UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

ANDREA ŠETINA

MAGISTRSKA NALOGA

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Univerza v *Ljubljani*
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ANDREA ŠETINA

DEVELOPMENT OF AN IN VITRO DIALYSIS SYSTEM FOR BLOOD PURIFICATION OF AMIODARONE

RAZVOJ IN VITRO SISTEMA ZA HEMODIALIZO AMIODARONA

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»Eppur si muove.« (Galileo Galilei)

STATEMENT

I hereby state, that I have done this master's thesis on my own under the mentorship of doc. dr. Jurij Trontelj and dr. Fabrice Pirot, MCU-PH.

Andrea Šetina

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POVZETEK

Amiodaron je antiaritmično zdravilo, ki se pogosto uporablja v bolnišnicah. Spojina je zelo slabo topna v vodi, topnost pa je v parenteralnih farmacevtskih oblikah bistveno izboljšana z miceli polisorbata 80 (npr. »Amiodarone Bioniche«). Dejstvo, da je več kot 96% amiodarona vezano na plazemske proteine, se odraža v nepredvidljivi farmakokinetiki in ozkem terapevtskem oknu (1-2.5 mg/L), zaradi česar lahko pride do nevarnih povišanj koncentracij amiodarona in aktivnega metabolita desetilamiodarona. Učinkovina se presnavlja z jetrnimi encimi (predvsem CYP3A4), a se zelo počasi izloča zaradi porazdeljevanja v tkiva. Zdravilo lahko povzroča številne neželene učinke, med najbolj nevarne zaplete pa spadajo življenjsko ogrožajoče ventrikularne tahikardije, ki so posledica podaljšanega QT intervala. Akutna ali kronična toksičnost se kaže predvsem kot motnje vida, pljučne ali ščitnične funkcije ter okvara jeter. Protistrup žal ne obstaja, prav tako pa zaradi obsežne vezave na plazemske proteine ne pride v poštev hemodializa. Postopek hemodialize, s katero se zdravijo pacienti z okvarjeno ledvično funkcijo, v določenih primerih pa tudi zaradi zastrupitev, temelji na metodi ravnotežne dialize, novejši načini prečiščevanja krvi preko zunanjega krvnega obtoka pa so zasnovani na dializatu, ki vsebuje albumin. Raziskujejo se tudi možnosti uporabe dializata s ciklodekstrini.

Namen dela je bil razvoj in vitro sistema za hemodializo amiodarona, ki bi omogočil sproščanje učinkovine, ki je v velikem deležu (62.1%) vezana na albumin, ter posledično prehajanje prostih molekul preko membrane v dializno tekočino. Z uporabo ravnotežne dialize in analizo učinkovine v vzorcih z RP-HPLC/UV metodo želimo dokazati, da je afiniteta amiodarona do micelov polisorbata 80 večja kot do albumina, prav tako pa bomo preučili afiniteto omenjene učinkovine do parenteralne emulzije.

Eksperimentalno delo je torej temeljilo na postopku ravnotežne dialize, ki je potekal tako, da smo na eno stran celulozne membrane z velikostjo por 3500 Da postavili medij s čisto substanco ali micelarno obliko zdravila (»Amiodarone Bioniche«) s koncentracijo 50 mg/L, ali pa raztopino amiodarona vezanega na albumin (s koncentracijo 500 mg/L), na drugo stran pa 5% raztopino glukoze, 0.9% raztopino NaCl, raztopino polisorbata 80 s koncentracijo stokrat višjo od kritične micelarne koncentracije, ali pa parenteralno emulzijo (»Medialipide 2%«). Vsakič smo odvzeli po 0.5 mL vzorca, vse raztopine so bile zaščitene s folijo, saj na svetlobi pride do razpada amiodarona, dializa ob mešanju z magnetnim mešalom pa je potekala 3 oziroma 7 ur, pri konstantnem volumnu. Vzorčenje v

akceptorskem mediju je potekalo vsako uro (oziroma vsakih 20 minut v prvi uri), v donorskem pa le na začetku in koncu poskusa. Vzorce, ki so vsebovali albumin, smo pred HPLC analizo oborili z acetonitrilom in centrifugirali, vzorce z emulzijo pa smo razredčili z mobilno fazo in filtrirali. V nadaljevanju smo razvili nov in vitro sistem za dializo s črpalko in z dializatorjem, kjer je donorski medij predstavljala raztopina amiodarona v albuminu, receptorski medij pa dializna tekočina (»Diasol, 7158«). Uporabili smo membrano z večjo velikostjo por (35-40 kDa), ki pa še vedno ni presegala velikosti albumina. Analiza vzorcev je potekala z RP-HPLC/UV metodo, s C8 kolono Luna 150x4.60 mm ter z mobilno fazo iz 70% acetonitrila in 30% pufra (KH_2PO_4), s pretokom 1.5 mL/min, odziv pa smo merili pri valovnih dolžinah 210 in 240 nm. Za kvantifikacijo različno koncentriranih vzorcev smo uporabili dve umeritveni premici, ki sta zajemali koncentracijski območji od 0.5 do 25 in od 5 do 50 mg/L.

V primeru raztopine amiodarona v glukozi sta slaba topnost in nespecifična vezava povzročali preveč težav, da bi metodo uspešno validirali, zato smo na podlagi standardnih raztopin iz »Amiodarone Bioniche« (redčenje s 5% raztopino glukoze) določili in validirali na podlagi ICH smernic dve umeritveni premici, eno z višjim in eno z nižjim koncentracijskim območjem (odzivi merjeni pri 240 nm).

Iz dobljenih koncentracij smo izračunali maso in delež amiodarona, ki je prešel v akceptorski medij. Glede na to, da prehajanje membrane sledi kinetiki prvega reda, smo iz kinetičnega profila za vsak poskus določili konstanto dialize ter maksimalno količino učinkovine, ki je prečkala membrano.

a) Pri poskusu dialize amiodarona in micelarne oblike zdravila s 5% raztopino glukoze v akceptorskem mediju smo opazili, da akceptorske koncentracije po 3 urah dosežejo plato, čeprav se ravnotežje na podlagi določenih koncentracij na donorski in akceptorski strani navidez nikoli zares ne vzpostavi. Pri tem smo iz donorske raztopine uspeli očistiti 27.8% amiodarona (oziroma 24.3% amiodarona v primeru micelarne oblike). Prav tako smo pri poskusih z micelarno obliko amiodarona, pri kateri je na konstanto dialize in prehod preko membrane vplivala tudi micelarna kinetika in pri kateri je bolj do izraza prišel premik vode v donorski medij, zaznali manjše izgube kot s čisto spojino.

b) Pri poskusu dialize amiodarona vezanega na albumin z miceli polisorbata 80 v akceptorskem mediju smo ugotovili, da so akceptorske koncentracije skoraj dvakrat večje,

kot če uporabimo samo 5% raztopino glukoze, kar kaže na veliko afiniteto učinkovine do micelov. V glukozo je iz donorske raztopine prešlo 3.0%, v raztopino z miceli pa 9.1% učinkovine (zaradi slabe topnosti le-te poskusi v 0.9% raztopini NaCl niso bili uspešni).

c) Ko je bila v akceptorskem mediju namesto micelov emulzija, so akceptorske koncentracije presegle tiste z raztopino glukoze, a so bile še vedno manjše kot v poskusu z miceli polisorbata (v emulzijo je prešlo 4.4% učinkovine). Omenjene ugotovitve niso dovolj zanesljive, ker smo zaradi redčenja z mobilno fazo in posledične flokulacije izgubili precejšen delež oljne faze emulzije.

d) Tudi v novem in vitro dializnem sistemu s črpalko z običajnim dializnim akceptorskim medijem (Diasol) so bili rezultati podobni kot pri b). V dializno raztopino je po 80 minutah prešlo 10.5% amiodarona. Zaradi strojnih instrumentalnih težav s HPLC in omejenega časa nismo mogli izvesti dialize s polisorbatom 80.

Iz rezultatov izhaja, da je amiodaron izkazal določeno afiniteto do parenteralne emulzije in še večjo afiniteto do micelov polisorbata 80, zaradi česar se je učinkovina sprostila z albumina, prečkala membrano in se vključila v micele. Statistični testi (ANOVA) so pokazali ponovljivost poskusov, signifikantne razlike so se pojavile le pri dializi amiodarona s 5% raztopino glukoze. Ena izmed glavnih težav tekom poskusov je bilo to, da nismo uspeli dokazati ravnotežja med donorsko in akceptorsko raztopino na podlagi izmerjenih koncentracij na obeh straneh membrane, izgube oziroma slaba masna bilanca, v poskusih z albuminom pa je prihajalo do večanja koncentracije v donorski raztopini. V različnih poskusih smo opazili tudi precejšnje razlike med izmerjenimi koncentracijami v donorskih raztopinah, čeprav bi le-te morale biti približno enake.

Naša raziskava torej odkriva možnost novega sistema za hemodializo v primeru hude zastrupitve z amiodaronom, in sicer z dializno tekočino, ki vsebuje raztopino polisorbata 80 s koncentracijo višjo od kritične micelarne koncentracije. Ne glede na to pa bi v prihodnjih raziskavah zagotovo morali poskusiti izboljšati občutljivost analizne metode, da bi lahko uporabili relevantno oziroma pričakovano koncentracijsko območje pri predoziranju. Nadalje, poskus bi morali izvesti s krvjo ali vsaj s krvno plazmo in optimalizirati ostale pogoje dialize, kot so npr. pretok, membrane in volumni.

Ključne besede: amiodaron, dializa, vezava na proteine, hemodializa, polisorbat 80, miceli, HPLC

ABSTRACT

Amiodarone is an antiarrhythmic agent, frequently used in hospitalized patients, and since more than 96% of the drug binds to the plasma proteins, the following unpredictable pharmacokinetics and a narrow therapeutic index (1-2.5 mg/L) sometimes result in severe cases of intoxication. However, there is neither an antidote nor a possibility of dialysis treatment due to the protein binding of the drug. Donor compartment of the applied dialysis study contained amiodarone (pure substance), a generic micellar form of the drug (Amiodarone Bioniche) or an amiodarone albumin solution, while the receptor compartment contained glucose 5% solution, NaCl 0.9% solution, polysorbate 80 solution at a concentration a hundredfold higher than its critical micellar concentration, or a parenteral emulsion. The compartments were separated by a regenerated cellulose dialysis membrane with a low molecular weight cut-off (3500 Da). Further on, we developed a different in vitro study with a dialysis system consisting of a pump and a hemodialysis column with larger pore size (35-40 kDa). The samples were submitted to RP-HPLC/UV analysis, with a mobile phase consisting of 70% acetonitrile and 30% of potassium monophosphate buffer solution. Due to solubility problem and non-specific absorption, two calibration curves for Amiodarone Bioniche were established, covering the concentration range from 0.5 to 50 mg/L. According to the results of the study, amiodarone showed some affinity towards the intravenous lipid emulsion and, most importantly, its high affinity towards the micelles of polysorbate 80, resulting in the release of the drug bound to albumin, passing the dialysis membrane and binding to the micelles. However, glucose 5% solution appeared to be a better choice of medium than NaCl 0.9% solution, due to the low solubility of amiodarone. Therefore our study implies a possibility of a new dialysis method by using a dialysis solution containing polysorbate 80 at a concentration higher than its critical micellar concentration for blood purification of amiodarone in case of a severe intoxication.

Key words: amiodarone, dialysis, protein binding, hemodialysis, polysorbate 80, micelles, HPLC

ABBREVIATIONS

ACE	angiotensin-converting-enzyme
ALT	alanine transaminase
ALP	alkaline phosphatase
AM	amiodarone
AST	aspartate transaminase
ATP	adenosine triphosphate
AV node	atrioventricular node
CMC	critical micellar concentration
CRP	C-reactive protein
CV	coefficient of variation
CYP	cytochrome enzymes
DEA	desethylamiodarone
DEHP	di-2-ethylhexyl phthalate
ECG	electrocardiogram
EMA	European Medicines Agency
EMR	electron magnetic resonance
FDA	Food and Drug Administration
FEV	forced expiratory volume
GC	gas chromatography
GGT	gamma-glutamyl transpeptidase
HDL	high-density lipoprotein

HPLC	high-performance liquid chromatography
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IL-6	interleukin 6
IR	infrared
I.V.	intravenous
LC	liquid chromatography
LD_{50}	median lethal dose
LDL	low-density lipoprotein
LOD	limit of detection
LOQ	limit of quantification
logP	partition coefficient
MARS	molecular adsorbent and recirculating system
Mr	relative molecular mass [g/mol]
MS	mass spectrometry
MWCO	molecular weight cut-off
NMR	nuclear magnetic resonance
pKa	dissociation constant
PTFE	polytetrafluoroethylene
ROS	reactive oxygen species
RP-HPLC	reversed phase high-performance liquid chromatography
T_3	triiodothyronine

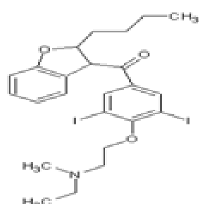
T_4	thyroxine
Th1, Th2	lymphocyte T-helper cells
TLC	thin-layer chromatography
TSH	thyroid-stimulating hormone
UV	ultraviolet

1 INTRODUCTION

1.1 AMIODARONE

1.1.1 Heart and dysrhythmias

AM is a potent agent for the treatment of severe arrhythmias, with various direct effects on cardiac cell system, which is controlled by noradrenaline and acetylcholine binding to the respective receptors. Action potential travelling through heart conducting system consists of five phases: fast depolarisation by opening of Na^{+} and Ca^{2+} channels, partial repolarisation by opening of K^{+} channels and plateau are followed by repolarisation and pacemaker phase. Myocardium contraction takes place after Ca^{2+} enters the cells and binds to troponin C, causing the cross-bridging of myosin to actin. Cardiac disrhythmias are a serious medical condition, appearing because of delayed after-depolarisation, re-entry of ions due to partial conduction block, ectopic pacemaker activity or heart block, and are divided by the site of origin (atrial or supraventricular, and ventricular) and by heart rate (tachycardia in case the heart rate is increased and bradycardia when it is decreased). Based on electrophysiological effects, antidysrhytmic drugs are divided into four classes, as proposed by Vaughan and Williams in 1970. [1]



Picture 1: Amiodarone.

AM is classified as Class III antidysrhythmic, but it shows the characteristics of all four classes. AM was discovered in 1962 and was at first intended to be used for its anti-anginal properties, as reported by Charlier et al. in 1968. [1, 2, 3] As a Class III antiarrhythmic, it prolongs heart action potential and thus slows the conduction, which can be noticed as a prolonged QT interval on ECG, as it prolongs repolarisation and refractory period by blocking some of the K^{+} channels. It also blocks voltage-sensitive Na^{+} channels and decreases maximal rate of depolarisation like Class I agents (disopyramide, lidocaine, flecainide). Moreover, it shows beta-adrenoreceptor antagonistic activity like Class II antiarrhythmics (propranolol). In addition, it shows negative chronotropic effects, similar

as Class IV drugs (phenylalkylamine type verapamil), through blocking of L-type voltage-sensitive Ca^{2+} channels, and negative dromotropic effects in the sinus and AV node, representing the heart conducting system, by blocking of Ca^{2+} and K^{+} channels, resulting in the slowing of conduction. On top of that, AM decreasing of ATP utilization through the inhibition of Na^{+}- and K^{+}-activated ATPase results in vasodilatory action and subsequently decreased cardiac workload and myocardial oxygen comsumption. [1, 4] In clinical practice AM is used on a regular basis to handle supraventricular and ventricular tachycardias, including tachycardia associated with Wolff-Parkinson-White syndrome, hemodynamically unstable ventricular tachycardia, life-threatening recurrent ventricular fibrillation, atrial flutter or atrial fibrillation, and it also forms a part of chronic treatment for patients with implanted defibrillators or pacemakers. [1, 4, 5] Unlike class I and III antiarrhythmic drugs, AM does not carry the risk of increased mortality caused by a proarrhythmic effect, including in patients with heart failure. [1, 6] However, its effectiveness has been somewhat overshadowed by serious adverse effects, especially since there are few alternative treatment options – dronedarone with a similar benzofuran structure or sotalol as a non-selective beta antagonist. [1]

1.1.2 Clinical use and pharmacokinetics

Due to life-threatening nature of dysrhythmias, AM therapy should be initiated in hospital with acces to monitoring facilities, when patient's health condition could not be improved by other antiarrhythmics. While almost instantaneous antiarrhythmic action is expected if patient is submitted to I.V. treatment, therapeutic response after oral administration is usually noticed in a extended period of one to three weeks, or sooner with administration of loading doses. Therapeutic response shoud be carefully monitored when changing from I.V. to oral administration and in drug transfer from one antiarrhythmic to AM or vice versa, usually requiring a dose reduction. Effectiveness of AM treatment in a patient without any arrhythmic manifestation on ECG is assessed by exercise or programmed electrical stimulation (PES). [4, 5]

Pharmacokinetic profile of AM is non-linear with poorly understood relationship between drug concentration and therapeutic response. Due to slow and variable absorption, peak serum concentration is not achieved before 3 to 12 hours after oral administration, with bioavailability as a consequence of first-pass metabolism varying from 33 to 65%. [4] Since AM is a lipophilic substance and a weak base, absorption takes place mostly in the

lower gastrointestinal tract where it remains nonionized in a greater extent due to higher pH value, is facilitated by bile secretion and affected by food. AM being a small lipophilic molecule with a polar region that can interact with hydrophilic moieties of membrane integral proteins can readily pass most of the membrane lipid bilayers by passive diffusion. [7] AM is metabolized to the major active metabolite DEA mostly by CYP3A4 in the gut wall and liver. Genetic polymorphism and interindividual variability in this enzyme activity could be the reason for highly variable bioavailability. [4] Once in blood, more than 96% of AM binds to plasma proteins, but it also willingly passes capillary cell walls and enters extravascular compartments, resulting in accumulating in numerous highly perfused organs like heart, lung, kidney, liver and spleen, and in fatty tissues, exhibiting two-phase elimination profile, with a quicker initial elimination from well-perfused tissues, followed by a terminal phase with a very slow elimination from poorly perfused tissue compartments. [4, 7] Concentration at the site of the action could not be linearly related to free drug concentration in plasma and a temporal delay of 11 minutes between vascular concentration and effect has been reported, most likely due to the transport to the site of action or due to postreceptor events. [3] The mean terminal half-life after steady-state being attained has been reported to be approximately 53 days, and for the main metabolite DEA it was even longer. [4] Moreover, it has been proposed that the active metabolite, reaching very high myocardial levels, can increase ventricular fibrillation treshold by a greater amount than its parent drug. [8] Four half-lives are usually deemed necessary to approach steady state (or sooner with loading doses), with therapeutic drug level ranging from 1 to 2.5 mg/L, with plasma ratio of metabolite to parent drug after chronic treatment being around 1:1. The concentrations above 2.5 mg/L are already deemed toxic, possibly resulting in life-threatening dysrhythmias. Both, AM and DEA, are eliminated by liver and bile, are excreted mainly in faeces, and they both also cross the placenta and appear in breast milk, limiting use in pregnant or nursing women. [4] After a single I.V. infusion of AM, serum levels decline quickly after the end of the infusion, but secondary peaks can appear on I.V. concentration curves, most probably due to enterohepatic circulation and excretion into the bile. [4, 9]

