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A SELECTIVE AND SENSITIVE LIQUID CHROMATOGRAPHIC/ TANDEM MASS SPECTROMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF OSELTAMIVIR AND ITS METABOLITE OSELTAMIVIR CARBOXYLIC ACID IN HUMAN PLASMA FOR BIOAVAILABILITY OR BIOEQUIVALENCE STUDIES

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ABSTRACT

Selective and sensitive liquid chromatographic/tandem mass spectrometric method was developed and validated for the simultaneous estimation of Oseltamivir and its metabolite Oseltamivir carboxylic acid in Human plasma containing CPDA as an anticoagulant. A plasma sample of 0.5ml was extracted by single step solid phase extraction method using Oasis® HLB (30 mg/cc) cartridges and peak of interest on Hydrosphere column C18 (150 x 4.6 mm, particle size 5µm, pore size 12nm) using a isocratic flow of Acetonitrile and 0.1% Formic acid (90:10, v/v) as mobile phase. The quantitation was carried out using a triple quadruple API-3200 LC/MS/MS with positive electro spray ionization in multiple reaction-monitoring (MRM) mode. The parent to product ion transitions for Oseltamivir, Oseltamivir carboxylic acid & Oseltamivir-d3 (internal standard) were m/z 313.20 > 166.00; 285.30 > 138.20; 316.40 > 228.01, respectively. The method was validated as per US FDA guidelines to establish selectivity, sensitivity, matrix factor, linearity of response, accuracy, precision, recovery, stabilities, dilution integrity, ruggedness reinjection reproducibility and extended batch verification. The weighted ($1/x^2$) calibration curves were linear over a range of 2.08 ng/mL to 241.12 ng/mL for Oseltamivir and 10.8 ng/mL to 1251.8 ng/mL for Oseltamivir carboxylic acid with coefficient of correlation (r) >0.9994 for Oseltamivir and >0.9982 for Oseltamivir carboxylic acid in a course of precision & accuracy batches. The mean % recovery of spiked

quality control samples was >83% and >72% for Oseltamivir and Oseltamivir carboxylic acid, respectively, while the % recovery of Oseltamivir-d3 in spiked samples was >72%.

Keywords: *Oseltamivir, Oseltamivir carboxylic acid, LC-MS/MS, Method validation.*

INTRODUCTION

Oseltamivir is a potent neuramidase inhibitor effective against both influenza A and influenza B. Oseltamivir phosphate [(3R,4R,5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)1-cyclohexene-1-carboxylic acid,ethyl ester, phosphate (1:1)] is an ethyl ester prodrug of the active metabolite, oseltamivir carboxylate. After oral administration, Oseltamivir (OSE) is readily absorbed from gastro intestinal track and rapidly converted; predominately by hepatic esterases to active metabolite oseltamivir carboxylate (OCA). At least 75% dose reaches the systemic circulation as OCA, which is a selective inhibitor of influenza virus neuraminidase enzymes with the possibility of alteration of virus particle aggregation and release. OCA is eliminated entirely (>90%) by renal excretion [1-3].

Many analytical methods have been reported for the simultaneous estimation of OSE and OCA in different biological matrices e.g., rat plasma [4, 5], cerebro-spinal fluid, brain [5], rodent blood by dried blood spot technique (DBS) [6], human plasma [7-9], saliva [7], urine [7] and serum [10]. Gholamreza et al, reported HPLC-UV method for estimation of OCA in human serum [10]. Kaneeti R et al. developed and validated method in plasma by solid phase extraction procedure using Chromopack (C18) column, with acyclovir as an internal standard [11]. Lindergardh et. al employed off-line solid phase extraction method for estimation in plasma, saliva and urine, using ZIC-HILIC column with mobile phase in gradient mode [7]. Kromdijk et al, validated a method in plasma containing fluoride EDTA as an anticoagulant [12].

The published methods were either nonspecific for a typical bioavailability / bioequivalence (BA/BE) study or there were issues of compliance [4-7, 10, 13]. The published methods, lack parameters like matrix factor [4, 11, 13], extended batch verification [4-11, 13], stock solution stability [4-7, 13]. The precision and accuracy batch should contain enough test samples to mimic the analytical batch of subject sample analysis in BA /BE study. There were not enough long batches to represent the analytical batch in the published articles. FDA may challenge this deficiency in study specific audits where there may be issues with the ruggedness [14].

