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Simultaneous determination of urinary tryptophan, tryptophan-related metabolites and creatinine by high performance liquid chromatography with ultraviolet and fluorimetric detection

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ABSTRACT

A high performance liquid chromatography method with ultraviolet and fluorimetric detection has been developed for the simultaneous determination of urinary creatinine (Cr), tryptophan (Trp) and three Trp-related metabolites including kynurenine (Kyn), kynurenic acid (Kyna) and 5-hydroxyindole-3-acetic acid (5-HIAA). Samples were pretreated by centrifugation after a freeze–thaw cycle to remove protein and other precipitates. Separation was achieved by an Agilent HC-C18 (2) analytical column and a gradient elution program with a constant flow rate 1 mL/min at an ambient temperature. Total run time was 30 min. Cr, Kyn and Kyna were measured by a variable wavelength detector at wavelengths 258 nm, 365 nm and 344 nm respectively. Trp and 5-HIAA were measured by a fluorescence detector with an excitation wavelength of 295 nm and an emission wavelength of 340 nm. This allowed the determination of Kyn/Cr, Kyna/Cr, Trp/Cr and 5-HIAA/Cr concentration ratios in a single run on the same urine sample. Good linear responses were found with correlation coefficient (r) > 0.999 for all analytes within the concentration range of physiological level. The limit of detection of the developed method was: Cr, 0.0002 g/L; Kyn, 0.1 μmol/L; Kyna, 0.04 μmol/L; Trp, 0.02 μmol/L and 5-HIAA, 0.01 μmol/L. Recoveries from spiked human urine were: Cr, 93.0–106.4%; Kyn, 97.9–106.9%; Kyna, 98.5–105.6%; Trp, 96.7–105.2% and 5-HIAA, 96.1–99.7%. CVs of repeatability and intermediate precision of all analytes were less than 5%. This method has been applied to the analysis of urine samples from normal subjects.

1. Introduction

Tryptophan (Trp) is an essential amino acid, which plays an important role in protein synthesis and acts as a precursor of many biologically active substances such as kynurenine (Kyn), kynurenic acid (Kyna), 5-hydroxytryptamine (serotonin, 5-HT), etc. Kyn is an endothelium-derived relaxing factor involved in blood pressure regulation during inflammation [1]. Kyna was demonstrated to be an endogenous antagonist at the glycine site of the N-methyl-D-aspartate (NMDA) as well as at the alpha 7 nicotinic cholinergic receptors [2]. 5-HT is involved in a variety of physiological processes, including smooth muscle contraction, blood pressure regulation and both peripheral and central nervous system neurotransmission [3]. Recently, a report suggested that 5-hydroxyindole-3-acetic acid (5-HIAA), the major metabolite of 5-HT, was responsible for thermal hyperalgesia [4]. Many efforts are still being made to further investigate the physiological functions and roles in pathogenesis of these compounds.

Urine is the primary physical fluid for excreting water-soluble chemicals from the bloodstream. Aside from water, urine contains an assortment of inorganic salts and organic compounds including proteins, hormones and a wide range of metabolites. Changes in the composition of urine could indicate metabolic issue of the body. As urine is able to be collected non-invasively, in clinical medicine, it is often used for the diagnosis of diseases by examining the organic or inorganic substances. Abnormal levels of urinary Trp-related metabolites were found in patients with carcinoid [5], breast cancer [6] and autistic symptom [7]. In an animal model, an increase of Kyna with a decrease in Trp was reported in a chronic unpredictable mild stress model of depression [8]. Because concentrations of the substances in urine fluctuate with the time of day and the intake of water, it is usual to analyze 24-h totals rather than

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measuring actual concentrations at any time. But the collection of 24-h urine is not convenient.

In clinical analysis, creatinine (Cr) is frequently considered to be the best natural internal standard for normalizing the excretion of other metabolites in urine. The ratio of analyte to Cr was used as a reference, enabling freedom from the rigid classical procedures of collecting 24-h urine [9,10].

