CHAPTER III

MATERIALS AND METHODS

MATERIALS

Reference product

Miacalcic© Nasal Spray 200 IU, Batch no. H3034 Manufacture date 07-2003, Expiration date 07-2006, Novartis Pharma AG Inc., Basle, Switzerland

Reagents

1. Normal saline 0.9% Injection, Lot No. 39-406-XL2, General Hospital Products, Bangkok, Thailand
2. Acetonitrile HPLC grade, Lot no.0210046, Labscan, Bangkok, Thailand
3. Calcitonin (salmon) (EP 1997, 2002) or salmon calcitonin I, acetate salt, Lot.No. 0547992, Manufacture date 01-02-2002, Retest date 02-2004, Bachem AG, Bubendorf, Switzerland (purity 99.6% w/w, water content = 4.2 % w/w, acetate content = 11.0%, peptide content = 84.6 % w/w)
4. Hydrochloric acid AR grade, Lot No. 03010087 Labscan, Bangkok, Thailand
5. Methanol HPLC grade, Lot No. 03030181, Labscan, Bangkok, Thailand
6. N-acetyl-cys[1] calcitonin EPCRS (European Pharmacopoeia Control Reference C0200010 Batch No.1, Council of Europe/ European Directorate for the Quality Medicines (EDQM), Strasbourg, France
7. Phosphoric Acid, Lot. No. H9C063, Merck, Darmstadt, Germany
8. Salmon calcitonin EPCRS (European Pharmacopoeia Control Reference Standard), C0200000 Batch No.5 (declared content of $C_{145}H_{240}N_{44}O_{48}S_2$ 1.00 mg per vial), Council of Europe/ European Directorate for the Quality Medicines (EDQM), Strasbourg, France
9. Sodium dihydrogen orthophosphate AR grade, Lot. No.A768946, Merck, Darmstadt, Germany
10. Tetramethylammonium hydroxide pentahydrate AR grade, Lot. No. 420275/1, Fluka, Switzerland

Nasal Spray Device Components

1. Actuator 29 nasal spray integrated insert, Product No. 58405, Batch No. 80013576, Ing. Erich Pfeiffer GmbH, Radolfzell, Germany
2. Glass bottle (crimp 20), Klarglas 3.5 mL, Product No. 33713, Batch No. 80013576, Ing Erich Pfeiffer GmbH, Radolfzell, Germany
3. Spray pumps (0.091 mL per actuation), crimp diameter 20 mm, Product No. 49013, Batch No. 80013576, Ing. Erich Pfeiffer GmbH, Radolfzell, Germany

Apparatus

1. Analytical balance, AG285, Metler Toledo, Switzerland
2. Analytical microbalance, seven digit, MT5 Metler Toledo, Switzerland
3. Crimping machine, Mary Commercial Suppliers Co., Ltd., Bangkok, Thailand
4. Digital pH meter, Orion 420A, Orion AG, Massachusetts, USA
5. Gamma counter, RIASTAR, Packard Instrument Company, USA
6. HPLC column Vydac ® 201 TP 54, a specialty reversed phase stainless steel column consisting of octadecylsilyl silica gel (C18 hydrocarbon chains bonded to TP silica), 5μm particle size with 300 A° pore size, 250 x 4.6 mm ,Grace Vydac, Hesperia, CA, USA
7. Laminar air flow hood, model Airone 100-GS, Safelab Systems Ltd., Nailesed, Great Britain
8. Mixer, Luckham multimix major, Germany
9. Osmometer, Osmomat-030-D, Gonotec, Berlin Germany
10. Hot air oven, Memmert, Schwabach, Germany
11. Refrigerated centrifuge, rotor RC 30, Sorvall Instrument, Dupont, Germany
12. RIA kits for salmon calcitonin, Lot. No. 10313 Expire date 30-12-03, Diagnostic systems laboratories, Inc., Webster, Texas, USA
13. Vortex mixer (Vortex-Genie, Scientific industries, Inc., USA
14. Vortex mixer, SP Multitube vortexer, Scientific product, Baxter, USA
15. High performance liquid chromatograph, pumps (model LC- 10A), a communication bus module (model CBM-10A), an autoinjector (model

SIL – 10A), a column oven (model CTO-10A), a UV detector (model SPD-10A) Shimadzu, Japan

16. Column Sonicator, model Bransonic 221, Branson, USA
17. Micropipets, Gilson Medical Electronics S.A., France
18. Glassware

METHODS

1. Assay of Salmon Calcitonin Powder (Standardization)

Firstly, the lyophilized powder of salmon CT (Bachem®, Switzerland) was determined for peptide content by reverse- phase gradient, high performance liquid chromatography (HPLC) according to the method of European Pharmacopoeia (2002) and British Pharmacopoeia (2002). It was described as follows:

1.1 Preparation of Standard Reference Solution

The whole content of the reference standard in the vial (salmon calcitonin EPCRS, with a declared content of $C_{145}H_{240}N_{44}O_{48}S_2$ of 1.00 mg) was dissolved in mobile phase A to obtain a concentration of 1.0 mg/mL. It was subsequently diluted to obtain a concentration of 40 μg/mL using the same mobile phase A.

1.2 Preparation of Test Solution

10 mg of salmon CT powder (Lot No. 0547992, Bachem®) was accurately weighed and dissolved with mobile phase A in a 10 mL-volumetric flask. The volume was then adjusted with mobile phase A and the resultant solution was used as a stock solution (1.0 mg/mL). 4.0 mL of the stock solution was pipetted into a 100.0 mL-volumetric flask and diluted to volume with mobile phase A. The final concentration was 40 μg/mL.

1.3 Preparation of Mobile Phases

Mobile phase A: 3.26 g of tetramethylammonium hydroxide pentahydrate was dissolved in 900 mL of water, and the pH was adjusted to 2.5 with orthophosphoric acid. The solution was subsequently mixed with 100 mL of acetonitrile, filtered and degassed.

