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Note

Improved method for the evaluation of 3-hydroxyproline and 4-hydroxyproline in the urine

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The hydroxyprolines (Hyp) are amino acids characteristic of collagen. The most abundant isomer, 4-Hyp, is found in all the types of collagens whereas a position isomer, 3-Hyp, absent from most collagens, exists in minor quantities in type I collagen and in higher amounts in basement membrane collagen [1] or, as recently described, in a non-basement membrane collagen found in the renal cortex [2]. 3-Hyp constitutes a marker of basement membranes or kidney collagens. The reader is referred to general reviews such as ref. 3 for a description of the various types of collagens.

We described several years ago a specific method for the determination of 3-Hyp which necessitated two preliminary steps of ion exchange followed by amino acid chromatography on a Beckman amino acid analyzer [4]. This technique permitted us to gather more than 300 results from normal and pathological urines and to describe an increase of urinary 3-Hyp with a significant decrease in the molar ratio 4-Hyp/3-Hyp in all the cases of polycystic kidney disease which were studied [5]. Unfortunately, this technique was time-consuming and tedious.

In a previous paper [6], we introduced a general technique sensitive to the picomole level for the evaluation of 4-Hyp and 3-Hyp in collagen hydrolysates or in cell cultures. In this paper, we describe an adaptation of this method to urine. It is ten times faster than the former method, far more sensitive, and gives results in excellent correlation with it.

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The new analytical procedure comprises preliminary separation of the hydroxyprolines from some interfering organic substances by fast chromatography through a column of Biorex 70 resin, the removal of inorganic salts by Dowex 50W-X2 chromatography, and fluorometric detection comprising derivatization with the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and thin-layer chromatographic separation of the fluorescent derivatives of Hyp on silica-gel plates, followed by quantitation with a recording spectrofluorometer.

MATERIALS AND METHODS

The usual reagents, all of the analytical grade, were purchased from Prolabo, Paris, France. Silica-gel 60 plates (ref. 5721) without fluorescent indicator were obtained from Merck (Darmstadt, G.F.R.), and NBD-Cl was from Aldrich-Europe (Beerse, Belgium). Biorex 70 and Dowex 50-X2 resins were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Ammonium formate was obtained from Sigma (St. Louis, MO, U.S.A.) and standard amino acids from Calbiochem (Los Angeles, CA, U.S.A.), except 3-Hyp which was prepared in the laboratory [7, 8].

Prior to analysis, the samples of urine are stored at —80°C and deproteinized by heating at 100°C for 2 min and centrifuging at 1500 *g* for 10 min. We verified the absence of any Hyp in the sediment. One millilitre of supernatant is mixed with 1 ml of 12 *M* hydrochloric acid and hydrolysed in a sealed tube for 18 h at 105°C. The hydrolysates are twice evaporated to dryness under vacuum and the residue is dissolved in 1 ml of 0.01 *M* ammonium formate buffer, pH 6.4. This solution is centrifuged and 200 µl of the clear supernatant are loaded on the top of the column of resin Biorex 70 prepared as follows.

Ion-exchange chromatography on Biorex 70

A 3 × 0.5 cm column of Biorex 70, 50—100 mesh (total capacity 3.3 mequiv.) is equilibrated in the Na^+ form by passage of 5 ml of 0.5 *M* sodium hydroxide. The resin is washed with 10 ml of distilled water and then with 20 ml of 0.1 *M* phosphate buffer, pH 6.4. Finally, the column is washed with 5 ml of 0.01 *M* ammonium formate solution. The sample of urinary hydrolysate is deposited at the top and the resin is eluted with 5 ml of the same buffer. In these conditions, the neutral and acidic amino acids are not bound to the resin and appear in the first 3 ml of effluent.

Ion-exchange chromatography on Dowex 50W-X2

A 3 × 0.5 cm column of the resin Dowex 50W-X2, 50—100 mesh (total capacity 1.2 mequiv.), is equilibrated in the H^+ form by passage of 20 ml of 2 *M* hydrochloric acid followed by distilled water until the pH of the effluent is neutral. The first 3 ml of effluent of the first column are acidified to pH 5.0 by addition of 100 µl of 0.1 *M* formic acid and this solution is passed through the Dowex 50 column. After the fixation of amino acids, the column is washed with 10 ml of distilled water. Then, the amino acids are eluted with 10 ml of a triethylamine—ethanol—water (20:40:40, v/v) solution. The eluate is

evaporated to dryness under a stream of nitrogen and the residue dissolved in 0.1 ml of distilled water.

