DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR THE SIMULTANEOUS QUANTITATIVE ANALYSIS OF OXCARBAZEPINE AND ITS METABOLITE 10-HYDROXYCARBAZEPINE IN KEDTA PLASMA

KASHIF UL HAQ1, NITESH KUMAR2
1Faculty of Health Sciences, Department of Pharmacy, Sri Venkateshwar University, Gajraula, Amroha, 2Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science & Animal Husbandry, Rewa, India. Email: Haq.kashif@gmail.com

Received: 22 Nov 2013, Revised and Accepted: 02 Feb 2014

ABSTRACT

Objective: To develop and validate a sensitive, selective and reproducible method for the estimation of oxcarbazepine (OXC) and its pharmacologically active metabolite 10-hydroxycarbazepine (MHD) in human K EDTA (Ethylene Diamine Tetra Acetic Acid) plasma.

Methods: The drugs of interest were extracted by solid phase extraction technique, using HLB cartridges (30mg/1cc). Chromatographic separation was achieved in less than 3.0 minutes on reverse phase C8 column, using isocratic elution with acetonitrile-buffer solution (0.1% formic acid in water) in proportion of (70:30, v/v). Tandem mass spectrometer was used to detect the positive ions, using APCI (Atmospheric Pressure Chemical Ionization) probe.

Results: The validated method was linear for OXC (0.032-7.044 µg/ml) and MHD (0.103-15.054 µg/ml) and the coefficient of correlation (r) for the drug and the metabolite were better than 0.99. The method was fully validated, complying FDA (Food and Drug Administration), EMEA (European Medicines Agency) guideline and recommendations of AAPS (American Association of Pharmaceutical Scientists) white papers demonstrating Selectivity, Sensitivity, Matrix factor, Precision and Accuracy, Linearity, Aqueous stability, Stability in biological matrix, Dilution Integrity, Rejection Reproducibility, Recovery, Ruggedness and Extended Batch Verification.

Conclusion: The validated method is applicable for Bioavailability/ Bioequivalence and Pharmacokinetic studies.

Keywords: Oxcarbazepine; 10-hydroxycarbazepine; LC-MS/MS; APCI; Bioavailability, Bioequivalence.

INTRODUCTION

Oxcarbazepine (OXC) is an antiepileptic drug that is quickly replacing carbamazepine. It is a ketonologue of carbamazepine, with a ketonic group in position 10 and with similar action mechanism. It's a new antiepileptic drug (AED) which is structurally related to Carbamazepine (CBZ) but differing in several important aspects, notably metabolism and induction of metabolic pathways. Consequently, OXC has fewer drug-drug interactions compared with CBZ. The OXC has been reported with reduced allergic potential.

The limitations of CBZ includes toxicity in sensitive individuals, auto induction, which requires dose adjustment when therapy is initiated, and chronic hepatic induction, producing drug interactions when CBZ is used with AEDs and other drugs that undergo hepatic metabolism. One of the two main products of CBZ microsomal metabolism, CBZ-10, 11-epoxide (formed by oxidation of double bond between C-10 and C-11), appears to provide antiepileptic efficacy but contributes significantly to clinical toxicity.

Oxcarbazepine is essentially a prodrug after administration, it is completely absorbed by the gastrointestinal tract and completely (96-98%) converted by an arylketone reductase, a cytosolic enzyme of hepatic cell, into its main and pharmacologically active metabolite monohydroxy derivative (MHD). 10,11-dihydro-10-hydroxy-5H-dibenzo[b,f]azepine-5-carboxamide have shown to exert antiepileptic activity by blockade of voltage-dependant sodium channels in the brain. Oxcarbazepine is rapidly reduced by cytosolic enzymes in the liver to MHD, which is responsible for the pharmacological effect of the drug. In plasma after a single oral administration of Oxcarbazepine the mean apparent elimination half life (t1/2) of MHD in adults was 8-9 h. Food has no effect on the bioavailability of the highest strength of the final market tablet (600 mg). A steady state MHD displays predictable linear pharmacokinetics to doses ranging from 300 to 2400 mg.

