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Determination of the potent antiprotozoal compound atovaquone in plasma using liquid–liquid extraction followed by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A specific and robust method is presented for the determination of atovaquone in plasma. Atovaquone is a potent antiprotozoal compound for use in immunocompromised patients who are intolerant of conventional therapies. The method involves a liquid–liquid extraction of the compound into hexane modified with 2% (v/v) isoamyl alcohol. The processed extracts are analysed by reversed-phase high-performance liquid chromatography with ultraviolet detection at 254 nm. The assay has a limit of quantification of 0.1 μg/ml and is linear between 0.1 and 50 μg/ml. The method has been applied to many clinical studies and has been demonstrated to be precise and accurate with high sample throughput. Atovaquone is not significantly metabolised in humans.

Keywords: Atovaquone

1. Introduction

Atovaquone (2-[trans-4-(4′-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone, Fig. 1) is one of a series of 2-substituted hydroxynaphthoquinones and was initially developed as a treatment for malaria. The action of these compounds involves inhibition of the mitochondrial electron transport cytochrome complex bc_1 which is linked to pyrimidine biosynthesis via ubiquinone [1]. The in vitro biological activity of atovaquone indicated that plasma concentrations of 100 ng/ml would be sufficient for efficacy as an anti-malarial treatment and for this reason a sensitive quantitative analytical method for determining concentrations in plasma was required. This method involved liquid–liquid extraction of acidified plasma followed by derivatization and capillary gas chromatography (GC) with electron capture detection (ECD) [2].

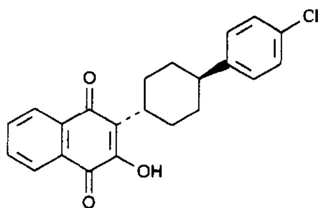

Fig. 1. Structure of atovaquone.

Atovaquone was subsequently shown, in vitro, to be active against other clinically important protozoa,

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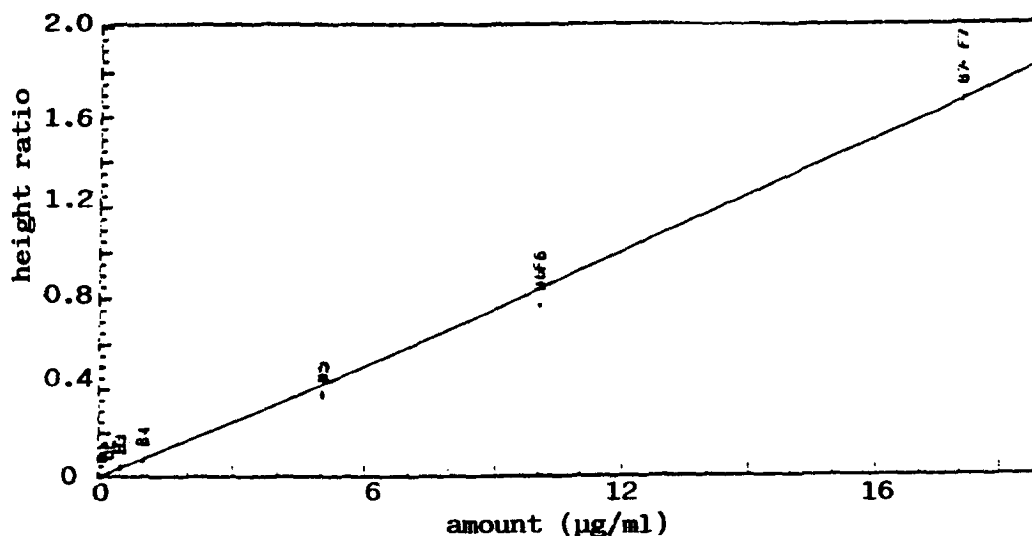

Fig. 2. Calibration curve. Curve fit, linear weighted $1/x$;, intercept, 0.0056; gradient, 0.0285; coefficient of determination, 0.9993.

