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Liquid chromatography–electrospray ionisation mass spectrometry method for the determination of escitalopram in human plasma and its application in bioequivalence study

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Abstract

A novel liquid chromatographic–electrospray ionisation mass spectrometric (LC–ESI-MS) method has been developed for the determination of escitalopram, an antidepressant in human plasma using paroxetine as internal standard. The method involved liquid–liquid extraction of the analyte from human plasma with a mixture of diethyl ether and dichloromethane (70:30, v/v). The chromatographic separation was achieved within 7.0 min by using 2.0 mM ammonium acetate (pH 5.0)–acetonitrile (54:46, v/v) as mobile phase and a ODS YMC^{TM} AQ 150 mm × 4.6 mm analytical column; the flow-rate was 1.0 ml/min. Ion signals *m/z* 325.0 and 330.0 for escitalopram and internal standard, were measured in the positive mode. A detailed validation of the method was performed as per USFDA guidelines and the standard curves were found to be linear in the range of 1.0–200 ng/ml with a mean correlation coefficient more than 0.99. The absolute recovery was more than 75% for both escitalopram and internal standard. The method was simple, sensitive, precise, accurate and was successfully applied to the bioequivalence study of escitalopram in healthy, male, human subjects.

Keywords: Escitalopram; Validation; Bioequivalence

1. Introduction

Escitalopram is the *S*-enantiomer of racemic citalopram. It is highly selective serotonin re uptake inhibitor antidepressant, developed for the treatment of depression and anxiety disorders [1]. Preclinical studies have demonstrated that the therapeutic activity of citalopram resides in escitalopram and the *R*-enantiomer is approximately 30-fold less potent than escitalopram [1]. Escitalopram exhibits linear pharmacokinetics and its half life in human is 27.0–32.0 h. It has a low potential for drug–drug interactions.

Quantification of citalopram—the racemic form, has been performed in the past [2–19] using HPLC coupled with UV or fluorometric detection employing the two prevalent technique of sample pre-treatment, i.e., liquid–liquid extraction (LLE), solid phase extraction (SPE) and also by direct injection of plasma into HPLC without any sample pre-treatment, i.e., online sample purification, pre-concentration and separation. The lower limit of quantitation (LLOQ) for all the reported methods ranged from 2.0 to 30.0 ng/ml.

The objective of the present investigation was to develop a simple and novel method for the determination of escitalopram in human plasma, employing liquid chromatography with electrospray ionization mass spectrometric (LC–ESI-MS) detection. The scope of the method was limited to the bioequivalence study of escitalopram formulations in healthy, male, human subjects only and was not applicable for pharmacokinetic studies in patients. The study was conducted in order to obtain marketing approval for escitalopram formulation. The analytical method employed for the quantitative determination of drug in biological matrix plays a significant

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role in the evaluation and interpretation of bioequivalence data. Therefore, a complete validation of analytical method was performed in accordance with USFDA guidelines [20] to yield reliable results that could be satisfactorily interpreted.

2. Experimental

2.1. Materials

Reference standards of escitalopram oxalate (purity, 99.98%) and paroxetine HCl (purity, 99.96%) were prepared in house (Cadila Health Care Ltd., Ahmedabad, India). Acetonitrile (HPLC grade) was obtained from Merck, Darmstadt, Germany. Formic acid was supplied by E. Merck (India) Ltd., Mumbai, India. Diethyl ether and dichloromethane were procured from Merck Limited, Mumbai, India. Human plasma was obtained from Gujarat Blood Bank, Ahmedabad, India. HPLC Type II Water from Millipore's Milli-Q System was used throughout the analysis.

2.2. Stock and working solution preparation

In order to prepare stock solution of escitalopram, 64.5 mg of escitalopram oxalate was dissolved in 50.0 ml of a mixture of water and methanol (50/50, v/v). This solution was further diluted in the same diluent to obtain two different concentrations: 100.4 and 10.04 μg/ml of escitalopram. The two solutions were appropriately diluted in mixture of water and methanol (50:50, v/v) so as to obtain working solutions for calibration standards as; 4016.0, 3012.0, 2008.0, 1204.8, 602.4, 200.8, 100.4, 40.2 l and 20.0 ng/ml; and working solutions for quality control samples as: 3212.8 ng/ml (HQC, high quality control), 1606.4 ng/ml (MQC, medium quality control) and 60.2 ng/ml (LQC, low quality control).

