CHAPTER IV

RESULTS AND DISCUSSION

1. Assay of Salmon Calcitonin Powder (Standardization of Raw Material)

The lyophilized powder of salmon CT was determined for the net peptide content ($C_{145}H_{240}N_{44}O_{48}S_2$, excluding water and acetic acid) by reverse-phase gradient HPLC according to the method of the European Pharmacopoeia 2002, which is identical to the British Pharmacopoeia 2002.

The content of salmon CT in the test solution was calculated by comparing the peak area of its chromatogram with that of the reference solution (salmon calcitonin EPCRS). The retention time of both the test and reference salmon CT solutions were about 18.6 min. The salmon CT peak was not interfered by any peaks from the mobile phase or the buffer salt. The chromatograms of the reference and the test solutions dissolved in mobile phase A are shown in Figure 13.

From the calculation by Equation I, it was found that the content of salmon CT (Bachem® Lot No. 0547992), expressed as % net peptide, was 84.45 $\pm$ 1.00 % w/w. Percent assay (purity) was then calculated according to Equation II using the values of the net peptide content obtained from Equation I and the water and the acetic acid contents provided by the manufacturer (4.2 % and 11.2 % w/w, respectively, Bachem® 's certificate of analysis, Appendix B). It was found to be 99.59 $\pm$ 1.18 %, which was within the limits of 90.0–105.0% as specified in the European Pharmacopoeia 2002 (and British Pharmacopoeia 2002) for salmon CT raw material. The values of the net peptide content and percentage assay obtained from the analyses were similar to those reported in the Bachem®'s certificate. The analytical results are provided in Table 12 whereas the calculations of the individual values are provided in Appendix A.

Table 12 Content of salmon CT expressed as % net peptide and % assay (purity).

Assay No.	% Net Peptide Content	% Assay (purity)
1	85.28	100.57
2	84.55	99.71
3	83.16	98.06
4	83.74	98.75
5	85.53	100.86
Mean	84.45	99.59
S.D.	1.00	1.19
% C.V.	1.19	1.19

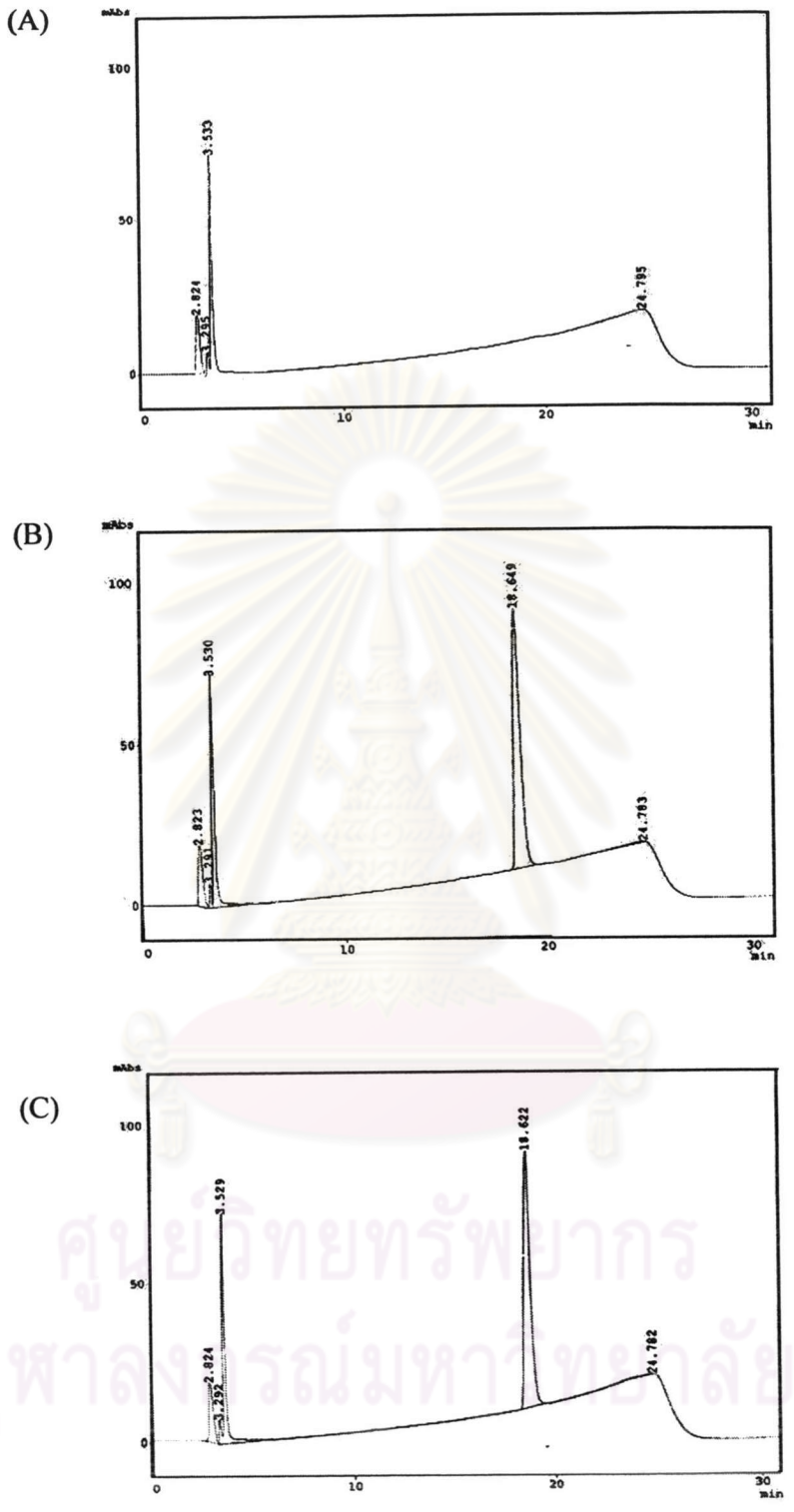


Figure 13. HPLC chromatogram of salmon CT.

(A) Blank; (B) Reference solution; and (C) Test solution. The retention time of salmon CT is 18.6 min.

2. Assay Validation of Salmon CT

2.1 Standard Calibration Curves

The standard calibration curve was done everyday before the analysis. Figure 14 shows one of the calibration curves of salmon CT in 0.1 M solution of sodium dihydrogen orthophosphate pH 4.0. The curve was obtained by plotting the peak areas of the HPLC chromatograms versus the known concentrations of salmon CT (Table 13). The standard solutions contained varying concentration of salmon CT from 1 to 40 µg/mL. These concentrations were used to perform the calibration curves in all experiments. The curves were linear and passed near the origin.

The linear regression equation for this curve was Y = 45516 X - 40981, where X and Y were the concentration (in µg/mL) and the peak area, respectively. The coefficient of determination (r^2) was 0.9997. All other standard curves gave similarly good linearity with the r^2 values in the range of 0.99 – 0.999. Consequently, these linear equations were used to determine the salmon CT content in the nasal preparations.

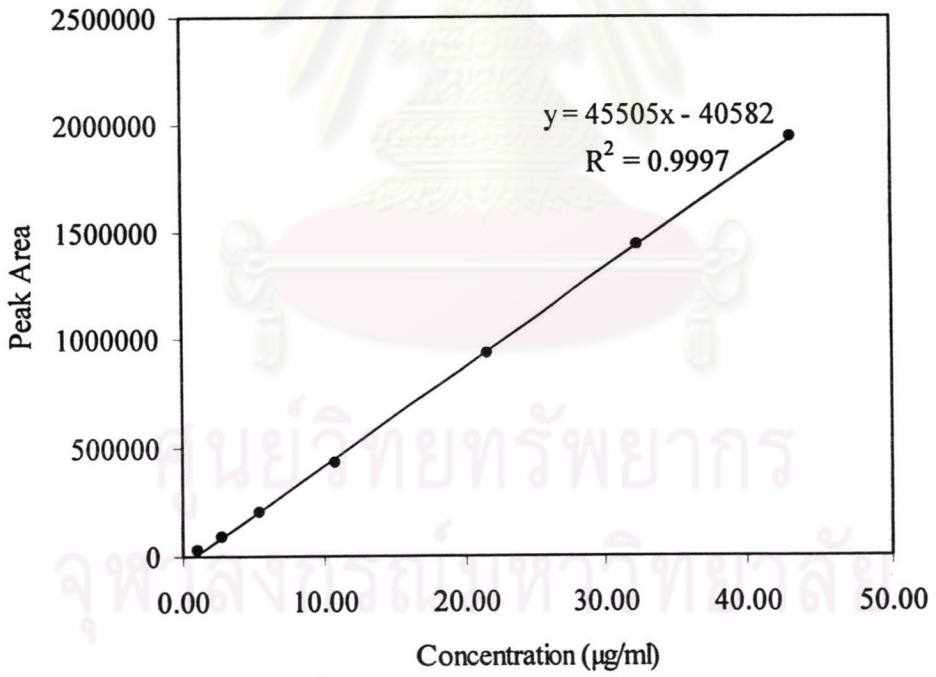


Figure 14 Representative calibration curve of salmon CT at different concentrations.

Table 13 Peak areas of salmon CT standard solutions for the construction of calibration curve in Figure 14.

Standard no	Concentration (μg/mL)	Peak area of salmon CT*	S.D.	% C.V.
1	43.20	1932775.67	831.69	0.04
2	32.40	1434468.33	23279.19	1.62
3	21.60	937110.33	17701.17	1.89
4	10.80	428502.00	3898.27	0.91
5	5.40	197878.67	1524.10	0.77
6	2.70	88787.67	627.81	0.71
7	1.08	27181.00	537.44	1.98

* Each data point is the means of three determinations.

* The individual data are shown in Appendix C.

2.2 Accuracy

The accuracy of the analytical method for salmon CT nasal preparations is shown in Table 14. With the exception of the lowest concentration studied (1 μg/mL), the percent analytical recovery of salmon CT was within ± 10 % of the nominal concentrations for concentrations between 5 and 40 μg/mL, with the values ranging from 93.04 to 102.57 %. The average percent recovery at 1 μg/mL concentration was 115.66 % whereas the C.V. was from 1.12 to 1.44 % at all concentrations.

Although the percent recovery at the lowest concentration was too much deviated from the nominal value indicating insufficient accuracy at this concentration, this posed no analytical problems because it was very unlikely that the concentration of salmon CT in the nasal preparations would drop down to this level. The concentration of salmon CT in the nasal solution with 100 I.U. per spray was about 220 μg/mL, a value much greater than all the standard concentrations used in the assay. In fact, appropriate dilution of the preparation to about 40 μg/mL was required before injecting into the HPLC.

In addition, the linearity of method was also evaluated by plotting the estimated concentration (amount found) versus the actual concentration (amount added) using the data from Table 14. The graph is shown in Figure 15. It can be seen from this figure that the graph was highly linear, with the R value (correlation coefficient) of 0.999, indicating a very good correlation.

2.3 Precision

The results of the within-run and between-run precision are shown in Table 15 and Table 16, respectively. The % C.V. for the within-run precision was from 1.19 to 1.53 %. Slightly higher variation was observed for the between-run precision, which gave % C.V. of 1.26 – 1.76 %. However, both the within- and between- run precision values were within the acceptable range of ± 2 % (WHO,1996).

In addition, repeated injections of 33 μg/mL standard solution also demonstrated very closeness of results, with mean estimated concentration of 33 ± 0.30 μg/mL and % C.V. of only 0.90% (Table 17). Since the analytical method employed here did not have an internal standard, adequate repeatability of injection was necessary to ensure reliable data.

Table 14 Accuracy data for the HPLC determination of salmon CT.

Standard No.	Actual concentration (μg/mL)	Estimated concentration (μg/mL)	% Recovery	% C.V.
1	40.64	40.50 ± 0.53	99.66 ± 1.31	1.31
2	20.32	20.84 ± 0.30	102.57 ± 1.48	1.44
3	10.16	9.82 ± 0.11	96.61 ± 1.08	1.12
4	5.08	4.73 ± 0.06	93.04 ± 1.21	1.30
5	1.02	1.18 ± 0.01	115.66 ± 1.44	1.25

* Results are the means of five determinations.
* The individual data are given in Appendix C.

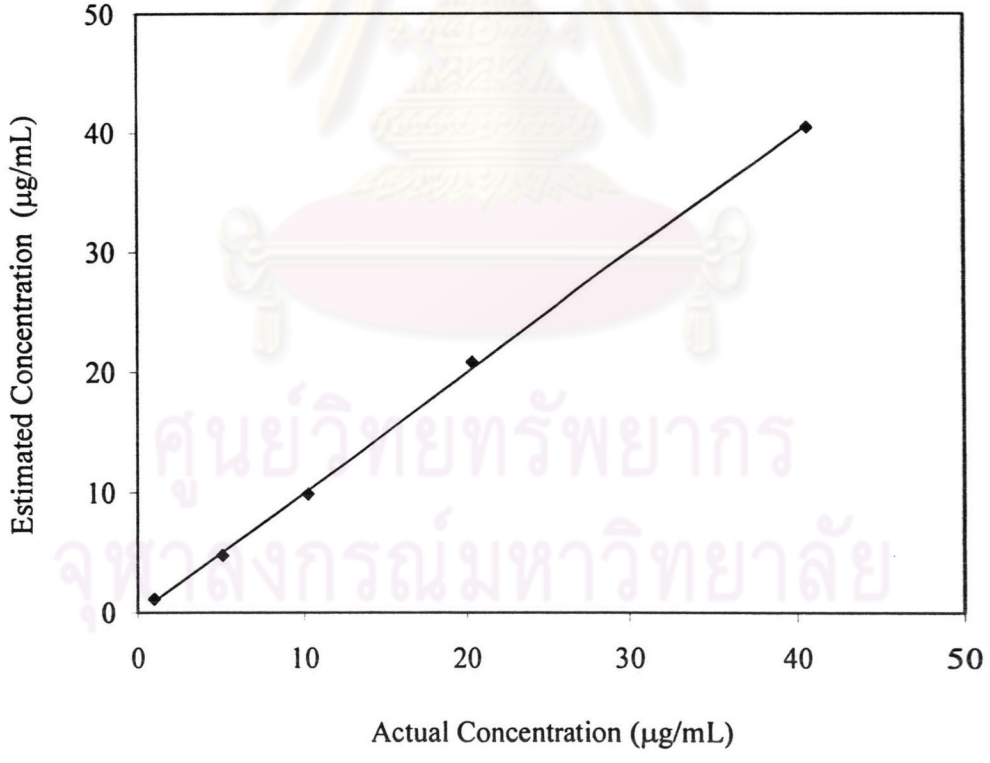


Figure 15 Diagram showing Linearity of Method (R = 0.999)

Table 15 Within-run precision data for HPLC determination of salmon CT.

Standard no.	Standard concentration (μg/mL)	Peak area*	S.D.	% C.V.
1	40.0	1911635.20	22745.20	1.19
2	10.0	369333.40	5227.20	1.42
3	1.0	39246.00	598.70	1.53

* Results are means of five determinations.
* The individual data shown in Appendix C.

Table 16 Between-run precision data for HPLC determination of salmon CT.

Standard no.	Standard concentration (μg/mL)	Peak area*	S.D.	% C.V.
1	40.0	1836555.40	23230.82	1.27
2	10.0	445948.87	7647.71	1.72
3	1.0	40060.20	703.23	1.76

* Results are mean of five determinations
* Individual data shown in Appendix C.

