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Note

Isolation of an additional inhibitor of glucose utilization in renal insufficiency: pseudouridine

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A huge number of compounds have been isolated from sera or urine of patients in renal insufficiency (RI). They accumulate because of increased altered metabolism and/or decreased excretion in urine. However, there are no data on their eventual biological activity and they are just listed under the heading "waste products". However, it is probable that at least a proportion participate in the altered metabolism of RI. To define this relationship a metabolic or functional abnormality is chosen and a sensitive test used. This test is used step by step during the purification procedure until a satisfactorily purified compound is isolated for structural analysis.

One of the significant alterations in RI is the inhibition of glucose utilization [1,2]. A sensitive in vitro test was developed that enables the measurement of glucose utilization [3] and is satisfactorily insensitive to small variations in salt and NH_4^+ concentrations. Two inhibitors have been isolated [4,5], but additional inhibitory activity was still present in the urine of RI patients. This inhibitor has now been isolated and structurally defined. This paper describes the isolation procedure.

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EXPERIMENTAL

Urine samples

Urine from patients with serum creatinine concentrations over 300 μM was collected for 24 h and stored at $-20\,^{\circ}C$ until lyophilized. The lyophilized urine sample was then diluted with 0.03 *M* acetic acid. The diluted urine was filtered and loaded onto the column.

Urine fractionation

The filtered sample from 150 ml of urine was applied to a 200 cm × 2.6 cm I.D. column filled with Sephadex G-15 (Pharmacia, Uppsala, Sweden) and eluted with 0.03 *M* acetic acid adjusted to pH 5 with ammonia. The flow-rate used was 2.0 ml/min. Fractions were pooled according to the absorbance maxima continuously detected at 254 nm with Uvicord S (LKB, Bromma, Sweden).

The lyophilized inhibitory sample dissolved in a small amount of water was applied to a 150 cm × 2.6 cm I.D. Sephadex G-15 column equilibrated and eluted with 0.03 *M* acetic acid. The eluate was detected by measuring the absorbance at 254 nm and collected in the fraction collector FCC 60 (Laboratorní přístroje, Prague, Czechoslovakia).

The lyophilized inhibitory fraction was further purified on a preparative reversed-phase 20 cm × 1.6 cm I.D. column packed with Separon SGX C_{18}, 60 μm particle size (Tessek, Prague, Czechoslovakia), by step-gradient elution using (A) 0.2 *M* acetic acid, (B) 0.2 *M* acetic acid–methanol (80:20, v/v), and (C) 0.2 *M* acetic acid–methanol (60:40, v/v). This eluate was detected at 254 nm.

The lyophilized inhibitory fraction was diluted with water and loaded onto a 30 cm × 0.9 cm I.D. Dowex 50W-X8 column (Serva, Heidelberg, F.R.G.) in the H^+ cycle eluted with water. The eluate was collected according to 254-nm maxima.

This inhibitory fraction was lyophilized and dissolved in a small amount of 0.02 *M* boric acid adjusted to pH 9 with ammonia. The sample was applied to a 15 cm × 0.9 cm I.D. Dowex 1-X8 (Serva) in the Cl^- cycle equilibrated with 0.5 *M* boric acid adjusted to pH 9 with ammonia and washed with water. The elution was carried out by linear salt gradient with 200 ml of 0.02 *M* boric acid adjusted to pH 9 in the mixer chamber, and 200 ml of 0.1 *M* ammonium bicarbonate were adjusted to pH 9 in the reservoir.

The lyophilized sample was applied to a 30 cm × 0.9 cm I.D. Dowex 50W-X8 column in the H^+ cycle and eluted with water. The eluate was detected at 254 nm.

Purity

The purity of isolated inhibitor was tested by high-performance liquid chromatography (HPLC) on an analytical reversed-phase column (15 cm × 0.33

cm I.D.) packed with Separon SGX C_{18}, 8 μm particle size (Laboratorní přístroje). The mobile phase was 0.2 *M* acetic acid–methanol (95:5, v/v) and the flow-rate was 1.0 ml/min. A rapid spectral detector (LKB) was used for spectral analysis of the isolated fraction and synthetic pseudouridine (Sigma, St. Louis, MO, U.S.A.).

Structure

The ^{13}C proton decoupled and APT (attached proton test) spectra were recorded on a Varian VXR 300 NMR spectrometer (Varian, Palo Alto, CA, U.S.A.) in 0.1 *M* ^2H_2O 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 [$^2H_6$]dimethylsulphoxide was used as internal reference.

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 pseudouridine. 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.

RESULTS

Isolation of the inhibitor

The concentrated and filtered urine was fractionated by gel chromatography (Fig. 1). All the lyophilized fractions were tested on rat hemidiaphragms. The fraction with $K_{av}=0.85$ inhibited the glucose utilization.

