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Determination and assay validation of pinosylvin in rat serum: application to drug metabolism and pharmacokinetics

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Abstract

A method of analysis of pinosylvin in biological fluids is necessary to study the kinetics of in vitro and in vivo metabolism and determine its concentration in natural products. A novel and simple high-performance liquid chromatographic method was developed for simultaneous determination of pinosylvin and products of its metabolism in rat serum and liver microsomes. Serum, or microsomes (0.1 mL) were precipitated with acetonitrile after addition of the internal standard, 7-ethoxycoumarin. Separation was achieved on an amylose tris 3,5 dimethylphenylcarbamate column (150 mm × 4.6 mm, ID, 5 μm) with UV detection at 308 nm. The calibration curves were linear ranging from 0.5 to 100 μg/mL. The mean extraction efficiency was >99%. Precision of the assay (coefficient of variation) was <10%, including the limit of quantitation (0.5 μg/mL). Bias of the assay was lower than 15%. The limit of detection was 100 ng/mL for a 0.1 mL sample. The assay was successfully applied to both the in vitro and in vivo metabolic kinetic study of pinosylvin. Three metabolites of pinosylvin, two oxidative and one glucuronidated, have been identified. The two oxidative metabolites of pinosylvin have been identified as E- and Z-resveratrol.

Keywords: Reversed-phase HPLC; UV-detection; Kinetics; Pinosylvin

1. Introduction

Pinosylvin (*trans*-3′,5′-dihydroxystilbene) $C_{14}H_{12}O_2$, M_W 212 (Fig. 1A) is a naturally occurring stilbene present in the wood pulp of pine and eucalyptus trees, and is present in tea oils and herbal remedies [1–4]. The biological function of pinosylvin in trees is to protect the tree against severe biotic stress. Pinosylvin functions as a phytoalexin, and is secreted in situations in which the tree is susceptible to infection by fungal agents. Pinosylvin possesses potent anti-fungal activity against a wide assortment of fungi [5,6].

Pinosylvin is a promising chemopreventative agent with anti-leukemic activity and is being extensively studied in various cancers including colorectal and liver cancers. Pinosylvin is structurally similar to the anti-cancer stilbene resveratrol, which is found in red wine [7]. Much data has been generated showing potent anticancer activity of resveratrol across many cancer cell lines [7]. Given the similarity in structure of resveratrol, it is possible that pinosylvin also possesses potent anticancer activity (Fig. 1B). Currently, there are no validated analytical methods to quantify pinosylvin in biological matrices. Hence, there are no pharmacokinetic data on the rate and extent of its metabolism or bioavailability.

In order to elucidate the metabolism kinetics of pinosylvin, knowledge of its metabolic pathways in biological fluids is of considerable importance. To our knowledge, no study has been published characterizing the in vitro metabolism of pinosylvin in healthy tissues, and there is no pharmacoki-

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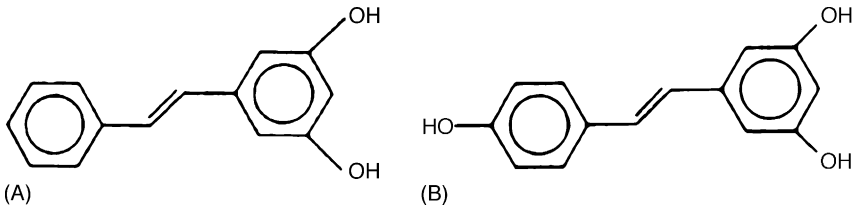

Fig. 1. (A) Structure of pinosylvin; (B) structure of resveratrol.

netic information or validated assays to measure pinosylvin described in the literature. Before performing studies of biotransformation, development of a selective and sensitive assay for pinosylvin is necessary. The present study describes a selective, isocratic reversed-phase HPLC method for the determination of pinosylvin and its metabolites in rat serum and its application to in vitro and in vivo kinetic studies.