Several high performance liquid chromatography (HPLC) methods have been developed to determine Cr and Trp-related metabolites in biological samples. Usually, ultraviolet (UV) detection is employed to determine Cr [11–13] and Kyn [14]; fluorimetric detection is used to measure Trp, Kyna and 5-HIAA [5,14,15]. HPLC with other detections such as electrochemistry [16–18], laser-induced fluorescence [19] and mass spectrometry [20–23] were also reported to have been used to determine these substances in biological samples.

The aim of the present work was to develop an HPLC method for the simultaneous quantitative measurement of Cr, Trp, Kyn, Kyna and 5-HIAA in urine using serial UV and fluorimetric detection. The performance of the method was evaluated in terms of accuracy, linearity, precision and limit of detection.

2. Experimental

2.1. Chemicals

Cr, Kyn, Kyna, 5-HT and 5-HIAA were purchased from Sigma. Trp was obtained from BDH Chemicals Ltd (England). Methanol and acetonitrile were of HPLC/SPECTRO grade and obtained from TEDIA Company Inc. (USA). Other chemicals and solvents were of analytical grade or biological reagent and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ultra-pure water was obtained through a distilled water system made of quartz and was used for the preparation of all solutions.

Stock solutions (1 mmol/L for Kyn, Kyna, 5-HT, Trp and 5-HIAA; 25 g/L for Cr) were prepared by dissolving the substances in distilled water. They were then kept frozen at −40 °C. A standard mixture containing 5 g/L Cr; 25 μmol/L Kyn; 50 μmol/L Kyna; 250 μmol/L Trp and 125 μmol/L 5-HIAA was obtained by diluting stock solutions with distilled water. Working standard solutions were made with appropriate dilution of the standard mixture with distilled water prior to use.

2.2. Apparatus

The HPLC equipment was an Agilent 1100 series LC system (Agilent Technologies, Germany) composed of 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 mm × 0.18 mm i.d. PEEK Tubing. An Agilent HC-C18 (2) column was employed as an analytical column (250 mm × 4.6 mm i.d.; 5 μm particle size).

2.3. Sample collection and preparation

Human urine was collected in 1.5-mL Eppendorf tubes and frozen at −20 °C. They were analyzed within 1 month after collection. Samples were thawed before use and centrifuged at 12,000 rpm for 5 min at 4 °C to remove protein [24] and other precipitates. Each supernatant (30 μL) was mixed with 120 μL distilled water (5-fold dilution) in a micro-sampling vial placed in a screw-cap vial specific for the autosampler of the HPLC system. The sample vials were then tightly capped to prevent oxidation by exposure to air.

Table 1
HPLC elution program.

Gradient time (min)	Eluent A (%)	Eluent B (%)
0	100	0
2	100	0
12	20	80
17	20	80
18	0	100
24	0	100
25	100	0
30	100	0

2.4. Chromatographic conditions

Separation was achieved by a gradient elution program (Table 1) with a constant flow rate 1 mL/min at an ambient temperature. Mobile phase consisted of eluent A (20 mmol/L NaAc, 30 mmol/L HAc and 3% methanol) and eluent B (20 mmol/L NaAc/HAc, 10% methanol and 10% acetonitrile). They were prepared daily, filtered through a 0.45-μm membrane filter and further degassed by sonication for 20 min. The VWD was set at 258 nm, changed to 365 nm at 9 min and changed again to 344 nm at 14 min for the determination of Cr, Kyn and Kyna respectively. Fluorimetric detection was performed with excitation at 295 nm and emission at 340 nm for the measurement of Trp and 5-HIAA. The volume per injection was 50 μL and the total time for urine sample analysis was 30 min. Concentrations of the analytes were calculated from peak areas. An HP Chemstation software was employed to control the HPLC system and process the chromatographic data.

