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Short communication

Simultaneous determination of homoeriodictyol-7-*O*-β-D-Glccopyranoside and its metabolite homoeriodictyol in rat tissues and urine by liquid chromatography–mass spectrometry

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Abstract

A liquid chromatographic–mass spectrometric (LC–MS) method was developed and validated for the simultaneous determination of homoeriodictyol-7-*O*-β-D-glycoside (HEDT-Glc) and its active metabolite homoeriodictyol (HEDT) in rat tissues and urine. The analytes and internal standard (dihydromyricetin, IS) were detected by using negative atmospheric pressure chemical ionization mass spectrometry in selected ion monitoring (SIM) mode at *m/z* 464, 301 and 319 for HEDT-Glc, HEDT and IS, respectively. These compounds were eluted on a Luna reverse phase column. The mobile phase was a methanol–water mixture (70:30, v/v) containing 0.1% of formic acid at a flow rate of 0.8 ml/min. The limit of quantification (LOQ) for both HEDT-Glc and HEDT was 10 ng/ml and their limit of detection (LOD) was 1 ng/ml. Calibration curves were linear ($r > 0.995$) over a wide range of the analytes in tissues and urine. The mean extraction recoveries were ≥75.6% for HEDT-Glc and ≥82.4% for HEDT from biological matrixes. Accuracy, expressed as the relative error, ranged from −4.0% to 3.8% for HEDT-Glc and from −2.8% to 4.7% for HEDT. The method was successfully applied to the estimation of HEDT-Glc and its metabolite HEDT in rat tissues and urine.

Keywords: Homoeriodictyol-7-*O*-β-D-glycoside; Homoeriodictyol; HPLC–MS

1. Introduction

Homoeriodictyol-7-*O*-β-D-Glccopyranoside (flavanone 4′,5,7-trihydroxy-3′-methoxy, -7-β-D-Glccopyranoside, HEDT-Glc, Fig. 1A), a platelet-activating factor (PAF) receptor antagonist [1], has a number of potential clinical applications such as treating acute myocardial infarction, ischemic heart disease [2], and anti-inflammatory conditions [3–5]. In addition, HEDT-Glc exhibits antioxidant activity which would be useful for the treatment of diseases mediated by reactive oxygen species [6]. Because its biological activities are beneficial to human health, the pharmacokinetics (PK) of HEDT-Glc needs to be studied in detail. Originally, we used high-performance liquid chromatography with ultraviolet detection (HPLC–UV) to study the PK of HEDT-Glc in rat plasma and tissues: after intravenous administration of HEDT-Glc to rats, the AUC and CL_{tot} were 16.04 ± 3.19 μg h/ml and 0.85 ± 0.17 l/kg/h, respectively, while the $t_{1/2,\alpha}$ and $t_{1/2,\beta}$ were 0.06 ± 0.01 and 1.27 ± 0.31 h, respectively; HEDT-Glc was eliminated quickly from the blood and mainly distributed to the liver and small intestine [7].

In recent studies, we found that HEDT-Glc was readily hydrolyzed to the free aglycone homoeriodictyol (4′,5,7-dihydroxy-3′-methoxyflavanone, HEDT, Fig. 1B) in vivo. HEDT is known to possess various pharmacological effects such as inhibiting platelet aggregation and increasing coronary flow rate [8]. It is also high cytotoxic to HeLa cells (IC_{50} 4.0 μg/ml) [9]. HEDT (5 μg) also inhibits the growth of the phytopathogenic fungus *Cladosporium cucumerinum* [10]. Furthermore, HEDT may even prevent certain cancers because of selective inhibition of CYP1B1 (IC_{50} 0.24 mmol), which activates carcinogens [11].

Although HEDT-Glc and HEDT exhibit many important pharmacological activities, there is only limited information in the literature regarding its determination. Dorothee et al. have investigated the stereoisomeric separation of HEDT by capillary electrophoresis and determination of interconversion barriers [12]. Miyake et al. have reported an HPLC-UV method for the

