ORIGINAL ARTICLE

Development and validation of a HPLC method for the determination of *trans*-resveratrol in spiked human plasma

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J. Adv. Pharm. Tech. Res.

ABSTRACT

A simple, accurate, precise, sensitive, and reproducible high-performance liquid chromatography method was developed for the determination of Resveratrol (*trans*-3, 4′,5-trihydroxystilbene) in human plasma using liquid-liquid extraction. Caffeine was employed as an internal standard (IS). However, little information is known about its distribution in the organism generally because of the lack of accurate and precise detection methods. The chromatographic separation was achieved on a Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) at room temperature in isocratic mode, and the column effluent was monitored by UV detector at 306 nm. The mobile phase used was methanol: phosphate buffer (pH 6.8 adjusted with 0.5% (v/v) orthophosphoric acid solution in Milli-Q water) (63:37%, v/v) at a flow rate of 1.0 ml/min. Nominal retention times of *trans*-resveratrol and IS were 3.94 and 7.86 minutes, respectively. Limits of detection and Limits of quantification of *trans*-resveratrol were 0.006 μg/ml and 0.008 μg/ml, respectively. This method was linear over the range of 0.010 to 6.4 μg/ml with regression coefficient greater than 0.9998. The inter- and intra-day precisions in the samples, 0.010, 3.2 and 6.4 μg/ml of *trans*-resveratrol was in the range 0.63 to 2.12% relative standard deviation (RSD) and 0.46 to 1.02% RSD, respectively. Resveratrol was found to be stable for a period of 15 days on storage at -20°C. The method was found to be precise, accurate, and specific during the study.

Key words: Caffeine, high-performance liquid chromatography, human plasma, *trans*-resveratrol

INTRODUCTION

In recent years, there has been a budding interest in *trans*-resveratrol (3, 4′,5-trihydroxystilbene), a phytochemical occurring naturally in high to moderate quantities in various foods including grapes, peanuts, and wine.[1] The structure of *trans*-resveratrol is shown in Figure 1. *Trans*-resveratrol holds a broad range of pharmacological properties without harmful effects and is well known for its antioxidant, anti-inflammatory, analgesic, cardioprotective, neuroprotective, anti-aging, and anticancer activities.[2] It inhibits the oxidation of low-density lipoprotein and platelet aggregation,[3] and protects isolated rat hearts from

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Access this article online

Quick Response Code:

Website: www.japtr.org

DOI: 10.4103/2231-4040.97296

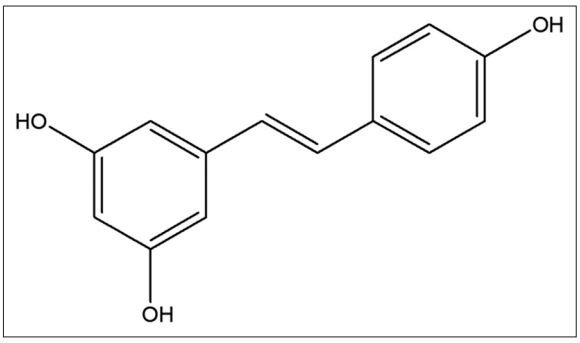

Figure 1: The chemical structure of *trans*-resveratrol

ischemia-reperfusion injury. The so-called French paradox stimulated the interest in resveratrol in wine.[4] Resveratrol subsists as *trans* and *cis* isomers. The *trans* isomer exists in fruits and plants, but in red wines a small amount of *cis* isomer has been detected and it is supposed that the *cis* isomer is derived by isomerization from the *trans* isomer during the fermentation of grapes.[5]

Several methods have been presented in the literature to determine the concentration of *trans*-resveratrol in wines and very few high-performance liquid chromatography (HPLC) methods are reported for *trans*-resveratrol in spiked human plasma. But, drawbacks of these methods are cost effective and time consuming and may cause *trans*-resveratrol to change to its *cis* form. In addition, these methods lack sensitivity, with the detection limits always in μM range.[6-14]

The main objective of this work was to develop a simple with adequate sensitivity, selectivity, precision, and accuracy for the determination of *trans*-resveratrol in human plasma in a comparatively short time with high linearity. The HPLC method can abet in the measurement of *trans*-resveratrol in routine monitoring, if indispensable, in common laboratories. The method offers the advantage in terms of time consuming, cost saving, and robustness. The second objective is to validate the method as per the International Conference on Harmonisation guidelines (ICH) [Q2 (R1)].