Stock solution of the internal standard was prepared by dissolving about 28.5 mg of paroxetine HCl in 25.0 ml of water and methanol (50:50, v/v) mixture. About 1.0 ml of this solution was further diluted to 10.0 ml in the same diluent to obtain a stock solution of 0.1 mg/ml. About 5.0 ml of the stock solution was diluted to 100.0 ml to obtain a working solution of 5.0 μg/ml of internal standard. All solutions were stored at 2–8 °C.

2.3. Preparation of calibration standards and quality control sample

To 950.0 μl of the drug free human plasma, 50.0 μl of working solutions of escitalopram and internal standard were added to yield final respective concentrations as 200.80, 150.60, 100.40, 60.24, 30.12, 10.04, 2.01 and 1.00 ng/ml of escitalopram and 250.0 ng/ml of internal standard in human plasma. QC samples (160.64, 80.32 and 3.01 ng/ml) were prepared in a similar manner. All samples were vortexed for 3.0 min and subjected to liquid–liquid extraction as in Section 2.4.

2.4. Sample preparation

After spiking 50.0 μl of the solution of internal standard to 1000.0 μl of plasma samples, liquid–liquid extraction was performed. To 3.0 ml of the sample, a mixture of diethyl ether and dichloromethane (70:30, v/v) was added and vortexed for about 3.0 min. After allowing to settle for 5.0 min., about 2.0 ml of the supernatant organic layer was transferred to the evaporation tube. The supernatant was evaporated to dryness in the thermostatically controlled water-bath maintained at 40 °C under the stream of nitrogen for about 15.0 min. After drying, the residue was reconstituted in 300.0 μl of acetonitrile–water mixture (50:50, v/v).

2.5. Chromatographic and MS conditions

Chromatography was performed on LC–MS system from Shimadzu Corporation, Kyoto, Japan. The system consisted of LCMS-2010A liquid chromatograph mass spectrometer and SIL-HT*c* autosampler. The data acquisition was carried out on LC–MS solution version 2.04-H3 software from Shimadzu Corporation, Kyoto, Japan. Chromatographic separation was achieved on ODS YMC^{TM} AQ 150.0 mm × 4.6 mm 5.0 μ analytical column maintained at 35 °C. The mobile phase consisting of 2.0 mM ammonium acetate (pH 5.0 with formic acid) and acetonitrile (54:46, v/v) was delivered at a flow rate of 1.0 ml/min with split ratio of 8:2. About 10.0 μl of sample was injected into LC–MS. Curved desolvation line (CDL) and BLOCK temperature was 250 °C. The nitrogen gas flow was maintained at 1.5 l/min for nebulization and 10 l/min for drying purpose using a nitrogen generator (Peak scientific instruments, USA). Single ion monitoring (SIM) of the ions was carried in positive mode and the ion signals; *m/z* 325.0 and 330.0, were measured for escitalopram and internal standard, respectively. A representative chromatogram is exhibited in Fig. 1. Quantitation of the analytes in human plasma was based on the ratio of the detector response of escitalopram versus internal standard.

2.6. Bio-analytical method validation

2.6.1. Linearity and lower limit of quantitation (LLOQ)

In order to establish the linearity of the method, a series of calibration standards ranging from 1.0 to 200.80 ng/ml were prepared as described previously in Section 2.3. Four linearity curves containing eight non-zero concentrations were analyzed. Ratio of detector response for escitalopram to internal standard was used for regression analysis. Each calibration curve was analysed individually by using least square weighted (1/*X*) linear regression. All the curves were forced through zero (i.e., *Y* intercept was made zero). Back calculations were made from the calibration curves to determine the concentration of escitalopram in each calibration standard.

