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Pre-formulation studies of resveratrol

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Abstract

Context—Resveratrol, a natural compound found in grapes, has potential chemotherapy effects but very low oral bioavailability in humans.

Objective—To evaluate the solubility, pH stability profile, plasma protein binding (PPB) and stability in plasma for resveratrol.

Methods—*Solubility* of resveratrol was measured in 10 common solvents at 25 °C using HPLC. The *solution state pH stability* of resveratrol was assessed in various United States Pharmacopeia buffers ranging from pH 2 to 10 for 24 h at 37 °C. Samples were analyzed up to 24 h. Human PPB was determined using ultracentrifugation technique. Standard solutions of drug were spiked to blank human plasma to yield final concentrations of 5, 12.5 or 25 μg/mL for determination. Finally, *stability* of resveratrol in human and rat plasma was also assessed at 37 °C. Aliquots of blank plasma were spiked with a standard drug concentration to yield final plasma concentration of 50 μg/mL. Samples were analyzed for resveratrol concentration up to 96 h.

Results—Resveratrol has wide solubility ranging from 0.05 mg/mL in water to 374 mg/mL in polyethylene glycol 400 (PEG-400). Resveratrol is relatively stable above pH 6 and has maximum degradation at pH 9. The mean PPB of resveratrol is 98.3%. Resveratrol degrades in human and rat plasma in a first-order process with mean half lives of 54 and 25 h, respectively.

Conclusion—Resveratrol is more soluble in alcohol and PEG-400 and stable in acidic pH. It binds highly to plasma proteins and degrades slower in human then rat plasma.

Keywords

Natural compound; physiochemical evaluation; plasma protein binding; polyphenol; solubility; stability

Introduction

Resveratrol (trans-3,5,4′-trihydroxystilbene) is a polyphenolic compound that is synthesized in grapes, berries, peanuts and plants to respond to ecological strain and pathogenic infection[1,2]. This natural compound has been of great interest to many because of its cardiovascular benefits due to its presence in red wine. In addition, numerous cell culture studies have revealed its potential health benefits such as: anti-aging, anti-oxidative, anti-diabetic, anti-inflammatory, anti-obesity, neuroprotective and cardio-protective activities[1–4].

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Previous studies have shown resveratrol to also have pharmacological activities as a chemopreventive agent[1–4]. Resveratrol has demonstrated several cellular mechanisms to prevent tumor initiation, promotion and development in the process of carcinogenesis[5]. Although this dietary compound seems promising, studies revealed the pharmacokinetic parameters do not show the same advantages as its pharmacological activities[1].

Resveratrol is a weak acid consisting of two phenols joined together by a double bond. Due to the deprotonation of the hydroxyl groups present in the compound, resveratrol has three acidic dissociation constants ($pK_{a1,2,3}$ = 8.8, 9.8 and 11.4)[6]. This natural compound has poor water solubility (0.05 mg/mL) with a partition coefficient of 3.1[7]. Earlier studies demonstrated solid state of resveratrol to be stable regardless of changing different temperature, humidity and light conditions[8]. However, the solubility in other solvents and stability at various pH is not fully understood.

It is known that resveratrol has very low oral bioavailability[9]. For example, after oral administration of a 0.2 mg dose of resveratrol in humans, there was less than 5 ng/mL of this compound detected at any time point in the six volunteers[9]. Previous studies suggest that high dose of resveratrol is not sufficient for systemic levels of chemoprevention suggesting that resveratrol undergoes significant biotransformation via conjugation pathways[10]. However, other mechanisms such as rapid degradation in plasma, high binding to tissues or low binding to the plasma could occur. Therefore, it is still unclear of the degradation kinetics of resveratrol in biological medium. To further investigate resveratrol rapid elimination *in vivo*, we desired to see whether plasma would cause rapid degradation of this compound. Past reports have found very low concentrations of drug in plasma, and it is suspected that this compound may be highly bounded to plasma and or tissue, or rapidly degraded in systemic circulation. However, there is no evidence in the literature to support these hypotheses. Therefore, our study is to investigate the plasma protein binding (PPB) and to evaluate the *in vitro* stability in plasma, as well as the solubility and solution state pH stability of resveratrol.

Materials and methods

Materials

All chemicals and solvents used in this study were HPLC grade. Resveratrol (Figure 1) standard powder, warfarin standard powder, HPLC water and acetonitrile, castor oil, cremophor-EL, soybean oil and Tween 80 were all purchased from Sigma Aldrich Co. (St. Louis, MO). Sodium dihydrogen phosphate monohydrate and polysorbate 80 were obtained from Biochemika Fluka (Buchs, Switzerland). Potassium biphthalate was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Potassium phosphate monobasic and sodium hydroxide were acquired from Fisher Scientific (Fair Lawn, NJ). Phosphoric acid and alcohol reagent were purchased from EMD Chemicals (Gibbstown, NJ). Labrasol and Labrafil were provided by Gattefossé (Nanterre, France) as a gift. Polyethylene glycol 400 (PEG-400) was provided by Wood Scientific (Houston, TX) and boric acid granular was purchased from Professional Compounding Centers of America, Inc. (Houston, TX). Potassium chloride was procured from Matheson Coleman & Bell (Cincinnati, OH). De-ionized water was obtained using a Milli-Q analytical deionization system from Millipore

Corporation (Bedford, MA). To complete the pre-formulation experiments, a centrifuge 5417 C from Eppendorf (Hauppauge, NY) and a precision heated water bath purchased from Lab Care America (Winchester, VA) were both utilized.

