Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 34 No. 2, 78-86, 2021
https://doi.org/10.5806/AST.2021.34.2.78

Determination of cut-off value by receiver operating characteristic curve of norquetiapine and 9-hydroxyrisperidone concentrations in urine measured by LC-MS/MS

Seon Yeong Kim, Dong Won Shin, and Jin Young Kim★

Forensic Genetics and Chemistry Division, Supreme Prosecutors' Office, Seoul 06590, Korea

(Received November 9, 2020; Revised January 13, 2021; Accepted January 28, 2021)

Abstract: The objective of this study was to investigate urinary cut-off concentrations of quetiapine and risperidone for distinction between normal and abnormal/non-takers who were being placed on probation. Liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was employed for determination of antipsychotic drugs in urine from mentally disordered probationers. The optimal cut-off values of antipsychotic drugs were calculated using receiver operating characteristic (ROC) curve analysis. The sensitivity and specificity of the method for the detection of antipsychotic drugs in urine were subsequently evaluated. The area under the ROC curve (AUC) was 0.927 for norquetiapine and 0.791 for 9-hydroxyrisperidone, respectively. These antipsychotic drugs are classified readily in the ROC curve analysis. The cut-off values for distinguishing regular and irregular/non-takers were 39.1 ng/mL for norquetiapine and 67.9 ng/mL for 9-hydroxyrisperidone, respectively. The results of this study suggest the cut-off values of quetiapine and risperidone were highly useful to distinguish regular takers from irregular/non-takers.

Key words: cut-off value, antipsychotic drugs, ROC curve, LC-MS/MS

1. Introduction

Violent crimes caused by mental disorder in Korea, such as Gangnam Station murder in 2016, Gangbuk Samsung Hospital murder of a psychiatrist in 2018, arson and murder at Jinju apartment in 2019, have continuously increased and have emerged as a social problem. According to the 2019 Analytical Statistics on Crime published by the Supreme Prosecutors' Office of the Republic of Korea, criminals with mental disorders are defined as criminals with insanity (those who have schizophrenia), mental retardation (those who are indecisive or unstable), and other mental disorders (those who have bipolar or personality disorder) at the time of the crime. In the past five years (2014 – 2018), the number of criminals with mental disorders in Korea was 6,301 in 2014, 7,016 in 2015, 8,343 in 2016, 9,089 in 2017, and 7,304 in 2018; the 2018 data present a lower value than that from the previous year.[1] Except in 2018, crimes with mental disorders have steadily increased, and the proportion of violent crimes such

★ Corresponding author
Phone : +82-(0)2-535-4173 Fax : +82-(0)2-535-4175
E-mail : paxus@spo.go.kr

as murder, robbery, arson, and sexual violence reached about 10 % of all crimes. The recidivism rate of the criminals with mental disorders is 65.3 %, which is 20% higher than 42.3% of the general population as of 2018. Therefore, if left unattended, the possibility of the criminals with mental disorders becoming repeat offenders is high.

The 'treatment supervision and treatment order system' was enforced based on the "Treatment Supervision Act" to prevent recidivism of the criminals with mental disorders and for them to receive systematic treatment under the management of government agencies.[2] The treatment order system is a system in which probation and parole officers systematically manage drug administration to treat the psychiatric disorders of the criminals with mental disorders within the national treatment and management network in order to help their rehabilitation and to prevent recidivism.[3] The effect of therapeutic treatment of mental disorders has been scientifically proven through extensive research. In particular, consistent drug treatment significantly reduces symptoms and recurrence rates of mental disorders, thereby enabling a patient to live a normal life.[4] According to a previous study, more than 50 % of patients with insanity relapsed within one year if they stopped the drug treatment. Another study found that among 53 patients with insanity who were stable after treatment with antipsychotic drugs for at least one year, 78 % of them relapsed within 1 year and 98 % of them relapsed within 2 years when the drug treatment was stopped.[5,6] Therefore, a process for confirming whether criminals with mental disorders are consistently taking prescribed drugs can be an important strategy that helps the rehabilitation of such criminals and strengthens the social safety net through their medication management.

