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An analytical methodology to quantify the incorporation of enzymes in polyalkylcyanoacrylate nanoparticles based on size exclusion chromatography

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Abstract

The performances of different methods of quantification of protein (methods based on direct spectrophotometric and spectrofluorimetric analysis, chemical reactions and liquid chromatography) to quantify the amount of enzyme incorporated into polyalkylcyanoacrylate nanoparticles, were compared. A methodology based on size exclusion chromatography was selected. The performances of the analytical method to quantify the enzymes L-asparaginase and superoxide dismutase in different polymerization media of poly-isobutilcyanoacrylate, were evaluated. The quantification of superoxide dismutase in the presence of esterase, enzyme used to solubilize nanoparticles, was attempted. An adequate separation between enzyme and the other components of polymerization media was achieved, so the selectivity of the method is adequate to the quantification of an enzyme in polymerization medium, either before or after polymerization. Although lack of selectivity of the column to separate enzymes was observed. The retention time of L-asparaginase and superoxide dismutase in polymerization medium are, respectively, 7.4 and 7.5. Linear correlation between peak area and enzyme concentration were observed for both enzymes in the concentration range from 10 to 80 μg ml^{-1}, either before or after polymerization and in different polymerization media. This SE-HPLC analytical methodology is adequate to determine the degree of incorporation of enzymes in polyalkylcyanoacrylate nanoparticles as evidenced by the linear responses of the chromatographic method, the reproductibility of repeated sample injections and the precision of the quantification of enzyme concentration. © 1997 Elsevier Science B.V.

Keywords: L-asparaginase; Superoxide dismutase; Nanoparticles; Size exclusion HPLC; Polyalkylcyanoacrylate; Surfactants

1. Introduction

The design of protein delivery systems, mainly colloidal carriers, is becoming an important field with the increased availability of therapeutic ac-

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tive proteins and the need for better bioavailability, made possible by the advances in biotechnology processes [1,2]. Analytical methodologies suitable for the analysis of proteins included in complex matrices, supporting the development of colloidal carriers for proteins, are particularly relevant in enzyme incorporation. The performance and quality of the analytical methods can limit the development of optimized protein delivery systems [3].

Polyalkylcyanoacrylate nanoparticles are one of the colloidal carriers with potential to improve the delivery of therapeutic active molecules [4]. A large amount of work in drug incorporation with this type of nanoparticles have been published [5], but there have been only a few publications on the incorporation of peptides in polymeric colloidal drug carriers [6–9]. In the case of incorporation of non-protein drugs several methods are used to characterize the systems [10], but generally the free drug is quantified in the supernatant and the total drug measured either by complete dissolution of nanoparticles suspension or in the initial polymerization medium.

To optimize the technological parameters in the preparation of polyalkylcyanoacrylate nanoparticles in order to maximize the immobilization of proteins/enzymes (preserving their biological and catalytic activities), the quantification of proteins in polymerization media plays a very important role. Several methods can be used to quantify proteins [11], although the complexity of the composition medium can reduce the number of possibilities. Polyalkylcyanoacrylate nanoparticles are prepared by emulsion polymerization where the anionic polymerization takes place in micelles [5]. Several adjuvants are used and the characteristics of nanoparticles strongly depend on the composition of the polymerization medium [12]. The analytical methodology to quantify protein either in the initial polymerization medium or in the supernatant after centrifugation can also be dependent on the chemical nature of the adjuvants used.

In the present paper several methods of quantification of proteins used in the analysis of two enzymes (L-asparaginase and superoxide dismutase) during their incorporation in poly-isobutylcyanoacrylate nanoparticles, are compared. The performances of a size exclusion HPLC (SE-HPLC) analytical methodology to quantify both enzymes in different polymerization media are reported.

2. Materials and methods

2.1. Chemicals and enzymes

Isobutylcyanoacrylate (IBCA) was a gift from Loctite, Dublin, Ireland. Superoxide dismutase (EC. 1.15.1.1.) was from Sigma. L-asparaginase (EC.3.5.1.1) was from Merck Sharp and Dome. Other reagents were of analytical grade.