2.5. Stability test for 5-HIAA

One milliliter urine sample from a healthy adult was centrifuged after a freeze–thaw cycle according to Section 2.3. The supernatant was transferred to four 1.5-mL Eppendorf tubes, 200 μL per tube. Each of them was diluted five times with a different solution: (1) distilled water; (2) 0.4 mol/L $HClO_4$, then neutralized by K_2CO_3; (3) a solution containing 0.4 mol/L $HClO_4$, 1.25 mmol/L oxalic acid and 3.75 mmol/L L-cysteine; and (4) 0.4 mol/L $HClO_4$. Each test solution was prepared freshly before its first injection and immediately aliquotted into 6 sample vials which were then tightly capped to prevent oxidation by the air. Every 4 h, one of them was injected onto the column to avoid the possibility of the metal injector needle contributing to the degradation of 5-HIAA [25]. The first injection of each test solution was considered as 0-h time point and used to normalize the data from other time points for the same solution.

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 the correlation coefficient (r) by linear regression analysis and test whether slopes of linear regression curves were significantly different between aqueous and standard-addition standards. Significance was determined as $P < 0.05$.

3. Results and discussion

3.1. Chromatographic conditions

Urine samples are often diluted one hundred times before Cr analysis with UV detection at a sensitive wavelength [11,12] because of the high concentrations typically present. This would greatly reduce the concentration of other analytes at the same time. As some of the analytes are in very small amount in urine even without any dilution, it is necessary to reduce the sensitivity for the

