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Determination of resveratrol and its sulfate and glucuronide metabolites in plasma by LC-MS/MS and their pharmacokinetics in dogs

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Abstract

An analytical approach for the determination of *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) and its glucuronide and sulfate conjugates in dog plasma by LC-MS/MS (without enzymatic hydrolysis of the conjugates) was validated to support pre-clinical toxicological and pharmacological studies. The approach required two independent sample extractions and consequent instrument runs. Samples for resveratrol determination were prepared by protein precipitation with acetonitrile; acetonitrile-methanol was used instead for resveratrol metabolites. Chromatographic separation was performed using a C18 column (30 × 2.0 mm) at a flow rate of 0.25 mL/min. For resveratrol the mobile phase consisted of A: 5 mM ammonium acetate in water-isopropanol (98:2, v/v) and B: methanol-isopropanol (98:2, v/v) and for metabolites the mobile phase was modified as follows: A: 0.1% (v/v) formic acid in water and B: 0.1% (v/v) formic acid in acetonitrile. Total run time was 12 min for each run with retention times of about 4–5 min for all analytes. A turbo ion spray source was used operating in negative mode for resveratrol and resveratrol sulfate and in positive mode for resveratrol glucuronide. Calibration curves were linear from 5 to 1000 ng/mL for resveratrol and its glucuronide, and 10 to 2000 ng/mL for resveratrol sulfate. Linearity was assessed using the internal standard method for resveratrol and the external standard method for the metabolites. Method accuracy was 90 to 112% of the true value for all analytes with precision of 9 %RSD or less for all validation experiments. The validated method was applied to a preclinical toxicology study in dogs after oral administration (200 to 1200 mg/kg) of the agent. Peak plasma resveratrol concentration (C_{max}) for most animals was observed within 1 to 5 h of dosing, with group mean values in the 1.7 to 9.9 µg/mL (7.5 to 43 µM) range. Area under the plasma concentration-time curve (AUC) mean values for resveratrol ranged from 3.6 to 44 h*µg/mL for all study groups and were generally proportional to the dose, with no consistent statistically significant changes observed for gender or number of doses. Mean molecular-weight adjusted ratios of resveratrol metabolites to resveratrol for AUC ranged from 1 to 9 for resveratrol glucuronide and from 2 to 11 for resveratrol sulfate.

Keywords

Resveratrol; Metabolites; Dog; Plasma; Pharmacokinetics

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Introduction

The polyphenolic natural product *trans*-resveratrol (5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol; Fig. 1; referred to hereafter as resveratrol), found in grapes, berries, peanuts and other foodstuffs, continues to be the subject of numerous studies probing into its diverse properties and potential health benefits. Literature about its varied biological activity derived from in-vivo and in-vitro studies and about its potential as a disease-preventing agent is extensive and includes cancer prevention, cardioprotection and life span prolongation [1-7]. However, in spite of the large volume of published research, complete understanding of resveratrol's fundamental properties as a therapeutic remains elusive [8-10]. For example, resveratrol's health benefits when ingested in the diet; the efficacy of supplementation for disease prevention; and its therapeutic window and toxicity at the high doses proposed as necessary for beneficial effects have yet to be fully researched. Resveratrol's low oral bioavailability and fast first-pass metabolism have been reported in species including human, mouse, rat, rabbit and pig [11-16]. Specifically, oral bioavailability was reported as 1.5% in rabbit [15] and up to 30% in rat [13]. In human studies, resveratrol plasma levels have been reported at below the limit of quantitation or low levels after oral administration of the agent [17]. Although metabolic profiles may differ, all species have in common an acceptable absorption after oral administration followed by fast conversion of the agent to glucuronide and sulfate metabolites, with plasma levels of the metabolites larger than those of the parent compound. Whether the prevalent metabolites are biologically active [8,18-19] or serve as a systemic repository of the agent through, for example, enterohepatic recirculation [14] is not completely understood. It is therefore apparent that the complete assessment of resveratrol's potential as a therapeutic should include not only the agent's pharmacokinetics but that of its prevalent conjugate metabolites as well.

