International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 11, Issue 4, 2019

Original Article

VALIDATION OF ANALYTICAL METHOD OF 2,5-HEXANEDIONE ON URINE BY GAS CHROMATOGRAPHY

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Received: 19 Feb 2019, Revised and Accepted: 24 May 2019

ABSTRACT

Objective: The purpose of this study was to obtain a valid analytical method for determining the level of 2,5-hexanedione in the urine of oil industry workers.

Methods: Gas Chromatography (GC) was employed to analyze 2,5-hexanedione in the urine. The analysis was done using HP-5 (Crosslinked methyl siloxane) capillary columns 30 m x 0.320 mm long, film thickness 0.25 µm. The temperature of the detector temperature was 300 °C, and the injector temperature was 250 °C. The helium gas flow rate was 2 ml/min. The detector was Flame Ionization Detection (FID). Parameters of system suitability test and validation were obtained.

Results: This study results that the method of analysis 2,5-hexanedione in urine by Gas Chromatography (GC) confirm the requirements of the validation method with a linearity was 0.99963, accuracy was in the range of 99.16% to 114.13%, the precision with % coefficient of variation was 1.65% to 5.16%, % coefficient variation of specificity was 0.027%, limit of detection was 0.054 µg/ml and limit of quantification was 0.18 µg/ml.

Conclusion: The proposed GC method meets the acceptance criteria of validation parameters and can be applied for routine analysis.

Keywords: 2,5-hexanedione, Gas chromatography, Hexane, Validation

DOI: http://dx.doi.org/10.22159/ijap.2019v11i4.32687

INTRODUCTION

Population growth was increasing rapidly. It led to the birth of the industrialization era. In this era, the development of science and technology was more advanced. One impacts of science and technology is the use of chemicals in many work processes. Currently, various chemicals are widely used in industries, such as food additives, pesticides, metals and compounds, as well as various organic chemicals including organic solvents [1]. One of the most widely used organic solvents in the industry is hexane.

Hexane is a non-polar solvent [2]. Hexane is a very good and cheap solvent. These compounds are found in glue, varnish, paint, and ink. Commercially, hexane is used to extract vegetable oil from various grains such as soybeans and cottonseed. These compounds are also widely used in the pharmaceutical and cosmetic industries [3].

Hexane is very volatile and will be metabolized in the body. The main metabolite of hexane is 2,5-hexanedione [3]. The mice that given 1% 2,5-hexanedione for 4 w caused neuropathy, systemic toxicity and testicular toxicity [4]. Toxic effects on the nervous system are associated with the bond between the 2,5-hexanedione metabolite with DNA, RNA and important proteins [5].

The body also could deactivate neurotoxic compounds by conjugating with the available hydroxyl groups to glucuronide and sulfate [6]. These conjugates then quickly eliminated in urine and bile. Toxicological studies of Cardona *et al.* (1966) showed that there was a correlation between exposure of hexane and 2,5-hexanedione in the urine. Therefore, the amount of 2,5-hexanedione in the urine can be used as a biomarker against hexane exposure [7].

2,5-hexanedione can be measured as a free metabolite or as total metabolite after acid hydrolysis. In 2011, the American Conference of Governmental Industrial Hygienists (ACGIH) determined the recommended biological exposure index for total 2,5-hexanedione was 5 mg/l and for free 2,5-hexanedione was 0.4 mg/l [8].

The toxic effect of 2,5-hexanedione is related to its concentration, therefore a quantitative 2,5-hexanedione analysis method is required in biological samples. 2,5-hexanedione analysis using HPLC was done by Gori *et al.* (1995) [9]. However, in the process of analysis required the derivatization process with 2,4-DNPH [10]. Derivatization involves a chemical reaction between an analyte with a reagent to change the physics-chemical properties of an analyte. This process required a longer analysis time. The novelty of this study, the analysis of 2,5-hexanedione was done using Gas Chromatography, no derivatization process was required. The method was simple, selective, and sensitive for the determination of 2,5-hexanedione in the urine.

