HPLC Determination and Pharmacokinetic Study of Homoeriodictyol-7-*O*-β-D-glucopyranoside in Rat Plasma and Tissues

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Homoeriodictyol-7-*O*-β-D-glucopyranoside (HEDT-Glu) was isolated from *Viscum coloratum* and identified by MS, ^{1}H- and ^{13}C-NMR. A HPLC method was developed for determination of HEDT-Glu in rat plasma and tissues. All biological samples were pretreated by protein precipitation with acetone. Vanillin was selected as internal standard. The mobile phase consisted of methanol–water–glacial acetic acid (45 : 55 : 0.5, v/v/v). Good linearity were observed over the concentration ranges of 0.1—200.0 μg·ml^{-1} in rat plasma and 0.05—5.0 μg·ml^{-1} in tissues. Both intra- and inter-day precisions of HEDT-Glu, expressed as the relative standard deviation, were less than 13.1%. Accuracy, expressed as the relative error, ranged from −0.8 to 5.4% in plasma and from −5.6 to 9.4% in tissues. The mean extraction recovery of HEDT-Glu was above 73.17% in biological samples. The described assay method was successfully applied to the pre-clinical pharmacokinetic study of HEDT-Glu. After intravenous administration of HEDT-Glu to rat, *AUC* and CL_{tot} were 16.04±3.19 μg·h·ml^{-1} and 0.85±0.17 l·kg^{-1}·h^{-1}, respectively. $T_{1/2,\alpha}$ and $t_{1/2,\beta}$ were 0.06±0.01 h and 1.27±0.31 h, respectively. HEDT-Glu was cleared from the blood and mainly distributed to the liver and small intestine.

Key words homoeriodictyol-7-*O*-β-D-glucopyranoside; *Viscum coloratum*; rat plasma; tissue; HPLC determination; pharmacokinetics

Homoeriodictyol-7-*O*-β-D-glucopyranoside (flavone 4′,5,7-trihydroxy-3′-methoxy, -7-β-D-glucopyranoside, HEDT-Glu, Fig. 1) was firstly synthesized by Hoerhammer *et al*.[1] Afterward it was isolated from the traditional Chinese medicinal herb *Viscum coloratum*[2] and *Viscum album*.[3] HEDT-Glu can inhibit platelet aggregation induced by platelet-activating factor[4] (PAF, a biologically potent phospholid mediator of diverse actions, is highly expressed in the pathologic states such as endotoxemia, septic shock, inflammatory joint disease, collagen-vascular syndromes, allergic disease, asthma and hypotension, *etc*.[5]). Phytochemical investigations indicated that *Viscum coloratum* demonstrated a remarkably inhibitory effect on PAF. HEDT-Glu was the responsible part of anti-PAF action of *Viscum coloratum*.[6] It is the potent PAF receptor antagonist which had the similar mechanism to Ginkgoides and kadsurenone.[7] In addition, Yao H. *et al*. reported HEDT-Glu had shown antioxidative activities. It would be useful for the treatment of various diseases mediated reactive oxygen species.[8] Substantial investigations indicated that HEDT-Glu had many clinical applications such as treating acute myocardial infarction, ischemic heart disease,[9] and protecting inflammatory conditions.[10—12]

Up to now, although many pharmacodynamic studies of HEDT-Glu have been reported,[13,14] no analytical procedure has been published for the quantification of HEDT-Glu in biological specimen. We firstly developed a specific, reproducible and accurate HPLC method for HEDT-Glu determination in rat plasma and tissues. It can be applied to extensive human pharmacokinetic studies.

