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PROTEIN PRECIPITATION METHOD FOR DETERMINATION OF OXCARBAZEPINE AND ITS 10-MONOHYDROXY DERIVATIVE IN HUMAN PLASMA BY LC-MS/MS

Margaryan T.S.[1,2], Zakaryan H.A.[1], Armoudjian Y.G.[1], Gevorgyan A.M.[1], Aleksanyan A.A.[1], Sargsyan M.S.[1], Harutyunyan A.S.[1], Alaverdyan H.R.[1], Mikayelyan A.V.[1]*

[1] DarmanTest Laboratories LLC, Yerevan, Armenia
[2] Scientific Research Center, Yerevan State Medical University after M. Heratsi, Yerevan, Armenia

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Abstract

Protein precipitation method for the determination of oxcarbazepine and its major active metabolite 10-monohydroxy derivative in human plasma by liquid chromatography tandem mass spectrometry was established. Analytes were extracted from human plasma samples by protein precipitation with acetonitrile. Analyte separation was done using Phenomenex Kinetex™ Biphenyl (2.6 µm, 100×2.1 mm) column using isocratic elution with a mobile phase of 5 mM ammonium bicarbonate (42.5%) and methanol (57.5%) at a flow rate of 0.3 mL/min and an injection volume of 5 µL. The detection was performed on a triple quadrupole mass spectrometer by multiple reaction monitoring mode to monitor the precursor-to-product ion transitions of m/z 253.1 → 180.0, 257.2 → 184.1 for oxcarbazepine and oxcarbazepine-D4, and 255.1 → 194.2, 259.2 → 198.2 for 10-monohydroxy derivative and 10,11-Dihydro-10-hydroxy carbamazepine-D4 in positive electrospray ionization mode, correspondingly. The method was validated over a concentration range of 20-8000 ng/mL for oxcarbazepine and 37.5-15000 ng/mL for 10-monohydroxy derivative on SCIEX Triple Quad 4500 MS System. Total run time was 4 min. Sample preparation was conducted in ice water bath because of instability of oxcarbazepine in plasma at room temperature. The method was validated in accordance with U.S Food and Drug Administration and European Medicines Agency guidelines. Method validation has been proved by a variety of tests for matrix effects, extraction efficiency, selectivity, linearity, sensitivity, precision, recovery and stability and can be successfully applied for the bioequivalence/pharmacokinetic studies of oxcarbazepine and its 10-monohydroxy derivative. This method has been designed for bioequivalence study for formulations containing 600 mg of oxcarbazepine.

Keywords: *oxcarbazepine, MHD, LC-MS/MS, plasma, bioequivalence, pharmacokinetics.*

Introduction

Oxcarbazepine (OCBZ) is a carbamazepine derivative, anticonvulsant which is used for the treatment of partial seizures [*Degen P et al., 1994; Schmidt D, Elger C, 2004*]. After oral administration it is rapidly and almost completely metabolized to the biologically active 10-monohydroxy derivative (MHD) through a reduction of the carbonyl group [*Shorvon S, 2000; May T et al., 2003*]. After single oral dose of OCBZ (30 *mg/kg*) in patients with epilepsy the maximum peak plasma concentration (C_{max}) of OCBZ (6.89 *µg/mL*) and MHD (26.83 µg/mL) reaches after 2 and 6 hours correspondingly [*Kim D et al., 2012*]. Following oral administration of 600 *mg* of OCBZ mean Cmax of parent drug and metabolite were about 1.25 and 6.8 *µg/mL* [*Bhatt M et al., 2011*].

This method has been designed for bioequivalence study for formulations containing 600 mg of OCBZ. According to U.S. Food and Drug Administration (US FDA) guidance on OCBZ [*FDA, 2008*] for the bioequivalence studies OCBZ and active metabolite 10-monohydroxy derivative (MHD) in plasma should be measured using an achiral assay. Based on pharmacokinetic data review, for 600 *mg* single oral dose of OCBZ it is sufficient to have

Address for Correspondence:
Astghik V Mikayelyan
DarmanTest Laboratories
LLC, 6/1 Abelyan street, Yerevan 0038, Armenia
Tel.: +374(77)288267
E-mail: mikastgh@gmail.com

lower limit of quantification (LLOQ) of 20 and 40 *ng/mL* for OCBZ and MHD correspondingly.

