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ASSOCIATION OF NETILMICIN SULPHATE TO POLY(ALKYLCYANOACRYLATE) NANOPARTICLES: FACTORS INFLUENCING PARTICLE DELIVERY BEHAVIOUR

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ABSTRACT

The entrapment of Netilmicin sulphate (NTS) in polyalkylcyanoacrylate nanoparticles could be of therapeutic interest particularly in the treatment of infection that localise in the macrophages. In fact, aminoglycosides are excluded from macrophages and therefore represent an ineffective therapy for the treatment of typhoid fever, despite the excellent *in vitro* activity. In this case the colloidal delivery system ensures the NTS targeting to the macrophages. In this paper it is shown that the various formulation conditions are capable of influencing: trapping capacity, particle size, molecular weight distribution. The nanoparticle preparation was carried out using three different methods in the presence of Tween 80 and Pluronic F68, as non-ionic surfactant. The incorporation method consisting in adding the drug to the polymerisation medium led to very low values of NTS entrapment; in addition, formation of instable suspension was obtained increasing the NTS concentration over 5 mg/ml. The other two methods consisting in the absorption of the drug in the polymeric network of the nanoparticles showed higher NTS entrapment values. The presence of the drug in the polymerisation medium influenced both particle size and polymer chain molecular weight, providing an increase in both values. These results indicate that arranging preparation conditions, it is possible to obtain a nanoparticle delivery device

presenting the most suitable behaviour as a function of the therapeutic aim and administration route.

INTRODUCTION

The biological effectiveness of aminoglycoside antibiotics against certain bacterial strain has prompted their therapeutic application for the treatment and prophylaxis of different types of infections. The aminoglycoside antibiotics underlined a particular efficiency in the treatment of some micro-organism diseases frequently occurring in patients suffering from acquired immunodeficiency syndrome (1). Furthermore, the efficacy of this therapeutic class against gram-negative bacteria involved in eye infections, has also been proved (2). The aminoglycoside administration by the intravenous route is more or less unable, depending on the drug, to provide bactericidal biological action at the infection site without causing unacceptable toxic side-effects at the level of the bodily system. Therefore, to provide a high biological selectivity of these drugs it is of great importance if the in *vivo* therapeutic action is to be improved.

It was proposed that colloidal drug delivery systems could be used as carrier of antibiotics and chemotherapeutic agents in order to enhance their therapeutic index (3-7). Since submicroscopical particles used for drug delivery are cleared from the blood and concentrated in fixed tissue macrophages (the reticuloendothelial system) (8-13), it could be said that antibiotic-loaded colloidal delivery device would be particularly useful for the treatment of infections that are localised in macrophages (7, 14-15). The advantage of colloidal system-entrapment over free antibiotics should be particularly evident for antimicrobial agents such as aminoglycosides, which are excluded from macrophages and therefore represent an ineffective therapy for the treatment of typhoid fever in spite of the excellent in *vitro* activity (16). Intracellular antimicrobial activity against *Mycobacterium avium* infection in human monocyte derived macrophage cultures, has been demonstrated (17). For nephrotoxic drugs such as gentamicin, another advantage of colloidal delivery system entrapment, is the decreased renal excretion, which avoids a rapid blood clearance by means of glomerular filtration and hence the renal tubule exposition to potentially toxic concentrations of the drug. When entrapped in vesicles or particellar colloidal drug carriers, aminoglycodides follow the biological fate of the carrier system being mostly removed by the liver and spleen (18).

The theoretical therapeutic advantages of antibiotic-loaded vesicular carriers has been demonstrated in several investigations, in which infected animal models with facultative intracellular pathogens were used (14, 19-22).

Among the colloidal drug delivery systems, the polymeric carriers have been put forward in recent years. Poly(alkyl-2-cyanoacrylate) (PACA) nanoparticles are solid colloidal particles that have recently become popular as pharmaceutical dosage forms owing to their biocompatibility, biodegradability and capability of controlling drug distribution in the body (23-24). The biological fate of such particles following intravenous administration, is dependent upon physico-chemical characteristics such as particle size, polymer molecular weight and surface charge

(25-26). These properties of PACA nanoparticles depend on the polymerisation conditions employed during the preparation process. In fact, variation in the formulation parameters may lead to the formation of PACA nanoparticles which, not only present different particle size and surface charge, but are also of different molecular weight polymer chains which may also influence the distribution and degradation rate of the particles (27-29). It is well known that the length of the alkyl chain of the monomer affects the degradation rate of the polymer bulk and the release of trapped drugs (30), whereas the influence of the molecular weight distribution was not clearly shown for these colloidal systems.

