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Journal of Chromatography A, 987 (2003) 367–374
JOURNAL OF CHROMATOGRAPHY A
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Pharmacokinetic study of free mangiferin in rats by microdialysis coupled with microbore high-performance liquid chromatography and tandem mass spectrometry

Ling Lai[a,b], Lie-Chwen Lin[c], Jer-Huei Lin[a], Tung-Hu Tsai[b,c,*]

[a]*National Laboratories of Foods and Drugs, Department of Health, Executive Yuan, Taipei 115, Taiwan*
[b]*Institute of Traditional Medicine, National Yang-Ming University, Taipei 112, Taiwan*
[c]*National Research Institute of Chinese Medicine, Taipei 112, Taiwan*

Abstract

Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthen-9-one) has been isolated from the herbal root of *Anemarrhena asphodeloides* Bung showing antioxidative, antiviral, and anticancer effect. An in vivo microdialysis sampling method coupled to microbore high-performance liquid chromatography (HPLC) was employed for continuous monitoring of free mangiferin in rat blood. Microdialysis probes were inserted into the jugular vein/right atrium and brain striatum of Sprague–Dawley rats, and mangiferin at doses of 10, 30 or 100 mg/kg were then administered via the femoral vein. Dialysates were collected every 10 min and injected directly into a microbore HPLC system. Mangiferin was separated by a reversed-phase C_{18} microbore column (150×1 mm) from dialysate within 10 min. The mobile phase consisted of acetonitrile–0.05% phosphoric acid–tetrahydrofuran (10:75:15, v/v/v) with a flow-rate of 0.05 ml/min. The wavelength of the UV detector was set at 257 nm. The limit of quantification for mangiferin was 0.05 μg/ml and in vivo recovery of mangiferin at concentrations of 1, 5 and 10 μg/ml was in range of 37.7–39.8%. The results indicate that the pharmacokinetics of mangiferin at doses of 10–30 mg/kg reveals a linear relation, while doses of 30–100 mg/kg show a nonlinear pharmacokinetic phenomenon. Mangiferin was undetectable in brain dialysate. The proposed method provides a technique for rapid and sensitive analysis of free mangiferin in rat blood and further application in pharmacokinetic study. Furthermore, the metabolites of mangiferin in the rat bile were confirmed by LC electrospray ionization (ESI) tandem mass spectrometry (MS–MS).

Keywords: Microdialysis; Pharmacokinetics; Mangiferin

1. Introduction

Mangiferin (Fig. 1) is a natural glucosyl xanthone, which occurs widely in the bark of *Mangifera indica* [1], the root of *Anemarrhena asphodeloides* Bung

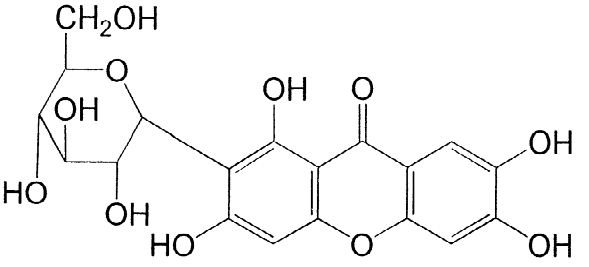

Fig. 1. Chemical structure of mangiferin.

*Corresponding author. National Research Institute of Chinese Medicine, 155-1, Li-Nong Street Section 2, Shih-Pai, Taipei 112, Taiwan. Tel.: +886-2-2820-1999x8091; fax: +886-2-2826-4276.
E-mail address: thtsai@cma23.nricm.edu.tw (T.H. Tsai).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01415-2

(Chinese herbal name: Zhi-Mu) [1], the leaf of *Hibiscus liliastrum* [2], as well as different angiosperm families and ferns [3–5]. Recent studies indicate that mangiferin has wide ranges of pharmacological activities including antioxidant [6], antidiabetic [7], anti-HIV [8], antitumor [8], hepatoprotective [6], antiviral [8,9], and anticancer [10] actions. Mangiferin has also been used for nervous debility by providing monoamine oxidative inhibition in traditional Indian medicine [11]. Previous reports have demonstrated that high-performance liquid chromatography (HPLC) is a suitable instrument for the determination of mangiferin in herbs [12], traditional Chinese herbal prescriptions [13] and biological fluids [14,15]. However, no previous studies have attempted to monitor the free form of mangiferin in biological fluids.

