Validation of atovaquone plasma levels by liquid chromatography-tandem mass spectrometry for therapeutic drug monitoring in pediatric patients

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ABSTRACT

Background: Atovaquone has traditionally been used as an antiparasitic and antifungal agent, but recent studies have shown its potential as an anticancer agent. The high variability in atovaquone bioavailability highlights the need for therapeutic drug monitoring, especially in pediatric patients. The goal of our study was to develop and validate the performance of an assay to quantify atovaquone plasma concentrations collected from pediatric cancer patients using LC-MS/MS.

Methods: Atovaquone was extracted from a 10 µL volume of K2-EDTA human plasma using a solution consisting of ACN: EIOH: DMF (8:1:1 v:v:v), separated using reverse-phase chromatography, and detected using a SCIEX 5500 QTrap MS system. LC-MS/MS assay performance was evaluated for precision, accuracy, carryover, sensitivity, specificity, linearity, and interferences.

Results: Atovaquone and its deuterated internal standard were analyzed using a gradient chromatographic method that had an overall cycle-time of 7.4 min per injection, and retention times of 4.3 min. Atovaquone was measured over a dynamic concentration range of 0.63 – 80 µM with a deviation within ± 5.1 % of the target value. Intra- and inter-assay precision were ≤ 2.7 % and ≤ 8.4 %, respectively. Dilutional, carryover, and interference studies were also within acceptable limits.

Conclusions: Our studies have shown that our LC-MS/MS-based method is both reliable and robust for the quantification of plasma atovaquone concentrations and can be used to determine the effective dose of atovaquone for pediatric patients treated for AML.

Introduction

Atovaquone is a hydroxynaphthoquinone, a structural analog to protozoan ubiquinone [1]. This broad-spectrum antiprotozoal drug is well known for its antiparasitic activity and is approved by the Food and Drug Administration (FDA) to be used in combination with proguanil hydrochloride for prophylaxis and treatment of malaria [2]. Due to its antifungal properties, atovaquone is also FDA approved for the prevention and treatment of Pneumocystis jirovecii pneumonia (PJP) and commonly used to prevent PJP in immunocompromised patients, including human immunodeficiency virus (HIV)-infected and cancer patients [3,4]. Currently, the National Comprehensive Cancer Network (NCCN) guidelines recommend atovaquone for antipneumocystis prophylaxis in patients that are intolerant to trimethoprim/
sulfamethoxazole (TMP/SMX) [5,6].

Observations of low relapse rates in cancer patients taking atovaquone for PJP prophylaxis has led to further studies of its potential anticancer properties [7]. Although the exact mechanism of action for atovaquone remains to be fully known, studies have shown that atovaquone exerts its effect on active myeloid leukemia (AML) cells through several mechanisms, including downregulation of gp130 cell-surface expression, which is required for signal transducer and activator of transcription 3 (STAT3) expression activation [7]. It has also been shown that atovaquone promotes eukaryotic initiation factor 2α (eIF2α) phosphorylation, induction of activating transcription factor 4 (ATF4) and regulated in development and DNA damage responses 1 (REDD1), inhibition of mammalian target of rapamycin (mTOR), and inhibition of mitochondrial oxidative phosphorylation, resulting in cellular stress, growth arrest and apoptosis of cancer cells [8].

Currently, AML treatment in pediatric patients mainly relies on high-dose chemotherapy to achieve remission [9]. The combination of agents, such as cytarabine and daunorubicin, shows highly myelosuppressive effects that are accompanied with undesired side effects and serious risks of toxicity, including infection and cardiac dysfunction [9,10]. A study in HIV positive children has shown that daily administration of atovaquone for PJP prophylaxis is well tolerated [11]. It has also been shown to be efficacious in preventing PJP infection in pediatric patients with leukemia [12]. Atovaquone’s potential as an anticancer therapy has prompted studies in pediatric AML patients to assess its benefits beyond PJP prophylaxis in patients receiving standard chemotherapy [10]. An ongoing multi-institution study is exploring the tolerability of combining atovaquone with standard pediatric AML treatment. To elucidate atovaquone’s utility in cancer therapy, evaluation of plasma levels achieved in combination with other medications is needed.

Atovaquone, a highly lipophilic compound, is currently formulated as a liquid suspension to address its poor drug solubility [13]. Despite changes in formulation, its absorption remains variable and dependent on dietary fat content, limiting its bioavailability [4]. The drug shows complex pharmacokinetics, including high protein binding, extremely long elimination half-life, potential enterohepatic circulation, and drug interactions [13]. While atovaquone is a cost-effective, widely accessible, and promising anticancer drug, the difficulty in predicting its plasma concentration for a specific dose highlights the need for therapeutic drug monitoring (TDM) methods. Atovaquone’s accurate quantification allows the identification and rapid adjustment of subtherapeutic doses in patients taking atovaquone for PJP prophylaxis and will shed light into the understanding of the optimal dosing to exploit its anticancer potential. The goal of our study was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that can reliably measure atovaquone plasma levels for TDM in pediatric patients.