including *Pneumocystis* and *Toxoplasma* [1,3,4]. Efficacy against *P. carinii* pneumonia (PCP), an opportunistic infection common in acquired immune deficiency syndrome (AIDS), has also been demonstrated in human immunodeficiency virus (HIV)-infected men, who show few adverse effects with this treatment [5,6]. The plasma concentrations required to achieve efficacy in PCP are in the tens of μg/ml compared to 100 ng/ml for effective treatment of malaria. Hence, the limit of quantification required to support development of this drug for its PCP indication was 100 ng/ml, rather than 10 ng/ml for malaria. For this reason the greater sensitivity of the GC method was no longer required and a rapid and simpler method for quantitation in plasma was developed using high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Chemicals

Atovaquone and 59C80 [*trans*-2-hydroxy-3-(4-phenylcyclohexyl)-1-4,naphthoquinone, internal standard] are products of The Wellcome Foundation (Dartford, UK).

Acetic acid, ammonium acetate, dimethyl formamide (DMF), dimethyldichlorosilane [a 2% (v/v) solution in 1,1,1-trichloroethane], ethyl acetate and formic acid (all AR grade) and trifluoroacetic acid (TFA, Spectrosol grade) were obtained from BDH (Poole, UK). Acetonitrile, hexane and methanol (all HPLC grade) were obtained from Rathburn Chemicals (Walkerburn, UK) and isoamyl alcohol (IAA), (98%, v/v), was obtained from Sigma (Gillingham, UK).

2.2. Apparatus

The equipment consisted of a modular HPLC system including an autosampler (WISP Model 710B, Waters, Millipore, MA, USA) a single pump (Model 880-PU, Jasco, Great Dunmow, UK) and a variable-wavelength UV detector (Spectroflow Model 773, Kratos Analytical, Manchester, UK). A VG Multichrom Data Acquisition System (VG Data Systems, Altringham, UK) was used for calculation of results.

2.3. Chromatography

The chromatographic system consisted of a single stainless steel guard cartridge (10 × 2.1 mm I.D.,

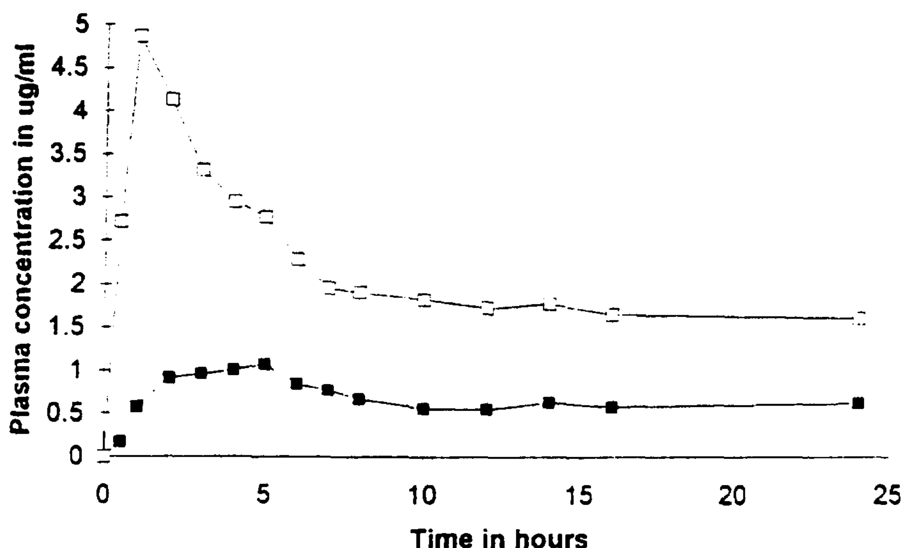

Fig. 3. Mean plasma atovaquone concentrations from a study into the effect of particle size on absorption in healthy male volunteers. ■ = 250 mg of standard formulation suspension; □ = 250 mg of fluid formulation suspension.

with mixed reversed-phase packing, Chrompack, London, UK) and two analytical Chromspher C_8 cartridge columns (100 × 3 mm I.D., 5 μm particle size, Chrompack) contained within a single cartridge column-housing. Chromatographic analyses were performed at ambient temperature with a mobile phase of acetonitrile and 0.4% (v/v) aqueous TFA (70:30, v/v) at a flow-rate of 0.6 ml/min. The UV detection wavelength used was 254 nm and sample aliquots of 10 μl were injected onto the chromatographic system.

