
Validated HPLC Method for the Determination of Clozapine in Rat Serum and its Application to Pharmacokinetics

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An isocratic reverse phase high performance liquid chromatographic method with UV absorbance detection was developed for quantification of clozapine in rat serum. This method involves a single solvent extraction step with ethylacetate. Celecoxib served as the internal standard. The analytes were separated by HPLC using C18 Wakosil II RS (SGE) analytical column (5 μ m particle size; 250 \times 4.6 mm ID). Mobile phase comprised of methanol-water-triethylamine (75:25:0.5, v/v/v). The eluent was monitored at 254 nm by UV absorbance detection. Retention times of clozapine and celecoxib were 10.5 and 7.9 min, respectively. The mean absolute recovery value was about 75.8 to 79.9 %, while the intra day and inter day coefficient of variation values were in the range of 2.1 to 5.7 %. The calibration was linear over a concentration range of 100-4000 ng/ml. Accuracy ranged from 96.8 to 99.7 %. The method was used to study the pharmacokinetics of clozapine after an intravenous bolus (10 mg/kg) and oral (20 mg/kg) administration of clozapine solution to rats.

Clozapine, a dibenzodiazepine derivative, is an atypical antipsychotic agent used in the treatment of schizophrenia¹⁻². The advantage of clozapine over conventional neuroleptics is lack of extrapyramidal side effects (EPS) and minimal effect on prolactin secretion³. However, use of clozapine has been limited by the risk of agranulocytosis in a small percentage of population⁴. Therapy with clozapine needs frequent hematological monitoring. Clozapine is rapidly absorbed orally with a bioavailability of 0.27 and extensively metabolized by hepatic microsomal enzymes (CYP 1A2 and CYP 3A4) that forms N-desmethyl and N-oxide metabolites⁴.

Several analytical methods have been developed for quantification of clozapine in human and rat serum. The methods include gas chromatographic detection⁵⁻⁶, mass spectroscopic detection⁷ and radioimmunoassay⁸. These techniques require long analysis time or expensive equipment. High performance liquid chromatography (HPLC) methods with ultraviolet detection have also been

developed. These methods involved liquid-liquid⁹⁻¹⁵ or solid phase extraction¹⁶⁻¹⁹ of the samples. This paper describes a simple and precise HPLC method for quantification of clozapine in rat serum. Sample clean up process involves a single step liquid-liquid extraction with ethylacetate. This method is applied to estimate the pharmacokinetics of clozapine after a single i.v. bolus and oral administration of clozapine solution to rats.

MATERIALS AND METHODS

Clozapine was a kind gift from SPARC, Baroda. Celecoxib was obtained from Panacea Biotech, Chandigarh. Methanol and ethylacetate was purchased from Qualigens Chemicals, Mumbai. Triethylamine, glacial acetic acid and other chemicals were of analytical reagent grade. Double glass distilled water was used during the entire HPLC procedure.

Standard solutions:

Primary stock solutions of 1 mg/ml of clozapine and celecoxib were prepared in methanol and stored at 4°. Working stock solutions of 0.5, 1, 2, 5, 10 and 20 μ g/ml of

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clozapine and IS of 30 µg/ml were prepared from primary stock solutions. Appropriate volumes of working stock solutions were added to serum in the preparation of calibration curves. Calibration samples were prepared by addition of 20 µl of working stock solutions of clozapine to 100 µl of rat serum. Samples for determination of recovery, precision and accuracy were prepared by addition of appropriate volumes of working stock solutions to bulk rat serum to get different concentrations (100, 250, 500 and 1000 ng/ml) and stored at -20°.

Extraction procedure:

To 100 µl of serum, 20 µl of celecoxib solution (600 ng) and 200 µl of 2M sodium hydroxide were added and mixed well. To this 4 ml of ethylacetate were added and vortexed for 5 min followed by centrifugation at 3500 rpm for 15 min. The organic phase was separated and evaporated under reduced pressure. The residue was reconstituted in 50 µl of mobile phase and 20 µl was injected to HPLC column.

