Journal of Associated Medical Sciences 2024; 57(3): 65-74

JAMS
Journal of Associated Medical Sciences

Scopus Indexed & Thai-Journal Citation Index Centre (TCI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Development of a simple HPLC method for the determination of urinary O-aminohippuric acid (OAH) and an establishment of OAH reference interval

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ARTICLE INFO

Article history:
Received 7 May 2024
Accepted as revised 19 June 2024
Available online 29 June 2024

Keywords:
HPLC, OAH, reference intervals

ABSTRACT

Background: O-aminohippuric acid (OAH) is considered a low-abundance urinary fluorescent metabolite with the potential to be an innovative lung cancer biomarker. There is a lack of simple methods for measuring urinary OAH metabolite, and the measurement needs to be normalized by urinary creatinine, which is produced at a constant rate. Thus, the newly developed method must be able to determine urinary creatinine and OAH simultaneously in the same run.

Objective: This work aimed to develop and validate a simple high-performance liquid chromatography (HPLC) method for measuring urinary OAH and to establish the reference intervals of OAH in healthy individuals.

Materials and methods: We synthesized OAH standard in a simple route and optimized simple HPLC method for simultaneous measurement of creatinine and OAH in a single run. Analysis was performed on a RP-C18 column with a gradient elution system of acetonitrile - ammonium acetate buffer (pH 6.5, 100 mM). After implementing optimal conditions, the procedure was compiled according to the International Council for Harmonisation (ICH) validation parameters. The developed method was used for the establishment of reference intervals of a total of 120 random urine samples of healthy individuals.

Results: The linear range of the calibration curve for creatinine and OAH were 1-1000 µg/mL and 0.1-100 µg/mL, respectively. The recoveries ranged for both metabolites were between 91.35 % and 109.12%. The relative standard deviations (RSDs) for the intra-day and inter-day results ranged from 0.11-0.66 % to 0.16-1.73 %, respectively. The limits of detection (LOD) and quantification (LOQ) were 0.258 µg/mL and 0.783 µg/mL for creatinine, while OAH was 0.045 µg/mL and 0.137 µg/mL, respectively. The method was successfully applied to establish reference intervals of OAH in healthy individuals and was defined as 0.420-2.287 mmol/mol creatinine.

Conclusion: According to various validated parameters, the proposed method was proven to quantify urinary OAH and creatinine in a single run. It can also be analyzed noninvasively without additional sample processing. Reported herein is the first establishment of OAH reference intervals in healthy individuals, which may benefit the utilization of OAH as a noninvasive biomarker for lung cancer detection in the future.

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doi: 10.12982/JAMS.2024.048
E-ISSN: 2539-6056

Introduction

The composition of O-aminohippuric acid (OAH) includes a metabolite of tryptophan and glycine, and it is believed to originate from kynurenine (KYN) through anthranilic acid.[1] Tryptophan plays a vital role as an essential

amino acid in the synthesis of proteins and serves as a pivotal regulator in cancer progression.[2] Its metabolites undergo processing through the kynurenine pathways, significantly impacting immune response regulation.[3,4] The main enzymes, indolamine-pyrrole 2,3-dioxygenase (IDO)1, IDO2, and tryptophan 2,3-dioxygenase (TDO) are responsible for the KYN pathway activities.[5,6] Moreover, cancer cells predominantly rely on glycine for their growth and multiplication. Lung tumor-initiating cells demonstrate increased glycine levels derived from enhanced glycolysis and glutaminolysis, subsequently converting into deleterious metabolites.[7,8]

The extensive use of tryptophan and glycine represents cancer cells' potential metabolic reprogramming trait.[9,10] However, the exact pathway of OAH formation in the urine of lung cancer patients is still unknown. OAH could be generated through tryptophan metabolism, producing anthranilic acid, which is then conjugated with glycine. Therefore, elevated OAH levels in lung cancer patients may be attributed to increased tryptophan and glycine metabolism in cancer cells.[1]