Mobile phase B: 1.45 g of tetramethylammonium hydroxide pentahydrate was dissolved in 400 mL of water and the pH was adjusted to 2.5 with orthophosphoric acid. The solution was subsequently mixed with 600 mL of acetonitrile, filtered and degassed.

1.4 High Performance Liquid Chromatographic (HPLC) Conditions

The chromatographic procedure was carried out using Shimadzu HPLC gradient system consisting of:

Two HPLC pumps (model LC- 10A), a communication bus module (model CBM-10A), an autoinjector (modol SIL – 10A), a column oven (model CTO-10A), a UV detector (model SPD-10A) and a computerized integrator.

Column: Vydac ® 201 TP 54, a specialty reversed phase stainless steel column consisting of octadecylsilyl silica gel (C18 hydrocarbon chains bonded to TP silica), 5µm particle size with 300 A° pore size, 250 x 4.6 mm (Grace Vydac, Hesperia, CA, USA).

Flow rate: 1.0 mL/min.

Detector: UV spectrophotometer set at 220 nm

Temperature: 65°C

Injection volume: 50µl

Retention time: ~ 18 – 21 min for salmon CT

Chromatographic system: The column was equilibrated with a mixture of 72 volumes of mobile phase A and 28 volumes of mobile phase B, followed by a gradient elution program as described below:

Table 4 The gradient elution program operated for salmon CT

Time (min)	Mobile phase A % v/v	Mobile phase B % v/v	Comment
0 – 30	72 → 48	28→ 52	Linear gradient
30 – 32	48 → 72	52 → 28	Switch to initial eluent conditions
32 – 55	72	28	Re- equilibration

. 50 µl of the reference and the test solutions was subsequently injected and the peak areas were calculated from the chromatograms. The content of salmon CT ($C_{145}H_{240}N_{44}O_{48}S_2$) in the test solution was calculated from the peak areas in the chromatograms obtained from the test solution and the reference solution (salmon calcitonin EPCRS) and expressed as the content of net peptide $C_{145}H_{240}N_{44}O_{48}S_2$ in the test powder. Both the European and British Pharmacopoeias (EP 2002, BP 2002) state that salmon CT contains not less than 90.0% and not more than the equivalent of 105.0 % w/w of the peptide $C_{145}H_{240}N_{44}O_{48}S_2$, calculated with reference to the anhydrous, acetic acid-free substance. Also, by convention, for the purpose of labeling salmon CT

preparations, both pharmacopoeias state that 1 mg of salmon CT ($C_{145}H_{240}N_{44}O_{48}S_2$) is equivalent to 6,000 I.U. of biological activity.

Thus, the net content of salmon CT, expressed as % peptide excluding water and acetic acid contents ($C_{145}H_{240}N_{44}O_{48}S_2$), was calculated by Equation I as follows:

$$\% \text{ Net peptide content} = \frac{A_{SMP} \times M_R\,(mg) \times V_{SPM}\,(mL) \times 100}{A_R \times M_{SMP}\,(mg) \times V_R\,(mL)} \quad \text{...Eq. I}$$

Where:

A_{SMP} = peak area of salmon CT in the sample chromatogram

M_R = mass of reference substance used in preparing reference solution

= declared content of $C_{145}H_{240}N_{44}O_{48}S_2$ in salmon CT EPCRS vial (1.00 mg)

= 1.00 mg net peptide (no water and acetic acid)

V_{SPM} = volume of sample solution (mL)

100 = conversion factor to percent

A_R = peak area of reference chromatogram

M_{SMP} = mass of salmon CT used in sample solution (mg)

V_R = volume of reference solution (mL)

To obtain % assay (purity) of salmon CT, the net peptide content (percent net peptide obtained from the above HPLC analysis) was compared with the unassayed peptide content (percent peptide remaining after subtracting the water and acetic acid contents) according to the following formula:

$$\% \text{ Assay (purity)} = \frac{\% \text{ Net peptide content} \times 100}{(100 - H_2O - AcOH)} \quad \text{...Eq. II}$$

Where

H_2O = water content of the test substance in % w/w = 4.2% w/w

AcOH = acetic acid content of the test substance in % w/w = 11.1% w/w

The contents of water and acetic acid were taken from Bachem's certificate of analysis for salmon CT, Lot No. 0547992. The percent net peptide content was needed during the preparation of the nasal spray such that the correct quantity of salmon CT, available as hydrated acetate salt, was used to produce solutions with desired peptide activity, i.e., 100 and 200 I.U. per actuation.

2. Assay of Water and Acetic Acid Contents

Water content by Karl Fisher Titration

The water content of salmon CT was determined by volumetric Karl Fisher Titration

Instrument:

Karl Fisher Titrator

Determination of the tritrant concentration:

20 mg of the tritrimetric standard (Sodium tartrate dehydrate) was accurately weighted and was immediately dissolved in 50 mL of pretitrated hydranal solvent. The solution was titrated using hydranal titrant. The end point will be found automatically, and the equivalence factor was displayed in percent (m/m) water.

Titration:

20 mg of salmon CT was accurately weighted and immediately dissolved in 35 mL of pretitrate hydranal solvent. The solution was titrated using hydranal titrant. The end point will be found automatically, and the equivalence factor was displayed in percent (m/m) water.

Acetic content by HPLC

The acetic content of salmon CT was determined by reverse phase HPLC using uv detection at λ =210 nm

Sample preparation:

12.50 mg of salmon CT was accurately weighted in duplicate, dissolved in 5 mL volumetric flask and diluted to volume with water.

Acetate standard preparation:

Stock standard solution: 0.8 g of potassium acetate standard was accurately weighted, dissolved in a 500 mL volumetric flask and diluted to volume with water.

Standard solution: the stock standard solution was diluted to volume with water in 100 mL volumetric flasks to prepared the following standard solutions (approximately 100, 200, 300, 400, 500 µg/mL of acetic acid)

Comparison solution: 250 mg of potassium acetate standard was accurately weighted, dissolved in 500 mL volumetric flask and diluted to volume with water.

