Development and Validation of LC-MS/MS Method for the Quantification of Oxcarbazepine in Human Plasma Using an Experimental Design

Gedela SRINUBABU,*[,a] Bandaru Veera Venkata RATNAM,[b] Allam Appa RAO,[a] and Medicherla NARASIMHA RAO[a]

[a] *International Center for Bioinformatics, Center for Biotechnology, Andhra University College of Engineering; Visakhapatnam 530003, India: and* [b] *Department of Neurology, Johns Hopkins University School of Medicine; Pathology 233/Meyer 222, 600 North Wolfe Street, Baltimore, MD 21287, U.S.A.*

Received June 27, 2007; accepted October 19, 2007; published online November 5, 2007

A rapid tandem mass spectrometric (MS-MS) method for the quantification of Oxcarbazepine (OXB) in human plasma using imipramine as an internal standard (IS) has been developed and validated. Chromatographic separation was achieved isocratically on a C18 reversed-phase column within 3.0 min, using a mobile phase of acetonitrile–10 mM ammonium formate (90 : 10 v/v) at a flow rate of 0.3 ml/min. Quantitation was achieved using multiple reaction monitoring (MRM) scan at MRM transitions *m/z* 253>208 and *m/z* 281>86 for OXB and the IS respectively. Calibration curves were linear over the concentration range of 0.2—16 μg/ml (*r*>0.999) with a limit of quantification of 0.2 μg/ml. Analytical recoveries of OXB from spiked human plasma were in the range of 74.9 to 76.3%. Plackett–Burman design was applied for screening of chromatographic and mass spectrometric factors; factorial design was applied for optimization of essential factors for the robustness study. A linear model was postulated and a 2^3 full factorial design was employed to estimate the model coefficients for intermediate precision. More specifically, experimental design helps the researcher to verify if changes in factor values produce a statistically significant variation of the observed response. The strategy is most effective if statistical design is used in most or all stages of the screening and optimizing process for future method validation of pharmacokinetic and bioequivalence studies.

Key words column liquid chromatography; mass spectrometry; oxcarbazepine; experimental design; robustness; validation

Oxcarbazepine (OXB) is a newer antiepileptic drug indicated for the treatment of partial seizures as both monotherapy and combination therapy in adults and children with epilepsy. At present, determinations of OXB have been established by the use of HPLC-UV spectrometry,[1—3] or LC-APCI-MS mass-spectrometry.[4] However, none of these methods achieved the quick quantification and identification of OXB in a single run. Although methods for simultaneous determination of OXB and some of antiepileptic drugs has been described,[5—11] they were not particular interest since it takes too long chromatographic run time especially when applied to OXB, and had low sensitivity; it appeared that no assay existed for determination of the OXB using HPLC-MS/MS. The assay described here requires small mobile phase and sample volume, short chromatographic run and is sensitive, specific and fully validated.

Optimization of an MS response includes methods to improve the abundance of a specific precursor or product ion, or yielding as comprehensive or selective measurements as possible (Table 1). Maximization of the signal or the signal-to-noise ratio (S/N) of a particular ion can be used for quantitative optimization, but choosing qualitative optimization criteria are less trivial.

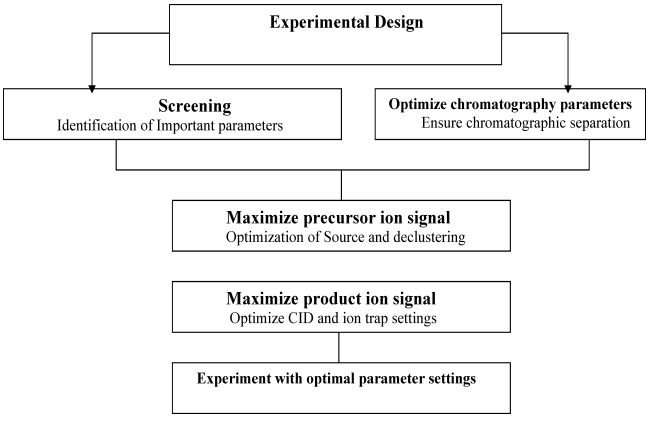
Optimization techniques of particular interest are factorial design and central composite design, for screening of the important parameters Placket–Burman design can be useful. Chemometric approaches for optimization of ESI or MS/MS parameters also occur in literature.[12,13] In this present study Plackett–Burman and factorial designs are employed for screening of essential parameters and optimization respectively.

