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Journal of Chromatography B, 753 (2001) 309–314

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of byak-angelicin and oxypeucedanin hydrate in rat plasma by column-switching high-performance liquid chromatography with ultraviolet detection

Kazuhisa Ishihara[a,*], Miwako Fukutake[b], Takayuki Asano[b], Yasuharu Mizuhara[b], Yoko Wakui[b], Toshihiko Yanagisawa[b], Hideo Kamei[b], Shigeru Ohmori[a], Mitsukazu Kitada[a]

[a]*Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan*
[b]*Safety & Drug Metabolism Department, New Drug Discovery Laboratory, Tsumura & Co., 3586 Yoshiwara, Ami-machi, Inashikigun, Ibaraki 300-1192, Japan*

Received 18 April 2000; received in revised form 22 October 2000; accepted 30 October 2000

Abstract

A simple and sensitive column-switching HPLC method was developed for the simultaneous determination of two furocoumarin compounds, byak-angelicin and oxypeucedanin hydrate, which are the main components of hot water extract of *Angelica dahurica* root (AE), in rat plasma. Plasma sample was simply deproteinated with perchloric acid. After centrifugation, the supernatant was injected into a column-switching HPLC system consisting of a clean-up column (Symmetry Shield RP 8, 20×3.9 mm I.D.) and analytical column (Symmetry C_{18}, 75×4.6 mm I.D.) which were connected with a six-port switching valve. The flow-rate of the mobile phase (acetonitrile–water, 20:80) was maintained at 1 ml/min. Detection was carried out at wavelength 260 nm with a UV detector. The column temperature was maintained at 40°C. The calibration curves of byak-angelicin and oxypeucedanin hydrate were linear over the ranges 19.6 to 980 ng/ml (r^2>0.997). The accuracy of these analytes was less than 4.4%. The intra- and inter-day relative standard deviations of byak-angelicin and oxypeucedanin hydrate were within 12.0% and 12.7%, respectively. The present method was applied for the analysis of plasma concentration from rats after administration of AE.

Keywords: Byak-angelicin; Oxypeucedanin hydrate

1. Introduction

The hot water extract of Angelicae Dahuricae Radix (AE), the dried roots of *Angelica dahurica* Benth. et Hook. (*Umbelliferae*), has been used as a natural remedy since ancient times in Chinese herbal medicine. AE has been reported as having the protective activity against dexamethasone-induced disorders, liver protective activity, antimicrobial activity, anti-inflammatory activity and antimutagenic activity [1]. Main active components of AE, byak-angelicin {9-(2,3-dihydroxy-3-methyl-

*Corresponding author. Safety & Drug Metabolism Department, New Drug Discovery Laboratory, Tsumura & Co., 3586 Yoshiwara, Ami-machi, Inashikigun, Ibaraki 300-1192, Japan. Fax: +81-298-892-158.

E-mail address: ishihara_kazuhisa@mail.tsumura.co.jp (K. Ishihara).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00569-7

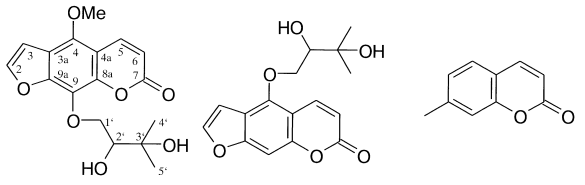

Fig. 1. Structures of byak-angelicin, oxypeucedanin hydrate and internal standard (7-methylcoumarin).

butoxy) - 4 - methoxy - 7H - furo[3,2 - *g*][1]benzopyran-7-one} and oxypeucedanin hydrate {4-(2,3-dihydroxy - 3 - methylbutoxy) - 7H - furo[3,2 - *g*][1]benzopyran-7-one}, are furocoumarins (Fig. 1). These compounds have been reported to have pharmacological effects such as activation of ACTH-induced lipolysis [2], inhibition of insulin-induced lipogenesis, inhibition of compound 48/80-induced histamine release [3] and inhibitory effect on cytochrome P-450 activity [4].

Regardless of the clinical use of AE, there was no previous pharmacokinetic study of the biologically active component of AE. Therefore, we tried to clarify the pharmacokinetic profiles of byak-angelicin and oxypeucedanin hydrate.

In this paper, we report the development of the sensitive method of determining the plasma concentrations of byak-angelicin and oxypeucedanin hydrate using a column-switching technique and an application for the pharmacokinetic study on byak-angelicin and oxypeucedanin hydrate after administration of AE to male rats.

