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QUANTITATION OF HARRINGTONINE AND HOMOHARRINGTONINE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Harringtonine and homoharringtonine are naturally occurring alkaloids with demonstrated antineoplastic activity against certain types of leukemias in cell cultures, experimental animals, and initial clinical trials. Sample preparation consists of addition of the internal standard (one compound used as the internal standard for the other), solvent extraction with methylene chloride, washing with ammonium formate, and evaporation to dryness. The residue is dissolved in the mobile phase (40% methanol—60% 0.1 *M* ammonium formate) and an aliquot is chromatographed on a μC_{18} reversed-phase column (flow-rate 1.5 ml/min). Peaks are detected with a spectrophotofluorimeter by monitoring the emission at 320 nm with excitation wavelength of 280 nm. Limit of detection is 10 ng/ml (20 n*M*) for both compounds; reproducible quantitation can be made to 30 ng/ml (60 n*M*).

INTRODUCTION

Extracts from a yew-like coniferous tree (*Cephalotaxus hainanensis* Li) have been used for the treatment of tumors in the Fujian province in China for a long time. Paudler et al. [1] isolated several active principles from a related plant. Among the esters of cephalotaxine several exhibited activity against L-1210 lymphoid leukemia and P-388 lymphocytic leukemia [2]. The alkaloids that can be isolated from the genus *Cephalotaxus* have been reviewed [3].

Harringtonine and homoharringtonine (Fig. 1) have been characterized [4] and partial synthesis has been attempted [5, 6]. They have been tested in a variety of experimental tumor systems; a clinical brochure on homoharringtonine summarizes available toxicological as well as antitumor activity informa-

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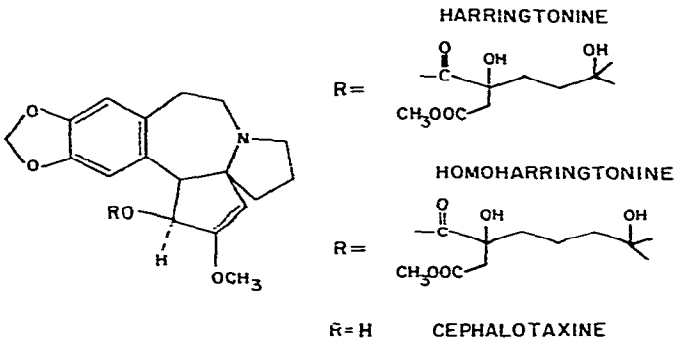

Fig. 1. Chemical structures of harringtonine and homoharringtonine, derivatives of cephalotaxine.

tion [7]. After encouraging initial clinical trials in China [8], both drugs are now being used in several cancer treatment centers in China [9]. Current research with these drugs, including the proposed mechanism of action, has been reviewed [10]. Homoharringtonine is now available in the U.S.A. for Phase I clinical trials, and harringtonine is also expected to be studied in humans.

Analytical methodologies are obviously required for the study of the pharmacokinetics and metabolism of these drugs. Some of the active principles in crude alkaloid extracts were characterized by electron ionization mass spectrometry after gas chromatographic separation [11]. A mass spectrometric method, utilizing chemical ionization, has been developed for the analysis of both drugs [12]. The authors are not aware of any other published method for the quantitation of these drugs. The present method utilizes high-performance liquid chromatography (HPLC) in a simple technique requiring equipment frequently available in laboratories supporting Phase I clinical trials.

EXPERIMENTAL

Drugs and reagents

Pure harringtonine and homoharringtonine, and also radiolabeled harringtonine, were supplied by the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, People's Republic of China). The composition of the pure drugs was confirmed by precise mass measurements: molecular weights agreed within 3—4 millimass units with calculated values. The purity of the compounds was 97—99% as determined by HPLC and thin-layer chromatography. The tritium-labeled harringtonine (universal labeling) had a specific activity of 454 mCi/mg and a radiochemical purity of 99% (purified by HPLC).

All solvents were of HPLC grade: methanol was from Fisher Scientific (Pittsburgh, PA, U.S.A.), water and other solvents were of "distilled in glass" quality from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium formate was purchased from Sigma (St. Louis, MO, U.S.A.); the Aquafluor scintillation cocktail was from New England Nuclear (Boston, MA, U.S.A.).