Materials and methods

Reagents and materials

Optima™ LC/MS-grade acetonitrile (ACN), methanol (MeOH), water, acetic acid, isopropanol (IPA), absolute ethanol (EtOH) and charcoal stripped K2-EDTA human (CSEH) plasma were all obtained from Fisher Scientific (Waltham, MA). Atovaquone, dimethyl formamide (DMF), and ammonium acetate were obtained from Millipore-Sigma (Burlington, MA). The internal standard, [2H2]-atovaquone, was obtained from Santa Cruz Biotechnology (Dallas, TX). The aqueous mobile phase (A) consisted of 0.01 % acetic acid in water (v/v), the organic mobile phase (B) consisted of 0.01 % acetic acid in methanol (v/v), and the needle wash consisted of IPA:MeOH:ACN: DMF (45:22.5:22.5:10 v/v/v/v). The chromatographic method included column heating at 45°C, autosampler tray chilling at 10°C, a mobile phase flow rate of 200 µL/min, and gradient elution program specified as follows: 0–1 min, 2 % B; 1–3 min, 2–100 % B; 3–6 min, 100 % B; 6–6.1 min, 100–2 % B; 6.1–7 min, 2 % B. The overall cycle-time per injection was 7.4 min, the operational backpressure for the system was typically at 1,500 psi at initial conditions, and the retention time for atovaquone and the IS was 4.3 min (see Fig. 1).

A TurboIonSpray® probe was installed in the Turbo V™ ion source and was operated in negative ion mode with the following conditions: Curtain Gas (Cur), 20 psi; IonSpray Voltage (IS), −4500 V; Ion Source Gas 1 (GS1), 50 psi; Ion Source Gas 2 (GS2), 50 psi; Temperature (TEM), 450°C. The MS system parameters were as follows: Collision Gas (CAD), “High”; Q1 and Q3 resolution, unit/unit; MS/MS/MW Dwell Time, 100 ms per transition; Declustering Potential (DP), −100 V; Entrance Potential (EP), −10 V; Collision Exit Exit (CXP), −12 V. The molecule-specific transitions used for quantification and qualification are listed in Appendix A.
The instrument was calibrated using Sciex PPG calibration standard and calibrated to the manufacturer’s specifications. Data were acquired with Sciex Analyst Software (v1.6.2). The data were analyzed using Sciex MultiQuant Software (v3.0.1).

Sample preparation and extraction procedures

The protocol was approved by the Baylor Institutional Review Board and since these were scavenged anonymized samples that were not collected specifically for this research study, waiver of consent was obtained. Patients’ blood was collected in K₂-EDTA tubes (lavender top). Plasma was separated after centrifugation and stored refrigerated or frozen at −20 °C for up to one week, according to previously reported stability [14]. The CSEH plasma, QC standards, and patient samples were brought to room temperature prior to analysis. Reagent and plasma blank samples each received a 20 µL volume of water or blank CSEH plasma, respectively. The blank-IS standard sample received a 10 µL volume of blank CSEH plasma and a 10 µL volume of the WIS solution. Each calibration standard, QC standard, or patient sample received a 10 µL volume of the respective standard or sample and a 10 µL volume of the WIS solution. Every sample received a 1.98 mL volume of the extraction solvent, was vortex-mixed at 2,000 RPM for 2 mins, and then was centrifuged at 2,000g for 5 mins. A 20 µL volume of the extracted sample supernatant was diluted further in a 480 µL volume of the extraction solvent in the glass autosampler vial, and a 2 µL volume was injected on the LC-MS/MS system.

Method validation

The method was validated for precision, accuracy, carryover, sensitivity, specificity, linearity, and interferences. Each QC level (low: 1.88 µM, medium: 7.5 µM, high: 60 µM atovaquone) was run 10 times to assess precision within-run and in duplicates across eight different days to assess precision between-run. Precision was expressed in terms of coefficient of variation (%CV) and considered acceptable if CV was < 20 %.

A total of 20 patient plasma samples collected on K₂-EDTA and stored at −20 °C were received from an external laboratory that measured atovaquone using HPLC with UV fluorescence detection. The samples were compared over a range of 0.11 – 92.85 µM. To assess accuracy, instrument comparison was performed using the external laboratory.

