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Development and *in vitro* evaluation of topically applied cinnamic acid formulations

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The aim of this study was to formulate microcapsulated cinnamic acid (CN) formulations in w/o and o/w emulsions and to compare their release profiles with w/o, o/w and w/o/w emulsions without microcapsules through cellulose acetate, cellulose nitrate membranes and excised rat skin. The experimental design approach was used for understanding the influence of different formulation factors on release characteristics and to optimise most accurate formulating parameters. The data obtained in this study were subjected to statistical analysis by a repeated measures ANOVA, following Bonferroni multiple comparisons test. A significantly different release was observed due to the core : wall ratio of microcapsules. Emulsions containing microcapsules with 2 : 1 core : wall ratio were found to be ideal according to the factorial design, dissolution and diffusion studies. The type of emulsion was found to be a very important factor affecting the release profile of CN. The results described in this study indicate that microencapsulation of CN in w/o emulsion could be suggested as an effective carrier for CN to achieve sustained release and to protect the drug. Moreover, w/o/w emulsions were also useful carriers for prolonged release by encapsulating the substances in their internal aqueous phase and they also protect drugs and their preparation and application are easy. By the way, in the case of o/w emulsion any significant difference was observed. The type of the membrane also affected release. According to the release results, rat skin was found to be significantly different from the synthetic membranes.

1. Introduction

Emulsions have been widely used for topical delivery of drugs. They serve as carriers of many drugs by dissolving drugs in a dispersed phase (Nakano 2000; Ferreira et al. 1995). Multiple emulsions which are vesicular and complex systems are interesting carriers for such applications. These systems have some advantages, such as the protection of the entrapped substances in the inner aqueous phase and the modulation of their release rate to improve product efficacy and the incorporation of several actives in the different compartments. Multiple emulsions may facilitate application, prevent the irritation and lead to formulations characterized by their efficient, light, non-greasy and non-sticky textures (Silva Cunha et al. 1995; Muguet et al. 2001). However, many other hybrid systems have been discussed in which one of the phases has been modified to improve stability and to slow or control the release of actives. Systems like microcapsules or microspheres-in-oil-in-water or in-water-in-oil have been mentioned as multiphase phase systems (Garti 1996).

Microcapsules are multiparticulate delivery systems and are prepared to obtain sustained or controlled drug delivery, to improve bioavailability or stability and to reduce side effects. The microencapsulation process is generally performed by forming a polymeric matrix or coating layer around a particular compound in order to protect its biological activity from environmental factors and to enhance its physicochemical stability. The microencapsulation techniques, such as spray drying, extrusion, freeze drying, cocrystallization and phase separation (coacervation) have been widely used for encapsulating active materials (Higuera-Ciapara et al. 2004; Özyazıcı et al. 1997; Yoo et al. 2006).

Complex coacervation can be induced in systems containing carboxymethylcellulose (CMC), a water soluble cellulose derivative and aluminium sulphate which does not have any toxic effect and have been used in therapy (Ertan et al. 1994; Arıca et al. 1997; Mathew and Speaker 1998). The technique was selected to prepare microcapsules due to its simplicity, low cost, success with poor aqueous solubility drugs and the microcapsule production of relatively high drug loading.

Cinnamic acid (CN) acts as a free radical scavenger, carcinogen inhibitor, antimicrobial and antiviral agent (Letizia et al. 2005; Villano et al. 2005). The cutaneous metabolism must be taken into account when studying the skin penetration of cinnamic compounds. Even if it was stated that CN is nonsensitizing, it is handled in the form of microcapsules in this study to prevent the irritation and to protect its biological activity from environmental factors and enhance its physicochemical stability.

The aim of the present investigation was to prepare topical emulsions containing CN microcapsules to provide the extended release and to improve the stability of CN. The *in vitro* release of CN through cellulose acetate, cellulose nitrate membranes and excised rat skin from simple emulsions (w/o and o/w), a multiple emulsion and microcapsulated simple emulsions were investigated in a factorial design approach for understanding the influence of different formulation factors on release characteristics.

Experimental design is an established method to study the action of selected parameters which allows to evaluate the effects of a number of different factors simultaneously. The application of experimental design to microcapsules has been reported before (Devay et al. 1984; Sevgi et al. 1994). It offers a good degree of accuracy and the possibility of detecting interactions between factors. In addition, the stability of the formulations were determined. Also, their antimicrobial activity was observed.

2. Investigations, results and discussion

The codes and the main characteristics of formulations are shown in Tables 1 and 2, respectively. The emulsion type was confirmed by conductivity analysis. The thermal stability tests performed at 25 ± 1 °C and 40 ± 1 °C showed that the formulations were stable during a periode of 3 months. The addition of microcapsules did not comprimise the structure of the skin creams.

The minimum inhibition concentration (MIC) of CN was found to be 10–20 μg/mL according to the microbiological studies. This value was taken into account when choosing the amount of CN added to the formulations. In addition, the antimicrobial activity of CN was also determined in correlation with release and it was indicated that the released amounts of CN from the formulations were in the range of those recommended for the antimicrobial effect.

CN is slightly soluble in water and thus should be applied using emulsion and microencapsulation technology to facilate its solubility in aqueous medium, to control its release and to protect CN from unfavorable enviroment.