Metabolomics offers a direct measurement of the metabolic profile of an organism, offering a more accurate approach to detecting changes in metabolism. In recent years, studying metabolites in biological specimens has been a subject of great interest.[11,12] Metabolomics experiments can be subdivided into targeted (limited and predefined subset) and untargeted analyses (broader approach).[10] Urine become the most frequently used biological fluid for metabolomics research for different cancer types since it has additional benefits over blood, such as being rich in metabolites, easy to handle, and accessible in substantial quantities, all without the need for invasive collection procedures.[12,13] Besides, it contains diagnostically important metabolite OAH, and its levels in urine samples from lung cancer patients were significantly higher than those in non-cancer.[1] Under a stable condition, the overall daily excretion of creatinine is directly proportional to the total body creatinine content and the total mass of skeletal muscle.[14] To increase the accuracy of the analysis, the concentration of urine constituents was standardized using urine creatinine levels to normalize urine volume variations.[15]

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) based techniques are currently utilized in metabolomics studies.[7] The quantification of OAH in the previous survey utilized high-performance liquid chromatography coupled with mass spectrometry and nuclear magnetic resonance (HPLC-MS/NMR).[1] These methods used separation via HPLC before detection, and OAH levels were adjusted by creatinine with an enzymatic reaction in all subjects. These approaches, however, include ion suppression, interference with other ions, and low throughput.[1,15,16] Given the current state of technologies and the diverse physiological properties of metabolites, no single separation or detection technique can simultaneously identify all metabolites within a urine sample.[3,8]

The present work aimed to develop and validate high-performance liquid chromatography (HPLC) to determine urinary metabolites OAH and creatinine simultaneously. This process consists of a simple sample preparation enabling chromatographic separation. There were no reports of OAH reference intervals in non-cancer patients, and the previous study just compared the levels statistically; the developed method was successfully applied to establish reference intervals of OAH in healthy individuals, which might be helpful to differentiate healthy and diseased patients in clinical settings.[17-20]

Materials and methods

Chemicals and reagents

Creatinine, isatoic anhydride, glycine hydrochloride, and deuterated methanol were purchased from Sigma-Aldrich, St.Louis, MO, USA. HPLC grade acetonitrile (purity, minimum 99.8%) was obtained from RCI Labscan Limited, Thailand. Ammonium acetate was purchased from BDK Chemicals Limited, Poole, England. ARIOSO water purifier (Human Corporation, Korea) was the source of type I water, which was employed in preparing samples and mobile phase throughout the study.

Instrumentation

HPLC was performed with Agilent Infinity Lab LC series 1260 Infinity II, USA, equipped with a diode array detector (G7115A), fluorescent detector (G7121A), vial sampler (G7129A), and a quaternary pump (G7111B). The analytical column used was an EC-C18 reversed-phase column with a length of 250 mm, an internal diameter of 4.6 mm, and a bead size of 4 µm. ChemStation version 1.1.1 system control software was used for data. Microcentrifugation of samples was carried out in a Kubota 5200 Japan centrifuge. Thin-layer chromatography (TLC) was performed with Merck precoated silica-gel 60 F_{254} plates. NMR spectra were measured with 500-MHz Bruker spectrometer FT-IR (Fourier Transform Infrared Spectroscopy).

Preparation of Standard Solution for Creatinine and OAH

Creatinine (0.03 gm) and OAH (0.003 gm) were transferred into a 10 mL volumetric flask and filtrated by nylon syringe filter pore size 0.22 µm. The stock standards were stored at 4 °C until further use. A series of calibration standards were prepared in the concentration range of 0.1-1000 µg/mL for creatinine and 0.1-100 µg/mL for OAH. Quality controls for creatinine were 1000 µg/mL (high-level quality control, HQC), 500 µg/mL (middle-level quality control, MQC), and 250 µg/mL (low-level quality control, LQC). For OAH, quality controls were 1 µg/mL (HQC), 0.5 µg/mL (MQC), and 0.25 µg/mL (LQC). All solutions were diluted with ultra-pure water and stored at 4 °C. The standards on HPLC were further analyzed for different validation parameters.

Sample Collection and Preparation for HPLC Analysis

The healthy volunteers, who had normal results in urine analysis, were selected. The left-over urine samples

were collected from the PROMT Health Center, Chiang Mai University. Ethical approval was obtained from the Ethics Review Committee, Faculty of Medicine, University of Chiang Mai, Thailand**.** Demographic characteristics were provided by the clinic. One hundred and twenty left-over random urine samples from healthy volunteers were collected and centrifuged at 315 g for 10 minutes. The supernatant was transferred to sterile tubes and was diluted by 10-fold dilution with deionized water. After that, a 2-mL aliquot of this mixture was passed through a 0.22- µm nylon syringe filter, and the filtrate was collected for HPLC analysis.

