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Application News

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Clinical Research / LCMS-8040

Direct Determination of Plasma Free Amino Acids by Combined MRM-SIM Method on LC/MS/MS

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❑ Introduction

Unbound or free amino acids in human blood and urine are important metabolites, which are either ingested or synthesized endogenously in human body. The levels and profiles of the so-called plasma free amino acids are determined clinically for diagnosis of specific diseases and researches of cancer biomarker discovery [1-3]. Analyses of amino acids by HPLC and LC-MS methods are well-established unavoidably with pre-column derivatization to improve chromatographic separation and detection sensitivity. A direct LC/MS/MS analysis method for amino acids without derivatization is adopted in this study, which could be achieved essentially due to use of a combined mode column [4-5]. We described here a method and performance of quantification of twenty free amino acids in human plasma and serum using a combined MRM-SIM mode on triple quadrupole LC/MS/MS. The purpose of incorporating SIM mode in the method is to enhance the detection sensitivity of a few very small amino acids like glycine, which MRM transition is rather poor leading to low sensitivity.

❑ Experimental

Analytical conditions

An LCMS-8040 triple quadrupole system coupled with an UFLCXR was employed in this work. A novel combined-mode *Amino Acid* column [4] was used with an optimized gradient elution program for twenty genetically encoded amino acids. The details of HPLC and MS conditions are compiled into Table 1.

Table 1: Analytical conditions of amino acids on LCMS-8040

Column	Amino Acid Column (100 x3 mm,3µm)
Mobile Phase	A: ACN/THF/25mM ammonium formate/formic acid: 9/75/16/0.3 B: ACN/100mM ammonium formate: 20/80
Elution Program	Gradient elution, 0%B (0-3.0 min), 0-17%B (3.0-9.0 min), 17-100%B (9.0-16.0min), 100 %B (16.0-19.0 min), 0%B (19.50 min)
Flow Rate	0.6 mL/min
Oven Temp.	35 ºC
Injection	2 µL
Interface	ESI
MS Mode	MRM & SIM mode, Positive
Block Temp.	400 ºC
DL Temp.	300 ºC
Nebulizing gas	N_2, 1.5 L/min
Drying gas	N_2, 15 L/min

Preparation of standards and bio-samples

Twenty amino acid standards (see Table 2) in powders were obtained from Sigma Aldrich. The compounds were dissolved in 0.1 mol/L HCl aqueous solution except for cystine and glutamine in 1.0 mol/L HCl solution, to obtain individual stock solutions. A mixed standard was prepared from the stocks and was diluted serially using Milli-Q water to various concentrations as calibrants. Pooled human plasma and serum were obtained from certified suppliers. The bio-sample was deproteinized by adding mixed solvent of MeOH-ACN (50:50) in a ratio of 3 to 1 (solvent to sample), followed by shaking for 3 mins and centrifugation at 13,000 rpm for 10 mins. The supernatant was transferred and filtered with 0.2µm syringe filter before LC/MS/MS analysis.

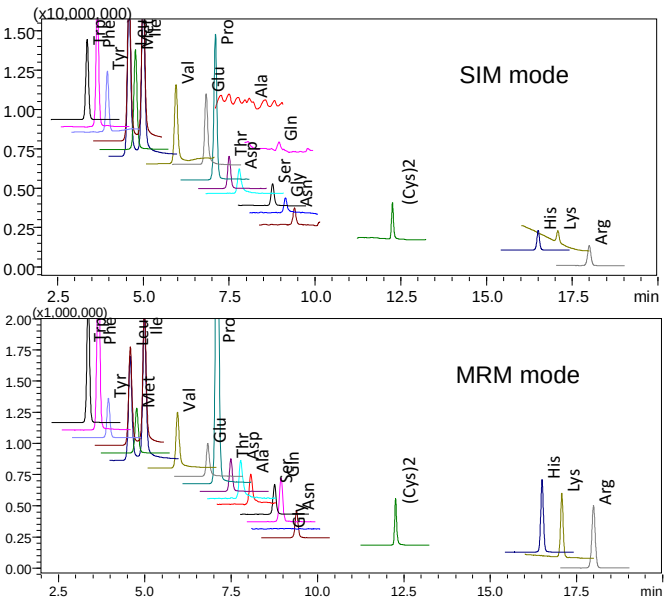

Figure 1: MRM-SIM chromatograms of twenty genetically-encoded amino acids in a mixed standard with 50µM each. Injection vol: 2µL