This isolated inhibitor was desalted by gel chromatography using 0.03 *M* acetic acid without adjusted pH (Fig. 2A). The lyophilized fraction was separated by preparative reversed-phase chromatography (Fig. 2B). Inhibitory activity was found in the fraction eluted with 0.2 *M* acetic acid without methanol.

Good separation of the inhibitory fraction could be achieved by ion-exchange chromatography. Both cation exchangers (Fig. 3A) and anion exchangers (Fig. 3B) were used.

The isolated inhibitor was desalted by cation-exchange chromatography, and the fraction deprived of ammonium ions was lyophilized and prepared for spectral analysis.

Purity and structure

The isolated fraction from last procedure was tested by analytical reversed-phase HPLC. This fraction was found to be homogenous and the purity was over 96%.

Spectral analysis of this fraction and synthetic pseudouridine showed no

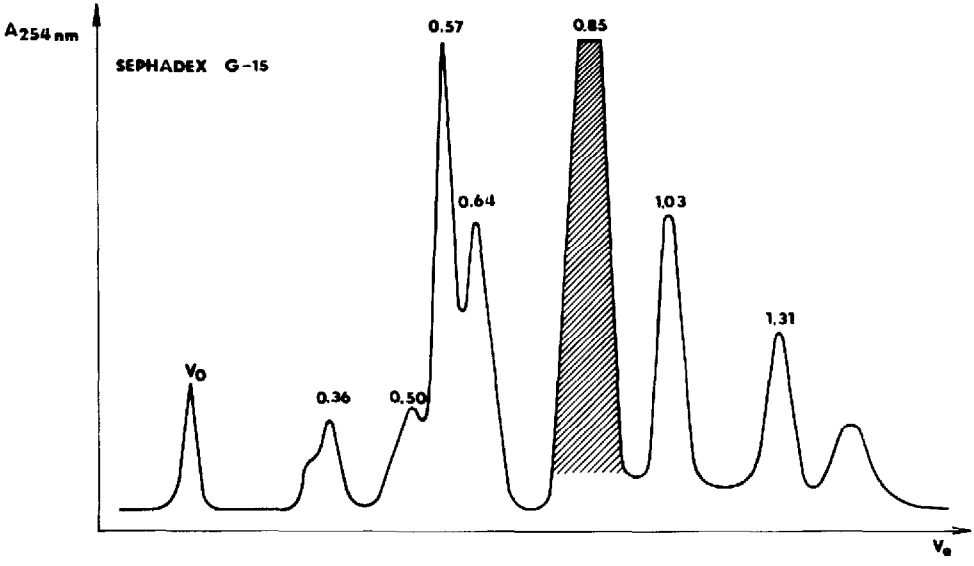

Fig. 1. Gel permeation chromatography on a 200 cm × 2.6 cm I.D. Sephadex G-15 column. Buffer, 0.03 *M* acetic acid, pH 5. The numbers above the peaks are K_{av} values calculated for each peak [$K_{av} = (V_e - V_0)/(V_t - V_0)$].

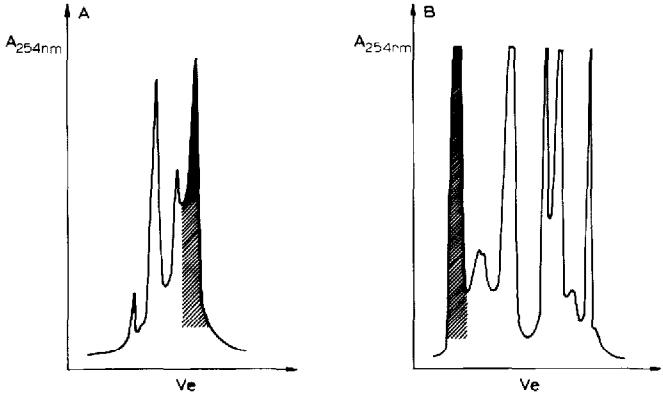

Fig. 2. (A) Gel chromatography on a 150 cm × 2.6 cm I.D. Sephadex G-15 column; buffer, 0.03 *M* acetic acid. (B) Step-gradient preparative reversed-phase separation of isolated inhibitor (20 cm × 1.6 cm I.D. Separon SGX C_{18} column, 60 μm particle size); buffers, (a) 0.2 *M* acetic acid, (b) 0.2 *M* acetic acid–methanol (80 20, v/v); (c) 0.2 *M* acetic acid–methanol (60:40, v/v)

differences in their spectra (Fig. 4). The NMR spectrum of this isolated fraction was identified as the spectrum of pseudouridine (Fig. 5).

