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Validation of Amphetamine and Methamphetamine Measurement Method by Gas Chromatography-Mass Spectrometry

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Abstract: Drug abuse is still very common in Indonesia, including in West Sumatra, which will result in psychological changes in users. A reliable method is needed to be able to detect the presence of narcotics and their metabolites in human specimens. The purpose of the study was to validate the mass spectrophotometric gas chromatography method for the measurement of amphetamine and metamphetamine. The benefits of research to ensure that the analytical method is accurate, specific, reproducible and resistant to the range of analytes to be analysed. The research method is by measuring the sample with GCMS (gas chromatography mass spectrometry) and then conducting a linearity test, detection limit test, accuracy test and precision test. The matrix used is urine specimen, spike is done with amphetamine and methamphetamine standards. From the results of the validation of amphetamine and methamphetamine measurement methods obtained MDL (method detection limit) 3.10 µg/L, LoD (limit of detection) 2.962 µg/L and LoQ (limit of detection) 2.962 µg/L. of Quantification) 9.873 µg/L. In the measurement of methamphetamine MDL 7.072 µg/L, LoD 6.757 µg/L and LoQ 22.253 µg/L. Accuracy of amphetamine and methamphetamine measurement methods 101.9% and 95.2% and reproducibility of amphetamine and methamphetamine measurements 3.83% and 6.743%. Measuring range and linearity of measurement of amphetamine and methamphetamine 25.0 µg/L - 200 µg/L with r = 0.9972 and 0.9992. Based on these data, the amphetamine and methamphetamine measurement method with gas chromatography-mass spectrometry is sensitive to measure amphetamine and methamphetamine in urine and fulfils the required criteria..

Keyword: MDL, LoD, LoQ, Linearity, Recovery, Reproducibility, Spike Matrix.

INTRODUCTION

The case of use and abuse of narcotics and psychotropic drugs in West Sumatra is the type of shabu-shabu (BNN, 2018). A mixture of several chemicals that are formulated into

white and clear crystals that are able to cause excessive energetic effects on the wearer and in a long time can cause addictive effects or dependence which is often called shabu-shabu (Widelia, I, 2012). *Amphetamine* or *methamphetamine* works to stimulate nerve function resulting in users becoming very active, this substance is contained in shabu-shabu (Novita, 2011; Widelia, 2012).

Shabu-shabu abuse can be subject to criminal sanctions, both against users and dealers. The process of proving the existence of shabu-shabu abuse is done through laboratory examination. This laboratory examination aims to find the presence of active compounds or metabolites of active compounds contained in specimens from users. (Law No. 32 Year 2007). The process of laboratory proof of shabu-shabu abuse can be done by examining specimens such as blood, urine, saliva, sweat and hair. Urine is the preferred specimen used for laboratory examination of shabu-shabu because urine specimens are easy to obtain and the concentration of *amphetamine* or *methamphetamine* contained in urine is higher than in other specimens. (Alfian and Taufik, 2017).

Amphetamine and *metaphetamine* compounds contained in urine can be detected by *immunochromatography* (ICT), thin layer chromatography (KLT) or *gas chromatography mass spectrometry* (GCMS). The examination of *amphetamine* and *methamphetamine* by *immunocromatography* and thin layer chromatography methods is qualitative while the examination method with GCMS can be qualitative or quantitative so that in the examination of shabu-shabu abuse the recommended laboratory examination method is the GCMS method (Alfian and Taufik, 2017).

To ensure the validity and performance of the test method, especially in proving shabu-shabu abuse, the examination method used must be a method that has been proven to be valid so that the method must first be validated. Method validation is a series of laboratory tests using the same specimens as the specimens to be examined to prove that the method is able to demonstrate performance that meets predetermined requirements. (ISO 17025, 2005).

According to Haryanto (2011), the reliability of a method can be known by verifying the method by measuring the method against the parameters of detection limit as *method detection limit (*MDL), *limit of detection (*LOD) and *limit of quantification* (LOQ), accuracy as percent *recovery (*%R), precision as *repeatability* or *reproducibility* (%RSD), linearity as coefficient r and measuring range. (ISO 17025 2005; Haryanto, 2011).

