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ORIGINAL ARTICLE

Thermoreversible Gel Formulation for the Intranasal Delivery of Salmon Calcitonin and Comparison Studies of *In Vivo* Bioavailability

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ABSTRACT

Objectives: We developed original thermoreversible (sol-gel) formulations of salmon calcitonin (sCT) for nasal applications. The sol-gel has been compared with commercial intranasal sprays *in vitro* and *in vivo* studies. The aim of studying sol-gel form is to arrange the viscosity of formulations for a reversible adequate fluidity at different temperatures. This situation may facilitate the use of drugs as sprays and increase the bioadhesive ability to mucosa.

Materials and Methods: Characterization of optimum formulations was studied. Validated analytical assays determined the number of sCT. An approximately equal number of commercial and sol-gel dosages were sprayed into the nostrils of the rabbits. Blood samples were collected from the ear veins of rabbits and determined by enzyme immunoassay plates. These plates were evaluated by Thermo Labsystem Multiscan Spectrum at 450 nm. Thanks to Winnonlin 5.2, pharmacokinetic data were evaluated by a non-compartmental method.

Results: The absolute bioavailability of the formulation at pH 4 and the commercial product (CP) was compared by evaluating the primary pharmacokinetic data area under the curve $0 \rightarrow t_{last}$. The absolute bioavailability of the commercial intranasal spray was measured 1.88 based on maximum concentration (C_{max}) assessment. C_{max} of the sol-gel formulation pH 4 was calculated as 0.99 and the relative bioavailability was obtained 53.3%.

Conclusion: *In vivo* pharmacokinetic data of sol-gel formulation with pH 3 showed significantly higher volume of distribution parameter than the CP (111167>35408). It is thought that the formulation adhered to the nasal mucosa releases sCT slowly and less.

Key words: Salmon calcitonin, thermoreversible gel, sol-gel, bioavailability, nasal spray

Introduction

Human calcitonin hormone (hCT), which consists of 32 amino acids, produced in the thyroid gland regulates calcium levels in the body by increasing the bone calcium level and decreasing the blood calcium level. Salmon calcitonin (sCT) is structurally very similar to human calcitonin, which was synthesized in 1969. It contains the same amino acids as the human calcitonin, however, it differs in terms of the amino acid sequence.[1]

The purity of calcitonin preparations varies based on the type (natural or synthetic) or production process. The hypocalcemic effect of calcitonin was ranked in the order of increasing strength as follows: sCT>eCT>hCT>pCT. sCT is more potent and to last longer than eel, human, and porcine calcitonin.[1,2] sCT inhibits the formation and the interactions of osteoclasts and prevents bone resorption. For this purpose, it is commonly used in formulations for osteoporosis, Paget's disease, and hypercalcemia, especially in the postmenopausal period.[3-5]

The biological activity of 1 mg of sCT is reported to be 6000 international unit (IU).[3] It is rapidly absorbed from nasal mucosa and its bioavailability *via* this route is approximately 3% (0.3-

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30.6%) compared to IM administration.[6] The recommended daily dose of sCT was 200 units with adequate Ca^{++} and vitamin D supplementation. Nasal application is performed with a dosimeter nasal spray pump.[7,8]

Intranasal administration is a suitable route for drugs that require a systemic action and undergo liver first-pass action (by enzymatic and acidic degradation). The molecular weight of most of the existing drugs (peptides or proteins) that penetrate systemic circulation by nasal administration varies between 1000 and 3400. Bioavailability of these drugs (intranasal formulations) is approximately 10% as compared to injection forms.[9,10]

The active substance and excipients used in nasal formulations do not irritate the nasal mucosa. Most of the peptides used in intranasal formulations are used for the treatment of chronic diseases; hence, their reliability is important.[11]

Nasal mucosa is a large capillary-rich area for drug absorption. Absorption and bioavailability values of drugs depend on the molecular structure of the active substance, the drug formulation, the tested species, and, if any, additional delivery devices. Generally, in intranasal formulations containing low molecular weight drugs, the bioavailability is relatively high, while the variability is low. In contrast, the bioavailability for high molecular weight drugs is relatively low, whereas the variability is high.[12,13]

One of the factors affecting drug penetration through nasal mucosa is the viscosity of nasal secretion. Approximately 15-20 mL of mucus is produced daily inside the nose, and the mucus layer is renewed almost every 10-30 minutes. If the mucus layer is thick, contact with the cilia decreases, thereby decreasing the mucociliary clearance. Drug penetration is influenced by the duration of contact of the drug with the mucosa. Mucociliary clearance mechanism, enzymatic degradation, and low permeability of nasal epithelium constitute the most important barriers for peptide/protein structured drugs. Viscous gel formulations that adhere to the mucosa (mucoadhesive) were developed with the aim of increasing the contact duration between the drug and the mucosa. Hence, it is also reported that the releasing of mucoadhesive drugs can be increased to enhance the bioavailability. On the other hand, they may be convenient for drugs even in low doses. It is difficult for high-viscosity gel formulations to provide the correct dosage and proper drug distribution in the nasal cavity. Bioadhesive applications increase the residence time of the drug on the mucosa in terms of patient comfort, compliance, and use. In this regard, alternative formulations such as thermogels may be good choices.[14,15]

Different types of polymers are used in thermogel formulations. Poloxamer 407 (Lutrol F 127) has been used in many thermogel formulations. It is a polymer material with many options for transdermal, ocular, topical, and implant application routes. Polyethylene oxide-polypropylene oxide-polyethylene oxide as triblock polymer can form transparent and temperature-sensitive sol-gels. It is chemically inert and has low toxicity.[16,17] Thermosensitive formulations have the advantage in terms of enhancing, controlling, and sustaining sCT for hypercalcemic effects.[18,19]

In this study, it was aimed that the homogeneous and fluid sol-gel formulation below room temperature should be sufficiently viscous to adhere to mucus at intranasal temperature. Thus, the sprayed drug will be prevented from flowing into the nasal passage or downwards. To research the bioavailability of sCT, thermogel nasal formulations at different pHs have been examined. It is thought that the temperature sensitive pharmaceutical form will allow controlled release of sCT. The formulations are designed to convert from solution to gel form at 34-36 °C, which is the intranasal temperature. The design with active ingredient transformed from solution form into gel form was compared with the commercial product (CP). Determination of the bioavailability and *in vivo* studies of sCT were carried out with enzyme immunoassay-enzyme-linked immunosorbent assay (EIA-ELISA) method.[20-23]

MATERIALS AND METHODS

sCT was purchased from Bachem, Switzerland. Potassium dihydrogen phosphate was purchased from J.T Baker, Netherlands. Sodium dihydrogen phosphate dihydrate, dipotassium hydrogen phosphate, polyethylene glycol (PEG) 1500, sodium dihydrogen phosphate, triethylamine, and *ortho*-phosphoric acid were purchased from Merck, Germany. Acetic acid was bought from Riedel de Haen, Germany. Poloxamer 407 was acquired from BASF, Germany. All other chemicals used were of analytical grade and used without further purification.