Chromatographic conditions:

Column	Spherisorp ODS, 5μm (250 x 4.6mm)
Temperature	Ambient
Elution	Isocratic elution
Mobile phase	0.10 M H_3PO_4 + 0.007 M NH_3 in Water: methanol = 50:1(v/v)
Flow rate	1.5 mL/min
Detection	UV at λ =210 nm

Calculation:

The acetic acid concentration of sample solution was calculated by means of linear regression.

3. Preparation of Salmon CT Nasal Solutions (100and 200 IU per actuation)

Two salmon CT nasal formulations were prepared as follows:

1. Salmon CT lyophilized powder (Lot No. 0547992, Bachem, Switzerland), previously standardized against salmon calcitonin EPCRS, was accurately weighed using a seven digit microbalance (Mettler Toledo MT5, Switzerland). This amount has been calculated in excess by taking into account the contents of water and acetate present in the powder as well as to compensate for the loss of peptide during the filtration process (10 % overage) such that the desired activity per spray could be achieved.

2. Salmon CT was then dissolved in 0.9 % normal saline for injection.

3. Benzalkonium chloride was then added as preservative to make the final concentration of 0.1 mg /mL

4. 0.1 N hydrochloric acid was subsequently added to adjust the pH to approximately 3.50 -3.70.

5. The osmolarity was then adjusted to about 290 – 310 mOsmol by the gradual addition of NaCl and checked with osmometer. The volume of the solution was finally adjusted with normal saline to 500 mL

6. The solution was filtered through a 0.2 μm cellulose acetate membrane (Minisart®, Sartorius, Goettingen, Germany) under laminar-airflow hood for sterilization and then 2.0mL of the solution was filled in each of the pre-sterilized Klarglas bottles (Figure10, part no.1), which was specially designed to fit with the spray pump.

7. The sterile spray pump (Figure 10, part no. 2) was placed on the top of the filled bottle before being crimped with the crimping machine as seen in Figure 11.

8. The pump was then fitted with an actuator (Figure 10, part no.3) before being covered with the protective cap (Figure 10, part no. 4)

9. Finally the finished product was labeled with batch no., concentration in IU, and the manufacture date.

10. Vials of the finished product were divided in half to be kept at 4°C and 30 °C for quality testing. For 200 IU formulations the amount of salmon CT was doubled as shown in Table 5. Both formulations (100 and 200 IU/spray) were prepared in duplicate (2 batches each) for quality control and stability testing.

Table 5 Formulas of salmon CT nasal solutions (100 and 200 IU per spray)

Ingredient	Salmon CT Nasal Solution 100 IU per spray	Salmon CT Nasal Solution 200 IU per spray
Salmon CT	0.219 mg	0.438 mg
Amount with 10 % excess	0.241 mg	0.482 mg
Isotonic adjusting agent	Qs	qs
pH adjusting agent	Qs	qs
Preservative	0.1 mg	0.1 mg
0.9% Normal Saline qs	1 mL	1 mL

Table 6 Tests and specifications for finished product of salmon CT nasal sprays (Both 100 and 200 IU per spray). Each test was performed in duplicate for all batches.

Tests	Real-time storage at 4°C (month)				Accelerate temperature at 30 °C (month)				
	0	3	6	12	0	1	2	3	4
1. Assay									
1.1 Salmon calcitonin	/	/	/	/	/	/	/	/	/
1.2 Calcitonin C	/	/	/	/	/	/	/	/	/
1.3 Related peptide	/			/	/				/
2. Uniformity of mass	/	/	/	/	/	/	/	/	/
3. pH	/			/	/				/
4. Sterility	/			/	/				/
5. Osmolarity	/			/	/				/
6. Clarity	/	/	/	/	/	/	/	/	/
7. Other tests									
7.1Particle size distribution	/				/				
7.2 Spray angle and spray pattern test	/				/				
7.3 Leak test	/				/				

/ represent the test being conducted at particular time point.

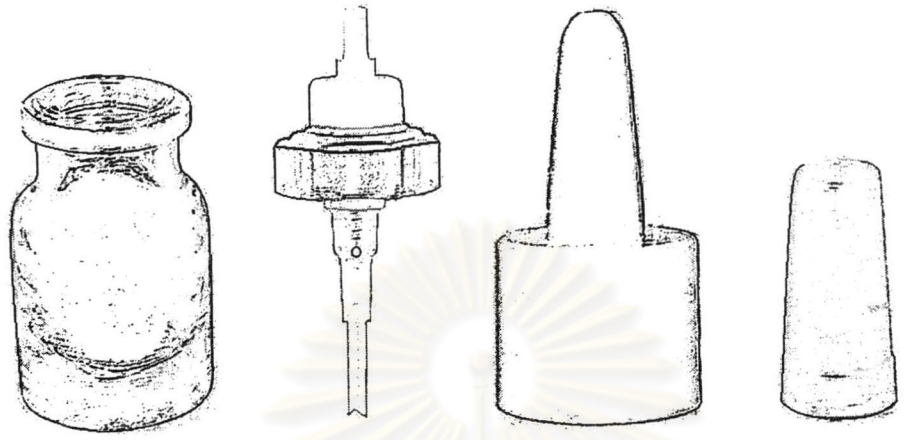

Figure 10 Parts of nasal spray bottle (Ing. Erich Pfeiffer GmbH, Radolfzell, Germany); 1 = Klarglass bottle 3.5 mL, 2 = Spray pump (crimp diameter 20 mm), 3 = Actuator, 4 = Actuator Cover

