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Short communication

Identification and determination of four metabolites of mangiferin in rat urine

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Abstract

Four metabolites of mangiferin were firstly isolated and identified from rat urine. The structures of the four metabolites were determined to be 1,3,7-trihydroxyxanthone (M-1), 1,3,6,7-tetrahydroxyxanthone (M-2), 1,3,6-trihydroxy-7-methoxyxanthone (M-3) and 1,7-dihydroxyxanthone (M-4), respectively. A simple and specific analytical method for determination of the four metabolites in rat urine was developed by high performance liquid chromatography (HPLC). Quercetin was employed as an internal standard. The correlation coefficients of the calibration curves were higher than 0.997, both intra- and inter-day precision of four metabolites were determined and their R.S.D. did not exceed 10%. The accuracy and linear range had been investigated in detail. The cumulative urinary excretions of the four metabolites were measured and the possible metabolic pathway of the metabolites was discussed.

Keywords: Mangiferin; Metabolite; HPLC; Urine

1. Introduction

The rhizome of *Anemarrhena asphodeloides* has been used as a traditional Chinese medicine for thousands of years. Its active xanthone compound mangiferin (1,3,6,7-tetrahydroxyxanthone-C-2-β-D-glucoside, Fig. 1), has been demonstrated to have various pharmacological effects, such as anti-diabetic [1,2], radioprotection [3], antioxidant [4], hepatoprotective [5] and anti-allergic [6]. Our previous study of its pharmacokinetics in rat showed that mangiferin was slowly absorbed and intensely metabolized [7]. One of the metabolites of mangiferin has been isolated by incubation with a bacterial mixture of human feces and identified as norathyriol (M-2) [8], which showed various pharmacological effects, including anti-inflammatory [9], vasorelaxation [10], and anti-platelet [11]. Previous study reported some of these metabolites from rat bile by LC–MS/MS method [12]. Another study reported two metabolites of mangiferin–euxanthone and its glucuronide were detected in rabbit's urine [13]. This study describes the extraction, isolation and identification of these metabolites and a validated HPLC method for the determination of the accumulation of four metabolites of mangiferin in rat urine.

2. Experimental

2.1. Materials

Mangiferin was isolated from *Anemarrhena asphodeloides Bge*. in our laboratory. Purity was about 98% determined by HPLC method, and the internal standard quercetin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). HPLC-grade methanol was purchased from Merck Company (Merck, Darmstadt, Germany). All other analytical grade reagents were from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, PR China). Water was doubly distilled in the laboratory. The formation for oral administration of mangiferin was prepared by mixing mangiferin with sodium carboxymethylcellulose (CMC-Na) water solution (0.5%, CMC-Na/H_2O, g/g), the final concentration of mangiferin in CMC-Na water solution was 10 mg/mL. Macroporous resin D101 was purchased

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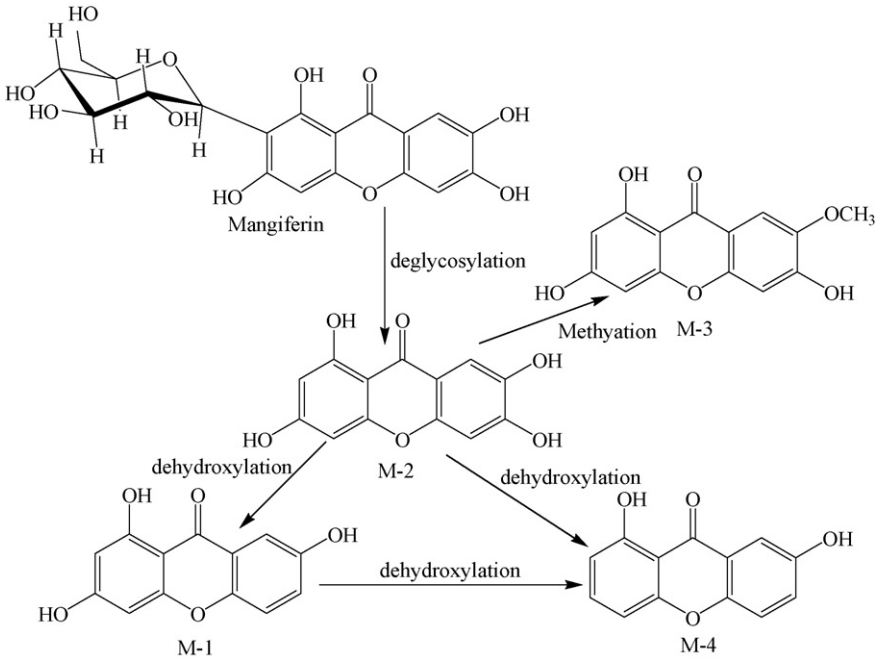

