Eurasian Journal of Analytical Chemistry
ISSN: 1306-3057 OPEN ACCESS 2018 13 (6): 76-82

Development and Validation of HPLC Method for Estimation of Oxcarbazepine in Spiked Human Plasma: Synthesis and Characterization of Its Active Metabolite

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Received 14 September 2018 ▪ Revised 23 October 2018 ▪ Accepted 24 November 2018

Abstract: A simple, precise and accurate HPLC-UV method was developed and validated for the quantitation of oxcarbazepine (OXZ) in human plasma. The OXZ and erlotinib (ERL) (internal standard) were extracted from human plasma using liquid-liquid extraction. The extraction efficiency of drug and internal standard from plasma were evaluated in different organic solvents and effect of pH modifier were studied. The separation was achieved on C18 (250 × 4.6 mm, 5 µm) column using methanol: 20 mM potassium phosphate buffer (pH 3.0) (60:40 %, *v/v*) at 237 nm. The flow rate was kept constant at 1mL/min throughout the analysis. To select the optimal calibration model, different weighting factors were evaluated. The calibration model with weighing factor, $1/x^2$was found optimal with minimum % relative error and most random distribution around *x*-axis. The calibration curve was linear in the range of 100-3200 ng/mL. The method was validated as per US-FDA guidelines with respect to selectivity, accuracy, precision, % recovery and stability. Further, the 10,11-dihydro-10-hydroxy carbamazepine (10-MHD); active metabolite of OXZ was synthesized and characterized by GC-MS. The extraction efficiency of10-MHDin human plasma was compared with OXZ using optimized LLE method.

Keywords: Oxcarbazepine, 10-MHD, RP-HPLC, Spiked human plasma, GC-MS.

INTRODUCTION

OXZ (10, 11-dihydro-10-oxo-5*H*-dibenzo [b,f]azepine-5-carboxamide)[1], a 10-keto analogue of carbazepine (Figure 1). It is an anticonvulsant drug which acts by blocking voltage sensitive Na^+ channels, increases potassium conductance and moderate calcium channel function[2].

Literature survey revealed several analytical methods for OXZ in biological fluids includes, estimation of OXZ and its metabolites in human serum [3] as well as in cerebrospinal fluid [4], LCMS/MS methods [5,6], estimation of OXZ with carbamazepine and its metabolites[7, -8], OXZ in presence of other antiepileptic drugs[9-13].

The objective of the present work was to develop a simple and economic HPLC bioanalytical method for quantitation of OXZ in pooled sample of human plasma, to study the effect of various organic solvents and pH modifiers on the extraction efficiency of drug from plasma by liquid-liquid extraction(LLE), to study different calibration model and suggest the suitable calibration model which passes the test of homoscedasticity and to validate the method according to US-FDA guidelines.

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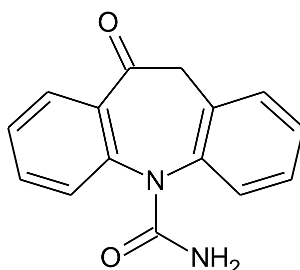

Figure 1: Chemical structure of Oxcarbazepine

MATERIALS AND METHODS

OXZ was kindly provided as a gift sample from Ajanta Pharma Ltd, Mumbai, India. Human plasma was procured as a gift sample from NDMVP's Dr. VasantraoPawar Medical College, Hospital and Research Centre Nashik, India and Pooled sample was prepared by mixing plasma from six different sources (gender, age, *etc*). HPLC grade methanol and potassium dihydrogen ortho phosphate were purchased from SD Fine Chemicals, Mumbai, India. Freshly prepared double distilled water was prepared from All Glass Double Distilled Assembly, purchased from Borosil, Mumbai, India. Membrane filter papers DURAPORE 0.45 µ x 47 mm were purchased from MiliporePvt.Ltd., Bengaluru, India.

Thework was carried out on HPLC system consisted of two pumpsmodel: PU-2080 Plus (JASCO Corporation, Tokyo, Japan) equipped with 100 µL Rheodyne loop injector (7725*i*). Separation was performed using Phenomenex HypercloneC18 analytical column (250 x 4.6 mm, 5µm) and detection was carried out on UV-2075 detector (JASCO Corporation, Tokyo, Japan) using Borwin Chromatography software (Version 1.50). Other instruments used in analysis includes, Bath sonicator (PCI Analytics Pvt. Ltd., Mumbai, India), pH meter (Model: 362, Systronics India Ltd., Ahmedabad, India), Electronic Balance (AUX 220, Shimadzu Corporation, Japan), High speed cooling Centrifuge (Model: C-24 BL, Remi Sales and Engineering Ltd., Mumbai, India), Micro pipettes (Thermo scientific, India) and Vortex Mixer (Remi Sales and Engineering Ltd., Mumbai, India).

