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ARTICLE

Determination of Dihydroresveratrol in Rat Plasma by HPLC

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Dihydroresveratrol is a metabolite of *trans*-resveratrol formed in the intestine by the hydrogenation of the double bond by microflora. The aim of the present study was to validate a method to measure dihydroresveratrol in rat plasma and then to quantify its plasmatic concentration after the oral administration of 60 mg/kg to Sprague–Dawley rats. Dihydroresveratrol was extracted from acidified plasma with a C18 cartridge, eluted with methanol, and concentrated prior to HPLC analysis with diode-array detection (HPLC-DAD) at 276 nm. The method was validated by spiking blank plasma samples with pure dihydroresveratrol, obtaining a linear correlation and good interday and intraday precisions, expressed as coefficient of variation (<7%). The average recovery was 96.7% and the limit of detection was 275 nM. The oral administration of dihydroresveratrol to rats and its subsequent detection, along with dihydroresveratrol glucuronide and sulfate, provides evidence of its absorption and metabolism.

KEYWORDS: Dihydroresveratrol; HPLC-DAD; method validation; rat plasma; resveratrol

INTRODUCTION

Dihydroresveratrol (*trans*-3,5,4′-trihydroxybibenzyl) (**Figure 1**) belongs to the family of stilbenoids, which are a class of plant secondary metabolites produced in a number of unrelated species. Dihydroresveratrol has been identified as a phytoalexin in *Orchidaceae* (*1*), in *Cannabis sativa* L. (*2*), and in the tuber of *Dioscorea dumetorum*, where its production seems to be elicited by fungal infection (*3*). In addition, this phytochemical has been found in *Maackia amurensis*, a major component of the Russian hepatoprotective preparation Maksar (*4*). The functions of stilbenoids in plants include constitutive and inducible defense mechanisms, plant growth inhibitors, and dormancy factors (*5*).

Dihydroresveratrol was also detected in humans as a metabolite of *trans*-resveratrol (*6*), a natural antioxidant with beneficial effects on health (*7*). After the oral administration of 25 mg of ^{14}C-resveratrol, Walle et al. (2004) identified in urine two resveratrol monoglucuronides, two monosulfates, and interestingly, dihydroresveratrol glucuronide and sulfate (*6*). The latter may have been produced by the intestinal microflora, since it involved the hydrogenation of the aliphatic double bond of *trans*-resveratrol. Later, dihydroresveratrol and its sulfate conjugate were detected in rats (*8*). Recently, a study that evaluated the interactions of this polyphenol with animal-associated bacteria identified dihydroresveratrol as a metabolite produced by *Eggerthella lenta* and *Bacteroides uniformis*. Both of these microorganisms have been isolated from human fecal samples, thus supporting the involvement of intestinal flora in the synthesis of this metabolite (*9*). The information regarding the human health protecting properties of dihydroresveratrol is limited. This compound has been reported to have antiproliferative effects in human prostate cancer cells (*10*, *11*). Moreover, dihydroresveratrol is a potassium channel modulator (*12*), an antioxidant, and it inhibits DNA synthesis (*13*).

Because the reduction of the stilbenic double bond of *trans*-resveratrol appears to be produced by the intestinal microflora, dihydroresveratrol might be absorbed through the colonic epithelium to reach blood and finally be excreted in urine. The presence of dihydroresveratrol in plasma has yet to be reported, which could be attributed not only to the lack of accurate methods but also to the absence of a commercially available pure standard. Here we present a simple, accurate, and sensitive method for identifying and quantifying dihydroresveratrol in rat plasma by means of solid-phase extraction, followed by HPLC analysis with photodiode array detection. The performance of the method was assessed by oral administration of dihydroresveratrol to Sprague–Dawley rats and its measurement in plasma.