Chromatographic conditions

A HPLC method from a previous study by Katsagonis et al.[11] was adopted and modified to estimate resveratrol concentrations in the various pre-formulation studies. The HPLC-UV instrument used to analyze resveratrol included 717 plus auto sampler, 600 pump and 2487 dual λ absorbance detector, all from Waters Corporation (Milford, MA). In conjunction with this HPLC-UV system, a XTerra MS C_{18} column with dimensions of 150 × 4.6 mm i.d. packed with 3.5 μm particles (Waters Corporation) was operated. An isocratic elution was used consisting of 20 mM of potassium phosphate monobasic in acetonitrile and water. The two solutions were mixed at a ratio of 50:50% v/v, respectively. The final pH was adjusted to 3.0 with phosphoric acid and then filtered using 0.45 μm membrane filter (Waters Corporation). The wavelength used was 300 nm. The flow rate was set to 1.0 mL/min, and the injection volume was 25 μL. Warfarin was used as internal standard (IS) for this assay.

The stock solution of resveratrol (1 mg/mL) was prepared by dissolving 0.01 g of resveratrol in 10 mL of acetonitrile and stored at −80 °C. Six working standard solutions ranging from 1.56 to 200 μg/mL were individually prepared daily by diluting the stock solution with the mobile phase. Warfarin (50 μg/mL) in acetonitrile was applied as the IS. The standard curve was created by plotting the peak area ratio of resveratrol to IS against the known concentration of resveratrol. The limit of detection (LOD) and the limit of quantification (LOQ) were evaluated based on a signal-to-noise ratio of 6. The accuracy and precision of intra-day and inter-day assay were determined by analyzing samples (n = 6) with three different concentrations (5, 35 and 75 μg/mL) using a standard curve constructed on the same day or three sequential days.

Solubility

To gain an array of knowledge about the solubility of resveratrol, 10 common solvents were selected and evaluated at room temperature using the shaker method. Briefly, an excess amount of resveratrol was added to each amber scintillation vial containing 5 mL of each solvent. Next, the mixtures were shaken vigorously at room temperature up to 27 d to reach drug saturation; 100 μL of sample was removed from each vial and centrifuged for 10 min. Samples were then diluted with mobile phase and analyzed by the established HPLC method above. To prevent photodegradation, all samples were protected from light and performed in triplicate.

Solution state pH stability

To illustrate the behavior of the resveratrol in acidic, neutral and basic conditions, next we investigated the solution state pH profile for the drug at a pH range of 2.0–10 in a 37 °C water bath while secluded from light. Hydrochloric acid, acid phthalate, neutralized phthalate, phosphate and alkaline borate buffer solutions (0.2 M) were prepared according to United States Pharmacopeia (USP) guidelines. Using 1 mg/mL of resveratrol stock solution, an aliquot of drug was added to an amber scintillation vial in a 1:10 ratio of drug to buffer (*n*

= 3). The mixture was vortexed for 30 s, and vials were immersed in the 37 °C water bath restricted from light. At predetermined time points, 100 μL of sample was removed and 100 μL of mobile phase was added. Immediately following, the mixtures were stored at −80 °C until analysis. After the last sample was removed, all samples were completely unthawed, and the degradation of resveratrol was assessed using the HPLC method listed above.

Plasma protein binding

Due to the rapid loss of resveratrol *in vivo* from our preliminary studies, we characterized the PPB using the ultracentrifugation technique in human plasma at 37 °C. Human plasma was provided by healthy volunteers from the Gulf Coast Regional Blood Center of Houston, TX. Male Sprague Dawley rat plasma was procured from Harlan Laboratories of Houston, TX. In brief, 80 μL of standard drug solutions were spiked to 270 μL of blank human plasma in amber vials to yield final concentration of 5, 12.5 and 25 μg/mL. Thenceforward, the drug and plasma blend were vortexed for one minute and then incubated for 30 min into a 37 °C water bath while protected from light. Finally, the mixture was placed into ultrafiltration devices (n = 6) and centrifuged for 10 min. For examination, 100 μL unfiltered sample was processed by protein precipitation through the addition of 20 μL of IS and 180 μL of acetonitrile. Sample was centrifuged for 10 min, and 100 μL of supernatant was removed. Both concentrated filtrate and unfiltered samples underwent HPLC analyses holding the same chromatographic conditions describe above. Percent PPB was calculated by the following equation:

$$\text{Percent plasma protein binding}\% = \frac{\text{concentrated unfiltered sample}}{\text{total concentrated filtrate} + \text{unfiltered sample}} \times 100\%$$

Stability in plasma

To further investigate the behavior of resveratrol in biological medium, the stability profile of resveratrol in human and rat plasma at 37 °C up to 96 h was implemented. In short, an 80 μL of resveratrol stock solution (500 μg/mL) was spiked to 720 μL of blank rat and human plasma (n = 6). The drug and plasma mixture were vortexed for one minute and then incubated into a 37 °C water bath while protected from light. A 100 μL sample was extracted from each batch at 0, 24, 48, 72 and 96 h in triplicate from each batch. Thereafter, samples underwent protein precipitation by the addition of 180 μL of acetonitrile and then centrifugation for 10 min at 16 400 rounds per minute (rpm); 100 μL of supernatant was removed and placed in an amber microcentrifuge tube and stored in −80 °C until examination. Upon analysis, 20 μL of IS was added to each completely unthawed sample and vortexed for 30 s while protected from light. Finally, 25 μL of sample was injected into the HPLC for analysis using the method listed above.