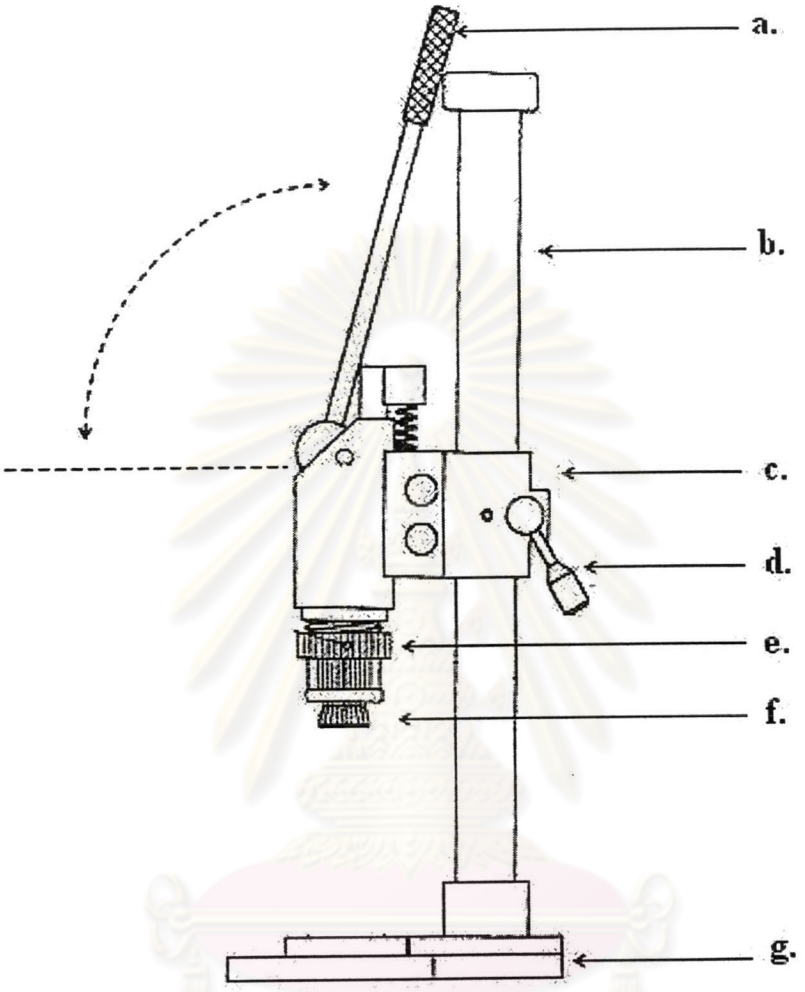

Figure11 Crimping Machine (Mary Commercial, Thailand); a. Arm, b. Body, c. Height Adjustment d. Locked valve, e. Tighten adjustment, f. Crimp control, g. Base.

4. Stability Test

The stability studies consisted of real-time testing under recommended storage condition (long term storage at 4 °C) and accelerated stability testing at 30 °C. Test conditions followed the Guidelines for Stability Testing of Pharmaceutical Products set by World Health Organization (WHO Technical Report series, No.863, 1996).

The two batches of each formula were sampled in accordance with a predetermined schedule (Table 7).

Table 7 Stability testing schedule of real-time and accelerated studies

Storage temperature (°C)	Sampling (month)
30	0, 1, 2, 3, 4
4	0, 3, 6,12

Vials of both strengths of salmon CT nasal sprays (100 and 200 IU) were divided into two groups. The first 40 vials were kept at 30 °C in a controlled temperature oven for 4 months and the similar quantity was kept in the refrigerator at 4 °C for 12 months. All of them were covered with aluminum foil to protect from light. Sample vials were taken periodically from two production batches to determined salmon CT content as well as other tests such as uniformity of mass, clarity, osmolarity, sterility and pH according to the sampling schedule in Table 6.

5. Content of Salmon CT in the Nasal Sprays

The amount of salmon CT in each delivered dose was determined by applying the assay method of salmon CT injection as well as the general monograph for the nasal spray solution as directed in the British Pharmacopoeia (BP 2002). It was described as follows:

5.1 Preparation of Test Solution

The nasal spray bottles were removed from the controlled temperature storage place (30 °C or 4 °C). They were placed for 30 min to reach the ambient temperature. The first spray was discharged once to waste; waiting for not less than 5 sec and the second spray was discharged again. The procedure was repeated for a further

spray. The next delivered-doses of ten sprays were collected into a 10 mL glass bottle and then they were transferred, with appropriate rinsing with the medium, to a 10-mL volumetric flask and diluted to volume with 0.1 M sodium dihydrogen orthophosphate (adjusted to pH 4.0 with orthophosphoric acid) and mixed gently. The final concentration of the test solution was about 100 I.U. of salmon CT ($C_{145}H_{240}N_{44}O_{48}S_2$) per mL for 100 I.U. strength, and 200 I.U. of net peptide per mL for 200 I.U. strength, respectively.

5.2 Preparation of Working Standard

About one mg of salmon CT (Lot. No. 0547992, Bachem), previously standardized against the salmon calcitonin EPCRS, was accurately weighed, dissolved and diluted with 0.1 M sodium dihydrogen orthophosphate solution (adjusted to pH 4.0 with orthophosphoric acid) to obtain a solution with a concentration equivalent to the net peptide ($C_{145}H_{240}N_{44}O_{48}S_2$) of 40 μg/mL. Since one mg of salmon CT ($C_{145}H_{240}N_{44}O_{48}S_2$) was defined as equivalent to 6,000 I.U. of biological activity, the activity of the standardized Bachem's salmon CT powder could be calculated by multiplying the net peptide content (in mg) with factor 6000.

5.3 Preparation of Mobile Phases

The mobile phase A was prepared by mixing 100 mL of 0.363 % w/v solution of tetramethylammonium hydroxide pentahydrate with 150 mL of acetonitrile. The solution was adjusted to pH 2.5 with orthophosphoric acid, filtered through a 0.5 μm membrane filter or finer porosity and finally degassed. The mobile phase B was prepared by mixing 450 mL of 0.402 % w/v solution of tetramethylammonium hydroxide pentahydrate with 50 mL of acetonitrile. The pH was adjusted to 2.5 with orthophosphoric acid followed by filtration through a 0.5μm membrane and degassing.