Owing to these challenges a regulatory compliant method was developed; which was also simple and easy to use. It's a time efficient method, since it did not involve steps like drying and reconstitution. The method was modified based on the fact that human plasma contains esterase enzymes. Butyrylcholinesterase, paraoxonase and albumin esterase present in human plasma are responsible for the esterase hydrolysis of the ester drugs [15]. Lindergardh et al, concluded that plasma esterase activity can cause significant degradation of Oseltamivir phosphate in blood/plasma samples under conditions likely to be encountered during the clinical studies and during assay preparation. Use of esterase inhibitor is suggested as possible remedy [16]. Esterase inhibitor (Dichlorvos) [17] was used to prevent the ester hydrolysis of OSE to its active metabolite. The validation protocol was followed as per the recommendations of United States Food and Drug Administration (USFDA) and European Medicines Agency (EMEA).

MATERIALS AND METHODS

Chemicals and reagents

Reference standards Oseltamivir phosphate (purity: 99.5%), OCA (purity: 97.2%) and internal standard Oseltamivir-d3 (OSE-d3) (purity: 99.70%) were obtained from USP, Synfine Research (Canada) and Varada Biotech (P) Ltd (Mumbai, India) respectively. Human plasma from healthy volunteers, with CPDA (citrate phosphate dextrose adenine) as anticoagulant, was obtained from CRTS Hospital Med V, El Jadida, Morocco. HPLC-grade acetonitrile from Rankem (India) and methanol from Sigma Aldrich (USA). Analytical grade Ammonium acetate, formic acid and liquor ammonia from Panreac (Barcelona, Spain). HPLC grade water was obtained from a Milli-Q gradient A10 purifier from Millipore Corporation (Massachusetts, USA). SPE Oasis® HLB (30mg/1cc) cartridges were purchased from Waters (Dublin, Ireland).

Preparation of calibration curve and quality control samples

Standard stock solutions for Oseltamivir phosphate, OCA and OSE-d3 (internal standard) were prepared in methanol to get a concentration of approximately 1mg/ml. The mother stock was diluted with methanol: water (50:50 v/v) to get the working calibration solutions of appropriate concentrations. Dichlorvos was added to the plasma (200 µg/ml) and properly mixed. The working solutions for calibration curve and quality control samples were spiked to get the concentration (2.08-241.12 ng/mL) for OSE, and (10.8-1251.8 ng/mL) for OCA. Quality control samples at concentrations (2.09 ng/mL) LLOQQC, (5.84 ng/mL) LQC,

(117.99 ng/mL) MQC and (181.52 ng/mL) HQC for OSE and (11.6 ng/mL) LLOQQC, (32.4 ng/mL) LQC, (655.3 ng/mL) MQC and (1008.1 ng/mL) HQC for OCA were prepared. These spiked calibration curve standards and quality control samples were aliquoted and stored into -70°C deep freezer until the analysis.

Plasma Sample Preparation

The sample preparation involved offline SPE technique to extract drug from the plasma samples using Oasis® HLB cartridges (30mg/1cc). The samples were retrieved from the deep freezer, thawed at room temperature and homogenized with vortex mixer. To 500µL aliquot of plasma, 50µL of internal standard working solution (approximately 5000 ng/mL) was added and vortexed. This was followed by addition of 500µL of 2mM ammonium acetate buffer (pH 2.5) and vortexing. The cartridges were conditioned with 1 mL of methanol followed by 1 mL Milli-Q water. Samples were loaded on the SPE cartridge. Post loading phase, cartridges were washed twice with 1mL of washing solution (Formic acid: Milli-Q water, 5:95 v/v). The samples were eluted with 500 µL of elution solution (0.1% Formic acid: acetonitrile, 10:90 v/v). The samples were transferred to the vials for analysis.

Liquid Chromatography and mass spectrometric conditions

The sample volume of 5 µL was separated using hydrosphere C18 (150 x 4.6 mm, particle size 5µm, pore size 12nm, YMC Co. Ltd, Japan) column on a Perkin-Elmer HPLC (200 series) system. The instrument was configured with vacuum degasser, binary pumps and autosampler. An isocratic flow of Acetonitrile and 0.1% Formic acid (90:10, v/v) at 0.500 mL/min was optimized to achieve the separation. Autosampler was maintained at 10ºC ± 2.0 ºC. The samples were detected using API 3200 triple qudrupole mass spectrometry (Applied Biosystems/MDS Sciex, Canada) equipped with electrospray ionization (ESI+) source, operating in the positive ion mode.

