ELSEVIER

Journal of Chromatography B, 689 (1997) 404–408

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of pyridinium crosslinks in serum An optimization of sample preparation

P. Špaček*, H. Hulejová, M. Adam

Rheumatological Institute, Na Slupi 4, 128 50 Prague 2, Czech Republic

Received 12 March 1996; revised 18 July 1996; accepted 25 July 1996

Abstract

Urinary pyridinoline (UPD) and deoxypyridinoline (UDPD) are selective markers in kinetic studies of mature collagen degradation in connective tissue, especially in bone. In patients with renal dysfunction, the determination of UPD and UDPD is not entirely reliable, while in anuretic patients it is impossible. As renal dysfunction is considered a risk factor for bone diseases, it is essential to determine both markers directly in the serum (SPD and SDPD). Due to the high serum concentrations of proteins, which during acid hydrolysis are converted to amino acid hydrochlorides, the system butanol–water is sometimes separated into two phases during sample preparation. Should this fact not be taken into account, the usual sample processing on a cellulose sorbent could yield substantially lower false results. This calls for some preventive measures: to ensure the homogeneity of the system containing *n*-butanol it is recommended to add an appropriate third component, e.g. methanol.

Keywords: Serum pyridinoline; Serum deoxypyridinoline; Pyridinium crosslinks; Collagen

1. Introduction

Bone turnover is a continuous life-long process, with osteoblasts promoting the formation of new bone collagen and osteoclasts stimulating its degradation. A disequilibrium of the two processes results in pathological bone changes. A consequence of bone mass destruction is the release into the bloodstream of certain metabolites, which are finally concentrated in the urine. Since urinary hydroxyproline as a marker of bone degradation has some disadvantages [1–3], more specific pyridinium crosslinks, pyridinoline (PD) and deoxypyridinoline (DPD), present solely in mature collagen (they stabilize its structure by the formation of covalent bonds between its chains), are being more frequently used for the monitoring of this process. These crosslinks are determined in the urine as UPD and UDPD [4–6]. The PD:DPD ratio in bone tissue is approximately 3.5:1 [7,8], while cartilage contains more PD – a more frequently occurring tissue crosslink [9,10].

One of the many factors responsible for disorders of bone collagen is chronic renal failure [11]. Various bone diseases (e.g. osteoporosis, osteosclerosis, osteomalacia etc.) may also occur during repeated long-term haemodialysis. Unfortunately, some of these patients are completely anuretic, which totally precludes the determination of both UPD and UDPD. Even in urinating patients the use of urinary values as markers of bone collagen degradation is ques-

*Corresponding author.

PII S0378-4347(96)00358-1

tionable because of substantially reduced glomerular filtration.

For all these reasons we have decided to elaborate a high-performance liquid chromatography (HPLC) method for PD and DPD determination in serum (i.e. SPD and SDPD levels), primarily in patients with impaired renal function. In healthy subjects the two crosslinks are eliminated by the kidneys and we assume that their serum concentrations are too low to serve as reliable markers of bone mass degradation. In the course of our study we have established that most complications, often resulting in false low results, occur during sample preparation. The actual HPLC is essentially identical with the HPLC for UPD and UDPD determination we have described in previous papers [12,13].

An HPLC method for the determination of SPD and SDPD in osteoarthrosis and Paget's disease was published [14], indicating substantially higher values of both markers than those found in healthy subjects. A recently published study, which used the enzyme-linked immunosorbent assay (ELISA) method for assessing bone degradation in haemodialysed patients [11], presents SPD values higher by several orders of magnitude than the values found in healthy individuals.

2. Experimental

2.1. Chemicals

We received pure PD and DPD from Prof. Robins, enabling us to compare the purity of our own standards and to verify their identity. We have used the following chemicals: HCl, acetic acid, *n*-butanol, sodium sulphate, sodium hydroxide and methanol (p.a. grade, Lachenma, Brno, Czech Republic). Microgranular cellulose CC31 was purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2. Sample preparation