There are many published LC methods for determination of OCBZ and MHD in biological fluids where solid phase extraction or liquid-liquid extraction were used as a sample preparation [*Souppart C et al. 2001; Levert H et al., 2002; Maurer H et al., 2002; de Sousa Maia M et al. 2007; Kimiskidis V et al., 2007; Srinubabu G et al., 2008; Bhatt M et al., 2011*]. As it is known, protein precipitation (PPT) is the simplest, easiest and fastest sample preparation method. There are few published paper were PPT was used. In one paper 50 *ng/mL* and 1.5 µg/mL was established as an LLOQ for OCBZ and MHD correspondingly, by using 100 µL of sample volume on C18 column with gradient elution [*Kim D et al., 2012*]. There is only one paper which was published recently, dedicated for bioequivalence studies, where PPT with LC-MS/MS detection was used [*Mano Y, 2018*]. Published stability data is contradictory. Instability of OCBZ in plasma at room temperature is well known. In some papers up to 6-7 hours of bench-top stability was approved [*de Sousa Maia M et al. 2007; de Jesus Antunes N et al., 2013; Mano Y, 2018*]. Limited stability at -20^oC was also reported [*Souppart C et al., 2001; Maurer H et al., 2002*]. During our method development we could not validate stability of OCBZ in plasma at room temperature for more than 1.5 hour. Freeze thaw stability (2 hours of thaw cycle) and 15 days at -20 ^oC were also failed. Even when samples were stored at -70 ^oC freeze thaw stability failed. To bypass stability issues of OCBZ following conditions were applied: sample preparation in ice water bath, -70 ^oC for sample storage and fast freeze with dry ice for freeze thaw samples.

This paper demonstrates a simple, sensitive and rapid method for simultaneous determination of OCBZ and MHD in human plasma. Presented method was fully validated in accordance with US FDA [*FDA, 2018*] and European Medicines Agency (EMA, 2011) guidelines and can be successfully applied for the bioequivalence/pharmacokinetic studies of OCBZ.

Materials and methods

Chemicals and reagents/Materials and reagents: OCBZ (purity 97%) (Fig. 1A) and Oxcarbazepine-D4 (OCBZ IS) (purity 99.9%) were received from Toronto Research Chemicals (Toronto, Canada). MHD (purity 97%) (Fig. 1B) and 10,11-Dihydro-10-hydroxy Carbamazepine-D4 (MHD IS) (purity 99.2%) were received from TLC Pharmaceutical Standards Ltd (Ontario, Canada). Co-medications were represented in Cotinine, Ibuprofen, Caffeine, Nicotine and Acetylsalicylic acid. HPLC grade acetonitrile, methanol, isopropanol, water and 99% grade ammonium bicarbonate were purchased from Carl Roth (Germany). For validation study 6 plasma matrices (K2EDTA as an anticoagulant) were used and were obtained from "ASTGHIK" Medical Center Yerevan, Armenia. The fresh whole blood, hemolytic blood and hyperlipidemic plasma were received in DTL premises.

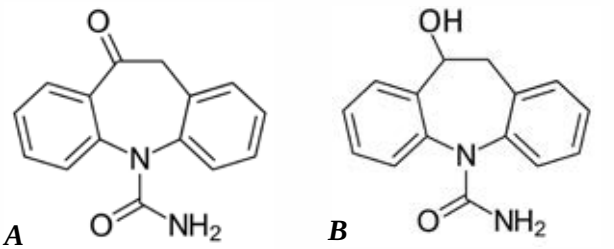

Figure 1. *Structural formula of Oxcarbazepine (A) and 10-monohydroxy derivative (B)*

Instrumentation and chromatographic conditions: Plasma samples were analyzed using Shimadzu Nexera X2 UHPLC system coupled with Sciex Triple Quad 4500 MS/MS (Toronto, Canada) equipped with an electrospray ionization (ESI) source (Sciex, Toronto, Canada). Chromatography separation was carried out on Phenomenex KINETEX™ Biphenyl (2.6 *µm*, 100×2.1 *mm*, 100Å) column at 40°C. The autosampler temperature was 4°C. The mobile phase was 5 *mM* Ammonium Bicarbonate (42.5%) and Methanol (57.5%). The flow rate of 0.3 *mL/min*, the run time was 4 min with an injection volume of 5 *µL*. Average retention times were 1.76, 1.78, 2.67 and 2.71 for MHD IS, MHD, OCBZ IS and OCBZ correspondingly. Typical chromatogram of the analytes is shown in Figure 2. Mass spectrometry parameters are presented in table 1. The data were acquired and analyzed by Analyst software version 1.6.2 (Sciex, Toronto, Canada).