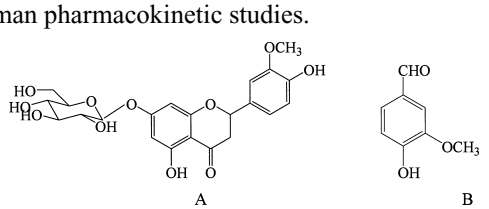

Fig. 1. Chemical Structures of HEDT-Glu (A) and Vanillin (Internal Standard, B)

MATERIALS AND METHODS

Chemicals *Viscum coloratum* was obtained from Tianyitang traditional Chinese medicine store (Shenyang, China). HEDT-Glu was isolated and purified in our laboratory. Methanol (HPLC grade) was purchased from Concord Tech Reagent Company (Tianjin, China). All other reagents were of analytical grade from Shenyang Damao Chemical Reagent Factory (Shenyang, China). Distilled water, prepared from demineralized water, was used throughout the study. Vanillin (Internal standard, IS) was obtained from E. Merck (Darmstadt, Germany). Polyamide was purchased from Taizhou sijia Biochemistry and Plastic factory (Taizhou, China).

Animals Male and female pathogen-free Wistar rats (200—240 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 standard laboratory food and water for at least 3 days before the experiments.

Instruments and Chromatographic Conditions The chromatographic system (Shimadzu, Kyoto, Japan) consisted of a pump (LC-10AT*vp*), an UV detector (LC-10A*vp*), a column oven (TC-100) and a LC-Workstation (Anastar). Chromatography was performed on a Diamonsil C_{18} column (200 mm×4.6 mm i.d., particle 5 μm, Dikma, Beijing, China). A mobile phase consisted of methanol–water–glacial acetic acid (45 : 55 : 0.5, v/v/v). The flow-rate was 1.0 ml·min^{-1}. Ultraviolet detection wavelength was set at 284 nm and the column temperature was kept at 30 °C. Identification of HEDT-Glu was carried out by MS (Finnigan TSQ), ^{1}H- and ^{13}C-NMR spectra (Bruker AVANCE-300).

Isolation and Purification of HEDT-Glu Branches and leaves of *Viscum coloratum* were splintered. After removal of lipophilic extractives using solvent extraction with ligarine,

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the hydrophilic extractives were extracted with *n*-butanol.[15] HEDT-Glu (96—98%) can then be obtained by chromatography on polyamide column and by crystallization from the methanol. The substance was submitted to instrumental analysis using ^{1}H-, ^{13}C-NMR and MS.

Preparation of Standard Solutions Stock solution of HEDT-Glu (1.0 mg·ml^{-1}) and IS (500.0 μg·ml^{-1}) were prepared in methanol. The solution of HEDT-Glu was then serially diluted with methanol to achieve standard working solutions at the concentrations of 0.1, 0.5, 2.0, 10.0, 50.0, 100.0 and 200.0 μg·ml^{-1} for plasma and at the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.5 and 5.0 μg·ml^{-1} for tissues. Quality-control (QC) solution for plasma (0.5, 10.0, 160.0 μg·ml^{-1}) and for tissue (0.1, 0.5, 2.0 μg·ml^{-1}) were independently diluted. IS working solution for plasma (25.0 μg·ml^{-1}) and for tissues (10.0 μg·ml^{-1}) were prepared by diluting the 500.0 μg·ml^{-1} stock standard solution in methanol, respectively.

Sample Preparation Plasma samples of 100 μl (tissues samples of 200 μl) from rats were transferred to tubes which were added 500 μl (1000 μl) acetone and 50 μl internal standard. This mixture was vortex-mixed for 1 min. After centrifuging for 3 min at 12000 rpm, the supernatants were evaporated to dryness under a stream of nitrogen at 40 °C. The residues were reconstituted in 100 μl mobile phase, vortex-mixed for 20 s, and then centrifuged for 3 min at 12000 rpm. The supernatants of 10 μl were injected into the HPLC system for analysis.

