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Short communication

Sensitive capillary chromatography mass spectrometric methods for the determination of salcatonin in human biological matrices

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Abstract

New methods employing capillary liquid chromatography in combination with time-of-flight mass spectrometry (μLC-TOF/MS) were developed for the rapid determination of salcatonin in human urine and plasma. The present approaches utilize $^{13}C_6$-leucine (19)-labeled salcatonin as internal standard, small matrix volumes and simple sample preparation procedures. They allow TOF/MS to be used as a highly selective detector for providing accurate quantitation of salcatonin. Data acquisition was performed in enhanced mode optimizing the signal for the triply charged species of salcatonin and its internal standard. We demonstrate that the determination of salcatonin is straightforward and reliable and can be performed with excellent linearity ($R^2 > 0.999$), precision and accuracy over the concentration ranges of 2.9–290 pmol/mL in human urine, and 7.3–730 pmol/mL in human plasma.
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1. Introduction

Calcitonin is a hypocalcemic factor secreted from the parafollicular "C" cells of the thyroid gland originating from the neural crest [1]. Many vertebrates express calcitonin and secretion is regulated by subtle changes in serum calcium levels. Gastrointestinal peptides, estrogens and Vitamin D also regulate its secretion. The physiological role of endogenous calcitonin in calcium homeostasis is not completely understood. Human calcitonin (hCT) is a useful biomarker in the diagnosis and monitoring of medullary thyroid carcinoma [2].

Salcatonin (sCT) is the salmon variety of calcitonin whose precursor is a 136-residue polypeptide. In vivo processing of the precursor results in a 32 residue active peptide, with a molecular weight of 3429.71 Da whose features include a disulfide bridge and C-terminal amidation (Fig. 1). Salcatonin is used to reduce pain from Paget's disease [3] and bone malignancies [4] by direct action on the central nervous system.

The determination of drugs and biomolecules from complex biological matrices may be performed by a variety of analytical techniques. Typically, the most sensitive bioanalytical quantitative methods commonly used are radioimmunometric and enzyme-linked immunosorbent assays. However, the antibody-based methods are time consuming and costly to implement, and may suffer from cross-reactivity towards other antigens. Recently, several reports have appeared advising that caution be exerted in interpreting results from immunoassays for the determination of hCT from serum [2]. Martinetti and coworkers [2] concluded that the analytical accuracy of hCT is flawed even if new, highly specific antibodies were utilized. LC methods with ultraviolet detection have been reported for the determination of sCT, but these are inadequate for trace detection [5,6].

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Fig. 1. Salcatonin peptide is comprised of 32 amino acid residues with a 1–7 disulfide bridge and C-terminal amidation. The leucine amino acid residue at position 19 is $^{13}C_6$-labeled.

Mass spectrometric techniques can be applied towards the high sensitivity qualitative and quantitative study of many compounds, especially proteins and peptides. Developments in mass spectrometer design have resulted in improved detection of biomolecules approaching the levels routinely achieved with immunoassays without the corresponding shortcomings. Recently, several LC ESI/MALDI TOF methods incorporating stable-labeled isotope tags have been developed to examine relative protein concentration [7,8]. There has been limited use of stable-labeled proteolytic peptides or polypeptides for analytical assays of proteins [9]. A recent review by Julka and Regnier [10] provides further information on application of stable isotope techniques for protein and peptide determination.

Qualitative mass spectrometric approaches have been used to characterize human or salmon calcitonins in different biological matrices [11,12]. A LC-MS method was reported by Song et al. [13] for the quantitative determination of sCT in rat and dog serums without internal standard, which makes accurate quantitation more challenging than with our approach.

Here we describe the first μLC-TOF/MS assays employing labeled internal standard for the determination of sCT in microliter volumes of human urine or human plasma with low pmole/mL lower limit-of-quantitation (LLOQ). Our results demonstrate that this approach can be used to rapidly generate quantitative data about the absolute concentration of polypeptides in biological matrices. The approach may be directly and dependably applicable to many current bioanalytical needs in pharmacokinetic studies, degradation product assessment and other areas related to biology, clinical chemistry and proteomics.

2. Materials and methods

2.1. Reagents and chemicals

All solvents and reagents were HPLC grade and were used without further purification.

Salcatonin was purchased from Bachem, King of Prussia, PA, USA. Synthetic $^{13}C_6$-labeled salcatonin was purchased from SynPep (Dublin, CA, USA). The purity of the synthetic labeled peptide was assessed by μLC with UV and mass spectrometric detection. No extraneous peak was detected in the UV and total ion mass chromatograms. These data and complementary information provided by the supplier indicated that the purity of both compounds was >99.9%. Human urine and EDTA salcatonin-free plasma, previously screened for infectious pathogens, were purchased from Biological Specialty Corporation (Colmar, PA, USA).

2.2. Calibration standards and quality control samples

Stock solutions of salcatonin and $^{13}C_6$-salcatonin were prepared in a mixture of acetonitrile: 0.1% TFA 1:1 (v/v) and stored at −20 °C. Prior to use, blank urine and plasma samples were screened for the presence of potential interfering compounds at the retention time and *m*/*z* of salcatonin and $^{13}C_6$-labeled salcatonin using the extraction methods and μLCMS analysis procedures described below. Calibration standards and quality control (QC) samples were freshly prepared using the following procedure: blank human urine and plasma samples were allowed to thaw on ice. Two hundred microliter aliquots were placed in individual 1.5 mL eppendorf tubes. Samples were spiked with appropriate amounts of salcatonin spiking solutions prepared by serial dilution of the stock solutions. The calibration standards and QC samples were used to assess precision and accuracy and determine the LLOQ. Sets of five calibration standards ranging from 10 to 1000 ng/mL (urine) and from 25 to 2500 ng/mL (plasma) were prepared in triplicate. For the urine assay, QC samples at low (30 ng/mL), medium (400 ng/mL) and high (700 ng/mL) concentrations were prepared as described above. In the case of the plasma assay, the concentration of the QC samples was 75, 1000 and 1750 ng/mL, respectively.

3. Sample preparation procedures

3.1. Urine

In a typical experiment, 20 μL of the internal standard (ISTD) solution spiked at a concentration of 10 μg/mL was added to 200 μL aliquots of freshly prepared calibration standard and QC samples in blank human urine so as to obtain a final ISTD concentration of 910 ng/mL. The resulting mixture was vortexed and centrifuged at 16,000 rpm for 1 min. A portion of the supernatant was transferred to a standard HPLC vial containing a 100 μL glass insert. One μL was applied directly to the μLCMS. For the 10 ng/mL LLOQ sample, this corresponds to an on-column injection of 9.1 pg or 2.65 fmole of sCT.

3.2. Plasma

Fifty microliters of a 10 μg/mL ISTD spiking solution was added to 200 μL aliquots of freshly prepared calibration standard and QC samples so as to obtain a final ISTD concentration of 2.0 μg/mL. Proteins were precipitated by the addition of 200 μL acetonitrile and the mixture was centrifuged at 16,000 rpm for 1 min. The supernatant was transferred into a 1.5 mL eppendorf tube and lyophilized. The dry residue was reconstituted in 5 μL of a mixture of acetonitrile:water 0.1% TFA (1:1, v/v), and the resulting solution transferred into a HPLC vial containing a 100 μL glass insert. One microliter was applied directly to the μLCMS. If we assume that the recovery of sCT is quantitative, this corresponds to an on-

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