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QUANTITATION OF HOMOHARRINGTONINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic procedure was developed for the quantitation of homoharringtonine in plasma Harringtonine was used as an internal standard, and 1 ml of sample was required The single-step extraction with dichloromethane resulted in almost 100% recovery for homoharringtonine and harringtonine Analysis was performed on a reversed-phase CN column with amperometric detection Chromatography was completed in 12 min At an oxidation potential of +1 0 V, the detection limit was 1 ng/ml at a signal-to-noise ratio of 2 The mean analytical recovery for homoharringtonine was 99 5% The within-run precision and between-run precision were both less than 11% The method is equally applicable for plasma or serum, and it has been demonstrated to be applicable for study of the pharmacokinetics of homoharringtonine in patients suffering from acute non-lymphocytic leukaemia

INTRODUCTION

Homoharringtonine (HHT) is an alkaloid isolated from several species of *Cephalotaxus*, which are plants native to South China (Fig. 1). HHT was first isolated, purified and characterized by Powell and co-workers in 1969 [1] and 1970 [2]. HHT has been shown to have anti-neoplastic activity [3,4], the

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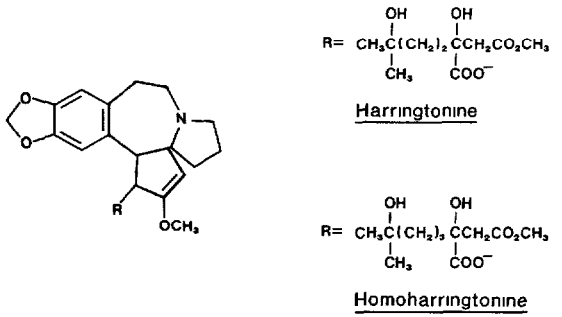

Fig 1 Structures of homoharringtonine (HHT) and harringtonine (HT)

mechanism of which may be due to protein biosynthesis inhibition, with a secondary inhibition of DNA biosynthesis [5,6]. Since 1976 HHT has been incorporated into frontline chemotherapy for the treatment of acute non-lymphocytic leukaemia in China [3] Several reports on phase I [7,8] and phase II [9] clinical trials on HHT conducted in the U.S A have been published. In our hospital, HHT is being used for the treatment of leukaemia as a last resort when all established routine therapies had failed.

Very little is known about the pharmacokinetics of HHT. One reason is because of the lack of a suitable analytical procedure for HHT in plasma. Jui and Roboz [10] developed a high-performance liquid chromatographic (HPLC) procedure with fluorometric detection for the determination of HHT and harringtonine (HT). The reproducibility of the technique was illustrated by regression analysis of the calibration curve Precision data on between-run assays were not presented. The reported detection limit (established at a signal-to-noise ratio of 2) and quantitation limit (signal-to-noise ratio of 6) were 10 and 30 ng/ml, respectively The method is applicable for monitoring serum concentrations following therapeutic doses, but it lacks the sensitivity required for single-dose pharmacokinetic study Roboz et al. [11] have also reported a chemical ionization mass spectrometric technique for HHT and HT The detection limit, at 10 ng/ml, is similar to that of their HPLC procedure. Another gas chromatographic–mass spectrometric method has been reported by Spencer et al [12] Their method was used primarily for quantitation of HHT in plant extracts, and its application for pharmacokinetic studies with serum or plasma samples is uncertain. An HPLC method that separates radioactive HHT from plasma extract has been described by Savaraj et al [13]. Their method necessitates the use of a large amount of unlabelled HHT as tracer for UV detection The sensitivity of their method is high (5 ng/ml), and the method had been used to study HHT pharmacokinetics in humans [13] and dogs [14]. However, it is not applicable for pharmacokinetic studies with unlabelled HHT.

This report describes a sensitive, reliable and rapid HPLC method with amperometric detection for the analysis of HHT in plasma.

EXPERIMENTAL

Instrumentation

The analysis was performed with a high-performance liquid chromatograph consisting of a Waters 6000A pump, a U6K injector, and an M370 data module (Waters Assoc., Milford, MA, U.S.A) A Bioanalytical Systems (West Lafayette, IN, U.S.A) TL-5A electrochemical flow-cell with a glassy carbon electrode, an RE-1 Ag/AgCl reference electrode and an LC-4B amperometric controller were utilized for peak detection. The temperature of the column was maintained at 40°C with a Kontron MS830 block heater (Kontron Instruments, Zurich, Switzerland). The analytical column was a 5-μm Lichrosorb CN steel column (12.5 cm×4 mm I.D.) from E. Merck (Darmstadt, F.R G.). Type HA filters of pore size 0.45 μm were from Millipore (Yonezawa, Japan).

