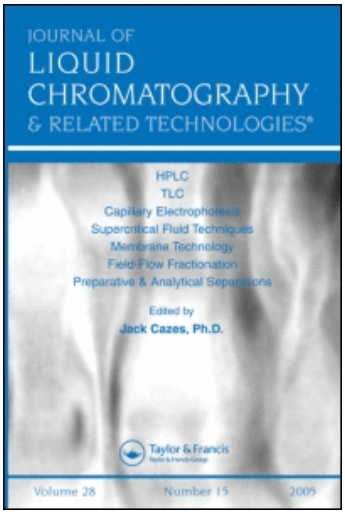

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P. Arbault[a]; E. Gineyts[b]; M. Grimaux[a]; P. Seguin[a]; P. D. Delmas[b]
[a] Cis Bio International, Bagnols sur Cèze, Cedex, France [b] INSERM Unité 234, Hôpital E. Herriot, Lyon, Cedex, France

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A RAPID PROCEDURE FOR PURIFYING LARGE AMOUNTS OF PYRIDINOLINE CROSSLINKS OF BONE

P. ARBAULT[1]*, E. GINEYTS[2], M. GRIMAUX[1],
P. SEGUIN[1], AND P. D. DELMAS[2]
[1]*Cis Bio International*
BP 175
30203 Bagnols sur Cèze Cedex, France
[2]*INSERM Unité 234*
Hôpital E. Herriot
69437 Lyon Cedex 03, France

ABSTRACT

HPLC assessment of urinary Pyridinoline (Pyr) and Deoxypyridinoline (Dpyr) requires the use of large amounts of purified Pyr and Dpyr as external standards. We have developped a procedure for large-scale pyridinoline (Pyr and Dpyr) purification from sheep bone combining successively gel filtration, partition chromatography and semi-preparative HPLC. After bone powder (500 g) hydrolysis in 6N HCl (5 liters), the concentrated hydrolysate (600 ml) was separated by gel filtration on a Biogel P2 column (2.4 liters), allowing the elimination of 95% of impurities and the reduction of the pyridinolines solution to 150 ml. Then partition chromatography was carried out on CF1 cellulose where non-polar contaminants were suppressed. Finally, drawing on analytical HPLC knowledge, an isocratic semi-preparative HPLC was developed using a reversed phase C_{18} column (250 mm x 10 mm) with HFBA as the ion pairing agent. The last impurities were thus eliminated, and the Pyr was separated from the Dpyr. By this sequence of processes, 15 mg of pyridinoline and 1.8 mg of deoxypyridinoline were purified. This optimized procedure allows the large-scale production of Pyr and Dpyr from large amounts of bone or other tissue in a relatively short time, and requires only conventional biochemical reagents.

* *Author to whom correspondence should be addressed.*

INTRODUCTION

In recent years, new markers have been developed for the diagnosis and management of metabolic bone diseases. Bone formation can be evaluated by serum measurement of osteocalcin (1,2) or alkaline phosphatase (2). Bone resorption was first assessed through the determination of urinary hydroxyproline whose level increases in cases of bone degradation, such as in osteoporosis or Paget's disease (3). However, this marker cannot be considered as bone-specific since it may be influenced by gelatin in the diet, by extra-osseous collagen synthesis, and by complement activation (4,5). This lack of specificity makes hydroxyproline a poorly sensitive marker for monitoring bone resorption. Since the identification of pyridinoline (Pyr) by Fujimoto (6,7) and of deoxypyridinoline (Dpyr) by Ogawa (8), several authors have investigated the interest of these collagen pyridinium crosslinks as markers for metabolic bone disease.

Deoxypyridinoline is not specific for type I collagen of bone, but it is found in large amounts in bone only. Pyridinoline is present in large concentrations in tissues like bone (type I collagen), cartilage (type II collagen), and in several other connective tissues such as aorta and tendon (9,10). In adult human bone, the molar ratio of Pyr to Dpyr is about 3.5 : 1. In urine, the Pyr/Dpyr ratio is usually similar to that found in bone, indicating that urinary pyridinolines seem essentially to originate from bone collagen breakdown (11). First clinical results demonstrated that urinary Pyr and Dpyr increase occured in many diseases such as Paget's disease (12,13), osteoporosis (14,15), hyperparathyroidism (12,13,16), or cancers with bone metastasis (17).

