ELSEVIER

Journal of Chromatography B, 744 (2000) 9–17

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Automated solid-phase extraction method for the determination of atovaquone in plasma and whole blood by rapid high-performance liquid chromatography

N. Lindegårdh[a], Y. Bergqvist[a,b,*]

[a]*Dalarna University College, S-781 88 Borlänge, Sweden*

[b]*Unit of Tropical Pharmacology, Division of Clinical Pharmacology, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden*

Received 14 October 1999; received in revised form 29 February 2000; accepted 22 March 2000

Abstract

A bioanalytical method for the determination of atovaquone in plasma and whole blood by solid-phase extraction and high-performance liquid chromatography has been developed and validated. A structurally similar internal standard was added and protein was precipitated from plasma and whole blood with acetonitrile before being loaded on to a C_8 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 precisions for plasma and whole blood were 2.2% and 1.9% respectively at 12 μ*M* and 6.0% and 5.6% respectively at 0.75 μ*M*. The inter-assay precisions for plasma and whole blood were 1.4% and 2.1% respectively at 12 μ*M* and 4.9% and 3.4% respectively at 0.75 μ*M*. The lower limit of quantification in plasma and whole blood were 150 n*M*. The limit of detection in plasma and whole blood were 30 n*M*.

Keywords: Atovaquone

1. Introduction

Atovaquone (ATQ), a hydroxynaphthoquinone, is a highly lipophilic potent inhibitor of various protozoa. 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 antimalarial compound, Proguanil [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]. ATQ is more than 99.9% bound to plasma proteins [3,4]. ATQ works by binding to a polypeptide in the mitochondrial respiratory chain, thereby blocking electron transport in the dihydroorotate dehydrogenase and inhibiting the pyrimidine biosynthetic pathway. Since the malaria parasites are unable to salvage preformed pyrimidines, which humans can, this leads to blocking of the parasite-replication [5]. Several clinical trials for malaria

*Corresponding author. Tel.: +46-23-778843; fax: +46-23-778050.

E-mail address: ybq@du.se (Y. Bergqvist)

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00221-8

treatment/prophylaxis have been conducted and therapeutic plasma concentrations have been about 5 μg/ml≈14 μ*M* [1].

Traditionally, the extraction of ATQ from plasma has mainly been performed by liquid–liquid extraction techniques and HPLC [6–8], which is quite time consuming. A protein precipitation-HPLC method has recently been published [9] but the method showed low precision because of possible HPLC column degradation due to protein not removed in the precipitation step. Extraction of ATQ from whole blood has never been published.

The aim of this work was to develop and validate an automated solid-phase extraction (SPE)-HPLC method for the assay of ATQ in plasma and whole blood. The assay has been validated with respect to accuracy, precision, linearity, selectivity, stability and recovery according to Ref. [10]. The automated SPE-HPLC method shows excellent precision, it is rapid and simple and would save a lot of time for analytical laboratories.

2. Experimental

2.1. Chemicals and materials

ATQ, 566C80, and the internal standard (I.S.), 59C80, *trans*-2-hydroxy-3-(4-phenylcyclohexyl)-1,4-naphthalenedione, were obtained from Glaxo Wellcome (Hertfordshire, UK). The structures are shown in Fig. 1. Acetonitrile (HPLC-grade), methanol (pro analysi) and dimethylformamide (pro analysi) were obtained from Merck (Darmstadt, Germany).

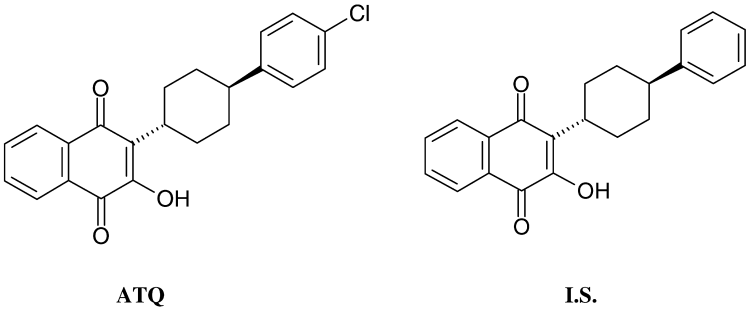
Deionised water was obtained from a Milli-Q deionised water system (Millipore, Bedford, MA, USA). The phosphate buffer solutions (0.01 *M*) were prepared by mixing different amounts of potassium hydroxide and potassium-dihydrogen-phosphate with Milli-Q deionised water.

