ELSEVIER

Journal of Chromatography B, 665 (1995) 295–302

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

# Rapid reversed-phase high-performance liquid chromatographic method with double derivatization for the assay of urinary hydroxyproline

Vincenza Bianchi*, Luigi Mazza

*Laboratory of Clinical Pathology, City Hospital, Via Venezia 18, 15100 Alessandria, Italy*

First received 21 September 1993; revised manuscript received 2 November 1994; accepted 2 November 1994

**Abstract**

An HPLC method with two derivatizations, the first with *o*-phthaldehyde in order to eliminate interferences due to some primary amino acids eluting with retention times similar to those of hydroxyproline and the second with dabsyl chloride, was developed and evaluated. Calibration graph linearity, influence of agitation and temperature on the preparation of the first derivative and the influence of the detection wavelength were assessed. The analysis time is shorter in comparison with other available methods, and therefore this method is suitable for laboratories that analyse both small and large series of samples.

## 1. Introduction

Hydroxyproline (HYP), a hydroxylated imino acid, contained almost exclusively in collagen [1], is today considered as an important bone metabolism marker [2]. Its urinary excretion increases in all physiological and pathological cases that involve an increase in osteoclastic activity [3]. The cell is able to sythesize HYP due to proline-4-monooxygenase, which acts on the proline residues only after their incorporation in a polypeptide chain [4], even if a large part is introduced into the organism by means of the diet (meat and fish).

All methods for the determination of total HYP in urine require hydrolysis [5,6] of the excreted oligopeptides in which HYP is contained. Manual spectrophotometric methods show low reproducibility owing to the complicated procedures involved whereas methods based on high-performance liquid chromatography (HPLC) seem to be much more reliable [7–11]. HYP requires derivatization to make it detectable using UV–Vis or fluorescence detectors. The stability of derivatives is a very important factor; those used for spectrophotometric determination are stable for many hours, whereas fluorescence declines rapidly. This is the reason for their poor utility for large series of samples. One of the most commonly used derivatives for spectrophotometric determination is dimethylaminoazobenzene-4-sulphonyl chloride (dabsyl chloride) [8–10], which reacts with the amino acid to form a sulphonamide with two

* Corresponding author.

0378-4347/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
*SSDI* 0378-4347(94)00518-4

absorption maxima at 436 and 471 nm. As dabsyl chloride is not specific for a single amino acid, the HPLC traces contain several peaks of amino acids eluting with retention times similar to that of HYP [12].

This paper describes the evaluation of an assay procedure for urinary HYP that involves double precolumn derivatization, the first with *o*-phthalaldehyde (OPA) and the second with dabsyl chloride (DABS-Cl), followed by HPLC. The first derivatization step eliminates the interferences due to primary amino acids, and the DABS-Cl derivative allows the use of a 471-nm detection wavelength. We also compared actual results with those obtained using only DABS-Cl.

## 2. Experimental

### 2.1. Instrumentation

The HPLC system (Bio-Rad Labs, Hercules, CA, USA) consisted of a Model 1350 pump, Model AS 100 autosampler and Model 1706 UV–Vis detector. The system was completed with a degasser, gradient monitor, dry oven for the column and precolumn and Model 3393 HP integrator. The analytical column was a reversed-phase ODS column (150 × 4.6 mm I.D., 5 μm) with a Microguard RP precolumn (30 × 4.6 mm I.D., 10 μm) all from Bio-Rad Labs.

### 2.2. Chemicals

Hydrochloric acid, sodium hydroxide, anhydrous sodium carbonate, citric acid monohydrate, sodium phosphate dodecahydrate, *o*-phthalaldehyde, *trans*-4-hydroxy-L-proline and dimethylaminobenze-4-sulphonyl chloride were obtained from Sigma (St. Louis, MO, USA), acetonitrile and methyltaurine from Merck (Darmstadt, Germany) and quality control urine (Lyphocheck Quantitative Urine) from Bio-Rad Labs. Ultrapure water was prepared with a Milli-Q system (Millipore, Milford, MA, USA).