The purpose of the study was to validate the mass spectrophotometric gas chromatography method for the measurement of amphetamine and metamphetamine. The benefits of the study are to ensure that the analytical method is accurate, specific, reproducible and resistant to the range of analytes to be analysed. The novelty of this research is to use the SPE technique where the advantages of the extraction process are more perfect, the separation of the matrix from the analyte is more efficient and the use of less solvent. To increase sensitivity and selectivity in sample analysis, the SPE method can be combined with other methods such as Chromatography (GC-MS) UV-Vis Spectrophotometer, and HPLC. Being a solution to replace other techniques as the latest technique that works more efficiently (Rahmatia, T.U, 2016).

METHODS

This research is a laboratory observation study which is done by measuring the reference material or control material *amphetamine and methamphetamine* which is dispensed into urine specimens and measured by gas chromatography mass spectrometry. The research was conducted at the UPTD Health Laboratory of West Sumatra Province. This study used urine as a specimen obtained from patients who performed *amphetamine* and *methamphetamine* examinations either with indications or without indications of shabu-shabu use with a urine volume of 20-50 ml each. *Spike* samples are urine specimens added

with *amphetamine* and *methamphetamine* standard solutions with a concentration of 100 µg/L. Samples without spike is urine from patients who are not added to the standard solution of *amphetamine* and *methamphetamine*. Each measurement of the research variables was repeated 7 times. (KAN, 2014).

Tools and Materials

The tools used for research consisted of a rotary evaporator, oven, 10 ml volumetric flask, volume pipette, heating block, tube rack, 3 mL syringe, measuring pipette, pointed base reaction tube, micro pipette, 10 uL micro syring, Solid Phase Extraction (SPE) C.18 200 mg, Gas Chromatography Mass spectrophotometry, HP 5 MS capillary column, Helium gas. The materials needed for the study were phosphate buffer pH 0.1 M pH 6.0, sodium periodate 0.3 M, methanol extra pure, distilled water, acetic acid, dichlormethane, isopropanol, ammonia, dimethyl formamide.

Work Procedure

1. Amphetamine and Methamphetamine Testing, SPE Column Preparation, Prepare a Solid Phase Extraction (SPE) column, flushing the SPE with 3 ml of methanol and then with 3 ml of distilled water.
2. Sample Preparation, Pipette 2.0 ml of urine sample into a test tube, add 5 µL of internal standard solution, add 1 ml of 0.1 M phosphate buffer pH 6.0 and 1 ml of 0.35 M sodium periodate. Homogenise with a portek, incubate at room temperature for 20 minutes.
3. Extraction, The prepared sample is slowly (drop by drop) passed into the prepared SPE column. Wash the SPE column with 3 mL of distilled water, wash the column with 1 mL of acetic acid and wash the column with 3 mL of ethanol. Wait about 3 minutes.
4. Elution, Dichlormethane mixed reagent was prepared: Isopropanol: Ammonia: (78:20:2). Elute the SPE column after extraction with 3 mL of the reagent mixture and collect into a taper bottom test tube and continue with concentration.
5. Concentration of Eluent, Into the eluted solution, add 30 µL Dimethylformamide, evaporate at < 40o C until the volume remains 100 µL, check with GC MS.

Examination of Samples by Gas Chromatography-Mass Spectrometry.

Set up and measure the extract urine specimen by Gas Chromatography Mass Spectrometry with the following instrument conditions: Oven temperature : Programme temperature: Starting temperature 80o C, increased by 10o C/minute until reach a temperature of 280o C, hold 3 minutes. Injectors : Split, temperature 240o C. Gas : Helium (He) Gas pressure 125 psi, velocity 25 cm/sec, column gas velocity of 1 ml/min. Column : Capillary Column, 30 metres long and ID: 0.2 µm, column diameter 0.25 mm, column type: HP 5 MS, solid phase: 95% dimethyl polixiloxane. Injection volume : 2 µL.

Linearity Test, Pipetted *amphetamine* and *methamphetamine* standard mother liquor (1000 µg/L) each: 0.5 ml; 1.0 ml; 2.0 ml; 4.0 ml and 8.0 ml into a 10 ml volumetric flask. Add methanol solution up to the limit mark and homogenised. The concentration of the standard solution was 50 µg/L; 100 µg/L 200 µg/L 400 µg/L and 800 µg/L. Each standard solution was measured by GCMS and recorded RT and peak peak area of *amphetamine* and *metaphetamine.* Make it into the standard calibration curve of *amphetamine* and *methamphetamine.*

Detection Limit Test

1. Prepare a urine sample that is indicated not to contain *amphetamine* and *methamphetamine* of $\pm$ 50 ml.

2. Pipette 2.0ml of *amphetamine* and *methamphetamine* standardised mother liquor (100µg/L) and place into a 50ml volumetric flask. Add urine sample up to the limit mark and homogenise. The concentration of *amphetamine* and *methamphetamine* in urine is 4.0 µg/L (***spike sample***).
3. Urine samples without spike and spike samples were examined for *amphetamine* and *methamphetamine* levels by GCMS with 7 repetitions each.
4. Calculate the concentration of *amphetamine* and methamphetamine in urine samples without spike and spike samples and calculate the MDL, LoD and LoQ of the method.