MHD is subsequently metabolized by two different ways. The predominant one is MHD-glucuronide conjugation mediated by the UDP-glucuronyl transferase, while a minor amount is oxidized into its pharmacologically inactive metabolite, 10,11-trans-hydroxycarbazepine (DHD). 10,11-dihydro-10,11-trans-dihydroxy-5H-dibenzo[b,f]azepine-5-carboxamide. Both OXC and MHD do not show self induction characteristics and, moreover do not seem to influence, by inhibition or by induction, the P450 cytochrome system, catalyst for the metabolic way of many drugs, avoiding problem for interactions with other substances in eventual multiple therapies. The OXC has a minor tendency to interact with other drugs it can be administered both as monotherapy and polytherapy in association with other antiepileptic drugs.

FDA no binding recommendation suggests, the BE (Bioequivalence) study should be performed on 600 mg Trileptal tablets to healthy male volunteer under fasting conditions. The mean Cmax for MHD was 34µmol/L, while the corresponding median Tmax reported was 4.5 hours. The reported Cmax for single dose (600mg) tablet study performed on healthy male volunteers, under fasting conditions was [3.15+ 6.6 µmol/L]. The calibration range of the analytical method should cover the expected concentration of both fasting and fed studies.

Various analytical methods were reported for the estimation of oxcarbazepine and its metabolite using LC (Liquid Chromatography) or LC/MS (Liquid chromatography/Mass spectrometry) procedures. These methods are less specific than LC/MS/MS methods and also are time consuming because of long chromatographic analysis or complex sample preparation procedures. Liquid chromatography tandem mass spectrometry method is considered the gold standard to implement procedures to analyze drugs in biological samples; in fact LC/MS/MS detection permits high specificity for qualitative and quantitative analysis of several compounds. Moreover, MS/MS detection allows a reduction of time in sample clean-up and chromatographic separation. A significant number of the reported quantitation methods are in plasma, a couple of them in serum.
There were two basic objectives of the study viz, developing a method in K.EDETA plasma. In the current industry practice K.EDETA Vacutainers are used during the clinical phase, due to its relative behavior with blood cells and also, since it does not dilute the samples, one of the objections raised by FDA with other anticoagulants.

The second objective of the study was to develop a regulatory compliant bioanalytical method for the simultaneous estimation of OXC and MHD. A fully validated method is not available, which completely adheres to FDA/EMA guidelines, AAPs whitepapers and current industry practices for the drugs. The method was optimized on Liquid Chromatography tandem with Triple Quad API-3000 unit. The result demonstrates, the compliance to the FDA guidelines and its applicability to BA/BE (Bioavailability/Bioequivalence) and Pharmacokinetic studies.

**MATERIALS AND METHODS**

**Reagents and Chemicals**

Free samples of Oxcarbazepine and carbamazepine were provided by (Arbo Pharmaceutical Limited, India) and 10-hydroxy oxcarbazepine was procured from (Synfine Research, Canada). HPLC grade solvents like; acetonitrile, methanol and formic acid were supplied by (Renkem Limited, India). Purified water from Milli-Q (Millipore Advantage A10) instrument was used. Extraction and purification was performed on Oasis ® HLB 1cc (30 mg) extraction cartridges from (Waters, Ireland).

**Instrumentation**

The LC-MS/MS instrument consisted of liquid chromatography unit from Shimadzu, Japan (Model LC10). Binary pumps (LC 10ADp Series), Column Oven (CTO-10 ASVP), Auto injector (SIL-10A) with System controller (SCL-10AP) tandem with triple quad API-3000 from MDS Scien Canada, using API probe in positive ion mode. Analyst software 1.4.1 was used for data processing.

**Chromatographic Conditions**

Sample injection (5µL) was separated and chromatographed at a flow rate of 0.650 mL/minute on Hypersil Gold (50x4.6) mm, 3 µm column, using Acetonitrile: buffer solution (70:30, v/v) mobile phase. The buffer solution was 0.1% formic acid. The column oven and auto sampler temperatures were maintained at 35 ± 2°C and 5 ± 1°C respectively.

