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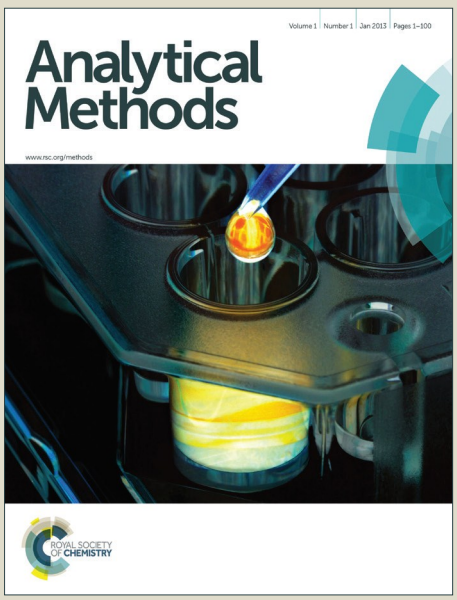

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Simultaneous quantitation of nine kinds of (D)-and (L)-amino acid enantiomers by HPLC-MS/MS: Application to the quality control of amino acid tablets

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Abstract

A simple and sensitive HPLC-MS/MS method was developed for simultaneous determination of nine kinds of (D) - and (L)-amino acid enantiomers in amino acid tablets. 7-chloro-4-nitrobenzoxadiazole (NBD-Cl) was selected as derivatization reagent, and the derived amino acid enantiomers were enantioseparated on Sumichiral OA-2500S (250 mm×4.6 mm, 5 μm) column, using a mobile phase composed of acetonitrile-methanol (50:50, v/v) containing 0.5% formic acid at the flow rate of 1.0 mL·min-1 with split ratio of 1:3. Sensitive detection was performed with 4000 Qtrap MS/MS system in electrospray-ionization source by negative mode. The calibration curves for the determination of all the nine pairs of amino acid enantiomers showed good linearity (R2 > 0.999) over the investigated ranges from 0.15 to 30 μg·ml−1 for the (D)-enantiomers and from 3.14 to 620 μg·ml−1 for the (L)-enantiomers, respectively. The assay was reproducible with overall intra- and inter-day variations of less than 7.7%. The detection for (D)-amino acid enantiomers was selective and sensitive, and trace amount of them could be detected and quantified, even if in the presence of massive corresponding (L)-enantiomers. The validated method was successfully applied to simultaneous quantitation of the nine kinds of (D) - and (L)-amino acid enantiomers in commercial tablets.

1. Introduction

Amino acids are essential for life, and have many functions in metabolism[1, 2]. Amino acid-induced protein synthesis leads to increased muscle mass/function and physical functioning, a factor that positively affects quality of life. Several amino acids are precursors of brain neurotransmitter synthesis including serotonin, dopamine, norepinephrine, relevant for mood and behavior. In clinical practice, amino acid supplement has hepatoprotective effects[3], as well as improvement in porto-systemic encephalopathy[4] and depressive symptoms[5], etc. So, amino acid has particularly important function in our daily life.

With the exception of glycine, all encoded protein amino acids have at least one chiral center (enantiomers) or even two (epimers). (L)-Amino acids and their derivatives are significant biochemical compounds that constitute the building blocks of proteins, neurotransmitters, hormones, and nucleic acids, thus play a very important role in nutrition, medicine, and agriculture. While (D)-amino acids, which are virtually nonexistent for protein synthesis, are undesirable in food for nutritional reasons. Since superfluous (D)-amino acid may lead to overloading of the (D)-amino acid oxidase system, loss of digestibility (due to racemization), loss of essential amino acids, eventually superfluous (D)-amino acid are toxic over the long-term[6]. Therefore it is crucial to control the levels of (D)-amino acids in preparations in order to avoid potential toxic effects and keep the balance of D-amino acids in mammal.

However, (D)- and (L)-amino acid enantiomers have not been distinguished in commercial amino acid tablets, and (D)-amino acid enantiomers have not been focused on, either. Amino acid content assay methods using titration were provided in the United States Pharmacopoeia (potentiometric titration, the USP 34-NF 29), the British Pharmacopoeia (BP2010), Japan's Specifications and Standards for Food

Additives Monograph (JFA 7th edition), the Korea Pharmacopoeia (KP 9th edition), the Korea Food Additives Code 2011 (KFA), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The amino acid contents were expressed as (L)-amino acids without concerning the presence of (D)-enantiomers. Therefore this resulted in poor correlation between amino acid titration data and content data of (L)-amino acid[7]. Analytical methods based on high performance liquid chromatography (HPLC)[8, 9], capillary electrophoresis[10, 11], enzymatic[12] and immunochemical biosensors[13], etc. were reported. Comprehensive reviews on analysis of amino acid enantiomers were given[14, 15]. Some of these methods had disadvantages like low precision, bad repetitiveness, and difficulty in precise quantification. Recently, HPLC/mass spectrometry (MS) based methods have been developed for chiral analysis of amino acids aiming for improved method selectivity and detection sensitivity[16-19]. However, as far as we are aware, most of these developed methods are dealing with enantioseparation of certain amino acids or single-enantiomer content determinations, and there is still a big challenge to simultaneous determination of various amino acids enantiomers. Consequently, it is significant to establish a more accurate and sensitive method which could differentiate (D)-enantiomers from (L)-enantiomers and simultaneous determine both the two types of amino acid enantiomers.