### 2.3. Sample preparation

Control urine and samples were prepare by adding 1 ml of 37% HCl and 0.1 ml of internal standard (65.5 mg/l methyltaurine) to 1 ml of urine. The standard was prepared by adding 0.9 ml of ultrapure water and 1 ml of 37% HCl to 0.1 ml of hydroxyproline solution (65.5 mg/l). The following step was hydrolysis at 100°C for at least 15 h. The hydrolysates were diluted with ultrapure water in the ratio of 1 : 4. Then 0.8 ml of 0.5 *M* NaOH and 0.8 ml of 0.25 *M* $Na_2CO_3$ were added to 0.1 ml of diluted hydrolysates so as to obtain a pH of 9. The first derivatization reaction was carried out by adding 0.2 ml of *o*-phthalaldehyde (5 g/l in acetonitrile) to 0.5 ml of diluted and buffered hydrolysates. The second derivatization was carried out by mixing 0.15 ml of the previous samples with 0.2 ml of dabsyl chloride (5 g/l in acetonitrile) and heating for about 10 min at 70°C in a dry oven. The prepared samples and standard were finally diluted with 0.5 ml of mobile phase before injection in the HPLC system.

### 2.4. Mobile phase preparation

A 1-l volume of mobile phase was prepared with 0.05 *M* citric acid (330 ml). 0.01 *M* sodium phosphate (380 ml) and acetonitrile (290 ml). This solution was filtered under vacuum before use.

### 2.5. Chromatographic conditions

The column was thermostated at 60°C, the mobile phase flow-rate was 1.5 ml/min and the detection wavelength was 471 nm. The elution control unit was programmed in such a way that for about 4 min mobile phase was eluted, then for 1 min acetonitrile and finally for about 10 min mobile phase again. The integrator was set up to measure peak heights. The HYP concentration was evaluated based on the internal standard for HYP peak-height ratio.

### 2.6. Comparison of methods

Urine samples (160) provided by patients undergoing a collagen-poor diet were analysed and the results were compared to those obtained by analysing the same urine with a method evaluated in a previous study [12], which involved only dabsyl chloride derivatization.

## 3. Results

Fig. 1 shows the chromatograms for the standard and urine samples. The retention time of the internal standard is about 3.7 min, whereas that of HYP is about 5 min. Apart from the solvent front, it can be seen that in the urine sample no other substances apart from those of analytical interest were eluted; linearity was verified by preparing and analysing aqueous solutions in 6 $M$ HCl with different HYP concentrations. The values obtained with these samples are given in Table 1.

Table 1
Evaluation of the method linearity

| Theoretical concentration (HYP added) (mg/ml) | Concentration found (mg/ml) | Difference (%) |
|---|---|---|
| 6.6 | 6.7 | +1.5 |
| 16.5 | 16.1 | −2.5 |
| 33.0 | 32.1 | −3.0 |
| 66.0 | 68.1 | +3.0 |
| 132.0 | 128.0 | −3.0 |
| 195.5 | 198.8 | +1.6 |
| 229.6 | 237.6 | +3.5 |
| 264.0 | 273.2 | +3.5 |
| 330.0 | 315.6 | −4.0 |
| 660.0 | 690.0 | +4.5 |
| 1320.0 | 1382.1 | +4.5 |