Association of aminoglycoside antibiotics to polymeric carriers to improve the poor bioavailability of these drugs after ocular administration (31) may be of therapeutic interest for topical treatment of infections. It is worth noting that PACA nanoparticles have been proposed for ophthalmic applications due to their adhesive properties (32-33) and therapeutic response (34-35).

These considerations have prompted us to study the entrapment of netilmicin into polyethyl- and polyisobuthylcyanoacrilate nanoparticles. Various formulation parameters were evaluated, i.e. trapping capacity, factors influencing the particle size, molecular weight distribution, effects of the presence of different non-ionic surfactants during the preparation procedure.

MATERIALS

Ethyl-2-cyanoacrylate (ECA) and isobuthyl-2-cyanoacrylate (ICA) were used for the monomer in the nanoparticle preparation (Sigma, St. Louis, MO, U.S.A.). Polyethylen-polypropylene glycols polymer (Pluronic F68) (Fluka, Buchs, Switzerlad), polyoxyethylene sorbitan-monooleate (Tween 80) (Merck, Darmstadt, Germany) were used as non-ionic surfactants. Polymethylmethacrylates (Polymer Laboratories Inc., Amherst, MA, U.S.A.) were used as standards for molecular weight determination of polyethyl- (PECA) or polyisobuthylcyanoacrylate (PICA). Netilmicin sulphate (NTS) was a gift from SIFI (Italty); its purity was greater than 95 %, as assayed by HPLC analysis. Double distilled water was used. All Other reagents and solvents were of analytical grade (Carlo Erba, Milano, Italy).

METHODS

Nanoparticle Preparation

PECA and PICA nanoparticle preparations were carried out by means of emulsion polymerisation technique (36-38), following three different procedures for drug loading.

Method A. The incorporation method (29) consisted in solubilizing the desired amount of NTS in 50 ml of a filtered (0.2 μm membrane filter) aqueous solution of 10^{-3} N HCl containing one of the non-ionic surfactants (0.5 % w/v), the pH value of the polymerisation medium was of 3. An aliquot (normally 0.6 ml) of ECA or ICA monomer was added drop by drop during a period of 8 min under mechanical stirring (1000 rpm). After the polymerisation was completed (usually 3

h), the colloidal particle suspensions were brought to a pH value of 7 by adding 0.2 N NaOH. Stirring continued for 2 h. The nanoparticle milky suspensions were filtered through a sintered glass funnel (grade 4, pore size 11-16 μm).

Method B. The absorption method was carried out as described elsewhere (39). Briefly, the polymerised and neutralised nanoparticle suspensions prepared in the absence of the drug, were purified by three centrifugations, suspending the pellet in water each time. The final suspension was freeze-dried and then resuspended over a range of NTS concentrations (0.1-20 mg/ml) for 2 h in order to determine the absorption isotherms.

Method C. NTS at various concentrations was included at the polymerisation stage as described for the method A. At the end the nanoparticle suspensions were freeze-dried to enhance the drug polymer association. The absorption isotherm of these formulations were similarly determined after lyophilization.

Incorporation Capacity Determination

To separate the untrapped amount of NTS, the nanoparticle suspensions were centrifuged (mod. J2-21, Beckman, Fullerton, CA, U.S.A.) at 31,100 g (Beckman JV-20.1 rotor, 17,000 rpm) for 1 h. The concentration of NTS in the supernatant and degraded sediment was determined by HPLC analysis. The chromatographic apparatus consisted of a Varian model 9010 solvent delivery pump (Varian Associates Inc., Walmut Creek, CA, U.S.A.), a Rheodyne model 7125 syringe loading injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 10 μl loop, a Varian model 9050 variable wavelength ultraviolet UV-Vis detector operating at 360 nm and a Varian model 4400 reporting integrator. The analysis procedure was performed on a Hypersil ODS reverse-phase C_{18} analytical column (5 μm, 160 mm · 4.6 mm ID., Shandon Southern, Runcorn, U.K.). The mobile phase was a solvent mixture of acetonitrile-water (68:32 v/v), and was delivered at 1.0 ml/min. The mobile phase was filtered through 0.2 μm microporous PTFE membrane filters (Millipore, U.S.A.) and degassed by ultrasonication prior to use. The determination was carried out at room temperature. The data were calculated from the linear regression of an external standard of NTS, relating peak area and concentration.