Experimental

Chemicals and Reagents OXB, and imipramine (Internal standard), were kindly provided by Glenmark Pharmaceuticals (Mumbai, India). HPLC-grade acetonitrile, methanol, formic acid and diethyl ether were purchased from Qualigens (Mumbai, India). Blank human plasma was harvested after 5 min centrifugation at 4000×***g*** and stored at −20 °C until use.

Instrumentation The HPLC system consisted of an HP1100 binary pump, auto sampler, column oven, and online degasser (Agilent Technologies, Palo Alto, CA, U.S.A.). The analytical column used was reversed-phase symmetry C18 (75 mm×4.6 mm i.d. 3.5 μm). A mobile phase of 10 mM ammonium formate/ACN (10 : 90, v/v) was pumped at a flow rate of 0.3 ml/min and the injected sample volume to whole over the method is 10 μl. The LC elute was introduced directly into an Applied Biosystems triple quadrupole mass spectrometer API 4000 (MDS-Sciex, Toronto Canada, software: Analyst 1.4.1) equipped with an electrospray ionization source. The triple quadrupole mass spectrometer was operated in the positive ion mode and the multiple reaction monitoring (MRM) chromatograms obtained were used for quantification. MRM transitions of *m/z* 253→208, *m/z* and 281→86 were respectively optimized for OXB and Imipramine. The

Table 1. Experimental Parameters Possibly Affecting the Performance of an Online LC-MS/MS Analysis



* To whom correspondence should be addressed. e-mail: srinubabuau6@gmail.com

Table 2. Tandem Mass Spectrometer Main Working Parameters

S. No.	Parameter	Value
1	Source temperature	350
2	Dwell time pertransition (MS)	200
3	Ion Source gas1 (psi)	6
4	Ion Source gas2 (psi)	8
5	Curtain gas	8
6	Collison gas (Electrode potential)	10
7	Ion spray voltage (V)	5000
8	Declustering potential (V)	35 (Analyte) and 25 IS
9	Collision energy (V)	27 (Analyte) and 25 IS.
10	Collision cell exit potential (V)	10 (Analyte) and 13 IS
11	Focusing potential	120 (Analyte) and 90 IS

detailed mass spectrometer conditions were tabulated (Table 2).

Preparation of Calibration Standards and Quality Control Samples Primary stock solutions were prepared by dissolving the compounds or internal standard in methanol. Appropriate dilutions of the stock solutions with water–acetonitrile (50 : 50), v/v) were made subsequently in order to prepare the working solutions in the range 0.2—16 μg/ml. Two different series of stock solutions were prepared from different weights for calibration standards (Cs) and quality control samples (QCs). The I.S. working solution is used at a concentration of 5 μg/ml. All the solutions were prepared in polypropylene flasks and stored in darkness in temperatures between 2 and 8 °C. Cs and QCs in the concentration range of 0.2—16 μg/ml were prepared for the assessment of calibration, accuracy and precision, quality control and stability by spiking 0.18 ml of drug-free human plasma with the appropriate volume of working solutions. The samples were prepared as described below.

Sample Preparation Appropriate volume of I.S. working solution was added to Cs, QCs or actual samples followed by 2.5 ml of tertiary butyl methyl ether (TBME) in glass tubes. The tubes were placed on a horizontal shaker for 5 min at a velocity of 250 rpm. After 5 min centrifugation at 4000 ***g*** at 20 °C, the organic layer was transferred into another glass tube. The solvent was evaporated to dryness at 40 °C under a nitrogen stream. The residue was dissolved in mobile phase followed by vortex mixing and this solution was transferred to a conical polypropylene insert inside amber glass micro vials. All liquid transfers were done manually using RAININ-Gilson electronic pipettors (Villiers-le-Bel, France).

Validation. Selectivity Ten human plasma from ten individual lots were extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent response at the retention times of OXB and I.S. was compared to the response at the lower limit of quantitation (LLOQ) for OXB and to the response at the working concentration for I.S.

Calibration and Sample Quantification Calibration standards at levels of 0.2, 0.5, 1.5, 3.4, 6.8, 13.2 and 16.0 μg/ml (n=2, at each level) were extracted and assayed as described above, on three different days. Calibration curves (y=ax+b), represented by the plots of the peak-area ratios (y) of OXB to I.S. *versus* the concentration (x) of the calibration standards, were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model. Actual, quality control and stability samples were calculated from the resulting area ratio of OXB and the regression equation of the calibration curve.

