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COMMUNICATIONS

METHOTREXATE-LOADED NANOPARTICLES: ANALYSIS OF DRUG CONTENT AND STUDY OF THE MATRIX STRUCTURE

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ABSTRACT

Entrapment of methotrexate in biodegradable colloidal systems was studied. Nanospheres were characterized in terms of morphometrical properties, such as particle diameter and particle size distribution. The ability of two surfactants, and dextrans of different molecular weights to improve the entrapment of the drug in a polymeric matrix was also studied. The presence of sodium lauryl sulphate in the polymerization medium increased the drug content in the nanospheres, and this effect was more significant when Dextran T70 was added to the formulation. Thermal methods such as thermogravimetry, differential scanning calorimetry and thermomicroscopy were applied, in order to study the possible interactions between the polymer and the drug. Crystalline methotrexate domains were observed in the polybutylcyanoacrylate matrix by thermomicroscopy.

INTRODUCTION

In recent years a number of efforts have been made to develop more rational approaches to specific cancer therapy, based on the concept of drug targeting. More recently a solid submicronic drug carrier of a polymeric nature in the nanometer size range (nanoparticles) has been proposed as biological target (1).

Since their introduction into clinical practice Methotrexate (MTX), have had an increasingly important role in the treatment of a greater number of localized and metastasized cancers. The toxic effects of this drug include bone marrow depression, renal failure, hepatotoxicity and pulmonary diseases (2). Several attemps to reduce the side effects of methotrexate include its encapsulation in

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liposomes (3), its association with fibrinogen formulation (4), incorporation to alkylcyanoacrylates (5-6) or its conjugation with serum albumin (7).

The aim of this study is to improve the entrapment of methotrexate to polyalkylcyanoacrylates (PACA) by analysing the influence of stabilisers, such as surface active agents and dextrans, added to the polymerization medium, on the methotrexate PACA association. Morphometrical properties were determined by photon correlation spectroscopy. The association efficiency of this drug was evaluated by high performance liquid chromatography (HPLC). In order to shed more light on the structure of PACA-methotrexate nanoparticles, thermal analyses were carried out (8).

EXPERIMENTAL

Materials

Butyl-2-cyanoacrylate (BCA) was given by Sichel-Werke GmbH (Germany). Isobutyl-2-cyanoacrylate (IBCA) and Dextrans T10 and T500 were purchased from Sigma. Dextrans T40 and T70 purchased by Pharmacia Fine Chemical (Sweden). Methotrexate (4-amino-10 methyl folic acid) and sodium lauryl sulphate (SLS) from Sigma. Poloxamer 188 (Surfoxid® F7068) was supplied by Tecneco (Spain) were also used.

Preparation of nanoparticles

PACA nanoparticles were prepared as described by Couvreur et al. (1,9). For the formulation 1.2 mg of MTX were added to 6 mL of the polymerization medium (HCl 0.001 N) containing dextran of different molecular weights, ranged from 10 to 500 kd respectively, and different amounts of sodium lauryl sulphate (0-0.30%) or Poloxamer 188 (0-0.60%), prior to incorporation of the monomer (BCA or IBCA). Afterwards, the monomer (60 µL) was added dropwise and the mixture was stirred for 3 hours at room temperature.

Nanospheres characterization

The particle size distribution of the nanospheres was determined by Photon Correlation Spectroscopy in a Malvern Autosizer II C (Malvern Instruments, Malvern, UK) (10). Morphologycal examination of nanoparticles was performed using Transmission Electron Microscope (TEM), Philips 1011, following negative staining with phosphotungstic acid (11).

Methotrexate content in the nanoparticles was determined by HPLC, using a Hewlett Packard HP 1090 instrument with UV detector. Determination of drug linked to the nanoparticles was carried out by separating free drug from drug-loaded nanoparticles, by ultracentrifugation of the suspension at 40000 rpm for two hours in a Kontron ultracentrifuge. MTX was mesured in both sediment (linked MTX) and supernatant (free MTX). Drug binding was expressed as the percentage of drug initially dissolved in the polymerization medium, associated with the carrier.

