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Optimization of Zn^{2+}-containing mobile phase for simultaneous determination of kynurenine, kynurenic acid and tryptophan in human plasma by high performance liquid chromatography

Jianxing Zhao*, Pingjin Gao, Dingliang Zhu

Shanghai Key Laboratory of Vascular Biology at Ruijin Hospital and Shanghai Institute of Hypertension, Shanghai Jiao Tong University School of Medicine, 197 Ruijin 2nd Road, Shanghai 200025, China

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ABSTRACT

In the present work we have developed a standard-addition HPLC method using a mobile phase containing low concentration of $ZnAc_2$ to determine physiological level of kynurenine (KYN), kynurenic acid (KYNA) and tryptophan (TRP) in human plasma simultaneously. The method greatly improved the sensitivity of KYNA, the resolution of KYNA and TRP, and avoided clotting risk caused by high concentration of $ZnAc_2$ in mobile phase. Samples were deproteinized by addition of equal volume of 0.6 mol/L $HClO_4$. Analytes in supernatants were separated by an Agilent HC-C18 (2) analytical column; an aqueous mobile phase containing 20 mmol/L NaAc, 3 mmol/L $ZnAc_2$ and 7% acetonitrile at flow rate of 1.0 mL/min. Detections were performed by a variable wavelength detector at wavelength 365 nm for KYN and a fluorescence detector at wavelengths excitation 344 nm and emission 398 nm for KYNA and TRP. Good linear responses were found with $r^2 > 0.999$ for all analytes within the concentration range of physiological levels. The limit of detection of the developed method was 0.03 μmol/L, 0.9 nmol/L and 0.4 μmol/L for KYN, KYNA and TRP respectively. Recoveries from spiked human plasma were 95.4–99.7% for KYN, 98.9–104% for KYNA and 96.5–100.2% for TRP. All CVs for the repeatability and intermediate precision were less than 5%. We conclude that the developed method is helpful for the research investigations in KYN pathway of TRP metabolism.

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1. Introduction

Tryptophan (TRP) is an essential amino acid, which plays an important role in protein synthesis, and a precursor of many biologically active substances. TRP is metabolized in mammals via different pathways, a major route being the kynurenine (KYN) pathway in both the peripheral and central systems. Enzymes catalyzing reactions in KYN pathway have been demonstrated to be involved in many diseases and disorders, where imbalances in TRP and kynurenines have been found. Degradation of TRP by a cytokine induced indoleamine 2,3-dioxygenase (IDO) to formyl kynurenine seemed to enhance when the cellular immune system was activated [1]. Kynurenine aminotransferase (KAT) catalyzes the synthesis of kynurenic acid (KYNA), an endogenous antagonist at the glycine site of the N-methyl-D-aspartate (NMDA) as well as at the alpha 7 nicotinic cholinergic receptors, which has been demonstrated a highly neuroactive metabolite whose impairment is associated with a number of severe diseases and disorders in nervous system [2,3]. Besides, KYNA was also seemed to be involved in the pathogenesis of hypertension [4] and diabetes [5]. Therefore, monitoring the activities of these enzymes are becoming increasingly important and measurements of TRP and kynurenines in plasma are of great interest in determining proper diagnosis and medication of the relative diseases.

Several high performance liquid chromatography (HPLC) methods have been developed to determine TRP, KYN and KYNA in biological samples. Normally ultraviolet (UV) detection was employed to determine KYN [6–11] and fluorescence detection was used to measure KYNA and TRP [5–10,12–17]. Besides, some other techniques were also reported to determine TRP and kynurenines, e.g., measuring KYN and TRP by coulometric detection [18]; KYNA by capillary electrophoresis with laser-induced fluorescence detection [19]; and TRP and related compounds by high performance liquid chromatography–mass spectrometry (HPLC–MS) [20–22].

Physiological level of KYNA in blood and tissues are too low to be measured accurately by its native fluorescence. Analytical procedures have mainly been based on the specific chelation of KYNA with Zn^{2+} to yield an intense fluorescence. Some authors [14,17,23,24] delivered Zn^{2+} through post-column. Others [5,9,10,25–29] simply used a Zn^{2+}-containing mobile phase by

* Corresponding author. Tel.: +86 21 64740165; fax: +86 21 64740165.
E-mail address: jzhao021@yahoo.com (J. Zhao).