Accuracy and Precision Intra-day accuracy and precision were evaluated by analysis of QCs at levels of 0.5, 8.0, 12.0 μg/ml (n=6 at each level) on the same day. These levels were chosen to demonstrate the performance of the method and to determine the lower limit of quantitation (LLOQ) of the method. The upper limit of quantitation (ULOQ) was given by the highest level of the calibration curve.

Carryover Test Carry over test is performed with extracted blank plasma sample and high QC sample. Initially extracted sample was injected followed by two times high QC sample (with IS) were injected, again blank plasma extracted was once again injected, the area obtained at third step is 0.064% of high QC, which is within the acceptance criteria.

Experimental Design Experimental design techniques are powerful tools for the exploration of multivariate systems. In particular, statistical design using response surface methodology (RSM) is a way of choosing experiments efficiently and systematically to give reliable and coherent information. RSM is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response. The final step in RSM is to find a suitable approximation for the true functional relationship between response Y and the set of independent variables. Usually, a low order polynomial in some region of the independent variables is employed. If the response is well modeled by linear function of the independent variables, then the approximating function is the first order model

$$Y=\beta_0+\beta_1x_1+\beta_2x_2+\cdots+\beta_kx_k+E$$

If there is curvature in the system, then a polynomial of higher degree must be used, such as the second order model.

$$Y=\beta_0+\sum_{i=1}^{k}\beta_ix_i+\sum_{i=1}^{k}\beta_{ii}x_i^2+\sum_{i<j}\beta x_ix_j+E$$

All RSM problems utilize one or both of these models. RSM is a sequential procedure. The eventual objective of RSM is to determine the optimum operating conditions for the system or to determine a region of the factor space in which operating requirements are satisfied. As demonstrated by Srinubabu *et al.* during the validation of robustness and intermediate precision for voriconazole[15] and pramipexole[16] in pharmaceutical dosage forms using response surface methodology, the use of experimental design during optimization made the validation process easier and more cost effective.

Plackett–Burman Design The Plackett–Burman (PB) design[17] is a two-level design for examining parameters in k=n+1 runs. Plackett and Burman rediscovered designs that had previously been given by Raj Chandra Bose and K. Kishen at the Indian Statistical Institute, Kolkata.[18] PB designs are factorial designs that examine up to N−1 factors in N (multiple of four) experiments.

Factorial Designs In statistics, factorial designs are experimental designs consisting of a carefully chosen subset (fraction) of the experimental runs of a full factorial design.[19] The subset is chosen so as to exploit the sparsity-of-effects principle to expose information about the most important features of the problem studied, while using a fraction of the effort of a full factorial design in terms of experimental runs and resources.

Results and Discussion

Mass Spectra Analysis The full scan mass spectra of OXB and IS after direct injection in mobile phase are presented in Figs. 1 and 2. The predominant protonated molecules found were (MH^+–$CONH_2$) m/z 208 for OXB and m/z 86 for IS. The mass spectrometric parameters were optimized to obtain the higher signal for the selected ions at 208. The method was fully validated using ion 208.

Selectivity and Matrix Effect Selectivity was assessed by comparing chromatograms of 10 different lots of blank human plasma with the corresponding spiked plasma. No significant interference from endogenous substances with mirtazapine or olanzapine was detected. Typical retention times for imipramine, OXB are 2.49 and 2.74 respectively (Figs. 3a, b). Limit of detection for the present study is 35 ng/ml.

The matrix effects are evaluated by spiking blank plasma sample extracts with neat standards at low and high concentrations. Figure 3c represents the chromatogram of extracted blank plasma. Assaying as many as ten different lots of plasma, the calculated matrix effects were within the range of 5—10%. Thus the ion suppression and enhancement from plasma matrix was negligible for this method.

Recovery The mean recovery after liquid–liquid extraction with TBME was 76% (range 74—77%). These results suggested that there was no relevant difference in extraction recovery at different concentration levels for OXB. I.S. recovery was also tested and was 94% at the working concentration of 5 μg/ml.

