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Quantitative determination of *trans*-polydatin, a natural strong anti-oxidative compound, in rat plasma and cellular environment of a human colon adenocarcinoma cell line for pharmacokinetic studies

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Abstract

A simple, accurate, precise, specific and reproducible high-performance liquid chromatography (HPLC) method was developed for determination of *trans*-polydatin, a natural strong anti-oxidative compound, in rat plasma and cell suspension. The assay procedure involved simple liquid–liquid extraction, the supernatant liquid was added an equal volume of water to avoid solvent effect. The detection of the analyte peak was achieved by monitoring the eluate using a UV detector set at 303 nm. The analysis used a Hypersil ODS2 C18 column (5 μm, 4.6 mm × 250 mm) and methanol/distilled water as the mobile phase (flow rate = 1 mL/min). A total analytical run was achieved within 6.0 min and calibration curve was linear over a wide concentration range of 0.25–40 μg/mL for plasma sample and 1.0–500 μM for cell suspension, the coefficients of correlation were 0.9997 and 0.9999 or better, respectively. There was 80.7 ± 7.86%, 96.8 ± 3.20% and 102.7 ± 9.72% recovery from 0.5, 10, and 40 μg/mL plasma samples, respectively. Intra- and inter-batch accuracy and precision were acceptable for the both matrices. The RSD of intra- and inter-day assay variations were all less than 10%. Both analyte and IS were stable in the battery of stability studies, freeze–thaw cycles. The described assay method was applied to pharmacokinetic studies in rats and a human colon adenocarcinoma cell line (Caco-2) successfully. The application of the assay to determine the pharmacokinetic is described.

Keywords: *trans*-Polydatin; HPLC; Plasma concentration; Pharmacokinetics

1. Introduction

Polydatin (3,4′,5-trihydroxystibene-3-β-mono-D-glucoside, Fig. 1), is a polyphenolic phytoalexin with strong anti-oxidative activity which is isolated from a variety of plant species and synthesized [1]. One of the richest sources of this compound is *Polygonum cuspidatem*, a weed that is used in traditional Chinese and Japanese medicines. Polydatin can inhibit platelet aggregation, lower blood cholesterol, enhance blood flow in capillaries and act as a tranquilizing agent [2–5].

There had many analytical methods including gas chromatography (GC)-coupled with MS and HPLC [6–10] been reported for determining *trans*-polydatin contained in the plants or red wine. However, no bioanalyitical method has been developed for *trans*-polydatin quantification up to now, since the plasma or other biosamples' concentrations were always much lower than that in the plants or pharmaceutical preparations.

Although *trans*-polydatin was widely used and many studies have reported its biological effects, but little works has been done on the pharmacokinetics of *trans*-polydatin in animals or

Abbreviations: HPLC, high performance liquid chromatography; IS, internal standard; QC, quality control; LLOQ, lower limit of quantification; RE, relative error; RSD, relative standard deviation; CV, coefficient of variation; AE, absolute recovery; Caco-2, human colon adenocarcinoma cell line

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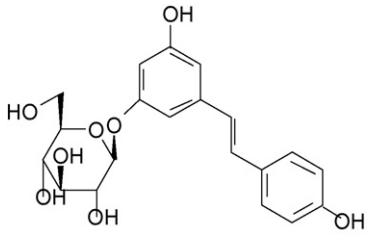

Fig. 1. The chemical structure of *trans*-polydatin (*trans*-3,4′, 5-trihydroxystibene-3-β-mono-D-glucoside).

in human body, especially we do not know whether it can be absorbed and the speed of absorption, distribution and elimination.

In recent years, there has been an increased interest in cellular pharmacokinetics. It may be regarded as absorption, distribution, metabolism and excretion of the drug in the cellular environment. Cellular pharmacokinetic studies involve the dynamic process, Meanwhile, parameters exhibit the special necessity for monitoring the intracellular drug concentration to guide for the clinical therapy.

