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Determination of *trans*-resveratrol and its metabolites in rat serum using liquid chromatography with high-resolution time of flight mass spectrometry

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ABSTRACT

In this study we developed a sensitive method using high performance liquid chromatography (HPLC) coupled to electrospray ionization (ESI) with high resolution time of flight (TOF) mass spectrometry (MS) for the determination of naturally occurring antioxidant *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, RES). This method enabled an investigation of a relationship between tumor growth in rats and concentration of RES and its primary metabolites, *trans*-resveratrol-3-O-sulfate-3-*O*-sulfate (R3S) and *trans*-resveratrol-3-*O*-β-D-glucuronide (R3G), in rat serum after RES exposure (5 or 25 mg/kg/day). RES levels in rat serum were near the limit of detection, showing concentrations of 4 ± 1 and 12 ± 4 ng/mL for low and high-dose exposure, respectively. Compared to RES, higher concentrations were found for its metabolites (R3G: 4.8 ± 0.3 and 6.8 ± 0.3 μg/mL; R3S: 0.27 ± 0.09 and 0.34 ± 0.04 μg/mL, respectively). Using TOF, for the first time, we measured the matrix affected limits of detection (LODs) in plasma (3.7, 82.4, and 4.7 ng/mL for RES, R3G, and R3S, respectively), which were comparable to those reported in previous work using HPLC tandem mass spectrometry, but with a benefit of a full mass spectral profile.

The ability to acquire data in full scan mode also revealed other isomers of R3S. The additional novelty of our study is in synthesis and application of deuterated recovery standards enabling accurate and precise quantification. In order to develop a robust method, the ESI conditions were optimized using a multilevel full factorial design of experiments.

1. Introduction

trans-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) (RES) is a polyphenol found in many foods, such as red wine, peanuts and berries [1]. RES and its metabolites have been reported to exhibit anticancer, analgesic, cardioprotective, and neuroprotective effects [1]. Numerous animal studies have already been performed to evaluate the benefits of RES [6,7]. A majority of animal studies involving RES have been employing rats [8,9], as reviewed by Park and Pezzuto [6], while use of other animals, such as pigs [5,10] and dogs [11] have been reported. The doses of RES in these studies varied greatly, between 1 and 1200 mg/kg/day [5,8–11]. The specific RES metabolites i.e., *trans*-resveratrol-3-*O*-β-D-glucuronide (R3G) and -3-O-sulfate *trans*-resveratrol-3-*O*-sulfate (R3S) sulfate and glucuronide were shown to be the most abundant primary resveratrol metabolites in rat serum [2–5]. Long-term clinical studies are still necessary to investigate its effectiveness in humans.

Various HPLC–MS methods have been employed to detect and quantify RES and its metabolites in blood and tissue samples as summarized in Supplementary Table 1 [2–5,8–27]. The

Abbreviations: RES, trans-resveratrol; R3G, trans-resveratrol-3-*O*-β-D-glucuronide; R3S, trans-resveratrol-3-O-sulfate; RtS, resveratrol trisulfate; MeOH, methanol; ACN, acetonitrile; HPLC-ESI-HRMS, high pressure liquid chromatography–electrospray ionization–high resolution mass spectrometry; RS, recovery standard; IS, internal standard; E2, estradiol; DOE, design of experiments; FIA, flow injection analysis.

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constituents of the mobile phase employed generally consisted of various mixtures of methanol (MeOH) [12–19] or acetonitrile (ACN) [2–5,8–11,20–26] with water and electrolytes such as acetic acid [4,26,27], ammonium acetate [12,15,18,19,27], or formic acid [2,5,9–11,14,17,20]. For the MS analysis, electrospray ionization (ESI) was typically used in negative mode, with voltages ranging from 2500 to 5000 V [2,4,5,10–24,26]. The limits of quantification were between 0.1 and 63 ng/mL [5,8,11,14,16–19,22,23,25,27]. The variety of conditions and matrices reported precludes the selection of the most sensitive method. Moreover, reasons for the selection of a particular electrolyte, its concentration, or the ESI voltages were generally not provided.

HPLC with tandem MS/MS has generally been employed for quantification and confirmation of the identity of various resveratrol species [2–5,8–11,13–15,17–23,25–27]. To our knowledge, liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) has not been used for quantification of resveratrol species, and only one study used HPLC-HRMS for identification of such compounds [5].