Data analysis

All pre-formulation studies for resveratrol were evaluated using SigmaPlot 10.0 (San Jose, CA). The calculated half-lives were obtained using first-order equation of kinetics.

Results

Chromatographic methods

The HPLC method was used for the analysis of resveratrol in all pre-formulation experiments. Figure 2 shows a representative HPLC-UV chromatogram of resveratrol and IS along with a sample calibration curve. It is evident that resveratrol and IS peaks are completely separated in the chromatogram. The retention time for resveratrol and IS was 2.7 and 7.0 min, and the chromatographic run time was 8 min, respectively. Standard curves in solution were prepared with resveratrol to yield a linear (r^2 = 0.999) calibration curve in solution at concentrations of 1.5, 10, 25, 50 and 100 μg/mL (Figure 2). These results indicate the simplicity and specificity of this assay method. The LOD and LOQ of resveratrol in the HPLC assay were found to be 0.39 μg/mL and 0.78 μg/mL, respectively. Intraday and interday assay accuracy and precision values for resveratrol in solution (5, 35 and 75 μg/mL) were all within 15%, the acceptable limit, suggesting the method was both precise and accurate. This method validation confirmed that our HPLC method for resveratrol was optimized for the following pre-formulation studies.

Solubility

The solubility of resveratrol in common mediums are presented in Figure 3. It ranges from 0.05 mg/mL in water to 373.85 mg/mL in PEG-400. Resveratrol is not soluble in water. This polyphenol compound contained the highest solubility in alcohol (87.98 mg/mL) and PEG-400 (373.85 mg/mL).

Solution state pH stability

The solution state pH stability profile is presented in Figure 4: the solution state stability of resveratrol in various 0.2 M buffers for 8 d. More than 70% of the original concentration of resveratrol remained in acidic and neutral conditions at 193 h. Resveratrol appears to be least stable in basic conditions. Less than 2% of resveratrol remained at 50 and 80 h at pH 8, 9 or 10. It is observed that resveratrol demonstrates first-order degradation in all USP buffers with the exception of pH 10. Resveratrol appears to have a biphasic degradation pattern in this basic environment.

The pH-rate profile for resveratrol was constructed by plotting rate constants versus pH (Figure 5). The rate constants obtained from the slopes of the curves were used to calculate the half-lives at various pH (Table 1). The s-shape rate profile suggests rapid degradation at pH 8–10 compared to the slower degradation at pH 2–7, respectively. The calculated half-lives ranged from 262.5 days at pH 5 to 0.072 days at pH 9.

Plasma protein binding

The PPB of resveratrol in human plasma at 37 °C through the ultracentrifugation process was consistently 98% over three concentrations of 5, 12.5 and 25 μg/mL (Table 2). Resveratrol demonstrated high PPB over these various concentrations.

Stability in human or rat plasma

Stability results are summarized in Figure 6. We observed resveratrol to be unstable in human and rat plasma samples at 37 °C up to 96 h. Furthermore, resveratrol followed first-order degradation kinetics in both human and rat plasma with a calculated mean half-life of 54 and 25 h, respectively.

Discussion

Resveratrol has gained great interest to researchers over the past couple of years due to its high concentration in red wine and cardioprotective effects known as ‘‘French paradox’’[9,12]. Although this unique polyphenol has had many favorable results after *in vitro* studies, there are a number of outstanding questions of how the drug behaves as well as disposes once administered *in vivo* that result in an unfavorable pharmacokinetic profile. Furthermore, there are very limited physiochemical characteristics of this natural compound. These unanswered questions and vague information could be the lacking knowledge that is keeping this natural compound from having a clinical significance for the prevention of cancer.

We have characterized the solubility profile of resveratrol and we found it to be soluble in 9 of the 10 tested solvents with the exception of water. Our data is consistent with prior findings in water and alcohol[5,13]. According to the USP[14] description and relative solubility chart, we found resveratrol to be freely soluble in the nonionic PEG-400 and soluble in alcohol. The other natural oils, surfactants and co-surfactants were either very slightly soluble, slight soluble or sparingly soluble. All of our solvents with the exception of water proven to be soluble.

Solubilities of resveratrol in these solvents were probably due to the lipophilicity of resveratrol ($\log P_{o/w} = 3.1$). Some drugs maybe very soluble in nature due to extensive hydrophobic hydroxyl group interactions[15]. We discovered that PEG-400 and alcohol are good solubilizing agents for resveratrol.

Data from our pH stability studies suggest that resveratrol is reasonably stable in acidic mediums up to pH 6. The compound starts to rapidly degrade at neutral and basic conditions due to basic hydrolysis. This is consistent with the behavior of a phenol[16]. Phenols are weak acids and consist of an aromatic ring with an attached hydroxyl group[16]. Resveratrol, a phenol like compound, becomes ionized when the pH increases in the basic medium and causes the compound to be unstable resulting in rapid degradation. The highest degradation was observed at pH around 8–9. It appears that the drug is most stable at pH 5 and 6 with estimated half-lives at about 196 and 263 d, respectively. Our results suggest that resveratrol may be formulated in a medium with pH of less than 6. It is also known that resveratrol undergoes photodegradation upon exposure to UV light[17]. As a result, all experiments were protected from light. In addition, resveratrol undergoes oxidation; however, this process does not occur rapidly[18].

