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Short communication

Differential extraction of artemether and its metabolite dihydroartemisinin from plasma and determination by high-performance liquid chromatography

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Abstract

A method is described for the separation of artemether (ARM) from its metabolite dihydroartemisinin (DHA) and determination by HPLC. The basis of the separation is differential extraction of the drugs from plasma as a function of plasma pH. Hexane extracted ARM from basified plasma and both ARM and DHA from normal plasma. Derivatized extracts were chromatographed on a 5-μm ODS column with water–acetonitrile (40:60) as mobile phase and detected at 254 nm. The method removes the need for expensive absorption cartridges (BondElut). Chromatography has been improved and the elution time shortened in comparison with previous methods.

1. Introduction

Artemether (12β-methyldihydroqinghaosu, ARM, Fig. 1A) is a semi-synthetic antimalarial derived from qinghaosu (QHS), a natural product of a Chinese herb *Artemisia annua*. Unlike other antimalarial drugs, QHS and its derivatives are nitrogen-free sesquiterpenes, containing a peroxide linkage which confers activity against the malarial parasite. The bioavailability of QHS is poor following oral administration [1]. The derivative ARM and its metabolite dihydroartemisinin (DHA, Fig. 1B) have improved bioavailability and efficacy against the parasite [2]. ARM is initially converted to DHA in vivo [3]. Methods have been reported for measuring QHS and its derivatives in physiological fluids by chemical assay [4,5] and by HPLC [6,7], al-

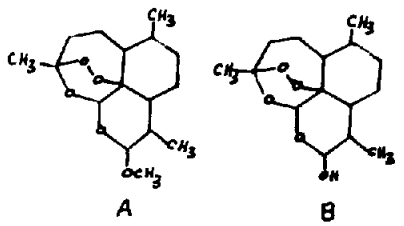

Fig. 1. Structures of (A) artemether and (B) dihydroatemisinin.

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though they are unable to measure ARM and DHA individually. An HPLC method that determines ARM and DHA individually has recently been reported by Thomas et al. [8]. While working with this method, we noticed in preliminary experiments that differential extraction of ARM and DHA could be achieved by changing the pH of the plasma, a finding which forms the basis of this paper.

2. Experimental

2.1. Derivatization of ARM and DHA

Artemisinin compounds do not absorb in the UV region, but can be readily reduced to α,β-unsaturated decalone and α,β-unsaturated aldehydes which have a specific absorption peak at 254 nm [9]. Derivatization was performed by heating the compounds at 53°C in 5 *M* HCl for 45 min as described by Idowa et al. [7].

2.2. Reagents

ARM, DHA and progesterone (internal standard; I.S.) were kindly supplied by Dr. G. Edwards, Liverpool University, UK. Acetonitrile, hexane and methanol (HPLC grade), hydrochloric acid and sodium hydroxide (both AnalaR grade) and ammonia (sp. gr. 0.91) were obtained from BDH (Poole, UK).

2.3. Collection of plasma

Whole blood was obtained from volunteers known not to have taken ARM and drawn slowly by venepuncture through a wide-bore needle to avoid haemolysis. The blood was immediately transferred into a heparinized tube, mixed and centrifuged. Plasma was removed to plain plastic tubes (Sterilin) and stored at −20°C.

