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# Estimation of *p*-coumaric acid as metabolite of E-6-*O*-*p*-coumaroyl scandoside methyl ester in rat plasma by HPLC and its application to a pharmacokinetic study

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**Abstract**

A rapid and simple high-performance liquid chromatographic (HPLC) method has been developed for the determination of *p*-coumaric acid in rat plasma and applied to a pharmacokinetic study in rats after administration of a prodrug, *E*-6-*O*-*p*-coumaroyl scandoside methyl ester, isolated from *Hedyotis diffusa* (Willd.). Sample preparation involved protein precipitation with acetonitrile. The supernatant was then injected onto a DiamonsilTM $C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm). The mobile phase consisted of acetonitrile–water (21:79, v/v) with 1% glacial acetic acid. The UV detector was set at 310 nm. The lower limit of quantification of *p*-coumaric acid in rat plasma was 0.02 μg/mL. The calibration curves were linear over the concentration range 0.02–5 μg/mL with correlations greater than 0.999. The assay procedure was applied to the study of the metabolite pharmacokinetics of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester in rat.


*Keywords:* *E*-6-*O*-*p*-Coumaroyl scandoside methyl ester; *p*-Coumaric acid; Pharmacokinetics; Metabolite

## 1. Introduction

*p*-Coumaric acid (Fig. 1c), as a phenolic acid, is an antioxidant which is implicated for the prevention of pathologies, such as colon cancer [1,2] and cardiovascular diseases [3]. It has been reported that there is a high efficient intestinal absorption of *p*-coumaric acid in vivo [4]. In addition, in vitro studies have demonstrated that *p*-coumaric acid is transported across human intestinal Caco-2 cells [5,6].

Recently a prodrug, *E*-6-*O*-*p*-coumaroyl scandoside methyl ester (Fig. 1a), was isolated from *Hedyotis diffusa* (Willd.), which can be further hydrolyzed to *p*-coumaric acid in vivo. Following intragastric administration in rats, the prodrug could not be detected in plasma due to extensive metabolism. Since no previous pharmacokinetic study of this ester of *p*-coumaric acid has been reported, *E*-6-*O*-*p*-coumaroyl scandoside methyl ester is worthy of further evaluation.

To study the pharmacokinetics of the prodrug in vivo, a sensitive, precise and accurate assay method is essential. A method has been reported for the analysis of *p*-coumaric in rat plasma by HPLC-ECD [4]. We are the first to develop a simple, rapid, sensitive, and selective HPLC-UV method for the determination of *p*-coumaric acid in rat plasma and we have used this method successfully in an intragastric pharmacokinetic study of *p*-coumaric acid as metabolite of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester in rats.

## 2. Experimental

### 2.1. Materials and reagents

*E*-6-*O*-*p*-coumaroyl scandoside methyl ester was separated and purified from an ethanol extract of *H. diffusa* Willd and its structure was confirmed by UV, IR, MS and NMR spectroscopy. The purity was 96.3% as determined by HPLC. *p*-Coumaric acid was also separated from *H. diffusa* Willd. Tinidazole (TNZ, Fig. 1b) used as internal standard (I.S.) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Glacial acetic acid was of analytical grade and acetonitrile was of chromatographic grade from Corncord Tech (Tianjing, China). Healthy Wistar rats were

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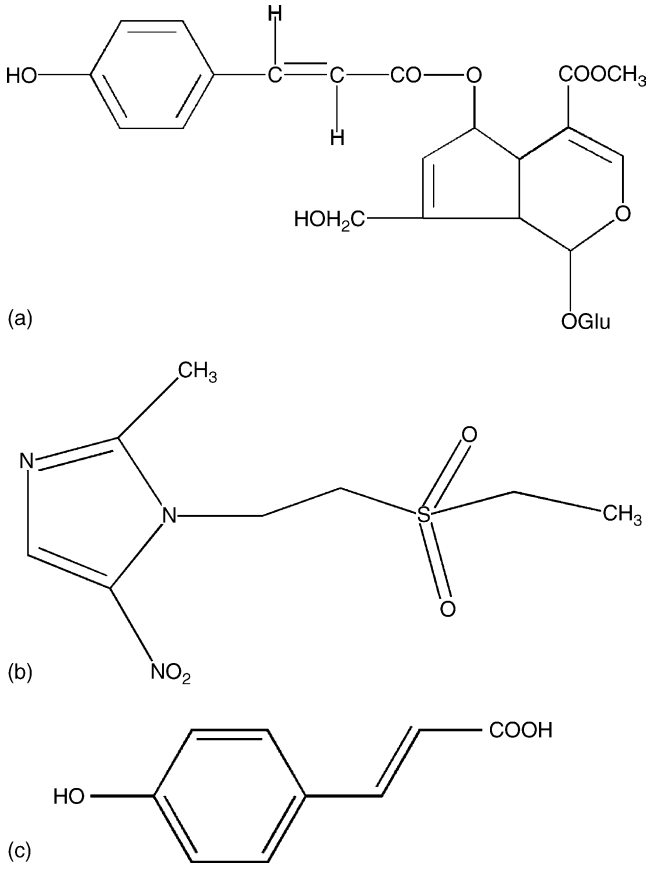


Fig. 1. Structures of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester (a); TNZ (b); *p*-coumaric acid (c).

obtained from the Experimental Animal Center of Shenyang Pharmaceutical University.

