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Physical and chemical evaluation of liposomes, containing artesunate

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Abstract

As artesunate has a rapid onset of therapeutic effect and quick elimination, frequent administration is required, especially in the treatment of malaria. Such treatment courses led to bad patients' compliance, leading to high recrudescence rate. Therefore, slow release preparations seemed to be a logical approach in artesunate monotherapies, as can be developed with liposomal suspensions, especially for parenteral administration. Thus, the aim of this study was to develop sterile liposomes. The suspension was evaluated on its chemical/physical stability, including chemical degradation and crystallization of artesunate, and release capacities, by use of the dialysis technique. The maximal encapsulation degree of artesunate without crystals was 1.5 mg in 300 mg lipids per ml suspension, containing egg-phosphatidylcholine/cholesterol in a molar ratio of 4:3. The highest stability was obtained with a phosphate buffer of pH 5, which could be expected, as artesunate is almost totally encapsulated. But by reason of instability in water, the suspension containing artesunate 1 mg/ml was preferred, as the encapsulation efficiency is 100%. The in vitro release test proves that artesunate is reversibly encapsulated in liposomes. A method for sterile production of liposomes at lab-scale level is also presented.

Keywords: Liposomes; Artesunate; Sterile production; Malaria

1. Introduction

Artesunate is a water-soluble derivative of artemisinin. It has been shown to be highly active against multi-drug-resistant Falciparum malaria in several regions dealing with high resistance rate against the classic antimalarials [1].

Artesunate has a rapid onset of therapeutic effect after extensive biotransformation of artesunate into dihydroartemisinin [2,3] which is likely to provide beneficial therapeutic outcomes in severe or cerebral malaria [4]. But it eliminates very quickly after intake (only 6 h after oral administration) [1]. Therefore, artesunate should be administered several times a day and at least 5 days [4] to achieve a radical cure rate. Such treatment courses led to reduce patients' compliance [4], being associated with high recrudescence rate.

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Therefore, slow release preparations, as well as for oral as for parenteral use seemed to be a logical approach in artesunate monotherapies. The availability of artesunate in a parenteral formulation is especially advantageous for the treatment of severe malaria, including cerebral malaria, which generally tends to be caused by a Falciparum infection. It is even an effective route of administration in semi- or unconscious patients and avoids administration failure owing to vomiting, caused by the disease [4].

Parenteral formulations, having controlled release capacities [5], even in treatment of malaria [6,7], are the liposomal suspensions. Liposomes are submicroscopic particles, generally composed of glycerophospholipid bilayers, mainly phosphatidylcholine, which can be obtained by extraction of egg yolk, for example. These are mostly stabilized with cholesterol [8,9].

Encapsulated drugs can be released slowly from the liposomes at the injection site after subcutaneous and intramuscular injection. But this depends strongly from the size of the liposomes [10]. Once in the blood stream, cells of the mononuclear phagocyte system located in liver, spleen and lymph nodes primarily clear liposomes [11].

These slow release characteristics of liposomes were extensively applied in cancer therapy [12–14], especially for drugs with short therapeutic effect, requiring frequent dosage regimens [15].

Moreover, lower drug administration frequency leads a lower incidence of systemic side effects [16,17] in comparison to the classic treatments. Other advantages of liposomes over free drug formulations are minimization of immune reactions, protection of the encapsulated drug to degradation and increasing solubility of it in aqueous solutions. Many studies showed the efficacy and safety of these preparations [18–21].

Several researchers investigated the capability of liposomes to encapsulate artemisinin derivatives [22,23] and their in vivo results suggest the usefulness of these formulations in oral and IV treatment [24].

Therefore, the setup of this investigation was to develop a parenteral slow release formulation with artesunate as active compound via liposomes. The main purpose was to develop a liposomal suspension, which contains artesunate as much as possible at the most suitable pH, in which artesunate can be kept stable. Later on, formulation was evaluated in terms of chemical/physical stability, including chemical degradation and crystallization of artesunate. Finally, the release capacities of the liposomal suspension by use of a dialysis technique were investigated.

2. Experimental

2.1. Analytes, excipients and reagents

The analyte artesunate was purchased from HelmAG (Hamburg, Germany). The excipients of the liposomal preparation were cholesterol (Chol; Mr 387) from Merck (Darmstadt, Germany) and egg-phosphatidylcholine (EPC; Mr 775) from Lipoid (Ludwigshafen, Germany). Organic solvents used for the preparation of liposomes and standard solutions for analysis, and reagents were ethanol 70% (v/v), dichloromethane (analytical grade) and dodecanol (reagent grade) from Merck (Darmstadt, Germany), acetonitrile (HPLC grade) from Biosolve (Valkenswaard, The Netherlands), chloroform and methanol, both from Labscan (Brussels, Belgium). The following products applied in the preparation of liposomal buffers were all obtained from Merck (Darmstadt, Germany): $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$, citric acid$\cdot H_2O$, tris(hydroxy-methyl-aminomethane), HCl 32% (g/g) and NaCl.

