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RESEARCH PAPER

Solid Phase Extraction as an Innovative Separation Method for Measuring Free and Entrapped Drug in Lipid Nanoparticles

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ABSTRACT

Purpose Contrary to physical characterization techniques for nanopharmaceuticals (shape, size and zeta-potential), the techniques to quantify the free and the entrapped drug remain very few and difficult to transpose in routine analytical laboratories. The application of Solid Phase Extraction (SPE) technique was investigated to overcome this challenge.

Methods The separation of free and entrapped drug by SPE was quantitatively validated by High Performance Liquid Chromatography. The developed protocol was implemented to characterize cyclosporine A-loaded 120 nm-sized lipid nanoparticles (LNPs, Lipidot®) dispersed in aqueous buffer. The colloidal stability was assessed by Dynamic Light Scattering (DLS).

Results Validation experiments demonstrated suitable linearity, repeatability, accuracy and specificity to quantify residual free, entrapped and total drug. For the investigated LNPs, the method revealed a very limited shelflife of the formulation when stored in an aqueous buffer at 5°C and even more at elevated temperature. Nevertheless, the DLS measurements confirmed the stability of nanoparticles during SPE in a suitable concentration range.

Conclusions SPE, when successfully validated, represents a valuable tool for drug development and quality control purposes of lipid-based nanopharmaceuticals in an industrial environment.

KEY WORDS cyclosporine · lipid nanoemulsion · nanoparticles · separation techniques · solid-phase extraction

ABBREVIATIONS

ACN	Acetonitrile
API	Active pharmaceutical ingredient
CSA	Cyclosporine A
CV	Coefficient of variation
DAD	Diode Array Detector
DLS	Dynamic Light Scattering
EDCD	Entrapped Drug Content Determination
F0	Fraction 0
F1	Fraction 1
F2	Fraction 2
FDCD	Free Drug Content Determination
HPLC	High Performance Liquid Chromatographie
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LNP	Lipid nanoparticle
LOD	Limit of Detection
LOQ	Limit of Quantification
MeOH	Methanol
MWCO	Molecular Weight Cut-Off
n. a.	Not available/not applicable

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NP	Nanoparticle
PBS	Phosphate Buffered Saline
PdI	Polydispersity Index
PEG	Polyethylene Glycol
R^2	Determination coefficient
RP	Reverse Phase
rpm	Revolutions per minute
RSD	Relative Standard Deviation
SPE	Solid Phase Extraction
TDCD	Total Drug Content Determination
TFA	Trifluoroacetic acid 0.5% (*v*/*v*)

INTRODUCTION

In the scientific literature, nanomedicines are often considered as a promising field that could offer many advantages over small molecules. Many nanoproducts are currently under clinical trial or have already been approved, such as Abraxane® (paclitaxel) Ferumoxytol® (iron supplement) or Amphotec® (amphotericin B) for instance. However, the path until the final status of "approved" appears to be harder as hoped (1). The complexity of the nanotechnologies for human health is such that time consuming missteps are unfortunately very common (2). In particular, suitable techniques for the physicochemical characterization of the product are of the highest importance to reliably interpret results of the next studies (-*in vivo* tests, toxicity, *etc*.). The shape, the size or the zeta-potential of nanoparticles are key parameters for which many techniques (3) are already available and thoroughly investigated. On the contrary, far fewer techniques can be implemented in routine to investigate the distribution of the Active Pharmaceutical Ingredients (API) within the different compartments of the nanoformulation, essentially to distinguish between the free and entrapped drug. Dialysis methods are common for this purpose but the interpretation of the results is often not so obvious (4). Other separation techniques like Cross-Flow-Filtration, Field-Flow Fractionation, Ultracentrifugation, Capillary Electrophoresis, Size Exclusion Chromatography or Hydrodynamic Chromatography have been proposed in the scientific literature (5). Yet, simple and reliable methods, as required for quality control purposes, may not be generally feasible for any nanosystem. In this aim, pressure ultrafiltration was already proposed as a suitable separation technique for *in vitro* drug release tests (6). Further investigations revealed that pressure ultrafiltration for liposomes could instantaneously completely achieve the separation whereas other techniques failed (7).

For the present study, the SPE technique was selected because routine analysis can easily be performed at a later stage, as it is the case for pressure ultrafiltration. It generally allows the full automation of the analytical procedure and hence, a strong reproducibility. Moreover, SPE has already been successfully applied for liposomes (8,9), suggesting that it may be suitable for lipid nanoparticles (LNP) as well. This paper deals with the development of SPE methods to quantify separately free and entrapped cyclosporine A in a patented nanocarrier: Lipidot® (10). The colloidal stability of the 120 nm-sized LNPs passed through SPE-cartridges was verified by monitoring the particle size. The subsequent quantification of the API was performed by High Performance Liquid Chromatography (HPLC). The developed protocol as presented in Fig. 1 consisted of a tripartite SPE-method including a Total Drug Content Determination method (TDCD), an Entrapped Drug Content Determination method (EDCD) and a Free Drug Content Determination method (FDCD).

After validation of the analytical procedure according to the ICH guideline Q2A (11), the protocol was implemented for the Lipidot® formulation. Finally, a 48 h-stress test at elevated temperature and stirring speed was performed to evaluate the stability of the nanoformulation.

