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Analysis of human parathyroid hormone (1–84) products
Separation of a major impurity in synthetic products by ion-pairing reversed-phase high-performance liquid chromatography

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Abstract

Human parathyroid hormone (1–84) is a naturally occurring polypeptide that acts as the major regulator of calcium ion homeostasis. It can be efficiently produced through both synthetic and biosynthetic routes and, as such, highly selective analytical methods are required for the detection of a wide range of impurities. Herein we report on the development of an ion-pairing reversed-phase HPLC method for the analysis of human parathyroid hormone and the separation of impurities including a major, unidentified impurity detected in synthetic preparations. This impurity could not be resolved using trifluoroacetic acid-based methods generally used for monitoring purity levels in commercial products. Separation conditions consisted of a gradient elution of 0.155 *M* sodium chloride containing 0.037 *M* sodium pentanesulfonate, pH 5.6, as mobile phase A and acetonitrile as mobile phase B. Separations were carried out on an octadecylsilyl silica column maintained at 50°C. Both column temperature and pH of mobile phase A significantly affected the separation of the major impurity. The major impurity eluted after the main human parathyroid peak and was detected in the two commercial synthetic products analyzed. Several minor impurities eluting before and after the main peak were also detected. Purity levels measured by the developed HPLC method (method C) were similar to those previously measured by capillary electrophoresis. Analysis of purified recombinant human parathyroid hormone did not show the presence of this impurity. This method offers a significant advantage for the purity assessment of human parathyroid hormone. Crown Copyright © 2000 Published by Elsevier Science B.V.

Keywords: Parathyroid hormone; Hormones; Peptides

1. Introduction

Human parathyroid hormone (1–84) (hPTH) is a naturally occurring polypeptide composed of 84 amino acids [1], with overall basic properties (isoelectric point, pI>9). It has important biological activity as the major regulator of calcium ion homeostasis [2] and is currently under study in human clinical trials. Efficient production methods have been reported through solution [3] and solid-phase [4] peptide synthesis as well as through recombinant DNA techniques [5]. Although synthetic hPTH has been shown to have biological activity similar to that of recombinant hPTH, larger doses (~29%) of the synthetic product were required to obtain comparable levels of activity [6].

Several hPTH-related variants have been iden-

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tified. In a recent study, an unidentified major impurity (>20%), detected by capillary zone electrophoresis (CZE), has been reported in commercial synthetic products [7]. Oxidized and deamidated forms have also been reported in synthetic and biosynthetic products and shown to have decreased biological activity [8–10]. Because of the potential presence of such a wide range of impurities highly selective analytical methods are required to adequately assess the purity of hPTH preparations.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is one of the more versatile chromatographic modes for the study of proteins and their impurities, owing in part to advances made in microparticulate stationary phases and to the development of a wide range of columns of differing selectivities [11]. The separation of moderately to highly basic proteins ($pI>9$) on reversed-phase silica-based columns requires particular attention since such supports are most stable at acidic pH ($2<pH<6$). Under such acidic conditions most of the protein amino groups are protonated, conferring upon it a high ionic character that reduces its retention with the hydrophobic column support. Thus, separations are frequently carried out by addition of an ion-pairing agent to the mobile phase in order to provide sufficient interactions between the substrate and the chromatographic support [12]. This process also prevents excessive protein adsorption onto the column. In this study, we report on the development of an ion-pairing RP-HPLC method using sodium pentanesulfonate (PSA) as ion-pairing agent for the purity analysis of hPTH preparations.

2. Experimental

2.1. Materials

Synthetic hPTH (1–84) was purchased from Star Biochemicals (Torrance, CA, USA), lot P34890, and Bachem Bioscience (King of Prussia, PA, USA), lot ZN760. Reference to synthetic products A or B in the text does not correspond to the order presented here and is intended to preserve anonymity. Biosynthetic hPTH was generously supplied by Dr. Gordon Willick (Institute of Biological Sciences, National Research Council of Canada, Ottawa, Canada). All reagents, buffers and solvents were HPLC or molecular biology grade. RP-HPLC columns were Vydac C_{18}, 218TP54, 250×4.6 mm I.D. purchased from Chromatographic Specialities (Brockville, Canada).