2. Experimental

2.1. Chemicals and materials

AE was prepared by immersing *A. dahurica* root in 12-fold (w/w) volume of distilled water and boiling at 95°C for 60 min, then passing the liquid through a filter (JP100 mesh). The filtrate was evaporated to one-quarter volume under reduced pressure, then spray-dried. The concentrations of byak-angelicin and oxypeucedanin hydrate were 4.35 and 3.72 mg/g, respectively. Byak-angelicin and oxypeucedanin hydrate were isolated from AE. Briefly, 50 g of AE was extracted with 2.5 l of MeOH and centrifuged. Then supernatant fraction was applied to repeated chromatography over DIAION HP 20 (Mitsubishi Kasei, Tokyo, Japan), C.I.G. Si-50 (Kusano Kagakukikai, Tokyo, Japan) and YMC-GEL ODS-AM (YMC, Tokyo, Japan).

Byak-angelicin was identical comparing the spectral data with those reported in the literature [5,6]. ^{1}H-Nuclear magnetic resonance (NMR) (399.65 MHz in C^2HCl_3, TMS as internal standard); δ 1.29, 1.32 (each 3H, s, H-4′, 5′), 2.61 (1H, s, 3′-OH), 3.47 (1H, br. dd, J=4.0 Hz, 2′-OH), 3.84 (1H, ddd, J=2.4, 4.0, 10.2 Hz, H-2′), 4.19 (3H, s, –O–CH_3), 4.27 (1H, dd, J=7.9, 10.2 Hz, H-1′), 4.60 (1H, dd, J=2.7, 10.2 Hz, H-1′), 6.29 (1H, d, J=9.8 Hz, H-6), 7.02 (1H, d, J=2.4 Hz, H-3), 7.64 (1H, d, J=2.4 Hz, H-2), 8.13 (1H, d, J=9.8 Hz, H-5). ^{13}C-NMR (100.4 MHz in C^2HCl_3); δ 25.05, 26.66 (each q, C-4′, C-5′), 60.73 (q, –O–CH_3), 71.51 (s, C-3′), 76.00 (d, C-2′), 76.08 (t, C-1′), 105.3 (d, C-3), 107.5 (s, C-4a), 112.9 (d, C-6), 114.5 (s, C-3a), 126.8 (s, C-9), 139.4 (d, C-5), 143.9 (s, C-8a), 144.9 (s, C-4), 145.2 (d, C-2), 150.2 (s, C-9a), 160.1 (s, C-7), Electron impact mass spectrometry (EI-MS) m/z [int., %]: 334.10480 ($C_{17}H_{18}O_7$, calculated 334.10525) [9.7], 319 [1.4], 245 [1.4], 232 [base], 217 [74.9], 59 [21.2].

Oxypeucedanin hydrate was identical on the basis of NMR spectral analysis and by comparing MS fragmentation with those reported in the literature [6]. ^{1}H-NMR (399.65 MHz in C^2HCl_3, TMS as internal standard); δ 1.10, 1.17 (each 3H, s, H-4′, 5′), 2.17 (1H, s, 3′-OH), 2.87 (1H, br. s, 2′-OH), 3.91 (1H, m, H-2′), 4.45 (1H, dd, J=7.8, 9.8 Hz, H-1′), 4.55 (1H, dd, J=3.0, 9.8 Hz, H-1′), 6.30 (1H, d, J=9.8 Hz, H-6), 6.99 (1H, dd, J=0.9, 2.4 Hz, H-3), 7.61 (1H, d, J=2.4 Hz, H-2), 8.17 (1H, d, J=9.8 Hz, H-5). ^{13}C-NMR (100.4 MHz in C^2HCl_3); δ 25.17, 26.69 (each q, C-4′, C-5′), 71.65 (s, C-3′), 74.49 (t, C-1′),76.51 (d, C-2′), 94.9 (d, C-9), 104.7 (d, C-3), 107.3 (s, C-4a), 113.13 (d, C-6), 114.3 (s, C-3a), 138.9 (d, C-5), 145.3 (d, C-2), 148.5 (s, C-4), 152.5 (s, C-8a), 158.1 (s, C-9a), 161.0 (s, C-7), EI-MS m/z [int., %]: 304.09461 ($C_{16}H_{16}O_6$, calcu-

lated 304.09469) [18.3], 202 [base], 174 [20], 59 [61].

The purities of these compounds were up to 98% by high-performance liquid chromatography (HPLC). 7-Methylcoumarin was purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile (HPLC grade), methanol (HPLC grade) and perchloric acid (60%) were obtained from Wako (Osaka, Japan).