2.2. Apparatus

Spectrophotometric analysis were performed using a spectrophotometer: Shimadzu UV-160A. Fluorimetric analysis were performed using a spectrofluorimeter: Hitachi F3000. Separations by ultra centrifugation were performed using an ultracentrifugue: Beckman XL90 equipped with a swinging bucket rotor SW 55 Ti.

The chromatographic system consisted of a programmable solvent module Beckman 126, an injector fitted with a 50 μl loop, a programmable

Table 1
Comparison of several methods of quantifying proteins in a polymerization medium used to obtain isobutylcyanoacrylate nanoparticles

Analytical method	Main limitation/viability
Photometric analysis	High background due to polymerization medium
Fluorimetric analysis	High background due to polymerization medium
Lowry method	Turbidity
Biuret method	A calibration with supernatant of unloaded nanoparticles is required; high lower limit of detection
RP-HPLC	Aggregation of enzyme in the mobile phase
SE-HPLC	Viable

Polymerization medium of composition C (as described in methods).

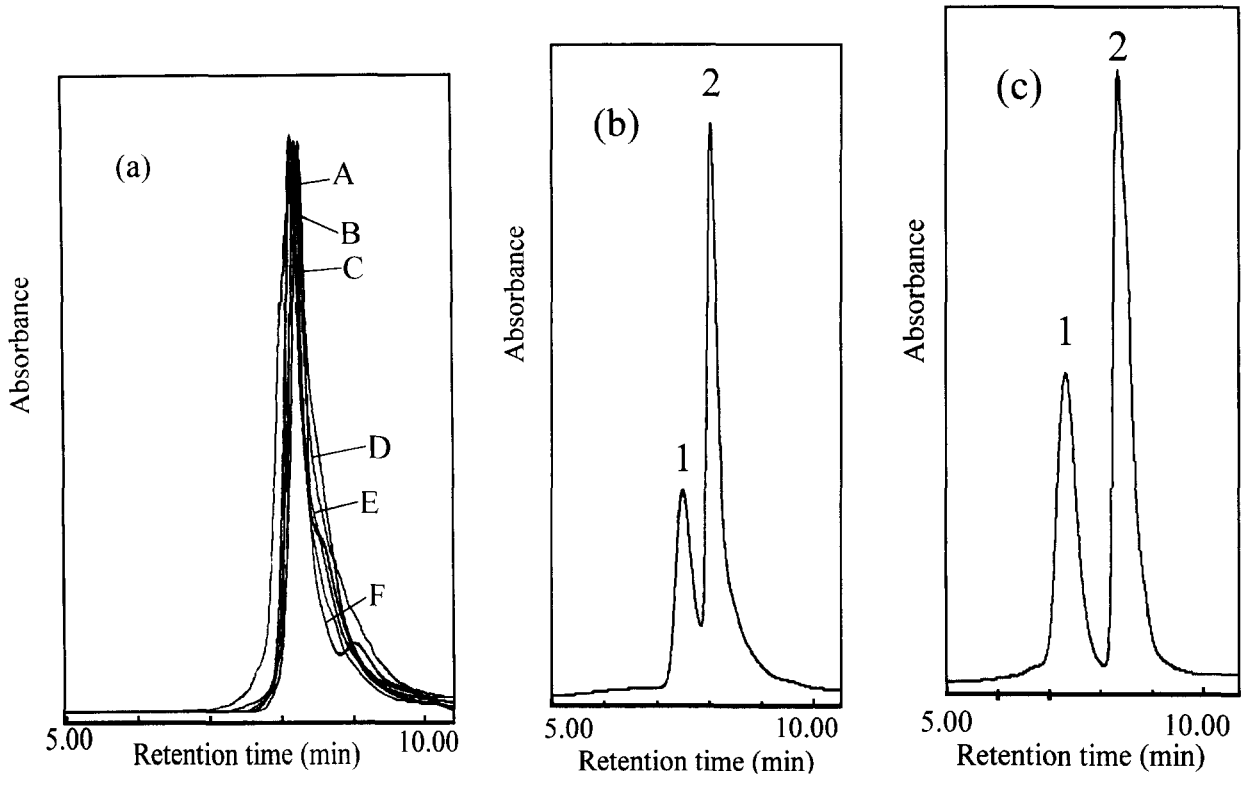

Fig. 1. Elution profiles of: (a) polymerization medium of compositions A–F; (b) superoxide dismutase (peak 1) in polymerization medium of composition C (peak 2), before polymerization (c) L-asparaginase (peak 1) in polymerization medium of composition C (peak 2), before polymerization. (See methods for composition of polymerization media and chromatographic details).

