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A liquid chromatography/tandem mass spectrometry method for determination of obatoclox in human plasma

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Abstract

We describe a selective and highly sensitive high-performance liquid chromatography-electrospray ionization-collision induced dissociation-tandem mass spectrometry (HPLC-ESI-CID-MS/MS) assay for the pan-antiapoptotic BCL-2 family inhibitor, obatoclox, in human plasma. Fifty μ L plasma specimens were prepared by addition of extraction solution consisting of Mobile Phase A (10mM ammonium formate (aq) titrated to pH of 3.0 with formic acid) : Mobile Phase B (100% methanol) (20:80, v,v) and internal standard followed by centrifugation. HPLC separations were performed on a Waters, YMC-PackTM, ODS-AQTM S-3 analytical column with LC mobile phase A and B. Linearity and sensitivity was assessed over a linear range of 0.04-25 ng/ml at eleven concentrations. The lower limit of quantification for obatoclox was 0.04 ng/mL. The intraday precision based on the standard deviation of replicates of quality control (QC) samples ranged from 0.9 to 5.1% and the accuracy ranged from 98.9 to 106.8%. Stability studies performed replicate sets of QC samples (0.1 ng/mL, 2.5 ng/mL, and 15 ng/mL) showed that obatoclox in human plasma was stable at room temperature for 24h as well as at -80 °C for 1m and 2y. Stability was also demonstrated after 3 freeze/thaw cycles (RT to -80 °C). The analytical method showed excellent sensitivity, precision, and accuracy. This method is robust and has been successfully employed in a Children's Oncology Group Phase 1 Consortium study of obatoclox in children with cancer.

Keywords

Obatoclox; LC-MS/MS; Plasma; Pediatric; Cancer

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1. Introduction

BCL-2 family proteins are key anti-apoptotic regulators in the intrinsic (mitochondrial) cell death pathway. Bcl-2 has been found to be overexpressed in several cancers and overproduction of the Bcl-2 protein has also been found to prevent cytotoxicity caused by chemotherapy and radiation[1-4]. Therefore therapeutic strategies have been developed to modulate the function of these pro-survival proteins.

Obatoclax (GX15-070) 2-{2-[(3, 5-dimethyl-1*H*-pyrrol-2-yl)methylene]-3-methoxy-2*H* pyrrol-5-yl}-1*H*-indole is a pan-anti-apoptotic BCL-2 family small molecule inhibitor. Obatoclax binds to the BH3 binding pocket of Bcl-2 family of proteins leading to disruption of their anti-apoptotic function[5]. Preclinical investigations have demonstrated that obatoclax can augment the effect of chemotherapy as well as cause cell death as a single agent. In clinical trials, obatoclax is reconstituted in polyethylene glycol (PEG) 300 and polysorbate 20 and diluted in 5% dextrose. The final solution is 11.5% PEG 300, 0.5% polysorbate in 5% dextrose administered as intravenous infusion over 3 hours. Obatoclax is currently being evaluated in combination with doxorubicin and vincristine in a phase 1 trial of pediatric patients with relapsed and refractory solid tumors or leukemia.

The objective of this investigation was to develop and validate a simple, selective and sensitive LC-MS/MS method for the quantification of obatoclax in human plasma to support an early phase pharmacokinetic study of obatoclax in pediatric patients. The pharmacokinetics of obatoclax as a single agent is being assessed in the clinical trial. To our knowledge this is the first published method for the detection of obatoclax in human plasma.

2. Experimental

2.1 Reagent and chemicals

2-{2-[(3, 5-dimethyl-1*H*-pyrrol-2-yl)methylene]-3-methoxy-2*H* pyrrol-5-yl}-1*H*-indole (GX 15-070, obatoclax) (MW 317.38) and GX 15-070-d5 were provided by Gemin X Pharmaceuticals (Malvern, PA USA) [Figure 1]. The different lots of drug-free (blank) human plasma were obtained from the blood bank at The Children's Hospital of Philadelphia. HPLC-grade methanol and ammonium formate was purchased from Fisher-Scientific (Pittsburgh, PA, USA) and reagent-grade formic acid (~96%) and dimethylsulphoxide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Deionized water was prepared using a Milli-Q water purifying system from Millipore Corp. (Bedford, MA, USA).