Preparation of calibration standards and quality control samples: The stock solutions for OCBZ (1.6 *mg/mL*), MHD (3 *mg/mL*) and OCBZ IS, MHD IS as an internal standards (IS) (0.5 *mg/mL*) were prepared in acetonitrile. Working solutions for calibration curve standards, quality controls (QC)

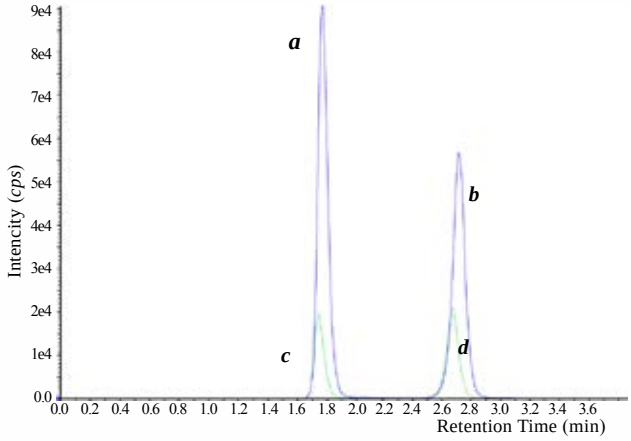

Figure 2 *Chromatograms of 10-monohydroxy derivative (a) and Oxcarbazepine (b) and its internal standards (c, d) respectivelly*

were prepared from stock solutions, by dilutions with acetonitrile. OCBZ/MHD IS-Precipitation Solution (400/200 *mg/mL*) was prepared in acetonitrile. Calibration standards were freshly spiked for every batch. QC samples were prepared in bulk by spiking appropriate amounts of working solutions into blank human plasma with 1:50 ratio. Final concentrations in human plasma of the calibration standards were 20, 40, 100, 260, 800, 2000, 4000, 8000 *ng/mL* for OCBZ and 37.5, 75, 187.5, 487.5, 1200, 3750, 7500, 15000 *ng/mL* for MHD. Five QC: lower limit of quantitation quality control (LLOQ QC), low quality control (LQC), medium quality control (MQC), high quality control (HQC) and out of calibration curve quality control (OCC QC), levels were used during method validation. The concentrations of QC samples (LLOQ QC, LQC, MQC, HQC and OCC QC) in plasma were 20, 60, 400, 6400, 16000 *ng/mL* for OCBZ and 37.5, 112.5, 750, 12000, 30000 *ng/mL* for MHD. All stock solutions and working solutions were stored at 4°*C*, QC samples were stored at -70°C. Independent stock solutions were used to prepare calibration standards and QC samples.

Sample preparation: Before analysis, the plasma sample was thawed in ice water bath. 300 *µL* of IS precipitation solution was added into 100 *µL* plasma sample for protein precipitation. After centrifuging at 8000 *rpm* for 5 minutes at 4°C, 100 µL of supernatant was diluted with 1000 *µL* acetonitrile:water (9:41) mixture in a glass vial. 5 *µL* was injected into LC–MS/MS system for analysis.

Method validation: Method validation was performed to evaluate the sensitivity, selectivity, linearity, precision, accuracy, ruggedness, hyperlipidemia effect, carry over, matrix effect, maximum batch size, dilution integrity and various stability tests according to US FDA [*FDA 2018*] and EMA guidelines.

Results and discussion

Method Validation Results: Selectivity (Blank Check, Interference Check, Metabolite and concomitant medications interference)

Six independent blank lots were screened for possible interference at the retention time of the analytes and the ISs. The interference in the blank was calculated using the peak area of the analyte

Table 1

MS parameters

Parameter	Analytes		Internal Standarts	
	OCBZ	MHD	OCBZ D4	MHD D4
MRM transition	253.1→180.0	255.1 – 194.2	257.2→184.1	259.2 – 198.2
Declustering potential, V	81	65	81	56
Collision energy, V	39	27	39	27
Entrance potential, V	10			
Collision cell exit potential, V	8			
Dwell time per transition, ms	100			
Curtain gas, psi	40			
Collision gas, psi	8			
Ion spray voltage, V	5500			
Source temperature, °C	425			
Nebulizer gas (GAS1), psi	40			
Heater gas (GAS2), psi	40			

and the IS at their respective retention times in the LLOQ sample.

An extracted solution of the Analyte at approximately the highest standard concentration was prepared without the IS in triplicate and injected to observe any possible interference at the retention time of the IS. An extracted solution of the IS at approximately the concentration equivalent to the IS concentration were prepared without the Analyte in triplicate and injected to observe any possible interference at the retention time of the Analyte.

