Preparation and Characterization of a Polyurethane Nanocarrier Used for Mixtures of Betulin and Fatty Acids

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Betulin is one of the main components of birch bark which possess a wide range of biological activities (antiinflammatory, antiviral, and anticancer actions). Free fatty acids are often used in mediteranean diets due to their potential to reduce the blood concentration of low-density lipoproteins. In the present study, betulin - fatty acid mixtures were obtained and were encapsulated inside polyurethane nanostructures. There were studied the size and stability of the nanostructures and the thermal behaviour of samples. In vitro cells culture assays were used to observe the cytotoxic potential of synthesized products and Balb/c Nude mice were used to evaluate the skin irritation potential of these products. The results suggest that were obtained nanostructures containing betulin-fatty acid mixtures with a good size and stability, which did not present any skin irritation potential.

Keywords: betulin, fatty acid, polyurethane, drug-delivery system

The continuous increasing number of patients with different cancers leads to a remarkable series of changes in cancer drug discovery over the last two decades. Nowadays, the new targets reflect the humans increasing understanding of epigenetic and genetic modifications which are responsible for the initiation and malignant progression of cancers [1]. Free fatty acids are an important energy source for bodies and they also act as signaling molecules [2].

Specifically designed studies are necessary to observe the mechanisms of the glucose-free fatty acids cycle in carcinoma cells. K.F. Andersen and his team indicate a free fatty acids dependent metabolic boost/switch of glucose uptake in prostate cancer, with patterns reflecting the true heterogeneity of this disease. Their study also supports observations stating that saturation/equilibrium in prostate cancer cell uptake of glucose and acetate in a fixed setting is achieved within a period of 2 h [3].

Many plants and animal products are natural sources of oleic acid ($C_{18}H_{34}O_2$, OA, fig. 1a), an omega-9 fatty acid, and this is the reason why it is considered one of the healthier sources of fat in human diet. Health experts recommend it to obtain healthy foods and diet products in place of animal fats. Researchers often obtain oleic acid from triglycerides in order to conduct health studies.

Most experts agree that OA is one of the better fats for humans to consume. It decreases the amount of total cholesterol by increasing blood concentrations of lipoproteins with high densities and decreasing the concentration of lipoproteins with low densities. It was observed that OA can slow the development of heart diseases and also promotes the production of antioxidants, which can trap harmful free radicals in the body. These are the reasons why OA is used in homeopathic treatments for lowering cholesterol. Depending on the physician and the patient needs, OA may be prescribed in capsule form or patients may simply be advised to add more natural sources to their diets.

The well-known anticancer activity of olive oil is based on its OA content; this unsaturated fatty acid specifically regulate the oncogenes. J.A. Menendez and his team observed the ability of OA to repress the transcriptional activity of Her-2/neu gene [4]. A. Soto-Guzman demonstrated that OA induces extracellular-signal related kinase 1/2 activation and then activating protein-1-DNA complex formation in a fashion dependent of epidermal growth factor receptor and Src kinase activity in MCF-7 breast cancer cells. In addition, proliferation induced by OA is restricted to breast cancer cells and it is dependent upon the activation of ERK1/2 and matrix metallo-proteinases [5].

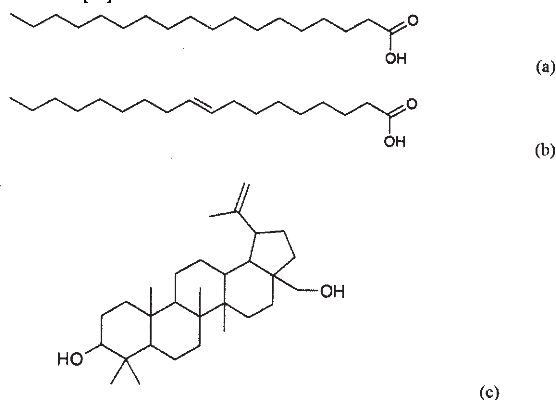

Fig. 1. The chemical structures of: (a) OA, (b) SA, (c) Bet

N. Navarro-Tito and his research team demonstrated that stimulation of breast cancer cells with OA encourages phosphorylation of the focal adhesion kinase, as it was observed by specific antibodies which can recognize the phosphorylation state at Tyr-397 and Tyr-577. They also observed that OA promotes the migration of MDA-MB-231 cells [6].