MATERIALS AND METHODS

Materials

Suppocire NB™ was purchased from Gattefosse S.A. (Saint-Priest, France). Myrj™ S40 (PEG 40 stearate, 1980 Da) and Super Refined Soybean Oil were obtained from Croda Uniquema (Chocques, France). Lipoid® S75 (soybean lecithin at 69% of phosphatidylcholine) provided by Lipoid GmbH (Ludwigshafen, Germany). All these excipients are pharmaceutical grade and used as received. Acetonitrile (ACN) and methanol (MeOH) were provided by LiChrosolv®, orthophosphoric acid 85% by BDH Prolabo® and trifluoroacetic acid 99.9% by EMD Millipore Corporation. All reagents were HPLC-grade. The cyclosporine A (CSA) was supplied by RTC Pharma. Highly purified water was produced using a Milli-Q® Gradient A10 from EMD Millipore Corporation. Lipidot® are formulated using a VCX750 Ultrasonic processor from Sonics (Newtown, USA) equipped with a 3 mm-diameter microtip. The balance was an AX205 from Mettler Toledo, the water bath was the type 19 of Julabo, the centrifuge was a Minispin from Eppendorf and the vortex was a Vortex-Genie 2 from Scientific Industries. The quantifications of the active pharmaceutical ingredient (API) were performed by high performance liquid chromatography (HPLC) using an Agilent 1100 Series system from Agilent Technologies with a degasser, oven and DAD-detector. The HPLC-column was an XTerra® RP-18 (5 µm×150 mm×4.6 mm) from Waters. The separation of the drug from nanoparticles was carried out using a SPE system from Supelco: Visiprep™ 12-Port Vacuum Manifolds. The SPE-cartridges Supelclean™ LC-18 SPE tube (1 mL, 60 Å pore size, 45 µm particle size) were provided by Supelco. The nanoparticle size experiments were performed using a Zetasizer Nano ZS from Malvern Instrument equipped

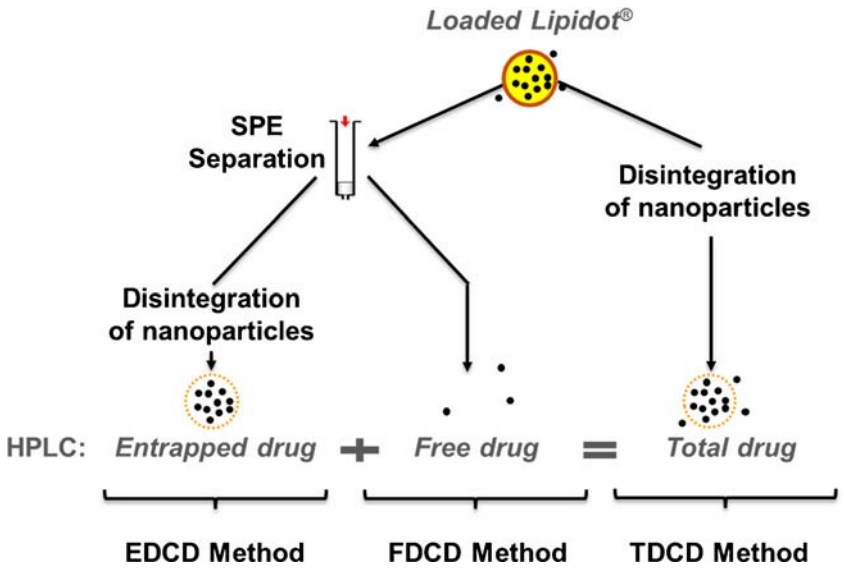

Fig. 1 Simplified process description of the tripartite SPE-method.

with a 532 nm green laser source. The stress test was carried out using the Manual Diffusion Test System equipped with a 7 mL vertical diffusion cell provided by Hanson Research.

Lipidot® Formulation

The formulation of conventional Lipidot® has been previously described elsewhere (12,13). The lipid phase was prepared by mixing lipophilic ingredients: wax, oil and Lipoid S75 phospholipids, whereas the aqueous phase was composed of the hydrophilic PEG surfactant, MyrjS40, solubilized in 1× PBS aqueous buffer. After melting the oil phase and homogeneous mixing with the aqueous phase at 45°C, both phases were crudely mixed and sonication cycles are performed during 20 min with intervals of 10 s "Pulse On" and 30 s "Pulse Off". The purification step was carried out overnight using dialysis (1× PBS, MWCO: 12 kDa, regenerated cellulose membrane, Spectra/Por®). Lipid nanoparticles (LNP) were finally formulated at a theoretical total concentration of lipids of 60 mg/mL and then filtered through a 0.22 µm cellulose Millipore membrane. Typically, for 120 nm-sized formulation, particles are composed of the 43% (*w*/*w*) of dispersed phase (860 mg of total excipients for 2 mL-volume of formulation) with a surfactant/core ratio of 0.43 and a lecithin/PEG surfactant weight ratio of 0.21. Concerning the manufacturing of the CSA-loaded LNPs, an appropriate amount of CSA in absolute ethanol (45 µL of 63.7 mg/mL of CSA stock solution) was initially added to the oily phase (local concentration of CSA in oily phase: 0.48% *w*/*w*), and then the solvent was evaporated under argon flow. The nanoparticles encapsulating CSA were then formulated as above described. The purification step by dialysis was extended until 72 h to leave time non-entrapped CSA to remove itself due to its low solubility in aqueous buffer. CSA drug was initially loaded until a concentration of 210 µg/mL and a total lipid concentration of 60 mg/mL. The blank nanoparticles were prepared without drug corresponded to the placebo formulation.

Preparation of the SPE and HPLC Mobile Phases

The solution of trifluoroacetic acid 0.5% (*v*/*v*) (hereafter named TFA), was prepared diluting trifluoroacetic acid 99.9% with highly purified water. The solution of ACN:H_3PO_4 5 mM (75:25; *v*/*v*) and the solution of MeOH:H_3PO_4 5 mM (75:25; *v*/*v*) were prepared mixing phosphoric acid 5 mM solution with respectively ACN and MeOH. The solution of phosphoric acid 5 mM was prepared by dilution of orthophosphoric acid 85% with highly purified water.

