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# Insulin-Loaded SLN Prepared with the Emulsion Dilution Technique: In Vivo Tracking of Nanoparticles after Oral Administration to Rats

M. Trotta, M. E. Carlotti, M. Gallarate, G. P. Zara, E. Muntoni e L. Battaglia

**Abstract**: Insulin-loaded solid lipid nanoparticles (SLN) were prepared according to a solvent dilution method from O/W emulsions using isovaleric acid as organic phase. Insulin was derivatized with fluorescein isothyocianate (FITC) obtaining a fluorescent marker to be used in in vivo experiments. FITC-insulin and native insulin–loaded SLN were quite similar with regard to their mean sizes and encapsulation efficiency. SLN intestinal uptake was then investigated administering FITC-insulin loaded SLN on healthy male Wistar rats. Significant drug accumulation within intestinal lymphatic system was recovered, but the immune system seems to play an important role in SLN degradation: further studies are necessary to improve the results on blood glucose level.

## INTRODUCTION

Insulin is a peptide hormone and it is the only drug that is effective for Type I diabetes treatment, which needs substitutive therapy, but frequently it becomes essential also for severe Type II diabetes. Since insulin is not absorbed after oral administration because of its molecular weight (5800 Da) and of its degradation by pepsin and trypsin in gastrointestinal tract, it has always been administrated by subcutaneous injection.

Insulin pulmonary administration is now entering diabetes therapy, but the best solution to meet patients' compliance is always the oral administration. Several attempts have been made for enhancing insulin intestinal uptake, either by colon release forms,[ 1 ] or by lymphatic uptake of colloidal pharmaceutical forms: microemulsions,[ 2 ] nanocrystals,[ 3 ] and nanoparticles.[ 4 ]

Among nanoparticles, solid lipid nanoparticles (SLN) could be considered the best choice for lymphatic uptake, owing to their lipid nature, as documented in literature.[ 5-7 ] Lymphatic uptake involves many steps: after initial absorption by Peyer's patches,[ 8 ] particles with diameters smaller than 5 μm can be collected by mesenteric lymph nodes, where they come in contact with lymphocytes. For this reason colloidal systems are used as adjuvant in vaccine oral delivery;[ 9 ] the mesenteric lymph nodes content is then drained by the lymph, which leads to venous circulation through lymphatic duct.

Our research group has previously developed a technique that, applying the solvent dilution method to an O/W emulsion with isovaleric acid as internal phase, allows to encapsulate insulin in solid lipid nanoparticles (SLN).[ 10 ] This method has proved to be suitable to obtain insulin entrapment in the lipid core without damaging its physicochemical stability; moreover, SLN entrapment proved not to reduce insulin biological activity, as confirmed by in vivo experiments.

The aim of this study is to evaluate the intestinal absorption of insulin after SLN duodenal administration to rats, and to track its biological route, investigating its possible effects on glucose level. Fluorescein isothyocyanate (FITC) was conjugated with insulin in order to obtain a fluorescent probe to be monitored in lymph, blood and tissues after administration.

## EXPERIMENTAL

### Materials

Bovine pancreas insulin, sodium taurodeoxycholate (TDC) and Histopaque 1083 were from Sigma (Dorset, UK), FITC and cholesterol were from Fluka (Buchs, Switzerland), glyceryl monostearate (Tegin M–GMS) was from Goldschmidt (Essen, Germany), soy lecithin (Epikuron 200) was from Lucas Meyer (Hamburg, Germany), isovaleric acid (IVA), was from Aldrich (St Louis, MO, USA), methanol and acetonitrile were from Carlo Erba (Milano, Italy), deionized water was obtained by a MilliQ system (Millipore, Bellerica, MA, USA); all other chemicals were analytical grade and used

without any further purification; male Wistar rats weighing 250 grams were used for in vivo experiments. All animals experiments were carried out in accordance with the European Committee Council Directive of 24 November 1986 (86/609/EEC) and the protocol was approved by the local Ethic Committee of the University of Torino.