Reagents

All reagents were of analytical grade or better Dibasic sodium phosphate was supplied by Sigma (St. Louis, MO, U S A). HPLC-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, U S.A.) Dichloromethane (AnalaR grade) was from BDH (Poole, U.K) HHT was supplied in 1.0-ml ampoules containing 1 0 mg equivalents of HHT (First Medicine Factory, Hangzhou, China). HT was supplied in 2.0-ml ampoules containing 2 0 mg equivalents of HT (China National Chemical Import and Export Corp., Fujian Branch, Fujian, China).

Standard solutions

HHT from 1.0 mg/ml ampoules was used as stock standard solution This HHT stock solution was divided into 100-μl aliquots, which were stored at −70°C until use.

A solution of HT in dichloromethane was prepared by extracting the contents of an HT ampoule three times with 20 ml of dichloromethane. The extracts were pooled. The concentration of HT in this extract was established by comparing its concentration with that of an ampoule by HPLC. The HT extract was then diluted with dichloromethane to give a HT stock solution of 12 μg/ml. It was stored as 1-ml aliquots at 4°C. On each day of analysis an extraction solution was prepared by dilution of 150 μl of HT stock solution to 250 ml with dichloromethane to give a final concentration of 7.2 ng/ml. An HT working solution was prepared by removing a second volume of 150 μl from the same HT stock solution This was dried under a stream of nitrogen and reconstituted with 1 ml of mobile phase. The concentration of HT in the working solution was 1.8 μg/ml

Blood sample collection

Blood samples (3 ml) were collected from the forearm of a patient via an indwelling catheter, into tubes containing sodium EDTA Plasma was separated by centrifugation and stored at −70°C until analysis.

Sample preparation

To 1 ml of plasma placed in a 75 mm×12 mm disposable borosilicate glass tube (Corning Glass Works, Corning, NY, U.S A.) were added 3 ml of extraction solution. The glass tube was stoppered by a disposable plastic cap. The mixture was shaken mechanically for 1 min at 240 strokes/min and centrifuged for 10 min at 1000 *g*. The top aqueous phase was aspirated and discarded. From the remaining organic phase, an aliquot of ca. 2 5 ml was transferred to another glass tube. The extract was evaporated to dryness under a stream of nitrogen gas at 40°C. The residues were reconstituted with 100 μl of mobile phase. Insoluble residues were removed by centrifugation at 10 000 *g* for 1 min. An 80-μl aliquot of the supernatant was injected onto the column

Chromatography

The mobile phase was acetonitrile–phosphate buffer (15 85, v/v) The phosphate buffer was 15 m*M* dibasic sodium phosphate in water, adjusted to pH 6.8 with phosphoric acid and filtered through a 0.45-μm filter. The mobile phase was degassed by purging with helium gas for 1 min prior to use.

The analytical column was maintained at 40°C. At a flow-rate of 1.2 ml/min, column pressure was 7.6 MPa. The working glassy carbon electrode was held at an oxidation potential of +1.0 V versus the Ag/AgCl reference electrode. The controller output was set at a sensitivity of 50 nA full scale with filter at 1 Hz.

Calibration and calculations

On each day of analysis a series of calibration solutions was prepared by mixing HHT stock solution, HT working solution and mobile phase. The HHT concentration in the calibration standards was from 11.25 to 900 ng/ml The HT concentration was 180 ng/ml.

Quantitation was by means of peak-height ratio between HHT and HT. The calibration curve was constructed by regression analysis of data obtained from the calibration solutions. A correction factor was used to compensate for the difference in recovery of HHT and HT, as well as the differences in the concentration of HT in the calibrators and that added to plasma sample. This correction factor was calculated as follows:

$$\text{correction factor} = \frac{\text{recovery of HT}}{\text{recovery of HHT}} \times \frac{[\text{HT}] \text{ in plasma}}{[\text{HT}] \text{ in calibrator}}$$

Recoveries for HT and HHT were found to be 102.0 and 98.3%, respectively.