Principle of the Tripartite SPE-Method for Lipid Nanoparticles

In order to avoid any "aging effect", the samples were stored at 5°C for less than 1 week. Prior to SPE test, the samples were left without handling until equilibration at room temperature. As described in Table I, media were successively introduced into the SPE-cartridges for cleaning and equilibration prior to introduction of the samples of formulation. The nanoparticles were eluted in a first fraction (F1) adding TFA as eluent into the SPE-cartridge. The free drug was eluted in a second fraction (F2) using ACN as a second eluent. The pressure in the vacuum chamber during the elution of LNPs (Step 3) was adjusted, resulting in a flow rate of 1 mL/min.

The nanoparticles eluted in F1 could further be disintegrated to release entrapped drug. Briefly, the disintegration step consisted in mixing one volume of eluted nanoparticles with two volumes of acetonitrile. The mixture was

Table I SPE Overall Plan

Step	Medium	Volume	Pressure	Fraction	Container	Function
1	ACN	≈6 mL	≈ – 50 kPa	F0	Waste	Cleaning
2	TFA	≈4 mL	≈ – 50 kPa			Equilibration
3	Sample	1 mL	≈ – 20 kPa	F1	5 mL-VF1	Elution of NPs
4	TFA	≈3 mL	≈ – 50 kPa			
5	ACN	≈4 mL	≈ – 50 kPa	F2	5 mL-VF2	Elution of API

The volumetric flasks were made to the marks after elution using the same medium as used for elution

ACN acetonitrile, *TFA* trifluoroacetic acid 0.5% (v/v), *Sample* Nanoformulation to be tested, *NPs* nanoparticles, *API* active pharmaceutical ingredient, *VF1* volumetric flask #1, *VF2* volumetric flask #2

vortexed and centrifuged at 10,000 rpm for 10 min. The released drug in supernatant was quantified using a suitable HPLC method (EDCD method).

The free drug eluted in F2 could be directly quantified by HPLC (FDCD method).

The total drug content was determined for a sample without being passed through SPE but directly prepared as previously described in the disintegration step. The released entrapped drug and the free drug were quantified by HPLC (TDCD method).

Assessment of the Colloidal Stability of Lipidot® Following SPE Protocol

Size distribution of Lipidot® was investigated by DLS to monitor the colloidal integrity of the nanoparticles after being applied SPE method. Prior to SPE, the sample of placebo Lipidot® was diluted using TFA until 3, 6, 10, 15, 30 and 60 mg/mL LNPs. Each dilution of the set was further eluted by SPE as described in Table I until step 4. The fractions F1 were collected and the particle sizes were assessed using the Zetasizer Nano ZS. The solution of TFA was used as blank and placebo Lipidot® as negative control. Before measurement, samples were diluted in TFA to a dispersed phase weight fraction of 1 mg/mL in order to avoid multiple scattering effects. All samples were prepared in duplicate and analyzed in triplicate. The Z-average diameter (Size, nm) and polydispersity index (PdI) of the lipid nanoparticles were extracted from the second cumulant of the correlation function of the intensity distribution. Each result was the mean of three independent measurements performed at 25°C, at a fixed angle of 173°.

SPE Separation Methods and Validation by HPLC Analysis

The quantitative validation of the SPE method for the FDCD was performed for a set of placebo Lipidot® diluted with highly purified water solutions spiked with different proportions of cyclosporine A. A CSA stock solution was prepared in duplicate dissolving the powder of CSA in acetonitrile until a concentration of 2842 µg/mL. A first CSA dilution set was then prepared by dilution of the stock solution using ACN until 284, 426, 710, 852, 1136, 1421 and 1705 µg/mL CSA. The dilution set was further diluted with a dilution factor of 50 using highly purified water, so that the resulting dilution set contained 2%, (*v*/*v*) acetonitrile, *i.e.,* less than the limit of 5% above which LNPs disintegrate (unpublished DLS results). The sample of placebo Lipidot® was added until a concentration of 9.6 mg/mL, *i.e.,* until a LNP dilution factor of 6.25 (dilution set designated by "CSA + placebo Lipidot® for FDCD" in Table II). In addition, another dilution set ("CSA for FDCD" in Table II) was prepared without placebo Lipidot®. Each sample of both the dilution sets of CSA solutions and CSA-spiked placebo LNPs was investigated in triplicate by solid phase extraction according to the validation plan presented in Fig. 2 and using the SPE protocol previously described in Table I.

Validation FDCD Method

The FDCD method further consisted in maintaining the fractions F2 of eluted CSA in a water bath for 5 min at 37°C. Aim of this step was to dissolve the precipitate formed by co-eluted excipients present in the extern phase of the Lipidot® formulation. The CSA was quantified by HPLC using the setups for FDCD (see in Table III), compatible with the presence of co-eluted excipients. The specificity of the CSA separation was assessed from the fraction F1: the eluted LNPs were disintegrated as previously described and analyzed by HPLC to verify the absence of CSA in this fraction. The HPLC method used the setups for EDCD and TDCD (see in Table III), compatible with the presence of excipients released after disintegration of the nanoparticles.