2. Experimental

2.1. Chemicals and reagents

7-Ethoxycoumarin, halothane, resveratrol, total protein reagent, protein standard solution, monosodium glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate (β-NADP) sodium salt hydrate, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). Pinosylvin was purchased from Sequoia Research Products Ltd., Oxford, United Kingdom. HPLC grade methanol, acetonitrile, and water were purchased from J. T. Baker (Phillipsburg, NJ, USA).

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT pump, a SIL-10AF autoinjector, a photodiode-array SPD-10A VP UV/VIS spectrophotometric detector and an SCL-10A system controller. Injection volume was 50 μL. Data collection and integration were accomplished using Shimadzu EZ start 7.1.1 program software.

The analytical column used was an amylose tris 3,5 dimethylphenylcarbamate (150 mm × 4.6 mm, ID, 5 μm) (Chiral Technologies Inc., Exton, PA, USA). The mobile phase consisted of acetonitrile and 0.1% phosphoric acid (42:58, v/v), filtered and degassed under reduced pressure prior to use. Separation was carried out isocratically at ambient temperature, and a flow rate of 0.500 mL/min, with UV detection at 308 nm.

2.3. Mass spectrometry conditions

Samples were applied to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using negative ion electrospray under similar chromatographic conditions to those mentioned above with the exceptions that an Agilent 1100 series HPLC system (Palo Alto, CA) was employed, consisting of: autosampler, binary pump, degasser, and UV detector and phosphoric acid was omitted as a modifier while 1 mM $(NH_4)HCO_3$ was used to maintain neutral pH. The mass spectrometer was operated under conditions optimized for pinosylvin at the chromatographic flow conditions (0.5 mL/min) as follows: The Ionspray needle was maintained at −4500 kV, with nitrogen as drying gas 1 (setting 40), drying gas 2 (setting 25), curtain gas (setting 10), and collision gas (setting 4). The turbospray interface was maintained at 400 °C. The declustering potential (DP), collision energy (CE), and exit potential (EP) were optimum at 30 V, 45 eV and 10 V, respectively. Both the Q1 and Q3 quadrupoles were maintained at unit resolution (0.7 Da width at half height). Characteristic fragmentation reactions include *m/z* 212.2 → 193.2 for pinosylvin, *m/z* 387.2 → 212.2 for pinosylvin glucoronide, and for resveratrol *m/z* 227.2 → 219.2.

2.4. Stock and working standard solutions

Methanolic stock solutions of pinosylvin (1 mg/mL) and 7-ethoxycoumarin (1 mg/mL) were prepared. The 7-ethoxycoumarin solution was subsequently diluted with methanol to make a working internal standard (IS) solution of 25 μg/mL. These solutions were protected from light and stored at −20 °C between uses, for no longer than 3 months. Calibration standards in serum were prepared daily from the stock solution of pinosylvin by sequential dilution with blank rat serum, yielding a series of concentrations namely 0.1, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/mL, in four replicates.

Quality control (QC) samples were prepared from the stock solution of pinosylvin by dilution with blank rat serum to yield target concentrations of 0.5, 1.0, 10.0, 50.0 and 100.0 μg/mL. The QC samples were divided into 0.5 mL aliquots in screw-capped test tubes and stored at −20 °C before use.

2.5. Sample preparation

0.5 mL of internal standard solution (25 μg/mL) was added to working standards or samples (0.5 mL). The mixture was precipitated with 1.0 mL ice-cold acetonitrile and was centrifuged at 8000 × *g* for 5 min using Beckman microfuge. Following transfer of the supernatant to new vials,

the residue was placed in sample vials 50 μl of the supernatant was injected onto the column.

2.6. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using six different concentrations, namely 0.5, 1, 5, 10, 50 and 100 μg/mL. The between-run precision and accuracy of the assays were estimated from the results of six replicate assays of QC samples of 6 different days within 1 week. The precision was evaluated by calculating the coefficient of variation (CV) using ANOVA. The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration. The values of CV and bias should be within 15%, at all concentrations tested [8].