EXPERIMENTAL

Preparation of Standard Stock Solutions of Oxcarbazepine and Internal Standard

The standard stock solutions, 100 µg/mL of OXZand 100 µg/mL ERL, respectivelywere prepared in methanol

Chromatographic Conditions

Chromatographic analysis was carried out on C18 Phenomenex Hyperclone column (250 × 4.6 mm, 5 µm) with mobile phase consisting of methanol: Potassium phosphate buffer (60:40 % *v/v*) at flow rate of 1 mL/min. The detection was carried out at 237 nm.

LLE Experiments and Preparation of Samples

In five different 15 mL glass test tubes, 1 mL of human plasma was taken, in it, 125 µL quantity of standard stock solution of OXZ and ERL were added. The solutions were mixed for 3 min using vortex-mixer. In each tube, five different organic solvents, *tert*-butyl methyl ether (TBME), ethyl acetate, tetrahydrofuran (THF), chloroform and dichloromethane were added. The solutions were made acidic or alkaline by adding 400 µL of 1 % formic acid or 1 % NaOH and vortex-mixed for 5 min. Further, the mixtures were centrifuged at 3000 rpm using cooling centrifuge at 4 °C and the supernatant organic layer were transferred to Eppendorf tubes. The organic solvents were evaporated to dryness under the stream of nitrogen and reconstituted to 500 µL with mentioned mobile phase and injected in HPLC

The extraction efficacy of the drug was evaluated by comparing the area of unextracted drug to the extracted drug sample.

Preparation of Calibration Standards and Quality Control (QC) Samples

The standard stock solution of OXZ was appropriately diluted with methanol to obtain six different working standard solutions of concentrations from 0.1 to 3.2 µg/mL. In each glass test tube containing 1 mL of plasma, 125 µL of working standard solutions of OXZ and 125 µL of standard solution of ERL were added to obtain calibration standards of concentrations; 100, 200, 400, 800, 1600, and 3200 ng/mL, each containing 1500 ng/mL of ERL as internal standard. The extraction procedure was executed similarly as mentioned earlier and the response factor(RF) was determined (RF = area of OXZ/ area of IS). The

resulting RF were plotted against the corresponding calibration standard and subjected to regression analysis.

Three QC samples were prepared as procedure mentioned above containing concentrations of 500 ng/mL, 1500 ng/mL and 3000 ng/mL of OXZ with 1500 ng/mL of internal standard, representing low-quality control (LQC), middle-quality control (MQC) and high-quality control (HQC) samples, respectively.

Selection of Calibration Model and Range

Linear regression models with different weighting factors (w) were evaluated to obtain the optimal regression model. The calibration model with w = 1 (unweighted linear regression) and with $w = 1/x$, $1/x^2$ and $1/\sqrt{x}$were studied against the % relative error (% RE) and variability around x- axis (homoscedasticity). The model with minimal % RE and homoscedasticity was considered optimal with least error and further used in the validation studies.

Method Validation

The developed method was validated as per the recommendations of US FDA Guidance for Industry: Bioanalytical Method Validation[14] with respect to selectivity, accuracy, precision, recovery and stability studies.

To prove the selectivity of the method, the response of 100 ng/mL LLOQ concentration was compared with the responses plasma samples obtained from six different sources.

To study accuracy and precision, five replicates of each QC samples (LQC, MQC and HQC) were studied for five successive days. For each day, the concentration of OXZ was determined from the calibration equation obtained on the same day. The results of accuracy and precision were expressed in % RE and % RSD, respectively.

