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Short communication

Selective and sensitive liquid chromatographic assay of amodiaquine and desethylamodiaquine in whole blood spotted on filter paper

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Abstract

We have developed a sensitive, selective and reproducible reversed-phase HPLC method with ultraviolet detection (340 nm) for the simultaneous quantification of amodiaquine (AQ) and its major metabolite, desethylamodiaquine (AQm) in a small volume (200 μl) of whole blood spotted on filter paper. The method involves liquid–liquid extraction with diethyl ether followed by elution from a reversed-phase phenyl column with an acidic (pH 2.8) mobile phase (25 mM KH_2PO_4–methanol; 80:20% (v/v) +1% (v/v) triethylamine). Calibration curves in spiked whole blood were linear from 100–2500 ng/ml ($r^2 \geq 0.99$) for AQ and 200–2500 ng/ml ($r^2 \geq 0.99$) for AQm. The limit of detection was 5 ng for AQ and 10 ng for AQm. The relative recovery at 150 ng/ml of AQ ($n = 6$) was 84.0% and at 300 ng/ml of AQm the relative recovery was 74.3%. The intra-assay coefficients of variation at 150, 600 and 2250 ng/ml of AQ and 300, 600 and 2250 ng/ml of AQm were 7.7, 8.9 and 6.2% (AQ) and 10.1, 5.4 and 3.9% (AQm), respectively. The inter-assay coefficient of variation at 150, 600 and 2250 ng/ml of AQ and 300, 600 and 2250 ng/ml of AQm were 5.2, 8.1 and 6.9% (AQ) and 3.3, 2.3 and 4.6% (AQm). There was no interference from other commonly used antimalarial and antipyretic drugs (chloroquine, quinine, sulfadoxine, pyrimethamine, artesunate, acetaminophen and salicylate). The method is particularly suitable for pharmacokinetic studies in settings where facilities for storing blood/plasma samples are not available.

Keywords: Amodiaquine; Desethylamodiaquine

1. Introduction

Amodiaquine (AQ) is a 4-aminoquinoline anti-malarial drug, effective against chloroquine-resistant *Plasmodium falciparum* infections [1,2]. Two major metabolites of AQ have been identified, namely desethylamodiaquine and 2-hydroxydesethylamodiaquine [3]. The drug is rapidly and extensively metabolised mainly to the pharmacologically active derivative, desethylamodiaquine (AQm), which is probably responsible for most of the therapeutic effect [2,4].

There has been a renewed interest in AQ [5–14] especially for use in combination with other anti-malarials. Several analytical techniques that have been reported for quantifying AQ and AQm [1,15,16] have large sample volumes (1.0–2.0 ml) [1,15,17] or low recoveries for both AQ and AQm [1,2,13,18]. All of them are not suitable for pharmacokinetic studies of amodiaquine in settings where there are no facilities for storing (freezing) blood or plasma samples. There are also concerns in some laboratories about handling liquid blood samples, due to the prevalence of HIV in some regions. We report a simple, sensitive and selective HPLC method that requires a sample volume of 200 μl of whole blood spotted and dried on filter paper for the determination of both AQ and AQm. Samples stored on filter paper are easily transported from the field and minimises the amount of storage space needed, which can be an issue in resource poor countries. We have applied this method to study the disposition of AQ and AQm in a healthy subject after a single 600 mg oral dose of AQ.

2. Experimental methods

2.1. Chemicals and reagents

Amodiaquine dihydrochloride dihydrate and quinidine base (QD) were obtained from the Sigma Chemical

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Company (St. Louis, MO, USA). Desethylamodiaquine diphosphate dihydrate was a gift from Professor Steve Ward, Liverpool School of Tropical Medicine, Liverpool, UK. HPLC-grade methanol, diethylether, orthophosphoric acid, triethylamine and dichlorodimethylsilane were purchased from BDH, Poole, England. Potassium dihydrogen orthophosphate (AnalaR® grade) was also obtained from BDH.

2.2. Preparation of standard solutions

A stock solution of AQ (1 mg of the base/ml) was prepared by dissolving the dihydrochloride salt in 10 ml of methanol while AQm stock solution (1 mg of the base/ml) was prepared by dissolving the diphosphate salt in water. Stock solutions were stable for 2 months when stored at −20 °C. Internal standard (QD) stock solution (1 mg/ml) was prepared by dissolving QD base in methanol. Working solutions (100, 10 and 1 μg/ml) were prepared by appropriate serial dilution of the stock solutions with methanol (AQ and QD) or water (AQm).