2.2. Standard solutions and spiked samples

Byak-angelicin and oxypeucedanin hydrate were dissolved in methanol (1.0 mg/ml, each) as stock solution and working standard solution were co-diluted in methanol. Spiked plasma was prepared on the day of analysis by adding 10 μl of the working standard solution to 500 μl of blank rat plasma, giving final concentrations of 19.6, 49.0. 98.0, 196 and 980 ng/ml, for both byak-angelicin and oxypeucedanin hydrate. Spiked plasma samples for quality control were prepared to 19.6, 98.0 and 980 ng/ml by the same manner and stored at −20°C until analysis.

2.3. Plasma sample collection

Seven-week-old male Sprague–Dawley rats were purchased from Charles River Japan (Atsugi, Japan). Animals were housed under controlled conditions (23±2°C, 55±20% relative humidity and 12 h light–dark cycle) and were fed standard laboratory chow with water ad libitum. AE was orally administered at a dose of 1 g/kg to male rats. Blood samples were taken from the femoral artery using cannulated polyethylene tubing (PE-10) with sodium heparin at a concentration of 100 IU/ml 5, 15, 30, 60, 90, 120, 240 and 360 min after administration. Obtained plasma was stored at −20°C until analysis. Animal experiments were performed in accordance with the institutional guidelines after obtaining the permission of the Laboratory Animal Committee of Tsumura, Ibaraki, Japan.

2.4. Instruments

Analyses were performed on an HPLC system consisting of the Shimadzu LC-10 series (Shimadzu, Kyoto, Japan), i.e., two LC-10A pumps, an SIL-10AXL autosampler, a CTO-10A column oven, a DGU-12A degassing unit, and an SPD-10A UV detector. The chromatogram was transferred from SPD-10A to CLASS LC10 integrating software via a CBM-10A communication bas module. The instrument arrangement for the automated column-switching system is shown in Fig. 2. A pre-column used for on-line sample preparation was a Symmetry Shield RP 8 (20×3.9 mm I.D., 5 μm; Waters, Milford, MA, USA). A Symmetry C_{18} column (75×4.6 mm I.D., 3.5 μm; Waters) was used as the main separation column. The same mobile phase (acetonitrile–water, 20:80) was used for pumps A and B. The flow-rate was maintained at a 1 ml/min. Detection was carried out at 260 nm with a UV detector. The column temperature was maintained at 40°C.

2.5. Analytical procedure

2.5.1. Step 1

To prepare the samples for assay, 7-methylcoumarin solution (as internal standard, 20 μg/ml in MeOH, 0.05 ml, Fig. 1) and 60% perchloric acid (0.05 ml) were added to the plasma samples (0.1 ml). After vigorous shaking, the mixture was centrifuged at 15 000 rpm for 10 min at 4°C and 50 μl of the supernatant was injected onto the pre-column. At the time of the sample injection, the column-switching valve was placed in position I. Hydrophilic interfering substances were washed to waste.

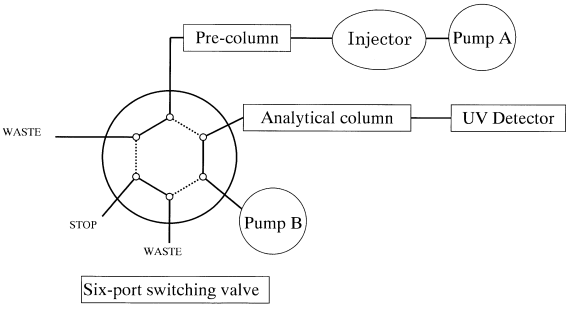

Fig. 2. Schematic diagram of column-switching HPLC system. Solid line; position I. Dashed line; position II.

2.5.2. Step 2

The switching valve was shifted to position II at 1.7 min for connecting the pre-column to the separation column, thus eluting fractions containing the target compounds from the pre-column to the separation column.

2.5.3. Step 3

The switching valve was shifted to position I again at 3.0 min after sample injection for disconnecting the pre-column from the separation column. Hydrophobic interfering substances were washed to waste from the pre-column.

2.6. Assay validation

The calibration curves for byak-angelicin and oxypeucedanin hydrate were generated by plotting peak height ratios (y) of these analytes to internal standard versus sample concentrations (x), and by applying the weighted linear least-square regression procedure using $1/x^2$ as the weight factor.

The intra- and inter-day relative standard deviations (RSDs) were estimated by one-way analysis of variance (ANOVA) for the calculated concentration of quality control plasma using Microsoft Excel (Microsoft).

