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Research Article

DEVELOPMENT AND VALIDATION OF METHAMPHETAMINE ANALYSIS IN SALIVA USING GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

YAHDIANA HARAHAP[1]*, MUHAMMAD REZQI HAKIM[1], KUSWARDANI SOEDIGDO[2]

[1]Bioavailability/Bioequivalence Laboratory, Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, Indonesia. [2]BNN's Drugs Testing Laboratory, Jakarta, 12940, Indonesia. Email: yahdiana03@yahoo.com

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ABSTRACT

Objective: The aim of this study was to develop and validate analytical methods for determining methamphetamine in saliva using gas chromatography-tandem mass spectrometry (MS).

Methods: The chromatography conditions were DB MS-5 capillary columns with a length of 30 m, inner diameter of 0.25 mm, mobile phase of Helium gas 99.999%, flow rate of 0.8 mL/min, detection of MS at m/z values of 58.00 and 91.00, respectively, and ephedrine HCl as the internal standard.

Results: The validation of analytical methods for methamphetamine satisfies the validation criteria by the EMEA Guidelines 2011. Bioanalytical methods obtained were linear in the concentration range from 15.0 to 300.0 ng/mL with r>0.9999. Sample preparation was done using liquid–liquid microextraction with cyclohexane, supernatant residue was dried and reconstituted with approximately 100 µL of methanol.

Conclusion: The method was successfully applied to saliva samples of methamphetamine users with levels in the range of test.

Keywords: Methamphetamine, Optimum, Validation, Human saliva, Ephedrine HCl.

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INTRODUCTION

Methamphetamine, known as *shabu* in Indonesia, is a very strong central nervous system stimulant from the amphetamine group [1,2]. To determine the evidence of methamphetamine abuse, a test to detect methamphetamine in the body is required. Methamphetamine levels in the body are usually detected using the blood and urine [1-4]. However, saliva is simpler to obtain and more efficient sample for methamphetamine tests in the body [4].

Narcotics are substances or drugs, derived from herbs or synthetic, that can cause altered levels of consciousness, loss of sensation, reduction in pain, and addiction [1]. Narcotics are also needed to treat certain diseases but, if misused, can be disadvantageous to the person and, moreover, to the community. In the year 2013, the amount of confiscated *shabu* rapidly increased compared with that in the year 2012 [5]. This shows that methamphetamine is a drug which is used a lot in society.

In Indonesia, drug abuse is a criminal act and needs to be judged by the law; thus, its intake needs to be proven. Until now, urine, blood, hair, and other body part tests have been performed to determine the presence of the drug in the body. The oral liquid or saliva test is a fast and non-invasive alternative to these tests. The main analyte is suspected to not undergo metabolism in the saliva. In addition, while collecting blood samples requires professional skills, collecting saliva does not [6].

Unlike urine samples, saliva can be collected under supervision without disturbing privacy. Most drugs are bound strongly to blood protein, based on the consideration that only free fractions of a drug are pharmacologically active. Saliva contains only a small part of the free fraction of drug. This can be filtrated from the salivary tissue, including the capillary wall, basal membrane, and epithelial cells of salivary glands [6].

Methods to analyze methamphetamine in saliva samples using gas chromatography-tandem mass spectrometry (GC-MS) are still limited. According to other studies, the maximum concentration of methamphetamine in saliva can reach 300 ng/mL 4 h after drug usage, whereas the maximum concentration in blood plasma can reach 35 ng/mL 4 h after usage [7]. Meanwhile, maximum concentration of methamphetamine in urine can reach 4500 ng/mL 16 h after usage [8]. Therefore, an analysis method that is sensitive and selective is needed if saliva is to be used as a biological test sample.

According to a previous research, methamphetamine can be extracted from saliva using a liquid–liquid extraction method. Along with the development of an analysis instrument, the modern analytical chemistry trend has shifted to simplification and minimalization of sample preparation. Currently, many innovative microextraction techniques have been developed which can simplify the sample preparation procedure and increase quality and sensitivity of the analysis. In the previous studies, liquid–liquid extraction was combined with GC, capillary electrophoresis, and high-performance liquid chromatography [9-11]. This technique was suitable for forensic examination of drug abuse using minimal sample volume and short analysis time.

