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Determination of *trans*-Resveratrol in Plasma by HPLC

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***trans*-Resveratrol (*trans*-3,5,4′-trihydroxystilbene), a phenolic compound present in grapes, wines, and peanuts, has been reported to have health benefits including anticarcinogenic effects and protection against cardiovascular diseases. Despite its importance, little is known about its bioavailability in both humans and animals. A fundamental step for this evaluation consisted in measuring this stilbene in blood. In the present work, a simple and rapid HPLC method with diode array-UV detection has been developed. Resveratrol contained in plasma was purified by solid-phase extraction using a C_{18} cartridge. The sample was rinsed with water and methanol−water (25:75 v/v), and *trans*-resveratrol was finally eluted with methanol. The collected fraction was evaporated under nitrogen and analyzed by HPLC. The method was validated by obtaining a linear correlation, a detection limit of 20 μg/L, and a good precision with a coefficient of variation of 2.85%. *trans*-Resveratrol administered orally to rats was detected in plasma. With this procedure, excellent separation of *trans*-resveratrol is achieved, thus allowing a rapid analysis of the sample for absorption, distribution, and metabolism studies.**

In developed countries, one out of every four people will eventually suffer cancer, claiming over 6 millions lives each year worldwide. *trans*-Resveratrol (3,5,4′-trihydroxistilbene) (Figure 1) has been reported to be a cancer chemopreventive agent inhibiting cellular events associated with tumor initiation, promotion, and progression,[1] acting through a dose-dependent inhibition of free-radical formation, inhibition of cyclooxygenase, and anticarcinogenic effects on mouse mammary cultures. More recently, Mgbonyeby[2] confirmed that this compound is a potential chemopreventive agent for breast cancer. Furthermore, it has been reported that *trans*-resveratrol inhibits platelet aggregation, alters eicosanoid synthesis, and modulates lipoprotein metabolism.[3,4,5]

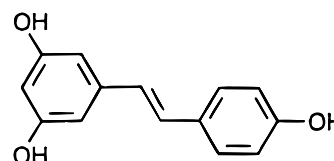

Figure 1. Chemical structure of *trans*-resveratrol.

The dried roots of *Polygonum cuspidatum* used in Asian folk medicine for treating lipid disorders and atherosclerosis[6] contain resveratrol and its glucosides, as their main active components. However, red wine seems to be the main dietary source of this compound, because it is present in much higher levels in red[7] than in rosé and white wines.[8]

Little is known about the absorption, distribution, and metabolism of *trans*-resveratrol in humans and in animals. The first step in *in vivo* physiological and pharmacokinetic studies is to develop a method to measure *trans*-resveratrol in plasma.

The present study describes a method to determine *trans*-resveratrol in human plasma by applying HPLC analysis with diode array detection. The preparative approach to the plasma extraction by means of a C_{18} cartridge allows a sensitive and rapid determination of this stilbene in a biological fluid. To assess the performance of this method *in vivo*, resveratrol was administered orally to experimental animals and it was detected in plasma, thus providing evidence of the absorption of *trans*-resveratrol.

EXPERIMENTAL SECTION

Instruments. A UV−visible recording spectrophotometer Shimadzu UV-160A (Kyoto, Japan) was used in the preliminary

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steps of the analysis. A 1050 Hewlett-Packard (HP) (Palo Alto, CA) gradient liquid chromatograph equipped with an automatic injector, a Nucleosil (Tracer) C_{18} 120 (25 × 0.4 cm, 5 μm) reversed-phase column with a precolumn of the same material, at 40 °C, and a HP 1050M diode array UV–visible detector, coupled to a HP Chem Station was used to determine *trans*-resveratrol.

Reagents. *trans*-Resveratrol was provided by PharmaScience (Montreal, Canada). Methanol and acetonitrile were obtained from SDS (Barberà del Vallès, Spain), and glacial acetic acid came from Panreac (Montcada i Reixac, Spain). All solvents were HPLC grade. Millipore (Milli-Q Plus) ultrapure water, filtered through a 0.22 μm filter, was used in all experiments.