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addition of 0.1–0.5 mol/L $ZnAc_2$ with or without pH adjustment. So high concentration of $ZnAc_2$ would cause technical solubility problem and clotting risk which could hamper HPLC procedures and results [8,25]. Though acidifying mobile phase with HAc could reduce the clotting risk, low pH would cause severe interference with the formation of the chelate complex of KYNA–Zn^{2+} and reduce fluorescence intensity [19].

Column-switching HPLC seemed to be an attractive technique to separate impurities from matrix for the determination of KYNA in biological samples [14,30]. The HPLC system consisted of two octadecyl silica (ODS) columns. KYNA was separated on the first ODS column and trapped on an anion-exchange column by changing the position of a six-port valve then introduced into the second ODS column. Subsequently, KYNA was detected fluorometrically as a fluorescence complex formed with Zn^{2+} which was pumped through post-column. The procedure of this technique was a little bit complicated and not always necessary if KYNA could be separated from other compounds.

The aim of the present work is to develop a sensitive and accurate HPLC method with a modified elution buffer for the simultaneously quantitative measurements of KYN, KYNA and TRP in human plasma with UV and fluorescence detections. Standard-addition method was employed to compensate the matrix interference. The performance of the method was evaluated in terms of accuracy, linearity, precision and limit of detection.

2. Experimental

2.1. Chemicals

KYN and KYNA were purchased from Sigma and TRP was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other chemicals and solvents were of analytical or chromatographic grade. Double distilled water was used for the preparation of all solutions.

The stock standard solutions of all compounds were 1 mmol/L. KYN and TRP were dissolved in distilled water. KYNA was dissolved in 1.5 mmol/L NaOH. A mixed standard solution was made by the above stock standard solutions with the concentrations of KYN, 50 μmol/L; KYNA, 500 nmol/L and TRP, 500 μmol/L. All of these standard solutions were kept in −20 °C. Working standard solutions were prepared freshly before use by serial dilution of the mixed standard solution with 0.6 mol/L $HClO_4$. Concentration levels for the construction of six-point calibration curves were 1 μmol/L, 2 μmol/L, 4 μmol/L, 6 μmol/L, 8 μmol/L, 10 μmol/L KYN; 10 nmol/L, 20 nmol/L, 40 nmol/L, 60 nmol/L, 80 nmol/L, 100 nmol/L KYNA and 10 μmol/L, 20 μmol/L, 40 μmol/L, 60 μmol/L, 80 μmol/L, 100 μmol/L TRP.

2.2. Apparatus

The HPLC equipment was an Agilent 1100 series LC system (Agilent Technologies, Germany) composed of a G1322A vacuum degasser; G1311A quaternary pump, G1313A autosampler, G1316A thermostated column compartment; G1314A variable wavelength detector (VWD) and G1321A fluorescence detector (FLD). VWD and FLD were connected with a 150× 0.18 mm i.d. PEEK Tubing. An Agilent HC-C18 (2) column was employed as an analytical column (250× 4.6 mm i.d.; 5 μm particle size). A Thermo Varioskan Flash spectral scanning multimode reader (Thermo Electron Corporation, Finland) was used to observe the effect of Zn^{2+} at wavelengths excitation 344 nm and emission 398 nm.

2.3. Sample collection

Human plasmas were obtained from 80 local healthy residents who were told not to eat anything 12 h before blood collection in the morning (mean age: 44 years, range: 23–69 years). Venous blood samples were collected in heparin sodium anticoagulant tubes and centrifuged at 4000 rpm for 5 min. The plasmas were stored at −20 °C until processing. A pooled plasma was made for the preparation of standard-addition working curve and precision test of the method.

2.4. Standard-addition working curve and sample preparation

150 μL pooled plasma was mixed with equal volume of each diluted standard solution (see Section 2.1) in several 0.5-mL Eppendorf tubes. Mixtures were vortexed and centrifuged at 12,000 rpm for 5 min in room temperature to precipitate and separate protein. 150 μL of each mixed standard-addition supernatant was transferred into a micro-sampling vial placed in a screw-cap vial specific for the autosampler of the HPLC system. Volumes of 100 μL were injected onto the column for the calibration curves to calculate KYN, KYNA and TRP concentrations in human plasma.