In principle, 3.0 ml serum are transformed to 2.0 ml of its hydrolysate, and of this 1.0 ml is used for preliminary fractionation on cellulose-packed columns. Two approaches are possible: (1) the serum is first evaporated to dryness in a rotary vacuum evaporator (SeedVac, Philadelphia, PA, USA) and the evaporation residue is then hydrolysed in 2.0 ml 6 *M* HCl (110°C, 16 h, N_2 atmosphere), or (2) the serum is first hydrolysed after mixing with an equal volume of concentrated HCl (35%), the hydrolysate is evaporated to dryness and the residue is reconstituted in 2.0 ml deionized water. In both instances 1.0 ml of the resulting hydrolysate is used for preliminary fractionation on cellulose columns, being mixed with 5.0 ml *n*-butanol–acetic acid (4:1, v/v) and with e.g. 0.5 ml methanol. The resulting solution is transferred onto a refining column packed with 1.0 ml (sedimented volume) of microgranular cellulose CC31. A major part of the undesirable admixtures is removed by washing the column with 25.0 ml of *n*-butanol–acetic acid–water (4:1:1, v/v). The partially purified crosslinking elements are washed out of the cellulose with 3.0 ml deionized water and the eluate is then vacuum-evaporated. The residue is then dissolved in 200 μl of the mobile phase, and 20 μl are used for the injection loop of the HPLC column (Fig. 1).

2.3. HPLC

The HPLC determination alone of SPD and SDPD differs only slightly from the method for UPD and UDPD determination, which we have published elsewhere [12,13]. The only differences are: (a) the mobile phase is an acetate buffer (pH 3.35) containing 0.45 *M* sodium sulphate (75:25, v/v), (b) the HPLC oven temperature is 48°C, (c) the excitation

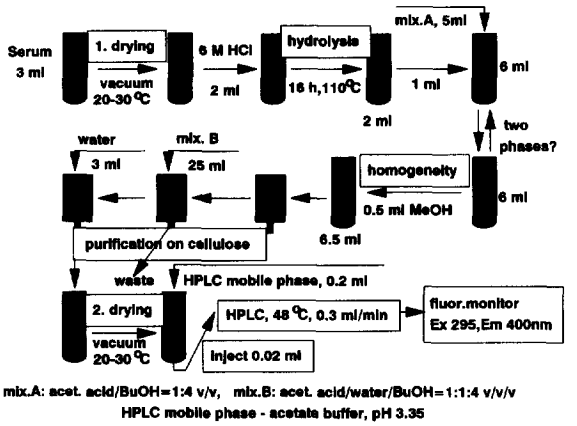

Fig. 1. Review of serum sample preparation.

and emission spectra are 297 and 400 nm respectively, (d) the sample loop volume is 20 μl and (e) the achieved detection limit is 200 fmol.

2.4. Apparatus

The HPLC liquid chromatograph SP 8100 (Spectra Physics, San Jose, CA, USA) with an autosampler (SP 8110) was equipped with a computing integrator ChromJet (SP 4400) from the same manufacturer. It was connected to a fluorescence detector Shimadzu RF 535 (Shimadzu, Kyoto, Japan).

3. Results and discussion

Serum levels of both pyridinium crosslinks were measured in a group of chronically dialysed patients and compared with relevant values found in osteoporotic subjects with functioning kidneys, whose serum values, if at all measurable, may be taken as practically normal. Although both groups tend to have significantly higher UPD and UDPD values [13], in the first group, because of impaired renal function, this is not necessarily a genuine marker of osteoresorption dynamics. Moreover, no such data are obtainable from anuretic patients.

Our preliminary results for a group of postmenopausal women suffering from osteoporosis yielded SPD values approximately two orders of magnitude lower than the corresponding UPD values. To allow the use of this method for the determination of serum concentrations of the two crosslinks, we have to substantially enhance its sensitivity, e.g. either by using a large volume of the analysed serum or by improving performance e.g. by increasing the number of theoretical plates of the column. For reasons of economy we have decided to work with a greater HPLC sample injected and a larger serum volume treated. Unfortunately, this procedure is burdened by higher concentrations of undesirable admixtures, i.e. amino acid hydrochlorides, resulting in the acid hydrolysis of the serum sample.

Because of the greater serum volume, the refining process on cellulose is more time- and solvent-consuming. In the case of a thermodynamic equilibrium, the system water–*n*-butanol is, at 20°C, miscible to a limited extent, the aqueous layer containing 6.5% *n*-butanol and the butanol layer containing 19.8% water [15]. The tendency towards demixing is, in the case of the serum, most probably supported by the already mentioned high content of amino-acid hydrochlorides. In some cases this may cause a more or less obvious separation of the system into two phases. Hydrochlorides of the pyridinium crosslinks (they too are amino acids) are, as quaternary salts, highly polar. During separation on cellulose columns they tend to concentrate in the aqueous phase and this may result in substantial loss of these compounds, although in some instances the volume of the separated aqueous phase is barely visible. Fig. 2 shows what happens when this fact is disregarded; however, in this case the two-phase separation was clearly visible.

