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Edetate calcium disodium nanoparticle dry powder inhalation: A novel approach against heavy metal decorporation

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ABSTRACT

Objective was to develop and characterize nano-edetate calcium disodium (Ca-Na_2EDTA) dry powder inhaler (DPI), and assess its in vitro and in vivo deposition using pharmacoscintigraphy techniques. Factors influencing nanoparticle formation including concentration of drug, polymer solution and stirring rate were determined. Optimized formulation was characterized with the help of SEM, TEM and Malvern Zetasizer studies. Any change in physical characteristics after nanosizing was determined by FT-IR, XRD and DSC studies. Anderson cascade impaction showed that nano Ca-Na_2EDTA exhibited significantly higher respirable fraction of $67.35 \pm 2.27\%$ and $66.40 \pm 2.87\%$ by scintigraphic and spectroscopic analysis respectively, as compared to $10.08 \pm 1.17\%$ and $9.36 \pm 1.02\%$ respectively for micronized form. Ventilation lung scintigraphy done in 12 volunteers showed significant increase in drug delivery till alveolar region with nano Ca-Na_2EDTA. The developed formulation may have a role in neutralizing heavy metal toxicity through inhalation route, including radio-metal contamination.

1. Introduction

Heavy metal poisoning remains a widespread problem in many of the industrial nations (Grandjean, 2010; Halatek et al., 2009). Exposure to these toxicants is not only confined to occupational workers but also to general population at large (Passos and Mergler, 2008). A phenomenal increase in the use of radionuclides in healthcare, food processing and energy production has greatly increased the vulnerability to radionuclide accidents, such as that seen in Japan recently. Another major concern with respect to heavy metal toxicity that cannot be overlooked is the increased threat perception of a radiological device being used by rouge states or terrorist organizations that may lead to significant radio-metal contamination of the affected population, mainly through inhalation and ingestion. The challenge of removing such contaminants from the body assumes greater significance considering that in vivo radio-metal deposition even in miniscule quantities may impart significant radiation dose to body's target organs depending upon their half-lives.

Although use of chelating agents against metal toxicity was employed decades back, introduction of ethylene diamine tetraacetic acid (EDTA) as a chelator is considered as a breakthrough in chelation therapy (Kalia and Flora, 2005). Use of EDTA and other chelating agents is the generally accepted treatment for lead and other heavy metal poisoning (James et al., 2007; Henge-Napoli et al., 2000; Goyer, 1995). However, clinical utility of EDTA and related member of the family, diethylene triamine pentaacetic acid (DTPA) has been severely compromised by the need to administer them intravenously through slow infusion.

As already mentioned, inhalation is a major route through which heavy metal toxicity can occur. Therefore it seemed logical to us to have a formulation that traps the metal at the portal of entry so that it can be excreted without absorption, thereby helping in minimizing its toxicity. Aim of the present study was therefore to provide an easy and effective chelation therapy against heavy metal toxicity using Ca-Na_2EDTA dry powder inhalation (DPI). In order to reach deeply into the lung parenchyma and further facilitate systemic absorption of a drug, dry powder aerosols need to be in submicron range and should be deposited in sufficient quantity for sustained action (Bhavna et al., 2009; Shekunov et al., 2007). In the present study we have made nanoparticles of Ca-Na_2EDTA for dry powder inhalation, characterized them using various analytical tools including in vivo lung penetration tests, and compared it with commercially available micronized powder.

One more highlight of the study is the use of gamma scintigraphy in development and evaluation of the present formulation; a tech-

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nique which we have successfully used in the development of a few of our other drug formulations including DPIs (Rajpal et al., 2010; Ali et al., 2009; Bhavna et al., 2009; Rajpal et al., 2009). We quantified total and regional lung deposition of inhaled Ca-Na_2EDTA DPI after radiolabeling it with Technetium-99m (Tc-99m). In vitro as well as human studies suggest significant deposition of the chelator at intended sites using the developed formulation.

2. Materials and methods

2.1. Reagents and chemicals

Calcium-disodium ethylenediamine tetraacetic acid (Ca-Na_2EDTA) was procured from Sigma Chemical Company, St. Louis, MO, USA, while all other chemicals and reagents were purchased from Merck India Ltd., Mumbai. Technetium-99 m pertechnetate was obtained from BRIT, BARC, India.

2.2. Nanoparticles preparation by precipitation technique

Nano Ca-Na_2EDTA was prepared by nanoprecipitation method. Ca-Na_2EDTA was dissolved in polyvinyl alcohol (PVA) solution at a definite concentration, and the solution was poured into water-immiscible non-solvent (Isopropyl alcohol) under continuous stirring till a cloudy suspension was formed. Precipitation was formed immediately upon mixing. The effect of process parameters, such as stirring rate, amount of drug, PVA concentration as well as method of drying (spray drying vs. vacuum rotary evaporator) on the properties of formed particles, particularly size and stability, were investigated. The freshly formed nanoparticles were dried under vacuum at 50 °C and characterized.

2.3. Characterization of Ca-Na_2EDTA nanoparticles

2.3.1. Particle size, morphology, and particle size distribution

The optimized nanoparticles of Ca-Na_2EDTA were examined by SEM (Leo 435VP; Cambridge, United Kingdom) for determining the surface morphology and particle size. Samples were fixed and coated under vacuum with gold in a Balzers SCD020 sputter coater unit (BAL-TEC GmbH, Witten, Germany) in an argon atmosphere at 50 mA for 100 s.

Morphological analysis of the Ca-Na_2EDTA nanoparticles was also performed using transmission electron microscopy (TEM, Philips CM-10, and USA). Samples of the nanoparticles suspension (5–10 μl) were dropped onto Formvar-coated copper grids. After complete drying, the samples were stained using 2% (w/v) phosphor tungstic acid. Digital Micrograph and Soft Imaging Viewer software was used for image capture and analysis, including particle sizing.

Particle size distribution was measured using photon correlation spectroscopy (Zetasizer, HAS 3000; Malvern Instruments, Malvern, United Kingdom). The dispersions were diluted with acetone and measured in a cuvette taking into account the refractory index of the dispersion medium. Each analysis was performed in triplicate.