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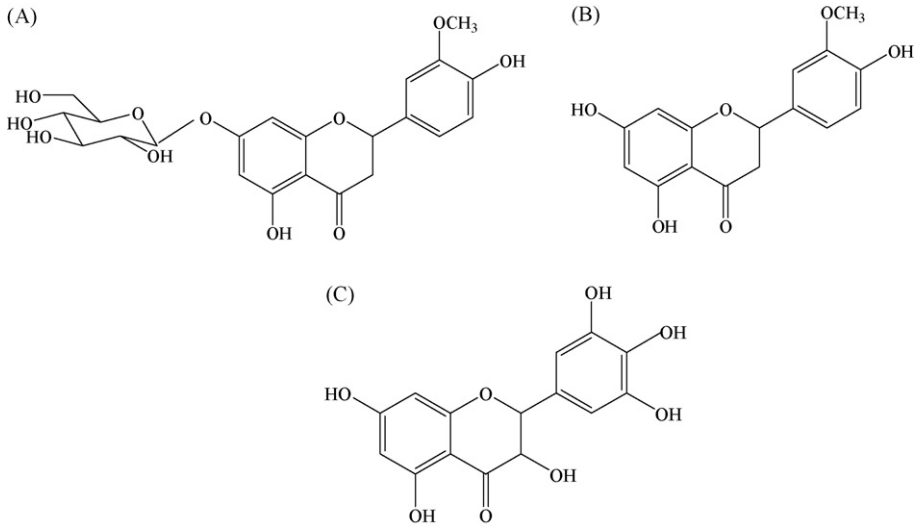

Fig. 1. Chemical structures of HEDT-Glc (A), HEDT (B) and dihydromyricetin (IS) (C).

determination of HEDT. After ingestion of flavanone glycosides and aglycones in lemon, HEDT is present in human plasma as the Glccuro- and sulfo-conjugates and it reaches a maximum concentration at 1 h [13]. In order to clarify the PK mechanisms of HEDT-Glc and HEDT, a specific, reproducible, and accurate method was developed for the simultaneous determination of HEDT-Glc and its metabolite HEDT following intravenous (i.v.) administration of HEDT-Glc. This could be applied to characterize the PK profile, and explore the relationship between the PK and pharmacodynamic (PD) effects of these compounds.

2. Experimental

2.1. Reagents and chemicals

HEDT-Glc, HEDT and dihydromyricetin (internal standard, IS) were isolated and purified in our laboratory. The substances were identified by ^{1}H, ^{13}C NMR and MS. The data were consistent with their standard specifications [14–16]. Methanol (HPLC grade) was purchased from Dima Technology Inc. (Richmond Hill, USA). All other reagents were of analytical grade and obtained from Tianjin Damao Chemical Reagent Factory (Tianjin, China). Distilled water, prepared from demineralized water, was used throughout the experiments.

2.2. Animals

Male and female pathogen-free Wistar rats (200–235 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University and the protocol was approved by the Animal Ethics Committee of this institution. The rats were fed with a standard laboratory diet and water for at least 3 days before the experiments.

2.3. Instrumentation

The HPLC system consisted of a DGU-14 AM degasser, two Shimadzu 10ADvp Pumps, a high pressure mixer and a Shimadzu 10ATvp Autoinjector (Shimadzu, Kyoto, Japan). A Shimadzu 2010 liquid chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an atmospheric pressure chemical ionization (APCI) interface, and a Q-array-Octapole-Quadrupole mass analyzer (QoQ system) was used in the study.

2.4. Standard solutions

An HEDT-Glc stock solution (1 mg/ml) was prepared in methanol. It was serially diluted with water to 0.1, 0.2, 0.5, 1.0, 5.0, 10 and 20 μg/ml solutions for tissues and 0.1, 0.2, 0.5, 1.0, 5.0, 20 and 100 μg/ml solutions for urine. Quality-control (QC) solutions of HEDT-Glc for tissues (0.2, 1.0, and 16 μg/ml) and urine (0.2, 1.0, and 80 μg/ml) were independently diluted. An HEDT stock solution (1 mg/ml) was also prepared in methanol. This solution was then serially diluted with water to give a series of working standards of 0.1, 0.2, 0.5, 1.0, 5.0, 20 and 100 μg/ml solutions for both tissues and urine. QC solutions of HEDT for both urine and tissues (0.2, 1.0, and 80 μg/ml) were independently diluted. The dihydromyricetin (IS) was also prepared as a stock solution of 1 mg/ml in methanol. It was diluted with water to give a working standard of 100.0 μg/ml solution.

2.5. Chromatographic conditions

Chromatographic separation of the analytes was performed on an ODS column (Luna, 150 mm × 4.6 mm i.d., particle size 5 μm, Phenomenex, USA), equipped with an ODS guard column (Security Guard, Phenomenex, USA). In order to determine HEDT-Glc and HEDT, the mobile phase was a methanol–water

mixture (70:30, v/v) containing 0.1% formic acid at a flow rate of 0.8 ml/min. The retention times for HEDT-Glc, HEDT and IS were 2.5, 3.8 and 2.5 min, respectively, with an overall runtime of 4.5 min.

