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LC assay for salmon calcitonin in aerosol formulations using fluorescence derivatization and size exclusion chromatography

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Abstract: A highly sensitive LC procedure was developed that utilizes fluorescence derivatization and detection coupled with size exclusion chromatography for the analysis of salmon calcitonin in salmon calcitonin aerosols. The LC procedure uses fluorescamine derivatization to label the primary amino groups of the peptide. The derivatization procedure is completely automated by an autosampler capable of pre-column mixing. Size exclusion chromatography is performed using a Supelco G2000 SWXL column. The method can be used to assay the amount of salmon calcitonin delivered per actuation of an aerosol unit. The procedure is simple, accurate, and precise and can detect as little as 2 ng ml^{-1} concentrations of salmon calcitonin.

Keywords: *Salmon calcitonin; aerosol; HPLC; fluorescence derivatization; fluorescamine; size exclusion; chromatography.*

Introduction

Salmon calcitonin (SCT) is a 32 amino acid peptide that is highly potent relative to other calcitonins [1]. Calcitonins are naturally occurring hormones secreted by the thyroid glands of mammals and by the ultrimobranchial glands in non-mammalian species. Calcitonins have been shown to promote the adsorption and inhibit the loss of calcium in bone [1]. To this end, pharmaceutical formulations of sCT, such as parentals, aerosols and inhalers, are designed to efficiently administer sCT to the blood stream in order to regulate calcium levels. Aerosol formulations of sCT can be particularly efficacious drug delivery systems of a potent drug and, therefore, require sensitive methods for analysis.

A sensitive LC procedure that uses fluorescence derivatization and size exclusion chromatography was developed for the analysis and testing of salmon calcitonin in aerosol formulations. Depending on the particular experimental protocol, testing of aerosol formulations can involve several analytical procedures to assess performance, potency, stability, accuracy and uniformity of the formulation. Performance testing of aerosols assess the accuracy of the delivered dose per actuation of an aerosol and is one of the most critical tests performed on the formulation. Yet, for sCT aerosols, analytical procedures developed for performance testing must be capable of assaying less than 1 $\mu g\ ml^{-1}$ of sCT.

LC methods using reversed-phase chromatography have been developed for the analysis of the stability, potency, and purity of calcitonin drug substances [2–5]. These methods have all used UV detection at 220–210 nm which is sensitive to the peptide bonds. Salmon calcitonin comprises one tyrosine residue as the only aromatic amino acid present in its sequence and, therefore, has no native fluorescence or appreciable UV activity.

Consequently, HPLC methods utilizing UV detection do not afford high sensitivity of analysis in sCT. Theoretical estimates of HPLC on-column detection limits of 20 ng [2] and 8 ng [5] of calcitonin have been reported for the drug substance. However, in the presence of a complex matrix, sensitive detection of sCT will be more difficult, especially when performed at 210–220 nm. Fluorescence derivatization could dramatically improve the sensitivity of detection for sCT in a matrix of formulation excipients; however, to

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date, no fluorescence derivatization procedure has been reported for sCT.

Experimental

Reagents and chemicals

Fluorescamine and benzalkonium chloride (BAC) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and were used as received. The sCT used in this study was a synthetic peptide with the identical residues and sequence as calcitonin of salmon origin. The sCT drug substance was synthesized and purified by LC by Armour Pharmaceutical Co. (Kankakee, IL, USA). HPLC-grade methanol and acetonitrile were used and analytical-reagent grade dibasic sodium phosphate and 85% phosphoric acid were used in the buffers. The water used was HPLC grade. The 0.1 M phosphate buffer was prepared by titrating 0.1 M Na_2HPO_4 to pH 7.5 with 85% phosphoric acid.

Sampler preparation and diluent

The diluent consisted of a solution containing BAC (0.1% w/v)–acetonitrile (70:30, v/v). Acetonitrile was used in the diluent to prevent precipitation of the fluorescamine reagent. Benzalkonium chloride was added to the diluent to inhibit glass adsorption of the peptide. Low recoveries of sCT in dilute solutions in glass containers were observed and attributed to adsorption. Benzalkonium chloride is a cationic surfactant that effectively deactivated surface adsorption sites on glass.