1.1.3 Drug interactions and adverse effects

Since AM is a substrate of CYP3A4 and CYP2C8, an inhibitor of CYP3A4, CYP2C9, CYP2D6 and the P-glycoprotein multidrug efflux transporter, various drug interactions

affecting the respective plasma levels can take place. [4, 7] In addition, diarrhea, hypokalemia and hypomagnesemia can lead to a greater chance of arrhythmic events. [4, 5] Adverse reactions of AM are neither dose-dependent nor easily predicted, but can be so severe that sometimes even a discontinuation of drug might be required. Most common adverse effects are neurological (peripheral neutropathy, tremor), pulmonary (decreased diffusion capacity) and gastrointestinal disturbances (frequent at high loading doses), followed by hepatic changes, dermatologic disorders (photosensitivity, blue-grey discolouration of skin), visual abnormalities (corneal microdeposits) and thyroid dysfunction. Cardiovascular events represent a great deal of concern due to possible occurrence of torsade de pointes, a life-threatening polymorphic form of ventricular tachycardia, which can be triggered by concomitant use of drugs prolonging QT interval. [5] Additionally, hypotension, bradycardia, fever, thrombocytopenia, and cardiac arrest can appear upon I.V. administration, but slower rate of infusion can prove favourable in diminishing hypotension. Moreover, injection site reactions were reported to cause pain, erythema and phlebitis. [4, 5] Last but not least, considering a very long half-life of AM, it is of great significance to take in account that adverse effects and potential for drug interactions persevere as long as several months after withdrawal of AM. [4] What is more, due to the accumulation of iodine-containing metabolites, patients can remain exposed to iodine up to two years after drug discontinuation. [10] Nevertheless, risk of sudden death should be also considered when thinking of withdrawal of AM. [5]

1.1.4 Toxicity

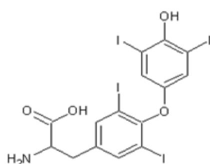
Eventhough very high doses have been determined to cause acute toxicity (acute oral LD_{50} in mice and rats is greater than 3000 mg/kg and acute I.V. LD_{50} is 150 mg/kg), AM can develop severe toxic effects during chronic treatment. In addition, AM embriotoxicity with infrequent congenital thyroid disorders and delayed ossification has been established in animal studies, while mutagenicity and teratogenicity studies were negative. However, direct connection between toxicity and dosage or treatment duration of AM is not well-established. Moreover, AM half-life cannot be accurately determined due to zero order elimination, as typically observed in case of intoxications, and non-linear pharmacokinetic profile. [4, 7] Toxicity of AM affects several organs, but since there is no antidote or a possibility of dialysis due to protein binding of the drug, an intoxicated patient can be submitted only to symptomatic and supportive treatment. [4] While kidney, liver and

gastrointestinal tract are somewhat protected by their reserve functional capacity, heart is especially vulnerable to the toxic effects of foreign compounds due to decreased levels of superoxide dismutase and catalase which normally form a part of cellular protective mechanisms. Particularly influenced by harmful effects of AM are well-perfused organs like heart, lungs and liver, thyroid gland for its similarity to the endogenous hormone, and eye's retina, most probably due to phospholipidosis, as described below. [4, 7]

Phospholipidosis is one of the possible toxic responses to foreign compounds, taking place in cells with high cell membrane turnover, such as macrophages and organs and tissues with high synthesis of lipids, such as retina. [7] It has been deemed to be caused by cationic amphiphilic drugs with an amine group forming a part of the cationic side chain. As soon as AM enters the acidic medium of lysosomes by passive diffusion, being a weak base it becomes ionized and remains entrapped without the ability to pass the lysosomal membrane. As the proposed mechanism for AM suggests, the antiarrhythmic induces phospholipidosis by the inhibition of lysosomal phospholipase A2 through the insertion into the inner lysosomal membrane. [11] Similar phenomenon of lysosomal overloading has already been described for aminoglycoside antibiotics exerting basic activity. [12] Accumulation of AM in lysosomes eventually results in disrupted catabolism of phospholipids and the occurrence of myeloid bodies in cytoplasm (corneal microdeposits or foamy alveolar macrophages in pulmonary phospholipidosis). [11]

Although pulmonary toxicity is rather rare in incidence, it can result in acute as well as chronic toxicity, assessed by lung screening or oxygen levels and FEV measurements. Pulmonary damage treatment is approached by corticosteroids like prednisone. Risk factors are high cumulative or daily dose, duration of therapy, age, male gender, pre-existing lung or renal disease, and high concentration of inspired oxygen in surgery. [13] It has been shown that apoptotic effects of AM and DEA on alveolar epithelial cells could be impeded by angiotensin receptor blockers or ACE inhibitors. [14] Hypersensitivity pneumonitis demands immediate withdrawal of AM. It emerges earlier in the course of therapy and it exhibits CD8+ lymphocytosis. On the other hand, oxygen radicals formation and phospholipidosis are distinctive of interstitial or alveolar pneumonitis, where dose reduction of AM may be discussed before drug discontinuation, and it seems that formation of lipid or drug radicals eventually leading to fibrosis could be substantially decreased by the antioxidative effect of alpha-tocopherole. [4, 15]

Thyroid toxicity arises from the structual similarity of AM with the endogenous thyroid hormone T_4, which likewise holds two iodine atoms. The thyroid reacts with acute inhibition of iodine organification as AM inhibits type 1 deiodinase, which converts the precursor T_4 into T_3, and due to this so-called Wolff–Chaikoff effect, T_4 and T_3 production rates decrease, while TSH consequently increases. This leads to altered iodine levels, rise in cholesterol levels, fall in gene expression of LDL receptor in liver, thyroid effects of AM on heart like bradycardia and DEA impact on the expression of T_3-dependent genes. AM-induced hypothyroidism emerges preferentially in iodine-sufficient areas and in females with autoimmune (Hashimoto's) thyroiditis, resulting in preserved radioiodine uptake. After discontinuation patients are treated with potassium perchlorate. [2]



Picture 2: Thyroid hormone (T_4).

On the other side, AM-induced thyrotoxicosis occurs preferentially in iodine-deficient regions and in males, and it is distinctive for low TSH and high T_4 levels, with low or absent radioiodine uptake. It can be classified as type 1 iodide-induced thyrotoxicosis, best treated by potassium perchlorate in combination with thionamides, while type 2 or destructive thyrotoxicosis is best treated by prednisone – discontinuation of AM may not be necessary. [6] A possible alternative to AM antiarrhythmic treatment is dronedarone, because it shows no thyroid effects. [2]

Hepatic damage caused by phospholipidosis and fibrosis can result in fulminant hepatitis or cirrhotic liver disease and can be assessed by up to three-fold increase in ALT, AST and GGT levels. Higher levels are reached also by total bilirubin and ALP. [4, 16]

What is more, severe side effects were reported to develop in paediatric population with severe bradycardia and hypotension arising from the overdose during an I.V. administration of micellar formulation, which has been attributed to potent vasodilator and negative inotropic effect of polysorbate 80 and poor benzyl alcohol metabolic pathway development in newborns, resulting in the metabolite (benzoic acid) accumulation. [17] Recent studies examine a possibility of AM-induced mitochondrial dysfunction and ROS

production, antithrombotic action of AM and its potential inhibition of protein expression for K^+ channels. [18, 19]

In order to avoid toxic outcomes, regular monitoring includes ECG, control over QT interval prolongation and electrolyte disturbances, pulmonary, liver and thyroid function tests and ophtalmologic evaluation, up to one year after drug withdrawal. [14, 20] New methods of assessing toxic responses are being deployed, for instance physical condition evaluation or measuring of Th1/Th2 ratio – an immunological parameter linked to collagen production and fibrosis. [21, 22] Fortunately, cases of AM intoxication are not very often in clinical practice. According to the Poison Control Centre, between 2012 and 2013 only one such patient has been treated in the University Medical Centre Ljubljana. Following concomitant AM and digoxin therapy, the patient experienced severe bradycardia.

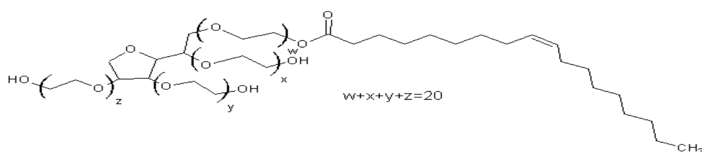
1.1.5 Formulation

Salt selection is an important step in drug formulation process for reaching a higher dissolution rate and a better solubility, subsequently decreasing the need of high-dose drug application. Since AM has a basic centre that can form salts with acids, AM hydrochloride was formulated. Hydrochloride is a common salt choice although reports have shown it does not always increase solubility due to common-ion effect of chloride. Moreover, this salt shows low pH for aqueous solutions and high hygroscopicity. [23] Chemical name of AM hydrochloride is (2-butyl-3-benzofuranyl) [4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl]methanone hydrochloride with a molecular formula $C_{25}H_{29}I_2NO_3$,HCl and CAS number 199774-82-4. This amphiphilic molecule consists of a lipophilic benzofurane heterocycle with two heavy iodine atoms bound to a phenyl group which carries a short hydrocarbon chain with a polar terciar amine group and has a high Mr (682 g/mol). Because the substance is sensitive to photodegradation, it should be kept protected from light, at the temperature below 30 °C. [24] Low solubility in water, determined to be 0.25 mg/mL, as in contrast to solubility in ethanol 96% of 30 mg/mL, presents a considerable challenge for a suitable formulation formation providing a suitable solubility in body fluids. The value of apparent pKa is 6.56 and logP 5.95, demonstrating its high lipophilicity. Maximal lipid solubility is allegedly reached between pH 3.5 and 5.5 and since absorption of basic substances is greater in duodenum and jejunum, gastric emptying time and changes of pH are critical factors for absorption. [4, 25, 26] Due to low solubility and high permeability, AM belongs to Class 2 drugs by Biopharmaceutical Classification

System, where the rate limiting step is not the absorption, but the release from the dosage form and solubility in gastric fluid. [27]

Several AM preparations are available, including tablets and I.V. infusion. [24] AM Bioniche is a generic micellar formulation for I.V. application. Concentrates for injections or infusions are sterile solutions intended for injection or infusion after dilution and they provide sterility, isotonicity, appropriate pH of solution, solubility and adequate antimicrobial properties of the final product. [28] The following excipients form a part of this I.V. solution: polysorbate 80 (E433), benzyl alcohol and water for injections. Polysorbate 80 as a non-ionic surfactant enhances the solubility, while benzyl alcohol is used as a conservans. [29, 30] The solution pH value is 3.5-4.5 and should not be refrigerated or freezed. Despite some reports claiming stability in NaCl 0.9% solution, AM micellar formulation remains deemed incompatible with saline solution and it may be administered only in a 5% w/v glucose I.V. infusion. [5, 31] Chemical and physical in-use stability has been demonstrated for 24 hours at room temperature. [5] Micellar formulation minimizes precipitation of AM, otherwise leading to prolonged contact of the drug with the vein wall cells, phlebitis and pain. Those inconveniences can be likewise avoided with rapid dilution of the formulation by slow and deep vein injection, producing a lower effective concentration at the site of injection. [32]

Polysorbate 80 or Tween 80 is a monooleate polyoxyethylene derivate (sorbitan mono-9-octadecenoate), a nonionic detergent with Mr of 1310 g/mol, easily soluble in water. The surfactant has a low CMC (0.012 mM), so it can easily form large micelles, by the aggregation of 60 molecules and with Mr amounting to 76000 g/mol. It is compatible with most materials, except DEHP. [33] Tween 80 is metabolized by blood esterases, exerts enhanced LDL endocytosis and is recognized as P-glycoprotein inhibitor. [34, 35] Nonetheless, it shows potential acute health effects at high doses, observed as cardiac changes (most commonly hypotension). [17]



Picture 3: Polysorbate 80.

Colloidal properties of the mere AM substance have been thoroughly researched and have yielded a relatively high CMC of 0.5 mg/ml. The reason lies in amphiphilic properties of AM alone, which shows surfactant properties and which, in sufficiently high drug concentrations, is capable of creating bilayer micelle formations, exerting a certain order in water solutions and thus forming liquid crystals. Formation of aggregates in certain order leads to increased viscosity properties and transitions between different liquid and solid states. Micellization is an entropy-driven process, caused by the liberation of water molecules of the AM backbone, and can be induced by the presence of impurities. The described phenomenon of liquid crystals formation is seen also in other amphiphilic structures such as cell membrane bilayers. [36, 37]

1.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Several methods of identification of AM assesed from pharmaceutical formulations or biological specimens are known, among them IR and UV spectroscopy, high performance TLC, NMR, EMR, titrations (for pure form analysis), GC and coupled methods like HPLC/UV or HPLC/MS. [26] Nevertheless, LC-MS is the method used to determine AM serum levels in many medical laboratories. [38]

HPLC is a well-known accurate and precise analytical method recognized by Pharmacopoeia. [39] In RP-HPLC the analysed substance is eluted at a certain retention time, determined by kinetic and thermodynamic distribution between non-polar stationary phase and mobile phase, which is often mixed with water or buffer solution in order to control the ionization of weak acids or weak bases like AM. AM has shown notably improved solubility in organic solvents such as acetonitrile (0.31889 g / 100 mL), compatible with RP-HPLC. Knowing that weak bases are soluble when ionized, or when pH is lower than pKa value, and taking in account that the determined pKa of AM is 6.56, it is estimated acidic pH is optimal for sample preparation of biological specimens by protein precipitation. Maximum absorption band of AM and its main metabolite DEA is at 240 nm. [40] However, bathochromic shift was observed from pH 6.0 to higher values. [25] To avoid any peak changes, the solvent has to be the same in the sample as in the standard solutions, so the samples are preferably diluted in mobile phase. Moreover, the concentration range should be carefully chosen to avoid dilution or concentration of samples, leading to more errors. [23] Sample concentrations are determined from the

calibration curve, obtainted from standard solutions with known concentrations of AM, which has to be validated and comply either with Pharmacopoeia, FDA, EMA or the ICH Guidelines, including specificity, linearity, range, accuracy, precision, LOD and LOQ. [41]

Since pharmaceutical formulations cannot be injected directly, sample preparation is an important step in HPLC analysis. In the first place it is essential to assure a good solution of the solute in the selected solvent, using mechanical stirring or an ultrasonic bath, but more complex samples containing proteins have to be adequately treated, for example with organic solvent like acetonitrile, causing protein precipitation. Following centrifugation, the free drug concentration is assesed from the supernatant. Since multiple excipients like emulsifiers and co-emulsifiers can result in interferences and lipid compounds can damage the column, it is necessary to break the emulsion before injection, for example with liquid-liquid extraction. Decomposition of AM is allegedly enhanced by shaking and sample stability appears problematic since aqueous solutions of 5 mg/L have been reported to decrease in concentration of 20% after 12 minutes standing at 20 °C. [26]

1.3 DIALYSIS

Equilibrium dialysis is a widespread experimental technique, commonly used for the separation of two compounds, the study of interactions between molecules and drug release or protein binding studies. [42] It consists of two chambers, divided by a semipermeable membrane with a selected MWCO which limits molecular passage. According to Fick's first diffusion law, the rate at which equilibrium is reached is governed by the difference in concentration of the observed compounds at both sides of the membrane. The dialysis membrane is the rate-limiting step of this first order diffusion process, which can be accellerated by higher concentration gradient or larger pore size. Tipically, plasma containing the drug is on one side of the membrane called also the donor compartment, while the dialysate or so called receptor compartment containing the buffer is placed in the other dialysis chamber. Even if the volume of receptor compartment is smaller than the donor, best effort is made to assure sink conditions in both compartments so there is no back diffusion. [43] However, these experiments can be quite time consuming and subsequently it is necessary to consider drug degradation, changes in pH and bacterial growth. Besides, some proteins could result damaged in view of lasting agitation stress. Supposing the equilibrium has been reached, the measurements should not

be largely affected by non-specific adsorption. Protein binding studies constantly face variations in the unbound drug fraction due to volume shift, because osmotic pressure of the plasma proteins causes the flow of fluid from the buffer side to the plasma side. Since dilution is not always the same, plasma might change its binding properties in an unpredictable way, but volume shift might be evaded by the addition of dextran, diminishing the difference in osmotic pressure. [44, 45] Furthermore, maintaining an appropriate pH value prevents the precipitation of poorly soluble drugs like AM, which could lead to overestimation of bound fraction. [46] The Gibbs–Donnan effect results in the flow of small ions to the plasma compartment in order to achieve electroneutrality, as charged proteins are too big to pass the membrane. Instead of being limited to unbound drug fraction measurements, some new variations of dialysis, like comparative equilibrium dialysis where the receptor phase contains plasma from other species instead of a buffer, are being developped with the intention of measuring the total drug concentration. To avoid the numerous drawbacks of dialysis, another alternative can be approached when determining protein binding, for example gel filtration, ultracentrifugation or a rapid technique of ultrafiltration. [44, 45]

1.4 HEMODIALYSIS

Hemodialysis is a blood purification method, where patient's blood flow is connected to an extracorporeal blood circuit through a (usually intrajugular) veno-venous access in form of a arteriovenous graft, fistula or catheter, in order to replace impaired renal function determined by severely decreased glomerular filtration rate. [47, 48] Conventionally it is used for subjects suffering from chronic kidney disease and inability to excrete toxic metabolites or regulate blood electrolyte levels. Short daily or long nocturnal hemodialysis can be conducted in home or hospital environment, with unfractioned heparine added during the treatment to avoid the risk of blood coagulation. [47] Unlike protein-bound compounds, small water soluble molecules like urea or creatinine and ions from blood can pass across the selective semipermeable dialysis membrane by diffusion concentration gradient, which is increased by different flow rate of blood and dialysate (for instance 300 and 500 mL/min), dialysate running in countercurrent direction, and biocompatible synthetic, cellulosynthetic or substituted cellulose membranes with different permeability of solutes, depending on membrane thickness, surface and MWCO reflecting pore size. [47, 49, 50] There is a vast choice of different (often polysulfonate or polyacrylonitrile)

membranes, varying from high-efficiency membranes with higher clearances of small molecules to high-flux membranes with high porosity and water permeability, as required for hemofiltration. Since larger compounds like beta2-microglobuline can cross the high-performance membranes with higher MWCO, selectivity has to be maximized in order to avoid albumin losses. Ultrafiltration provides better clearances than diffusion alone, but it can be accompanied by retrofiltration, which can be circumvented by an adequate I.V. volume replacement either before or after dialysis, at the same time presenting certain risks like less efficient treatment and thrombosis. [47, 50] A sterile ultrapure bicarbonate-based dialysate frequently comes in form of a concentrate which needs to be diluted with sodium hydrogen carbonate solution, directly before treatment or in-line, and contains Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, acetate, bicarbonate and glucose, but since dialysates slightly differ in composition, attention should be given particularly to K^+ concentration. [47, 50, 51] There are several variations of hemodialysis, namely conventional hemodialysis, hemofiltration for elimination of even larger molecules, combinated hemodiafiltration or peritoneal dialysis, which is particulary interesting because dialysis membrane is replaced by a serosal membrane peritoneum, while hyperosmolar dialysate, enabling both diffusion and ultrafiltration, is injected in peritoneal cavity. Another possibility is the method of fresh congelated plasma exchange called plasmapheresis, conducted mostly for emergency indications like an acute digoxin poisoning. [47]

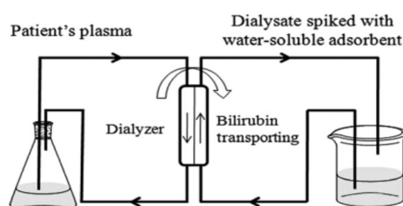
Oral post-dialysis therapy usually includes vitamin supplementation (water-soluble vitamin C and B-group), iron and erythropoietin injections for anemia. [47, 52] Complications to be avoided are electrolyte disturbances and metabolic acidosis or alkalosis, bone disease, thrombocytopenia, vascular calcification, bleeding and infections. Hemodynamic instability and hypotension can appear between two intermittent treatments, particularly due to ultrafiltration-stimulated hypovolemia. [47] Signs of inflammation, like an elevated CRP or IL-6 level and a decreased albumine concentration, should be treated carefully, since they have been connected to higher mortality in patients with pre-existing pathology. [47, 53] All the materials must be sterile, the fistula needles are siliconised to avoid blood coagulation and maximal care should be taken to prevent endotoxin uptake, incuding disinfection with NaOCl solution. [51] Overnight dialysis three times a week (lasting for 8-10 hours) or short daily dialysis six times a week (lasting 2-3 hours) are considered as the

most comfortable choice, with the least adverse events and less post-dialysis fatigue. [47, 50]

1.4.1 Blood purification systems

Several blood purification systems have been developped for detoxification and elimination of toxic albumin-bound substances like bilirubin, bile salts, ammonium and free radicals in acute hepatic insuficiency, acute-on-chronic or fulminant hepatic failure, leading to further complications like hepatic encephalopathy, acute renal failure and multiple-organ failure. Biological or hybrid bio-artificial devices with immortilised hepatocytes in bioreactors mimic not only the excretory but also the synthetic function of liver, while non-biological extracorporeal systems like charcoal hemodiadsorption or MARS provide only blood purification, subsequently representing a bridge to a required liver transplantation and thus leading to a lower mortality rate. [54] Molecular adsorbent and recirculating system (MARS) connects three circles – blood, albumin and dialysate compartment. Blood water soluble and albumin-bound molecules below 50 kDa can pass across the high-flux polysulfone membrane into the albumin compartment, which is followed by albumin entering the third circle, allowing small molecules to exchange with the bicarbonate dialysate through a low-flux polysulfone membrane, like in conventional hemodialysis, and before it is recirculated, albumin regenerates on a charcoal column and an anionic exchange resin column. The treatment duration was empirically estimated to 6-8 hours. Although it can be of great use even after liver transplantation in case of graft dysfunction or severe pruritus due to the accumulation of bile salts, the main drawbacks are high cost, microbial risk in albumin medium and discarding the kit after use. [54, 55, 56] In Prometheus system with even higher clearances than MARS, blood first passes across albumin-permeable membrane into the albumin compartment and then across the high-flux membrane into the dialysate compartment, while albumin regenerates like previously described. [57] Single pass albumin dialysis (SPAD) is also based on countercurrent albumin dialysis, only that the albumin-containing dialysate is discarded after passing the high-flux hemodiafilter, contributing to higher cost. [54] A new, bilirubin-removing in vitro system has been reported by applying a cyclodextrin-based dialysate. Beta-cyclodextrin as a complexing agent, with the ability to capture lipophilic drugs into its hydrophobic internal cavity, was attached to polyethylenimine matrix with a covalent bond, forming a water-soluble large polymer, while the low MWCO of the triacetate

cellulose membrane prevented adsorbent leaking into blood. It has been shown that cyclodextrin binds more dialysate than albumin, due to strong interaction with bilirubin, and on top of that it offers a lower cost for continuous replacing of dialysate. [58] This innovative approach opened the research field in blood purification techniques to new ideas.