2.6. Liquid chromatography and mass spectrometry conditions

Mass spectrometric conditions were optimized to obtain maximum sensitivity. The analytes were ionized in the negative APCI interface of a mass spectrometer. Mass spectra were obtained at a dwell time of 0.2 s in SIM mode and 1 s in scan mode. The curve dissolution line (CDL) voltage was fixed at 10.0 kV; interface temperature 400 °C; CDL temperature 200 °C; heat block temperature 200 °C; detector voltage 1.40 kV; nebulizing gas flow rate 2.5 l/min. The analytes were monitored at *m/z* 464, 301, and 319 for HEDT-Glc, HEDT and IS, respectively. Data collection, peak integration, and calculations were performed using LC–MS Solution Version 3.0.

2.7. Sample preparation

Two hundred microlitres of samples (tissues homogenates or urine) were transferred to a 10 ml polypropylene tube followed by the addition of 20 μl IS working standard solution, 500 μl ammonium dihydrogen phosphate buffer solution (pH 5.0), and 3 ml ethyl acetate. After vortexing for 5 min followed by a 5 min centrifugation at 3000 × *g* in a bench top centrifuge, the organic phase was transferred to another 10 ml polypropylene tube, and evaporated to dryness under nitrogen at 40 °C. Residues were reconstituted before analysis with 100 μl of methanol–water (70:30, v/v) and transferred to autosampler microvials for injection and analysis. Samples with drug concentrations above the calibration range were diluted with the corresponding blank matrix prior to sample extraction.

2.8. Method validation

The calibration curve was prepared by adding 20 μl IS and different concentrations of HEDT-Glc and HEDT to blank matrices. The calibration curve of HEDT-Glc/HEDT consisted of seven concentration points (0.01–2.0/0.01–10.0 μg/ml in rat tissues and 0.01–10.0/0.01–10.0 μg/ml in urine). Calibrations were performed by least-squares linear regression of the peak-area ratios of the analytes to the IS versus the nominal standard concentration with a weighted (1/square of concentration) factor [17]. Quantitation was based on the peak area ratios of the analytes against that of IS.

The QC samples at low, medium and high concentrations and the limit of quantitation (LOQ) samples were analyzed to determine the accuracy and precision of the proposed method. Intra-day and inter-day accuracy and precision were determined over a period of 3 consecutive days with 6 replications at each concentration per day ($n = 18$). The precision was evaluated as the relative standard deviation (R.S.D.), while the accuracy was expressed as the relative error (RE) [18,19]. The limit of detection (LOD) was considered to be the lowest concentration that can be discriminated from the baseline level with signal intensity at least three times greater than the background level.

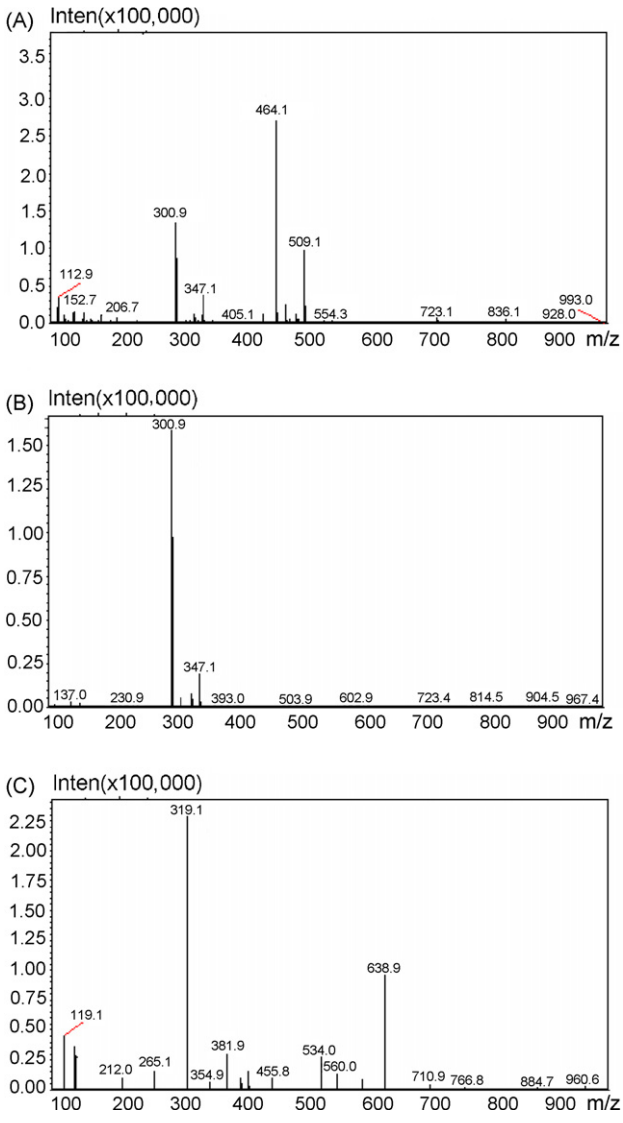

Fig. 2. The product ion mass spectra of HEDT-Glc (A), HEDT (B) and IS (C).