Sample preparation procedure was carried out by mixing 150 μL plasma with equal volume of 0.6 mol/L $HClO_4$ in a 0.5-mL Eppendorf tube and treated as the way mentioned above.

2.5. Chromatographic conditions

The mobile phase was composed of 20 mmol/L NaAc, 3 mmol/L $ZnAc_2$ and 7% acetonitrile without any extra pH adjustment. It was prepared daily, filtered through a 0.45-μm membrane filter and degassed by an ultrasonic equipment for 20 min. The flow rate was 1 mL/min and the volume per injection was 100 μL. The wavelength of VWD was set at 365 nm; FLD conditions were excitation 344 nm with detection at emission 398 nm. Separation was achieved at an ambient temperature. The total time for plasma sample analysis was 40 min because after the elution of TRP at about 13.4 min, there were still several unknown impurity peaks that should be eluted before a new injection for next sample analysis. The concentrations were calculated from peak areas. An hp Chemstation software was employed to control HPLC system and process chromatographic data.

2.6. Statistical analysis

Data were analyzed as mean ± standard deviation (SD) and evaluated statistically by the Student's *t*-test. GraghPad Prism software was used to calculate correlation coefficients by linear regression analysis and test whether slopes of linear regression curves were significantly different. Significance was determined as $P < 0.05$.

3. Results and discussion

3.1. Fluorescence intensity of KYNA–Zn^{2+} is reduced in either HAc or NaOH solution

To optimize pH condition of the mobile phase for the formation of KYNA–Zn^{2+} chelation, we observed the fluorescence intensity of the solutions containing 5 μmol/L KYNA, 0.1 mol/L $ZnAc_2$ and 0–20 mmol/L HAc or NaOH. As Zn^{2+} reacted with OH^- to form $Zn(OH)_2$ precipitate, these samples were centrifuged and the supernatants were used for the determination. Measurements were carried out by a Thermo Varioskan Flash spectral scanning multimode reader as described in Section 2.2. The results showed that the fluorescence intensity of KYNA–Zn^{2+} was influenced by either HAc or NaOH (Fig. 1). Fluorescence intensities kept decreasing with the increase of acidity of the solution and reduced about 80% in the presence of 20 mmol/L HAc. This result proved Hansen's observation [19] that severe fluorescence quenching problem of KYNA–Zn^{2+} happened in acidic solutions. 2.5–20 mmol/L NaOH

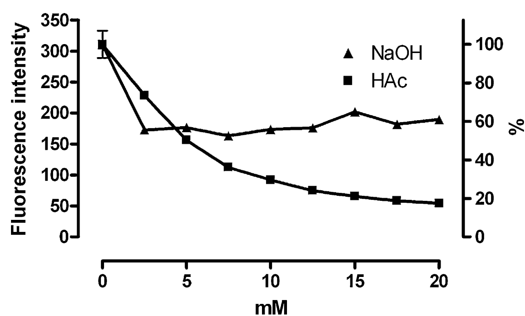

Fig. 1. Fluorescence intensities of KYNA–Zn^{2+} in HAc and NaOH solutions. Fluorescence intensities of kynurenic acid (KYNA) were determined by a Thermo Varioskan Flash spectral scanning multimode reader at wavelengths excitation 344 nm and emission 398 nm with a 96-well black plate. The volume of each test solution was 200 μL. Results are expressed as mean ± SD of duplicate determinations.

caused 40–50% reduction of the fluorescence intensity without typical concentration-dependency.

