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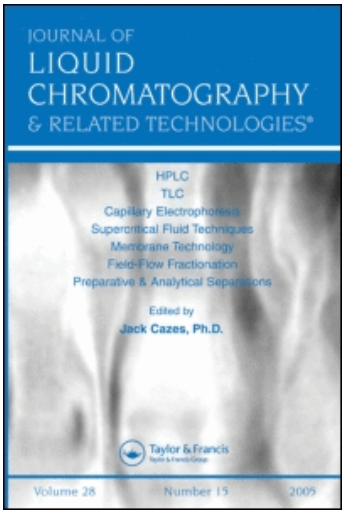

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T. H. Tsai[ab]; S. Y. Hsu[a]; C. J. Chou[a]; T. R. Tsai[c]; C. F. Chen[ad]
[a] Department of Pharmacology, National Research Institute of Chinese Medicine Shih-Pai, Taipei, Taiwan [b] Institute of Traditional Medicine Yang-Ming University Shih-Pai, Taipei, Taiwan [c] Graduate Institute of Pharmaceutical Sciences, Kaohsiung, Taiwan [d] Department of Pharmacology, National Yang-Ming University Shih-Pai, Taipei, Taiwan

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DETERMINATION OF DIHYDRO-5,6-DEHYDROKAWAIN IN RAT PLASMA BY HPLC AND ITS PHARMACO-KINETICS APPLICATION

T. H. Tsai[1,2]*, S. Y. Hsu[1], C. J. Chou[1],
T. R. Tsai[3], C. F. Chen[1,4]

[1] Department of Pharmacology
National Research Institute of Chinese Medicine
Shih-Pai, Taipei 11221, Taiwan

[3] Graduate Institute of Pharmaceutical Sciences
Kaohsiung 807, Taiwan

[2] Institute of Traditional Medicine
[4] Department of Pharmacology
National Yang-Ming University
Shih-Pai, Taipei 11221, Taiwan.

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ABSTRACT

A simple high performance liquid chromatographic method was developed to study the pharmacokinetics of dihydro-5,6-dehydrokawain (DDK) in the rat plasma. After addition of an internal standard (osthole), plasma was deproteinized by acetonitrile for sample clean-up. The drugs were separated on a reverse phase column and detected by UV detection at a wavelength 281 nm.

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Acetonitrile-water-diethylamine (50:50:0.1, v/v/v, pH 3.0 adjusted by orthophosphoric acid) was used as a mobile phase. It was applied to the pharmacokinetic study of DDK in rats after a dose of 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.

INTRODUCTION

Alpinia speciosa K. Schum. (Zingiberaceae) is used as a folk medicine in Taiwan for the treatment of vomiting, dyspepsia and gastric ulcers. Dihydro-5,6-dehydrokawain (DDK, Fig. 1) was isolated from the root of *Alpinia speciosa* rhizoma.[1] It has been demonstrated that DDK has a protective effect on various experimental gastric ulcers (30-250 mg/kg), and can markedly inhibit gastric secretion (30-200 mg/kg).[2-4] The biologically active constituent was reported to have antiplatelet action due to the inhibition of thromboxane A_2 formation.[5] It was recently reported that DDK possesses an analgesic effect via non-opiate pathway.[6] Determination of kawain and its derivatives have been reported by gas chromatography-mass spectrometry.[7] However, the spectrum identification of DDK from plasma and its pharmacokinetic properties have not been studied. In this work, we developed a high performance liquid chromatographic (HPLC) method with photodiode-array and UV detection to determine the concentration of DDK in rat plasma and its related pharmacokinetic profile.

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MATERIALS AND METHODS

Chemicals And Reagents

DDK and osthole (internal standard)[8] were extracted from the rhizome of *Alpinia speciosa* and *Angelica pubescens*, respectively. Identification and purity of DDK and osthole were compared with an authentic compound by ^{13}C-NMR (Bruker, Germany), infra-ray and HPLC/photodiode-array detection (Fig. 2). Acetonitrile and orthophosphoric acid (85%) were obtained from E. Merck (Darmstadt, Germany). Triple deionized water (Millipore Corp., Bedford, MA, USA) was used for all preparations.