Derivatization and thin-layer chromatography

To the previous solution are added 0.1 ml of a 3.0 *M* solution of triethylamine in ethanol and 0.1 ml of a 0.03 *M* solution of NBD in ethanol. The mixture is decanted and left in the dark in an incubator for 30 min at 65°C.

The NBD-amino acids are separated by thin-layer chromatography on silica-gel plates. For the evaluation of 3-Hyp, 5-μl samples of the unknown derivatized solutions are spotted in triplicate on the starting line 1 cm from the inferior edge of the plate. Standards of NBD-3-Hyp prepared simultaneously are deposited on both sides of the unknown sample in the range 20—100 pmoles. After drying the spots under a stream of nitrogen, the plate is predeveloped in methanol to obtain a thinner linear spot. This predevelopment permits better separations. It is stopped when the spots are located 2 cm from the lower edge. Prior to development, the plates are activated by heating at 65°C for 10 min. They are developed in a glass tank previously saturated with the solvent chloroform—acetone—methanol—tributylamine (60:20:5:15, v/v). After 1 h (when the front has moved up to 12 cm from the starting line), the plates are dried in an oven at 65°C for 5 min. They are stored in the dark up to the time of the fluorometric evaluation.

When 4-Hyp is to be measured, the same derivatized sample may be used, but 4-Hyp is far more concentrated in the urine than 3-Hyp so the sample must be diluted by adding 19 volumes of a 50% ethanol solution to 1 volume of the derivatized solution prior to thin-layer chromatography. Also, the separation of 4-Hyp is improved by the use of a different solvent for developing the thin-layer plates, i.e. acetone—toluene—methanol—triethylamine (40:40:15:5, v/v).

The preliminary treatment by Biorex 70 and Dowex 50 allows the perfect separation of spots of 3-Hyp or of 4-Hyp from any interfering amino acid. We checked the identity of the spots by supplementing the urine with standards of 4-Hyp or of 3-Hyp.

The plate fluorescence is recorded with a Farrand spectrofluorometer Model Mark I equipped with a thin-layer-plate recording attachment. The excitation wavelength is set at 340 nm with an additional violet filter absorbing the light over 500 nm and a slit of 1 cm length and 0.5 nm width. The emitted light is read at 525 nm with an additional yellow filter to absorb radiation under 450 nm. For the calculation of fluorescence, it is necessary to measure the width of the Hyp peak at its half-height and its height from the baseline, in order to calculate the surface. When the deposit is rigorously standardized, the measurement of the height furnishes a reproducible value of the concentration. There is a linear relationship between the surface of the peak and the amount of Hyp deposited on the plate in the range 1—100 pmoles [6]. The correlation with the Beckman column chromatography method was checked by linear regression analysis.

RESULTS AND DISCUSSION

In this paper, we present an adaptation to urine of a general method for the

evaluation of the isomers of Hyp which was described more completely elsewhere [6].

Chromatography on Biorex 70 is necessary for the elimination of basic amino acids such as histidine and methylhistidine which migrate with the same mobility as 4-Hyp on thin-layer plates. Chromatography on Dowex 50W-X2 permits the elimination of inorganic ions which interfere with the final thin-layer chromatography. Fig. 1A shows that a known amount of 3-Hyp is quantitatively eluted in the first 3 ml of the effluent from the Biorex 70 column, and Fig. 1B shows that 3-Hyp is completely eluted from Dowex 50W-X2 by 10 ml of triethylamine—ethanol solution.

