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Automated solid-phase extraction method for the determination of atovaquone in capillary blood applied onto sampling paper by rapid high-performance liquid chromatography

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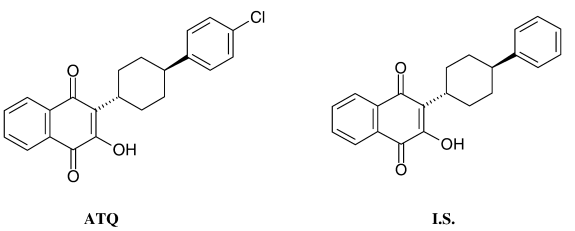
Abstract

A bioanalytical method for the determination of atovaquone in 100 μl blood-spots by solid-phase extraction and high-performance liquid chromatography has been developed and validated. Atovaquone was extracted from the sampling paper in 0.2 *M* phosphoric acid and a structurally similar internal standard was added with acetonitrile before being loaded onto a C8 end-capped solid-phase extraction column. Atovaquone and internal standard were analysed by high-performance liquid chromatography on a C_{18} J'Sphere ODS-M80 (150×4.0 mm) column with mobile phase acetonitrile–phosphate buffer, 0.01 *M*, pH 7.0 (65:35, v/v) and UV detection at 277 nm. The intra-assay precision was 2.7% at 12.00 μ*M* and 13.5% at 1.00 μ*M*. The inter-assay precision was 3.3% at 12.00 μ*M* and 15.6% at 1.00 μ*M*. The lower limit of quantification was 1.00 μ*M*. The limit of detection was 0.50 μ*M*.

Keywords: Atovaquone

1. Introduction

Atovaquone (ATQ) (Fig. 1), is a chemical analogue of coenzyme Q (ubiquinone), a cellular component which plays a essential role in mediating electron transfer between mitochondrial respiratory enzyme complexes [1]. The compound was initially developed as an antimalarial compound but has also been shown to be effective for the treatment of *Pneumocystis carinii pneumonia* (PCP) in patients with the acquired immunodeficiency syndrome (AIDS) [2]. Recently, the compound has received great interest because of its high efficacy against *Plasmodium falciparum*, the cause of severe and lethal malaria, when combined with another antima-



ATQ I.S.

Fig. 1. Structures of ATQ and I.S.

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larial compound, Proguanil [3]. ATQ is highly lipophilic and more than 99.9% bound to plasma proteins [4,5]. Active analogues of coenzyme Q inhibit the mitochondrial respiratory activity and it is suggested that ATQ disrupts the mitochondrial membrane potential of *Plasmodium falciparum* [6].

Several clinical trials for malaria treatment/prophylaxis have been conducted and in a pharmacokinetic study therapeutic plasma concentrations were between 2 and 5 μ*M* [7].

Traditionally, the extraction of ATQ from plasma has been performed by liquid–liquid extraction techniques or protein precipitation and HPLC [8–11], which has a few disadvantages as it is quite time consuming and shows low precision. A method with improved precision for the determination of ATQ in plasma and venous blood with solid-phase extraction and HPLC has recently been published [12].

For field studies of antimalarials, it is necessary to have a simple, rapid and accurate sampling technique for the determination in blood. Dried capillary blood spots provides a suitable delivery system since the finger prick technique is rapid and simple. Furthermore, the transportation of the dried blood spots does not require the same controlled temperature conditions as plasma or venous blood samples. Other antimalarials have been assayed before in dried capillary blood spots, for example proguanil and cycloguanil [13].

The aim of this work was to develop and validate an automated solid-phase extraction (SPE) HPLC method for the assay of ATQ in 100 μl blood spots applied onto sampling paper. The assay has been validated with respect to accuracy, precision, linearity, selectivity, stability and recovery according to [14].

2. Experimental

2.1. Chemicals and materials

ATQ, and the internal standard (I.S.), *trans*-2-hydroxy-3-(4-phenylcyclohexyl)-1,4-naphthalenedione, were obtained from Glaxo Wellcome (Hertfordshire, UK). The structures are shown in Fig. 1. Sampling paper ET 31 Chr was obtained from Whatman (Maidstone, UK). Acetonitrile (HPLC-grade), methanol (pro analysi) and dimethylformamide (pro analysi) were obtained from Merck (Darmstadt, Germany).