Table 19 Repeatability of HPLC injection

No. of Injection	Peak Area	Interpolated concentration (μg/mL)
1	1528597	33.37
2	1536843	33.62
3	1551010	33.92
4	1549793	33.89
5	1566866	34.26
6	1539865	33.68
Mean	1545496	33.79
S.D.	13399	0.30
% C.V.	0.87	0.90

3. Assay of Percent Labeled Amount of Salmon CT in the Nasal Sprays

After preparation, all 4 nasal spray batches were determined for their initial salmon CT content. Each batch was periodically tested to assay for salmon CT percent labeled amount and other properties according to the sampling schedule given in Table 6. The percent labeled amount of salmon CT in all batches were calculated by extrapolating the peak areas from the standard curves taking into account the dilution factor associated with the sample preparation. The data of the initial percent labeled amount and at different storage times (means of two determinations) are summarized in Table 18. As seen from this table, the initial values of all batches (both 100 and 200 IU dose) were well within the acceptable 90.0 – 115.0 % range, which is the assay limit required for salmon CT injections BP 2002.

4. Stability of Salmon CT Nasal Sprays

4.1 Percent Labeled Amount

The time profiles of the change in the percent labeled amount of peptide during storage at 30 °C (elevated condition) and at 4 °C (recommended condition) are depicted in Figures 16 and 17, respectively.

From Figure 16 and the data in Table 18, it can be seen that salmon CT nasal sprays retained more than 90% of its peptide dose per actuation when stored at 30° C for 2 months. After 2 months the content further dropped but not below the 90% lower limit, with the average values after 4-month storage at this elevated temperature of 91.65, 91.74, 90.79 and 90.01% for the four batches, respectively. This suggested a good stability of the nasal preparations in terms of the peptide content delivered per spray, which can be kept at ambient or room temperature for at least 4 months regardless of the strength (100 or 200 IU per spray).

The preparations were also stable for at least 12 months when kept at 4° C, which is a real storage temperature recommended for the salmon CT nasal sprays (Figure 17 and Table 18). The percent labeled amounts after 12 months were 107.70, 107.38, 107.49, and 107.49 for the four batches, respectively. These values remained relatively unchanged from the initial values and were all well within the acceptable range of 90.0 – 115.0 % labeled amount.

Table 18 Percent labeled amount of salmon CT at different storage condition times.

Formulation	% labeled amount of Salmon CT nasal spray at 30 °C				
	Day 0	1 mo.	2 mo.	3 mo.	4 mo.
Batch I	110.74	106.85	98.08	91.01	91.65
Batch II	110.41	109.46	97.86	90.18	91.74
Batch III	108.94	104.27	97.41	93.57	90.79
Batch IV	108.46	102.16	96.32	92.19	90.01
Formulation	% labeled amount of Salmon CT nasal spray at 4 °C				
	Day 0		3 mo.	6 mo.	12 mo.
Batch I	110.74		110.49	109.77	107.70
Batch II	110.41		110.17	109.96	107.38
Batch III	108.94		108.10	108.37	107.49
Batch IV	108.46		107.04	108.21	107.79

* Each value is a mean of two determinations.

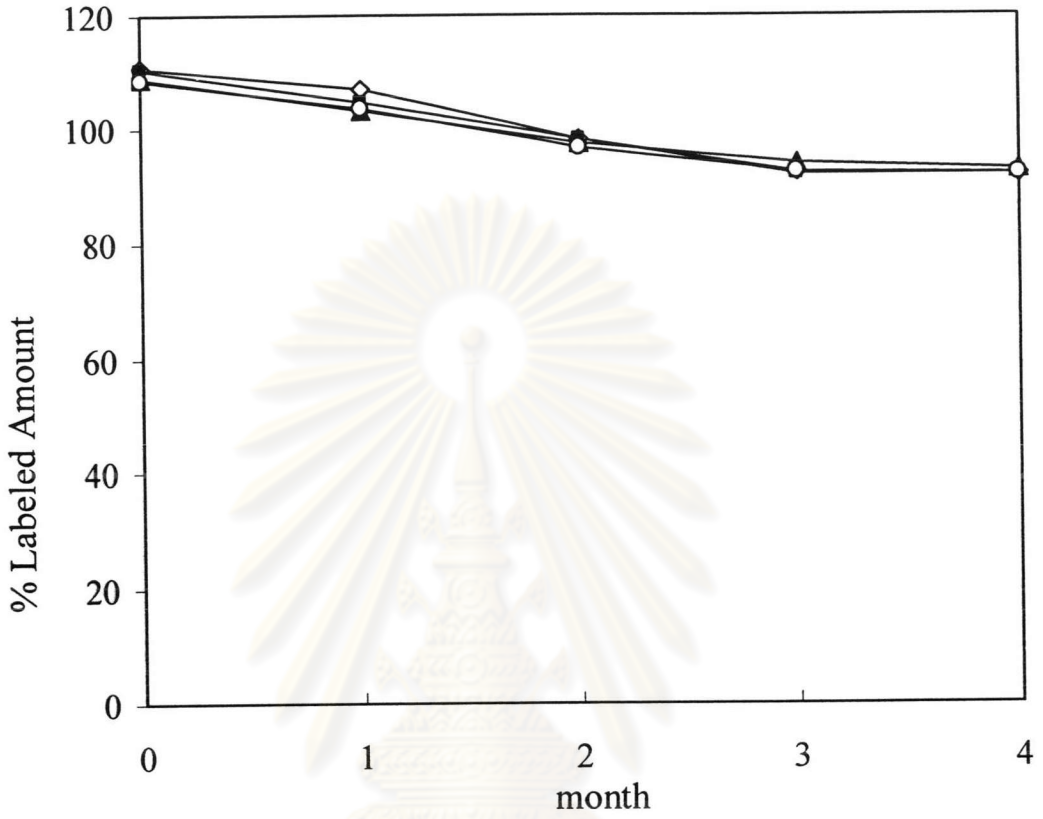


Figure 16 Percent labeled amount of salmon CT nasal sprays during storage at accelerated condition (30°C).

—◇— Batch I (100 IU/spray)

—■— Batch II (100 IU/spray)

—▲— Batch III (200 IU/spray)

—○— Batch IV (200 IU/spray)

* Each value is a mean of two determinations.

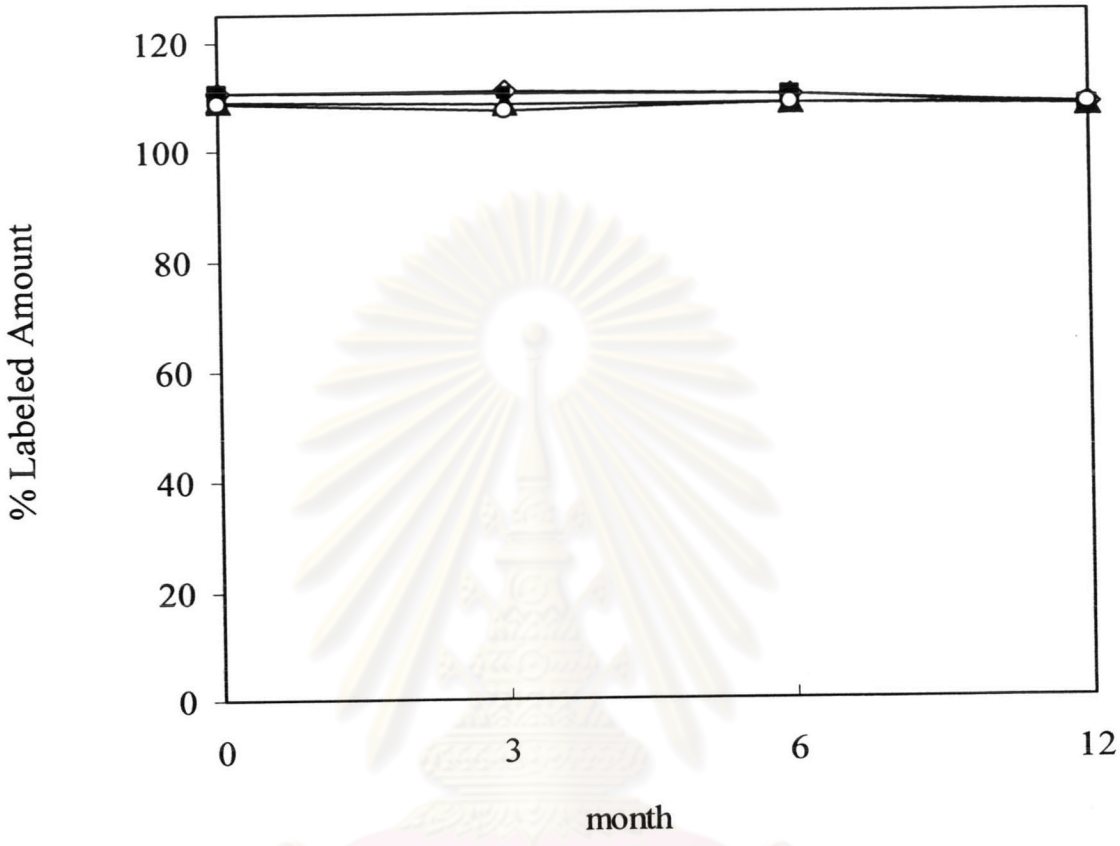


Figure 17 Percent labeled amount of salmon CT nasal sprays during storage at recommended condition (4° C).

—◇— Batch I (100 IU/spray)

—■— Batch II (100 IU/spray)

—▲— Batch III (200 IU/spray)

—○— Batch IV (200 IU/spray)

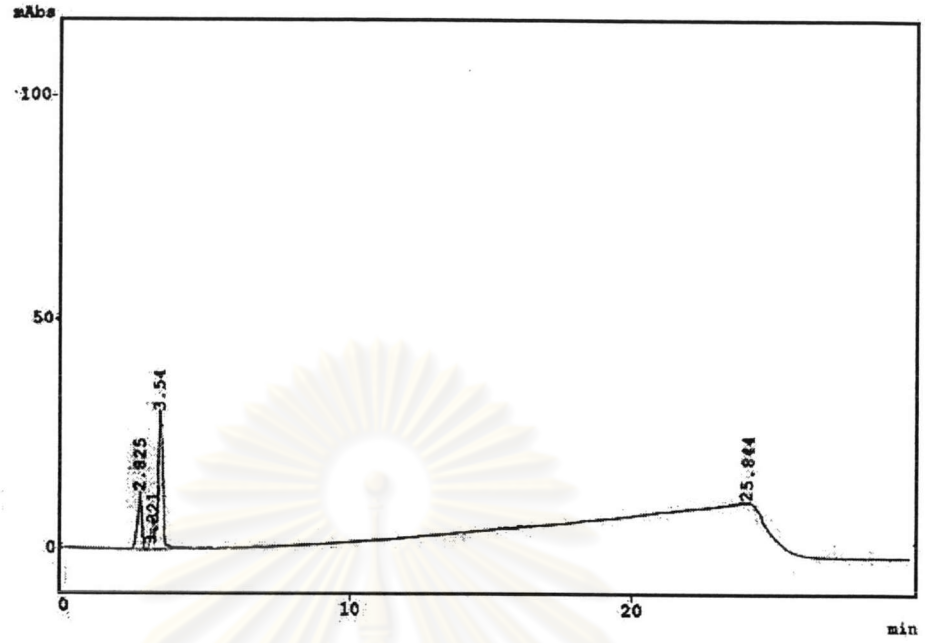
* Each value is a mean of two determinations.

4.2 Calcitonin C

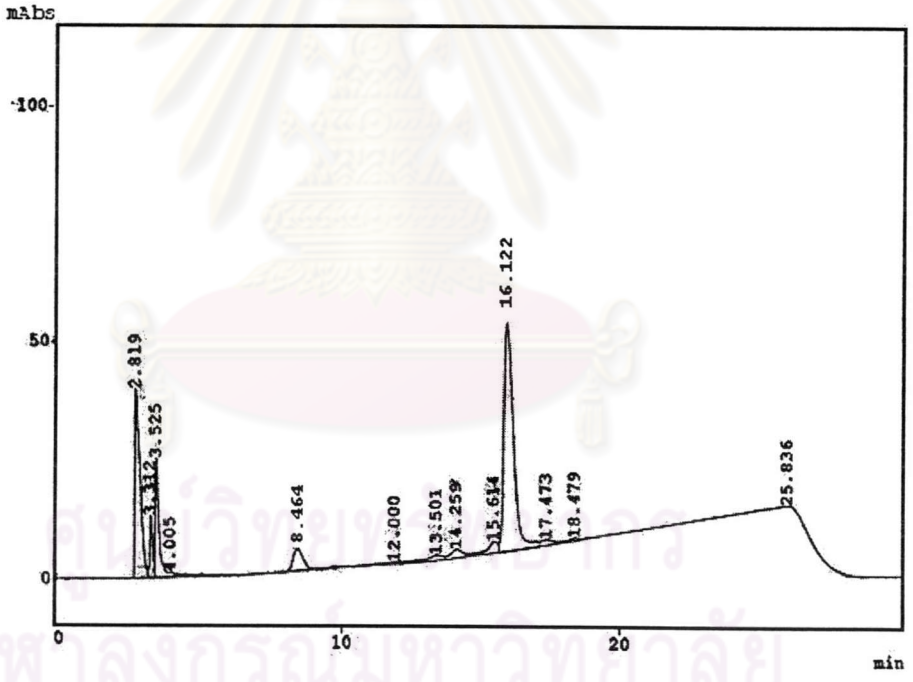
Figure 18 shows the chromatogram of calcitonin C reference solution, which was obtained using the same HPLC conditions as that for the assay of percent labeled amount. As defined by BP, calcitonin C is a heat-generated degradation product of salmon CT. The reference standard of calcitonin C can be obtained by oven-heating the product solution at 75 °C for 15 hr. It is identified as the largest peak in the chromatogram to elute after the buffer salts but before the principal salmon CT peak. From Figure 18, calcitonin C showed a retention time of 8.46 min compared to 16.12 min for salmon CT, giving a relative retention time of 0.53, which fell within the acceptable BP range of 0.5 – 0.6. Because the solution was heated before HPLC injection to accelerate the degradation, several other unidentifiable degradation products were also present. However, their peaks were much smaller and did not interfere with calcitonin C or salmon CT. In addition, the resolution factor between the calcitonin C and salmon CT peaks was calculated to be 15.3. This was greater than 3.0, a minimum value required for validity of the test as specified in the monograph of salmon CT injection BP 2002.

The analyses of percent calcitonin C in the nasal sprays after storage at 30 °C and 4 °C are presented in Table 19. All the nasal preparations passed the same BP limits required for salmon CT injections, which state that the area of the peak corresponding to calcitonin C must not be greater than 7% of the total peaks (salmon CT plus all the degradation product peaks). All four batches of the nasal sprays demonstrated very low or negligible amount of calcitonin C regardless of the storage conditions. The extent of calcitonin C was smaller than 7% limit indicating a good stability of the nasal spray formulations without major degradation at 30 °C for 4 months whereas at 4 °C the calcitonin C was not occur for 12 months, respectively.

A.



B.



Retention Time16.122 min = salmon CT

Retention time 8.464 min = Calcitonin C

Figure 18 HPLC chromatograms of calcitonin C reference solution.(A) Blank; (B) Calcitonin C reference solution

Table 19 The extent of calcitonin C in the salmon CT nasal sprays upon storage at different temperatures

Formulation	% Peak area of calcitonin C at 30 °C				
	Day 0	1 mo.	2 mo.	3 mo.	4 mo.
Batch I	NO	0.95	2.05	3.79	4.32
Batch II	NO	1.02	2.06	3.71	4.35
Batch III	NO	0.99	2.02	3.54	4.32
Batch IV	NO	0.92	1.90	3.42	4.35
Formulation	% Peak area of calcitonin C at 4 °C				
	Day 0		3 mo.	6 mo.	12 mo.
Batch I	NO		NO	NO	NO
Batch II	NO		NO	NO	NO
Batch III	NO		NO	NO	NO
Batch IV	NO		NO	NO	NO

Each value = Mean of two determination

NO = not occur

* The individual data are given in Appendix F.