To confirm whether a criminal who received a treatment order is taking the prescribed drugs, treatment order fulfillment is verified by conducting a periodic drug test. This test has become an effective tool to preemptively prevent the act of arbitrarily stopping the drug treatment because it involves periodically analyzing the presence of antipsychotic drugs in urine samples of the criminals with the treatment order.

The drug test confirms compliance with the drug treatment to treat mental disorders through the detection and quantification of parent antipsychotic drugs and their metabolites from the urine samples of the subjects. Analytical methods such as liquid chromatography (LC), gas chromatography–mass spectrometry (GC-MS), capillary electrophoresis, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) have been utilized for the test.[7-12] In this study, LC-MS/MS was applied because it is useful for analyzing metabolites with relatively high polarity in biological samples and enables to reduce sample pretreatment time as this method does not require a derivatization process.

The verification of "normal" compliance with the treatment based on antipsychotic drugs is one of the important tools to prevent recidivism of criminals with mental disorders and to allow their rehabilitation through treatment. However, it is difficult to clearly distinguish between normal compliance and non-compliance, that is, not taking or intermittently taking the prescribed drugs, based on the qualitative and quantitative analysis results obtained through the drug test. Therefore, an approach that sets the cut-off value of drug compounds was sought to establish the criteria for objective classification between normal and abnormal compliances.[13,14] To verify the accuracy of such classification, the receiver operating characteristic (ROC) curve method has been commonly utilized. Two indicators, sensitivity and specificity, are used to predict the cut-off value and determine accuracy of the test that produces results that are divided into two groups corresponding to compliance and non-compliance with the drug treatment. The ROC curve is advantageous for this approach because it allows the reduction of evaluation errors as the curve is plotted on the basis of information such as accuracy, sensitivity, specificity of the set values.[15] In addition, the ROC curve method involves a simple procedure and allows setting of criteria in a facile manner, facilitating its acceptable utilization in the field of drug testing.

The purpose of this study is to determine the

optimal cut-off value of drug metabolites to determine normal compliance with the treatment involving the target drug. The metabolite concentration of the parent drug is considered as a variable to set the criteria using the ROC curve method. This method is utilized for determining compliance with antipsychotic-drug-based treatment for the subjects who received the treatment order.

2. Experiment

2.1. Chemicals and reagents

Quetiapine, norquetiapine, risperidone, and 9-hydroxyrisperidone, which are standard substances used for standard solution preparation, were purchased from Cerilliant (Austin, TX, USA). Quetiapine-d_8 and risperidone-d_4, which are standard substances used for internal standard solution preparation, were purchased from Cerilliant. The solvents used for LC-MS/MS, water, methanol, 0.1 % formic acid in water and 0. 1% formic acid in methanol, were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and all these solvents were LC-MS grade. The stored standard and internal standard solutions were prepared by diluting with methanol at concentrations of 200 μg/mL and 20 μg/mL, respectively, and used as sequential dilutions as needed. All standard and internal standard solutions were stored at -20 °C until use.

2.2. Urine samples

Blank urine samples were acquired through consent from laboratory researchers who had never consumed antipsychotic drugs. Moreover, urine samples were acquired from criminals with mental disorders whose main disease is insanity and those who are subject to treatment supervision termination or those who are subject to treatment order. The urine samples from 631 people were analyzed, which was requested for evaluation by probation offices in metropolitan areas such as Seoul and Suwon between November 2019 and September 2020. The age distribution of the requested samples was 18 – 80 years, and gender distribution was 480 males (76.1 %) and 151 females (23.9 %). Prior to the analysis, the samples that were not suitable for the analysis (1.001 < specific gravity < 1.003, 2.0 mg/dL ≤ creatinine concentration < 20.0 mg/dL) were excluded by measuring the specific gravity and creatinine concentration to verify dilution and replacement of the samples. All urine samples were stored at 4 °C until analysis.

2.3. LC-MS/MS conditions

LC analysis was performed using an Agilent 1290 Infinity LC System (Santa Clara, CA, USA) consisting of a vacuum degasser, a binary pump, an automatic sample injector, and a column oven. Waters Xselect HSS T3 (2.1 mm × 150 mm, ø 2.5 μm) was used as the column. The mobile phase was consisted of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in methanol (solvent B). The flow rate of the mobile phase was set to 200 μL/min and gradient elution was performed for 19 min. At this time, the ratio of mobile phase B started at 30 %, increased to 50 % until 2 min, increased to 90 % by 10 min, and then maintained for 5 min. After that, the mobile phase was changed to the initial condition and maintained for 4 min to stabilize it.