Linearity Linear calibration curves were obtained with a coefficient of correlation (r) usually higher than 0.998 (Table

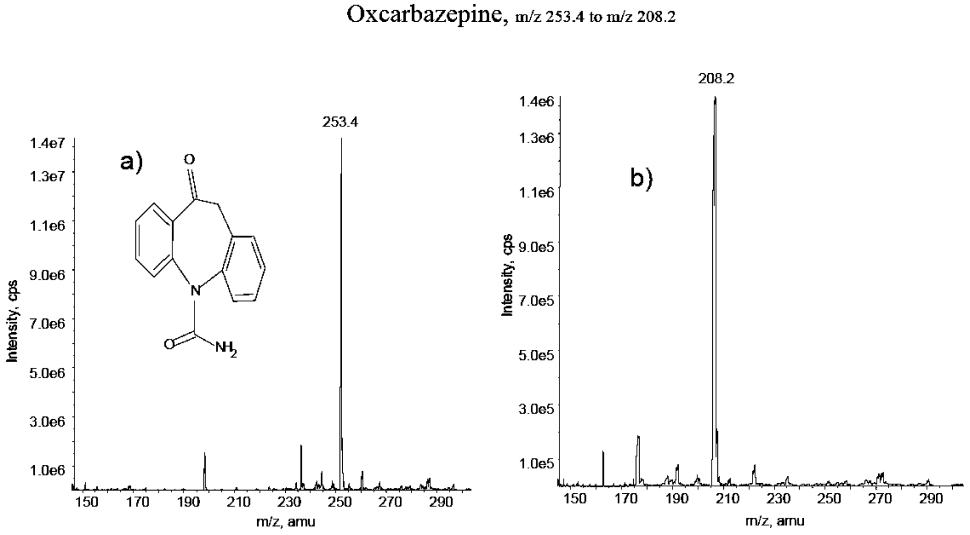


Fig. 1. Mass Spectra of the (a) Oxzcarbazepine Precursor and (b) Major Oxcarbazepine Fragment

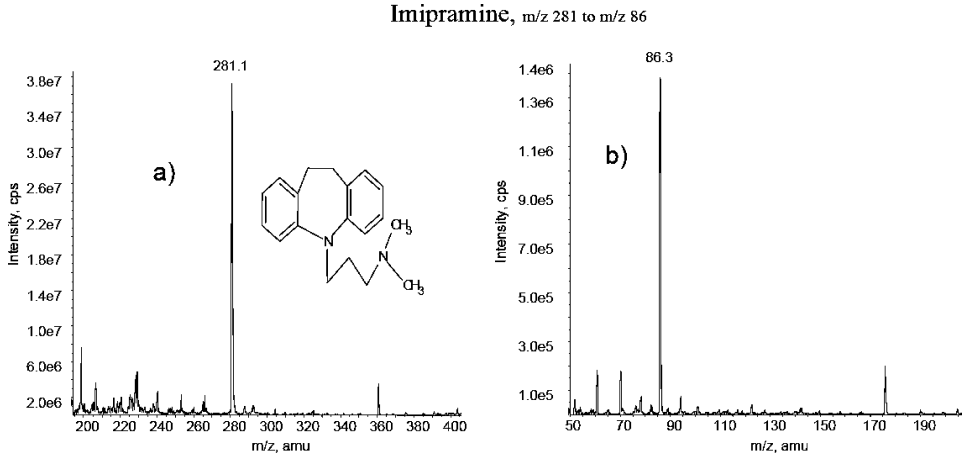


Fig. 2. Mass Spectra of the (a) Imipramine Precursor and (b) Major Imipramine Fragment

3). For each calibration standard level, the concentration was back calculated from the linear regression curve equation.

Precision and Accuracy The LLOQs were defined as the lowest drug concentration, which can be determined with an accuracy of 80—120% and a precision ≤20% on a day-to-day basis.[20] The results (Table 4) satisfactorily met the acceptance criteria: mean accuracy within 85—115% and C.V.≤15% (80—120% and ≤20% at LLOQ). LLOQs were set at 0.5 μg/ml.

Dilution Integrity Dilution integrity was performed at middle quality control sample with two times dilution (2T) and four times dilution (4T), the results are tabulated in Table 5.

Stability OXB was stable for at least 3 months in both actual and spiked human plasma samples when frozen at or below −20 °C. The mean (±S.D.) recoveries for actual samples (from the first determination), were 104±8% (n=6) for OXB. OXB was stable for at least 6 h at room temperature in spiked human plasma samples; the mean recoveries from the nominal concentration were 95—106%.

OXB in working solutions were found to be stable for at least 2 weeks at +2—8 °C; the mean recoveries (n=3) from the nominal concentrations were 101 and 88% for OXB, at 8.0 μg/ml. They were also found to be stable in working solutions for at least 6 h at room temperature in darkness; the mean recoveries (n=3) from the nominal concentration OXB at LQC concentration and at HQC concentration were at 0.2, and 16.0 μg/ml. The results obtained were within the acceptance criteria (Table 5).

