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Quality analysis of salmon calcitonin in a polymeric bioadhesive pharmaceutical formulation: Sample preparation optimization by DOE

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ABSTRACT

A sensitive and selective HPLC method for the assay and degradation of salmon calcitonin, a 32-amino acid peptide drug, formulated at low concentrations (400 ppm m/m) in a bioadhesive nasal powder containing polymers, was developed and validated.

The sample preparation step was optimized using Plackett–Burman and Onion experimental designs. The response functions evaluated were calcitonin recovery and analytical stability. The best results were obtained by treating the sample with 0.45% (v/v) trifluoroacetic acid at 60 °C for 40 min. These extraction conditions did not yield any observable degradation, while a maximum recovery for salmon calcitonin of 99.6% was obtained. The HPLC-UV/MS methods used a reversed-phase C_{18} Vydac Everest column, with a gradient system based on aqueous acid and acetonitrile. UV detection, using trifluoroacetic acid in the mobile phase, was used for the assay of calcitonin and related degradants. Electrospray ionization (ESI) ion trap mass spectrometry, using formic acid in the mobile phase, was implemented for the confirmatory identification of degradation products. Validation results showed that the methodology was fit for the intended use, with accuracy of 97.4 ± 4.3% for the assay and detection limits for degradants ranging between 0.5 and 2.4%.

Pilot stability tests of the bioadhesive powder under different storage conditions showed a temperature-dependent decrease in salmon calcitonin assay value, with no equivalent increase in degradation products, explained by the chemical interaction between salmon calcitonin and the carbomer polymer.

1. Introduction

Calcitonin is a 32-amino acid polypeptide hormone with an intramolecular disulphide bridge between cysteine residues at positions 1 and 7, and an amidated carboxy-terminal. It was first discovered in 1962 by Copp and Cheney [1–3]. Human calcitonin plays an important role in calcium metabolism as it reduces blood calcium levels, opposing the effects of parathyroid hormone. It is also a potent inhibitor of osteoclastic bone resorption [4]. In order to exert this biological activity, following three structural features are necessary: disulphide bridge between residues 1 and 7; eight specific amino acid residues at the N-terminus and a proline amide moiety at the C-terminus [5]. Other mammals and fish also produce calcitonin. For medical purposes, only salmon, eel and porcine calcitonin are applicable. The sequences, all containing the three essential structural features, of these aforementioned calcitonins are presented in Table 1. Salmon calcitonin (sCT), which differs in 16 amino acid residues compared to human calcitonin, is about 20–30 times more potent than human calcitonin, which is the second most potent of all calcitonin species [6]. Salmon calcitonin is indicated for treatment of postmenopausal osteoporosis and Paget's disease of the bone, either alone or in combination with other drugs [7].

Commercially, sCT is produced either by chemical synthesis or by microbial processes using recombinant DNA technology [8,9]. Given the polypeptide nature of sCT, parenteral administration is common [10]. Alternatively, liquid nasal spray administration is preferred by most patients. However, the biological availability after nasal administration of the sCT solution relative to parenteral injection is considerably lower, *i.e.* 3–5%. In order to further improve therapy, research has focused on alternative administration routes, such as colonic, rectal, buccal, pulmonary and oral administration [11–15]. Nevertheless, sCT is currently only available as an injectable form, *e.g.* Miacalcic® (Novartis; 1997 FDA approved) and as a liquid nasal spray, *e.g.* Fortical® (Unigene, 2005 FDA approved). Novel controlled release formulations based on a polymer matrix

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Table 1
Calcitonin species sequences.

