

Measurement of endogenous Na^+, K^+ -ATPase inhibitors in human plasma and urine using high-performance liquid chromatography

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This study was undertaken to assess endogenous Na^+, K^+ -ATPase inhibitors in both plasma and urine in the same subjects. Samples were chromatographed on reverse-phase HPLC using an acetonitrile gradient and the eluent screened using Na^+, K^+ -ATPase inhibition and cross-reaction with anti-digoxin antibodies. The donors were divided into inhibiting and non-inhibiting subjects using a previously described method, plasma action on ouabain binding and on Na^+, K^+ -ATPase activity. Three Na^+, K^+ -ATPase inhibitors (1P, 2P and 3P) were detectable in plasma; the antibodies cross-reaction of the peaks 2P and 3P were larger than that of peak 1P. The peaks 2P and 3P were significantly higher in inhibiting subjects as compared to non-inhibiting subjects. The 24-h urine is resolved into two peaks inhibiting Na^+, K^+ -ATPase activity (1U and 2U). Peak 2U cross-reacted with anti-digoxin antibodies to a greater extent than peak 1U and is significantly larger in inhibiting subjects in terms of Na^+, K^+ -ATPase inhibition. These data support the heterogeneity of human Na^+, K^+ -ATPase inhibitor in both plasma and urine.

$(\text{Na}^+ + \text{K}^+)$ -ATPase	Endogenous $(\text{Na}^+ + \text{K}^+)$ -ATPase inhibitor	Plasma inhibitor	Urine inhibitor
Reverse-phase HPLC			

1. INTRODUCTION

An increased activity or concentration of an endogenous Na^+, K^+ -ATPase inhibitor or inhibitors has been associated with hypertension in humans [1–3] and animals [4]. While the existence of this endogenous factor(s) is generally accepted, the number and structure of the inhibitor(s) is yet to be determined. For example, two inhibitors were found in the plasma of salt-loaded dogs [5,6], 3 in the plasma of healthy humans [7], 4 in the plasma of patients with fulminant hepatic failure [8] and only 1 in the plasma of uremic patients [9]. In contrast, only a single active substance has been identified in the urine of salt-loaded humans [10–14] or salt-loaded dogs [15]. The confusion is compounded by the fact that plasma and urine samples of the same subjects have not been compared.

The analytical approaches to the separation

and determination of the Na^+, K^+ -ATPase inhibiting substances have also varied. The reported methods include chromatography on Sephadex G-25 [8,9,13], ion-exchange chromatography [7], and combinations of ion-exchange and reverse-phase chromatography [5,6,15]. The same method has not been applied to both plasma and urine.

We report the development of a reverse-phase high performance liquid chromatographic (HPLC) method coupled with biological tests for the isolation of endogenous Na^+, K^+ -ATPase inhibitors and the application of this method to plasma and urine samples from the same subjects. The study included subjects with inhibiting and non-inhibiting plasma [2,16,17]. The main findings are: (i) inhibiting and non-inhibiting plasma contain 3 fractions which inhibit Na^+, K^+ -ATPase, two of which cross-react with anti-digoxin antibodies; (ii) the enzyme active fractions in the inhibiting plasma are

significantly larger than those of the non-inhibiting plasma; (iii) the corresponding urine samples contain two inhibiting fractions, one of which is antibody active; and (iv) the peak inhibition of the enzyme activity of antibody active fraction is significantly greater for subjects with inhibiting plasma.

2. MATERIALS AND METHODS

2.1. *Patients and subjects*

Essential hypertensive patients and normotensive subjects were used in the study. The essential hypertensive patients came from the Hypertension Unit of Necker Hospital and had been off medication for at least one week. They were on regular Na^+ diet (100–120 mmol/24 h). Their blood pressures exceeded 160–90 mmHg when measured according to the recommendations of the WHO [18]. The normotensive subjects, both with and without genetic history of hypertension, were also free of any form of medication for at least a week.

2.2. *Sample preparation*

Blood samples were collected in chilled, heparinized tubes between 9 and 11 a.m. Since it had previously been reported that the endogenous Na^+, K^+ -ATPase inhibitor is heat stable [2,7], the plasma was immediately separated from the cells ($3000 \times g$, 5 min, 4°C) and boiled for 15 min. The time between blood sampling and the beginning of the boiling never exceeded 15 min. After cooling, the resulting clot was disrupted and centrifuged for 20 min at 4°C at $10\,000 \times g$. The supernatants were collected and stored at -20°C until analysis.

A total 24-h urine, covering the day previous to the blood sampling, was collected from participants. A 10 ml aliquot was freeze-dried and then stored at -20°C . The samples were reconstituted using 1 ml of bidistilled water just before analysis.

