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Tissue distribution of puerarin and its conjugated metabolites in rats assessed by liquid chromatography tandem mass spectrometry

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Abstract

Puerarin (an isoflavone *C*-glucoside from kudzu root) has been the focus of several studies investigating its potential effects on health benefits. In this study, we determined single dose tissue distribution of puerarin and its metabolites in order to examine whether they undergoes selective uptake by specific organs. Puerarin was administered orally (50 mg/kg) to rats and the concentration of puerarin in tissue compartments was determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Puerarin was widely distributed in rat tissues with highest concentrations in lungs (799 ± 411.6 ng/gram wet tissues). In addition, we examined the excretion of puerarin into the bile. LC-MS/MS analysis of bile samples collected after infusing puerarin directly into the portal vein indicated that puerarin was excreted into the bile predominantly in the form of unconjugated puerarin. This report identifying puerarin in several organs including kidney and pancreas may explain its beneficial effects in diabetes.

Keywords

Puerarin; LC-MS/MS; tissue distribution; metabolites

Introduction

Puerarin (4′-7-dihydroxy-8-β-D-glucosylisoflavone), the chemical structure shown in Fig. 1, is the most abundant isoflavone *C*-glucoside isolated from traditional Chinese medicine (TCM) Pueraria Radix (the root of the kudzu *Pueraria lobota*). It has been shown to have

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beneficial effects on cardiovascular, neurological and hyperglycemic disorders (Xu and Zho, 2002; Zhu et al., 2004; Hsu et al., 2003). Kudzu is reported to be used in combination with other medicinal plants such as Danshen (*Salvia miltiorrhiza*) and Notoginseng (*Panax notoginseng*). These products have been shown to have beneficial effects on atherosclerosis (Sieveking et al., 2005) and acute ischemic myocardial injury (Wu et al., 2007). Similarly, a TCM preparation called Naodesheng which consists of five kinds of common crude drugs including puerarin and ginsenosides has been reported to be effective in the treatment of cardiovascular diseases (Yu et al., 2006). The therapeutic effectiveness of these herbal mixtures is believed to be the result of different herbs acting collectively or synergistically rather than as single active agents (Keyler et al., 2002).

We demonstrated that puerarin significantly improves glucose tolerance in C57 BL/6J ob/ob mice, an animal model of Type 2 diabetes mellitus, blunting the rise in blood glucose levels after i.p. administration of glucose (Meezan et al., 2005). In our study, puerarin administered by intraperitoneal injection was four times more bioavailable than via the oral route of administration. Puerarin was rapidly absorbed intact and the maximum blood concentration was obtained within 1 h after oral administration (Prasain et al., 2007). Interestingly, unlike the isoflavone *O*-glucosides, unmodified puerarin was the major component in the blood and urine, indicating that phase II metabolism is not the major metabolic pathway for puerarin excretion (Prasain et al., 2004).

Li et al. (2006) reported the pharmacokinetics and tissue distribution following oral administration of very high doses of puerarin and a puerarin:phospholipid complex (400 mg/kg) using a reverse-phase HPLC method. However, the use of high acute dose of puerarin may have saturation effects and alters the uptake and metabolism. Furthermore, there is no evidence for bioavailability of puerarin's metabolites in tissues using a sensitive LC-MS/MS method. It also remains unknown whether puerarin is eliminated through the bile and thereby undergoes extensive enterohepatic circulation as for other isoflavones (Sfakianos et al., 1997) or directly excreted into the urine.

It is generally accepted that the biological effects of xenobiotics are related to the forms in which they circulate and concentrate in the target cells. To assess the potential mechanism of action of puerarin and/or metabolites, it is essential to understand their bioavailability in the target tissues. As part of our continuing study on the cardiovascular and metabolic effects of puerarin in spontaneously hypertensive rats, we analyzed tissue distribution of puerarin and its metabolites in these rats and determined whether puerarin could be transported into bile using a sensitive LC-MS/MS method.

Materials and methods

Materials

Puerarin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and apigenin from Indofine (Somerville, NJ). All HPLC solvents and reagents were purchased from Fisher (Norcross, GA) and were of HPLC grade.

Puerarin administration and sample collection

Male spontaneously hypertensive rats (SHR, n = 6, body weight = 250–300 g, 8–10 weeks of age; Harlan Sprague-Dawley Inc., Indianapolis, IN) were housed in a controlled environment at 23°C and 55% relative humidity under a 12 h dark-light cycle, with free access to soy-free custom diet TD86369 (Harlan Teklad, Wisconsin, MD) and tap water for one week. All experimental procedures were conducted in accordance with Institutional Animal Care and Use Committee of the University of Alabama, Birmingham, and National Institutes of Health guidelines. After overnight fasting, puerarin was orally administered by gavage (50 mg/kg body weight) to each animal.