Method Validation Calibration, Precision, and Accuracy: Samples spiked with HEDT-Glu at seven concentrations were prepared in duplicate and analyzed in three separate analytical runs. Calibration curves were constructed using weighted ($w=1/x^2$) least-squares linear regression analysis of the observed peak area ratios of HEDT-Glu and IS *versus* the respective standard concentration.[16] The unknown samples concentrations were calculated from the linear regression equation of the peak area ratio against concentrations of the calibration curve. The QC samples at low, medium and high levels and the limit of quantification (LOQ) sample were analyzed to access the accuracy and precision of the proposed method. In order to determine intra-day accuracy and precision, 6 duplicates were sampled on the same day. Inter-day accuracy and precision were determined over a period of 3 days with 6 duplicates per day ($n=18$). The precision was evaluated as the relative standard deviation (RSD), while the accuracy was expressed as the relative error (RE).[17,18]

Extraction Recovery and Analyte Stability: The extraction recoveries were determined at three concentration levels by comparing the analyte peak areas, obtained from the QC ($n=6$) after extraction, with those obtained from the corresponding unextracted reference standards prepared at the same concentrations. The stability of HEDT-Glu in rat plasma and tissues were investigated under a variety of storage conditions: performing three cycles of freeze (−20 °C)—thaw (room temperature), 24 h storage at room temperature and under −20 °C freezer at least one month.

Application of the Method Plasma Samples: Each rat ($n=6$) was fasted for 12 h, with free access to water, during the experiments and administered injection of 13.2 mg·kg^{-1} HEDT-Glu *via* the tail vein. The blood samples were collected at 0, 0.016, 0.083, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, and 5.0 h after intravenous (i.v.) administration and centrifuged to obtain plasma.

Tissue Samples: The rats were decapitated 0.083, 0.25 and 1.0 h after i.v. administration of HEDT-Glu. The brain, liver, kidney, spleen, pancreas, lung, heart, skeletal muscle, stomach, small intestine, and fat were immediately removed, blotted onto filter papers and weighted. The tissues were minced in saline and homogenized with homogenizer. The homogenate were stored at −20 °C.

The plasma (tissues) concentrations of HEDT-Glu at different time were expressed as mean±S.D. All data of plasma concentrations were subsequently processed by the computer program 3p97 (Practical Pharmacokinetic Program, China) to determine the compartment models and pharmacokinetic parameters.

RESULTS AND DISCUSSION

Structural Identification of HEDT-Glu HEDT-Glu was a whitish crystal and its mp was 158—160 °C. HCl–Mg, Molish and $NaBH_4$ reactions were all positive, indicating a glycoside of flavanone. MS (*m/z*): 177, 274, 302 (aglycone), 464 (M^+). The date given by ^{1}H-NMR (DMSO) δ ppm were 2.76 (1H, dd, $J=4$, 18 Hz, C_3 *cis*-H); 3.22—3.47 (m, glycoside of H, OH and C_3 *trans*-H); 5.04 (1H, m, indicating glycoside H-1), 3.79 (3H, s, C_3'-OCH_3); 5.49 (1H, dd, $J=4$, 12 Hz, C_2-H); 6.17 (2H, s, $C_{6,8}$-H); 6.90 (1H, d, $J=8.5$ Hz, C_5'-H); 6.92 (1H, dd, $J=8.5$, 2 Hz, C_6'-H); 7.11 (1H, d, $J=2$ Hz, C_2'-H); 9.18 (1H, s, C_4'-OH); 12.07 (1H, s, C_5-OH). ^{13}C-NMR (DMSO) δ ppm: 156.5 (C-2), 138.5 (C-3), 178.2 (C-4), 161.0 (C-5), 98.0 (C-6), 165.3 (C-7), 92.7 (C-8), 155.4 (C-9), 105.4 (C-10), 121.9 (C-1′), 112.2 (C-2), 149.1 (C-3′), 148.7 (C-4′), 115.0 (C-5′), 123.3 (C-6′), 59.9 (OCH_3), 56.3 (OCH_3), 56.0 (OCH_3), 99.6 (C-1″), 73.2 (C-2″), 77.2 (C-3″), 69.7 (C-4″), 77.0(C-5″), 60.7(C-6″). Based on these data, the compound was identified as HEDT-Glu with formula of $C_{22}H_{24}O_{11}$ and molecular weight of 464, which were consistent with the standard data.[19]