2.2. Instrumentation and chromatographic conditions

The HPLC system used in the assay consisted of a LKB 2150 HPLC pump (Pharmacia Biotech, Uppsala, Sweden) and a Gilson 231 injector with Dilutor 401 (Gilson, Middleton, WI, USA). The detector was a Spectroflow 757 Absorbance detector (Kratos, Ramsey, NJ, USA) set at 277 nm. Data acquisition was performed using Chromatography station for windows 1.7 (DataApex Ltd, Prague, The 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)



ATQ **I.S.**

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

using C_8 extraction columns containing octyl silica as support (1 ml, 100 mg, IST, Hengoed, Glamorgan, UK). The ASPEC system uses a positive air pressure instead of vacuum to get the liquids through the columns.

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

Concentrated stock solutions of ATQ and I.S. (500 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 plasma and whole blood to yield spiked calibration standards ranging from 0.25 to 15 μ*M*. The calibration standards were prepared in batches of 20 ml and stored in 0.5 ml aliquots at about −17°C. Calibration curves were prepared using 7 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 precision in plasma and whole blood were prepared at three concentrations (0.75, 3, 12 μ*M*) in batches of 20 ml and stored in 0.5 ml aliquots 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. Plasma samples

To 500 μl plasma, 500 μl I.S. (15 μ*M*) in acetonitrile were added and the tube contents were mixed for 10 seconds. The tubes were then left to stand for 10 min followed by centrifugation at 13 400 *g* for 10 min. The liquid phase was decanted into new tubes, 3000 μl phosphate buffer (0.01 *M*, pH 9) were added and all of it were then loaded on to a SPE column. The extraction procedure for plasma on the ASPEC XL is shown in Table 1. One hundred μl of the eluate were injected into the HPLC system

2.4.2. Whole blood samples

To 500 μl whole blood, 500 μl Milli-Q deionised water were added and the tubes were shaken gently for about 2 min to achieve haemolysis of the blood cells. After that, 1000 μl I.S. (7.5 μ*M*) in acetonitrile were added and the tube contents were mixed for 10 s. The tubes were then left to stand for 10 min, followed by centrifugation at 13 400 *g* for 10 min. The liquid phase was decanted into new tubes, 4000 μl phosphate buffer (0.01 *M*, pH 9) were added and all of it were then loaded on to a SPE column. The extraction procedure for whole blood on the ASPEC

Table 1
ASPEC procedure for the extraction of plasma and whole blood

SPE-step	Liquid dispensed	Dispensing volume (μl)	Dispensing flow-rate (ml/min)	Pressuring air volume (μl)	
				Plasma	Whole blood
Conditioning	Methanol	2000	3	200	200
	Buffer 0.01 *M*, pH 9	2000	3	599	500
Sample	Plasma	4000[a]	1	700	–
Loading	Whole blood	6000[a]	1	–	700
Washing	Buffer 0.01 *M*, pH 9	2000	3	0	0
	Methanol: Buffer 0.01 *M*, pH 9 (30:70, v/v)	2000	2	500	500
Elution	Acetonitrile: Buffer 0.01 *M*, pH 7 (70:30, v/v)	300	1	0	0
	Acetonitrile: Buffer 0.01 *M*, pH 7 (70:30, v/v)	300	1	500	700

[a] The volume after protein precipitation 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.

XL is shown in Table 1. One hundred μl of the eluate were injected into the HPLC system

2.5. Assay validation

2.5.1. SPE elution profile

To obtain the most effective washing- and elution-solvent composition, an elution profile for methanol was made. One ml ATQ at 10 μ*M* in phosphate buffer (0.01 *M*, pH 9) was loaded on to a SPE column and then washed with deionised water. Eluted fractions with an increasing content of methanol were collected and analysed. The cumulative relative value was plotted against the content of methanol.