**Mass Spectrometry**

The Triple Quad API-3000 Mass Spectrometer was operated with API probe in positive ion mode. The source gas i.e. nebulizer (NEB) and curtain (CUR) gas were optimized at 11, 10 while CAD gas (Collision Associated Dissociation) at 4 psi. The Source temperature was regulated at 475°C and Needle current (NC) at 4 amp. The different voltages which were optimized for desired results were Enterprising potential (EP-10V), Dechannelling potential (DP (35V) for OXC, MHD and CBZ. Focusing Potential [FP (286V-OXC), (136V- MHD) & (136V-CBZ)], Collision Energy [CE (19V-OXC), (11eV- MHD) & (27eV-CBZ)]. The Collision exit potential [CEP (15V-OXC), (10V-MHD) and (13V-CBZ)]. The LLOQ was optimised with Unit: Unit resolution in Q1 and Q3.

**Preparation of stock solutions**

The stock solution of OXC, MHD and CBZ were prepared in methanol to give the final concentration of approximately 1 mg/ml. The actual concentration was corrected using potency, molecular weight and actual amount of the weight. The stock solutions were stored at refrigerated temperature of 2-8°C until use. For CC/QC (Calibration Curve/Quality Control) spiking, stock solutions of OXC and MHD were further diluted using dilaicnt solution methanol: water: (50:50, v/v). IS stock dilution were diluted to approximately 10.000 µg/mL using diluent solution methanol: water: (50:50, v/v).

**Preparation of calibration standards and quality control samples in plasma**

Different plasma lots were screened for any significant interference at the RT (Retention Time) of drug, metabolite or internal standard. The interference free batches were further subject to matrix factor determination. Batches showing no significant interference and ion enhancement/suppression were pooled together for the spiking of CC/QC samples.

Calibration curve standards and quality control samples were prepared by spiking drug free pooled plasma. Stock dilution were prepared from the mother stock and spiked to obtain CC concentrations of 0.032, 0.075, 0.252, 0.629, 1.258, 3.145, 6.289 and 7.044 µg/ml for OXC and 0.103, 0.206, 0.822, 1.645, 3.290, 6.580, 13.159, 15.054 µg/ml for MHD. Spiked QC samples were obtained at concentration for lower limit of quantitation quality control (LLOQC= 0.033), lower quality control (LQC=0.082), middle quality control (MQC=0.319) and high quality control (HQC=5.266) for OXC and (LLOQC=0.106), (LQC=0.265), (MQC=6.617) and (HQC=11.028) for MHD. Spiked CC/QC samples were stored in -70°C deep freezer, except QC samples stored at -25°C deep freezer for stability at -25°C. Six sensitivity samples were also spiked separately (0.032-OXC & 0.103-MHDµg/ml) and stored in -70°C deep freezer, until analysis.

**Sample Preparation**

Aliquot 200 µl of plasma sample, added 25 µl of IS (approximately 10.000 µg/mL) and vortexed well. Solid Phase Extraction Method (SPE) was used to extract the drugs of interest and sample cleanup. The step followed is mentioned hereunder;

Step 1: Conditioned the HLB cartridge (30 mg/1cc) with 1.0 mL methanol. 1.0 mL of Milli-Q water, Step 2: Loaded sample, Step 3: Washed the cartridges with 1.0 mL of Milli-Q, followed by 1.0 mL of 5% methanol, Step 4: Eluted the sample with 1.0 mL methanol, Step 5: Evaporated the eluate to dryness at 40°C under constant pressure in nitrogen evaporator, Step 6: Reconstituted the samples in 200 µl mobile phase and transferred into HPLC vials for analysis.

**Validation Procedures**

A full validation was performed in K.EDETA plasma according to current FDA guideline on Bioanalytical Method Validation [22] and EMA [23].

**Selectivity**

Seven K.EDETA plasma lots obtained from different sources were screened for significant interference at the RT of drug/metabolite and internal standard.

A double blank was prepared from each lot of plasma, one without any drug or internal standard, while the other spiked with LLOQ and internal standard, processed and analyzed as per the defined method.

In the drug free blank sample, area response at RT of drug/metabolite should be <20% of the corresponding LLOQ area. The area response at the RT of internal standard should be <5 % of IS area in corresponding LLOQ sample.