Deionised water was obtained from a Milli-Q deionised water system (Millipore, Bedford, MA, USA). The phosphate buffer solutions (0.01 and 0.1 *M*) were prepared by mixing different amounts of potassium hydroxide and potassium-dihydrogenphosphate with Milli-Q deionised water.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a LKB 2150 HPLC pump (Pharmacia Biotech, Uppsala, Sweden) and a Gilson 234 auto-injector (Gilson, Middleton, WI, USA). The detector was a Jasco UV-970 Intelligent UV/Vis detector (Jasco Corporation, Tokyo, Japan) set at 277 nm. Data acquisition was performed using Chromatography station for Windows 1.7 (DataApex Ltd, Prague, Czech Republic) on an AT&T (Dell Computer Corporation, Bracknell, Berkshire, UK) Pentium 90 MHz personal computer. The mobile phase was acetonitrile–phosphate buffer, 0.01 *M*, pH 7 (65:35, v/v) with a flow-rate of 1.0 ml/min through a C_{18} J'Sphere ODS-M80 column, 5 μm (150×4.0 mm I.D.), (YMC, Wilmington, NC, USA), protected by a short guard column Haicart Kromasil C_{18}, 5 μm (15×3.2 mm I.D.), (Higgins Analytical, Mountain View, CA, USA).

2.2.1. Solid-phase extraction

Extraction was carried out on an automated SPE system, ASPEC XL (Gilson, Middleton, WI, USA) using C8 end-capped extraction columns containing octyl silica as support (1 ml, 100 mg, IST Ltd, Hengoed, Glamorgan, UK). The ASPEC system uses positive air pressure instead of vacuum to get the liquids through the columns.

2.3. Preparation of calibration standards and samples for determination of accuracy and precision

Concentrated stock solutions of ATQ and I.S. (100 μ*M*) were prepared in methanol-dimethylformamide (99:1, v/v). The solutions were stored at about +4°C. Appropriate amounts of the stock solution of

ATQ were added to blank venous blood to yield spiked calibration standards ranging from 1 to 20 μ*M*. The calibration standards were prepared in batches of 10 ml, applied in 100 μl aliquots onto sampling paper, dried and stored at about −17°C. Calibration curves were prepared using seven different concentrations of ATQ. The peak-height ratio ATQ to I.S. against concentration of ATQ with non-weighted linear regression was used for quantification.

Samples for determination of accuracy and precision in venous blood were prepared at three concentrations (1.00, 4.00, 12.00 μ*M*) in batches of 10 ml, applied in 100 μl aliquots onto sampling paper, dried and stored at about −17°C. The lower limit of quantification (LOQ) was selected at the concentration at which the assay precision was within 15% and the signal-to-noise ratio exceeded 10:1. The limit of detection (LOD) was determined at the concentration at which the signal-to-noise ratio exceeded 3:1.

2.4. Sample preparation

2.4.1. Venous blood spot samples

The dried blood spots were cut into small pieces and transferred to polypropylene tubes, to which 2.0 ml of 0.2 *M* phosphoric acid was added. The tube contents were vortex-mixed and shaken on a mechanical shaker for 90 min. After that, 2.00 ml I.S. (1 μ*M*) in acetonitrile was added and the tube contents were vortex-mixed again. The tubes were then left to stand for 10 min, where after 5.00 ml phosphate buffer (0.1 *M*, pH 9) was added followed by centrifugation at 5400 *g* for 2 min. The liquid phase was decanted into new tubes and all of it was then loaded on to a SPE column. The extraction procedure on the ASPEC XL is shown in Table 1. The eluates were partly evaporated at about 20°C for 10 h down to about 300 μl, and then 100 μl of the eluates were injected into the HPLC-system.

