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Note

Direct injection of highly protein-bound compounds by column-switching high-performance liquid chromatography

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Determination of drugs or endogenous compounds by high-performance liquid chromatography (HPLC) in complex biological samples, such as blood plasma, usually involves sample pretreatment by for example solvent extraction or deproteinization. However, these conventional sample pretreatments are sometimes laborious and also the least accurate part of the analytical procedures. To avoid the disadvantages of sample pretreatment, HPLC methods incorporating on-line sample clean-up by solid-phase extraction using column-switching technique have been developed [1–6] and have allowed direct injection of plasma or serum samples. The method, which can easily be automated, has been widely acknowledged to improve both the accuracy and the simplicity of the analysis. There are occasional drawbacks, such as skewed peaks or incomplete recovery of some compounds [1,7].

Strategies for the precolumn [8] or using the ion-pair technique to increase the retention volume on the precolumn [9] have been described to prevent recovery problems caused by low adsorption of compounds on the precolumn, which can lead to breakthrough during the purge step. Strong binding of the compounds to different plasma proteins also causes problems, and attempts to overcome these have included decreasing the protein binding by dilution or by addition of a displacer [10,11].

This paper describes a simple technique for direct plasma injection to determine strongly protein-bound compounds in plasma by using HPLC with col-

umn switching. The influence on recovery of the ratio of free to total (free plus protein-bound) was examined using a series of indole ring compounds as models.

EXPERIMENTAL

Sample solution

We used some tryptophan (Trp) metabolites as model compounds, since they contain a series of (acidic, neutral and basic) indole ring structures. Also, some Trp metabolites are known to bind to plasma proteins to various extents: for example, the percentage of free Trp in human serum was reported to be ca. 20% [12] and that of free serotonin in human platelet-poor plasma was reported to be ca. 70% [13]. Trp metabolites were spiked at a concentration of $5 \cdot 10^{-7} M$ each (except for Trp, $5 \cdot 10^{-6} M$) in phosphate-buffered saline (PBS, pH 7.4), 6% bovine serum albumin (BSA) solution or human dialysed plasma. BSA (Fraction V powder from Sigma, St. Louis, MO, U.S.A.) was dissolved in 0.1 M phosphate solution (pH 7.4 or 3.0). Free Trp metabolite fractions from 6% BSA solution were obtained by ultrafiltration of 0.5 ml BSA solution at 1500 g for 20 min using Ultracent (Tosho, Tokyo, Japan).

Mixed human plasma was obtained from heparinized plasma of normal volunteers ($n=10$), and the free fraction was obtained by ultrafiltration using Ultracent as before.

Column-switching HPLC for direct plasma injection

Two columns were used, one a short precolumn (40 mm × 4 mm I.D.) packed with protein-coated ODS [14], and the other an analytical column (150 mm × 4.6 mm I.D.) packed with TSKgel ODS-80TM (5 μm, Tosho). The protein-coated ODS, in which the outer surface of the small pore (100 Å) packing was coated with denatured plasma proteins, has reversed-phase characteristics for small molecules but has no affinity for proteins. The preparation and characteristics of the protein-coated ODS are described in detail elsewhere [14]. The precolumn was used not only as guard column for the analytical column but also for the chromatographic clean-up of the samples (trapping the Trp metabolites on the precolumn and washing out proteins). The HPLC system was assembled in our laboratory.

To demonstrate the influence of protein (albumin) on recovery, 100 μl of sample solution were directly injected into the precolumn using one of two purge solvent systems: either a neutral purge solvent of 0.1 M phosphate buffer (pH 7.4) or an acidic purge solvent of 0.1 M phosphate solution (pH 3.0). After injection of 100 μl of sample solution, the precolumn was washed with the purge solvent for an additional 6 min at a flow-rate of 0.9 ml/min, to drain out proteins and hydrophilic compounds. Then the precolumn was connected in the 'flow-through mode' to the analytical column to allow the trapped com-

pounds to be separated on the analytical column at a flow-rate of 1.3 ml/min by stepwise elution of increasing acetonitrile content: 4% acetonitrile in 0.1 M phosphate solution (pH 3.3) for 32 min; 20% acetonitrile in 0.1 M phosphate solution (pH 4.5) for 12 min; 30% acetonitrile in 0.1 M phosphate solution (pH 4.5) for 10 min. The precolumn of protein-coated ODS remained effective for several months, when it could be regenerated simply. Between runs the precolumn was regenerated by elution with purge solvent. Fluorescence detection was carried out using a Model RF-530 fluorometer (Shimadzu, Kyoto, Japan), set at 287 nm (excitation) and 340 nm (emission).