Mass spectrometry was conducted on a SCIEX 4000 triple-quadrupole mass spectrometer (AB Sciex, Foster city, CA, USA) equipped with an electrospray ionization device. The positive electrospray ionization mode was used, and the amounts of gas supplied were nebulizing gas 55, curtain gas 42, turbo ion spray heater gas 50, and collision gas 10 (arbitrary unit). The turbo-gas temperature was set to 550 °C, and the ionization voltage was set to 5 kV. The multiple reaction monitoring (MRM) mode was utilized for the quantitative and qualitative analyses, and nitrogen was used as a collision gas for the fragmentation of precursor ions. One quantitative ion pair and two qualitative ion pairs were selected as MRM ion pairs for each analyte, and one quantitative ion pair was selected as an MRM ion pair for each internal standard. The MRM ion pairs were set as follows: For the analyte, quetiapine: *m/z* 384→253/*m/z* 384→221/*m/z* 384→279; norquetiapine: *m/z* 296→210/ *m/z* 296→253/ *m/z* 296→183; risperidone: *m/z* 411→194/

m/z 411→159/ *m/z* 411→166; and 9-hydroxyrisperidone: *m/z* 427→207/ *m/z* 427→110/ *m/z* 427→69. For the internal standard substance, quetiapine-d_8: *m/z* 392→258 and risperidone-d_4: *m/z* 415→195. Other parameters such as the ionization method, selected ion pair, and mass spectrometer variable conditions used in this study were based on the results obtained by Kim *et al*.[16]

Herein, the ratio of the peak height of the quantitative ion pair and the qualitative ion pair was calculated using an MRM chromatogram. When the analyte is determined LC-MS/MS, the deviation of the height ratio of the qualitative ion pair to the quantitative ion pair generally accepted by the forensic science laboratory is set to within ± 30 %.[17]

2.4. Sample pretreatment

A Waters Oasis PRiME HLB cartridge (3 mL, 60 mg) was loaded with 1 mL of urine sample that was centrifuged at 4,500 g for 3 min. After washing by flowing 1 mL of 20 % methanol, the column was dried for 10 min under vacuum. The analytes were extracted by elution with 1 mL of methanol under 20 kPa pressure. Then, the dried extracts were reconstituted in 1 mL of the mobile phase and a 5 μL aliquot was injected into the LC-MS/MS system.

2.5. Method validation

Selectivity and limit of quantitation were determined by analyzing 10 different blank samples. A calibration curve of the quantitative range for the urine samples was plotted for the quetiapine, risperidone, and 9-hydroxyrisperidone concentrations of 0.1 – 50 ng/mL and the norquetiapine concentration of 0.5 – 50 ng/mL. Retention times and peak areas were confirmed from the MRM chromatograms of the samples pretreated according to the abovementioned method, and a calibration curve was plotted with the ratio of the peak area of the analyte to that of the internal standard. The applied weighting factor (w_i) was $1/x^2$. After adding the standard solution of the analyte to the blank sample, quality control (QC) samples (n = 6) corresponding to the lower limit of quantification, low, medium, and high concentrations within the quantitative range were treated according to the abovementioned pretreatment method and analyzed. Precision and accuracy were calculated from the measured concentration values. Measurements of precision and accuracy were repeated for 3 days. To measure the concentration beyond the upper limit of the quantitative range, dilution integrity in the range of 5 to 50 folds was measured. Using the QC samples corresponding to low and high concentrations, the stability of the analytes was evaluated using an automatic sample injector both for 8 h at room temperature and for 7-14 days at 4 °C. The effectiveness of the analysis was confirmed by measuring the recovery rate and matrix effect using the QC samples corresponding to low, medium, and high concentrations.[18]

2.6. Statistical analysis

The ROC curve analysis was applied to determine the optimal cut-off value for the metabolite concentration of the parent drug to divide the groups into normal compliance group and non-compliance group, that is, the group that did not take it or that took it irregularly. The cut-off value represents a fraction that accurately predicted compliance for what was genuinely taken (true positive rate, sensitivity) and a fraction that mispredicted what was taken (false positive rate, 1-specificity). The cut-off value was selected at the high levels of both sensitivity and specificity on the ROC curve. To evaluate the performance of the classification model in the ROC curve, the area under the ROC curve (AUC) was calculated. Statistical analysis of the measured values in this study was performed using the R-3.6.3 program (www.r-project.org).

