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Phosphate-gradient high-performance liquid chromatographic method for the analysis of synthetic salmon calcitonin

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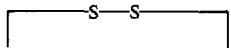
ABSTRACT

A gradient reversed-phase high-performance liquid chromatographic method was developed for the quantitative analysis of salmon calcitonin. A 0.054-*M* potassium monobasic phosphate buffer was modified with an acetonitrile–methanol (4:1) mixture as the mobile phase. Several gradient slopes are used during the separation and analysis so that potential peptidal component impurities in salmon calcitonin samples can be separated without wasting analysis time. The method is applicable to the determination of salmon calcitonin in the peptide portion of the sample as well as in the total sample. The analytical results are expressed as percent chromatographic purity instead of activitiy units.

INTRODUCTION

Salmon calcitonin (sCT) is a synthetic 32 amino acid peptide (Fig. 1) that is used primarily for the treatment of post-menopausal osteoporosis and Paget's disease. Other indications include general osteoporosis, osteogenesis imperfecta, and as an analgesic.

Isocratic high-performance liquid chromatographic (HPLC) separations of sCT were reported previously [1–3] but without any details about the chromatographic efficiency. The Merrifield synthesis process, while very efficient at synthesizing the 32 amino acid peptide chain, also produces a significant number of impurity peptidal components which are similar to salmon calcitonin. While most of these components are removed by chromatographic purification steps [1], it was necessary to develop an analytical gradient HPLC system capable of a separation based on very slight differences in component polarities. At the same time, it was desirable to save time. It was decided to concentrate our efforts on separating the peptidal components which have



Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-
Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-
Pro-Ary-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH2

Fig. 1. Structure of salmon calcitonin

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similar polarity —and therefore similar HPLC retention times— as sCT. The remaining components, mostly non-peptidal, would be eluted at the beginning of the chromatogram, while retained peptide-like components would be eluted at the end of the gradient. By modifying the initial mobile phase conditions and employing fast and shallow gradient profiles [4], it was possible to effect nearly complete separation of sCT from the other peptidal components of the sample within a reasonable time, *i.e.*, < 1 h.

EXPERIMENTAL

A modular HPLC system was used for the analysis. It consisted of a Spectra-Physics Model 8700 gradient pumping system with a dynamic mixer (San Jose, CA, U.S.A.) and a Beckman Model 501 Automatic sample injector (Fullerton, CA, U.S.A.). An LDC Spectromonitor III variable-wavelength UV detector (Rivera Beach, FL, U.S.A.) was used to monitor the column effluent. Samples were separated on a B&J OD5 (octadecylsilane, 5 μm) 25 cm $\times$ 4.6 mm I.D. column, obtained from American Scientific Products (McGaw Park, IL, U.S.A.). Data were collected on a Hewlett-Packard HP 1000 minicomputer with CALS software from CIS-Beckman (Waldwick, NJ, U.S.A.).

Doubly distilled water was obtained from an in-house distillation system. Chrompure HPLC-grade acetonitrile and methanol as well as the potassium monobasic phosphate were obtained from Burdick & Jackson (McGaw Park, IL, U.S.A.). sCT standards and samples were obtained from the peptide synthesis department at Armour Division.

