Acknowledgements
The authors wish to thank the staff of Simbec Research Ltd., Merthyr Tydfil, who conducted the bioavailability studies in volunteers, and Drs. W. Jansen and A. Osterspey of the University of Cologne, who carried out the investigations on patients, for their interest in this work. The skillful technical assistance of Mrs. C. Reinbach-Diekhof and Mrs. Schneider is gratefully acknowledged.

References

(1) Blasini, R., Froer, K. L., Blümel, G., Rudolph, W., (1982) Herz 7, 250–258.
(2) Parker, J. O., Fung, H.-L., Rugirello, D., Stone, J. A (1983) Circulation 68, 1074–1080.
(3) Rudolph, W., Dirschinger, J., Hall, D., Blasini R., Reiniger G., Kraus F. (1984) International Symposium Nitrates '84, Düsseldorf (FRG).
(4) Taylor, T., Chasseaud, L. F., Major, R., Doyle E., Darragh, A. (1981) Biopharm. Drug. Disp. 2, 255–263.
(5) Abshagen, U., Betzien, G., Endele, R. Kaufmann, B. (1981) Eur. J. Clin. Pharmacol. 20, 269–275.
(6) Steudel, H., Steudel, A., Bonn, R. (1983) in Mononitrat (Köhler, E., Noack E., Schrey A., Weiß M., ed.), pp. 38–45, Dr. C. Wolf und Sohn, München.
(7) Laufen, H., Aumann, M., Leitold, M. (1983) Arzneim.-Forsch/ Drug Res. 33, 980–984.
(8) Chasseaud, L. F. (1983) Z. Kardiol. 72 (Suppl. 3), 20–23.
(9) Steudel, H. C., Volkenandt, M., Steudel, A. T. (1983) Z. Kardiol. 72 (Suppl. 3), 24–28.
(10) Mannebach, H., Ohlmeier, H., v. Möllendorff, E., Gleichmann U., Abshagen, U. (1981) Med. Welt 32, 517–520.
(11) Förster, H., Lippold, B. C. (1982) Pharm. Act. Helvet. 57, 345–349
(12) Bechgaard, H. (1982) Acta Pharm. Technol. 28, 149–157 and literature cited therein.
(13) Lippold, B. C., Förster H. (1982) Pharm. Ind. 44, 735–740.
(14) Zerbe, H. (1985) Herz, Kreisl. in press.
(15) Maddock, J., Lewis, P. A., Woodward, A., Massey, P. R., Kennedy, S. (1983) J. Chromatogr. Biomed. Appl. 272, 129–136.
(16) Brockmeier, D. (1981) Arzneim.-Forsch./Drug./Res. 31 (II) 1746–1751.
(17) Voegele, D., Brockmeier, D., v. Hattingberg, M., Lippold, B. C. (1983) Acta Pharm. Technol. 29, 167–174.
(18) Wagner, J. G., Nelson, E. (1963) J. Pharm. Sci. 52, 610–611.
(19) Meier, J., Nüesch, E., Schmidt, R. (1974) Eur. J. Clin. Pharmacology 7, 429–432.
(20) Beckett, A. H. (1981) in Nitrates III (Lichtlen, P. R., Engel, H.-J., Schrey, A., Swan, H. J. C., ed.) pp. 61–65, Springer, Berlin-Heidelberg-New York.
(21) Lippold, B. C. (1982), Pharm. Ztg. 127, 2167–2173.

Molecular Weights of Free and Drug-Loaded Nanoparticles

Luc Vansnick[1], Patrick Couvreur[1,2], Dominique Christiaens-Leyh[1] and Michel Roland[1]

Received: April 9, 1984; accepted: August 31, 1984.

Abstract: This study demonstrates that the molecular weight of polyalkylcyanoacrylate nanoparticles can be modified by the composition of the polymerization medium, the nature of the monomers and the drug to be linked to the carrier.
The influence of a surfactive agent is particularly important because polymers of very high molecular weight have been obtained. Likewise, polyalkylcyanoacrylate molecular weight distribution has been greatly modified after binding doxorubicin to nanoparticles. These long polymers could induce important changes in carrier degradation, in bound drug bioavailability and in polymer excretion rate. Some additional findings have been added concerning the state of polyalkylcyanoacrylate polymer during the degradation process.

