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An HPLC with evaporative light scattering detection method for the quantification of PEGs and Gantrez in PEGylated nanoparticles

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Abstract

A rapid and precise HPLC method with evaporative light scattering detection (ELSD) for the separation and quantification of polyethyleneglycol 2000 (PEG 2000), polyethyleneglycol 6000 (PEG 6000) and poly(methyl vinyl ether-*co*-maleic anhydride) (Gantrez) in a nanosized pharmaceutical formulation has been developed. Separation was carried out on a PL aquagel-OH 30,8 μm column (300 mm × 7.5 mm), in a gradient elution with methanol–water as mobile phase at a flow rate of 1 ml/min. Quantification was determined in supernatants of PEGylated nanoparticles and the quantification limits were found to be 0.075 mg/ml for polyethyleneglycols and 0.25 mg/ml for Gantrez. The precision did not exceed 8% and accuracy range for PEGs (−11.50 and 10.61%) and Gantrez (−12.18 and 14.81%) were always within the acceptable limits. The amount of polyethyleneglycol associated to nanoparticles was also calculated by a Nuclear Magnetic Resonance Method (^{1}H NMR). Likely, for both PEGs, a good relationship between both techniques was found. In summary, the developed HPLC technique provides an alternative for the routine and rapid analysis of PEGs and Gantrez in nanoparticle formulations.

Keywords: HPLC; ELSD; Polyethyleneglycol; Gantrez; Nanoparticles

1. Introduction

Polyethylene glycols (PEGs) are condensation polymers of ethylene oxide and water with the following general formula: $H(OCH_2CH_2)_nOH$, where n is the average number of repeating oxyethylene groups. These polymers vary in consistency from liquid to solid, depending on the molecular weight, indicated by a number following the name [1].

PEGs have solvent and dispersing properties and can act as surfactants. In addition, they are regarded as nontoxic and nonirritant materials [1]. For these reasons, they are widely employed in a variety of pharmaceutical products including parenteral, topical, ophthalmic, oral and rectal preparations. Liquid PEGs are used as suspending agents or to adjust the viscosity and consistency of other types of vehicles [2]. When used in conjunction with other surfactants, polyethylene glycols can also act as emulsion stabilizers [3]. In solid dosage formulations, higher molecular weight polyethylene glycols can enhance the effectiveness of tablet binders, impart plasticity to granules and act as lubricants, particularly for soluble tablets [4,5]. In film coating, solid grades of polyethylene glycol are also widely used as plasticizers in conjunction with the film forming polymers [6].

More recently, PEGs have been used as linker for conjugation with drugs in order to modify their biological behaviour in vivo. In fact, the link of PEG chains to biotherapeutics (PEG conjugates) may prolong their plasma retention time, renders them more resistance to proteases and less immunogenic [7–9]. In this context, PEG-coated nanoparticles have also been developed over the past years since they can offer a number of advantages for drug delivery purposes. This is due to their ability to minimise the recognition of these carriers by the cells of the monocyte–macrophage system and, thus, prolong their cir-

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culation in the blood after intravenous administration [10–12]. Furthermore, it appears that the PEG chains may facilitate the diffusion of nanoparticles across the mucus network, protecting the different mucosas (i.e. oral, pulmonar, ocular and nasal), and therefore facilitate the absorption and bioavailability of drugs [13–15].

In any case, the type of PEG employed and the density of PEG chains in the surface of nanoparticles are important factors in the development of these drug delivery systems [10,14,15]. Consequently, the quantification of PEG moieties associated to drug carrier is a key point in order to characterize these pharmaceutical dosage forms. However, PEG compounds are difficult to quantify by conventional and current techniques. Therefore, UV techniques can only be used after derivatization of PEGs with a chromophore [16–18]. A more reliable quantitative method is afforded by ^{1}H NMR, which can be directly applied to intact samples [14]; although the sensibility of the technique is not very high. On the other hand, analytical HPLC techniques have been applied to the characterization of some polyethylene glycol derivatives by using a refractive index detector (RID) [19,20] or mass spectroscopy [21]. Another possibility to quantify PEGs by HPLC techniques may be the use of an ELSD detector. These detectors, which popularity has increased considerably since its introduction in the early 1980s, have the ability to detect any non-volatile compounds regardless of their structural characteristics [22], including pharmaceutical excipients [22,23], drugs [24–26] and inorganic counter ions [27].