2.7. Recovery

Recovery of pinosylvin from rat serum was assessed ($n = 6$) at 0.5, 1.0, 5, 10, 50 and 100 μg/mL. A known amount of pinosylvin was spiked into 0.1 mL rat serum to give the above concentrations. The proteins present in the serum were precipitated with 1 mL ice-cold acetonitrile and the supernatant was analyzed by HPLC following centrifugation at $8000 \times g$ for 5 min. The extraction efficiency was determined by comparing the peak area ratio (PAR) of pinosylvin to IS in the serum matrix to that in water.

2.8. Stability of pinosylvin samples

The stability of pinosylvin samples were assessed under five different conditions. The stability of pinosylvin in rat serum at room temperature ($22 \pm 1\,^{\circ}C$) and at $-20\,^{\circ}C$ was investigated using QC samples of five concentration levels, 1, 5, 10, 50 and 100 μg/mL in four replicates.

The freeze–thaw stability of pinosylvin was evaluated at six concentrations 0.5, 1, 5, 10, 50 and 100 μg/mL, using QC samples. These samples were analyzed in triplicate without being frozen at first, and then stored at $-20\,^{\circ}C$ and thawed at room temperature ($22 \pm 1\,^{\circ}C$) for three cycles.

The stability of pinosylvin in reconstituted extracts during run-time in the HPLC auto-injector was investigated, using pooled extracts from QC samples of three concentration levels, 1, 10 and 50 μg/mL. Samples were kept in the sample rack of the auto-injector and injected into HPLC system every 6 h, from 0 to 24 h, at the temperature of auto-injector ($26 \pm 1\,^{\circ}C$).

The stability of reconstituted extracts was also tested at $-20\,^{\circ}C$ for 1 week. The reconstituted extracts of six concentrations, 0.5, 1, 5, 10, 50 and 100 μg/mL were allocated in injection vials, stored at $-20\,^{\circ}C$ and injected onto the column on days 0 and 1.

The stability of reconstituted extracts was also tested at $-20\,^{\circ}C$ for 1 day. The reconstituted extracts of six concentrations, 0.5, 1, 10, 50 and 100 μg/mL were allocated in injection vials, stored at $-20\,^{\circ}C$ and injected onto the column on days 0 and 1.

The light stability of pinosylvin in stock solution was also tested at room temperature for 1 day. Samples were exposed to laboratory (fluorescent overhead) illumination for up to 24 h and injected onto the column from time 0 to 24 h post-illumination.

2.9. Stability of pinosylvin in rat serum

Pinosylvin was incubated in rat serum at $37.0 \pm 0.1\,^{\circ}C$ in a thermostatically controlled shaking water bath. Prior to the kinetic study, the incubation media were equilibrated to the temperature of the study. Kinetic studies were initiated by the addition of a stock solution of pinosylvin to incubation media, yielding an initial concentration of 10 μg/mL. At predetermined time intervals, samples (0.5 mL) were removed and the reaction was stopped by adding equal volume of ice-cold acetonitrile and mixing immediately. Samples were analyzed by HPLC following centrifugation at $8000 \times g$ for 5 min using Beckman microfuge.

2.10. Pharmacokinetics

A male Sprague Dawley rat (325 g) was anaesthetized using halothane and a silastic catheter was cannulated into the right jugular vein. The animal was placed in a metabolic cage, allowed to recover overnight and fasted for 12 h before dosing. On the day of experiment, the animal was dosed intravenously with pinosylvin (10 mg/kg). Serial blood samples (0.25 mL) were collected at 0, 1, 10 min, 0.25, 0.5, 1, 2, 4, 6, 12 and 24 h. After each sample collection, the cannula was flushed with 0.25 mL of saline. Following centrifugation of the blood samples, serum was collected and stored at $-70\,^{\circ}C$ until analyzed. The experimental animal protocols were approved by the Institutional Animal Care and Use Committee of Washington State University.