Picture 4: Cyclodextrin-based dialysis system. [58]

1.4.2 Micelles and emulsion as part of dialysis solution

Following the concept of cyclodextrin blood purification system, it appears appealing to study different colloidal drug carriers for potential use in hemodialysis.

Micelles are thermodynamically stable association colloids which can – under certain conditions – form spontaneously through a balance of different intermolecular forces and are normally intended to control wetting, stability, bioavailability or achieve a better targeting for ligand-specific drugs. For instance, AM micellar formulation is already well-established in clinical practice and a micellar formulation for paclitaxel I.V. infusions has been studied for a long time. [59, 60] The low milimolar CMC value of polysorbate 80 suggests nanoscopic micelles are stable and the rate of micellar dissociation is related to sustained drug release from long-circulating drug carriers, which can be described by biexponential equation suggesting second order rate process due to the practically zero order burst release preceding the regular diffusion process. [59, 61] They consist of hydrophilic or polar heads, controlling micelle growth, and hydrophobic tails, resulting in anisotropic water distribution, allowing drug distribution based on its polarity. Further on, the polymeric chains of polysorbate 80 are believed to prevent opsonization and recognition by the macrophages, allowing the micelles to circulate longer. The concentration higher than CMC should be ensured upon dilution in blood stream. Drug entrapment in micelles, procured by micelle-based dialysate solution, also depends on temperature which attributes to higher thermal activity or pH of solution in relation with drug pKa value. [59]

Another interesting view of blood purification would be a system based on lipid emulsion dialysate, examined for the potential of capturing AM from the blood compartment. Generally speaking, parenteral lipid emulsions have been formulated to enhance solubility and bioavailability of lipophilic or amphiphilic poorly water soluble drugs, for sustained release and site specific delivery – for instance, brain targeting with an HIV inhibitor capable of passing the blood-brain-barrier – or to limit drug degradation. [33, 60, 62]. Emulsion is a dispersion of two immiscible liquids and a thermodynamically instable system, stabilized by one or more emulsifiers (lecithin being one of the most common choices) separating the outer water phase from the oils in the core lipid phase, and characterized by (nanometer order) droplet size, zeta potential, viscometric properties and buffering capacity. Apart from triglycerides and emulsifiers, an emulsion for I.V. application consists of isotonicly adjusted aqueous phase and ionic, antioxidant, antimicrobial, chelating and buffering agents, providing isotonicity and sterility, but also biocompatibility and biodegradability. Colloidal particles of emulsion are rapidly eliminated from blood since they are taken up by macrophages and liver metabolism with HDL and LDL fatty acid transfer. Events seeked to be avoided are demulsification, prompted by centrifugation or, as described by DLVO theory, by decreased kinetic stability leading to fusion of particles called creaming or coalescence. [62]

However, the crucial problem with hemodialysis of protein-bound drugs like AM is their strong unwillingness to unbind from plasma proteins. Albumin is the most abundant plasma protein carrying various endogenous compounds like peptide hormones and many lipophilic xenobiotics. Protein concentration can be altered during a disease state, leading to several medical conditions like dilutional hyponatriemia. [45, 63] In physiological conditions, impermeable negatively charged albumin contributes to elevated plasma osmolality, since it attracts Na^+ and K^+ ions which can pass the membrane using passive transport or actively with Na^+-K^+ ATPase – unlike water, in which case capillary hydrostatic pressure opposes its movement into the plasma space. [63] Human serum albumin has multiple binding sites for neutral and negatively charged hydrophobic compounds or polar ligands, but AM has only one binding site. [64, 65] Many drugs are reversibly bound to plasma proteins by non-covalent bonds. Hence, less than 4% of AM remains free once in the blood, because a very high share of the drug (62.1%) is tightly bound to albumin, and the rest probably binds to beta-lipoprotein. [66]

2 AIM

The aim of this study is the development of an in vitro dialysis system for blood purification of AM, which is an antiarrhythmic agent with a high degree of plasma protein binding, amounting to more than 96%, and resulting in unpredictable pharmacokinetics and narrow therapeutic index (1-2.5 mg/L). Patients on AM might experience higher blood concentrations than expected as well as severe intoxications. Moreover, there is neither an antidote nor a possibility of dialysis treatment due to extensive protein binding of the drug.

Firstly, we are going to study the possibility of blood purification of AM by using an in vitro dialysis study in which we will apply the »QuickSep« system, with donor compartment, corresponding to blood compartment in real hemodialysis, containing AM (pure substance), AM Bioniche (generic micellar form of the drug) or an AM albumin solution, and the receptor compartment containing glucose 5% solution, NaCl 0.9% solution, micelles of polysorbate 80 solution or diluted parenteral emulsion Medialipide 20%. Both compartments shall be separated by a semipermeable membrane. Later we are going to apply another in vitro (»mini-hemodialysis«) study, using a dialysis system with a pump, where donor and receptor compartments will be separated by a real dialysis column. In both studies the samples obtained from the receptor and donor compartment are going to be analysed by RP-HPLC/UV method.

AM is a lipophilic substance, very likely to have a high affinity towards polysorbate 80, possibly resulting in the release of the drug bound to albumin, free AM passing the dialysis membrane and binding of the drug to the micelles of polysorbate 80. Based on this hypothesis, we are going to make an attempt at development of an in vitro dialysis method, using a dialysis solution containing polysorbate 80 at a concentration a hundredfold higher than its CMC. Finally this could lead to the development of a new hemodialysis method for blood purification of AM and other protein-bound drugs. Similarly we are going to explore the affinity of drug towards the dialysis solution containing an oil-in-water emulsion, potentially proposing yet another blood purification method. The studies could be of great value for hospital treatment of patients with a severely increased blood concentration of AM, which would be the ultimate goal of the conducted study.

3 METHODS

3.1 MATERIALS

3.1.1 CHEMICALS

- Amiodarone Hydrochloride; RT-corp, Sigma-Aldrich, Fluka Analytical, LOT P500164
- Amiodarone Bioniche 50 mg/mL; Bioniche Pharma, LOT 120995
- Vialebex, solution pour perfusion 200 mg/mL; LFB Biomedicaments (human albumin), LOT 09L13392
- Vialebex, solution pour perfusion 40 mg/mL; LFB Biomedicaments (human albumin)
- Glucose 5%, solution isotonique pour perfusion; B. Braun, LOT 12402412
- NaCl 0.9%, solution isotonique pour perfusion; B. Braun, LOT 12512405
- Montanox 80 (Polysorbate 80); Seppic, LOT A61141
- Medialipide 20%, emulsion pour perfusion; B. Braun, LOT 130758081
- Potassium phosphate monobasic (KH_2PO_4); Sigma-Aldrich, Inc., Batch 106K0209
- Acetonitrile HPLC grade, CAS=75-05-8; Ficher Scientific UK, LOT 1225322
- Diasol 7158, solution acide concentree pour hemodialyse, dilution 1:45; Baxter, LOT 13A1517
- Sodium bicarbonate, $NaHCO_3$; Coopération pharmaceutique française, LOT G5056

3.1.2 APPARATUS

- QuickSep
- Spectra/Por Dialysis Membrane (molecular porous membrane tubing; MWCO=3500 Da)

- Magnetic stirrer
- Balance
- Flasks
- Pipettes
- Syringes
- Whatman Syringe Filter (PTFE, 0.2 μm)
- Vortex
- Centrifuge
- Vacuum pump
- Whatman Membrane filters (cellulose acetate, 0.45 μm)
- Ultrasonic cleaner
- HPLC column: Luna 5 μm, C8 (2); 100 Å, 150x4.60 mm, Phenomenex, P/No. 00F-4249-E0; S/No. 649372-10
- Dialysis column: AN 69 XT; LOT 181062, 05/98, 256 F
- Pump: Masterflex; Easy-Load, L/S, Cole-Parmer, model 7518-00
- 3 way stopcock; CAIR LGL, LOT 11B14

3.2 METHODS

3.2.1 HPLC analysis

We performed reversed phase chromatography with HPLC column Luna 5 μm, C8 (2) (100 Å, 150x4.60 mm, Phenomenex, Serial No. 649372-10) and a mobile phase consisting of 70% of acetonirile and 30% of potassium monophosphate buffer solution (KH_2PO_4), at the flow rate of 1.5 mL/min and at ambient temperature. The analysis was performed on HPLC Agilent instrument (Agilent Technologies 1200 series), with a dual-head

reciprocating pump and a fixed loop injection. The variable wavelength UV detector was set at 210 and 240 nm, volume of injection was 50 µL and the time of analysis was 7 min. When the analysis was finished, the system was washed with acetonitrile/water (70/30) mixture and a mixture of acetonitrile/water (50/50) was applied for the storage of the HPLC column. Both of the mentioned mixtures were mixed in-line.

Mobile phase preparation:

For the preparation of 1 L of mobile phase, we weighed 2.205 g of KH_2PO_4 and added 300 mL of ultrapure water. We used magnetic agitation for 10 minutes to achieve the solubilization and then we filtered the solution, using a vacuum pump and a Whatman membrane filter (cellulose acetate, 0.45 µm). We added 700 ml of acetonitrile (HPLC grade) to the filtered solution and shaked the mixture which was afterwards left into an ultrasonic bath for 45 minutes. Hence, the mobile phase was not mixed in-line, but manually.

Picture 5: HPLC system.

Calibration curve:

To obtain a calibration curve for pure substance (AM Sigma) and micellar formulation (AM Bioniche), we first prepared a solution of AM Sigma and Bioniche at a concentration of 50 mg/L, and then we prepared diluted solutions (in glucose 5% as sample solvent) at different concentrations, as it follows: 40, 30, 25, 20, 10, 7.5, 5, 2.5, 1, 0.75 and 0.5 mg/L. Taking in account that AM is prone to degradation due to its light sensitivity, we used silver foil to protect the solutions from the light. We took 0.5 mL of every solution, transferred it into a viale and injected it into the HPLC system. A calibration curve was

derived from the relation between the concentration and the corresponding concentration-dependent response. The signals as peak areas were integrated manually.

Sample preparation:

- Adequacy of sample matrix

Since it is recommended to use uniform sample matrix, equivalent to the solvent in the standard solutions used to create a calibration curve, in order to obtain reliable responses, all the samples from receptor compartment (as well as certain samples from donor compartment – AM Sigma or Bioniche diluted in glucose 5%) contained glucose 5% solution. Thus, the receptor compartment contained glucose 5% solution, or polysorbate 80 solution and emulsion diluted in glucose 5% medium, providing the same matrix for all the samples taken from the receptor compartment.

- Albumin precipitation of samples

Samples from the donor compartment containing albumin, taken in the beginning and in the end of the experiment, underwent precipitation before being submitted to HPLC analysis.
First, we added 300 µL of acetonitrile to 100 µL of sample. Then we vortexed and centrifuged the samples for 3 minutes (at 2400*x*-g). After the centrifugation we took 100 µL from the supernatant and added 400 µL of acetonitrile, filtered the mixture through a syringe filter (0.2 µm) into a microvial and then we performed HPLC analysis of the samples.

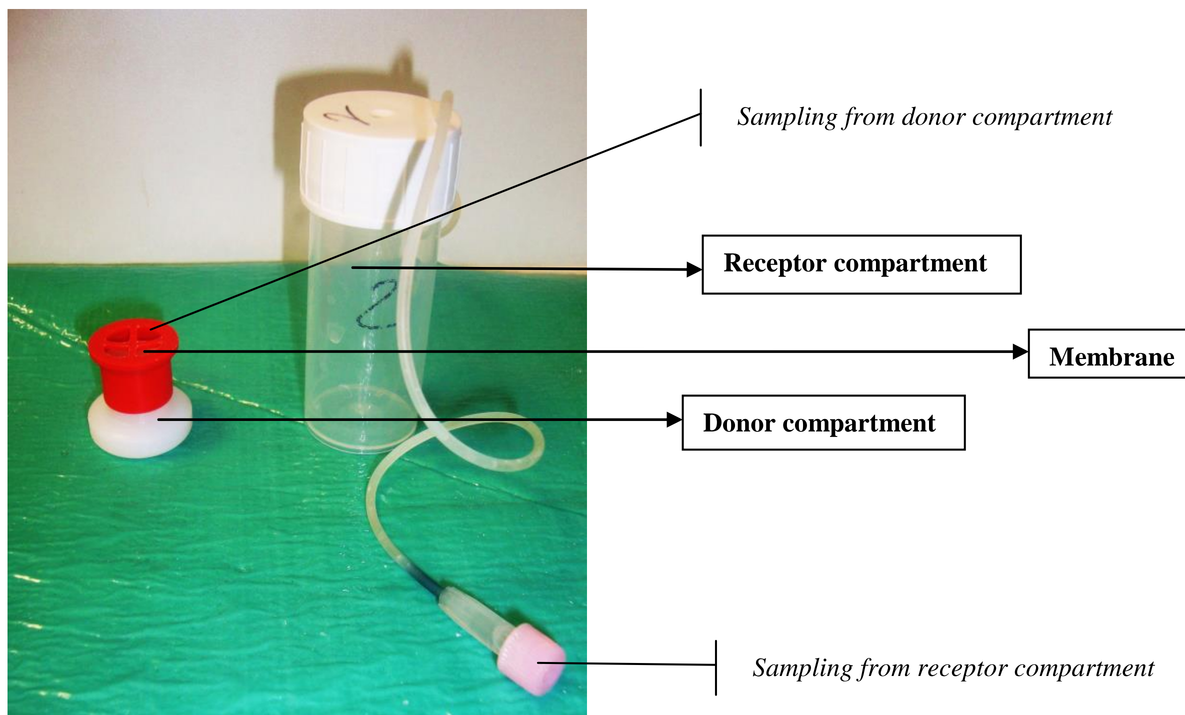
- Dilution with mobile phase

In assays with emulsion, each sample (0.5 mL) obtained from the receptor compartment was diluted with 0.5 mL of mobile phase. Filtration by a syringe filter (0.2 µm) into microvials was necessary due to sample precipitation. It was more convenient to use microvials instead of vials due to the small volume of the filtrate.

3.2.2 Dialysis (QuickSep)

To perform an equilibrium dialysis, we used the QuickSep system, consisting of donor and receptor compartment (propylene flasks), both separated by a regenerated cellulose dialysis

membrane, previously saturated in receptor solution medium. The volume of donor compartment was 1 mL, while the volume of receptor compartment was 10 mL. The membranes with MWCO of 3500 Da needed to be treated with precaution to avoid perturbation of its consistence.



Picture 6: QuickSep system representing donor (left) and receptor compartment (right). The dialysis membrane was installed between the red and white part of donor compartment (left); afterwards the donor compartment was placed into the flask representing the receptor compartment (right).

Dialysis was performed with magnetic agitation at 135 rpm. The time of dialysis at the beginning of our study was 7 hours and later only 3 hours. All the solutions of AM needed to be protected from light. The samples from receptor compartment (with volume of 10 mL) were taken at constant volume – each time we took 0.5 mL of sample, we injected into the receptor compartment 0.5 mL of the corresponding receptor medium. The samples from donor compartment (with volume of 1 mL) were taken only in the beginning and in the end of the experiment. Sampling was performed with a 1 mL syringe and sample volume was 0.5 mL, as mentioned above. As recommended when dealing with AM, we used medical gloves.

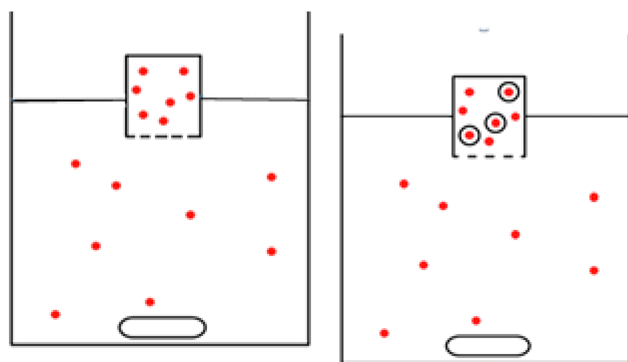
Picture 7: Dialysis with QuickSep system.

A. Dialysis study of AM Bioniche compared to AM Sigma

We prepared two donor compartments, filled with a solution of AM Hydrochloride (AM Sigma) with a concentration of 50 mg/L. For the preparation of this solution we weighed 5 mg of AM Sigma into a 100 mL volumetric flask and added glucose 5% solution up to 100 mL. To achieve optimum solubilization, we used magnetic agitation and ultrasonic vibrations for approximately 5 minutes.

The other two donor compartments were filled with a diluted solution (1/1000 dilution) of AM Bioniche, solution for injections (50 mg/mL): we took 100 µL of AM Bioniche, solution for injections, and 9.9 mL of glucose 5% solution to obtain »solution 1«; then we added 9 mL of glucose 5% solution to 1 mL of the »solution 1« to obtain the solution at the desired concentration of 50 mg/L.

All four receptor compartments were filled with glucose 5% solution.



Picture 8: The first donor compartment contains AM Sigma solution and the second one AM Bioniche solution, while the receptor compartment in both cases contains glucose 5% solution. Red dots represent AM and circles represent the micelles of polysorbate 80.

Before launching the experiment we immersed the membranes into the media corresponding the receptor phase (glucose 5% solution) to obtain saturation of the membranes. We performed the experiment during 7 hours, while sampling at constant volume (0.5 mL) every one hour. Having finished the experiment we washed the dialysis system with glucose 5% solution.