A number of references are available with validated methods for the determination of resveratrol in plasma from multiple species [14,20-25]. Additionally, resveratrol metabolite identification following oral administration of the agent has been performed for multiple species, with results consistently identifying mono-glucuronides and mono-sulfates as two of the prevalent metabolites [13-14,20,26-27]. Published methodologies for the determination of resveratrol metabolites in plasma and tissues include HPLC with UV detection [11,20-21,28-29] and tandem mass spectrometry [14,30]. With few exceptions [28-29], in these earlier methods enzymatic hydrolysis of the conjugates and indirect determination from the increases observed in the concentration of resveratrol were necessary to estimate the degree of Phase II metabolism. HPLC methods are long, approximately 25 min per run, and sample preparation is demanding, often requiring solid phase extraction (SPE) to purify the extracts and remove interferences. The indirect approach using LC-MS/MS with selective hydrolysis requires three separate extractions and its accuracy depends heavily on the selectivity of the enzymatic hydrolysis and the extent to which the reaction is carried out. A direct determination approach for resveratrol mono-sulfate by LC-MS/MS was published earlier; however, resveratrol mono-glucuronide concentrations were estimated using the parent compound calibration curve and method validation results were not provided [26].

In this work, we present a fully validated LC-MS/MS analytical approach for the determination of resveratrol and of resveratrol glucuronide and resveratrol sulfate without previous enzymatic hydrolysis to free resveratrol. The chromatographic separation of resveratrol is based on an HPLC method published earlier [20] and optimized here for throughput and performance using tandem mass spectrometry detection. This modified approach was first validated for resveratrol and later validated for the direct determination of

resveratrol mono-glucuronide and mono-sulfate metabolites after additional modifications using a separate protein precipitation sample preparation and instrumental analysis. The approach was applied to examine the pharmacokinetics of resveratrol, resveratrol glucuronide and resveratrol sulfate in dogs after a 13 week oral administration of resveratrol, a study that was performed as part of the preclinical investigation of resveratrol as a candidate chemopreventive agent by the US National Cancer Institute.

1. Experimental

1.1. Chemicals and reagents

Resveratrol (Sochinaz SA, Vionnaz, Switzerland) was provided by the Division of Cancer Prevention, National Cancer Institute (Bethesda, MD, USA). *Trans*-resveratrol-3-O- β -D-glucuronide (Toronto Research Chemicals, Inc., North York, Ontario, Canada) and *trans*-resveratrol-3-O-sulfate (SynFine Research, Inc., Richmond Hill, Ontario, Canada) were used as reference standards for the determination of conjugated resveratrol. *Trans*-resveratrol- $^{13}\text{C}_6$ (Toronto Research Chemicals, Inc.) was used as the internal standard for the determination of resveratrol. Acetonitrile, methanol, isopropanol, ammonium acetate and formic acid (all HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade water was generated using a PURELAB Ultra system from ELGA (Lowell, MA, USA) followed by filtration with a Millipore (Billerica, MA, USA) 0.25 μm filter. Blank dog plasma was obtained from Bioreclamation Inc. (Westbury, NY, USA).

1.2. Animals

Twenty male and twenty female healthy naïve beagle dogs (approximately five months old; Ridgland Farms, Inc., Mount Horeb, WI, USA) were held in quarantine for approximately 4 weeks and observed daily for survival and general health status. Prior to randomization into experimental groups, each animal underwent a detailed physical examination to ensure suitability as a test animal. Dogs were housed individually in pens in accordance with standard protocols [31]. Animal rooms were held within a temperature range of approximately 20-25°C and a humidity range of approximately 30-70%. Fluorescent lighting in the animal rooms was provided on a cycle of 12 h of light followed by 12 h of darkness. Each dog was provided with 400 g of commercial chow (Certified Canine Diet 2021C, Harlan Teklad, Madison, WI, USA) daily for a minimum of 2 h during the treatment period. Drinking water was available *ad libitum* by automatic watering systems.

1.3. Preparation of analytical stock and standards solutions

Stock solutions of resveratrol, *trans*-resveratrol- $^{13}\text{C}_6$, resveratrol-3-O- β -D-glucuronide and resveratrol-3-O-sulfate were prepared in methanol at 1, 0.1, 0.2 and 0.4 mg/mL concentrations, respectively, and stored at -20°C when not in use. Stock standards were prepared by further diluting the stock solutions in methanol-water (50:50, v/v) to 50, 100, 200, 500, 2000, 5000 and 10000 ng/mL for resveratrol and its glucuronide, and to 100, 200, 400, 1000, 2000, 4000, 10000 and 20000 ng/mL for resveratrol sulfate. The internal standard working solution was prepared by diluting the stock solution with acetonitrile to a concentration of 25 ng/mL.

