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A robotics-based liquid chromatographic assay for the measurement of atovaquone in plasma

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Abstract

A precise and specific robotics-based liquid chromatographic (LC) method for measuring atovaquone concentrations in plasma was developed and validated, and the method was compared with an existing manual LC method. The compound was isolated from plasma by liquid-liquid extraction, separated by reversed-phase LC, and quantitated against an internal standard with UV detection. Least-squares linear regression with 1/concentration^2 weighting was used as the calibration model. The range of the calibration curve for the assay under routine conditions was 0.25–50 μg ml^{-1}. No endogenous interferences with the compound or the internal standard were noted in either untreated human plasma or in plasma from patients enrolled in Phase III clinical trials of atovaquone. The accuracy of the assay (determined as the percent bias) ranged from −4.8% to −9.4% in the validation runs. The intra- and interassay precisions (determined as the relative standard deviation) were less than 6.8% and 6.4%, respectively. The contribution of an internal standard on assay accuracy and precision also was examined. Interassay variability was marginally improved by the incorporation of an internal standard to the assay; accuracy and intra-assay precision were essentially unchanged. A paired *t*-test between estimates of atovaquone concentrations in healthy volunteer and HIV + patient human plasma samples assayed by the automated and manual methods demonstrated no significant difference ($p = 0.31$) between the values determined by each method.

Keywords: Atovaquone; Comparison with manual sample preparation; Reversed-phase HPLC; Robotic sample preparation; Validation of internal standard

1. Introduction

Atovaquone (*trans*-2-[4-(4-chlorophenyl)-cyclohexyl]-3-hydroxy-1,4-naphthalenedione, 566C80; Fig. 1(a)) is a hydroxynaphthoquinone structurally related to ubiquinone. It is a potent inhibitor of the electron transport chain in *Pneumocystis carinii*, *Toxoplasma gondii*, and *Plasmodium species* [1–3], and is thought to act by inhibiting several metabolic enzymes linked via ubiquinone. The compound is under investigation for the treatment of malaria and toxoplasmosis and has been approved in the US, Canada, and several European countries for the treatment of *P. carinii* pneumonia (PCP) [4–6]. Clinical trials have shown that atovaquone is equivalent to trimethoprim/sulfamethoxazole for treatment of PCP, and that therapeutic success and steady-state plasma concentrations of atovaquone are closely correlated [6].

Initially, a gas chromatographic method for measuring plasma concentrations of atovaquone up to 10 μg ml^{-1} was devised [7]. Subsequently, the sample work-up for that assay was combined with liquid chromatography (LC) to provide an assay that offered a faster, and more direct, method of quantitation [8].

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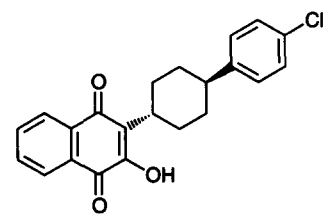
This method consisted of liquid–liquid extraction, separation by reversed-phase LC, and quantitation against an external standard curve with UV detection (254 nm). The upper and lower limits of quantitation were 100 and 0.25 μg ml^{-1}, respectively. The accuracy of the assay (determined as the percent bias) ranged from −7% to +2%. The intra- and interassay variability (expressed as the coefficient of variation) were less than 8%. Slight modifications to the chromatography conditions and the incorporation of an internal standard led to a second, similar LC assay [9]. Ongoing clinical trials with atovaquone and studies of alternative formulations continue to generate numerous samples for analysis. Therefore, a robotics-based automated LC method was developed and validated to supplement the manual analysis method and to expand our bioanalytical capacity for atovaquone. Robotic automation has been applied successfully to several LC assays with various modes of sample preparation [10–14]. Benefits of robotic analyses include decreased labor and increased sample throughput, with a potential cost reduction for bioanalyses.

Several alterations to the atovaquone assay were necessary during the adaptation of the manual method to a robotic method. In particular, the nonchlorinated analog of atovaquone (*trans*-2-hydroxy-3-(4-phenylcyclohexyl)-1,4-naphthalenedione, 59C80; Fig. 1(b)) was used as an internal standard added just prior to the final reconstitution step. As recommended by Haefelfinger [15], a critical assessment of the value of the internal standard on assay variability was made as part of the validation of the method. This report describes the experiments that were conducted (1) to validate the robotics-based automated method, (2) to cross-validate it with the existing manual assay, and (3) to assess the value of using the internal standard.

(a)



(b)

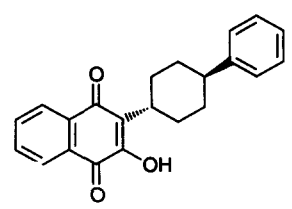


Fig. 1. Structure of (a) atovaquone and (b) 59C80.

