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Short communication

Determination of tryptophan and its kynurenine pathway metabolites in human serum by high-performance liquid chromatography with simultaneous ultraviolet and fluorimetric detection

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic method for the simultaneous determination of tryptophan and four metabolites of the kynurenine pathway (kynurenine, 3-hydroxykynurenine, kynurenic acid and 3-hydroxyanthranilic acid) in human serum is described. This new method, which uses both isocratic elution and two on-line connected programmable ultraviolet and spectrofluorimetric detectors, allows the determination of these metabolites, in the physiological ranges, with satisfying specificity and sensitivity within 30 min.

Keywords: Tryptophan; Kynurenine; 3-Hydroxykynurenine; Kynurenic acid; 3-Hydroxyanthranilic acid.

1. Introduction

Mammalian peripheral tissues metabolized approximately 90% of tryptophan (L-TRP) by the kynurenine pathway [1]. Interestingly, in the central nervous system (CNS) two metabolites are neuroactive compounds: kynurenic acid (KYNA) and quinolinic acid. Recent studies have implicated the kynurenine pathway metabolites in the pathogenesis of several neurologic disorders, particularly in neurodegenerative diseases [2–11].

Different HPLC methods for serum determination of L-TRP [12,13] alone, or L-kynurenine (KYN) and 3-hydroxy-DL-kynurenine (3-HKYN) [6,9,14], or KYNA [15] have been already reported. However, few HPLC methods described the simultaneous determination of L-TRP and its kynurenine pathway metabolites, with UV and fluorimetric detections [16,17]. The purpose of our study was to develop a rapid isocratic-elution HPLC method for the measurement in biological

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samples of L-TRP and its metabolites at physiological levels in a single run.

2. Experimental

2.1. Instrumentation

HPLC analysis were performed using a Waters 600E pump and gradient controller (Waters Chromatography Corp., Milford, MA, USA) and a Rheodyne injection valve with a 100-μl fixed sample loop (Model 7125, Rheodyne, Cotati, CA, USA). The analytical column was a 100 mm × 4.7 mm I.D., packed with Partisphere 5 μm C_{18} (Whatman, Clifton, NJ, USA). Separations were achieved at ambient temperature by isocratic elution at a flow-rate of 1.0 ml/min. The detection and the quantification were carried out with a Waters 470 scanning fluorescence detector connected on line with a Waters 994 programmable photodiode array detector. The fluorescence excitation and emission wavelengths were set to 320 and 420 nm respectively at the beginning of the run (bandwidths 18 nm). Eight minutes later, excitation and emission wavelengths were changed to 254 and 404 nm. UV signals were then monitored at 230 and 365 nm (bandwidths 3 nm). These optimized conditions were determined by recording fluorescence spectra with a stop-flow technique and UV spectra from 200 to 400 nm. Acquisition and processing of the chromatograms were performed using a Baseline 820 Workstation software [version 3.31 (Waters)], on a NEC APC IV microcomputer (NEC Informations Systems, Boxborough, MA, USA). The concentrations were determined as the peak-height measurement against external standards.

2.2. Reagents

The reference metabolites investigated [L-TRP, KYN, 3-HKYN, KYNA and 3-hydroxyanthranilic acid (3-HANT)] and zinc acetate were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, acetic and perchloric acids were obtained from Merck (Darmstadt, Germany).

The mobile phase consisted of 50 m*M* acetic acid, 250 m*M* zinc acetate (pH 4.9) with 1% (v/v) acetonitrile. The mobile phase was prepared daily and filtered through a 0.45-μm Millipore filter (Millipore, Milford, MA, USA).

2.3. Sample preparation

Millimolar stock solutions of each standard were prepared and stored at −80°C. These solutions were stable for at least six months. Working solutions were freshly diluted as required.

Human sera were obtained from 35 healthy volunteers (mean age: 36.1 years, range: 18–59 years). Venous blood samples were collected in tubes without anticoagulant after overnight fasting. The samples were centrifuged (3500 *g*, 15 min, 4°C) and the sera stored at −80°C until processing. Samples were deproteinized by addition of 50 μl of 2.4 *M* perchloric acid to 0.5 ml of serum. After centrifugation (3500 *g*, 15 min, 4°C), the supernatant was transferred into a glass tube and 100 μl of filtered (0.22-μm Millipore filter) supernatant were injected onto the column. Injections of acidic supernatants in the mobile phase did not harm the C_{18} column [6].

