Thesis for the degree Master of Pharmacy

DEVELOPMENT OF IMPROVED BENDAMUSTIN-LIPOSOMES

By

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Tove Julie Evjen

INDEX

1 ABSTRACT

Bendamustin is an alkylating anticancer agent which is currently in routine use for the treatment of different types of cancer. The drug is very unstable in serum due to hydrolysis; the half life of the first part of the serum elimination curve is about 6-10 minutes. The rapid degradation of the drug in serum impairs its cytostatic action within a short period of time, and frequent application of relatively high doses is required. This, in turn, leads to dose-limiting systemic toxicity. Incorporation of bendamustin into liposomes might be a promising way to prolong its half life in plasma, and thus improve the efficiency and toxicity profile of the drug. Up to now only a few attempts to incorporate bendamustin into liposomes are found in literature. However, none of these have been successful and reached clinical practice. Recently, a new technique for liposome preparation, dual asymmetric centrifugation (DAC), has been suggested which is suitable for making liposomes immidiatly prior to application (bed-side preparation). In a previous study a protocol for liposomes made of phosphatidyl choline and cholesterol was developed and used for direct entrapment of bendamustin. However, the formulation turned out to be unstable in terms of rapid efflux of bendamustin out of the liposomes.

In this study, a new liposomal formulation of bendamustin was developed using the DAC. The intention was to improve the stability of the liposome formulation by obtaining a reduced leakage of drug. Liposome release of incorporated drug was investigated by incubation of liposomes under physiological conditions; 37 °C, with further assay of the samples with respect to loss of incorporated drug over time. For this, cation-exchange chromatography and RP-HPLC was used. It turned out that bendamustin in its zwitterionic form tended to diffuse through the lipid-membrane more readily than both the cationic and anionic forms. In order to reduce the amount of zwitterionic molecules, and hence efflux, a buffered system with a pH of 2.0 in the liposome interior was chosen. Compared to the previous formulation, the new formulation showed an increase in encapsulation efficiency as well as a slower efflux of drug when incubated in phosphate buffered saline pH 7.4 at 37 ºC; EE= 61% ± 2 as compared to 44 % ± 3 and t1/2= 3 h as compared to 1.5 h.

Stability of the new formulation was assayed with respect to intact bendamustin as well as lipid at 23 °C over 24 hours. For this RP-HPLC and HPTLC were used, respectively. The results showed that the formulation is stable enough to be used within the same day as a bed-side preparation. Furthermore, stability of the new bendamustin-liposomes was compared to the free drug in cell culture medium at 37 ºC. Unfortunately, the bendamustin-liposomes showed a minor improvement in stability as compared to the free bendamustin only; the half life was prolonged to 20 minutes for the liposomes (14 minutes for the solution). Finally, an attempt of active loading of bendamustin was performed by the means of a pH gradient between the liposome interior and exterior. An EE of 14 % was observed. Further optimization of the protocol will be needed to render active loading an attractive alternative.

2 ABBREVATIONS

CH	Cholesterol
EE	Encapsulation efficiency
EPC3	Hydrogenated egg phosphatidyl choline
EPC3/CH	Mixture of hydrogenated egg phosphatidyl choline and cholesterol
HPLC	High performance liquid chromatography
i.v	Intravenous
LUV	Large unilamellar vesicle
MLV	multilamellar vesicle
m/m	Mass ratio
Mw	Molecular weight
PBS	Phosphate buffered saline
PC	Phosphatidyl choline
PCS	Photon correlation spectroscopy
P.I.	Polydispersity index
RES	Reticulo endothelial system
RP-HPLC	Reversed phase high performance liquid chromatography
SD	Standard deviation
SUV	Small unilamellar vesicle
t ½	Half- life
VPG	Vesicular phospholipid gel
v/v	Volume ratio

3 INTRODUCTION

Cancer is the uncontrolled growth and spread of cells that may affect almost every tissue of the body. Normally proliferation and apoptosis of body cells is a strictly controlled process. A normal cell turns into a cancer cell because of one or more mutations in its DNA, which the body is not able to repair. Instead of apoptosis, the cells outlive normal cells and continue to grow and divide to form new abnormal cells. The cancer cells might spread by the blood-stream to other parts of the body where they begin to grow and replace normal tissue. This process is called metastasis [1].

Cancer is a disease that is affecting us all. More than 11 million people worldwide are diagnosed with cancer every year [2]. According to the Cancer registry in Norway, 24 228 new cases of cancer was detected in 2005. Of these 12706 were males and 11522 females. One third of every Norwegian will get cancer in the course of life [3]. The frequency of different types of cancer is dependent on different factors as sex and age. Overall, prostate cancer is the most common cancer type among males, and breast cancer among women [3].

The incidence of cancer in Norway has increased drasticaly the last decades (figure 1). Fortunately, today more than half of those who get a diagnose, survive cancer. The current therapy for cancer is based predominantly in surgical removal of tumours, radiotherapy and chemotherapy with antineoplastic drugs. A lot of research is carried out, with the intention to improve the efficacy of the treatment. Some of the goals are to make the antineoplastic drugs more potent, selective and less toxic to normal tissue.

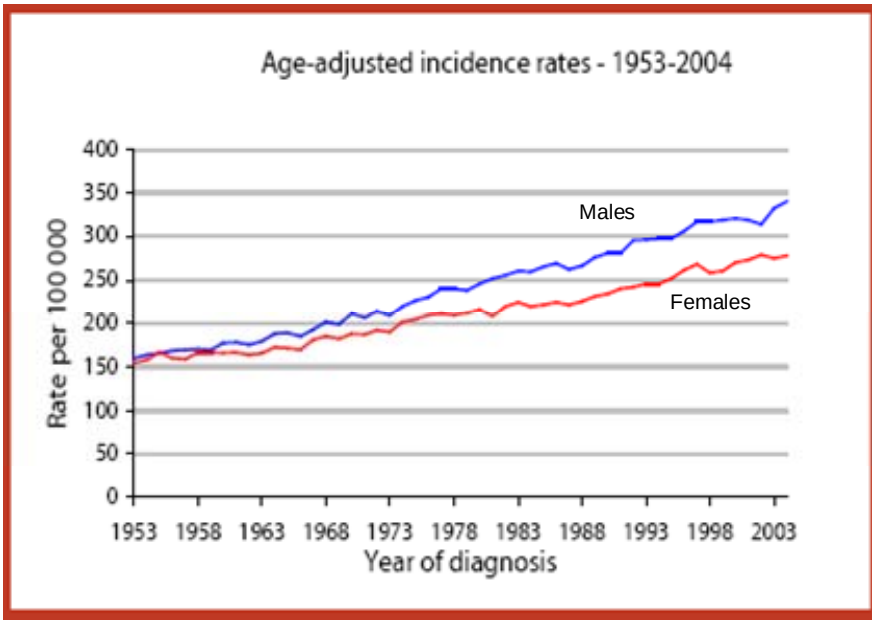


Figure 1: Age adjusted incidence rate, all cancer types combined, 1953-2004. Figure taken with permission from: www.kreftregisteret.no, Cancer in Norway 2004, part 3 fig 3.1.1.

3.1 Chemotherapy

Chemotherapy is often only possible treatment in non solid tumours and metastases spread in the body [4]. Cytotoxic drugs apply to any drug that inhibit cell division and are potentially useful in cancer chemotherapy. The main cytotoxic drugs can be divided into the following categories [1]:

- Alkylating agents, which act by forming covalent bonds with DNA and thus impeding DNA replication.
- Antimetabolites, which block or subvert one or more of the metabolic pathways involved in DNA synthesis.
- Plant derivatives, which affect microtubule function and hence reduce cell division.

3.2 Alkylating agents

Alkylating agents are developed from mustard gas (dichlorethylsulfid) which was used as the first time during the 1st world war as a war fare agent. The gas was yellow-brown in colour and had an odour resembling mustard, which is how it got its name. Mustard gas was the most lethal of all poisonous chemicals used during the war. To day sulphur mustard is regulated under the 1993 Chemical Weapons Convention [5].

Despite the tragic history, mustard gas was the beginning of the modern era of cancer chemotherapy. After the war, two pharmacologists, Louis S. Goodman and Alfred Gilman, observed that people exposed to mustard gas had revealed profound lymphoid and myeloid suppression. They reasoned that this agent could be used to treat lymphoma, since lymphoma is a tumour of lymphoid cells. After injection of less toxic nitrogen mustard into a patient with non-Hodkin`s lymphoma, a dramatic reduction in the patient`s tumor masses was observed. This was the first step to the realization that cancer could be treated by pharmacological agents [6].

Modifications of the dichlorethylsulfid- molecule have been made in order to reduce its reactivity and toxicity, resulting in alkylating cytostatics used in chemotherapy. The sulfid has been exchanged with less reactive nitrogen, and different alkyl groups and aromatics have been added in order to withdraw electrons away from nitrogen, thus making it less reactive and toxic to normal tissue. (figure 2 and 3)

Figure 2: Sulfur mustard

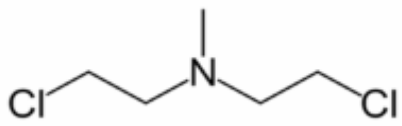


Figure 3: Nitrogen mustard

Alkylating agents contain chemical groups that can form covalent bonds with particular nucleophilic substances in the cell, and thus impeding DNA replication. Most alkylating agents have two alkylating groups and can cross-link two nucleophilic groups and cause intra or interchain cross-linking. This interfere transcription as well as replication. The resulting DNA damage triggers apoptosis of the cells [1].

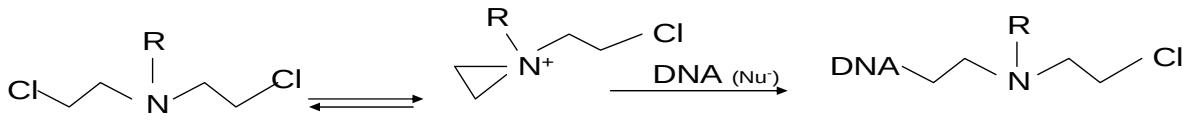


Figure 4: Mechanism of action alkylating agents, where one of the two alkylating groups binds to a nucleophilic base (Nu^-) in DNA.

3.3 Bendamustin

Bendamustin is an alkylating cytostatic drug which was developed in the early 1960s at the Microbiological and Experimental Therapy group in Jena, Germany. The idea was to combine a purine and amino acid antagonist with an alkylating nitrogen mustard group; bifunctional alkylating agent. In addition alcanecarboxylic acid was added to provide water- solubility [7], [8].

Chemical properties

The chemical name of the active ingredient Bendamustinhydrochloride:
(5-[bis (2-chloroethyl)-amino]-1-methyl-2-benzimidazole) butyric acid hydrochloride.

Formula:
$C_{16}H_{18}C_{l2}N_3O_2 \times HCl$

Molecular weight: 394.7 g/mol

Calculated pKa values [9];
4.5 ± 0.10 (most acidic, 25 °C)
6.3 ± 0.3 (most basic, 25 °C)

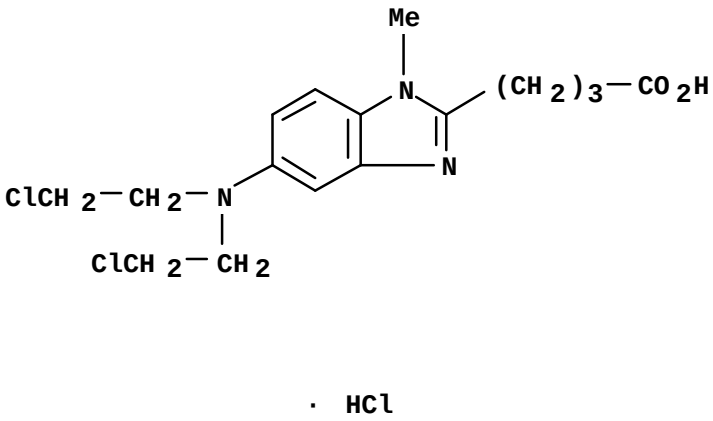


Figure 5: Structural formula of bendamustinhydrochloride.

Mechanism of action

Bendamustin is a bifunctional alkylating agent with antineoplastic and cytocidal properties. The efficacy is attributed mainly to crosslinking of the DNA single and double strands by alkylation. It is not yet known whether the benzimidazole ring possesses additional antimetabolite properties [10], [11], [12].

Indications

Today bendamustin is marketed under the name Ribomustin®.

Ribomustin® is registered as single- agent therapy or in combination with other antineoplastic drugs for the treatment of the following malignancies [13]:

- Breast cancer
- Hodkin`s disease
- Non-Hodkin`s lymphoma
- Plasmocytoma
- Chronic lymphocytic leukaemia

Stability and pharmacokinetics

Bendamustin is very unstable in water due to hydrolysis into monohydroxy- and dihydroxy-bendamustin [14]. The drug is available as a freeze- dried powder. Bendamustin- solution is administered intravenously using 0.9 % sodium chloride solution as a diluent. The presence of sodium chloride in the infusion is slowing down the hydrolysis of the drug. (Stability in 0.9 % sodium chloride 0.25 mg/ml; 4 ºC t90= 120 h, 23 ºC t90= 9 h [15]). The reason for the increased stability in sodium chloride solution is most probably due to the presence of chloride ions working as competitative inhibitors for the nucleophilic H_2O molecules, and hence reducing degradation of the chlorine bounds of the molecule. Hydrolysis and stability of bendamustin is pH dependent. Hydrolytic cleavage of the chlorine bonds is fast at pH values above 6 [14]. Bendamustin is a zwitterionic molecule with two pKa values: 4.6 and 6.3. Low pH will give rise to a protonation of the carboxylic group and the amino-group(s), whilst a high pH results in deprotonation.

In serum bendamustin is bound to plasma proteins, mainly albumin, to the extent of 95 %.[16], [17], [18]. The distribution volume is 15.8-20.5 L at steady state. The drug is extremely unstable in serum, due to hydrolysis. Following bolus injection, the plasma level follows a biphasic exponential pattern. The elimination half life of the alfa phase is between 6 and 10 minutes, and the terminal half life (beta phase) between 28 and 36 minutes. The substance is primarily metabolised in the liver and eliminated by the kidneys [17], [18].

Entrapment of drug within liposomes is a way to protect the drug from its systemic environment. Some advantages with liposomes as drug formulations are their ability to

prolong half-life of unstable drugs in serum and thus improve its activity toward cancer cells. Liposomal entrapment of the bendamustin might be a way to reduce hydrolysis of the drug in serum, and hence prolong its half life.

3.4 Liposomes

Liposomes are spherical vesicles which enclose an aqueous core by a membrane composed of lipid molecules, usually phospholipids. Liposomes are spontaneously formed when lipids are dispersed in aqueous solution by mechanical energy. The vesicles formed may consist of one or more concentric bilayers (lamellae), and have a size range from tens of nanometers to tens of micrometers. The composition of an aqueous core as well as a lipid membrane gives the liposome an ability to incorporate both hydrophilic and lipophilic drugs. For recent review see [19].

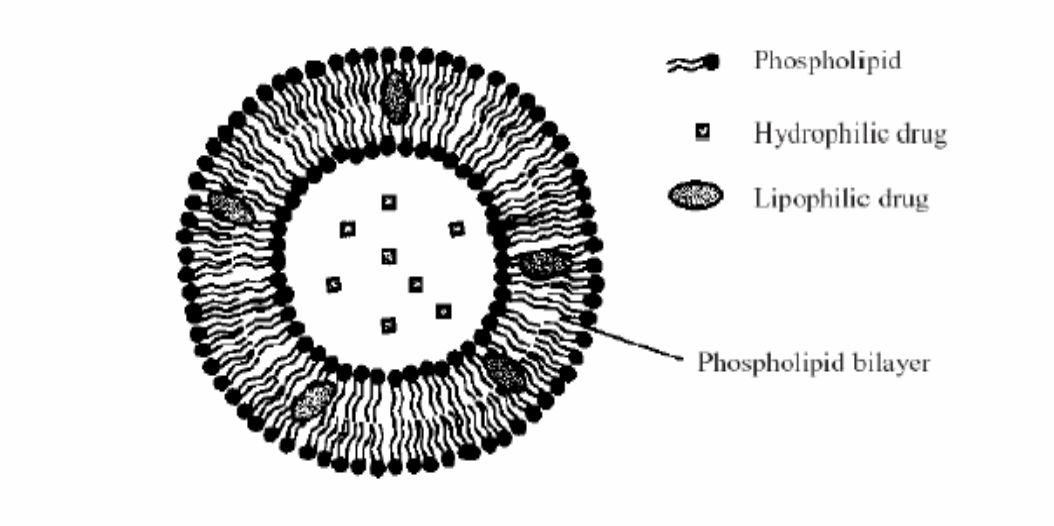


Figure 6: Liposome representing incorporation and encapsulation of lipophilic and hydrophilic drugs. Figure is taken with permission from Elsvier publisher.

The choice of lipids for liposomal drug carriers is related to the desired stability of the liposome formulation, and the drug to be loaded into the liposomes. The most common phospholipid used in liposomal drug carriers is phosphatidylcholine (PC). For recent review see [20]. PC can be derived synthetically or from natural sources as egg and soya. PC is an amphiphilic molecule in which a glycerol bridge links a pair of hydrophobic alkyl hydrocarbon chains with a hydrophilic polar headgroup phosphorylcholine [19]. These molecules are not soluble in water, but instead of solutions they form colloidal dispersions.

The hydrophilic part tends to be in contact with water, whilst the hydrophobic hydrocarbon chains prefer to be shielded against water in the interior of the structures. In aqueous media, under the influence of mechanical agitation these lipid bilayers are forming closed sealed vesicles; liposomes [21].

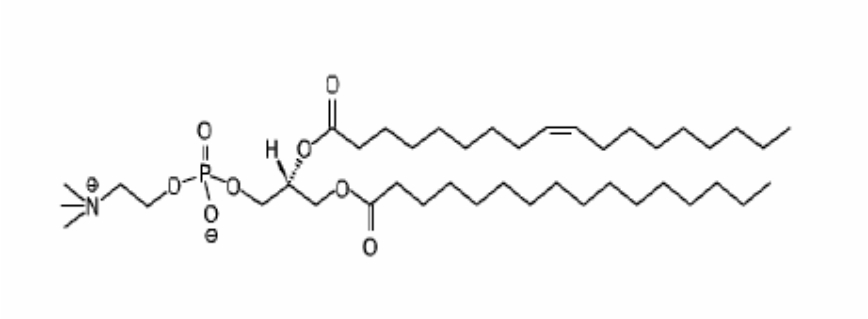


Figure 7: Structural formula of phosphatidyl choline

Liposomal membranes can exist in different physical phases depending on the transition temperature of the membrane. Transition temperature is a given temperature where the lipid membrane goes over from a rigid to a more flexible state which is more permeable. The length and degree of saturation of the alkyl chains mainly determines the transition temperature of the membrane. For recent review see [20]. Egg PC has a phase transition temperature between -15 and -7 ºC, i.e. is in the "fluid"-state at ambient temperature [19].

Usually when drugs are incorporated into liposomes one wants to prevent leaking and premature loss of drug through the membrane. Hydrophilic compounds with a low Mw are most prone to leak out of the liposomes due to the concentration gradient between the inside and the outside of the liposomes. A normal way of preventing leakage by making the membrane more rigid is the use of cholesterol (CH). CH is a flat, rigid molecule which is added to the lipid in order to make a tighter packing of the membrane, and hence loss of entrapped drug is reduced. CH can be incorporated in very high mixing ratios, up to a CH: PC mole ratio of close to 1:1 [20].

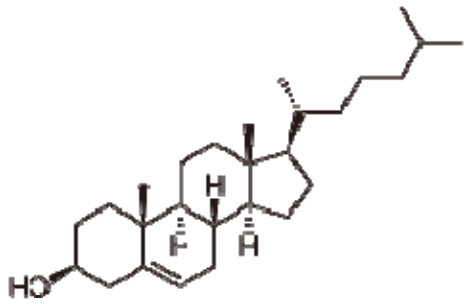


Figure 8: Structural formula of cholesterol

Phosphatidylcholine and cholesterol are both very important components in most natural membranes. The ability of liposomes to mimic the behaviour of natural membranes, and also to be degraded by the same pathways, makes them a very safe and efficacious vehicle for medical applications. For recent review see [19].

Classification of liposomes

Liposomes are often classified according to their size. This is partly because different sizes are manufactured in different ways, and partly because different applications demand particle sizes in a certain size-range [19].

Usually, liposomes belong to one of the following categories [19];

- Multilamellar vesicles (MLVs): Usually consist of a polydisperse population of vesicles covering a size range of 100-1000 nm. The vesicles contain several up to hundreds of concentric lamellae. (Vesicles containing just a few concentric lamellae are sometimes called oligo-lamellar liposomes.)

- Large unilamellar vesicles (LUVs): These vesicles have a size above 100 nm, and normally consist of one concentric lamella.