4.3 Related Peptide

The related peptide content of the two nasal formulations (4 batches in total) was evaluated by HPLC using a different chromatographic system previously explained. N-acetyl-cys-1 calcitonin is a major related peptide of salmon CT and is used for the quality control of the raw material and the finished product in BP and EP. It also serves as a reference substance for HPLC method validation. The chromatograms of N-acetyl-cys-1 calcitonin EPCRS are shown in Figure 19. It gave a retention time of about 25 min, whereas salmon CT peak eluted slightly earlier at about 23 min. The relative retention time of N-acetyl-cys-1 calcitonin EPCRS, when mixed with the salmon CT sample (solution 2 in Chapter III, section 6), was in the range of 1.093 – 1.127. These values were close to the relative retention time of 1.15 suggested by BP 2002. Also, the resolution factor between the salmon CT and N-acetyl-cys-1 calcitonin EPCRS peaks was calculated to be 6.0, whereas the symmetry factor for the related peptide was 1.0 The two values were within the acceptable range (greater than 5.0 for the resolution factor and less than 2.5 for the symmetry factor), thereby indicating a good validity of the test.

After HPLC method validation, the extent of the related peptide in the products was evaluated by determining the areas of all the secondary peaks, including N-acetyl-cys^{1}-calcitonin, in the chromatogram of the diluted spray samples (solution 1 in Chapter III, section 6) in relation to the total peak areas. The values are shown in Table 20. From this table, it can be seen that the percent related peptide in the samples slowly increased with time regardless of the formulation and storage condition. However, the values of these combined secondary peaks were less than 5 % in all cases and no individual secondary peak gave value greater than 3 %. Thus, all the four batches of the nasal sprays passed the test for related peptide. They also demonstrated a good stability as the extent of the related peptides did not exceed the limits even after storage at an elevated temperature.

Table 20 Relative retention time of N-acetyl-cys-1 calcitonin EPCRS and the percent related peptide found in the salmon CT nasal sprays

A. Initial (Day 0)

Formulation	Relative retention time	% Related peptide by total area
Batch I	1.112	0.87
Batch II	1.111	0.83
Batch III	1.119	1.09
Batch IV	1.120	1.11

B. After 4-month storage at 30 °C

Formulation	Relative retention time	% Related peptide by total area
Batch I	1.109	1.20
Batch II	1.114	1.56
Batch III	1.117	1.49
Batch IV	1.113	1.42

C. After 6-month storage at 4 °C

Formulation	Relative retention time	% Related peptide by total area
Batch I	1.126	1.85
Batch II	1.127	1.86
Batch III	1.093	2.60
Batch IV	1.120	2.58

* The individual data are given in Appendix F.

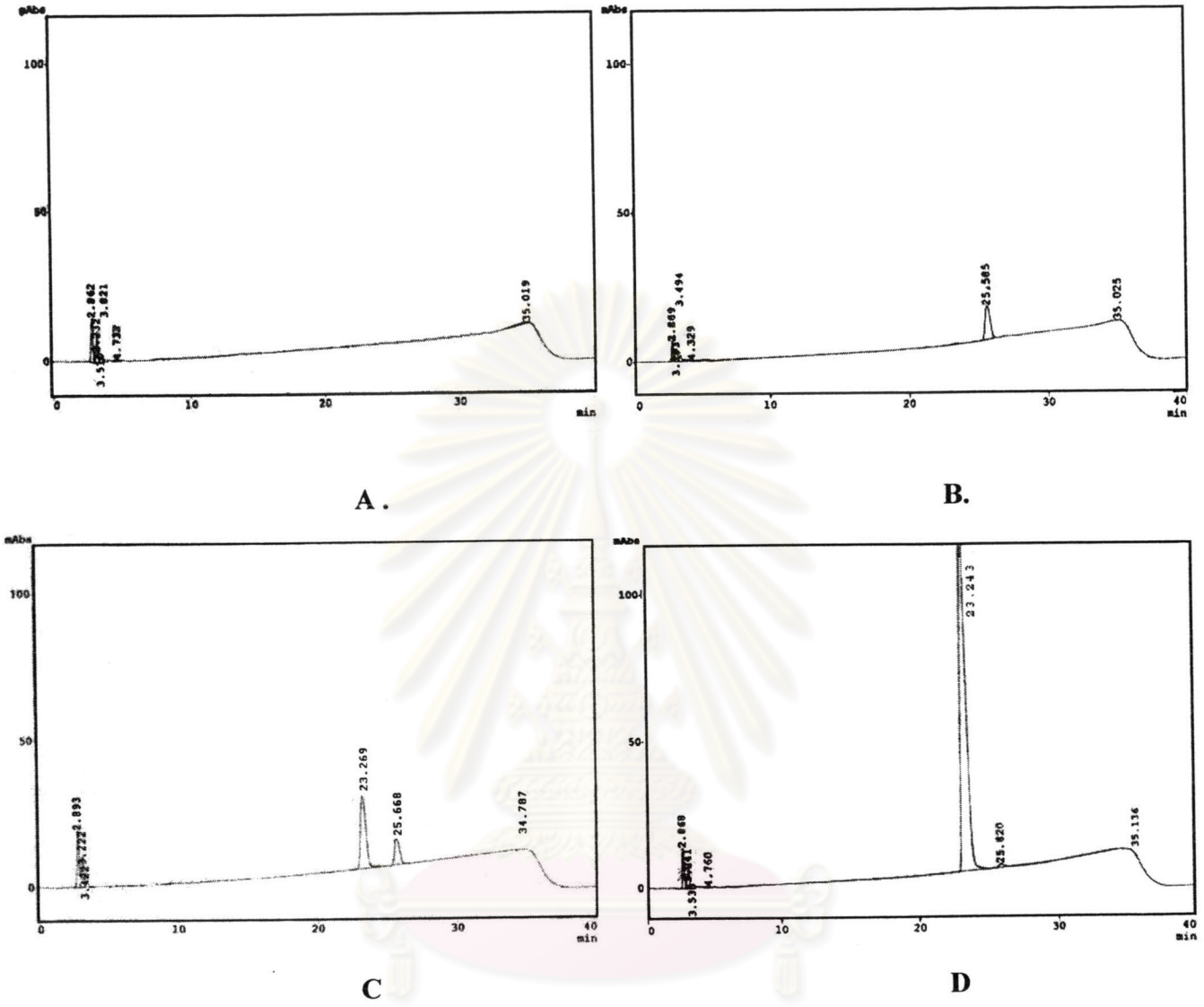


Figure 19 Chromatograms showing related peptide and salmon CT peaks.

(A) Blank

(B) N-acetyl-cys[1]-calcitonin EPCRS (retention time 25.585)

(C) Solution 2: Salmon CT nasal spray (retention time 23.265) mixed with N-acetyl-cys[1]-calcitonin EPCRS (retention time 25.668)

(D) Solution 1: Salmon CT nasal spray sample without N-acetyl-cys[1]-calcitonin EPCRS (retention time of salmon CT = 23.243 and intrinsic N-acetyl-cys[1]-calcitonin = 25.820)

4.4 Acidity

All nasal spray preparations had the acidity between 3.3-3.7, the most stable pH condition for salmon CT solutions (Lee,1992). Moreover, this range was reported to be acceptable for the nasal preparation of salmon CT because it was not irritating to the nasal cavity (Kurose, 1987; Hussain, 1990; and Kagatani et al., 1996). The pH of nasal preparations also closed to the BP 2002 specification for salmon CT injection, which requires the product to have the pH between 3.9 and 4.5. The pH data of the nasal sprays at different storage times and temperatures are presented in Table 21.

Table 21 pH of the nasal spray preparations stored at 30 °C and 4 °C.

Formulation	pH			
	Day 0	4 months (30°C)	6 months (4°C)	12 months (4°C)
Batch I	3.50 ± 0.006	3.49 ± 0.006	3.50 ± 0.006	3.50 ± 0.006
Batch II	3.51 ± 0.010	3.51 ± 0.012	3.50 ± 0.006	3.51 ± 0.006
Batch III	3.53 ± 0.006	3.53 ± 0.006	3.54 ± 0.012	3.52 ± 0.010
Batch IV	3.53 ± 0.015	3.53 ± 0.006	3.53 ± 0.020	3.54 ± 0.006

* Each value = Mean ± S.D. (n = 3)

4.5 Osmolarity

The osmolarity of all batches of the nasal sprays were tested prior to filtration and bottling (day 0) and also at the end of the stability testing period. The results were in the isotonic range of 290 - 310 mOsm/kg (Dau, Zia and Needham, 1997). The osmolarity data of the nasal sprays are given in Table 22.

Table 22 Osmolarity of the nasal spray preparations stored at 30 °C and 4 °C.

Formulation	Osmolarity (Osmol/Kg)			
	Day 0	4 months (30°C)	6 months (4°C)	12 months (4°C)
Batch I	0.299 ± 0.0020	0.298 ± 0.0017	0.299 ± 0.0021	0.298 ± 0.0010
Batch II	0.301 ± 0.0006	0.301 ± 0.0017	0.299 ± 0.0026	0.299 ± 0.0021
Batch III	0.304 ± 0.0015	0.302 ± 0.0029	0.300 ± 0.0021	0.302 ± 0.0010
Batch IV	0.299 ± 0.0015	0.301 ± 0.0017	0.300 ± 0.0026	0.302 ± 0.0006

* Each value = Mean ± S.D. (n = 3)

4.6 Clarity

By visual inspection, all the nasal spray preparations were observed to be colorless, transparent, and free from any visible particles and fibers during the entire storage period (4 months at 30 °C and 12 months at 4 °C, Tables 23 and 24).

Table 23 Clarity of the nasal spray preparations after storage at 30 °C

Formulation	Time (months)				
	0	1	2	3	4
Batch I	Clear	Clear	Clear	Clear	Clear
Batch II	Clear	Clear	Clear	Clear	Clear
Batch III	Clear	Clear	Clear	Clear	Clear
Batch IV	Clear	Clear	Clear	Clear	Clear

Table 24 Clarity of the nasal spray preparations after storage at 4 °C

Formulation	Time (months)			
	0	3	6	12
Batch I	Clear	Clear	Clear	Clear
Batch II	Clear	Clear	Clear	Clear
Batch III	Clear	Clear	Clear	Clear
Batch IV	Clear	Clear	Clear	Clear

4.7 Sterility Test

Samples of nasal spray preparations were sent to Microbiology Department at Siriraj Hospital to test for sterility. All the four batches passed the test after storage at both 4 and 30 °C as reported in Table 25. The test specimen gave negative results after 14 days of incubation

Table 25 Sterility test results after storage at 4 and 30 °C.

Formulation	Amount of sampling	Storage time			
		Day 0	4 months (30 °C)	6 months (4 °C)	12 months (4 °C)
Batch I	10	Pass	Pass	Pass	Pass
Batch II	10	Pass	Pass	Pass	Pass
Batch III	10	Pass	Pass	Pass	Pass
Batch IV	10	Pass	Pass	Pass	Pass

4.8 Uniformity of Mass (Weight per Spray)

The results of the average weight per spray (mean of ten sampled nasal spray bottles) are shown in Table 26. The purpose of this test is to ensure the reproducibility of the mechanical function of the metered dose spray components, especially when the valve is actuated by hand. According to the BP 2002 general monograph for the nasal spray solution, the products comply with the test if not more than two of the individual values deviate by more than 25 per cent from the average value and none deviates by more than 35 per cent. The results showed that none of the individual values were more than 25 percent of the average value at all determination times. Thus, the data clearly demonstrated that all four batches of the nasal spray preparations were able to deliver the labeled amount of the solution accurately and reproducibly, even at accelerated storage condition (30 °C).

Table 26 Uniformity of mass (averaged weight per spray of ten bottles) determined at different storage times and temperatures.

Formulation	Averaged weight per spray (gm) At different storage times			
	Day 0	4 months (30 °C)	6 months (4 °C)	12month (4°C)
Batch I	0.09015 ± 0.002	0.09006 ± 0.004	0.0907 ± 0.002	0.09013 ± 0.003
Batch II	0.09034 ± 0.002	0.09036 ± 0.003	0.0907 ± 0.003	0.09015 ± 0.003
Batch III	0.09126 ± 0.001	0.09118 ± 0.003	0.0910 ± 0.002	0.09030 ± 0.001
Batch IV	0.09137 ± 0.001	0.09075 ± 0.003	0.0906 ± 0.002	0.09101 ± 0.002

Each value = Mean ± S.D. (n = 10 bottles).

5. Leak Test

Leak test of the sample spray bottles was performed both at Mary Commercial Supplier Co., Ltd., Thailand, and at Erich Pfeiffer GmbH, Germany. Both companies reported that the crimped nasal spray products had satisfactory tightness between the spray pump and the bottle. The leak test is critical for other tests such as particle size measurement, droplet size distribution, as well as uniformity of mass and sterility tests. If the products demonstrated any leakage, the results of the above-mentioned tests would not be valid. The results of leak test are shown in Table 27.

Table 27 Leak test results.

Formulation	Amount of sampling	Amount of passed samples
Batch I	25	25
Batch II	25	25
Batch III	25	25
Batch IV	25	25

6. Droplet Size Measurement and Droplet Size Distribution

The tests were performed at Erich Pfeiffer GmbH, Germany, employing the *in vitro* tests recommended by US Food and Drug Administration (US FDA) Guidance for Industry 2002 Nasal spray and inhalation solution suspension, and spray drug products chemistry, manufacturing, and control document. The results of all tested bottles (n=25 per batch) were expressed in term of ranges for the D10, D50, D90 and span as defined in the above Guidance (see Chapter III p.52). The particle size distribution curves give a normal distribution. The median particle size (D50) of both formulations were in the range of 26.54 to 45.44 μm. For effective nasal delivery the particle or droplet with size between 10 -50 μm to be able to be deposited in the upper respiratory tract, for subsequent permeation (Mygind,1998). Particles with

larger size than this will be removed, whereas those smaller than 2 μm will be exhaled. Since all the distribution curves displayed a normal or Gaussian distribution, the median particle size (D50) could be regarded as the mean particle size.

The span value, which is a simple measurement of the width of the distribution, was calculated by (D90 –D10/D50). The average span number of placebo A and B were 3.89 $\pm$ 2.53 and 5.04 $\pm$ 3.65, respectively. The results of the droplet size measurement are shown in Table 28 and the representative curve of the droplet size distribution taken from one bottles is illustrated in Figure 20. The individual data for each formulation are given in Appendix H (Table 67 and 68).