Stock solutions of harringtonine and homoharringtonine were prepared by dissolving 1.0 mg of each in 0.2 ml of 0.1 *N* hydrochloric acid at room temperature. The solutions were diluted with HPLC grade water and neutralized with

0.1 *M* sodium hydroxide. These stock solutions could be kept in a refrigerator for two weeks. Aliquots of these stock solutions were diluted with HPLC grade water as needed for spiking serum samples or for addition as internal standards. For the animal experimentation, the drugs were weighed as dry powder (provided without excipients) and prepared as described above for the stock solutions, except that sterile saline solution (0.85%) was used instead of water; these solutions were prepared immediately prior to injection into the animals.

Instrumentation

HPLC was carried out using a system consisting of a single pump (Model 110A, Altex, Berkeley, CA, U.S.A.), a manual injector (Model U6K, Waters Assoc., Milford, MA, U.S.A.), a spectrophotofluorimeter (Model FS-970, Schoeffel/Kratos Co., Westwood, NJ, U.S.A.), a 10-mV potentiometer recorder (Omniscribe, Houston Instruments, Houston, TX, U.S.A.), and an electronic filter amplifier (Model 1021A, Spectrum, Newark, DE, U.S.A.). The filter was placed in series with the spectrophotofluorimeter and was used to improve the quality and intensity of the signals.

Samples were evaporated to dryness with a nitrogen evaporator (N-Evap, Organomation Assoc., Shrewsbury, MA, U.S.A.). The spectrophotofluorimeter used in the initial experiments to study the pure compounds was a Model MPF-3 instrument from Perkin-Elmer Co. (Stamford, CT, U.S.A.). Radioactivity counting was made with a conventional scintillation counter (Model 32-55 Tricarb, Packard, New York, NY, U.S.A.). High-resolution mass spectra were obtained with a Model ZAB-1F mass spectrometer system (VG Analytical, Altrincham, Great Britain).

High-performance liquid chromatography

A stainless-steel column (30 cm × 3.9 mm I.D.) filled with a reversed-phase C_{18}-type material of 10-μm particle size (μBondapak C_{18}, Waters Assoc.) was the analytical column. A guard column, filled with Bondapak C_{18}/Corasil (Waters Assoc.), was placed between the injector and the analytical column. The guard column was changed after 75—100 runs or as needed.

The mobile phase was 40% methanol—60% ammonium formate buffer (0.1 *M*, pH 6.8) which was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and thoroughly vacuum-degassed prior to use. The flow-rate was 1.5 ml/min, resulting in an average pressure of 14 MPa in the chromatographic column.

The spectrophotofluorimeter was operated at an excitation wavelength of 280 nm, and emission was monitored at 320 nm. Detector sensitivity was usually 0.05 μA (time constant: 9). The particular settings of filtering and gain on the electronic filter-amplifier were adjusted as needed by the quantity of the drug present; typical settings were 0.02 Hz for cut-off and 5 for the gain.

Sample preparation

Serum samples were obtained from whole blood after clotting for 10—15 min at room temperature and centrifuging at 1000 *g* for 15 min. Plasma samples were obtained with heparin with no preservatives added. Either serum or plasma may be used in the technique developed (Fig. 2).