2.3. *Determination of inhibiting and non-inhibiting plasma*

Inhibiting and non-inhibiting plasma were determined according to their capacity to inhibit both dog kidney Na^+, K^+ -ATPase activity [16,17] and ouabain binding to human erythrocytes [2,16]. In brief, the plasma inhibition of Na^+, K^+ -ATPase was assessed using a plasma concentration of 2; and the plasma effect on ouabain binding was de-

termined at a dilution of 1 to 20. The positive and negative urines were obtained from people having inhibiting and non-inhibiting plasma, respectively.

2.4. *High-performance liquid chromatography*

The plasma and urine samples were chromatographed using a gradient-capable HPLC system (LKB-Product AB, Bromma, Sweden) equipped with a UV detector set at 226 nm. The chromatography was carried out on a 5μ Lichrosorb RP18 column (Merck, Darmstadt) using elution gradients of 0–50% acetonitrile in water at a flow rate of 1 ml/min. The mobile phase contained 0.1% trifluoroacetic acid and all the solvents were J.T. Baker HPLC grade (Sochibo, Boulogne-sur-Seine). Three $200\text{-}\mu\text{l}$ injections of each sample were chromatographed under these conditions and the eluent was collected in one-minute fractions. The fractions were freeze-dried, reconstituted to 1/10 of the initial volume with 10^{-3} M acetic acid and tested.

2.5. *Bio-assays*

The HPLC residues were assessed for their capacity to inhibit Na^+, K^+ -ATPase activity and to cross-react with anti-digoxin antibodies. The plasmas were then tested at a final concentration of 1 and 3 times the initial concentration for enzyme activity and antibodies, respectively. The urines were assessed at a final concentration and 10 and 30 times the initial concentration for the enzyme activity and antibodies, respectively.

2.6. *Na^+, K^+ -ATPase*

Dog kidney Na^+, K^+ -ATPase ($1.6\mu\text{g}$ protein, EC 3.6.1.3, Sigma) was incubated at 37°C for 45 min in the following medium: 80 mM Tris-HCl, 1 mM EGTA, 2 mM ATP (vanadate free, Sigma), $0.02\mu\text{Ci/ml}$ [$\gamma\text{-}^{32}\text{P}$]ATP (3000 Ci/mmol, Amersham), 3 mM MgCl_2 , 100 mM NaCl, 5 mM KCl, pH 7.4. The fractions from HPLC were assessed at a dilution of 1 to 10 in the incubating medium volume of $120\mu\text{l}$. Blanks were obtained by parallel incubation performed in the absence of K^+ and the presence of 0.1 mM ouabain. The reaction was stopped by sudden cooling at 4°C and by addition of cold perchloric acid (10% final concentrations). Then, 0.5 ml of cold charcoal suspension (20%, w/v) was added, and after 5 min the mixture was spun down for 3 min at $15\,000 \times g$. The resulting

supernatant was analyzed for its ^{32}P content in a liquid scintillation counter. The difference between the incubation in the absence and presence of ouabain was considered as the Na^+, K^+ -ATPase activity which represented 95–98% of the total enzyme activity. The effects of various HPLC fractions were expressed as percentages of inhibition of the ouabain-sensitive ATPase in the HPLC patterns. The activities of various peaks inhibiting Na^+, K^+ -ATPase were expressed by the sums of the percentage of inhibition (higher than 10%) of tubes present in the peak, divided by the number of these tubes, and also by the maximum inhibitory value obtained in each peak.

2.7. Antibodies

The procedure for measurement of digoxin immunoreactivity was essentially that described in [19] using a kit from NEN. The effect of various HPLC fractions were expressed as digoxin equivalent concentration in nm/ml of HPLC fractions for the HPLC patterns. Since a non-parallelism was obtained between the digoxin standard curves and the fractions dilution curves, it is not possible to calculate a digoxin equivalent concentration in the peaks.

3. RESULTS

The boiled plasma supernatants from inhibiting (positive, $n = 5$) and non-inhibiting (negative, $n = 4$) plasma were analyzed by reverse-phase HPLC using an acetonitrile-water gradient. The resulting chromatograms contained 3 peaks (1P, 2P, 3P) which inhibited Na^+, K^+ -ATPase and cross-reacted with antibodies raised against digoxin (fig.1). The peaks eluted at acetonitrile concentrations of 23, 29 and 34%, respectively. A fourth peak which cross-reacted with antibodies but did not inhibit Na^+, K^+ -ATPase was also detected (acetonitrile = 36%).

