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A Novel Sublingually Applied Melatonin Nanoemulsion Ensures Rapid Onset of Action

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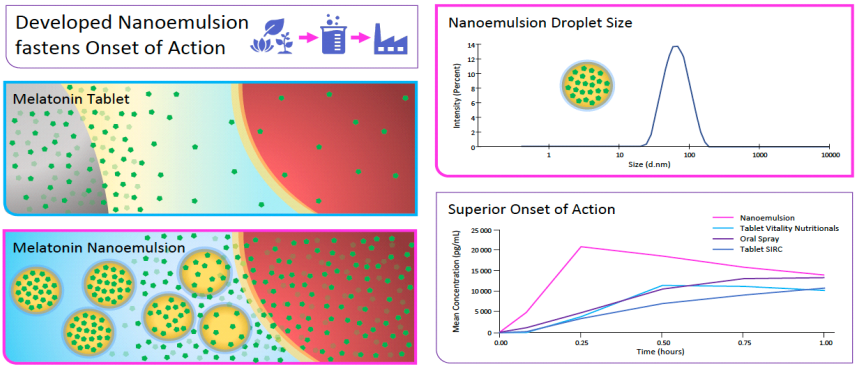
Abstract

When consuming pharmaceuticals or supplements, the pharmacokinetic parameters like bioavailability, maximum concentration and onset of action are of uttermost interest. This is especially true when it comes to the pineal gland hormone melatonin being involved in the sleep-wake cycle and is therefore applied to alleviate sleep disturbances. To reach the sleeping state quickly, high bioavailability combined with fast onset of action is needed. Herein, we present the development of a non-irritating nanoemulsion with a droplet size smaller than 60 nm for sublingual use. Scalability of the production process for commercial use was achieved. Furthermore, the pharmacokinetic parameters of the formulation were compared with a standard oral tablet of melatonin (both with a dose of 5 mg melatonin) in an open label, balanced, randomized, single-dose, two-treatment, two-sequence, two-period, two-way crossover study (n = 12). Comparing the pharmacokinetic parameters of the oral tablet and the spray formulation, the sublingual nanoemulsion leads to the highest bioavailability and up to two times faster onset of action in all cases. Clinical study number: SLS-CT-0003-22-MELA.



Keywords: *Melatonin, Nanoemulsion, Bioavailability, Sublingual, Fast onset, First-pass-effect, In human, Area under the curve, Lipid-based drug delivery system*

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1. Introduction

The lack of bioavailability of potential drugs has been a problem in the field of pharmaceutics for a long time and the need to solve this problem is even getting more urgent with up to 90% of potential drugs in the pipelines being poorly water-soluble (Huang *et al.*, 2021). In addition, other parameters contributing to poor bioavailability can be a low permeability and a high extraction of the drug in the liver (hepatic first-pass effect). These parameters are especially important when it comes to peroral formulations. While the peroral route is the preferred way of drug intake by adults, it is a rather challenging one for a potential drug. First the drug has to dissolve in the (gastro-) intestinal fluids, then it has to permeate through the intestinal membrane to subsequently reach the liver passage.

To overcome this issue, several formulation techniques acting as enabling technologies have been developed over the years, one of them being the delivery of the drug via a nanoemulsion. Nanoemulsions are kinetically stable dispersions of oil in water with a droplet size around 100 nm or less (Nouf Aljabri *et al.*, 2022). The oil droplets do not only contain the hydrophobic drug and therefore enhance its solubility, they also protect the drug from the environment (e.g., gastric pH, intestinal enzymes). When nanoemulsions are considered as a potential dosage form, the main focus generally lies on the first part of the above, the enhancement of the drugs solubility to enhance overall bioavailability. This is mainly because of the lipid phase being able to dissolve the poorly water-soluble drug. In addition, because of the small droplet size, nanoemulsions are characterized by an enormous interfacial area which influences the transport of the drug through the membrane and into the bloodstream and/or the lymphatic route. This indeed increases the bioavailability, but it does also influence other pharmacokinetic parameters like Tmax (Lawrence and Rees, 2000; Kawakami *et al.*, 2002; Kawakami *et al.*, 2002; Huang *et al.*, 2021).

Due to the properties mentioned above nanoemulsions are considered especially suited for the delivery of biopharmaceutical classification system (BCS) II and IV drugs, which show poor water solubility (BCS II and IV) and low permeability (BCS IV) (Hardeland *et al.*, 2006). Fortunately, every drug that shows sufficient solubility in the nanoemulsions lipid phase can be formulated as a nanoemulsion and can therefore benefit from pharmacokinetic enhancements enabled by this dosage form as well. A very promising approach to positively shape pharmacokinetics with nanoemulsions is the absorption of the drug via the sublingual route, which can be done when the droplet size is sufficiently small enough. This way of application, not only avoids the first-pass effect but also enables a faster onset of action of the drug since the nanoemulsion can permeate through the mucosal lining and can then enter systemic circulation by passing the vena cava. Melatonin (N-acetyl-5-methoxytryptamine) is a tryptophan-derived, circulating hormone synthesized and released by the pineal gland. Its precursor is the aminoacid L-Tryptophane, which gets 5-hydroxylated (5-HTP), decarboxylated (serotonin), N-acetylated (N-acetylserotonin) and O-methylated to melatonin (Zamfir Chiru *et al.*, 2014).

It obtains low levels during the day with concentrations of 0-20 pg/mL and elevated levels at night with concentrations of 40-100 pg/mL following a circadian rhythm due to the presence or absence of light being the trigger factor for its release. Being in the dark leads to an increase in the melatonin secretion. Over a normal sleep duration and time, melatonin peaks around 2-5 am in the plasma (Zisapel *et al.*, 2005). Its physiological opponent is cortisol, which also shows a circadian rhythm. Cortisol levels are low during the night and start increasing in the early hours before wakening. It peaks 20-40 min after awakening, a process known as cortisol awakening response (CAR). In the elderly, the secretion of melatonin in the night delays while there is an earlier onset of cortisol production. It is discussed in the literature, that this process in part might be the reasons why elderly show poorer sleep quality (Steptoe and Serwinski, 2016; Bartoli *et al.*, 2012).

Melatonin has been extensively used in the treatment of primary sleep disorders (Zdunska *et al.*, 2021; Brown *et al.*, 2009). This therapeutic indication is of great interest since sleep disturbances affect up to 30% of the population in the western hemisphere. Due to its multifaceted nature, melatonin's health benefits are not limited to the circadian rhythm, but include antioxidant effects, anti-aging, anti-inflammatory and neuroprotective effects, as well as mood regulation and control of cardiovascular diseases, obesity, and diabetes (Pandi-Perumal *et al.*, 2007; Li *et al.*, 2016; Oxenkrug *et al.*, 2001; Li *et al.*, 2016; Pandi-Perumal *et al.*, 2005; Chenevard *et al.*, 2008; Pandi-Perumal *et al.*, 2013; Agil *et al.*, 2015; Agil *et al.*, 2020; Reiter *et al.*, 2020). In

mitochondria, it works as a radical scavenger (Harpsøe *et al.*, 2015). Accordingly, administering exogenous melatonin has demonstrated a number of clinical effects and has also been investigated in a number of different conditions, like breast cancer, tinnitus and for pain management (Grant *et al.*, 2009; Hosseinzadeh *et al.*, 2019; Xie *et al.*, 2020; Touitou, 2001).