Poor tissue specificity of pharmacologically active agents is a serious obstacle to their effective use. Formulations such as polyalkylcyanoacrylate nanoparticles could serve to improve the selectivity of these agents (1, 2). The use of nanoparticles increased the anticancer activity of dactinomycin against an experimental subcutaneous sarcoma of the rat (3). Furthermore, the possibility of significantly reducing the toxicity of doxorubicin by fixing it on nanoparticles has been demonstrated (4). More recently, whole body autoradiography performed on Lewis Lung carcinoma bearing mice showed an accumulation of the carrier in the tumoral tissue (5).

It is important to determine the molecular weights of the polymers forming nanoparticles, because the length of the polymer chain could greatly modify the body distribution of the carrier as well as the degradation rate of the polymer (6) and thus the bioavailability of the carried drug. Furthermore, it has been demonstrated that polyalkylcyanoacrylate nanoparticles undergo an enzymatic ester hydrolysis on the side chain without breaking the polymer (7). Therefore the polymers should retain the same number of monomeric subunits after dissolution. Heavier fractions are expected to remain longer in the body and the accumulation of such polymers of high molecular weight could also induce toxic side effects.

Some of the data presented in this paper partially confirm the preliminary results obtained by El-Egakey et al. (8). However, we have noted further unexpected results in our experiments. Moreover, this publication specifies the influence of all preparation conditions, the influence of the drug to be absorbed, and the state of the polyalkylcyanoacrylate polymer during the process of degradation.

Materials and Methods

Nanoparticle Preparation and Characterization

Free nanoparticles were prepared with two different monomers: isobutylcyanoacrylate (IBC)[1] and hexylcyanoacrylate

[1] Laboratoire de Pharmacie Galénique, Avenue E. Mounier, 73.20, 1200 Bruxelles, Belgium
[2] To whom correspondence should be addressed

[1] Bucrylat®, Ethicon GmbH, Norderstedt, Germany.

(HCA)[2]. 100 µl of monomer were added to 10 ml of various polymerization media, under continuous mechanical stirring. After 4 h (IBC) or 24 h (HCA) polymerization, an homogeneous milky suspension was obtained. The size of the resulting particles was estimated by measuring laser light scattering[3].

Nanoparticles prepared for molecular weight estimations were made in media of various pH containing various amounts of a surface active agent (polyoxyethylene-polyoxypropylene[4]) or of a suspending agent (dextran 40). Drug loaded nanoparticles were prepared according to the same method after dissolution of Dactinomycin[5] (DACT) (50 µg/ml) or Doxorubicin[6] (DOX) (100 to 750 µg/ml) in the polymerization medium.

Determination of DOX linked to nanoparticles was carried out by fluorimetry[7] according to a previously described method (4). For the determination of DACT linked to nanoparticles, 20 µl of ^{3}H-DACT (specific activity: 322 GBq/mmol; radioactive concentration: 18.5 MBq/ml) was added to the polymerization medium before adding the monomer. After polymerization, the suspension was centrifuged at 20000 rpm[8] and radioactivity measured by scintillation counting in both sediment (linked DOX) and supernatant (free DOX).

Chemical Degradation of Nanoparticles

Polyisobutylcyanoacrylate (PIBCA) nanoparticles were prepared in accordance with the method described above by adding 500 µl of monomer in 50 ml of a polymerization medium containing 5 % w/v dextrose, 0.3 % dextran 40, adjusted to pH 3 with 0.1 N HCl. Water was added to the resulting suspension in order to obtain a volume of 100 ml. The pH was then quickly adjusted to pH 11 with N NaOH solution. Under continuous mechanical stirring, the suspension was maintained in an incubation cell at pH 11 by using a pH stat system[9] that released 0.5 N NaOH solution.

At various degradation times, 5 ml samples were immediately mixed with 5 ml pH 7 buffered solution in order to stop the alkaline degradation. The resulting solution was centrifuged for 1 h at 20000 rpm. The sediment was vacuum-dried and dissolved in 5 ml of tetrahydrofuran for GPC analysis. sobutanol produced during the degradation process was determined following a gas chromatographic method previously described (7).