The free form of a drug is considered the pharmacologically active portion because it can diffuse out of the vascular compartment through cell membranes to reach target tissues. Furthermore, plasma protein binding can significantly affect important pharmacokinetic processes, such as distribution and elimination by renal and/or hepatic mechanisms. Thus, for many drugs, the free drug concentration has important pharmacokinetic and pharmacodynamic implications [16]. In the present study, we used a microdialytic sampling technique coupled with a microbore liquid chromatographic system to examine the free mangiferin in rat blood. In addition, we utilized this sampling and assay system to investigate the pharmacokinetics of free mangiferin in rat blood following its intravenous administration (10, 30 and 100 mg/kg). Furthermore, in order to investigate the metabolism of mangiferin, a single bolus intravenous dose of 100 mg/kg of mangiferin was administered to a rat, and the bile dialysate was then collected to analyze its metabolite using LC–MS–MS.

2. Experimental

2.1. Chemicals

Mangiferin was purchased from Sigma (St. Louis, MO, USA). The chromatographic solvents and reagents were obtained from BDH (Poole, UK). Triple deionized water from Millipore (Bedford, MA, USA) was used for all preparations. Authentic mangiferin stock solutions 100 and 50 μg/ml with methanol were stored refrigerated (nominal 4 °C) in amber glass vessels for up to 2 months. Aliquots of these working stock solutions were dilute to produce quality control samples containing mangiferin at nominal concentrations of 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml.

2.2. Animals

Adult male Sprague–Dawley rats (280–320 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and were allowed to acclimate to their environmentally controlled quarters (24±1 °C and 12:12 h light–dark cycle) for at least 5 days before experimentation. The rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and remained anesthetized throughout the experimental period. The femoral vein was exposed for drug administration, and the rat's body temperature was maintained at 37 °C with a heating pad.

2.3. Liquid chromatography

HPLC was performed with a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), a Rheodyne Model 7125 injector equipped with a 10-μl sampling loop and an ultraviolet detector (Linear Model 340, San Jose, CA, USA). Separation was achieved by a reversed-phase C_{18} microbore column (Inertsil-2, 150×1 mm I.D.; particle size 5 μm, GS Sciences, Tokyo, Japan). The mobile phase consisted of acetonitrile–0.05% phosphoric acid–tetrahydrofuran (10:75:15, v/v/v) with a flow-rate of 0.05 ml/min, and the optimal wavelength was 257 nm. Output data from the detector were integrated using an EZCHROM chromatographic data system (Scientific Software, San Roman, CA, USA).

2.4. Liquid chromatography–tandem mass spectrometry

LC–MS–MS analysis was performed using a Waters 2690 with a 996 photodiode array detector (PDA) together with an automatic liquid chromato-

graphic sampler and an injector (Bedford, MA, USA) system hyphenated to a Micromass Quattro Ultima tandem quadrupole mass spectrometry (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). The separation of LC–MS–MS was achieved using a reversed-phase C_{18} column (4.6× 150 mm) (Agilent, USA). The mobile phase was kept constant at 0.2 ml/min. Mobile phases A and B were acetonitrile and 2% acetic acid solution, respectively. The flow gradient was initially 10:90, linearly ramped to 30:70 for 20 min, and then returned to 10:90 for 5 min. The volume of injection was 5 μl. For operation in MS–MS mode, the mass spectrometer with an orthogonal Z-spray electrospray interface was used. Infusion experiment was performed using a Mode 22 multiple syringe pump (Harvard, Holliston, MA, USA). During the analyses, the ESI parameters were set as follows: The capillary voltage was 2.98 kV for negative ionization mode. The desolvation temperature was 350 °C and the source block temperature was 117 °C. The cone voltages of *m*/*z* 421, 435, 501 and 611 were 80, 80, 80 and 101 V and the collision voltages were 2, 25, 27 and 28 eV, respectively. The nebuliser and desolvation gas flows were 50 and 603 l/h, respectively. The collision gas was Argon 99.998% (Sanfu Chem., Taipei, Taiwan) with a pressure of 1.41×10^{-3} mbar in the collision cell. All LC–MS–MS data were processed by the MASSLYNX version 4.0 NT Quattro data acquisition software.