3. Results and discussion

3.1. Chromatography and column-switching procedure

A heart-cut technique using column-switching HPLC was a very simple and time saving method, because it eliminates the intermediate off-line steps such as further purification of the extracting procedure [7–9]. In the column-switching technique, the appropriate switching timing is important to obtain a robust recovery. The robustness of the recovery was confirmed when the mobile phase was changed to ±1% from the set-up concentration (Fig. 3). The timing of switching for connecting the pre-column to the separation column was fixed at 1.7 min, because the recovery of the first peak, oxypeucedanin hydrate, was ca. 100% even though the acetonitrile concentration increased by 1% from the set-up concentration. Similarly, the timing of switching for disconnecting the pre-column was fixed at 3.0 min, because the recovery of the last peak, internal standard, was ca. 100% when the concentration of acetonitrile was 19%.

Fig. 4 shows the chromatograms of blank rat plasma (A), rat plasma containing 196 ng/ml of byak-angelicin and oxypeucedanin hydrate (B) and rat plasma obtained at 30 min after oral administration of AE (C). There was no interfering peak at the

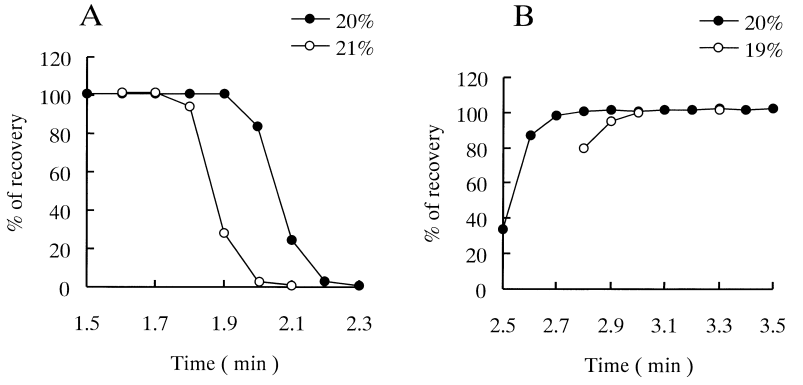

Fig. 3. Relationship between recovery and switching time for byak-angelicin and oxypeucedanin hydrate determination. Closed circles show the recovery when the set-up concentration of acetonitrile (20%) was used, and open circles show the recovery when the acetonitrile concentration was changed to ±1%. (A) The timing of switching for connecting the separation column. Recovery for oxypeucedanin hydrate (first peak). (B) The timing of switching for disconnecting the separation column. Recovery for 7-methylcoumarin (third peak).

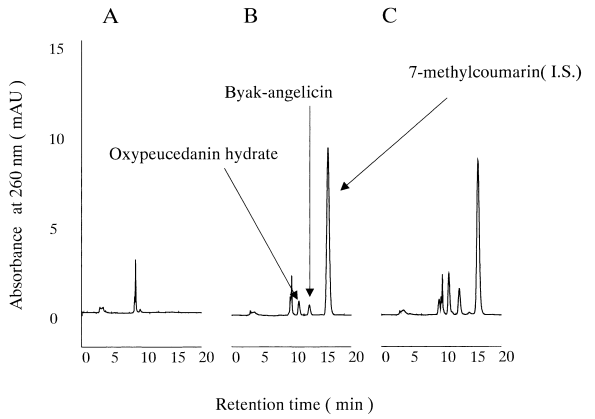

Fig. 4. Chromatograms of (A) blank plasma, (B) blank plasma spiked with byak-angelicin and oxypeucedanin hydrate (196 ng/ml, respectively) and (C) plasma sample from a rat at 30 min after the oral administration of AE (1 g/kg).

retention time of byak-angelicin, oxypeucedanin hydrate or internal standard.

3.2. Calibration curve, precision and accuracy

The linearities of byak-angelicin and oxypeucedanin hydrate were evaluated over concentration ranges of 19.6 to 980 ng/ml. The typical calibration curves of byak-angelicin and oxypeucedanin hydrate were given by the following equations, $y=3065x+1.13$ ($r^2=0.997$) and $y=2196x+1.24$ ($r^2=0.998$), respectively.