Table 1

The molecule-specific MS/MS parameters for [37Cl]⁻-atovaquone and the internal standard, [²H₄]-atovaquone. Rows 1 and 3 show quantifier transitions (bold) and rows 2 and 4 show qualifier transitions.

| Q1 (m/z) | Q3 (m/z) | Compound                  | CE (eV) |
|---------|---------|---------------------------|--------|
| 365.0   | 337.2   | Atovaquone (Quantifier)    | −42    |
| 365.0   | 201.2   | Atovaquone (Qualifier)     | −44    |
| 371.1   | 343.1   | [²H₄]-Atovaquone (Quantifier) | −42  |
| 371.1   | 203.1   | [²H₄]-Atovaquone (Qualifier) | −52   |

* CE, collision energy; eV, electron-volts.
results as reference method and were compared to the method developed in our laboratory. The total allowable error was 30 %. Deming regression analysis, bias, and error index were also assessed. An additional five plasma samples collected on K2-EDTA and stored refrigerated were sent to an external laboratory that used LC-MS/MS to measure atovaquone and the results were compared with our laboratory results.

Carryover was evaluated via injection of the highest calibrator concentration (80 µM) followed by two blanks and this set-up was repeated three times. Carryover was also assessed using the high QC (60 µM) followed by two blanks. Percentage of carryover was calculated with the equation below and was considered acceptable if it was < 1 %.

$$\% \text{ carryover} = \frac{\text{total peak area of 2 post}_80 \mu M \text{ calibrator (or high QC) blanks}}{\text{peak area of } 80 \mu M \text{ calibrator (or high QC)}} \times 100$$

To assess limit of detection (LOD), the lowest calibrator (0.63 µM) was serially diluted (1:2) in CSEH plasma and each dilution was run in triplicates. Serial dilutions were stopped when atovaquone was not detected in 2/3 replicates. Limit of quantification (LOQ) was assessed by measuring the lowest calibrator 10 times. The acceptability criterion for the LOQ was a CV < 20 %. Analytical specificity was assessed by spiking a sample containing 10 µM of atovaquone with 10 µM of chloroquine diphosphate salt (structurally similar to atovaquone) from Sigma-Aldrich and extracted as an unknown. A ± 20 % deviation from the target value was considered acceptable. Linearity was evaluated by running each calibrator in replicates of six and calculating its accuracy. A sample accuracy ranging within 80 - 120 % for each calibrator was considered acceptable. Additionally, to extend the clinical reportable range to 400 µM, a 1:5 dilution of the highest calibrator (80 µM) in CSEH plasma was run in triplicate and deviation within 20 % was considered acceptable to validate the dilution. In addition, ion suppression studies were performed using post-spiked calibrators prepared in blanked plasma and compared to blank solvent. There was no ion suppression of the analyte and IS at the level of calibrators used.

Lastly, hemolysate interference material and triglyceride-rich lipoprotein material (TG) from a Sun Diagnostics Assurance Interference Kit were spiked prior to extraction into separate samples that had charcoal stripped plasma as the base pool with atovaquone at a final concentration of 10 µM. Hemolysate material had a final hemoglobin concentration of 1000 mg/dL and TG had a final concentration of 1500 mg/dL. A percentage difference within ± 20 % was considered acceptable.

Results

Mass spectra

Using a rapid protein precipitation, requiring a sample volume of 10 µL, the sample was processed for atovaquone quantification. The overall cycle-time per injection was 7.4 min. Fig. 1 shows the extracted ion chromatograms (XIC) of atovaquone and [3H4]-ataovaquone (IS) contained in a mid-level quality control sample (Fig. 1A, 1B) and in a pediatric AML patient sample (Fig. 1C, 1D). Fig. 1A and 1C show the XIC chromatograms for the quantifying (black line, white fill) and qualifying (black line, black fill) MS/MS transitions for atovaquone, while Fig. 1B and 1D show the XIC chromatograms for the quantifying (dashed black line, white fill) and qualifying (dashed black line, horizontal lined pattern) MS/MS transitions for the IS, [3H4]-ataovaquone. LC separation of atovaquone and the IS resulted in retention times of 4.3 min. The optimal qualifier transitions identified by the mass analyzer were m/z 365.0 → 337.2 for atovaquone and m/z 371.1 → 343.1 for the IS, while the qualifier transitions were m/z 365.0 → 201.2 for atovaquone and m/z 371.1 → 203.1 for the IS (Table 1).

Validation

The three QC levels, when run 10 times, gave a within-run CV of 2.7, 1.9, and 2.5 % for concentrations of 1.88, 7.5, and 60 µM, respectively. For the same concentrations, the between-run of samples run in duplicate over eight days resulted in CVs of 6.5, 6.4, and 8.4 % (Table 2). When the accuracy of the method developed in our laboratory was assessed by comparison with the measurements from an external laboratory that was using HPLC, Deming regression statistics resulted in a correlation coefficient (r) of 0.9730, slope of 1.121 and an intercept of −0.87. Of the 20 samples that were run for method comparison, 12 were within the 30 % total allowable error. The average absolute bias was 2.04 µM, with a range of −6.46 - 20.50 µM. The average error index was 0.47, with a range of 1.78 – 6.36. The largest error index occurred at a concentration of 0.11 µM. From the five samples that were sent to another external laboratory that uses LC-MS/MS to measure atovaquone, 4/5 were within the 15 % error. The sample that was outside the 15 % error was below the reporting limit of the external laboratory. For this small set of samples, r was 0.9989, the slope was 1.086 and the intercept was −0.51.

