Spring 6-2022

The Detection of Clozapine in Oral Fluid via a Kinetic Interaction of Microparticles in Solution (KIMS) Assay

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A thesis presented in partial fulfillment of the requirements for the degree of

Master of Sciences in Forensic Science.

John Jay College of Criminal Justice

City University of New York

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May 2022

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Acknowledgements

I would like to extend my sincere thanks to my advisors Dr. Richard Stripp and Dr. Damon Borg for all of their assistance, guidance, and patience throughout my time in this master's program and during this research project. Their knowledge and experience have inspired me, and their feedback has encouraged me to become a better student and individual. Without them, this project would not have been possible.

I would also like to extend my gratitude to the final member of my thesis committee, Teeshavi Acosta. Teeshavi has been a great help to me not just with this project but throughout my time at John Jay College. She has been supportive and encouraging to me and I will always appreciate that.

I want to give a big thank you to my family, who have shown me unconditional love and support. A special thank you to my father who has always pushed me to be the best student and person I can be. He has encouraged and supported me every step of the way. To my brother, thank you for always sticking by my side and showing up when I needed you the most. Without them, I would not be where I am today.

Lastly, I would like to thank my friends, classmates, and the entire Forensic Science department at John Jay College. I will forever cherish the great memories and laughs we had. These past two years at John Jay have had its ups and downs but a big thank you to all of them for supporting me in one of my biggest accomplishments yet.

Abstract

In recent years, the use of antipsychotic medications has rapidly increased. Most of these medications have a high risk potential. Due to this risk potential, patients who are prescribed these medications have to be monitored. Therapeutic drug monitoring (TDM) has become extremely important in the clinical environment. Physicians rely on TDM to make sure patients are adhering to their prescription and to make decisions about whether the medication is working. Clozapine is a very popular antipsychotic used in the treatment of schizophrenia. The current method for TDM for clozapine is a blood test. This causes challenges for physicians, who need to perform frequent TDM tests, because collecting blood samples is an invasive procedure. This study focused to validate a method for detecting clozapine in oral fluid using a KIMS Assay. This method allows for a non-invasive sample collection and presents rapid and accurate results. This method passed multiple validation tests, including linearity, accuracy and precision, carryover potential, interfering substances, and the evaluation of authentic positive and negative samples. The linearity had a limit of quantification (LOQ) range between 50 ng/ml and 1,000 ng/ml. The imprecision was <15%. There was carryover potential with samples that have concentrations above 1,000 ng/ml. None of the 21 substances tested were found to interfere with the method. This method reported the correct number of positive and negatives when compared to a traditional method for detecting clozapine. A rapid, precise, and accurate method for the determination of clozapine in oral fluid using a KIMS assay was validated.

Table of Contents

Acknowledgements i

Abstract ii

1. Introduction 1

2. Materials and Methods 7

3. Results and Discussion 17

4. Conclusion 23

References 25

1. Introduction

In the 1950's, antipsychotic drugs were introduced into medicine. The term antipsychotic encompasses many different substances. It includes any drug used for the resolution of psychosis. Antipsychotic drugs are thought of as the first actual effective medicine to treat mental illness. Before the 1950s, treatment for mental illnesses was merely suppressing or controlling a mentally ill person's taxing behavior. Antipsychotic drugs are more beneficial because they target the root disease that causes the behavior (Moncrieff, 2013).

The use and prescription of antipsychotic medications has become increasingly more popular over the years. During the 1990s, atypical antipsychotic drugs were introduced. Clozapine was considered the archetypal atypical antipsychotic because it was around in the 1970s and resurfaced in the 1990s (Moncrieff, 2013). The development of new atypical antipsychotics was modeled after clozapine. There are currently only six atypical antipsychotics commercially available in the United States. These include clozapine, risperidone, olanzapine, quetiapine, ziprasidone, and aripiprazole. Atypical antipsychotics are less likely than typical antipsychotics to cause extra pyramidal effects (Farah, 2005).

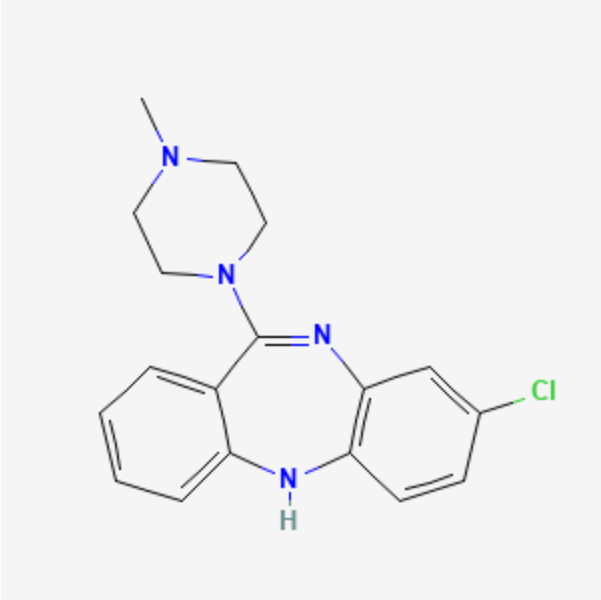

Figure 1.1. Chemical structure of Clozapine (National Center for Biotechnology Information, 2022).

