Journal of Chromatography, 490 (1989) 186–192
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4668

Note

Inhibition of glucose utilization in uremia by hippurate: liquid chromatographic isolation and mass spectrometric and nuclear magnetic resonance spectroscopic identification

VIERA SPUSTOVÁ* and PETER ČERNAY

Center of Clinical Pharmacology, Medical Bionics Research Institute, 833 08 Bratislava (Czechoslovakia)

and

IGOR GOLIER

Central Laboratory of Chemical Technique, Faculty of Chemical Technology, 812 37 Bratislava (Czechoslovakia)

(First received October 4th, 1988; revised manuscript received January 2nd, 1989)

Uremic subjects suffer from inhibited glucose utilization caused by insulin resistance [1]. The inhibition was localized at the post-receptor level [2,3], and one of the inhibitors was identified to inhibit glucose utilization at the P-fructokinase level [4]. However, the comparison of isolation protocols with another crude inhibitor participating in insulin resistance [5,6] excluded their identity. As a result, an intensive study of serum and urinary inhibitory activities was started. One of them was found to be caused by hippurate, and this paper presents the evidence on its significance.

EXPERIMENTAL

Urine samples

Overnight urine collections were obtained from patients with serum creatinine concentrations over 300 μM. The samples were collected without preservatives and stored at $-20\,^{\circ}C$ until freeze-dried. A freeze-dried urine sample

0378-4347/89/$03.50

was dissolved in a small amount of 0.03 *M* acetic acid. After gentle mixing for 10 min, the sample was filtered and applied to the column.

Urine fractionation

A 150-ml filtered sample of urine was applied to a 200 cm × 2.6 cm I.D. Sephadex G-15 column (Pharmacia, Uppsala, Sweden) equilibrated and eluted with 0.03 *M* acetic acid at a flow-rate of 2 ml/min. The absorbance at 254 nm (A_{254}) was continuously registered with Uvicord S (LKB, Bromma, Sweden), and the eluate was collected with a fraction collector FCC 60 (Laboratorní přístroje, Prague, Czechoslovakia). Collected fractions were pooled according to A_{254} maxima.

The freeze-dried inhibitory fraction obtained was dissolved in a small amount of water and applied to a 40 cm × 2.6 cm I.D. Dowex 50W-X8 column (Serva, Heidelberg, F.R.G.) in H^+ cycle equilibrated and eluted with water. According to A_{254} the eluate was collected in a fraction collector.

The freeze-dried inhibitory fraction was also applied to a preparative reversed-phase 20 cm × 1.6 cm I.D. column filled with Separon SGX C_{18}, 60 μm particle size (Tessek, Prague, Czechoslovakia). The mobile phase was 0.2 *M* acetic acid–methanol (8:2, v/v). The pH of the solution was adjusted to 6.5 by the addition of concentrated ammonia. A_{254} was continuously registered with the Uvicord S.

Inhibitor purity and structure

Purity. The purity of the inhibitor was tested on a modular high-performance liquid chromatographic (HPLC) system (Gilson, Middleton, WI, U.S.A.) with an analytical reversed-phase column (15 cm × 0.33 cm I.D.) filled with Separon SGX C_{18}, 8 μm particle size (Laboratorní přístroje) at 240 nm and the same mobile phase as in the preparative mode. A Rapid Spectral Detector (LKB) was used for spectral analyses of the isolated fraction and synthetic hippurate.

Structure. The ^{13}C proton decoupled spectra were recorded on a Varian VXR 300 spectrometer (Varian, Palo Alto, CA, U.S.A.) in 0.1 *M* [$^2H_6$]dimethylsulphoxide solution and 5 mm O.D. tubes at 75.4 MHz. The spectral width of 16 kHz was chosen with 64 000 data points. The molecular mass of the inhibitor was determined by mass spectrometry on a System 5985 gas chromatograph–mass spectrometer (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Biological activity

The inhibition of glucose utilization was tested in vitro on rat hemidiaphragm (striated muscle) incubated in Krebs–Henseleit bicarbonate medium containing 5 m*M* glucose with or without the addition of the tested fraction or hippurate. Incubation was performed in a Dubnoff shaker under an atmosphere of O_2–CO_2 (0.95:0.05) at 37 °C for 1 h. The details of the procedure and

the effect of hippurate on glucose utilization have been published elsewhere [8].