2.2. HPLC methods

HPLC separations were performed on a SpectraSystem (Thermo Separation Products, Mississauga, Canada) consisting of a P4000 quaternary pump system, an AS 3000 autosampler and a UV6000LP diode array detector. Mobile phases were filtered through 0.45-μm membranes (Millipore). Data were collected between 200 and 360 nm and chromatograms were recorded at 230 nm. Samples were dissolved in water to give a concentration of approximately 1 mg/ml and 20-μl aliquots were injected unless otherwise indicated.

2.2.1. Method A

HPLC method A was adapted from the conditions described in the certificate of analysis supplied by Star Biochemicals for the purity evaluation of hPTH. Mobile phase A was 0.1% trifluoroacetic acid (TFA) in 5% aqueous acetonitrile and mobile phase B was 0.1% TFA in 80% aqueous acetonitrile. The system was equilibrated with a mixture A–B (68:32) until a stable baseline was obtained. Separations were carried out using a gradient of 32% to 52% mobile phase B over a period of 20 min at a flow-rate of 1.5 ml/min. The chromatographic column was maintained at 20°C.

2.2.2. Method B

HPLC method B was derived from conditions used in method A. Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in 60% aqueous acetonitrile. The system was equilibrated with a mixture A–B (70:30) until a stable baseline was obtained. Separations were performed using a stepwise gradient in the following manner: from 30% to 50% mobile phase B over a period of 29 min, then from 50% to 60% mobile phase B over a period of 40 min. The flow-rate was 1.0 ml/min. The chromatographic column was maintained at 50°C.

2.2.3. Method C

HPLC method C corresponds to the optimized separation conditions described in this article.

Mobile phase A consisted of an aqueous solution of 0.155 *M* sodium chloride containing 0.037 *M* sodium pentanesulfonate, adjusted to pH 5.6 with dilute HCl or NaOH, and mobile phase B was acetonitrile. The system was equilibrated with a mixture A–B (70:30) until a stable baseline was obtained. Separations were conducted using a linear gradient from 30% to 40% mobile phase B over a period of 30 min at a flow-rate of 1.0 ml/min. The chromatographic column was maintained at 50°C. Column reconditioning was performed by washing the column with 0.1 *M* nitric acid–isopropanol (1:4) for a minimum of 4 h followed by column re-equilibration using initial separation conditions. Experimental details relating to specific conditions used during method development are presented in the text and figure legends.

2.3. Capillary zone electrophoresis

CZE separations were carried out using the conditions described previously [7].

3. Results and discussion

The purity of hPTH, and particularly that of synthetic preparations, is usually monitored by RP-HPLC with methods using TFA–acetonitrile-based mobile phases such as those reported by Goud et al. [4] and Bennett et al. [13]. A typical chromatogram of synthetic hPTH product A obtained under separation conditions similar to those used by manufacturers (method A) is presented in Fig. 1a. The separation was qualitatively and quantitatively similar to that of the manufacturer as reported in the certificate of analysis. Modification of the elution conditions as described under HPLC method B, and which included the use of a shallower gradient step and increased column temperature, resulted in the appearance of a shoulder on the downward slope of the main peak, indicating the presence of a major impurity (Fig. 1b) that co-eluted with the main peak under method A separation conditions. Further attempts at improving the separation through modifications of the TFA concentration (between 0.01–0.1 *M*) or other parameters were unsuccessful. New chromatographic conditions were required to separate the major impurity. The following discussion relates to the development of separation conditions as described under HPLC method C.