### 2.2. Chromatographic system

The HPLC system consisted of a LC-10A system (Shimadzu, Kyoto, Japan) equipped with a LC-10AD pump, a variable UV detector model SPD-10A UV–vis and an Anastar workstation (Tianjin, China). The mobile phase was a mixture of acetonitrile–water (21:79, v/v) containing 1% glacial acetic acid. Separations were performed on a DiamonsilTM $C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm) at room temperature. The effluent was monitored by UV detection at 310 nm at a flow rate of 1.0 mL/min.

The mobile phase was filtered through a 0.45 μm cellulose membrane filter (Auto Science, Tianjing, China), and degassed under reduced pressure before use.

### 2.3. Preparation of calibration standards and quality control samples

Stock solutions of *p*-coumaric acid (20 μg/mL) and tinidazole (5 μg/mL) were prepared in acetonitrile and stored in light-protected glass bottles at 4 °C. Solutions of *p*-coumaric acid with concentrations of 0.02, 0.1, 0.2, 0.5, 1, 2, 5 μg/mL were prepared by serial dilution of stock solutions with acetonitrile. Each blank rat plasma sample was spiked with TNZ solution and different concentrations of *p*-coumaric acid to prepare a series of standards for the calibration curve. Quality control (QC) samples were prepared at low (0.02 μg/mL), medium (1 μg/mL) and high (5 μg/mL) concentrations in the same way as the plasma samples for calibration. All the samples were stored at −20 °C until analysis.

### 2.4. Pharmacokinetic studies in rats

Six Wistar rats (body weight 220 ± 20 g) were fasted for 12 h with free access to water during the experiment. The rats were then given a dose of 20 mg/kg *E*-6-*O*-*p*-coumaroyl scandoside methyl ester in a vehicle consisting of 10% trimethylene glycol and 90% sterile saline solution by intragastric administration (i.g.). Blood samples (0.5 mL) were collected from the suborbital vein into heparinized tubes at 0.33, 0.67, 1, 1.5, 2, 3 and 4.5 h following drug administration.

All blood samples were immediately centrifuged for 10 min at 12,000 × *g*, and the plasma was transferred into clean tubes. Then, to 100 μL plasma, 100 μL I.S. solution (5.1 μg/mL) and 100 μL acetonitrile were added, followed by vortex mixing for 90 s and centrifuging at 12,000 × *g* for 10 min. The upper organic phase was transferred into labeled clean tubes and a 20 μL aliquot of the solution was injected into the HPLC system for analysis.

## 3. Results and discussion

### 3.1. Specificity

Typical chromatograms of blank plasma, blank plasma spiked with *p*-coumaric acid and I.S., and rat plasma at 0.67 h after administration of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester are presented in Fig. 2. *p*-Coumaric acid and the I.S. were eluted at 9.4 and 12.8 min, respectively. There were no interfering peaks at the retention times of *p*-coumaric acid and I.S.

### 3.2. Calibration and validation

The calibration curve for the determination of *p*-coumaric acid was linear over the range of 0.02–5 μg/mL with a mean correlation coefficient of 0.9992. The linear regression of the curve for the peak area ratio versus concentration was weighted by $1/x^2$ (the reciprocal of the square of the *p*-coumaric acid concentration).The mean (±S.D.) regression equation from replicate calibration curves on different days was:

$$y = (0.591 \pm 0.007)x + (0.0351 \pm 0.003)$$

where $y$ is the peak area ratio of *p*-coumaric acid to the I.S. and $x$, the plasma concentration of *p*-coumaric acid.

The lower limit of quantitation (LLOQ) was defined as the lowest concentration measured with an imprecision of less than 20% (coefficient of variation, cv) and accuracy of 80–120%. The LLOQ was found to be 0.02 μg/mL for *p*-coumaric acid in rat plasma.

