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Disposition of asarone after intravenous administration to rabbits assessed using HPLC

T. H. TSAI*, C. M. CHEN†, C.-F. CHEN†‡, *National Research Institute of Chinese Medicine, Taipei, Taiwan, †Graduate Institute of Pharmaceutical Sciences, Taipei Medical College, Taipei, Taiwan, and, ‡Department and Institute of Pharmacology, National Yang-Ming Medical College, Taipei, Taiwan, Republic of China

Abstract—A simple and sensitive high-performance liquid chromatography (HPLC) method for the determination of asarone in rabbit plasma has been developed. Up to 0·1 mL of plasma containing asarone was deproteinated by acetonitrile, which contained an internal standard (indomethacin). The supernatant was injected into a Nucleosil 7C18 column using acetonitrile-water-triethylamine (55:45:0·1 v/v, pH 5·4–5·5, adjusted with orthophosphoric acid) as the mobile phase and UV detection at 257 nm, followed by UV spectrum identification (between 200 and 380 nm) with a photodiode array detector. The method is rapid, easily reproduced, selective and sensitive. It was applied to pharmacokinetic studies of asarone in rabbit, after 5, 10, or 20 mg kg^{-1} intravenous administration. Rapid distribution followed by a slower elimination phase was observed from the plasma concentration–time curve. The plasma disposition at each dose fitted well to a two-compartment open model and the terminal disposition became much slower as the dose was increased, suggesting a nonlinear dose-dependent plasma asarone disposition.

Asarone is the active principle contained in *Acorus calamus* Linn, (Chinese name: Shuichangpu) or in *Acorus gramineus* Soland, (Chinese name: Shichangpu) about 12·75% or 8·8–13·7%, respectively (Wu 1986a, b). Tranquilizing effects (Dandiya & Menon 1965; Menon & Dandiya 1967; Bannerjee 1967), and expectorant effects (Gracza 1981) of asarone have been reported. However, a chromosome-damaging effect (Abel 1987) and a teratogenic effect (Jimenez et al 1988) of asarone were also reported.

Several methods for the determination of asarone isomers have been described. They include spectrofluorometric detection (Wojtowicz 1976), gas liquid chromatography (Dyer et al 1976; Dyer 1977), gas liquid chromatography–mass spectrometry (Liddle & de Smedt 1978), gas chromatography with selected-ion monitoring, mass spectrometry (Galli et al 1984), and HPLC (Curro et al 1987). However, no pharmacokinetic data obtained from experimental animals or man have been reported. In this paper, we report an HPLC method, using indomethacin as internal standard, for the determination of asarone in rabbit plasma and apply this to a pharmacokinetics study.

Materials and methods

Chemicals and reagents. Asarone (1,2,4-trimethoxy-5-propenylbenzene, *trans* isomer) was purchased from Sigma (St Louis, MO, USA). Indomethacin (internal standard), HPLC grade acetonitrile, methanol, orthophosphoric acid and triethylamine were purchased from E. Merck (Darmstadt, Germany). Polyethylene glycol 200 was purchased from Wako Pure Chemicals (Tokyo, Japan). Stock solutions of asarone were made in methanol at a concentration of 1 mg mL^{-1} and stored at 4°C. Quality remained dependable for at least one month. Stock solutions of asarone were diluted to 0·1, 0·01, and 0·001 mg mL^{-1} with methanol before use.

Correspondence: C.-F. Chen, Department and Institute of Pharmacology, National Yang-Ming Medical College, Taipei 112, Taiwan, Republic of China.

Chromatography conditions. The HPLC system consisted of a Waters U6K injector, a Waters 990 photodiode array detector, and a Waters 510 chromatographic pump (Milford, MA, USA). The separation was performed on a Macherey-Nagel ODS column (Nucleosil 7C18, 250 mm × 8 mm o.d. × 4 mm i.d., 7 μm particle size, Duren, Germany) fitted with a column inlet filter (0·5 μm × 3 mm, Rheodyne, USA). The mobile phase was acetonitrile–water–triethylamine (55:45:0·1, v/v, pH 5·4–5·5 adjusted with orthophosphoric acid), at a flow rate of 1·0 mL min^{-1}. The detection was monitored at 257 nm and the wavelengths scanned between 200 and 380 nm by the photodiode array detector.