Fig. 1. Structure of mangiferin metabolites and possible metabolic pathways for their production.

from the Chemical Plant of NanKai University (Tianjin, China). Sephadex LH-20 (Amersham, Biosciences) and Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd.) were used for column chromatography. Silica gel GF_{254} (0–40 μm, Qingdao Haiyang Chemical Co. Ltd.) was used for TLC analysis.

2.2. Liquid chromatography

The HPLC system consisted of a Waters 600E pump, a Waters 2487 UV–vis detector set at 254 nm, a LC workstation for data collection. In order to prepare the metabolites, a 200 μL injection loop and a Inertsil C_{18} reversed-phase column (5 μm, 250 mm × 10 mm) protected by an RP18 (5 μm) guard column (from GL Sciences Inc.) were used. The semi-preparative HPLC condition: the flow rate was 2.5 mL/min; mobile phase component A was acetonitrile, and B was water; the column was eluted with a linear gradient of 46–48% A over 0–13 min, 48–50% A over 13–16 min, 50–60% A over 16–30 min, then returned to 46% A at 35 min immediately.

A 20 μL injection loop and a Diamonsil C_{18} reversed-phase column (5 μm, 200 mm × 4.6 mm) protected by an RP18 (5 μm) guard column (from GL Sciences Inc.) were used for analytic use. The analytic HPLC condition: the flow rate was 1 mL/min; mobile phase component A was methanol, and B was water with 0.1% phosphoric acid; the column was eluted with a linear gradient of 45–60% A over 0–15 min, 60–80% A over 15–25 min, and the composition was maintained 80% A for 5 min, then returned to 45% A at 30 min immediately.

2.3. Animals and treatment

Male Sprague–Dawley rats (200–220 g) were obtained from the Laboratory Animal Center of Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, PR China). They were kept in an environmentally controlled breeding room for at least 3 days before experimentation, fed with standard laboratory food and water and fasted overnight but with access to water before the test.

In order to isolate the metabolites, 60 rats were used for collecting urine sample. Aqueous solutions of mangiferin were orally administrated to rats at a dose of 120 mg/kg. Rats were then immediately kept in individual metabolic cages. Urine samples were collected at times of 6, 12, 24, 30, 36, 48 and 60 h after dosing. These urine samples were combined together and stored at −20 °C.

Another six rats were used for studying the accumulative character of the metabolites in rat urine. These rats were administrated the same dose of mangiferin as mentioned above and kept in individual metabolic cages immediately. Urine samples were collected at times of 1, 2, 4, 6, 10, 12, 24, 36, 48 and 60 h after dosing, and 1 mL urine was withdrawn from each time plot and centrifuged for 5 min at 3552 × *g*. The supernatant was transferred to labeled plastic vials. The rest of the urine was stored in a large flask. All these urine were stored at −20 °C until analysis was carried out.