3.2. Fluorescence quenching problem of KYNA–Zn^{2+} in acidic solutions is partially reversible after neutralization

Next we measured fluorescence intensities of 5 μmol/L KYNA in 0.1 mol/L $ZnAc_2$ solution containing either 50 mmol/L HAc or 0.3 mol/L $HClO_4$, which were commonly used for pH adjustment of the mobile phase and deproteinization of blood samples. Their neutralized solutions were also measured to see if the quenching problem of KYNA–Zn^{2+} in acidic solutions was reversible after neutralization. An acid free solution was analyzed for the comparison. Data were normalized by 5 μmol/L KYNA in H_2O. The measurements were carried out by the same method mentioned above. As shown in Fig. 2, the fluorescence intensity of 5 μmol/L KYNA increased about 29-fold in the presence of 0.1 mol/L $ZnAc_2$. This increase of fluorescence intensity dropped down in acidic solutions. Only 2.7-fold increase was found with addition of 50 mmol/L HAc. The fluorescence intensity of KYNA was even lower than in its H_2O solution when 0.3 mol/L $HClO_4$ was used. After neutralization with NaOH, the fluorescence intensities re-increased to about 15-fold for both solutions once treated with HAc or $HClO_4$. This result indicated that the fluorescence quenching problem of KYNA–Zn^{2+} in acidic solutions was partially reversible after neutralization.

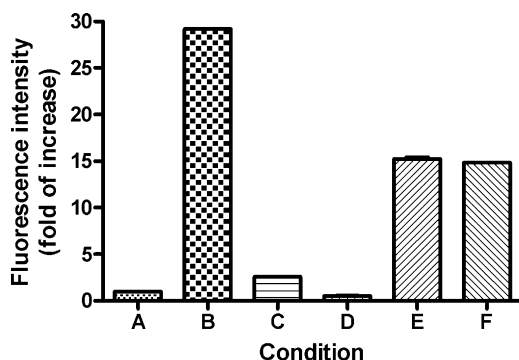

Fig. 2. Effect of neutralization on acid-induced fluorescence quenching of KYNA–Zn^{2+}. Fluorescence intensities of kynurenic acid (KYNA) were determined by a Thermo Varioskan Flash spectral scanning multimode reader at wavelengths excitation 344 nm and emission 398 nm with a 96-well black plate. The volume of each test solution was 200 μL. Results are expressed as mean ± SD of duplicate determinations. Condition: A = 5 μM KYNA; B = A in 0.1 M $ZnAc_2$; C = B in 50 mM HAc; D = B in 0.3 M $HClO_4$; E = C neutralized with NaOH; F = D neutralized with NaOH. Data were normalized by *A*.

3.3. Low concentration of Zn^{2+} in NaAc mobile phase greatly improves the sensitivity of KYNA and the separation of KYNA and TRP

To avoid clotting risk, concentration of $ZnAc_2$ included in mobile phase should be reduced to a very low extent. To enhance the fluorescence intensity of KYNA by its reaction with Zn^{2+}, the acidic mobile phase should be avoided using according to the evidences above. We next tested mobile phases composed of 20 mmol/L NaAc, 7% acetonitrile and 0–5 mmol/L $ZnAc_2$ without any addition of HAc in the determination of KYNA and TRP by HPLC with fluorescence detection. Our hypothesis was based on the assumption that KYNA would be completely separated on the column from $HClO_4$. Then, KYNA would react with Zn^{2+} to produce KYNA–Zn^{2+} chelate complex, which dramatically increase the fluorescent yield of KYNA, in an optimized mobile phase. This hypothesis was proved with great success and satisfaction. Chromatographic conditions were based on Section 2.5. A standard solution containing 20 nmol/L KYNA and 20 μmol/L TRP was mixed with equal volume of 0.6 mol/L $HClO_4$ as the way for deproteinization process of plasma samples. Fig. 3A illustrated the chromatograms. The efficiency of $ZnAc_2$ (E_{ZnAc_2}) was calculated according to the equation: $E_{ZnAc_2} = F_{FI}/C_{ZnAc_2}$, where F_{FI} was the fold of increase of the fluorescence intensity and C_{ZnAc_2} was the concentration of $ZnAc_2$ (mmol/L). As shown in Fig. 3B, with the increase of $ZnAc_2$, fluorescence intensities enhanced significantly. When $ZnAc_2$ concentration reached to 5 mmol/L, KYNA signal increased as 17-fold as that of Zn^{2+} free mobile phase. On the contrary, E_{ZnAc_2} were decreased gradually. The higher concentration of $ZnAc_2$ used in mobile phase, the lower E_{ZnAc_2} would be produced. The E_{ZnAc_2} were 6.6, 5.0, 4.2, 3.7 and 3.5 corresponding to 1 mmol/L, 2 mmol/L, 3 mmol/L, 4 mmol/L, and 5 mmol/L $ZnAc_2$ in mobile phase. The method reported by Lan-Gan et al. [29] used 500 mmol/L $ZnAc_2$, which produced 50-fold increase of the fluorescence intensity. Its E_{ZnAc_2} was only 0.1, which was much lower than that by using low concentration of $ZnAc_2$.