Enantioseparation of amino acid enantiomers has been the focused area of our group and a method to separately determinate enantiomeric purity of a series of (L)-amino acids with pre-column derivatization and chiral stationary phase was developed by us[20]. On the basis of the previous study, a new method was achieved by pre-column derivatization with 7-chloro-4-nitrobenzoxadiazole and detected with 4000 Qtrap mass spectrometry. This proposed method was more sensitive and

convenient owing to the merits of the derivatization and mass spectrometry detection. Take D-Ala as an example, in our formerly developed HPLC method, the LOD was 300 ng·ml^{-1} and LOQ was 100 ng·ml^{-1} [20]. And in the analysis of underivatized chiral amino acids by liquid chromatography–ionspray tandem mass spectrometry using chiral stationary phase, the LOD was 40 ng·ml^{-1} [21]. While in the proposed method, it was 5 ng·ml^{-1} and 1 ng·ml^{-1}, respectively for LOD and LOQ. Furthermore, it enabled simultaneously determination of 9 kinds of amino acid enantiomers for the first time, including alanine (Ala), leucine (Leu), valine (Val), proline (Pro), phenylalanine (Phe), methionine (Met), serine (Ser), threonine (Thr) and tyrosine (Tyr). All of the nine investigated amino acids are primary components in commercail amino acid preparations, and have important physiological activities for human[22-24]. Furthermore, after fully validation according to the guidance of ICH, the method was successfully applied to simultaneous quantitation of the nine kinds of amino acid enantiomers in the tablets.

2. Materials and methods

2.1. Chemical and reagents

Four kinds of commercial amino acid tablets were purchased from market in China. (D)-, (L)-alanine (Ala), (D)-, (L)-leucine (Leu), (D)-, (L)-valine (Val), (D)-, (L)-proline (Pro), (D)-, (L)-phenylalanine (Phe), (D)-, (L)-methionine (Met), (D)-, (L)-serine (Ser), (D)-, (L)- threonine (Thr) and (D)-, (L)- tyrosine (Tyr) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 7-chloro-4-nitrobenzoxadiazole (NBD-Cl) was purchased from ACROS ORGANICS (Belgium). Methanol and acetonitrile of HPLC grade were provided by Fisher Chemicals (Fair Lawn, NJ, USA). All the other reagents were of analytic grade. Redistilled and deionized water was used throughout the study.

2.2 Instrumentation and chromatographic conditions

Chromatographic runs were carried out using an XR LC-20 AD Prominence™ HPLC system equipped with a binary pump, a degasser, an auto sampler, a thermostatted column compartment (Shimadzu, Japan) and a 4000 QTRAP™ MS/MS system from AB Sciex equipped with a turbo ion spray source (Foster City, CA,USA). All the operations, the acquiring and analysis of data were controlled by Analyst (version 1.6, AB Sciex, USA). Enantiomers were enantioseparated on Sumichiral OA-2500S column (250 mm×4.6 mm, 5 μm) protected by a matched Pirkle-type chiral stationary phase column pre-filter (10 mm×4 mm, 5 μm) at 40 °C. Analysis was completed with an isocratic elution of 0.5% formic acid in 50% acetonitrile (A) and 50% methanol (B). Efficient and symmetrical peaks were obtained at a flow rate of 1.0 $ml \cdot min^{-1}$ with split ratio of 1:3 and a sample injection volume of 2 μL. The detection of the analytes was performed in the multiple reaction monitoring mode (MRM) using an electrospray negative ionization (ESI^-) under following conditions: Ion Spray Voltage 5500 V, Vacuum Gauge ($10e^5$) 2.8 Torrand source temperature 550 °C. Nitrogen was used as gas 1, gas2 and curtain gas at a flow rate of 50, 50 and 30 psi, respectively. The quantitative parameters were shown in Table 1.

2.3 Preparation of standard and calibration solutions

Nine tested (D)-amino acids stock solutions (I) were prepared by dissolving accurately weighed amounts of approximately 10 mg of each compound in 10 mL of demineralized water (1.00 $mg \cdot ml^{-1}$). While nine tested (L)-amino acids stock solutions (II) were prepared in water at the concentration of 10.0 $mg \cdot ml^{-1}$. The stock solutions (III) of racemic amino acid enantiomers were prepared at a concentration of 1 $mmol \cdot L^{-1}$ in water.

All the stock solutions were stored in a refrigerator (4 ^oC) protected from light.

The calibration standards at six levels were prepared by appropriately mixed and further diluted stock standard solutions.

2.4 Preparation of sample

A number of ten accurately weighed tablets were ground into a fine powder using a glass mortar and pestle. A quantity of the powder was transferred to 10 mL volumetric flasks. Then, 5 mL of demineralized water was added. After standing for 30 min under ultra-sonication at ambient temperature, the flask was adjusted to volume and mixed well. The resulting solution was filtered using 0.22 μm filter membrane. The filtered solution was transferred into another clean tube and submitted to NBD-Cl derivatization procedure.

2.5 Derivatization procedure

The 100 μL sample solution was alkalinized by adding borate buffer solution (100 $mmol \cdot L^{-1}$ at pH 9.0), followed by 100 μL NBD-Cl (16 $mmol \cdot L^{-1}$) diluted with methanol. The mixture was vortexed and heated at 60 °C in the water bath for 1 h protected from light, followed by cooling at 4 °C in refrigerator. After heating, the mixture was evaporated to dryness under nitrogen. The residue was reconstituted in 0.1 mL mobile phase, vortex-mixed for 3 min, then sonicated for 3 min followed by being centrifuged at 12 000 rpm for 5 min. Finally, the 2 μL supernatant was injected for HPLC-MS/MS analysis. For blank sample, 100 μL water was used instead of sample solution.

2.6 Method validation

The method was fully validated according to ICH guidelines for validation of analytical procedures.

