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High-performance liquid chromatographic assay for the measurement of atovaquone in plasma

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Abstract

A rapid and efficient isocratic high-performance liquid chromatographic assay for the measurement of atovaquone in plasma has been developed and validated. The drug was extracted from plasma with organic solvents, assayed on a C_1 column with a mobile phase of methanol–0.1% acetic acid (70:30, v/v), and detected by ultraviolet absorbance at 254 nm. Recovery of atovaquone from plasma was greater than 85%. Intra- and inter-assay variability were less than 8%, and the average accuracy of the assay (expressed as % bias) ranged from −7.4 to +2.2%. The upper and lower limits of quantitation were 100 and 0.25 μg/ml, respectively. Measurement of atovaquone in spiked plasma control samples during routine runs of clinical trial samples confirmed the reliability of the assay.

1. Introduction

The hydroxynaphthoquinone, atovaquone (*trans* - 2 - [4 - (4 - chlorophenyl) - cyclohexyl] - 3 - hydroxy - 1,4 - naphthalenedione, 566C80), is a potent, selective inhibitor of a variety of medically important protozoa, including *Pneumocystis carinii*, *Toxoplasma gondii*, and *Plasmodium* species [1]. Atovaquone is structurally related to ubiquinone and is thought to act at the cytochrome bc_1 (complex III) site of the mitochondrial electron transport chain by indirectly inhibiting several metabolic enzymes linked via ubiquinone [2]. The compound was initially under development as an antimalarial compound [3,4], but its activity against *P. carinii* and *T. gondii* in *in vitro* models [5,6] and in experimentally induced *P. carinii* pneumonia (PCP) infections in rats [1] prompted further development for therapy for PCP and toxoplasmosis infections in immunocompromised (especially AIDS) patients. Phase I, I/II, and III clinical trials have been completed [7–10], and atovaquone was shown to be equivalent to trimethoprim/sulfamethoxazole in overall therapeutic outcome against PCP [11]. The phase III trial also demonstrated that the incidence of therapeutic success with atovaquone was directly correlated with steady-state plasma concentrations of the compound [11].

The structure of atovaquone is shown in Fig. 1; it is a yellow crystalline solid with a molecular

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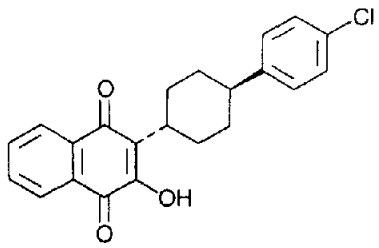

Fig. 1. Chemical structure of atovaquone.

mass of 366.84. It is poorly soluble in a variety of organic solvents, and its aqueous solubility is <0.2 μg/ml. Atovaquone is highly lipophilic and, like many other hydroxynaphthoquinones, is poorly bioavailable in experimental animals and humans [12]. A reliable, accurate, sensitive, and specific analytical method for the quantitation of atovaquone in plasma was required both for determining plasma concentrations in preclinical pharmacology, toxicology, and disposition studies and for defining therapeutic concentrations in clinical trials.

In the early stage of the compound's development, a gas chromatographic method for measuring plasma concentrations of atovaquone was devised and reported [13]. Because of the number of manipulations involved in the extraction and derivatization steps, a more direct, less time-consuming procedure was needed. The HPLC assay reported in this paper was originally developed and validated to support preclinical toxicology, pharmacology, and disposition experiments in animals. As the compound progressed from the preclinical stage to early phase I clinical trials, the method was modified to meet the different demands of clinical studies (*e.g.* larger number of samples covering a broader concentration range) and was transferred from research laboratory to routine drug assay laboratory. An evolution of the assay paralleled the progress of atovaquone through its preclinical and clinical development. The validation experiments and results reported in this paper reflect the evolving nature of a typical analytical method in a pharmaceutical research setting. The initial validation experiments (model selection, specificity, recovery, accuracy, and precision of the assay) and stability studies were performed on a single HPLC system by one analyst. After the assay was transferred to the routine assay laboratory, it was performed on a slightly different HPLC system by several analysts. The validity of the method as performed in the routine assay laboratory was then verified; results from both laboratories were used to establish the validity of the method and are reported in this paper.