2. Materials and methods

2.1. Chemicals

Atovaquone and 59C80 were obtained from Compound Registration, Burroughs Wellcome Co., RTP, NC. All solvents used for extraction and chromatography were LC grade (EM Science, Inc., Gibbstown, NJ; Mallinckrodt, Inc., Paris, KY). Dimethylformamide (99.9%), 3-methyl-1-butanol (99 + %), acetic acid (99.8%), and trifluroacetic acid (99 + %) were purchased from Aldrich (Milwaukee, WI). Potassium phosphate monobasic–sodium hydroxide buffer (pH 7.0, 0.05 M) was purchased from Fisher Scientific (Pittsburgh, PA). Untreated human plasma was obtained from volunteers who were not taking any medication prior to donation.

2.2. Instrumentation

An AD-2 analytical balance (Mettler Instrument Corp., Highstown, NJ) was used to weigh atovaquone and 59C80 for the preparation of standard and internal standard solutions. Positive displacement pipettors (SMI, American Dade, Miami, FL) were used to prepare standard solutions and to spike plasma. Automated sample processing was accomplished by a Zymate Laboratory Automation System (Zymark Corp., Hopkinton, MA), featuring a robotic arm with a general purpose hand (ZP900-1) and a pipette hand (ZP912-1), system V-15 controller, two power and event controllers (ZP830), two master laboratory stations (ZP510), capping section (ZP412), test tube dispenser (ZP030-1), dilution station (SPL 132), vortex mixer (ZP620), liquid/liquid extraction station (ZP740), centrifuge (ZP710-2), tumble mixer (ZP650-1), and a turbo-style evaporator (ZP630-1). The robot also included a RC-250S balance (Sartorius Corp., Edgewood, NY); Fig. 2 shows a bench-layout of the total robotic system. The LC sysetm consisted of a ZP311 50-μl fixed-loop injector and an

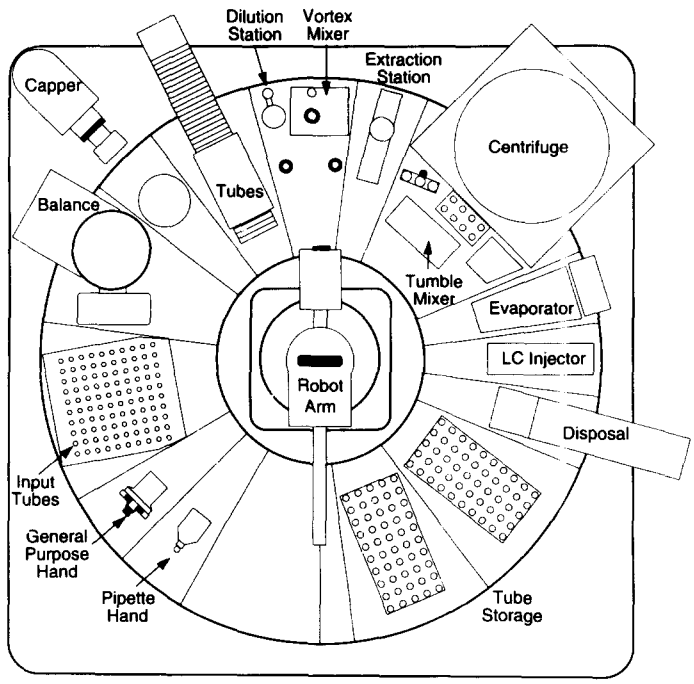


Fig. 2. Configuration of the Zymate Laboratory Automation System.

Encore 46198-D HPLC pump (Zymark), and a 490E variable wavelength detector (Waters Associates, Milford, MA). Two Chromspher C_8 glass LC columns (5 μm, 100 × 3 mm, Chrompack Inc., Raritan, NJ) in series were used for the analysis. The columns and a reversed-phase guard column (10 × 2 mm, Chrompack) were coupled in a stainless steel housing. A 2-μm precolumn filter (Upchruch Scientific, Oak Harbor, WA) was placed in-line between the injector and the columns. The LC system was programmed to deliver acetonitrile–0.4% trifluoroacetic acid (pH 2.0) (65:35, v/v) at a flow rate of 0.6 ml min^{-1}. The UV detector wavelength was set at 254 nm (based on an absorbance maximum of 250 nm for atovaquone in this mobile phase). The Zymate Laboratory Automation System was programmed to inject 50 μl from each specimen at 11-min intervals. Data were collected and analyzed with VG Multichrom software (Fisons Instruments Inc., Beverly, MA) and a VMS operating system (vs. 5–20) on a VAX 6320 (Digital Equipment Corp., Maynard, MA).