Differential scanning calorimetry analyses (DSC) were carried out on samples (free nanoparticles, MTX nanoparticles, MTX and SLS) of 3 to 5 mg on a Meettler analyzer (FP800, Paris, France) with an FP5 programmer. The samples were heated at a rate of 10°C/ min from 20 to 340°C in an atmosphere of nitrogen.

Thermogravimetric analyses (TG) were performed from 20 to 300°C, using a Dupont 990 analyzer (Wilmington, DE). Samples of 0.5 to 3 mg were heated at a rate of 10°C/min.

Thermomicroscopy analyses (TM) were carried out using a polarizing microscope LEITZ SM POL with a video camera Sony. Samples were heated initially at a rate of 10°C/min and then at 2°C/min when the sample began to melt.

RESULTS AND DISCUSSION

Effect of surfactants

The effect of surface active agents on the morphometrical properties was studied in the presence of a suspending agent: dextran T40. The choice of dextran molecular weight was made in a attempt to obtain a low polydispersity index of nanoparticles, as described in elsewhere (12).

In absence of SLS, when only dextran T40 was used as stabilizer, a high mean size of nanoparticles was obtained, for PIBCA nanoparticles. On the eher hand, in presence of SLS the size of BCA nanoparticles varied from 155.8 to 293.1 nm, and no linear relationship was found between size and amounts of SLS added according to described by Müller (13). Variations in the size were also achived when another monomer as IBCA was used, varying average diameter of particles from 171.9 to 300.4 nm. Slight differences in the average size of MTX-nanoparticles related with the monomer used (BCA or IBCA) were found by applying Peritz's F test (14). The mean particle size of methotrexate loaded PIBCA nanoparticles is higher than drug loaded PBCA nanoparticles. These differences between monomers were more significant for very small concentrations of SLS (0.005%-0.05%) and for high concentrations of the surface active agent (0.23%-0.30%).

The polydispersity index (Q) indicates a narrow size distribution for PBCA or PIBCA nanoparticles when surfactant added to polymerization medium varies from 0.030 to 0.100, similar to a monodisperse standard latex (Q=0.05). In absence of surfactant, larger and more agglomerated particles were obtained (Q=0.070-0.173), similar to slightly broader distribution. The difference in width of the distribution can be attributed to the conditions used during the process of polymerization (0.5% dextran T40 and 0 - 0.30% SLS). In this way, in absence of SLS, the action of dextran 40 as stabilizer may not be enough to reduce polydispersity index of samples. Significant differences ($P< 0.05$) in polydispersity index due to monomer used were found only in the absence of the surfactant agent. The morphology of these nanoparticles, studied by Transmission Electron Microscopy (TEM) (**Figure 1**) was similar for both monomers used.

Different particle diameters and polydispersity was observed when different amounts of Poloxamer 188 (0-0.60%) were used as surface active agent. In this case the mean size varied from 210 or 173 nm to 428 or 635 nm for PBCA and PIBCA nanoparticles respectively. The mean size of MTX-nanoparticles was higher when the monomer used was IBCA. These differences are statistically significant for all the concentrations studied. Values of polydispersity higher than 0.1 were found at concentrations of surfactant higher than 0.1% and lower than the critic micellar concentration (CMC) for the two monomers analysed. This suggests that the range of Poloxamer 188 is limited.