Recently, our research group has developed PEG-coated nanoparticles based on the simple reaction in an aqueous medium of PEG chains with a copolymer of methyl vinyl ether and maleic anhydride (Gantrez AN) [7]. In this paper, we report a new method for the quantification of different PEGs and Gantrez in the same sample. For this purpose, an HPLC method with evaporative light scattering detector (ELSD) was developed and fully validated.

2. Materials and methods

2.1. Chemicals, reagents and solutions

Poly(ethylene glycol) with M_w of 2000 and 6000 Da (PEG 2000; PEG 6000) were provided by Fluka (Switzerland). Poly(methylvinylether-*co*-maleic anhydride) or PVM/MA (Gantrez® AN 119; M_w 200,000) was a gift from ISP (Barcelona, Spain). Acetone and ethanol were obtained by VWR Prolabo (Fontenay-sous-Bois, France). Methanol (HPLC grade) by Merck (Darmstadt, Germany). Deionized reagent water (18.2 MΩ resistivity) was prepared by a water purification system (Wasserlab, Pamplona, Spain). Nitrogen gas (ultra-pure, >99%) was produced using an Alltech nitrogen generator (Ingeniería Analítica, Barcelona, Spain).

2.2. Calibration standards

Stock solutions of PEGs and PVM/MA (with a concentration of mg/ml) were separately prepared by dissolving 10 mg of either PEGs or PVM/MA in 10 ml of acetonitrile. Eight standard solutions of PEGs (0.05, 0.075, 0.1, 0.15, 0.17, 0.2, 0.22 and 0.25 mg/ml) were prepared by dilution of the stock solution with appropriate volumes of acetonitrile. Similarly, the PVM/MA standard solutions (0.25, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/ml) were prepared by dilution of stock solution with appropriate volumes of acetonitrile.

2.3. Instrumentation and chromatographic conditions

The apparatus used for the HPLC analysis was a model 1100 series Liquid Chromatography, Agilent (Waldbronn, Germany) coupled with an evaporative light scattering detector, ELSD 2000 Alltech (Illinois, USA). An ELSD nitrogen generator Alltech was used as the source for the nitrogen gas. Data acquisition and analysis were performed with a Hewlett-Packard computer using the ChemStation G2171 AA program. Separation was carried out at 40 °C on a PL aquagel-OH 30 column (300 mm × 7.5 mm; particle size 8 μm) obtained from Agilent Technologies (GB, United Kingdom). ELSD conditions were optimized in order to achieve maximum sensitivity: the drift tube temperature was set at 110 °C, the nitrogen flow was maintained at 3 l/min and the gain was set to 1. The mobile phase composition was a mixture of methanol (A) and water (B) in a gradient elution at a flow rate of 1 ml/min. The gradient started with a methanol–water phase (50:50, v/v) for 6 min. Two minutes later (time: 8 min) the composition of the mobile phase was methanol–water 40:60 (v/v). At 10 min it changed to a methanol–water mixture of 25:75 (v/v) and at 12 min to 10:90 (v/v). The re-equilibration of the column was performed during the following 2 min (mobile phase: 50:50 methanol–water).

2.4. Sample preparation

The nanoparticles (size of about 200 nm) were prepared using the method previously established for pegylation of PVM/MA nanoparticles [14]. Thus, 100 mg of PVM/MA copolymer and a variable amount of PEG (5–25 mg) were dissolved and stirred in acetone (5 ml) for 1 h. After their incubation, 10 ml of a hydroalcoholic mixture (1:1, v/v) was added to the organic phase. The solvents were eliminated under reduced pressure (Buchi R-144, Switzerland) and nanoparticles were purified by twice centrifugation at 17,000 rpm for 20 min (Sigma 3K30, Germany) and finally lyophilized (Genesis 12EL, Virtis, USA).

