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Short communication

Determination of Z-butylidenephthalide in plasma by high-performance liquid chromatography

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Abstract

An HPLC assay is described for the determination of Z-butylidenephthalide (Z-Bdph) in plasma. Plasma samples were cleaned up by extraction with 2% chloroform in *n*-hexane. Z-Bdph was separated on a normal-phase silica column with a mobile phase of chloroform–*n*-hexane (1:1). The limit of quantitation with UV detection at 254 nm for Z-Bdph in plasma was 0.01 μg/ml. The recovery of Z-Bdph by organic solvent extraction of plasma was 99.5%. The intra-day and inter-day coefficients of variation and relative errors for Z-Bdph determination in plasma were both less than 10%. The present method was applied to pharmacokinetic studies of Z-Bdph in plasma after intravenous administration to rabbits.

Keywords: Z-Butylidenephthalide

1. Introduction

Butylidenephthalide (Bdph) (Fig. 1) is an non-specific spasmolytic agent isolated from the rhizoma of *Ligusticum chuaxiong* Hort. (*Ligusticum wallichii* Franch.) and *Cnidium officinale* Makino [1,2]. Inhibition of rabbit platelet aggregation by Bdph has been reported [3] and it has been suggested that Bdph is potentially useful as an antianginal agent [4,5]. *Ligusticum chuaxiong* is widely used in traditional Chinese medicine as a smooth muscle relaxant to treat headaches, abdominal pain and cardiovascular and gynaecological diseases [6]. A pharmacokinetic study of Bdph in experimental animals or humans has not been reported in the literature. In this paper, we report the development of an assay for measuring Z-Bdph in plasma by high-performance liquid chromatography and the application of the

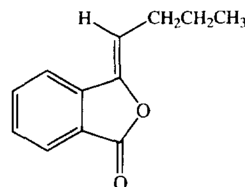

Fig. 1. Structure of Z-butylidenephthalide (Z-Bdph).

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assay to a pharmacokinetic study of Z-Bdph in rabbits.

2. Experimental

2.1. Chemical and reagents

A mixture of *E*- and *Z*-isomers (15:85) of Bdph was synthesized by a previously described method [7] in our laboratory. Pure Z-Bdph was isolated by column chromatography on silica gel and eluted with 2% diethyl ether in *n*-hexane. Dibutylphthalate, used as an internal standard, was purchased from Tokyo Kasei (Tokyo, Japan). Sodium heparin was obtained from Sigma (St. Louis, MO, USA). *n*-Hexane and chloroform were HPLC grade (Alps, Taipei, Taiwan).

2.2. Instrumentation and chromatographic system

The HPLC system consisted of a Model L-6000 pump (Hitachi, Tokyo, Japan), a Model 875 UV monitor (Jasco, Tokyo, Japan), a Rheodyne Model 7125 injector (Cotati, CA, USA) and a Model D-2500 integrator (Hitachi, Tokyo, Japan). The stationary phase utilized a normal-phase silica column (5 μm, 250×4 mm I.D.) (Lichrospher Si-60, E. Merck, Dramstadt, Germany) and performed at ambient temperature. The mobile phase consisted of *n*-hexane–chloroform (1:1, v/v). The chromatogram was monitored by UV detection at a wavelength of 254 nm, with a sensitivity setting of 0.001 AUFS. The flow-rate of the mobil phase was 1 ml/min.

2.3. Preparation of plasma samples

Plasma samples (0.2 ml) were mixed with 20 μl of dibutylphthalate solution in ethanol (150 μg/ml) as an internal standard and 2 ml of 2% chloroform in *n*-hexane. The mixture was vortex-mixed for 1 min. The organic layer was collected and evaporated to dryness under nitrogen at 50°C. The residue was reconstituted with 10 μl of the mobile phase and then injected into the HPLC system.

2.4. Preparation of calibration graph for Z-Bdph in plasma

Stock solutions of Z-Bdph in ethyl alcohol were prepared at concentrations of 1, 2, 4, 20, 40, 200, 500, 1000 and 2000 ng/μl. Blank plasma (0.2 ml) was spiked with 2 μl of Z-Bdph stock solutions at final concentrations of 0.01, 0.02, 0.04, 0.2, 0.4, 2, 5, 10 and 20 μg/ml. The spiked plasma samples were then treated as described in Section 2.3.