B. Dialysis study of AM bound to albumin passing into a polysorbate 80 solution

We prepared four donor compartments, each filled with a solution of AM Sigma in an albumin solution (Vialebex, 200 mg/mL), with a concentration of 500 mg/L (5 mg of AM in 10 mL of albumin solution). It is no coincidence the chosen concentration was as high as 500 mg/L, which is much above the expected therapeutic drug levels. Since we could not detect a large part of drug bound to albumin and since dilutions with organic solvent were needed in order to achieve albumin precipitation, forming a part of sample preparation, we started the assay by preparing a solution of AM in an albumin solution at a very high concentration, with the intention to obtain reliable detection, with sample concentrations falling into our detection range. To achieve optimum solubilization, we used magnetic agitation and ultrasonic vibrations for approximately 5 minutes.

Two of the receptor compartments were filled with glucose 5% solution and the other two flasks contained a solution of polysorbate 80 (Montanox 80) in glucose 5%, with a concentration of 1.668 g/L (417 mg of polysorbate 80 in 250 mL of glucose 5% solution). The concentration of polysorbate 80 was at least a 100 times greater than its CMC, allowing to assume that micelles of polysorbate 80 would be successfully formed.

For the preparation of 250 mL of polysorbate 80 solution in glucose 5% solution:

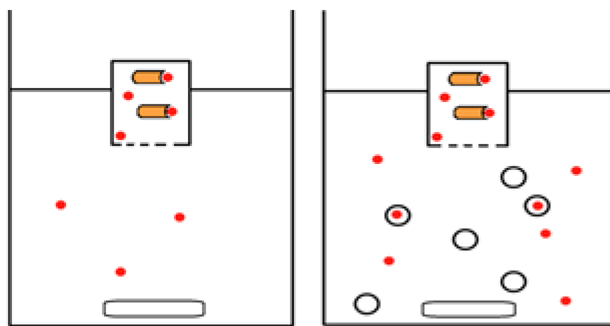
c(polysorbate 80)=100xCMC=100 x 0.012 mM=1.2 mM

MM(polysorbate 80)=1310 g/mol

c(polysorbate 80)=1310 g/mol x 0.0012 M=<u>1.572 g/L</u>

Theoretically, for 250 mL: m(polysorbate 80)=<u>393 mg</u>

We used magnetic agitation for 10 minutes to achieve optimal solubilization of polysorbate 80 in glucose 5% solution.



Picture 9: Donor compartments contain AM in albumin solution, while the first receptor compartment consists of glucose 5% medium and the second receptor compartment of polysorbate 80 solution (100xCMC). Red dots represent AM, yellow particles albumin and circles represent the micelles of polysorbate 80.

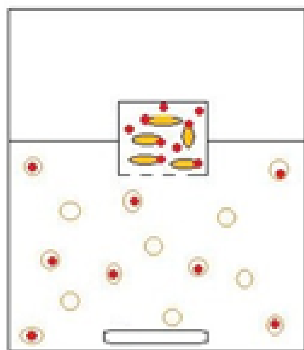
Before launching the experiment we immersed the membranes into the media corresponding the receptor compartment (glucose 5% solution or NaCl 0.9% for flask 1 and 2 and polysorbate 80 solution for flask 3 and 4) to obtain saturation of the membranes. We performed the experiment during 3 hours, while sampling at constant volume every 20 minutes, and every 30 minutes during the last hour of the experiment.

Having finished the trial, we washed the dialysis system with glucose 5% solution (flask 1 and 2) and polysorbate 80 solution (flask 3 and 4).

C. Dialysis study of AM bound to albumin passing into an emulsion (Medialipide 2%)

We prepared four donor compartments, each filled with a solution of AM Sigma in an albumin solution (Vialebex, 200 mg/L), with a concentration of 500 mg/L (5 mg of AM in 10 mL of albumin solution). Again, the concentration was this high with the intention to obtain reliable detection, as explained above. To achieve optimum solubilization, we used magnetic agitation and ultrasonic vibrations for approximately 5 minutes.

We prepared 200 mL of diluted emulsion Medialipide 2%: we put 20 mL of parenteral lipid emulsion Medialipide 20% into a flask and then we added glucose 5% solution up to 200 mL. All of the four receptor compartments were filled with 10 mL of Medialipide 2%.



Picture 10. The donor compartment contains AM Sigma in albumin solution, while the receptor compartment contains oil-in-water parenteral emulsion Medialipide 2%.

Before launching the experiment we immersed the membranes into the media corresponding the receptor phase (Medialipide 2%) to obtain saturation of the membranes. We performed the experiment during 3 hours, while sampling at constant volume every 20 minutes, and every 30 minutes during the last hour of the experiment.

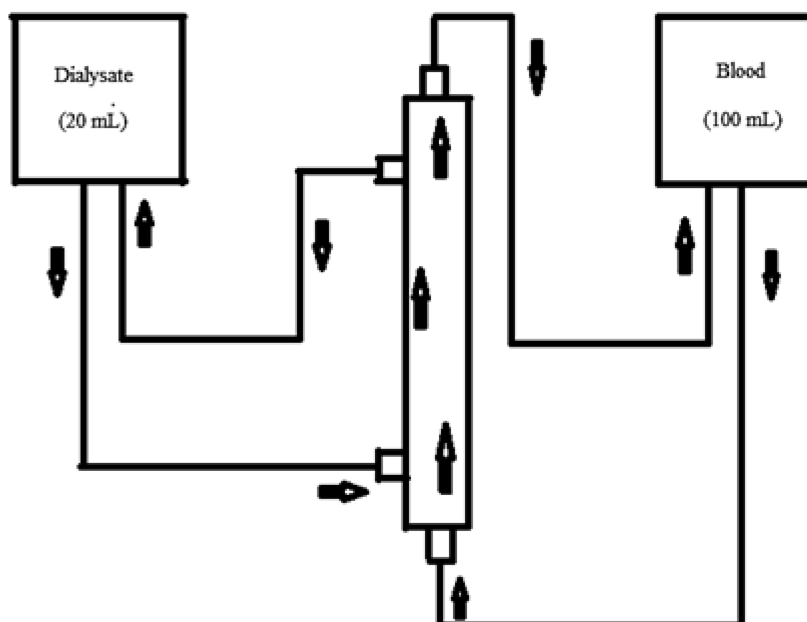
Having finished the experiment we washed the dialysis system with Medialipide 2%.

3.2.3 Mini-hemodialysis system

To perform a dialysis study we used a dialysis system imitating the real hemodialysis performed in a hospital. The dialysis system consisted of receptor compartment, containing dialysis solution, and blood or donor compartment, containing solution of AM in an albumin solution, both separated by a dialysis column. The MWCO of the polyacrylonitrile membrane was 35-40 kDa. A peristaltic pump worked at a flow rate estimated at 23.7 mL/min and 10.6 mL/min for the receptor and donor compartment, representing dialysate and blood compartment, respectively. All the solutions of AM were protected from light. The minimal volume of the system (column and tubing) was estimated at 20 mL. The time of dialysis at the beginning of our study was 3 hours.

The samples from the receptor compartment were taken at constant volume (each time we took 0.5 mL of sample, we injected 0.5 mL of receptor medium into the receptor compartment) and the samples from the donor compartment were taken only in the beginning and in the end of the experiment. Sampling was performed with a 1 mL syringe and, like always when dealing with AM, we used also medical gloves. Before use the system was washed with an ethanol/distilled water (v/v=50/50) solution and distilled

water. All used materials were sterile in order to achieve the best approximation of a real hemodialysis system.



Picture 11: Mini-hemodialysis system. The left side represents the dialysate compartment and the right side represents the blood (donor) compartment.

A. Dialysis study of AM bound to albumin passing into a dialysis solution

100 mL of a solution of AM Sigma in an albumin solution (Vialebex, 40 mg/mL), with a concentration of 500 mg/L (50 mg of AM in 100 mL of albumin solution) represented the donor compartment:

We weighed 50 mg of AM and added 20 mL of albumin solution (Vialebex, 40 mg/mL). Again, the concentration was as high as 500 mg/L in order to obtain reliable detection, as explained before. To achieve optimum solubilization, we used ultrasonic vibrations for 10 to 15 minutes. Then we transfered the obtained solution with a syringe into the same flask of Vialebex (40 mg/mL, 100 mL).

To prepare the receptor compartment, we diluted the acidic dialysis concentrate (Diasol, 7158; dilution 1/45) with bicarbonate solution ($NaHCO_3$) and purified water:

We added 174.4 mg of bicarbonate and 44 mL of purified water to 1 mL of acidic concentrate to obtain 45 mL of dialysis solution. For the receptor phase we used 20 mL of the prepared dialysis solution.

We performed the experiment during 80 minutes, while sampling at constant volume every 20 minutes. Having finished the experiment, we washed the dialysis system with dialysis solution.

Picture 12: Mini-hemodialysis system.

4 RESULTS

4.1 Calibration curve for AM Bioniche – HPLC analysis

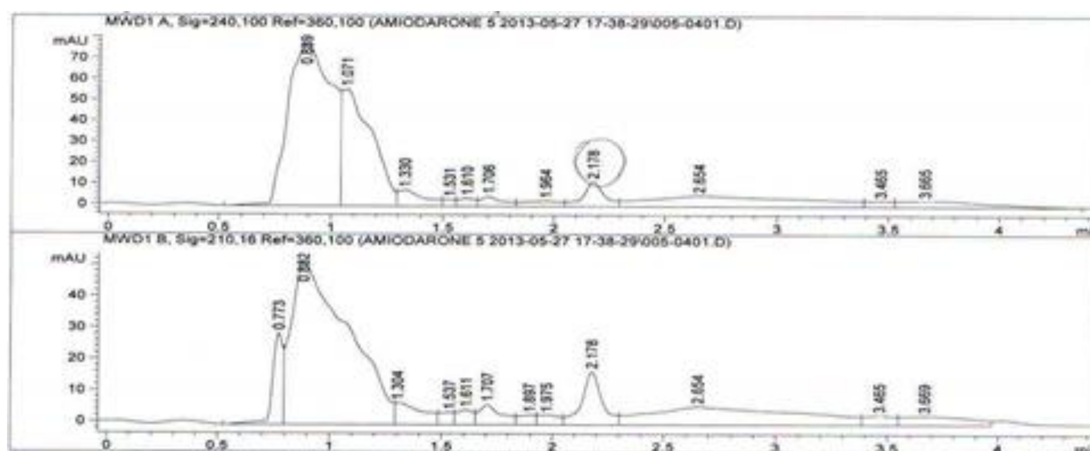
Since solubility appears to be the crucial step for successful HPLC analysis, low solubility of AM was tackled with magnetic agitation and ultrasonic vibrations, but the problem persisted, thus preventing the formation of calibration curve for AM Sigma. However, calibration curve was effectively obtained for the micellar formulation AM Bioniche. Since

it was not possible to establish one uniform calibration curve for the whole concentration range of the conducted experiments, two calibration curves were formed, with a minor difference in slope value.

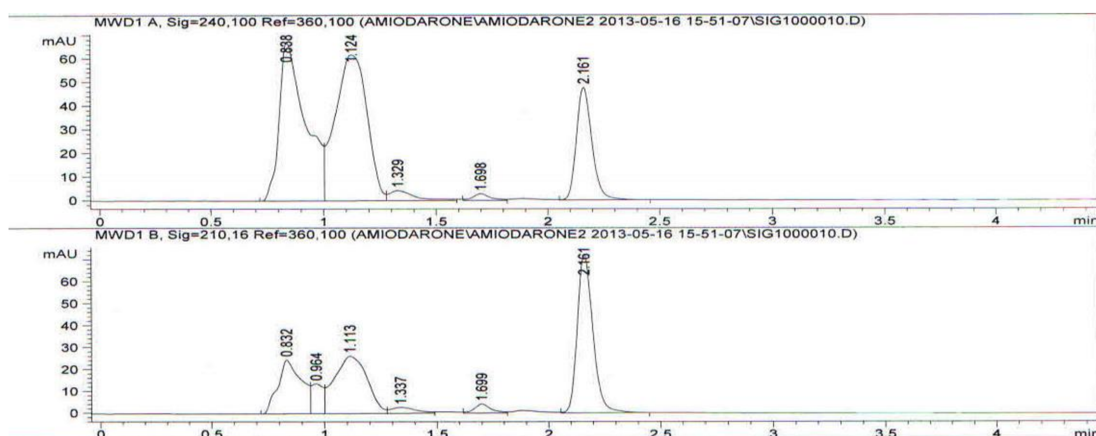
The equation of calibration curve corresponds to y = ax + b, hence for the lower curve of AM Bioniche: **y = 65.78x – 42.657** at 210 nm and **y = 42.575x – 25.218** at 240 nm (x stands for signal or area, and y for concentration). The equations determined at **240 nm** was chosen for the calculations of the sample concentrations, due to better compliance with all of the required ICH guidelines and demands, including better recovery, and adequate LOD and LOQ. [41] However, better interday precision and slightly lower LOD and LOQ were observed at 210 nm.

<u>The lower calibration curve for AM Bioniche</u> (**y = 42.575x – 25.218;** at **240 nm**) was validated for specificity, linearity and range, accuracy, precision (intra- and interday), LOD and LOQ. The obtained values are shown in Table E (Appendix).

Under the previously described conditions AM was eluted at approximately 2.1 minutes, with no or only negligeable variations of peak retention times, proving the repeatability of the separation. AM peaks were also separated from other peaks, showing appropriate selectivity, as depicted in Pictures 13 and 14.



Picture 13: HPLC chromatograms for AM in polysorbate 80 solution. The peaks at retention time of 2.17 min correspond to AM.



Picture 14: HPLC chromatograms for AM Sigma in glucose 5% solution. The peaks at retention time of 2.16 min correspond to AM.

Linearity was studied in the concentration range from 0.5 to 25 mg/L. Determination coefficient (R^2) for the proposed equation was 0.997 and for the others curves was always greater than 0.99. Accuracy was verified by calculating recovery, considered acceptable when the values fall between 90 and 110%. Three concentrations measured three times, at three different days, complied with this requirement, though some deviations were seen at 0.5 and 2.5 mg/L. Precision was measured as intraday repeatability (precision), studied as areas of three injections of the same sample on the same day, expressed with coefficient of variation (CV). It is clear CV was under 5% for every point of the calibration curve, with the exception of the lowest concentration. As for interday precision, the measured values appear lower than 8%, but only at higher concentrations. LOD was 0.16 mg/L and LOQ 0.47 mg/L, both calculated from standard deviation of y-intercept (σ) and slope (S).

- $LOD = 3,3 \times \sigma / S$
- $LOQ = 10 \times \sigma / S$

The upper calibration curve for AM Bioniche (**y = 48.923x – 92.463** at **240 nm**) equally showed good linearity with R^2 for the proposed equation amounting to 0.998 (for the other curves was always greater than 0.989), accuracy and intraday precision, while interday precision was even better than for the lower equation. The obtained values are shown in Table F (Appendix). As expected, LOD and LOQ values, both again calculated from standard deviation of y-intercept and slope, were higher – 2.17 mg/L and 6.57 mg/L, respectively. However, some deviations were noticed at the low end of the curve.

HPLC analysis had to be conducted simultaneously with dialysis experiments due to problems with providing stability of the samples. The substance is prone to precipitation due to low solubility, or degradation if not protected from light, and it showed significant instability when freezed, as well as in NaCl 0.9% solution, as it follows from the results from Table B (Appendix; observe the pronounced fall in donor compartment concentration from time 0 to time 7h in NaCl 0.9% solution, where almost no AM passed the dialysis membrane.). Thus, the samples were submitted to HPLC analysis immediately after sampling.

4.2 Dialysis studies

A. Dialysis study of AM Bioniche compared to AM Sigma

From the results gathered in Table A (Appendix), we can see that the concentrations increase very quickly in the first hour of dialysis and we can also notice the quantity of AM in donor compartment after 7 hours of dialysis greatly diminished in comparison with the quantity at the beginning of the assay, due to AM passing the dialysis membrane. As observed in Figure 1, the numbers constantly rise with time, reaching almost a constant value after 3 hours.

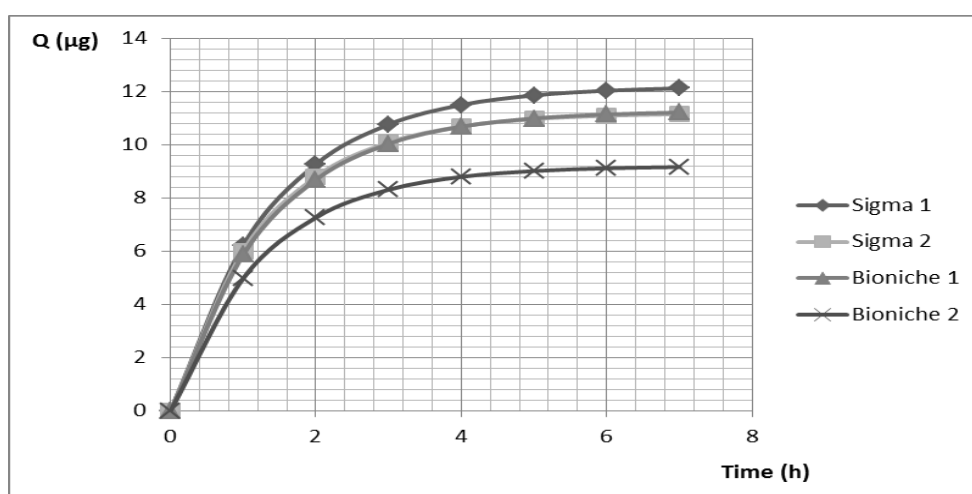


Figure 1. Relation between time of dialysis and quantity of AM Sigma (Sigma 1 and 2) and Bioniche (Bioniche 1 and 2) in the receptor compartment. Dialysis was performed over 7 hours in glucose 5% receptor medium.

The quantity of AM Sigma in donor compartment after 7 hours of dialysis was lower than the quantity in donor compartment containing AM Bioniche, according to Table A

(Appendix). We also attempted to estimate the losses of the studied substance during the assay, hence estimating their impact on the obtained results. Comparing the quantities of AM Sigma with the quantities of AM Bioniche, larger losses were noticed when referring to AM Sigma, while only minor losses were seen in the flasks containing AM Bioniche.

B. Dialysis study of AM bound to albumin passing into a polysorbate 80 solution

The results presented in Table C (Appendix) show a rapid rise in concentrations in the receptor compartment at the beginning of the conducted experiment. However, we can also notice the quantity of AM in donor compartment after 3 hours of dialysis is somewhat higher than at the beginning of the assay.

As it follows from Figure 2, not only can we observe all the values constantly increase with time until they reach a plateau, but also the quantities in the receptor compartment containing polysorbate 80 solution exceeded almost by twofold the ones in the glucose 5% solution. This implies a high affinity of the drug towards the micelles of polysorbate 80, causing AM to be released from albumin, to pass across the membrane and to enter receptor compartment containing the mentioned surfactant.

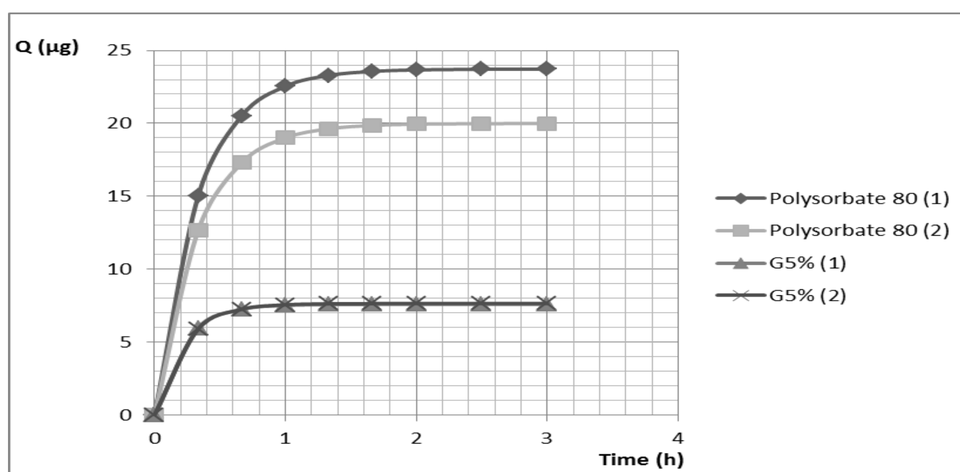


Figure 2. Relation between time of dialysis and quantity of AM Sigma in the receptor compartment. Dialysis was performed over 3 hours in glucose 5% receptor medium (G5% (1) and (2)) and polysorbate 80 solution (100xCMC) in glucose 5% medium (Polysorbate 80 (1) and (2)), while the donor compartment contained AM Sigma in albumin solution.