Accuracy Test

1. Prepare a urine sample that is indicated not to contain *amphetamine* and *methamphetamine* as much as ± 100 ml, enter into two 50 ml volumetric flasks.
2. Pipette 2.0 ml of *amphetamine* and *methamphetamine* standardised mother liquor (1000 µg/L), into a 50 ml volumetric flask. Add urine sample up to the limit mark and homogenise. The concentration of *amphetamine* and *methamphetamine* in urine is 200 µg/L (***spike sample***).
3. Urine samples without spike and spike samples were examined for *amphetamine* and *methamphetamine* levels by GCMS with 7 repetitions each.
4. Calculate the *amphetamine* and *methamphetamine* concentrations of urine samples without spike and spike samples, calculate the accuracy as recoveries (%R).

Precision Test

a. Prepare a 20-30 ml urine sample that is indicated to contain *amphetamine* and *methamphetamine.*
b. Check the *amphetamine* and *methamphetamine* levels of urine samples by GCMS with 7 repetitions.
c. Calculate the concentration of *amphetamine* and *methamphetamine in the* urine sample, calculate the precision as relative standard deviation (%RSD).

Measuring Range Limit, The measuring range limit is obtained from the lowest limit and the highest limit of standard solution concentration on the calibration curve which still shows the regression coefficient (r) ≥ 0.995. To conclude the fulfilment of the method performance measurement results is done by comparing the measurement results with the test method acceptance requirements, namely:

Table 1. Test Method Acceptability Requirements

Variables	Measurement Result	Available at Acceptability
Linearity and Measure Range	Regression coefficient - r	r > 0,990 : Linear
Detection Limit	MDL	< Quality Standard
	LoD	< Quality Standard
	LoQ	-
Accuracy	% R	85-115%
Precision	% RSD	< 20%

Source: Haryanto, 2014

RESULT AND DISCUSSION

Results

Identification of Amphetamine and Methamphetamine Compounds

The identification of amphetamine and methamphetamine compounds was analysed by gas chromatography-mass spectrometry with the following settings.

Oven temperature : Temperature programme: Initial temperature 80o C, increase 10^{o} C/minute until it reaches 280^{o} C, hold 3 minutes.

Injectors : Split, temperature 240o C.
Gas : Helium (He) Gas pressure 125 psi, velocity 25 cm/s, column gas velocity 1 ml/min.
Column : Capillary Column, length 30 metres and ID: 0.2 µm, column diameter 0.25 mm, column type: HP 5 MS, solid phase: 95% dimethyl polixiloxane.
Injection volume : 2 µL.

From the measurements taken, the retention time of amphetamine and methamphetamine compounds was obtained as shown in the chromatogram below.

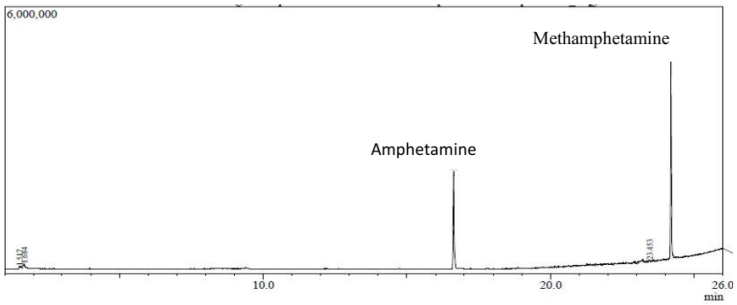

Figure.1. Peak chromatogram of Amphetamine and Methamphetamine

From the chromatogram above, the retention time is obtained as follows.

Table. 2. Retention Time of Amphetamine and Methamphetamine Compounds measured by Gas Chromatography Mass Spectrometry.