Validation of Chromatographic Methods Assay Specificity and LOQ: The specificity of method was demonstrated by comparing chromatograms obtained from plasma (tissues) samples of rats, each as a blank and a spiked sample. Figures 2 and 3 show that the peaks of compound in plasma and tissues were not interfered by endogenous substances, respectively. The retention time of internal standard and HEDT-Glu were approximately 6.80 and 9.90 min, respectively. The LOQ was 1 ng in plasma samples and was 0.5 ng in tissues samples.

Calibration, Precision, and Accuracy: Good linearity were observed over the concentration range of 0.1—200.0 μg·ml^{-1} in rat plasma and 0.05—5.0 μg·ml^{-1} in tissues. For the samples prepared in plasma, kidney, spleen, liver, and small intestine, the mean regression equation were $y=0.040x+0.0035$ ($r=0.9965$); $y=0.1125x-0.0148$ ($r=0.9981$); $y=0.1103x-0.0027$ ($r=0.9945$); $y=0.108x+0.0017$ ($r=0.9974$); $y=0.1102x-0.0258$ ($r=0.9932$), respectively, where y is the peak area ratio and x is the concentration. In this assay, the intra- and inter-day precision in plasma ranged from 3.4 to 7.5% and from 1.9 to 9.7%, respectively. The results of tissues never exceeded 13.1%. The accuracy

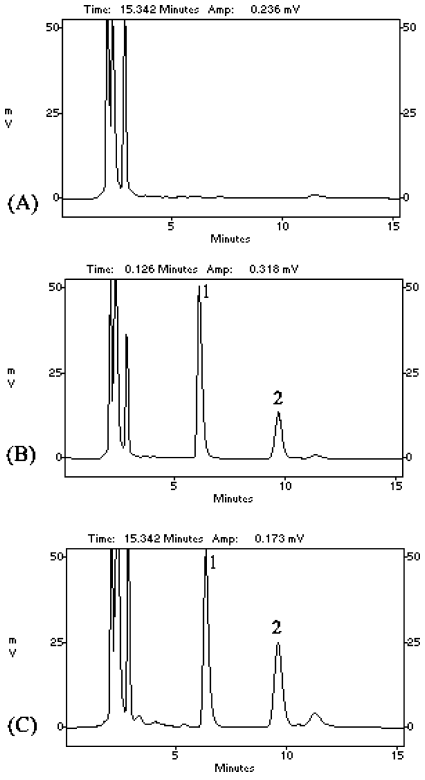

Fig. 2. Representative Chromatograms of HEDT-Glu

(A) A blank rat plasma sample; (B) a blank rat plasma sample spiked with HEDT-Glu (concentration at 10.0 $\mu g \cdot ml^{-1}$) and (C) a rat plasma sample 0.25 h after intravenous administration of 13.2 $mg \cdot kg^{-1}$ HEDT-Glu. Peak 1 and 2 refer to IS (12.5 $\mu g \cdot ml^{-1}$) and HEDT-Glu, respectively.