Limited pharmacokinetic studies are available with respect to the *in vivo* behavior of resveratrol. Even though there are a number of studies that have shown the efficacy of this compound, there are few correlations of pharmacokinetic data and the *in vivo*

pharmacodynamics outcomes. For example, less than 5 ng/mL of resveratrol was detected at all time points after a 25 mg oral dose of resveratrol in human volunteers[9]. Because of resveratrol disappearance, it is not clear what can cause this behavior. We suspect that this challenge could be caused by rapid degradation in the plasma, high binding to the tissues or low binding to the plasma. These hypotheses lead us to investigate the PPB in resveratrol. Our results found high binding of resveratrol to plasma proteins, which rules out the possibility of the drug rapidly disappearing from the systemic circulation. On the other hand, when we look at the stability of this polyphenol, we found that resveratrol has apparent first order degradation in both human and rat plasma demonstrating a mean apparent degradation half-lives of 54 and 25 h, respectively. These half-lives indicate that resveratrol is not immediately degraded by plasma. Therefore, other mechanisms may exist in terms of the rapid disappearance of resveratrol *in vivo*, such as rapid metabolism in the liver by cytochrome P450, high tissue bindings, etc. Further investigation is needed to explore the mechanisms involve in the disappearance of this compound.

Conclusion

In our study, we investigated the solubility of resveratrol in various solvents, solution state pH stability, PPB and stability in human plasma. The natural compound had the highest solubility in alcohol and PEG-400. Resveratrol was stable in acidic conditions. Resveratrol is 98% plasma protein bound. This polyphenol is unstable in human and rat plasma with calculated half-lives of 54 and 25 h, respectively. Our pre-formulation studies provide additional understanding about the physical and chemical state of resveratrol.

Acknowledgments

Declaration of interest

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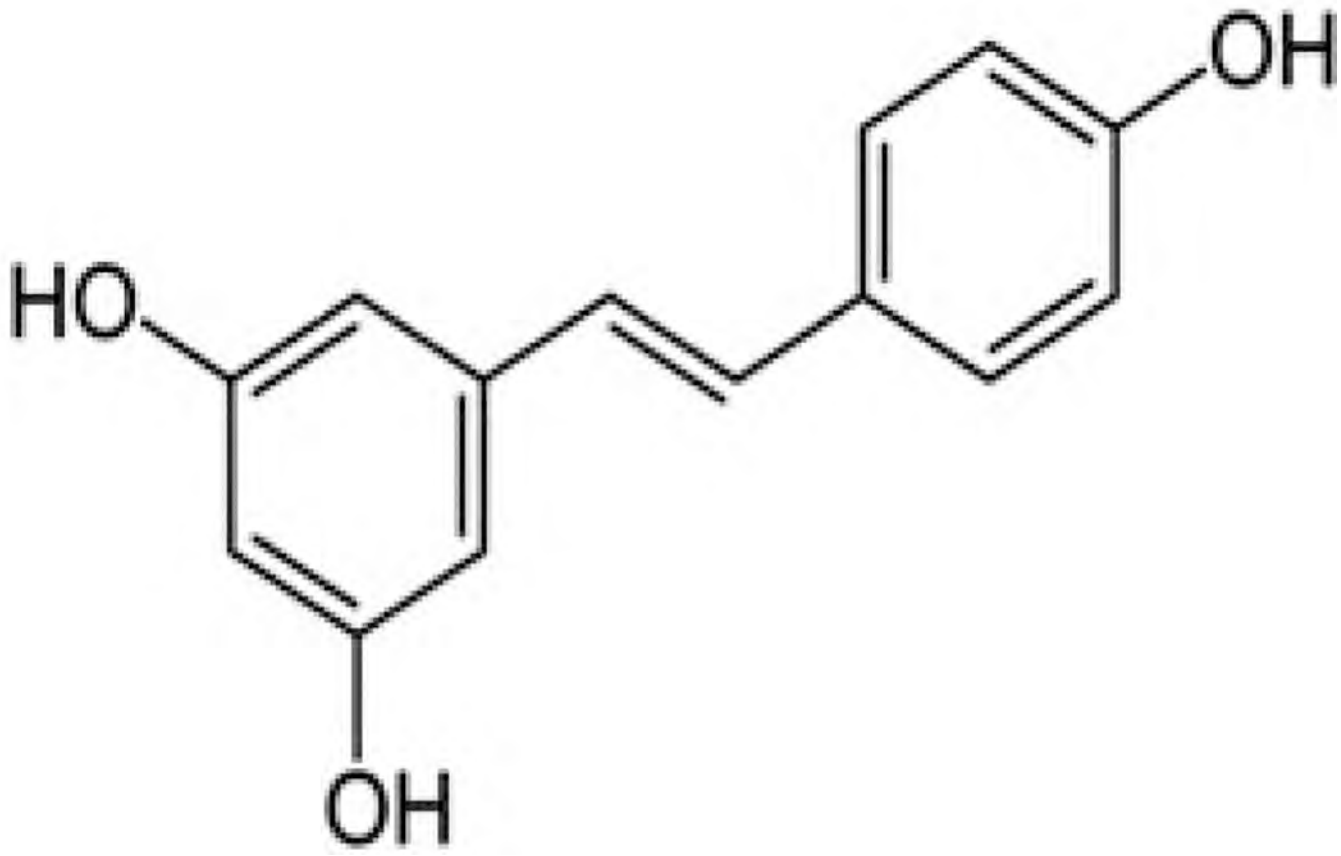

Figure 1.
Chemical structure of resveratrol.