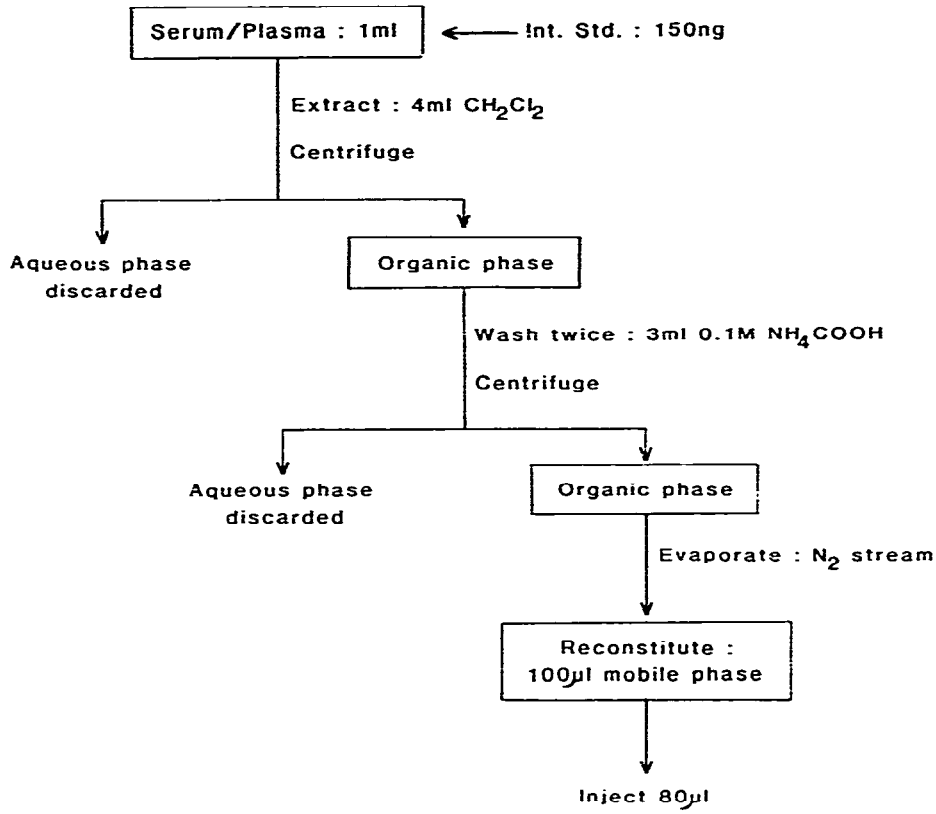

Fig. 2. Scheme of the procedure of extraction of harringtonine and homoharringtonine from serum or plasma.

Starting with a sample volume of 1 ml, the first step was the addition of the internal standard: harringtonine and homoharringtonine served as internal standard for each other. In most experiments the level of the internal standard was 150 ng/ml. The samples were next extracted with 4 ml of methylene chloride. After vortexing for 1 min, the samples were placed in a centrifuge at 50,000 *g* for 15 min at 4°C. After discarding the aqueous layer (top layer), the methylene chloride extract was washed twice with 0.1 *M* ammonium formate buffer. Next, the organic layer was evaporated with a gentle flow of nitrogen in a water bath maintained at 40°C. The dry residue (white or pale yellow color) was dissolved in 100 µl of mobile phase and an aliquot of 80 µl was injected into the liquid chromatograph.

Recovery experiments

Harringtonine was universally labeled with tritium by gas exposure. It was purified by HPLC using the same type of column and mobile phase utilized for analytical work. For the recovery experiments, 6 µg of unlabeled harringtonine was spiked by adding labeled harringtonine corresponding to 3605—17,094 counts. Seven different concentrations were used within this range and all samples were analyzed in duplicate. The spiked samples were extracted as described

for sample preparation. The dry residues were dissolved in 100 μl of mobile phase; 8 ml of scintillation cocktail were added and the mixtures were counted.

Calibration samples

Pure samples of harringtonine and homoharringtonine, dissolved in the mobile phase, were used to establish retention times, to determine sensitivities and detection limits for the pure compounds, and also for routine daily checking of instrument performance.

An appropriate amount of internal standard (usually 150 ng/ml) was added to every calibration sample prior to sample preparation. The same amount of internal standard was added to all samples within a set. Calibration curves were established by spiking normal serum samples with increasing quantities of the drug. A blank sample, i.e. no drug added, was always included in the calibration sets. To compensate for possible experimental errors, a full set of calibration samples (spiked) were analyzed with every set of samples from the experimental animals and from patients undergoing experimental chemotherapy. The concentration range of the drugs in the calibration set covered the expected range in the samples (30–150 ng/ml).

RESULTS AND DISCUSSION

Spectrophotofluorimetry

When harringtonine and homoharringtonine were dissolved in 0.1 *M* hydrochloric acid or methanol and their fluorescence properties were investigated using a conventional spectrophotofluorimeter, maximum emission sensitivity was observed at 320 nm for excitation wavelengths of 290 and 292 nm. It was also observed, in agreement with expectations based upon structure, that these compounds do not have particularly favorable fluorescence properties. However, both selectivity and sensitivity, and also overall performance, were still found to be superior with spectrophotofluorimetry than techniques based on UV absorption. As discussed later, sensitivity that is adequate for initial clinical trials could be obtained when the detector signal was improved electronically. It was also observed that the optimum excitation wavelength (in terms of highest attainable sensitivity) should best be determined individually for the particular spectrophotofluorimetric detector (and overall HPLC system) employed; any value in the 280–292-nm range may be found optimal.