A correlation of more than 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was to be accepted [20] as the lower limit of quantitation

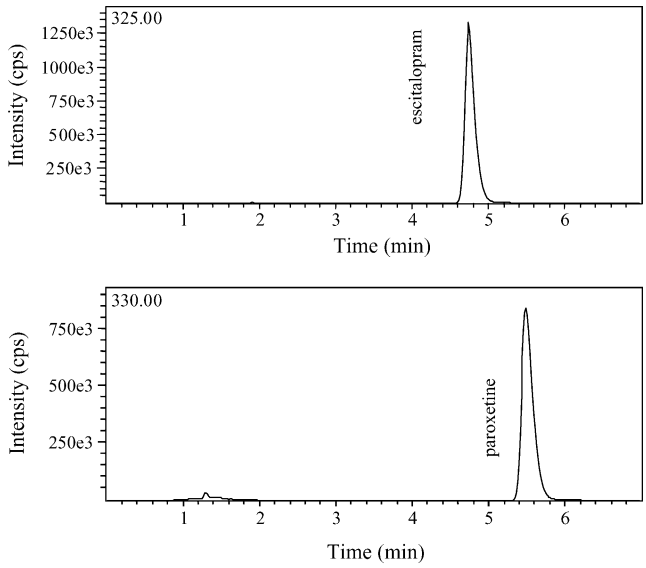

Fig. 1. Representative LC–MS chromatogram in human plasma.

(LLOQ) if the analyte response in the standard was five times more than that of drug free (blank) extracted plasma. In addition, the analyte peak in LLOQ sample should be identifiable, discrete, and reproducible with a precision of 20.0% and accuracy within 80.0–120.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0%. It was desirable that a minimum of six non zero standards, including LLOQ, met the above criteria.

2.6.2. Specificity

Six randomly selected control blank human plasma samples were processed by the similar liquid–liquid extraction procedure and chromatographed to determine the extent to which endogenous plasma components may contribute to the interference at retention time of analyte and internal standard.

2.6.3. Recovery (extraction efficiency) from plasma

The extraction efficiency of escitalopram was evaluated by comparing the mean detector response of six quality control (QC) samples of low (3.01 ng/ml), mid (80.32 ng/ml) and high (160.64 ng/ml) concentrations to mean detector response of six standard solutions of equivalent concentration. Similarly, the recovery of internal standard was evaluated by comparing the mean detector response of six plasma samples to mean detector response of standards solution of the internal standard at similar concentration. As per the acceptance criteria [20] the recovery of the analyte need not be 100.0%, but the extent of recovery of an analyte should be consistent, precise and reproducible.

2.6.4. Accuracy and precision

For determining the intra day accuracy and precision, replicate analysis of plasma samples of escitalopram in human plasma was performed on the same day. The run consisted of a calibration curve and six replicates of each LLOQ, low, mid and high quality control samples. The inter day accuracy and precision were assessed by analysis of five precision and accuracy batches on different occasions. The precision of the method was determined by calculating the percent coefficient of variation (%CV) for the concentrations obtained for different determinations. For the evaluation of precision, the deviation of each concentration level from the nominal concentration was expected to be within ±15.0% except for the LLOQ, for which it should not be more than 20.0% [20]. Similarly, the mean accuracy should not deviate by ±15.0% of the nominal concentration except for the LLOQ where it should not deviate by more than ±20.0% of the nominal concentration.

2.6.5. Stability

2.6.5.1. Long-term stability. Six aliquot each of low and high QC samples were kept in deep freezer at −70 ± 5 °C for 36 days. Thereafter, the samples were processed and analyzed along with precision and accuracy batch and the concentrations thus obtained were compared with nominal values. All values within ±15.0% of the nominal concentration qualified the test.

2.6.5.2. Short-term stability. Six aliquots each of the low and high un-extracted QC samples were kept at ambient temperature (25 ± 5 °C) for 8 h in order to establish the short-term stability of escitalopram in human plasma. Thereafter, the samples were processed and analyzed. The concentrations thus obtained were compared with the nominal values of QC samples and the samples were considered stable if the deviation from the nominal concentration was within ±15%.