Validation EDCD Method

Concerning the EDCD method, the validation focused on the step following the elution of LNPs in F1, since spiking placebo

Table II Dilution Sets Prepared for the Validation of the Tripartite-SPE Method

	CsA for FDCD		CSA + placebo Lipidot® for FDCD		CSA + placebo Lipidot® for EDCD		CSA + placebo Lipidot® for TDCD	
	CSA (μg/mL)	LNPs (mg/mL)	CSA (μg/mL)	LNPs (mg/mL)	CSA (μg/mL)	LNPs (mg/mL)	CSA (μg/mL)	LNPs (mg/mL)
120%	34.10	0.00	34.10	9.60	2.27	9.60	2.27	1.92
100%	28.42[a]	0.00[a]	28.42	9.60	1.89	9.60	1.89	1.92
80%	22.74	0.00	22.74	9.60	1.52	9.60	1.52	1.92
60%	17.05	0.00	17.05	9.60	1.14	9.60	1.14	1.92
50%	14.21	0.00	14.21	9.60	0.95	9.60	0.95	1.92
30%	8.53	0.00	8.53	9.60	0.57	9.60	0.57	1.92
20%	5.68	0.00	5.68	9.60	0.38	9.60	0.38	1.92
0%	0.00	0.00	0.00	9.60	0.00	9.60	0.00	1.92

For the EDCD method, the data given in the table do not correspond to entrapped CSA because of the technical impossibility to spike drug inside nanoparticles. The value of the entrapped concentrations (from 0.00 to 2.27 μg/mL) were hence calculated from the concentration of CSA spiked in ACN (from 0.00 to 3.41 μg/mL), *i.e.,* using a factor 1.5. For the TDCD method, the data were calculated as for the EDCD method

[a] The 100%-labelled sample of "CSA for FDCD" was used as reference solution for the quantification of CSA by HPLC. The reference solution was prepared in duplicate

Lipidot® with entrapped CSA was not feasible. Placebo LNPs were diluted using highly purified water until a concentration of 9.6 mg/mL LNPs (*i.e.*, LNP dilution factor 6.25) and were further investigated in triplicate according to the overall SPE-plan presented in Table I, until step 4, including the volume adjustment of 5 mL-VF1 (*i.e.*, LNP dilution factor 5). Afterward, 0.5 mL of F1 were mixed with 1 mL of a dilution set of CSA in acetonitrile containing 0.57, 0.85, 1.42, 1.71, 2.27, 2.84 and 3.41 μg/mL drug (dilution set designated by "CSA + placebo Lipidot® for EDCD" in Table II). The resulting mixtures were shaken using the vortex to disintegrate the placebo LNPs and centrifuged for 10 min at 10,000 rpm

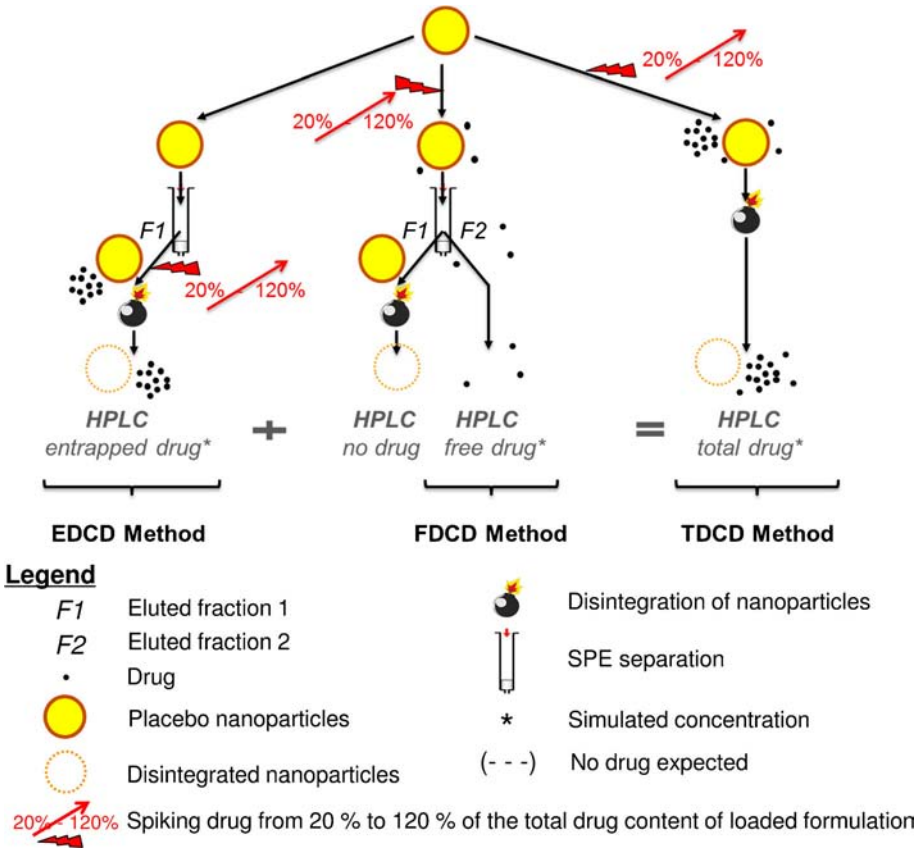

Fig. 2 Validation plan for the tripartite SPE method.

Table III Setups of the HPLC Methods Used for FDCD, EDCD and TDCD

Parameters	Setups for FDCD	Setups for EDCD and TDCD
Column	XTerra®, RP-18 (5 μm × 150 mm × 4.6 mm)	
Elution mode	Isocratic	
Mobile phase	ACN:H_3PO_4 5 mM (75:25, *v/v*)	MeOH:H_3PO_4 5 mM (75:25, *v/v*)
Flow	1.0 mL/min	1.2 mL/min
Wavelength detection	205 nm	
Temperature	55°C	
Injection volume	30 μL	50 μL
Run time	7 min	10 min
Retention time	3.10 min (±0.05 min)	6.55 min (±0.10 min)

to precipitate the excipients and to only collect the CSA in supernatant solution. The HPLC setups used for EDCD are presented in Table III.

