Journal of Chromatography, 583 (1992) 131–136
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6559

Short Communication

Selective determination, in plasma, of artemether and its major metabolite, dihydroartemisinin, by high-performance liquid chromatography with ultraviolet detection

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(First received June 17th, 1992; revised manuscript received August 28th, 1992)

ABSTRACT

A sensitive and selective reversed-phase high-performance liquid chromatographic method for the determination of artemether and its major metabolite dihydroartemisinin in plasma has been developed. It involves extraction of plasma with dichloromethane, solid-phase separation of the two analytes and acid decomposition prior to chromatography on a C_{18} Spherisorb column with a mobile phase of acetonitrile–water (50:50, v/v). Run time is 30 min. The assay satisfies all of the criteria required for use in clinical pharmacokinetic studies.

INTRODUCTION

The antimalarial artemether is a semi-synthetic derivative of the sesquiterpene lactone, artemisinin [1]. These agents are potent antimalarials with efficacy against both drug-sensitive and drug-resistant strains of *Plasmodium falciparum* and are of use in the treatment of life-threatening cerebral malaria. Metabolic studies, *in vitro*, have shown that the major metabolite of artemether is dihydroartemisinin (Fig. 1). This is a hemi-acetal retaining the peroxide group essential for antimalarial activity and appears to be a more potent antimalarial than artemether [2]. It is now thought that artemether is rapidly and extensively metabolised to dihydroartemisinin and that it is this metabolite which is primarily responsible for antimalarial potency.

Pharmacokinetic data on each of these compounds is scanty, due to the lack of suitably sensi-

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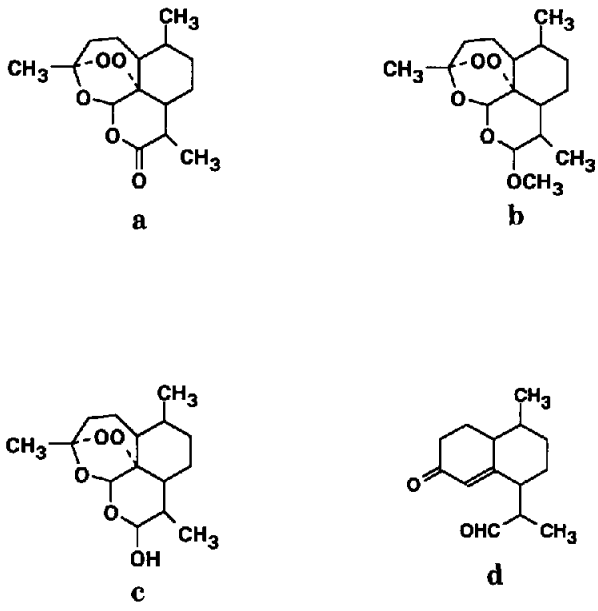

Fig. 1. Chemical structures of (a) artemisinin, (b) artemether, (c) dihydroartemisinin and (d) the acid decomposition product of both artemether and dihydroartemisinin.

tive and specific analytical methodology. Previously in this laboratory [3], we have reported a method for the determination of arteether, a similar synthetic derivative of artemisinin using high-performance liquid chromatography (HPLC) followed by ultraviolet (UV) detection. This involves an acid decomposition with hydrochloric acid to produce a UV-absorbing product, an α,β-unsaturated decalone [8-methyl-5-(2-propanalyl)decalin-4-ene-3-one, Fig. 1]. Examination of their chemical structure suggests that artemether and dihydroartemisinin would be expected to undergo this reaction when incubated with acid under the same conditions. For reasons stated above it is important to quantitate selectively both artemether and dihydroartemisinin in order to determine accurately the pharmacokinetics of this drug and establish the relationship between plasma concentration and antimalarial effect. Therefore to determine selectively artemether and dihydroartemisinin in plasma requires physical separation prior to acid derivatization. We now report a method of separation using solid-phase extraction which accomplishes this.

EXPERIMENTAL

Materials

Progesterone, the internal standard, was obtained from Sigma (Poole, UK). Artemether and dihydroartemisinin were gifts from Dr. P. Buchs (SAPEC, Lugano, Switzerland). All organic solvents were of HPLC grade and were obtained from Fisons (Loughborough, UK) as were hydrochloric acid, sodium hydroxide and anhydrous sodium sulphate. Aminopropyl Bond Elut cartridges (500 mg, 2.8 ml) were supplied by Jones Chromatography (Hengoed, UK).

HPLC was performed on a Spectra Physics (Hemel Hempstead, UK) system incorporating an SP8770 isocratic pump, a Spectra 100 UV detector and an SP8780 autosampler. A 10 cm × 4.6 mm I.D. C_{18} (5 μm) ODS Spherisorb column (Capital HPLC, West Lothian, UK) was used for the separation.