For HPLC quantitation, supernatants recovered during the purification step were diluted to 10 ml in water and stored at −20 °C until analysis. A 1 ml aliquot of the supernatants were transferred to auto sampler vials, capped and placed on the HPLC auto sampler. A 20 μl aliquot of the supernatant was injected onto the HPLC column.

2.5. Quantitation

The peak area ratio between the corresponding PEG or PVM/MA versus the corresponding analytes concentration was plotted for this purpose. For PEG 6000 and PVM/MA, calibration curves were determined by polynomial regression analysis. For PEG 2000, a potential relationship between PEG concentra-

tion and ELSD response was observed. For polyethyleneglycols, the following concentrations were selected: 0.075, 0.1, 0.15, 0.17, 0.2, 0.22, 0.25, 0.3, 0.5, 0.6 and 0.75 mg PEGs/ml. In the case of Gantrez, the calibration curve ranged from 0.25 to 1 mg PVM/MA/ml.

2.6. Validation

The method was fully validated by analysis of calibrators prepared at different concentrations. In supernatants, for PVM/MA, the range between 0.25 and 1 mg PVM/MA/ml was analysed. For both PEGs, the range between 0.075 and 0.75 mg/ml was studied. The quality control samples were prepared as a single batch on the same day at each concentration. The precision, accuracy and reproducibility were also determined. The precision was expressed as the coefficient of variation (CV). Within-day variability was determined by measuring five replicate measurements at four concentration levels for Gantrez (0.25, 0.3, 0.6, and 0.8 mg/ml) and at three concentration levels for polyethylene glycols (0.2, 0.5 and 0.7 mg/ml for PEG 2000 and 0.1, 0.3 and 0.5 mg/ml for PEG 6000). Similarly, between-day variability, on three different days, was determined by repeated analysis of four quality control samples with the same nominal concentration value. Accuracy was determined according to the following equation:

$$\text{difference from theoretical value}\,(\%) = \frac{X - C_T}{C_T} \times 100 \qquad (1)$$

where X is the estimated concentration of the analysed molecule and C_T is the theoretical concentration. To be acceptable, all the differences should be lower than 15%.

The limit of detection (LOD) was defined as the lowest concentration of analytes able to be clearly detected and can be calculated as three times the variation in the measured response (signal/noise ratio = 3). The limit of quantitation (LOQ) was defined as the lowest drug concentration quantifiable and was estimated as 10 times the variation in the measured response (signal/noise ratio = 10). In this work, LOD and LOQ were determined by serial dilution of sample preparations containing PEGs and Gantrez.

2.7. Application of the method

This analytical HPLC method using ELSD was applied to determine the PEGs and PVM/MA content in nanoparticles. The amount of PEGs associated to nanoparticles was calculated as the difference between the initial PEGs and the amount of PEGs recovered in the supernatants. Similarly, the amount of PVM/MA was estimated by the difference in the same way. The associated PEG was expressed in μg PEG/mg NP and calculated as follows:

$$\text{PEG content}\,(\mu g/mg) = \frac{\text{amount of PEG in nanoparticles}\,(\mu g)}{\text{PVM/MA nanoparticle yield}\,(mg)} \qquad (2)$$

where the nanoparticle yield was the addition of PEG and Gantrez amounts determined by HPLC.

For comparisons, the amounts of PEGs associated to the nanoparticles were also determined by nuclear magnetic resonance method (^{1}H NMR) (Brucer Avance 400, Germany) as described previously [14]. Thus, exactly weighted amounts of the PEGylated nanoparticles (5 mg) were dissolved in deuterated DMSO (0.45 ml) and the spectra were obtained at $n_s = 19{,}200$. ^{1}H NMR spectra of free PEGs were performed using the same ratio and experimental conditions. The quantity of PEG-attached to the nanoparticles was calculated by the ratio between peak areas of the protons of ethylene units (3.51 ppm) detected in the spectra of PEGylated nanoparticles and in the spectra of free PEGs, respectively.

3. Results

3.1. LC-ELSD method validation

Under the chromatographic conditions described previously, the different PEGs and Gantrez were well resolved within 14 min. The retention time for Gantrez was 4.48 ± 0.06, 7.71 ± 0.01 min for PEG 2000 and 6.92 ± 0.02 min for PEG 6000.