The particular aerosol formulations used were formulated to deliver 7.8 μg of sCT per actuation. This potency is equivalent to 36 international units for the synthetic lot of sCT used, as determined by the compendial assay of the drug substance [6]. Dose delivery from the valve testing was performed on the aerosol units by collecting 10 actuations into a 100 ml bottle containing 40 ml of diluent. The contents of the bottle were then quantitatively transferred to a 100 ml volumetric flask which was filled to volume with diluent and assayed. The samples were assayed against a standard prepared at the same concentration. The final concentrations of the samples and standards were approximately 0.78 μg ml^{-1}.

Equipment and conditions

Chromatography was conducted using a Hitachi (Tokyo, Japan) L-600 pump and a Hitachi AS-4000 Autosampler. Fluorescence detection was performed using a LDC Fluoromonitor III (Milton Roy, Riviera Beach, FL, USA).

Fluorescence excitation wavelengths were selected using a 370 nm UV pass filter and emission was filtered by a 420 nm cut-off filter. Data acquisition and processing were performed using a Waters Expert Ease chromatography system (Waters Chromatography Division, Milford, MA, USA).

Separations were achieved using a Supelco (Bellefonte, PA, USA) G2000 SWXL (300 × 7.8 mm i.d.) column. Also, a guard column (Supelco SWXL 40 × 6.0 mm i.d.) was placed between the injector and the analytical column. The mobile phase was composed of phosphate buffer (pH 7.5, 0.1 M)–acetonitrile–methanol (60:30:10, v/v/v) and was delivered at a flow rate of 0.7 ml min^{-1}.

The fluorescence excitation and emission spectra were obtained using a Perkin–Elmer 650-40 Fluorescence Spectrometer (Perkin–Elmer Corp., Norwalk, CT, USA) using a 150 W xenon lamp light source. Excitation and emission monochromators were set for a 2 nm bandpass.

Derivatization procedure

The fluorescamine derivatization reaction was performed in a manner similar to procedures reported previously [7, 8]. The sample and standard solutions were first made basic by the addition of a concentrated phosphate buffer (0.1 M) followed by the addition of a concentrated fluorescamine solution in acetonitrile (1.5%, w/v). Concentrated reagents were used to keep dilution of the analytical solution to a minimum and thereby to provide optimal sensitivity and reproducibility. Fluorescamine was used in approximately a 50 fold excess of the total amino groups available for labelling. In the presence of trace levels of sample and excipients, the low buffering capacity of the final solution was sufficient to stabilize pH.

The derivatization procedure was totally automated by the AS-4000 autosampler which performed the fluorescamine reaction prior to each injection. This procedure is illustrated in the flow diagram shown in Fig. 1. A 1.0 ml aliquot of a sample or standard solution in an autosampler vial was made basic by the addition of 50 μl of pH 7.5 0.1 M phosphate buffer. This solution was mixed by rapid

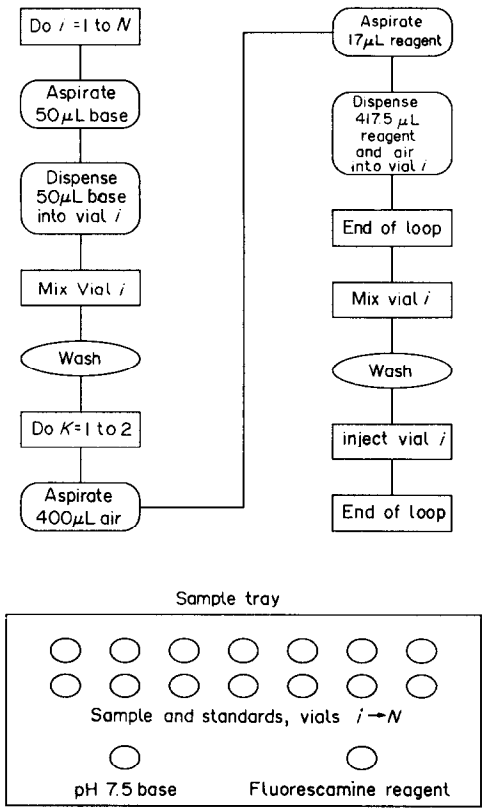

Figure 1
Flow chart schematic of derivatization programme using AS-4000 autosampler and illustration of sample tray arrangement. Vials *i* through *N* refer to standard and sample solutions. See text for further details.

