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Short communication

Capillary zone electrophoresis for the determination of atovaquone in serum

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Abstract

A rapid and simple capillary zone electrophoresis (CZE) method has been developed for the determination of atovaquone in serum. The drug was extracted from equine serum–chloroform (1:3, v/v) at greater than 80% recovery and assayed in buffer containing 25 m*M* sodium borate (pH 9.1) and 25% acetonitrile. A 100 μm I.D. fused-silica capillary was used and the detection was by UV-diode array at 254 nm; the migration time was approximately 8 min. Intra- and inter-assay variabilities were less than 7.8% and 5.8%, respectively, and the accuracy of the assay (expressed as % bias) ranged from 4.5 to −5.2%. The working assay range was from 2 to 100 μg/ml. This sensitivity could be increased by concentrating during the extraction procedure. Replacement of acetonitrile with 75 m*M* surfactant 3-(dimethyldodecylammonio)propanesulfonate gave similar sensitivity and provided an additional option to facilitate the separation of atovaquone on multiple-drug samples. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Atovaquone (Fig. 1) is a potent hydroxy-naphthoquinone with approved use in the USA, Canada and several European countries for the treatment of *Pneumocystis carinii* pneumonia [1–3] in acquired immunodeficiency syndrome (AIDS) patients intolerant to trimethoprim/sulfamethoxazole. Its potent antiprotozoal activity against *Plasmodium*, *Pneumocystis* and *Toxoplasma* [4–6] had

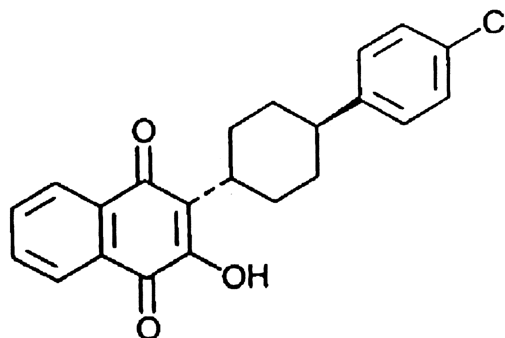

Fig. 1. Chemical structure of atovaquone {*trans*-2-[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone}.

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prompted further investigations including clinical trials for treatment of *T. gondi* encephalitis in AIDS patients [7]. A previous study of atovaquone disposition in humans yielded no evidence of metabolites [5]. To date, the assays published for atovaquone are limited to complex gas chromatographic methods [8] and high-performance liquid chromatography (HPLC) methods with multiple sample preparation and extraction procedures [9,10]. To our knowledge, there has not been a capillary electrophoresis (CE) method published. Therefore, we describe here a capillary zone electrophoresis (CZE) method for the determination of atovaquone in serum. This CZE method, which requires only a single extraction step and has an equivalent order of sensitivity to HPLC methods, should facilitate the determination of atovaquone concentrations in serum and potentially other body fluids in clinical trials.

2. Experimental

2.1. Reagents

Atovaquone (566C80) was provided by Glaxo Wellcome (Research Triangle Park, NC, USA). All solvents used for extraction and chromatography were HPLC-grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). Reagent-grade sodium borate was also purchased from Fisher Scientific and dimethyldodecylaminopropanesulfonate (DAPS) was obtained from Aldrich (Milwaukee, WI, USA).

2.2. Preparation of calibration standards

A concentrated stock solution of atovaquone (1 mg/ml) was prepared in acetonitrile, and calibration standards were made at concentrations of 0, 1, 2, 5, 10, 20, 50, 100 and 500 μg/ml by diluting appropriate amounts of the stock solution with acetonitrile. For spiked serum standards, 100 μl of the above standards ranging from 10 μg/ml to 1 mg/ml was added to 0.9 ml of normal equine serum to yield spiked calibration standards ranging from 1 to 100 μg/ml. Blank serum with 10% acetonitrile was used as zero spiked standard. Calibration curves on standards were made by least-square linear regression of the peak areas of the calibration standards ($n=5$ replications at each concentration) on the concentrations of the calibration standards.

