

Development and validation of high performance liquid chromatographic method for analysis of clozapine

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Abstract: In this study a rapid, simple and sensitive assay to quantify clozapine in human plasma by using reverse phase high performance liquid chromatographic method has been developed. Clozapine was extracted from human plasma using a mixture of chloroform: n-hexane 50:50 employing liquid- liquid extraction method. The calibration curve was found to be linear in the concentration range of 25-800 ng/ml. The inter day and intra day assay accuracy and precision fulfilled the criteria specified by USFDA, Guidance for industry: bioanalytical method validation. Clozapine was found to be stable in human plasma after 6 h incubation at room temperature, 50 days storage at -27°C and freeze thaw cycles, as well as after reconstitution with mobile phase after 24 h of storage in refrigerator. The validated method offers the advantage of using minimum injection volume (25µl) and plasma sample volume (300µl). The extraction method is simple and single step with no back extraction step, thus, making this method applicable to determination of pharmacokinetic profiles and parameters.

Keywords: HPLC, chromatography, clozapine, analytical method validation.

INTRODUCTION

Clozapine, 8-chloro-11-(methyl-1-piperazinyl)-5H-dibenzo[b,e] diazepine (fig. 1), is the first atypical antipsychotic for the treatment of both positive and negative symptoms of schizophrenia (Byerly *et al.*, 1996). This benzodiazepine derivative is a serotonergic (5-HT₂) antagonist and dopaminergic (D₂) antagonist with affinity towards histamine, adrenergic and cholinergic receptors. Clozapine is preferred in comparison to classical neuroleptics as it causes lower rate of extrapyramidal side effects and hyperprolactinemia (Lieberman *et al.*, 1992). However, it can cause severe agranulocytosis and due to this problem it was withdrawn from markets in all countries in 1975 (Heikkila *et al.*, 1977). Because of its high efficacy in certain schizophrenic patients, it has been reintroduced but it is used with caution and follow up of safety parameters (e.g. blood granulocyte count). Infact, National Institute for Clinical Excellence 2002 recommends the use of atypical antipsychotic drugs as the first line treatment for newly diagnosed schizophrenia and the use of clozapine in resistant schizpherenia. Several bioanalytical methods to quantitate clozapine have been reported. They include gas chromatography (Markowitz *et al.*, 1995), High Performance Liquid Chromatography (HPLC) (Raggi *et al.*, 2001; Shen *et al.*, 2002; Sachse *et al.*, 2006). The HPLC methods using both liquid- liquid extraction (Aravagiri *et al.*, 2001) and solid phase extraction (Niederlaender *et al.*, 2006) have been reported. HPLC techniques for quantitative analysis of clozapine that have been reported generally require large

plasma sample volume (~1ml) (Akerman, 1997; Avenoso *et al.*, 1998; Weigmann *et al.*, 2001) greater injection volume into HPLC system (40-125µl) (Shen *et al.*, 2002; Weigmann *et al.*, 2001; Micolini *et al.*, 2007) and tedious sample extraction procedure (Guitton *et al.*, 1997; Avenoso *et al.*, 1998). In this study we developed an isocratic reverse phase HPLC method, which is simple, sensitive, and robust and uses a simple extraction method, small plasma volume for analysis and additionally requires very less amount of sample to be injected into HPLC system. This method has been validated according to CDER guidelines.

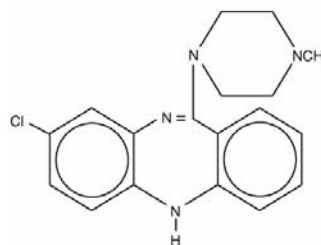


Fig. 1: Structure of clozapine

MATERIALS AND METHODS

Clozapine was purchased from Sigma Aldrich Chemie, GmbH, Germany. Amitriptyline was a gift sample from Yashica Pharmaceuticals Pvt. Ltd., Maharashtra, India. Methanol (HPLC grade) and disodium hydrogen orthophosphate were obtained from SD Fine Chemicals Ltd., Mumbai. Tris(hydroxymethyl)amino-methane,

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orthophosphoric acid and hydrochloric acid were obtained from Qualigens Fine Chemicals, Mumbai. Blank human plasma and HPLC grade water obtained from Ranbaxy Research Laboratories. Chloroform, n-hexane were all HPLC grade (Rankem, Ranbaxy Fine Chemicals Ltd.). All reagents and chemicals were of analytical grade and used as received.