From the carryover studies, no signal was detected in the analyte retention time window of 2/3 post-calibrator blanks and 2/3 high QC blanks. The percentage carryover in the other post-calibrator blank and high QC blank were both < 0.2 %. LOQ resulted in a CV of 9.0 % for the 10 points measured with the lowest calibrator.

For evaluation of analytical specificity, the acceptable criterion was an interference of the non-target compound below 20 %. When 10 µM of chloroquine were spiked in a sample with 10 µM atovaquone, the

| Table 2 | Within-run and between-run precision summaries. |
|---------|-----------------------------------------------|
|         | Mean   | SD    | %CV   |
| Within-run (n = 10) |        |       |       |
| Low QC (1.88 µM) | 1.756  | 0.047 | 2.7 % |
| Medium QC (7.5 µM) | 7.694  | 0.147 | 1.9 % |
| High QC (60 µM) | 60.389 | 1.507 | 2.5 % |
| Between-run (n = 16) |        |       |       |
| Low QC (1.88 µM) | 1.856  | 0.120 | 6.5 % |
| Medium QC (7.5 µM) | 8.023  | 0.517 | 6.4 % |
| High QC (60 µM) | 62.190 | 5.251 | 8.4 % |

| Table 3 | Analytical reportable range. |
|---------|--------------------------------|
| Calibrator Expected concentration (µM) | Mean concentration (µM) | % Accuracy |
| 1 | 0.63 | 0.625 | 99.2 |
| 2 | 1.25 | 1.212 | 96.9 |
| 3 | 2.5 | 2.627 | 101.1 |
| 4 | 5 | 5.135 | 102.7 |
| 5 | 10 | 10.425 | 104.3 |
| 6 | 20 | 20.447 | 102.2 |
| 7 | 40 | 41.335 | 103.3 |
| 8 | 80 | 82.668 | 103.3 |
The combination of atovaquone’s limited bioavailability and difficulty in predicting its plasma concentration with the differences of drug pharmacokinetics in children, highlights the need for TDM in this population. Reliable measurements of atovaquone in plasma of pediatric patients could be used to inform clinicians of the optimal effective dose to treat AML patients to potentially replace some of the standard chemotherapeutics and minimize side effects in children undergoing treatment for AML. Our study describes the development and validation of a highly sensitive LC/MS/MS assay for the quantification of atovaquone in plasma samples. The assay requires only 10 µL of plasma, which is suitable for pediatric populations. This volume requirement is considerably lower than previously developed mass spectrometry-based assays (25–500 µL) [14–16]. The total run-time for each sample analysis in our assay was 7.3 min, which is longer than what has been reported in previous studies (1.3 – 2.5 min), but fulfills the throughput requirements of our laboratory.

The assay has a limit of quantification of 0.63 µM, far below the desired plasma levels for treatment of PJP (40 µM) [8]. Atovaquone quantification also demonstrated an acceptable inter-assay precision of < 8.4% over the range 0.63 – 80 µM. While the use of atovaquone as an anticancer agent in pediatric populations is still in clinical trials, with a CRR up to 400 µM, physicians will be able to account for the large variations in the plasma drug concentrations in children and more precisely tailor the effective dose of atovaquone for pediatric patients treated for AML. When comparing samples with results obtained from an external laboratory that employed HPLC for the measurement of atovaquone plasma concentrations, 8/20 samples were outside of the 30% error margin. Nevertheless, the Deming regression analysis showed acceptable correlation and linearity (r: 0.9730, slope: 1.121, intercept: −0.87), based on the different methodologies compared. It is also worth noting that our method has a higher sensitivity than the HPLC method. Comparison of five samples with an external laboratory that uses LC-MS/MS further demonstrated acceptable performance of our method (4/5 samples within the 15% error, r: 0.9989, slope: 1.086, intercept: −0.51).

Our study shows that LC-MS/MS is an accurate, and reliable method for quantification of atovaquone in plasma. The implementation of this assay in our clinical laboratory will allow TDM of pediatric AML patients at our institution. Quantification of the achieved plasma concentration of this drug will further contribute to understanding the benefits of atovaquone, not only as prophylaxis and treatment of PJP in this pediatric population, but as an anticancer agent.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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