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Thermoreversible Gel Formulation For Intranasal Delivery of Salmon Calcitonin And Comparision Studies of *In Vivo* Bioavailability

Short title: Intranasal solgel formulation of salmon calcitonin

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Abstract
Purpose we developed original thermoreversible (solgel) formulations of salmon calcitonin (sCT) for nasal applications. The solgel has been compared with the commercial intranasal spray in vitro and in vivo studies. Aim of studying solgel form is to arrange viscosity of formulations for a reversible adequate fluidity in different temperatures. This situation may facilitate use of drug as spray and increase bioadhesive ability to mucosa.
Methods Characterization of optimum formulations were studied. Validated analytical assays determined the amount of sCT. The approximately equal amount of commercial and solgel dosages were sprayed nostrils of the rabbits. Blood samples collected from the ear veins of rabbits and determined by EIA (Enzyme Immunoassay Plates). These plates evaluated by Thermo Labsystem Multiscan Spectrum at 450 nm. Thanks to Winnonlin 5.2, pharmacokinetic data were evaluated by non-compartmental method.
Results The absolute bioavailability of the formulation at pH 4 and the commercial product was compared by evaluating the primary pharmacokinetic data AUC0→tlast. The absolute bioavailability of the commercial intranasal spray was measured 1.88 based on the Cmax assessment. Cmax of solgel formulation pH:4 was calculates as 0.99 and the relative bioavailability was obtained 53.3%.
Conclusion In vivo pharmacokinetic data of solgel formulation with pH:3 showed significantly higher Vd parameter than commercial product (111167 > 35408). It is thought that the adhered formulation to the nasal mucosa releases sCT slowly and less.
Keywords: 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 and 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 order of increasing strength as follows: sCT>eCT>hCT>pCT. sCT has been shown to be more potent and to last longer than eel, human, and porcine calcitonin [1,2]. Salmon calcitonin inhibits the formation as well as the interactions of osteoclasts and also prevents bone resorption. For this purpose, it is commonly used in formulations for osteoporosis, Paget's disease and hypercalcemia especially in postmenopausal period [3-5].

The biological activity of 1 mg of salmon calcitonin is reported to be 6000 IU (3). It is rapidly absorbed from the nasal mucosa and its bioavailability via this route is approximately 3% (0,3-30,6%) compared to IM administration [6]. The recommended daily dose of sCT is 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 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 the systemic circulation by nasal administration varies between 1000-3400. The bioavailability of these drugs (intranasal formulations) is approximately 10% as compared to the injection forms [9,10].

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

The 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 where as the variability is low. On the contrast, the bioavailability for high molecular weight drugs is relatively low where as the variability is high [12,13].

One of the factors affecting drug penetration through the 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, the contact with the cilia decreases, thereby decreasing the mucociliary clearance. Drug penetration is influenced by the contact duration of the drug to 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) are developed with the aim of increasing the contact duration between the drug and the mucosa. Hence it is also reported that releasing of the mucoadhesive drug can be increased in order 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 utilization. In this regards, 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 a 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 of sCT for hypercalcemic effect [18,19].
In this study, it was aimed that the homogeneous and fluid solgel formulation below room temperature should be viscous enough to adhere to mucus at intranasal temperature. Thus, the sprayed drug will be prevented from flowing into the nasal passage or downwards. In order to research the bioavailability of salmon calcitonin, thermogel nasal formulations in different pH have been examined. It is thought that the temperature-sensitive pharmaceutical form will allow the controlled releasing of sCT. The formulations are designed to convert from solution to gel form at 34-36 ºC, which is the intranasal temperature. The design with the active ingredient that transformed from solution form to gel form was compared with the commercial product. Determination of the bioavailability and *in vivo* studies of sCT were carried out with Enzyme Immunoassay (EIA-ELISA) method [20-23].

Materials and Methods

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

Quantification of sCT using HPLC

The quantification of sCT was carried out using a HPLC system (HP Agilent 1100, ABD) equipped with an injector, quaternary pump, autosampler, column oven and UV detector. sCT contains thirteen different amino acids, but the determination assay uses a single peak to sCT detection. [24].
For the determination of sCT, chromatography parameters such as C18 column (4.6 x 250 mm) (conditioned at ambient temperature), mobile phase that was composed of Mobile phase A (20 mL tetramethylammonium hydroxide (10%), 880 mL distilled water and 100 mL acetonitrile) and Mobile phase B (8 mL tetramethylammonium hydroxide in 392 mL distilled water and 600 mL acetonitrile) were used. Each mobile phase was adjusted with orthophosphoric acid to pH 2.5 (using a pH meter) and degassed for 30 minutes prior to use. Mobile phase was pumped at a flow rate of 1 mL/minute, injection volume of 50 µL and a 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 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 they were 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, 2200 IU / mL sCT was added to the formulations. The composition of the 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 carried out with a calibrated pycnometer with a volume of 5.442 mL. The empty weight of the pycnometer is 14.875 g. The weights of the formulations that filled the pycnometer at 25 °C were measured. At the end of three replicates studies, the ratio of average weight to volume was evaluated as the density of the formulations.