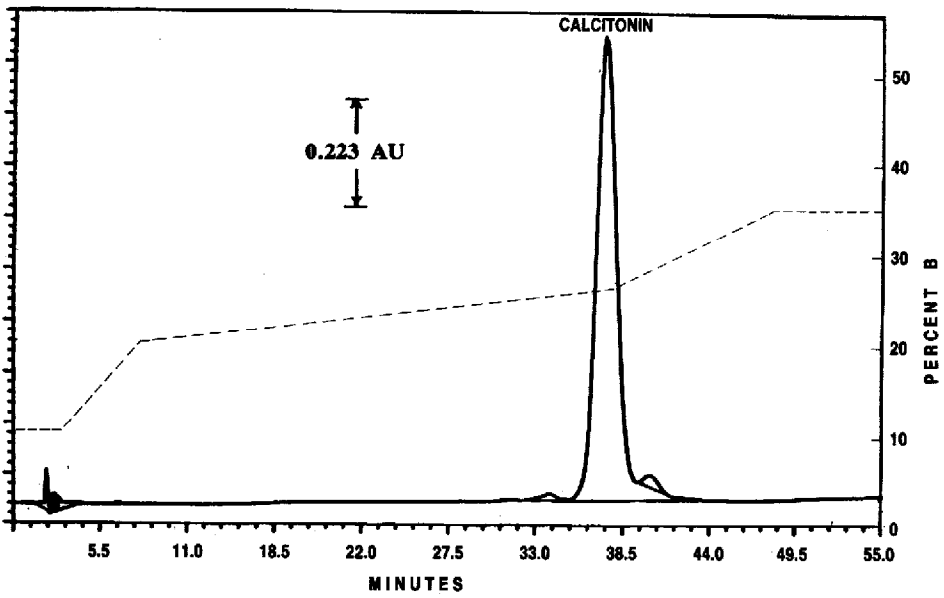

Fig. 2. Typical separation of salmon calcitonin as described in text. CALS integration system factors set to perform tangent skim of peaks immediately following the salmon calcitonin peak.

Phosphate buffer

Potassium monobasic phosphate solution (0.054 *M*) was adjusted to a pH of 2.86 with 85% phosphoric acid. After filtration through a 0.45-μm PTFE filter (Alltech, Deerfield, IL, U.S.A.), three volumes of the buffer were mixed with one volume of the organic phase to make mobile phase A.

Organic phase

Acetonitrile–methanol (4:1). A portion of organic phase was mixed with the phosphate buffer to make mobile phase A and the rest was used as mobile phase B.

Gradient elution

The gradient controller on the Spectra-Physics 8700 pumping system was used to make the gradient profile as shown in Fig. 2. The flow-rate was kept constant at 1.0 ml/min. After a 15-min equilibration time, a blank gradient was run in order to equilibrate the system, and to ascertain that no unexpected peaks (primarily from the aqueous buffer source) would be eluted during the gradient separation.

Approximately 2 mg of each of the sCT samples and standards were dissolved in enough buffer–organic (64:36) mixture to make 10 ml of solution; 100-μl sample injections were made into the HPLC system.

RESULTS AND DISCUSSION

A typical separation of a sCT sample is shown in Fig. 2. With this gradient system sCT has a retention time of *ca.* 38 min. During the critical part of the separation, the amount of the organic component could vary by only 0.1875% per

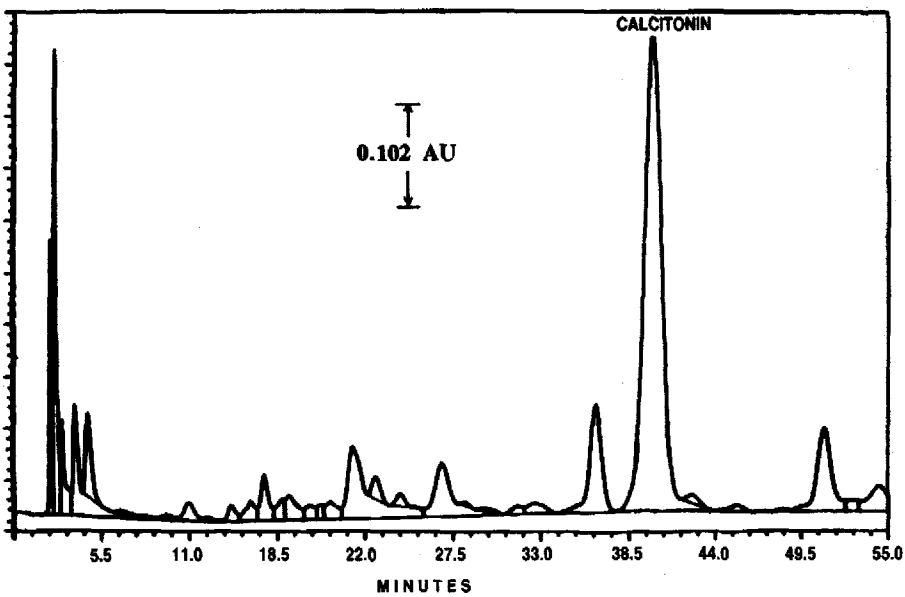

Fig. 3. Separation of a crude extract of sCT. (For conditions, see text.)

minute (0.25% per minute of mobile phase B). This is on the same order as that recommended for the gradient elution of peptide digests [5]. A tangent skim of the peaks immediately following on the tail of the sCT peak produces the most accurate area of the sCT peak as well as better peak symmetry than either a perpendicular drop or forcing a baseline at the valley points.