We found that low concentration of Zn^{2+} in NaAc mobile phase not only significantly enhanced the fluorescence intensity of KYNA, but also helped the separation for KYNA and TRP efficiently (Fig. 3C). When using Zn^{2+} free mobile phase, peaks of KYNA and TRP were so close that complete separation of these two compounds could not be achieved. The presence of $ZnAc_2$ in the mobile phase induced lower retention time of KYNA and higher retention time of TRP. Thus peaks of KYNA and TRP were completely separated. A concentration of 3 mmol/L $ZnAc_2$ in the mobile phase was finally chosen in the determination of KYN, KYNA and TRP in human plasmas. The reasons were (1) sufficient sensitivity to measure KYNA in all plasma samples; (2) the lower concentration of $ZnAc_2$, the less risk of clotting; and (3) good separation of KYNA and TRP.

3.4. Linearity and slope of standard working curves

A series of mixed standard solutions were used for the construction of six-point calibration curves at concentration levels: 1 μmol/L, 2 μmol/L, 4 μmol/L, 6 μmol/L, 8 μmol/L, 10 μmol/L KYN; 10 nmol/L, 20 nmol/L, 40 nmol/L, 60 nmol/L, 80 nmol/L, 100 nmol/L KYNA and 10 μmol/L, 20 μmol/L, 40 μmol/L, 60 μmol/L, 80 μmol/L, 100 μmol/L TRP. Standard-addition working curves were performed by spiking the sample before deproteinization. The signal intensities of all analytes were proportional to the concentrations in these ranges by linear regression and r^2 of all aqueous and standard-addition working curves were >0.999. Confidence interval analysis on the difference for the slopes of linear regression curves between aqueous and standard-addition indicated no significance for KYN and extreme significance for KYNA and TRP (Table 1).

Table 1
Slope of the standard working curves.

Compound	Range (μmol/L)	Slope		*P*
		Aqueous	Standard-addition	
Kynurenine	1–10	9.69	9.86	0.12
Kynurenic acid	10–100 (nmol/L)	8.32	6.86	<0.001
Tryptophan	10–100	31.35	28.68	<0.001

