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Evaluation of two fluorescent probes for the characterization of W/O/W emulsions

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Abstract

The aims of this work were to select the most convenient fluorescent probe for the characterization of the W/O/W emulsion and to propose a new technique in order to study the release mechanism from the W/O/W emulsion. For this purpose fluorescence was compared with the well established conductimetric measurements using $MgSO_4.7H_2O$ as electrolyte. Two fluorescent molecules were evaluated: 5,(6)-carboxyfluorescein (5,(6)-CF) and 1,3,6,8, pyrene tetrasulfonic acid, tetrasodium salt (PTSA). After their introduction in the inner aqueous phase of the W/O/W emulsions; their stability (in aqueous solutions) to temperature and to light, their sensitivity at different pH conditions and to the presence of the constituents of the inner aqueous phase, their partition between the different phases of the emulsions and their influence on rheological properties of the emulsions were analysed. PTSA was finaly selected as the most convenient probe due to its high stability at different conditions and its excellent yield of encapsulation. After the release studies, a satisfying correlation between HPLC fluorescence and conductimetry was obtained. Both techniques confirm that the release mechanism of the multiple emulsion is swelling-breakdown.

Keywords: W/O/W multiple emulsion; Fluorescent probes; PTSA; 5,(6)-CF; Release mechanism; Swelling-breakdown

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1. Introduction

Water/oil/water (W/O/W) multiple emulsions are emulsions of emulsions. They consist of a W/O type emulsion as a dispersed phase and an aqueous emulsifier solution as a dispersion medium. The W/O/W multiple emulsions, due to their oily membrane which separate two aqueous phases, can be considered as liquid membrane systems.

Nowadays, multiple emulsions are coming into use more extensively in the pharmaceutical and cosmetic fields, since they permit the protection of entrapped substances (Shichiri et al., 1974) and the sustained release (Omotosho et al., 1986; Mishra and Pandit, 1990; Vaziri and Warburton, 1994; Nakhare and Vyas, 1994). The use of different tracers, the electrolytes such as $MgSO_4$ (Grossiord et al., 1993; Terrisse et al., 1994) and NaCl (Brodin et al., 1978; Matsumoto and Kohda, 1980), the sugars such as glucose (Matsumoto et al., 1976; Tomita et al., 1982; Kawashima et al., 1992), the polyols such as sorbitol (Ohwaki et al., 1992), the dyes such as new coccine (Ohwaki et al., 1993) and sulphane blue (Law et al., 1986) enables us to study the yield (Matsumoto et al., 1976; Magdassi et al., 1984; Terrisse et al., 1993), the release (Brodin et al., 1978; Omotosho et al., 1986; Mishra and Pandit, 1990; Nakhare and Vyas, 1994) and the permeation of entraped solutes (Matsumoto and Kohda, 1980; Tomita et al., 1982). Nevertheless, their characterization and the release studies are still difficult due to their various and complex structure.

Fluorescent probes were widely used for the characterization of new pharmaceutical drug delivery systems such as liposomes (Rolston et al., 1981; Goldbach et al., 1995), nanoparticles (Oppenheim and Stewart, 1979) etc. The fluorescent tracer technique, in theory, presents several interesting possibilities: only a small amount of fluorescent probes are necessary for the studies, an easy image analysis can be achieved by fluorescence microscopy and this is a very sensitive method of determination. Nevertheless only a few studies were performed on multiple emulsions using fluorescence probe techniques (Davis and Walker, 1983).

Consequently, this work has two aims: to select the most convenient fluorescent probe for the characterization of the multiple emulsion and to propose a new fluorescent technique based on the best fluorescent probe in order to study its release from the multiple emulsion.

2. Materials and methods

2.1. Materials

Two hydrophilic fluorescent markers were evaluated; 5,(6)-Carboxyfluorescein (5,(6)-CF) and the 1,3,6,8-pyrene tetrasulfonic acid, tetrasodium salt (PTSA) (Table 1). They were purchased from Sigma, USA and Molecular probes, USA, respectively. Hepta hydrated magnesium sulfate ($MgSO_4.7H_2O$), L-ascorbic acid and *N*-acetyl L-cysteine, were purchased from Merck, Germany. Demineralized water was used. Abil EM 90® (polysiloxane polyalkyl polyether copolymer) was from Goldschmidt AG, France. Generol 122 E-25® (PEG-25 Soya sterol) was from Henkel, France and Brij 58® (POE-20 Cetyl alcohol) was from I.C.I Surfactants, France. Triglycérides 5545^R (Caprylic/capric triglycerides) was obtained from Dubois, France. All materials employed were of analytical grade and used without further purification.

