

## Determination of homogentisic acid in human plasma by GC-MS for diagnosis of alkaptonuria

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(Received July 15, 2015; Revised September 21, 2015; Accepted October 14, 2015)

## GC-MS를 이용한 혈장 중 호모겐티식산의 분석; 알카톤뇨증의 진단

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(2015. 7. 15. 접수, 2015. 9. 21. 수정, 2015. 10. 14. 승인)

**Abstract:** Alkaptonuria, a rare inherited metabolic disease, is characterized by a lack of homogentisate dioxygenase and accumulation of homogentisic acid (HGA), leading to homogentisic aciduria, arthritis, and ochronosis. In this study, a rapid analytical method, without an expensive and tedious solid phase extraction step, was developed to quantify HGA in plasma using GC-MS. HGA-spiked pooled plasma samples were subjected to liquid-liquid extraction (LLE) with ethyl acetate, followed by trimethylsilyl derivatization (TMS) and GC-MS quantification using selected ion monitoring. The formation of TMS derivative of the 1 carboxylic and 2 hydroxyl functional groups was performed by reacting BSTFA (with 10% TMCS) for 5 min at 80 °C. For selected ion monitoring, quantification and confirmation ions were determined based on specific ions ( $m/z$  384,  $m/z$  341 and  $m/z$  252) of the TMS derivative of HGA. Calibration curves of pooled normal plasma specimens showed a linear relationship in the range of 1-100 ng/ $\mu$ L. The precision and accuracy were within a relative standard deviation (RSD) of 1 to 15% and a bias of -5 to 25%. Recoveries were obtained in the range of 99-125% and 95-115% for intra-day and inter-day assay, respectively, at 2, 20 and 80 ng/ $\mu$ L. The limit of detection (LOD) and limit of quantification (LOQ) were 0.4 ng/ $\mu$ L and 4 ng/ $\mu$ L, respectively. No homogentisic acid was excreted from normal Korean plasma samples. Collectively, the results from the present study suggest that this method could be useful for routine diagnosis and therapeutic monitoring of alkaptonuria patients with excellent sensitivity and rapidity.

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**요약:** 알카プト뇨증은 극히 드문 희귀 유전성 대사이상질환으로, 호모겐티세이트 디옥시게나제의 부족이나 결손으로 인한 질환이며 호모겐티식산 (HGA)의 축적을 특징으로 한다. 임상적으로는 호모겐티식산 혈증, 관절염 및 퇴자병 (ochronosis) 등을 보인다. 본 연구는 고가이고 시간이 많이 걸리는 고체상 추출 단계 없이 신속하게 우수한 감도로 혈장 중 HGA의 정량분석을 위해 개발되었다. 통합 혈장 중 100  $\mu\text{L}$ 를 취하여 에틸아세테이트로 액체-액체 추출(LLE)한 후, 트리메틸 실릴 유도체 (TMS) 화한 후 GC-MS로 정량하였다. 카르복실기 및 히드록실 관능기의 TMS 유도체화의 형성은 80 °C에서 5 분 동안 BSTFA (10% TMCS 포함)로 반응시켜 수행 하였다. GC-MS의 선택 이온 모니터링을 위한 HGA의 선택 이온은  $m/z=384$ ,  $m/z=341$ ,  $m/z=25$ 로 설정 하였다. 통합 혈장에 여러 농도로 표준품을 첨가하여 검토한 검량선은 1-100 ng/ $\mu\text{L}$  범위에서 직선성은  $R^2=0.9991$ 로 양호하게 나타났다. 통합 혈장에 HGA를 2, 20과 80 ng/ $\mu\text{L}$  농도를 첨가하여 회수율, 정확도와 정밀도를 측정하였을 때, 일간내 회수율은 99-125% 였고, 일간의 회수율은 95-115% 였고, 정확도는 1-15% 이내로 양호하였다. LOD와 LOQ는 각각 0.4 ng/ $\mu\text{L}$ , 4 ng/ $\mu\text{L}$ 로 관찰되었다. 한국인 정상 혈장으로부터 HGA를 분석하였을 때, 적용된 모든 시료에서 HGA는 검출되지 않았다. 결론적으로 본 연구에서 개발된 분석 방법을 이용하면 알카プト뇨증 환자를 신속하게 진단할 수 있으며 그 치료과정에서의 HGA 모니터링에 유용하게 응용할 수 있음을 시사한다.