Validation TDCD Method

The TDCD method was validated by diluting the placebo Lipidot® using TFA until equivalent concentration of LNP to Fractions F1 in EDCD methods (1.92 mg/mL LNPs). As used for the validation of the EDCD method, 0.5 mL of this latter diluted solution were mixed afterward with 1 mL of the same dilution set of CSA in acetonitrile (dilution set designated by "CSA + placebo Lipidot® for TDCD" in Table II). The mixtures were vortexed and centrifuged as previously described for the EDCD method and then analyzed using the same HPLC method (see Table III).

Implementation of the Tripartite SPE Method for a Sample of Cyclosporine A-Loaded Lipidot® Formulation and Stress Test

The SPE protocol was implemented for a sample of cyclosporine A-loaded 120 nm-sized Lipidot® as previously validated. After equilibration at room temperature and dilution of the sample (dilution factor 6.25 in highly purified water), the FDCD and the EDCD methods were implemented directly whereas for the TDCD method, a dilution in TFA with factor 1:5 was applied. All samples were prepared five times ($n=5$).

The protocol was implemented as well for the nanoformulation without the dilution step in highly purified water, namely 1 mL of Lipidot® product was placed in the SPE cartridge at step 3 (see Table I) for the FDCD and EDCD methods and the product was directly diluted in TFA (dilution factor 1:5) for the TDCD method.

Regarding the stress conditions, 7 mL of cyclosporine A-loaded Lipidot® formulation were placed in the acceptor compartment of the vertical diffusion cell used without membrane as 1-compartment cell. The stress test was carried out for 48 h at 37°C with a magnetic stirring of 500 rpm. The stressed product was analyzed as previously without the dilution step in highly purified water.

RESULTS AND DISCUSSION

The developed protocol is based on the reverse-phase SPE concept, relying on the Van der Waals interactions between a hydrophobic analyte and a hydrophobic solid stationary phase (SPE-cartridge). This phenomenon leads to a stronger retention of the lipophilic analyte whereas the lipid nanoparticles are eluted faster by a hydrophilic mobile phase due to their outer hydrophilic PEG shell. The separation between the API and LNPs is further enhanced by the significant size difference of the two entities. Indeed, the stationary phase consists of beads containing pores smaller than the nanoparticle diameter but larger than the API as molecular entity. Based on these two mechanismes, PEG-shell lipid nanoparticles loaded with hydrophobic drugs or contrast agents can be eluted in a first step by using TFA as a hydrophilic mobile phase. In a second step, the retained drug can be eluted using ACN as a more lipophilic mobile phase. This technique is consequently suitable to separate and quantify either the remaining non-entrapped drug fraction after a given manufacturing process, to detect a leakage of drug during storage on the shelf, or even to monitor drug release during an *in vitro* performance test. Moreover, such separation of the API from the particles takes only few minutes whereas other techniques, like *e.g.*, dialysis, may require several days, and thus might be of the same time scale as the expected drug release from the carrier. Contrary to polymer or inorganic nanoparticles, considering the metastable character of lipid nanoparticles when dispersed in aqueous buffer, techniques based on separation-precipitation cannot be applied. Methods requiring organic solvents would destabilize the interface of droplets resulting in a biphasic system (oil and water parts). Consequently, solid phase extraction turned out to be the most suitable separation method for lipid nanoparticles.

Assessment of the Colloidal Stability of Lipidot® Following SPE Protocol

As colloidal nanoemulsions, Lipidot® formulations are composed of lipid droplets surrounded by lecithin and coated with PEG surfactant with Z-average diameters from 50 to 150 nm to render colloidal stability, reported with a shelf-life in suspension over 1 year (12). Because their interaction with the stationary phase materials could potentially affect the integrity of the nanoparticles it was necessary to confirm their colloidal stability after the SPE process to validate its use as a separation method. Such experiment was consequently performed using a Zetasizer Nano ZS after SPE process. It was firstly shown that the median size and Polydispersity Index (PdI) for different dilutions of LNPs in TFA (control dilutions) did not significantly differ one to another. This finding demonstrated that acid medium did not destabilize the LNPs. Secondly, considering the concentrations from 6 to 60 mg/mL, the Polydispersity Index (PdI) after SPE remained constant whatever the dilution and was roughly the same as for the control (see Fig. 3 and Table IV). It was consequently assumed that the lipid nanoparticles were not disintegrated during SPE. However, as concern the size, the Lipidot® seemed to swell with the dilution factor. It was supposed that an excess of excipient after manufacturing remains in the extern phase of the formulation. These excipients may stabilize the lipid particles, until the formulation is too diluted. At 3 mg/mL, the Lipidot® were probably not stable enough to tolerate the mechanical stress caused by the SPE and 6 mg/mL might be the limit where the Lipidot® (or the PEG-shell of the Lipidot®) expand without disintegrate. Consequently, the Table IV suggested that the acceptable nanoparticle concentration range is 6 to 60 mg/mL.

Validation of the Tripartite SPE Method

The tripartite SPE method actually consists of preparative and analytical methods for the quantification of the total API (TDCD), the entrapped API (EDCD) and the free API (FDCD). The dilution set of CSA prepared without LNPs for the validation of the FDCD method was practically not turbid in the selected cyclosporine A concentration range. However, for concentrations greater than 35–40 μg/mL (before SPE), the turbidity increased, precipitates were observed and the validation test failed (RSD>>5%). The validation of FDCD succeeded using highly purified water as dilution medium until 34.10 μg/mL (see results in Table V). The linearity and repeatability were slightly better in presence than in absence of Lipidot®, obviously reflecting the improved solubility of cyclosporine A by this formulation.

