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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF AN ENDOGENOUS DIGOXIN-LIKE IMMUNOREACTIVE SUBSTANCE IN UREMIC SERUM

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SUMMARY

A reversed-phase high-performance liquid chromatographic method has been applied to the analysis of an endogenous digoxin-like immunoreactive substance present in uremic serum and in urine from healthy subjects An endogenous digoxin-like immunoreactive substance was eluted as a single peak and was considered to be a less polar substance The analysis of urine suggested that an endogenous digoxin-like immunoreactive substance present in uremic serum may be excreted by the kidneys and accumulated in uremia due to renal impairment

INTRODUCTION

An endogenous digoxin-like immunoreactive substance(s) (DLIS) has recently been documented in neonates, pregnant women and in patients with renal failure [1—5] The presence of DLIS in both normal human plasma and urine was also reported [6] Gruber et al [7] reported the existence of an endogenous digitalis-like substance in plasma of volume-expanded dogs They suggested that this substance may be the putative natriuretic hormone [8]

Soldin et al. [9] suggested that many steroids and lipids give false positive results for digoxin radioimmunoassay (RIA) It has also been suggested that bile salts may be examples of a class of endogenous compounds that non-specifically alter the reactivity of digoxin with its antibody in patients with combined hepatic and acute renal failure [10] Heazlewood et al [11], using a fluorescence polarization immunoassay, found no detectable DLIS in patients with renal failure They suggested that glycoproteins or glycosylated proteins,

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which are known to be elevated in uremia, may be related to the interference of digoxin RIA.

In this study we tried to separate DLIS present in serum of dialysed patients and in urine from healthy subjects by the method of high-performance liquid chromatography (HPLC)

EXPERIMENTAL

Sample and treatment

Serum samples were obtained from five dialysed patients not receiving digitalis preparations and three healthy subjects Apparent serum digoxin levels of patients were > 0 1 ng/ml In healthy subjects, levels were at or near zero. Serum was obtained by venipuncture and allowed to clot in a siliconized glass tube at room temperature and stored at −50°C until use Serum was deproteinized with an equal volume of 30% trifluoroacetic acid. After centrifugation, the supernatant was applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U S A) Urine was filtered through a 0 5-μm filter, after which the urine was applied to a Sep-Pak C_{18} cartridge The cartridge was washed with 10 ml of distilled water and eluted with 5 ml of 80% acetonitrile containing 0 1% heptafluorobutyric acid (HFBA) The eluate was lyophilized and subjected to HPLC analysis

Chromatography

A Shimadzu Model LC-3A HPLC system (Shimadzu, Kyoto, Japan) was used, which included a Model SIL-1A injector, a Model GRE-2B linear gradient former and a Model SPD-2A variable-wavelength UV detector equipped with an 8-μl flow cell Reversed-phase HPLC analysis was performed by the use of a LiChrosorb RP-18 column (5 μm, 25 × 0 46 cm I D , Merck, Darmstadt, F R.G.) The elution was done by a linear gradient of acetonitrile containing 0 1% HFBA at a flow-rate of 0 8 ml/min The column effluent was monitored by UV absorbance at 210 nm and collected in a fraction collector

Radioimmunoassay

Digoxin RIA was performed with the use of a kit purchased from Clinical Assays (Cambridge, MA, U S A) following manufacturer's instructions. The manufacturer's information suggested that the sensitivity of this kit is 0 09 ng/ml A previous report [2] had demonstrated that this digoxin RIA kit has a high degree of cross-reactivity to DLIS present in uremic serum

Each fraction from the HPLC column was lyophilized and resuspended in the zero digoxin standard serum

Statistics

The results were expressed as means ± the standard error of the mean (S.E M) or means of duplicates, and the probability was determined by using the Student *t*-test