The derivatization of the drug necessary for the determination of the NTS content in the various samples was carried out employing 1-fluoro-2,4-dinitrobenzene (FDNB) as derivatizing agent (40).

Dimensional Analysis and Molecular Weight Determination

The technique used for the particle size and molecular weight (MW) determination was light-scattering (LS). The LS apparatus was entirely assembled in our laboratories and consisted of a He-Ne Melles-Griot (78180 Montigny-le-Bretonneux, France) mod. 05-LNP-991 laser (70 mW), equipped with a holding system PC 8 Malvern (Worcestershire WR14 1AQ) thermostated at 21±0.01 °C (Haake F3-R, Berlin, Germany), a microcontrol precise mechanical goniometer and an optical system Melles-Griot f. 150. The photomoltuplier was a RCA mod. C 31034 (Burleigh, Fishers, NY, U.S.A.), cooled at -30 °C.

The LS method employed for particle size analysis was the photo-correlation spectroscopy (PCS) (41), performed at a scattering angle of 90° and 50°. The scattering signals were correlated by a Malvern 4700 particle analyser connected to an Olivetti 240 computer. For the analysis each sample of milky suspension was diluted with filtered (0.2 µm filter) double-distilled water until an optimal nanoparticle concentration was obtained.

The PECA and PICA molecular weights of the various nanoparticle preparations were calculated by determining their translation diffusion coefficient by means of the LS technique. In fact, a relationship runs between the translation diffusion coefficient (D) extrapolated at null concentration and the MW (Mark Houwink equation):

Eq. 1 $$D = K_d \cdot M^{-b}$$

where K_d is a constant depending on the polymeric nature, solvent and temperature, M is the molecular weight and b is a value in a range from 0.5 to 0.6. In order to obtain the K_d and b values a polymethylmethacrylate (physico-chemical properties very close to the sample polymers) set was employed as a reference. The straight-line calibration of polymethylmethacrylate polymeric solutions in acetonitrile-methanol (4:1 v/v) at known MW, presented a linear regression of 0.99987. Therefore, it was possible measuring the D distribution value to know the MW distribution, according to the following equations (28, 42-43):

Eq. 2 $$F(k,t) = P(\Gamma) \exp(-\Gamma t)^2$$

in which $\Gamma = k^2 D$, and so Eq. 2 can be rewritten to give the expression:

Eq. 3 $$F(k,t) = F(M)\, M^2 \exp(-k^2 K_d M^{-b} t)^2$$

where F (k,t) is the autocorrelation function of the scattering light intensity with a transfer moment k. The MW distribution F (M) is achieved inverting F (k,t). The LS analysis for the determination of MW distribution was performed at a scattering angle of 30°, a temperature of 21 °C and a wavelength of 632.8 nm.

The nanoparticle samples for MW analysis were prepared by centrifuging 25 ml of the particle colloidal suspension, discharging the surnatant and resuspending the pellet in 10ml filtered (0.2 µm pore size) double-distilled water. This procedure was repeated four times to wash out all aqueous soluble materials coming from the polymerisation (NTS, salts, unabsorbed surfactants). Then, the pellet was suspended in 10 ml of water and exhaustively dialysed against 800 ml of water. At the end, the purified nanoparticle suspensions were poured into a round-bottomed flask and lyophilised. The required amount of the PECA and PICA dried powder was solubilized in an organic mixture of acetonitrile-methanol (4:1 v/v) before the determination of MW distribution.

Results and Discussion

The drug-polymer association is a very important limiting factor in the application of polymeric delivery system for the pharmacological treatment of various diseases. An optimal entrapment of a drug onto PACA nanoparticles normally requires a certain hydrophilic nature of the pharmaceutical active substances, although the process of association seems to be quite difficult for true polar molecules, as well as NTS, due to the hydrophobicity of the polymeric network of the nanoparticle carrier (30). For this reason, different preparation procedures were used employing at the same time drug solutions at different concentrations, in order to improve the NTS loading into and/or onto the PECA or PICA nanoparticles.

The often used standard preparation process, incorporation method (method A), achieved very low values of NTS entrapment into PECA and PICA nanoparticles, prepared either in the presence of Tween 80 or in the presence of Pluronic F68 (table 1 and 2). The scarce NTS loading could be probably due to the highly polar nature of the drug molecule.