Compound Name	Mean ± SD (minutes)
Amphetamines	16,659 ± 0,017
Methamphetamine	24,243 ± 0,028

The mean and standard deviation of the retention time of the *amphetamine compound* was 16.659 ± 0.017 minutes and that of the *methamphetamine* compound was 24.243 ± 0.028 minutes.

Method Linearity

The linearity of the method of determining *amphetamine* and *methamphetamine* from urine specimens by gas chromatography is known by determining the intensity of the gas chromatographic response in the form of an area to standard solutions of *amphetamine* and *methamphetamine* with different concentrations. From this linearity test, a regression equation is obtained which serves to calculate the sample concentration.

The linearity of the *amphetamine* and *methamphetamine* calibration curves was measured over a concentration range of 25.0 ug/L to 200 ug/L.

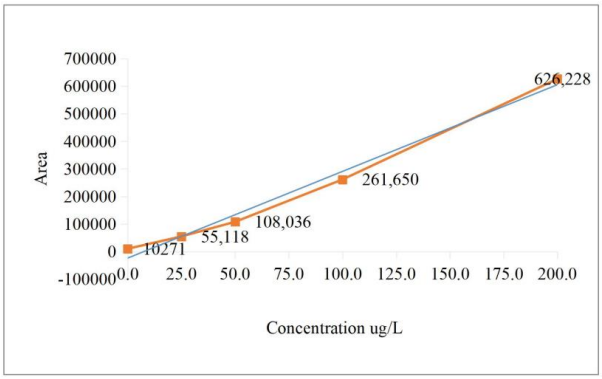

Figure 2. Linearity curve of concentration to area relationship of Amphetamine compound by gas chromatography.

The data from the chromatographic intensity measurement of the *methamphetamine* standard is shown below.

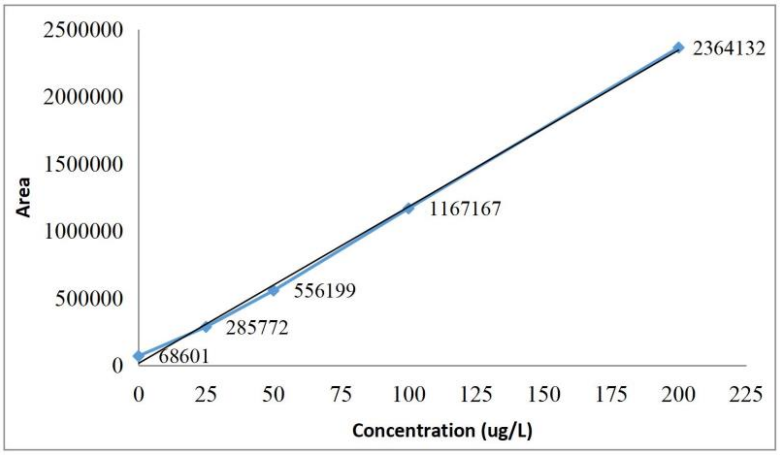

Figure 3. Linearity curve of concentration-area relationship of Methamphetamine compound by gas chromatography.

From the above curves, the regression coefficient of the *amphetamine* standard curve is r = 0.9972 with the equation y = 3143x - 23517 and the regression coefficient of the *methamphetamine* standard curve is r = 0.9992 with the equation y = 11654x + 14303.

Method Detection Limit

Data from the measurement of the detection limit of the determination method of *amphetamine* and *methamphetamine* from urine specimens by GCMS as shown in the table below.

Table 3. Measurement Data of Detection Limit of Amphetamine and Methamphetamine Method

Test Parameters	Concentration (µg/L)	
	Amphetamines	Methamphetamine
n	7	7
Mean	13,391	8,494
SD	0,987	2,252
MDL	3,10	7,072
LoD	2,962	6,757
LoQ	9,873	22,523

Methods Detection Limit is calculated with the equation 3.14 times the standard deviation at 95% confidence level. While LoD and LoQ were calculated as 3 times and 10 times the standard deviation. Based on table 3, the method detection limit as MDL of *amphetamine* is 3.10 µg/L, LoD = 2.962 ug/L and LoQ = 9.873 ug/L. The detection limit of *methamphetamine* as MDL is 7.072 µg/L, LoQ = 6.757 ug/L and LoQ is 22.523 µg/L.

Method Accuracy

The accuracy of the method in this study states the accuracy of the measured concentration of *amphetamine* or *methamphetamine* metabolites from the reference concentration obtained by adding *amphetamine* and *methamphetamine* standards with known concentrations to the urine sample (*spike sample*). Accuracy is calculated as percent recovery (%R).