Instrumentation used for the manual processing of samples included a reciprocal shaker (Ederbach Corp., Ann Arbor, MI), RC3B Sorvall centrifuge (Du Pont Co., Wilmington, DE), and TurboVap LV evaporator (Zymark Corp.). The LC system consisted of an LC9560 pump and LC9523 UV detector (IBM Instruments Inc., Danbury, CT), and a 715 WISP autoinjector (Waters). Chromatographic separation was performed on a Supelcosil LC-1 column (5 μm, 150 × 4.6 mm, Supelco, Bellefonte, PA) with an inert C8 guard column cartridge (10 × 4 mm, Keystone Scientific, Bellefonte, PA) and a 2-μm precolumn filter (Upchurch Scientific). The LC system was programmed to deliver methanol–0.1% acetic acid (pH 3.7) (75:25, v/v) at a flow rate of 1.0 ml min^{-1} and UV detection at 254 nm. Specimens (50 μl) were injected at 7-min intervals, and data collected and analyzed identically to the automated system.

2.3. Preparation of calibration standards, spiked controls, and interval standard solution

Separate concentrated stock solutions for controls (A1, 1 mg ml^{-1} atovaquone) and calibration standards (S1, 1 mg ml^{-1} atovaquone) were prepared in methanol–dimethylformamide (99:1, v/v). Stock solution A2 (0.1 mg ml^{-1} atovaquone) was prepared by diluting a 1-ml portion of concentrated stock solution A1 with methanol-1% acetic acid (pH 3.1) (4:1, v/v). Stock solution S2 (0.5 mg ml^{-1} atovaquone) was prepared by diluting a 5-ml portion of concentrated stock solution S1 with methanol–1% acetic acid. Stock solution S3 (0.05 mg ml^{-1} atovaquone) was prepared by

Table 1
Comparison of the relative standard deviation (%) of spiked control and internal standard peak area and the correlation coefficient between paired areas

Control ($\mu g\ ml^{-1}$)	$S_{a,rel}$ [a] (%)	$S_{b,rel}$ [b] (%)	r	$2rs_{a,rel}$	$S_{b,rel} < 2rs_{a,rel}$
0.596	3.70	2.37	0.835	6.18	Yes
1.96	4.05	3.19	0.877	7.11	Yes
5.66	7.97	4.32	0.835	13.31	Yes
16.7	3.62	3.62	0.740	5.36	Yes

[a] $n = 18$ spiked control replicates.
[b] $n = 18$ internal standard replicates.

Table 2
Comparison of accuracy (%bias) and precision (RSD) of the atovaquone assay in human plasma with and without correction by the internal standard 59C80

Nominal concentration ($\mu g\ ml^{-1}$)	Internal standard	Assayed concentration [a] ($\mu g\ ml^{-1}$)	Intra-assay RSD [b] (%)	Interassay RSD (%)	Bias [c] (%)
0.596	w	0.501 ± 0.0187	3.57	1.34	−15.9
	wo	0.484 ± 0.0308	4.52	5.31	−18.7
1.96	w	1.89 ± 0.128	2.71	7.35	−3.88
	wo	1.88 ± 0.143	3.56	7.96	−3.88
5.66	w	5.80 ± 0.0759	5.53	0.622	2.47
	wo	5.83 ± 0.0933	6.48	2.44	2.93
16.7	w	16.9 ± 0.862	2.92	4.99	1.22
	wo	16.6 ± 1.09	3.45	6.61	−0.312

[a] Mean ± SD ($n = 18$).
[b] $n = 6$ per assay run.
[c] $\text{Bias} = \dfrac{\text{assayed conc.} - \text{nominal conc.}}{\text{nominal conc.}} \times 100.$

diluting a 0.5-ml portion of concentrated stock solution S1 with methanol–1% acetic acid. Concentrated internal standard stock solution ($\approx 0.6\ mg\ ml^{-1}$ 59C80) was prepared in methanol–dimethylformamide. Internal standard solution (0.1 $\mu g\ ml^{-1}$ 59C80) was prepared by diluting a portion of concentrated internal standard stock solution with isopropanol. The internal standard solution was stored at room temperature in an amber bottle.

Calibration standards ranging from 0.25 to 50 $\mu g\ ml^{-1}$ (six concentrations, 20-ml pools) were prepared. The appropriate amount of the 1, 0.5, and 0.05 $mg\ ml^{-1}$ standard stock solutions (0.04–1 ml) were added to untreated human plasma. Plasma control samples spiked at four concentrations (20-ml pools) were prepared by the addition of the appropriate amounts of the 1 and 0.1 $mg\ ml^{-1}$ control stock solutions (0.12–0.80 ml) to untreated human plasma. Each calibration standard and spiked control pool was divided into 1-ml portions and stored at −70°C.