Method Development and Validation

Firstly, a retrosynthetic analysis of O-aminohippuric acid has been performed. Then, the optimized method for the simultaneous estimation of creatinine and OAH has been validated as per the ICH guidelines, consisting of evaluating system suitability, specificity, linearity, and range, the lower limit of detection (LOD), the lower limit of quantification (LOQ), accuracy, precision (intra- and inter-day), and robustness of creatinine and OAH.[21,22]

Establishment of Reference Intervals

Using the validated HPLC method, the current process was employed to establish a reference interval of potential lung cancer urinary biomarkers, OAH levels, in healthy populations.

Statistical Analysis

Linear regression tests were performed using Microsoft Excel 2018, and the respective calibration curve equation was concluded. The data were expressed as the mean±SD and 95% confidence interval. Statistical analysis was performed using SPSS 19.0. The Kolmogorov-Smirnov test evaluated the data distribution with $p>0.05$. If the data were normally distributed, the reference intervals were expressed as mean±1.96 SDs.

Results

Chemical Synthesis of OAH Standard

The previous synthesis of O-aminohippuric acid relied on multi-step synthetic routes.[23,24] As shown in Figure 1, we identified two commercially available precursors, isatoic anhydride and glycine hydrochloride, as key starting materials.

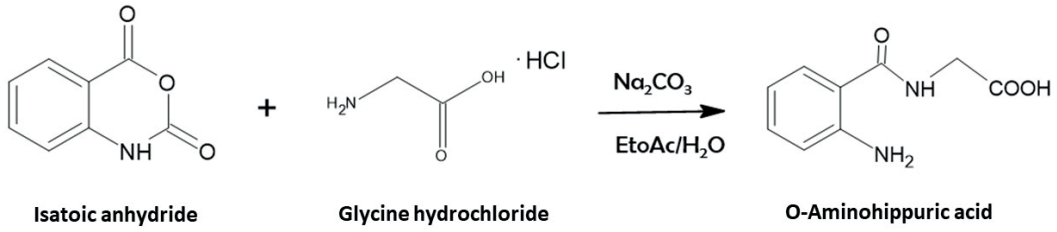

Figure 1*. Retrosynthetic analysis of O-aminohippuric acid using isatoic anhydride and glycine hydrocholride.*

After subjecting isatoic anhydride and glycine hydrochloride in the presence of sodium carbonate, TLC analysis of a crude product showed two new spots with retention factor (R_f) of 0.23 and 0.94, respectively. The spot with R_f of 0.94 corresponds to the hydrolysis product of isatoic acid, anthranilic acid. The spot with R_f of 0.23 corresponds to the targeted product, a higher polar compound. In addition, the R_f of 0.71 in the first lane corresponds to the starting. The three spots in the co-sport lane serve as a control, and all these spots were visualized under ultraviolet (UV) light 254 nm in the lamp chamber material shown in Figure 2(A). We purified the target compound by a simple wash with ethyl acetate and hexane. After washing with ethyl acetate and hexane several times, TLC analysis of a washed sample showed only one spot with R_f of 0.37 illustrated in Figure 2(B). The purity of a purified sample was rechecked with high-performance liquid chromatography. To our delight, the chromatogram of a purified sample showed one significant peak of O-aminohippuric acid at 13.38 retention time (RT) in Figure 2(C). In contrast, anthranilic acid impurity was insignificant at 10.44 retention time, with a peak area of less than 10 mAU*s, accounting for 95% purity. Despite a small amount of impurities, the chromatogram results from TLC and HPLC agree with each other after purification and can be accepted.

Finally, the structure of OAH was analyzed by ^{1}H-NMR spectroscopy using methanol-d_4 (CD_3OD) as a solvent in which the ^{1}H-NMR spectrum is shown in Figure 2 (D). The chemical shift (δ) at 7.52 (dd, *J*=7.9, 1.5 Hz, 1H), 7.21 (dd, *J*=8.4, 7.1, Hz, 1H), 6.77 (d, *J*=8.2 Hz, 1H), 6.65 (t, *J*=7.5 Hz, 1H) are ascribed to aromatic protons. The chemical shift (δ) of 4.06 (s, 2H) corresponds to methylene protons of glycine units. It should be noted that the protons of the amino group ($-NH_2$) could not be observed by ^{1}H-NMR spectroscopy. Altogether, ^{1}H-NMR spectroscopy could be used to confirm the structure of synthetic O-aminohippuric acid and the purity of our synthetic compound, which is sufficient for use in HPLC analysis.