**Matrix Factor (MF)**

Six plasma batches, previously screened and found to be clean of any significant interference at the RT of drugs and IS were used for matrix factor experiment. The matrix effect was performed by post extraction spiking method’. Two aliquots were taken from each plasma lot and processed. The processed samples from each plasma lot were spiked with LQC and HQC. The samples were also spiked with IS dilution. Next aqueous comparison samples corresponding to final concentration of LQC and HQC were also prepared. Matrix factor was calculated as per the formula given below;

\[ MF = \frac{\text{Peak response in presence of matrix ions/Peak response in absence of matrix ions}}{\text{Variability of the matrix factor, as measured by coefficient of variation (CV), should be <15%}.} \]

The ratio of matrix factor of drugs and IS yields IS-normalized MF for the analyte.

**Linearity**

Eight non zero calibration standards were prepared having concentration of 0.032, 0.075, 0.252, 0.629, 1.258, 3.145, 6.289 and
7.044 µg/ml for OXC, also 0.103, 0.206, 0.822, 1.645, 3.290, 6.580, 13.159 and 15.054 µg/ml for MHD.

The data from three precision and accuracy batches were subjected to goodness of fit analysis using 1/x and 1/x² weighing factor. Deviation from nominal concentration should be within +20% for LLOQ and within ±15% for the other concentrations.

At least 75% of non-zero standards should meet the criteria, including LLOQ and ULOQ standard. Linear coefficient of correlation (r) should be ≥0.98.

**Accuracy and Precision**

The precision of the assay was measured by the percent coefficient of variation (% CV) for the concentration at LLOQC, LQC, MQC and HQC level during the validation. Six replicates at each QC level, in a single analytical run constitute one PA (Precision and Accuracy) batch. Three batches were run to calculate the Inter and Intra Batch precision and accuracy.

Three batches run on two different days provide the Inter batch estimate. Deviation from nominal concentration should be within +20% at LLOQC and within ±15 % for the other concentrations. A minimum of 67% of the total QC and 50% at each concentration level should pass the % nominal criteria. The % CV at each concentration level should be ±15%, except LLOQC (≤ 20%).

**Sensitivity**

Sensitivity of the method is defined as the lowest concentration of analyte that can be measured with acceptable limit of precision and accuracy. Five LLOQ samples were run and back calculated against a calibration curve. The % nominal and % CV should be within ±20% and <20%, respectively.

**Carry over**

Two processed blank samples were injected following ULOQ of each calibration curve. The permissible interference at the RT of each analyte should be 20% of LLOQ, while area response at the RT of IS should be <5 % of mean IS area in the calibration curve.

**Stability**

There were two different stability considerations; stability of analyte in stock solution and stability of analyte in plasma under different conditions, both the conditions were investigated.

Stability of mother stock in refrigerator (2-8°C) and stock dilution stability at room temperature were compared with fresh solution. Six replicates each from stability and comparison stock were injected to compare their response. The percent change should be within ±7 %, Quality control samples for bench top stability (BT) at room temperature, freeze thaw stability (FT) after three successive freeze & thaw cycles, long term stability (LT) in -25°C & -70°C deep freezers and autosampler stability (AI) at 5°C were back calculated against 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%.

% Change = [(Mean response of comparison samples - Mean corrected response of stability samples) / Mean response of comparison samples] x 100

Correction Factor = Concentration of Comparison Dilution/ Concentration of Stability Dilution

Corrected Response = Mean Stability dilution response x Correction Factor

**Reinjection Reproducibility**

A Precision and accuracy batch was reinjected after a period of storage into the autosampler to assess the reinjectibility of the samples. The batch should follow the acceptance criteria of a precision and accuracy batch.

**Ruggedness**

To demonstrate the method can withstand minor changes in reagents/solutions and chromatographic condition, ruggedness of method was investigated. A precision and accuracy batch was processed with fresh reagent and solutions. The analytical column was replaced with an identical column, with a different serial number. The batch should follow the acceptance criteria of a precision and accuracy batch.