2.4. Extraction and isolation

The collected urine samples (approximately 2600 mL in total) was concentrated to almost dryness, the residue was suspended in methanol and deposited for few hours. The deposit was not needed and the supernatant was removed and evaporated to dryness, then the residue was dissolved in water and partitioned with *n*-butanol three times; the *n*-butanol layer was subjected to D101

and eluted with H_2O/EtOH stepwise. The 30% and 50% EtOH eluting fractions were further subjected to Sephadex LH-20 with a MeOH–H_2O system (8:2), and then the eluted fractions were combined and subjected to a silica gel chromatography column with a cyclohexane-2-propylalcohol system (20:1,13:1,7:1). The fraction eluted by cyclohexane-2-propylalcohol (20:1) yielded metabolite M-1 (40 mg), the fraction eluted by cyclohexane-2-propylalcohol (13:1) and cyclohexane-2-propylalcohol (7:1) were finally purified by semi-preparative HPLC to yield 16 mg of M-2 and 10.8 mg of M-3. The fraction eluted from the D101 resin by 95% EtOH was directly subjected to Sephadex LH-20 by MeOH–H_2O system (8:2) and purified by semi-preparative HPLC to yield 8.4 mg of M-4. The purity of these metabolites was higher than 98% checked by HPLC method.

2.5. Calibration procedure

Stock solutions of 52.0 μg/mL for M-1, 60.0 μg/mL for M-2, 24.0 μg/mL for M-3, 16.8 μg/mL for M-4 and 30.5 μg/mL for quercetin were prepared in volumetric flasks in methanol individually. Urine calibration solutions were prepared by adding 5, 20, 50, 200 and 600 μL of M-1 stock solution; 5, 20, 50, 100 and 200 μL of M-2 stock solution; 10, 50, 100, 200 and 400 μL of M-3 stock solution and 10, 50, 100, 300 and 600 μL of M-4 stock solution into 1.5 mL Eppendorf tube, respectively, all these solutions were spiked with 100 μL of internal standard stock solution. All standard working solutions were evaporated into dryness at 40 °C in vacuum drying oven. The residue was reconstituted in 200 μL blank urine to prepare the calibration standards. The urine samples were treated with 400 μL methanol, mixed and centrifuged for 10 min at 8000 rpm at 5 °C. The supernatant was transferred into labeled Eppendorf tubes and evaporated to dryness at 40 °C in vacuum drying oven. The residue were reconstituted in 200 μL of methanol and then centrifuged at 12,000 rpm for 5 min. A volume of 20 μL of each supernatant was analyzed by HPLC to obtain the calibration curve.

Quality control (QC) samples were prepared in the same way as calibration standards with blank urine, and the concentrations of each analyte were at the low, middle and upper limits of quantification.

2.6. Method validation

2.6.1. Accuracy and precision

Intra-day accuracy and precision (each $n = 5$) were evaluated by analysis of QC samples at different times on the same way. Inter-day accuracy and precision (each $n = 5$) were determined by repeated analyses of QC samples three times per day at three concentration levels over three consecutive days. The concentration of each sample was determined using calibration standards prepared on the same day. Precision of the method was determined by the relative standard derivation (R.S.D.), and accuracy was determined by the relative recovery. Signal-to-noise ratio (S/N) of 3 was regarded as the limit of detection (LOD) and S/N ≥ 10 was regarded as the limit of quantification (LOQ) for four metabolites.

2.6.2. Recovery

The extraction recovery was determined by comparing the peak areas obtained from QC samples (without adding internal standard solution) with the unextracted standard working solutions at the same concentration in the same solvent.

2.6.3. Stability

The compound stability for 0, 6, 12 and 30 h at room temperature in urine was evaluated by repeated analysis at medium concentration of QC samples.

3. Results

3.1. Structure identification

Metabolite 1: 1,3,7-trihydroxyxanthone, yellow amorphous powder. EI-MS *m/z*: 244$[M]^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.20 (1H, d, $J = 1.7$ Hz, H-2), 6.38 (1H, d, $J = 1.7$ Hz, H-4), 7.29 (1H, dd, $J = 2.8$ and 9.0 Hz, H-6), 7.42 (1H, d, $J = 2.8$ Hz, H-8), 7.48 (1H, d, $J = 9.0$ Hz, H-5). ^{13}C NMR (125 MHz, DMSO-d_6): δ 162.5 (C-1), 97.8 (C-2), 165.5 (C-3), 93.8 (C-4), 157.6 (C-4a), 149.1 (C-4b), 119.1 (C-5), 124.5 (C-6), 153.8 (C-7), 108.0 (C-8), 120.5 (C-8a), 102.0 (C-8b), 179.7 (C–*C*=O). These data were well consistent with that of the literature [14].

