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Quantitative determination of 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone (HMTC) in rat plasma by HPLC-MS/MS and its application to a pharmacokinetic study

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Abstract: 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone (HMTC) is a newly synthesized chalcone that affects proliferation, cytotoxic potential and apoptosis in human leukemia cells. However, no validated determination method has been described so far for HMTC in biological samples. Thus, we developed a liquid chromatographic method using a tandem mass spectrometry to determine HMTC in rat plasma. Liquid-liquid extraction with ethyl acetate was used for the clean-up procedure. The analyte was separated on a reversed-phase column with mobile phase of distilled water and acetonitrile (2:8, v/v, including 0.1 % formic acid). The ion transition of the precursor to the product ion was principally deprotonated ions $[M-H]^-$ at m/z 356.8 → 327.2 for HMTC. This analytical method was successfully applied in pharmacokinetic study of HMTC after intravenous administration in rats.

Key words: 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone, HPLC-MS/MS, pharmacokinetics, rats

1. Introduction

Chalcones, 1,3-diaryl-2propen-1-ones, are one of the major metabolites of plants that belong to the flavonoid family. The chalcones are widely distributed in fruits, vegetables, spices, tea and soy based foodstuff[1] and exhibit many pharmacological activities, such as anti-inflammatory,[2] antioxidant,[3] and cytotoxic[4] effects. Many chemical entities have been synthesized due to the variety of substitution potentials in the diaryl positions. Moreover, the synthesis of chalcones is relatively simple: a condensation of a phenone and an aldehyde under alkaline conditions.

Whilst Sale *et al*.[5] introduced a methylenedioxy group in the ring B in *Fig.* 1, which allows the substance to be activated by the tumor-selective enzyme activity with relatively low intrinsic toxicity associated the group in ring A and synthesized many

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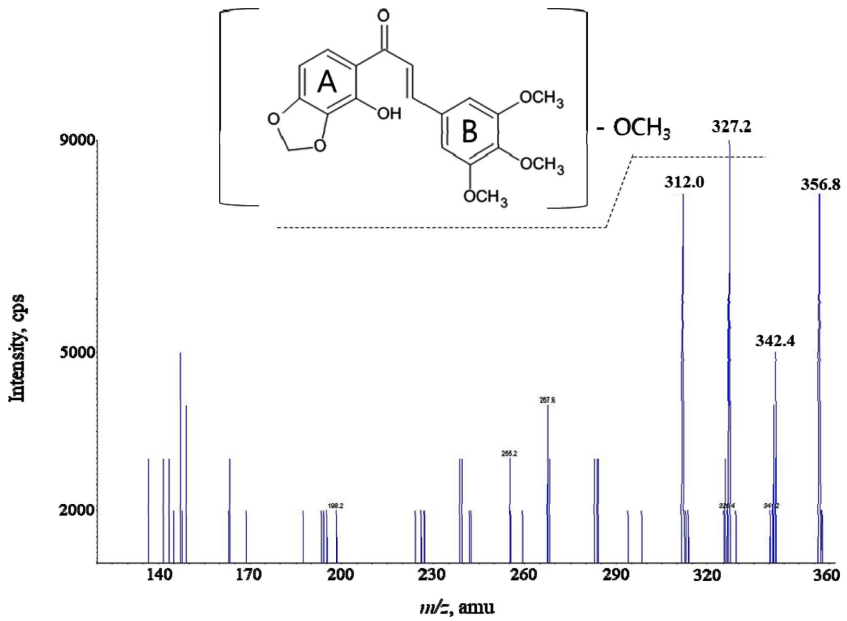

Fig. 1. Product ion mass spectrum and proposed fragment ion of 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone.

derivatives.[6-8] HMTC (2'-hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone) exerts down-regulation of cancer cell proliferation and cytotoxic potential and apoptosis in human leukemia cells.

The pharmacokinetic characteristics of a new chemical entity with pharmacological activity should be investigated *in vivo* early in the drug development process. Systemic exposure can provide important information about the dosing regimens and medication routes. Therefore, HMTC was given here enterally and intravenously to rats, and the plasma concentrations were serially monitored. In advance, a sensitive and validated quantification method was developed by using a high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS).

2. Experimental

2.1. Chemicals and reagents

HMTC was synthesized in our laboratory, as previously reported. The chemical structure of chalcones was confirmed using nuclear magnetic resonance (NMR) spectroscopy.[6,8] Diclofenac, formic acid, ethyl acetate, dimethylacetamide (DMA), polyethyleneglycol 400 (PEG400), normal saline and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Seoul, Korea); acetonitrile was obtained from Burdick & Jackson (Muskegon, MI, USA). All other chemicals and solvents were of the highest analytical grade available.