detector module Beckman 166, with variable-wavelength photometric detector fitted to 220 nm. The system was controlled using the software package System Gold from Beckman, the chromatographic data being collected and treated using this system.

2.3. Chromatographic conditions

The chromatographic analysis was performed at ambient temperature (approximately 22°C). The mobile phase consisted of an aqueous buffer ($K_3PO_4 \cdot 7H_2O$, (0.1 M) and NaCl (0.1 M), pH = 7). A LiChrospher 300Diol SE-HPLC column (10 μm particle size, 250 × 4 mm i.d.) and a guard column LiChrospher 100Diol (5 μm, 4 × 4 mm i.d.) from Merck were used. The flow rate was maintained at 0.3 ml min^{-1}. The detection was performed at 220 nm.

2.4. Preparation of nanoparticles

Nanoparticles were prepared by emulsion polymerization of isobutylcyanoacrylate (4 h under magnetic stirring) in an adequate polymerization medium as described in [13]. Polymerization media with different compositions were used: medium A–citric acid (1 mM) containing glucose (5%), dextran 40 (0.5%); medium B–citric acid (1 mM) containing glucose (5%), dextran 40 (0.5%), Symperonic F68 (0.5%); medium C–citric acid (1 mM) containing glucose (5%), dextran 40 (0.5%), Symperonic F68 (1%); medium D–HCl 1 mM containing glucose (5%), dextran 40 (0.5%), Symperonic F68 (2%); medium E–citric acid (1 mM) containing glucose (5%), dextran 40 (0.5%), Symperonic F68 (2%); medium F–HCl 1 mM containing Symperonic F68 (2%). To prepare enzyme-loaded nanoparticles 100 to 1200 μg of

Table 2
Linear regression equations correlating peak area as a function of concentration of enzyme in polymerization media of different composition

Enzyme (concentration range)	Medium	Linear regression parameters[a]				Corr. coef.
		Slope	(Slope std.)	Interception	(Inter. std.)	
L-Asparaginase (10–80 μg ml^{-1})	C	1.051	(0.0205)	1.529	(0.888)	0.9992
Superoxide dismutase (10–80 μg ml^{-1})	A	0.759	(0.0119)	−0.613	(0.681)	0.9993
	B	0.721	(0.0145)	0.589	(0.824)	0.9990
	C	1.070	(0.0102)	0.787	(0.579)	0.9997
	D	1.003	(0.00699)	−0.217	(0.398)	0.9999
	E	0.783	(0.0164)	−0.963	(0.935)	0.9990
	F	0.875	(0.0181)	−0.842	(1.04)	0.9990

[a]Five different concentration standards, in the range 10–80 μg ml^{-1}, were used for each curve. For each concentration four within-day replicates of peak area were used. (See methods for other chromatographic details).

enzyme were added per ml of polymerization medium.

2.5. Sample preparation

Samples of polymerization medium before the addition of the monomer were diluted with polymerization medium to achieve an enzyme concentration in the calibration range in order to be injected in the HPLC apparatus. Samples of polymerization medium after preparation of nanoparticles were ultracentrifuged to separate the colloidal particles (600 μl of enzyme suspension added to 4.4 ml of water ultracentrifugated at 100 000 × g during 2 h in swinging basket rotor) and the supernatant diluted with polymerization medium, if necessary.

For HPLC evaluation, all the samples were filtered through membranes of 0.2 μm pore size before injection. Samples of 100 μl were injected and 50 μl were retained in the loop.

2.6. Calibration curves

Calibration curves were obtained using different working standard solutions of enzyme prepared independently by dissolving appropriate amounts of enzyme in each polymerization medium. Calibration standards were: 10, 20, 40, 60 and 80 μg ml^{-1}.