Thus, to develop a highly sensitive, precise and accurate analytical method to monitor the amount changes in biological specimen is an urgent requirement raised for the pharmacokinetic studies. In this paper, a simple and sensitive HPLC method to determine the concentration of *trans*-polydatin in biological specimens including rat plasma and cell suspension was developed and applied to the subsequent pharmacokinetic studies. This method was fully validated for its specificity, accuracy, precision, and sensitivity, and was successfully applied to the pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

trans-Polydatin was kindly donated by Bio-sep Biotechnique Stock Co., Ltd. (Xian, China) with high purity (>98%), methanol and acetonitrile were HPLC grade (Tedia, USA). Caffeine, used as internal standard was supplied by the Medical Chemistry Department of China Pharmaceutical University. Purity was found to be more than 99% for both the compounds. Acetonitrile and methanol (HPLC-grade) were purchased from Tedia (USA). All aqueous solutions including the HPLC mobile phase were prepared with Milli Q grade water. The control rat plasma was obtained from the experiment animal breeding center of Southeast University.

2.2. Chromatography

The HPLC system consisted of a Shimadzu LC-10AT pump (Kyoto, Japan) and a Shimadzu SPD- 10 A VP ultraviolet detector (Kyoto, Japan). The data were acquired and processed using HS Chromatography Data System software of Hzep company from Hangzhou, China. The chromatographic separation was performed using a Hypersil ODS_2 C_{18} analytical column (5 μm, 4.6 mm × 250 mm) from Elite Analytical Instruments (Dalian City, China). The mobile phase consisted of a mixture of 0.5% (v/v) acetic acid in methanol and distilled water (40:60, v/v), filtered through a 0.45 μm nylon membrane and ultrasonically degassed prior to use. The mobile phase was delivered at a flow-rate of 1.0 mL/min. The eluate was monitored by an ultraviolet detector set at 303 nm, the maximal absorption for *trans*-polydatin and the same wavelength was found adequate for monitoring the internal standard. The attenuation was 0.001. Temperature used for HPLC was unregulated ambient.

2.3. Cell culture

DMEM media and fetal calf serum were purchased from Gibco. The human colon adenocarcinoma cell line Caco-2 was purchased from the American Type CμLture Collection (Rockville, MD, USA). The cells were maintained in DMEM with 2 g/L $NaHCO_3$ supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL) at 37 °C in a humidified 5%CO_2 atmosphere, were used for the cell experiments. For uptake studies, cells were cultured in 24-well plastic culture plates and used on the 7th day after seeding.

2.4. Preparation of quality-control samples

Standards and QC stock solutions of *trans*-polydatin was prepared in 50% methanol and water. Appropriate dilutions of *trans*-polydatin were made in methanol to produce working stock solutions. Stock solutions of IS caffeine (CA) (0.436 mg/mL) were prepared in methanol. All stock solutions were stored away from light at approximately 4 °C for 2 months. Calibration samples were prepared by spiking with the appropriate amount of the analyte and IS on the day of analysis into 50 μL of control rat plasma. QC samples for the determination of recovery, precision and accuracy were prepared by spiking control rat plasma in bulk at appropriate concentrations (0.5, 10, 40 μg/mL of *trans*-polydatin) and 50 μL volumes were aliquoted into different tubes and, depending on the nature of the experiments, were stored at −20 °C until analysis.

2.5. Sample pretreatment

Plasma and cell suspension samples were stored at −20 °C until analysis. To 50 μL of samples, 10 μL of IS was added and mixed for 30 s. After addition of 100 μL of acetonitrile, the mixture was vortexed for 1 min and centrifuged at 10000 × *g* for 10 min. The organic layer (100 μL) was separated and 100 μL of water was added in to avoid solvent effects. After mixing, 25 μL of the supernatant was injected for analysis.

2.6. Calibration curves

Calibration curves were acquired by plotting the peak area ratio of *trans*-polydatin: IS against the nominal concentration of calibration standards. The working standards (a) at 1.25, 2.5, 5.0, 25, 50, 100, 200 μg/mL for rats plasma and (b) at 5.0, 25, 50, 250, 500 μM for cell suspension were prepared by further dilution in water, and then stored at −4 °C. The calibration standards were prepared by spiking the matrices with 10 μL cor-

responding working solutions, IS was added in samples before analysis.