2.4. Preparation of standard solutions

Atovaquone (10 mg) was accurately weighed and dissolved in 50 ml of 1% (v/v) DMF in methanol (DMF–methanol) to give a primary stock solution having a nominal concentration of 200 μg/ml. Dilutions of this were made with DMF–methanol to give working solutions of approximately 50, 10 and 2.5 μg/ml, respectively. The 59C80 internal standard solution was prepared by dissolving 59C80 in DMF–methanol to give a nominal concentration of

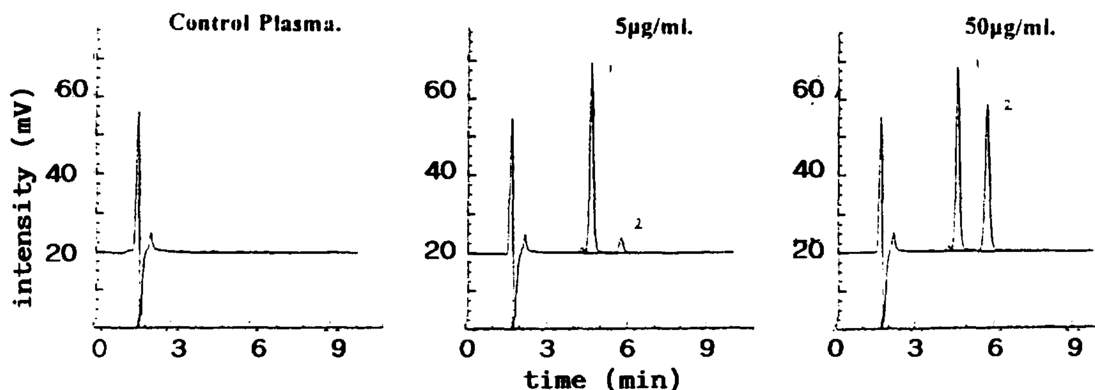

Fig. 4. Typical chromatograms of extracted control and test plasma. Peaks: 1 = 59C80 internal standard; 2 = atovaquone.

200 μg/ml. These solutions are stable for 5 weeks when stored at +4°C.

2.5. Preparation of plasma samples

To prevent analyte adsorption to glassware, all glass tubes were treated with dimethyldichlorosilane prior to use. Calibration samples in the range of 0.1 to 50 μg/ml plasma were prepared by the addition of appropriate volumes of atovaquone stock solutions into glass tubes. The solvent was removed by evaporation under a stream of nitrogen, the internal standard was added (20 μl) followed by the addition of drug free plasma (0.5 ml). For study samples and for quality control samples, 0.5 ml of plasma was placed in glass tubes and 20 μl of internal standard was added. All samples were then vortex-mixed for 10 s and acetic acid (0.5 ml, 0.5 *M*) and IAA in hexane (5 ml, 2%, v/v) was added. Samples were mixed on a flatbed shaker for 1 h and the aqueous and organic phases separated by centrifugation (3000 *g*, 10 min). The organic supernatant was transferred to a glass centrifuge tube and evaporated, with heating at 60°C, under a stream of nitrogen. The dry residue was resuspended with 200 μl of methanol and transferred to autosampler vials ready for analysis.

2.6. Calibration

The results were calculated from the ratio of the peak heights of atovaquone and its internal standard, 59C80. Linear regression ($y = mx + C$) with a weighting factor of $1/x$ was used. The weighting factor reduces the residuals of the lower concentrations and improves the inter-day reproducibility at these concentrations.