2.7. Quantification of protein by direct methods

Direct spectrophotometric analysis were performed by the measurement of light absorption of tyrosine and tryptophan residues of proteins at 280 nm. Direct fluorimetric analysis were performed by the measurement of proteins fluorescence intrinsic emission detected at 340 nm, for an excitation wavelength of 280 nm.

2.8. Quantification of protein by methods based on chemical reactions

Quantification of protein by the Lowry procedure were performed according with conventional Lowry method [14] and to one modified procedure to quantifications in the presence of detergents [15].

Quantification of protein by the biuret method were performed according to a modification of the original method to increase its sensitivity [16].

2.9. Calculation of the efficiency of incorporation

The efficiency of incorporation (EI) of enzymes in nanoparticles of polyisobutylcyanoacrylate was determined by the ratio: EI = (enzyme incorporated in nanoparticles/total initial enzyme) × 100.

Table 3
Precision data for the enzyme superoxide dismutase in polymerization medium of composition C

Quantity of enzyme added to 1 ml of medium of composition C (μg)	Mean enzyme concentration quantified by this assay (μg ml^{-1} ± S.D.)	R.S.D. (%)
24.0	24.4 ± 0.78	3.2
43.2	43.6 ± 0.96	2.2
62.4	62.3 ± 1.13	1.8
80.8	80.8 ± 1.31	1.6

Each enzyme concentration represents the mean of four within day replicates.

3. Results and discussion

The aim of this work was to develop and optimize an analytical procedure to quantify enzymes incorporated in isobutylcyanoacrylate nanoparticles. The performance of several methods for quantification of proteins was evaluated. Photometric and fluorimetric analysis, both simple and non-destructive procedures, were compared with methods based on chemical reactions and with liquid chromatography. A comparison between the different methods for quantification of proteins in polymerization media is shown in Table 1. Photometric analysis are based on characteristic ultraviolet absorption spectrum of proteins in solution, due to tyrosine and tryptophan aminoacid residues of the protein, with a maximum close to 280 nm. Although, the presence of compounds in the polymerization medium that absorb at 280 nm, interfere with the determination of protein. Fluorimetric analysis are based on the intrinsic fluorescence of proteins, mainly due to their tryptophan residues, with emission of fluorescence at 330–340 nm, when excited at 280–290 nm. This procedures is more sensitive than the absorption measurement, but severe interference by compounds present in the polymerization medium was observed.

According to the sensitivity required to quantify protein after polymerization, especially when a high efficiency of incorporation is achieved and only small quantities of enzyme remain in the medium of polymerization, the points stated in Table 1 indicate the selection of SE-HPLC.

The criteria for the selection of the chromatographic column for SE-HPLC were, simultaneously, the adequate properties for the separation by size exclusion in non-denaturating conditions and the lowest price of the column. A study of the characteristics of several columns, according to literature, was performed [18]. Either in the selection of the column or in the selection of the operational conditions it was considered that the fractionation limits are directly related with the particle size and the pore size of the column particles, the sample volume, the column length and the composition of the mobile phase. The selection of mobile phase was based on literature data for other SE-HPLC columns [18]. Salt concentrations above 0.1 M are recommended to minimize possible ionic interactions between proteins and the support, NaCl concentration between 0.1 and 0.5 M is recommended for true SE-HPLC [18]. Preliminary results (non shown) using the selected column, mobile phases with pH 7 and different salt composition, sample volumes from 25 to 75 μl and flow-rates from 0.1 to 0.5 ml min^{-1}, pointed to the selected conditions.

The performances of SE-HPLC to quantify the enzymes L-asparaginase and superoxide dismutase in polymerization media of different composition, before and after polymerization, were studied. Fig. 1 shows typical elution profiles for different polymerization media and for the enzymes in one of the polymerization media, before the addition of the monomer.