2.6.1 Linearity, limits of detection (LOD) and quantitation (LOQ)

Appropriate amounts of amino acid stock solutions (I) were diluted with water to give

six concentrations covered from 0.5 to 10 μg·mL^{-1} for D-Ala, 1.5 to 30 μg·mL^{-1} for D-Leu, 0.25 to 5 μg·mL^{-1} for D-Val, 0.6 to 12 μg·mL^{-1} for D-Pro,1.5 to 30 μg·mL^{-1} for D-Phe, 0.6 to 12 μg·mL^{-1} for D-Met, 0.19 to 3.8 μg·mL^{-1} for D-Ser, 0.15 to 3 μg·mL^{-1} for D-Thr, 1.5 to 30 μg·mL^{-1} for D-Tyr, and 7.2 to 144 μg·mL^{-1} for L-Ala, 12.4 to 248 μg·mL^{-1} for L-Leu, 10.0 to 200 μg·mL^{-1} for L-Val, 31 to 620 μg·mL^{-1} for L-Pro, 14.7 to 294 μg·mL^{-1} for L-Phe, 3.26 to 6.52 μg·mL^{-1} for L-Met, 3.14 to 62.8 μg·mL^{-1} for L-Ser, 4.5 to 90 μg·mL^{-1} for L-Thr, 13.2 to 264 μg·mL^{-1} for L-Tyr, respectively. Linearity was evaluated by linear least-squares regression analysis. Limits of quantification (LOQ) were calculated the lowest concentration producing signal-to-noise ratio of at least 10:1. Limits of detection (LOD) were calculated as the lowest concentration producing signal-to-noise ratio of at least 3:1

2.6.2 Accuracy

The accuracy of the method described for the determination of amino acid enantiomers in tablets were calculated as the percent recoveries. And the recovery studies were carried out by adding known quantities of standards at three different levels to the pre-analyzed sample, 80%, 100% and 120% of the amino acids concentration level of the tablets. Three replicates were prepared for each concentration level. Measured values were compared with the theoretical concentration. Recovery for pharmaceutical formulations should be within the range 100 ± 10%. The relative standard deviations (R.S.D.) values of individual measurements were also determined.

2.6.3 Precision

The precision of the proposed method was determined by repeatability (intra-day) and intermediate precision (inter-day). The intermediate precision was studied by comparing the assays on different days (3 days). Six sample solutions were prepared

and assayed daily.

2.6.4 Stability

The stability of amino acid enantiomers in sample solutions was tested using the sample solutions at the six different time of duration at stored temperature (4 ^{o}C) protected from light. The samples were examined at 0, 2, 4, 8 and 12 h after derivatization. The R.S.D. of injection sample solutions at different time was paid close attention to.

2.6.5 Specificity

The specificity was evaluated by comparing chromatograms of blank sample added water instead of sample solutions, the amino acid standard solutions and the amino acid tablet solutions. All the chromatograms were examined to demonstrate the absence of interference with the auxiliary material of amino acid tablets and the derivatization reagents. And the retention times of analytes and IS in standard solutions should be equal to those in samples.

3. Results and discussion

3.1 Optimization of derivatization conditions

A standard solution (III) of nine kinds of (D) - and (L)-amino acid enantiomers (1 mmol·L^{-1}) were used to investigate the optimum derivatization conditions. The derivatization reaction of amino acid and NBD-Cl proceeded under alkaline conditions. During the process of reaction, amino acid reacted with NBD-Cl to give a kind of derivatives compound, i.e. amino acid-NBD. The reaction diagram for amino acid derivatization of amino acid with NBD-Cl was shown in Fig. 1.

The effects of concentrations of derivatization reagent, pH values, derivatization temperatures and time on derivative intensity have been paid close attention to. To optimize the derivatization conditions, a single-factor test was performed. Since

different ratios of NBD-Cl to amino acid could influence the degree of derivatization, different concentrations of derivatization reagent from 1 mmol·L^{-1} to 5 mmol·L^{-1} were studied whereas the concentration of amino acid was fixed at 1 mmol·L^{-1}. The result was shown in Fig. 2A. The derivatization reagent, four times of amino acid concentration, was used in the recommended procedure which provided almost maximum and constant peak areas as for all the nine kinds of amino acids.

Different pH values could also affect derivative intensity and it was a critical factor for the derivatization reaction. Thus the pH values ranging from 7.5 to 10.0 were performed to find the best derivative pH value. As shown in Fig. 2 B, it was found that peak areas of the derivatives reached plateau at pH 8.5. As a result, the optimum pH value of borate buffer solution of 8.5 was selected.

The effects of derivatization temperatures (40, 50, 60 and 70 ^{o}C) and reaction time (50, 60, 70, 80 and 90 min) on derivatization yields were investigated, and the results were displayed in Fig. 2C and Fig. 2D, respectively. The time effect studies were carried out to a maximum of 90 min. The maximum and constant peak areas were obtained after reacting for 60 min, and there were no significant changes in peak areas with increasing time. Hence, the appropriate reaction time was decided to be 60 min. As for the derivatization temperature, it was examined from 40 to 90 ^{o}C. Adduct yields increased indistinctively before derivatization temperatures >70 ^{o}C and it decreased after derivatization temperatures >70 ^{o}C. This may be due to the decomposition of the adducts. Therefore, 70 ^{o}C was taken as the optimal derivatization temperature for the derivatization process.

3.2 Optimization of chromatographic and mass conditions

Under the electrospray ionization conditions, a greater sensitivity was achieved for nine kinds of amino acid enantiomers in the negative mode (ESI^{-}) than in the positive

mode (ESI^+). The optimal conditions for the triple-quad analyzer were determined by direct infusion of the standard solutions into the mass spectrometer. Some parameters such as gas1, gas2, curtain gas, IS voltage, DP, CE and CXP were optimized in automatic runs under evaluation of the ionic sensitivity. The other parameters were adopted for the recommended value of the instrument.

To optimize the peak shapes and retention times of nine kinds of amino acid enantiomers and IS, the varying proportions of methanol–acetonitrile system were investigated. The best results in terms of enantioresolution and retention of the enantiomers were obtained with the 50% acetonitrile composition (acetonitrile -methanol, v/v), whereas continuing increase of acetonitrile more than 50% resulted in a small decrease of the enantioresolution. The addition of a small amount of formic acid to the mobile phase was proved to improve peak symmetry and shape of amino acid enantiomers. For this reason, different proportions of formic acid (0.05%, 0.1%, 0.3% and 0.5%) were added into acetonitrile-methanol system to investigate the effect. With the increase of the amount of formic acid, the run time was gradually shortened, but the enantioresolution would be decreased. Finally, a mobile phase composed of acetonitrile-methanol (50:50, v/v) containing 0.5% formic acid was selected for sufficient ionization response, good peak symmetry and proper retention time for the analytes and IS.