Preparation of Plasma Samples. Blood samples from fasted healthy individuals were collected by venipuncture into blood collection tubes containing EDTA-K_3 (Blood Bank, Hospital Clínic i Provincial de Barcelona, Spain). Blood was centrifuged at 2700 rpm (Beckman, model TJ-6 centrifuge, rotor TH-4 with buckets), at 4 °C for 15 min, and the plasma was immediately removed from the cells and mixed with known amounts of stock solution of *trans*-resveratrol and stirred for 1 min.

Solid-Phase Extraction (SPE). *trans*-Resveratrol was extracted from plasma in a reversed-phase C_{18} Sep-Pak Classic Cartridge for manual operation (WAT051910, Waters, Milford, MA). To avoid changes in the separation efficiency due to variations in the flow rate, this variable was carefully controlled. The cartridge was conditioned at a flow rate of 4–5 mL/min, and the analyte was loaded, washed, and eluted at 1.5–2 mL/min.

The column was conditioned prior to use with 4 mL of methanol, followed by 10 mL of water. Plasma (1 mL) was slowly loaded onto the Sep-Pak, and the cartridge was rinsed with 10 mL of water and then 3 mL of 25% (v/v) methanol. The adsorbed fraction containing *trans*-resveratrol was then eluted with methanol and collected in a tube. The eluted fraction was evaporated under a nitrogen stream and redissolved in water to a final volume of 0.5 mL. Finally, it was passed through a Whatman inorganic membrane filter, Anotop 10 Plus, 0.2 μm (Maidstone, England), into a sealed amber vial. Sample preparation was performed in dim light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form.

HPLC Procedure. *trans*-Resveratrol in the eluted fraction was determined using a Nucleosil reversed-phase column C_{18}, 120 (25 × 0,4 cm) with a 5 μm particle size and a precolumn of the same material as the stationary phase. The injection volume was 100 μL. The temperature of the column oven was 40 °C. The mobile phase included solvent A, consisting of glacial acetic acid in water, pH 2.0, and solvent B, 20% phase A/80% acetonitrile. The flow rate was 1.5 mL/min.

Separation was effected with gradient elution, starting at 0 min with 78% solvent A (22% solvent B) up to 10 min, decreasing to 0% solvent A (100% solvent B) at 13 min, and followed by washing and reconditioning the column.

The chromatogram was recorded according to the retention time. It was monitored at 306 nm, where the absorbance of *trans*-resveratrol presents its maximum. *trans*-Resveratrol was identified by spectroscopic analysis with diode array-UV detection from 220 to 400 nm. The results of the analysis are expressed in milligrams of *trans*-resveratrol per liter.

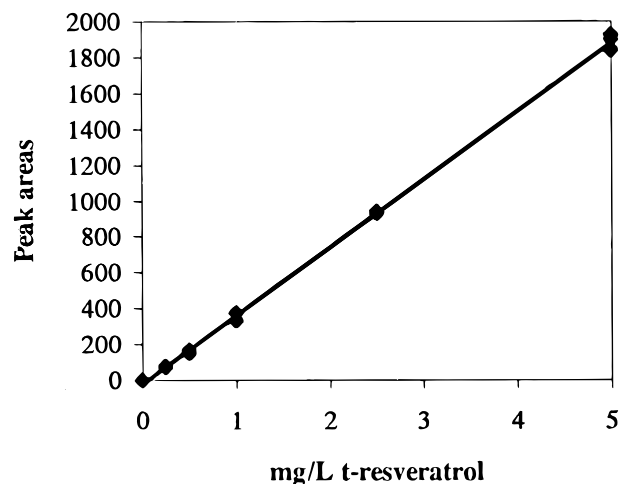

Figure 2. Calibration curve of *trans*-resveratrol in plasma. Individual values were represented. Regression line was calculated by the least-squares method, and the equation and correlation coefficient (r) was $y = 381.73x - 44.93$; $r = 0.9985$.