When a drug applied to the skin the release of active compound from the vehicle and its penetration through the skin barrier are two important effects which may becomerate-limiting steps. These processes are closely related, and both are dependent on the physical properties of the drug, vehicle and barrier (Röpke et al. 2002; Laugel et al. 1998).

In this work CMC was used as polymer and aluminium sulphate as the coacervating agent for preparing microcapsules. CMC incompatibilities have been reported with soluble salts of Al and some other metals such as Fe, Zn, Ag, Cu, Bi and aluminium sulphate does not have any toxic effect (Ertan et al. 1994). Its solutions have been used in therapy and the industrial applicability of this method was very simple and rapid. The aluminium sulphate solution can be used repeatedly.

The particle size of microcapsules affects the release rates of the drug from the microcapsules. Therefore particle sizes of the prepared microcapsules are determined by sieve analysis in a mechanical shaker. The highest yield was found as 95.5% with the particle size 53–125 μm at the end of the sieve analysis. It's known that the amount of the active substance changes depending on the microcapsule's particle size and core : wall ratio (Ertan et al. 1997; Özyazıcı et al. 1997).

The encapsulation efficiencies were calculated as 78.4; 75.7; 89.5% for 1 : 1, 1 : 2 and 2 : 1 core : wall ratios, respectively, and were determinded to be significantly different ($p < 0.05$). The encapsulation efficiency was affected by the ratio of core and wall materials. Highest entrapment was obtained with 2 : 1 core : wall ratio. CN-loaded microcapsules having a fairly high yield between 84.3 and 89.5% were obtained.

The determination of dissolution rate in the receptor medium was carried out to verify that the solubility of CN in this medium did not constitute a limiting factor in the absorption process. The *in vitro* disssolution rate of microcapsulated CN increased in the following order: 1 : 1 < 1 : 2 < 2 : 1 with the amount of 63.4, 67.3, 93.6% respectively. The solubility of CN in phosphate buffer solution (pH 7.4) was 0.5 mg/mL.

The *n*-octanol/buffer partition coefficient (P_o) is commonly used to reflect the lipophilicity of a compound (Comer and Tam 2001). The best vehicle for topical controlled release would be the one which contributes to a reversible decrease in the stratum corneum resistance and allows the controlled diffusion of molecules in the vehicle itself (Laugel et al. 1998). Our results indicated that CN had affinity to phosphate buffer solutions with the value of 0.76 as it was stated in the solubility studies, too.

The factorial design model was applied to the evaluation of the diffusion rate of emulsions with and without microcapsules. The data obtained in this study were subjected to statistical analysis by repeated measures ANOVA, following the Bonferroni multiple comparisons test. P value of less than 0.05 was considered as evidence of a significant difference. A four factor experiment with a repeated measure on last factor ($4 \times 2 \times 3 \times 8$) was performed, in order to show the effect of core : wall ratio (V1), type of the emulsion (V2), type of the diffusion barrier (V3) on the *in vitro* release rate of CN. These factors are easy to

Table 1: Codes of the formulations

Vehicles	Formula code
W/O	F1
O/W	F2
W/O + MC	F3
O/W + MC	F4
W/O/W	F5

* MC: The microcapsules having 2 : 1 core : wall ratio

Table 2: Characteristics of the emulsions

Emulsions	Microscopic aspect	Macroscopic aspect	Conductivity	Stability	
				25 °C	40 °C
w/o	Simple globules 2–3 μm	Homogeneous, compact, white	0.02 μS	>3 m	3 m
o/w	Simple globules <2 μm	Homogeneous, very compact, white	25 μS	>3 m	3 m
w/o/w	Multiple globules 8–12 μm	Homogeneous, very compact, white	19 μS	>3 m	3 m

Table 3a: Results of the interactions between time and the other factors

Source	DF	Sum of squares	Mean of squares	F	P
Time	7	36891.711	5270.244	1050.902	0.000*
Time*V1	21	171.661	8.174	1.630	0.391
Time*V2	7	125.602	17.943	3.578	0.001*
Time*V3	14	2929.897	209.278	41.731	0.000*
Error	455	2281.812	5.015		

* The mean difference is significant at the 0.05 level.
V_1: core : wall ratio (%1 CA, 2 : 1 MC, 1 : 1 MC, 1 : 2 MC)
V_2: Type of the emulsion (w/o, o/w)
V_3: Type of the diffusion barrier (cellulose acetate membrane, cellulose nitrate membrane, rat skin)

Table 3b: Results of statistical analysis by ANOVA ($4 \times 2 \times 3 \times 8$)

Source	DF	Sum of Squares	Mean of Squares	F	P
Corrected model	23	7617.735[a]	331.206	163.395	0.000*
Intercept	1	62813.043	62813.043	30987.822	0.000*
V_1	3	690.057	230.019	113.476	0.000*
V_2	1	82.077	82.077	40.491	0.000*
V_3	2	5338.846	2669.423	1316.918	0.000*
$V_1 \times V_2$	3	694.829	82.266	114.261	0.000*
$V_1 \times V_3$	6	493.595	8.476	40.585	0.000*
$V_2 \times V_3$	2	16.951	50.230	1.181	0.021*
$V_1 \times V_2 \times V_3$	6	301.380	2.027	24.780	0.000*
Error	48	97.297			
Total	72	70528.075			
Corrected total	71	7715.032			

[a] $r^2 = 0.987$
* The mean difference is significant at the 0.05 level

control. Furthermore they were expected to have a pronounced effect on the release rate of CN. When a drug is applied to the skin, the release of active compound from the vehicle and its penetration through the skin barrier are two important effects which may become rate-limiting steps. These processes are closely related, and both are dependent on the physical properties of the drug, vehicle and barrier.