Tonicity studies

The 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 a pipette and placed in the cooling cell. The osmolarities of each formulation were studied in three replicates.

Refractive index measurements

The ATAGO RX 7000α 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. Three replicate measurements were made for each of the formulations and the average refractive indices were observed.

Conductivity measurements

The Mettler Toledo instrument used was first calibrated with a conductivity standard solution. The calibration measurement is recorded as 1413 µS/cm ± 2%. The conductivity of the formulations (prepared in 100 mL) was measured in three replicates in a beaker and the average conductivity of each formulation was evaluated.

Viscosity measurements

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

Evaluation of chromatogram

The formulations were placed in the donor phase as 1 mL (2200 IU/mL) for the transition study. The amount of sCT diluated 5 mL of the receiving phase was. The possible maximum amount for determination would be 2200 IU/5 mL (=440 IU/mL= 73.3 µg/mL). Therefore, formulations containing 75 µg/mL sCT were loaded and analyzed separately in three replicates. 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 repeatability parameter 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 11000 IU sCT in 5 mL. The formulations were kept in capped vials for 3 months at + 4 ºC. The samples were also examined for physicochemical properties at predetermined time intervals (1. day, 1. week, 1. month and 3. month). The concentrations of the formulations were determined by HPLC. Samples to be examined for stability at +4 °C were 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 cellulose membrane (MW:25.000 Da) by diffusion tube method specifically designed for our study. The system consist of two parts: the transmitter and the 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 place in the lower beaker. Formulations (containing approximately 2200 IU/mL sCT) and commercial product were

placed over the membrane as the donor phase. The system was placed in a water bath at 37 ± 0.5 ºC with magnetic stirrer. The receiving phase was mixed with the help of the 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. Fresh pH 7.4 phosphate buffer had been added to the medium as much as the sample taken. The transition studies was carried out in three replicates.

In vivo studies

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

Nasal spray dosimeter evaluation

The metered dose spray systems of the developed formulations were compared with the commercial nasal preparation in terms of spraying amounts (gravimetrically). The sprays were weighed on a scale with a precision of 0.0001g 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 commercial product to examine whether there was a difference in the dose of 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 0-100 ng, the amount of sCT to be determined by EIA in rabbit plasmas should be within this range. In a similar study, when 2000 IU sCT was applied IN to rabbits, sCT was determined between 0-100 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 µg precision to obtain 1000 IU/puff at each spraying, with 11917 µg sCT. That is, the formulations were designed to contain 14300 IU/mL sCT. 10084 µg sCT was weighed and loaded into 5 mL of the commercial product bearing in mind 5 mL of commercial product contains 11000 IU. The formulations prepared were analyzed in HPLC with an injection volume of 10 µL using the same analytical method and their concentrations 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 from the Faculty of Medicine Experimental Animal Production Center. Before the studies, ethics approval was obtained from the Ege University Faculty of Medicine Animal Ethics Committee (2009-95).

The rabbits were divided into four groups: three females and three males in each group. The rabbits were fasted for 24 hours 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 sample was taken from the six rabbits per group at 0 minute, 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, one puff in each nostril of the rabbits. IV administration was carried out into the venous vein of the rabbit's ear using an insulin needle. After the applications, 1 mL of blood samples were taken at 15, 30, 45, 60, 75, 90, 105, 120, 180th minutes. Blood samples were taken with 22 G1 cannulas and put into EDTA tubes. These samples were then centrifuged at 4°C for 15 minutes (at 1600 rpm) and the plasma

separated. The plasma samples were stored at -80 °C for evaluation by enzyme immunoassay (EIA).

Quantification of sCT in rabbit plasma

Enzyme Immunoassay (EIA) Kit was used for assay of sCT. The immunoplate in the ready kit was pre-coated with a secondary antibody and nonspecific binding sites were blocked. The log/logit curve of the optical density versus the concentration is determined with standards of known concentrations. Unknown concentrations are estimated by means of extrapolation. The kits were kept in room temperature before use. The assay buffer included in the kit was diluted with 950 mL of distilled water. Five different concentrations were obtained by diluting with the assay buffer from the 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 rehydrated also by mixing with 5 mL 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 was added to all wells except for the blank, respectively. Plates were covered with acetate plate sealer and incubated for 2 hours at room temperature with 300 cycles on a shaker. At the end of this period, the 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 covered again with acetate. The plates were incubated for one hour 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 µL of 2N HCl to the wells. The plate was analyzed using Thermo Labsystem Multiscan Spectrum at 450 nm.

