Rapid Quantification of Resveratrol in Mouse Plasma by Ultra High Pressure Liquid Chromatography (UPLC) Coupled to Tandem Mass Spectrometry

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Objective: The objective of this study was to develop a rapid and sensitive method for the quantification of resveratrol, a polyphenolic compound with multiple health beneficial effects, in mouse plasma.

Methods: We used reversed-phase ultra high pressure liquid chromatography with tandem mass spectrometry detection for the determination of resveratrol levels in mouse plasma. An Agilent Zorbax Eclipse Plus C_{18} column (2.1 mm x 50 mm, 1.8 µm) was used as the stationary phase. The mobile phase consisted of a gradient formed using 1 mM ammonium fluoride and methanol.

Results: Using this improved method, we obtained a retention time of 2.2 min and a total run time of 5 min, for resveratrol. The calibration curve for resveratrol showed a linear range from 0.5 to 100 ng/mL. The average coefficient of variation was 6% for interday variation and 4% for intraday variation. The recovery for resveratrol in mouse plasma was 85 ± 10% (mean ± standard deviation).

Conclusion: The method presented herein allows a rapid and very sensitive quantification of resveratrol in mouse plasma at concentrations as low as 500 ppt. [*P R Health Sci J 2014;33:151-156*]

Key words: Resveratrol, Quantification, UPLC, Plasma, Mass spectrometry

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a polyphenol particularly enriched in red wine, and also found in berries, grapes, and peanuts. It has been widely studied during the past years due to its antioxidant, anti-aging, anti-inflammatory, cardioprotective, neuroprotective, antidiabetic, and anticancer properties (1). Among resveratrol's health benefits, its cardioprotective and anticancer qualities have received special attention. Resveratrol acts as a cardioprotective agent by a plethora of activities that prevent atherosclerosis and coronary heart disease. Resveratrol: 1) inhibits low density lipoprotein oxidation, a primary event in the initiation of atherosclerosis; 2) inhibits platelet aggregation, allowing for the rapid repair of injuries in the vascular endothelium; 3) suppresses proliferation of smooth muscle cells and pulmonary aortic endothelial cells, which is necessary for atherogenesis; and 4) induces nitric oxide synthase (2).

The anticancer properties of resveratrol have been demonstrated both *in vitro* and *in vivo* by many research groups. Resveratrol inhibits all stages of tumorigenesis by modulating cell division and growth, apoptosis, angiogenesis and metastasis (3,4). Additionally, resveratrol has proven to be effective as a chemosensitization agent against several types of cancer. Resveratrol's ability to chemosensitize cancers to therapy has been attributed to the regulation of many signaling molecules including drug transporters, cell survival and cell proliferation regulators, and members of the nuclear factor kappa B and signal transducer and activator of transcription 3 signaling pathways (5).

Resveratrol has the capability to act in an estrogenic or antiestrogenic manner to inhibit or promote breast cancer progression, dependent on concentration, and can bind to and regulate gene transcription through estrogen receptor α and β isoforms (6-8). Therefore, resveratrol is of particular interest for gynecological cancers, such as breast cancer. Our laboratory has demonstrated that resveratrol, similar to estrogen, regulates actin structures and focal adhesions that are relevant for breast cancer cell migration and invasion. We reported an inhibitory role for high concentrations of resveratrol and a promotional role for low concentrations of resveratrol in signaling to the actin cytoskeleton and breast cancer cell migration (9-11). Moreover, we recently reported that resveratrol increases tumor growth and metastases from mammary fat pad tumors, established with

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human breast cancer cells, in nude mice (12). Therefore, it is critical to identify the effective concentrations of resveratrol in the circulation following consumption of resveratrol-rich foods or dietary supplements.