Results obtained suggest that the mean size of MTX-nanoparticles containing SLS was lower than the average size of nanoparticles containing Poloxamer 188. The polydispersity index of nanoparticles prepared with SLS was similar to that of a monodisperse system. Although anionic surfactant agents are not suitable for injectable preparations (15), for nanoparticle preparation an anionic agent, SLS, was

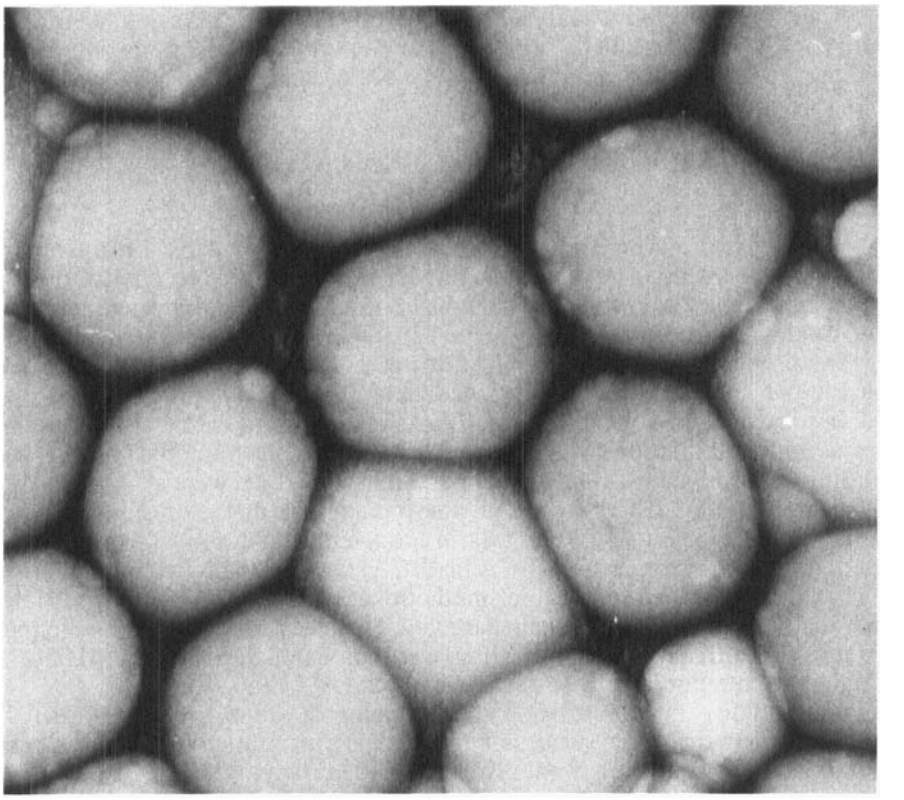

FIGURE 1
Transmission electron micrographs showing methotrexate-PBCA nanoparticles.

chosen, since the amount remaining in the nanoparticles after centrifugation is minimal. Moreover, the adverse effects of antineoplastic agents may also reduced, tanks to optimization of their use.

MTX entrapped in nanospheres, determined by HPLC when different amounts of SLS (0-0.30%) were added to the polymerization medium (Dextran T40 0.5%) is shown in **Figure 2**. The content of MTX in nanoparticles increases at concentrations of surfactant ranging between 0.010-0.10%. Lower and higher concentrations produce a decrease in the association efficiency. In this way, nanoparticles prepared with only dextran T40 (0.5%) as stabilizer contain a very low quantity of drug (0.73% for PBCA or 2.97 % for PIBCA nanoparticles). The fact that the addition of small amounts of surfactant can improve the quantity of drug entrapped by the polymer particles was also reported by other authors (16). This can be attributed to the role of surface active agents probably acting as primary stabilizers (17).

The amount of MTX loaded-nanoparticles was highly influenced by the concentration of sodium lauryl sulphate added to the formulation, as described for