Quantification of sCT using high performance liquid chromatography (HPLC)

Quantification of sCT was carried out using HPLC system (HP Agilent 1100, ABD) equipped with injector, quaternary pump, autosampler, column oven and ultraviolet (UV) detector. sCT contains 13 different amino acids, but the determination assay uses a single peak for sCT detection.[24]

For determination of sCT, chromatography parameters such as C18 column (4.6 x 250 mm) (conditioned at ambient temperature), mobile phase composed of phase A (20 mL tetramethylammonium hydroxide (10%), 880 mL distilled water and 100 mL acetonitrile), and phase B (8 mL tetramethylammonium hydroxide in 392 mL distilled water and 600 mL acetonitrile) were used. Each mobile phase was adjusted with *ortho*-phosphoric acid to pH 2.5 (using a pH meter) and degassed for 30 min before use. The mobile phase was pumped at a flow rate of 1 mL/minute, injection volume of 50 µL and UV detector wavelength of 210 nm was used. The quantification method was carried out within the concentration range of 1-75 µg/mL. This analytical method was validated in terms of linearity, specificity, accuracy, precision, robustness, and stability based on International Council on Harmonisation (ICH) guidelines.

Preparation and characterization of thermoreversible formulations

Sol-gels were prepared using mixtures of PEG 1500 and Lutrol F 127 (LF 127) in different proportions with three different pH phosphate buffers (pH 3-5) (Table 1). Magnetic stirrers were placed in the formulations and mixed at a speed of 300 rpm to ensure homogeneity. Gelation temperatures of the formulations were investigated from +4 ˚C to 40 ˚C (with temperature increase interval of 0.5 ˚C) by stirring at 300 rpm in a cooled water bath. For the preparation of sCT loaded formulations, 2.200 IU/mL sCT were added to the formulations. The composition of investigated formulations is presented in Table 1 below.

The amount of PEG 1500 was fixed at 2% in order to determine the gelation temperatures with different poloxamer ratios. Gelling corresponding to the intranasal temperature was followed at intermediate concentrations of the F4 formulation in three pH (Table 2). The formulations were characterized immediately after removal from storage conditions at +4 ˚C.

Density of formulations

This study was conducted using a calibrated pycnometer with a volume of 5.442 mL. The empty weight of the pycnometer was 14.875 g. The weights of the formulations that filled the pycnometer at 25 ˚C were measured. At the end of three replicate studies, the ratio of average weight to volume was evaluated as the density of formulations.

Tonicity studies

Knauer-Semi-Micro Osmometer was first calibrated at "0" miliosmol/kg. For this study, 0.15 mL of pure water was placed in the measuring cup with pipette and placed in the cooling cell. The osmolarities of each formulation were studied in three replicates.

Refractive index measurements

ATAGO RX 7000 a device was first calibrated using pure water. The device was calibrated, when the refractive index reached 1.330 after the chamber of the device was filled up with pure water till the limit. Triplicate measurements were made for each formulation and the average refractive indices were observed.

Conductivity measurements

Mettler Toledo instrument used was first calibrated with conductivity standard solution. The calibration measurement was recorded as 1.413 s/cm ± 2%. The conductivity of the formulations (prepared in 100 mL) was measured in triplicates in a beaker and the average conductivity of each formulation was evaluated.

Viscosity measurements

Viscosity was measured at temperatures between 4 and 40 ± 0.5 ˚C. Briefly, 35 mL of the formulations was placed into the sample measuring cup. Viscosity measurements were taken between 4 ˚C and 40 ˚C. The viscosity measurement (at each temperature) was carried out every 30 seconds in a vibroviscometer to follow the changes related to temperature. The experiment was repeated for each sample in reversely from 40 ˚C to 4 ˚C.

Table 1. Compositions of formulations

Formulation	PEG 1500 % *w/v*	Lutrol F 127 % *w/v*	Phosphate buffer (pH: 3)	Phosphate buffer (pH: 4)	Phosphate buffer (pH: 5)
F1	10	25	+	+	+
F2	7.5	22.5	+	+	+
F3	5	20	+	+	+
F4	2	17.5	+	+	+
F5	1	15	+	+	+

(+) means; all formulations have been prepared according to ratio of excipients and pH
PEG: Polyethylene glycol

Table 2. Composition of optimized formulations

Formulation	LF 127 % *w/v*	PEG 1500 % *w/v*	Phosphate buffer (pH: 3)	Phosphate buffer (pH: 4)	Phosphate buffer (pH: 5)
F4a	18	2	+	+	+
F4b	17	2	+	+	+
F4c	16.8	2	+	+	+
F4d	16.5	2	+	+	+
F4e	16.2	2	+	+	+

(+) means; all formulations have been prepared according to ratio of excipients and pH
PEG: Polyethylene glycol

Evaluation of chromatogram

The formulations were placed in the donor phase as 1 mL (2200 IU/mL) for the transition study. Optimized formulations containing sCT were diluted with 5 mL of the receiving phase. The possible maximum amount for determination would be 2200 IU/5 mL (=440 IU/mL= 73.3 µg/mL). Therefore, formulations containing 75 g/mL sCT were loaded and analyzed separately in triplicates. Evaluation was made to examine, whether the peaks of the excipients in the formulation interfere with the peak of the active substance.

Stability studies

Short-term stability assessment of sCT was carried out based on the repeatability parameters included in the validation studies. The sCT (25 µg/mL in pH 7.4 phosphate buffer) inside the same vial was analyzed 10 times. Monthly stability assessment was performed for formulations containing 11,000 IU sCT in 5 mL. The formulations were kept in capped vials for 3 months at +4 ˚C. The samples were also examined for physicochemical properties at predetermined time intervals (1st day, 1st week, 1st month, and 3rd month). The concentrations of formulations were determined by HPLC. Samples to be examined for stability at +4 ˚C was stored in the refrigerator. Samples to be examined at 25 ± 0.5 ˚C (60 ± 5% RH) and 40 ± 0.5 ˚C (75 ± 5% RH) were stored in climate cabinets.