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❑ Results and Discussion

A. Combined MRM-SIM method and performance

In this study, a combined MRM-SIM method was created and applied for analysis of the 20 amino acids, which results are illustrated in Figure 1. Incorporating SIM (Q3) mode into a MRM method is to improve the sensitivity of a few small amino acids, especially glycine (Gly), which MRM sensitivity is rather poor. To determine LOD and LOQ of the method in MRM and SIM modes, mixed standards of 0.5, 1.0 and 5.0 µM were prepared and injected to LC/MS/MS. As shown in Table 2, the LOQs in MRM mode are at 0.12~0.81 µM for ten amino acids and 1.0~7.2 µM for eight amino acids. The LOQs of Ala in MRM mode are 13.7 µM, and that of Gly is as poor as 528 µM. While, the LOQs of Ala and Gly in SIM mode are 132 µM and 17.6 µM, respectively. Based on above, a final MRM-SIM method for quantification includes 19 MRM and 1 SIM (Gly) channels. The Individual peaks of the amino acids at concentrations closer to the LOQs are displayed in Figure 2. Figure 3 shows a few selected calibration curves, which were established for quantification of the 20 amino acids in plasma and serum samples. The calibration curves range for 10~100 µM, with good linearity of R^2>0.992 (Table 2).

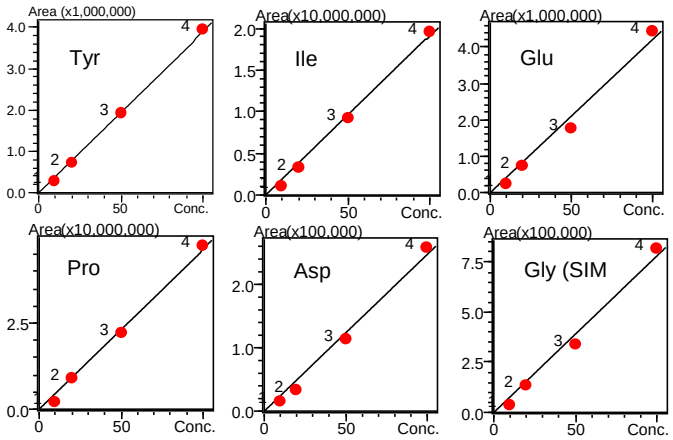

Figure 3: Selected calibration curves of amino acid standards in MRM mode except Gly in SIM mode. Injection vol: 2µL.