Metabolite 2: 1,3,6,7-tetrahydroxyxanthone, yellow amorphous powder. EI-MS *m/z*: 260$[M]^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.15 (1H, d, $J = 1.6$ Hz, H-2), 6.33 (1H, d, $J = 1.8$ Hz, H-4), 6.83 (1H, s, H-5), 7.36 (1H, s, H-8). ^{13}C NMR (125 MHz, DMSO-d_6): δ 162.3 (C-1), 97.6 (C-2), 164.4 (C-3), 93.6 (C-4), 157.4 (C-4a), 151.2 (C-4b), 102.5 (C-5), 154.1 (C-6), 143.8 (C-7), 107.8 (C-8), 111.8 (C-8a), 101.6 (C-8b), 178.8 (C–*C*=O). The structure of M-2 was further substantiated by the literature [15].

Metabolite 3: 1,3,6-trihydroxy-7-methoxyxanthone, yellow amorphous powder. EI-MS *m/z*: 274$[M]^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.87 (3H, s, OMe-C-7), 6.19 (1H, d, $J = 1.4$ Hz, H-2), 6.38 (1H, d, $J = 1.4$ Hz, H-4), 6.94 (1H, s, H-5), 7.43 (1H, s, H-8). ^{13}C NMR (125 MHz, DMSO-d_6): δ 162.3 (C-1), 97.8 (C-2), 164.7 (C-3), 93.8 (C-4), 157.4 (C-4a), 152.2 (C-4b), 102.8 (C-5), 154.8 (C-6), 146.2 (C-7), 104.9 (C-8), 111.5 (C-8a), 101.6 (C-8b), 178.8 (C–*C*=O), 56.0 (C-O*C*H$_3$). These data were well consistent with those of in literature [14,16].

Metabolite 4: 1,7-dihydroxyxanthone, yellow amorphous powder. EI-MS *m/z*: 228$[M]^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 6.80 (1H, d, $J = 8.2$ Hz, H-4), 7.07 (1H, d, $J = 8.2$ Hz, H-2), 7.38 (1H, dd, $J = 8.1$ and 3.0 Hz, H-6), 7.47 (1H, d, $J = 3.0$ Hz, H-8), 7.58 (1H, d, $J = 8.1$ Hz, H-5), 7.72 (1H, t, $J = 8.3$ Hz, H-3). ^{13}C NMR (125 MHz, DMSO-d_6): δ 160.7 (C-1), 109.7 (C-2), 137.3 (C-3), 107.4 (C-4), 154.0 (C-4a), 149.5 (C-4b), 119.5 (C-5), 125.6 (C-6), 156.0 (C-7), 107.9 (C-8), 120.6 (C-8a), 108.0 (C-8b), 181.6 (C–*C*=O). The structure of M-4 was well supported by those data in the literature [17].