A slight difference in the drug-polymer association was observed between particles prepared in the presence of Tween 80 or Pluronic F68; in fact, the latter presented drug content values a little bit higher than particle system with Tween 80. This behaviour was underlined in both PECA and PICA nanoparticles (table 1 and 2). The reason could be due to the property of Pluronic F68 to lead not only to the entrapment of a drug in the nanoparticle core but also to a great accumulation near the particle surface that, being highly hydrophilic, is capable of loading a great deal of a polar drug up to saturation. Whereas, Tween 80 seemed to achieve an incorporation due to the distribution of the drug in the polymeric bulk without any enrichment in any part of the PACA nanoparticle (44).

The influence of the drug concentration of the polymerisation medium on the loading capacity of PECA and PICA nanoparticles prepared with method A was also studied. The results listed in table 3 showed that the increase of NTS concentration in the polymerisation medium led to a slight enhancement of the drug content for both polymers and surfactants, up to a NTS concentration of 5 mg/ml. After this point, no improvement of drug content was observed and, moreover, the formation of an instable suspension and large unwanted agglomerates were observed at higher drug concentration (20 mg/ml). This effect could be due to the interference in the synthesis and deposition of the oligomeric chains caused by the presence of NTS.

PACA nanoparticles are a suitable drug delivery device of great therapeutic interest not only for systemic application through the injectable route but also for topical pharmaceutical application, as well as for ophthalmic use against eye infections. When ophthalmic application is desired, the drug should be mostly absorbed onto the surface of the colloidal carrier despite being incorporate within the polymeric bulk of the nanoparticles (34, 45). The sorption isotherms were determined following the preparation method B and C, as reported in the methods section. The following variables on NTS absorption were investigated: NTS

TABLE 1

Entrapment Capacity of PECA Nanoparticles Prepared in the Presence of Tween 80 or Pluronic F68 (0.5 % w/v) following the three Preparation Procedures at a NTS Concentration of 1 mg/ml.

Preparation method	Tween 80		Pluronic F68	
	Association[a] (%)	Drug Content[b] µg/mg PECA	Association[a] (%)	Drug Content[b] µg/mg PECA
A	3.12	3.9 ± 0.3	4.08	5.7 ± 0.2
B	21.04	26.3 ± 1.6	16.40	20.5 ± 1.1
C	38.24	47.8 ± 1.3	34.96	43.3 ± 1.9

[a] Each value is the average of five different experiments.
[b] Mean value ± the standard deviation of five different experiments.

TABLE 2

Entrapment Capacity of PICA Nanoparticles Prepared in the Presence of Tween 80 or Pluronic F68 (0.5 % w/v) following the three Preparation Procedures at a NTS Concentration of 1 mg/ml.

Preparation method	Tween 80		Pluronic F68	
	Association[a] (%)	Drug Content[b] µg/mg PECA	Association[a] (%)	Drug Content[b] µg/mg PECA
A	4.72	5.9 ± 0.1	6.80	8.5 ± 0.3
B	32.08	26.3 ± 1.6	16.40	20.5 ± 1.1
C	60.72	75.9 ± 1.8	52.08	65.1 ± 3.7

[a] Each value is the average of five different experiments.
[b] Mean value ± the standard deviation of five different experiments.

TABLE 3

Drug loading (μg/mg polymer) of PECA and PICA Nanoparticles obtained with the Preparation Method A in the presence of Tween 80 or Pluronic F68 and at different NTS Concentrations in the Polymerisation Medium.

NTS conc. (mg/ml)	PECA[a]		PICA[a]	
	Tween 80	Pluronic F68	Tween 80	Pluronic F68
0.1	1.5	2.1	2.2	3.9
0.5	2.3	3.5	3.7	6.7
1.0	3.9	5.7	5.9	8.5
2.5	4.5	7.1	6.3	9.1
5.0	6.3	7.9	7.5	9.7
10.0	6.1	8.1	7.6	9.5
20.0	6.3	8.3	7.6	9.6

[a] Each value is the average of three different experiments.

concentration in the polymerisation medium, type of monomer, kind of non-ionic surfactants employed during the preparation process.