2.5.2. Protein precipitation

ATQ is more than 99.9% protein bound [3,4] and it is necessary to precipitate the proteins to achieve reasonable recovery. Spiked ATQ samples at 5 μ*M* in a mixture of phosphate buffer (pH 9)/plasma were processed both with and without protein precipitation with acetonitrile. 500 μl samples with phosphate buffer/plasma, were mixed to achieve a concentration of buffer in the samples ranging from 0 to 100% in steps of ten.

2.5.3. Accuracy, intra- and inter-assay precision

The accuracy and precision of the method was estimated by analysis of spiked plasma and whole blood at three concentrations (0.75, 3, 12 μ*M*) in triplicate for five days. Concentrations were determined using a calibration graph prepared on the day of analysis. Intra- and inter-assay precisions were calculated. Accuracy in plasma was also determined by cross-validation with an earlier published HPLC method [9].

Nine samples in duplicate and five calibration samples were processed with both methods.

2.5.4. Linearity

Calibration curves were constructed using seven 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.

2.5.5. Extraction recovery

The extraction recovery was determined from the plasma and whole blood samples used for the precision study. Three concentrations (0.75, 3, 12 μ*M*) in triplicate were analysed for five days. The recovery of extracted samples was compared with a dilution in the same way in mobile phase as the actual dilution during the extraction. The non-extracted samples were injected directly into the HPLC system. Peak heights of the non-extracted samples were compared to the SPE samples from plasma and whole blood.

2.5.6. Stability

Both ATQ and I.S. have earlier shown good stability in plasma [8,11]. The stability in acetonitrile:phosphate buffer solutions has never been investigated. Eight ml spiked plasma at an ATQ and I.S. concentration of 10 μ*M* was portioned into 16 aliquots, which were loaded on to the SPE columns. Eight of the 16 eluates (acetonitrile:phosphate buffer, 0.01 *M*, pH 9 (70:30, v/v)) were kept at about 20°C and the rest at about −17°C. One sample from each group was analysed simultaneously during a five day time period.

2.5.7. Selectivity

Blank plasma and whole blood from six different healthy volunteers were analysed, and the chromatograms were examined for endogenous compounds that could interfere with the quantification of ATQ. Several antimalarials, e.g. chloroquine, mefloquine, pyrimethamine, sulphadoxine, proguanil, 4-chlorophenylbiguanide, cycloguanil, amodiaquine and pyronaridine were injected directly into the HPLC system, and the chromatograms were examined to establish that their retention times would not interfere with those of ATQ and I.S.

2.5.8. Quality control

Quality control (QC) samples in plasma were prepared at three concentrations (0.75, 3, 12 μ*M*) in batches of 20 ml and stored in 0.5 ml aliquots at about −17°C. The QC samples were analysed together with clinical plasma samples in every assay