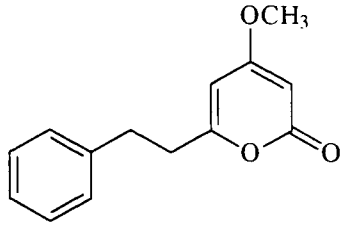

Figure 1. Chemical structure of dihydro-5,6-dehydrokawain (DDK).

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Apparatus And Chromatography

The HPLC system consisted of an autosampler (SIC model 23, Tokyo, Japan), a variable wavelength UV-VIS detector (Soma, Tokyo, Japan) and a chromatographic pump (Waters model 510). Separation was achieved on a reverse phased Nucleosil 5C8 column (250 x 4 mm, particle size 5 μm, Macherey-Nagel, Duren, Germany). The mobile phase was acetonitrile-water-diethylamine (50:50:0.1, v/v/v; pH 3.0 adjusted by orthophosphoric acid), and the flow rate was 1.0 mL/min. DDK was monitored at a wavelength of 281 nm throughout the experiments. The system was operated at room temperature (25°C).

Animals

Male Sprague-Dawley rats (250-300 g) were obtained from the Laboratory Animal Center at the National Yang-Ming University. These animals were specifically pathogen free and kept in our own environmentally controlled quarters (temperature maintained at 24 ± 1 °C and with 12:12 light-dark cycle) for at least 1 week before use. Standard laboratory food and water was available continuously, except when food was withdrawn 18 hours prior to experimentation.

Blood Sampling

Rats were anesthetized with intraperitoneal urethane 0.8 g/kg. Additional dosages of 0.08 g/kg urethane was given when the rat showed signs of awakening during the experiment period. Blood samples (0.3 mL) were collected via cardiac puncture[9,10] at interval of 2.5, 5, 10, 15, 20, 30, 45, 60, 90,

120 and 180 min after intravenous administration of DDK (5 mg/kg). Data from these sample were used to construct pharmacokinetic profiles by plotting concentration of DDK in plasma versus time.

Treatment Of Plasma Samples

Each blood sample collected was transferred to a heparinized microcentrifuge tube and centrifuged at 8,000 *g* for 3 min (Eppendorf Model 5402, Germany). The resulting plasma (0.1 mL) was then mixed with 0.2 mL of acetonitrile containing osthole (2 μg/mL) as an internal standard. The denatured protein precipitate was separated by centrifugation at 8000 *g* for 3 min and a 20 μL aliquot of the supernatant was directly injected into the HPLC. The same sample handling process was used for recovery and precision determination.

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Recovery

Plasma samples were spiked with DDK at concentrations ranging from 0.1 to 2 μg/mL. The resulting peak area ratio (DDK : internal standard) were compared to the standards prepared in acetonitrile.

Precision

Precision over the entire working dose range was determined by replicate analyses of plasma samples (n=4) spiked with three different concentrations (0.1, 1, or 2 μg/mL) of DDK. To determine intra-assay variance, quadruplicate assays were carried out on the same samples at different times during the day. Inter-assay variance was determined by assaying the spiked samples in quadruplicate on days 1, 2, 4, and 6 after spiking. Coefficients of variation (CVs) were calculated from these values.

Pharmacokinetic Analysis

A calibration curve was constructed based on the analysis by HPLC of various concentrations of DDK spiked in rat plasma. The concentrations of DDK in rat plasma after i.v. administration was determined from the peak area by using the equation for linear regression from the calibration curve. All data

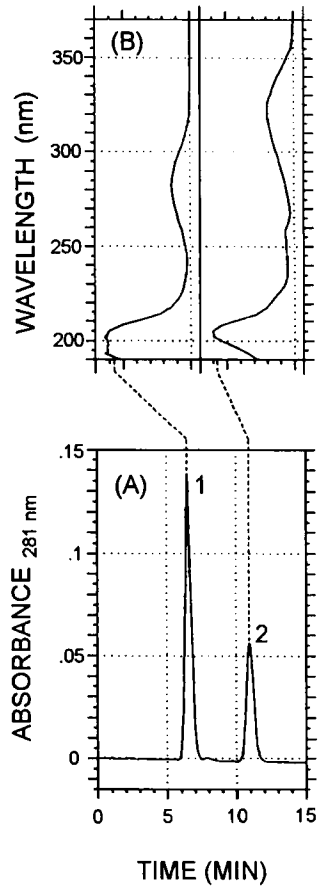

Figure 2. Chromatogram (A) and UV spectra (B) of authentic DDK and osthole (internal standard), measured by a photodiode-array detector (Waters, Model 990). 1: DDK, 2: osthole.