Recovery experiments

A scheme of the sample preparation technique is shown in Fig. 2. Radioactively labeled harringtonine was used to establish the efficiency of the method developed. Unlabeled harringtonine was spiked as described, and seven different concentrations were analyzed in duplicate. Recovery was determined by taking the ratios of the initial counts added with respect to counts obtained after the entire sample preparation procedure was carried out. There was no need for corrections because the quenching of the mobile phase was below 6%. The overall recovery was determined as 71.2% ± 2.04% with a range of 68.2–74.1%. This was considered to be adequate for Phase I clinical trials.

Detection limits, quantitation

The limit of detection of pure harringtonine and homoharringtonine is approximately 0.8 ng of injected material. The limit of detection (detectability) in serum is defined as the amount of substance needed to produce a peak height of the particular drug analyzed twice that of the noise level in the blank sample. Fig. 3A shows that there is no interference in the blank at the retention times of harringtonine (6.0 min) and homoharringtonine (8.5 min). Fig. 3B shows the detection of 10 ng/ml (20 n*M*) for both drugs in human plasma. It is noted that there is a negative peak of rather high intensity just before the harringtonine peak. The intensity of the negative peak appeared to vary from sample to sample. The one shown in Fig. 3 represents a case of a "large" negative peak; usually they are much smaller. At any rate, the appearance of this peak does not affect the quantitation of harringtonine. It is also noted that the negative peak does not occur in mice or rat plasma (see Fig. 5). Finally, it is noted that the limit of detection can only be reached with the full utilization of the filter-amplifier accessory. At these very low levels it is often necessary to make several runs at various cut-off frequencies and gain settings to provide the best signal-to-noise ratio.

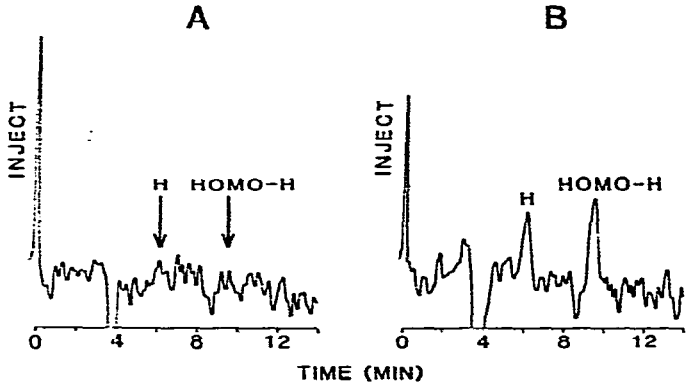

Fig. 3. Detection limit of harringtonine (H) and homoharringtonine (HOMO-H). A = blank serum with no drugs added; B = detection of 10 ng/ml (20 n*M*) of each drug added.

The limit of quantitation was established as three times that of the limit of detection, i.e. 30 ng/ml (60 n*M*), for both harringtonine and homoharringtonine. An illustration of this is shown in Fig. 4. Here different settings were used on the filter-amplifier to demonstrate the variations obtainable in noise levels. It was concluded that the sensitivity of the technique was adequate for the expected use in Phase I trials in all except the lowest initial dose levels. It may be possible to extend detectability by producing a derivative of the compounds with enhanced fluorescence. This, however, would involve more complex sample preparation and will probably not be needed because blood levels greater than 60 n*M* are expected with therapeutic doses.

Quantitation of either drug was accomplished with the aid of calibration curves consisting of a plot of observed peak height ratios of the drug analyzed/internal standard against known amounts of the drug used in spiking the cali-

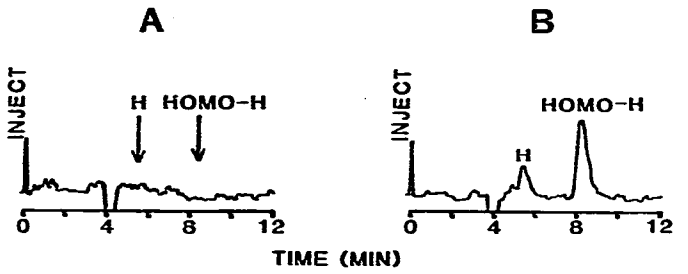

Fig. 4. Quantitation limit of harringtonine (H) and homoharringtonine (HOMO-H). A = blank serum with no drugs added; B = quantitation of 30 ng/ml (60 n*M*) of H in serum with 150 ng/ml HOMO-H added as internal standard.