HPLC method development

HPLC Instrumentation and chromatographic conditions

The Shimadzu HPLC system consisted of a model LC-10AT vp pump, a model SIL 10AD auto-injector, a SCL 10A vp system controller configured with a 4°C cooler and a model CTO 10A vp UV detector. The stationary phase was C18 Nova_Pak[®] column (3.9mm X 150mm and pore size of 4µm). The column oven temperature was 40°C. The mobile phase consisted of 10mM disodium hydrogen orthophosphate (containing 1% triethyl amine): methanol in the ratio of 50:50 v/v at a flow rate of 1ml/min and UV detection performed at 254nm. It was then mixed in a sonicator (Brason-5510, Brason Ultrasonics Corporation, USA) for 15min after which the pH was adjusted to 5±0.2 with orthophosphoric acid. The 10mM disodium hydrogen orthophosphate containing triethyl amine thus prepared was then sonicated with HPLC grade methanol (50:50 v/v) for 30min to get the mobile phase which could be used and stored for 3 days. The overall run time for the HPLC system was 20min.

Preparation of stock solution and standard working solution

Stock solution of clozapine (1 mg/ml) was prepared in HPLC grade methanol. This was further diluted with 50:50; methanol: water prior to spiking. For calibration curve, dilutions of clozapine were prepared in blank plasma to cover the range of 25-800 ng/ml. Solutions of amitriptyline, imipramine and olanzaine were screened for their use as internal standard (IS). 0.05 mg/ml stock solution of IS was prepared with methanol and stored below 10°C. This was diluted to a concentration of 500 ng/ml with 50% v/v methanol. Quality control samples (ICH guidelines) of lower limit of quantification (LLOQ), lower quality control (LQC), middle quality control (MQC) and higher quality control (HQC) were prepared from a separate stock solution in the same manner as calibration standards. All the samples were stored at -20°C.

Sample extraction procedure

Aliquots of 300 µl plasma samples containing 50 µl IS were extracted with 150µl tris buffer (pH 10.6) for 1min followed by addition of 5ml of extracting solvent (chloroform:n-hexane, 50:50). The samples were shaken in reciprocating shaker at 100rpm for 20 min followed by centrifugation at 4000 rpm for 5 min at 4°C. 3.5ml of supernatant was removed and evaporated to dryness under a stream of nitrogen for 30 min at 50°C. The resulting

residue was reconstituted with 300µl of mobile phase and vortexed for 5min. This was then transferred to 1ml autosampler vials and 25µl was injected into HPLC for analysis. Besides using the above extraction solvent ethyl acetate, tert-butyl methyl ether and n-hexane were also experimented for their use as extracting solvents.

HPLC method validation

Linearity of calibration curve

The clozapine concentration in the working solution for calibration curve, after spiking were in the range of 25 to 800 ng/ml. Calibration curve was obtained by plotting peak area ratio of clozapine/IS to spiked clozapine theoretical concentration in blank plasma. Linearity of the developed method was assessed using weighted least square analysis of standard plot. The minimally acceptable correlation coefficient (r^2) for calibration curve was 0.99 or greater. Using calibration curve the limit of detection (LOD) and lower limit of quantification (LLOQ) were calculated using the formula $3.3\sigma/S$ and $10\sigma/S$, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Specificity and selectivity

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms obtained after injecting six lots of blank plasma with plasma spiked with LLOQ concentration of clozapine and IS.

System suitability

Six injections of aqueous mixture of clozapine and IS, 500 ng/ml each were injected into HPLC column for analysis and their mean peak area ratio (analyte:IS) were found.