The extraction recoveries of HEDT-Glc and HEDT from tissues or urine were determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 18$) with the response of analytes from non-extracted standard solutions at equivalent concentrations.

2.9. Stability

The stability of HEDT-Glc and HEDT in rat tissues and urine was studied under a variety of storage conditions: performing three cycles of freezing (−20 °C)—thawing (room temperature), 24 h storage at room temperature and in a freezer at −20 °C for at least 1 month.

2.10. Method application

2.10.1. Tissue samples

The rats were fasted for 12 h, with free access to water. They were then decapitated at 0.083, 0.25 and 1.0 h after i.v. administration of HEDT-Glc and the brain, liver, kidney, spleen, pancreas, lung, heart, skeletal muscle, stomach, small intestine, fat, ovary, uterus and testis were collected. These tissue samples were gently blotted with absorbent paper to remove surface blood and weighed. Then the tissues were minced in saline and homogenized in a homogenizer and the homogenates were stored at −20 °C.

2.10.2. Urine samples

Following i.v. administration of 13.2 mg/kg HEDT-Glc, rat urine was collected in seven fractions over a period of 60 h. The total volume of each portion of urine was measured, and aliquots were taken and stored at −20 °C until analysis.

3. Results and discussion

3.1. Liquid chromatography and mass spectrometry

HEDT-Glc, HEDT and IS were analyzed by MS in APCI negative ion mode without any problems. ESI was also tested,