MATERIALS AND METHODS

Chemicals and Reagents

trans-resveratrol was kindly donated by Sami Labs (Bangalore, India). Caffeine was supplied by Himedia (Mumbai, India). Purity was found to be more than 99% for both the compounds. Methanol was purchased from Finar Chemicals, Ahmedabad (Gujarat, India) and orthophosphoric acid was purchased from Merck (Mumbai, India). All these solvents were HPLC grade. Other chemicals used were HPLC and analytical grade and obtained from Himedia (Mumbai, India). Water used for the preparation of aqueous mobile phase in all experiments was passed through a Milli-Q water purification system supplied by Millipore (Bangalore, Karnataka, India) filtered through a 0.22 μm filter. The human blood was obtained from the Unique Blood Bank Center, Bangalore, India.

Special Precautions

All laboratory procedures involving the manipulations of *trans*-resveratrol were performed in dim light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form and were stored at room temperature, protected from light.

Instrumentation

A gradient high-performance liquid chromatograph from Shimadzu (Japan), HPLC Class VP series with two LC-10ATVP pumps, SPD-10AVP variable wavelength programmable UV-vis detector, SCL-10AVP system controller and Shimadzu Class VP version 6.12 SP2 data station system was used. Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) equipped with a guard column (4 mm × 3 mm × 5 μm) (Torrance, CA, USA) was used for the present analysis.

Separation of Plasma from Human Blood

Human blood was taken in 3 ml heparinized eppendorf tubes and centrifuged at 10, 000 rpm for 10 minutes using cold centrifuge (Remi Model TC 650 D) to extract the plasma. The plasma was stored in a deep freezer at −4°C until analysis.

Preparing the Standard Master Stock Solution

Individual clear stock solutions of *trans*-resveratrol and internal standard (IS) were prepared at 1 mg/ml concentration. Standard solutions of *trans*-resveratrol were prepared in mobile phase in which the concentration of *trans*-resveratrol was known. For *trans*-resveratrol, 10 mg powder was accurately weighed, dissolved in mobile phase, and volume adjusted up to 100 ml with mobile phase. IS, caffeine was prepared in a 100 ml volumetric flask by dissolving 10 mg powder in methanol to get a concentration of 10 μg/ml. All stock solutions were stored away from light at approximately 4°C and used within seven days.

Preparing the Control Plasma Samples

Preparation of plasma samples 0.010 to 6.4 μg/ml *trans*-resveratrol and 2.0 μg/ml caffeine IS were prepared in 10 ml volumetric flasks by spiking pooled drug-free plasma with known amounts of stock solutions, aliquoted and stored at −20°C.

Optimization of Plasma Extraction Method

To develop an efficient and reproducible extraction method from human plasma for *trans*-resveratrol, various solvents *viz*., acetonitrile, methanol, and acetone were used for extraction. Different compositions of solvents were attempted but no momentous result observed. The application of protein precipitation procedure has been exposed to be good choice in the extraction of *trans*-resveratrol. Acetonitrile as precipitating agent gave excellent recovery and reproducibility from human blood plasma for bioanalytical method development of *trans*-resveratrol and IS.[15]

Plasma Sample Extraction and Processing

To 100 μl of drug-free plasma was spiked with *trans*-resveratrol and IS. 20 μl of the caffeine solution was added and 50 μl of the trans-resveratrol solution was added to the drug-spiked plasma. Final concentration of IS was 2.0 μg/

ml. After the addition of 200 µl of acetonitrile as precipitating agent to the drug-spiked plasma, the solutions were vortex-mixed for 1 minute followed by centrifugation at 10, 000 rpm for 10 minutes using a cold centrifuge Remi Model TC 650 D and organic layer transferred to heparinized eppendorf tubes. The organic layer (100 µl) was separated and 50 µl aliquot was injected onto the chromatographic system for analysis.

Chromatographic Conditions

The samples were chromatographed on a Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) column. The mobile phase used was methanol: phosphate buffer (pH 6.8 adjusted with 0.5% (v/v) orthophosphoric acid solution in Milli-Q water) (63:37%, v/v) filtered through a 0.22 µm nylon membrane and ultrasonically degassed prior to use. The mobile phase was delivered at a flow rate of 1.0 ml/min. The injection volume was 50 µl. The eluate was monitored by an ultraviolet detector set at 306 nm. The maximal absorption for *trans*-resveratrol and the same wavelength were found adequate for monitoring the IS. The temperature used for HPLC was ambient.