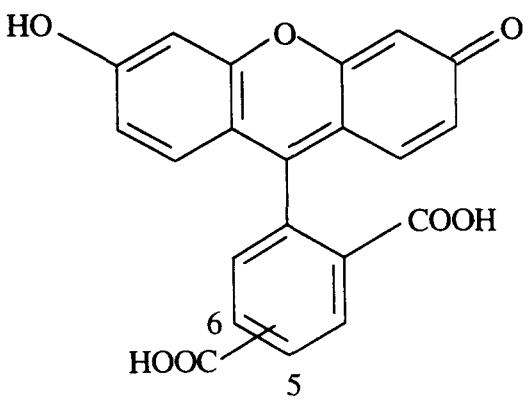
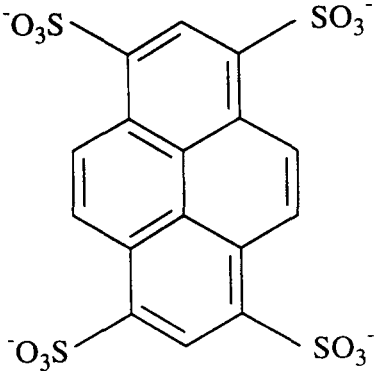
2.2. Solutions

In each case, 10^{-4} M stock solution of fluorescent marker was used and diluted as required.

2.3. Preparation of the emulsions

The multiple emulsions were prepared using a two-step emulsification procedure as previously described (Raynal et al., 1993). In all cases fluorescent probes were introduced in the inner aqueous phase of the W/O/W multiple emulsions. The basic formula of multiple emulsion is as follows:

Table 1
Some characteristics of the two water soluble fluorescent probes studied

	5,(6)-CF	PTSA
Chemical formula		
Molecular weight	376.3	610
Wavelenghts of detection	λ_{ex}= 485 nm λ_{em}= 515 nm	λ_{ex}= 375 nm λ_{em}= 404 nm

W/O primary emulsion	% w/w
Triglycerides 5545®	24%
Abil EM 90®	8%
$MgSO_4.7H_2O$	0.7%
Fluorescent probe[a]	X%
Active substances[b]	Y% and Z%
Demineralized water	sq 100%
W/O/W multiple emulsion	
Primary emulsion	80%
Brij 58®	1.4%
Generol 122 E-25®	0.6%
Demineralized water	sq 100%

[a]Sufficient amount (w/w %) for a 10^{-4} M fluorescent probe solution

[b]L-ascorbic acid (2%, w/w) and *N*-acetyl-L-cysteine (0.5%, w/w)

2.4. Assays

2.4.1. Microscopic analyses

The multiple character of the emulsions and the location of the fluorescent probes were observed by an optical microscope (Wild and Leitz, France) at 1250 magnification after dilution in water. The microscope was equipped with special optical filters adapted for each fluorescent probes.

(1) For 5,(6)-CF: a band pass (450–490 nm) excitation filter and cut off emission filter at 515 nm (Leica, Germany)

(2) For PTSA: a band pass (340–380 nm) excitation filter and cut off emission filter at 430 nm (Leica, Germany)

2.4.2. Conductimetric analysis

This analysis consisted in directly measuring the conductivity on the external aqueous phase in order to monitor the release of the electrolytic species entrapped in the internal aqueous phase. The conductivity was measured with SDM 3 conductimeter (Radiometer, Jarre-Jacquin, France). The assay was carried out at 20 ± 1°C on the diluted (1/20 in demineralized water) multiple emulsion samples under magnetic stirring. A calibration curve of the $MgSO_4.7H_2O$ was performed. The slope of the calibration curve allows the determination of the mass fraction $\beta(t)$ released at a time 't':

$$\beta(t) = M(t)/M_0$$

where M_0 is the initial incorporated mass of the $MgSO_4.7H_2O$ and $M(t)$ is the mass present in the external phase at given time t.