Depending on the interaction between time and the other factors, each time point was evaluated as $4 \times 2 \times 3$ factorial design. Tables 3a, 3b show the results of the statistical analysis. As it was obtained from ANOVA results, main effects of each factor and their interactions were also found to be significant ($p < 0.05$). Thus, V_1, V_2 and V_3 have been found as important factors effecting the release rate of active substance.

The *in vitro* release methods measure drug/vehicle interactions, which affect release characteristics. It has been reported that *in vitro* diffusion models can be useful as basic test systems for comparing vehicles (Barry 1988; Yener et al. 2003). The *in vitro* release of CN through artificial membranes and rat skin was studied for 8 h from 0.05 M phosphate buffer (pH 7.4). CN were found stable under these conditions (Smith et al. 2000). The *in vitro* releases of CN were studied for 24 h. After 24 h, the release patterns were found almost same with the results of 8 h. So we showed only the results of 8 h in the figure.

A significant difference was observed between the release of the simple w/o and o/w emulsions containing microcapsules with different core : wall ratios from cellulose nitrate, cellulose acetate membrane and excised rat skin following Bonferroni multiple comparisons test (Fig. 1). Emulsions containing microcapsules with a 2 : 1 core : wall ratio showed the highest release. An increasing of the core : wall ratio gave a lower release due to the increasing of thickness of the microcapsule wall and liberation of active ingredient was incomplete. As the concentration of the polymer in the system decreased, the release rate of CN increased. This shows that the release rate is controlled by the wall thickness. Sustained release through microencapsulation depends on forming a diffusion barrier around the drug particles through which the drug must pass to reach the external media. A diffusion controlled process is responsible for the release of CN from the microcapsules (Mortada et al. 1987; Sevgi et al. 1994).

Emulsions having microcapsules with 2 : 1 core : wall ratio were found to be ideal according to the factorial design, dissolution and diffusion studies. So, the release results of

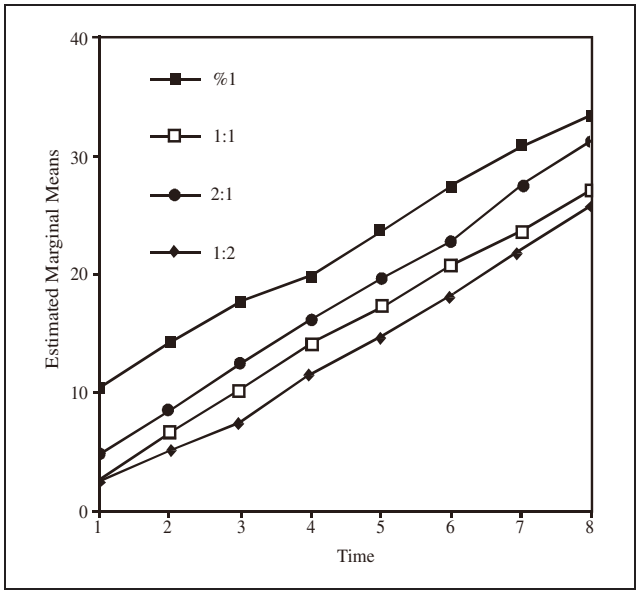

Fig. 1: Multiple comparisons of %1 free CA, 2 : 1 MC, 1 : 1 MC, 1 : 2 MC based on estimated marginal means

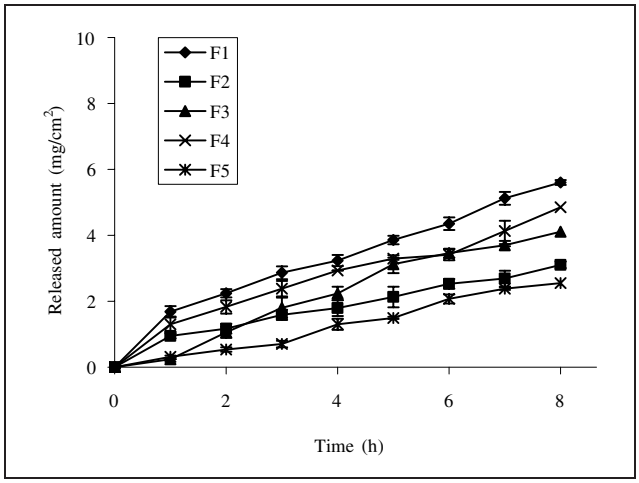

Fig. 2: Release profiles of CN incorporated in different formulations, through cellulose acetate membrane