Clozapine is a tricyclic benzodiazepine that is mainly used in the treatment of schizophrenia. Today, it is specifically used for severely ill patients with treatment-resistant schizophrenia (National Center for Biotechnology Information, 2022). This is a form of schizophrenia that cannot be treated with traditional neuroleptics (e.g., haloperidol and chlorpromazine). Clozapine is less likely to cause extrapyramidal side effects and it has a low effect on prolactin secretion, but it poses a high risk due to the high chance that patients on clozapine will experience agranulocytosis. This makes frequent monitoring of patients on clozapine necessary.

Patients being treated with any antipsychotic medication, but especially clozapine, needs to monitored to make sure they have the correct levels of the drug in their system to reach its highest therapeutic potential without harm. This is called therapeutic drug monitoring (TDM). Therapeutic concentrations of clozapine are determined by observing the dose to effect

relationship over a multiple week period. This period is often 6 to 8 weeks long. During these weeks, the dose given to the patient is increased gradually until the therapeutic range is reached (Freeman and Oyewumi, 1997). Therapeutic drug monitoring will observe and help maintain that the patient's clozapine levels are within therapeutic range.

Quantitative clozapine testing, used for therapeutic drug monitoring, is typically done with serum samples as of right now. The clinical treatment of clozapine heavily relies on the serum testing. It helps physicians decide whether the treatment is working and if they need to change the dosage they are prescribing to their patients. There are a few problems with relying on serum testing. Firstly, the procedure for testing the serum can vary from laboratory to laboratory which can cause doubt in its accuracy. Secondarily, collecting a blood sample from patients is an invasive process. Due to this, the TDM testing cannot always be done as regularly as it may need to be done.

Immunoassays play a very big and important role in therapeutic drug testing. The method used for TDM at any given laboratory is chosen for a number of reasons. Some of these reasons include staffing, equipment, time required to complete the tests, the cost and medical benefits to the patient. TDM is one of the fastest growing sections of clinical medicine (Boguslaski & Burd, 1983). This is because the technology is changing and new methods are appearing. Liquid chromatography tandem mass spectrometry (LC-MSMS) is one of the most widely used and accepted methods for TDM, but more recently immunoassays have been emerging and becoming more routine to use. Immunoassays are usually cheaper, quicker, and more easily performed compared to LC-MSMS. Immunoassays are quantitative and have shown to be sensitive, precise, and accurate, just like the traditional LC-MSMS methods.

The basic principle of an immunoassay is based on the interaction of an antigen with a corresponding antibody. The antigen is labelled with a measurable marker which allows the results of a sample to be compared with a calibration curve made from known standards. The drug being tested for and the labelled antigen compete for the binding sites on an antibody. Immunoassays are separated by whether separation of bound and free antigen is required before a signal can be measured. The two types of immunoassays are homogeneous and heterogeneous. Homogeneous immunoassays do not require the separation process like heterogeneous immunoassays do. Most commonly, for TDM, homogeneous immunoassays are used (Borg, 2020).

The fact that homogeneous assays do not require a separation process allows the immunoassays to be more easily automated. Automated chemistry analyzers have become popular for running TDM tests. This allows for fast, quantitative results that can be reported to the physicians and patients sooner. These automated systems are capable of analyzing thousands of samples per day (Borg, 2020). Chemistry analyzers use spectrophotometry to measure the absorbance of a sample and converts it to a reportable value. For this research, an automated Kinetic Interactions of Microparticles in Solution (KIMS) assay was used.

A KIMS assay is a homogeneous assay with the basic principle being that the amount of agglutination is inversely proportional to the amount of drug present in a sample. Agglutination is the clumping of particles. For a KIMS assay, microparticles have target drug conjugated onto them. The antibodies attach to these drug-coated microparticles and produce agglutinates. This leads to an increase in sample absorbance. When there is drug present in the sample, the drug competes for the attachment of antibodies and this prevents the formation of agglutinates. This leads to a decrease in sample absorbance (Borg, 2020).

The development of point-of-care (POC) tests is important for the treatment of schizophrenia with clozapine. POC tests are diagnostic tests that can be performed in a clinical setting where a patient is being treated. According to Kalaria and Kelly, the FDA restricted the distribution of clozapine even though it was one of the only treatments that showed effectiveness in patients with treatment-resistant schizophrenia. TDM needs to be done on any patient who was prescribed clozapine. One of the main reasons this was so difficult to maintain were that patients did not adhere to the frequent blood work required for TDM. Having a POC test would create a fast method of obtaining a smaller specimen from patients and it would also provide faster results (Kalaria & Kelly, 2019).