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Stearic acid ($C_{18}H_{36}O_2$, SA, fig. 1b) is an essential saturated fatty acid that was found in vegetables and animal oils. SA is a naturally occurring fat that is present in all of our bodies. Since free fatty acids were previously suggested to trigger hepatocyte apoptosis [7], M. Vinciguuerra and his team investigated whether OA affects HepG2 cells apoptosis and viability. In their study, there was proved that OA decreases the cell viability and increases the activity of caspase-3 when its concentration is greater than 0,1mM.Palmitoleic and linoleic acid act similarly to OA by increasing the proliferation and apoptosis, while saturated fatty acids, as SA, do not affect proliferation and are highly pro-apoptotic [8].

Betulin (Bet, fig 1c) is a well-known natural lupane triterpene with various pharmacological activities [9]. It is a compound that is widely distributed in nature and can be easily extracted from birch bark, a side product of forest industry [10], and betulin-derived compounds have most widely been studied for their anticancer activity. Betulin is a promising chemotherapeutic agent for the treatment and prevention of cancer because of its selective cytotoxicity against many different tumor cells [11].

The aim of this research was to obtain polyurethane nanostructures with mixtures of Bet with OA and SA and to characterize these products.

Experimental part

Materials, equipment and methods

Raw materials

Bet was extracted from the bark of *Betula pendula* using a procedure described in the literature by C.A. Dehelean and her group [12]. OA and SA were purchased from Sigma-Aldrich.

The raw materials for the synthesis of polyurethane nanostructures were the following: isophorone-diisocyanate (IPDI) from Aldrich, ethylene glycol (EG) from Lach-Ner (Czech Rep.), 1,4-butanediol (BD) from Carl Roth GmbH (Germany), polyethylene glycol, M=200 (PEG) and solvent (acetone) from Merck (Germany). Emulsifier (Cremophor EL) was obtained as donation from University of Szeged (Hungary).

All reagents were used without any previous purification.

Sample preparation and characterization

There were obtained Bet - fatty acid mixtures 1:2 (w/w) using acetone as solvent and an ultrasonic processor UP200S (Hielscher Ultrasound Technol., Germany) three hours daily for one week. There were prepared three different samples: Bet in acetone as control (sample 1), Bet:OA in acetone (sample 2), and Bet:SA in acetone (sample 3); 1:30 dilution ratio was used in order to assure the complete solubilization of mixtures in acetone. There was confirmed on infrared spectra that no ester groups were formed between the hydroxyl groups from Bet and the carboxyl groups from the fatty acids.

The protocol for obtaining of polyurethanes nano-structures was already described in the literature in previous papers [13-15]. It is a multi-step procedure, as follows:

- preparing of the organic phase: 0.5 mlL sample described previously and 1 mL emulsifier (Cremophor EL) were added to 20 mL solution 10% of isophoro-nediisocyanate in acetone and were magneticaly stirred at 400 rpm and 45°C for 2 h;
- preparing of the aqueous phase: 40 mL aqueous solution 10% of EG, BD and PEG (2:2:1, v/v) mixture were magnetically stirred at 400 rpm and 45°C for 2 h;
- mixing of the two phases: the organic phase was rapidly injected into the aqueous phase under magnetical stirring at 900 rpm and 60°C;
- finishing of chemical reaction: stirring was continued at 900 rpm and 60°C for other four hours to ensure the maturation of nanostructures walls;
- purification of the products: acetone and water were removed by keeping the obtained suspensions as thin layers at 60°C in the oven for 12 h. The final powders were repeatedly washed, centrifuged, and dried using a water-acetone mixture (1:1, v/v).

The samples containing polyurethanes nanostructures loaded with active substances were labeled as: nanostructures with Bet (sample 4), nanostructures with Bet:OA mixture (sample 5), and nanostructures with Bet:SA mixture (sample 6).

The size and the charge of the polyurethane nanostructures loaded with active substances were measured using a CordouanZetasizer equipment (Cordouan Technol., France) containing a Vasco Particle Size Analyzer and a Wallis Zetapotential Analyzer. For this purpose, aqueous solutions (1:100 w/v) were used; the measurements were carried out three times for each sample. The following Vasco Particle Size Analyzer parameters were chosen: temperature (25°C), time interval (between 10-15 μs), and number of channels (400-450), laser power (between 70-80%), acquisition mode (continuous), and analysis mode (Pade-Laplace). The following Wallis Zetapotential Analyzer parameters were chosen: cuvette type (plastic, with a wavelength from 380 to 780 nm, visible spectrum), temperature (25°C), resolution (medium, 0.8 Hz), and Henry function (Smoluchowski).