RESULTS

The chromatograms of serum extracts and apparent digoxin levels of eluate

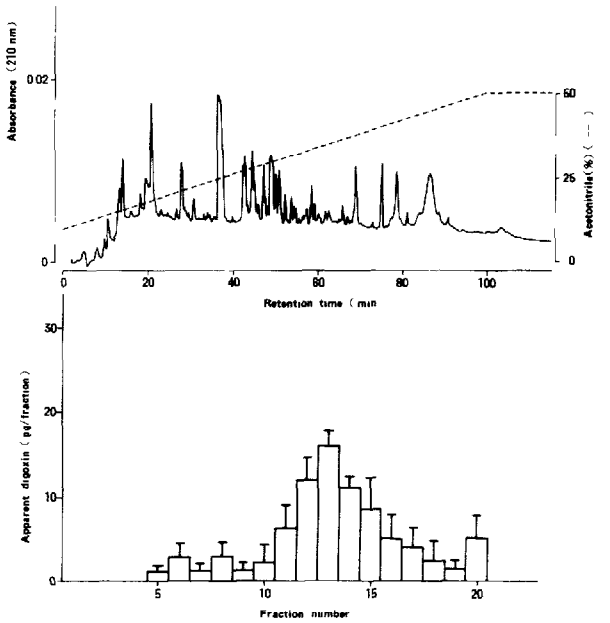

Fig 1 Typical chromatogram of serum extracts from healthy subjects (upper) and apparent digoxin levels of eluate fractions (lower) (n = 3) Eluate fractions were collected at 5-min intervals

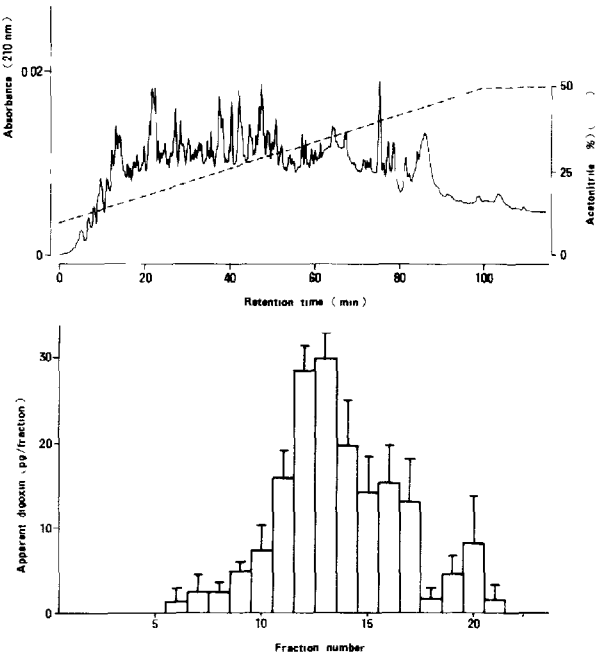

Fig 2 Typical chromatogram of serum extracts from uremic patients (upper) and apparent digoxin levels of eluate fractions (lower) (n = 5) Other conditions are the same as in Fig 1 Significance of differences from apparent digoxin levels of eluate fractions of healthy subjects (Fig 1, lower) (*) $P < 0.05$, (**) $P < 0.01$

fractions, representing an original serum volume of 10 ml, were shown in Figs. 1 and 2. Many fractions showed digoxin-like immunoreactivity in both uremic and normal serum samples However, apparent digoxin levels of fractions 12 and 13 of uremic serum samples were significantly higher than those of normal serum samples Then, pooled uremic serum (30 ml) was separated by the same conditions as in Figs. 1 and 2, and fractions 12 and 13 were combined, lyophilized and rechromatographed on a LiChrosorb RP-18 column. The results (shown in Fig 3) demonstrated that DLIS present in uremic serum is eluted as a single peak.

Quantitative estimation of the recovery of DLIS from the Sep-Pak C_{18} cartridge could not be performed, but preliminary experiments showed that the Sep-Pak cartridge is suitable for extraction and condensation of DLIS present in both serum and urine (data not shown)