Chromatographic conditions:

The chromatographic system consisted of a Shimadzu LC-10AT solvent delivery pump equipped with a 20 µl loop and Rheodyne sample injector and SPD-10A VP dual wavelength UV/Vis detector. The column used was C18 Wakosil II RS (SGE) analytical column (5 µm particle size; 250x4.6 mm ID). The mobile phase consisted of methanol-water-triethylamine (75:25:0.5, v/v/v), pH of aqueous phase is adjusted to 6.5 with glacial acetic acid and was used at a flow rate of 1 ml/min. The eluate was monitored at 254 nm. The sensitivity was set at 0.001 AUFS. The data was recorded and calculated using Winchrome software.

Linearity and limit of quantification:

The calibration samples were prepared by addition of appropriate amount of clozapine and IS to 100 µl of control rat serum on the day of analysis. The LOQ was defined as the lowest concentration at which the RSD and deviation from the nominal concentration were less than 20 %.

Precision:

Samples for the determination of precision were prepared by appropriate spiking control rat serum in bulk to get concentration of 100, 250, 500, 1000 and 2000 ng/ml. At each concentration, 100 µl aliquots were distributed into screw capped tubes and stored at -20°. For intra day precision five replicates at each concentration were processed similar to sample preparation. For inter day precision, the samples were analyzed for five different days. The preci-

sion of the method at each concentration was calculated as RSD.

Recovery and accuracy:

The assay recoveries of the clozapine were assessed at concentrations of 100, 250, 500 and 1000 ng/ml. Six replicates of each concentration were extracted according to method described above. The recovery from the serum samples was determined by using formula: Absolute recovery = (Peak area of clozapine from serum sample)/(Mean peak area of the clozapine by direct injection)x100. The accuracy of the method was estimated by expressing mean calculated concentration as a percentage of the spiked/nominal concentration.

Application to study pharmacokinetics in rats:

All animal experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee, euthanasia and disposal of carcass in accordance with guidelines. Male Wistar rats weighing 180-230 g were used for the study. Before experimentation, they were kept in groups of three. They were fed with standard rat chow, with water *ad libitum*. Animals were fasted over night prior to experiment. Clozapine (100 mg) was dissolved in 250 µl of 1.2 N HCl and further diluted to 10 ml with 0.9 % sodium chloride solution²⁰. Clozapine solution was administered intravenously through rat tail vein (10 mg/kg), and by oral gavage (20 mg/kg). Blood samples (300 µl) were collected through tail vein at 15, 30, 45, 60, 90, 120 and 240 min and allowed to clot and were centrifuged for 15 min at 5000 rpm. The serum was separated and stored at -20° until analysis. Pharmacokinetic parameters were estimated by non-compartmental analysis using Win Nonlin software.

RESULTS AND DISCUSSION

Typical chromatograms corresponding to individual rat blank serum, clozapine added to controlled serum (4 µg/ml), serum sample of the rat obtained after 15 min of i.v. bolus administration and 45 min of oral administration of clozapine solution were shown in fig. 1. The specificity of the method was confirmed by observing no endogenous interfering peaks in blank serum at the retention times of clozapine and celecoxib. Both the analyte and internal standard were well separated with retention times of 10.5 and 7.9 min, respectively. Celecoxib was selected as an internal standard because it is well extracted (recovery 80.5±3.6 %, n=5) and elutes after a suitable retention time (7.9 min) at which no endogenous serum peaks were observed and well separated with clozapine peak in the chromatogram.