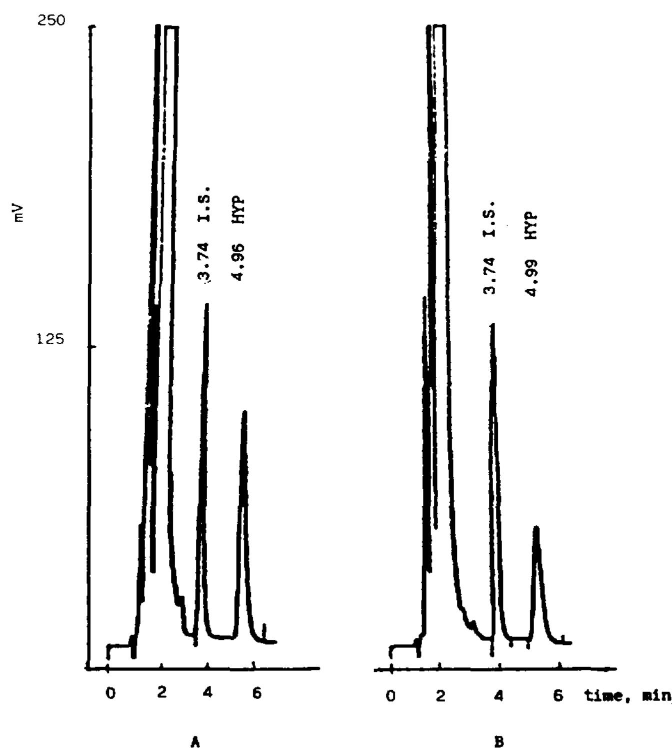


Fig. 1. (A) Standard chromatogram and (B) sample chromatogram. I.S. = Internal standard; HYP = hydroxyproline. For chromatographic conditions, see text.

Table 2
Evaluation of the method recovery: HYP added to the initial sample containing 33.7 mg/ml

| Amount added (mg/ml) | Theoretical amount (mg/ml) | Amount found (mg/ml) | Difference (%) |
|---|---|---|---|
| 0.0 | 33.7 | 34.5 | +2.3 |
| 5.0 | 38.7 | 37.5 | −3.1 |
| 10.0 | 43.7 | 44.8 | +2.5 |
| 20.0 | 53.7 | 51.9 | −3.3 |
| 50.0 | 83.7 | 86.2 | +3.0 |
| 100.0 | 133.7 | 138.4 | +3.5 |

The recovery was verified with a urine sample that after three determinations gave an average result of 33.7 g/l; known amounts of HYP were added to this sample in order to obtain samples with five different HYP concentrations. The amounts of HYP found in these samples are reported in Table 2. The precision and accuracy were evaluated by analysing a commercial quality control urine (Lyphocheck Quantitative Urine), as shown in Table 3.

The influence of the reaction time necessary for the preparation of the first derivative was verified by mixing the sample with the first derivatization agent and then carrying out vortex

