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Short communication

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

Mike Aguiar[a], Bernard F. Gibbs[a,b], Robert Massé[a,*]

[a] *MDS Pharma Services, Applied R&D, Bioanalysis Department, 2350 Cohen Street, Montreal, Que., Canada H4R 2N6*
[b] *Endocrinology Division, Faculty of Medicine, McGill University, Montreal, Que., Canada*

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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.

Keywords: Salcatonin; Capillary chromatography; Time-of-flight mass spectrometry; Quantitation; Isotope dilution; Plasma; Urine

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].

* Corresponding author. Tel.: +1 514 333 0042x4196; fax: +1 514 335 8347.
E-mail address: robert.masse@indsinc.com (R. Massé).

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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-

column injection of 1.0 ng or 291.5 fmole for the 25 ng/mL LLOQ sample.

3.3. Capillary liquid chromatography conditions and instrumentation

Gradient HPLC methods were used for chromatographic separation on an Agilent 1100 capillary liquid chromatography system equipped with an in-line degasser and a diode array detector (Agilent Technologies, Palo Alto, CA, USA) set to detect at 206 and 280 nm. A Zorbax SB-C18 300Å 250 mm × 0.3 mm i.d. capillary column was used. The gradients employed two mobile phases (A and B). Mobile phase A was a 0.1% TFA in water and mobile phase B consisted of 0.1% TFA in acetonitrile. All samples were chromatographed at a flow rate of 5 μL/min. Chromatographic conditions were optimized separately for urine and plasma samples. Urine samples were chromatographed using a 10 min linear gradient where mobile phase B was increased from 10 to 70% B and maintained for 10 min to ensure that all major components in the sample had eluted, as revealed by the UV signal at 206 nm. Only then was the solvent composition reset to the starting conditions (i.e. 90% A/10% B) and the system allowed to re-equilibrate. For plasma samples, the same 10–70% B gradient was extended over a period of 30 min for adequate chromatographic resolution of the analyte of interest from extraneous peaks.

3.4. Mass spectrometric analysis

The effluent from the LC column was directly coupled to the electrospray source of a QSTAR Pulsari hybrid tandem time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) operated in the enhanced TOF-MS mode, scanning positive ions from *m/z* 800–1300 Da. Typically, a voltage of 5.5 kV was applied to the electrospray needle for ionization, while declustering potentials were adjusted to 50 V and curtain gas was set at 25 psig. The mass spectrometer was mass calibrated prior to sample analysis using cesium iodide (Cs^+ *m/z* 132.9054) and an octapeptide (*m/z* 829.5398). MS parameters were optimized by infusing a reference solution of the analyte.

3.5. Quantitation

Extracted ion chromatograms for the mass ranges of *m/z* 1144–1145 (salcatonin) and *m/z* 1146–1147 ($^{13}C_6$-labeled salcatonin) were obtained for all samples. The mass spectrum of the peak of interest in the extracted ion chromatograms was used to verify that the signal was as a result of the triply charged $[M + 3H]^{3+}$ ions. Quantitation was based on the peak height of the signal for the triply charged species of salcatonin and its $^{13}C_6$-labeled analog used as internal standard. Standard calibration curves were constructed by plotting the corresponding peak height ratios against five standard concentrations of salcatonin spiked in blank human urine or human plasma and analysed by least square linear regression using a Microsoft Excel spreadsheet.

4. Results and discussion

4.1. Mass spectrometry of salcatonin and $^{13}C_6$-leucine-labeled salcatonin

Salcatonin and its $^{13}C_6$-leucine-labeled synthetic analog were examined by capillary LC TOF/MS to confirm their identity by high mass accuracy and the expected mass increment due to the stable $^{13}C_6$-labeling of salcatonin. As illustrated in Fig. 2a and b, both peptides have similar mass spectral profiles dominated by a triply-charged ion at 1144.82 and 1146.53 Da, respectively. The high relative intensity of the $[M + 3H]^{+3}$ ion and the absence of extensive product ions was achieved by selecting appropriate mass spectrometric operating parameters. The distribution of the isotopic peaks for both $[M + 3H]^{+3}$ ions (Fig. 3a and b) verifies their charge state and indicates the absence of any mass overlap between the two compounds, thus confirming that the isotopic purity of the ^{13}C-labeled analogue was >99.9%. Although tandem MS multiple reaction monitoring (MRM) experiments provide better signal to noise ratios with crude samples than TOF/MS methods, the high mass resolution of the latter technique provides for unambiguous identification of the analyte and unique selectivity of detection, particularly in complex biological matrices.