The instrument was controlled by Analyst software (version 1.5). The source and compound parameters are summarized in Table 1 for OSE, OCA and OSE-d3. The samples were acquired in multiple reaction monitoring (MRM) mode, with transitions at m/z 313.20→166.00, m/z 285.30→ 138.20, m/z 316.40 →228.01 for OSE, OCA and OSE-d3, respectively. The run time was 5.5 minutes, with retention time of 2.0, 2.1 and 2.0 minutes were observed for OSE, OCA and OSE-d3, respectively (Fig.1).

Table 1 Optimized parameters for mass spectrometry					
Source & Gas Parameters		**Compound Parameters**			
Curtain gas (psi)	10		OSE	OCA	OSE-d3
Ion spray voltage (volts)	5500	Declustering potential (volts)	20	23	35
Heater gas (psi)	50	Entrance potential (volts)	5	5	7
Source temperature (°C)	550	Collision energy (volts)	24	24	38
Nebulizer gas (psi)	55	Collision cell exit potential (volts)	12	11	16
Collision associated dissociation gas (psi)	3	Dwell time (msec)	200	200	200

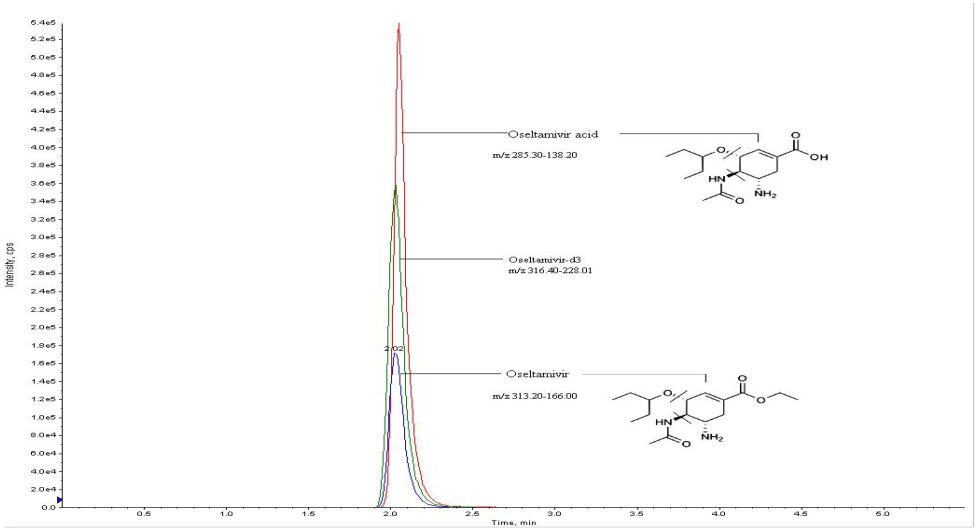

Fig. 1 LC-MS/MS TIC chromatogram of OSE, OCA and OSE-d3

Method Validation

The full method validation was performed in accordance with FDA and EMEA guidelines [18, 19]. The parameter investigated were selectivity, matrix effect, precision and accuracy, linearity, sensitivity, reinjection reproducibility, ruggedness, stabilities in plasma (auto sampler, freeze thaw, bench top, post processing and long term), aqueous stabilities (standard stock solution stability in refrigerator and room temperature, stock dilution stability), recovery and extended batch verification. The analytical range was based on the expected plasma concentration [1]. Spiked calibration curve (8 points) and quality control samples (4 levels) were prepared as mentioned in 'Plasma sample preparation' section.

Selectivity

The selectivity of the method in presence of inherent endogenous matrix components, decomposition products or metabolites etc was evaluated by screening six blank plasma

batches collected from 6 different volunteers. Blank and LLOQ samples were prepared from each plasma batches as per the processing method. The peak response in blank samples at the retention time of analyte(s) and IS should < 20% of the response for the LLOQ sample and < 5 of the area response of IS in LLOQ samples, respectively.