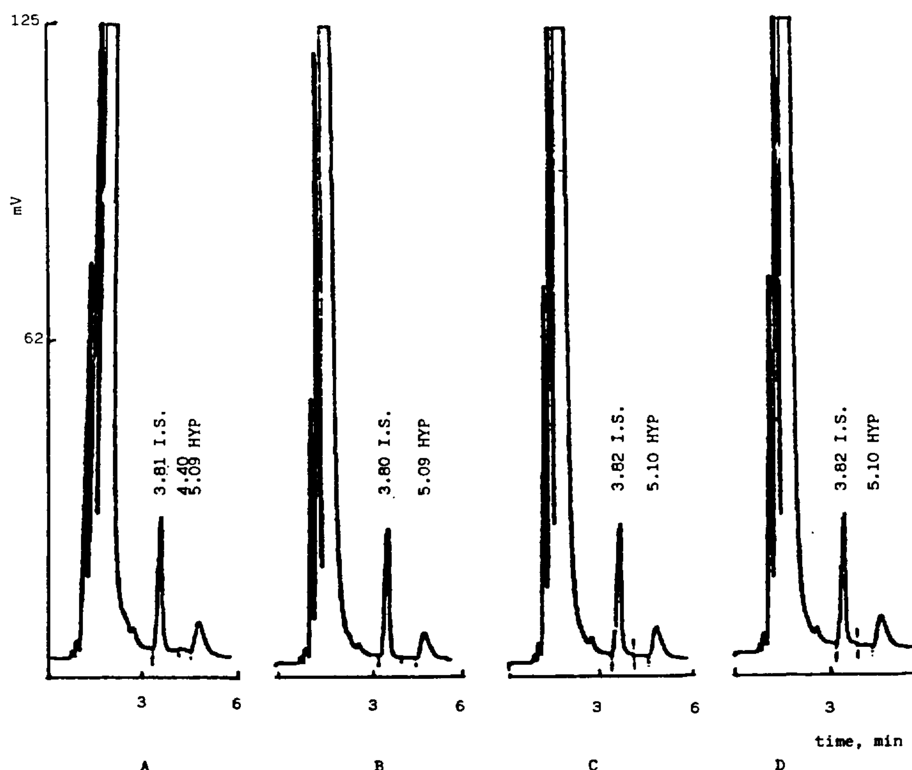


Fig. 2. Evaluation of agitation time, (A) without agitation; (B) agitation for 10 s; (C) agitation for 20 s; (D) agitation for 30 s on a vortex mixer.

Table 3
Evaluation of the precision and accuracy, with normal and pathological urine samples used for quality control

| Parameter | Within-run | | Between-run | |
|---|---|---|---|---|
| | Normal urine | Pathological urine | Normal urine | Pathological urine |
| *Precision* | | | | |
| No. of samples | 10 | 10 | 10 | 10 |
| Mean value (mg/ml) | 16.56 | 113.92 | 17.03 | 115.38 |
| S.D. (mg/ml) | 6.67 | 5.51 | 1.01 | 6.02 |
| C.V. (%) | 4.45 | 4.52 | 4.97 | 4.88 |
| *Accuracy* | | | | |
| HPLC mean value | | 16.56 | | 113.9 |
| Acceptable values | | 14.82–18.30 | | 102.92–124.94 |
| Theoretical spectrophotometric mean value | | 13.0 | | 88.0 |
| Theoretical acceptable values | | 10.4–15.6 | | 70.4–105.6 |
| Experimental vs. theoretical value (%) | | 27 | | 29 |

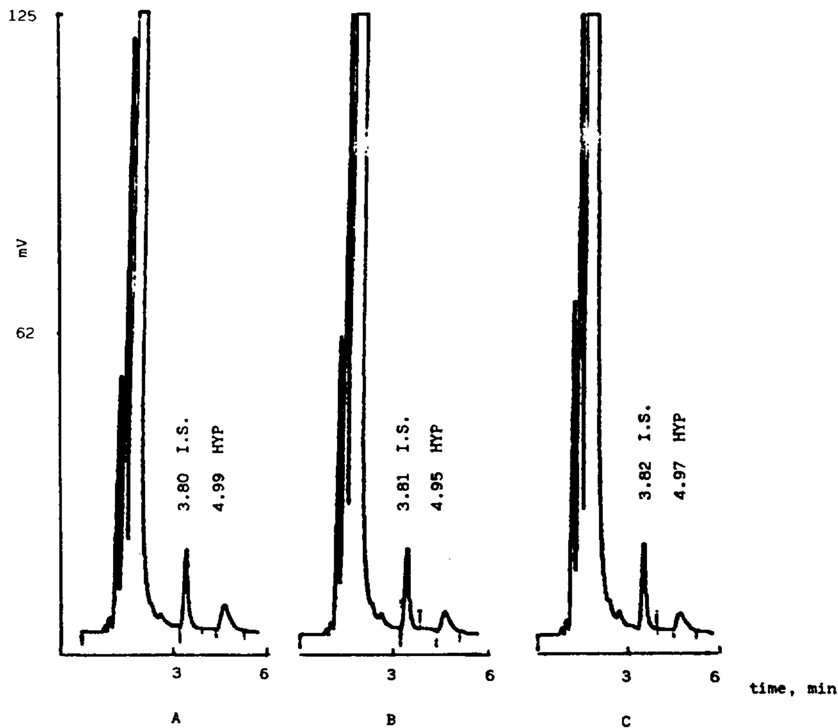


Fig. 3. Effect of the temperature during the preparation of the second derivative. (A) Room temperature; (B) 70°C for 5 min; (C) 70°C for 10 min.