Animals. Male New Zealand albino rabbits, 2·5–3·0 kg, were obtained from the Laboratory Animal Center at the National Taiwan University. These animals were kept in environmentally controlled quarters, with the temperature maintained at 24 ± 1°C and a 12 h light dark cycle, lights on 0700–1900 h for at least one week before use with free access to water and standard laboratory chow. Eighteen hours before the experiments, food was withheld.

Sample preparation. An 0·5 mL blood sample was directly withdrawn from the ear vein of conscious rabbits minimally restrained in a rabbit holder. Blood samples were collected at

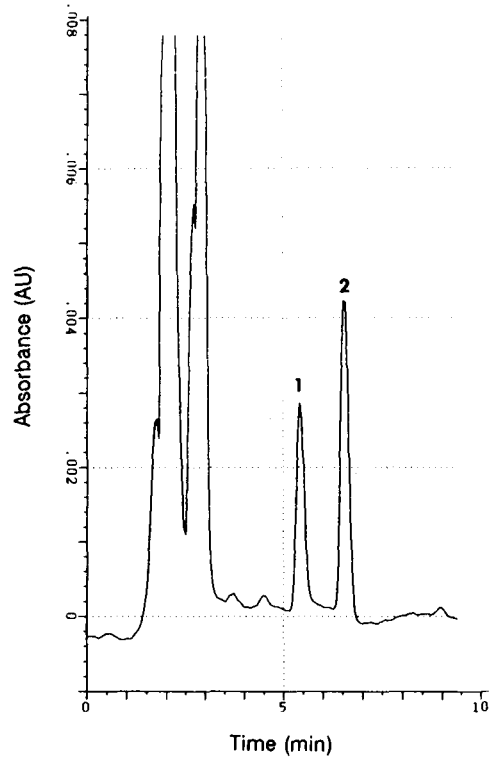

FIG. 1. Chromatogram of asarone (8 μg mL^{-1}) with indomethacin as internal standard in rabbit plasma. 1: indomethacin, 2: asarone.

2·5, 5, 10, 15, 30, 45, 60, 90 min, 2, 3, 4, 6, 8, and 12 h after intravenous administration of asarone (5, 10 or 20 mg kg^{-1}). Polyethylene glycol 200 in saline (50%) was used as vehicle for intravenous injection. Six animals were used for each dose tested. Each blood sample was transferred to a heparinized microfuge tube and centrifuged at 8000 *g* for 5 min (Eppendorf 5402). The resulting plasma (0·1 mL) was mixed with a 0·2 mL portion of acetonitrile, which contained 1 $\mu g\ mL^{-1}$ of internal standard. The denatured protein precipitate was separated again at 8000 *g* for 5 min. The supernatant (20 μL) was directly injected into the HPLC for analysis. The same sample handling process was employed throughout the experiments.

Calculation. The ratio between the peak area of the drug analysed and that of the internal standard was calculated by using a calibration curve, obtained after analysis of blank samples with added concentrations of 0·5 to 50 $\mu g\ mL^{-1}$ asarone and a constant amount of internal standard (20 ng indomethacin). The equation of the curve was $y = 0{\cdot}131x - 0{\cdot}004$ for rabbit plasma. The correlation coefficient value was 0·999.

Results and discussion

Under the conditions described above, the retention times of indomethacin and asarone were 5·43 and 6·54 min, respectively (Fig. 1). Fig. 2A shows the chromatograms and the spectra from a plasma sample taken 30 min after 10 mg kg^{-1} intravenous asarone, using indomethacin as the internal standard. Fig. 2B shows the authentic spectra of indomethacin and asarone. The spectra obtained in the mobile phase showed absorption maxima at 215, 257, and 314 nm for asarone, and at 206, 263, and 320 nm for indomethacin, and were identical to the spectra from pure compounds, indicating no interference from endogenous substances.

Recovery. For recovery studies, 1 mL portions of blank plasma with added 0·5, 5, or 50 μg asarone (n = 4) were extracted as described above. The internal standard was added to the eluate. Peak area ratios of the extracts were compared with those obtained from direct injection of the residue of the methanolic standard solutions. The recoveries were between 92 and 95% (coefficient of variation: % CV = 4·3 to 7·2) for the plasma.