**Key words:** Homogentisic acid, Alkaptonuria, Inherited metabolic disease, Gas chromatography-mass spectrometry

## 1. Introduction

Alkaptonuria is a rare autosomal recessive metabolic disease characterized by excretion of an abnormal amount of homogentisic acid (HGA), a metabolite of phenylalanine and tyrosine, due to the deficiency of homogentisate 1, 2-dioxygenase, which readily converts HGA to maleylacetoacetic acid.<sup>1</sup> The lack of dioxygenase enzyme leads to a dramatic elevation in HGA levels in the blood and urine (Fig. 1).<sup>1</sup> In Slovakia and the Dominican Republic, the incidence of alkaptonuria has been reported to be as high as 1 in 19,000; however, the worldwide incidence is much lower at 1 in 250,000.<sup>2,3</sup>

The three major manifestations of alkaptonuria include dark urine, arthritis in the large weight-bearing joints, and black ochronosis (bluish-black pigmentation in the connective tissue) of the cartilage and collagenous tissue.<sup>4</sup> Discoloration of urine-filled diapers is the earliest clinical symptom. The complications of this disease include stone formation in kidneys, gall bladder, prostate, and salivary glands; rupture of tendons, muscles, and ligaments; and cardiovascular manifestations such as aortic valve disease and cardiac arrhythmia.<sup>5</sup>

For quantitative and semi-quantitative analysis of HGA, the currently available technologies include enzymatic spectrophotometry,<sup>6</sup> high-performance liquid chromatography (HPLC),<sup>7</sup> mid-infrared spectrometry,<sup>8</sup> liquid chromatography with mass spectrometry (LC-MS/MS),<sup>9</sup> and gas chromatography mass spectrometry (GC-MS).<sup>10</sup> Trimethylsilyl derivatives are the most commonly employed silylation derivatization procedures for GC-MS analysis.<sup>11</sup> Until now, reports on derivatization techniques with HGA have included trimethylsilyl (TMS)<sup>10</sup> and *tert*-butyldimethylsilyl (TBDMS) derivatives.<sup>12</sup> Most reports require a relatively large volume of derivatizing reagent<sup>10</sup> and a long reaction time.<sup>12</sup> The present study demonstrated a more effective quantitative analysis method for plasma HGA using GC-MS combined with fast and simple LLE.

## 2. Experimental Method

### 2.1. Materials

Homogentisic acid ((2, 5-dihydroxyphenyl) acetic acid) was purchased from TCI Co. (Tokyo, Japan). All other chemicals and organic solvents including acetonitrile and methanol were analytical reagent

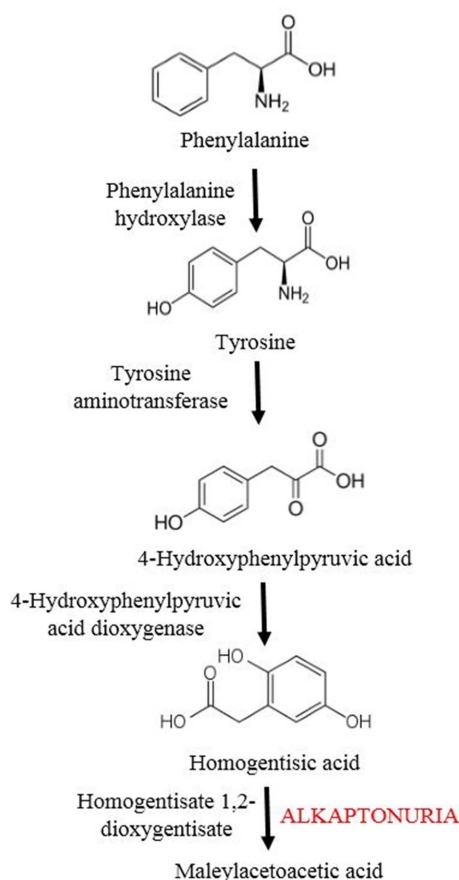


Fig. 1. Enzyme defect in alkaptonuria.

grade and purchased from J. T. Baker or Yakuri Pure Chemical Co., Ltd. (Osaka, Japan). Tropic acid was used as an internal standard and was purchased from Sigma-Aldrich. REGISIL®, a combination of BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and 10% TMCS (trimethylchlorosilane), was used as a derivatization reagent and was purchased from Sigma-Aldrich (MA, USA). Triethylamine (TEA) was purchased from Sigma-Aldrich. Distilled water was prepared using a Millipore-Milli Q™. Thermo vap (TAITEC model DTU-2C) (Tokyo, Japan) was used for evaporation and derivatization. A shaker (TAITEC, Tokyo, Japan) and a centrifuge (Eppendorf model 5424) (Hamburg Germany) were used for mixing and centrifuging the specimens in different steps.