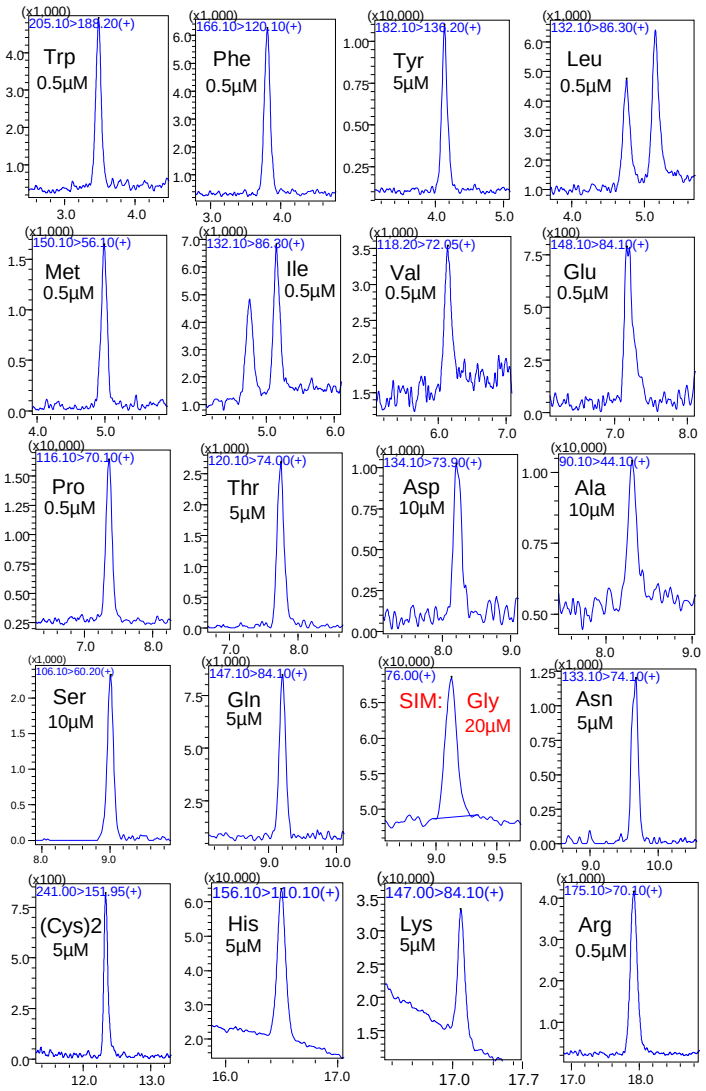

Figure 2: Individual MRM peaks of amino acid (except glycine in SIM) at low concentrations. Injection vol: 2µL.

Table 2: Summary of calibration range, linearity and detection sensitivity of a combined MRM-SIM method for amino acids on LCMS-8040

No	Name	m/z	Ret Time (min)	Range (µM)	R^2	LOD (µM)	LOQ (µM)
1	Trp	205.1>188.2	3.42	10-100	0.998	0.05	0.16
2	Phe	166.1>120.1	3.74	10-100	0.999	0.04	0.12
3	Tyr	182.1>136.2	4.06	10-100	0.999	0.55	1.7
4	Leu	132.1>86.3	4.69	10-100	0.999	0.13	0.4
5	Met	150.1>56.1	4.91	10-100	0.999	0.08	0.25
6	Ile	132.1>86.3	5.10	10-100	0.999	0.09	0.28
7	Val	118.2>72.0	6.09	10-100	0.998	0.17	0.52
8	Glu	148.1>84.1	7.06	10-100	0.992	0.09	0.26
9	Pro	116.1>70.1	7.29	10-100	0.998	0.07	0.21
10	Thr	120.1>74.0	7.68	10-100	0.995	0.27	0.81
11	Asp	134.1>73.9	8.05	10-100	0.997	2.39	7.2
12	Ala	90.1>44.1	8.25	10-100	0.992	4.52	13.7
13	Ser	106.1>60.2	8.94	10-100	0.999	0.63	1.9
14	Gln	147.1>84.1	9.13	10-100	0.998	0.84	2.6
15	Gly	76.0 (SIM)	9.38	10-100	0.994	5.8	17.6
16	Asn	133.1>74.1	9.56	10-100	0.994	0.34	1.0
17	(Cys)2	241.0>151.9	12.31	10-100	0.994	0.43	1.3
18	His	156.1>110.1	16.57	10-100	0.992	0.73	2.2
19	Lys	147.0>84.1	17.15	10-100	0.994	1.13	3.4
20	Arg	175.1>70.1	18.10	10-100	0.999	0.09	0.26

B. Direct analysis of free amino acids in human plasma and serum

One of the purpose of this study is to evaluate the robustness of the method for direct quantification of the 20 free amino acids in biological samples, i.e., human plasma and serum, without pre-column derivatization and additional clean-up except deproteinization and filtration. Both MRM and SIM quantification methods were applied and the results were compared with each other for actual biological samples.