Bioavailability assessments

The results of all the plasma samples taken from rabbits after nasal administration of the prepared formulations were subjected to pharmacokinetic analysis in comparison to the commercial product administered via IV and nasal route. For pharmacokinetic analysis using WinNonlin Version 5.2, Pharsight Corporation program, the data of each rabbit and the 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 intravenously (iv). The total area below the blood concentration-time curve when the drug is given intravenously (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 (AUC) of the formulation to that of the reference and multiplying by 100 [26,27].

Results

Quantification of sCT concentration using HPLC

The linear equation of the calibration curve was determined as $y=16{,}989x + 30{,}097$ with $R^2= 0.9946$. The sensitivity of the method was evaluated in terms of limit of detection (LOD) (0.276 µg/mL) and the limit of quantification (LOQ) (0.836 µg/mL). The coefficients of variation for repeatability and reproducibility were lower than 2%. Relative standard deviation of both accuracy and precision values were less than 8%. Specific peak of sCT was

detected clearly in an irreproachable manner. The retention time of the peak of the active substance is 14.2 minute as shown in **Figure 1** below.

Preparation and characterization of thermoreversible formulations

The temperature of the nasal mucosa is about 32-34 ºC. The temperature of the entrance part of the nose and nasopharynx is stated to be 31 and 36 ºC, respectively. The temperature increases from the nasal vesticulate to the back. Not any 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.

Density of formulations

Optimum formulation contains approximately 2200 IU/mL sCT. The density, tonicity, refractive indices and conductivity values of the selected formulations (related with F4e and F4d formulations) are shown in **Table 5, 6, 7** and **8** respectively**.** In **Table 5** the standard deviations between repetitiative measurements are quite small, indicating homogeneity in formulations.

Tonicity studies

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

Refractive index measurements

From **Table 7**, the refractive index measurements vary between 1.33 – 1.36. These values suggest the clarity of the prepared formulations.

Conductivity measurements

The conductivity values of optimum formulations were determined in the range of 860 – 1935 µS/cm. Due to the fact that the pH of the formulations varies between pH 3-5 and the quantities LF 127 and PEG 1500 are different, the measured values are not the same. As shown in **Table 8,** the conductivity of the researched formulations increases in turn: 4A> 4B> 3A> 3B> 5A> 5B.

Viscosity measurements

The vibroviscometer used in this study makes measurements using the 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 and with high accuracy/repeatability. Given that measurements can be taken from low viscosity to high viscosity, studies were carried out without the need for many spindles compared to conventional rotational viscometers [32].

Viscosity comparison between formulations kept at 34 ºC (which is the nasal mucosa temperature) is given in **Figure 2**.

Evaluation of chromotogram

The amount 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. The highest RSD was found as 3.31 %. Formulations have been found to be under repeatable experimental conditions. The quantities determined during the analysis (according to FDA and ICH validation criteria) are less than 15% repeatability and are appropriate [35-37].

Stability studies

The stability results of the sCT solution examined at room temperature for 220 minutes are given in **Table 10**. Mucosiliary clearens occur on average every 20 minutes due to nasal application. [38]. 10 consecutive analysis stability assessment processes correspond to approximately 11 mucosilyer clearens. % Ratios of the samples taken at room temperature (based on the value of the first concentration) are shown in **Table 11**.

Table 11 demonstrated that a 10 % of the initial concentration was lost within 120' (min). Based on these results, we limited the period of the *in vitro* studies to 120 minutes.
The short term and monthly stability evaluation of the 3A, 3B and 4A, 4B formulations and the commercial product were carried out at 4 ± 0.5 C and 25 ± 0.5 ºC, respectively. **Figure 3** and **4** compare the stability of various concentrations (with respect to time) at 4 ± 0.5 °C and 25 ± 0.5 ºC, respectively.

In **Figure 3**, it was observed that the commercial product (CP) and the 3A formulation showed similar stability at 4 ± 0.5 ºC at the end of 30 days. Meanwhile, both the commercial product and formulations could not maintain their stability within the 30-day period.
SCT was not detected in formulations and commercial products stored at 40 °C and 75% humidity on the 7th, 15th and 30th days.