2.4.3. Rheological analyses

The rheological analyses were carried out at 20 ± 0.1°C with a controlled stress rheometer CSL100 (Carri-Med, Rheo, France) equipped with a cone plate geometry. Two different analyses were performed: viscoelastic oscillatory analysis (on a non-diluted emulsion sample) and steady state flow analysis (on a 1/5 diluted emulsion sample).

(1) Viscoelastic oscillatory analysis. In this experiment, a sinusoidal stress was applied and the evolution of the main viscoelastic parameters such as G^* (shear modulus), δ (loss angle) and G'' (loss modulus) were recorded on increasing stress (τ) but at fixed frequency (1 hz) and temperature (25°C) (Grossiord et al., 1993). For low stress values, a 'plateau' region was observed and this profile was the index of the unmodified structure of the multiple emulsion. After a critical value of the stress $(\tau_0)_c$ which is defined by the highest value of the G'', an increase of δ value and a decrease of G^* value appeared. Then certain viscoelastic oscillatory parameters were noted as follows: calculated G^*_m and δ_m in 'plateau' region. This profile of multiple emulsions defines the limit between elastic strain and viscous flow (Grossiord et al., 1993). The oscillatory analysis provides information as to whether the introduction of trace amounts of fluorescence probes have an influence or not, on the structure of the multiple emulsions.

(2) Steady state flow analysis. During this experiment, the diluted sample is subjected to a constant shear rate ($100\ s^{-1}$) and the evolution of the viscosity coefficient (η) is recorded (Grossiord et al., 1993). The steady state flow analysis gives information about the evolution of the volume fraction of the multiple emulsions during the time.

2.4.4. Direct spectrofluorimetry

Steady state fluorescence measurements were performed with luminescence spectrophotometer (Perkin Elmer LS 50B, France) using a 10 mm quartz cuvette. Excitation and emission slits were set at 5 nm and 2.5 nm, respectively. Each measurements were performed in triplicate at 20 ± 2°C and the samples were prepared in the required pH conditions.

2.4.5. HPLC analyses

Multiple emulsion was diluted (1/20) in demineralized water and vortexed in order to homogenize the sample. Then it was centrifugated for 15 min at 3500 rpm. The aqueous lower layer was then pipetted and filtered through a 0.45 μm cellulosic filter (Millex HV, Milipore) in order to remove all the remaining W/O droplets. Then, collected cleared external phase was assayed for fluorescent probe content by reverse-phase HPLC. The HPLC device (Shimadzu/Kyoto, Japan) consisted of a pump (LC6A), a fluorescent detector (RF 551) and an integrator (CR5A). The samples were injected through the column with a 20 μl Rheodyne loop injector. The column used, is a C-18/5 μm Nucleosyl (Interchim N5C18-15F, 4.6 mm i.d. and 150 mm long).

The HPLC method for the quantification of the 5,(6)-CF requires a post column system (a mixing tee), since the marker was not fluorescent under low pH conditions used in the chromatography. The mobile phase consisted of methanol/water (60:40, v:v), pH 3.9, adjusted by acetic acid and pumped at a flow rate of 0.8 ml/min. The post column reagent was a solution of sodium hydroxide (10^{-2} mol/l) pumped at a flow rate of 0.4 ml/min. The fluorescence detection was performed

with $\lambda_{ex} = 485$ nm and $\lambda_{em} = 515$ nm setting. Under these conditions two peaks were eluted with retention time of 3 and 3.5 min respectively, since 5,(6)-CF molecule is a mixture of two isomers.

The mobile phase for the quantification of PTSA consisted of methanol/water/40% tetrabutylammonium hydroxide (70:25:5, v/v), adjusted at pH 7.6 with pure acetic acid and pumped at a flow rate of 1 ml/min. The fluorescence detection was performed at $\lambda_{ex} = 375$ nm and $\lambda_{em} = 404$ nm. Under these conditions the retention time of PTSA was 4.5 min.