2.2. Preparation procedures of and evaluation methods on liposomal suspensions containing artesunate

2.2.1. Preparation of liposomal suspensions

The preparation method for lab-scale batches of liposomes for investigation was adapted from the "film-hydration method" [8,25]. To investigate the encapsulation capacity of artesunate, a great number of different liposomal suspensions were prepared, altering the concentration of the lipids, the concentration of artesunate or the pH of the buffer used to hydrate the film. Therefore, stock solutions of EPC 180 mg/ml, Chol 140 mg/ml and

artesunate 10 mg/ml were prepared in chloroform. Series of liposomes were prepared using concentrations of 100, 200 and 300 mg/ml lipids, containing EPC and cholesterol in a molar ratio of 4:3, and up to 6 mg/ml of artesunate. An appropriate volume of each stock solution was mixed with each other and eventually diluted with chloroform to obtain the end volume of the mixture. The lipids and the drug were then deposited as a thin film in a round bottom flask by rotary evaporation under nitrogen. Therefore, a Rock 'n' Roller from Snijders Analysers (Tilburg, The Netherlands) was used, which enabled us to evaporate several flasks at the same time and to adjust the position of the bottles to a more horizontal position, as soon as a part of the solvent has evaporated. Residual solvents were removed by an overnight vacuum evaporation in the Secfroid lyofilizator (Vanderheyden, Brussels, Belgium). The films were than hydrated by the addition of an appropriate volume of isotonic buffer of a certain pH. The solution was homogenized in an overhead shaker (Heidolph, Swabach, Germany) by gently turning overnight to ensure the formation of the liposomal suspension.

Three buffers of, respectively, pH 5, 7 and 9 were prepared, containing: phosphate buffer, pH 5, 0.05 M: 2.952 g $Na_2HPO_4 \cdot 12H_2O$, 0.816 g citric acid $\cdot H_2O$, 0.1055 g NaCl in 100 ml water; phosphate buffer, pH 7, 0.05 M: 0.42 g $NaH_2PO_4 \cdot 2H_2O$, and 1.43 g $Na_2HPO_4 \cdot 12H_2O$, 0.4495 g NaCl in 100 ml water, and the tris(hydroxy-methyl-aminomethane) buffer, pH 9, 0.05 M: 0.605 g Tris, 25 ml 0.2 N HCl and 730 mg NaCl in water to 100 ml. Each buffer was adjusted to its pH with HCl or NaOH 0.1 N, as necessary. To prepare empty liposomes, the same procedure was followed without addition of artesunate stock solution.

The production of a sterile liposomal suspension is slightly modified, compared with the method for lab-investigational liposomes. Therefore, extra solvents, materials and instruments were used such as alcohol 70%, dichloromethane; a laminar airflow chamber (LAF), a PTFE 0.22 μm antibacterial filter of Millipore (Molheim, Germany) and a sterile vacuum pump container (Filtration Duran glass container, WITT Scott Cie). Sterility test on the liposomal suspension was performed by the Department of Clinical Biology of the hospital of the Free University of Brussels (AZ-VUB, Brussels, Belgium). Glass serum vials, capped with an aluminum cap crimper (Fempress H 207, Schwarz, Switzerland), were used as packaging for the sterile liposomal suspensions.

2.2.2. Investigational methods for the physical and chemical stability of liposomal suspensions

2.2.2.1. Microscopical evaluation of the liposomal suspensions. After preparation, which takes at least 2 days, the presence of crystals in the liposome preparations was assessed within 1 week thereafter by counting the crystals within 20 squares of a Burker counting cell of $0.0025 \times 0.0025 \times 0.1$ mm^3 (W. Schreck, Hofheim, Germany) using an optic microscope (Carl Zeiss, Oberkochen, Germany), equipped with a standard Junior 2 monocular micrometer. During this test, liposomal suspensions were kept at 4 °C.

2.2.2.2. Incorporation capacity, trapping efficiency and chemical stability of artesunate in buffers and in liposomes. As artesunate is known to be unstable in aqueous solutions, investigation was performed on its stability in the presented buffers. Therefore, buffer solutions, saturated with artesunate after shaking during 5 days were tested on degradation products of the active compound. To evaluate the behavior of the active compound in a liposomal suspension, the trapping efficiency (TE) was determined. As well as the total artesunate content in the liposomal suspension and the free, "not entrapped" content were investigated as a function of time.

To determine the exact total content, an exactly weighted amount of liposomal suspension (500 mg) was vigorously mixed with 250.0 μl methanol; 1.50 ml acetonitrile was dropwise added to precipitate the lipids. The solution was then centrifuged during 15 min at 3000 rpm ($1512 \times g$) with a refrigerated centrifuge Mistral 400 (Fisons, Onshore, UK). The supernatant was injected in the HPLC system after appropriate dilution. The same procedure was used to prepare the samples for the

stability investigation of artesunate in the liposomal suspension.