In vitro release studies

In vitro release studies were carried out using a cellulose membrane (MW: 25.000 Da) using a diffusion tube method specifically designed for our study. The system consists of two parts, *e.g.* transmitter and receiver compartment, and the membranes are left in contact with the receiver compartment. The membranes (3.81 cm^2) are placed tightly between the donor and receiver compartments.

5 mL of pH 7.4 phosphate buffer was receiver phase and tool placed in the lower beaker. Formulations (containing approximately 2200 IU/mL sCT) and CP was placed over the membrane as donor phase. The system was placed in a water bath at 37 ± 0.5 ˚C with magnetic stirrer. The receiving phase was mixed with help of magnetic stirrer at a speed of 300 rpm during the operation. The amount of active substance from the samples was determined at regular intervals by HPLC. The fresh phosphate buffer (pH 7.4) was added to the medium as much as the sample taken. The transition studies were carried out in triplicates.

In vivo studies

CP and the developed formulations must be in the same package to compare their applications to nostrils of rabbits. For this purpose, pediatric packaging of another CP was used. Nasal spray dosimeter control was performed before drug administration.

Nasal spray dosimeter evaluation

Metered dose spray systems of the developed formulations were gravimetrically compared with commercial nasal preparations in terms of spraying amounts. The sprays were weighed on a scale with a precision of 0.0001 g and, then, the amount of each puff was determined gravimetrically by pressing ten times in succession after each puff. The standard deviations and relative standard deviations of the weights were calculated and compared statistically.

The same spray was used for both the developed formulations and the CP to examine, whether there was a difference in the dose of the nasal spray used. Their performance was examined gravimetrically and evaluated statistically in a similar way.

Content uniformity

Since the sensitivity of the kit to be used in the method is between 0 and 100 ng, the amount of sCT to be determined by EIA in rabbit plasma should be within this range. In a similar study, when 2000 IU sCT was applied *IN* to rabbits, sCT was determined between 0 and 100 ng/mL in RIA analysis of serum samples.[25] With this goal in mind, before administering to the rabbits, 5 mL of our formulations were weighed on a balance with 1 g precision to obtain 1000 IU/puff at each spraying with 11.917 g sCT. That is, the formulations were designed to contain 14.300 IU/mL sCT. 100.84 g sCT was weighed and loaded into 5 mL of the CP bearing in mind 5 mL of CP contains 110.00 IU. The formulations prepared were analyzed by HPLC with an injection volume of 10 L using the same analytical method, and their concentrations were checked before application.

Drug administration to rabbits

Drugs were administered to 24 New Zealand-type white rabbits of both sexes (weighing between 2.5 and 3 kg). Rabbits were provided by Faculty of Medicine, Experimental Animal Production Center. Before the studies, ethic approval was obtained from the Ege University, Faculty of Medicine Animal Ethics Committee (2009-95).

The rabbits were divided into four groups consisting of three females and three males in each group. The rabbits were fasted for 24 h before drug administration. During the studies, anesthesia was administered by giving *IM* 50 mg/kg ketamine and 5 mg/kg xylazine.[21]

1 mL of blood samples was taken from six rabbits *per* group at 0 min before even the first administration. The sprays were applied to the nose after being sprayed into the air once. The application consisted of two squeezes and one puff in each nostril of the rabbits. *IV* administration was carried out into the venous vein of rabbit ear using insulin needle.

After the applications, 1 mL of blood samples was taken at 15, 30, 45, 60, 75, 90, 105, 120, and 180 minutes. Blood samples were taken with 22 G1 cannulas and put into EDTA tubes. These samples were then centrifuged at 4 ˚C for 15 min (at 1600 rpm) and the plasma was separated. The plasma samples were stored at -80 ˚C for evaluation by EIA.

Quantification of sCT in rabbit plasma

EIA kit was used for the assay of sCT. The immunoplate in the ready kit was pre-coated with secondary antibody and non-specific binding sites were blocked. The log/logit curve of the optical density *versus* the concentration is determined using standards at known concentrations. Unknown concentrations are estimated by extrapolation.

The kits were maintained at room temperature before use. Assay buffer included in the kit was diluted with 950 mL of distilled water. Five concentrations were obtained by diluting with assay buffer from standard solution at a concentration of 1000 ng/mL (Table 3 below). The primary antibody was rehydrated by mixing with 5 mL assay buffer. Likewise, the biotinylated peptide was also rehydrated by mixing with 5 mL of assay buffer. The positive control was rehydrated with 200 µL assay buffer and centrifuged. 50 µL assay buffer (for total binding), 50 µL standard solutions from less diluted to more diluted and 50 µL of positive control were placed in the first row of the well plates from top to bottom. 50 µL of plasma samples were added to the remaining wells of the plates, respectively. 25 µL of rehydrated primary antibody and rehydrated biotinylated peptide were added to all wells except for the blank, respectively. Plates were covered with acetate plate sealer and incubated for 2 h at room temperature with 300 cycles on a shaker. At the end of this period, inside of the plates was poured out and the wells were washed four times with assay buffer. 100 µL of mixture containing 12 µL of SA-HRP and 12 mL of assay buffer was added to each well and the plates were covered again with acetate. The plates were incubated for 1 h at room temperature at 300 rpm while shaking. Then, the wells were emptied by washing the wells four times with assay buffer. 100 µL of TMB substrate solution was placed in each well and the plates were kept in the dark at 300 cycles on a shaker until the wells were discolored. The reaction was terminated by adding 100 L of 2 N HCl to the wells. The plate was analyzed using a Thermo Labsystem Multiscan Spectrum at 450 nm.

Bioavailability assessments

The results of all plasma samples taken from rabbits after nasal administration of the prepared formulations were subjected to pharmacokinetic analysis compared to the CP administered *via IV* and nasal route. For pharmacokinetic analysis, using WinNonlin version 5.2, Pharsight Corporation program, the data of each rabbit, and pharmacokinetic parameters stated below were evaluated.

