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Plasma protein binding of frusemide in renal failure rabbits: investigation of endogenous protein binding inhibitors

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The reduction mechanism of frusemide-protein binding in the plasma of renal failure was investigated. The drug-albumin binding was inhibited by the low molecular-weight fraction obtained from acute renal failure rabbits, suggesting the presence of the inhibitors in the plasma. Further, this fraction was divided into six subfractions by Bio-Gel P-2. Fractions II and V_2 showed significant inhibition of the protein binding of frusemide. Among uraemic toxins, four indole derivatives markedly inhibited the protein binding. Analysis by hplc confirmed that the concentration of indican was markedly increased in acute renal failure rabbit plasma. It is suggested that this compound could be one of the major inducers of the protein binding defect.

Disease states often alter the binding of drugs. The decrease of drug-protein binding in renal failure has been widely reported (Grafnetterova et al 1976; Goto et al 1980; Yoshitomi et al 1983), while the mechanism remains unresolved. Hypoalbuminaemia is often caused by renal failure, but it cannot fully account for the decrease in the degree of protein binding. Two major hypotheses have been described to explain this. One is that there is structural change of the binding protein (i.e. albumin) (Shoeman & Azarnoff 1972; Boobis 1977; Erill et al 1980), the other is that certain endogenous inhibitors of drug binding are accumulated in the plasma. The latter hypothesis is supported by the fact that the plasma protein binding is recovered after the charcoal treatment at acidic pH (Craig et al 1976) or by extraction with hydrophobic resins (Depner & Gulyassy 1980), anion-exchange resins (Lichtenwalner et al 1982) or n-butyl chloride (Lichtenwalner et al 1981).

Previously Yoshitomi et al (1983) demonstrated the protein binding defect in rabbits with acute renal failure (ARF) and the existence of the protein binding inhibitors in their plasma was suggested. We have set out to clarify the presence of inhibitors in the low molecular-weight fraction from ARF rabbit plasma. Attempts at identifying the inhibitor were also made.

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MATERIALS AND METHODS

Chemicals

Frusemide was kindly supplied by Hoechst Japan Ltd, Tokyo. Crystalline bovine serum albumin (BSA), fraction V, was purchased from Wako Pure Chemical Industries Ltd, Osaka and was electrophoretically pure. Indole derivatives and other chemicals were of reagent grade and were used without further purification.

Acute renal failure induced by $HgCl_2$

Male albino rabbits (2·5–3·5 kg) were used. ARF was produced by an intramuscular injection of $HgCl_2$ (10 mg kg^{-1}) in the femoral region, as described by Yoshitomi et al (1983). Forty-eight hours after the HgCl administration, blood urea nitrogen (BUN) and plasma creatinine concentration, but not free fatty acid level, were markedly elevated. Whole blood was collected from the artery into heparinized tubes and centrifuged to obtain the plasma. ARF plasma (BUN, 80–150 mg dl^{-1}) was pooled and stored at −20 °C before use.

Fractionation of rabbit plasma by gel chromatography

(1) For separation of the low molecular-weight fraction (LMF) from normal or ARF plasma, a Sephadex G-50 (fine, Pharmacia) column (4·6 × 36 cm; flow rate, 18 ml h^{-1}) was used. Thirty-five ml of normal or ARF plasma was added to the column and eluted with purified water. The absorbance at 280 nm was monitored and 6 ml fractions were collected. All eluates were separated into three

fractions; globulin fraction (G), albumin fraction (A), and LMF. Each fraction was lyophilized. G and A were diluted with phosphate buffer (1/15 M, pH 7·4) to one tenth concentration of the original plasma. LMF was reconstituted to equal concentration with the original plasma for the binding experiments.

(2) Further fractionation of LMF was by use of a Bio-Gel P-2 (200–400 mesh, Bio-Rad Laboratories) column (1·5 × 75 cm; flow rate, 7·5 ml h^{-1}). Lyophilized LMF was reconstituted with 2 ml of water, applied to the column, and then eluted with purified water. Forty drop fractions were collected and the absorbance of each fraction was monitored at 280 nm. They were divided into six parts as shown in Fig. 3. Each of them was lyophilized.

Molecular weight was estimated from the elution volume of standard substances (cyanocobalamin and glutathione (both reduced and oxidized forms)).