2.3.2. Fourier transform infrared spectrometry (FT-IR)

FT-IR spectra were recorded with an FT-IR spectrometer (JASCO, Easton, Maryland) in the range 370–4000 cm^{-1} using a resolution of 4 cm^{-1} and 10 scans so as to evaluate the molecular states of commercially available crude Ca-Na_2EDTA and the optimized nano Ca-Na_2EDTA formulation. Samples were diluted by mixing with KBr powder and pressed to obtain self-supporting discs.

2.3.3. X-ray powder diffraction (XRD) analysis

The physical states of unprocessed drug and optimized nano Ca-Na_2EDTA formulation were characterized to assess the crystallinity of the formulation. Phase identification was conducted using an X-ray diffractometer (Shimadzu XRD-6000, Shimadzu, Kyoto, Japan) with CuKα radiation at a scanning speed of 0.05° per minute.

2.3.4. Differential scanning calorimetry (DSC)

The phase transition of unprocessed and processed drug was analyzed by DSC (Pyris 6; PerkinElmer, Massachusetts) at a heating rate of 10 °C per minute from 30 to 190 °C. A dry nitrogen purge of 27 ml/min was used in the process.

2.4. Radiolabeling of nano Ca-Na_2EDTA

The surface of particulate Ca-Na_2EDTA was covalently complexed with Tc-99m using stannous based reduction technology developed from our laboratory (Mittal et al., 2008). Radiolabeling efficiency of Ca-Na_2EDTA with Tc-99m was characterized on the basis of serum and saline stability up to 24 h as per previously reported methods using a gamma counter (Capintec, USA) (Bhavna et al., 2009; Singh et al., 2003). Radiolabeled Ca-Na_2EDTA (micronized as well as nanosized) was filled into respective HPMC capsules after passing through 200# mesh (micronized) or 400# mesh (nanosized) for further studies.

2.5. In vitro analysis using Anderson Cascade Impactor

Five capsules of ^{99m}Tc-Ca-Na_2EDTA (each containing equivalent of 15 mg Ca-Na_2EDTA) were run through Anderson Cascade Impactor (ACI) (Copley Scientific, Nottingham, UK) to study aerodynamic particle deposition in vitro. Respirable fractions of nano and micronized Ca-Na_2EDTA were calculated and compared using scintigraphy as well as UV spectroscopy. The ACI consisted of initiation port (IP) and the preseparator (PS), seven stages and a final collection filter. The stages were coated with polypropylene glycol dissolved in isooctane (0.5%, w/w). The stages were left to dry under ambient room conditions for 30 min. Experiments were conducted at an air flow rate of 60 l min^{-1} for 5 s. After actuation, the wash solutions from different parts were collected and quantitated for drug content by scintigraphy and UV spectroscopy at 243 nm. Respirable particle fraction (RF) and emission dose (ED) were calculated to describe the inhalation properties of DPIs. The measurements were performed in three replicates.

2.6. In vivo gamma scintigraphy

A total of twelve healthy volunteers were recruited for ventilation scintigraphy study (mean age 35 years, 22–54 years). The clinical protocol was approved by the institutional Human Ethics Committee (Reg. No. INM/TS/IEC/008/07). Prior to dosing, subjects were trained to inhale dummy DPIs deeply and to retain the breath following deep inspiration for 10 s. Vital signs were recorded before and 30 min after each dose and before discharging the subject from the study centre. Adverse events were monitored throughout the study.

The studies were conducted on a dual head gamma camera system (Symbia T2, Siemens, Erlangen, Germany). A single DPI capsule containing freshly prepared nano Ca-Na_2EDTA (15 mg) mixed with tracer quantity of Tc-99m labeled nano Ca-Na_2EDTA (1.5-2MBq) was given to each volunteer for inhalation with the help of a standard, commercially available rotacap device after obtaining radioactivity count rate of the DPI capsule on the gamma camera. Immediately upon inhalation (5 min), a two-dimensional static scintigraphy image of the chest region was obtained for 300 s covering the highest and lowest point of radioactivity distribution

(oropharynx to stomach). Count rate of the used capsule and Rotacap device was noted. Additional images of the chest were taken at 2 and 4 h. This was done to observe the movement of radioactivity, if any. All images were recorded on a computer system assisted with the software Entegra Version-2. For comparison, the same volunteers were made to inhale micronized Ca-Na_2EDTA (15 mg) mixed with tracer quantity of Tc-99m labeled micronized Ca-Na_2EDTA (1.5-2MBq) on another day and the scintigraphy procedure was repeated as described above. Mixing tracer quantity of 99mTc-labeled Ca-Na_2EDTA with un-labeled Ca-Na_2EDTA particles in the DPI formulations was done on the basis of isotope dilution principle which states that the flow and distribution of radiolabeled compound with non-radiolabeled compound follows same pattern as parent molecule/formulation within the body and/or biological membrane (De Hevesy, 1944; Fassett, 1995). This also helped in minimizing unwarranted radiation exposure to the volunteers.

2.6.1. Scan analysis

Images acquired on the same time scale ensured that the count statistics comparison between different scans were valid. Region-of-Interest was drawn around the oropharynx, esophagus, stomach, and whole lung for obtaining count statistics. The lung region was again subdivided into central, intermediate and peripheral sections which translate predominantly into respiratory tree, mixed and alveolar region, respectively (Newman et al., 1989). Since swallowing action continuously transports radioactivity deposited in oropharynx into stomach, counts deposited in these two organs were integrated to represent a single compartment. Respiratory fraction, the fractions of radiolabeled drug deposited in the central, intermediate and peripheral lung, was calculated in the initial and subsequent images. Visual comparison between the lung images was done to record movement of the deposited drug with time from one compartment to another.

2.7. Statistical analysis

Data are expressed as mean ± SD. Unpaired *t*-test was applied for the calculation of significance at $p < 0.05$ using GraphPad Instat version 3.00 for Windows XP, GraphPad Software, San Diego, California, USA.