3.3. Method validation

This method was estimated to give evidence of its applicability for its intended purpose with adequate validation characteristics.

3.3.1 Linearity, limits of detection (LOD) and quantitation (LOQ)

Calibration curves for nine kinds of amino acid enantiomers were constructed by plotting concentration versus the ratio of peak area normalizations of different amino

acid and IS and showed good linearity in the different ranges, respectively. Linear regression analysis of the curves was tabulated in Table 2. High coefficients of correlation were obtained and the intercepts of the curves were not notably different from zero. In spite of significant differences of the linearity ranges between (D) - and (L)-amino acid enantiomers, the proposed method could still be generally suitable for simultaneous determination of (D) - and (L) - amino acids enantiomers.

Certain analytical parameters such as LOD and LOQ values were calculated by computing the processed of integrated peak from HPLC–MS/MS, and the results were also listed in Table 2. It could be found, the lowest points on the calibration curves of (D)-amino acid enantiomers were in accordance with the levels of the LOQ, whereas for the (L)-amino acid enantiomers, the lowest points on calibration curves were significantly higher than the LOQs. This can be attributed to the fact that there were large amounts of (L)-amino acid enantiomers and small amounts of (D)-amino acid enantiomers in the amino acid tablets, and the ranges of calibration curves should be suitable to the concentrations of practical products. If necessary, the scope of the calibration curves of (L)-amino acid enantiomers could also be expanded in further research.

3.3.2 Accuracy

The accuracy of the method was evaluated by the mean recovery obtained from three levels. At each level of amino acid concentration three determinations were performed. The mean recoveries were presented in Table 3 to prove the developed method could be considered accurate over the range investigated.

3.3.3 Precision

To validate the precision of the proposed method, both the repeatability and intermediate precision were investigated, and were expressed as R.S.D of a set of

results. Results were presented in Table 4, which indicated good repeatability and low inter- day variability with R.S.D maximum of 7.7%

3.3.4 Stability

The R.S.D values of one derivative sample at 0, 2, 4, 8 and 12 h within a single day was less than 9.1%, which proved that the derivative sample was stable over a 12 h period.

3.3.5 Specificity

No significant interference was observed at the retention time of the nine kinds of amino acid enantiomers and IS. With the help of HPLC system, high selectivity of MRM mode and appropriate mobile phase, a total analysis time of less than 34 min was achieved which could put high-throughput analysis for nine pairs of enantiomers to practice. Typical MRM chromatograms of blank sample, amino acid standard solution and amino acid tablet sample solution were shown in Fig. 3. It can be seen there was no interference at the migration times of the enantiomers between standard solutions sample and tablet samples, and all the nine pairs of enantiomers were enantioseparated very well.

3.4 Method Application

The developed method was successfully applied to simultaneously determine the nine kinds of (D) - and (L)-amino acid enantiomers in tablets. The results obtained for the nine kinds of (D) - and (L)-amino acid enantiomers were given in Table 5. In the four kinds of commercial amino acid tablets, both (D) - and (L) - enantiomers of all the nine kinds of amino acids were detected in the amino acid tablets. Among them the (L)-enantiomers were the main existent form, while the amounts of (D)-enantiomers were little and varied differently according to the kinds of amino acid and brands of the commercial tablets. Furthermore, this more accurate and sensitive

enantioseparation method for simultaneously determination of (D)-and (L)-amino acid enantiomers could provide the scientific basis for the detection of amino acid enantiomers in vivo and the clinical tests. The application in vivo is currently underway in our group.

4. Conclusion

In this paper, a new HPLC-MS/MS method for the simultaneous assay of nine kinds of (D) - and (L) amino acid enantiomers in tablets was reported. This method was simple, reliable, linear, accurate, and sensitive as well as cost-effective for quantitative analysis of amino acid enantiomers in tablet. In the measured commercial amino acid tablets, (L)-amino acid was the main existence, whereas there was also little corresponding (D)-amino acids present. The developed method can be easily and conveniently adopted for a routine analysis of (D) - and (L)-amino acids in tablets. In addition, the method could be applied in vivo and the clinical tests in the long run.