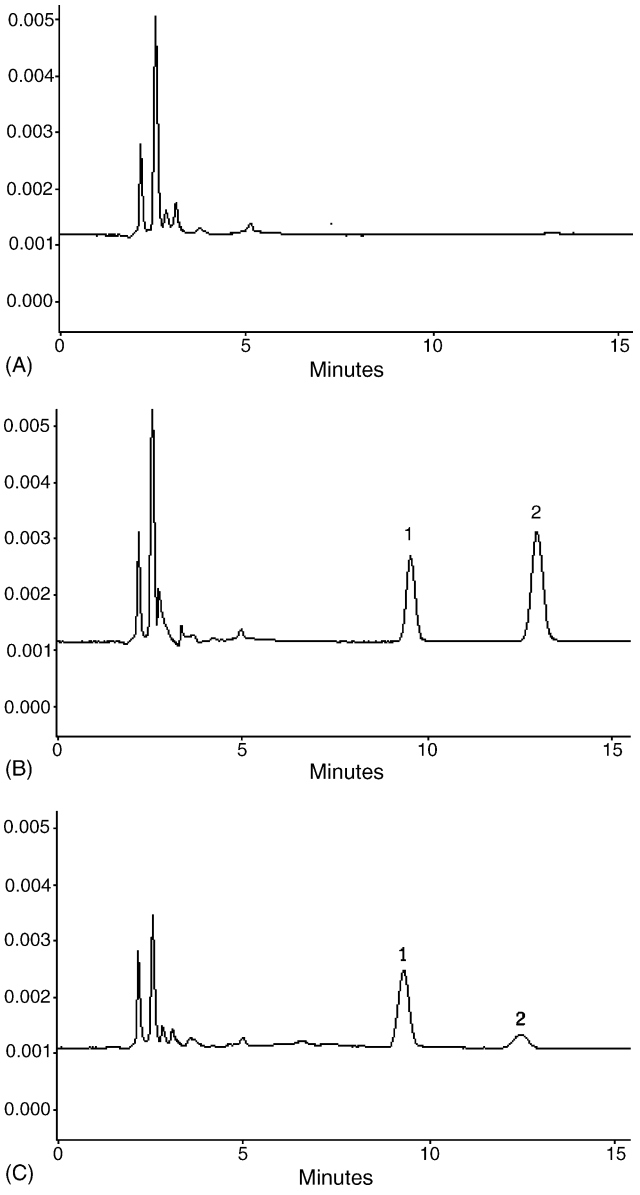


Fig. 2. Representative chromatography of blank plasma (A); plasma spiked with I.S. (TNZ, 5.1 μg/mL) and *p*-coumaric acid (1.0 μg/mL) (B); a plasma sample at 0.67 h after administration of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester (C). Peak 1: TNZ; peak 2: *p*-coumaric acid.

The accuracy and precision of the method were evaluated with QC samples at concentrations of 0.02, 1, 5 μg/mL. The results are shown in Table 1.The intra-day and inter-day precision of the QC samples were satisfactory with R.S.D.s less than 11.7%. The determined values deviated from the nomial concentration with R.E. less than 5.0%.

Table 1
Precision and accuracy of the HPLC-UV method to determine *p*-coumaric acid in rat plasma ($n = 15$)

| Concentration (μg/mL) | | Relative error (%) | Intra-day R.S.D. (%) | Inter-day R.S.D. (%) |
|---|---|---|---|---|
| Added | Found | | | |
| 0.02 | 0.021 | 5.0 | 11.7 | 9.3 |
| 1.0 | 0.964 | −3.6 | 4.9 | 4.4 |
| 5.0 | 5.11 | 2.2 | 5.3 | 3.8 |

Table 2
Pharmacokinetic parameters of *p*-coumaric acid as metabolite in rats after intragastric administration of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester ($n = 6$)

| | |
|---|---|
| $C_{max}$ (μg/mL) | 0.236 ± 0.047 |
| $T_{max}$ (h) | 1.2 ± 0.3 |
| $T_{1/2}$ (h) | 1.3 ± 0.5 |
| $K_e$ ($h^{-1}$) | 0.6 ± 0.2 |
| $AUC_{0-t}$ (μg min/mL) | 23.8 ± 5.1 |
| $AUC_{0-\infty}$ (μg min/mL) | 26.0 ± 4.7 |
| MRT (h) | 2.2 ± 0.5 |
| CL (mL/min) | 874.8 ± 145.1 |

The extraction recoveries of *p*-coumaric acid from the spiked plasma samples were calculated at low (0.02 μg/mL), medium (1 μg/mL), and high (5 μg/mL) concentrations. Recovery was calculated by comparing the observed concentrations with the spiked concentrations. The mean extraction recoveries of *p*-coumaric acid at the three concentrations were 95.6 ± 6.3, 98.2 ± 3.5 and 98.5 ± 4.4%. The extraction recovery of the I.S. was 97.9%.