aspiration and dispensing of the solution by the syringe of the autosampler. A total of 35 μl of a 1.5% fluorescamine solution in acetonitrile was then added. Since the fluorescamine reaction occurred in less than a few seconds [8], addition of fluorescamine was done in two 17.5 μl steps. Each addition of fluorescamine was followed by immediate bubbling to ensure sudden and rapid mixing. Immediate bubbling of the solution was achieved by first pulling 400 μl of air into the syringe followed by 17.5 μl of fluorescamine solution. The total 417.5 μl of fluorescamine and air was then dispensed into the sample or standard solution using a high syringe speed. Final mixing was achieved by rapidly aspirating then dispensing 450 μl of the solution. After derivatization, the solution was injected immediately since the sCT-fluorescamine product was unstable in the reaction medium on an hourly time scale. The volume injected was 100 μl.

Results and Discussion

Fluorescamine derivatization

Fluorescamine derivatization exhibits several desirable characteristics amenable for sensitive but routine assay procedures of pharmaceutical formulations of sCT. Under basic conditions, fluorescamine reacts with primary amines within seconds at room temperature to form a highly fluorescent product with quantum yields in the range of approximately 0.1–0.4 [7, 8]. The reagent is also fluorogenic in that, once reacted with amines, any excess reagent is subsequently hydrolysed to a non-fluorescent compound [7]. Other commercially available amine specific fluorogenic reagents such as dansyl chloride [9] and 7-fluoro-4-nitrobenzofurazan (NBD-F) [10] require heat and also react with water producing potential interferences. Also, the amine specific fluorogenic reagent, ortho-phthaldialdehyde (OPA), is unsuitable since it forms highly unstable derivatives with peptides that also have low fluorescence yields [11].

Salmon calcitonin has three primary amines available for labelling by fluorescamine as shown in Fig. 2. It is not known whether all three amines of sCT are labelled, though, previous studies have shown a strong correlation between the degree of labelling by fluorescamine and the number of primary amines of a protein [12]. The fluorescence spectrum of sCT-fluorescamine, Fig. 3, shows an excitation maximum around 390 nm and an emission maximum 470 nm and is similar to spectra of other fluorescamine derivatized

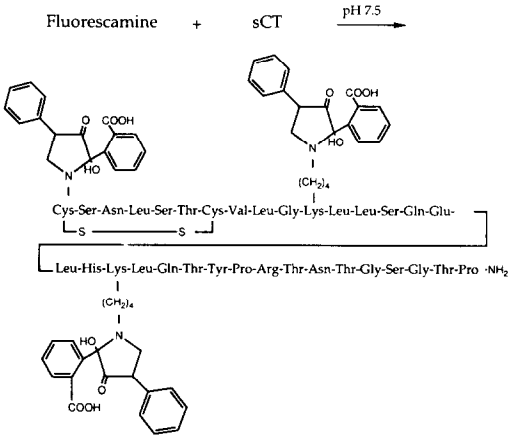

Figure 2
Reaction of sCT with fluorescamine. Fluorescamine labeling of N-terminal cysteine and side chain of two lysine residues is shown.

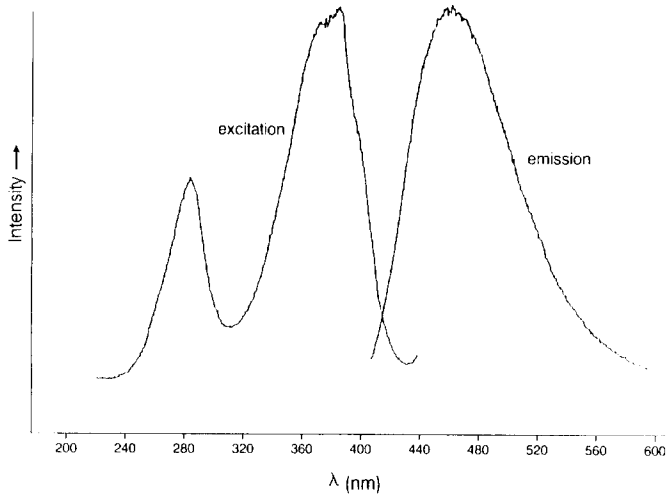

Figure 3
Excitation and emission spectra of sCT-fluorescamine fluorescence at pH 7.5.

amines [8]. Optimal fluorescence was observed at pH 7.5 for sCT-fluorescamine. At much lower pH levels, diminished fluorescence intensity of sCT-fluorescamine was observed.

