Journal of Chromatography, 612 (1993) 187–190
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6653

Determination of tryptophan in human serum by high-performance liquid chromatography with pre-column fluorescence derivatization using phenylglyoxal

Eijiro Kojima, Masaaki Kai and Yosuke Ohkura

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

(First received September 1st, 1992; revised manuscript received October 27th, 1992)

ABSTRACT

A high-performance liquid chromatographic method based on pre-column fluorescence derivatization with phenylglyoxal is described for the sensitive and selective quantification of total (free plus albumin-bound) and free tryptophan in serum. Serum was deproteinized with perchloric acid for total tryptophan and by ultrafiltration for free tryptophan, and then subjected to fluorescence derivatization. The reaction mixture was separated on a reversed-phase column by isocratic elution and the tryptophan derivative was then detected by fluorimetry. The method was at least ten-fold more sensitive than other conventional chromatographic methods with no derivatization. The lower limit of determination (signal-to-noise ratio = 3) for endogenous tryptophan in human serum was 72 nmol/l, which corresponds to 200 fmol on-column.

INTRODUCTION

Tryptophan (Trp) in serum has been quantified in studies concerning medical diagnoses [1–4] and metabolism [5]. Various methods have been reported for the determination of Trp in serum: spectrofluorimetry [6] utilizing norharman formation of Trp with formaldehyde in the presence of an oxidant; isocratic high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [7] or native fluorescence detection [8] and with pre-column derivatization on the basis of the norharman formation for fluorescence detection [9]; column-switching HPLC with native fluorescence detection [10]; and gas chromatography–mass spectrometry (GC–MS) [11].

In these methods, chromatography has been necessary to separate Trp from various interfering substances in complex biological samples. However, HPLC methods [7,8,10] involving no derivatization for detection often show unsatisfactory reliability in routine quantitative assays of endogenous Trp, because of the limited selectivity and low sensitivity (submicromolar concentration), though the methods permit simple and ready assays. The GC–MS method [11] affords the most reliable determination of Trp concentration in biological samples, but it needs tedious clean-up procedures before the derivatization for volatilizing and GC. The HPLC method [9] with pre-column fluorescence derivatization using formaldehyde and hexacyanoferrate as oxidizing agents can selectively detect Trp at nanomolar concentrations in serum. However, using this method tryptamine cannot be separated from Trp and fluoresces with a peak height that corre-

Correspondence to: Y. Ohkura, Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812, Japan.

0378-4347/93/$06.00

sponds to 24% of that from Trp at an equimolar concentration.

We have recently reported that phenylglyoxal (PGO) is a fluorogenic reagent that is more selective for Trp than the formaldehyde and hexacyanoferrate reagents. It provides a fluorescent compound, 1-(1-hydroxybenzyl)-β-carboline (β-HBC) under acidic conditions [12] and is suitable for the manual spectrofluorimetric determination of Trp at submicromolar concentrations in human serum [13]. We herein report the application of the PGO reaction to HPLC with pre-column fluorescence derivatization of Trp in order to obtain a sensitive and reliable method for the determination of free and total (free plus albumin-bound) Trp in serum.

EXPERIMENTAL

Reagents and solutions

PGO hydrate was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade. PGO solutions (60 m*M*) was prepared in dimethyl sulphoxide. The solution was stable for at least two weeks at room temperature. Phosphate buffer (pH 2.0) was prepared by adjusting the pH of 50 m*M* sodium dihydrogenphosphate with 50 m*M* phosphoric acid.

Serum deproteinization

Total Trp. A portion (10 μl) of serum was mixed with 30 μl of water (or a standard aqueous solution of Trp for the calibration graph) and 200 μl of 0.3 *M* perchloric acid. After centrifugation at 1000 *g* for 5 min, the supernatant was used for fluorescence derivatization.

Free Trp. Serum (10 μl) was mixed with 10 μl of water (or a standard aqueous solution of Trp for the calibration graph) and 80 μl of saline, and then ultrafiltered through a membrane filter (Ultrafree; molecular mass exclusion limit, 30 000; Nihon Millipore, Tokyo, Japan). The filtrate was used for the derivatization.

Pre-column derivatization

Total Trp. A portion (100 μl) of the acid-deproteinized sample was mixed with 50 μl of 60 m*M* PGO. The mixture was heated at 100°C for 15 min. The reaction mixture (100 μl) was subjected to HPLC.

Free Trp. A portion (10 μl) of the ultrafiltered sample was mixed with 150 μl of 0.3 *M* perchloric acid and 100 μl of 60 m*M* PGO. The mixture was heated as for free Trp. An aliquot (100 μl) of the reaction mixture was used for HPLC.