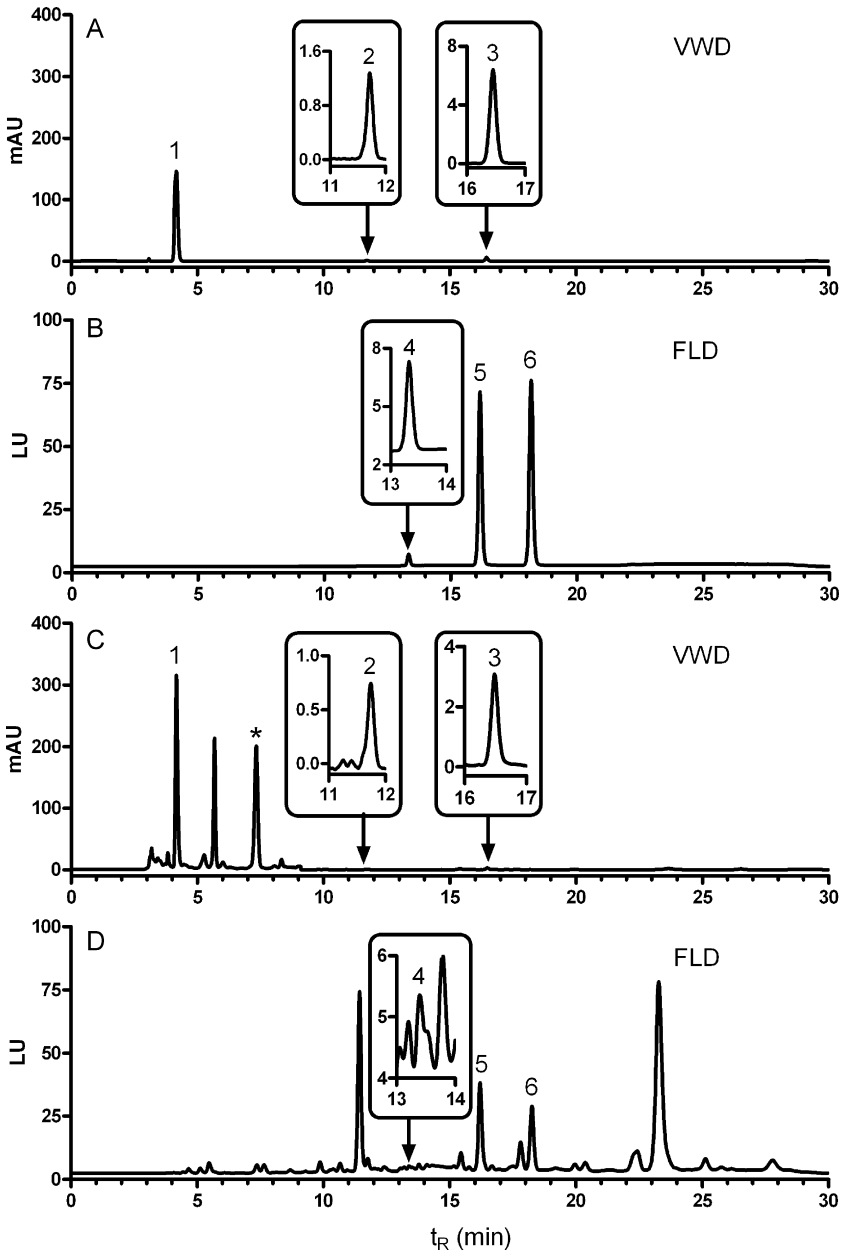

Fig. 1. Chromatograms of standard (A, B) and normal urine (C, D). Detections were performed by a variable wavelength detector (VWD) and a fluorescence detector (FLD). Injected amounts of the standard were: creatinine (Cr), 8 μg; kynurenine (Kyn), 40 pmol; kynurenic acid (Kyna), 80 pmol; 5-hydroxytryptamine (5-HT), 4 pmol; tryptophan (Trp), 400 pmol; and 5-hydroxyindole-3-acetic acid (5-HIAA), 200 pmol. Calculated concentrations of the analytes in urine were: Kyn, 0.25 mmol/mol Cr; Kyna, 0.37 mmol/mol Cr; Trp, 2.00 mmol/mol Cr; and 5-HIAA, 0.64 mmol/mol Cr. 1 = Cr, 2 = Kyn, 3 = Kyna, 4 = 5-HT, 5 = Trp and 6 = 5-HIAA; * = uric acid.

determination of Cr. After comparing the chromatograms of urine at different wavelengths, we selected 258 nm as the analytical wavelength which was much less sensitive than those recommended in published papers for the determination of Cr. The wavelength was changed to 365 nm at 9 min for the determination of Kyn and then changed to 344 nm at 14 min for the determination of Kyna. 5-HT, Trp and 5-HIAA were measured by fluorimetric detection using the wavelengths recommended by Kema et al. [5].

Cr or Trp-related metabolites could be separated by a reverse phase column with a constant elution, but the composition of the mobile phase for their separation was quite different. To solve this issue, we used a gradient elution technique, which enabled Cr, Kyn, Kyna, Trp and 5-HIAA in urine to be well separated within 30 min. Fig. 1 illustrates the chromatograms of standard solution (Fig. 1A and B) and urine (Fig. 1C and D). The retention time (min) of the analytes were: Cr, 4.18 ± 0.00 (VWD); Kyn, 11.73 ± 0.02 (VWD); 5-HT, 13.37 ± 0.04 (FLD); Trp, 16.18 ± 0.03 (FLD); Kyna 16.47 ± 0.02 (VWD) and 5-HIAA, 18.22 ± 0.05 (FLD). This gradient program also accelerated the elution of the unknown peaks after 5-HIAA and shortened the analysis time.

It was our wish to measure 5-HT simultaneously with the other analytes but the separation of it was not good from other peaks in urine matrix (Fig. 1D). It was omitted from further measurement because the poor resolution influenced its accurate integration.

3.2. Stability of 5-HIAA

5-HIAA is known to be unstable due to its rapidly oxidative breakdown, especially in an acidic solution. To permit automated analysis of a high amount of samples, it was recommended that antioxidants be used for protecting 5-HIAA from degradation during the analytical procedure [26]. Urine samples need be pretreated

Table 2
Slope, intercept and correlation coefficient (r) of the standard working curves.