3. Results

Nanoparticles of Ca-Na_2EDTA in the optimum range were developed after parameter standardization using precipitation method. Different methods were used for drying the preparation. Spray drying method resulted in average particle size in the range of 100–300 nm, but the yield achieved by this technique was very low (1–5%). Rotary evaporator method was eventually used, which gave the average particle size in the desired range of 200–400 nm with a yield of 95–99%.

3.1. Factors influencing nanoparticle formation of Ca-Na_2EDTA

At higher and lower drug concentration on both sides of a narrow window of drug solution concentration, no fine particles were formed (window: 0.1–1.0 g/ml) (Table 1). Below 0.1 g/ml no particles were formed, whereas a sticky mass resulted at the higher end of the window. Similarly, 1% concentration of the PVA solution was found to be optimum, where fine particles of desired size range were formed (Table 2). The viscosity increased with the increasing drug/PVA concentration, hindering diffusion between solution and non-solvent.

Table 3 shows the effect of stirring rate on size distribution of particles. The size of the Ca Na_2EDTA particles formed by nanoprecipitation was inversely related to stirring rate. At 600 rpm the mean size of particles was 5.2 μm, whereas it was reduced to 0.32 μm at 1400 rpm. High stirring rates seemed to enhance the mass transfer and rate of diffusion between the multiphase, which induced high homogeneous supersaturation and rapid nucleation to produce smaller drug particles. Moreover, there seems to be a threshold speed above which any further increase in the stirring speed has no influence on the particle size.

Table 1
Effect of Ca-Na_2EDTA concentration on size of particle.

$CaNa_2$EDTA concentration (g/ml) in 1% polymer solution	Mean particle size[a] (PSD) (μm)	
	600 (rpm)	1400 (rpm)
1.5	Sticky mass	Sticky mass
1.0	10.0 (6.5–16)	Sticky mass
0.5	4.0 (6.2–3.0)	0.62 (0.9–0.5)
0.1	0.9 (1.2–0.5)	0.35 (0.2–0.4)
0.05	Clear solution	Clear solution

[a] Mean particle size results of three determinations from three different batches as determined by Zetasizer.

3.2. Bulk characterization

3.2.1. Particle size, morphology, and particle size distribution

The particle size, morphology, and particle size distribution of optimized formulation were evaluated by SEM, TEM, and Malvern Zetasizer. The SEM and TEM images of Ca-Na_2EDTA nanoparticles are shown in Fig. 1. Photomicrographs of the formulation suggested that nano Ca-Na_2EDTA particles were evenly round in shape with mean particle size of 350 nm (≥20% particles below then 100 nm), as compared to 3–4 μm in case of micronized Ca-Na_2EDTA.

Particle size distribution (PSD) of nano Ca-Na_2EDTA measured on a Malvern Zetasizer is shown in Fig. 2. Mean particle size obtained was about 350 nm. Commercially available micro Ca-Na_2EDTA had a significantly higher mean particle size (3–4 μm; $P < 0.05$).

3.2.2. FT-IR analysis

The spectra of the two particle types were found to be similar as shown in Fig. 3A. Close agreement between the spectra of micro Ca-Na_2EDTA and nano Ca-Na_2EDTA suggests that there were no gross changes in the structure of Ca-Na_2EDTA induced by the nanoprecipitation process.

3.2.3. XRD analysis

The XRD patterns are shown in Fig. 3B. The pattern illustrates that the peak positions of nanosized sample were same as that of the commercially available micronized Ca-Na_2EDTA, suggesting that nanoprecipitation did not affect the physical characteristics of Ca-Na_2EDTA. Moreover, peaks of nanosized Ca-Na_2EDTA had lower intensities as compared to those of the micronized one,

Table 2
Effect of polymer concentration on size of particle.

Concentration of PVA solution (%)	Mean particle size[a] (PSD) (μm)	
	600 rpm	1400 rpm
15.0	Sticky mass	Sticky mass
10.0	13 (11.5–17)	Sticky mass
5.0	4 (5.2–3.0)	0.62 (0.4–0.8)
1.0	1.2 (1.8–0.55)	0.34 (0.2–0.4)
0.5	Clear solution	Clear solution

Ca Na_2EDTA concentration: 0.1 g/ml.
[a] Mean particle size results of three determinations from three different batches as determined by Zetasizer.

Table 3
Effect of stirring speed on size of particle.

Parameter	Stirring speed (rpm)				
	600	800	1000	1200	1400
Observation	Micronized with needle shape fiber	Micronized	Become micronized on settling	Nanosized	Nanosized
Particle size range (μm)	2–10	1–4	0.5–2	0.1–0.6	0.08–0.5
Mean particle size[a] (μm)	5.2	2.87	1.16	0.45	0.32

Ca Na_2EDTA concentration: 0.1 g/ml in 1% polymer solution.
[a] Mean particle size results of three determinations from three different batches as determined by Zetasizer.