TDM testing could be performed more regularly and more easily if a different biological matrix could be tested, like oral fluid. Being able to quantify a person's clozapine levels with oral fluid would make the process much simpler and more accessible. It takes a lot of the hassle out of frequent testing for patients being treated with clozapine. It would be a less invasive collection procedure, and it would also speed up the overall process of the test. Patients would be more likely to adhere with TDM because the oral fluid collection process is much more convenient than the blood sample collection process is. One downfall to using oral fluid is the fact that it is not considered to be as stable of a biological sample as blood is. The Forensic Science International Journal published a study looking at the stability of different antipsychotics in multiple biological samples. This study showed that all of the antipsychotic analytes were stable in human plasma at least 1 year at 20°C, 2 weeks at 2-8°C, and for 3 days at room temperature. The analytes in oral fluid were not stable for as long a human plasma, but they still proved to be stable long enough for testing. The study showed that all analytes, including clozapine, were stable in oral fluid for 2 months at -20°C, 1 week at 2-8°C, and 2 days at room

temperature (Fisher et al., 2013). Though, it is stable for a shorter amount of time, it would still simplify the process of TDM testing for patients. The goal of this research was to validate a method to detect clozapine in oral fluid.

In order for a method to be validated, certain standards have to be met. There are many different organizations that give suggested guidelines that a method must meet. Some of these organizations include the US Food and Drug Administration (FDA) (FDA, 2019), Scientific Working Group for Forensic Toxicology (SWGTOX) (Scientific Working Group for Forensic Toxicology, 2013), and the College of American Pathologists (Sarewitz, 2013), and many more.

In a study published in the Journal of Pharmaceutical and Biomedical Analysis, an HPLC-MS method was developed and validated, just following the FDA guidelines for the quantification of seven antipsychotics, including clozapine. The method was validated with tests that looked at selectivity, carryover, matrix effects, variability of peak areas, process efficiency, extraction recoveries, stability, potential interference, and the plasma dilution effect. (Choong et al., 2009). These tests are part of the standards that needed to be met according to the FDA and after analyzing the results of these tests, this method was deemed suitable for TDM of these antipsychotic medications. But not every validation needs to follow guidelines given from one specific organization. Rosado et al. developed and validated a method for the quantification of many antipsychotics in human plasma and oral fluid. Their procedure used solid phase extraction and GC-MSMS analysis. In their work, this method was fully validated. They followed the validation guidelines recommended by the FDA, SWGTOX, and the International Conference on Harmonization (ICH). The validation protocol tested the method's selectivity, linearity, limits of detection and quantification, precision and accuracy, and recovery (Rosado et al., 2018). Every

laboratory has the opportunity to develop and then follow their own combination of the guidelines suggested by the many different organizations.

This research was done at Cordant Health Solutions Laboratory, where the guidelines were specifically established for this laboratory. The guidelines for Cordant Health Solutions are a hybrid combining ones from the Society of Forensic Toxicology, the FDA, and the College of American Pathologists. The guidelines for Cordant Health Solutions include the following tests: linearity, accuracy and precision, carryover potential, interfering substances, and the evaluation of authentic positive and negative samples (true positives test).

Linearity testing is the establishment of an analytical measurement range (AMR). An AMR is the range of values that the method can directly measure on the specimen without dilution, concentration, or any other pretreatment that is not part of the normal assay preparation. Accuracy testing is the verification that the method results agree with the actual concentration of a sample. Precision testing is ensuring that the method results are reproducible. Carryover potential testing looks for the appearance of unintended analyte signal in a sample following the analysis of a positive sample. Interfering substances tests look for any substances that may alter the clozapine test results. The evaluation of authentic positive and negative samples tests the same samples on an already validated method to confirm that the new method gives the same results. In the validation of the method using a Kinetic Interaction of Microparticles in Solution (KIMS) assay for the detection of clozapine, these tests were all completed and analyzed to see if the method is suitable for TDM.

2. Materials and Methods

2.1 Reagents and Supplies

The following materials were purchased through Cordant Health Solutions from Saladax Biomedical, Inc. (Bethlehem, PA): MyCare Psychiatry Clozapine Assay Kit, MyCare Psychiatry Calibrator Kit 2, and MyCare Psychiatry Control Kit 2. The MyCare Psychiatry Clozapine Assay Kit is designed for human serum, so the calibrator and control kits were just used as references in developing the calibrators and controls used in this method. The reagents from the MyCare Psychiatry Clozapine Assay Kit were used in this method.

The following materials were obtained directly from the supplies at Cordant Health Solutions Laboratories (Huntington, NY): synthetic oral fluid, dichloromethane, isopropyl alcohol, ammonium hydroxide, formic acid, and antipsychotic drug standards with clozapine concentrations at 10,000 ng/ml and 100,000 ng/ml.

2.2 Equipment and Instrumentation

All equipment used for this research was property of Cordant Health Laboratories. The instrumentation used was at Cordant Health Laboratories. Instrumentation used included an Olympus AU400 automated clinical chemistry analyzer and an Agilent 1290 liquid chromatograph paired with a 6460 triple quadrupole mass selective detector.