The published analytical methods are often modified to suit the conditions with the equipment and materials available in the testing laboratory. This modification should be validated to ensure proper testing of the method of analysis [11]. Therefore, validation of the 2,5-hexanedione analysis method using gas chromatography is expected to quantify the 2,5-hexanedione level in the urine sample.

MATERIALS AND METHODS

Materials

Standard of 2,5-hexanedione (Sigma-Aldrich), dichloromethane (Sigma-Aldrich), sodium sulphate anhydrate (EMSURE®), aquabidest, pooled urine.

Tools

The tools used in this study were a set of GC (Agilent Technologies®) with Flame Ionization Detector (FID), crosslinked methyl siloxane, centrifugation (Eppendorf AG®), analytical balance scale (Mettler Toledo®), spatel, glass vial (Agilent Technologies®), vortex (Digisystem®), flask, test tube, micropipette 10-100 µl and 100-1000 µl (ACURA 825®), and glass tools commonly used in Analytical Laboratory. Data analysis and interpretation using Microsoft Excel 2013 64 bit software and online GC Software and Agilent Technologies GC offline.

Preparation of standard 2.5-hexanedione

Standard of 2,5-hexanedione was prepared by dissolved 5 mg standard with 10 ml dichloromethane, Then the standard solution was diluted to obtain the concentration of 0.1; 0.2; 0.4; 1,2; 2 µg/ml.

Preparation of pooled urine

Pooled urine prepared by mixing urine with aquabidest with a ratio of 20:80. The prepared urine is used as a matrix in the analysis by spike method.

Preparation of spiked urine

Standard of 5 mg 2,5-hexanedione was prepared, then added 20 ml of pooled urine. The solution was diluted to obtain the concentration of 0.1; 0.25; 0.5 and 2 µg/ml.

GC condition

The chromatographic system was optimized by injecting 2 µg/ml standard under the following conditions [12]: Columns: HP-5 (Crosslinked methyl siloxane) capillary columns, 30 m x 0.320 mm long, film thickness 0.25 µm, detector temperature 300 °C, injector temperature 250 °C, column temperature was programmed from 30 °C to 325 °C. The initial temperature of 30 °C was held for 3 min, gradually increased to 60 °C at a rate of 6 °C/min and held for 5 min. Then the temperature was increased to 90 °C at a rate of 15 °C/min. Helium gas flow rate: 2 ml/min with detector Flame Ionization Detection (FID) and the injection volume was 1 µl.

System suitability test

The system suitability test was performed by injecting a standard solution of 2 µg/ml under optimum conditions. Then determined the Retention Time, Tailing Factor (TF), Resolution (Rs), Number of Theoretical Plate (N), and High-Efficiency Theoretical Plate (HETP) [13].

Validation of analysis method

Validation methods include linearity, accuracy, specificity, the limit of detection, and limit of quantification [14].

Linearity

The linearity was determined from the standard curve. Preparation of the standard curves with external standard method by preparing the standard of 2,5-hexanedione at concentration 0,1; 0.2; 0.4; 1,2; 2 µg/ml.

Accuracy

Accuracy was done by prepare the spike urine solution with 3 different concentrations (0.1, 0.5, and 2 µg/ml). Each concentration was triplicate. Then, determined the recovery. Recovery (%CV) should be between 80-120% [14].

Precision

The precision test was performed by prepare the spike urine solution with 3 different concentrations (0.25, 0.5 and 2 µg/ml). Each concentration was triplicated. The value of precision was expressed by the Relative Deviation Standard (RSD) response. The RSD should be ≤ 2.0% [14].