For the determination of the free artesunate, approximately 1 ml suspension was centrifuged at 4000 rpm (2500 × *g*) for 20 min and an aliquot of the supernatant was analyzed with HPLC.

2.2.2.3. Release of the artesunate from the liposomes. To check the release of artesunate from liposomes, an in vitro dialysis technique using a Kontron-Diapack model 4000 apparatus (Kontron Instruments, Zürich, Switzerland) was performed. The donor and the acceptor compartment were separated with a Teflon TE 35 membrane filter, 0.2 μm, ∅ 50 mm (Schleicher and Schüll, Dassel, Germany), previously impregnated with dodecanol (Merck, Darmstadt, Germany). The donor compartment is filled with 2 ml of the liposomal suspension, containing artesunate. Two milliliters of empty liposomes, containing the same content of lipids as the suspension to investigate, was injected in the acceptor compartment. The dialysis is performed during 24 h at a velocity of 2 units on the instrument in a thermostatic room of 25 °C. A certain amount (150 μl) of each compartment was then subjected to HPLC after sample treatment, similarly as for the determination of the total content of artesunate, using 150 μl methanol.

2.2.2.4. Removal of artesunate in the determination of hydroperoxides in EPC by the modified International Diary Federation method. As artesunate disturbs the modified International Diary Federation (IDF) method [26], the Sep-Pak mini-column extraction method, presented in the work of New [8], which is applied to clean the EPC from the liposomal suspension, should be evaluated for the separation of artesunate from the EPC sample. One milliliter of a (50 mg/50 ml) solution of artesunate in chloroform, exactly weighted, was put on the Isolute® silica gel 3 ml mini-column (IST International, mid-Glamorgan, UK) after conditioning. Different extraction methods, as presented in Table 2, were performed. The sample was taken after each extraction step and then dried under nitrogen stream and redissolved in an appropriate amount of mobile phase and then subjected to HPLC. A standard solution was treated similarly, with the exception of the extraction steps.

2.3. HPLC methods for the determination of artesunate and other liposomal components

The HPLC system used through this study consists of an Uvikon HPLC pump 420, an Uvikon capillary detector and a Merck Hitachi D 2500 chromato-integrator and a Rheodyne 20 μl loop (Cotati, CA).

All artesunate solutions were analyzed following this HPLC method: a Lichrocart C18 column (250 mm, 4 mm) filled with Lichrospher 100 (5 μm) particles (Merck, Darmstadt, Germany), a mobile phase consisting of 50% acetonitrile HPLC grade from Biosolve (Valkenswaard, The Netherlands) and 50% MQ-water (v/v), a flow set at 1 ml/min, a detection wavelength at 215 nm and an injection volume of 20 μl.

The method of Lang [27] was applied to investigate the passage of EPC and cholesterol through the applied membrane in the in vitro dialysis test.

Standards of EPC (2 mg/ml) and cholesterol (1 mg/ml) were prepared in methanol. One milliliter of empty liposomes, containing only EPC and cholesterol, was diluted in methanol to 100 ml. Twenty microliters of all these solutions and citrate–phosphate buffer of pH 5 were injected in the same HPLC system as for artesunate, using a Lichrocart C18 column (250 mm, 4 mm) filled with Lichrospher 100 (5 μm) particles (Merck, Darmstadt, Germany) and pure methanol as mobile phase at a flow rate of 1 ml/min. Detection was also performed at 215 nm.

3. Results and discussion

3.1. Incorporation capacity and trapping efficiency for artesunate into liposomes

To study the incorporation capacity of artesunate in liposomes, series of liposomal suspensions were prepared, which contain different contents of lipids and artesunate and which are dispersed in

Table 1
Crystals and free artesunate (F) in different liposomal preparations

pH	Lipids (mg/ml)	Concentration of artesunate (mg/ml)							
	First series	2	[UP]%		4	[UP]%		6	[UP]%
5	100	n.i.	–		n.i.	–		n.i.	–
	200	n.i.	–		n.i.	–		n.i.	–
	300	n.i.	–		n.i.	–		n.i.	–
7	100	++	33.4		+++	26.2		+++	26.8
	200	++	29.0		++	27.5		+++	25.8
	300	++	26.1		++	23.8		+++	24.0
9	100	++/+++	38.3		++/+++	39.3		++/+++	31.4
	200	++/+++	32.1		+++	28.1		+++	30.3
	300	++/+++	28.6		+++	25.3		h. η	h. η
	Second series	1	[UP]%	TE%	1.5	[UP]%	TE%	2	[UP]%
5	100	+	n.i.	–	+	1.4	–	++	6.0
	200	+	11.2	–	+++	0.4	–	+++	0.4
	300	–	~0.0	100.0	–	2.0	98.0	+++	8.8
7	100	+	16.5	–	+	16.8	–	+++	n.i.
	200	++	20.4	–	+	12.8	–	+++	1.6
	300	–	n.i.	n.i.	–	2.9	87.1	+++	~0.0
9	100	+	11.1	–	+	2.1	–	+++	3.0
	200	+	24.1	–	+	3.2	–	+++	1.1
	300	–	h. η	h. η	–	h. η	h. η	+	0.9