2.4. Sample preparation

Calibration standards, control samples, and healthy volunteer and clinical HIV+ patient samples were prepared individually for anlaysis by the Zymate Laboratory Automation System or by the manual method as described previously [8]. For the automated assay, plasma samples in polypropylene tubes were placed in a temperature-controlled rack (15°C) accessible to the robot arm. Plasma (0.1 ± 0.01 ml) was delivered into a 16×100 mm culture tube with the pipette hand. Potassium phosphate monobasic–sodium hydroxide buffer (1 ml) and extraction solvent (hexane–3-methyl-1-butanol, 98:2, v/v, 5 ml) were added by the Master Laboratory Station. The tube was capped, mixed by inversion in a tumble mixer for 11 min, and centrifuged for 11 min at 1500 r.p.m. After removal from the centrifuge, a 2-ml portion of the organic layer was withdrawn at the liquid/liquid extraction station.

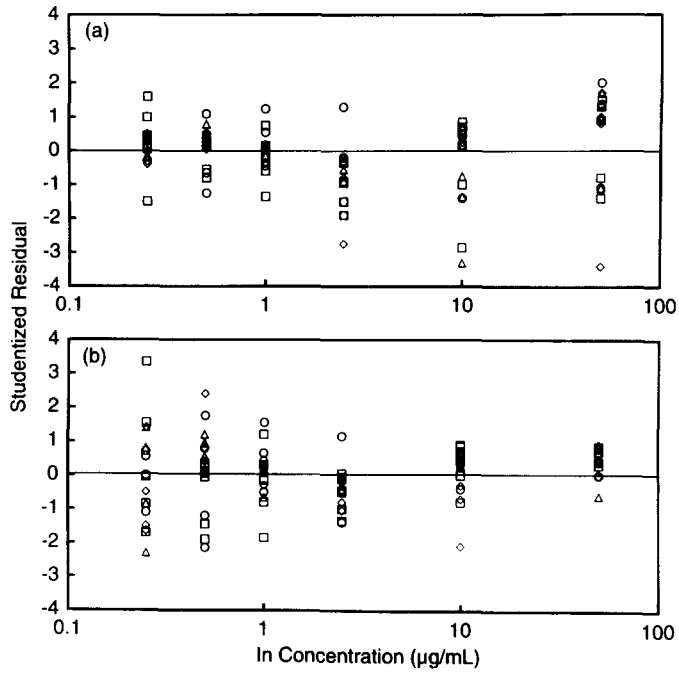


Fig. 3. Plots of the Studentized residuals vs. ln concentration for the weighted (a) 1/concentration and (b) 1/concentration^2 least-squares linear regressions across four assay runs ($n = 18$ at each of six concentrations; different symbol for each assay run).

Isopropanol (0.8 ml) and internal standard solution (0.2 ml) were dispensed by the Master Laboratory Station into a fresh tube, and the 2-ml portion of the organic layer from the extraction tube was added. The solvents were evaporated in a Turbo-Vap (5 psi, 50 ± 5°C) for 11 min, reconstituted with 0.5 ml of extraction solvent, and re-evaporated for 11 min. Each specimen was reconstituted with 0.2-ml methanol–1% acetic acid (pH 3.1) (4:1, v/v) added by the Master Laboratory Station and vortexed for 15 s. Injections were made at 11 min intervals, after the initial time lag (≈ 1 h) for preparation of the first sample.

2.5. Calculations

A least-squares linear regression model was fitted to the peak area ratio (calibration standard to internal standard) and concentration data determined from the calibration standards. The concentrations of atovaquone in control and untreated human and clinical patient samples were calculated from the equation of the regression line.

2.6. Internal standard

The usefulness and influence of the internal standard, 59C80, was examined. Eighteen replicates of four spiked controls (0.596, 1.96, 5.66 and 16.7 µg ml^{-1}, $n = 6$ at each concentration over three assay runs) were assayed against the internal standard. The peak areas (mean and standard deviation) for each spiked control concentration and the corresponding values for the internal standard were determined, and the relative standard deviation calculated. As suggested by Haefelfinger [15], the usefulness of the internal standard, 59C80, was assessed by the relationship: $s_{b,rel} < 2rs_{a,rel}$ where $s_{a,rel}$ is the relative standard deviation for atovaquone peak areas, $s_{b,rel}$ is the relative standard deviation for 59C80 peak areas, and r is the correlation coefficient between the peak areas of the compounds. In addition, accuracy and precision were determined with and without the internal standard to determine the influence of the internal standard on these parameters. The measured values for each concentration were averaged, and the percentage bias was calculated to estimate the accuracy. Analysis of variance (ANOVA) was performed on the combined data from the four sets of control samples to partition the total observed variance of the assay into intra-assay variance, or random error, and interassay variance, or the error between runs [16]. Precision was expressed as the intra- and interassay relative standard deviation (RSD).

2.7. Calibration model selection

Spiked calibration standards (range = 0.25–50 μg ml^{-1}, $n = 6$ at each of six concentrations) were extracted and assayed. A least-squares linear regression model with four weighting schemes (unweighted, 1/concentration, 1/concentration^2, and log–log transformed) was fitted to the concentration–peak area ratio data to verify the appropriate model and weighting. The residuals at each concentration were calculated, and a plot of the Studentized residuals vs. concentration was inspected for homogeneity of variance in the response across the concentration range, and for random distribution of the residuals around a value of zero [17].