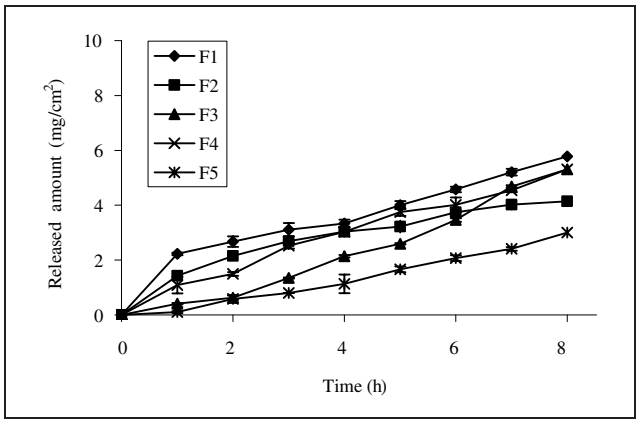

Fig. 3: Release profiles CN incorportated in different formulations, through cellulose nitrate membrane

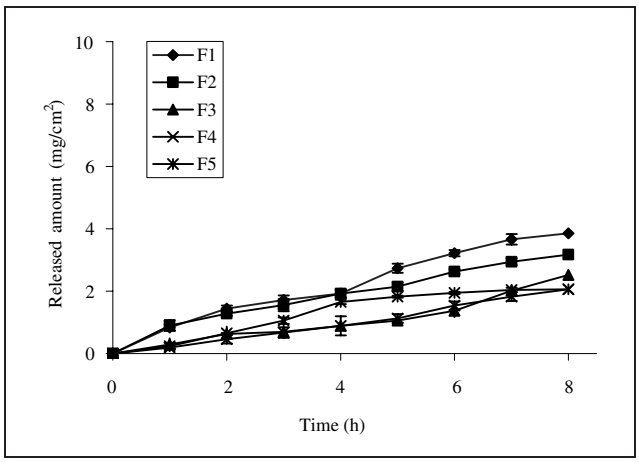

Fig. 4: Release profiles of CN incorporated in different formulations, through rat skin

these formulations were compared with the release results of the w/o/w multiple emulsion which was reported to be a prolonged release system by various authors (Laugel et al. 1998; Omotosho et al. 1996; Okochi and Nakano 2000). The release profiles of CN from the w/o and o/w simple emulsions with microcapsules having 2 : 1 core : wall ratio and without microcapsules and w/o/w multiple emulsion through different membranes are shown in Figures 2–4 and Table 4.

Differences in release were observed due to the vehicles. The comparison of release results of w/o emulsion with microcapsulated w/o emulsion showed a prolongation of release by microencapsulation from 5.6, 5.7, 3.8 mg/cm^2 to 3.6, 4.6, 2.0 mg/cm^2 for cellulose acetate, cellulose nitrate membrane and rat skin, respectively. Therefore it appears that the microcapsules could be used to prepare a sustained release preparation of CN and the release is a function of both the formulation factors and membrans. By the way, in the case of o/w emulsion any significant difference was observed between the microcapsulated formulations.

Three factor experiments with a repeated measure on the last factor ($3 \times 3 \times 8$) design were used for evaluating the release from emulsions without microcapsules. The *in vitro* release of free CN, from w/o, o/w and w/o/w emulsions were found to be significantly different. The results of release studies and variance analysis clearly shows that the type of emulsion has a pronounced effect on the release rate of CN (Fig. 5). The release profiles indicated the rank order of w/o, o/w simple emulsions and w/o/w multiple emulsions. Release rate and amount of CN are dependent on the solubilizing effect of the vehicle. When all formulations were evaluated according to the amount released, w/o simple emulsions showed the highest release while w/o/w multiple emulsions showed the lowest release with the values of 2.9, 3.1, 1.5 mg/cm^2 for cellulose acetate, cellulose nitrate membrane and rat skin, respectively. In the case of w/o emulsion the location of CN in the external oil phase was available for the diffusion. The lipophilic surfactants of w/o emulsions might also facilitate the release process. In this study, Span 80 was thought to enhance the release of CN from w/o emulsion. It has been shown that nonionic surfactants increased the skin penetration of some actives. The authors also hypothesized that the nature of the medium could influence the interaction between nonionic surfactants and the skin barrier (Nokhodchi et al. 2002).

In contrast, the diffusion of CN from w/o/w and o/w emulsion was slower. This result was in agreement with a better encapsulation of CN in the inner phase of the emulsion, unavailable for diffusion. In addition, due to the partition coefficient of CN, it was concluded that the release could be reduced if there was a great propensity for the

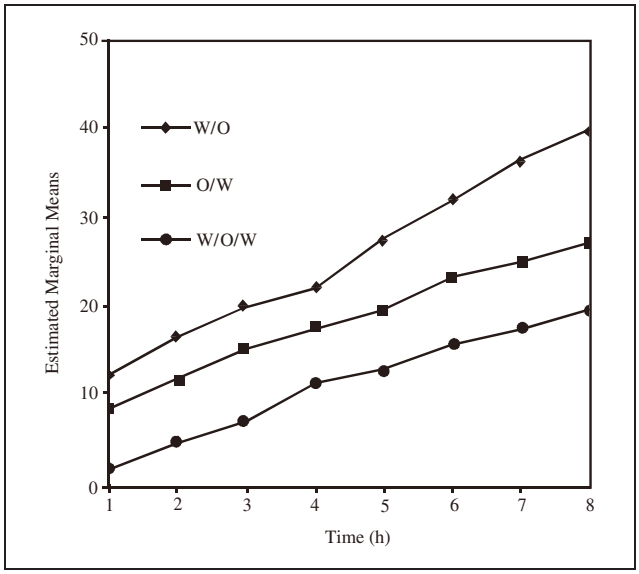

Fig. 5: Multiple comparisons of w/o, o/w and w/o/w type emulsions based on estimated marginal means