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References

1. P. Fürst, *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism*, 2009, **4**, e62-e65.
2. Joint WHO/FAO/UNU Expert Consultation, World Health Organ. Tech. Rep. Ser., 2007,***935***,1-265
3. K. Hoffmann, M. W. Buchler and P. Schemmer, *Clin. Nutr.*, 2011, **30**, 143-147.
4. E. L. Metcalfe, A. Avenell and A. Fraser, *Clin. Nutr.*, 2014, **33**, 958-965.
5. M. Rondanelli, A. Opizzi, N. Antoniello, F. Boschi, P. Iadarola, E. Pasini, R. Aquilani and F. S. Dioguardi, *Clin. Nutr.*, 2011, 30, 571-577.
6. M. Friedman, *J. Agr. Food Chem.*, 1999, **47**, 3457-3479.
7. M. S. Lee, F. N. Khan, S. C. Shin, E. D. Jeong, H. G. Kim, M. J. Kim, Y. J. Cho, J. Y. Cha, M. H. Hyun and J. S. Jin, *Food Chem.*, 2012, **135**, 343-347.
8. T. Toyo'oka , N. Tomoi, T. Oe. and T. Miyahara, *Anal.Biochem.*, 1999, **276**, 48-58.
9. Y. Gogami, K. Okada and T. Oikawa, *J. Chromatogr. B*, 2011, **879**, 3259-3267.
10. A. Giuffrida, G. Maccarrone, V. Cucinotta, S. Orlandini and A. Contino, *J. Chromatogr. A*, 2014, **1363**, 41-50.
11. F. Kitagawa and K. Otsuka, *J. Chromatogr. B*, 2011, **879**, 3078-3095.
12. L. Frattini, E. Rosini, L. Pollegioni and M. S. Pilone, *J. Chromatogr. B*, 2011, **879**, 3235-3239.
13. L. D. Mello, L. T. Kubota , *Food Chem.*, 2002, **77**, 237-256.
14. K. Hamase, A. Morikawa, K. Zaitsu, *J. Chromatogr. B*, 2002, **781**, 73-91.
15. K. Imai, T. Fukushima,T. Santa, H. Homma, K. Hamase, K. Sakai and M. Kato, *Biomed. Chromatogr.*, 1996, **10**, 303-312.
16. K. Kinoshita, S. Jingu and J. Yamaguchi, *Anal. Biochem.*,2013, **432**, 124-130.
17. J. Alt, C. Rojas, K. Wozniak, Y. Wu, D. Ferraris, T. Tsukamoto and B. Slusher, *Anal. Biochem.*, 2011, **419**, 106-109.
18. C. Muller, J. R. Fonseca, T. M. Rock, S. Krauss-Etschmann and P. Schmitt-Kopplin, *J. Chromatogr. A*, 2014, **1324**, 109-114.
19. Y. Xie, G. M. Alexander, R. J. Schwartzman, N. Singh, M. C. Torjman, M. E. Goldberg, I. W. Wainer and R. Moaddel, *J. pharmaceut. biomed.*, 2014, **89**, 1-5.
20. Y. Cui, Z. Jiang, J. Sun, J. Yu, M. Li, M. Li, M. Liu and X. Guo, *Food Chem.*, 2014, **158**, 401-407 .
21. K. Petritis, C. Elfakir and M. Dreux, *J. Chromatogr. A*, 2001, **913**, 331-340.
22. Y. Nagat ,R. Masui, T. Akino, *Experientia*, 1992, **48**, 986-988.
23. A. Panatier, D. T. Theodosis, J. P. Mothet, B. Touquet, L. Pollegioni, D. A. Poulain and S. H. Oliet, *Cell*, 2006, **125**, 775-784.
24. H. Wolosker, K. N. Sheth, M. Takahash, J. P. Mothet, R. O. Brady, JR,C. D. Ferris, and S. H. Snyder, *Proc. Natl. Acad. Sci. USA*, 1999, **96**,721-725.

Table captions

Table 1. List of selected MRM parameters, declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) for each analyte and IS

Table 2. Linearity, LOQ and LOD for 9 kinds of (D)- and (L)-amino acid enantiomers

Table 3. Experimental values obtained in the recovery test for 9 kinds of (D)- and (L)-amino acid enantiomers from tablets solutions spiked with standard solution

Table 4. Experimental values of the 9 kinds of (D) - and (L)-amino acid enantiomers obtained for a commercially available sample method

Table 5. Stability of 9 kinds of (D)- and (L)-amino acid enantiomers obtained for a commercially available sample at 4 $^{\circ}$C for 12 h

Figure captions

Fig. 1 Derivatizing reaction of NBD-Cl with amino acid

Fig. 2 Influence of different factors on derivative reaction. (A) Influence of NBD-Cl concentration; (B) Influence of pH value; (C) Influence of temperature; (D) Influence of time

Fig. 3 MRM chromatograms of a blank sample (A), standard solution sample (B) and amino acid tablet dosage form (C). MRM transitions named m/z 251.4→207.1, 266.8→222.8, 277.0→232.9, 279.9→235.8, 280.9→237.0, 292.6→249.0, 311.9→267.9, 327.7→283.9, 342.9→298.9, 341.0→204.7 for Ala, Ser, Pro, Val, Thr, Leu, Met, Phe, Tyr and IS, respective.

Table 1. List of selected MRM parameters, declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) for each analyte and IS

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	DP	EP	CE	CXP
Ala	251.4	207.1	-34.1	-4.8	-11.0	-6.0
Leu	292.6	249.0	-47.0	-5.7	-10.0	-19.0
Val	279.9	235.8	-40.0	-4.7	-11.0	-8.0
Pro	277.0	232.9	-37.0	-5.0	-7.0	-5.0
Phe	327.7	283.9	-38.0	-5.0	-10.5	-7.0
Met	311.9	267.9	-44.0	-3.0	-11.5	-9.0
Ser	266.8	222.8	-35.0	-3.5	-10.0	-5.0
Thr	280.9	237.0	-33.0	-2.7	-11.0	-5.0
Tyr	342.9	298.9	-43.0	-3.2	-12.0	-7.0
IS	341.0	204.7	-36.0	-4.0	-15.9	-3.0

Table 2. Linearity, LOQ and LOD for 9 kinds of (D)- and (L)-amino acid enantiomers

Analyte	Concentration range ($\mu g \cdot mL^{-1}$)	Regression equation	R^2	LOQ ($ng \cdot mL^{-1}$)	LOD ($ng \cdot mL^{-1}$)
D-Ala	0.5-10	$y=311.4x+2.770\times10^{-2}$	0.9994	5	1
D-Leu	1.5-30	$y=906x+5.527\times10^{-1}$	0.9993	15	3
D-Val	0.25-5	$y=351.2x+1.020\times10^{-2}$	0.9994	12.5	2.5
D-Pro	0.6-12	$y=260.2x+4.230\times10^{-2}$	0.9996	300	150
D-Phe	1.5-30	$y=115.6x+3.380\times10^{-2}$	0.9991	150	30
D-Met	0.3-6	$y=158.9x-9.40\times10^{-3}$	0.9990	300	150
D-Ser	0.19-3.8	$y=956x+2.680\times10^{-2}$	0.9991	190	95
D-Thr	0.15-3	$y=632.8x-3.150\times10^{-2}$	0.9992	150	75
D-Tyr	1.5-30	$y=89.4x+4.860\times10^{-2}$	0.9990	1500	750
L-Ala	7.2-144	$y=326.9x+7.683\times10^{-1}$	0.9992	5	1
L-Leu	12.4-248	$y=905x+1.240\times10^{-1}$	0.9990	15	3
L-Val	10-200	$y=353.8x-5.8a05\times10^{-1}$	0.9991	12.5	2.5
L-Pro	31-620	$y=260.3x+4.170\times10^{-2}$	0.9990	300	150
L-Phe	14.7-294	$y=118.9x+4.859\times10^{-1}$	0.9992	150	30
L-Met	3.26-65.2	$y=153.9x-7.190\times10^{-2}$	0.9999	300	150
L-Ser	3.14-62.8	$y=961x+7.574\times10^{-1}$	0.9991	190	95
L-Thr	4.5-90	$y=633.6x+6.420\times10^{-2}$	0.9991	150	75
L-Tyr	13.2-246	$y=177.4x+2.678\times10^{-1}$	0.9993	1500	750