A variety of internal standards (ISs) have been used in the HPLC–MS analysis of RES and its metabolites. The ISs were often compounds of different chemical structure from RES [2,6,9,10,12,14,15,17–20,23], and only two studies reported the use of $^{13}C_6$-RES as an IS, which was added before extraction to correct the RES recoveries [18,11]. In these studies, $^{13}C_6$-RES was used as the IS for RES determination but not for its metabolites. The application of the ^{13}C RES analog enables more accurate monitoring of RES behavior during sample processing but the cost is rather high and thus may be prohibiting. The application of a deuterated RES is more economical than that of the ^{13}C RES analog, has not previously been reported as an IS, nor as a possible recovery (surrogate) standard.

In this study, we optimized an HPLC-ESI-HRMS method to determine levels of RES and its metabolites, R3G and -3-O-sulfate *trans*-resveratrol-3-*O*-sulfate R3S (the target species are shown in Table 1). To increase the method precision, deuterated standards of RES, R3G, and R3S were synthesized and used as recovery standards (RSs) added prior to the sample preparation in combination with an IS (pinosylvin), which was added before injection. This combination of RSs and IS (used frequently in environmental studies) allowed for an improved understanding of repeatability issues (i.e., whether the issues arise from sample preparation or analysis). The developed method was applied to rat serum samples following their exposure to RES.

2. Experimental

2.1. Materials

RES (≥99%), pinosylvin (97%) and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). ACN, MeOH, acetic acid, and ammonium acetate (all LC–MS quality) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was obtained using a Direct-Q 3 UV water purification system (Millipore Corporation, Billerica, MA, USA). Toluene-d_8 was purchased from Cambridge Isotope Laboratories, other starting materials for the synthesis (3,5-dihydroxybenzoic acid, iodomethane, triethylphosphite) were purchased from Sigma-Aldrich.

2.2. Synthesis and purification of RES analogs and deuterated RSs

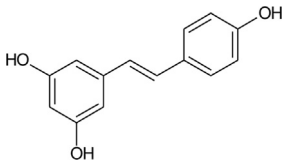
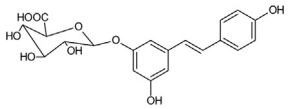
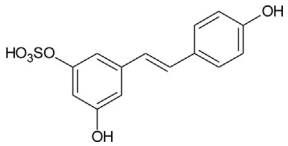
R3G and R3S were synthesized using a previously developed procedure for non-deuterated analogs [29]. Resveratrol-d_5 (RES-d_5), along with its conjugates (R3S-d_5 and R3G-d_5), were prepared using a modification of this procedure (described in detail in Supplementary materials), based on the condensation of 3,5-dimethoxybenzyl diethylphosphonate and anisaldehyde-d_5 (Fig. 1). Anisaldehyde-d_5 was prepared from readily available commercial toluene-d_8, relying on the reported highly selective NaY zeolite catalyzed *para*-bromination, followed by radical benzylic bromination [30].

Synthesized R3G and RES-d_5 were purified using solid phase extraction (SPE), with a Waters Sep-Pak C_{18} cartridge. R3G, in 0.5 mM acetic acid, was eluted with 10% and 20% MeOH and RES-d_5, in 90 mM acetic acid, was eluted with 40% ACN; their HPLC purity was 98% and 89%, respectively. Synthesized R3S, R3S-d_5, and R3G-d_5 were dissolved in 100% MeOH, which resulted in HPLC purity of 99%, 90%, and 86%, respectively. No non-deuterated compounds were observed in the mass spectra of the deuterated analogs.

2.3. Sample preparation

Standards for the ESI optimization were solutions consisting of RES, R3G, or R3S (4 μg/mL each) in a range of electrolyte concentrations (0–10 mM ammonium acetate or 0–100 mM acetic acid) in 50% MeOH/H_2O or 50% ACN/H_2O. For the analysis of serum samples, calibration solutions were in the range 2–70 ng/mL of RES and R3S, and 0.2–6.7 μg/mL of R3G dissolved in the mobile phase. Calibration of RSs was in the range 2–125 ng/mL of RES-d_5 and R3S-d_5, and 0.1–5.0 μg/mL of R3G-d_5 in the same solvent system.

Table 1
Target analytes, their structures, the mass of the ions used for quantification, and determination of LODs.

Compound	Formula	Structure	Quantification ion [M-H]$^-$ (m/z)	Confirmation ion (m/z)
trans-resveratrol (RES)	$C_{14}H_{12}O_3$		227.07137	228.07765
trans-resveratrol-3-*O*-β-D-glucuronide (R3G)	$C_{20}H_{20}O_9$		403.103456	227.07137
-3-O-sulfate *trans*-resveratrol-3-*O*-sulfate (R3S)	$C_{14}H_{12}O_6S$		307.028182	227.07137