For the EDCD and TDCD methods, the results of the validation were satisfying regardless of the very low selected concentrations. The areas corresponding to CSA in the chromatograms (see Fig. 4) were low for the EDCD and TDCD methods but still acceptable. All relevant peaks were separated with a sufficient resolution and peak symmetry for a reproducible integration. Both methods presented very wide and high injection peaks attributed to excipients released after destruction of LNPs and immediately eluted by HPLC. However, further peaks were observed for TDCD but not for EDCD, suggesting that other excipients of the formulation were not eluted in the fraction F1 but instead co-eluted with CSA in the fraction F2. Additional peaks were actually observed for FDCD, but did not affect the quantitation of the drug related peaks.

Tripartite SPE Method for a Lipidot® Nanoformulation—Stress Test

A protocol of characterization based on the SPE technique was previously designed, developed and validated using placebo lipid nanoparticles. Its implementation was also performed using the loaded Lipidot® formulation. The tripartite SPE method was able to determine the concentrations of entrapped CSA (88.9 μg/mL), free CSA (67.5 μg/mL) and total CSA (163.8 μg/mL). Considering the LNPs remained dispersed in an aqueous solution during the storage, a release of drug may have started before the samples were analyzed, even stored at 5°C. Moreover, comparing the experimental total CSA concentration to the theoretical concentration (210.0 μg/mL), an encapsulation efficiency of the manufacturing process could be calculated (78%) to highlight the loss of non-encapsulated drug removed over intensive dialysis during the manufacturing process. The protocol was further implemented using a diluted formulation of Lipidot® in order to assess the influence of a dilution on the release of CSA. The free CSA represented 41% of the total concentration for the samples prepared without dilution, *vs.* 82% for the diluted samples. The entrapped CSA was quantified as well and represented 54% for undiluted sample *vs.* 11% for diluted samples. The methods for free and entrapped CSA could successfully reveal a dilution-effect and were almost complementary. However, according to the results summarized in Table VI, the repeatabilities (coefficient of variation) were very satisfying for the total and free CSA content determination method but not optimal for the entrapped CSA. Consequently, when the complementarity between the three methods was not completely achieved, the unidentified amount should be to be related to the entrapped CSA. As concern the results of the stress test at 37°C and 500 rpm, a proportion of 68% free CSA was observed after the stress test *vs.* 41% free CSA without the test. The result suggested a sustained release potential for the Lipidot® formulations. All the results of the tests performed for CSA-loaded Lipidot® formulation are given in Table VI.

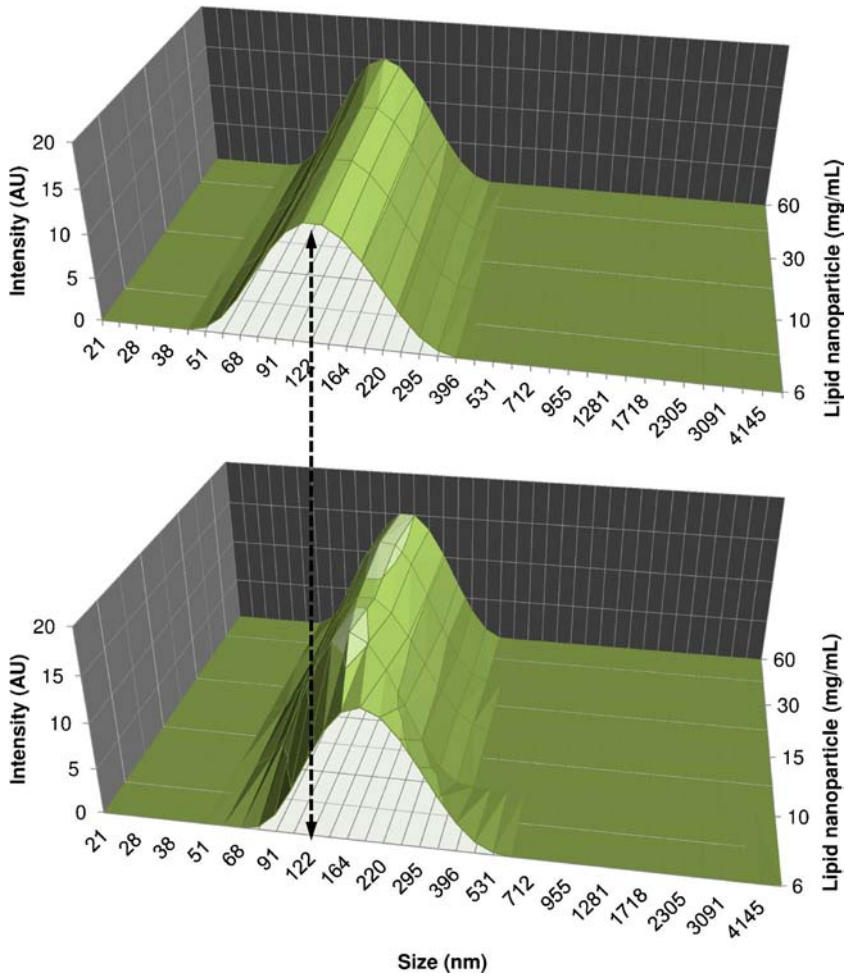

Fig. 3 Particle distribution for different concentrations of Lipidot® samples before and after SPE. The control set of LNPs (*top*) was diluted until same concentration as the set of eluted LNPs through SPE (*bottom*). The size distribution remained constant for the whole control set (6, 10, 30 and 60 mg/mL). After SPE, the modal size remained not affected for the concentrations higher than 10 mg/mL but was shifted towards higher values at 6 mg/mL.