3. Results and Discussion

3.1. LC-MS/MS analysis

When the analytes were identified through LC-MS/MS, the permissible range of the peak height ratio on the MRM chromatogram was set within ± 30 % of the average value of the ratio of the quantitative ion peak height to the qualitative ion

Table 1. MRM transitions for the analytes, MRM ratios and acceptable ratio ranges

Compound	MRM 1	MRM 2	Ratio (mean)	Acceptable ratio range (± 30 %)
Quetiapine	*m/z* 384→253	*m/z* 384→221	2.5	1.7-3.2
		m/z 384→279	6.3	4.4-8.2
Norquetiapine	*m/z* 296→210	*m/z* 296→253	1.3	0.9-1.7
		m/z 296→183	2.5	1.8-3.3
Risperidone	*m/z* 413→194	*m/z* 413→159	13.4	9.3-17.4
		m/z 413→166	15.1	10.6-19.7
9-Hydroxyrisperidone	*m/z* 427→207	*m/z* 427→110	4.3	3.0-5.5
		m/z 427→69	7.8	5.5-10.1

pair peak height obtained by analyzing low- and high-concentration QC samples with the analyte added to the blank sample (n = 74) and authentic urine samples. *Table* 1 shows the ratio of peak heights of two qualitative ion pairs to one quantitative ion pair for quetiapine, norquetiapine, risperidone, and 9-hydroxyrisperidone.

3.2. Method validation

Selectivity, detection limit, calibration curve, precision and accuracy, dilution integrity and stability were evaluated to confirm the effectiveness of the LC-MS/MS method of the study. Based on the analysis result of urine samples (n = 10) from those who did not take drugs to confirm the selectivity, no interfering substances influencing the analysis were found. The coefficient of determination (r^2) that represents the linearity of the calibration curve for the analytes in the set quantitative range was ≥ 0.997.

The detection and quantification limits for quetiapine, norquetiapine, risperidone, and 9-hydroxyrisperidone were 0.03 – 0.15 ng/mL and 0.1 – 0.5 ng/mL, respectively. The intra-day and inter-day accuracies were -9.6 – 9.0 %; the precision was within 9.1 % of the detection limit for the QC samples corresponding to lower limit of quantification, low, medium, and high concentrations. When the QC samples were diluted 5-50 folds to confirm the dilution integrity, the accuracy was between -10.6 and 10.5 % and the precision was within 8.9 %. The accuracy was obtained between ±15 % and precision was within 15 %, suggesting that the results were satisfactory. The stability of the analytes in the samples determined using three conditions (at room temperature for 8 h, in the 4 °C refrigerator for 14 days, and in the automatic sample injector for 3 days) were confirmed because the deviations were within 11.3 %. Upon measuring the recovery rate and the matrix effect for the analytes, the values were found to have met the evaluation criteria.

3.3. Forensic applications

The urine samples (n = 631) requested for analysis were analyzed according to the pretreatment and instrumental analysis methods that were previously set. The main antipsychotic drugs detected in the urine samples were in order of quetiapine (n = 311), risperidone (n = 229), aripiprazole (n = 134), and olanzapine (n = 100). It was found that quetiapine and risperidone, which accounted for > 58 % of the total drugs, were the most frequently prescribed drugs for probation subjects who received the treatment order (*Fig.* 1).