In this study, the aim of this was to determine the concentration of methamphetamine in saliva samples using GC-MS with microextraction methods, using cyclohexane as a solvent extractor and ephedrine HCl as an internal standard. This method can hopefully be applied in the forensic laboratory to test for methamphetamine abuse.

MATERIALS AND METHODS

Equipment

GC-MS (Shimadzu GCMS-QP2010 Ultra), equipped with DB-5 MS Capillary Column (0.25 mm x 30 m; 0.25µm), data processing software (Windows Software) and a computer (HP).Other equipments were microcentrifuge (Spectrafuge 16M), analytical balance (Sartorius), ultrasonic mixer (Mmert, vortex, and micropipette (Eppendorf)

Materials

Materials used in the research were as follows: Methamphetamine standard (Cerrilant); internal standard ephedrine HCl (NADFC); methanol, cyclohexane, chloroform, and NaOH were purchased from

Merck; Aquabidest (Ikapharmindo); control saliva sample, and saliva sample from a drug user (Indonesian National Narcotics Agency).

Standard solution, internal standard, and quality control (QC) solution

Methamphetamine standard (0.1 mL) was pipetted into a 10.0 mL volumetric flask. The substance was dissolved in 2 mL of methanol; then, methanol was added to the limit of the flask. The concentration of standard solution was 0.01 mg/mL. Ephedrine was used as internal standard and was weighed 5.0 mg and then added to a 5.0 mL volumetric flask. The substance was dissolved in methanol (2 mL), and then, methanol was added to the limit of the volumetric flask. The internal standard solution obtained had a concentration of 1.0 mg/mL. The internal standard solution and standard solution were then stored at 4°C.

Instrumentation and chromatography conditions

GC was performed at a column temperature of 280°C and flow rate of 0.8 mL/min. The mobile phase used was helium 99.999%. Injection volume was 1 µL.

The ionization parameters were as follows: Capillary pipe voltage, 3.5 kV; ion source temperature, 230°C, interface temperature, 250°C; and solvent cut time, 1.5 min. The m/z value obtained was the ratio of the main ion molecular weight and product ion molecular weight. The m/z values used in the methamphetamine and ephedrine HCl analyses were 58.00 and 91.00, respectively.

After determining optimum conditions for methamphetamine analysis, mixed solutions of methamphetamine and internal standard were injected 6 times and coefficients of variation (CV) for retention times and areas under the curve for each substance were then calculated as peak area ratio (PAR), which a CV should be <2.0%. From this experiment, PAR was obtained with a CV of <2.0% (Table 1).

Sample preparation

Sample preparation was performed with saliva containing methamphetamine. The solvent containing 300 µL cyclohexane and 100 ng/mL internal standard ephedrine HCl was added into the tube. The tube was sonicated at 55°C for

7 min. The tube was then centrifuged for 5 min at 3000 rpm. The supernatant was pipetted to other tube to be evaporated. The residue was reconstituted with 100 µL methanol and mixed by vortex for 10 s. The solvent was subsequently inserted into the autosampler vial, and 1 µL of the sample was injected into the GC-MS system (Fig. 1).

Bioanalysis method validation

The lower limit of quantification (LLOQ) was determined by dissolving the lowest concentration from the methamphetamine calibration curve (15 ng/mL) until it was halved and then quartered. The concentration of the solution was then calculated by injecting it into the GC-MS. This assay was replicated 5 times. From the calculated concentration, the percentage difference (% diff) system and CV were obtained with a requirement of <±20%.

A calibration curve of standard solution was prepared by dissolving the standard solution (methamphetamine, 1000 ng/mL) until a concentration of 15–300 ng/mL was obtained. The methamphetamine solution was then dissolved with saliva to obtain a concentration range of 20–300 ng/mL. The correlation coefficient (r) of the linear regression line equation was calculated to obtain a linearity curve. The result showed that %diff obtained A was not exceeding ±15% for all concentrations, except LLOQ. LLOQ value obtained was not exceeding ±20%.