RESULTS AND DISCUSSION

Isolation of the inhibitory activity

The acid filtrate of the concentrated urine was fractionated by gel permeation chromatography (Fig. 1). All the UV-absorbing fractions were collected and freeze-dried, and individual peaks were tested on rat hemidiaphragms. Just two fractions inhibited glucose utilization, one with $K_{av}=0.57$, containing a previously isolated inhibitor [4], and another one with $K_{av}=1.03$. This structurally undefined fraction was purified further.

The freeze-dried fraction was separated by cation-exchange chromatography (Fig. 2a). The cation resin was also effective for separating some anions or uncharged substances. The inhibitory fraction did not bind to the resin and was eluted with water as the last fraction. No inhibitory activity was found in the fractions eluted with 2 *M* ammonia.

Reversed-phase chromatography on a C_{18} column purified the inhibitory activity further (Fig. 2b).

Purity and structure

The fraction from the C_{18} column was found to be homogenous (Fig. 3) by analytical reversed-phase HPLC with a purity over 96%, and the remaining

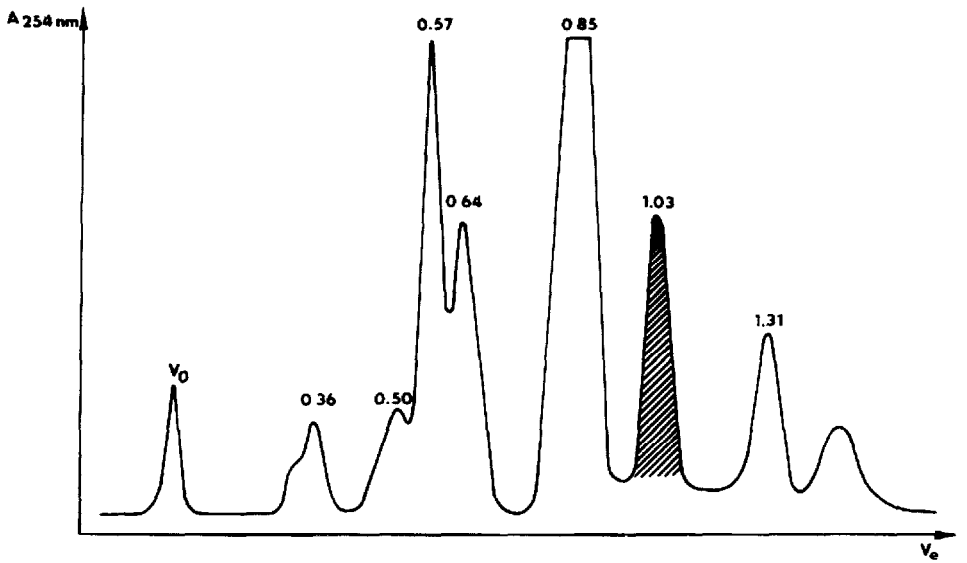

Fig. 1. Gel permeation chromatography on a 200 cm × 2.6 cm I.D. Sephadex G-15 column, eluted with 0.03 *M* acetic acid. The numbers above the peaks are K_{av} values calculated for each peak [$K_{av}=(V_e-V_0)/(V_t-V_0)$]. The shaded peak marks the inhibitory activity.