- Small unilamellar vesicles (SUVs): These are defined as the smallest phospholipids vesicles possible. Their size is below 100 nm, and they consist most often of one lamella. SUVs have a relatively homogenous population with regard to size.

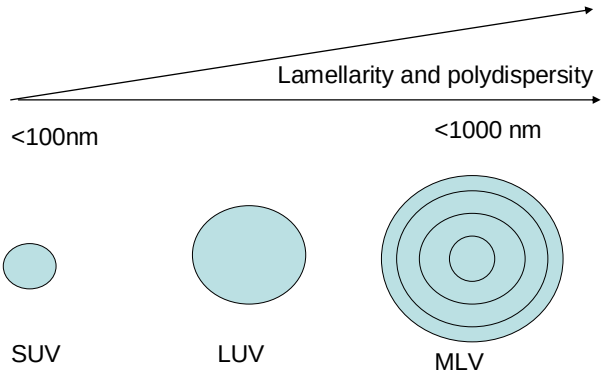


Figure 9: Size, size-distribution and lamellarity of liposomes.

Stability of liposomes

During storage, physical stability might alter the particle size of liposomes. Aggregation and fusion are two unfavourable processes in this respect. Fusion is the occasion when vesicles fuse together and make bigger liposomes. Aggregation is the phenomenon where liposomes are forming aggregates oftenly accompagnied by sedimentation or flotation, but is on the contrary to fusion a reversible process which can be resolved by agitation or stirring [20].

Two other unfavourable processes regarding stability of liposomes are oxidation and hydrolysis of lipids. The resulting chemical degradation of the phospholipids leads to short-chain phospholipids and lyso-derivatives in the membrane, respectively. Hydrolytic cleavage of the ester linkage of PC results in lyso-phophatidylcholine (Lyso-PC). The consequences of such reactions might be increased permeability of the bilayers as well as fusion or aggregation. Different precautions can be taken to prevent chemical degradation. In the case of oxidation, choice of phospholipid, optimal temperature for storage and absence of light and oxygen are of importance. The main defence against hydrolysis is selection of optimal pH, buffer and lyophilized lipids if possible [22].

It is not much known to which impact presence of Lyso-PC arising from hydrolysis has on safety in vivo. However, all biological membranes contain small amounts of lyso-PC as a consequence of natural membrane turnover [23].

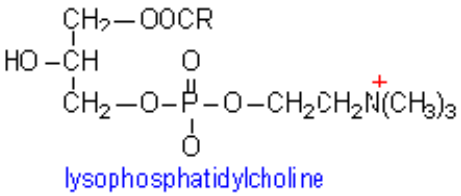


Figure 10: Structural formula of Lyso-phosphatidylcholine.

3.5 Vesicular phospholipid gels

As the name is insinuating, vesicular phospholipid gels are gels formed by phospholipids-vesicles (VPG). The dispersion does not contain any gelifying agents. It must neither be confused with the term "gel-phase" used for phospholipid bilayer systems below their transition temperature. VPGs have simply got its name because of its gel-like rehological behaviour, deriving from phospholipids vesicles which are so tightly packed that a steric interaction between them occurs. Their consistence can be described as viscous to semi-solid, with a highly ordered homogenous appearance [24].

VPGs differ from conventional liposomes in that they contain very high lipid concentrations. Like liposomes, VPGs can be made of a single phospholipid, mainly phosphatidyl choline, or a mixture of two or more lipids that upon minimal amounts of water swell and form vesicles when subjected to mechanical agitation. The vesicles formed are densely packed and appear uniform and small in size (mostly unilamellar) [25].

Because of the consistence of the VPG and the steric interaction between the vesicles, the aqueous volume inside and outside the vesicles are the same. This results in no concentration gradient between the aqueous core and the water phase surrounding the vesicles. When conventional liposomes are stored and uncaptured drug are removed from the dispersion, entrapped drug tends to leak out of the liposomes. Since there is no concentration gradient in VPGs, as long they are not diluted, such leakage is prevented, and the vesicles do retain a constant amount of drug during long-term storage [25]. (Figure 11)

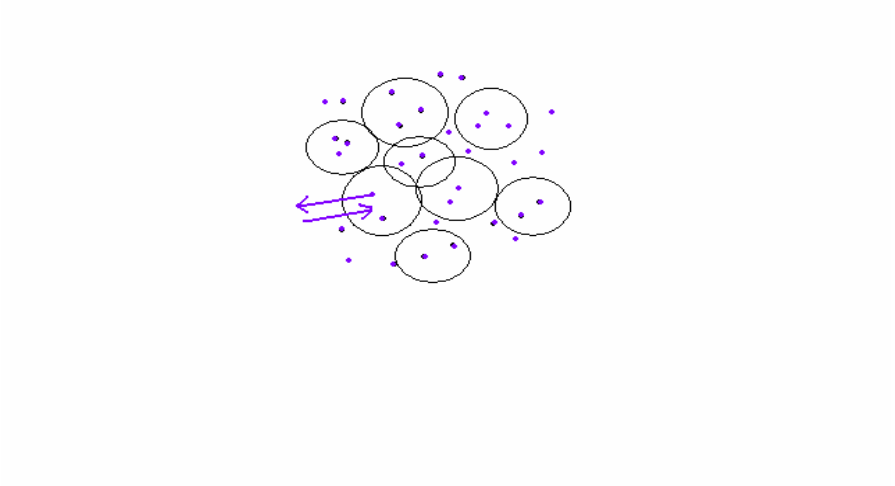


Figure 11: Vesicular phospholipids gels reveal the same aqueous volume inside and outside the vesicles resulting in no concentration gradient for the incorporated drug.

Another advantage with VPGs is their ability to entrap higher percentages of drug than conventional liposomes. This is due to a greater lipid content which results in more liposomes per unit volume, and hence an increased ratio of the aqueous part entrapped into liposomes compared to the total volume. The vesicles can be loaded with hydrophilic, amphiphilic and lipophilic drugs in different ways [24]. The most common technique for preparing VPGs of small and uniform vesicle sizes is high- pressure homogenisation. VPGs can be transferred into conventional small-sized liposome dispersions by addition of excess aqueous medium and gentle mechanical agitation shortly before use [25].

3.6 Liposomes in anticancer therapy

Liposomes in cancer therapeutic are maybe the most important, but also one of the most complex fields of liposome applications. The main motive for developing liposomes as new drug delivery systems was the rather unspecific action of all known antineoplastic drugs against tumour cells, giving a low ratio of therapeutic to toxic effects [4]. The intention with liposomes as drug delivery formulations is that they should circulate in blood reaching specific targets, as sites of inflammations and solid tumors. Normal cells will hence be less affected, resulting in a reduction in toxicity and side effects. Up to now intravenous administration is the most promising route for liposomal formulations in anticancer therapy [4].

The role of liposome size in anticancer therapy

The size, size-distribution and lamellarity of liposomes play an important role in their use as drug delivery formulations, not least in anticancer therapy.

MLVs are the largest type of liposomes although their entrapped volume and hence the ability to entrap drug is not necessarily biggest. Once they are infused they are rapidly recognized by the immune system and taken up by macrophages which remove them from the circulation. Liposomes of intermediate size (LUVs) have a better chance of escaping the reticulo-endothelial system (RES), and hence have the ability to stay in the circulation for a longer period of time. The small liposomes (SUVs) show the shortest circulation time in blood due to capillary extravasations. However, this gives them the opportunity to reach targets outside the vasculature such as solid tumors. For recent review see [20].

The accumulation of SUVs into solid tumours is based on dissimilarities of healthy and cancerous tissues; Solid tumours are dependent on higher vascular blood supply than normal tissue, because of the high turnover of cells. At the same time, the endothelial walls of blood vessels in tumours are more permeable than normal endothelial linings because of an increased number of big gaps. This gives SUVs, as for all nanoparticles, the ability to penetrate into solid tumours, but not healthy tissues. At the same time the liposomes stay longer in the tumour due to reduced lymphatic drainage in tumour tissue [20]. This phenomenon is called enhanced permeability and retention effect of liposomes in solid tumour tissue. (Figure 12)

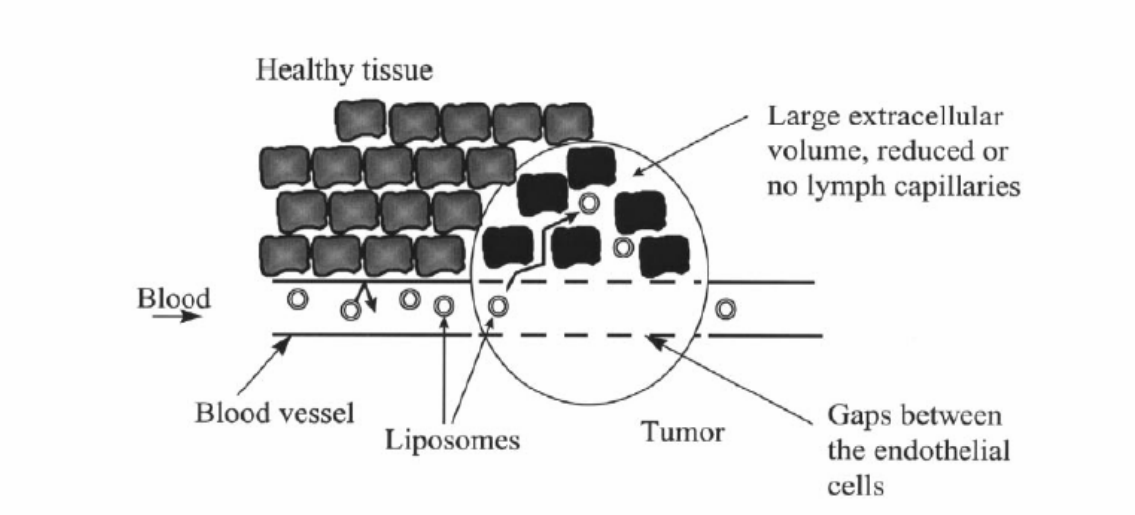


Figure 12: The enhanced permeability and retention effect of liposomes in solid tumour tissue. Figure is taken with permission from Elsvier publisher.

3.7 Challenges with bendamustin-liposomes

Alkylating agents have seldom been used in liposomal formulations. Water soluble alkylating agents did not show any improvement in therapeutic effects, and sometimes the drug stability was decreased [4]. In a previous study by Fichtner I et al, different alkylating agents, including bendamustin, were encapsulated into liposomes in order to investigate toxicity, stability and effect of the liposomal formulations compared to the free drugs. The conclusion was that only certain lipophilic alkylating drugs were suitable in liposomal formulations. Encapsulation of bendamustin did not show any advantages because of its instability in water [26]. Whether this is an effect of insufficient encapsulation and/or stability of the liposomes used remains unclear. From the stability data in literature it is obvious, however, that aqueous bendamustin- preparations are not suitable for storage.

Massing and co-workers suggested in a previous study a new technique for bed-side preparation of bendamustin- liposomes by dual asymmetric centrifugation (DAC) [27]. An optimal protocol for EPC3/CH- liposomes was developed for entrapment of bendamustin. The entrapping efficiency was good (42% ± 4), and the particle size of the liposomes was small (62.5 nm ± 3.5). The bendamustin-liposomes were shown to be stable enough for application within one day. However, a rapid efflux of drug out of the liposomes was observed under physiological conditions, resulting in an impaired activity of the drug towards cancer cells in culture.

4 AIM

The purpose of this project was to develop an improved liposomal formulation for bendamustin, using the DAC- technology. The goal was to find an optimal pH for the liposome formulation regarding stability and efflux of the drug, and thus prolong its half life under physiological conditions.

5 MATERIALS AND METHODS

5.1 Chemicals

Table 1: Lipids

Name of lipid	**Article number**	**Producer**
EPC3/CH 998 (molar ratio: 55/65)	Charge: 899259-1	LIPOID Germany

Table 2: Chemicals

Chemicals	**Article number**	**Producer**
Acetonitril	1.14291.2500	Merck, Darmstadt, Germany
AG 50W-X8 Resin	142-1441	BioRad Laboratories Inc. Munchen, Germany
Ammonia solution 25 % (p.a)	1.05432.1000	E.Merck,Darmstadt, Germany
Chloroform (Suppra solv)	1.02432.1000	E.Merck,Darmstadt, Germany
Copper(II)sulfate pentahydrate (p.a)	1.02790.1000	E.Merck,Darmstadt, Germany
di-Kaliumhydrogenphosphat trihydrat	1.05099.1000	Merck, Darmstadt, Germany
di-sodium hydrogenphosphate	1.06580.0500	Merck, Darmstadt, Germany
Dulbecco`s Modified Eagle`s Medium,modified formulation	30-1002	ATCC®, USA
Ethanol 96 %	5054.5	Merck, Darmstadt, Germany
Hydrochloric acid 37 %	1.00317.1000	Merck, Darmstadt, Germany
Potassium chloride	1.04936.0500	Merck, Darmstadt, Germany

Kalium di-hydrogenphosphate	4873.1000	Merck, Darmstadt, Germany
Lyso-phophosphatidylcholine palmitoyl (purity 99%)	L 5254	Sigma, Steinheim, Germany
Methanol (LiChrosolv)	1.06018.2500	E.Merck,Darmstadt, Germany
n-Hexane; C_6H_{14} (p.a)	1.00573.2500	E.Merck,Darmstadt, Germany
Phosphoric acid 85 %; H_3PO_4 (p.a)	1.00573.2500	E.Merck,Darmstadt, Germany
2- Propanol (p.a)	1.02790.1000	E.Merck,Darmstadt, Germany
Ribomustin®		Ribosepharm
Sodium chloride	1.06404.0500	Merck, Darmstadt, Germany
Sodium chloride (extra pure)	1.06400.1000	E.Merck,Darmstadt, Germany

5.2 Equipments

Table 3: Equipments

Equipment	**Type**	**Manufacturer**
Accusizer	PSS SWS 788	PSS Nicomp, California USA
Analytic balance	Mettler AT26119 Delta Range N85171	Mettler Toledo, Giessen Germany
Autosampler	Waters 717 Autosampler (717003252)	Waters, Eschborn, Germany
Centrifuge, model J2-HC	JGY93J11	Beckman coulter Gmbh
Chromatogram Immersion device III		Camag, Berlin, Germany
DC-Automatic TLC Sampler	000214	Camag, Berlin, Germany

Eppendorf tubes (2 ml)	0020120.094	Eppendorf, Germany
Glass beads - 1mm	BBI-8541809	B. Braun Biotech Int. GmbH, Melsungen, Germany
Glass chamber		Camag, Berlin, Germany
Heating/drying oven	901001894	Binder, Tuttlingen, Germany
Heater plate	93913	Desaga,Heidelberg, Germany
HPLC- system		
Waters 717 Autosampler	717003252	Waters, Eschborn, Germany
Detector	Waters 486 Tunable Absorbance Detector MX4MM8469M	Waters, Eschborn, Germany
Waters 625 LC System Pump	MX5MM5736M	Waters, Eschborn, Germany
Pump control module	PCM MX5HM7044M	VWR Brucksal, Germany
Pre- column	Lichrospher 60, RP select B 4*4 mm (5 µm)	Waters, Eschborn, Germany
Column	LiChrospher 60, RP- Select B	VWR, Brucksal, Germany
Column oven	CHM-012670	Waters, Eschborn, Germany
Temperature controller	Waters Temperatur Contol Module MX4MM6879M	Waters, Eschborn, Germany
Software	Empower Pro 2, version 6.00.00.00	Waters, Eschborn, Germany

HPTLC-plates, Silicagel 60	1.05626	Merck, Germany
Injection vial	10 ml, glass vial	
Injection vial	38 ml brown glass ISO 8362-4-JOH	ZSCHEILE & Kinger GmbH
Laboratory balance	Mettler PM 4000 (N88736)	Mettler Toledo, Giessen
Mini column separation	LiChrolut incl. PTFE frits and glass columns	Merck, Darmstadt, Germany
Mixer, Vibrax VXR	00,059656	Electronic, Germany
Photon correlation spectroscopy- PCS	Submicron Particle sizer Model 370 (CW370)	Nicomp Particle Sizing systems, California, USA
pH meter	Microprocessor PMX 3000	WTW GmbH Weilheim, Germany
SpeedMixer	DAC 150 FVZ (DAZ)	Hauschild, Hamm
Stirrer	Ikawag Ret-GS	IKA Werke GmbH & co. KG Staufen, Germany
Sterile Filter	Disposable filter holders 0,45µm Sterile, pyrogenfree	Schleicher &schnell GmbH Dassel, Germany
TLC scanner II, UV-detection system	991225	Camag, Berlin, Germany
Vials/reaction tubes 16×100 mm (with teflonlined screw stoppers)	358646	Wheaton Millville, USA
Vortexer	Reax 2000	IKA Werke GmbH & co. KG Staufen, Germany

5.3 Media and solutions

All the following solutions are given in examples of 1 L volume:

0. 9 % Sodium Chloride solution

- Used as hydration medium for preparation of VPGs, dilution medium for PCS measurements, and in efflux experiments.

1. Sodium Chloride 9.0 g
2. Distilled water ad 1000. 0 g

1.8 % Sodium Chloride solution

- Used for preparing bendamustin-solution.

1. Sodium Chloride 18.0 g
2. Distilled water ad 1000. 0 g

Concentrated Sodium Chloride solution

- Used for preparing ion-exchange columns

1. Sodium Chloride 360.0 g
2. Distilled water ad 1000.0 g

The Sodium Chloride was dissolved in distilled water by heating and stirring. The solution was filtrated through a 0.45 µm sterile filter into a flask for storage.

Ethanol/Methanol (90/10 v/v)

- Used for dissolving liposomes.

1. 96% Ethanol 711.0 g
2. Methanol 79.0 g

Mobile phase HPLC method cholesterol

Acetonitrile/methanol/acidic water (67/30/3 v/v/v):

1. Acetonitrile 527 g
2. Methanol 237 g
3. Distilled water containing 3 drops of phosphoric acid 3 g

Mobile phase HPLC method Bendamustin

1)

- Di-kaliumhydrogenphosphate- Trihydrate: 2. 3 g
- Distilled water ad 1000. 0 g
- Phosphoric acid adjust to pH 2.3

Di-kaliumhydrogenphosphate-trihydrate (pH 2.3) / methanol (58/42 v/v):

580.0 g of 1) ad 331.8 g Methanol

PBS 150 mM (containing 10mM phosphate) pH 7.4

- Used as aqueous medium for re-dispersion of liposomes.

1. Sodium chloride 8.00 g
2. Kalium chloride 0.20 g
3. Di-sodium hydrogenphosphate 1.44 g
4. Kalium di-hydrogenphosphate 0.24 g

Ad 1 L distilled water

The solution corresponded to a pH of 7.4.

PBS 150 mM (containing 30 mM phosphate) pH 2.0

- Used as a hydration medium for preparation of VPGs for active loading.

1. Kalium-dihydrogenphosphate 4.026 g
2. Sodium Chloride 6.856 g
3. Kalium Chloride 0.200 g

- Ad 1.0 L distilled water.
- Adjust pH to 2.0 with Hydrochloric acid.

The same recipe was used for re-dispersion medium for the active loading experiment, except that the pH was adjusted to 4.5 with Hydrochloric acid.

PBS 300 mM (containing 20 mM phosphate) pH 2.0

- Used for preparation of bendamustin-solution.

1. Kalium-dihydrogenphosphate 2.68 g
2. Kalium chloride 0.40 g
3. Sodium chloride 16.00 g

Ad 1.0 L distilled water
Adjust pH to 2.0 with Hydrochloric acid

Dulbecco`s Modified Eagle`s medium (DMEM), Modified Formulation

Addition:
- 10 % Fetalt bovint serum
- 1% Penicillin
- 1 % Glutamine
- 1 % Fungisone

5.4 Preparative methods

5.4.1 Preparation of VPGs by dual asymmetric centrifugation

Theory

Dual-asymmetric centrifugation (DAC), also called speed-mixing, is a technique which is widely used by dentists to mix fillings out of two components. Recently the DAC has been presented as a homogenisation method for liposome preparation.

DAC differs from normal centrifugation in that it contains two centrifugal units; one main rotator which is connected to a rotary arm containing the sample vial. While the main rotary basket is constantly pushing the sample material outwards due to centripetal forces, the additional rotation of the sample- containing- vial around its own vertical axis moves the sample material towards the centre of the centrifuge due to adhesion forces between the sample and the vial. The combination of the two contra movements result in shear forces, and hence in homogenisation.

Optimal homogenisation is seen for viscous materials. This is simply due to the fact that inward movement of sample is dependent on the transfer of the adhesion forces into the sample, and this transfer is best for viscous materials. An optimal liposome production using DAC- homogenisation is obtained by using a viscous blend of phospholipids and buffer. The resulting vesicular phospholipid gels can be further diluted into liposome dispersions if required.