Selectivity of the method was determined by preparing saliva which contained the standard methamphetamine solution at the LLOQ concentration, and then, sample preparation was performed at 15 ng/mL. The value of %diff and % CV was not to exceed ±20%. The test was conducted using saliva from six different sources, and for each of these, a blank measurement was done with the LLOQ concentration and replicated twice.

Accuracy and precision were determined by preparing a standard solution of methamphetamine at the concentration of LLOQ, QCL, QCM, and QCH. Sample preparation was performed using saliva, and 1 µL of the solution injected to the GC-MS system. Accuracy was calculated from the %diff value to see the relationship between the concentrations obtained from measurement compare to the actual concentration. The test was conducted within-run and between-run. Accuracy was calculated using at least four concentrations of standard solution, with each of them replicated 5 times. The within-run test was conducted 3 times on at least two different days. Value %diff and % CV were not to exceed ±15% for all concentrations, except LLOQ (not exceeding ±20%). Value of recovery was calculated by comparing the area of the extraction result to the area of standard solution of the same concentration.

Carryover was performed by preparing the blank saliva solution and samples which contained methamphetamine at upper LOQ (ULOQ). Aliquot from the extraction process of blank saliva (1 µL) was injected into the GC-MS system after injecting the standard solution which contained methamphetamine at the concentration of the ULOQ. Peak area of methamphetamine and the internal standard that appeared in the blank was observed. This test was replicated 5 times.

The integrity of dilution was determined by preparing a sample saliva which contained methamphetamine at the concentration of 225 ng/mL. The solution was dissolved with blank saliva until halved and also a quarter of the concentration was acquired. The dilution solution was then prepared, and 1 µL of the final solution was injected into the GC-MS system. The test was replicated 5 times to acquire accuracy and precision.

The matrix effect was determined by preparing a blank saliva for sample preparation. The acquired supernatant was then added with methamphetamine of low concentration (QCL) and high concentration (QCH) and, thus, added the internal standard (100 µg/mL). The final solution (1 µL) was then injected into the GC-MS system. After that, the standard methamphetamine solution was prepared at low concentration (QCL) and high concentration (QCH) and the internal standard at

Table 1: System suitability test results*

Data No.	Retention time (min)		Area (µV.s)		
	Methamphetamine	IS	Methamphetamine	IS	PAR
1	5.428	6.946	1025946	218530	4.6947
2	5.434	6.946	1019306	222837	4.5742
3	5.426	6.946	1022766	222863	4.5892
4	5.427	6.946	1035906	225997	4.5837
5	5.426	6.944	1027644	225618	4.5547
6	5.428	6.945	1026313.6	223169	4.5993
Mean±SD	5.428±0.00	6.945±0.00	1026313.6±6237.21	223169±2988.28	4.5993±0.0549
CV (%)	0.06	0.01	0.61	1.34	1.1941