3. Results and discussion

3.1. Precision and accuracy

The intra-day precision of the method was determined by replicate analyses ($n = 6$) of drug free plasma spiked at nominal atovaquone concentrations of 0.1, 0.2, 1, 5, 20 and 50 μg/ml. Concentrations were interpolated from the resulting weighted ($1/x$) linear regression graph ($y = 0.03857x + 0.00095$). A summary of the results obtained is presented in Table 1. These results show that the method is both precise and accurate over the full dynamic range tested. During operational use the calibration range is often restricted to the expected concentrations of the unknowns. For single doses of atovaquone the upper concentration is normally 20 μg/ml and for multiple dosing 50 μg/ml with a raised lower concentration of 1 μg/ml. Samples exceeding 50 μg/ml are diluted to the desired range using control plasma. Most batches of control plasma contain a minor peak which co-chromatographs with atovaquone and results in a small positive intercept on the calibration curve Fig. 2.

Table 1
Intra-assay precision

Atovaquone (μg/ml)	n	Mean interpolated value (μg/ml)	Standard deviation (μg/ml)	C.V. (%)	Bias (%)
0.095	6	0.083	0.0101	12.1	−12.5
0.191	5	0.169	0.0167	9.9	−11.4
0.953	5	0.987	0.0473	4.8	+3.5
4.764	5	5.437	0.5701	10.5	+14.1
19.85	6	21.871	1.5322	7.0	+10.0
51.610	5	48.962	2.0796	4.3	−5.1

The inter-day precision and accuracy were estimated by replicate analyses ($n = 3$) carried out on consecutive days. These results are a combination of both the intra- and inter-day precision and are presented in Table 2.

This method has been used to analyse plasma samples from many phase I/II clinical studies during which a large number of quality control samples were also processed. A summary of the quality control results obtained from these studies is presented in Table 3. These results were obtained from more than 200 assays and provide the most conclusive gauge of the combined intra-/inter-assay precision and accuracy of the method.

3.2. Stability

Quality control samples spiked with atovaquone were shown to be stable for at least three months when stored at −20°C. Atovaquone was also shown

Table 2
Inter-assay precision and accuracy

Day	Low (0.105 μg/ml)	Medium (4.921 μg/ml)	High (49.21 μg/ml)
1	0.100	5.053	47.779
	0.122	5.261	49.757
	0.127	5.107	48.133
2	0.125	5.237	47.400
	0.125	5.089	49.607
	0.111	5.005	48.603
3	0.141	5.231	47.647
	0.126	5.154	50.374
	0.114	4.616	52.356
Mean	0.121	5.084	49.076
S.D.	0.0116	0.1963	1.6110
C.V. (%)	9.6	3.9	3.3
Bias (%)	+15.4	+3.3	−0.3

to be stable in human plasma after inactivation of HIV and hepatitis B (HBV) viruses in human plasma, by dilution with phosphate buffered saline (1:5, v/v) followed by heat treatment (60°C for 18 h). The compound is extremely stable and clinical samples have been transported frozen from many clinical centres throughout the world without degradation.

3.3. Application

One application of the method is illustrated in Fig. 3 which shows the plasma profiles obtained from two formulations tested in a study to determine the effect of particle size on absorption of atovaquone after oral administration to healthy male volunteers []. In addition, typical chromatograms of control and test human plasma samples are shown in Fig. 4. With increasing clinical trials in 'high risk' groups i.e. HIV and HBV sero positive subjects, plasma heat treatment is carried out at the Clinical Study Centre or once they have been received at these laboratories.

The described method has been used to analyse a large number of samples effectively and efficiently. The extracts are clean, ensuring good chromatography, minimal maintenance and chromatographic processing. The method is simple and robust and has been successfully transferred to other laboratories.

Table 3
Operational precision and accuracy from compiled quality control data

QC concentration (μg/ml)	*n*	Mean (μg/ml)	C.V. (%)	Bias (%)
0.50	22	0.509	6.7	+1.5
0.82	72	0.858	18.2	+4.3
1.00	87	1.110	16.8	+11.0
5.00	49	5.180	13.1	+3.6
5.00	32	4.750	12.5	−5.0
5.01	21	5.746	5.7	+14.7
5.03	27	5.100	13.7	+1.4
10.00	48	9.627	15.6	−3.7

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