C. Dialysis study of AM bound to albumin passing into an emulsion (Medialipide 2%)

Figure 3 shows a slightly higher affinity of AM towards Medialipide 2% parenteral emulsion when compared to glucose 5% medium. However, the values for emulsion receptor medium do not approach the ones obtained with polysorbate 80 solution in glucose 5% medium, at a concentration 100-fold higher than its CMC. Table D (Appendix) reveals the quantity of AM in donor compartment after 3 hours decrease and are accompanied by only minor losses.

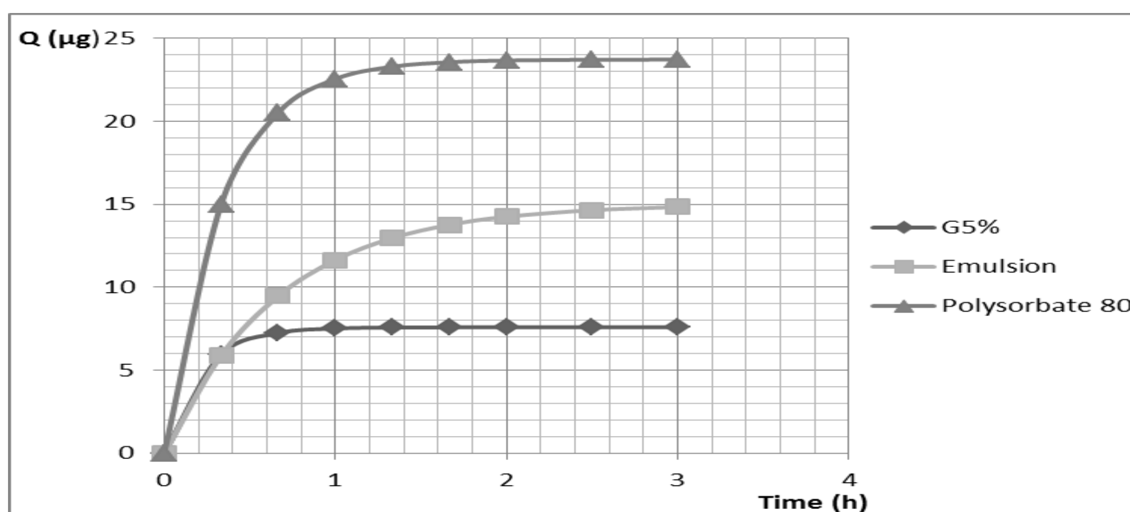


Figure 3. Relation between the time of dialysis and quantity of AM Sigma in the receptor compartment. Dialysis was performed over 3 hours in glucose 5% and emulsion receptor medium, as well as polysorbate 80 solution (100xCMC) in glucose 5% medium, while the donor compartment contained AM Sigma in albumin solution.

Unfortunately, truly reliable results could not be obtained from this study since samples from emulsion receptor medium underwent flocculation, observed upon dilution of the samples with mobile phase and resulting in low recovery due to partial analyte partition in the oil phase. This phenomenon leads to underprediction of the AM concentration in receptor medium.

4.3 Mini-hemodialysis study

As it can be noticed from Figure 4, it seems that the free fraction of AM bound to proteins readily passes the membrane in the dialysis column and enters the receptor compartment containing diluted solution of Diasol. The willingness to pass the membrane and enter the receptor compartment with Diasol appears to be similar to the one from the earlier QuickSep study with glucose 5% receptor medium, since the calculated quantities are fairly comparable.

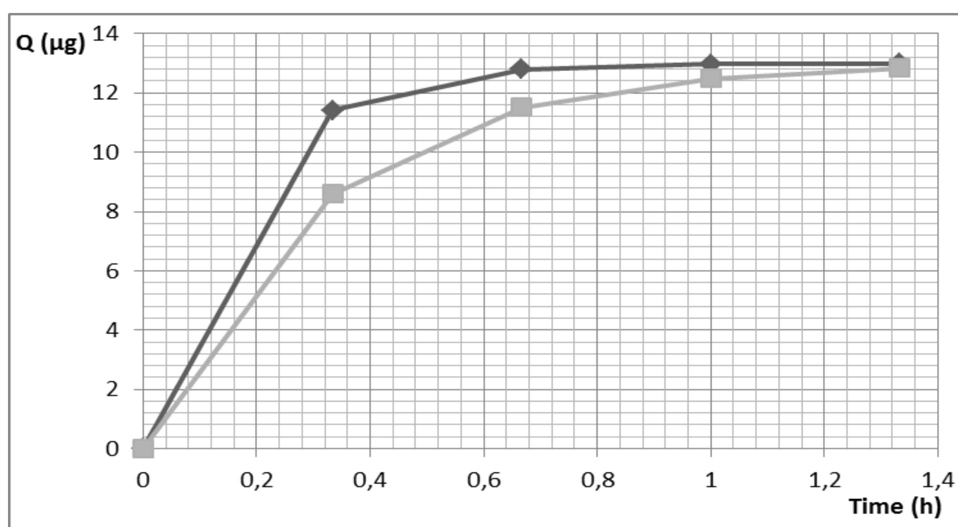


Figure 4: Relation between time of dialysis and quantity of AM Sigma in the receptor compartment. Dialysis was performed over 80 minutes in Diasol receptor medium (diluted acidic concentrate), while the donor compartment contained AM Sigma in albumin. The two lines represent two parallels of the same experiment.

4.4 Statistics

Two-way ANOVA test with replication, calculated in Excel and with the level of confidence being 0.05, was conducted for the assessed measurements. The results are gathered in Table G (Appendix). Although the statistics revealed significant difference between the parallel values for AM Sigma passing into glucose 5% solution (p=0.0098), there was no significant difference between measurements obtained on two different days for AM Bioniche passing into glucose 5% solution (the calculated F value was lower than the critical F value; p=0.0841). However, no significant differences were detected between both, AM Sigma and AM Bioniche, passing into glucose 5% medium. The same was

perceived for AM bound to albumin passing into glucose 5% or polysorbate 80 solution, as well as emulsion receptor medium. However, the parallel values for AM bound to albumin passing into polysorbate 80 receptor medium (p=0.0505) differ to a greater extent, compared to glucose 5% medium (p=0.7776).

5 DISCUSSION

5.1 Calibration curve for AM Bioniche – HPLC analysis

Our goal was to prepare two calibration curves, one for AM Sigma and one for AM Bioniche. Since AM shows great instability, all the standard solutions were kept protected from light and HPLC analysis was conducted immediately after dilution. However, when we prepared dilutions of AM Sigma and Bioniche, we were only able to successfully validate the calibration curve for AM Bioniche. The slope of the calibration curve for AM Sigma at the same wavelength appeared quite different, which was not what we would have expected when analysing the same substance. The possibility of substance degradation was rejected because no additional peaks were noticed in the chromatograms, but the principal culprit could be the water solubility problem of AM Sigma, since this is a pure substance – unlike AM Bioniche which is a micellar formulation with improved solubility characteristics. However, the difference in slopes cannot be entirely attributed to a solubility problem because discrepancies were apparent not only at higher, but also at lower end of the concentration range. Hence, considering the high lipophilicity of AM, it is very likely its non-specific adsorption caused the mentioned inconsistency of the slope values obtained from AM Sigma (pure, non-micellar drug formulation) compared with the ones from AM Bioniche, posing more than a few problems during validation of the respective calibration curves.

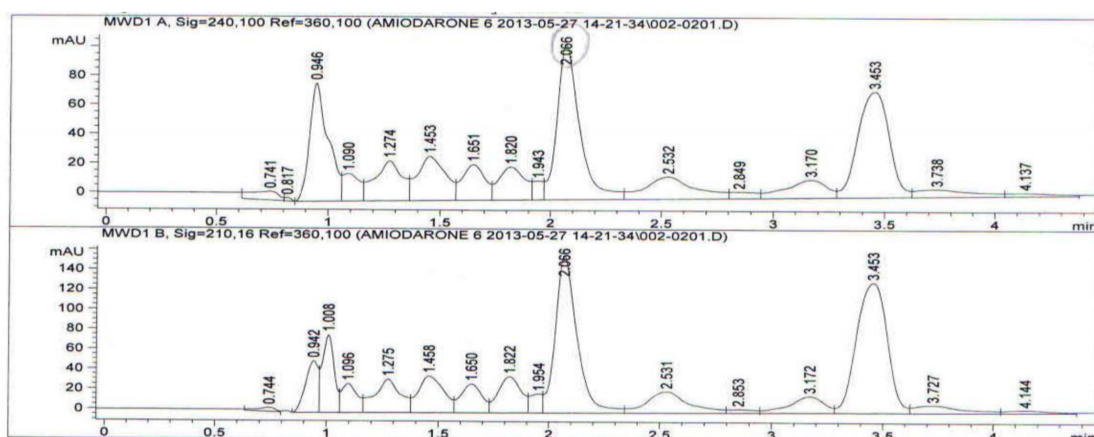
Two curves for AM Bioniche had to be established due to inability of creating simply one calibration curve (in which case the validation failed in the lower concentration range). The calibration curves were made of more than 6 points and eventhough some deviations were perceived especially at the low concentration end, as showed in Tables E and F (Appendix), AM Bioniche calibration curves meet the requirements of ICH guidelines, using more than the recommended minimum of 9 determinations over a minimum of 3 concentration levels. [41] LOD and LOQ are suitable for our dialysis method, since the

measured area values obtained in our sample analysis exceed the mentioned LOQ. Responses measured as areas were higher at 210 nm than those at 240 nm, and also showed better interday precision and only slightly lower LOD and LOQ, proving better sensitivity, but due to better intraday precision (better repeatability) and better recoveries when measured at 240 nm, we decided to use the respective calibration curve to calculate the concentrations from our samples. This wavelength was also determined as absorption maximum for AM. [40, 67] The upper calibration curve for AM Bioniche was not forced through zero, thus yielding more accurate and precise results. However, it has a greater slope and even more negative intercept than the lower curve. The deviations and poor compliance with requirements of certain points (like 2.5 mg/L) can be blamed on dilution errors while pipetting. As anticipated, greater deviations as well as greater CV were seen at low concentrations.

Polysorbate 80 exerts a large UV/VIS absorption (over the 200-300 nm range) and a broad chromatographic elution which can interfere with the retention time of the studied drug, but solutions with a concentration lower than 3% have showed a local minima in absorbance at 210 nm. Also, polysorbate 80 build-up can negatively impact the column performance, but this can be solved with multiple washings removing the retained surfactant. [29] Glucose UV absorption spectra gave maximum absorption at 197 nm, but also at 250 nm. [68] Plasma protein albumin showed maximum absorption at 250-300 nm, absorption being attributed to aromatic amino acids tyrosine, tryptophan and phenylalanine. [69] Nevertheless, in RP-HPLC hydrophilic molecules like glucose and polysorbate 80 are quickly eluted, which also applies to benzylic alcohol (a conservans in AM Bioniche formulation) due to its low lipophility. The chromatograms were easily interpreted and no interferences were noticed since both glucose or polysorbate 80 were eluted sooner than AM, with peaks approximately at 0.8, 1.1 and 1.3 minutes, or approximately at 0.8 minutes, respectively. Substantially more lipophilic AM is eluted approximately at 2.1 minutes. The remaining albumin peaks after the sample clean-up produced some interferences, but those peaks do not interfere with the peak corresponding to AM and do not present difficulties in determining the response of AM, which is eluted at slightly lower retention time than 2.1 minutes, as depicted in Picture 15. Since there were no additional peaks in the chromatograms for donor compartment, we can also conclude that polysorbate 80 from the receptor compartment does not enter the donor

compartment, which is a preferable outcome, because it is certainly not welcome for a greater extent of this exogenous substance to enter patient's blood stream when forming part of dialysate in real hemodialysis, even if the substance is physiologically compatible and can even be administered I.V.

According to other sources, acetonitrile mixed with potassium monophosphate buffer solution with a concentration of 10 mmol/L, giving a pH of 3.5 (in ratio of 62:38, respectively) seems to be an appropriate choice of mobile phase for AM separation on a C8 column. [40] Hence, the mobile phase we used in our analysis was a mixture of 70% of acetonirile and 30% of buffer solution (KH_2PO_4). First, we prepared a mixture of 50% of acetonirile and 50% of buffer solution (KH_2PO_4) and performed the HPLC analysis at 1 mL/min, but then we changed the proportion of the constituents to 70% of acetonirile and 30% of buffer solution (KH_2PO_4). We also increased the flow rate to 1.5 mL/min, resulting in lower retention time of the substance and lower time of analysis; the backpressure at the conditions mentioned above was around 75 bar. The resolution of the applied HPLC analysis method was suitable, causing no troubles in defining the peak of analysed substance.



Picture 15: HPLC chromatograms for AM in albumin solution. The peaks at retention time of 2.07 min correspond to AM.

5.2 Dialysis study of AM Bioniche compared to AM Sigma

The purpose of this study was to observe the differences and similarities between the dialysis of pure substance (AM Sigma) and AM Bioniche as a generic form of the drug,

where AM forms part of the lipophilic core of the micelles. Hence, the water solubility of the drug is increased, which results in better detection, since only a well-solubilized substance should be submitted to HPLC analysis. AM Bioniche is consisted of AM, polysorbate 80 as a micelle-forming polymer and it also contains benzylic alcohol, but since both excipients are eluted sooner than AM, they do not interfere with the peaks that belong to the analysed substance.

The equations used to calculate the concentrations of AM were $y = 42.575x - 25.218$ for all the samples except the ones from donor compartment where (due to greater concentrations) the upper calibration curve had to be applied ($y = 48.923x - 92.463$). Further on, from those concentrations the respecitve quantities were calculated:

*m=c*V; V(receptor)=10 mL and V(donor)=1 mL*

Since it is easier to determine whether our results were affected by losses of substance when dealing with quantities instead of concentrations, we decided to present a cumulative profile of drug quantity passing into the receptor compartment. Although we were sampling at constant volume, which allows us a rather simple calculation of concentrations, we had to consider a correction due to mass loss during the sampling in the receptor compartment. The respective corrected mass was calculated by adding the sum of concentrations determined in all of the preceding samples, multiplied by sample volume being 0.5 mL, to the mass determined for the respective sample.

$m(corr) = m + (\sum c) * V$

Dividing the mass from the receptor compartment by the mass from the donor compartment at the beginning of the assay (considered as the overall mass of AM) allowed us to calculate the respective percentage of quantities.

Adding the mass from the receptor compartment after 7 hours to the mass from the donor compartment after 7 hours gave us the total quantity which turned out to be slightly different from the mass from the donor compartment calculated at the beginning of the assay (considered to be the overall mass of AM), giving us percentages lower than 100% (81.7% and 89.7% for AM Sigma and AM Bioniche, respectively). Although analytical

error during the sampling could not be completely excluded, it is most likely that the observed loss of substance is due to the adsorption of the lipophilic drug to dialysis membrane and other surfaces as well as solubility problem. However, if we look into the percentages presented in Table A (Appendix), it is clear that the difference between the total quantity and the mass from the donor compartment at the beginning of the assay is larger when dealing with AM Sigma. In this case it is possible that lower values were measured because the solubilized substance concentration in the receptor and/or donor compartment was decreased due to an adsorption problem, but not due to the degradation of the substance, since no additional peaks that could be attributed to AM degradation products, have been observed.

Regarding the concentrations in the receptor compartment (Table A), at first we can see a rapid rise of AM concentrations in receptor compartment, but the curve does not really reach a plateau, as seen in Figure 1, because with every sampling a small quantity of substance is removed, resulting in slight fluctuations of concentrations with time.

The mass of AM Bioniche in donor compartment is higher than the mass of AM Sigma, while the mass of AM Bioniche in the receptor compartment is slightly lower. Beside the observational error, a possible explanation of this occurence could be the kinetics of the micellar formulation of AM Bioniche, which shows biphasic release, resulting in burst release of AM from the micelles, followed by quasi-stationary equilibrium in donor compartment between the free drug and the drug bound to micelles. In drug release process a practically zero order burst release precedes the first order process of diffusion through the carrier, thus this type of kinetics can be described by biexponential equation suggesting second order rate process. Burst release is faster for small particles and high drug dose, but it is also accellerated by the remainders of free surfactant or free drug in the aqueous phase. [61] The established equilibrium in donor compartment lowers drug thermodynamical activity and activity gradient in the membrane, thus lowering dialysis rate. Hence, dialysis process following first order rate constant is controlled by permeation across the dialysis membrane, which is the dialysis rate limiting step, but also by equilibrium distribution constant in donor compartment, where the colloid (or in our case the micelles) is in equilibrium with its dispersion medium (the free fraction of AM). [43] This implies that the concentration and the quantity of AM Bioniche in donor compartment

are slightly higher due to equilibrium between the free fraction of AM and the micellar fraction of the same drug. The great affinity of AM towards micelles of polysorbate 80, certainly followed by a high equilibrium distribution constant in donor compartment, is affecting the potential of AM to pass the membrane, consequently resulting in smaller quantity of the drug found in the receptor compartment. Therefore the receptor quantities of AM Bioniche were slightly lower in comparison with AM Sigma.

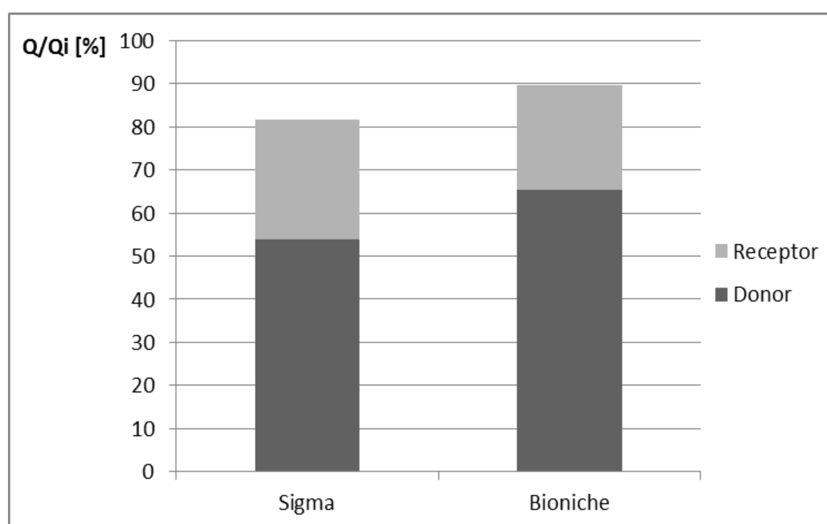


Figure 5. Shares of AM Sigma and AM Bioniche in donor and receptor compartment containing glucose 5% solution, after 7 hours of dialysis.