2.4. Extraction procedure and derivatization

A 100-ng amount of progesterone as internal standard (10 μl of a 10 μg/ml solution) was added to 0.5-ml volumes of plasma containing drug in 10-ml glass culture tubes (Corning) using a 25-μl capacity Microlitre glass syringe (SGE, Ringwood, Australia). A 0.5-ml volume of ammonia solution (ARM assay) or 0.5 ml of water (ARM + DHA assay) was added and the tube contents were mixed on a vortex mixer for 10 s. Hexane (5 ml) was added, the tubes were capped and the contents mixed on a Stuart SBI tube rotator (Jencons) at a rate of 30 inversions/min for 10 min, followed by centrifugation (2000 *g* for 5 min) to separate the phases. The upper, organic phase was removed into a clean tube and evaporated to dryness under nitrogen (East African Oxygen, Nairobi, Kenya) at 37°C. Samples were reconstituted in 1 ml of methanol by vortex mixing and 1 ml of 5 *M* HCl was added. The tubes were transferred into a water-bath at 53°C for 45 min. After cooling to room temperature, the samples were re-extracted into 5 ml of hexane and the extract was washed (vortex mixing for 15 s; centrifugation at 2000 *g* for 5 min) once with 5 *M* sodium hydroxide solution and once with distilled water. The upper, organic layer was removed into a clean tube and evaporated to dryness as above. Samples were reconstituted in redistilled methanol (50 μl) and aliquots of 20 μl were injected on to the column.

2.5. Chromatography

Chromatography was performed using an Isochrom delivery system (SpectraSystem P 1000; Spectra-Physics) fitted to a Rheodyne valve injector (20-μl loop) and connected to a stainless-steel column (Hypersil 5 ODS, 25 cm × 4.6 mm I.D.; Wellington House, Macclesfield, UK) preceded by a guard column (CN precolumn, RP-18 endcapped 5 μm, 10 mm × 4.6 mm I.D., Waters Assoc., Milford MA, USA). Column effluent was monitored by variable wavelength UV detector (model SpectraSystem UV 1000; Spectra Physics) set at 254 nm. Mobile phase consisted of water–acetonitrile (40:60, v/v) flowing at 2.0 ml/min, which generated an operating pressure of ca. 110 bar.

2.6. Validation of differential extraction of ARM and DHA

The differential extraction of ARM and DHA from plasma was assessed on solutions of the compounds in plasma in the concentration range 0–600 ng/ml. Stock standard solutions of ARM and DHA (1 mg/ml in methanol, stored at −20°C) were used to prepare working standard solutions (10 μg/ml in saline), which were added to 0.5-ml aliquots of pooled human plasma. In the first experiment (total assay), extraction was performed at concentrations of 0, 50, 100, 200, 400 and 600 ng/ml for each of ARM, DHA and ARM–DHA (1:1). In the second experiment (ARM assay) similar plasma–drug aliquots were extracted, but the plasma was first basified with ammonia as described. Following extraction, samples were derivatized and chromatography was conducted as described.

2.7. Recovery, calibration and reproducibility

The recovery of ARM and the I.S. was assessed by adding 25, 50, 75, 100 and 200 ng of ARM to 0.5-ml aliquots of drug-free plasma, adding 100 ng of I.S., extracting from basified plasma and conducting derivatization and chromatography as described. The recovery of total drug (ARM + DHA) was assessed by adding the above amounts of each compound to plasma, extracting from normal plasma and following the same procedure as above. Both ARM and ARM + DHA were quantified via the peak-height ratio (PHR) in comparison with the I.S. Recovery was assessed as the difference between quantifications of extracted samples and samples injected directly on-column. Each recovery experiment was replicated five times. Calibration graphs were prepared by the addition of either ARM or ARM + DHA (stoichiometrically equal amounts) to plasma as before, at concentrations of 0, 50, 100, 200 and 400 ng/ml. These solutions were treated in the same way as test samples and extracted together with the unknowns in each run. In assays performed on plasma samples containing both ARM and DHA, DHA concentrations were defined as the differences between assay results for ARM and total drug (ARM + DHA). The subtraction of ARM from DHA + ARM does not limit the sensitivity of DHA as both compounds have equal physicochemical responses to the chromatographic conditions described. The intra-assay reproducibility was assessed at ARM concentrations of 50 and 400 ng/ml ($n = 6$) and at the same concentrations of ARM and DHA in combination ($n = 6$). Inter-assay reproducibility was assessed weekly over 1 month using the same solutions, which were stored at −20°C between tests ($n = 5$).