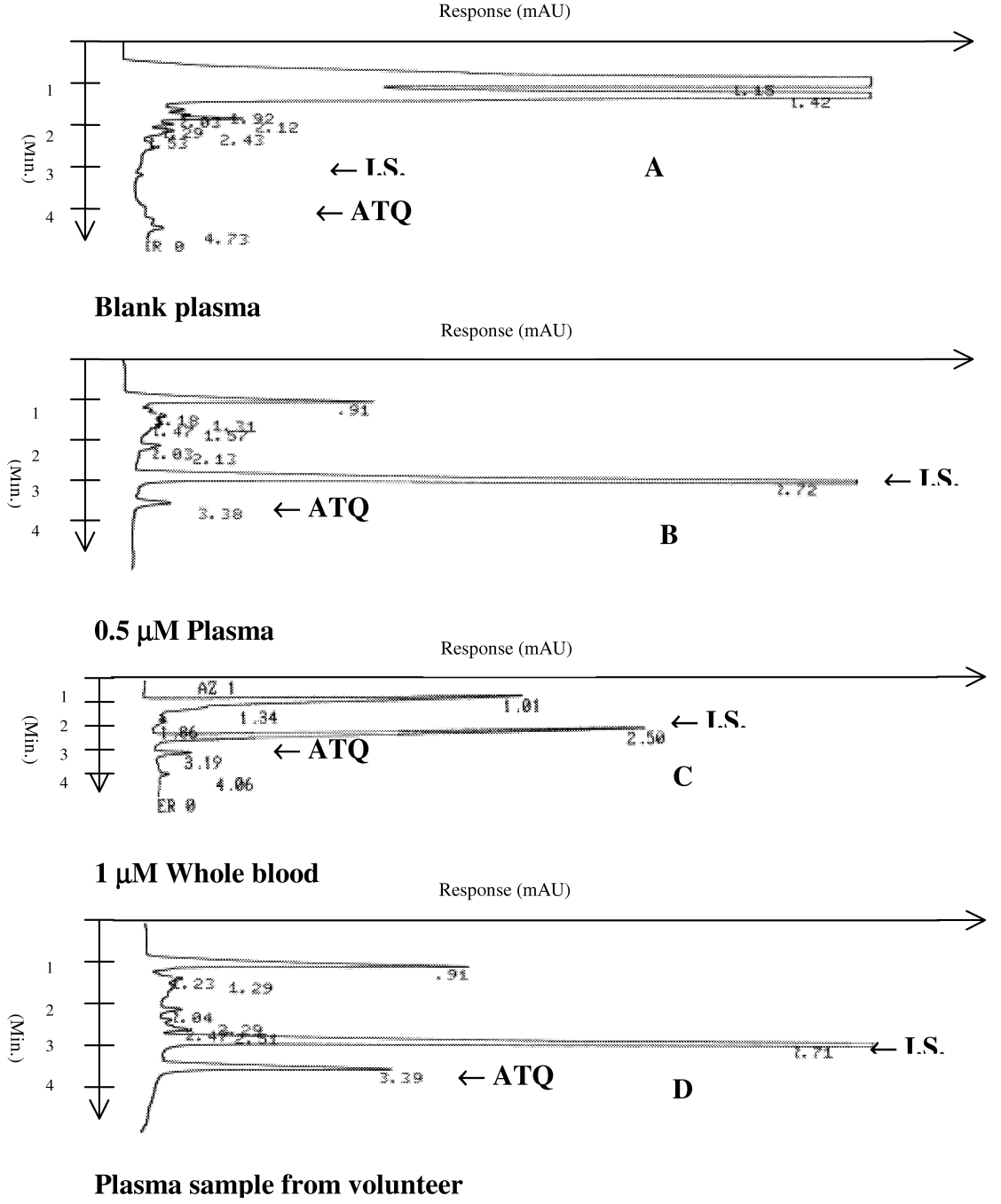


Fig. 2. Chromatograms from blank plasma, plasma spiked with ATQ (0.5 μ*M*), whole blood spiked with ATQ (1.0 μ*M*) and plasma from a patient who received a tablet of Malarone® (250 mg ATQ). For SPE and HPLC conditions see Experimental Section. The figures beside the peaks are retention times in minutes and responses in mAU.

run over a period of two months in a clinical pharmacokinetic study of ATQ and proguanil.

3. Results and discussion

ATQ is highly lipophilic and almost insoluble in water (<0.3 μM) [12]. Thus, absorption in man is greatest when the drug is taken with high-fat foods [3,12]. The manufacturer states that ATQ is extensively (99.9%) bound to plasma proteins [4]. Studies have been done which confirm the fact that ATQ would not be metabolised in humans. It has also been shown that ATQ has negligible excretion in urine (<0.6%) because of the very high plasma protein binding level [3,4,13]. The structure of ATQ (Fig. 1) includes a hydroxyl group that acts as a weak acid with a calculated pK_a≈5.0 [14]. Retention times of ATQ and I.S. depend particularly upon pH and acetonitrile content of the mobile phase. Thus, increase of 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 4 min and a very good separation between ATQ, I.S. and endogenous compounds as well as other common antimalarials in the solvent front. Fig. 2 shows HPLC chromatograms from blank plasma, plasma spiked with ATQ (0.5 μM), whole blood spiked with ATQ (1.0 μM) and plasma from a patient who received a tablet of Malarone® (250 mg ATQ).

3.1. SPE elution profile

Several different SPE reversed-phase columns (C_2, C_4, C_6, C_8, C_{18}) were tested to achieve high recoveries and clean chromatograms. The C_8 column gave acceptable recoveries with clean chromatograms and no endogenous interferences.