Table 28 Droplet size measurement of salmon CT nasal spray 100 IU and 200 IU (placebo A and B)

Sample	Percentage of droplet less than 10 µm (%)	D_{10} (µm)	D_{50} (µm)	D_{90} (µm)	Span
Placebo A	3.05 ± 1.21	14.79 ± 1.52	34.36 ± 5.14	151.18 ± 95.79	3.89 ± 2.53
Placebo B	3.07 ± 0.74	14.50 ± 0.80	34.34 ± 3.07	189.03 ±132.33	5.04 ± 3.65

* Each value = Mean $\pm$ S.D. (n = 25 bottles)

* D_{10} = 10% of the droplet diameters are smaller than the indicated value
* D_{50} = 50% of the droplet diameters are smaller than the indicated value
* D_{90} = 90% of the droplet diameters are smaller than the indicated value
* Span = $[(D_{90}-D_{10})/D_{50}]$
* Placebo A; representative salmon CT nasal sprays 100 IU per actuation
* Placebo B; representative salmon CT nasal sprays 200 IU per actuation

| Pfeiffer |

User Name: Leichenauer Jutta

Security Level: 1

Sample Details

Sample ID: 0302021/147
Sample File: FEB03
Sample Path: C:\SIZERD~1\2003\
Sample Notes: Customer
Articel:49013 Actuator 58405
Medium: Saline Solution Placebo B
Pump No.9

Run Number: 9
Record Number: 147

Measured: Fri 21. Feb 2003 10:48
Analysed: Fri 21. Feb 2003 10:49
Result Source: Analysed

System Details

Range Lens: 1000 mm
Presentation: 3QAA
Analysis Model: Compressed Range
Modifications: None

Beam Length: 14.30 mm
[Particle R.I. = (1.3000, 0.0000); Dispersant R.I. = 1.0000]

Sampler: None

Obscuration: 24.4 %
Residual: 0.300 %

Result Statistics

Distribution Type: Volume
Mean Diameters:
D [4, 3] = 62.29 um

Concentration = 0.0193 %Vol
D (v, 0.1) = 15.43 um
D [3, 2] = 30.89 um

Density = 1.000 g / cub. cm
D (v, 0.5) = 39.86 um
Span = 2.415E+00

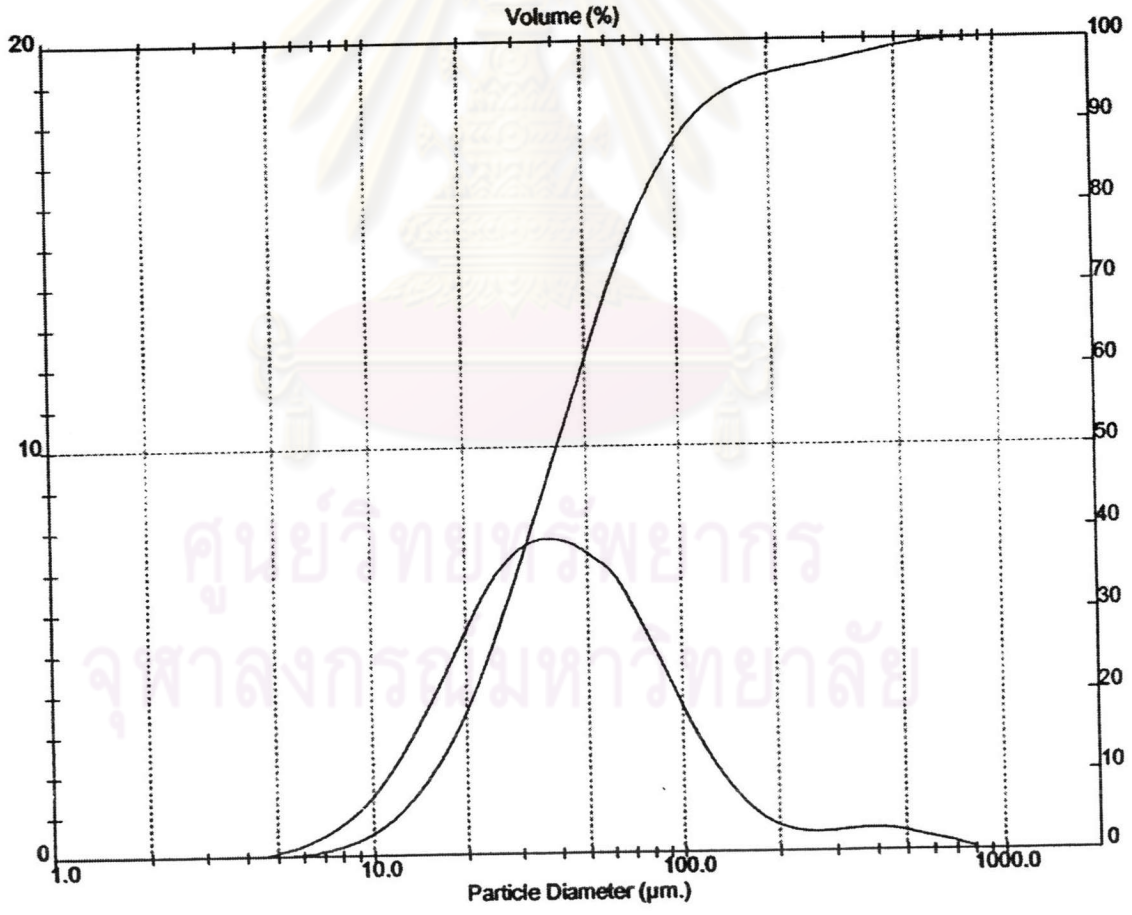
Specific S.A. = 0.1943 sq. m / g
D (v, 0.9) = 111.68 um
Uniformity = 9.636E-01

Size (um)	Volume Under %
10.00	2.81
30.00	35.71

Size (um)	Volume Under %
50.00	61.24
100.00	87.66

Size (um)	Volume Under %
500.0	99.08

Size (um)	Volume



Malvern Instruments GmbH.
Herrenberg, Germany
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Mastersizer S long bed Ver. 2.19
Serial Number: 32913-120

21 Feb 03

Figure 20 Representative particle size distribution of calcitonin nasal spray (placeboB)

7. Spray Pattern Test

Samples of the finished nasal sprays (n=25 per batch) were tested for their spray patterns by Erich Pfeiffer GmbH, Germany. Spray pattern testing allows the cross-sectional uniformity of the spray shape to be evaluated at specified distances away from the pump orifice tip. The measurement parameters were as recommended by the US FDA guidance for industry: Nasal spray and inhalation solution suspension, and spray drug products chemistry, manufacturing, and control document (2002). The spray pattern obtained were relatively uniform in shape and density. The mean diameter of the spray pattern, which is the average of 25 bottles, was found to be 38.04 ± 3.33 mm and 36.00 ± 4.19 mm for Placebo A (representative of salmon CT nasal spray 100 IU) and Placbo B (representative of salmon CT nasal spray 200 IU), respectively. The individual spray patterns show homogeneous distribution of droplets with round distribution. The ovality ratio, which is the ratio of Dmax to Dmin was calculated to be 1.27 ± 0.12 and 1.24 ± 0.11 for A and B . These ratios are within the generally accepted range of 1.0 – 1.3, indicating a good uniformity of the spray pattern in which the slightly greater than 1.0 value indicated the ellipsoidal shape of the spray. Spray angle, which reflects the shape of the plume, was determined from the maximum diameter of the spray pattern and the distance from the paper (30mm). The mean spray angles were 64.65° ± 4.50 and 61.69° ± 5.82 for A and B, respectively. The data of the spray pattern test and spray angle are given in Table 29 and the representative of spray shapes are illustrated in Figure 21.

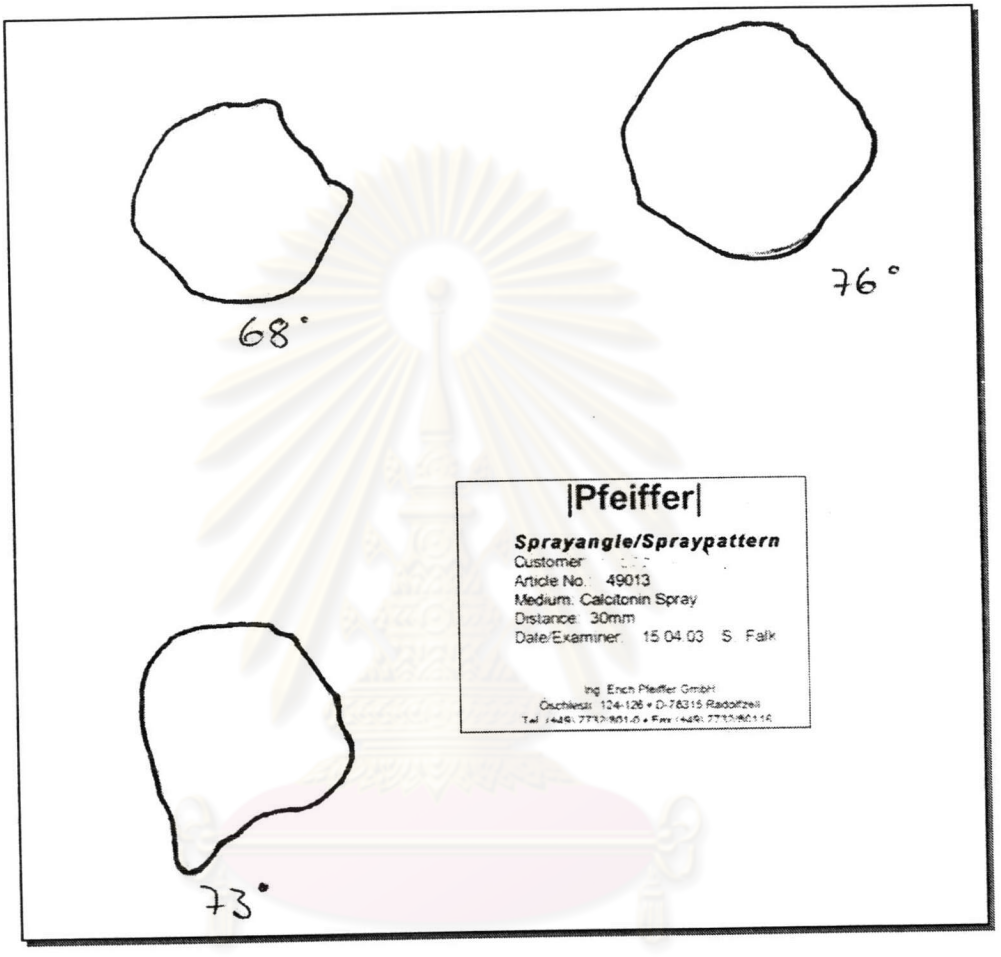

Figure 21 Typical shape of spray pattern at spray angles of 68°, 76° and 73°.

Table 29 Average diameters at different spray angles of salmon CT nasal spray 100 IU and 200 IU (placebo A and B)

Sample	Dmin (mm)	Dmax (mm)	Mean Diameter (mm)	Angle	Ovality Ratio
Placebo A	33.50 ± 3.59	42.58 ± 3.92	38.04 ± 3.33	64.65 ± 4.50	1.27 ± 0.12
Placebo B	32.08 ± 4.07	39.92 ± 4.91	36.00 ± 4.19	61.69 ± 5.82	1.24 ± 0.11

* Each value = Mean ± S.D. (n = 25 bottles)
* Dmin = Smallest Diameter
* Dmax = Largest Diameter
* Mean Diameter = [(Dmin + Dmax)/2]
* Spray angle = $180 - 2\theta$, where $\tan\theta = 2h/Dmax$; h=Distance between plate and spray nozzle(h= 30mm)
* Placebo A; representative salmon CT nasal sprays 100 IU per actuation
* Placebo B; representative salmon CT nasal sprays 200 IU per actuation
* Individual data shown in Appendix H.

8. In Vivo Study

8.1 Analysis of Salmon CT in Plasma

To prove that salmon CT is absorbed intranasally into the circulation, measurements of plasma salmon CT level following nasal administration were made and compared to the innovator's product (Miacalcic Nasal Spray, strength 200 IU per actuation). The lot number, manufacturing date, expiration date and other specifications of the innovator's product used in this study are given in Table 30. For this part of study, the highest strength (200 IU per actuation) was used and batch no. III of the prepared nasal sprays (200 IU) was arbitrarily chosen as the test product.

Table 31 shows the in vitro assay results of the innovator's product used in this study in comparison with the test product. It can be seen from this table that both products passed the in vitro testing with respect to the initial percent labeled amount, calcitonin C, related peptide, clarity and uniformity of mass. Thus, it can be concluded that the test products, especially the 200 IU strength (batch III and IV), were pharmaceutically equivalent to the innovator's product. Consequently, the test product prepared in this project was considered suitable for further in vivo bioequivalence study.

Healthy twelve male subjects were enrolled in this study based on passing the physical examination and showing normal blood and urine chemistry. Their demographic data are provided in Table 56 in Appendix E. The results of their blood and urine chemistry are given in Table 57 in Appendix E together with the normal ranges.

Table 30 Test Products

Product	Brand Name	Strength	Batch no.	Mfg. Date	Exp. Date
Innovator's Product	Miacalcic (Novartris)	200 IU/puff	H3034	07-2003	07-2006
Test's Product	Salmon CT Nasal spray	200 IU/puff	200-III	05-2003	Not indicated

Table 31 Comparison of the initial percent labeled amount and other *in vitro* quality parameters between the innovator's and the test nasal spray products (strength 200 IU per spray).

Quality Parameters**	Miacalcic Nasal Spray (Innovator's Product) Mfg. 07-2003	Test Product (Batch III) Mfg. 05-2003	Test Product (Batch IV) Mfg. 05-2003
Percent labeled amount	104.42	108.37	108.21
Calcitonin C (%)	NO	NO	NO
Related peptide (%)***	0.968	1.09	1.11
Clarity	Clear	Clear	Clear
Uniformity of Mass	0.09*	0.091 ± 0.001	0.091 ± 0.001

* Package inserts information.

** In vitro evaluation comparing the test products (III, IV) with the innovator's product was performed simultaneously (at 6 months after preparation of the test products)

*** Related peptide evaluation comparing the test products (III, IV) with the innovator's product was performed simultaneously, at the initial of production (day0).

Plasma salmon CT was quantitated by radioimmunoassay (RIA) technique using a standard RIA kit developed by Diagnostics Systems Laboratories (Webster, TX). All the analyses were performed in duplicate. Figure 22 is a representative standard RIA curve for salmon CT. It is a semilogarithmic plot of the ratio of percent bound (B) to unbound (Bo) radiolabeled salmon CT (%B/Bo) on a linear scale versus standard salmon CT concentration on a logarithmic scale. The curve was sigmoidal indicating the saturable nature of the binding process. The standard curve was prepared for every experiment and all the curves obtained were similar, suggesting a low variability between assays. The best-fitted line was determined using a least square regression analysis. For example, the best-fitted equation for standard curve in Figure 22 was $y = 91.685e^{-0.0052x}$ ($r^2 = 0.9906$). Data for construction calibration curve are shown in Appendix E.

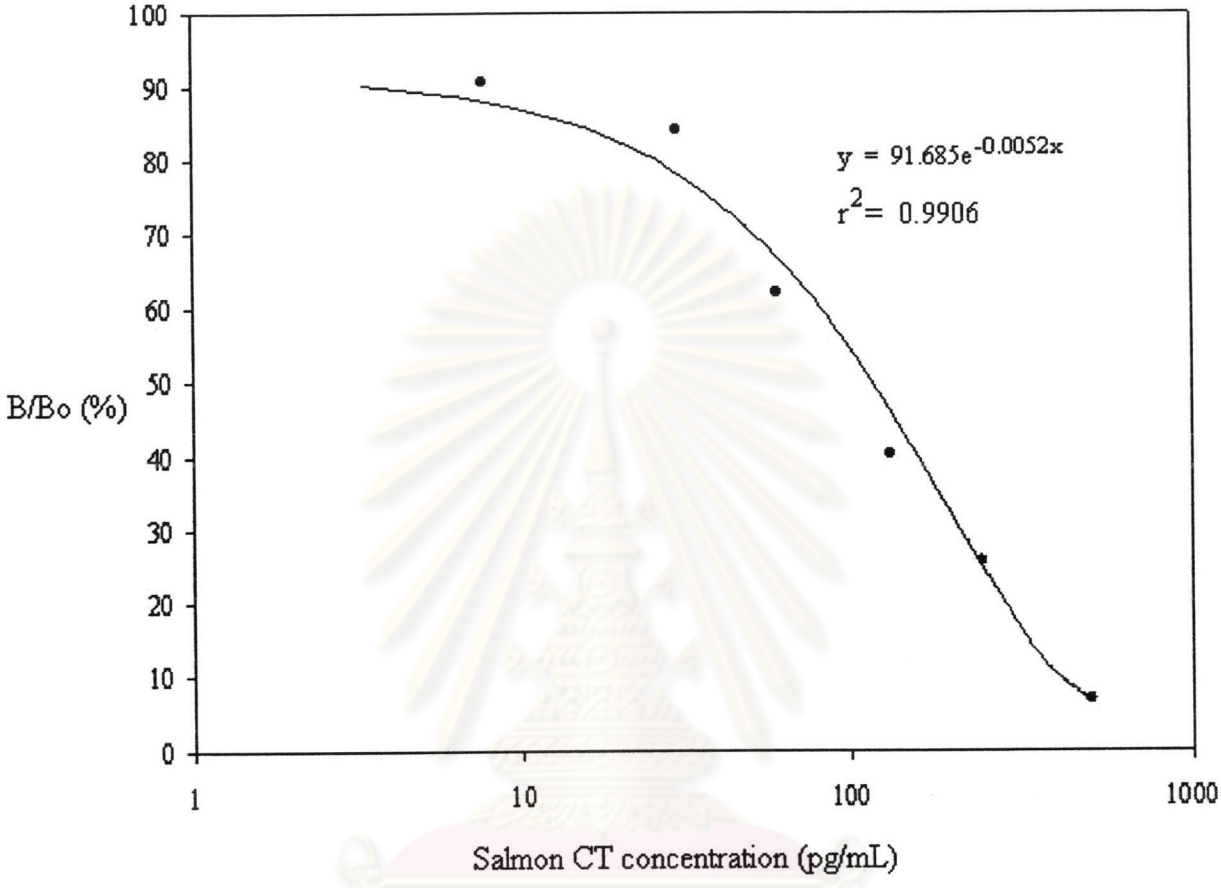

Figure 22 Representative standard RIA curve for salmon CT.