2.5. Method validation

The accuracy of the method was verified with respect to selectivity, limits of detection and quantification, recovery, linearity, as well as accuracy and precision. Selectivity was verified with six independent blank samples of dialysate. The limit of detection was determined at a signal-to-noise ratio of 3. The limit of quantification is defined as the lowest analyte concentration that can be measured with a stated level of confidence. In practice, it is the lowest concentration in the calibration curve. All calibration curves of mangiferin were constructed prior to the experiments with correlation values of at least 0.995. The intra- and inter-variabilities for mangiferin were assayed (six replicates) at 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml on the same day and on 6 sequential days, respectively. Accuracy (% Bias) = $[(C_{obs} - C_{nom})/C_{nom}] \times 100$. The precision (relative standard deviation; RSD) was calculated from the observed concentrations as follows: RSD = [standard deviation (SD)/C_{obs}]×100. Accuracy (Bias) and precision (RSD) values within ±15% covering the range of actual experimental concentrations were considered acceptable [17].

2.6. Microdialysis procedures

Blood, brain and bile microdialysis systems consisted of a microinjection pump (CMA/100, Stockholm, Sweden), a fraction collector (CMA/140) and microdialysis probes. The dialysis probes for blood (1 cm in the length for dialysis) and brain (0.3 cm in the length for dialysis) [18] were made of silica capillary in a concentric design. The tip of microdialysis probes were covered by dialysis membranes (Spectrum, 150 μm outer diameter with a cut-off at nominal molecular mass of 13 000, Laguna Hills, CA, USA). The blood microdialysis probe was positioned within the jugular vein/right atrium (toward the heart) and then perfused with anticoagulant citrate dextrose, ACD solution (citric acid 3.5 m*M*; sodium citrate 7.5 m*M*; dextrose 13.6 m*M*) at a flow-rate of 1.2 μl/min.

The bile duct microdialysis probes were constructed in our own laboratory [19,20]. A 7-cm dialysis membrane was inserted into a polyethylene tubing (PE-60; 0.76 mm I.D.; 1.22 mm O.D., Clay-Adams, NJ, USA). The ends of the dialysis membrane and PE-60 were inserted into a silica tubing (40 μm I.D., 140 μm O.D., SGE, Australia) and PE-10 (0.28 mm I.D., 0.61 mm O.D.), respectively. Both the ends of the tubing and the union were cemented with epoxy. At least 24 h were allowed for the epoxy to dry. After bile duct cannulation, the probe was then perfused with Ringer's solution at a flow-rate of 1.2 μl/min.

After the implantation of the blood and bile microdialysis probes, the rat was immobilized in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The skull was surgically exposed, and a hole was trephined into the skull according to stereotaxic coordinates [21]. The brain microdialysis probe was placed into the right striatum (0.5 mm anterior to the bregma, 3.0 mm lateral to the midline

and 7.0 mm lower to the tip). The brain microdialysis probe was perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+; pH 7.0) at a flow-rate of 1.2 μl/min. Brain dialysates were collected by a fraction collector (CMA/140) at 10-min intervals. The position of each brain microdialysis probe was verified at the end of the experiments. Following a 2-h postsurgical stabilization period, mangiferin (10, 30 and 100 mg/kg) was intravenously administered via the femoral vein. Each animal received only one dose. Then, aliquots of 10 μl of blood, brain and bile dialysates were collected by the fraction collector (CMA 140) and then injected onto the microbore chromatographic system.

2.7. Recovery of microdialysate

For in vivo recovery determinations, a retrograde calibration technique was used, in which the blood microdialysis probe was inserted into the rat jugular vein, under anesthesia with sodium pentobarbital. Mangiferin solution of 1, 5 and 10 μg/ml was perfused through the probe at a constant flow-rate (1.2 μl/min) using the infusion pump. After a 2-h postsurgical stabilization period, the inlet ($C_{\text{perfusate}}$) and outlet ($C_{\text{dialysate}}$) concentrations of mangiferin were determined by HPLC. The in vivo recovery ($\text{Recovery}_{\text{in vivo}}$) of agents across the microdialysis probe was calculated by the following equation [21]:

$$\text{Recovery}_{\text{in vivo}}\,(\%) = \frac{C_{\text{perfusate}} - C_{\text{dialysate}}}{C_{\text{perfusate}}} \times 100$$

2.8. Pharmacokinetic analysis

To evaluate the suitability of the assay for pharmacokinetic studies, 10, 30 or 100 mg/kg of mangiferin were intravenously administered to rats. Six animals were used in each dosage. Pharmacokinetic calculations were performed using the observed data. All data were subsequently processed by the pharmacokinetic program, WINNONLIN standard version 1.1 (Pharsight, Mountain View, CA, USA), for the calculation of pharmacokinetic parameters according to the noncompartmental model.