Synthesis and Characterization of Active Metabolite of Oxcarbazepine

OXZ is a pro drug and undergoes enzymatic reduction at its keto group, resulting to the formation of monohydroxyl derivative, 10-hydroxy-10, 11-dihydrocarbamazepine (10-MHD) which is an active metabolite of OXZ. To synthesize the 10-MHD, in to three necked flask, equipped with reflux condenser, mechanical stirrer and thermometer, about 0.6 g of OXZ, 5 g NaOH and 5 g Zn powder were taken. The resulting mixture was stirrer with increasing temperature to 70 °C. After 3 h, when the temperature was commenced to fall, reaction mixture was filtered and warmed with 10 mL of hot rectified spirit. Filtrate was poured in to a beaker containing about 50 g of crushed ice and acidified with conc. HCl to form crude technical compound. The compound formed was filtered, recrystallized from alcohol. The chemical reaction of OXZ to 10-MHD is depicted in Figure 2.The synthesized product was confirmed by GC-MS.

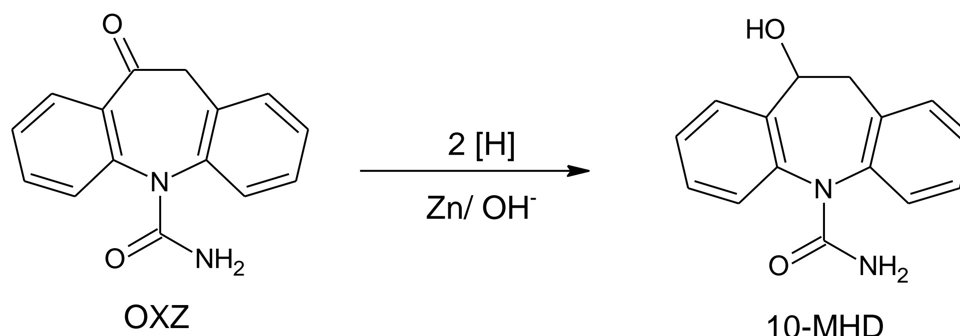

Figure 2: Reaction of OXZ to 10-MHD

The optimized LLE procedure was adopted and the % recovery of 10-MHD from human plasma was calculated and compared with the % recovery of OXZ.

RESULT AND DISCUSSION

Optimization of Chromatographic Conditions

To obtain optimum chromatographic conditions, initially mobile phases with water as an aqueous phase were tried with methanol and acetonitrile as organic phase. But because of partial ionization of OXZ, the peak splitting was observed. Phosphate buffer was tried to prevent the partial ionization of OXZ. It was found that mobile phase comprising of methanol: 20 mM potassium phosphate buffer (pH 3.0) (60:40 %, v/v) gave desired retention and resolution at flow rate 1 mL/min when C18 column used as a

stationary phase. Good response was obtained for both OXZ and ERL at 237 nm. The retention time for OXZ and ERL were obtained at 4.657 min and 6.525 min, respectively.

Optimization of LLE Method

To obtain the clear extract with good recovery of OXZ and ERL, different organic solvents; *tert*-butyl methyl ether, TBME, THF and dichloromethane were tried. Compared to other organic solvents tried, 42.12 % and 69.26 % recovery was obtained in TBME. Also, when 1 % formic acid and 1 % NaOH were tried as pH modifier with TBME, the % recovery of OXZ was increased to 66.67 % and 79.36 % for ERL, respectively with clear extract. No significant % change in recovery for OXZ and ERL obtained with 1 % NaOH.

The representative chromatogram of OXZ with ERL and blank plasma sample extracted in 5 mL TBME and 400 µL of 1% Formic acid was presented in Figure3 and the representative chromatogram of MQC sample in Figure 4.

Calibration Curve Experiments

Data obtained from the calibration experiments (Table 1) were subjected to linear regression analyses to obtain optimal calibration model.

The degree of association between x and y variables were expressed by r (coefficient of correlation) for each regression model. With unweighted linear regression model, the F *(experimental)* found to be 40.47 which is significantly higher than F *(tabulated)* = 5.05 ($F_{5,5}$), which revealed that the variance is not evenly distributed over the calibration standard range and results in the heteroscedasticity.

Table 1: Area ratio from calibration experiment

CC No.	Amount of drug (ng/mL)	RF (mean ± SD, n=6)
CC-1	100	0.0604 ±0.0044
CC-2	200	0.1093 ±0.0052
CC-3	400	0.2052 ±0.0190
CC-4	800	0.3451 ±0.0373
CC-5	1600	0.6294 ±0.0362
CC-6	3200	1.3308 ±0.0226

Further, the data of calibration standards was subjected to weighted linear regression with $w = 1/x$, $1/x^2$, $1/\sqrt{x}$. The optimal linear regression model was selected on the statistical evaluation of % RE and *one-way* ANOVA as presented in Table 2.