Thermal behaviour of samples was studied using a Mettler-Toledo DSC1 instrument (Mettler-Toledo, Switzerland) between 30-300°C in an oxidative atmosphere using aluminum crucibles and a 5 degree/min heating speed.

For *in vitro* study on cells culture, human dermal fibroblasts (HDFa, Invitrogen, USA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal calf serum (FCS, PromoCell, Germany) and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell, Germany). Cells were maintained at an atmosphere of 5 % CO_2 at 37 °C.

During the viability assay: Alamar Blue *in vitro* analysis, the human dermal fibroblasts HDFa (Invitrogen, USA) were seeded onto a 96-well microplate (5000 cells/plate) and attached to the bottom of the well overnight. After 24 h, 150 μL of new medium containing DMEM supplemented with 20% FCS, and 1% Pen/Strep mixture and the tested substances were added. After an incubation of 24 h and 48 h, respectively, 15 μL of the Alamar Blue solution was added and the cells were incubated for 4 h at 37°C. Finally, the samples were analyzed using a spectrophotometer. The wavelengths were 570 nm, 600 nm respectively; wells with untreated cells were used as controls. All *in vitro* experiments were performed on microplates with at least four parallel wells. Dimethyl-sulfoxide (DMSO) was used to prepare stock solutions of evaluated substances, and the highest DMSO concentration (0.1 %) of the medium did not have any significant effect on cell proliferation. The concentration of stock solution was 10 mM, the dilution rate was 1:1000, and the final concentration was 10 μM.

Cells viability was calculated using the following formula [16]:

$$\frac{\varepsilon ox2\ A1 - \varepsilon ox1\ A2}{\varepsilon red1\ A2 - \varepsilon red2\ A1} \cdot 100 \qquad (1)$$

where:

εox1 = molar extinction coefficient for oxidized Alamar Blue at 570 nm

εox2 = molar extinction coefficient for oxidized Alamar Blue at 600 nm

εred1 = molar extinction coefficient for reduced Alamar Blue at 570 nm

εred2 = molar extinction coefficient for reduced Alamar Blue at 600 nm

A1 = absorbance of tested cells at 570 nm

A2 = absorbance of tested cells at 600 nm

Annexin V-FITC (MiltenyiBiotec, Germany) and propidium iodide (PI) staining solution (BD Biosciences, USA) were used in the cell death flowcytometric studies (apoptosis). 10^6 cells were washed in 1 × Annexin V Binding Buffer (BD Pharmigen), centrifuged at 300 g for 10 min, resuspended in the same solution and incubated with 10μL of Annexin V-FITC for 15 min in the dark. The cells were washed with 1 mL specific binding buffer and centrifuged, and then the cell pellet was resuspended in 500 μL binding buffer. 1 μg/mL of PI solution was added immediately prior to analysis by flow cytometry.

Six Balb/c Nude, homozygote, five weeks old, healthy male mice, purchased from Charles River (Sulzfeld, Germany) were used in this research. The following standard conditions, which are rules of the National Institute of Animal Health (NIAH), were used for mice during the evaluations: repeated cycles of 12 h light-dark, water and food *ad libitum*, a temperature of 24±1°C, and a humidity above 55%. The samples were applied on the mice skin daily for one week and after each application, skin parameters evaluation was performed within 60 min. All the measurements were carried out with a Multiprobe Adapter System (MPA5) from Courage&Khazaka Electronics, Germany, equipped with: a Tewameter®TM300 probe, a Mexameter®MX18 probe, and a Corneometer® CM825 probe. All measurements were done in triplicate, by the same person, in a narrow range of temperature (24±1°C) and air humidity (55±3%) at the same hour in the morning. The obtained values were prepared as differences between an area were sample was applied and a reference area (fig. 2).

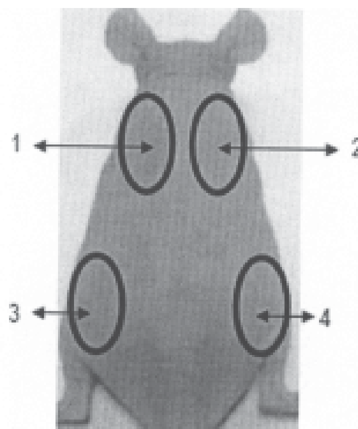


Fig. 2. Scheme of procedure for obtaining values of skin parameters

The areas 1 and 3 were used for sample application while the areas 2 and 4 were used as reference. The formula used to obtain the differences between values was: $\Delta p = [(p_1-p_2)+(p_3-p_4)] / 2$, where p_i means skin parameter as TWL, erythema or moisture of *stratum corneum*. There were used only positive values even if some differences were negative (in the case of moisture of *stratum corneum*).