2.7. Precision and accuracy

To calculate intra-day and inter-day accuracy and precision, QC samples were reanalyzed five times in a single batch and once in three consecutive bathes, respectively. The determined concentrations, which were obtained from a calibration curve prepared on the same day, were used to evaluate the method accuracy and precision. The accuracy was expressed as the relative error (RE) according to the equation: RE (%) = 100% × (measured concentration − spiked concentration)/spiked concentration, and the precision was evaluated by the coefficient of variation (CV). The intra-assay precision and accuracy were estimated by analysing five replicates containing *trans*-polydatin at three different QC levels, i.e. 0.5, 10 and 40 μg/mL. The inter-assay precision was determined by analyzing the three levels of QC samples on five different runs. The criteria for acceptability of the data included accuracy within ± 15% RE from the nominal values and precision within 15% CV [11,12].

2.8. Stability experiments

The stability of *trans*-polydatin and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 6 h (at 5 °C) after the initial injection. The peak areas of the analyte and IS obtained at initial cycle were used as the reference to determine the relative stability at subsequent points. Freezer stability of *trans*-polydatin in rat plasma was assessed by analyzing the QC samples stored at −20 °C for at least 1 month. The stability of *trans*-polydatin in rat plasma following repeated freeze–thaw cycles was assessed using QC samples spiked with *trans*-polydatin. The samples were stored at −20 °C between freeze–thaw cycles. The samples were thawed by allowing them to stand at room temperature for approximately 1 h. After drawing out the required volume, the samples were then returned to the freezer. The stability of *trans*-polydatin was assessed after three freeze–thaw cycles. The samples were processed using the same procedure as described in Section 2.4. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% DEV) and precision (i.e. 15% RSD) [11,12] and there were no statistically significant differences between in two compared parameters. To assess the final stability decision, assay values were evaluated with tests: two-way ANOVA.

2.9. Extraction recovery

Two sets of standards were analyzed containing the analyte at three different concentrations 0.5, 10 and 40 μg/mL. One set was prepared in rat plasma and the other set was prepared in methanol (neat set). The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. If one depicted the peak areas obtained in neat solution standards as *A* and the peak areas for extracted QC samples as *B*, the AE values were calculated as follows:

$$\mathrm{AE}(\%) = \frac{B}{A} \times 100\%$$

The recovery value was calculated at the various concentrations of *trans*-polydatin.

2.10. Application of the assay

2.10.1. Pharmacokinetics in rats

Five male Sprague–Dawley rats (210–240 g) were obtained from experiment animal breeding center of Southeast University. Animals were housed under controlled conditions (20 ± 2 °C, H50 ± 20%) with a natural light–dark cycle. They were allowed to adapt to the housing environment for at least 1 week prior to study. The animals were fasted overnight (12 h) and had free access to water throughout the experimental period. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. The rats were anesthetized with i.p. administered urethane (1 g/kg), and then underwent cannulation of the jugular vein and using PE50 polyethylene tubing (inner diameter, 0.58 mm; out diameter, 0.965 mm; Becton Dickinson & Co.(Parsippany, NJ). After the operation, each rat was given *trans*-polydatin at a single dose of 10 mg/kg in rats by intravenous injection administration. Blood samples (about 100 μL) were collected in heparinized 1.5 mL polythene tubes immediately before and 0, 2, 5, 10, 15, 30, 45, 60, 90, 120 and 180 min after dosing, and were at once centrifuged at 800 × *g* for 10 min at 4 °C. A 50 μL volume of plasma was finally harvested and, stored at −20 °C until analysis. The pharmacokinetic parameters were calculated using 3P87 program [13].

2.10.2. Cellular pharmacokinetic: trans-polydatin uptake measurement

The applicability of this assay was examined in a cellular pharmacokinetic study of *trans*-polydatin in a human colon adenocarcinoma cell line (Caco-2). Cellular uptake experiments were carried out according to the method described previously [14,15]. *trans*-Polydatin uptake was examined by cell incubation over different times and at two different temperatures (37 and 4 °C). Briefly, cells were incubated with 20 μM *trans*-polydatin for different times, and at 2, 4, 6, 8, 10, 30 min, post-dose the intracellular concentrations of *trans*-polydatin were determined. An aliquot of cell lysate was used in parallel to determine cellular protein concentration by the Bradford protein assay [15], and the intracellular concentrations of *trans*-polydatin were expressed as nM/mg protein.