HPLC-GPC Determination

A gel permeation liquid chromatograph[10] fitted with refractive index detector[11] was used. Columns of µ styragel of 100, 500 and 1000 Å were used simultaneoulsy. Tetrahydrofuran, with a solvent flow of 2 ml/min was used as eluant. 50 mg of nanoparticles were dissolved in 10 ml of tetrahydrofuran. This solution was filtered through a 0.45 µm filter and 150 µl were injected into the chromatographic system. The chromatograms were registered and the peak surfaces integrated on a printer fitted with GPC calculation capacity[12].

Polyethyleneglycol standards with molecular weight ranging from 106 to 19100 were used for column calibration. A calculation method proposed by Waters (9) was adopted in which the number average molecular weight ($\overline{M}_n$) and the weight average ($\overline{M}_w$) molecular weight were calculated according to the following equations 1 and 2:

$$\overline{M}_n = \frac{\Sigma Qi}{\Sigma(Qi/Mi)} \quad (1)$$

$$\text{and } \overline{M}_w = \frac{\Sigma(Qi \cdot Mi)}{\Sigma Qi} \quad (2)$$

where Qi represents the amount of polymer having a molecular weight Mi. The polymer molecular weight distribution was estimated by calculating the dispersity coefficient ($d = \frac{\overline{M}w}{\overline{M}n}$

Reference solutions of dextran 40 were analysed by the GPC method in order to demonstrate that this stalilizer does not interfere with the cyanoacrylate polymer.

In our experimental conditions we observed no peak corresponding to dextran 40 probably because it does not dissolve in tetrahydrofuran.

Results

Free Nanoparticles

Influence of the Presence of a Surfactant

PIBCA nanoparticle samples were prepared in a 0.2 % dextran 40 polymerization medium with and without polyoxyethylene-polyoxypropylene as a surfactant. Fig. 1 shows the chromatograms of both preparations at pH 4. Only one peak was noticed in the preparations made without a surface active agent. This proved that the analyzed polymer was homogeneous. Furthermore, the symmetry of the peak suggested a uniform distribution of the molecules with a calculated molecular weight ranging between 500 and 1000. The low molecular weights of nanoparticles show that they consist of numerous little oligomeric subunits rather than one or a few long polymer chains.

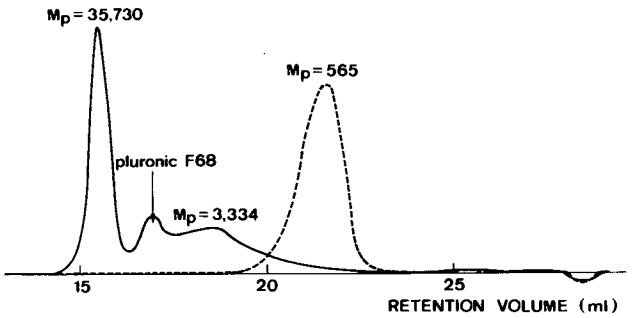

Fig. 1 GPC chromatographic profiles of PIBCA nanoparticles made with (0.8 %) (—) and without (---) polyoxyethylene-polyoxypropylene as surfactant. Nanoparticles sizes: 0.12 µm (—) and 0.22 µm (---).

[2] Weyl Chemische Fabrik GmbH, Mannheim-Waldhof, Germany.
[3] Nano-sizer®, Coulter® Electronics, Harpenden, England.
[4] Pluronic F68, Marles-Kuhlmann-Wyandotte, Paris, France.
[5] Lyovac Cosmegen, Merck Sharp Dohme B.V., Haarlem, Netherlands.
[6] Adriblastina, Montedison Farmaceutica Benelux, Brussels, Belgium.
[7] Vitatron Fluorimeter, type UFD, Vitatron, Netherlands.
[8] Beckman Centrifuge, Model J21C, Beckman Instruments, Palo Alto, California, USA.
[9] pH M 61 pH meter, abu 12 autoburette, TTT 60 titrator, Radiometer Copenhague, Denmark.
[10] M-45 solvent delivery system, model U6-K universal LC injector, Waters Ass., Milford, USA.
[11] R-401, Differential refractometer, Waters Ass., Milford, USA.
[12] M 730 Data module, Waters Ass., Milford, USA.