2.2 Liquid chromatography and tandem mass spectrometry analysis

2.2.1 Liquid chromatography conditions—The Shimadzu HPLC system consisted of two LC-20AD delivery pumps, a DGU-20A5 Shimadzu vacuum degasser, a SIL-20AC Shimadzu autosampler and a CBM-20A system controller (Shimadzu Scientific Instruments; Columbia, MD, USA). HPLC separations were performed on a Waters, YMC-Pack™, ODS-AQ™ S-3 analytical column (4.6mm × 50 mm, 3 μm, 120Å). LC mobile phase A consisted of 10mM ammonium formate (aq) titrated to pH of 3.0 with formic acid and mobile phase B was 100% methanol. The gradient and flow rates were as follows: 0.00-2.00 minutes mobile

phase A 25%, mobile phase B 75%, flow rate 0.8 mL/min; 2.01-3.99 minutes mobile phase A 0%, mobile phase B 100%, flow rate 1.0 mL/min; 4.00-6.49 min mobile phase A 25%, mobile phase B 75%, flow rate 1.2 mL/min; 6.50 min returned to initial conditions. An injection volume of 25 μ L was used for each analysis. To minimize carryover the autosampler was washed with methanol: dimethylsulphoxide (60:40, v/v). The column and autosampler were maintained at 50° C and 10° C, respectively. To further reduce the impact of carryover, two blanks were run after high standards (25 ng/mL) and high QCs (15 ng/mL). An electronic valve actuator with a Rheodyne selector valve was used to divert LC flow to waste when no data acquisition was taking place. Flow was diverted to mass spectrometer from 0.5 to 4.5 min.

2.2.2 MS/MS Parameters—Samples were analyzed with an Applied Biosystems 4000 QTRAP tandem mass spectrometer in electrospray ionization mode. Software for controlling this equipment, acquiring and processing data was Analyst version 1.4.2 software (MDS Sciex; Toronto, Canada). The positive ion mode for MS/MS analysis was selected. Nitrogen was used as the nebulizer, auxiliary, collision and curtain gases. Obatoclox and the internal standard (GX 15-070-d5) were detected by tandem mass spectrometry using multiple reactions monitoring (MRM) with a dwell time of 250 ms and 125 ms, respectively. For the determination of the precursor and product of ion spectra a solution of 1 mg/mL obatoclox or internal standard in mobile phase was infused directly into the ion sources with a Harvard Apparatus syringe pump at a flow rate of 10 μ L/min. The mass transitions were: obatoclox, m/z 318.1 $\rightarrow$ 286.1; obatocloxIS-323.1 $\rightarrow$ 291.1.

The conditions for ionization of obatoclox and internal standard were optimized using individual standard solutions, each at 500 ng/mL, which were infused by a syringe pump through a Tee device at a flow rate of 10 μ L/min into the stream of mobile phase eluting from the LC column through a mixing Tee and then into the turbo spray source, to mimic the LC-MS/MS conditions. The main working parameters of the mass spectrometer were: collision activated dissociation (CAD) gas, high; curtain gas, 12; ion source gas (GS1), 30; ion source gas (GS2), 30; ion Spray voltage (IS), 5000 V; source temperature 500 °C. The optimized declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP) were set at 90 V, 10.7 V, 41 eV, 10 V for both obatoclox and its internal standard.