RESULTS AND DISCUSSION

One of distinctive characteristics of plasma or serum is its high protein concentration (6–8% in plasma). In the HPLC analysis of plasma by direct injection using the column-switching technique, the purge solvent is generally free from organic solvent in order to prevent precipitation of plasma proteins, and an aqueous purge solvent such as water or buffer has been commonly used in order to trap compounds on the precolumn and also to wash out proteins. We therefore examined the influence of the pH of aqueous purge solvents on the recovery of Trp metabolites spiked in BSA solution or in human dialysed plasma (plasma protein fraction).
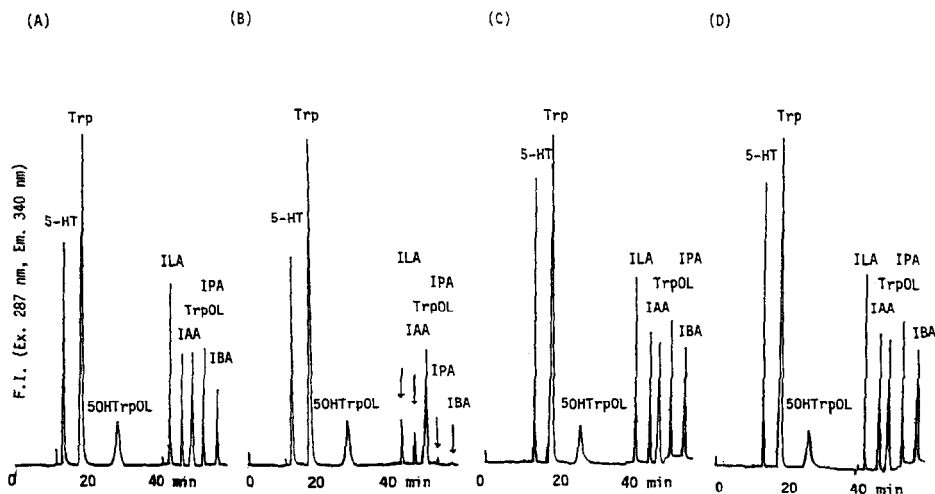

Fig. 1. Chromatograms of Trp metabolites in PBS (A and C) and in 6% BSA solution (B and D) by column-switching HPLC using either (A and B) neutral purge solvent (pH 7.4) or (C and D) acidic purge solvent (pH 3.0). For chromatographic conditions see Experimental. Peaks: 5-HT = serotonin; Trp = tryptophan; 5OHTrpOL = 5-hydroxytryptophol; ILA = indole-3-lactic acid; IAA = indole-3-acetic acid; TrpOL = tryptophol; IPA = indole-3-propionic acid; IBA = indole-3-butyric acid.

Fig. 1 shows chromatograms of Trp metabolites spiked in aqueous buffer (pH 7.4) and 6% BSA solution (pH 7.4), in which the samples were introduced onto the precolumn using either neutral (pH 7.4) or acidic (pH 3.0) purge solvent. Some Trp metabolites, such as serotonin (5-HT) or tryptophol (TrpOL), showed quantitative recoveries from 6% BSA solution. However, the relatively hydrophobic, acidic Trp metabolites, such as indole-3-lactic acid (ILA) or indole-3-propionic acid (IPA), showed poor recoveries when neutral purge solvent (pH 7.4) was used. Similar results were obtained with human dialysed plasma spiked with Trp metabolites.

In order to explain these phenomena, the percentage of free Trp metabolites in 6% BSA solution was determined by ultrafiltration, followed by HPLC. As shown in Table I, the hydrophobic, acidic Trp metabolites were strongly bound to BSA in neutral medium (pH 7.4) and showed low recoveries by direct plasma injection with the neutral purge solvent. But tryptophol, which is strongly bound, showed quantitative recovery with the neutral purge solvent, possibly owing to rapid dissociation between the bound and free forms. In acidic medium (pH 3.0), the percentage of free Trp metabolites increased, and the recoveries of all of Trp metabolites by direct plasma injection with the acidic purge solvent were quantitative. It appears that two factors, the dissociation constant and the rate of dissociation between free and protein-bound forms, may be important for the success of direct plasma injection with column-switching HPLC.

TABLE I

TRAPPING EFFICIENCY OF TRYPTOPHAN METABOLITES FROM NEUTRAL OR ACIDIC MEDIUM

Each Trp metabolite was spiked in 6% BSA solution; $n = 5$.

Compound	Symbol	Neutral medium (pH 7.4)		Acidic medium (pH 3.0)	
		Percentage free	Recovery[a] (%)	Percentage free	Recovery[b] (%)
Serotonin	5-HT	70	99	97	100
L-Tryptophan	Trp	71	98	91	101
5-Hydroxytryptophol	5-OHTrpOL	20	100	59	100
D,L-Indole-3-lactic acid	ILA	N.D.[c]	17	42	100
Indole-3-acetic acid	IAA	N.D.	15	37	99
Tryptophol	TrpOL	N.D.	100	50	100
Indole-3-propionic acid	IPA	N.D.	8	25	100
Indole-3-butyric acid	IBA	N.D.	4	19	100

[a]For purge solvent, 0.1 M phosphate buffer (pH 7.4) was employed.
[b]For purge solvent, 0.1 M phosphate solution (pH 3.0) was employed.
[c]N.D. = not determined.