2. Experimental

2.1. Reagents

Atovaquone was provided by Compound Registration, Burroughs Wellcome Co. (Research Triangle Park, NC, USA). All solvents were HPLC-grade and were obtained from EM Science (Gibbstown, NJ, USA). Glacial acetic acid and dimethylformamide (99.9% pure) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Potassium phosphate buffer (0.05 *M*, pH 7.0) was obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Instrumentation and chromatographic conditions

The HPLC system used in the initial development and validation of the assay consisted of a Waters Associates (Milford, MA, USA) Model 600 Multisolvent Delivery System, an autoinjector (Waters WISP Mode 712B), and a programmable multiwavelength detector (Waters Model 490E). The HPLC system used in the routine assay of atovaquone consisted of an IBM LC/9560 liquid chromatograph (Danbury, CT, USA), an autoinjector (Waters WISP Model 712), and a programmable multiwavelength detector (IBM Model LC/9523). For both systems, the UV detector was set at 254 nm; UV_{max} for atovaquone in the HPLC mobile phase was 251 nm. Detector output was monitored and analyzed with an in-house chromatography data acquisition program, which was operated by a Digital Specialties DS-80Z microcomputer (Carrboro, NC, USA). For all of the initial development and validation experiments and some of the routine assays, chromatographic separations

were performed on a Supelcosil LC-8-DB column (15 cm × 4.6 mm I.D., particle size 5 μm, Supelco, Bellefonte, PA, USA) protected by an inert C_8 guard column cartridge (1 cm × 4 mm I.D., Keystone Scientific, Bellefonte, PA, USA) and a precolumn filter (2 μm, Upchurch Scientific, Oak Harbor, WA, USA). The mobile phase of acetonitrile–1% acetic acid (85:15, v/v) was pumped at a flow-rate of 0.8 ml/min. The injection vehicle was methanol–1% acetic acid (80:20, v/v). A Supelcosil LC-1 column (15 cm × 4.6 mm I.D., particle size 5 μm) protected by an inert C_1 guard column cartridge (1 cm × 4 mm I.D., Keystone Scientific) and a precolumn filter (2 μm, Upchurch Scientific) was also used in the routine assay of atovaquone. For this column, a mobile phase of acetonitrile–0.1% acetic acid (60:40, v/v) or methanol–0.1% acetic acid (70:30, v/v) pumped at a flow-rate of 1.0 ml/min was used.

2.3. Preparation of calibration standards and quality control samples

In the initial development and validation of the assay, calibration standards were prepared for each assay run. A concentrated stock solution of atovaquone (0.3 mg/ml) was prepared in methanol–dimethylformamide (99:1, v/v). Stock solutions A (0.1 mg/ml) and B (0.01 mg/ml) were made by diluting appropriate amounts of the stock solution with injection vehicle. Appropriate amounts (0.01–0.05 ml) of stock solutions A or B, blank plasma (0.1 ml), and potassium phosphate buffer (1.0 ml) were combined in disposable tubes to yield spiked calibration standards (duplicates at each concentration) ranging from 0.5 to 50.0 μg/ml. For routine assays, calibration standards were prepared in batches of 30–40 ml at each concentration and stored in 1-ml aliquots at −40°C. Subsequent to the initial validation of the assay over the concentration range of 0.5 to 50 μg/ml, the calibration curve was extended to a range of 0.25 to 100 μg/ml. Quality control (QC) samples were prepared at three concentrations (1.96, 5.66, and 16.67 μg/ml) from stock solutions that were made separately from those used to prepare the calibration standards. The concentrations of the QC samples were relevant to the atovaquone concentration range of clinical interest. The routine accuracy and precision (*i.e.* quality control) of the assay were important to monitor at concentrations between 2 and 20 μg/ml, because that was the concentration range associated with therapeutic failure or success. Plasma concentrations near 17 μg/ml were associated with a high rate of therapeutic success [11].

2.4. Sample preparation

Atovaquone was extracted from plasma with a solvent mixture of hexane–isoamyl alcohol (98:2, v/v). Plasma (0.1 ml), phosphate buffer (1.0 ml), and extraction solvent (5.0 ml) were combined in a disposable screw-cap tube with a teflon-lined cap, shaken on a reciprocal shaker for 15 min, and centrifuged (1000 *g*, 5 min). A portion (2.0–4.0 ml) of the extract was removed and combined with isopropyl alcohol (1.0 ml) in a disposable glass test tube. The solvent was evaporated under a stream of nitrogen, and the sample was reconstituted by vortex-mixing for 10 s in HPLC injection vehicle (0.2 ml). A portion (0.05 ml) of each sample was injected into the column.