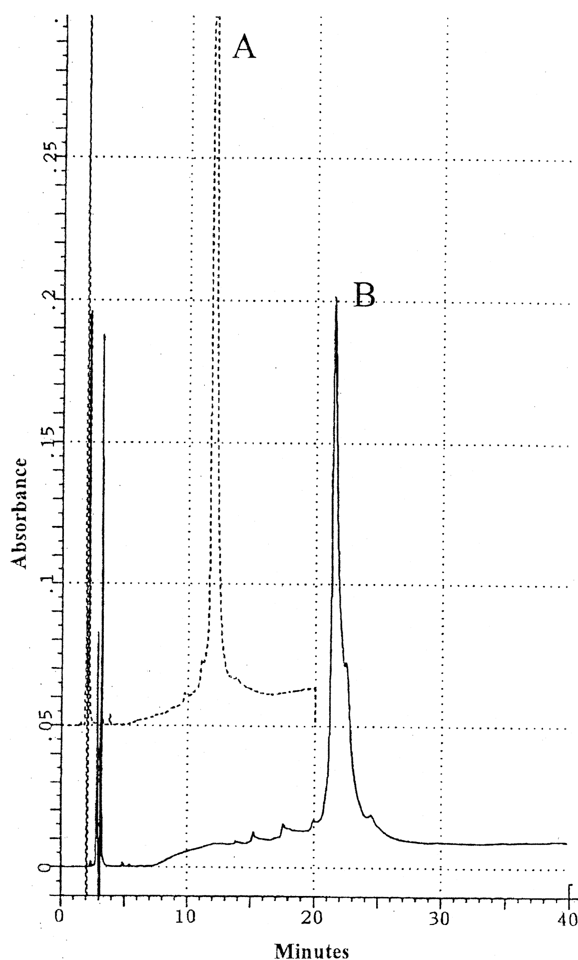

Fig. 1. Chromatograms of hPTH synthetic product A using (A) HPLC method A conditions and (B) HPLC method B conditions. Chromatograms were obtained at 220 nm.

3.1. Mobile phase salt

Previous studies have demonstrated the successful use of sodium chloride as mobile phase salt for the purification of bovine PTH [14] and hPTH [15] by RP-HPLC. We studied the elution of hPTH after

addition of sodium chloride at concentrations varying between 0.05 and 0.2 *M* to mobile phase A and using acetonitrile as mobile phase B over a shallow gradient range (30–40% B over 29 min, 0.35%/min). In all cases hPTH was eluted with adequate peak shape at retention times between 10 and 20 min. In the absence of ion-pairing agent minor impurity peaks eluting prior to the main peak were separated and shoulders on the downward slope of the main peak were visible as shown in Fig. 2A. An increase in salt concentration resulted in shorter retention times (data not shown), a situation that can be explained on the basis of the separation mechanism being partly dependent on an ion-exchange process [12]. The salt concentration giving rise to the best peak shape and the highest sensitivity was found to be 0.155 *M* sodium chloride. It has been suggested that the salt plays a crucial role in preventing excessive adsorption of the protein to the silica matrix, hence improving the sensitivity [16].

3.2. Selection of ion-pairing agent

As discussed above, although TFA has been widely used as ion-pairing agent for the analysis of hPTH and other proteins, it was found to be of little value for the separation of the major impurity. The selection of other ion-pairing agents to be studied was based on considerations of the basic character of hPTH (p*I*>9) and the pH range suitable for use with a silica-based column (2<pH<6). Thus, at acidic pH, the protein would carry considerable positive charge due to the protonation of its amino groups which would suggest that anionic ion-pairing agents would be suitable to provide efficient interactions between the substrate and the stationary phase [12]. Several ion-pairing agents were studied and while anionic species such as acetate and propionate and the cationic triethylammonium phosphate failed to resolve the impurities, alkylsulfonates proved successful. PSA was found to provide the best results in