3. Results and discussion

Retention time of mangiferin was 6.0 min (Fig. 2A). Peak-areas of mangiferin were linear over a concentration range of 0.05–10 μg/ml in blood dialysates. Linear regression of the peak area for analyte versus concentration was performed using unweighting linear regression. The regression equation was $y = 2.43 \times 10^{-6}x + 8.54 \times 10^{-4}$, where x is the peak area and y is the analyte concentration. The correlation coefficient was higher than 0.999, indicating good linearity. The limit of quantitation was 0.05 μg/ml. Intra- and inter-assay precision and accuracy of mangiferin fell well within the predefined limits of acceptability (<15%) (Table 1). Fig. 2A shows the chromatogram of standard mangiferin (1 μg/ml). None of the observed peaks in a blank blood dialysate interfered with mangiferin, as shown in Fig. 2B. Fig. 2C shows a typical chromatogram of a blood dialysate which contains mangiferin (0.49 μg/ml) collected 120 min after mangiferin administration (100 mg/kg, i.v.). The in vivo recovery was approximately 37.7–39.8%, as shown in Table 2.

The dialysate samples collected over the first 2 h were discarded to allow recovery from the acute effects of the surgical procedure. The microdialysis

Table 1
Intra- and inter-assay accuracy and precision values for mangiferin

Nominal concentration (μg/ml)	Observed concentration (μg/ml)[a]	RSD (%)	Accuracy (% Bias)
Intra-assay ($n=6$)			
0.05	0.046±0.004	8.7	−8.0
0.10	0.096±0.005	5.2	−4.0
0.50	0.501±0.007	1.7	0.2
1.00	1.00±0.014	1.4	0.0
5.00	5.01±0.052	1.0	0.2
10.00	9.99±0.022	0.2	−0.01
Inter-assay ($n=6$)			
0.05	0.047±0.007	14.9	−6.0
0.10	0.097±0.007	7.2	−3.0
0.50	0.502±0.004	0.8	0.4
1.00	1.002±0.011	1.1	0.2
5.00	5.01±0.02	0.4	0.2
10.00	9.99±0.01	0.1	−0.01

[a] Observed concentration data are expressed as rounded mean±SD.

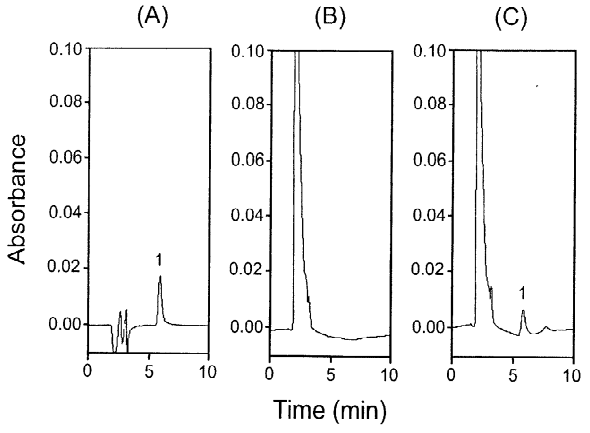

Fig. 2. Typical chromatograms of (A) standard mangiferin (1 μg/ml) (B) blank blood dialysate, and (C) blood sample containing mangiferin (0.49 μg/ml) collected from jugular vein at 120 min after mangiferin administration (100 mg/kg, i.v.).

