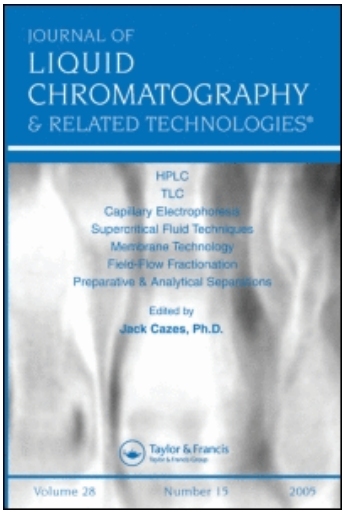

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Determination of Aristolochic Acid in Rabbit Plasma by High-Performance Liquid Chromatography with Photodiode-Array Ultraviolet Detection and Its Pharmacokinetics Application

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DETERMINATION OF ARISTOLOCHIC ACID IN RABBIT PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE-ARRAY ULTRAVIOLET DETECTION AND ITS PHARMACOKINETICS APPLICATION

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ABSTRACT

A simple and sensitive high-performance liquid chromatographic method for the determination of aristolochic acid (AA) in rabbit plasma has been developed. Up to 0.1 ml of plasma containing AA was deproteinized by acetonitrile, which contained an internal standard (indomethacin). The supernatant was injected onto a COSMOSIL 5C18-AR column (5 μm) using acetonitrile-0.1% phosphoric acid (60:40, v/v, pH 2.5-2.8) as the mobile phase. UV detection at 227 nm was followed by ultraviolet spectrum identification (among 200 and 380 nm) with a photodiode-array detector. The method was rapid, easily reproduced, selective and sensitive. It was applied to pharmacokinetic studies of AA in rabbit, after a 5 mg/kg intravenous administration. A biphasic phenomenon with a rapid distribution followed by a slower elimination phase was observed from the plasma concentration-time curve. Compartmental analyses yielded a two-compartment model.

INTRODUCTION

Aristolochic acid (AA), a constituent of Aristolochia species (Chinese name: Madoulin), has been used for medicinal purposes since the Greco-Roman period or ancient Chinese. Very recently, however, the Federal Health Office withdrew the license for all drugs containing AA because of the well-founded suspicion that aristolochic acid may be a very potent carcinogen. AA was shown to be mutagenic agent in 1982 [1]. In a serial study, aristolochic acid was shown to bind covalently to the exocyclic amino group of purine nucleotides in DNA and was strongly carcinogenic in the rat [2]. Although Madoulin was a popular herbal medicine in traditional Chinese preparations, the potential toxic effects of AA has been largely overlooked. Therefore, a convenient determination method and pharmacokinetics study would be very important. Only a few methods, using thin layer chromatography [3,4], fluorometric and GLC analyses [5] and ultraviolet spectrophotometric [6], have been described for the determination of AA. The present study was undertaken to develop a rapid procedure for the determination and identification of AA in plasma, after protein precipitation, with UV detection and UV absorption spectra.

MATERIALS AND METHODS

Reagents

AA (Fig. 1) was extracted from *Aristolochia fangchi* Wu. Acetonitrile, methanol, indomethacin (internal standard), and phosphoric acid were obtained

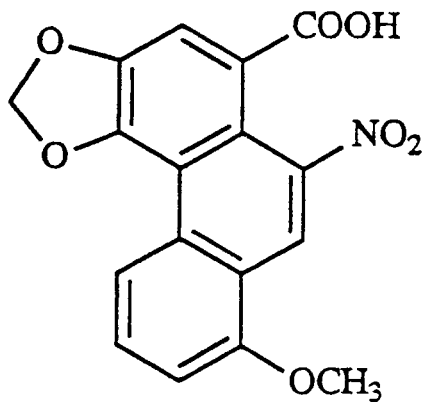

Fig. 1 Structure of aristolochic acid.

from E. Merck (Darmstadt, Germany). Thricely deionized water (Millipore, Bedford, MA, USA) was used for all preparations. The stock standard solution of AA was prepared by dissolving 10 mg of AA in 100 ml of methanol. Indomethacin was dissolved in acetonitrile at a concentration of 100 ng/ml and stored at 4 °C. These solutions were stable for at least one month.

Chromatographic Conditions

The high-performance liquid chromatographic (HPLC) system consisted of a Rheodyne 7125 injector (Cotati, CA, USA), a Waters 990 photodiode-array detector and a Waters 510 chromatographic pump (Milford, MA, USA). Separation was achieved at room temperature (25 °C) on a reversed-phase COSMOSIL 5C18-AR column, 4.6 X 150 mm, 5 μm particle size, fitted with a column inlet filter of 3 mm x 0.5 μm (Rheodyne). The mobile phase was a acetonitrile-water (60:40, v/v, pH 2.5-2.8 adjusted by phosphoric acid) at a flow-rate of 1.0 ml/min.