PAR: Peak area ratio, CV: Coefficients of variation, SD: Standard deviation

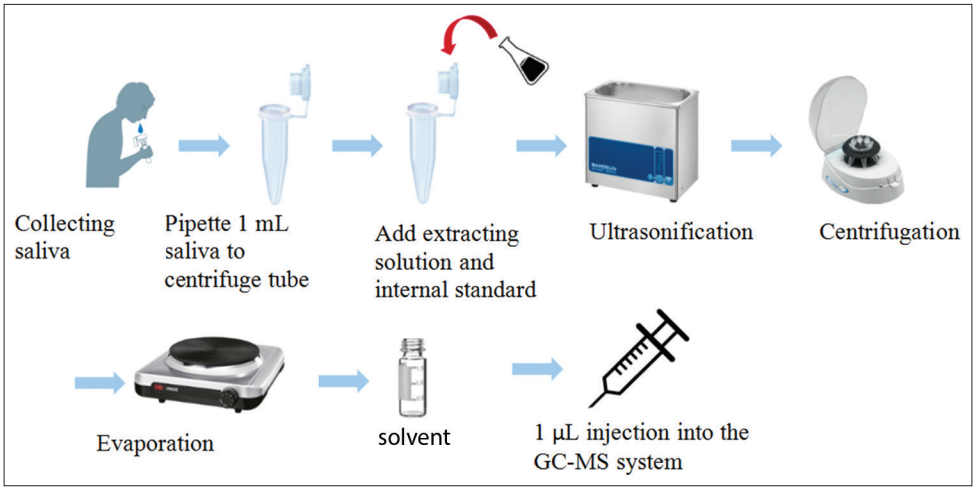

Fig. 1: Working scheme of salivary sample preparation

100 µg/mL. The solution (1 µL) was then injected into the GC-MS. The matrix effect was obtained by calculating the matrix factor, which was to compare the peak area of methamphetamine and internal standard within the saliva and the peak area of methamphetamine and internal standard within the standard solution. The matrix factor was normalized by the internal standard and then counted by dividing the matric factor of analyte with the matrix factor of internal standard. The matrix effect was fulfilled the criteria because % CV value for each concentration not exceeding 15%.

The stability test included stock solution stability, long-term stability (30 days), and short-term stability (0, 6, and 24 h) and autosampler stability (0 and 24 h). For the stock solution stability test, a QC sample of methamphetamine at low and high concentrations was prepared and stored at −20°C. For the short-term stability test, saliva was kept at room temperature. Autosampler stability was performed by reserving the sample, which was injected at 0 and 24 h.

RESULTS AND DISCUSSION

Determining the LLOQ

The value of LLOQ was determined by preparing a calibration curve of a standard solution with concentrations of 15–300 ng/mL. The 15 ng/mL concentration of standard solution was considered as the temporary LLOQ, and then, this was repeated 5 times. The value of %diff and CV at the concentration of 15 ng/mL fulfilled the accuracy and precision criteria, which was <±20%. Subsequently, the solution was diluted to half (7.5 ng/mL) and five replicas were made, but the results did not fulfil the criteria.

Linearity/calibration curve

For analyzing methamphetamine in saliva, a calibration curve was prepared with a range of 15–300 ng/mL. The calibration curve was prepared using blank saliva (saliva without the analyte and internal standard), zero sample (saliva with the internal standard), and non-zero saliva (saliva with the analyte and internal standard) at seven concentrations: 15, 30, 50, 75, 100, 200, and 300 ng/mL.

Calibration curve analysis was conducted by looking at the linearity parameter (r>0.9990) and accuracy (%diff not exceeding ±15%, except for LLOQ concentration [not exceeding ±20%]). One of the measurements of the calibration curve is shown in Fig. 2. The calibration curve produced the following linear regression equation: y=0.0004+0.0005x with r=0.9999; where x is the methamphetamine concentration (ng/mL) and y is PAR between methamphetamine and internal standard ephedrine HCl.

A calibration curve was prepared for every analysis to minimalize the possibility of measurement error because of the changing conditions

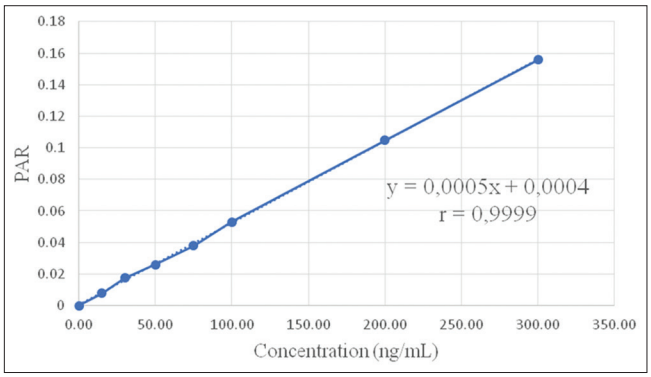

Fig. 2: Methamphetamine calibration curve

of the GC-MS system on different days. Therefore, the calibration curve had to fulfil the precision criteria every time a calibration curve was made, by looking at the CV value of each of the concentrations of the calibration curves (not exceeding ±15%; except LLOQ, which was not exceeding ±20%). According to data, the CV value that was obtained did not exceed ±15% for all concentrations, including LLOQ, with a mean r of 0.9990 (Fig. 2).