Compound	Range (μmol/L)	Aqueous				Standard-addition				Difference between slopes
		Slope	*X*-intercept	*Y*-intercept	*r*	Slope	*X*-intercept[a]	*Y*-intercept[a]	*r*	
Cr	0.2–2 (g/L)	1613 ± 22	−0.033	52.95	0.9996	1524 ± 18	−0.007	11.07	0.9998	NS
Kyn	1–10	2.497 ± 0.029	−0.149	0.372	0.9997	2.486 ± 0.038	−0.061	0.152	0.9994	NS
Kyna	2–20	6.383 ± 0.022	−0.030	0.192	1.000	6.398 ± 0.059	0.069	−0.440	0.9998	NS
Trp	10–100	15.69 ± 0.08	−0.452	7.099	1.000	15.24 ± 0.22	−0.714	10.88	0.9997	NS
5-HIAA	5–50	37.31 ± 0.16	−0.242	9.010	1.000	36.74 ± 0.38	0.153	−5.635	0.9998	NS

Abbreviations: Cr, creatinine; Kyn, kynurenine; Kyna, kynurenic acid; Trp, tryptophan; 5-HIAA, 5-hydroxyindole-3-acetic acid; NS, not significant.
[a] Basal values of the original sample have been deducted for calculating the intercept of each standard working curve.

Table 3
Recoveries of Cr, Kyn, Kyna, Trp and 5-HIAA in human urine (n = 4).

Spike value	Urine 1	Urine 2	Urine 3	Urine 4	Recovered (mean ± SD)	Recovery (mean ± SD%)	CV (%)
Cr (g/L)							
0	0.86	0.70	0.56	1.08			
0.2	1.06	0.92	0.79	1.29	0.21 ± 0.02	106.4 ± 8.3	7.8
0.4	1.26	1.13	0.92	1.49	0.40 ± 0.03	99.9 ± 6.9	6.9
0.8	1.64	1.52	1.37	1.86	0.80 ± 0.02	99.6 ± 2.8	2.8
1.2	1.99	1.86	1.73	2.23	1.15 ± 0.02	96.1 ± 1.5	1.5
1.6	2.35	2.23	2.11	2.59	1.52 ± 0.02	95.1 ± 1.5	1.6
2	2.71	2.58	2.44	2.90	1.86 ± 0.03	93.0 ± 1.4	1.5
Kyn (μmol/L)							
0	1.45	1.33	1.02	1.02			
1	2.55	2.44	2.13	1.97	1.07 ± 0.08	106.9 ± 7.8	7.3
2	3.53	3.23	3.16	3.16	2.07 ± 0.11	103.3 ± 5.6	5.4
4	5.62	5.41	4.87	4.99	4.02 ± 0.14	100.4 ± 3.5	3.4
6	7.42	7.08	7.01	6.81	5.88 ± 0.12	97.9 ± 2.0	2.0
8	9.26	9.22	8.88	9.35	7.97 ± 0.24	99.7 ± 3.0	3.0
10	11.63	11.17	10.58	10.94	9.87 ± 0.25	98.7 ± 2.5	2.5
Kyna (μmol/L)							
0	4.97	4.87	2.24	4.06			
2	6.96	6.96	4.53	6.15	2.11 ± 0.13	105.6 ± 6.3	5.9
4	8.98	8.82	6.26	8.15	4.02 ± 0.06	100.4 ± 1.4	1.4
8	12.80	12.53	10.29	12.04	7.88 ± 0.17	98.5 ± 2.2	2.2
12	16.73	16.63	14.33	16.01	11.89 ± 0.16	99.1 ± 1.3	1.3
16	20.81	21.14	18.28	20.05	16.03 ± 0.18	100.2 ± 1.1	1.1
20	25.19	24.72	21.92	23.94	19.91 ± 0.23	99.5 ± 1.1	1.2
Trp (μmol/L)							
0	34.66	22.77	17.58	10.32			
10	45.36	33.21	28.40	20.45	10.52 ± 0.31	105.2 ± 3.1	2.9
20	55.62	43.15	37.90	30.71	20.51 ± 0.30	102.6 ± 1.5	1.5
40	73.84	62.03	57.16	50.22	39.48 ± 0.32	98.7 ± 0.8	0.8
60	93.53	81.29	76.23	69.04	58.69 ± 0.14	97.8 ± 0.2	0.2
80	112.07	100.30	95.80	89.73	78.15 ± 0.91	97.7 ± 1.1	1.2
100	130.36	119.87	114.62	107.37	96.72 ± 0.68	96.7 ± 0.7	0.7
5-HIAA (μmol/L)							
0	12.84	7.27	16.64	8.23			
5	17.39	12.18	22.04	13.30	4.98 ± 0.35	99.7 ± 6.9	7.0
10	22.22	17.07	26.58	18.13	9.76 ± 0.26	97.6 ± 2.6	2.6
20	32.91	26.23	36.06	27.83	19.52 ± 0.46	97.6 ± 2.3	2.4
30	42.27	36.01	45.73	37.20	29.06 ± 0.29	96.9 ± 1.0	1.0
40	51.89	45.62	55.58	47.38	38.87 ± 0.36	97.2 ± 0.9	0.9
50	61.22	55.05	64.93	56.01	48.06 ± 0.32	96.1 ± 0.6	0.7