1.4. Extractions

Plasma samples (100 μL) were transferred to 2-mL microcentrifuge plastic vials (placed in ice and protected from light) and mixed with 10 μL of stock standards (for plasma calibrators and QC samples) and 1 mL of internal standard solution in acetonitrile. The samples were vortex-mixed for 1 min, centrifuged at 4 °C in a Sorvall RC 5C Super Speed centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 8000 $\times g$ for 10 min to remove precipitated proteins, and the supernatant was transferred to a clean tube and dried under

nitrogen flow at room temperature (about 25 °C). After evaporation was complete, the residue was reconstituted in 100 µL of methanol with five min of sonication, added to 400 µL of water, vortex-mixed and centrifuged again. The resulting supernatant was transferred to a sample vial for instrumental analysis. For the determination of conjugated resveratrol metabolites, the protein precipitation procedure was performed instead with 1 mL of acetonitrile-methanol (1:1, v/v) solution. All other steps were the same.

1.5. Instrumentation

Samples were analyzed on an API 3000 MS/MS system (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE, USA) and run by Analyst™ 1.4.2 software.

Separation of resveratrol from plasma components was achieved using a Luna 3 µm C18(2) 100Å 30 × 2.0 mm column (Phenomenex, Torrance, CA, USA). The column was maintained at room temperature, the flow rate was 0.25 mL/min, and the injection volume was 25 µL. For the resveratrol determination method, the mobile phase consisted of A: 5 mM ammonium acetate in water-isopropanol (98:2, v/v) and B: methanol-isopropanol (98:2, v/v). The mobile phase gradient was as follows: after injection, initial conditions with Solvent A at 90% were held for 0.5 min, decreased to 5% in 3.5 min and held constant for 5 min, returning to initial conditions for another 3 min of reequilibration time. The mobile phase was changed for the analysis of resveratrol metabolites as follows: A: 0.1% (v/v) formic acid in water and B: 0.1% (v/v) formic acid in acetonitrile. All other chromatographic conditions were the same.

Retention times of resveratrol, resveratrol glucuronide and resveratrol sulfate were approximately 5.2, 4.0 and 4.9 min, respectively. Total run time was 12 min for each analytical run.

A turbo ion spray interface was used as the ion source, operating in negative ion mode for the resveratrol determination and with polarity switch for the metabolites determination (negative ion mode for resveratrol sulfate and positive mode for resveratrol glucuronide). Acquisition was performed in multiple reaction monitoring (MRM) mode using m/z 227 ($[M-H]^-$) → 185 (loss of 42 (C_2H_2O) [32]; 233 ($[M-H]^-$) → 191 (loss of 42 (C_2H_2O); 405 ($[M+H]^+$) → 229 (resveratrol, $[M+H]^+$); and 307 ($[M-H]^-$) → 227 (resveratrol, $[M-H]^-$) at unit resolution for resveratrol, resveratrol- $^{13}C_6$, resveratrol glucuronide and resveratrol sulfate, respectively. Ion spray voltage and collision energy were -3000 and -30 V, 4500 and 24V, and -4500 and -24 V for resveratrol, resveratrol glucuronide and resveratrol sulfate, respectively. The collision gas was nitrogen; the ion spray temperature was 450 °C; and dwell time was 200 ms.

1.6. Method validation

Method validation was performed following the FDA's *Guidance for Industry: Bioanalytical Method Validation* [33]. Selectivity, linearity, precision, accuracy, recovery and stability were used to assess assay performance.

Selectivity was assessed by analyzing extract from six individual animals for the presence of analytical interferences and comparing the results to those obtained from spiking blank plasma sources with the analytes at the lowest limit of quantitation (LLOQ; 5 ng/mL for resveratrol and resveratrol glucuronide, 10 ng/mL for resveratrol sulfate).

Linearity was assessed using the internal standard method for resveratrol and the external standard method for its conjugated metabolites and up to eight calibrators prepared with blank dog plasma and analyte concentrations in the 5 to 1000 ng/mL (resveratrol and its

glucuronide) and 10 to 2000 ng/mL (resveratrol sulfate) ranges. Curves were built from peak areas ratios to the internal standard (resveratrol) or peak areas (resveratrol metabolites) using least-squares linear regression with a $(1/x^2)$ weighting factor that was chosen based on goodness-of-fit criteria, including coefficient of determination (r^2), the back-calculated concentration of individual calibrators, and minimization of the intercept value.