Supplementary Information and Future Studies

Quantitative Determination of Lipid Nanoparticles

Quantification methods based on UV-spectrophotometry were developed for LNPs. The amount of LNPs before and after SPE could be compared using such methods. Unfortunately, the UV absorption not only depends on the concentration of the particles but on the size as well (because of the light scattering). Since Lipidot® are polydispersed systems, no absolute reliable quantification method is to be expected. This is the reason why these methods were not published in this paper. Other methods for the quantification of the Lipidot components are currently under development by HPLC analysis using specific separation and detection such as Evaporative Light Scattering detector (ELSD) suitable for non chromogenic molecules like lipid ingredients. The mixture of wax and soybean oil composing of the oily core of Lipidot induces high level of complexity of glycerides to be identified and to be separated for accurate quantification.

Table IV Z-Average Diameter (nm) and Polydispersity Index (PdI) for Different Concentrations of Lipidot® Samples Prepared by SPE or Not (Control)

LNP concentration (mg/mL)	Z-average diameter (nm)		PdI	
	Control	SPE	Control	SPE
3	n. a.	2849.0	n. a.	0.747
6	123.3	169.3	0.125	0.129
10	124.8	137.0	0.117	0.097
15	n. a.	130.9	n. a.	0.141
30	124.1	125.8	0.114	0.106
60	124.4	123.8	0.125	0.106

Preliminary Tests for the Preparation of the SPE-Plan

The choice of the SPE-cartridge was based on the theoretical property of the stationary phase to totally retain the drug at a first stage of the separation, whereas lipid nanoparticles can be totally eluted using an appropriate solution, and to allow at a

Table V Validation of the Tripartite SPE Method

Parameters	FDCD[a]	FDCD[b]	EDCD[b]	TDCD[b]
Linearity				
• Range (μg/mL)	1.14–6.82	1.14–6.82	0.38–2.27	0.38–2.27
• R^2	0.9991	0.9991	0.9992	0.9997
• LOQ (μg/mL)	0.41	0.17	0.26	0.16
• LOD (μg/mL)	0.14	0.06	0.09	0.05
• RSD (%)	3.97	1.71	2.56	1.67
Repeatability ($n = 3$)				
• CV (%) at 20%	5.59	2.13	0.93	1.33
• CV (%) at 100%	1.15	3.88	1.58	1.06
• CV (%) at 120%	1.95	1.68	1.02	1.56
Accuracy ($n = 3$)				
• 20%	99.9–111.7	98.3–103.0	100.9–102.8	100.9–103.3
• 100%	98.4–100.8	94.7–102.1	99.8–103.0	100.5–102.6
• 120%	99.3–103.0	99.2–103.1	100.1–103.0	100.4–103.4
Specificity	No interfering peaks were observed at the retention time of the API			

The given concentrations correspond to the samples prepared for HPLC analysis and should be multiplied by a factor 5 for FDCD and a factor 15 for EDCD and TDCD to calculate the concentration of the sample before preparation. Linearity performed with 20, 30, 60, 100 and 120% of the simulated free API concentration, 100% corresponding to 5.684 μg/mL CSA for FDCD method and to 1.920 μg/mL CSA for both EDCD and TDCD methods