The cumulative relative value in the eluted fractions was plotted against the content of methanol (Fig. 3). It is preferable to have as strong a washing solvent as possible, and as weak an elution solvent as possible to minimize interferences. As seen in Fig. 3 it is possible to wash with at least 30% methanol without losing recovery. The minimal concentration of methanol that is required for maximum elution of ATQ is about 60% methanol in Milli-Q water. Since the HPLC column contains octadecyl silica phase that is more retentive than the octyl silica phase in the SPE column, a slightly modified HPLC mobile phase is very suitable to use as an elution solvent.

3.2. Protein precipitation

ATQ has a very strong binding to plasma proteins. To obtain higher recovery in the SPE extraction, it was necessary to precipitate the proteins. A significant decrease in recovery from 100% to about 10% was seen for the samples without protein precipitation. Protein precipitation with 50% acetonitrile increased the recovery to about 80% for the samples containing plasma solely. This shows us how im-

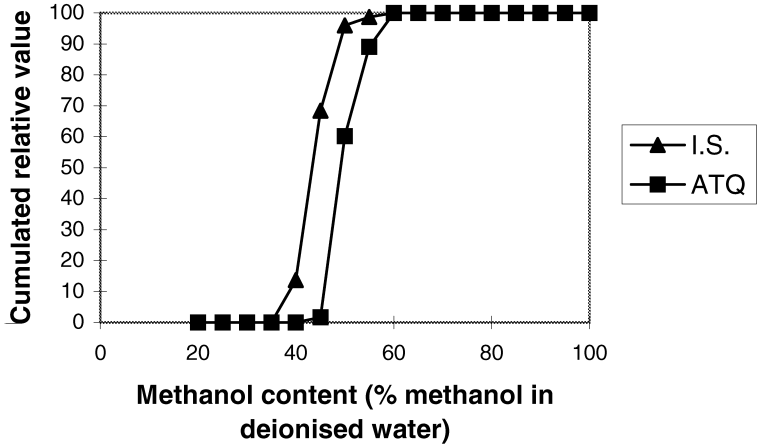


Fig. 3. Elution profile for ATQ and I.S. on an IST C_8 column.

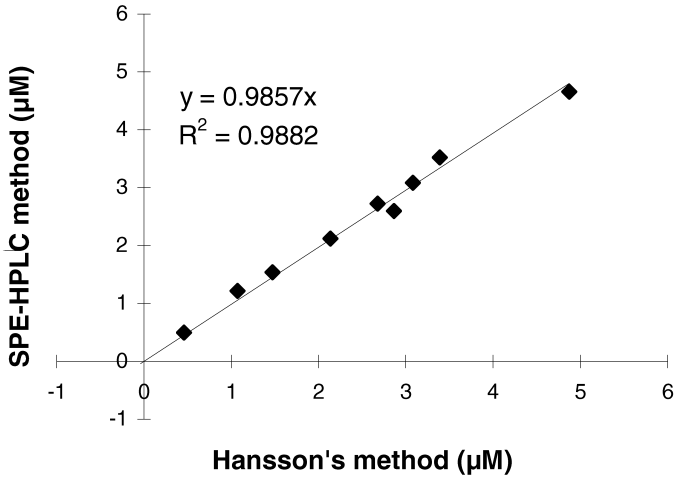


Fig. 4. Cross-validation accuracy test between an earlier published HPLC-method i.e. Hansson et al. [9] (*x*) and the presented SPE-HPLC method (*y*).

portant it is to have calibrators spiked in the same matrix as the samples.

3.3. Accuracy, intra- and inter-assay precision

The results from the cross-validation accuracy test are presented in Fig. 4. Both HPLC methods gave similar results. The precision and accuracy of the assay is summarized in Table 2. At the LOQ 150 n*M*, the signal-to-noise ratio was greater than 10:1 and intra- and inter-assay precisions were less than 15% for both plasma and whole blood. At the LOD 30 n*M*, the signal-to-noise ratio was greater than 3:1. These limits could easily be decreased since only

Table 2
Accuracy, intra- and inter-assay precision for the determination of ATQ in plasma and whole blood

