Direct Determination of Polydatin and Its Metabolite in Rat Excrement Samples by High-Performance Liquid Chromatography

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Simultaneous determination of polydatin and its metabolite in excrement samples using high-performance liquid chromatography (HPLC) with UV detection was accomplished. After extracted them by C_{18} solid phase extraction, the samples were separated on a reversed-phase column. Detection wave-lengths were set at 306 nm. The separation was carried out with a gradient elution. The mobile phase was acetonitrile–water (containing 0.1% formic acid) at a flow rate of 1.0 ml·min^{-1}. The identities of the peaks were accomplished by comparing retention times, UV and mass data with reference compounds under the same conditions. The standard curve was rectilinear in the range of 0.803—642.6 μg·ml^{-1} (r=1.0000) for polydatin, 0.407—325.8 μg·ml^{-1} (r=1.0000) for resveratrol. The recoveries of the markers listed above were 102.2% and 97.3%, respectively. The verified method can be used to determine the contents of two compounds in samples of stomach, small intestine, caecum, and large intestine (including excrement) of rats fed with polydatin. The analytical results demonstrated that the metabolism of polydatin is mainly processed in the intestines; polydatin can be transformed into resveratrol by de-sugaring process.

Key words polydatin; resveratrol; metabolite; intestinal flora; gastrointestinal; excrement sample

The essentials of Traditional Chinese Medicine (TCM) for disease treatment are their chemical substances. And the bioavailabilities of some substances are lower. Modern studies prove that many components of TCM can be metabolized by intestinal bacteria, and produced a series of products. These products carry out pharmacological effect after being absorbed.[1—12] Researches on the biotransformation of TCM components in intestinal flora bear a great significance to guide the development of new medicine and clinic reasonable application of medicines and wardship.

Polygonum cuspidatum is one of TCM in common use with its root and rhizome, which has been officially listed in Chinese Pharmacopoeia for a long time.[13] Polydatin is supposed to be one of the main effective elements of *Polygonum cuspidatum*. Pharmacological studies and clinical practice have demonstrated that polydatin has many biological functions, and widely applied in the control of quality for the *Polygonum cuspidatum* products.[14—18] *Polygonum cuspidatum* is mostly taken orally, but what metabolites polydatin is metabolized and what kind of metabolites are to be absorbed and exert pharmacological effect are still not deeply researched and reported. In this study, a quality assessment method for the simultaneous determination of polydatin and its metabolite was established. And the gastrointestinal metabolic transforming of polydatin through observation of the biology modifying of polydatin exerted by rat intestinal bacteria *in vivo* was studied. The results show that the metabolism of polydatin is mainly processed in the intestines; polydatin can be transformed into resveratrol by de-sugaring process. This research can provide reference to the methodological research on the effective metabolic parts by using TCM.

Experimental

Materials and Reagents HPLC grade acetonitrile was purchased from Tedia (Fairfield, OH, U.S.A.). Distilled water was supplied by Wahaha (Hangzhou, China) and formic acid was obtained from Shanghai Chemical Company (Shanghai, China). Standard substance of polydatin and resveratrol (Fig. 1) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); other chemical reagents were all analytical grade.

Standard solutions of polydatin and resveratrol were prepared at 642.6 and 325.8 μg·ml^{-1} in methanol respectively and stored in refrigeration before they were used to prepare the working solutions.

A drug-free excrement sample collected from a healthy SPF Wistar rat in our laboratory was used to make blank samples and spiked excrement samples containing standard solutions. The excrement samples of metabolism were obtained from following experiments. Nine healthy SPF Wistar rats were divided into three groups. After an overnight (about 12 h) fast, they were orally fed by the standard solution of polydatin based on 240 mg·kg^{-1} and put them into metabolic case to continue fasting. They drank freely sugar-brine water (containing 5% glucose and 0.9% sodium chloride) during fasting. They were slain and taken out the substances as samples in their stomach, small intestine, caecum, large intestine and excrement at 20 min, 90 min, 240 min, respectively.

HPLC System and Conditions Analyses were performed with Agilent series 1100 HPLC instrument equipped with a quaternary pump, a diode-array detector, an autosampler, a column compartment, and Agilent Chemistation of LC and LC/MS systems (California, U.S.A.). Samples were separated on an Agilent Zorbax SB-C_{18} column (250×4.6 mm, 5 μm particles, Agilent) together with a C_{18} guard column. The mobile phase was a gradient prepared from acetonitrile (component A) and water containing 0.1% formic acid (component B). The elution program was designed as follows: 0—15 min, 15% (A) to 20% (A); 15—40 min, 20% (A) to 45% (A). The flow-rate of the mobile phase was set at 1.0 ml·min^{-1} at room temperature and detection wave-lengths were set at 306 nm.