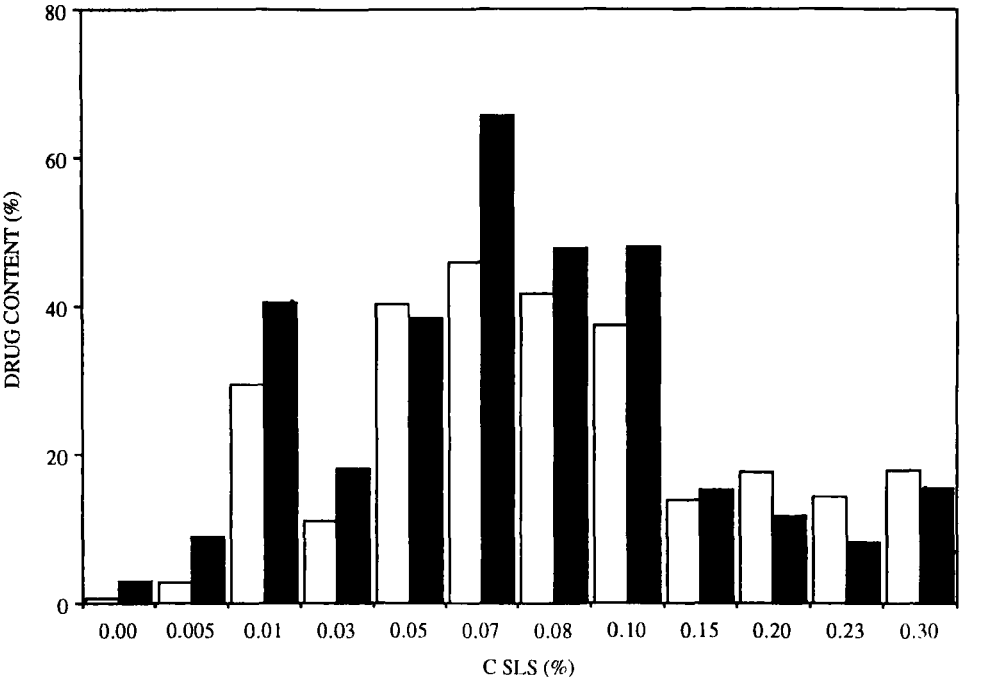

FIGURE 2
Drug content of methotrexate in PBCA □ or PIBCA ■ nanoparticles in function of sodium lauryl sulphate (SLS) concentration.

other drugs (18) The increase of association efficiency of MTX with increasing SLS in the polymerization medium suggests the formation of a complex between surfactant and drug, which leads a to change in the hydrophobic/hydrophillic ratio (19). Ionic surfactants can be used to increase the dissolution rate of drugs in different dosage forms. Najijb et al. (20) suggested that the observed increase in dissolution with the incorporation of ionic surfactant like SLS could be attributed to the ability of the surface active agent to reduce the interfacial tension between the solid and the dissolution medium and hence improve the wettability of the drug particles. The possible mechanism is considered to involve the formation of polymer-surfactant complexes or micelles, through the formation of bonds between the surfactant ions and the drug. The highest percentage of MTX entrapment was attained for surfactant concentration of around 0.08% (62.35±1.90 % for BCA and 72.03±3.13 % for IBCA. In general PIBCA nanoparticles showed a significant higher association efficiency than PBCA nanoparticles.

Low amounts of drug payloaded (with a maximum value of 16.90 for 0.03% of Poloxamer 188) was reported for nanoparticles made with Poloxamer 188. This could be attributed to the non ionic character of the surfactant.

In view of results obtained the incorporation of an anionic surface active agent as SLS in the polymerization medium improves the association efficiency of MTX to the colloidal system more than the presence of a non ionic surfactant such as

