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Determination of tryptophan and its metabolites in human plasma and serum by high-performance liquid chromatography with automated sample clean-up system

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SUMMARY

An automated high-performance liquid chromatographic method that incorporates direct injection of biological samples followed by chromatographic sample clean-up in a precolumn is described for the determination of tryptophan and its metabolites in human plasma and serum. The system gave reproducible data with a coefficient of variation of less than 3% with a sample size of 100 μl of human plasma. The major tryptophan metabolites found in 100 μl of human plasma were kynurenine, indolelactic acid, indoleacetic acid, indolepropionic acid, serotonin and 5-hydroxyindoleacetic acid. The level of tryptophan and kynurenine in individuals was constant in comparison with other metabolites. Analysis of samples from normal controls, diabetics, gravida and their foetuses showed a tendency for tryptophan metabolites to be low in maternal plasma.

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INTRODUCTION

In man, tryptophan (Trp) is metabolized by two major pathways, either through kynurenine or via a series of indoles. Many of these metabolites are biologically active and the importance of Trp and its metabolites in both physiological and pathological states has been reviewed [1,2]. Measurement of Trp and its metabolites in biological samples has been actively investigated in many laboratories.

High-performance liquid chromatography (HPLC) has become a useful tool for the analysis of biologically active compounds owing to its ability to separate these compounds with closely similar structures [3–5]. Prior to injection of biological samples into the HPLC system, sample pretreatments such as solvent extraction or deproteinization have usually been required and these have been rate-limiting steps, being the most laborious and least accurate part of the analytical procedures. In order to avoid the disadvantages of conventional sample pretreatments, various methods of sample pretreatment have been developed. For the purpose of injecting biological samples such as plasma or serum directly into a column, an HPLC system based on on-line solid-phase extraction of analytes in a column has been introduced using the column-switching technique [6–8] or new columns [9,10] for pretreatments. One of the latter [9] is termed "protein-coated ODS", in which the outer surface of the pores is coated with denatured plasma proteins to ODS and the internal surface of the pores remains intact ODS. The other [10] is termed an "ISRP" column, in which the outer surface of the pores is hydrophilic glycerylpropyl-bonded groups and the functional group of the internal surface of the pores is a hydrophobic polypeptide (glycine-L-phenylalanine-L-phenylalanine). These columns are to be designed to have characteristics of reversed-phase columns for small molecules with a non-adsorptive outer surface to proteins. In previous papers, we have described an HPLC system with the column-switching technique for the determination of Trp metabolites which allows the direct injection of biological samples such as plasma or serum [7,11].

The aim of this study was the development of a method for the simultaneous determination of Trp and its metabolites for routine use in clinical chemistry laboratories. In this paper, some fundamental factors such as the variation of their levels in individuals was studied using a fully automated HPLC system assembled with an autosampler and data processors. In addition, some clinical plasma samples were also analysed in a preliminary study to evaluate the roles of Trp metabolites in human plasma and serum.

EXPERIMENTAL

Chemicals and reagents[a]

Trp metabolites were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals, including components of elution solvents, were obtained from Yoneyama Pharmaceutical Industry (Osaka, Japan). All reagents were of guaranteed grade and used as received.

HPLC system

Trp metabolites were analysed by direct-injection HPLC with the column-switching technique using two columns. The first was a short precolumn of protein-coated ODS [12] for deproteinization and also for trapping Trp metabolites. The second was an analytical ODS column. A 100-μl volume of plasma or serum sample was injected onto the precolumn, which was equilibrated with the purge solvent. After washing for 6 min with the purge solvent, the precolumn was connected with an analytical ODS column in the flow-through mode. The metabolites were separated on the analytical column by stepwise elution with 0.1 *M* phosphate solution with increasing acetonitrile content. The HPLC system was analogous to that in our previous report [11], but an autosampler (Model AS-48; Tosoh, Tokyo, Japan) and data processors (Chromatopac C-R3A; Shimadzu, Kyoto, Japan) were incorporated to allow fully automated analysis. The total system was controlled by a sequence programmer (Model SCY-PO; Omron, Tokyo, Japan).