Results and discussions

The suspensions which contain submicron particles often present a complex particle size distributions; the size distribution can be broad (polydisperse) or the suspension can consist of several distinct particle populations of varying size (multimodal) [17]. The diluted aqueous solutions (1:100, w/v) of polyurethane nanostructures were analyzed by a Vasco Particle Size Analyzer in order to examine the size distribution (fig 3-5).

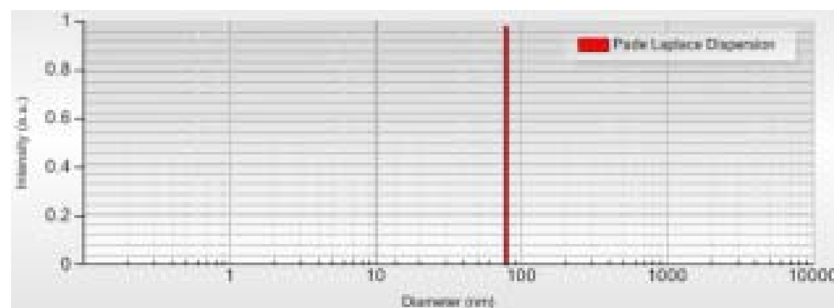


Fig. 3.The average diameter of polyurethane nanostructures (sample 4)

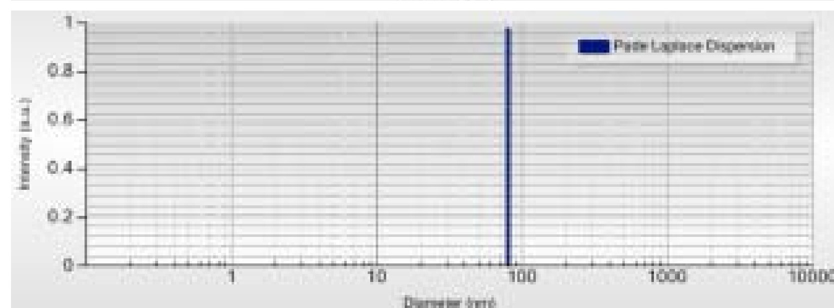


Fig. 4.The average diameter of polyurethane nanostructures (sample 5)

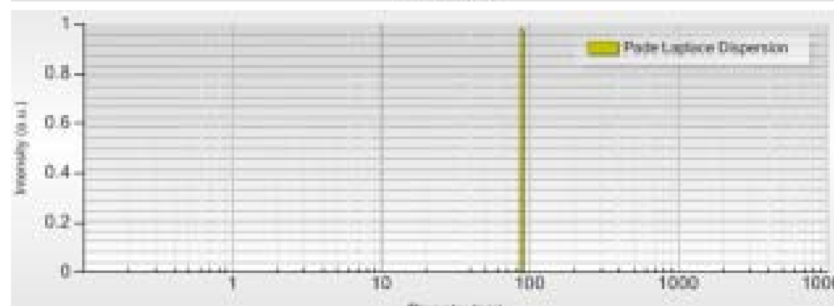


Fig. 5.The average diameter of polyurethane nanostructures (sample 6)