Analysis of fraction V_2 by ion-paired reversed phase hplc

Fraction V_2 obtained by Bio-Gel P-2 from ARF plasma was analysed by hplc (Gasukuro Kogyo, Model 570B, Tokyo) in a reversed phase with a Unisil Q column (10 μm, 30·0 cm × 4·0 mm i.d., Gasukuro Kogyo). A detection system was a multichannel spectrophotometric detector (Shimadzu, Model SPD-M1A, Kyoto) and the mobile phase was 30% methanol in 0·02 M phosphate buffer (pH 7·5) containing 5 mM tetra-n-butyl ammonium bromide and the flow rate was 1 ml min^{-1}.

Drug-protein binding measurements

The equilibrium dialysis method described by Goto et al (1977) was used. A phosphate buffer (1/15 M, pH 7·4) was used for all binding experiments. The equilibrium was reached within 6 h at 30 °C. In all binding experiments, BSA concentration was 0·345%.

In any discussion of drug-protein binding, the primary binding site is usually of prime importance, so we analysed only frusemide binding to the primary site.

Analytical methods

Frusemide concentration was measured by the spectrofluorometric method of Yoshitomi et al (1983). Protein was assayed by the method of Lowry et al (1951), using BSA as a standard. BUN was determined by a diacetylmonoxime method (Ameno & Kameoka 1969).

RESULTS

Effect of LMF on protein binding of frusemide

ARF plasma was divided into three fractions; G, A and LMF by Sephadex G-50, where G and A mainly contain globulin and albumin, respectively. Fig. 1 shows the Sandberg–Rosenthal plot for frusemide binding to each fraction. The drug binds mainly to A and the binding to G is negligible. When LMF was added to A, the binding constant, K, decreased significantly as in the case of patients of renal failure. This suggests the presence of frusemide-protein binding inhibitors in the LMF from ARF plasma.

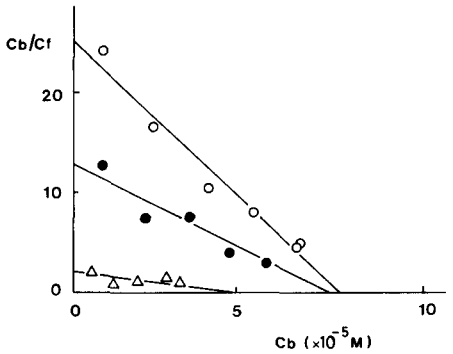

FIG. 1. Sandberg–Rosenthal plots for frusemide binding to gel-filtrated fractions of acute renal failure rabbit plasma. ○, albumin fraction; △, globulin fraction; ●, albumin fraction + low molecular-weight fraction.

When rabbit albumin fraction A was replaced by BSA, K was similarly decreased by the addition of LMF (Fig. 2). Thus the use of BSA is possible for the investigation of the binding inhibitors.

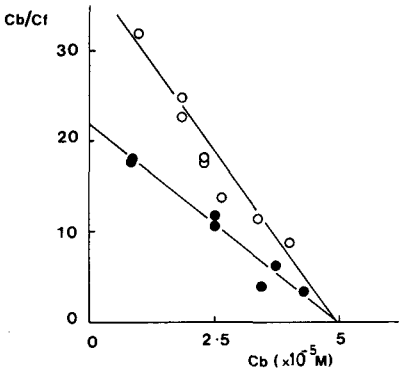

FIG. 2. Sandberg–Rosenthal plots for frusemide binding to bovine serum albumin with (●) or without (○) low molecular-weight fraction (LWF) from acute renal failure rabbit plasma.

Fractionation of LMF by Bio-Gel P-2 and the effects on frusemide-BSA binding

LMF obtained from normal or ARF rabbit plasma by Sephadex G-50 was further fractionated by Bio-Gel P-2. As shown in Fig. 3, each peak was significantly higher compared with that from LMF from normal rabbit plasma, indicating an increase of many low molecular-weight substances in the ARF plasma.

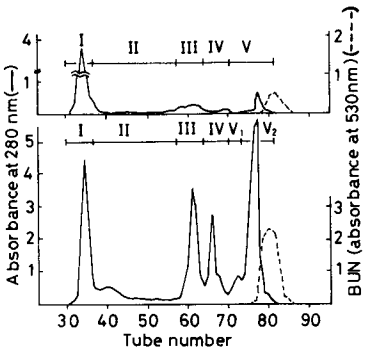

FIG. 3. Typical chromatograms for low molecular-weight fraction from normal (A) or acute renal failure (B) rabbit plasma obtained by Bio-Gel P-2.