Reproducibility. Reproducibility was tested on plasma which contained either 0·5, 5, or 50 $\mu g\ mL^{-1}$ asarone. Within-day coefficients of variation were 6·87, 4·51, and 2·23%, respectively. Between-day coefficients of variation were 8·68, 6·97, and 4·03%, respectively, over a period of 6 days.

Sensitivity. With the 990 photodiode array detector, the detection limit of plasma asarone was 100 $\mu g\ mL^{-1}$ (signal-to-noise ratio = 4).

Selectivity. The complementary use of the photodiode array detector for identifying the analysed compound by its retention time/wavelength absorbance is more reliable than the UV detector which gives only the retention time value. A chromatogram of a blank plasma showed no background interference from endogenous constituents.

Pharmacokinetic studies. A statistical nonlinear regression program was accessed through a JANA program (SCI Software Inc., Lexington, KY, USA) for the kinetic analysis. A two-compartment open model with elimination from the central compartment was proposed and validated through the program to explain the apparent biphasic disposition of asarone in plasma after intravenous injection as viewed in Fig. 3. The

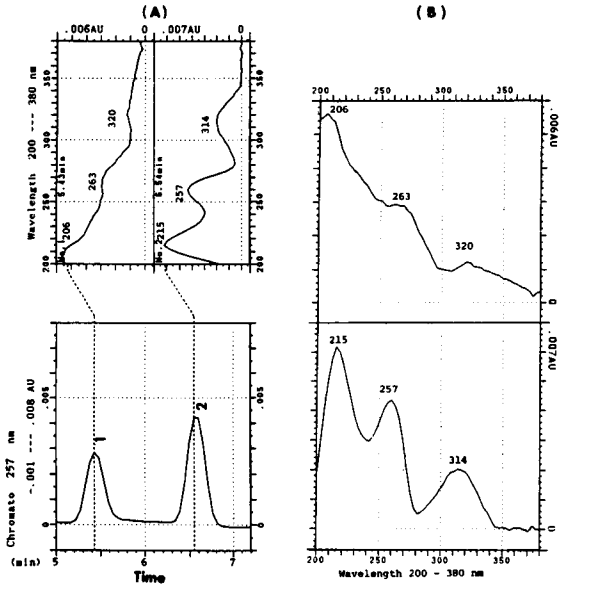

FIG. 2. A. Chromatogram and spectra of asarone in a rabbit plasma sample 30 min after 10 mg kg^{-1} intravenous asarone. B. The authentic UV spectra of indomethacin and asarone. 1: indomethacin, 2: asarone.

plasma concentration as a function of time can be described by the following equation:

$$C = Ae^{-\alpha t} + Be^{-\beta t}$$

where C is the plasma concentration of asarone at time t; A and B are constants related to the first order distribution between central and peripheral compartments; and α and β are exponents, representing the distribution and elimination phases. Two-compartment pharmacokinetic model analysis yielded the following equations:

$$C = 13{\cdot}71e^{-7{\cdot}94t} + 3{\cdot}53e^{-1{\cdot}56t}$$
$$C = 28{\cdot}80e^{-10{\cdot}43t} + 5{\cdot}95e^{-0{\cdot}91t}$$
$$C = 75{\cdot}96e^{-5{\cdot}91t} + 8{\cdot}28e^{-0{\cdot}52t}$$

for intravenous injection of 5, 10, or 20 mg kg^{-1} of asarone, respectively. k_{12} and k_{21} are rate constants for transfer between the central and peripheral compartments, and k_{10} is the elimination rate constant.

