Communications to the Editor

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DIRECT ENRICHMENT OF TRYPTOPHAN AND ITS METABOLITES IN PLASMA ONTO A PRE-COLUMN FOLLOWED BY REVERSE PHASE HPLC ANALYSIS

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A reverse phase method combined with direct pre-column enrichment and HPLC is proposed for the analysis of tryptophan (Trp) and its metabolites in plasma. A pre-column of protein-coated ODS did not adsorb plasma proteins, but quantitatively adsorbed Trp and its metabolites with an indole ring from acidified plasma in the presence of trichloroacetate anion. After elution of plasma proteins with phosphate buffer, the enriched metabolites were separated by an ODS analytical column by stepwise elution method. The HPLC analysis with native fluorescent detection showed several peaks corresponding to Trp, 5-hydroxy tryptophol, serotonin (5-HT), 5-hydroxy indole-3-acetic acid (5-HIAA), indole-3-acetic acid (IAA) and indole-3-propionic acid (IPA). The peaks were identified by fluorescent and electrochemical characterization. The peak heights were proportional to the injected volume of plasma samples (50-500 µl), and the recovery of the spiked metabolites (2 nmol each/ml plasma) was almost quantitative (99-102%) with good reproducibility (within-run c.v., less than 3%). The present method is simple and reproducible enough for routine analysis of total Trp and its metabolites in plasma.

KEYWORDS —— protein-coated reverse phase column; tryptophan metabolites; direct injection of plasma sample; pre-column enrichment in HPLC; 5-hydroxy tryptophol; serotonin; 5-hydroxy indole-3-acetic acid; indole-3-acetic acid; indole-3-propionic acid

The importance of Trp and its metabolites in both normal and pathological states has been reviewed.[1-3] Trp is metabolized by two major pathways, either through kynurenine or through a series of indoles. A simple enrichment method of Trp-metabolites in biological samples was not available until our recent report[4] on the method for enriching metabolites with an indole ring in deproteinized plasma. Recently, we found that a protein-coated ODS column[5] could adsorb Trp-metabolites directly from acidified plasma in the presence of trichloroacetate anion. In this communication we present an advanced method for the direct enrichment and analysis of Trp and its metabolites with an indole ring in plasma by the use of pre-column enrichment followed by reverse phase HPLC analysis.

An HPLC apparatus with a stepwise elution system was assembled in our laboratory. Two columns were used; one was a small pre-column (4 x 40 mm) of protein-coated ODS[5] for the enrichment and deproteinizing. The other column was an analytical column

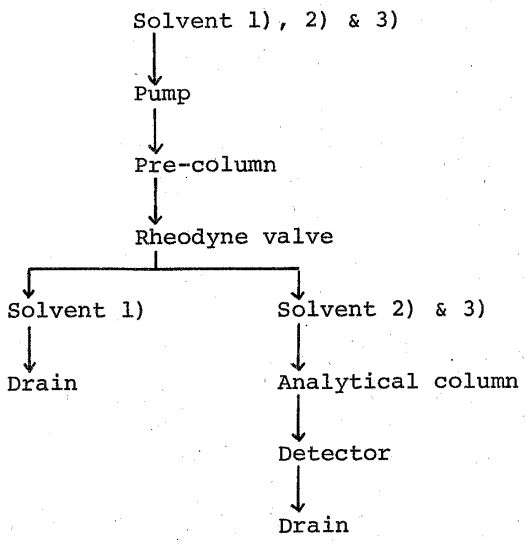

Fig. 1. Illustrated Flow Diagram
solvent 1), 2) & 3); see Fig. 2.

of ODS (TSK LS-410, 5 μm, Toyo Soda, Tokyo, Japan). A flow direction switch valve (model 7010, Rheodyne, C.A., U.S.A.) was placed between the pre-column and the analytical column as shown in Fig. 1. A fluorophotometer, RF-500 LC (Shimadzu, Kyoto, Japan), was usually used as the detector, and an electrochemical detector, EC-8 (Toyo Soda), was also used to identify the peaks.

