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Journal of Chromatography B, 857 (2007) 332–336
JOURNAL OF CHROMATOGRAPHY B
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Short communication

Determination of scopoletin in rat plasma by high performance liquid chromatographic method with UV detection and its application to a pharmacokinetic study

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Received 9 May 2007; accepted 14 July 2007
Available online 22 July 2007

Abstract

A rapid and simple high-performance liquid chromatographic (HPLC) method has been developed and validated for determination of scopoletin in rat plasma using psoralen as internal standard. Chromatographic separation was achieved on a C_{18} column using methanol and distilled water (49:51, v/v) containing 0.05% (v/v) phosphoric acid as mobile phase. The UV detector was set at 345 nm. The calibration curve was linear over the range of 0.165–9.90 μg/ml with a correlation coefficient of 0.9994. The recovery for plasma samples of 0.165, 1.32 and 6.60 μg/ml was 93.2%, 95.9% and 95.5%, respectively. The RSD of intra- and inter-day assay variations was less than 6.7%. This HPLC assay is a precise and reliable method for the analysis of scopoletin in pharmacokinetic studies.

Keywords: Scopoletin; HPLC; Plasma; Pharmacokinetics

1. Introduction

Scopoletin, a 6-methoxy-7-hydroxycoumarin as shown in Fig. 1, was isolated from the stems of *Erycibe obtusifolia* Benth. which was usually used in traditional Chinese medicine for rheumatic arthritis therapy. It was also the main component of many plants such as *Nicotiana glauca*, *Lycium chinense*, *Angelica dahurica* and *Trigonella foenum-graecum* roots [1–4]. Previous studies indicated that scopoletin possessed many biological effects. When orally administered to levo-thyroxine-treated rats, scopoletin (1 mg/kg) decreased the levels of serum thyroid hormones and glucose as well as hepatic glucose-6-phosphatase activity, suggesting its potential to regulate hyperthyroidism and hyperglycemia. It also inhibited hepatic lipid peroxidation and increased the activity of antioxidants, superoxide dismutase and catalase [5]. Findings from our laboratory pointed out that scopoletin (50, 100, 200 mg/kg, i.p.) exhibited an immediate and dose-dependent hypouricemic effect in potassium oxonate-induced hyperuricemic mice through decreasing uric acid production and a uricosuric mechanism [6]. In vitro, scopoletin (1–50 μg/ml) inhibited the release of pro-inflammatory cytokines (TNF-a, IL-1β and IL-6) and PGE_2 from LPS-stimulated RAW 264.7 cells [7]. It (100, 200, 400 μg/ml) inhibited the proliferation of human prostate adeocarcinoma cells by inducing cell cycle arrest and increase apoptosis [8]. Scopoletin (10 μg/ml) induced cell proliferation of T lymphocytes from lymph nodes of C3H mice [9]. In isolated rat aortic rings, scopoletin (26–520 μM) inhibited to approximately the same extent the contractions induced by a variety of substances, including phenylephrine, potassium chloride, serotonin and $PGF_2\alpha$ [10].

To detect the quantity of scopoletin in plants and medical preparations, several analytical methods have been developed during the last decade. High-performance liquid chromatography (HPLC) [11–13] and thin layer chromatography (TLC) [14] are most widely used. These methods were developed to determine higher concentrations of scopoletin in samples with comparatively simpler matrix. The main drawbacks of existing HPLC methods with UV detections are either long elution time (>15 min) or lack of internal standard [12,13] or

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doi:10.1016/j.jchromb.2007.07.023

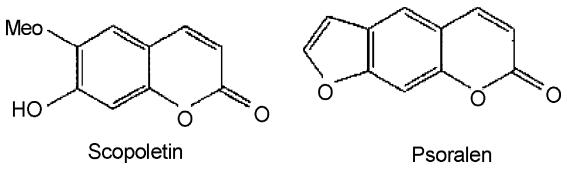

Fig. 1. Chemical structures of scopoletin and psoralen (internal standard).

need a gradient of the mobile phase [11]. They are unsuitable for in vivo microanalysis in relatively complex matrix such as rat plasma. A more rapid, more sensitive and well-validated detection method is necessary.

In the present study, we first established a simple and rapid HPLC method with ultraviolet detection for the quantification of scopoletin in rat plasma. After fully validated, the method was successfully applied to the pharmacokinetic study of scopoletin in rats. The novelty of the method include simple sample treatment with methanol precipitation, centrifugation and direct injection of the clear supernatant to the HPLC system, short analysis time (less than 15 min) with isocratic elution, suitable internal standard, low limit of detection (0.05 μg/ml), good precision (less than 7%) and high recovery (greater than 93%).