Following a midline incision, the bile duct was exposed and was cannulated with PE-10 tubing, tied and secured with 5-0 silk (Ethicon, Somerville, NJ) under isoflurane anesthesia. During experiments, the body temperature of rats was maintained at 37°C. Bile was collected in 10 min in tervals over 2 h. Rats were euthanized by cervical dislocation following isoflurane anesthesia and blood was collected via heart puncture using heparin as anticoagulant. Plasma was isolated by centrifugation of the blood at 3,000 × g for 5 min and stored at −20°C. After perfusion with ice-cold normal saline, the liver, kidney, spleen, pancreas, lungs, eyes and heart were dissected and immediately frozen in liquid nitrogen.

Portal vein infusion

To determine the excretion of puerarin in the bile, it was infused into the portal vein of an adult rat under isofluorethane anesthesia. The portal vein was exposed and a 27-gauge stainless steel syringe needle, connected to PE10 tubing, was inserted. Puerarin was infused into the portal vein at 0.5 μg/min for 60 min in 140 mM NaCl containing 10 mM sodium taurocholate using a Harvard syringe pump. Super glue was used immediately to seal the puncture point. Bile was collected in 5 min intervals during the first 30 min and then at 10-min intervals over the next two and half hours.

Sample preparation

The tissue samples (wet weight 1–7 g) were minced and added to (2–14 ml) methanol containing 1% acetic acid. The tissue was homogenized with a Tissue Tearor homogenizer (Biospace Product Inc., Racine, WI). The samples were vortex mixed and tumbled for 2 h at room temperature. The homogenate was centrifuged at 3,000 × g for 10 min and the supernatant was removed and dried under air and reconstituted in 80% methanol in water (200 μl).

Sample preparation and quantification of puerarin in biological samples were performed as described before (Prasain et al., 2007). Briefly, each plasma sample (20 μl) was diluted by adding 80 μl of methanol containing 1% acetic acid, vortex-mixed and centrifuged for 10 min at 3,000 × g to precipitate proteins. In the case of bile, each sample (5–20 μl) was diluted 3–4 fold with methanol containing 1% acetic acid, vortex mixed and centrifuged as described above. The supernatant solutions were directly transferred to HPLC auto samplers and injected (10 μl) into the mass spectrometer as described below to analyze the samples.

A calibration curve containing 2, 20, 50, 200, 500, 2000 and 5000 ng/ml of puerarin was generated. The correlation coefficient of each standard curve was greater than 0.99. This allowed selective and specific quantitative data to be obtained for puerarin in samples. In the case of tissue samples, the method was not validated because there was not enough material available for complete validation procedure. Thus, quantification results in tissues are approximate.

Liquid chromatography-mass spectrometry

LC-MS/MS analyses of rat plasma, bile and tissue extracts were performed using a system consisting of a model SIL-HT refrigerated Shimadzu autosampler and HPLC instrument (Shimadzu Scientific Instruments, Inc. Columbia, MD), and an API 4000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada). Chromatography was carried out on a reversed-phase Phenomenex Synergi 4 micron Fusion-RP80 column (150 × 2.0 mm i.d.) pre-equilibrated with 10 mM ammonium acetate (NH_4OAc). The mobile phase consisted of acetonitrile-water (10:90, v/v) in 10 mM NH_4OAc with a flow rate of 0.2 ml/min. Multiple reaction monitoring (MRM) was used to perform mass spectrometric quantification of puerarin. The column effluent was introduced into the mass spectrometer using atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) in the negative mode for puerarin and conjugated metabolites of puerarin, respectively. Nitrogen was used as nebulizer, gas 1 and curtain gas. The nebulizer current and temperature were 5 amps and 500°C, respectively. The collision gas (N_2) was set at high and collision energy was −45 eV. The MRM analysis for puerarin was conducted by monitoring the precursor ion to product ion transition *m/z* 415/267. Puerarin's phase I and phase II metabolites were also characterized by MRM of anticipated mass transitions (Q1/Q3 transitions) *m/z* 591/415 (puerarin glucuronide), 495/415 (puerarin sulfate), 431/283 (monhydroxylated puerarin), 429/415 (methyl puerarin), and 253/223 (daidzein). The LC-MS-MS system was controlled by BioAnalyst 1.4.1. software (Applied Biosystems/MDS Sciex).

Results

Tissue distribution of puerarin and its metabolites

LC-MS/MS with ESI turbo ionspray analysis was performed on rat tissues, bile and plasma samples collected following oral administration of puerarin (50 mg/kg). Multiple MS/MS experiments (MRM, MS/MS and neutral loss scan) were carried out to identify the metabolites of puerarin (e.g., glucuronidated, sulfated and hydroxylated). This enabled rapid identification, characterization and confirmation of Phase I (functionalization reactions such as oxidation and hydrolysis) and Phase II (conjugation reactions such as glucuronidation and sulfation) metabolites in a single LC-MS/MS run.

