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Determination of Unbound Urinary Amino Acids Incorporated with Creatinine Normalization by LC-MS/MS Method with CLAM-2000 Online Sample Pre-treatment

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Introduction

Free or unbound amino acids are important metabolites in human blood and urine [1]. The profile of unbound amino acids in urine are the reference indication of metabolic imbalances and amino acid transport disorders as well as dietary protein adequacy and assimilation. Creatinine produced by muscle metabolism is excreted in the urine, which can be used to normalize the metabolite levels to compensate the large variation due to different intakes of water and fluid food [2-4]. The aim of this study is to develop a reliable LC-MS/MS method for quantitation of 22 free amino acids and creatinine in urine samples. A derivatization-free LC-MS/MS amino acid method [5] with stable isotope labelled IS was employed. An on-line sample pre-treatment module CLAM-2000 coupled with LC-MS/MS makes the analysis fully-automated, which enables from adding internal standards, sample and solvent mixing, shaking for protein-crash and filtration to transferring the final sample solution to LC-MS/MS for analysis.

Experimental

A stock solution of 22 amino acids (AA) and creatinine (CRE) were prepared from powder standards. Two isotope-labelled amino acid standards, phenylalanine (ring-d5) and serine (2,3,3-d3) were added to the samples as internal standard by CLAM-2000 prior to LC-MS/MS analysis. A total of 28 urine samples were collected from health individuals of different ages and genders and used for amino acid analysis in this study. The CLAM-2000 is an automated on-line sample pre-treatment module (Shimadzu Corporation), which was employed for urine sample preparation automatically for a connected LC-MS/MS system. The automated operation includes adding IS, sample-solvent mixing, shaking and filtration etc. The prepared sample was subsequently transferred to LC-MS/MS triple quadrupole system (LCMS-8040) for analysis (Figure 1). An Amino Acid Column (100x3mm, 3µm) was adopted for separation of the 22 compounds with an optimized gradient elution program. The detailed LC and MS/MS conditions are compiled in Table 1.

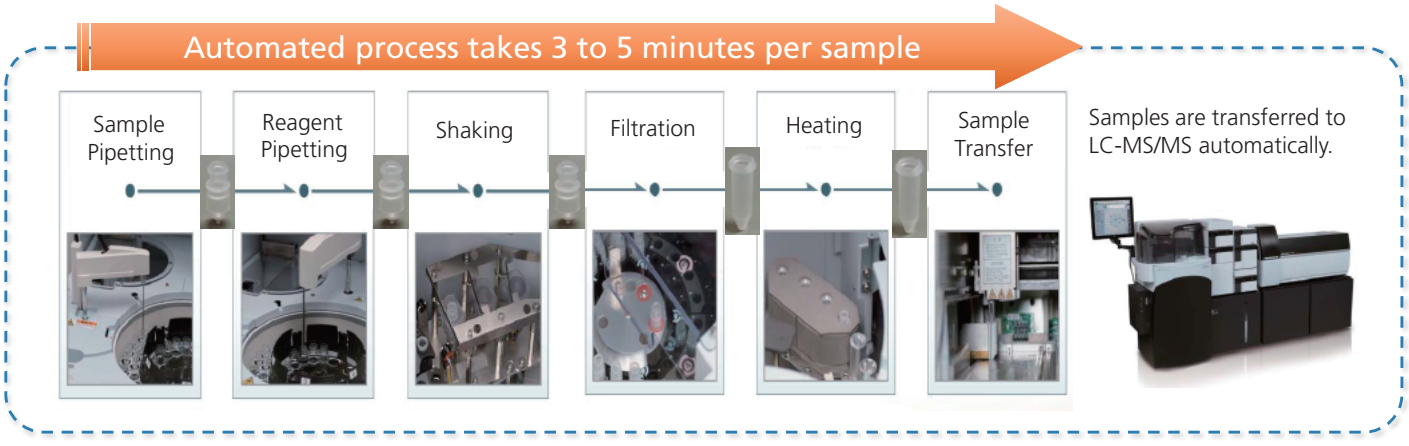

Figure 1: Workflow of CLAM-2000: a fully automated sample preparation module coupled with LCMS-8040

