82/18 (60 min).

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