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High-performance liquid chromatography spectrometric analysis of *trans*-resveratrol in rat plasma

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Abstract

A HPLC method for determination of *trans*-resveratrol concentrations in rat plasma was developed. Plasma samples were treated with acetonitrile to deposit proteins. The analysis used a Hypersil ODS_2 C_{18} column (5 μm, 4.6 mm × 250 mm) and methanol/distilled water as the mobile phase (flow-rate = 1 mL/min). The UV detection wavelength was 303 nm, and chlorzoxazone was used as the internal standard. The calibration curve was linear over the range of 0.02–40 μg/mL with a correlation coefficient of 0.9997. This concentration range corresponds well with the plasma concentrations of resveratrol in pharmacokinetic studies. There was 98.7%, 91.3% and 84.4% recovery from 0.02, 0.4 and 40 μg/mL plasma samples respectively. The R.S.D. of intra- and inter-day assay variations were all less than 12%. This HPLC assay is a quick, precise and reliable method for the analysis of resveratrol in pharmacokinetic studies.

Keywords: Resveratrol; HPLC; Rat; Plasma concentration

1. Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene, Fig. 1), is a polyphenolic phytoalexin with strong anti-oxidative activity which is present in grapes [1], grape juice, red wine and other plant extracts [2,3]. Extracts containing resveratrol have been used for treating and preventing cardiovascular disease [4–10], and these applications are consistent with many of the biochemical properties observed for resveratrol. It inhibits the oxidation of low-density lipoprotein, platelet aggregation [11], and protects isolated rat hearts from ischemia reperfusion injury. The so-called French paradox stimulated the interest in resveratrol in wine [12–14].

To control the quantity of resveratrol in wine or foods, several analytical methods have been developed during the last decade. The most widely used of them are gas chromatography (GC)–coupled with MS [15–17], capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) [18]. Resveratrol is widely used as a food additive and its pharmacological effects have been studied extensively, but little work has been done on its pharmacokinetics and there is no analytical method for resveratrol in plasma. In this contribution we present a HPLC method to accurately measure resveratrol in rat plasma which will enable its pharmacokinetics to be studied.

2. Experimental

2.1. Chemicals and reagents

trans-Resveratrol was kindly donated by Bio-sep Biotechnique Stock Co., Ltd. (Xian, China) with high purity (>98%), methanol and acetonitrile were HPLC grade (Tedia, USA). Chlorzoxazone, used as the internal standard, was supplied by the Medical Chemistry Department of China Pharmaceutical University. Other reagents were highest purity commercially available.

2.2. Apparatus

The HPLC system consisted of a Model Shimadzu LC-10AT pump (Shimadzu Company, Japan) and a Shimadzu Model SPD-10 A UV–vis detector. The apparatus was connected to

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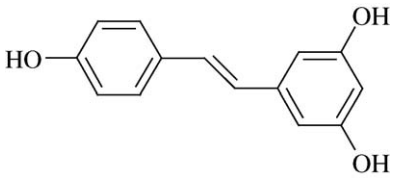

Fig. 1. The chemical structure of resveratrol (*trans*-3,5,4′-trihydroxystilbene).

a personal computer with a HS Chromatography Data System software of Hzep company from Hangzhou, China.

2.3. Chromatographic conditions

The chromatographic separation was performed using a Hypersil ODS_2 C_{18} analytical column (5 μm, 4.6 mm × 250 mm) from Elite Analytical Instruments (Dalian City, China). The mobile phase consisted of a mixture of 0.5% (v/v) acetic acid in methanol and distilled water (52:48, v/v), filtered through a 0.45 μm nylon membrane and ultrasonically degassed prior to use. The mobile phase was delivered at a flow-rate of 1 mL/min, the detection wavelength was 303 nm, the attenuation was 0.001, and the injection volume was 25 μL. Temperature used for HPLC was unregulated ambient.

2.4. Plasma samples

Venous blood samples (200 μL) were withdrawn into the heparinized tubes, then centrifuged at 800 × *g* for 10 min. The plasma obtained was stored at −20 °C until analysis.

2.5. Standard solutions

Stock solutions of *trans*-resveratrol (1.0 mg/mL) and the internal standard, chlorzoxazone (CZ) (0.232 mg/mL), were prepared in methanol and stored at 4 °C within 2 months.

2.6. Extraction procedures

To 50 μL of plasma in an Eppendorf tube, 10 μL of the internal standard working solution was added. After briefly mixing, 100 μL of acetonitrile was added, vortexed for 1 min and centrifuged at 800 × *g* for 10 min. One hundred microliters of the supernatant was transferred to a second tube and 100 μL of water was added to avoid solvent effects. After mixing, a 25 μL aliquot was injected into the HPLC system for analysis.