The selectivity of the assay was studied by the analysis of supernatants of PVM/MA nanoparticles. Under these chromatographic conditions, no interferences were observed and the resolution of the peaks was satisfactory. For Gantrez, this value

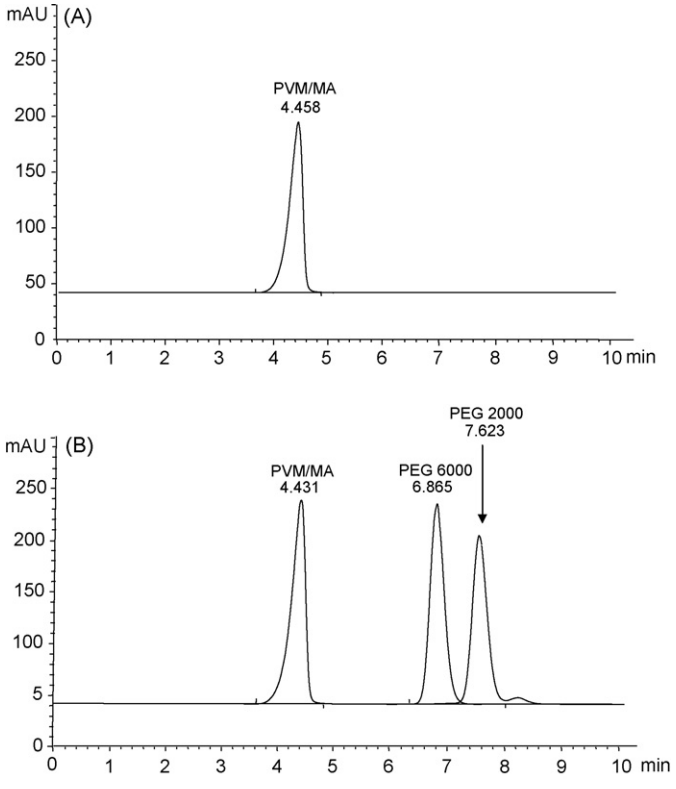

Fig. 1. Chromatogram obtained from the analysis of a PVM/MA solution in acetonitrile at 0.6 mg/ml (A) and a mixture of PEG 2000 and PEG 6000 at the same concentration (B).

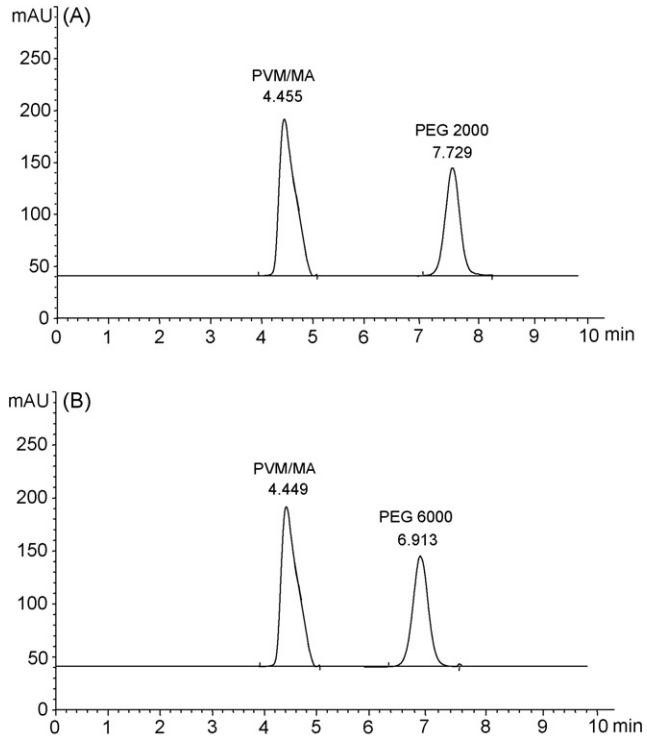

Fig. 2. Chromatograms resulting from the analysis of supernatants obtained during the purification step of the preparation process of PEG 2000: Gantrez nanoparticles (A) and PEG 6000: Gantrez nanoparticles (B). PVM/MA: poly(methyl vinyl ether-*co*-maleic anhydride); PEG 2000: polyethyleneglycol 2000; PEG 6000: polyethyleneglycol.

was calculated to be 2.020 ± 0.736. For PEGs, the resolution of peaks was found to be as follows: 2.535 ± 0.374 for PEG 2000 and 2.637 ± 0.383 for PEG 6000. Figs. 1 and 2 show some chromatograms of this study.