Urinary Pyr and Dpyr are currently quantified, after acid hydrolysis and cellulose separation, by reversed phase high performance liquid chromatography (HPLC), where both crosslinks are detected by their natural fluorescence (9,10,12-19). For Pyr and Dpyr measurements in tissues, Eyre (9,10) and Black (18) developed HPLC methods with internal standards, respectively pyridoxamine and pyridoxine. But for urinary assays all the authors work with Pyr and Dpyr as external standards (12-19) which require large amounts of both molecules.

This paper describes a procedure for large-scale purification of pyridinoline and deoxypyridinoline, involving three steps with successively gel filtration, partition chromatography and semi-preparative HPLC.

MATERIALS AND METHODS

Bone powder preparation

Metacarpal bone was collected from a nine-year old sheep. Bone (500 g) was frozen in liquid nitrogen, broken into one centimeter square pieces with a hammer and crushed into a fine powder with a Retsch mill (Bioblock, Illkirch, France), under liquid nitrogen. Lipids were eliminated by washing the powder several times with acetone and water. Demineralization was carried out by stirring the powder in a 0.5 M EDTA solution pH 7.4 (five liters) during 24 hours. This solution was centrifuged and the pellet was pooled and dried.

Bone powder hydrolysis

Envisaging large-scale hydrolysis, we adjusted the bone powder concentration in the hydrolysis solution and measured the pyridinoline release: one to ten grams of bone powder were mixed with 100 ml of 6N HCl solution, and hydrolysis was performed in a glass bottle at 110 °C under agitation for 24 hours. After hydrolysis, one milliliter samples were taken and analyzed for their free pyridinoline (Pyr and Dpyr) contents by HPLC after analytical cellulose purification (described hereafter).

Large scale production: five hundred grams of bone powder in five liters of 6N HCl solution were heated at 110 °C under reflux during 24 hours. The hydrolysate was concentrated under reduced pressure and by heating at 45°C, to 600 ml of solution.

Gel filtration

A first analytical run was carried out to monitor the pyridinoline elution: 50 ml of concentrated hydrolysate were injected into a K 50/30 column (Pharmacia, St Quentin en Yvelines, France),containing 400 ml of Biogel P2 superfine gel (400

mesh) (Biorad, Paris, France). The elution was performed with a 250 ml/hour flow rate using ultraviolet (UV) detection at 280 nm. Twelve milliliter fractions were collected and analyzed by analytical HPLC to measure their pyridinoline concentration.

Large-scale process: about 300 ml of hydrolysate were processed in a BP 113 column (Pharmacia) containing 2.4 liters of Biogel P2 fine gel (200-400 mesh). Elution was carried out at a 900 ml/hour flow rate and followed up by UV detection at 280 nm. The 600 ml of hydrolysate was purified in two runs.

Pyridinoline fractions were pooled and concentrated under reduced pressure by heating at 45°C, to 150 ml of solution.

Partition chromatography

For these steps, a CF1 cellulose slurry was prepared by mixing 1000 ml of 1-butanol, 250 ml of acetic acid, 250 ml of water (4:1:1 solvent mixture), and 100 grams of Whatman CF1 cellulose powder (Touzart et Matignon, Vitry sur Seine, France).

Analytical cellulose column: successively, five hundred microliter fractions of hydrolysate, gel filtration solutions, or of cellulose solutions were mixed with 500 µl of acetic acid and 2 ml of 1-butanol. They were processed in a ten milliliter polypropylene column (Bioblock) containing 5 ml of cellulose slurry. After three washes with 5 ml of 4:1:1 solvent mixture, the pyridinoline fraction was collected with 2 ml of water. The residual 1-butanol fraction was eliminated by pipetting, and the water fraction was removed by evaporation using a Savant speed-vac apparatus (Bioblock).

Large scale cellulose: one hundred and fifty milliliters of pyridinoline solution were mixed together with 450 ml of CF1 cellulose slurry, 600 ml of 1-butanol, and 150 ml of acetic acid in a polypropylene bottle, and left for three hours under shaking. The slurry was passed through a G2 filter, then the recovered cellulose was suspended in 600 ml of 4:1:1 solvent mixture, left under shaking for 30 min, and refiltered on a G2 filter. After repeating this washing step three times, the

cellulose was suspended in 400 ml of water to release pyridinolines, left thirty minutes under shaking, and passed through a G2 filter after which the solution was collected for pyridinoline analysis by HPLC. This release step was repeated once more and the pyridinoline fractions were pooled and their water content evaporated. Filtrate from each step was analyzed by HPLC for pyridinoline yield assessment.