5.4 HPLC Conditions

The chromatographic procedure was carried out using the same HPLC instruments (Shimadzu, Japan) and column (Vydac C18, 300A° wide pore, 5 μm particle size, 250 x 4.6 mm, Vydac, USA) as in section 1.5 but with the following conditions:

Flow rate	: 1.0 mL/min.
Detector	: UV spectrophotometer set at 220 nm.
Temperature	: 40°C
Injection Volume	: 50μL
Retention time	: ~ 18 – 21 min for salmon CT

The gradient elution program: The column was equilibrated with a mixture of 35 volumes of mobile phase A and 65 volumes of mobile phase B, followed by a gradient elution program as described below.

Table 8 The gradient elution program operated for salmon CT in nasal solution.

Time (min)	Mobile phase A % v/v	Mobile phase B % v/v	Comment
0 – 21	35 → 57	65→ 43	Linear gradient
21 – 32	57 → 35	43 → 65	Switch to initial eluent conditions
32 – 50	35	65	Re- equilibration

The average content of salmon CT ($C_{145}H_{240}N_{44}O_{48}S_2$) per actuation was calculated by firstly interpolating the concentration of the peptide in the test solution from the standard calibration curve. Secondly, the peptide concentration (μg/mL) was converted into total amount (μg) by multiplying with factor 10. The average amount per spray was then obtained by dividing the total amount with factor 10. After multiplying with factor 6, the averaged amount delivered per spray was finally converted to biological units delivered per spray.

5.5 Standard Calibration Curve

One mg of previously standardized salmon CT (Lot. No. 0547992, Bachem) was accurately weighed, dissolved and adjusted to 10.0 mL with 0.1 M solution of sodium dihydrogen orthophosphate (pH 4.0) to obtain a stock solution with peptide ($C_{145}H_{240}N_{44}O_{48}S_2$) concentration of 0.1 mg/mL.

Aliquots of stock solution was transferred by pipets at volumes of 400, 300, 200, 100, 50, 25 and 10 μl into 1-mL volumetric flasks and diluted to volume with

0.1 M solution of sodium dihydrogen orthophosphate (pH 4.0). The final concentration of each solution was 40, 30, 20, 10, 5, 2.5, and 1 μg/mL, respectively.

All these standard solutions were analyzed following the same procedure as described earlier. The peak areas of salmon CT ($C_{145}H_{240}N_{44}O_{48}S_2$) were plotted versus their known concentrations and fitted to straight line using linear regression analysis.

5.6 Assay Validation

5.6.1 Accuracy

Five concentrations of standard salmon CT in 0.1 M sodium dihydrogen orthophosphate (pH 4.0) were analyzed for peak areas (five replicates for each concentration). The concentrations were then obtained by interpolation from the standard calibration curve and compared with the respective nominal values. The accuracy was calculated from percent recoveries, the value (% Recovery) should be within 80 -120 %.

5.6.2 Precision

Within run precision

Three concentrations of standard salmon CT (40, 10 and 1 μg/ml) in 0.1 M sodium dihydrogen orthophosphate (pH 4.0) were analyzed within the same day (five replicates per concentration). The percent coefficient of variation (C.V.) of estimated concentration was determined at each concentration level. The precision determined at each concentration level should not exceed 2% of the C.V.

Between run presicion

Three concentrations of standard salmon CT (40, 10 and 1 μg/ml) in 0.1 M sodium dihydrogen orthophosphate (pH 4.0) were analyzed on five different days. The percent coefficient of variation (C.V.) of the estimated concentration was determined at each concentration level after five days. The precision determined at each concentration should not exceed 2% of the C.V.

In addition, a standard salmon CT was prepared at ~30 μg/ml in the same buffer. This concentration was near the concentration of the test solution. It was injected 6 times and the resulting peak areas were interpolated from the standard curves. The average and S.D. of the estimated concentration was obtained to test for respectively of the method.

6. Related Peptide in the Nasal Sprays

Since there is no specific monograph for salmon CT nasal preparations, other testing specifications apart from the percent labeled amount and uniformity of mass per spray were taken from the monograph for calcitonin (salmon) injection BP 2002. As the two dosage forms are similarly present as a clear solution, many of the testing specifications required for the injection can be applied to the nasal spray solution such as the contents of related peptide and calcitonin C (see section 7). The related peptide content of the two nasal formulations was evaluated by HPLC. The chromatographic system, as applied from calcitonin (salmon) injection BP 2002, was as follows:

Column: a stainless steel column (25 cm x 4.6 mm) packed with octadecylsilyl silica gel for chromatography, 5µm

Mobile phase A: 3.26 g of tetramethylammonium hydroxide pentahydrate was dissolved in 900 mL of water and the pH was adjusted to 2.5 with orthophosphoric acid. The solution was then mixed with 100 mL of acetonitrile

Mobile phase B: 1.45 g of tetramethylammonium hydroxide pentahydrate was dissolved in 400 mL of water and the pH was adjusted to 2.5 with orthophosphoric acid. The solution was subsequently mixed with 600 mL of acetonitrile

Detection wavelength: 220 nm

Flow rate: 1.0 mL/min

Temperature: 65 °C

Chromatographic system: The column was equilibrated with a mixture of 72 volumes of mobile phase A and 28 volumes of mobile phase B. The gradient was carried out according to the gradient elution program as described below:

Table 9 The gradient elution program operated for related peptide.

Time (min)	Mobile phase A % v/v	Mobile phase B % v/v	Comment
0 – 30	72 → 48	28→ 52	Linear gradient
30 – 32	48 → 72	52 → 28	Switch to initial eluent conditions
32 – 55	72	28	Re- equilibrattion

For solution (1) the content of a nasal spray bottle was diluted with mobile phase A to give a final concentration of 40 μg/mL of salmon CT. For solution (2), the content of a vial of N-acetyl-cys[1] calcitonin EPCRS was dissolved in 0.4 mL of mobile phase A, diluted to 10 mL with mobile phase A and finally, 25 μl of solution (1) was added to obtain solution (2). 50 μl of solutions (1) and (2) was separately injected into the chromatograph.

