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Parenteral water/oil emulsions containing hydrophilic compounds with enhanced in vivo retention: formulation, rheological characterisation and study of in vivo fate using whole body gamma-scintigraphy

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Abstract

The preparation and characterization of parenteral water-in-oil (w/o) emulsions with a potential for sustained release of hydrophilic drugs was described with emphasis on rheological behaviour and spreading phenomenon after intramuscular (i.m.) injection in rabbit thigh muscle. Both steady state and dynamic rheological parameters were investigated showing Newtonian behaviour at low fraction of disperse phase ratio as opposed to viscoelastic and pseudoplastic behaviour at high fraction of disperse phase. Disappearance and spreading behaviour of hydrophilic radioactive markers, aprotinin (6512 g/mol) and pertechnetate (193 g/mol) entrapped in w/o emulsions from an i.m. injection site was studied by whole body gamma-scintigraphy. The retention of entrapped aprotinin 24 h postinjection was $83 \pm 5\%$ for a low spreading emulsion and $76 \pm 6\%$ for a high spreading emulsion. The corresponding values for pertechnetate were 50 ± 11 and $23 \pm 2\%$, respectively. The relatively long retention times were suggested to be related to the good physical stability properties of the present emulsions. It was concluded that the presented w/o emulsions are promising vehicles for sustained release of hydrophilic drugs from an i.m. injection site.

Keywords: Sustained release; W/o emulsions; Intramuscular administration; Rheology; Spreading mechanism; Disappearance rates

1. Introduction

Many biotechnologically produced drugs are characterised by poor oral bioavailability due to gastrointestinal degradation or first-pass effect. In addition the plasma half-life is typically short. To

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circumvent these delivery problems, a great variety of parenteral prolonged drug delivery systems have been developed. The depot effect may vary from a few hours to several months depending on the formulation e.g. crystal suspensions such as protamine zinc insulin, biodegradable polymeric implants/microspheres (Smith et al., 1990), gels (Katakam et al., 1997), osmotic pumps (Ranade, 1990), emulsions (Hashida et al., 1977a) and liposomes (Titulaer et al., 1990). In the present study, water-in-oil (w/o) emulsions are investigated. W/o emulsions are flexible systems, to which release properties can be adjusted by several parameters such as volume fraction of disperse phase, droplet size and osmotic gradients (Bjerregaard et al., 1999a). By careful choice of oil phase and surfactants, w/o emulsions can be prepared which are easily administered through a fine needle.

Parenteral w/o emulsions for prolonged release of encapsulated drugs have been known of at least since 1956, where Freund described prolonged release of antigens from a w/o emulsion, known as Freunds Complete Adjuvant, FCA (Freund, 1956). However, the Freund-type emulsions are highly irritating even in the absence of antigen (Woodard, 1989). In addition, mineral oil, one of the oil phase constituents, is not metabolised and may produce chronic inflammation (Symmers, 1955; Bomford, 1981). Other w/o emulsions with bioacceptable excipients have been developed. However, so far prolongation of drug absorption from an intramuscular/subcutaneous injection site with w/o emulsions (Nakamoto et al., 1975; Hashida et al., 1977a; Davis et al., 1987; Bello et al., 1994) and the related water-in-oil-in-water emulsions (Omotosho et al., 1989) has only been moderate. Hence, absorption half-lives in the above mentioned studies have been 2 h at most. Physical stability of the emulsions is typically not quoted in these studies and might be a possible limiting factor for disappearance of drug from the injection site.

Another limitation with liquid formulations after intramuscular (i.m.) injection, is the variability of spreading and dispersion of the drug vehicle as opposed to, for example, implants. The extent of spreading/dispersion determines the total surface area available for release of drug and degradation of the vehicle. The absorption rate from various vehicles is believed to be proportional to the total surface area (Ballard, 1968; Zuidema et al., 1994). The spreadability and/or disappearance rate of vehicles at parenteral injection sites is suggested to be related to the injection site and rheological properties of the vehicle (Schultz et al., 1998), injection volume (Hashida et al., 1980; Schultz et al., 1998), interfacial tension between body fluids and vehicle (Omotosho et al., 1989), mechanical stimuli due to massaging (Hashida et al., 1980; Trubetskoy et al., 1998) or muscle contraction and speed/pressure of injection. The primary aim of the present study was to formulate and test parenteral w/o emulsions with sustained release properties. Secondly, to compare intramuscular spreading and disappearance of w/o emulsions of various viscosities using the non-invasive technique of gamma-scintigraphy.

2. Materials and methods

2.1. Materials

Fractionated coconut-oil (Viscoleo), a medium chain triglyceride–oil (MCT–oil), was supplied from H. Lundbeck A/S (Denmark). The polymeric surfactant, triglycerol polyricinolate-6 (TG PR-6), was kindly donated by Danisco Ingredients (Denmark), while the cosurfactant, Span 80 (sorbitan monooleate), was obtained from Sigma Chemical Company (USA). The aprotinin, Trasylol®, used for radioactive labelling was optained from Bayer. Bovine recombinant aprotinin for cold labelling of the emulsions was kindly donated by Novo Nordisk (Denmark). The $^{99}Mo/^{99m}Tc$ generator, Amertec II, was purchased from Nycomed Amersham (UK). Dulbecco's phosphate buffered saline (PBS) without magnesium and calcium was from Life Technologies (USA). Sephadex G25F for gelfiltration of radioactive labelled compounds was obtained from Pharmacia (Sweden). All other chemicals were of reagent grade.

Table 1
Composition of radioactive labelled w/o emulsions (% w/w)

	I	II
Saline, containing either aprotinin, glucose or pertechnetate	30.0	60.0
Sorbitan monooleate	1.0	1.0
Triglycerol polyricinolate-6	3.5	3.5
MCT–oil	65.5	35.5

2.2. Labelling procedure

Aprotinin, a protease inhibitor (Mw: 6512 g/mol) was labelled with technetium-99m. This is a 140 keV monoenergetic gamma-emitter with a short half-life ($t_{1/2} = 6.01$ h). Labelling of aprotinin was done according to Aprile et al. (1995). A 150 μl volume of aprotinin, Trasylol®, 20 000 KIU/ml, was adjusted to pH 10.5 with 1.9 ml of glycine buffer containing 0.1 mg stannous chloride under nitrogen atmosphere. One ml of Tc-99m pertechnetate solution, freshly eluted from a sterile ^{99}Mo-^{99m}Tc generator, was added to the conjugate solution and incubated for 20 min. Finally, the conjugate solution was sterile filtrated. Labelling efficiencies were assured with both TLC and gel chromatography. In general, there was no free pertechnetate and less than 4% of total radioactivity originating from hydrolyzed ^{99m}Tc.