Variation of Trp metabolite level in the same individuals

Heparinized plasma samples were used to study the variation in the concentration of Trp metabolites in plasma samples from fasting individuals over four weeks in the same three individuals.

Analysis of clinical samples

Heparinized plasma samples from four groups of fasting individuals were analysed by the proposed HPLC method. The groups were normal controls, diabetics (type II and type III), and gravida and their foetuses at delivery. The controls ($n=31$) were healthy volunteers. Diabetic patients were type II ($n=51$) and type III ($n=89$), typed according to the WHO criterion, by the glucose tolerance test (75 mg). Maternal blood ($n=20$) and umbilical venous blood ($n=17$) were obtained at delivery.

[a]Abbreviations used: Trp, tryptophan, 5-OH-Trp, 5-hydroxytryptophan; 5-HT, serotonin (5-hydroxytryptamine); 5-OH-TrpOL, 5-hydroxytryptophol; 5-HIAA, 5-hydroxyindole-3-acetic acid; ILA, indole-3-lactic acid; IAA, indole-3-acetic acid; TrpOL, tryptophol, IPA, indole-3-propionic acid; IBA, indole-3-butyric acid; 3-OH-Kyn, 3-hydroxykynurenine; Kyn, kynurenine; 3-OH-AA, 3-hydroxyanthranilic acid; XA, xanthurenic acid; KA, kynurenic acid; QA, quinaldic acid, AA, anthranilic acid.

RESULTS AND DISCUSSION

Chromatography of Trp metabolites

Typical chromatograms of Trp metabolites in human plasma and serum are shown in Fig. 1. The eluent from the analytical ODS column was monitored with three detectors (fluorescence, spectrophotometric and electrochemical). Metabolites in a series of indoles were determined by native fluorescence (excitation at 287 nm, emission at 340 nm) and metabolites through kynurenine were determined by UV spectrophotometry at 350 nm. As the level of 5-HIAA in human plasma or serum was low, it was mainly determined by electrochemical detection on glassy carbon at an applied voltage of 0.6 V. Assignment of each chromatographic peak was checked with the relative peak-height ratio by the three detectors as discussed previously [11].

When a 100 μl of human plasma or serum were analysed, the detected metabolites were kynurenine, indolelactic acid, indoleacetic acid, indolepropionic acid, 5-hydroxyindoleacetic acid and serotonin in addition to tryptophan.

Reproducibility

The precision of the method using 100 μl of human control plasma (mixed plasma from twenty volunteers) was excellent, with a coefficient of variation (C.V.) ranging from 0.9 to 3.3%, being best for Trp (0.9% at x=69.6 nmol/ml) and the poorest for 5-HIAA (3.3% at x=27 pmol/ml) (Table I).

In view of its simplicity and accuracy, the method could be useful for the routine determination of total (free+bound to plasma proteins) Trp metabolites in plasma and serum samples. Over 100 samples could be analysed without deterioration of the efficiency of the protein-coated ODS precolumn.

Variation of Trp metabolite level in the same individuals

Table II shows the variation of the level in heparinized plasma from fasting subjects over four weeks from the same three individuals (A, B and C). The level of Trp and Kyn in the same individuals was relatively constant with a C.V. of less than 10% of the mean values. The variation of IAA or IPA was larger than that of Trp or Kyn. However, there is a possibility that intestinal bacteria might affect the IPA level [13].