2.3 Preparation of standards and quality control (QC) samples

Two independent obatoclox stock solutions were prepared. Standard solutions were prepared from one stock solution and QC samples were prepared from the other. The primary stock solutions of obatoclox were prepared by dissolving obatoclox in methanol:dimethylsulphoxide solution (50:50, v/v) producing a concentration of 500 μ g/mL. The primary stock solution was diluted in human plasma to prepare secondary and tertiary stock solutions of 2000ng/mL and 80 ng/mL, respectively. Working solutions of eleven standards containing obatoclox concentrations of 0.4, 0.8, 1.2, 4, 8, 12, 40, 80, 120, 180, 250 ng/mL were prepared by adding the appropriate volumes of secondary and tertiary stock solutions into 1.5 mL eppendorf tubes containing plasma. Working solutions of three QC concentrations were prepared in the same manner by adding appropriate volumes of

secondary and tertiary stock solutions to obtain concentrations of 1, 25, 150 ng/mL, representing low, medium and high QCs. In followup studies we included a lower limit of quantitation (LLOQ) QC (0.4 ng/mL). The standard and QC working solutions contained a maximum of 0.05% and 0.03% spiked solvent, respectively. The primary internal standard stock solution was prepared by dissolving d5-obatoclox in methanol:dimethylsulphoxide solution (50:50, v/v) to produce a concentration of 500 µg/mL. Secondary IS stock solution was prepared by adding 50 µL to extraction solution consisting of Mobile Phase A: Mobile Phase B (20:80, v,v) to a final concentration of 5 µg/mL. All stock solutions as well as the standard and quality control working solutions were stored at -80° C, protected from light. Standard and quality control samples of obatoclox were freshly prepared at the time of analysis by dilution in human plasma to final concentrations of 0.04-25ng/mL and 0.04-15 ng/mL, respectively. Working solution of IS was also freshly prepared at the time of analysis by dilution in extraction solution to a final concentration of 0.75 ng/mL.

2.4 Sample preparation

To 50 µL of human plasma sample, 250 µL of extraction solution (consisting of Mobile Phase A: Mobile Phase B (20:80, v,v) and 0.75 ng/mL of internal standard) was added in a 1.5 mL Eppendorf tube. The sample was vortexed for 90 seconds and then stood for approximately 2 minutes. This procedure was repeated and then the sample was centrifuged at -16,100 rcf at 0° C for 15 minutes. 200 µL of the supernatant was aliquoted and added to 96-well plate. Immediately prior to analysis, the 96-well plate was centrifuged at -2100 rcf at 4° C for 15 minutes.

2.5 Method validation

Method validation and documentation were performed according to guidance set by the United States Food and Drug Administration (FDA) for bioanalytical method validation[6]. This method was validated in terms of linearity, specificity, lower limit of quantitation (LLOQ), recovery, intra- and inter-day accuracy and precision, and stability of the analyte during sample storage and processing procedures. Each analytical run included a double blank sample (without internal standard), a blank sample (with internal standard), eleven standard concentrations for calibration, and replicate sets of QC samples: low (LQC) 0.1 ng/mL, medium (MQC) 2.5 ng/mL, and high (HQC) 15 ng/mL. Follow-up studies included a LLOQ QC of 0.04 ng/mL.

2.5.1 Linearity and sensitivity—For the evaluation of the linearity of the standard calibration curve, the analyses of obatoclox in plasma samples were performed on three independent days using fresh preparations. The calibrations curves were prepared over a linear range of 0.04-25 ng/ml at eleven concentrations of 0.04, 0.08, 0.12, 0.4, 0.8, 1.2, 4, 8, 12, 18, 25 ng/mL. Each calibration curves consisted of a double blank sample, a blank sample (with IS), a plasma blank sample, the eleven-calibrator concentrations, and six replicate sets of QC samples (LQC, MQC, and HQC). Another blank sample was analyzed immediately following the highest concentration standard in each run to monitor for carry-over of obatoclox or the internal standard.

The calibration curve was developed using the following criteria: (1) the mean value should be within $\pm 15\%$ of the theoretical value, except at the LLOQ, where it should not deviate by more than $\pm 20\%$; (2) the precision around the mean value should not exceed a 15% coefficient of variation (CV), except for LLOQ, where it should not exceed a 20% CV. (3) at least 75% of the non-zero standards of each nominal concentration should meet the above criteria; (4) the correlation coefficient (r) should be greater than or equal to 0.98.