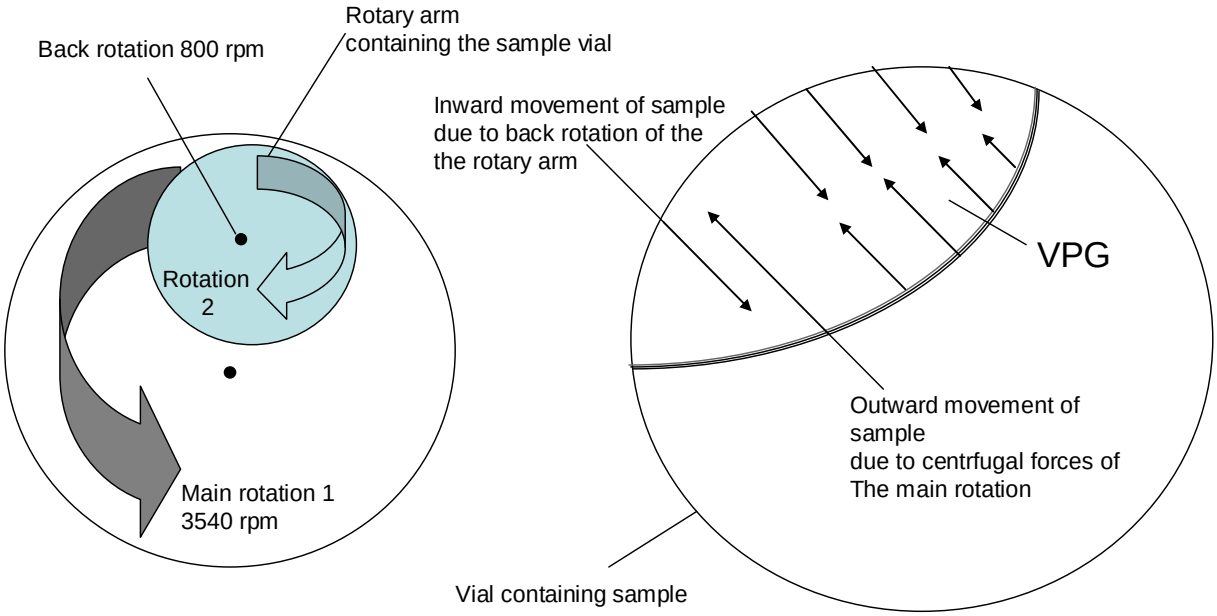


Figure 13: Schematic drawing of the principals behind the mechanical forces involved in dual asymmetric centrifugation.

Producing liposomes by DAC offers various advantages:

- Sterile production and handling with toxic compounds is easy because preparation can be handled within a closed vial. The apparatus itself does not have to be cleaned before use.

- VPGs are produced, which result in high entrapping efficiencies, especially for water soluble compounds.

- Preparation of small batch sizes as well as bigger ones is possible.

- Preparation of liposomes is relatively fast.

- Small debit to thermo- labile and unstable compounds.

- The DAC is mobile, easy and safe to use

Picture 1: Speed-Mixer

Picture 2: Speed-Mixer containing a sample vial.

In a previous experiment by Cicko, a protocol for production of EPC3/CH liposomes was developed [27]. For this investigation of variables which were thought to influence the particle size of liposomes prepared by DAC was carried out; different batch sizes, ratio of lipids and water, rotation-speed and time of speed-mixing, as well as size and amount of glass beads. The optimum of these parameters listed in table 4, is used in my further experiments.

Table 4: Overview of different variables investigated in order to determine their influence on liposome size during speed-mixing.

Parameters investigated	**Variables**	**Optimum***
Lipid amount	10, 25, 35, 40, 45, 50 %	35- 40 %
Duration of agitation /time of speed-mixing	1, 5, 10, 20, 30, 40, 50 min	30 min (no difference in longer time of speed-mixing)
Speed of agitation in rpm	1000, 2000, 2500, 3000, 3540 rpm	3540 rpm
Batch size	0.25, 0.5, 1.0, 2.0, 3.7 g	0.25- 3.7 g
Amount and size of dispersion aids	0.25- 0.3, 0.4-0.6, 1.0, 3.0 mm	1 mm in diameter, 100 % amount of the batch size

* Optimum; The smallest liposome size and size-distribution obtained

Experiment

Preparation of VPG:

The lipid mixture hydrogenated egg phosphatidyl choline and cholesterol (Epc3/CH) was blended with 0.9 % sodium chloride solution in the ratio of 40:60 m/m. Glass beads, in the size of 1 mm in diameter, were added in equal amounts with respect to the total weight of the batch size prepared. The VPGs were prepared by speed-mixing with maximum rotation speed; 3540 rounds per minute (back rotation 800 rpm) for 30 min.

Re-dispersion of VPG:

The VPGs were re-dispersed into liposome dispersions by adding aqueous medium in the ratio of 1:3. The solution was added 4 times, and speed- mixed for 30 seconds at 3540 rpm in between each added amount.

i.e: For a batch size of 500 mg, 250 µl aqueous medium was added 4 times. In between each added amount, the VPG was speed mixed for 30 seconds at 3540 rpm.

5.4.2 Preparation of bendamustin-containing VPGs

5.4.2.1 Bendamustin-VPG formulation 1

Preparation of bendamustin- solution:

20 mg Ribomustin® powder, containing 45 % (m/m) bendamustin, was dissolved in 0.5 ml distilled water, and further diluted with 0.5 ml 1.8 % (v/v) sodium chloride solution.

A 500 mg loaded VPG batch was prepared by adding 200 mg EPC3/CH, 300 mg of the bendamustin- solution and 500 mg glass beads (1 mm in diameter) to an ISO 8362-4-J0H 38 ml injection vial. The blend was speed- mixed for 30 min at 3540 rpm.

5.4.2.2 Bendamustin-VPG formulation 2

The new bendamustin-VPG was prepared after the same recipe as for bendamusin-VPG formulation 1 described above, except that for preparation of the bendamustin solution 300 mM PBS (containing 20 mM phosphate) pH 2.0 was used instead of 1.8 % sodium chloride solution.

5.4.3 Active loading of bendamustin

Theory

Active loading is a process where drugs containing ionisable groups or display both lipid and water solubility can be introduced into liposomes after formation of the liposomes [28]. Such drugs are often difficult to retain inside the liposomes by normal means, since their lipophilicity leads to a passage through the membrane, readily, and thus equilibrating between the liposome interior and exterior. In an active loading, the conditions in the vesicle interior and in the outer aqueous phase are arranged in a way that the drug is capable of diffusing in

from the exterior, and once it reaches the interior it becomes ionized. This is often arranged by a pH difference between the inside and the outside of the membrane. After the solute enters the liposome by diffusion through the membrane in its uncharged form, it is converted to its ionized form. Consequently, the drug is unable to escape from the liposome, because its lipophilicity is highly reduced [28].

Experiment:

An attempt of an active loading of bendamustin was carried out after the following procedure;

Preparation of VPG:

A 500 mg VPG batch was prepared by adding 200 mg EPC3/CH, 300 mg 150 mM PBS (containing 30 mM phosphate) pH 2.0 and 500 mg 1 mm glass beads to an ISO 8362-4-30H 38 ml injection vial. The blend was speed- mixed for 30 min at 3540 rpm.

The VPG was re-dispersed with 150 mM PBS (containing 30 mM phosphate) pH 4.5 in the ratio of 1:3, by adding 250 µl of the solution four times and speed-mixing at 3540 rpm for 30 seconds in between each added amount.

Preparation of bendamustin-solution;

20 mg Ribomustin® was dissolved in 0.5 ml distilled water and further diluted with 0.5 ml 0.9 % sodium chloride solution.

Addition of bendamustin solution to the liposome dispersion:

300 µl of the bendamustin- solution was added drop wise under continuously stirring of the re-dispersed VPG. The liposome-dispersion was kept on gentle stirring for 30 minutes at room temperature; 23 ºC.

After 30 minutes 100 µl of the liposome dispersion was added to an ion exchange column (3 parallels), according to section 6.2, and dissolved with ethanol/methanol (90/10 v/v) in the ratio of 1:100. As a reference 100 µl of the loaded liposome dispersion from the same VPG, containing the overall content bendamustin, was diluted in ethanol/methanol (90/10 v/v) (3 parallels).

Triplicates of the eluates and references were then ready to be injected and measured on to the HPLC system. Entrapped amount bendamustin within liposomes could be calculated according to equation 2 under section 5.5.2. 3 VPGs were prepared as parallels.

5.5 Analytical methods

5.5.1 Characterization of particle size by PCS

Theory:

Photon Correlation Spectroscopy (PCS) is an analytical tool to determine the size and size distribution of sub-micron particles suspended in a medium. The method is based on dynamic light scattering, and takes advantage of the time dependence of intensity fluctuation in scattered laser light due to Brownian motion of particles. The diffusion rate of particles varies according to their molecular weight and size. Small particles will diffuse more rapidly than bigger particles and will consequently give a more rapid fluctuation of scattered light intensity. Only a few changes in the position of a particle will give rise to significant changes in the phase to the scatter light waves [29], [30].

A laser light beam is sent through a cuvette containing the sample of interest. A detector, placed usually at 90º angle to the incident beam, is detecting the scattered laser light from the sample. The temperature must be held stable in order to ensure that viscosity is not changing and hence the diffusion rate of the particles. Based on a mathematical function called correlation, the diffusion coefficient is determined, and the particle radius can be calculated using the Stokes- Einstein equation. Depending on the power of the laser, it is possible to measure particles in the range of about 3 nm up to about 3 μm [29], [30].

Fitting and interpretation of the results

Depending on the size-distribution of the sample, the software evaluates the data using two different models; The Gaussian model and the Nicomp model. The Gaussian model states how well a fit is approaching a normal distribution. The goodness of the fit is given by the Chi squared. If the data perfectly follow a normal distribution the Chi squared is close to 0. If the Chi squared is > 3, the data do not show a good fit to the normal distribution, and interpretation of the data should be done by the more advanced Nicomp model [31], [32].

The Nicomp model is suitable for polydisperse samples having two or more distinct populations of different particle sizes. The model then calculates a mean of several populations of particles in the sample. As an indication of how stable the results of the analysis are, the PCS software gives a value for Fit error. For reliable results using the Nicomp model, the fit error should be below 1.5 and preferably near 0 [31], [32].

Polydispersity index (P.I.) is stating how broad the distribution is around the mean particle size. Highly polydisperse samples, containing particles with different sizes, will show a high P.I. value close to 1. For liposome formulations the P.I. is often of importance because different applications demand a particle size in a certain size-range. A low P.I. value, close to 0, is therefore most often desirable. However, based on empirical data, liposome dispersions in general do often show a certain polydispersity.

For reliable results one has to make sure that there is no particular presence of contaminants in the sample. This can be seen in the Gaussian model as the “baseline adjust” and in the Nicomp model as the “residual”. These parameters should be close to zero [32].

Channel width or channel sampling time is a conception used to describe the width of the autocorrelator channel intervals the sequence of photon pulses from the detector is divided into [31]. The sample time should ideally correspond roughly to the frequency of signal fluctations. The channel width can be adjusted as required, depending on the particle size and distribution in the sample. Choosing a too small channel width, the bigger particles in the sample, with the slowest fluctations, will be excluded from the measurement. Subsequently, a too broad channel width will often result in only a few channels responding to scattered light. The choice of channel width is important in order to ensure that sufficient information is acquired to enable a full plot of the correlation curve. A too small or too big channel width might result in an auto correlation function that decreases too fast or too slow, respectively. Consequently, the results can be incorrect with respect to particle size and size distribution [32].

In order to ensure high degree of statistical accuracy, the amount of photon pulses data collected by the auto correlator, used to calculate the auto correlation function, should ideally

exceed one million (1000 K) [33]. The amount of data collected is dependent on the time of measuring as well as the selected channel width [29].

Experiment:

Re-dispersed VPGs were further diluted with 0.9 % sodium chloride solution by manual shaking until reaching a suitable intensity level of 250-350 KHz as stated in the Nicomp user manual [32].

Before measurements were performed the instrument parameters were set according to the values listed in table 5. To ensure statistical accuracy, two cycles of 10 min were run for each sample in order to collect a big amount of collected data.

Table 5: PCS parameters

Parameters	**Value**
Temperature	23 C°
Viscosity	0.933 cp
Liquid index of refraction	1.333
Intensity set point	300 ± 50
Channel width	Auto set, 20, 10 and 5 μs

5.5.2 Determination of ratio of entrapped/unentrapped bendamustin

Theory

In order to determine encapsulation efficiency of bendamustin or incorporated bendamustin within liposomes over time, the external bendamustin in the outer aqueous phase of the liposome-dispersion has to be removed. One method suited for this is ion- exchange chromatography.

Ion exchange chromatography relies on charge-charge interactions between the charges of dissolved spices and the charges immobilized on the resin used. For bendamustin, cation-exchange chromatography was used. Cation- exchange chromatography is based on the fact that positively charged ions bind to a negatively charged resin. External bendamustin in the outer aqueous phase will bind to the resin in the column, while incorporated bendamustin

within the liposomes will not. Hence, only the liposomal bendamustin will pass through the column and can be collected in a flask.

Experiment

The following ion-exchange method was the first method used for separating liposomal bendamustin from external bendamustin. (The method was taken from personal communication within the working group of Massing at the Tumour Biology Centre, Freiburg, Germany.)

Preparation of the Lichrolut cation exchange column:

- PTFE-frits were placed within the glass column.
- 1g of AG-50W-X8 Resin was added to the glass column.
- The column was filled with distilled water and left still in 5 minutes in order to get sedimentation of the resin.
- Excess water was removed by vacuum until only the top of the resin was covered with water.
- The glass column was loaded three times with 1.0 ml filtrated concentrated sodium chloride solution, and thereafter flushed three times with 1.0 ml distilled water. Finally the glass column was dried by full vacuum for 5 minutes.

Separation of liposomal bendamustin from external bendamustin by ion-exchange chromatography

- 50 µl of the liposome dispersion was added dropwise to the column followed by vacuum suction.
- The column was flushed five times with 125 µl distilled water under continuous vacuum suction.
- The eluate, containing only liposomal bendamustin, was collected in a flask.

In order to correct for potential loss of liposomes on the columns (liposomes not coming through the resin), cholesterol in the eluate was measured and compared to a reference sample from the same sample set, not applied to the column. The amount cholesterol in the Epc3/CH powder is 27.3 % per weight. After re-dispersion of the VPG and further dilution of 1:100, the theoretically concentration of cholesterol in the sample is 364 µg/mg. If all the liposomes have gone through the column and been collected in the flask, the cholesterol concentration in

the eluate should be the same as for the reference, which in turn should correspond to about the theoretical amount of cholesterol. As a correction for possible loss of liposomes on the columns, cholesterol recovery was measured using equation 1.

Equation 1:

$$\text{Cholesterol recovery \%} = (\text{Cholesterol in eluate} / \text{Cholesterol in reference}) \times 100$$

Approaches for optimization of ion exchange chromatography regarding cholesterol recovery. Cholesterol recovery in the liposome-eluates separated by the columns prepared as described above, should be quite equal, and not vary between the columns used. Moreover, previous experience from similar ion-exchange chromatography methods has shown stable cholesterol recoveries above 80 %. Different approaches were carried out in order to optimize the cation-exchange method for bendamustin described above, regarding cholesterol. These approaches are described in section 6.2.

Quantification of bendamustin and cholesterol by Reversed-Phase High performance Liquid Chromatography- RP-HPLC

Theory

HPLC is a form of column chromatography used to separate components of a mixture by the variety of chemical interactions between the substance being analyzed, analyte, and the chromatography column, stationary phase. Reverse phase chromatography consists of a non-polar stationary phase and a moderately polar mobile phase, and is used in these experiments to separate both bendamustin and cholesterol from other components in the sample such as ethanol/methanol and lipids. A small volume of the sample being analyzed is injected into the stream of the mobile phase which is pumped through the column at high pressure. When the sample is passing through the column, non polar molecules interact with the stationary phase and are retarded [34].

In order to quantify the bendamustin or cholesterol concentration in the samples of interest, a series of known concentrations are injected onto the HPLC for detection. The chromatographs of these known concentrations will give a series of peaks that correlate to the concentration of

the compound injected. A calibration curve is generated by calculating the area under the peak. Using a calibration curve, unknown concentration of bendamustin or cholesterol in the sample can be executed [34].

Experiments

The liposome eluate, separated from external bendamustin by the optimized ion exchange chromatography method described in section 6.2, was diluted with ethanol/methanol (90/10 v/v) in the ratio of 1:100 in order to dissolve the liposomes.

As a reference, an aliquot from the same liposome dispersion, containing the overall bendamustin content, was diluted in ethanol/methanol (90/10 v/v) in the ratio of 1:100. The bendamustin concentration as well as the cholesterol concentration in both the eluate and in the reference could subsequently be determined by RP-HPLC. Throughout all the experiments triplicates for both the eluates as well as references were prepared.

Quantification of bendamustin by RP-HPLC

The parameters used for quantification of bendamustin by RP-HPLC are listed in table 6.

Table 6: HPLC parameters used for quantification of bendamustin

Column	Lichrospher 60 RP-select B (5µm) 250 × 4 mm
Packing material column	C-8
Injection volume	10 µl
Mobile phase	PBS(di-Kaliumhydrogenphosphate-Trihydrate) pH 2.3 / Methanol 58/42 (v/v)
Flow rate	1.0 ml/min
Column temperature	30 °C
UV detection wavelength	254 nm
Retention time	Ca 10 min
Calibration range	2- 64 µg/ml 6 calibrators: 2, 4, 8, 16, 32, 64 µg/ml

A calibration series of bendamustin was developed by plotting the area under the curve (AUC) from the peaks versus the concentration of the samples.

Preparation of a calibration series bendamustin:

Stock solution:

5 mg Ribomustin® was dissolved in 2.5 ml distilled water and further diluted with 2.5 ml 1.8 % sodium chloride. This results in a bendamustin concentration of 0.455 mg/ml in the stock solution.

Dilution 1:

1.760 ml of the stock solution was added to a 10 ml graduated flask. The flask was filled up with 0.9 % sodium chloride solution. The concentration of bendamustin in this dilution is 80 µg/ml. 6 calibrators were prepared as listed in table 7.

Table 7: Calibrators bendamustin

Calibrator nr	Concentration µg/ml	Ad dilution 1	Fill up with 0.9 % Sodium-chloride solution to
1	2	125 µl	5 ml
2	4	250 µl	5 ml
3	8	500 µl	5 ml
4	16	1000 µl	5 ml
5	32	2000 µl	5 ml
6	64	4000 µl	5 ml

As seen from figure 14, the calibration series reveals a good linear fit with a R^2 value of 0.999995.

(The range of R^2 values for all of the calibration lines for bendamustin prepared throughout the experiments were: 0.99665 – 0.999998.)

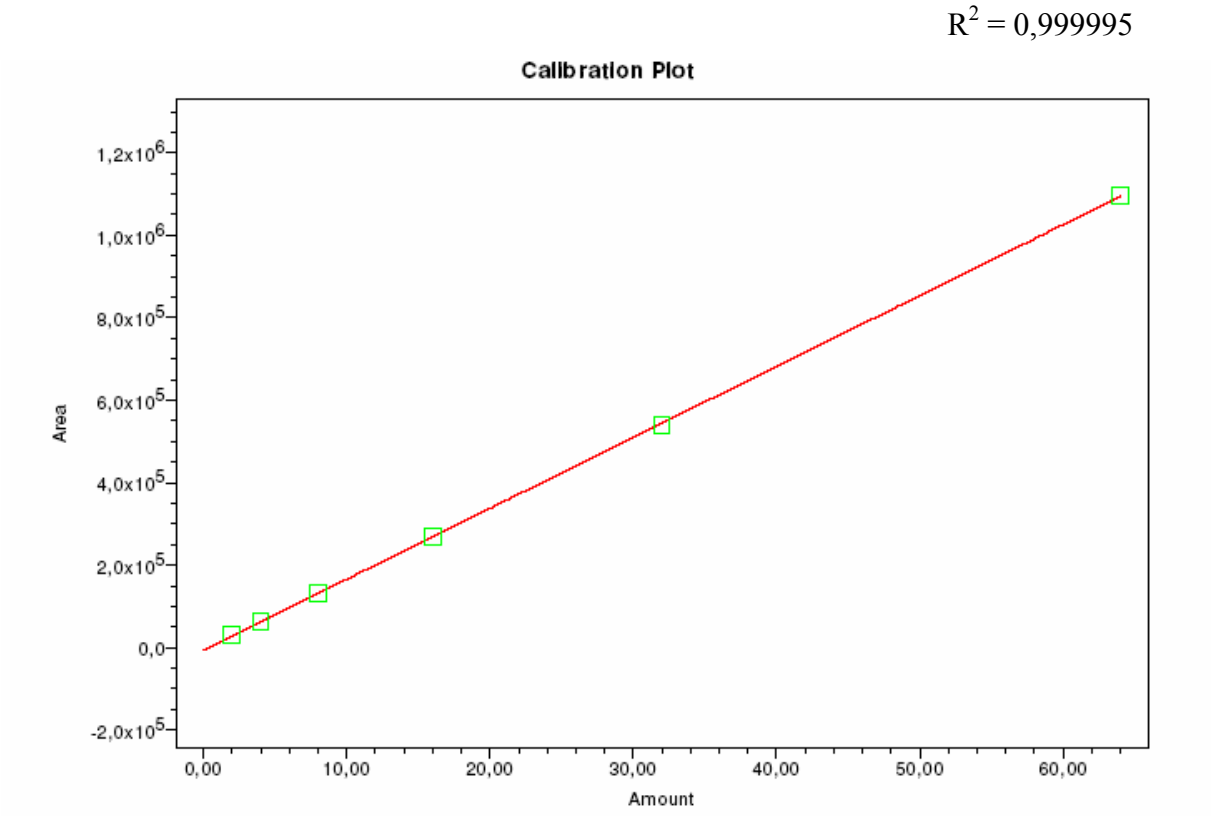


Figure 14: Calibration line for bendamustin, showing AUC over amount bendamustin in µg/ml.