The relative retention time of N-acetyl-cys1 calcitonin in solution (2) should be about 1.15 relative to the principle peak. The test was not valid unless the resolution factor between the peaks corresponding to calcitonin and N-acetyl-cys1 calcitonin was at least 5.0 and the symmetry factor for the N-acetyl-cys1 calcitonin peak was not greater than 2.5.

In the chromatogram obtained with solution (1), the area of any secondary peak is not greater than 3 % of the total area of all the peaks and the sum of the areas of any such peaks is not greater than 5 % of the total area of all the peaks by normalization. Any peak with an area less than 0.1% of that of the principal peak was disregarded.

7. Calcitonin C

Both nasal formulations were evaluated for calcitonin C, which is the heat-generated degradation product of calcitonin. The HPLC procedure was the same as that used for determining the peptide content in the nasal sprays (sections 5.3 – 5.4). To obtain the reference solution of calcitonin C, the entire content of a nasal spray bottle was removed and transferred to a glass test tube. The tube was oven-heated at 75 °C for 15 hours prior to injecting into the chromatograph.

Passing criteria: In the chromatogram obtained with this solution the peak due to calcitonin C is the largest peak to elute after the injection buffer salts and before the principle peak with a relative retention to that of salmon CT of between 0.5 and 0.6.

The test is not valid unless the resolution factor between the peaks due to calcitonin C and salmon CT from this chromatogram is at least 3.0. Also, in the chromatogram obtained with the test solution (section 5.1) the area of any peak corresponding to calcitonin C is not greater than 7 % by normalization.

8. Acidity

Before filtration, the two nasal formulations were measured for their acidity with a pH meter. If necessary, the pH was adjusted to a preferable range of 3.5 to 3.9. The pH

was also rechecked for stability evaluation, i.e., after 4-month storage for samples kept at 30 °C and after 6 months for samples kept at 4 °C.

9. Osmolarity

The two nasal formulations were measured for their osmolarity prior to filtration. If they were not within the isotonic range of 290 – 310 mOsm/kg, the tonicity was adjusted by NaCl addition. At the end of the stability testing period the samples were also rechecked for their osmolarity.

10. Clarity

The clarity of the solution was observed by visual inspection. The solution must be colorless, transparent and free of any visible particles and fibers.

11. Sterility Test

Ten vials of each formulation were sampled and sent to the Microbiology Department, Siriraj Hospital for sterility test. Details of the test procedure are provided in Appendix G. The results were sent back within 14 days and the numbers of contaminated samples were recorded.

12. Uniformity of Mass

The samples must comply with the BP 2002 protocol for metered-dose nasal spray solution according to the following procedure:

- Take the two formulations (ten bottles each) from their storage conditions and allow them to reach the ambient temperature
- Remove the protective cap and discharge a bottle of each formulation once to waste, waiting for not less than 5 seconds and discharge again to waste
- Repeat this procedure for a further actuation.
- Weigh the mass of the bottle, discharge once to waste and weigh the remaining mass of the bottle
- Calculate the difference between the two weights
- Repeat the whole procedure for a further nine bottles and calculated the average of ten bottles

They comply with the test if not more than two of the individual values deviate by more than 25 percent from the average value and none deviates by more than 35 percent.

13. Droplet Size Measurement

Both salmon CT nasal preparations (100 and 200 IU) were sent to Erich Pfeiffer GmbH, Germany to test for the droplet size and droplet size distribution using laser diffraction method. Sample products were sprayed at an angle of 60° into the laser beam. Droplet size distribution are determined in terms of ranges for the D10, D50, D90, span [(D90-D10)/D50], and percentage of droplets less than 10 μm. The value D10, D50 and D90 are the volume median diameters, these are 10 %,50% and 90 % respectively of the total volume of liquid is in drops of smaller diameter and 90 %, 50 % and 10 % respectively is in drops of larger diameter. When the distribution is normal or Gaussian distribution the value of median diameter, D50 will exactly illustrate the mean of droplet size.

According to CDER, 2002 Guidedance for Industry, Nasal spray and Inhalation solution, Suspension, and Spray Drug Products Chemistry, Manufacturing, and Controls Documentation, it is possible to use the placebo formulations which contain the same characteristics to substitute for the drug products. The placebo A and B were prepared for use as the representative nasal spray solution in the same specifications (pH osmolarity and same preservative) as salmon CT nasal preparation 100 and 200 IU, respectively.

14. Spray Pattern

Spray pattern was determined by assessing the shape and size of the spray after actuating the spray pump at pre-determined angles on a collection surface (like a piece of paper). Both salmon CT nasal preparations (100 and 200 IU) were sent to Erich Pfeiffer GmbH, Germany, for testing. The test solution was first dyed with 0.2% methylene blue to make the spray pattern visible. After firing the spray pump onto the paper surface. The longest (Dmax) and the shortest (Dmin) diameters of each stain were measured by opitical scanning and image analyses. The size of the stain was obtained by averaging the values of Dmax and Dmin whereas the spray shape was indicated by the ovality ratio, which is the ratio of Dmax to Dmin as shown in Figure 12.

For valid assessment of the spray pattern, the testing conditions must be specified like the spray distance between the nozzle and the collection surface, number of sprays per spray pattern, position and orientation of the collection surface relative to the nozzle, as well as the visualization procedure. The acceptance criteria for the spray pattern include the shape (e.g., ellipsoid with a relatively uniform density) as well as the size of

the pattern (the ratio of the longest to the shortest axes should lie within a specified range, e.g., 1.00 - 1.30).