2.6.5.3. Autosampler stability. In order to establish the autosampler stability of escitalopram in human plasma matrix, six aliquots each of low and high QC samples were kept in autosampler maintained at 15 °C, for about 30 h. Thereafter, samples were analyzed and the concentrations thus obtained were compared with the nominal values. A deviation of more than ±15.0% was undesirable.

2.6.5.4. Freeze–thaw stability of frozen plasma samples. Effect of three freeze and thaw cycles on stability of plasma samples containing escitalopram was determined by subjecting six aliquots each of low and high-unextracted quality control samples (previously frozen at −70 ± 5 °C) to three freeze–thaw cycles. After the completion of third cycle, the samples were analyzed and the experimental concentrations were compared with the nominal values. The samples qualified the test if the deviation from the nominal value was within ±15.0%.

2.6.5.5. Solution stability. For determining the solution stability, working solutions of 80.2 ng/ml of escitalopram and 5.0 μg/ml of internal standard was kept at 2–8 °C for 30 days. Thereafter, the mean detector response for escitalopram from

five replicate chromatographic runs was compared to that of mean detector response of freshly prepared solution of same concentration. The samples qualified the criteria of stability if the deviation was within ±2.0%.

2.7. Study design

The bioequivalence of two oral formulation of escitalopram oxalate 20 mg tablet of Cadila Health Care Ltd., Ahmedabad, India versus Lexapro tablets containing 20 mg of escitalopram oxalate of Forest laboratories Inc., USA was conducted using an experimental design [21] of two way crossover single blind and randomized study. The study was conducted on 14 healthy male subject after they had been informed of the purpose protocol and risk of the study. All subjects gave written informed consent and local ethics committee approved the protocol. The study was conducted strictly in accordance with the current Good Clinical Practices (GCP), International Conference on Harmonization (ICH), Indian Council of Medical Research (ICMR) and USFDA guidelines [21]. The protocol for the study was approved by Institutional Review Board (IRB), which constituted of a panel of medical practitioners. The subject were not allowed to consume any other medicine or alcohol for at least 8 days during the study. The health of the participant was judged by studying their clinical history, physical examination and laboratory tests, i.e., hematology, biochemistry serology, urine analysis, ECG, X-ray, ability to communicate efficiently with study person and willingness to adhere to the protocol requirement. A wash out period of 15 days was observed between the two phases of the study. Blood samples were withdrawn at 0, 1, 2, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 10, 12, 18, 24, 72, and 120 h after the oral administration of the dose. Samples were centrifuged and plasma was separated and stored at $-70 \pm 5\,^{\circ}C$ until analyzed.

2.8. Pharmacokinetic and statical analysis

The descriptive statistics for pharmacokinetic parameters were computed using WinNonlin Professional Software—version 4.0.1. The pharmacokinetic parameters; maximum plasma concentration (C_{max}), time point of maximum plasma concentration (T_{max}), area under the plasma concentration–time curve from 0 h to the last measurable concentration (AUC_{0-t}), area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$), elimination rate constant (λ_Z) and half-life of drug elimination during the terminal phase ($t_{1/2}$) were determined. The comparison of the pharmacokinetic parameters and analysis of variance (ANOVA) was carried out using SAS® Release 8.2 (SAS Institute Inc., USA) for untransformed and ln-transformed pharmacokinetic parameters—C_{max}, AUC_{0-t} and $AUC_{0-\infty}$. Intra Subject variability and power were calculated for untransformed and ln transformed pharmacokinetic parameters using root mean square error computed by PROC GLM. Based on the statistical results of 90.0% confidence intervals for the ratios of the means of ln-transformed pharmacokinetic parameters; C_{max}, AUC_{0-t} and $AUC_{0-\infty}$, conclusions were drawn as to whether the test product was bioequivalent to the reference product. Bioequivalence was to be concluded if the 90.0% confidence interval for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ fell within the bioequivalence range of 80.0–125.0% [22] (Fig. 2).