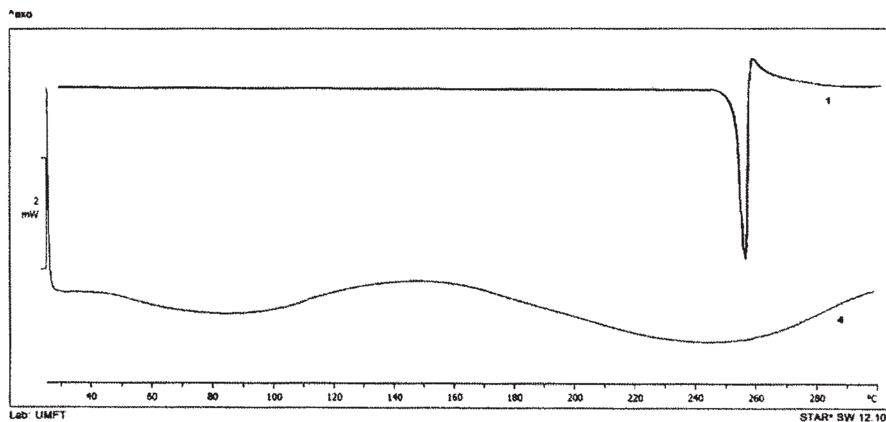


Fig. 6.DSC curves for sample 1 vs. sample 4

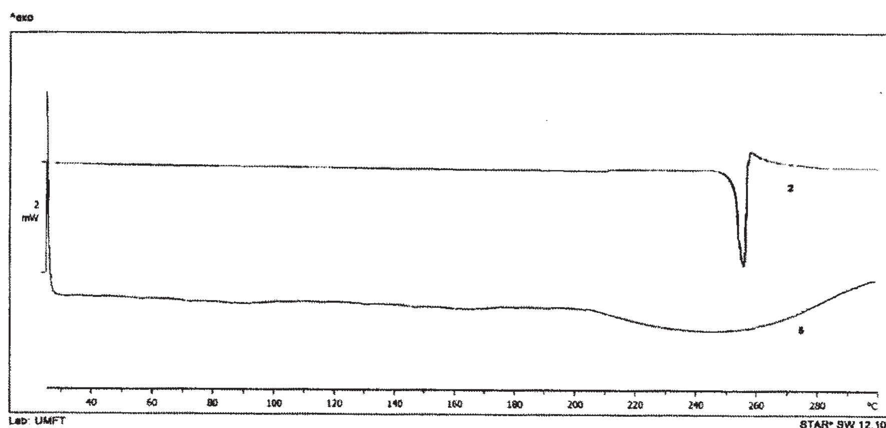


Fig. 7.DSC curves for sample 2 vs. sample 5

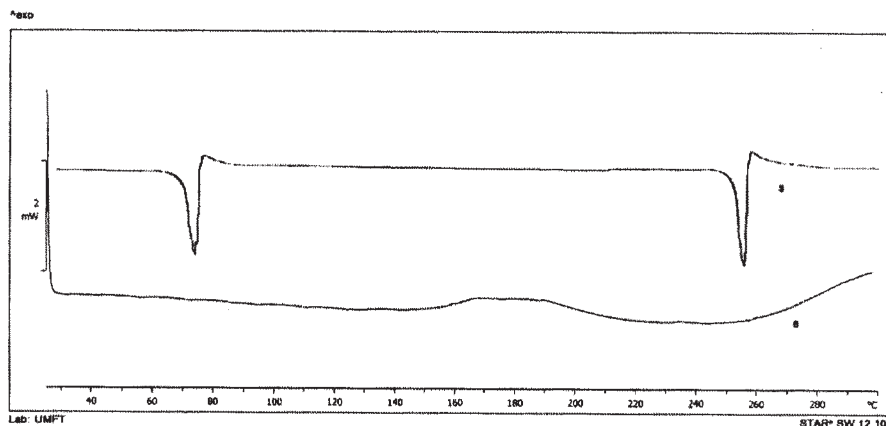
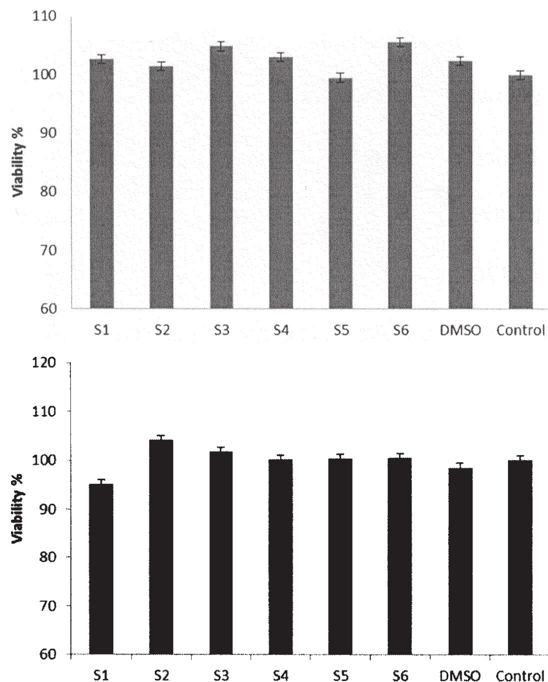


Fig. 8.DSC curves for sample 3 vs. sample 6

The active substances loaded in the three samples with polyurethane nanostructures do not influence the diameter of particles; the three samples present sizes between 87-95 nm as it can be seen in figure 3-5. There was obtained only one population in each case and the polydispersity index values (0.1-0.2) indicate that homogeneous samples were obtained. The recorded Zeta potential values were situated to the border between stable-unstable particles: 31.5 ± 3.2 mV (sample 4), 29.1 ± 2.7 mV (sample 5), and 30.2 ± 2.3 mV (sample 6). Scientific researchers mention that particles, with a Zeta potential within the range of 20-30 mV, have a medium stability degree, while particles, with a Zeta potential above 30 mV, present a high stability degree [18].