4.2. Human urine method

Our minimal sample preparation approach was ideal since it minimizes the degradation of the analytes. The μLC-QSTAR combination provides sufficient resolving power for the clean separation and identification of the analytes. Specificity, linearity, accuracy and precision of salcatonin quantitation were assessed for the 10–1000 ng/mL concentration range with a 1 μL on-column sample requirement which correspond to the injection of 2.65 fmole for the LLOQ sample. Specificity was assessed in three different batches of blank urine, in which the ionic background noise at the retention time of salcatonin was less than 25% of the height of the LLOQ calibration standard peak. A representative total ion chromatogram of a 10 ng/mL LLOQ sample is shown in Fig. 4a. The corresponding salcatonin extracted ion chromatogram at *m/z* 1144–1145 (Fig. 4b) shows the peak of interest with a signal to noise ratio of about 10:1, whereas that of the internal standard at *m/z* 1146–1147 is displayed in Fig. 4c. The lower limit of detection was approximately 3.6 ng/mL or 3.6 pg (1.05 fmole) on-column. The fact that salcatonin and its ^{13}C-labeled analog co-elute greatly minimized potential differences in ionization efficiency (e.g. matrix effect) which are likely to arise in non-isotope dilution methods where the internal standard and the analyte of interest display different chromatographic behavior. This specific feature of our