An example of a typical chromatogram of a sample of bendamustin is shown in figure 15. The peak in this chromatogram corresponds to a concentration of bendamustin of 64 µg/ml.

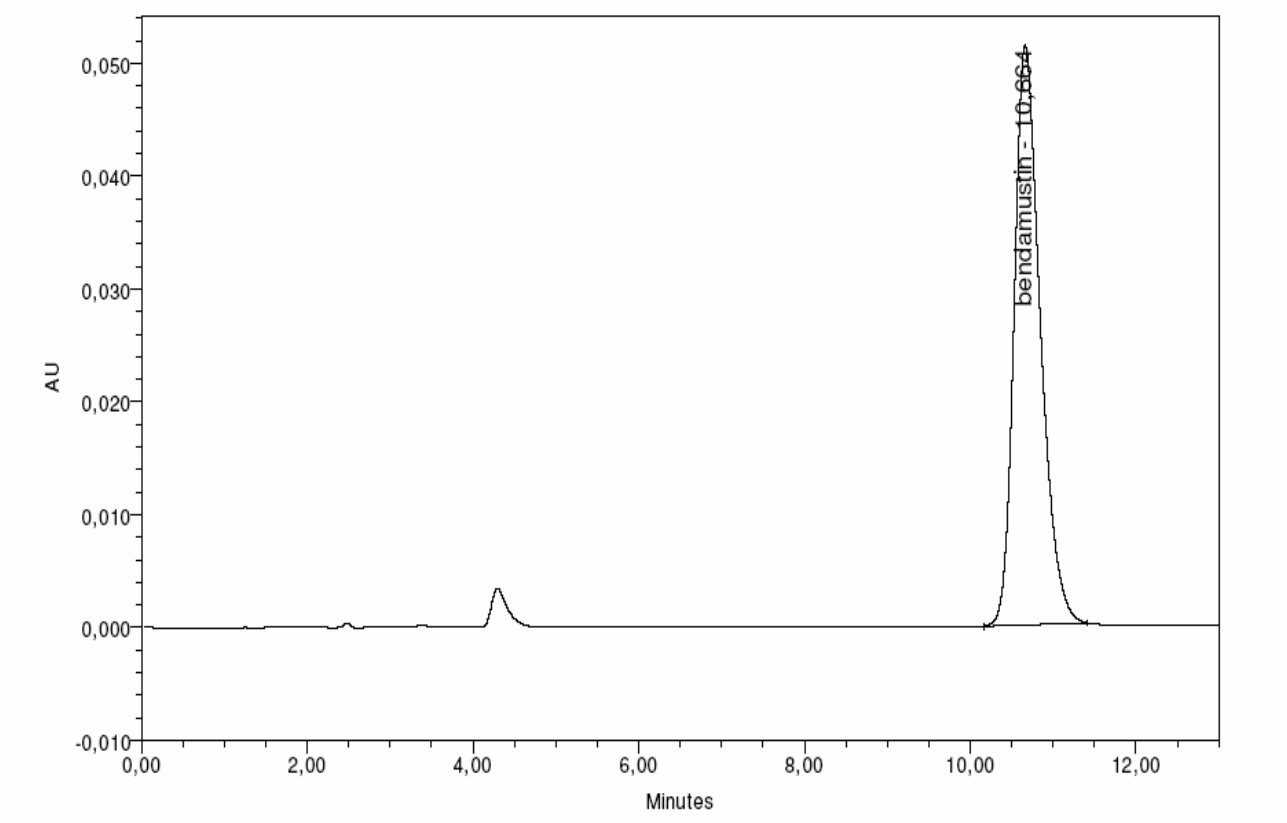


Figure 15: Example of a typical chromatogram of bendamustin.

Quantification of Cholesterol by RP-HPLC

Measurement of cholesterol was carried out as a correction factor for the ion-exchange chromatography.

The parameters used for quantification of cholesterol by RP-HPLC are listed in table 8.

Table 8: HPLC parameters used for quantification of cholesterol

Column	Lichrospher 60 RP-select B (5µm) 250 × 4 mm
Packing material column	C-8
Injection volume	10 µl
Mobile phase	Acetonitrile/methanol/acidic water (67/30/3 v/v/v)
Flow rate	1 ml/min
Column temperature	40 ºC
UV detection wavelength	215 nm
Retention time	Ca 6 min
Calibration range	100-800 µg/ml 5 calibrators: 100, 200, 300, 400, 800 µg/ml

A calibration series of cholesterol was developed by plotting the area under the curve (AUC) from the peaks versus the concentration of the samples.

Preparation of calibration series cholesterol:

14 mg cholesterol was dissolved in 5 ml ethanol/methanol (90/10 v/v), resulting in a concentration of cholesterol of 2660 µg/ml.

Stock solution:

The 2660 µg/ml cholesterol solution was further diluted to yield in a stock solution containing 2500 µg/ml cholesterol. From the stock solution 5 calibrators were prepared as shown in the table 9.

Table 9: Calibration series cholesterol

Calibrator nr	Concentration µg/ml	Ad stock solution µl	Fill up with ethanol/methanol (90/10 v/v) in ml
1	100	200	5
2	200	400	5
3	300	600	5
4	400	800	5
5	800	1600	5

As seen from figure 16, the calibration series reveals a good linear fit with a R^2 value of 0,999691. The range of R^2 values for all of the calibration series prepared for cholesterol used throughout the experiments were: 0.996708- 0.999694.

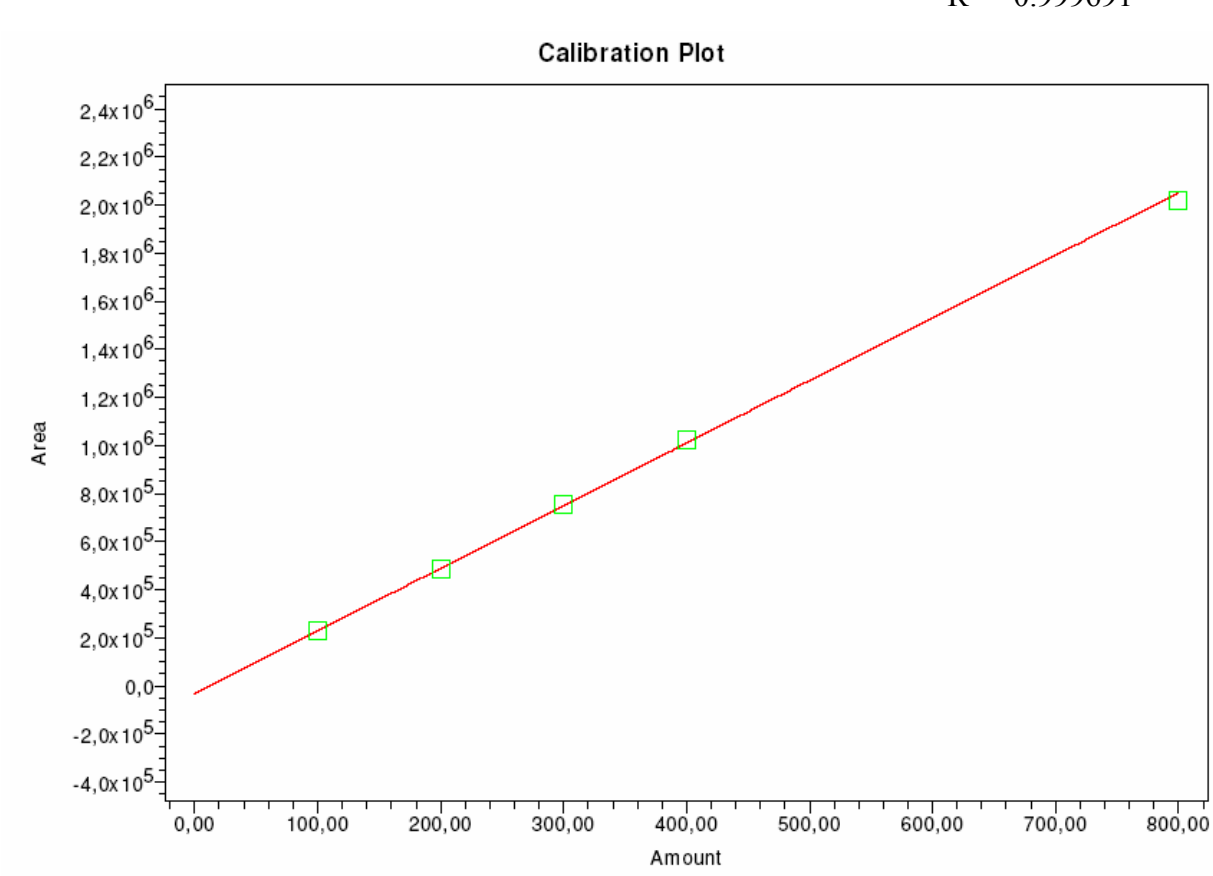


Figure 16: Calibration line cholesterol.

An example of a typical chromatogram of cholesterol is shown in figure 17. The peak in this chromatogram corresponds to a cholesterol concentration of 363,048 µg/ml.

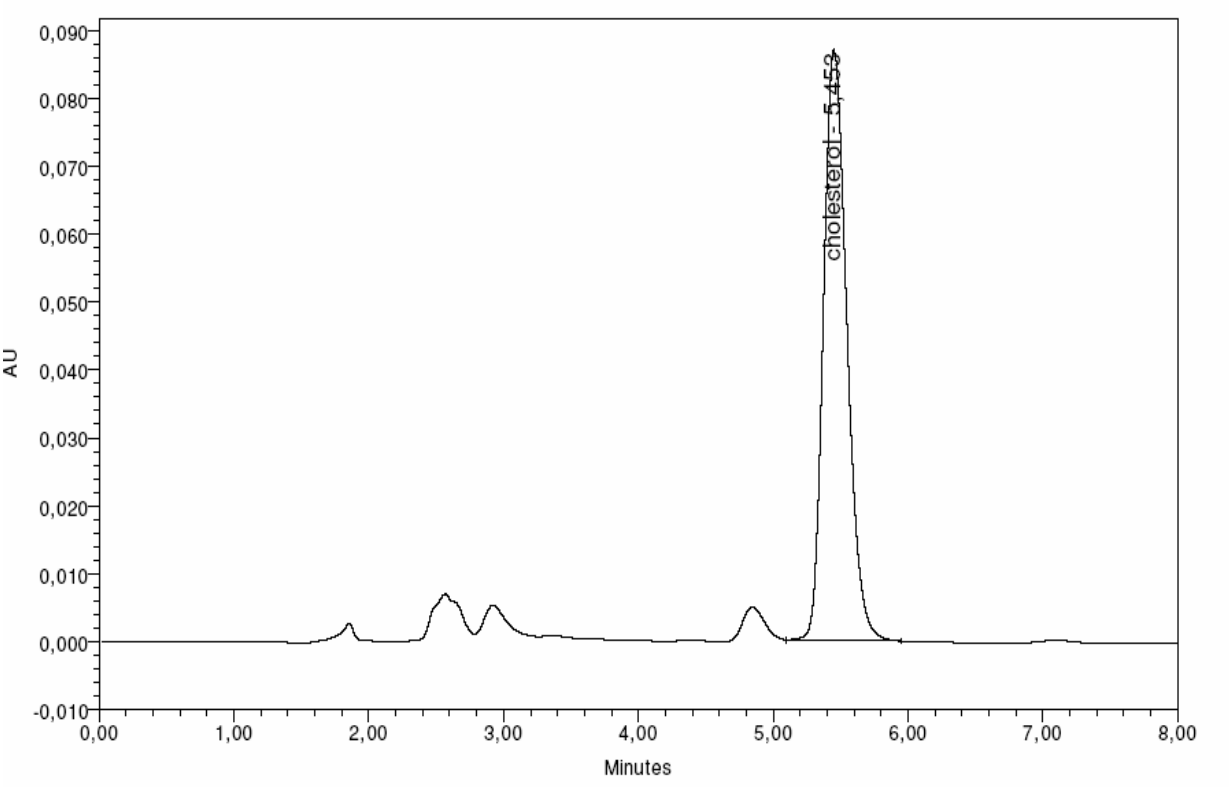


Figure 17: Example of a typical chromatogram of cholesterol.

Encapsulation efficiency or entrapped amount liposomal bendamustin was determined using the following formula:

Equation 2:

$$EE\ \% = 100 \Big/ \frac{\text{(reference bendamustin)}}{\text{(reference cholesterol)}} \times \frac{\text{(eluate bendamustin)}}{\text{(eluate cholesterol)}}$$

5.5.3 Degradation of bendamustin during speed-mixing.

Degradation of bendamustin during speed-mixing of the loaded VPGs was investigated as follows;

Bendamustin-VPG formulation 1 as well as bendamustin-VPG formulation 2 were prepared according to section 5.4.2.1 and 5.4.2.2 respectively, and re-dispersed with 0.9 % sodium chloride solution according to section 5.4.1.

Immediately after re-dispersion of the VPGs, aliquots of the liposome dispersion were dissolved in ethanol/methanol (90/10 v/v) in the ratio of 1:100. The overall bendamustin-content in the re-dispersed VPGs was measured on RP-HPLC and compared with the theoretical concentration bendamustin in the liposome dispersion diluted 1:100.

Triplicates for each of 3 VPGs were prepared and measured as parallels.

5.5.4 Stability of bendamustin-solution at pH 7.4

Stability of bendamustin in pH 7.4 at 23 ºC and 37 ºC was measured after the described method;

Preparation of Bendamustin/ PBS solution:
5 mg Ribomustin® was dissolved in 20 ml 150 mM PBS buffer (containing 10 mM phosphate), resulting in a concentration of 0.114 mg/ml bendamustin.

The solution was further diluted with the same PBS buffer to yield in a bendamustin-concentration of 32 µg/ ml, which is a concentration within the calibration line.

The solution was incubated at 23 ºC as well as at 37 ºC. After the following time points an aliquot was taken out and injected onto the HPLC system: 0, 5, 10, 15, 20, 30, 40 and 60 minutes. Triplicates were prepared for each time point and remaining intact bendamustin was measured on HPLC according to section 5.5.2

5.5.5 Efflux experiments of liposomal bendamustin

Release experiments of liposomal bendamustin were carried out. Loaded liposomes were incubated at 37 ºC and at various time points the samples were assayed with respect to loss of incorporated drug. All the efflux experiments were carried out after the following general description;

A loaded VPG was prepared and re-dispersed into liposome dispersions in the ratio of 1:3 by adding 250 µl aqueous medium four times, and speed-mixing for 30 seconds at 3540 rpm in between each added amount.

The liposome dispersion was incubated in eppendorf cups at 37 ºC. At different time-points aliquots of the liposome dispersion were applied to ion-exchange columns, according to section 6.2 for removal of external bendamustin.

Both, the liposomal bendamustin and references containing the overall bendamustin content from the same sample set were determined at each time point. In order to crack the liposomes the samples were collected in graduated flasks and diluted with ethanol/methanol (90/10 v/v) in the ratio of 1:100.

Triplicates from the liposome dispersion were prepared at each time point. (eluates from 3 columns as well as 3 references) For practical reasons, maximum 5 time points were measured in one run. Several VPGs were therefore prepared, and amount of liposomal bendamustin was determined at different time points for the respective VPGs, until the efflux-curve was executed. (The VPGs had shown to be reproducible regarding EE as well as particle size, and was therefore expected to show the same efflux kinetics.)

The samples were injected into the RP-HPLC system for quantification of bendamustin as well as cholesterol before the lipsomal bendamustin per time point was calculated using equation 2 as described under section 5.5.2

5.5.6 Stability experiments

Stability of the new bendamustin liposome-formulation was investigated in respect to degradation of bendamustin and lipids upon storage in room-temperature 23 °C, as well as stability in cell culture medium at 37 °C.

5.5.6.1 Stability of liposomal bendamustin at room temperature

A bendamusin-VPG formulation 2 was prepared according to section 5.4.2.2 and re-dispersed in the ratio of 1:3 with 150 mM PBS (containing 10 mM phosphate) pH 2.0, by adding 250 µl of the PBS solution four times and speed-mixing at 3540 rpm for 30 seconds in between each added amount.

Aliquots of the re-dispersed VPG were incubated in eppendorf cups at 23 ºC (room temperature) for the following time points: 0, 1, 2, 16, 21 and 24 hours.

At the respective time points the samples were dissolved with ethanol/methanol (90/10 v/v) in the ratio of 1:100, and injected onto the RP-HPLC system in order to measure intact bendamustin. Triplicates were prepared at each time point.

5.5.6.2 Quantification of Lyso-phosphatidylcholine by High Performance Thin Layer Chromatography- HPTLC.

Theory HPTLC

The principle of Thin-layer chromatography of lipids is, as for all chromatographic methods, the fact that two different, immiscible phases can be used to separate compounds based on the compounds characteristics and their respective affinities for the phases.

For phospholipids the stationary phase is most often silica gel which is moderately hygroscopic and consists of spherical granules coated with a layer of tightly bound water. The mobile phase is usually a mixture of organic solvents including chloroform. Under these conditions, phospholipids are separated principally according to differences in their head groups, which in the liquid organic phase will have unlike affinities for the hydrophilic solid phase. As the liquid phase runs through the solid phase, the dissimilar phospholipids will be

retained to different extent by the solid phase, and will consequently spread out at different distances behind the solvent font [35].

Most often lipids are visualized by means of specific bands which are sprayed on to the plate. Together with standards of known concentrations, the samples of interest can be detected by an UV detection system. Accordingly, quantification of the lipids of interest can be executed [35].

Experiment

Stability of lipids in the bendamustin-liposomes compared to "empty" liposomes was investigated after the following method;

Sample preparation and incubation:

One bendamustin- VPG was prepared according to section 5.4.2.2. As a reference an "empty" VPG was prepared according to section 5.4.1 by using 150 mM PBS pH 2.0 as aqueous medium. Both the "empty" VPG and the loaded VPGs were re-dispersed in the ratio of 1:3 with 150 mM PBS pH 2.0, by adding 250 µl of the PBS solution four times and speed-mixing at 3540 rpm for 30 seconds in between each added amount.

The liposome dispersions were stored at 23 ºC /room temperature for the following time-points; 0 min, 1 hour, 2 hours and 24 hours.

At the respective time-points 90 µl of the gel-dispersions were diluted with 0.9 % sodium chloride solution to yield in a final concentration of 8 mM, and mixed for 2 minutes at 200 rpm. The samples were immediately extracted in order to stop any further reaction.

Duplicates for each of the two VPGs were prepared and measured in the same way.

Extraction:

The samples were transferred to 10 ml glass tubes. 2 ml chloroform/methanol (2:1 v/v) were added and mixed for 5 minutes at 1000 rpm/min. The tubes were centrifuged for 10 minutes at 4 ºC, 4000 rpm, before the chloroform phase was pipetted to another glass vial and evaporated to dryness at 40 ºC under a weak steam of nitrogen. The remaining water phase was extracted the same way for two more times, and the chloroform phase was always collected in the same

tube and evaporated. The samples were then re-dissolved in Hexan/2-Propanol/H_2O 40/50/8 (Concentration factor 2 to 3.1).

HPTLC:

The HPTLC plates used were pre-run with mobile phase $CHCl_3$/MeOH/H_2O/NH_3 (65/25/4/0.4 per volume). Approximately 7 µl of each sample were sprayed as bands automatically with a TLC sampler III. The distanced between the 14 mm bands were 2.6 mm. Application were executed with a spraying speed of 70 nL/s and a horizontal speed of 7 mm/sec.