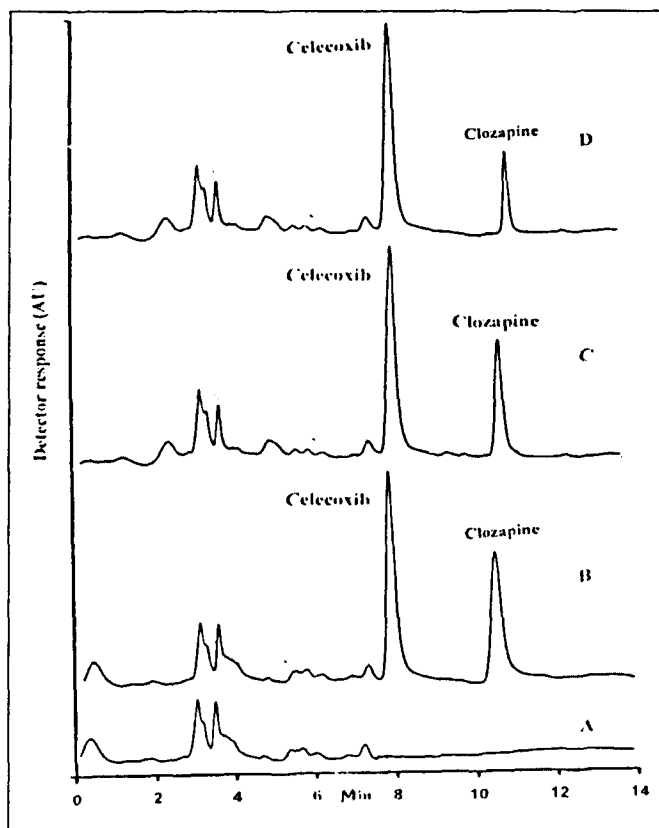


Fig. 1: Typical HPLC chromatograms

(A) Blank serum, (B) Serum containing 4 µg/ml clozapine and 6 µg/ml IS (C) serum samples collected after 15 min of i.v. dosing (10 mg/kg), respective clozapine concentration was 2.53 µg/ml and (D) Serum samples collected after 45 min of oral dosing (20 mg/kg), respective clozapine concentration was 1.09 µg/ml.

The inclusion of triethylamine (0.5 %) in the mobile phase improved the peak shape of the clozapine. pH of the mobile phase also affected the retention time of clozapine. The retention time of the clozapine was prolonged with the increased pH. A pH of 6.5 was found to be optimum for obtaining well separated clozapine peak with that of the IS. Sodium hydroxide (2 M) was added in the processing of samples to increase the extraction efficiency by increasing pH of the serum sample. At higher pH, octanol-water partition-coefficient value of clozapine is increased (octanol-water partition-coefficient of clozapine is increased from 0.4 to 1500 as the pH increased from 2 to 8)⁵. System suitability parameters for the method were as follows. Theoretical plates for clozapine and internal standard were 6787 and 7144, respectively. Peak asymmetric factor was less than 1.2 for both clozapine and internal standard and reso-

lution between clozapine and internal standard was 5.2.

Haring *et al.*, described a HPLC method with UV detection that include a laborious extraction procedure, with linearity from 50 to 1000 ng/ml¹¹. In all other liquid-liquid extraction methods¹²⁻¹⁵ two to three steps were involved with percentage recoveries 72-95 %. In the present method, single step extraction method was developed that has comparable recovery (70-84 %) and intra day and inter day coefficient of variation less than 6 %. The present method is less sensitive (LOQ 50 ng/ml) comparing to previous methods but simple with short extraction procedure. Therapeutic range of clozapine was reported is 100-800 ng/ml²¹. Therefore the present method is also applicable to therapeutic monitoring of clozapine.

The ratio of peak area of clozapine to that of IS was used for the quantification of the clozapine in serum samples. The calibration curves were linear in the concentration range 100 to 4000 ng/ml. The equation of the calibration curve obtained from six points was $y=0.1021x+0.023$, ($R^2=0.9972$). The limit of quantification (LOQ), established by determining the concentration of four spiked calibration standards having a reproducibility with a relative standard deviation (RSD) less than 20 % and an accuracy of 80 to 120 % was found to be 50 ng/ml. Using this method it is possible to further increase the sensitivity by increasing the injection volume.

The intra day precision of the assay was determined by analyzing five fortified serum samples at each concentration and for the determination of inter day precision the samples were analyzed on five different days. The intra and inter day RSD values (Table 1) were within the limits (<15 %) specified²². The recovery of the clozapine from serum was estimated at 100, 250, 500 and 1000 ng/ml concentrations. Serum samples containing clozapine and IS were extracted with ethylacetate and analyzed. Similar concentrations of clozapine in methanol were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak area of clozapine obtained from extraction of serum samples with that of mean peak area of clozapine obtained by direct injection. The absolute recoveries ranged from 75.8 to 79.9 % (Table 2). The accuracy of the HPLC method was verified by comparing the concentration of clozapine measured in extract with the actual concentrations added.