3.4. Comparison of drug concentration distribution by gender

Statistical analysis was performed to examine whether there was a difference in the distribution of drug concentrations according to gender. As a statistical method to verify the difference in mean between two groups, the independent sample *t*-test (independent *t*-test) is generally used when the measured values are equal variances and have a normal distribution, whereas the Wilcoxon rank sum test, which is a nonparametric

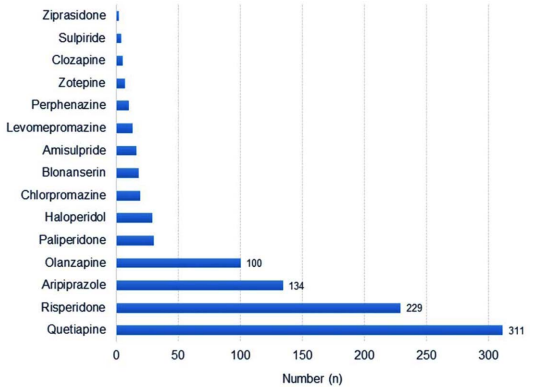

Fig. 1. Frequency distribution of antipsychotic drugs on mentally disordered probationers.

method, is applied when it is difficult to assume a normal distribution. Currently, the test methods for verifying normality and homoscedasticity are the Shapiro-Wilk test and Fisher's F test.[19] In this study, the Shapiro-Wilk test was conducted. whose results revealed that normality cannot be assumed. Therefore, the Wilcoxon rank sum test was performed on the measured values for analyzing gender-based distribution of two antipsychotic drugs.[20] As a result of the analysis of the samples that can be classified according to gender among the requested samples, the p-values of quetiapine (53 males/12 females) and risperidone (76 males/18 females) were 0.3144 and 0.6343, respectively. Therefore, there was no difference in the mean between the two groups according to gender (*Fig*. 2).

3.5. ROC analysis and optimal cut-off value

For classifying the compliance with the drug treatment by using the ROC curve method, sensitivity and specificity are analyzed to facilitate the division of classification into two groups on the basis of the characteristics of the ROC curve. To this end, the ROC curve method was conducted by dividing treatment compliance for each drug into normal and abnormal compliance (*Fig.* 3). *Table* 2 shows the

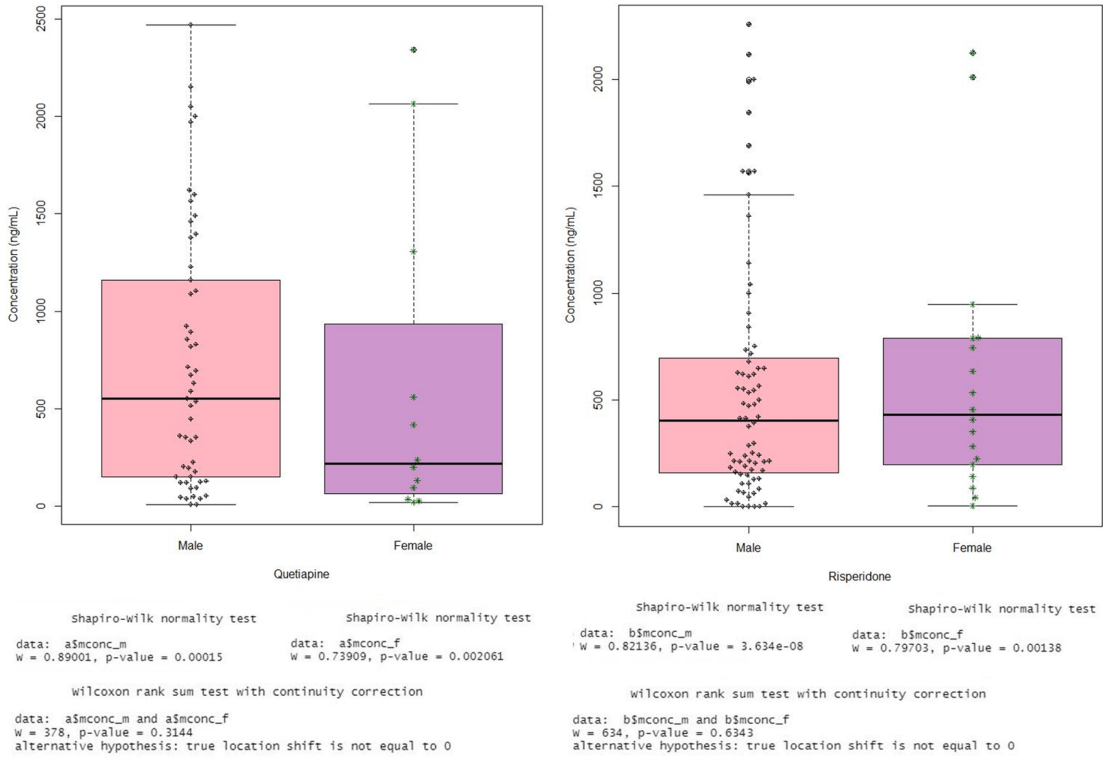

Fig. 2. Boxplot and statistical analysis of the quantitative results for forensic urine samples.