8.2 Determination of Bioavailability Parameters (AUC, Cmax and Tmax)

The salmon CT standards, as provided by the RIA kit manufacturer, were from 7.5 to 500.0 pg/mL. After correction for the non-specific binding (NSB), the value of B/Bo was calculated for each plasma sample and this value was then interpolated from the standard curve to obtain the concentration of salmon CT in plasma. If the estimated value were less than the limit of quantitation (7.5 pg/mL), it would be regarded as non-quantifiable (NQ). The estimated plasma data of the individual subjects are given in Table 32 and 33. The plasma salmon CT concentration was also measured prior to drug administration (t = 0 min) for each subject. This value was then subtracted from the plasma concentration at all the subsequent times to correct for the cross-reactivity of the assay, since the antibody in the test kit may interact with the intrinsic human CT in plasma thereby yielding a small "baseline" value (although the extent of such cross-interaction was extremely small, < 2%). The plasma data after baseline (or zero time) correction are given in Tables 34 and 35 for the test and the innovator's products, respectively, whereas the plasma salmon CT concentration-time profiles of the individual subjects are graphically shown in Figures 23 - 34.

The mean plasma profiles of the twelve subjects are illustrated in Figure 35. Trapezoidal rule was used to estimate the AUC values whereas the Cmax and Tmax values were directly taken from the plasma salmon CT concentration – time profiles of the individual subjects. The values of AUC, Cmax and Tmax are given in Table 36, 37, 41 and 44 (tmax). None of the subjects withdrew from the study or exhibited any signs of allergy and adverse drug reactions to both products throughout the study period. This indicated a good tolerance of the subjects to both products.

It is interesting to note that the plasma concentration-time profiles of most subjects exhibited a smaller, secondary peak following the main peak. This phenomenon was observed in both the innovator's and the test products. However, the extent of the secondary peak varied from subject to subject. For example, secondary peak was distinctively high and well separated from the main peak in subjects no. 2, 7, 9, 11, and 12 after receiving the test nasal spray, as well as in subjects no. 4, 5, and 11 after administration of the innovator's product. The rest of the subjects, however, showed a more flattened, less distinctive secondary peak with a shoulder-like shape.

The reason as to the observation of this phenomenon was not clearly known. It could be due to an enterohepatic circulation behavior of the drug or simply due to idiosyncratic behavior of the drug in plasma. Moreover, in subject no. 5 (Figure 27) there was an extra peak that was observed before the main peak and could not be clearly explained. It is possible that a small fraction of the dose administered to this subject might have reached the systemic circulation before the rest of the dose, resulting in the observation of a smaller peak coming out before the main peak. However, after all the data from the 12 subjects had been combined and averaged, the mean plasma drug concentration – time profile showed only a single peak as seen in Figure 35, indicating a rapid, single absorption phase. This profile was in agreement with other researchers, who also reported a single peak in the mean plasma concentration – time profile following nasal administration of salmon CT in healthy volunteers (Lee et al., 1994, Kurose et al., 1987, Buclin et al., 1987)

Table 32 Plasma Salmon CT Interpolated Concentrations (pg/ml) of 12 Subjects Following Intranasal Administration of The Test Preparation (200 IU per actuation) at 400 IU dose.

(NQ = Non-quantifiable; The estimated value < 7.5 pg/ml which was the LLOQ).

Time (min)	Subj. 1	Subj. 2	Subj. 3	Subj. 4	Subj. 5	Subj. 6	Subj. 7	Subj. 8	Subj. 9	Subj. 10	Subj. 11	Subj. 12	Mean	S.D.	% C.V.
0	NQ	11.98	9.89	8.30	10.11	8.01	7.88	9.23	NQ	7.96	9.00	11.26	9.36	1.44	15.34
5	29.09	12.2	28.57	55.70	73.70	35.09	31.99	29.61	31.82	41.82	35.81	53.75	38.26	16.07	42.01
10	70.39	144.95	63.86	70.52	48.94	38.07	38.34	46.92	47.62	46.66	103.78	129.29	70.78	36.12	51.03
15	111.3	74.76	112.76	135.84	139.01	120.98	141.84	140.26	118.98	118.17	140.61	115.36	122.49	19.19	15.67
20	51.19	30.64	106.95	111.66	131.73	101.23	48.26	34.67	73.83	42.2	51.31	22.64	67.19	36.68	54.58
30	48.55	54.72	33.03	82.33	73.39	18.85	31.38	28.96	5.02	37.65	23.42	26.33	38.64	22.48	58.18
45	17.42	19.68	23.04	17.61	23.49	23.23	51.61	25.09	28.38	30.46	50.34	31.54	28.49	11.43	40.13
60	6.69	22.79	23.88	24.43	25.28	19.95	24.32	31.51	15.56	32.76	21.01	30.75	23.24	7.21	31.01
90	7.36	19.06	28.02	17.22	16.44	9.55	18.25	34.53	25.36	24.78	15.72	15.45	19.31	7.71	39.92
120	12.57	13.84	20.89	12.98	15.10	13.94	26.52	14.06	8.68	11.38	8.57	11.61	14.18	5.03	35.49
180	NQ	15.23	14.51	11.56	11.07	8.70	11.84	8.44	NQ	NQ	NQ	8.42	11.22	2.66	23.66

Table 33 Plasma Salmon CT Interpolated Concentrations (pg/ml) of 12 Subjects Following Intranasal Administration of The Innovator's Preparation (Miacalcic© 200 IU per actuation) at 400 IU dose.
(NQ = Non-quantifiable; The estimated value < 7.5 pg/ml which was the LLOQ).

Time (min)	Subj. 1	Subj. 2	Subj. 3	Subj. 4	Subj. 5	Subj. 6	Subj. 7	Subj. 8	Subj. 9	Subj. 10	Subj. 11	Subj. 12	Mean	S.D.	% C.V.
0	9.77	9.85	11.02	10.73	8.99	11.09	9.31	10.77	8.47	10.16	9.92	10.42	10.04	0.82	8.20
5	11.29	21.49	56.12	48.51	32.69	47.15	50.48	39.19	51.57	51.27	78.32	72	46.67	18.91	40.51
10	80	101.75	130.31	136.4	99.44	88.64	63.14	101.84	107.58	141.88	103.39	107.42	105.15	22.76	21.64
15	140.14	143.84	109.15	84.81	145.83	123.59	129.9	119.58	135.55	123.79	142.5	139.37	128.17	17.70	13.81
20	37.8	32.64	72.55	68.01	94.19	55.5	89.3	55.42	70.5	54.41	39.8	71.86	61.83	19.50	31.53
30	23.39	31.82	39.28	46.88	31.86	43.12	36.77	45.45	35.35	45.95	54.22	40.03	39.51	8.33	21.08
45	23.16	30.05	34.72	58.05	21.91	26.16	21.88	27.64	18.1	23.53	24.68	15.65	27.13	10.98	40.48
60	22.14	28.39	16.8	33.86	35.04	18.76	26.39	16.12	18.68	25.26	22.48	18.31	23.52	6.40	27.22
90	24.26	25.81	18.92	30.84	31.48	15.39	24.95	16.38	11.91	10.4	10.54	18.02	19.91	7.46	37.47
120	18.83	14.98	17.52	19.36	14.52	15.4	15.69	15.26	11.02	13.07	12.54	13.91	15.18	2.48	16.31
180	NQ	8.06	9.76	16.16	NQ	9.59	13.36	13.66	7.91	13.65	11.67	12.39	11.25	2.86	25.47

Table 34 Plasma salmon CT concentrations (pg/ml) of 12 subjects following intranasal administration of the Test preparation (200 IU per actuation) at 400 IU dose.

(ND = Not detectable after baseline correction).

Time (min)	Subj. 1	Subj. 2	Subj. 3	Subj. 4	Subj. 5	Subj. 6	Subj. 7	Subj. 8	Subj. 9	Subj. 10	Subj. 11	Subj. 12	Mean	S.D.	% C.V.
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
5	29.09	0.22	18.68	47.40	63.59	27.08	24.11	20.38	31.82	33.86	26.81	42.49	30.46	15.85	52.04
10	70.39	132.97	53.97	62.22	38.83	30.06	30.46	37.69	47.62	38.70	94.78	118.03	62.98	34.73	55.14
15	111.30	62.78	102.87	127.54	128.90	112.97	133.96	131.03	118.98	110.21	131.61	104.10	114.69	19.75	17.22
20	51.19	18.66	97.06	103.36	121.62	93.22	40.38	25.44	73.83	34.24	42.31	11.38	59.39	36.99	62.28
30	48.55	42.74	23.14	74.03	63.28	10.84	23.50	19.73	5.02	29.69	14.42	15.07	30.84	21.73	70.49
45	17.42	7.70	13.15	9.31	13.38	15.22	43.73	15.86	28.38	22.50	41.34	20.28	20.69	11.65	56.30
60	6.69	10.81	13.99	16.13	15.17	11.94	16.44	22.28	15.56	24.80	12.01	19.49	15.44	5.01	32.46
90	7.36	7.08	18.13	8.92	6.33	1.54	10.37	25.30	25.36	16.82	6.72	4.19	11.51	7.99	69.39
120	12.57	1.86	11.00	4.68	4.99	5.93	18.64	4.83	8.68	3.42	ND	0.35	8.13	5.19	63.81
180	ND	3.25	4.62	3.26	0.96	0.69	3.96	ND	ND	ND	ND	ND	2.79	1.61	57.59

Table 35 Plasma salmon CT concentrations (pg/ml) of 12 subjects following intranasal administration of the Innovator's product (Miacalcic© 200 IU per actuation) at 400 IU dose.

(ND = Not detectable after baseline correction).

Time (min)	Subj. 1	Subj. 2	Subj. 3	Subj. 4	Subj. 5	Subj. 6	Subj. 7	Subj. 8	Subj. 9	Subj. 10	Subj. 11	Subj. 12	Mean	SD.	%CV.
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	1.52	11.64	45.10	37.78	23.70	36.06	41.17	28.42	43.10	41.11	68.40	61.58	36.63	18.78	51.27
10	70.23	91.90	119.29	125.67	90.45	77.55	53.83	91.07	99.11	131.72	93.47	97.00	95.11	22.48	23.64
15	130.37	133.99	98.13	74.08	136.84	112.50	120.59	108.81	127.08	113.63	132.58	128.95	118.13	18.20	15.40
20	28.03	22.79	61.53	57.28	85.20	44.41	79.99	44.65	62.03	44.25	29.88	61.44	51.79	19.73	38.09
30	13.62	21.97	28.26	36.15	22.87	32.03	27.46	34.68	26.88	35.79	44.30	29.61	29.47	7.99	27.10
45	13.39	20.20	23.70	47.32	12.92	15.07	12.57	16.87	9.63	13.37	14.76	5.23	17.09	10.61	62.10
60	12.37	18.54	5.78	23.13	26.05	7.67	17.08	5.35	10.21	15.10	12.56	7.89	13.48	6.71	49.80
90	14.49	15.96	7.90	20.11	22.49	4.30	15.64	5.61	3.44	0.24	0.62	7.60	9.87	7.59	76.93
120	9.06	5.13	6.50	8.63	5.53	4.31	6.38	4.49	2.55	2.91	2.62	3.49	5.13	2.19	42.74
180	ND	ND	ND	5.43	ND	ND	4.05	2.89	ND	3.49	1.75	1.97	3.26	1.38	42.18

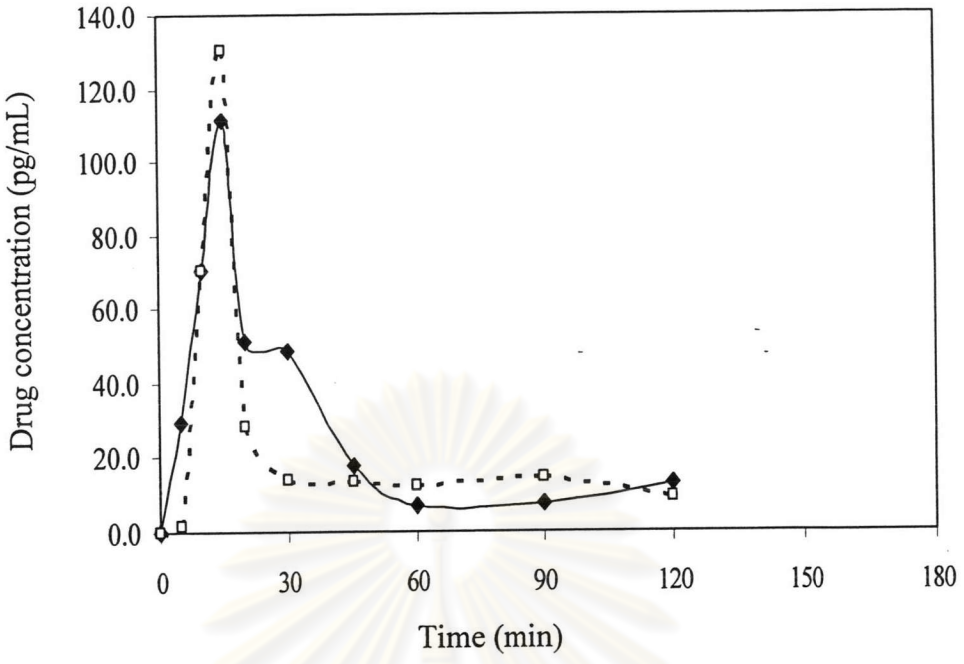

Figure 23 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.1 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---).

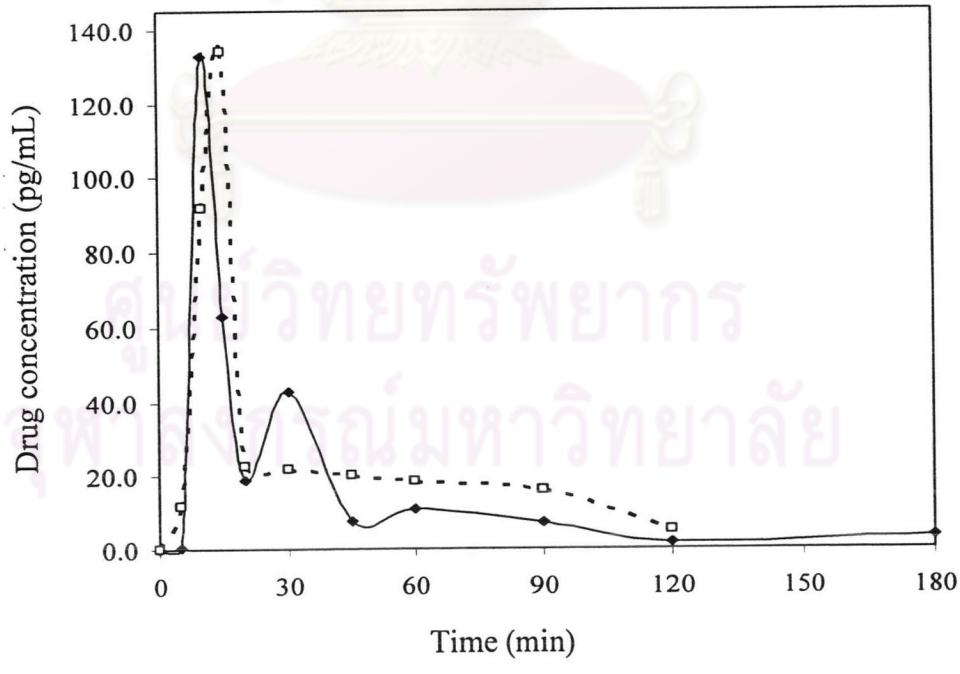

Figure 24 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.2 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---).