Product	Sequence[a]	Molecular formula	Monoisotopic mass (g/mol)
Salmon calcitonin	H-Cys[1]-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro[32]-NH_2	$C_{145}H_{240}N_{44}O_{48}S_2$	3429.7132
Human calcitonin	H-Cys[1]-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro[32]-NH_2	$C_{151}H_{226}N_{40}O_{45}S_3$	3415.5787
Porcine calcitonin	H-Cys[1]-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Ser- Ala-Tyr-Trp-Arg-Asn-Leu-Asn-Asn-Phe-His-Arg-Phe-Ser-Gly-Met-Gly-Phe-Gly-Pro-Glu-Thr-Pro[32]-NH_2	$C_{159}H_{232}N_{46}O_{45}S_3$	3601.6441
Eel calcitonin	H-Cys[1]-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-Thr-Pro[32]-NH_2	$C_{146}H_{242}N_{43}O_{47}S_2$	3413.7308
Rat calcitonin	H-Cys[1]-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Leu-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ser-Ile-Gly-Val-Gly-Ala-Pro[32]-NH_2	$C_{148}H_{228}N_{40}O_{46}S_3$	3397.5892

[a] All contain a S–S bridge between Cys^1 and Cys^7.

from which the API diffuses have several advantages: reducing the number of administrations results in increased patient compliance, more constant drug levels are achieved over a longer period (incl. the night) and rapid clearance of sCT is reduced. An example of such a novel formulation comprises salmon calcitonin (400 ppm) in a starch and carbomer containing matrix, which was specially developed for the nasal administration of vaccines and peptides [16,17]. After nasal application of 25 mg powder, a theoretical amount of 60 IU sCT, equivalent to 10 μg [8], is delivered in the nasal cavity. Given the low amount of sCT, *i.e.* 60 IU (or 10 μg) sCT per 25 mg powder (400 ppm), potential degradation products in the sample will be present in even lower concentration ranges.

Several methods are described for sCT assay, which can be divided into three major groups: direct fluorescence [11,18], immunoassay [19,20] and LC-UV/MS methods [5,8,21–25] (see supplementary Table S-1 for detailed overview). However, all these methods were evaluated for liquid samples (drug solutions and/or biological fluids), requiring much less or almost no sample preparation. Moreover, most of these methods do not consider salmon calcitonin related degradants (see supplementary Table S-2). Hence, these methods are neither directly usable nor well suited for stability studies of sCT in solid dosage forms.

The goal of this research was to develop a suitable method for quantification and degradation profiling of sCT in a powder. Because of the low salmon calcitonin concentration formulated in a polymeric bioadhesive matrix, emphasis was given towards the sample preparation step. Using this developed method, the short-term stability of the novel nasal formulation under different storage conditions was evaluated. These results are indicative for a chemical interaction between the peptide and the carbomer polymer.

2. Materials and methods

2.1. Materials

Acetonitrile (LC–MS grade) was purchased from Fisher Scientific (Doornik, Belgium). Water was purified using an Arium 611 purification system (Sartorius, Gottingen, Germany) yielding ≥18.2 MΩ cm quality water. LC–MS grade formic acid and trifluoroacetic acid were obtained from Fluka (Bornem, Belgium). Synthetic salmon calcitonin as well as the other species-derived calcitonins were bought from Bachem (Bubendorf, Switzerland). The nasal bioadhesive powder was obtained by adding a premanufactured mixture of Amioca® starch and Carbopol® 974P carbomer (National Starch and Chemical Company, Bridgewater, NJ, USA) to distilled water, 2 M sodium hydroxide (VWR, Haasrode, Belgium) and Miacalcic® (100 IE/ml) (Novartis Pharma, Vilvoorde, Belgium). Next, this aqueous dispersion is freeze-dried, thus obtaining the lyophilized nasal powder [26]. One milligram of powder contains 0.395 μg salmon calcitonin, 47.399 μg glacial acetic acid, 47.399 μg sodium acetate, 177.747 μg sodium chloride, 148.123 μg Amioca® starch and 578.937 μg Carbopol® 974P.

2.2. Liquid chromatography

The HPLC apparatus consisted of a Waters Alliance 2695 separations module and a Waters 2496 Photodiode array detector with Empower 2 software for data acquisition (all Waters, Milford, MA, USA). For sCT assay, a Vydac Everest RP-C_{18} (250 mm × 4.6 mm I.D., 5 μm particle size, 300 Å) column (Grace Vydac, Hesperia, CA, USA) was used in an oven set at 40 °C. The flow rate was set at 1.0 ml/min and the linear gradient used was as follows (where *A* = 0.1% (w/v) MS grade trifluoroacetic acid (TFA) in water and *B* = 0.085% (w/v) MS grade trifluoroacetic acid in MS grade acetonitrile): *t* = 0 min, *A*:*B* (73:27, v/v); *t* = 0–20 min, *A*:*B* (63:37, v/v); *t* = 20–21 min, *A*:*B* (73:27, v/v); *t* = 21–40 min, *A*:*B* (73:27, v/v). The sample compartment was thermostated at 20 °C and UV quantification was performed at 195 nm.