Interference from concomitant medications (Nicotine, Cotinine, Ibuprofen, Caffeine and Acetylsalicylic acid) was evaluated by spiking Blank and LQC samples with the concomitant medications to obtain final concentrations of 1 µg/mL for nicotine and cotinine, 50 µg/mL for ibuprofen and acetylsalicylic acid and 10 µg/mL for caffeine appropriately.

The maximum interference at the retention time of OCBZ, MHD, OCBZ IS, and MHD IS were 2.4%, 2.6%, 0.0% and 0.1% correspondingly. Typical chromatograms of analytes in Blank and LLOQ samples are shown in figures 3 and 4

Calibration curve and sensitivity: Linear regression with 1/×2 weighting factor was the simplest model, which describes the concentration-response relationship. The calibration curve was linear over the concentration range of 20-8000 *ng/mL* for OCBZ and 37.5-15000 *ng/mL* for MHD. The average %Accuracy for calibration standards except of the LLOQ were between 97.4% - 101.1% for OCBZ and 97.8% - 102.0% for MHD. The average %Accuracy for LLOQ was 99.9% and 99.3% for OCBZ and MHD correspondingly. The highest %CV of the calibrators except of the LLOQ was 3.0% for both analytes. %CV values of the LLOQ were 0.3% for OCBZ and 0.8% for MHD.

Precision and accuracy: Seven different precision and accuracy batches were analyzed on seven different days. The %CV and %Accuracy of QCs at each level among nine batches (inter-assay) were within acceptable limits for both OCBZ and MHD. The %Accuracy of LLOQ QC was 97.5% and 96.8%, %CV was 3.2% and 4.9% for OCBZ and MHD correspondingly. For the rest of QCs %Accuracy was within 97.2% - 97.3% and 94.3% - 95.3% ranges and the highest %CV was 3.2% and 4.9% for OCBZ and MHD correspondingly. The %CV and %Accuracy of QCs at each level within each batch (intra-assay) was within acceptable limits for nine batches. For seven batches the %Accuracy of LLOQ QC was within 93.2% - 101.6% and 89.2% - 103.7% ranges, and the highest %CV was 3.4% and 3.1% for OCBZ and MHD correspondingly. For the rest of QCs %Accuracy was within 92.7% - 101.2% and 88.3% - 97.5% ranges, the highest %CV was 2.9% and 2.0% for OCBZ and MHD correspondingly. Results are presented in table 2.

Matrix effect: Matrix effects were investigated using six lots of blank matrix from individual donors. For each Analyte and IS, the matrix factor was calculated for each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with Analyte after extraction), to the peak area in the absence of matrix (pure solution of the Analyte). The IS normalized matrix factor (MF) was also calculated by dividing the MF of the Analyte by the MF of the IS. This determination was done in three replicates at LQC and HQC concentrations.

The %CV of IS-normalized MF for OCBZ LQC

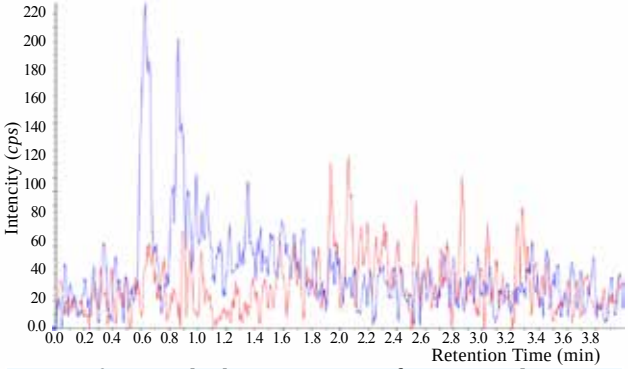

Figure 3 *Typical Chromatogram of OCBZ and MHD in Blank sample*

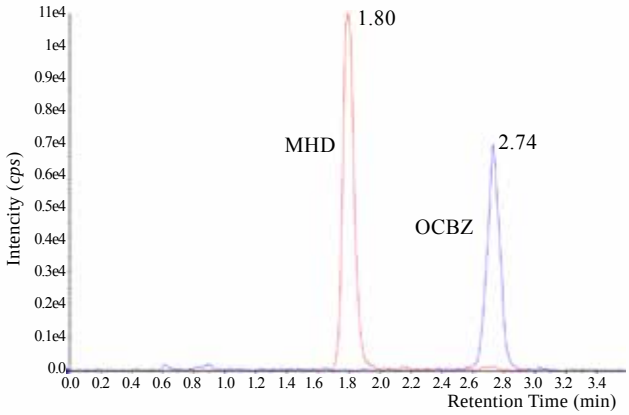

Figure 4 *Typical Chromatogram of OCBZ and MHD in LLOQ sample*

Table 2

OCBZ and MHD Inter-assay (overall) Precision and Accuracy Results for Batches 1 through 7