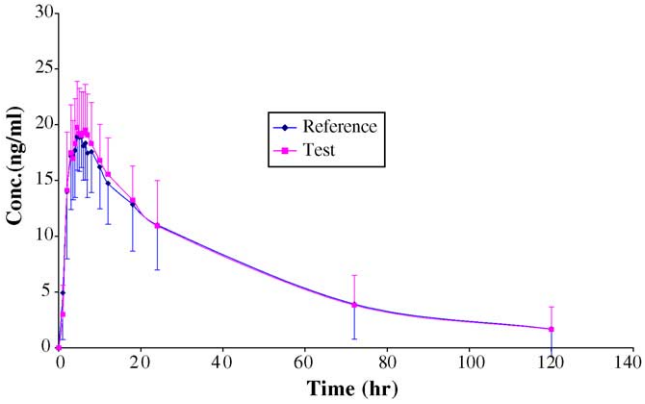

Fig. 2. Mean plasma concentrations vs. time graph of escitalopram after administration of test and reference formulations to healthy, adult, male human subjects under fasting condition.

2.9. Quality assurance

All clinical data generated during the course of the study, including the clinical, bioanalytical and statistical operations as well as the reports generated, were subjected to rigorous quality audits by quality assurance department.

3. Results and discussion

3.1. Bio-analytical method validation

3.1.1. Linearity and lower limit of quantitation

Calibration curves were found to be linear over the range of 1.0–200.80 ng/ml with the lower limit of quantitation of 1.0 ng/ml. The co-efficient of correlation were found to be better than 0.99 for all the four calibration curves analysed. Table 1 exhibits the mean concentrations obtained for the calibration curves.

3.1.2. Specificity

No interference was observed in six different lots of drug free human plasma samples used for analysis, at the retention times of either analyte or internal standard.

3.1.3. Recovery (extraction efficiency) from plasma

The extraction efficiency of escitalopram from human plasma was found to be between 75.33 and 79.92% as presented in Table 2. The recovery of internal standard was 79.22%.

Table 1
Summary of calibration standards of escitalopram in human plasma

Concentration added (ng/ml)	Mean concentration found (ng/ml)	CV (%)	Accuracy (%)	*n*
1.00	1.08	6.88	107.75	4
2.01	1.83	7.7	90.8	4
10.04	9.52	8.29	94.82	4
30.12	28.67	4.14	95.19	4
60.24	58.34	2.24	96.85	4
100.40	98.03	1.78	97.64	4
150.60	144.72	0.44	96.1	4
200.80	198.29	1.51	98.75	4

Table 2
Extraction efficency (recovery) of escitalopram from human plasma

QC samples	Concentration added (ng/ml)	Extraction recovery (%)	CV (%)	*n*
Low	3.01	79.92	5.53	6
Mid	80.32	75.33	6.08	6
High	160.64	78.00	3.60	6

3.1.4. Accuracy and precision

The intra day accuracy of the method was between 99.88 and 107.81% with a precision of 1.22–4.86%. (Table 3). The inter day accuracy was between 98.42 and 107.30%, (Table 3) with a %CV of 3.57–9.70. The data indicates that the method possessed adequate repeatability and reproducibility.

3.1.5. Stability

3.1.5.1. Long-term stability. Escitalopram was stable at −70 ± 5 °C for 36 days (long-term stability) in human plasma. The accuracy for the LQC and HQC samples was 96.01 and 94.84% over the stability testing period in deep freezer at −70 ± 5 °C (Table 4).

3.1.5.2. Short-term stability. Escitalopram was found to be stable for eight hours in human plasma at ambient temperature (25 ± 5 °C). The accuracy was 95.14 and 100.66% at the two concentrations studied (Table 4).

3.1.5.3. Autosampler stability. In the autosampler maintained at 15 °C, the plasma samples of escitalopram were stable for 30 h with the accuracy percent of 111.76 and 101.34 at the two concentration levels studied (Table 4).

3.1.5.4. Freeze–thaw stability of frozen samples. Three freeze–thaw cycles had no effect on the stability of the of the frozen plasma samples of escitalopram as apparent from the percent accuracy and %CV data depicted in Table 4.

3.1.5.5. Solution stability. Working solutions of escitalopram and internal standard were found to be stable for 30 days at 2–8 °C.

3.2. Statistical evaluation of pharmacokinetic parameters

The pharmacokinetic comparison between the two formulations was made in terms of extent and rate of absorption.