Pertechnetate, $^{99m}TcO_4^-$ (Mw: 193 g/mol), was also used as a small molecular weight marker. This is the chemical stable form of technetium in aqueous media (Hjelstuen, 1995).

2.3. Preparation of w/o emulsions

The w/o emulsions were prepared by dropwise addition of the aqueous phase to the oil phase under agitation with a magnetic stirrer. The composition of the emulsions is given in Table 1. The concentration of hydrophilic markers in the water phase of emulsions with 30% w/w aqueous phase was either 40 mM D-glucose, 13.4 mM aprotinin or freshly eluted radioactive pertechnetate corresponding to approximately 600 MBq. The concentrations of aprotinin and pertechnetate in the aqueous phase were reduced by half in emulsions with 60% w/w% aqueous phase. Emulsification was achieved with an Ultra Turrax T-25 (Janke and Kunkel, Germany) equipped with dispersing element S25N-25G agitating at 13 500 rpm for 3×1 min. The emulsions were further treated with an ultrasound probe (Branson, USA) equipped with a microtip at an output value of 2 for 3×1 min. Emulsification was carried out in an ice-cold water bath.

2.4. Factorial design

W/o emulsions were prepared as described above. The effect of homogenisation power, phase ratio and temperature on mean droplet size of the dispersed aqueous phase was studied by a two level full factorial design. The levels of the factors are listed in Table 2. The emulsions were stored at 4°C until the mean droplet size was determined by photon correlation spectroscopy (PCS) approximately 24 h after preparation. The PCS system consisted of a Malvern Zetasizer 4 (Malvern Ltd., UK) with a helium–neon laser (wavelength = 633 nm) and a 7032 Multi-8 correlator. About 1–3 μl

Table 2
Factorial design: variables and factor levels

Factor	Factor level		
	Low (−)	Center (0)	High (+)
X_{power}: Homogenisation power (arbitrary units)	2	–	3
X_Φ: Amount of aqueous phase (% w/w)	30	50	60
X_T: Temperature of emulsion during homogenisation (37°C)	0	–	37

of emulsion was dispersed in 1 ml of isopropylmyristate (viscosity = 4.9 mPas) previously saturated with water. Measurements were carried out at 25°C and at a scattering angle of 90°. All sizes given are z-average-mean values.

2.5. In vitro release

The hydrophilic marker molecules, [^{3}H]glucose (Mw: 180 g/mol) and aprotinin (Mw: 6512 g/mol), were released into a PBS buffer, preserved with 0.05% w/v sodium azide, using a Hanson Transdermal diffusion cell system (Hanson Research Co., USA) at a temperature of 37°C. A weighed amount of emulsion (approximately 200 mg) was placed on 3.7 ml of PBS pH 7.4 without any membrane separating emulsion and buffer. The interfacial area between sample and release buffer was 0.67 cm^2. The release buffer was agitated with a magnetic bar. Samples were regularly withdrawn during a period of 72 h and were replaced with fresh PBS. All glassware used in experiments with aprotinin was siliconized with Aquasil™ (Pierce, USA). Glucose was quantified by liquid scintillation with a Tricarb counter (Packard, the Netherlands). Aprotinin was quantified with a trypsin inhibition test using Z-lysine thiobenzylester as substrate.

2.6. Rheological characterisation of emulsions

The rheological characteristics of the emulsions, except the oscillatory measurements, were examined with a controlled stress Carri-Med CLS^2 100 rheometer (TA Instruments) using cone-plate geometry. Cone radius and angle were 4 cm and 1°, respectively. Two types of analysis were conducted with the controlled stress rheometer. (i) Shear stress–shear rate experiments, where shear viscosities for two w/o emulsions with 30 and 60% w/w disperse phase, respectively, were obtained at increasing stresses followed by decreasing stresses; and (ii) Temperature sweeps. Emulsions with various phase ratios were measured at constant shear stress while increasing the temperature from 4 to 40°C by 1°C/min.

The viscoelastic parameters of two w/o emulsions with 30 and 60% w/w disperse phase, respectively, were investigated with a Bohlin VOR Rheometer (Bohlin Reologi, Sweden), a controlled rate rheometer, in the dynamic oscillation mode. The measuring system used was a concentric cylinder, C14, maintained at 37°C. The elastic (G') and loss (G'') moduli were measured during an oscillation frequency sweep. Measurements were carried out in the linear viscoelastic region verified by a strain sweep.

2.7. Interfacial tension measurements

A K10 tensiometer with a platinum–iridium Du Noüy ring (Krüss GmbH, Germany) was used to determine interfacial tension between rabbit plasma and oil phase and a range of serial dilutions of oil phase (1.4% w/w sorbitan monooleate, 5.0% w/w triglycerol polyricinolate-6, 93.6% w/w MCT–oil) with MCT–oil. The Du Noüy ring (circumference, 59.9 mm) was cleaned with acetone, and lightly flamed prior to each measurement. The experiments were conducted at 37°C after approximately 30 min of equilibrium of the interface. All measurements were carried out in duplicate.

2.8. In vivo spreading and disappearance studies

Female New Zealand White rabbits weighing 2.8–3.5 kg were used in all experiments. Two emulsions with a water fraction of 30 and 60% w/w and a viscosity of 50 and 380 mPas at 37°C, respectively, were injected in rabbit hind leg muscle, *vastus lateralis*, in a cross-over design. In the cross-over design, three rabbits were injected bilaterally in the hind legs with an emulsion containing 30 and 60% w/w disperse phase, respectively. The injected volume was 300 μl, corresponding to approximately 60 MBq of radioactive marker. The injection area, 3 cm proximal to the knee joint, was shaved prior to injection. Injections were made with a 0.5×16 mm needle and a 1 ml Hamilton syringe at an angle of 90°. The injection speed was kept as constant as possible. A uniform injection depth of 10 mm was controlled by a needle stop.