It was observed that the calibration model with $w = 1/x$ and $1/x^2$ shows the less % RE but the F (*experimental*) value was found much more less for $1/x^2$ as compared to F (*experimental*) of $1/x$, which revealed that the linear regression model with $w = 1/x^2$ was suitable to homogenize the variance of residual. Thus, it was decided to adopt a weighted linear regression model with $w = 1/x^2$ for the calibration range of 100-3200 ng/mL of OXZ.

Table 2: Regression parameters of the calibration curve generated for each weighing factor

Model No.	*w*	*b*	*a*	*r*	% RE	F value
1	1	0.0004	+0.0232	0.995	40.47	25.68
2	1/x	0.00040	+0.0251	0.996	1.44×10^{-13}	0.0250
3	$1/x^2$	0.00041	+0.0209	0.992	3.90×10^{-12}	2.45×10^{-05}
4	$1/\sqrt{x}$	0.00040	+0.0257	0.985	-14.90	0.8026

Method Validation

In validation studies, five times response was obtained for LLOQ samples (45554.58 ± 20273.77 µV.sec, n = 6) compared to the blank pooled plasma samples (1496.58 ± 145.18 µV.sec, n = 6), proved the selectivity of the method at 100 ng/mL with absence of interference at the retention times of OXZ or ERL.

Results of accuracy and precision (Table 3) revealed good % recovery of OXZ and ERL with minimal % RE and % RSD, confirms the accuracy and precision of the method with the selected calibration model.

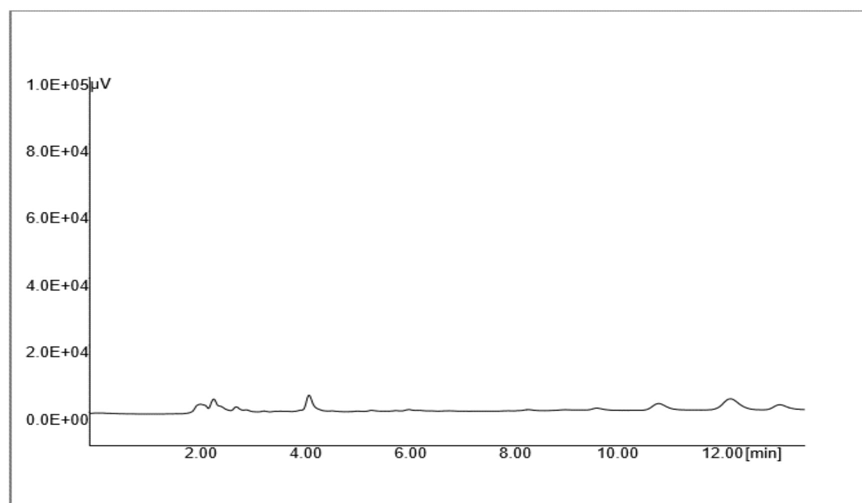

Figure 3: Chromatogram of blank plasma extract showing a lack of significant interference at the retention times of Oxcarbazepine and Erlotinib (IS)

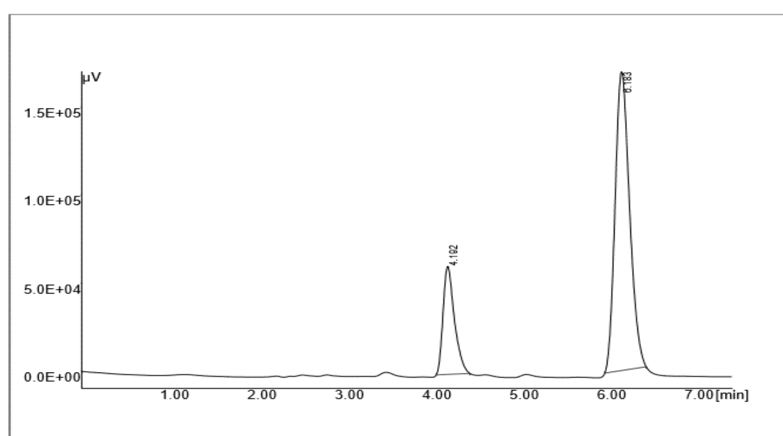

Figure 4: Representative Chromatogram of MQC sample of Oxcarbazepine showing at the retention times of (4.192 min) and (IS) Erlotinib (6.183 min)