Limit of quantification and limit of detection

Limit of detection (LOD) and Limit of Quantification (LOQ) was obtained by the determination based on the standard deviation and slope. LOD and LOQ were calculated by the following formula [13]:

$$\text{LOD} = \frac{3 \text{ x SD}}{\text{slope}}$$

$$LOQ = \frac{10\ x\ SD}{slope}$$

Note:

SD = Standard deviation of standard curve intercept

LOD = Limit of Detection

LOQ= Limit of Quantification

Specificity

Specificity was determined by analyzing a standard solution 0.4 µg/ml and a spike urine 0.5 µg/ml. The specificity was determined by comparing the retention time of the standard 2.5-hexanedione chromatogram and the spike solution chromatogram and then determined the value of coefficient variation [15].

Preparation of test solution

Test solution was prepared by mixing 2.5 ml of 2,5-hexanedione standard solution with 2.5 ml dichloromethane, then vortex it. The solution was centrifuged at 3000 rpm for 10 min. Its organic phase was taken, then 500 mg of anhydrous sodium sulphate was added. It was centrifuged again at 3000 rpm for 10 min. Take the dichloromethane phase. Inject to GC-FID.

RESULTS AND DISCUSSION

Preparation of pooled urine

Pooled urine preparation needs to be done because the urine pooled will be used as a matrix for spike solutions. Pooled urine was made by mixing urine with aquabidest with a ratio of 20:80.

Preparation of spiked urine

Spike urine preparation was used for the analysis of validation parameters such as accuracy, precision, specificity and system suitability testing. Spike urine preparation was done by added the standard into the pooled urine to obtain the required various concentration for the analysis. Urine was spiked into several concentrations of 0.1; 0.25; 0.5 and 2 µg/ml.

Optimization of GC condition

The optimum condition of GC for 2,5-hexanedione was using column temperature programmed at 30 °C-325 °C and helium gas flow rate in column 2 ml/min. Used also H_2 gas to heat the FID detector.

System suitability test

System suitability test was a series of experiments conducted to ensure that a method will produce acceptable accuracy and precision. The chromatogram of an analyte using the optimum condition can be seen at fig. 1.

The parameters used to determine the suitability of the system in this study include retention time (RT), theoretical plate (N), high-efficiency theoretical plate (HETP), tailing factor (TF) and resolution (Rs) in standard solution 5 times [13]. From the system suitability test, the CV RT, N, HETP, and RS values appropriated with the system suitability parameters. The result of the system suitability test can be seen in table 1.

Validation of the analytical method

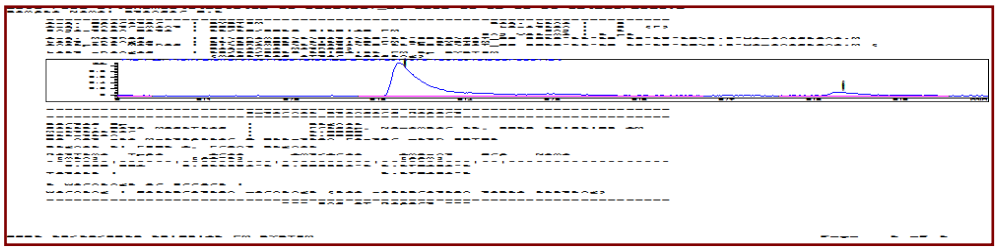
Validation of the analytical method was used to ensure that the methods used corresponding with the requirements in use so that the results obtained are acceptable and reliable. In this research, the validation parameters used are linearity, accuracy, precision, the limit of detection, the limit of quantification, and specificity [16].

Linearity

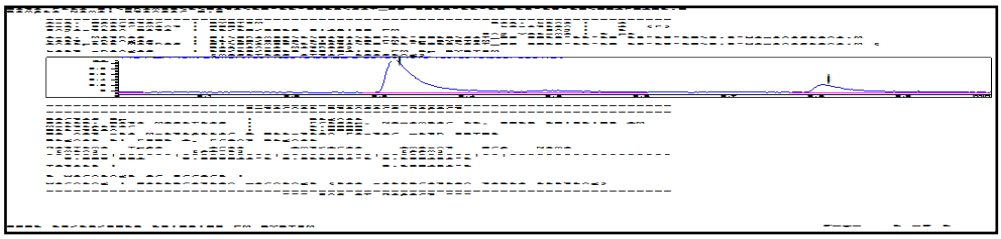
Linearity test results obtained from the equation of 2,5-hexanedioe calibration curve y = 4.0526x+0.0787 with the value of correlation coefficient 0.99963. Analysis method was valid if linearity parameter was>0.99 [15].