The animals were housed in individual cages and had free access to food and water. Further-

more, they were free to exercise between image acquisitions. Each experiment was performed in triplicate.

Disappearance and spreading of the hydrophilic gamma emitter from the injection site as followed with an Argus gamma camera, ADAC (Netherlands) during a 24-h period. Upper body planar images were recorded in a 128×128-pixel matrix using an ultrahigh resolution 140 keV collimator. The image acquisition time was varied between 1 and 5 min according to radioactivity at the injection site. During imaging the rabbits were restrained. Any movements during imaging meant that the image had to be recollected. Each rabbit was imaged together with a reference source, a glass container with a diameter of 28 mm containing 300 μl of radioactive labelled emulsion dispersed in 10 ml oil.

The amount of gamma activity present at the injection site and in the reference was obtained from computer generated images. Data were corrected for decay by dividing the counts in the injection site area with the counts in the reference source area.

The percentage of emulsion remaining at the injection site was calculated relative to the first image recorded 5 min post-injection.

The extent of spreading of emulsions after i.m. injection was determined from the computer generated images as described by Schultz et al., 1998. A line was drawn through the unit area (pixel) with highest radioactivity and across the widest distribution of radioactivity (Fig. 1A). Next, the profile of radioactivity along the line was depicted in a diagram. The extent of spreading was then defined as the width of the curve at the level of 10% of the highest radioactivity per pixel (Fig. 1B).

Statistical analysis of results from the spreading study was performed using the Students *t*-test for paired data.

The in vivo spreading of emulsions with 30 and 60% w/w disperse phase was also investigated qualitatively. A volume of 100 μl of the emulsions, coloured with Sudan Black, was injected central in rabbit *triceps brachii* muscles in a depth of 10 mm using the same technique as described above. Rabbits were killed 2 h post-injection by

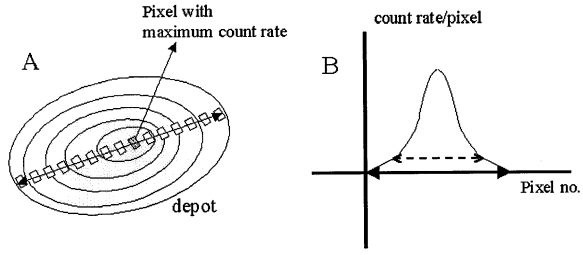

Fig. 1. Illustration of the method to characterise the in vivo spreading of radioactive labelled w/o emulsions in a quantitatively manner. (A) Insertion of a line across the widest distribution of radioactivity and through the pixel with highest count rate on a gamma camera image of the injection site. (B) The number of counts in each pixel under the line is depicted in a graph. The extent of spreading is taken as the width of the curve (broken line) at 10% of the maximum radioactivity as originally shown by Schultz et al. (1998).

an intravenous injection of pentobarbital. The *triceps brachii* muscles were dissected and examined macroscopically. The muscles were frozen in isopentane, cooled to -78°C with dry ice. Approximately 8 mm thick cross-sectional discs, containing the injection site, were cut with a saw from the frozen muscles. The discs were mounted on cryostat microtome holders. Ten micrometer thick cryo-sections were cut and sampled on microscope glass slides. The sections were exposed to OsO_4 vapours for 24 h at room temperature in a sealed jar to stain lipids. The sections were counter-stained with toluidine blue and examined by light microscopy.

2.9. Data analysis: factorial experiment

Size data from the factor experiment were analysed with Modde 4 software (Umetri AB, Sweden) using a multiple linear regression model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + e,$$

where Y is the response parameter of interest, i.e. mean droplet diameter of the emulsions, β_0 is a constant (arithmetic mean), β_1 to β_{23} are model coefficients determined by the multiple linear regression analysis and e is the residual error.

Table 3
Experiments of the factorial design: set-up and results (± standard deviations, $n = 2$)

Emulsion ID	Fraction of disperse phase (% w/w)	Homogenisation power (arbitrary units)	Temperature (°C)	Mean droplet diameter (nm)
A	60	2	0	246 ± 3
B	60	3	0	230 ± 5
C	60	2	37	259 ± 7
D	60	3	37	229 ± 11
E	50	2	0	243 ± 2
F	50	3	0	200 ± 2
G	50	2	37	252 ± 5
H	50	3	37	197 ± 1
I	30	2	0	216 ± 3
J	30	3	0	175 ± 7

3. Results and discussion

3.1. Factorial formulation design

For an emulsion drug delivery system to be successful, it should be possible to obtain a reproducible droplet size and size distribution. In the present study, the effect on mean droplet size of homogenisation power, temperature and phase ratio between the aqueous and oily phases were studied in a factorial experimental design. By changing these three parameters, the initial mean droplet diameter of the w/o emulsion could be varied between 175 and 259 nm as shown in Table 3.

The results from the factorial experiment (Table 3) can be well explained by a multiple regression model including all statistical terms ($R^2 = 0.9712$). However, the predictive power of a regression model employing all statistical terms is poor ($Q^2 = 0.3840$). By excluding the statistically insignificant coefficients i.e. all interaction terms and the temperature coefficient from the regression model, the predictive power is strongly improved ($Q^2 = 0.8624$) and the regression coefficient is only slightly reduced ($R^2 = 0.9256$). The coefficients of the reduced regression model are shown in Fig. 2. This result shows that emulsions can be prepared in a reproducible manner from given values of homogenisation power and phase ratio between water and oil.