The plates were developed in a glass tank with the mobile phase, run distance: 9.0 cm. After development, plates were dried for 10 minutes at 180 ºC on a heating plate.

Visualizing of lipids occurred by dipping the plate 3 times for 2 seconds in a copper sulphate solution (14.7 % w/v) in phosphoric acid (10 % v/v). The plates were then dried in an oven at 160 ºC for 6 minutes.

The spots were read quantitatively using a TLC-Scanner II at 530 nm with a wolfram lamp in reflexion mode at the following conditions:

- Speed of measurement: 0.1 mm/s
- Sensitivity: Ca. 125
- Span Ca. 7
- Monochromator 30 nm
- Slit: 0.2 × 12.0 mm

Calibration line

A calibration range from 1 to 5 % Lyso-PC according to the reference amount of 8 mM EPC3 was prepared as follows;

Lyso-PC stock solution:

10 mg Lyso-PC was dissolved in 20 ml 0.9 % Sodium chloride solution.

Lyso-PC dilution 1

The stock solution was further diluted to yield in a concentration of 1000 µM.

Table 10: Calibration solutions Lyso-PC

Calibrators	Addition of dilution 1 (µl)	Filled up to (ml) with NaCl solution	Soluition conc. (µM)	Corresponding degradation EPC3 (%) *
1	800	10	40	0.5
2	1600	10	80	1.0
3	2400	10	160	2.0
4	3200	10	320	4.0
5	4000	10	400	5.0
6	4800	10	480	6.0

* Reference concentration EPC3 is 8000 µM

For each analysis 1 ml calibrator was used. After extraction procedures the calibrators were re-dissolved in 320 µl Hexane/2-Propanol/H_2O 40/50/8 (concentration factor 3.1)

5.5.6.3 Stability of bendamustin-liposomes in comparison to free bendamustin- solution upon incubation in cell culture medium.

Stability of the new bendamustin-liposome formulation was tested and compared to the free drug in cell culture medium as follows;

- *Incubation of bendamustin-liposome formulation 2 in cell culture medium:*
Bendamustin-VPGs formulation 2 were prepared according to section 5.4.2.2, and re-dispersed in cell culture medium in the ratio of 1:6, by adding 250 µl of the medium four times, and speed-mixing for 30 seconds at 3540 rpm in between each added amount. Then, 1.5 ml of the same medium was added to the dispersion and speed-mixed for another 30 seconds in order to get a proper liposome suspension.

Aliquots of the sample were added to eppendorf cups and incubated at 37 ºC for the following time points: 0, 5, 15, 20, 30, 60, 120 and 240 minutes.

At the respective time points the aliquots were dissolved with ethanol/methanol 90/10 (v/v) in the ratio of 1: 100. The diluted samples were then centrifuged for 10 minutes at 4 ºC. This was done in order to separate proteins in the medium from the samples, which are prone to clog in the HPLC- system during measurement. Triplicates were prepared for the respective time- points for each of in total 3 VPGs. Intact bendamustin at each time point was quantified on HPLC according to section 5.5.2.

- *Incubation of free bendamustin- solution in cell culture medium:*
20 mg Ribomustin® powder was dissolved in 0.5 ml distilled water, and further diluted with 0.5 ml 300 mM PBS pH 2.0. (This bendamustin-solution is the same as used for preparation of the bendamustin-VPGs.)

300 µl of the bendamustin-solution was added to 2.7 ml cell culture medium pH 7.6, in order to get the same dilution ratio as for the liposome samples.

Aliquots of the sample were added to eppendorf cups and incubated at 37 ºC for the following time points: 0, 5, 15, 20, 30, 60 and 120 minutes.

At each time point the solutions were diluted in ethanol/methanol 90/10 (v/v) and centrifuged for removal of proteins in the same way as for the liposome dispersions described above. Triplicates at each time point were prepared for in total 3 bendamustin-solutions incubated in medium. Intact bendamustin were assayed on HPLC according to section 5.5.2.

6 RESULTS AND DISCUSSIONS

6.1 Size and size- distribution of liposomes prepared by DAC

Size and size-distribution of "empty" VPG batches prepared in two vials with different radius and size.

The extent of mechanical energy applied during preparation of liposomes, is known to have impact on the size and size-distribution of liposomes [24]. One important factor which is affecting the centrifugal force on a basket which is rotating is its radius. The bigger the radius of the basket, the stronger is the force exerted on its contents. It is therefore reason to assume that the radius and size of sample-vials used during speed- mixing could influence the size and size- distribution of the liposomes formed.

The following VPG batches were prepared according to section 5.4.1; 100, 200 and 500 mg. Each batch- size was prepared in both a 38 ml glass vial (diameter 5 cm) and in a 10 ml glass vial (diameter 2 cm). 3 parallels of each batch- size were prepared in both of the two vials in order to investigate reproducibility of the size and size-distribution of VPGs prepared by DAC. The VPGs were re-dispersed in the ratio of 1:3 with 0.9 % sodium chloride solution, before the samples were further diluted and measured on PCS according to section 5.5.1.

For all the PCS measurements, only channel width 5 µs and 10 µs were fittable by the Gaussian model. Autoset and Channel width 20 µs showed a Chi squared > 3. This indicated that the samples revealed a polydisperse particle- size distribution; i.e presence of liposomes of different sizes. The data was neither, for most of the measurements, fittable by the Nicomp model (Fit error > 1.5).

Presence of bigger particles in a sample measured has the tendency to overshadow smaller particles, and the mean diameter calculated by the software is bigger than the actually ones. In order to calculate the data from a rather polydisperse sample by a Gaussian distribution, the software suggest to reduce the channel width, taking only a proportion of the total signal into calculation of the diffusion coefficient. The drawback with this is that the correlation function plot created is not as long as desired, and might give values derived from the diffusion coefficient that is not so accurate [35]. However, this inaccuracy is most probably systematic making the results of the different batches prepared in their respective vials still comparable. Moreover, the results should give a rough estimate of the size and size-distribution of the liposomes prepared.

The data collected were for the Autoset channel width above 1000 K for all measurements. For Channel width 10 µs and 5 µs the amount of data were lower; range 186-592 K.

As presented in figure 18, there was no difference in the mean particle- size distribution for the different VPG batches prepared in the two vials. The results show that one can achieve a liposome size of about 60 nm by speed-mixing 100 mg, 200mg and 500mg VPG batches for 30 min (3540 rpm), using either the 38 ml -or the 10 ml glass container.

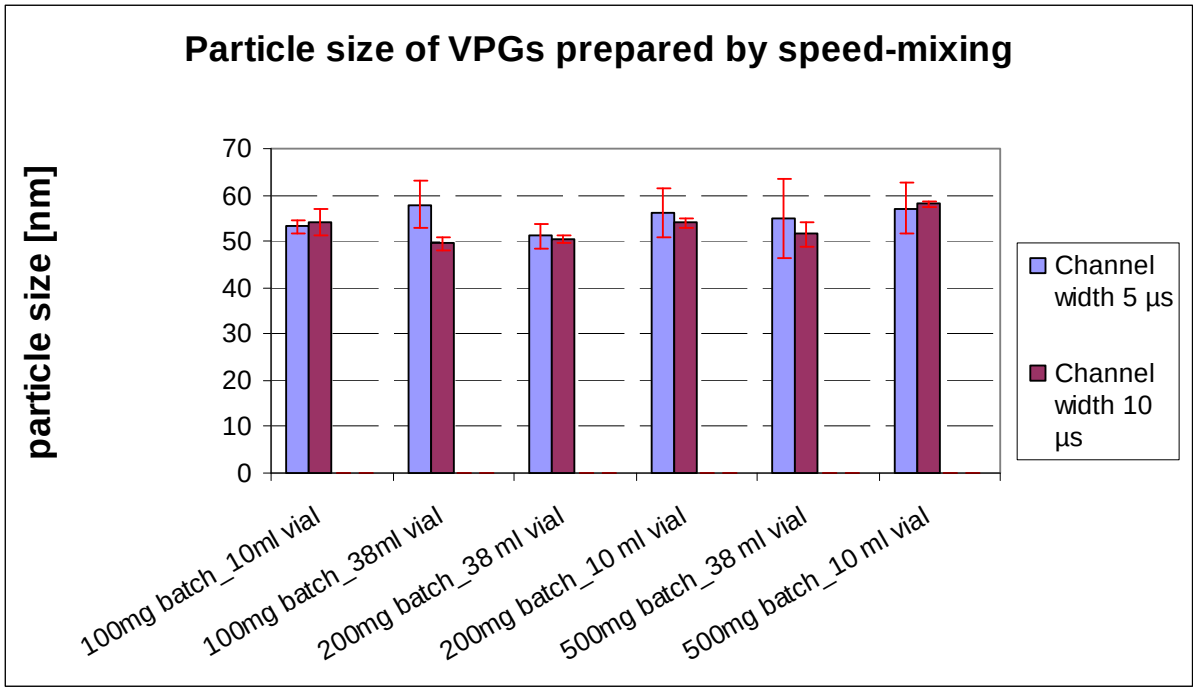


Figure 18: Mean particle size of different VPG batches prepared by speed-mixing in two vials with different radius and size.

One explanation why there is not seen any difference in particle size between the VPGs prepared in the two vials might be that the mean diameter calculated is not a sensitive indicator able to indicate potensial differences, which in turn makes the applied analytical method suboptimal. Moreover, it might be that the difference in radius between the two vials is not big enough to make a significant difference on the results.

As seen from figure 19 the polydisperity indexes for the different VPGs prepared show quite high values roughly above 0.5.

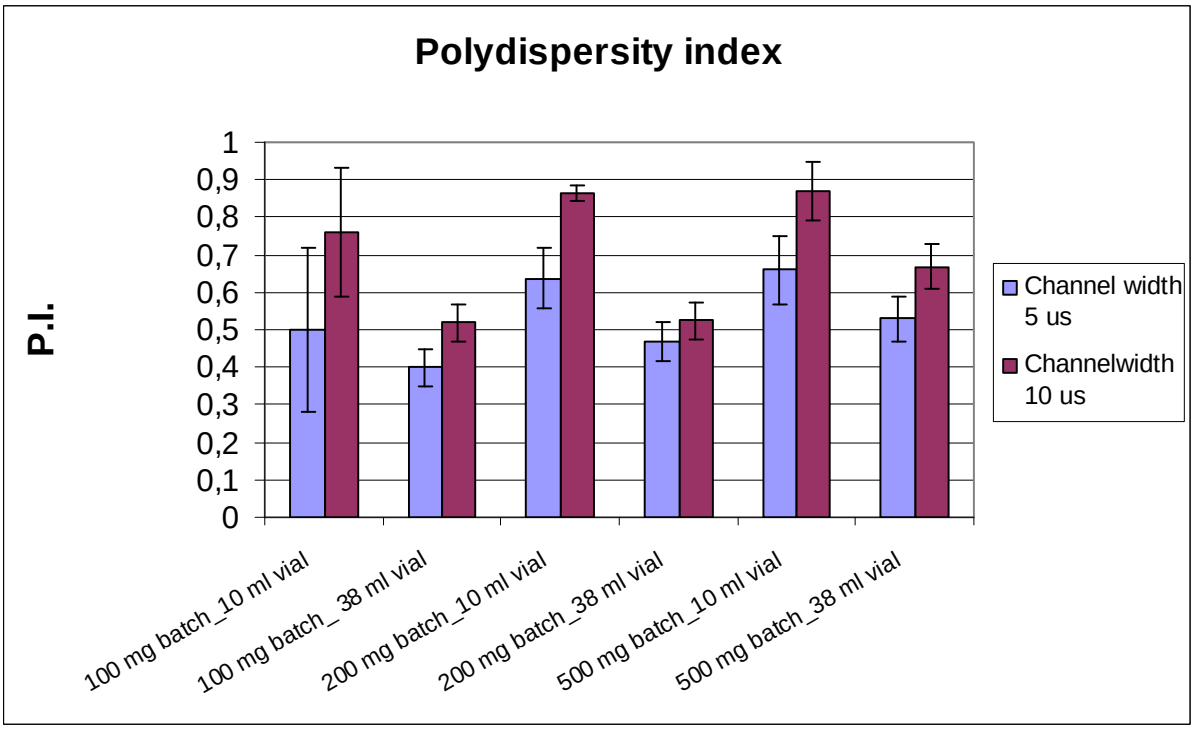


Figure 19: Polydispersity index of different VPGs batches prepared by speed-mixing in two vials with different size.

The polydispersity indexes indicate that the samples contain a rather broad particle- size distribution. As seen from figure 19 there is a tendency of smaller P.I. values for the VPG batches prepared in the biggest vial. This indicates that there might be a difference in size-distribution between the VPGs prepared in the different vials. In the bigger vial the sample material might be more evenly forced towards the walls of the vial, resulting in a more homogenous particle size distribution. In addition, the standard deviations between the VPGs prepared in the biggest vial seem to be a bit smaller than for the VPGs prepared in the

smallest vial, thus indicating more reproducible results regarding size-distribution of the liposomes. Based on these findings, the 38 ml vial should be chosen in preference to the 10 ml vial for the respective batches investigated.

Size and size-distribution of bendamustin-liposomes

Particle size of the bendamustin-liposomes was investigated in order to see if the presence of bendamustin within the liposomes, as well as the low pH of 2.0 in the new bendamustin-liposome formulation 2, could alter the size of the vesicles in comparison to the "empty" liposomes. Bendamustin-liposome formulation 1 and 2 were prepared according to section 5.4.2.1 and 5.4.2.2 and measured on PCS according to section 5.5.1.

The results presented in table 11 show a particle-size of about 60 nm for both of the bendamustin-liposome formulations as well as for the "empty" liposomes.

Table 11: Particle size and size-distribution of "empty" VPGs in comparison to bendamustin-VPG formulation 1 and bendamustin-VPG formulation 2. (Channel width 10 µs)

VPG formulation	**Mean particle size (nm) and SD**	**P.I. and SD**
Bendamustin-VPG formulation 1	62.8 ± 2.0	0.53 ± 0.01
Bendamustin-VPG formulation 2	60.5 ± 1.3	0.50 ± 0.01
"Empty" VPGs	51.6 ± 3.0	0.70 ± 0.06

The liposome size and distribution was found not to be affected by presence of bendamustin within the liposomes. This results corresponds to a previous measurement carried out by Cicko[27].

There is a chance, however, that the particle size of the new bendamustin-liposome formulation 2 might change after long term storage due to the pH of 2.0 in the VPG. The low pH might alter the lipid membrane and accelerate physical instability processes such as fusion of liposomes, when diluted [22]. Consequently, particle size might increase over time. However, considering the fact that the formulation is intended as a bed-side preparation, this should not be a problem regarding stability of the formulation.

6.2 Optimization of ion exchange chromatography method for bendamustin

The first cation- exchange chromatography method used, as described under section 5.5.2, showed a great variance in cholesterol recovery between the liposome-eluates separated from the columns prepared; SD 16.7 %. In addition the recoveries were relatively low; mean cholesterol recovery calculated from the eluates was 49.8 % (See appendix for details). The great variance in cholesterol recovery in the eluates separated from the columns indicated that the cation- exchange method was suboptimal. Three different approaches of improving the method by obtaining a higher cholesterol recovery as well as a lower variance in cholesterol between the eluates were carried out as described below.

Approach 1

A VPG was prepared and re-dispersed in 0.9 % sodium chloride solution according to section 5.4.1. The same ion-exchange method described under section 5.5.2 was carried out with the exception that all the amounts for both column- preparation as well as for sample application and flushing of sample was doubled; resin, sodium chloride solution and water used for column preparation, liposome sample as well as water used for flushing of the liposomes.

The eluates and the references (not applied to the columns), were diluted with ethanol/methanol (90/10 v/v) in the ratio of 1:100 in order to dissolve the liposomes. Cholesterol in the samples was measured on RP-HPLC and cholesterol recovery was calculated using equation 1 according to section 5.5.2

The results showed considerable less variance in cholesterol recoveries between the eluates from the columns. In addition the recovery was considerable higher; Mean cholesterol recovery calculated from equation 1 under section 5.5.2 was; 74 % ± 8.5 (See appendix for details).

A SD of 8.5 % is an acceptable variance in cholesterol recovery between the eluates. The ratios between the critical parameters as amount of sample, resin, and water for flushing were the same in this approach as in the first method described in section 5.5.2, and in this respect the cholesterol recovery should theoretically be the same for the two methods. There could be a problem, however, that the amount of sample applied to the columns in the first method,

was too small to handle, and therefore better results were seen when double amount of sample was used.

Approach 2

Although lower variance in cholesterol recovery between the eluates was obtained in approach 1, the cholesterol recovery was still a bit low; 74 %. One theory explaining the rather low cholesterol recovery could be suboptimal dissolution of liposomes in the eluates in dilution 1:100 of ethanol/methanol (90/10 v/v), even though the solutions seemed clear. The theoretical concentration of cholesterol in this dilution is 364 µg/ml. The references from the same sample set, not applied to columns, showed a cholesterol concentration of about 100 % of the theoretically cholesterol value in the respective liposome dilution (See appendix for details). This stated that the liposomes were totally dissolved in the reference samples. In the eluates, however, the amount of water which was added in the flushing step could result in a dissolution problem of the liposomes.

In order to investigate if low cholesterol recoveries were due to suboptimal dissolution of the liposome-eluates, the following approach was carried out;

A VPG was prepared and re-dispersed with 0.9 % sodium chloride solution according to section 5.4.1. 100 µl of the liposome sample was added to each of two 10 ml graduated flasks. The liposomes in the one flask was diluted with 1.25 ml distilled water, which was the same amount of water used during the flushing step of ion-exchange chromatography, and then further diluted with ethanol/methanol (90/10 v/v). The liposomes in the other flask was diluted only with ethanol/methanol (90/10 v/v). Both of the liposome samples yielded in a dilution of 1:100. Triplicates were prepared for both the sample containing water, as well as for the reference, before cholesterol was measured on RP-HPLC according to section 5.5.2.

The results indicated, however, no difference in the cholesterol concentration between the samples containing water and the references (diluted only in ethanol/methanol); Cholesterol concentration of 399 µg/ml ± 4.2 for the samples containing water, and 402 µg/ml ± 7,2 for the references (See appendix for details). These cholesterol values were within the range of the theoretical cholesterol concentration of 364 µg/ml, in the respective dilution.

Based on these findings the liposomes in the eluates, containing 1.25 ml water + ethanol/methanol (90/10 v/v) were properly dissolved. Hence, the reason for the low cholesterol recovery was not due to suboptimal dissolution of the liposome- eluates.

Approach 3

Another explanation for the low cholesterol recoveries could be that the amount of water used for flushing was too small, leading to suboptimal flushing of liposomes through the resin of the columns. More water for flushing would lead to a dissolution problem of the liposomes in dilution 1:100. Furthermore, a higher dilution with ethanol/methanol would result in a bendamustin concentration under the UV detection range, and was therefore not possible. However, by reducing the resin amount, and keeping the water amount stable, more water per resin would be obtained for flushing.

First, columns were prepared according to section 5.5.2. Then resin capacity was checked in order to see if 1 g of resin was sufficient for keeping all the external bendamustin attached to the resin. For this 4 mg Ribomustin® was dissolved in 0.5 ml distilled water and further diluted with 0.5 ml 1.8 % sodium chloride solution, yielding in the same bendamustin-concentration as for the re-dispersed VPG; 1.82 mg/ml bendamustin.

Furthermore, VPGs were prepared and re-dispersed with 0.9 % sodium chloride solution according to section 5.4.1. The amount of liposome-sample applied to the columns as well as the water amount used for flushing was doubled; 100 µl of the liposome dispersion was applied to each of the columns. The columns were then flushed five times with 250 µl distilled water under continuous vacuum suction.

All the eluates and references were dissolved in ethanol/methanol (90/10 v/v) in the ratio of 1:100. Potential bendamustin in the eluates from the resin-capacity test was quantified on RP-HPLC. Cholesterol was measured for the liposome-eluates on RP-HPLC before cholesterol recovery was calculated using equation 1 according to section 5.5.2.

The results from the resin-capacity test showed no detected bendamustin in the eluates measured on HPLC, which stated that all of the bendamustin was left in the resin, and concenquencly the resin-capacity was satisfying.

The variance in cholesterol recoveries from the liposome-eluates showed a low standard deviation, in addition to increased recovery; 86 % ± 3. Hence, the reason for the low cholesterol recoveries was due to suboptimal water per resin used in the flushing step of the liposomes. The improved ion-exchange method for bendamustin, described below, was used further on in this study;

Improved Ion- exchange chromatography method for bendamustin:

Preparation of the Lichrolut cation exchange column:

- PTFE- frits were placed within the glass column
- 1 g of AG 50W-X8 Resin was added to the glass column
- The column was filled with distilled water and left still in 5 minutes in order to get a sedimentation of the resin
- Excess water was removed by vacuum, but there was left enough water to cover the top of the resin
- The glass column was loaded with filtrated saturated sodium chloride solution 1.0 ml × 3, and thereafter flushed with 1.0 ml × 3 distilled water. Finally the glass column was dried by vacuum for 5 minutes.