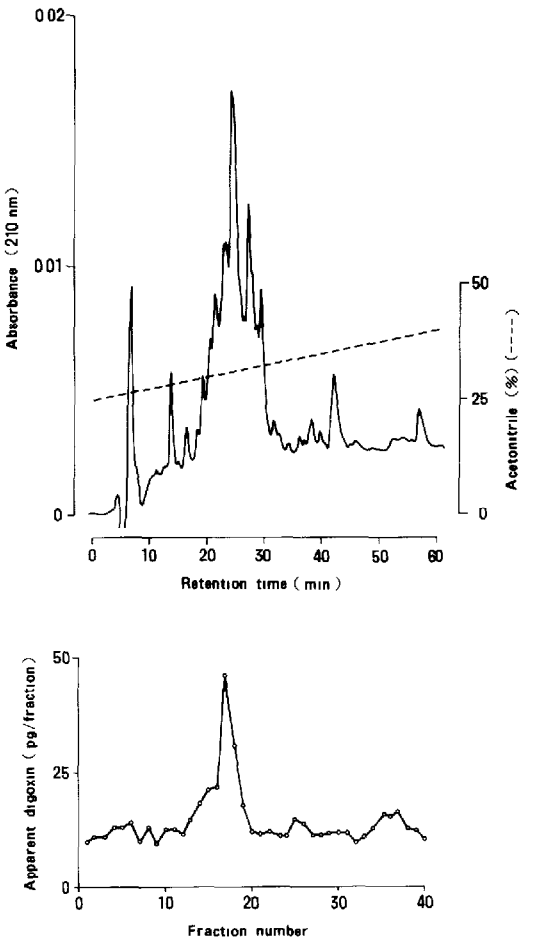

Fig 3 Typical chromatogram of fractions 12 and 13 from pooled uremic serum (upper) and apparent digoxin levels of eluate fractions (lower) Eluate fractions were collected at 1 5-min intervals Values of apparent digoxin were means of duplicates

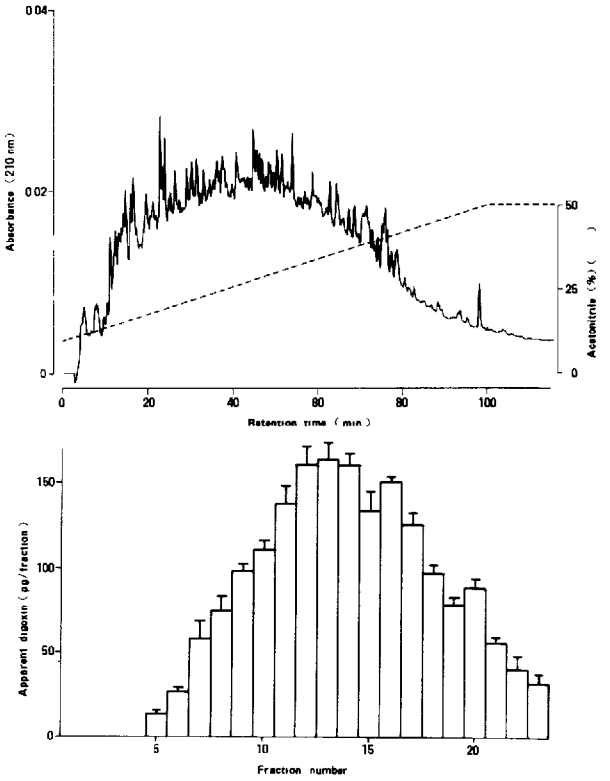

Fig 4 Typical chromatogram of urine samples (upper) and apparent digoxin levels of eluate fractions (lower) (n = 3) Other conditions are the same as in Fig 1

