Determination of Clozapine in Human Plasma by High – Performance Liquid Chromatography with UV – VIS Detector

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ABSTRACT

A specific reversed- phase high-performance liquid chromatographic method has been developed for the simultaneous determination of clozapine in human plasma. Diazepam was used as an internal standard. The drug from human plasma were extracted by liquid-liquid extraction with diethyl ether. The analysis was performed on a C18 analytical column with UV – VIS detector at 250 nm and acetonitrile-methanol-0.5% triethylamine (40:10:50) was used as mobile phase. It was found to be linear linear over the concentration range of 25 to 2000 ng/ml and extraction recovery was more than 80% . The coefficients of variation (CV) for intraday and interday assay were found to be less than 5%. The limit of quantification (LOQ) was 25 ng/ml. This analysis method was successfully used in pharmacokinetic and bioequivalence study of clozapine in schizophrenic patients.

Key words: Clozapine, Plasma analysis, HPLC, Determination, Pharmacokinetic

INTRODUCTION

Clozapine, a dibenzodiazepine derivative (piperazine – substituted tricyclic antipsychotic agent) (McEvoy, 2005) is used in the treatment of schizophrenia in patients who do not respond or are tolerant to the other antipsychotic drugs.

Clozapine has a yellow crystalline powder and melting point at 183°C and is slightly soluble in water. The structure of clozapine is 8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e] [1,4] diazepine and molecular weight is 326.8 (McEvoy, 2005). The chemical structures of clozapine and diazepam are similar and are presented in Figure1.
A simultaneous determination of clozapine and other antipsychotic drugs in plasma by HPLC with UV-detector has been developed for application to management of acute toxicity (Garcia et al., 2003). The analysis was performed on Xterra™MC C18 column and and acetonitrile – phosphate buffer was used as mobile phase. Gupta (1995) used solid phase extraction and HPLC analysis for clozapine, performed on C8 column and acetonitrile-0.1%tetramethyl-ammonium perchlorate was used as mobile phase.

The purpose of the present study was to develop a simple HPLC analysis method for clozapine in human plasma determination with high specificity/selectivity, sensitivity, accuracy, precision and reproducibility.

**MATERIALS AND METHODS**

**Chemicals and reagents** Clozapine reference standard was obtained from Jurichem (Germany). Diazepam was supplied by Ranbaxy Laboratories (New Delhi, India). HPLC grade methanol (Fisher, United Kingdom), isocratic grade acetonitrile (Merck, Darmstadt, Germany), analytical grade diethyl ether (Labscan, Bangkok Thailand), triethylamine (Merck, Hohenbrunn Germany), di – potassium hydrogen phosphate powder, K2HPO4 (Fluka, Switzerland). Deionized water from a milli Q apparatus was used in this study. Plasma was purchased from a local blood bank to generate a drug – free plasma pool.

**Apparatus and chromatographic conditions** The HPLC system consisted of a model LC–10ATvp pump (Shimadzu, Kyoto, Japan), a model DGU-14A degasser (Shimadzu, Kyoto, Japan), a model SIL–10ADvp auto injector (Shimadzu, Kyoto, Japan). Seperation was achieved on ODS hypersil (5 µm) cartridge column (125 x 4.0 mm I.D) (Agilent Tecnologies, U.S.A) and detected with UV-VIS detector model SPD-10Awp (Shimadzu, Kyoto, Japan) at wavelength 250 nm.

The part of extraction consisted of a model 2601 multi – tube vortexer (Scientific Manufacturing Industries, U.S.A), a model Z 383 K centrifuge (Hermle Labortechnik, Germany) and a model 4322100 vortex – evaporator (Buchler Instruments a Labconco Company).

![Clozapine](image1.png)

![Diazepam](image2.png)

**Figure 1.** Chemical structures of clozapine and diazepam as internal standard.
The mobile phase consisted of acetonitrile – methanol - triethylamine (0.5%,pH 5.5 ) (40:10:50 ) . It was filtered with 0.25 μm membrane filter (Sartorius, Germany) before use. Chromatography was performed at ambient temperature. Flow rate was 1.0 ml/min and injection volume was 50 μl.