[UP]%, % of the total amount of artesunate in the upper phase after centrifuging the liposomal suspension; TE%, encapsulation efficiency; h. η (high viscosity), suspension is too viscous for analysis; n.i., not investigated. Number of crystals: –, no crystals; +, 1–5 crystals; ++, 6–10 crystals; +++, more than 10 crystals.

buffers of different pHs, as figured in Table 1. Since artesunate has acidic characteristics owing to its carboxyl function, we can expect influence of the pH of the buffer on the incorporation capacity of artesunate into liposomes. Addition of α-tocopherol (vitamin E), usually present as antioxidant in lipids, was omitted as it possibly interferes with the pharmacological effect of artesunate, which is based on the reductive effects of its peroxide bridge [28,29].

In the first series, the concentration of artesunate varied between 2 and 6 mg/ml buffer added. This means that the final concentration of artesunate was 2, 4 or 6 mg/1.1 ml suspension when 100 mg/ml lipids were used, and per 1.2 or 1.3 ml suspension, when respectively 200 and 300 mg lipids were used. The density of the lipid fraction equaled 1, as cholesterol and EPC, respectively, have a density higher and lower than 1.

To simplify the expression of the concentration of active compound in the suspension, it will be mentioned as mg/ml buffer added.

The results are shown in Table 1, in which the amount of detected crystals per 20 squares was estimated for each prepared suspension. Even the fraction of artesunate, which is not encapsulated, was determined. From these results, TE was calculated, using the following formula:

$$\text{Trapping efficiency} = \frac{\text{TotalAS} - \text{FreeAS}}{\text{TotalAS}} \times 100\%,$$

in which TotalAS is the total concentration of artesunate added for the preparation of the liposomes and FreeAS the total concentration dis-

solved in the upper aqueous phase after centrifugation of the liposomal suspension.

This is only valuable for crystal-free suspensions, which means that the TE should be determined for liposomal suspensions, containing maximal 1.5 mg/ml. The highest value was found for the liposomal suspension, containing 1 mg artesunate in 300 mg lipids in buffer of pH 5.

The table shows that in the first series of liposomal suspensions, crystals were seen in all the suspensions. Fig. 1 represents artesunate crystals, suspended in some water (A) and in the liposomal suspension (B).

So, we can conclude that the concentrations of artesunate were too high to prepare liposomal suspensions. The amount of crystals increased sharply with increasing artesunate concentration and lowering the lipid concentration, as expected.

The following series were prepared with lower artesunate concentrations, between 1 and 2 mg. The results given in Table 1 show that at least 1.5 mg artesunate per ml suspension can be incorporated in liposomes. Therefore, 300 mg lipids per ml suspension are essential. The buffer influences the TE of artesunate, which is the lowest at pH 9. This can be explained by the fact that artesunate has a carboxyl function in its structure and dissolves easily in alkaline solutions. And so, at pH 5 much more acidic molecules could be found than at the other pH's, which are lipophilic and thus, are preferably entrapped in the lipid fraction. As conclusion, several liposomal suspensions can incorporate 1.5 mg/ml artesunate, independent of the buffer.

For further selection of the best liposomal suspension, chemical stability of artesunate should be taken into account, as the active compound is known to be unstable in aqueous solutions.

3.2. Chemical stability of artesunate at different pHs and in the liposomes

In a pretest to estimate the solubility and so, the TE of artesunate in the liposomal suspension, the supernatant of buffer solutions, oversaturated with artesunate, were controlled on the stability of the active compound. The following results were found: 29.7 mg% at pH 5, 24.0 mg% at pH 7, and 36.6 mg% at pH 9. This suggests the degradation of artesunate already after 5 days, as our analyte has acidic properties and would thus much easier soluble in alkaline solutions. Much higher concentrations were expected for pH 7 and 9. As demonstrated in Fig. 2, several additional peaks were noticed compared with the HPLC chromatogram of pH 5 and the standard.

This can explain our results, as it proves degradation of artesunate [30]. These findings have consequences on the selection of the best liposomal suspension. Indeed, the suspension in which the TE reaches 100% should be preferred owing to the instability of the active compound.