MATERIALS AND METHODS

Chemicals and Reagents. Dihydroresveratrol was provided by Biopharmalab SL (Alicante, Spain). Stock solutions of 1 mM dihydroresveratrol in ethanol 20% (v/v) were prepared weekly and stored at room temperature. Acetonitrile and methanol were purchased from J. T. Baker (Deventer, Netherlands) and acetic acid was provided by Scharlau Chemie SA (Barcelona, Spain). All solvents were HPLC grade. Ascorbic acid (ref A5960) was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals used were analytical grade and obtained from Sigma-Aldrich. Reversed-phase C_{18} Sep-Pak classic cartridge for manual operation (ref WAT051910) were purchased from Waters (Milford, MA). Water used in all experiments was passed through a Milli-Q water purification system (18 MΩ) (Millipore, Milan, Italy).

Instrumentation. A Concentrator 5301 (Eppendorf Iberica SL, San Sebastian de los Reyes, Spain) was used to evaporate methanol from samples. The determination of dihydroresveratrol and its metabolites was carried out using a gradient liquid chromatograph Agilent model 1100

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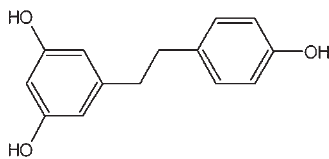

Figure 1. Chemical structure of dihydroresveratrol.

(Agilent Technologies, Palo Alto, CA) equipped with an automatic injector, a Synergi Fusion-RP 80A column (250 mm × 4.6 mm; 4 μm; Phenomenex, Torrance, CA) with a C18 guard column cartridge and a diode array UV−visible detector. Agilent Chemstation software controlled all the equipment and performed the data processing. An Applied Biosystems API 3000 triple quadrupole mass spectrometer (Applied Biosystems, PE Sciex, Concord, Ontario, Canada), equipped with a Turbo IonSpray source in the negative ion mode, was used to obtain the mass spectrometry.

Solid-Phase Extraction of Dihydroresveratrol from Plasma Samples. Blood was collected from Sprague−Dawley rats by cardiac puncture and was placed in tubes containing EDTA-K_3 as anticoagulant. Samples were centrifuged at 1500*g* for 15 min at 4 °C, and the plasma was immediately separated from cells. Plasma (200 μL) was acidified with 6 μL of acetic acid and stirred in a vortex for 2 min. Prior to use, the Sep-Pack cartridge used for the extraction was conditioned with 4 mL of methanol followed by equilibration with 10 mL of water. Then plasma was slowly loaded into the cartridge, followed by 5 mL of water. The dihydroresveratrol contained in the cartridge was eluted with 4 mL of methanol. Ascorbic acid (10 μL) at 15% were added to the eluate as an antioxidant, which was then evaporated at 45 °C to a final volume of 400 μL. Finally, it was placed in a sealed amber vial for HPLC analysis.

HPLC and HPLC-MS Analyses. The temperature of the column oven was set at 40 °C, and the injection volume was 100 μL. The mobile phase included solvent A consisting of an acetic acid solution (3%, v/v) and solvent B, which was a mixture of phase A:acetonitrile (20:80, v/v). The flow rate was 1.5 mL/min. The gradient elution was: min 0 with 22% solvent B to min 2; 2−6 min, linear gradient from 22 to 30% B; 6−14 min, linear from 30 to 50% B; 14−18 min, increasing to 60% B; 18−25 min, linear from 60 to 100% B; followed by washing and reconditioning the column.

The chromatograms were obtained at 276 nm at which the absorbance of dihydroresveratrol presents a maximum (**Figure 2**). The compound was identified using comparative retention times of pure standard and photodiode array spectra (from 200 to 400 nm). Quantification of dihydroresveratrol was performed using standard curves constructed after spiking relevant concentrations of this compound in blank plasma. As dihydroresveratrol metabolites were not available, their quantities were calculated based on the assumption that recovery characteristics and relationship between peak area ratios and concentrations were the same as those for dihydroresveratrol.