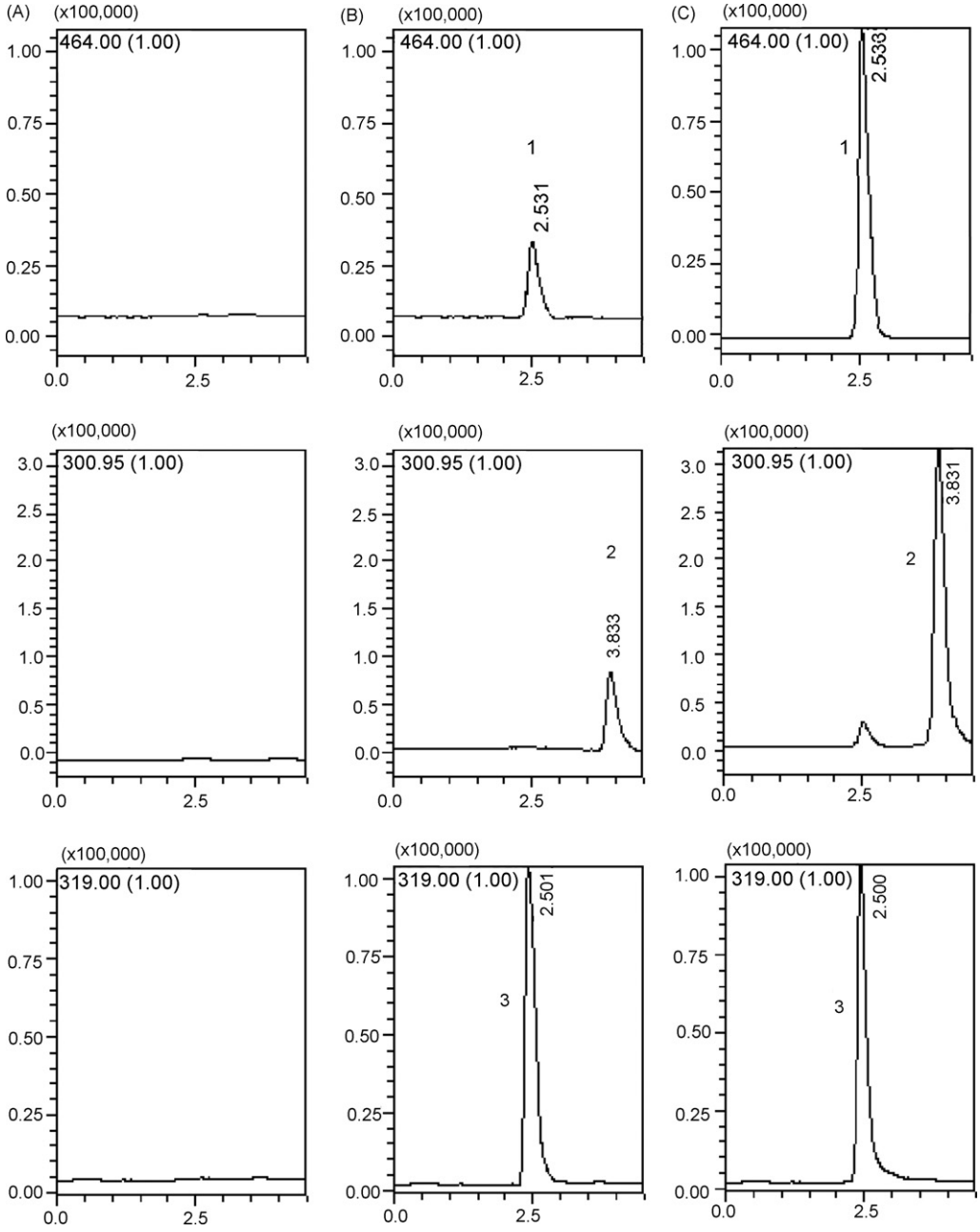

Fig. 3. Chromatograms of blank liver (A), liver spiked with HEDT-Glc (10 ng/ml), HEDT (10 ng/ml) and IS (10 μg/ml) (B) and an extracted liver sample from a rat (0.083 h after i.v. administration of 13.2 mg/kg HEDT-Glc) containing HEDT-Glc (0.65 μg/ml), HEDT (1.98 μg/ml) along with IS (10 μg/ml) (C). 1: HEDT-Glc; 2: HEDT; 3: IS.