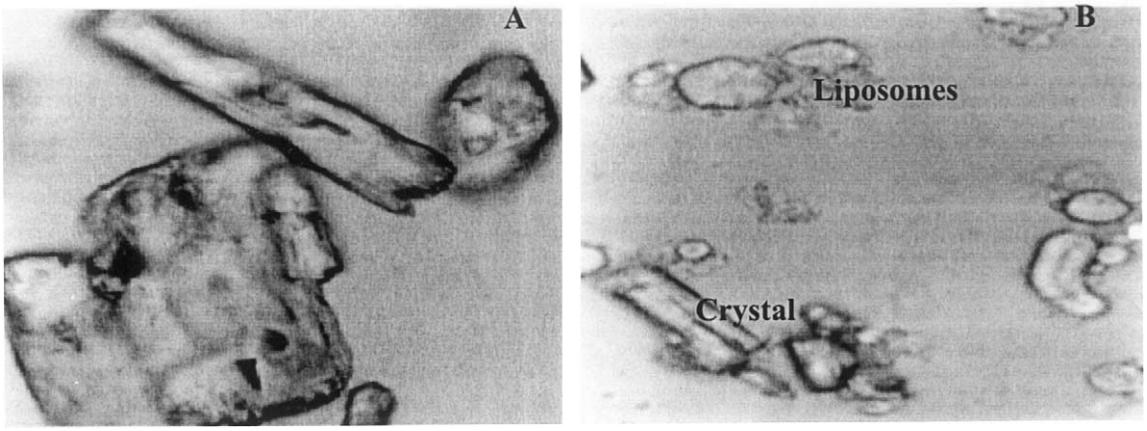

Fig. 1. Microscopic picture of crystals of not dissolved artesunate in an aqueous solution (A) and in a liposomal suspension (B) (magnification: 400 ×).

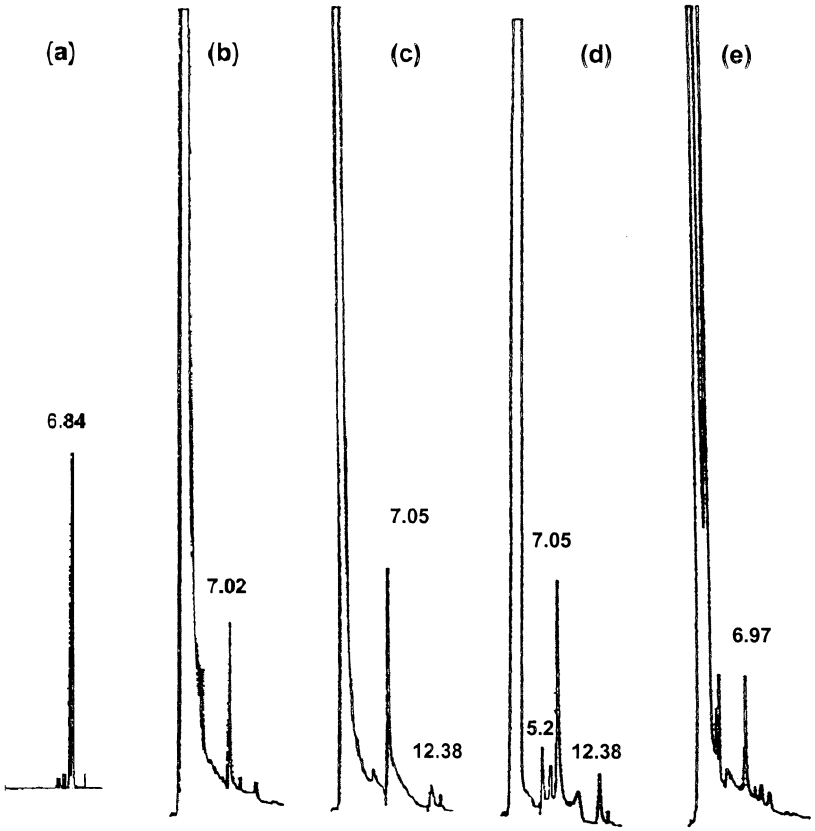

Fig. 2. HPLC chromatogram of a standard of artesunate in ACN (100 mg%) (a), the undiluted supernatant of pH 5 (b), 7 (c) and 9 (d) and the supernatant of AS in liposomes in buffer pH 5.

The best suspension is the one containing 1 mg artesunate and 300 mg lipids in 1 ml buffer of pH 5. Short-term stability investigation on this liposomal preparation revealed that it remains stable for at least 10 days.

Therefore, a liposomal suspension should be selected in which artesunate is preferably and totally incorporated. And when long-term stability is controlled, the one with 1.5 mg/ml artesunate was not selected as it still contains 2% free artesunate, which can degrade.

The most safe suspension is thus the one containing 1 mg/ml artesunate containing 300 mg lipids in pH 5, having a TE of 100%.