2.7. Application

Sprague–Dawley rats (210–240 g) were obtained from experiment animal breeding center of Southeast University. Animals were housed under controlled conditions (20 ± 2 °C, RH 50 ± 20%) with a natural light–dark cycle. They were allowed to adapt to the housing environment for at least 1 week prior to study. Diet was prohibited for 6 h before the experiment while water was taken freely. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Each rat was given *trans*-resveratrol at a single dose of 20.0 mg/kg by oral administration. Blood samples (about 200 μL) were collected in heparinized 1.5 mL polythene tubes immediately before and 0, 2, 5, 10, 20, 30, 45, 60 and 90 min after dosing, and were at once centrifuged at 800 × *g* for 10 min at 4 °C. A 50 μL volume of plasma was finally obtained, and stored at −20 °C until analysis.

3. Results

3.1. Validation

3.1.1. Chromatography spectrum

The selectivity was studied by analyzing blank plasma samples. The chromatogram of blank plasma extract (Fig. 2A) did

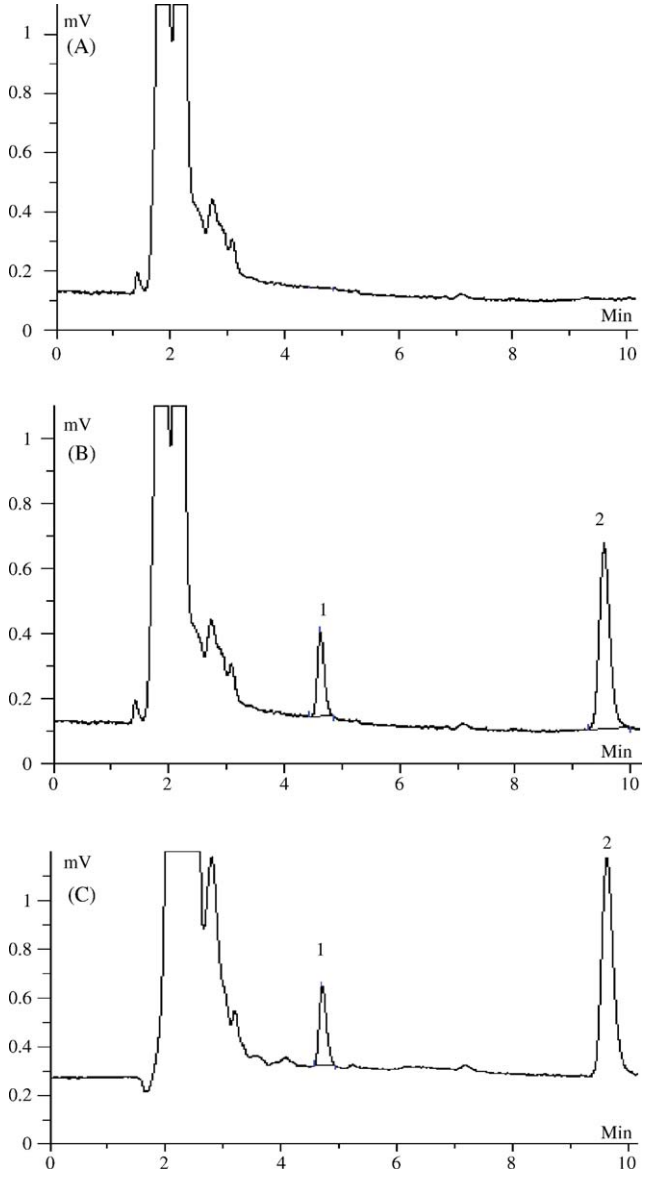

Fig. 2. Typical HPLC-UV chromatogram of blank plasma (A), blank plasma spiked with *trans*-resveratrol (B), and a plasma sample obtained from a rat that was injected intravenously with 20 mg/kg of *trans*-resveratrol (C). The labeled chromatographic peaks indicate *trans*-resveratrol (peak 1) and the internal standard (peak 2).

not show any interfering components. A typical chromatogram of a drug-free plasma sample spiked with *trans*-resveratrol and internal standard is shown in Fig. 2B. The chromatogram of a plasma sample from a rat that received 20 mg/kg *trans*-resveratrol intravenously is shown in Fig. 2C. The retention times of *trans*-resveratrol and internal standard were 4.8 and 9.5 min, respectively.

3.1.2. Extraction efficiency

The absolute recovery of *trans*-resveratrol from plasma was calculated by comparing the peak area obtained from extracts of spiked plasma samples to that obtained from the direct injection of known amounts of standard solutions of *trans*-resveratrol. The overall extraction yields of 0.02, 0.40 and 40.0 μg/mL *trans*-resveratrol in plasma were above 80% (Table 1). The data proved the suitability of the extraction method for use with plasma samples.