Two plasma (P-1 and P-2) and one serum (S-1) samples were used and each sample was duplicated (a & b) from sample preparation to LC/MS/MS analysis. The SIM and MRM chromatograms of P-1(a) are displayed in Figure 4. The quantification results are summarized in Table 3. Noted that, the concentrations of amino acids listed in the table are the results multiplying the dilution factor (DF = 4) incurred in the sample preparation step. It can be seen that the method is capable of quantification of the 20 amino acids in both MRM and SIM modes except glycine. The MRM and SIM results are in agreement with each other for most amino acids. However, there are certain discrepancies between SIM and MRM results for some amino acids, e.g., alanine in S-1(a & b). The big difference may be due to its concentration in the sample beyond the linear range of the calibration curves. Furthermore, the MRM and SIM results for the plasma and serum samples are compared in more detailed. Figures 5-7

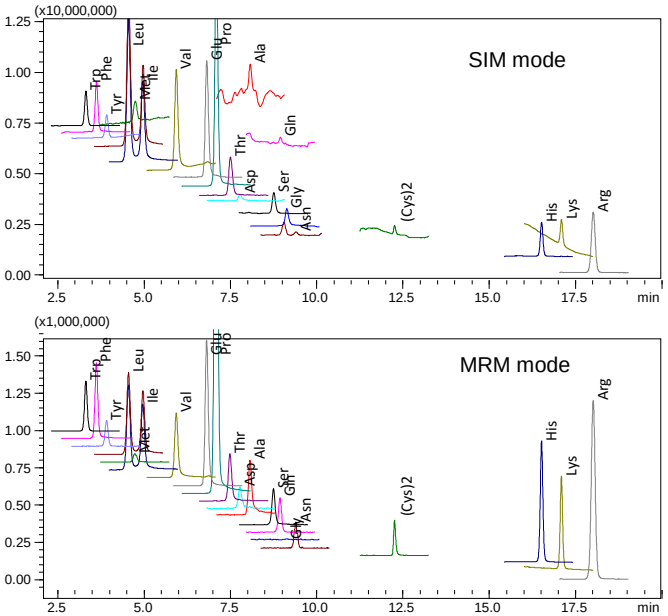

Figure 4: Chromatograms of human plasma P-1(a) in SIM & MRM modes acquired in same analysis on LCMS-8040. Injection vol: 2 uL.

show the peak-to-peak comparison for three amino acids, Gln, Gly and Met. For glutamine (Figure 5), the MRM peak is much more sensitive than the SIM peak which was interfered by the high baseline. As a result, the SIM mode sensitivity and analysis accuracy are decreased. On the other hand,

Table 3: Quantification results of free amino acids in human plasma and serum (pooled) by direct MRM-SIM method on LCMS-8040 using combined mode Amino Acid column. (N.D. = Not Detected)