2.4. Statistical analysis

Correlations and regressions were analysed by the least squares method (Statview software on Macintosh).

3. Results and discussion

The chromatographic analyses were performed with three chromatograms: UV absorbance at 230 nm and 365 nm and fluorescence intensity at two coupled excitation and emission wavelengths. A chromatographic analysis of an aqueous standard solution is presented in Fig. 1 and a typical chromatographic analysis of human serum in Fig. 2. The retention times of 3-HKYN, 3-HANT, KYN, L-TRP and KYNA were respectively 3.33, 3.57, 5.91, 12.39 and 18.79 min. The

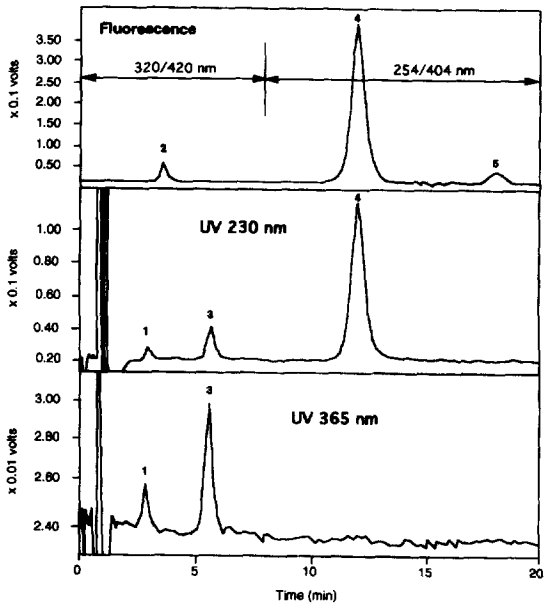

Fig. 1. Chromatographic analysis of an aqueous standard solution. Peaks: 1, 0.2 μM 3-HKYN; 2, 100 nM 3-HANT; 3, 1.0 μM KYN; 4, 25 μM L-TRP; 5, 50 nM KYNA.

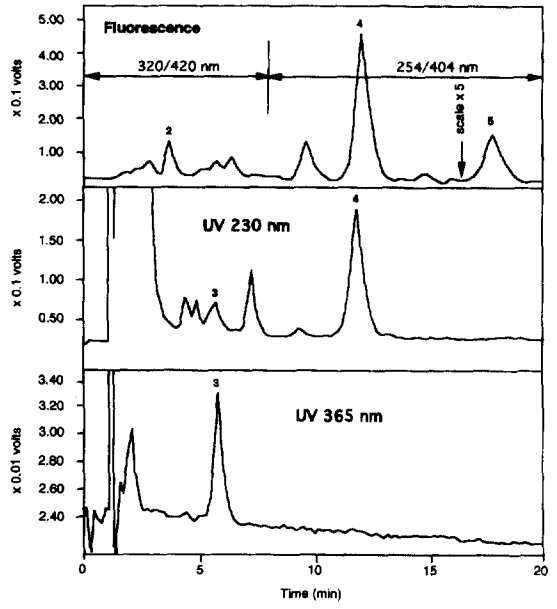

Fig. 2. Chromatographic analysis of a typical human serum. Peaks: 2, 3-HANT; 3, KYN; 4, L-TRP; 5, KYNA.

identification of the peaks was accomplished by use of two procedures: (a) by spiking samples with reference compounds, and (b) by analysing the spectral properties of the metabolites: UV absorbance ratio at 230 nm and 365 nm for KYN and 3-HKYN, and ratio of the UV absorbance (230 mm) to fluorescence intensity (254/404 nm) for L-TRP (not detectable at 365 nm at physiological levels).

Recovery tests were performed in triplicate by spiking the sample before deproteinization with 0.2 μM 3-HKYN, 100 nM 3-HANT, 1.0 μM KYN, 25 μM L-TRP and 50 nM KYNA. Results were not significantly different from 100% [respectively $100 \pm 1.0\%$, $95 \pm 2.6\%$, $97 \pm 2.3\%$, $98 \pm 2.2\%$ and $101 \pm 3.1\%$ (mean $\pm$ S.D.)].