As can be seen in MRM chromatogram of puerarin-treated kidney sample (Fig. 2A), a prominent peak eluting at t_R 6.46 min corresponded to intact puerarin (Prasain et al., 2007; Prasain at al., 2004). For the detection of puerarin's phase I and phase II metabolites, the MRM mass transitions *m/z* 591/415 (puerarin glucuronide), 495/415 (puerarin sulfate), 431/283 (monhydroxylated puerarin), 429/415 (methyl puerarin), and 253/223 (daidzein)

were used. A broad peak appearing at t_R 5.73 min corresponded to puerarin glucuronide (Fig. 2A). Two well separated sharp peaks with mass transition *m/z* 495/415 at t_R 5.98 and 6.77 min indicated the presence of two isomers of puerarin sulfates. We also detected monohydroxylated derivatives of puerarin with mass transition *m/z* 431/311 at t_R 6.40 and 6.92 min (data not shown). No methyl puerarin was detected in the kidney. The detection of aglycone daidzein was inconclusive.

Similarly, MRM chromatograms of liver, lungs and pancreas indicated the presence of glucuronides, sulfates and monohydroxylated metabolites of puerarin (Figs. 2B–D). In addition to two puerarin sulfate conjugates, two glucuronide conjugates of puerarin (a major peak at t_R 5.70 min and a minor peak at t_R 6.31 min) were detected in these samples. The different conjugation sites (7 and 4' positions of puerarin) of the sulfate or glucuronic acid group result in the different retention behaviors under these HPLC conditions.

The major peak eluting earlier was considered to be puerarin 7-O-β-glucuronide and the latter puerarin 4'-O-β-glucuronide on the basis of comparison of the relative retention times of daidzein glucuronides (Clarke et al., 2002). This is also in accord with the detection of puerarin 7-*O*-β-D-glucuronide as a biliary metabolite of puerarin (Yasuda et al., 1994). Similarly, the major MRM peak (*m/z* 495/415) eluting earlier in the tissue samples was assigned to be puerarin 4'-O-β-sulfate which is supported by the previous detection of this metabolite in puerarin-treated rat bile and the relative retention time behavior of daidzein sulfates (Yasuda et al., 1994; Fang et al., 2002; Clarke et al., 2002). However, there could be an opposite scenario for glucuronidation at the 7-position due to the presence of sterically hindered *C*-glucoside at the 8-position of puerarin. Therefore, further characterization of these conjugates based on NMR interpretation of standards is needed to fully establish their structures. Puerarin was detected by MRM in the heart and eye samples, but was not quantified due to its very low concentration. No puerarin and its metabolites were detected in the spleen.

For further identification of puerarin's conjugated metabolites, we performed MS/MS of the *m/z* 591 and 495 ions. MS/MS of the *m/z* 591 ion yielded characteristic product ions *m/z* 415 and 295 due to the subsequent losses of 176 (glucuronic acid moiety) and 120 amu (Fig. 3A). Interestingly, in the case of puerarin, the product ion *m/z* 267 is the base peak, whereas the conjugated puerarin showed *m/z* 295 as the base peak. The neutral losses of 176 and 120 amu are indicative of glucuronide and *C*-glucoside moieties, respectively (Prasain et al., 2004; Brownsill at al., 1994; Zhang et al., 2000; Lafaye et al., 2004; Clarke et al., 2002). Thus, this metabolite was proposed to be a glucuronide conjugate of puerarin. MS/MS of m/z 495 showed characteristic product ions obtained from the subsequent neutral losses of 80 and 120 amu from this ion (Fig. 3B). The product ion at *m/z* 80 is a typical fragment for aromatic sulfate conjugate and ions at *m/z* 295 and 267 are formed from a puerarin moiety.

Quantification of intact puerarin in tissues

Because tissues of rats had been perfused with saline, they contained no residual blood containing puerarin. The highest concentration of puerarin was found in the lungs (799 ± 411 ng/gram). The puerarin concentrations in kidney, liver and pancreas were 270 ± 74, 45 ± 11 and 66.09 ± 47.69 ng/gram tissues, respectively. The method was not validated for

tissue samples, since there was not enough material available for complete validation procedure. Thus, quantification results in tissues are approximate.