Linearity, accuracy and precision and sensitivity

To evaluate the linearity, precision and accuracy of the method, three validation batches were processed and analyzed, two on the same day and the third on a different day. Each precision and accuracy batch consisted of blank plasma, blank with IS and eight non-zero concentrations. The calibration curve was followed by two carry over blanks and six replicates each of LLOQQC (Lower Limit of Quality Control), LQC (Lower Quality Control), MQC (Middle Quality Control) and HQC (High Quality Control). The linearity was determined by weighted least square regression analysis of standard plot with eight point standard curve, during a course of three precision and accuracy batches. The mean % accuracy should be within ±15% of the actual value except for LLOQ, where it should not deviate by more than ±20%. The precision determined at each concentration level should not exceed 15% of relative standard deviation (R.S.D.) except for the LLOQ, where it should not exceed 20% of R.S.D.

Sensitivity is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision [18]. Six LLOQ were processed and back calculated against calibration curve standards. The % nominal should be ± 20% and the RSD should be ≤ 20%. The analyte signal to noise ratio (S/N) should be at least 5 times [19].

Matrix effect

Matrix effect can be determined in terms of matrix factor; post-extraction addition technique was used for determining matrix factor at LQC and HQC concentrations. 12 sets of blank plasma samples were processed from 6 plasma lots, 2 aliquots from each lot. From each of the processed sample, 75 µL of sample was withdrawn and 25 µL aqueous dilution of LQC containing OSE and OCA were added to 6 samples similarly 25 µL aqueous dilution of HQC containing OSE and OCA were added to other 6 samples. 50 µL of IS dilution was added to all the 12 processed samples. The neat (non matrix based) samples were prepared using 425 µL of Elution Solution, adding 25 µL aqueous dilution LQC and HQC respectively containing OSE and OCA and 50 µL of IS dilution. MF was calculated in each lot of matrix

as per the following formula: *MF = Peak Area in Presence of Matrix Ions / Peak Area in Absence of Matrix Ions* RSD for MF must be <15%.

Extraction Recovery

The percentage recovery of OSE and OCA was determined at low, middle and high concentration by comparing the mean peak area response of replicates of extracted QC samples with the mean peak area response of replicates of aqueous QC samples. The percentage recovery of OSE-d3 was determined by comparing the mean peak area response of OSE-d3 in extracted QC samples with the mean peak area response of aqueous OSE-d3 sample [19]. % Recovery= (Mean peak area response of extracted sample) / (Corrected mean peak area response of unextracted sample)*100

The recovery is deemed acceptable, if RSD is < 20% for % recovery between low, middle and high QC concentrations.

Dilution Integrity

Six quality control samples for dilution integrity were prepared by spiking in excess to their ULOQ concentration, approximately 7.5 times (1815.20 ng/mL) and 8 times (10080.9 ng/mL) for OSE and OCA, respectively. Six samples were diluted 10 times (DI-10) using similar plasma and processed as per the processing method. Diluted samples were back calculated against a calibration curve using dilution factor (DI-10). Within batch precision of the QC samples should be <15% and accuracy should be within ± 15% of their nominal value. At least 67% of the total QC samples should meet the above acceptance criteria.

Stability

Stability of the analytes was investigated, mimicking the conditions that clinical samples may undergo during the course of analysis. It was ensured that every step taken during sample preparation, analysis and storage did not affect the concentration of the analytes [20].

Stability in plasma

Stability of OSE and OCA in plasma under different storage conditions was investigated. Under each exercise, a set of 6 LQC and 6 HQC undergoes a different storage condition, prior to its analysis against a freshly prepared calibration curve. 67% of the total QC, including minimum 50% at each concentration (Low & High) should be within ±15% of the nominal values. The % CV should be ≤ 15 % [18, 20].

Freeze and thaw stability was determined for three freeze thaw cycles (1st cycle after 24 hours and the 2nd and 3rd cycles after 12 hours), autosampler stability at 10°C was assessed for a storage period for 43 hours, post extraction (wet extraction) stability was assessed for the processed samples for a storage period for 67 hours in the refrigerator at 2-8°C. Bench top stability at room temperature for 7 hours (bench top stability) and long term stability in -70 °C deep freezer was also investigated. All the stability samples were analyzing against freshly prepared calibration curve.

Stability in aqueous solutions

Stability of analytes in different solvent and storage conditions, were mimicked for mother stock and stock dilution. Stock solution stability in refrigerator (SRF) for 13 days, Stock solution stability at room temperature (SRT) for 26 hours, stock dilution stability (SDS) at room temperature for 21 hours was carried out for OSE, OCA and OSE-d3. Six replicates of stability and comparison samples were injected. % change was calculated as per the given formula [20].