Accuracy

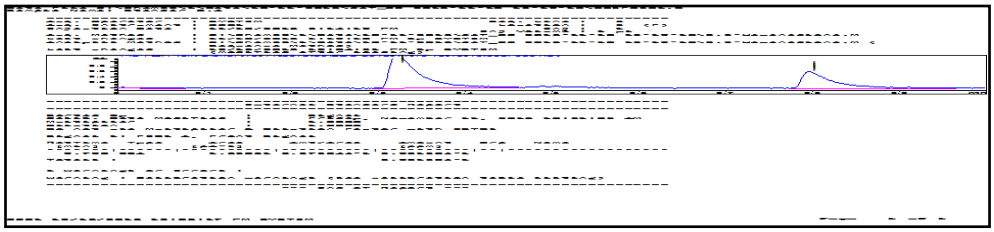
The accuracy of an analytical procedure describes the closeness between the measured value and the value received either the convention value or the reference value, or the actual value [13]. The calculation of % recovery can be seen in table 2.



(a)



(b)



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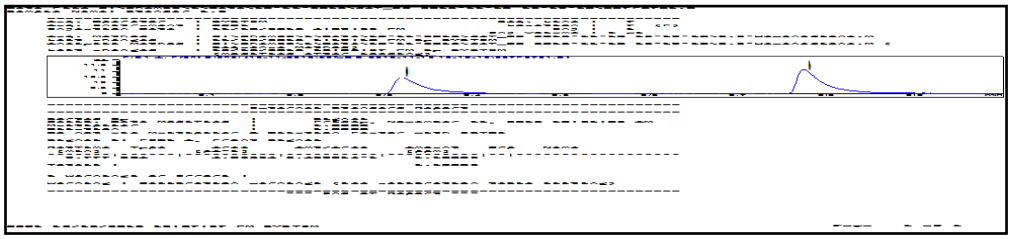

(d)

Fig. 1: Chromatogram of 2,5-hexanedione with concentration (a) 0,1 µg/ml (b) 0,2 µg/ml (c) 0,4 µg/ml (d) 1,2 µg/ml

Table 1: System suitability test results

Parameters	Results
% CV RT	0.013+0.001
N	806388.60+68.89
HETP	0.00372+0.0008
TF	3.20+0.54
Rs	13.231+2.34

Number of experiments = 5, Note: CV: Coefficient variation, RT: Retention time, N: Number of Theoretical Plate, HETP: High-Efficiency Theoretical Plate, TF: Tailing factor, Rs: Resolution

Table 2: Accuracy test results

Theoritical concentration (µg/ml)	Recovery (µg/ml)	% Recovery	Average % recovery
0.1	0.120	120	114.13+10.16
	0.120	120	
	0.102	102.4	
0.5	0.580	116	114+07.21
	0.600	120	
	0.530	106	
2	2.040	102	99.16+03.68
	1.940	95	
	2.010	100.5	

Note: Number of experiments = 3

Accuracy was obtained by calculating the recovery of urine spike solution. The recovery was obtained by calculating the difference between the concentrations of the results obtained with the blanks. The % recovery in the three concentrations shows that in all three concentration appropriated with the requirements of the accuracy parameter, % recovery requirement is 80-120% [15].

Precision

Precision was a measure of the repetition of a homogenous sample measurement series. This test can be performed by preparing three different concentrations of the target analytical concentration [13]. The results of calculation %CV of the test of the precision can be seen in table 3.