Table 4: Results of release studies

Formulation	Diffusion barrier	Amount of drug released (mg/cm^2)	Jss ($mg/cm^2/h$)	D (cm^2/s)	r^2
F1 (%1 CA, w/o)	Cellulose acetate	5.601 ± 0.169	0.715	1.080×10^{-10}	0.995
	Cellulose nitrate	5.777 ± 0.111	0.671	8.060×10^{-11}	0.986
	Rat skin	3.857 ± 0.139	0.566	0.00109	0.973
F2 (%1 CA, o/w)	Cellulose acetate	3.170 ± 0.173	0.398	2.029×10^{-9}	0.993
	Cellulose nitrate	4.145 ± 0.281	0.426	7.292×10^{-11}	0.976
	Rat skin	3.106 ± 0.169	0.416	0.00077	0.994
F3 (2 : 1 MC, w/o)	Cellulose acetate	3.699 ± 0.239	0.644	4.596×10^{-10}	0.968
	Cellulose nitrate	4.684 ± 0.144	1.003	1.117×10^{-10}	0.989
	Rat skin	2.017 ± 0.173	0.401	0.00177	0.907
F4 (2 : 1 MC, o/w)	Cellulose acetate	4.132 ± 0.194	0.594	1.265×10^{-10}	0.974
	Cellulose nitrate	4.540 ± 0.315	0.749	2.019×10^{-10}	0.980
	Rat skin	1.824 ± 0.147	0.344	0.00298	0.979
F5 (%1 CA, w/o/w)	Cellulose acetate	2.935 ± 0.266	0.366	1.395×10^{-10}	0.928
	Cellulose nitrate	3.196 ± 0.453	0.402	1.537×10^{-10}	0.967
	Rat skin	1.586 ± 0.215	0.283	0.00355	0.939

active material in the phase which it had an affinity. This suggests the importance of the vehicle, especially the nature of the external phase in case of emulsion on the diffusion of molecules. The microcapsules were not added to the w/o/w multiple emulsions because of the multiple emulsions prolonged effect as well.

It was demonstrated that encapsulating the fragile or biodegradable molecules in the internal phase of w/o/w multiple emulsions protects them against degradation (Tedajo et al. 2005). Therefore, when CN was introduced in the inner phase of w/o/w multiple emulsions it was also possible to protect them against environmental factors such as light degradation.

When diffusion barriers were compared by using Bonferroni multiple comparisons test, rat skin were found to be significantly different from the synthetic membranes ($p > 0.05$) (Fig. 6). The release through cellulose nitrate membrane was higher than the release through cellulose acetate membrane and excised rat skin for all formulations. This observation could be explained by the polar structure of cellulose and lipophilic barrier role of the skin (Laugel et al. 1998).

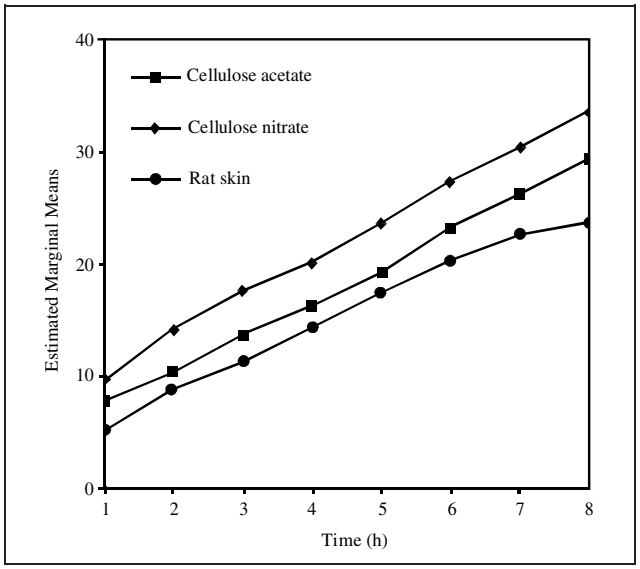

Fig. 6: Multiple comparisons of the diffusion barriers based on estimated marginal means

It was concluded that CN is able to permeate through biological membranes. Physicochemical and structural features of this biophenol may be taken into consideration to justify its capability to interact with model membranes (Castelli et al. 1999).

3. Experimental

3.1. Materials

Cinnamic acid was obtained from Fluka. Parafin liquid (Birpa, Turkey) used as oil phase in emulsions. The lipophilic surfactants were Span® 80 (Merck) and Abil®EM (Goldschmid, France), the hydrophilic surfactants were Tween®80 (Merck) and Synperonic®PE/F127 (ICI, France).