**Recovery**

There should not be significant difference between the recoveries at different concentration level. A mixture of drug and metabolite, representing 100% extraction at low, middle and high concentrations were prepared and serve as non-extracted ‘comparison samples’. 6 QC samples each at LQC, MQC and HQC level were extracted as per the method. They serve as the extracted ‘recovery samples’. Inject 6 replicates of the ‘comparison’ samples and 6 replicates of the ‘recovery samples’ at each concentration level. Calculate the percent recovery as follows:

% Recovery = [Mean peak area response of recovery samples/ Mean Peak area response of comparison sample] x 100

Comparison sample were prepared by taking 10 µL drugs (5% Spiking) + 25 µL (IS dilution) + 165 µL mobile phase to obtain an equivalent of extracted concentration.

The % recovery, mean % recovery and % CV among mean % recoveries at low, medium and high levels were calculated. % recovery for (IS) was calculated from the data of 18 comparison and recovery samples. The % CV among % recoveries at Low, Middle and High QC should be <20%.

**Dilution Integrity (DI)**

The drug free matrix was spiked to obtain the plasma concentration (5.266 & 11.028 µg/ml) for OXC and MHD, respectively. Six replicates of dilution integrity QC samples were diluted ten times (DI-10) with similar blank matrix and processed as per the method. The samples were back calculated against a calibration curve by applying dilution factor of (DI-10). The % nominal and precision should be within ±15% and <15%, respectively.

**Extended Batch Verification**

Extended batch verification is performed to establish the analytical batch size for the method. It further demonstrates the ruggedness of the method under the given experimental conditions.

Six batches of test samples (QC interspersed between blank samples), processed and analyzed against a calibration curve. Evaluate the results for each batch separately. At least 67% of the QC samples including minimum 50% at each concentration level should be within ±15% of nominal values.

**RESULTS AND DISCUSSION**

**Mass Spectrometry**

The protonated molecule of OXC and MHD (253.20 & 255.10) were selected to generate the product ions. The ion transition (253.20——236.10 m/z) OXC & (255.10——237.00 m/z) MHD were selected for two fundamental reasons; stable, reproducible response and interference free blanks. The internal standard transition (237.10——-194.10 m/z) was also optimised.

During the development phase under the experimental conditions, significant interference was observed at (253.3——180) & (255——194) [20] and (253.2——208.3) & (255.2——194.4) [24], reported by other investigators.

For the transition (253.20——236.10 m/z) OXC, (255.10——237.00 m/z) MHD and (237.10——-194.10 m/z) IS, linearity was established for OXC but not for MHD.
The observation was investigated and it was concluded that the parent mass of the IS (237.10 amu) and product ion of MHD (237.00 amu) are having a difference of only 0.1 amu. It should have never been a problem theoretically and also since the dual time was sufficient. Also, the combination of ions for MHD (255.10→237.00) and IS (237.10→194.10) were different. Later, the run time and the column were optimised in order to differentiate their elution time. They were eluting at the same RT. The problem was solved with varying thier elution time. The S/N (Signal/Noise) ratio and chromatography was optimised at LLOQ level.

**Selectivity**

All screened seven batches were free from significant interference from endogenous substances. Selectivity of the method in presence of known concomitant drugs will be performed after the completion of clinical phase. Possible interference from decomposition products, back conversion of the metabolite will be covered and reported under incurred sample reanalysis at the study level. Fig. 1 & 2, illustrates the representative chromatogram for blank and LLOQ.

**Matrix factor (MF)**

Matrix factor experiment was performed by ‘post processing spiking method’ at LQC & HQC level. The % CV of MF for the drugs was <15%. The IS Normalised MF was calculated and reported. The results are summarised in Table 1.

**Linearity**

The calibration curve data was subjected to goodness of fit analysis, resulting $1/c^2$ to be the best fit for regression. The coefficient of correlation ($r$) was >0.99 for calibration range OXC (0.052-7.044 µg/ml).
and MHD (0.103-15.054 µg/ml). The Weighting chart for OXC and MHD is illustrated in Fig. 3 & 4, respectively.

**Precision and accuracy**

The Intra and Inter batch precision were within ±15% at all the concentration levels, except LLOQQC (±20%). The % nominal calculated for a batch and between batches were within ±15% at all the concentrations, except LLOQQC (±20%). The assay performance is summarized in Table 2.