Table 3. Experimental values obtained in the recovery test for 9 kinds of (D)- and (L)-amino acid enantiomers from tablets solutions spiked with standard solution

Analyte	Sample concentration ($\mu g \cdot mL^{-1}$)	Concentration of added standard ($\mu g \cdot mL^{-1}$)	Recovery (%)	RSD (%)	Mean Recovery(%)
D-Ala	0.7132	0.5706	103.1	3.4	103.0
		0.7132	103.6	1.1	
		0.8559	102.2	2.3	
D-Leu	1.858	1.487	101.6	2.4	103.6
		1.858	104.7	2.3	
		2.230	104.6	2.5	
D-Val	0.5812	0.4650	101.1	1.4	104.2
		0.5812	103.6	2.6	
		0.6975	107.9	1.1	
D-Pro	2.291	1.833	102.0	2.8	102.0
		2.291	99.7	2.0	
		2.749	104.2	0.2	
D-Phe	2.776	2.221	102.8	5.7	104.4
		2.776	106.1	2.8	
		3.331	104.5	3.2	
D-Met	0.3195	0.2556	101.9	3.0	102.7
		0.3195	103.6	3.1	
		0.3833	102.4	2.0	
D-Ser	0.4723	0.3778	98.7	2.2	103.1
		0.4723	104.9	1.9	
		0.5671	105.6	3.2	
D-Thr	0.1233	0.09861	98.0	6.5	101.7
		0.1233	105.2	2.7	
		0.1479	106.4	3.1	
D-Tyr	2.384	1.907	98.0	6.5	103.2
		2.384	105.2	2.7	
		2.861	106.4	3.1	
L-Ala	13.21	10.57	105.3	1.4	99.1
		13.21	98.2	3.5	
		15.85	93.9	0.3	
L-Leu	46.88	37.50	94.8	2.0	95.0
		46.88	95.2	2.1	
		56.25	95.0	3.6	
L-Val	28.67	22.94	96.2	4.0	98.4
		28.67	99.1	3.1	
		34.41	99.8	2.4	
L-Pro	130.4	104.3	98.0	1.0	98.8
		130.4	98.6	1.3	
		156.5	99.7	5.4	
L-Phe	61.14	48.91	99.2	2.9	98.9
		61.14	99.3	5.0	
		73.37	98.3	5.0	
L-Met	11.28	9.020	104.8	2.4	99.2
		11.28	95.1	1.2	
		13.53	97.8	5.0	
L-Ser	15.17	12.14	99.4	1.9	98.3
		15.17	98.3	1.9	
		18.21	97.0	0.8	
L-Thr	13.83	11.06	99.3	1.5	102.9
		13.83	103.8	5.2	
		16.59	105.7	2.3	
L-Tyr	55.55	44.44	96.4	0.9	97.7
		55.55	97.4	1.5	
		66.66	99.2	4.4	

Table 4. Experimental values of the 9 kinds of (D) - and (L)-amino acid enantiomers obtained for a commercially available sample method

Analyte		RSD (%)[a]	RSD (%)
D-Ala	Day1	3.7	2.9
	Day2	2.9	
	Day3	2.4	
D-Leu	Day1	7.4	5.9
	Day2	6.0	
	Day3	4.3	
D-Val	Day1	6.5	5.5
	Day2	5.7	
	Day3	4.9	
D-Pro	Day1	4.4	3.7
	Day2	4.6	
	Day3	2.1	
D-Phe	Day1	4.8	3.7
	Day2	2.7	
	Day3	3.9	
D-Met	Day1	6.0	4.6
	Day2	4.1	
	Day3	3.9	
D-Ser	Day1	6.7	4.7
	Day2	4.4	
	Day3	3.0	
D-Thr	Day1	6.7	4.3
	Day2	3.2	
	Day3	2.6	
D-Tyr	Day1	6.0	3.9
	Day2	3.1	
	Day3	2.7	
L-Ala	Day1	4.8	4.2
	Day2	4.1	
	Day3	4.2	
L-Leu	Day1	6.3	5.7
	Day2	6.7	
	Day3	5.0	
L-Val	Day1	5.2	4.5
	Day2	4.5	
	Day3	4.6	
L-Pro	Day1	4.5	3.7
	Day2	3.6	
	Day3	3.7	
L-Phe	Day1	7.3	6.7
	Day2	3.7	
	Day3	6.6	
L-Met	Day1	5.4	4.1
	Day2	3.5	
	Day3	3.9	
L-Ser	Day1	6.6	5.0
	Day2	4.5	
	Day3	4.7	
L-Thr	Day1	7.7	6.0
	Day2	5.8	
	Day3	4.0	
L-Tyr	Day1	6.0	4.5
	Day2	4.0	
	Day3	4.0	

[a] mean of 3 determinations

Table 5. Content of 9 kinds of (D) - and (L)-amino acid enantiomers obtained for a commercially available Sample ($mg \cdot g^{-1}$)