The entire system has to be homogenized by the addition of another appropriate solvent well mixing with both water and *n*-butanol, e.g. methanol. We have to verify, however, whether this system with a modified polarity will not influence the irreversible sorption of the crosslinks on cellulose and thus result in losses during their determination. Fig. 3 demonstrates that an addition of almost 50% methanol has practically no effect on SPD determination. In this instance the scale of methanol addition is intentionally greater than in Fig. 4.

Fig. 4 demonstrates that the drying of the hydrolysate (first three anterior columns) results in

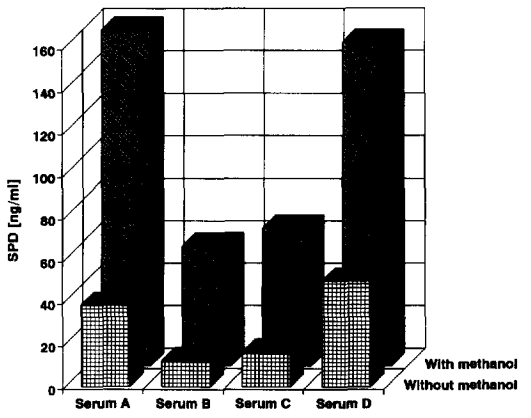

Fig. 2. Losses in pyridinoline determination, due to inappropriate processing, especially when the separation of the system into two phases (see Section 2) is disregarded.

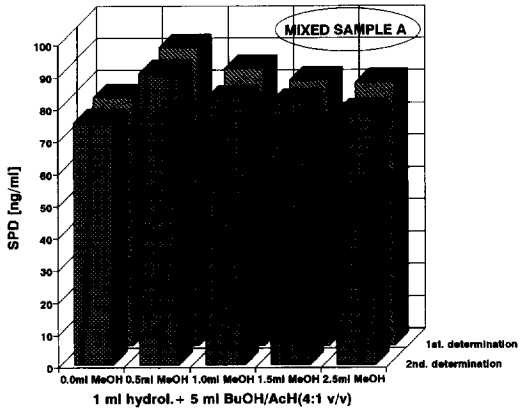

Fig. 3. Effect of methanol addition on the irreversible sorption of crosslinking elements during their refining on cellulose (see Section 2).

lower SPD values (compare first three posterior columns), probably due to the chemical disintegration under high temperatures and long drying period. In this figure we can also see the losses due to the absence of methanol (last three columns). It should be emphasized that compared with Fig. 2 the values in Fig. 3 and Fig. 4 concern samples in which the phase separation was barely visible and, consequently, the addition of methanol had relatively little impact. For methodological reasons we have used in Fig. 3 and Fig. 4 a "mixed sample" obtained by pooling individual sera.

Fig. 5 shows higher mean SPD values in urinating and anuretic dialysed patients as compared with

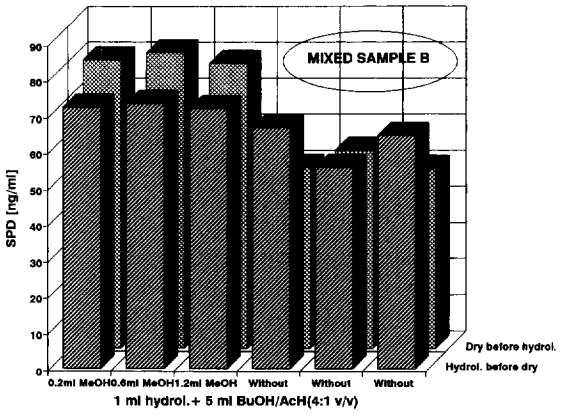

Fig. 4. Effect of the sequence of serum sample drying and hydrolysis on the results of pyridinoline determination.

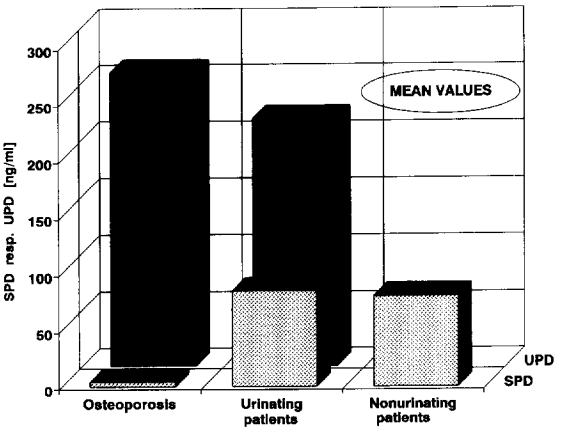

Fig. 5. Mean values of serum and urinary pyridinoline in patients with a renal disease (both urinating and anuretic patients) and osteoporosis.

patients with osteoporosis and normal renal functions (anterior columns). As an approximation we may say that 80 ng/ml correspond to 200 pmol/ml. The SPD values, as compared with UPD values (posterior columns), in osteoporotic patients are approaching the sensitivity limit (approx. 200 fmol) of the method.