Extracts at concentrations of LQC and HQC μg/ml were found to be stable on the autosampler at 10 °C for at least 12 h. Arithmetic mean recovery values after three freeze–thaw cycles were between 95—105% of the nominal value for LQC and HQC respectively (Table 6).

Dry Extract Stability Dry extract stability was conducted with one set of calibration curve standards and three sets of quality control samples. For this, sample preparation was performed upto the evaporation step, after this the evaporation samples were stored at 4 °C. After 48 h which depends upon molecule stability, the samples were reconstituted with 200 μl mobile phase. The results are tabulated in Table 6.

Robustness Robustness testing is a part of method validation.[21] Nowadays, method validation and robustness testing have become increasingly important. Especially in the pharmaceutical industry, extensive method validation is required in order to meet the strict regulations set by the regulatory bodies. The international conference on harmonization

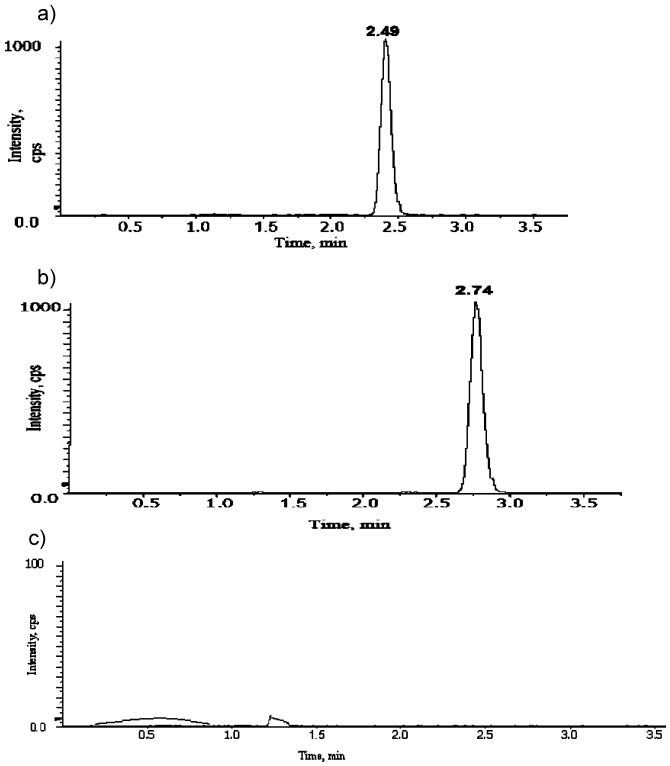


Fig. 3. (a) Chromatogram for Imipramine, (b) Chromatogram for Oxcarbazepine and (c) Chromatogram for Blank Human Plasma

of technical requirements for the registration of pharmaceuticals for human use guidelines define robustness as: "The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage". Robustness testing should be performed during the development of an analytical method to show its reliability when small variations occur in method parameters, such as the operating conditions for chromatographic methods, various chromatographic factors and detector conditions. When measurements are affected by these deliberate variations, precautions should be taken to ensure that the analytical method is valid and robust when complying with these precautionary measures.

For robustness study the factors selected have to reflect potential changes that may occur during validation process. Screening designs are often applied in the evaluation of the robustness of a method, they allow screening a relatively large number of factors f in a minimal number of experiments ($N \geq f+1$). As shown in the Tables 7.1 and 7.2, in this study two level Plackett–Burman design was employed at two levels (-1, $+1$) for the evaluation of essential qualitative factors from the percentage of acetonitrile in mobile phase (%ACN), flow rate (FR), auto sampler temperature (AST), column oven temperature (COT), declustering potential (DP), collision energy (CE), exit potential (EXP). The ANOVA results such as p-values 0.0367 for FR, 0.0106 for % ACN and 0.160 for DP (Table 7.3) indicated that these factors are more important than other factors indicated above. The $R^2=0.9983$ indicated that the model was fit for the applied experimental design, further optimization should have to be performed to report critical values.

Factorial optimization design[22] may be useful for this type of optimization study, because it creates empirical model equations that correlate the relationship between variables and response(s). Factorial design has the following advantages: (a) to allow a complete study where all interaction ef-

Table 3. Linearity Related Results for the Quantitative Determination of OXB by the Proposed Method

Statistical parameter	
Concentration range (μg/ml)	0.2—16.0
Regression equation	$y=0.21359x-0.0693$
Correlation coefficient (r)	0.9998
Stand error on estimation (S_e)	0.09293
Standard deviation on slope (S_b)	0.00215
Standard deviation on intercept (S_a)	0.04428
Limit of quantification (LOQ) (μg/ml)	0.1

Table 4. Accuracy for OXB

Concentration (μg ml^{-1})	Recovery (%)[a)]	RSD (%)
0.2	95.4	3.92
8.0	102.6	3.49
16.0	96.9	2.56
Mean	99.6	3.32

a) Mean of six determinations.