The larger amount of NTS entrapped onto the PACA nanoparticle prepared with methods B and C, compared to the drug loading capacity values obtained with the method A, indicated that the freeze-drying procedure allowed a better interaction between NTS and PACA polymeric network. The comparison of the absorption isotherms achieved by the absorption of the drug after the freeze-drying procedure or by the addition of NTS during the polymerisation process (preparation methods B and C) was reported in figures 1 and 2. Up to a concentration of 5 mg/ml preparation method C was able to give a higher drug-polymer association. Above this concentration method C showed a flattening in the absorption isotherm coupled with an increase of the nanoparticle mean size (fig. 3 and 4), which may explain this particular trend; in fact, the bigger the particle size, the less is the surface available for the drug absorption. As for method A, very large unwanted agglomerates were detected at a NTS concentration of 20 mg/ml. Method B showed that for both PECA and PICA nanoparticles prepared in the presence of Tween 80, an almost linear increase in the loaded drug as a function of the concentration of NTS solutions employed for the absorption experiments in the range from 0.1 to 20 mg/ml. The situation was different in the presence of Pluronic F68 that showed a lower absorption efficiency, probably due to the hindrance of the drug-polymer interaction, owing to the alteration of the particle surface caused by the thickness of the hydrophilic shell of the non-ionic surfactant.

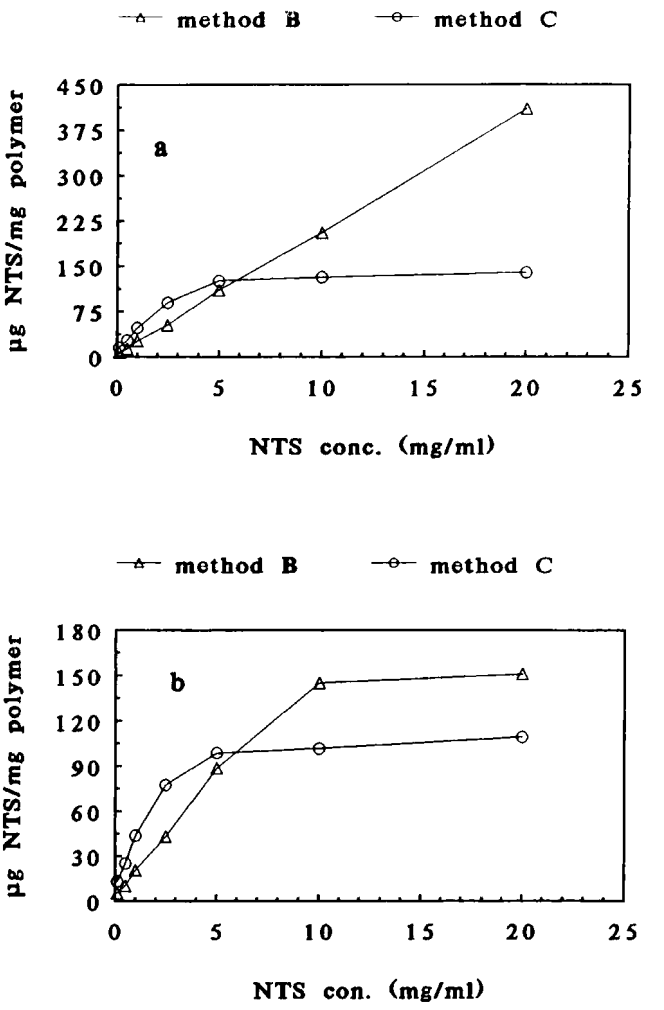

FIGURE 1

Absoption isotherm of PECA nanoparticles obtained with the preparation method B and C in the presence of Tween 80 (a) and Pluronic F68 (b).