The LC–MS apparatus, used for sCT degradation profiling, consisted of a Thermo Spectra System SN4000 interface, a Spectra System SCM 1000 degasser, a Spectra System P1000XR Pump, a Spectra System AS3000 autosampler and a Finnigan LCQ Classic ion trap mass spectrometer in positive ion mode (all Thermo, San José, CA, USA) equipped with a Waters 2487 dual wavelength UV detector (Waters, Milford, MA, USA) and Xcalibur 2.0 software (Thermo, San José, CA, USA) for data acquisition. ESI was conducted using a needle voltage of 4.5 kV. Nitrogen was used as the sheath and auxiliary gas with the heated capillary set at 250 °C. Mass spectra, which were deconvoluted manually, were obtained by selected ion monitoring (SIM) at 858.8, 859.9, 863.3, 866.8, 869.3 and 902.2 amu (± 0.5 amu), corresponding with sCT and the most abundant degradation products, respectively. LC–MS analysis for degradation profiling was performed using a Vydac Everest RP-C_{18} (250 mm × 4.6 mm I.D., 5 μm particle size, 300 Å) column (Grace Vydac, Hesperia, CA, USA) in an oven set at 30 °C. The gradient used for the separation was as follows (where *A* = 0.1% (w/v) MS grade formic acid (FA) in water and *B* = 0.1% (w/v) MS grade formic acid in MS grade acetonitrile): *t* = 0 min, *A*:*B* (80:20, v/v); *t* = 0–60 min, *A*:*B* (65:35, v/v); *t* = 60–61 min, *A*:*B* (80:20, v/v); *t* = 61–80 min, *A*:*B* (80:20, v/v). The flow rate was set at 1.0 ml/min and sample compartment was thermostated at 20 °C. The injection volume used was 100 μl.

2.3. Design of experiments

Different experimental designs were used to obtain a suitable sample preparation procedure.

Table 2
Plackett–Burman screening design results.

Exp. #	Factors											sCT recovery (%)
	HPβCD (mg/ml)	Dummy 1	*T* (°C)	Dummy 2	Time (h)	Dummy 3	Steps	Dummy 4	Mixing speed (rpm)	Dummy 5	FA conc (%v/v)	
– Level	0	n.a.	50	n.a.	1	n.a.	1	n.a.	300	n.a.	1	n.a.
+ Level	10	n.a.	70	n.a.	2	n.a.	2	n.a.	600	n.a.	5	n.a.
1	+	+	–	+	+	+	–	–	–	+	–	53.72
2	–	+	+	–	+	+	+	–	–	–	+	75.13
3	+	–	+	+	–	+	+	+	–	–	–	36.26
4	–	+	–	+	+	–	+	+	+	–	–	54.08
5	–	–	+	–	+	+	–	+	+	+	–	50.09
6	–	–	–	+	–	+	+	–	+	+	+	53.96
7	+	–	–	–	+	–	+	+	–	+	+	92.92
8	+	+	–	–	–	+	–	+	+	–	+	83.16
9	+	+	+	–	–	–	+	–	+	+	–	71.58
10	–	+	+	+	–	–	–	+	–	+	+	73.86
11	+	–	+	+	+	–	–	–	+	–	+	80.43
12	–	–	–	–	–	–	–	–	–	–	–	43.32
Effect	11.28	9.09	1.03	10.65	7.37	10.65	−0.11	2.04	3.01	3.95	25.07	

Critical effect value: 20.85.

2.3.1. Plackett–Burman screening design

In order to evaluate the effects of different extraction variables on the sCT recovery from the polymeric matrix, a Plackett–Burman screening design was initially used. The selected variables include (low–high levels) hydroxypropyl-β-cyclodextrin (HPβCD) concentration (0–10 mg/ml), incubation temperature (50–70 °C) and time (1–2 h), number of steps used (1–2), mixing speed (300–600 rpm) and formic acid concentration (1–5% v/v).