Biliary excretion of puerarin

We performed a pilot study to understand whether puerarin undergoes an efficient enterohepatic circulation. Time-dependent biliary excretion of puerarin in a rat following portal vein infusion of puerarin is shown in Fig. 4. Integration of the total biliary output recovered at the end of the experiment revealed that only 18.1% of infused puerarin could be accounted for in the bile (infused amount = 30.3 μg; recovered amount = 5.5 μg). The highest amount of puerarin was excreted in the 30 min bile sample. Interestingly, only 0.17% of total unmetabolized puerarin was excreted into bile during 2 h after oral administration. As can be seen in Fig. 5, puerarin was excreted into the bile mostly as unmetabolized puerarin with small amounts of conjugated metabolites during 2 h after oral administration of puerarin. This suggests that biliary excretion of puerarin does not represent a major route of elimination, although a small amount of the material is excreted as metabolites. A neutral loss scan of 176 was performed to profile all glucuronides and only *m/z* 591 and its sodiated ion *m/z* 613 were detected. The plasma concentration of puerarin was 1035 ± 232 ng/ml.

Discussion

Current interest in puerarin is based on emerging evidence suggesting its health beneficial effects (Xu and Zho, 2002; Zhu et al., 2004; Hsu et al., 2003; Meezan et al., 2005). Pharmacokinetic studies from this laboratory have shown that puerarin is rapidly absorbed into the blood, reaching a maximum and then declining within 1 h (Prasain et al., 2007). In an attempt to establish whether puerarin is distributed in rat tissues after single oral dose of puerarin and in what form(s), sensitive LC-MS/MS techniques were used to detect and directly measure the concentration of puerarin.

The present study shows that orally administered puerarin is rapidly absorbed from the intestinal compartment to the blood and widely distributed to various organs such as liver, kidney, lungs, pancreas, heart and eyes. Of the organs tested, the highest concentration of puerarin was in lungs, exceeding even that in kidney. A previous report indicates that quercetin and its metabolites also distribute in the highest concentrations in the lungs (de Boer et al., 2005). The significance of puerarin accumulation in these organs is not known yet; however, the lung may be a site for discovery of biomarkers of puerarin effects.

Previous study from our laboratory revealed that puerarin is absorbed as the intact glucoside and blunts the rise in blood glucose concentration after oral and i.p. administration of glucose (Meezan et al., 2005). We propose that the mechanism for this action involves two sodium-dependent glucose cotransporters (SGLT1 and/or SGLT2). It is also possible that puerarin suppresses renal tubular glucose reabsorption by inhibiting renal SGTL2, thereby increasing urinary glucose excretion. To improve our understanding of the mechanism of puerarin’s action on glucose metabolism, it is necessary to know whether puerarin or its metabolites are accessible to the major metabolizing organs. In the present study,

accumulation of puerarin in kidney tissues was found, which supports our hypothesis that puerarin could be a substrate for SGLT2 in kidney.

LC-MS/MS analysis of pancreatic tissue of rats treated with puerarin showed the presence. This indicated that puerarin penetrates into the pancrease and may be effective in preventing islet cells from the toxic action of reactive oxygen species in diabetes (Xiong et al., 2006). In our previous study, we have demonstrated that puerarin is bioavailable in brain tissues (Prasain et al., 2004). It is assumed that blood-brain barrier-specific influx transporters may be involved for the transport of puerarin into the brain.

The data from this study demonstrate that small amount of puerarin is excreted in bile along with puerarin glucuronide and puerarin sulfate. These data suggest that hydrolysis of puerarin in this model does not take place before it is excreted into bile. This is in contrast to the isoflavone *O*-glucoside genistin which undergoes intestinal hydrolysis before its absorption (Sfakianos et al., 1997; Prasain et al., 2006); puerarin is β-glucosidase-resistant. It is interesting to note that isoflavones like genistein and daidzein undergo extensive metabolism in the gut and liver, which might affect their biological properties. It has been reported that sulfation of genistein, with the associated loss of hydroxyl groups, decreases its antioxidant activity and its effect on platelet aggregation, inflammation, cell adhesion and chemotaxis (Rimbach et al., 2003; Turner et al., 2004). In this context, the presence of low levels of phase I and phase II metabolites of puerarin in body fluids and tissues may have special relevance to its health beneficial effects.

In summary, the present study demonstrated that a single oral administration of puerarin to rats results in a wide distribution of puerarin to the most of the organs, being highest in lungs. In addition, it provides preliminary evidence on biliary excretion of puerarin and its metabolites in rats which may be useful for designing toxicology and pharmacology studies and for interpreting the results of these experiments. Tissue distribution of puerarin requires further examination, particularly, the long-term tissue distribution of puerarin after chronic exposure in animals to different concentrations of puerarin.