Table 2
In vivo microdialysate recovery of mangiferin in rat blood

Concentration (μg/ml)	Recovery (%)
1	37.7±0.7
5	39.8±3.2
10	37.8±3.0
Average	38.4

Data are expressed as means±SD ($n=6$).

sampling technique and liquid chromatographic detection were then applied to the pharmacokinetic characterization of mangiferin in rats (Table 3). Fig. 3 shows the free mangiferin pharmacokinetic curve in rat blood during mangiferin administration (10, 30 and 100 mg/kg, i.v., $n=6$ for each dose). Pharmacokinetic parameters of mangiferin are shown in Table 3. The results indicate that the elimination half-life, mean residence time, steady-state volume of distribution and total body clearance between the two doses of 10 and 30 mg/kg did not differ significantly whereas the AUC was proportionally related to the dose, showing a linear pharmacokinetic phenomenon.

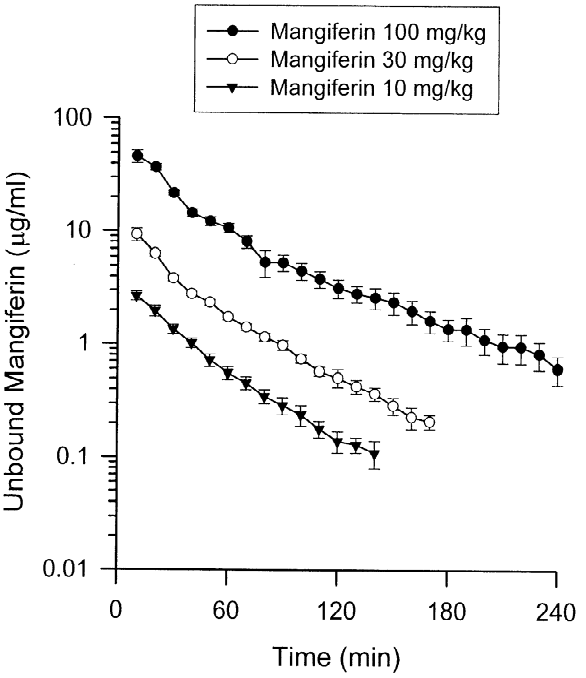

Fig. 3. Mean free level of mangiferin in rat blood after mangiferin administration (10, 30, and 100 mg/kg, i.v.). Data are expressed as mean±S.E.M. ($n=6$).

However, when the dose of mangiferin was increased from 30 to 100 mg/kg, the AUC in rats

Table 3
Pharmacokinetic parameters following mangiferin administration (10, 30 and 100 mg/kg, i.v.)

Parameters	10 mg/kg	30 mg/kg	100 mg/kg
AUC (min μg/ml)	122.9±17.1	452.5±39.8	2424.2±196.7*
$t_{1/2,\beta}$ (min)	28.5±3.5	35.0±3.5	47.8±4.1*
V_{ss} (l/kg)	3.0±0.3	2.5±0.3	2.0±0.3
Cl (ml/min/kg)	90.1±12.6	69.1±6.5	43.2±4.3*
MRT (min)	35.0±2.52	35.9±2.8	45.8±4.8*

Data are expressed as means±S.E.M. ($n=6$). AUC, area under the concentration versus time curve; $t_{1/2,\beta}$, elimination half-life; V_{ss}, steady-state volume of distribution; Cl, clearance; MRT, mean residence time.
*, Significantly different ($P<0.05$) from the dose of 10 and 30 mg/kg (Student's t-test).

increased greater than that in direct proportion with the dose. In addition, the terminal half-life ($t_{1/2,\beta}$) was prolonged at the higher dose. These results suggest that the pharmacokinetics of mangiferin in rats between the doses of 30 and 100 mg/kg is nonlinear. Several factors may affect the elimination half-life of drug at higher dose such as saturation of biodegradation. Consideration of free drug concentration, the percentage of protein bound and tissue bound may affect the free drug elimination half-life. In general, the percentage of protein binding is a constant which is concentration unrelated. In our previous studies, the same nonlinear pharmacokinetic phenomena were observed in other herbal ingredients of some commonly used herbal medicines, such as glycyrrhizin [22], and glycyrrhetinic acid [23]. In cases like these, administration of a large dose may retard drug elimination and prolong its effect [24].

