**STUDIES ON VARIATION OF TRYPTOPHAN METABOLITES LEVEL IN RABBIT PLASMA
WITH PREPARATION PROCEDURES BY HIGH PERFORMANCE LIQUID
CHROMATOGRAPHY**

Ikue MORITA, Tsutomu MASUJIMA, Hisanobu YOSHIDA[®], and Hideo IMAI

Institute of Pharmaceutical Sciences, Hiroshima University School of
Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima-shi, Hiroshima 734,
Japan

Reversed phase high performance liquid chromatography with direct injection of plasma samples was applied to evaluate the variation of plasma tryptophan (Trp) metabolites level with sample preparation procedures. Fresh heparinized rabbit plasma showed the peaks corresponding to serotonin (5-HT), 5-hydroxy indole-3-acetic acid (5-HIAA), indole-3-acetic acid (IAA), indole-3-lactic acid (ILA), indole-3-propionic acid (IPA), kynurenine (Kyn), kynurenic acid (KA) in addition to tryptophan (Trp). By standing plasma at 37°C, 5-HT trended to decrease about 5%/h, but other metabolites levels were constant for at least 24 h. By standing platelet rich plasma (PRP) for 6 h, 5-HT and 5-HIAA increased. In addition, 5-hydroxy tryptophol (5-OH TrpOL), which was not found as a common metabolite in fresh plasma, was detected. By standing whole blood for 6 h, almost all metabolites except IAA and IPA increased by several times. Serum levels of 5-HIAA, 5-OH TrpOL and especially 5-HT were higher than that of plasma. Consequently, the plasma samples should be prepared just after collecting blood for the analysis of Trp-metabolites level.

Tryptophan (Trp) is metabolized by two major pathways, either through kynurenine or through a series of indoles, as shown in Fig. 1. Many of these metabolites are biologically active, and the importance of Trp and its metabolites in both normal and pathological states has been reviewed¹⁾²⁾. However, there are two major difficulties for the analysis of minute Trp-metabolites in biological samples. First, there is no suitable method concurrently useful for both metabolites through kynurenine and a series of indoles pathways. Secondly, Trp-metabolites level might be varied during a sample preparation step; e.g., 5-HT could be released from platelets or enzymatically converted.

We have reported that high performance liquid chromatography (HPLC) with direct plasma injection has some advantages over a conventional HPLC³⁾⁴⁾. For example, increasing of sample volume and accurate total amount analysis (free + bound to plasma proteins) are possible.

Recently, we have modified our previous method⁴⁾ for the enrichment analysis of both metabolites through kynurenine and a series of indoles pathways in plasma⁵⁾. Using this method, we studied the variation of Trp-metabolites in rabbit heparinized plasma with sample preparation procedures.

MATERIALS AND METHOD

Chemicals

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

Sample preparation

Blood was drawn into a plastic syringe with or without heparin as an anti-coagulant. The blood without anti-coagulant was allowed to clot at room temperature for 20 min, then centrifuged at $1500 \times g$ for 10 min at room temperature. The supernatant was taken out and used as serum. Blood with heparin (0.1 mg/ml) was centrifuged at $150 \times g$ for 15 min at room temperature to sediment blood cells except platelets. The upper phase suspension was taken out and used as platelet rich plasma (PRP). Blood with heparin was centrifuged at $1500 \times g$ for 10 min at room temperature, and the supernatant was taken out and used as plasma.

Incubation

Plasma, PRP or whole blood was put into plastic tubes, and incubated (stood) for 2-24 h at 37°C . After the incubation, PRP and whole blood were centrifuged at $1500 \times g$ for 10 min at room temperature to obtain plasma. One hundred μl of plasma sample was directly injected to HPLC to analyze the Trp-metabolites level.

HPLC apparatus

Two columns were used; one was a 40 mm $\times$ 4 mm I.D. pre-column packed with protein-coated ODS³⁾⁶⁾ for enrichment of Trp-metabolites in plasma and also for deproteinization (plasma proteins were eluted out with initial aqueous solution). The other column was a 150 mm $\times$ 4.6 mm I.D. column packed with native ODS (Chemcosorb ODS-H, Chemco, Osaka, Japan) for the separation of Trp-metabolites⁴⁾.