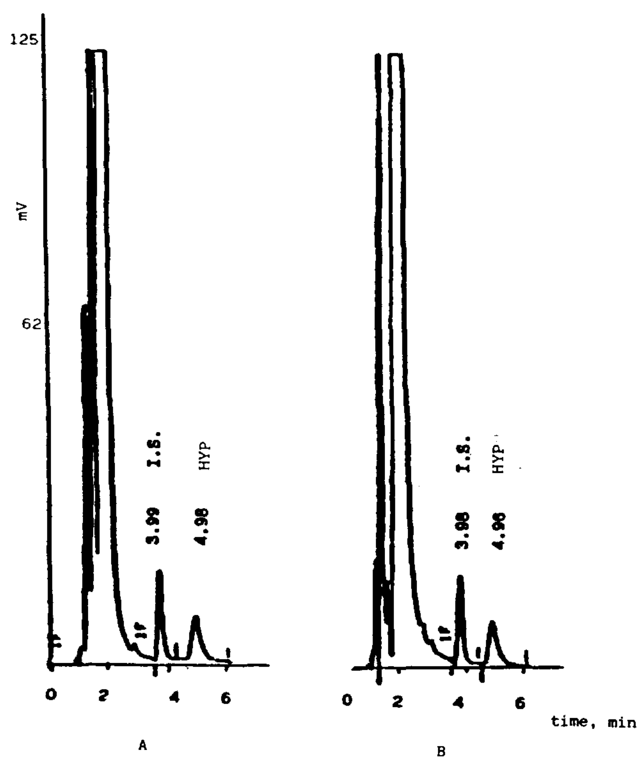


Fig. 4. Effect of detection wavelength: (A) 471 nm; (B) 436 nm.

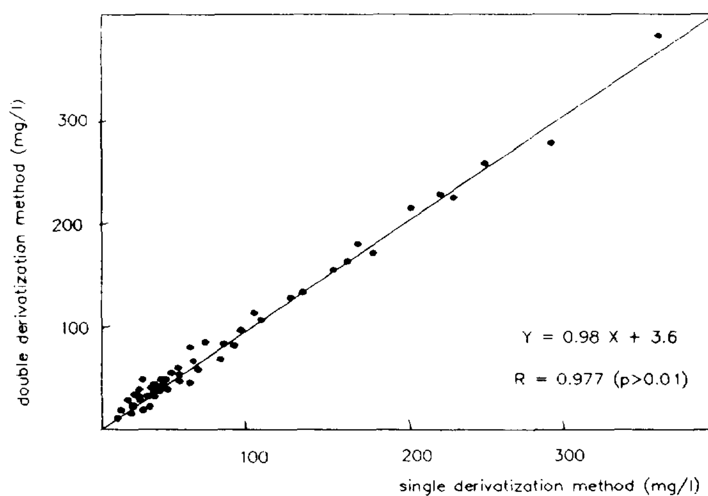


Fig. 5. Comparison between single and double derivatization methods.

mixing for 10, 20 and 30 s at room temperature (Fig. 2). The influence of temperature during the preparation of the second derivative was evaluated without agitation but by heating for 5 and 10 min at 70°C (Fig. 3). The same urine sample was analysed with detection at the two DABS-Cl absorption maxima, 471 and 436 nm (Fig. 4).

HYP concentrations determined in 160 urine samples by the present method were compared with those obtained with an analogous HPLC method, but with single derivatization (Fig. 5).