**Stock and standard solutions** Stock solutions of clozapine were prepared by dissolving 25 mg in 25 ml methanol to yield a final concentration of 1 mg/ml and stored at 4°C until analysis. This stock solution was used to prepare a set of working standard by diluting in deionized water. A 100 μl of each concentration of working standard was pipetted into 900 μl of drug-free human plasma to yield concentrations of 25, 50, 100, 250, 500, 1000 and 2000 ng/ml. In the same manner, plasma quality controls (QC) which stock solution of clozapine QC sample was separately prepared at concentration of 1 mg/ml. Three concentrations of QC sample (75, 375 and 1500 ng/ml) were prepared in plasma and included in every analytical run. Internal standard solution (diazepam) was prepared in methanol and working internal standard was diluted with deionized water. A 100 μl of internal standard working solution (10 μg/ml) was added to a 1000 μl of drug-free human plasma to yield concentration of 1000 ng/ml. Plasma standards were extracted by the process mentioned above before injection into the HPLC system.

**Analytical procedure** 1 ml aliquot of the spiked plasma or human plasma sample was pipetted into a test-tube and 100 μl of 10 μg/ml diazepam (internal standard) was mixed. 1 ml of 50 mM phosphate buffer pH 7.0 was added into plasma sample. The mixture was extracted with 3 ml of diethyl ether. The samples were closed with a cap. After that, the content were vortex-mixed for 10 minutes and centrifuged at 4,000 rpm for 10 minutes. Then 2 ml of organic supernatant was collected into clean test tube and evaporated to dryness under reduced pressure at 35°C. The residue was reconstituted with 250 μl of mobile phase and 50 μl was injected on to the HPLC column.

Validation of the analysis method including specificity/selectivity, extraction recovery, accuracy and precision, sensitivity and stability of the sample was performed according to the Guidance for Industry: Bioanalytical Method Validation (Guidance for Industry, 2001).

**Specificity and selectivity** Peaks of drug and internal standard were separated from other interfering peaks in blank plasma by comparing the chromatograms of the following samples:
1. blank plasma of 6 normal volunteers
2. standard solution of clozapine and the internal standard (diazepam)
3. blank plasma spiked with clozapine and the internal standard

**Recovery (extraction efficiency)** Extraction recoveries of clozapine and diazepam from human plasma were determined by comparison of HPLC responses (peak area) from extracted sample (quality control sample), containing known amounts (75, 375, 1500 ng/ml for clozapine and 1000 ng/ml for diazepam), to those from unextracted and directly injected standard, spiked with the same amount.

**Accuracy and Precision** Accuracy and precision of the method were determined by five replicate analysis of known clozapine concentration over the
calibration curve. Interday (between – run ) and intraday (within-run) accuracy were expressed as percentage from spiked concentration, using following equation:

\[
\% \text{ Accuracy} = \left( \frac{C_{\text{obs}}}{C_{\text{spike}}} \right) \times 100
\]

Where \( C_{\text{obs}} \) is the observed concentration for each standard, and \( C_{\text{spike}} \) is the spiked theoretical concentration. Intraday (within-run) precision was studied by analyzing 5 sets of plasma spiked with clozapine on the same day. Interday (between – run) precision was determined by quantifying the observed concentrations of quality control (QC) sample at three concentration levels, i.e., 75 ng/ml (low conc.), 375 ng/ml (medium conc.), and 1500 ng/ml (high conc.) on 5 different days along with a daily – prepared standard curve. Interday and intraday precision of the method was expressed as the percent coefficient of variation (\%CV) of the mean peak ratio for each standard or QC sample.

**Calibration curve** Calibration curves were constructed in the range of 25 – 2000 ng/ml. The curves were obtained daily for 5 days by plotting between the peak area ratio and concentrations of standards.

**Stability** To determine the influence of different times on the stability of drug, clozapine was spiked to blank plasma at the concentrations of 75, 375 and 1500 ng/ml, each portion of spiked plasma was repeatedly analyzed five times for clozapine concentration \((n=5)\) under different storage conditions at \(-40^\circ\text{C}\). The first portion (initial concentration) was immediately extracted and analyzed as mentioned above, another portion was extracted and analyzed at time described under the following stability studies. The stability of clozapine was expressed as observed concentration of clozapine comparing with initial concentration \((t=0)\). This study will be referred to in detail as follows.