**FITC-Insulin**

*Synthesis*

FITC-insulin was synthesized slightly modifying a method described in literature:[ 11 ] briefly 6 mmoles insulin were dissolved in 60 ml 0.1 M sodium carbonate; 18 mmoles FITC were dissolved in 6 ml methanol; the solutions were mixed and allowed to react for 12 hours. The selective precipitation of the fluorescent peptide was performed by bringing pH to 4.5 with 0.1 M HCl: the sample was then centrifuged at 14,000 rpm for 15 minutes with an Allegra64 centrifuge (Beckman Coulter, CA, USA); the precipitate was washed with 5 ml methanol to withdraw unreacted FITC. After centrifugation the precipitate was dried under vacuum overnight.

*HPLC Analysis*

FITC-insulin was analyzed by a HPLC method in alkaline condition, partially modified from literature. [12].Native insulin and its fluorescent derivative were separated by using a CH3CN/pH 8.0 0.1 M phosphate buffer (11.5:88.5) with a C18 Chromo system 150 × 4.4 mm column: peaks were analyzed both with RF-551 fluorimeter (Shimadzu, Kyoto, Japan) (λexc = 494, λem = 517) and SPD-10A spectrophotometer (Shimadzu, Kyoto, Japan); pump was a LC9 (Shimadzu, Kyoto, Japan); data were collected with a CR5 Chromatopac (Shimadzu, Kyoto, Japan). The LOQ, defined in the presented experiment as the lowest insulin concentration that can be measured routinely with acceptable precision and accuracy was 0.080 µmol ml−1; the LOD, defined as the lower detection limit was 0.040 µmol l−1.

A SE-HPLC method was also employed in the evaluation of entrapment efficiency in SLN, using a TSK gel G2000SWXL (Tosoh Bioscience, Stuttgart, Germany) and adapting a method described in literature.[ 13 ] The mobile phase was CH3CN/0.05% TFA (10:90). Detector was a SPD-10A spectrophotometer (Shimadzu, Kyoto, Japan). The LOQ was 1.0 µmol ml−1; the LOD was 0.40 µmol l−1.

**Solid Lipid Nanoparticles**

*Preparation*

SLN were prepared according to the emulsion dilution technique.[ 10 ] The composition of the O/W emulsion which was diluted to obtain SLN is reported in Figure 1. SLN were prepared with a) insulin in order to evaluate the effect on glucose level and b) with FITC-insulin to track the drug uptake after duodenal administration to rats.

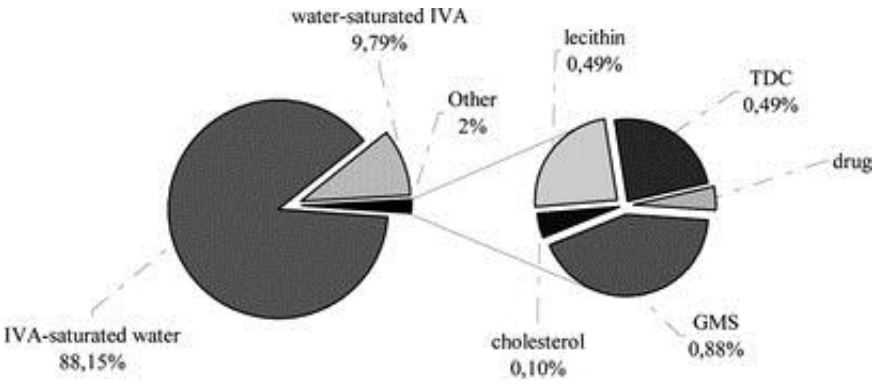


**FIG. 1** Schematic representation of O/W emulsion.

*Characterization*

Mean particle size was determined with a 90Plus Particle Size Analyzer (Brookhaven Instrument Corporation, Holtsville, NY, USA).

*Encapsulation Efficiency*

Encapsulation efficiency, that is, the ratio between the amount of drug entrapped in SLN and the amount of drug used for the preparation of nanoparticles, was calculated by extracting the peptide from SLN with a procedure described in a previous work.[ 10 ] and then analyzing by SE-HPLC. HPLC fluorimetric method was used to confirm encapsulation efficiency of FITC-insulin loaded SLN.

