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# Simultaneous determination of imperatorin and its metabolite xanthotoxol in rat plasma and urine by LC–MS/MS and its application to pharmacokinetic studies

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ABSTRACT

An accurate, precise, selective, and sensitive liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of imperatorin (IMP) and its metabolite, xanthotoxol (XAN), in rat plasma and urine samples. The analytes, along with psoralen as an internal standard, were determined by multiple reaction monitoring (MRM) operated in the positive electrospray ionization (ESI) mode. Chromatographic separation was performed on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) with a mobile phase consisting of 0.1% formic acid solution and 0.1% formic acid in methanol at a flow rate of 0.3 mL/min. The run time was 6 min per sample and the injection volume was 5 μL. The method had a lower limit of quantification (LLOQ) of 0.25 ng/mL for IMP in plasma and urine, and 1 ng/mL for XAN in urine. The linear calibration curves were fitted over the range of 0.25–1000 ng/mL for IMP in plasma, 0.25–1000 ng/mL for IMP in urine, and 1–1000 ng/mL for XAN in urine, with correlation coefficients greater than 0.995. The inter- and intra-day accuracies (relative error, RE%) were between −8.5% and 3.5%, and the precisions (relative standard deviation, RSD%) were less than 10.0% for all quality control samples (QCs). The analytes were extracted from rat plasma and urine samples using a liquid-liquid extraction method with the extraction recovery in the range of 60.3–79.1%. A good stability of the analytes was observed in all the analysis procedures. The method was successfully validated and applied to determine the pharmacokinetics of IMP in rat plasma and, for the first time, the metabolite kinetics of IMP to XAN in rat urine after IMP administration.



## 1. Introduction

Imperatorin (IMP), an active natural furocoumarin, is extracted from many herbal medicines, including *Casimiroa edulis* (Rutaceae) [1,2], *Glehnia littoralis* (Apiaceae) [3–5], and *Angelica dahuricae* (Apiaceae) [6–10]. IMP has been reported to have numerous potent pharmacological actions, including anti-inflammation [3,11], antibacterial [12], anti-allergic [13,14], beneficial cardiovascular effects [1,15–17], and neuromodulation [18–20].

Some reports have been studied on the isolation and quantitation of IMP, and determination of IMP pharmacokinetics (PKs). Various methods, including GC–MS [21], HPLC–UV [22,23], SPE–HPLC [24], and LC–MS [4,25–29] have been developed. Most of these methods displayed low sensitivity with lower limits of quantification (LLOQ) in the range of 1–40 ng/mL. Other methods were more sensitive; however, they were time-consuming because they included a long total run time per injection [28] or a complex sample pretreatment [26].

Since metabolite identification is an integral part of drug screening and development, there have also been reports regarding IMP metabolites. In 2012, IMP and its two metabolites, IMP hydroxylate and IMP epoxide, were first identified and quantified in dog plasma after an intravenous (IV) administration of IMP (5 mg/kg) by using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) [25]. In 2013, Qiao et al. [30] reported the urinary metabolites of IMP after an oral administration of IMP (80 mg/kg) by using LC–triple TOF–MS. In this study, a total of 51 IMP metabolites were identified in rat urine, including 44 phase I and 7 phase

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II metabolites. Among them, xanthotoxol (XAN) displayed clearly the strongest response. Recently, IMP metabolites were screened and identified for the first time in rat bile after an oral administration of IMP (100 mg/kg) using UPLC–QTOF–MS/MS [31]. Herein, a total of 32 metabolites were detected and tentatively identified from bile samples, and possible metabolic pathways were subsequently proposed and summarized. The study concluded that the M1 metabolite with the precursor ion at m/z 203.04 ($C_{11}H_7O_4$, loss of $C_5H_8$, 68 Da less than that of IMP) was the basis for numerous metabolites in the proposed metabolic pathways of IMP. This precursor ion and the fragment ions at m/z 185.02, 175.04, and 147.13 were consistent with those of XAN reported in previous studies [26,30]. These suggested that XAN may be one of the main metabolites of IMP and it would be informative to use XAN as one of the marker compounds for IMP PKs.

XAN and IMP were simultaneously determined for the first time in rat plasma after an oral administration of IMP (80 mg/kg) using HPLC–ESI–MS [26] coupled with hollow fiber liquid phase microextraction (HF-LPME). This method had the advantage of excellent sample cleanup ability; however, as mentioned above, it was time-consuming since a complex pretreatment procedure followed by the extraction steps. Moreover, the hollow fibers had to be cut to an appropriate length and sealed, which limited the implementation of HF-LPME in routine laboratories and applications. Therefore, a new method for determination of IMP and XAN needed to be developed.

To date, there have been no published reports on the PKs of IMP and its metabolite, XAN, in rat urine following administration of IMP. The present study aimed to explore a rapid, selective, and sensitive LC–MS/MS method for the simultaneous determination of IMP and its metabolite, XAN, in rat plasma and urine after an oral and IV administration of IMP at a dose of 3 mg/kg to rats.