2.4.2. Assay validation

2.4.2.1. Accuracy, intra- and inter-assay precision. The accuracy and precision of the method were estimated by analysis of spiked venous blood in 100 μl spots applied onto sampling paper at three concentrations (1.00, 4.00, 12.00 μ*M*) in triplicate for 5 days. Concentrations were determined using a calibration graph prepared on the day of analysis and intra- and inter-assay precisions were calculated.

2.4.2.2. Linearity. Calibration curves were constructed using seven calibration standards and were obtained by calculating the peak-height ratios of ATQ to the internal standard against the corresponding concentration. Linear calibration curves were generated by non-weighted linear regression analysis and obtained over the standard concentration range.

2.4.2.3. Extraction recovery. The extraction recovery was determined from the samples used for the precision study. Three concentrations (1.00, 4.00, 12.00 μ*M*) in triplicate together with nine blank

Table 1
ASPEC SPE procedure for the extraction of venous blood applied onto sampling paper

SPE-step	Liquid dispensed	Dispensing volume (ml)	Dispensing flow-rate (ml/min)	Pressuring air volume (ml)
Conditioning	Methanol	2.00	3	0.20
	Buffer 0.01 *M*, pH 9	2.00	3	0.50
Sample	Venous blood	9.00[a]	1	0.70
Loading				
Washing	Buffer 0.01 *M*, pH 9	2.00	3	0
	Methanol:Acetonitrile:Buffer 0.01 *M*, pH 9 (20:5:75, v/v)	2.00	2	0.50
Elution	Acetonitrile:Buffer 0.01 *M*, pH 7 (70:30, v/v)	0.30	1	0
	Acetonitrile:Buffer 0.01 *M*, pH 7 (70:30, v/v)	0.30	1	0.70

[a] The volume after the extraction from the paper is less than this volume. This is the volume that is in the programme for ASPEC XL. This means that the whole liquid phase is loaded on to the SPE column.

venous blood spots were analysed for 5 days. The eluates from the blank samples were spiked at the same nominal concentration as the precision samples. The peak-height ratios of ATQ to the internal standard of the spiked blank eluates were compared to those of the precision samples. In this way, any effect of the matrix does not complicate interpretation of recovery data [15].

2.4.2.4. Stability. Both ATQ and I.S. have earlier shown good stability in plasma [10,16]. The compounds have also shown to be stable in acetonitrile:phosphate buffer solutions such as the eluate solution [12]. Spiked venous blood at an ATQ and I.S. concentration of 5.00 μ*M* was applied onto sampling paper in 100 μl aliquots. The blood spots were stored at different temperatures (−86°C, about 4°C, about 23°C and 60°C) and both in airtight plastic bags and exposed to air.

2.4.2.5. Selectivity. Blank capillary blood from six healthy volunteers was analysed and the chromatograms were examined for endogenous compounds that could interfere with the quantification of ATQ. Selectivity for several antimalarials has earlier been investigated for the same HPLC-method without interference for the quantification of ATQ [12].

2.4.2.6. Drug levels in capillary blood versus venous blood. The presented method was applied to the determination of ATQ in three healthy volunteers in a study to compare drug levels in capillary blood and venous blood. Each volunteer was given a single oral dose of Malarone® (250 mg ATQ) and the concentration of ATQ in both capillary- and venous blood were determined at 1, 2, 3, 5 and 7 h after the dose.

3. Results and discussion

ATQ is highly lipophilic and almost insoluble in water (<0.3 μ*M*) [17]. Thus, absorption in man is greatest when the drug is taken with high-fat foods [4,17]. The manufacturer states that ATQ is extensively (99.9%) bound to plasma proteins [5]. There is negligible metabolism of ATQ in man and the main route of elimination is via the liver. Only the parent compound is recovered in the bile and faeces with only small amounts eliminated via the urine (<0.6%) because of the very high plasma protein binding level [4,5,18]. The structure of ATQ (Fig. 1) includes a hydroxyl group that acts as a weak acid with a calculated $pK_a \approx 5.0$ [19]. In an earlier published method the SPE columns used were common C_8 [12]. By using C_8 (end-capped) it was possible to get less interference from possible basic compounds occurring in the sample. Retention times of ATQ and I.S. depend particularly upon pH and acetonitrile content of the mobile phase. Thus, increases in pH and acetonitrile content decrease the retention times. A mobile phase consisting of acetonitrile–phosphate buffer, 0.01 *M*, pH 7 (65:35, v/v) gave retention times less than 5 min and a very good separation between ATQ, I.S. and endogenous compounds as well as other common antimalarials in the solvent front.