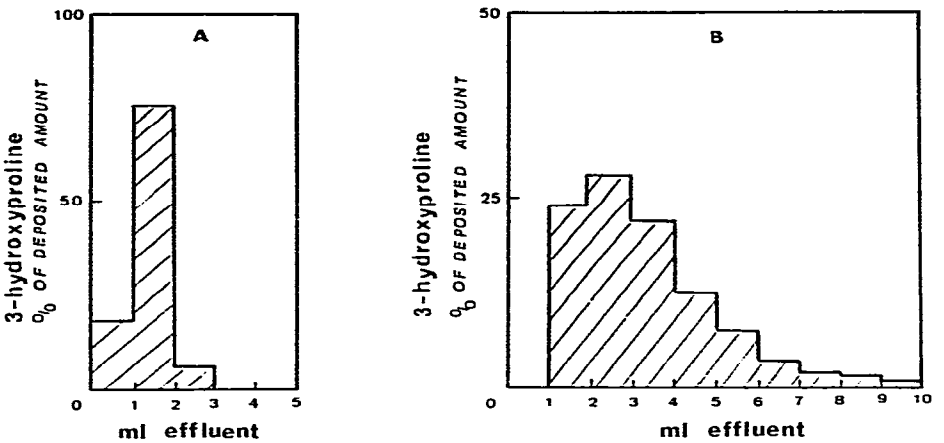

Fig. 1. Recovery of standard 3-Hyp from the preliminary stages of chromatography. (A) Column of Biorex 70 (Na^+). Elution by 0.01 *M* ammonium formate, pH. 6.6. (B) Column of Dowex 50W-X2 (H^+). Elution by 10 ml of triethylamine—ethanol—water (20:40:40, v/v).

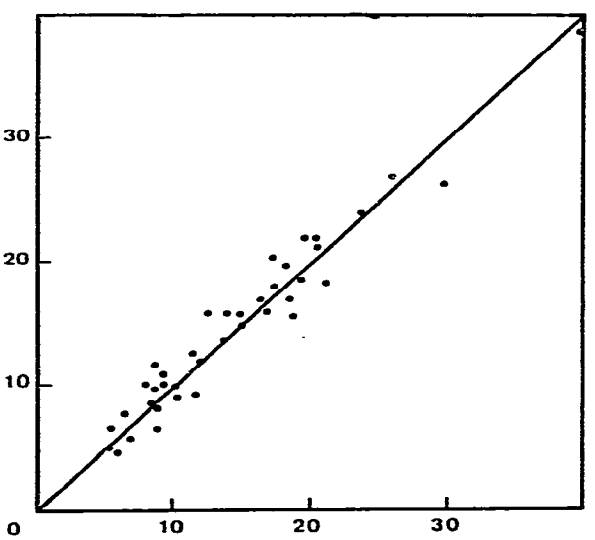

Fig. 2. Correlations of the concentrations of 3-Hyp measured in 39 human urines by ion-exchange chromatography (former method) and thin-layer chromatography (present method). $y = 0.97x + 0.36$, $r = 0.97$, $n = 39$.

In twenty experiments in which known amounts of 3-Hyp were successively chromatographed on the two columns, the final recovery was 99 ± 1.5% (mean ± 1 S.D.). Thirty-nine urine samples from normal or pathological subjects were analyzed in parallel by the former and the newer methods. As shown in Fig. 2, an excellent correlation was found, with $r = 0.97$, demonstrating that both methods may be used with the same reference values. Figs. 3 and 4 show the records of chromatograms used for the evaluation of 3-Hyp and 4-Hyp, respectively. These records demonstrate that the separation of 3-Hyp is complete with the first solvent and that of 4-Hyp with the second solvent. The two isomers would not be completely separated from interfering amino acids by a single solvent. This difficulty has no practical significance because the high concentration of 4-Hyp in the urine compared to 3-Hyp would necessitate in any case separate thin-layer chromatography of the two isomers to be performed.

The interest of the new technique is its speed and sensitivity. Ten evaluations can be performed within a day. The method is sensitive to the picomole level, which means that under the conditions described it is a hundred times more sensitive than the former method.

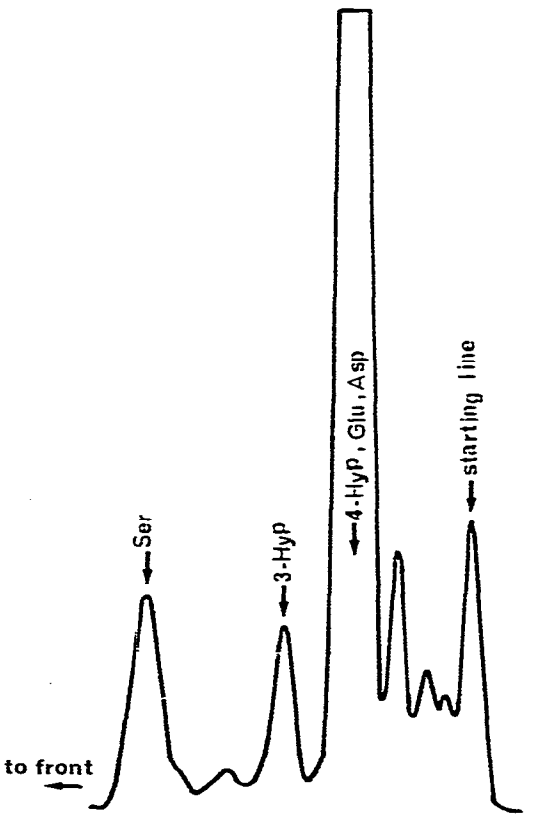

Fig. 3. Scan of thin-layer chromatogram showing the peak of NBD-3-Hyp from an hydrolysate of urine. Solvent: chloroform—acetone—methanol—tributylamine (60:20:5:15, v/v). Development for 1 h at 20°C. Spectrofluorometer Farrand Mark I, sensitivity 0.03, scan speed 150 mm/min.