ID#	Name	m/z	Ret. Time	Concentration of Amino Acid (μM) in Plasma and Serum sample					
				P-1(a)	P-1(b)	P-2(a)	P-2(b)	S-1(a)	S-1(b)
1	Trp	205.1	3.32	69.1	70.5	66.1	55.0	16.8	18.4
		205.1>188.2	3.31	66.8	67.4	67.4	55.5	16.6	17.6
2	Phe	166.1	3.62	54.8	61.4	64.2	56.5	43.1	42.4
		166.1>120.1	3.62	58.0	61.7	66.0	56.2	41.9	44.9
3	Tyr	182.1	3.92	56.0	56.4	60.8	45.0	37.4	47.7
		182.1>136.2	3.91	55.7	56.8	65.4	47.8	39.2	43.6
4	Leu	132.1	4.55	138.1	143.4	138.6	126.3	213.1	232.4
		132.1>86.3	4.55	138.5	137.3	143.1	128.0	206.7	223.6
5	Met	150.1	4.75	1.6	1.8	2.5	2.0	0.3	0.4
		150.1>56.1	4.73	1.6	1.7	2.5	2.1	0.3	0.4
6	Ile	132.1	4.97	79.0	82.1	82.4	73.9	46.6	50.8
		132.1>86.3	4.97	76.7	75.7	79.0	71.5	42.6	43.2
7	Val	118.2	5.93	181.0	180.8	179.6	166.0	145.6	156.9
		118.2>72.1	5.93	185.5	189.0	200.7	179.5	156.4	161.9
8	Glu	148.1	6.82	636.7	670.9	520.7	405.1	443.8	475.6
		148.1>84.1	6.81	635.6	672.1	534.5	410.8	460.5	497.5
9	Pro	116.1	7.09	241.0	247.2	234.2	201.2	192.1	211.4
		116.1>70.1	7.09	238.8	244.7	247.5	202.6	192.9	209.5
10	Thr	120.1	7.5	174.2	192.2	169.8	146.8	196.3	214.8
		120.1>74.0	7.49	140.3	151.6	136.1	101.1	172.0	190.4
11	Asp	134.1	7.78	38.4	43.0	33.4	24.3	120.6	139.1
		134.1>73.9	7.78	29.8	38.0	35.6	25.1	127.6	140.4
12	Ala	90.1	8.07	479	468.7	364.7	322.4	504.5	581.9
		90.1>44.1	8.07	464.9	485.8	375.4	371.5	693.2	801.2
13	Ser	106.1	8.76	158.3	167.8	136.4	96.0	273.3	302.1
		106.1>60.2	8.75	166.5	171.8	145.8	95.0	291.5	319.4
14	Gln	147.1	8.95	85.6	96.5	45.2	33.6	126.2	127.8
		147.1>84.1	8.94	122.4	127.0	68.8	44.2	198.0	205.5
15	Gly	76.0	9.13	321.9	321.6	248.0	187.3	266.7	297.6
		76.0>30.1	9.14	N.D	N.D.	N.D.	N.D.	N.D.	N.D.
16	Asn	133.1	9.40	14.9	17.4	11.3	10.8	11.5	13.7
		133.1>74.1	9.39	12.9	15.0	10.8	8.2	12.0	12.5
17	(Cys)2	241	12.26	9.6	9.4	7.1	4.9	4.8	5.6
		241.0>152.0	12.25	11.0	10.4	8.6	5.8	4.4	4.7
18	His	156.1	16.51	126.9	118.7	37.5	63.7	39.5	36.0
		156.1>110.1	16.51	131.6	123.1	38.8	65.2	42.6	38.0
19	Lys	147	17.09	287.1	274.8	60.5	119.6	333.4	331.2
		147.0>84.1	17.09	297	290.9	62.0	135.2	347.5	335.0
20	Arg	175.1	18.01	516.1	458.1	64.7	227.7	325.1	254.6
		175.1>70.1	18.01	521.5	458.6	64.7	230.0	327.2	250.4

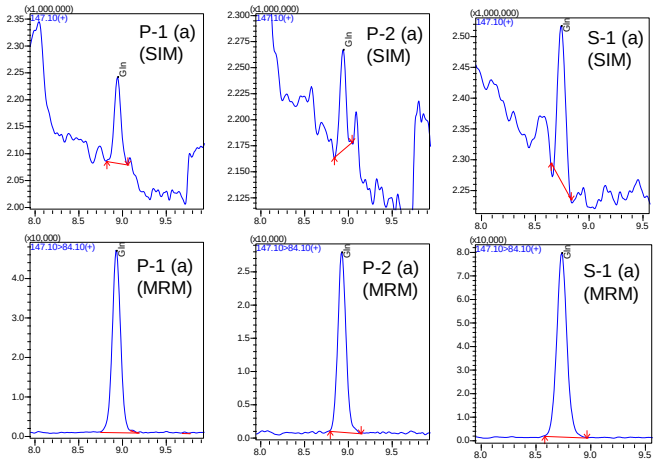

Figure 5: Glutamine (Gln) peaks in SIM mode (top) and MRM mode (bottom) in plasma and serum samples. Injection vol: 2µL.