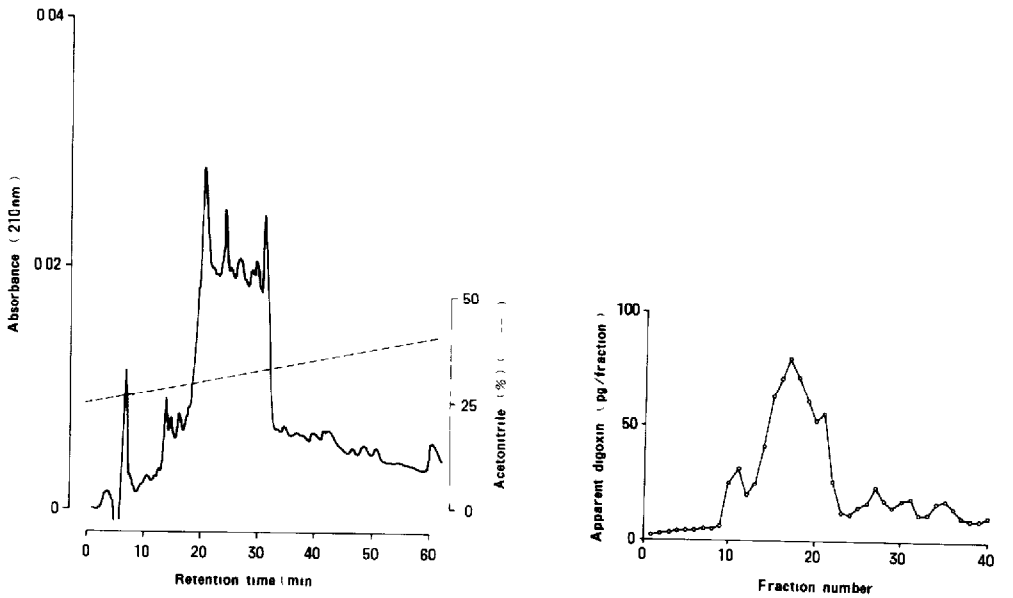

Fig 5 Typical chromatogram of fractions 12 and 13 from urine samples (left) and apparent digoxin levels of eluate fractions (right) Other conditions are the same as in Fig 3

Urine samples from healthy subjects, representing an original urine volume of 50 ml, were separated and rechromatographed by the same conditions as serum samples (Figs 4 and 5) The elution profile of DLIS present in urine was similar to that of DLIS present in uremic serum (Fig 5).

Glycochenodeoxycholic acid and glycocholic acid, which are major bile acids in serum, were chromatographed on a LiChrosorb RP-18 column The elution was performed by the same conditions as in Figs. 1 and 2 The retention times of glycochenodeoxycholic acid and glycocholic acid were 93 and 77 min, respectively.

DISCUSSION

In recent years, several attempts have been made to isolate endogenous Na^+,K^+-ATPase inhibitor(s), the putative natriuretic hormone [8], which has digitalis-like immunoreactivity [7, 12–14] Several authors have suggested that a digitalis-like factor(s) is related to sodium metabolism and hypertension in both humans and animals [15—18]

Whether or not the interference of digoxin RIA seen in uremic serum [2, 10] is related to an endogenous substance(s), we tried to separate DLIS present in uremic serum by HPLC techniques Our results suggested that DLIS found in uremic serum may be a single endogenous substance (Figs. 2 and 3) The results shown in Figs. 4 and 5 strongly suggested that DLIS present in uremic serum may be excreted by the kidneys and accumulated in uremia due to renal impairment [19] However, Graves et al. [2] reported that DLIS does not seem to accumulate with increased renal impairment, because many undialysed patients with severe uremia showed little or no apparent digoxin levels

The results by HPLC analysis indicated that DLIS present in uremic serum is a less polar substance, and HPLC behaviours are similar to those of the endogenous digitalis-like factor found in rat heart [20] or endogenous Na^+,K^+-ATPase inhibitors, designated as 2P and 2U, detected in hypertensive patients [21] However, HPLC behaviours of DLIS present in uremic serum differ from the endogenous ouabain-like substance separated by Shimori et al. [22] or the digitalis-like substance reported by Gruber et al [7] Recently, Crabos et al [21] have suggested the heterogeneity of Na^+,K^+-ATPase inhibitors and DLIS present in both plasma and urine

The chemical nature of DLIS present in uremic serum was not well characterized in this study, but a digitalis-like substance separated from volume-expanded dogs was thought to be a peptidic substance [7] Graves et al [2] suggested that DLIS present in uremic serum is a so-called uremic "middle molecule", as postulated in the middle molecule hypothesis [23]. The uremic middle molecule is thought to be peptidic substances [24] We have reported that medium-sized peptides are accumulated in uremia and several peptides are unique to uremia at a low picomole level [24] However, DLIS detected in serum of rats with cardiac overload is thought to be a steroid [25] In addition, the highest content of DLIS in adrenals is reported [17]

It was suggested that bile salts non-specifically alter the reactivity of digoxin with its antibody, perhaps by a detergent effect [10] No patients in this study showed hepatic dysfunction and the elution position of DLIS differs

from glycochenodeoxycholic acid and glycocholic acid, which are major bile acids in serum

The clinical significance of DLIS present in uremic serum was also not elucidated in this study, but DLIS present in serum of neonates was shown to inhibit Na^+,K^+-ATPase activity by measurement of ^{86}Rb uptake in erythrocytes [20] Gudson et al [4] suggested the possibility that DLIS may play a role in the pathophysiology of pre-eclampsia

Further study is needed, however, to establish the chemical nature and the clinical significance of DLIS accumulated in uremic serum, which is now in progress in our laboratory

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