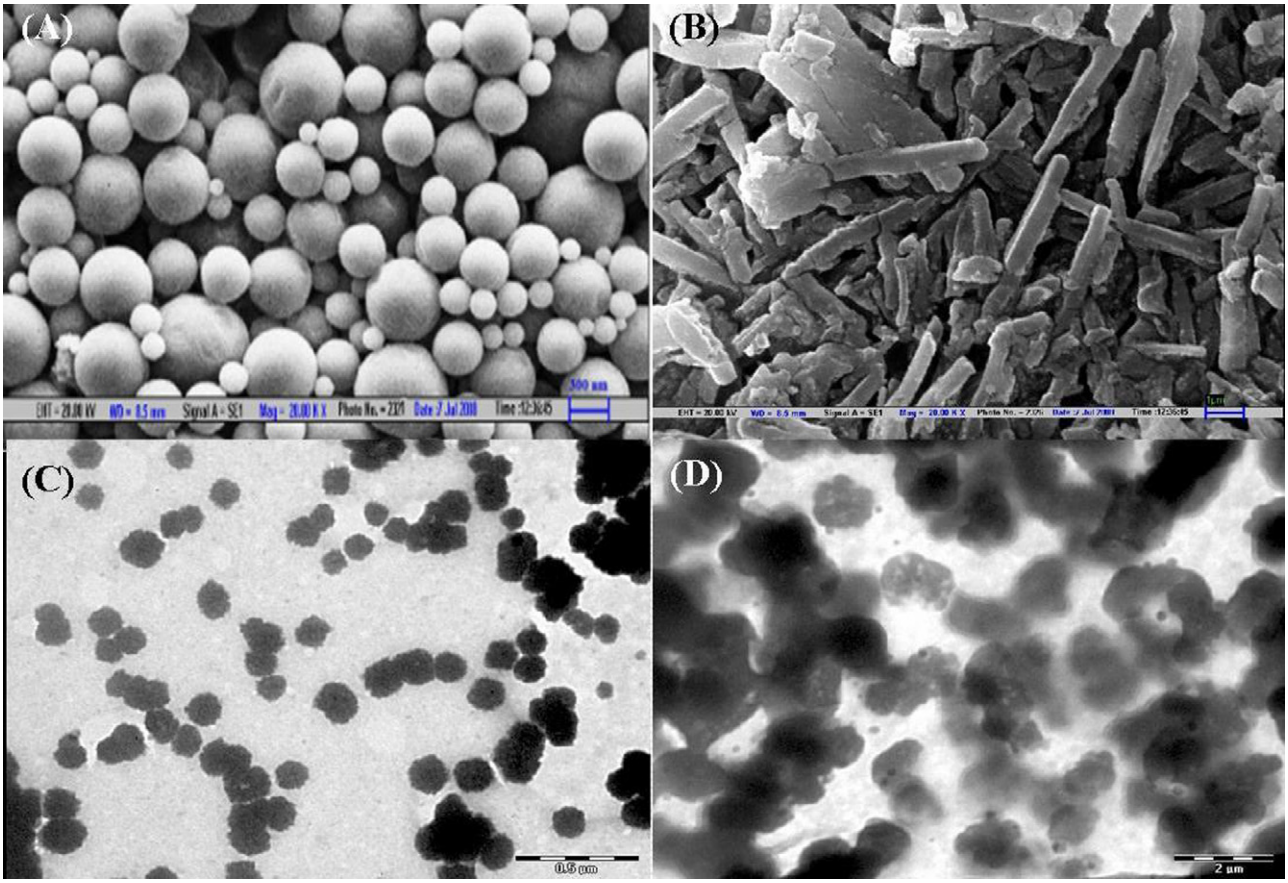

Fig. 1. SEM photograph of (A) nano Ca-Na_2EDTA, (B) micronized Ca-Na_2EDTA; TEM photograph of (C) nano Ca-Na_2EDTA, (D) micronized Ca-Na_2EDTA.

indicating higher crystallinity in crude Ca-Na_2EDTA. Since pharmaceuticals having lower crystallinity often result in higher solubility and bioavailability (Sarkari et al., 2002) decreased crystallinity in nano Ca-Na_2EDTA is expected to enhance its solubility and bioavailability.

3.2.4. DSC analysis

In order to further confirm the physical state DSC was performed, the results of which are shown in Fig. 3C. The overlapping spectra suggest that nano Ca-Na_2EDTA has largely the same form as the parent micronized Ca-Na_2EDTA with no change in melt-

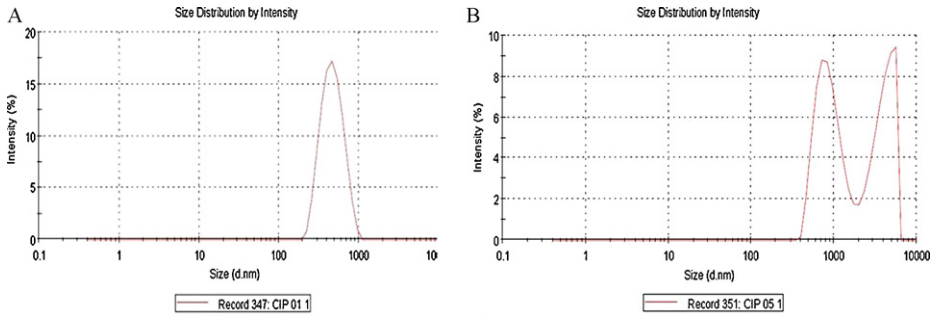

Fig. 2. Particle size distribution by Malvern Zetasizer (A) nano Ca-Na_2EDTA and (B) micro Ca-Na_2EDTA.