Heparinized plasma was mixed with 1/30 volume of 20% hydrogen chloride to adjust the pH to about 3 and was injected onto the pre-column, which was equilibrated with 0.1 M phosphate buffer (pH 3.0) containing 0.5% TCA (trichloroacetic acid). Then, the pre-column was washed with the same buffer. The phosphate buffer and plasma proteins were drained and then the flow direction valve was switched to allow the metabolites to be eluted through the analytical column with stepwise elution by 0.1 M phosphate buffer (pH 3.0) containing 10% and 30% acetonitrile. The TCA anion was important for the enrichment of 5-HT, which forms a lipophilic ion-pair in solution. However, the TCA anion in the pre-column was sufficient for the analysis of at least up to 500 μl of plasma, so the TCA anion was not added to the plasma samples in this experiment. The acidic condition was important for the enrichment of the acidic metabolites such as 5-HIAA or IPA to prevent their dissociation or reduce their adsorption to albumin in the plasma by the conformational change of albumin (N-F transition).[6] The injection of more than 200 μl of neutral plasma samples showed the trends of peak broadening for acidic metabolites.

Typical chromatograms of rabbit and human plasma are shown in Fig. 2. Plasma or the plasma spiked with metabolites showed the peaks of the same retention time and of the same symmetry as the standard metabolites. By the multi-parallel detection method,[7] the peaks showed similar fluorescent (excitation and emission spectrum) and electrochemical (applied voltage versus peak height) characteristics as the standard metabolites. These results indicate that the peaks in Fig. 2 are obviously Trp-metabolites. The identification of 5-OH Trp in plasma was not reliable by the present method.[8]

As shown in Fig. 3, the height of each peak was proportional to the injected volume of plasma samples (50-500 μl), and this result indicates that the enrichment by the present method is reliable.

As shown in Table I, the recovery of the spiked metabolites (2 nmol each/ml plasma) was almost quantitative (99-102%) with good reproducibility (within-run c.v., less than 3%, n=5), and the present method is superior to the conventional method including the mannual deproteinizing step such as with TCA.