Table 5. Dilution Integrity

Concentration (μg ml^{-1})		Recovery (%)[a)]	RSD (%)
Middle conc.	(8.0)	96.3	3.5
2T	(4.0)	98.2	4.2
4T	(2.0)	103.1	3.8

a) Mean of six determinations.

Table 6. Stability

Stability parameter	Standard concentration (μg/ml)	% Recovery[a)]
Bench top (6 h)	0.2	97.50
	16.0	96.56
Freeze thaw 1st cycle	0.2	97.5
	16.0	96.3
2nd cycle	0.2	95.5
	16.0	85.93
3rd cycle	0.2	96.5
	16.0	86.0
Autosampler (18 h)	0.2	99.0
	16.0	96.75
Long term (30 d)	0.2	94.5
	16.0	93.75
Stock solution (at room temp, 12 h)	8.0	95.12
Stock solution at 2—8 °C for 2 weeks	8.0	94.15
Dry extract	0.2	95.1
	16.0	96.43

a) Mean of six determination.

Table 7.1. Denominations for Plackett–Burman Design

%ACN in mobile phase	Flow rate	Auto sampler temp.	Column oven temp.	Declusturing potential	Collision energy	Exit potential	Level
85	0.2	5	25	30	25	8	−1
95	0.4	15	35	40	30	12	1

Table 7.2. Experimental Plan for Plackett–Burman Design and Obtained Responses

%ACN in Mobile phase	Flow rate	Auto sampler temp	Column oven temp	Declusturing potential	Collision energy	Exit potential	Level
−1	−1	1	1	−1	−1	1	1.63
1	−1	1	−1	1	−1	−1	1.65
−1	1	−1	−1	1	−1	1	1.72
−1	1	1	−1	−1	1	−1	1.70
1	−1	−1	−1	−1	1	1	1.66
1	1	−1	1	−1	−1	−1	1.73
1	1	1	1	1	1	1	1.77
−1	−1	−1	1	1	1	−1	1.65
1	1	1	1	1	1	1	1.78

Table 7.3. ANOVA Results for Plackett–Burman Design

SS	Mean	F	*p*	Factor
0.001763	0.001763	35.2667	0.106204	%ACN
0.014963	0.014963	299.2667	0.036759	FR
0.000003	0.000003	0.0667	0.839139	AST
0.000403	0.000403	8.0667	0.215518	COT
0.000750	0.000750	15.0000	0.160861	DP
0.000403	0.000403	8.0667	0.215518	CE
0.000403	0.000403	8.0667	0.215518	EXP

fects are estimated; and (b) to give an accurate description of an experimental region around a center of interest with validity of interpolation with minimum runs.[23] In Factorial k factors requires 3^k factorial runs, symmetrically spaced at $\pm\alpha$ along each variable axis, and at least one center point. In order to study the variables at no more than three levels (−1, 0, +1), three factors were considered: percentage v/v of acetonitrile (x_1); flow rate ml min^{-1} (x_2) and collision energy (x_3). The ranges examined were small deviations from the method settings and the corresponding responses in the peak area ratio considered (Y) were observed. A factorial design with 10 experiments including two center points, the experimental plan and the corresponding responses are reported in Table 8.2. All experiments were performed in randomized order to minimize the effects of uncontrolled factors that may introduce a bias on the response. A classical second-degree model with a cubic experimental domain was postulated. Experimental results were computed by statistica.[24] The coefficients of the second-order polynomial model were estimated by the least squares regression. The equation model for Y (found peak area ratio) was as follows:

$$Y = 1.762729 + 0.015000x_1 + 0.026594x_2 + 0.003333x_3 + 0.004094x_1^2 + 0.001667x_2^2 + 0.006594x_3^2$$

The model was validated by the analysis of variance (ANOVA). The statistical analysis showed (Table 8.3) that the model represents the phenomenon quite well and the variation of the response was correctly related to the variation of the factors (R^2=0.9988). Figure 4 shows the influence

Table 8.1. Chromatographic Conditions and Range Investigated During Robustness Testing

Variable	Optimized value	Range investigated
Mobile phase (ACN/buffer)	90 : 10	85—95
Flow rate (ml min^{-1})	0.3	0.2—0.4
Collision energy	27	25—30