2.3. Extraction procedure

To each ml of spiked serum, 3 ml of chloroform was added to perform a single-step extraction. The mixture was agitated vigorously for 30 s and then placed on a reciprocal shaker for 10 min before it was centrifuged at 2060 *g* for 10 min. The organic layer was aspirated and evaporated in a speed vacuum concentrator (Speed Vac Plus SC110A, Savant, Holbrook, NY, USA) until dryness and then reconstituted with 1 ml of acetonitrile before CZE injection.

2.4. Instrumentation and CZE chromatographic conditions

The development and validation of the assay was performed by an automated CZE system (Beckman P/ACE 5510) with a diode-array UV detector (190–450 nm) operating under System Gold software program (Beckman, Fullerton, CA, USA). Uncoated 100 cm×100 μm I.D. fused-silica capillary columns purchased from J & W Scientific (Folsom, CA, USA) were used at 23°C. The applied voltage was 30 kV and UV detection was performed at 254 nm with bandwidth set at 80 nm. Before each run, the column was pre-rinsed with 0.1 *M* sodium hydroxide as column regenerator for 5 min, followed by HPLC water for 3 min. Next, the column was pre-treated with run buffer for 3 min, followed by hydrostatic injection of sample at the anodic end for 2 s.

CZE was carried out in two different run buffers. Sodium borate (25 m*M*) with 25% acetonitrile, pH 9.1, was freshly prepared on the day of each experiment. Replacement of 25% acetonitrile with 75 m*M* surfactant DAPS at pH 9.1 to exclude the use of organic solvent as run buffer was also tested in our methods.

2.5. Assay validation

Accuracy and precision were determined at three spiked concentrations (2, 5 and 50 μg/ml) and assessed by carrying out four replicate analyses daily for 3 days. Recovery of extraction procedure was

carried out in triplicate at two concentrations (5 and 50 μg/ml). Accuracy was expressed as percentage bias from the nominal concentration. One-way analysis of variance (ANOVA) was used to determine the intra- and inter-day variance. The precision was expressed as the relative standard deviation (RSD) of the means from the quadruplicated runs. The limit of detection was set as three-times the average baseline noise level.

3. Results

Representative electropherogram of 50 μg/ml atovaquone extracted from spiked serum is shown in Fig. 2b. With 25% acetonitrile and 25 m*M* sodium borate (pH 9.1) as run buffer, two UV absorbance peaks of 221 nm and 277 nm were evident when the detection wavelength was set at range 254±40 nm. The retention time of atovaquone was typically 8 to 9 min, and no endogenous interference was noted.

3.1. Linearity and limit of detection of assay

With atovaquone standards prepared in acetonitrile, linearity and detection limit were determined. A standard curve was constructed by comparing the mean of five replicates of varying concentrations to the corresponding peak. The standard curve was linear in the range of 1 to 100 μg/ml. Peak area (y) could be expressed by the equation $y=0.075x+0.165$ ($R^2=0.994$) where x is the concentration in μg/ml. Higher linearity was found at concentrations below 20 μg/ml with $y=0.106x+0.033$ ($R^2=0.997$). The limit of detection, which was three-times the baseline noise level, was estimated to be 1 μg/ml. Although peak height signals at 1 μg/ml were distinct from baseline noise, the intra-assay variation exceeds 20% (data not shown) and therefore the working assay range was considered from 2 to 100 μg/ml.

3.2. Recovery

The recovery of atovaquone from normal serum was 82±2% at a concentration of 5 μg/ml and 89±5% at the 50 μg/ml level ($n=3$). The RSD value at each concentration was 2.5% and 5.1%, respectively.