Peak 1P was present in both positive and negative plasma and there was no significant difference between the peaks from the two plasmas when the maximum or average inhibition of Na^+, K^+ -ATPase was measured (table 1). Peaks 2P and 3P were also present in all plasmas, however, the maximum and average Na^+, K^+ -ATPase inhibition measured for these peaks in the positive plasmas were signifi-

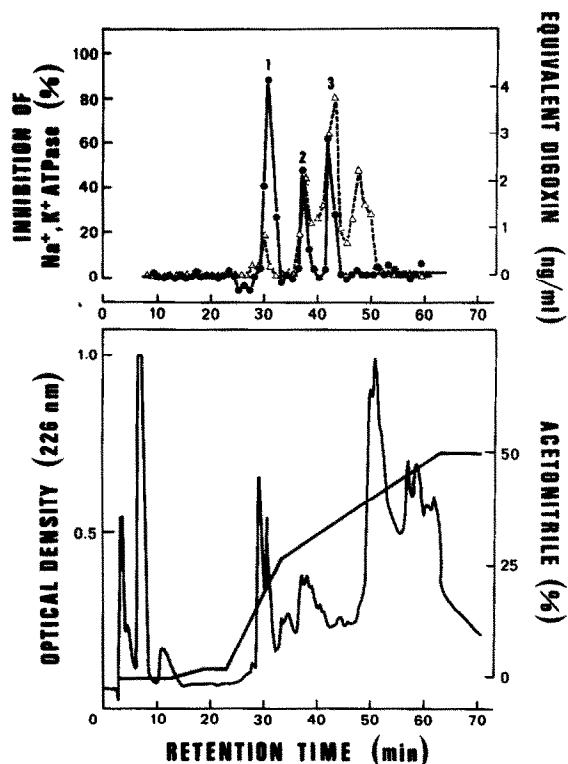


Fig.1. HPLC of 200- μl injection of supernatant of boiled plasma on 5 μ Lichrosorb RP18 (250 \times 4 mm). Chromatography was developed using an acetonitrile gradient (lower panel, —). The optical density was recorded at 226 nm (lower panel, —). 1-min fractions were collected, freeze-dried and assessed for their capacity to inhibit dog kidney Na^+, K^+ -ATPase activity (upper panel, ●—●) and to cross-react with anti-digoxin antibodies (upper panel, Δ — Δ).

cantly higher than the values found in the negative plasmas (table 1).

Urine samples from subjects with inhibiting (positive urine, $n = 3$) and non-inhibiting (negative urine, $n = 3$) plasmas were also analyzed by reverse-phase HPLC using an acetonitrile-water gradient. The resulting chromatograms contained two peaks (1U, 2U) which inhibited Na^+, K^+ -ATPase and cross-reacted with antibodies raised against digoxin (fig.2). The peaks eluted at acetonitrile concentrations of 18 and 28%. A third peak which cross-reacted with antibodies but did not inhibit Na^+, K^+ -ATPase was also detected (acetonitrile = 38%).

Peak 1U was present in the urine from all the subjects and there was no significant difference in

Table 1

Peak	Positives ^a					Negatives ^a				
	Subjects	Plasma		Urine		Subjects	Plasma		Urine	
		b	c	b	c		b	c	b	c
1	1	71	57	66	40	6	90	68	40	36
	2	88	73	100	86	7	19	18	13	13
	3	85	59	—	—	8	12	12	21	16
	4	15	15	—	—	9	40	32	—	—
	5	90	52	17	16					
	mean	69.8	51.2	61.0	47.6	mean	40.2	32.5	24.7	35.1
	±SE	15.8	15.8	29.5	25.1	±SE	20.3	14.5	9.8	5.9
2	1	72	56	57	42	6	12	12	0	0
	2	88	57	48	37	7	15	14	14	12
	3	88	50	—	—	8	18	15	21	17
		84	65	—	—	9	13	13	—	—
		49	31	36	26					
	mean	76.2 ^d	51.6 ^d	47.0 ^f	21.4	mean	14.5	13.5	11.7	9.4
	±SE	8.3	6.3	7.4	9.0	±SE	1.5	0.7	7.6	6.0
3	1	75	54			6	24	21		
	2	61	39			7	13	12		
	3	88	53			8	17	17		
	4	62	48			9	83	36		
	5	63	45							
	mean	69.8 ^f	47.8 ^e			mean	29.2	21.5		
	±SE	5.8	3.1			±SE	13.2	6.0		

^a Positive subjects (giving inhibiting plasma) and negative subjects (giving non-inhibiting plasma) were determined according to the capacity of boiled plasma supernatant to inhibit dog kidney Na⁺,K⁺-ATPase [16,17] and ouabain binding to human erythrocytes [2,16]

^b Maximum value of Na⁺,K⁺-ATPase inhibition for each peak

^c Average of the inhibition of the Na⁺,K⁺-ATPase activity of each peak. The data were analyzed using the Student *t*-test, positives vs negatives: ^d *P* < 0.001; ^e *P* < 0.01; ^f *P* < 0.02

— Indicates that the urines of this subject were not analyzed.

maximum or average Na⁺,K⁺-ATPase inhibition between this peak in the positive urine and the corresponding peak in the negative urine (table 1). Peak 2U was present in all the positive urines but was only detected in 2 out of the 3 negative urines. The maximum value of the Na⁺,K⁺-ATPase inhibition for peak 2U in the positive urine was significantly greater than the corresponding value for the negative urine. However, there was no significance in the average inhibition of these peaks.