2.8. Stability of atovaquone and 59C80

The stability of atovaquone in standard solution, during freeze–thaw cycles, and during heat-inactivation has been reported previously [8]. The stability of the internal standard solution (59C80 in isopropanol) was evaluated over an eight-week period. The internal standard solution was prepared and stored in an amber container at room temperature (expected conditions during routine use). Portions ($n = 3$) of this solution were assayed on the first day, and at one, five, and eight weeks after preparation. On each analysis day, a fresh stock standard solution of 59C80 was prepared and assayed in triplicate. The chromatograms were examined for extraneous peaks, which would have been a qualitative indication of degradation of 59C80. As a quantitative measure of internal standard stability, the ratio of the average peak area of the stored solution compared to that of the fresh solution at each time interval was calculated.

Table 3
Stability of 59C80 in isopropanol

Time (weeks)	Stored 59C80 peak area [a] (mV s)	Fresh 59C80 peak area [b] (mV s)	Ratio [c]
0	245 ± 2.74	251 ± 12.6	0.976
1	252 ± 16.3	248 ± 5.95	1.02
5	254 ± 2.07	251 ± 6.73	1.01
8	231 ± 2.99	248 ± 7.82	0.931

[a] Mean ± SD, $n = 3$.
[b] Mean ± SD, $n = 3$.
[c] Ratio = $\frac{\text{stored 59C80 peak area}}{\text{fresh 59C80 peak area}}$.

2.9. Specificity

Human plasma from untreated volunteers (predose and spiked) and predose clinical HIV+ patients were extracted and assayed as described to ascertain that the method was specific for 59C80 and atovaquone. Chromatograms from these experiments were inspected to determine if endogenous substances would interfere significantly with the integration of the 59C80 and atovaquone peaks.

2.10. Accuracy and precision

Spiked control plasma samples (0.596, 1.96, 5.66, and 16.7 μg ml^{-1}, $n = 4$ at each concentration over three assay runs) were assayed to determine the accuracy and precision of the method. The measured values for each concentration were averaged, and the percentage bias was calculated to estimate the accuracy. The combined data from the four sets of control samples were subjected to ANOVA to determine the within- and between-day variance, and the precision was expressed as the intra- and interassy RSD.

Spiked control plasma samples (0.600, 15.0, and 40.0 μg ml^{-1}, $n = 2$ at each concentration over eight assay runs) were assayed to determine the accuracy and precision of the method during routine analysis of clinical samples. The measured values for each concentration were averaged, and the percent bias was calculated to estimate the accuracy. The combined data from the eight sets of control samples were treated as described above, and the precision was expressed as the intra- and interassay RSD.

2.11. Analyses of unknown samples

Spiked plasma samples treated as unknowns (0.40, 4.0, 8.0, 20, and 40 μg ml^{-1}) were prepared in 4-ml pools, separated into duplicate portions, and assayed by the automated method ($n = 5$ replicates in two assay runs) and the manual method ($n = 3$ replicates in a single run). The percent bias was calculated for each method.

Human plasma samples (95 untreated for healthy volunteers, 153 heat-inactivated from HIV+ patients) that had been assayed previously by the manual method were reassayed with the automated method. Concentration estimates for the human plasma samples by each method were compared to determine any sig-

nificant difference between the methods and to examine the ability of the automated system to process heat-inactivated plasma samples. The relationship between the paired values was examined by geometric regression of the automated assay estimates on the values determined by the manual assay [18]. A geometric regression of dependent variables on independent variables indicates the absence of a systematic bias if the 95% confidence interval (CI) for the slope includes the value unity, and demonstrates the absence of a fixed bias if the 95% CI for the y-intercept includes the value zero. The pairs of estimated values also were ln-transformed and analyzed with a paired t-test. Similar to the determination of bias, a paired t-test demonstrates no significant difference between the paired values if the 95% CI includes the value zero. In addition, the percentage difference between the two determinations was plotted as a function of ln (average concentration) to assess whether any differences in the values obtained with each method varied as a function of concentration. The average concentration was calculated as $(C1 + C2)/2$ and the percentage difference as $\ln (C1/C2) \times 100$, where C1 was the manual method value and C2 was the automated method value.

