Journal of Chromatography, 338 (1985) 417—421
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2436

Note

Rapid large-scale isolation of biologically active molecules using reversed-phase "flash" chromatography: initial purification of endogenous Na^+,K^+-ATPase inhibitors from human urine

I.W. WAINER*,*

Division of Drug Chemistry, U.S. Food and Drug Administration, Washington, DC 20204 (U.S.A.)

and

M. CRABOS and J.F. CLOIX

U7 INSERM, Department of Pharmacology, Hôpital Necker, 161 rue de Sèvres, 75015 Paris (France)

(First received July 19th, 1984; revised manuscript received September 26th, 1984)

An increased activity or concentration of an endogenous Na^+,K^+-ATPase inhibitor or inhibitors has been reported to be associated with hypertension in humans [1—3] and animals [4]. The analytical approaches to the separation of these Na^+,K^+-ATPase-active substances has varied. The reported methods include chromatography on Sephadex G-25 [5, 7], ion-exchange chromatography [8] and combinations of ion-exchange and reversed-phase chromatography [9—11]. While these methods have been utilized on an analytical scale, they have not been successfully adapted to the preparative scale needed for isolation and identification of these substances.

We have recently reported a reversed-phase high-performance liquid chromatographic (HPLC) method for the isolation on an analytical scale of endogenous Na^+,K^+-ATPase inhibitors from human plasma and urine [12]. This method utilizes an octadecyl support and an acetonitrile—water gradient. We have now adapted this method for preparative work using "flash" chromatography [13], which is a low-pressure-driven hybrid of conventional gravity column chromatography and high-pressure preparative systems. The chromatography was carried out using a 450-ml heavy walled glass column packed with

*Present address: U7 INSERM, Hôpital Necker, 161 rue de Sèvres, 75015 Paris, France.

0378-4347/85/$03.30

40-μm octadecylsupport, a stepwise acetonitrile—water gradient and low nitrogen pressure. In this manner we have been able to process 1—2 l of unconcentrated human urine in a single run with a significant initial purification of the desired inhibitors.

EXPERIMENTAL

Apparatus

The chromatography was carried out using a glass column (52.7 cm × 33 mm I.D.) fitted with a pressure-regulating flow controller and a 500-ml solvent reservoir; the column was filled with 100 g of 40-μm octadecyl reversed-phase column packing (J.T. Baker, Deventer, The Netherlands). The chromatographic fractions were collected using an LKB 2211 Superrac (LKB, Bromma, Sweden) and freeze-dried in a Lyovac GT2 (Leybold-Heraeus, France). Sodium, potassium and calcium ion concentrations in the collected fractions were measured by flame ionization using an Eppendorf Gerätebau (Netheler and Hinz, Hamburg, F.R.G.).

Materials

HPLC-grade water, methanol, acetonitrile and trifluoroacetic acid were purchased from J.T. Baker.

Biological samples

Blood samples and total 24-h urines were collected from essential hypertensive patients and normotensive subjects. The essential hypertensive patients came from the Hypertension Unit of Necker Hospital and had been off medication for at least one week. The normotensive subjects were also free of any form of medication for at least a week.

The blood samples were tested for their capacity to inhibit both dog kidney Na^+,K^+-ATPase activity [14, 15] and ouabain binding to human erythrocytes [2, 14]. On the basis of these tests each subject was placed in one of three groups: inhibiting hypertensive (I), inhibiting normotensive (II) and non-inhibiting (III). The urine samples were pooled by group and chromatographed.

Chromatography

The mobile phases were prepared using HPLC-grade solvents and contained 0.1% trifluoroacetic acid.

Before each chromatographic run, the column was washed with 500 ml of methanol followed by 500 ml of water. A pooled urine sample (1—2 l containing 0.1%, v/v trifluoroacetic acid) was placed on the column and chromatographed using a step-gradient of: 0, 15, 25, 30, 35, 50 and 100% acetonitrile in water. A flow-rate of 15 ml/min was maintained using 2—3 bars nitrogen pressure; 30-ml fractions were collected.

Bioassays

The HPLC fractions were freeze-dried and the residues reconstituted to 1/30 of the initial collected volume with 10^{-3} *M* acetic acid. The HPLC residues were then assayed for their capacity to inhibit Na^+,K^+-ATPase activity

following previously published procedures [8, 12] and for their phosphate [16] and sodium, potassium and calcium ion content. Inhibiting fractions were also tested for cross-reactivity with antidigoxin antibodies following the method described by Smith et al. [17].