Absolute bioavailability: It is the ratio of the amount of an active substance that is administered into the blood circulation by any method to the amount of active substance that enters the blood circulation when administered *IV*. The total area below the blood concentration-time curve when the drug is given *IV* (completely) is considered to be 100%.[26,27]

Relative bioavailability: It is the comparison of the rate and degree of absorption of the active ingredient from the test and reference dosage forms applied in the same way. It is obtained by comparing the blood/plasma concentration-time profile [area under the curve (AUC)] of the formulation to that of the reference and multiplying by 100.[26,27]

RESULTS

Quantification of sCT concentration using HPLC

Linear equation of the calibration curve was determined as y= 16.989x + 30.097 with R^2= 0.9946. Sensitivity of the method was evaluated in terms of the limit of detection (0.276 µg/mL) and the limit of quantification (0.836 µg/mL). Coefficients of variation for repeatability and reproducibility were lower than 2%. Relative standard deviations of both accuracy and precision values were less than 8%. The specific peak of sCT was detected clearly in an irreproachable manner. Retention time of the peak of the active substance was 14.2 min, as shown in Figure 1 below.

Preparation and characterization of thermoreversible formulations

Temperature of the nasal mucosa is about 32-34 °C, while temperature of the entrance part of the nose and nasopharynx is stated to be 31 and 36 °C, respectively. The temperature increases from the nasal vesticulate to the back. No temperature difference was reported between the nostrils.[28,29] Taking all this into account, we designed our formulation to transform into sol-gels at 34 °C.

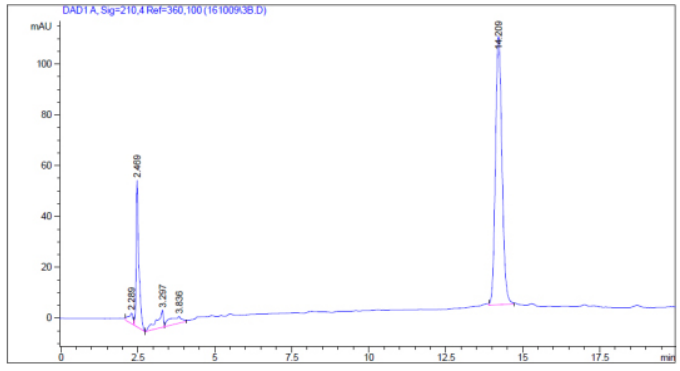

Figure 1. HPLC chromatogram of salmon calcitonin

Table 3. Concentrations of standart solutions for ELISA determination

Standard no	Volume of standard (µL)	Buffer (µL)	Concentration (ng/mL)
Stock	1000 µL	-	1000.0
Std 1	100 µL stock	900	100.0
Std 2	100 µL Std 1	900	10.0
Std 3	100 µL Std 2	900	1.0
Std 4	100 µL Std 3	900	0.1
Std 5	100 µL Std 4	900	0.01

Std: Standard

The density of formulations

The optimal formulation contains approximately 2200 IU/mL sCT. Density, tonicity, refractive indices, and conductivity values of the selected formulations (related with F4e and F4d formulations) are shown in Tables 4-8, respectively. In Table 5, standard deviations between repetitiative measurements are quite small indicating homogeneity in formulations.[30]

Tonicity studies

NaCl solution (0.9%), which has the same isotonia as blood plasma, has an osmolarity of 300 mOsm (less than that of the investigated formulations in Table 6), indicating that all the prepared formulations are hypertonic.

Refractive index measurements

From Table 7, refractive index measurements varied between 1.33 and 1.36. These values suggest the clarity of prepared formulations.[31]

Conductivity measurements

Conductivity values of optimum formulations were determined in the range of 860-1935 S/cm. Because pH of the formulations varied between pH 3-5 and the quantities LF 127 and PEG 1500 are different, the measured values were not the same. As shown in Table 8, conductivity of the researched formulation increases in turn: 4A>4B>3A>3B>5A>5B.

Viscosity measurements

Vibroviscometer used in this study makes measurements using tuning fork vibration method. With the aid of sensor plates vibrating at a frequency of 30 Hz, it is possible to make continuous measurements in a dynamic measuring range with high accuracy/repeatability. Given that measurements can be taken from low to high viscosity, studies were carried out without the need for many spindles compared to conventional rotational viscometer.[32] Viscosity comparison between formulations

Table 4. Gelling temperatures of various gel formulations

Formulations	Phosphate buffer pH: 3	Phosphate buffer pH: 4	Phosphate buffer pH: 5
F4a	25 °C	25 °C	25 °C
F4b	29.5 °C	28 °C	26.5 °C
F4c	30.5 °C	28.5 °C	31 °C
F4d	31 °C (3B)	31 °C (4B)	31 °C (5B)
F4e	36 °C (3A)	35.0 °C (4A)	32 °C (5A)

Table 5. Density of the investigated formulations

Formulations	Weight I (g)	Weight II (g)	Weight III (g)	Average weight (g)	SD	% RSD	Density (g/mL)
3A	5.573	5.578	5.567	5.573	0.006	0.099	1.024
4A	5.596	5.593	5.590	5.593	0.003	0.054	1.028
5A	5.605	5.609	5.598	5.604	0.006	0.099	1.030
3B	5.603	5.595	5.604	5.601	0.005	0.088	1.029
4B	5.615	5.613	5.616	5.615	0.002	0.027	1.032
5B	5.576	5.578	5.583	5.579	0.004	0.065	1.025

RSD: Relative standard deviation, SD: Standard deviation

Table 6. Tonicity measurements of formulations

Formulations	1. Trial (mOsm)	2. Trial (mOsm)	3. Trial (mOsm)	Average (mOsm)	SD	% RSD
3A	360	350	360	356.7	5.8	1.6
3B	360	370	360	363.3	5.8	1.6
4A	380	380	380	380.0	0.0	0.0
4B	380	390	380	383.3	5.8	1.5
5A	390	380	400	390.0	10.0	2.6
5B	420	410	410	413.3	5.8	1.4

RSD: Relative standard deviation, SD: Standard deviation

kept at 34 °C, which is the nasal mucosa temperature, is given in Figure 2. Based on viscosity of the formulations, which increases with temperature, the flow property is observed to be a thixotropic system in non-Newtonian flow. It is suitable for sol-gel form.[33,34]

The evaluation of chromatogram

Number of sCT in the formulations determined using HPLC is given in Table 9. It was observed that the excipients did not interfere with 75 µg/mL of active substance. Highest relative standard deviation (RSD) was found to be 3.31%. Formulations have been under repeatable experimental conditions. The quantities determined during the analysis according to Food and Drug Administration (FDA) and ICH validation criteria are less than 15% repeatability and are appropriate.[35-37]

Stability studies

Stability results of the sCT solution examined at room temperature for 220 min are given in Table 10. Mucociliary clearens occur on average every 20 min due to the nasal application.[38] Ten consecutive analysis stability assessment processes correspond to approximately 11 mucosilyer clearens. Percentage ratios of the samples taken at room temperature based on the value of the first concentration are shown in Table 11.