3. Results and discussion

3.1. Chromatography

ARM and DHA derivatives and the I.S. were resolved to the baseline throughout the concentration range 0–600 ng/ml, with retention times of 4.5 min for the derivative compound and 5.5 min for the I.S. (Fig. 2). There was no interference in the assay from phenobarbitone (used to prevent fits in severe malaria), the antipyretic drug paracetamol or the antimalarials quinine, quinidine, chloroquine, sulfadoxine pyrimethamine, proguanil, cycloguanil, chlorproguanil or chlorcycloguanil. The lowest detectable concentration of ARM and DHA was 10 ng/ml in a 0.5-ml sample, which at 1.00 AUFS consistently produced a peak larger than four times background noise.

3.2. Recovery, calibration and reproducibility

The recovery of ARM was 90 ± 10.5% (mean ± S.D.), of total drug 98 ± 5.7% and of the I.S. 96 ± 6.5% ($n = 5$ in all instances). The calibration graphs were linear for extractions from normal and basified plasma ($r^2 > 0.9900$). Differential extraction was demonstrated by the change in slope (b) of the calibration graphs. The ARM calibration graphs had the same b value of 0.0014 in both normal and basified plasma. Although the DHA graph had a slope of

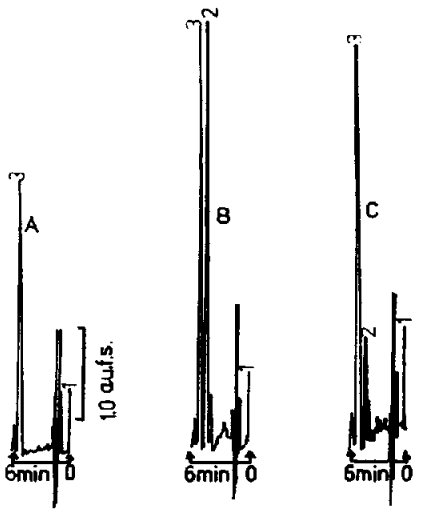

Fig. 2. Chromatograms of (A) an extract of blank plasma containing 100 ng of progesterone as I.S., (B) an extract of plasma containing 600 ng/ml of ARM and 100 ng of I.S. and (C) an extract of plasma from a patient following an intramuscular injection of a 3.2 mg/kg loading dose of ARM (84 ng/ml) and 100 ng of I.S. Peaks: 1 = start; 2 = ARM/DHA derivative; 3 = I.S.

0.0015 in normal plasma, there was no extraction in basified plasma. However, in a 1:1 combination (ARM–DHA) the slope changed from 0.0015 in normal plasma to 0.0007 in basified plasma.

The intra-assay coefficients of variation (C.V.s) at 50 and 400 ng/ml for ARM were 6.4% and 4.7% and for ARM–DHA at the same concentrations of 5.4% and 4.4%, respectively. The inter-assay C.V.s at 50 and 400 ng/ml for ARM were 7.5% and 1.1% and for ARM–DHA 1.4% and 5.6%, respectively.

Our study shows that both ARM and DHA are extracted quantitatively from normal plasma whereas only ARM is extracted from basified plasma. This effect permits the determination of both species in plasma down to a concentration of 10 ng/ml, although the mechanism remains obscure. We thought that differences between ARM and DHA in the extent of binding to plasma protein might account for the differential extraction, but this was not the case, as similar results were obtained when plasma was replaced by phosphate buffer. It therefore appears that at pH $\geq$ 12 there is a change in the chemical structure of DHA which confers increased polarity and preferential partitioning into the aqueous phase during extraction with hexane, and that the same change does not occur with ARM. Alternatively, interaction of ammonia and DHA could result in the formation of an adduct which on derivatization yields a compound with different HPLC characteristics to those of α,β-unsaturated decalone.

Although the chemical basis of the observed effect is unknown, we have shown pH-dependent differential extraction to be a reliable basis for a simplified separation of drug and metabolite, prior to determination of the derivative by HPLC. The method is quantitative, and may have application in the measurement of physiological concentrations of this important antimalarial in man, which are currently unknown.

Acknowledgements

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