2.11. Rat liver microsomes preparation

Male rat liver microsomes were prepared from adult male Sprague-Dawley rats using a previously published procedure [9,10]. The fresh rat livers were cut from euthanized rats and put into ice-cold saline, weighed, and minced. Samples were homogenized using a motorized homogenizer (four strokes) in ice-cold homogenization buffer (50 mM pH 7.4 potassium phosphate buffer, 250 mM sucrose, 1 mM EDTA) and centrifuged at $7700 \times g$ for 15 min at $4\,^{\circ}C$. The supernatant collected was then centrifuged again at $18{,}500 \times g$ for 15 min at $4\,^{\circ}C$. After the pellet was discarded, the supernatant was centrifuged again at $85{,}600 \times g$ for 1.0 h at $4\,^{\circ}C$ to yield microsome pellets. The microsomes were resuspended in microsome washing buffer (10 mM pH 7.4 potassium phosphate buffer, 0.1 mM EDTA, and 150 mM KCl) and centrifuged again at $85{,}600 \times g$ for 1.0 h at $4\,^{\circ}C$ to yield microsomes. The microsome pellet was then resuspended in 250 mM sucrose,

aliquoted into vials (0.5 mL/vial), and stored at −80 °C until use.

2.12. Microsome protein concentration

Protein concentration of microsomal protein was determined using a protein assay (Bio-Rad, Hercules, CA), using bovine serum albumin as standard.

2.13. Phase I metabolism

Studies of metabolic kinetics of pinosylvin were conducted in the presence of cofactors which included 10 mM $MgCl_2$ and an NADPH-generating system (7.5 mM glucose 6-phosphate, 0.3 mM β-NADP and 0.42 unit/mL glucose-6-phosphate dehydrogenase), in 100 mM phosphate buffer containing 1 mM EDTA (pH 7.4) under carbogen gas at 37.0 ± 0.1 °C in a shaking (75 rpm) water bath. The parent drug was added as a methanolic stock solution of 1.0 mg/mL (at a volume of 0.5% in the final incubation mixtures) and was pre-incubated in the incubation buffer for 5 min at 37 ± 0.1 °C. The reaction was initiated by adding the cofactors. At pre-determined time intervals, samples (0.5 mL each) were withdrawn and the reaction was terminated immediately by adding 50 μL of 94% acetonitrile/6% glacial acetic acid. Samples were then extracted and analyzed by HPLC.

2.14. Phase II metabolism

The incubation procedures for measuring uridine diphosphate-glucuronosyltransferase (UGT) activities using microsomes were as follows: (1) microsome (final concentration ≈0.05 mg protein/mL) was mixed with each of the following: magnesium chloride (0.88 mM), saccharolactone (4.4 mM), and alamethicin (0.022 mg/mL). 42 μM of pinosylvin in a 50 mM potassium phosphate buffer (pH 7.4) was added as the substrate and finally uridine diphosphoglucuronic acid (3.5 mM) was added to activate the reaction. (2) The mixture was incubated at 37 °C for 10, 20, 30, or 60 min. (3) The reaction was stopped by the addition of 50 μl of 94% acetonitrile/6% glacial acetic acid.

2.15. Data analysis

Metabolism products were identified by their relative retention times to the IS on HPLC chromatograms. Quantification was based on calibration curves constructed using PAR of pinosylvin to IS, against pinosylvin concentrations using unweighted least squares linear regression. The percentage of metabolism products was estimated as the ratio of PAR of metabolite to PAR of patent drug at time zero. The apparent decomposition rate constants (k_{app}) were estimated from the slope of log-linear phase of declining concentration versus time plots. The half-lives ($t_{1/2}$) were calculated using the following equation: $t_{1/2} = 0.693/k_{app}$. Data were expressed as the mean ± standard deviation (S.D.) of replicate determinations. Pharmacokinetic parameters were estimated using WinNonlin (version 1.0).

3. Results and discussion

3.1. Chromatography

There were no interfering peaks co-eluted with the compounds of interest (Fig. 2A). Separation of pinosylvin and the internal standard in rat serum were achieved successfully. The retention times of pinosylvin and IS were approximately 14 and 22 min, respectively (Fig. 2B).

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in rat serum, linearity, limit of quantitation (LOQ), limit of detection (LOD), freeze–thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. Various conditions of HPLC were tested to achieve the best resolution of pinosylvin and other degradation products. The retention times of analytes were found to be very sensitive to the percentage of acetonitrile in the mobile phase. The optimal separation was achieved when the combination of acetonitrile and phosphoric acid was 42:58 (v/v) and the flow rate was 0.5 mL/min.