TABLE II

TRYPTOPHAN METABOLITE LEVELS IN MIXED HUMAN PLASMA

Metabolite	Total[a]		Free[b]	
	Native plasma	Acidified plasma	Native plasma	Acidified plasma
Tryptophan (nmol/ml)	63.1	62.9	12.8	56.6
Indolelactic acid (pmol/ml)	530	530	N.D.[c]	250
Indoleacetic acid (pmol/ml)	1300	1320	100	720
Indolepropionic acid (pmol/ml)	1030	1020	N.D.	210

[a]Total level in 100 μl of human plasma was determined by the present HPLC method using acidic purge solvent.
[b]Free level in ultrafiltrated plasma was determined by the present HPLC method using acidic purge solvent.
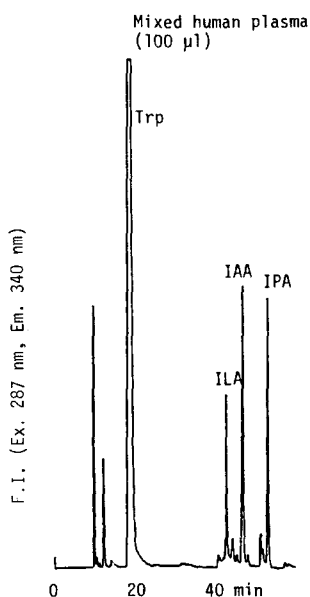[c]N.D. = not determined (< 10 pmol/ml).

Fig. 2. HPLC analysis of Trp metabolites in human plasma by the direct plasma injection using acidic purge solvent. Conditions as in Experimental, except for the addition of 0.5% trichloro-acetic acid to the acidic purge solvent (pH 3.0) as an ion-pair reagent, to ensure the retention of hydrophilic metabolites such as serotonin on the precolumn during the purge step [9]. Abbreviations as in Fig. 1.

HPLC with the acidic purge solvent was applied to measure Trp metabolites in human plasma. Peaks of Trp metabolites spiked in plasma showed the same peak sharpness with standard Trp metabolites, and recoveries calculated from the peak height of all Trp metabolites were close to 100% with good reproducibility, similar to the results for the acidic medium (Table I). So values obtained by the HPLC method using the acidic purge solvent could be estimated as total (free plus protein-bound) amounts. As shown in Table II, free and total levels of Trp metabolites in mixed human plasma were measured. The level of free Trp metabolites in acidified plasma was also increased.

Fig. 2 shows typical chromatograms of Trp metabolites in human plasma: the peak heights were proportional up to 200 μl of untreated (native) blood plasma injection (Fig. 3). This means that injected plasma samples were diluted with the acidic purge solvent to be acidified in the precolumn. To prevent the peak-splitting of acidic, hydrophobic compounds such as IPA over 200 μl of plasma injection, the pH of untreated blood plasma should be changed to acidic (ca. pH 3) by adding 1/30 volume of 20% hydrogen chloride prior to injection of plasma samples onto the precolumn.

This simple technique thus enables the injection of untreated non-diluted blood plasma on liquid chromatographic columns. Strongly protein-bound compounds in plasma can be determined by column-switching HPLC and by
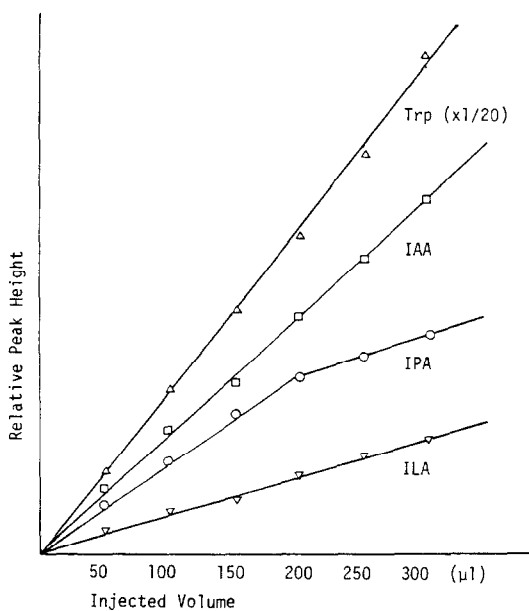

Fig. 3. Peak height of Trp metabolites versus injected volume of untreated human plasma. Chromatographic conditions and abbreviations as in Fig. 2.

using an acidic purge solvent, it is possible to recover all target compounds quantitatively and determine them as total (free plus protein-bound) amounts in plasma, regardless of the protein-binding ratio. Our results indicate that free and bound compounds behaved similarly during partitioning on the pre-column in the acidic medium because of rapid dissociation between the free and protein-bound forms. Thus acidification of the plasma sample effectively decreased the influence of albumin, which is a major protein in plasma, on the recovery by causing the albumin to undergo conformational change (N–F transition) [15].

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