3.3. Chemical stability of the liposomal components in the liposomal suspension

The most important degradation products of EPC, which exist after lipid peroxidation, should be evaluated in the development of liposomal suspensions. Lipid peroxidations assessed mostly by measuring principally the hydroperoxides and dienes, as they are the initial degradation products [31]. For both degradation products, methods were described in literature namely the modified IDF method, including an SPE (solid-phase extraction) technique to measure the hydroperoxides [8,26] and the UV measurement of conjugated

dienes at 233 nm [8,32]. To analyze these products, cholesterol and even the small content of vitamin E, always added to EPC to protect against oxidation, should be separated from the EPC. As artesunate has oxidative properties owing to the peroxide bridge and its mechanism of antimalarial activity in vivo is based on an iron-dependent free radical generation [33], which is caused by the reaction between the peroxide bridge and Fe^{2+} in the intraparasite heme [34], one could expect interference in the analysis. Indeed, determination of hydroperoxides is based on the oxidation of Fe^{2+} into Fe^{3+}, which will be complexed with thiocyanate. It is thus quite clear that artesunate will interfere. Therefore, an experiment was set up to evaluate whether the SPE technique, used as pretreatment of the EPC sample, can be applied on the liposomal suspension, containing artesunate.

The extraction technique for the removal of vitamin E, presented by New [8], namely a solid-phase extraction fulfilled in two steps (Fig. 3): firstly, removal of the aqueous part of the liposome' suspension by a two-step elution on a Waters Seppak-C_{18} column; and secondly, removal of vitamin E and cholesterol from the EPC with chloroform on a silica gel column; then, the "vitamin E-free" EPC was eluted with methanol.

Following the presented procedure (Fig. 3), the greatest part of artesunate is estimated to be extracted as the lipids in the first step. Then, in the second step, artesunate should follow the extraction way for vitamin E and cholesterol. The investigation for the elimination of artesunate from EPC is thus especially performed in this step.

The major change made to the system of New [8] was the process to elute the lipids. Firstly, 10 ml was put drop by drop to wash the column. This step was repeated with the same amount of chloroform.

As desired, 100% artesunate should be eluted with this fraction. Even the methanol fractions, which are applied to elute EPC from the column, were investigated in the presence of artesunate.

In a second step, 20 ml chloroform was put per 2 ml on the column to wash it. Between each step, chloroform was almost totally removed from the column under vacuum with the Microlut extraction unit. The amount of artesunate, remaining on the column, was then eluted with methanol. This fraction should be as low as possible.

Afterwards, we investigated whether a column can extract consecutively several samples, always

Table 2
Influence of the elution process with chloroform on the behavior of artesunate on a Seppak-C_{18} column

Elution process with chloroform	Fraction in	
	Chloroform phase (%)	Methanol phase (%)
10 ml dropwise	44.02	50.45
10 × 2 ml chloroform		
First sample	72.06	5.32
Second sample	94.97	4.44

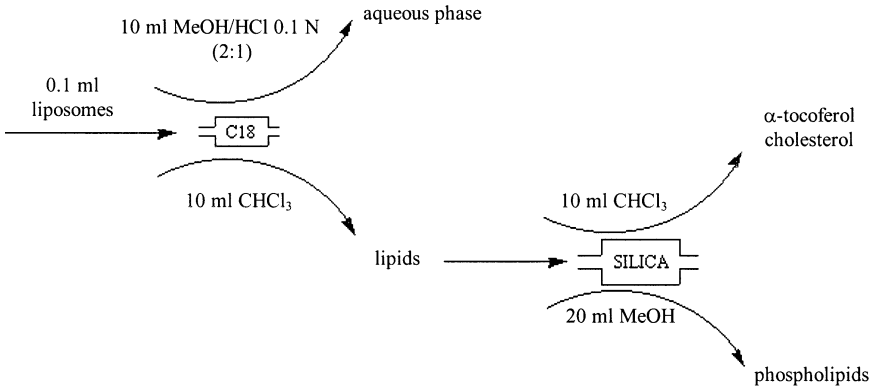

Fig. 3. Seppak solid-phase extraction of lipids from a liposomal suspension as presented by New [8]and Lang [27].

alternated with a conditioning step. A second set of samples was investigated to test the recycling of the columns. Results are presented in Table 2.

The following conclusion could be made from the results in Table 2. As the artesunate fraction was only 5%, we can conclude that the methanol phase is nearly free of artesunate and this is the phase from which EPC will by extracted from the silica column. These findings were obtained by enhancing the chloroform content, normally used for the separation of vitamin E and cholesterol from the liposome and even the way of eluting these compounds, e.g. 10×2 ml chloroform instead of drawing up the whole volume of 10 ml on the column.

3.4. In vitro release of artesunate from the liposomes

Since artesunate has a high affinity for the lipids in the liposomal suspension at pH 5, we are unsure whether it can be easily released from the liposomes. In order to determine the release of artesunate from the liposomes, we performed a series of in vitro dialysis experiments. The first problem that occurred here was the choice of the acceptor medium. The acceptor medium should contain a solvent in which artesunate dissolves easily, in order to obtain SINK conditions. Since artesunate is poorly soluble in the citrate–phosphate buffer at pH 5, this solvent could not be used. The use of organic solvents is also unlikely, as they will pass the membrane and damage the liposomes, enhancing the release of artesunate. Cortesi et al. [35] used empty liposomes in their investigation. If we want to do so, we should test whether the liposomes are passing through our selected membrane, a lipophilic one.