Calibration Curves

Calibration curves were attained by plotting the peak area ratio of *trans*-resveratrol:IS against the nominal concentration of calibration standards. The concentrations of *trans*-resveratrol used were 0.010-6.40 µg/ml, respectively.

Validation of the Developed Method

All the validation studies were carried out as per ICH guidelines [Q2 (R1)] by six consecutive replicate injections of the sample and standard solutions. Selectivity is the ability of the analytical method to differentiate and quantify the analyte in the presence of other expected components in the sample. Sensitivity was determined by analyzing control human plasma in replicates ($n = 6$) spiked with the analyte at the lowest level of the calibration standard, that is 0.010 µg/ml.

Accuracy and precision of the quality control (QC) samples were calculated using the calibration curve. Assay precision was assessed by expressing the standard deviation of the measurements as a percentage of the average value. The accuracy was estimated for each spiked control by comparing the nominal concentration with the assayed concentration.

Extraction Recovery

The recovery of *trans*-resveratrol and IS were determined. The recovery of *trans*-resveratrol was determined at concentration of 0.010, 3.2 and 6.4 µg/ml and for IS was determined at a concentration of 2.00 µg/ml. Six replicates at each concentration level with peak area response from non-extracted control samples prepared at the same concentration level were prepared and injected into the HPLC system.

Precision and Accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing *trans*-resveratrol at three different levels, i.e., 0.010, 3.2 and 6.4 µg/ml. The inter-assay precision was determined by analyzing the three levels on six different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and precision within 15% relative standard deviation (RSD).[16,17] For intra-day, accuracy and precision at each concentration were assayed on the same day. The inter-day accuracy and precision were evaluated for three subsequent days.

Limit of Detection and Limit of Quantification

The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were determined at 3.3 and 10 times the baseline noise, respectively.[18]

Ruggedness

From the stock solution, sample solutions of *trans*-resveratrol (0.010 µg/ml, 2 µg/ml, 4 µg ml and 6.4 µg/ml) were equipped and analyzed by two different analysts employing analogous operational and environmental surroundings. The peak area was calculated for identical concentration solutions six times.

Stability experiments

The stability of *trans*-resveratrol in solution as well as plasma matrix was evaluated. The stock solution stability was evaluated at room temperature for 10 hours for 15 days and these were compared with freshly prepared stock solution. The stability of *trans*-resveratrol and IS in the injection solvent was determined intermittently by injecting replicate preparations of processed samples for up to 4 hours after the initial injection. Repeated freeze-thaw cycles were assessed using human samples spiked with *trans*-resveratrol. The samples were stored at –20°C between freeze-thaw cycles. The samples were thawed by allowing them to stand at room temperature for 30 minutes. The samples were then kept in the freezer following drawing out the required volume. The stability of resveratrol isomers was assessed after three freeze-thaw cycles. The samples were treated using the similar procedure as described in the sample preparation section.[19,20]

RESULTS AND DISCUSSION

High-performance Liquid Chromatography Method Development and Optimization

Column chemistry, solvent type, solvent strength, detection wavelength, and flow rate were varied to determine the chromatographic conditions giving the best separation. The mobile phase conditions were optimized so that the components were not interfered from the solvents and excipients.

After trying different column, the final choice was the reversed phase Phenomenex C18 column of stationary phase giving satisfactory resolution and run time. Mobile phase and flow rate selection was based on peak parameters *viz.*, height, area, tailing, theoretical plates, capacity factor and resolution.

Method Validation

Validation

Short elution time, good separation between *trans*-resveratrol and IS, and baselines with low background were accomplished by using a reversed phase Phenomenex C18 column with low carbon load. C18 analytical columns with different carbon loads showed different selectivity. Reducing the pH of phosphate buffer and alter the ratio of organic to aqueous phase reduced the retention times of *trans*-resveratrol and IS. Finally, a higher pH buffer with gradient elution was found to give the best results. Reducing the pH further gave grubby extracts. Though the drugs and IS have λ-max at same wavelengths, 306 nm was selected as both the molecules had good absorption at this wavelength and interference from endogenous substance was also relatively less.

The proposed method is suitable for quantification of *trans*-resveratrol and IS in human plasma samples. It showed specificity, since I.S. and drug were well resolved and no interfering peaks from endogenous components of normal plasma were observed, as can be seen from Figure 2.