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 our own environmentally controlled quarters. The temperature maintained at 24 ± 1 °C and a 12-h light-dark cycle (light 07:00-19:00 h) for at least one week before use. Water and standard laboratory chow were given ad libitum until 18 h before the experiments which time only food was withdrawn.

Sample preparation

A 0.5 ml blood sample was directly withdrawn from the ear vein of conscious rabbits, which were minimally restrained in a rabbit holder. Blood samples were collected at time intervals of 5, 10, 15, 30, 45, 60, 90 min and 2, 3, 4, 6, 8 h after intravenous administration of AA (5 mg/kg). Six animals were used for the test. The blood sample was then transferred to a heparin rinsed microfuge tube and centrifuge at 8000 g for 5 min (Ependorf 5402). The resulting plasma (0.1 ml) was mixed with a 0.2 ml portion of acetonitrile, which contained 100 ng/ml indomethacin as internal standard. The denatured protein precipitate was separated again by centrifuging at 8000 g for 5 min. The supernatant (20 μl) was directly injected onto the HPLC system for analysis.

RESULTS

Peak Identifications and Linearity

Fig. 2 shows the chromatogram and UV spectra of authentic AA and indomethacin. The peaks corresponding to AA and indomethacin were confirmed by the retention times and the UV spectra obtained with photodiode-array

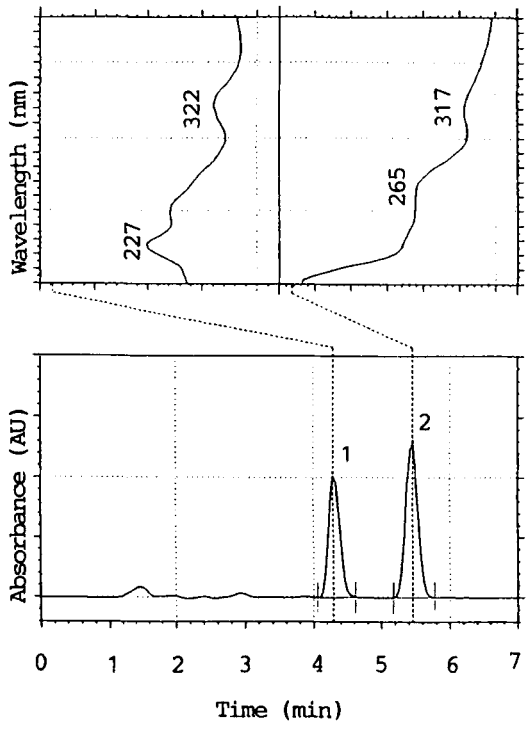

Fig. 2 Chromatogram and UV spectra of authentic aristolochic acid and indomethacin. 1: aristolochic acid. 2: indomethacin.

detection. The retention times of AA and indomethacin were 4.30 and 5.46 min, respectively. The main spectral characteristics were absorption maxima at 227 and 322 nm for AA, and at 265 and 317 nm for indomethacin. Fig. 3A shows the blank rabbit plasma and no background interference from endogenous constituents. Fig. 3B shows the rabbit plasma spiked with AA (2 μg/ml). Fig. 3C shows the plasma sample obtained after 5 mg/kg of AA intravenous administration, then spiked with internal standard (indomethacin), whose concentration was 5.82 μg/ml. The equation of the calibration curve was y =

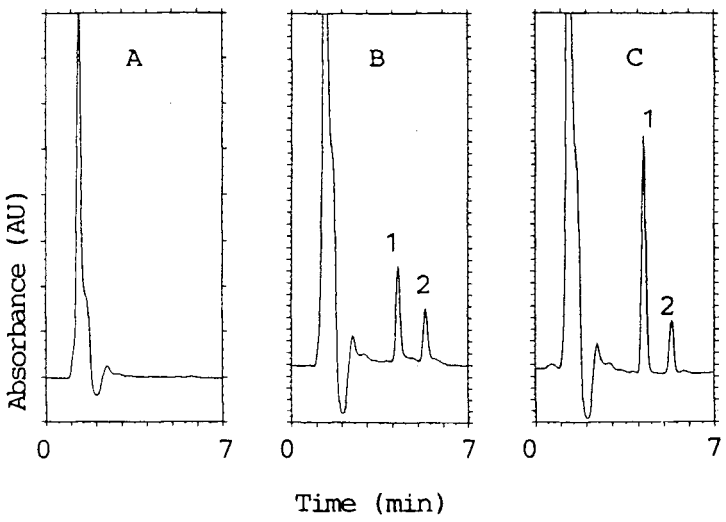

Fig. 3 Chromatogram of aristolochic acid in rabbit plasma. (A) Blank plasma. (B) Blank plasma spiked with indomethacin and aristolochic acid (2 μg/ml). (C) Plasma sample obtained after a 5 mg/kg aristolochic acid intravenous administration, and then spiked with the internal standard, the concentration of aristolochic acid was 5.83 μg/ml.

0.637x - 0.011 for rabbit plasma (1 to 100 μg/ml), where x is the amount of compound analyzed and y is response in peak-area ratio. The correlation coefficient was 0.999.