The elimination half-life ($t_{\frac{1}{2}\beta}$) of asarone increased from 0·45

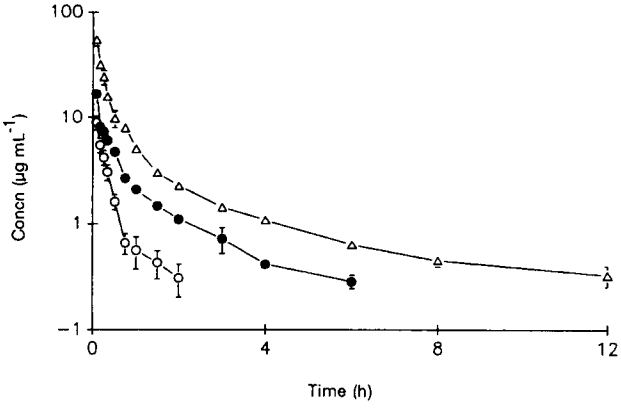

FIG. 3. Plasma concentration–time curve for asarone following intravenous administration of 5 (○), 10 (●), or 20 (△) mg kg^{-1} to rabbits. (n = 6 for each dose.)

Table 1. Estimates of pharmacokinetic parameters using a two-compartment open model with elimination from the central compartment, when a dose of 5, 10, or 20 mg kg^{-1} asarone was intravenously administered to male rabbits.

	Dose (mg kg^{-1})		
Parameters	5	10	20
A ($\mu g\ mL^{-1}$)	13·71 ± 0·58	28·80 ± 1·48[a]	75·96 ± 3·80[b]
B ($\mu g\ mL^{-1}$)	3·53 ± 0·28	5·95 ± 0·54[a]	8·28 ± 1·79[b]
α (h^{-1})	7·94 ± 0·66	10·43 ± 0·74	5·91 ± 0·65
β (h^{-1})	1·56 ± 0·08	0·91 ± 0·07[a]	0·52 ± 0·11[b]
k_{10} (h^{-1})	4·37 ± 0·53	3·76 ± 1·84[a]	2·89 ± 0·15[b]
k_{12} (h^{-1})	2·29 ± 0·33	5·05 ± 0·45[a]	2·42 ± 0·32[c]
k_{21} (h^{-1})	2·83 ± 0·07	2·53 ± 0·21	1·32 ± 0·28[b]
$t_{\frac{1}{2}\beta}$ (h)	0·45 ± 0·03	0·79 ± 0·08[a]	1·27 ± 0·21[b]
AUC ($\mu g\ h\ mL^{-1}$)	4·01 ± 0·43	10·25 ± 0·53[a]	29·73 ± 2·25[b]
Vd ($mL\ kg^{-1}$)	292·35 ± 12·30	276·34 ± 15·57	262·50 ± 9·78
CL ($L\ h^{-1}\ kg$)	1·28 ± 0·16	0·99 ± 0·05[a]	0·69 ± 0·05[b]

Data are expressed as mean ± s.e.m. (n = 6). [a] $P < 0·05$ as compared with 5 mg kg^{-1}. [b] $P < 0·05$ as compared with 5 or 10 mg kg^{-1}. [c] $P < 0·05$ as compared with 10 mg kg^{-1}.

to 1·27 h following the increase in intravenous bolus dose from 5 to 20 mg kg^{-1}. A greater than proportional increase in area under the concentration–time curve (AUC) of asarone was observed following increase in the dose. The apparent volume of distribution (Vd) was unaffected but the apparent total body clearance (CL) decreased from 1·28 to 0·69 for the increased dose. The estimates of these pharmacokinetic parameters based on the two-compartment open model were calculated from the best fitting coefficients and exponents using the PCNONLIN program (SCI Software Inc., Lexington, KY, USA) and listed in Table 1.

The asarone we used for the pharmacokinetics study was the *trans* form. Tsai & Chen (1991a, b) showed that the pharmacokinetics of α and β forms of glycyrrhetinic acids were different; differences in the pharmacokinetics between the *trans* and *cis* forms of asarone remain to be elucidated.

In conclusion, the UV spectrum identification, extraction and chromatographic procedures described in this study allow the quantitation of asarone from rabbit plasma. The decline in plasma of asarone was generally biexponential at each intravenous dose (5, 10 or 20 mg kg^{-1}), but the terminal disposition became much slower as the dose was increased. The apparent total body clearance decreased significantly with increase in dose but the apparent volume of distribution was unaffected. A greater than proportional increase in plasma asarone concentration was observed with increase of dose, suggesting a dose-dependence of asarone disposition. The results indicated that the pharmacokinetics of asarone was nonlinear (Shargel & Yu 1985).

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