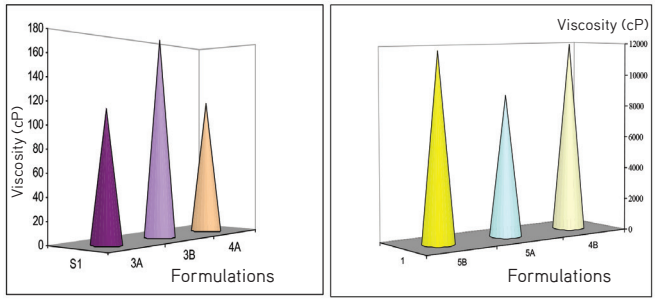

Figure 2. Viscosity measurements of various formulations

Table 7. Measurement of refractive indices

Formulations	Indice I	Indice II	Indice III	Average indice	SD	% RSD
3A	1.3582	1.3582	1.3582	1.3582	0.0	0.0
3B	1.3588	1.3588	1.3588	1.3588	0.0	0.0
4A	1.3302	1.3302	1.3302	1.3302	0.0	0.0
4B	1.3352	1.3352	1.3352	1.3352	0.0	0.0
5A	1.3582	1.3582	1.3582	1.3582	0.0	0.0
5B	1.3587	1.3587	1.3587	1.3587	0.0	0.0

RSD: Relative standard deviation, SD: Standard deviation

Table 8. Conductivity measurements of formulations

Formulations	I (µS/cm)	II (µS/cm)	III (µS/cm)	Average (µS/cm)	SD	% RSD
3A	1620	1620	1620	1620.0	0.0	0.0
3B	1123	1121	1123	1122.3	1.2	0.1
4A	1935	1936	1935	1935.3	0.6	0.0
4B	1850	1850	1852	1850.7	1.2	0.1
5A	881	881	881	881.0	0.0	0.0
5B	864	863	864	863.7	0.6	0.1

RSD: Relative standard deviation, SD: Standard deviation

Table 9. Concentrations of the investigated formulations

Formulations	I. amount µg/mL	II. amount µg/mL	III. amount µg/mL	Average µg/mL	SD	% RSD
3A	86.75	86.36	85.33	86.15	0.73	0.85
3B	86.51	84.33	82.87	84.57	1.83	2.17
4A	73.70	72.47	73.28	73.15	0.63	0.85
4B	67.22	69.31	69.08	68.54	1.15	1.67
5A	80.30	82.17	76.96	79.81	2.64	3.31
5B	85.38	83.97	85.79	85.05	0.95	1.12

RSD: Relative standard deviation, SD: Standard deviation

Table 11 demonstrated that 10% of the initial concentration was lost within 120 min. Based on these results, we limited period of the *in vitro* studies to 120 min.

Short term and monthly stability evaluation of 3A, 3B, and 4A, 4B formulations and CP was carried out at 4 ± 0.5 ˚C and 25 ± 0.5 ˚C, respectively. Figures 3 and 4 compared stability of various concentrations (with respect to time) at 4 ± 0.5 ˚C and 25 ± 0.5 ˚C, respectively.

In Figure 3, it was observed that CP and the 3A formulation showed similar stability at 4 ± 0.5 ˚C at the end of 30 days.

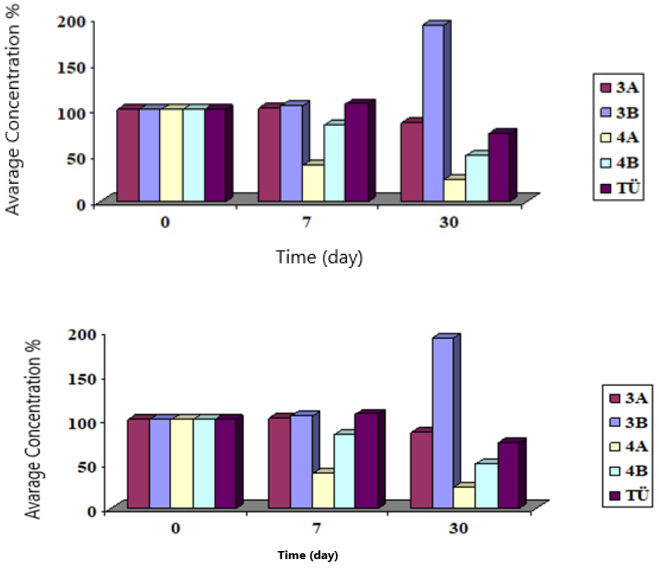

Figure 3. Stability results in terms of mean % concentration *vs.* time (day) at 4 ± 0.5 ˚C

Meanwhile, both CP and formulations could not maintain their stability within the 30 day period.

sCT was not detected in formulations and CP stored at 40 ˚C and 75% humidity on the 7th, 15th, and 30th days.

In vitro release study results

A graph of the cumulative amount of active substance in the receiving phase of the diffusion experiment setup *vs* time is shown in Figure 5. sCT amounts were determined by HPLC. The active ingredient could not be determined in 5A and 5B formulations during diffusion studies. Stability problem was encountered due to the degradation of sCT in a short time at pH 5.[39,40]

The prepared formulations and CP are known to be stable for at least 90 min. Hence, sCT amount expressed as percentage release ± SD in the formulations, and CP was investigated for a period of 90 min (Table 12). CP was noted to be superior in terms of transition as compared to the prepared formulations: 3A>4A>3B>4B.

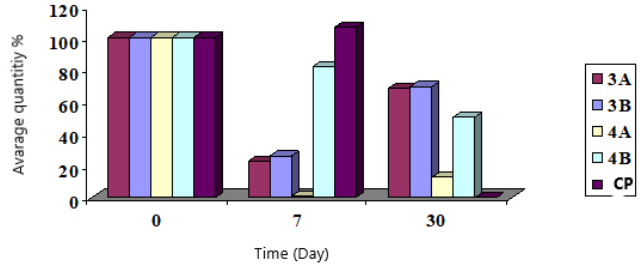

Figure 4. Stability results in terms of mean % concentration *vs.* time (day) at 25 ± 0.5 ˚C

Table 10. Results of short-term stability studies (n: 10 time points) of sCT in HPLC

% Concentration	22 min	44 min	66 min	88 min	110 min	132 min	154 min	176 min	198 min	220 min	Average	SD	% RSD
100	117.3	115.1	113.2	111.8	109.8	108.2	105.0	102.3	98.3	93.3	107.4	7.69	7.16

sCT: Salmon calcitonin, HPLC: High performance liquid chromatography, RSD: Relative standard deviation, SD: Standard deviation