Calibration Curve. A stock solution of 200 mg/L *trans*-resveratrol dissolved in 20% (v/v) methanol was stored in an amber bottle, at room temperature in the dark. The calibration curve was obtained with pooled human plasma spiked with known amounts of the stock solution: 0.25, 0.50, 1.00, 2.50, and 5.00 mg/L. The analyte was extracted by following the protocol described above using solid-phase extraction. The calibration curve was obtained by plotting the peak area of each compound against the concentrations of the analyte and performing a linear regression. The calibration curve is shown in Figure 2.

Animal Studies. *trans*-Resveratrol was administered orally to male Sprague–Dawley rats (200–250 g) at 2 mg/kg, and the animals were killed at 15, 30, and 45 min after administration. Animal treatment was in full accordance with the European Community Guidelines for the care and management of laboratory animals. Blood was taken by cardiac puncture and transferred to a tube containing EDTA-K_3 as anticoagulant. Plasma was obtained by centrifugation, and the analyte was extracted using the protocol described above.

RESULTS AND DISCUSSION

Optimization of Solid-Phase Extraction. The effect of experimental variables was evaluated to develop a rapid, specific, and precise method for measuring *trans*-resveratrol in plasma.

In the preliminary analysis, three different solvents generally used in reversed-phase SPE were assessed for the rinsing and the elution steps: acetonitrile, ethyl acetate, and methanol. Methanol was selected because it gave the best recovery and resolution chromatogram. *trans*-Resveratrol eluted with acidified 1 M methanol gave the same peak areas as those obtained with methanol.

The optimal amount of water and the percentage of methanol in water that was sufficient to clean the sample without eluting the analyte were evaluated. Therefore, the washout fractions were monitored with the UV–visible spectrophotometer from 250 to 420 nm. The optimal volume of water for the first rinsing step was studied over a range of 5–15 mL and found to be 10 mL.

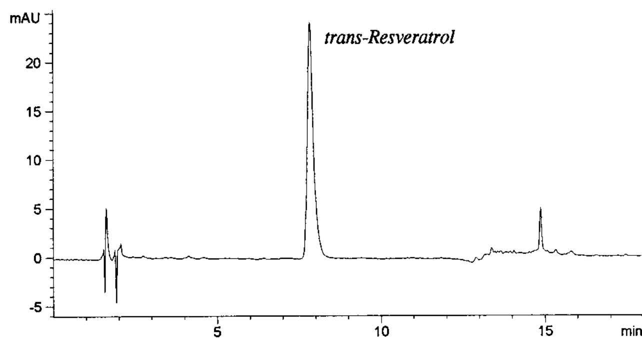

Figure 3. HPLC chromatogram at 306 nm of *trans*-resveratrol in plasma, after purification.

Different amounts of methanol in water were assessed for the second rinsing step: 5, 10, 15, 20, 25, and 30%. A concentration of 25% methanol proved to be the optimal solution to eliminate all possible polar interferences of plasma without eluting *trans*-resveratrol.

To improve the sensitivity of the analytical method and to confirm the detection of low amounts of *trans*-resveratrol, the eluted volume was concentrated under nitrogen and redissolved with water to a final volume of 0.5 mL.

Moreover, a cartridge was loaded with a known amount of the stock solution of *trans*-resveratrol diluted in water, to confirm that no analyte was lost in any step of the procedure, and all the fractions obtained after the two rinsing and the elution steps were injected in the HPLC. The analyte was completely recovered when 100% methanol was loaded in the cartridge, and no *trans*-resveratrol was detected in the washout fractions.

Method Validation. The method was validated according to *The United States Pharmacopoeia*.[9] The analytical performance parameters considered were precision, linearity, selectivity (specificity), sensitivity, and recovery.