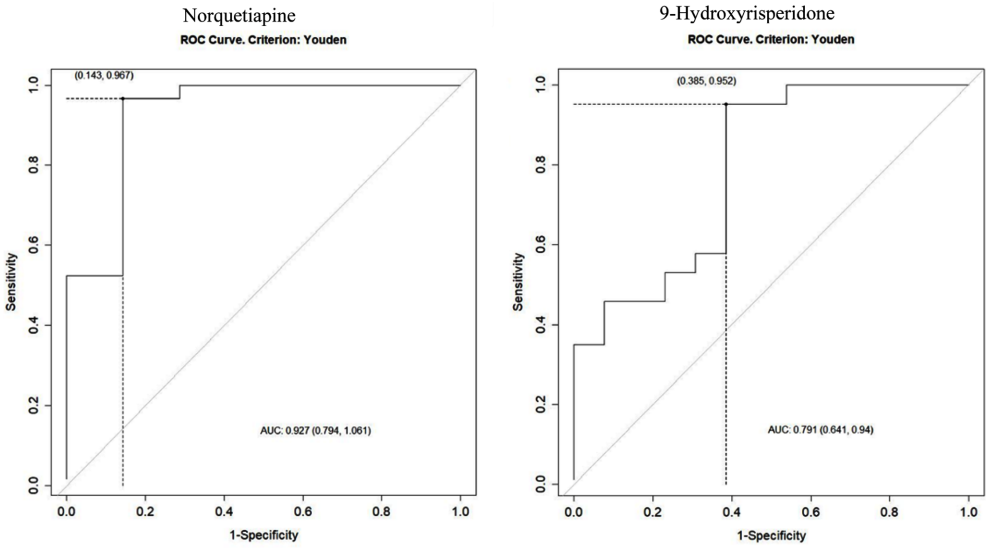

Fig. 3. ROC curve analysis identifying optimal cut-off values of target compounds.

Table 2. ROC curve analysis for determination of urinary cut-off values

Antipsychotic drug	Optimal cut-off value (ng/mL)	Sensitivity	Specificity	AUC	
				Area	95 % CI (confidence interval)
Norquetiapine	39.1	0.967	0.857	0.927	0.794-1.061
9-Hydroxyrisperidone	67.9	0.952	0.615	0.791	0.641-0.940

measured optimal criterion values and AUC values for the performance evaluation of the classification model. Data that met the criteria for determining the compliance and for confirming the analyte were used for the analysis. 9-Hydroxyrisperidone was drawn closer to a straight line than norquetiapine on the ROC curve from the results for norquetiapine (n = 68) and 9-hydroxyrisperidone (n = 97). When the AUC value determined from the ROC curve is closer to 1, the accuracy is high. However, when this value is around 0.5, it is difficult to assess the accuracy of the classification model. The AUC values from the graph were 0.927 for norquetiapine and 0.791 for 9-hydroxyrisperidone, which were relatively higher than 0.5. Therefore, there is a high possibility of classifying compliance or non-compliance with the prescribed drug treatment. In this study, the optimal criterion value calculated from the ROC curve was 39.1 ng/mL for norquetiapine and 67.9 ng/mL for 9-hydroxyrisperidone.

As shown in *Fig.* 1, because the number of data available for analysis was limited for aripiprazole, olanzapine, and other drugs, whose prescription frequency gradually decreases compared to that of quetiapine and risperidone, the cut-off value was not set. Therefore, it is necessary to expand the range of drugs by adding more data in the future.

4. Conclusions

In this study, LC-MS/MS was conducted to establish the optimal cut-off value for antipsychotic drugs quetiapine and risperidone present in urine samples. Moreover, the existence of a difference in distribution between the two groups according to gender for each drug was verified. By comparing the AUC values of two antipsychotic drugs obtained through ROC curve analysis, norquetiapine showed a higher AUC value than 9-hydroxyrisperidone. It was found that, in the case of risperidone rather than quetiapine, it is relatively difficult to distinguish between compliance and non-compliance with the drug treatment. Therefore, further research for accuracy improvement should be conducted by adding variables such as the dose, frequency, and properties of the target drug.