Each calibration curve was constructed by plotting the analyte to internal standard peak area ratio (y) against the analyte concentrations (x). The calibration curves were fitted using a least-square linear regression model $y=ax+b$, weighted by $1/x$ using the KaleidaGraph® software. The resulting parameters were used to determine backcalculated concentrations, which were then statistically evaluated.

2.5.2 Specificity—The specificity was defined as non-interference at retention times of obataclax from the endogenous plasma components and no cross-interference between obataclax and internal standard using the proposed extraction procedure and LC-MS/MS conditions. Six different lots of blank (obataclax free plasma) were evaluated with and without internal standard and low QC (0.1 ng/mL) to assess the specificity of the method. Specificity was also further tested at LLOQ QC (n=6), concentration (0.04 ng/mL).

2.5.3 Precision and accuracy—Precision and accuracy of the method was assessed over a linear range of 0.04-25 ng/ml at eleven concentrations of 0.04, 0.08, 0.12, 0.4, 0.8, 1.2, 4, 8, 12, 18, 25 ng/mL. The intra and inter-assay precisions were determined using the CV (%), and the intra and inter-assay accuracies were expressed as the percent difference between the measured concentration and the nominal concentration. The % accuracy of the method was expressed by the formula: % accuracy = (measured concentration)/(nominal concentration) $\times$ 100. Intra-assay precision and accuracy were calculated using replicate (n=6) determinations for each concentration of the spiked plasma sample during a single analytical run. Inter-assay precision and accuracy were calculated using replicate (n=6) determination of each concentration made on three separate days (total n=18).

2.5.4 Recovery (extraction efficiency) and matrix effect—The extraction efficiency of obataclax was determined by analyzing three replicates of obataclax plasma samples at three QC concentrations of 0.1, 2.5, and 15 ng/mL, respectively. Recovery was calculated by comparing the peak areas of obataclax added into blank plasma and extracted using the protein precipitation procedure with those obtained from obataclax spiked directly into post-protein precipitation solvent at the three QC concentrations. Matrix effect can affect the reproducibility from the analyte or the internal standard of the assay. The matrix effect, or intensity of ion suppression or enhancement is caused by co-eluting matrix components. The matrix effect of obataclax and IS were calculated using the following formula: % matrix effect = $(A/B) \times 100\%$. A represents the corresponding peak areas of the analytes in spiked plasma post-precipitation and B represents peak responses of the pure standards prepared in mobile phases. A value of $>100\%$ indicated ionization enhancement, and a value of $<100\%$ indicated ionization suppression. The matrix effect was tested on the three QC concentrations and three individual lots of blank plasma were evaluated (data not shown). In clinical trials, obataclax formulation contained PEG 300 (11.5%). Based on the formulation

volume and the average approximate blood volume of study subjects, we estimated the physiologically relevant concentration of PEG300 to be 0.5%. Therefore, the effect of PEG on the quantitation of obatoclax was evaluated by comparing the accuracy of QCs with 0.5% PEG spiked QCs at physiologically relevant concentrations.

2.5.5 Stability study—The stability of obatoclax in human plasma was assessed by analyzing replicates QC samples at concentrations of 0.1, 2.5, and 15 ng/mL, during the sample and storage procedure. For all stability studies, freshly prepared and stability testing QC samples were evaluated by using a freshly prepared standard curve for the measurement. The short-term stability was assessed after exposure of the plasma samples to room temperature for 24 hours. The free/thaw stability was determined after three freeze/thaw cycles (room temperature to -80 °C). The concentrations obtained from all stability studies were compared to freshly prepared QC samples that were at room temperature for 4h, and the percentage concentration deviation was calculated. In addition, long-term stability was assessed for QC samples stored at -80 °C for 1 month and 2 years. The analytes were considered stable in human plasma when the concentration difference was less than 15% between the freshly prepared samples and the stability testing samples.