However, when nanoparticles were prepared in presence of the surface active agent, the chromatogram was quite different (Fig. 1). In fact, 3 different peaks were observed: the middle peak corresponded to the polyoxyethylene-polyoxypropylene, whereas the two other peaks corresponded to a bimodal distribution of the molecular weights. The molecular weight at the top of the peak (Mp) corresponding to the heaviest fraction was found to be above 30000, while the Mp of the lightest one was approximately 3000.

Polyhexylcyanoacrylate (PHCA) nanoparticles were prepared in a medium containing various concentrations of polyoxyethylene-polyoxypropylene. The measurement of polymer molecular weights indicated that up to a concentration of 0.05 % the surfactant increased the length of the PHCA chain. No further increase was observed beyond this value.

The average molecular weights are given in Table I. They correspond to the low molecular weight peak without influencing the peak with a molecular weight above 30000. Indeed, a carrier consisting of small oligomeric subunits may be of greater interest.

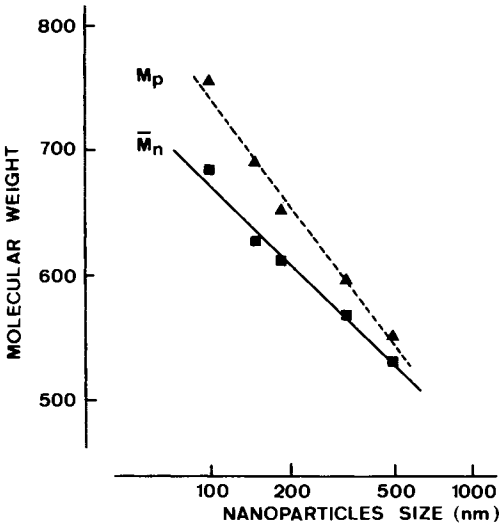

Fig. 3 Correlation between the average molecular weights of PIBCA and the size of nanoparticles.

Table I. Average Molecular Weights of PHCA Nanoparticles Made with Various Concentrations of Polyoxyethylene-Polyoxy-Propylene as a Surfactant. Nanoparticles Sizes: 0.08 µm to 0.13 µm

% W/V Pluronic F68	Molecular weights M_p	$\bar{M}_n$	$\bar{M}_w$	d
0.8	3,052	2,393	3,106	1.3
0.2	3,063	1,506	4,154	2.76
0.05	3,369	2,299	4,595	1.99
0.01	2,181	2,035	2,509	1.23
0.005	2,181	1,656	2,133	1.29
0.001	2,001	1,589	1,869	1.18

Influence of pH

PIBCA nanoparticles were prepared in a polymerization medium containing dextrose 5 % at various pH. The average molecular weights of the polymer increased with the pH (Fig. 2) as the nanoparticle sizes decreased. Indeed, it was observed that high molecular weight polymers form small size nanoparticles and vice versa (Fig. 3).

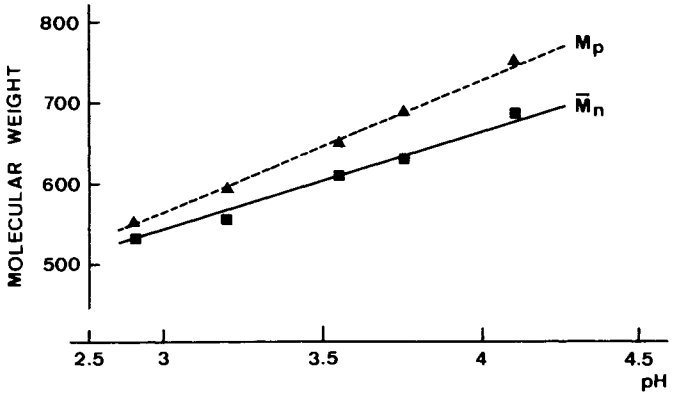

Fig. 2 Influence of the pH of preparation on the average molecular weights of PIBCA nanoparticles.