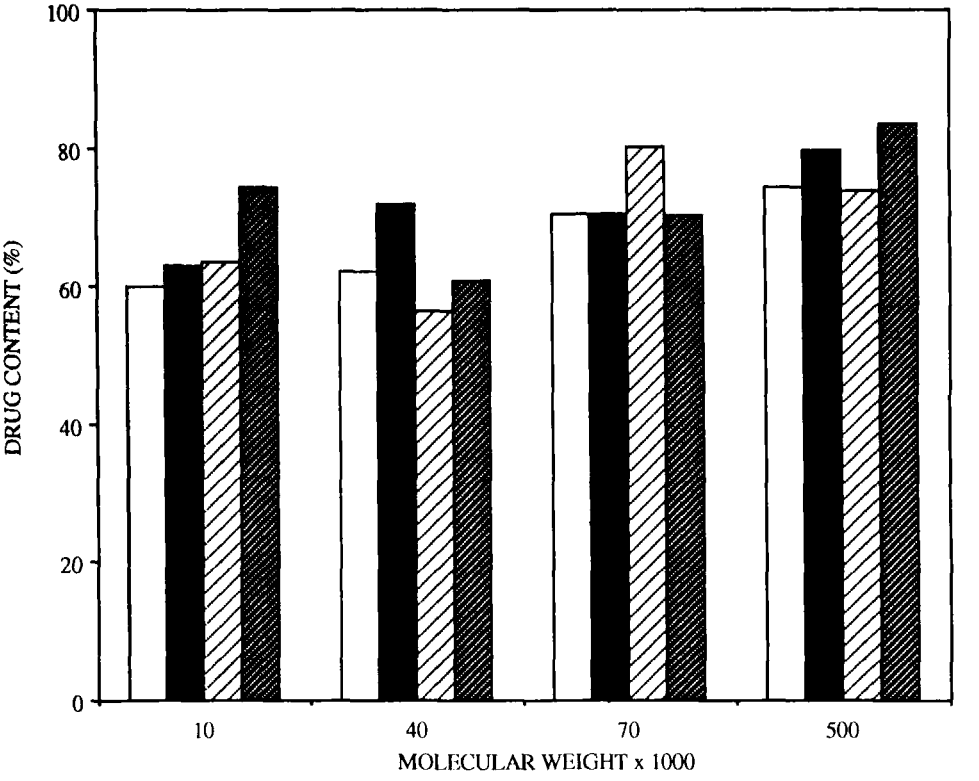

FIGURE 3

Drug content of methotrexate in PBCA nanoparticles as a function of type of dextran (0.5 or 1 %) included in the polymerization medium. Methotrexate-PBCA nanoparticles(DEX-0.5%) □ ; Methotrexate-PIBCA nanoparticles (DEX-0.5%) ■ ; Methotrexate-PBCA nanoparticles (DEX-1%) ▨ ; Methotrexate-PIBCA nanoparticles (DEX-1%) ▩ .

Poloxamer 188. This fact is probably due to the chemical structure of the drug and to the characteristics of the surface active agent.

Effect of dextrans

The effect of molecular weights of dextran on morphometrical properties and association efficiency was also investigated. Averages MTX nanoparticle size of three assays with dextran of different molecular weights at concentrations of 0.5 and 1% for a drug's invariable concentration (0.2 mg/mL) were studied. All the formulations analysed show an average particle diameter less than 314 nm, suitable for parenteral administration. This can be attributed to the presence of the surface active agent (SLS 0.08%), which contributes with dextran to stabilizing the formulation. An increase in size of methotrexate-PBCA and methotrexate-PIBCA nanoparticles when molecular weights increase was observed, but a linear relation