Calibration standards and QC samples were prepared and extracted as described above with each set of unknown samples. One set of calibration standards was assayed at the start and a duplicate set was assayed at the end of the HPLC run; QC samples (duplicates at each concentration) were interspersed throughout the HPLC run. As suggested in a recent consensus report on bioanalytical methods validation [14], a portion of stock solution B (0.5 μg) was injected at regular intervals to serve as a system suitability check (*i.e.* to ensure that the system was performing as expected).

2.5. Calculations

Calibration curves were obtained by weighted least-squares linear regression (LSLR) of the peak areas of the calibration standards (duplicates assayed at each of six concentrations) on the concentrations of the calibration standards. Concentrations of atovaquone in the QC and

unknown samples were calculated from the equation of the regression.

2.6. Assay validation

Calibration model selection

The appropriate regression model for the assay was determined from several sets of calibration standards that were assayed as described above. The concentration and peak-area data were fitted to LSLR models with weighting factors of 1/(concentration) and 1/(concentration)^2. The residuals plots obtained from these regressions were inspected for homogeneity of variance in the response (peak area) across the concentration range and for random distribution of the residuals around a residual value of zero [15]. In addition, back-calculated concentrations for the calibration standards were determined, and percent relative concentration residuals (%RCR) were calculated according to the formula described by Karnes and March [16]. The LSLR model and weighting scheme that produced the lowest total %RCR was chosen.

Stability of atovaquone

Atovaquone's stability in stock solution was examined over a three-week period. Portions of a stock solution ($n = 4$, 0.5 μg in 0.05 ml) prepared on the first day of the test period were stored at room temperature in the dark. Once each week, concentrations of atovaquone in this original stock solution were then calculated from a standard curve derived from a freshly prepared stock solution.

The stability of atovaquone in frozen plasma stored at −40°C was examined over a six-month period in assays run with freshly prepared calibrators. The stability of the drug in plasma that underwent four freeze-thaw cycles also was investigated. Portions of spiked plasma ($n = 5$, 25 μg/ml) were assayed on the day of preparation, and the remainder of the plasma was frozen at −40°C. The plasma was thawed, re-assayed, and refrozen over a four-day period.

Before routine assay of samples from HIV-positive subjects, plasma is heated to 56–58°C for 5 h to inactivate the virus [17]. The stability of atovaquone during heat inactivation was examined in spiked human plasma. Samples (2.0 ml) of the spiked plasma ($n = 7$) were pipetted into screw-capped polypropylene vials; a portion of each sample was removed to serve as the non-heat-treated control. The remaining sample was placed in a hot air oven (56°C) for 5 h. The ratio of the peak area of atovaquone in the heat-treated sample to that in the control was calculated to assess atovaquone stability during the heat treatment.

Recovery

Spiked plasma samples (5.2, 26.0, and 52.0 μg/ml; $n = 5$ at each concentration) were extracted, and the recovery of atovaquone from plasma was determined from a calibration curve prepared with standards that were injected directly into the HPLC system.

Specificity

Blank plasma samples from untreated volunteers and predose plasma samples from patients were analyzed in routine assay runs, and the chromatograms were examined for compounds that would interfere with the quantitation of atovaquone. In addition, four drugs that are commonly taken by HIV-positive patients (pentamidine, zidovudine, sulfamethoxazole, and trimethoprim) were injected directly into the HPLC system to determine their retention times relative to atovaquone.

Accuracy and precision

The accuracy and precision of the method were estimated by assaying spiked plasma at two concentrations (3.79 and 37.89 μg/ml) in separate assays on seven days. Accuracy (expressed as % bias) was calculated as shown in Table 2. One-way analysis of variance (ANOVA) was used to partition the total observed variance of the assay into its two components, within-assay variance (random error) and between-assay variance (error associated with differences in day-to-day conditions) [18]. Precision was expressed as the coefficient of variation (C.V.) of the means from these runs. In addition to the specific validation experiment designed to estimate accuracy and within- and between-day precision,

quality control charts were maintained during the course of the clinical trials, and these results were used to monitor assay precision during the routine use of the assay.