We also noticed a difference between the mass in the donor compartment at the beginning of the assay and in the end of the assay (after 7 hours of dialysis), which can be explained by water molecules passing the membrane and leaving or entering the donor compartment. This is why the mass of the donor compartment appeared to be slightly changing thorought the assay. To understand this, we have to realize that all the molecules intend to reach an equilibrium, with the same concentration on both sides of the membrane, which is a thermodynamically favourable occurence. The molecules move from the receptor compartment to the donor compartment or the other way around, depending on their concentration in the compartments. Since the water molecules are the most numerous ones, their movement can be roughly detected as a mass increase or decrease in the observed compartment. Hence, the weight of donor compartment increased after having finished the dialysis experiment due to water shift. This was proven by weighing the donor compartment at the beginning of the assay and in the end of the assay, after 7 hours of

dialysis. Dividing the weights gave us ratio values lower than 1 (for one assay the numbers were 0.90261 and 0.92498 for flasks with AM Sigma or 0.87772 and 0.88676 for flasks with AM Bioniche). It is obvious the difference was greater in the donor compartments containing AM Bioniche, so the respective drug concentrations seem to be influenced by water shift to a higher extent. Apparently the donor compartment with polysorbate 80 micelles attracted more water molecules than AM Sigma alone. In consequence this could result in incorrect assumptions of lower amount of drug in donor and higher amount of drug in receptor compartment, leading to under- or overestimation of drug quantities in each compartment. In view of the decribed water shift it is possible to predict that the real proportion of AM Bioniche passing into receptor compartment is even lower than presented in our calculations from Table A (Appendix). Nevertheless, the lower percentages of overall drug mass (especially regarding AM Bioniche), calculated after 7 hours of dialysis, could also be attributed to water shift.

When performing a dialysis, it is expected that after reaching the equilibrium, the concentrations of the diffusable substances on both sides of the membrane will reach equal values, because this is a thermodynamically favourable occurence, lead by concentration gradient. Although it is clear that approximately 20 to 30% (Q/Qi column in Table A, Appendix) of AM passed the membrane, entering the receptor compartment, while 50 to 70% of the drug remained in the donor compartment, it is also clear that the concentration in the receptor compartment is considerably lower than the concentration in the donor compartment. One of the possible reasons for not reaching an equilibrium could be the fact we have not performed dialysis long enough to reach a steady state. However, regarding the respective figures, we can see that the values after 3 hours do not increase in a significant way, in fact we could even say that they reach a plateau. Obviously the equilibrium has not been established probably due to the properties of the dialysis system, for instance the fact the receptor volume was 10-fold higher than the volume of donor compartment. It is more difficult to provide sink conditions in a small donor volume and accurately measuring of the concentration can result troublesome, especially considering the water shift. More errors and misleading calculations can also arise from inaccurate and imprecise sampling procedure, for instance when sampling at the compartment walls.

However, it is very likely the equilibrium was actually attained, but as it appears, it was not possible to detect with the proposed method, due to the imprecise detection of AM in donor compartment, leading to overestimation of AM. When referring to AM Bioniche, both micellar and free AM were detected, but it is important to notice it was only the free fraction which took part in the equilibrium between the receptor and donor compartment. Likewise, in donor compartments containing AM Sigma, it was the sampling from an oversaturated solution that lead to the higher detection of AM. To estimate whether the equilibrium has been attained or not, it would be essential to accurately determine the real AM concentrations in donor compartment, excluding the micellar fraction of AM Bioniche and avoiding the problems arising from sampling from an oversaturated solution of AM Sigma.

In earlier studies it has been shown that dialysis method is not very reliable when measurements were performed on colloidal particles that exhibit rapid release kinetics. This could concern our study of AM Bioniche and its micellar liberation. Since dialysis is commonly used as a reference method for ultrafiltration, in future work it would be appropriate to compare the dialysis results with the results obtained from an ultrafiltration method. [45] This would allow us to confirm the accuracy of our dialysis assay, especially because an ultrafiltration process at low pressure has been suggested when dealing with drug release of hydrophobic molecules like AM instead of dialysis method. [70] With dialysis we can only measure the free fraction of AM, without the drug entrapped in micelles. The usual limitation to the measurement of the free drug concentration with fluorescence or UV spectroscopy can be circumvented by bathocromic shift method, which is an improved spectroscopy method for determining both, the free drug and drug-carrier concentration, without separating them. The method is based on the sum of free drug absorbance and drug-carrier absorbance with different wavelength maximum and extinction coefficient. [70] Apart from dialysis, drug release can be observed with more sophisticated techniques like separation methods of centrifugation and filtration, in situ or continuous flow methods. [61] Also, to improve the reliability of the conducted study, we could try adding an osmolar agent, for example dextran, preventing the water from moving between compartments.

5.3 Dialysis study of AM bound to albumin passing into a polysorbate 80 solution

Polysorbate 80 is known as a non-ionic surfactant and micelle-forming agent, where micelles are formed at a concentration higher than its CMC (0.012 mM), and as being successfully implemented in the micellar formulation for I.V. administration, yielding notably better solubility characteristics, AM willingly binds to polysorbate 80 micelles. The main goal of this study was to explore whether the affinity of AM towards the micelles of polysorbate 80 is greater than its tendency to remain bound to albumin, possibly suggesting a great potential for development of a new blood purification method of AM.

The equation for the lower calibration curve was used to calculate all the concentrations of AM ($y = 42.575x - 25.218$). As previously described in the earlier study of AM Sigma and Bioniche, the respective quantities were calculated from the corresponding concentrations and a cumulative profile of drug mass passing into the receptor compartment was presented, while considering the correction due to mass loss during the sampling in the receptor compartment. Further on, we also calculated the respective percentage of quantities, like presented in Table C (Appendix).

Adding the mass from the receptor compartment after 3 hours to the mass from the donor compartment after 3 hours gave us the total mass which surprisingly turned out to be higher from the mass in the donor compartment at the beginning of the assay, considered to represent the overall mass of AM. This was observed to a greater extent if the medium in the receptor compartment was polysorbate 80 solution. One possible reason could be the higher value of AM concentration in donor compartment due to observational error during sampling, resulting in the respective percentages for the donor compartment being higher than 100%. A more believable explanation for the higher AM concentration in donor compartment would be the water leak from the same compartment, resulting in higher concentration of AM. However, water transfer into receptor compartment is not very likely considering the theory behind the Gibbs-Donnan equilibrium, which has an important physiological role of osmolality modulation in body fluid compartments. This can be observed under certain conditions where distribution of Na^+ and Cl^- ions in plasma and interstitial fluids is altered in order to preserve electroneutrality. Because of the presence of negatively charged, impermeant proteins like albumin in plasma space, diffusible cation

concentration is higher and diffusible anion concentration is lower in plasma compartment. Hence, the total osmolar concentration is slightly greater in plasma compartment and this is why more water moves into plasma than it would be predicted on the basis of the protein concentration alone. Nevertheless, in physiological conditions, plasma osmolality is maintained at a slightly greater osmolality than the interstitial fluids due to capillary hydrostatic pressure opposing the osmotic movement of water into plasma space. [63] On the contrary, there was no capillary hydrostatic pressure under our dialysis conditions, so the water molecules were able to enter the donor compartment with negatively charged impermeant albumin. Disintegration of albumin-drug complexes leading to higher free fraction of AM, exposed to the previously mentioned solubility problem or non-specific adsorption, due to mechanical stress or chemical reactions, is also rather unlikely, since there is no obvious reason for this to happen.

The remaining albumin and the albumin-drug complexes in the samples containing albumin (from the donor compartment) were precipitated by acetonitrile, in volume proportions as suggested in certain scientific reports. [72] Considering the fact that the addition of acetonitrile makes AM unbind from albumin and the dilution factor, the theoretical concentration of AM in the precipitated samples taken at the beginning of the assay is 25 mg/L.

- *Precipitation by acetonitrile:*
- *Dilution in 1/4: 100* µL *(sample V) + 300* µL *(acetonitrile)=400* µL
- *Centrifugation*
- *Dilution in 1/5: 100* µL *(supernatant) + 400 µL (acetonitrile)=500 µL*

- *c(AM in albumin)=0.5 mg/mL=500 mg/L*
- *500/4 mg/L=125 mg/L (the sample was diluted in 1/4)*
- *125/5 mg/L=25mg/L (the supernatant was diluted in 1/5)*

Eventhough albumin concentration in blood normally stays between 35 and 53 mg/mL, we used a solution with an even higher albumin concentration (Vialebex, 200 mg/mL),

providing that our presumption of the substance being truly bound to the protein is justified. [45] It is believed that 62.1% of AM is bound to albumin, but considering the high concentration of albumin, but the percentage could be even higher due to high albumin concentration used in donor compartment. [66]

The obtained concentration calculated from the lower calibration curve is lower than the concentration calculated above (25 mg/L) most likely due to solubility problem and non-specific adsorption.

$c(free\ AM)_{measured}$*=288/20 mg/L=*<u>*14.4 mg/L*</u> *(considering dilution factor is 4x5=20)*

Regarding the concentrations in the receptor compartment presented in Table C (Appendix), a rapid rise of values was seen at first and then the concentrations settled, but the curve never really reached the plateau, because with every sampling we removed a small quantity of substance, resulting in a slight decrease and increase of concentration with time. Comparing the numbers from Table A with the values from Table C, we can conclude that AM concentrations in receptor compartment containing glucose 5% solution are lower than the respective concentrations for AM Sigma in glucose 5% medium, measured in our earlier study. The lower receptor compartment concentrations were expected due to albumin binding, resulting in a small free drug concentration in donor compartment, reducing the share of drug which is able to pass the membrane.

It is crucial to point out the notably higher AM concentrations in the receptor compartment containing polysorbate 80 solution, compared to those in glucose 5% medium, as presented in Figure 6. Due to great affinity of drug towards the micelles of polysorbate 80, the free fraction of AM apparently willingly passes the membrane and enters the receptor compartment containing polysorbate 80 solution at a concentration higher than its CMC. When polysorbate 80 is dissolved in water at concentrations above its CMC (0.012 mM), it forms aggregates known as micelles. Usually, in a micellar solution the aggregation number (for polysorbate 80 it amounts to 60) is approximately constant for a broad total concentration range (up to about a hundred times the CMC), with the number of micelles varying. In our study polysorbate 80 solution was prepared at the concentration a hundred

times higher than its CMC, providing that the polymer will most certainly form micelles. The micelle-forming ability is important not only for the aimed integration of AM into micelles, but it is also especially important from the pharmacological point of view, since upon dilution with a large volume of the blood, considering I.V. administration, only stable micelles of surfactants with low CMC value will still exist and will not dissociate into monomers, nor will they precipitate in the blood. [59] Curiously, drug concentration in donor compartment rised after 3 hours of dialysis, which can most likely be attributed to sample inhomogeneity when sampling from an overly saturated AM Sigma solution in donor compartment. It is of paramount importance to realize that due to high affinity of AM towards the micelles, a part of AM from the albumin-drug complexes was probably no longer bound to albumin and being free, released from albumin, it would therefore be able to pass the membrane. However, sampling from donor compartment containing AM-albumin complexes as well as free drug fraction, forming an oversaturated AM solution, could likely result in an overestimation of AM. The inovative blood purification system described by Wang et al. also showed that affinity of bilirubin towards cyclodextrin-based dialysate overcame its tendency to remain bound to albumin. [58] Thus, it is reasonable to conclude that AM shows higher affinity for the micelles of polysorbate 80 than for albumin, causing the drug to unbind and enter the receptor compartment, which is definitely the outcome we have hoped for.

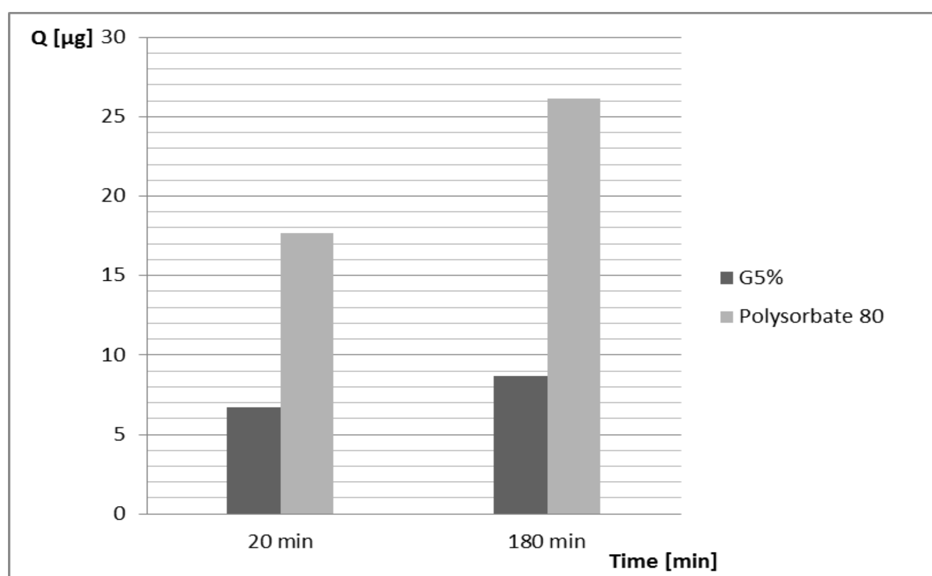


Figure 6. Mass of AM in receptor compartment containing glucose 5% solution or the micelles of polysorbate 80 (after 20 minutes and 3 hours of dialysis).

We tried to perform dialysis under the same conditions, only this time we used saline solution for the receptor compartment. We prepared a polysorbate 80 solution in NaCl 0.9% medium, at concentration a hundred times exceeding its CMC. The results gathered in Table B (Appendix) show the assay failed to give reliable results regarding dialysis performance, because we could not detect the substance in the receptor compartment containing NaCl 0.9% medium, most probably due to instability or, more precisely, due to the lack of solubility of AM in NaCl 0.9%. Based on the fact that AM molecule has a basic centre, we can claim that in acidic medium a larger proportion of the substance stays protonated, while in basic medium the molecule is deprotonated. We also know the substance tends to be soluble when protonated. Since pH value of glucose 5% solution is between 3.5 and 6.5 and thus lower than the pH of saline solution, we can expect the substance to be positively charged and well soluble in glucose 5% medium, allowing a normal HPLC detection. [73] This is why glucose 5% solution was a better choice of medium than NaCl 0.9% solution. AM Bioniche micellar formulation was also reported to have pH of 3.5-4.5 and should be administered in a 5% w/v glucose I.V. infusion. [5]

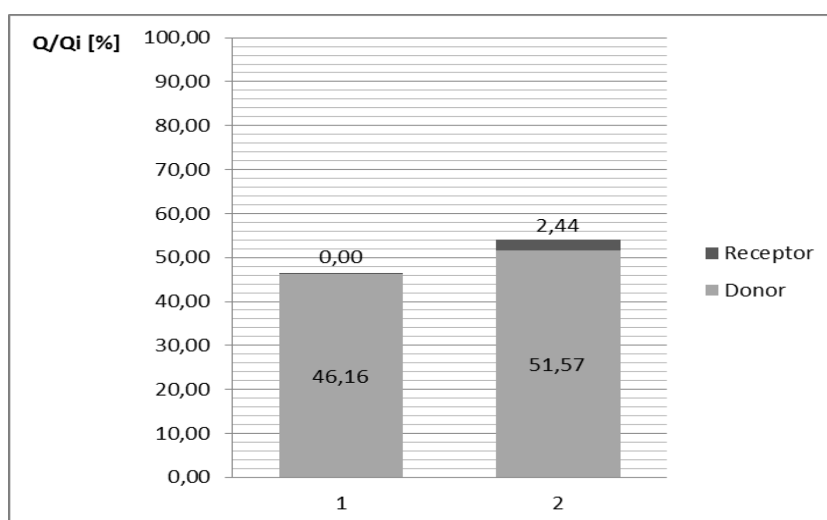


Figure 7. Shares of AM in each compartment after 3 hours of dialysis. Both donor compartments contain AM bound to albumin, while the receptor compartment contains NaCl 0.9% solution (1) or polysorbate 80 solution in NaCl 0.9% medium at 100xCMC (2).

Figure 7 clearly shows that AM exhibits great insolubility in NaCl 0.9% medium, making it almost impossible to detect any AM. Although still facing great losses of substance, the detection was slightly better when micelles of polysorbate 80 were present, but in spite of

some reports claiming the opposite, we clearly proved that (otherwise physiologically suitable) saline solution is undoubtedly not an appropriate medium for AM. [31]

Moreover, regarding Table B (Appendix), we can see the concentration of AM in donor compartment (876 mg/L) largely exceedes the theoretical value (500 mg/L). Although the accuracy of the calculations of AM concentrations in 0.9% NaCl medium is deemed somewhat questionable, since the calibration curves were established from standard solutions based on a different medium (glucose 5% solution), it is assumed it was the sample inhomogeneity, originating from the exceeded solubility product, that lead to the observed large overestimation of AM.

For future research we should try weighing donor compartments to assess the impact of water shift regarding mass changes, due to possibilty of water transfer into albumin-containing donor compartment. However, like previously suggested, this could be avoided by addition of dextran. What is more, instead of measuring the free fraction of AM, it would be interesting to apply a bathocromic shift method – a spectroscopy method for determining both, free drug and drug-protein concentration, without separating them, since drug-albumin complexes also showed UV shift towards a longer wavelength. [65] This could yield more accurate conclusions on unbinding of albumin-bound AM when facing the receptor compartment with polysorbate 80 micelles, leading to better understanding of AM behaviour during dialysis intended for a potential blood purification.

5.4 Dialysis study of AM bound to albumin passing into an emulsion (Medialipide 2%)

Medialipide is an emulsion for infusion (oil-water emulsion, where water is the external phase), otherwise known under name Lipofundin MCT/LCT 20%, contains soya-bean oil, medium-chain triglycerides, glycerol and lecithin, and is used for parenteral nutrition of hospitalized patients. A diluted emulsion is injected into patient's blood flow to deliver the necessary amount of lipids. [74] Since AM is a lipophilic molecule with a very low water solubility, it is assumed it would exert higher affinity towards lipidic medium of emulsion in comparison to a water-based medium.

The equation for the lower calibration curve was used to calculate all the concentrations of AM (y = 42.575x – 25.218). Like previously described in the earlier studies, the respective quantities were calculated from corresponding concentrations and a cumulative profile of drug mass passing into the receptor compartment was presented, while considering the correction due to mass loss during the sampling in the receptor compartment. Further on, we also calculated the respective percentage of quantities, like presented in Table D (Appendix). Regarding the concentrations in the receptor compartment, at first we can notice a more gradual rise in quantity and afterwards the values approach, but do not actually reach, the plateau, like presented in Figure 8.

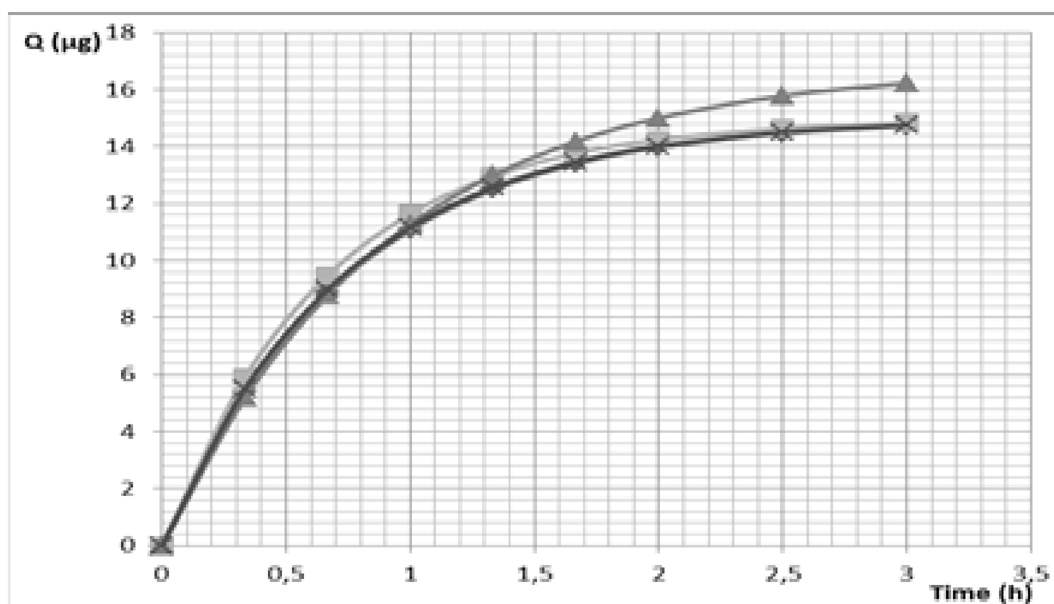


Figure 8. Relation between time of dialysis and quantity of AM Sigma in the receptor compartment. Dialysis was performed over 3 hours in emulsion diluted in glucose 5% medium, while the donor compartment contained AM Sigma in albumin solution.