Analysis of clinical samples

The method was applied to the determination of Trp metabolites in clinical samples, and the preliminary results are summarized in Table III. Among the Trp metabolites found, the level of 5-HT was easily affected by the anticoagulant used in the sample preparation and the deviation of its levels was larger than that of other metabolites (data not shown). The values given in Tables I and II for 5-HT in human plasma are relatively high compared with the lowest previous reports [14,15], probably owing to platelet contamination of the

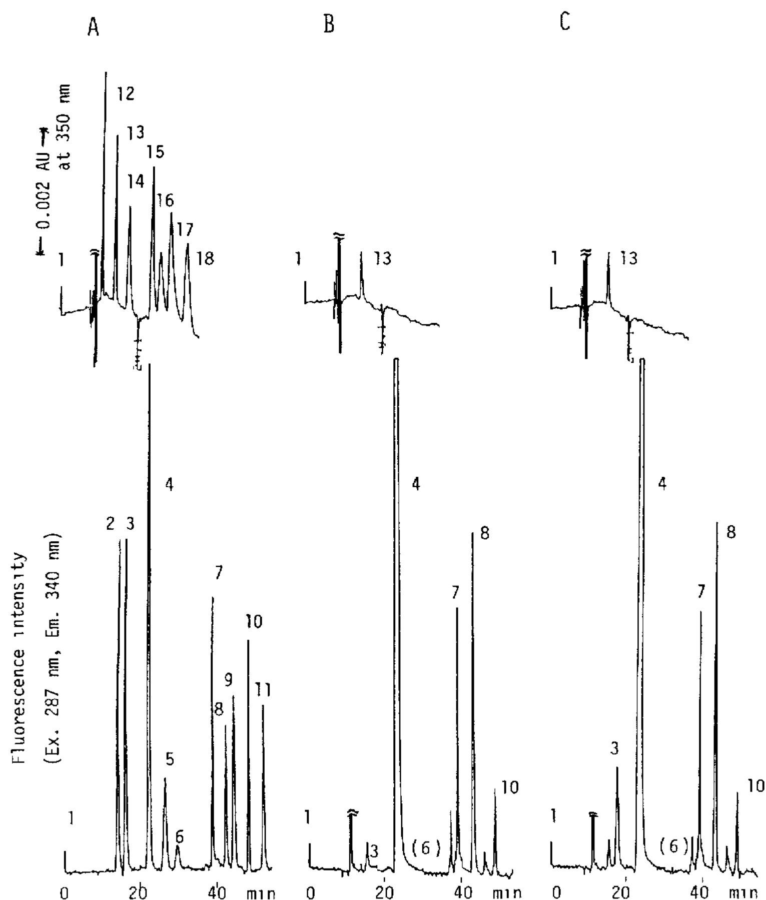

Fig. 1. Typical chromatograms of Trp metabolites in human plasma and serum. Samples: (A) standard Trp metabolites (Trp, 1 nmol; other indole metabolites, 100 pmol; 3-OH-Kyn, Kyn, XA and KA, 500 pmol; 3-OH-AA, QA and AA, 2 nmol); (B) human plasma (100 μl); (C) human serum (100 μl). Peaks: 1 = injection marker; 2 = 5-OH-Trp; 3 = 5-HT; 4 = Trp; 5 = 5-OH-TrpOL; 6 = 5-HIAA; 7 = ILA; 8 = IAA; 9 = TrpOL; 10 = IPA; 11 = IBA; 12 = 3-OH-Kyn; 13 = Kyn; 14 = 3-OH-AA; 15 = XA; 16 = KA; 17 = QA; 18 = AA. Columns: protein-coated ODS (40 mm × 4 mm I.D.) precolumn; analytical ODS (Chemcosorb ODS-H, 7 μm) column (200 mm × 4 mm I.D.). Eluents: (1) 0.1 *M* phosphate buffer (pH 2.0) containing 1.0% TCA, 6 min, 0 9 ml/min (for purge solvent); (2) 0.1 *M* phosphate solution (pH 3.3) containing 1.5% acetonitrile, 10 min, 1.3 ml/min; (3) 0.1 *M* phosphate solution (pH 3.3) containing 4% acetonitrile, 17 min, 1.3 ml/min; (4) 0.1 *M* phosphate solution (pH 4.5) containing 20% acetonitrile, 10 min, 1.3 ml/min; (5) 0.1 *M* phosphate solution (pH 4.5) containing 30% acetonitrile, 10 min, 1.3 ml/min. Analytical column temperature, 35 °C.