Carboxymethylcellulose (CMC) used in coacervation was obtained from Sigma. Aluminium sulphate solution (Horasan Kimya, Turkey) was used as coacervation solution. All other chemicals used for experiments were of analytical reagent grade.

Cellulose acetate membranes (por size: 0.2 μm Sartorious AG, Germany) and cellulose nitrate membranes (pore size: 0.45 μm Sartorious AG, Germany) were rinsed with phosphate buffer solution (pH: 7.4) and soaked in the receptor fluid for 10 min.

For *ex vivo* studies the abdominal furs of rats were removed. Skin samples in full thickness were cut, removed and washed with water. Fat and connective tissues were carefully removed. Thickness of the rat skin was measured by micrometer and found as 0.59 ± 0.04 mm.

3.2. Preparation of emulsions

A two-step process was used to prepare the multiple emulsions (Ferreira et al. 1995). The formula was 24% paraffin oil, 4% Abil EM©90, 0.8% Synperonic PE/F©127, 0.7% $MgSO_4 \cdot 7H_2O$, 70.5% distilled water, by weight.

The simple emulsions (o/w and w/o) were formulated by adding aqueous phase to the oil phase containing CN. In the case of o/w emulsion, aqueous phase contained 3.25% Tween80, 0.375% carbomer, 0.3% triethanolamine and oil phase consisted of 1.75% Span80 while W/O emulsion was containing 5% Span80 in oil phase. In all of the formulations the concentration of CN was 1%. This concentration was chosen because of being within the range of those recommended for the antioxidant and antimicrobial effect.

3.3. Preparation of microcapsules

The microcapsules with different core : wall ratios (1 : 1, 1 : 2 and 2 : 1) were formulated with carboxymethylcellulose-aluminium sulphate by means of a coacervation-phase separation technique (Ertan et al. 1994). CMC was one of the most used coacervation agent in microcapsule formulations with no side effects (Koh and Tucker 1988; Ertan et al. 1997).

CMC (1 g) was dissolved in 20 g of boiling water and allowed to hydrate for 12 h at room temperature. 1 g CN was suspended in this viscous polymer solution and stirred with a propeller type agitator (Ika-Werk, Germany). Coacervates were prepared at 37 °C by adding the 4% aluminium sulphate solution to the CMC solution dropwise. The microcapsules were then separated by filtration, washed 6–7 times with distilled water and dried at room temperature for 24 h. All microcapsule formulations were prepared in triplicate. Microcapsules dried at room temperature were then weighed and the yield of microcapsule preparation was calculated.

3.4. Sieve analysis

Separation of the microcapsules into various size fractions was carried out using a mechanical sieve shaker (Retsch, Germany). The sieves were shaken for a period of about 10 min, and then the particles on the screen were weighed. The procedure was carried out three times for each product.

3.5. Percentage yield value

The percentage yield value is defined as the quantity of microcapsules produced as a function of loaded drug and polymer.

3.6. Determination of encapsulation efficiency of microcapsules

Microcapsules amount equivalent to 1% CN were weighed accurately and dissolved in phosphate buffer (pH 7.4). Drug concentration was determined by UV spectrophotometry at 269 nm (n = 4). To determine the encapsulation efficiency, the following practical relationship was used:

$$\text{Encapsulation efficiency} = \left[\frac{\text{drug entrapped (b)}}{\text{theoretical drug content (a)}}\right] \times 100$$

3.7. In vitro dissolution rate experiments

The USP XXII rotating basket method was used in the disolution rate experiments. The mesh size of the basket was 40. The rotating speed used was 100 rpm. The dissolution media used was phosphate buffer (pH 7.4). At various time intervals the samples were taken and replaced by an equal volume of dissolution phosphate buffer (pH 7.4). Microcapsules with 1 : 1, 1 : 2 and 2 : 1 core : wall ratios were used in the *in vitro* dissolution studies. The samples were then assayed spectrophotometrically (Shimadzu UV-1208, Japan) at 269 nm. The determination was carried out in triplicate and the calibration curve was used for the determination of the amounts dissolved.

3.8. Partition coefficient determination

The partition coefficient (P_o) determination experiments (Gao et al. 2005) were carried out in aqueous phosphate (pH 7.4) buffer solution for CN. The buffer solution was pre-saturated with *n*-octanol. The drug substances were dissolved in this buffer solutions at concentrations of about 10^{-5} M. The buffer phase: *n*-octanol ratio was adjusted to 1 : 1. Six vials of the drug substance was sealed and agitated in a magnetic stirrer for about 6 h, at 600 rpm. After stirring the vials the phases were separated by centrifugation at 3000 rpm for 20 min (Kim et al. 2001; Mrestani et al. 2004). Samples, before and after partition, were quantified using UV–Vis spectrophotometry, at 269 nm. The average partition coefficient was obtained from three separate experiments.

3.9. Characteristics of the emulsions

Macroscopically, the emulsion appearances were checked every day for any sign of phase separation or microbial contamination. The conductivity of the emulsions was measured with a conductimeter (Jenway 4071, U.K.) in order to discriminate the emulsion type. An optical immersion microscope at 1000× magnification after diluting the emulsions was used for the microscopic observations.
Stability was tested at 25 ± 2 °C and 40 ± 2 °C at equal time intervals via clarity, particle size and centrifuge test.