Linearity, Limit of Detection, and Limit of Quantification

The peak area ratios of drug to IS for the calibration standards were proportional to the concentration of each drug in plasma over the range tested. The calibration curves were linear over the range of 0.010 to 6.4 μg/ml resulted in the regression equation $y = 0.1732x + 0.0203$ ($r^2 > 0.9998$). The LOD and LOQ of the calibration graph were 0.006 μg/ml and 0.008 μg/ml.

Selectivity

The selectivity was studied by analyzing blank plasma samples. The chromatogram of blank plasma [Figure 2] did not show any interfering compound. A typical chromatogram of a drug-free plasma sample spiked with *trans*-resveratrol and IS is shown in Figure 3. The retention times of *trans*-resveratrol and IS were 3.94 and 7.86 minutes, respectively.

In the chosen chromatographic conditions, no interfering endogenous compound peak was observed at the retention time of peaks of interest as evaluated by chromatograms of blank human plasma and plasma spiked with resveratrol and IS. Both *trans*-resveratrol and IS were well separated with retention times of 3.94 and 7.68 minutes, respectively.

Extraction Recovery

The absolute recovery of *trans*-resveratrol from plasma was calculated by comparing the peak area obtained from extracts of spiked plasma samples and the peak area obtained from the direct injection of known amounts of standard solutions of *trans*-resveratrol. The overall extraction yields of 0.010, 3.2, and 6.4 μg/ml *trans*-resveratrol in plasma were 93 to 98% [Table 1]. The study illustrated that at least 10minutes of centrifugation at 10, 000 rpm was needed for protein denaturization completely when 100 μl acetonitrile were added to 50 μl plasma sample.

Precision and Accuracy

The precision and accuracy data for the analytical procedures are shown in Table 2. Intra-day and inter-day precision (%R.S.D.) of the methods were lower than 10% and were within the acceptable limits to meet the guidelines for United States Pharmacopeial norms method validation which is considered to be within 15% (RSD).[21-23] The accuracy of both the methods was also good with the deviation between the nominal concentration and calculated concentration for *trans*-resveratrol well below the limits of ±15%. Precision and accuracy data indicated that the methods to extract *trans*-resveratrol from plasma and tissues are highly reproducible and robust. The values of precision and accuracy were acceptable in view of the international recommendation that the precision and accuracy should not exceed 15%.[24-26]

Ruggedness

When the method was performed by two different

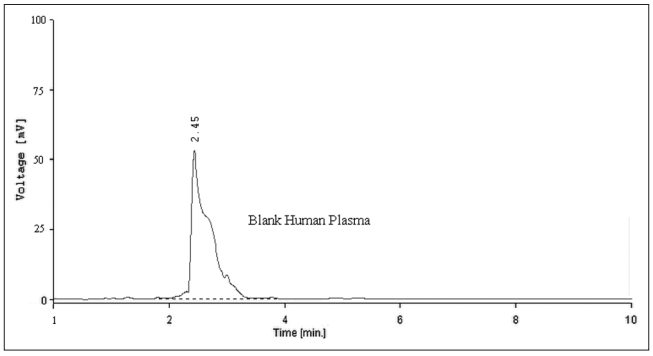

Figure 2: HPLC chromatograms of control blank Human plasma

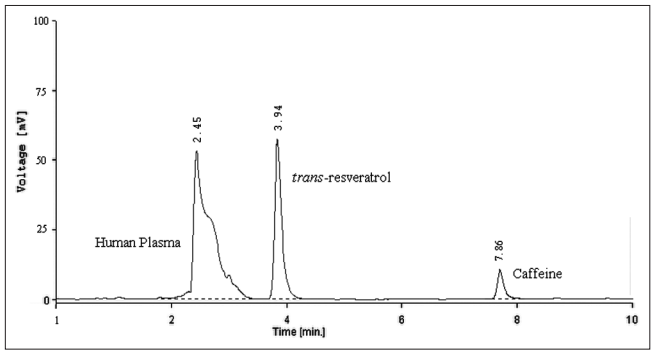

Figure 3: HPLC chromatograms of human plasma spiked with *trans*-resveratrol and caffeine

analysts under the same experimental and environmental conditions, it was found to be rugged. The contents of the drug were not adversely affected by these changes as evident from the low values of % RSD (2%), indicating ruggedness of the method as shown in Table 3. %RSD values of less than 2% were acquired for repetitive measurements and operators.