In addition to, the spray angle was determined from the maximum diameter of the spray (Dmax) and the distance between the nozzle and collection surface (Figure 12).

For the nasal spray solutions, the test can be performed on a placebo formulation contained inside the same type of pump and bottle. The results, if passed, can substitute for the release testing of spray pattern for the drug product (CDER, 2002).

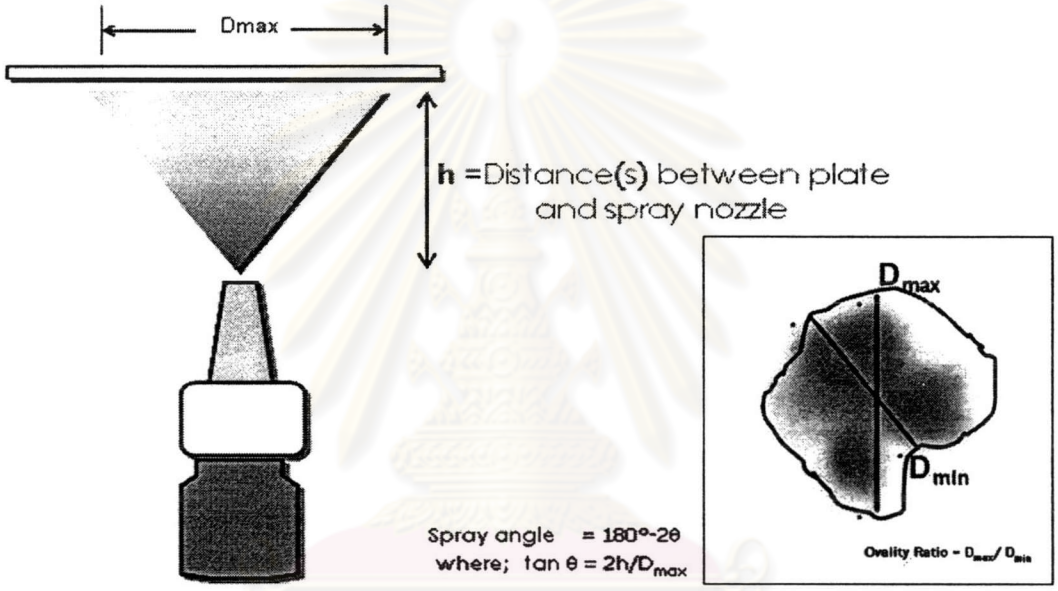

Figure 12 Image analysis of spray pattern and spray angle.

15. Leak Test

After crimping, 10 bottles of salmon CT nasal spray were tested for leakage at the gasket between closure and bottle as well as for the pump tightness by placing samples at 300 mbar under vacuum bell jar for 5 min. The tests were conducted and reported by Mary Commercial Suppliers Co., Ltd., Thailand.

16. In Vivo Study

The methodology used in this study followed the recommendations described in "Guideline for Bioequivalence Study of Generic Drugs", Drug Control Department, Office of Food and Drug Administration, Thailand, 2000. The study was a randomized, two-way crossover with a Latin square design. The details are described as follows:

16.1. Products

Miacalcic® nasal spray manufactured by Novartis® (Batch no.H3034 Mfg. date 07-2003 Exp. date 07-2006) was assigned as the innovator's product whereas the 200 IU strength of salmon CT nasal formulation (Batch no. 200-III Mfg. date 05-2003) was assigned as the test product.

16.2. Subjects

Twelve healthy Thai male volunteers participated in this study. Their demographic data are presented in Appendix E and Table 56. Prior to testing, all subjects had to pass physical examination and clinical laboratory tests. Information was provided to all subjects, explaining the risks and benefits. Written informed consents were obtained before study initiation. The protocol was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of Chulalongkorn University. The volunteers were asked to take no medication as well as to abstain from alcoholic preparations and cigarettes for at least two weeks before and during the entire study period.

16.3. Inclusion Criteria

1. Healthy Thai male volunteers with age ranging from 18 to 45 years and body mass index between 18 to 24 kg/m^2
2. Normal physical and laboratory chemical tests
3. No history of gastrointestinal, pulmonary, hepatic, renal, allergic diseases or any other disorders that affect bioavailability of the drug
4. Non-smokers and without alcohol or drug abuse
5. No history of allergic reactions to calcitonin

16.4. Exclusion Criteria

1. Failure or refusal to complete the study
2. Having any respiratory abnormalities, infection or allergies during study
3. Allergic or having adverse drug reactions to calcitonin

16.5. Dose and drug administration

One spray of 200 IU of salmon CT formulation or Miacalcic® nasal spray was given in each nostril of the individual subjects at 8.00 am after an 8-hr overnight fast. The total dose administered was 400 IU per subject. No food or drink was permitted until 4 hours after dosing. Subjects were administered according to the following instructions:

- Blow the nose so that the nostrils are clear
- Clean each nostril by swapping with a cotton bud
- Tilt the head slightly forward
- Close one nostril by gently pressing against the side of nose with the subject's index finger
- Insert the tip of the nasal spray into the other nostril
- Press the pump quickly and steadily so that one actuation is discharged into the nostril
- Remove the spray from the nostril and breathe out through the mouth
- Tilt the head backward to allow the spray to drain into the back of the nose
- Repeat the above procedure for the other nostril to complete dosing

16.6. Subject Monitoring

Any unusual symptoms were observed by a physician particularly during the first 3 hr. The subjects were periodically questioned throughout the study period for any unwanted side effects. If adverse drug reactions occurred, they would be recorded in the case record form and the subjects would be diagnosed and treated by the physician.

16.7. Experimental Design

The study was conducted in a randomized, two-way crossover design. Each subject was randomly assigned to receive the two products (A and B) with 1-week washout period between each administration according to the schedule shown in Table 10.

Table 10 Sequences of drug administration by Latin square design.

