Rapid Liquid Chromatography/Tandem Mass Spectrometer (LCMS) Method for Clozapine and its Metabolite N-Desmethyl Clozapine (Norclozapine) in Human Serum

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Clozapine is indicated for the treatment of schizophrenia and related psychotic disorders. Several methods have been developed for monitoring Clozapine levels; however, they possess limited specificity and are often laborious. This study describes a simple liquid chromatography/tandem mass spectrometer (LCMS) method in human serum. The ion transitions monitored were m/z 327, 270, 296 for Clozapine, m/z 313, 192, 227 for Norclozapine and m/z 328, 271 for Loxapine.

Key words: Clozapine; Norclozapine; LCMS

The assay is linear (25–1000 ng/ml) and showed a good correlation ($r = 0.98$) within the analytical range of 79–1210 ng/ml in human serum. This assay is highly specific and sensitive for the simultaneous measurements of Clozapine and Norclozapine. The simplification of this assay makes it ideal for high throughput analyses of the patient samples in a routine clinical laboratory staffed with general medical technologists. *J. Clin. Lab. Anal.* 23:394–398, 2009.

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INTRODUCTION

Antipsychotic medications are one of the fastest growing products in the pharmaceutical industry. This upsurge is primarily a result of the transition from the conventional or first generation antipsychotics (Chlorpromazine, Haloperidol, Phenothiazine, etc.) to a newer second generation antipsychotics (Clozapine, Olanzapine, Risperidone, Quetiapine) (1). Clozaril (Clozapine; MW 326.83) is an atypical antipsychotic drug, primarily used for the management of severely ill schizophrenic patients who fail to show adequate response to the standard drug treatment for schizophrenia (2). This drug is also effective in reducing the risk of suicide in patients who are classified as being at “high risk” for suicidal behavior. Clozapine is approximately 97% bound to serum proteins and peak Clozapine levels are reached in approximately 1–6 hr following the oral dosage. The elimination half-life is 4–16 hr once steady state has been reached. Clozapine is completely metabolized before excretion and approximately 50% of the administered dose is excreted in the urine and 30% in the feces. Therapeutic drug monitoring of Clozapine may be of clinical value in certain situations, such as poor clinical response, signs of toxicity, onset of seizures, changes in concurrent medication,

caffeine or nicotine, liver disease and suspected non-compliance.

There are several methods published for the analyses of Clozapine and its metabolites using gas chromatography (GC) (3–5), GC-mass spectrometry (GCMS) (6–8), liquid chromatography (LC) (9) and high performance liquid chromatography (HPLC) (10–29). All aforementioned methods are often laborious, use tedious extraction procedures and have long run times with varying accuracies and precision. Both liquid (30) and solid phase extraction procedures (31,32) are reported for the determination of Clozapine and its metabolite using liquid chromatography/tandem mass spectrometer (LCMS). In this study, we describe a simple and rapid method for the determination of Clozapine and Norclozapine using LCMS technology deemed suitable for routine clinical laboratory. We have used this method for patient monitoring for more than 4 years with good patient management.

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EXPERIMENTAL

Solvents and Chemicals

HPLC quality reagents (acetonitrile, methanol, ammonium acetate, formic acid) were purchased from Sigma-Aldrich, MO. Clozapine and Norclozapine standards were purchased from the UTAK Laboratories, Inc (Valencia, CA) and Internal Standard Loxapine was purchased from the Altech (Grace, Deerfield, IL).

Instruments and Columns

Clozapine and Norclozapine testing was performed by LCMS methodology using Quattro micro triple quadrupole tandem mass spectrometer fitted with a Z-spray ion source (Micromass, Waters Corporation, MA) and Waters 2795 Alliance HPLC system. The separation was achieved using a Symmetry C18 column (2.1×100 mm) purchased from the Waters Corporation, MA. System control and the data acquisition were performed by Mass Lynx NT 4.0 software with automated data processing using the QuanLynx software. Calibration curves are constructed using linear least squares regression with $1/X$ weighting for the multiple reaction monitoring (MRM) quantification using QuanLynx was performed by spectral peak height ratio to a known concentration of Loxapine. Clozapine and Norclozapine analysis was performed in the MRM mode of the MS with a dwell time of 0.15 sec per channel using the following transitions; m/z 327 > 270, 327 > 296 (Clozapine) 313 > 192, 313 > 227 (Norclozapine) and 328.2 > 271.2 (Loxapine, IS).

Sample Preparation and Methodology

Two hundred microliters of serum was mixed with an equal volume of acetonitrile, vortexed for 15 sec, and then separated via centrifugation for a period of 5 min. One hundred micro liters of IS (at a concentration of 200 ng/ml) was added to the equal volume of supernatant, briefly vortexed and transferred to HPLC vials. Ten microliters of this solution was injected. The mobile Phase 1 (A) consisted of 2 mmol ammonium acetate/0.1% formic acid in water and mobile Phase 2 (B) consisted of 2 mmol ammonium acetate/0.1% formic acid in methanol. In order to achieve separation, 80% of (A) and 20% of (B) was pumped for a period of 5 min at a constant flow rate of 0.350 ml/min. Maintaining this same flow rate, the gradient flow was then switched to 5% (A) and 95% (B) for a period of 30 sec. After this, the initial gradient concentrations are restored (80% (A), 20% (B)).