4. DISCUSSION

Our results are in good agreement with the previously described heterogeneity of human plasmatic Na⁺,K⁺-ATPase inhibitors [5–8] and the increasing level of circulating Na⁺,K⁺-ATPase inhibitors in hypertensives [1–3]. The identification of two urinary Na⁺,K⁺-ATPase inhibitors, however, is not consistent with previous reports of a single inhibitor [10–14]. This discrepancy could be

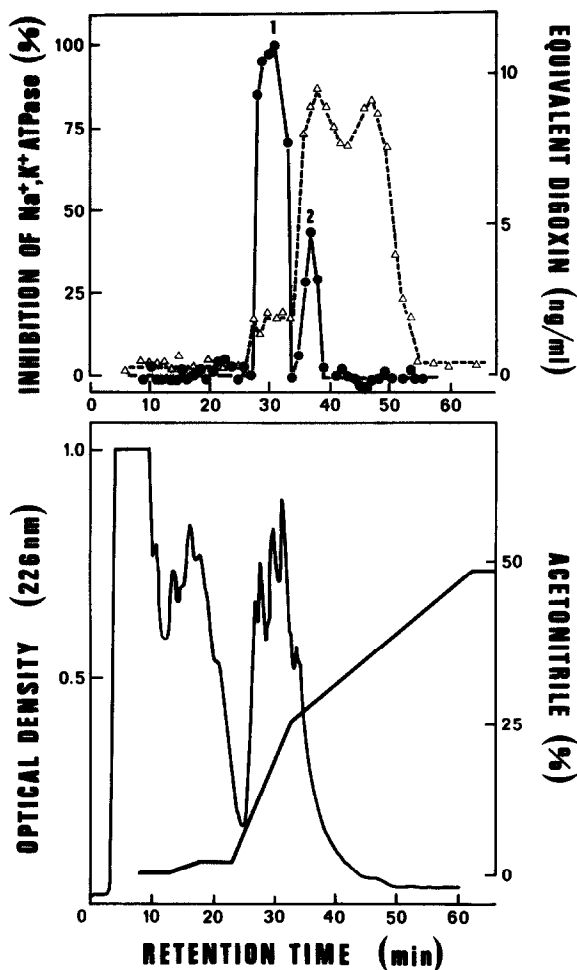


Fig.2. HPLC of 200- μ l injection of 10 times concentrated urine on 5 μ Lichrosorb RP18 (250 \times 4 mm). Chromatography was developed as in fig.1.

due to methodological differences arising from the employment here of gradient-elution reverse-phase HPLC.

Our data suggest a connection between the amount of Na⁺,K⁺-ATPase inhibitors found in the plasma and urine of the same subject. This is consistent with the observed correlation between the Na⁺,K⁺-ATPase level in plasma measured by ouabain binding and in urine measured by affinity chromatography in the same subject [21,22].

The significant difference in the maximum and average Na⁺,K⁺-ATPase inhibition for peaks 2P and 3P in inhibiting plasma vs non-inhibiting plasma and the corresponding difference in maxi-

mum inhibition for peak 2U in urine leads to the conclusion that the first peaks are not related to hypertension while peaks 2P, 3P and 2U appear to be specific for this disease.

Unfortunately, this tentative conclusion is only supported by the results of the Na⁺,K⁺-ATPase inhibition. It was not possible to draw the same conclusion using the cross-reaction with anti-digoxin antibodies since the digoxin standard curves were not parallel to peak dilution curves, and it was not possible to estimate the equivalent digoxin concentration of the plasmatic and urinary peaks. However, in both fluids, the cross-reaction of the first peak (1P, 1U) with antibodies raised against digoxin is lower than that of the other peaks. Therefore, these data supported the possible immunological similarity between the endogenous Na⁺,K⁺-ATPase inhibitors and digoxin.

The retention times and eluting acetonitrile percentage of peaks 1P and 2P are close to those of peaks 1U and 2U, respectively. Nevertheless, this comparison is not sufficient to support the identity of peaks 1P and 1U, and 2P and 2U. If the peaks 1P and 2P are similar to the peaks 1U and 2U, respectively, it is possible that both peak 2P and peak 2U may represent a metabolite of the peak 3P. The further purification and identification of these peaks, as well as peak 3P, is currently in progress.

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