were subsequently processed by the computer program PCNONLIN (SCI Software Inc., Lexington, KY). The data for the area under the curve of concentration in plasma versus time ($AUC_{0\text{-}inf}$) were calculated by the trapezoidal method and extrapolated to infinite time.

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RESULTS

Under the conditions described above, the retention times of DDK and osthole (internal standard) were found to be 6.49 and 11.62 min, respectively (Fig. 2A). The main characteristic spectral data obtained in the mobile phase were shown as an absorption maxima at 203 and 281 nm for DDK and at 203 and 323 nm for osthole (Fig. 2B).

The recoveries of DDK from rat plasma were found to be 93.19, 93.27, and 95.85 % for the concentrations 0.1, 1, and 2 μg/mL, respectively. The reproducibility of the method was also defined by examining both intra- and inter-assay variabilities. The intra-assay CVs for DDK at concentrations of 0.1, 1, and 2 μg/mL were 6.64, 6.48, and 4.82%, respectively, and the inter-assay CVs for DDK at the same concentrations were 7.33, 5.97, and 4.98%, respectively.

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To determine the linearity and the detection limit of the HPLC method, rat plasma samples spiked with six different concentrations of DDK (0.05-5 μg/mL) were analyzed. The peak area ratios (DDK to osthole) were linearly related to the concentration of drug and the equation for the regression line for DDK was found to be $y = 2.4361x - 0.0568$ (r^2=0.999).

The limits of the lowest concentration on the standard curve which can be measured with acceptable accuracy (C.V. <20 %). The lower practical limit of quantification was 0.05 μg/mL. Under the procedure described above, the detection limit for DDK, at a signal-to-noise ratio of 3, was 0.01 μg/mL in rat plasma.

Fig. 3(A) shows the chromatogram of blank rat plasma. No discernible peaks were observed within the time frame in which DDK and osthole were detected. Fig. 3(B) shows the chromatogram of rat plasma spiked with DDK (0.5 μg/mL) and internal standard. Fig. 3(C) shows the chromatogram of DDK (0.61 μg/mL) sample obtained 20 min after i.v. administration of DDK (5 mg/kg) to a rat.

The data from the dose fit best into a two-compartment open model by the computer program PCNONLIN. The following equation applies into a two-compartment pharmacokinetic model:

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

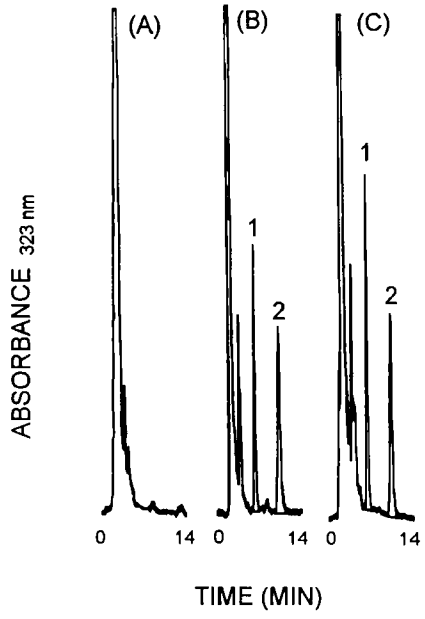

Figure 3. Chromatograms of DDK in rat plasma: (A) blank plasma. (B) spike DDK (0.5 μg/mL) and osthole (internal standard). (C) plasma sample 20 min after a 5 mg/kg i.v. administration of DDK (0.61 μg/mL). 1: DDK; 2: osthole.