Dihydroresveratrol and its conjugates were identified by mass spectrometry. The ion spray voltage was 3500 V, with nitrogen as the nebulizer gas, 10 (arbitrary units), and curtain gas, 15 (arbitrary units). The detecting conditions were optimized with a standard solution of dihydroresveratrol in the presence of LC mobile phase, as follows: declustering potential, −70 V; focusing potential, −200 V; drying gas (N_2) heated to 400 °C and introduced at a flow rate of 5000 cm^3/min. Mass spectra were acquired in the 100−500 *m*/*z* range.

Method Validation. The solid-phase extraction (SPE) of dihydroresveratrol from rat plasma followed by HPLC analysis was validated according to *The United States Pharmacopoeia* (2008) (*14*). Blank rat plasma was obtained by cardiac puncture from rats that had not received dihydroresveratrol. Aliquots of the pooled plasma were stored at −20 °C until the analyses were performed, then 200 μL of plasma were spiked with known amounts of dihydroresveratrol and subsequently stirred in a vortex for 1 min before being extracted as indicated above.

Linearity. Spiked plasma samples containing increasing concentrations of dihydroresveratrol: 5, 10, 25, 50, 75, and 100 μM were analyzed according to the procedure described above. Integrated peak areas were plotted against analyte concentration, and linear regression was performed by the least-squares method.

Precision. The precision of the analytical method was determined by assaying a sufficient number of plasma samples (n = 4−6) at six different concentrations of dihydroresveratrol ranging from 5 to 100 μM and was expressed as relative standard deviation (RSD). The intraday precision was determined by analyzing the spiked samples prepared within a day, whereas the interday precision assessed spiked samples prepared on three different days. Peak areas were considered for the calculation of the concentration and to establish the precision.

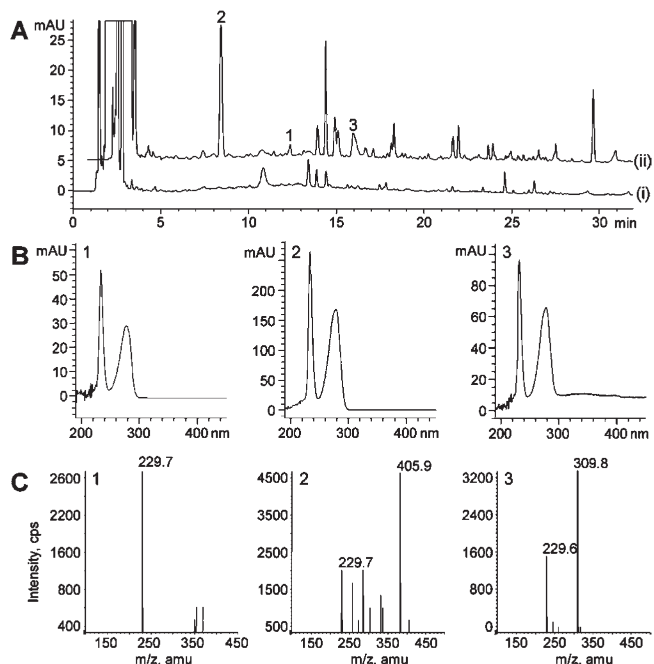

Figure 2. HPLC chromatograms and UV and mass spectra of dihydroresveratrol and its glucuronide and sulfate conjugates. (**A**) HPLC chromatogram at 276 nm of (i) blank plasma and (ii) plasma of rats administered with 60 mg/kg of dihydroresveratrol. Peaks of (1) dihydroresveratrol and its conjugates (2) glucuronide and (3) sulfate are indicated. (**B**) UV spectra obtained by diode-array detection of (1) dihydroresveratrol, (2) glucuronide and (3) sulfate. (**C**) Full-scan product ion mass spectra of (1) dihydroresveratrol, (2) glucuronide, and (3) sulfate.

Recovery. Recoveries of dihydroresveratrol from plasma were measured by spiking 200 μL of blank samples at final concentrations of 5, 10, 25, 50, 75, and 100 μM. Absolute recoveries were calculated by comparing the peak area ratio from spiked samples to those of the corresponding concentrations injected directly into the HPLC system without extraction.