HPLC-MS System and Conditions Mass spectrometry was used to confirm the chromatographic profile obtained by diode-array detector. Finnigan Surveyor High Performance Liquid Chromatography System, Finnigan TSQ Quantum Discovery Triple Tandem Quadrupole Mass Spectrometer and Electrospray Ionization (Thermo Electron, U.S.A.) were used for HPLC-MS analysis. Identification was achieved using full-scan mode at a mass

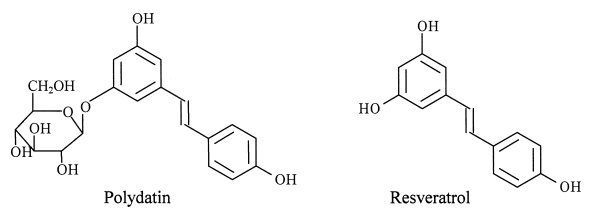

Fig. 1. The Chemical Structures of Compounds

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range of *m*/*z* 40—650. Automatic sampler was used. The mobile phase was a gradient prepared from water containing 0.02% v/v ammonia (component A) and acetonitrile (component B). The elution program was designed as follows: 0—15 min, 85% (A) to 55% (A). The flow-rate of the mobile phase was set at 0.2 ml·min^{-1} at room temperature. Injection volume was 1.0 μl.

Pretreatment of Excrement Samples Twenty-five milliliters of 50% ethanol solution was added to excrement sample to homogenize. After centrifugation, 10 ml supernatant clear solution was fetched and evaporated. The remainder then was dissolved into certain amount of water and extracted by C_{18} solid-phase extraction (Alltech, 200 mg, 4.0 ml) which was prewashed successively with 1.0 ml of methanol and 2.0 ml of water. The cartridge was rinsed with 2.0 ml water and eluted with methanol. An aliquot of 10 μl eluate was injected into the HPLC.

Calibration Curves, Limits of Quantification A methanol stock solution, which contained polydatin and resveratrol, was prepared and diluted to an appropriate concentration to make spiked excrement sample solutions for the construction of calibration curves. The calibration curves were constructed by plotting the mean peak areas *versus* the concentration of standards. The lowest concentration of working solution was diluted with methanol to yield a series of appropriate concentrations, and the limit of quantification under the chromatographic conditions were separately determined at signal-to-noise ratio (S/N) of 10.

Precision, Accuracy and Stability The measurement of intra-day and inter-day variability was utilized to determine the precision of this newly developed method. The intra-day variation was determined by analyzing three concentrations in sextuple the spiked excrement samples within 1 d. While for inter-day variability test, the solution was examined three concentrations in sextuple for consecutive 3 d. The relative standard deviation (RSD) was taken as a measure of precision. Recovery test was used to evaluate the accuracy of the developed assay. Accurate amounts of the two standards were added to drug-free excrement sample, and then extracted and analyzed as described above. Each sample was analyzed in sextuple. The average percentage recoveries were evaluated by calculating the ratio of detected amount *versus* added amount. Stability of sample solution was tested at room temperature. The sample solution was analyzed within 27 h. The analytes showed very stable (RSD 1.5%) over the tested period.

Results and Discussion

Validation As summarized in Table 1, the calibration curves obtained by plotting the peak-area against the concentration of substances showed a good linear correlation at the indicated concentration range with correlation coefficients of 1.0000.

The results of precision and accuracy evaluation for polydatin and resveratrol are shown in Table 2. Recoveries from excrement were 102.2% and 97.3% for polydatin and resveratrol, respectively. The limits of quantification for polydatin and resveratrol were found to be 0.803 and 0.407 μg·ml^{-1} (signal-to-noise ratio: 10), respectively.

Stability of the stock solution of sample was checked and found to be stable for 27 h.

Assay Characteristics A typical chromatogram of the compounds of interest: polydatin and resveratrol analyzed by the established method was shown in Fig. 2. The peaks appearing at 14.5 and 26.8 min were identified as polydatin and resveratrol, respectively. No peak was observed which interfered with the retention times of the compounds of interest.