2. Experimental

2.1. Reagents and chemicals

Scopoletin was separated and purified from an ethanol extract of *E. obtusifolia* Benth. and its structure was confirmed by UV, IR, MS and NMR spectroscopy. The purity was 99.5% as determined by HPLC. Psoralen used as internal standard (IS) was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol was obtained from Hanbon Sci. & Tech. (Jiangsu, China). Other chemicals used were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrument and chromatographic conditions

HPLC analysis was carried out on an Agilent 1100 Series HPLC (Palo Alto, CA, USA) with UV detector. A Shim-pack CLC-ODS C_{18} analytical column (5 μm, 4.6 mm × 150 mm, Shimadzu Corporation, Kyoto, Japan) protected by a compatible guard column was used. The column was maintained at 25 °C. A mobile phase consisted of a mixture of 0.05% (v/v) phosphoric acid in methanol and distilled water (49:51, v/v) was used throughout the analysis at a flow rate of 1.0 ml/min. The ultraviolet (UV) detector was set at 345 nm.

2.3. Preparation of calibration standards and quality control samples

Primary stock solutions of scopoletin and psoralen were prepared in methanol at concentrations of 110 and 115 μg/ml, respectively. These primary stock solutions were sealed and stored at 4 °C until used. The secondary working stock solution of scopoletin was prepared by diluting with methanol from primary stock solution. The working internal standard solution was prepared by diluting the primary stock solution with methanol giving a concentration of 5.75 μg/ml. Calibration standards of scopoletin were prepared by serial dilution with blank rat plasma yielding final concentrations of 0.165, 0.33, 0.66, 1.32, 1.65, 3.30, 6.60, and 9.90 μg/ml. Quality control (QC) samples were prepared at low (0.165 μg/ml), medium (1.32 μg/ml) and high (6.60 μg/ml) concentrations in the same way as the plasma samples for calibration.

2.4. Sample preparation

One hundred microlitres of rat plasma samples (calibration standards, QC samples, and pharmacokinetic plasma samples) were mixed with 200 μl methanol containing 5.75 μg/ml of psorale. The mixture was vortexed for 1 min and then centrifuged at 12,000 rpm for 10 min in a 1–15 K centrifuge (Sigma, Germany) at 10 °C. Then 60 μl of supernatant was injected into the HPLC system.

2.5. Method validation

2.5.1. Linearity of calibration curves

Calibration curves were constructed from working standard solutions of scopoletin at concentration range 0.165–9.90 μg/ml by plotting peak-area ratio (Y) of scopoletin to the internal standard, psoralen, versus scopoletin concentration (X). The scopoletin samples were prepared in five replicates. The regression parameters of slope, intercept, and correlation coefficient were calculated by weighted ($1/x^2$) least-squares linear regression analysis of Y versus X.

2.5.2. Accuracy and precision

The accuracy and precision (presented as relative standard deviation, RSD) of this analytical method were evaluated using QC samples. QC samples were prepared as described above. The solution of a certain concentration was injected into the HPLC system for quantitative determination five times a day to evaluate intra-day precision. The same procedure was performed once a day for 5 consecutive days to determine inter-day precision. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration.

2.5.3. Recovery

Spiked plasma samples were prepared at concentrations of 0.165, 1.32 and 6.60 μg/ml, and assayed as described above. Recovery (extraction efficacy) was calculated by comparing the peak-area of the extracted sample to that of the unextracted standard solution containing the same concentration. The recovery of internal standard from plasma was determined at a concentration of 5.75 μg/ml by the same method.

2.5.4. Stability

Analyte stability in plasma was tested using QC samples for three freeze–thaws, long-term, short-term and post-preparative

stabilities. The freeze–thaw stability of the analyte was determined over three freeze–thaw cycles within 3 days. In each freeze–thaw cycle, the spiked plasma samples were frozen for ~24 h at −20 °C and thawed at room temperature. The long-term stability was evaluated after keeping the plasma samples frozen at −20 °C for 1 month. For the short-term stability, frozen plasma samples were kept at room temperature for 6 h before sample preparation. The stability of the prepared plasma samples was tested after keeping the samples at room temperature for 24 h. The samples were analyzed and the results were compared with those obtained for freshly prepared samples. For the acceptance criterion of stability, the deviation compared to the freshly prepared standard should be within ±15%.

