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A simplified method for isolation of pure pseudouridine C from human urine

In the method for isolation of pseudouridine described by COHN *et al.*[1], urine was freed from cations and anions by successive passage through columns of strong-acid and weak-base ion-exchange resins. The neutral substances, including pseudouridine, which pass through both these columns were then applied in an alkaline borate solution to a strong-base ion-exchange resin and the uncharged or the monoionic compounds were eluted with a sodium borate–ammonium bicarbonate buffer. Pseudouridine which under these conditions exists as a doubly charged borate complex was subsequently eluted with ammonium bicarbonate. Substitution of sodium borate–ammonium bicarbonate buffer by a single, volatile buffer[2] offers no advantage.

The suitability of this procedure for isolating pure pseudouridine C (5-β-D-ribofuranosyluracil) might be questioned because COHN[3] has shown that unnatural isomers can be obtained from pseudouridine by treatment with strong acid, a condition encountered in the urinary effluent from strong-acid ion-exchange resin.

To exclude this possibility DLUGAJCZYK[4] separated neutral substances containing pseudouridine from salts by means of a charcoal column, and further fractionated them on an anion-exchange resin which essentially accords with COHN's method[5].

The present paper describes a simple procedure for the isolation of isomer-free pseudouridine C. The latter adsorbed on a charcoal column by a simplified method similar to DLUGAJCZYK's[4], was separated from neutral substances by paper chromatography and further purified on a small column of anion-exchange resin.

Materials and methods

Twenty-four-hour urine specimens, obtained from laboratory personnel, or from patients maintained on a normal diet, were kept at about 4° during collection time and prior to use. Before pouring columns, fines were removed from charcoal, and from ion-exchange resins. Dowex-50W and Dowex-1, both 8% cross-linked and 200–400 mesh, were obtained from Sigma. All other reagents were Fisher products. All evaporations were performed under reduced pressure at 40–45°.

Experimental

Isolation of crude pseudouridine. One half of the 24-h urine specimen was filtered free of precipitated uric acid by suction on a large buchner funnel and the clear filtrate passed by gravity (average speed 10 ml/min) through a column 2 cm in diameter and 12 cm long, prepared from 20 g dry weight activated cocoanut charcoal (50–200 mesh). The column was not allowed to run dry during the packing and subsequent procedures. After all the urine had passed through the column, the latter was washed free of chloride ions with 200 ml of distilled water, and the adsorbed material was eluted from the column with 700 ml of 10% aqueous pyridine. The eluate was concentrated to dryness in a flash evaporator at 40° and the residue extracted twice with 10 ml water. The aqueous fraction after standing overnight at 4° was centrifuged, the supernate concentrated to an oily consistency and diluted with 5–6 volumes of ethanol. The precipitated material was removed by centrifugation, and one half of

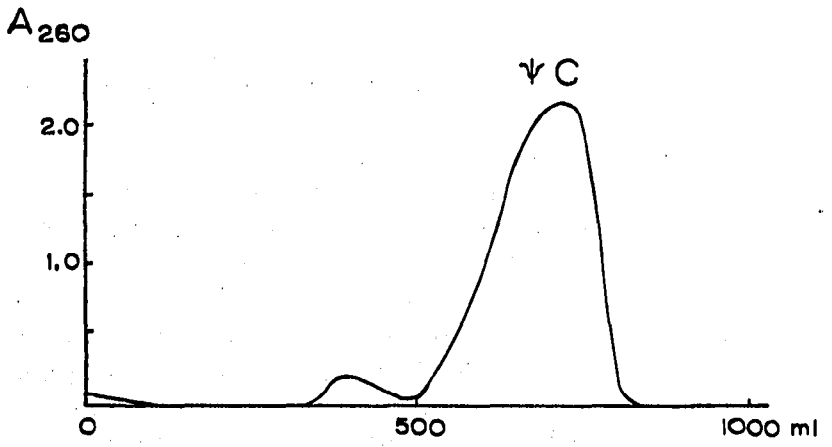

Fig. 1. Chromatography of pseudouridine C (ψC) on a Dowex-1 X8 (HCO_3^- form, 200–400 mesh) column (1 × 5 cm) equilibrated with borate buffer. Elution with linear salt gradient, starting with 0.02 *M* ammonium borate buffer pH 9 in the mixing chamber and 0.05 *M* ammonium bicarbonate in the reservoir.