In Vitro Release Study results
A graph of cumulative amount of active substance (in the receiving phase of the diffusion experiment setup) vs time is shown in **Figure 5**. The sCT amounts were determined by HPLC. The active ingredient could not be determined in the 5A and 5B formulations during the diffusion studies. Stability problem was encountered due to the degradation of sCT in a short time at pH 5.
The prepared formulations and the commercial product are known to be stable for at least 90 minute. Hence, the sCT amount (expressed as % release ± SD) in the formulations and the commercial product was investigated for a period of 90 minute (**Table 12**). The commercial product was noted to be superior in terms of transition as compared to the prepared formulations: 3A>4A>3B>4B.
Formulations 5A and 5B in pH 7.4 buffer could not be analyzed in any way. This situation was evaluated as a 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, whiles a 3B and 4B formulations were excluded due to gelation at lower temperatures. Furthermore, the sCT diffusion from the 3B and 4B formulations was limited. Numerous trials were conducted using diffusion tube method and high variations were observed in the results of the 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 for the 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 uniformities 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, 2.8416 respectively.
Content uniformity of the investigated formulations
The F test was used to determine whether there was a general difference between the applications. After the F test, groups with differences were determined using the Tukey HSD method. Tukey HSD method was also used to compare the formulations in pairs. Significant differences between formulations are shown with asteriks "***" in **Table 13** below [41].
Spr: The original spray of the commercial product,
CP: Spray device in which the commercial product is placed in order to be compared in the same apparatus

3A: The spray device in which the 3A formulation is placed in order to be compared in the same apparatus
4A: The spray device in which the 4A formulation is placed in order to be compared in the same apparatus

Concentration of the administered formulations

After 2000 IU intranasal administration in immunoassay study, the highest concentration of sCT detected in rabbit plasma was 40 ng/mL [25]. Since the sCT detection range of our EIA kit is 0-100 ng/mL, the formulations were designed to administer 1000 IU/puff to the rabbits. The formulations were prepared by loading of sCT in 14300 IU/mL and 71500 IU/5mL. In order to eliminate any other factors for the sake of accuracy, 11917 µg 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 the dose content of the formulations was supported by analytical method. The dosage of the formulations was selected based on the analytical method.

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

Quantification of sCT in rabbit plasma

The method used for the quantification of sCT in rabbit plasma is ELISA. ELISA is a bioanalytical method in which the 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 sometimes 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 hours. Anesthesia was administered before the application and was continued based on the motility of the rabbits in order to avoid deviations in blood draws. A catheter was inserted in the rabbits so that blood samples could be collected conveniently, accurately and on time after the injection. In cases where blood cannot be obtained from the ear veins during frequent blood withdrawals, blood was taken from the ear arteries rather. 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 the right and left nostrils of the rabbits; and blood samples were taken at the stated time periods. The plasma was separated from the blood and stored at -80 ºC until quantification using EIA.

The pharmacokinetic constants of the iv and intranasal application of the investigated formulations (3A and 4A) and the commercial product are obtained using a program called WinNonlin Version 5.2, Pharsight Corporation. The findings of the pharmacokinetic study using this program are shown in **Table 15** (standard dose application was not applied in practice).

Table 15 compares AUC0→tlast values (the primary pharmacokinetic parameter) of the investigated formulations. Considering that the initial sCT amount in the commercial product is approximately 1.5 times that of the 4A formulation, then the area under the curve will increase in the order: **4A>CP>3A**. Critical parameters were found to be similar and comparable between formulation 4A and CP.

As seen in Table 16, the absolute bioavailability of the intranasal application of the commercial product is 0.856, which is within the range of 0.3-3 stated in the literature (28, 42). The absolute bioavailability of formulation 4A was 0.743, which is very close to the commercial product even though the initial concentration of formulation 4A is 1,5 times less than the commercial product. For 3A formulation, despite the positive results obtained during

in vitro studies, the *in vivo* results (*i.e.,* bioavailability result) was less significant.

Bioavailability of 3A formulation is not in the same level as 4A and CP.

Table 18 contains data and bioavailability results of the applications in terms of primary pharmacokinetic parameter (AUC0 → tlast value).

In **Table 17,** the relative and absolute bioavailability was evaluated based on the secondary pharmacokinetic parameter (AUC0 → ∞).

The data from **Table 17** has revealed that the absolute bioavailability of the commercial product and the 4A formulations are similar: AUC0→∞ value is 0.254 and 0.234, respectively. On the other hand, the bioavailability of 3A formulation is only about 35% of the mentioned bioavailability values (*i.e.,* AUC0→∞ value is 0.157).

Relative and absolute bioavailability assessment based on Cmax was measured in similar way as AUC. The relative/ absolute bioavailability data based on Cmax are presented in **Table 18**. The absolute bioavailability of commercial product administered via nasal route was found to be 1.88 (see **Table 18**) using Cmax 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 (gelling agent) in combination with 2% PEG 1500 were explored**.** Polyethylene glycol 1500 (PEG) is used in the formulation due to its water-solubility and amphibian feature. It is also non-toxic and non-immunogenic. PEG has been approved by the FDA and is used as a carrier in many food, cosmetic and injectable, topical, rectal and nasal applied pharmaceutical products. It also has a penetration enhancing effect. The ratio of PEG1500 used as 2% is to reduce variability in temperature transformation. But 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 which tend to have gelation temperature closer to the lower limit of the nasal mucosa temperature were coded as 3B, 4B, and 5B. These coded formulations are selected for further investigations.