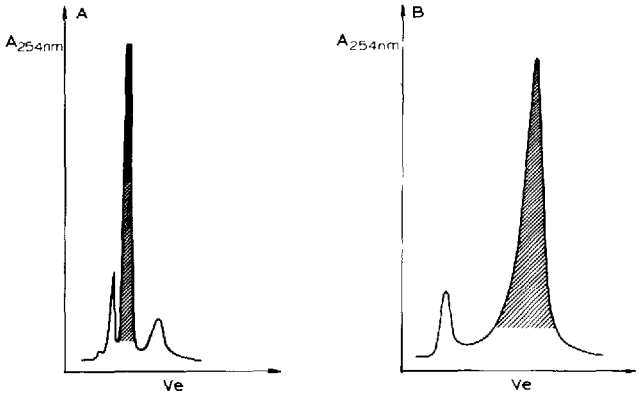

Fig. 3. Separation of the inhibitory fraction by ion-exchange chromatography: (A) cation e change and (B) anion exchange

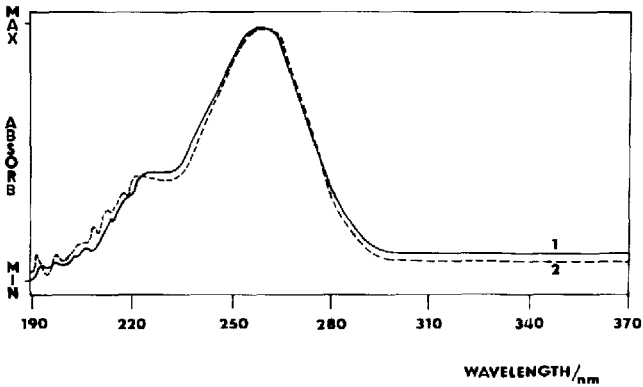

Fig. 4. Spectral analysis of (1) synthetic pseudouridine and (2) isolated inhibitory fraction.

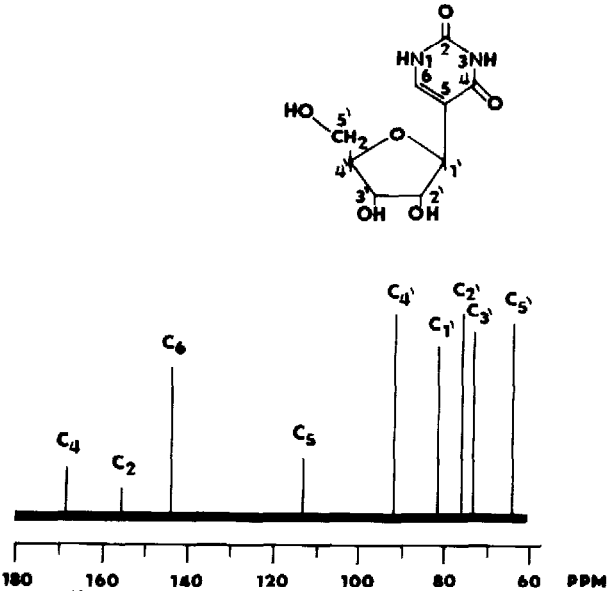

Fig. 5. ^{13}C NMR spectrum of isolated inhibitory fraction.

DISCUSSION

Classical strategy, i.e. the combination of gel permeation, ion-exchange and reversed-phase chromatography, was exploited for the isolation. However, because of the biological testing after each purification step and residual sensitivity at high salt concentrations, anion-exchange chromatography was used for the desalting with simultaneous partial purification.

The isolated and defined pseudouridine is a metabolic end-product, which is excreted in increased amounts in the urine of cancer patients [6,7]. Moreover, recently it was found to be accumulated in interstitial fluid of patients in RI [8]. However, no data were published about its effects on glucose utilization. This is a completely new finding, which appears to be worth elucidating in further studies.

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REFERENCES

1 R Dzúrik and V. Spustová, Czechoslov. Med., 7 (1984) 191.
2 D. Smith and R.A DeFronzo, Kidney Int., 22 (1982) 54.
3 R. Dzúrik and E. Valovičová, Clin. Chim. Acta, 30 (1970) 137.
4 R. Dzúrik, V. Hupková, P. Černáček, E. Valovičová and T.R Niederland, Clin Chim. Acta, 46 (1973) 77.
5 V. Spustová, P. Černay and I. Golier, J. Chromatogr., 490 (1989) 186.
6 A.C. Schoots, P.G G. Gerlag, A.W. Mulder, J.A.G. Peeters and C A.M.G. Cramers, Clin. Chem., 34 (1988) 91.
7 D. Drahovský, A. Winkler and J. Škoda, Neoplasma, 12 (1965) 561.
8 F. Salvatore, M. Savoia, T. Russo, L. Sacchetti and F. Cimino, in F. Cimino, G.D. Birkmayer, J V. Klavins, E. Pimentel and F. Salvatore (Editors), Human Tumor Markers, Walter de Gruyter, Berlin, New York, 1987, p. 451.