2.3 Preparation of Calibrators and Quality Controls

The preparation of calibrators and quality controls (QCs) were made using synthetic oral fluid and antipsychotic drug standards. The antipsychotic drug standards had concentrations of clozapine at 10,000 ng/ml and 100,000 ng/ml. The calibrators and OCs were prepared with a dilution factor of 4. This is because the oral fluid sample collection devices that are used for this method dilutes the samples by a factor of 4 with a buffer. This is based on the Quantisal oral

fluid collection device. A different volume of the clozapine standard was spiked into 10.0 ml synthetic oral fluid for each calibrator and each QC. The exact volumes for how each individual calibrator and QC was made is shown in table 2.1. For example, the level 1 calibrator was prepared by combining 12.5 µl of 10,000 ng/ml clozapine standard with 10.0 ml of synthetic oral fluid. This made the level 1 calibrator have a final clozapine concentration of 50.0 ng/ml.

Table 2.1. Volumes of clozapine standard needed for each calibrator and quality control in 10.0 ml synthetic oral fluid with a dilution factor of 4.

Calibrator/QC	Stock Concentration (ng/ml)	Spiking Volume (µl)	Working Concentration (ng/ml)	Working Volume (ml)
Level 1 Cal	10,000	12.5	50.0	10.0
Level 2 Cal	10,000	25	100.0	10.0
Level 3 Cal	100,000	6.25	250.0	10.0
Level 4 Cal	100,000	12.5	500.0	10.0
Level 5 Cal	100,000	25	1000.0	10.0
QC1	10,000	20	80.0	10.0
QC2	100,000	7.5	300.0	10.0
QC3	100,000	20	800.0	10.0

A blank sample was also prepared, which was just 10.0 ml of synthetic oral fluid without any antipsychotic drug standard spiked into it. These blank, calibrator, and QC samples were used during every validation test.

2.4 Linearity Test

The blank, the 5 calibrators, and the 3 QCs were used as samples for the linearity test. The 5 calibrators and the blank were run on the chemistry analyzer once per day over a course of 5 different days. The resulting data was used to determine the range.

2.5 Inter-day and Intra-day Precision Test

Inter-day precision tests used the blank sample, the 5 calibrators, and the 3 QCs as samples. The inter-day test took place over a course of 5 different days. All the samples were run on the Olympus AU400 automated clinical chemistry analyzer. Each day, at the start of the testing, a calibration run and a QC run was completed and the results were confirmed to make sure everything was working properly. The blank and the 3 QC samples were run as patient samples on an "unknown" rack. This was done 5 times each day. The OD value and concentration results were collected for each run on each day. For inter-day precision tests, the average concentration, standard deviation of the concentrations, and %CV of the data collected between the 5 days were calculated for each QC.

Intra-day precision tests followed the same procedure of running the blank and the 3 QCs as patient samples, but the data was looked at differently. For intra-day precision tests, the average concentration, the standard deviation of the concentrations, and the %CV of the 5 runs done on 1 day were calculated for each QC. This was done once for each of the 5 days.

2.6 Carryover Potential Test

For the carryover validation test, new samples had to be made that had higher concentrations than the highest calibrator. Calibrator 5 had the highest concentration at 1,000 ng/ml. A sample that was 10x the concentration of calibrator 5 was made, with a concentration of

10,000 ng/ml. The spiking volumes to make the sample are shown in table 2.2. On an unknown rack, 5 samples were run as patient samples on the chemistry analyzer. These samples were a blank, calibrator 5, a blank, the 10x calibrator 5 sample, and another blank. The concentrations and OD values that resulted were collected.

Due to the results seen with the 10x calibrator 5 sample, it was diluted to have a concentration of only 5x the concentration of calibrator 5. This dilution was done but taking 5.0 ml of the 10,000 ng/ml sample and adding 10.0 ml of blank synthetic oral fluid to it, leading to a sample with a concentration of 5,000 ng/ml. A blank, calibrator 5, a blank, the 5x calibrator 5 sample, and another blank were run again as patient samples. The concentrations and OD values from each sample were collected.

Table 2.2. Volume of clozapine standard needed for a 10,000 ng/ml sample made in 10.0 ml synthetic oral fluid with a dilution factor of 4.

Sample	Stock Concentration (ng/ml)	Spiking Volume (μl)	Working Concentration (ng/ml)	Working Volume (ml)
10x Calibrator 5	100,000	250	10000.000	10

2.7 Interfering Substances Test

For the interference validation test, an interference cocktail had to be made. The interference cocktail included 21 different drugs. Table 2.3 shows a list of the drugs and their corresponding concentrations in the interference cocktail. One 2.0 ml interference cocktail was made with all the drugs in it. This 2.0 ml interference cocktail was made by spiking different amounts of each drug into synthetic oral fluid. The spiking amount for each drug was based on

the stock concentration available for that drug. Table 2.4 shows the different spiking volumes needed for each drug to reach its target concentration in the interference cocktail This was separated into 2 1.0 ml samples. One of them was left as it was, with no clozapine in it. The other was spiked with clozapine to have a clozapine concentration of 1,000 ng/ml. These 2 samples were run on the chemistry analyzer and their clozapine concentrations were recorded.