The experiments were conducted on placebo matrix, consisting of a starch and carbomer mixture, spiked with Miacalcic® at 400 ppm sCT. The volume of the extraction solvent, comprising the extraction levels as indicated in the design setup (see Table 2), was kept constant at 1.6 ml. After incubation, test samples were analyzed as mentioned in Section 2.4. The response factor used was sCT recovery. Statistical analysis was performed on the results by comparing the effects of each variable calculated to the critical E value ($P = 0.05$), derived from the dummy effect values.

2.3.2. Onion design

In order to obtain higher sCT recovery values, a sample extraction procedure containing trifluoroacetic acid (pKa = 0.3), a stronger acid compared to formic acid (pKa = 3.8), was developed. Three different extraction variables were included (low–high range): trifluoroacetic acid concentration (0.1–0.75% v/v), incubation temperature (20–70 °C) and incubation time (30–90 min). An Onion design was selected seeing the large experimental space and the ability to obtain a quadratic model with a limited number of experiments. An overview of the applied design is presented in Table 3. The response factors evaluated were sCT recovery and degradation. The experiments were conducted on a placebo matrix, consisting of a starch and carbomer mixture, spiked with Miacalcic® at 400 ppm sCT. After incubation, corresponding to the Onion design conditions, test samples were analyzed as mentioned in Section 2.4. From the Onion design results, a mathematical recovery model was deduced using the partial least squares (PLS) technique.

2.3.3. Plackett–Burman robustness evaluating design

In order to evaluate the robustness of the sample methodology obtained from the Onion design model, a Plackett–Burman design was formulated. The variables were identical as those evaluated in the Onion design and three different levels (–/0/+) were selected, *i.e.* TFA concentration (0.45, 0.55 and 0.65 (% v/v)), temperature (50, 55 and 60 °C) and incubation time (40, 45 and 50 min). The '0' level corresponds with the optimal extraction levels as deduced from the mathematical recovery model obtained from the Onion design (see Section 2.3.2), and was used to experimentally confirm the

Table 3
Onion design.

Exp. #	TFA conc. (% v/v)	*T* (°C)	Time (min)	Experimental recovery (%)[a]	Degradation (%)[b]	Calculated sCT recovery (%)[c]
1	0.2625	32.5	45	73.16	–	72.99
2	0.5875	57.5	45	92.21	–	94.62
3	0.5875	32.5	75	87.90	–	85.13
4	0.2625	57.5	75	94.23	5.7	89.62
5	0.1000	20.0	30	46.69	–	53.65
6	0.7500	20.0	30	75.70	–	70.78
7	0.1000	70.0	30	85.11	–	79.77
8	0.7500	70.0	30	85.50	16.11[d]	96.90
9	0.1000	20.0	90	49.70	–	60.78
10	0.7500	20.0	90	67.99	–	77.92
11	0.1000	70.0	90	89.14	–	86.90
12	0.7500	70.0	90	109.10	7.17[e]	104.03
13	0.1000	45.0	60	67.83	–	70.28
14	0.4250	20.0	60	60.31	–	74.78
15	0.4250	45.0	30	82.53	–	84.27
16	0.4250	45.0	60	103.84	–	87.84

[a] Recovery was calculated as follows: (peak area sCT test solution/peak area sCT reference solution) × 100.
[b] Degradation (%) was calculated using area normalization relative to the sum of all areas (calcitonine and degradants). Reporting threshold: 0.2%.
[c] Calculated by Onion design model.
[d] Identified as deamidated sCT.
[e] Identified as sCT epimer.

Table 4
Confirming Plackett–Burman design TFA extraction.