3.2. Linearity

Standard curves were constructed by plotting the peak area ratio of *trans*-resveratrol to the internal standard against the concentration of *trans*-resveratrol. The standard curve for *trans*-resveratrol was linear over the range 0.02–40.0 μg/mL. The standard curve was calculated by linear regression, according to the following formula: $Y = aX + b$, where Y is the peak area ratio of drug to internal standard, a (the slope) and b (the y-intercept) are constants, and X is the *trans*-resveratrol concentration (μg/mL). Typical values for the regression parameters a, b and r (correlation coefficient) were calculated to be 0.6135, 0.0029 and 0.9997, respectively ($n = 5$).

3.3. Limits of detection and quantitation

The limit of quantification (LOQ) was the lowest point on the calibration curve which could be detected with a variation of less than 15%. The minimum detectable concentration of *trans*-resveratrol was determined to be 0.02 μg/mL.

3.4. Precision and accuracy

The intra-day assay variations were determined by analyzing five 50 μL aliquots of spiked plasma samples containing 0.02, 0.40, 40.0 μg/mL of *trans*-resveratrol. The inter-day assay variations were determined by analyzing 50 μL aliquots of spiked plasma samples in duplicates on five separate days. The data proved good precision and accuracy of the method, as shown in Table 2.

Table 1
Recovery of *trans*-resveratrol from rat plasma ($n = 5$)

Concentration (μg/mL)	Recovery (mean ± S.D., %)	R.S.D. (%)
0.02	98.7 ± 2.7	2.8
0.40	91.3 ± 3.5	3.9
40.0	84.4 ± 2.8	3.4

Table 2
The intra- and inter-day precision of the method ($n = 5$)

Concentration (μg/mL)	Intra-day (mean ± S.D.)	R.S.D. (%)	Inter-day (mean ± S.D.)	R.S.D. (%)
0.02	0.020 ± 0.0023	11.9	0.023 ± 0.0014	6.3
0.40	0.382 ± 0.015	3.8	0.388 ± 0.021	5.3
40.0	43.37 ± 3.70	8.5	41.85 ± 0.63	1.5

3.5. Application of the method

The applicability of the assay procedure is illustrated in Fig. 3, which shows the average plasma concentration–time curve of *trans*-resveratrol after intravenous injection (20 mg/kg) in five SD rats.

4. Discussion

In the last ten years, many methods used to measure resveratrol in wines have been presented [19–22]. These include gas chromatography (GC/GC–MS), capillary electrophoresis (CE) and HPLC. Most GC methods require derivatization with bis (trimethyl-silyl) trifluoroacetamide to enhance volatility [15,23]. The extraction and derivatization procedures are time-consuming and may cause the *trans*-resveratrol to change to its *cis*-form [24]. Most CE methods reported for measuring resveratrol in wine were able to detect resveratrol at 0.2–1.0 μM, but sample concentration can be a factor limiting the sensitivity of CE because injection volumes are very small (nanoliters) and the narrow diameter of the capillary results in a short light path [25].

Based on the number of reports in the literature, HPLC appears to be the most widely used method for quantifying resveratrol in wines [16,18,26–29]. The solid-phase extraction (SPE) approach is always used to gain cleaner samples [30–32]. This allows the limit of detection to be lowered 10-fold [33], but it is time-consuming and is not economical when used to measure a large number of plasma samples in pharmacokinetic studies. In the protocol presented here, double the volume of acetonitrile was added to plasma in order to remove proteins and hence obtain a high assay specificity and sensitivity, and the sample volumes is so small that the plasma get less dilution than use SPE. This assures that the assay is suitable for the investigation of resveratrol pharmacokinetics (Fig. 3).

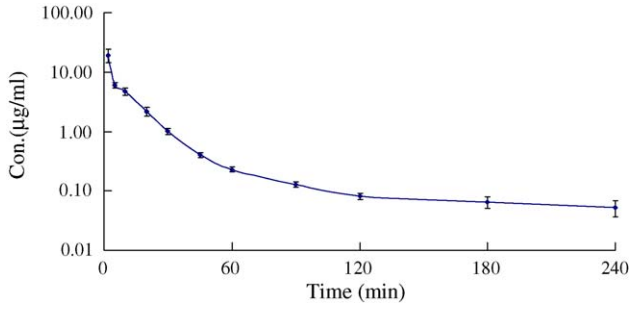

Fig. 3. Mean plasma concentration-time profile of *trans*-resveratrol in rats after 20 mg/kg intravenous administration ($n = 5$).

In the development phase of this work, the supernatant of the acetonitrile extraction was directly injected into the chromatographic system. The solvent effect was serious and induced distorted chromatographic peaks which were not suitable for calculating the concentration. By adding an equal volume of water to the supernatant acetonitrile extraction, the solvent effect was avoided and symmetrical chromatographic peaks were obtained.

5. Conclusion

In conclusion, a simple, rapid and reliable assay method was developed and validated for the determination of resveratrol in rat plasma. The sensitivity, accuracy, linearity, stability, and specificity of the method were acceptable for determination of resveratrol in plasma samples in the pharmacokinetic study.

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