**In Vivo Studies**

*FITC-Insulin-Loaded SLN Intestinal Uptake*

An amount of 1.5 ml of FITC-insulin loaded SLN, concentrated by dialysis up to 1.0% w/v lipid and 0.1% w/v drug (0.1 mg/mg lipid), were administered through a surgically implanted duodenal cannula to adult male Wistar rats (weight 250 g), which were fasted for 12 hours prior of the experiment, but allowed access to water ad libitum.

Two different studies were performed on male Wistar rats: 1) analysis of drug uptake in lymph and lymph nodes, and 2) analysis of drug recovered in blood. Each analysis was performed on three rats and data were represented as mean ± standard deviation.

As reference FITC insulin suspension in normal saline was administered at the same concentration.

*Lymph*

Lymph samples (0.5 ml) were collected for 3 hours after SLN administration by means of a catheter surgically positioned in the lymphatic duct.

Collected lymph was observed by fluorescence microscope (Leica DM 2500, Wetzlar, Germany) at λexc = 494 λem = 517, and then centrifuged at 14,000 rpm for 5 minutes with 5417 Centrifuge (Eppendorf, AG, Hamburg, Germany). The supernatant was added of an equal volume of methanol

to precipitate lymph proteins, and then centrifuged at 14,000 rpm for 5 minutes: the clear solution was transferred to a glass vial and dried under nitrogen to avoid fluorescence quenching of FITC-insulin due to the organic solvent. Finally, the residue was dissolved in 200 µl 0.01 M pH 9.0 phosphate buffer and analyzed by HPLC-fluorimetry for FITC-insulin. The precipitate was extracted overnight with 1 ml methanol, then 1 ml water was added and the sample was centrifuged at 14,000 rpm for 5 minutes: the clear solution was transferred to a glass vial and dried under nitrogen, as described above; finally the residue was dissolved in 200 µl 0.01 M pH 9.0 phosphate buffer and analyzed by HPLC-fluorimetry for FITC-insulin.

*Lymph Nodes*

At scheduled times (3, 4, and 5 hours after administration) rats were sacrificed by CO2-induced euthanasia and mesenteric lymph nodes were surgically removed.[ 6 ]

An amount of 1 ml methanol was added to weighted lymph nodes and kept under stirring overnight; 1 ml water was then added and the sample was centrifuged at 14,000 rpm for 5 minutes: the clear solution was dried under nitrogen, as described before; finally the residue was dissolved in 200 µl 0.01 M pH 9.0 phosphate buffer and analyzed by HPLC-fluorimetry for FITC-insulin.

*Blood*

At scheduled times after SLN administration, blood samples (0.5 ml) were collected by means of a catheter surgically positioned in the jugular vein within 6 hours and treated as follows in two distinct series of experiments. First, they were centrifuged at 14,000 rpm for 5 minutes. The supernatant was analyzed as described as above for the lymph, while the cellular fraction was dried under vacuum and then extracted overnight with methanol as described for lymph precipitate. Second, they were centrifuged in gradient with Histopaque 1083 to isolate lymphocytes from other blood cells:[ 14 ] lymphocytes, forming a layer in centrifuge tube, were withdrawn and suspended in water, centrifuged again and then dried under vacuum overnight.

*Native Insulin-Loaded SLN Administration*

Native insulin-SLN were washed and concentrated by dialysis as described before for FITC-insulin loaded SLN; 0.5 ml SLN, corresponding to 0.5 mg drug were then administered through a duodenal cannula to 3 healthy adult Wistar male rats (weight 250 g), which were fasted for 12 hours prior of the experiment, but allowed access to water ad libitum.

Blood samples were collected by means of a catheter surgically positioned in the jugular vein, within 6 hours and serum glucose level was determined by the enzymatic test Glucosio-GOD-PAP (Roche, Basel, Switzerland) using a Boehringer Mannheim/Hitachi 912 Analyzer.