Precision and accuracy were determined from three validation runs with dog plasma quality control (QC) samples ($n = 6$) spiked at the LLOQ (5 ng/mL) and at low (12 ng/mL), mid (400 ng/mL) and high (800 ng/mL) concentrations for resveratrol and its glucuronide and corresponding concentrations of 10, 24, 800 and 1600 ng/mL, respectively, for resveratrol sulfate. Within-run precision and accuracy were assessed from the results from a single day, while between-run precision and accuracy were determined from the results from the three validation runs on different days. Dog plasma extraction recovery of resveratrol and metabolites was determined by comparison of peak area results of dog plasma QC samples ($n = 6$) to peak area results of extracted blank dog plasma samples spiked post extraction with analyte concentrations at the same levels. For matrix effect evaluation, the peak areas were also compared to those from the corresponding standards prepared in the reconstitution solvent.

Bench-top stability was determined by analyzing low and high level QC samples for the analyte concentrations after 4 and 24 h of storage at ambient temperature and comparing the results to those obtained from freshly prepared samples. The 24 h stability experiment was repeated for resveratrol under refrigerated conditions (approximately 4 °C). Similar experiments were performed for the long-term stability evaluation of the analytes in dog plasma after 50 days storage at -70 °C. Freeze-thaw stability of the low and high level QC samples was determined at -70 °C and over three cycles. Stability was also determined for low and high level QC samples that were extracted and stored in the instrument autosampler under refrigerated conditions (approximately 4 °C); sample extracts were injected on the LC-MS/MS instrument and the concentrations determined after approximately 6 days of storage using freshly prepared calibrators.

To evaluate the impact of sample dilution on the method's accuracy and precision, six replicate QC samples were prepared in blank dog plasma at 4000 ng/mL (resveratrol and its glucuronide) and 8000 ng/mL (resveratrol sulfate) and diluted 5-fold with additional blank plasma prior to analysis.

1.7. Application to a pre-clinical toxicological study

Resveratrol was administered to beagle dogs to evaluate the toxicity and pharmacokinetics of the agent following daily oral administration for 91 consecutive days. Animals were randomly assigned to a control or one of three treatment groups at the end of the quarantine period using a computerized body weight stratification procedure that produced similar group mean body weight values. Body weights for the animals assigned to the study ranged from 5 to 10 kg. The study groups, each consisting of four male and four female dogs, received capsules with neat doses of resveratrol at 0, 200, 600 and 1200 mg/kg (control, low, mid and high groups, respectively).

Blood samples for determination of plasma levels of resveratrol, resveratrol glucuronide and resveratrol sulfate were collected from all treated animals after the first dose and during the last week (week 13) of the study. Blood samples (approximately 3 mL) were collected from the jugular or cephalic vein into Vacutainer tubes (Fisher Scientific, Pittsburgh, PA, USA) containing EDTA at 9 time points (0, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h post-dose). Tubes were inverted several times to mix; placed on ice until centrifuged to separate plasma

(within 1 h); transferred into storage tubes (0.5 mL); and stored frozen (approximately -70°C) until analyzed.

1.8. Pharmacokinetics analysis

Plasma concentration-time profiles of resveratrol, resveratrol glucuronide and resveratrol sulfate for individual animals were analyzed using a noncompartmental model for extravascular administration with WinNonlin® Professional Edition software, Version 5.0.1 (Pharsight Corporation, Mountain View, CA, USA). Pharmacokinetic parameters reported for all target analytes included: time to maximum plasma concentration (T_{max}), peak plasma concentration (C_{max}), area under the plasma concentration-time curve (AUC), and elimination half-life ($t_{1/2}$). Clearance (CL/F) and apparent volume of distribution (V_z/F) were calculated only for resveratrol. AUC from time zero to the last measured concentration (AUC_{0-t}) was estimated by the linear trapezoidal rule up to C_{max} , followed by the log trapezoidal rule for the remainder of the curve. AUC extrapolated to infinity ($AUC_{0-\infty}$) is defined as $AUC_{0-t} + C_t / \lambda_z$, where λ_z is the disposition rate constant estimated using log-linear regression during the terminal elimination phase and C_t is the last measurable plasma concentration. Statistical comparisons of the data to assess changes due to dose level, gender, or study day were performed using Systat software (version 10.2). Data was analyzed via analysis of variance (ANOVA) followed, as necessary, by the *post hoc* Tukey's test (inter-group comparison) or via t-test (Day 1 vs. Week 13 and male vs. female); $p \leq 0.05$ (all comparisons); data was log-transformed (with the exception of $t_{1/2}$ and T_{max}); C_{max} and AUC parameters were normalized to the body surface area dose (i.e., mg resveratrol/m²) prior to log-transformation [34].