All these effects have to be seen in the greater concept of the decline of melatonin secretion over lifetime. Since this means that a major antioxidant and radical scavenger is missing later in life, it has been discussed that aging is in part secondary to pineal gland insufficiency. This would be in accordance with the free radical theory of aging (Rozencwaig *et al.*, 1987; Touitou *et al.*, 2001). When it comes to melatonin's major therapeutic use, the treatment of sleep disorders, a rapid onset of action is needed to ensure the therapeutic outcome since time awake spent waiting for melatonin to kick in is time lost for sleep. To achieve this, sublingually applied nanoemulsions seem to be the best option. Orally administered and ingested melatonin is almost completely absorbed. However, up to 90% of blood melatonin is cleared by the liver where it is metabolized by hydroxylation to 6-hydroxymelatonin, conjugated with sulfuric or gluconic acid and excreted in the urine. This makes its half-life of 30-60 minutes very short and the bioavailability of 15% very low. Additionally, large interindividual variations of the pharmacokinetic parameters of melatonin are reported in the literature (Andersen *et al.*, 2016; Suhner *et al.*, 2009).

Taking the information about the dosage form and the drug together, nanoemulsion seems like a perfect fit for melatonin. The bioavailability can be enhanced by the avoidance of the first-pass effect and in addition, Tmax can be reached earlier because of the enhanced permeation of the drug-loaded lipid droplets through the mucosal lining. This should lead to a fast and high rise of the plasma levels, enabling a fast onset of action, in this case meaning onset of sleep. Therefore, we herein present the development of a non-irritating nanoemulsion with a droplet size smaller than 60 nm for sublingual use. To the best of our knowledge, this is the first report of a nanoemulsion with a relevant melatonin content of 5 mg in 1 ml tested in humans.

2. Materials

The tested formulations have been created including the following ingredients: Glycerol (Gustav Hees GmbH), Ethanol (Brüggemann Alcohol Heilbronn GmbH), Phosphatidylcholin P75 (Lipoid GmbH), Red Palmoil (Gustav Hees GmbH), Copaibaoil (Endlos Fit GmbH), Orangepeeloil (SanaBioGmbH), α-tocopherol (Denk Ingredients BTSA), Sunflower oil (Gustav Hees GmbH), Melatonin (Aplantis GmbH), Melatonin (Hebei Four Leaf Clover Biotech Co.), MCT-Oil (IOI Oleo).

The formulations have been manufactured in laboratory scale using the Disperser (Steinberg Systems), XStream Lab Homogenizer 2000 (GEA) or LM10 Microfluidizer (Microfluidics International Corporation). Industrial scale production was performed using the Panther NS300GL (GEA). Particle size analysis was performed and analyzed with the Malvern ZetaSizer Nano ZS (Malvern Panalytical Ltd.).

3. Methods

3.1. Development of the Nanoemulsion

For the nanoemulsion the oil phase was premixed containing 0.5 mass % melatonin, sunflower oil, orange peel oil as well as α-tocopherol as an antioxidant compound. The water phase was prepared using soy lecithin phosphatidylcholine P75, ethanol, glycerin and water. Both premixes were dispersed to a homogeneous mixture by stirring for 30 minutes. To receive a small droplet size, the mixture was then processed using a XStream Lab Homogenizer 2000 (1200 bar Inj-Cha, 50 bar CCMS) for 7 cycles.

3.2. Stability Tests (Warm Storage)

To analyze the stability of the nanoemulsion under accelerated conditions, the formulation was stored at 40 °C for two months. Dynamic light scatter (DLS)-measurements were performed after manufacturing and after each month. For these measurements 0.1 g of each formulation was diluted with demineralized water to a total mass of 30 g. The resulting dispersion was stirred with a magnetic stirrer for 2 minutes, afterwards the DLS-measurement was performed. The prototype was considered as stable if a monodisperse size distribution

was detected. Following pharmaceutical standards after 1 month stability at 40 °C, a prototype is considered to be at least stable for 12 months at room temperature. Furthermore, the prototypes were assessed optically (by eye) and olfactory.

3.3. Droplet Size Measurement with Dynamic Light Scattering (DLS)

Particle size and distribution were determined using the Malvern ZetaSizer Nano ZS analyzing a 1:300 dilution of the prepared nanoemulsion in demineralized water. Analysis was performed using the Malvern software detecting the z-average and the polydispersity index (PDI) of the measured particles.

3.4. Morphology of the Nanoemulsion using Cryo Electron Microscopy (Cryo-EM)

Cryo-EM imaging was performed with a JEOL JEM-2100 transmission electron microscope (JEOL GmbH, Eching, Germany). Cryo-EM specimens were prepared by applying a 4 µL droplet of sample suspension to lacey carbon-coated copper TEM grids (200 mesh, Electron Microscopy Sciences, Hatfield, PA) and plunge-freezing them into liquid ethane using an FEI vitrobot Mark IV set at 4 °C and 95% humidity. Vitrified grids were either transferred directly to the microscope cryo transfer holder (Gatan 914, Gatan, Munich, Germany) or stored in liquid nitrogen. All grids were glow-discharged before use. Imaging was carried out at temperatures around 90 K. The TEM was operated at an acceleration voltage of 200 kV, and an objective lens defocus of about 1.5-2 µm was used to increase the contrast. Cryo-EM micrographs were recorded with a bottom-mounted 4*4k CMOS camera (TemCam-F416, TVIPS, Gauting, Germany) at a magnification of 50000 ×, corresponding to a pixel size of 2.32 Å at the specimen level.

3.5. Microbiological Stability

Microbial stability testing was performed by Eurofins Consumer Product Testing GmbH (Hamburg, Germany). The nanoemulsion was inoculated with *S. aureus, P. aeruginosa, C. albicans* and *A. brasiliensis*. Microbial count was analyzed at day 0, 2, 7, 14 and 28.

Furthermore, the not inoculated product was incubated with growth medium at 25 °C (for the detection of yeast and mold), 30 °C (for the detection of *Bacillus cereus* and the total aerobic microbial count) and 37 °C (for the detection of *enterobacteriaceae* and *coagulase* positive *staphylococcus*). In addition, the nanoemulsion was tested for the presence of *Salmonella ssp*. via PCR.

3.6. Technology Transfer

For Technology transfer the melatonin 0.2% formulation was premixed with a total volume of 60 liters. For homogenization the mix was processed using the Panther NS300GL.

3.7. Study Design and Clinical Procedure

The study was initiated after written approval obtained from the Ethics Committee and was conducted as per the National Ethical Guidelines for Biomedical and Health Research Involving Human Participants ICMR. The study center was Spinos Life Science and Research Private Limited located in No. 29 A, "Krishna Madhuravanam", Vellakinar Pirivu, Thudiyalur, Coimbatore-641029, Tamil Nadu, India. Written informed consent was obtained from each subject.

This study was an open label, balanced, randomized, single-dose, two-treatment, two-period, two-way crossover, 24-hour bioavailability study of melatonin 5 mg in 1 mL nanoemulsion developed and manufactured by TINY Technologies GmbH, Kleiner Kielort 6-8, 20144 Hamburg, Germany as test product and melatonin 5 mg sublingual tablets Vitalis Nutritionals manufactured by Vision Healthcare B.V. Postbus 5038, 2000 GA, Haarlem, The Netherlands as reference product in healthy, adult human subjects under fasting conditions.

The nanoemulsion developed by TINY Technologies consists of an oil phase with sunflower oil, orange peel oil and α-tocopherol and a water phase with soy lecithin, ethanol, glycerin, water and melatonin. Both premixes were dispersed to a homogeneous mixture. To receive a small droplet size the mixture was then processed using a XStream Lab Homogenizer 2000. During the screening visit, subjects' medical history and

concomitant therapies were reviewed and eligibility was determined based on the inclusion and exclusion criteria. Height, weight, pulse rate, and blood pressure were measured and BMI was calculated. For measures of general health status, blood samples were collected for hematology and biochemistry analysis.

Subjects returned to the clinic after 14 days of restrictions to melatonin and related foods for randomization and test period. Subjects were housed in the facility from 36 hours pre-dose to 24 hours post-dose. After check-out, a washout period of at least 7 days between the successive dosing days was performed. The clinical study was conducted over a period of 11 days. Each enrolled subject was assigned a randomization code according to the respective randomization list. The pharmacist packed the products for each individual subject (as per randomization schedule). The products were coded with a randomization number, study number, name of product, Batch/Lot No., expiry date labeled "Treatment R or T" and later administered.