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Table 1: Analytical conditions of twenty two amino acids without derivatization on LCMS-8040 and CLAM-2000

Column	: Intrada Amino Acid (100 mmL x 3 mmID, 3µm)
Flow rate	: 0.6 mL/min
Mobile phase	: A: ACN / THF / 25mM ammonium formate / FA = 9 / 75 /16 / 0.3 (v) B: ACN / 100mM ammonium formate = 20 / 80
Elution mode	: Gradient elution, 0-3min (0% B) → 9min (17% B) → 16-18.5min (100% B) → 19min (0% B)
Oven temp.	: 35°C
Injection vol.	: 5.0 µL
Interface	: ESI
MS mode	: Posi, MRM
Block temp.	: 400°C
DL temp.	: 250°C
CID gas	: Ar (230kPa)
Nebulizing gas flow	: N_2, 2 L/min
Drying gas flow	: N_2, 15 L/min

Results and Discussion

Quantitative method for 22 AA and CRE on CLAM-LC-MS/MS platform

The 20 proteinogenic amino acids (AA), citrulline (Cit) and ornithine (Orn) as well as creatinine (CRE) are the targeted analytes in urine in this study. This is because citrulline and ornithine are the dietary amino acids in the urea cycle along with arginine. A MRM method for quantitative analysis of the 22 AA and CRE (creatinine) was established with IS (internal standard) method as summarized in Table 2. In a previous study [5], it was observed that glycine exhibited low peak intensity and sensitivity in MRM mode (76.1>30.1). Thus, SIM mode (m/z 76.1) was selected for glycine. Figure 2 shows the MRM chromatograms of the 22 AA and CRE mixed standards obtained on CLAM-LC-MS/MS platform. For calibration curve construction, a calibrant series of eight levels (0.1, 0.5, 1, 2, 5, 10, 50 and 100 µM) were prepared and analysed. A few selected calibration curves by IS method are shown in Figure 3. The accuracy and repeatability (based on area) of the method with 10 uM mixed standards are shown in Table 2 and the results obtained with urine samples (not shown in the table) are also satisfied.

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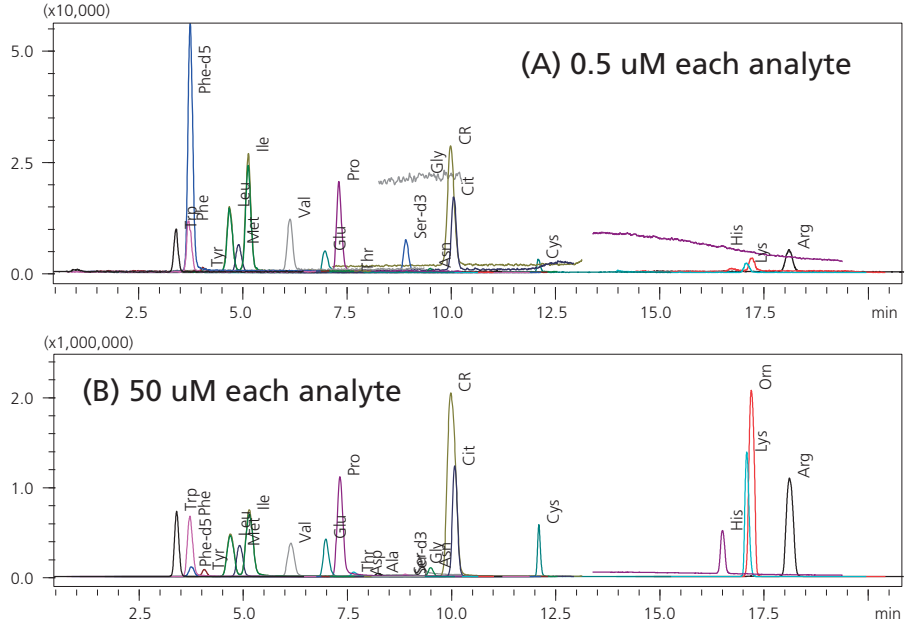

Figure 2: MRM (SIM for Gly) chromatograms of 22 AA and CRE in mixed standards with (5µL inj. volume).