Correction factor = (Concentration of fresh standard solution) / (Concentration of stability Standard solution)

Corrected response = Stability stock response * Correction factor

% Change = [(Mean response of comparison samples – Mean corrected response of stability Samples) / Mean response of comparison samples] X 100

% Change in stability should be within ± 7%.

Reinjection reproducibility, ruggedness and extended batch verification

Reinjection reproducibility was evaluated to determine the possibility of reanalysis, in case of instrument failure by re-injecting the already injected and analyzed samples. A precision and accuracy batch was reinjected after storage of 21 hours in autosampler [19, 20].

Ruggedness was measured to study the impact of small variations under the laboratory conditions on the analytical method. A precision and accuracy batch was processed using freshly prepared solutions, processed and analyzed by a different analyst. The change is the chromatographic conditions were accomplished by replacing the analytical column with identical column, but with different lot number [21].

Extended batch verification was performed by analyzing two analytical batches against a calibration curve, anticipating the batch size in the intended study. Each processed batch

constitutes of 2 QC sets (LQC, MQC, HQC) interspersed among 42 blank plasma samples. For each batch, at least 67% of total QCs, including 50% at each concentration level, should be within ±15% of their nominal values. This exercise further demonstrates the ruggedness of the method [14].

RESULTS AND DISCUSSION

Representative chromatograms for OSE, OCA and OSE-d3 are illustrated in the Fig. 1; adequate (S/N) ratio and response was achieved for the analytes.

Selectivity and Sensitivity

The selectivity of the method was investigated by comparing chromatograms of six different sources of human plasma. No significant peaks were observed at the retention times of OSE, OCA and OSE-d3 in human blank plasma. Representative chromatograms of blank plasma and blank plasma spiked with OSE and OCA are shown in Fig. 2 (A) and Fig. 2 (B) respectively.

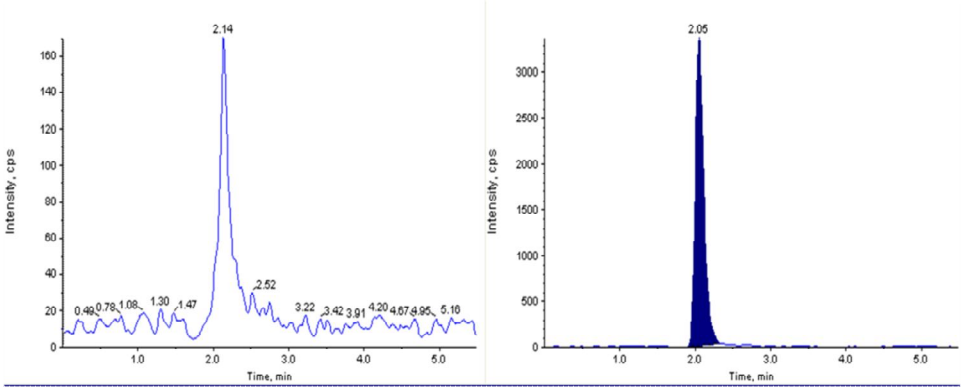

Fig. 2 (A) Representative LC-MS/MS chromatograms of blank plasma and LLOQ for OSE.

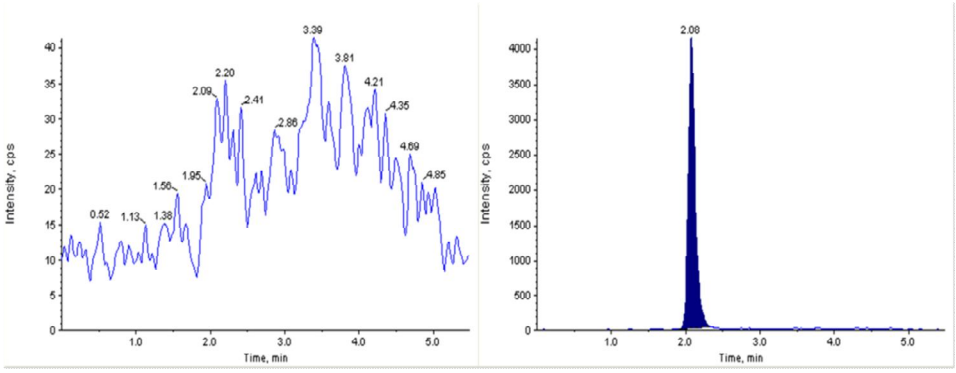

Fig. 2 (B) Representative LC-MS/MS chromatograms of blank plasma and LLOQ for OCA.