Separation of liposomal bendamustin from external bendamustin by ion-exchange chromatography

- 100 µl of the loaded liposome dispersion was added drop wise to the column followed by vacuum suction.
- The column was flushed five times with 250 µl of distilled water under continuous vacuum suction.
- The eluate, containing liposomal bendamustin, was collected in a flask, while the external Bendamustin was left in the column.

6.3 Encapsulation efficiency of bendamustin

Encapsulation efficiency for both bendamustin- liposome formulation 1 as well as the new bendamustin- liposome formulation 2 was determined according to section 5.5.2.

The respective liposome formulations were prepared according to section 5.4.2.1 and 5.4.2.2. The two formulations differed only in the composition of the bendamustin-solution used for direct loading of the VPGs. For bendamustin-liposome formulation 1 Ribomustin® was dissolved in water and diluted with 0.9 % sodium chloride solution to yield in a pH of 3.0. Whilst for the new bendamustin-liposome formulation 2 Ribomustin® was dissolved in water and diluted with PBS pH 2.0. (The concentration of bendamustin in both of the solution was the same; 9.09 mg/ml) Both of the loaded VPG-formualtions were re-dispersed in 0.9 % sodium chloride solution, yielding in a pH of about 4.5 for the outer aqueous phase of the liposome dispersions.

Encapsulation efficiency of bendamustin- liposome formulation 1

As shown in table 12, the encapsulation efficiency of bendamustin-liposome formulation 1 was 44 ± 3.2 %.

Table 12: Encapsulation efficiency of bendamustin-liposome formulation 1

Parallels	**EE % (mean of triplicates ± SD)**
VPG 1	42 ± 1.6
VPG 2	48 ± 3.9
VPG 3	43 ± 1.9
Mean VPGs and SD	44 ± 3.2

The encapsulation efficiency found corresponds to a previous finding by Cicko, where the EE was determined to 41.5% ± 4.1 % [27].

The EE is quite high comparing to many other liposome formulations. One reason for this is that the formulation prepared is using a vesicular phospholipid gel as intermediate. VPGs contain greater lipid content than conventional liposomes, resulting in more liposomes per unit volume, and hence an increased ratio of the aqueous part entrapped within liposomes as compared to the total volume [24]. This in turn will result in high EE.

Furthermore, a certain amphiphilic characteristic of bendamustin will probably besides entrapment of drug within the aqueous core as well leads to some interaction with the lipid membrane. This might also add to the high EE of the drug.

Encapsulation efficiency of the new bendamustin- liposome formulation 2

As seen from table 13 the new bendmustin-liposome formulation 2 showed a EE of 61 %. This was an increase from the previous formulation by about 20 %.

Table 13: Encapsulation efficiency for the new bendamustin-liposome formulation 2

Parallels	**EE % (mean of triplicates ± SD)**
VPG 1	60 ± 3.3
VPG 2	63 ± 0.5
VPG 3	59 ± 3.5
Mean VPGs and SD	61 ± 2.4

For VPGs the EE of hydrophilic compounds is determined by the ratio of vesicle core volume as compared to overall aqueous volume in the VPG at the time of VPG formation.
The theoretical maximum EE can be predicted if the phospholipid concentration, the vesicle diameter and the aqueous volume are known[24]. For VPGs consisting mainly of small unilamellar vesicles with a phospholipid concentration of 400 mg/g, which is the case for the bendamustin-liposomes, the maximum EE should be about 40 % [24]. However, experimental values may deviate from theory. It is difficult to achieve experimentally the theoretically predicted maximum encapsulation efficiency and true SUVs since vesicle size and lamellarity often are quite inhomogeneous. EE will also vary with type of drug and lipid used. Previous studies for the hydrophilic marker calcein encapsulated into SUVs with a phospholipid concentration of 400 mg/g, showed an EE of about 30-40 % [24]. Considering this an EE of 60 % for bendamustin within the new liposome formulation is quite high.

The new bendamustin-liposome formulation 2 did only differ from bendamustin- liposome-formulation 1 by a decreased pH within the aqueous core. The type of lipid, ratio lipid concentration /aqueous medium, as well as preparation technique was the same. Moreover, as

shown in section 6.1 both of the formulations showed to have the same size and size-distribution. Hence, the increase in EE was most probably not a question of vesicle size.

An explanation for the increased EE could be that there occurs some active loading of bendamustin during re-dispersion of the VPG. The zwitterionic molecules in the outer aqueous phase might diffuse through the lipid membrane, and when they reach the buffered pH of 2.0 inside the liposome, the molecules get protonated and are retained.

For further investigation of active loading see section 6.7

6.4 Degradation of bendamustin during speed-mixing.

Degradation of bendamustin during preparation of the VPGs by speed-mixing was investigated for both bendamustin-liposome formulation 1 and the new bendamustin-liposome formulation-2 according to section 5.5.3.

The results presented in table 14 and 15, showed a low degradation of bendamustin during speed-mixing for both of the formulations; 93.7% ± 4.1 intact bendamustin for bendamustin-liposome formulation 1 and 97.5 % ± 2.2 intact bendamustin for bendmustin- liposome formulation 2 were left immediately after preparation of the VPGs. In addition, the preparation seemed to give reproducible results regarding degradation of the compound; rather low standard deviations < 5 %. Moreover, the difference observed in amount intact bendamustin after speed-mixing for the two liposome formulation was not significant.

Table 14: Intact bendamustin after speed-mixing of bendamustin-VPG formulation 1

Parallels	**Intact bendamustin % (mean of triplicates and SD)**
VPG 1	90.8 ± 1.3
VPG 2	98.5 ± 5.3
VPG 3	92.0 ± 1.5
Mean of parallels and SD	93.7 ± 4.1

Table 15: Intact bendamustin after speed-mixing of bendamustin-liposome formulation 2.

Parallels	**Intact bendamustin % (mean of triplicates and SD)**
VPG 1	96.3 ± 0.8
VPG 2	100.0 ± 0.7
VPG 3	96.3 ± 1.0
Mean of parallels and SD	97.5 ± 2.2

The results confirm that there is minimal degradation of bendamustin during speed-mixing. Thus, dual asymmetric centrifugation is a suitable technique for liposome preparation of the highly sensitive compound bendamustin.

6.5 Efflux experiments

Stability of bendamustin-solution at pH 7.4

In order to check whether it makes sense to run efflux experiments in PBS pH 7.4, stability of bendamustin upon incubation in PBS pH 7.4 at 23˚C and 37˚C was investigated according to section 5.5.4

As shown in figure 20 bendamustin was quite unstable in PBS pH 7.4; half life was observed after about 10 minutes at 37 ˚C and 15 minutes at 23 ˚C/ room temperature.

The retention time of bendamustin during HPLC measurement gave a small time- gap between injection onto the HPLC and until the samples were measured on the HPLC- UV

detector. This could be a reason for the relatively high standard deviations between the triplicates; 5-10 %.

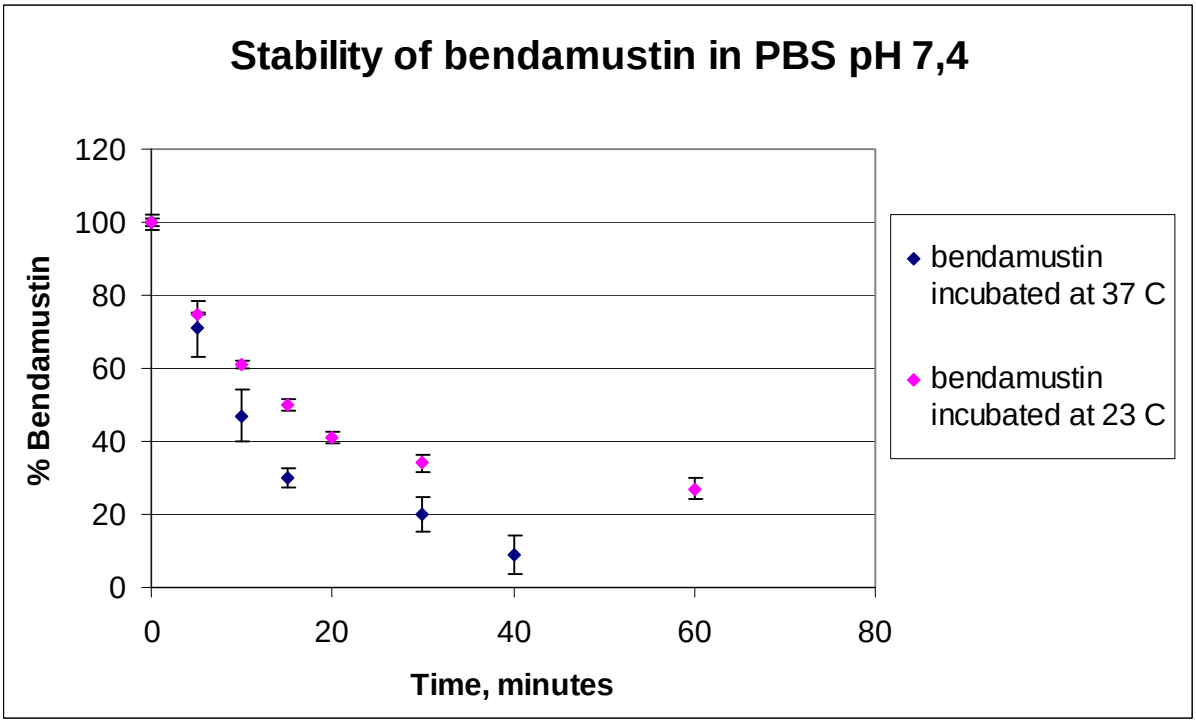


Figure 20: Remaining intact bendamustin upon incubation in PBS pH 7.4 at 37 ºC and 23 ºC over time.

Normally, hydrolysis of bendamtustin follows first order kinetics [15]. The reason why this is not seen here is unclear. The rapid degradation of bendamustin observed was probably due to pH. PBS does not contain any strong nucleophiles which might degrade bendamustin. At high pHs > 6, the bendamustin is expected to exist mainly in its anionic form, resulting in increased hydrolysis and hence in inactivation of the drug. This seems to be in accordance with a study done by Von G. Hesse which found that there was a fast hydrolytic cleavage of the chlorine bond at pH values > 6 [14].

6.5.1 Efflux kinetics of bendamustin-liposome formulation 1 upon incubation in sodium chloride solution.

Upon incubation of a bendamustin-VPG in physiological pH; 7.4, there will exist a situation were the pH inside and outside the liposomes are different resulting in unequal degradation rate of the drug in the two compartments. The bendamustin-solution used for preparation of the bendamustin-VPG formulation 1 was measured to reveal a pH of 3.0. Hence, this was probably the pH within the aqueous core of the liposomes. Upon re-dispersion and incubation in PBS pH 7.4 the external bendamustin in the outer aqueous phase will subsequently be degraded much faster than for the incorporated drug due to a higher pH. Because of the unstable system with references containing both liposomal and external bendamusin, it was difficult to investigate efflux kinetics alone. Therefore, it did not make sense to determine the overall content of bendamustin over time.

In order to overcome this problem, and to know only the efflux of drug out of the liposomes per time, a more stable system was chosen; where the drug is rather stable both inside as well as outside the liposomes within the respective time period measured. For this, 0.9 % sodium chloride solution was used as the outer aqueous phase, which corresponds to a pH value in the outer aqueous phase of about 4.5. This pH reveals a minimal degradation of bendamustin over the time-period the experiment lasted [15].

Efflux of liposomal bendamustin was carried out after the general describtion in section 5.5.5. Bendamustin solution pH 3.0 was directly loaded into liposomes resulting in bendamustin-VPG formulation 1 as described in section 5.5.4.1. The VPG was re-dispersed with 0.9 % sodium chloride in ratio 1:3 yielding in a pH of 4.5 in the outer aqueous phase. The liposome dispersion was incubated at 37 ºC for the following time-points: 0, 10, 20, 30, 40, 60, 90 and 120 minutes. At the respective time-points amount entapped/unentrapped liposomal bendamustin was executed.

The result presented in figure 21 showed no efflux within the time period measured.

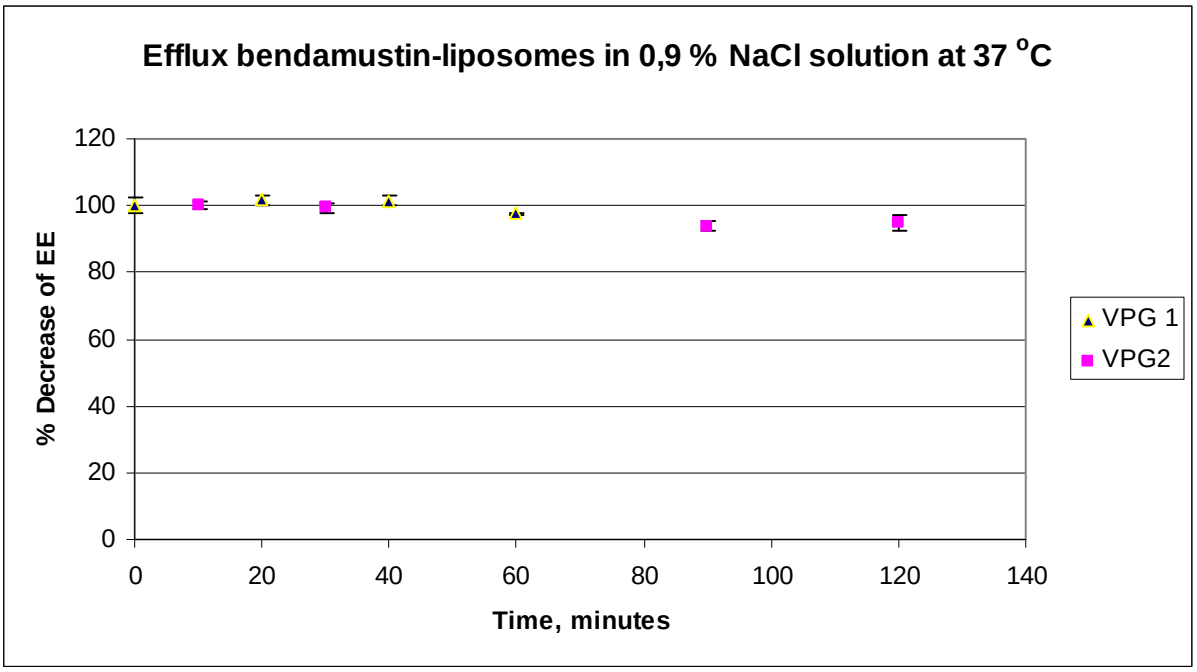


Figure 21: Remaining intact bendamustin within bendamustin-liposome formulation 1 upon re-dispersion in 0.9 % Sodium Chloride Solution and incubation at 37 ºC over time. The liposome interior revealed a pH of 3 whilst the liposome exterior yielded in a pH of about 4.5.

A possible explanation why there is not obeserved efflux in this system could be due to the difference in pH inside and outside the liposomes. Bendamustin has two pKa values; 4.5 and 6.3. Above pH 4.5 the carboxylic acid group on the molecule is deprotonated and reveals a minus charge. Below pH 6.3 the amino group(s) are protonated resulting in positively charged molecules. Molecules with a net charge are restricted from diffusion over lipid membranes [36]. Bendamustin exists in a charged form in the whole pH range. However, between pH 4.5 and pH 6.3 bendamustin is in a zwitterionic form. In this form there might act ionic forces between the carboxylic group and the amino group on the molecule, resulting in a ring formation. (Figure 22) This formation might act like a sterical shielding of the charges and give rise to an increased lipophilicity of the molecules, which in turn might result in a more rapid diffusion over the lipid membrane when the molecules are in the zwitterionic form.

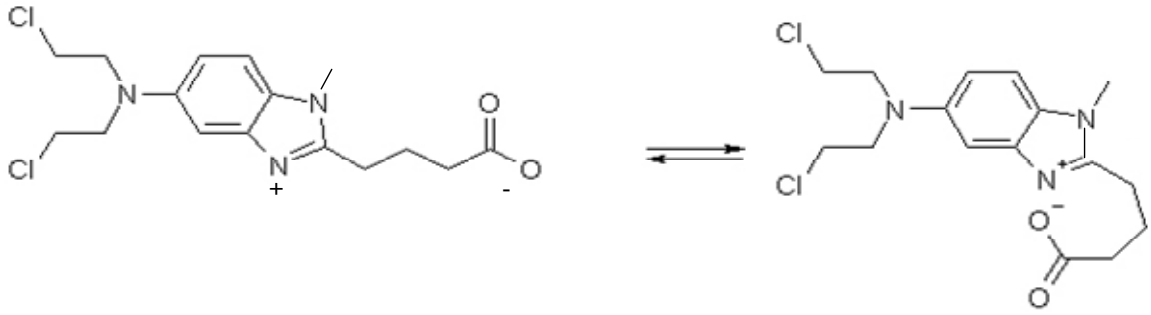


Fig 22: Possible ring formation of the bendamustin molecule in the zwitterionic form.

The difference in pH outside and inside the liposomes, will lead to a different total percentage of zwitterionic molecules in the two compartments. The bendamustin solution in the aqueous core was measured to have a pH of about 3. After re-dispersion of the VPG with sodium chloride solution in the ratio of 1:3, the pH in the outer aqueous phase was measured to be about 4.5. According to Hendelson-Hasselbach equation the percentage of cationic (amino-protonated) bendamustin inside the liposomes at pH 3 is 97 %, which means that 3 % of the molecules exist in the zwitterionic form at this pH. In the outer aqueous phase, however, the pH is about 4.5, resulting in a concentration of zwitterionic molecules of 50 %. Although the volume- ratio of the aqueous core and the outer aqueous phase in the liposome-dispersion is about 1:8, the number of zwitterionic molecules per volume is higher in the outer aqueous phase than in the aqueous core of the liposomes.

Under the assumption that both the cationic and anionic forms are not readily permeable whereas the zwitterionic form is permeable, such a pH gradient (pH 3 inside and pH 4.5

outside) is regarded to a situation where a gradient of the zwitterionic form occurs eventually leading to a net efflux of bendamustin from the outer to the inner aqueous volume. However, if this is the case, then there should be an increase in incorporated amount bendamustin over time. This was not seen in the results. An explanation for this might be due to a change in the pH gradient over time because of no buffer system. When some of the zwitterionic molecules have diffused into the liposomes, the pH in the liposome interior might be increased, resulting in loss of the pH gradient, and equilibrium between the liposome interior and exterior occurs.

Another possible explanation to this might be that there is equilibrium between the ring form and the "normal" zwitterionic bendamustin molecules, leading to a difference in diffusion rate for the two forms over the membrane. Consequently, not all of the zwitterionic molecules may penetrate the membrane at equal speed.

6.5.2 Efflux of bendamustin-liposome formulation 1 upon incubation in PBS pH 2.0

In order to investigate whether the theory of back-diffusion of zwitterionic bendamustin into the liposomes, explained under section 6.5.1, is correct, the same efflux experiment was repeated except that sodium chloride solution as re-dispersion medium was replaced by PBS pH 2.0. According to the theory, this experiment should give a rapid efflux of bendamustin out of the liposomes, because a pH of 2.0 in the outer aqueous phase would lead to a decrease in zwitterionic bendamustin molecules able to diffuse back into the lipsomes.

The results presented in figure 23 show a drastically efflux in the system; 40 % of the encapsulated bendamustin had leaked out after approximately 15 minutes. The curve shows an exponential fit with most leakage within the first ten minutes. However, after about 20 minutes the curve seems to reach equilibrium, and there is no further efflux observed after this time- point.