Table 8.2. Experimental Plan for Robustness Testing Using Factorial Design and Obtained Responses

Flow rate	% ACN	CE	Peak area ratio
1	0	−1	1.76
−1	0	1	1.73
0	0	0	1.812
0	1	−1	1.79
1	−1	0	1.76
−1	1	0	1.74
−1	−1	−1	1.72
0	−1	1	1.79
1	1	1	1.76
0	0	0	1.815

Table 8.3. ANOVA Table Factorial Design

Factor	SS	MS	F	*p*
FR	0.007816	0.003908	298.8623	0.000353
MP	0.000220	0.000110	8.4079	0.058907
DP	0.000414	0.000207	15.8368	0.025450

of each of the variables studied in the OXB as a response. Optimized critical values are tabulated in Table 8.1.

Intermediate Precision/Ruggedness The intermediate precision is a measure of precision between repeatability and reproducibility and it should be established according to the circumstances under which the procedure is intended to be used. The analyst should establish the effects of random events on the precision of the analytical procedure. The intermediate precision is obtained when multiple analysts, using multiple columns, on multiple days in one laboratory,[15,16] perform the assay. In order to study these effects simultane-

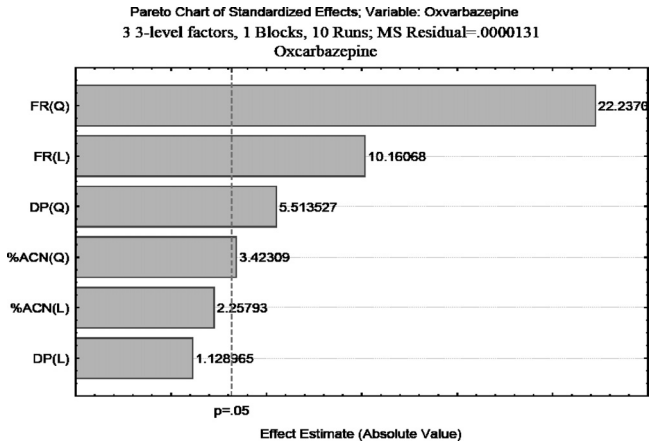


Fig. 4. Pareto Chart of Standard Effects

ously, a multivariate approach was used.

The considered variables included analysts (1 and 2), equipment (Column 1 and 2) and days (1 and 2). The considered response was the found drug peak area ratio. A linear model ($y=b_0+b_1x_1+b_2x_2+b_3x_3$) was postulated and a 2^3 full factorial design was employed to estimate the model coefficients. Each experiment was repeated three times in order to evaluate the experimental error variance. The analyses were carried out in a randomized order according to the experimental plan reported in Table 9. The concentration of mirtazapine was about $8\,\mu g\,ml^{-1}$. No considered factor was found significant for the assumed regression model. The RSD found (7.92%, $n=24$) was acceptable, indicating an acceptable precision of the analytical procedure.

Conclusion

Compared with other methods, HPLC-MS/MS improved the specificity and sensitivity while shortening the analytical time of the samples. The liquid–liquid extraction technique simplified the preparation of the samples. The main aim of the study was to establish a HPLC-MS method that was suitable for determination of OXB in plasma. To the best of our knowledge, this method meets the request need in the present pharmacokinetic studies, bioequivalence studies of the OXB. In particular, the use of experimental design for the improvement of accuracy and precision is a very attractive goal, and the use of experimental design during validation constitutes a basic feature of multivariate optimization, which if appropriately used can solve several problems and constitutes a powerful tool in the hands of researchers.

Competing Interests: The authors have no competing interests; further local ethical committee approved the work.

Acknowledgments Authors are thankful for partial financial support from IIT up gradation- (AUCE (Autonomous)) grants.