The mean droplet diameter, d_m, can then be described by Eq. (1):

$$d_m = 217.1 \text{ nm} + 22.8X_\phi - 18.5X_E, \quad (1)$$

where X_ϕ and X_E are the factor level of fraction of disperse phase and homogenisation power, respectively. Surprisingly, the temperature range employed in the present study did not have any statistically significant effect on droplet size, although it is known that the physical properties of especially non-ionic surfactants are sensitive to temperature in such a manner that they become more lipophilic at increasing temperatures. This phenomenon has extensively been described in the

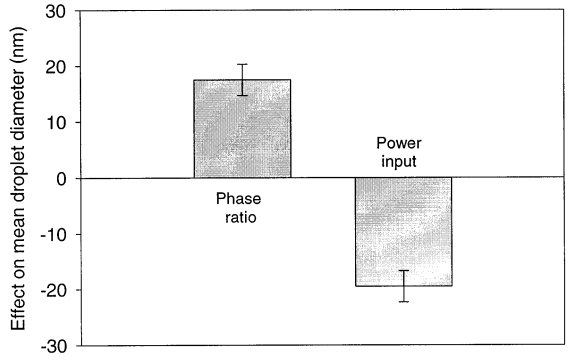

Fig. 2. Graphical representation of statistically significant regression coefficients ($P < 0.05$) from the multiple linear regression analysis. The regression coefficients indicate the effect on mean droplet diameter by changing one of the factors from a center to a high level value. Abbreviations: phase, amount of aqueous phase; power, homogenisation power.

phase inversion temperature-theory by Shinoda (1967). Both sorbitan monooleate and triglycerol polyricinolate-6 are non-ionic lipophilic surfactants, but the relatively narrow temperature interval employed in the present study might not influence the lipophilicity significantly. The rheological characteristics of the oil phase are also significantly influenced by temperature, as shown in Section 3.3. An increased viscosity of the oil phase will increase the shearing forces during homogenisation but also energy dissipation as heat. In the present study, the different effects of temperature on droplet size might counterbalance each other.

Volume fraction of disperse phase had a significant effect on the final droplet size ($P < 0.001$). An increased droplet concentration might shift the balance between formation and coalescence of drops towards coalescence because of more frequent collisions between emulsion droplets.

As expected, increasing power input during homogenisation reduced the mean droplet size. Gopal (1968) described a relationship between homogenisation power and the maximum droplet size under isotropic turbulence:

$$d_{\max} \propto \mathrm{E}^{-2/5}(\rho/\gamma)^{-3/5}, \quad (2)$$

where $d_{\max}$ is the diameter above which particles are likely to be broken up, E is energy intensity, γ is the interfacial tension and ρ is density. A good relation is found between the effect of energy intensity on droplet size, according to Eq. (2), and experimental data, assuming proportionality between $d_{\max}$ and mean droplet diameter determined by photon correlation spectroscopy. The theoretical reduction in mean droplet diameter increasing the homogenisation power from 2 to 3 (arbitrary units) is 15.0%. The corresponding observed reduction in mean droplet diameter was 15.6% calculated from the regression model.

3.2. Interfacial tension

Interfacial tension between the oil phase of the emulsion and plasma together with rheological parameters are potentially indicative factors of how the emulsion is spread and dispersed in tissue. The interfacial tensions between rabbit plasma and serial dilutions of oil phase at 37°C

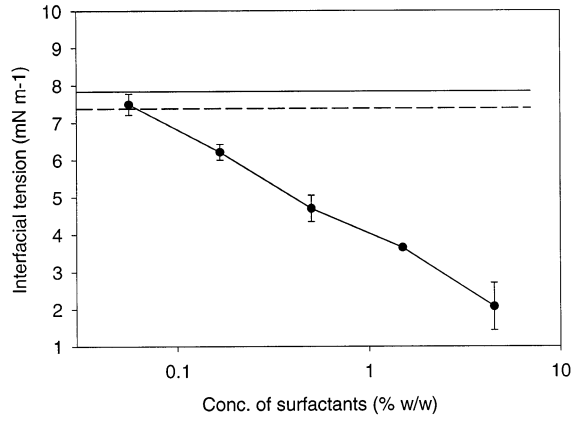

Fig. 3. Interfacial tension between rabbit plasma and oil with various concentrations of the surfactant mixture (sorbitan monooleate: triglycerol polyricinolate-6, 2:7) used in the preparations of emulsions. A solid and dashed line shows the interfacial tension between rabbit plasma and MCT–oil or w/o emulsion with 30% w/w aqueous phase, respectively.

are illustrated in Fig. 3. A linear decrease in the interfacial tension with logarithm of concentration of a 2:7 surfactant mixture (sorbitan monooleate:triglycerol polyricinolate-6) is observed. Surprisingly, the interfacial tension between plasma and emulsion with 30% w/w aqueous phase and 4.5% w/w surfactants is almost the same as between plasma and MCT–oil, although a surplus of surfactants should be present in the oil phase of the emulsion according to Bjerregaard et al. (1999a). In fact, the interfacial tension between the emulsion and rabbit plasma corresponds to the interfacial tension between plasma and a 81-fold dilution of the oil phase containing 6.4% w/w surfactants with MCT–oil. The interfacial tension between a w/o emulsion with 60% w/w aqueous phase and plasma could not be measured with the Du Noüy Ring, because the viscosity of the emulsion was too high. The interfacial tension is probably even higher than that of the thinner emulsion, because of the higher droplet concentration relative to the amount of surfactants. This indicates that most of the surfactants are bound to the primary water droplets and not available to stabilise new interfaces after 30 min of equilibrium. Consequently, the surfactants are not expected to have any significant influence on the initial spreading/disper-

Table 4
Temperature dependence of viscosity parameters of w/o emulsions with various fractions of disperse phase according to Eq. (3), where A is the pre-exponential term E_v is the 'activation' energy required to initiate flow

Fraction of disperse phase (% w/w)	A $\times 10^8$ (Pa s)	E_v KJ/mol	Regression coefficient R^2
0	3.50	33.5	0.9983
10	3.66	34.1	0.9986
20	4.42	34.4	0.9984
30	5.54	34.8	0.9986
40	6.50	35.7	0.9989
50	9.80	36.3	0.9990
60	10.7	38.8	0.9996

sion process on the present w/o emulsion compared to MCT–oil without any surfactants.

3.3. Rheological characterisation

The temperature dependency of viscosity of w/o emulsions with various phase ratios was investigated. A practical linear relation was observed between the logarithm of viscosity and temperature for both the oil phase and w/o emulsions with a water fraction up to 60% w/w. It is known that an Arrhenius-type equation often apply to the relation between viscosity and temperature of Newtonian fluids (Radebaugh, 1996):

$$\eta = A^* e^{Ev/RT}, \quad (3)$$

where A is a pre-exponential term that is a function of molecular weight and molar volume of the fluid, R is the gas constant, T is absolute temperature and E_v is the 'activation' energy required to initiate flow between molecules. The results from an analysis of the viscosity of the various emulsions according to the Arrhenius-type equation are shown in Table 4. It is interesting that this relation also applies to a heterogeneous system like a w/o emulsion. The significant temperature dependency of viscosity is important for the handling and administration of an emulsion. Injectability or the ease of injecting non-aqueous liquid formulations through a hypodermic needle is found to be linear related to the reciprocal of viscosity (Chien et al., 1981).