Each analysis was done in triplicate and collected data were represented as mean ± standard deviation and compared with data obtained after subcutaneous injection, reported in a previous work.[ 10 ]

## RESULTS AND DISCUSSION

### FITC-Insulin

Synthesis of labeled peptide was performed with a conjugation yield, calculated as the ratio between labeled peptide and the total (labeled and not labeled) peptide recovered at the end of the synthesis, was of 73%.

### SLN Characterization

FITC-insulin and native-insulin loaded SLN were quite similar with regard to their mean diameters and polydispersion index and only a slight reduction in entrapment efficiency was noted for FITC-insulin respect to native insulin (Table 1). These results strengthen the possibility to use the fluorescent derivative of insulin to track the peptide-loaded SLN after oral administration.

| SLN | Mean diameter (nm) | Polydispersion | Entrapment efficiency (%) | |
|---|---|---|---|---|
| | | | SEC-UV | C18-fluorimeter |
| Insulin loaded | 596 ± 23 | 0.20 | 66.0 ± 4.1 | |
| FITC-insulin loaded | 559 ± 15 | 0.22 | 61.7 ± 2.9 | 57.8 ± 5.5 |

**TABLE 1** Size distribution and entrapment efficiency of FITC-insulin and insulin loaded SLN

### In Vivo Studies

Fluorescence microscope images in Figure 2 show a SLN suspension (A) and a lymph sample (B) collected 3 hours after FITC-insulin SLN administration. Many fluorescent particles, quite similar to SLN simply dispersed in water are clearly visible in the biological sample.

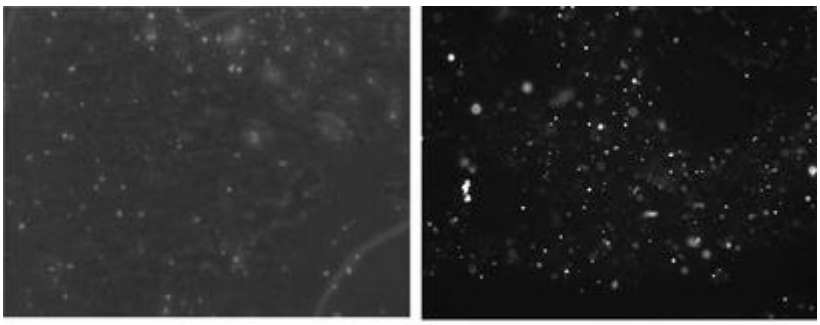


**FIG. 2** Fluorescence microscope micrographs of SLN suspension (A) and lymph concentrated samples (B).

The evidence of the presence of FITC-insulin in lymph was further confirmed by lymph nodes and lymph analysis. Interestingly, comparing the amounts of FITC-insulin in lymph nodes at different times after SLN administration, a decreasing trend from 120 mg/g to less than 1 mg/g in 2 hours was observed (Figure 3).