**Sensitivity**

The observed S/N ratio was >5 throughout validation exercise. The precision and accuracy for sensitivity samples [LLOQ=0.032µg/ml (OXC) & 0.103 µg/ml (MHD)] were within ±20% limit. The LLOQ for OXC and MHD were less as 1.6% and 1.4% of their reported Cmax [25]. The results are summarised in table 3.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Conc (µg/mL)</th>
<th>Mean MF ± S. D</th>
<th>% CV for MF</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXC</td>
<td>0.082</td>
<td>0.826±0.038</td>
<td>4.60</td>
</tr>
<tr>
<td></td>
<td>5.266</td>
<td>0.835±0.029</td>
<td>3.44</td>
</tr>
<tr>
<td>MHD</td>
<td>0.265</td>
<td>0.977±0.044</td>
<td>4.50</td>
</tr>
<tr>
<td></td>
<td>11.028</td>
<td>0.972±0.022</td>
<td>2.24</td>
</tr>
</tbody>
</table>

**Fig. 3: Weighting chart for OXC**

**Fig. 4: Weighting chart for MHD**

Table 2  Intra-day and Inter-day precision and accuracy

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Level</th>
<th>Nominal (µg/ml)</th>
<th>Mean Concentration (µg/ml)</th>
<th>Mean % nominal</th>
<th>% CV</th>
<th>Mean Concentration (µg/ml)</th>
<th>Mean % nominal</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXC</td>
<td>LLOQQC</td>
<td>0.033</td>
<td>0.034</td>
<td>103.03</td>
<td>1.86</td>
<td>0.034</td>
<td>103.37</td>
<td>5.85</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>0.082</td>
<td>0.086</td>
<td>104.63</td>
<td>2.70</td>
<td>0.085</td>
<td>103.78</td>
<td>2.73</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>3.159</td>
<td>3.095</td>
<td>97.97</td>
<td>3.45</td>
<td>3.108</td>
<td>98.39</td>
<td>5.23</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>5.266</td>
<td>5.234</td>
<td>99.40</td>
<td>4.52</td>
<td>5.260</td>
<td>99.88</td>
<td>5.17</td>
</tr>
<tr>
<td>MHD</td>
<td>LLOQQC</td>
<td>0.106</td>
<td>0.114</td>
<td>107.86</td>
<td>10.45</td>
<td>0.117</td>
<td>109.91</td>
<td>8.33</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>0.265</td>
<td>0.271</td>
<td>102.08</td>
<td>4.22</td>
<td>0.278</td>
<td>104.78</td>
<td>5.08</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>6.617</td>
<td>6.146</td>
<td>92.88</td>
<td>3.90</td>
<td>6.354</td>
<td>96.02</td>
<td>4.39</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>11.028</td>
<td>10.525</td>
<td>95.44</td>
<td>2.91</td>
<td>10.906</td>
<td>98.90</td>
<td>3.77</td>
</tr>
</tbody>
</table>
The % interference at the RT of drugs was <20% throughout the validation. The % interference at the RT of IS was always <5%.

**Stability**

In a study where the results are directly influenced by analyte stock solutions, it is recommended that the acceptance difference between the absolute responses of fresh stock and aged stock be tighter (within 5 - 7%), rather (15 - 20%) normally applied to bioanalytical results [26].

The % Change for Stock solution stability at refrigerated temperature range (2-8°C) and stock dilution stability at room temperature were within ±7%. Majority of the methods reported % stability, instead of % change [14, 20, 27 & 28].

Stability of drugs in plasma, which includes BT, FT, LT and AI stability were within the acceptance limit. Results are demonstrated in table 4 & 5