concentrated supernate was applied on the shorter side of Whatman 3 MM 18¼ × 22½ in. chromatography paper. After 60–65 h of descending chromatography in the solvent system *n*-butanol–acetic acid–water (4:1:1), pseudouridine, the only major UV light absorbing band, had moved about 4/5 of the length of the paper. The paper was dried in air and the pseudouridine band cut out and eluted with about 60 ml water. The identity of pseudouridine can be verified by measuring the ratio of the absorbances at 290 and 260 nm at pH 12–13, which should be in the range of 2.

Purification of pseudouridine. The eluate from one sheet of paper, corresponding to 25 % of a 24-h urine collection, was made basic by addition of 0.5 ml 1.5 *M* ammonium hydroxide and poured on a 1 × 5 cm column of Dowex-1 X8 (HCO_3^- form, 200–400 mesh) which had been treated previously with 20 ml H_3BO_3 (0.5 *M*), adjusted to pH 9.0 with NH_4OH, and washed with 20 ml water.

Elution was carried out by the modified procedure of Cohn[3,5] in which a linear salt gradient with 500 ml 0.02 *M* boric acid adjusted to pH 9.0 with ammonia in the mixing chamber and 500 ml 0.05 *M* ammonium bicarbonate in the reservoir was used. Fractions (10 ml) were collected at 10 min intervals and the absorption measured with a Beckman DU spectrophotometer at 260 nm (Fig. 1). Pseudouridine, the only major peak, began to emerge when approx. 500 ml buffer had passed through the column and was collected in the next 270 ml. It differed from two other minor peaks by having a strong bathochromic effect in the alkaline region. The tubes containing pseudouridine were pooled and concentrated to dryness under reduced pressure at about 40°. The residue was redissolved several times in a small amount of water and concentrated to dryness in order to remove ammonium bicarbonate. The final residue was dissolved in 10 ml water and passed through a column of 1 ml Dowex-50W X8 (H^+ form, 200–400 mesh) to remove residual ammonium ions. Effluent and washings were concentrated to dryness and boric acid was removed by repeated addition of methanol and evaporation. The residue was dissolved in 3 ml water and traces of insoluble material and coloration removed by passage through 0.5 ml of Dowex-1 X8 (acetate form, 200–400 mesh). On concentration to dryness, 12.4 mg crystalline pseudouridine melting at 213–215° was obtained. It was recrystallized

from 95 % ethanol, passed again through Dowex-1-acetate and recrystallized a second time from ethanol. Yield of pure pseudouridine C 8.8 mg, micro m.p., K. 223–224° (reported 223–224°, *cf.* ref. 6); A 290/260 at pH 2: 0.070 (reported 0.066, *cf.* ref. 2), A 290/260 at pH 12: 2.29 (reported 2.26, *cf.* ref. 2).

Discussion

Pure pseudouridine C was isolated from human urine in practically quantitative yield by using less than 10 % of the ion-exchange resin required by published methods. Absence of other isomers in isolated pseudouridine C was verified by chromatography on a column of Dowex-1-bicarbonate according to COHN's method[3].

The procedure described here can be readily scaled up. For purification of crude pseudouridine, isolated from three 24-h urine specimens, a column of 1.5 × 36 cm of Dowex-1-bicarbonate proved satisfactory when a total of 8 l of borate–bicarbonate buffer was used.

The crude pseudouridine, as eluted from paper, contained by weight more than 50 % contaminants. Direct recrystallization from 90 % ethanol and twice from water, as recommended by DLUGAJCZYK AND EILER[7], resulted in a low yield of pseudouridine melting at 219–221°.

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