Accuracy and precision

Accuracy was measured by analysis of six determinations per concentrations. Accuracy was expressed as % nominal concentration (%NC) and was calculated by dividing the concentration obtained during analysis with that of nominal concentration (NC) and expressing as percentage. Similarly precision was calculated using six sets each of HQC, MQC, LQC and LLOQ. It was expressed as %coefficient of variance (%CV) and calculated as the ratio of standard deviation (SD) to mean and expressing as percentage. Intra day precision and accuracy were tested by analysis of six identically spiked plasma samples for four different concentrations (LLOQ, LQC, MQC and HQC) on the same day. Interday precision and accuracy were calculated from repeated analysis of identically spiked plasma samples on three successive days for the different concentrations.

Recovery

Percentage recovery for analyte and IS were determined to test the efficiency of extraction procedure. This was

done by comparing the mean peak response of extracted samples with that of non extracted samples to which the analyte had been added at the same nominal concentration (representing 100% recovery) just before injection. Recovery experiments were carried out at three concentrations of clozapine namely LQC, MQC and HQC.

Stability

a) Freeze thaw stability

It was assessed by assaying six replicates of QC samples at low and high concentration previously frozen and thawed over 3 cycles against freshly spiked calibration standards. The samples were frozen at -27°C for at least 24h followed by unassisted thawing at room temperature. The samples were again frozen at -27°C for 24h and thawed at room temperature. This freeze thaw cycle was repeated and after 3 complete cycles the samples were analyzed.

b) Bench top stability

This was determined by evaluating six replicate samples

at LQC and HQC concentrations by keeping the samples at 25°C (on bench) for 6h and comparing them with freshly prepared calibration standards.

c) Aqueous solution stability

Aqueous solution stability of drug and IS were assessed at MQC by storing the stock solution at 10°C for 24 h and comparing the area response of stored solution with freshly prepared solution.

d) Long term stability

The storage time for long term stability should exceed the time between date of first sample collection and date of last sample analysis. Therefore, keeping in view this fact the samples were stored for 50 days. Six replicate samples at low and high concentration were stored in cold room (-27°C) for 50 days. After allowing the samples to thaw they were analyzed in terms of peak area ratio of drug to IS against freshly spiked calibration standards.

e) Stock Solution Stability

Stock solution stability of drug and IS were evaluated by injecting a dilution of 544.8 ng/ml and 505.3 ng/ml of