Precision and Recovery

For recovery studies, 1 ml portions of blank plasma were spiked with 1, 10, and 100 μg of AA (n = 4) and extracted as described above. The recoveries were among 92 and 97 % (coefficient of variation: percentage CV = 2.7-7.1) for plasma.

Reproducibility

The reproducibility of the method was indicated by the intra-day CVs of 6.34, 4.92, and 3.89 %. The inter-day CVs were 6.91, 6.54, and 4.34 % over a period of six days for the three concentrations (1, 10, and 100 μg/ml) tested.

Sensitivity

With the photodiode-array detector, the detection limit established for plasma AA was 80 ng/ml (signal-to-noise ratio = 3). Thus, UV detection was considered a suitable procedure for pharmacokinetic studies of AA.

Pharmacokinetics

The estimates of these pharmacokinetic parameters based on the two-compartment open models. They are calculated from the best fitting coefficients and exponents using the JANA and PCNONLIN programs (purchased from SCI Software, Lexington, KY, USA) and have been listed in Table 1. The biphasic disposition of AA has demonstrated in Fig. 4. The plasma concentration as a function of time and can be described by the following equation:

$$Cp = Ae^{-at} + Be^{-bt}$$

Where Cp is the plasma concentration of AA at time t, A and B denote two pre-exponential constants consisting of the first-order distribution between central and peripheral compartments. The a and b are two exponents, representing the distribution and elimination phases.

The elimination half-life ($T_{1/2,b}$) of AA from the central compartment as indicated in the terminal phase of the plasma concentration-time curve was determined from the equation:

$$T_{1/2,b} = 0.693/b$$

TABLE 1

Estimates of pharmacokinetic parameters according to a two-compartment open model with elimination following intravenous administration of a dose of 5 mg/kg aristolochic acid to male rabbits (n = 5)

Parameter (units)	Estimate
A, (μg/ml)	71.65 ± 3.42
B, (μg/ml)	37.39 ± 10.89
a, (1/h)	5.21 ± 1.26
b, (1/h)	1.47 ± 0.17
k_{10}, (1/h)	2.55 ± 0.28
k_{12}, (1/h)	1.52 ± 0.40
k_{21}, (1/h)	2.91 ± 0.66
$T_{1/2,b}$, (h)	0.51 ± 0.09
AUC, (μg h/ml)	42.57 ±1.72
Vol, (ml/kg)	49.42 ± 7.02

Data are expressed as mean ± SEM

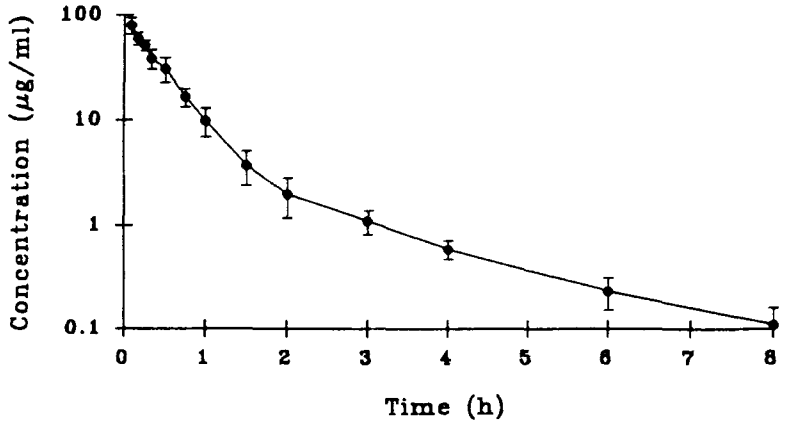

Fig. 4 Plasma concentration-time curve for aristolochic acid intravenous administration of 5 mg/kg to rabbits (n = 5).

DISCUSSION

An ultraviolet spectrophotometric method [6] with polyamide TLC for the isolation and detection of AA from biological specimens in rats has previously been reported. Also, using fluoresence [5], 20-50 ng/ml of AA can be determined following reduction to the lactam. Furthermore, in a GLC assay [5], the minimum detectable concentration was 0.01 mg/ml. However, complementary uses of photodiode-array detection for identifying the analyzed compound by its retention time and wavelength absorbance are more reliable than the spectrophotometric method or HPLC-UV detection.

The present investigation clearly shows that with our HPLC system provides a simple and sensitive method for plasma sample determination. It should be noted that in the reverse-phase systems the resolution of indomethacin (internal standard) and AA of pH of the mobile phase is very important. The separation was improved and the retention time was shortened as the pH decreased. It appeared that conditions were optimal at a acetonitrile-water composition of 60:40 (v/v) at pH 2.5-2.8, adjusted by phosphoric acid. This condition was adopted for subsequent assays.

In conclusion, the UV spectrum identification, extraction and chromatographic procedures described in this study allow the quantitation of AA from rabbit plasma. The pharmacokinetic study of AA (5 mg/kg, intravenously) was characterized by the two-compartment open model.

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