3. Results

3.1. Validation

3.1.1. Selectivity and chromatography

Under current conditions, *trans*-polydatin and IS were clearly separated chromatographically with the retention times of 4.0

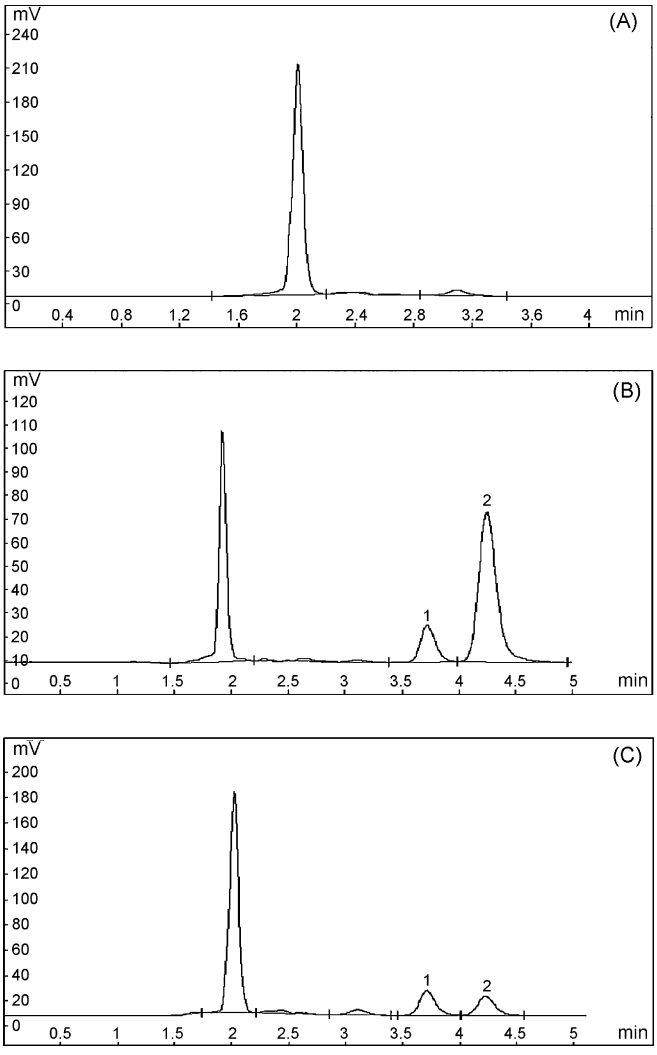

Fig. 2. Typical HPLC-UV chromatogram of blank plasma (A), blank plasma spiked with *trans*-polydatin (B), and a plasma sample obtained from a rat that was injected intravenously with 10 mg/kg of *trans*-polydatin (C). The labeled chromatographic peaks indicate *trans*-polydatin (peak 1) and the internal standard (peak 2).