The symmetry of the peaks was acceptable in both Gantrez and PEGs. For PEGs, the values were always near 1 (0.905 ± 0.176 for PEG 2000 and 0.887 ± 0.112 for PEG 6000). In the case of Gantrez, the symmetry value of the peak was lower (0.535 ± 2.79) because of the small tail that can be observed at the end of the peak.

3.1.1. Polyethylene glycols

3.1.1.1. Sensitivity of the assay. Detection (LOD) and quantification (LOQ) limits of the HPLC assay were found to be 0.001 and 0.075 mg/ml, respectively, determined by the analysis of the peak baseline noise in six blank samples.

3.1.1.2. Linearity of the assay. As a first approach, linearity was studied by plotting a standard curve from the ratios between peaks areas of each PEG. Finally, linearity was determined by the peaks areas of PEG 2000 and 6000 versus the corresponding PEGs concentration. A relationship between chromatographic response and PEG concentration was observed on three different days over the range 0.075–0.75 mg/ml (see Table 1). In all cases, polynomial regression for PEG 6000 and potential regression for PEG 2000, displayed correlation coefficients greater than 0.999 and 0.992, respectively. In addition, relative error in each concentration was calculated in the mean curve and did not exceed 8% in all cases. The absence of a linear response when ELSD is used appears to be a characteristic of this detector [28].

Table 1
Standard curves for polyethylene glycols in acetonitrile

	N	Regression equation	*r*
PEG 2000			
Day 1	10	$y = 5537.3x^{1.5625}$	0.9924
Day 2	10	$y = 7838.4x^{1.7187}$	0.9983
Day 3	10	$y = 5900.9x^{1.7872}$	0.997
PEG 6000			
Day 1	10	$y = 6137.2x^2 + 517.06x + 8.3982$	0.9996
Day 2	10	$y = 5966.5x^2 + 600.87x + 17.519$	0.9995
Day 3	10	$y = 7647.7x^2 + 1060.1x - 9.072$	0.9998

3.1.1.3. Accuracy of the assay. Accuracy values during the same day (intra-day assay) at low, medium and high concentrations of both PEGs were always within the acceptable limits (−11.50 and 10.61%) at all concentrations tested. Table 2 summarizes these results.

3.1.1.4. Precision of the method. To calculate the precision of the method, "within-day" and "between-day" test were performed. These data clearly indicate that the assay method was reproducible within the same day. From these results (Table 3), it also appears that the analytical method was reproducible between different days.

3.1.2. PVM/MA

In the same way as PEGs, the assay performance of PVM/MA was also assessed.

3.1.2.1. Sensitivity of the assay. The LOD of PVM/MA, determined as three times the variation in measured response (S/N = 3), was calculated to be 0.0002 mg/ml and the estimated LOQ was calculated to be as low as 0.25 mg/ml (S/N = 10).

3.1.2.2. Linearity of the assay. The assay exhibited linearity between the response (y) and the corresponding concentration of Gantrez (x), over the range 0.25–1 mg/ml. A polynomial regression displayed correlation coefficient greater than 0.998 (Table 4).

