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International Journal of Pharmaceutics 114 (1995) 215–220

international journal of pharmaceutics

Post-column fluorescence HPLC for salmon calcitonin formulations

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Received 29 March 1994; modifed version received 4 July 1994; accepted 6 July 1994

Abstract

Post-column fluorescence HPLC was developed for nanogram level salmon calcitonin (sCT) assay for the in vitro evaluation of sCT formulations. A gradient reversed-phase C_{18} column was adopted and fluorescamine was used as a fluorescence derivatizing reagent. A practical calibration range could be constructed over a range of 10–600 ng per injection and is approx. 100-times more sensitive than conventional UV detection. The method was successfully applied for the quantitation of sCT from biodegradable microspheres, injection and nasal spray formulations.

Keywords: Salmon calcitonin; HPLC; Post-column fluorescence HPLC; Fluorescamine; Fluorescence derivatization

1. Introduction

Salmon calcitonin (sCT) is a polypeptide comprised of 32 amino acid residues and is one of the most potent forms among calcitonins available for clinical use (e.g., human, salmon, porcine, chicken and eel calcitonins). sCT is used primarily for the treatment of postmenopausal osteoporosis and Paget's disease (Azria, 1989).

For the design of sCT delivery systems and their pharmaceutical evaluation, reversed-phase HPLC has become the preferred analytical method (Heinitz et al., 1988; Lee et al., 1991a; Mayer et al., 1991). However, the HPLC with UV detector often lacks the requisite sensitivity for quantitation at or below the microgram level per injection. A practical HPLC used for sCT only covers a range of 1–15 μg per injection (Buck and Maxl, 1990; Lee et al., 1991b, 1992). Therefore, it is critical to develop a more specific and sensitive method for performing precise and extensive evaluation of sCT formulations, in terms of preformulation, manufacturing process control, in vitro drug release, adsorption test and stability kinetic studies as well as quality control (Celebi et al., 1990; Lee et al., 1992; Calis et al., 1993; Tsai et al., 1993). In this study, we developed a simple post-column fluorescence HPLC method suitable for in vitro evaluation of sCT formulations at the nanogram level using fluorescamine for fluorescence derivatization.

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SSDI 0378-5173(94)00248-7

2. Materials and methods

2.1. Materials

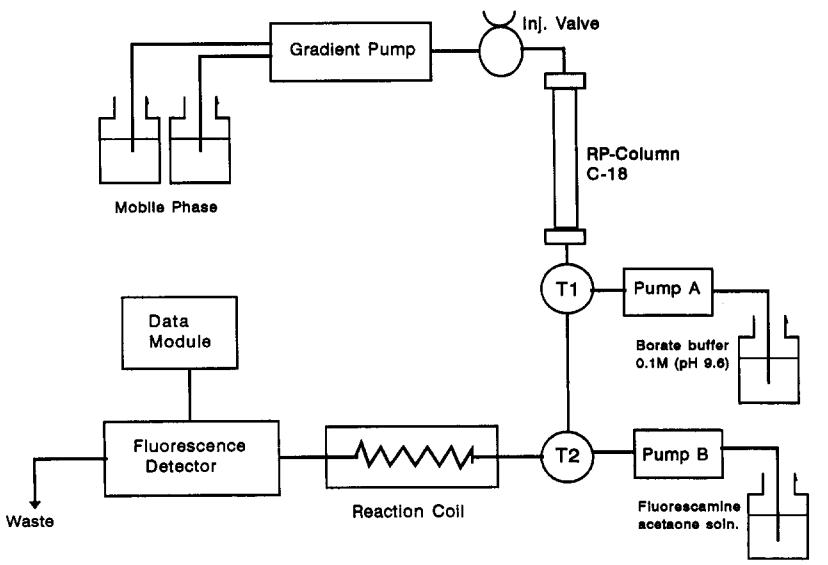
Synthetic salmon calcitonin (cyclic) was obtained from Bachem Inc. (Torrance, CA, U.S.A.) and fluorescamine from Sigma (St. Louis, MO, U.S.A.). HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Fisher (Pittsburgh, PA, U.S.A.) and Pierce (Rockford, IL, U.S.A.), respectively. Commercial sCT formulations of injection (Miacalcic® 50 IU, sCT 50 IU/ml, Sandoz, no. 130MFD0292) and nasal spray (Miacalcic® Nasal 100, sCT 1100 IU/ml, Sandoz, no. 027MFD1192) were purchased from Sandoz Korea. Biodegradable sCT microspheres were prepared with poly(glycolide-co-lactide) (PGL, 50:50, Mol. Wt 34 000, Beringer-Ingelheim, Germany) and characterized as described previously (Mehta et al., 1994). Other reagents and chemicals were of reagent grade.