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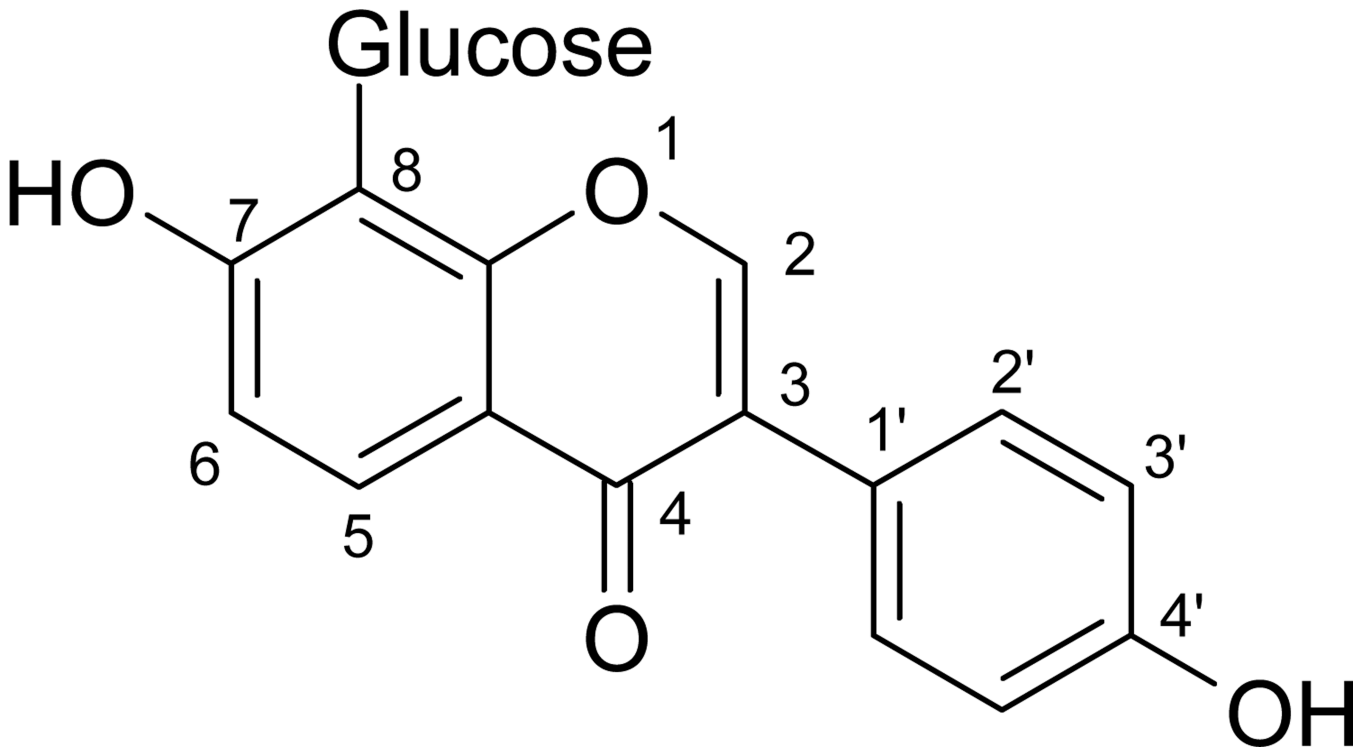

Figure 1.
Chemical structure of puerarin (molecular formula $C_{21}H_{20}O_9$)