3. Results

Representative chromatograms of blank plasma, plasma spiked with atovaquone near the lower limit of quantitation (0.5 μg/ml), and plasma from a patient who received atovaquone are shown in Figs. 2a–c. The retention time of atovaquone was typically 6–7 min, and no endogenous interferences were noted. As the HPLC column aged and the retention time of atovaquone decreased, the composition of the mobile phase was adjusted to compensate for the change in retention. Columns were replaced when the amount of methanol in the mobile phase reached 60%, the peak shape deteriorated, or baseline resolution was lost.

3.1. Calibration model selection

Residuals plots from the calibration data fitted to an LSLR model indicated that the variance associated with the response (peak area) was homogeneous throughout the concentration range when the data were weighted by 1/(concentration) or 1/(concentration)^2 functions. Examination of the residuals plots during the validation runs revealed that a better fit to the model was achieved with the 1/(concentration)^2 weighting function for most of the calibration curves. Back-calculated concentrations of the standards and %RCR values from routine assay runs suggested that a good fit was achieved weighting by either 1/(concentration) or 1/(concentration)^2.

Examination of the residuals plots for each assay also influenced the assignment of the upper and lower limits of quantitation. The highest and lowest calibration standards in the run were assessed for their fit to the weighted LSLR model. Also, the variance in response for those standards was compared to the variance for the other calibration standards. For most routine

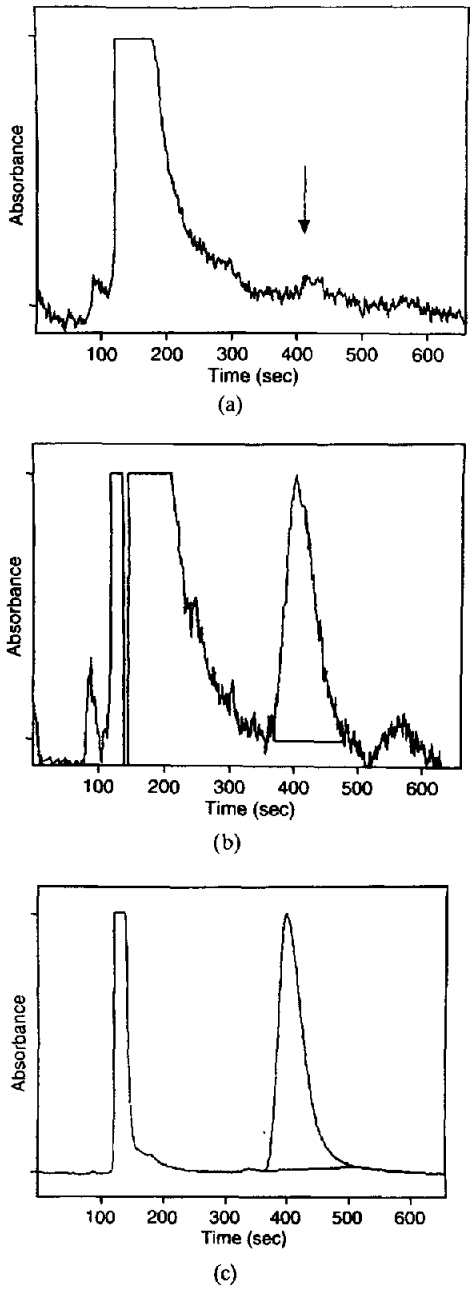

Fig. 2. (a) Chromatogram of a blank plasma extract. The arrow indicates the retention time of atovaquone. Column LC1; Mobile phase, methanol–0.1% acetic acid (70:30, v/v); flow-rate, 1.0 ml/min; detection, UV at 254 nm (0.003 AUFS). (b) Chromatogram of an extract from plasma spiked at 0.5 μg/ml (0.003 AUFS). (c) Chromatogram of a plasma extract from a patient receiving atovaquone; the concentration of atovaquone in this sample was 18.7 μg/ml (0.015 AUFS).

runs, the upper and lower limits were set at 100 and 0.25 μg/ml, respectively.