Table 3
Intra day and inter day accuracy of escitalopram in human plasma

Accuracy and precision	QC samples	Concentration added (ng/ml)	Mean concentration found (ng/ml)	CV (%)	Accuracy (%)	*n*
Intra day	LLOQ	1.00	1.06	4.86	106.33	6
	Low	3.01	3.25	3.06	107.81	6
	Mid	80.32	83.10	1.22	103.46	6
	High	160.64	160.44	1.72	99.88	6
Inter day	LLOQ	1.00	1.05	9.70	104.50	24
	Low	3.01	3.23	4.79	107.30	24
	Mid	80.32	81.46	3.92	101.42	24
	High	160.64	158.10	3.57	98.42	24

Table 4
Summary of stability of escitalopram in human plasma

Stability	Concentration added (ng/ml)	Mean concentration found (ng/ml)	CV (%)	Accuracy (%)	*n*
Long term (36.0 days)	3.01	2.89	2.09	96.01	6
	160.64	152.35	3.20	94.84	6
Short term (8.0 h)	3.01	3.03	2.92	100.66	6
	160.64	152.83	1.17	95.14	6
Auto sampler (30.0 h)	3.01	3.36	10.15	111.76	6
	160.64	162.8	6.67	101.34	6
Freeze–thaw	3.01	2.91	3.83	96.68	6
	160.64	145.86	1.79	90.80	6

Table 5
Mean pharmacokinetic parameters and 90.0% confidence interval for escitalopram, after the administration of an oral dose of 20 mg of test and reference formulations to healthy human volunteers

Pharmacokinetic parameters	Reference formulation (mean ± S.D.)	Test formulation (mean ± S.D.)	Confidence limit 90.0%
T_{max} (h)	5.07 ± 1.70	4.71 ± 1.31	78.03–117.30
C_{max} (ng/ml)	20.50 ± 3.70	21.00 ± 4.33	96.80–107.63
AUC_{0-t} (ng h/ml)	797.64 ± 378.57	792.69 ± 348.26	81.24–120.86
$AUC_{0-\infty}$ (ng h/ml)	998.67 ± 504.89	985.14 ± 441.53	81.64–117.75
$T_{1/2}$ (h)	36.06 ± 12.91	34.15 ± 16.88	69.29–116.78
λ_Z (1/h)	0.02 ± 0.01	0.03 ± 0.01	85.63–144.33

3.2.1. Rate of absorption

The pharmacokinetic parameters C_{max} and T_{max} indicate the rate at which the drug is absorbed in vivo. The mean C_{max} for reference and test formulations were 20.50 ± 3.70 and 21.00 ± 4.33 ng/ml (Table 5), respectively. The two one-sided 90.0% confidence interval for the ratios of the ln-transformed means of C_{max} was found to be 96.80–107.63% (Table 5) complying the acceptance criteria required for the conclusion of bioequivalence. The mean T_{max} for reference and test formulations were 5.07 ± 1.70 and 4.71 ± 1.31 h (Table 5), respectively.

3.2.2. Extent of absorption

Area under the plasma concentration–time curve from 0 h to the last measurable concentration (AUC_{0-t}) and area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$) define the extent of exposure of the drug to the body. The mean (AUC_{0-t}) for reference and test formulations were 797.64 ± 378.57 and 792.69 ± 348.26 ng h/ml and the mean values for ($AUC_{0-\infty}$) were 998.67 ± 504.89 and 985.14 ± 441.53 ng h/ml (Table 5), respectively. The two one-sided 90.0% confidence interval for the ratios of the ln-transformed mean for AUC_{0-t} and $AUC_{0-\infty}$ was 81.24–120.86 and 81.64–117.75% (Table 5), respectively, which complied the criteria of 80.0–125.0%, required for the conclusion of bioequivalence. The power of the test for untransformed pharmacokinetic parameters: C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ was 99.9, 67.4, 68.9%, respectively and than for ln-transformed pharmacokinetic parameters, respectively.