Extraction of artemether and dihydroartemisinin from plasma

Dichloromethane (3 ml) was added to plasma (1 ml) in screw-capped glass tubes and extracted by mechanical tumbling (10 min) and centrifuged (3000 *g*, 5 min). The organic phase was removed and collected. This process was repeated, and the combined organic phases were evaporated under nitrogen at 37°C. The samples were then reconstituted in hexane (1 ml) prior to solid-phase separation.

Solid-phase separation of artemether and dihydroartemisinin

Solid-phase extraction was achieved by drawing the samples through the cartridge under vacuum and collecting the eluates in glass tubes. Cartridges were primed initially with methanol (1 ml) and then conditioned with hexane (1 ml). During each wash, care was taken to ensure that the sorbent bed did not dry.

Sample mixtures were loaded on to the cartridges and passed through the column, under vacuum (71 kPa) for 10 s, and the eluate was collected. The cartridges were washed with methyl *tert.*-butyl ether (1 ml), and the eluate was col-

lected. The combined eluate (eluate a) was transferred to clean test tubes. The cartridges were then washed with methanol (2 × 1 ml) and the methanol eluates were transferred to clean glass tubes (eluate b). Following evaporation to dryness under nitrogen at 37°C, the samples were reconstituted in methanol (1 ml) prior to acid decomposition. Progesterone (50 ng, 50 μl of a 1 μg/ml stock solution), the internal standard, was added.

Acid decomposition

The acid decomposition step is a modification of that described previously [3] and involves adding 1 ml of 5 *M* hydrochloric acid to the reconstituted sample and placing the tubes in a water bath at 53°C for 15 min for the determination of artemether and 45 min for the determination of dihydroartemisinin.

The products of the reaction were then extracted from the aqueous phase using dichloromethane (3 ml) by mechanical tumbling for 10 min. The aqueous phase was then removed and discarded. To neutralise any remaining acid, 1 ml of 5 *M* sodium hydroxide solution was added and thoroughly mixed with the sample before the aqueous phase was again removed. Finally, distilled water (1 ml) was added to the tubes and the mixture agitated, before the organic phase was placed in clean tubes, and dried with 0.5 g of anhydrous sodium sulphate before centrifugation (350 *g*, 5 min). Finally, the organic phase was transferred into clean tubes and evaporated to dryness under nitrogen at 37°C. The residue was reconstituted in methanol (150 μl) prior to chromatography.

Chromatographic separation and UV detection

The mobile phase consisted of acetonitrile–water (50:50, v/v) flowing at 0.7 ml/min at ambient temperature. The reconstituted sample (50 μl) was injected on to the column. There was a 30-min run time between injections. Detection was at 254 nm, using an attentuation equivalent to 0.001 a.u.f.s. on a chart recorder set at 10 mV and a chart speed of 2 mm/min. The artemether and dihydroartemisinin acid decomposition products eluted at 10.5 min while progesterone had a retention time of 17 min.

Efficiency of separation

Artemether (1 μg, $n = 3$) and dihydroartemisinin (1 μg, $n = 3$), as solutions in hexane (1 ml), were applied separately to aminopropyl cartridges, and the solid-phase separation procedure was performed on each sample as described above. The resultant eluates were analysed using HPLC separation. From this the amount of analyte in each wash and therefore the level of cross-contamination between washes could be determined.

Calibration

Standard curves were generated by the addition of known quantities of artemether and dihydroartemisinin (0–300 ng) to a fixed amount of the internal standard (progesterone; 50 ng) in drug-free plasma. Samples were analysed as described above and the peak-height ratio of either artemether or dihydroartemisinin acid decomposition product to internal standard was plotted against the corresponding mass of drug. Peak-height ratios of unknowns were treated similarly and concentrations determined from the standard curves.

Assay specifications

Inter- and intra-assay variation (*i.e.* assay precision) was determined by analysis of samples of drug-free plasma to which had been added artemether and dihydroartemisinin to produce final concentrations of 25, 50 and 100 ng/ml for each analyte. The samples were stored at −20°C prior to analysis. Accuracy was assessed as the deviation of measured values from the predicted concentrations.

Clinical study

A healthy volunteer after giving full informed consent received artemether (200 mg) as a single oral dose. Venous blood was removed prior to dosage and at 1, 2, 3, 4, 6, 8, 10, 12 and 16 h thereafter. Following centrifugation (3000 *g*, 10 min), the plasma was removed and frozen prior to analysis.