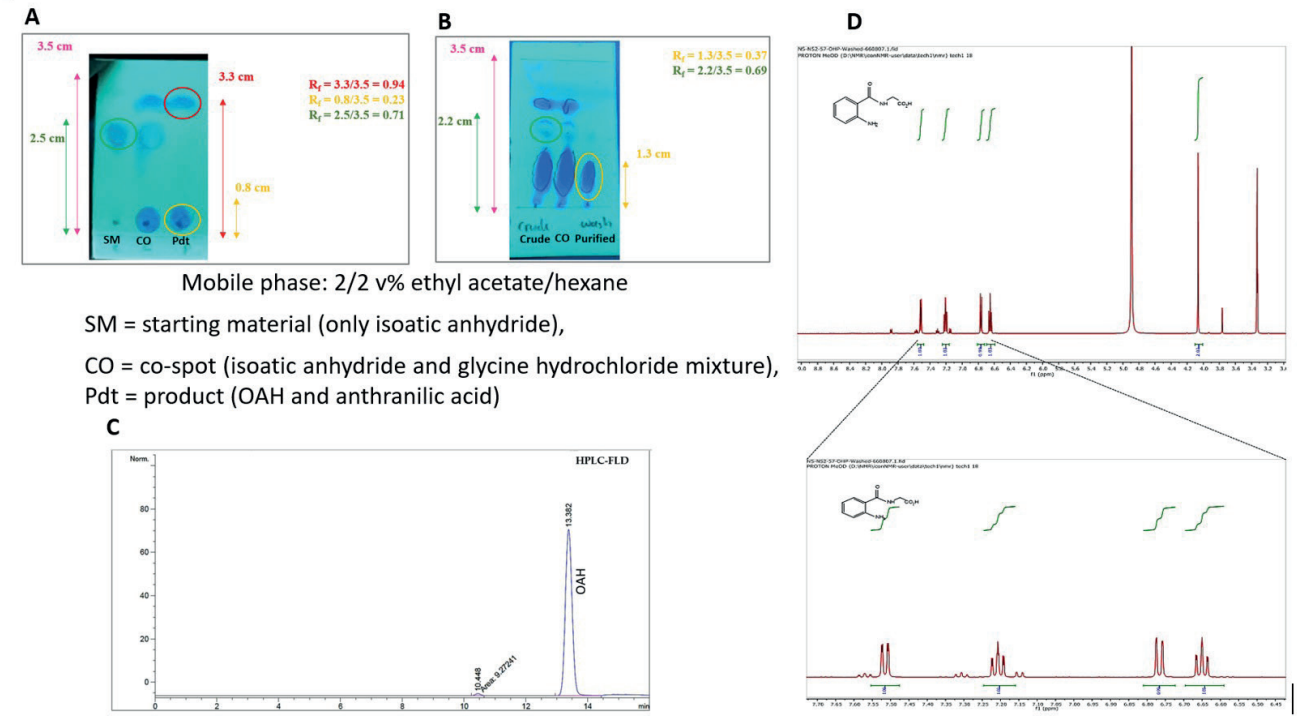

***Figure 2**. Chemical synthesis for OAH standard. A: TLC chromatogram of crude OAH, B: TLC chromatogram of washed sample OAH, C: HPLC chromatogram of washed sample OAH, D: ^{1}H-NMR spectrum (500MHz, CD_3OD) of OAH.*

HPLC method development and validation

After many trials had been tested, the gradient program, mobile phase composition, buffer pH, and column temperatures were adjusted, and the best-optimized condition was obtained. It was applied from 0 to 30% solvent B (acetonitrile) in solvent A (100 mM ammonium acetate, pH 6.5) with a 27-min gradient time (time-B (%): 0-1, 12-1, 13-30, 19-30, 20-1, 27-1). The flow rate was 1.0 mL/min at 40 °C. The detection wavelengths for both analytes were obtained the maximum sensitivity. For OAH detection using a fluorescent detector, the excitation wavelength was 340 nm, and the emission wavelength was 430 nm. For creatinine detection using diode array detector, the wavelength was 235 nm. The column temperature was 40 °C, and the sample injection volume was 10 µL.[1,18,25-27] Then, the developed method was validated according to ICH guidelines.