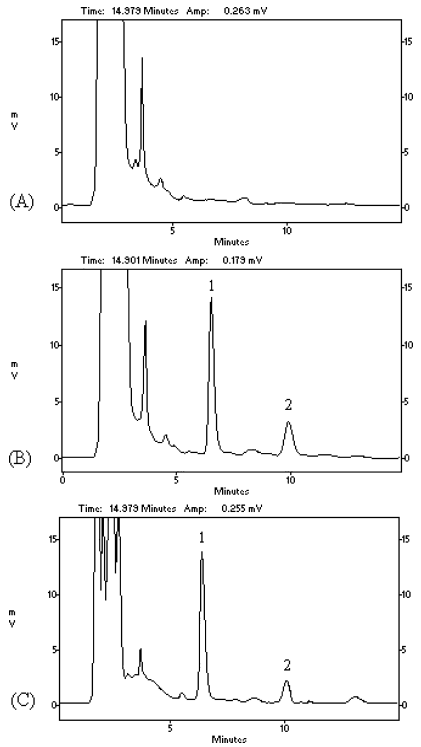

Fig. 3. Representative Chromatograms of HEDT-Glu

(A) A blank rat liver sample; (B) a blank rat liver sample spiked with HEDT-Glu (concentration at 2.0 $\mu g \cdot ml^{-1}$) and (C) a rat liver sample 0.083 h after intravenous administration of 13.2 $mg \cdot kg^{-1}$ HEDT-Glu. Peak 1 and 2 refer to IS (5.0 $\mu g \cdot ml^{-1}$) and HEDT-Glu, respectively.

Table 1. Precisions and Accuracies for Analysis of HEDT-Glu in Rat Plasma and Tissues ($n=6$)

	Concentration spiked ($\mu g \cdot ml^{-1}$)	Intra-day concentration		Inter-day concentration		Accuracy RE (%)	Recovery (%) (mean±S.D.)
		Found (mean±S.D.)	RSD (%)	Found (mean±S.D.)	RSD (%)		
Plasma	0.50	0.509±0.026	7.5	0.496±0.009	1.9	−0.8	82.93±6.81
	10.00	10.48±0.342	4.2	10.204±0.99	9.7	2.0	85.39±5.23
	160.00	168.31±1.717	3.4	168.67±11.91	7.1	5.4	87.06±5.39
Liver	0.10	0.103±0.016	10.9	0.109±0.025	13.1	9.4	73.17±3.96
	0.50	0.479±0.036	7.5	0.485±0.015	3.2	−3.1	81.46±5.20
	2.00	2.223±0.208	10.1	2.061±0.119	5.8	3.1	77.68±2.99
Kidney	0.10	0.094±0.007	5.7	0.095±0.002	2.2	−5.6	79.11±1.02
	0.50	0.524±0.029	7.6	0.095±0.037	7.0	4.8	80.06±6.44
	2.00	2.182±0.143	8.7	0.095±0.257	12.5	3.1	75.39±4.92
Spleen	0.10	0.103±0.008	8.6	0.102±0.003	3.2	2.0	76.11±2.87
	0.50	0.512±0.040	7.9	0.507±0.014	2.8	1.5	73.96±6.89
	2.00	2.042±0.154	7.9	1.959±0.185	9.5	−2.1	81.93±2.16
Small intestine	0.10	0.097±0.007	6.6	0.099±0.005	5.6	−0.2	75.93±4.44
	0.50	0.534±0.029	5.7	0.516±0.041	7.9	3.3	79.87±3.68
	2.00	2.109±0.143	7.0	2.052±0.138	6.8	2.6	78.93±7.03

ranged from −0.81 to 5.42% in plasma and from −5.6 to 9.4% in tissues. Related data are given in Table 1. These data suggested that the method was accurate and reproducible for the determination of HEDT-Glu in rat plasma and tissues.

Extraction Recovery and Analyte Stability: The mean extraction recoveries of HEDT-Glu was above 73.17% in biological samples. The extraction recovery of IS recovery was found 82.0±5.8%. Related data are given in Table 1.

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

Applications of the Analytical Method in Pharmacokinetic Studies After i.v. injection of 13.2 $mg \cdot kg^{-1}$ HEDT-Glu to rats, plasma concentrations of HEDT-Glu were determined by the described HPLC. Figure 4 shows the mean plasma concentration–time curve of HEDT-Glu ($n=6$).