From Fig. 4 it is clear that the viscosity of the present w/o emulsion is very sensitive to the volume fraction of disperse phase and offers a great span of various viscosities. The increase in viscosity is especially high at volume fractions of disperse phase close to a theoretical critical volume fraction, ϕ_o, above which layers of closed-packed spheres can no longer freely slip past one another. For a monodisperse emulsion, ϕ_o, was found to be 0.6046 (Princen and Kiss, 1986). The relative viscosity at the various phase ratios shown in Fig. 4 was satisfactorily described by the hard-sphere model suggested by Krieger (1972):

$$\eta_{rel} = [1 - (\phi/\phi_{max})]^{-[\eta]\phi max}, \quad (4)$$

where $[\eta]$ is the intrinsic viscosity, which has a theoretical value of 2.5 for rigid spheres and ϕ_{max} is the maximum packing fraction, which is equal to 0.64 for random packing and 0.74 for hexagonal packing of monodisperse spheres (Tadros,

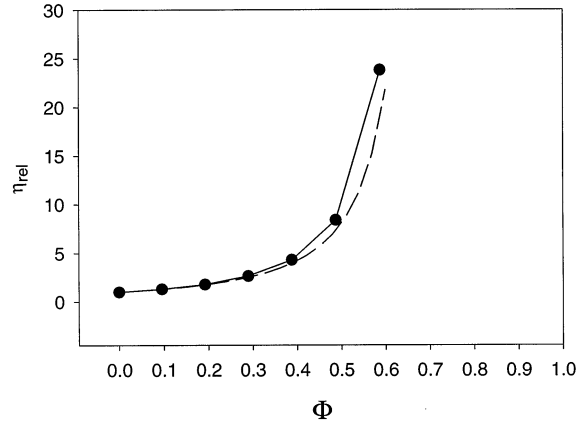

Fig. 4. Relative viscosity, η_{rel}, versus volume fractions of disperse phase, ϕ, for w/o emulsions (●, experimental results, dotted line represents theoretical values according to Eq. (4)).

1994). In the present study ϕ_{max} was estimated from linear plots of $\eta^{-0.5}$ versus ϕ assuming that the emulsions behave similarly to hard sphere dispersions. Extrapolation to $\eta^{-0.5} = 0$ (i.e. $\eta = \infty$) gave a ϕ_{max} value of 0.74.

Other factors, such as interactions between droplets, have to be considered. Concentrated w/o emulsions with identical phase ratios ($\phi = 0.688$) and prepared from the same oil and aqueous phase, but stabilised with different emulsifiers, show quite different relative viscosities (Sherman, 1968). W/o emulsions stabilised by sorbitan monooleate and polyglycerol polyricinolate showed a relative viscosity of 15.14 and 11.76 at $\phi = 0.688$, respectively (Sherman, 1968). In the present study, the relative viscosities were 23.82 at $\phi = 0.588$. These variations in the relative viscosities are partly mediated through different interactions between emulsion droplets, in particular in concentrated w/o emulsions where the droplets are in close contact. For example, it has been suggested that polymeric surfactant molecules on droplet surfaces in concentrated emulsions are forced to interpenetrate (Tadros, 1994; Gasperlin et al., 1997).

Fig. 5 illustrates that the thin emulsion with 30% w/w disperse phase exhibits Newtonian behaviour, while the thick emulsion with 60% w/w disperse phase exhibits a pseudoplastic behaviour. This is recognised by shear thinning of the thick emulsion, which has also been observed for other concentrated w/o emulsions (Gasperlin et al., 1997). Greater entanglement of the polymeric surfactant in the emulsion with 60% w/w disperse phase at lower than at higher shear stress can be one explanation. This is analogous to macromolecules in solution. Furthermore, an increasing degree of alignment and deformation of droplets is expected at increasing shear rate. The superimposed up and down curves on Fig. 5 indicate an unaltered structure which was not changed by the shear cycles. The viscosity of an emulsion with 60% w/w disperse phase at an i.m. injection site may then vary depending on the tension affected by the tissue during, for example, muscle contraction.

The Newtonian behaviour of the emulsion with 30% w/w disperse phase indicates that the

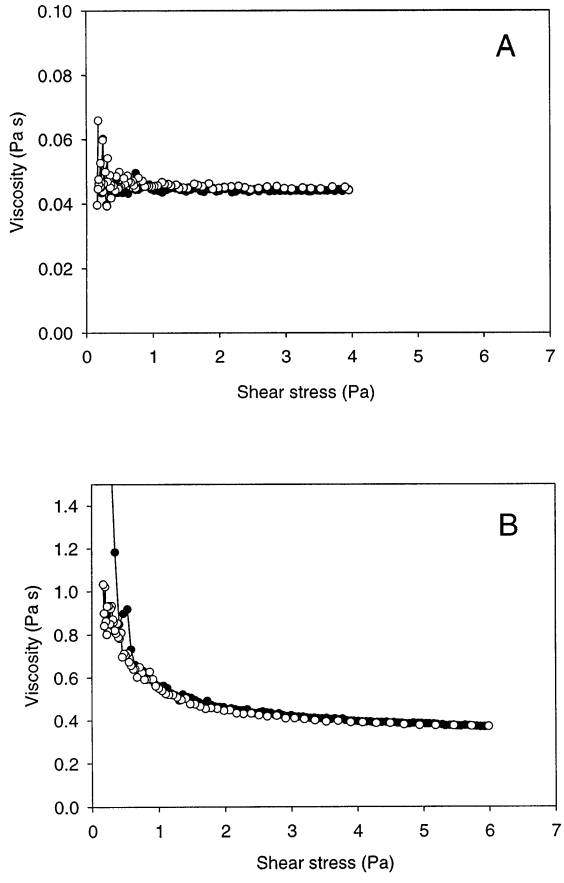

Fig. 5. Stress sweep of w/o emulsions with (A) 30 and (B) 60% w/w disperse phase, respectively. Up regulation (●) and down regulation (○) of shear stress.

droplets of the emulsions do not aggregate to any significant extent, since aggregates would break at high shear rates/shear stress (Sherman, 1968). This is consistent with microscopy of the coarse pre-emulsion, showing discrete droplets without any tendency to agglomeration.