Therefore, a dialysis test was performed, including the suspension of empty liposomes, containing 300 mg lipids in the donor compartment, against a buffer of pH 5 in the acceptor compartment.

An HPLC analysis as presented by Lang [27], for the determination of the liposomal excipients was performed, using a C18 column and pure methanol as mobile phase.

Fig. 4 represents the chromatograms of an EPC, a cholesterol standard, a pure solution of buffer pH 5 and the solutions of the acceptor and donor compartments.

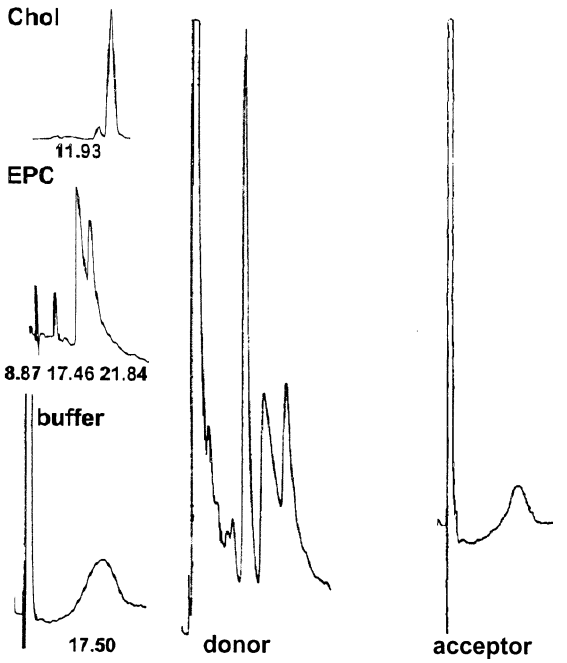

Fig. 4. Chromatograms of cholesterol standard in methanol (100 mg/100 ml) (CHOL), an EPC in chloroform (100 mg/100 ml) (EPC), a solution of buffer of pH 5 (buffer) and the solutions of the donor compartment (donor) and the acceptor compartment (acceptor).

From the color of the solution in the acceptor compartment, we could suggest that there was no passage of liposomes as it was a clear solution and not creamy colored as liposomal suspensions. The HPLC results prove our suggestion that the liposome is not passing through the membrane. None of the peaks of EPC or cholesterol are appearing in the chromatogram, which represents only a peak of the buffer.

So, our dialysis system is suitable to investigate the release of artesunate from liposomal suspension. Different suspensions, all prepared in buffer of pH 5, were investigated to test whether the amount of lipids or the content of active compound influences the release rate or velocity. Table 3 represents the results of the dialysis test.

The results of this experiment, summarized in Table 3, indicate that artesunate can be released from liposomal suspension but it is influenced by

Table 3
Release results of artesunate from the liposomal suspensions, performed with a dialysis technique after 24 h

Content per ml		% AS in donor compartment
100 mg lipids	1 mg AS	55.23
200 mg lipids	1 mg AS	66.77
300 mg lipids	1 mg AS	79.16
300 mg lipids	1.5 mg AS	54.02

several factors, for instance, the content of the lipids. The release of artesunate is decreasing with increasing lipid concentration. Assuming that artesunate is not absorbing on the membrane, only 30.8% artesunate was released from 300 mg lipids in 1 ml liposomal suspension, compared with almost 50% from liposomal suspension, containing 100 mg/ml lipids, in which the equilibrium was thus reached.

Even the content of artesunate seemed to enhance the release velocity, which can suggest that a part of artesunate, the same quantity for each preparation, containing the same content of lipids, is strongly associated with the liposome, but at least reversible; the other part, which is higher in the liposomal suspension, containing 1.5 mg/ml artesunate, releases quite easy. As artesunate is released from the liposomes, owing to reversible bindings, the purpose of our study is already reached, except from sterility.

3.5. Sterile preparation of lab-scale batches of liposomes

Liposomes cannot be sterilized by exposure to high temperatures, and are also sensitive to various types of irradiation, as well as chemical-sterilizing agents. The only way to sterile a liposomal suspension is by filtration, if it consists of liposomes sufficiently small to pass easily through a 0.22 μm filter [36]. For larger ones, every stage must carried out under aseptic conditions; the initial organic solutions of lipids can be filtered [8]. The whole procedure for sterile production in the lab is similar as the one used for the investigated liposomal suspensions, but should be adapted at certain levels as discussed below.

First of all, we should work under extreme conditions of sterility, which means in a laminar air flow chamber (LAF). But not all instruments are transferable to the LAF chamber. So, special connections with sterile filters between apparatus and the sample are made. Fig. 5 shows some of these steps and the adaptations made for a sterile production.