Based on spectrophotometer analysis of pinosylvin reconstituted in mobile phase prior to HPLC analysis, UV detection was set at 308 nm.

3.2. Linearity, LOQ and LOD

An excellent linear relationship ($r^2 = 0.9997$) was demonstrated between PAR of pinosylvin to IS and the corresponding serum concentrations of pinosylvin over a range of 0.5–100 μg/mL.

The mean regression line from the validation runs was described by pinosylvin (μg/mL) = PAR × 0.9884 + 0.1548. The LOQ of this assay was 0.5 μg/mL in rat serum with the corresponding relative standard deviation and bias of 0.8 and 6%, respectively. This calibration curve was cross-validated with QC samples of pinosylvin in microsomes. The back-calculated concentration of QC samples in these matrices was within the acceptance criteria. The LOD of pinosylvin was estimated to be 0.1 μg/mL in rat.

3.3. Precision, accuracy and recovery

The within- and between-run CV calculated during replicate assays ($n = 6$) of pinosylvin in rat serum were <10% over a wide range of pinosylvin concentrations. The intra- and inter-run bias assessed during replicate assays varied between −7.1 and 14.0%. Precision and accuracy studies indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for pinosylvin from rat serum varied from 99 to 100%. High recovery of pinosylvin from rat serum suggested that there was negligible loss