Subjects were checked in the facility two days prior to dosing to standardize the subjects. Before dosing blood samples under fasting conditions (0 hours) were collected prior to treatment administration. Subjects were given one tablet of the reference with 240 mL of water or 1 mL nanoemulsion of the test product with 1 mL of water (to rinse the syringe the nanoemulsion was applied with to ensure the full dose) according to their sequence allocation. The nanoemulsion had to be contained under the tongue for 1 minute and was swallowed afterwards. Treatments were administered in the presence of clinic personnel to ensure compliance. Subjects remained at the clinic for post-dose blood samples. Meanwhile, subjects were provided with standardized very low-melatonin meals throughout the study and the food consumed at each meal was recorded. Subjects were allowed to leave the clinic upon completion of blood sampling. Also see study flow chart (Figure 3) for overview and summary of the study procedure.

3.8. Subjects

Twelve adult male subjects between 18 and 45 years of age were enrolled in the study. Healthy subjects with a body mass index (BMI) of 18.50-29.99 kg/m^2, who were non-smokers or who had stopped smoking for more than three months, were included in the study. Subjects were excluded from the study if they had used prescription or over-the-counter products containing melatonin within 14 days prior to the study check-in and during the trial; had any major illness in the last three months; had a history of difficulty in swallowing or a history of hypersensitivity to melatonin, or any other condition which may have adversely affected the subject's ability to complete the study or its measures or which posed significant risk to the subject, additionally, subjects agreed to consume a very low melatonin diet during the study.

3.9. Determination of Plasma Melatonin Concentrations

Blood samples collected at pre-dose (0.00 hours) and post-dose 0.08, 0.25, 0.50, 0.75, 1.00, 01.50, 2.00, 3.00, 4.00, 5.00, 6.00, 8.00, 12.00, 16.00 and 24.00 hours were analyzed for the pharmacokinetics of plasma melatonin. Blood was collected in a 6 mL pre-labeled K2EDTA calculated and displayed over time. For comparability with the sublingual oral spray and oral tablet by SIRC only data from 6 hours post-dose were considered. The tube was centrifuged at 3800 RPM for 10 minutes at 2 °C to 8 °C to separate the plasma. Plasma melatonin measurements were performed as per method standard operation procedure.

3.10. Pharmacokinetic and Statistical Analysis

The following parameters were assessed: Maximum observed concentration (C_{max}), time of maximum concentration (T_{max}), area under the curve (AUC_{0-t}). Arithmetic mean and standard deviation (SD) were calculated and statistical analyses were performed using Graph pad Prism software.

3.11. Comparison with Previously Reported Clinical Study by Bartoli et al.

The clinical study was described in Bartoli *et al.* (2013) and was performed as a single-dose, open-label, crossover study where the subjects were randomly assigned to receive 5 mg of a sublingually applied oral spray by Medestea SpA or oral melatonin tablets by SIRC. Study design, as well as melatonin dose, main pharmacokinetic parameters, the sublingual application of the oral spray and the oral tablet made this a suitable comparative study. The number of subjects (8 compared to 12) and the time of sample collection after IP administration (6 hours compared to 24 hours) should be considered as differences between the two studies.

4. Results

4.1. Development of a Nanoemulsion

Producing a nanoemulsion with small z-average and high stability over time we tested different production methods as well as different formulations to develop a melatonin nanoemulsion with a fast onset of action and a high bioavailability which can also be produced in high quantities. To receive a nanoscale droplet size the oil/water emulsion has to be exposed to a high amount of pressure. Therefore, after dispersing the two phases with a stirrer to a homogenous mix, it was processed with high pressure. Here, two different devices, a LM10 microfluidizer and a XStream Lab Homogenizer 2000 were tested. Developing an experimental design leading to a small z-average but also considering economical effects as time and machine wear different numbers of processing cycles and different pressures were evaluated. For this purpose a standard formulation without any active ingredient was created containing water, glycerol, red palm oil, orange peel oil and copaiba oil. It was processed 9-times and after each processing cycle a sample was withdrawn and analyzed for z-average and PDI. The shown experimental data were received using the LM10 Microfluidizer with 18500 PSI. As Table 1 depicts, no significant decrease in droplet size was obtained after more than 7 cycles. Using the XStream Lab Homogenizer 2000, nanoemulsions resulted in comparable droplet sizes (Figure 1A). This data shows that both devices equally function. In perspective of the small volume per hour being processed by the microfluidizer we used the XStream Lab Homogenizer 2000 for further experiments.

Another factor highly influencing the droplet size is the applied pressure. Thus, different pressures in the injection chamber (Inj-Cha) were tested with the Cavitation Cloud Modulating System (CCMS) always kept at 50 bar. 600, 800, 1000 or 1200 bar were evaluated for 7 cycles and after each run samples were analyzed (Figure 1A). In all cases nanoscale formulations were reached by that time. Using 600 bar the size of ca. 125 nm was reached, while a strong decrease was observed applying 800 bar. However, smallest droplets (ca. 80 nm) were created using 1200 bar in the injection chamber (Figure 1A). Thus, for future formulations 7 cycles with 1200 bar in the injection chamber and 50 bar in CCMS were used. Relying not only on one technique defining the droplet size we executed visual Cryo-EM of our formulation without active ingredient (Figure 1B). A homogenous distribution of the droplet size was detected. Further, the z-average of 60 nm after manufacturing measured for our melatonin could be verified (Figure 1B, C and Table 2).

Analyzing our standard 60% glycerol nanoemulsion for microbial contamination showed a reduction of the microorganisms in the nanoemulsion to under 10 colony forming units per gram (cfu/g) of the formulation for *S. aureus, P. aeruginosa*. For *C. albican*s and *A. brasiliensis* no cfu were detectable on day 2 and 7. However, on day 14 and 28 cfu's were detectable, but the amount was under 10 cfu/g. The incubated growth media with the nanoemulsion showed a count of below 10 cfu/g for every of the in the methods part mentioned microorganisms. *Salmonella ssp.* could not be detected via PCR. Hence, we were able to successfully develop a macrobiotically and physically stable nanoemulsion.

Having developed the used methods we now tested different substitutions and melatonin concentrations increasing the bioavailability of the dissolved drug. With the goal to develop the best formulation in terms of small size and high stability, different types and concentrations of lecithin were tested. It was found that soy

Table 1: Analysis of the Decrease in Droplet Size within an Increasing Number of Processing Cycles using the LM10 Microfluidizer. No Significant Change in Size was Observed after 7 Cycles

Number of Cycles	z-Average	PDI
1	97.87	0.160
3	75.54	0.150
5	64.71	0.177
7	58.99	0.177
9	56.15	0.150