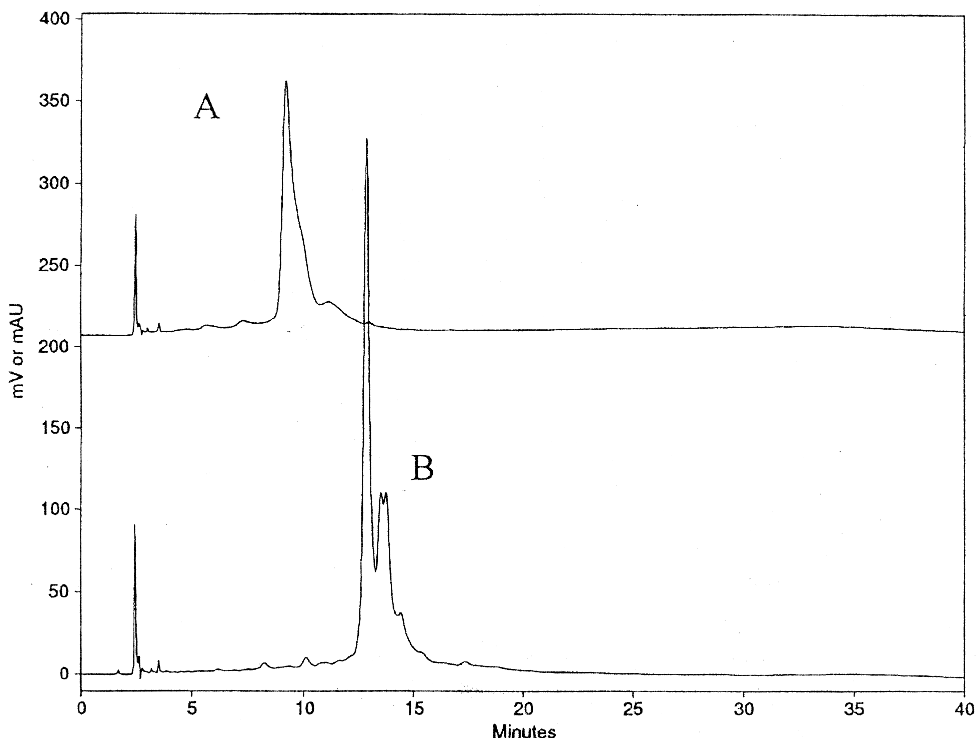

Fig. 2. Separation of hPTH synthetic product A using as mobile phase A 0.155 *M* sodium chloride (A) in the absence of ion paring agent and (B) with 0.037 *M* sodium pentanesulfonate as ion-pairing agent (method C). Gradient conditions were as described in the text.

terms of peak shape, sensitivity, retention and resolution. The addition of PSA to mobile phase A containing 0.155 *M* sodium chloride led to increased peak retention, indicating that the substrate interacted with the stationary phase through the ion-pairing agent. The optimal PSA concentration was found to be 0.037 *M* giving rise to separation of the major impurity (Fig. 2B). While no separation was observed at lower PSA concentrations, no improvement on the separation was obtained at higher concentrations.

The increased retention of the major impurity peak suggested that it was more hydrophobic than hPTH, a feature that was consistent with partial structural information showing that it was a closely-related variant containing an additional methyl group [7].

3.3. Influence of pH and column temperature

Separation by CZE had previously demonstrated that charge differences between hPTH and the major impurity were maximized at pH 5.8 [7]. During the development of HPLC method C, the pH was also found to have a major influence on the separation. At pH<4 no separation was obtained as exemplified in Fig. 3A showing a chromatogram obtained at pH 3.4, where the major impurity appears as shoulder. Increased resolution was obtained upon increasing the pH of mobile phase A and optimal resolution was attained at pH 5.6, as demonstrated in Fig. 2B under optimal conditions. This situation is readily explained in terms of the increased charge difference between the two species at pH>4 resulting in increased retention of the major impurity. At pH<4, such closely related basic compounds are likely fully ionized and bear very similar overall charges. By increasing the pH, charge differences are maximized due to differences in their respective pK_a and lead to dissimilar interactions with the ion-pairing agent and the chromatographic support.