System Suitability

The relative standard deviation (RSD) (%) values of RT and peak area for creatinine and OAH were <2.0, indicating excellent repeatability of replicate injections on the integral HPLC system used. The tailing factor never exceeded 2 in all peaks, demonstrating reasonable peak regularity (acceptance limit is <2.0); and the number of theoretical plates was always >2000 in all chromatography runs, ensuring good columns efficacy throughout the developed separation process reported in Table 1.

Table 1. Results of system suitability parameter

Parameters	Value		% RSD	
	Creatinine	OAH	Creatinine	OAH
	Mean±SD (N=6)	Mean±SD (N=6)		
Theoretical plate	19549.2±331.7	23522.2±380.70	1.69	1.62
Tailing factor	0.76±0.013	1.18±0.01	1.71	1.18
RT (min)	3.848±0.03	14.04±0.11	0.78	0.78
Peak area	2257±18.42	440.5±5.06	0.82	1.15

Note: RSD: relative standard deviation, RT: retention time, SD: standard deviation.

Specificity

The optimized analytical method successfully detected and evaluated OAH even in the presence of minor impurities. The chromatographic specificity of the process was demonstrated by the absence of significant interfering peaks at the RT of creatinine and OAH (3.87 and 14.05 mins) upon comparison of blank chromatograms. This outcome suggests that the peaks corresponding to the analytes were pure, thereby confirming the specificity of the method.

Linearity and Range

Linearity was verified through the analytical curve using four concentration levels and evaluated in five injections.[21,28,29] The calibration curves were linear in the specified ranges of 0.1-1000 µg/mL for creatinine and 0.1-100 µg/mL for OAH. The correlation coefficient (r^2) for creatinine and OAH was 1 with linear regression equations Y =37.051 and Y =7.6284, respectively, in Figure 3.

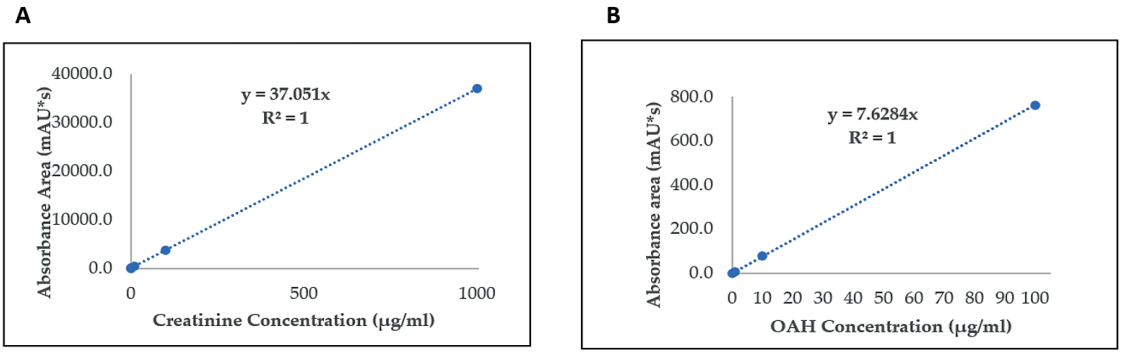

Figure 3*. Calibration curves of standard creatinine (A) and OAH (B).*

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ are used to calculate the sensitivity of the developed method.[29,30] The LOD were 0.258 and 0.045 µg/mL for creatinine and OAH, respectively. Moreover, the LOQ was 0.783 and 0.137 µg/mL for OAH and creatinine, respectively. These values are adequate for accurate and precise detection and quantification of OAH and creatinine.

Accuracy

Accuracy represents the closeness of the agreement between the mean values obtained from the series of measurements by the method and the actual value.[28,31] The corresponding HQC, MQC, and LQC values of creatinine and OAH were spiked into the normal urine sample, and percentage recovery was calculated along with standard deviation, which fell into 109.12%, 97.68%, 97.1% for creatinine and 91.35%, 94.0%, 96.39% for OAH, respectively. The relative standard deviation (RSD) was less than 2%, indicating a high accuracy of this method.