HPLC

Analytical HPLC: pyridinoline and deoxypyridinoline were assayed by the HPLC method, as previously described by Uebelhart (1990)(12). The HPLC system included LKB 2150 pumps and LKB 2152 LC controller (Pharmacia). The reversed-phase column was a Lichrospher 100 RP 18 endcapped, 5 µm beads diameter, 125 mm x 4 mm column (Merck, Nogent sur Marne, France). The detector was a LS II Filter Fluorimeter (Perkin-Elmer, St Quentin en Yvelines, France) with a 300 nm excitation filter and a 395 nm emission monochromator. The samples were diluted in a 1% heptafluorobutyric acid (HFBA) (Sigma, La Verpilliere, France) solution and eluted isocratically with a 15% acetonitrile - 85% water -10 mM HFBA solution, at a flow rate of 1 ml/min. Purified human Pyr and Dpyr prepared and calibrated as previously described (12) were used as external standards.

Semi-preparative HPLC: the preparative HPLC system consisted of two Shimadzu LC8A pumps (Touzart et Matignon), a SCL6B system controller (Touzart et Matignon) and a LKB 2510 Uvicord SD detector (Pharmacia). The semi-preparative HPLC conditions were previously optimized using a Lichrospher RP 18 endcapped 250 mm x 4 mm column (Merck).

The dried pyridinoline fraction was dissolved in 15 ml of a 20% HFBA solution. Pyridinoline and deoxypyridinoline were separated from final endogenous contaminants by reversed-phase HPLC on a Lichrospher RP 18 endcapped, 5µm bead diameter, 250 mm x 10 mm column (Merck). The separation was performed isocratically at a 5 ml/min flow rate, using a solvent mixture of 15% acetonitrile - 85 % water 10mM HFBA. After 25 min, the acetonitrile gradient was brought up to 100% in 10 min to wash the column. The elution was monitored with a 280 nm

UV detector. Finally, Pyr and Dpyr were quantified using the analytical HPLC procedure and their respective spectral characteristics were controled by excitation and emission spectra with an LS 50 luminescence spectrometer (Perkin Elmer).

Statistical analysis

Results are expressed as mean ± 1 standard deviation (SD). Student's t test was used for comparison of two means.

RESULTS AND DISCUSSION

In his report, D. Eyre (9) described the purification of Pyr from 40 g of cartilage which had been hydrolyzed in 2 liters of 3N HCl. Using the same experimental conditions, 25 l of 6N HCl solution would have been necessary to carry out the hydrolysis of 500 g of bone powder. Our investigations demonstrate that the hydrolysis can be performed at a concentration of 100 g of bone/l with a good pyridinoline release yield compared to classical conditions at 10 g/l concentration (see table n° 1). The hydrolysis solution volume can thus be reduced to 5 liters for 500 g of bone.

After this step, the hydrolyzate contained a mixture made of amino acids and mineral salts. Prior to the CF1 cellulose step, a gel filtration on Biogel P2 was added to eliminate the smallest molecular weight particles and to reduce volume before the cellulose step. The Biogel P2 chromatograms (see figure n° 1) were similar for the large scale application and the Biogel P2 development, showing a good extrapolation of this step. Pyridinolines were eluted from the first fractions and most of the smallest molecules could be eliminated. At 280 nm, the peak area calculation showed that 95% of impurities were eliminated. In the large scale production, the volume was reduced to 150 ml. Thus the cellulose purification could be performed with small 1-butanol volume and container, which would allow manipulation under a classical laboratory hood and so avoid inhalation of 1-butanol, a toxic solvent. Although the volume was reduced to 150 ml, the final HPLC step could not take place with so much solution. Therefore, a CF1 cellulose step had to be performed to eliminate other contaminants and to reduce volume.

TABLE 1

Hydrolysis Solution: Influence of Bone Powder Concentration on the Amount of Pyridinoline Released.

Hydrolysis conditions	*n*	mg of released Pyr for 100g of hydrolyzed bone (Mean ± 1 SD)
1g of bone powder /100 ml HCl 6N	4	4.58 ± 0.59
10g of bone powder /100ml HCl 6N	4	5.39 ± 0.76[a]

a: non significant difference.