Under sensitivity experiment, the observed precision and mean % nominal for OSE and OCA were 2.84 %, 86.30 % and 6.91 % and 95.83%, respectively. S/N ratio for both the analytes was found to be > 5 throughout the validation (Fig. 3).

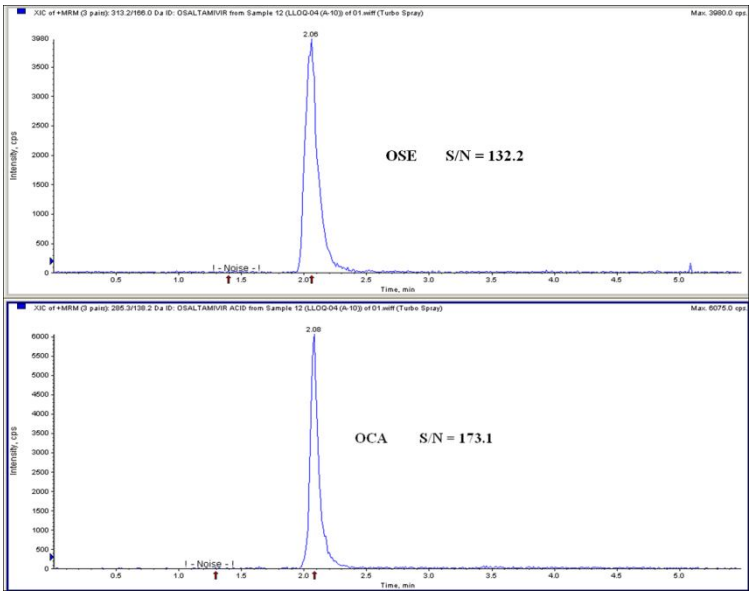

Fig. 3 Representative LC-MS/MS chromatograms of LLOQ for OSE and OCA with signal to noise ratio.

Matrix effect

The presence of unmonitored, co-eluting compounds from the matrix may affect the detection of analytes, even in assays with high selectivity. MF at LQC and HQC level for OSE and OCA were calculated and found to be between 0.90-1.32. The R.S.D. of the mean of MF varied between 2.53-8.51%. The result indicates the matrix components did not alter the intensity of OSE and OCA ion peaks in mass spectrometric analysis. Table 2 summarizes the results.

Table 2 Results for matrix factor and recovery (n=6)

Matrix Factor				
Analyte	**QC Conc. (ng/mL)**	**Mean MF**	**R.S.D. for MF**	
OSE	**5.84**	1.32±0.11	8.51	
	181.52	1.10±0.07	5.98	
OCA	**8.02**	0.94±0.05	4.90	
	752.64	0.90±0.02	2.53	
Recovery				
Analyte	**QC Conc. (ng/mL)**	**% Recovery**	**Mean % Recovery**	**R.S.D. for % Recovery**
OSE	**5.84**	84.13	83.79±2.59	3.10
	117.99	86.19		
	181.52	81.04		

OCA	**32.4**	80.30	79.36±1.14	1.44
	655.3	79.68		
	1008.1	78.09		
OSE-d3	**250.00**	74.91		

Extraction recovery

The recovery for both OSE and OCA was consistent and reproducible at different concentration levels. The mean % recovery for OSE and OCA was 83.79% and 79.36% respectively. The recovery for OSE-d3 was 74.91%. The R.S.D for recovery at different concentration level was within acceptable limit. The simple offline SPE procedure used in this method was simple and efficient to extract drugs from human plasma. Table 2 summarizes the results for recovery.

Linearity

The calibration curve was linear for a range (2.08-241.12 ng/mL) for OSE and (10.8-1251.8 ng/mL) for OCA. The goodness of fit results showed $1/X^2$ to be the best fit weighting for linear regression. The coefficient of correlation (r) was consistently $\geq$ 0.9988 for OSE and $\geq$ 0.9974 for OCA for precision and accuracy batches. The back calculated calibration standards were within the limit of acceptance ±20% for LLOQ and ±15% for other standards.

Accuracy and Precision

Intra batch precision ranged between 3.30% to 5.19% for OSE and 2.75% to 6.09% for OCA. Inter-batch precision ranged from 1.52% to 10.73% for OSE and 3.41% to 7.78% for OCA. The % accuracy ranged between 96.25 to 107.43% for OSE and 95.58 to 112.10% for OSE. The results are summarized in Table 3.