Chromatography

The separation requirements for the LC method to measure dose delivery from the valve of sCT aerosols involved filtering the labelled peptide from many small molecular weight substituents present in the final analytical solution. The final analytical solution contained benzalkonium chloride in the diluent, possible impurities of sCT, and aerosol excipients such as lubricants and carriers. LC methods using reversed-phase chromatography have been developed for the assay of sCT aerosols but have not shown sufficient selectivity or sensitivity for the quantitation of sCT in aerosol sprays at low doses. Matrix interferences, using either UV or fluorescence detection, limit the sensitivity of reversed-phase HPLC methods to detection limits of approximately 1 $\mu g\ ml^{-1}$ for sCT aerosols. The unique physical discrimination based on size afforded by size exclusion chromatography efficiently separates sCT-fluorescamine from the matrix. Size exclusion chromatography yields high separation efficiencies for sCT-fluorescamine and separates the labelled peptide, which has a molecular weight in the range of 4000, from the smaller substituents in the matrix. On the other hand, derivatized peptide impurities with a similar molecular weight as sCT will not be resolved from sCT-fluorescamine. However, analysis of impurities and degradation products of the active are normally performed by a LC assay of the total content of the aerosol unit.

A typical chromatogram for a standard preparation of sCT at a concentration of 0.78 $\mu g\ ml^{-1}$ is shown in Fig. 4. The sCT-fluorescamine derivative was excluded by the column and elutes in 13.0 min which corresponded to an elution volume of approximately 9.1 ml. The sCT-fluorescamine peak was symmetrical indicating ideal size exclusion behaviour and had approximately 7000 theoretical plates. Fluorescent impurities in the diluent and fluorescamine reagent eluted after 15 min, in the interstitial volume of the column. A chromatogram of a sample solution is shown in Fig. 5. The chromatogram of the sample showed additional eluants in the interstitial volume which are fluorescamine labelled excipients from the aerosol formulation.

Validation and detection limit

Precision was measured by performing 10 replicate derivatizations, each followed by LC analysis. The average integrated peak area and standard deviation for the replicate derivatization and chromatographs of a standard solution were calculated. The procedure exhibited good reproducibility with a RSD of 1.4% (n = 10). This high degree of precision demonstrates the capabilities of the instrument to perform the microscale derivatization procedure in a reproducible manner.

The system was observed to be linear over

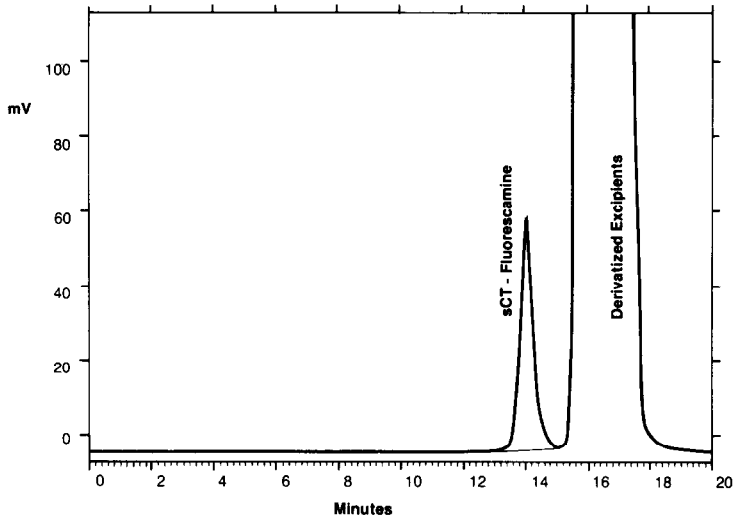

Figure 4
Chromatogram of fluorescamine derivatized sCT standard solution.