Table 2.3. Interference cocktail sample list.

	Analyte	**Concentration (ng/ml)**
1	Amphetamine (d-methamphetamine)	1,000
2	Cannabinoids	1,000
3	Barbiturates	1,000
4	Carisoprodol	1,000
5	Cocaine (Benzoylecgonine)	1,000
6	Cotinine	1,000
7	Ethyl Glucuronide (ETG)	1,000
8	Fentanyl	1,000
9	Gabapentin	1,000
10	Methadone	1,000
11	Meperidine	1,000
12	Opiates(Morphine)	1,000
13	Oxycodone	1,000
14	Phencyclidine (PCP)	1,000
15	Propoxyphene (PPX)	1,000
16	Tricyclic antidepressants (TCA)-Nortriptyline))	1,000
17	Buprenorphine	1,000
18	Pentatonic Acid (Spice)	1,000
19	Ecstasy(MDMA)	1,000
20	6-AM(6-Acetylmorphine)	1,000
21	Benzodiazepine (Oxazepam)	1,000

Table 2.4. Volumes of the different drug standards needed to make the interference cocktail in 2.0 ml of synthetic oral fluid with a dilution factor of 4.

Drug	Stock Concentration (ng/ml)	Spiking Volume (µl)	Working Concentration (ng/ml)	Working Volume (ml)
Amphetamine (d-methamphetamine)	100,000	5	1,000	2
Cannabinoids	100,000	5	1,000	2
Barbiturates	100,000	5	1,000	2
Carisoprodol	10,000	50	1,000	2
Cocaine (Benzoylecgonine)	100,000	5	1,000	2
Cotinine	100,000	5	1,000	2
Ethyl Glucuronide (ETG)	10,000	50	1,000	2
Fentanyl	100,000	5	1,000	2
Gabapentin	100,000	5	1,000	2
Methadone	100,000	5	1,000	2
Meperidine	100,000	5	1,000	2
Opiates(Morphine)	100,000	5	1,000	2
Oxycodone	100,000	5	1,000	2
Phencyclidine (PCP)	100,000	5	1,000	2
Propoxyphene (PPX)	100,000	5	1,000	2
Tricyclic antidepressants (TCA)-Nortriptyline))	100,000	5	1,000	2
Buprenorphine	100,000	5	1,000	2
Pentatonic Acid (Spice)	10,000	50	1,000	2
Ecstasy(MDMA)	100,000	5	1,000	2
6-AM(6-Acetylmorphine)	100,000	5	1,000	2
Benzodiazepine (Oxazepam)	100,000	5	1,000	2

2.8 True Positives Test

For true positives testing, 15 unknown samples were received. These samples were ran on the chemistry analyzer and their concentrations were recorded.

These same 15 samples had to be run on LC-MSMS for comparison. For the LC-MSMS run, calibrators and QCs also had to be prepared.

2.8.1 LC-MSMS Calibrators and QCs

For this test, 7 calibrators needed to be prepared and 5 QCs needed to be prepared. These were prepared the same way as the ones used on the chemistry analyzer (section 2.3). The exact concentrations and volumes needed to make these are shown in table 2.5. Once prepared, these sample would have to undergo the same procedures as the 15 unknown patient samples.

Table 2.5. Volumes of clozapine standard needed for each calibrator and quality control in 5.0 ml synthetic oral fluid with a dilution factor of 4.

LC-MSMS Calibrator Spiking Table				
	Stock Concentration (ng/ml)	**Spiking Volume (µl)**	**Working Concentration (ng/ml)**	**Working Volume (ml)**
Level 1	100	31.25	2.5	5
Level 2	1,000	12.5	10.0	5
Level 3	1,000	62.5	50.0	5
Level 4	10,000	12.5	100.0	5
Level 5	10,000	31.25	250.0	5
Level 6	10,000	62.5	500.0	5
Level 7	100,000	12.5	1000.0	5

LC-MSMS QC Spiking Table				
NC	1,000	0	0.0	5
PC1	1,000	12.5	10.0	5
PC2	1,000	31.25	25.0	5
PC3	10,000	12.5	100.0	5
PC4	10,000	31.25	250.0	5

2.8.2 Sample Preparation

All of the samples, calibrators, and QCs that were going to be run on the LC-MSMS had to undergo an extraction first. This was done by solid-phase extraction (SPE). The SPE followed these steps: conditioning, sample loading, washing, drying, and elution. First a sample mixture was made by combining 200 µl of each of the samples with 100 µl internal standard and 1.0 ml sodium phosphate buffer (pH 6.0).

The type of columns used for the SPE were Trace-B SPE columns. The columns were conditioned with 500 µl methanol and 500 µl deionized water. The sample mixture was then added to the column. Following that, the columns were washed with 3.0 ml of deionized water, 3.0 ml 0.1 M acetic acid, and 3.0 ml 25% methanol. The columns were dried under pressure for 14 minutes. The samples were eluted from the columns with an elution solvent (see section 2.8.3) into 750 µl autosampler vials.