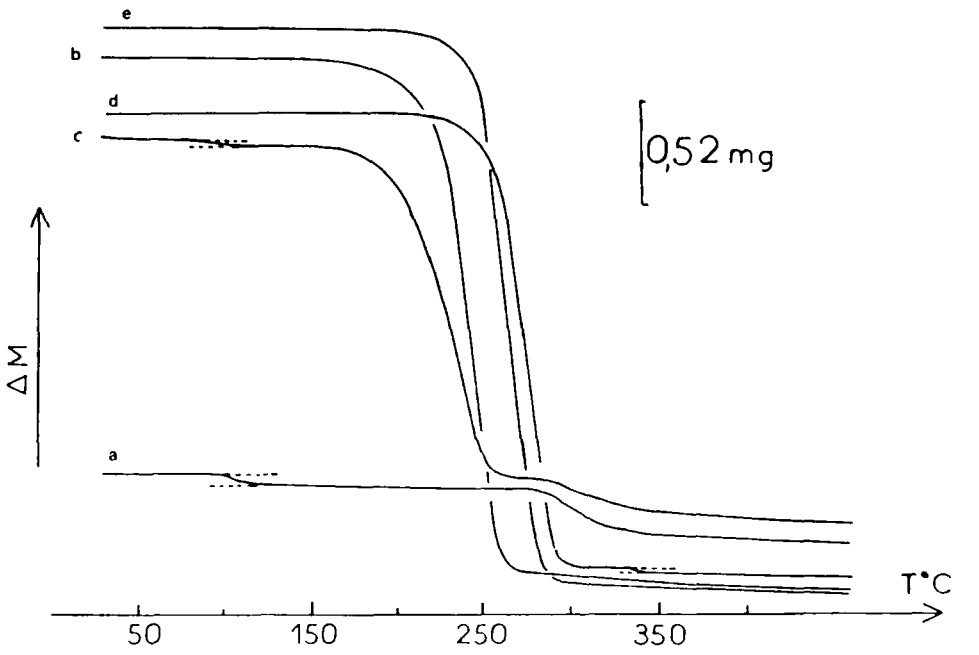

FIGURE 4
Thermogravimetric courves obtained with: a) methotrexate, b) PBCA nanoparticles , c) PBCA nanoparticles + methotrexate, d) methotrexate-PBCA nanoparticles, e) SLS.

between nanoparticle size and dextran molecular weight was only found for high concentrations of polysaccharide (1%) when IBCA was used as monomer. Significant differences in the average size of MTX-loaded nanoparticles related with monomer used were detected for different dextrans analysed. In general, PIBCA nanoparticles are larger than PBCA nanoparticles.

The variations in the average polydispersity index of three assays carried out at the same experimental conditions were studied. All the formulations analysed showed a polysdispersity less than 0.1 as could be expected in a monodisperse system. Only PIBCA nanoparticles containing dextran 500 at 1% showed a polydispersity higher than 0.1. Differences in average polydispersity index of same kinds of dextran were also described by Douglas et al. (12). It can be explained by the mechanism of nanoparticle preparation that involves an emulsion-polymerization procedure (9). Results of Peritz's test in general did not show significant differences due to different monomers used (BCA, IBCA).

Different kinds of dextran in the MTX loaded nanoparticles (concentration in the polymerization medium: 0.5 and 1%) are shown in **figure 3**. The MTX content increases when the molecular weights of polysaccharides increase for both monomers assayed (BCA, IBCA). In general for all the dextrans, MTX PIBCA nanoparticles have a higher association efficiency than the MTX PBCA nanoparticles. By applying Student's "t" test to the results, significant differences in

TABLE 1

Thermogravimetry and Kinetics descomposition results.

Sample	Sample weight (mg)	Decomposition phase	begin (°C)	end (°C)	Lose of mass (mg)	Kinetics (mg/min)
PBCA nanoparticles	1.792	1	81	100	0.064	0.25
+		2	157	277	1.696	3.40
MTX free	0.576	3	277	350	0.192	0.35
		4	350	400	0.040	
		residue*			0.344	
MTX nanoparticles	2.496	1	200	314	2.304	6.00
		residue*			0.192	
PBCA nanoparticles	2.784	1	150	375	2.624	7.00
		residue*			0.064	
MTX	0.736	1	50	150	0.064	0.25
		2	260	325	0.256	0.57
		residue*			0.416	
SLS	0.294	1	175	300	2.752	5.00
		residue*			0.192	

*Residue evaluated at 400°C.

drug association efficiency related with monomers used (BCA or IBCA) were found for dextran T40 and T500.

The results obtained suggest that the drug content is influenced by the molecular weight of dextran, probably due to the higer number of hydroxyl groups present in the polysaccharide. Therefore, dextran 70 seemed to be useful in the preparation of nanoparticles to achieve homogeneus polymer distribution and uniformity of nanoparticle sizes (21).