Precision

Precision represents the closeness of agreement among a series of measurements obtained from multiple sampling.[28] The peak areas were obtained by injecting HQC, MQC, and LQC for inter-day and intra-day studies. The results of both intra-day and inter-day findings ensure the high repeatability and precision of the developed method. All data were expressed in % RSD, less than the acceptable limit (<2.0%). Results for intra-and inter-day precision for OAH and creatinine are given in Table 2.

Table 2. Results of the precision parameter.

Parameters	Intra-day (Repeatability)		Inter-day (Intermediate precision)	
	Mean area±SD (N=6)	RSD (%)	Mean area±SD (N=6)	RSD (%)
Creatinine				
HQC	34344.0±52.16	0.15	34308.9±53.39	0.16
MQC	17141.1±18.94	0.11	17161.6±29.03	0.17
LQC	8533.2±10.05	0.12	8518.8±14.48	0.17
OAH				
HQC	7.1±0.05	0.66	7.2±0.09	1.73
MQC	3.6±0.02	0.65	3.6±0.05	1.67
LQC	1.8±0.01	0.60	1.8±0.01	1.29

Note: HQC: high-level quality control, MQC: middle-level quality control, LQC: low-level quality control.

Robustness

In an analytical method, robustness measures its reliability, characterized by its capacity to remain unaffected by minor, intentional changes in method parameters.[32] In all cases, creatinine and OAH peaks were symmetric, and the RSD (%) of a number of the theoretical plates (NTP) and tailing factor (TF) values were <2% with varying parameters. Results for robustness are presented in Table 3.

Table 3. Overview of robustness parameter.

Variables	Value	Conc. (µg/mL)	Peak area Mean±SD (N=3)	RSD (%)	RT (min) Mean±SD (N=3)	RSD (%)
Creatinine						
Flow rate (mL/min)	0.9	300	13747.1±113.88	0.83	4.16±0.05	1.23
	1	300	12408.57±44.6	0.36	3.80±0.04	1.15
	1.1	300	11229.53±80.65	0.72	3.68±0.01	0.27
Wavelength	233	300	12434.4+24.07	0.19	3.88±0.01	0.15
	235	300	12408.57±44.6	0.36	3.80±0.04	1.15
	237	300	12143.30±73.71	0.61	3.85±0.05	1.31
pH	6	300	11400.67±104.36	0.92	3.82±0.01	0.30
	6.5	300	12404.63±43.47	0.35	3.80±0.04	1.15
	7	300	12486.43±51.25	0.41	3.82±0.02	0.55
OAH						
Flow rate (mL/min)	0.9	100	813.87±3.35	0.41	14.51±0.06	0.42
	1	100	753.47±3.25	0.43	14.26±0.24	0.98
	1.1	100	659.60±0.87	0.13	13.59±0.03	0.19
Wavelength	338, 428	100	809.67±0.84	0.10	14.15±0.04	0.33
	340, 430	100	753.47±3.25	0.43	14.26±0.14	0.98
	342, 432	100	717.30±11.17	1.56	14.17±0.15	1.08
pH	6	100	756.93±6.38	0.84	13.57±0.15	1.13
	6.5	100	753.47±3.25	0.43	14.26±0.14	0.98
	7	100	796.07±12.12	1.52	13.61±0.08	0.57

Stability

The percentage of the original amount of both analytes remaining in the urine sample was calculated for different days at different temperatures. More than 80% of these two metabolites at various concentration levels remained in the urine sample for 20 days at -20 °C and -80 °C. However, HQC and LQC of OAH levels reduced to less than 80% at 4°C for 20 days.

Establishment of reference intervals

Table 4 summarizes the demographic characteristics of healthy population involved in this study. The optimized conditions were applied to quantify the OAH level of 120 urine sample. An example of HPLC chromatogram for the measurement of OAH and creatinine is shown in Figure 4.

Table 4. Sample characteristics of all samples presented in the study.