QC Level	NC (*ng/mL*)	Intra-batch (first batch)				Inter-batch			
		n	MCC. (*ng/mL*)	Accuracy (%)	CV (%)	n	MCC (*ng/mL*)	Accuracy (%)	CV (%)
OCBZ									
lower limit	20	6	19.16	95.6	1.8	42	19.54	97.5	3.2
low	60	6	57.32	95.4	1.2	42	58.45	97.3	2.1
mid	400	6	371.22	92.7	0.8	42	389.92	97.3	2.7
high	6400	6	5952.16	92.9	0.7	42	6226.58	97.2	3.0
MHD									
lower limit	37.5	6	35.07	93.5	3.1	42	36.29	96.8	4.9
low	112.5	6	103.61	92.1	0.7	42	107.25	95.3	2.1
mid	750	6	662.18	88.3	1.3	42	707.17	94.3	3.2
high	12000	6	10804.22	90.0	1.0	42	11355.66	94.6	2.9

Note: NC – Nominal concentration, MCC - Mean Calculated concentration, CV - coefficient of variation, QC - quality control

and HQC samples prepared from 6 matrices were 1.4% and 1.1%, and for MHD LQC and HQC were 1.5% and 0.8% correspondingly. The results indicate that after IS normalization matrix effect is within acceptability limits.

Recovery: Recovery pertains to the extraction efficiency of a bioanalytical method within the limits of variability. The recovery of Analytes from human plasma was evaluated by comparing the peak area ratios of 6 replicates of extracted QC samples, versus the mean peak area ratios of 6 replicates of post-extraction spiked samples representing 100% recovery. For this experiment the IS was spiked after extraction to compensate the variation in chromatography and MS/MS detection. The evaluation was assessed at low, medium and high QC concentrations. The recovery of ISs was evaluated by comparing the IS/Analyte peak area ratios of 6 replicates of extracted samples versus the mean IS/Analyte peak area ratio of 6 replicates of post-extraction spiked samples representing 100% recovery. The evaluation was assessed at a single concentration equivalent to that of the ISs in MQC samples. For this test the Analytes were spiked at MQC level after extraction to compensate the variation in chromatography and MS/MS detection.

The %CV of recovery samples for OCBZ was NMT 1.6% and for OCBZ IS was NMT 1.5%. The %CV of recovery samples for MHD was NMT 1.0% and for MHD IS was NMT 0.9%. The recovery of OCBZ and OCBZ IS was 102.1% - 103.1% and 104.4 % correspondingly. For MHD and MHD IS the recovery was 99.8% - 101.8% and 99.8% correspondingly.

Carry over: The carry over effect was monitored prior to the injection of every analytical run as part of system suitability. Additionally, the carry over effect was investigated by injection of three replicates of Blank sample and Diluent after OCC QC sample. OCC is 2 times the ULOQ. The maximal carry over for OCBZ after OCC QC sample was 0.3%. The maximal carry over for MHD after OCC QC sample was 5.0%. For the IS Carry Over test values were NMT 0.1% for OCBZ IS and NMT 0.0% for MHD IS.

Ruggedness: The ruggedness test was performed during precision and accuracy batch testing by four different chemists on two different columns.

Hyperlipidemia Effect: The impact of the high level of lipids was assessed by employing an additional lot of Analyte free lipemic matrix for the preparation of each of the low and high QC samples. Six replicates at each of the low and high QC sample concentration levels and one Blank sample were prepared and assayed for this matrix lot. The plasma is considered lipemic (hyperlipidemic), if the triglyceride concentration is more than 300 *mg/*

dL. The mean values of the OCBZ lipemic LQC and HQC samples were 93.9% and 95.0% for %Accuracy and NMT 1.2% for %CV. The mean values of the MHD lipemic LQC and HQC samples were 94.5% and 94.3% for %Accuracy and NMT 1.2% for %CV. Interference from the lipemic Blank was 0.2 % at the retention time of the OCBZ and 0.4% at the retention time of MHD, 0.0% at the retention time of OCBZ IS and 0.0% at the retention time of MHD IS. Hence, lipemic samples can be assayed for OCBZ and MHD determination by the described method.

