A simple, simultaneous and reliable HPLC method was developed for analysis of clozapine that is a widely prescribed drug especially for treatment of schizophrenia, but may have fatal toxicities in some cases. Its two metabolites are norclozapine and clozapine N-oxide in human plasma. Chromatographic conditions and detection parameters were adjusted in consequence of optimization study. An isocratic high-performance liquid chromatography method with ultraviolet detection at 220nm was utilized. Analytes are concentrated from plasma by liquid–liquid extraction with ethyl acetate, n-hexane and isopropylalcohol (80:15:5,v/v/v) which allows to obtain good extraction yields (>80%) for all analytes. Separation was performed on a C18 reversed-phase column using a mixture of acetonitrile and 62.4mM phosphate buffer (containing 0.3%triethylamine, pH4.5) at the ratio of 40:60(v/v). Method showed linearity with excellent correlation coefficients ($r^2>0.999$) for each analyte. The relative standard deviations and relative standard errors calculated to present precision and accuracy between and within-day assay were less than 4% for low concentrations. The method was specific and sensitive with detection limits of 23.6 $\mu$g/L, 19.3 $\mu$g/L and 23.6 $\mu$g/L for clozapine, norclozapine and clozapine N-oxide respectively. The procedure described is relatively simple, precise, and applicable for routine therapeutic drug monitoring especially in psychiatry clinics or in toxicology reference laboratories.

Key words: Clozapine, Clozapine-N-oxide, Norclozapine, HPLC-UV, Optimization.

Klozapin ve Major Metabolitlerinin İnsan Plazmasında Analizi İçin Bir YPSK-UV Metodunun Optimişasyonu ve Validasyonu

Bu çalışmada özellikle şizofreni tedavisini için yaygın şekilde reçete edilmeye başlanan ancak bazı vakalarla toksisitesi ölümcül olabilen klozapin analizi için basit ve güvenir bir YPSK yöntemi geliştirilmiştir. Klozapinin insan plazmasındaki iki ana metaboliti norklozapin ve klozapin N-oksit’dir. Kromatografik analiz koşulları ve deteksiyon parametreleri optimizasyon çalışmasının sonucunda belirlenmiştir. UV dedektörülü, izokratik yüksek performanslı sıvı kromatografisi analiz yöntemi 220nm dalga boyunda kullanılmıştır. Analitler plazmadan etilasetat, n-heksan ve izopropil alkol (80:15:5, v/v/v) karışımı ile sıvı-sıvı ekstraksiyon yöntemile konseptre edilmiş, ekstraksiyon verimi %80’un üzerinde ölçülmüştür. Ayırmı C18 ters-faz kolon ile, 40:60 (v/v) orandında asetonitril ve 62.4 mM fosfat tamponu (%0.3 trietilamin, pH 4.5) kullanılarak yapılmıştır. Metot her analit için mükemmel korelasyon katsayısı ile ($r^2>0.999$) doğrulukla göstermiştir. Günler arası ve gün içi kesinlik ve doğruluk için ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen ölçülen çözüm.
INTRODUCTION

Clozapine (CLZ) is classified structurally as a dibenzodiazepine derivative. It is known as an atypical antipsychotic agent and displays efficacy in management of schizophrenia and treatment of other psychotic disorders. Especially for the cases of life time prevalence of schizophrenia, CLZ is an effective choice among the antipsychotics (1). Although it has a high antipsychotic and therapeutic potential, dose dependent epileptic seizures (2) and serious risk of fatal agranulocytosis (3) limits the wider use of CLZ. According to the statistics recently reviewed, incidence of clozapine-induced agranulocytosis ranges up to 21 cases per 1000 patients in one year (4). Therefore, frequent hematological monitoring and drug monitoring is toxicologically important and recommended during the treatment. CLZ undergoes extensively oxidative metabolism in the liver via cytochrome P450 enzyme system yielding several derivatives, mainly two principle metabolites: norclozapine (N-desmethylclozapine) (NCLZ), and clozapine N-oxide (CLZNO) (5) (Figure 1). NCLZ, as the major metabolite, is known to be pharmacologically active and stable whereas CLZNO is known as the minor and unstable metabolite that one third of it may be reduced back to CLZ (6). Including the reversible metabolism of CLZNO, person-to-person differences in the CYP enzyme activity is one of the important contributors to steady-state clozapine and active metabolite NCLZ concentrations (7) in human. Studies have revealed that peak plasma concentrations range from 1.1 to 3.6 hours; and elimination half-life also varies from 9.1 to 17.4 hours due to inter-patient variability in pharmacokinetic parameters (3). Consequently, the necessity for therapeutic drug monitoring for clozapine and its metabolites had been accrued which becomes conspicuous subject among the analytic toxicologists and clinicians.