Table 3: Summary for Intra-batch and Inter-batch precision and accuracy, reinjection reproducibility and ruggedness results

Nominal conc.(ng/mL)	Mean calculated conc.(ng/mL)	Accuracy (%)	R.S.D.	Mean calculated conc.(ng/mL)	Accuracy(%)	R.S.D.
OSE						
	Intra-batch (N=6)			**Inter-batch (N=18)**		
2.09	2.13±0.11	102.05±5.23	5.19	2.10±0.30	100.74±10.81	10.73
5.84	6.27±0.17	107.43±2.95	2.74	6.25±0.26	107.16±4.48	4.18
117.99	119.53±3.50	101.30±2.96	3.30	123.40±4.64	104.59±3.93	1.52
181.52	174.72±5.76	96.25±3.17	3.30	183.87±8.22	101.29±4.53	4.47
	Reinjection reproducibility (N=6)			**Ruggedness (N=6)**		
2.09	1.78±0.16	85.18±7.62	8.95	2.00±0.09	95.85±4.44	4.64
5.84	5.79±0.30	99.12±5.11	5.16	6.21±0.16	106.34±2.66	2.50

117.99	118.49±3.47	100.43±2.94	2.92	118.62±3.51	100.53±2.98	2.96
181.52	175.35±2.87	96.60±1.58	1.64	176.16±2.59	97.04±1.43	1.47
OCA						
	Intra-batch (N=6)			**Inter-batch (N=18)**		
11.6	12.58±0.77	108.40±6.58	6.09	11.96±0.93	103.07±8.02	7.78
32.4	36.32±1.40	112.10±4.32	3.85	35.70±1.20	110.18±3.76	3.41
655.3	651.66±30.97	99.44±4.73	4.75	665.36±32.54	101.54±4.97	4.89
1008.1	963.60±26.46	95.58±2.62	2.75	986.89±40.68	97.90±4.03	4.12
	Reinjection reproducibility (N=6)			**Ruggedness (N=6)**		
11.6	10.67±0.27	92.06±2.31	2.51	10.72±0.64	92.39±5.55	6.00
32.4	33.63±1.59	103.76±4.86	4.68	33.60±1.90	103.70±5.87	5.66
655.3	627.78±20.05	95.80±3.06	3.19	649.32±23.64	99.09±3.61	3.64
1008.1	931.05±31.61	92.36±3.14	3.40	964.98±18.09	95.72±1.80	1.88

Dilution Integrity

The diluted samples were analyzed against a calibration curve applying dilution factor (DI-10). Mean % accuracy and RSD found to be 101.91%, 4.40 for OSE and 97.61, 2.45 for OCA. The samples can be diluted up to 10 times, with consistent and reproducible results. None of the researchers have reported dilution integrity experiment. The method is equally applicable for quantifying the concentrations outside the calibration curve and at low plasma volume.

Reinjection reproducibility, ruggedness and extended batch verification

The % accuracy for the reinjected batch ranged from 85.18–100.43% for OSE and 92.06%-103.76% for OCA, while precision ranged between 1.64-8.95% for OSE and 2.75-6.09% for OCA. The method exhibited the degree of ruggedness under the experimental conditions. The % accuracy ranged from 95.85-106.34% for OSE and 92.39-103.70% for OCA and the precision varied from 1.47-4.64% for OSE and 1.88-6.00% for OCA.

In extended batch verification, 67% of QC samples, including at least 50% at each concentration level were within ±15% of their respective nominal values for each analytical batch. Based on the results, approximately 96 samples can be run against a single calibration curve during the clinical sample analysis. Evaluation of this parameter is standard norm of any bioanalytical method validation, however missing in the reported methods Table 3 summarizes the results for reinjection reproducibility and ruggedness.

Stability in Plasma and aqueous solutions

The results for bench top stability at ambient temperature (20-30^{0}C) for 7 hours were found within the acceptance limit. Long term stability samples were stable in − 70^{0}C deep freezer

for 27 days. The samples kept into the autosampler at 10^0C were stable for 43 hours. Freeze–thaw stability results showed that OSE and OCA were stable for at least three freeze–thaw cycles. Post processed samples kept in refrigerator at 2-8^0C were stable for 67 hours without any significant change. Table 4 summarizes the results of stability studies in plasma samples carried out under various conditions.