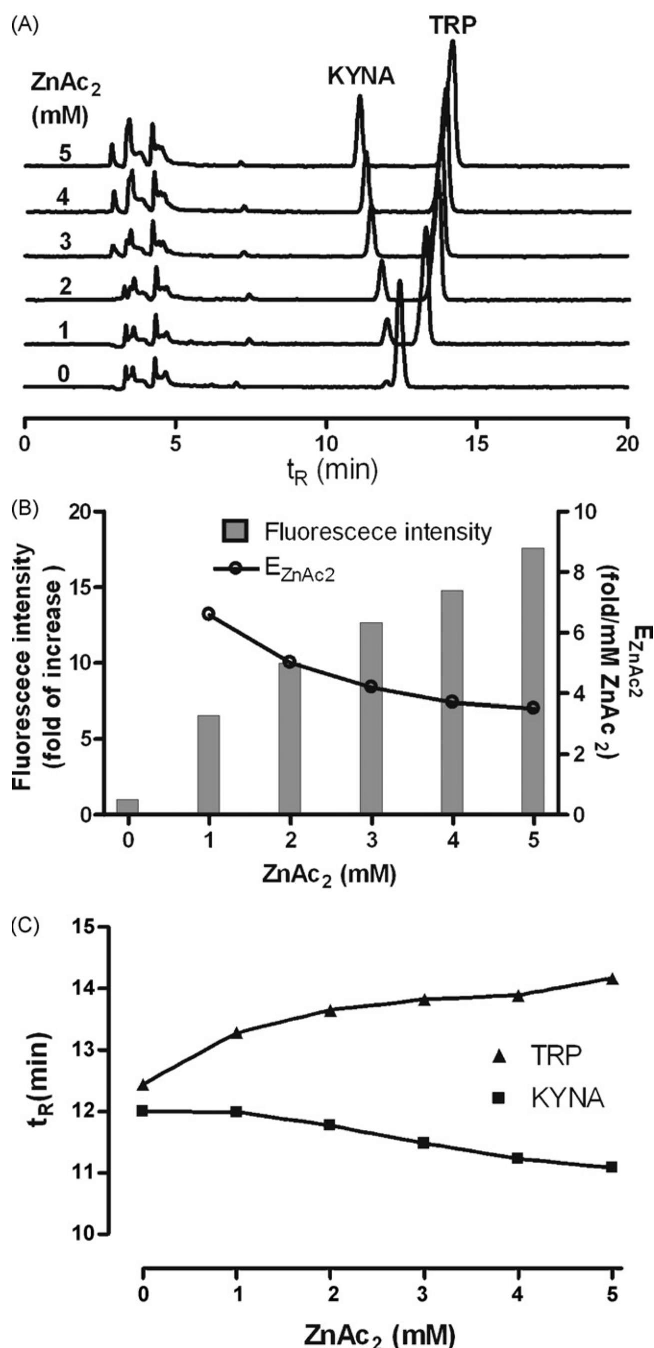

Fig. 3. Effect of Zn^{2+} in NaAc mobile phase. A standard solution containing 20 nmol/L kynurenic acid (KYNA) and 20 μmol/L tryptophan (TRP) was analyzed by HPLC. The analytical column was an Agilent HC-C18 (2). Mobile phase consisted of 20 mmol/L NaAc, 7% acetonitrile and 0, 1, 2, 3, 4 or 5 mmol/L $ZnAc_2$. The flow rate was 1.0 mL/min. Detection was performed by a fluorescence detector at wavelengths excitation 344 nm and emission 398 nm. A illustrates the chromatograms. B is the fold increase of fluorescence intensity normalized by Zn^{2+} free mobile phase and the efficiency of $ZnAc_2$ at different concentration level. C shows the separation of KYNA and TRP.

This was because of the matrix interferences occurring during analytical detection and influenced the sensitivity of KYNA and TRP in plasma-matched solutions. For accurately determining KYNA and TRP in plasma, we used standard-addition method to compensate the matrix effect.

3.5. Limit of detection

Limit of detection (LOD) was determined as signal-to-noise ratio of 3 with an injection of 100 μL. The LOD of KYN was 0.03 μmol/L which was not influenced by Zn^{2+}. Fluorescence intensity of TRP was increased a little bit in the presence of Zn^{2+} and the LOD of TRP was 0.4 μmol/L with 3 mmol/L $ZnAc_2$ in mobile phase. LODs of KYNA were measured by using 0–5 mmol/L $ZnAc_2$ in mobile phases. The results showed dose-dependent effects of Zn^{2+} on LOD of KYNA at the testing concentration range. As shown in Table 2, the LOD of KYNA was 0.6 nmol/L with 5 mmol/L $ZnAc_2$ included in the mobile phase. This value of LOD was comparable to the reported method using the mobile phase consisted of 0.25 mol/L $ZnAc_2$, 50 mmol/L HAc [9]. When 1 mmol/L $ZnAc_2$ was used in the mobile phase, the corresponded LOD of KYNA was 1.7 nmol/L, which was even better than the method using the mobile phase consisted of 0.1 mol/L $ZnAc_2$ and 50 mmol/L HAc [10]. This was a remarkable improvement for the determination of ultra-trace level of KYNA in biological samples. Though it was reported [29] that LOD of KYNA could reach to 0.05 nmol/L by using mobile phase containing 0.5 mol/L $ZnAc_2$, so high concentration of Zn^{2+} would cause severe clotting risk [8].

3.6. Accuracy and precision

Evaluation of accuracy was carried out by six-point recovery test. Known quantities of KYN (1–10 μmol/L), KYNA (10–100 nmol/L) and TRP (10–100 μmol/L) were spiked to plasma before deproteinization. Concentrations of the analytes from the original plasma $C_A(O)$ and recovered from the spiked plasma $C_A(O+S)$ were obtained via standard-addition calibration curves. The equation of recovery (%) = $[C_A(O+S) - C_A(O)]/C_A(S) \times 100$. $C_A(S)$ was the concentration of spike value. Mean of each concentration point was calculated based on the determinations of 5 individual workday. The results are shown in Table 3 and indicate that the described method has efficient recovery (95.4–99.7% with CV 0.5–4.5% for KYN; 98.9–104% with CV 0.5–5.1% for KYNA; and 96.5–100.2% with CV 0.4–7.7% for TRP).