3. Results and discussion

3.1. Selection of the most appropriate fluorescent probe

3.1.1. Influence of the manufacturing temperature of primary emulsion and of the light on the aqueous solutions of fluorescent probes

The stability of the fluorescent probes under real manufacturing and storage conditions of the W/O/W emulsions was first explored. For this purpose, 10^{-7} M of 5,(6)-CF in 10^{-2} M sodium hydroxide solution and a 10^{-7} M PTSA in aqueous solution were prepared. Use of sodium hydroxide was due to the fact that the 5,(6)-CF is scarcely soluble in pure water and it needs a buffer or an alkaline solution (pH > 7) for its complete dissolution. Then, the samples were heated to 80 ± 1°C (to mimic the maximum temperature reached in the manufacturing process of primary emulsion) then, cooled to 20 ± 1°C and exposed to direct sun-light during one week in order to evaluate the effect of the temperature and the light on the stability of the fluorescent probes. Both samples were then assayed by direct spectrofluorimetry. As shown in Fig. 1; 5,(6)-Cf was more sensitive to light and temperature than PTSA since no significant change (< 10%) on the emission intensity values for this latter compound was recorded.

3.1.2. Influence of pH on the stabilty of the fluorescent probes

The constituents of the inner aqueous phase make this solution acidic (pH 2.45). Because of this low pH condition, the effect of pH on the stability of fluorescence probes was first explored. The experiment was only performed on the aqueous solutions of PTS since 5,(6)-CF is an acidic pH indicator. In addition, its sensitivity to different pH conditions was largely discussed in the literature (Thomas et al., 1979; Barbet et al., 1984, Graber et al., 1986). Different PTSA solutions, between pH 2 and pH 12, were assayed by direct spectrofluorimetry. They were not significantly sensitive (average signal variation < 10%) to pH changes.

3.1.3. Influence of inner phase constituents on the fluorescence emission of the probes

As is well known, 5,(6)-CF is nearly non-fluorescent at low pH and the acidic pH of inner aqueous phase (pH 2.45) corresponds to the molecular form of 5,(6)-CF (pKa 6.45). Accordingly, it appears from Fig. 2(a) that the fluorescence emission of 5,(6)-CF drastically decreased in the presence of L-ascorbic acid (Vitamin C) and *N*-acetyl-L-cysteine (NAC). On the other hand and although the self-quenching is one of the characteristics of the 5,(6)-CF, this phenomenon was not observed in this set of experiments and this was certainly due to the use of very low concentrations (maximum 2, 5×10^{-7} M).

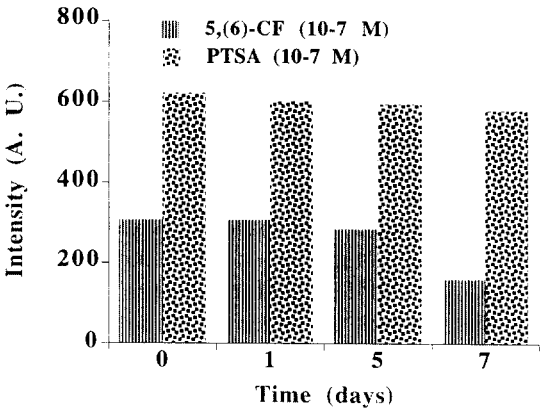


Fig. 1. Influence of the light and the manufacturing temperature of the emulsions on the aqueous solutions of the fluorescent probes (5,(6)-CF and PTSA).

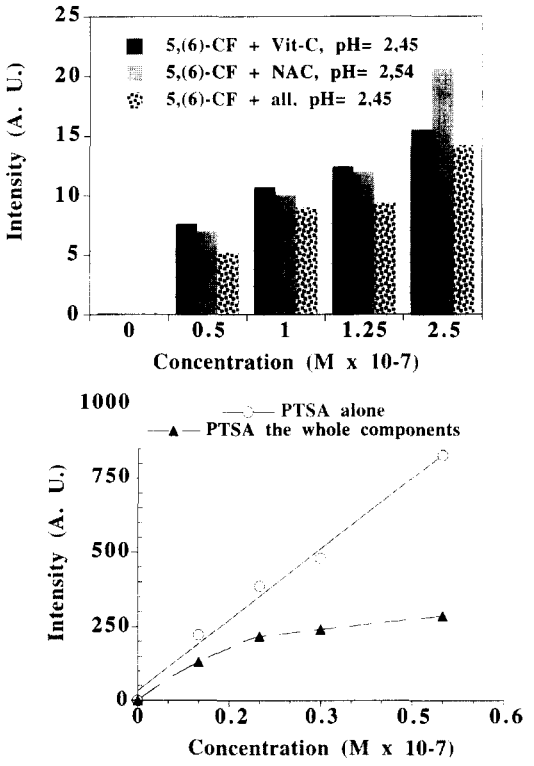


Fig. 2. (a) Influence of the inner aqueous phase constituents on the fluorescence emission of 5,(6)-CF. (b) Influence of the inner aqueous phase constituents on the fluorescence emission of PTSA.