3. Results

3.1 Linearity and sensitivity

The method was validated at the above criteria and found to be linear from 0.04 to 25 ng/mL. A representative calibration curve for obatoclax is shown in Figure 2. The correlation coefficient from inter-day analysis was found to be greater than 0.99 in all cases. The LLOQ was 0.04 ng/mL, demonstrating a %CV of less than 10% (precision) and an accuracy of greater than 98%, with an average signal-to-noise (S/N) ratio of 40. A representative chromatogram of double blank, LLOQ, internal standard and a pediatric pharmacokinetic sample are shown in Figure 3. There was a carryover of 0.08 % for obatoclax. No significant carryover was observed for the IS. Therefore a precaution is required when this method is used to measure samples with a concentration of < 0.1 ng/mL.

3.2. Precision and accuracy

At the eleven calibration standards, the inter-day precision ranged from 2.0-6.7% and the accuracy ranged from 88.6-104.9% (Table 1). All subsequent validation results are depicted in Table 2. The intraday precision ranged from 0.9-5.1% with the accuracy ranging from 98.8-106.8. The inter-day precision ranged from 3.2-7.1% with the accuracy ranging from 102.4-113.3%. These data confirm that the present method has a satisfactory accuracy, precision and reproducibility for the quantification of obatoclax throughout a wide dynamic range.

3.3. Recovery

At 0.1, 2.5, and 15 ng/mL QC concentrations the percent extraction recoveries (mean $\pm$ % SD) after five replicates were 65.6 ± 4.4 , 65.1 ± 1.4 , and 62.3 ± 1.4 , respectively (Table 2). Data indicated that the extraction efficiency for obatoclax using protein precipitation not concentration dependent and was satisfactory for quantification using an internal standard.

3.4. Assay specificity and ionization suppression (matrix effect)

No significant differences were observed between the pure standards and the postextracted spiked samples, illustrating that the HPLC separation conditions had little effect by background signal of plasma after simple protein precipitation sample preparation. In our initial studies, the specificity was tested at low QC level (0.1 ng/mL) in six different lots of plasma. The accuracies ranged from 103.0 to 112.0% (mean accuracy and precision were: $107.5 \pm 2.7\%$). In the follow-up studies we have further tested specificity at LLOQ (0.004 ng/mL) QCs (n=6) and the results showed that accuracy and precision were acceptable (mean accuracy and precision were: 100.4 ± 9.1). These studies confirm that the assay had acceptable specificity. Accuracies for obatoclox QCs with and without PEG were identical suggesting that use of stable isotope-labeled obatoclox internal standard effectively canceled out ion suppression effects of PEG at physiologically relevant concentrations (0.5% PEG300). This is consistent with previously reported results that the ion suppression effects of matrix components is effectively canceled out by the stable isotope-labeled internal standard, as long as they are co-eluted from the LC column[7].

3.5. Analyte stability

The stability of obatoclox was investigated to cover expected conditions during sample preparation and storage for all samples, which include stability data from freeze/thaw and bench top, as well as short-term and long-term stability (Table 3). The precision for freeze/thaw samples ranged from 5.4-10.7% and the remaining analyte ranged from 86.1-93.6%. The results indicated that the analyte was stable in plasma for three cycles when stored at -80 °C and thawed to room temperature. The precision for 24h stability at room temperature ranged from 5.4-10.7% and the remaining analyte ranged from 97.0-99.8% (Table 3). The precision for stability over 1 month and 2 year at -80 °C ranged from 7.9-12.5% and 8.1-17.3%, respectively. The remaining analyte over 1m and 2y at -80 °C ranged from 92.4-103.0% and 83.4-101.3%, respectively. This indicated reliable stability under the experimental conditions of the analytical runs.