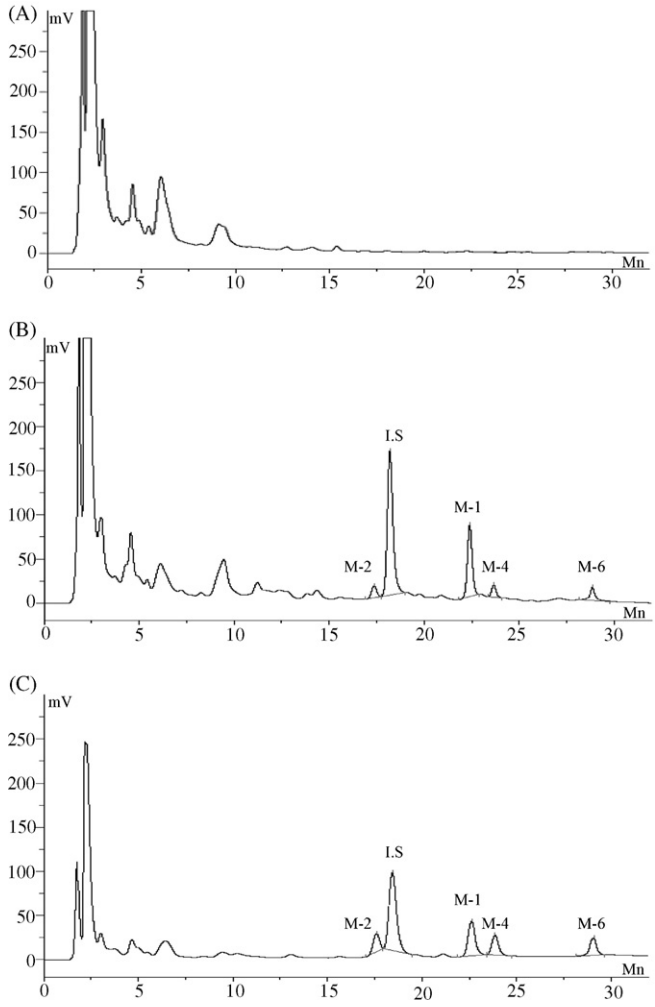

Fig. 2. Chromatograms for the analysis of the four metabolites of mangiferin in rat urine. (A) Blank rat urine sample; (B) urine sample from rat spiked with I.S. 2 h after oral administration of mangiferin; (C) urine sample spiked with four metabolites of mangiferin and I.S. standards.

3.2. Method selectivity

The separated peaks of mangiferin's four metabolites and I.S. (Fig. 2) revealed that no interfering peaks were detected. This indicated the selectivity of the elaborated procedure was satisfactory.

3.3. Calibration curves

The calibration curves were prepared for four mangiferin metabolites in urine in the range of 1.3–156.0 μg/mL for M-1, 1.5–60.0 μg/mL for M-2, 1.2–48.0 μg/mL for M-3 and 0.84–50.40 μg/mL for M-4, all that covered the levels following the administration of a single dose of 120 mg/kg mangiferin. The urine standard curve was described by the following equations: M-1, $y = 20.5x + 0.0255$, $r = 0.9981$; M-2, $y = 7.6849x + 0.0208$, $r = 0.9978$; M-3, $y = 12.744x + 0.0517$, $r = 0.9971$; M-4, $y = 8.72994x + 0.0196$, $r = 0.9994$, where y is the peak area, x the concentration, and r is the correlation coefficient. The LOQ of four metabolites in urine were defined as the lowest concentration on the standard curve for which the assay precision was reflected by R.S.D. $\leq 10\%$, and it amounted to 1.30 μg/mL for M-1, 1.50 μg/mL for M-2, 1.20 μg/mL for M-3 and 0.84 μg/mL for M-4. The limits of detection (LOD) were 0.76 μg/mL for M-1, 0.90 μg/mL for M-2, 0.84 μg/mL for M-3 and 0.59 μg/mL for M-4, as a signal-to-noise ratio of 3.

3.4. Recovery

Blank urine samples were spiked with three different concentrations of each substance (Table 1). These spiked urine samples were processed using the procedures described above for the calibration standards.