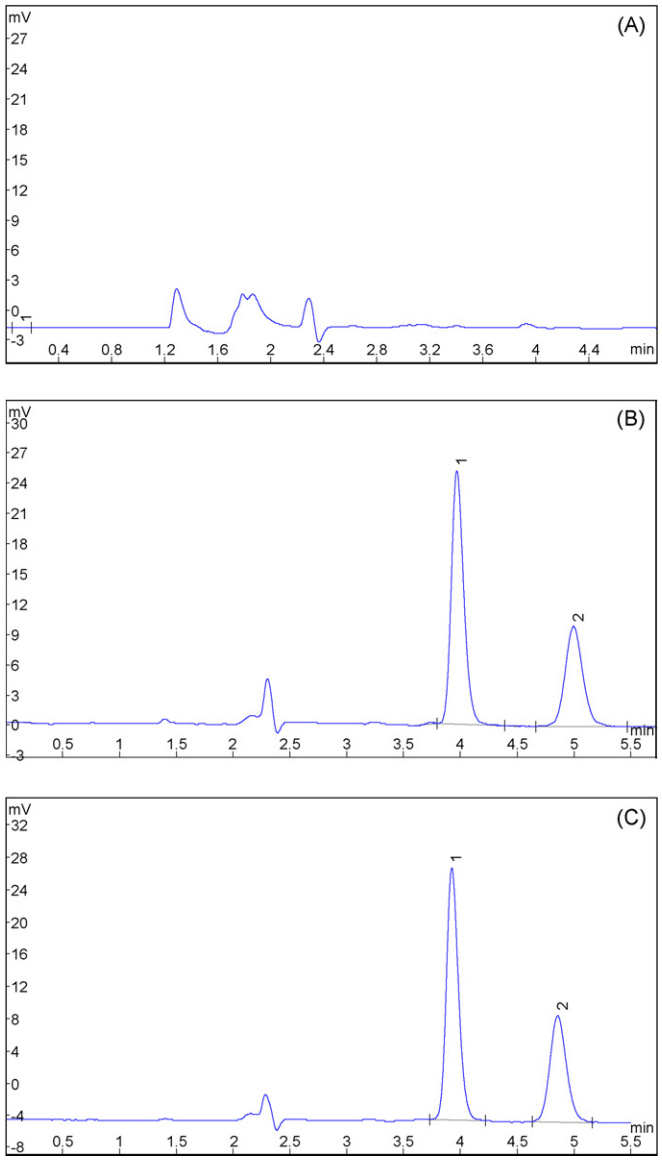

Fig. 3. Typical HPLC-UV chromatogram of cell suspension (A), blank cell suspension spiked with *trans*-polydatin (B), and a cell sample obtained from cells that were incubated with 20 μM *trans*-polydatin (C). The labeled chromatographic peaks indicate *trans*-polydatin (peak 1) and the internal standard (peak 2).

and 5.2 min, respectively. Results of HPLC analysis of six different randomly selected drug-free blank plasma and cell suspensions showed few or no interfering peaks presented at the retention times of either target or internal standard (Figs. 2 and 3).

3.1.2. Extraction efficiency

The absolute recovery of *trans*-polydatin from plasma was calculated by comparing the peak area obtained from extracts of spiked plasma samples to that obtained from the direct injection of known amounts of standard solutions of *trans*-polydatin. The overall extraction yields of 0.5, 10, and 40.0 μg/mL *trans*-polydatin in plasma were above 80% (Table 1). The data proved the suitability of the extraction method for use with plasma samples. The study showed that at least 10 min of centrifugation at 10,000 × *g* were needed for protein denaturization completely where 100 μL acetonitrile was added to 50 μL plasma sample, where acetonitrile acted as deproteinization agent. A smaller volume of water was used here, which was advantageous in increasing the sensitivity of the method. In the development phase of this work, the supernatant of the acetonitrile extraction was directly injected into the chromatographic system. The solvent effect was serious and induced distorted chromatographic peaks which were not suitable for calculating the concentration. By adding an equal volume of

Table 1
Recovery of *trans*-polydatin from rat plasma ($n = 5$)

Concentration (μg/mL)	Recovery (Mean ± SD %)	RSD (%)
0.50	80.7 ± 7.86	9.74
10.00	96.8 ± 3.20	3.31
40.00	102.7 ± 9.72	9.46

Table 2
The intra-day and inter-day precision of the method for *trans*-polydatin ($n = 5$)

	Spiked concentrateion (μg/mL)	Measured concentrateion (mean ± SD, μg/mL)	Precision (RSD, %)[a]	Accuracy (relative error, %)[b]
Intra-day	0.5	0.480 ± 0.032	6.67	−4.00
	10	10.165 ± 0.255	2.51	1.65
	40	41.284 ± 1.389	3.36	3.21
Inter-day	0.5	0.470 ± 0.033	7.02	−6.00
	10	9.450 ± 0.323	3.42	−5.50
	40	38.544 ± 1.625	4.33	−6.14

[a] RSD = 100% × (SD/mean).
[b] Relative error $r = 100\% \times$ (measured concentrateion − spiked concentrateon)/spiked concentration.

water to the supernatant acetonitrile extraction, the solvent effect was avoided and symmetrical chromatographic peaks were obtained.