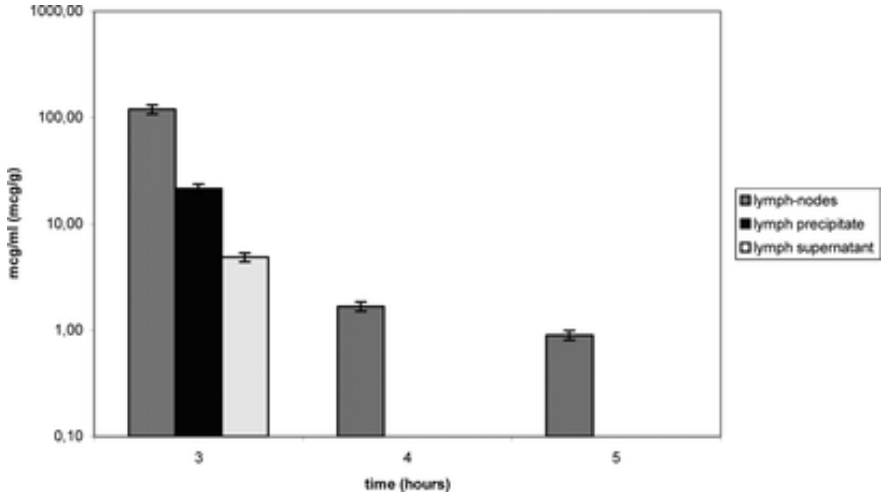


**FIG. 3** FITC-insulin recovery in lymph and lymph nodes.

Despite a relevant FITC-insulin amount was recovered in the lymphatic system, it should be noticed that only a small fraction of the drug (less than 20% of the total 26.5 µg/ml) was detected as "free" in lymph supernatant, which was then drained directly in the blood stream through the lymphatic duct.

On the contrary, no labeled peptide was recovered either in lymph nodes or in lymph after administration of FITC-insulin suspension in normal saline. Surprisingly, no FITC-insulin amount was recovered in plasma, while the same drug was detected in blood cellular fraction (see Figure 4). By means of gradient centrifugation, it was evidenced that lymphocytes were involved in FITC-insulin uptake, since peptide was specifically recovered within these cells.

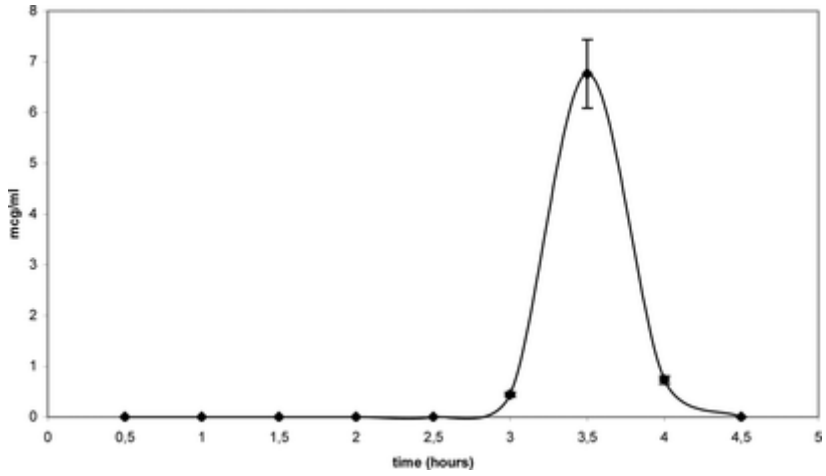


**FIG. 4** FITC-insulin recovery in blood.

All these data suggest a possible drug uptake by phagocytes, leading to drug degradation. This could explain the lack of a significant glucose level reduction after duodenal administration of

native insulin-loaded SLN. Figure 5 shows the change in blood glucose levels in rats that received the native insulin-loaded SLN suspensions.