2. Method validation results

2.1. Specificity

No significant peaks interfering with the quantitation of resveratrol and metabolites were detected in the chromatograms of blank dog plasma at the method's LLOQ for the three analytes. Fig. 2 shows representative chromatograms of blank dog plasma extract, LLOQ (5 ng/mL or 10 ng/mL for resveratrol sulfate), and a 200 ng/mL calibrator (400 ng/mL for resveratrol sulfate) in dog plasma for each of the analytes.

2.2. Calibration curves

Calibration curves were linear from 5 to 1000 ng/mL (resveratrol and glucuronide) and 10 to 2000 ng/mL (resveratrol sulfate). Calibrators that fell outside the range of 85 to 115% were not used to calculate the standard curve (two instances for the 200 ng/mL calibrator for resveratrol glucuronide). Mean r^2 values were 0.995 or greater for all analytes. Representative regression equations for the calibration curves were $y = 0.0365x + 0.0031$, $y = 1780x + 876$ and $y = 6340x + 1380$ for resveratrol, resveratrol glucuronide and resveratrol sulfate, respectively. The mean accuracy (% of true value) of individual calibrators used to determine the calibration curves ranged from 97 to 103%, 93 to 112% (106% without the 200 ng/mL calibrator), and 97 to 108% of the true value for resveratrol, resveratrol glucuronide and resveratrol sulfate, respectively. Between-run precision [% relative standard deviation (RSD)] for the back-calculated calibrator concentrations ranged from 1 to 8% for the three analytes.

2.3. Precision and accuracy

Precision and accuracy experiment results are presented in Table 1. For all QC samples and analytes the method precision was 9 % RSD or less. Accuracy was within 15% of the true value for all determinations.

2.4. Recovery

Average recovery for the low, mid and high QC replicate samples was 95, 98 and 90; 74, 77 and 73; and 83, 81 and 78% for resveratrol, resveratrol glucuronide, and resveratrol sulfate, respectively. Precision ranged from 4 to 7, 2 to 9 and 3 to 9% RSD for resveratrol, resveratrol glucuronide, and resveratrol sulfate, respectively. Comparison of the peak areas of the above experiments to those from the analytes in the reconstitution solvent did not show any significant matrix ion suppression.

2.5. Lower limit of quantitation

Results for the LLOQ determination experiments are included in Table 1. For samples prepared at the LLOQ, method precision was 7 %RSD or less for all analytes. Accuracy was within 15% of the true value for all determinations.

2.6. Stability studies

The experiments designed to evaluate the bench-top stability of the target analytes in dog plasma after 4 or 24 h at room temperature had recoveries within $\pm 10\%$ of their nominal concentration for all analytes and concentrations, with the exception of resveratrol after 24 h (the low and high QC samples degraded to 65 and 49%, respectively, of their initial concentrations) and resveratrol glucuronide (high QC, 4 h time period, 113% recovery). Recovery of resveratrol in dog plasma improved under refrigerated conditions, at 96 and 95%, respectively, of the initial concentrations for the low and high QC samples after 24 h. The changes after three freeze-thaw cycles for the low and high QC samples were within $\pm 10\%$ of their nominal concentrations. The changes after six days of storage of the QC sample extracts in the instrument refrigerated autosampler were within $\pm 10\%$ of their nominal concentrations. No degradation was observed for resveratrol or metabolites in dog plasma after 50 days storage at -70°C . Results for all stability experiments performed are presented in Table 2.

2.7. Sample dilution

For the 6-replicate set of QC dog plasma samples prepared at 4000 or 8000 ng/mL and diluted 5-fold before analysis, accuracy was 98.3, 101 and 106% of the target value, with a precision of 2, 3 and 1% RSD for resveratrol, resveratrol glucuronide and resveratrol sulfate, respectively.

3. Pharmacokinetics results

Representative individual animal plasma concentrations (ng/mL) of resveratrol and its metabolites are presented graphically for males and females from the low, mid and high dosing groups in Fig. 3A, B and C, respectively. Group mean value ranges for resveratrol, resveratrol glucuronide and resveratrol sulfate are summarized in Table 3.

3.1. Resveratrol results

Following oral administration, C_{max} for most animals was observed within 1 to 5 h of dosing; with group mean values in the 1.7 to 9.9 $\mu\text{g/mL}$ (7.5 to 43 μM) range. Statistical comparisons indicated proportional increases with the dose; however, no other consistent statistically significant changes were observed. Although T_{max} appeared to shift to larger values with the dose, no consistent statistically significant changes were observed related to dose, gender, or study day. Mean $t_{1/2}$ values for the different study groups were similar (ranging from 2 to 4 h). No statistically significant changes were observed for $t_{1/2}$ related to dose level, gender, or study day. AUC mean values ranged from 3.6 to 44 $\text{h}\cdot\mu\text{g/mL}$ for all study groups and were proportional to the dose. V_z/F mean values ranged from 120 to 440

L/kg; CL/F mean values ranged from 22 to 110 L/h/kg. No consistent statistically significant changes were observed for V_z/F or CL/F.