Accuracy. First, 500 μL of plasma and 500 μL of water as control were spiked with dihydroresveratrol at concentrations of 10 and 50 μM. The samples were purified by SPE and analyzed by HPLC as described. Accuracy was calculated by comparing the values for the plasma samples and control: results are expressed as percentages of analyte recovered.

Sensitivity. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated by measuring the analytical background response, running six blanks of plasma using the maximum sensitivity allowed by the system. The signal-to-noise ratio was used to determine the LOD, and it was estimated as the concentration of dihydroresveratrol in plasma samples that generated a peak with an area at least 3 times higher than the baseline noise. LOQ was considered to be 10 times the standard deviation of the six blank samples analyzed using the maximum sensitivity allowed by the system. The LOQ was subsequently validated by the analysis of six plasma samples known to be near the LOQ.

Selectivity. The presence of interfering substances in blank plasma at the retention time of dihydroresveratrol as well as its glucuronide and sulfate conjugates was evaluated. The selectivity of the method was determined by comparing the chromatograms of blank plasma with the corresponding spiked plasma samples.

Animal Studies. Male Sprague−Dawley rats (200−250 g) were housed three per cage, in a temperature-controlled room, with a light−dark cycle of 12 h and free access to water and a commercial rat chow. Animal treatment was in full accordance with the European Community

Table 1. Precision, Accuracy, and Recovery of *trans*-Resveratrol in Spiked Rat Plasma Samples

dihydroresveratrol (μM)	precision (% RSD) intraday	interday	recovery (%)
5 (n = 6)	3.05	3.50	96.8 ± 5.9
10 (n = 6)	5.85	5.14	95.2 ± 6.9
25 (n = 6)	1.30	6.62	95.8 ± 2.2
50 (n = 6)	5.16	6.66	97.5 ± 5.6
75 (n = 6)	5.23	5.60	96.5 ± 6.7
100 (n = 6)	1.73	3.34	98.3 ± 2.7

Guidelines for the care and management of laboratory animals. Dihydroresveratrol dissolved in water was administered orally by gavage to overnight-fasted rats at a dose of 60 mg/kg, except for the control group, which was given only water. Rats were fasted overnight and anesthetized by intramuscular injection of 90 mg/kg ketamine (Imalgene 1000, Merial Laboratorios SA, Barcelona, Spain) and 10 mg/kg xylacine (Rompun 2%, Química Farmaceutica Bayer SA, Barcelona, Spain). Blood was withdrawn by cardiac puncture and transferred to a tube containing EDTA-K3 as anticoagulant, at 10 and 30 min after the administration. Immediately after obtaining the plasma by centrifugation at 1500g for 15 min at 4 °C, the analyte was purified using the SPE procedure described above.

Statistical Analysis. Data are reported as the mean ± SEM. A commercially available package (Prism version 4.02; GraphPad Software Inc., San Diego, CA) was used for all statistical tests. Data were evaluated by one-way ANOVA and post hoc Bonferroni's Multiple Comparison tests (Graph Pad Prism). A $p < 0.05$ level was taken as significant.

RESULTS

Method Validation. The analytical performance parameters assessed for the overall assay were linearity, precision, accuracy, sensitivity, and selectivity.

Linearity. The response was linear in the range of concentrations evaluated from 5 to 100 μM, giving an equation of $y = 8.75x - 1.26$ (n = 31) and a regression coefficient of 0.998.

Precision. Intraday and interday precision (% RSD) ranged from 1.30% to 6.66% (**Table 1**) and were within the acceptable limits to meet the guidelines for bioanalytical method validation, which is considered to be ≤20% (*15*). The precision data indicate that the analytical method is repeatable.

Recovery. The extraction recoveries of dihydroresveratrol were assayed in plasma samples at six different concentrations (**Table 1**). The mean recovery in plasma was 96.7 ± 5.0%, thus indicating the high extraction efficiency of this procedure.