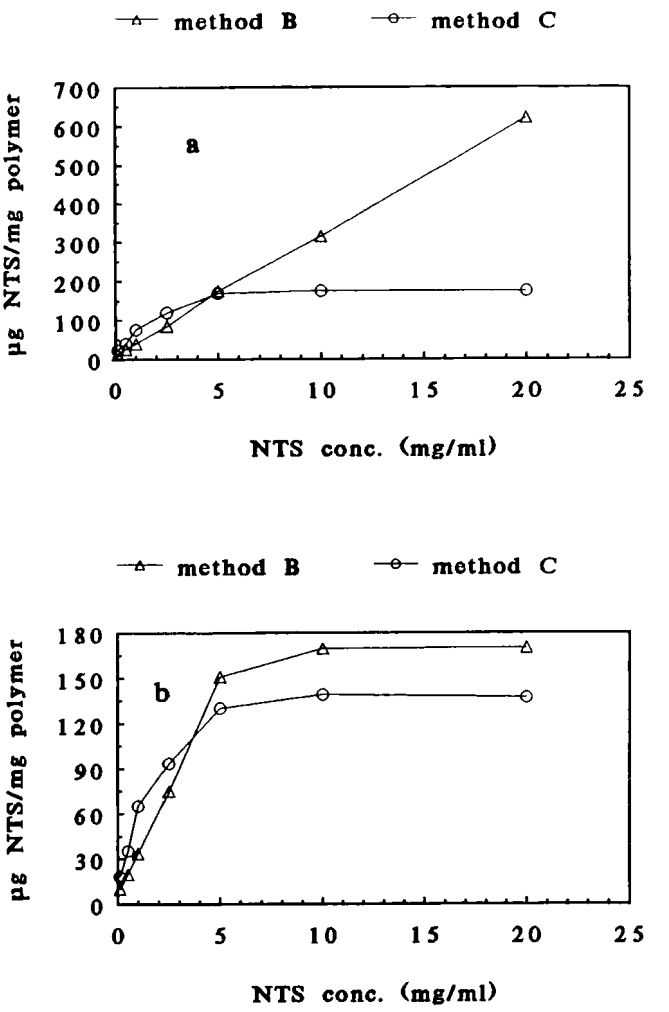

FIGURE 2

Absoption isotherm of PICA nanoparticles obtained with the preparation method B and C in the presence of Tween 80 (a) and Pluronic F68 (b).

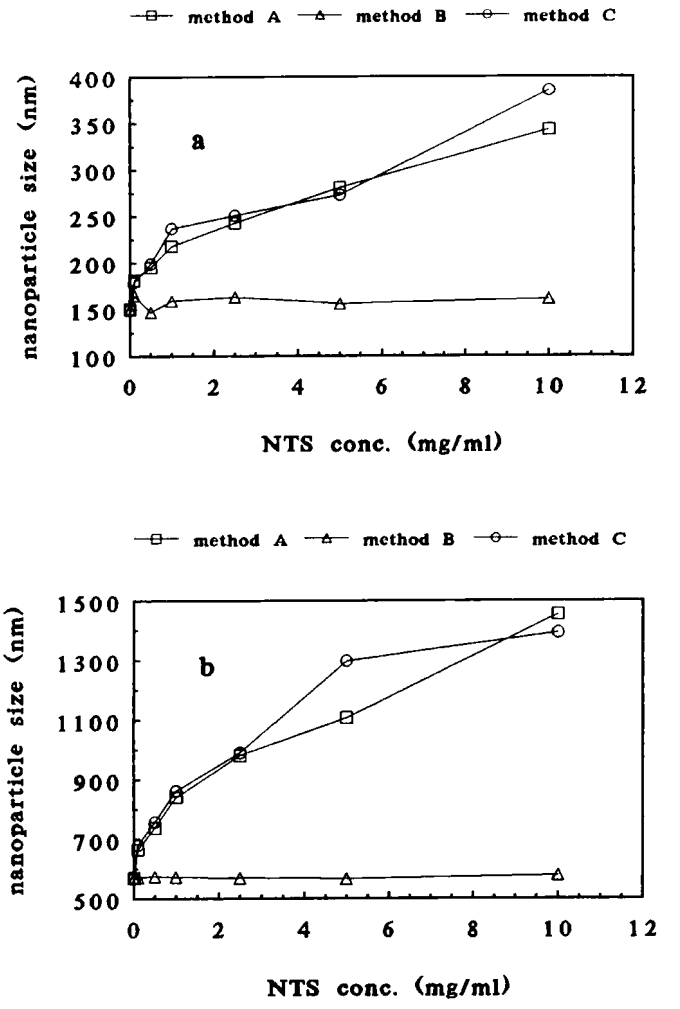

FIGURE 3

Influence of NTS concentration on size of PECA nanoparticles prepared in the presence of Tween 80 (a) or Pluronic F68 (b).