Table 11. % Ratios of samples during a short-term stability studies

Time (min)	0	20	40	60	80	100	120	140	160	180
Ratio (%)	100	98	96.6	95.6	93.9	92.5	89.8	87.4	84.1	79.5

Table 12. % *In vitro* release of sCT with respect to time (min)

Time (min)	3A	3B	4A	4B	CP
0	0.0	0.0	0.0	0.0	0
10	34.4 ± 39.9	1.0 ± 1.2	9.8 ± 17.1	ND	4.5 ± 1.5
30	72.7 ± 24.0	2.7 ± 3.0	18.8 ± 21.1	ND	74.2 ± 16.8
60	62.3 ± 16.8	5.6 ± 6.0	22.5 ± 36.7	ND	78.8 ± 25.2
90	50.7 ± 29.5	4.6 ± 1.5	13.7 ± 10.4	0.2 ± 0.3	73.3 ± 16.9

sCT: Salmon calcitonin, ND: Not determined, CP: Commercial product

Formulations 5A and 5B in pH 7.4 buffer could not be analyzed in any way. This situation was evaluated as stability problem.

In vivo studies

According to *in vitro* studies, 3A and 4A formulations were used considering that they have better performance in terms of membrane permeation as compared to other formulations. 5A and 5B formulations were excluded from the studies due to stability problems, while 3B and 4B formulations were excluded due to gelation at lower temperatures. Furthermore, sCT diffusion from 3B and 4B formulations was limited. Numerous trials were conducted using diffusion tube method and high variations were observed in the results of transition studies.

Nasal spray dosimeter evaluation

The average gravimetric assessments of original drug, 3A and 4A formulations were determined as 0.0739, 0.0720, and 0.0701, respectively. SD values were less than 0.0015 and RSD values were less than 2. Based on the results, it could be said that dose uniformity were achieved in liquid form at the time of pressing. The average gravimetric assessments of original drug after spraying from its original packaging was defined as 0.0927. SD and RSD values were determined as 0.0026 and 2.8416, respectively.

Content uniformity of the investigated formulations

F-test was used to determine, whether there was a general difference between the applications. After F-test, groups with differences were determined using Tukey Honest Significant Difference (HSD) method. Tukey HSD method was also used to compare the formulations in pairs. Significant differences between formulations are shown with asterisks "***" in Table 13 below.[41]

Spr: The original spray of CP

CP: Spray device, in which CP is placed to be compared in the same apparatus

3A: The spray device, in which the 3A formulation is placed to be compared in the same apparatus

4A: The spray device, in which the 4A formulation is placed to be compared in the same apparatus

The concentration of administered formulations

After 2000 IU intranasal administration in an immunoassay study, highest concentration of sCT detected in rabbit plasma was 40 ng/mL.[25] Since sCT detection range of our EIA kit was 0-100 ng/mL, the formulations were designed to administer 1000 IU/puff to the rabbits. The formulations were prepared by loading sCT in 14.300 IU/mL and 71.500 IU/5 mL. In order to eliminate any other factors for the sake of accuracy, 11.917 g sCT was weighed and loaded into 5 mL formulation and the amount of loaded sCT was determined using HPLC before each *in vivo* study. By the way, dose content of the formulations was supported by an analytical method. Dosage of formulations was selected based on the analytical method.

Results of analytical assay of the formulations loaded with sCT before applying to rabbits are given in Table 14. Volume of the spray released from each puff was found to be approximately 0.07 mL.

Quantification of sCT in rabbit plasma

The method used for quantification of sCT in rabbit plasma is ELISA. ELISA is a bioanalytical method, in which substance to be analyzed is measured depending on the antigen-antibody relationship. Sometimes, it is used to quantitatively detect the presence of antigen/antibody in a matrix and occasionally, it is used to detect the amount of analyte in samples of unknown concentration based on calibration curve.

Bioavailability assessments

Before starting the bioavailability studies, the rabbits to be treated were allowed to fast for 24 h. Anesthesia was administered before the application and was continued based on the motility of the rabbits to avoid deviations in blood draws.

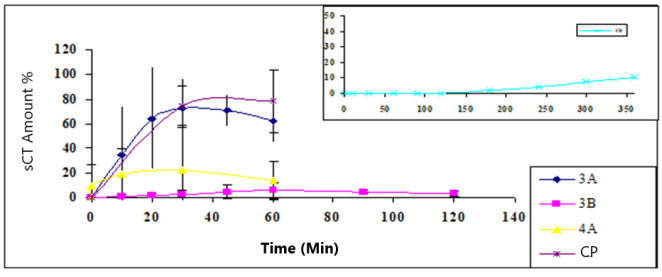

Figure 5. % Salmon calcitonin, release *versus* time (min)

Table 13. Statistical evaluation of dosage differences in sprays

Evaluation of gravimetric dosage differences of sprays at 0.05 level				
Compared formulations	**Differences of averages**	**95% confidence limit**		**Evaluation**
spr - CP	0.019	0.017	0.021	***
spr - 3A	0.021	0.019	0.023	***
spr - 4A	0.023	0.021	0.025	***
CP - 3A	0.002	0.0001	0.004	
CP - 4A	0.004	0.002	0.006	
3A - 4A	0.002	0.0001	0.004	

CP: Commercial product

A catheter was inserted in the rabbits so that blood samples could be collected conveniently, accurately, and on time after injection. In cases where blood could not be obtained from the ear veins during frequent blood withdrawals, blood was taken from the ear arteries. For some of the rabbits, blood was withdrawn from the heart, instead of the veins, due to their complex anatomical structure (*e.g.* no prominent ear veins located) or difficulty in obtaining blood.

The formulations were applied to right and left nostrils of rabbits and blood samples were taken at the stated time periods. The plasma was separated from the blood and stored at -80 °C until quantification using EIA.

Pharmacokinetic constants of *IV* and intranasal applications of the investigated formulations (3A and 4A) and CP were obtained using a program called WinNonlin version 5.2 (Pharsight Corporation). Findings of the pharmacokinetic study using this program are shown in Table 15. Standard dose application was not applied in practice.

Table 15 compares AUC 0 → last values (the primary pharmacokinetic parameter) of the investigated formulations. Considering that initial sCT amount in the CP is approximately 1.5 times that of 4A formulation, then AUC will increase in the order: 4A>CP>3A. Critical parameters were found to be similar and comparable between formulations 4A and CP.