The intra- and inter-assay coefficients of variation (C.V.s) measured (10 replicates) were respectively 4.7 and 2.0% for 3-HANT (75 nM), 2.3 and 3.5% for KYN (1.8 μM), 4.4 and 4.1% for L-TRP (40 μM), 5.2 and 4.5% for KYNA (32 nM). Taken together, results of the recovery tests and the C.V.s allowed us not to use an internal standard as previously described [5,6,9,10,12,14,16,17]. Thus, the use of an internal standard will be preferred in multiple step methods [13].

Linearity and detection limits were determined by analysis of standard solutions from 0 to 100 μM for 3-HKYN and KYN, from 0 to 10 μM for 3-HANT, from 0 to 1000 μM for L-TRP and from 0 to 1000 nM for KYNA. The signal intensities were proportional to concentrations in these ranges. The detection limits, as signal-to-noise ratio of 3 with an injection volume of 100 μl, are presented in Table 1. Our detection limits, especially for KYN, 3-HANT and KYNA, were lower than those already published [16,17], which were in the picomolar range. This new technique can be successfully used to measure basal levels and not only in tryptophan loading tests. We achieved lower detection limits by (a) optimization of sample preparation and detection conditions and (b) the choice of the mobile phase. Zinc acetate, which greatly enhanced the fluorescence of KYNA [18], was incorporated in the mobile phase as described by Wu et al. [11]. Finally, this mobile phase contributed to the

Table 1
Fluorescence (FL) and UV detection limits (signal-to-noise ratio of 3 and injection volume of 100 μl)

Compound	FL (λ ex./λ em., nm)[a]	UV (λ, nm)
3-HKYN	Not fluorescent	0.1 μM (230) 0.2 μM (365)
KYN	Not fluorescent	0.1 μM (230) 0.2 μM (365)
3-HANT	1.0 nM (320/420)	1.0 μM (230) 5.0 μM (365)
L-TRP	0.03 μM (254/404)	1.0 μM (230) 1.0 mM (365)
KYNA	0.5 nM (254/404)	>1.0 μM (230) >1.0 μM (365)

[a] ex. = excitation; em. = emission.

realization of an "on-column derivatization", which is more convenient than the post-column method [5,10,18] and without any damage for the detection of the other compounds. In contrast to Caruso et al. [16], the separation of L-TRP and its kynurenine pathway metabolites was done using an isocratic elution.

The serum concentrations in our healthy fasting subjects are reported in Table 2. The concentrations were in good agreement with those reported previously for L-TRP [7,12,15,19], KYN [6,14] and KYNA [15]. To our knowledge, no reference values for 3-HANT in human serum were reported in the literature. In all sera analysed, 3-HKYN was undetectable according to Holmes et al. [6], using a similar HPLC method with UV detection (365 nm). Better sensitivity for 3-HKYN could be reached with electrochemical detection [9,15]. No relationship was noted between 3-HANT and L-TRP but L-TRP and KYN, KYN and KYNA, and finally L-TRP and 3-HKYN were linearly linked ($r = 0.334$, $r = 0.332$ and $r = 0.462$, respectively, $p < 0.05$). These data confirmed the metabolic relationships between these kynurenine pathway compounds.

Table 2
Trytophan and its metabolites in 35 healthy human sera

Compound	Concentration		
	Mean	Range	S.D.
3-HKYN (μM)	<0.1	—	—
KYN (μM)	1.35	0.7–3.0	0.26
3-HANT (nM)	79	15–209	53.6
L-TRP (μM)	38	25–53	7.1
KYNA (nM)	23	6–54	10.3

4. Conclusions

Our isocratic-elution HPLC method allows the simultaneous, accurate and reproducible determination of tryptophan in human serum and most of its metabolites in the kynurenine pathway in a total analysis time of less than 30 min. Our data confirmed the metabolic relationships between these kynurenine pathway compounds and we believe that this simple procedure will be useful in characterizing various acquired or inborn diseases related to abnormalities in the metabolism of tryptophan.

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