After preparation of PHCA nanoparticles at various pH in the presence of polyoxyethylene-polyoxypropylene, a dramatic molecular weight increase was observed for the higher pH values (Fig. 4). Therefore, for a preparation pH value of 4, the chromatographic profile of the resulting polymer showed a peak corresponding to a molecular weight (M_p) above 30000. Furthermore, in several chromatograms, a peak of lower molecular weight revealed the presence of residual monomer in some of the nanoparticle samples. This indicated that the polymerization process was not fully completed.

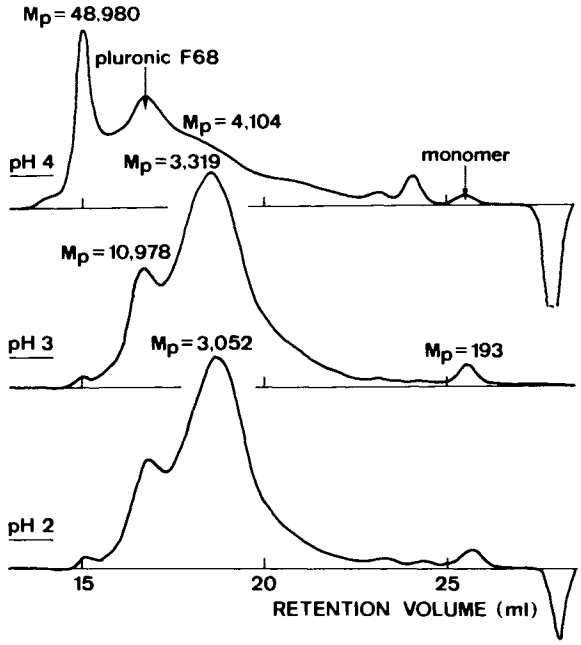

Fig. 4 GPC chromatographic profiles of PHCA nanoparticles made in various pH media containing polyoxyethylene-polyoxypropylene (0.8 %).

Influence of Dextran 40

Nanoparticles were made with and without using dextran 40 as suspending agent in the polymerization medium. The dextran free nanoparticle suspension produced polymers of low molecular weight and large size nanoparticles (0.45 to 0.50 μm) (Table II). Furthermore, we noticed that the presence of dextran in the polymerization medium produced a more homogeneous suspension and particle sizes (0.22 μm) that are easier to reproduce.

Table II. Average Molecular Weights of PIBCA Nanoparticles Made with or without Dextran 40 as Suspending Agent.

Polymerization medium	Molecular weights			Polydispersity
	M_p	$\overline{M}_n$	$\overline{M}_w$	d
Glucose 5 % pH = 3	554	589	633	1.12
Glucose 5 % Dextran 40 0.3 % pH = 3	895	685	798	1.17

Influence of the Monomer

Molecular weight measurements of PIBCA and PHCA nanoparticle suspensions showed that when prepared in pluronic free media, the PHCA molecular weights were higher than those obtained with the PIBCA preparations. Furthermore, the number of monomeric subunits in the polymer chain was calculated to be higher for the PHCA polymer (Table III). For both monomers, the presence of a surfactant in the polymerization medium gave higher molecular weight polymers with an equivalent number of monomeric subunits.

Table III. Molecular Weights of PIBCA and PHCA Nanoparticles and the Corresponding Numbers of Monomeric Subunits (n). Polymerization media: (1) Dextrose 5 %, Dextran 40 0.3 %, pH = 3 (size = 0.22 to 0.24 μm); (2) polyoxyethylene-polyoxypropylene 0.8 %, Dextran 40 0.2 %, pH = 2 (size = 0.08 to 0.12 μm); (3) citric acid 0.5 %, Dextran 40 1 %, Dextrose 4 % (size = 0.17 to 0.20 μm); (4) Dextrose 2 %, Dextran 40 0.3 %, phosphate buffer pH 3 (size: 0.22 to 0.25 μm)

Polymerization medium	PIBCA		PHCA	
	Mp	n	Mp	n
1	962	6.3	2,100	11.6
2	2,815	18.1	3,052	17
3	845	5.5	1,804	10
4	962	6.3	2,082	11.5

Drug Loaded Nanoparticles

Considering the significant change of polymeric molecular weights of nanoparticles by modifying the polymerization conditions, it was important to determine the influence of bound drug on the molecular weight of polyalkylcyanoacrylate nanoparticles. PIBCA nanoparticles were prepared in various polymerization media containing either DACT (50 μg/ml) or DOX (750 μg/ml).