## 2.2. Specimen collection and preparation of standard solution

Plasma specimens for analysis were collected in polyethylene bottles from three healthy volunteers (18-30 years of age). The plasma specimens were immediately stored at -20°C until the analyses. All control subjects were subjected to the same diagnostic procedure at the same facility.

Stock solutions of homogentisic acid and tropic acid were dissolved in methanol (1,000 µg/mL) and stored in a refrigerator until analysis. Each stock solution was further diluted to 1-100 ng/µL with methanol as working solution.

## 2.3. Instrument and analytical conditions

We used a Hewlett-Packard-6890 Series II GC-MS system (PA, USA) consisting of a Model 6890N gas chromatograph, a Model 5973N mass-selective detector (a HP Hewlett-Packard 3365 MSD Chemstation), and a Model 7683 series injector. An HP-5 Column (30 m × 0.25 mm I.D., 0.25 µm) was purchased from Agilent Technologies. Helium as a carrier gas was set to a flow rate of 0.8 mL/min. The GC oven temperature was programmed from 80 °C (2 min hold) to 120 °C at a rate of 30 °C/min, to 160 °C at a rate of 20 °C/min, and finally to 300 °C (3 min hold) at a rate of 30 °C/min, with a total run time of 8.5 min. The temperatures of the inlet and the interface were 280 °C and 300 °C, respectively. The MS was executed in selected ion monitoring (SIM) mode with a dwell time of 100 ms. The molecular ion ( $[M]^+$ ) was selected as the conformation ion (CI), and the ion with highest abundance was chosen as the quantification ion (QI).

One-µL aliquots of the final derivative were injected into the GC-MS ion source at a split ratio of 10:1.

## 2.4. Sample preparation and derivatization

A 200 µL plasma specimen was combined with 600 µL of methanol in an eppendorf tube. The mixture was centrifuged at 5,000 rpm at room temperature for 3 min. The precipitate was discarded, and the supernatant was transferred to a glass tube. Then, 50 µL of tropic acid (100 ng/µL) was added to

100  $\mu\text{L}$  of the plasma sample in a glass tube as the internal standard. Sodium chloride was added until saturation. Two mL of ethyl acetate was vortex mixed for 10s and centrifuged at 2,000 rpm for 3 min, and then the upper layer was transferred to another glass test. Moisture free extract (sodium sulfate) was evaporated to dryness at 80  $^{\circ}\text{C}$  under a gentle stream of nitrogen.

The TMS derivative of carboxylic and hydroxyl functional groups was derived by adding 50  $\mu\text{L}$  of REGISIL and 50  $\mu\text{L}$  of 1% trimethylamine in acetonitrile. After a gentle vortex mixing for 1 min, the mixture was then reacted at 80  $^{\circ}\text{C}$  for 5 min. The completed TMS derivatives were cooled for 5 min and transferred to a vial containing a low-volume insert (200  $\mu\text{L}$ ) for analysis with GC-MS.

### 2.5. Calibration

The calibration was performed by spiking pooled plasma specimen with 1, 3, 10, 30, and 100  $\text{ng}/\mu\text{L}$  of HGA. An internal standard (50  $\mu\text{L}$  of tropic acid (100  $\text{ng}/\mu\text{L}$ )) was also added to the pooled plasma specimens.

### 2.6. Validation

Our method was validated according to the US Food and Drug Administration (FDA) bioanalytical method validation guidelines.<sup>13</sup>

The analyte concentration at which the signal-to-noise ratio was greater than 3 was chosen as the limit of detection (LOD), and that greater than 10 was chosen for limit of quantitation (LOQ).<sup>13</sup> We evaluated the matrix effect for HGA, which was determined by calculating the ratio of the peak area in the presence of matrix to the peak area in the absence of matrix at low, medium, and high concentrations.<sup>13</sup>

The carryover effect was evaluated through the injection of the high concentration calibrator followed by a blank injection. Accuracy for intra-day and inter-day assay was measured by the following equation;  $[\text{measured concentration} - \text{apparent concentration}] / [\text{apparent concentration}] \times 100\%$ .<sup>14</sup> Precision for intra-day and inter-day assay was calculated using standard spiked pooled plasma and was expressed as coefficient of variation (CV). Recovery tests were

performed by comparing quantitative result of extracted and non-extracted spiked plasmas after fortified with low (2  $\text{ng}/\mu\text{L}$ ), medium (20  $\text{ng}/\mu\text{L}$ ), and high (80  $\text{ng}/\mu\text{L}$ ) concentration of HGA, respectively.