Abbreviations: Cr, creatinine; Kyn, kynurenine; Kyna, kynurenic acid; Trp, tryptophan; 5-HIAA, 5-hydroxyindole-3-acetic acid.

Table 4
Precision of the method.

Compound (μmol/L)	Repeatability		Intermediate precision		Difference between results
	Mean ± SD	CV%	Mean ± SD	CV%	
Cr (g/L)	0.86 ± 0.01	0.65	0.85 ± 0.02	2.09	NS
Kyn	1.57 ± 0.03	2.08	1.56 ± 0.07	4.38	NS
Kyna	4.73 ± 0.08	1.71	4.65 ± 0.13	2.90	NS
Trp	33.40 ± 0.31	0.92	33.71 ± 0.53	1.58	NS
5-HIAA	12.96 ± 0.08	0.63	13.26 ± 0.48	3.58	NS

Abbreviations: Cr, creatinine; Kyn, kynurenine; Kyna, kynurenic acid; Trp, tryptophan; 5-HIAA, 5-hydroxyindole-3-acetic acid; NS, not significant.

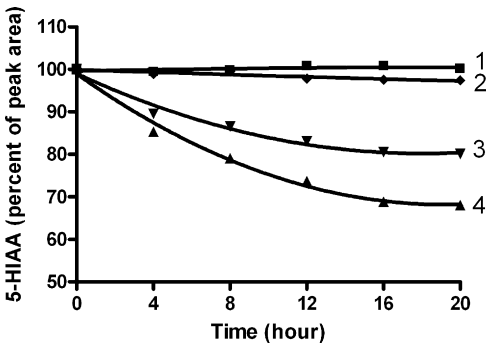

Fig. 2. Stability of 5-hydroxyindole-3-acetic acid. Supernatant from a centrifuged urine was diluted five times with (1) distilled water; (2) 0.4 mol/L $HClO_4$, then neutralized by K_2CO_3; (3) a solution containing 0.4 mol/L $HClO_4$, 1.25 mmol/L oxalic acid and 3.75 mmol/L L-cysteine; and (4) 0.4 mol/L $HClO_4$. 5-Hydroxyindole-3-acetic acid (5-HIAA) was analyzed by HPLC with fluorimetric detection. First injection of each test solution was considered as 0-h time point and used to normalize the data of other time points of the same solution.