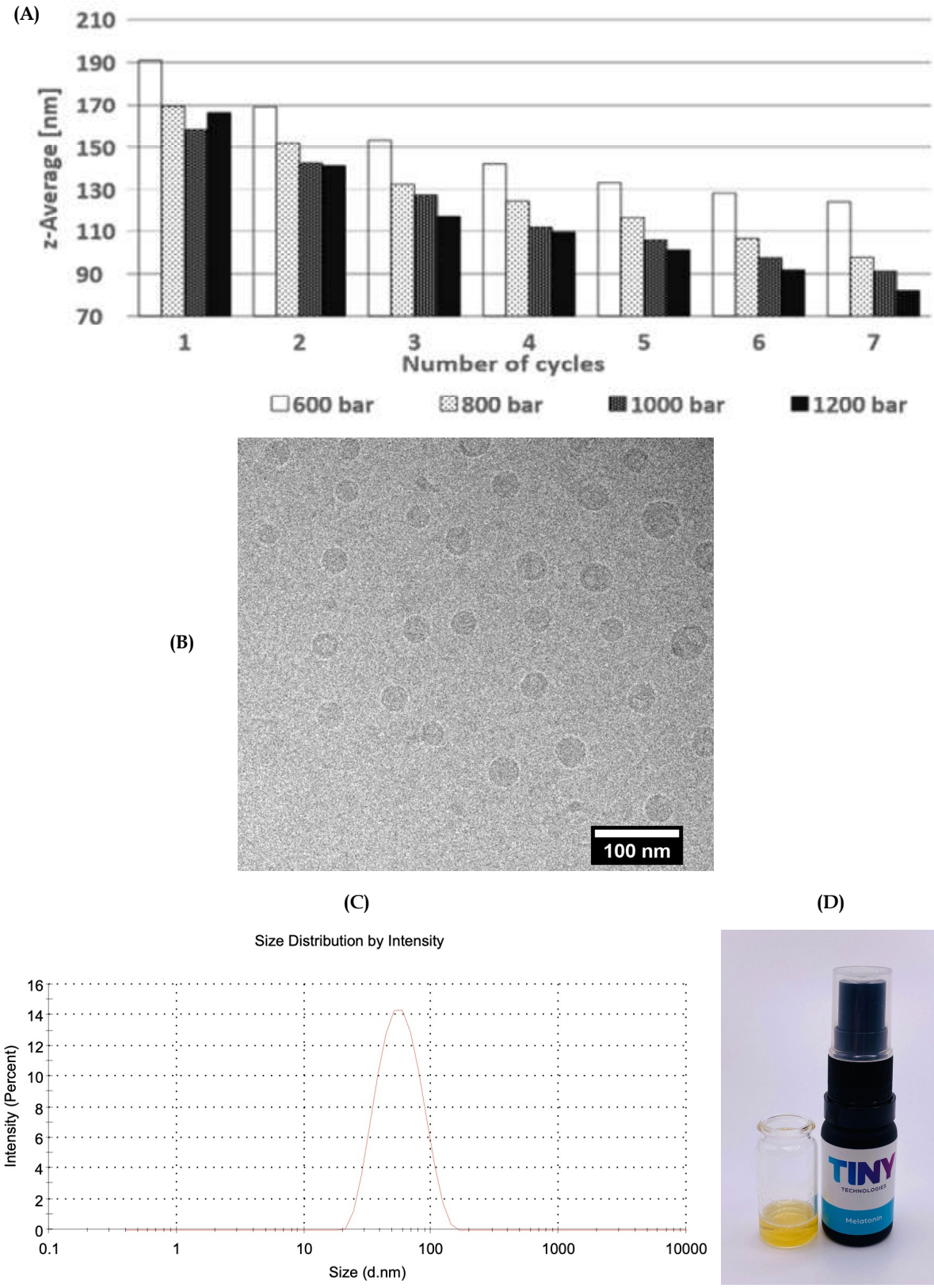

Figure 1: Nanoemulsion Development. A) Investigation of the Needed Pressure in the Injection Chamber Leading to a Small Droplet Size. The Lowest z-Average of 81.92 nm was Reached with 1200 bar after 7 Cycles. B) Cryo-EM Image of Nanoemulsion. The Average Size is 50 nm. C) Size Distribution Intensity of Melatonin 0.5%, 60% Glycerol on Date of Manufacture. D) Picture of Melatonin 0.5%, 60% Glycerol

lecithin with a phosphatidylcholine fraction of 75% dissolved in ethanol leads to the best results. Further, we added α-tocopherol as an antioxidant to the oil phase, prohibiting oxidation of the active ingredient. First, we were able to stabilize 0.2% melatonin in our delivery system, having a z-average of 45.92 nm after manufacturing. Second, we increased the melatonin concentration to 0.35% and even to 0.5% gaining a similar droplet size smaller than 60 nm and a PDI lower than 0.25, showing a monodisperse peak (Table 2, Figure 1C). The final product is shown in Figure 1D. The stability of the two formulations were evaluated with the same parameters keeping the samples at 40 °C for up to three months. Even though the average size of the droplets increased, the distribution decreased. Third, we reduced the glycerol content in the water phase by half. To our surprise it not only spares the machine from abrasion but also increases stability maintaining a small droplet size after one month at 40 °C storage. A size smaller than 150 nm was still measured after 2 months of warm storage, respectively the prototypes are considered stable at room temperature for 18 months in an unopened light protected container (Table 2). This was also the case loading the delivery system with melatonin, allowing practical dosing and adaptation of the system depending on usage (Table 2).

Supplements are becoming a constantly increasing value in society. Thus, developing a highly bioavailable melatonin product with fast onset which can be produced in high quantities is of high interest. Transferring our technology to large scale we produced 60 kg of melatonin 0.2% nanoemulsion using the Panther NS3006L. Analyzing the droplet size, comparable results were obtained as in a small-scale production, as a z-average of 69.23 nm and a PDI of 0.204 were achieved. This data shows that the here developed formulation is easily used for upscaling.

Table 2: Nanoemulsions with Different Melatonin Concentrations have been Developed. Melatonin 0.2%, 0.35% and 0.5% Each with 60% Glycerol Keep a Monodisperse Droplet Size even after 1 Month Stress Test but Grow in Size Over Time. A Better Long Term Stability was Reached with Halving the Glycerol Concentration

Product Name	Manufacturing Date		Stresstest 1 Month		Stresstest 2 Months		Stresstest 3 Months	
	z-Average [nm]	PDI	z-Average [nm]	PDI	z-Average [nm]	PDI	z-Average [nm]	PDI
Melatonin 0.2% 60% glycerol	45.92	0.113	93.08	0.126	575.3	0.21	n.a.	n.a.
Melatonin 0.35% 60% glycerol	50.67	0.183	105.0	0.129	n.a.	n.a.	576.2	0.206
Melatonin 0.5% 60% glycerol	52.03	0.111	123.1	0.92	n.a.	n.a.	368.7	0.332
Melatonin 0.2% 30% glycerol	48.23	0.249	51.63	0.198	n.a.	n.a.	161.8	0.108

4.2. Clinical Study

Analyzing the benefits of the developed nanoemulsion a comparative bioavailability study was carried out to evaluate the pharmacokinetics of a 5 mg melatonin nanoemulsion developed and manufactured by TINY Technologies GmbH (Kleiner Kielort 6-8, 20144 Hamburg, Germany) and Vitality Nutritionals melatonin 5 mg oral tablets manufactured by Vision Healthcare B.V. (Postbus 5038, 2000 GA, Haarlem, The

Netherlands), in healthy, adult human subjects under fasting conditions and also to monitor the safety and tolerability of a single dose. Study design was an open label, balanced, randomized, single-dose, two-treatment, two sequence, two-period, two-way crossover bioavailability study. A Study flow chart is depicted in Figure 2. All subjects (n = 12) completed the study. There was no in-house adverse event reported, hence concluded as safe and tolerable for human use. One post study adverse event displayed as elevated SGPT (ALT) level which was categorized as mild in severity SGPT (ALT) levels were normal after the repeat assessment five days later. This event could not be attributed to either the nanoemulsion or the tablet. Collected pharmacokinetic blood samples were analyzed for plasma melatonin concentrations as per validated LC-MS/MS method. Later, the analyzed parameters were compared with a previously reported clinical study with similar study design and procedure. Blood melatonin levels of each of the 12 subjects over time (24 hours) are shown in Figure 3A for the nanoemulsion (5 mg in 1 ml) and in Figure 3B for the oral tablet (5 mg) by Vitalis Nutritionals. The mean area under the curve ($AUC_{0\text{-}6h}$) was calculated and plotted in Figure 4A with the nanoemulsion resulting in the highest value (35,593 pg*h/ml) even though no significant difference was observed (Table 3 and Figure 4A). The $AUC_{0\text{-}6h}$ of the test nanoemulsion was 1.31x higher than of the oral