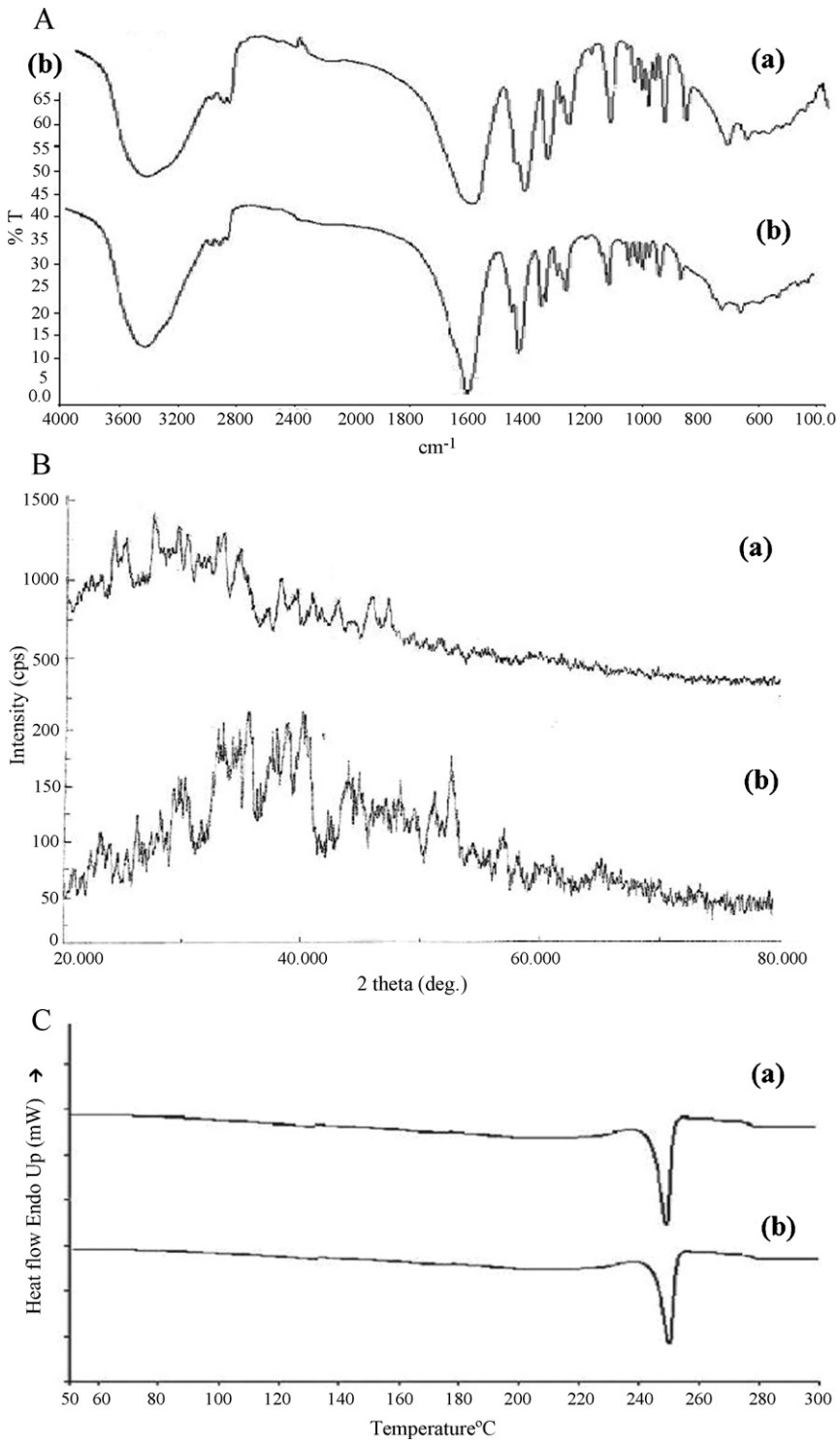

Fig. 3. Characterization of nano Ca-Na_2EDTA through (A) FT-IR, (B) XRD and (C) DCS thermo grams and its comparison with micronized Ca-Na_2EDTA, where 'a' represents micronized drug and 'b' represents nanosized drug.

ing point, indicating that there was no apparent addition of any impurity during the manufacturing process.

3.3. Radiolabeling and stability of nano Ca-Na_2EDTA

Ca-Na_2EDTA was covalently complexed with Tc-99m using stannous based reduction technology developed from our laboratory (Mittal et al., 2008). The radiolabeling efficiency for nano as well as micronized Ca-Na_2EDTA at the time of complexation was consistently >92%. Serum stability studies showed 95% and 94% radiolabeling efficiency of Ca-Na_2EDTA after 6 and 24 h respectively, indicating the stability of the labeled product (Fig. 4).

3.4. In vitro analysis using Anderson Cascade Impactor

Comparative results of % drug deposition pattern of micronized and nano Ca-Na_2EDTA on ACI are shown in Table 4. Nano Ca-Na_2EDTA had respiratory fraction of 66.5% as compared to 10.23%

Table 4
Andersen cascade impactor (ACI) results for Ca-Na_2EDTA DPI formulations measured using an air flow rate of 60 l min^{-1} (mean ± S.D., $n = 6$).

Particle size attributes	Nano Ca-Na_2EDTA		Micro Ca-Na_2EDTA	
	Scintigraphy	UV	Scintigraphy	UV
Emitted dose (%)	96.30 ± 1.24	95.12 ± 1.52	91.31 ± 1.72	90.29 ± 1.58
Respiratory Fraction (%)	67.35 ± 2.27	66.40 ± 2.87	10.08 ± 1.17	9.36 ± 1.02
Total recovery (%)	97.92 ± 1.41	97.22 ± 1.42	96.68 ± 3.43	96.02 ± 2.34

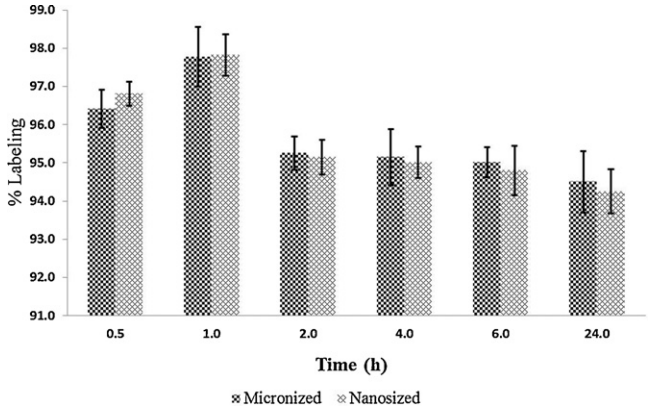

Fig. 4. Serum stability study to ascertain radiolabeling efficiency of Ca-Na_2EDTA particles with technetium-99 m at different time intervals.

for the micronized drug. The mean emitted dose for micronized Ca-Na_2EDTA was 90.29 and 91.31% by UV and scintigraphy methods respectively, while for the nanosized formulation it was 95.12 and 96.3% respectively. Mean total recovery for micronized and nanosized drug was 96 and 98% respectively by both analytical methods.

3.5. *In vivo gamma scintigraphy*

No abnormal symptoms were observed in the subjects during and after the study. The inhalations were well tolerated by the subjects. Ventilation lung scintigraphy in healthy human volunteers done just after inhalation (5 min) of nano Ca-Na_2EDTA and micronized Ca-Na_2EDTA on different days showed significant increase in delivery of the drug in lungs till the alveolar region with nano-formulation in comparison to the micronized one (Fig. 5).