Table 2
Polyethyleneglycol analysis: accuracy of the method, expressed as relative error in percent

	Concentration added (mg/ml)	Concentration found (mean ± S.D.; mg/ml)	Relative error (%)
PEG 2000	0.2	0.21 ± 0.007	6.66
	0.5	0.55 ± 0.026	10.61
	0.7	0.72 ± 0.059	3.66
PEG 6000	0.1	0.08 ± 0.011	−11.50
	0.3	0.28 ± 0.018	−5.42
	0.5	0.49 ± 0.014	−1.21

Table 3
Polyethyleneglycol analysis: between- and within-day variability of the HPLC method

	Concentration added (mg/ml)	Concentration found (mean ± S.D.; mg/ml)	
		Between-day variability ($n=5$) (mean ± S.D.; CV)	Within-day variability ($n=5$) (mean ± S.D.; CV)
PEG 2000	0.2	0.18 ± 0.023 (21.23)	0.21 ± 0.007 (5.84)
	0.5	0.45 ± 0.037 (13.59)	0.55 ± 0.026 (7.47)
	0.7	0.61 ± 0.033 (9.06)	0.72 ± 0.059 (13.28)
PEG 6000	0.1	0.09 ± 0.004 (11.97)	0.08 ± 0.011 (2.78)
	0.3	0.26 ± 0.022 (12.68)	0.28 ± 0.018 (6.25)
	0.5	0.45 ± 0.026 (8.40)	0.49 ± 0.014 (9.79)

Table 4
Standard curve for PVM/MA in acetonitrile

	N	Regression equation	r
Day 1	8	$y = 3777.6x^2 + 702.72x + 160.77$	0.9984
Day 2	8	$y = 1634.9x^2 + 2012.7x - 85.582$	0.9984
Day 3	8	$y = 446.59x^2 + 3843.1x + 360.65$	0.998

Table 5
PVM/MA analysis: accuracy of the method, expressed as relative error in percent

Concentration added (mg/ml)	Concentration found (mean ± S.D.; mg/ml)	Relative error (%)
0.25	0.21 ± 0.008	−12.18
0.3	0.31 ± 0.022	6.09
0.6	0.68 ± 0.028	14.81
0.8	0.80 ± 0.028	0.42

3.1.2.3. Accuracy and precision of the method. Accuracy values calculated as the percentage difference between the expected and measured concentrations were within −12.18 and 14.81% (see Table 5). The results for intra-assay variability and between-day precision for our samples are summarized in Table 6. These data clearly indicate that the assay method is reproducible within the same day and between different days.

3.2. Application of the method

The described method has been used for the quantification of Gantrez and polyethyleneglycol in the nanoparticles. This method is also useful to estimate the yield of the fabrication process of the nanoparticles, which was found to be 78.51 ± 2.97, similar to that obtained by gravimetry [14]. On the other hand, the influence of the PEG-bulk concentration on the pegylation of nanoparticles was also studied. Fig. 3 summarizes these results.

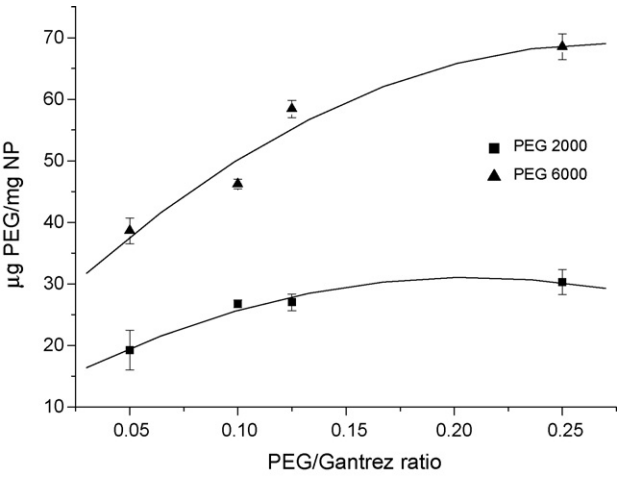

Fig. 3. Influence of the PEG-bulk concentration (expressed as PEG/Gantrez ratio) on the pegylation of the nanoparticles (in µg PEG/mg NP). Data shows mean ± standard deviation ($n=3$).

As it can be observed, for both types of PEGs, the pegylation degree (associated PEG to nanoparticles) increased by increasing the amount of PEG incubated with the copolymer before the formation of nanoparticles. However, at a PEG/Gantrez ratio higher than 0.125, a plateau was reached. Under these conditions, the amount of PEG 6000 associated to nanoparticles was found to be 68.52 ± 2.09. For PEG 2000, this value was 30.28 ± 2.03.