3.5. Precision and accuracy

The intra-day and inter-day accuracies were estimated and the studied concentrations (QC samples) were lower than 10%, as indicated by the respective values of R.S.D. These show that the

Table 1
The recovery of four metabolites in three different concentrations ($n = 5$)

Compound	Spiked concentration (μg/mL)	Measured concentration (mean ± S.D.) (μg/mL)	Recovery (%)
1,3,7-Trihydroxyxanthone (M-1)	5.20	3.89 ± 0.22	74.83
	17.00	14.11 ± 0.83	83.02
	52.00	45.24 ± 1.72	87.00
1,3,6,7-Tetrahydroxyxanthone (M-2)	6.00	4.54 ± 0.36	75.66
	20.00	16.68 ± 0.46	83.42
	60.00	51.43 ± 1.44	85.72
1,3,6-Trihydroxy-7-methoxyxanthone (M-3)	4.00	3.21 ± 0.13	80.30
	24.00	20.02 ± 1.29	83.44
	48.00	41.49 ± 2.15	86.43
1,7-Dihydroxyxanthone (M-4)	4.20	3.29 ± 0.17	78.31
	16.80	14.03 ± 0.55	83.40
	50.40	43.32 ± 1.73	85.96

Table 2
Intra-day precision and accuracy of four metabolites in rat urine ($n = 5$)

Compound	Spiked concentration (μg/mL)	Measured concentration (mean ± S.D.) (μg/mL)	Accuracy (%)	R.S.D. (%)
1,3,7-Trihydroxyxanthone (M-1)	5.20	5.01 ± 0.01	96.43	2.49
	18.20	17.52 ± 0.14	96.30	7.85
	52.00	52.79 ± 0.16	101.5	3.00
1,3,6,7-Tetrahydroxyxanthone (M-2)	6.00	6.40 ± 0.03	106.7	5.34
	21.00	20.39 ± 0.11	97.13	5.59
	60.00	64.86 ± 0.31	108.1	4.76
1,3,6-Trihydroxy-7-methoxyxanthone (M-3)	3.60	3.93 ± 0.03	109.4	2.58
	24.00	24.53 ± 0.10	102.2	4.10
	48.00	51.67 ± 0.18	107.6	3.48
1,7-Dihydroxyxanthone (M-4)	4.20	4.00 ± 0.02	95.19	3.76
	16.80	17.08 ± 0.10	101.7	5.78
	50.40	51.27 ± 0.08	101.7	1.47

Table 3
Inter-day precision and accuracy of four metabolites in rat urine ($n = 5$)

Compound	Spiked concentration (μg/mL)	Measured concentration (mean ± S.D.) (μg/mL)	Accuracy (%)	R.S.D. (%)
1,3,7-Trihydroxyxanthone (M-1)	5.20	5.09 ± 0.04	97.86	8.14
	18.20	18.24 ± 0.32	100.2	4.49
	52.00	52.01 ± 0.05	100.01	1.03
1,3,6,7-Tetrahydroxyxanthone (M-2)	6.00	5.79 ± 0.01	96.45	2.23
	21.00	19.61 ± 0.03	93.39	1.31
	60.00	57.98 ± 0.07	96.63	1.26
1,3,6-Trihydroxy-7-methoxyxanthone (M-3)	3.60	3.86 ± 0.01	107.1	8.87
	24.00	22.89 ± 0.10	109.0	7.84
	48.00	47.29 ± 0.10	98.52	2.10
1,7-Dihydroxyxanthone (M-4)	4.20	4.33 ± 0.01	103.1	2.55
	16.80	16.40 ± 0.13	97.62	8.02
	50.40	51.00 ± 0.28	101.2	5.43

method is quite precise. Moreover, the small difference (≤10%) noted between added levels and the estimated concentrations have documented an appropriate accuracy of the elaborated method. The results are presented in Tables 2 and 3.

3.6. Stability

The coefficients of variation (CV%) of M-1, M-2, M-3 and M-4 in 30 h at room temperature were 5.4, 4.7, 4.1 and 5.9, respectively. The results showed good stability of these four metabolites.