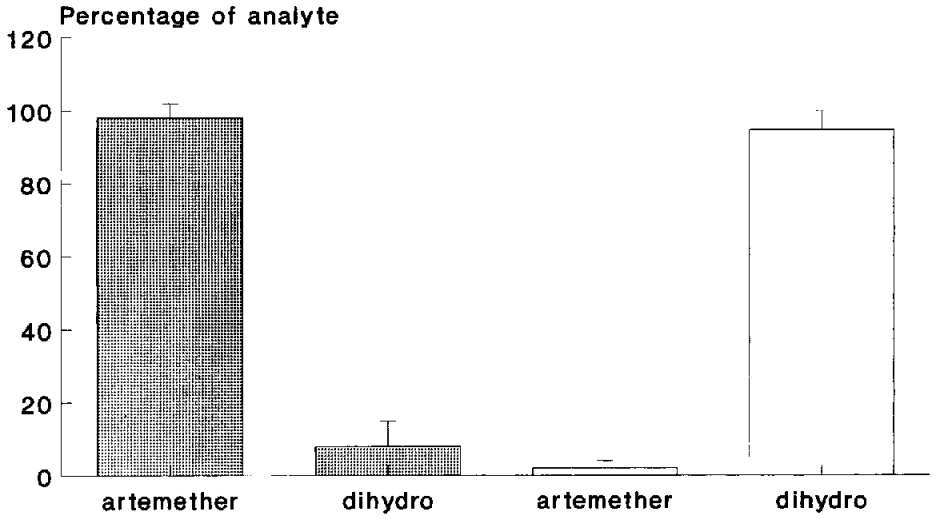

Fig. 2. Percentage elution of each analyte in eluate a (filled bars) and eluate b (open bars).

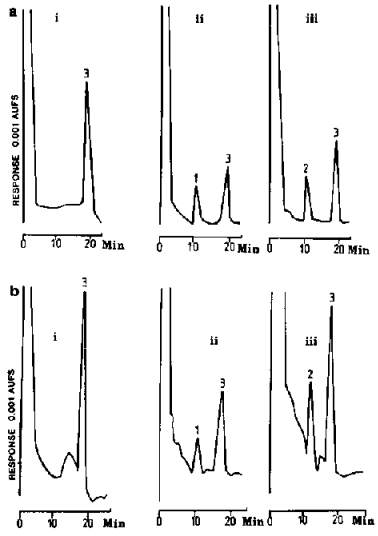

Fig. 3. (a) Chromatograms of extracts of (i) drug-free plasma containing internal standard (50 ng), (ii) 1 ml of plasma containing artemether (100 ng) and internal standard (50 ng), obtained from solid-phase eluate a and (iii) 1 ml of plasma containing dihydroartemisinin (100 ng) and internal standard (50 ng), obtained from solid-phase eluate b. Peaks: 1 = artemether acid decomposition product; 2 = dihydroartemisinin acid decomposition product; 3 = internal standard. (b) Chromatograms of extracts of (i) a pre-dose plasma sample from a healthy volunteer prior to dosing with artemether, (ii) a plasma sample from the same volunteer taken 2 h post dose with artemether (49 ng/ml), obtained from solid-phase eluate a and (iii) the same plasma sample (2 h post dose) showing levels of dihydroartemisinin (203 ng/ml), obtained from solid-phase eluate b. Peaks: 1 = artemether acid decomposition product; 2 = dihydroartemisinin acid decomposition product; 3 = internal standard.

RESULTS AND DISCUSSION

The percentage of each analyte in each wash is shown in Fig. 2: 98 ± 4% of the artmether present in a sample elutes with eluent a, while 92 ± 5% of the dihydroartemisinin elutes with eluent b. The level of cross-contamination (8 ± 7% for dihydroartemisinin contamination of the initial washes and 2 ± 2% for artemether contamination of the final wash) is sufficiently low for this method to be considered viable.

Calibration curves were linear in the range 0–300 ng/ml artemether and dihydroartemisinin (r = 0.992 and r = 0.991, respectively). These observations demonstrate the suitability of the method to measure artemether and dihydroartemisinin over the range of concentrations expected to occur *in vivo*.