Exp. #	Factors							sCT recovery (%)
	Dummy 1	TFA conc. (% v/v)	Dummy 2	T (°C)	Dummy 3	Time (min)	Dummy 4	
− Level	n.a.	0.45	n.a.	50	n.a.	40	n.a.	n.a.
+ Level	n.a.	0.65	n.a.	60	n.a.	50	n.a.	n.a.
1	+	+	−	−	+	−	+	86.71
2	−	+	+	+	−	−	+	94.03
3	−	+	−	+	+	+	−	92.74
4	+	−	−	+	−	+	+	96.29
5	+	+	+	−	−	+	−	79.90
6	+	−	+	+	+	−	−	99.63
7	−	−	+	−	+	+	+	83.25
8	−	−	−	−	−	−	−	84.51
9	0	0	0	0	0	0	0	92.45
10	0	0	0	0	0	0	0	94.95
11	0	0	0	0	0	0	0	92.75
Effect	2.00	−2.57	−0.86	12.08	1.90	−2.57	0.87	

Critical effect value: 6.49.

predicted sCT recovery. Considering the evaluation of the model robustness, the experimental space around the optimal extraction levels is kept relatively small. Also, 4 dummy variables were included in the 11 experiments comprising the design setup (see Table 4). The experiments were conducted using a placebo matrix, consisting of amioca starch and carbomer mixture, spiked with 60 IU of Miacalcic®. Samples were incubated with 1.6 ml extraction solvent corresponding to aforementioned incubation times, temperatures and trifluoroacetic acid concentrations as specified in the design. After incubation, test samples were treated as mentioned in Section 2.4. The response factor used was sCT recovery.

2.4. Solutions

2.4.1. Reference solution

The reference solution was prepared by adding 1 ml of concentrated extraction solvent (*i.e.* aqueous solution containing 16% v/v acetonitrile (for solubility purposes) and 0.72% v/v trifluoroacetic acid) to 0.6 ml of Miacalcic® 100 IU/ml, thus resulting in a concentration of 60 IU sCT per 1.6 ml.

2.4.2. Test solution

The test solution was prepared by accurately weighing approximately 25 mg of the nasal sCT formulation in a 2.0 ml LoBind Eppendorf tube. To this sample 1.6 ml extraction solvent containing 10% v/v acetonitrile and 0.45% v/v trifluoroacetic acid was added. The test solution was subsequently incubated for 40 min at 60 (±2) °C, whilst shaking at 1400 rpm using a thermomixer comfort (Eppendorf, Hamburg, Germany). After cooling, the solution was centrifuged for 15 min at 20,000 × *g* before analysis by the HPLC and LC–MS method.

2.5. Final method evaluation

2.5.1. Forced degradation conditions

Salmon calcitonin was subjected to different stress conditions to validate the selectivity of the LC–MS identification method. Stress samples were prepared by dissolving 200 μg sCT in 670 μl peptide solvent. The peptide solvent consisted of 95 volume parts 0.1% (m/v) formic acid in H_2O and 5 volume parts 0.1% (m/v) formic acid in acetonitrile. Hereof, 100 μl was added to 100 μl phosphate buffer pH 3, 100 μl phosphate buffer pH 4, 100 μl citrate–phosphate buffer pH 6 solution and 100 μl 0.1% (v/v) aqueous hydrogen peroxide-phosphate buffer pH 7.4 solution, respectively. The acid stress solutions were heated at 70 °C, whilst the oxidation stress sample was incubated at room temperature. The total incubation period for all stress samples comprised 24 h.

Using additional dilution steps, the final concentration of stressed as well as unstressed control samples was 60 IU sCT (or 10 μg) per 1.6 ml.

2.5.2. Quantitative validation

Basic validation of the final sCT assay method, comprising a sample preparation step and HPLC-UV analysis, was performed. Following characteristics were evaluated: accuracy, linearity, precision and range. The experiments were conducted on 3 reference samples, *i.e.* 54, 60 and 66 IU sCT (corresponding with 90, 100 and 110% of the theoretical 60 IU/25 mg label claim) and 3 spiked placebo solutions, *i.e.* 15 mg of placebo powder spiked with the respective reference solutions. All samples were prepared as indicated in Section 2.4. The reference solutions were injected in duplicate, the spiked placebo solutions in triplicate.