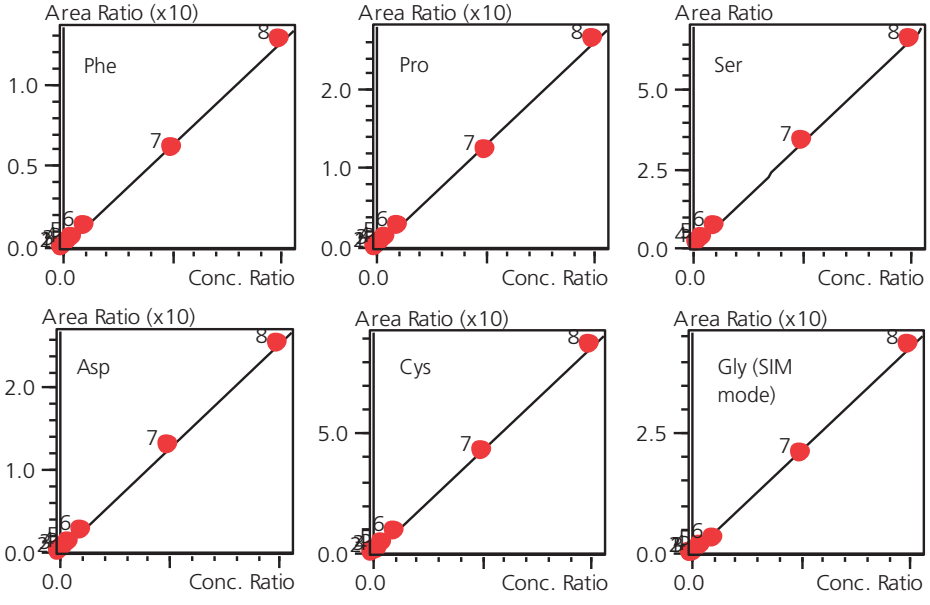

Figure 3: Representative calibration curves of eight levels at from 0.1 µM to 100 µM on CLAM-LC-MS/MS.

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Table 2: Summary of MRM quantification method and performance for analysis of 22 AA and CRE on CLAM-2000 coupled with LC-MS/MS: calibration range, linearity, LOQ, accuracy and repeatability (%RSD)