	Added (µ*M*)	mean±SD	Plasma			Whole blood	
			RSD (%)	% Deviation (found versus added)	mean±SD	RSD (%)	% Deviation (found versus added)
Intra-assay (*n*=15)	0.75	0.68±0.04	6.0	−9.3	0.82±0.05	5.6	9.3
	3	2.94±0.05	1.7	−2.0	3.19±0.11	3.4	6.3
	12	11.97±0.26	2.2	−0.3	12.34±0.23	1.9	2.8
Inter-assay (*n*=5)	0.75		4.9			3.4	
	3		1.5			3.4	
	12		1.4			2.1	

Table 3
Mean parameters of the calibration curves for ATQ

	$y = mx + b$		Correlation coefficient (r±SD)
	m (mean±SD)	*b* (mean±SD)	
Plasma (*n*=6)	0.0686±0.0005	−0.007±0.004	0.999±0.006
Whole blood (*n*=4)	0.0599±0.0009	−0.0003±0.0072	0.999±0.012

Table 4
Recovery of ATQ and I.S. from whole blood and plasma ($n=12$)

	Concentration (μM)	Whole blood (mean±SD) (%)	Plasma (mean±SD) (%)
ATQ	0.75	98.2±5.5	91.3±7.6
	3	90.1±5.3	83.7±3.5
	12	89.2±2.8	81.1±1.4
I.S.	10	85.7±4.6	81.4±3.0

100 μl of the eluate from the SPE was injected. Indeed, the therapeutic range of ATQ in plasma is about 10–14 μM [1].

3.4. Linearity

Linear calibration curves were obtained with correlation coefficients (r) greater than 0.99; mean calibration curve parameters are reported in Table 3.

3.5. Recovery

The absolute recovery is presented in Table 4. The recoveries of ATQ and I.S. from plasma were between 80 and 92% and from whole blood were between 85 and 98%. We have tested different protein precipitation solvents according to [15]. Since ATQ is a highly lipophilic weak acid, we cannot use acid precipitation due to co-precipitation of ATQ.

3.6. Stability

There were no significant changes (<5%) in the measured peak heights for the analytes (10 μM) in the SPE-eluate stored at about 20°C compared to those stored at about −17°C. It was shown that the analytes were stable in the SPE-eluate at about 20°C for at least 120 h. Both ATQ and I.S. have earlier shown good stability in plasma including freeze–thaw stability [8,11].

3.7. Selectivity

None of the analysed antimalarial drugs interfered with the retention times of the analytes. No endogenous interference from plasma or whole blood was observed. A typical HPLC chromatogram from blank plasma is presented in Fig. 2.