**Reinjection reproducibility**

A precision and accuracy batch, which was subjected to storage in autosampler for 25 hours at 5°C, was re-injected. The statistics demonstrated the reinjectibility of the sample within 25 hour. The precision, % nominal were within limits of a precision and accuracy batch.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LLOQ (µg/ml)</th>
<th>Replicate</th>
<th>Observed Conc. (µg/ml)</th>
<th>% Nominal</th>
<th>Mean</th>
<th>Mean % nominal</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXC</td>
<td>0.032</td>
<td>1</td>
<td>0.032</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.032</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.033</td>
<td>103.13</td>
<td>0.033</td>
<td>102.50</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.033</td>
<td>103.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.034</td>
<td>106.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MHD</td>
<td>0.103</td>
<td>1</td>
<td>0.103</td>
<td>100.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.105</td>
<td>101.94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0.105</td>
<td>101.94</td>
<td>0.104</td>
<td>100.78</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.104</td>
<td>100.97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>0.102</td>
<td>99.03</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stability</th>
<th>Analyte</th>
<th>Nominal concentration (µg/mL)</th>
<th>Mean concentration (µg/mL)</th>
<th>% Nominal</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze thaw (3 cycles)</td>
<td>OXC</td>
<td>0.082</td>
<td>0.080</td>
<td>97.36</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>MHD</td>
<td>0.265</td>
<td>0.263</td>
<td>99.37</td>
<td>12.01</td>
</tr>
<tr>
<td>Bench Top (7 hours)</td>
<td>OXC</td>
<td>0.082</td>
<td>0.082</td>
<td>100.41</td>
<td>5.83</td>
</tr>
<tr>
<td></td>
<td>MHD</td>
<td>0.265</td>
<td>0.276</td>
<td>104.09</td>
<td>4.15</td>
</tr>
<tr>
<td>Long term -70 Degree (15 days)</td>
<td>OXC</td>
<td>0.082</td>
<td>0.081</td>
<td>99.19</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td>MHD</td>
<td>0.265</td>
<td>0.277</td>
<td>104.53</td>
<td>6.24</td>
</tr>
<tr>
<td>Long term -25 Degree (15 days)</td>
<td>OXC</td>
<td>0.082</td>
<td>0.081</td>
<td>98.99</td>
<td>5.31</td>
</tr>
<tr>
<td></td>
<td>MHD</td>
<td>0.265</td>
<td>0.271</td>
<td>102.08</td>
<td>3.27</td>
</tr>
<tr>
<td>Auto sampler (48 Hours)</td>
<td>OXC</td>
<td>0.082</td>
<td>0.079</td>
<td>96.34</td>
<td>2.53</td>
</tr>
<tr>
<td></td>
<td>MHD</td>
<td>0.265</td>
<td>0.278</td>
<td>104.72</td>
<td>5.33</td>
</tr>
</tbody>
</table>
Ruggedness
The ruggedness of the method was demonstrated under the subjected change of solutions/reagents and chromatographic column. The precision, % nominal were within the acceptance limit for a PA batch.

Recovery
The mean % recovery was 71.31% and 84.69% for OXC and MHD respectively. The % recovery for IS was 86.73%. Precision among % recoveries at Low, medium and higher QC level were 3.44% and 1.79% for OXC and MHD, respectively.

Dilution Integrity
The sample dilution (D1-10) was reproducible for both the drugs (OXC:5.266, MHD:11.028 µg/ml), when diluted with similar blank matrix. The % nominal was within ±15% and the observed precision was within ±15%. This demonstrates that the sample can be diluted up to 10 times and yet the results are predictable and reproducible.

Extended Batch Verification
The method validation precision and accuracy run should contain enough test samples to mimic the actual run time of a production batch; this is a current industry standard and FDA expectation [29]. Six batches, totaling 288 samples were run against a calibration curve. The batch size was defined at SPE unit. A batch contained 2 QC set (LQC, MQC and HQC) and 42 plasma samples, processed together. In a batch, 2/3 of the total QC samples and 1/2 at each concentration level were within ±15% for accuracy. None of the previously reported method established this parameter.

CONCLUSION
The very objective of the study was to interpret the guidelines correctly, to understand the current expectation of the regulatory bodies based on guidelines, and to perform the experiment on these understandings. The full validated was compliant to, not only FDA/EMEA guidelines, but also take an account of AAPS whitepapers and other guidelines. The method is applicable to BA/BE and pharmacokinetics studies.

REFERENCES
17. Souppart C, Decherf M, Humbert H, Maurer G. Development of a high throughput 96-well plate sample preparation method for the determination of Trileptal (oxcarbazepine) and its...