to remove protein and other impurities prior to injection onto the column. The simplest way was centrifugation after a freeze–thaw cycle [24,27]. Another common method is using a protein precipitant, such as $HClO_4$ [28,29], trichloroacetic acid (TCA) [30] and acetonitrile [31,32]. Our preliminary experiments showed that the separation of the acetonitrile-treated sample was not good and that a TCA-treated sample had a little bit of interference with Cr. They were omitted from further testing. The detailed arrangement of the stability test was described in Section 2.5. Considering the influence of the sample matrix, the evaluation was carried out using endogenous 5-HIAA in a real urine sample. Fig. 2 shows the results of the 20-h stability experiment which indicated that $HClO_4$ greatly accelerated the degradation of 5-HIAA. At the 20-h time point, the relative value (percent of peak area) dropped to 68% (Fig. 2, line 4). The recommended antioxidant (a mixture of oxalic acid and L-cysteine) [26] showed some effect to slow this down but could not completely prevent the degradation of 5-HIAA in the presence of $HClO_4$ (Fig. 2, line 3). It seemed that 5-HIAA was very stable in neutral solution. No obvious loss of 5-HIAA was found in urine treated by $HClO_4$, then neutralized with K_2CO_3 (Fig. 2, line 2) or diluted with distilled water (Fig. 2, line 1). The latter way was employed for the further use in our experiments because it was easy and avoided error from unnecessary chemical procedures.

3.3. Linearity and slope of standard working curves

A series of mixed standard solutions were used for the construction of six-point calibration curves. The concentration range was in accordance with physiological levels. Standard-addition working curves were constructed by spiking urine with mixed standard solutions. Statistical parameters for the linear regression are summarized in Table 2. In all cases, no statistical difference was found when a comparison of slopes between the aqueous standard and the standard-addition calibration curve was made. This meant that direct standardization with aqueous calibration curves were available for the determination of Cr, Kyn, Kyna, Trp and 5-HIAA in urine samples.

3.4. Accuracy, precision and limit of detection

Evaluation of accuracy was carried out by six-point recovery test. Known quantities of analytes were spiked to urine sam-