With relative noninvasive and nonconsumptive sampling method, microdialysis is especially beneficial to biological fluid and brain tissues. Microdialysis sampling of small molecular substances is uniquely suitable for continuous monitoring of free drugs in biological fluids because the larger protein-bound molecules are excluded. Monitoring of free of drug concentrations is a relatively new concept; however, it has been suggested as the most appropriate approach for many drugs [16]. The ultrafiltration method [25] is one of another method to obtain protein-free analytes.

In order to investigate blood–brain barrier (BBB) penetration and hepatobiliary excretion of mangiferin in rat, the multiple microdialysis probes were simultaneously implanted into the brain striatum and bile duct, respectively. However, the parent molecule of mangiferin was not detected in the bile and brain regions for all dosage treatments. These results suggest that mangiferin cannot cross the BBB or is restricted by its detection limit.

Mangiferin was undetected in the bile dialysate, therefore, the forward biotransformation including phases I and II were investigated using LC–MS–MS. Fig. 4 shows the UV spectrum (A) and the mass spectrum (B) from tandem mass spectrometry where the peaks of m/z 421, 331 and 301 corresponds to mangiferin in ESI negative mode.

Phases I and II metabolites of mangiferin were considered in bile dialysate. Fig. 5 shows the chromatogram of bile sample after mangiferin administration (100 mg/kg, i.v.), illustrating four major peaks (I–IV) at retention times (t_R) of 3.09, 3.54, 6.79 and 8.37 min. Because the difference between the above four metabolites and mangiferin is located in the side chains, the UV absorbance of the metabolites and mangiferin are all the same. Subsequent LC–MS–MS analyses were performed in order to

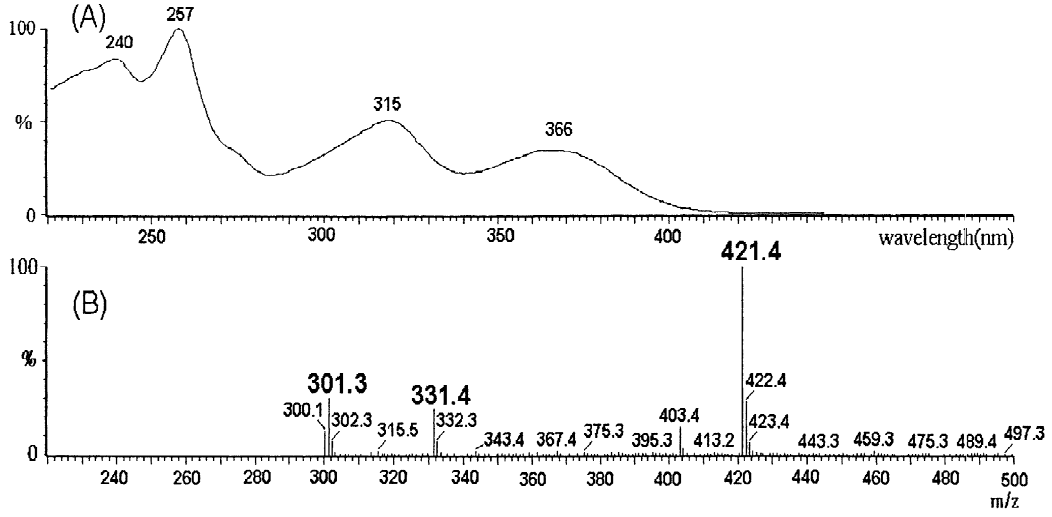

Fig. 4. Full scan of authentic mangiferin (A) UV wavelength from photodiode array and (B) the relative abundance of mass spectrum from tandem mass spectrometry.

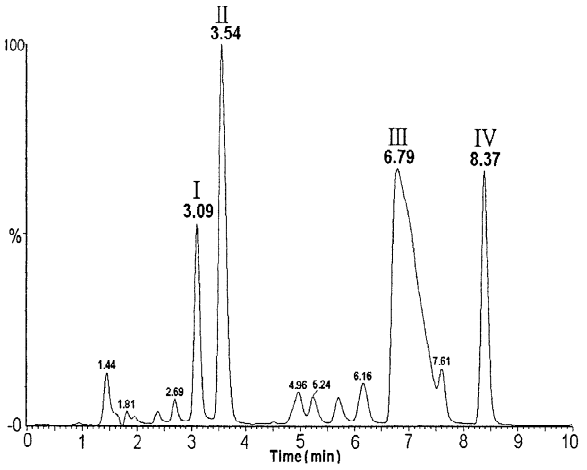

Fig. 5. LC–MS chromatogram of bile dialysate after mangiferin administration (100 mg/kg, i.v.) illustrating four major peaks (I–IV) at retention times (t_R) of 3.09, 3.54, 6.79 and 8.37 min.