No.	Name	RT (min)	MRM transition (m/z)	IS Calibration Curves and Quantitation Performance				
				Range (μM)	R^2	LOQ (μM)	Accuracy (%)	%RSD (n=6)
1	Trp	3.39	205.10>188.20	0.1-100	0.998	0.14	93.5	2.1
2	Phe	3.67	166.10>120.10	0.1-100	0.999	0.12	99.4	1.6
3	Tyr	3.99	182.10>136.20	0.5-100	0.996	0.78	89.3	0.7
4	Leu	4.62	132.10>86.30	0.1-100	0.998	0.14	98.2	1.5
5	Met	4.81	150.10>56.10	0.1-100	0.999	0.11	104.5	1.6
6	Ile	5.11	132.10>86.30	0.1-100	0.998	0.10	97.7	1.0
7	Val	6.06	118.20>72.05	0.5-100	0.997	0.26	98.8	2.8
8	Glu	7.01	148.10>84.10	0.1-100	0.996	0.12	101.2	2.3
9	Pro	7.26	116.10>70.10	0.1-100	0.999	0.07	104.6	2.2
10	Thr	7.62	120.10>74.00	1-100	0.997	0.9	103.0	4.3
11	Asp	7.81	134.10>73.90	5-100	0.994	5.0	102.5	4.6
12	Ala	8.16	90.10>44.10	1-100	0.995	1.5	103.4	3.3
13	Ser	8.91	106.10>60.20	1-100	0.999	1.6	97.2	3.3
14	Gln	9.10	147.10>84.10	0.1-100	0.999	0.1	106.6	11.8
15	Gly	9.30	76.1 (SIM)	2-100	0.998	4.6	100.3	6.7
16	Asn	9.38	133.10>74.10	0.5-100	0.999	0.66	99.3	3.4
17	Cit	9.98	176.00>70.10	0.1-100	0.997	0.10	101.2	4.6
18	CRE	10.08	114.00>44.00	0.1-100	0.996	0.12	99.3	2.6
19	Cys	12.13	241.00>151.95	0.1-100	0.999	0.12	99.1	8.4
20	His	16.52	156.10>110.10	2-50	0.991	2.2	103.2	7.4
21	Lys	16.95	147.00>84.10	0.5-50	0.996	0.5	109.0	12.9
22	Orn	17.21	133.10>70.05	0.5-50	0.995	0.7	113.3	16.2
23	Arg	17.97	175.10>70.10	0.5-50	0.993	0.3	105.5	8.6

Notes: (1) LOQ values refer to the AA concentrations in neat solution; (2) Accuracy and repeatability values refer to 10 μM level

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Automated batch-run for analysis of 22 AA in 28 urine specimens

A total of 28 urine specimens were collected from 14 male and 14 female health volunteers age between 20 and 30 years old. The urine samples were stored in sealed plastic tubes at -20°C. The samples were analysed in a sequenced batch-run on the CLAM-2000 coupled with LCMS-8040. The automated pre-treatment of each sample on CLAM-2000 involves: (a) pipetting 40 µL of urine, 120 µL of organic solvent (MeOH/ACN) and 40 µL of IS, (b) votexing the mixture, (c) vacuum filtering to obtain clear sample solution and (d) transferring to the autosampler and injecting to LCMS-8040.

A batch-run includes (A) Blank, (B) Calibration series, (C) QCs and (D) samples. The sequence starts from a blank (milli-Q water), followed by calibration series → bank → QC low and QC high → blank → 8-10 samples → blank → QCs → 8-10 samples. The blank was used to ensure no sample carry-over, while QC low and QC high were placed between different group of samples to monitor the loss during sample preparation and ensure the reliability of the results. There was not significant matrix effect of the method as observed in a separate study.

Total AA and Total Creatinine-Normalized AA (TCNA)

Figure 3(a) shows the results of total 22 amino acid (AA) and creatinine (CRE) of 28 urine specimens. The variation of both total AA and CRE are significant, e.g., the total AA scattering in 543~16,640 uM and CRE in 438~2,261 uM. Figure 3(b) displays their molar of Total AA / CRE, named as Total Creatinine-Normalized AA (TCNA). It can be seen that the 28 urine specimens can be classified into three groups according to the TCNA ratios:

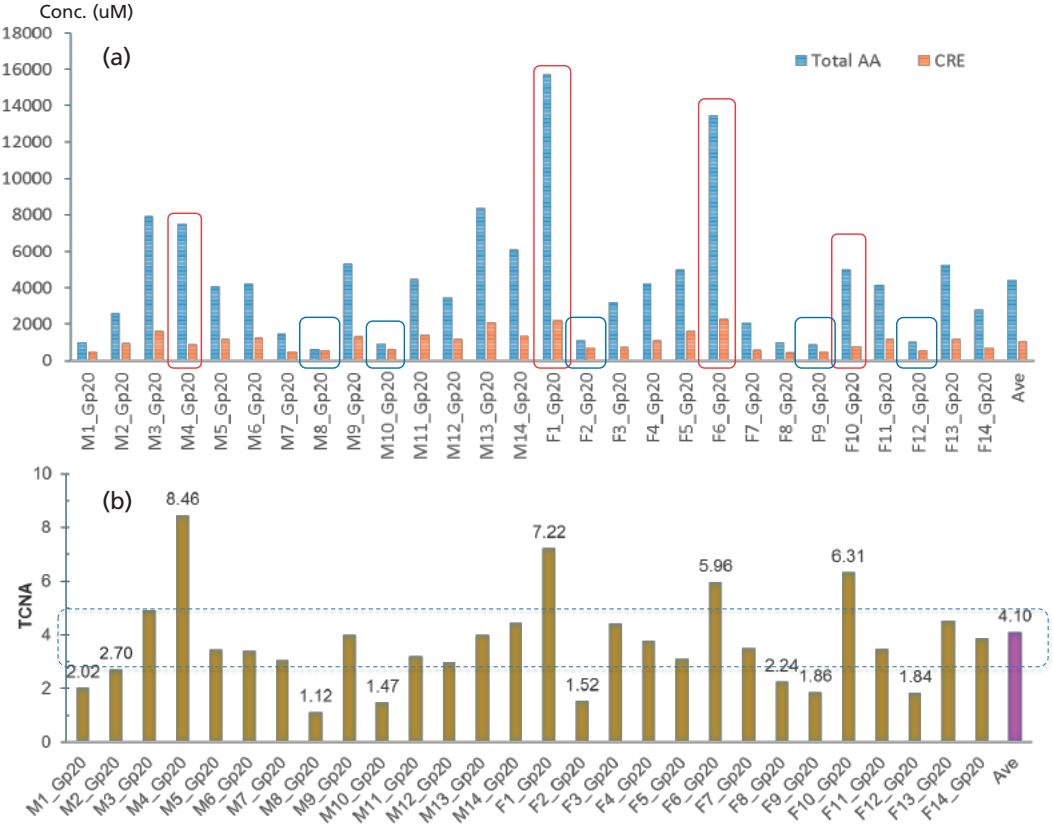

Figure 3: (a) Total AA and creatinine (uM) and (b) Total creatinine-normalized AA (TCNA) of 28 urine specimens from 14 male and 14 female health volunteers age at 20~30 years old

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Table 3: P values of the 21 AA variables between two groups identified according to different TCNA

Amino Acid	TCNA (R) = 1.12~3.25			TCNA (R) = 3.45~8.46			*P-value*
	Ave	Max	Min	Ave	Max	Min	
Trp	3.06	5.96	1.20	4.62	7.82	1.95	0.0056
Phe	2.64	4.00	0.96	4.05	6.32	2.24	0.0005
Tyr	4.73	9.36	1.34	8.04	22.30	3.40	0.0135
Leu	3.35	6.06	0.88	4.07	9.60	0.68	0.1273
Met	0.54	1.48	0.11	1.05	2.20	0.26	0.0022
Ile	1.63	3.38	0.36	2.00	4.11	0.13	0.1477
Val	2.88	4.98	1.48	3.23	5.40	1.26	0.1478
Glu	2.17	4.67	0.52	3.04	10.49	1.16	0.2625
Pro	0.83	1.72	0.14	0.58	1.22	0.13	0.0339
Thr	4.95	10.19	2.08	8.44	19.91	2.95	0.0067
Asp	1.27	4.11	0.11	1.11	6.14	0.06	0.3003
Ala	25.22	51.57	5.90	52.95	114.75	25.96	0.0013
Ser	23.23	42.77	6.12	40.03	90.00	5.20	0.0115
Gln	32.78	51.31	18.40	43.17	67.25	31.47	0.0254
Gly	61.36	125.16	25.27	128.67	249.43	36.18	0.0032
Asn	4.36	7.87	1.00	10.11	24.02	1.07	0.0032
Cys	3.62	6.84	1.19	7.07	22.97	1.87	0.0133
His	49.94	128.20	16.69	140.26	290.32	65.77	0.0000
Lys	3.15	13.77	0.51	24.17	101.00	2.45	0.0075
Orn	0.30	1.18	0.07	1.60	6.60	0.14	0.0088
Arg	1.18	3.74	0.18	2.88	9.47	0.19	0.0092