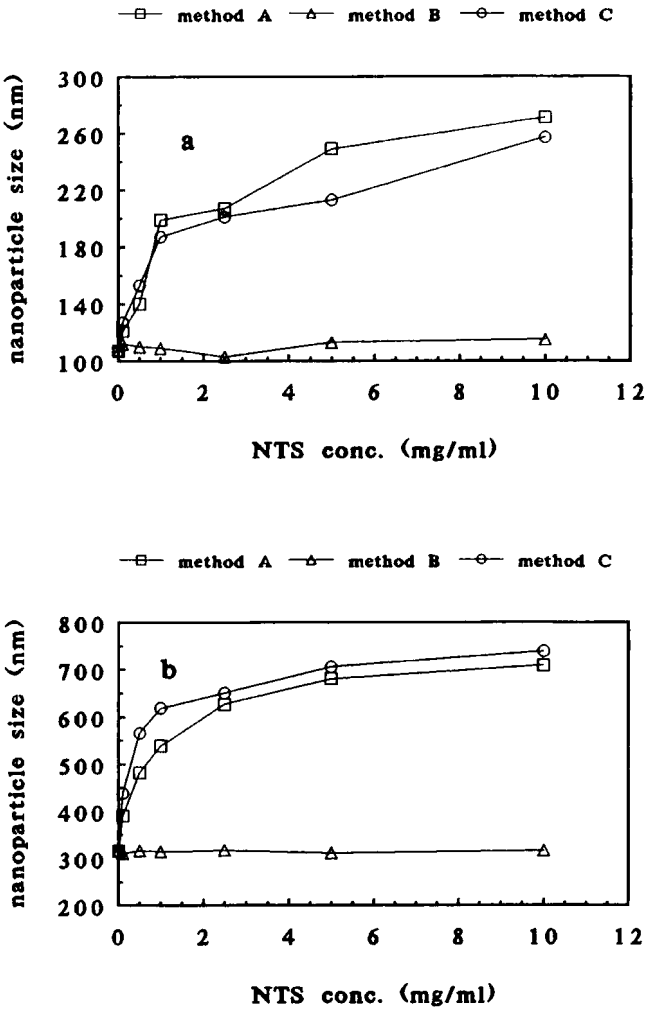

FIGURE 4

Influence of NTS concentration on size of PICA nanoparticles prepared in the presence of Tween 80 (a) or Pluronic F68 (b).

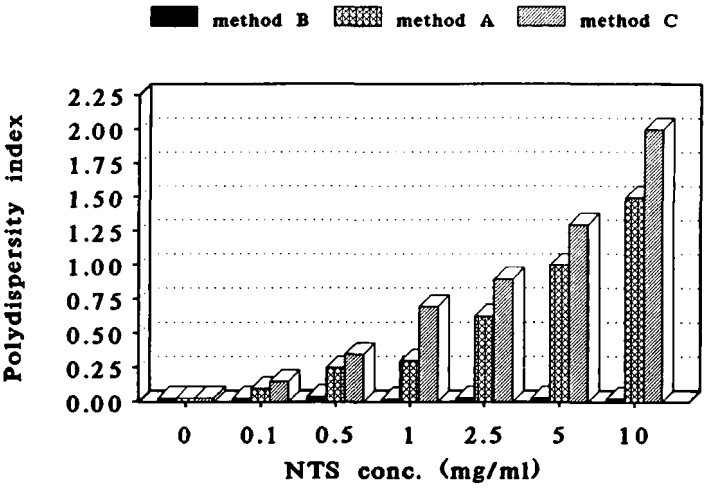

FIGURE 5

Polydispersity index values of PECA nanoparticles prepared in the presence of Tween 80 with the three preparation methods as function of NTS concentration.

These results seemed to be in agreement with previous reported observations (45-46). Besides the preparation methods mentioned, the concentration of the drug and the non-ionic surfactant, a difference in the NTS entrapment was detected between PECA and PICA nanoparticles. The latter ensured higher drug content values than PECA systems (tables 1-3; figures 1 and 2), probably due to the highly porous polymeric structure which enhances the NTS loading, achieving a greater surface available for the absorption phenomenon than PECA nanoparticles do.

The variables: preparation method, drug concentration, non-ionic surfactant, greatly influenced the particle size. Preparation method B (figures 3 and 4) showed a constant PECA and PICA nanoparticle size as a function of the NTS concentration employed in absorption experiments. The absence of the drug during the polymerisation medium ensured no interference with the nucleation process and polymerisation leading to a constant particle size and molecular weight of the polymeric chains (data not reported). The only difference in size and molecular weight was due to the surfactant used during the nanoparticle formation (figures 3 and 4; table 4). The PECA and PICA nanoparticles prepared with the method A and C were greatly influenced in size (figures 3 and 4) and molecular weight values (table 4) by the concentration of NTS which was used during the ECA and ICA polymerisation, being able, therefore, to interact with both monomer polymerisation and chains nucleation. The polydispersity index values (figure 5) of PECA nanoparticles also showed dependence on the preparation method and NTS concentration. Method B gave highly monodisperse colloidal suspensions, unaffected by the NTS concentration used during preparation. Whereas, method A