Resveratrol's bioavailability is relatively low; however, due to its therapeutic potential, many efforts are currently directed towards the generation of novel delivery systems for resveratrol (13,14). Since resveratrol's dual estrogenic/antiestrogenic role appears to be concentration dependent, it is important to delineate its anticancer effects at a broad range of concentrations. Correlation of the physiological effects of resveratrol in organisms with circulating plasma levels may be used as a prognostic tool for resveratrol effects *in vivo*. However, thus far detection and quantification of low levels of resveratrol has been limited by the sensitivity of the available methods. Sensitivity of detection of resveratrol in plasma and urine becomes extremely important, especially for the study of cancer promotion by low levels of resveratrol. To address this need, we developed a rapid ultra high pressure liquid chromatography (UPLC)-tandem mass spectrometry (MS) method that requires minimal sample preparation and allows the quantification of resveratrol in mouse plasma at lower levels and/or shorter retention times than those previously reported by similar methodologies (15-19).

Materials and Methods

Chemicals and reagents

Resveratrol (Figure 1) was purchased from LKT Laboratories, St. Paul, MN. Deuterated resveratrol (resveratrol-d_4) (Fig. 1) from Cayman Chemical, Ann Arbor, MI, was used as internal standard (IS). Liquid chromatography (LC)-MS CHROMASOLV® grade ≥99.9% methanol and acetonitrile (ACN) and ≥99.99% ammonium fluoride were purchased from Sigma–Aldrich, St Louis, MO. Mouse plasma containing sodium citrate was obtained from Equitech-Bio, Inc, Kerrville, TX.

Instrument

The UPLC MS/MS system consisted of an Agilent 1290 dual pump chromatograph with a 6460 Triple Quad LC/MS (Agilent Technologies, Santa Clara, CA). Agilent MassHunter Workstation software was used to control both the instrument and data acquisition. Qualitative and quantitative data analysis was performed with Agilent Mass Hunter Workstation Software, Version B.04.00/Build 4.0.225.0 for QQQ.

Standard solutions

Stock solutions of resveratrol were prepared at concentrations of 1 mg/mL, 100 µg/mL, and 1 µg/mL. The IS stock solution was prepared at 4 µg/mL. All stock solutions were prepared in 50% methanol and stored at -20°C in the dark. Solutions of resveratrol in mouse plasma were prepared by diluting the stock solutions prepared in 50% methanol. Plasma solutions were prepared at concentrations of 0.5, 1, 5, 10, 50, and 100 ng/mL resveratrol for the calibration curve; and at concentrations of 7, 25, and 70 ng/mL resveratrol for calibration control. Once prepared, plasma solutions were dispensed into 100 µL aliquots and stored at -80°C.

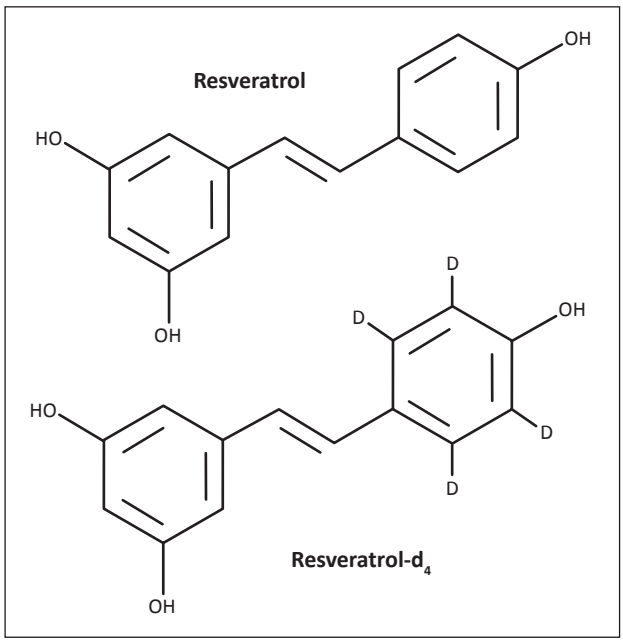

Figure 1. Structures of resveratrol and resveratrol-d_4. The structures of resveratrol and of the internal standard (deuterated resveratrol or resveratrol-d_4) are presented.