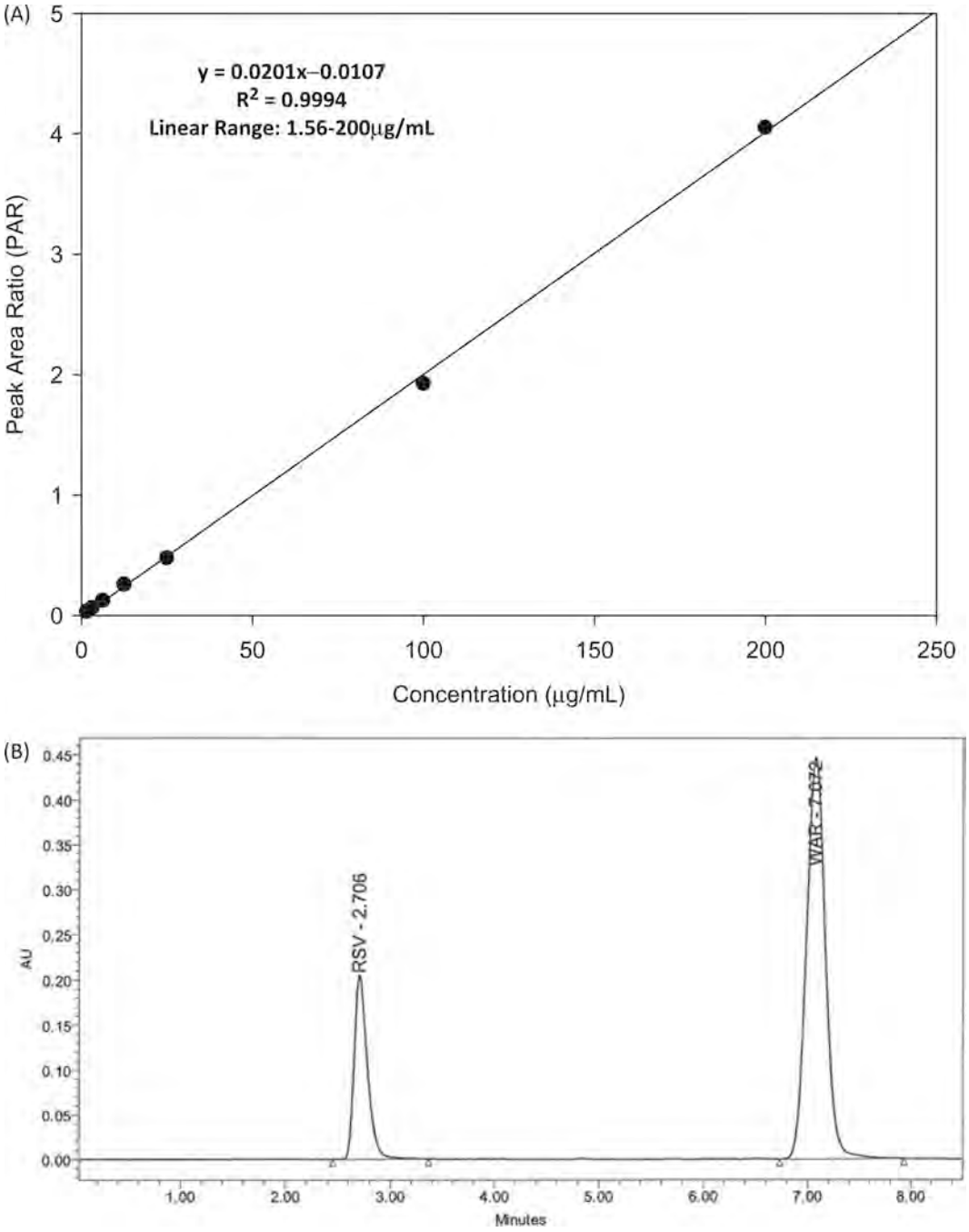

Figure 2.

(A) Calibration curve for resveratrol and internal standard in solution.

(B) Representative chromatogram of resveratrol 6.25 µg/mL and internal standard in solution.