The emulsions were further characterised by dynamic rheology in the linear viscoelastic region. Fig. 6 shows the frequency dependency of dynamic modulus.

The storage modulus, G', represents energy stored in the system during oscillation and is due to elastic deformation of structures in the emulsion. The loss modulus, G'', characterises the viscous properties. For both emulsions G'' was dominating. Both emulsions are consequently

characterised as fluids. The G' modulus was negligible compared to G'' for the emulsion with 30% w/w disperse phase, which then can be described as an entirely viscous material. This is consistent with the Newtonian behaviour in Fig. 6. However, the magnitude of G' and G'' was almost equal for the emulsion with 60% w/w disperse phase indicating viscoelastic properties of the emulsion. The viscoelastic properties of the emulsion with 60% w/w disperse phase are also recognised by a phase difference significantly lower than 90° between the sinusoidal stress wave and the resulting strain. The elastic modulus depends on the interaction forces, attractive or repulsive, between droplets and their nearest neighbours (Sherman, 1967; Tadros, 1994). In w/o emulsions the interaction forces are governed primarily by steric interactions (Tadros, 1994). The change

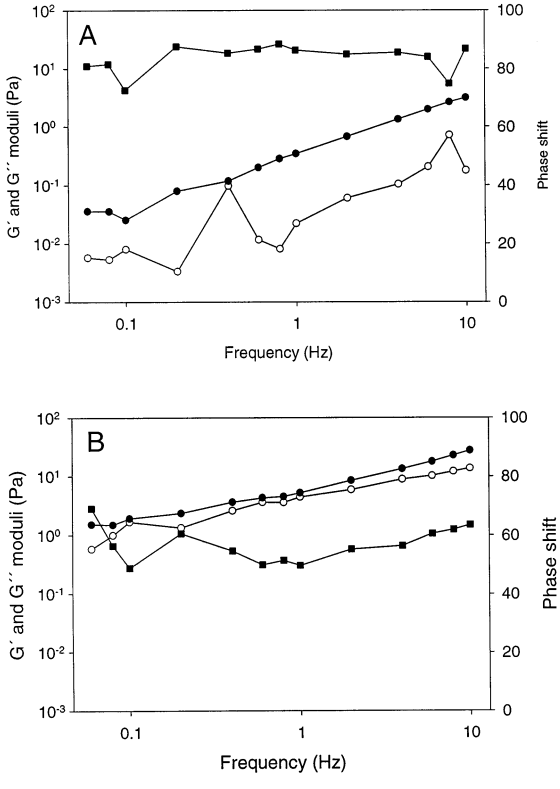

Fig. 6. Dynamic rheological analysis of w/o emulsions with 30% (A) and 60% (B) w/w disperse phase, respectively. Elastic modulus, G' [○], loss modulus, G'' [●], and phase shift (■) versus frequency.

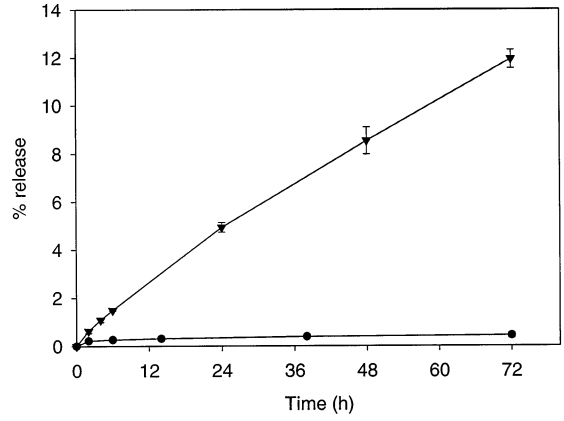

Fig. 7. Cumulative release of glucose, Mw: 180 g/mol (▼) and aprotinin, Mw: 6512 g/mol (●) from w/o emulsions with 30% w/w disperse phase at 37°C ($n = 3$, $\pm$ standard deviation). Release media was phosphate buffered saline, pH 7.4.

from predominantly viscous to predominantly elastic response is influenced by the ratio of the adsorbed thickness of surfactant, δ, relative to the droplet radius, R (Tadros, 1994). In the present study, the shift to an predominantly elastic system was almost achieved considering the small differences between the G' and G''. Tadros (1994) observed this transition with a w/o emulsion, when the volume fraction of disperse phase exceeded 0.67.

3.4. In vitro release

Results from the release study of w/o emulsions with 30% disperse phase are shown in Fig. 7. The amounts of glucose and aprotinin released after 72 h of release were, respectively, 11.9 ± 0.7 and 0.4 ± 0.1%, reflecting the different physicochemical properties of the two model compounds. Glucose is very hydrophilic and has a molecular weight of only 180 g/mol. Aprotinin is a positively charged protein at physiological pH (isoelectric point at pH 10.5), also very hydrophilic, but with a molecular weight of 6512 g/mol. Consequently, aprotinin does not readily distribute into the oil phase and may primarily be released from the emulsion by membrane rupture, i.e. fusion between emulsion droplets and the release media. In contrast, glucose may primarily be released by

diffusion through the oil phase, because glucose has a very small, but finite, solubility in the oil phase. These data are consistent with previous results obtained with glucose and inulin, a polysaccharide, in an identical emulsion system (Bjerregaard et al., 1999a).

3.5. Spreading studies

Fig. 8 shows the relative spread of radioactivity in *vastus lateralis* following 300 μl injections of emulsion. The data are normalised with the mean of the first measurement of the emulsion with 60% w/w disperse phase containing aprotinin. The curves for emulsions with same phase ratio/viscosity are practically superimposed indicating a good reproducibility of results. It is further observed that the low viscosity emulsion, immediately after injection, spreads to a higher degree than the emulsion with 60% w/w disperse phase. After the initial fast spreading (i.e. the first 5 min post-injection) a much slower but steady spreading takes place during the next 2–3 h post-injection probably mediated by muscle movement. Then no more spreading occurs, and the position of emulsions or dispersions of emulsion seem fixed, as estimated from the profiles of radioactivity on gamma images (not shown).