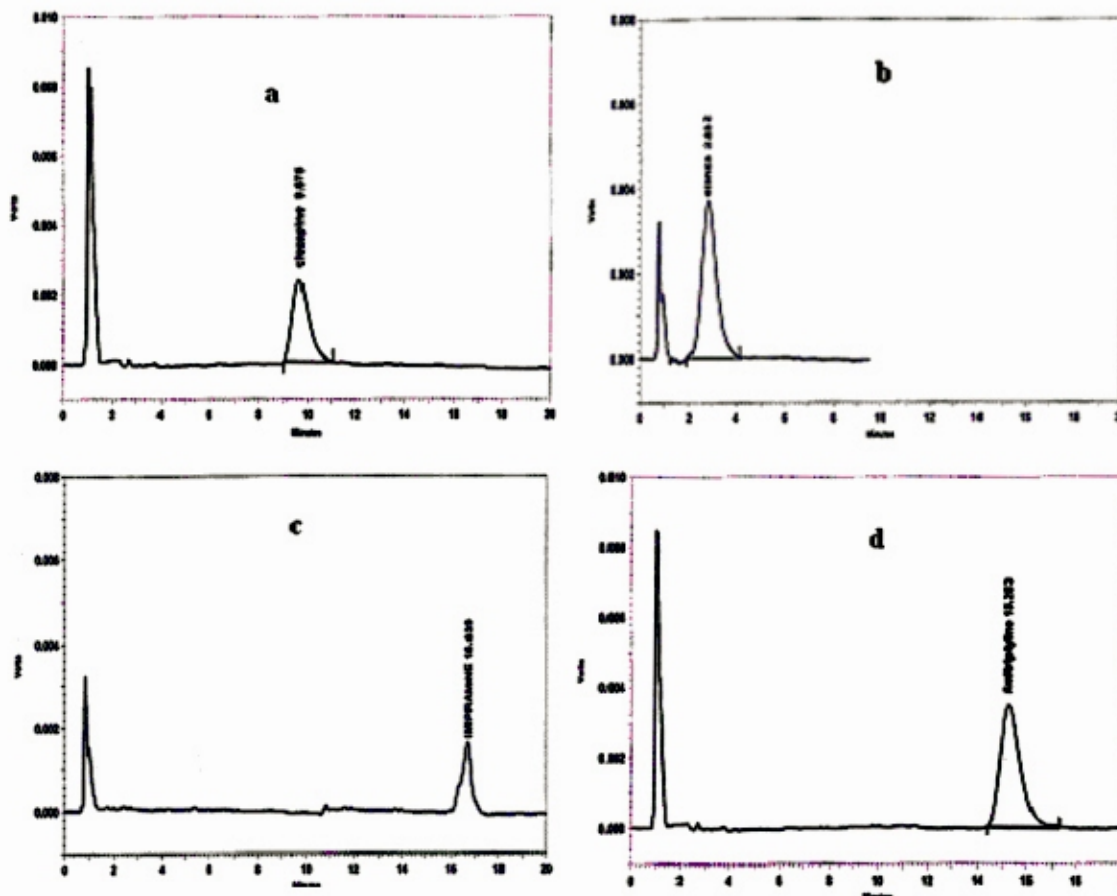


Fig. 2: HPLC Chromatograms of different internal standards evaluated during method development. a-chromatogram of simple clozapine without internal standard, b, c, d- chromatograms of olanzapine, imipramine and amitriptyline, respectively.