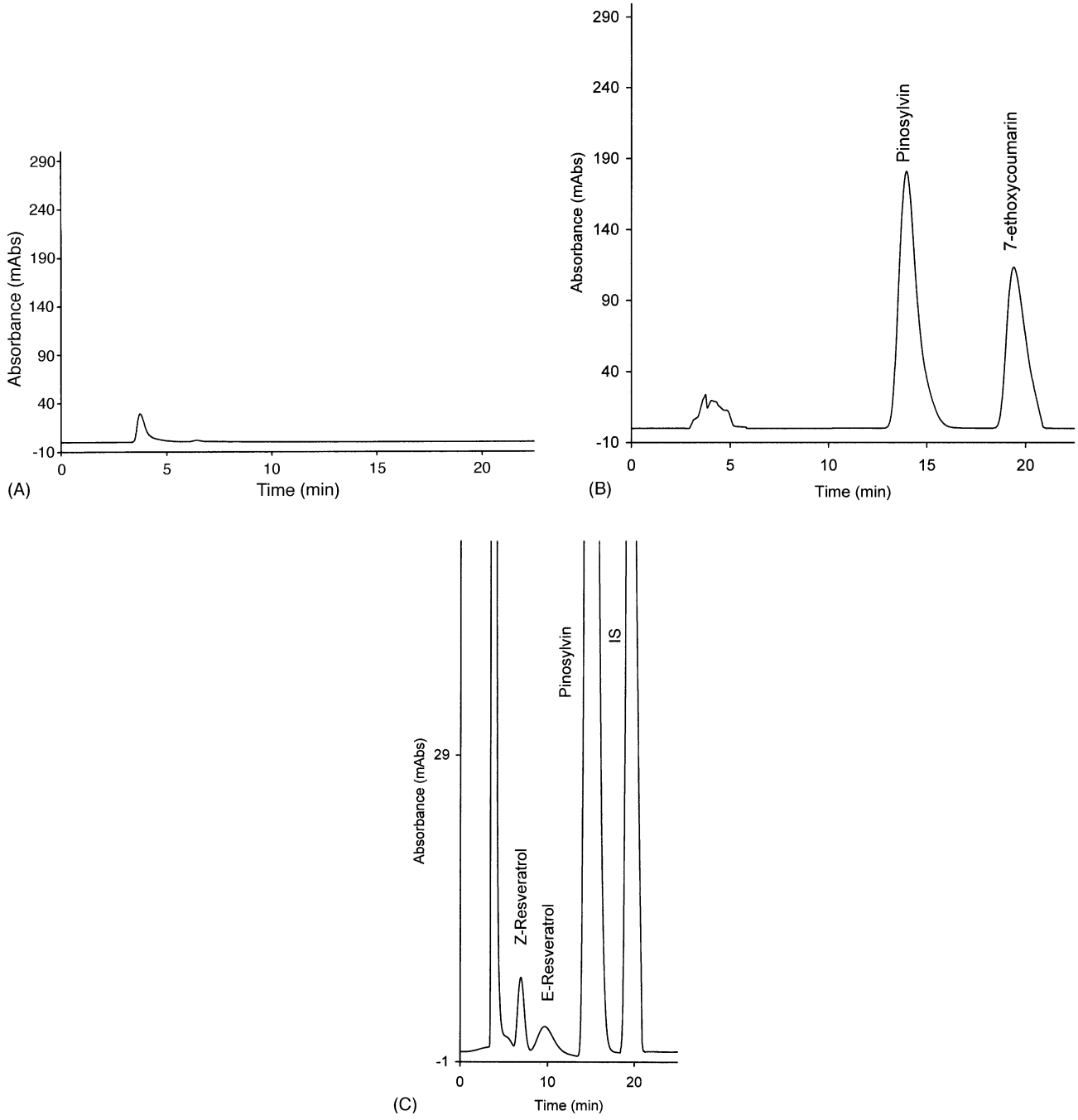

Fig. 2. Representative chromatograms of (A) blank serum demonstrating no interfering peaks co-eluted with the compounds of interest. (B) Rat serum containing 7-ethoxycoumarin (internal standard) and pinosylvin with concentration of 10 μg/mL. (C) Rat liver microsome containing pinosylvin, 7-ethoxycoumarin, and E- and Z-resveratrol formed under a NADPH-generating system.

during the protein precipitation process, and the efficiencies of extraction of pinosylvin and IS were comparable.

3.4. Stability of pinosylvin samples

No significant degradation was detected after the samples of pinosylvin in rat serum were stored at room temperature for 3 h, or in a freezer at or below −20 °C for 4 weeks, or after undergoing one freeze–thaw cycle. Under ambient conditions for 3 h, there was >99% of pinosylvin recovered across concentrations. When stored in a freezer at −20 °C, recoveries of pinosylvin were <99% after 1 and 4 weeks. The recoveries were >99% following three freeze–thaw cycles in all concentrations tested (1, 10 and 100 μg/mL). There was no significant decomposition observed after the reconstituted extracts of pinosylvin were stored in the auto-injector at room

temperature for 24 h or in freezer at −20 °C for 1 week. The measurements were >99% of the initial values for all concentrations during the storage in the auto-injector at room temperature for 24 h. When stored in a freezer at −20 °C, the recovery was >99% within 1 week at all concentrations investigated.

3.5. Metabolism of pinosylvin in rat liver microsomes under a NADPH-generating system

The HPLC method has been applied to the determination of pinosylvin and its metabolic products in the phase I metabolic kinetic study of pinosylvin in rat liver microsomes. Pinosylvin was added individually to microsomes in concentration of 10 μg/mL. Following the incubation of pinosylvin as parent drug at 37 °C in rat liver microsomes with an NADPH-generating system two minor extra peaks were detected at approximately 7 and 9 min, respectively (M1 and M2) suggesting minimal oxidative metabolism is apparent (Figs. 2C and 3). Further analysis employing mass spectrometry revealed these two minor oxidative metabolites to be Z- and E-resveratrol, with *m/z* fragmentation patterns of 227.1. Stock solutions of resveratrol (10 μg/mL dissolved in methanol) were run using the developed pinosylvin assay. Resveratrol isomers eluted at approximately 7 and 9 min, which corresponds to the elution time of Z- and E-resveratrol, respectively. The amount of each metabolite detected increased over time (Fig. 3).

3.6. Pharmacokinetics of pinosylvin in rats

The HPLC method has been applied to the determination of pinosylvin in pharmacokinetic studies in rats. There are no previously published studies or information of the pharmacokinetics of pinosylvin in any species. Following administration of pinosylvin there was an apparent terminal elimination half-life of 10 min for the parent compound (Fig. 4). One previously unidentified metabolite (M2) was apparent at approximately 4 min. This metabolite has an *m/z* fragmentation pattern of 387.3 and is consistent with glucuronidation. The pharmacokinetics of pinosylvin appears to be qualitatively very similar to previous reports of resveratrol in the rat where a glucuronide metabolite is also present in plasma [11].