3.2. Stability

The concentration of atovaquone in stock solution stored at room temperature was stable during the three-week test period; differences between the measured and nominal concentrations were less than 10%. Atovaquone was stable in plasma frozen at −40°C over a period of at least six months. For the three concentrations studied (1.96, 5.66, and 16.67 μg/ml), the daily assayed concentrations of atovaquone were within 11% of the nominal concentrations. No trends in concentrations were observed; the overall averages of the assayed concentrations were within 2% of their nominal concentrations. The compound was also stable during repeated freeze-thaw cycles. The average assayed concentration measured after each freeze-thaw cycle was within 10% of the nominal concentration, indicating that plasma could be frozen and thawed at least four times with no appreciable change in the concentration of atovaquone. The heat-inactivation process applied to plasma samples from HIV-positive patients also did not affect the concentration of atovaquone in plasma. The peak-area ratios (heat-inactivated:control) of the spiked human plasma samples ranged from 0.848 to 1.179. The average difference between the peak areas in the samples before and after heat inactivation was −1.2%.

3.3. Recovery

The recovery of atovaquone from human plasma is shown in Table 1. Greater than 85% of the compound was recovered at the three concentrations studied. The C.V. value at each concentration was less than 5%.

3.4. Accuracy and precision

The accuracy of the assay, expressed as % bias, estimated from assay of control samples (3.79 and 37.89 μg/ml) is presented in Table 2. The average % bias was 2.16% for the high control and −7.39% for the low control. Within-assay precision was estimated at 7.4% and 5.4% for the low and high controls, respectively. The between-assay precision was estimated to be 3.9% for the low controls, and 0.4% for the high controls. The ANOVA results showed that essentially all of the variability seen in the assay of the high control samples in these validation runs was accounted for by the within-assay variability [18]. These results were extended by analysis of the QC data from the assay of patient plasma samples obtained in clinical trials. In one such study [11], intra-assay precision for the low, mid, and high QC samples was calculated as 4.9, 2.7, 3.8%, respectively. Inter-assay precision over the 13-month course of analyzing samples from that trial was 2.8, 4.2, and 4.5% for the low, mid, and high QC samples.

Table 1
Recovery of atovaquone from plasma

Nominal concentration (μg/ml)	Recovery[a] (%)	C.V. (%)
5.2	88.8	2.1
26.0	87.8	3.3
52.0	85.5	4.7

[a] $n = 5$ samples at each concentration.

3.5. Specificity

Plasma samples from untreated volunteers contained no endogenous peaks that interfered with the quantitation of atovaquone. Peaks associated with pentamidine, zidovudine, trimethoprim, and sulfamethoxazole were well resolved from atovaquone (data not shown). Extracts of predose plasma samples from patients in clinical trials were also free of interfering substances; fewer than 1% of the samples from clinical trials showed interferences in the assay for atovaquone.

4. Discussion

A method for the measurement of plasma concentrations of atovaquone that is specific,

Table 2
Accuracy and precision of the assay

Day	Nominal concentration (μg/ml)	Assayed concentration[a] (μg/ml)	C.V. (%)	Bias[b] (%)
1	37.89	37.87 ± 1.26	3.33	−0.05
	3.79	3.51 ± 0.39	11.11	−7.39
2	37.89	39.88 ± 3.13	7.85	5.25
	3.79	3.83 ± 0.11	2.87	1.06
3	37.89	39.19 ± 0.57	1.45	3.43
	3.79	3.46 ± 0.23	6.65	−8.71
4	37.89	38.24 ± 1.43	3.74	0.92
	3.79	3.35 ± 0.07	2.09	−11.61
5	37.89	38.56 ± 1.98	5.13	1.77
	3.79	3.28 ± 0.23	7.01	−13.46
6	37.89	39.79 ± 3.39	8.52	5.01
	3.79	3.58 ± 0.22	6.14	−5.54
7	37.89	37.45 ± 1.12	2.99	−1.16
	3.79	3.57 ± 0.39	10.92	−5.80
Overall	37.89	38.71 ± 2.09		2.16
	3.79	3.51 ± 0.29		−7.39
Within-assay	37.89		5.39	
	3.79		7.39	
Between-assay	37.89		0.36	
	3.79		3.95	

[a] $n = 5$, mean ± S.D.
[b] Bias = (assayed conc − nominal conc)/nominal conc × 100.

rapid, and simple to perform has been described. Validation experiments have shown that the assay has good accuracy and precision, and an internal standard was deemed unnecessary. Assay of patients' predose plasma samples generated in clinical trials indicated that interferences caused by endogenous substances or concomitant medications were seen in fewer than 1% of patients. Because the HPLC run duration is short, an analyst can readily assay 100 plasma samples per day. The stability of atovaquone during heat inactivation of the human immunodeficiency virus enables the drug to be quantitated after minimizing the HIV-related health hazard to laboratory personnel.