3.1. Accuracy, intra- and inter-assay precision

The precision and accuracy of the assay is summarized in Table 2. At the LOQ 1.00 μ*M*, the signal-to-noise ratio was greater than 10:1 and the intra-assay precision was about 15%. At the LOD 0.50 μ*M*, the signal-to-noise ratio was greater than 3:1. These limits could easily be decreased since only 100 μl of the partly evaporated eluate (300 μl) from the SPE was injected. Indeed, the therapeutic range of ATQ in plasma is about 10–14 μ*M* [3].

3.2. Linearity

Linear calibration curves were obtained with correlation coefficients 0.999±0.056 ($r \pm$SD), slope 0.032±0.005 (mean±SD) and intercept 0.0085± 0.0071 (mean±SD). The calibration curves were obtained during 5 days.

3.3. Extraction recovery

The absolute extraction recovery is presented in Table 3. The recoveries of ATQ from venous blood applied onto sampling paper were between 42 and 54%. The recovery of I.S. were 92.2±2.8 (mean±SD). The difference between ATQ and I.S. in recovery is probably due to the fact that ATQ is

Table 2
Accuracy, intra- and inter-assay precision for the determination of ATQ in 100 μl venous blood spots

	Added (μ*M*)	Found±S.D (μ*M*)	RSD (%)	% Deviation (found versus added)
Intra-assay (*n*=15)	1.00	1.16±0.16	13.8	16.0
	4.00	4.05±0.12	3.0	1.3
	12.00	12.06±0.32	2.7	0.5
Inter-assay (*n*=5)	1.00		15.6	
	4.00		2.8	
	12.00		3.3	

dried within the blood on the paper and I.S. is added with acetonitrile. The recovery of I.S. is similar to previous results [12]. High recovery is desirable but as long as adequate sensitivity, precision and accuracy are achieved, the extent of recovery should not be an issue in bioanalytical method development and validation [15].

3.4. Stability

Both ATQ and I.S. have earlier shown good stability in plasma including freeze–thaw stability [10,16]. The stability of ATQ applied onto sampling paper stored at four different temperatures in airtight plastic bags and exposed to air is presented in Fig. 2. ATQ seemed to be stable at all test temperatures except for 60°C and 23°C when exposed to air. All the samples that were kept in airtight plastic bags formed some kind of decomposition product, probably due to leaching from the plastic bag. However this did not interfere with the quantification of ATQ. Earlier published recommendations for transportations of dried blood spot specimens says that they should not be packaged in airtight plastic bags. This could cause moisture accumulation that may damage the dried blood spot test substances because of the lack of air exchange. In addition, various chemicals that may adversely affect the test substances in the dried blood spots could leach from the plastics and thus cause incorrect analytical test results [20]. However, since ATQ seemed to be stable at 23°C when kept in airtight plastic bags but not when exposed to air, the recommendations after this experiment must be to store the blood spots in plastic bags during transportation.

Table 3
Recovery of ATQ from venous blood applied onto sampling paper (*n*=12)

	Concentration (μ*M*)	Venous blood (%) (mean±SD)
ATQ	1.00	53.8±4.4
	4.00	42.1±2.8
	12.00	45.2±2.6

3.5. Selectivity

No endogenous interference from venous blood was observed. The selectivity for several antimalarials was investigated for the same HPLC-method and none of them showed any interfering effect for the quantification of ATQ [12].

3.6. Drug levels in capillary blood versus venous blood

Fig. 3 shows chromatograms 1 h, 3 h and 7 h after a single oral dose of Malarone® (250 mg ATQ). The simultaneously sampled capillary and venous blood, from the experiments with volunteers, showed a systematic difference in ATQ-level (Fig. 4). For 13 samples with a venous level range from 0.95 to 6.03 μ*M*, the average was 29 percent higher in the venous blood, but with an excellent linear correlation (r=0.981 in weighted Deming regression).