The column temperature was also found to have a marked influence on the separation. Fig. 3B shows a

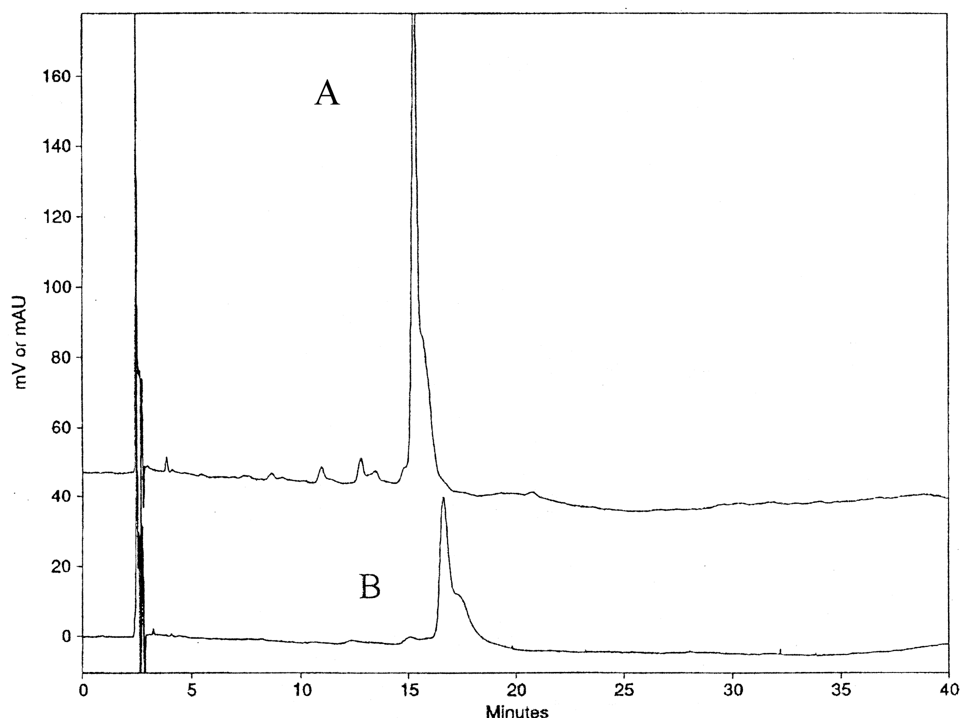

Fig. 3. Influence of pH and temperature on the separation of hPTH synthetic product A using elution conditions as described for method C: (A) mobile phase A at pH 3.4 and (B) column temperature of 30°C.

chromatogram obtained at 30°C. Under these conditions, both the separation and sensitivity of the method are reduced. Separations performed at 50°C gave the best resolution and sensitivity.

Thus, the optimal conditions determined by this method were: mobile phase A consisting of 0.155 *M* sodium chloride–0.037 *M* PSA, pH 5.6, and mobile phase B consisting of acetonitrile. The gradient elution was carried out from 30 to 40% B over 29 min. However, repeated injections under these conditions led to rapid deterioration of column efficiency, a situation that is likely due to non-specific adsorption of the protein to the silica support. Column reconditioning using a mixture of 0.1 *M* nitric acid–isopropanol (1:4) was found to be necessary and was carried out on a regular basis.