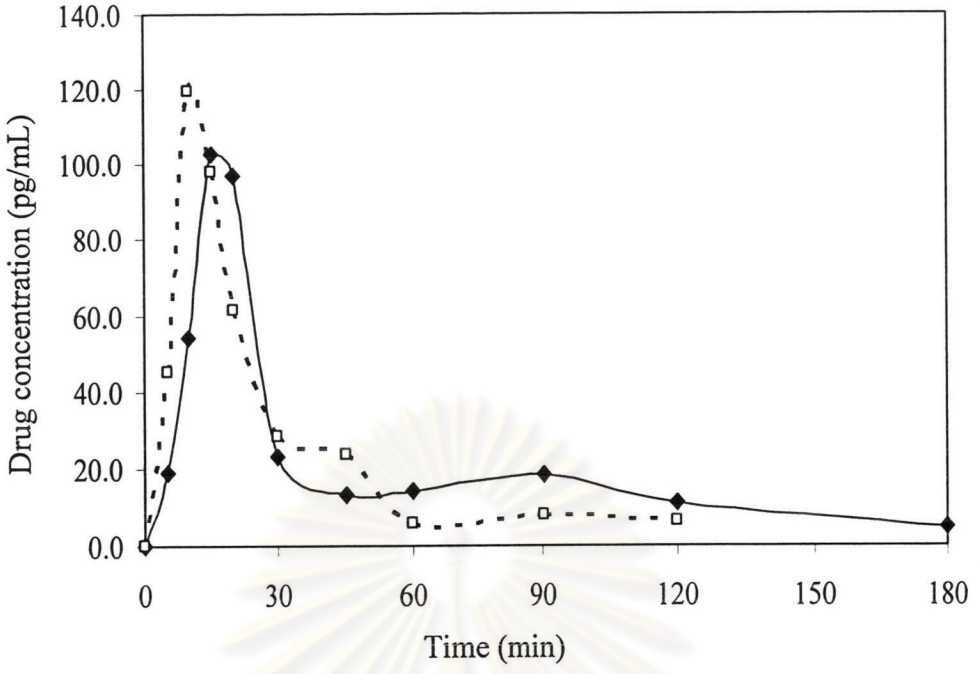

Figure 25 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.3 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---).

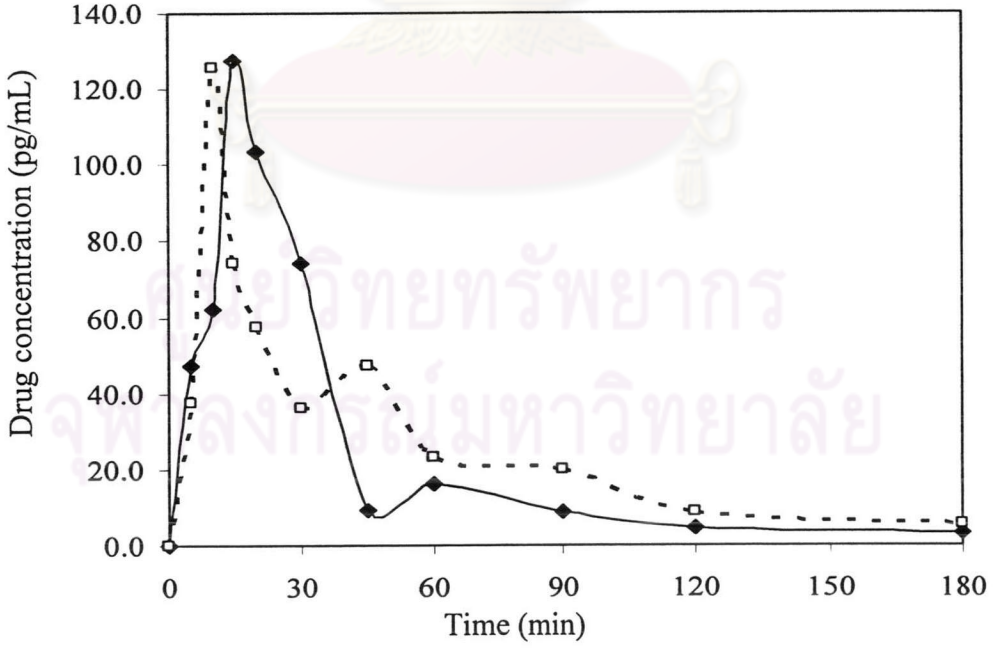

Figure 26 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.4 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---).

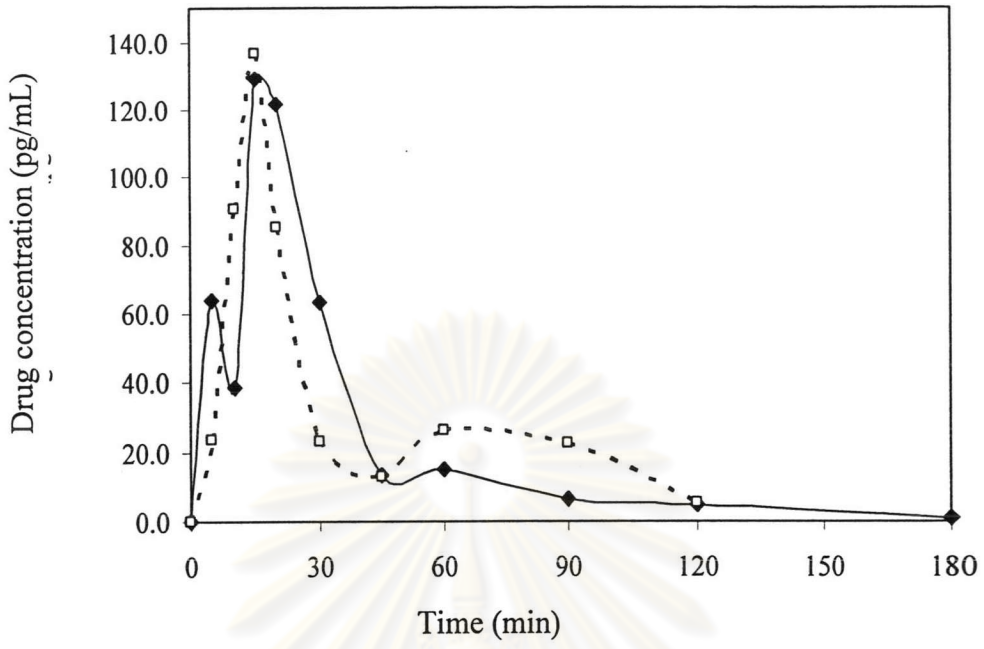

Figure 27 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.5 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---).

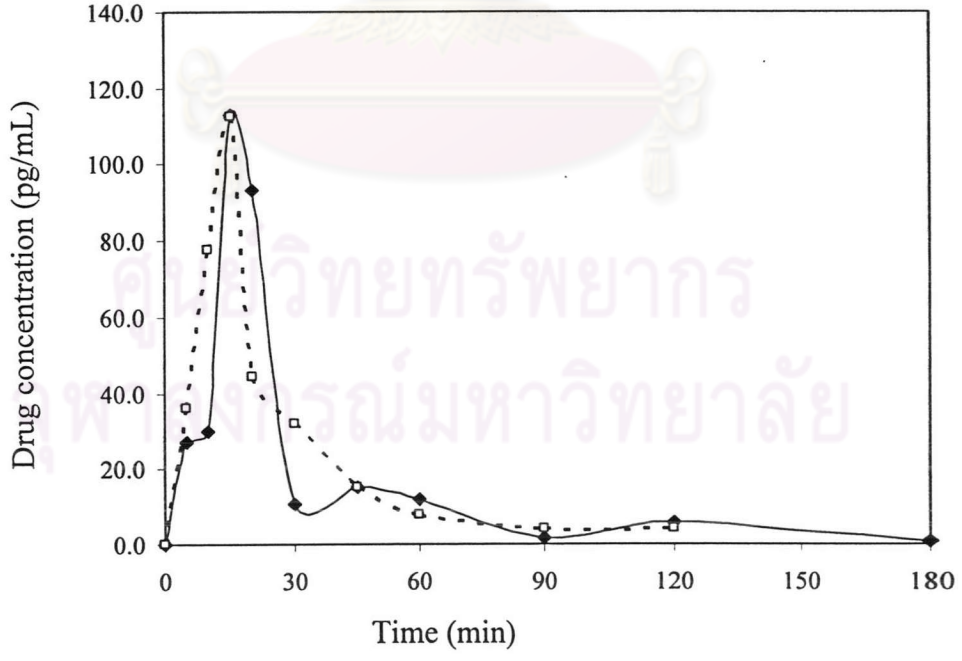

Figure28 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.6 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---)

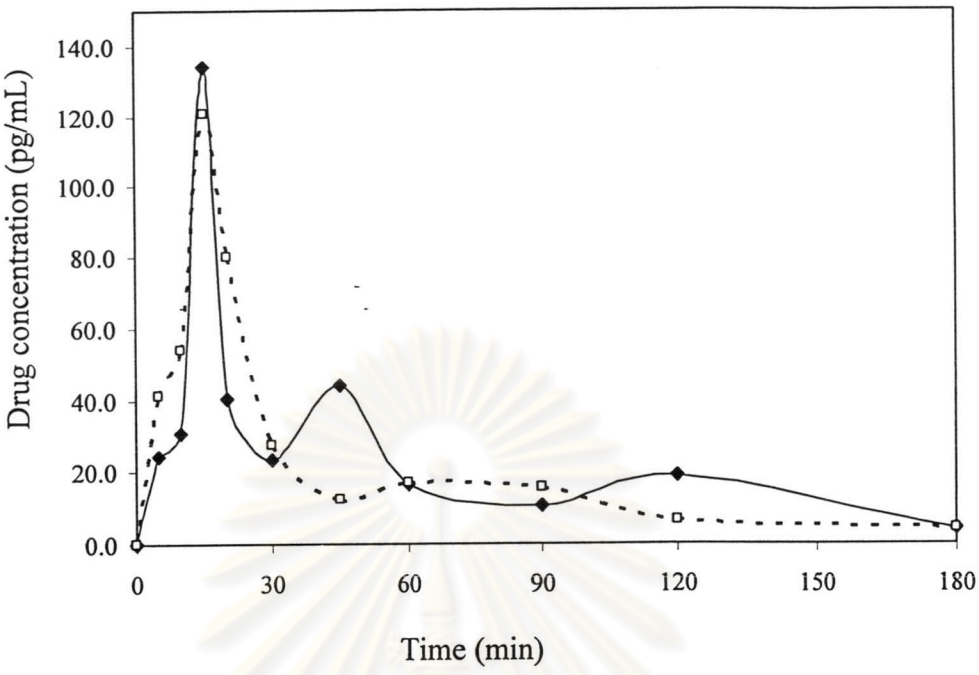

Figure 29 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.7 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---)

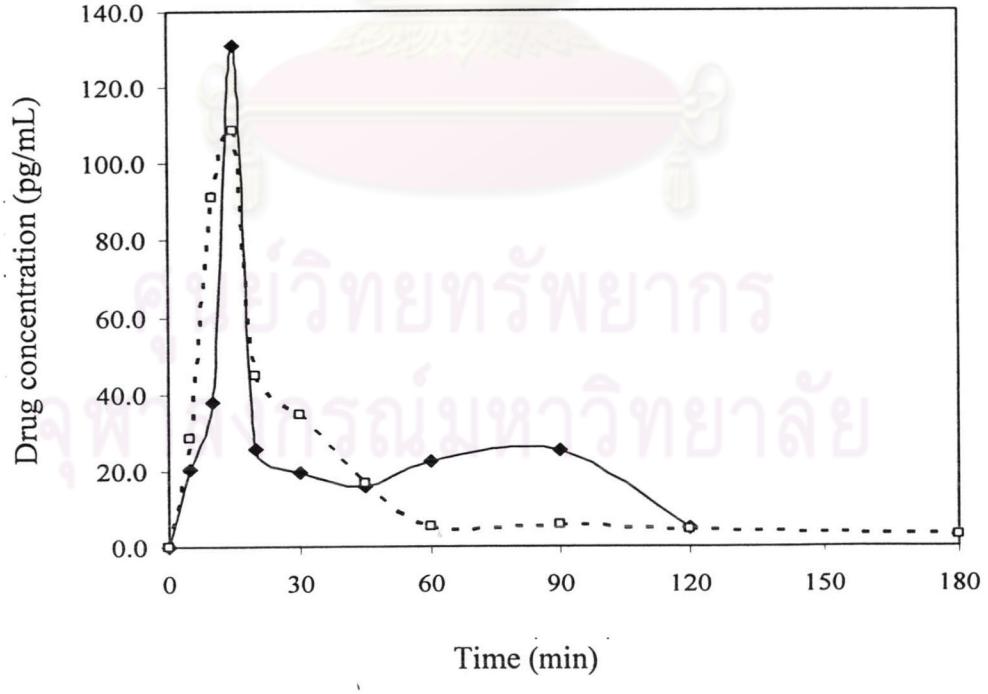

Figure 30 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No 8 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---)

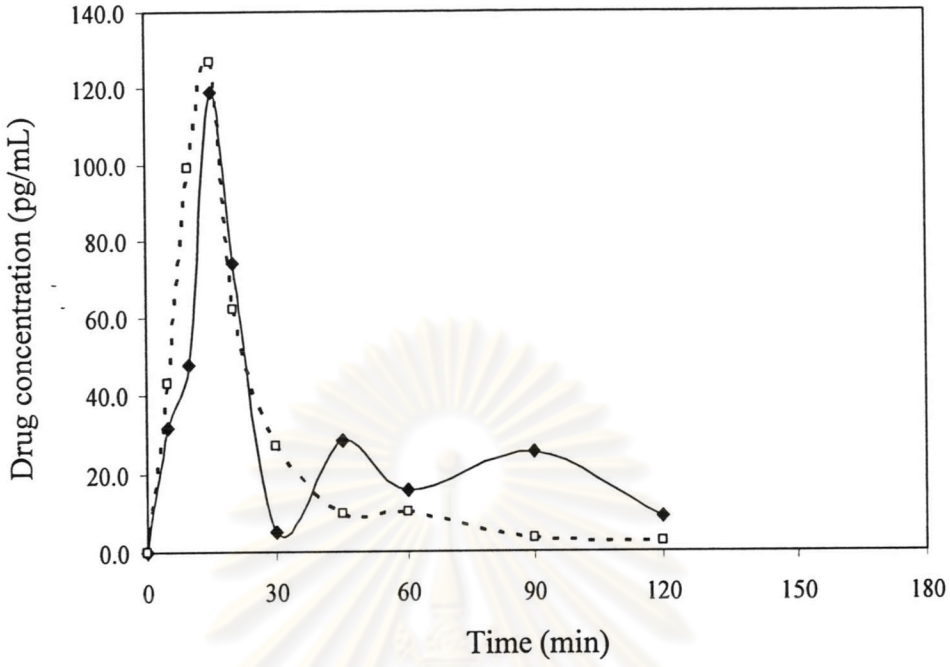

Figure 31 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.9 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---)