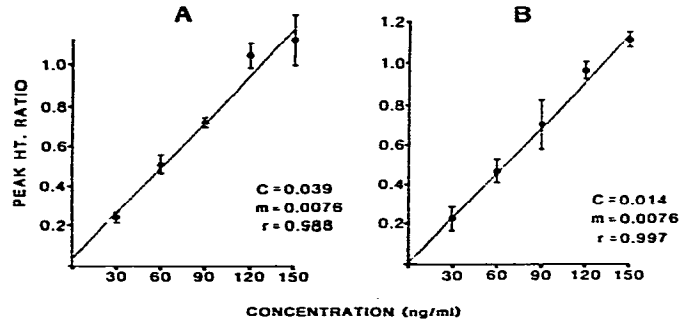

Fig. 5. Calibration curves for the quantitation of harringtonine (H) and homoharringtonine (HOMO-H) in human serum. The drugs served as internal standards for each other. Ordinates: peak height ratios of drugs measured/internal standard. Reproducibility of individual points is indicated for $n = 5$. The values c, m, and r were obtained by conventional regression analysis.

bration samples. The calibration curves (Fig. 5) were straight lines passing through or very near the origin with correlation coefficients (r) routinely in the 0.988–0.997 range. The reproducibility of the technique for both drugs in human plasma is illustrated in Fig. 5 ($n = 4$).

Applications

The technique is now being used for quantitation of both harringtonine and homoharringtonine in experimental studies (both mice and rats) and will be applied for patient monitoring when Phase I clinical trials commence. Fig. 6 illustrates a typical analysis of harringtonine in BDF1 mice with a dose of 3 mg/kg drug injected intraperitoneally. The amount of homoharringtonine added as internal standard was 150 ng/ml. Using a calibration curve, the amount of harringtonine in this sample was determined to be 149 ng/ml. (It is noted that the blank here did not exhibit the negative peak which usually appears in human samples.) Fig. 6 shows the analysis of a sample collected 20 min after dosing. Maximum blood level was attained in 5 min, and by 120 min the level was below detectability. It was estimated that the half-life in this case was approximately 15 min, in agreement with determinations made by radioactivity

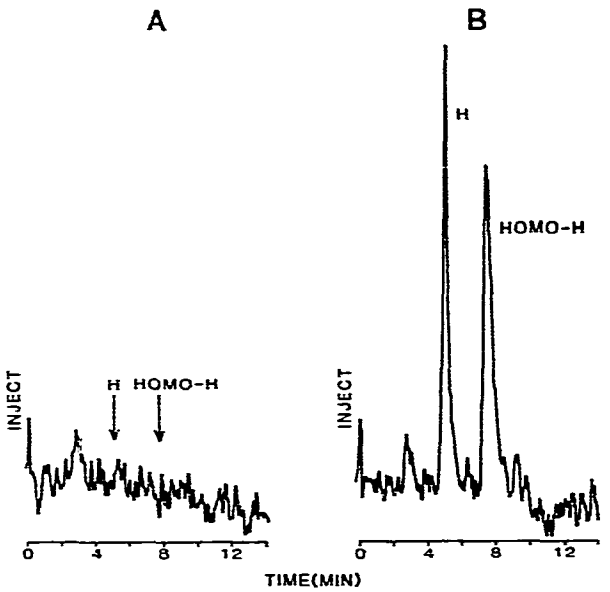

Fig. 6. Quantitation of harringtonine (H) in mouse serum. A = blank serum, no H or internal standard (homoharringtonine, HOMO-H) added. B = analysis of sample 20 min after the intraperitoneal injection of 3 mg/kg H. The amount of H present = 149 ng/ml.

measurements (Han, unpublished data). Pharmacokinetic data on experimental animals will be published elsewhere.

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