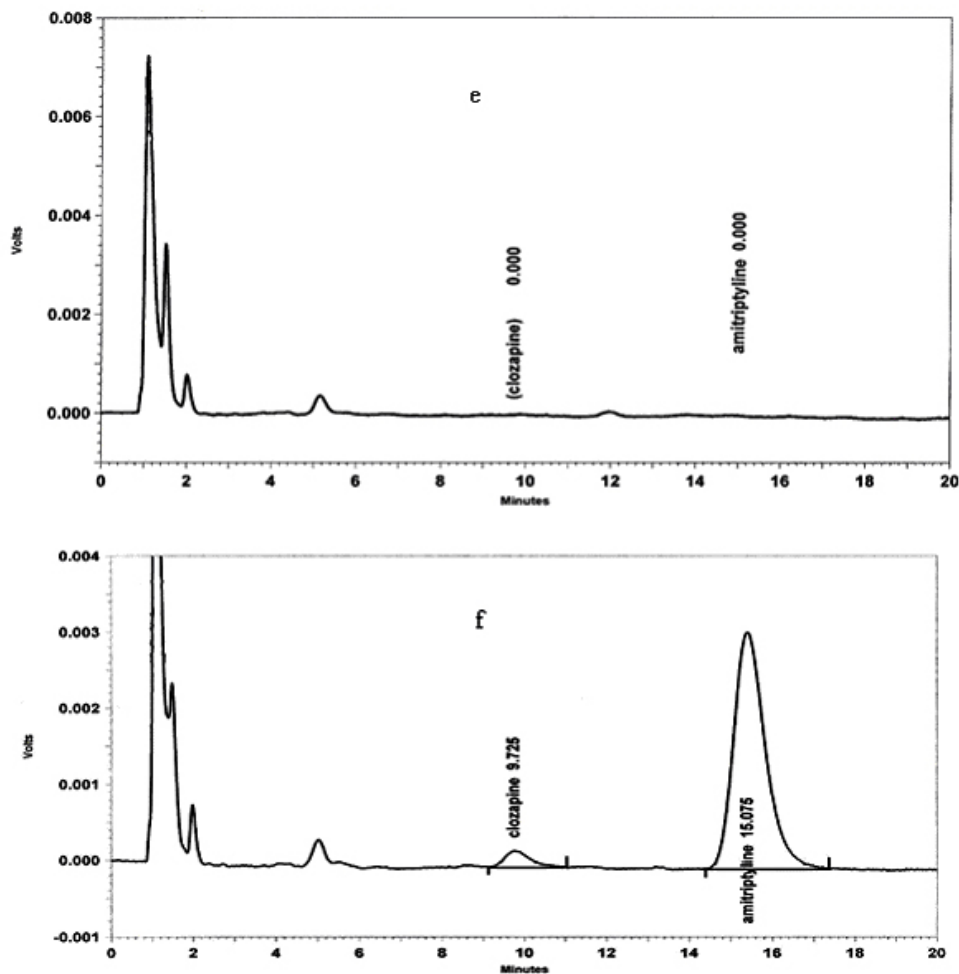


Fig. 3: Chromatogram of (e) blank plasma and (f) plasma spiked at lower limit of quantification (LLOQ concentration) of clozapine along with amitriptyline as internal standard

clozapine and IS respectively made from 30 days old stock stored below 10°C and comparing with freshly prepared stock solution of the respective compounds.

f) Post operative stability- Stability of non reconstituted sample

It was determined by processing six replicates of low and high concentration (LQC and HQC) samples and keeping the processed sample prior to reconstitution step with mobile phase in refrigerator for 24h. After 24h samples were reconstituted with mobile phase and injected. The peak areas obtained were compared with those obtained for freshly processed calibration standards.

g) In- injector stability

This was assessed by extracting six replicates of QC sample at low and high concentration (LQC, HQC) and putting the processed samples in auto sampler. The samples were injected after 96h and the results were compared with freshly spiked samples.

h) Aqueous solution stability

This was validated at MQC by storing the stock solution at 10°C for 24h and comparing the area response of stored solution with freshly prepared solution.

Ruggedness

Ruggedness of the method developed was tested by processing six replicates of LLOQ, LQC, MQC and HQC, and analyzed against fresh calibration standards by different analysts, with different columns of same manufacturers in different HPLC systems.

Anticoagulant effect

Anticoagulants acetate citrate dextrose phosphate (ACDP) and ethylenediamine tetra acetic acid (EDTA) were used in this study. Six LQC and six HQC samples were analysed (using plasma in which different anti coagulants were added) for validating both ACDP and EDTA, the results were compared with freshly spiked samples with respective anti coagulants.

RESULTS

Method development

The scan of stock solution of clozapine (1 µg/ml in methanol: water (50:50)) in the UV region of 200-400nm yielded a UV absorption maxima of clozapine at 254nm. Further HPLC analysis was carried out at this wavelength (fig. 2). Table 1 summarizes the various chromatographic parameters that were developed in this method.

Table 1: Optimized chromatographic parameters for clozapine estimation

Parameter	Chromatographic condition
Column	Nova-Pak [®] C-18 (3.9mm X150mm)
Mobile phase	50:50 10mM disodium hydrogen orthophosphate containing (1% triethyl amine):methanol, 50:50 at pH 5±0.2
Internal standard	Amitriptyline
λ _{max}	254nm
Plasma sample required for extraction	300µl
Extraction method	Liquid- Liquid Extraction using chloroform: n-hexane (50:50) with tris buffer (pH 10.6)
Flow rate	1.0 ml/min
Retention time Clozapine	9.7±0.1min
Amitriptyline	15.2±0.1min
Run time	20min
Injection volume	25µl

Method validation

Linearity of calibration curve

The calibration curve for clozapine was found to be linear in the range of 25-800 ng/ml. The linearity of method was determined by weighted least square regression analysis of standard plot with weighting factor of $1/x^2$. The r^2 value was 0.99 during the course of validation. The equation for the line was $y=0.0056x + 0.007$. The value of limit of detection (LOD) and lower limit of quantification (LLOQ) as calculated from calibration curve (using formula specified in experimental section) was found to be 10 ng/ml and 25 ng/ml, respectively. From the LLOQ value the LQC, MQC and HQC samples selected were 75 ng/ml, 300 ng/ml and 600 ng/ml, respectively.