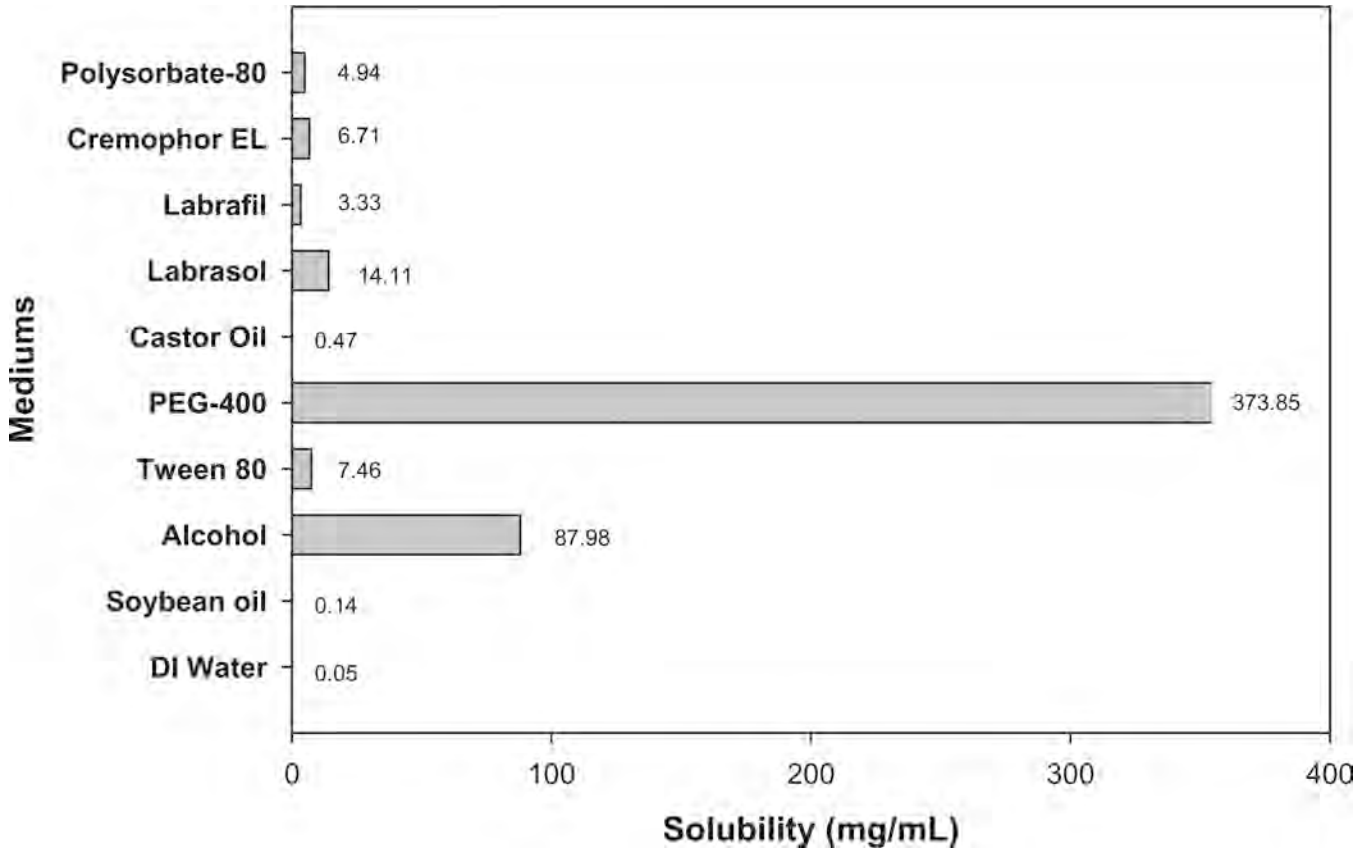

Figure 3.
Solubility of resveratrol in various solvents. Data expressed as mean, $n = 3$.