Chromatograms of extracts of drug-free plasma and plasma to which artemether and dihydroartemisinin (100 ng/ml) had been added and of human plasma pre and 2 h post oral administration of artemether are shown in Fig. 3. The plasma extract (3 ml) from the human volunteer showed peaks of retention time 10.5 min for both artemether and dihydroartemisinin, and the internal standard peak at 17 min. The concentrations of artemether and dihydroartemisinin determined in samples to which predetermined

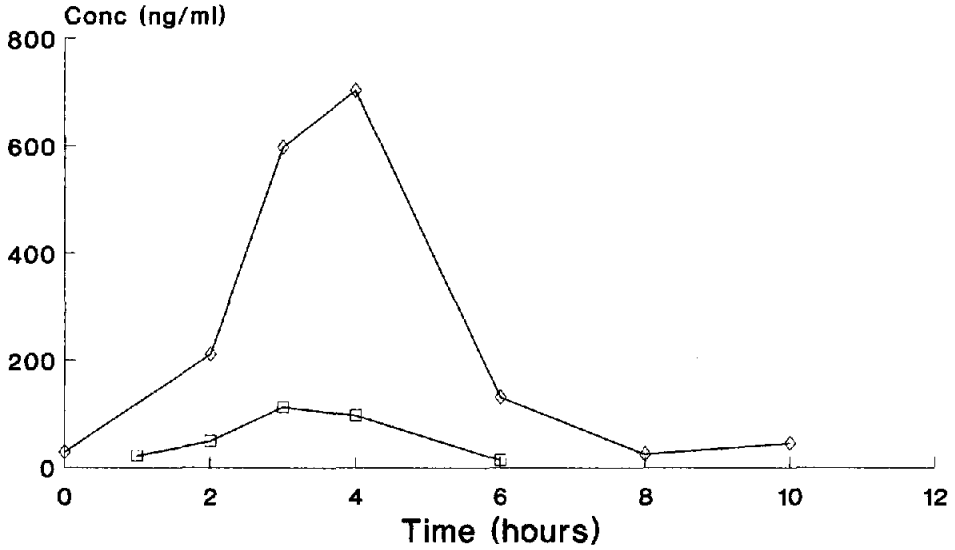

Fig. 4. Graph of plasma concentration *versus* time for artemether (□) and its major metabolite, dihydroartemisinin (◇), in a healthy volunteer after receiving a single oral dose of artemether.

quantities of each analyte had been added are reported in Table I. Intra- and inter-assay coefficients of variation of the analysis of spiked plasma samples were, respectively, 8.0 and 14.2% at 25 ng/ml, 10.7 and 5.6% at 50 ng/ml and 12.35 and 5.5% at 100 ng/ml for artemether. The corresponding values for dihydroartemisinin were 8.0 and 14.7% at 25 ng/ml, 9.8 and 8.2% at 50 ng/ml and 9.9 and 11.0% at 100 ng/ml.

TABLE I

INTRA-ASSAY AND INTER-ASSAY MEANS ± STANDARD DEVIATIONS FOR SPIKED PLASMA SAMPLES CONTAINING ARTEMETHER AND DIHYDROARTEMISININ AT KNOWN CONCENTRATIONS

Concentration added (ng/ml)	Concentration found (ng/ml)	
	Artemether	Dihydroartemisinin
Intra-assay (n = 6)		
25	26 ± 8.0	27 ± 8.0
50	54 ± 10.0	53 ± 9.8
100	95 ± 13.0	101 ± 11.0
Inter-assay (n = 6)		
25	24.1 ± 14.2	25 ± 3.7
50	50 ± 5.6	51.6 ± 8.1
100	100 ± 5.5	100 ± 9.9

The assay was applied to the determination of artemether and dihydroartemisinin in a healthy volunteer following a single oral dose (200 mg). The plasma concentration–time profiles for artemether and dihydroartemisinin is shown are Fig. 4. Peak concentrations for artemether (112 ng/ml) and dihydroartemisinin (702 ng/ml) were attained within 3 and 4 h post dosage, respectively. Metabolite levels rose very quickly after dosing and, at maximum, were over seven times the concentration of artemether at the corresponding time. Artemether levels fell below the level of detection (25 ng) after 5 h while dihydroartemisinin concentration remained above the sensitivity level for 12 h.

In conclusion, we have reported a procedure for the separation of artemether from dihydroartemisinin which, in association with a previously established and validated method [3], would appear to be suitable for pharmacokinetic studies in man. The separative method has a high degree of selectivity for each compound resulting in low levels of cross-contamination and as such provides the first HPLC–UV method for the accurate determination of both analytes at therapeutically relevant concentrations. This method is currently being applied to the analysis of clinical samples.

ACKNOWLEDGEMENTS

C.G.T. is supported by a Project Grant from the Medical Research Council. This work received financial support from the UNDP/World-Bank/ W.H.O. Special Programme for Research and Training in Tropical Diseases.

REFERENCES

1 D. Klayman, *Science*, 228 (1985) 1049.
2 I. S. Lee and C. D. Hufford, *Pharmacol. Ther.*, 48 (1990) 345.
3 O. R. Idowu, G. Edwards, S. A. Ward, M. L'E. Orme and A. M. Breckenridge, *J. Chromatogr.*, 493 (1989) 125.