Following elution, the samples were placed on a concentrator and evaporated to dryness at 40°C. Once dry, they were reconstituted with 100 µl of mobile phase A1 (see section 2.8.2), capped, and vortexed.

2.8.3 Solutions and Solvents

The internal standard used in this procedure for clozapine was clozapine-d4. Both, this and the sodium phosphate buffer were obtained from Cordant Health Solutions Laboratories.

The 25% methanol was prepared with deionized water at a 1:4 methanol to water ratio.

The elution solvent was dichloromehane:isopropyl alcohol:ammonium hydroxide (70:26:4). This was prepared daily.

Mobile phase A1 was 0.1% formic acid. This was made by adding 1.0 ml reagent grade formic acid to a 1,000 ml volumetric flask that was filled to volume with LC-grade water. This was obtained already made from Cordant Health Solutions laboratories.

The deionized water, methanol, acetic acid, dichloromethane, isopropyl alcohol, ammonium hydroxide, and formic acid were all obtained at Cordant Health Solutions Laboratories.

2.8.4 LC-MSMS Parameters

2.8.4.1 LC Parameters

The injection volume is 10 µl. There are 2 mobile phases; A1 and B1. Mobile phase A1 is 0.1% formic acid. Mobile phase B1 is methanol. The column is an Agilent InfinityLab Poroshell 120 SB-C8 2.1 x 50 mm, 2.7 µm. The column temperature is 50°C.

2.8.4.2 Triple Quadrupole MS Parameters

The acquisition mode is ESI (+). The ESI parameters are shown in table 2.7. Multiple reaction monitoring (MRM) is used. The data for clozapine and clozapine d4, clozapine's internal standard, from MRM is shown in table 2.6.

Table 2.6. Multiple reaction monitoring data table.

Compound Name	Precursor Ion	Product Ion	Fragmentor	Collision Energy	Cell Accelerator Voltage	Dwell	Polarity
Cloazpine d4	331.2	272	140	20	3	400	Positive
Clozapine	327.2	296	100	20	3	400	Positive
Clozapine	327.2	270.1	100	20	3	400	Positive

Table 2.7. ESI source parameters.

Gas Temperature	350°C
Gas Flow	10 L/min
Nebulizer Pressure	50 psi
Capillary Voltage	3,500 V
Nozzle Voltage	500 V
Sheath Gas Temperature	300°C
Sheath Gas Flow	11 L/min

3. Results and Discussion

3.1 Method Validation

The converted KIMS assay method to detect clozapine was fully validated using the following tests: linearity, accuracy and precision, carryover potential, interfering substances, and an evaluation of true positive samples.

3.2 Linearity

During linearity testing, an analytical measurement range was established. The range was establish using a logarithmic calibration curve. The linearity range was between 50 ng/ml and 1,000 ng/ml. The limit of detection (LOD) was determined to be 50 ng/ml, the same

concentration as the lowest non-zero calibrator. The lowest non-zero calibrator (50 ng/ml) is also the lower limit of quantification (LLOQ). The upper limit of quantification (ULOQ) was determined to be equal to the highest calibrator at a concentration of 1,000 ng/ml.

3.3 Inter-day and Intra-day Precision

Inter-day precision testing compared the OD value results of the 3 QCs over the course of 5 days. Intra-day precision testing looks at the results of 5 runs of the QCs within each individual day. Using the concentration data collected during these tests, the relative standard deviation (RSD), or %CV, was calculated. The goal was for the %CV to be less than 10%, though it was acceptable for the %CV to be between 10%-15%. The %CV could not be greater than 15% for the method to be considered precise. The standard deviation and % error were also looked at to compare the data between the different runs. For the inter-day precision test, the %CV for the low QC was found to be 13.82%, the medium QC 9.95%, and for the high QC 8.34%, as shown in table 3.2. Inter-day precision testing confirms that the instrument is precise between days and not just when running the samples back to back in one day. As seen in table 3.1, all of the %CV values passed the requirements for intra-day precision testing also. This method is considered to be precise.

Table 3.1. Results of Intra-day precision testing.

		Day 1	Day 2	Day 3	Day 4	Day 5
Low QC	**Average**	87.92	78.30	71.64	71.33	79.4
	SD	10.17	11.09	5.84	10.45	10.58
	%CV	11.57	14.17	8.15	14.65	13.32
	%Error	8.99	3.71	10.67	34.68	8.94
Medium QC	**Average**	270.72	288.66	274.24	258.33	300.84
	SD	31.92	23.43	7.78	11.14	16.88
	%CV	11.79	8.12	2.84	4.31	5.61
	%Error	1.05	8.03	12.84	2.63	4.10
High QC	**Average**	737.26	744.6	780.44	804.62	751.84
	SD	48.97	56.22	71.94	69.44	20.44
	%CV	6.64	7.55	9.22	8.63	2.72
	%Error	1.86	9.37	6.52	3.78	9.37

Table 3.2. Results of Inter-day precision testing.