Specificity and selectivity

Fig. 3 represents the chromatograms of blank plasma and chromatograms obtained from plasma spiked with clozapine at LLOQ and IS.

System suitability

For validating system suitability the mean of area ratio (analyte: IS) for six injections of drug and IS was found to be 0.5, the % CV calculated was 0.7. Thus, the method developed passed the system suitability test as the %CV was less than 2%.

Accuracy and precision

The results of different precision accuracy batches conducted on different days are given in table 2. Intra batch precision for three precision accuracy batches was in the range of 2.2-3.9%, 1.8 to 3.9% and 2.8 to 3.9%, respectively. The interbatch precision was in the range of 1.8-3.9%. The intrabatch accuracy for batch 1, 2 and 3 (table 2) were 89.0- 98.4%, 89-99.7% and 87.6-99.5%, respectively. The values for interbatch accuracy were in the range of 87.6- 99.7% (table 2).

Recovery

To test the efficiency of extraction, mean peak area response of extracted samples from spiked serum samples were compared with non extracted samples, analyzed at LQC, MQC and HQC concentrations. The data representing the % recovery of clozapine is summarized in Table 3. The mean % recovery of amitriptyline was found to be 79.8%. Thus, the extent of recovery for both analyte and IS was consistent, precise and within limits of variability.

Table 3: Recovery (%) of clozapine at different QC levels

QC Sample	% Recovery of Clozapine
LQC	84.70
MQC	86.10
HQC	84.10
Mean	84.97
S.D. (+/-)	1.03
C.V.	1.21

Stability

Results of stability studies are shown in Table 4. The results of freeze thaw stability studies showed that the concentration of clozapine after three repeated cycles of freezing at -27°C for 24h followed by thawing at room temperature was 93.4-97.4% of the nominal concentration. This indicated that the solution can withstand processing delays without compromising the stability. The method was also validated in terms of short term stability as the drug concentration was found to be ~ 95% in comparison to the freshly spiked samples when exposed to bench top conditions at room temperature. The concentration of drug and IS were found to be 99.8% and 99.2% of the nominal concentration, respectively when stored at 10°C for 24h. Thus, indicating that the method exhibits short term stability as the analyte were stable for sufficient time at room temperature and therefore can withstand the time period between the retrieval of sample from cold room upto its final processing which is done at room temperature. The stock solution of drug and IS were also found to exhibit long term stability. The concentration of samples stored for 50 days were 94.9- 95.5% of nominal concentration. Validation of method in terms of long term stability implies that the analyte can

Table 4: Stability studies of six replicates at different concentrations

Stability	Freeze thaw		Bench top		Long term		Post- preparative		In-injector	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
QC Sample concentration										
Mean± S.D	75.7 ± 0.7	615.7 ± 9.4	73.9 ± 2.3	590.9 ± 17.9	73.4 ± 2.3	596.9 ± 17.7	75.71 ± 0.7	582.9 ± 18.7	74.9 ± 1.9	582.9 ± 12.7
% C.V	0.9	1.5	3.1	3	3.1	3	0.9	3.2	2.5	2.2
NC	81.1	632.1	77.6	618.7	77.3	625	81.8	609.7	79.6	602.8
% NC	93.4	97.4	95.2	95.5	94.9	95.5	92.5	95.6	94.1	96.7

SD- Standard Deviation, CV- Coefficient of variance, NC- Nominal Concentration

Table 5: Effect of anticoagulant on clozapine estimation and ruggedness of the developed method

	Anticoagulant effect				Ruggedness			
	ACDP		EDTA		LLOQ	LQC	MQC	HQC
	LQC	HQC	LQC	HQC				
Mean± S.D	70.9 ±2.1	599.9 ±15.7	67.9 ±2	605.9 ±13.7	27.7 ±0.8	67.9 ±2.3	288 ±10	605.9 ±30.2
% CV	3	2.6	2.9	2.3	2.9	3.4	3.5	5
NC	75.5	625.5	71.6	629.2	27.8	71.4	322.1	639.8
% NC	93.9	95.9	94.8	96.3	99.6	95.1	89.4	94.7

ACDP: Acetate citrate dextrose phosphate, EDTA: Ethylenediamine tetra acetic acid, SD- Standard Deviation, CV- coefficient of variance, NC- Nominal concentration.

with stand processing from time of sample collection from volunteer upto its final processing for analysis.