2.5. Administration of Z-Bdph to rabbits by intravenous route

Z-Bdph was dissolved in 2.5% ethyl alcohol in saline solution and injection into the ear vein of male albino rabbits weighing 1.6~3.4 kg at doses of 10, 30 and 90 mg/kg. Blood samples (0.5 ml) were withdrawn at 1, 2.5, 5, 7.5, 10, 15, 20, 30, 60, 90, 120, 180, 240, 300, 420, 540 and 720 min. Plasma was then separated by centrifugation at 6000 *g* for 10 min at 20°C and immediately stored under protection from light at −10°C until analysis.

3. Results and discussion

Due to the high lipophilicity of Z-Bdph, a stationary phase of silica gel was selected for HPLC separation. The retention times of Z-Bdph and the dibutylphthalate, internal standard were 5.4 min and 7.9 min, respectively. Plasma samples were cleaned up by organic solvent extraction to eliminate interference from endogenous substances before injection into the HPLC column. Chromatograms are shown in Fig. 2. The calibration curve for 0.2 ml samples of Z-Bdph in plasma was linear over the range of 0.01~20 μg/ml and could be expressed by the equation $y=1.224x-0.046$ ($r^2=0.9996$). Spiked plasma samples of Z-Bdph were stored at −10°C under protection from light and no significant degradation of Z-Bdph was found during storage for a 2-month period. Therefore, Z-Bdph plasma samples were assayed within one week. The limit of quantitation of Z-Bdph in plasma by HPLC was 0.01 μg/ml. The coefficient of variation (C.V.) and the relative error (R.E.) of the mean of measured concentrations served as measures of the accuracy and precision for

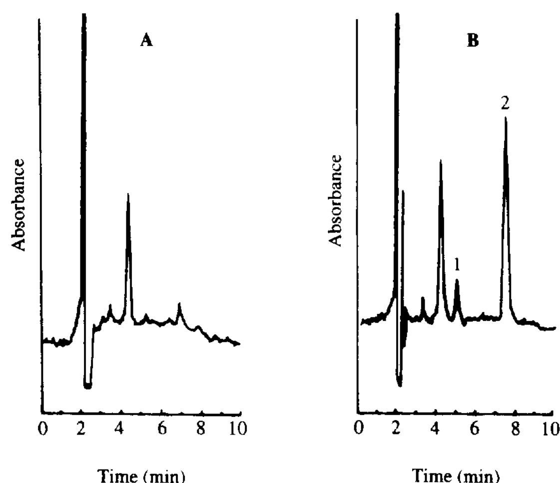

Fig. 2. Chromatograms of (A) drug-free plasma and (B) Z-Bdph plasma sample (0.021 μg/ml) obtained 60 min after intravenous injection of 10 mg/kg of Z-Bdph into a rabbit. Peak: 1=Z-Bdph, 2=internal standard.

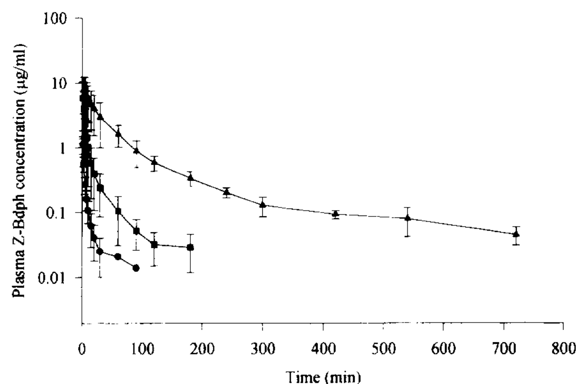

Fig. 3. Plasma drug concentration versus time curves of Z-Bdph in rabbits after intravenous dose of (▲) 90 mg/kg (n=5), (■) 30 mg/kg (n=8) and (●) 10 mg/kg (n=8). Data are expressed as mean±S.D.