Kuile et al. observed a similar phenomenon with halofantrine and desbutyl-halofantrine but not with mefloquine [21] and proposed as an explanation, that capillary blood is contaminated with interstitial tissue fluids, containing lower concentrations of the antimalarials. We find this theory far-fetched, however, as a possible admixture would vary randomly, prohibiting

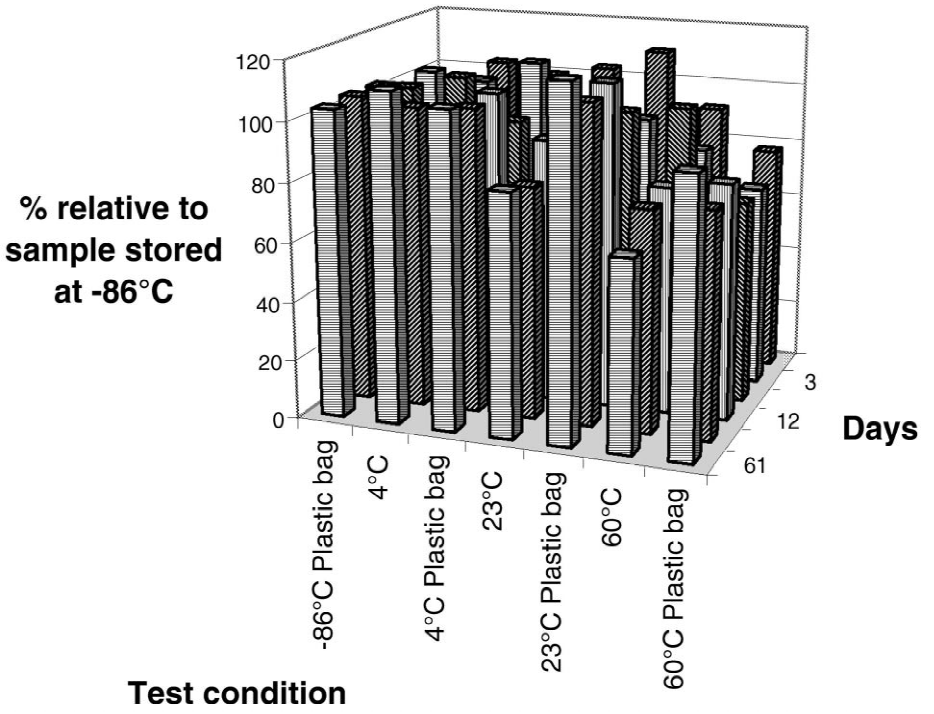

Fig. 2. Stability data for spiked venous blood samples (5.00 μ*M*) applied onto sampling paper in plastic bags and exposed to air stored at –86°C, about 4°C, about 23°C and 60°C.

the demonstrated high degree of correlation. Furthermore, the interstitial tissue fluid would have lowered the mefloquine concentration in capillary blood as well as the concentration of halofantrine and desbutyl-halofantrine. Other antimalarials concentration in venous blood compared to capillary blood have been investigated with varying results. Quinine has also proved to have lower concentrations (about 8 percent) in capillary blood than in venous blood [22], while lumefantrine proved to have higher concentrations in capillary blood than in venous blood (about 31 percent) [23]. Sulfadoxine and tafenoquine showed no significant differences between measurement in both biological fluids [24,25]. The differences between levels of ordinarily measured high molecular analytes in capillary and venous blood is less than 5 percent with lower content of lipids and lipoproteins in capillary blood. Kupke et al. proposed as an explanation that capillary blood is not only contaminated with interstitial fluid, but also affected by filtration and reabsorption mechanisms across the walls of small vessels. These mechanisms leads to protein transport into the interstitial fluid and hence into the lymf fluid which would lower the content in the capillary blood [26,27]. It may thus be, that the small, but consistent, matrix-difference between capillary and venous blood, diminishes the distribution into the capillary blood. Irrespective of possible explanations, the difference must be taken in account in experimental planning. Venous and capillary sampling should *not* be mixed in an experiment. The good correlation permits comparison of venous and capillary analyzed experiments when the constant bias is considered.