## 3. Results

The linear dynamic range of HGA standard solution was in the range of 1.0 to 100  $\text{ng}/\mu\text{L}$ .

TMS derivatization reactions of the 1 carboxylic and 2 hydroxyl functional groups of HGA were quantitatively completed by adding BSTFA including 10% TMCS with 1% TEA in acetonitrile (Fig. 2).

The chromatogram of the HGA-3TMS derivatives

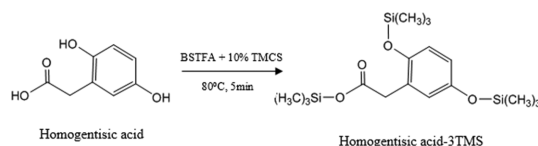


Fig. 2. Reaction scheme of TMS derivative for homogentisic acid.

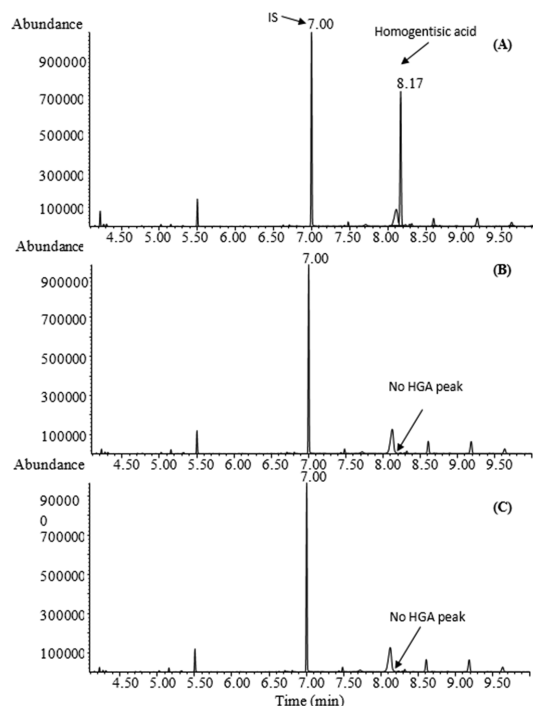


Fig. 3. GC-MS profile of homogentisic acid-TMS derivatives. (A) HGA standard, (B) normal plasma (male), (C) normal plasma (female).

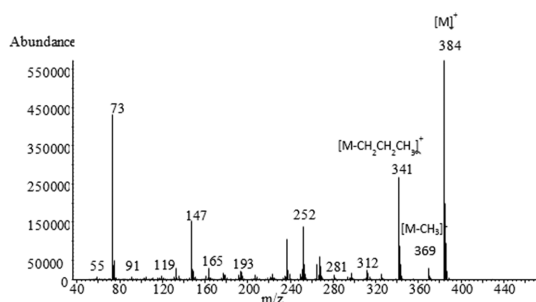


Fig. 4. Mass spectrum of TMS derivative for homogentisic acid.

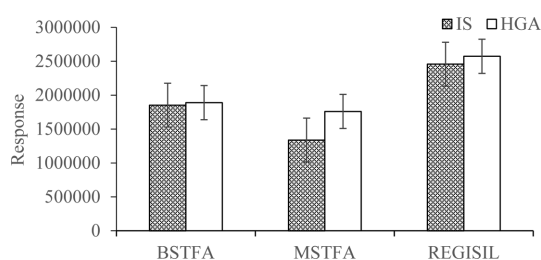


Fig. 5. Optimization of derivatization reagent for homogentisic acid.

was well resolved from plasma matrix (Fig. 3A).

The mass spectrum was obtained at electron impact ionization mode in full scan with a scan range from 50 to 550  $m/z$ . Molecular ion ( $m/e$  384), and other fragmentation ion of HGA, and trimethylsilyl ion ( $[\text{Si}(\text{CH}_3)_3]^+$ , 73  $m/z$ ) were confirmed concurrent with library matching (Fig. 4).

To optimize the response of derivatizing reagents for HGA, different TMS reagents (BSTFA, MSTFA and REGISIL®) were compared with its respective responses (Fig. 5). Among these three reagents, REGISIL showed the highest response for HGA and internal standard. Optimum reaction temperature for HGA was compared and then 80 °C was chosen as generally known (data not shown). Optimum derivatization reaction time was chosen as 5 min with REGISIL at 80 °C, showed highest peak intensity (Fig. 6).