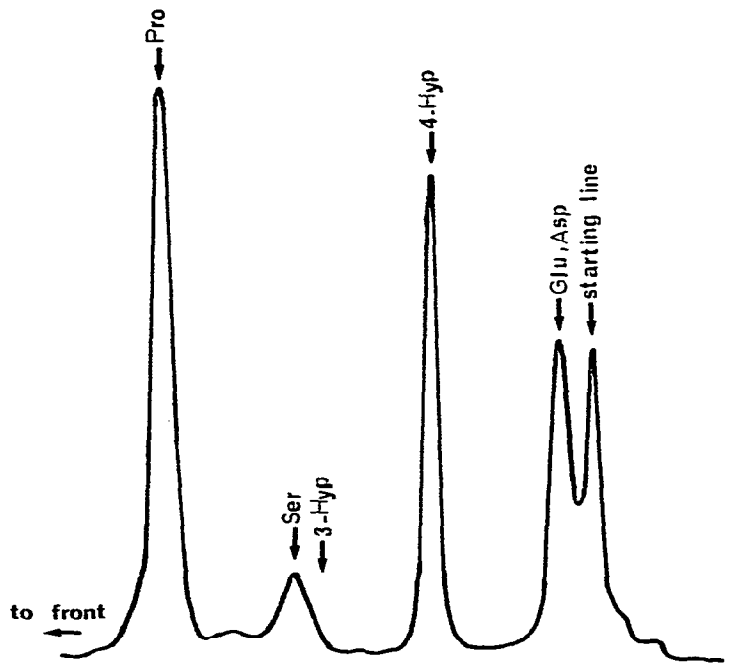

Fig. 4. Scanning of thin-layer chromatogram showing the peak of NBD-4-Hyp from an hydrolysate of urine. Solvent: acetone—toluene—methanol—triethylamine (40:40:15:5). Scanning conditions as in Fig. 3.

TABLE I

REFERENCE VALUES FOR THE CONCENTRATIONS OF 3-Hyp AND 4-Hyp IN HUMAN URINE

Values are the mean of 20 determinations ± 1 S.D.

	3-Hyp (μmol per 24 h)	4-Hyp (μmol per 24 h)	Ratio 4-Hyp/3-Hyp
Adults	12.5 ± 3.5	225.7 ± 61.5	18.0 ± 1.7
Children	6.0 ± 4.8	237.0 ± 201	39.0 ± 7.3
Teenagers	15.2 ± 6.4	517.0 ± 108	34.5 ± 7.5

Up to now, two methods for the evaluation of 3-Hyp in the urine have been described: one by Adams et al. [9] and the other by our group [4]. Both papers furnished nearly identical reference values (Table I). Both methods are tedious which prevents them from being used routinely. The present technique is fast enough for routine use. It permits not only the measurement of 3-Hyp but also of 4-Hyp with excellent precision. It must be emphasized that the amounts of 4-Hyp found with this technique are always 15% lower than the values given by the conventional techniques using chloramine T oxidation and coupling with *p*-methylaminobenzaldehyde (data not shown). This latter technique is probably less specific than the fluorometric one.

The semiological usefulness of urinary 3-Hyp evaluation firstly resides in the constancy of its increase in cases of polycystic kidney disease [10], while the

ratio 4-Hyp/3-Hyp is decreased. A second application of this evaluation lies in the fact that 3-Hyp exists only in the triple helical part of definitive interstitial collagen, not in the procollagen N-terminal extensions, while 4-Hyp exists in the N-terminal extension as well as in collagen itself. This provides a means of differentiating between increases in urinary 4-Hyp due to pure catabolism of collagen (4-Hyp and 3-Hyp are increased in parallel) and increases in urinary 4-Hyp due to an enhancement of collagen biosynthesis (4-Hyp increases because it comes from the N-terminal extension of procollagen, while 3-Hyp remains at the usual level).

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