An HPLC apparatus with stepwise elution was assembled in our laboratory. The apparatus, as shown in Fig. 2, consisted of two pumps (Pumps A and B). Flow direction was controlled by a model 7125 sample injection valve (Rheodyne, C.A.,

Fig. 1 Some tryptophan metabolites

U.S.A.), a model 7010 flow direction switching valve (Rheodyne), a model 8V solvent selector (Kyowa Seimitsu, Tokyo, Japan) and a sequence programmer SCY-PO (Omron, Tokyo, Japan).

Metabolites in indole pathways were detected by native fluorescence (Ex. 287 nm, Em. 340 nm) with a model RF-530 fluorometer (Shimadzu, Kyoto, Japan). The indole compounds were also detected by an electrochemical

detector, EC-8 (Toyo Soda, Tokyo, Japan) to identify the peaks (applied voltage was 0.6 V or 0.9 V). Metabolites in kynurenine pathways were detected at 350 nm with a model UV-8 spectrophotometer (Toyo Soda, Tokyo, Japan). Native fluorescence detection (Ex. 370nm, Em. 470nm or Ex. 330 nm, Em. 380 nm) was also used to identify Kyn or KA.

Fig. 2 Block diagram of HPLC system

Chromatographic condition

Plasma or serum sample was mixed with 1/30 volume of 20% hydrogen chloride to adjust the pH at around 2, and was injected onto the pre-column, which was equilibrated with 0.1 M phosphate buffer (pH 2.0) containing 1.0% trichloroacetic acid (TCA) by pump A. Then, the column was washed with the same buffer in order to elute out plasma proteins and other hydrophilic compounds. The Trp-metabolites were trapped on the top of the pre-column. Then, the pre-column was connected in a manner of flow through mode with the analytical column to allow the trapped Trp-metabolites to be eluted through the analytical column. The adsorbed Trp-metabolites were separated with stepwise elution of 0.1 M phosphate solution containing 1.5%, 4%, 20% and 30% acetonitrile, successively, at 35°C by pump B.

RESULTS AND DISCUSSION

Comparison of plasma with serum

Chromatograms of Trp-metabolites in rabbit plasma and serum are shown in Figs. 3-b and 3-c. Rabbit heparinized plasma sample showed the peaks corresponding to 5-HT, 5-HIAA, ILA, IAA, IPA, Kyn and KA in addition to Trp. The presence of the indole compounds were also identified by electrochemical detection (applied voltage 0.6 V or 0.9 V), and Kyn and KA by native fluorescence detection (Kyn, Ex. 370 nm, Em. 470 nm; KA, Ex. 330 nm, Em. 380 nm). These data were not shown. In general, serum levels of 5-HT, 5-HIAA and 5-OH TrpOL were higher than plasma levels. One of the reasons might be the release of 5-HT from platelets during serum preparation from whole blood. Accordingly, it seems to be reasonable that the serum level of 5-HT was higher than that of the plasma level, and that 5-HIAA and 5-OH TrpOL which are

Fig. 3 Typical chromatograms of Trp-metabolites in rabbit plasma and serum

Samples: (a) standard metabolites (Trp 1 nmol; other indole metabolites 100 pmol; metabolites in kynurenine pathways 200 pmol)

(b) rabbit plasma (100 μ l)

(c) rabbit serum (100 μ l)

Elution: 1) 0.1 M phosphate buffer (pH 2.0) containing 1.0% TCA, 6 min 0.9 ml/min

2) 0.1 M phosphate solution (pH 3.3) containing 1.5% CH_3CN , 1 min 1.3 ml/min

3) 0.1 M phosphate solution (pH 3.3) containing 4.0% CH_3CN , 23 min 1.3 ml/min

4) 0.1 M phosphate solution (pH 4.5) containing 20% CH_3CN , 10 min 1.3 ml/min

5) 0.1 M phosphate solution (pH 4.5) containing 30% CH_3CN , 10 min 1.3 ml/min

Detection: metabolites in indole pathways; native fluorescence (Ex 287 nm, Em 340nm)

metabolites in kynurenine pathways; UV spectrophotometry at 350 nm

Signals: 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 (tryptophol), 10; IPA, 11; IBA (indole-3-butylic acid;

internal standard), 12; 3-OH Kyn, 13; Kyn, 14; 3-OH AA, 15; XA, 16; KA, 17; AA

metabolites from 5-HT (see Fig. 1) were also higher.

Furthermore, it was difficult to obtain the same serum levels of 5-HT, 5-HIAA and 5-OH TrpOL from the same rabbit. The reason might be the different degree or quantity of platelets involved in serum preparation. Thus, it was concluded that serum samples were not suitable for the study of Trp-metabolites in biological processes.