3.2. Metabolites results

Resveratrol metabolized rapidly to resveratrol glucuronide and resveratrol sulfate, producing similar plasma concentration time profiles; no significant changes were apparent for T_{max} between resveratrol and its metabolites.

Resveratrol glucuronide— T_{max} mean values (2 to 7 h) were similar to those observed for resveratrol and also appeared to increase with the dose. Mean C_{max} values were in the 3.8 to 210 $\mu\text{g/mL}$ range and statistical comparisons indicated proportional increases with the dose. Mean $t_{1/2}$ values were comparable to those for resveratrol and did not change significantly among the study groups. AUC mean values ranged from 7.2 to 560 $\text{h}\cdot\mu\text{g/mL}$ for all study groups and were mostly proportional to the dose.

Resveratrol sulfate— T_{max} mean values for resveratrol sulfate were similar to those observed for resveratrol. Mean C_{max} values were in the 17 to 30 $\mu\text{g/mL}$ range and did not change significantly among the study groups. Mean $t_{1/2}$ values ranged from 4 to 9 h and were, in general, larger than those for resveratrol. AUC mean values ranged from 40 to 170 $\text{h}\cdot\mu\text{g/mL}$ for all study groups and although in general they increased with the dose, the changes were not proportional for Day 1 and less apparent for Week 13.

Mean molecular-weight adjusted ratios of resveratrol metabolites to resveratrol for AUC ranged from 1 to 9 for resveratrol glucuronide and from 2 to 11 for resveratrol sulfate. Although the AUC ratio for resveratrol glucuronide appeared to increase with the dose, the changes were not statistically significant. The statistical comparisons for resveratrol sulfate AUC ratios indicated a general decrease with the dose.

4. Discussion and Conclusions

4.1. Method development

Initial attempts in our laboratory to use the resveratrol plasma extraction and chromatographic conditions for the conjugated metabolites analysis were unsuccessful due to poor reproducibility. Recovery for resveratrol sulfate improved from about 45% to approximately 75% with the use of acetonitrile-methanol for the extractions; although still not optimal, this proved consistent and reproducible. In addition, we found better method performance for resveratrol glucuronide with the mass spectrometer operating in positive mode and with the addition of formic acid in the mobile phase. A reference standard for resveratrol di-sulfate, another conjugated metabolite found in multiple species, was not commercially available at the time of the study, thus the method could not be validated for this metabolite.

4.2. Assay performance

As presented, the determination of resveratrol and target metabolites required two independent sample extractions and consequent instrument runs. Although the approach worked acceptably, the assay throughput could be substantially improved by combining the sample preparation and instrumental analysis into a single method for all analytes; in our work we could not find the right analysis conditions to achieve this. The utilization of deuterated analogs of the Phase II metabolites as internal standards, not commercially available at the time of the study, could further improve the method's performance. Additionally, if resveratrol di-sulfate reference material were to become available, the method could also be validated for its determination.

4.3. Pharmacokinetics of resveratrol in dogs

The plasma concentration individual results and concomitant concentration-time profiles for this study contained several irregularities based on expected pharmacokinetics. Some animals showed occasional delayed absorption, while others presented sudden increases in plasma concentrations approximately 8 h after oral administration, with significant prolongation of the terminal elimination half-life. This last observation is consistent with results from similar studies with resveratrol in other species and is attributed to enterohepatic recirculation [13-14]. The limited number of blood collection time points in the 8 to 24 h post-dose period for this study did not allow the modeling and estimation of the two apparent elimination phase rate constants believed to exist in the pharmacokinetic profile of resveratrol. Therefore, the pharmacokinetic parameters presented correspond to the estimation of the average elimination rate constant as permitted by the experimental data.

There were no consistent statistically significant changes in the $C_{\max}$ or AUC group mean values between Day 1 and Week 13 that would indicate evidence of induction or accumulation for either resveratrol or its metabolites. Similarly, statistical comparisons of $C_{\max}$ or AUC group mean values did not provide consistent evidence of differences among experimental groups due to gender. For resveratrol and resveratrol glucuronide, statistical comparison of AUC data indicated proportional increases with the dose for Day 1; however, for Week 13 AUC increases lacked proportionality to the dose. For resveratrol sulfate, changes for AUC on Day 1 were not proportional to the dose increases, while at Week 13, AUC changes were proportional to the dose changes, with one exception [low vs. high (females)].