Precision of the method was tested by analysis repeatability and intermediate precision of KYN, KYNA and TRP in a pooled plasma at physiological level. Analysis repeatability was carried out by consecutive measurements of ten specimens deproteinized separately. Intermediate precision was tested by measuring analytes in triplicate on 5 individual workday with all samples deproteinized separately. Calculation of intermediate precision was based on the mean of triplicate determinations in each day. CVs of KYN, KYNA and TRP of either repeatability or intermediate precision were less than 5%. *t*-Test was applied to examine whether the results between repeatability and intermediate precision differed significantly at the 95% confidence level limit. There were no statistically significant differences for all analytes (Table 4).

Table 2
Limit of detection[a] of kynurenic acid.

Main components of the mobile phase			pH	Injection (μL)	Limit of detection (nmol/L)	Reference
$ZnAc_2$ (mmol/L)	NaAc (mmol/L)	HAc (mmol/L)				
0	20	–	7.5	100	9.2	Our experiment results
1	20	–	6.5	100	1.7	
2	20	–	6.5	100	1.1	
3	20	–	6.5	100	0.9	
4	20	–	6.5	100	0.7	
5	20	–	6.5	100	0.6	
250	–	50	4.9	100	0.5	[9]
100	–	50		100	2	[10]
500	50	–	6.2	20	0.05	[29]

[a] Signal/noise ratio = 3.

Table 3
Recoveries of kynurenine, kynurenic acid and tryptophan in human plasma (n = 5).

Spike value	Measured (mean ± SD)	Recovery (mean ± SD%)	CV (%)
Kynurenine (μmol/L)			
1	0.95 ± 0.04	95.4 ± 4.3	4.5
2	1.92 ± 0.03	95.9 ± 1.4	1.4
4	3.86 ± 0.07	96.5 ± 1.7	1.8
6	5.93 ± 0.08	98.9 ± 1.3	1.3
8	7.95 ± 0.05	99.4 ± 0.7	0.7
10	9.97 ± 0.05	99.7 ± 0.5	0.5
Kynurenic acid (nmol/L)			
10	10.4 ± 0.5	104 ± 5	5.1
20	19.8 ± 0.7	98.9 ± 3.4	3.4
40	40.1 ± 1.5	100.1 ± 3.8	3.8
60	60.7 ± 1.2	101 ± 2	2.0
80	80.3 ± 1.0	100.4 ± 1.2	1.2
100	99.8 ± 0.5	99.8 ± 0.5	0.5
Tryptophan (μmol/L)			
10	9.65 ± 0.71	96.5 ± 7.1	7.4
20	19.3 ± 1.5	96.6 ± 7.5	7.7
40	39.1 ± 1.2	97.8 ± 3.0	3.1
60	60.0 ± 1.1	100.1 ± 1.8	1.8
80	80.1 ± 0.9	100.2 ± 1.1	1.1
100	99.3 ± 0.4	99.3 ± 0.4	0.4

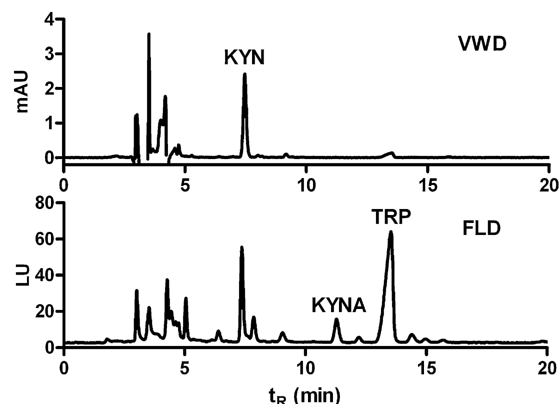

Fig. 4. Chromatograms of kynurenine, kynurenic acid and tryptophan in human plasma. An Agilent HC-C18 (2) column was employed as an analytical column. Mobile phase containing 20 mmol/L NaAc, 7% acetonitrile and 3 mmol/L $ZnAc_2$ at flow rate of 1.0 mL/min. Detections were performed by a variable wavelength detector (VWD) absorbance at 365 nm for kynurenine (KYN) and a fluorescence detector (FLD) at wavelengths excitation 344 nm and emission 398 nm for kynurenic acid (KYNA) and tryptophan (TRP) determinations. Detected concentrations of KYN, KYNA and TRP of this specimen were 2.23 μmol/L, 22.6 nmol/L and 46.2 μmol/L respectively.