The behavior of the PTSA was different than the 5,(6)-CF in the presence of the constituents of the inner aqueous phase. Although it was not significantly sensitive ($< 10\%$) to pH changes, either low or high, an important decrease of fluorescence intensity of PTSA was observed in the presence of the constituents of the inner aqueous phase as shown in Fig. 2(b). Although remaining unclear, this could be explained by the formation of complexes at the fundamental or excited state of PTSA with one of the inner phase constituents present in considerable excess (L-ascorbic acid w/w, 2%, *N*-acetyl-L-cysteine w/w, 0.5% and $MgSO_4$ w/w, 0.7%) in comparison to PTSA. On the other hand, the possibility of direct spectroscopic interference by absorption of a part of the excitation light at 335 nm by the large excess of L-ascorbic acid cannot be excluded. Whatever the case, and according to these results, direct fluorescence measurements of PTSA should be considered as inappropriate. Consequently, to overcome this problem the fluorescent probe determination needs to be measured by HPLC.

3.1.4. Partition coefficient studies

The partitioning of the studied probes between the aqueous phase and the oily phase of the emulsion was examined. The fluorescent markers were determined by HPLC in order to overcome the problems raised above. The 5,(6)-CF and the PTSA are both very hydrophilic compounds (depending of the pH for the first one) and it was expected that the loss of probes from the aqueous phase would be very limited. The results are summarized in Table 2. After 48 h, for all samples, more than 20% of the 5,(6)-CF migrated to the oily phase. In contrast, there was almost no measurable loss for the PTSA for all samples over the same time. These results were confirmed by fluorescence microscopy. The W/O/W emulsion marked with the 5,(6)-CF, shows a yellow fluorescence emission at the external aqueous phase due to the diffused 5,(6)-CF from the inner aqueous phase. This is certainly due to the partition coefficient of 5,(6)-CF in acidic pH (i.e. with ascorbic acid and its anti-oxidant). In contrast, for the emulsion marked with the PTSA only the aqueous inner globules were fluorescent. These results showed that 5,(6)-CF was not a convenient tracer (for this formulation) for investigating the release mechanism of our W/O/W multiple emulsion for-

Table 2
Partition coefficient (in percent) of the fluorescent probes studied ($n = 4$)

		5,(6)-CF	PTSA
Without surfactant	Marker alone	$21.4 \pm 0.3\%$	$1.6 \pm 0.3\%$
	Marker +actives	$21.3 \pm 0.3\%$	$1.0 \pm 0.3\%$
With surfactant	Marker alone	$21.2 \pm 0.3\%$	$1.0 \pm 0.3\%$
	Marker +actives	$23.2 \pm 0.3\%$	$1.6 \pm 0.3\%$

Table 3
Influence of incorporation of fluorescent probes on the viscoelastic oscillatory parameters of the multiple emulsions

	Without probes	5,(6)-CF (10^{-4} M)	PTSA (10^{-4} M)
g^*_m	167	309	190
δ_m	14	11	11
τ_c	21	31	46
ϵ_c	0.3	0.2	0.4

mula, since it was sensitive to light, to temperature, to acidic pH caused by the inner aqueous phase constituents and it diffused partially through the oily membrane. For this reason, the release studies will be performed only with PTSA.