As seen in Table 16, absolute bioavailability of the intranasal application of CP is 0.856, which is within the range of 0.3-3, stated in the literature.[28,42] Absolute bioavailability of formulation 4A was 0.743, which is very close to CP, even though the initial concentration of formulation 4A is 1.5 times less than CP. For 3A formulation, despite the positive results obtained during *in vitro* studies, *in vivo* results (*i.e.* bioavailability result) were less significant.

Bioavailability of 3A formulation is not at the same level as 4A and CP. Table 16 contains data and bioavailability results of the applications in terms of primary pharmacokinetic parameters (AUC 0 → last value). In Table 17, the relative and absolute bioavailability was evaluated based on the secondary pharmacokinetic parameter (AUC 0 → ∞). The data from Table 17 revealed that absolute bioavailability of CP and the 4A formulations are similar: AUC 0 → ∞ value is 0.254 and 0.234, respectively. On the other hand, bioavailability of 3A formulation is only about 35% of the mentioned bioavailability values (*i.e.,* AUC 0 → ∞ value is 0.157).

Table 14. Quantity of active ingredient in formulations prior to application

Formulations	Rabbit no	Amount (µg/mL)	Application dose (µg)	Application dose (IU)
3A	R1, R2	3270.1	457.8	2746.8
	R3	3401.7	476.2	2857.2
	R13, R14, R15	3078.5	431	2586
4A	R4	2133	298.6	1791.6
	R5, R6	2090.6	292.7	1756.2
	R16, R17, R18	2597.3	363.6	2181.6
CP *IN*	R19, R20, R21	3237.8	453.3	2719.8
	R22, R23, R24	3169.6	443.7	2662.2
CP *IV*	R7, R8, R9, R10, R11, R12	-	1.66	10

CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal, IU: International unit

Table 15. The obtained pharmacokinetic data of sCT administered *via* different routes

Parameters	CP (*IV*)	CP (*IN*)	3A	4A
$t_{1/2}$ (min)	308.9 ± 399.8	84.3 ± 121.5	227.4 ± 148	75.7 ± 49.6
t_{max} (min)	30 ± 30	75 ± 15	105 ± 68.4	57.5 ± 33.4
C_{max} (ng/mL)	17.4 ± 21.5	87.4 ± 95.1	4.8 ± 2.1	37.3 ± 75.6
AUC 0 → t_{last} (min*ng/mL)	786.5 ± 330.9	1838.6 ± 1588.8	466.9 ± 140.2	1231.1 ± 1653.6
AUC 0 → ∞ (min*ng/mL)	3059.5 ± 3221.7	2120.9 ± 1398.9	1279.1 ± 413.5	1477.4 ± 1548.0
Vd (mL)	207.5 ± 96.2	35408.4 ± 53569.3	111167.1 ± 75645.9	40247.5 ± 28583.0
Cl (mL/min)	0.964 ± 0.57	274.2 ± 141.3	395.6 ± 186.6	358.6 ± 187.1
MRT0 → last (min)	73.8 ± 15	61.9 ± 16.7	93.8 ± 6.0	77.1 ± 8.9
MRT 0 → ∞ (min)	474.1 ± 575.8	122.5 ± 197.1	363.2 ± 195.8	146.7 ± 75.8

sCT: Salmon calcitonin, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal, C_{max}: Maximum concentration, AUC: Area under the curve, V_d: Volume of distribution

Relative and absolute bioavailability assessments based on maximum concentration (C_{max}) were measured in a similar way as AUC. The relative/absolute bioavailability data based on C_{max} is presented in Table 18.

Absolute bioavailability of CP administered *via* nasal route was found to be 1.88 (see Table 18) using C_{max} value as a primary pharmacokinetic parameter.

DISCUSSION

Among the investigated formulations, F4 gelled at a temperature close to the nasal mucosa temperature (*i.e.* 34 ˚C) as desired. For this reason, new formulations based on F4 with modified ratios of Lutrol F-127 as gelling agent along with 2% PEG 1500 were explored. PEG 1500 was used in the formulation due to its water-solubility and amphibian features. It is also non-toxic and non-immunogenic. PEG has been approved by FDA and is used as a carrier in many food, cosmetic, injectable, topical, rectal, and nasal applied pharmaceutical products. It also has penetration enhancing effect. The ratio of PEG 1500 used as 2% is to reduce the variability in the temperature transformation. However, this application is not a PEGylation.[30,31]

According to Table 4, formulations with gelation temperature closer to the nasal mucosa temperature are coded as 3A, 4A, and 5A. Similarly, formulations that tend to have gelation temperature closer to the lower limit of the nasal mucosa temperature are coded as 3B, 4B, and 5B.[13] These coded formulations were selected for further investigations.

Based on viscosity of the formulations, which increases with temperature, the flow property is observed to be a thixotropic system in non-Newtonian flow. It is suitable for sol-gel form.[33,34] Viscosity of formulations with decreasing temperatures from 40˚C to 4˚C was found to be the same as 34 ˚C. Moreover, formulations 3A, 3B, and 4A exhibited a slower gelation transformation.

Nasal formulations should be between 4.5 and 6.5 based on nasal pH.[13] However, pH of commercially available preparations of sCT is around 3. Given that sCT is stable at pH between 3 and 4, pH of the nasal mucosa is around 5. Our formulations were prepared at pH 3, 4, and 5 in order to examine effect of pH on the stability as well as bioavailability.[39-41]

During the diffusion studies, sCT was not detected in the samples or CP after 24 h. Besides, no sCT amount could be determined in the formulation with pH: 5, indicating that the molecule is very unstable in such environments. Stability of commercially available product in transition and storage conditions was also found as very sensitive to be delicate.

When the results of transition studies were evaluated, it is seen that variation between the samples is high. Despite the large

Table 16. Relative and absolute bioavailability data based on AUC 0 → t_{last} values

	AUC 0 → t_{last} (min*ng/mL)	AUC 0 → t_{last}/dose	Absolute bioavailability	Relative bioavailability
CP (*IV*)	786.5	473.8	-	-
CP (*IN*)	1838.6	4.06	0.856	-
3A	466.9	1.05	0.222	25.9
4A	1231.1	3.52	0.743	86.7

AUC: Area under the curve, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal

Table 17. Relative and absolute bioavailability data based on AUC 0 → ∞ values

	AUC 0 → ∞	AUC 0 → ∞ /dose	Absolute bioavailability	Relative bioavailability
CP (*IV*)	3059.5	1843.1	-	-
CP (*IN*)	2120.9	4.68	0.254	-
3A	1279.1	2.89	0.157	61.8
4A	1477.4	4.31	0.234	92.1

AUC: Area under the curve, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal

Table 18. Relative and absolute bioavailability data based on C_{max} values

	C_{max}	C_{max}/dose	Absolute bioavailability	Relative bioavailability
CP (*IV*)	17.4	10.5	-	-
CP (*IN*)	87.4	0.195	1.88	-
3A	4.8	0.011	0.11	5.64
4A	37.3	0.104	0.99	53.3

C_{max}: Maximum concentration, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal

number of trials, consistent values could not be obtained even with the same formulation. It can be said that the release in formulation A is more balanced than in formulation B based on their viscosity. Moreover, release of formulation B was slower than that of formulation A.