Table 4. Percent Recovery of Amphetamine and Methamphetamine from Urine Samples Measured by Gas Chromatography Mass Spectrometry.

Test Parameters	Concentration (µg/L)	
	Amphetamines	*Methamphetamine*
n	7	7
Sample mean	23,678 ug/L	40,570 ug/L
Sample mean + spike	73.466 ug/L	135,787 ug/L
Target value	50.0 ug/L	100 ug/L
% R	101,9	95,2

The average concentration of Amphetamine in urine samples (n=7) was 23.678 ug/L and the average concentration of Metamphetamine in urine samples (n=7) was 40.570 ug/L. Recovery of Amphetamine and methamphetamine by means of urine spike obtained 101.9% and 99.3%.

Method Precision

Method precision was measured as reproducibility using urine samples positive for Amphetamine and Metamphetamine with 7 repetitions (n=7).

Table.5. Relative Percent Standard Deviation of Amphetamine and Methamphetamine from Urine Samples Measured by Gas Chromatography Mass

Test Parameters	Concentration (µg/L)	
	Amphetamines	*Methamphetamine*
N	7	7
Highest concentration	100,79	177,48
Lowest concentration	91,94	148,84
Sample mean	95,5	165,293
SD	3,653	11,145
%RSD	3,83	6,743

From table 5 the mean and standard deviation of *amphetamine* concentration in the sample is 95.5 $\pm$ 3.653 ug/L and the mean and standard deviation of *metamphetamine* is 165.293 + 11.145 ug/L. Reproducibility as % RSD of *amphetamine* is 3.83% and *metamphetamine* is 6.743%,

Measure Range Method

The measuring range of *amphetamine* and *methamphetamine* determination with GCMS in this study states the lowest concentration to the highest concentration of *amphetamine* and *methamphtamine* measured and is linear. Measurement data of *amphetamine* and *methamphetamine* calibration curves as shown in Figure 2 and 3. Based on these data, the working range of concentration determination of *amphetamine and methamphetamine is* 25.0 to 200 ug/L and is linear with coefficient r = 0.9972 and 0.9992.

Discussion

Identification of Amphetamine and Methamphetamine Compounds

Amphetamine and *methamphetamine* are active ingredients contained in shabu-shabu which is widely used in drug abuse cases. The results of this study obtained *amphetamine* and *methamphetamine* compounds from standards and samples with retention times of 16.659 minutes and 24.243 minutes. Research conducted by Widelia (2012) on *methamphetamine* compounds from urine samples after being analysed by GCMS obtained a retention time of 1.2 minutes with mass spectrometry fragments having *finger prints* at *m/z*. Another study on the optimisation of GCMS methods in the detection of *amphetamine* and

methamphetamine obtained a retention time of 14.24 minutes for *amphetamine* and 14.68 minutes for *methamphetamine*. (Bonchev G, *et al*, 2017). From some of these studies, the retention time data obtained varied. These varying retention times are due to differences in the chromatographic gas conditions used in the analysis of *amphetamine* and *methamphetamine*. The main factors that affect the retention time in gas chromatography analysis are oven temperature, column type and gas flow in the column. (Komang AGD, et al, 2016; Zul A, et al, 2017).

Linearity and Measuring Range Method

Linearity is one of the most important parameters in test method validation. Linearity is the ability of the analytical method that provides a proportional response between concentration and instrument response (Harmita, 2004). In the analysis with gas chromatography, the linearity of the standard calibration curve can be seen from the area of the gas chromatographic response at each concentration of the measured standard solution. The results obtained regression coefficient (r) = 0.9972 for amphetamine and r = 0.9992 for metamphetamine with a standard concentration of 25.0 to 200 ug/L which indicates that the standard calibration curve is linear. According to Haryanto (2011), a calibration standard curve is linear if the coefficient r > 0.995.

The correlation coefficient value close to +1 indicates a strong positive correlation between variables while based on the coefficient of determination (R2) of 0.9901 which indicates that variable X (concentration of standard solution) affects variable Y (area) by 99.01%. (Darmapatni KAG, et al, 2016). In research on the analysis of amphetamine, methamphetamine, 3,4 methylenedioxy amphtamin and 3,4 methylenedioxymetamphtamin from hair samples also obtained a standard calibration curve that is linear with a regression coefficient (r2) > 0.997. (Lin DL, *et.al*, 2005).