Stability

trans-resveratrol was shown to be stable in frozen plasma at –20°C for at least three freeze-thaw cycles and was found to be stable when stored at –20°C for at least 10 days. After storage for 10 days, *trans*-resveratrol remained unchanged, based on peak areas in comparison with freshly prepared solution of *trans*- resveratrol [Table 4].

CONCLUSION

A simple and sensitive method for the determination of *trans*-resveratrol, a novel antioxidant, in spiked human plasma by HPLC was developed and validated. Protein precipitation method was employed for sample preparation followed by chromatographic separation and UV detection. No interfering peaks were observed at the elution times of *trans*-resveratrol and IS. Adequate specificity, precision, and accuracy of the proposed method were demonstrated over the concentration range of 0.010 to 6.4 μg/ml. The method was accurate, reproducible, specific, and provided excellent separation and enable the quantification of *trans*-resveratrol in human plasma. The small volume of plasma required, the simplicity of separation procedure, and the short run time

Table 1: Recovery of *trans*-resveratrol and Internal standard from spiked human plasma samples (*n* = 6)

Plasma concentration of *trans*-resveratrol (μg/ml)	Recovery of extraction (%) (mean ± S.D., *n* = 6)	Plasma concentration of IS (μg/ml)	Recovery of extraction (%) (mean ± S.D., *n* = 6)
0.010	96.51 ± 4.63		97.42 ± 2.15
3.2	93.80 ± 3.22	2.00	92.28 ± 1.27
6.4	98.11 ± 1.47		94.89 ± 3.08

Table 2: Intra- and inter-day precision and accuracy determination of *trans*-resveratrol concentration in spiked human plasma samples (*n* = 6 at each concentration for intra-day and *n* = 6 days for inter-day precision)

Spiked concentration (μg/ml)	Measured concentration (mean ± S.D)		Precision (%RSD)		Accuracy (relative error, %)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
0.010	0.0098 ± 0.0001	0.0094 ± 0.0002	1.02	2.12	–2.0	–6.0
3.2	3.01 ± 0.014	2.99 ± 0.019	0.46	0.63	–5.93	–6.56
6.4	6.23 ± 0.045	6.01 ± 0.086	0.72	1.43	–2.65	–6.09

%RSD = 100% x (SD/mean); Relative error r = 100% (measured concentration – spiked concentration)/spiked concentration, RSD - Relative standard deviation

Table 3: Values of ruggedness studies of the developed method

Drug	Concentration μg/ml	Analyst one amount Found% ± SD (*n* = 6)	%RSD	Analyst two amount found% ± SD (*n* = 6)	%RSD
Trans-resveratrol	0.010	0.0095 ± 0.0001	1.05	0.0092 ± 0.0001	1.08
	2.00	1.987 ± 0.011	0.55	1.976 ± 0.018	0.91
	4.00	3.874 ± 0.028	0.72	3.952 ± 0.009	0.22
	6.00	5.921 ± 0.064	1.08	5.851± 0.027	0.46

RSD - Relative standard deviation

Table 4: Stability studies of *trans*-resveratrol in human plasma

Spiked concentration (μg/ml)	Stability	(mean ± S.D), *n* = 6	Accuracy (%)
0.010	0hr	0.010 ± 0.003	
	3F/T cycles	0.0095 ± 0.001	95
	10 days at–20°C	0.0089 ± 0.001	89
3.2	0hr	3.2 ± 0.05	
	3F/T cycles	2.97 ± 0.03	92.81
	10 days at–20°C	2.85 ±0.06	89.06
6.4	0hr	6.4 ± 0.23	
	3F/T cycles	6.28 ± 0.31	98.12
	10 days at–20°C	6.03 ± 0.46	94.21

F/T, freeze–thaw, Accuracy (%) = Mean assayed concentration/mean assayed concentration at 0 h × 100%.

construct this method suitable for rapid and regular analysis.

ACKNOWLEDGEMENTS

Authors are thankful to Prof. B.G. Shivananda, Principal, Al-Ameen College of Pharmacy, Bangalore, for his kind support throughout this research work. Authors are also grateful to Dr. Pritee Paliwal, Sr. Manager-Technical Support, Sami Labs, Bangalore, India for providing the gift sample of *trans*-resveratrol.

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How to cite this article: Singh G, Pai RS, Pandit V. Development and validation of a HPLC method for the determination of *trans-resveratrol* in spiked human plasma. J Adv Pharm Tech Res 2012;3:130-5.

Source of Support: Nil, **Conflict of Interest:** Nil.