The peak area calibration plot gave a linear regression with a correlation coefficient of 0.9983 with x and y intercepts of -0.25 (μg/ml) and 0.123 (area counts), respectively.

As the tangent skimming peak integration process always brings into question the reproducibility of the system, a series of ten standard sCT injections were monitored during an extended analytical run of 20 sCT samples. The standard peak areas were reproducible with relative standard deviation of 0.64%. There were no problems with the ability of the CALS software to reproducibly determine when the detector signal returned to the prescribed baseline. The small peak on the tailing side of the sCT peak also was reproducibly "skimmed" from the sCT peak without any difficulty. With a relative standard deviation (R.S.D.) of only slightly less than 1%, it is also likely that we are at the limit of the ability of the Beckman 501 autosampler to inject 100 μl of sample reproducibly after flushing the sample loop and connecting tubing.

The retention time of the sCT peak has a tendency to change slightly from day-to-day and run-to-run. We have noticed that two factors in particular are responsible for this variance, *i.e.*, freshness of the mobile phase and the column condition. Over a 13 h analytical run, the retention time of sCT would increase by 1 min. The lengthening of the retention time for sCT during the assay is likely the result of the preferential evaporation of the organic components from mobile phase A during the continuous helium sparging as refilling the mobile phase reservoirs with fresh mixtures reduces the retention time to that obtained originally. The retention times of sCT on three different B&J OD5 columns ranged from 35.5 to 39.8 min. As these columns had various histories prior to their use for the separation of sCT, the variation observed was not considered significant.

The effects of the pH and the organic content of the initial mobile phase conditions on the retention times was investigated. Of primary concern was any effect on the retention time of sCT. While the procedure is quite specific about the pH of the phosphate buffer prior to mixing, it was found that varying the pH of the buffer from 2.80 to 2.90 while holding the organic content constant resulted in very little change of the sCT retention time. However, modifying the organic content of mobile phase A from 24.5 to 25.5% produces a considerable change in the retention time. A difference of 0.5% in the organic solvent concentration can produce as much as a 5.7% change in the retention time. This is not entirely unexpected, as the gradient slope is sufficiently shallow during the major part of the separation that the difference in retention times is roughly equivalent to the time it would take for the gradient to catch up to the mobile phase composition at the time of the sCT elution during a normal assay. sCT will be eluted only when the mobile phase composition is within a narrow range as the retention times of molecules with higher molecular weight are much more sensitive to slight changes in organic composition than are lower molecular-weight molecules [6]. However, the separation of sCT from other peptidal components does not deteriorate with this change in retention time.

This procedure is suitable for the determination of sCT as either a percent of the

entire sample or as a percent of only the peptidal portion of the sample. The determination of the peptidal portion of the sample requires the assumption that the peaks near the void volume are non-peptidal and due to salts, solvents, etc., from the synthesis and sample preparation of sCT. Non-peptidal assignments also are based on the chromatographic profile of the excipients expected in the material. A well-characterized primary reference standard is then used to determine the amount of sCT as a percent of the entire sample. For some highly purified sCT samples it was quite easy to determine a peptidal purity of 98.5% as sCT and a total sample analysis of 93.5% as sCT.

The resolving power of this system is demonstrated by Fig. 3, which shows the separation of a crude extract of sCT. The crucial portion of the separation is its ability to resolve impurities following the elution of sCT from the sCT peak. With the CALS software it is possible to perform a tangent skim of the impurities from the sCT peak and thus provide the best approximation to the peak area of sCT. If need be, the integration parameters can be modified to obtain the best fit for the peak area determination of both sCT and the impurities being monitored.

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