Based on the 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 forms [33,34]. The viscosity of formulations with decreasing temperatures from 40 ºC to 4 ºC was found to be the same as 34 ºC. Moreover, Formulations 3A, 3B and 4A showed slower gelation transformation.

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

During the diffusion studies, sCT was detected in neither the samples nor the commercial product after 24 hours. Also, no sCT amount could be determined in the formulation with pH:5, indicating that the molecule is very unstable in such environment. The stability of the commercially available product in transition and storage conditions was also found as very sensitive to be delicate.

When the results of the transition studies were evaluated, it is seen that the variation between the samples is high. Despite the large number of trials, consistent values could not be obtained even with the same formulation. It can be said that the release in formulations A is more balanced than in formulations B based on their viscosity. Moreover, the release of formulations B was slower than formulations A.

Using the Tukey HSD method, a difference (within 95% confidence interval) was observed between the spray original spray of the commercial product and the spray investigated spray

(intended to be used during the application). By 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 the spray applications there was no difference between formulations and commercial nasal product spray homogeneity at the 95% confidence interval as seen in Table 13. This indicate the reliability of the selected spray device to be used during the bioavailability studies.

The international unit (IU) (used for sCT here) 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 the international reference preparation of calcitonin [1]. Based on this, the spraying volume was taken as 0.14 mL for each nostrils to achieve the targeted single dose of 1000 IU. The standardization of the sCT dose is very complicated due to precision in the weighings as well as slight volume differences in the prepared formulations.

ELISA method is generally a heterogeneous non-competitive application. The primary antibody corresponding to the analyte of interest is usually detected on the multi-well plate or solid plastic surface. The biological sample is dispensed onto the multi-well plate and the detected antibody captures the analyte to be measured. Excess analyte is removed by washing. The antigen-antibody complex is determined with 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 takes place with a specific substrate solution suitable for the enzyme. At this stage, the amount of the colored product is determined spectrophotometrically [23].

The area under the curve (AUC) and Cmax values (main parameters in bioavailability assessment) of the commercial products and the formulations were calculated separately using Winnonlin program after each application to six rabbits. To compensate for the differences between the doses administered to rabbits, the values were calculated based on the doses administered.

The absolute bioavailability of commercial product administered via nasal route was found to be 1.88 (see **Table 18**) using Cmax value as a primary pharmacokinetic parameter. This finding is in agreement with the data presented in literature [38]. Among the developed formulations, the 4A formulation has an absolute and relative bioavailability of 0.99 and 53.3%, respectively: the bioavailability value is half that of the commercial product as expected. This could be explain 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 the commercial product.

Conclusion

In this study, a solgel formulation that is in liquid form at +4 C (storage condition) and gel form at intranasal temperature was developed. When sprayed into the nose, the developed formulation adheres to the mucosa. Taken into account the fact 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. The fluidity of the product is reduced where it is squeezed, allowing the released of active substance. sCT is stable at pH 3-4, and considering that no sCT was detected in formulations with pH:5, it shows that the sCT molecule is very unstable in this pH.

In an *in vitro* release study, formulations 3A and 4A showed faster and more permeation than the commercial product at 10 minutes. This situation can be attributed to the transition of the formulations in the first few minutes. Nevertheless, 3A and the commercial product showed

similar *in vitro* release capability. Overall, formulations A were found to be more stable and have better *in vitro* release capability as compared to formulations B based on their viscosity. The commercial product and the developed formulations were further investigated *in vivo* studies. The standard dose was provided with a spray head suitable for rabbit noses. The pharmacokinetic data has revealed that the commercial product is able to reach the Cmax value in 30 minutes. Meanwhile the tmax value (in EIA analysis) was found to be 0 in two of the six rabbits administered intravaneously, whiles 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 as a result of the analysis method used given that the same problem was encountered in another study [25]. Based on AUC0→t_{last} value (the primary pharmacokinetic parameter), the absolute bioavailability of the commercial product administered via intranasal route was found to be 0.856, which is within the range of 0.3-3 stated in the literature [20,25,42]. The absolute bioavailability value for formulation 4A (*i.e.,* 0.743) was very close to the value of commercial product. As for the 3A formulation, the absolute and relative bioavailability was found to be 0.222 and 25.9%, respectively, which is below the values of the commercial product and the 4A formulation: this result is in contrast with the positive results obtained in the *in vitro* studies. Similar situation was observed using the secondary pharmacokinetic parameter, AUC0→∞: the absolute bioavailability of the commercial product and the 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 the commercial product shows an absolute bioavailability of 1.88 based on the Cmax value (which is 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 a relative bioavailability of 53.3%, half as much as the commercial product. The absolute bioavailability of 0.11 and the relative bioavailability of 5.64% for the 3A formulation is less than the bioavailability values obtained with AUC parameters. This is thought to be due to the slow releaser of sCT from the gel form. The obtained Vd (Virtual volume of distribution) value (from pharmacokinetic data) indicates greater plasma protein binding of the solgel formulations. Post IV and IN administration, the Vd values for the commercial product was much smaller than the values of the developed formulations. Based on this results, it was suggested that bioavailability assessment using pharmacodynamic parameters (other than EIA and RIA method) would be more meaningful during data evaluation.