Analyte	Brand A	Brand B	Brand C	Brand D
D-Ala	0.07	0.10	0.10	0.18
D-Leu	0.18	0.26	0.15	0.47
D-Val	0.05	0.07	0.06	0.09
D-Pro	0.20	0.59	0.34	0.43
D-Phe	0.25	0.38	0.13	0.77
D-Met	0.03	0.04	0.02	0.06
D-Ser	0.04	0.07	0.08	0.14
D-Thr	0.01	0.03	0.00	0.05
D-Tyr	0.21	0.32	0.19	0.76
L-Ala	1.13	1.30	0.87	1.73
L-Leu	4.04	4.76	1.70	6.12
L-Val	2.46	2.78	1.15	3.47
L-Pro	3.24	3.62	1.48	4.56
L-Phe	5.20	6.27	1.80	7.57
L-Met	0.96	1.17	0.42	1.53
L-Ser	1.28	1.45	0.98	1.78
L-Thr	1.18	1.33	2.09	1.74
L-Tyr	4.75	5.89	0.76	8.34

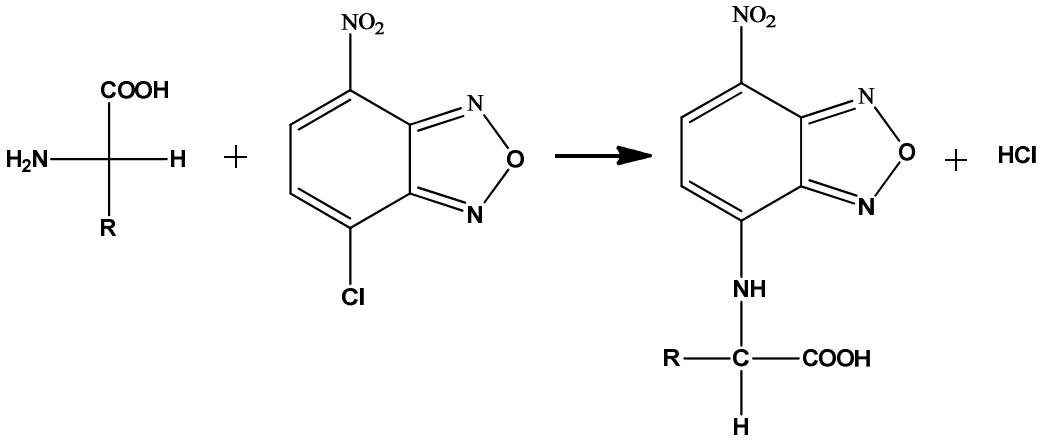
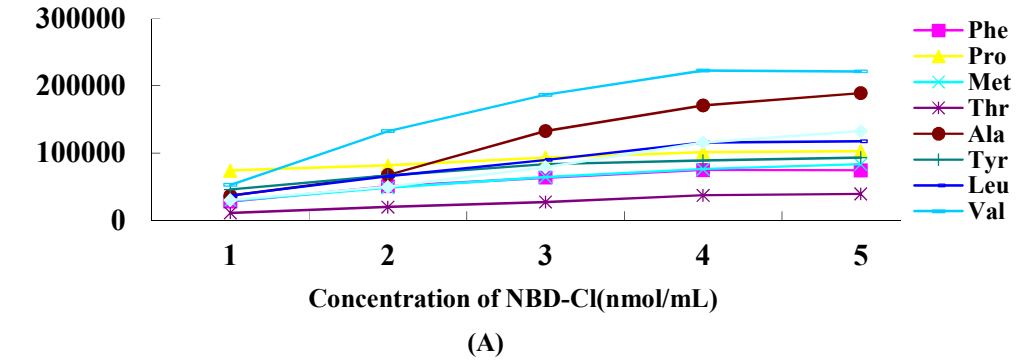
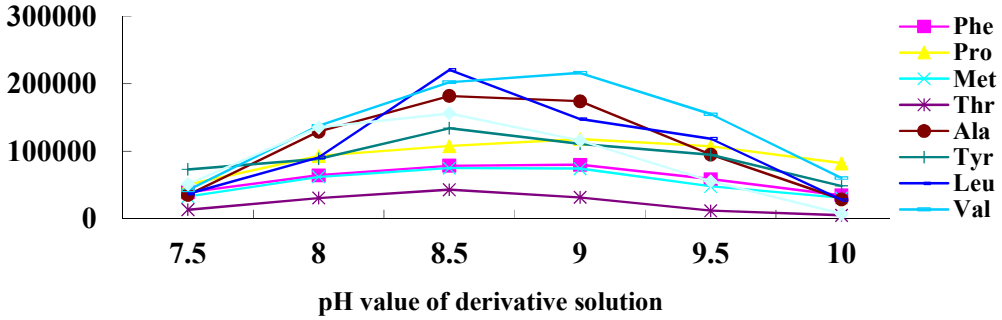


Fig. 1



(A)



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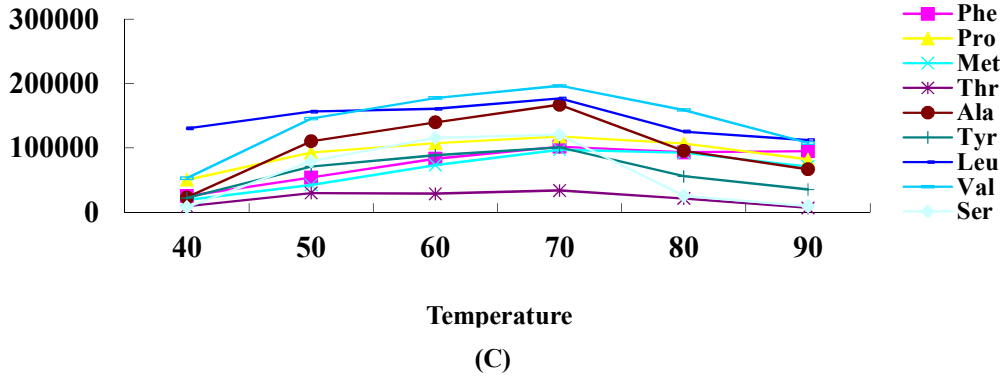