2.6. Drug sample stability

The short-term stability of the sCT nasal powder stored under different conditions was determined. Samples were stored in freezer (−35 °C), in refrigerator (5 °C) and at room temperature (20 °C) during 6 weeks. Salmon calcitonin assay was performed using the optimized methods (see Sections 2.2 and 2.4). The different stability samples were also subjected to LC-UV/MS degradation profiling.

3. Results

3.1. Method development

3.1.1. Liquid chromatography

The influence of column temperature, *i.e.* 30, 37.5 and 45, on the sCT analysis using the HPLC-UV method was evaluated. The three conditions were compared based on symmetry factor, plate count, resolution, peak to valley ratio and peak capacity. At elevated column temperatures, *i.e.* 37.5 and 45 °C, a plateau is reached for plate count (N_0 and N_g) and peak capacity, and therefore, the intermediate, *i.e.* 40 °C was chosen as column temperature for further calcitonin assay.

Due to increased ion suppression with trifluoroacetic acid, formic acid containing mobile phases were used for MS detection. Salmon calcitonin, analyzed using aforementioned HPLC-UV and LC–MS methods, was characterized by a retention time of approximately 13.6 and 17.2 min respectively. A HPLC-UV chromatogram of sCT in a 60 IU/1.6 ml concentration is depicted in supplementary Fig. S-1.

3.1.2. Plackett–Burman screening design

Highest sCT recovery results, *i.e.* 92.92, 83.16, 80.43 and 75.13% were obtained with Plackett–Burman experiments Nos. 7, 8, 11 and 2, respectively, which were characterized by high formic acid concentration, *i.e.* 5% (v/v). These findings were supported by observing a statistically significant ($P < 0.05$) positive effect for 5% (v/v) formic acid vs. 1% (v/v) formic acid. Hydroxypropyl-β-cyclodextrin concentration, temperature, time, number of steps used and mixing speed had no statistical significant effect on recovery in the investigated ranges (see Table 2).

Although reasonable sCT recovery values were obtained in some high level formic acid samples, the average recovery value of 76.58% was clearly insufficient. This could be the result of electrostatic interactions between calcitonin and the carbomer polymer. Therefore, the use of a stronger acid like TFA (pKa = 0.3) compared to formic acid (pKa = 3.8) was evaluated, as this is expected to yield higher sCT recovery values.

3.1.3. Onion design

The sCT recovery and degradation formation, after using different extraction variable levels, are given in Table 3. The observed degradants in the test solutions, when compared to those obtained with the reference solution are: an epimer in test solution 12, deamidated sCT in test solution 8 and an unidentified degradation product in test solution 4 with a relative retention time of 0.98. Because only three degradation products were observed, no mathematical model could be established for the degradation response. Therefore, we left the 'degradation' response out of the model, but kept in mind that those specific variable levels led to a degradation product.

The initial PLS model, resulting from the Onion design, which included all interactions and second degree terms, was optimized by deleting non-significant second order and interaction effects based upon *P*-values ($P > 0.05$). The final sCT recovery model, characterized by R^2 and Q^2 values of 0.800 and 0.599, respectively, thus obtained within the experimental space was:

$$\text{sCT recovery}(\%) = 8.5670 \times \text{TFA conc.} + 13.0586 \times \text{temp.} + 3.5659 \times \text{time} - 8.9959 \times (\text{TFA conc.})^2 + 87.8381$$

The coefficients of this mathematical model were scaled and centered, meaning that they are no longer expressed according to the original measurement scales of the extraction variables, but have been re-expressed to relate to the coded −1/−0.5/0/+0.5/+1 units, whereby the minimal and maximal extraction variable levels (see Section 2.3.2) correspond to the −1 and +1 unit, respectively. This scaling and centering of the coefficients enhances the interpretability of the model [27]. For example, the constant term of 87.84% recovery relates to the calculated recovery at the design center-point.

The sCT recovery model was visualized by means of a contour plot as depicted in Fig. 1. Extraction levels which yielded degradants are visualized by 'X' in the plot. Given the degradation formation during experiment 4, comprising a 75 min incubation time, and given the minor increase in sCT recovery during 60 min vs. 30 min incubation time, *i.e.* 3.57%, the intermediate incubation time of 45 min was chosen. At extreme TFA concentration and incubation temperature, a degradant was formed (experiments 8 and 12). Hence, the most robust extraction levels, deduced from the contour plot, are 0.55% (v/v) trifluoroacetic acid concentration, incubation temperature of 55 °C and total incubation time of 45 min, yielding a calculated sCT recovery of 93.24%.