Table 3: Precision test results

Theoretical concentration (µg/ml)	Measurable (µg/ml)	Average	%CV
0.25	0.33	0.33+0.0082	2.47
	0.34		
	0.32		
0.50	0.58	0.57+0.0294	5.16
	0.60		
	0.53		
2.00	2.04	2.00+0.033	1.65
	1.96		
	2.01		

Number of experiments = 3

The calculation was performed on the concentration obtained from spiked urine. In the test, urine blanks are also measured to know whether there was 2,5-hexanedione content in it or not. Based on the research, the urine blank does not contain 2,5-hexanedione. Based on the results of the research, the % of the coefficient of variation obtained ranged from 1.65 to 5.16%. The criteria for receiving precision depends on the concentration of the analyte. For unit 1 µg/ml (ppm), % CV should not be more than 11% [13]. Based on these criteria, this study was appropriate with the precision requirement.

Limit of quantification and limits of detection

Limit of Detection (LOD) indicates the smallest number of analyte in the sample that can give a significant response, while the Limit of Quantification (LOQ) indicates the smallest number of analyte in the sample that can appropriate with the accuracy and precision criteria [14]. Based on the calculation results, the limit of detection was 0.054 µg/ml and the limit of quantification was 0.18 µg/ml. Result of LOD and LOQ can be seen in table 4.

Table 4: LOD and LOQ results

Concentration (µg/ml)	Width Area (y)	Averages	LOD	LOQ
0.1	0.33	0.33+0.010	0.054	0.18
	0.32			
	0.34			
0.2	0.34	0.34+0.010		
	0.33			
	0.35			
0.4	0.32	0.32+0.005		
	0.33			
	0.32			
1.2	0.58	0.58+0.010		
	0.57			
	0.59			
2	0.60	0.60+0.015		
	0.62			
	0.59			

Number of experiments = 3

Table 5: Specificity test results

Concentration	Retention time (min)
Standard (0.4 µg/ml)	9.798
Standard (0.4 µg/ml)	9.801
Standard (0.4 µg/ml)	9.807
Spike (0.5 µg/ml)	9.803
Spike (0.5 µg/ml)	9.801
Spike (0.5 µg/ml)	9.801
Average	9.801
SD	0.002
% CV	0.027

Number of experiments = 3

Specificity

Specificity tests were performed to know the appropriateness of an analyte in the presence of other components in the sample matrix, such as impurities, product degradation, and matrix components. The results of the specificity test can be seen in table 5.

Table 5 shows % CV of the standard and spike solution was 0.027%. It shows that there was no significant change. Therefore, this method can be used as a specific analysis for 2,5-hexanedione.

The proposed method gives a simple and sensitive method for the determination of 2,5-hexanedione. The previous methods need the

derivatization process. Maestri *et al.* [17] had used dansyl hydrazine; 1,3-diacetyl benzene (1,3-DAB) to react with 2,5-hexanedione before analyzed using high-performance liquid chromatography with fluorescence detection. Gori *et al.* [9] has developed the analytical method for the determination of 2,5-hexanedione with acid hydrolysis and derivatization step using 2,4-dinitrophenylhydrazine at 70 °C for 20 min. Moreover, the detection limit from this study was 0.054 µg/ml, was more sensitive than the previous study from Fedtke and Bolt [12] that was 0.12 µg/ml.

CONCLUSION

The optimum condition for the analytical method of 2,5-hexanedione by GC (Gas Chromatography) was carried out by using HP-5 (crosslinked methyl siloxane) capillary columns, 30 m x 0.320 mm long, film thickness 0.25 µm, detector temperature was 300 °C, injector temperature 250 °C, column temperature was programmed from 30 °C to 325 °C. The helium gas flow rate was 2 ml/min, and the injection volume was 1 µL.

ACKNOWLEDGMENT

The authors acknowledge the facilities from PT. Prodia OHI.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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