Various assays have been developed for determination of clozapine in biological matrices. A number of methods based on gas chromatography, high performance liquid chromatography and immunoassay are published. Radioimmunoassay analysis for clozapine and its metabolites is one of early applications (8), but not common due to its limited sensitivity and specificity in comparison with chromatographic methods (9). Gas chromatography methods for determination of clozapine and its metabolites have also been carried out by a variety of detection techniques (10, 11) for the analysis of biological material including tissues (12). In fact, GC technique is a common choice of analytic chemists – toxicologists because of its high sensitivity especially when coupled with the mass spectroscopy detection. However, in the case of CLZ detection, GC assays are not common preferences due to thermal stability problems leading CLZNO to decompose quantitatively (13). Hence, liquid chromatographic methods, gathering the advantages of mild analytic conditions and sensitivity, stand out markedly among others for determination and monitorization of clozapine and its metabolites (9). In recent years coupling of LC to MS has provided a useful and rugged technique for analysis of drug compounds and an alternative to GC-MS. On the other hand, it is certainly not the cheapest solution for analysis of pharmaceuticals (14). Since HPLC-UV assays are inexpensive and widely utilized, it has appeared to fit best for performing simultaneous separation, quantification and clinical monitoring of CLZ and its metabolites as primary concern of this paper.

Number of comprehensive researches about HPLC-UV methods for determination of clozapine and its metabolites has been reported (15-26). This study intends to optimize the analytical conditions by modifying parameters of previously proposed methods, and to develop and validate more precise, rugged and accurate method for the determination of clozapine and its two major metabolites in human plasma which would be suitable for routine use in clinical practice. Hence, the novelty of the present study relies basically on method developing via optimization of analytic parameters and validation of the optimized assay. For this purpose, a simple and reliable HPLC method was established in order to assess clozapine and its two major metabolites in human plasma by adjusting analytical conditions to optimum parameters.
EXPERIMENTAL

Chemicals and reagents

Potassium dihydrogen phosphate, sodium hydroxide, orthophosphoric acid and HPLC-grade methanol were purchased from Merck (Darmstadt, Germany). Ethyl acetate, n-hexane, isopropyl alcohol and triethylamine were obtained from Sigma (St. Louis, MO, USA). The standards of pharmaceuticals, clozapine norclozapine, clozapine n-oxide, and chlorpromazine hydrochloride (Internal standard) were also purchased from Sigma (St. Louis, MO, USA).

Instrumentation and optimized chromatographic conditions

The separation and quantification were performed by HP Agilent 1100 (Santa Clara, CA, USA) high-performance liquid chromatography (HPLC) system, equipped with a UV detector. Optimum analytic conditions were set after an optimization procedure was performed for column selection, content of mobile phase (MP) and wavelength. Prior to optimization, a standard assay for benzodiazepine determination offered by the manufacturer was used and each parameter was adjusted while others were fixed.

The analysis were carried out with a system consist of an isocratic pump, manual injector with a loop volume of 20 µL, and a Waters C18 column with diameters 3.5 µm, 4.6 x 150mm. UV detection was adjusted to 220 nm. The mobile phase, composed of acetonitrile and 62.4mM phosphate buffer (40:60, v/v) at pH 4.5, containing 0.3 % (v/v) triethylamine was filtered through a 0.45 µm membrane (Alltech, IL, USA) and degassed in ultrasound bath for 30 min. An isocratic solution was performed at a flow rate of 1.0 mL/min and at room temperature. Peak areas were measured and calculations were carried out considering the internal standard (IS) peak ratios.

Selection of internal standard

Discovery of chlorpromazine in the 1950’s was one of the most significant breakthroughs for the treatment of schizophrenia until the advent of atypical antipsychotics such as clozapine (7). Besides the physicochemical-molecular proximity, similarities in

![Figure 1: Chemical structures of chlorpromazine (I.S), clozapine and its two major metabolites norclozapine and clozapine N-oxide](image_url)
pharmacological purpose of chlorpromazine with clozapine are noteworthy. On the other hand, also, the chromatographic pre-study with chlorpromazine displayed confident results to be used as an internal standard for this study.

Preparation of stock solutions and workings standards

Stock solutions and working standards of all analytes and internal standard were prepared in methanol solution at the concentration of 50 mg/L. CLZ, NCLZ and CLZNO were mixed in methanol forming 5 different working standards at ascending concentrations (1, 2.5, 5, 10 and 20 mg/L for CLZ; 1, 1.5, 3, 6 and 12 mg/L for NCLZ; and 1, 2.5, 5, 7.5 and 10 mg/L for CLZNO). Working standards were prepared weekly, and were used to spike blank plasma samples daily, prior to analysis. Blank human blood samples were collected from Ankara University Faculty of Medicine-Serpil Akdağ Blood Center, centrifuged at 3000 g for 5 min to separate the plasma. Plasma samples, and all working solutions were stored at -20°C until the analysis were carried out; were checked chromatographically for purity before experiments, were utilized as quality control specimens and were checked for the stability before and after the injections of every sample set.