4. Discussion

We developed an obatoclox assay that has simple and rapid sample preparation and a selective and sensitive LC-MS/MS method capable of analyzing small volumes of plasma samples needed for use in pediatric subjects. The LC-MS/MS assay reported here accurately and precisely quantitates obatoclox concentration in 50 µL plasma specimens with a limit of quantification of 0.04 ng/mL. The intra-day and inter-day coefficients of variation were less than 5.2 and 7.2%, respectively, at all concentrations tested. With linearity up to 25 ng/mL, the assay is well suited for use in pediatric pharmacokinetic studies. We are currently utilizing this assay to support a Children's Oncology Group Phase 1 Consortium study of obatoclox.

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Highlights

- We describe a HPLC tandem mass spectrometry assay for obatoclax, a BCL-2 inhibitor.
- The analytical method showed excellent sensitivity, precision, and accuracy.
- This method is being used in a phase 1 study in children with relapsed cancer.

Obatoclax (GX15-070)
Molecular Formula: $C_{20}H_{19}N_3O$
Molecular Weight: 317.38

Figure 1.
Molecular structure of obatoclax. Internal standard is deuterium labeled obatoclax (GX 15-070-d5).

Figure 2.
Calibration Curve for obatoclox in human plasma.

Figure 3. Chromatograms of obatoclox in human plasma. (A) Double blank; (B) LLOQ 0.04 ng/mL (MRM: 318.1/286.1); (C) Internal Standard (MRM: 323.1/291.1); (D) Representative chromatogram of pharmacokinetic sample from the clinical study.

Table 1
Precision and accuracy and precision of back-calculated obatoclax calibration standards
in human plasma

Nominal Concentration (ng/mL)	Measured Mean Concentration (ng/mL) (n=6)	CV (%) (n=6)	Accuracy (%) (n=6)
0.04	0.0390	6.2	97.6
0.08	0.0709	6.7	88.6
0.12	0.109	3.8	91.0
0.4	0.403	5.7	100.6
0.8	0.819	3.6	102.4
1.2	1.23	2.6	102.8
4	3.78	3.1	94.5
8	7.79	3.0	97.4
12	11.9	4.5	98.8
18	18.9	3.6	104.9
25	24.7	2.0	98.9

Table 2

Summary of validation outcomes for obatoclox in human plasma

QC Nominal Conc. (ng/mL)	Intra-Day (n=6)			Inter-day (3 days) (n=18)			Extraction Recovery (n=6)			PEG Effect* (n=6)		
	Mean	CV (%)	Acc (%)	Mean	CV (%)	Acc (%)	Rec (%)	SD (%)	CV (%)	PEG Effect (%)	SD	CV (%)
0.1	0.107	5.1	106.8	0.113	7.1	113.3	65.6	4.4	6.7	98.7	9.7	9.8
2.5	2.62	1.4	104.8	2.63	3.2	105.2	65.1	1.4	2.1	108.9	6.0	5.5
15	14.8	0.9	98.9	15.4	4.1	102.4	62.3	1.4	2.3	113.0	8.1	7.2

* The effect of PEG in samples was determined by comparing obatoclox QCs with and without PEG ($([QC_{PEG}]/[QC]) \times 100$) where 100% PEG effect refers to no effect of PEG on the measured obatoclox concentration.

Table 3
Summary of stability outcomes for obatoclax in human plasma

Nominal Conc. (ng/mL)	Stability RT 24h (n=5)			Stability 1month (-80 ° C) (n=8)			Stability 2years (-80 ° C) (n=8)			Stability, three cycles freeze (-80 ° C)/thaw (n=6)		
	Mean	CV (%)	Remaining Analyte (%)	Mean	CV (%)	Remaining Analyte (%)	Mean	CV (%)	Remaining Analyte (%)	Mean	CV (%)	Remaining Analyte (%)
86.1	0.103	10.7	98.9	0.100	7.9	103.0	0.0981	8.1	101.3	0.0897	10.7	86.1
2.5	2.60	5.4	97.0	2.46	11.2	93.8	2.19	17.3	83.4	2.51	5.4	93.6
5	14.6	10.1	99.8	13.4	12.5	92.4	14.6	8.3	100.9	13.0	6.1	89.0