Method Detection Limit

Methods Detection Limit is calculated with the equation 3.14 times the standard deviation at 95% confidence level. While LoD and LoQ are calculated as 3 times and 10 times the standard deviation (Haryanto, 2011). Based on the data in table 3. method detection limit as MDL on amphetamine analysis is 3.10 µg/L, LoD is 2.962 µg/L and LoQ is 9.873 µg/L. While in the analysis of methamphetamine, MDL is 7.072 ug/L, LoD is 6.757 ug/L and LoQ is 22.523 ug/L.

The method detection limit is one of the parameters that must be determined by the laboratory to state the smallest concentration limit that can be measured quantitatively by the method used in the laboratory. (ISO/IEC 17025:2017). This method detection limit can also be used as an illustration of the sensitivity of the method used in the laboratory to measure specimens because laboratories are not allowed to report test results of zero point zero or not detected without stating the detection limit concentration.

In the research of Lin DL *et al* (2005) on the analysis of *amphetamine, methamphetamine, 3,4 methylenedioxyamphtamine and 3,4 methylenedioxymetamphtamine* from hair samples by gas chromatography mass spectrometry obtained detection limit LoD 0.05 ng/mg and LoQ 0.1 ng/mg. Based on data from several studies above, that the detection limit of amphetamine and methamphetamine measurement methods with gas chromatography mass spectrometry shows a detection limit that is not too different and with a very small concentration.

Method Accuracy

The standard concentrations of *amphetamine* and *methamphetamine* dispensed into the urine sample in this recovery determination were 50 µg/L for *amphetamine* and 100 ug/L for

methamphetamine. The matrix used is human urine, so that any interference from the matrix contained in the urine can be monitored for its effect on the measurement results. Based on table 4. above, the recovery of *amphetamine* and *methamphetamine* determination from urine specimens by GCMS is 101.9% and 95.2%.

The results of this study indicate that the recovery of the test method used meets the specified requirements of 85% <%R< 115%). In addition, to determine the limit of acceptability of a test recovery can be calculated based on the recovery formula from Hortwiz. According to Hortwiz's theory that the recovery of a measurement is influenced by the concentration of the element being measured. (Haryanto, 2011).

Method Precision

Based on table 5. above, the % RSD reproducibility of the determination of *amphetamine* and *methamphetamine* from urine specimens with GCMS on amphetamine analysis is 3.83% while on methamphetamine analysis is 6.743%. According to the limit of acceptability of the test method with a concentration of ug / L the limit of acceptability of reproducibiliti or repeatibility is %RSD <20%.

In determining precision, reproducibility data is better than repeatibiliti because the bias of measured data due to differences in measurement time can be monitored while repeatibility cannot be monitored so that it only shows instantaneous data. (Haryanto, 2011).

According to Haryanto (2011) in Horwitz theory, that the tolerance limit of % RSD measurement of a compound is influenced by the concentration of the compound being measured. In this theory, it is said that the smaller the concentration of the compound, the greater the error that can occur so that the tolerance of % RSD acceptance requirements also becomes greater.

Test data with small %RSD values have better precision and accuracy, so these data sets are very sensitive to differences in values and easily fall into the outlier category. Factors that affect the precision and accuracy of data are the selection of test methods, personnel competence, calibration or verification of test equipment and the use of appropriate chemicals. Precision and accuracy of test data determine the level of laboratory competence, this can be achieved if the quality management system has been implemented effectively and consistently (Ulfiati R, et al, 2017).

CONCLUSION

1. The measuring range of *amphetamine and methamphetamine* was 25 µg/L to 200 µg/L and showed a linear curve with a regression coefficient (r) of 0.9972 and 0.9992.
2. The detection limit of *amphetamine* measurement by gas chromatography-mass spectrometry method as MDL = 3.10 µg/L, LoD = 2.962 µg/L and LoQ = 9.873 µg/L.
3. The detection limit of *methamphetamine* measurement by gas chromatography-mass spectrometry method as MDL = 7.072 µg/L, LoD = 6.757 µg/L and LoQ = 22.253 µg/L.
4. The accuracy of the amphetamine and methamphetamine measurement method by gas chromatography-mass spectrometry as percent recovery (%R) was 101.9% and 95.2%.
5. The precision (reproducibility) of the amphetamine and methamphetamine measurement method by gas chromatography-mass spectrometry as percent relative standard deviation (%RSD) was 3.83% and 6.74%.

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