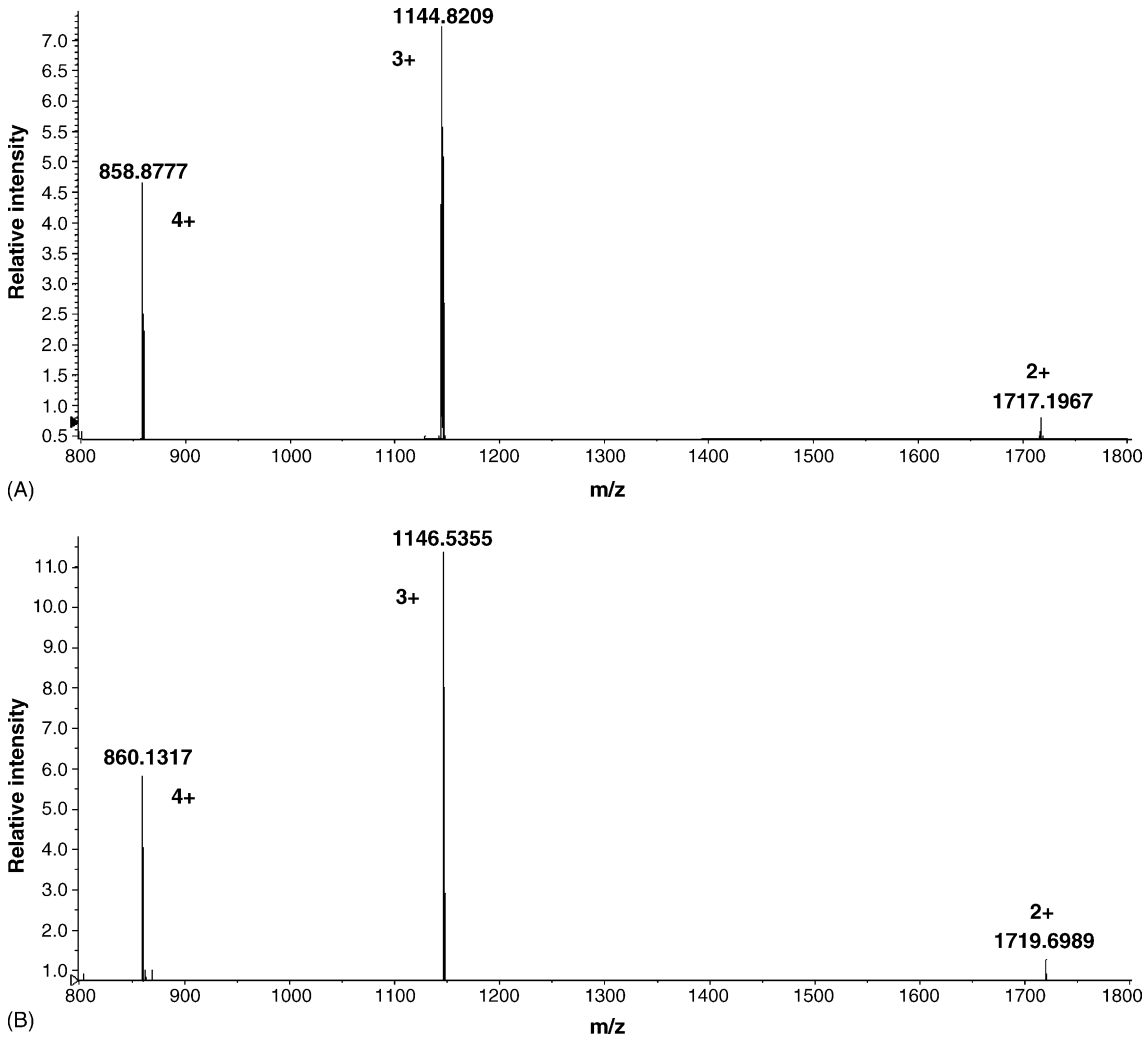

Fig. 2. ESI TOF/MS of (A) salcatonin and (B) $^{13}C_6$-salcatonin obtained by μLC-TOF/MS analysis of a reference solution. Data displays only the range from 800 to 1800 Da that contained the multi-charged species of interest.

approach maximizes the precision and accuracy of the measurements.

A linear regression expressed by the equation $y = 0.00011x + 0.0157$ gave an excellent fit for the detector response to concentration relationship with a coefficient of correlation (R^2) of 1.0000. Calibration standards were measured with an accuracy (% bias) ranging from 100.5 to 106.4%. Three replicates of QC samples afforded within-run % bias of 105.7, 111.8 and 110.4% for low, medium and high QC concentrations respectively (Table 1). These results demonstrate that salcatonin can be measured with good precision and accuracy in human urine using a crude sample preparation procedure combined with μLC-TOF/MS.

4.3. Human plasma method

The determination of salcatonin in plasma was more challenging due to the very complex nature of this matrix. Although the sample preparation procedure was straightforward, chromatographic resolution of salcatonin from abundant endogenous matrix components was achieved using an extended gradient μLC method. A representative total ion chromatogram of a 25 ng/mL (7.3 pmole/mL) LLOQ sample is shown in Fig. 5a, along with the corresponding extracted ion chromatograms of salcatonin (Fig. 5b) and its $^{13}C_6$-leucine-labeled analog (Fig. 5c). Even with the extended gradient, a 10-fold decrease was observed in the detection of internal standard signal of the plasma extracts relative to the urine samples as illustrated in Figs. 4c and 5c where 1 and 100 ng of internal standard was applied to the system, respectively. This difference was most likely due to severe ion suppression by the complex matrix of the precipitated plasma samples. The intensity of the background noise arising from co-eluting material in blank plasma samples was found to be lower than 25% that of the LLOQ sample so that a signal to noise ratio of 5/1