The concentration of HT added via the extraction solution was equivalent to a plasma concentration of 21.6 ng/ml. The concentrations of HT in the calibrators were 180 ng/ml. This resulted in a correction factor of 0.125. Each HHT concentration calculated from the calibration curve was multiplied by this correction factor to give plasma concentrations for all unknown samples

Hydrodynamic voltammograms

Hydrodynamic voltammograms of HHT and HT were constructed using a mixture containing 100 ng/ml HHT and HT in mobile phase. A 80-μl volume of this mixture was injected onto the column. The peak heights for HHT and HT at various oxidation potentials were recorded

Method validation

The absolute recovery of HHT by the extraction procedure was studied by adding into plasma 10, 70 or 140 ng/ml HHT The absolute recovery measures the extraction efficiency of the procedure and was calculated by comparison of the peak heights of the extracted standards with the peak heights of the standards in mobile phase injected directly onto the column. The absolute recovery of HT was studied at three different specified concentrations using a similar procedure.

The analytical recovery measures the accuracy of the method and was evaluated by adding known amounts of HHT at two different concentrations to HHT-free plasma samples from nine individuals. The results were expressed as the mean observed concentration divided by the expected concentration and multiplied by 100

The within-run precision was determined by extracting and assaying replicate samples from three pools of plasma with different concentrations of HHT The between-run precision was determined by assaying aliquots of three pools of plasma, which were stored at $-70\,^{\circ}C$ over a period of three months

RESULTS

Hydrodynamic voltammograms

The hydrodynamic voltammograms are shown in Fig. 2A. The electrochemical response of HHT was higher than that of HT at all electrode potentials At an oxidation potential between +0.8 and +1 2 V, HHT and HT display similar oxidation profiles. The relative responses between HHT and HT were fairly constant at oxidation potentials between +0.9 and +1 05 V (Fig. 2B).

Chromatography

The effect of the mobile phase composition on the separation was studied The retention time and peak height of HHT were influenced by the ionic strength, pH and acetonitrile concentration of the mobile phase. When the

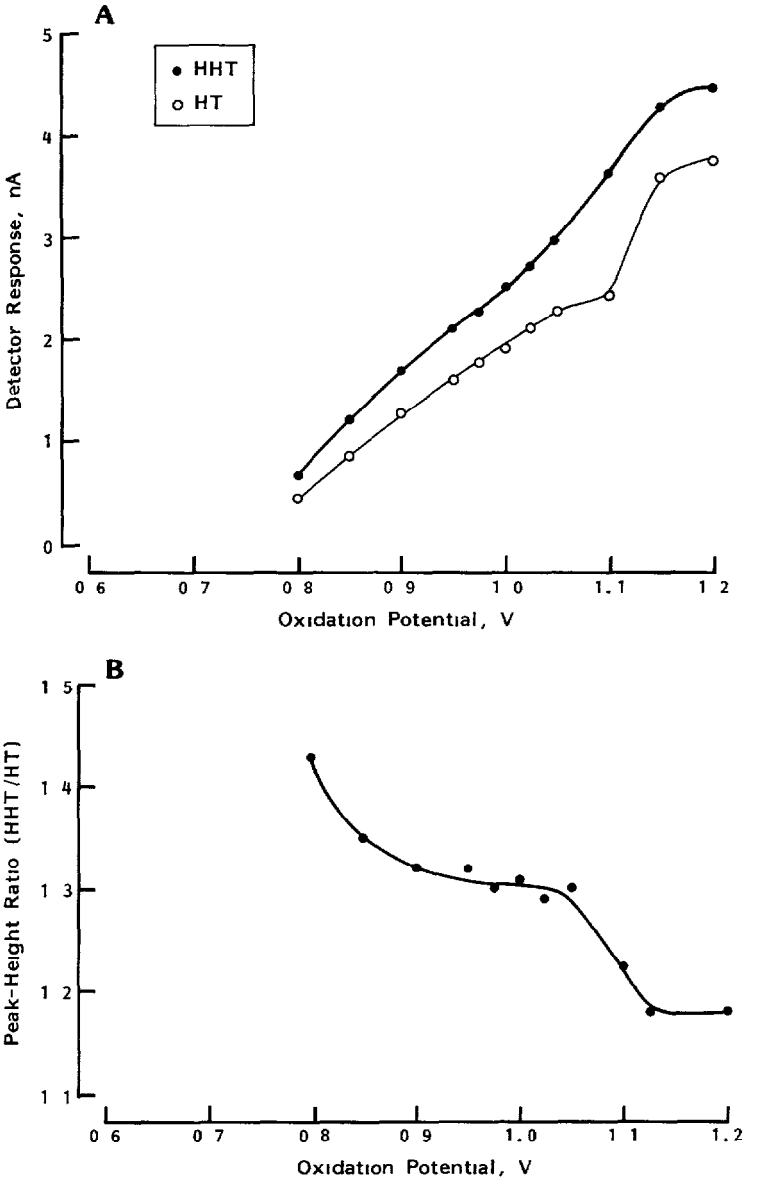

Fig 2 (A) Hydrodynamic voltammograms of HHT and HT (B) Relative response between HHT and HT at different oxidation potentials