RESULTS AND DISCUSSION

The chromatograms resulting from the flash chromatography of inhibiting urine from hypertensive patients (I), inhibiting urine from normotensive subjects (II) and non-inhibiting urine (III) are presented in Fig. 1. Each sample contained a large number of fractions which inhibited Na^+,K^+-ATPase activity. The sodium, potassium, calcium and phosphate ion concentrations of the inhibiting fractions were determined, and significant concentrations were found in fractions 1—20. Since these ions are known to affect Na^+,K^+-ATPase activity [18, 19] the fractions were discarded and are not included in the chromatograms.

The remaining inhibitory fractions were assessed for their capacity to cross-react with antidigoxin antibodies. Cross-reactivity with antidigoxin antibodies coupled with the capacity to inhibit Na^+,K^+-ATPase activity has been used as an indicator of the presence of endogenous Na^+,K^+-ATPase inhibitors [9, 12, 20, 21]. In this manner, the fractions eluted with 15% acetonitrile in the mobile phase (urines I, II and III) and the fractions eluted with 30—35% acetonitrile in the mobile phase (urines I and II) were found to contain in the desired activities. Thus, there appears to be a significant difference between inhibiting and non-inhibiting urine based on the presence or absence of activity in the fractions eluted with 30—35% acetonitrile in the mobile phase.

These results are consistent with our previous data [12] comparing the concentration of endogenous Na^+,K^+-ATPase inhibitors in inhibiting and non-inhibiting urines. The samples were analysed on an analytical scale using a reversed-phase octadecyl column and an acetonitrile—water gradient. The resulting chromatograms contained two peaks which inhibited Na^+,K^+-ATPase activity and cross-reacted with antidigoxin antibodies. The peaks eluted at acetonitrile concentrations of 18% and 28%.

The inhibitory capacity of the first peak (18% acetonitrile) was the same for both the inhibiting and non-inhibiting urine. However, the inhibitory activity of the second peak (28% acetonitrile) was significantly greater in the inhibiting urines. These results suggest, as do the results from the preparative-scale flash chromatography, that the second peak contains a substance which is specific for essential hypertension.

The endogenous Na^+, K^+-ATPase inhibitors are present in minute concentrations in the plasma and urine. An initial accumulation of the crude material is necessary before further purification and eventual identification is possible. Flash chromatography using a reversed-phase octadecyl support and an acetonitrile step gradient appears to be a viable and reproducible first step for the rapid, large-scale initial purification of these inhibitors. It is now routinely used in our laboratory.