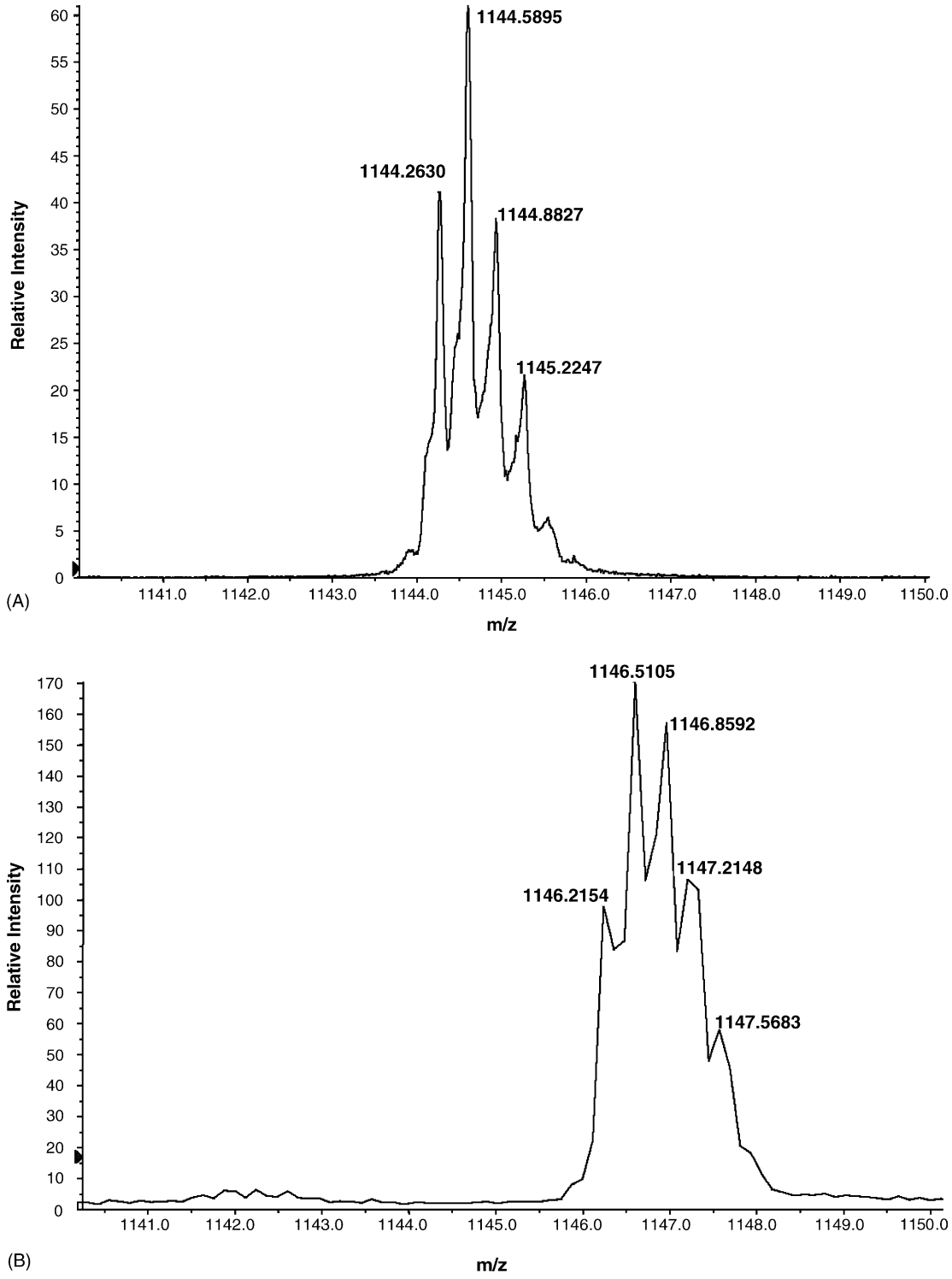

Fig. 3. Isotopic distribution of for the triply charged species of (A) salcatonin and (B) $^{13}C_6$-leucine labeled salcatonin internal standard. The monoisotopic ions at *m/z* 1144.2630 and *m/z* 1146.2154 were measured with an accuracy of 22.2 and 19.4 ppm from their respective theoretical values.