## 2. Experimental

### 2.1. Reagents and materials

Reference standards for IMP and XAN were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Psoralen reference standard (internal standard, IS) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Heparin sodium (25000 IU/5 mL) was purchased from Choongwae Pharm. Co. (Seoul, Korea). Ethanol and ethyl acetate (HPLC grade), methanol and water (LC/MS grade) were purchased from Fischer Scientific (Fair Lawn, NJ, USA). Water used throughout the study, except for LC–MS/MS assays, was produced in-house using a Milli-Q Plus Ultra-Pure Water System (Millipore Co., Milford, MA, USA). All other chemicals and solvents were of analytical grade or the highest quality available.

### 2.2. LC–MS/MS analysis

The LC–MS/MS system used was a Shimadzu liquid chromatography (LC) system coupled to a Shimadzu mass spectrometer (LCMS–8040, Shimadzu Corp., Kyoto, Japan). A 5 μL aliquot of extracted samples was injected onto an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm; Waters, Milford, MA, USA) for separation. The mobile phase consisted of (A) 0.1% formic acid solution and (B) 0.1% formic acid in methanol (v/v) at a flow rate of 0.3 mL/min. The gradient elution was as follows: 0–0.25 min, 35% B; 0.25–0.60 min, 35–55% B; 0.60–2.25 min, 55% B; 2.25–2.75 min, 55–60% B; 2.75–4.0 min, 60% B; and 4.0–6.0 min, 35% B (v/v). The column and autosampler tray were maintained at 40 °C and 15 °C, respectively. The total run time was 6 min per sample. IMP, XAN, and IS were eluted at approximately 4.1, 1.6, and 1.9 min, respectively.

The MS detection was operated in the positive electrospray ionization (ESI) mode using multiple reaction monitoring (MRM) transitions (Fig. 1). The capillary voltage, desolvation, and ion source temperature were 1.88 kV, 400 °C, and 250 °C, respectively. The drying and nebulizing gas flow rates were 12 and 2.5 L/min, respectively. Nitrogen was used in all cases. The dwell time was 100 ms for all compounds. Other MS/MS detection conditions are represented in Fig. 1.

System management and hardware interface for data acquisition were carried out using the LabSolutions V5.53 SP3 software package from Shimadzu (Shimadzu Corp., Kyoto, Japan).

### 2.3. Preparation of standard solutions and quality control samples (QCs)

An appropriate amount of IMP and XAN was accurately weighed and each was dissolved separately in methanol to make the standard stock solutions at strengths of 0.052 and 0.056 mg/mL, respectively. These were diluted in methanol at appropriate ratios to prepare standard curves with concentration ranges of 2.5–10000 ng/mL for IMP and 10–10000 ng/mL for XAN. The IS solution was diluted in methanol to a final concentration of 500 ng/mL. For method validation, each analyte was prepared at three (low, medium, and high) concentration levels (IMP: 7.5, 500, and 8000 ng/mL; XAN: 25, 500, and 8000 ng/mL). All solutions were stored at −20 °C for later use.

Calibration standards and QCs were prepared by spiking a 10 μL aliquot of the standard solutions with a 100 μL aliquot of plasma or urine samples. These samples were then treated as real plasma and urine samples.

### 2.4. Sample extraction

IMP and XAN were extracted from rat plasma and urine samples using a liquid-liquid extraction method. Briefly, a 100 μL aliquot of each sample was sequentially mixed with 10 μL of the IS solution (500 ng/mL) and 10 μL of methanol (the volume corresponding to that of working solution for calibration curve and QCs) in an Axygen microtube (Axygen, Inc., Union, CA, USA). The mixture was then vortexed for 10 min with 1 mL of ethyl acetate using a vortex mixer (Vortex-Genie 2 Mixer, Scientific Industries, Inc., Bohemia, NY, USA) for extraction. After centrifugation at 10000 rpm for 5 min (Centrikon T-2070, Kontron, Switzerland), 950 μL of the upper layer was transferred to another microtube. The organic phase was evaporated to dryness at room temperature with a gentle stream of nitrogen under vacuum. The obtained residue was reconstituted in 100 μL of methanol, vortexed for 1 min, and centrifuged at 10000 rpm for 5 min. A 5 μL aliquot of the supernatant was then injected into the LC–MS/MS system for analysis.

### 2.5. Method validation

Validation experiments and run acceptance criteria were based on the United States Food and Drug Administration Bioanalytical Method Validation Guidance [32].

#### 2.5.1. Selectivity

The method selectivity was assessed to ensure that there was no interfering signal in the samples, particularly at the expected retention time of the analytes. The chromatogram of blank samples from six different sources was compared with those spiked with the analytes at the LLOQs and the samples after drug administration.