2.2. Characterization of product ions using MS/MS

Standard solutions of HMTC and diclofenac (the internal standard; IS) were prepared in methanol (1 mg/mL) and serially diluted. Then, 100 ng/mL of each solution was infused into the mass spectrometer at 10 μL/min to characterize its precursor and product ions using both positive and negative ion modes. The major ions fragmented were scanned by ramping the collision energy, and the major mass spectrometry peaks were used to quantify HMTC and the IS.

2.3. Analytical system and chromatographic conditions

An API 4000-LC/MS/MS system (AB SCIEX, Framingham, MA, USA) with an electrospray ionization interface was used. The compounds were

separated on a reversed-phase column (Poroshell EC-C_{18}, 50 × 2.1 mm internal diameter, 2.7 μm particle size; Agilent, Cork, Ireland) at 30 °C; the mobile phase consisted of purified water and acetonitrile (2:8, v/v) including 0.1 % formic acid, and was set to 0.2 mL/min using an HP 1260 series pump (Agilent, Wilmington, DE, USA).[9,10] The running time and the injection volume were 3 min and 5 μL, respectively.

The turbo ion spray interface was maintained at 4500 V and 450 °C. The precursor ion of HMTC was monitored in positive and negative ion modes. Multiple reaction monitoring was used to quantify the precursor and its product ions, and the ratio of the peak areas for each substance was calculated. All data were processed by Analyst software (ver. 1.5.2; Applied Biosystems, Foster City, CA, USA).

2.4. Stability of HMTC in rat plasma

Three plasma samples at 50 and 800 ng/mL of HMTC were prepared to test its stability. Ascorbic acid (5 mg/mL) as the final concentration in plasma samples was added to ensure its stability in rat plasma.[11] The short- and long-term stabilities were examined after storage at room temperature for 3 h and -70 °C for 2 weeks, respectively. Freeze-thaw stability was evaluated after three freeze-thaw cycles (-70 °C to room temperature). The post-extraction stability was assessed after 24 h of storage at 10 °C. The stability in various conditions was assessed by comparing the peak ratios with those of freshly prepared samples, and within 15 % accuracy was considered acceptable.

2.5. Calibration curve and plasma sample preparation

HMTC standard solution was serially diluted in methanol to yield solutions of 100 ng/mL to 10 μg/mL. Then, 10 μL of each sample was added to 90 μL of drug-free plasma to get calibration standards at 10, 20, 50, 100, 200, 500, and 1000 ng/mL. Ascorbic acid (55 mg/mL, 10 μL) was added at the mixture to ensure the plasma stability at room temperature.

Acetonitrile (50 μL including the IS 100 ng/mL) and ethyl acetate (1 mL) were added to the calibration standards. The mixtures were stirred vigorously for 3 min and centrifuged at 10,000 g for 10 min at 4 °C. Supernatant was transferred into a new tube and evaporated under reduced pressure at 50 °C. The residue was reconstituted with 30 μL of the mobile phase. The reconstitute was filtered, and 5 μL was injected into the LC-MS/MS system. Linear regression was performed to derive a calibration curve based on the ratio of the area under the peak for HMTC to that of the IS at each concentration.

Plasma samples (100 μL) from the animal study were prepared the same way as the calibration standards. The concentrations of real samples were obtained by an inverse calculation using the calibration curve.

2.6. Validation procedure

Blank plasma samples obtained from five individual rats were screened to determine specificity. Quality controls were prepared at four concentrations (10, 50, 200, and 800 ng/mL for lower limit of quantification, and low, intermediate, and high concentrations, respectively). The intra- and inter-day assay precision and accuracy were estimated using a calibration curve to predict the concentration of quality controls. Acceptable ranges were ±20 % for the lower limit of quantification and ±15 % for the other quality controls. Recovery was calculated by dividing the peak areas of quality controls pre-spiked by those post-spiked. A possible matrix effect was examined by dividing the peak areas of quality controls post-spiked by those in neat solutions.

2.7. Animal study

Ten male, 7-week-old Sprague-Dawley rats weighing 240-250 g were used. The animal room was maintained at 23 °C, with a relative humidity of 50 ± 10 %, 10-20 air change/h, and a light intensity of 300 Lux under a 12 h/12 h light-dark cycle. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Chung-Ang University. All animals were cared for in accordance with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Ten

rats were randomly divided into two groups (five rats in each group) for intravenous and intragastric administration of HMTC. HMTC was suspended in corn oil for intragastric administration (10 mg/kg, 1 mL/kg), and dissolved in a mixture of normal saline, dimethylacetamide, polyethyleneglycol 400, and dimethylsulfoxide (3.5:3:3:0.5, v/v) for intravenous dosing (1 mg/kg, 1 mL/kg).