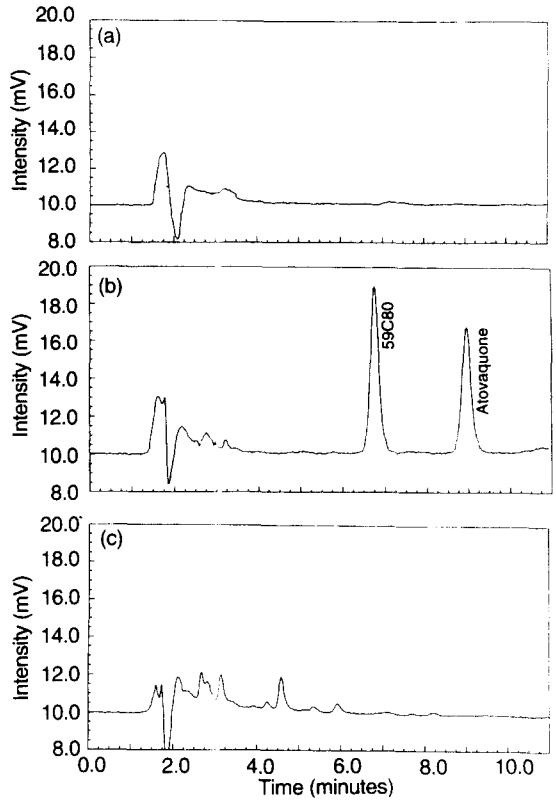


Fig. 4. Representative chromatograms of extracted (a) predose untreated human plasma, (b) spiked human plasma, and (c) predose clinical HIV+ patient plasma.

3. Results

3.1. Internal standard

Table 1 contains the relative standard deviation (RSD) and correlation coefficient values determined for the spiked controls and corresponding 59C80 peak areas. The RSD of the internal standard peak areas was less than or equal to the RSD of the spiked control peak areas. The correlation coefficient ranged from 0.74 to 0.88. The RSD of the internal standard peak area was lower than $2r$ (RSD spiked control) at each concentration. This property, according to Haefelfinger, is a necessary (but not sufficient) prerequisite for an internal standard to contribute to improving the precision of a chromatographic assay [15]. A comparison of the assay accuracy and precision with and without the influence of 59C80 is shown in Table 2. The inclusion of the internal standard had a minimal effect on assay accuracy and intra-assay precision. However, the interassay RSD was decreased by an average of 2% across the range of spiked controls.

3.2. Calibration model selection

The plots of the Studentized residuals vs. concentration for the least-squares linear regression with various weighting schemes were examined. Inclusive plots of the Studentized residuals weighted 1/concentration and 1/concentration^2 across four assay runs as shown in Figs. 3(a) and 3(b), respectively. A factor 1/concentration^2 was determined to be an appropriate weighting for the calibration model. The plots reveal that the residuals were distributed normally around a residual value of zero, and the variance of the residuals across the concentration range was similar with the two weighting schemes. The deviation tended to be greatest at higher concentrations with the 1/concentration weighting and greatest at lower concentrations with the 1/concentration^2 weighting.

3.3. Stability

The apparent concentration of the internal standard, 59C80, remained stable when stored

Table 4
Accuracy (%Bias) and precision (RSD) of the automated atovaquone assay in human plasma

Nominal concentration ($\mu g\ ml^{-1}$)	Assayed concentration [a] ($\mu g\ ml^{-1}$)	Intra-assay RSD [b] (%)	Interassay RSD (%)	Bias [c] (%)
0.596	0.540 ± 0.0300	5.4	2.2	−9.4
1.96	1.84 ± 0.160	6.8	6.4	−6.1
5.66	5.13 ± 0.210	2.0	4.2	−9.4
16.7	15.9 ± 0.200	1.1	0.73	−4.8

[a] Mean ± SD ($n = 12$).
[b] $n = 4$ per assay run.
[c] $\text{Bias} = \frac{\text{assayed conc.} - \text{nominal conc.}}{\text{nominal conc.}} \times 100.$

Table 5
Comparison of spiked unknowns assayed by the manual and automated atovaquone assays

Nominal conc. ($\mu g\ ml^{-1}$)	Manual			Automated			
	Assayed conc [a] ($\mu g\ ml^{-1}$)	Bias [b] (%)	Intra-assay RSD (%)	Assayed conc [c] ($\mu g\ ml^{-1}$)	Bias (%)	Intra-assay RSD (%)	Inter-assay RSD (%)
0.40	0.45 ± 0.0058	12	1.3	0.45 ± 0.037	13	7.7	3.6
4.0	3.9 ± 0.13	−3.1	3.4	3.8 ± 0.026	−3.7	2.2	0.60
8.0	7.9 ± 0.080	−0.91	1.0	7.6 ± 0.044	−4.5	1.5	1.4
20	21 ± 0.84	3.1	4.0	21 ± 0.12	2.9	1.6	1.0
40	40 ± 0.18	−0.7	0.46	42 ± 0.28	6.1	1.9	1.0

[a] Mean ± SD ($n = 3$).
[b] $\text{Bias} = \frac{\text{assayed conc.} - \text{nominal conc.}}{\text{nominal conc.}} \times 100.$
[c] Mean ± SD ($n = 5 \times 2$ assay runs).

protected from light at approximately 25°C during an eight-week period. The ratios of the peak areas are presented in Table 3. No additional peaks were observed in the chromatograms, and a decrease in the peak area of only $\approx 5\%$ over the eight-week period was evident.