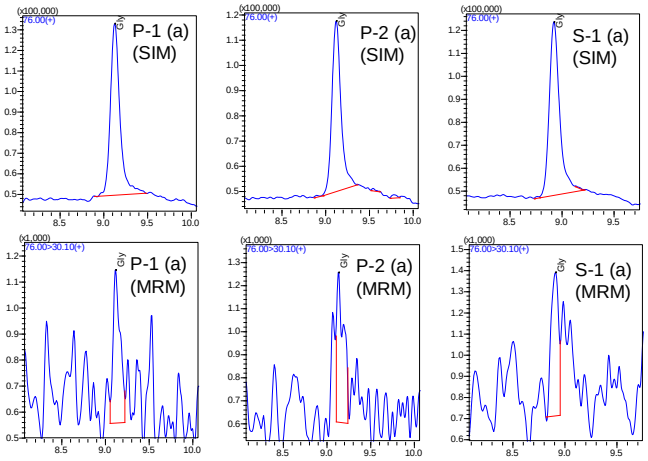

Figure 6: Glycine (Gly) peaks in SIM mode (top) and MRM mode (bottom) in plasma and serum samples. Injection vol: 2µL

glycine exhibits very poor MRM peak intensity and sensitivity, while its SIM mode sensitivity is much higher (Figure 6). As a result, the quantification result of glycine in SIM mode is much better in accuracy and reliability. Methionine was found to be the lowest level amino acid in the plasma and serum samples. As shown in Figure 7, both MRM and SIM peaks are well accepted and the quantitative results (Table 3) are in good agreement.

The amino acid profiles in these pooled human plasma and serum samples used in this study are not meaningful clinically. This study focuses on method evaluation to prove the applicability and robustness of the analysis method for plasma and serum samples. The profiles obtained, which show certain levels of differences from those reported in literatures [1-3], fall in a pattern, e.g., the levels of Glu, Ala and Arg in the plasma and serum samples are very high, while Met and (Cys)2 levels are very low compared to other amino acids. It is also noted that the Gln levels in all the samples are lower than that reported in literature [1-3].

It was reported [2] that in clinical analysis of free amino acids in human plasma, Glu, Asp and Cys are not measured because of their instability in blood samples. While, the rest 17 amino acids as well as citrulline (Cit) and ornithine (Orn) are determined [2]. In addition, quantification of creatinine in clinical plasma and urine is often required for determining the levels of specific amino acids per unit amount of creatinine in the sample. The current MRM-SIM method has been proven to be suitable for determining all of the amino acids as well as Cit, Orn and creatinine (results are not displayed).

❑ Conclusions

Using a novel amino acid column, a combined MRM-SIM method has been established for detection and quantification of 20 free amino acids in plasma and serum. This novel column can separate effectively the amino acids without need of pre-column derivatization, which allow direct analysis of

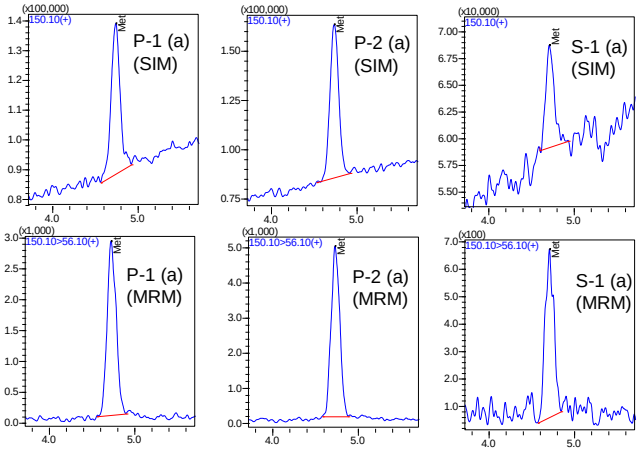

Figure 7: Methionine (Met) peaks in SIM mode (top) and MRM mode (bottom) in plasma and serum samples. Injection vol: 2µL

amino acids on LC-MS. The advantages of a combined MRM-SIM method on LC/MS/MS are the higher sensitivity for glycine in SIM mode and overall enhanced reliability, robustness and accuracy in comparison with the only MRM method or SIM method.

❑ References

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