identify unambiguously the daughter ion spectra of the peaks in bile dialysate. Fig. 6A shows the fragment ions of peak I (Fig. 5; t_R 3.09 min) at m/z 611 which may be caused by methylation (m/z 14) and follows glucuronidation (m/z 176) with m/z of 611 (421+14+176) (Fig. 6A). Other fragment ions at m/z 435, 345 and 315 were assumed to be $[421+CH_3]^-$, $[331+CH_3]^-$ and $[301+CH_3]^-$, respectively.

Fig. 6B shows the fragment of peak II (Fig. 5; t_R 3.54 min) at m/z 501 which may be derived from sulfation (m/z 80) with m/z of 501 (421+80) (Fig. 6B). This result suggests that the phase II sulfation may occur in the biotransformation of mangiferin. Fig. 6C and D represents the fragment of peaks III and IV with the same m/z 435 at t_R 6.79 and 8.37 min (in Fig. 5), respectively, which may be caused by the methylation. The fragment ions (m/z) of 345 and 315 were assumed to be $[331+CH_3]^-$ and $[301+CH_3]^-$, respectively.

The hydroxylation of mangiferin at positions 1, 3, 6 and 7 may be processed through *O*-methylation (m/z 14) at hydroxylated group to produce methylmangiferin with m/z) of 435 (421+14) (Fig. 6C and D).

In summary, a rapid and sensitive microbore liquid chromatographic system for the determination of mangiferin in rat blood was developed. This method has the advantages of less tissue damage;

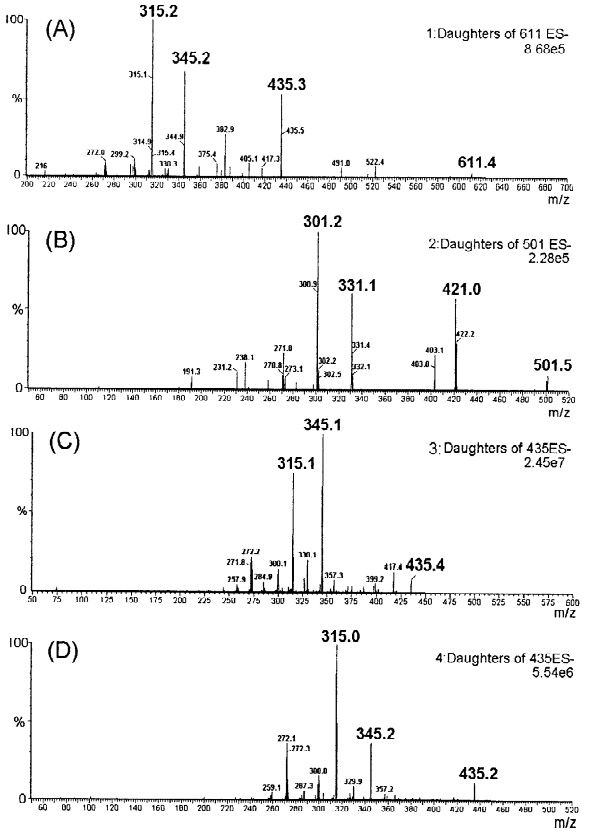

Fig. 6. Fragment daughter ionic chromatograms of (A), (B), (C) and (D) are correlated to the peaks of I, II, III and IV, respectively, in Fig. 5.

fewer animals needed, no biological fluid loss, and exhibit no endogenous interference, with sufficient sensitivity for the measurement of free mangiferin in the blood dialysates. This model may be useful in performing in vivo mechanistic studies of the pharmacokinetics of mangiferin's free aspect. In addition, the LC–MS–MS was used to identify the possible pathway of metabolism. These results suggest that the possible biotransformation of mangiferin includes methylation and phase II sulfation and glucuronidation.

Acknowledgements

This study was supported in part by research grants (NSC90-2113-M-077-002; NSC90-2320-B-

077-005) from the National Science Council, Taiwan.

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