After the division of LMF into six parts as shown in Fig. 3, each fraction was lyophilized and its effect on frusemide-BSA binding was investigated. Data were analysed by a double-reciprocal plot (Fig. 4, Table 1). Fraction I contains mainly albumin which was contaminated at the separation step by Sephadex G-50, so that the experiment was not performed. Straight lines for the data are consistent with the following well-known equation:

$$1/r = 1/(n \cdot K \cdot C_f) + 1/n$$

where r is the molar ratio of bound frusemide to BSA, n is the number of binding sites, K is the binding constant, and C_f is the unbound concentration of frusemide. In all cases, n was unchanged, but with fractions II and V_2, K was significantly decreased, suggesting the presence of competitive inhibitors in both fractions.

Table 1. Effect of Bio-Gel P-2 fractions on the binding parameters for frusemide-bovine serum albumin (BSA) binding.

Fraction	Mol. wt	Normal n	Normal K ($\times 10^5$ M^{-1})	Renal failure n	Renal failure K ($\times 10^5$ M^{-1})
II	3000–900	0·96	9·34	1·07	4·92
III	about 600	0·94	7·85	0·89	7·09
IV	about 440	0·82	8·16	0·87	6·24
V_1	about 290	—[a]		0·87	6·73
V_2	about 200	0·88	7·52	0·90	3·34

Each fraction obtained from 1 ml of plasma was added to 0·345% BSA.
[a] V_1 Fraction was negligible in normal rabbit plasma.

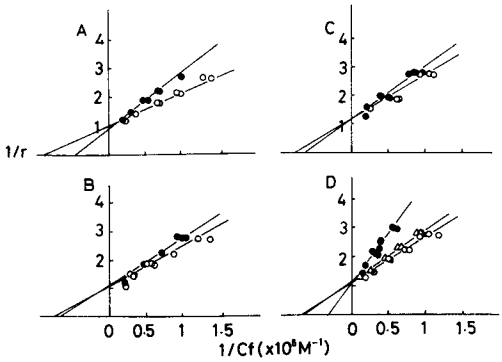

FIG. 4. Double reciprocal plots for frusemide binding to bovine serum albumin (BSA) containing each fraction obtained by Bio-Gel P-2 from normal (○) or acute renal failure (●) rabbit plasma. (A), Fraction II; (B), Fraction III; (C), Fraction IV; (D), Fraction V, where △ represents Fraction V_1 and ● represents Fraction V_2. Each fraction obtained from 1 ml of original plasma was added to 1 ml of BSA solution.

Effect of several uraemic toxins on frusemide binding to BSA

In renal failure, various endogenous metabolic products known as 'uraemic toxins' accumulate in the blood. In order to investigate the participation of these in the drug-protein binding defect, some were tested for their ability to inhibit frusemide-BSA binding and the results are summarized in Table 2. Urea, creatinine, guanidinoacetic acid and guanidinosuccinic acid did not affect frusemide-BSA binding, but four indole derivatives, indole acetic acid (IAA), indole butyric acid (IBA), indole lactic acid (ILA) and indican significantly inhibited the binding. These indole derivatives showed concentration-dependent inhibition (Fig. 5). Analysis of the inhibition by the double-reciprocal plot indicated that all

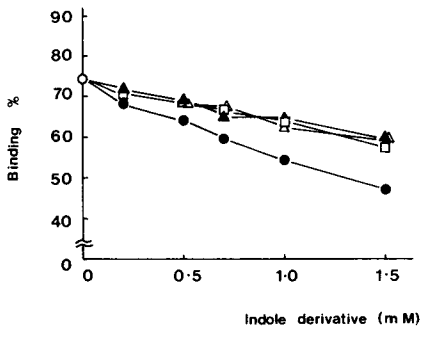

FIG. 5. Inhibition of the frusemide-bovine serum albumin (BSA) binding by indole derivatives as a function of their concentrations. BSA concentration was 0·345% and frusemide concentration was 1×10^{-4} M. ○, control; □, indole acetic acid; ●, indole butyric acid; △, indole lactic acid; ▲, indican.

Table 2. Effect of uraemic toxins on frusemide-bovine serum albumin (BSA) binding.