As shown in Fig. 3, these compounds were eluted with excellent separation without any interference by endogenous

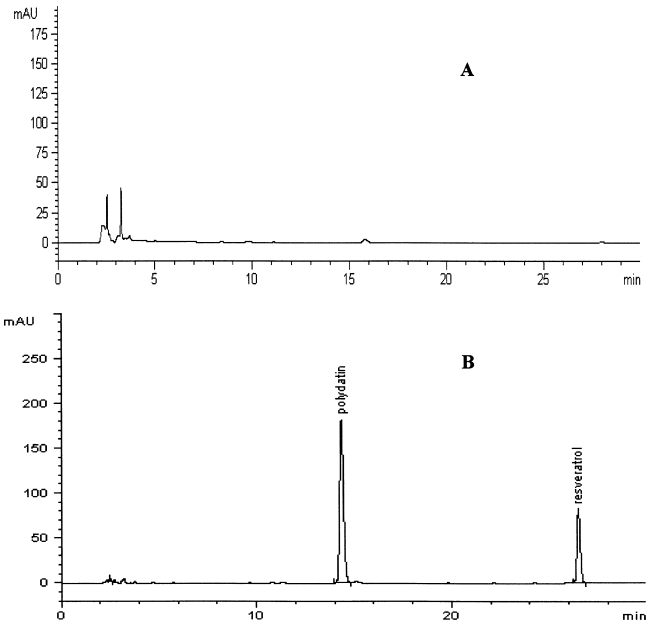

Fig. 2. Chromatograms of (A) Blank Excrement and of (B) Blank Excrement Spiked with Polydatin (37.67 μg·ml^{-1}) and Resveratrol (26.33 μg·ml^{-1})

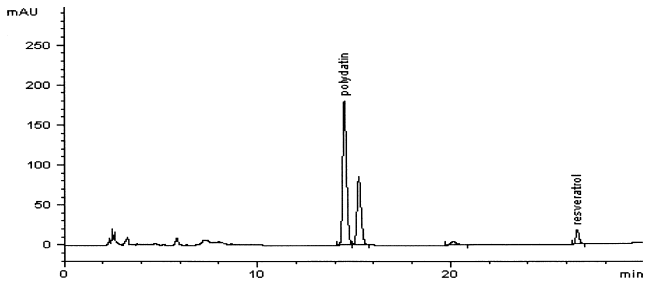

Fig. 3. Chromatogram Obtained from Gastrointestinal Sample of Rat Fed with Polydatin in 240 min

Table 1. Calibration Curves and LOQ of Two Analytes

Analyte	Calibration	*r*	Linear range (μg·ml^{-1})	LOQ (μg·ml^{-1})
Polydatin	y=43.30x+13.82	1.0000	0.803—642.6	0.803
Resveratro1	y=77.54x−27.25	1.0000	0.407—325.8	0.407

y: peak area; *x*: contents of reference compound.

Table 2. Precisions and Recoveries of the Two Analytes

Analytes	Concentration spiked (μg·ml^{-1})	Intra-day RSD (%)	Inter-day RSD (%)	Recovery (%)	Mean recovery (%)	RSD (%)
Polydatin	8.032	3.5	0.6	99.5		
	160.6	3.6	1.3	103.7	102.2	4.3
	642.6	4.5	0.2	103.5		
Resveratrol	4.072	1.6	1	90.1		
	162.9	3.0	2.4	99.9	97.3	6.5
	325.8	4.7	1.1	101.9		

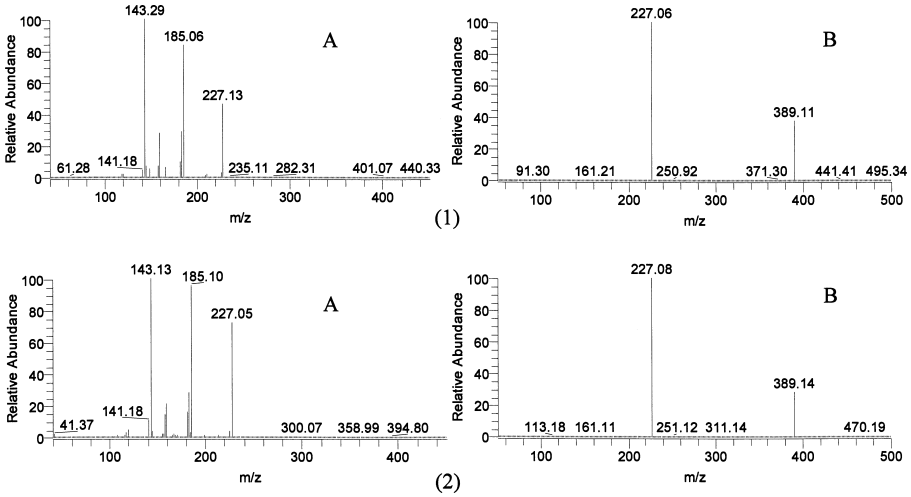

Fig. 4. Spectra of (1) Standards and of (2) Rat *in Vivo* Intestinal Sample (A, Polydatin; B, Resveratrol)

Table 3. The Content of Polydatin in Different Sections (mg±S.D.)