2.2. Post-column HPLC system

As shown in Scheme 1, the post-column HPLC system consisted of one ternary gradient pump (ACS, Cheshire, U.K.) for gradient solvent delivery and two isocratic pumps (Model 307, Gilson, France and Mini-pump, Milton Roy, U.S.A.) for feeding both buffer and fluorescamine solutions, a Rheodyne 7125 injector, an ODS column (10 μm, 4.6 mm × 25 cm, Bio-Rad, Richmond, U.S.A.), a post-column reactor (CRX390, tube length and diameter, Pickering, U.S.A.), a fluorescence detector (Model 484, Waters, Milford, MA, U.S.A.) and a computing integrator (C-R6A, Shimadzu, Japan).

2.3. Post-column derivatization procedure

sCT was eluted from the column and then derivatized with fluorescamine for fluorescence detection. A linear gradient was employed: 30% A:70% B to 55% A:45% B over 25 min. Mobile phase A was a 0.1% TFA/acetonitrile solution and mobile phase B was 0.1% TFA/distilled water. The injection volume was 10 μl and the flow rate was 1.5 ml/min. The eluent was adjusted to pH 9.2 with 0.1 M borate buffer (pH 9.6; flow rate, 0.3 ml/min) and post-column derivatized with fluorescamine solution (80 mg/l in acetone; flow rate, 0.2 ml/min) at 30°C. The fluorescence detector was set with a 395 nm interference filter and a 455 nm cut-off filter for



Scheme 1. Schematic diagram of the post-column HPLC system for salmon calcitonin.

excitation and emission, respectively. A standard curve was constructed for each series of determinations over a range of 10–600 ng of sCT per injection. For calibration, sCT was dried over a P_2O_5 desiccator when water was not detected by the Karl Fisher method.

2.4. Assay of sCT from formulations

Miacalcic® 50 IU (sCT injection) and Miacalcic® Nasal 100 (sCT nasal spray) were diluted 2- and 22-fold, respectively, with 35% acetonitrile adjusted to pH 3.3 with 0.1% TFA before injection into HPLC. For PGL microspheres, sCT was extracted with 35% acetonitrile adjusted to pH 3.3 with 0.1% TFA and centrifuged and the supernatant was subjected to HPLC injection.

3. Results and discussion

There has been a great deal of interest surrounding the use of derivatization in HPLC for improving selectivity and sensitivity. In particular, post-column fluorescence derivatization in HPLC for peptides and proteins offers a number of advantages over other possible derivatization approaches, pre-column methods (Windisch et al., 1992), in terms of sensitivity, precision, ease of automation and the lack of necessity for a single reaction pathway. However, these schemes require more care in experimental design of fluorogenic reagent selection and optimizing reaction conditions for post-column derivatization.

Conventional amine specific fluorogenic reagents such as dansyl chloride and 7-chloride-4-nitrobenzofurazan require heat and also react with water producing potential interferences. Another popular amine specific fluorogenic reagent, *o*-phthaladehyde, is unsuitable since it forms highly unstable derivatives with peptides that also have low fluorescence yields. However, fluorescamine has several desirable characteristics for post-column fluorescence derivatization in HPLC for peptides and proteins: fluorescamine reacts with primary amines within seconds at room temperature under basic pH conditions to form a highly fluorescent product and any excess reagent is subsequently hydrolysed to a non-fluorescent compound (DeBernardo et al., 1974; Wehr, 1991).

In general, the reaction of fluorescamine with peptide amino groups is pH-dependent (Udenfriend et al., 1972). To optimize derivatization procedure of the eluted sCT with fluorescamine, post-column reaction conditions, e.g., fluorescamine concentration, reaction temperature and pH were examined as shown in Fig. 1 and described under section 2.3 in detail. A typical chromatogram of a standard of sCT is shown in Fig. 2.

Fluorescence contour spectra of both eluents of sCT conjugated to fluorescamine and blank were measured. As shown in Fig. 2, the sCT-fluo-