Sample preparation

A total of 2.5 µL of IS (4 µg/mL stock solution) were added to 100 µL of plasma to obtain a final IS concentration of 100 ng/mL. Then, 400 µL of ACN were added and the sample was vortex-mixed for 5 s and spun at 9,000 x g (4°C) for 10 min to precipitate proteins. Following protein precipitation, samples were centrifuged for 10 minutes at 3,300 rpm at 4°C, the supernatant was carefully transferred to a glass tube and the solvent was dried at room temperature using a vacuum concentrator. Once dry, samples were reconstituted in 100 µL of 50% methanol, filtered through a 0.45 µm nylon filter, and transferred to appropriate vials for injection into the UPLC MS/MS system.

Ultra high pressure liquid chromatography

Chromatographic separation was achieved by injecting 5 µL of sample into a Zorbax Eclipse Plus C18 column (2.1 x 50 mm, 1.8 µm, Agilent Technologies, Santa Clara, CA). The column temperature was set to 40°C. The mobile phase consisted of a gradient of 1 mM ammonium fluoride in distilled water (solvent A) and 100% methanol (solvent B) at a flow of 0.5 mL/min. The gradient elution was carried out as follows: 0 min, 0% B; 2 min, 30% B; 2.5 min, 70% B; 2.6 min, 95% B; 3.5 min, 95% B;

and finally 3.6 min, 30% B for re-equilibrating. Retention time for both resveratrol the reseveratrol-d_4 was 2.22 and 2.17 min, respectively.

Mass spectrometry

An Agilent 6460 employing electrospray ionization (ESI) with Jet Stream technology was used in the negative ionization mode. The instrument settings were as follows: time filtering width 0.07 s; gas temperature 350°C; gas flow 10 L/min, nebulizer gas 20 psi; sheath gas heater 400°C; sheath gas flow 12 L/min; capillary voltage 4000 V; cell accelerator voltage (CAV) 7 V; dwell time 100 ms; and collision energy (CE) 12 V for resveratrol quantifier (227→185), 20 V for resveratrol qualifier (227→143), and 24 V for the IS (231→147).

Results and Discussion

Resveratrol analysis and detection

Resveratrol and resveratrol-d_4 were eluted at 2.22 min and 2.17 min respectively with 70% percent of organic solvent B through a C18 column set at 40°C (Figure 2). For quantification, negative ESI was used with ammonium fluoride at 1 mM as the aqueous solvent, which has been previously reported to substantially improve ionization in the negative ESI mode (20). Only resveratrol and IS precursor ions with mass to charge (m/z) of 227 and 231, respectively, and at unit resolution were allowed from MS1 to MS2. Product ions 185, 143, and 147 m/z with unit resolution were detected and later used as resveratrol quantifier peak, resveratrol qualifier peak, and IS peak, respectively. The run time was only 5 min for each sample, representing a great improvement over previously described methodologies for quantification of resveratrol in plasma (16,17).

Linearity

Six calibration runs for resveratrol in mouse plasma were performed using standards of 0.5, 1, 5, 10, 50, and 100 ng/mL resveratrol, containing IS at 100 ng/mL. All calibration curves were linear over the measured range, and are defined by the following equation $y = (0.018289 \pm 0.000683)x + (-0.0009212 \pm 0.0016098)$ (mean ± standard deviation (SD) for slope and