Serial images of the lung field showed proximal movement of radioactivity deposited in lungs through the tracheo-bronchial tree. A semi quantitative analysis was done by making separate region of interest (ROI) on area showing uptake (Lung, esophagus and gastrointestinal tract). The lungs were further divided into peripheral (alveolar region), intermediate (small air ways) and proximal regions (larger airways). The uptake ratios were obtained for peripheral, intermediate and proximal regions vs total lung (i.e. activity deposited in whole lung). The initial image at 5 min showed greater deposition of nano Ca-Na_2EDTA in lung parenchyma, especially in the peripheral region, as compared to micronized Ca-Na_2EDTA. Mathematically, deposition of inhaled drug at 5 min interval was 23, 38.7 and 38.3% respectively for peripheral, intermediate and proximal regions in case of nano Ca-Na_2EDTA, while it was 17.3, 45 and 37.7% for the micronized drug. Images taken 4 h post-inhalation showed movement of the radiolabeled drug from proximal airways upward and then to the GIT due to physiological mucociliary movement. At 4 h, percentage of inhaled drug in peripheral, intermediate and proximal compartments of the lung was 2, 3.4 and 2.6% for nano Ca-Na_2EDTA, while it was 0.47, 0.64 and 0.59% for the micronized form. No radioactivity was seen in the lungs at 24 h in case of micronized Ca-Na_2EDTA, whereas 0.24, 0.31 and 0.27% deposition of nanoformulation was seen in peripheral, intermediate and proximal regions respectively. The scintigraphic scans suggested reverse movement of the drug from the periphery (alveolar region) to the gastrointestinal tract (GIT) through the tracheo-bronchial region and oropharynx by

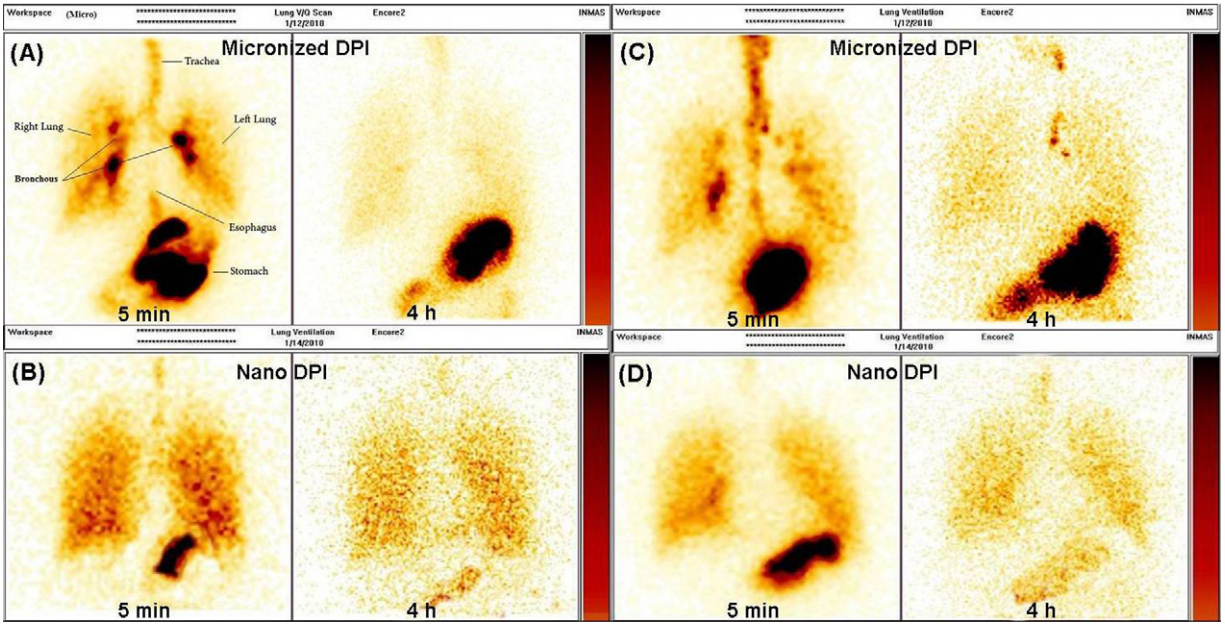

Fig. 5. Human scintigraphy images of Tc-99m labeled micronized/nano Ca-Na_2EDTA in two healthy volunteers at 5 min and 4 h showing distribution of the drug into oral cavity, tracheobronchial tree, lungs and stomach. While (A) and (C) represents distribution of micronized drug in the 2 volunteers, images (B) and (D) represent distribution of nanosized drug in the same 2 volunteers on a different day.

normal physiological route. From the values of amount of drug deposited in different compartments of the lung given above, as well as from qualitative assessment of the scintigraphy scans, it was found that on an average radioactivity in the alveolar and tracheo-bronchial region of each volunteer decreased in the range of 80–120% in 4 h after DPI administration due to movement of Ca-Na_2EDTA from lungs to GIT. Radioactivity was also seen in the blood pool from the early images with a rising trend suggesting that a fraction of the drug was directly transferred across the alveolar membrane to the systemic circulation, more so in the case of nanoformulation.

4. Discussion

This work successfully describes the parameter optimization to obtain Ca-Na_2EDTA nanoparticles through precipitation method, characterization of nano Ca-Na_2EDTA DPI formulation in comparison to the parent micronized form, followed by ventilation scintigraphy in healthy human volunteers. The study is probably the first wherein a DPI antidote formulation of Ca-Na_2EDTA has been made and characterized. The study did not aim to evaluate the therapeutic potential of developed formulation at this stage, but rather focused on its per cent deposition in lungs to counter inhaled heavy metal toxicity. The results of this study shall help in dose-fixation and subsequently form the basis of initiating phase-1 trials of the formulation.