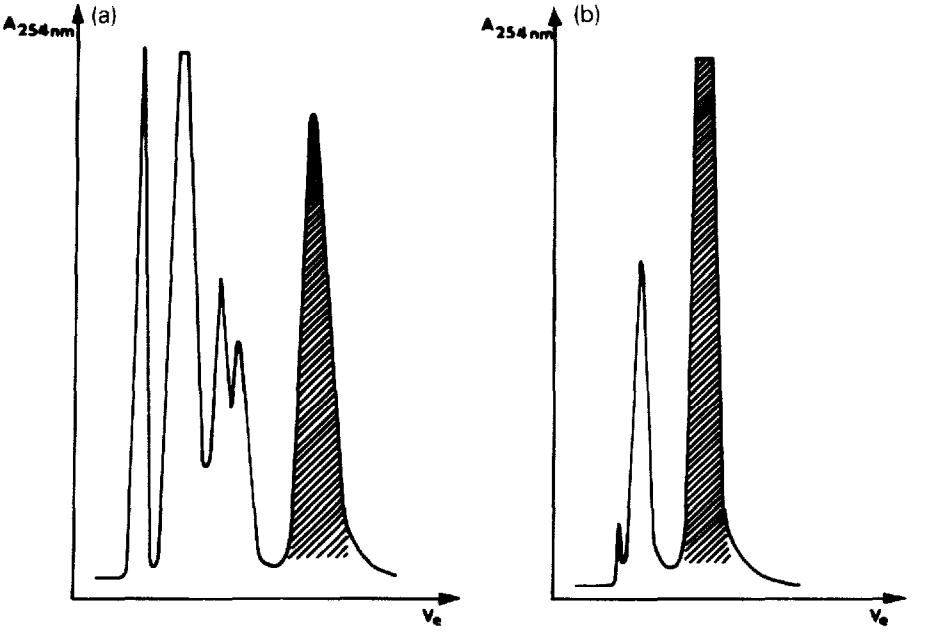

Fig. 2. Separation of inhibitory activity obtained from Sephadex G-15 fractionation using (a) cation resin (40 cm×2.6 cm I.D. Dowex 50W-X8 column, eluted with water) and (b) reversed-phase chromatography (20 cm×1.6 cm I.D. Separon SGX C_{18} column, 60 μm particle size, eluted with 0.2 *M* acetic acid–methanol, 8:2, v/v, pH 6.5).

impurities were removed by repeated chromatography under the same conditions.

Spectral analysis of the isolated fraction and synthetic hippurate revealed no differences in their spectra (Fig. 4). The NMR spectrum (Fig. 5) pointed unambiguously to the hippurate, and this conclusion was verified by the analysis of synthetic hippurate. The molecular mass was determined by MS (m/z^+ 179).

Hippurate effects

Hippurate is a well known conjugation product, which was supposed to be just an endogenous end-product of benzoate compounds. This conjugate is extremely effectively excreted by the kidney. Now, it appears that it inhibits glucose utilization at concentrations found in the sera of uremic subjects [7]. Moreover, it interferes with the protein binding of various drugs, increasing their free-form concentrations, and interferes with the excretion of organic anions in the proximal tubule of the kidney. These, as well as other effects, make hippurate a very intensively studied compound [8–11].

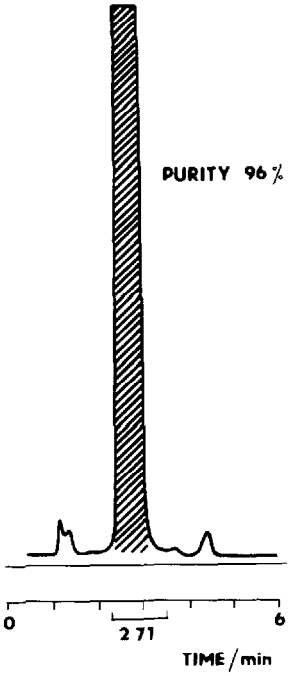

Fig. 3 Analytical high-performance liquid chromatography on a 15 cm×0.33 cm I.D. Separon SGX C_{18} column (7 μm particle size).

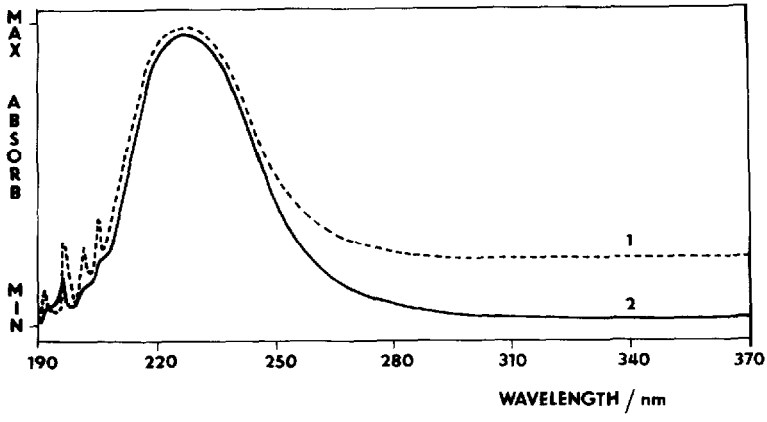

Fig. 4. Spectral analysis of (1) synthetic hippurate and (2) isolated inhibitory fraction.