During the initial development of the assay, a Supelco LC 8-DB HPLC column was used for the analysis of atovaquone. Most of the assay validation experiments were performed with this column. Unfortunately, atovaquone eluted as a badly tailing peak when the original column was replaced with other LC 8-DB columns. Because atovaquone was strongly retained by many nonpolar stationary phase columns, a relatively polar reversed-phase column, C_1, was chosen to replace the LC 8-DB column. Use of the C_1 column reduced atovaquone's retention time and improved its peak shape. As our experience with the C_1 column grew, we discovered that the retention of atovaquone decreased as the column aged, which resulted in problems with the resolution of the drug and endogenous substances. To maintain the resolution, we progressively decreased the proportion of methanol in the mobile phase. When the proportion of methanol reached 60%, the difference in methanol content between the mobile phase and the injection vehicle caused the atovaquone peak shape to degrade [19], and the column was replaced with a new C_1 column. In our experience, more than

2000 samples could be analyzed before an analytical column needed to be replaced.

Much of the variability in this method occurred at the step where the extract was reconstituted in the injection vehicle. Proportions of methanol in the injection vehicle less than 70% resulted in erratic recovery of the compound and increased variability of the assay. In addition, we discovered that decreasing the sample vortex time in the reconstitution step to less than 10 s increased the variability of the method, especially if the concentration of atovaquone in the sample was above 50 μg/ml. Standardizing the length of the vortex-mixing to 10 s stabilized the precision of the method.

The use of an internal standard was not pursued in the development and validation of this assay. Although internal standards are frequently incorporated into HPLC assays, it is usually done by force of habit, and a critical evaluation of an internal standard's contribution to assay accuracy and precision is rarely conducted [20,21]. Use of an internal standard would require evaluation of the potential improvement the internal standard might make [21] as well as additional validation work to show purity, stability, recovery, and specificity of the internal standard. (The fact that AIDS patients are usually taking several medications at once makes specificity a heightened concern in an assay of a drug such as atovaquone.) Careful attention to the assay procedure for this HPLC method has resulted in an assay that is as accurate and precise as most other methods that incorporate an internal standard.

Simple nonweighted LSLR is not a valid calibration model for most HPLC assays that employ UV detection, because the absolute error of the response (peak area) usually increases with the concentration of the analyte [15,16]. When the assumption of constant variance of the response across the concentration range is violated [15,16,18], weighted LSLR can be used to solve this problem. Weighted LSLR produces a calibration line that more accurately predicts the concentrations of unknowns, especially those at the lower end of the calibration range [22]. For routine assays, the number of replicates at each concentration was too small to obtain meaningful residuals plots from the data, therefore, the weighting factor that minimized the %RCR values for the calibration standards was used [16].

The changing demands on an analytical assay during its development, validation, and routine use occasionally call for modifications to be implemented in the assay procedures. The magnitude of each change and its effect on the validity of the assay must be carefully assessed, so that appropriate revalidation experiments can be designed and carried out. Careful monitoring of the assay by the routine analyst allows early detection of problems in the method. The routine analyst and the developer of the assay can then collaborate to solve the problems and perform appropriate experiments to validate the modified assay [23].

5. Conclusion

A simple, accurate, and efficient high-performance liquid chromatography method for measurement of atovaquone in plasma has been developed and validated. The assay, which is less time-consuming than a previous gas chromatographic method, was validated for the plasma concentration range expected in patients being treated for PCP and toxoplasmosis infections. It has been used successfully to assay plasma samples from preclinical and phase I, II, and III clinical trials. The simplicity and reliability of the assay make it a valuable tool in the clinical laboratory for the measurement of plasma concentrations of atovaquone in patients being treated for PCP and toxoplasmosis.

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