Dilution Integrity: Dilution integrity was validated by diluting six replicates of OCC QC and six replicates of MQC with blank biological matrix using dilution factor 5. For OCBZ and MHD the mean value of diluted samples was within ±15.0% of the nominal value (102.6% and 113.3%) and %CV was within 15.0% (0.6% and 1.1%) correspondingly. Thus, when the calculated concentration of the samples is above the upper limit of quantification, samples can be diluted 5 times, if necessary, to fit within the calibration curve.

Stability: The stability of the analytes was investigated under a variety of storage and process conditions for both Analytes. All established stabilities for the analyte were summarized in table 3 for OCBZ and MHD. Freeze-Thaw (3 cycle), Short-Term Room Temperature (2 hours in ice water bath), Long-term at -700C (37 days) stability of analyte in human plasma were assessed by analyzing six replicates (six different tubes) of QC samples at the low, high and OCC concentrations. Freeze-Thaw samples before placing back to freezer were fast frozen with dry ice. Processed sample stability at room temperature (4 hours) and autosampler stability (4°C nominal for 48 hours) were assessed by six replicates of QC at low and high concentrations. All stability tests were calculated along with a freshly extracted calibration curve and QC samples. Reinjection reproducibility was assessed within autosampler stability period.

Stability of OCBZ and MHD in human whole blood were assessed by comparing six replicates of stability whole blood samples, maintained in

Table 3

Stability Results for OCBZ and MHD

		OCBZ				MHD			
Stability	QC Level	NC (*ng/mL*)	MCC (*ng/mL*)	Accuracy (%)	CV (%)	NC (*ng/mL*)	MCC (*ng/mL*)	Accuracy (%)	CV (%)
Bench Top Stability (2 hours)	*low*	60.08	68.08	113.3	3.4	112.51	124.44	110.6	2.8
	high	6408.98	7004.21	109.3	2.8	12000.69	12920.93	107.7	3.2
	out of CC	16022.46	17753.04	110.8	1.6	30001.72	32643.68	108.8	1.1
Freeze Thaw Stability (3 cycle)	*low*	60.08	62.67	104.3	2.2	112.51	114.46	101.7	1.9
	high	6408.98	6428.84	100.3	1.4	12000.69	11908.17	99.2	1.8
	out of CC	16022.46	16478.36	102.8	0.7	30001.72	30512.94	101.7	1.5
Auto-sampler Stability (48 hours)	*low*	60.08	52.82	87.9	1.4	112.51	102.76	91.3	1.1
	high	6408.98	5767.18	90.1	0.6	12000.69	10836.47	90.3	1.1
Processed Sample Stability (4 hours)	*low*	60.08	57.28	95.3	2.1	112.51	101.93	90.6	1.2
	high	6408.98	6130.04	95.6	1.2	12000.69	10907.80	90.9	1.4
Long-term stability (at -70°C for 37 days)	*low*	60.08	64.11	106.7	1.8	112.51	119.39	106.1	1.0
	high	6408.98	6652.19	103.8	1.3	12000.69	12435.53	103.6	1.0
	out of CC	16022.46	16262.45	101.5	1.7	30001.72	30204.56	100.7	1.0

Note: *NC – Nominal concentration, MCC - Mean calculated concentration, CV - coefficient of variation, QC - quality control, CC - calibration curve*

an ice water bath for 2 hours, versus six replicates of comparison samples at both low and high QC concentrations taken from aliquots at the beginning of experiment.

Maximum Batch Size: The maximum batch size was tested by including in one analytical batch more samples than the expected maximal batch size during subject sample analysis. The maximum batch size was determined by analyzing a batch containing CCS samples and LQC, MQC and HQC samples. CCS samples were injected at the start and same CCS samples were reinjected at the end of the run. Overall number of injections of QC, system suitability and calibration curve samples within this run was 289 samples.

Conclusion

Simple PPT method with LC-MS/MS for determination of OCBZ and MHD in human plasma has been described. Method validation has been proved by a variety of tests for matrix effects, extraction efficiency, selectivity, linearity, sensitivity, precision, recovery and stability. Sample preparation was conducted in ice water bath because of instability of OCBZ in plasma at room temperature. The method was validated in accordance with US FDA and EMA guidelines and can be successfully applied for the bioequivalence/pharmacokinetic studies of OCBZ and MHD. This method has been designed for bioequivalence study for formulations containing 600 mg of OCBZ.

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