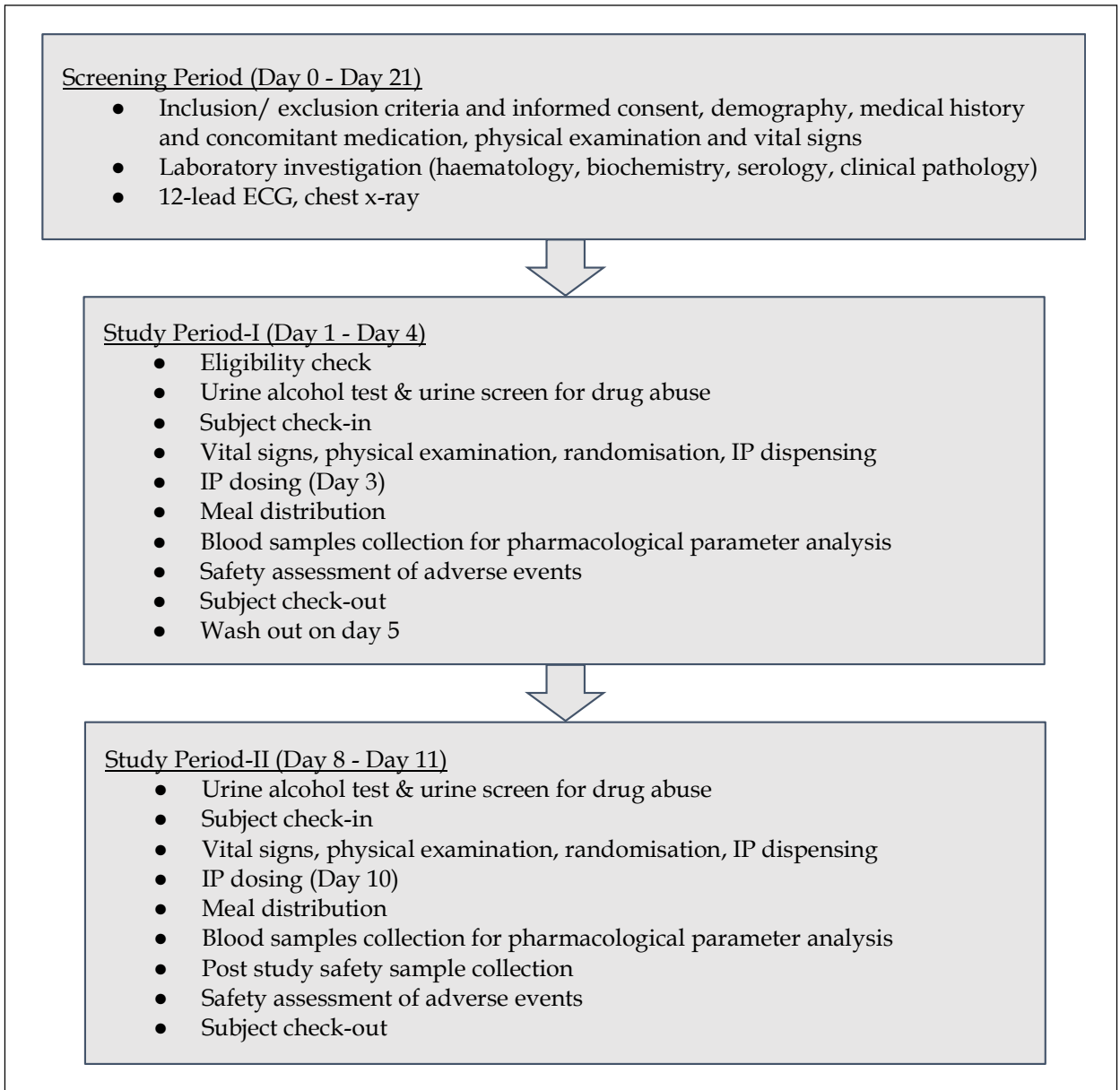

Figure 2: Study Flow Chart. The Study Assessments and Procedures are Displayed in a Flow Chart. The Study Started with a Screening Period, followed by the Study Periods I and II where the Investigational Products (IP) were Administered and Blood Samples were Collected and Analyzed

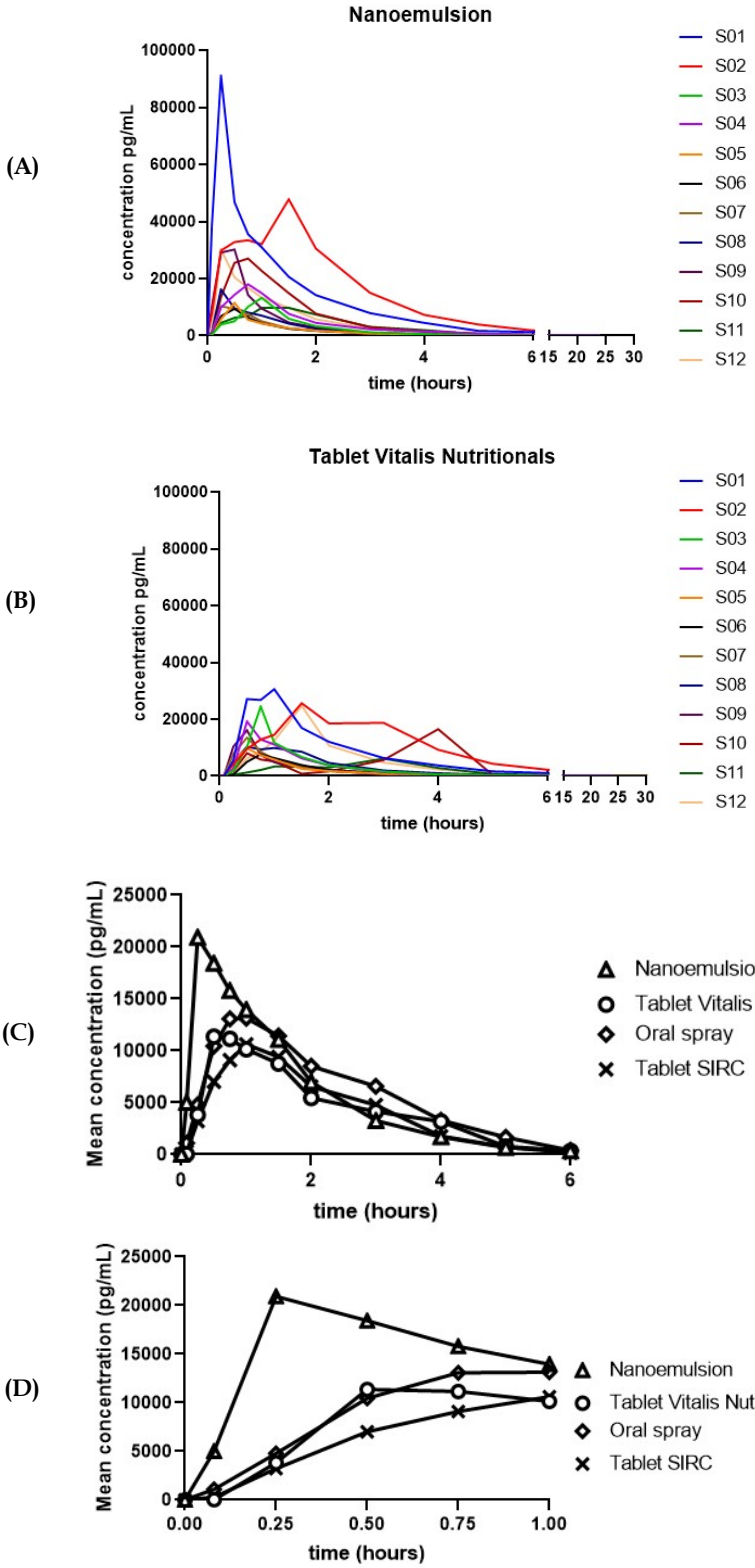

Figure 3: Melatonin Blood Concentrations. A) The Melatonin Blood Concentrations (pg/mL) over the Sample Collection Period of 24 hours are Depicted for all 12 Subjects after Administration of the Nanoemulsion by TINY. B) Melatonin Blood Concentrations of Oral Tablet Manufactured by Vitalis Nutritionals. C) Mean Melatonin Blood Concentrations (pg/mL) were Calculated and Displayed over 6 hours after for both Products (TINY Nanoemulsion and Vitalis Nutritionals Oral Tablet), as well as for Investigational Products (Oral Spray Oral Tablet) from a Comparable Study by Bartoli. D) Close-up of the Data for the First hour