	Time		
	20 min	90 min	240 min
Stomach	1.90±1.86	2.09±1.64	0.051±0.053
Small intestine	1.54±1.52	1.97±1.38	1.75±1.80
Large intestine	0.014±0.005	0.018±0.016	0.42±0.41
Caecum	0.002±0.000	0.013±0.014	1.21±1.02

Table 4. The Content of Resveratrol in Different Sections (mg±S.D.)

	Time		
	20 min	90 min	240 min
Stomach	0.081±0.051	0.14±0.09	0.013±0.007
Small intestine	0.62±0.63	0.37±0.36	0.62±0.66
Large intestine	0.002±0.001	0.006±0.005	0.83±0.03
Caecum	0.008±0.003	0.006±0.004	1.92±0.62

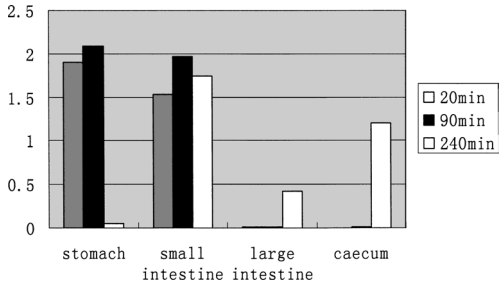

Fig. 5. The Content of Polydatin in Different Sections

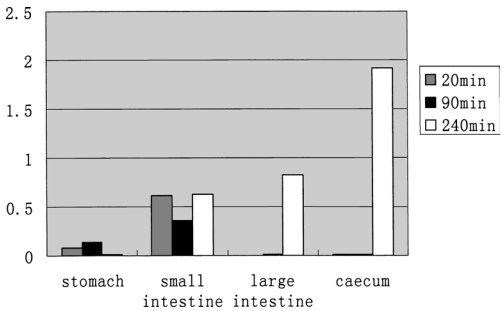

Fig. 6. The Content of Resveratrol in Different Sections

impurities.

Peak Identifications Considering that the chromatographic peaks could not be identified unambiguously only by retention time and UV spectra in a considerable complex matrix, HPLC-MS was used as a necessary supplement for confirmation of peak identification. Thus, peaks were identified by three means in this study: (1) by comparing the retention times of the unknown peaks with reference compounds, (2) by comparing the UV data with reference compounds, and (3) by comparing the MS data with reference compounds (Fig. 4).

Sample Analysis This newly proposed HPLC-DAD-MS method was subsequently applied to the simultaneous determination of polydatin and its metabolite resveratrol in contents samples of stomach, small intestine, caecum and large intestine (including excrement) obtained from rats fed with polydatin. Their contents were summarized in Tables 3, 4, and Figs. 5, 6.

The content of polydatin was very high in stomach in the first 90 min and reduced to very low after 240 min. In large intestine and caecum, the content was low in the first 90 min and increased significantly after 240 min. The content of resveratrol was low in stomach but was high in small intestine and saw significant increase in large intestine and caecum after 240 min. The result indicated that polydatin was metabolized into resveratrol in intestine.

Four hours after the rats were *in vivo* fed with polydatin, the medicaments in the body were mostly discharged and the glycosidic bond was to some extent hydrolyzed because the surplus glycoside was affected by the stomach conditions.[19,20] Through transferring from small intestine to large intestine, relative content of polydatin was decreased greatly and the resveratrol was relatively increased. The analytical results obtained also indicated that polydatin was hydrolyzed by the impact of micro-organic enzyme in the intestine and shucked off a molecule of glucose into resveratrol.

Conclusions

A simple and reproducible method for the simultaneous determination of polydatin and its metabolite resveratrol in excrement was established employing the combination of a solid-phase extraction and reversed-phase HPLC.

This method was successfully evaluated in terms of linearity, precision and LOQ. This is the first report of polydatin and its metabolite resveratrol being detected using HPLC in the excrement sample. HPLC-MS System and Conditions Mass spectrometry was used to confirm the chromatographic profile obtained by DAD detection.

In this article, it had been confirmed that rat intestinal bacteria hydrolyzed the glycosidic bond of polydatin. Polydatin can be easily metabolized by intestinal bacteria and the main metabolite is resveratrol.

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