The retention times of L-asparaginase and superoxide dismutase in the different polymerization media are, respectively, 7.4 and 7.5 with relative standard deviations, for both enzymes, smaller than 0.7 and 1%, respectively for within-day and between-day determinations. The precision of repeated simple injections, evaluated as the relative standard deviations of peak area for the minimum

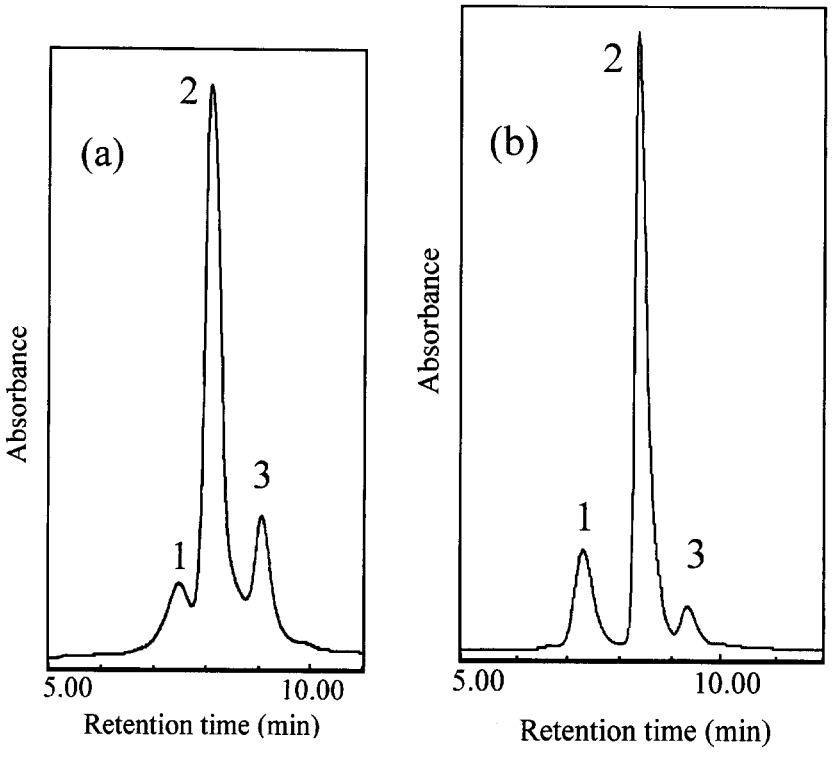

Fig. 2. Chromatograms of polymerization medium after separation from enzyme loaded nanoparticles: (a) polymerization medium separated from superoxide dismutase loaded nanoparticles; (b) polymerization medium separated from L-asparaginase loaded nanoparticles. In both chromatograms the enzyme is at peak 1 and the other components of supernatant at peaks 2 and 3. (See methods for other chromatographic details).

and maximum enzyme concentration, varies from 3 to 0.6% and from 6 to 1%, respectively, for within-day and for between-day determinations.

Standard curves using peak area as a function of enzyme concentration, for both enzymes, were linear over a range of enzyme concentration from 10 to 80 μg ml^{-1}. Parameters of linear regressions correlating peak area with enzyme concentration are in Table 2.

The precision of the method was calculated as the relative standard deviation (R.S.D.) of quantifications for standard enzyme solutions of superoxide dismutase in medium of composition C (Table 3). The relative standard deviations for within day determinations of enzyme concentrations from 24 to 80 μg ml^{-1} ranged from 3.2 to 1.6%.

Chromatograms of L-asparaginase and superoxide dismutase in polymerization medium, after polymerization and separation from nanoparticles, can be observed in Fig. 2. After polymerization a third peak is observed in polymerization medium either of unloaded nanoparticles or enzyme loaded nanoparticles.

The quantification of superoxide dismutase in nanoparticles solubilized by esterase was attempted. The chromatograms of esterase and of solubilized enzyme loaded nanoparticles are in Fig. 3.

The chromatograms in Fig. 3 show the complexity of the sample after enzymatic degradation of enzyme-loaded nanoparticles and the lack of selectivity of the chromatographic column for the separation of enzymes by molecular weight (MW of superoxide dismutase and esterase are 30 and 60 kDa, respectively). This characteristic of the column was also evidenced by the very small difference of retention time observed for superox-

ide dismutase and L-asparaginase, enzymes with very different molecular weights (30 and 130 kDa, respectively). Meanwhile, the selectivity of this column is adequate for the separation between proteins and small molecular weight molecules, as evidenced by the observed separation between proteins and the other components of the polymerization medium and by the linear correlation between the enzyme peak area and the concentration of enzyme.