very low (1.12 ~ 2.24), very high (5.96 ~ 8.46) and the rests (2.70 ~ 4.92) closer to the average of 4.10.

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AA profile and statistics analysis

Statistics analysis of 21 AA (Cit is excluded) in urines based on the TCNA model are shown in Table 3 and Figure 4. A best grouping according to TCNA (R) is found at R=1.12~3.25 and 3.45~8.46 based on p-value evaluation. With this assumption, most AA exhibit statistically significant differences *(P<0.05)* except five AA which *p-values* are greater than 0.05. Further multivariate analysis (PCA) reveals that the two groups identified from *p-value* evaluation are distinct, and the few very high values of TCNA (5.96~8.46) are the outliers. This result indicates that the TCNA value can be regarded as a key factor in analysis of AA profile, which may provide a new approach in characterization of AA profiles linking with diagnosis of disease and health conditions [4].

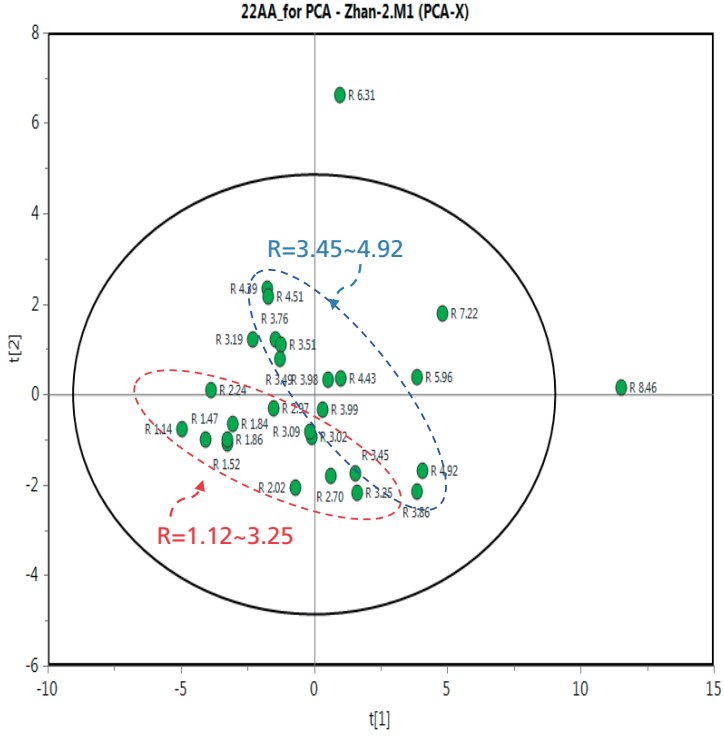

Figure 4: PCA score plot of TCNA (R) for 21 amino acids of 28 urine specimens which are classified in two groups according to TCNA using SIMCA P+ program.

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Conclusions

A fully-automated method for quantitative analysis of 22 amino acids (AA) and creatinine (CRE) in urines was established on a novel platform of CLAM-LC-MS/MS. An Intrada Amino Acid column adopted allows the analysis of AA without derivatization by LC/MS/MS. Twenty-eight urine specimens from health volunteers were analysed and the results were used to calculate total creatinine-normalized AA (TCNA), from which two distinct groups of urines are identified. Multivariate analysis of the AA profiles of the 28 specimens confirms the significant importance of the classification based on TCNA, which may provide a new approach in characterization of AA profiles linking with diagnosis of disease and health conditions.

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