Dactinomycin (DACT) Loaded Nanoparticles

The molecular weight measurements after linkage of DACT to nanoparticles showed no change either in the length of the polymer chain or in the size of the obtained nanoparticles (Fig. 5b).

Doxorubicin (DOX) Loaded Nanoparticles

After linking of DOX to nanoparticles, the increase in molecular weights of the polymer as well as that of the molecular weights dispersity was significant. Furthermore, nanoparticle sizes were increased and less homogeneous. The molecular weight increase of the polymer depended on the DOX concentration in the polymerization medium (Table IV). Even at a low DOX concentration, a peak appeared that corresponded to a heavy polymer chain (Fig. 5a.).

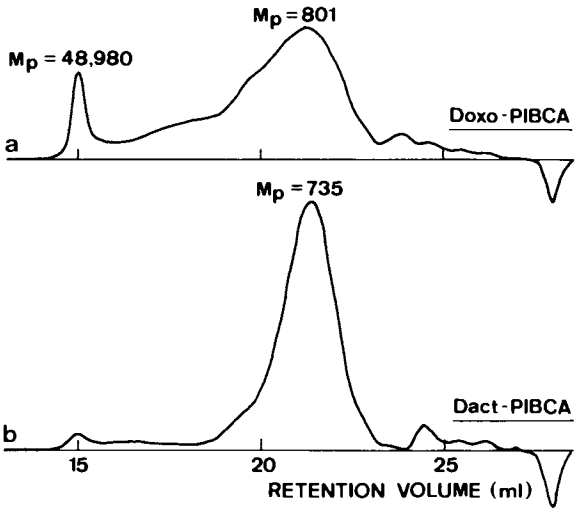

Fig. 5 GPC chromatographic profiles of PIBCA nanoparticles loaded with DOX (a) and DACT (b).

Table IV. Average Molecular weights of PIBCA Nanoparticles Loaded with Increasing Concentrations of DOX

Doxorubicin concentration (μg/ml)	Molecular weights			Polydispersity
	M_p	$\overline{M}_n$	$\overline{M}_w$	
0	838	744	925	1.24
100	855	861	1,128	1.31
200	855	894	1,467	1.64
400	855	937	1,830	1.95
600	915	983	2,040	2.07
750	1,771	1,188	2,744	2.31

Chemical Degradation of Nanoparticles

During the degradation process, nanoparticle samples (5 ml) were removed and centrifuged at 20000 during 1 h. The sediment was then dissolved in tetrahydrofuran and the molecular weight distribution was determined. The data showed that the molecular weight of the polymer did not decrease proportionally to the time of degradation. Indeed, the polymer was dissolved without the appearance of an insoluble polymer with intermediate weight (Fig. 6). Isobutanol produced after hydrolysis of the ester function (Fig. 7) was measured and tallied with the disappearance of the nanoparticles solid polymer (Fig. 6). Indeed, after 240 min degradation, 36 % of all ester functions were hydrolysed while very small amounts of polymer remained solid.