3.4. Specificity

Representative chromatograms of extracted predose and spiked untreated human plasma are shown in Figs. 4(a) and 4(b), respectively. No endogenous peaks were presented in untreated human plasma samples that would interfere with 59C80 or atovaquone peak quantitation. Similarly, no interference from endogenous and exogenous compounds was seen with predose clinical HIV+ patient plasma samples (Fig. 4(c)).

3.5. Accuracy and precision

The results from the analysis to estimate the accuracy and precision of the automated assay from these validation experiments are presented in Table 4. The percent bias of the assay ranged from −5% to −9%. The intra-assay precision ranged from 1 to 7%, and the estimates of the interassay precision ranged from 1 to 6%.

The comparison between the manual and automated method estimates of the spiked samples treated as unknowns is shown in Table 5. The range of the percent bias was similar between the manual and automated method (−1 to 12% and −4 to 13%, respectively for these spiked samples). The percent bias was greatest at the lowest concentration in both assays; otherwise, no trend was apparent across the concentration range. The precision of each assay method also was examined with these

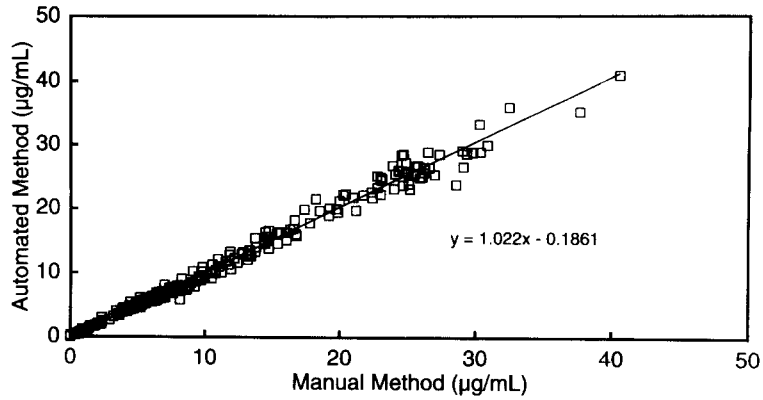


Fig. 5. Geometric regression of concentrations determined by the automated method on concentrations determined previously by the manual method for clinical human plasma samples.

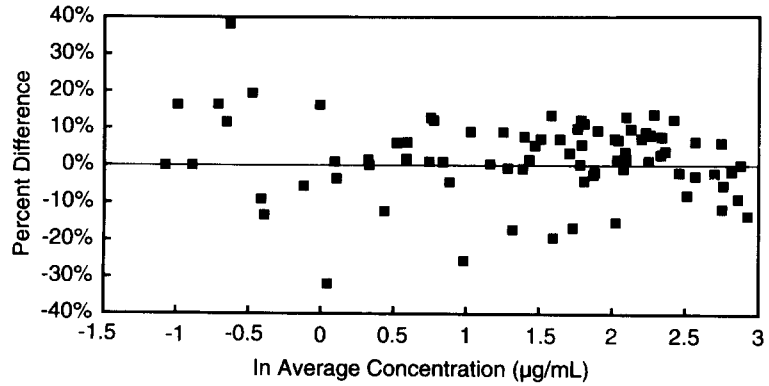


Fig. 6. Plot of the percent difference between estimates determined by the manual and automated methods as a function of the average concentration of untreated plasma samples.

data. In this experiment, the intra-assay precision for the manual method was $< 4\%$. The automated assay yielded an intra-assay precision value of $< 8\%$ and interassay precision value of $< 4\%$.

Measured concentrations of the high, medium, and low quality control samples obtained in the analyses of atovaquone in routine runs with the automated method provided data for accuracy and precision extimates of the method during routine use (data not shown). With these data, the method showed an 8% bias and an intra-assay RSD value that ranged from 5 to 14%. Intra-assay variability accounted for all the assay variability seen with the assay of the two higher controls [16]; the lowest control yielded an interassay RSD value of 5%.

3.6. Analyses of unknown samples

No marked difference was seen in the "head-to-head" comparison of untreated and heat-inactivated sample concentrations determined by the manual and automated methods. Geometric regression [18] between estimates of untreated and heat-inactivated plasma samples (Fig. 5, $n = 238$) assayed by each method yielded a line described by the equation $y = 1.022x - 0.1861$. The 95% CI for the slope was 1.008–1.037, and the 95% CI for the y-intercept was -0.398 to $+0.0255$. A paired t-test between estimates of untreated and heat-inactivated plasma samples assayed by each method demonstrated no significant difference (95% CI was -0.20 to $+0.064$, $p = 0.31$) between the automated and manual methods. The composite plots of the average concentration vs. the percentage difference for untreated (Fig. 6) and heat-inactivated clinical samples (Fig. 7) show that no trend across the concentration range was evident, and the average percent difference between the two methods was close to zero.