ionic strength of the mobile phase was decreased from 50 to 10 m*M*, the retention time of HHT increased and the peak height decreased. At ionic strengths less than 10 m*M*, the peak shape deteriorated and became too broad for adequate resolution. When the pH of the mobile phase was increased from 5 to 7,

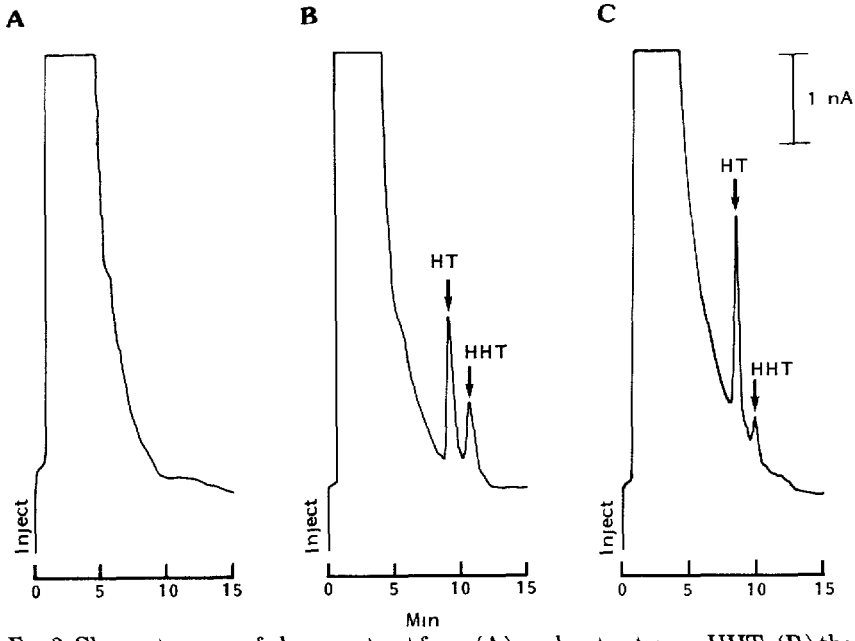

Fig 3 Chromatograms of plasma extract from (A) a subject not given HHT, (B) the same plasma spiked with 10 ng/ml HHT and HT, and (C) a patient receiving 2 mg/m^2 HHT intravenously The concentration of HHT was calculated to be 3 0 ng/ml

the retention time and peak height of HHT also increased. At high concentrations of acetonitrile, the sensitivity was greater but the resolution deteriorated, with a corresponding decrease in the retention time.

Using the mobile phase specified in Experimental, optimal separation was obtained. Typical chromatograms of plasma extracts are presented in Fig. 3. The retention times for HT and HHT were 8.6 and 10 min, respectively. No endogenous peak was detected following the elution of HHT No interfering peak with a retention time similar to that of HHT or HT was found in plasma from twenty healthy volunteers and from ten leukaemia patients who were on therapy other than HHT. Based on a signal-to-noise ratio of 2, plasma with an HHT concentration of less than 1 ng/ml cannot be detected by this method.

Extraction efficiency

Results for absolute recoveries of HHT and HT are shown in Table I. The recovery of HHT and HT from plasma showed no significant difference at the concentrations studied ($p > 0.05$, Student's t-test). Thus extraction efficiency of HHT and HT was independent of their concentration in plasma. The mean recovery of HHT from plasma was 98.3% (S.E.M. = 1 2, $n = 34$), and for HT it was 102.0% (S.E.M. = 0.8, $n = 34$)

The absolute recovery was also evaluated by spiking HHT and HT to paired

TABLE I

ABSOLUTE RECOVERY

Known amounts of HHT and HT were added at three different concentrations to plasma Samples were extracted and chromatographed as described The peak heights from the extracts were compared with those obtained from injection of standard solutions

Compound	Concentration (ng/ml)	n	Recovery (mean ± S E M) (%)
HHT	140	12	98 4 ± 1 4
	70	11	96 8 ± 1 3
	10	11	100 0 ± 3 1
HT	140	12	102 6 ± 1 2
	70	11	102 4 ± 0 5
	10	11	100 9 ± 2 3

drug-free plasma and serum collected from ten healthy donors. The mean recovery from plasma and serum was 99.16% (S.E.M. = 0.8) and 98.0% (S E.M. = 0.8), respectively, for HHT, and 100.4% (S.E M. = 0.7) and 100.7% (S.E.M. = 0.9), respectively, for HT. There was no significant difference in the absolute recoveries of HHT and HT from plasma and serum ($p > 0.05$, Student's paired t-test).