Trp and its metabolites have rather high affinity for plasma proteins, but

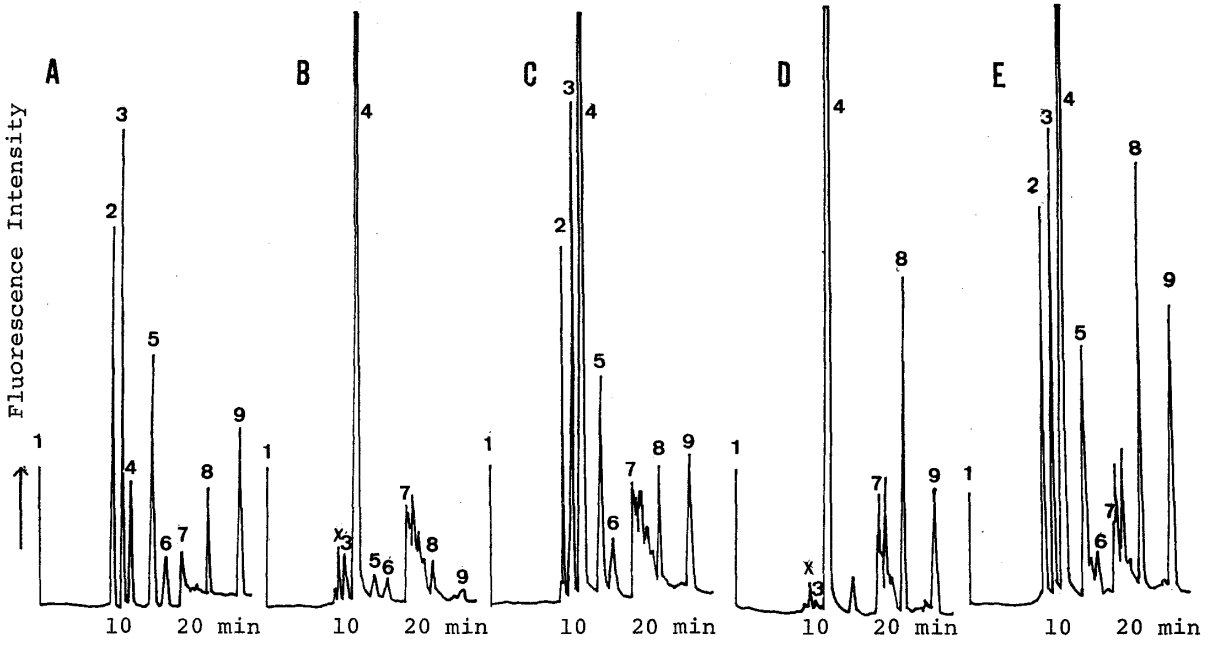

Fig. 2. Typical Chromatograms of Rabbit and Human Plasma

column: pre-column, 4 x 40 mm of protein-coated ODS (20-32 μm).
analytical column, 4 x 150 mm of native ODS (5 μm).
elution : 0.9 ml/min, 25°C.
1) 0.1 M phosphate buffer (pH 3.0) containing 0.5% TCA, 6 min.
2) 0.1 M phosphate buffer (pH 3.0) containing 10% CH_3CN, 10 min.
3) 0.1 M phosphate buffer (pH 3.0) containing 30% CH_3CN, 12 min.
samples : A) standard metabolites (200 pmol each). B) rabbit plasma (200 μl).
C) rabbit plasma spiked with metabolites (ca. 1 nmol each/ml plasma, 200 μl).
D) human plasma (200 μl).
E) human plasma spiked with metabolites (1 nmol each/ml plasma, 200 μl).
detection : native fluorescence (Ex. 287 nm, Em. 340 nm).
signals : 1) injection marker. 2) 5-OH Trp. 3) 5-HT. 4) Trp. 5) 5-OH tryptophol.
6) 5-HIAA. 7) buffer change artifact. 8) IAA. 9) IPA. X) see note 8).

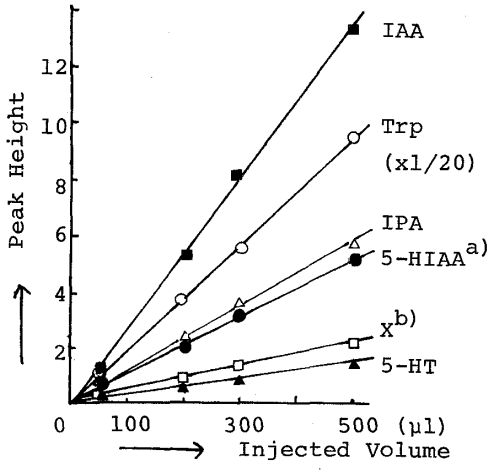

Fig. 3. Peak Heights of Trp-Metabolites versus Injected Volume of Human Plasma

a) 5-HIAA was spiked to human plasma (2 nmol/ml plasma).
b) Peak indicated by X in Fig. 2 (see note 8).

their recovery was almost quantitative. Thus it is noteworthy that the total amounts of the metabolites in the plasma were recovered, whether they were free or bound to plasma proteins.

In conclusion, the protein-coated ODS column is useful for the direct enrichment of Trp-metabolites with an indole ring in plasma when coupled with reverse phase HPLC analysis, in terms of accuracy, reproducibility and simplicity. Determination of Trp-metabolites in normal biological fluids is in progress in our laboratory.

Table I. Recovery of Spiked Trp-Metabolites

	Recovery, %[a] (c.v. %)	
	Present Method n=5	5% TCA[b] n=5
5-OH Trp	100.5 (2.7)	73.0 (6.7)
5-HT	102.0 (2.2)	66.0 (7.2)
Trp	100.0 (1.6)	72.0 (5.1)
5-HIAA	99.0 (2.2)	58.0 (4.5)
IAA	101.0 (2.1)	49.0 (3.5)
IPA	99.6 (2.7)	35.0 (3.8)

a) Human plasma was spiked with metabolites (2 nmol each/ml plasma) and 100 µl samples were analyzed to calculate the recovery.

b) The spiked plasma was mixed with an equal volume of 10% TCA, and the neutralized supernatant (pH about 3) was analyzed.

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8) The peak, indicated by X in Fig. 2., had the same retention time as 5-OH Trp. However, when the elution solvent was 0.1 M phosphate buffer (pH 3.0) containing 3% acetonitrile, the peak X was resolved into 2 or 3 peaks by electrochemical detection, and by this elution system the peak corresponding to 5-OH Trp was not always present. More detailed study is needed to determine whether some plasma contains 5-OH Trp or not.

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