4. Conclusion

A validated method using SPE and HPLC for the determination of ATQ in 100 μl blood spots applied onto sampling paper was developed. A mobile phase consisting of acetonitrile:phosphate buffer, 0.01 *M*, pH 7 (65:35, v/v) gave retention times for ATQ and I.S. of less than 5 min. The assay has proven to be

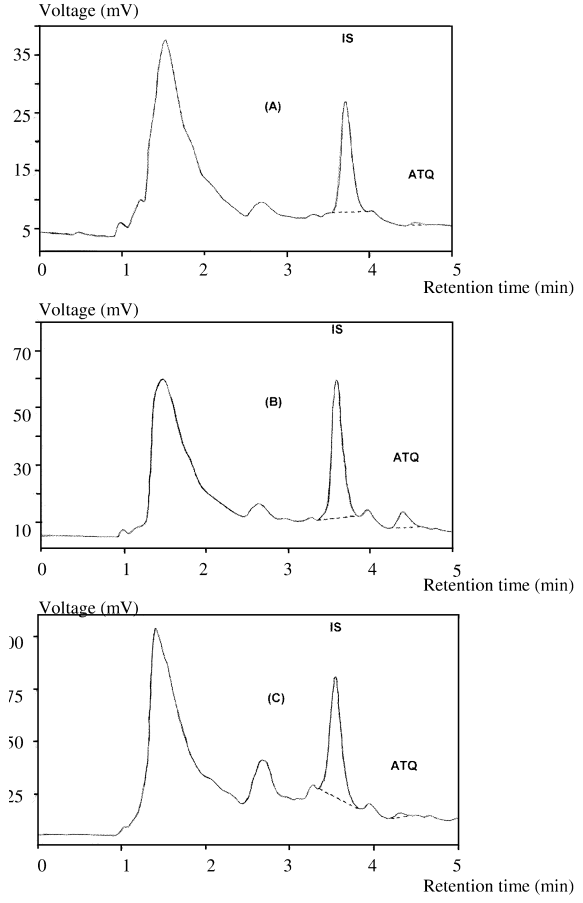

Fig. 3. Chromatograms from capillary blood after a single dose of Malarone® (250 mg ATQ). One hour after dose (A), 3 h after dose (B) and 7 h after dose (C). For SPE and HPLC conditions see Experimental. The figures besides the axis are retention times in minutes and responses in mV.

accurate, reproducible, selective and will be very suitable for field studies of ATQ.

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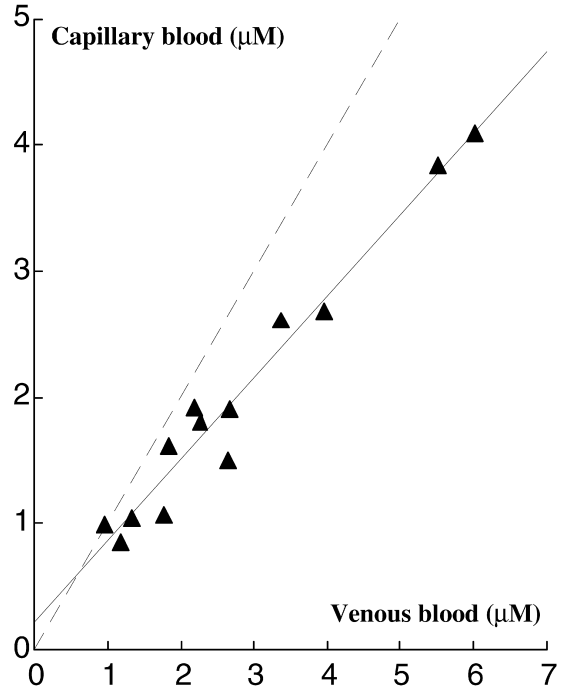

Fig. 4. Relationship between simultaneously sampled capillary blood applied onto sampling paper and venous blood concentrations of ATQ. Full drawn line: Correlation. Dashed line: $y=x$.

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