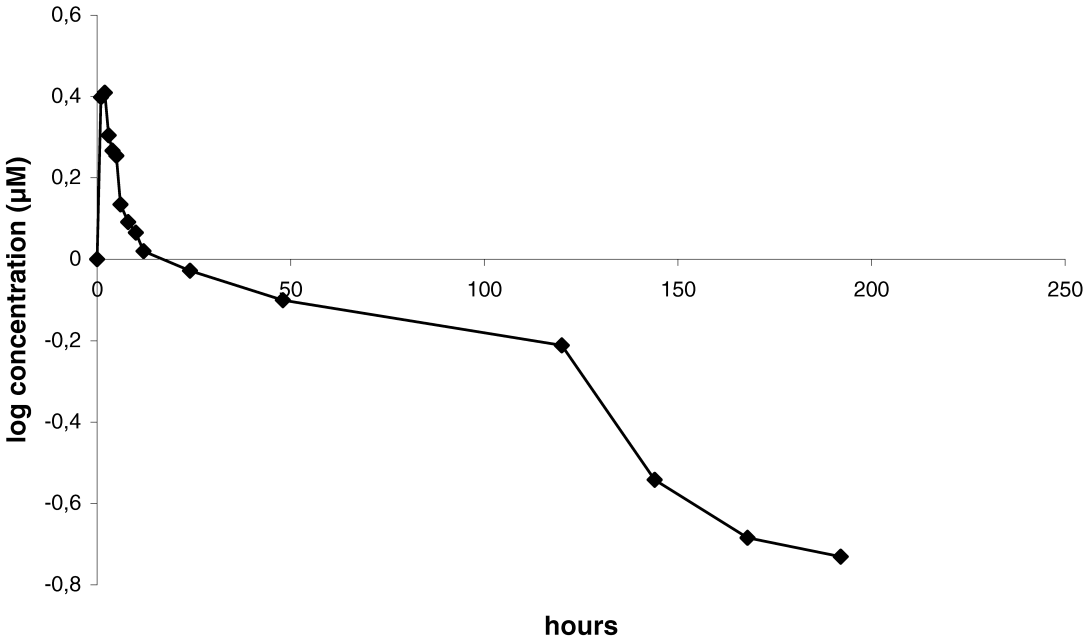


Fig. 5. Plasma concentration of ATQ as a function of time after a single oral dose of Malarone® (250 mg ATQ) to a healthy volunteer.

Table 5
Quality control in a pharmacokinetic study of ATQ in plasma ($n=15$)

Concentration ATQ (μM)	Mean±SD	RSD (%)
0.75	0.82±0.05	5.1
3	2.92±0.08	2.9
12	11.78±0.17	1.5

3.8. Quality control

The presented method was applied to the determination of ATQ in twelve healthy volunteers in a clinical pharmacokinetic study. The concentration of ATQ in plasma as a function of time for one of the volunteers after a single oral dose of Malarone® (250 mg ATQ) is shown in Fig. 5.

The precision results from the QC samples analysed together with the clinical plasma samples are presented in Table 5. The precision results give an estimation of the clinical applicability of the presented method.

4. Conclusion

A validated method using SPE and HPLC for the determination of ATQ in plasma and whole blood 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. less than 4 min. The assay has proven to be accurate, reproducible, selective and applicable to clinical pharmacokinetic studies. The SPE procedure provides improved sample clean-up and permits greater selectivity in the assay of ATQ.

Acknowledgements

Thanks to Dalarna University College, Sweden for supporting this investigation. We gratefully acknowledge the suggestions of Dr. Anders Björkman, Karolinska Hospital, during the preparation of this manuscript.

References

[1] A. Sabchareon, P. Attanath, P. Phanuaksook, P. Chanthavanich, Y. Poonpanich, D. Mookmanee, T. Chongsuphajaisiddhi, B.M. Sadler, Z. Hussein, Trans. R. Soc. Trop. Med. Hyg. 92 (1998) 201.
[2] W.T. Hughes, V.L. Gray, W.E. Gutteridge, V.S. Latter, M. Pudney, Antimicrob. Agents Chemother. 34 (1990) 225.
[3] R.J. Artymowicz, V.E. James, Clin. Pharm. 12 (1993) 563.
[4] GlaxoWellcome, Wellvone product information (1999), Gothenburg, Sweden.
[5] M. Fry, M. Pudney, Biochemical Pharmacol. 43 (1992) 1545.
[6] D.V. DeAngelis, J.D. Long, L.L. Kanics, J.L. Woolley, J. Chromatogr. B 652 (1994) 211.
[7] S.L. Hannan, G.A. Ridout, A.E. Jones, J. Chromatogr. B 678 (1996) 297.
[8] S.D. Studenberg, J.D. Long, J.H. Woolf, C.J. Bruner, D. Wilson, J.L. Wooley, J. Pharma. Biomed. Anal. 13 (1995) 1383.
[9] A.G. Hansson, S. Mitchell, P. Jatlow, P.M. Rainey, J. Chromatogr. B 675 (1996) 180.
[10] R. Causon, J. Chromatogr. B 689 (1997) 175.
[11] D.V. DeAngelis, J.D. Long, L.L. Kanics, J.L. Woolley, J. Chromatogr. Biomed. Appl. 652 (1994) 211.
[12] P.E. Rolan, A.J. Mercer, B.C. Weatherley, T. Holdich, H. Meire, R.W. Peck, G. Ridout, J. Posner, Br. J. Clin. Pharmac. 37 (1994) 13.
[13] P.E. Rolan, A.J. Mercer, E. Tate, I. Benjamin, J. Posner, Antimicrob. Agents Chemother. 41 (1997) 1319.
[14] ACD/pKa DB, version 1.2, Advanced Chemistry Development Inc., Toronto, Ontario, Canada.
[15] R.D. McDowall, J. Chromatogr. 492 (1989) 3.