The purpose of our study was to adapt our HPLC method for UPD determination and to optimize the sample preparation so as to ensure that the method will provide, with sufficient accuracy and sensitivity, relevant SPD values. Briefly: 3 ml of serum are evaporated to dryness; the evaporated residue is hydrolysed for 16 h at 110°C in 2 ml 6 *M* HCl; after filtration 1 ml of the hydrolysate is mixed with 5 ml *n*-butanol–acetic acid (4:1, v/v) and with 0.5 ml methanol to prevent possible phase separation. The mixture is fed into the cellulose column, washed with 25 ml *n*-butanol–acetic acid–water (4:1:1, v/v), the sorbed crosslinks are washed out with 3 ml water, dried and reconstituted in 200 μl of the mobile phase and 20 μl are injected into the HPLC column.

All results from this study are in ng/ml serum. It will most probably be necessary to establish to what extent the higher serum levels of the pyridinium crosslinks are due to a deteriorated renal function and what is the effect of a possible bone disease on these values. Changes in renal filtration are reflected in higher serum creatinine values. When assessing

the intensity of collagen metabolism in patients with renal insufficiency, it would probably be appropriate to relate the SPD values to serum creatinine concentrations, thus to some extent ruling out the impact of renal function. This step can, however, be fully accurate only when both the pyridinium crosslinks and creatinine are eliminated by the kidneys in exactly the same way, i.e. when their clearance is identical. This remains to be verified. At worst, we have to consider these values as only approximative. We hope to investigate this problem in our future work.

Acknowledgments

The authors wish to thank J. Vachtenheim, MD, who was instrumental in obtaining the collection of sera and Ms K. Čadová for her invaluable technical assistance.

References

[1] P. Bettica, L. Moro, S.P. Robins, A.K. Taylor, J. Talbot, F.R. Singer and D.J. Baylink, Clin. Chem., 38 (1992) 2313.
[2] P. Arbault, E. Gineyts, M. Grimaux, P. Sequin and P.D. Delmas, J. Liq. Chromatogr., 17 (1994) 1981.
[3] P.D. Delmas, Am. J. Med., 91 (1991) 59.
[4] S.P. Robins, D. Black, C.R. Paterson, D.M. Reid, A. Duncan and M.J. Siebel, Eur. J. Clin. Invest., 21 (1991) 310.
[5] A. Colwell, R.G.G. Russell and R. Eastell, Eur. J. Clin. Invest., 23 (1993) 341.
[6] S. Kamel, M. Brazier, V. Neri, C. Picard, L. Samson, G. Desmed and J.L. Sebert, J. Bone Miner. Res., 10 (1995) 1385.
[7] L.J. Beardsworth, D.R. Eyre and I.R. Dickson, J. Bone Miner. Res., 5 (1990) 671.
[8] P.J. Stone, J.H. Korn, H. North, E.V. Lally, L.C. Miller, L.B. Tucker, S. Strongwater, G.L. Snider and C. Franzblau, Arthritis Rheum., 38 (1995) 517.
[9] D.R. Eyre, T.J. Koob and K.P. Van Ness, Anal. Biochem., 137 (1984) 380.
[10] D.R. Eyre, Methods Enzymol., 144 (1987) 115.
[11] P. Urena, A. Ferreira, V.T. Kung, C. Morieux, P. Simon, K.S. Ang, J.C. Souberbielle, G.V. Segre, T.B. Drueke and M.C. Vernejoul, J. Bone Miner. Res., 10 (1995) 932.
[12] A. Lichý, J. Macek and M. Adam, J. Chromatogr., 563 (1991) 153.
[13] P. Špaček, H. Hulejová and M. Adam, Klin. Biochem. Metab., 2 (1994) 239.
[14] I. James, C. Crowley and D. Perret, J. Chromatogr., 612 (1993) 41.
[15] J. D'Ans, in E. Lax (Editor), Taschenbuch für Chemiker und Physiker, Springer Verlag, Berlin, 1943, p. 959.