or greater was routinely achieved. The normalized peak heights were shown to be linear with concentration over the 25–2500 ng/mL range and the corresponding relationship is expressed by the equation $y = 0.0109x + 0.0179$ with a coefficient of correlation (R^2) equal or better than 0.9998. The mean back-calculated concentrations of the calibration standards were between 76.3 and 100.1% and QC samples prepared at 75, 1000 and 1750 ng/mL salcatonin were

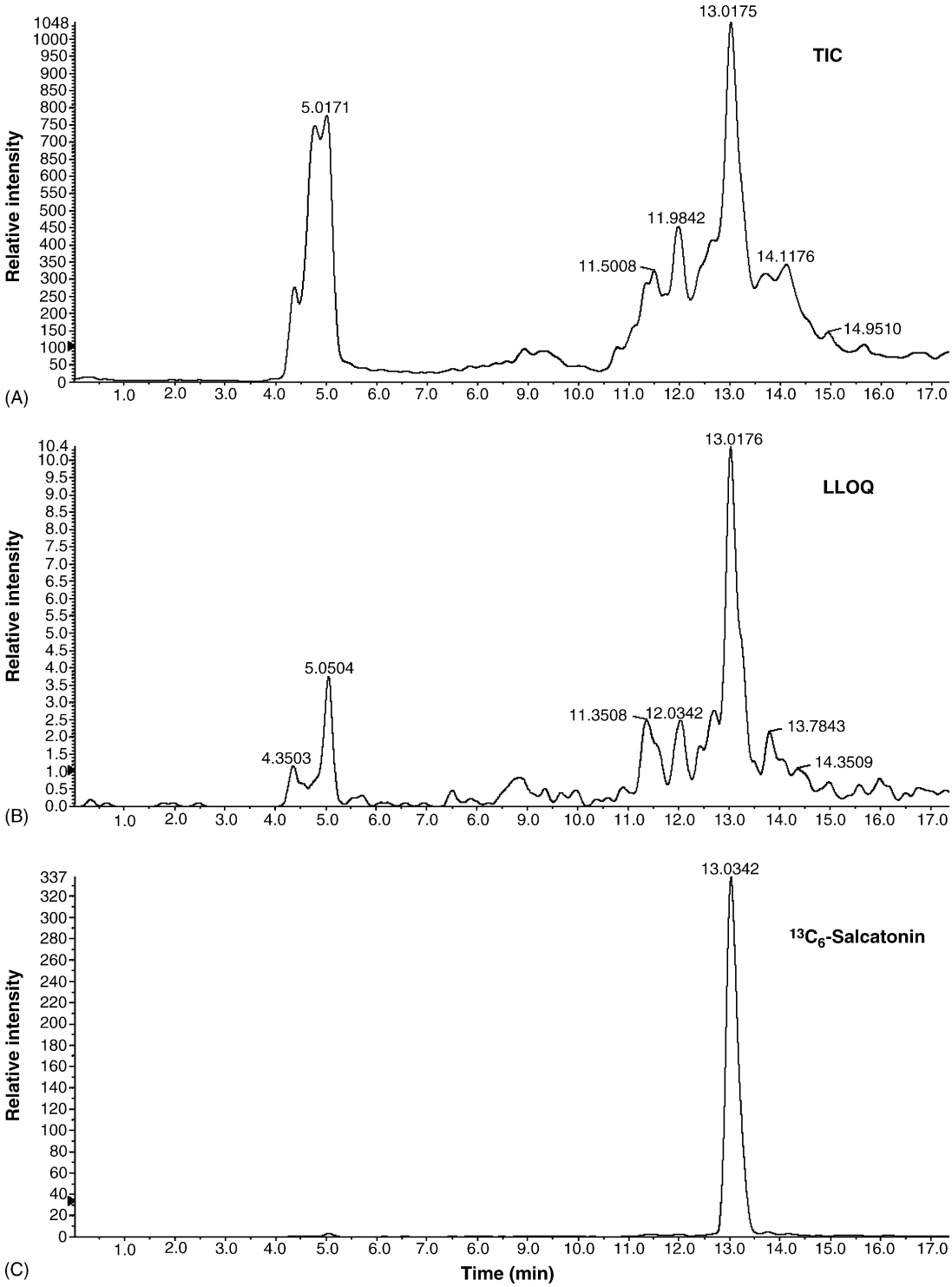

Fig. 4. Analysis of a lower limit of quantitation (10 ng/mL) for salcatonin in human urine: (A) μLC TOF/MS total ion chromatogram obtained by scanning positive ions from 800 to 1300 Da, (B) extracted ion chromatogram for salcatonin over the mass range 1144–1145 Da and (C) extracted ion chromatogram for the $^{13}C_6$-leucine labeled salcatonin internal standard over the mass range of 1146–1147 Da. Conditions: mobile phase A was a 0.1% TFA solution in water and mobile phase B consisted of 0.1% TFA in acetonitrile, a 10 min linear gradient was used at a flow rate of 5 μL/min where mobile phase B was increased from 10 to 70% B and maintained for 10 min.