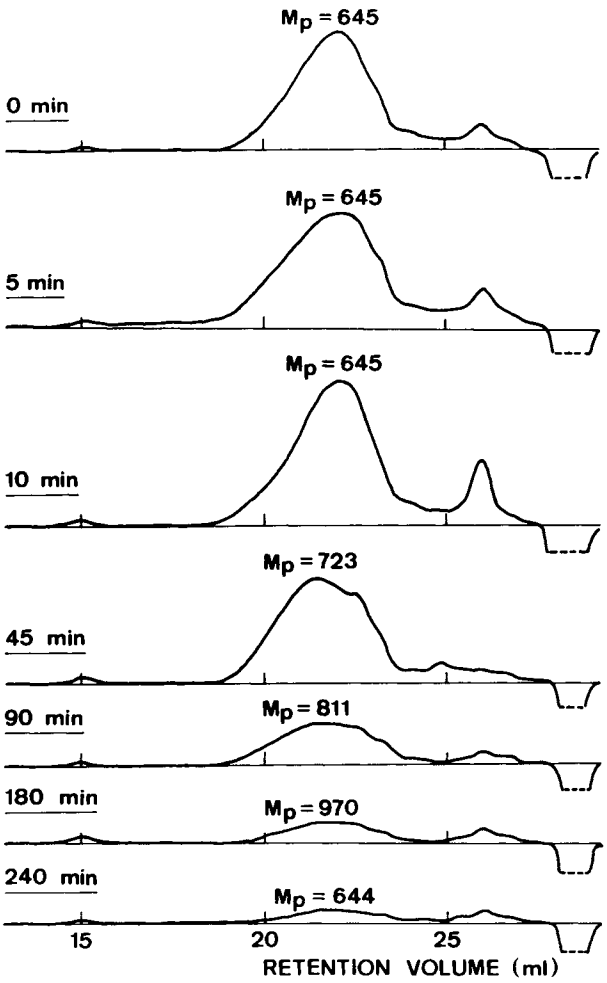

Fig. 6 GPC chromatographic profiles of PIBCA nanoparticles insoluble fraction at various degradation times (pH 11).

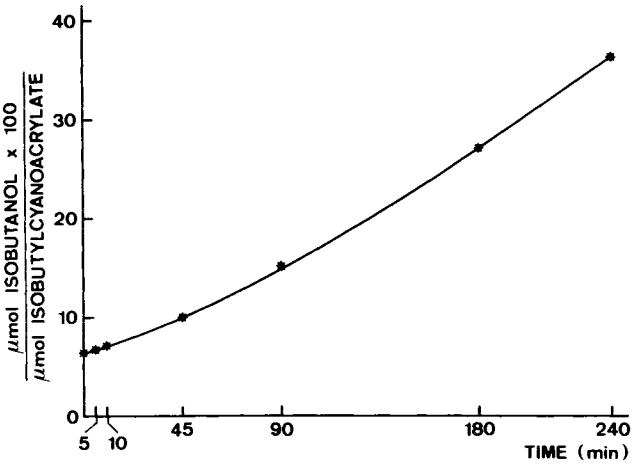

Fig. 7 Isobutanol production at various nanoparticles degradation times.

Discussion

The presented data show that the molecular weight of polyalkylcyanoacrylate nanoparticles can be significantly modified not only by the polymerization medium (surfactant, suspending agent and pH) but also by the nature of the monomer used and by the drug linked to them.

The influence of a surface active agent was particularly important because polymers of very high molecular weight were obtained. Indeed, a peak corresponding to a value above 30000 appeared and became more prominent as the pH of the preparation was increased. These observations are of interest because long polymers could induce important changes in carrier degradation (6), in bound drug bioavailability and in polymer excretion rate. As a result toxic overload of the polymer in RES cells might occur (10).

An increase in the pH induced an increase in polyalkylcyanoacrylate molecular weights and a decrease in nanoparticle sizes. This observation combined with the use of surfactant at a chosen concentration allows one not only to obtain polymers of selected molecular weights but also nanoparticles of selected sizes. Furthermore, at low pH values, attention must be given to the possible presence of residual monomers that could induce toxic effect.

Dextran seemed to be useful in the preparation of nanoparticles to achieve homogeneous polymer distribution and uniformity of nanoparticle sizes. However, after intravenous administration of dextran, there is a possibility of an anaphylactic reaction described previously (11).

Therefore attempts are now in progress to avoid the use of dextran in the nanoparticle formulation while maintaining the same characteristics. In surfactant free media, the nature of the monomer also influences the length of the polymer. Indeed, HCA produced a polymer chain with a number of monomeric subunits generally ranging between 10 and 12, while IBC gave polymers with a maximum of 6 subunits.