Precision

Within-day and between-day precision for Clozapine and Norclozapine was determined by testing three levels of the

matrix matched control solutions (UTAK, Valencia, CA). Within-day precision was performed by taking 20 consecutive measurements at each level. Between-day precision was determined by testing with control solutions for ten consecutive days. Precision testing was performed on two analyzers. The mean, SD and CVs were calculated.

Linearity and Recovery

Linearity performance of the LCMS analyzers for the Clozapine and Norclozapine was analyzed by five different linearity standards (UTAK, Valencia, CA).

Method Comparison

Clozapine and Norclozapine values from both the LCMS and HPLC were compared with blood specimens routinely submitted for Clozapine analyses to our laboratory. This study was approved by the human subjects' committee institutional review board of UMass Medical School.

Statistical Analyses

Precision, linearity, accuracy and method comparison data were analyzed using the EP Evaluator, release 8.0 software (D.G. Rhoades Associates, Inc, Kennett Square, PA).

RESULTS AND DISCUSSION

Chromatogram and Mass Spectrum Ions

Figure 1 shows the chromatograms of the Clozapine, Norclozapine and Loxapine. The separation was achieved rapidly within 5 min and the peaks show good separation. Figure 2 shows the mass spectrum for Clozapine, Norclozapine and Loxapine, respectively.

Precision

Within and between-day variations using the three levels of matrix matched control material showed an acceptable variations at all levels. For low control, the mean and within assay and between-day CVs for Clozapine are 116 and 1.8%, 4.7% and for Norclozapine 83 and 2.3%, 6.2%, respectively. For Medium control, the mean and within assay and between-day CVs for the Clozapine are 325 and 4.6%, 6.7% and for Norclozapine 83 and 4.4%, 6.8%, respectively. For high control, the mean and within assay and between-day CVs for Clozapine are 607 and 2.1%, 4.1% and for Norclozapine 83 and 2.5%, 3.8%, respectively.

Linearity and Recovery

The assay showed a good linear response, from 25 to 1000 ng/ml of Clozapine and Norclozapine. The linear

Fig. 1. Chromatogram of Clozapine, Norclozapine and Loxapine. *x*-axis shows time in minutes and *y*-axis shows the percentage elution in the HPLC.

Fig. 2. Electro spray mass spectrum of Clozapine (270.17; 296.11), Norclozapine (192.23; 227.19) and Loxapine (271.11). *x*-axis shows *m/z* ratio and *y*-axis shows the percentage.

regression equation for clozapine ($Y = 0.9986X + 4.6204$) and Norclozapine ($Y = 0.9928X + 6.1356$), respectively. The recovery of all the levels tested was within the allowable total error of 10%.

Method Comparison

Figure 3 shows the comparison of Clozapine levels on 80 blood specimens submitted to the laboratory by both HPLC and LCMS methods. Overall, a good correlation ($r = 0.98$) was observed in the range evaluated (79–1210 ng/ml). Table 1 shows the mean plasma levels of Clozapine and Norclozapine in both the male and female schizophrenic patients treated with Clozapine. The mean percent ratio between Clozapine and Norclozapine in both males and females is about 50% with no significant difference. A large variation exists in the levels of Clozapine and Norclozapine in both males and females. Due to its complex metabolism, significant inter and intraindividual variations exist in Clozapine levels (Smoking, hepatic metabolism, gastric absorption, age and possibly gender). Clozapine has two primary metabolites, Norclozapine and Clozapine *n*-oxide. One third of Clozapine *n*-oxide can be converted back to Clozapine. This reversible metabolism of *n*-oxide to Clozapine may partially account for the wide

interpatient variability seen in Clozapine plasma concentrations. Also, Clozapine binds principally to alpha-acid glycoprotein (AAG) (33). Mean plasma AAG levels can increase rapidly in response to systemic tissue injury, inflammation or infection. Increased plasma AAG levels provide possible explanation for the findings of abnormally high total levels of plasma Clozapine levels in some patients in the absence of clinical features of toxicity (34). Pharmacological testing has shown the desmethyl metabolite to have only limited activity. Therapeutic concentrations of Clozapine correlate well with Norclozapine concentrations, but the formation of Norclozapine is a saturable process under conditions of overdose. Were it not for its side effects, Clozapine would be the first-line treatment; however the rare but potentially lethal side effects of agranulocytosis and myocarditis relegate it to third-line use. Clozapine-induced hematotoxicity at the therapeutic dose range is not directly related to plasma concentrations of Clozapine or Norclozapine (35). Plasma levels of Clozapine do correlate with EEG changes and are thereby a good indicator of electrophysiological reactions to the drug (36,37).

CONCLUSION

The analysis of Clozapine and Norclozapine by LCMS method reported here in this study is very simple and rapid, makes it ideal to adopt in a routine clinical laboratory. This highly specific and sensitive LCMS method has been successfully applied for routine patient monitoring for more than 4 years.

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Fig. 3. Comparison of levels of Clozapine by HPLC and LCMS in 80 patients treated with Clozapine. The slope (0.94), intercept (1.52) and correlation ($r = 0.98$) and range tested (79–1210 ng/ml).

TABLE 1. Mean Plasma Levels of Clozapine and Norclozapine in Schizophrenic Patients Treated with Clozapine

	Males ($n = 311$)			Females ($n = 159$)		
	Mean	CV	Range (ng/ml)	Mean	CV	Range (ng/ml)
Clozapine	498	67	48–2588	554	64	50–1782
Norclozapine	221	69	65–852	239	58	31–364
Ratio %	49.8		9–229	50		13–142

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