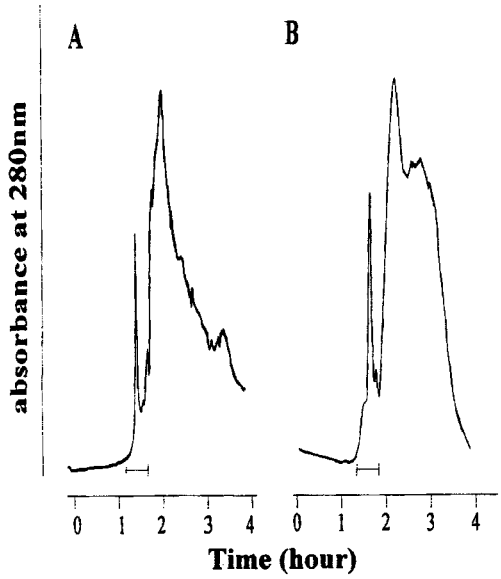

FIGURE 1. Typical chromatograms of gel filtration step. **A**: Biogel P2 development. **B**: large-scale Biogel P2. Bars indicate fractions containing pyridinolines.

TABLE 2

Cellulose Purification: Amount of Pyridinoline Present in each Solution (Fixation, Washes, Release).

The Pyr amount was assessed after each step of this purification. HPLC measurements were performed in the aqueous fractions of the 4:1:1 solutions after fixation, washing, and in the purified pyridinoline solution.

	Aqueous fraction recovered in the 4:1:1 solution after fixation	Aqueous fraction recovered in the 4:1:1 solution after washes	Aqueous fraction containing purified pyridinolines
Pyridinoline	10.6%	11.0%	78.4%

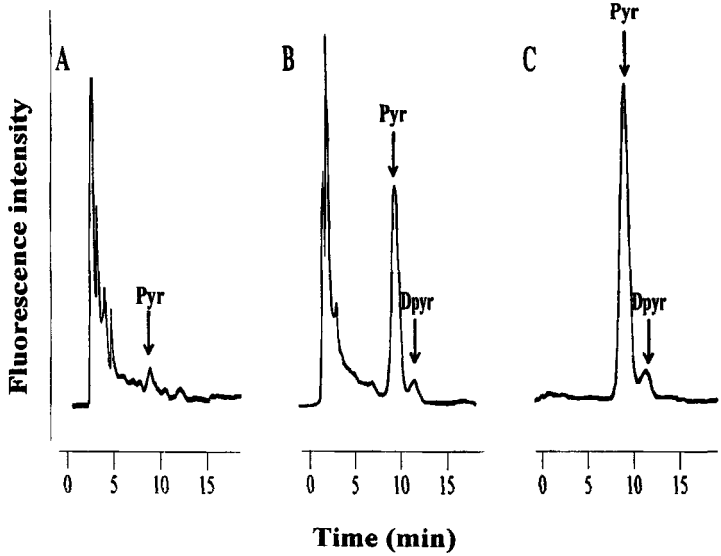

FIGURE 2. Analytical HPLC chromatograms for pyridinoline fractions after bone powder hydrolysis (**A**), gel filtration (**B**) and partition chromatography (**C**). Column: Lichrospher 100 RP 18 endcapped, beads diameter 5 µm (125 mm x 4 mm). Solvent: 85% water-15% acetonitrile 10 mM HFBA.

TABLE 3

Semi-preparative HPLC: Influence of Quantity of HFBA in the Injection Solution.

The amount of HFBA in the injection solution was increased and the pyridinoline rate in the first fractions (Elution time: 2-5 min) of the semi-preparative HPLC was monitored by analytical HPLC method.

HFBA	Pyridinoline (% of total) in the first HPLC fractions
1%	42%
3%	30%
5%	22%
10%	10%
20%	5%

Yields were studied (table n° 2) demonstrating that about 78% of Pyr was recovered after this step. Analytical HPLC before and after cellulose showed that many contaminants were eliminated (figure n° 2).