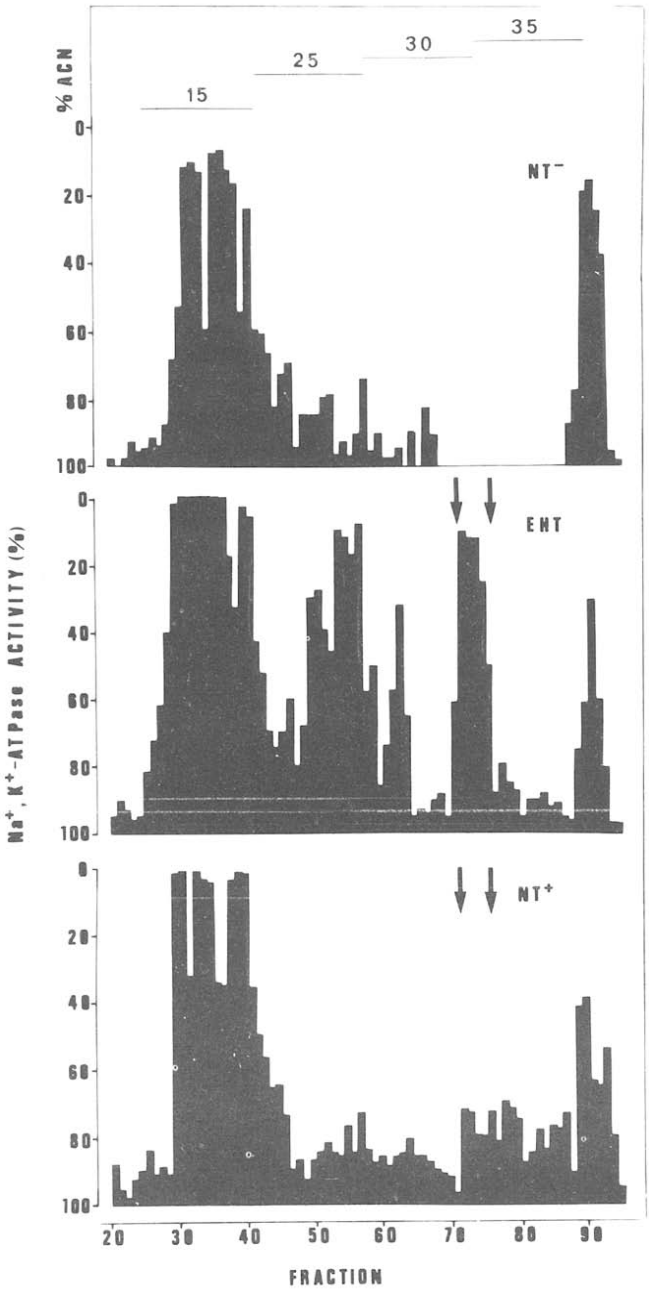

Fig. 1. Na^+,K^+-ATPase inhibitory activity of fractions from the flash chromatography of pooled urines from essential hypertensive patients (I, EHT), normotensive subjects with inhibiting plasma (II, NT^+) and normotensive subjects without inhibiting plasma (III, NT^-). Particular activity found in urine of EHT patients and NT^+ subjects lies inside arrows. These are profiles of repeated chromatographic runs (n = 12). ACN = Acetonitrile.

ACKNOWLEDGEMENT

The authors would like to thank Luuk Beumer, J.T. Baker Chemicals, for his suggestions and assistance.

REFERENCES

1 J.M. Hamlyn, R. Ringel, J. Schaeffer, P.A. Levinson, B.P. Hamilton, A.A. Kowarski and M.P. Blaustein, Nature, 300 (1982) 650.
2 M.A. Devynck, M.G. Pernollet, J.B. Rosenfeld and P. Meyer, Brit. Med. J., 287 (1983) 631.
3 G.A. MacGregor, S. Fenton, J. Alaghband-Zadeh, M. Markandu, E. Roulston and H.E. de Wardener, Brit. Med. J., 283 (1981) 1355.
4 F.J. Haddy and H.W. Overbeck, Life Sci., 19 (1976) 935.
5 R.B. Sewell, R.D. Hughes, L. Poston and R. Williams, Clin. Sci., 63 (1982) 237.
6 J.J. Bourgoignie, K. Hwang, E. Ipakchi and N.S. Bricker, J. Clin. Invest., 53 (1974) 1559.
7 J.E. Sealey, J.D. Kirshman and J.H. Laragh, J. Clin. Invest., 48 (1969) 2210.
8 J.F. Cloix, G. Dagher, M. Crabos, M.G. Pernollet and P. Meyer, Experientia, in press.
9 K.A. Gruber, I.M. Whitaker and V.M. Buckalew, Nature, 287 (1980) 743.
10 K.A. Gruber and V.M. Buckalew, Proc. Soc. Exp. Biol. Med., 159 (1978) 463.
11 A. Licht, S. Stein, C.W. MacGregor, J.J. Bourgoignie and N.S. Bricker, Kidney Int., 21 (1982) 339.
12 M. Crabos, I.W. Wainer and J.F. Cloix, FEBS Lett., 176 (1984) 223.
13 W.C. Still, J. Org. Chem., 43 (1978) 2933.
14 J.F. Cloix, M.A. Devynck, J.L. Elghozi, L.A. Kamal, L.C. Lacerda-Jacomini, P. Meyer, M.G. Pernollet, J.B. Rosenfeld and H. De The, J. Hypertension, 1 (S2) (1983) 11.
15 H. De The, M.A. Devynck, J.B. Rosenfeld, M.G. Pernollet, J.L. Elghozi and P. Meyer, J. Cardiovasc. Pharmacol., 6 (1984) S49.
16 B. Swynghedauw, P. Bouveret and V. Piguet, Ann. Biol. Clin., 28 (1970) 159.
17 T.W. Smith, V.P. Butler and E. Haber, N. Engl. J. Med., 281 (1969) 1212.
18 A. Askari, W.H. Huang and P.W. MacCormick, J. Biol. Chem., 258 (1983) 3453.
19 A.M. Brown and V.L. Lew, J. Physiol., 343 (1983) 455.
20 D. Klingmuller, E. Weiler and H.J. Kramer, Klin. Wochenschr., 60 (1982) 1249.
21 S. Balzan, A. Clerico, M.G. Del Chicca, N. Montoli and S. Ghione, Clin. Chem., 30 (1984) 450.