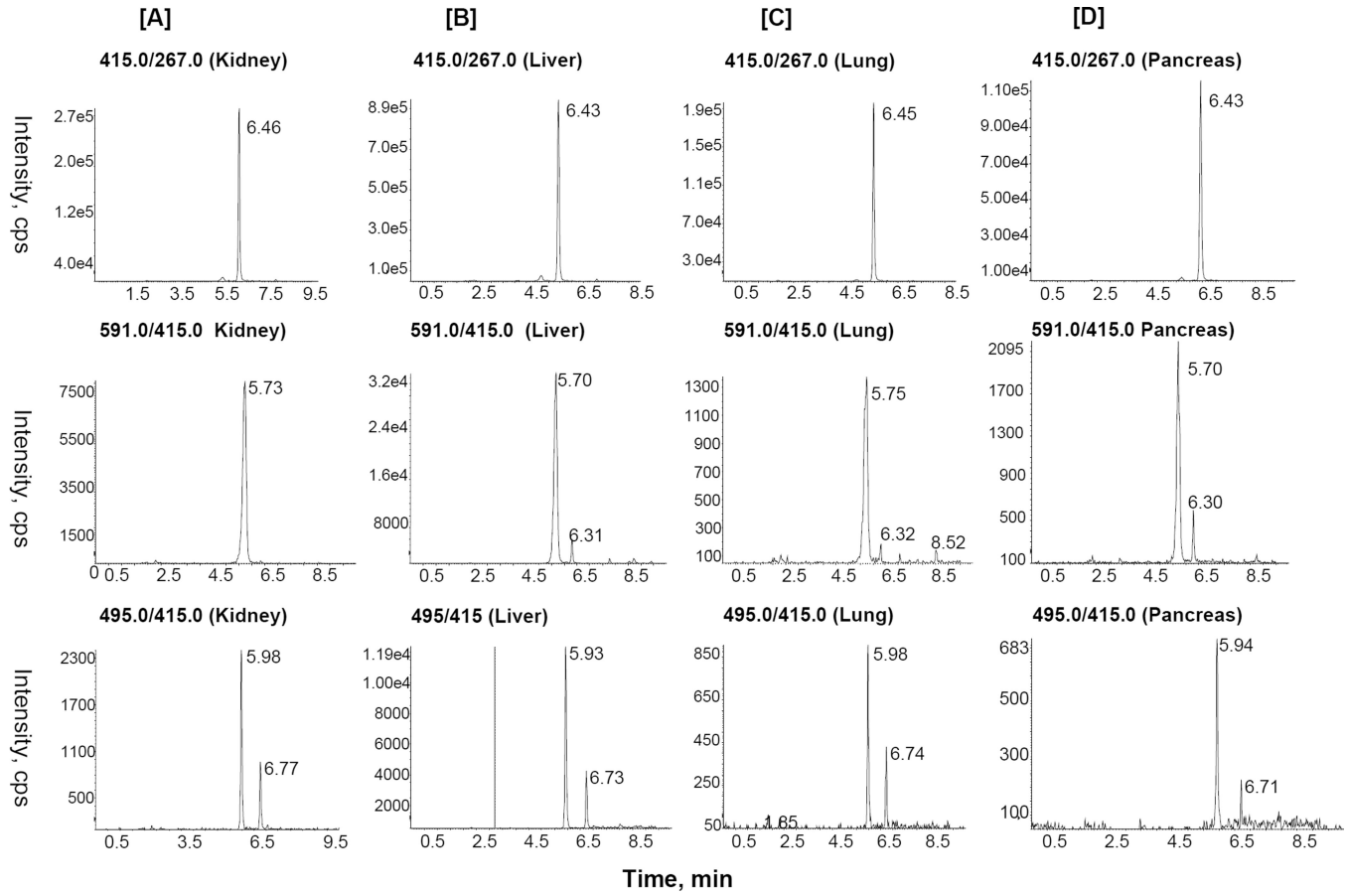

Figure 2.

Representative MRM ion chromatograms with transitions *m/z* 415/267, 591/415, and 495/415 obtained from rat [A] kidney, [B] liver, [C] lungs, [D] pancreas samples 2h after oral administration of puerarin.