Finally, Fig. 4 shows the comparison of pegylation data obtained by HPLC and by ^{1}H NMR. For both types of PEGs, a good relationship between both techniques was found. For PEG 2000 nanoparticles, the correlation coefficient between both techniques was $r > 0.991$. For PEG 6000 nanoparticles, r was 0.949.

Table 6
PVM/MA analysis: between- and within-day variability of the HPLC method

Concentration added (mg/ml)	Concentration found (mean ± S.D.; mg/ml)	
	Between-day variability ($n=5$) (mean ± S.D.; CV)	Within-day variability ($n=5$) (mean ± S.D.; CV)
0.25	0.23 ± 0.013 (7.43)	0.21 ± 0.008 (3.47)
0.3	0.29 ± 0.020 (8.89)	0.31 ± 0.022 (7.95)
0.6	0.60 ± 0.020 (5.56)	0.68 ± 0.028 (6.53)
0.8	0.74 ± 0.029 (6.46)	0.80 ± 0.028 (5.97)

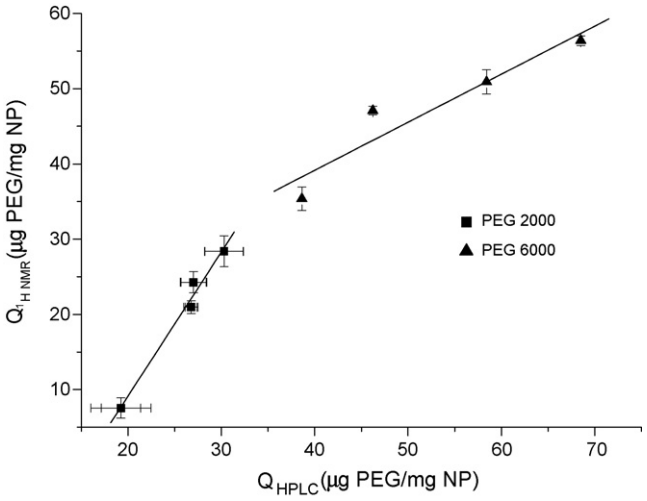

Fig. 4. Comparison between values of pegylation obtained by HPLC and ^{1}H NMR.

4. Discussion

The main objective of our research was to develop an analytical method to permit the simultaneous quantitation of polyethylene glycols and Gantrez on a new nanosized pharmaceutical formulation. Gantrez is a copolymer of poly(methyl vinyl ether-*co*-maleic anhydride) characterized by its ability to easily react in aqueous medium with molecules containing –NH_2 or –OH residues [17]. This fact permits the development of new nanoparticulate systems with different physico-chemical or biological properties. PVM/MA nanoparticles coated with PEG combine the properties of polyethylene glycols to prolong the residence time of the drug at the site of absorption with the ability of PVM/MA nanoparticles to modify drug release and bioadhesion in specific regions of the gastrointestinal tract. In this context, the association of PEGs and Gantrez nanoparticles provides a good strategy in order to increase the loading capacity of unstable and polar drugs with low membrane permeability and control their release from these pharmaceutical devices.

The amount of PEG associated to Gantrez can be directly quantified by ^{1}H NMR after lyophilization of PEGylated nanoparticles. However, during the preparation process of these nanoparticles, this technique cannot be applied and no information is available in order to verify the correct and adequate synthesis of these particles. In this context, we have developed a routine and rapid technique of PEGs and Gantrez in the supernatants obtained during the purification step of the preparative process to both check the adequate formation of these drug delivery systems and calculate the amount of PEG associated to nanoparticles and their yield.

4.1. Optimization of the chromatographic system

In this work, different packing materials were tested for the separation of PEGs by HPLC. Normal-phase chromatography for PEG characterization is performed on bare silica and on the so-called bonded phases [29]. However, bare silica columns usually offer poor reproducibility and peak asymmetries. Other strategies for PEG analysis involved the use of cyanopropyl columns [16] or ion-exchange columns [20]. In the former, the association of this column with a sodium perchlorate–acetonitrile gradient has been proposed for the chromatographic separation of amino-derivatives of PEGs [16]; although, the analysis of mixtures between different PEGs remains difficult [11]. In the latter, the presence of buffered phases or ion-pair agents (associated with ion-exchange columns) can induce the aggregation and the precipitation of polymers or macromolecules employed in the preparation of the nanoparticles [21]. Moreover, although PEG compounds without ionizing groups do not interact with the matrix of this column and their retention is expected to be independent of different salt concentrations, ELSD detector does not allow the use of any kind of buffered mobile phase.