The stability of *p*-coumaric acid in rat plasma was investigated through three freeze-thaw cycles using the QC samples. The relative errors at the three different concentrations studied were 7.3, 4.6 and −3.1%. The stability of the analyte in plasma and mobile phase was investigated. *p*-Coumaric acid was shown to be stable in rat plasma at room temperature for at least 8 h (R.E. < −3.7%) and in the reconstitution mobile phase at room temperature for 24 h (R.E. < −8.9%). All procedures were carried out under light-protected conditions.

### 3.3. Pharmacokinetic applicability

The described analytical method was used to analyze plasma samples following the administration of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester at a dose of 20 mg/kg rat body weight. The standard pharmacokinetic parameters, namely: the maximum plasma concentration ($C_{max}$), time point of maximum plasma concentration ($T_{max}$), half-life of drug elimination during the terminal phase ($T_{1/2}$), apparent elimination rate constant ($K_e$), area under the plasma concentration–time curve from 0 to the last measurable concentration ($AUC_{0-t}$), area under the plasma concentration–time curve from 0 to infinity ($AUC_{0-\infty}$),

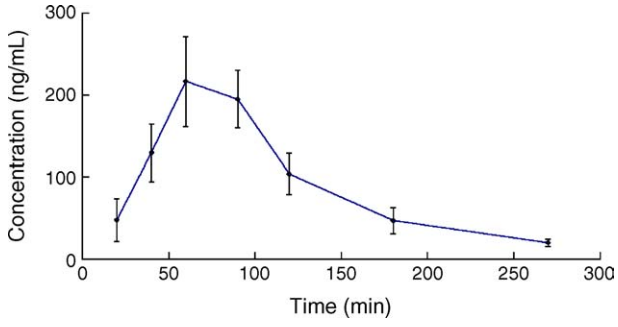


Fig. 3. Plot of the mean concentration of *p*-coumaric acid as metabolite in the plasma of rats ($n = 6$).

mean residence time (MRT) and clearance (CL), were estimated using non-compartmental analysis of Topfit (version 2.0, Thomae GmbH, Germany). The pharmacokinetic parameters are presented in Table 2.The mean plasma concentration–time profile is illustrated in Fig. 3.

## 4. Discussion

The UV spectrum of *p*-coumaric acid has two absorption maxima at 233 and 310 nm. However, interferences from endogenous substances were observed at 233 nm. A detection wavelength of 310 nm proved to be the most suitable since this allowed us to obtain the greatest sensitivity with minimal interference.

The extraction of plasma samples was optimized by the use of a protein precipitation step with acetonitrile. Carrying out protein precipitation of plasma samples was more convenient and time saving than liquid–liquid extraction and solid-phase extraction, and resulted in the least amount of interference with endogenous compounds, while retaining a high extraction efficiency. Other organic solvents, such as methanol and ethyl acetate, were also investigated in our preliminary studies but produced endogenous interference and/or variability in recovery.

The selection of the internal standard was an arduous and hard process. TNZ was finally selected because it had a suitable retention time and was well resolved from the target analyte in our mobile phase.

Although electrochemical detection is more sensitive for the determination of *p*-coumaric acid in cells [5], the actual limit of detection (20 ng/mL in plasma) by UV fulfills all the requirements for pharmacokinetic studies. Compared with the HPLC-EC method [4] without an internal standard, the proposed method, which does not require gradient elution and an expensive, sophisticated electrochemical detector, can be used in many labs for routine measurements.

The absorption and elimination of *p*-coumaric acid were extremely fast in rats ($T_{max}$, 10 min; $T_{1/2}$, 15.9 min) after direct administration of *p*-coumaric acid [4]. However, *p*-coumaric acid as a metabolite was absorbed and eliminated more slowly ($T_{max}$, 1.2 h; $T_{1/2}$, 1.3 h) after administration of the prodrug, *E*-6-*O*-*p*-coumaroyl scandoside methyl ester, probably due to its hydrolyzed diffusion in vivo.

## 5. Conclusion

The HPLC-UV method has been used for monitoring and evaluating the plasma profiles and pharmacokinetics of *p*-coumaric acid as metabolite of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester in rats after intragastric administration of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester. The assay has proven to be rapid and simple with each sample requiring no more than 15 min analysis time. The analysis method is precise and accurate, and has a within-and between-day precision in the range −3.6 to 5.0% (R.E.) and an accuracy of 89.3–102.9% (R.S.D.) for QCs at low, medium and high concentrations. The pharmacokinetic study of *E*-6-*O*-*p*-coumaroyl scandoside methyl ester in rats demonstrated that the ester is a perfect prodrug for *p*-coumaric acid. The ester was found to be much more stable than *p*-coumaric acid in solutions and a suitable formulation can be easily obtained.

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