Uraemic toxin	% Bound
None	73·5
Urea	73·0
Creatinine	72·8
Guanidinoacetic acid	72·4
Guanidinosuccinic acid	73·4
Indole acetic acid	58·7
Indole butyric acid	53·9
Indole lactic acid	61·1
Indican	59·8

The concentrations of BSA, frusemide and uraemic toxins were 0·345%, 1×10^{-4} M and 1×10^{-3} M, respectively.

indole derivatives inhibit the binding competitively and the order of the inhibitory capacity was ILA < IAA < indican < IBA (Fig. 6). Thus indole derivatives may be candidates, which cause the decrease of frusemide-protein binding in ARF plasma.

Analysis of fraction V_2 by hplc

To analyse fraction V_2 from LMF, an ion-paired reversed-phase hplc system equipped with Shimadzu SPD-M1A detector was used. This detection system can monitor the ultra-violet to visible region, continuously. A chromatogram for the fraction from 200 to 340 nm is shown in Fig. 7. The peak detected at 17 min from the injection was identified as indican by retention time and the uv spectrum. Although indican cannot be detected in normal rabbit plasma or fraction V from normal rabbit plasma, the mean concentration in ARF plasma was 0·20 ± 0·03 mM (mean ± s.e., n = 4). None of the other indole derivatives, IAA, IBA and ILA were detected in normal or ARF plasma.

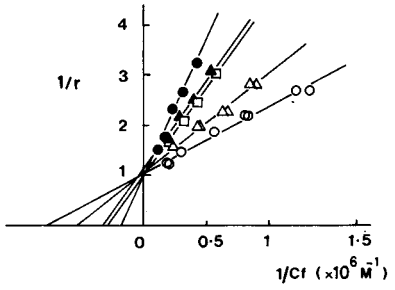

FIG. 6. Double reciprocal plots for frusemide-bovine serum albumin (BSA) binding in the presence of indole derivatives. The concentration of indole derivatives was 1×10^{-3} M and BSA concentration was 0·345%. Keys are the same as in Fig. 5.

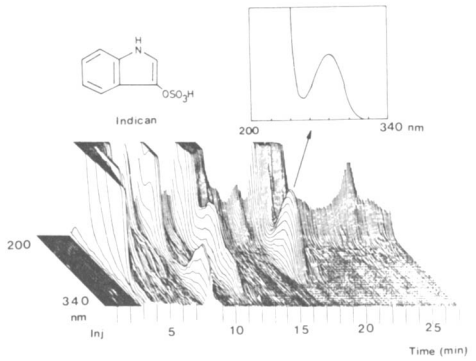

FIG. 7. High performance liquid chromatogram of Fraction V_2 by multi-channel spectrophotometric detector and uv spectrum of indican.

DISCUSSION

As an hypothesis to explain the decreased binding of acidic drugs in renal failure, it has been claimed that certain endogenous metabolic products accumulated in the plasma might affect the drug-protein binding. That the addition of LMF from ARF plasma caused the decrease of frusemide-BSA binding suggests the presence of the binding inhibitor in LMF. Furthermore, since the binding capacity of the albumin fraction from ARF rabbit plasma was normal, the structural change or modification of albumin such as carbamylation may not participate in the frusemide-protein binding defect.

Fractions II and V_2 obtained from ARF plasma by Bio-Gel P-2 inhibited frusemide-BSA binding significantly. Therefore, two or more inhibitors may exist in ARF plasma. One of these inhibitors, indican, was confirmed to be present in fraction V_2 from ARF plasma by hplc. Indican concentration in ARF plasma was 0·20 mM, which effectively inhibited frusemide-protein binding (Fig. 5). (In patients in renal failure, plasma indican concentration may be raised up to about 0·2 mM, Ludwig et al 1968.) Therefore indican plays an important role in the frusemide-protein binding defect in ARF plasma.

Fraction II also inhibited frusemide-BSA binding suggesting the presence of frusemide-protein binding inhibitors in this fraction, which are not identified yet. Fraction II contains many ninhydrin-positive substances on tlc and their speculated molecular weight are 900–3000. Thus there may be peptides consisted of several amino acids which act as the inhibitor.

Recently, Lichtenwalner et al (1981) reported that the drug-protein binding inhibitor is n-butyl chloride-extractable and its molecular-weight is

approximately 500 or less. But our result showed that the inhibitors, both in fractions II and V_2, are water-soluble compounds and the molecular-weight of each fraction is 900–3000 and about 200, respectively. The results indicate the possibility of the presence of several inhibitors with different physicochemical properties and their simultaneous inhibition of the drug-protein binding.

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