Table 3: Pharmacokinetic Parameters Cmax (pg/mL), T_{max} (hours) and AUC0-6h (pg*h/mL) for the Nanoemulsion, Oral Tablet by Vitalis Nutritionals (Tablet VN), Oral Spray and Tablet by SIRC. The Arithmetic mean (mean) and Standard Deviation (SD) were Calculated for 12 Subjects (Nanoemulsion and Tablet VN) or 8 Subjects (Oral Spray and Tablet SIRC, Bartoli et al). The Melatonin Dose was 5 mg

Parameters	Nanoemulsion		Tablet VN		Oral Spray		Tablet SIRC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C_{max} [pg/mL]	26235	23485	17041	7948	17200	9300	12400	6600
T_{max} [h]	0.63	0.39	1.25	1.13	0.71	0.42	0.63	0.20
AUC_{0-6h} [pg*h/ml]	35593	33091	27189	19938	28666	15308	19654	12947

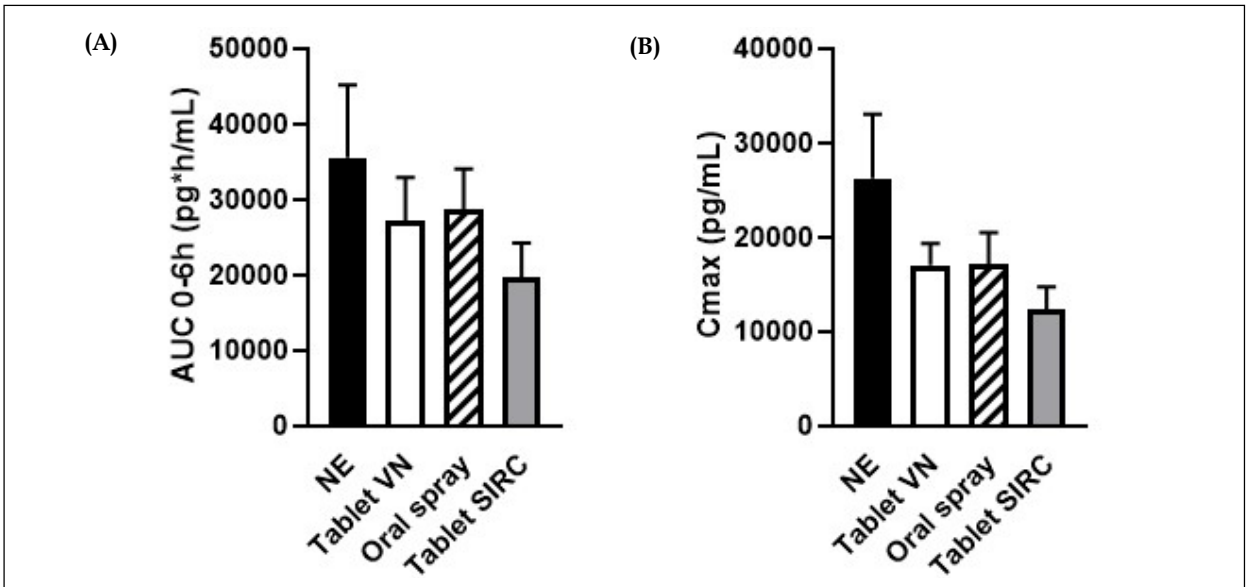

Figure 4: Pharmacological Parameters. The Melatonin Blood Concentrations over Time Allowed the Calculation and Assessment of the following Pharmacological Parameters that are Displayed as mean with Standard Deviation. A) Area Under the Curve (AUC 0-6h). B) Maximum Concentrations (C_{max})

tablet by Vitalis Nutritionals (27,189 pg*h/ml), 1.24x higher than of the oral spray (28,666 pg*h/ml) and 1.81x higher than of the tablet by SIRC (19,654 pg*h/ml) (Table 3). Mean maximum melatonin blood concentrations C_{max} were calculated and displayed in Figure 4B with again considerably higher values observed after administration of the nanoemulsion (26,253 pg/ml). However, no statistical significance was reached due to high standard deviation when the nanoemulsion was compared to the oral tablet ($p > 0.05$). The rapid onset of action of melatonin in the nanoemulsion compared to the oral tablet by Vitalis Nutritionals was verified in Figure 4D, in which the time of the maximum melatonin concentration T_{max} can be seen. T_{max} is 2x faster after administration of the nanoemulsion compared to the oral tablet by Vitalis Nutritionals, but only 1.2x faster than the oral spray, and as fast as the oral tablet by SIRC (Table 3).

First appearance of melatonin in the plasma is found after 5 minutes (0.08 h) in all but one subject (equal to 91,7%) when the nanoemulsion is applied. On the contrary, the first appearance of melatonin is found after 5 minutes in only one participant (equal to 8,3%) when the oral tablet is applied. In addition, the sublingual nanoemulsion led to a tighter T_{max} corridor in the subjects (0.25 h-1.5 h, range 1.25 h) with 33% of all participants reaching T_{max} at 0.25 h and only one participant (8 %) with T_{max} 1.5 h. This leads to a mean T_{max} of 0.63 h with a standard deviation of 0.39 h. The oral tablet showed a much broader corridor (0.5 h-4 h, range 3.5 h) with a larger standard deviation of T_{max} (1.25 h) of 1.13 h. Here, 42% participants reached T_{max} at 0.5 h (earliest), whereas one participant (8%) reached T_{max} at 4 h (latest). This shows the considerably quicker and more uniformly reaching of the peak concentration throughout all participants when the nanoemulsion is applied.

With regard to the height of the melatonin peak, the mean peak height is 45% higher when the nanoemulsion is applied. The highest peak measured in a participant was even 197% enhanced compared to the peak when the oral tablet was applied. This shows the ability of nanoemulsions to not only enable fast onset but also high peak concentrations.

On the interindividual level it can be seen that, if the participants are categorized in a top (2), bottom (3) and intermediate (rest = 7) group, these groups behave the same with the different supplements. The 3 participants with the lowest AUC for the reference product are also the ones showing the lowest AUC when the nanoemulsion is applied. The same is true for the 2 participants with the highest AUC. On the individual level, if the 80-125% deviation from the AUC of the reference product is considered as the bioequivalence criteria, 83% of the participants (10 out of 12) show a bioequivalent or even enhanced outcome by AUC when the nanoemulsion is applied. This is also evident with regard to the overall enhancement of the AUC by 31% and the median enhanced by 26%.

5. Discussion and Conclusion

In this study we developed a nanoemulsion with high physical and microbial stability. This delivery system can be loaded with different concentrations of an active ingredient as it was shown with 0.2-0.5% melatonin. Further we showed that our technology can easily be used for upscaled production. This comparative bioavailability study was carried out to evaluate the pharmacokinetics of a 5 mg melatonin nanoemulsion developed and manufactured by TINY Technologies GmbH and Vitality Nutritionals melatonin 5 mg oral tablets manufactured by Vision Healthcare B.V. (Postbus 5038, 2000 GA, Haarlem, The Netherlands), in healthy, adult human subjects under fasting conditions and also to monitor the safety and tolerability of a single dose. For the nanoemulsion investigated in this study, all major pharmacokinetic parameters (AUC, C_{max}, T_{max}) showed superiority compared to the other formulation tested in this study. Superiority was also shown when the data was compared to the study of Bartoli *et al.*

All of these pharmacokinetic properties of the nanoemulsion mentioned above are necessary for a superior therapeutic use of melatonin. For the therapy of chronobiotic disorders like jet lag, it is known that a fast onset and a rapid increase of melatonin levels in plasma are needed to synchronize suprachiasmatic nucleus and to initiate sleep (Suhner *et al.*, 2009). The nanoemulsion reported herein is capable of achieving the required performance more holistically than the other formulations mentioned in this paper, by increasing all major pharmacokinetic parameters (C_{max}, T_{max}, AUC), as can be seen in Table 3 where the arithmetic mean (mean) and standard deviation (SD) of the pharmacokinetic parameters were calculated for 12 subjects (nanoemulsion and tablet VN) or 8 subjects (oral spray and tablet SIRC, Bartoli *et al.*).