Table 4: Summary of stability exercises carried out in plasma samples (n=6)

Stability (N=6)	Nominal conc.(ng/mL)	Mean calculated conc.(ng/mL)	Accuracy(%)	R.S.D.
	OSE			
Freeze thaw (3 cycles)	**5.84**	6.24±0.34	106.76±5.84	5.47
	181.52	185.81±4.07	102.36±2.24	2.19
Bench Top (7hours)	**5.84**	6.06±0.31	103.74±5.32	5.13
	181.52	184.02±2.36	101.38±1.30	1.28
Wet extract (67hours)	**5.84**	6.08±0.33	104.08±5.64	5.41
	181.52	179.73±2.38	99.01±1.31	1.32
Long term (27 days)	**5.84**	6.16±0.27	105.48±4.66	4.42
	181.52	182.76±4.63	100.69±2.55	2.53
Autosampler (43hours)	**2.09**	2.07±0.14	99.20±6.61	6.66
	5.84	6.39±0.09	109.45±1.61	1.47
	117.99	133.13±9.22	112.83±7.81	6.92
	181.52	205.92±6.32	113.44±3.48	3.07
	OCA			
Freeze thaw (3 cycles)	**32.4**	32.58±2.70	100.57±8.34	8.29
	1008.1	1015.22±17.31	100.71±1.72	1.71
Bench Top (7hours)	**32.4**	31.82±2.27	98.20±6.99	7.12
	1008.1	1007.57±11.32	99.95±1.12	1.12
Wet extract (67hours)	**32.4**	31.12±1.13	96.04±3.50	3.64
	1008.1	998.72±28.93	99.07±2.87	2.90
Long term (27 days)	**32.4**	33.10±2.69	102.16±8.30	8.13
	1008.1	1051.37±30.80	104.29±3.06	2.93
Autosampler (43hours)	**11.6**	11.68±0.74	100.72±6.39	6.35
	32.40	33.88±1.24	104.58±3.84	3.67
	655.3	678.47±69.46	103.54±10.60	10.24
	1008.1	999.90±31.25	99.19±3.10	3.12

Stock solution stability of OSE, OCA and OSE-d3 was generated at RT (26 Hours) and refrigerated temperature (2-8 ^{0}C) for 13 days. The % change observed were -1.42, -0.94, 5.41 (RT) & -1.32%, -2.63% and 5.82% at refrigerated temperature. Most of the investigators reported % stability instead of % change. However, it is recommended that the acceptable difference between the absolute responses of the fresh stock solutions and aged stock solutions be tighter (within 5-7%) than the normally applied to bioanalytical results (i.e. within 15-20 %) [22].

Stock dilution stability at RT was generated for 21 hours. The % changes were within the acceptance criteria and were -0.11, -0.71 & 6.30 for OSE, OCA and OSE-d3, respectively. The results are illustrated in Table No 5.

Table 5: Summary of stability exercises carried out in aqueous solutions (n=6)

	SRF (N=6)	SRT (N=6)	SDS (N=6)
	OSE		
Mean peak area response (stability sample)	721989.8±9574.19	2234600.0±475158.38	2309132.7±28066.70
Mean peak area response (comparison sample)	701905.2±4873.83	2185856.0±31013.97	2288123.2±20502.47
% Change	-1.32	-1.42	-0.11
	OCA		
Mean peak area response (stability sample)	1781358.3±1771575.5	4557769.8±1084555.82	4745945.3±87877.35
Mean peak area response (comparison sample)	1726096.7±13555.66	4537799.3±78395.57	4736122.5±58129.30
% Change	-2.63	-0.94	-0.71
	OSE-d3		
Mean peak area response (stability sample)	1013275.8±13021.18	2223758.5±651268.63	2459737.5±16697.55
Mean peak area response (comparison sample)	1074143.5±14619.23	2345185.7±42081.43	2308440.7±19844.51
% Change	5.82	5.41	-6.30

SRF: Stock solution stability in refrigerator; SRT: Stock solution stability at room temperature; SDS: stock dilution stability

CONCLUSION

The method validation was based on the basic understanding of regulatory body (FDA/EMEA) and current industry practice. Even though it was a direct elution method, the method was selective, sensitive and with appreciable degree of precision and accuracy. The method is applicable for bioequivalence/bioavailability and pharmacokinetic studies.

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