Due to dilution during the precipitation of samples from donor compartment by acetonitrile, conducted in the same way as described before, the theoretical concentration of AM in the precipitated samples taken at the beginning of the assay, considering the dilution factor, is again 25 mg/L.

500 mg/L/20=25 mg/L

However, like already explained, the measured concentration calculated from the lower calibration curve is lower than the concentration calculated above (25 mg/L), most likely due to solubility problem and non-specific adsorption.

c(free AM)$_{measured}$*=376.34/20 mg/L=*<u>*18.82 mg/L*</u> *(considering dilution factor is 4x5=20)*

The difference between the theoretical and the measured value in donor compartment was higher in the previous assay (with the micelles of polysorbate 80 in receptor compartment), where the measured AM concentration amounted to 14.4 mg/L. The discrepancies probably arise from the previously mentioned solubility problem and non-specific adsorption, resulting in an unpredictable drug detection.

Adding the mass from the receptor compartment to the mass from the donor compartment after 3 hours gave us the total quantity which turned out to be lower from the mass from the donor compartment at the beginning of dialysis (considered to be the overall mass of AM), implying there were some losses affecting the detected concentrations.

According to Table D (Appendix), the amount of AM in the receptor compartment also appears higher than the respective values for albumin-bound AM passing into glucose 5% medium (Appendix; Table C, column G5%). However, the respective quantity is still lower than the one from the receptor compartment containing the micelles of polysorbate 80 (Appendix; Table C, column Polysorbate 80). Based on the fact that AM is a lipophilic substance, it is expected that it would be preferably distributed to lipophilic environment such as the oil phase of an oil/water emulsion, which lead us to the assumption that AM exhibits considerable affinity towards oil-water emulsion.

Also, we can conclude the emulsion does not pass the membrane since there were no additional peaks in the chromatogram for the samples from donor compartment after 3 hours of dialysis, which is a preferable outcome, because we would not like to perturb blood lipids concentrations of the dialysed patient. The emulsion consists of nanometer-sized droplets which cannot pass the membrane due to their size exceeding the small MWCO of the membrane (3500 Da). Moreover, even provided that the emulsion's stability was perturbed, it is not likely that the particles would be transferred to donor compartment

due to processes originating from kinetic instability (like coalescence), resulting in the formation of even larger particles that cannot pass the membrane. In conclusion, apparently the emulsion does not show any tendency to pass the membrane. However, in future assays it would be convenient to test its tendency to pass into human blood, normally containing different lipids and lipidic components at a certain concentration, under conditions similar to the real hemodialysis.

If the samples from the receptor phase were injected directly into the HPLC system, a great amount of interferences would appear in the obtained chromatograms, hampering the precise defining of peak areas of AM and severely damaging or even destroying HPLC column. The interferences and additional peaks in the chromatogram would be caused by triglycerides and other lipids from the emulsion. Also, it is likely that the emulsion would flocculate after being injected in the HPLC system, most likely resulting in build-up of an adsorbed layer on column packing, producing additional peaks and changing the overall chromatography. To avoid those inconveniences, we decided to break the emulsion by liquid-liquid extraction, diluting the samples from the receptor phase with our mobile phase (dilution 1/2). However, the mobile phase we used (acetonitrile/KH_2PO_4 buffer solution=70/30, v/v) changed the structure of emulsion, causing its flocculation. The emulsions are a thermodynamically instable system, prepared by a specific technique which enables vast energy input and are stabilized with a surfactant, but by adding our mobile phase into the emulsion, the lipophility of the system was increased, resulting in the increase of attractive forces between the droplets of the dispersed oil phase. [62] Subsequently, following the flocculation, we could clearly see the white particles at the bottom of our mixture. This is why it was necessary to filter the mixtures (by using a syringe filter; 0.2 µm) before injecting the samples into the HPLC system. Having filtered the samples, we lost most of the oil phase, trapped in the flocculated particles that could not pass through the filter.

Although we have already assumed AM exerts high tedency towards emulsion Medialipide 2%, the percentages of substance passing across the membrane, plotted in Figure 9, do not confirm those predictions. It seems the share of AM entering the receptor compartment containing emulsion is rather similar to the one for glucose 5% medium, implying that

statements on high affinity of AM towards emulsion were at least exaggerated if not inaccurate, probably due to imprecise calculations, possibly affected by volume shift.

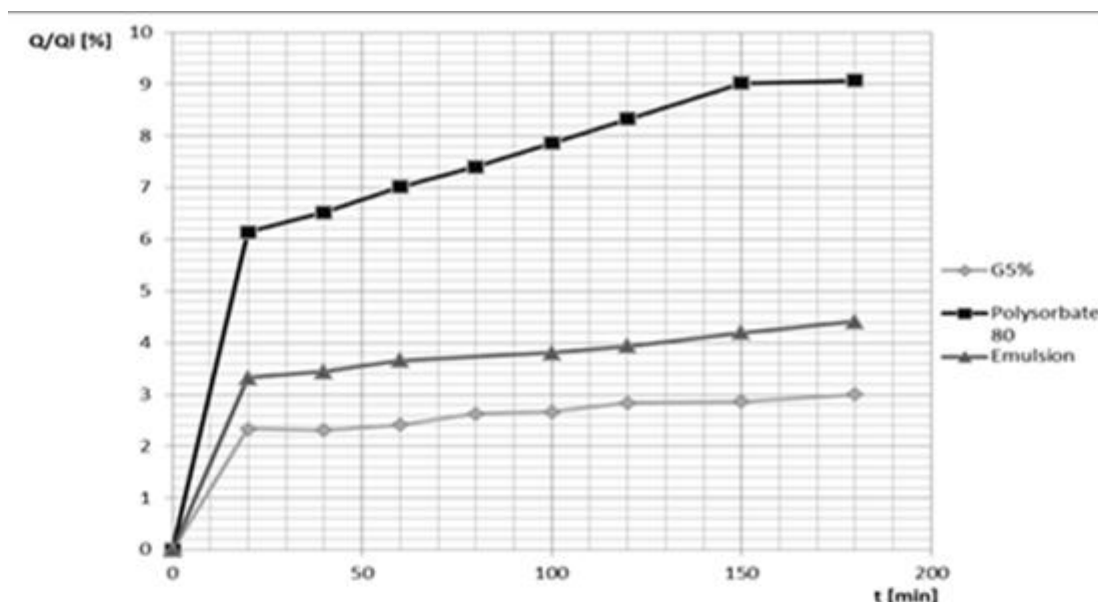


Figure 9. Relation between time of dialysis and share of AM Sigma in the receptor compartment. Dialysis was performed over 3 hours in glucose 5% and emulsion receptor medium, as well as polysorbate 80 solution (100xCMC) in glucose 5% medium, while the donor compartment contained AM Sigma in albumin solution.

In future work it would be appropriate to repeat the conducted study to obtain more reliable and accurate results. Also, we could try other techniques to extract the AM trapped in the flocculated oil phase (for example, the samples from the receptor compartment could be extracted on solid phase extraction sorbent). Eventhough AM has shown some affinity towards emulsion, a dialysate based on emulsion would pose quite a few stability problems, due to its properties of a thermodynamically instable system.

5.5 Mini-hemodialysis system

The following mini-hemodialysis system was developped with the purpose of adapting dialysis conditions to the ones applied in real hemodialysis procedures. We used a real dialysis column consisting of a polyacrylonitrile membrane, with larger pore size than in the previous QuickSep assays. MWCO of 35-40 kDa (in contrast to the previously used

membrane with MWCO of 3500 Da) would allow larger molecules like beta2-microglobulin to pass across the membrane into the receptor compartment, unlike albumin which is not able to leave the donor compartment due to its size of 68 kDa.

The system was washed with a diluted ethanol solution to prevent microbial growth and the dialysate was prepared, like in a hospital hemodialysis treatment, from diluted acidic concentrate (Diasol). A peristaltic pump caused AM in albumin solution to move at the flow rate of approximately 11 mL/min and dialysate to run at more than two-fold flow rate (approximately 24 mL/min), but due to the properties of this specific pump, we could not provide countercurrent direction of dialysate flow. However, larger pore size, as well as higher dialysate flow rate providing a considerable concentration gradient, result in higher rate of diffusion, thus reaching values comparable to those from QuickSep study in a shorter time period, as already seen in Figure 4.

In upcoming assays it would be of great importance to study the impact of countercurrent dialysis flow. Furthermore, micelles of polysorbate 80 should be included into the dialysate, with the goal to prove that AM exhibits great affinity towards the micelles, resulting in quick transfer across the polyacrylonitrile membrane of dialysis column into the receptor compartment. Also, it would be reasonable to use human blood instead of AM in albumin solution and to assess the impact of the suggested blood purification method on blood levels of other (lipophilic) substances. Some of the considerations will be addressed further on.

5.6 OVERALL REVIEW ON DIALYSIS

Since the ultimate goal of this study is to develop a completely new hemodialysis system for blood purification of the drug AM, we applied an in vitro dialysis method as a part of preliminary studies. We observed the relation between time and quantity of AM in the receptor compartment, while using different donor and receptor media. The obtained data was plotted in Excel, thus creating figures showing the mentioned relation between time and quantity. The passage of the drug through the dialysis membrane dividing receptor and donor compartment is deemed to be the rate limiting step of the process, following first

order kinetics. Dialysis constant can be calculated from the drug concentration profile, showing the kinetics of dialysis process. To create the figures showed in this report, we used quantities calculated in a cumulative way instead of the respective concentrations. The equation which describes this dialysis process and which we used to plot our figures is **$Q=Qmax(1-e^{-k*t})$**, with *k* being the dialysis constant, *t* representing the time at which sampling took place (x axis), *Q* being the corresponding cumulative quantity (y axis) and *Qmax* being the maximum value of quantity. The corresponding constants and Qmax values were also calculated by solver add-on module, minimizing the deviation of experimental measurements from the proposed model.

Each type of the assay was repeated at least two times on two different days. As presented in the respective figures in Appendix (Figure 10, 11, 12, 13), we can roughly estimate that our study generally shows intraday precision as well as some interday precision. This was confirmed with ANOVA tests, although the deviation of results was more pronounced in assays with polysorbate 80 solution in the receptor compartment (Figure 11). Nevertheless, the studies should be repeated several times to obtain more reliable information on its applicability.

Table I. Table shows the average maximal quantity of AM in receptor medium (Qmax) and the average dialysis constant (k) for the dialysis process equation: $Q=Qmax(1-e^{-k*t})$. Data are presented as a mean value +/- SEM (standard error of the mean).

Dialysate	Donor compartment	Qmax	k [1/h]
G5%	Amiodarone Sigma (albumin sol.)	8,15 +/- 0,468	4,69 +/- 0,113
Polysorbate 80	Amiodarone Sigma (albumin sol.)	19,45 +/- 1,594	3,29 +/- 0,187
G5%	Amiodarone Sigma (G5% sol.)	12,32 +/- 0,912 *	0,73 +/- 0,017 *
G5%	Amiodarone Bioniche (G5% sol.)	10,31 +/- 0,614	0,70 +/- 0,075
G5%	Amiodarone Sigma and Bioniche (G5% sol.)	10,97 +/- 0,632	0,75 +/- 0,015

As we can see in Table 1, the dialysis rate konstant (k) for polysorbate 80 as a receptor medium is only marginally lower than that of glucose 5% (3.29 vs 4.69 1/h). This is consistent with the hypothesis that the rate limiting step of the equilibrium process is the passive diffusion of free AM through the dialysis membrane. However, the slightly lower constant for polysorbate 80 as receptor medium is a likely consequence of the previously

described colloidal kinetics, resulting in the inclusion of AM into the micelles of polysorbate 80. On the other hand, the maximum passed quantity (Qmax) observed in polysorbate 80 medium is more than two times higher than in glucose 5% receptor medium, which is in accordance with the assumption of AM having a higher affinity towards polysorbate 80 micelles compared to aqueous glucose 5% solution. Bearing in mind that only the free fraction of AM can pass the dialysis membrane, and that free and protein-bound AM are in constant equilibrium, it is safe to assume that a part of the bound drug was unbound and then passed through the membrane. The more willing passage of AM towards the polysorbate 80 receptor medium indicates that this might be a promising new blood purification method.

Due to different dialysis and sampling conditions (since the kinetics of dialysis is influenced by the time of dialysis and the presence of albumin in donor compartment), the dialysis constants for AM Sigma and Bioniche alone passing into glucose 5% medium are notably lower than those of AM bound to albumin. But if we compare the dialysis constants of AM Sigma passing into glucose 5% with the ones representing AM Bioniche, we can notice a difference with a slightly lower dialysis constant of AM Bioniche. Furthermore, Qmax value is somewhat lower for the micellar formulation of AM Bioniche, which is most likely the consequence of micellar release which takes place in donor compartment, slowing the passage of AM into receptor compartment, and the affinity of the drug towards the micelless of polysorbate 80. However, although the membrane passage of the substance is hampered by the micellar release process, the membrane still represents the main rate limiting step, resulting in the first order kinetics.

To sum up, since the highly lipophilic AM is compatible with the applied materials (polypropylene flasks), we attributed the notable losses of substance during the experiment, observed in all assays, to drug solubility problem and drug adsorption onto the membrane or other surfaces. However, when dealing with albumin-bound AM in donor compartment, it can be assumed that obtained concentrations exceeding the overall drug concentration were not only due to imprecise calculations caused by water shift, encouraged by Gibbs-Donnan effect, but also due to sample inhomogeneity when sampling from an overly saturated AM Sigma solution in donor compartment. The general fact that the concentration of AM was never equal on both sides of the membrane, does not mean the equilibrium had not been attained, because the measurements were seen to practically

reach a constant value. Bearing in mind that only free fraction (but not micellar or protein-bound drug fraction) plays role in attaining the equilibrium between both compartments and considering the observed solubility problem, the main culprit for this phenomenon was probably the inability to accurately detect AM in donor compartment, resulting in its overestimation.

In view of utility and efficacy of blood purification, stable micelles of polysorbate 80 with low CMC seem to offer a better choice than emulsion-based dialysate. Although I.V. emulsion complies with sterility and other requirements for dialysis, and despite AM showing affinity towards Medialipide 2%, emulsions represent an unstable, rather easily perturbed system, thus bringing the concern of being affected by the high dialysate flow rate, which would definitely diminish the effectiveness of conducted hemodialysis. What is more, there is a high chance that larger membrane pore size would allow the emulsion constituents to enter blood compartment and disturb triglyceride and other lipid blood levels, presenting a whole new level of problems.

All in all, we have to take in account that the real conditions of hemodialysis differ from the ones we applied in our in vitro dialysis study. The membrane in the dialysis column used in real hemodialysis, like the one used in mini-hemodialysis study, has a larger MWCO and is performed at higher blood and dialysate flow rates, thus resulting in higher dialysis rate. Also, larger membrane pore size could lead to polysorbate 80 passing the membrane and entering the donor compartment, which is fortunately not very likely to happen due to the size of micelles (76 kDa), but is still obviously not an outcome we would wish for. [33] On the other hand, although it has been reported to cause hypotension, polysorbate 80 is luckily not extremely toxic and is frequently used in different I.V. formulations like for instance AM Bioniche. [5, 29] However, it would be important to choose a suitable membrane with reasonably low MWCO, providing a good selectivity and efficiency, and the need of I.V. volume replacement would depend on the porosity of membrane. Another important benefit is that micelle-based dialysate replacement would not be as costly as for instance albumin-based dialysate in MARS, but its saturation capacity remains to be determined. In future work we would also have to try applying polysorbate 80 solution into receptor compartment of mini-hemodialysis system and adapt the conditions of our dialysis assays in order to maximally approach the real conditions of hemodialysis. In the first place it would be necessary to establish countercurrent dialysate

flow and increase the flow rates. Nevertheless, it seems reasonable not to push the flow rates to very high values, in order to avoid additional adverse effects in already hemodynamically instable patients intoxicated with AM. Further on, it would be essential to prove stability of micelles when submitted to higher dialysate flow rates. Moreover, before including human plasma in donor compartment, it would be necessary to assess the interferences of DEA metabolite and other frequently concomitantly used cardiovascular drugs with AM, creating difficulties in drug determination by the proposed HPLC/UV analysis of suitably treated plasma samples. However, certain studies report no interferences were found in similar HPLC determinations of AM in biological fluids. [75, 76] Duration of hemodialysis treatment would have to be empirically established, but since one MARS treatment with albumin-based dialysate usually lasts between 6 and 8 hours, we can assume the time of blood purification with micelle-based dialysate would probably not be longer. In hemodialysis water-soluble vitamins have to be regularly supplemented, but in blood purification system with a dialysate based on micelles of polysorbate 80 we would also have to determine whether supplementation of fat-soluble vitamins (like vitamin A, D, E and K) would be required. In addition, other lipophilic and protein-bound substances (like bilirubin and several hormones) would have to be tested to determine whether they enter the receptor compartment, leading to disturbed blood levels of the respective substances. However, the disturbances might be bypassed by pre- or after-dialysis supplementation.

In general, an overdose on AM is treated symptomatically, including induced emesis and administration of positive inotropic or vasopressor agents, beta agonists and glucagon. Discontinuation of AM requires introduction of a different antiarrhythmic therapy. [4] AM is a potent antiarrhythmic for treatment of severe arrhythmias, which have been connected to sudden cardiac death, where the loss of heart function cannot be reversed not even after quick professional resuscitation. Eventhough this sudden cardiac event seems to be trending downward, this is not true for sudden cardiac death among young people, and having in mind the longer life expectancy, we can assume that the use of this specific medication and drugs in general should rise. [77, 78] The widespread use of AM, a drug with unpredictable pharmacokinetic profile, is likely to result in more cases of intoxication, hence a new hemodialysis method would be of great utility for severe acute or chronic toxic events, experienced by patients treated with AM, as well as it shows considerable

potential to be applied in hepatorenal syndrome, liver failure and for blood purification of many other lipophilic protein-bound drugs where conventional dialysis is not posssible.

Taking everything into consideration, besides showing a great potential for blood purification of protein-bound drugs, our study also opened new questions that need to be addressed in following researches, as listed below.

- Non-specific adsorption and solubility problem should be tackled in future assays, since this leads to various limitations of our study, as already discussed above, including:
    - Inability of forming a uniform calibration curve for AM Sigma and Bioniche.
    - Apparent inability to reach an equilibrium between donor and receptor compartment, not even after 7 hours of dialysis, and inability to attain mass balance between donor and receptor compartment.
    - Rise in AM concentration in donor compartment after having finished the experiment.
    - Inter-assay discrepancies of the measured AM concentration in donor compartment.
- Albumin concentration did not match the real conditions in blood, thus influencing the protein binding of AM, equilibrium between both compartments and the passage of AM across the membrane during our dialysis study with AM bound to albumin, and due to non-linear protein binding kinetics, our results cannot be extrapolated to lower, physiological albumin concentrations. Hence, it is suggested to test protein binding of AM with lower concentrations of albumin.
- Analytical method used for our experiments showed inadequate sensitivity, since it was only possible to apply our studies for a therapeutically irrelevant concentration range, with concentrations much higher than the expected drug levels in patients on AM. Thus, it would be necessary to make several adjustments to improve the sensitivity of our method or to use another method with better sensitivity, like LC-MS-MS.