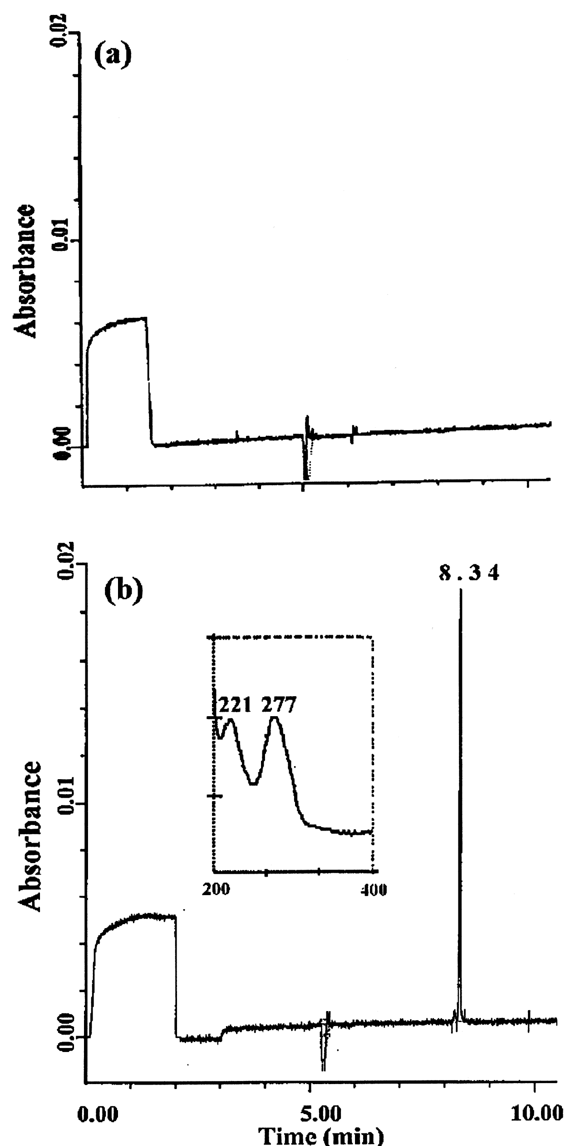

Fig. 2. Representative electropherogram of extracted (a) blank serum and (b) spiked 50 μg/ml atovaquone (migration time 8.34 min) and its UV spectrum (inset). The maximum absorbance with diode-array UV detection set at range 254±40 nm was at 221 nm and 277 nm. Buffer and run conditions: 25 m*M* sodium borate, 25% acetonitrile, pH 9.1. Voltage: 30 kV; injection time: 2 s.

3.3. Accuracy and precision

The results from the analysis of three different concentrations (2, 5 and 50 μg/ml) to estimate the accuracy and precision are presented in Table 1. The

Table 1
Accuracy and precision of CZE for atovaquone detection

Nominal concentration (μg/ml)	Accuracy		Precision[c]	
	Assayed concentration[a] (μg/ml)	Bias[b] (%)	Intra-assay RSD (%)	Inter-assay RSD (%)
2	2.18±0.02	4.5	7.71	5.73
5	4.34±0.18	−5.2	5.12	3.75
50	47.93±0.71	−4.1	1.55	2.02

[a] Values represent mean±S.E.M. from four samples (n=4).
[b] Bias=100×(assayed concentration−nominal concentration)/nominal concentration.
[c] n=4.

accuracy of the method, expressed as % bias, was −4.1% for 50 μg/ml and was within 5.3% for 2 and 5 μg/ml. The intra-assay precision was estimated at 7.7%, 5.1% and 1.6% for atovaquone concentrations of 2, 5 and 50 μg/ml, respectively. The inter-assay precision was estimated to be 2.02% for 50 μg/ml and less than 6% for 2 and 5 μg/ml. The ANOVA results showed no difference between inter- and intra-assay variations, to the contribution of overall variability of the CZE method.