QC Sample	Average Conc.	SD	RSD (%CV)
Low QC	77.96	10.77	13.82
Medium QC	273.76	21.76	7.95
High QC	766.73	62.10	8.10

3.4 Carryover Potential

During the carryover testing, blank samples were run after samples with high clozapine concentrations to see if the blank samples then appeared to have a clozapine concentration even though they didn't contain any clozapine. An acceptable amount of carryover is 50% of the lowest calibrator or less. In this method the lowest calibrator has a concentration of 50.0 ng/ml. That means the highest acceptable carryover amount is 25.0 ng/ml. The results of the carryover

tests are seen in table 3.3, with the unacceptable results highlighted in red. With a sample that had a clozapine concentration of 10,000 ng/ml, a significant amount of carryover was seen. The blank following this sample resulted having a clozapine concentration of 123.0 ng/ml. A sample with a 5,000 ng/ml clozapine concentration was also tested for carryover. An unacceptable amount of carryover was also seen with this sample. The blank following this sample resulted a clozapine concentration of 49.5 ng/ml. The 3 highest calibrators (calibrator 3, 4, and 5) were tested for carryover potential. No carryover was seen with calibrator 3 or calibrator 4. There was a slight but quantifiable carryover seen with calibrator 5. The blank after calibrator 5 resulted with a clozapine concentration of 18.5 ng/ml. This shows that at a concentration above 1,000 ng/ml there is a chance for carryover and any sample that tested positive after this high of a sample would need to be retested.

Table 3.3. Results of carryover potential testing.

	Concentration	**OD Value**
Blank	0	0.1953
Calibrator 3	246.3	0.1034
Blank	0	0.1943
Calibrator 4	514.6	0.0632
Blank	0.2	0.1922
Calibrator 5	945	0.0352
Blank	18.5	0.1874
5x Calibrator 5	3881.1	0.0016
Blank	49.5	0.1701
10x Calibrator 5	3332.6	-0.0021
Blank	123.0	0.1373

3.5 Interfering Substances

Interference testing looks at commonly taken substances to make sure they don't affect the clozapine results given by this method. 21 different substances were tested for this method. These substances can be seen in table 2.3. One sample was made with all of these substances at concentrations of 1,000 ng/ml and no clozapine. Another was made with all of these substances at 1,000 ng/ml and with clozapine, also at a concentration of 1,000 ng/ml. The sample without clozapine was tested with this method and resulted with a concentration of 0 ng/ml. The sample with clozapine resulted with a concentration of 913 ng/ml. This shows that there is no interference of these 21 substances with this method.

3.6 True Positives Test

True positive testing confirms that the method is reporting positive samples as positive and negative samples as negative. 15 blind samples were tested for clozapine using both the new KIMS assay method on the AU400 chemistry analyzer and a traditional method on the LC-MSMS. On the AU400 10 samples were reported positive for clozapine and 5 samples reported negative for clozapine. The LC-MSMS reported the same 10 samples as positive and the same 5 samples as negative (table 3.4). This means the method gives no false negatives and no false positives.

Table 3.4. The results of the true positives test.

		LC/MSMS	
		+	-
AU400	+	10	
	-		5

Table 3.5. Comparison of the concentrations of the 15 true positive testing samples.

Sample	AU400 (ng/ml)	LC/MSMS (ng/ml)	Percent Difference
1	121.9	123.7	1.5
2	0	0	0
3	419.8	446.9	6.3
4	733.4	770.1	4.9
5	1.1	0	200
6	0	0	0
7	705.4	642.1	9.4
8	554.2	564.8	1.9
9	395.9	372.0	6.2
10	0	0	0
11	1561.1	986.0	45.2
12	16.3	0	200
13	1002	1142.4	13.1
14	793.7	823.6	3.7
15	350.8	338.6	3.5

Even though both methods resulted the same positive and negative results, the concentrations they resulted for each sample were compared and the percent difference was looked at. The goal is the have the percent difference be less than 10% but a percent difference less than 15% is acceptable. As seen in table 3.5, 3 samples have unacceptable percent differences, but they are not actually unacceptable. Sample 5 is a negative sample and the AU400 measured it as having a concentration of 1.1 ng/ml, this is because there is slight carryover from the sample before it with a concentration above 700 ng/ml. Sample 12 also was a

negative sample that was affected by carryover from the sample before it. Sample 11 had a concentration that is above this method's ULOQ.

4. Conclusion

A rapid, precise, and accurate method for the determination of clozapine in oral fluid using a KIMS assay was developed and validated. We were able to directly test oral fluid samples without any pretreatment or preparation with an efficient method that allowed for accurate and fast results. A lower limit of quantification of oral fluid samples for this method was determined to be 50 ng/ml for clozapine. The KIMS assay was converted using a MyCare Psychiatry Clozapine Assay Kit which was originally used to test for the drug concentration in serum samples. This method used with serum is considered to be very rapid and able to increase physicians patient care (Saladax, 2020). Converting the method to be able to use oral fluid is not only beneficial to the physicians treating patients but it is also beneficial to the users of the MyCare Psychiatry Clozapine Assay Kits. As previously mentioned, oral fluid collection is much more convenient than blood collection for patients. The users of the test will have less preparation work to do with the samples and the process remains automated and accurate.