validation of the assay procedure. The inter-day and intra-day assay precision and accuracy for Z-Bdph in the spiked plasma samples at concentrations of 0.01, 0.1, 2 and 20 μg/ml are shown in Table 1. The C.V. and R.E. values were both less than 10%. The average recovery of Z-Bdph from solvent extraction of plasma was almost quantitative (99.5±6%) at six

Table 1
Precision and accuracy tests for Z-Bdph in rabbit plasma (n=6)[a]

Spiked concentration (μg/ml)	Measured concentration (μg/ml) (mean±S.D.)	C.V. (%)	R.E.[b] (%)
Inter-day			
0.01	0.011±0.001	9.09	+10
0.1	0.095±0.007	7.37	−5.0
2.0	2.05±0.04	1.95	+2.5
20.0	20.2±0.03	1.49	+1.0
Intra-day			
0.01	0.011±0.001	9.09	+10
0.1	0.108±0.009	8.33	+8.0
2.0	2.10±0.06	2.86	+5.0
20.0	20.3±0.4	1.97	+1.50

[a] Six analyses of the same samples at four different concentrations of Z-Bdph in rabbit plasma were performed.
[b] Relative error of the mean (%)=[(true concentration−mean measured concentration)/(true concentration)]×100 .

different concentrations (0.02, 0.04, 0.2, 0.4, 2.0 and 20 μg/ml) as compared with direct injection of Z-Bdph solution in mobile phase onto the HPLC system.

The assay for Z-Bdph was then applied to pharmacokinetic studies in rabbits using intravenous administration. The plasma concentration of Z-Bdph versus time after iv. doses of 10, 30 and 90 mg/kg is plotted in Fig. 3. The plasma Z-Bdph concentrations at dose of 90 mg/kg in rabbits can be described by a biexponential equation, $C=Ae^{-\alpha t}+Be^{-\beta t}$, where C is the plasma concentration of Z-Bdph at time t, A and B are hybrid constants, and α and β are exponents representing the distribution and elimination phases. The pharmacokinetic parameters of Z-Bdph based on a two-compartment open model are listed in Table 2. Only the distribution α-phase was observed in the plasma conc versus time curve when iv. doses of 10 and 30 mg/kg were administered to the animals. The AUC estimated from the plasma level–time curve by trapezoidal rule were 6.02, 46.42 and 366.56 μg min/ml at the iv. doses of 10, 30 and 90 mg/kg. A plot of the AUC versus dose for Z-Bdph exhibited an nonlinear dose-dependent pharmacokinetics and the result is illustrated in Fig. 4.

In conclusion, an HPLC assay for Z-Bdph has been developed that is simple, possesses high precision, accuracy and reproducibility and is suitable for the determination of Z-Bdph in plasma.

Table 2
Pharmacokinetic parameters of Z-Bdph following an i.v. bolus dose of 90 mg/kg to five rabbits

Pharmacokinetic parameter[a]	Value (mean ± S.D.)	Pharmacokinetic parameter[a]	Value (mean ± S.D.)
A (μg/ml)	6.58±4.0	$t_{1/2}(\alpha)$ (min)	18.73±7.92
B (μg/ml)	1.0±1.24	$t_{1/2}(\beta)$ (min)	138.60±55.54
α (h^{-1})	0.037±0.014	V_c (l)	11.87±7.54
β (h^{-1})	0.005±0.040	Cl (ml/min)	237.35±50.6
AUC/dose (min/l)	4.21±1.42	V_{ss} (l)	16.36±8.01

[a] Data were calculated using PCNONLIN computer software (SCI Software, Lexington, KY, USA).

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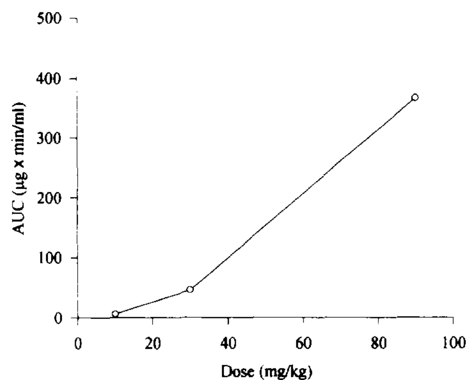

Fig. 4. Mean AUC versus dose for Z-Bdph after iv. administration to rabbits at three doses of 10, 30 and 90 mg/kg.

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