3.1.5. Influence of incorporation of the fluorescent probes on the viscoelastic oscillatory parameters of the multiple emulsion

It can be seen in Table 3 and Fig. 3 that especially 5,(6)-CF changed the viscoelastic oscillatory parameters of the multiple emulsion. They became more compact and more elastic (G^*_m and δ_m values). It was not surprising to obtain a more compact multiple emulsion by the addition of a trace amount of 5,(6)-CF. In fact, the presence of NaOH (10^{-2} M for dissolving the fluorescence probe, pH > 7) in addition to the constituents of the inner phase, created a high concentration gradient difference between two aqueous phases. Because of this osmotic pressure difference the size of the aqueous droplets increased during the preparation of the multiple emulsion due to a flux of certain part of the external aqueous phase towards the internal phase causing a more compact emulsion. In the case of PTSA, its influence seems less evident, a similar behaviour was found but with lower G^*_m value. Nevertheless, it was surprising to obtain such a change with a very low concentration gradient modification and this remains unclear.

3.2. Release studies of PTSA from multiple emulsion

Three main release mechanisms are possible for the delivery of the substances from the inner aqueous phase of the multiple emulsions. Release could be induced by the facilitated transport of lipophilic surfactants across the oily membrane (Sela et al., 1995); by the swelling-breakdown of the oily globules (Jager-Lezer et al., in press); by the simple diffusion of the substance across the oily membrane (Magdassi and Garti, 1984).

Our previous conductimetric experiments showed that the only possible release mechanism for the present formulation is the swelling-breakdown of the emulsion. Indeed, any transport observed by the inverse micelles was possibly due to the polymeric nature of the lipophilic surfactant since any change on the conductivity value was measured on the external aqueous phase of the iso-osmotically diluted multiple emulsion (1/20 in 1% glucose solution). When a W/O/W multiple emulsion was diluted under a hypo-osmotic condition (in demineralized water), the concentration gradient between all the dissolved species $\Delta C > 0$ is responsible for water flow from the external phase to the internal phase. This aqueous transport produces an increase of the internal microglobule sizes and therefore the oily globules swell until a critical size is reached. Beyond this critical size, the globules split by the breakdown of the oily membrane and release the entrapped molecules (Jager-Lezer et al., in press). After the dilution of the multiple emulsion, since the PTSA do not diffuse through the oily membrane (according to the previous partition studies), we can only observe a release of fluorescent marker by the destruction of this one. So, we can expect to measure roughly the same %ß values by HPLC and conductimetric analyses.

The release was evaluated by two different techniques:

(1) A complete separation of external aqueous phase was performed and the fluorescent probe was assayed by HPLC

(2) $MgSO_4.7H_2O$ was assayed directly on the diluted emulsions (1/20 with demineralized water) by conductimetry. This technique was applied only on the multiple emulsion formula without L-ascorbic acid and *N*-acetyl-L-cysteine. Since the electrode of the conductimeter is sensitive to all the electrolytical species present in the sample and the calculation of %ß become hazardous due to

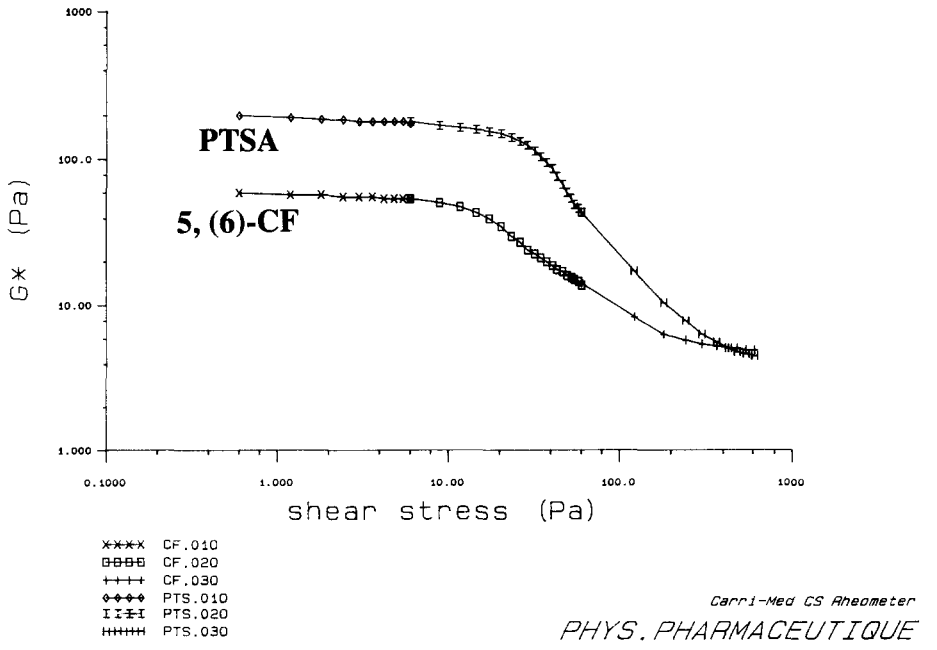


Fig. 3. Viscoelastic oscillatory rheograms of the 5,(6)-CF and PTSA.

the presence of these two molecules together with $MgSO_4.7H_2O$. Also, it is necessary to mention that the conductivity of the PTSA in an aqueous solution is negligible in relation to other electrolytical species present in the formula.