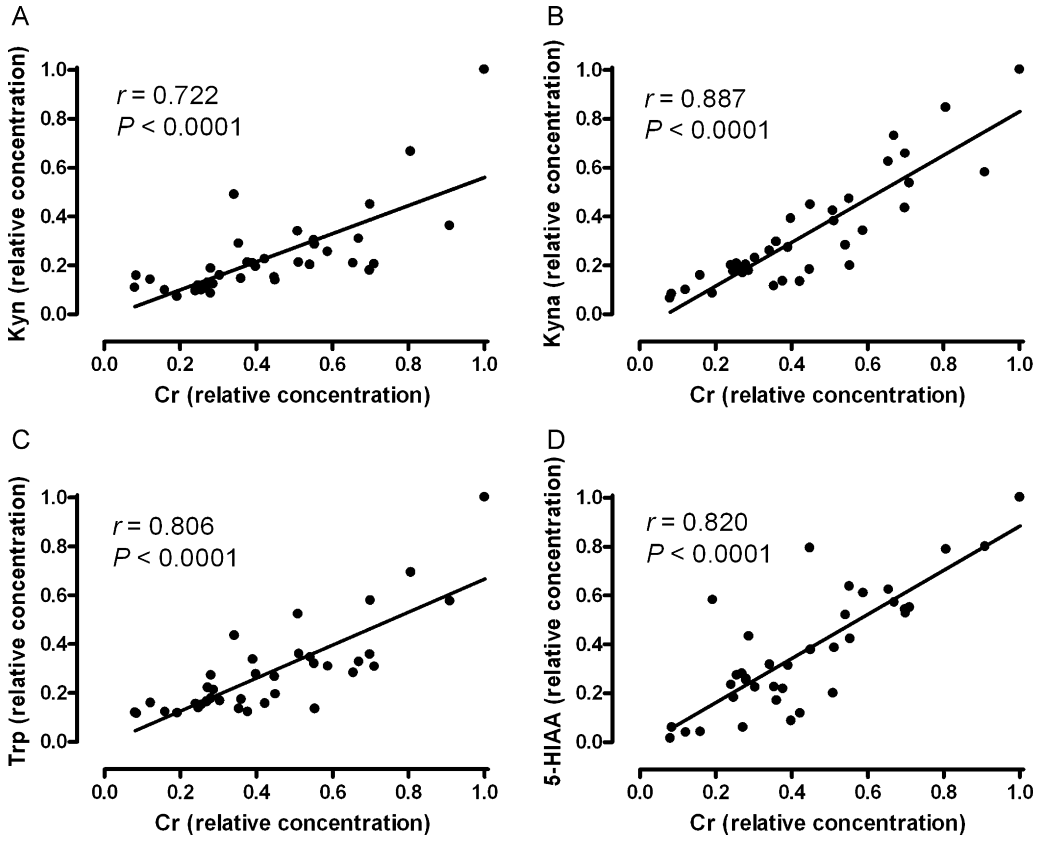

Fig. 3. Correlation between creatinine and other analytes in urine. Urine samples were collected randomly from 38 healthy Chinese adults. Creatinine (Cr), kynurenine (Kyn), kynurenic acid (Kyna), tryptophan (Trp) and 5-hydroxyindole-3-acetic acid (5-HIAA) were determined by HPLC with UV and fluorimetric detection. Data were normalized by the sample containing the highest concentration of Cr (relative concentration).

ple. Concentrations of the analytes from the original urine $C_A(O)$ and recovered from the spiked urine $C_A(O+S)$ were obtained via aqueous standard 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 and SD of each concentration point were calculated based on 4 independent experiments using different urine samples. Table 3 lists the results which indicated that the described method had efficient recovery (Cr, 93.0–106.4%; Kyn, 97.9–106.9%; Kyna, 98.5–105.6%; Trp, 96.7–105.2% and 5-HIAA, 96.1–99.7%).

Precision of the method was tested by repeatability and intermediate precision using a urine sample from a healthy adult. Repeatability was carried out by consecutive measurement of ten specimens. Intermediate precision was tested by measuring analytes in triplicate on 5 individual workdays. CVs of repeatability and intermediate precision of all analytes 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).

At a signal-to-noise ratio of 3, the limit of detection of the method for the analytes were: Cr, 0.0002 g/L; Kyn, 0.1 μmol/L; Kyna, 0.04 μmol/L; Trp, 0.02 μmol/L and 5-HIAA, 0.01 μmol/L.

3.5. Application

The developed method was applied to the determination of Kyn, Kyna, Trp, 5-HIAA and Cr in human urine. Samples (no dietary restriction) were collected randomly from 38 healthy Chinese adults (including 18 from males and 20 from females). Calculated concentrations [median (range)] in the urine samples were: Kyn, 0.21 (0.11–1.08) mmol/mol Cr; Kyna, 0.79 (0.33–1.15) mmol/mol Cr; Trp, 3.82 (1.08–9.08) mmol/mol Cr; and 5-HIAA, 0.94 (0.20–3.37) mmol/mol Cr. Trp and 5-HIAA were lower than those reported previously [5]. No data could be found from the published papers for the comparison of urinary Kyn and Kyna expressed as mmol/mol Cr.

A graphical representation of the correlation between Cr and other analytes is given in Fig. 3. Data were normalized by the sample containing the highest concentration of Cr (relative concentration). The result showed that Cr was positively correlated to the other analytes in urine. Strong correlations were observed between Cr and Kyna ($r = 0.887$), Trp ($r = 0.806$) and 5-HIAA ($r = 0.820$). A moderate correlation was observed between Cr and Kyn ($r = 0.722$). All *P* values were less than 0.0001.

4. Conclusions

We have successfully developed a method for simultaneously analyzing Trp, Kyn Kyna, 5-HIAA and Cr in human urine using HPLC with UV and fluorimetric detection. This allows the determination of Kyn/Cr, Kyna/Cr, Trp/Cr and 5-HIAA/Cr concentration ratios in a single run on the same urine sample. We believe that this method will be useful in routine use and research investigations.

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