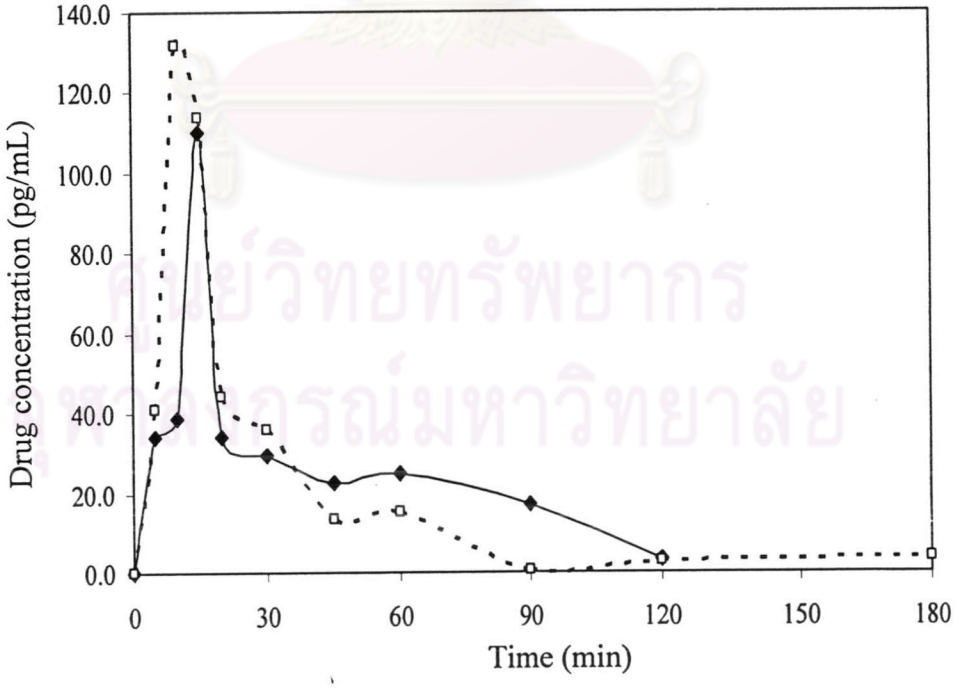

Figure 32 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.10 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---)

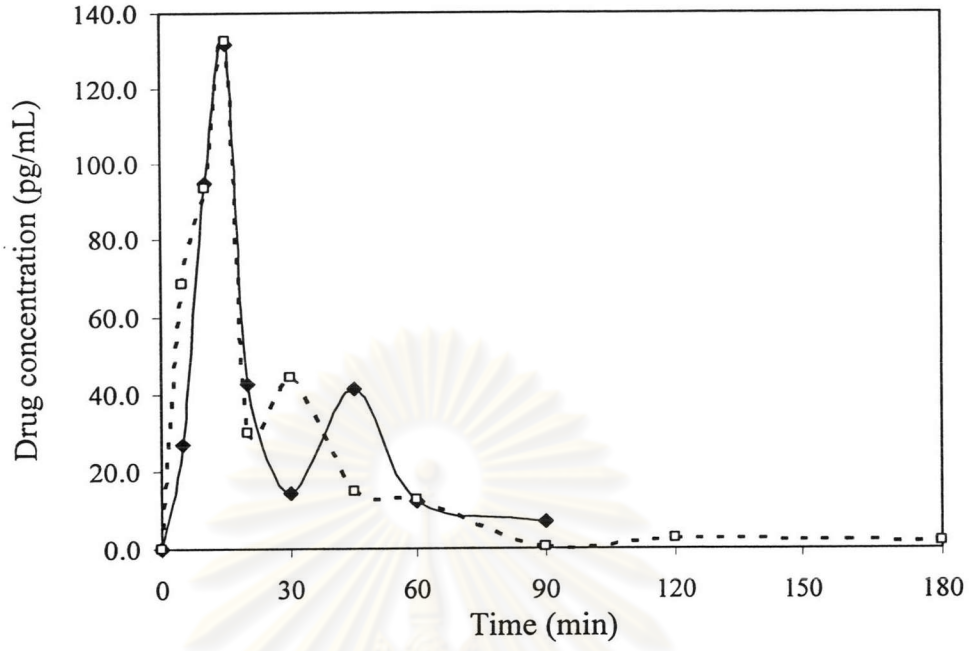

Figure 33 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.11 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---)

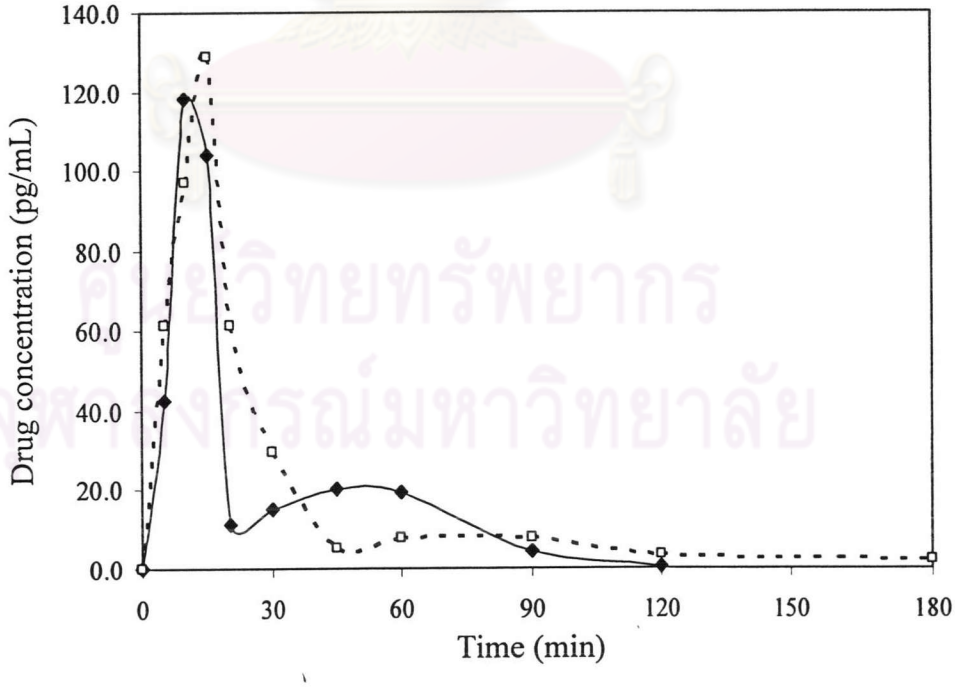

Figure 34 Plasma salmon CT concentration (pg/mL) versus time (min) of subject No.12 after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray). Test product (—◆—); Innovator's product (---□---)

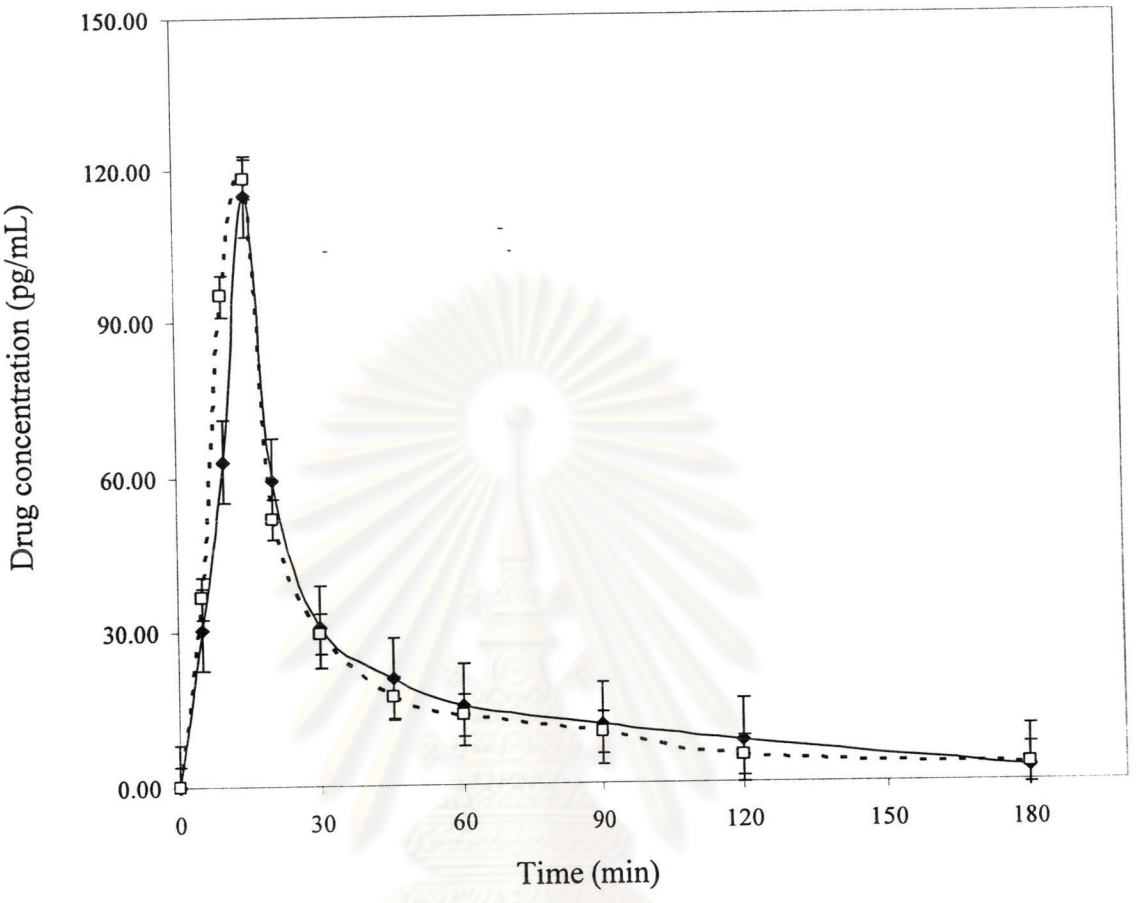

Figure 35 Mean plasma salmon CT concentration (pg/mL) versus time (min) after intranasal administration of 400 IU salmon CT (2 x 200 IU per spray) to healthy subjects. Test product (—◆—); Innovator's product (---□---).
Each value = Mean ± S.D. (n = 12 subjects).

8.3 Bioequivalence Evaluation

The individual numerical valus of AUC_{0-t}, $AUC_{0-\infty}$, Cmax and Tmax of salmon CT obtained with the test and the innovator's products are given in Tables 36, 37, 41 and 44, respectively. The parameters Tmax and AUC respectively reflect the rate and the extent of systemic absorption of a drug whereas the Cmax values represent both the rate and extent of drug absorption (Notari, 1987)

As previously discussed, these parameters are derived from the plasma drug concentration - time profile of the individual subjects. In the bioequivalence study, any drug product that is pharmaceutically equivalent to the innovator's product will also be considered bioequivalent if the 90% confidence intervals for the ratio of its logarithmically transformed AUC and Cmax values relative to the innovator's product are contained within 80.0 – 125.0 % bioequivalence range (Thai FDA). The natural logarithmically transformed data of AUC and Cmax are given in Tables 60 and 61 of Appendix E.

The bioavailability parameters obtained from this study are as follows:

Area under the plasma versus time curve (AUC)

For the extent of salmon CT nasal absorption, the mean AUC values from time 0 to the last detectable time point (AUC0-t) were 3,096.66 $\pm$ 579.14 and 3,032.56 $\pm$ 587.77 pg.min/mL for the test and innovator's product, respectively, as shown in Table 36. The individual values ranged from 2,406.44 to 3,965.06 pg.min/mL for the test product, and from 2,440.77 to 4,453.84 pg.min/mL for the innovator's product. The coefficient of variation (% C.V.) was 18.70 % for the test product, which was very close to 19.38 % for the innovator's product. The standard deviation (S.D.) values of the two products were also very similar (579.14 vs 587.77 pg.min/mL), indicating the same extent of data variation.

The total AUC value from time 0 to infinity ($AUC_{0-\infty}$) was also calculated for each subject by combining the AUC_{0-t} value with the area interpolated from the last detectable time point to the infinite time ($AUC_{last-\infty}$). The value of $AUC_{last-\infty}$ was estimated from the formula C_{last} / k_{el}, where C_{last} is the last quantifiable concentration and k_{el} was the elimination rate constant of salmon CT of the individual subject. The mean $AUC_{0-\infty}$ values were 3,300.91 $\pm$ 586.42 and 3,307.03 $\pm$ 586.85 pg.min/mL for the test and innovator's product, respectively, as shown in Table 37. The individual

values ranged from 2,548.96 to 4,028.54 pg.min/mL for the test product, and from 2,550.26 to 4,030.12 pg.min/mL for the innovator's product. As with AUC_{0-t}, the C.V. and S.D. values of $AUC_{0-\infty}$ were also very similar between the two products (17.77 vs 17.75 % for C.V. and 586.42 vs 586.85 pg.min/mL for S.D.). Since the C.V. and the S.D. are parameters related to the variability of the data, the two products thus demonstrated the same variation with respect to the AUC_{0-t} and $AUC_{0-\infty}$ values. The observation of similar C.V. or equal S.D. is important since it is one of the requirements for a valid statistical evaluation of data using the analysis of variance (ANOVA).

It is well known that several factors can affect the extent of nasal drug absorption, including the formulation factors (physicochemical properties) and the biological factors (pathophysiology of the nose, mucociliary clearance rate etc.) (Behl, 1998). Since only the healthy subjects were included in this study and none of them exhibited nasal symptoms such as colds or allergic rhinitis, the latter factors were not considered to greatly affect the nasal bioavailability of salmon CT as compared to the formulation factors. Even if these biological factors were significant, the nature of the crossover study design should be able to separate this variation since comparison of the bioavailability parameters will be made within the same subject. Also, the period effect was also balanced out by proper Latin-square design in which there would be equal number of subjects receiving the test and the innovator's products at any particular period. For example, during the first period of study there were 6 subjects who had been administered with the test product and the other 6 subjects were treated with the innovator's product. In the second period, the two groups were switched (crossed over) to the other product so that the same number of subject (6 for each group) was always assigned to receive either the test or innovator's product.

Therefore, if there had been any changes in the climate that might have caused some nasal symptoms to occur (such as common colds resulting from variation in weather), these changes would have affected both products equally during any particular period.

Results from the analysis of variance (ANOVA) for the two-way crossover design based on the natural log-transformed values of AUC (ln AUC) are provided in Tables 38 and 39 for AUC_{0-t} and $AUC_{0-\infty}$, respectively. For both AUC

parameters, ANOVA results showed that there were no statistically significant differences ($p > 0.05$) in the ln AUC values between the test and the innovator's products with respect to the formulation, period and sequence effect. Significant difference in the ln AUC values was found only for the subject effect ($p < 0.05$) indicating a significant inter-subject variability.

However, this subject-to-subject variation in the bioavailability parameters was not uncommon and had been expected due to a wide variation in the plasma salmon CT concentrations commonly observed among the subjects, as evidenced from the individual plasma concentration – time profiles.

The 90 % confidence interval for the ratio of AUC of the test product to the innovator's product, after natural logarithmic transformation followed by taking anti-log, was 95.07 – 109.30 % for the AUC_{0-t}, whereas it was 93.80 – 113.01 % for the $AUC_{0-\infty}$ (Table 40). This range was well within the acceptance criteria of 80.0 – 125.0 %. Therefore, the results indicated that the test product of salmon CT nasal spray was bioequivalent to the innovator's product with respect to the extent of nasal absorption.

Peak plasma concentration (C_{max})

Previous reports showed that the mean peak plasma concentrations of salmon CT achieved following 200 IU salmon CT nasal spray was about 76 pg/mL (Thamborg, 1990). In this study the mean peak plasma salmon CT levels after 400 IU dose were 121.70 $\pm$ 10.62 and 125.70 $\pm$ 8.72 pg/mL for the test and the reference products, respectively (Table 41). The individual values ranged from 102.87 to 133.96 pg/mL for the test product, and from 108.81 to 136.84 pg/mL for the innovator's product. The coefficient of variation of the two products (8.73 vs 6.94 %) as well as their S.D. values (10.62 vs 8.72 pg/mL) were also close to each other. This suggested that the Cmax data of the two products demonstrated the same variation, and that ANOVA could be used for further statistical evaluation.