Sequence	Subject No.	Period	
		1	2
1	1	A	B
	2	A	B
	3	A	B
	4	A	B
	5	A	B
	6	A	B
2	7	B	A
	8	B	A
	9	B	A
	10	B	A
	11	B	A
	12	B	A

* The subject number was randomly assigned to the individual volunteers.

A and B represent Miacalcic® and the test preparation of salmon CT, respectively

16.8. Sample Collection

7 mL of blood samples were withdrawn from a forearm vein of each subject using a disposable syringe at the following time points: 0, 5, 10, 15, 20, 30, 45 min, 1, 1.5, 2 and 3 hours after administration. All blood samples were collected in glass tubes containing lithium heparin, chilled at 0 °C. Within 30 min after collection, blood samples were centrifuged at approximately 300 rpm for 15 minutes. Following separation of plasma, the samples were divided into two aliquots and stored at – 20 °C until analysis.

16.9. Analysis of Salmon CT in Plasma

Plasma immunoreative salmon CT was quantitated by double antibody radioimmunoassay (RIA) using a commercial kit developed by Diagnostics Systems Laboratories (Webster, TX). Rabbit anti-salmon CT antibodies were used as primary antibodies and have demonstrated less than 2% w/w cross reactivity. The sigmoidal standard curve of B/Bo vs salmon CT concentration, typical of competitive binding assays was linearized using log transformation, whereas B and Bo represent the

radioactivity of the bound and total ???, respectively. The best-fit line was determined using a least square regression analysis. The RIA quantitation range was 7.5 – 250.0 pg/mL.

The assay procedures are as follows:

1. Polystyrene disposable tubes (12 X 75mm) were labeled and arranged in duplicate as total counts (TC) tubes, non-specific binding (NSB) tubes, salmon CT standards A-F tubes, controls level I, II and III tubes which contain low, medium and high concentrations of salmon CT. Plasma samples were also prepared in duplicate and labeled for their sampling times

2. Three hundred μL each of standards A to F (Table 11), controls and plasma samples was placed at the bottom of the respectively labeled tubes. To the NSB tubes 400 μL of 0 pg/mL salmon CT standard (standard A) was added.

3. One hundred μL of salmon CT antiserum was added to all tubes, except NSB and Total Count tubes.

4. All the tubes were gently vortexed for 1 – 2 seconds.

5. All tubes were incubated overnight for 16 – 24 hours at 4 °C.

6. One hundred μL of tracer (125 I-salmon CT) solution were added to all tube

7. All the tubes were again vortexed, covered and incubated overnight for 16 – 24 hours at 4 °C

8. One mL of precipitating reagent containing goat anti-rabbit gamma globulin serum in a buffer with polyethylene glycol was added to all tubes except the Total Count tubes. All tubes were immediately vortexed. The precipitating reagent must be shaken thoroughly before use

9. All tubes were incubated for 15 min at room temperature

10. All tubes, except Total Count tubes, were centrifuged for 15 – 20 min at 1500 x g (approximately 3000 rpm)

11. The supernatant of each tube except Total Count tubes was carefully decanted by simultaneous inversion with a sponge rack into a radioactive waste receptacle. The tubes were allowed to drain on absorbent material for 15 – 30 sec and gently blotted with pieces of cotton to remove any droplets adhering to the rim before returning to the upright position

12. The radioactivity in each tube was counted for one min using gamma counter

Table 11 Concentration of Ultra-sensitive salmon CT standards.

Ultra-sensitive salmon CT standard	Catalog no.	Concentration (pg/mL)
Standard A	3601	0
Standard B	3602	7.5
Standard C	3603	30
Standard D	3604	60
Standard E	3605	125
Standard F	3606	250

16.10. Data analysis

After the value in count per min (CPM) was obtained for each tube, a correction for non specific binding was made by subtracting the average NSB count from the average CPM of each tube to obtain average net CPM (Equation1).The percentage of bound radio-labeled antigen (%B/Bo) was then determined from the following formula:

$$\% \text{ B/Bo} = \frac{\text{Mean sample counts} - \text{NSB counts}}{\text{Mean count of 0 pg/mL Standard A - NSBcounts}} \times 100$$

%B/Bo for the salmon CT standard was then plotted on the Y-axis against the log of salmon CT concentration (X-axis) on semi-log graph paper. A standard curve was drawn through the mean of the duplicate points. The concentration of salmon CT in plasma samples was determined from the standard curve.

17. Evaluation of Bioequivalence

The bioequivalence of two preparations of salmon CT nasal spray was evaluated by comparing two bioavailability parameters AUC and C_{max}. Both parameters were logarithmically transformed prior to data evaluation as suggested by USP and US FDA.

17.1 Statistical Test

The log-transformed values of AUC and C_{max} of the two preparations were evaluated by analysis of variance (ANOVA) after a Latin square, two-way crossover

design at significance level (α) of 0.05. Following ANOVA, the 90% confidence interval was constructed for each of the bioavailability parameter.

17.2 Construction of 90% confidence interval

A 90% confidence interval of individual parameter ratio based on log-transformed data was constructed using an equation:

$$90\ \%CI = (X_T - X_R) \pm (t_{0.1,df} \times S.E.)$$

Where

X_T and X_R = Mean in AUC and mean in Cmax values of the test and innovator's product, respectively

$t_{0.1,df}$ = Tabulated t-value at $\alpha = 0.1$ and df of the MSE (Mean square error)

$$S.E. = \sqrt{2MSE/n}$$

Where; MSE is the mean square error obtained from the ANOVA table

$$\%\ \text{Upper limit} = [e^{(X_T - X_R) + (t_{0.1,df} \times S.E.)}] \times 100$$

$$\%\ \text{Lower limit} = [e^{(X_T - X_R) - (t_{0.1,df} \times S.E.)}] \times 100$$

The test product was considered to be bioequivalent to the innovator's product when the 90 % confidence interval of the ratio of the individual parameter (test product relative to innovator's product) was within 80.0 – 125.0 % range for salmon CT (CDER.1999).