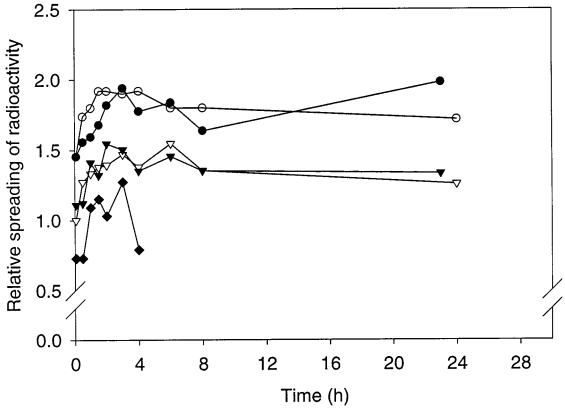

Fig. 8. Spreading of hydrophilic radioactive markers after injection of 300 μl of emulsion into rabbit thigh muscle, *vastus lateralis* ($n = 3$, $\pm$ standard deviation). The spreading is relative to the spreading of aprotinin encapsulated in emulsion with 60% w/w aqueous phase at $t = 5$ min postinjection. Legend: emulsions with aprotinin: 30% w/w aqueous phase (○) and 60% w/w aqueous phase (▽). A solution of aprotinin in PBS was also injected intramuscularly (◆), $n = 1$. Emulsions with pertechnetate: 30% w/w aqueous phase (●) and 60% w/w aqueous phase (▼). The viscosities of emulsions with 30% w/w and 60% w/w aqueous phase were approximately 50 and 390 mPas, respectively.

Surprisingly, the aqueous aprotinin solution apparently spreads to a lower extent than the emulsions despite of a lower viscosity. However, this can probably be related to extensive clearance of aprotinin in solution within the first 5 min after injection, hereby concealing the true extent of spreading.

Spreading of the low viscosity emulsions may have been restricted by the dimensions of the muscle (5–6 cm). However, there is, in general, a statistically significantly difference ($P < 0.05$) between the relative spreading of emulsion with 30 and 60% w/w disperse phase, respectively.

The data from the present study can be compared with a similar study by Schultz et al. (1998). Spreading of sesame oil and MCT–oil in rabbit hind leg, *vastus lateralis*, were investigated employing gamma scintigraphy. The viscosities of the two oils were 35 and 15 mPas, respectively. The two oils spread to the same extent, reflecting a too small span of viscosity values compared to the variability of this type of experiments. The relative spreading of these oils, 24 h post-injection, was equal to the spreading of the w/o emulsion in the present study with a viscosity of 50 mPas. The surfactants in the emulsion vehicle are not believed to have any influence on the initial spreading.

The qualitative spreading behaviour was examined by light microscopy of transverse sections of the *triceps brachii* muscles after injection of 100 μl emulsion. Both emulsions spread beneath the epimysial and fascial sheaths and within perimysial septa, as small ellipsoid droplets. The dimensions of these ellipsoids was rather uniform for emulsions with 30% w/w disperse phase with equatorial radii in the order of 150–350 μm (Fig. 9). However, the corresponding radii were up to approximately 1000 μm for thick emulsions with 60% w/w disperse phase. This indicates a more disruptive effect on the muscle tissue of the high viscosity emulsion and a smaller degree of dispersion. Moreover, a large fraction of the high vis-

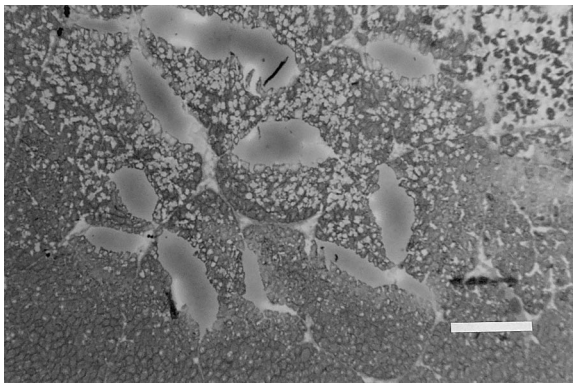

Fig. 9. Transverse section of frozen rabbit *triceps brachii* muscle 2 h postinjection of 100 μl of w/o emulsion with 30% w/w disperse phase. The emulsion was injected into the centre of the muscle in a depth of 10 mm. Bar, 500 μm.

cosity emulsion was found on the surface of the *triceps brachii* muscle, i.e. on the fascial sheath surrounding the muscle. This was not observed to the same extent with the low viscosity emulsions. Hence, high viscosity emulsions might not disperse well in the muscle and are instead squeezed back along the track of the needle or out between the muscle fibres in a disruptive manner. This behaviour of non-spreading injections has also been suggested by Oettingen et al. (1927).

The present results are consistent with microscopic observations done by Hashida et al. (1977a). This group found that w/o emulsions injected in rat thigh muscle were also finely dispersed between muscle fibers into droplets of various sizes. The diameters ranged from 1 to 30 μm. This is smaller than in the present study, which may be explained by the use of a different animal model and the use of hydrophilic surfactants facilitating the formation of multiple droplets.

Brown et al. (1944), found that i.m. administered oils had a tendency to accumulate along fascial sheaths, but was also observed to some extent between muscle bundles as oil cysts. Svendsen and Aaes Jorgensen (1979), observed that relatively large volumes of a low viscosity MCT–oil (1 ml/kg) injected in rabbit thigh muscle oil both could be found between the muscle fibres and around the muscle bundles. In rat muscle, the oil was mainly located to the connective tissue between the muscle bundles. Thus, it may be appropriate to distinguish between intramuscular and intermuscular depots, as suggested by Oettingen et al. (1927).