Table 9. Experimental Plan for Intermediate Precision Testing and Obtained Responses

No. exp.	Analyst	Instrument	Day	Peak area ratio
1	Analyst 1	Column 1	Day 1	1.71
2	Analyst 1	Column 1	Day 1	1.68
3	Analyst 1	Column 1	Day 1	1.69
4	Analyst 2	Column 1	Day 1	1.67
5	Analyst 2	Column 1	Day 1	1.68
6	Analyst 2	Column 1	Day 1	1.69
7	Analyst 1	Column 2	Day 1	1.72
8	Analyst 1	Column 2	Day 1	1.72
9	Analyst 1	Column 2	Day 1	1.70
10	Analyst 2	Column 2	Day 1	1.71
11	Analyst 2	Column 2	Day 1	1.72
12	Analyst 2	Column 2	Day 1	1.68
13	Analyst 1	Column 1	Day 2	1.68
14	Analyst 1	Column 1	Day 2	1.67
15	Analyst 1	Column 1	Day 2	1.67
16	Analyst 2	Column 1	Day 2	1.67
17	Analyst 2	Column 1	Day 2	1.71
18	Analyst 2	Column 1	Day 2	1.68
19	Analyst 1	Column 2	Day 2	1.68
20	Analyst 1	Column 2	Day 2	1.69
21	Analyst 1	Column 2	Day 2	1.66
22	Analyst 2	Column 2	Day 2	1.65
23	Analyst 2	Column 2	Day 2	1.66
24	Analyst 2	Column 2	Day 2	1.67

References and Notes

1) Kimiskidis V., Spanakis M., Niopas I., Kazis D., Gabrieli C., Kanaze F. I., Divanoglou D., *J. Pharm. Biomed. Anal.*, **43**, 763—769 (2007).
2) Juenke J. M., Brown P. I., Urry F. M., McMillin G. A., *J. Chromatogr. Sci.*, **44**, 45—51 (2006).
3) Levert H., Odou P., Robert H., *J. Pharm. Biomed. Anal.*, **28**, 517—523 (2002).
4) Kłys M., Rojek S., Bolechała F., *J. Chromatogr. B: Anal. Technol. Biomed. Lift Sci.*, **825**, 38—43 (2005).
5) Ma C. L., Jiao Z., Jie Y., Shi X. J., *Chromatographia.*, **65**, 267—272 (2007).
6) Yang J., Jiao Z., Shi X. J., *Chinese Pharma. J.*, **41**, 1899—1908 (2006).
7) Contin M., Balboni M., Callegati E., Candela C., Albani F., Riva R., Baruzzi A., *J. Chromatogr. B: Anal. Technol. Biomed. Lift Sci.*, **828**, 113—12 (2005).
8) Bugamelli F., Sabbioni C., Mandrioli R., Kenndler E., Albani F., Raggi M. A., *Anal. Chim. Acta*, **472**, 1—8 (2002).
9) Levert H., Odou P., Robert H., *Biomed. Chromatogr.*, **16**, 19—26 (2002).
10) Khoschsorur G. A., Fruhwirth F., Halwachs-Baumann G., *Chromatographia* **54**, 345—349 (2001).
11) Kumps A., *J. Liq. Chromatogr.*, **7**, 1235—1239 (1984).
12) Vaidyanathan S., Kell D. B., Goodacre R., *Anal. Chem.*, **76**, 5024—5032 (2004).
13) Riter L. S., Vitek O., Gooding K. M., Hodge B. D., Julian R. K., *J. Mass Spectrom.*, **40**, 565—579 (2005).
14) Charles L., Caloprisco S., Mohamed S., Sergent M., *Int. J. Mass Spectrom.*, **11**, 361—370 (2005).
15) Srinubabu G., Raju Ch. A. I., Sarath N., Kiran Kumar P., Seshagiri Rao J. V. L. N., *Talanta*, **71**, 1424—1429 (2007).
16) Srinubabu G., Jaganbabu K., Sudharani B., Venugopal K., Girizasankar G., Seshagiri Rao J. V. L. N., *Chromatographia*, **64**, 95—100 (2006).
17) Plackett R. L., Burman J. P., *Biometrika*, **33**, 305—311 (1946).
18) Bose R. C., Kishen K., *Sankhya*, **21**, 5—13 (1940).
19) Chen J., Sun D. X., Wu C. F. J., International Statistical Review, Special Issue on Statistics in Industry., **61**, 131—145 (1993).
20) Validation of Analytical Procedures, Q2A Definitions and Terminology, Guidelines prepared within the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use, ICH, 1995, pp. 1—5.
21) http://www.fda.gov/cder/guidance/4252fnl.pdf.
22) Box G. E., Hunter W. G., Hunter J. S., Hunter W. G., "Statistics for Experimenters: Design, Innovation, and Discovery," 2nd ed., John Wiley Sons, New York, 2005, pp. 134—143.
23) Douglas C. M., "Design and Analysis of Experiments," 5th ed., John Wiley Sons, New York, 2003, pp. 214—231.
24) Stat soft, Inc. (2001) Statistica data analysis system, Statistica software east 2300 14th street, Tulsa, Ok 74104.