The mean terminal half-life for test and reference formulations were 34.15 ± 16.88 and 36.06 ± 12.91 h, respectively (Table 5). The mean elimination rate constant for test product and reference product were 0.03 ± 0.01 and 0.02 ± 0.01 h^{-1}, respectively (Table 5). No adverse event was reported during the study.

4. Conclusions

The bioanalytical methodology described in this manuscript was specific, sensitive accurate and precise. The method employed HPLC coupled with electrospray ionization mass spectrometric detection (LC–ESI-MS). The method involved a simple sample preparation by liquid–liquid extraction followed by isocratic chromatographic separation. The LC–ESI-MS method was capable of estimating 1.0 ng/ml of escitalopram accurately in human plasma with high degree of reproducibility. The method was robust and was successfully applied to bioequivalence study of escitalopram in healthy, human subjects.

The analysis of pharmacokinetic parameters confirmed that the test product (escitalopram Oxalate tablets containing 20 mg escitalopram) manufactured by M/s. Cadila Healthcare Ltd., India was bioequivalent the reference product (Lexapro tablet containing 20 mg of escitalopram of M/s. Forest Laboratories Inc., USA) were bioequivalent in terms of rate and extent of absorption.

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References

[1] W.J. Burke, Expert Opin. Investig. Drugs 11 (10) (2002) 1477.
[2] E. Matsui, M. Hoshino, A. Matsui, A. Okahira, J. Chromatogr. B 668 (2) (1995) 299.
[3] D. Haupt, J. Chromatogr. B 685 (2) (1996) 299.
[4] O.V. Olesen, K. Linnet, J. Chromatogr. B 675 (1) (1996) 83.
[5] F.C. Kugelberg, B. Carlsson, J. Ahlner, F. Bengtsson, Chirality 15 (7) (2003) 622.
[6] L. Kristoffersen, A. Bugge, E. Lundanes, L. Slordal, J. Chromatogr. B 734 (2) (1999) 229.
[7] B. Carlsson, B. Norlander, J. Chromatogr. B 702 (1–2) (1997) 234.
[8] J. Macek, P. Ptacek, J. Klima, J. Chromatogr. B 755 (1–2) (2001) 279.
[9] D. Ohman, B. Carlsson, B. Norlander, J. Chromatogr. B 753 (2) (2001) 365.
[10] B. Rochat, M. Amey, H. Van Gelderen, B. Testa, P. Baumann, Chirality 7 (6) (1995) 389.
[11] J. Rampono, J.H. Kristensen, L.P. Hackett, M. Paech, R. Kohan, K.F. Ilett, Br. J. Clin. Pharmacol. 50 (3) (2002) 263.
[12] E. Oyehaug, E.T. Ostensen, B. Salvsen, J. Chromatogr. 227 (1) (1982) 129.
[13] L. Dalgaard, C. Larsen, Xenobiotica 29 (10) (1999) 1033.

[14] Z. Zheng, M. Jamour, U. Klotz, Ther. Drug Monit. 22 (2) (2000) 219.
[15] M. Kosel, C.B. Eap, M. Amey, P. Baumann, J. Chromatogr. B 719 (1–2) (1998) 234.
[16] J.P. Foglia, B.G. Pollock, M.A. Kirshner, J. Rosen, R. Sweet, B. Mulsant, Psychopharmacol. Bull. 33 (1) (1997) 109.
[17] D. Haupt, J. Chromatogr. B 685 (2) (1996) 299.
[18] B. Rochat, M. Amey, P. Baumann, Ther. Drug Monit. 17 (3) (1995) 273.
[19] P.P. Rop, A. Viala, A. Durand, T. Conquy, J. Chromatogr. 338 (1) (1985) 171.
[20] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veternary Medicine (CVM), May 2001, BP, Website: http://www.fda.gov/cder/guidance/index.htm.
[21] FDA Guidance for Industry, Biaoavaliability Studies for Orally Administered Drug–Products–General Considerations, US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), 2000, Website: http://www.fda.gov/cder/guidance/index.htm.
[22] FDA Guidance for Industry, Statistical Approaches to Establishing Bioequivalence, US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), 2001, Website: http://www.fda.gov/cder/guidance/index.htm.