Precision. The precision of the analytical method was determined by analyzing six aliquots of a homogeneous sample of plasma spiked with *trans*-resveratrol. Peak areas were considered for the determination. The precision, expressed as the coefficient of variation (CV), was 2.82%. This value was lower than those acceptable according to the CV established by Horwitz for intralaboratory analysis.[10]

Linearity. Calibration curves of *trans*-resveratrol were prepared to determine the linearity of the method. Integrated peak areas were plotted against analyte concentration, and linear regression was performed by the least-squares method. The calibration curves generated using plasma as matrix (Figure 2) showed a correlation coefficient of 0.9985.

Selectivity. To improve selectivity, *trans*-resveratrol was quantified at its maximum absorbance, which is 306 nm. The chromatogram at this wavelength shown in Figure 3 indicated that *trans*-resveratrol is well resolved and free from interference peaks. The use of a diode array detector allowed the confirmation of the identity of the chromatographic peak not only by its retention time but also by its spectrum (Figure 4).

Sensitivity. The limit of detection (LD) and the limit of quantification (LQ) were calculated by measuring the magnitude of the analytical background response, running six blanks using the maximum sensitivity allowed by the system, and calculating the standard deviation (SD) of this response. LD was estimated multiplying the SD by 3, while LQ was considered to be 10 times the SD. At 306 nm, the LD for *trans*-resveratrol was 7 μg/L and the LQ was 20 μg/L. The LQ was subsequently validated by the analysis of six standards at the LQ concentration.

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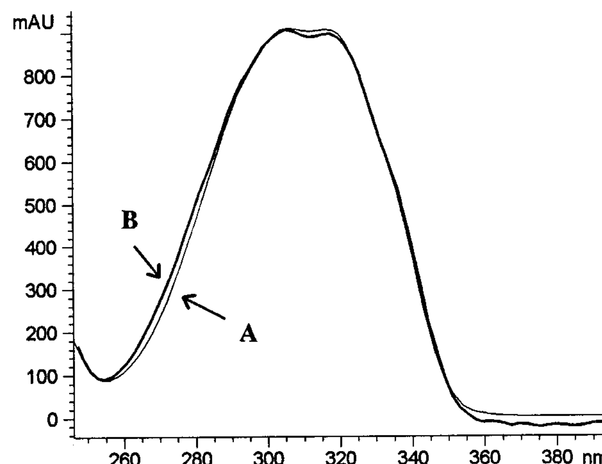

Figure 4. UV spectra of standard *trans*-resveratrol (A) and *trans*-resveratrol in plasma after purification (B) obtained by diode array detection.

To evaluate recovery, *trans*-resveratrol was added to plasma in the 0.25−2.5 mg/L concentration range. The samples were deproteinized and prepared for HPLC analysis as described in the Experimental Section, with an average recovery of 99%.

In contrast to the number of methods published to determine *trans*-resveratrol in natural products, there are few studies related to biological samples. Recently, the first assay to measure resveratrol in plasma, cells and low-density lipoproteins (LDL) after incubations in vitro was reported.[11] This method includes organic-phase extraction and derivatization prior to analysis by gas chromatography.

The method here described is suitable for analyzing plasma samples. It has the advantage that time-consuming procedures such as liquid−liquid extractions are not necessary. The preparation of the sample prior to HPLC injection requires 20 min. The subsequent HPLC run takes 18 min, and thanks to the automatic sample injector, up to 60 samples can be analyzed by HPLC every 24 h, including time for the reequilibration of the column. Linearity, precision, sensitivity, and recovery were highly satisfactory.

To assess the validity of the method, rats were administered with *trans*-resveratrol (2 mg/kg). Blood taken by cardiac puncture was analyzed as described, and the stilbene was detected in plasma and quantified, resulting in a concentration of 0.175 mg/L at 15 min after administration.

Until now, no data have been published concerning absorption, bioavailability, and tissue distribution of *trans*-resveratrol. Therefore, the physiological significance of this compound remains unclear. We have described a simple method to determine *trans*-resveratrol in plasma, and we have also detected for the first time

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the absorption of this stilbene by the intestine of rat and its presence in blood. Further experiments should be carried out in order to establish which circulating levels of this compound have to be reached in plasma to reproduce the *in vitro* chemoprotective effects of *trans*-resveratrol.

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