2.6. Pharmacokinetic study

Sprague-Dawley rats (210–250 g) were obtained from the Experimental Animal Center of China Pharmaceutical University. Animals were housed under controlled conditions (22 ± 2 °C, RH 50 ± 20%) with a natural light–dark cycle. They were allowed to adapt to the housing environment for at least 1 week prior to study. Diet was prohibited for 10 h before the experiment while water was taken freely. The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. After an intragastric (i.g.) administration of scopoletin (50 mg/kg), 0.3 ml blood samples *via* orbital veins were collected in heparinized 1.5 ml polythene tubes just before and 2, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after dosing, and were immediately centrifuged at 3000 rpm for 10 min at 4 °C. A 100 μl volume of plasma was finally obtained and stored at −20 °C until analysis.

3. Results

3.1. Specificity, linearity and sensitivity

Representative chromatograms were shown in Fig. 2. Under the chromatographic conditions described, scopoletin and IS were eluted with retention times of 5.1 and 10.3 min, respectively. Endogenous components in rat plasma did not give any interfering peaks. Calibration curves were established on five replicate experiments with scopoletin spiked to rat plasma demonstrated good linearity over a range of 0.165–9.90 μg/ml. The mean linear regression equation of the peak-area ratio (Y) versus scopoletin concentration (X) was typically of the form $Y = (a \pm \mathrm{SD}) + (b \pm \mathrm{SD})X$ and it was $Y = (-0.0150 \pm 0.0020) + (0.6664 \pm 0.0072)X$ with mean correlation coefficient of 0.9994 ± 0.0002. The lower limit of quantitation (LLOQ) of scopoletin in plasma was verified as 0.165 μg/ml, as this was the lowest concentration assessed at which the accuracy was between 85 and 115%, and a coefficient of variation lower than 15%. The lower limit of detection (LOD) was 0.05 μg/ml at a signal-to-noise ratio of 3.

3.2. Accuracy and precision

The intra- and inter-day precision and accuracy of scopoletin were shown in Table 1. The precision (RSD) was all less than 6.7%. The accuracy of scopoletin ranged from 95.6 to 99.1% for intra-day and 94.8–98.9% for inter-day, respectively. These results indicated that the present method had a good accuracy and precision.

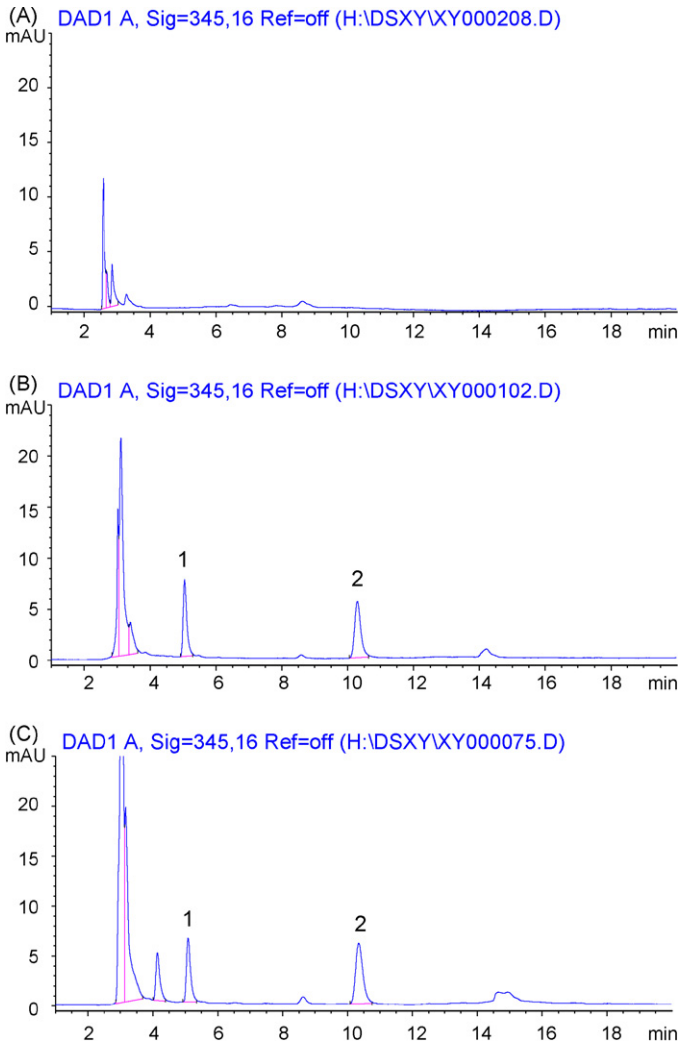

Fig. 2. Representative chromatograms of blank plasma (A); plasma spited with scopoletin (6–0.60 μg/ml) and IS (psoralen, 5.75 μg/ml) (B); plasma samples obtained at 15 min after intragastric administration to a rat with 50 mg/kg dose of scopoletin (C). Peak 1: scopoletin; peak 2: psoralen.