3.7. Chromatograms of plasma

The chromatographic analyses were performed according to Section 2.5. Typical chromatograms of human plasma are presented in Fig. 4. Retention times (min) were KYN: 7.53 ± 0.03; KYNA: 11.33 ± 0.05; TRP: 13.40 ± 0.06 calculated by using the same samples for the intermediate precision test. Though the retention times of KYNA and TRP would be easily influenced by the concentration of $ZnAc_2$ in the mobile phase, which could not be prepared exactly the same every time, the variation of the retention time of each analyte seemed very small. The identification of the peaks was accomplished by use of spiking samples with quantitative reference compounds (standard-addition method). Peaks of all analytes were well resolved from each other and no interference was found from endogenous substances.

It was reported that KYNA was hardly retained on the ODS column when pH of mobile phase was at around 6.2 [28], we did not find any such issue in our experiments. Besides, peak shapes of the analytes were much narrower than those in the published papers [9,29].

3.8. Application

The developed method was applied to the determination of KYN, KYNA and TRP in human plasmas. Table 5 listed the results and the ratios of KYN/TRP and KYNA/KYN. Data were found to be well within the ranges reported previously in human plasma and serum [9,21,31]. *t*-Test was applied to examine whether the concentrations of KYN, KYNA, TRP and the ratios of KYN/TRP, KYNA/KYN between males and females differed significantly at the 95% confidence level limit. It followed that the differences were statistically significant for all compounds but not statistically significant for the ratios of KYN/TRP and KYNA/KYN. As KYN, KYNA and TRP concentrations vary over a comparatively wide range, the ratios of

Table 4
Precision of the method.

Compound	Repeatability		Intermediate precision		*P*
	Mean ± SD[a]	CV%	Mean ± SD[a]	CV%	
Kynurenine	2.25 ± 0.05	2.3	2.20 ± 0.04	1.7	0.09
Kynurenic acid	23.1 ± 0.6	2.8	23.1 ± 1.1	4.7	0.92
Tryptophan	46.3 ± 1.2	2.5	47.5 ± 1.7	3.6	0.13

[a] Kynurenine and tryptophan: μmol/L; kynurenic acid: nmol/L.

Table 5
Plasma kynurenine, kynurenic acid and tryptophan in 80 Chinese people (mean ± SD).

	Male (n = 39)	Female (n = 41)	P
Kynurenine (μmol/L)	2.20 ± 0.35	1.76 ± 0.31	<0.001
Kynurenic acid (nmol/L)	24.7 ± 6.9	18.3 ± 4.5	<0.001
Tryptophan (μmol/L)	47.8 ± 6.5	40.0 ± 5.2	<0.001
Kynurenine/tryptophan (%)	4.7 ± 0.8	4.4 ± 0.8	0.22
Kynurenic acid/kynurenine (%)	1.1 ± 0.3	1.1 ± 0.2	0.19

KYN/TRP, KYNA/KYN may give reasonable evidences for the activities of IDO and KAT. There was no evidence of age-dependency in the examined plasmas.

4. Conclusions

The main advantage of this method lay in the use of a mobile phase providing an ideal condition for the chelation reaction of KYNA–Zn^{2+} which greatly improved the sensitivity of KYNA. The method avoided the quenching problem of KYNA–Zn^{2+} associated with fluorescence detection and the clotting risk caused by high concentration of $ZnAc_2$. It allows the simultaneous, reproducible determination of KYN, KYNA and TRP in human plasma with great satisfaction and hence enables direct insight into the TRP metabolism in a rapid and simple manner. We believe that this simple and effective procedure will be useful in routine use and research investigations.

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