Statistical comparison of mean AUC resveratrol glucuronide values normalized to resveratrol AUC values did not provide evidence of differences for the study groups. However, similar statistical comparisons performed for resveratrol sulfate indicated significant decreases relative to resveratrol AUC values with the dose for both Day 1 and Week 13.

Details of the toxicological observations for this dog study are presented elsewhere [35].

4.4. Comparison of resveratrol pharmacokinetics in dog, human and rat studies

Resveratrol pharmacokinetic results from experiments with a single oral dose in humans at 42 and 83 mg/kg (1500 and 3100 mg/m²; assuming 60 kg subjects) [36] and rats at 50 and 150 mg/kg (300 and 900 mg/m²) [13] as compared to our dog study at 200 to 1200 mg/kg (4000 to 24000 mg/m²) are as follows:

Mean $T_{\max}$ values in human and rat groups were 1.5 and 1.0 h, respectively, which compares well with 1.8 and 1.1 h (male and female, respectively) for our 200 mg/kg dog dose group, although increased to 3-5 h for the higher dose groups. Dose-normalized $C_{\max}$ mean values were 6.4 and 6.5, 1.5 and 5.6, and 4.3 to 13 ng/mL*kg/mg for human, rat and dog, respectively.

Mean $t_{1/2}$ values were 4.2 and 8.5 h for human, 12 and 3.6 h for rat and 1.9 and 3.8 h for dog. Dose-normalized AUC mean values were 19 and 16, 27 and 17, and 20 to 37 h*ng/mL*kg/mg for human, rat and dog, respectively.

Mean AUC ratios of resveratrol glucuronide to resveratrol were 13 and 14 (sum of glucuronide isomers), 8.6 and 8.8, and 1.8 to 3.4 for human, rat and dog, respectively. Similarly, the AUC ratios for resveratrol sulfate were 22 and 23, 9.3 and 18, and 2.9 to 11

for human, rat and dog, respectively. Although comparable, these results seem to indicate more extensive conjugation of the agent in humans.

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Highlights

- Plasma method validated for resveratrol metabolites without enzymatic hydrolysis
- Peak resveratrol plasma concentration in dogs observed within 1 to 5 h after oral dosing
- Resveratrol was rapidly metabolized to glucuronide and sulfate
- AUC ratios of resveratrol metabolites to resveratrol ranged from 1 to 11

Fig. 1.
Chemical structure of resveratrol

Fig. 2.

A. Resveratrol – blank dog plasma; B. Resveratrol – calibrator at the LLOQ (5 ng/mL) in dog plasma; C. Resveratrol – calibrator at 200 ng/mL in dog plasma; D. Resveratrol glucuronide – blank dog plasma; E. Resveratrol glucuronide – calibrator at the LLOQ (5 ng/mL) in dog plasma; F. Resveratrol glucuronide – calibrator at 200 ng/mL in dog plasma; G. Resveratrol sulfate – blank dog plasma; H. Resveratrol sulfate – calibrator at the LLOQ (10 ng/mL) in dog plasma; I. Resveratrol sulfate – calibrator at 400 ng/mL in dog plasma

Fig 3.

Representative plasma concentration-time curve for resveratrol, resveratrol glucuronide and resveratrol sulfate; A. male dog, 200 mg/kg/day group, Day 1; B. male dog, 600 mg/kg/day group, Day 1; C. male dog; 1200 mg/kg/day group, Day 1

Table 1
Within-run and between-run precision and accuracy for QC dog plasma samples

Nominal Concentration ^a (ng/mL)		Method Precision and Accuracy (%) ^{b, c}		
		Resveratrol	Resveratrol Glucuronide	Resveratrol Sulfate
5 or 10 (LLOQ)	Within-Run	98.2, 101, 105	103, 98.2, 109	100, 115, 109
	%RSD	4.8, 6.2, 6.2	3.0, 5.8, 4.3	3.8, 3.5, 3.3
	Between-Run	101	103	108
	%RSD	6.1	6.0	6.8
12 or 24 (Low QC)	Within-Run	91.9, 93.2, 98.5	97.4, 89.7, 96.5	92.9, 97.6, 95.5
	%RSD	6.5, 2.7, 9.1	1.0, 1.9, 3.2	1.7, 3.1, 4.7
	Between-Run	94.3	94.5	95.1
	%RSD	7.2	4.4	3.7
400 or 800 (Mid QC)	Within-Run	101, 102, 105	112, 107, 111	108, 104, 105
	%RSD	2.5, 1.7, 5.8	1.8, 1.7, 4.1	2.4, 5.4, 1.4
	Between-Run	102	110	106
	%RSD	4.0	3.3	3.6
800 or 1600 (High QC)	Within-Run	104, 103, 106	109, 105, 112	110, 106, 102
	%RSD	4.3, 2.3, 6.9	1.3, 3.6, 5.2	1.7, 5.0, 4.5
	Between-Run	104	109	106
	%RSD	4.8	4.3	4.7