In another study with eight pairs of plasma samples, the extraction time was increased from 1 to 5 min. The absolute recoveries following extraction for 1 and 5 min were 98.7% (S.E.M. = 0 9) and 98.1% (S.E.M. = 0.8), respectively, for HHT, and 101.5% (S.E.M. = 0.85) and 100.9% (S.E.M. = 1.0), respectively, for HT. Increasing the extraction time did not significantly change the absolute recovery of HHT and HT ($p > 0.05$, Student's paired t-test).

Linearity and calibration curves

The linearity of the method was studied by assaying a series of nine plasma samples in duplicate with HHT concentrations ranging from 1 to 500 ng/ml A linear relation between HHT/HT peak-height ratios and plasma HHT concentrations was observed Regression analysis produced the equation of $y = 0.0434x + 0.071$, with a correlation coefficient of 0.9977. This range is adequate for pharmacokinetic study. The highest recommended intravenous dose for HHT adopted by our hospital is 5 mg/m^2 At this dose, plasma concentration is not expected to exceed 200 ng/ml following HHT administration.

The daily calibration curve obtained by injecting pure HHT in the mobile phase was linear between 11.25 to 900 ng/ml. This corresponds to a concentration range of 1 4–113 ng/ml in plasma. The mean correlation coefficient of ten standard curves run over a period of three months was 0.9996, and the

mean regression equation was $y = 0.0053$ (S E M.$=0.0004$) $x + 0\ 0119$(S.E.M $= 0.0038$).

Accuracy and precision

The analytical recoveries of HHT at different concentrations are presented in Table II. The mean analytical recovery was 99.5%

Data on within-run and between-run precisions are presented in Table III The coefficient of variation ranged from 2 9 to 10 6% for within-run assays and from 3.8 to 11.1% for between-run assays over three months.

Pharmacokinetic applications

Serial plasma samples were collected from a leukaemia patient who had received a single dose of HHT (2 mg/m^2) as a 1-h intravenous infusion The plasma concentration–time curve is shown in Fig 4.

TABLE II

ANALYTICAL RECOVERY

Known amounts of HHT were added at two different concentrations to HHT-free plasma samples from nine individuals The results obtained were compared with the expected concentrations

Nominal concentration (ng/ml)	Determined concentration (ng/ml)	Recovery (mean ± S E M) (%)
20	19 6	98 2 ± 2 1
80	80 3	100 7 ± 2 9

TABLE III

PRECISION DATA

Replicate samples from three pools of plasma were assayed as a batch for the within-run precision study For between-run precision, aliquots of three pools of plasma were stored at −70 °C and eleven sets were assayed over a period of 90 days

Sample	*n*	HHT concentration (ng/ml)	C V (%)
Within-run			
A	12	109 4	4 0
B	8	57 2	2 9
C	12	13 3	10 6
Between-run			
X	11	115 5	7 1
Y	11	47 8	3 8
Z	11	10 8	11 1