2.3. Sample preparation and calibration curves

Drug-free whole blood was spiked with AQ or AQm and gently tumbled for 0.5 h to ensure complete mixing. Duplicate samples of spiked whole blood (200 μl) containing known concentrations of AQ (100–2500 ng/ml) and AQm (200–2500 ng/ml) were spotted on glass microfibre filter strips (GF/C Cat NO. 1822 915 Whatman®) and dried at room temperature for at least 12 h. If they were not to be assayed immediately the strips were stored in a microscope slide box at 4 °C.

2.4. Extraction procedure

The dried filter strip spots were cut into small pieces and put into 15 ml PTFE-lined screw-cap centrifuge tubes (previously cleaned and dried after rinsing in dimethyldichlorosilane solution). Methanol (50 μl) containing QD (internal standard, 500 ng) followed by 2.5 ml distilled water was added and the strips soaked while shaking for 0.5 h. Diethylether (5 ml) was then added and the mixture tumbled for 15 min on a Stuart STR4 rotator drive (Stuart Scientific, Bedfordshire, UK) followed by centrifugation (1500 g, 10 min). The upper organic phase was transferred to a clean 15 ml glass centrifuge tube and evaporated to dryness in a water bath (37 °C) under a gentle stream of white spot nitrogen gas. Samples were reconstituted in mobile phase (100 μl) and 50 μl aliquot injected onto the chromatographic system.

2.5. Recovery and reproducibility

Recovery of AQ and AQm was assessed by spotting duplicate 200 μl aliquots of whole blood spiked with 30 ng of AQ and 60 ng of AQm ($n = 6$ for each level) on filter paper, followed by overnight drying at room temperature. After addition of QD (500 ng), the samples were processed as described above. In another set of tubes equivalent amounts of AQ, AQm and internal standard (500 ng) were added directly into extracting solvent followed by evaporation of the solvent under a gentle stream of nitrogen. The residue was reconstituted and injected onto the chromatograph as described above. Recovery was assessed by comparing peak area ratios of AQ and AQm to internal standard for extracted whole blood samples, with those from equivalent amounts of AQ and AQm spiked directly into the organic phase.

Reproducibility of the assay was assessed by determining the intra- and inter-day coefficients of variation (%CV) for amounts of AQ and AQm in spiked whole blood (200 μl) and spotted on filter paper strips. Intra-assay (within-day) variability was assessed by analysing six replicates of whole blood samples spiked with 150, 600 and 2250 ng/ml of AQ and 300, 600 and 2250 ng/ml of AQm. Inter-assay (day-to-day) variability was assessed by analysing duplicates of each amount over a period of 5 days.

2.6. Selectivity

Various anti-malarial drugs (chloroquine and its metabolite desethylchloroquine, quinine, sulfadoxine, pyrimethamine, proguanil, cycloguanil, artesunate), and analgesics (acetaminophen, salicylate) used in management of severe malaria were evaluated for interference with the assay for AQ and AQm. Drug-free whole blood was spiked with therapeutic concentrations of these drugs followed by extraction and analysis as described.

2.7. Stability

After initially drying filter paper strips spotted with another set of whole blood samples spiked with AQ (30 ng; $n = 6$) and AQm (60 ng; $n = 6$) at room temperature overnight, the strips were stored in a microscope slide box at 4 °C and assayed for AQ and AQm after 30 days.

2.8. Chromatography

Chromatography was performed under isocratic conditions at ambient temperatures. An isochrom delivery system (Isochrom LC; Spectra Physics, San Jose, CA, USA) fitted with a Model 7125 Rheodyne (Cotati, CA, USA) valve injector (50 μl loop) was used. The column was a reversed-phase phenyl column (Synergi 4 μm Polar–RP, 150 × 4.6 mm; Phenomenex, Macclesfield, Cheshire, UK) preceded by a guard column (LiChrospher 100 RP–18e, 5 μm, 10 × 4.6 mm i.d.; Merck, Darmstadt, Germany). The mobile phase comprised 25 mM KH_2PO_4–methanol (80:20% (v/v)) containing 1% (v/v) triethylamine, and adjusted to pH 2.8 with

orthophosphoric acid. The flow rate was 1.2 ml/min, generating an operating back pressure of about 190 bar. The column effluent was monitored with a variable wavelength UV-Vis detector (model Spectra Series UV100; Spectra Physics, San Jose, CA, USA) set at 340 nm. Chromatographic peaks were recorded on an integrator (ChromJet CH-1 data integrator, Thermo Separation Products, San Jose, California). Peak area ratios (drug/internal standard) were plotted as a function of the known concentrations of the AQ and AQm. The results were analysed by least squares linear regression.