Uremic toxicity

More than twenty compounds were isolated from urine or serum of uremic patients [12], and some classical liquid chromatographic urine fractionations stressed the accumulation of many unidentified fractions or modulators [13].

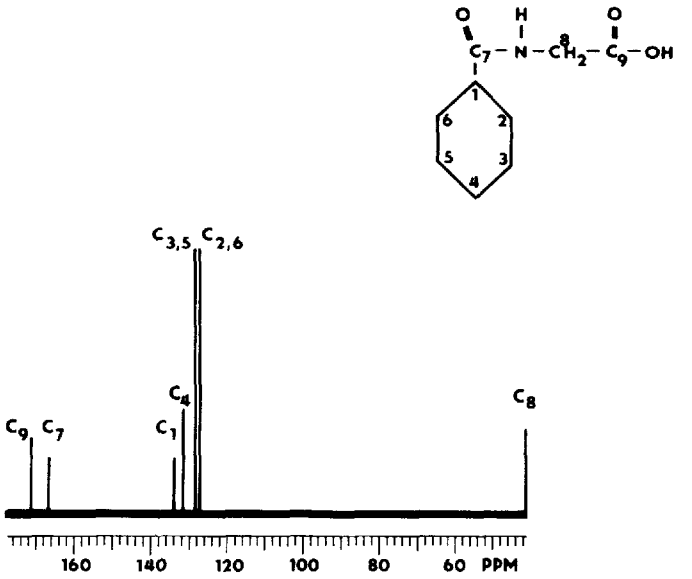

Fig. 5. ^{13}C NMR spectrum of isolated inhibitory fraction.

Most of them are of unknown significance, and therefore their descriptions have not attracted the interest of clinical nephrologists. It could, however, be suggested that the experience with hippurate will stimulate interest in further isolation and screening of their biological effects according to accepted criteria [14].

REFERENCES

1 D. Smith and R.A. DeFronzo, Kidney Int., 22 (1982) 54.
2 B.C. Maloff, M.L. McCaleb and D.H. Lockwood, Am. J. Physiol., 245 (Endocrinol. Metab., 8) (1983) E178.
3 F. Cecchin, O. Ittoop, M.K. Sinha and J.F. Caro, Am. J. Physiol., 254 (Endocrinol. Metab., 17) (1988) E394.
4 R. Dzúrik, V. Hupková, P. Černáček, E. Valovičová and T.R. Niederland, Clin. Chim. Acta, 46 (1973) 77.
5 L.S. Phillips, A.C. Fusco, T.G. Unterman and F. Del Greco, J. Clin. Endocrinol. Metab., 59 (1984) 764.
6 D. Powell, J. Bergström, R. Dzúrik, P.F. Gulyassy, D.H. Lockwood and L.S. Phillips, Am. J. Kidney Dis., 7 (1986) 292.
7 R. Dzúrik, V. Spustová and M. Geryková, in S. Ringoir, R. Vanholder and S.G. Massry (Editors), Uremic Toxins, Plenum Press, New York, London, 1987, p 105.
8 P.F. Gulyassy, A.T. Bottini, L.A. Stanfel, E.A. Jarrard and T.A. Depner, Kidney Int., 30 (1986) 391.
9 R. Vanholder, N. Vanlandschoot, S. De Smet and S. Ringoir, Abstracts XXIIIrd EDTA-ERA Congress, Budapest, June 29–July 3, 1986, p. 72.
10 J.V. Møller and M.I. Sheikh, Pharmacol. Rev., 34 (1983) 315.

11 G.A. Thompson and A. Meister, J. Biol. Chem., 255 (1980) 2109.
12 S. Ringoir, R. Vanholder and S.G. Massry (Editors), Uremic Toxins, Plenum Press, New York, London, 1987.
13 S.R. Burzynski, Physiol. Chem. Phys., 5 (1973) 437.
14 S.G. Massry, in S. Ringoir, R. Vanholder and S.G. Massry (Editors), Uremic Toxins, Plenum Press, New York, London, 1987, p. 1.