Kurose et al. (1987) studied the pharmacokinetics of salmon CT and found that the maximum plasma concentrations were reached 20 - 60 min after nasal administration of 400 IU salmon CT, giving the average Cmax value of 97.3 ± 22.6 pg/mL. Although their value was slightly lower than the result from this study, their data also showed greater variability with a two-fold S.D. value. Other researchers also reported the average Cmax values in the range of 150 - 200.pg/mL following the

same dose (400 IU) of intranasal salmon CT (Lee,1994 and Buclin,1987). These values are in relative agreement with the results of this study.

ANOVA for the two-way crossover design based on the natural logarithmically transformed data of Cmax (ln Cmax) is shown in Table 38. The individual data of ln Cmax is provided in Table 60 and 61 in Appendix E. ANOVA results showed that there were no statistically significant differences ($p > 0.05$) in the values of ln Cmax between the test and the innovator's product with respect to all effects, i.e., the formulation, period, subject and sequence effects.

90% Confidence interval for the ratio of Cmax (test product to innovator's product) was then constructed using the error mean square from the ANOVA table of ln Cmax. The confidence interval for the difference in the average ln Cmax values between the test and the innovator's products ($\ln C_{max\ test} - \ln C_{max\ innovator's}$) was first constructed. After taking anti-logarithm, the exact 90% confidence interval of the ratio of Cmax was then obtained, which was calculated to be 91.32 – 102.61 %, as seen in Table 43. This interval was well within the acceptable bioequivalence range of 80.0 – 125.0 % as recommended by both the US and the Thai FDA (2000)

Since Cmax is related to both the rate and extent of systemic absorption, passing the 90 % confidence interval test with respect to this parameter indicated that the test nasal spray was able to provide the systemic absorption of salmon CT at the rate and extent similar to the innovator's product. The 90 % confidence interval test results previously obtained for the AUC values further substantiate that the test formulation developed in this study (200 IU per actuation) was bioequivalent to the innovator's product at the same administered dose (400 IU).

Time to Peak Plasma Concentration (T_{max})

The time to peak plasma concentrations of salmon CT from this study was from 10 to 15 min for both the test and the innovator's products as seen in Table 44. Similar findings have been reported by Lee et al. (1994), who found that all the serum level versus time profiles after intranasal salmon CT administration were characterized by a rapid absorption phase, with 5 to 10 min T_{max}. It should be noted here that the Tmax values were not further analyzed by ANOVA since this parameter generally does not follow a normal distribution criterion important for parametric statistical analysis. Tmax may be analyzed by a non-parametric approach. However,

the bioequivalence guidelines issued by many regulatory agencies, including the US FDA and the Thai FDA, do not require statistical analysis of Tmax.

Elimination Half-life ($t_{1/2}$)

The mean elimination half-life ($t_{1/2}$) was 31.47 ± 7.48 min and 32.25 ± 8.60 min for the test and the innovator's products, respectively, as shown in Table 45. The individual values ranged from 22.14 to 47.26 min for the test product and from 17.70 to 44.41 min for the innovator's product. The values of % C.V. and S.D. were also similar between the two products indicating the same extent of variation in the half-life data. ANOVA was then performed at 5 % significance level and the results are given in Table 46. No significant differences ($p > 0.05$) were found in the half-life values with respect to all the effects tested, i.e., the product, the period, the subject and the sequence effects. The non-significant product effect after ANOVA indicated that the nasal administration of the two products resulted in the same elimination kinetics of salmon CT from the plasma.

Elimination Rate Constant (K_e)

Apart from the elimination half-life, the elimination rate constant (K_e) of salmon CT was found to be 0.0231 ± 0.0053 min^{-1} and 0.0232 ± 0.0072 min^{-1} for the test and the innovator's products, respectively (Table 47). The individual values ranged from 0.0147 to 0.0313 min^{-1} for the test product and from 0.0156 to 0.0392 min^{-1} for the innovator's product. ANOVA was also performed and the results are shown in Table 48. As with the half-life, no significant differences were found with respect to the product, period, subject and sequence effects ($p > 0.05$).

Summary of Bioequivalence Evaluation

The pharmacokinetic parameters for bioequivalence evaluation are summarized in Table 49, together with their 90 % confidence intervals for the ratio (test to innovator's). Two most important parameters in bioequivalence assessment are the AUC and Cmax, which in combination reflect the rate and extent of drug absorption into systemic circulation. From this table, it can be seen that the test nasal spray demonstrated bioequivalence to the innovator's product with respect to the AUC_{0-t}, $AUC_{0-\infty}$, and Cmax values. Their *in vitro* specifications were also similar, as both products passed the tests required by BP 2002 general monograph for nasal spray solutions as well as those adopted from the monograph for salmon CT injections. Other specifications, e.g. droplet size and size distribution as well as the

spray pattern, followed the guidance for industry for nasal spray and inhalation solution as set by the US FDA Center for Drug Evaluation and Research (CDER, 2002).

Table 36 Area under the plasma salmon CT concentration - time curve from time 0 to the last detectable time point (AUC_{0-t}), following intranasal administration of 400 IU salmon CT nasal spray to 12 subjects.

Subject no.	AUC_{0-t} (pg.min/mL)	
	Test Product	Innovator's Product
1	2865.93	2440.77
2	2406.44	2592.90
3	3584.32	2947.37
4	3965.06	4453.84
5	3845.07	3717.58
6	2515.88	2456.38
7	3833.60	3471.78
8	2953.66	2740.32
9	3275.37	2663.31
10	2689.56	2994.70
11	2754.69	2943.08
12	2470.33	2968.65
Mean	3096.66	3032.56
Min	2406.44	2440.77
Max	3965.06	4453.84
S.D.	579.14	587.77
%C.V.	18.70	19.38

Table 37 Area under the plasma salmon CT concentration - time curve from time 0 to infinite time ($AUC_{0-\infty}$), following intranasal administration of 400 IU salmon CT nasal spray to 12 subjects.

Subject no.	$AUC_{0-\infty}$ (pg.min/mL)	
	Test Product	Innovator's Product
1	3455.47	3472.54
2	2583.93	2590.79
3	3672.30	3674.70
4	4028.54	4030.12
5	3880.60	3881.52
6	2548.96	2550.26
7	3986.92	3990.77
8	3219.46	3227.71
9	3867.29	3882.59
10	2846.37	2851.88
11	2969.41	2976.64
12	2551.64	2554.83
Mean	3300.91	3307.03
Min	2548.96	2550.26
Max	4028.54	4030.12
S.D.	586.42	586.85
%C.V.	17.77	17.75

Table 38 Analysis of variance for two-way crossover study at $\alpha = 0.05$ of ln AUC_{0-t} following intranasal administration of 400 IU salmon CT nasal spray to 12 subjects.

Source of variation	d.f.	Sum of Square	Mean Square	F_{cal}	F_{tab}	Significant Level
Total	23	0.7186	--	--	--	
Sequence	1	0.0092	0.0092	0.149	4.96	NS
Subject (sequence)	10	0.6164	0.0616	6.933	2.98	S
Period	1	0.0018	0.0018	0.207	4.96	NS
Formulation	1	0.0022	0.0022	0.248	4.96	NS
Error	10	0.0889	0.00889	--	--	--

Where;

NS = Not significant difference ($p > 0.05$)

S = Significant difference ($p < 0.05$)

d.f. = Degree of freedom

F_{cal} = F value obtained from calculation

F_{tab} = F value taken from the upper 5% values of the F distribution table

Table 39 Analysis of variance for two-way crossover study at $\alpha = 0.05$ of ln $AUC_{0-\infty}$ following intranasal administration of 400 IU salmon CT nasal spray to 12 subjects.

Source of variation	d.f.	Sum of Square	Mean Square	F_{cal}	F_{tab}	Significant Level
Total	23	0.7594	--	--	--	
Sequence	1	0.0117	0.0117	0.201	4.96	NS
Subject (sequence)	10	0.5833	0.0583	3.679	2.98	S
Period	1	0.0007	0.0007	0.044	4.96	NS
Formulation	1	0.0051	0.0051	0.322	4.96	NS
Error	10	0.1585	0.01585	--	--	--

Where;

NS = Not significant difference ($p > 0.05$)

S = Significant difference ($p < 0.05$)

d.f. = Degree of freedom

F_{cal} = F value obtained from calculation

F_{tab} = F value taken from the upper 5% values of the F distribution table

Table 40 The exact 90 % confidence interval for the ratio of the area under the plasma concentration – time curve ($AUC_{test}/AUC_{innovator's}$) following intranasal administration of 400 IU salmon CT nasal spray (test and innovator's products).

Product	Average ln AUC_{0-t}	90% Confidence Interval
Test product (ln T)	8.02 ± 0.185	95.07 – 109.30%
Innovator's product (ln R)	8.00 ± 0.178	
Ratio of AUC_{0-t} (T/R) (anti ln of ln T – ln R)	1.020	
	Average ln $AUC_{0-\infty}$	
Test product (ln T)	8.09 ± 0.182	93.80 – 113.01%
Innovator's product (ln R)	8.06 ± 0.182	
Ratio of $AUC_{0-\infty}$ (T/R) (anti ln of ln T – ln R)	1.030	

Table 41 Peak plasma salmon CT concentration (Cmax) following intranasal administration of 400 IU nasal spray (test and innovator's products)to 12 subjects.

Subject no.	Cmax (pg/mL)	
	Test product	Innovator's product
1	111.30	130.37
2	132.97	133.99
3	102.87	119.29
4	127.54	125.67
5	128.90	136.84
6	112.97	112.50
7	133.96	120.59
8	131.03	108.81
9	118.98	127.08
10	110.21	131.72
11	131.61	132.58
12	118.03	128.95
Mean	121.70	125.70
Min	102.87	108.81
Max	133.96	136.84
S.D.	10.62	8.72
%C.V.	8.73	6.94

Table 42 Analysis of variance for two-way crossover study at $\alpha = 0.05$ of ln Cmax following intranasal administration of 400 IU salmon CT nasal spray to 12 subjects.

Source of variation	d.f.	Sum of Square	Mean Square	F_{cal}	F_{tab}	Significant Level
Total	23	0.1540	--	--	--	
Sequence	1	0.0012	0.0012	0.150	4.96	NS
Subject (sequence)	10	0.0804	0.0080	1.297	2.98	NS
Period	1	0.0040	0.0040	0.646	4.96	NS
Formulation	1	0.0063	0.0063	1.022	4.96	NS
Error	10	0.0620	0.0062	--	--	--

Where;

NS = Not significant difference ($p > 0.05$)

S = Significant difference ($p < 0.05$)

d.f. = Degree of freedom

F_{cal} = F value obtained from calculation

F_{tab} = F value taken from the upper 5% values of the F distribution table

Table 43 The exact 90 % confidence interval for the ratio of peak plasma concentration ($Cmax_{test}/Cmax_{innovator's}$) following intranasal administration of 400 IU salmon CT nasal spray (test and innovator's products).

Products	Average ln Cmax	90% Confidence Interval
Test product (ln T)	4.80 ± 0.089	91.32 – 102.61%
Innovator's product (ln R)	4.83 ± 0.071	
Ratio of Cmax (T/R) (anti ln of ln T – ln R)	0.970	

Table 44 Time to maximum concentration (T_{max}) of salmon CT following intranasal administration of 400 IU nasal spray (test and innovator's products).

Subject no.	T_{max} (min)	
	Test's Product	Innovator's Product
1	15	15
2	10	15
3	15	10
4	15	10
5	15	15
6	15	15
7	15	15
8	15	15
9	15	15
10	15	15
11	15	15
12	10	15
Mean	14.167	14.167
S.D.	1.95	1.95
%C.V.	13.74	13.74

Table 45 Elimination half-life ($t_{1/2}$) of salmon CT following intranasal administration of 400 IU nasal spray (test and innovator's products).

Subject no.	$t_{1/2}$ (min)	
	Test's Product	Innovator's Product
1	32.50	32.70
2	37.85	39.46
3	23.27	28.79
4	34.91	44.41
5	25.65	26.83
6	33.23	17.70
7	26.85	39.08
8	38.14	22.21
9	47.26	23.26
10	31.81	41.55
11	22.14	32.03
12	23.98	38.95
Mean	31.47	32.25
Min	22.14	17.70
Max	47.26	44.41
S.D.	7.48	8.60
%C.V.	23.78	26.65

Table 46 Analysis of variance for two-way crossover study at $\alpha = 0.05$ of ln elimination half life ($t_{1/2}$) following intranasal administration of 400 IU salmon CT nasal spray to 12 subjects.

Source of variation	d.f.	Sum of Square	Mean Square	F_{cal}	F_{tab}	Significant Level
Total	23	1.5204				
Sequence	1	0.0033	0.0033	0.06	4.96	NS
Subject (sequence)	10	0.5784	0.0578	0.62	2.98	NS
Period	1	0.0043	0.0043	0.05	4.96	NS
Formulation	1	0.0014	0.0014	0.01	4.96	NS
Error	10	0.9331	0.0933			--

Where;

NS = Not significant difference ($p > 0.05$)

S = Significant difference ($p < 0.05$)

d.f. = Degree of freedom

F_{cal} = F value obtained from calculation

F_{tab} = F value taken from the upper 5% values of the F distribution table

Table 47 Elimination rate constant (K_e) of salmon CT following intranasal administration of 400 IU nasal spray (test and innovator's products).

Subject no.	K_e (min^{-1})	
	Test product	Innovator's product
1	0.0213	0.0212
2	0.0183	0.0176
3	0.0298	0.0241
4	0.0198	0.0156
5	0.0270	0.0258
6	0.0209	0.0392
7	0.0258	0.0177
8	0.0182	0.0312
9	0.0147	0.0298
10	0.0218	0.0167
11	0.0313	0.0216
12	0.0289	0.0178
Mean	0.0231	0.0232
Min	0.0147	0.0156
Max	0.0313	0.0392
S.D.	0.0053	0.0072
%C.V.	22.82	30.97

Table 48 Analysis of variance for two-way crossover study at $\alpha = 0.05$ of ln elimination rate constant (K_e) following intranasal administration of 400 IU salmon CT nasal spray to 12 subjects.

Source of variation	d.f.	Sum of Square	Mean Square	F_{cal}	F_{tab}	Significant Level
Total	23	1.5170	--	--	--	--
Sequence	1	0.0026	0.0026	0.04	4.96	NS
Subject (sequence)	10	0.5760	0.0576	0.62	2.98	NS
Period	1	0.0046	0.0046	0.05	4.96	NS
Formulation	1	0.0011	0.0011	0.01	4.96	NS
Error	10	0.9354	0.0935	--	--	--

Where;

NS = Not significant difference ($p > 0.05$)

S = Significant difference ($p < 0.05$)

d.f. = Degree of freedom

F_{cal} = F value obtained from calculation

F_{tab} = F value taken from the upper 5% values of the F distribution table

Table 49 Principle Pharmacokinetic Parameters of Salmon CT following intranasal administration of 400 IU nasal spray (test and innovator's products)

Parameters	Product		90% confidence Interval
	Test's	Innovator's	
AUC_{0-t} (pg.min/mL)	3096.66 ± 579.14	3032.56 ± 587.77	95.07 – 109.30%
$AUC_{0-\infty}$ (pg.min/mL)	3300.91 ± 586.42	3307.03 ± 586.85	93.80 – 113.01%
Cmax (pg/mL)	121.70 ± 10.62	125.70 ± 8.72	91.32 – 102.61%
T_{max} (min)	14.167 ± 1.95	14.167 ± 1.95	--
$t_{½}$ (min)	31.47 ± 7.48	32.25 ± 8.60	--
K_e (min^{-1})	0.0231 ± 0.0053	0.0232 ± 0.0072	--

Each value = Mean ± S.D.