Linear range of calibration curve on HGA spiked on plasma ranged from 1.0-100.0 ng/ $\mu\text{L}$  with a correlation coefficient of 0.9991 ( $y=1.4863x-0.0072$ ), showing excellent linearity (Fig. 7) by linear regression analysis. Limit of detection and limit of quantification

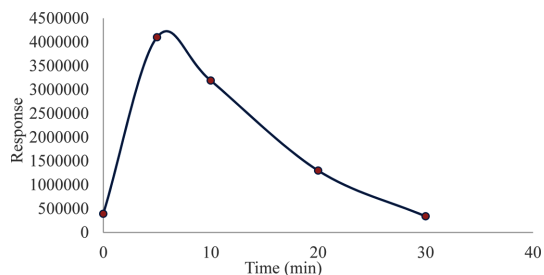


Fig. 6. Optimization of derivatization reaction time for homogentisic acid.

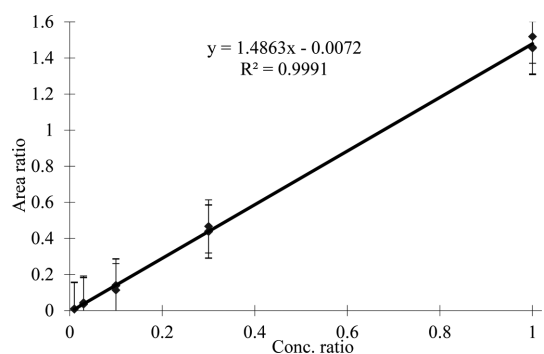


Fig. 7. Calibration curve of homogentisic acid for GC/MS-SIM analysis ( $n=3$ ).

were determined as 0.4 ng/ $\mu\text{L}$  and 4 ng/ $\mu\text{L}$ , respectively for developed method. To observe a carry-over effect, six blank plasmas were run after running the highest concentration of HGA and demonstrated no visible interference peak (data not shown).

Precision and accuracy of the method were evaluated on standard spiked plasma at 3 different concentration for intra-day and inter-day assays. The precision was within 1 to 15% of RSD. The accuracy was within -5 to 25% of bias. The lower the concentration, the higher the tendency of the bias. Overall the method was accurate enough for screening and diagnostic purposes (Table 1 and Table 2).

The matrix effect was evaluated at low (83.3% with 13% CV), medium (89.2% with 8% CV), and high concentrations (91.2 % with 7% CV) (Table 3). The coefficient of variance was less than 15%, showed a slight matrix effect, satisfying the validation criteria. Extraction recovery was in the range of 95-125%, showing reasonable recovery (Table 1 and Table 2).

Our developed method was applied to clinical

Table 1. Recovery and precision of the intra-day assay ( $n=3$ )

Conc. (ng/ $\mu$ L)	Conc. Calculated	Mean $\pm$ SD	Recovery (%)	Mean Recovery (%)	Precision RSD* (%)	Accuracy Bias (%)
2	2.3	2.5 $\pm$ 0.3	115	125	11	25
	2.4		120			
	2.8		140			
20	17.7	20.1 $\pm$ 2.0	88.5	101	11	0.5
	21.1		105.5			
	21.6		108			
80	76.7	80.0 $\pm$ 4.0	95.8	99.5	4	-0.5
	83.6		104.5			
	78.6		98.3			

\*RSD=SD/mean $\times$ 100.

Abbreviations: Conc., concentration, SD, standard deviation; RSD, relative standard deviation.

Table 2. Recovery and precision of the inter-day assay ( $n=3$ )

Conc. (ng/ $\mu$ L)	Conc. Calculated	Mean $\pm$ SD	Recovery (%)	Mean Recovery (%)	Precision RSD* (%)	Accuracy Bias (%)
2	1.9	2.3 $\pm$ 0.3	95	115	15	15
	2.5		125			
	2.5		125			
20	16.7	19.0 $\pm$ 2.0	83.5	95	10	-5
	20.1		100.5			
	20.1		100.5			
80	78.2	79.0 $\pm$ 0.7	97.7	99	1	-1
	79.6		99.5			
	78.9		98.6			

\*RSD=SD/mean $\times$ 100.

Abbreviations: Conc., concentration, SD, standard deviation; RSD, relative standard deviation.