To measure plasma concentrations, blood samples (250 μL) were collected from the subclavian veins at 2, 5, 15, and 30 min, and 1, 1.5, 2, 4, 6, 8, 10 and 12 h after intravenous administration, and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h after intragastric administration. The samples were heparinized, centrifuged at 10,000 g for 10 min, and plasma was transferred at a new tube and stored at -70 °C before analysis. Ascorbic acid (55 mg/mL, 25 μL) was added to a tube before taking the blood samples to ensure the stability in plasma.

The pharmacokinetic parameters were calculated from the time courses of plasma HMTC concentrations. The elimination rate constant (k) was estimated by linear regression of the log-transformed plasma concentration in terminal phase, and half-life was given by 0.693/k, and clearance (Cl) was calculated by dividing the dose by the area under the plasma concentration–time curve (AUC).

3. Results and Discussion

3.1. Quantification of HMTC

Fig. 1 shows the product ion mass spectrum and chemical structure of HMTC. Its precursor ion in negative ionization mode was superior to that in positive mode and appeared mainly as deprotonated ions $[M-H]^-$ at m/z of 356.8. The major product ion was scanned in Q3 after collision with nitrogen in Q2 at a m/z of 327.2 (declustering potential -105 ev, collision energy -30 ev, dwell time 150 ms). The transition from the IS precursor, $[M-H]^-$ to product ion occurred at 296.1→251.7 (collision energy -18 eV), as reported previously.[12,13]

Typical chromatograms of HMTC and the IS in rat plasma are shown in *Fig.* 2. No endogenous interference was found at the elution time of either substance (*Fig.* 2A). HMTC and the IS eluted at 1.15 and 1.05 min, respectively (*Figs.* 2B-2D).

The calibration curves for the analyte allowed us to quantitate the substance in plasma reliably. The ratio of the peak area of HMTC relative to that of the IS was correlated with the corresponding plasma concentration, and good linearity was shown (r^2 > 0.999). The intra- and inter-day assay precisions were ≤10.6 % and ≤11.2 %, respectively. The intra- and inter-day assay accuracies were 101.5-103.4 and

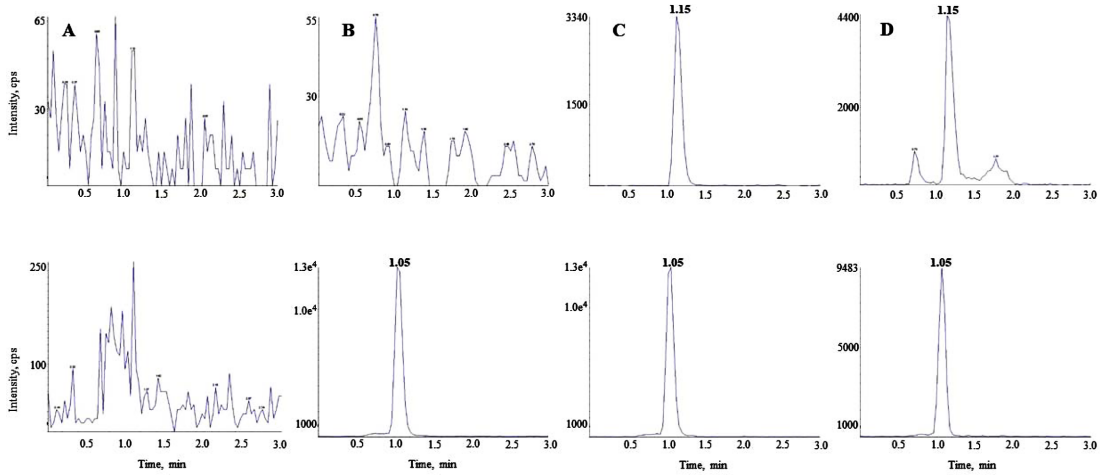

Fig. 2. Chromatograms of HMTC (top) and internal standard (bottom): double blank (A); zero blank (B); spiked plasma with 200 ng/mL of HMTC (C); a real sample was calculated (173 ng/mL) (D).