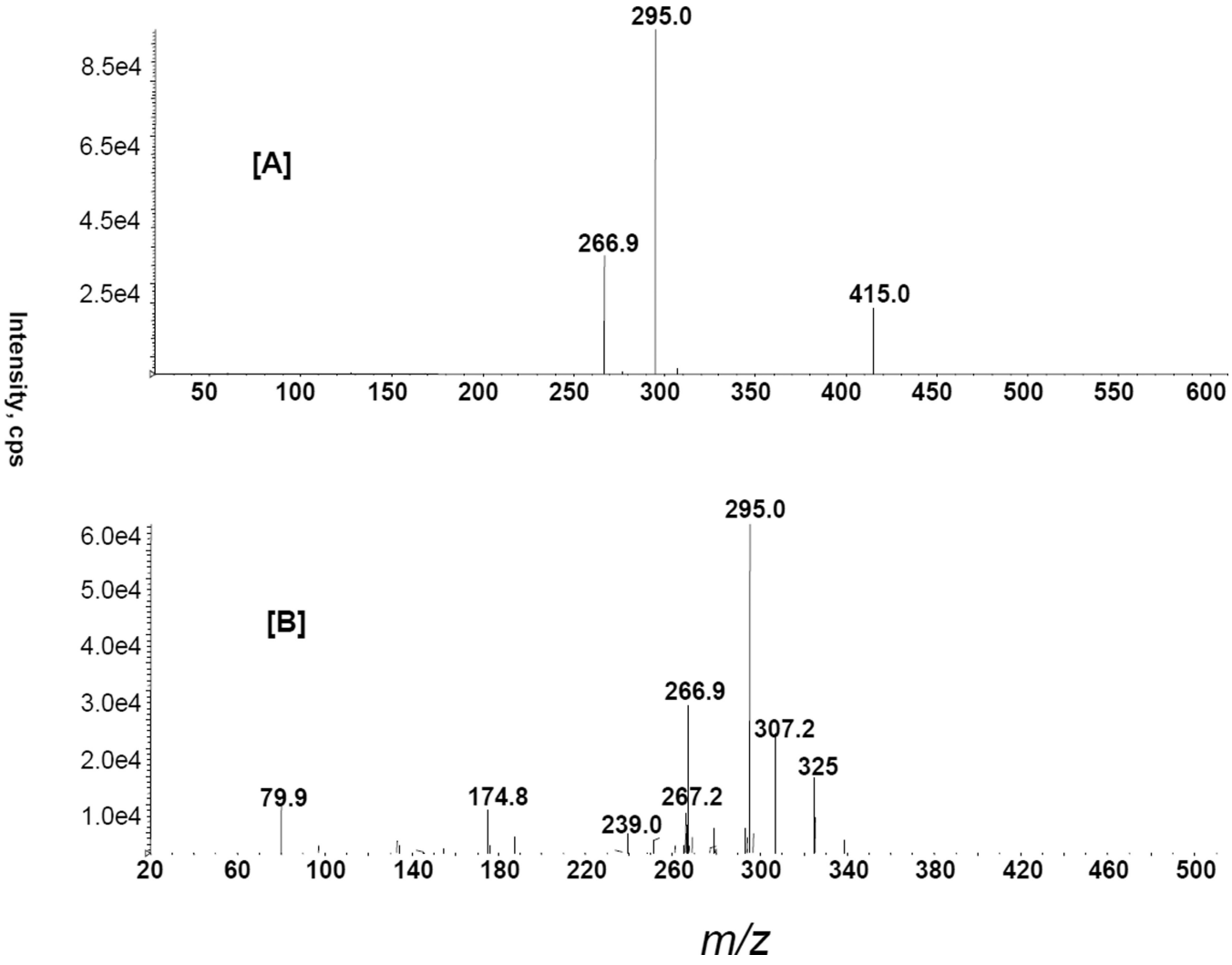

Figure 3.

Product ion spectra obtained from LC-MS/MS analysis of [A] *m/z* 591 and [B] *m/z* 495 in puerarin treated kidney and liver samples, respectively.

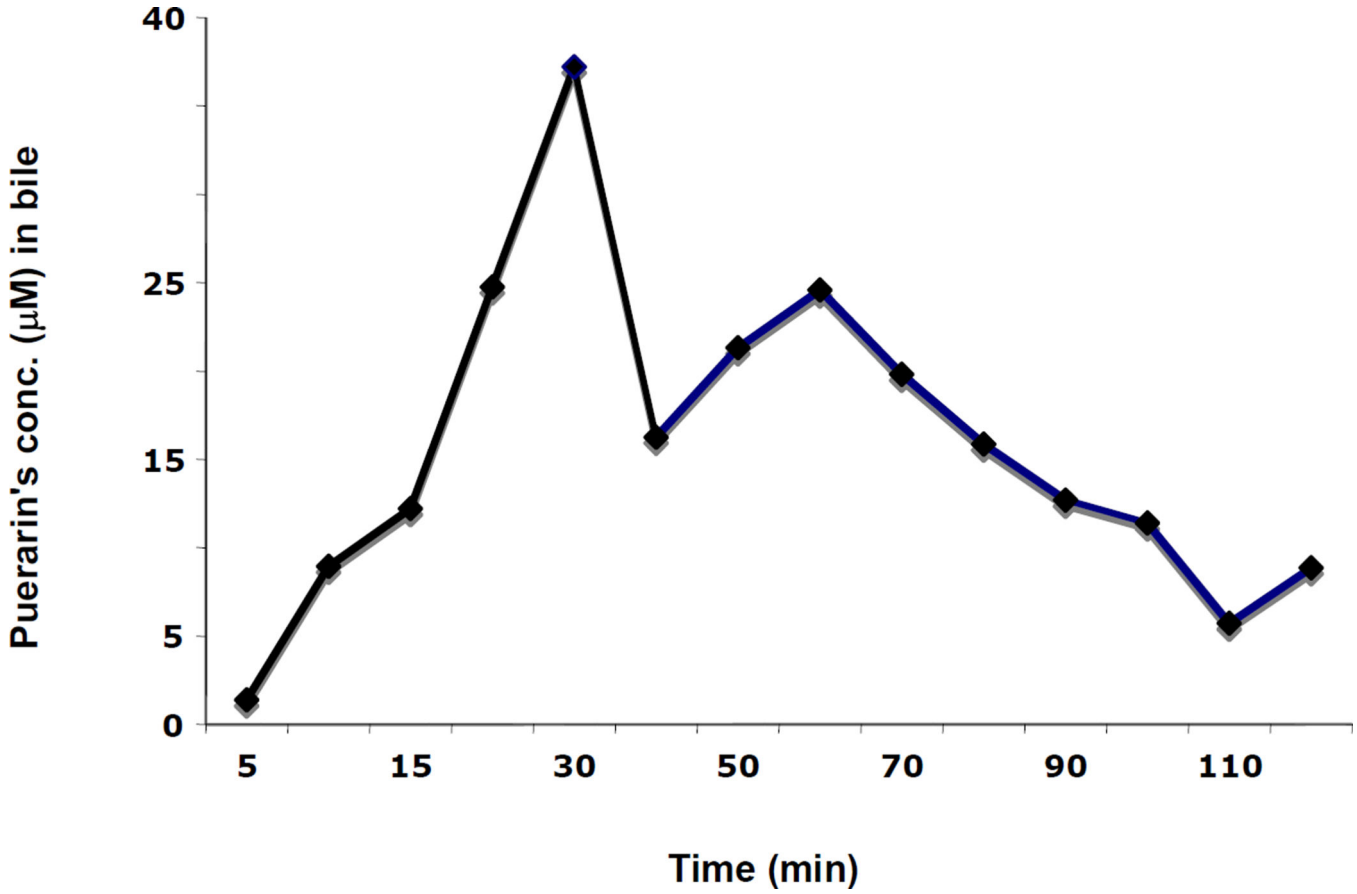

Figure 4.
Time-dependent biliary excretion of puerarin following infusion of puerarin into the portal vein. Experimental conditions are described in the Materials and Methods.