Table 1
Intra- and inter-day accuracy and precision ($n = 5$)

Concentration (μg/ml)		RSD (%)	Accuracy (%)
Added	Found (mean ± SD)		
Intra-day			
0.165	0.158 ± 0.009	5.7	95.6
1.32	1.294 ± 0.036	2.8	98.0
6.60	6.542 ± 0.076	1.2	99.1
Inter-day			
0.165	0.156 ± 0.011	6.7	94.8
1.32	1.290 ± 0.047	3.7	97.7
6.60	6.528 ± 0.130	2.0	98.9

Table 2
Recoveries of scopoletin and IS ($n = 5$)

Compound	Concentration (μg/ml)	RSD (%)	Recovery (%) (mean ± SD)
Scopoletin	0.165	3.9	93.2 ± 3.6
	1.32	1.9	95.9 ± 1.8
	6.60	3.3	95.5 ± 3.1
IS	5.75	4.2	91.7 ± 3.9

3.3. Recovery

Table 2 showed the recoveries of scopoletin and IS from rat plasma. The extraction efficacy of scopoletin at three concentrations ranged from 93.2 to 95.9%. The recovery of IS was 91.7% at the concentration of 5.75 μg/ml. The data proved the suitability of the extraction method for use with plasma samples.

3.4. Stability

The stability of scopoletin in rat plasma under different storage conditions was summarized in Table 3. The deviation of the mean test responses was within 1.7–6.3% of appropriate controls in all stability tests of scopoletin in rat plasma. Three freeze–thaw cycles of the quality control samples appeared to have no effect on quantification of the analyte. Quality control samples stored in a freezer at −20 °C remained stable for at least 1 month. No effect on quantification was observed for the short-term stability of the frozen samples kept at room temperature for 6 h. The extracted samples were analyzed after at least 24 h at room temperature. These studies suggested that rat plasma samples containing scopoletin could be handled under normal laboratory conditions without significant loss of the compound.

Table 3
Stability of scopoletin in rat plasma ($n = 5$)

Concentration (μg/ml)		RSD (%)	Accuracy (%)
Added	Found (mean ± SD)		
Three freeze and thaw cycles			
0.165	0.159 ± 0.007	4.6	96.4
1.32	1.280 ± 0.080	6.3	97.0
6.60	6.500 ± 0.108	1.7	98.5
1 month stability at −20 °C			
0.165	0.155 ± 0.009	6.1	93.9
1.32	1.234 ± 0.038	3.1	93.5
6.60	6.448 ± 0.126	2.0	97.7
At room temperature for 6 h			
0.165	0.162 ± 0.005	3.1	98.2
1.32	1.264 ± 0.066	5.2	95.8
6.60	6.410 ± 0.147	2.3	97.1
24 h at room temperature			
0.165	0.154 ± 0.006	3.9	93.3
1.32	1.250 ± 0.046	3.7	94.7
6.60	6.028 ± 0.365	6.1	91.3

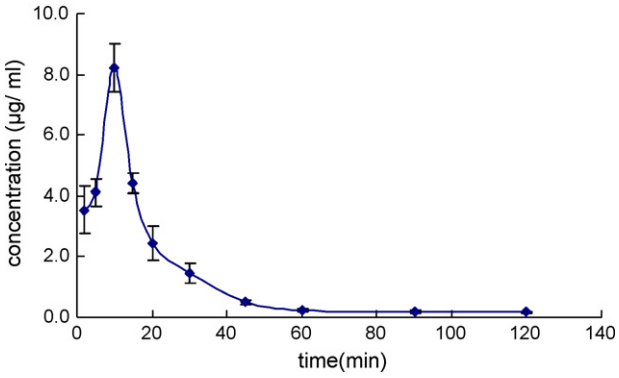

Fig. 3. Mean plasma concentration–time profile of scopoletin after oral administration of 50 mg/kg scopoletin in rats.