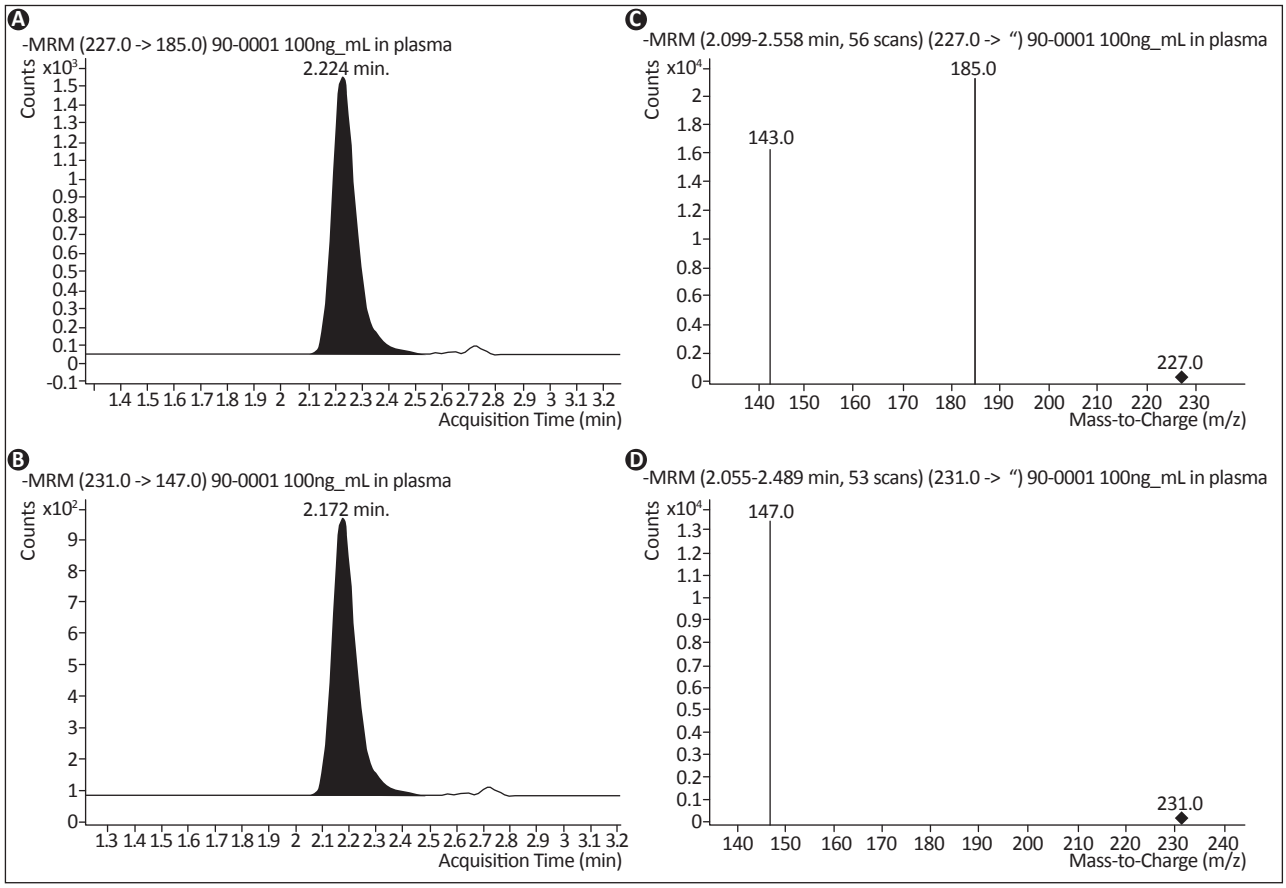

Figure 2. LC-ESI-MS/MS analysis of resveratrol in mouse plasma. Plasma samples were spiked with 100 ng/mL of resveratrol and resveratrol-d_4. MRM transitions for **A**. resveratrol (227→185), and the **B**. IS (231→147) are presented. Retention times were 2.22 and 2.17 min, respectively. **C**. Mass spectra for resveratrol, showing product ions 185, 143 m/z used as resveratrol quantifier peak, resveratrol qualifier peak, respectively. **D**. Mass spectra for the IS, showing product ion 147 m/z.

y intercept); and presented a correlation coefficient (r^2) of 0.99916 ± 0.00055 (mean ± SD) (Figure 3).