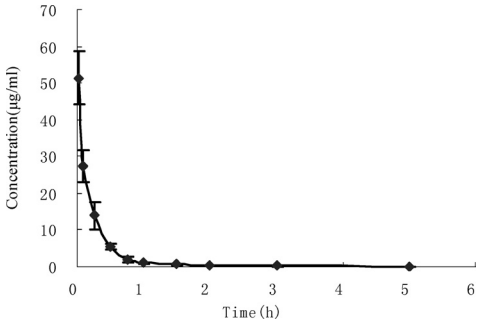

Fig. 4. Mean Plasma Concentration–Time Profile of HEDT-Glu after i.v. Administration of 13.2 mg·kg^{-1} HEDT-Glu to Wistar Rats

Table 2. Pharmacokinetic Parameters of HEDT-Glu Following after i.v. Administration 13.2 mg·kg^{-1} HEDT-Glu in Rats (n=6)

Parameters	Mean±S.D.
A (μg·ml^{-1})	58.24±18.04
B (μg·ml^{-1})	0.82±0.37
α (h^{-1})	12.94±2.70
β (h^{-1})	0.57±0.12
K_{12} (h^{-1})	2.99±1.81
K_{21} (h^{-1})	0.74±0.09
K_{10} (h^{-1})	9.78±1.61
$t_{1/2,\alpha}$ (h)	0.06±0.01
$t_{1/2,\alpha}$ (h)	1.27±0.31
AUC (μg·h·ml^{-1})	16.04±3.19
$AUMC$ (μg·h^2·ml^{-1})	2.10±0.71
MRT (h)	0.13±0.03
V_{dss} (l·kg^{-1})	0.11±0.03
CL_{tot} (l·kg^{-1}·h^{-1})	0.85±0.17

Plasma concentrations of HEDT-Glu in rat were detectable for at least 5 h after i.v. administration. The main pharmacokinetic parameters of HEDT-Glu, calculated by non-compartmental analysis, are summarized in Table 2. The area under the plasma concentration–time curve (AUC) was 16.04±3.19 μg·h·ml^{-1}. The elimination half-life at the α phases ($t_{1/2,\alpha}$) and β phase ($t_{1/2,\beta}$) were 0.06±0.01 h and 1.27±0.31 h, respectively. The elimination rate constant (Ke) was 9.78±1.61 h^{-1} and total clearance (CL_{tot}) was 0.85±0.171·kg^{-1}·h^{-1}. The distribution of HEDT-Glu in some tissues 0.083, 0.25, and 1.0 h after i.v. administration of 13.2 mg·kg^{-1} are summarized in Table 3. HEDT-Glu was cleared from the blood and mainly distributed to the liver and small intestine, but low levels of the drug remained in the blood even 5 h after i.v. administration.

CONCLUSIONS

For the first time, a simple and reliable HPLC method to determine HEDT-Glu levels in rat plasma and tissues was established and validated, which had been successfully applied to the pharmacokinetics studies. The developed assay showed acceptable precision, accuracy, linearity, stability and specificity. It can be applied to extensive human pharmacokinetic studies as well.

Table 3. Tissues Distribution of HEDT-Glu after i.v. Administration 13.2 mg·kg^{-1} HEDT-Glu in Rats (n=6)

Tissues	Mean±S.D. (μg·g^{-1})		
	0.083 h	0.25 h	1.0 h
Heart	0.181±0.071	N.D	N.D
Liver	0.679±0.143	0.417±0.065	0.318±0.067
Spleen	0.284±0.026	0.276±0.043	0.261±0.045
Lung	0.266±0.044	0.261±0.009	0.102±0.022
Kidney	0.383±0.152	0.621±0.267	N.D
Pancreas	0.238±0.081	0.224±0.110	0.561±0.154
Stomach	0.094±0.044	0.087±0.034	0.104±0.025
Small intestine	0.483±0.066	0.600±0.089	0.443±0.052
Skeletal muscle	0.152±0.074	N.D	N.D
Brain	N.D	N.D	N.D
Fat	0.114±0.102	0.079±0.022	0.120±0.043

N.D represents "not detected."

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