It can be seen in Fig. 4 that the release curves of PTSA by HPLC and of $MgSO_4.7H_2O$ by conductivity are almost superimposable. In addition, a quite satisfying correlation was obtained between two types of analyses (Fig. 5). Under hypoosmotic conditions, the release of only a small quantity ($< 2\%$) of PTSA, at the end of the 24 h is an index of the elasticity of the oily membrane. Our previous experiments showed that the yield of encapsulation of PTSA was extremely high, almost 100% (99.8%). So, the possibility of the release of PTSA by the rupture of the oily glob-

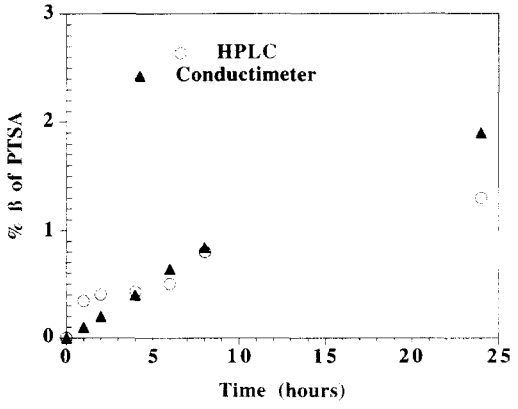


Fig. 4. Release of PTSA measured by HPLC and conductimetry.

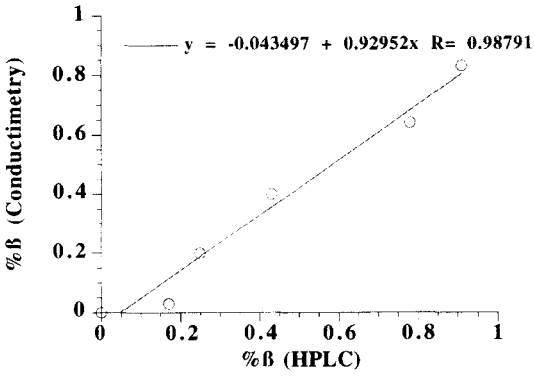


Fig. 5. Release correlation (%β) between HPLC and conductimetry values for PTSA.

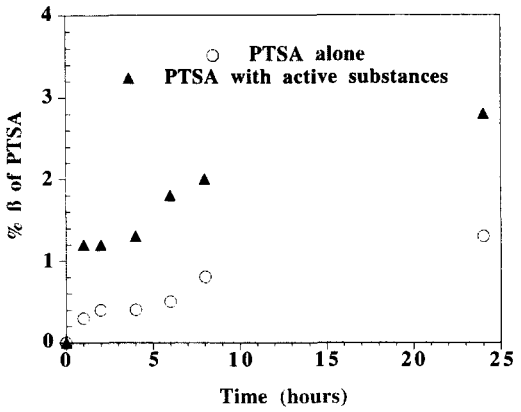


Fig. 6. Release of PTSA from different formulations measured by HPLC.

ules in the course of manufacturing could be eliminated. Consequently, these results demonstrated that PTSA could be released only by the swelling-breakdown mechanism.

As a consequence, the release of PTSA was 2-fold faster when the formulation contained L-ascorbic acid and *N*-acetyl-L-cysteine (Fig. 6). The presence of these substances caused a high concentration gradient difference between the two aqueous phases. For this reason, when the emulsion was diluted on the hypo-osmotic conditions, a faster water flux occurs from the external aqueous phase to the internal aqueous phase. So the oily membrane swelled and ruptured more rapidly. This result is well supported by the steady state rheological analyses (Fig. 7). For the formulae containing L-ascorbic acid and *N*-acetyl-L-cysteine, the swelling phase was sharper and faster due to the water flux. Once more, the delivery of this small amount of PTSA (< 3%) against the high concentration gradient difference between the aqueous phases demonstrates the elasticity and the stability of the oily membrane for this formulation. The polymeric nature of the lipophilic surfactant could be responsable for this exceptional behaviour.