HPLC system and its operation conditions

The HPLC system consisted of a Tosoh (Tokyo, Japan) 803 D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Hitachi F-1000 fluorescence spectrophotometer fitted with a 12-μl flow cell operating at an emission wavelength of 460 nm and an excitation wavelength of 385 nm. A column (150 mm × 4.6 mm I.D.) of TSK gel ODS-80T_M (particle size 5 μm; Tosoh) was used. The mobile phase, acetonitrile–50 m*M* phosphate buffer (pH 2.0)–water (33:35:32, v/v), was delivered at a flow-rate of 1.0 ml/min. The column temperature was ambient (25 ± 4°C). The concentration of Trp was evaluated on the basis of peak heights in chromatograms.

RESULTS AND DISCUSSION

Fluorescence derivatization and HPLC separation

Trp reacts with PGO in acid solution such as perchloric, hydrochloric and phosphoric acids [12]. Perchloric acid was useful not only for the derivatization of Trp but also for the deproteinization of the serum sample for the determination of Trp; 0.2–0.3 *M* acid in the reaction provided the maximum production of fluorescent derivatives from Trp. The other optimum conditions of the derivatization were almost identical to those described previously [12].

The reaction mixture was separated by reversed-phase HPLC with isocratic elution of aqueous mobile phase containing 33% (v/v) acetonitrile and 35% (v/v) 50 m*M* phosphate buffer (pH 2.0) (Fig. 1). The chromatogram obtained with the reaction mixture of Trp shows mainly three fluorescent peaks (peaks 1–3 in Fig. 1A).

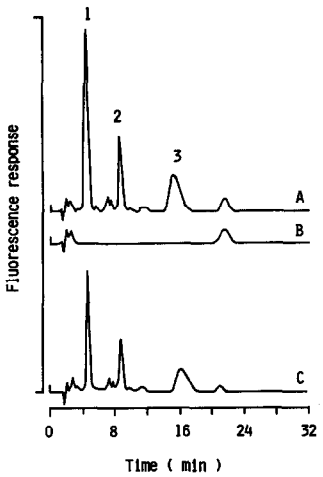

Fig. 1. Chromatograms of the reaction mixtures of (A) 100 μM Trp, (B) the reagent blank and (C) a serum sample containing 66.8 μM total Trp. For HPLC conditions, see Experimental section. Peaks: 1 = β-HBC; 2 and 3 = unknown fluorescent derivatives derived from Trp; others = by-products derived from Trp and/or phenylglyoxal.

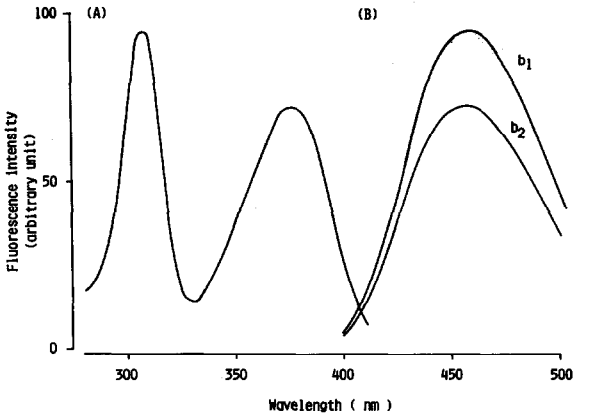

Fig. 2. Fluorescence excitation (A) and emission (B) spectra of the eluate of peak 1. Trp (100 μM, 10 μl) was subjected to derivatization. The emission spectra of b_1 and b_2 were obtained with irradiation at the excitation wavelenghts of 310 and 385 nm, respectively.

The retention times of peaks 1–3 were 4.6, 9.0 and 17.2 min, respectively. Peak 1, the highest peak, was ascribed to β-HBC by the retention time, co-chromatography with β-HBC and the fluorescence excitation and emission spectra of the peak eluate. The chemical structures of the components of peaks 2 and 3 remain unidentified. Peak 1 was used for the quantification.

The eluate of peak 1 fluoresced intensely at rather strongly acidic pH values; pH 2 was selected in the mobile phase. The fluorescence excitation maxima of the eluate were at 310 and 385 nm for the emission maximum at 460 nm (Fig. 2). On irradiating at 385 nm, the baseline noise was approximately one fifth lower than at 310 nm, though the peak height of the Trp derivative was approximately 77% at 385 nm in comparison with that at 310 nm: excitation at 385 nm was used for the fluorescence detection.

When tryptamine was subjected to the derivatization and separated under the same conditions as in Fig. 1, the tryptamine peak was found at the same retention time as for peak 1 in the chromatogram, but its height was approximately 1% of that given by Trp at an equimolar concentration (100 μmol/l). In addition, the tryptamine peak can be separated from the Trp peak when the acetonitrile concentration in the mobile phase is reduced to 25% (v/v) from the 33% (v/v) used for Fig. 1. In this case, the retention times of Trp and tryptamine derivatives were 12.8 and 11.2 min, respectively. No fluorescent peak was detected with other Trp-related compounds (100 μmol/l), such as serotonin, 5-hydroxytryptophan, indoleacetic acid and Trp-containing peptides (Trp-Trp, Ala-Trp and Lys-Trp-Lys).