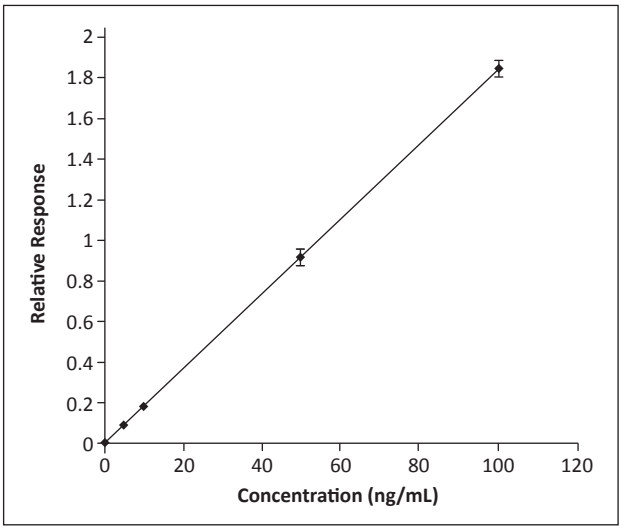

Figure 3. Calibration curve for resveratrol. The average of six calibration curves generated for resveratrol using Agilent software is presented as mean ± SD. Linearity was achieved for 0.5-100 ng/mL resveratrol. Each standard contained 100 ng/mL of resveratrol-d_4 (the IS).

Limit of detection and quantification, precision and accuracy

The limit of detection (LOD) and limit of quantification (LOQ) for resveratrol in plasma was 0.25 and 0.5 ng/mL, respectively. Interday and intraday precision and accuracy showed a coefficient of variation (CV) of 6 and 4% (in average) and a relative error (RE) of 1.3 and 1.4% (in average), respectively (Table 1).

Recovery

To calculate the percentage of recovery for resveratrol in mouse plasma, we used the ratio of the concentration calculated for the extracted standards versus the concentration calculated for the neat standards (5, 50, and 100 ng/mL in 50% methanol). To prepare the extracted standards, we used mouse plasma that underwent the sample preparation process but was reconstituted in resveratrol standards of 5, 50, and 100 ng/mL in 50% methanol (neat standards). Five replicates of each standard were prepared and each one was injected twice; all standards were assayed in the same analytical run. The percent of recovery for resveratrol in mouse plasma was high, as shown by the reported values of 82, 76, and 96% recovery for 5, 50, and 100 ng/mL, respectively; average percent of recovery was 85 ± 10% (Table 1).

Stability

Several tests were performed to evaluate the stability of resveratrol: benchtop and freeze/thaw stability in plasma and post-preparative reinjection reproducibility in reconstitution solvent were determined. For the benchtop stability test, 1 and 100 ng/mL resveratrol standards (5 replicates; each injected once) were thawed and, after 6 h at room temperature, subjected to the sample preparation protocol (see methods). Our results show a CV of 14 and 4% and a RE of 6 and 2% for the 1 and 100 ng/mL resveratrol standards, respectively (Table 1). On the other hand, the freeze/thaw stability test used 25 and 100 ng/mL resveratrol standards (one replicate; each injected 5 times) that underwent 5 freeze/thaw cycles (15 min at -80°C/15 min at room temperature), prior to sample preparation. The CV was 3 and 2% and the RE was 11 and 1% for 25 and 100 ng/mL resveratrol standards, respectively (Table 1). Finally, the calculated concentration of 25 and 70 ng/mL resveratrol standards (two replicates; each injected twice) at 0 h and after 96 h at room temperature were compared for the

Table 1. Method validation parameters for resveratrol in mouse plasma

	Resveratrol (ng/mL)								
Validation parameter	0.50	1	5	10	25	50	70	100	Average
	Mean value for calculated concentration or % recovery								
Interday precision and accuracy	0.51	1.01	4.95	9.67		49.74		100.50	
SD	0.06	0.13	0.24	0.49		1.16		1.89	0.66
%CV	11	12	5	5		2		2	6
%RE	2	1	1	3		1		0	1.3
Intraday precision and accuracy		1.01	5.15	9.67		49.89		100.28	
SD		0.11	0.09	0.37		0.96		2.24	0.75
%CV		11	2	4		2		2	4
%RE		1	3	3		0		0	1.4
Recovery (%) Benchtop stability			82			76		96	85 ± 10
		0.94						102.33	
SD		0.13						4.45	2.29
%CV		14						4	9
%RE		6						2	4
Freeze/thaw stability					27.70			98.89	
SD					0.78			1.58	1.18
%CV					3			2	2.5
%RE					11			1	6
Post-preparative reinjection reproducibility (% recovery after 96h)					99±7		98±6		