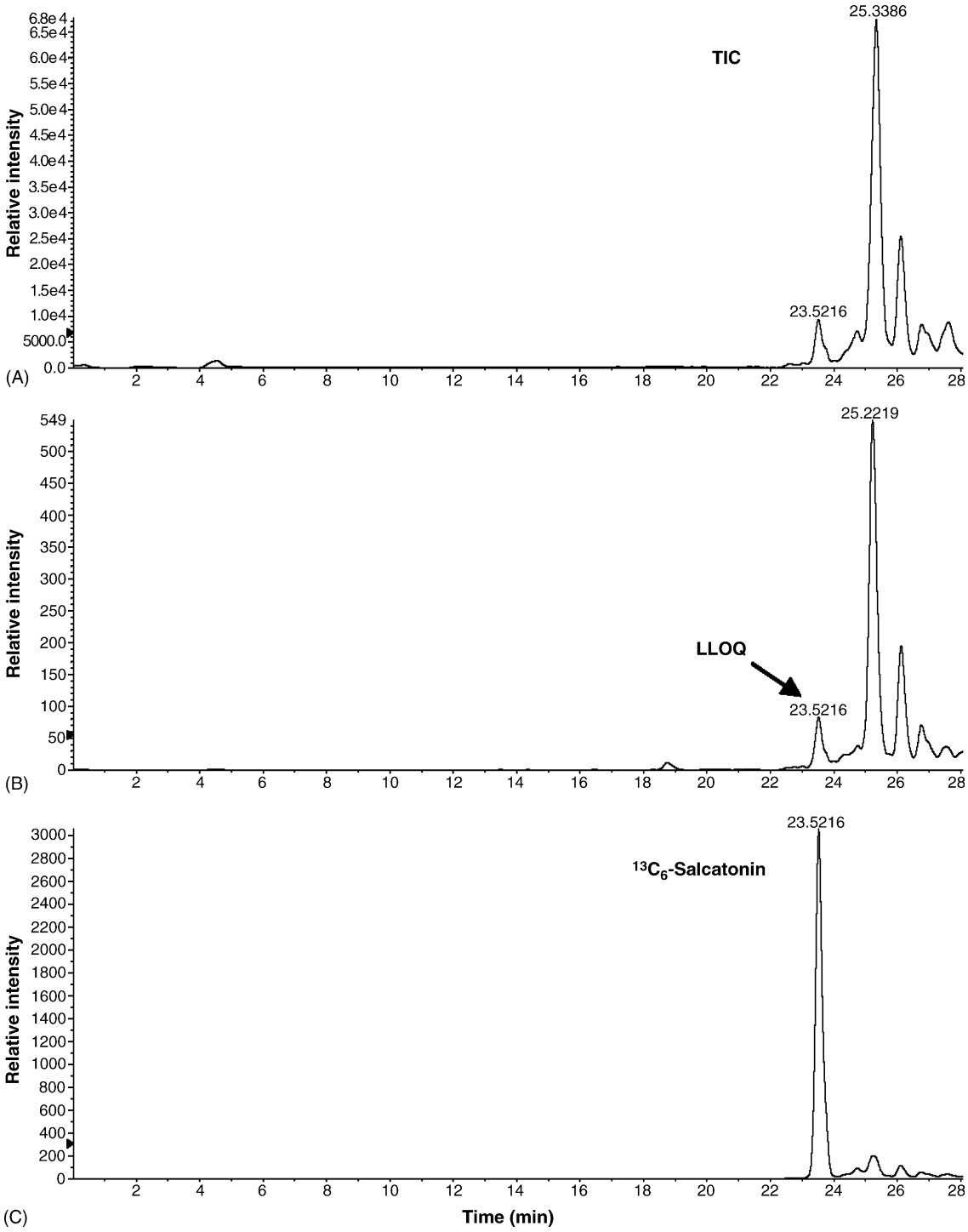

Fig. 5. Analysis of a lower limit of quantitation (25 ng/mL) for salcatonin in human plasma: (A) μLC-TOF/MS total ion chromatogram obtained by scanning positive ions from 800 to 1300 Da, (B) extracted ion chromatogram of salcatonin over the mass range 1144–1145 Da and (C) extracted ion chromatogram for the $^{13}C_6$-leucine labeled salcatonin internal standard over the mass range of 1146–1147 Da. Chromatographic conditions are as in Fig. 4 except that the gradient is over 30 min rather than 10 min.

analyzed with excellent precision and accuracy (Table 1) as indicated by their respective % standard deviation values ranging from 0.77 to 1.49% and % bias varying between 98.6 and 112.9%. Our μLC-TOF/MS method compared advantageously with that reported by Song et al. [13] where on-column injection of 200 pg (58.3 fmole) salcatonin was necessary to achieve a LLOQ of 10 ng/mL in rat and dog serum.

Table 1
Precision and accuracy data for human urine and human plasma calibration standards and QC samples

Calibration standards			QC samples			
Concentration (ng/mL)	Calculated concentration (ng/mL)[a]	Accuracy (% bias)[b]	Concentration (ng/mL)	Calculated concentration (ng/mL)[a]	% CV[c]	Accuracy (% bias)[b]
Human urine						
10	10.6	106.4	30	31.7	9.5	105.7
20	20.4	102.1	400	447.2	2.3	111.8
500	502.6	100.5	700	773.2	1.8	110.5
800	805.8	100.7				
1000	1009.1	100.9				
Human plasma						
25	19.1	−23.6	75	73.9	1.5	98.5
50	41.9	−16.2	1000	1129.1	0.8	112.9
1250	1284.1	2.7	1750	1841.1	0.8	105.2
2000	2002.1	0.1				
2500	2497.0	−0.1				

[a] Concentration results were rounded to one decimal place.
[b] % Bias is the calculated salcatonin concentration expressed as a percentage of its nominal concentration.
[c] % CV was calculated from three individual results at each salcatonin concentration.

5. Conclusion

The combination of capillary column chromatography with the use of a $^{13}C_6$-labeled salcatonin analog as an internal standard permits the development of versatile and straightforward assays. Optimal sensitivities were obtained with minimal fragmentation and quantitation was based on the triply charged species of salcatonin and its internal standard. Sample volume requirements were in the microliter range.

The data generated from both the plasma and the urine assays afforded similar precision and accuracy. The analytical approach employed can be used to monitor salcatonin in human urine and plasma since no significant interference by endogenous peptides or proteins were observed. Also, it could be applied to assess salcatonin purity and its degradation products in pharmaceutical preparations and in the course of stability studies. Whilst the instrumentation used can afford detection in the attomole range, the LLOQs presented in this paper were essentially limited by the intensity of chemical background, which was pronounced in plasma extracts. Affinity extraction methods such as immuno-precipitation combined with capillary or nano LC may provide solutions for the removal of the excessive chemical background noise observed in plasma samples, thus allowing the quantitation of salcatonin in the low fmole/mL range. The specificity of a mass spectrometric method with good sensitivity is an appropriate alternative to established methods for the measurement of salcatonin.

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