Determination of tryptophan in human serum

Trp occurs as free and albumin-bound forms in serum [14]. Therefore, serum deproteinization was performed by ultrafiltration for free Trp and acidic denaturation for total Trp, according to procedures reported by Eccleston [15]. Fig. 1C depicts a typical chromatogram obtained with a serum sample for the determination of total Trp. The pattern of the chromatogram is quite similar to that in Fig. 1A for the Trp standard. This result means that no fluorescent peak was derived from biological substances other than Trp in the serum sample. In the chromatography, tryptamine present in serum did not interfere with the determination of Trp, because the tryptamine

TABLE I

CONCENTRATIONS OF TOTAL AND FREE TRP IN SERA FROM HEALTHY SUBJECTS

Blood samples were collected at 10:30–11:00 from healthy volunteers who did not have a breakfast.

Sex	Age	Total Trp (μmol/l)	Free Trp (μmol/l)
Male	32	58.0	19.6
Male	27	64.4	17.8
Male	26	55.4	22.2
Female	24	68.0	15.7
Female	24	42.6	21.3
Female	21	55.9	14.4
Mean		57.4	18.5
Standard deviation		8.80	3.09

peak in the sample was not detected by HPLC. The tryptamine concentration in human serum is normally two or three orders of magnitude lower than that of Trp [9].

The calibration graphs were linear in both the presence and absence of serum up to at least 150 μmol/l (correlation coefficient, 0.998). However, the slopes of the lines varied slightly depending on the serum used. Therefore, the quantification of total and free Trp in serum should be performed by the standard addition method.

The reproducibility of the present method was examined by seven replicate determinations using a serum sample with a mean Trp concentration of 69 μmol/l. The relative standard deviation (R.S.D.) was 5.1%. The recovery of Trp in spiked serum was 111 ± 2.9% (total Trp) and 77 ± 3.2% (free Trp) (mean ± R.S.D., $n = 6$), respectively, which was measured after adding 10 μl of 100 μ*M* Trp to 10 μl of serum and allowing the mixture to stand for 15 min at room temperature (25°C). The limit of detection (signal-to-noise ratio = 3) was 72 nmol/l in serum (200 fmol on-column).

The determined values of total and free Trp in sera from six normal subjects are shown in Table I. The values of total and free Trp that were obtained by the present method agree with the reported data [6,13].

In conclusion, we have developed a highly sensitive and reliable HPLC method using the PGO reagent for the determination of Trp in human serum. The sensitivity of the proposed method is at least one order of magnitude higher than that of spectrofluorimetric methods [6,13], HPLC methods [7,8,10] with UV and native fluorescence detection and GC–MS [11], though it is similar to that of pre-column fluorescence derivatization HPLC [9] based on norharman formation. The present method should be used especially for the confirmation of abnormal concentrations of biogenic Trp obtained by other conventional methods in medical and physiological studies, because of its high selectivity for Trp.

REFERENCES

1 L. Branchey, S. Shaw and C. S. Lieber, *Life Sci.*, 29 (1981) 2751.
2 A. Coppen, E. G. Eccleston and M. Peet, *Lancet*, ii (1973) 60.
3 J. Lehman, *Acta Psychiatr. Scand. Suppl.*, 300 (1982) 1.
4 E. Rocchi, F. Farina, M. Silingardi and G. Casalgrandi, *J. Chromatogr.*, 180 (1986) 128.
5 P. J. Knott and G. Curzon, *Nature*, 239 (1972) 452.
6 W. D. Denckla and H. K. Dewey, *J. Lab. Clin. Med.*, 69 (1967) 160.
7 H. Gjerde, P. T. Normann and J. Morland, *Biomed. Biochim. Acta*, 46 (1987) 53.
8 T. Flatmark, S. W. Jacobsen and J. Haavik, *Anal. Biochem.*, 107 (1980) 71.
9 S. Inoue, T. Tokuyama and K. Takai, *Anal. Biochem.*, 132 (1983) 468.
10 I. Morita, T. Masujima, H. Yoshida and H. Imai, *Anal. Biochem.*, 151 (1985) 358.
11 H. Wegmann, H. C. Curtis and U. Redweik, *J. Chromatogr.*, 158 (1975) 305.
12 E. Kojima, M. Kai and Y. Ohkura, *Anal. Chim. Acta*, 248 (1991) 213.
13 E. Kojima, M. Kai and Y. Ohkura, *Anal. Sci.*, (1993) in press.
14 R. H. McMenamy, C. C. Lund, J. V. Marcke and J. L. Oncley, *Arch. Biochem. Biophys.*, 93 (1961) 135.
15 E. G. Eccleston, *Clin. Chim. Acta*, 48 (1973) 269.