Accuracy. The accuracy was determined for the overall assay by measuring the percentage of recovery after the addition of known amounts of standard to a control (water) and to a pool of blank plasma, at two different concentrations. The mean recoveries at 10 and 50 μM were 93.0 ± 2.1% and 98.9 ± 3.3%, respectively.

Sensitivity. The LOD was 275 nM on the basis of a signal-to-noise ratio of 3. The LOQ was 578 nM.

Selectivity. Dihydroresveratrol was well resolved and free from interference peaks. This compound was quantified at 276 nm, its maximum absorbance to improve selectivity, and the identity of the chromatographic peak was confirmed not only by its retention time but also by its spectrum (**Figure 2**).

Animal Studies. Dihydroresveratrol was orally administered to rats at a dose of 60 mg/kg, and blood was withdrawn at 5 and 30 min to assess the validity of the method. The representative HPLC profile (**Figure 2A**) was characterized not only by the presence of dihydroresveratrol but also by two more peaks with the same absorbance spectrum, which were identified by means of MS. The peak with retention time of 8.1 min gave a deprotonated molecular ion $[M - H]^-$ at m/z 405 (**Figure 2C**) with a dihydroresveratrol fragment at m/z 229, thus allowing its identification as dihydroresveratrol glucuronide. The second peak (retention time

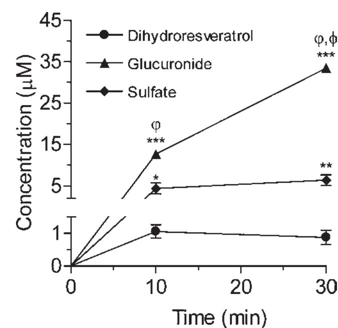

Figure 3. Plasmatic concentrations after the oral administration of 60 mg/kg of dihydroresveratrol. Data are presented as means ± SEM (n = 4), *p < 0.05, **p < 0.01, and ***p < 0.001, conjugates vs dihydroresveratrol. $^{\varphi}p$ < 0.001, glucuronide vs sulfate. $^{\Phi}p$ < 0.001, 30 min vs 10 min.

15.8 min) was identified as the sulfate conjugate because it showed a deprotonated molecular ion $[M - H]^-$ at m/z 309 (**Figure 2C**) and also the dihydroresveratrol fragment at m/z 229.

Dihydroresveratrol was detected in plasma at 10 and 30 min (**Figure 3**) with concentrations of 1.06 ± 0.20 and 0.88 ± 0.22 μM, respectively. However, its glucuronide conjugate was the most abundant compound in plasma, with concentrations of 12.6 ± 1.1 μM and 33.5 ± 0.9 μM, at 10 and 30 min, respectively. The sulfate conjugate was also present with 4.4 ± 1.3 μM and 6.4 ± 1.3 μM at 10 and 30 min, respectively.

DISCUSSION

The development of a method to measure dihydroresveratrol that provides insight into its plasmatic concentration and metabolism is not trivial. First, because it is a metabolite of *trans*-resveratrol, and although comprehensive data are available on the oral bioavailability of *trans*-resveratrol (*16−18*), there are still some unanswered questions in terms of the complete identification and quantification of its metabolites. In addition, given that the in vivo concentrations of individual metabolites are higher than those of the parent compound, *trans*-resveratrol conjugates might act as a pool from which free *trans*-resveratrol could be released in various tissues or be active on themselves in promoting many of the health benefits attributed to *trans*-resveratrol (*19*). The last could be the case of dihydroresveratrol, which is a phytoalexin like *trans*-resveratrol (*1*). Dihydroresveratrol is synthesized by the condensation of one molecule of dihydro-*p*-coumaroyl-CoA and three molecules of malonyl-CoA as an intermediate in the biosynthetic pathway of stilbenoids in plants (*20*). Although the biological properties of dihydroresveratrol have not been completely established, it shows antiproliferative activity in human prostate cancer cells, with an IC_{50} of around 25 μM (*10, 11*). Moreover, dihydroresveratrol displayed a higher potential as a potassium channel modulator than *trans*-resveratrol in mouse neuroblastoma cells (*12*). This activity has also been reported in other tissues in connection with antitumor (tamoxifen) and cardiac antiarythmic (tedisamil) agents.