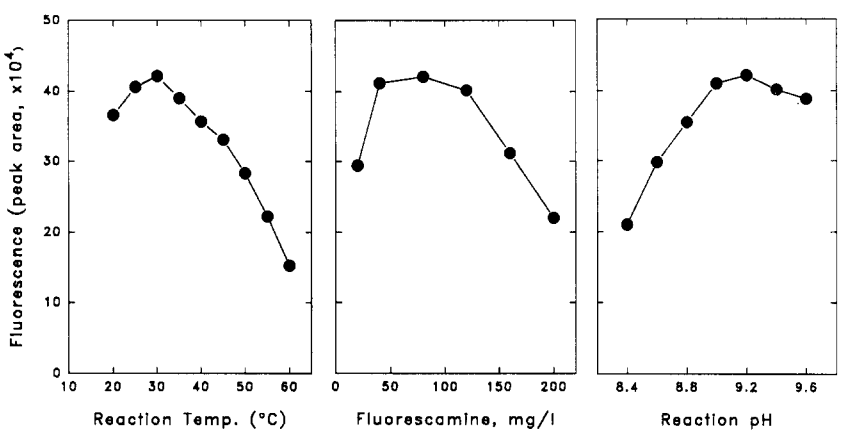


Fig. 1. Optimization of reaction conditions for post-column fluorescence HPLC of sCT.

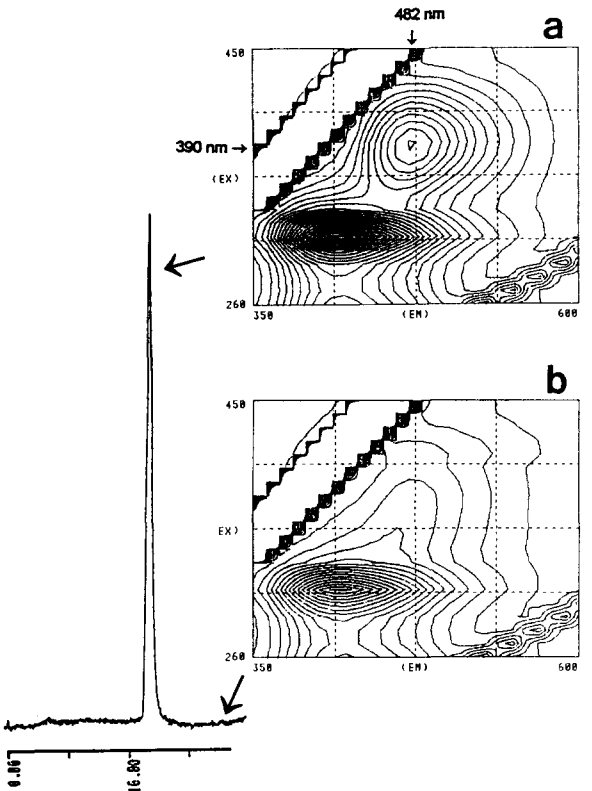


Fig. 2. Fluorescence chromatogram and contour spectra of eluents of post-column fluorescence HPLC of sCT. (a) Fluorescamine-sCT derivative eluent, (b) blank eluent.

rescamine conjugate demonstrates an excitation maximum around 390 nm and an emission maximum 482 nm which is quite different from that of blank eluent. Theoretically, sCT has three primary amines available for conjugating by fluorescamine, two lysine and one N-terminal cysteine residue.

With this post-column derivatization, the correlation of peak area ratio with sCT concentration was linear in the range of 10–600 ng sCT per injection and the correlation coefficients for the standard calibration curves were better than 0.999. The limit of quantitation, defined by a signal-to-noise ratio of about 3:1, was about 1 ng per injection, which is approx. 100 times more sensitive than conventional UV detection (Lee et al., 1992).

sCT samples stored under different pH conditions at 70°C were assayed by this post-column

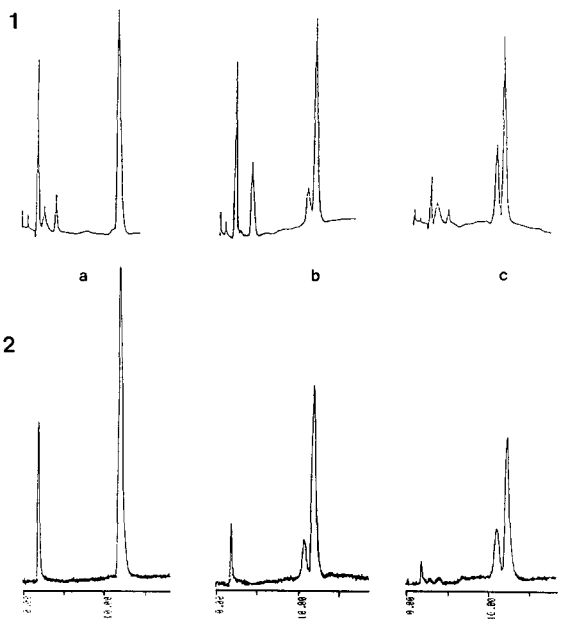


Fig. 3. Comparison of stability-indicating HPLC chromatograms of sCT stored under different pH conditions at 70°C. (1) UV detection, 220 nm, initial sCT 10 μg; scale, 0.1 A.U.; adopted from Lee et al. (1992). (2) Post-column fluorescence detection, initial sCT 0.5 μg. (a) pH 3.7, 5 h; (b) pH 6.0, 10 h; (c) pH 9.0, 1 h.