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In equation 1, A and B are the concentration (C) intercepts for fast and slow disposition phases, respectively, and α and β are disposition rate constants for fast and slow disposition phases, respectively. The K_{12} and K_{21} are micro rate constants between the central and peripheral compartments, and K_{10} as the elimination rate constant. The distribution half-life ($t_{1/2,\ \alpha}$) and elimination half-life ($t_{1/2,\ \beta}$) of DDK as shown in the initial phase and terminal phase of the plasma concentration-time curve was determined by the equation of $0.693/\alpha$ and $0.693/\beta$, respectively. Analysis of data after i.v. injection of DDK at 5 mg/kg yields equations 2 (and Fig. 4), respectively:

$$C = 1.79e^{-0.18t} + 0.92e^{-0.017t} \quad (2)$$

The pharmacokinetic parameters, as derived from these data and calculated by PCNONLIN program, are shown in Table 1.

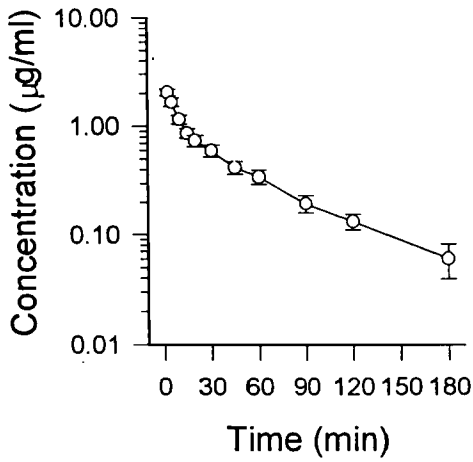

Figure 4. Plasma concentration-time curve after i.v. administration of DDK in rats at dose of 5 mg/kg.

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DISCUSSION

The complementary use of the photodiode-array detection for identifying the analyzed compound by its retention time / wavelength absorption is more reliable than the UV detector which gives only the retention time.[9-12] The photodiode-array detection allowed for observation of the full UV spectrum of each peak as it eluted from the chromatographic column. Hence the detection of other components could also be observed.

A statistical nonlinear regression program was accessed through the JANA and PCNONLIN programs for the kinetic analysis. The pharmacokinetic models (one *vs.* two compartment) were compared according to Akaike's information criterion (AIC)[13] and Schwartz criterion (SC)[14] and with minimum AIC and SC values were regarded as the best representation of the plasma concentration time course data. 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 DDK in rat plasma after iv administration.

The noncompartmental method for calculating disposition parameters of DDK are based on the theory of statistical moments.[15] The area under the concentration curve of a plot of the product of concentration and time versus time from zero time to infinity is often referred to as the area under the moment

Table 1

Pharmacokinetic Parameters of DDK in Rats after 5 mg/kg, Intravenous Administration.

Parameters	Estimate
A, μg/mL	1.79 ± 0.18
B, μg/mL	0.92 ± 0.14
α, 1/min	0.18 ± 0.025
β, 1/min	0.017 ± 0.002
K_{10}, 1/min	0.042 ± 0.003
K_{12}, 1/min	0.081 ± 0.014
K_{21}, 1/min	0.076 ± 0.013
Vol, 1/kg	1.87 ± 0.10
$t_{1/2,\alpha}$, min	4.18 ± 0.62
$t_{1/2,\beta}$, min	44.60 ± 5.73
Cl, 1/kg/min	0.079 ± 0.009
AUC, μg min/mL	67.56 ± 8.09
AUMC, μg min^2/mL	3710 ± 740
MRT, min	52.79 ± 5.02

Data are expressed as mean ± SEM. Cl: clearance. See text for other abbreviations.

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curve, AUMC.[15] The ratio of AUMC to AUC for DDK is a measure of its mean residence time (MRT).[16] MRT calculated after iv administration is the statistical moment analogy to drug elimination half-life. After administration of DDK (10 mg/kg, iv), MRT and $t_{1/2,\beta}$ were 44.60 and 52.79 min, respectively (Table 1).

Like half-life, MRT is a function of both distribution and elimination. Whereas, half-life tell us the time required to eliminate 50% of the dose, MRT_{iv} tells us the time required to eliminate 63.2% of the dose.

In conclusion, the present method allows a high selectivity and reliability. The relative simplicity permits its use for pharmacokinetic studies. Analysis of data after i.v. injection of DDK at 5 mg/kg yields a two-compartment pharmacokinetic model.

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