Accuracy, precision, and recovery test

At the within-run measurement of accuracy, the %diff value obtained for LLOQ concentration was within the range of −2.07%–+7.65% and for QC concentration was within the range of −6.10%–+6.97%. The within-run measurement of precision obtained a CV value of <5.14% for LLOQ and QC concentration. For the within-run measurement, the accuracy obtained for LLOQ concentration was a %diff value ranging from −19.81% to +18.87% and for QC concentration was from −4.43% to +8.77%. The between-run for precision the CV value was <15.5%. The values of %diff and CV for the within-run and between-run tests fulfilled the requirements for accuracy and precision.

Carryover

The result achieved from peak area percentage from blank saliva at methamphetamine retention time <8.33% with mean of 5.90%, so it fulfilled the requirement of <20%. The peak area percentage for blank saliva and ephedrine HCl retention time was <0.51% with a mean of 0.40%.

Dilution integrity

The methamphetamine solution in the saliva was made with QCH concentration (225.0 ng/mL). Then, dilution was done to halve the concentration to 112.50 ng/mL and halved again to 56.25 ng/mL. The

integrity test for methamphetamine dilution fulfilled the requirements of value %diff at the half QCH concentration (−6.48−− to tion w at the quarter of QCH (−8.30−− to tion w at the quarter was obtained was 2.11% for half ULOQ and 1.29% for quarter ULOQ. With this method, it can be concluded that dilution will not influence the accuracy and precision.

Selectivity

The selectivity test was performed using the analyte with LLOQ concentration (15 ng/mL) and blank saliva from six different sources. This was done to look at the ability of the analysis method in the quantitative measurement of LLOQ concentration and to observe any interference of blank saliva using different saliva. In this research, the value % analyte interference was obtained at 8.42–11.61% and fulfilled the requirement of <±20%, with a CV for LLOQ of 11.13%. Other than that, the percentage interferences of internal standard ephedrine HCl was obtained at 0%.

Matrix effect

The matrix effect of methamphetamine was 80.79% at QCL and 73.73% at QCH. That result showed that ionization suppression happened to the analyte which was possibly caused by the existence of many matrices other than the analyte that can disturb the ionization process during MS. The CV value from the analyte fulfilled the requirements of EMEA (CV value achieved from six different saliva sources not exceeding 15%).

Stability test

The short-term stability test of the stock solution was conducted for 24 h at room temperature, and the long-term stability test for 30 days at 4°C. The results obtained were: %Diff value for the stability of methamphetamine stock solution in short term: −0.14% to 0.16%; and %diff value for stability for ephedrine HCl stock solution in short term: −1.09–1.08%. This result shows that the stock solution of methamphetamine and ephedrine HCl was stable when kept at room temperature for at least 24 h. For long-term stability, value %diff for stock solution of methamphetamine was −0.08–+0.07% and for ephedrine HCl was −1.92−− to % and fo days storage at temperature of 4°C. Based on these results, the stock solution for methamphetamine and ephedrine HCl can be stored for at least 30 days.

The short-term stability test was performed by storing QCH and QCL at room temperature for 24 h and then observed for its stability at 0, 6, and 24 h. The %diff value after 24 h for QCL concentration starts from 7.21% up to 9.19% and for QCH start from 4.47% up to 9.03%. This showed that methamphetamine in saliva can be kept at room temperature for at least 24 h. The long-term stability test was done by keeping QCL and QCH at −4°C for 30 days and then analyzed at day 0 and day 30. According to the result, the value %diff at day 30 of QCL concentration was between −5.20% and −nd day This showed that methamphetamine in saliva was stable and can be stored at a temperature of −4°C for at least 30 days.

CONCLUSION

The developed method for determination methamphetamine in saliva was simple and easy with liquid-liquid microextraction. The method was valid and linear at the concentration range of 15.0–300.0 ng/mL with r >0.9999 and can be applied to saliva samples of methamphetamine users. Furthermore, the stock solution of methamphetamine and ephedrine HCl was stable when kept at room temperature for at least 24 h and can be stored for at least 30 days.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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