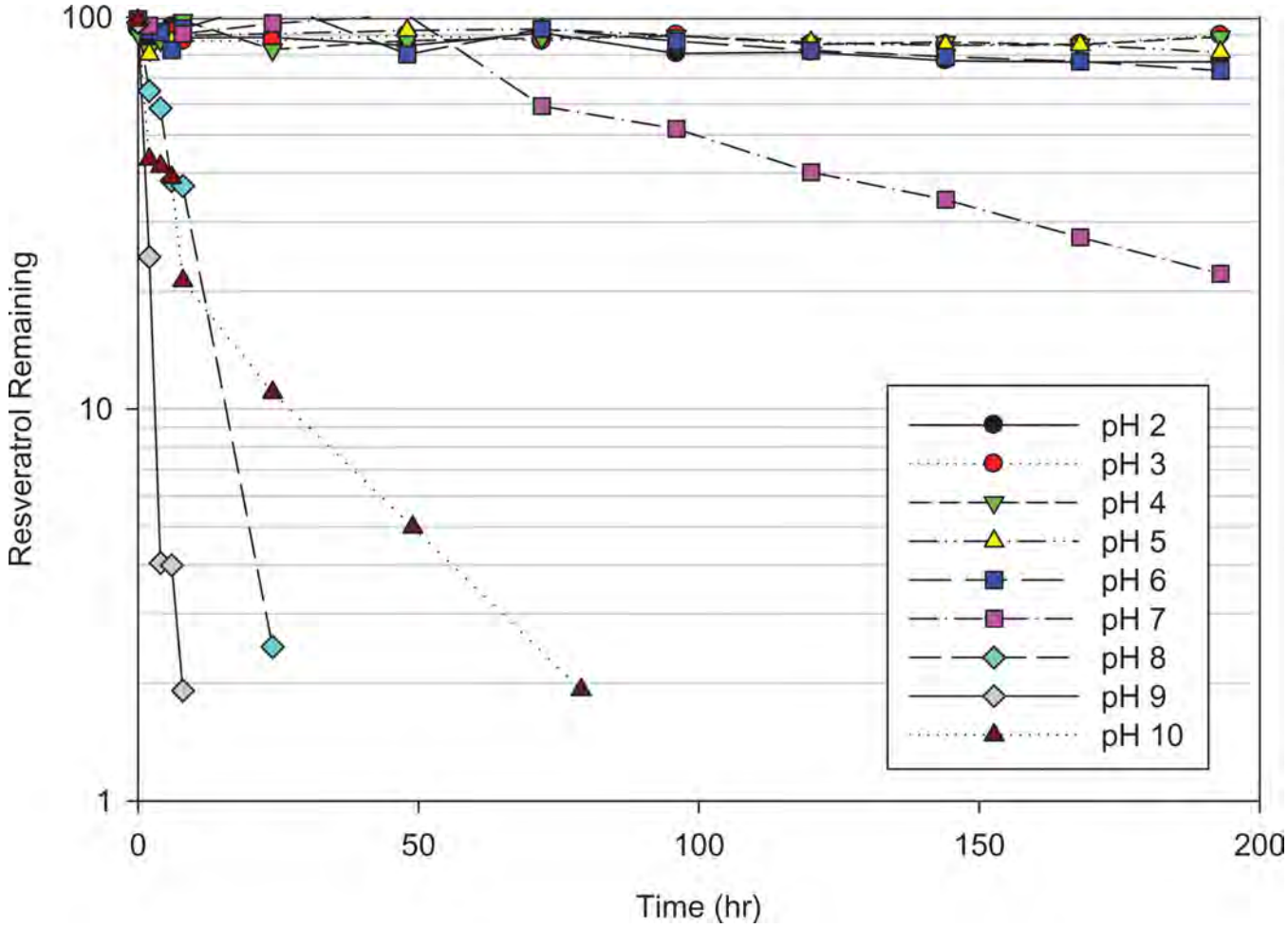

Figure 4.

Solution state pH stability of resveratrol in various USP buffers. Average (n = 3) Log% of drug remaining versus time for data presented in pH rate profile. Hydrochloric acid buffer solution used for pH 2, acid phthalate buffer solution used for pH 3 and 4, neutralized phthalate buffer solution used for pH 5, phosphate buffer solution used for pH 6 and 7 and borate buffer solutions used for pH 8–10.

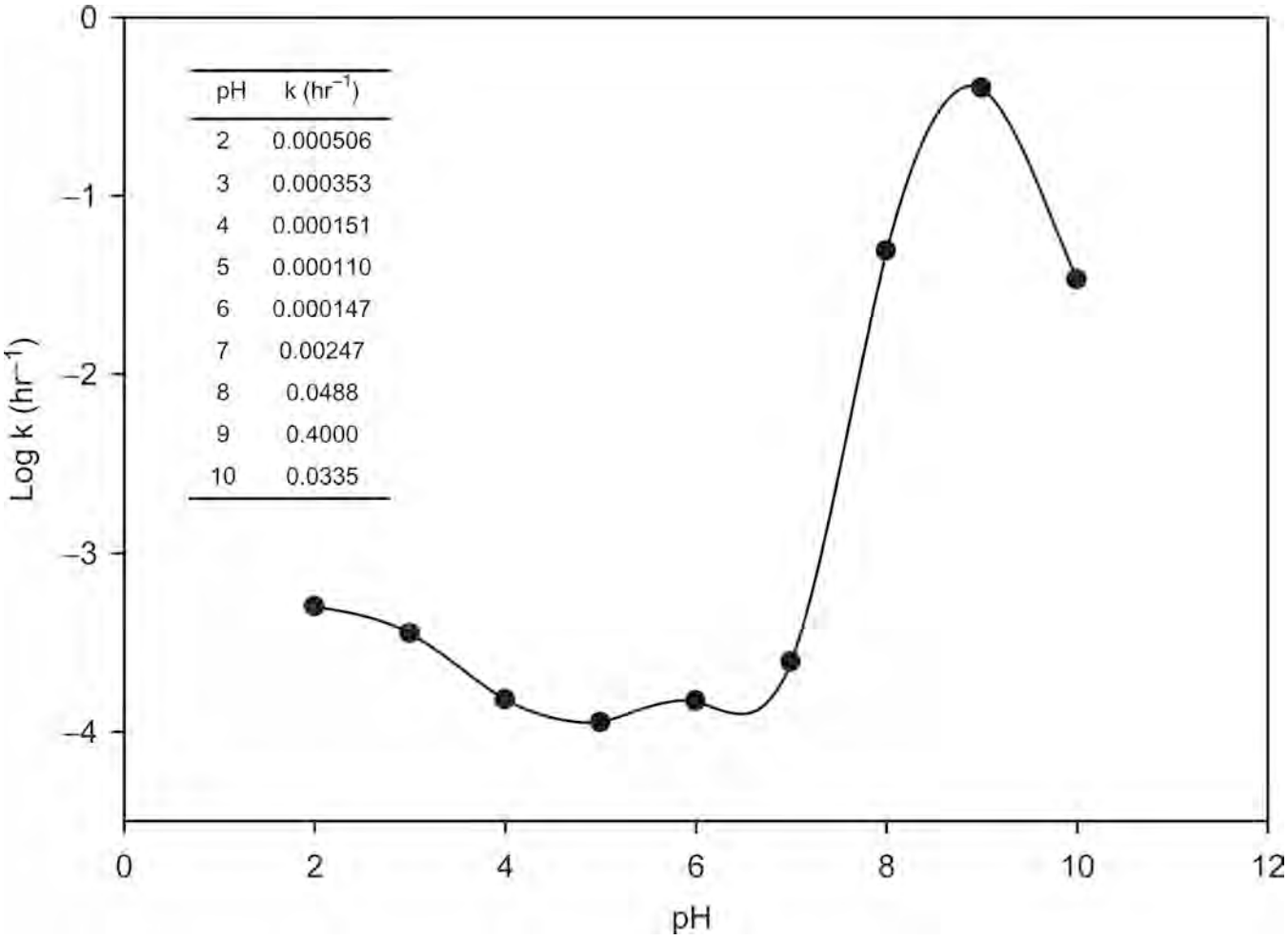

Figure 5.
pH-rate (log *k* versus pH) profile for resveratrol at 37 °C.