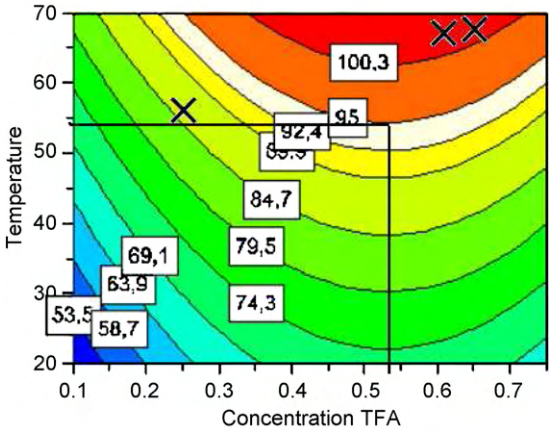

Fig. 1. Contour plot of sCT recovery model from Onion design (incubation time fixed at 45 min).

Sample extraction experiments using the extraction levels derived from the Onion design model, yielded an experimental average sCT recovery (±SEM, $n = 3$) of 93.38 (±0.79)%, hence confirming the calculated results from the Onion design model.

3.1.4. Plackett–Burman robustness evaluating design

The main effect of each extraction variable obtained with the Plackett–Burman robustness evaluating design is given in Table 4. The temperature factor has a positive significant effect ($P < 0.05$) on the recovery value. Therefore, it is concluded that the temperature has to be held within a narrow range (± 2 °C) around the preset temperature value during sample preparation. Incubation time and TFA concentration are found to be not statistically significant ($P > 0.1$), allowing greater variation during sample preparation. The highest recovery value, 99.63%, was found using following conditions: 0.45% (v/v) TFA–60 °C–40 min. HPLC-UV analysis of the sCT sample subjected to aforementioned extraction levels did not show any observable analytical degradation products. Therefore, these extraction variable levels were selected in final sCT assay and degradation determinations.

3.2. Method validation

3.2.1. Selectivity

When comparing unstressed calcitonin to the stressed samples (see Section 2.5.1), different degradation products were

Table 5
Calcitonin degradation products.

#	RRT	Tentative identification	SIM (*m*/*z*)	LoD (%)[a]	Condition[b]
1	1.00	Salmon calcitonin	858.8 ± 0.5	–	–
2	1.26	Epimer/deamidated product	859.9 ± 0.5	2.4	pH 3, 4, 6 and oxidation
3	0.49	Cys1-Ser2 hydrolysis product	863.3 ± 0.5	1.2	pH 3 and 4
4	0.91	Calcitonin trisulphide	866.8 ± 0.5	0.5	pH 4, 6 and oxidation
5	1.43	Acetylated calcitonin	869.3 ± 0.5	0.5	pH 3, 4, 6 and oxidation
6	1.77	Unidentified degradation product	902.2 ± 0.5	0.5	pH 6

[a] Compared to main sCT peak.
[b] Stress condition yielding the degradant.

observed, *e.g.* calcitonin epimer, deamidated calcitonin, calcitonin trisulphide, etc. An overview of these degradation products and additional analytical characteristics are given in Table 5. Further selectivity was found in the separation of sCT and following calcitonin species: human calcitonin (RRT: 1.04, SIM: 855.2 ± 0.5 amu); rat calcitonin (RRT: 1.01, SIM: 850.7 ± 0.5 amu) and porcine calcitonin (RRT: 1.45, SIM: 901.7 ± 0.5 amu). The relative retention time (RRT) of each species calcitonin was calculated compared to the main salmon calcitonin peak.