Fig. 2 shows the serum clozapine concentration-time profiles after i.v. (10 mg/kg) and oral (20 mg/kg) administra-

TABLE 1: INTRA AND INTER DAY PRECISION FOR CLOZAPINE IN SERUM

Theoretical Concentration (ng/ml)	Experimental concentration (ng/ml) (Mean±SD)	RSD (%)
Intra day (n=5)		
100	93.6±5.25	5.61
250	245±9.66	3.95
500	492±17.1	3.49
1000	977±24.3	2.48
2000	1953±41.6	2.12
Inter day (n=10)		
100	96.4±5.56	5.72
250	247±6.83	2.76
500	483±19.0	3.94
1000	970±29.3	3.02
2000	1961±42.7	2.18

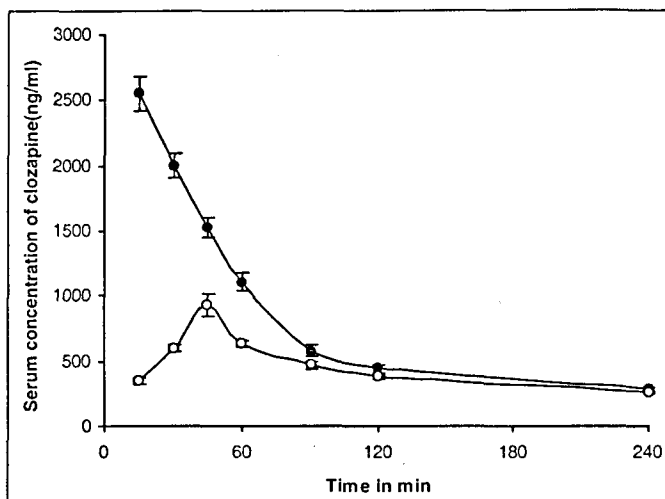


Fig. 2: Mean serum concentration-time profiles
Mean serum concentration-time profiles of clozapine after iv bolus (10 mg/kg) (●) and oral (20 mg/kg) (○) administration of clozapine solution to rats (n = 6).

TABLE 2: RECOVERY AND ACCURACY OF THE HPLC METHOD FOR THE CLOZAPINE

Theoretical concentration (ng/ml)	Absolute recovery (%)		Accuracy (%)	
	Mean±SD (n=6)	Range (min-max)	Mean±SD (n=6)	Range (min-max)
100	76.5±3.90	69.9-80.6	96.8±2.43	92.3-99.3
250	75.8±4.41	69.7-82.5	97.1±3.01	94.5-100.5
500	76.7±3.65	72.6-82.6	98.7±2.18	96.7-102.6
1000	79.9±3.62	73.6-84.2	99.7±1.80	6.8-101.6

tion of clozapine solution to rats. Area under the curve (0-∞) after i.v. administration was 4404.8±286.9 ng/ml/h, with half life of 2.34±0.63 h. Volume of distribution and clearance were estimated to be 7.56±1.63 l/kg and 2.28±0.142 l/h/kg, respectively. Whereas after oral administration, peak concentration (926.6±86.3 ng/ml) for clozapine was reached at 45 min and the half life was 2.89±0.43 h. The pharmacokinetic parameters AUC (0-∞) (2765.4±158.7 ng/ml/h), volume of distribution (9.48±1.4 l/kg) and clearance (2.27±0.0003 l/h/kg) were estimated according to non compartmental model.

However, terminal elimination half life of 1.5-1.6 h was reported after i.p. administration (10 mg/kg) of clozapine²³. In another study, it was reported that terminal elimination half lives were estimated to be 0.69 and 1.36 h after intravenous administration of clozapine 2.5 mg/kg and 5 mg/kg,

respectively^{12,20}. Thus clozapine is short lived in the rats than humans (half life 8-10 h)²⁴⁻²⁵.

In conclusion, the presented HPLC method is simple, precise and accurate method to determine clozapine concentration in serum. The convenience of this method is single step extraction which is inexpensive and time saving. This method has been used to study pharmacokinetics of clozapine in rats and same is applicable to therapeutic drug monitoring.

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