The DSC diagram of sample 1 (fig. 6) exhibit an endothermic peak around the temperature of 260°C, corresponding to the melting point of Bet. The melting point of OA (around 315°C) was not observed in the studied range (fig. 7); this is the reason why there were not obtained too many differences between figure 6 and figure 7. In figure 8, sample 3 presents two endothermic peaks which correspond to the melting point of SA (around 70 °C) and Bet (at 260 °C). There were not observed any peaks on the DSC curves of polyurethane nanostructures because the urethane groups from polymer chains begin to degrade over 300 °C. The absence of peaks from samples 4, 5, and 6 is an important evidence that the active substance (simple Bet or Bet-fatty acid mixtures) is well-protected inside the polyurethane nanostructures.

The samples were tested for their cytotoxic activity on human dermal fibroblasts HDFa. Figure 9 presents the viability of HDFa after exposure to the tested compounds for 24 h (a) and for 48 h (b). It can be observed that neither after 24 h, nor after 48 h the tested compounds had no major toxic activity on human dermal fibroblast cells. After 48 h, sample 1 (betulin) reduced the viability of cells with 5%, but the other samples had no cytotoxic activity compared to the control. The proapoptotic effect of the compounds was tested using Annexin V/FITC assay. As figure 9 show, none of the tested substances had a major proapoptotic effect on HDFa cells. The maximum percent of early apoptotic cells was 5.59 % for S6, while the maximum of the late apoptotic cells was 4.35 % for S1. S2 was observed to induce the lowest apoptosis to HDFa cells, only 2.95% of cells being early apoptotic and 1.72 % being late apoptotic. Taken together, Alamar Blue assay and Annexin V/FITC assay show that none of the compounds



a

b

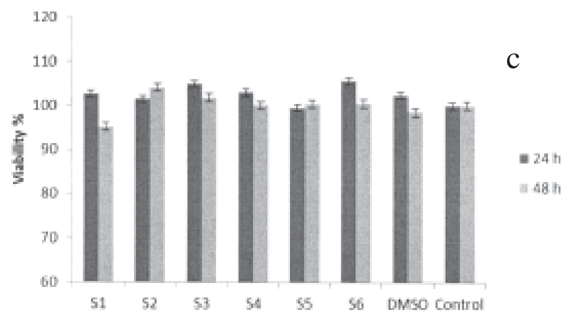


Fig. 9.HDFa viability after (a) 24 h , (b) 48 hours, (c) 24 and 48 h exposure on the tested compounds

present toxicity on normal human dermal fibroblast HDFa cells. These results can lead to the conclusion that all the formulations obtained could be safe for administration.

Visual observations of skin modifications was one of the best methods of assessing skin characteristics and health, but the development of novel tools which are able to reduce the interobserver variability was necessary. Nowadays, tewameter, skin pH-meter, sebumeter, mexameter, and corneometer measurements are noninvasive techniques used to evaluate the skin changes.

The evolutions of average values that included main parameters such as transepidermal water loss (TWL), erythema, and moisture of stratum corneum for each mouse are shown in figure 12-14.

In vivo evaluations of samples with Bet (1), mixtures of Bet with fatty acids (2, 3) and samples of polyurethane nanostructures with previously mentioned substances (4-6) were done based on two important features of nude mice skin: it is very sensitive and it has a penetration degree a few times greater than human skin [19]. This is why the mice skin can be used to evaluate the irritation potential of new synthesized chemical products.

TWL evaluations (which describe the rate at which vapours of water are lost from body through the skin) are very important for description of the barrier functionality [20]. It can be observed in figure 12 that higher values of TWL were recorded for all six samples, and the best values were obtained for samples containing polyurethane nanostructures (grey curves): between 0.4-0.7 g/cm^2/h after one week.