## 4. Discussion

The method presented here gives the possibility of the HPLC determination of urinary HYP in about 15 min, excluding the time for preparation of the two derivatives. This is possible because *o*-phthalaldehyde blocks the reactivity of all primary amino acids, so that HYP, which is an imino acid, is free to react with dabsyl chloride and therefore it can be determined at 471 nm without interferences. Moreover, the internal standard is not blocked by the first derivatization agent, thus allowing the use of an eluent richer in acetonitrile, i.e., with a stronger eluent and consequently a shorter retention time. The evaluation of the linearity of this method showed that even for concentrations of about 1320 mg/l (4–5 times higher than those found in the most marked pathological cases), the results differed by less than 5% from the theoretical values, probably because the reactivity of the primary amino acids was blocked by the first derivatization agent, leaving the dabsyl chloride added to the sample still available to HYP, which will be well determined even at very high concentrations. On the other hand, even at concentrations lower than those found in normal samples, the difference between the theoretical and true values is considerably lower than the clinically acceptable value (5%).

Recovery tests with increasing amounts of HYP added to the sample showed that our method allows both a good separation of HYP and high sensitivity, as variations in concentration up 5 mg/l can be seen. The precision (C.V. = 4.45% for normal control and 4.52% for the pathological control) is slightly greater than that between series (C.V. = 4.97% and 4.88%, respectively). This may be due to slight changes in the mobile phase composition.

The results obtained with our HPLC method were compared only with those in the literature obtained using a spectrophotometric methods. The accuracy differences found justify the use of the HPLC method. Both shaking and the temperature seem important in the preparation of the first derivative, because they favour a short reaction time, whereas heat seems to diminish the efficiency of the second derivatization reaction. In fact, without shaking, a peak (although very small) between the internal standard and HYP is seen, while brief vortex mixing (10–20 s) is sufficient to block primary amino acid functions totally. The comparison of the chromatograms obtained at 471 nm with those obtained at 436 nm shows that they are very similar both in peak number and in peak height. Only the solvent front is slightly modified, that at 436 nm being slightly wider.

A comparison between this method and an analogous method used until now showed a good correlation for both low and marked pathological values: $y = 0.98x + 3.6$, with $r = 0.9767$ ($p > 0.001$). Therefore, this method can be considered effective, reliable, sensitive and sufficiently rapid, despite the need for two derivatization steps and the hydrolysis of urine, which can be done overnight. Up to 20 samples can be analysed in a working day. This method seems very versatile and suitable for analysing both small and large series of samples.

## References

[1] D.J. Prockop, K.L. Kivirriko, L. Tuderman and N. Guzman, *N. Engl. J. Med.*, 301 (1979) 13.

[2] R. Smith and M. Elia, *Amino acid Analysis*, Halstead Press, New York, 1981, p. 225.

[3] W.J. Burtis and R. Lang, *Orthop. Clin. North Am.*, 15 (1984) 653.

[4] A. Lenhinger, *Biochemistry*, Zanichelli Press, Bologna, 1979, p. 180.
[5] C.D. Dawson, S. Jewell and W.J. Driskell, *Clin. Chem.*, 34 (1988) 1572.
[6] J. Podenphant, N.E. Larsen and C. Christiansen., *Clin. Chim. Acta.* 142 (1984) 145.
[7] C.A. Palmierini, C. Fini, A. Floridi, A. Morelli and A. Vedovelli, *J. Chromatogr.*, 339 (1985) 285.
[8] U. Turpeinen and U.M. Pomoell, *Clin. Chem.*, 31 (1985) 828.
[9] H. Hughes, L. Hagen and R.A.L. Sutton, *Clin. Chem.*, 32 (1986) 1002.
[10] P. Pang, K.C. Ho, G. Jones and C.K. Cheung, *J. Chromatogr.*, 386 (1987) 309.
[11] K. Nakazawa, H. Tanaka and M. Arima, *J. Chromatogr.*, 223 (1982) 313.
[12] V. Bianchi and L. Mazza, *Patol. Clin.* 928 (1991) 11.