plasma samples or release of 5-HT from platelets. Therefore, the levels of 5-HT in clinical samples are not shown. Maternal plasma showed a trend of low Trp metabolite levels among the clinical samples analysed. The maternal

TABLE I

REPRODUCIBILITY OF THE PRESENT HPLC METHOD ($n=13$, WITHIN-ASSAY)

Compound	Concentration (mean ± S.D.) (pmol/ml)	Coefficient of variation (%)
Trp	69600 ± 630	0.9
Kyn	1260 ± 30	2.4
5-HT	23 ± 0.80	3.5
ILA	710 ± 15.4	2.2
IAA	1370 ± 37.0	2.7
IPA	786 ± 18.8	2.4
5-HIAA	27 ± 0.86	3.2

TABLE II

VARIATION OF PLASMA Trp METABOLITE LEVELS IN THE SAME INDIVIDUALS OVER FOUR WEEKS

Trp metabolites in plasma from three fasting volunteers (A, B and C) were analysed.

Compound	A ($n=12$)		B ($n=11$)		C ($n=11$)	
	Mean ± S.D. (pmol/ml)	C.V. (%)	Mean ± S.D. (pmol/ml)	C.V. (%)	Mean ± S.D. (pmol/ml)	C.V. (%)
Trp	61900 ± 4600	7	67500 ± 4900	7	87300 ± 8500	10
Kyn	1140 ± 110	10	1470 ± 140	10	1850 ± 160	9
5-HT	23 ± 11	48	20 ± 10	50	13 ± 8	62
ILA	520 ± 59	11	605 ± 65	11	720 ± 180	25
IAA	2800 ± 610	22	2340 ± 712	30	2360 ± 300	13
IPA	750 ± 270	36	2530 ± 690	27	710 ± 220	31
5-HIAA	35 ± 8	23	40 ± 9	23	23 ± 5	22

plasma level of each Trp metabolite except IPA was always significantly lower than those in the foetal plasma. In the analysis of foetal plasma, a peak with the same retention time as standard 3-hydroxyanthranilic acid was observed in the chromatograms with electrochemical detection, although identification of the peak was not made at this stage. These findings might be interesting in view of correlations with the development of the foetus, and further studies are in progress.

In conclusion, the automated HPLC method using a column-switching technique was reproducible, and plasma Trp metabolites except for 5-HT were stable enough to be determined by the method. The method could be useful for the study of possible roles of Trp metabolites in biological fluids in both physiological and pathological states.

TABLE III

TRYPTOPHAN METABOLITE LEVELS IN HUMAN PLASMA

Group	Number of subjects	Trp		Kyn		ILA		IAA		IPA		5-HIAA	
		Mean ± S.D. (nmol/ml)	C.V (%)	Mean ± S.D. (pmol/ml)	C.V. (%)	Mean ± S.D. (pmol/ml)	C.V. (%)	Mean ± S.D. (pmol/ml)	C.V. (%)	Mean ± S.D. (pmol/ml)	C.V. (%)	Mean ± S.D. (pmol/ml)	C.V. (%)
Control	31	62.5 ± 13.5	22	1520 ± 350	23	717 ± 207	29	1980 ± 855	43	972 ± 765	79	30 ± 10	33
Diabetic (type II)	51	64.9 ± 11.5	18	1550 ± 350	23	749 ± 285	38	2050 ± 1230	60	790 ± 700	89	33 ± 11	33
Diabetic (type III)	89	68.5 ± 15.0	22	1500 ± 400	27	948 ± 590	62	1850 ± 960	52	953 ± 931	97	31 ± 11	35
Gravida	20	36.3 ± 10.9	30	950 ± 270	28	370 ± 170	46	620 ± 320	52	740 ± 600	81	42 ± 20	48
Foetus	17	74.5 ± 17.6	24	5130 ± 1500	29	1450 ± 450	31	2170 ± 730	34	710 ± 620	87	110 ± 40	36

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