[a] Results for CSA without placebo Lipidot®

[b] Results for placebo Lipidot® spiked with CSA

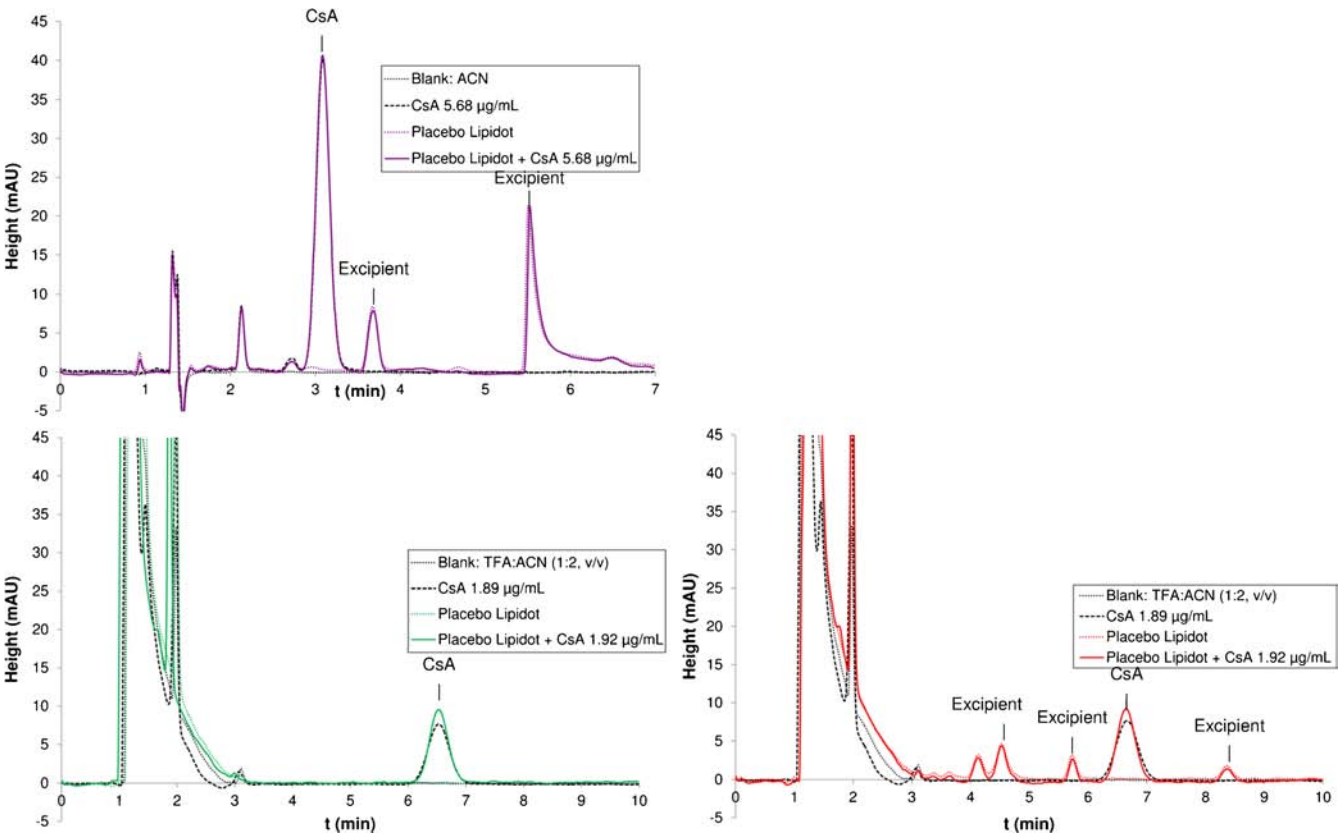

Fig. 4 Chromatogram of cyclosporine A quantified using the FDCD method (*violet*), EDCD method (*green*) and TDCD method (*red*).

Table VI Total, Entrapped and Free CSA Concentrations for the Loaded Lipidot® Formulation

	No dilution		Dilution 6.25		No dilution stress test	
	Mean (μg/mL)	CV (%)	Mean (μg/mL)	CV (%)	Mean (μg/mL)	CV (%)
Total CSA	163.8	1.1	155.5	1.1	178.8	1.1
%/total CSA	100		100		100	
Entrapped CSA	88.9	8.5	17.6**	15.3**	39.0	15.3
%/total CSA	54		11		22	
Free CSA	67.5*	6.3*	127.1	5.0	121.5	4.0
%/total CSA	41		82		68	
Total - Sum (entrapped CSA + free CSA)	7.4	n.a.	10.8	n.a.	18.3	n.a.
%/total CSA	5		7		10	

Dilution 1:1: sample not diluted prior SPE separation; Dilution 6.25: sample diluted prior SPE separation; Dilution 1:1 Stress test: sample stressed over 48 h, not diluted prior SPE. Experiments performed for $n = 5$

CV coefficient of variation, *n.a.* not applicable

* $n = 4$

** $n = 3$

second stage the total elution of the drug using an appropriate solvent. LC-18 SPE-cartridges, supposed to present this property, were consequently selected and tested for this study. The results performed using the current methods for the quantification of lipid nanoparticles were in line with this type of cartridge. However, if the separation is later revealed by more elaborate methods not to be complete, other SPE-cartridges may be tested for optimization. The first tests were performed using other acid solutions (HCl) as TFA and with lower concentrations of TFA as described in this paper but the retention of the lipidot® in the cartridge remained significant. The elution was actually complete at 0.5% TFA, though high concentrations of TFA are generally not recommended for the LC-18 SPE-cartridges. This is the reason why UV-spectrophotometry was used to compare the absorption spectrum of the eluents before and after SPE. The baseline became stable using more than 6 mL ACN for the cleaning and 4 mL TFA 0.5% for the equilibration. In this condition, the elution of particles (F1) and drug (F2) was assumed to be more reproducible.

Externe Phase of Lipidot® Formulation

The chromatogram FDCD presented extra peaks supposed to correspond to an excess of excipient in solution in the externe phase of the Lipidot® formulation. If this hypothesis is confirmed by other techniques such as HPLC-ELSD, successive SPE cycles will result in an extern phase free of stabilizing excipients. Consequently, the LNPs may not tolerate the stress of the SPE preparation, unless fresh stabilizing excipients are spiked in the extern phase. Further investigations are currently ongoing to analyze the extern phase of the formulation which may play a key role in the stability of Lipidot®.

CONCLUSION AND PERSPECTIVES

An accurate, repeatable, fast and automatable method based on SPE was developed for the separation and quantification of non-entrapped and entrapped cyclosporine A in the lipid nanoformulation Lipidot®. The results obtained by DLS have shown that nanoparticles were actually eluted in a first fraction. Furthermore, the validation of the technique by HPLC resulted in satisfying results revealing a reliable analytical performance of the method and usability for quality control purposes. At this stage of product developement, the SPE technique clearly demonstrated the complexity of the Lipidot® formulation and underlined the need of further characterization investigations. Indeed, *in vitro* drug release tests remain necessary to confirm the sustained release potential of Lipidot® and to elucidate the mechanism of release (*e.g.*, diffusion, erosion, melting, *etc.*). Further improvements towards a pharmaceutical product are required as well. Besides testing drug release and leakage during storage, respectively, the technique would also allow to quantify the amount of API removed during the purification step of the manufacturing process and thus the encapsulation rate and efficacy. Apart from the considerations related to the specific formulation, which served as an example for the study, the concept of SPE presents itself as promising tool for drug development and quality control purposes of lipid-based nanopharmaceuticals in an industrial environment.

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