The method developed was also validated for post preparative stability. The dried extracted sample, prior to reconstitution, when stored at 10°C for 24h, demonstrated concentration range of 92-95% concentration of the nominal concentration when analyzed after storage. From the CV values (table 4) it can be concluded that processing delays during analysis does not affect accuracy and precision of the method developed. Clozapine was also found to be stable in auto sampler after storage for 48h which exceeds the time period from injection of first sample to injection of final sample in a batch. Thus, there were no stability related issues even if the drug remains in the autosampler for some time period.

Ruggedness

The results of this analysis are shown in Table 5. With in batch precision were in range of 2.9- 5.0 and accuracy was found to be 89.4-99.6% which suggests that this method can be used in any laboratory and by any analyst without significant variation in accuracy.

Anti coagulant effect

The results shown in table 5 demonstrate that, changing the anticoagulant does not affect precision and accuracy of the method.

DISCUSSION

Addition of IS enhances the precision and reliability of the method by providing an internal check on extraction efficiency and by reducing technical artifacts such as potential variation in injection volume (Bohnert *et al.*, 2010). Therefore, IS was added in the analysis. Amitriptyline, olanzapine and imipramine were tested for use as IS, but satisfactory results were obtained only with amitriptyline, as splitting of peak and tailing were observed in the chromatogram of imipramine whereas, olanzapine eluted out very early (retention time 2.85 min) and the peak appeared near the initial interfering peaks. Thus amitriptyline with peak retention time of 15.2±0.1 min was chosen as IS.

For extraction of drugs from plasma samples a variety of solvents like ethyl acetate, tert-butyl methyl ether and n-hexane were used. However, with all these solvents interfering peaks near retention time (RT) of clozapine (9.7±0.1min) were observed, leading to reduced resolution. Therefore, these solvents were not used for extraction. However, no interference was observed when chloroform: n-hexane were used as extracting solvents.

Previous developed HPLC methods for clozapine require upto 1ml of plasma sample for analysis (Akerman *et al.*, 1997; Avenoso *et al.*, 1998; Weigmann *et al.*, 2001). The present method offers the advantage of using only 300µl

of sample for analysis, this is especially important as this would require very less amount of blood to be withdrawn from subjects thus permitting withdrawal of increased number of serial samples to be obtained from single subject if need be. Besides this, the injection volume is 25µl, which to the best of our knowledge is one of the lowest volumes reported for analysis of clozapine in human plasma.

The analytical method was found to be specific and selective as there was no interference at respective retention times of clozapine (9.717 min) and IS (15.250 min).

According to ICH guidelines a method is said to have appropriate accuracy if the mean value is within 15% of actual value except at LLOQ where it should not deviate by more than 20% and a method is said to be precise if % CV values should not exceed $\pm 15\%$ except for LLOQ where it should not exceed $\pm 20\%$ of CV. The results of intra and inter batch precision and accuracy for the method developed were found to be within the specified parameters and therefore, the developed method was said to be accurate and precise.

CONCLUSION

A sensitive, accurate and precise HPLC method was developed and validated for estimation of clozapine in human plasma. Simple, single step liquid-liquid extraction requiring no back extraction step was used to prepare samples. This method also offers an advantage of providing a clean solution for injection and thus prolongs the life of the column, which aids in method reproducibility. A Nova_Pak® C18 column was used for analysis of samples. The developed method is rapid and has the advantage of using very small plasma volume and injection volume for HPLC analysis. In addition the method has been fully validated according to FDA guidelines and was found to be accurate, precise, robust and rugged, additionally the drug, IS and stock solutions were found to be stable under a variety of conditions.

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