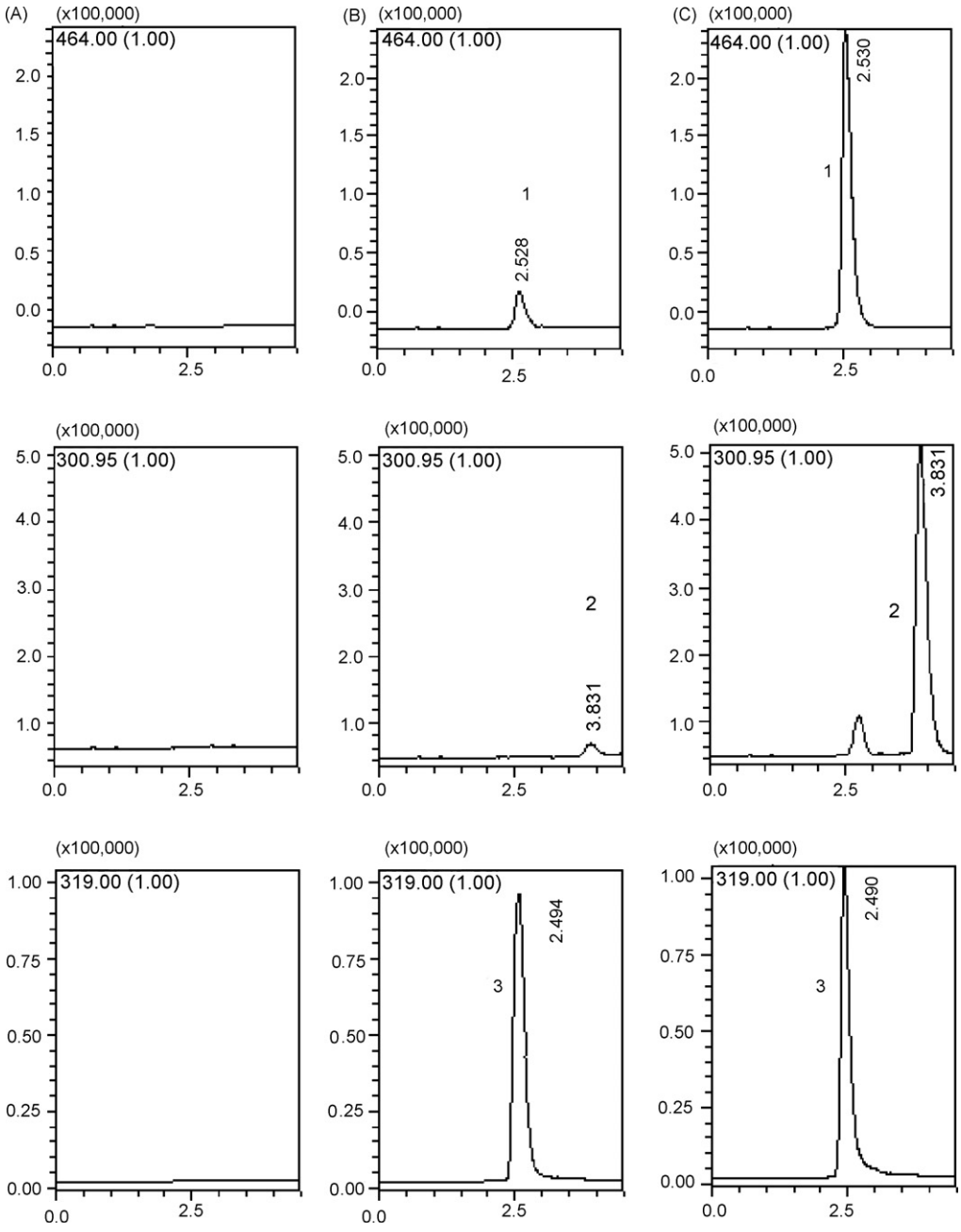

Fig. 4. Chromatograms of blank urine (A), urine spiked with HEDT-Glc (10 ng/ml), HEDT (10 ng/ml) and IS (10 μg/ml) (B) and an extracted urine sample from a rat (24 h after i.v. administration of 13.2 mg/kg HEDT-Glc) containing HEDT-Glc (4.47 μg/ml), HEDT (3.24 μg/ml) along with IS (10 μg/ml) (C). 1: HEDT-Glc; 2: HEDT; 3: IS.

and the sensitivity and specificity were substantially the same as APCI. However, ESI was not used since it had shown a non-linear detector response during preliminary tests over the same concentration range as used with APCI. Full scan negative ion mass spectra of HEDT-Glc, HEDT and IS yielded predominately fragments (Fig. 2) at *m/z* 464, 301 and 319, respectively. Typical chromatograms of spiked rat tissues and urine samples at the LOQ showed well-shaped and separated peaks (Figs. 3 and 4). The analytes were identified by comparing their retention time and mass spectra with standard solutions containing the corresponding compound. Selectivity was evaluated by comparing the extracted chromatograms of blank samples with those of the same matrices spiked with HEDT-Glc, HEDT at the LOQ and IS at the amount used in the procedure. No peaks interfering with the HEDT-Glc, HEDT and IS determination were observed (Figs. 3 and 4).

Formic acid was tested as a modifier in order to increase sensitivity. Addition of 0.1% formic acid enhanced the sensitivity by at least twofold compared with no additives. In addition, it was found that formic acid produced symmetric peaks.

The sample clean-up was based on a liquid/liquid extraction (LLE), optimized by testing the influence of different kinds of organic solvents (ethyl acetate, dichloromethane, and diethyl ether), solvent volumes, pH and times of extraction on the

Table 1
Calibrations for analysis of the HEDT-Glc and HEDT in rat tissues and urine ($n = 6$)

	HEDT-Glc			HEDT		
	Slope	Intercept	r	Slope	Intercept	r
Liver	0.1045	0.0805	0.9984	0.8427	0.0142	0.9993
Kidney	0.0679	0.0696	0.9991	0.7723	0.0321	0.9978
Spleen	0.0919	0.0846	0.9943	1.0046	−0.0021	0.9966
Small intestine	0.0815	0.0713	0.9967	0.9412	−0.0049	0.9949
Urine	0.1121	0.0781	0.9997	0.9478	0.0117	0.9980

recovery. Purification by solid phase extraction (SPE) with C_{18} and polymeric sorbents was also tested, but the recovery was not satisfactory. In brief, the best result was obtained with the procedure described in Section 2.7.

3.2. Assay validation

The current assay allows the quantitation of HEDT-Glc and HEDT over a wide concentration range. Standard curves of HEDT-Glc were linear with $r \geq 0.994$ at concentrations ranging from 0.01 to 2.00 μg/ml in rat tissues and from 0.01 to 10.0 μg/ml in rat urine. Standard curves of HEDT were linear with $r \geq 0.995$ at concentrations ranging from 0.01 to 10.0 ng/ml in both rat tissues and urine (Table 1). Precision and accuracy data confirmed the good reproducibility of the described method (Table 2). The LOQ for both HEDT-Glc and HEDT was 10 ng/ml and their LOD was 1 ng/ml. Good recoveries from tissues and urine, ranging from 75.6% to 88.1% for HEDT-Glc and from 82.4% to 92.8% for the HEDT, were obtained with a single and relatively fast LLE (Table 2). The mean extraction recovery of IS was 86.7%.