Using Tukey HSD method, a difference within 95% confidence interval was observed between the original spray of CP and the investigated spray intended to be used during the application. Using the same method, spray homogeneity was evaluated against our formulations by placing the commercial nasal spray content into the nasal spray to be administered. In spray applications, there was no difference between formulations and commercial nasal product spray homogeneity at 95% confidence interval as seen in Table 13. This indicates reliability of the selected spray device to be used during bioavailability studies.

IU used for sCT herein is defined as the amount of calcitonin that produces an equivalent reduction in blood calcium level in young rats under strictly defined experimental conditions within one hour as the injection of an ampoule or part of an ampoule of international reference preparation of calcitonin.[1] Based on this, spraying volume was taken as 0.14 mL for each nostril to achieve the targeted single dose of 1000 IU. Standardization of sCT dose is very complicated due to precision in the weighing and slight volume differences in the prepared formulations.

ELISA method is generally a heterogeneous non-competitive application. Primary antibody corresponding to the analyte of interest is usually detected on the multiwell plate or solid plastic surface. Biological sample is dispensed onto the multiwell plate and the detected antibody captures the analyte to be measured. Excess analyte is removed by washing. Antigen-antibody complex is determined at the conjugated antibody and its antigen by a two-step retention process. First, the enzyme labeled antibody goes to the analyte and binds to the antibody-antigen complex. The second incubation occurs with a specific substrate solution suitable for enzyme. At this stage, the amount of the colored product is determined spectrophotometrically.[23]

AUC and C_{max} values as main parameters in bioavailability assessment of CPs and the formulations were calculated separately using Winnonlin program after each application to six rabbits. To compensate for differences between the doses administered to rabbits, values were calculated based on the doses administered.

Absolute bioavailability of CP administered *via* nasal route was found to be 1.88 using C_{max} value as a primary pharmacokinetic parameter (Table 18). This finding agrees with the data presented in the literature.[38] Among the developed formulations, 4A formulation has an absolute and relative bioavailability of 0.99 and 53.3%, respectively; the bioavailability value is half that of CP as expected. This could be explained by the fact that the formulation in question is a controlled release hydrogel matrix type preparation designed to release sCT at a much slower rate and in lower quantities than CP.

CONCLUSION

In this study, a sol-gel formulation that is in liquid form at +4 °C as storage condition and gel form at intranasal temperature was developed. When sprayed into nose, the developed formulation adheres to the mucosa. Taken into account the fact that the formulation is in liquid form at the time of spraying, but turns into a gel only after being in contact with the mucosa. Dose uniformity was ensured during the development. Fluidity of the product is reduced, where it is squeezed, allowing the release of the active substance. sCT is stable at pH 3-4 and considering that no sCT was detected in formulations with pH 5. It shows that sCT molecule is very unstable at this pH.

In an *in vitro* release study, formulations 3A and 4A showed faster and more permeation than CP at 10 min. This situation can be attributed to the transition of formulations in the first few minutes. Nevertheless, 3A and the CP showed similar *in vitro* release capability. Overall, formulations A were found to be more stable and have better *in vitro* release capability compared to formulations B based on their viscosity.

CP and developed formulations were further investigated *in vivo* studies. The standard dose was provided with a spray head suitable for rabbit noses. Pharmacokinetic data revealed that CP was able to reach C_{max} value in 30 min. Meanwhile, t_{max} value in EIA analysis was 0 in two of six rabbits administered *IV*, while values such as 15, 45, and 75 min were found in the other rabbits. As this situation could not possibly be explained theoretically, it is likely because of the analysis method used given that the same problem was encountered in another study. Based on AUC $0 \rightarrow t_{last}$ value (the primary pharmacokinetic parameter), the absolute bioavailability of CP administered *via* the intranasal route was found to be 0.856, which is within the range of 0.3-3 stated in the literature.[20,25,42] Absolute bioavailability value for formulation 4A (*i.e.* 0.743) was very close to the value of the CP. As for 3A formulation, absolute and relative bioavailability was found to be 0.222 and 25.9%, respectively, which is below the values of CP and 4A formulation in contrast with the positive results obtained in the *in vitro* studies. A similar situation was observed using the secondary pharmacokinetic parameter, AUC $0 \rightarrow \infty$: absolute bioavailability of CP and 4A formulations were similar (*i.e.,* 0.254 and 0.234, respectively), whereas the 3A formulation has a bioavailability value (*i.e.,* 0.157), which is 35% of the mentioned bioavailability values.

Nasal administration of CP showed an absolute bioavailability of 1.88 based on C_{max} value as the primary pharmacokinetic parameter, which is in accordance with the literature. Among the developed formulations, formulation 4A has an absolute bioavailability of 0.99 and relative bioavailability of 53.3%, half as much as CP. Absolute bioavailability of 0.11 and relative bioavailability of 5.64% for 3A formulation is less than bioavailability values obtained using AUC parameters. This is thought to be due to slow release of sCT from gel form. The obtained virtual volume of distribution (V_d) value from pharmacokinetic data indicates greater plasma protein binding of the sol-gel formulations. Post *IV* and *IN* administration, V_d

values for CP were much smaller than the values of the developed formulations. Based on these results, it was suggested that bioavailability assessment using pharmacodynamic parameters other than EIA and the RIA method would be more meaningful during data evaluation.

Ethics

Ethics Committee Approval: Ege University Faculty of Medicine Animal Ethics Committee (2009-95).

Informed Consent: Not necessary.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.L.A., E.K., Concept: A.L.A., Design: A.L.A., Data Collection or Processing: A.L.A., G.Y.T., Analysis or Interpretation: A.L.A., E.K., L.D.K., Literature Search: A.L.A., G.Y.T., Writing: A.L.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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