The method validation tests done for this research showed that this KIMS assay method using a MyCare Psychiatry Clozapine Assay Kit could be sensitive and selective for clozapine in oral fluid. The MyCare Psychiatry Clozapine Assay Kit has previously been shown to be accurate and reliable in other studies. A study done validating the use of this kit with whole blood samples showed that this assay was specific for clozapine, had no interferences, and it showed agreement between the MyCare Psychiatry Clozapine Assay Kit results and LC-MSMS results (Sumanth et al., 2019). This study showed that the MyCare Psychiatry Clozapine Assay

Kit, originally used for serum sample, could be converted to be able to test whole blood samples and in this research, we showed that it could also be used to test oral fluid samples.

References

Baselt, R. C. (1987). *Analytical procedures for therapeutic drug monitoring and emergency toxicology*. PSG Publ. Co.

Boguslaski, R. C., & Burd, J. F. (1983). Analytical methods for therapeutic drug monitoring. *The American journal of medical technology*, *49*(8), 551–556.

Borg, D. (2020). Immunoassays [PowerPoint slides]. John Jay College of Criminal Justice.

Choong, E., Rudaz, S., Kottelat, A., Guillarme, D., Veuthey, J. L., & Eap, C. B. (2009). Therapeutic drug monitoring of seven psychotropic drugs and four metabolites in human plasma by HPLC-MS. *Journal of Pharmaceutical and Biomedical Analysis*, *50*(5), 1000–1008. https://doi.org/10.1016/j.jpba.2009.07.007

Farah A. (2005). Atypicality of atypical antipsychotics. *Primary care companion to the Journal of clinical psychiatry*, *7*(6), 268–274. https://doi.org/10.4088/pcc.v07n060

FDA. (2019). Criteria end guidance for the validation of chemical methods. *Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products*, 23. https://fda.report/media/121751/ValidationNucleicAcidSequenceBasedAnalysisFoodFeedCosmeticsVeterinary.pdf

Fisher, D. S., Partridge, S. J., Handley, S. A., Couchman, L., Morgan, P. E., & Flanagan, R. J. (2013). LC-MS/MS of some atypical antipsychotics in human plasma, serum, oral fluid and haemolysed whole blood. *Forensic Science International*, *229*(1–3), 145–150. https://doi.org/10.1016/j.forsciint.2013.02.010

Freeman, D. J., & Oyewumi, L. K. (1997). Will routine therapeutic drug monitoring have a place in clozapine therapy?. *Clinical pharmacokinetics*, *32*(2), 93–100. https://doi.org/10.2165/00003088-199732020-00001

Kalaria, S. N., & Kelly, D. L. (2019). Development of point-of-care testing devices to improve clozapine prescribing habits and patient outcomes. *Neuropsychiatric Disease and Treatment*, *15*, 2365–2370. https://doi.org/10.2147/NDT.S216803

Moncrieff. (2013). The Bitterest Pills The Troubling Story of Antipsychotic Drugs (1st ed. 2013.). Palgrave Macmillan UK. https://doi.org/10.1057/9781137277442

National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 135398737, Clozapine. Retrieved March 6, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Clozapine.

Rosado, T., Oppolzer, D., Cruz, B., Barroso, M., Varela, S., Oliveira, V., Leitão, C., & Gallardo, E. (2018). Development and validation of a gas chromatography/tandem mass spectrometry method for simultaneous quantitation of several antipsychotics in human plasma and oral fluid. *Rapid Communications in Mass Spectrometry*, *32*(23), 2081–2095. https://doi.org/10.1002/rcm.8087

Saladax. (2020, May 2). *Saladax biomedical launches clozapine test in the US after FDA Grants Market Authorization*. Saladax Biomedical. Retrieved June 1, 2022, from https://www.saladax.com/saladax-biomedical-launches-clozapine-test-in-the-us-after-fda-grants-market-authorization/

Sarewitz, S. J. (2013). 2013 CAP Accreditation Programs Audioconferences and Webinars: Accreditiation Requirements for Validation of Laboratory Tests. *College of American Pathologist*, 57.

Scientific Working Group for Forensic Toxicology. (2013). Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *Journal of Analytical Toxicology*, *37*(7), 452–474. https://doi.org/10.1093/jat/bkt054

Sumanth, A., Kozo, D., Courtney, J., Siegfried, N., Gill, R., Hilaire, M. R., Venkateswaran, A., Harney, R., Jarrah, J., Distefano, K., Salamone, S., & Baburina, I. (2019). Novel point of care testing device for rapid measurement of clozapine in whole blood. *Clinica Chimica Acta*, *493*(12), S658–S659. https://doi.org/10.1016/j.cca.2019.03.1388