4. Discussion

CZE has emerged as a popular method for the analysis of drugs in biological fluids and tissues in the past decade. The exceptional power of separation and resolution of complex mixtures of analytes form biological samples, rapid analysis time, economy of reagents, minimal sample preparation and miniscule sample requirements have made CZE an attractive method for both forensic and clinical laboratories. As a result, CZE analysis has been routinely employed as a complimentary tool to HPLC. The CZE method we developed for atovaquone assay is convenient with a single extraction step. Sensitivity of this assay is slightly lower than HPLC methods [7,9,11]. Lower sensitivity has been the major issue when comparing CZE with HPLC method due to the extremely small amount (nl) of samples employed in CZE separation. Several options are available to improve the detection limit. These include increasing sample injection time, larger-bore capillary, sample concentration, and modified Z-cell capillaries to increase detection pathlength [12]. In this method, a capillary with 100 μm I.D. was used rather than 75 μm I.D., which is normally the maximum workable capillary diameter for determination of trace levels of drugs [12]. It was then possible to improve the sensitivity by at least 20-fold by doubling the sample injection time to 4 s in addition to sample concentration by reconstituting the extracted sample in one-tenth of the original volume. These procedures lowered the limit of detection to be comparable to published HPLC methods [7,9] for atovaquone quantitation.

Reproducibility is usually another concern in CZE method development [13–15]. In this method, it was discovered that the retention time of atovaquone increased as the buffer aged, and the retention time was very sensitive to the change of sodium borate concentration. Therefore, it is recommended to prepare a large stock of sodium borate solution which can be mixed with acetonitrile fresh each day as needed before running samples. It is also suggested that the capillary be conditioned with 100 m*M* sodium dodecyl sulfate every 20 sample runs, in addition to the sodium hydroxide pre-rinse in each cycle to regenerate the capillary wall charge. Alternatively, our preliminary work showed that 75 m*M* DAPS could be used to replace acetonitrile to mix with sodium borate solution as run buffer without sacrificing the sensitivity of our assay (data not shown). The compatibility of the buffer to different surfactants would provide versatility to the method in separating possible structurally similar atovaquone-related compounds or samples containing multiple drugs, which is very common in AIDS patients.

This method also showed good precision and low variability at tested concentrations, which indicates that the rinsing and conditioning processes were

appropriate. Therefore, the use of an internal standard was not pursued in this method. Although use of internal standard may slightly improve the precision [9], it also required additional validation to show purity, stability, recovery and specificity of the internal standard.

Peak area plotted against sample concentration has been predominantly preferred over peak height in CE method development to ensure better linearity of standard curve [16,17]. Peak height measurements give reduced linear dynamic range due to band broadening effects at higher concentrations [12]. It was noticed that buffer constitution may also be a contributing factor in deciding whether peak area or peak height should be selected for constructing the standard curve. During this method development, we discovered that peak height measurement is less sensitive to minor changes in buffer composition. For example, when 50 μg/ml atovaquone was run on different combination of borate–acetonitrile (B–A) buffers, the peak heights (arbitrary unit, AU) were consistent at around $2.70 \cdot 10^{-3}$ AU (20 m*M* B–20% A), $2.77 \cdot 10^{-3}$ AU (20 m*M* B–25% A), and $2.8 \cdot 10^{-3}$ AU (15 m*M* B–15% A). The peak area, on the other hand, showed more variability with 0.39 AU (20 m*M* B–20% A), 0.31 AU (20 m*M* B–25% A) and 0.26 AU (15 m*M* B–15% A). Therefore, both sensitivity and precision must be considered in the decision to use peak area or peak height. We chose to use a combination of 25 m*M* borate–25% acetonitrile with peak area measurements due to the best yield of peak area at this combination without extensive prolongation of migration time. The migration time in this study is approximately 8 min; with only one peak in the electropherogram, it might be desirable to use shorter length capillary to further reduce migration time. However, in view of the application of this method to multiple-drug separations and other species, the capillary was used in its original length (1 m) in light of better resolution with a longer capillary. Subsequently, a higher voltage (30 kV) was applied to compensate for the reduced electroosmotic flow caused by organic modifier (acetonitrile) in the run buffer.

In conclusion, we have developed a simple, efficient CZE method for measurement of atovaquone in equine serum. The determination of drugs in biological samples in a simple, rapid and specific manner has far-reaching implications in pharmaceutical and medical science. The simplicity of the assay and possible application to both human patients and animals should make it a useful tool for the measurement of atovaquone concentration in clinical trials.

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