Table 3. Matrix effect ( $n=2$ )

Spiked Conc. (ng/ $\mu$ L)	Conc. without matrix	Conc. on matrix	Mean $\pm$ SD	RSD (%)	Matrix effect (%)	Mean $\pm$ SD	RSD (%)
2	1.8	1.5	1.7 $\pm$ 0.2	13.0	83.3		
20	22.3	19.9	21.1 $\pm$ 1.7	8.0	89.2	87.94 $\pm$ 4.12	4.67
60	62.9	57.4	60.2 $\pm$ 4.0	6.5	91.2		

specimens for obtaining Korean normal range of HGA. We measured HGA concentration of normal plasma specimens from healthy volunteers ( $n=20$ ). Healthy normal men ( $n=10$ ) and women ( $n=10$ ) did not show any HGA peak as shown in Fig. 3B and Fig. 3C.

#### 4. Discussion

Without time-consuming solid phase extraction,

the new method was validated under a simple sample preparation within a 8.5 min chromatographic separation. The mass spectrum of HGA shows the presence of a TMS derivative with molecular ion  $[M]^+$  at  $m/z$  384, the loss of a methyl ion ( $[M]^+-CH_3$ ) at  $m/z$  369 from molecular ion, and  $[M-CH_2CH_2CH_3]^+$  ion at  $m/z$  341 (Fig. 4).

Formula weight (FW),  $[M]^+$  ion, and relative retention time (RRT) versus internal standard were evaluated

for selected ion monitoring mode. The quantification ion (QI) was chosen from the ion showing the maximum intensity in the spectra, that did not interfere with ions from other matrix compounds. Generally the  $[M]^+$  ion was chosen as confirmation ion (CI). The identification of an HGA was based on the co-elution of selected ion species, the ratio of QI and CI on the GC-MS chromatogram of the plasma specimens. The ratio of peak intensity (QI/CI) is considered as another very important parameter for identification.

REGISIL is the combination of BSTFA and 10% TMCS. TMCS is a catalyst that speeds up the reaction between HGA and BSTFA. Sameeh *et al.* used 200  $\mu\text{L}$  of BSTFA for derivatization of HGA.<sup>10</sup> In the present study, 50  $\mu\text{L}$  REGISIL was sufficient for derivatization because of the catalytic properties of TMCS, which can be considered an advantage of REGISIL over other derivatizing reagents. We used 1% TEA in ACN as a solvent catalyst.

The calibration curve showed excellent linearity with a correlation coefficient of 0.9991. The acceptable precision and accuracy, reasonable matrix effect, and no carryover effects demonstrated the integrity of validation. Determination of the signal-to-noise ratio was performed by comparing the desired signal to the average noise around the signal. The LOD was determined at three times the noise level, and LOQ was determined at 10 times the noise level.

Generally, patients with alkaptonuria show high excretion of abnormal HGA. The HGA concentration in plasma of alkaptonuria patients was previously reported in the range of 33-39  $\mu\text{mol/L}$ ,<sup>7</sup> and that in urine was 0.46-1.5 g/24 hr.<sup>10</sup> The calculated LOD of our method was 0.4 ng/ $\mu\text{L}$  (approximately 2.38  $\mu\text{mol/L}$ ), which was sensitive enough to detect HGA in plasma of patient with alkaptonuria. LOQ determined in our method was 4 ng/ $\mu\text{L}$  (approximately 23.8  $\mu\text{mol/L}$ ), which is well below the LOQ reported (30  $\mu\text{mol/L}$ ) for LC-MSMS.<sup>9</sup> Thus, the proposed LOD and LOQ levels are sensitive enough for both diagnosis and follow-up testing for alkaptonuria patients

The main advantages of this method are the simple liquid-liquid extraction process without an expensive,

tedious solid phase extraction step with its great sensitivity and specificity. Secondly, the amount of derivatizing reagent used in this study was only 50  $\mu\text{L}$ , which is very low volume compared to that in other studies.<sup>10</sup>

## 5. Conclusions

We developed a simple, fast, and sensitive analytical method for the quantification of HGA in plasma using GC-MS/SIM. This method enabled fast analysis of HGA within 8.5 min with 5 min of derivatization reaction time, which is very much faster compared to previously published analytical approaches.<sup>10</sup> Thus, this method could provide a useful analytical and clinical tool for diagnosing monitoring the effect of treatment on HGA level in patients with alkaptonuria.

## Conflicts of Interest

The author have no potential conflicts of interest relevant to this article to report.

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