3.2.2. Quantitative validation

In order to evaluate the analytical sample stability, both spiked placebo and reference solutions were injected at fixed time intervals covering 2 and 8 h, respectively. No statistically relevant trend was observed, indicating suitable analytical stability at 20 °C. Furthermore, for both reference and spiked placebo solutions, a linear relationship between peak area and calcitonin concentration exists. By comparing the slopes of the two graphs, *i.e.* 9822 (95% CI: ±682) μAU × s × 1.6 ml/IU and 7117 (95% CI: ±2099) μAU × s × 1.6 ml/IU for reference and spiked placebo solutions, respectively, it was found that they were not statistically significant different. The limit of detection for sCT was calculated to be 4.83 IU/1.6 ml using the corresponding formula stipulated in the European Pharmacopoeia, *i.e.* signal–noise ratio of 3 [8].

The absolute sCT recovery values of the spiked placebo solutions, calculated as ratio between sCT peak area in spiked placebo solutions and sCT area in corresponding reference solutions, averaged (±SD, $n = 9$) 97.4 (±4.31)%. The precision for reference and spiked placebo solutions were calculated to be 4.33 and 3.34%. The accuracy (±SD, $n = 9$) was calculated to be 96.8 (±3.30)% by calculating the ratio between sCT concentration in spiked placebo solution (obtained by external calibration using 100% label claim reference solution) and the theoretical sCT concentration in the spiked placebo solutions. In order to evaluate the range, the theoretical sCT concentrations of spiked placebo solutions were compared to their respective sCT concentrations determined by external calibration with the 100% label claim reference solution. This yielded a sCT percentage (±SD, $n = 3$) of 85.03 (±1.33)% and 106.53 (±2.63)% for 90 and 110% label claim spiked placebo solutions, respectively. From this it was concluded that quantification in the 90–110% label claim range, *i.e.* 54–66 IU/1.6 ml, is possible.

3.3. Stability results of the new formulation

The relative salmon calcitonin assay values after 6 weeks incubation at −35, 5 and 20 °C were calculated to be 93.9, 83.9 and 69.8%, respectively (assay precision 4.3%). These results, obtained during a short-term stability study within a clinical development program, clearly revealed a temperature-dependent decrease in calcitonin assay. However, no significant degradation products were observed which could explain the large decrease in assay value vs. T_0. A potential hypothesis is a chemical interaction of the low quantity of peptide with the abundant quantities of carbomer carboxylic acid groups. Due to the low concentration of sCT in the formulation, this hypothesis could not yet be confirmed.

4. Discussion

While intrinsic degradation compounds of peptides are reasonably well described [28], interactions between carbomer polymers and active pharmaceutical ingredients (API) have rarely been described. Walker et al. reported a decrease in tryptic activity, due to the presence of carbomer [29]. Lucke et al. observed a salmon calcitonin acylation by lactic and glycolic acid, originating from poly(lactic-co-glycolic acid) polymer. Further experiments revealed amine groups of lysine and hydroxyl groups of tyrosine and serine as potential acylation targets [30]. In 2006, Bommareddy et al. attributed the slower release, when compared to non-electrolyte drugs, of weakly basic drug salts to an ionic interaction between protonated amines of these salts and the carbomeric carboxylates used as resin [31]. Coucke et al. observed a variability in the biological availability of metoprolol tartrate after administration of carbomer containing nasal powders, obtained through different manufacturing processes. This functionality phenomenon was attributed by the authors to the different physical properties, *i.e.* densities of the powder matrices as most probable explanatory hypothesis without considering a chemical interaction problem [17].

5. Conclusions

For the first time, a complete quality control of salmon calcitonin in a solid pharmaceutical powder is performed. A stability indicating method for assay and degradation profiling of low-level salmon calcitonin (400 ppm) in a bioadhesive polymeric formulation was developed. Onion and Plackett–Burman designs were used to develop and optimize the sample treatment and following optimized sample extraction parameters were obtained: 0.45% (v/v) TFA at 60 °C, during 40 min. This sample extraction results in an average recovery value of 97% and a precision of 4%, without any analytical degradation observed.

A pilot stability test under different storage conditions revealed a temperature-dependent decrease in salmon calcitonin assay, whilst no equivalent increase in degradation products was observed. A potential hypothesis explaining these observations is the chemical interaction between salmon calcitonin and the polymer present in the formulation, *i.e.* carbomer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.06.028.

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