2.9. Validation study

A predose blood sample (1 ml) was taken from a healthy volunteer who then received a single 600 mg oral dose of AQ (Camoquine; Parke-Davis, Dakar, Senegal) with 200 ml of water. Venous blood samples (1.5 ml) were obtained at various time intervals up to 24 h. Blood samples were collected into heparinised blood collection tubes. Aliquots of blood (200 μl) were spotted on filter paper and assayed (in duplicate) for AQ and AQm by the procedures described. To compare the filter paper method with liquid-liquid extraction, duplicate samples of the rest of the liquid blood (200 μl each) were spiked with the internal standard and extracted with the organic solvent as described above.

3. Results and discussion

3.1. Chromatography

AQ, AQm and QD (internal standard) were resolved to baseline with retention times of 6.8, 8.9 and 16 min, respectively (Fig. 1). This is in contrast to one previous method that depended on pH changes in the mobile phase to achieve good peak resolution between AQ and AQm [20]. In the method described here, good resolution between the peaks was achieved without the need to change the pH of the mobile phase. The limit of detection was 5 ng for AQ and 10 ng for AQm, respectively at a detector attenuation of 0.005 a.u.f.s.

3.2. Recovery, calibration curves and reproducibility

One previous study reported that extraction of AQ and AQm from whole blood samples spotted on filter paper was unsatisfactory due to the instability of AQ and AQm [19]. We initially tried using the Whatman® chromatography types 17CHR and ET 31 but found that the recovery of AQ and AQm was <20%. Based on these results, we changed to the glass microfibre filters and found that the percent recoveries at 30 of AQ and 60 ng of AQm (the low quality control concentrations that we used) were 83.9 and 74.3% ($n = 6$), respectively. The intra-assay coefficients of variation at 150,

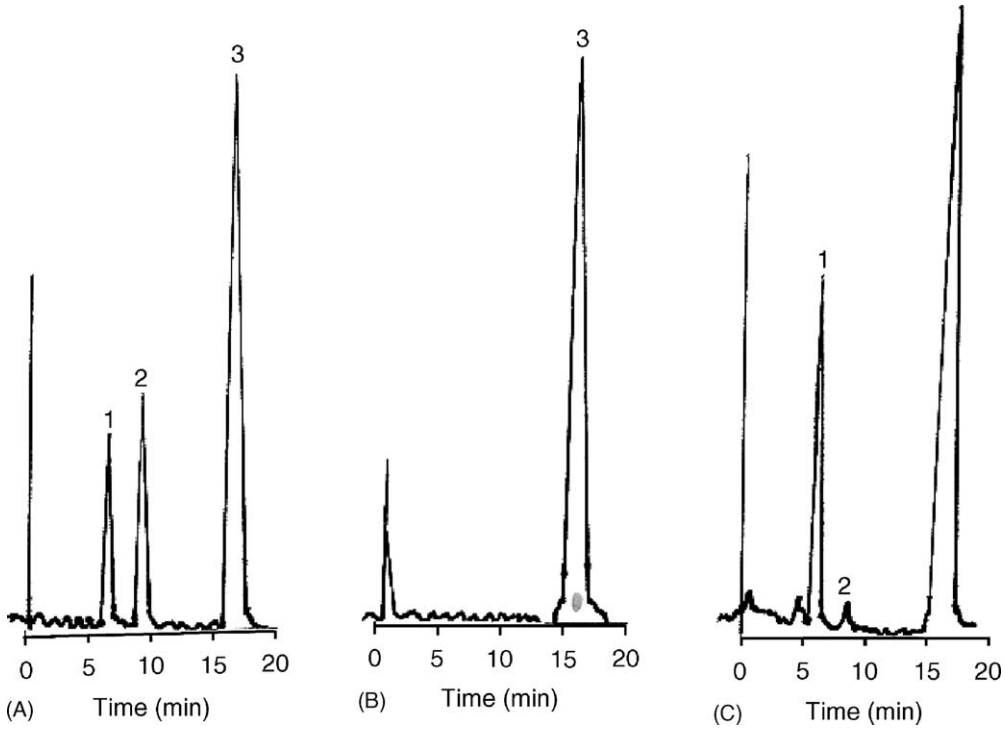

Fig. 1. Chromatograms of (A) direct injections of 16 ng each of AQ and AQm and 200 ng of QD (internal standard); (B) extracted predose whole blood sample of a healthy volunteer spiked with 500 ng of QD; and (C) extracted whole blood sample obtained from same patient obtained at 2 h following a 600 mg single oral dose of AQ and spiked with 500 ng of QD. AQ and AQm concentrations were 258.8 and 1137.5 ng/ml, respectively. Peaks: 1, AQm; 2, AQ; 3, QD.