The accuracy and the precision are summarized in Table 1. The accuracies of byak-angelicin and oxypeucedanin hydrate were less than 3.9 and 4.4%, respectively. The intra-day RSDs (repeatability) of byak-angelicin were between 1.0 and 3.4%. The inter-day RSDs (intermediate precision) of byak-angelicin were between 7.1 and 12.0%. Repeatability of oxypeucedanin hydrate was between 0.7 and 2.6%. Intermediate precision of oxypeucedanin hydrate was 7.5 and 12.7%. The limit of quantitation for both compounds was set to 19.6 ng/ml, which is the lowest concentration of the analytes.

3.3. Stability of byak-angelicin and oxypeucedanin hydrate in rat plasma under storage conditions

Stability of byak-angelicin and oxypeucedanin hydrate in rat plasma (19.6, 98.0 and 980 ng/ml) under storage condition was confirmed. The remaining percentages were over 94% for byak-angelicin and 99% for oxypeucedanin hydrate after 8 months under −20°C.

3.4. Application to biological samples

The present method was applied to the analysis of plasma concentration from rats after administration of AE. Fig. 5 shows the mean plasma concentration–time curves of byak-angelicin (A) and oxypeucedanin hydrate (B) after administration of AE at a dose of 1.0 g/kg. A 1-g amount of AE contained 4.35 mg of byak-angelicin and 3.72 mg of oxypeucedanin hydrate, respectively. The pharmacokinetic parameters are summarized in Table 2.

Table 1
Accuracy and precision for byak-angelicin and oxypeucedanin hydrate determination in rat plasma

	Added amount (ng/ml)	Found amount (ng/ml)	Accuracy (%)	Repeatability[a] (%)	Intermediate precision[b] (%)
Byak-angelicin	19.6	19.8	1.2	3.4	12.0
	98.0	94.2	−3.9	1.4	7.1
	980	979	−0.1	1.0	9.1
Oxypeucedanin hydrate	19.6	19.7	0.4	2.6	12.7
	98.0	93.7	−4.4	0.7	7.5
	980	974	−0.6	1.2	8.4

[a] RSD, degrees of freedom=6.
[b] RSD, degrees of freedom=4.

Table 2
Pharmacokinetic parameters of byak-angelicin and oxypeucedanin hydrate in rats after oral administration of AE (1 g/kg)[a]

Analyte	$AUC_{(0-20\ h)}$ (ng·h/ml)	C_{max} (ng/ml)	t_{max} (h)	Cl^c/F^d (l/h)	Vd^e/F (l/kg)
Byak-angelicin, 4.35 mg/g[b]	2850.9±915.7	850.2±260.3	1.25±0.27	1.68±0.58	3.88±1.40
Oxypeucedanin hydrate, 3.72 mg/g[b]	2365.8±645.4	578.4±218.3	0.38±0.31	1.75±0.56	7.38±1.66

[a] Each value represents the mean±SD of five or six animals.
[b] The value shows the concentration of byak-angelicin and oxypeucedanin hydrate in AE.
[c] Cl: Clearance.
[d] F: Availability.
[e] Vd: Distribution volume.

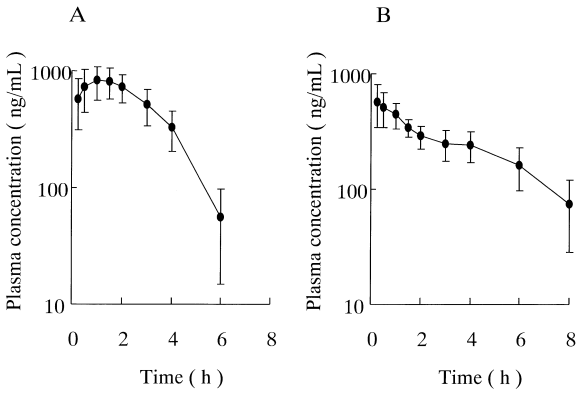

Fig. 5. Plasma concentration of byak-angelicin (A) and oxypeucedanin hydrate (B) in rat plasma after the oral administration of AE at a dose of 1 g/kg. Each value represents the mean±SD of six animals.

Both compounds showed rapid absorption. The mean plasma concentration–time curves of oxypeucedanin hydrate showed two peaked profiles.

4. Conclusion

A simple and sensitive column-switching HPLC method for the quantification of byak-angelicin and oxypeucedanin hydrate in rat plasma was developed. It was established that the accuracy and precision of the assay were satisfied by the proposed method. This method was successfully applied to evaluate the pharmacokinetics of byak-angelicin and oxypeucedanin hydrate in rats after administration of extract of Angelicae Dahuricae Radix.

Acknowledgements

We thank Dr. H. Nishimura for his help on the isolation of the byak-angelicin and oxypeucedanin hydrate.

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