3.6. Disappearance studies

The use of non-invasive imaging technique of gamma scintigraphy is in general well suited for quantitative studies of distribution of a radionucleide (Wilson et al., 1997). The true absorption rate can only be obtained by measuring the local amount of drug remaining (Sund and Schou, 1964). Gamma scintigraphy has previously been shown to be a valid method for following the disappearance of i.m. oily depots (Schultz et al., 1998). This method reduces the number of animals necessary for the study, since the same animal is observed throughout. Furthermore, an animal can be used as its own control in a cross-over design hereby circumventing inter-individual variations. The results from the disappearance study are shown in Fig. 10. Spreading of emulsion in the anterior or posterior direction from the gamma camera might shift individual disappear-

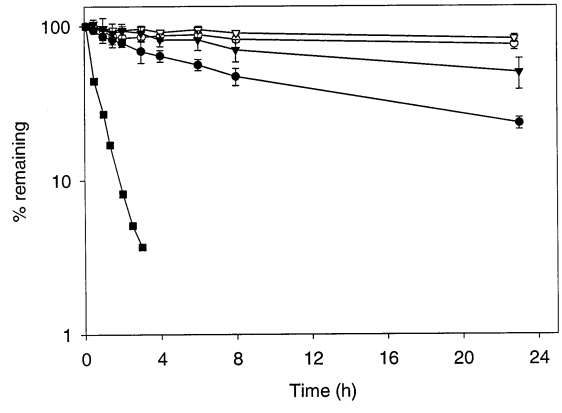

Fig. 10. Disappearance profiles of hydrophilic radioactive markers encapsulated in w/o emulsions with 30% w/w and 60% w/w aqueous phase, after injection of 300 μl of emulsion into rabbit thigh muscle, *vastus lateralis* ($n = 3$, ± standard deviation). Legend: emulsions with aprotinin: 30% w/w aqueous phase (○) and 60% w/w aqueous phase (▽). Solution of aprotinin in PBS (■), $n = 1$. Emulsions with pertechnetate: 30% w/w aqueous phase (●) and 60% w/w aqueous phase (▼).

ance profiles ± 10%, when the thickness of *vastus lateralis* and the absorption coefficient for gamma radiation from Tc^{99m} is considered. However, this effect will probably not be significant on disappearance profiles based on the average of several curves. The percentages of Tc-aprotinin remaining after 24 h were 76 ± 6 and 83 ± 5% for the emulsions with 30 and 60% w/w disperse phase, respectively. The corresponding values for pertechnetate were 23 ± 2 and 50 ± 11%, respectively. Disappearance rate seems to be biphasic with an initial fast disappearance followed by a slower phase. There might be a relation between the disappearance and the spreading of the emulsion. Consequently, the change from a fast to a slow disappearance rate 2 h post-injection is coincident with a change in spreading behaviour of the emulsion i.e. from a spreading state to a stationary state. The i.m. forces, e.g. hydrostatic pressure, that effectuate spreading might also partly destabilise the emulsion and increase absorption rate in the same way as massage of subcutaneously injected liposomes increase mobilisation of liposome-encapsulated drug into the blood (Trubetskoy et al., 1998).

The disappearance profiles of the two radioactive markers, Tc-aprotinin and pertechnetate, are very different. This may be explained by the strongly molecular weight dependent release properties observed in the in vitro release studies. Furthermore, there is a tendency that the radioactive markers incorporated in emulsions with 30% w/w disperse phase disappear faster from the injection site than in the corresponding emulsions with 60% w/w disperse phase. This can be explained by the lower spreading of the high viscous emulsion exposing a relatively smaller surface area where release of marker can take place.

The good physical stability of the present w/o emulsions, as shown by Bjerregaard et al. (1999b), might be the main reason for improved retention capacity of drug on an i.m. injection site compared to previous studies with w/o emulsions and the related w/o/w emulsions. Here, the half-life of disappearance has not exceeded 2 h as mentioned previously. The shortest half-life of disappearance in the present study was approximately 8 h. The relatively strong association of aprotinin, in particular, to the w/o emulsion indicates that the fate of the hydrophilic marker is linked to the disappearance and/or metabolism of the oil phase of the w/o emulsion.

Oily depots are suggested to disappear by the combined action of local metabolic degradation, absorption to the blood or lymphatics and phagocytosis (Ballard, 1968; Svendsen and Aaes Jorgensen, 1979; Howard and Hadgraft, 1983). At present, it is unclear which mechanism is dominating, although it seems unlikely that emulsion/oil is transported across blood capillaries via unspecific transport mechanisms, e.g. vesicles, in any significant amount. However, w/o emulsions and related systems have been known to enhance the transport of hydrophilic drugs and oil via the lymphatics (Hashida et al., 1977a,b; Nakamoto et al., 1975), especially after massaging of the injection site (Hashida et al., 1980). The lipophilic surfactants of w/o emulsions might facilitate the disappearance process, as shown by Omotosho et al. (1989). In that study, an increased concentration of lipophilic surfactant (Span 80) increased the disappearance rate of w/o/w emulsions. Similarly, Tanaka et al. (1974), showed that Span 80 increased the disappearance rate of methyl oleate from an i.m. injection site. In contrast, Hashida et al. (1980) found that lipophilic surfactants decreased the disappearance rate, but increased the lymphatic absorption of sesame oil. The surfactants were further shown to facilitate dispersion of w/o emulsions into finely divided multiple emulsions after i.m. injection. Hashida et al. (1980), hypothesised that the dispersion of a formulation might be the first step in lymphatic absorption. A high spreading emulsion may, therefore, be eliminated more efficiently by the lymphatics than a low spreading emulsion.

4. Conclusion

The present study has highlighted some properties of a parenteral w/o system. The emulsions can be prepared in a reproducible fashion. Thus, the size of emulsion droplets, and thereby release characteristics, can be controlled between certain limits via homogenisation power and the phase

ratio between the oil and aqueous phase. A molecular weight dependency of the in vitro release was observed. The same tendency was observed in the in vivo disappearance rates of pertechnetate and 99mTc-labelled aprotinin, respectively.

The long retention of marker molecules on the i.m. injection site was suggested to be related to the good physical stability of the emulsions.

The in vivo spreading and disappearance characteristics of the w/o emulsions were shown to depend on the relative amount of disperse phase in the emulsions and hereby viscosity. Increasing the water content of the emulsion increased the viscosity significantly. Both quantitative and qualitative differences were found in the spreading characteristics showing that low viscosity emulsion was more extensive and finely spread than the high viscosity emulsion.

Overall, it can be concluded that the presented w/o emulsions are promising vehicles for sustained release from an i.m. injection site.

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