3.4. Purity assessment of hPTH products

Commercial synthetic hPTH products were obtained from two manufacturers and analyzed using the optimized conditions. Chromatograms for synthetic products A and B are presented in Fig. 4 along with that of a purified recombinant hPTH product. Both synthetic products showed similar impurity profiles characterized by the presence of several minor impurities eluting prior to hPTH and the major impurity peak eluting after hPTH and consisting of several unresolved constituents. The chromatogram of the recombinant product did not show the presence of the major impurity. A comparative purity assessment of the two synthetic products was carried out by three different methods, that is, using the TFA-based HPLC method A, the optimized HPLC method C and by CZE [7]. Results are presented in Table 1. The purity levels measured by method C and by CZE were in good agreement for both products and, as predicted, were significantly lower that those measured by method A since this method cannot separate the major impurity. Furthermore, the

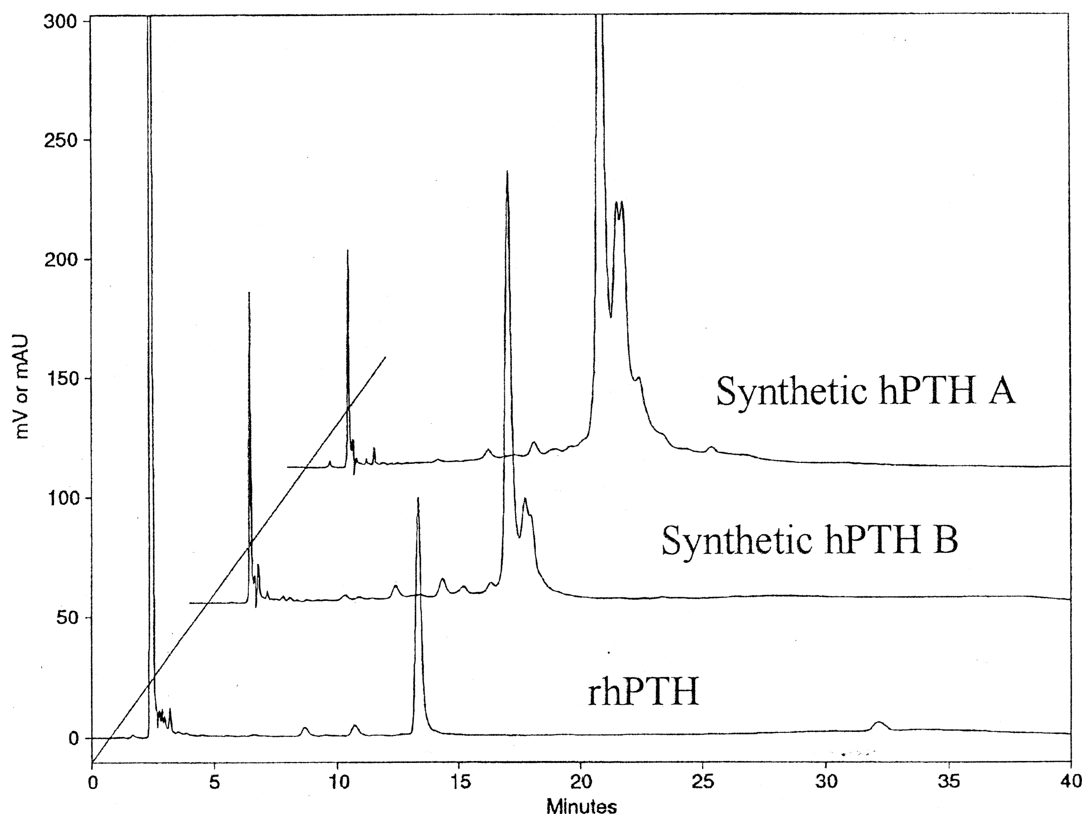

Fig. 4. Chromatograms of hPTH products obtained under optimized conditions of HPLC method C monitored at 220 nm.