3.3. Stability

The QC samples prepared using rat tissues and urine showed no significant degradation after three freeze-thaw cycles. In extracts, these compounds were stable for up to 24 h at ambient temperature. Also, there were no significant difference in tissues and urine after storage at −20 °C for up to 1 month. Stock solutions of the compounds in methanol were stable for up to 45 days at −4 °C.

3.4. Method application

The method was applied to the determination of HEDT-Glc and its metabolite HEDT in rat tissues and urine after i.v. administration of 13.2 mg/kg HEDT-Glc. The HEDT-Glc and HEDT concentration in some tissues are summarized in Table 3 and the major pharmacokinetic parameters are listed in Table 4. HEDT-Glc was distributed widely, but the concentrations were relatively low except in the liver and small intestine. At 0.083 h, the concentrations of HEDT-Glc were 0.65 ± 0.24 μg/g in the liver and 0.51 ± 0.07 μg/g in the small intestine. Similar to HEDT-Glc, HEDT was detected in many tissues. At 0.083 h, the kidney samples contained large amounts of HEDT (10.93 ± 2.92 μg/g); whereas the tissues extracts from liver, lung and stomach contained only moderate amounts of HEDT (1.98 ± 0.80–1.37 ± 0.25 μg/g). Only traces of HEDT were detected in the heart and skeletal muscle.

Table 2
Precision and accuracy for analysis of the HEDT-Glc and HEDT in rat tissues and urine ($n = 6$)

HEDT-Glc					HEDT				
Concentration spiked (μg/ml)	Inter-day R.S.D. (%)	Intra-day R.S.D. (%)	Accuracy (%)	Recovery% (mean ± S.D.)	Concentration spiked (μg/ml)	Inter-day R.S.D. (%)	Intra-day R.S.D. (%)	Accuracy RE (%)	Recovery% (mean ± S.D.)
Liver									
0.02	11.6	10.9	−1.4	79.6%	0.02	5.9	9.2	−0.6	88.7%
0.10	3.9	11.1	3.8	81.2%	0.10	12.6	10.9	1.3	85.6%
1.60	13.2	9.1	−2.7	75.6%	8.00	11.0	8.9	−1.0	83.9%
Kidney									
0.02	10.4	13.2	−1.8	82.8%	0.02	6.9	11.8	−2.8	85.6%
0.10	13.1	8.4	2.9	77.4%	0.10	4.2	12.2	0.6	84.9%
1.60	11.9	5.8	−4.0	79.3%	8.00	4.5	7.8	−0.8	83.6%
Spleen									
0.02	12.7	10.7	−1.6	80.5%	0.02	11.7	13.2	−1.0	86.9%
0.10	7.3	12.1	2.7	76.9%	0.10	8.8	11.5	1.3	85.6%
1.60	3.9	8.1	1.1	78.5%	8.00	6.9	9.6	−1.8	87.1%
Small intestine									
0.02	7.1	11.4	2.9	82.5%	0.02	13.6	14.3	−3.4	82.9%
0.10	4.5	6.8	0.1	81.6%	0.10	12.3	8.0	4.7	85.6%
1.60	3.6	9.6	−2.1	78.8%	8.00	5.5	4.1	0.3	82.4%
Urine									
0.02	3.6	9.2	−2.6	86.7%	0.02	14.3	10.4	−1.2	91.3%
0.10	14.9	3.4	1.5	84.3%	0.10	12.4	12.1	−0.4	92.8%
8.00	7.8	2.3	−2.2	88.1%	8.00	6.3	8.6	−0.2	89.5%

Table 3
Tissues distribution of HEDT-Glc and HEDT after i.v. administration 13.2 mg/kg HEDT-Glc in rat ($n = 6$)