At this stage, some contaminants were still present, and pyridinoline purification needed an HPLC step. Extrapolating the conditions of the analytical method, a large-scale HPLC was performed isocratically with the same solvent. First of all, the column length was increased two-fold, in order to obtain a good resolution for the separation between Pyr, Dpyr and other products. Similarly, HFBA was adjusted in the injection solution to improve the separation of pyridinolines from the remaining impurities. Using 1% HFBA, only 50% of the expected Pyr and Dpyr were separated from the contaminants. The remaining fraction of Pyr-Dpyr was eluted simultaneously in the first fractions (elution time 2-5 min) with the usual contaminants. When the amount of HFBA was brought up

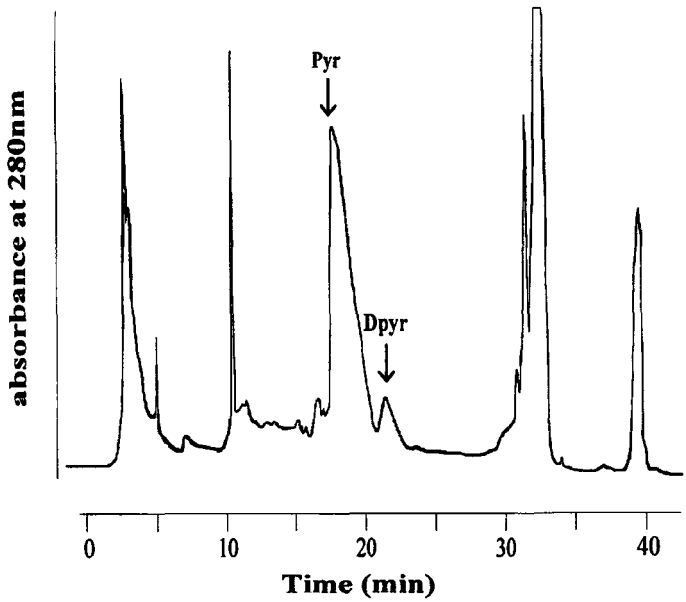

FIGURE 3. A typical chromatogram of semi-preparative HPLC for final step of pyridinoline purification. Column: Lichrospher 100 RP 18 endcapped, beads diameter 5µm (250 mm x 10 mm). Solvent A: 85% water-15% acetonitrile 10 mM HFBA. Solvent B: 100% acetonitrile. From 0 to 20 min: 100% A, then a linear gradient over 15 min from 0 to 100 % B.

from 3% to 20% (table n° 3), the Pyr-Dpyr presence in the first fractions decreased to reach 5%, which allowed a good pyridinoline recovery. It must be stressed that the amount of HFBA had to be increased either when the preparation contained a lot of impurities or when the quantity to be injected was greater. In our large-scale HPLC conditions (figure n°3), HFBA was fixed at 20%. Finally, in order to separate pyridinoline from deoxypyridinoline, the amount of HFBA in the solvent was studied. At 1.3 ml for 1 liter of 15% acetonitrile solution, Pyr and Dpyr were eluted respectively at 19.5 min and 21 min. These elution times became respectively 23 min and 26 min with 2 ml HFBA for 1 liter of solution, allowing perfect separation of the two crosslinks (see figure n° 4). The increasing amount of HFBA in the solvent mixture delayed the pyridinolines' elution by enhancing their reversed phase interactions.

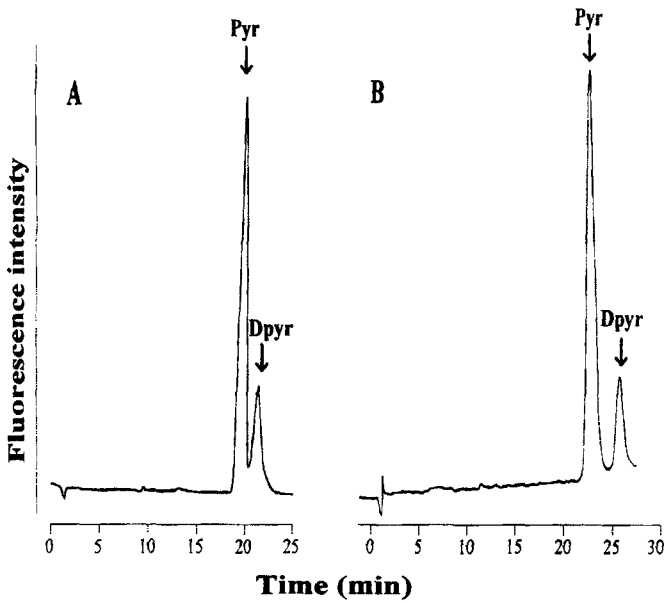

FIGURE 4. Pyridinoline (Pyr) and deoxypyridinoline (Dpyr) separation at 1.3 ml HFBA (**A**) or at 2 ml HFBA (**B**) in 15% acetonitrile solution.