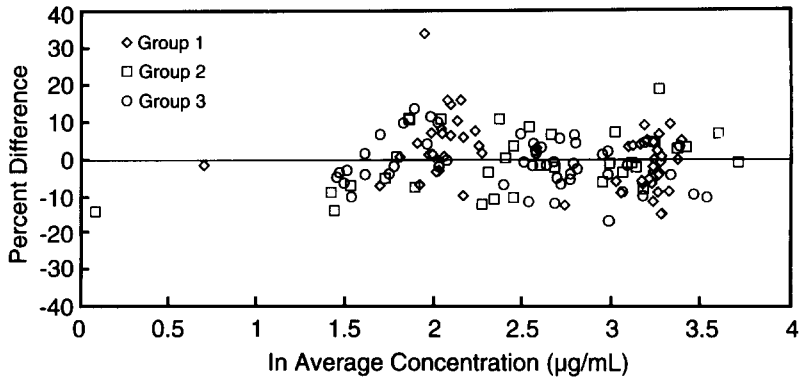


Fig. 7. Plot of the percent difference between estimates determined by the manual and automated methods as a function of the average concentration of heat-inactivated HIV + plasma samples across three assay runs (different symbol for each assay run).

4. Discussion

The development of an automated LC assay for the measurement of atovaquone in plasma was undertaken to reduce the manpower required for the assay and expand the bioanalytical resources that could be applied to atovaquone clinical trials. Sample preparation was virtually identical between the two assays, except that batch preparation was used in the manual method, and serial preparation was used in the automated method. The total number of specimens per run was limited to 99 by constraints of the current data acquisition system, which would yield an approximate total run time of 21 h. The operator time required for daily maintenance and sample preparation was 3–4 h; manual preparation of a full run would require 9–12 h. Several authors [10,11] have noted increased sample analyses (25–100%) and decreased manpower requirements after assay automation.

During the early development of the automated procedure, preliminary runs with the robot were characterized by unacceptable variability in the standard and control values. The inclusion of 59C80 as an internal standard in the reconstitution step was adopted from a similar plasma assay for atovaquone [9] to address this issue. As suggested by Haefelfinger [15], an evaluation of the potential improvement afforded by incorporating an internal standard was made. Calculations indicated that the presence of 59C80 as an internal standard offered the potential of a slight improvement in assay precision, based on a comparison of the relative standard deviation of the peak areas of atovaquone (spiked controls) and 59C80, and the correlation coefficient between the respective peak areas.

Experiments to support the validation of the method included model selection, stability of the internal standard solution (59C80 in isopropanol), specificity, determination of assay accuracy and precision, and a comparison of human plasma samples assayed by the manual and automated methods. Although the selected model was a $1/\text{concentration}^2$ weighted least-squares linear regression, a 1/concentration weighting also was appropriate. Although either weighting scheme would be acceptable, our experience [8] has been that $1/\text{concentration}^2$ weighting has minimized the percent relative concentration residuals more reliably then 1/concentration weighting [19]. Therefore, the least-squares linear regression, weighted by $1/\text{concentration}^2$, was chosen as the model to use for the estimation of atovaquone concentrations determined with the automated assay. The lack of interfering endogenous compounds stands in agreement with results from a more extensive evaluation of specificity conducted previously [8]. The accuracy and precision of the automated assay are also comparable to values reported by DeAngelis et al. [8] and Rolan et al. [9].

A crucial issue was the ability of the automated system to handle patient HIV+ plasma samples that had been heat-inactivated. Heat treatment may cause precipitate formation or other changes that can affect sample manipulation. Adjustments to these changes ordinarily made by the analyst in the course of the manual assay cannot be programmed into an automated robotics sequence. However, no significant difference was observed in the ability

of the automated system to process heat-inactivated samples, compared to untreated plasma samples from healthy volunteers. Values estimated by the automated assay for untreated and heat-inactivated plasma samples were compared to previous estimates determined with the manual method. The slope derived from the fitted line of the geometric regression was very close to unity, and the 95% CI values for the y-intercept included the value zero. In addition, the 95% CI values from the paired t-test also included the value zero. These results indicate that no unacceptable difference, in magnitude or percent bias, exists between estimates obtained by each method.

This report describes the validation of a robotics-based automated method for the measurement of atovaquone in plasma, and the cross-validation with a previous manual method. The automated method was precise, specific, and yielded results that were not significantly different from results determined by the manual assay. This earlier assay was developed to provide a simple and reliable method for the analysis of atovaquone in plasma samples. The development of an automated assay offers the potential for additional sample throughout and a reduction in manual labor requirements.

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