3.2. Linearity of calibration

Good linearity was observed.The following equations were derived from the calibration curves: $y = 1.0564C + 0.0407$ and $y = 0.1625C - 0.0199$, for *trans*-polydatin in plasma and cell suspensions, respectively, where y indicates the peak area ratios of analyte to internal standard and C represents the concentrations. The coefficients of correlation were 0.9997 and 0.9999 or better, respectively ($n = 5$).

3.3. Limits of detection and quantitation

The lower limit of quantification (LLOQ) for *trans*-polydatin in these matrices, which were defined as the concentration of analyte that gave a signal-to-noise ratio of 10 and both precision and accuracy less than or equal to 20%, were defined as the lowest standards on the calibration curves. The minimum detectable concentration of *trans*-polydatin was determined to be 0.05 μg/mL.

3.4. Precision and accuracy

The intraday assay variations were determined by analyzing five 50 μL aliquots of spiked plasma samples containing 0.5, 10, 40.0 μg/mL of *trans*-polydatin. The interday assay variations were determined by analyzing 50 μL aliquots of spiked plasma samples in duplicates on five separate days. The data proved good precision and accuracy of the method. As shown in Table 2, the intra-day precision of *trans*-polydatin were between 2.51 and 6.67% The accuracy were from −4.00 to 3.21%. the inter-day precision were between 3.42 and 7.02%, The accuracy were from −6.14 to −5.50%. The values of precision and accuracy were acceptable in view of the international recommendation that the precision and accuracy should not exceed 15% except at the LOQ, where they should be within ±20% [11]. The results indicated the method was reproducible.

3.5. Analyte stability

3.5.1. Freeze–thaw stability

Table 3 shows the results of the analyses of the QC samples following three freeze–thaw cycles. *trans*-Polydatin was shown to be stable in the frozen plasma at −20 °C for at least three freeze–thaw cycles (Table 3).

Table 3
Stability data of *trans*-polydatin quality controls in rat plasma

QC (spiked) concentration(μg/mL)	Stability	Mean ± SD[a] (μg/mL), $n = 4$	Accuracy (%)[b]	Precision (% CV)
0.5	0 h (for all)	0.53 ± 0.013		2.45
	3 F/T	0.47 ± 0.025	88.68	5.32
	6 h (in injector)	0.52 ± 0.015	98.11	1.76
	7 days at −20 °C	0.48 ± 0.023	90.57	3.75
10	0 h (for all)	9.87 ± 0.03		
	3 F/T	9.06 ± 1.06	91.79	7.59
	6 h (in injector)	9.55 ± 0.06	96.76	4.54
	7 days at −20 °C	9.27 ± 0.08	93.92	9.20
40	0 h (for all)	40.17 ± 0.27		
	3 F/T	36.85 ± 2.33	91.74	5.26
	6 h (in injector)	40.04 ± 0.15	99.68	2.26
	7 days at −20 °C	39.10 ± 1.37	97.34	7.47

QC, quality control; %CV, coefficient of variation; F/T, freeze–thaw.
[a] Back-calculated plasma concentrations.
[b] (Mean assayed concentration/mean assayed concentration at 0 h) × 100%.

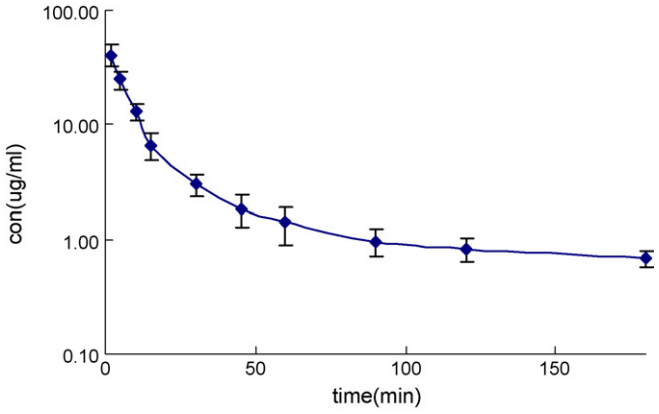

Fig. 4. Plasma concentrations of *trans*-polydatin after iv administration of 10 mg/kg in rats ($n = 5$).