Sample preparation

Plasma samples, provided from the healthy volunteers among laboratory crew of Ankara University Institute of Forensic Medicine, were pooled for experimental use. 800 µL of blank plasma was placed into clean glass tubes containing 100 µL of IS stock solution (30 mg/L). Each of these tubes 100 µL of CLZ, NCLZ and CLZNO mixture working standards were spiked, reaching the total volume to 1 mL and achieving 10 times diluted mixtures; yielding analyte concentrations as calibration samples.

Extraction

Liquid-liquid extraction method was used for clozapine and its metabolites. After plasma samples were spiked with the analytes and chlorpromazine (CPZ) as internal standard (IS), and pH were adjusted to 9 with 1 M NaOH. Before the extraction procedure, samples were centrifuged at 3000 g for 5 min. Extraction was performed twice with 3 mL of ethylacetate, n-hexane, isopropylalcohol (80:15:5 v/v/v). (24). Analytes were dried under N2 before dissolved in mobile phase prior to injection to HPLC.

RESULTS and DISCUSSION

Optimization of method

The optimization procedure for HPLC-UV analysis was performed on three basic parameters, namely column, mobile phase (MP) and wavelength.

In chromatography, even though selectivity is adjusted for peak spacing and maximum sample resolution, resulting an adequate separation, further improvement in separation may be possible by varying column condition such as column length so as to improve the column plate number (27). In this study, besides maintaining the good resolution, minimizing retention time was our first concern. Therefore, a C18 reversed phase column 150 mm in length was chosen and checked with standard benzodiazepine assay offered by the manufacturer.

Various HPLC techniques from the literature were tested based on the mobile phase; modifications were performed to increase the applicability in current lab conditions; and three MP’s were selected. Three different mobile phase were modified namely, MP1 (acetonitrile:methanol:phosphate buffer (24:12:64, v/v/v) at pH 4) (26), MP2 (acetonitrile:methanol:phosphate buffer (30:2:100, v/v/v) at pH 3.7) (15) and MP3 (acetonitrile:phosphate buffer (40:60, v/v) at pH 4.5) (19). Analyte in isocratic elution is controlled by varying mobile phase composition. The solvent strength (percentage of the organic solvent content) in mobile phase controls the retention time of the analyte and the organic solvent can have a dramatic effect on selectivity (28). Thus, in current study, the selection of the mobile phase as the corner stone of chromatographic separation was examined focusing on the organic solvent, ratio of total acetonitrile and methanol. At almost similar pH values of
three mobile phases, it was noteworthy that increasing ratio of the organic modifiers designated better chromatographic results. Figure 2 shows that the optimum chromatographic conditions with less retention time and more peak area, indicating better response for all analytes, were achieved with MP3, where there is highest portion of acetonitrile as a strong organic solvent in elution of pharmaceuticals. When organic solvents are considered separately, it is known that acetonitrile is stronger organic solvent than methanol. Hence, the higher response in MP1 than MP2 can be explained comparing them in terms of the ratio of acetonitrile to methanol.

In order to plot the parameters for simple demonstration and explanation, responses of all four analytes (including IS) were gathered and representative graphs were plotted using total response values (Figure 2 and 3). And appropriate separation and detection parameters constituted according to the resulting data of optimization procedure.

**Figure 2.** The effect of mobile phase content to peak area and the retention time of analytes in the chromatograms.

In UV detection, ideally a compound absorbs light at a unique wavelength which is known as lambda maximum ($\lambda_{\text{max}}$). However, $\lambda_{\text{max}}$ may vary depending on the dissolution of the analyte which can be effected by the type/content or pH of its diluent. Therefore detection should be performed at various wavelengths to accomplish a proper UV detection. In this study 6 different wavelengths for UV detection were tested according to the related literature (15-26). Responses at wavelengths ranging 220 nm to 280 nm are displayed in Figure 3. The UV detection at 220nm revealed the best response for detection of IS, clozapine and two major metabolites.

**Figure 3.** The response of UV detection signals versus wavelengths at the range of 220-280nm

**Validation of method**

The analytical method was validated as performed in previous studies (29) to demonstrate the selectivity, specificity, linearity, recovery, lower limit of detection (LLOD) and limit of quantification (LOQ). Intra- and inter-day validation protocol was applied considering reproducibilities of method and instrument to obtain accurate and precise measurements in agreement with Conference on Harmonization guidelines (30).