Analysis of matrix structure

In order to shed more light on the structure of the methotrexate-PBCA nanoparticles, DSC and TG studies were carried out. The results of the thermogravimetric analyses for the free methotrexate, PBCA nanoparticles without

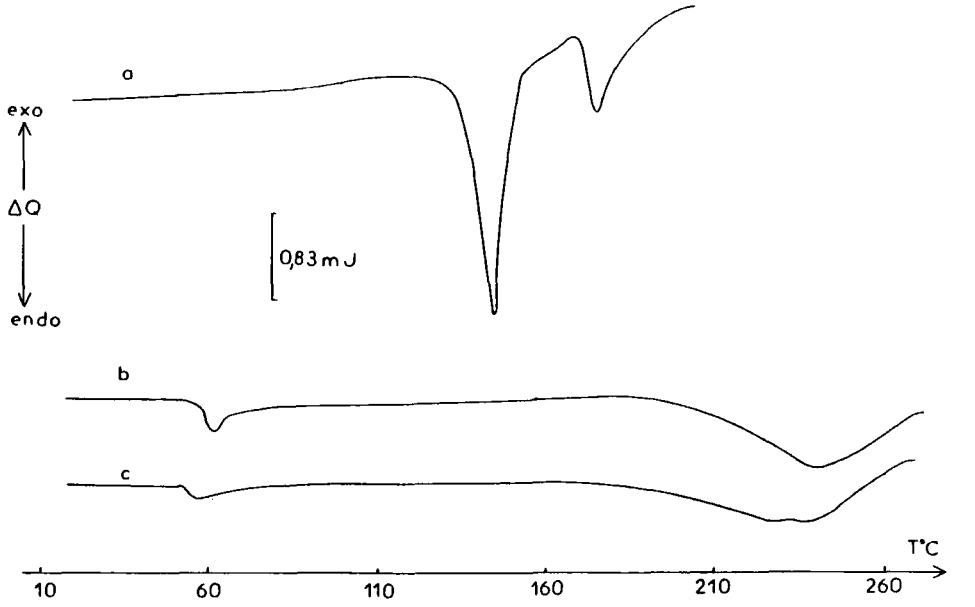

FIGURE 5
DSC scans obtained with: a) methotrexate, b) PBCA nanoparticles, c) methotrexate-PBCA nanoparticles.

drug, PBCA nanoparticles with added methotrexate (0.578 mg), methotrexate-PBCA nanoparticles and SLS are shown in **Figure 4** and **Table 1**.

Thermogravimetric curves obtained with the different nanoparticles (4b, 4c, 4d) reflect that when the drug is added to the white nanoparticles (curve 4d the losses of the mass are recovered. Curve 3a4, which corresponds to the methotrexate-PBCA nanoparticles, shows a difference of about 50°C at the decomposition temperature compared with the values obtained for the other curves (4b, 4c). This curve also reflects a very weak event between 350 and 400°C, which manifests the presence of about 0.12 mg of methotrexate inside the nanoparticle.

The SLS added to the polymerization medium of the nanoparticles does not show a great influence. The loss of mass corresponding to surface active agent occured at the same temperature.

The curves of the differential scanning calorimetry analyses (DSC), which give information about different components on nanoparticles, are shown in **Figure 5**. The methotrexate shows two endotherm events during the heat treatment (T1=139°C, T2=168°C; $\Delta H1 = 107.8$ J/g; $\Delta H2 = 36$ J/g). The first acute peak (139°C) corresponds to the evaporation of water and the second one to the melting/decomposition phase, which was also demonstrated by thermomicroscopy. Heating of methotrexate to more than 340°C did not show any event, corresponding to it decomposition.

Both the PBCA nanoparticles and the methotrexate-PBCA nanoparticles were subjected to the same heat-cool cycles (fig.5b, 5c). These thermal curves reflect two endothermic events, one peak at about 57°C (Ta) and the second, more acute and

wide, at 197°C (Tb). The enthalpy values associated with these events were: ΔHa = 12.6 + 0.9 J/g; ΔHb= 293+ 7 J/g and ΔHa = 11.3+ 0.9 J/g; ΔHb = 183+ 6 J/g respectively. After the cooling step the two events disappeared indicating the absence of any form of recrystallization.