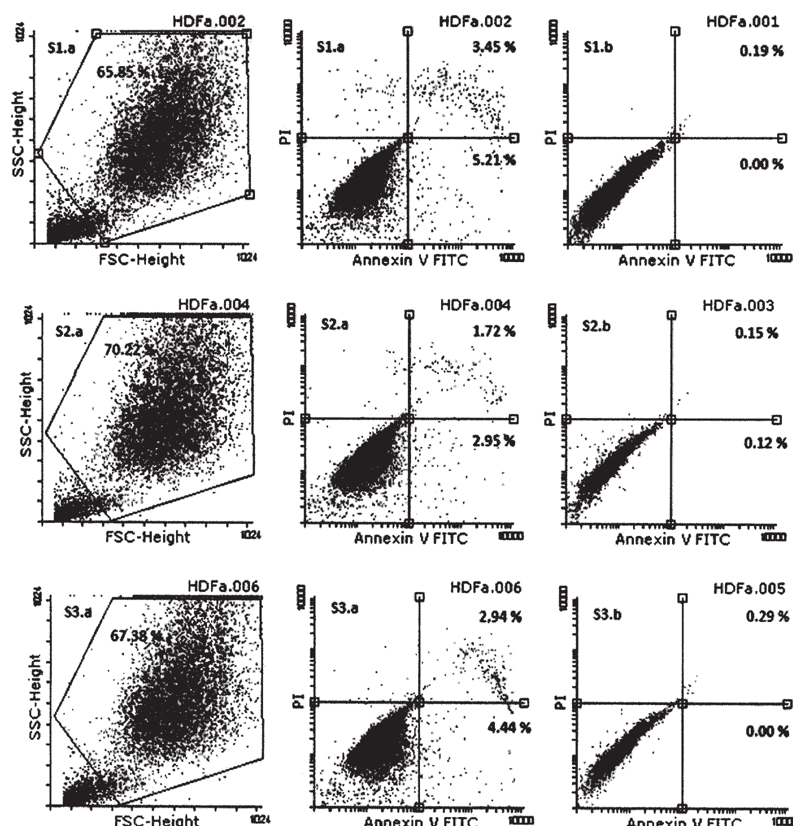


Fig. 10.Annexin pictures for samples 1-3

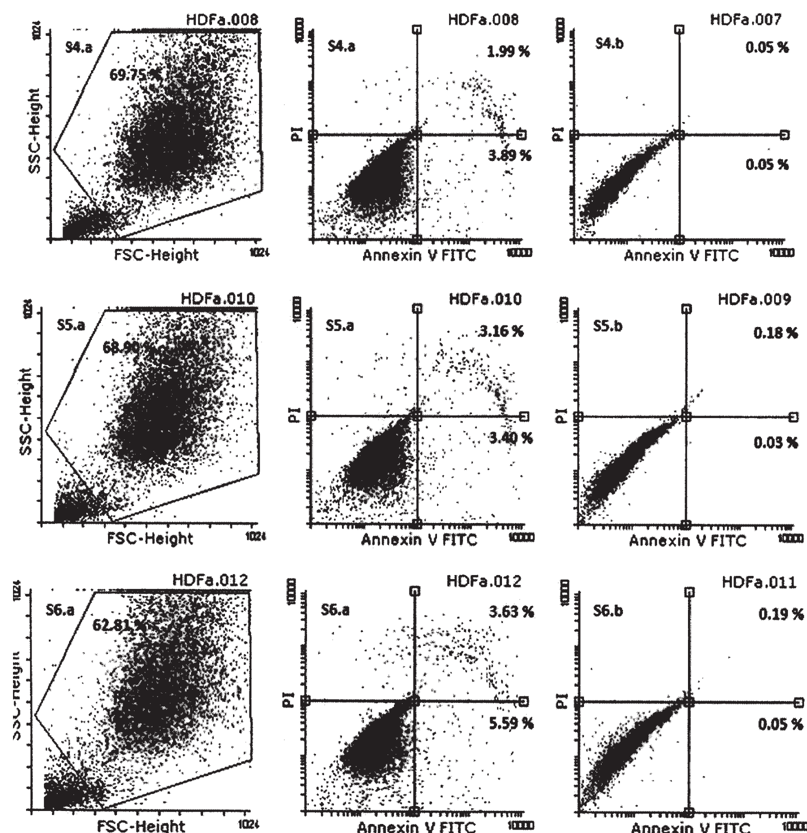


Fig. 11.Annexin pictures for samples 4-6

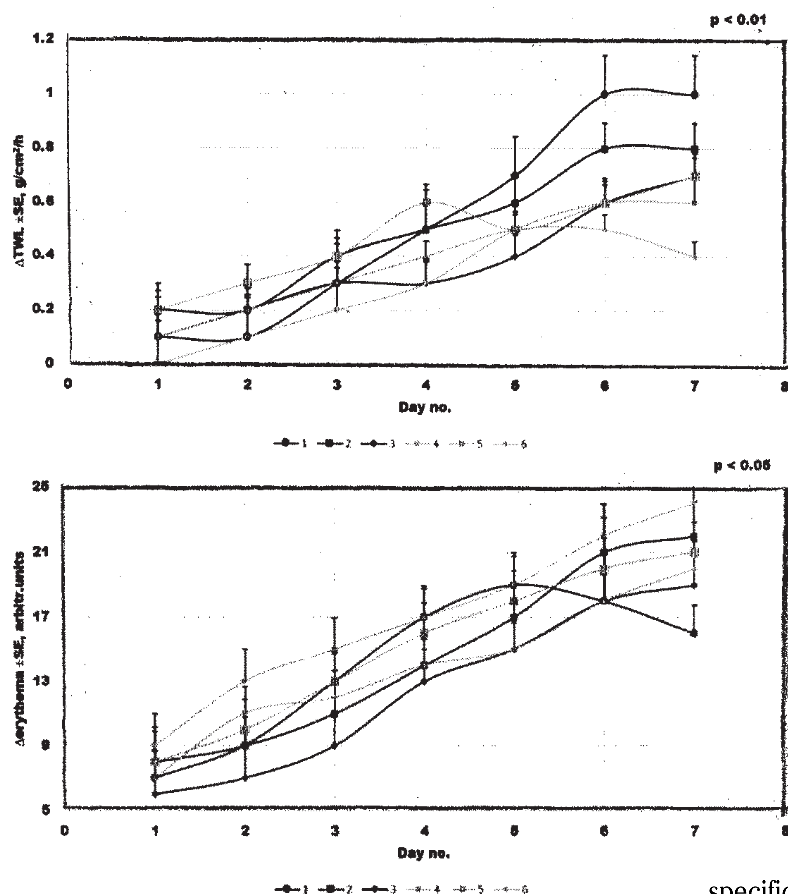


Fig. 12.Evolution of mice skin TWL

Fig. 13. Evolution of erythema

The Mexameter®MX18 is a skin probe developed by Courage-Khazaka to evaluate the content of melanin and erythema. The measurement is based on the absorption-reflection. The probe emits light at three specific wavelengths and a detector measures the light reflected by the skin; specific wavelengths which correspond to different absorption rates of pigment, are used to measure the melanin, while for the erythema measurement, the specific wavelengths correspond to the absorption peak of haemoglobin spectra [21].The erythema value increases very much and fast in the presence of any irritation given by an aggressive agent. The erythema values recorded in this seven days experiment have an easily growing trend for all the samples (fig. 13), but it can be considered as a normal trend for a skin treatment (the average increase is about 10-15 units / sample). This is the reason why it could

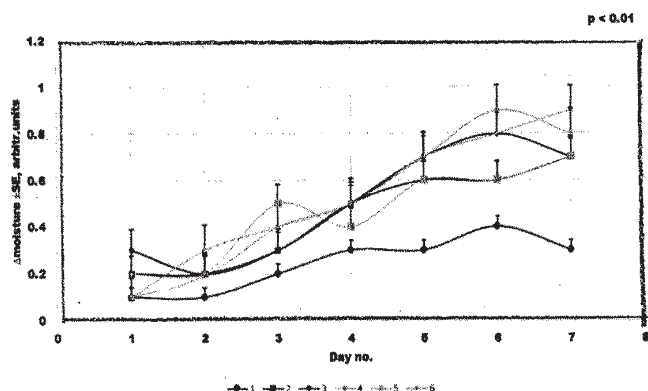


Fig. 14. Evolution of moisture of stratum corneum

be considered that these products does not present any irritation on the mice skin.

The measurement of moisture of stratum corneum, the skin surface, is based on capacitance measurement of a dielectric medium. This probe determines any change of dielectric constant and it contains a precision capacitor which measure the capacitance modifications due to changes of skin surface hydration state [21]. In this experiment it was observed that samples do not influence too much the moisture level of *stratum corneum*; the values are inside a narrow range, between 0.1-0.9 units (fig 14).

Conclusions

Betulin - fatty acid mixtures were obtained and were incorporated inside a polyurethane drug delivery system in order to facilitate their transfer through membranes. The polyurethane nanostructures presented good values for their size and stability. It was observed that the active compounds did not influence the carrier characteristics. Cells viability presents lower increases in the case of mixtures and nanostructures with mixtures compared to the values recorded for betulin. The evaluations of skin parameters shown that these products are not irritative: the TWL level was not increased too much, the erythema index increases just a little, and the moisture of *stratum corneum* did not decrease too much during experiment.

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