Table 4

Numeral (mwN) and mass average molecular weight (mwM) of PECA and PICA nanoparticles prepared according to the method c in the presence of Tween 80 and Pluronic F68 at different concentration of NTS in the polymerisation medium.[a]

NTS conc. mg/ml	PECA						PICA					
	Tween 80			Pluronic F68			Tween 80			Pluronic F68		
	mwN	mwM	PI[b]	mwN	mwM	PI[b]	mwN	mwM	PI[b]	mwN	mwM	PI[b]
0.0	1578	1835	1.16	3128	3515	1.12	2795	2995	1.07	7284	7550	1.04
0.1	1921	2425	1.26	3620	4120	1.14	3121	3720	1.19	7980	8530	1.07
0.5	2127	2778	1.31	4175	4951	1.19	3350	4280	1.28	8520	9120	1.07
1.0	2543	3250	1.27	4740	5730	1.21	3870	4950	1.28	9371	10135	1.08
2.5	2980	3995	1.33	5100	6715	1.32	4510	5775	1.28	10240	11005	1.07
5.0	3751	5185	1.38	5571	7245	1.30	4998	6719	1.34	10795	12080	1.12

[a] Each value represents the average of three different experimental measurements.
[b] Polydispersity index (PI) related to the molecular weight distributions. It was calculated as the ratio between the mass average molecular weight and (mwM) and the numeral average molecular weight (mwN).

and C always gave heterodisperse colloidal suspensions at an increase in drug concentration.

The increase of molecular weight values caused by the presence of NTS in the polymerisation medium could be explained keeping in mind the nanoparticle formation process proposed by Douglas et al. (47). The anionic polymerisation is started by nucleophilic molecules (hydroxyl ions and water), whereas the termination process is mainly due to the protons. The oligomeric chains clumping together form nuclei which are able to take up further oligomers and monomer molecules, undergoing an additional polymerisation process that achieves polymeric chains at much higher molecular weight. The presence of sulphate ions coming from NTS may be able to elongate this secondary polymerisation, furnishing polymers at higher molecular weights as a function of the drug concentration present in the medium (table 4).

The difference between the systems prepared in the presence of Tween 80 and Pluronic F68 could be caused by the fact that Tween 80 was able to form micelles and therefore polymerisation takes place just along the micelle surface hampering an extended secondary polymerisation which leads to lower molecular weight values than that obtained with Pluronic F68 (table 4). As a consequence of this hypothesis, the mean size of the nanoparticle prepared in the presence of Tween 80 was also smaller than that with Pluronic F68 (figure 3 and 4). Pluronic F68, whose micelle formation is controversial (47), ensured a more intense secondary polymerisation, obtaining higher molecular weight values (table 4) and mean size (figure 3 and 4). This behaviour could be due to the property of Pluronic F68 to produce around the growing nuclei a protective layer from the acid polymerisation environment, and reducing the rate of termination process.

Conclusions

The results, herein reported, indicate that the manufacturing process of PACA nanoparticles can be easily controlled to obtain the most suitable delivery device for the therapeutic application, e.g. absorption or incorporation preparation process may be chosen depending on a fast or slow drug release, or if a topical (ophthalmic route) or systemic application is required. Furthermore, the possibility of coating the particle surface is capable of ensuring different body distribution targeting the drug to various tissues, i.e. nanoparticles coated with Tween 80 are preferably taken up by the lungs (12), therefore, of therapeutic interest in respiratory infection diseases. PACA nanoparticles coated with Pluronic F68 are taken out of the bloodstream by liver and spleen, because these organs have fenestrated capillaries and are well perfused. Although this colloidal carrier does not pass through the intact endothelium, monocites and leukocyte cells are able to ingest colloidal particle. Hence, these cells that would be attracted by the inflammatory foci could come "armed" with NTS. Another possible way may be that capillary integrity could be compromised at site of inflammation, allowing the passage of NTS-loaded colloidal carrier which can selectively exert its therapeutic action.

Therefore, PACA nanoparticles-entrapped NTS may be an improved dosage form for the treatment of infections with obligate and facultative intracellular pathogens, that localise in the reticuloendothelial system.

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