Table 1
Comparative assessment of the purity levels and the major impurity levels in synthetic hPTH preparations using three analytical methods (values are expressed as percentages)

	Purity level			Major impurity level[b]	
	Method A (n=2)	Method C (n=4)	CZE (n=3)	Method C (n=4)	CZE (n=3)
Product A	>96.4 (99.3)[a]	55.1	56.7	43.1	36.0
(RSD, %)	–	±1.2	±0.3	±0.8	±1.1
Product B	>90.3 (90.9)[a]	66.1	66.9	26.1	26.9
(RSD, %)	–	±1.2	±6.1	±5.1	±0.5

[a] Values in parentheses indicate the purity level stated on the certificate of analysis from the manufacturer.
[b] Major impurity includes unresolved peaks eluting after the main peak.

levels of the major impurity detected in each product by method C and CZE were also in good agreement. Thus, for product A values of 43.2% and 36.0% were obtained by method C and CZE, respectively, and for product B values of 26.1% and 26.9% were obtained by method C and by CZE, respectively. These results provided convincing evidence that the major impurity detected by the present HPLC method C corresponded to the major impurity observed by CZE.

4. Conclusion

This study demonstrated the increased selectivity of HPLC method C using sodium chloride as mobile phase salt and PSA as an ion-pairing agent for the separation and quantitation of impurities present in hPTH preparations. The method allowed detection of impurities not separated under TFA-based RP-HPLC methods and confirmed the presence of a major impurity previously detected by CZE. On-going studies will attempt to fully characterize the major impurity detected in the two synthetic products and to examine the impurity profiles following controlled hPTH degradation.

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References

[1] H.T. Keutmann, M.M. Sauer, G.N. Hendy, J.L.H. O'Riordan, J.T. Potts, Biochemistry 17 (1978) 5723.
[2] J.T. Potts Jr., H.M. Kronenberg, M. Rosenblatt, Adv. Protein Chem. 35 (1982) 323.
[3] T. Kimura, M. Takai, K. Yoshizawa, S. Sakakibara, Biochem. Biophys. Res. Commun. 114 (1983) 493.
[4] N.A. Goud, R.L. Mckee, M.K. Sardana, P.A. Dehaven, E. Huelar, M.M. Syed, R.A. Goud, S.W. Gibbons, J.E. Fisher, J.J. Levy, J.A. Rodkey, C. Bennett, H.G. Ramjit, L.H. Caporale, M.P. Caulfield, M. Rosenblatt, J. Bone Miner. Res. 6 (1991) 781.
[5] H. Gram, P. Ramage, K. Memmert, R. Gamse, H.P. Kocher, Bio/Technology 12 (1994) 1017.
[6] O.K. Olstad, N.E. Morrison, R. Jemtland, H. Jüppner, G.V. Segre, K.M. Gautvik, Peptides 15 (1994) 1261.
[7] M. Girard, N. Mousseau, T.D. Cyr, J.-C. Ethier, 12th International Symposium on High-Performance Capillary Electrophoresis and Related Microscale Techniques, Palm Springs, CA, 23–28 January 1999, poster 338; manuscript in preparation.
[8] Y. Nabuchi, E. Fujiwara, H. Kuboniwa, Y. Asoh, H. Ushio, Pharm. Res. 14 (1997) 1685.
[9] Y. Nabuchi, E. Fujiwara, K. Ueno, H. Kuboniwa, Y. Asoh, H. Ushio, Pharm. Res. 12 (1995) 2049.
[10] G. Zaman, P.W. Saphier, N. Loveridge, T. Kimura, S. Sakakibara, M. Bernier, G.N. Hendy, Endocrinology 128 (1991) 2583.
[11] K. Benedeck, in: E.D. Katz (Ed.), High Performance Liquid Chromatography – Principles and Methods in Biotechnology, Wiley, Chichester, 1996, pp. 413–467.
[12] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd ed., Wiley, New York, 1997.
[13] H.P.J. Bennett, S. Solomon, D. Goltzman, Biochem. J. 197 (1981) 391.
[14] J.M. Zanelli, M.J. O'Hare, E.C. Nice, P.H. Corran, J. Chromatogr. 223 (1981) 59.
[15] T. Kimura, M. Takai, K. Yoshizawa, S. Sakakibara, Biochem. Biophys. Res. Commun. 114 (1983) 493.
[16] W.S. Hancock, J.T. Sparrow, J. Chromatogr. 206 (1981) 71.