These numbers are only comparative estimates. The exact calculation of these numbers is hazardous, because the calibration curve was based on polyethyleneglycols as standards. However, these values tally with those obtained by the end-function titration method. In fact, the quantitative determination of formaldehyde produced by a reverse Knoevenagel reaction at the end of each polymer molecule allows one to calculate a $\overline{M}_n$ value corresponding to 6 monomeric subunits for PIBCA.

While DACT did not seem to induce any change in nanoparticle molecular weights, the polyalkylcyanoacrylate chromatographic profile was greatly modified after DOX binding. This could be due to the intervention of DOX as initiator (by its free amine function) in the anionic polymerization of the cyanoacrylic monomer. The drug could also be covalently linked to the beginning of the polymer chain. Studies are now in progress to test this hypothesis.

During the nanoparticle degradation process, GPC data showed that a non-dissolved polymer fraction retained the same molecular weight. This suggested that the degradation pathway with formaldehyde production and progressive break-down of the polymer should be reconsidered (12). The results also confirm the hypothesis of Lenaerts et al. (7) that assumes the hydrolysis of the ester function with production of isobutanol and a resulting increase of polymer solubility, as polycyanoacrylate.

In comparison to the other particulate carriers described in the literature (13–15), polyalkylcyanoacrylate nanoparticles are an original colloïdal drug delivery system because they are built by numerous small oligomers rather than by one or a few macromolecules. Such oligomeric systems should be more easily cleared from the body and they would be more apt to avoid polymeric overload of the RES. Furthermore, the possi-

bility to prepare nanoparticles of selected size and selected molecular weights has been demonstrated. Finally, special attention must be given to the possible influence of nanoparticle bound drugs on the molecular weights and on the distribution of the polymeric chain that form the nanoparticles.

Acknowledgements
This work was sponsored by SOPAR S.A. Company and the Institut pour l'Encouragement de la Recherche dans l'Industrie et l'Agriculture (IRSIA). The support of the Fonds National de la Recherche Scientifique (FNRS) is greatly appreciated. The authors wish to thank Mr. Bulckens for his excellent technical assistance and Mr. Vandiest for the drawings.

References

(1) Couvreur, P., Roland, M., Speiser, P. (1982) US Patent 4, 320, 332.
(2) Couvreur, P., Kante, B., Roland, M., Guiot, P., Baudhuin, P., Speiser, P. (1979) J. Pharm. Pharmacol. 31, 331–332.
(3) Brasseur, F., Couvreur, P., Kante, B., Deckers-Passau, L., Roland, M., Deckers, C., Speiser, P. (1980) Europ. J. Cancer 16, 1441–1445.
(4) Couvreur, P., Kante, B., Grislain, L., Roland, M., Speiser, P. (1982) J. Pharm. Sci. 71, 790–792.
(5) Grislain, L., Couvreur, P., Lenaerts, V., Roland, M., Deprez-Decampeneere, D., Speiser, P. (1983) Int. J. Pharm. 15, 335–345.
(6) Vezin, W. R., Florence, A. T. (1980) J. Biomed. Mat. Res. 14, 93–106.
(7) Lenaerts, V., Couvreur, P., Christiaens-Leyh, D., Joiris, E., Roland, M., Rollman, B., Speiser, P. (1984) Biomaterials 5, 65–68.
(8) El-Egakey, M. A., Bentele, V., Kreuter, J. (1983) Int. J. Pharm. 3, 349–352.
(9) Sun, S. F., Wong, F. (1981) J. Chromatography 208, 253–259.
(10) Lenaerts, V., Nagelkerke, J. F., Van Berkel, T. J. C., Couvreur, P., Grislain, L., Roland, M., Speiser, P. (1984) J. Pharm. Sci. 73, 980–982.
(11) Briseid, G., Briseid, K. (1980) Acta Pharmacol. Toxicol. 47, 119–126.
(12) Leonard, F., Kulkarni, R. K., Brandes, G., Nelson, J., Cameron, J. J. (1966) J. Appl. Polym. Sci. 10, 259–272.
(13) Kreuter, J. (1978) Pharm. Acta Helv. 53, 33–39.
(14) Oppenheim, R. C. (1981) Int. J. Pharm. 8, 217–234.
(15) Kramer, P. A. (1974) J. Pharm. Sci. 63, 1646–1647.