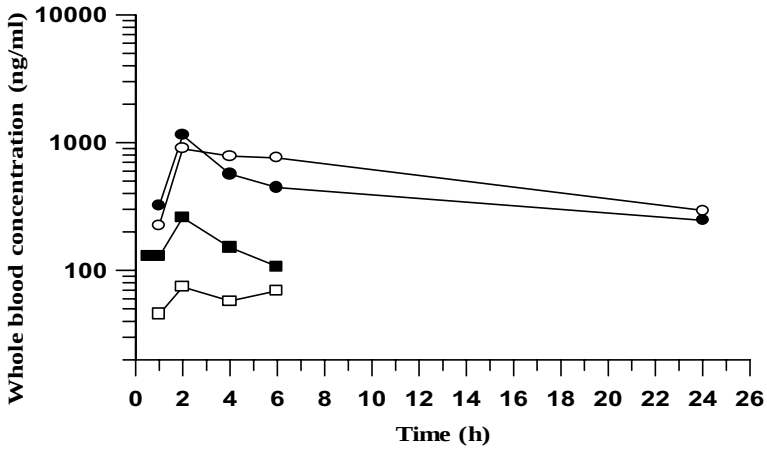

Fig. 2. Concentration vs. time profiles following administration of a 600 mg single oral dose of AQ. Desethylamodiaquine extracted from liquid whole blood (Aqm: open circles) and liquid whole blood spotted on filter paper (AQm: closed circles). Amodiaquine extracted from liquid whole blood (AQ: open squares) and whole blood spotted on filter paper (AQ: closed squares).

600 and 2250 ng/ml of AQ and 300, 600 and 2250 ng/ml of AQm were 7.7, 8.9 and 6.2% (AQ) and 10.1, 5.4 and 3.9% (AQm), respectively. The inter-assay coefficients of variation at the same concentrations were 5.2, 8.1 and 6.9% (AQ) and 3.3, 2.3 and 4.6% (AQm), respectively. The calibration curves in spiked whole blood were linear within the range 30–2500 ng/ml ($r^2 \geq 0.99$).

3.3. Stability

The stability experiments showed that both AQ and AQm were stable on the filter strips stored at 4 °C for up 30 days. At room temperature, the samples were stable for up to 24 h.

3.4. Validation study

The assay method was used to measure the concentrations of AQ and AQm in whole blood samples obtained from a volunteer following administration of a 600 mg single oral dose of AQ. Concentrations versus time profiles for AQ and AQm in whole blood spotted on filter paper are shown in Fig. 2. After oral dosage, AQm maximum concentration of 1137.5 ng/ml was achieved at 2 h, while maximum AQ concentration was 258 ng/ml at 2 h. The results from filter paper strips and liquid whole blood were comparable. The maximum concentrations determined in the present study were higher than previously reported values in which much larger blood volumes (up to 1 ml) had been used for assay [1,2,15,16,19,20]. They were also higher than those reported in a previous study [21] in which the volume of blood used was not specified. However, our in vivo results were from one volunteer and these discrepancies could partly be explained by inter-individual variability in the extent of absorption and metabolism of AQ to AQm.

In conclusion, we have described a HPLC method for the simultaneous determination of AQ and its major metabolite AQm that is specific, simple and rapid. It involves spotting blood onto filter paper strips, an obvious advantage in rural parts of Africa where storage and transportation of liquid whole blood can be very cumbersome and difficult. Another advantage is that handling dried whole blood is much safer than liquid blood, especially in resource-poor facilities. The use of the micro-fibre glass filter paper, as opposed to the other types of filter paper (Whatman® 17 CHR and ET 31) commonly used for the same purpose, increases the recovery of the drug and this paper has the added advantage of drying within 30 min making it easily manageable in a field setting. The assay requires a small sample volume (200 μl), which is important to clinical studies especially in small children. Together with previously reported methods for the assay of chloroquine/desethylchloroquine [18] and sulfadoxine-pyrimethamine [22] in filter paper-collected blood samples, the present assay method will facilitate the conducting of pharmacokinetic studies under field conditions.

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