**Specificity and selectivity**

The UV detection was set to a wavelength of 220 nm displaying optimum sensitivity. The method for plasma demonstrated excellent chromatographic specificity with no endogenous interference at the retention times of CLZ, NCLZ, CLZNO and the IS, (4.1, 3.5, 8.3 and 15.8 min. respectively). Figure 4 shows overlaid chromatograms of blank and spiked human plasma illustrating the high resolution with no interference and short separation time.
Linearity
After establishing the chromatographic conditions, calibration curves of CLZ, NCLZ and CLZNO in plasma were generated with concentration ranges of 100–2000 µg/L, 100–1200 µg/L and 100–1000 µg/L respectively. For each concentration 5 individual replicates were injected and linearity was obtained for each analyte with the correlation coefficients ($r^2$) over 0.999 (Figure 5).

Precision and accuracy
Precision, defined as relative standard deviation (RSD), was determined by five individual replicates at three different concentrations (n=5). Table 1 shows the RSD values of low, medium and high concentrations to present inter- and intra-day precision. Accuracy, defined as relative error (RE %), was also determined for the same concentrations of analytes (Table 1).
Table 1. Confidence parameters of validated method; intra-day and inter-day precision and accuracy for determination of clozapine (CLZ), norclozapine (NCLZ) and clozapine N-oxide (CLZNO) in human plasma samples

<table>
<thead>
<tr>
<th>Conc. (µg/L)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Obs.</td>
<td>Estimated conc.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( \bar{x} \pm SD ) (µg/L)</td>
</tr>
<tr>
<td>CLZ 100</td>
<td>5</td>
<td>100.13±4.66</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
<td>494.06±13.21</td>
</tr>
<tr>
<td>2000</td>
<td>5</td>
<td>1997.58±26.48</td>
</tr>
<tr>
<td>NCLZ 100</td>
<td>5</td>
<td>105.69±3.57</td>
</tr>
<tr>
<td>300</td>
<td>5</td>
<td>290.83±7.03</td>
</tr>
<tr>
<td>1200</td>
<td>5</td>
<td>1210.67±19.29</td>
</tr>
<tr>
<td>CLZNO 100</td>
<td>5</td>
<td>103.77±3.05</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
<td>509.85±13.21</td>
</tr>
<tr>
<td>1000</td>
<td>5</td>
<td>994.25±16.83</td>
</tr>
</tbody>
</table>
Recovery

The recovery of extraction procedures from human plasma was determined by comparing pre-extraction spikes with the post-extraction spikes of IS. Three individual replicates of spiked samples at mid-concentrations of CLZ, NCLZ and CLZNO (500 µg/L, 300 µg/L and 500 µg/L respectively) were prepared with and without internal standard (n=10). The extraction procedure was conducted as described previously. Peak area ratios were compared and recoveries were calculated as 80% and above for each analyte as tabulated in Table 2.

**Table 2. Recovery of extraction and limit values for detection and quantification of clozapine (CLZ), norclozapine (NCLZ) and clozapine N-oxide (CLZNO) from human plasma**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Extraction Recovery (%)</th>
<th>LLLOD (µg/L)</th>
<th>LOQ (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLZ</td>
<td>82.77</td>
<td>23.6</td>
<td>71.52</td>
</tr>
<tr>
<td>NCLZ</td>
<td>80.75</td>
<td>19.3</td>
<td>58.51</td>
</tr>
<tr>
<td>CLZNO</td>
<td>80.19</td>
<td>23.57</td>
<td>71.43</td>
</tr>
</tbody>
</table>

Limit of detection and quantification

Limit of detection (LOD) and lowest limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of the calibration curve, according to ICH guidelines (30) (LOD = 3.3σ/S, LOQ = 10σ/S where σ is the standard deviation of the response and S is the slope of the calibration curve). LOD and LOQ values were calculated and shown in Table 2.

CONCLUSIONS

In this study, simultaneous procedure of HPLC-UV method was proposed with simple extraction of sample yielding good recovery, selective UV detection with enhanced sensitivity and accuracy of determination for analysis of CLZ and its metabolites. Therefore, to achieve quality separation of analytes in a reasonable analysis time acceptable chromatographic factors were adjusted. The mobile phase composition and the pH were optimized. The mobile phase was a phosphate buffer adjusted to acidic pH and containing acetonitrile as the organic modifier. Baseline separation of the 3 analytes (and the IS) was achieved in less than 20 min. The method was validated in terms of reproducibility, sensitivity, accuracy, precision and detection limits in accordance with internationally accepted guidelines. Including the major metabolites of CLZ, analysis for all analytes demonstrates very precise and accurate results even for inter-day assays which allows determining therapeutic and toxic concentration levels.

In this work it is revealed that the established HPLC-UV method is suitable for the therapeutic drug monitoring of schizophrenic patients undergoing clozapine treatment, and applicable as a reference method in routine monitoring for toxicological and/or analytic purposes in clinics of psychiatry or elsewhere.

REFERENCES


Received:19.06.2014
Accepted:11.09.2014