Thermomicroscopy analyses, carried out above silicon oil, were used to demonstrate the different events that occur during the heat-treatment of the methotrexate. These analyses reflect a melting process at 105°C for the free drug. The yellow crystals of methotrexate become dark at 119°C and some bubbles can be seen. This effect was maximum at 127°C. More than 140°C the dark crystals decomposed.

The methotrexate-PBCA nanoparticles appear as thin translucent sheets and the crystals of methotrexate can be seen inside them. The thin sheets melt at about 93°C, which corresponds to the methotrexate melting process. This value was different to the melting temperature obtained in the DSC analyses. More than 224°C the crystals darkened and decomposed. In view of these results, the melting of MTX entrapped in nanoparticles is greatly influenced by the presence of polybutylcyanoacrylate (which acts as an impurity), in terms of melting temperatures, without speculating about any effect on the heats of melting. These results are in accordance with those obtained by Dubernet et al. (22) for ibuprofen-loaded ethylcellulose microspheres. Results of thermal analysis suggest that methotrexate was entirely dispersed in the polymeric matrix.

REFERENCES

1) P. Couvreur, B. Kante , M. Roland , P. Guiot, P. Baudin and P. Speiser, J. Pharm. Pharmacol., 31, 331 (1979).
2) P.A. Brimmell and D.J. Sams, J . Chromatogr., 413, 320 (1987).
3) L.D. Leserman , P. Machy and J. Barbet, Nature, 293, 226 (1981).
4) J. E. Dyr, K. Slavik and Z. Vodrazka, Thromb. Res., 31, 737 (1983).
5) P. Couvreur, B. Kante, M. Roland, P. Speiser, J. Pharm. Sci., 68, 1521 (1979).
6) M. Llovet, M.L. García, M.A. Egea, M. Espina, J. Valero, M.A. Alsina and O. Valls,*Vectorización de metotrexato en polialquilcianoacrilatos.* Reunión Nacional de Materiales Polímeros, Valencia (Spain), Juny, 1991.
7) G.W. Halbert, A.T. Florence, J.F.B. Stuart, J. Pharm. Pharmacol., 39, 871 (1987).
8) U. W. Gedde, Drug Development and Industrial Pharamcy, 16(17), 2465 (1990).
9) W.D. Harkins, J. Polym. Sci., 5, 217 (1950).
10) M.L. Mc.Connell, Anal. Chem., 53, 1007 A (1981).
11) S. A. C. Golud, B. Drake, C.B. Prater, A.L. Weisenhorn, S. Mannes, G.L. Keldderman, H.J. Butt, H. Hasina and P.K. Hansma, Ultramicroscopy, 33, 93 (1990).
12) S.J. Douglas, L. Illum and S.S Davis, J. Colloid Interface Sci., 103, 154 (1985).
13) R.H. Müller, Int. J. Pharm., 84, 1 (1992).
14) J.F. Harper, Comput. Biol. Med., 14 (4), 437 (1984).
15) M.J. Alonso, C. Losa, P. Calvo and J.L. Vila-Jato, Int. J. Pharm., 68, 69 (1991).

16) T. Harmia, P. Speiser and J.Kreuter, Pharm. Acta Helv., 62(12), 322 (1987).
17) T. Harmia, P. Speiser and J. Kreuter, J. Microencap., 3(1):3 (1986).
18) V. Guise, J. Y. Drovin , J. Benoit , J. Mahuteau, P. Dumont and P. Couvreur, Pharm. Res., 7(7), 736 (1990).
19) M.A. Egea, O. Valls, M.A. Alsina, M.L. García, C. Losa , J.L. Vila-Jato and M.J. Alonso, Int. J. Pharm. , 67, 103 (1991).
20) N.M. Najib, M. Suleiman and A. Malakh, Int. J .Pharm., 32, 229 (1986).
21) L. Vasnick, P. Couvreur, D. Christiaens-Leyh and M. Roland, Pharm. Res., 1, 36 (1985).
22) C. Dubernet, J.C. Rouland and J.P. Benoit, J.Pharm. Sci., 11(Vol 80), 1029 (1991).