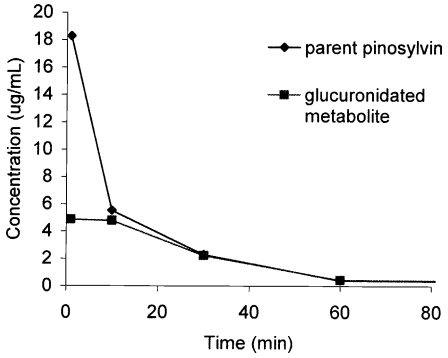

Fig. 4. Pharmacokinetic time course of pinosylvin in rat serum.

3.7. Metabolism of pinosylvin in rat liver microsomes under a UGT generating system

The HPLC method has been applied to the determination of pinosylvin and its metabolic products in the phase II metabolic kinetic study of pinosylvin in rat liver microsomes. Pinosylvin was added individually to microsomes in concentration of 10 μg/mL. Following the incubation of pinosylvin as parent drug at 37 °C in rat liver microsomes with the UGT enzyme, one major peak was determined at 4 min (M3). The amount of this metabolite detected increased over time (Fig. 5). This suggests glucuronidative metabolism is apparent, and was confirmed by mass spectrometry. To further lend evidence of glucuronidated metabolite formation, β-glucuronidase was added to a set of microsomal samples instead of acetic acid/acetonitrile stop solution. These samples were analyzed via HPLC along side of original microsomal samples exposed to the stop solution. HPLC analysis confirmed the absence of the glucuronidated metabolite. This same peak at the same retention time was also apparent in the rat serum and urine pharmacokinetic samples.

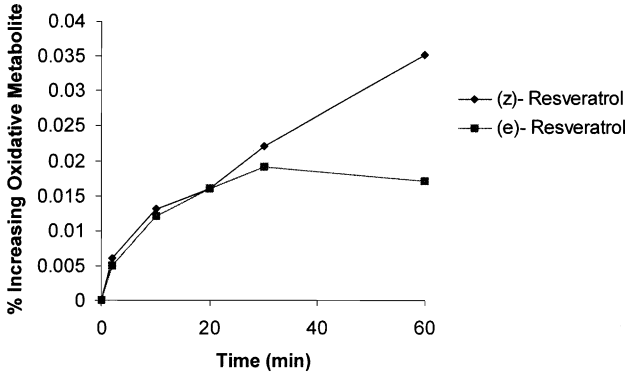

Fig. 3. Formation of E- and Z-resveratrol during phase I biotransformation in microsomes.

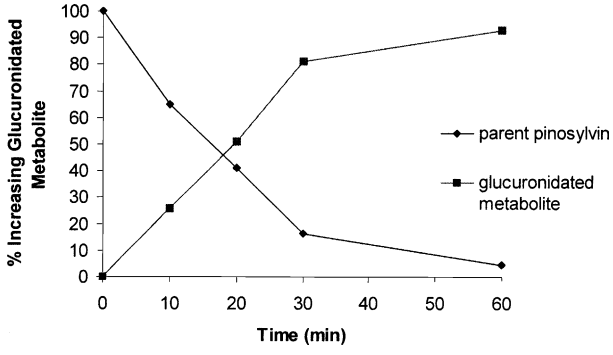

Fig. 5. Phase II microsomal metabolism of pinosylvin in rat liver microsome.

4. Conclusions

In summary, the developed HPLC assay is sensitive, reproducible and accurate and specific. It has been successfully applied to the study of pharmacokinetics and metabolism of pinosylvin in rats for the first time. Using this method large number of biological samples can be analyzed in a relatively short period of time. Further studies are ongoing in our laboratory to further characterize the metabolites of pinosylvin and other stilbenes and their pharmacological and toxicological activity.

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