Dihydroresveratrol is a scarcely known metabolite of *trans*-resveratrol. This lack of knowledge can be attributed, in part, to the absence of a commercially available pure standard, which hinders the development of sensitive methods for measuring this compound in plasma and body fluids. The present study validates a method that allows the measurement of dihydroresveratrol and its conjugates in rat plasma. The results show that the method is reliable, reproducible, and easy to apply to biological samples. Solid-phase extraction of dihydroresveratrol from plasma with a C18 cartridge was appropriate for its measurement because it avoided analyte losses and provided maximal sensitivity with minimal handling. Solid-phase extractions with different sorbents

were also used when dihydroresveratrol was detected in urine samples (*6*, *8*). However, these authors did not validate the methods, nor did they quantify dihydroresveratrol or its conjugates.

The average extraction recoveries were 96.7% in the six concentrations assayed in plasma. However, the LOD was 275 nM, which could be attributed to the poor UV absorbance of dihydroresveratrol. Indeed, the validation of the instrument for this compound gave an LOD of 50 nM and an LOQ of 170 nM (data not shown). The precision of the method was acceptable, as shown by the intraday and interday coefficients of variation which were below 15%, which is considered acceptable (*15*). Moreover, the HPLC method was reproducible and linear over a wide range of concentrations (5−100 μM). Consequently, the validation of the method showed good reproducibility, accuracy, precision, and recovery in the assays of dihydroresveratrol in rat plasma.

Once the method was validated, it was applied to the detection of dihydroresveratrol and its metabolites in rat plasma after the oral administration of 60 mg/kg. This compound was detected in plasma, along with its glucuronide and sulfate conjugates, which were identified by HPLC-MS. Dihydroresveratrol was extensively metabolized, and 30 min after its oral administration, the concentrations of its glucuronide and sulfate conjugates were 38- and 6-fold higher, respectively, than the parent compound. In a previous study by our group, the chemopreventive activity of *trans*-resveratrol in a rat model of colon cancer induced by 1,2-dimethylhydrazine was assessed. After the oral administration of 60 mg/kg of *trans*-resveratrol for 49 days, dihydroresveratrol was the most abundant compound in colon, followed by *trans*-resveratrol glucuronide and small amounts of *trans*-resveratrol and its sulfate conjugate (*21*). Consequently, dihydroresveratrol formed in the colon could be absorbed, thus reaching blood and urine as indicated previously (*6*, *8*).

Although the different *trans*-resveratrol conjugates have been comprehensively described (*16*), little is known about dihydroresveratrol. The hydrogenation of the aliphatic double bond of *trans*-resveratrol by the intestinal flora rendered a metabolite with different UV absorption properties from the parent compound. The poor sensitivity observed for dihydroresveratrol even when dissolved in water indicates that it might not be detected at low concentrations. In addition, the UV maximum changed from 306 nm for *trans*-resveratrol to 276 nm for dihydroresveratrol. Therefore, when monitoring samples for *trans*-resveratrol, unless the detectors were also set at 276 nm, dihydroresveratrol might have been unnoticed. Boocock et al. (2007) reported their inability to find dihydroresveratrol in their samples since neither their UV-HPLC conditions nor their LC-MS/MS system was optimized for its detection (*22*). Moreover, the different UV spectra obtained by diode-array detection could have constituted an additional drawback to the detection of this metabolite.

In conclusion, the HPLC method described herein possesses appropriate sensitivity, accuracy, and reproducibility for analysis of dihydroresveratrol in preclinical investigations. Furthermore, HPLC analysis enables the separation of dihydroresveratrol metabolites, and when coupled with tandem mass spectrometry, provides a useful tool for metabolism studies.

ABBREVIATIONS USED

LOD, limit of detection; LOQ, limit of quantification; SPE, solid-phase extraction; RSD, relative standard deviation.

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