**Freeze – thaw stability and long term stability** The study of freeze-thaw stability and long term stability were performed by analyzing the spiked plasma samples after 3 cycles of freeze-and-thaw and after being stored for 14 days, respectively.

**Bench-top stability** The study of bench top stability was performed by analyzing the spiked plasma samples after sitting on a laboratory bench at room temperature for 5 hours.

**Autosampler stability** The study of autosampler stability was performed by analyzing the spiked plasma samples immediately and then repeated after 24 hours.

**RESULTS AND DISCUSSION**

Good chromatographic separation was achieved by ODS hypersil \((5 \mu\text{m}, 125 \times 4.0 \text{ mm I.D})\) column, and mobile phase consisting of acetonitrile – methanol - triethylamine \((0.5\%,\text{pH 5.5}) (40:10:50)\) at flow rate of 1.0 ml/min. The optimum wavelength was detected with UV-VIS detector at 250 nm which had much better detector response.
No interfering peak was observed at the retention times of both clozapine and the internal standard. The retention times of either clozapine or the internal standard were approximately the same in all chromatograms (see Figure 2).

Figure 2. Representative chromatograms of blank plasma from a volunteer (A), Plasma standard containing 500 ng/ml clozapine (peak I) and internal standard (peak II). (B), Plasma standard containing 25 ng/ml Clozapine (peak I) and internal standard (peak II) (C).

The method was validated with regard to linearity, limit of detection and quantitation, recovery, precision, accuracy and specificity.

The lowest standard on the calibration curve should be accepted as the lower limit of quantification (LLOQ) when the response at LLOQ is at least 5 times greater than that of blank response and the analyte peak (response) should be identifiable, discrete and reproducible with high precision (% CV ≤ 20 and accuracy of 80 – 120%), the lowest concentration on the standard curve with acceptable accuracy, precision and variability was 25 ng/ml with the coefficient of variation (CV) of 4.1% and the accuracy of observed plasma clozapine concentration (n=5) from
the spiked concentration was 101.6%. Therefore, this concentration was accepted as the lower limit of quantification.

Peak area ratio of clozapine of calibration standard concentrations were proportional to the plasma concentration of clozapine in the range of 25-2000 ng/ml. Each concentration was tested 5 times. The coefficient of correlation ($r^2$), slope and intercept to linear regression line are reported in Table 1.

**Table 1.** Statistical data (n=5) for linearity of clozapine.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$r^2$</th>
<th>slope (mean±S.D)</th>
<th>intercept (mean±S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>clozapine</td>
<td>0.9998±0.0001</td>
<td>0.0009±0.0000</td>
<td>-0.0036±0.0024</td>
</tr>
</tbody>
</table>

The absolute recovery was calculated by comparing the areas under the peak obtained from standard working solutions with the peak areas from standard sample. The recovery of clozapine (75, 375, and 1500 ng/ml) and diazepam were 99.8±5.1%, 95.1±4.7%, 88.7±3.7 % and 99.2±3.3%, respectively. The results are shown in Table 2.

**Table 2.** Recovery of extraction for the analysis of clozapine and diazepam in human plasma. (n = 5)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentrations add (ng/ml)</th>
<th>% Recovery±(S.D.)</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>clozapine</td>
<td>75</td>
<td>99.8±5.1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>375</td>
<td>95.1±4.7</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>88.7±3.7</td>
<td>1.7</td>
</tr>
<tr>
<td>diazepam</td>
<td>1000</td>
<td>99.2±3.3</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Intraday assay precision of the method is illustrated in Table 3. It was estimated by assaying the quality control samples five times in the same analytical runs. The precision was less than 3.0% and accuracy ranged from 97.2 – 105.8% at all concentration levels.

Interday assay precision and accuracy was evaluated by processing a set of calibration curves and quality control samples (three levels analyzed five times, results averaged for statistical evaluation) in the same analytical runs (Table 3). The precision was less than 3.5% and accuracy ranged from 96.3-103.9% at all concentration levels.
Table 3. Precision and accuracy of the HPLC method for the analysis of clozapine in human plasma.