Nano Ca-Na_2EDTA was developed with characteristics favouring deeper penetration and retention in the lung spaces. Another important characteristic was the significant improvement in respirable fraction with the nanoformulation in favour of better lung deposition as compared with the micronized one. This observation is consistent with our findings for some of the other nanoformulations developed from our laboratory (Ali et al., 2009; Bhavna et al., 2009) and seems to be due to (a) reduction in size and (b) difference in shape of the nanoformulation, which is evenly spherical as a result of precipitation process, as compared to irregular shape of the micronized forms resulting from machining or grinding processes. Although many approaches have been attempted to produce nanosized drug powders (Dateand and Patravale, 2004; Pathak et al., 2004; Liversidge et al., 2003; Chattopadhyay and Gupta, 2001) we have used the precipitation technique, a commonly practiced method for preparation of nanoparticles with the advantages of lower operating temperature and higher product yield and stability (Ali et al., 2009; Rasenack et al., 2003). The DPI capsules in this study were freshly made. Although excipients like micronized lactose have been used to deliver optimum doses of nanoparticle DPI formulations and to prevent exhalation due to small particle size, our in vitro experiments along with scintigraphy data in all human volunteers showed fairly good retention of the formulation in different lung compartments. Use of any carrier system was also compromised because of the need for having a higher therapeutic dose of the drug (15 mg), and would have warranted an addition of much higher amounts of carrier to the DPI formulation than generally used, making it a slightly impractical solution. We are now working on the stability profile of the formulation, because our data have indicated water retention in the formulation in humid conditions. Particle sizing experiments at present suggest that the product is stable for 4 months (data not shown) though the manufacturing and storage conditions can have a significant bearing on the shelf-life.

Medical management of heavy metal toxicity has been a cause of concern in most industrial nations. The concern is more so because of the increased threat perception of a radiological device being used by rouge states or terrorist organizations that may lead to significant radio-metal contamination of the affected population, mainly through inhalation. It is estimated that in a radioactive scenario, a major portion of radioactivity incorporated in the human body shall originally come from inhalation route. It is therefore very important to have a formulation that can bind to the metal ions at the point of entry only so that they can be excreted out of the body without giving them an opportunity to accumulate into their respective target organs.

Apart from neutralizing at the local level, inhalation of nano Ca-Na_2EDTA DPI may also be considered for delivering the drug for systemic action. That respiratory route offers a means of rapid bioavailability is well described in the literature for many of the drugs, primarily due to extremely large surface area and thin epithelium barrier of the lungs (Ugwoke et al., 2005; Türker et al., 2004). We have earlier shown that size of a drug aerosol is the most important factor determining the biodistribution of a drug after inhalation, because smaller size ensures deeper penetration whereas larger particles tend to deposit in the proximal respiratory conduction pathway (Ali et al., 2009; Bhavna et al., 2009). Ventilation scintigraphy of nano Ca-Na_2EDTA DPI corroborated our earlier findings. It may be noted that carrier particles such as lactose in a DPI formulation have been suggested to form agglomerates with the drug, which deposit in the airways according to aerodynamic properties of the agglomerates rather than that of primary drug particles (Podczeck, 1998). In our study it is possible that not using any carrier has also led to a higher respirable fraction and deeper lung penetration. Although no attempt was made to carry out a pharmacokinetic study, there is sufficient literature to suggest that submicron-sized particles have a higher likelihood of biotransfer to blood through the pulmonary membrane as compared to microcolloids (which tend to deposit in the upper respiratory pathways), and molecular or gaseous drugs (which reach the alveoli but tend to be expired back in substantial quantity) (Ali et al., 2009; Bhavna et al., 2009).

Since pulmonary absorption is rapid, it will provide a faster onset of action as compared to oral and intramuscular dosing, apart from being one of the easiest routes of drug delivery for the victims or the rescue team entering a contaminated zone, including radioactive scenarios. This may circumvent the need for an intravenous administration of the drug, one of the major limitations of chelation therapy at present. Moreover, pulmonary drug delivery is needle-free, patient friendly, and painless. Between the fluid nebulization and DPI technologies, DPI is more suitable for emergency field use because it is easy to operate and does not require electricity or complicated delivery devices. Additionally, DPI allows for fast self-administration and mass use, an advantage not available with intravenous administration.

Despite proven safety and known clinical utility of the inhalation route, formulations for systemic action have only been marginally successful. This is because alveolar delivery of the inhaled drugs is only a small fraction of the internalized drug, typically 5–10%, whether one uses wet nebulization or DPI technology using microcolloidal drugs. This results in wastage, and more importantly, delayed absorption through the gut route. If higher therapeutic concentration is desired in the initial phase with microsized drug by inhalation route, this may result in toxicity. By enhancing respiratory fraction and deep tissue penetration, which become feasible by using nanoparticles, it is possible to optimize the inhalation route for faster systemic drug delivery. The nanoparticles should preferably be of relatively larger size to escape expiration, which is likely to increase with reducing size of the inhaled nanoparticles. Alternatively, the developmental process should include steps to enhance interaction of the nanosized drug particles with the alveolar membrane, particularly the epithelium.

Our findings along with already established role of Ca-Na_2EDTA as a heavy metal chelating agent suggest that (a) nano Ca-Na_2EDTA DPI technology has the potential to be used as an inhalation based

chelation therapy for neutralizing inhaled heavy metal ions at the portal of entry so that they can be excreted without absorption, (b) the novel formulation can probably be a medium for fast and sustained systemic absorption of the drug and may be developed into an alternative to intravenous administration, especially in emergency or field conditions, and (c) the formulation has the potential to be used as a preventive or therapeutic approach for members of rescue teams as well as victims in a radioactive fallout zone, and also for people working in mining and other such industries with high probability of metal ion exposure. A detailed pharmacokinetic study is in progress for establishing the therapeutic significance of the developed formulation.