3.10. Antimicrobial activity studies

The antimicrobial activity was determined by a microbroth dilution method using two Gram positive (*Staphylococcus aureus* ATCC 6538P and *Enterococcus faecalis* ATCC 29212) and two gram negative (*Escherichia coli* ATCC 11230 and *Pseudomonas aeroginosa* ATCC 27853) bacteria (Bozkır and Saka 2005; NCCL Standards 1997). For testing antifungal activities of the compounds (*Candida albicans* ATCC 90028) reference strains were used. Triptic Soy broth (Merck) and Sabouraud Dextrose broth (Merck) were used for testing bacterial and fungal strains, respectively. The compounds investigated were dissolved in phosphate buffer (pH 7.4) and added to the medium. Test microorganisms (10^6 cfu/mL) were added to each sample and test tubes were incubated 18–24 h at 35 ± 0.5 °C for testing bacterial strains. Minimal inhibitory concentrations (MIC) were defined as the lowest concentrations of the compounds that inhibited visible growth of the microorganism.

3.11. In vitro release studies

The dialysis cell method was used to determine the amount of the drug diffused from emulsions (Roseman 1981). The release of CN was investigated through a cellulose acetate, cellulose nitrate membrane and excised rat skin.
The membranes were mounted at the bottom of diffusion tube with a diameter of 3 cm and immersed in a beaker containing phosphate buffer (pH 7.4) maintained at 37 °C ± 0.5 that the bottom of the cell was slightly touching medium's surface. The membrane was previously allowed to hydrate in distilled water for 30 min and then in buffer solutions. Donor phases (1 g) including 1% (w/w) CN were applied to the membranes. Microcapsules in an amount equivalent to this concentration were introduced by dispersion into the simple w/o and o/w emulsions before application of emulsions on the membranes. The medium was agitated with a magnetic stirrer at 50 rpm. After application of the emulsions on membranes, samples were taken at specified time intervals from the receptor fluid. The effective diffusion area was 1.54 cm^2. The released amounts were determined spectrophotometrically at 269 nm and the averages of the release rates were calculated.
Permeability coefficients of the formulations were calculated according to Fick's Law of diffusion (Higuchi 1962):

$$Q = P^*A^*C_0{}^*t$$

where P is the permeability coefficient (cm/s), Q is the amount of the drug released (mg); A is the area of the diffusion membrane (cm^2); C_0 is the initial concentration of drug in the formulations (mg/cm^3); and t is the time.

3.12. Statistical analysis

The data obtained in this study were subjected to statistical analysis by a repeated measures ANOVA, following Bonferroni multiple comparisons test. A p value of less than 0.05 was considered as evidence of a significant difference. Four different factors were compared. The effects of the core : wall ratio, the type of emulsion, type of the diffusion barrier and time on release kinetics were studied by four factor experiments with a repeated measure on the last factor (4 × 2 × 3 × 8) design.

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References

Arica MY, Yiğitoğlu M, Lale M, Kök KN, Hasırcı V (1997) Controlled release of Aldicarb from carboxymethylcellulose microcapsules. Turk J Chem 21: 100–104.
Barry BW (1988) Topical preparations Pharmaceutics: The Science of Dosage Form Design, Ed.: Aulton ME, Churchill Livingstone, Inc., New York, pp. 381–411.
Bozkır A, Saka OM (2005) Formulation and investigation of 5-FU nanoparticles with factorial design based studies. Farmaco 60: 840–846.
Castelli F, Uccella N, Trombetta D, Saija A (1999) Differences between coumaric and cinnamic acids in membrane permeation as evidence by time-dependent calorimetry. J Agric Food Chem 47: 991–995.
Comer J, Tam K (2001) Pharmacokinetic Optimisation. In: Testa B, Waterbeemd HV, Folkers G, Guy R (eds.) Chapter 17, Drug Research, Wiley-VCH, Weinheim.
Devay A, Fekete P, Racz I (1984) Application of factorial design in the examination of tofizopam microcapsules. J Microencaps 1: 299–305.
Ertan G, Özer Ö, Baloğlu E, Güneri T (1997) Sustained-release microcapsules of nitrofurantoin and amoxicillin; preparation, *in-vitro* release rate, kinetic and micromeritic studies. J Microencaps 14: 379–388.
Ertan G, Sarıgüllü I, Karasulu Y, Erçakır E, Güneri T (1994) Sustained release dosage form of nitrofurantoin. Part 1. Preparation of microcapsules and *in vitro* release kinetics. J Microencaps 11: 127–135.
Ferreira LAM, Seiller M, Grossiord JL, Marty JP, Wepierre J (1995) Vehicle influence on in vitro release of glucose: w/o, w/o/w and o/w systems compared. J Control Release 33: 349–356.
Gao X, Yu CH, Tam KY, Tsang SC (2005) New magnetic nano-absorbent for the determination of *n*-octanol/water partition coefficients. J Pharm Biomed Anal 38: 197–203.
Higuera-Ciapara I, Felix-Valenzula L, Goycoolea FM, Argüelles-Monal W (2004) Microencapsulation of astaxanthin in a chitosan matrix. Carbohydrate Polymers 56: 41–45.
Higuchi WI (1962) Analysis data on the medicament release from ointments. J Pharm Sci 51: 802–804.
Kim K, Cho W, Chang S, Yong C, Lee C, Kim D (2001) Pharmacokinetics of a new antitumor 3-arylisoquinoline derivative, CWJ-a-5. Int J Pharm 217: 101–110.
Koh GL, Tucker IG (1988) Characterization of sodium carboxymethyl cellulose-gelatin complex coacervation by viscosity, turbidity and coacer-