As a first approach, a C8 and C18 columns were chosen, which were previously proposed for the resolution of low molecular weight PEGs in blood and urine [30,31]. However, chromatographic peaks displayed a poor resolution and did not show acceptable chromatographic separation between peaks. Finally, a size exclusion column (PL aquagel-OH 30,8 μm column; 300 mm × 7.5 mm) was chosen which gave optimum chromatographic separation of peaks with appropriate resolution at acceptable elution times for PEGs. The use of this column enabled us to determine PEGs and Gantrez in small sample volumes without the need of buffered mobile phases. This functionality and versatility enables the analysis of most neutral hydrophilic polymers, such as PEGs. In addition, the excellent stability of packing materials of this column allows the eluent to be modifying, while retaining the high column efficiency.

ELSD detector is based on the nebulisation of the column effluent to form an aerosol, followed by solvent evaporation in a heated drift tube, and then detection of the remaining non-volatile solute particles in the light scattering cell. Taking this in account, the mayor instrumental parameters affecting the signal response are the nebuliser-gas pressure and drift tube temperature. Concerning the nebuliser-gas pressure, droplets size is dependent on the gas flow rate. In general, large droplets are formed at low gas pressure, which results in spikes and noisy signals [28]. Therefore, larger droplets scatter more light and increase the sensitivity of the analysis. Thus, the use of a high gradient slope has been proved to be an advantage resulting in sharp peaks, and, in consequence, contributing to increase sensitivity [3]. In addition, the optimum nebuliser-gas pressure in this case was set at 3 bar. With respect to the drift tube temperature, the range of 87.5–112.5 °C was analysed and 110 °C was found to be the best temperature to obtain a good chromatographic response.

These findings demonstrate that this HPLC method is quite enough precise to separate and identify the two different peaks of PEGs and Gantrez found in chromatogram. Related to this, no interferences were observed, the symmetry of the peaks was acceptable and the resolution was satisfactory. In addition, the retention time of both Gantrez and PEGs were well resolved within 14 min. Calibration curves, polynomial regression for PEG 6000 and potential regression for PEG 2000, displayed correlation coefficients greater than 0.999 and 0.992,

respectively. Gantrez displayed a polynomial regression with correlation coefficient greater than 0.998. The symmetry of the peaks was also acceptable with values close to one and its resolution was also satisfactory, been higher than 2. Accuracy values during the same day at low, medium and high concentrations of PVM/MA and both PEGs were always within the acceptable limits and the results for intra-assay variability and between-day precision for our samples clearly indicate that the assay method is reproducible within the same day and between different days.

4.2. Application of the method

The reported method has been used for the determination of PEG content in PVM/MA nanoparticles, however, it can also be used to estimate the yield of the preparative process. The amount of polyethyleneglycol associated to the nanoparticles was determined by this HPLC method as well as by a Nuclear Magnetic Resonance Method (^{1}H NMR). Ideally, the same results would have been found by both techniques and, therefore, the ratios (pegylation by HPLC/pegylation by ^{1}H NMR) would have been equal to 1. These experimental ratios were found to be 0.94 for PEG 2000 and 0.83 for PEG 6000. So, from these results, it is possible to conclude that the data obtained by HPLC and ^{1}H NMR are similar.

Concerning the pegylation process, it was found that the amount of polyethyleneglycol associated to the nanoparticles significantly increased when the molecular weight of the polymer increased and by increasing the PEG/Gantrez ratio.

In conclusion, the HPLC method described in this study can be an interesting and appropriate tool for the routine analysis of PEGylated nanoparticles due to the simplicity of the technique, short time analysis and high sensitivity, accuracy and precision.

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