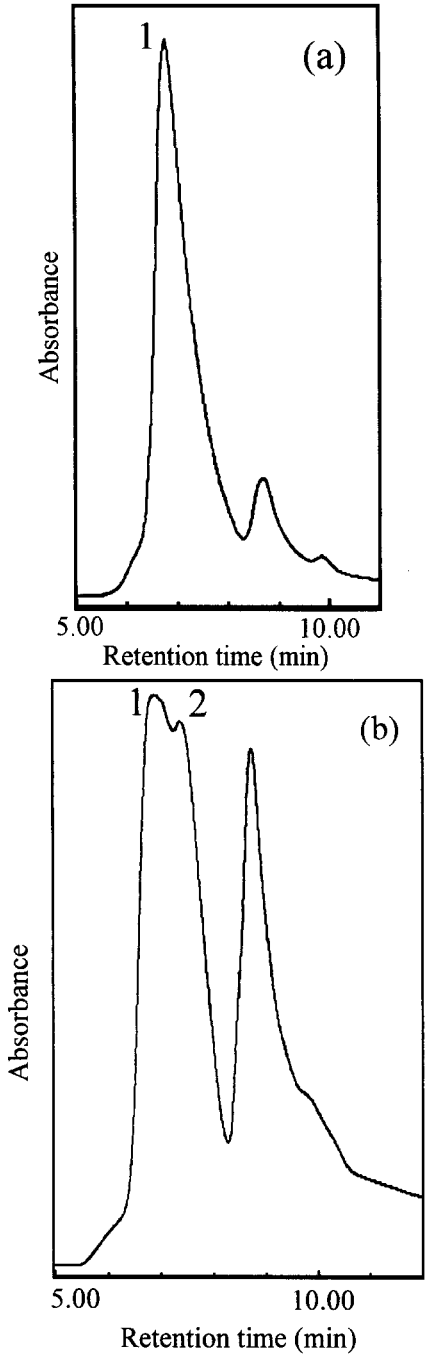

Fig. 3. Chromatograms of (a) esterase at peak 1 and (b) esterase and superoxide dismutase at peak 1 and 2, respectively. (See methods for other chromatographic details).

Examples of quantification in terms of efficiency of incorporation relating to superoxide dismutase in poly-isobutylcyanoacrylate nanoparticles, polymerized in medium of composition C, are presented in Table 4. The significative reduction of enzyme concentration after polymerization is consistent with the decrease of catalytic activity quantified in the supernatant [17].

In conclusion, the developed analytical methodology based on the separation between the components of the polymerization medium of nanoparticles and enzymes by SE-HPLC, exhibits the adequate performance to quantify enzyme in the polymerization media used during preparation of poly-isobutylcyanoacrylate nanoparticles. This was evidenced by the separation of enzymes from low molecular weight components, the linear responses of the chromatographic method (in the selected range of enzyme concentration and for the different polymerization media), the reproductibility of repeated sample injections and the precision of the quantification of enzyme concentration. This easy and rapid assay can help the optimization of parameters in the process of emulsion polymerization of polyalkylcyanoacrylates, assuming an important role in the development of colloidal drug carriers in order to maximize the immobilization of active enzyme in nanoparticles.

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Table 4
Efficiency of incorporation of superoxide dismutase in isobutylcyanoacrylate nanoparticles quantified by SE-HPLC

Enzyme quantified before polymerization		Enzyme quantified after polymerization		Efficiency of incorporation
Area	$\mu g\ ml^{-1} \pm$ S.D.	Area	$\mu g\ ml^{-1} \pm$ S.D.	(% ± S.D.)
27.67[a]	377 ± 12	27.98[c]	25.41 ± 0.79	93 ± 5.9
36.37[b]	997 ± 25	27.66[d]	251 ± 8.1	74 ± 4.3

[a]Dilution × 15.
[b]Dilution × 30.
[c]No dilution.
[d]Dilution × 10.

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