The melatonin dose was 5 mg. Although the results are not significant with regards to the population the sublingual melatonin nanoemulsion presented in this study can be of great therapeutic improvement on the individual level for specific patients suffering from sleep disorders with regard to bioavailability and onset of action. In those cases, a fast increase of melatonin combined with a high peak value could give rise to melatonins hypnotic properties and could therefore initiate sleep more rapidly (Suhner *et al.*, 2009). Therefore, the dosage form presented in this article can have a huge impact on the therapeutic outcome of melatonin. So far, most melatonin products on the market are solid dosage forms. Circadin 2 mg is a prolonged-release tablet approved in the EU for the short-term treatment of primary insomnia in patients aged 55 and older. It is a prescription only medicine. The dosage form is coated with Eudragit-RS, an insoluble but swellable polymer. Therefore, the dosage form cannot easily disintegrate in the stomach meaning it can only escape into the intestine when the stomach is empty and housekeeper waves are starting (Davis Stanley, 2005).

This means, the time between the application and the last intake of food can alter these pharmacokinetic parameters as well due to a possible retentive effect. If there is still food in the stomach, T_{max} of melatonin might only be achieved 2-3 h after intake. Which is a serious disadvantage for people, who tend to eat dinner rather late in the day.

Overall, the results of this study highlight the ability of sublingual applications to increase the bioavailability of melatonin. Further it shows the potential of nanoemulsions as efficient drug delivery systems. With a sufficiently small droplet size, the absorption of the drug via the sublingual route can be achieved under

avoidance of the first-pass effect and with a faster onset of action. The poor bioavailability of a large number of active substances has been a problem in the field of pharmaceutics for a long time. Nanoemulsions might therefore represent powerful tools to tackle this issue for many more substances.

With the focus on the drug melatonin, the sublingually applied nanoemulsion has shown the potential to address the issue of short-term sleep disturbance treatment more effectively than current market standard products since not only overall bioavailability, but also C_{max} was increased. Furthermore, and in this condition more important, T_{max} was decreased.

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References

Agil, A., El-Hammadi, M., Jiménez-Aranda, A., Tassi, M., Abdo, W., Fernández-Vázquez, G. and Reiter, R.J. (2015). Melatonin Reduces Hepatic Mitochondrial Dysfunction in Diabetic Obese Rats. *J Pineal Res.*, 59(1), 70-9. doi: 10.1111/jpi.12241. Epub 2015 May 14. PMID: 25904243.

Andersen, L.P., Werner, M.U., Rosenkilde, M.M., Harpsøe, N.G., Fuglsang, H., Rosenberg, J. and Gögenur, I. (2016). Pharmacokinetics of Oral and Intravenous Melatonin in Healthy Volunteers. *BMC Pharmacol Toxicol.*, 17:8. doi: 10.1186/s40360-016-0052-2. PMID: 26893170; PMCID: PMC4759723.

Aouichat, S., Navarro-Alarcon, M., Alarcón-Guijo, P., Salagre, D., Ncir, M., Zourgui, L. and Agil, A. (2021). Melatonin Improves Endoplasmic Reticulum Stress-Mediated IRE1α Pathway in Zücker Diabetic Fatty Rat. *Pharmaceuticals (Basel),* 14(3), 232. doi: 10.3390/ph14030232. PMID: 33800157; PMCID: PMC8001258.

Bartoli, A., De Gregori, S., Molinaro, M., Broglia, M., Tinelli, C. and Imberti, R. (2013). Bioavailability of a New Oral Spray Melatonin Emulsion Compared with a Standard Oral Formulation in Healthy Volunteers. *Journal of Bioequivalence & Bioavailability,* 04(07). doi.org/10.4172/jbb.1000120

Brown, G.M., Pandi-Perumal, S.R., Trakht, I. and Cardinali, D.P. (2009). Melatonin and its Relevance to Jet Lag. *Travel Med Infect Dis.*, 7(2), 69-81. doi: 10.1016/j.tmaid.2008.09.004. Epub 2008 Oct 31. PMID: 19237140.

Chenevard, R., Suter, Y. and Erne, P. (2008). Effects of the Heart-Lung Machine on Melatonin Metabolism and Mood Disturbances. *Eur J Cardiothorac Surg.*, 34(2), 338-43. doi: 10.1016/j.ejcts.2008.03.035. Epub 2008 May 1. PMID: 18455411.

Constantinides, P.P. (1995). Lipid Microemulsions for Improving Drug Dissolution and Oral Absorption: Physical and Biopharmaceutical Aspects. *Pharm Res.*, 12(11), 1561-72. doi: 10.1023/a:1016268311867. PMID: 8592652.

Fourtillan, J.B., Brisson, A.M., Gobin, P., Ingrand, I., Decourt, J.P. and Girault, J. (2000). Bioavailability of Melatonin in Humans after Day-Time Administration of D(7) Melatonin. *Biopharm Drug Dispos.*, 21(1), 15-22. doi: 10.1002/1099-081x(200001)21:1<15::aid-bdd215>3.0.co;2-h. PMID: 11038434.

Grant, S.G., Melan, M.A., Latimer, J.J. and Witt-Enderby, P.A. (2009). Melatonin and Breast Cancer: Cellular Mechanisms, Clinical Studies and Future Perspectives. *Expert Rev Mol Med.*, 5, 11:e5. doi: 10.1017/S1462399409000982. PMID: 19193248; PMCID: PMC4301735.

Hardeland, R., Pandi-Perumal, S.R. and Cardinali, D.P. (2006). Melatonin. *Int J Biochem Cell Biol.*, 38(3), 313-316. doi: 10.1016/j.biocel.2005.08.020. Epub 2005 Sep 27. PMID: 16219483.

Harpsøe, N.G., Andersen, L.P., Gögenur, I. and Rosenberg, J. (2015). Clinical Pharmacokinetics of Melatonin: A Systematic Review. *Eur J Clin Pharmacol.*, 71(8), 901-909. doi: 10.1007/s00228-015-1873-4. Epub 2015 May 27. PMID: 26008214.

Hosseinzadeh, A., Kamrava, S.K., Moore, B.C.J., Reiter, R.J., Ghaznavi, H., Kamali, M. and Mehrzadi, S. (2019). Molecular Aspects of Melatonin Treatment in Tinnitus: A Review. *Curr Drug Targets,* 20(11), 1112-1128. doi: 10.2174/1389450120666190319162147. PMID: 30892162.

Huang, Y., Yu, Q., Chen, Z., Wu, W., Zhu, Q. and Lu, Y. (2021). *In Vitro* and *In Vivo* Correlation for Lipid-Based Formulations: Current Status and Future Perspectives. *Acta Pharm Sin B.*, 11(8), 2469-2487. doi: 10.1016/j.apsb.2021.03.025. Epub 2021 Mar 21. PMID: 34522595; PMCID: PMC8424225.

Kawakami, K., Yoshikawa, T., Hayashi, T., Nishihara, Y. and Masuda, K. (2002). Microemulsion Formulation for Enhanced Absorption of Poorly Soluble Drugs. II. *In Vivo* Study. *J Control Release*, 81(1-2), 75-82. doi: 10.1016/s0168-3659(02)00050-0. PMID: 11992680.