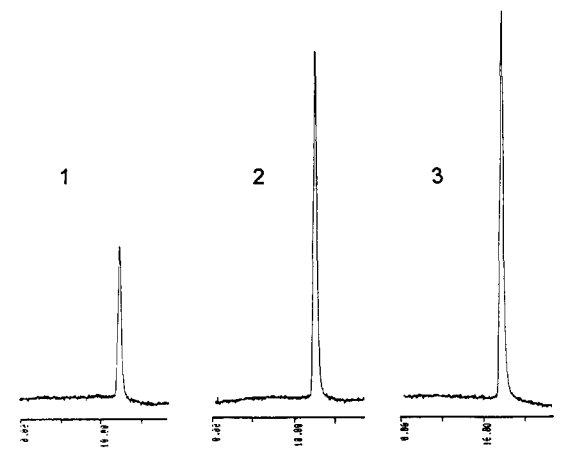


Fig. 4. Post-column fluorescence HPLC chromatogram of sCT from sCT formulations. (1) sCT injection (Miacalcic® 50 IU, sCT 50 IU/ml, Sandoz, no. 130MFD0292): diluted 2-fold with 35% acetonitrile adjusted to pH 3.3 with 0.1% TFA. (2) sCT nasal spray (Miacalcic® Nasal 100, sCT 1100 IU/ml, Sandoz, no. 027MFD1192): diluted 22-fold with 35% acetonitrile adjusted to pH 3.3 with 0.1% TFA. (3) PGL sCT microspheres: extraction with 35% acetonitrile adjusted to pH 3.3 with 0.1% TFA.

Table 1
Assay of sCT formulations by the post-column fluorescence HPLC system

Formulations	Claims, sCT	Found, sCT ($\pm$S.D., $n = 5$)
Miacalcic® 50 IU (Inj.)	50 IU/ml [a]	12.3 $\pm$ 0.4 μg/ml
Miacalcic® Nasal 100	1100 IU/ml [a]	264.3 $\pm$ 4.3 μg/ml
sCT microspheres	36.5 $\pm$ 2.9 μg/mg [b]	37.0 $\pm$ 2.0 μg/mg

[a] 1 μg of sCT is approximately equivalent to 4 IU of sCT.
[b] HPLC assay by UV detection (Lee et al., 1991b).

derivatization system and compared with conventional UV HPLC detection using the same analytical separation method without post-column derivatization. With 0.5 μg of sCT, which is about 1/20th of the sCT required by UV detection, similar stability-indicating HPLC chromatograms were obtained as shown in Fig. 3.

The post-column system was adopted to quantitate sCT from its formulations, e.g., commercial sCT injection and nasal spray and sCT microspheres made of PGL. As shown in Fig. 4, three chromatograms from sCT formulations do not differ from that of the sCT standard which indicates that possible ingredients in sCT formulations have no effect on the sCT assay by this post-column system. For dilution or extraction medium for sCT formulations, 35% acetonitrile adjusted to pH 3.3 with 0.1% TFA was used. To enhance extraction yields of sCT from sCT microspheres, TFA was added to the extraction medium and the pH was adjusted to 3.3 at which sCT is most stable (Lee et al., 1992). In general, extraction media play a major role in the extraction yield of a peptide from its polymeric matrix due to their possible interaction or adsorption with polymers and glass surfaces.

Direct comparison of biological and HPLC assay results of sCT is not possible generally (Buck and Maxl, 1990). However, assuming that 1 μg sCT is equivalent to approx. 4 IU (BP, 1988), the data for sCT injection and nasal spray listed in Table 1 seem to be satisfactory. For sCT microspheres, no differences between assaying data from UV detection and this post-column fluorescence system was found.

In conclusion, post-column fluorescence HPLC was developed for a nanogram level sCT assay which is approx. 100-times more sensitive than conventional UV detection using fluorescamine as a fluorescence derivatizing reagent. The quantitation of sCT from formulations, e.g., injection, nasal spray and biodegradable microspheres, was possible per unit dosage without additional concentration of sCT from the formulations. With this system, it may also be possible to perform more precise and extensive chemical and physical stability evaluation of sCT formulations as well as their content uniformity and dissolution testing over conventional HPLC methods.

Acknowledgement

This work was supported in part by the Reseach Center for New Drug Development in Seoul National University.

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