Table 1. Assay validation and stability of 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone in rat plasma

Concentration (ng/mL)	Intra-day	Inter-day	Conditions for stability test[c]	50 ng/mL	800 ng/mL
10	103.4 ± 11[a] (10.6)[b]	100.0 ± 8.2 (8.2)	Room temperature for 1 h	93.8 ± 10.9[d]	94.7 ± 6.9
50	103.1 ± 3.0 (2.9)	95.7 ± 10.7 (11.2)	3 freeze-thaw cycles	97.7 ± 6.4	85.5 ± 0.8
200	101.5 ± 4.5 (4.5)	96.3 ± 7.9 (7.0)	Post-extraction at 10 °C for 24 h	87.9 ± 6.1	105.4 ± 8.4
800	102.6 ± 3.3 (3.3)	102.6 ± 2.3 (2.2)	-70 °C for 2 weeks	92.9 ± 8.9	88.1 ± 8.1

[a]Accuracy (mean% ± s.d., n=5)
[b]Precision, relative standard deviation (%)
[c]Ascorbic acid (5 mg/mL as the final concentration) was added in plasma to ensure the stability of the substance.
[d]Accuracy in plasma under various conditions (mean% ± s.d., n=3)

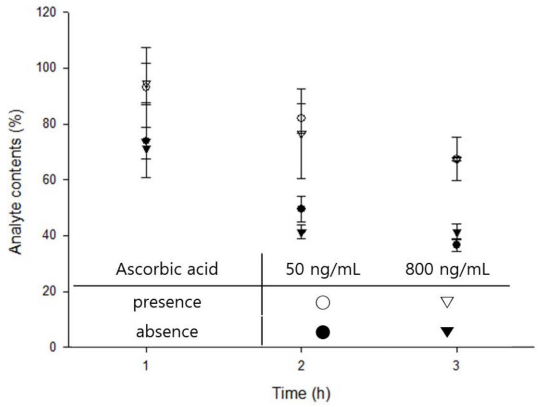

Fig. 3. Plasma stability of 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone at room temperature in the presence and absence of ascorbic acid.

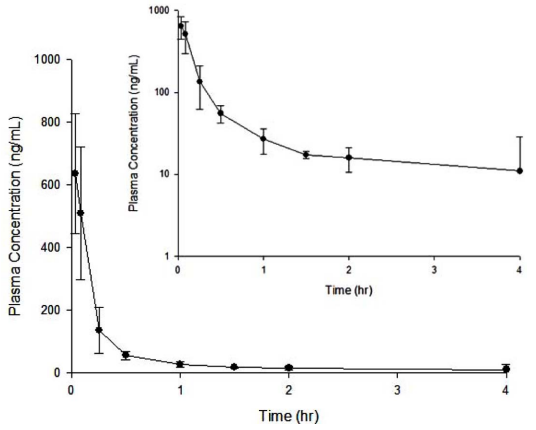

Fig. 4. Time course of plasma concentrations following intravenous (1 mg/kg) administration of HMTC in rats. The insert indicates a semi-logarithmic plot.

95.7-102.6 %, respectively (*Table* 1).

Although the stock solution of HMTC was stable in methanol, the substance in rat plasma is unstable at room temperature in the absence of ascorbic acid (*Fig.* 3). Ascorbic acid (5 mg/mL) can prolong its stability for 1 h, so the sample has to be prepared within 1 h in the presence of ascorbic acid. In the literature, as mentioned earlier (Sus *et al*., 2018), ascorbic acid improved the stability of 4-fluoro-3',4',5'-trimethoxychalcone similar to HMTC in biological samples.

As shown in *Table* 1, HMTC was stable under the other conditions. The substance remained intact in plasma for up to 2 weeks at -70 °C. No degradation was observed after three cycles of freezing and thawing. The stability of the compound in extracts was confirmed after 24 h of storage at 10 °C. The extraction recovery was 96.8 ± 3.4 %, and any matrix effect was negligible (98.9 ± 1.6 %), indicating that the present procedure is suitable not only to extract the analyte from plasma but also to minimize the endogenous materials in the matrix, which could affect the ionization of HMTC.

3.2. Pharmacokinetics of HMTC in rats

Time course of plasma HMTC concentrations is depicted in *Fig.* 4 after an intravenous administration at 1 mg/kg to rats. HMTC was bi-exponentially decayed with an elimination rate constant of 0.63 ± 0.23 h^{-1} at the terminal phase, and the half-life was accordingly calculated to be 1.02 ± 0.45 h. AUC_{inf} and clearance were 218 ± 43 ng·h/mL and 4.74 ± 1.06 L/h/kg, respectively.

On the other hand, HMTC shows very poor oral absorption: all plasma concentrations following oral

administration were under the limit of quantification. Because chalcones are insoluble in water, poor gastrointestinal absorption is predicted.

4. Conclusions

We have developed a bioanalytical method to determine 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone in rat plasma using high-performance LC–MS/MS. The time course of its plasma concentrations was characterized following intravenous administration to rats. The present method would be useful for conducting pharmacokinetic studies of 2'-Hydroxy-3',4'-methylenedioxy-3,4,5-trimethoxychalcone in animals.

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