3.5.2. Freezer stability

trans-Polydatin was found to be stable when stored at −20 °C for at least 7 days. Both accuracy and precision of QC samples in this evaluation were within the assay variability of ±15% (Table 3).

3.5.3. Ambient temperature stability

trans-Polydatin was shown to be stable at ambient temperature after 6 h for post-preparative samples (Table 3).

Table 3 shows the 90% confidence interval obtained by analysis of variance (ANOVA) for these parameters. No differences between the reference and test formulations were detected (a value of $p < 0.05$ was considered significant between the two groups), the compound was considered stabile.

3.6. Application of the method

3.6.1. Pharmacokinetics in rats

The established HPLC method was applied for the determination of *trans*-polydatin in the pharmacokinetic study after intravenous administration of a single dose to rats. The mean plasma concentration of *trans*-polydatin versus time profile is shown in Fig. 4. The pharmacokinetic data obtained are listed in Table 4. Following intravenous administration of the isomers, a rapid eliminate disposition was observed.

Table 4
Pharmacokinetic data for five rats after iv administration of 10 mg/kg *trans*-polydatin

Parameter	Mean ± SD
A (μg/mL)	68.19 ± 21.26
α (1/min)	0.008 ± 0.0001
B (μg/mL)	1.058 ± 0.039
β (1/min)	0.003 ± 0.001
K_a (min)	0.17 ± 0.016
MRT (min)	186.28 ± 40.34
AUC_t (min μg/mL)	481.84 ± 58.85
C_{max} (μg/mL)	38.65 ± 4.06
$t_{1/2\alpha}$ (min)	83.32 ± 0.97
$t_{1/2\beta}$ (min)	249.96 ± 2.93
V (L/kg)	0.15 ± 0.048
CL (L/kg min)	0.019 ± 0.003

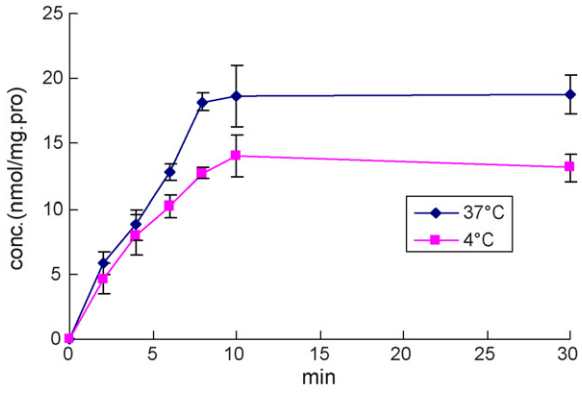

Fig. 5. Time-course of *trans*-polydatin uptake by Caco-2 cells. Caco-2 cells were incubated in the presence of 20 μM of *trans*-polydatin at 37 °C (♦) or at 4 °C (■) for indicated times. The uptake of trans-polydatin by Caco-2 cells was measured as described in Section 2. Data points are from a representative experiment among two and each point represents the mean of two determinations.

3.6.2. Cellular pharmacokinetic

The amounts of *trans*-polydatin taken up by the cells were determined after cell incubations with 20 μM *trans*-polydatin. This concentration was chosen in accordance with our previous results showing that it has no cell damage. In order to reveal a possible carrier-mediated transport of *trans*-polydatin, parallel experiments were performed at 37 and 4 °C (Fig. 5). At 37 °C, the intracellular concentration increased rapidly during the first 10 min according to a one-phase exponential association process, reaching near saturation after approximately 10 min and a maximal intracellular concentration reached about 18.69 nM/mg protein. A steady state was observed at 30 min after the initial phase. At 4 °C, the initial rate of the incorporation of *trans*-polydatin was about 1.6 times lower than that estimated at 37 °C.

4. Conclusion

The established HPLC method of determination of *trans*-polydatin was fully validated in terms of sensitivity, accuracy and specificity. A preliminary pharmacokinetics application proved further that the developed method based on HPLC detection was sensitive enough and reproducible for the pharmacokinetics study of *trans*-polydatin. Moreover, the sample preparation procedure is easy to operate and control. The assay could extend to some other cell lines and was proved to meet the requirements of cellular pharmacokinetic studies of *trans*-polydatin.

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