Table 4
Pharmacokinetic parameters for scopoletin in rats after oral administration (50 mg/kg, $n = 5$)

Parameter	Result (mean ± SD)
T_{max} (min)	10
C_{max} (μg/ml)	8.2 ± 0.8
$T_{1/2}$ (min)	14.1 ± 0.6
AUC_t (μg min/ml)	145.9 ± 11.8
K_e (min^{-1})	0.051 ± 0.005

3.5. Application of the method

The described method was applied to a pharmacokinetic study in rats. The mean plasma concentration–time curve after an oral dose of 50 mg/kg scopoletin was shown in Fig. 3. The main pharmacokinetic parameters of scopoletin were summarized in Table 4.

4. Discussion

The chromatographic condition for scopoletin was investigated to optimize for sensitivity and peak shape. As shown in Fig. 4, the UV spectrum of scopoletin had two absorption maxima at 228 and 345 nm, respectively. Detection at 228 nm had a higher sensitivity compared to that at 345 nm (0.85 times that at 228 nm). In plasma samples (Fig. 5), however, detection at 228 nm exhibited a lower chromatographic resolution (only 0.9),

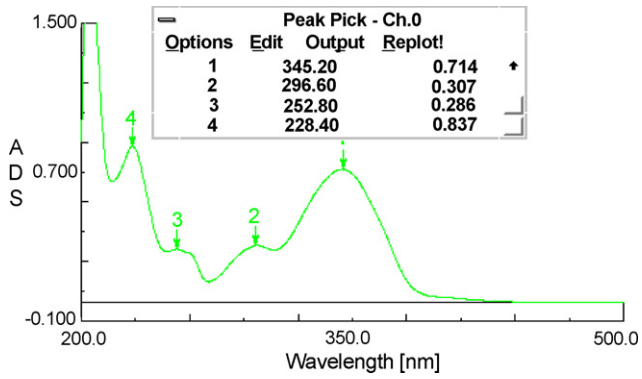

Fig. 4. Ultraviolet absorption spectrum of scopoletin.

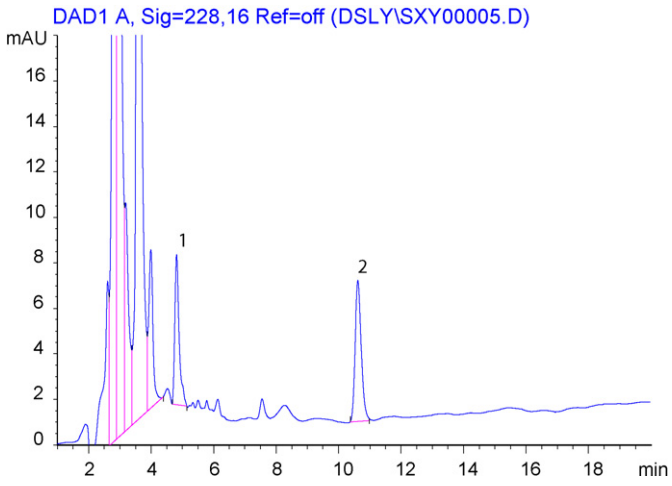

Fig. 5. Chromatogram with detection at 228 nm of plasma sample. Peak 1: scopoletin; peak 2: psoralen.

which meant there were significant biological interferences from endogenous plasma substances at this wavelength. To assure the specificity and sensitivity of this method, 345 nm was therefore chosen as detection wavelength in the current study.

Different mobile phase compositions were also screened. As a result, methanol and distilled water (49:51, v/v) containing 0.05% (v/v) phosphoric acid was chosen as the eluting solvent system so that desired separation and symmetry could be achieved.

The selection of the internal standard was an arduous and hard process. Psoralen was finally selected because it had a suitable retention time with no endogenous interferences and was well resolved from the target analyte in our mobile phase.

The extraction of plasma samples was also optimized in our preliminary studies by comparing protein precipitation reagents, such as methanol, acetonitrile and acetic ether, the results were satisfactory when methanol was used in protein precipitation.

5. Conclusion

In conclusion, we described a novel method for the quantitative determination of scopoletin in rat plasma, which is specific, accurate and precise, and could be easily implemented in routine practice. This method was successfully applied for the evaluation of pharmacokinetic profiles of scopoletin in rats.

Acknowledgements

This study was partially supported by the Open Grant of Jiangsu Key Laboratory for Molecular and Medical Biotechnology (no. 164070304301) and by the National Natural Science Foundation of China (no. 30672471).

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