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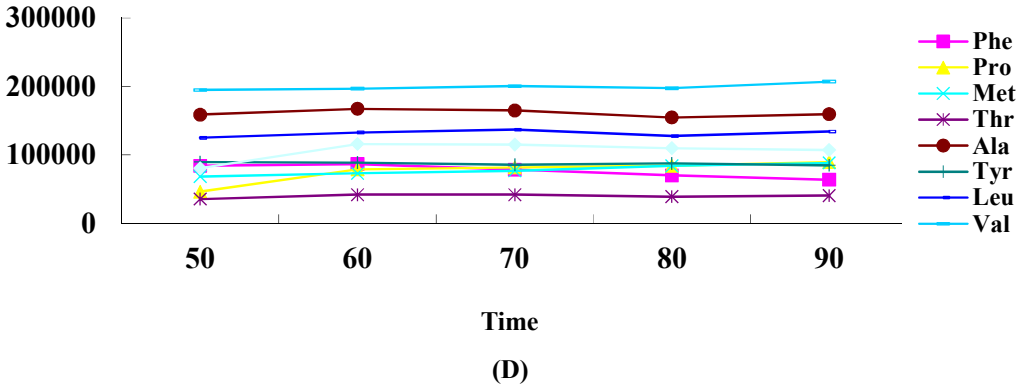


(D)

Fig. 2

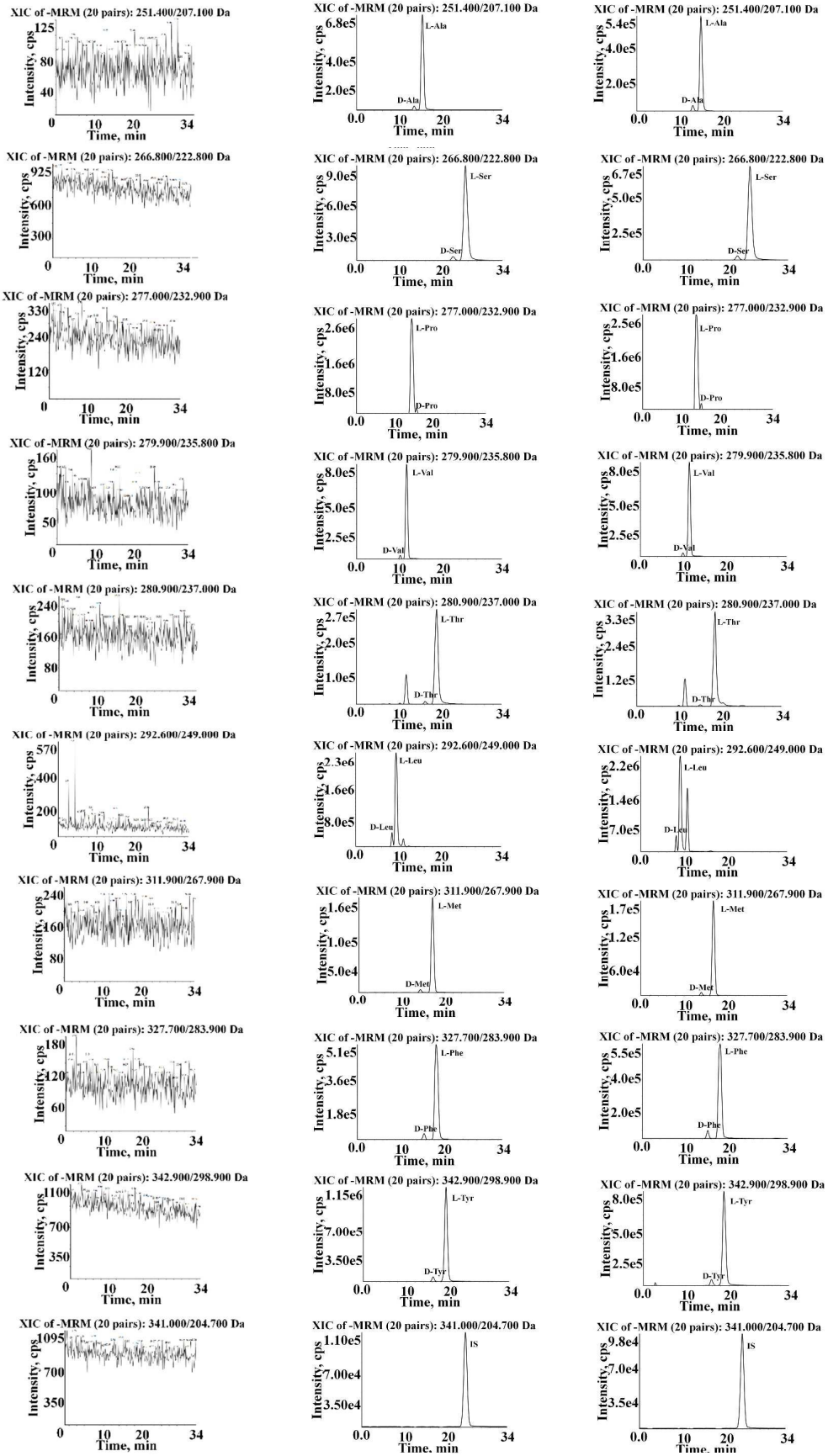
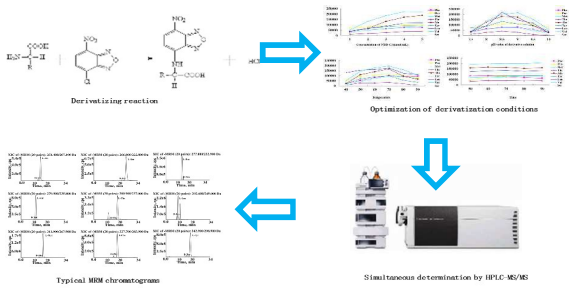


Fig. 3



A HPLC-MS/MS method was developed to simultaneous determination of nine kinds of (D)-and (L)-amino acid enantiomers and applied to control the quality of amino acid tablets