^a QC dog plasma samples were prepared at 5, 12, 400 and 800 ng/mL for resveratrol and its glucuronide and 10, 24, 800 and 1600 ng/mL for resveratrol sulfate.

^b For all experiments, the number of QC samples (*n*) was 6 and 18 for individual validation runs (within-run) and between-run, respectively.

^c Accuracy expressed as % of true value, precision as % relative standard deviation (RSD).

Table 2
Stability assessment in dog plasma and dog plasma extracts

Nominal Concentration ^a (ng/mL) - Time	Mean Measured (SD) Recovery (% of true value)		
	Resveratrol	Resveratrol Glucuronide	Resveratrol Sulfate
Plasma Bench-Top Stability (Room Temperature)			
12 or 24 - 4 hours	92.4 (2.6)	98.5 (1.8)	96.3 (6.6)
12 or 24 - 24 hours	65.1 (4.4)	94.1 (1.6)	102 (5.6)
800 or 1600 - 4 hours	93.9 (3.3)	113 (2.2)	92.3 (3.4)
800 or 1600 - 24 hours	49.0 (2.9)	106 (1.7)	95.2 (2.8)
Plasma Refrigerated Stability (4 °C)			
12 or 24 - 24 hours	95.5 (1.0)	<i>_b</i>	<i>_b</i>
800 or 1600 - 24 hours	95.2 (2.2)	<i>_b</i>	<i>_b</i>
Plasma Freeze-Thaw Stability (3 Cycles) ^c			
12 or 24	97.6 (10)	109 (1.5)	99.9 (6.0)
800 or 1600	102 (3.4)	105 (2.7)	90.7 (8.0)
Plasma Extract Autosampler Stability (4 °C)			
12 or 24 - 6 days	103 (9.3)	99.4 (5.8)	102 (12)
800 or 1600 - 6 days	106 (4.4)	95.4 (4.6)	97.9 (1.8)
Plasma Long Term Stability (-70 °C)			
12 or 24 - 50 days	101 (17)	100 (2.0)	105 (15)
800 or 1600 - 50 days	100 (2.9)	105 (16)	107 (2.7)

^a QC dog plasma samples ($n = 6$) prepared at 12 and 800 ng/mL for resveratrol and its glucuronide and 24 and 1600 ng/mL for resveratrol sulfate.

^b Experiment not performed for the indicated target analyte.

^c Plasma samples were kept frozen at -70 °C.

Table 3
Summary of Pharmacokinetic Results – Group Mean Value Ranges

Resveratrol Dose (mg/kg/day)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (µg/mL)	AUC ^a (h*µg/mL)	AUC Ratio ^b	V_z/F ^c (L/kg)	CL/F ^c (L/h/kg)
Resveratrol							
200	2-4	1-2	1.7-2.6	3.6-5.0	1	120-280	41-58
600	3-4	3-4	3.7-9.0	13-30	1	120-300	22-51
1200	3-4	3-5	5.1-9.9	21-44	1	130-440	28-110
Resveratrol Glucuronide							
200	1-5	2-3	3.8-12	7.2-17	1-2	-	-
600	2-3	2-5	19-110	48-260	2-5	-	-
1200	2-3	4-7	40-210	160-560	3-9	-	-
Resveratrol Sulfate							
200	4-8	2-3	17-23	40-58	8-11	-	-
600	4-7	2-3	20-28	83-120	2-7	-	-
1200	4-9	2-5	18-30	76-170	2-4	-	-

^a AUC = AUC_{0-∞} for Day 1 and AUC_{0-24hr} for Week 13

^b AUC ratio was calculated as (AUC/MW)_{metabolite}/(AUC/MW)_{resveratrol}, where MW=molecular weight: 228, 404 and 308 for resveratrol, resveratrol glucuronide and resveratrol sulfate, respectively.

^c V_z/F and CL/F were not determined for resveratrol metabolites.