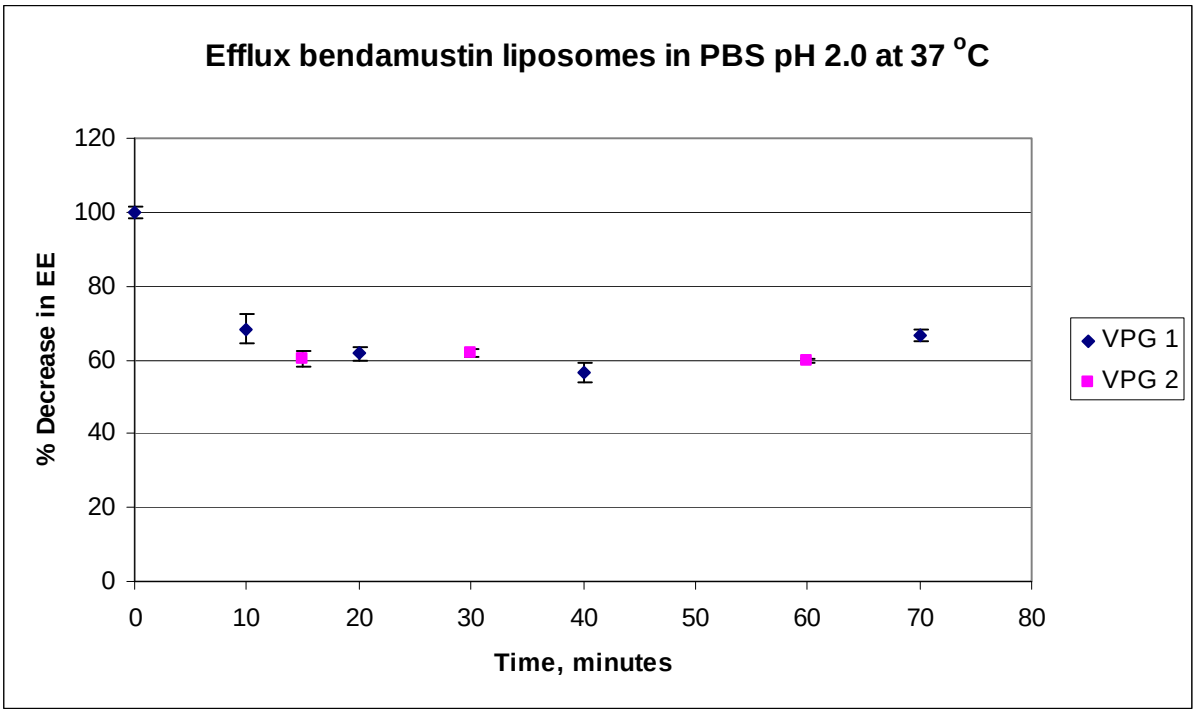


Figure 23: Remaining intact bendamustin within the bendamustin-liposome formulation 1 upon re-dispersion in PBS pH 2.0 and incubation at 37 ºC over time. The pH in the liposome interior reveals a pH of about 3.0, whilst the outer aqueous phase reveals a pH of 2.0.

An explanation why the curve is flattening off could be equilibrium of zwitterionic molecules between the inner and the outer aqueous phase. This might occur because the presence of hydrochloride ions from the Ribomustin® powder is making the solution more acidic inside the liposomes after some of the zwitterionic bendamustin has diffused out, resulting in loss of the pH gradient.

6.5.3 Efflux kinetics of bendamustin-liposome formulation 1 in comparison to bendamustin-liposome formulation 2 upon incubation in PBS pH 7.4

The findings in the efflux experiments above showed that the zwitterionic form of bendamustin tended to penetrate the lipidmembrane more readily than the cationic and anionic form. This in addition to the unstability of bendamustin at higher pH indicated that the optimal pH considering efflux and stability seems to be a further reduction of pH within the liposomes. By decreasing the pH in the aqueous core from 3 to 2, and holding the pH stable by adding a buffer system, will theoretically give a ten time reduction of zwitterionic molecules able to pass the lipid membrane. Hence, the efflux should be decreased. The new bendamustin-liposome formulation was prepared according to section 5.4.2.2.

As observed from the efflux experiments in section 6.5.1 and 6.5.2, efflux kinetics of liposomal bendamustin would partly rely upon the incubation medium used. And as already mentioned, incubation of the liposome dispersion in physiological pH of 7.4, will lead to a rapid degradation of external bendamustin, thus making it difficult to determine the overall bendamustin- content over time. The overall content bendamustin show the stability of bendamustin in the liposome- dispersion over time. Therefore, upon incubation of the bendamustin liposomes in PBS pH 7.4, only the overall bendamustin content at time- point 0 minutes can be used for calculation of the EE according to equation 2 under section 5.5.2. However, due to slow hydrolysis of bendamustin in low pH one could assume that possible decrease of liposomal bendamustin would be a result of efflux and not degradation. Subsequently, the efflux kinetics of bendamustin-lipsosome formulation 2 could be compared to the new bendamustin-liposomeformulation 2 upon incubation at physiological pH 7.4.

Bendamustin-liposome formulation 1 and 2 were prepared according to section 5.4.2.1 and 5.4.2.2. Both the VPGs were re-dispersed in the ratio of 1:3 with PBS pH 7.4 and incubated at 37 ºC over time before efflux was determined according to section 5.5.5.

Figure 24 shows the efflux kinetics of the two bendamustin-liposome formulations. For bendamustin-liposome formulation 1 half life of liposomal bendamustin was observed after 90 minutes, whilst the half life of formulation 2 was observed after 180 minutes. However, because of the higher EE of formulation 2, the amount intact bendamustin within the liposomes over time is considerable higher for formulation 2 than for formulation 1. After 3 hours there is left 30 % intact liposomal bendamustin within formulation 2, whilst for liposome formulation 1 30 % intact bendamustin is left after 1 hour.

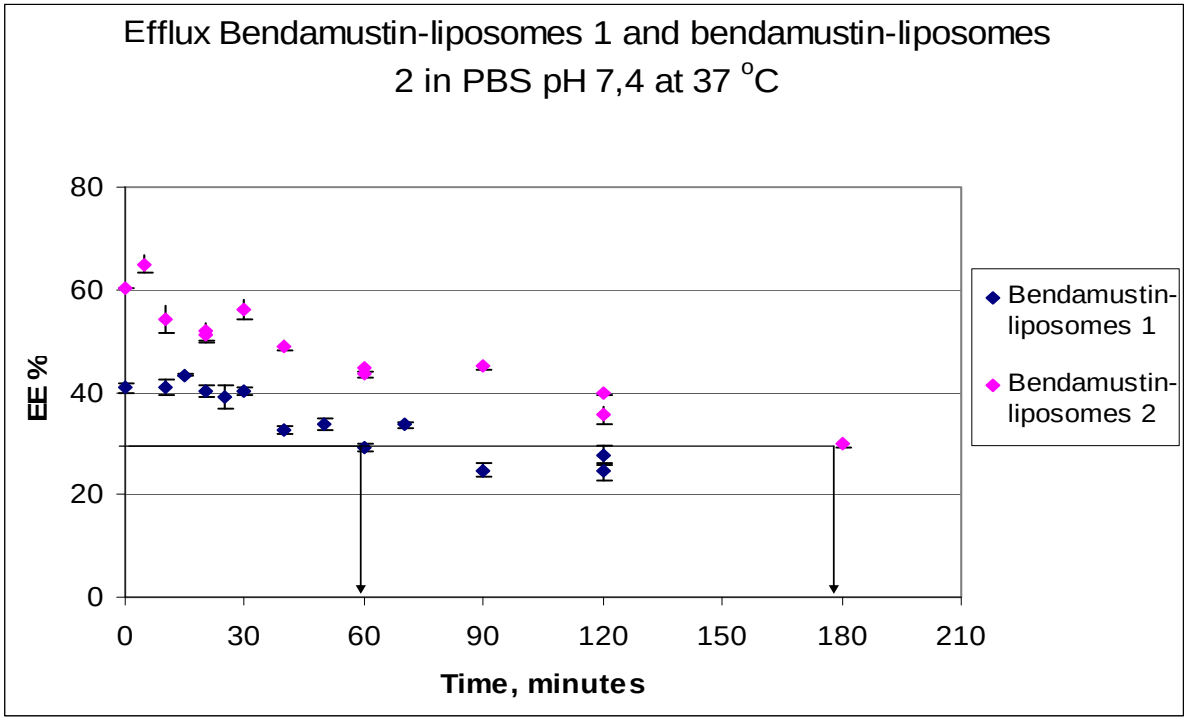


Figure 24: Efflux kinetics of bendamustin-liposome formulation 1 and 2 upon incubation in PBS pH 7.4 at 37 ºC over time.

For bendamutsin- liposome formulation 1 the efflux curve seems to flatten off after 90 minutes, which corresponds to the theory behind the results in section 6.5.2; there is no buffer within the liposomes, leading to an unstable pH inside the liposomes. After some of the bendamustin has leaked out, the pH in the aqueous core might decrease resulting in equilibrium of zwitterionic molecules between the two compartments. Consequently, efflux is reduced.

The result does not correspond to a previous finding by Cicko, where the half life of bendamustin in bendamustin liposome-formulation 1 was estimated to be about 18 minutes [27]. However, in this study, there was not given detailed descriptions of the methods used, which makes it difficult to ensure that the experiment was carried out in a similar way. i.e; no information is given about the composition of the PBS, its molarity and pH. In addition it seems like no parallels were carried out.

The reason why there is efflux in this system and not in the case where the liposomes where incubated in sodium chloride solution pH 4.5, showed in section 6.5.1 might be due to pH.

When the VPG is incubated in PBS pH 7.4, there is a concentration of zwitterionic bendamustin in the outer aqueous phase of 9 %. This is a considerable smaller percentage than in the case when the VPG was incubated with sodium chloride solution pH 4.5. In addition, the fast degradation of bendamustin in pH 7.4 in the outer aqueous phase would probably yield in a shift in equilibrium, and only a few intact molecules are able to diffuse back into the liposomes.

Considering the fact that a change in pH from 3 to 2 theoretically gives a ten time reduction in zwitterionic molecules, the reduction in efflux was not as high as expected. The reason for this is unclear.

The two improved effects for the new bendamustin-liposome formulation; reduced efflux rate as well as an increased EE result in more intact bendamusin over time, thus making the new formuation improved.

6.6 Stability experiments of the new bendamustin-liposome formulation

6.6.1 Stability of bendamustin

Stability of bendamustin relies partly upon pH [14]. In a previous study for a related compound; L-phenylalanine mustard, hydrolysis upon different pHs was executed. The result showed that hydrolysis increased with increasing pH [37]. As shown under section 6.5 bendamustin was fast degraded in pH 7.4 due to hydrolysis of the chlorine bonds. The new bendamustin-VPG formulation 2 revealed a pH of 2.0. Even upon entrapment within liposomes at low pH, bendamusin will not be suitable for long term storage due to degradation. However, considering the fact that the liposome formulation might be intended as a bed side preparation, the stability of bendamustin in the new liposome formulation was checked upon storage in room temperature over a time period of 24 hours as described in section 5.5.6.1

According to the European pharmacopeia, drugs should contain at least 90 % of the intact active ingredient when applied to a patient [38]. Following this standard will mean that the

liposomes have to be used within a time period where no more than 10 % bendamustin is degraded.

As seen from figure 25, the degradation rate of bendamustin shows a linear fit (R^2= 0.99), where 90 % intact bendamustin is left after about 20 hours. After 24 hours there is 87 % ± 2 intact bendamustin left.

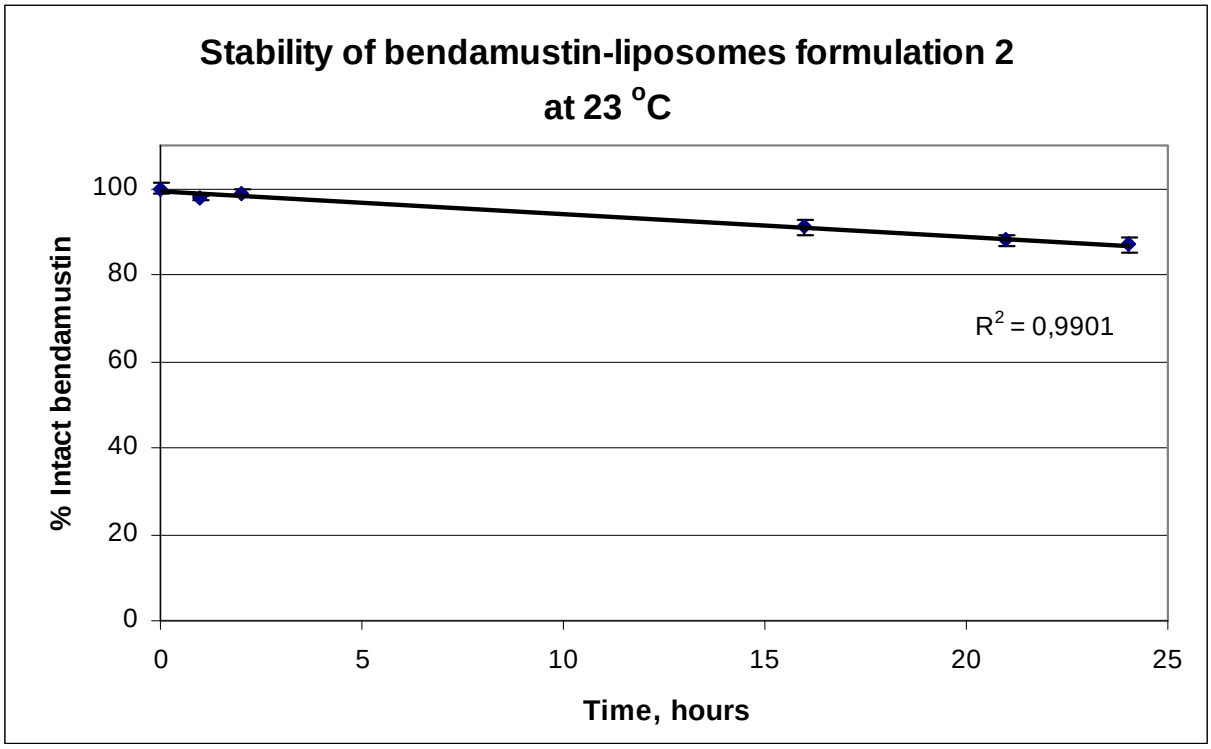


Figure 25: Remaining intact bendamustin in bendamustin-liposome formulation 2 upon incubation at room temperature/23 ºC over time.

The result shows that the formulation has to be prepared and used within the same day. Degradation would probably be a bit slower if the liposomes are stored at 4 ºC. Anyhow, this liposome formulation is only suitable as a bed-side preparation.

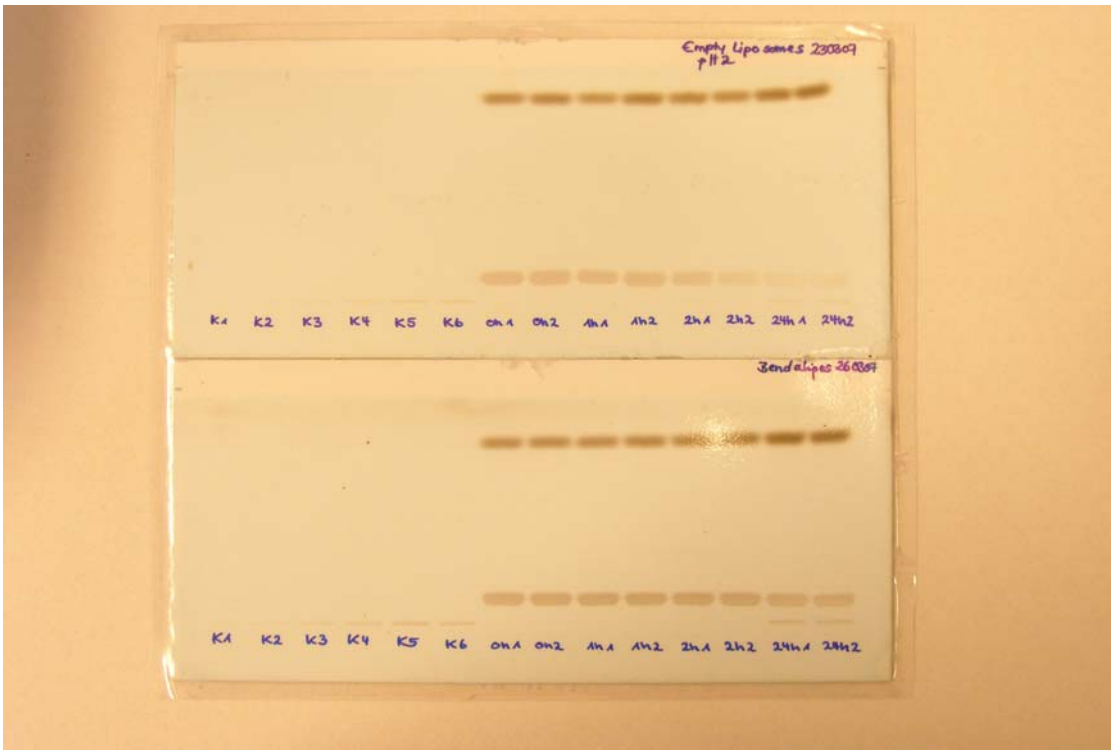
6.6.2 Stability of lipid

It is a well known fact that the reaction rate of hydrolysis of fatty acid chains is strongly dependent on pH and temperature. Extreme pH values; very acidic or alkali, as well as high temperatures result in a faster hydrolysis and degradation of the lipids [39].

Hydrolysis normally follows first order kinetics. In early stages of hydrolysis the structure of the liposomes are not affected. However, other characteristics such as permeability of the membrane are seriously altered. Small amounts of Lyso-PC might stabilise SUV because of their preferential distribution in the outer leaflet of the liposome. At advantage stages, however, the liposome integrity is disrupted because free fatty acid and lysolipid are detergent- like molecules and start to dissolve the membrane [39].

Due to the low pH of 2.0 within the new bendamustin-liposome formulation 2, stability of lipid; phosphatidylcholine, was investigated over 24 hours. In order to investigate whether bendamustin could influence on the rate of hydrolysis of the lipid membrane, a reference of "empty" liposomes were also prepared within PBS pH 2.0. Both the bendamustin-liposome dispersion as well as the "empty"- liposome dispersion were incubated at room temperature and assayed on HPTLC after the same recipe according to section 5.5.6.2.

The silica-plates used for sample application and detection of lyso-PC are shown in picture 3.



Picture 3: HPTLC plates; The upper plate in the picture shows the "empty" liposome samples, whilst the lowest shows the bendamustin-liposomes. Lyso-PC is seen as the narrow bands at the bottom of the plates.

The calibration series of Lyso-PC shown in figure 26 shows a good linear fit; $R^2 = 0.9949$.

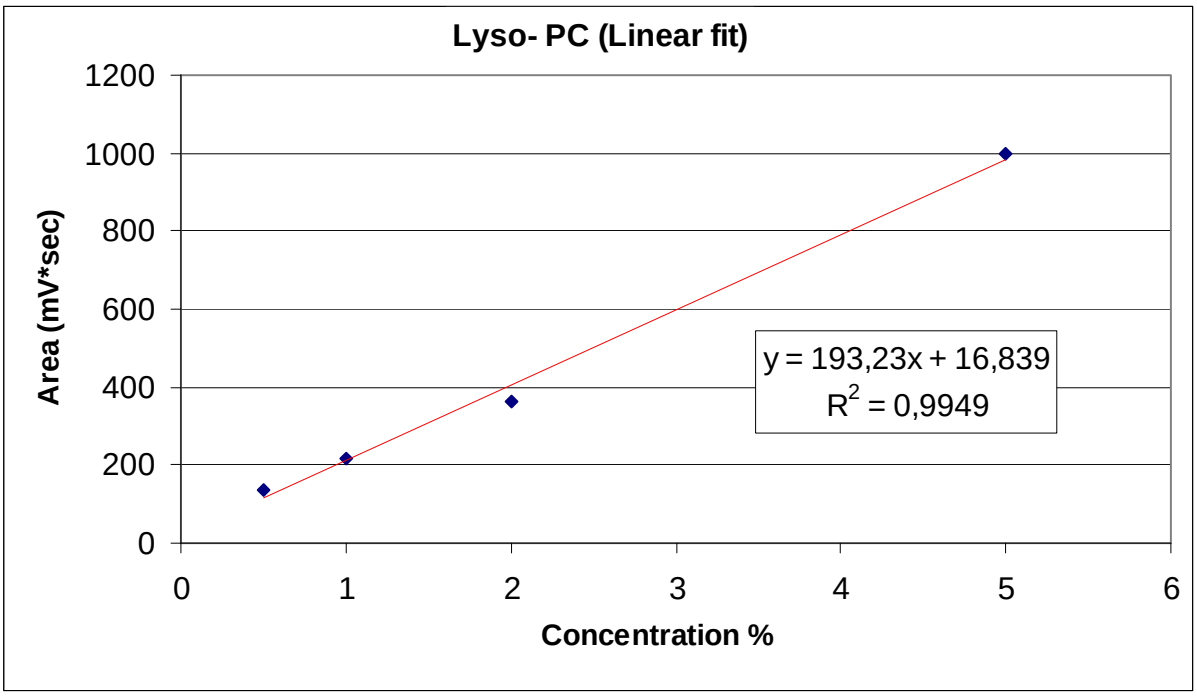


Figure 26: Calibration line Lyso-phosphatidylcholine

Ideally, in order to achieve higher statistically insurance of the results, triplicates of the VPGs should have been assayed. This was not accomplished due to the fact that 4 time points were measured for both of the VPGs, causing no space for application of additional samples on the HPTLC plates. However, as seen from table 16 and 17 the duplicates show very similar results, and hence should give a good indication of the tendency of the rate of hydrolysis of PC within both the bendamustin-containing liposomes and the "empty" liposomes.