vate wet weight and volume measurements. J Pharm Pharmacol 67: 151–158.
Laugel C, Baillet A, Youenang Piemi MP, Marty JP, Ferrier D (1998) Oil-water-oil multiple emulsions for prolonged delivery of hydrocortisone after topical application: comparison with simple emulsions. Int J Pharm 160: 109–117.
Letizia CS, Cocchiara J, Lapezynski A, Lalko J, Api AM (2005) Fragrance material review on cinnamic acid. Food Chem Toxicol 43: 925–943.
Garti N (1996) Influence of the formulation on the characteristics and stability of multiple emulsions. In: Grossiord, JL; Seiller, M (eds) Multiple Emulsions: Structure, Properties and Applications, Editions de Santé Paris, pp. 279–312.
Mathew E, Speaker TJ (1998) Influence of dextran molecular weight on capture in and release from decylamine carboxymethylcellulose capsules. J Microencaps 15: 675–680.
Mortada SAM, El Egaky AM, Motawi AM, El Khodery KA (1987) Preparation of microcapsules from complex coacervation of gantrez-gelatin. J Microencaps 4: 23–27
Mrestani Y, Mrestani-Klaus C, Bretschneider B, Neubert RHH (2004) Improvement of lipophilicity and membrane transport of cefuroxime using *in vitro* models. Eur J Pharm Sci 58: 653–657.
Muguet V, Seiller M, Barrat G, Özer Ö, Marty JP, Grossiord JL (2001) Formulation of shear rate sensitive multiple emulsions. J Control Release 70: 37–49.
Nakano M (2000) Places of emulsions in drug delivery. Advanced Drug Delivery Reviews 45: 1–4.
National comittee for clinical laboratory standards (1997) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved Standard. M27-A4, Villanova, PA.
Nokhodchi A, Nazemiyeh H, Ghafourian T, Hassan-Zadeh D, Valizadeh H, Bahary LAS (2002) The effect of glycyrrhizin on the release rate and skin penetration of diclofenac sodium from topical formulations. Farmaco 57: 883–888.
Okochi H, Nakano M (2000) Preparation and evaluation of w/o/w type emulsions containing vancomycin. Adv Drug Del Rev 45: 5–26.
Omotosho JA, Whateley TL, Florence AT (1996) Release of 5-FU from intramusculer w/o/w multiple emulsions. Biopharm Drug Disp 10: 180–186.
Özyazıcı M, Sevgi F, Ertan G (1997) Sustained release dosage form of nicardipine hydrochloride: application of factorial design and effect of surfactant on release kinetics. Drug Devel Ind Pharm 23: 761–770.
Roseman TJ (1981) Continuous flow bead-bed apparatus for suppositories. J Pharm Sci 70: 646–651.
Röpke CD, Kaneko TM, Rodrigues RM, Da Silva VV, Barros S, Sawada TCH, Kato JM, Barros BMB (2002) Evaluation of percutaneous absorption of 4-nerolidylcathecol from four topical formulations. Int J Pharm 249: 109–116.
Sevgi F, Özyazıcı M, Güneri T (1994) Sustained release dosage form of phenylpropanolamine hydrochloride (3). Application of factorial design. J Microencaps 11: 439–444.
Silva Cunha A, Grossiord JL, Puisieux F, Seiller M (1997): Insulin in w/o/w multiple emulsions: preparation, characterization and determination of stability towards proteases in vitro. J Microencaps 14: 311–319.
Smith CK, Moore CA, Elahi EN, Smart ATS, Hotchkiss SAM (2000) Human skin absorption and Metabolism of the contact allergens, cinnamic aldehyde and cinnamic alcohol. Toxicol Appl Pharmacol 168: 189–199.
Tedajo GM, Bouttier S, Fourniat J, Grossiord JL, Marty JP, Seiller M (2005) Release of antiseptics from the aqueous compartments of a w/o/w multiple emulsion. Int J Pharm 288: 63–72.
USP United States Pharmacopeia 24 – NF 19 (2000) Suppl. II, United States Pharmacopoeial Convention Rockville, USA.
Villano D, Soledad Fernandez-Pachon M, Troncoso AM, Carmen Garcia-Parilla M (2005) Comparison of antioxidant activity of wine phenolic compounds and metabolites *in vitro*. Anal Chim Acta 538: 391–398.
Yener G, Incegul T, Yener N (2003) Importance of using solid lipid microspheres as carriers for UV filters on the example octyl methoxy cinnamate. Int J Pharm 258: 203–207.
Yoo SH, Song YB, Chang PS, Lee HG (2006) Microencapsulation of α-tocopherol using sodium alginate and its controlled release properties. Int J Biol Macromol, in press.