	Total N=120	Male N=55 (45.8%)	Female N=65 (54.2%)	p^a
Age	47.5 (35.25-57.0)	48 (37-60)	45 (34-55.5)	0.142
21-60	100 (83.33%)	43 (35.83%)	57 (47.5%)	
≥61	20 (16.67%)	12 (10%)	8 (667%)	
Smoking Status				
Yes	16 (13.33%)	16 (13.33%)	0 (0%)	
No	104 (86.67%)	39 (32.5%)	65 (54.17%)	
Exercise				
Yes	41 (34.16%)	20 (22.5%)	21 (17.5%)	
No	79 (64.16%)	35 (23.33%)	44 (36.67%)	
Alcohol				
Occasionally	43 (35.84%)	27 (22.5%)	16 (13.34%)	
Never	77 (64.16%)	28 (23.33%)	49 (40.83%)	
BMI	22.1 (20.10-23.97)	23 (20.80-24.20)	21 (19.60-23.90)	0.253
Normal weight (≤22.9)	69 (57.5%)	27 (22.5%)	42 (35%)	
Overweight (≥23)	51 (42.5%)	28 (23.33%)	23 (19.17%)	

Note: BMI: body mass index, N: number of samples. Data are presented as N (%). The median (interquartile range) is also given for age and BMI. [a]: Independent t test, p<0.05 is statistically significant different between male and female.

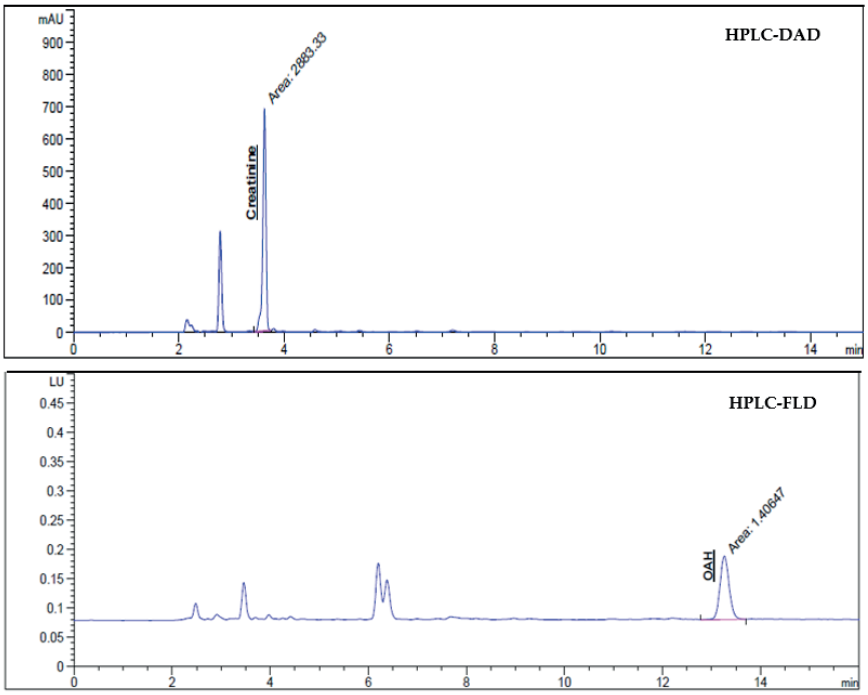

Figure 4. *HPLC chromatogram of human urine. Creatinine at retention time 3.62 mins detected by DAD detector, OAH at retention time 13.26 mins detected by FLD detector.*

The established reference intervals (RIs) for males was 0.469-2.244 mmole/mole creatinine, and females was 0.373-2.329 mmole/mole creatinine as shown in Figure 5. No difference was found when stratified by gender and age. Therefore, we expressed RIs for both, which were 0.420-2.287 mmole/mole creatinine.

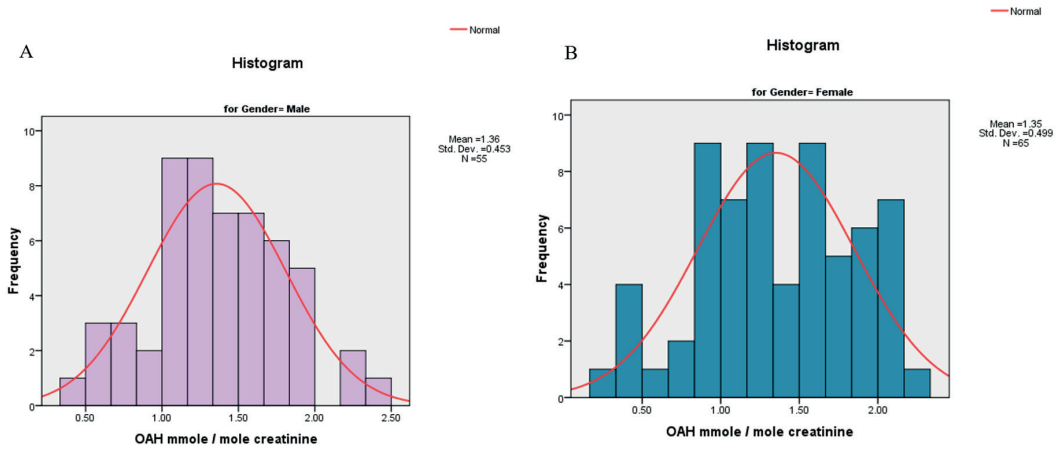

Figure 5. *Determination of reference intervals for OAH in males (A) and females (B).*