3.7. Urine accumulation study

After oral administration of mangiferin to rats, characteristics of the cumulative urinary excretion of four metabolites of mangiferin were determined in male SD-rat. The cumulative urinary excretions of four metabolites were 11.59 ± 2.46 mg for M-1; 1.97 ± 0.26 mg for M-2; 2.80 ± 1.19 mg for M-3 and 2.63 ± 0.90 mg for M-4. The maximum excretions of four metabolites of mangiferin into urine were observed from 12 to

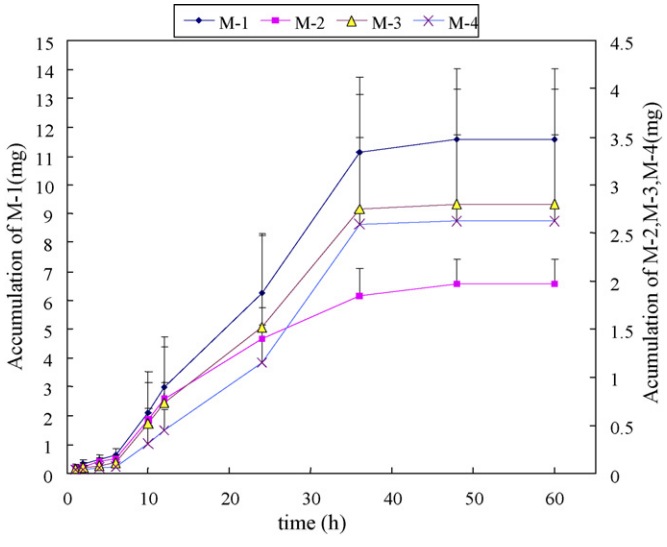

Fig. 3. The accumulation of four metabolites of mangiferin.

24 h for M-2, from 24 to 36 h for M-1, M-3 and M-4 (Fig. 3). Our previous study showed that the maximum excretion of mangiferin into urine was from 4 to 6 h [7]. These results suggest that mangiferin may possess a slow metabolic procession in the rat.

4. Discussion

In recent years, comparison of ESI-MS^n data and retention times of metabolites in HPLC with synthesized standards were usually to identify the structures of those metabolites [18–22]. Previous study has also reported some of metabolites from rat bile by LC–MS/MS method [12]. However, when the standards are difficult to synthesize, some metabolites' structure deduced only from LC/MS^n data may not be correct and believable. Therefore, structure elucidation of metabolites was an important task in drug metabolites study. In this study, four metabolites from rat urine have been isolated and identified. Our previous study has found that the extreme low concentration of mangiferin was detected in plasma and urine [7], which indicated that the majority of dosed mangiferin might have been metabolized. This could also be the signal that the pharmacological activities of mangiferin may not come from mangiferin itself but its metabolites, such as norathyriol.

M-1 was the major metabolite of mangiferin, whereas M-2 was the lowest metabolites of four metabolites. As one of the metabolites, norathyriol (M-2) has been reported as the unique metabolic transformation of mangiferin after subjected to intestinal bacterium, involving elimination of a *C*-glucosyl chain [8]. Therefore, as for the isolated four metabolites from rat urine, the possible metabolic way of mangiferin in rat could be deduced as following: mangiferin was firstly deglycosylated into unique metabolite M-2 by intestinal bacterium. Metabolite M-3 was the derivation of M-2 by way of the mechanism of methylation. Meanwhile, catalyzed by the dehydroxylase, M-2 was further transformed into M-1 and M-4, respectively. In the same way, M-1 may be transformed into M-4 by eliminating one hydroxyl group (Fig. 1).

However, other metabolites may also exist in rat urine, such as glucuronide conjugate, sulfate ester compound, cysteine *S*-conjugate. In order to substantiate these possible metabolites, our further study will focus on using the LC–MS^n method to study metabolites of mangiferin in rat urine. Meanwhile, we have also studied these isolated metabolites in rat plasma, the results showed that none of these four metabolites were detected by using the current analytic HPLC condition due to their low concentrations in plasma.

In conclusion, four metabolites of mangiferin were first isolated and identified from rat urine after orally administered. On the other hand, a simple, rapid and specific HPLC method for quantitive determination of the four metabolites of mangiferin was developed.

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