6 CONCLUSION

AM is a potent antiarrhythmic agent, frequently administered I.V. to hospitalized patients, but due to very high degree of plasma protein binding, resulting in unpredictable pharmacokinetics, the drug has a narrow therapeutic index. In order to improve treatment of intoxicated patients suffering from serious, often life-threatening conditions and despite the general belief that protein-bound substances cannot be dialysed, a possibility of development of a new blood purification system based on micelles of polysorbate 80 as part of dialysate has been studied. An in vitro dialysis study with QuickSep system proved that albumin-bound AM in donor compartment exhibits high affinity towards polysorbate 80 solution in receptor compartment, at a concentration a hundredfold higher than its CMC, believed necessary for the formation of micelles. According to the results of our dialysis study, in which the free drug fraction has been determined, AM unbinds from albumin, passes across the membrane, enters the receptor compartment and binds to the stable micelles of polysorbate 80. Tendency to enter the compartment containing the micelles was more than two times higher than in case of glucose 5% solution alone. All samples were submitted to HPLC analysis and the respective concentrations were calculated from two validated calibration curves. Nonetheless, detection of AM was especially challenging due to its low solubility and non-specific adsorption. AM was also proved unstable in saline solution. Furthermore, an I.V. emulsion has been tested in the role of dialysate. Although AM again showed some tendency towards the lipid parenteral formulation, the results are not as reliable as the ones with micelle-based dialysate, due to the loss of oil phase during sample preparation. However, due to hypothetically greater thermodynamical instability of emulsion, compared to the micelles of polysorbate 80, the latter seems to be a more clever choice, offering a low-cost dialysate replacement. Lastly, a mini-hemodialysis system with a real dialysis column with larger MWCO has been developed, with lower flow rates than the ones in real therapy and without dialysate running in countercurrent direction. With albumin-bound AM in donor compartment, AM levels in diluted acidic concentrate, representing a conventional dialysate, were sooner reached, but similar to those from the QuickSep study. The effect of polysorbate 80 micelles in dialysate remains to be examined in future assays, where the following suggestions should be considered:

- Improving sensitivity of the proposed analytical method.

- Adjustments needed for HPLC analysis of samples containing emulsion.
- Determining the solubility of AM in different mediums.
- Applying lower, relevant concentration range.
- Providing mass balance in dialysis experiments.
- Establishing countercurrent dialysis flow and adequate flow rates in our mini-hemodialysis system.

In conclusion, despite the mentioned limitations, a great potential has been discovered in this study, not only for treatment of AM intoxications, but also for blood purification of many other lipophilic protein-bound drugs where conventional dialysis is not posssible.

7 APPENDIX

Table A. Dialysis of AM Sigma and AM Bioniche in donor compartment against glucose 5% receptor medium. Time of dialysis with QuickSep system was 7 hours, while sampling was done every hour, at constant volume.

	t [h]	Response [mAU*s]		c [µg/mL]		m [µg]		m (corr) [µg]		Q/Qi [%]	
Medium		Sigma	Bioniche	Sigma	Bioniche	Sigma	Bioniche	Sigma	Bioniche	Sigma	Bioniche
Donor	0	2444,9	2435,9	51,86	51,68	51,86	51,68	51,86	51,68	100	100
Receptor	0	0	0	0	0	0	0	0	0	0	0
	1	9,94	5,21	0,83	0,72	8,26	7,15	8,26	7,15	15,92	13,78
	2	14,14	10,53	0,92	0,84	9,24	8,4	9,66	8,75	18,62	16,88
	3	0	14,36	0,59	0,93	5,92	9,3	7,26	10,49	14	20,23
	4	18,51	10,26	1,03	0,83	10,27	8,33	12,2	10,46	23,52	20,17
	5	3,66	5,1	0,68	0,71	6,78	7,12	9,74	10,08	18,78	19,44
	6	14,26	4,58	0,93	0,7	9,27	7	12,91	10,67	24,89	20,57
	7	16,64	9,84	0,98	0,82	9,83	8,23	14,39	12,61	27,75	24,31
Donor	7	1227,55	1506,55	26,98	32,68	26,98	32,68	26,98	32,68	53,96	65,37
Receptor+donor								41,38	45,29	81,72	89,68
Loss										18,28	10,32

Table B. Dialysis of AM Sigma in albumin solution in donor compartment against NaCl 0.9% and polysorbate 80 solution (100xCMC) in NaCl 0.9%. Time of dialysis with QuickSep system was 3 hours, while sampling was done every 20 minutes and every 30 minutes in the last hour of the assay, at constant volume.

	t [h]	Response [mAU*s]		c [µg/mL]		m [µg]		m (corr) [µg]		Q/Qi [%]	
Medium		NaCl 0.9%	Poly 80	NaCl 0.9%	Poly 80	NaCl 0.9%	Poly 80	NaCl 0.9%	Poly 80	NaCl 0.9%	Poly80
Donor	0	2050		876		876		876		100	100
Receptor	0	0	0	0	0	0	0	0	0	0	0
	1	0	27,43	0	1,24	0	12,37	0	12,37	0	1,41
	2	0	30,66	0	1,31	0	13,12	0	13,74	0	1,57
	3	0	33,51	0	1,38	0	13,79	0	15,72	0	1,8
	4	0	31,33	0	1,33	0	13,28	0	16,59	0	1,89
	5	0	28,86	0	1,27	0	12,7	0	17,34	0	1,98
	6	0	35,78	0	1,43	0	14,33	0	20,24	0	2,31
	7	0	34,43	0	1,4	0	14,01	0	21,35	0	2,44
Donor	7	846,3	948,49	409,4	457,4	409,4	457,4	409,4	457,4	46,16	51,57
Donor+receptor								409,4	478,76	46,16	54,01
Loss										53,84	45,99

Table C: Dialysis of AM Sigma in albumin solution in donor compartment against glucose 5% solution and polysorbate 80 solution (100xCMC) in glucose 5%. Time of dialysis with QuickSep system was 3 hours, while sampling was done every 20 minutes and every 30 minutes in the last hour of the assay, at constant volume.

	t [min]	Response [mAU*s]		c [µg/mL]		m (corr) [µg]		Q/Qi [%]	
Medium		G5%	Polysorbate 80	G5%	Polysorbate 80	G5%	Polysorbate 80	G5%	Polysorbate 80
Donor	0	587,26		288		288		100	
Receptor	0	0	0	0	0	0	0	0	0
	20	3,42	50,07	0,67	1,77	6,73	17,68	2,34	6,15
	40	1,72	50,88	0,63	1,79	6,66	18,76	2,32	6,52
	60	1,6	53,18	0,63	1,84	6,95	20,19	2,42	7,02
	80	2,87	53,95	0,66	1,86	7,57	21,29	2,63	7,4
	100	1,93	55,7	0,64	1,9	7,67	22,63	2,67	7,87
	120	2,73	57,31	0,66	1,94	8,18	23,96	2,84	8,33
	150	1,66	61,7	0,63	2,04	8,26	25,96	2,87	9,02
	180	1,99	57,96	0,64	1,95	8,65	26,11	3,01	9,07
Donor	180	588,08	613,5	288,1	300,04	288,1	300,04	100,13	104,28
Receptor+donor						296,75	326,15	103,14	113,36
Loss								-3,14	-13,36

Table D. Dialysis of AM Sigma in albumin solution in donor compartment against Medialipide 2% after filtration. Time of dialysis with QuickSep system was 3 hours, while sampling was done every 20 minutes and every 30 minutes in the last hour of the assay, at constant volume.

	t [min]	Response [mAU*s]	c [µg/mL]	m [µg]	m (corr) [µg]	Q/Qi [%]
Donor	0	775,91	376,34	376,34	376,34	100
Receptor	0	0	0	0	0	0
	20	4,11	1,38	13,78	13,78	3,66
	40	NA	0	0	0	0
	60	1,08	1,24	12,35	13,04	3,47
	80	1,09	1,24	12,36	13,66	3,63
	100	1,89	1,27	12,73	14,04	3,73
	120	2,04	1,28	12,8	15,36	4,08
	150	1,12	1,24	12,37	15,57	4,14
	180	1,75	1,27	12,67	16,49	4,38
Donor	180	687,79	334,94	334,94	334,94	89
Receptor+donor					351,43	93,38
Loss						6,62

Table E. Validation of the lower AM Bioniche calibration curve at the detection wavelength of 240 nm (y = 42.575x – 25.218).

	Day 1			Day 2			Day 3									Intraday			Interday		
c [µg/mL]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Average Response [mAU*s]	SD	CV [%]	Average Response [mAU*s]	SD	CV [%]
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0,5	0	0,59	118,46	0	0,59	118,46	5,42	0,72	143,92	5,4	0,72	143,83	5,14	0,71	142,61	5,32	0,16	2,94	1,77	3,07	173,21
0,75	8,55	0,79	105,75	11,63	0,87	115,4	8,42	0,79	105,35	8,05	0,78	104,19	7,78	0,78	103,34	8,08	0,32	3,97	9,42	1,93	20,46
1	19	1,04	103,86	33,83	1,39	138,69	20,87	1,08	108,25	20,54	1,07	107,48	19,88	1,06	105,93	20,43	0,5	2,47	24,42	8,18	33,5
2,5	45,7	1,67	66,63	48,18	1,72	68,96	56,86	1,93	77,11	53,71	1,85	74,15	52,39	1,82	72,91	54,32	2,3	4,23	49,4	4,44	8,98
5	183	4,88	97,69	175,25	4,71	94,17	181,29	4,85	97,09	177,39	4,76	95,18	174,21	4,68	93,68	177,63	3,55	2	178,54	3,82	2,14
7,5	265	6,81	90,75	262,53	6,76	90,11	287,55	7,35	97,95	282,68	7,23	96,43	274,15	7,03	93,75	281,46	6,78	2,41	269,52	10,39	3,86
10	435	10,81	108,1	470,86	11,65	116,52	385,65	9,65	96,5	384,19	9,62	96,16	381,21	9,55	95,46	383,68	2,26	0,59	429,86	43,82	10,19
20	759	18,42	92,12	777,97	18,87	94,33	880,48	21,27	106,37	873,89	21,12	105,59	860,31	20,8	104	871,56	10,28	1,18	802,89	60,21	7,5
25	1070	25,68	102,72	1073,4	25,8	103,22	1047,06	25,19	100,74	1032,58	24,85	99,38	1018,06	24,5	98,02	1032,57	14,5	1,4	1058,03	22,21	2,1
slope		42,131			42,602			43,501			43,04			42,432							42,57
intercept		-26,115			-22,434			-26,9349			-27,124			-27,257							-25,22
r2		0,993			0,991			0,997			0,997			0,997							0,997

Table F. Validation of the upper AM Bioniche calibration curve at the detection wavelength of 240 nm (y = 48.923x – 92.463).

	Day 1			Day 2			Day 3									Intraday			Interday		
c [µg/mL]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Response [mAU*s]	c (calc) [µg/mL]	recovery [%]	Average response [mAU*s]	SD	CV [%]	Average Response [mAU*s]	SD	CV [%]
5	182,73	5,63	112,5	175,25	5,47	109,44	181,29	5,6	111,91	177,39	5,52	110,32	174,21	5,45	109,02	177,63	3,55	2	178,54	3,82	2,14
7,5	264,56	7,3	97,3	262,53	7,26	96,75	287,55	7,77	103,57	282,68	7,67	102,24	274,15	7,49	99,92	281,46	6,78	2,41	269,52	10,39	3,86
10	435,03	10,78	107,82	470,86	11,51	115,15	385,65	9,77	97,73	384,19	9,74	97,43	381,21	9,68	96,82	383,68	2,26	0,59	429,86	43,82	10,19
20	759,15	17,41	87,04	777,97	17,79	88,96	880,48	19,89	99,44	873,89	19,75	98,76	860,31	19,48	97,38	871,56	10,28	1,18	802,89	60,21	7,5
30	1413,44	30,78	102,6	1324,68	28,97	96,56	1397,97	30,47	101,55	1363,98	29,77	99,23	1343,11	29,34	97,81	1368,35	27,69	2,02	1368,82	44,38	3,24
40	1859,08	39,89	99,73	1862,31	39,96	99,89	2001,97	42,81	107,03	1968,71	42,13	105,33	1932,77	41,4	103,49	1967,82	34,61	1,76	1896,4	61,87	3,26
50	2481,56	52,61	105,23	2435,9	51,68	103,36	2192,27	46,7	93,4	2142,81	45,69	91,38	2122,5	45,27	90,55	2152,53	35,89	1,67	2356,66	178,25	7,56
Slope	50,634			49,453			47,578			46,514			45,953						48,923		
Intercept	-118,92308			-103,79228			-57,751			-52,114			-54,157						-92,463		
r2	0,994			0,994			0,99			0,989			0,99						0,998		

Table G. Two-way ANOVA test with replication, reported as F value vs. F critical value, with respective p-values.

Dialysate	Donor compartment	F	F critical	p
G5%	Amiodarone Sigma (albumin sol.)	0,2545	3,4668	0,7776 (ns)
Polysorbate 80	Amiodarone Sigma (albumin sol.)	3,4534	3,4668	0,0505 (ns)
G5%	Amiodarone Sigma (G5% sol.)	6,0435	3,5546	0,0098
G5%	Amiodarone Bioniche (G5% sol.)	2,8503	3,5546	0,0841 (ns)
G5%	Amiodarone Sigma and Bioniche (G5% sol.)	2,7717	3,5546	0,0893 (ns)

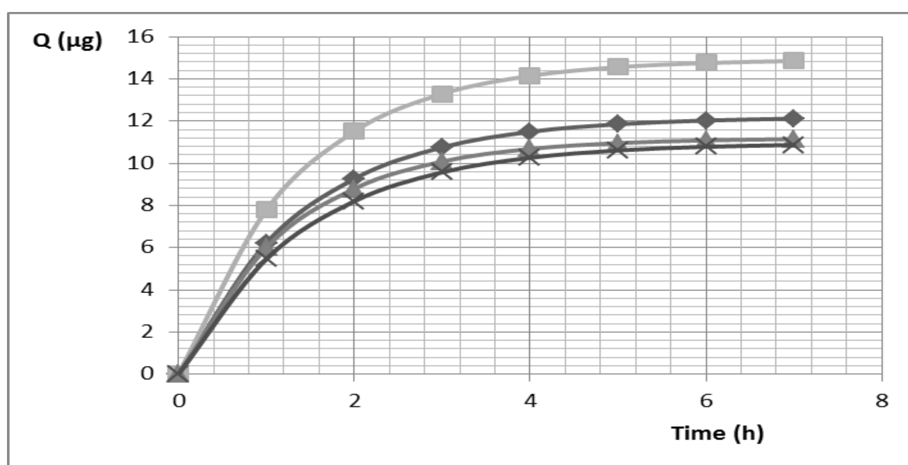


Figure 10. Four independent assays of dialysis were performed over 7 hours in glucose 5% receptor medium on different days (two assays were performed on each day). The figure shows the relation between time of dialysis and quantity of AM Sigma in the receptor compartment.

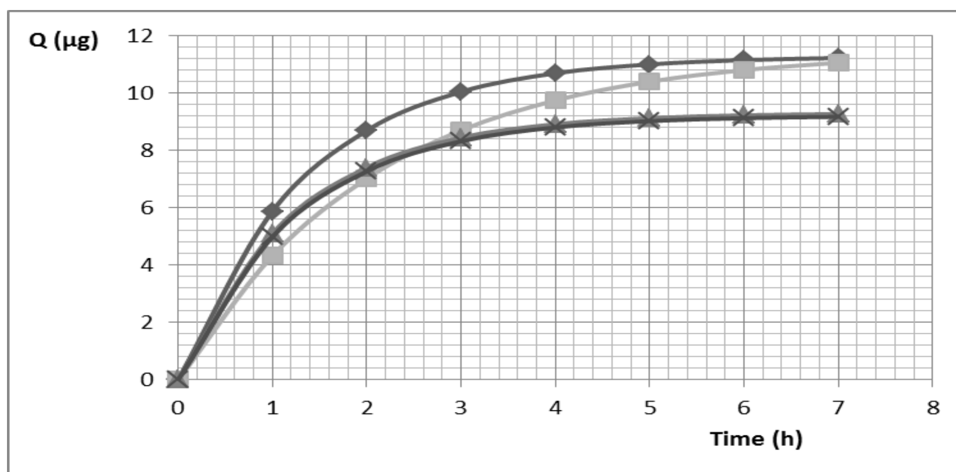


Figure 11. Four independent assays of dialysis were performed over 7 hours in glucose 5% receptor medium on different days (two assays were performed on each day). The figure shows the relation between time of dialysis and quantity of AM Bioniche in the receptor compartment.

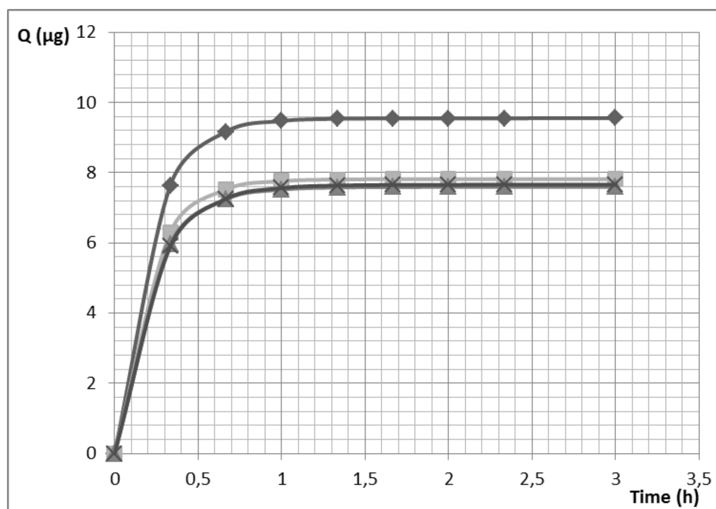


Figure 12. Four independent assays of dialysis were performed over 3 hours in glucose 5% receptor medium on different days (two assays were performed on each day), with donor compartment containing AM in albumin solution. The figure shows the relation between time of dialysis and quantity of AM Sigma in the receptor compartment.

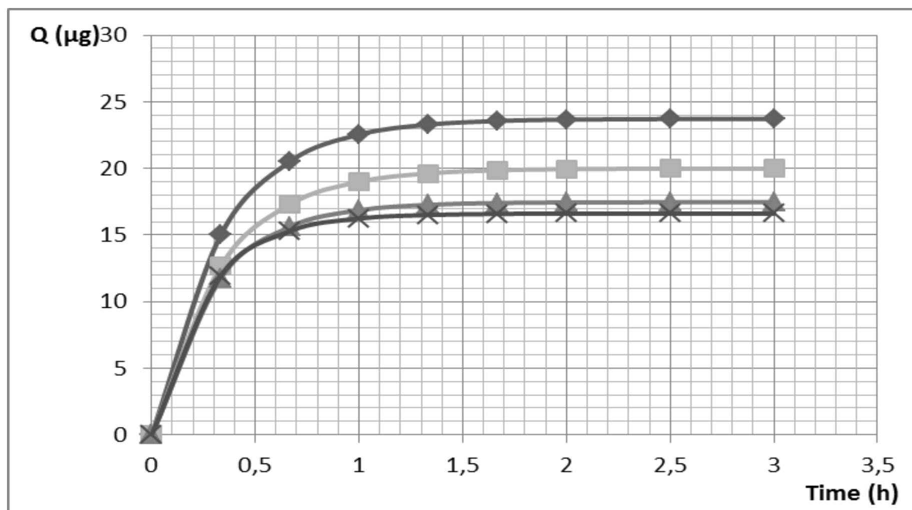


Figure 13. Four independent assays of dialysis were performed over 3 hours in polysorbate 80 receptor medium on different days (two assays were performed on each day), with donor compartment containing AM in albumin solution. The figure shows the relation between time of dialysis and quantity of AM Sigma in the receptor compartment.

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