Stability of whole blood

Whole heparinized rabbit blood was stood at 37°C, and an aliquot was periodically taken out, and after centrifugation at 1500 x g for 10 min at room temperature, the Trp-metabolites level in plasma was analyzed. As shown in Fig. 4-a, with increasing standing time as whole blood, the plasma levels of almost all of the Trp-metabolites, including metabolites in kynurenine pathways, were varied rapidly. The reason might be complicated, but at least the standing time as whole blood should be as short as possible.

Stability of PRP

As plasma contains a small number of platelets oftenly, the effect of platelets on Trp-metabolites level in plasma was studied by using platelet rich plasma (PRP) as the model sample. As shown in Fig. 4-b, with increasing standing time as PRP at 37°C, the plasma levels of 5-HIAA and 5-OH TrpOL increased proportionally. These phenomena might be the result of enzymatic conversion of 5-HT, which was released from platelets. In PRP, the 5-OH TrpOL level was higher than 5-HIAA in contrast to the case of whole blood, and a possible reason might be due to the difference of enzymatic activities involved. The reason for 5-HT level fluctuation (the similar phenomenon was also observed with whole blood) was not clear. At any rate, there was a trend that platelets released 5-HT, followed by conversion to 5-OH TrpOL and 5-HIAA. Accordingly, the plasma sample should contain as little platelets as possible, especially when the plasma sample has to be stored long time. The plasma containing platelets should not be stored in a freezer, because the platelets will be destroyed in a frozen state. The best way to obtain platelet poor plasma is to re-centrifuge the plasma sample mentioned in "method" section at the same condition.

Stability of plasma

In rabbit heparinized plasma sample, which was separated from blood cells just after blood collecting, the Trp-metabolites levels except 5-HT was constant for at least 24 h when it was stood at 37°C in contrast to PRP or whole blood. The 5-HT level showed the trend to decrease with time (ca. 5%/h) and after the disappearance of 5-HT, no significant 5-HIAA or 5-OH TrpOL peak was detected. In preliminary experiment, the addition of pargiline (monoamineoxidase inhibitor; 200 µg/ml plasma) or ascorbic acid (anti-oxidant; 30 µg/ml plasma) gave little or no effect. Rat heparinized plasma containing pargiline showed weak or no 5-HT degradative activity. The investigation of protection procedure for 5-HT in rabbit plasma is under progress in our laboratory.

Differences in animal source

In preliminary experiment, it was shown that, in contrast to rabbit blood, rat heparinized blood had no detectable activity of the formation of 5-HIAA or 5-OH TrpOL from 5-HT, and that rat heparinized plasma showed weak or no 5-HT degradative activity in the presence of pargiline. These data indicate that enzymatic activities should be carefully checked before the analysis of Trp-metabolites level in plasma in each species of animals.

Fig. 4 Effects of standing time of (a) whole blood, (b) PRP and (c) plasma on Trp-metabolites

All samples were stood at 37°C during this study.

Rabbit blood samples for (b) and (c) were the same origin, but for (a) was different.

Symbols; ● 5-HT, ○ 5-HIAA, ⊙ 5-OH TrpOL, ■ Trp, □ ILA, ▣ IAA, ▲ Kyn, △ KA

This research was supported in part by a Grant-in-Aid for Scientific Research from the Iatro Chemical Foundation.

REFERENCES

- 1) D. J. Boullin : "Serotonin in Mental Abnormalities", (1978), (Willey, New York).
- 2) G. Curzon, P. K. Bridges : J. Neurol. Neurosurg. Psychiatry., 33, 698 (1970).
- 3) H. Yoshida, I. Morita, T. Masujima, H. Imai : Chem. Pharm. Bull., 30, 2278 (1982).
- 4) H. Yoshida, I. Morita, T. Masujima, H. Imai : Chem. Pharm. Bull., 30, 3327 (1982).
- 5) I. Morita, T. Masujima, H. Yoshida, H. Imai : manuscript in preparation. (Abstract of 32nd Annual Meeting of Jpn. Soc. Anal. Chem., p. 468 (1983)).
- 6) H. Yoshida, K. Takano, I. Morita, T. Masujima, H. Imai : Jpn. J. Clin. Chem., 12, 312, (1983).

Keyword phases

direct plasma injection method for HPLC analysis; variation of tryptophan metabolites level with sample preparation procedures; tryptophan metabolites in kynurenine and indole pathways.

(Received February 20, 1984)