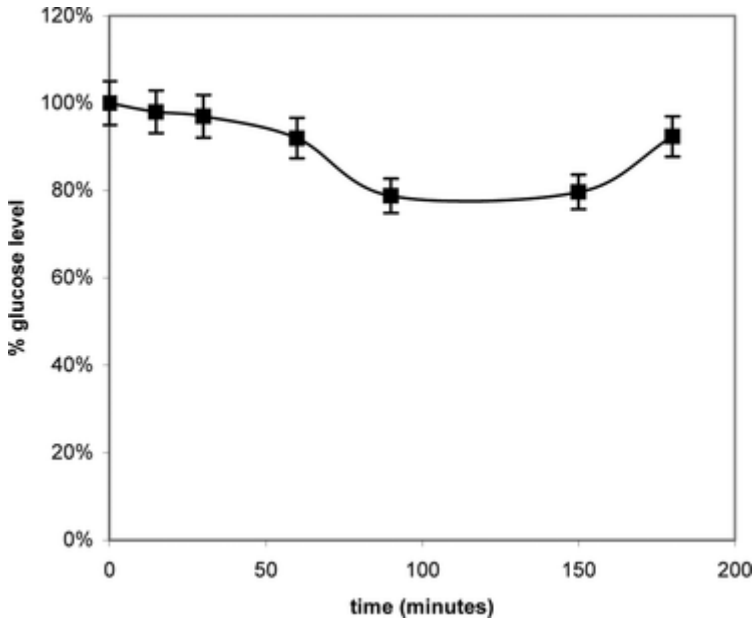


**FIG. 5** Blood glucose levels in rats after oral administration of insulin-loaded SLN suspension 50 UI/kg.

Considering the main results of oral administration to rats of FITC-insulin, a significant FITC-insulin uptake by gut lymphatic system can be demonstrated: drug accumulation in mesenteric lymph nodes and in lymph, particularly in its centrifuged fraction, shows that SLN are a promising drug delivery system for oral insulin.

Nevertheless, no significant drug amount can be detected in plasma: as a consequence, the effect on glucose was negligible if compared to that obtained after subcutaneous injection. This phenomenon might be explained starting from the consideration that a relevant amount of FITC-insulin was found only in blood cellular fraction nearly 3 hours after administration, corresponding to lymph drainage from the gut, while no drug was recovered in plasma. We hypothesized that the drug might be entrapped in some structures present in blood cellular fraction, and it can be recovered only after extraction. Gradient centrifugation experiments gave an explanation of this phenomenon, as drug was recovered in blood only in the lymphocytes layer.

Moreover FITC-insulin accumulated in lymph nodes decreases in a time-dependent manner, after 3 hour postadministration, corresponding to the time of drug appearance in blood. A probable mechanism should be summarized as follows: SLN are absorbed in the gut through the lymphatic pathway, insulin is quickly degraded by phagocytes accumulating in mesenteric lymph nodes; after nearly 3 hours, the phagocytes, which have entrapped the drug, appear in the systemic circulation, but insulin has been degraded before it can play its physiological effects. Considering in vivo administration of nano-micro particles, the data produced by several research groups in the last 25 years lead to the conclusion that both the extent of absorption and the mechanism of particle uptake at mucosal surfaces are dependent on a number of factors, such as particle diameter,

surface hydrophobicity, surface charge, shape and elasticity, specific targeting ligands (e.g., lectins), physical and chemical stability and other factors such as vehicle properties and volume.[ 15 ] Particularly, it is generally agreed that absorption increases with decreasing particle diameter. Studies on polystyrene latex in the range of 50 nm to 3 mm[ 16 ] revealed that maximal absorption occurred with particles ranging 50 to 100 nm in diameter, with particles above 1 mm being trapped in the Peyer's patches. These did not translocate to the systemic circulation. On the other side, in an in vitro experiment described in literature,[ 17 ] it was found that the number of particles internalized in phagocytes exhibited a dependence on particle size: particles with diameters between 2 and 3 μm were phagocytosized more readily than both smaller and larger particles.

Consequently, if 50 nm insulin-loaded SLN can be effective in reducing blood glucose level after oral administration,[ 18 ] larger nanoparticles, as those described in the present work, may be retained by the gut lymphatic system. In fact, encapsulation within nanoparticles can increase immune response to orally administered vaccines;[ 9 ] moreover, some authors[ 19 ] demonstrated that triglyceride-based solid lipid microparticles were efficiently internalized by macrophages in an in vitro experiment with a complete degradation after 24 hours. A recent report[ 20 ] describes the oral immunization of mice with 1.6 μm-diameter solid lipid microparticles containing a Japanese encephalitis antigen: they showed a good in vitro uptake by the intestinal M-cells. Although insulin is normally not considered an immunizing agent, SLN carrier can be recognized by the immune system and degraded by the reticulo-endothelial system.

## CONCLUSION

SLN showed to be a promising vehicle for insulin oral delivery, since they are absorbed by the intestinal lymphatic system, but size reduction and surface modification of nanoparticles will be necessary to help the uptake from lymph nodes to lymph and blood and to avoid degradation by phagocytes, in order to increase its therapeutic effect on glucose level.

***Acknowledgments***

This work was supported by a grant from the Italian Government (MIUR, Cofin 2004)

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