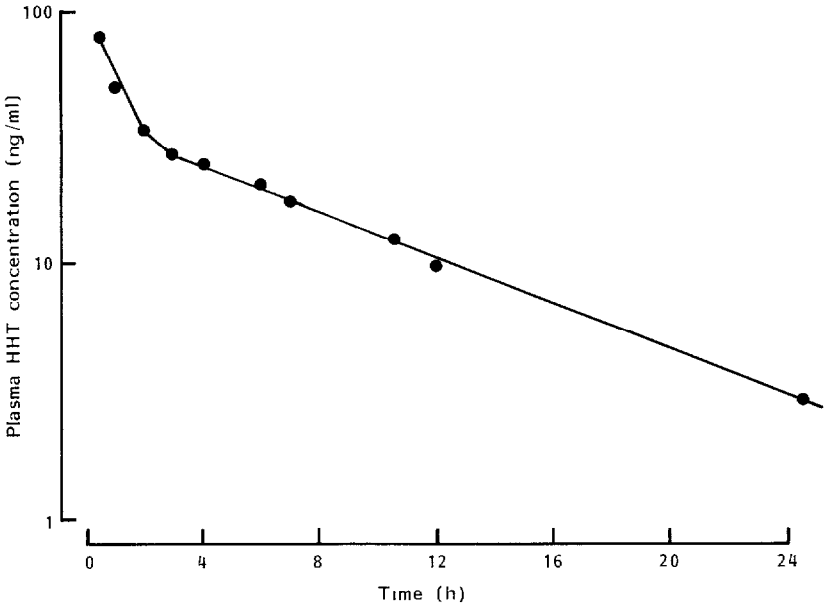

Fig 4 Serum HHT concentration profile in a patient following a 1-h intravenous infusion The dose was 2 mg/m^2 The patient was a 19-year-old Chinese male suffering from acute non-lymphocytic leukaemia

The disposition of HHT in this patient can be described by a two-compartment pharmacokinetic model, with a half-life of ca 0 4 h in the distribution phase and 7.5 h in the terminal phase The plasma HHT concentration 24 5 h after cessation of infusion was 3.0 ng/ml

DISCUSSION

An analytical HPLC method for the measurement of HHT in plasma was developed. HT was used as an internal standard in this method because it is structurally and chemically similar to HHT (Fig. 1) Consequently the extraction efficiency and chromatographic properties of HT are close to those of HHT. A further advantage of using HT as an internal standard is that it has similar profile in the hydrodynamic voltammogram to that of HHT (Fig. 2) The peak-height ratio of HHT to HT was stable in the oxidation potential range +0.9 to +1.05 V Within this range, any fluctuation in detector performance would not seriously affect the peak-height ratio of HHT to HT This would minimize error from the detector.

Detection of chromatographic eluents was performed with a glassy carbon electrode. The sensitivity for HHT was inadequate at potentials below +0.9 V. Although an oxidation potential of +1 2 V gave a higher signal for HHT, it was associated with excessive background peaks An oxidation potential of

+1.0 V was chosen for this study. This gives adequate sensitivity for HHT while minimizing potential interference from background noise.

The single-step liquid–liquid partition extraction for HHT with dichloromethane was found to be adequate. The extraction efficiency was close to 100% at the several widely distributed concentrations studied. Extracts prepared from HHT-free plasma yielded no endogenous peak with retention time similar to HHT or HT. Consequently no washing step or further purification procedure is required as described by other workers [10,13]. As HHT and HT were eluted within 10 min, and no detectable peak was observed afterwards, a short total chromatographic time of 12 min is adequate. The method is sensitive, and 1 ml of sample is enough for the analysis Both serum and plasma samples were found to be suitable for analysis

Chromatography of HHT on the CN column was found to be influenced by buffer strength, pH and acetonitrile concentration. In particular, the concentration of hydrogen ions was found to have a very significant effect on the separation. Since HHT is an alkaloid it becomes more non-polar as the pH increases from 5 to 7, and HHT is retained longer on the CN column. We found that the peak height of HHT also increased as the pH increased. However, pH values higher than 7 were not employed because column packings would be destroyed [15]. The composition of the mobile phase (15 m*M* disodium phosphate–phosphoric acid, pH 6.8, with 15% acetonitrile) was finally selected as a compromise between sensitivity and resolution, while still achieving a reasonably short analysis time.

The method was successfully applied to the analysis of plasma samples from a leukaemia patient after intravenous infusion of HHT. The half-lives determined from our patient were in agreement with those reported by Savaraj et al. [13], who used radiolabelled HHT. Our data, however, are preliminary and are part of a clinical study that will be reported elsewhere.

HT, the internal standard used in this study, had been shown to have pharmacological action against acute leukaemia and is used for therapy in China [3]. Previous analytical methods for HHT have also used HT as an internal standard [10,11]. These methods are capable of measuring HT in serum with HHT as the internal standard However, the reported detection limit for HT is also 10 ng/ml, and therefore the method is not adequate for single-dose pharmacokinetic studies. The lack of a suitable analytical method may be one of the reasons that there had been no report on the pharmacokinetics of HT. Our method can readily be adapted for the analysis of HT in plasma using HHT as the internal standard. The sensitivity, accuracy and precision of our assay for HT will be similar to that found for HHT.

In conclusion, the method described is sufficiently simple, sensitive, reliable and rapid for the determination of HHT in plasma samples It has been shown to be applicable to the pharmacokinetic study of the drug in human leukaemia patients

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