All glassware, tools and instrumentation, brought into contact with liposomes or their components during preparation were cleaned with alcohol 70% or sterilized and all manipulations were conducted in an LAF. The needed content of Chol and EPC and artesunate was separately dissolved in dichloromethane, as this organic solvent is generally accepted for the preparation of parenteral injections. These solutions were passed through a PTFE 0.22 μm antibacterial filter of Millipore. Afterwards, the organic solvent was evaporated under a sterile nitrogen flow on the Rock 'n' Roller in the LAF chamber. The complete removal of dichloromethane was performed with the lyophilizator. A sterile vacuum pump container, in which the liposomal samples were transferred under sterile conditions, was sealed and externally connected with the apparatus via a sterile plastic vacuum pump adapter bearing a sterile filter to prevent non-sterile air insert, when disconnecting the vacuum. For the hydration of the film, sterile phosphate buffer previously passed through a 0.22 μm antibacterial membrane filter, and sterile glass beads were added. The same procedure was then followed as for non-sterile liposomes. An aliquot of 1 ml was immediately checked for sterility. Sterility was proven for aerobe organisms. The results were negative for two different batches. The sterile liposomal suspensions were then transferred under sterile conditions into sterile glass serum vials, capped with an aluminum cap crimper and stored at 4 °C.

4. Conclusion

We are able to prepare artesunate liposomes with rather simple equipment using EPC and cholesterol as lipid components. Even sterile pre-

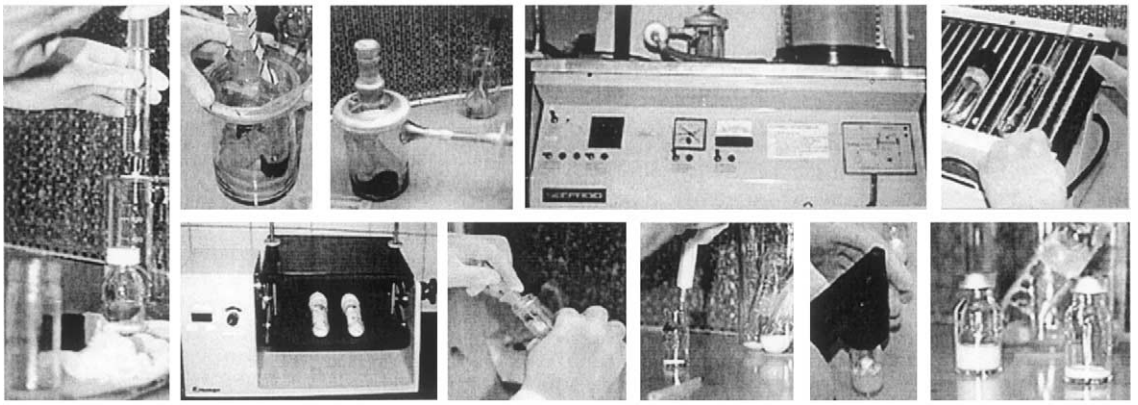

Fig. 5. Sterile production of lab-scale batches of liposomes.

paration at lab-scale level is possible. Different concentrations of artesunate and lipids in three buffers (pH 5, 7 and 9) were investigated. From these results, we could conclude that only in pH 5 artesunate seemed to be stable as it is almost totally incorporated in the liposomes and solubility in the aqueous phase is nil. Degradation studies on the liposomal suspension revealed at least 10 days of stability at 25 °C. The most stable liposomal suspension with the highest stability and the highest incorporation capacity is the one with 1.0 mg artesunate and 300 mg lipids per ml buffer of pH 5 added, as it has a TE of 100%.

The incorporation capacity results are comparable as those published by Chimanuka et al. [22] for artemether. The specific problem of artesunate, being unstable in aqueous solutions, is resolved by using the pH 5 buffer and the short-term stability results can overcome inconveniences that Batty et al. [30] suggested: artesunate should be administered by infusion rather than bolus injection; therefore, it should be stable in aqueous solutions for approximately 4 h at 30 °C.

The in vitro release test proves the release of reversibly bounded artesunate up to equilibrium or 30% within 24 h, depending on the content of lipids and artesunate. For topical application, it was already showed in literature [37–39] that topical administration is a good alternative for treatment with artemisinin derivatives with many advantages concerning liver passage, undesirable effects, sustained release characteristics. Liposomes seem to concentrate active compound locally on the skin as the liposome structure seemed to be lost when penetrating the first skin layer [40,41]. It could be interesting to continue investigation in this direction. To obtain a long-term stability in tropical conditions, one should investigate the stability-enhancing capacities of the lyophilization technique. Enhancing the incorporation capacities of these liposomal suspensions, other phospholipids or combinations can open new ways for in vivo applications. These aspects will be investigated in the near future.

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