determination of post-preparative reinjection reproducibility of resveratrol in reconstitution solvent. After 96 h, the percent of recovery was 99 ± 7% (mean ± SD) and 98 ± 6% for 25 and 70 ng/mL resveratrol standards, respectively (Table 1). These results suggest that resveratrol is stable after 6 h in plasma and after 96 h in reconstitution solvent at room temperature (Table 6). Additionally, resveratrol was proven to be stable after 5 freeze/thaw cycles. The robust stability of resveratrol greatly simplifies sample handling during preparation and allows for the preparation of multiple samples simultaneously.

Application of the method

The described method allows for the rapid quantification of low levels of resveratrol in mouse plasma. The reported methodology allows the quantification of resveratrol at lower levels and/or shorter retention times than previously reported methodologies (15-19). Our method has a total run time of only 5 min with a LOQ of 0.5 ng/mL or 2.19 nM. In comparison, other methods report longer run times and higher LOQs. For instance, the method published by Juan ME, et al. (21) to detect resveratrol in rat plasma by high pressure liquid chromatography (HPLC) has a run time of >20 min and a LOQ of 5.77 nM, the method reported by Singh G, et al. (22) to detect resveratrol in human plasma by HPLC has a run time of 10 min and a LOQ of 8 ng/mL, and the method reported by Menet MC, et al. (23) to detect resveratrol in mouse plasma by UPLC has a run time of <10 min and a LOQ of 25 nM. Our rapid and sensitive methodology is expected to enable a more accurate correlation between the plasma levels of resveratrol and its anti-cancer effects. This in turn, will provide a better understanding of the concentration-dependency of resveratrol's health beneficial properties. This method will be used in our studies to determine resveratrol's circulating plasma levels in mammary tumor-bearing immonucompromised mice orally treated with low and moderate concentrations of resveratrol.

Resumen

Objetivo: El objetivo del presente estudio consistía en desarrollar un método rápido y sensitivo para la cuantificación de resveratrol, un compuesto de tipo polifenólico con múltiples propiedades beneficiosas para la salud, en plasma de ratón. Métodos: Utilizamos cromatografía líquida con presión ultra alta acoplada a espectrometría de masa en serie, para la determinación de los niveles de resveratrol en plasma de ratón. Una columna Agilent Zorbax Eclipse Plus C_{18} (2.1 mm x 50 mm, 1.8 μm) fue utilizada como fase estacionaria. La fase móvil consistió de un gradiente de fluoruro de amonio 1 mM y metanol. Resultados: Obtuvimos un tiempo de retención de 2.2 min para resveratrol y el tiempo total de corrida fue de 5 min. La curva de calibración para resveratrol se comportó de manera lineal en un rango de 0.5 a 100 ng/mL. El coeficiente de variación promedio fue 6% para la variación entre días y de 4% para la variación en un mismo día. El porciento de recuperación de resveratrol en plasma de ratón fue de 85 ± 10% (promedio ± desviación estándar). Conclusión: El método que hemos desarrollado permite la cuantificación de resveratrol en plasma de ratón de manera rápida y sensitiva. Utilizando este método se pueden detectar cantidades de resveratrol tan bajas como 500 ppt.

Conclusion

Herein we have reported a reliable, rapid and sensitive method for the quantification of resveratrol in mouse plasma using an UPLC MS/MS system. The high recovery, speed, and sensitivity of the method combined with the reported stability of resveratrol are characteristics that render this method a practical tool for the identification and quantification of resveratrol. The described methodology can be further developed to quantify resveratrol in human plasma for pharmacokinetic studies and large scale clinical trials.

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