Conflict of interest statement

The authors declare that there are no personal or financial conflicts of interest with individuals or organization

Contributors

Neeraj Kumar was responsible for macro- and microplanning and acquisition of data; *Sandeep Soni* was responsible for acquisition of data, prepared the pharmaceuticals and helped in generating the data for scintigraphy experiments; *Abhinav Jaimini* was responsible for carrying out scintigraphy experiments and helped in manuscript writing; *Farhan Jalees Ahmad* was responsible for supervising the characterization studies with respect to the formulation and in interpretation of results; *Aseem Bhatnagar* was responsible for conception and design of the study and for analysis and interpretation of the data; *Gaurav Mittal* was responsible for conception and design of the study, drafting and revising the article for correctness of important intellectual content. All authors participated in the discussion and interpretation of the final results, contributed to the final paper, and approved the final version submitted for publication. The authors had full access to the data and take full responsibility for its integrity

References

Ali, R., Jain, G.K., Iqbal, Z., Talegaonkar, S., Pandit, P., Sule, S., Malhotra, G., Khar, R.K., Bhatnagar, A., Ahmad, F.J., 2009. Development and clinical trial of nano-atropine sulfate dry powder inhaler as a novel organophosphorous poisoning antidote Nanomedicine. NBM 5, 55–63.

Bhavna, Ahmad, F.J., Mittal, G., Jain, G.K., Malhotra, G., Khar, R.K., Bhatnagar, A., 2009. Nano-salbutamol Dry Powder Inhalation: a new approach for treating bronchoconstructive conditions. Eur. J. Pharm. Biopharm. 71, 282–291.

Chattopadhyay, P., Gupta, R.B., 2001. Production of antibiotic nanoparticles using supercritical CO_2 as antisolvent with enhanced mass transfer. Ind. Eng. Chem. Res. 40, 3530–3539.

Dateand, A.A., Patravale, V.B., 2004. Current strategies for engineering drug nanoparticles. Curr. Opin. Colloid Interface 9, 222–235.

De Hevesy, G., 1944. Some applications of isotopic indicators. Nobel Lecture, December 12, pp. 9–41.

Fassett, J.D., 1995. Elemental isotope dilution analysis with radioactive and stable isotopes. Pure Appl. Chem. 67, 1943–1949.

Goyer, R.A., 1995. Chelation of toxic metals: current interests. Environ. Health Perspect. 103, 988–989.

Grandjean, P., 2010. Even low-dose lead exposure is hazardous. Lancet 376, 855–856.

Halatek, T., Sinczuk-Walczak, H., Rabieh, S., Wasowicz, W., 2009. Association between occupational exposure to arsenic and neurological, respiratory and renal effects. Toxicol. Appl. Pharmacol. 239, 193–199.

Henge-Napoli, M.H., Stradling, G.N., Taylor, D.M., 2000. Decorporation of radionuclides from the human body. Radiat. Prot. Dosimetry 87, 9–17.

James, A.C., Sasser, L.B., Stuit, D.B., Glover, S.E., Carbaugh, E.H., 2007. Ustur whole body case 0269: demonstrating effectiveness of i.v. Ca-DTPA for Pu. Radiat. Prot. Dosimetry 127, 449–455.

Kalia, K., Flora, S.J.S., 2005. Strategies for safe and effective therapeutic measures for chronic arsenic and lead poisoning. J. Occup. Health 47, 1–21.

Liversidge, E.M., Liversidge, G.G., Cooper, E.R., 2003. Nanosizing: a formulation approach for poorly-water-soluble compounds. Eur. J. Pharm. Sci. 18, 113–120.

Mittal, G., Singh, T., Goomer, N.C., Bhatnagar, A., Lulla, A., Malhotra, G., Kashyap, R., Tripathi, R.P., 2008. Formulations and methods thereof for making radiolabeled dry powder. Patent No. 1536/DEL/2008.

Newman, S.P., Clark, A.R., Talalee, N., Clarke, S.W., 1989. Pressurised aerosol deposition in the human lung with and without an open spacer. Thorax 44, 706–710.

Passos, C.J., Mergler, D., 2008. Human mercury exposure and adverse health effects in the Amazon: a review. Cad Saude Publica 24, s503–s520.

Pathak, P., Meziani, M.J., Desai, T., Sun, Y., 2004. Nanosizing drug particles in supercritical fluid processing. J. Am. Chem. Soc. 126, 10842–10843.

Podczeck, F., 1998. The relationship between physical properties of lactose monohydrate and the aerodynamic behaviour of adhered drug particles. Int. J. Pharm. 160, 119–130.

Rajpal, S., Mittal, G., Sachdeva, R., Chhillar, M., Ali, R., Agrawal, S.S., Kashyap, R., Bhatnagar, A., 2009. Development of atropine sulphate nasal drops and its pharmacokinetic and safety evaluation in healthy human volunteers. Environ. Toxicol. Pharmacol. 27, 206–211.

Rajpal, S., Ali, R., Bhatnagar, A., Bhandari, S.S., Mittal, G., 2010. Clinical and bioavailability studies of sublingually administered atropine sulphate. Am. J. Emerg. Med. 28, 143–150.

Rasenack, N., Steckel, H., Muller, B.W., 2003. Micronization of anti-inflammatory drugs for pulmonary delivery by a controlled crystallization process. J. Pharm. Sci. 92, 35–44.

Sarkari, M., Brown, J., Chen, X., Swinnea, S., Williams 3rd, R.O., Johnston, K.P., 2002. Enhanced drug dissolution using evaporative precipitation into aqueous solution. Int. J. Pharm. 243, 17–31.

Shekunov, B.Y., Chattopadhyay, P., Tong, H.H.Y., Chow, A.H.L., 2007. Particle size analysis in pharmaceutics: principles, methods and applications. Pharm. Res. 24, 203–226.

Singh, A.K., Verma, J., Bhatnagar, A., Sen, S., Bose, M., 2003. Tc-99m Isoniazid: a specific agent for diagnosis of tuberculosis. World J. Nucl. Med. 2, 292–305.

Türker, S., Onur, E., Ozer, Y., 2004. Nasal route and drug delivery systems. Pharm. World Sci. 26, 137–142.

Ugwoke, M.I., Agu, R.U., Verbeke, N., Kinget, R., 2005. Nasal mucoadhesive drug delivery: background, applications, trends and future perspectives. Adv. Drug Deliv. Rev. 57, 1640–1665.