Tissues	HEDT-Glc (mean ± S.D.) (μg/g)			HEDT (mean ± S.D.) (μg/g)		
	0.083 h	0.25 h	1.0 h	0.083 h	0.25 h	1.0 h
Heart	ND	ND	ND	0.17 ± 0.019	0.15 ± 0.045	0.04 ± 0.066
Liver	0.65 ± 0.24	0.46 ± 0.06	0.32 ± 0.02	1.98 ± 0.80	0.92 ± 0.32	0.06 ± 0.02
Spleen	0.27 ± 0.03	0.28 ± 0.05	0.26 ± 0.05	0.54 ± 0.10	0.50 ± 0.18	0.21 ± 0.16
Lung	0.26 ± 0.05	0.36 ± 0.10	0.08 ± 0.02	1.61 ± 0.46	0.95 ± 0.28	0.37 ± 0.19
Kidney	0.42 ± 0.15	0.63 ± 0.27	0.11 ±0.08	10.93 ± 2.92	4.26 ± 1.44	1.03 ± 0.87
Pancreas	0.26 ± 0.18	0.22 ± 0.11	0.56 ± 0.15	0.23 ± 0.14	0.09 ± 0.04	0.03 ± 0.02
Stomach	0.09 ± 0.04	0.08 ±0.03	0.1 ± 0.02	1.37 ± 0.25	0.78 ± 0.26	0.42 ± 0.20
Small intestine	0.51 ± 0.07	0.63 ± 0.12	0.47 ± 0.05	0.22 ± 0.14	0.12 ± 0.04	0.15 ± 0.17
Skeletal muscle	0.15 ± 0.08	ND	ND	0.03 ± 0.01	0.08 ± 0.04	0.03 ± 0.02
Brain	ND	ND	ND	ND	ND	ND
Fat	0.114 ± 0.10	0.079 ± 0.02	0.120 ± 0.04	0.49 ± 0.17	0.32 ± 0.23	0.07 ±0.22
Ovary	ND	0.03 ± 0.02	ND	0.06 ± 0.01	0.03 ± 0.02	0.02 ± 0.02
Uterus	0.24 ± 0.07	0.32 ± 0.19	0.27 ± 0.07	0.22 ± 0.09	0.27 ± 0.07	0.04 ± 0.03
Testis	ND	ND	ND	ND	ND	ND

"ND" means "not detected".

Table 4
Pharmacokinetics of HEDT-Glc and HEDT in rats following i.v. administration at a dose of 13.2 mg/kg

	HEDT-Glc		HEDT	
	AUC_{0-t} (μg h/ml)	MRT_{0-t} (h)	AUC_{0-t} (μg h/ml)	MRT_{0-t} (h)
Heart	ND	ND	0.098	0.341
Liver	0.385	0.460	0.610	0.232
Spleen	0.248	0.529	0.353	0.396
Lung	0.217	0.337	0.709	0.365
Kidney	0.365	0.319	3.252	0.292
Pancreas	0.333	0.713	0.072	0.323
Stomach	0.082	0.579	0.630	0.407
Small intestine	0.508	0.496	0.130	0.552
Skeletal muscle	ND	ND	0.050	0.409
Brain	ND	ND	ND	ND
Fat	0.091	0.604	0.214	0.310
Ovary	ND	ND	0.026	0.431
Uterus	0.272	0.517	0.157	0.302
Testis	ND	ND	ND	ND

The excretion of HEDT-Glc into urine following by i.v. administration of 13.2 mg/kg to rats is presented in Fig. 5. The average cumulative excretion of HEDT-Glc in rat urine was 0.78%, 7.68%, 9.76%, 10.09%, 10.94%, 10.99% and 10.99% of the administered dose within 2.5, 6, 12, 24, 36, 48 and 60 h, respectively. The average cumulative excretion of HEDT in rat urine was 0%, 4.43% 5.40%, 5.48%, 5.59%, 5.66% and 5.69% of administered dose within 2.5, 6, 12, 24, 36, 48 and 60 h, respectively. Therefore, the total excretion of HEDT-Glc and HEDT over 60 h was 10.99% and 5.69%, respectively. Compared with the administered dose, the lower excretion levels of HEDT-Glc could be partially explained as a result of other potential metabolic pathways. Further studies of the metabolism of HEDT-Glc are currently ongoing in our laboratory.

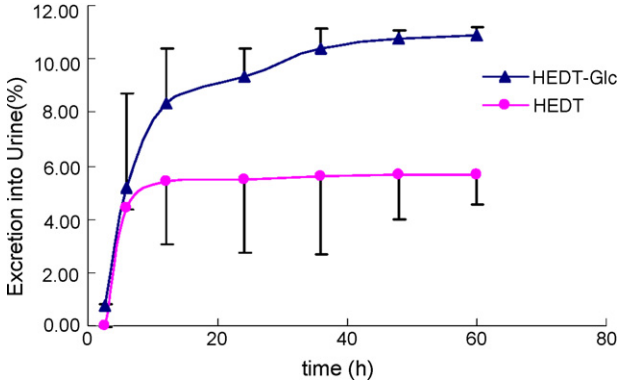

Fig. 5. Accumulation of excretion of HEDT-Glc and HEDT into urine.

4. Conclusion

For the first time, an LC–MS method for simultaneous determination of HEDT-Glc and its metabolite HEDT in rat tissues and urine has been developed. The method has acceptable sensitivity, precision, accuracy, selectivity, recovery and stability. It has been successfully applied to the PK study of HEDT-Glc and HEDT after i.v. administration of HEDT-Glc to rats, and it will also be of potential use for human PK studies of HEDT-Glc.

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