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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

Table 2. Composition of optimized formulations

Formulation	LF127 % w/v	PEG1500 % 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**	+	+	+

Table 3. Concentrations of standart solutions for ELISA determination

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

Table 4. Gelling temperatures of various gel formulations

Formulation	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

Formulation	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

Table 6. Tonicity measurements of formulations

Formulation	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

Table 7. Measurement of refractive indices

Formulation	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

Table 8. Conductivity measurements of formulations

Formulation	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

Table 9. Concentrations of the investigated formulations

Formulation	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

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**
100	117.3	115.1	113.2	111.8	109.8	108.2	105.0	102.3	98.3	93.3	107.4	7.6

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

ND : Not determined*

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	

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

Formulation	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

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

Parameter	CP (iv)	CP (in)	3A	4A
t1/2 (min)	308.9±399.8	84.3±121.5	227.4±148	75.7±49.6
tmax (min)	30±30	75±15	105±68.4	57.5±33.4
Cmax(ng/mL)	17.4±21.5	87.4±95.1	4.8±2.1	37.3±75.6
AUC 0→tlast (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

Table 16. Relative and absolute bioavailability data based on AUC0 → tlast values

	AUC 0→tlast (min*ng/mL)	AUC0→tlast /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

Table 17. Relative and absolute bioavailability data based on AUC0 → ∞ values

	AUC0→∞	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

Table 18. Relative and absolute bioavailability data based on Cmax values

	Cmax	Cmax/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

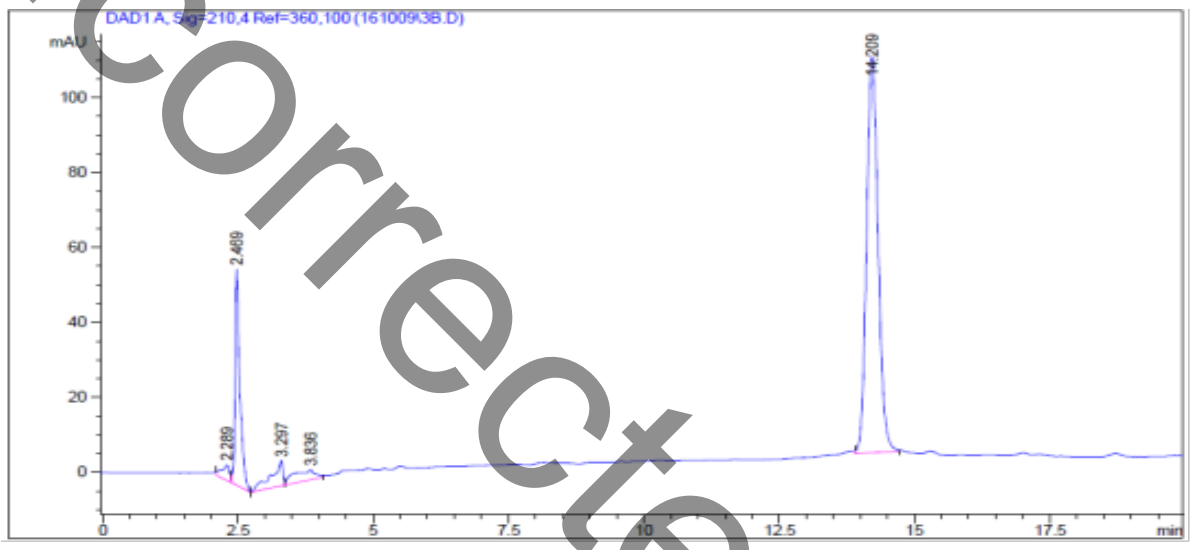

Fig 1. Chromatogram of sCT

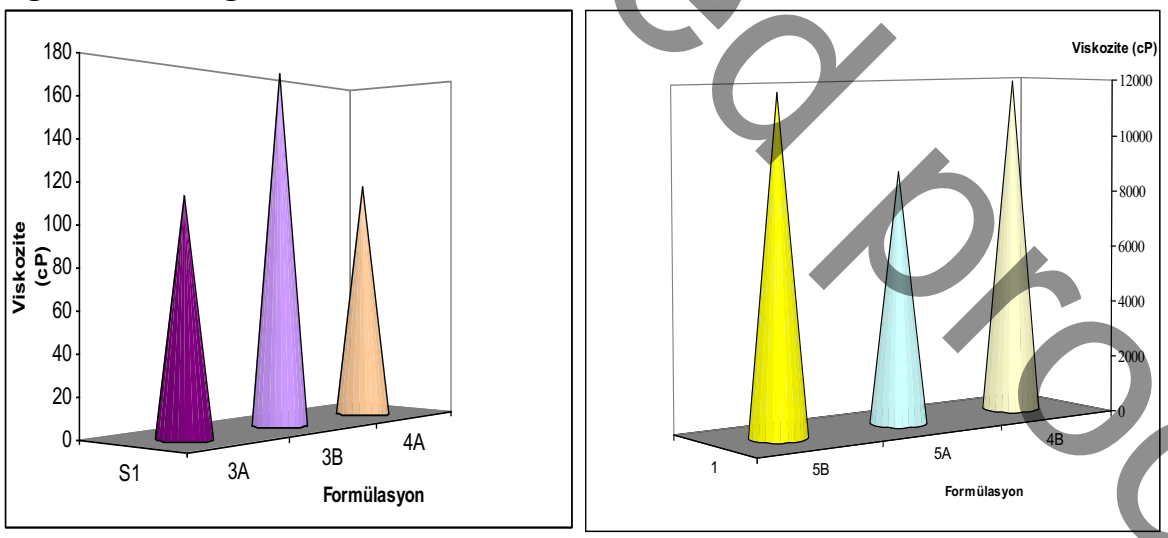

Fig 2. Viscosity measurements of various formulations

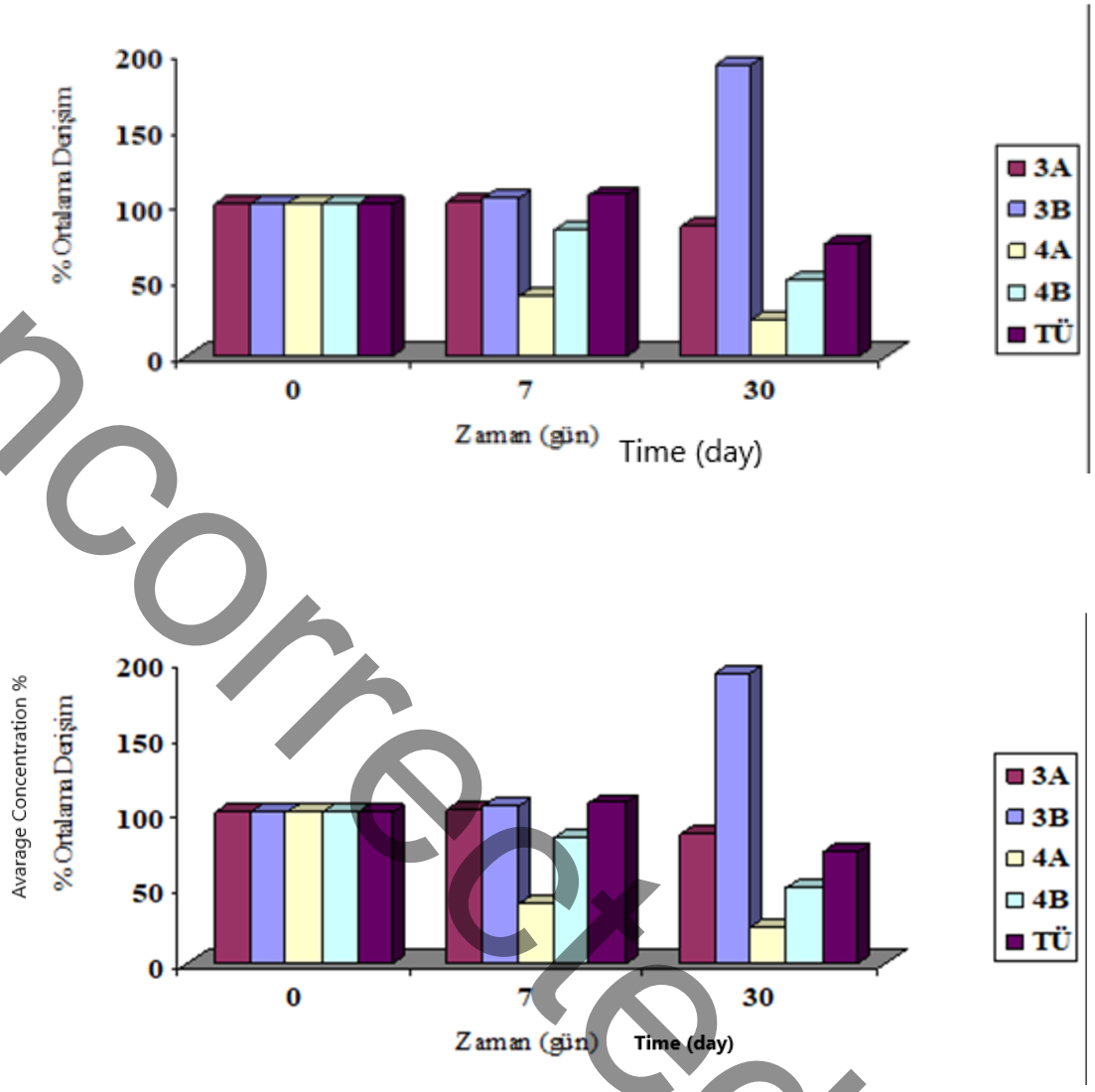

Fig 3. Stability results in terms of mean % concentration vs time (day) at 4 ± 0.5 ºC.

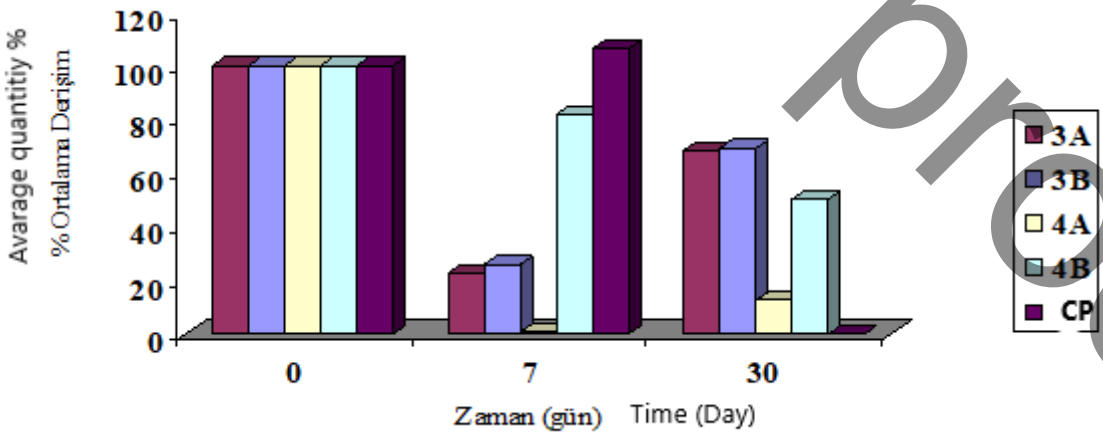

Fig 4. Stability results in terms of mean % concentration vs time (day) at 25 ± 0.5 ºC.

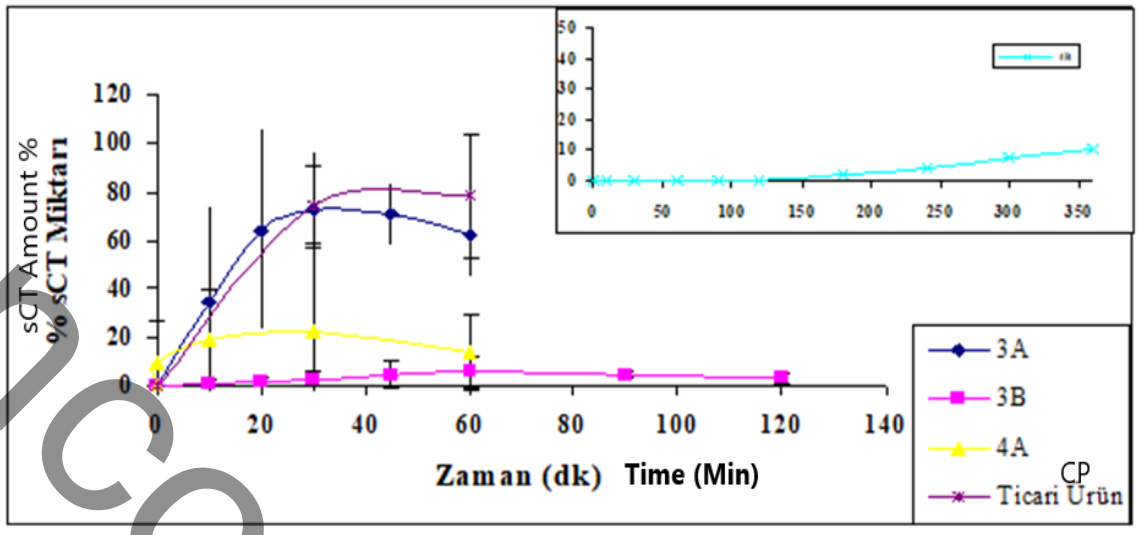

Fig 5. % sCT release versus time (min)