The results presented as % lyso-PC over time are shown in table 16 and 17. The amount of Lyso-PC in the bendamustin-liposome formulation was about 9 % after 24 hours. For the reference, the "empty" liposomes, the amount Lyso-PC after 24 hours was about the half; 4.4 %.

Table 16: Percentage Lyso-PC in "empty" liposomes over time.

Incubation-time hours	**Lyso PC % Replicate 1**	**Lyso PC % Replicate 2**
0	0,00	0,00
1	0,00	0,00
2	1,01	1,04
24	4,33	4,51

Table 17: Percentage Lyso-PC in the new bendamustin- liposome formulation over time

Incubation-time hours	**Lyso PC % Replicate 1**	**Lyso PC % Replicate 2**
0	0,00	0,00
1	0,00	0,00
2	1,23	1,20
24	9,01	8,68

Not surprisingly the amount of Lyso-PC deriving from the liposome formulation was rather high after 24 hours. This was most probably due to the low pH of 2.0 in the formulation, resulting in a rapid hydrolysis of the ester linkages of PC.

The bendamustin- liposomes showed a considerably higher amount of Lyso-PC than the "empty" liposomes after 24 hours. This indicates that bendamustin acts as an indirect or direct catalyst in the hydrolysis of the ester linkages of PC, and in that order contribute together with the low pH to the rapid degradation of the lipid-membrane.

Theoretically, a rise in temperature during speed-mixing could result in a faster hydrolysis of the lipids. However, no Lyso-PC was detected after 0 minutes, which was the first time point assayed after re-dispersion of the VPGs. Fortunately; this showed that preparation of the liposomes by speed-mixing is not a problem regarding lipid stability of the formulation. Thus, DAC is suitable as a bed-side preparation technique for bendamustin-liposomes.

It should be mentioned that the re-dispersion and incubation of the VPGs in PBS pH 2.0 would theoretically result in increased hydrolytic attack from the outer aqueous phase due to a higher volume on the outside of the liposomes, than if the undiluted VPG had been kept for storage. Consequently, storage of the undiluted VPG at a lower temperature such as 4 ºC, would probably lead to a decreased amount of Lyso-PC. However, practical reasons such as volume sampling made it difficult to incubate the VPG it self. Anyhow, the trend would probably be the same. The results indicate that the bendamustin-liposome formulation should be prepared and used within the shortest time possible.

6.6.3 Stability in cell culture medium.

Stability of the new bendamustin-liposome formulation was compared to the free drug upon incubation in cell culture medium at 37 ºC over time, as described in section 5.5.6.3.
Cell culture medium contains a various amount of proteins, amino-acids, inorganic salts and vitamins which all might affect the stability of the liposome formulation. As has become clear from several previous studies, addition of medium may have a profound effect of liposome stability [40].

As presented in table 18 and figure 27 the half life of the bendamustin-liposomes in medium was observed after about 20 minutes. This was not considerably longer than for the free bendamustin-solution which showed a half-life after about 14 minutes (table 18 and figure 28).

Table 18: Half-life of free bendamusin compared to the new bendamustin-liposome formulation 2, upon dilution in cell culture medium pH 7.6 and incubation at 37 ºC.

Formulation	**t ½ bendamustin**
Free bendamustin solution	14 min
Bendamustin- liposome formulation 2	20 min

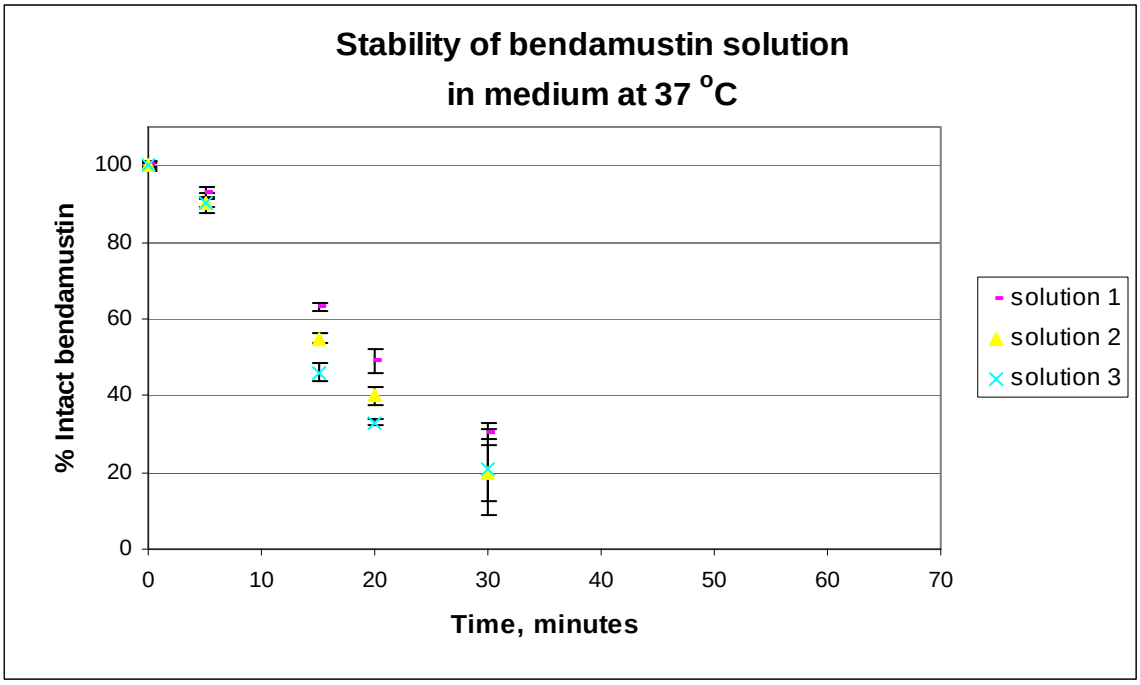


Figure 27: Remaining intact bendamustin in PBS solution upon dilution in cell culture medium pH 7.6 and incubation at 37 ºC over time.

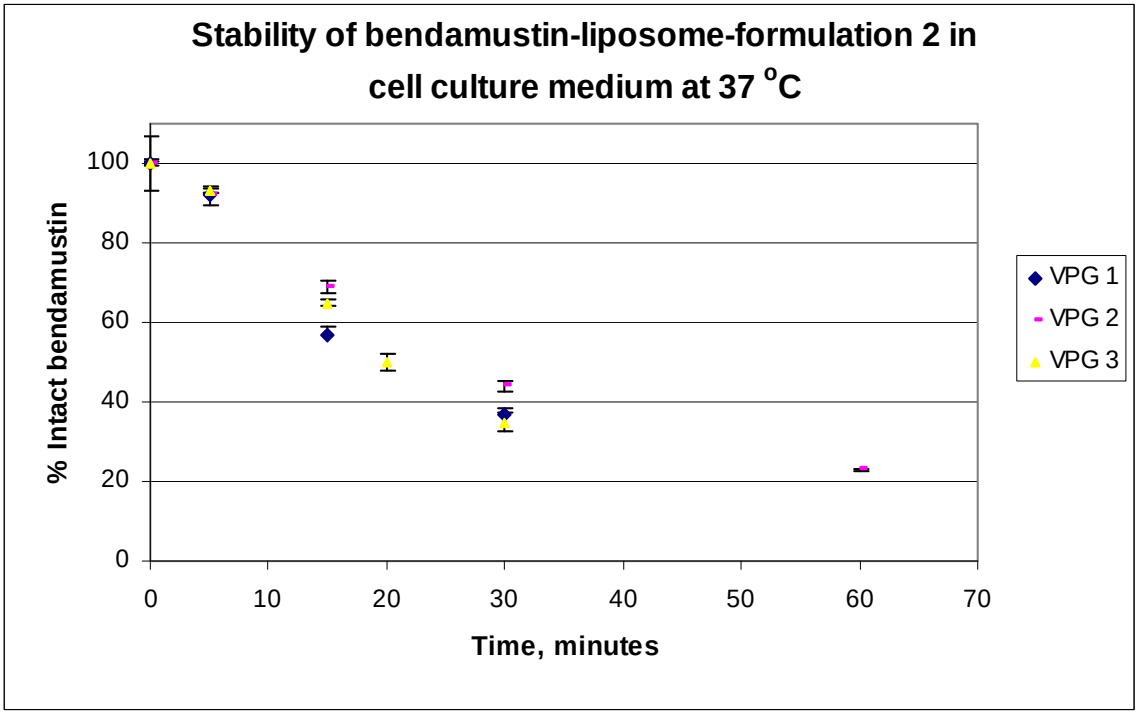


Figure 28: Remaining intact liposomal bendamustin in the new bendamustin-liposome formulation upon re-dispersion in cell culture medium pH 7.6 and incubation at 37 ºC over time.

Kinetics of liposome leakage does often follow a single exponential release profile, with most degradation within the first time period due to inactivation of external drug [41]. Even though this tendency is seen in figure 28, liposomal bendamustin was expected to have a prolonged release due to protection of the drug from the outer environment. However, as seen from the results the liposomal bendamustin was quickly degraded, indicating that the system is complex.

The stability of bendamustin in the liposome formulation is drastically decreased from what was seen upon incubation in PBS pH 7.4, where the half life was estimated to about 3 hours (See section 6.5.3). It is well known that liposomal leakage of drug in medium and plasma is increased from non-biological fluids, though the reasons for this are not fully fulfilled. Hence, an explanation for the rapid degradation of liposomal bendamustin might be a more permeable liposome membrane, resulting in increased loss of drug. However, stability, measured as the leakage rate, strongly depends upon the lipid composition. Presence of impurities, structural defects and rough surfaces are examples of factors making the surface easier to penetrate [41]. The lipid composition EPC3/CH used in the liposome formulation for

bendamustin is a well documented ideal mixture, shown to exhibit high mechanical stability for liposome preparations [41]. As mentioned in section 3.4 the presence of cholesterol increases the lateral packing in the liquid crystalline phase and eliminates phase boundaries in the fluid gel phase. In addition it maintains a smooth surface [41]. Thus, the lipid membrane in the bendamustin- liposomes should be quite stable.

Moreover, the volume used for re-dispersion and incubation in this experiment compared to the "PBS experiment" was the double, resulting in a higher concentration gradient between the inner and the outer aqueous phase of the liposomes; ratio about 1:15. However, this is probably only partly the reasons for the extremely rapid efflux of liposomal bendamustin.

The most obvious theory for the rapid degradation of liposomal bendamustin is the presence of proteins in the medium and opsonisation of the liposomes. Bendamustin is a strong plasma protein-binder; binding to the extent of 95 %, preferably to albumin[13]. Proteins, especially lipoproteins, might interact with the liposomes by simple charge-charge interactions and/or hydrophobic interactions, which in turn lead to protein adhesion or adsorption on the liposome surface or penetration into the liposome bilayer [41]. Bendamustin sitting in the lipid bilayer pointing out from the liposome surface, would be fast recognized and bound to the proteins in the medium. Proteins penetrating into the liposome bilayer might result in loss of both encapsulated and incorporated bendamustin in the membrane, as well as liposome disintegration might occur.

It is worth to mention that cell culture medium might represents a more liposomicidal environment than blood because of higher activity of proteins in cell-free solutions [41]. But then again, other components in blood and RES might contribute to liposome disintegration. Therefore, one should always be careful with extrapolating the results of in vitro experiments to an in vivo situation where the conditions are even more variable and complex. Moreover, it might be worthy to test the liposome-formulation in cell- assays and furthermore in vivo, to see if the efficiacy as well as toxicity of the bendamustin-liposomes in comparison to the free drug is improved.

6.7 Active loading of bendamustin

The results presented in section 6.5 have indicated that bendamustin in its zwitterionic form might have an increased lipophilicity making it capable of diffusing through the liposome membrane more readily than both the cationic and anionic forms. In that respect an active loading of bendamustin should be possible.

Bendamustin takes predominantly a zwitterionic form in the pH range between pH 4.5 and 6.3. The molecules in this pH-range are only sparingly soluble; calculated mass solubility in water at pH 4 = 0.86 g/L and at pH 6= 0.32 g/L [9]. This is of course a limitation, since active loading is dependent of concentration of drug in the outer aqueous phase. However, even with a low concentration of dissolved drug, the tendency of an active loading of drug should be seen if the zwitterionic molecules readily diffuse through the lipid membrane. At higher and lower pH, solubility increases (calculated mass solubility at pH 2.0 = 16 g/L and pH 8 = 9.3 g/L) [9]. Considering the fact that bendamustin exist in a charged form at all pHs, the reduction in solubility of bendamustin between pH 4 and 6, support the theory described under section 6.5; the drug exhibit, in the zwitterionic form, an increased lipophilicity due to a ring formation of the drug.

The active loading experiment was carried out according to section 5.4.3; “empty” VPGs with a high buffer-capacity pH 2.0 were prepared. The VPGs were subsequently re-dispersed with PBS solution pH 4.5 before bendamustin-solution was added drop-wise to the dispersion to hinder precipitation. The bendamustin would predominantly yield in a zwitterionic form in the outer aqueous phase, thus making it capable of diffusing into the liposome interior where it gets protonated due to the low pH, and in that order get retained.

As shown in table 19, the entrapped amount bendamustin was 14 ± 1%.

Table 19: Entrapped amount bendamustin, active loading experiment.

Parallels	**EE% (Mean and SD of internal triplicates)**
VPG 1	14.5 ± 0.3
VPG 2	12.7 ± 0.4
VPG 3	14.2 ± 0.6
Mean and SD	14 ± 1

The EE of 14 % observed in this experiment was lower than seen for other active loading processes such as for Doxorubicin [42].

Even though the concentration of bendamustin added to the liposome dispersion is quite low, the number of zwitterionic molecules per volume is higher than within the aqueous core of loaded lipoosmes due to pH. In this respect an influx of drug from the outer aqueous phase to the "empty" liposome interior should be just as fast as the other way around.

There will always be equilibrium of diffusion between the liposome interior and exterior. The net diffusion of drug is therefore dependent on a shift in equilibrium due to a concentration- and/or a pH gradient. In that respect one might assume that after a certain amount of bendamustin has diffused into the liposome interior, the buffer capacity could be exceeded and no longer sufficient. As a consequence the pH gradient is changed and the equilibrium of diffusion is shifted, resulting in no higher entrapped amount bendamustin within the liposomes.

However, all the parameters which might be critical for an active loading process, such as the highest bendamustin concentration possible without any precipitation, optimal buffer capacity, loading time and temperature, are not fully investigated. Especially is an active loading process often time-dependent, and one might assume that the loading time in this experiment was too short, leading in a suboptimal active loading. Optimum of the critical parameters important for obtaining a successful loading should be executed. Unfortunately, lack of time made it impossible to further investigate this topic.What can be concluded from these findings, however, is that bendamustin in its zwitterionic form is capable of diffusing through the liposome membrane to a certain extent.

7 CONCLUSIONS

The first step in this approach was to prepare bendamustin- liposomes by DAC according to a method developed by Cicko, and subsequently investigate leakage of drug out of the liposomes over time. The results from these experiments indicated that bendamustin in its zwitterionic form tended to diffuse through the liposome membrane more readily than both the cationic and anionic forms. This was assumed to be due to intra-molecular charge-charge interactions of bendamustin in its zwitterionic form, thus increasing its lipophilicity. Moreover, bendamustin showed to be unstable in its anionic form at pH; 7.4 due to hydrolysis.

A new bendamustin liposome formulation was developed. For this liposomes were prepared in a PBS pH 2.0, which should decrease the content of zwitterionic molecules able to leak out of the liposome interior. The new formulation showed an EE of 61 % ± 2.4, which was about 20 % higher than for the old formulation. Furthermore, efflux of the new formulation was compared to the old upon incubation in 150 mM PBS pH 7.4 at 37 °C over time. The results showed a prolonged half life of 3 hours in comparison to 1.5 hours, respectively. Based on these two positive effects, the new formulation showed a threefold increase of intact liposomal bendamustin over time.

Stability of the new formulation regarding degradation of bendamustin as well as lipids was investigated upon incubation at room temperature over 24 hours with subsequent assay on HPLC and HPTLC, respectively. 90 % intact bendamustin was left after about 20 hours, whilst a relatively fast hydrolysis of lipid was observed after 24 hours; 9 % lyso-PC. Bendamustin showed to increase the hydrolysis of phosphatidyl choline in the liposome membrane. This stated that the formulation should be prepared and used within the same day as a bed-side preparation. Unfortunately, upon incubation in cell culture medium at 37 °C, the liposomal bendamustin was quite rapidly degraded, and showed a minor increase in stability as compared to the free drug, t ½ ; 20 min and 14 min, respectively. This was assumed to be a result of opsonisation of the liposomes due to proteins in the medium, which in turn inactivated liposomal bendamustin. Finally, an attempt of an active loading for bendamustin was carried out by the means of a pH gradient between the liposome interior and exterior. Bendamustin was added to empty liposomes at a pH where the drug was in zwitterionic form, while the interior of the liposomes was acidic. An EE of 14 % was obtained. However, further investigation need to be done in order to see if optimization of the loading process is possible.

8 FUTURE PERSPECTIVES

At present time, further investigations of the new bendamustin-liposome formulation in cell culture assays and in vivo might not be relevant. The reason for this is the quite fast degradation of liposomal- bendamustin in cell culture medium, most probably due to adhesion and/or adsorption by proteins in the serum. However, a possible further approach might be to protect the liposomes from opsonisation by the means of membrane modifications. An alternative for this is coating the liposomes with a polymer such as polyethylene glycol, which might protect the liposomes from being opsonised and recognized by cells of the reticulo endothelial system, thus prolonging the half life of drug in serum.

Furthermore, it would be of interest to test if optimization of the active loading process of bendamustin is possible, giving a further increase in encapsulation efficiency of drug. For this, variations in the loading protocol such as concentration of bendamustin, optimal buffer-capacity and time of incubation should be investigated. Generation of an ammonium sulphate transmembrane gradient to the system might give precipitation of bendamustin in the liposome interior, which in turn might reduce leakage of drug out of the liposomes. Such a gradient has shown to be successful for active loading of Doxorubicin.

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10 APPENDICES

Appendix 1

Calculation percentage ionised bendamustin.

Henderson- Hasselbach equation:

pH= pKa + log ([A^-]/[HA]) or pH= pKb + log ([B^-]/[BH])

pKa values bendamustin;

pKa: 4.5

pKa: 6.3

Example

% ionisation pH 3 (aqueous core):

100/ (1+10^pKa-pH) = 100/ (1+10^ (4.5-3.0) = 3 %

3 % of the COOH group of bendamustin in the aqueous core is protonated, while 97 % is deprotonated.

100/ (1+10^pH-pKa) = 100/ (1+10^(3.0-6.3) = 99.95 %

99.95 % of the NH_2 groups of the bendamustin in the aqueous core are protonated,

0.05 % is neutral.

Approximately 3 % of bendamustin in the aqueous core is in a zwitterionic form at pH 3.0.

Appendix 2

Approaches of optimization of Ion exchange chromatography- Cholesterol values

Cholesterol values, first ion exchange method method used

Cholesterol values in the reference samples.

Parallels Reference samples	**Cholesterol µg/ml**
1	355.6
2	363.7
3	357.8
4	345.0
5	358.2
6	334.6
Mean	352.5
SD	10.7 or 3 %

Cholesterol values in eluates.

Parallels Eluates	**Cholesterol µg/ml**	**Cholesterol recovery %**
1	210.0	59.1
2	223.2	61.4
3	255.7	71.5
4	139.4	40.4
5	139.7	39.0
6	91.9	27.5
Mean	176.7	49.8
SD	62.4 or 35 %	16.7

Approach 1

Cholesterol values in references

Parallels Reference samples	**Cholesterol µg/ml**
1	351
2	342
3	378
Mean	357
SD	± 18.3 or 5 %

Cholesterol values in eluates.

Parallels Eluates	**Cholesterol µg/ml**	**Cholesterol recovery %**
1	281	79
2	266	75
3	218	61
4	244	68
5	300	84
6	255	71
7	300	84
Mean	266	74
SD	± 30.3 or 11 %	8.5

Approach 2

Cholesterol values samples

Parallels Samples-containing water	**Cholesterol µg/ml**
1	394
2	400
3	402
Mean	399
SD	± 4.2 or 1 %

Cholesterol values references

Parallels Samples- not containing water	**Cholesterol µg/ml**
1	396
2	400
3	411
Mean	402
SD	± 7.8 or 2%

Approach 3

Cholesterol values in references

Parallels Reference samples	**Cholesterol µl/ml**
1	376
2	364
3	372
Mean	371
SD	± 6.1 or 1.6 %

Cholesterol values in eluates

Parallels Eluates (Flushed five times with 250 µl distilled water)	**cholesterol µg/ml**	**Cholesterol recovery %**
1	305	82
2	316	85
3	330	89
4	317	85
5	331	89
Mean	320	86
SD	± 9.7 or 3 %	3