Discussion

This study aimed to identify and establish urinary biomarker OAH reference intervals with a simple method of high-performance liquid chromatography (HPLC). Although first-morning void urine samples are a better choice than random urine samples, the choices between them depend on the specific goals of the metabolomics study and practical considerations.[33] Since the current study aimed to establish reference intervals of OAH metabolite in a large-scale survey, first-morning void urine may be less convenient for participants. Random urine samples are easy to collect and often most cost-effective because they require less coordination and a close approach to routine analysis.

The previous synthesis of O-aminohippuric acid relied on multi-step synthetic routes and the use of highly toxic chemicals. In this study, we synthesized OAH with a simple route inspired by previous work. The synthesis was designed to eliminate the deprotection step of toxic carboxylic acid. In short, an efficient synthesis of OAH has been developed, and it was about 95% pure enough to use in HPLC analysis.

The recent method was never validated per guidelines, focusing on quantifying OAH with long analytical run times and low throughput. They mainly focused on the identification of OAH as a low-abundance urinary fluorescent metabolite as a potential lung cancer biomarker. Several steps are required for sample preparation, such

as solid phase extraction, precipitation, dilution, and filtration.[1] The creatinine concentration was analyzed using an enzyme-based method to normalize in each sample. We used simple HPLC method and rapid sample preparations, which allowed us to detect OAH, and creatinine concentration in each sample simultaneously, saving time and cost effectively.

Due to no significant difference being found by gender, reference intervals of OAH were expressed for both genders in the 0.420-2.287 mmole/mole creatinine range. The median concentration of OAH in healthy urine detected by HPLC was 1.335 mmole/mole creatinine. The median concentration of OAH in a previous study detected by mass spectrometry was 0.05 mmole/mole creatinine.[1] This is probably because detection could not differentiate other similar naturally fluorescent metabolites in urine from various factors such as food, drugs, and other amino acid tryptophan metabolites.[34,35] We selected participants according to our criteria and collected urine samples left over from the clinic that were reported as normal urine from urine analysis. However, samples were not collected under stringent conditions of diets, exercise, drugs, or hydration, which can be influenced by variations even though we did questionnaires or applied creatinine normalization.

A previous study identified a peak with a mass per charge ratio of mass spectrometry and confirmed using a commercially available standard.[1] Thus, a nuclear magnetic resonance (NMR) study is needed to confirm the presence of only OAH metabolites using selected peak fragments in HPLC. This is the first study to quantify and establish reference intervals of OAH levels in the urine samples of healthy people. However, the utility of these metabolites has not been evaluated in other cancers, and its potential to aid early diagnosis of lung cancer remains to be further assessed. The study's strength is simple sample preparation and immediate processing after urine collection to avoid storage bias. We have some limitations in proving the OAH level detection in lung cancer patient's urine. This would require us to obtain and analyze some time points. Thus, further studies are needed to quantify noninvasive urinary biomarkers OAH in lung cancer patients because these fluorescent metabolites may screen lung cancer and other cancers effectively.

Conclusion

In summary, we developed a simple and accurate RP-HPLC method for the simultaneous measurement of creatinine and OAH. The validation results proved that the process is accurate, precise, sensitive, and reproducible for targeted metabolomics studies. We interrogated urine for cancer biomarkers, and the method requires minimal preparation before being subjected to HPLC. The reference interval of OAH in healthy individuals was 0.420-2.287 mmole/mole creatinine. The results of this investigation suggest that the established approach may be evaluated in further steps to measure these metabolites in healthy individuals and other cancer patients in preclinical or clinical trials.

Conflicts of Interest

The authors declare no conflict of interest.

Funding

This work was funded by the Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand.

Acknowledgements

The first author is grateful for the CMU Presidential Scholarship, which supported the master's degree program in Medical Technology at the Faculty of Associated Sciences, Chiang Mai University.

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