4. Conclusion

A fluorescent tracer technique was developped to select the most appropriate fluorescent probe in

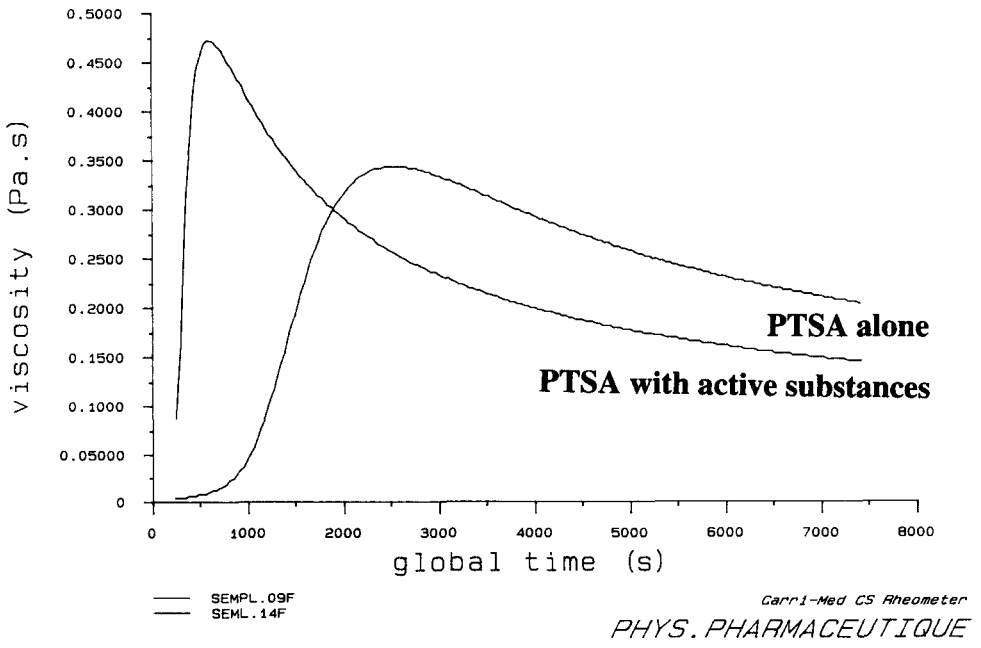


Fig. 7. Viscosity versus time rheograms of PTSA.

order to characterize W/O/W multiple emulsions and to study their release mechanism from the multiple emulsion.

5,(6)-CF is easily detectable by fluorescence microscopy, exhibiting a vivid yellow fluorescence emission, and easily quantified by direct spectroscopy and HPLC. Nevertheless, it is sensitive to light, temperature and the pH of the inner aqueous phase of the emulsion. In addition, it is an inappropriate fluorescent probe for studying the release mechanism of this multiple emulsion, since it diffuses through the oily membrane as a function of the pH of the inner aqueous phase.

PTSA is easily quantified either by direct fluorescence spectroscopy and HPLC. In addition, it is not sensitive to light, temperature and pH change. It does not diffuse through the oily membrane so it appears as a convenient tracer for studying the release mechanism of W/O/W multiple emulsion. Nevertheless it is less appropriate marker for the fluorescence microscopy due to its blue fluorescence emission.

The yield of encapsulation of the PTSA in the inner aqueous phase of the multiple emulsion is extremely high (almost 100%) and the release of the PTSA from the W/O/W multiple emulsion is very slow in all cases. This is certainly due to the exceptional stability and elasticity of the oily membrane, as well as its lack of sensitivity to the inner phase pH variation.

Comparison between the well established conductimetric release analysis with the proposed new fluorescent tracer technique shows a satisfying correlation. This work underlines the interest in a non-sensitive pH probe as a PTSA when the inner aqueous phase composed of several electrolytical species makes the conductimetric determinations defficient, and as a swelling-breakdown tracer when insight is required into the release mechanism of the active substance.

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