Using this pyridinoline purification process, about 15 mg of pyridinoline and 1.8 mg of deoxypyridinoline were purified from 500 g of bone powder. A brief estimation after hydrolysis showed that 500 g of sheep metacarpal bone contained potentially about 25 mg of Pyr and 2.5 mg of Dpyr. Thus, using the complete process, the final purification yield was about 60% for Pyr and 72% for Dpyr. Whilst, pyridinoline losses were generated throughout the three-step process, due to the analytical investigations carried out to optimize the purification conditions, the CF1 cellulose was indeed shown to be crucial for pyridinoline recovery: twenty two percent of total Pyr appeared to be in the 4:1:1 solution of fixation and washing steps (see table n° 2). These pyridinolines could be recovered by allowing the solution to decant at room temperature overnight. The aqueous phase is collected, evaporated, and can be dissolved in water for a new run in the CF1 cellulose step. Deoxypyridinoline represents about 10% of total Pyr-Dpyr in sheep bone. However, larger amounts could be obtained using human or chicken bone,

which contain about 25% and 50% of Dpyr respectively (9). In conclusion, a large scale pyridinoline production was developed by using three different purification steps in sequence. This process allows the purification of large quantities of pyridinoline and deoxypyridinoline from all types of tissues in a relatively short time.

REFERENCES

1 J. P. Brown, P. D. Delmas, L. Malaval, C. Edouard, M. C. Chapuy, P. J. M. Meunier, Lancet, 1: 1091-1093 (1984).

2 P. D. Delmas, Am. J. Med., 91(supl 5B): 59S-63S (1991).

3 A. C. Deacon, P. Hulme, R. Hesp, J. R. Green, M. Tellez, J. Reeve, Clin. Chim. Acta, 166: 297-306 (1987).

4 P. H. Weiss, L. Klein, J. Clin. Invest., 48: 1-5 (1969).

5 K. I. Kivirikko, Int. Rev. Connect. Tissue Res., 5: 93-163 (1970).

6 D. Fujimoto, Biochem. Biophys. Res. Commun., 76: 1124-1129 (1977).

7 D. Fujimoto, T. Moriguchi, T. Ishida, H. Hayashi, Biochem. Biophys. Res. Commun., 84: 52-57 (1978).

8 I. Ogawa, T. Ono, M. Tsuda, Y. Kawamishi, Biochem. Biophys. Res. Commun., 107: 1252-1257 (1982).

9 D. R. Eyre, "Collagen crosslinking amino-acids" in Methods in enzymology, L. W. Cunningham, ed., Academic Press Inc., New York, 1987, 144: 115-139.

10 D. R. Eyre, T. J. Koob, K. P. Van Ness, Anal. Biochem., 137: 380-388(1984).

11 Z. Gunja-Smith, R. J. Boucek, Biochem. J., 197: 759-762 (1981).

12 D. Uebelhart, E. Gineyts, M. C. Chapuy, P. D. Delmas, Bone Miner., 8: 87-96 (1990).

13 S. P. Robins, D. Black, C. R. Paterson, O. M. Reid, A. Duncan, M. J. Seibel, Eur. J. Clin. Invest., 21: 310-315 (1991).

14 D. Uebelhart, A. Schlemmer, J. S. Johansen, E. Gineyts, C. Christiansen, P. D. Delmas, J. Clin. Endocrinol. Metab., 72: 367-373 (1991).

15 P. D. Delmas, A. Schlemmer, E. Gineyts, B. Riis, C. Christiansen, J. Bone Miner. Res., 6: 639-644 (1991).

16 M. J. Seibel, F. Gartenberg, S. J. Silverberg, A. Ratcliffe, S. P. Robins, J.P. Bilezikian, J. Clin. Endocrinol. Metab., 74: 481-486 (1992).

17 C. R. Paterson, S. P. Robins, J. M. Horobin, P. E. Preece, A. Cushieri, Br. J. Cancer, 64: 884-886 (1991).

18 D. Black, A. Duncan, S. P. Robins, Anal. Biochem., 169: 197-203 (1988)

19 I. T. James, D. Perret, P. W. Thompson, J. Chromatogr., 525: 43-57 (1990).

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