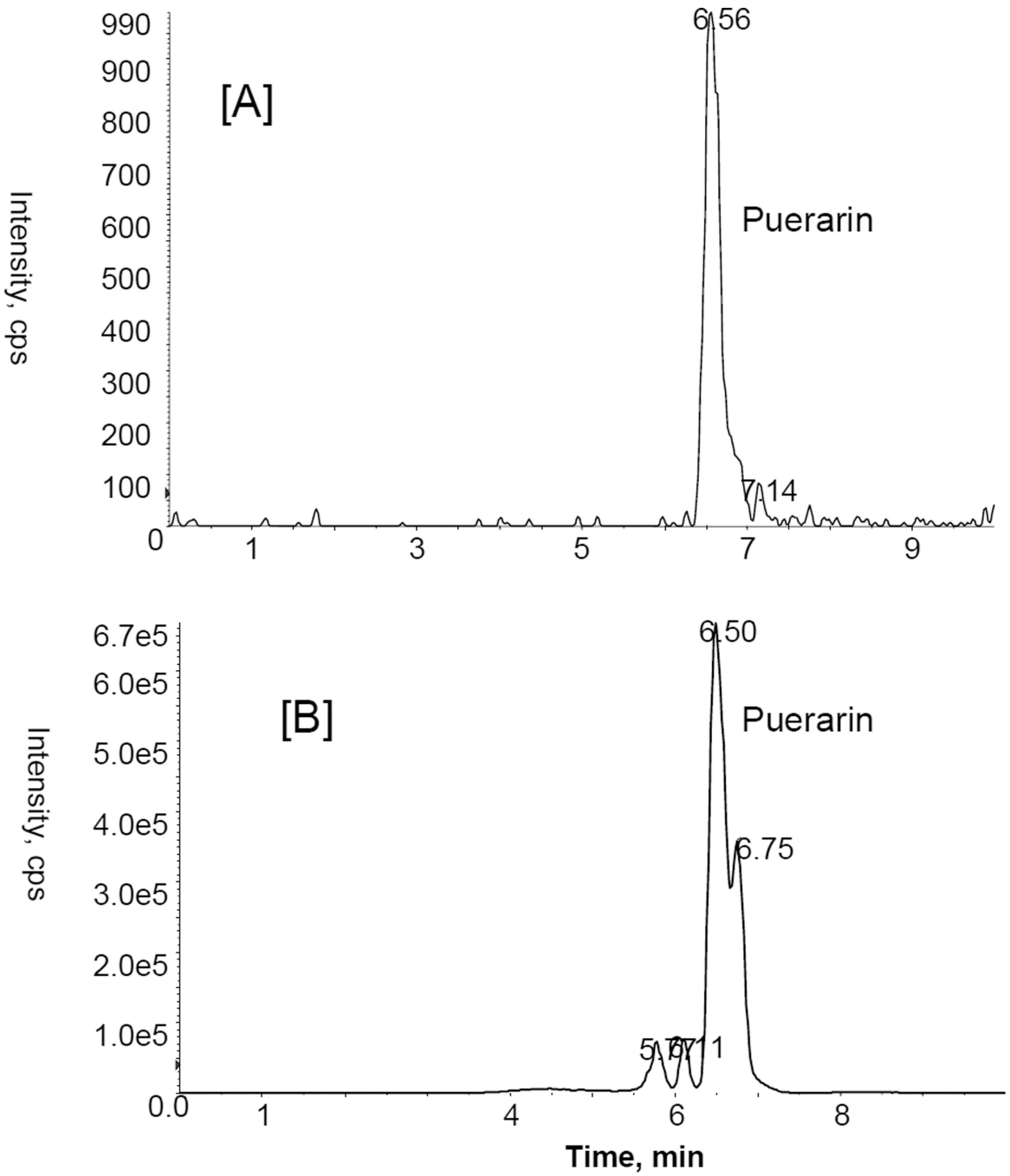

Figure 5.

MRM ion chromatograms for puerarin with mass transition *m/z* 416/267 at LLOQ 2 ng/ml [A]; bile sample collected at 120 min after oral administration of puerarin [B].