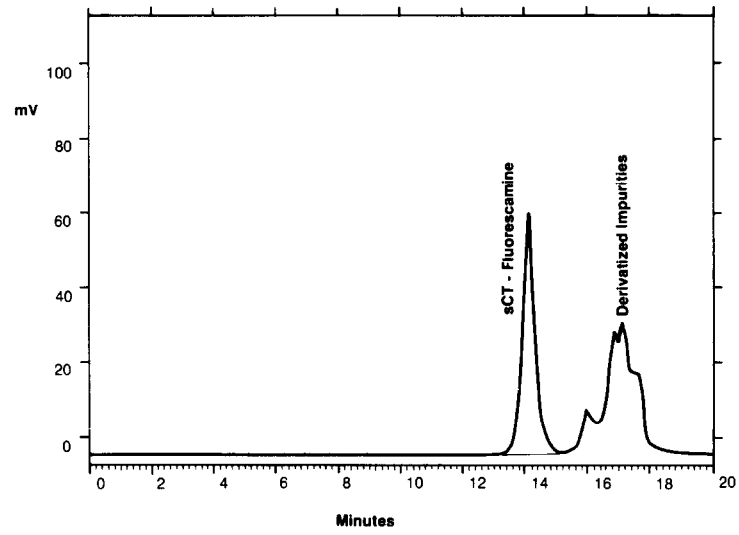

Figure 5
Chromatogram of fluorescamine derivatized sCT sample solution.

the range of 0.11–2.21 μg ml^{-1} as shown in Fig. 6. The calculated correlation coefficient was 1.000 and the studentized peak residuals for all data points were within a 2% absolute value of a linear fit over the range measured. The linearity of response indicated that the adsorption of sCT onto glass had been quenched by the BAC present in the diluent. Indeed, when BAC was not included in the diluent, non-linearities and low recoveries were observed due to glass adsorption.

Recovery of sCT was measured by preparing a series of dry powder placebo blends spiked with various amounts of sCT. The spiked placebos range from approximately 150 to 50% of the labelled amount of sCT specified by the formulation (0.78 μg ml^{-1}, Table 1). All spiked placebo preparations assayed within 98–106% of theoretical. The average recovery was 101.4% with a relative standard deviation of 0.3%.

In order to validate the procedure for the analysis of a sequence of samples over an extended period of time, the stability of sCT in the diluent was monitored for 24 h for both standard and sample preparations. The sCT fluorescamine integrated area showed no change due to degradation over this period.

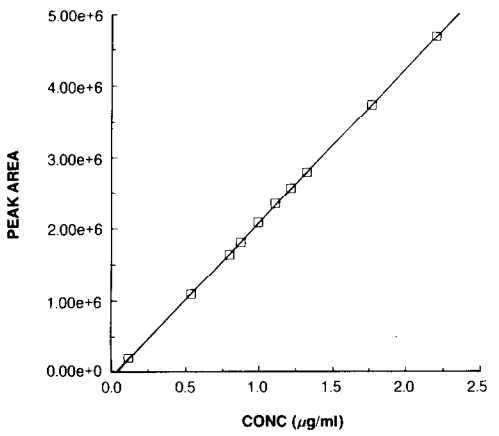

Figure 6
Linearity of response of sCT-fluorescamine. Linear regression:slope = 2.15×10^6, intercept = -5.45×10^4.

Table 1
Per cent recovery of spiked placebo preparations

Per cent spiked*	Per cent recovery
50.3	99.7
80.6	106.2
100.0	99.2
120.6	97.9
150.8	100.9

* 100% = 0.78 μg ml^{-1}.

Finally, the detection limit of the method, defined as three times the baseline noise, is 2 ng ml^{-1} for a sample preparation. This detection limit corresponds to 200 pg of the peptide injected on-column and is approximately two orders of magnitude more sensitive than UV detection using reversed-phase chromatography.

Conclusion

When appropriate, fluorescence derivatization in combination with LC is an excellent means of substantially increasing the sensitivity of an assay. However, fluorescence derivatization is rarely used in LC procedures for the analysis and quality assurance of pharmaceutical formulations. Such assay procedures must possess a high degree of simplicity and expediency to be useful for routine analysis. Traditionally manual pre-column or on-line post-column systems were cumbersome and complex. With the advent of LC sampling systems capable of robotic sample manipulations, derivatization techniques can now be done on a more routine basis. This technology has been exploited to develop a routine but highly sensitive assay procedure for the analysis of sCT in aerosol formulations.

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