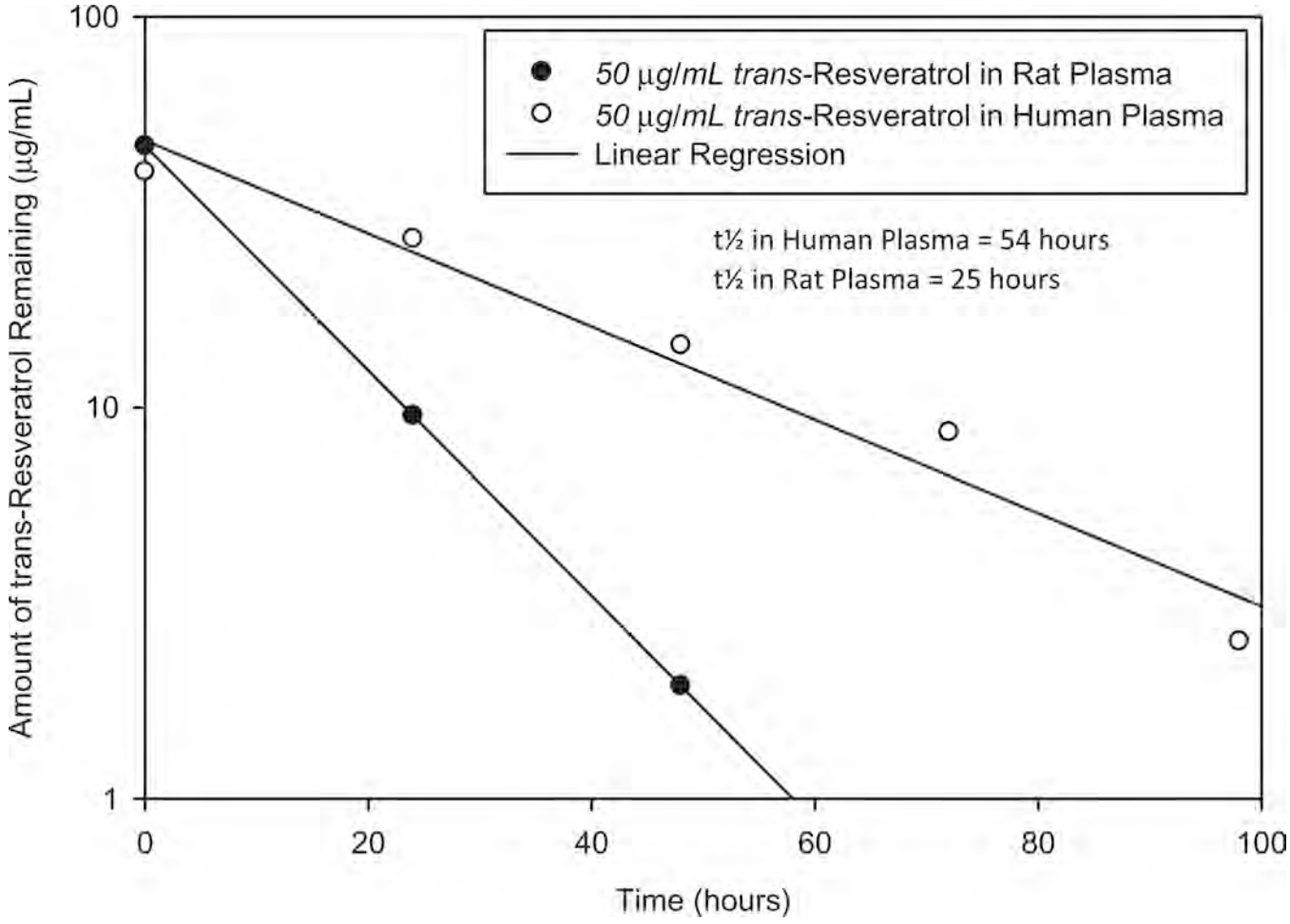

Figure 6.
Representative graph of stability of resveratrol in spiked human and rat plasma at 37 °C.

Table 1

Degradation rate constants (k), degradation half-lives ($t_{½}$) of resveratrol in various pH buffers.

pH	k (h^{-1})	$t_{½}$ (d)
2	0.000506	57.75
3	0.000353	81.79
4	0.000151	191.2
5	0.000110	262.5
6	0.000147	195.5
7	0.00247	11.6
8	0.0488	0.592
9	0.4000	0.0722
10	0.0335	0.8619

Table 2

Resveratrol plasma protein binding in spiked human plasma. Data expressed as mean ± SD.

Concentration (μg/mL)	Bound fraction (%) ±standard deviation (*n* = 6)
5	98.2 ± 0.41
12.5	98.5 ± 0.23
25	98.3 ± 0.33