Kawakami, K., Yoshikawa, T., Moroto, Y., Kanaoka, E., Takahashi, K., Nishihara, Y. and Masuda, K. (2002). Microemulsion Formulation for Enhanced Absorption of Poorly Soluble Drugs. I. Prescription Design. *J Control Release,* 81(1-2), 65-74. doi: 10.1016/s0168-3659(02)00049-4. PMID: 11992679.

Lawrence, M.J. and Rees, G.D. (2000). Microemulsion-Based Media as Novel Drug Delivery Systems. *Adv Drug Deliv Rev.,* 45(1), 89-121. doi: 10.1016/s0169-409x(00)00103-4. PMID: 11104900.

Li, R., Luo, X., Li, L., Peng, Q., Yang, Y., Zhao, L., Ma, M. and Hou, Z. (2016). The Protective Effects of Melatonin Against Oxidative Stress and Inflammation Induced by Acute Cadmium Exposure in Mice Testis. *Biol Trace Elem Res.,* 170(1), 152-64. doi: 10.1007/s12011-015-0449-6. Epub 2015 Jul 30. PMID: 26224376.

Li, Y., Zhang, J.J., Xu, D.P., Zhou, T., Zhou, Y., Li, S. and Li, H.B. (2016). Bioactivities and Health Benefits of Wild Fruits. *Int J Mol Sci.,* 17(8), 1258. doi: 10.3390/ijms17081258. PMID: 27527154; PMCID: PMC5000656.

Oxenkrug, G., Requintina, P. and Bachurin, S. (2001). Antioxidant and Antiaging Activity of N-Acetylserotonin and Melatonin in the In Vivo Models. *Ann N Y Acad Sci.,* 939, 190-9. doi: 10.1111/j.1749-6632.2001.tb03626.x. PMID: 11462771.

Pandi-Perumal, S.R., BaHammam, A.S., Brown, G.M., Spence, D.W., Bharti, V.K., Kaur, C., Hardeland, R. and Cardinali, D.P. (2013). Melatonin Antioxidative Defense: Therapeutical Implications for Aging and Neurodegenerative Processes. *Neurotox Res.*, 23(3), 267-300. doi: 10.1007/s12640-012-9337-4. Epub 2012 Jun 28. PMID: 22739839.

Pandi-Perumal, S.R., Srinivasan, V., Poeggeler, B., Hardeland, R. and Cardinali, D.P. (2007). Drug Insight: The Use of Melatonergic Agonists for the Treatment of Insomnia-Focus on Ramelteon. *Nat Clin Pract Neurol.*, 3(4), 221-228. doi: 10.1038/ncpneuro0467. PMID: 17410109.

Pandi-Perumal, S.R., Zisapel, N., Srinivasan, V. and Cardinali, D.P. (2005). Melatonin and Sleep in Aging Population. *Exp Gerontol.,* 40(12), 911-25. doi: 10.1016/j.exger.2005.08.009. Epub 2005 Sep 23. PMID: 16183237.

Reiter, R.J., Ma, Q. and Sharma, R. (2020). Melatonin in Mitochondria: Mitigating Clear and Present Dangers. *Physiology* (Bethesda), 35(2), 86-95. doi: 10.1152/physiol.00034.2019. PMID: 32024428.

Rozencwaig, R., Grad, B.R. and Ochoa, J. (1987). The Role of Melatonin and Serotonin in Aging. *Med Hypotheses.,* 23(4), 337-352. doi: 10.1016/0306-9877(87)90054-5. PMID: 2889131.

Steptoe A. (2016). *Cortisol Awakening Response*. Elsevier eBooks. 277-283. doi.org/10.1016/b978-0-12-800951-2.00034-0

Suhner, A., Schlagenhauf, P., Johnson, R., Tschopp, A. and Steffen, R. (1998). Comparative Study to Determine the Optimal Melatonin Dosage form for the Alleviation of Jet Lag. *Chronobiol Int.*, 15(6), 655-66. doi: 10.3109/07420529808993201. PMID: 9844753.

Touitou, Y. (2001). Human Aging and Melatonin. Clinical Relevance. *Exp Gerontol.*, 36(7), 1083-100. doi: 10.1016/s0531-5565(01)00120-6. PMID: 11404053.

Xie, S., Fan, W., He, H. and Huang, F. (2020). Role of Melatonin in the Regulation of Pain. *J Pain Res.*, 13, 331-343. doi: 10.2147/JPR.S228577. PMID: 32104055; PMCID: PMC7012243.

Zamfir Chiru, A.A., Popescu, C.R. and Gheorghe, D.C. (2014). Melatonin and cancer. *J Med Life,* 7(3), 373-374. Epub 2014 Sep 25. PMID: 25408757; PMCID: PMC4233441.

Zduñska, A., Cegielska, J., Zduński, S., Bialobrzewski, M. and Kochanowski, J. (2021). Variability in Melatonin Concentration in Blood Serum of Patients with Episodic Migraine: A Pilot Study. *Neurol Neurochir Pol.*, 55(1), 81-90. doi: 10.5603/PJNNS.a2020.0095. Epub 2020 Dec 11. PMID: 33306192.

Zisapel, N., Tarrasch, R. and Laudon, M. (2005). The Relationship Between Melatonin and Cortisol Rhythms: Clinical Implications of Melatonin Therapy. *Drug Development Research*, 65(3), 119-125. doi.org/10.1002/ddr.20014

Supplementary Information

S. Table 1: Melatonin Blood Plasma Concentrations are Indicated for each Time Point for the Nanoemulsion and the Oral Tablet by Vitalis Nutrionals (Tablet VN). Values are Presented as Mean (C pg/mL) and Standard Deviation (SD pg/mL)

Time (min)	Nanoemulsion		Tablet VN	
	C (pg/mL)	*SD (pg/mL)*	C (pg/mL)	*SD (pg/mL)*
0	0	*0*	0	*0*
5	5020	*10763*	25	*88*
15	20923	*24233*	3816	*3112*
30	18433	*12931*	11356	*6924*
45	15791	*10749*	11148	*7355*
60	13939	*9748*	10137	*7351*
90	11080	*12812*	8762	*8725*
120	6990	*8251*	5449	*5390*
180	3263	*4208*	4135	*5103*
240	1688	*2122*	3181	*4902*
300	671	*1138*	757	*1202*
360	337	*573*	342	*661*
480	67	*164*	59	*146*
720	0	*0*	0	*0*
960	0	*0*	0	*0*
1440	0	*0*	0	*0*

Supplementary Information

S. Table 2: Melatonin Blood Plasma Concentrations are Indicated for Each Time Point for the Oral Spray and Tablet by SIRC. Values are taken from Bartoli *et al.* and are Presented as Mean Values (C pg/mL) and Standard Deviation (SD pg/mL)

Time (min)	Oral Spray		Tablet SIRC	
	C (pg/mL)	*SD (pg/mL)*	C (pg/mL)	*SD (ng/mL)*
0	380	*470*	0	*0*
5	1090	*850*	290	*260*
10	4790	*3870*	3210	*3840*
20	10440	*7200*	7010	*5240*
30	13080	*6290*	9090	*4360*
40	13140	*5970*	10620	*5650*
60	11430	*4730*	9410	*7040*
90	8520	*5610*	6550	*5090*
120	6580	*4700*	4770	*3650*
180	3310	*3170*	1760	*1610*
240	1660	*2090*	720	*580*
360	390	*480*	180	*150*

S. Table 3: Ratios of the Pharmacokinetic Parameters C_{max}, T_{max}, and AUC0-6h by Dividing the Values of the Nanoemulsion (NE) by the Values of the Tablet by Vitalis Nutritionals (Tablet VN), the Oral Spray and the Tablet by SIRC (Bartoli *et al.*)

	NE/Tablet VN	NE /Oral Spray	NE/Tablet SIRC
C_{max}	1.54	1.53	2.12
t_{max}	0.50	0.89	1
AUC_{0-6h}	1.31	1.24	1.81

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