<table>
<thead>
<tr>
<th>Conc. Add (ng/ml)</th>
<th>Intraday studies (n=5)</th>
<th>Interday studies (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. Found mean±S.D (ng/ml)</td>
<td>%CV</td>
</tr>
<tr>
<td>75</td>
<td>72.9±1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>375</td>
<td>385.4±2.2</td>
<td>0.6</td>
</tr>
<tr>
<td>1500</td>
<td>1551.3±44.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The stability of clozapine in plasma was determined by periodic analysis of spiked samples. The results indicated less degradation which suggested that clozapine had a good stability in human plasma, either short – term or long – term stability test (Table 4).

Table 4. Stability of clozapine in human plasma (n = 5).

<table>
<thead>
<tr>
<th>Type of stability</th>
<th>Conc. (ng/ml) at t = 0, (mean±S.D)</th>
<th>Conc. (ng/ml) at t = t, (mean±S.D)</th>
<th>% Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze - thaw (3cycles)</td>
<td>72.9±1.1</td>
<td>73.2±0.6</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>385.4±2.3</td>
<td>353.6±5.0</td>
<td>-8.2</td>
</tr>
<tr>
<td></td>
<td>1587.5±44.5</td>
<td>1494.8±28.7</td>
<td>-5.8</td>
</tr>
<tr>
<td>Long - term (14 days)</td>
<td>72.9±1.1</td>
<td>71.2±1.5</td>
<td>-2.4</td>
</tr>
<tr>
<td></td>
<td>385.4±2.3</td>
<td>366.3±9.3</td>
<td>-5.0</td>
</tr>
<tr>
<td></td>
<td>1587.5±44.5</td>
<td>1591±16.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Short - term (5 hours)</td>
<td>72.9±1.1</td>
<td>74.1±1.8</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>385.4±2.3</td>
<td>376.2±8.8</td>
<td>-2.4</td>
</tr>
<tr>
<td></td>
<td>1587.5±44.5</td>
<td>1563.8±27.5</td>
<td>-1.5</td>
</tr>
<tr>
<td>Autosampler (24 hours)</td>
<td>72.9±1.1</td>
<td>70.5±1.9</td>
<td>-3.3</td>
</tr>
<tr>
<td></td>
<td>385.4±2.3</td>
<td>365.5±3.6</td>
<td>-5.2</td>
</tr>
<tr>
<td></td>
<td>1587.5±44.5</td>
<td>1553.1±46.6</td>
<td>-2.2</td>
</tr>
</tbody>
</table>

The one-step liquid-liquid extraction with diethyl ether was good enough to be an alternative extraction method for solid phase extraction that was used in the method published by Gupta (1995) since it used minimal steps of extraction process and had high extraction recovery. Garcia et al.,(2003) used acetonitrile – phosphate buffer as mobile phase, but this mobile phase could not give good chromatographic separation in our study. Thus, 0.5% triethylamine (pH 5.5), ion-pairing agent, was used in mobile phase in order to improve chromatographic separation.

The developed method was applied to determine plasma clozapine in multiple-dose pharmacokinetic and bioequivalence study in 25 schizophrenic male patients. Plasma samples were periodically collected up to 24 hours...
after oral administration of 100 mg clozapine tablet (Clozaril®) at steady state. Figure 3 illustrates the mean±SD plasma concentration time profile of clozapine, following an oral dose of 100 mg clozapine. The plasma level of clozapine reached the maximum in about 2.5 hours after administration and calculated half-life ($t^{1/2}$) of clozapine was 11.5±3.9 hours. The results are similar to those reported in AHFS Drugs Information (McEvoy, 2005).

**CONCLUSION**

This developed HPLC method for the determination of plasma clozapine concentration was found to be a highly effective method since it used one-step liquid-liquid extraction with diethyl ether which is simple with low cost of extraction and high extraction recovery. Moreover, this analysis method had high specificity, sensitivity, accuracy and precision and also had a good stability, either short-term or long-term. The analytical method was successfully used in multiple-dose pharmacokinetic and bioequivalence study of clozapine in schizophrenic patients.

**ACKNOWLEDGEMENTS**

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