Table 3: Result of accuracy and precision studies for OXZ

QC Level	Intra-day (n= 5)				Inter-day (n = 5)			% Recovery
	Conc. Added (ng/mL)	Mean conc. Found (ng/mL)	% RE	% RSD	Mean conc. Found (ng/mL)	% RE	% RSD	
LQC	500	497.48	1.09	4.06	501.82	-2.10	2.75	67.35
MQC	1500	1504.18	-4.18	1.18	1496.96	1.50	6.86	64.03
HQC	3000	2998.32	1.67	2.32	3007.15	-1.67	1.73	68.62
IS	---	---	---	---	---	---	---	79.36

The results of stability evaluation for OXZare presented in Table 4. From, the stability at room temperature for 6 h and stability at -20 °C indicated that OXZwas stable in human plasma.

Table 4: Results of stability studies for OXZ

QC Level	LQC			HQC		
Stability at room temp (Time)	**2 h**	**4 h**	**6 h**	**2 h**	**4 h**	**6 h**
% nominal	103.25	102.56	103.56	100.23	100.55	100.45
% RSD	4.03	4.07	5.33	3.72	2.65	4.39
Stability at -20 °C (Time)	**10 days**	**20 days**	**30 days**	**10 days**	**20 days**	**30 days**
% nominal	101.25	101.56	102.56	100.53	100.56	100.53
% RSD	4.07	5.05	4.77	1.86	6.98	5.86

Characterization and Extraction of 10-MHD

The synthesized compound was subjected to GC-MS analysis and the mass spectrum obtained is depicted in Figure5, which was confirmed as 10-MHD.

Further, when 10-MHD was extracted using the mentioned optimized LLE procedure, 69 % of recoverywas obtained. This indicates that the similar extraction procedure can be adopted for the quantitation 10-MHD.

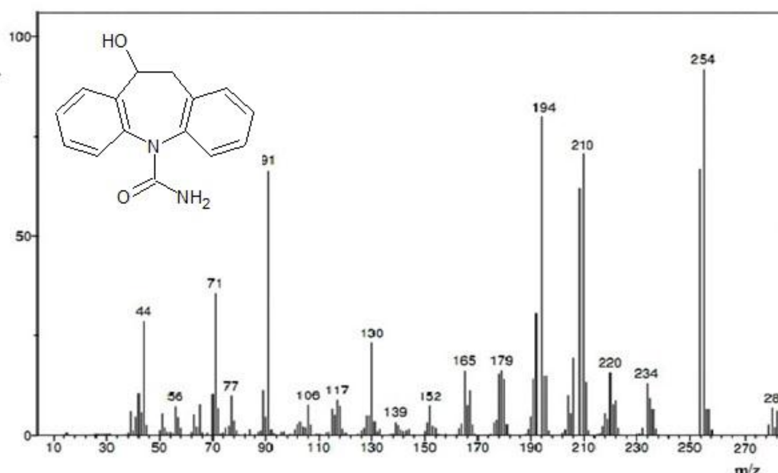

Figure 5: Mass spectrumof 10-MHD

CONCLUSION

In present study, a simple, accurate, reproducible and selective HPLC method was reported for the quantitation of OXZ in spiked human using ERL as an internal standard. Good recovery of drug and clear extract without any interferent was obtained when TBME solvent with 1 % formic acid was used for extraction. The heteroscedasticity obtained with linear regression model with w = 1 (unweighted linear regression) was minimized using weighted linear regression with different $w(1/x$, $1/x^2$ and $1/\sqrt{x})$.Minimum % RE with random distribution around x-axis was observed with linear regression model with w = $1/x^2$in the calibration standard range of 100-3200 ng/mL. Further, the method was validated as per US-FDA guidelines suggesting the method was in good agreement with selectivity, accuracy, precision, recovery and stability of OXZ in human plasma. Comparing the extraction efficiency of OXZ and its active metabolite, 10-MHD concluded that the developed LLE method can be used in the further quantitation of 10-MHD. Although, several methods have been reported for the quantitation of OXZ in human plasma, the present method is economic, does not require expensive instrumentation and can be performed at laboratory level for routine estimation of OXZ and 10-MHD in human plasma.

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