Acknowledgements

This study was supported in part by a grant from the National R&D program of Supreme Prosecutors' Office (SPO) and Ministry of Science and ICT (MSIT) of Korea. We thank Prof. Ki-Jung Paeng at Yonsei University for providing guidance in our work.

Supplemental Material

The R script for determining cut-off value by ROC curve of norquetiapine and 9-hydroxyrisperidone concentrations in urine is available at https://github.com/paxus11.

Conflicts of Interest

The authors declare no competing financial interest.

References

1. Supreme Prosecutors' Office, '2019 Analytical Statistics on Crime', Seoul, Korea, 2019.
2. S. I. Jang, *Regal Theory & Practice Rev.*, **4**, 103-128 (2016).
3. B. S. Kim, *Law Rev.*, **60**. 195-217 (2019).
4. J. H. Cho, S. H. Kang, S. J. Lee, J. G. Kim and M. J. Joo, *J. Korean Soc. Biol. Ther. Psychiatry*, **10**, 172-183 (2004).
5. H. A. Pincus, *Schizophr. Bull.*, **36**, 109-111 (2010).
6. H. Ascher-Svanum, D. E. Faries, B. Zhu, F. R. Ernst, M. S. Swartz and J. W. Swanson, *J. Clin. Psychiatry*, **67**, 453-460 (2006).
7. R. Mandrioli, L. Mercolini, D. Lateana, G. Boncompagni and M. Augusta Raggi, *J. Chromatogr. B*, **879**, 167-173 (2011).
8. M. Grapp, H. H. Maurer and H. Desel, *Drug Test. Anal.*, **8**, 816-825 (2016).
9. C. Danel, C. Barthélémy, D. Azarzar, H. Robert, J. P. Bonte, P. Odou and C. Vaccher, *J. Chromatogr. A*, **1163**, 228-236 (2007).
10. S. Feng, J. R. Enders, O. T. Cummings, E. C. Strickland, T. McIntire and G. McIntire, *J. Anal. Toxicol.*, **44**, 331-338 (2020).
11. G. J. Dear, I. J. Fraser, D. K. Patel, J. Long and S. Pleasance, *J. Chromatogr. A*, **794**, 27-36 (1998).
12. M. De Meulder, B. M. Remmerie, R. de Vries, L. L. Sips, S. Boom, E. W. Hooijschuur, N. C. van de Merbel and P. M. Timmerman, *J. Chromatogr. B*, **870**, 8-16 (2008).
13. Y. H. Lee, S. S. Kweon, J. S. Choi, J. A. Rhee, H. S. Nam, S. K. Jeong, K. S. Park, H. Y. Kim, S. Y. Ryu, S. W. Choi, B. H. Kim and M. H. Shin, *Kidney Blood Press. Res.*, **36**, 290-300 (2012).
14. K. Polanska, A. Krol, P. Kaluzny, D. Ligocka, K. Mikolajewska, S. Shaheen, R. Walton and W. Hanke, *Int. J. Environ. Res. Public Health*, **13**, 1216 (2016).
15. S. I. Pak and T. H. Oh, *J. Vet. Clin.*, **33**, 97-101 (2016).
16. S. Y. Kim, H. S. Kim, J. C. Cheong and J. Y. Kim, *J. Anal. Toxicol.*, **44**, 784-796 (2020).
17. S. P. Elliott, D. W. S. Stephen and S. Paterson, *Sci. Justice*, **58**, 335-345 (2018).
18. U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation

and Research (CDER) Center for Veterinary Medicine (CVM), 'Bioanalytical Method Validation-Guidance for Industry', 2018.
19. J. Rochon, M. Gondan and M. Kieser, *BMC Med. Res. Methodol*., **12**, 81 (2012).
20. R. R. Wilcox, *Biometrical J*., **32**, 771-780 (1990).

Authors' Positions

Seon Yeong Kim : Forensic Chemist
Dong Won Shin : Researcher
Jin Young Kim : Forensic Chemist