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Determination of oligonucleotide ISIS 2922 in nanoparticulate delivery systems by capillary zone electrophoresis

A. Arnedo[a], M.A. Campanero[b], S. Espuelas[a], M.J. Renedo[a], J.M. Irache[a,*]

[a]*Centro Galénico, Departamento de Farmacia y Tecnología Farmacéutica, Universidad de Navarra, 31008 Pamplona, Spain*
[b]*Servicio de Farmacología Clínica, Clínica Universitaria de Navarra, Universidad de Navarra, 31008 Pamplona, Spain*

Abstract

ISIS 2922 is an antisense oligonucleotide with antiviral activity against cytomegalovirus. However, its rapid degradation in biological fluids and its low capacity for diffusion across cell membranes limit its therapeutical use. One possibility to overcome these drawbacks consists of using nanoparticles as drug carriers. The aim of this study was to develop an analytical method for determining the amount of ISIS 2922 loaded into albumin nanoparticles. For this purpose, capillary zone electrophoresis (CZE) was performed on a fused-silica capillary filled with borate buffer (12.5 m*M*, pH 9.5). Paracetamol was used as an internal standard and a diode-array detection system was set at 270 nm. Under these conditions, the limit of quantitation of ISIS 2922 was 1.27 μg and the precision and accuracy of the method did not exceed 7%. Moreover, the use of paracetamol as internal standard and the quantification by means of a 'corrected area' procedure enabled us to reduce the peak variability and accurately determine the amount of oligonucleotide loaded in the albumin nanoparticles. In summary, this assay is a selective and sensitive CZE method for the accurate quantitation of ISIS 2922 oligonucleotide in albumin nanoparticles.

Keywords: Nanoparticles; ISIS 2922; Oligonucleotides; Albumin

1. Introduction

Antisense oligonucleotides are a new generation of drugs designed to hybridize to specific mRNAs, thereby inhibiting protein expression. Once hybridized to their RNA target, oligonucleotides can act by physical blockage of the site preventing the access and binding of various factors such as ribosomes [1,2]. In addition, the heteroduplex formed by the oligonucleotide and its target can be recognized by Rnase H and degraded [3]. This therapeutical approach provides a level of selectivity not available with traditional drugs and has been proved effective in a number of animal models [4]. Nowadays, different oligonucleotides are currently being evaluated as anticancer [5] and antiviral [6] agents. However, therapy with antisense oligonucleotides faces several problems [7], such as their rapid degradation in biological fluids by nucleases and their low capacity for passive diffusion across cell membranes.

A possible strategy to circumvent both stability and permeability problems is the use of nanoparticles as carriers for these molecules. Indeed, these colloidal drug carriers may protect oligonucleotides from the external medium and directly deliver these relatively large and hydrophilic molecules of a single DNA or RNA strand, into cells.

*Corresponding author. Tel.: +34-94-842-5600; fax: +34-94-842-5649.
E-mail address: jmirache@unav.es (J.M. Irache)

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ISIS 2922 is a phosphorothioate oligonucleotide, 21 nucleotide units in length, which has shown antiviral activity against cytomegalovirus via inhibition of the major immediate early gene [8]. This drug has a different molecular target than currently available therapies and, therefore, can be used to treat infections caused by resistant strains [9].

In this context, we have recently designed and prepared new ISIS 2922 pharmaceutical carriers based on albumin nanoparticles in order to minimise the drug sensitivity to nuclease degradation and to increase its cell uptake by modifying their biodistribution in the body. Then, the aim of this work was to develop a sensitive, specific and precise analytical method to determine the amount of this antisense oligonucleotide loaded into albumin nanoparticles.

A number of different studies have been performed for the analysis of oligonucleotides. In these studies, oligonucleotides were determined by liquid chromatography or electrophoresis. On the one hand, a number of new packing materials, such as stationary phases for HPLC, have been recently developed and tested for improving both the assay sensitivity and the separation and quantification of these drugs [10–12]. However, these chromatographic methods are based on the use of gradient elution techniques and require large volume samples.

Similarly, electrophoretic separations in polyacrylamide or agarose gels have several drawbacks such as poor resolution and sensitivity [13]. More recently, capillary electrophoresis (CE) has also been applied to the separation and quantification of oligonucleotides. In this context, capillary zone electrophoresis (CZE) [14,15] and capillary gel electrophoresis (CGE) [16–18] have recently gained popularity as a viable alternative to polyacrylamide gel electrophoresis for the separation of these drugs. Although CGE is the most suitable technique for the separation and accurate quantitation of oligonucleotides [19], it remains a time-consuming and troublesome task to optimise the separation conditions. Moreover, the performance of the gel filled capillaries decreases over time, and their lifetime is very limited [20].

Finally, CZE has been used for the separation of oligonucleotides [14,15]. However, due to recent advances in the performance of CE instruments, it is reasonable to extend this latter technique to the quantification of oligonucleotides.

2. Experimental

2.1. Chemicals and reagents

Oligonucleotide ISIS 2922 (5′-GCGTTTGCTCTT-CTTCTTGCG-3′) was provided by Pharmacia Biotech (Cambridge, UK). High-purity water (Milli-Q, Millipore) was used for sample and buffer preparation. Buffers were composed of sodium tetraborate decahydrate (Borax) purchased from Panreac (Barcelona, Spain) and sodium hydroxide from Sigma (Madrid, Spain). Albumin fraction V from bovine serum, methanol and chlorhydric acid were from Merck (Darmstadt, Germany), glutaraldehyde was from Sigma, and ethanol absolute was from Prolabo (Fontenay, France).

2.2. Solutions

Buffer borate (12.5 m*M*, pH 9.5) was prepared by dissolving 4.76 g borax in 1000 ml high-purity water. Then, the pH of this solution was adjusted to 9.50 by a freshly prepared 0.1 *M* sodium hydroxide solution. The resulting buffer was filtered through a 0.22-μm pore size filter.

A stock solution of ISIS 2922 (1.02 mg/ml) was prepared by dissolving 1.02 mg oligonucleotide in double-distilled water. Working-standard solutions were prepared in water from the stock solution. The amount of ISIS 2922 for the standard curve samples ranged between 1.27 and 152.5 μg.

Paracetamol was used as the internal standard (I.S.). For this purpose, 50 mg paracetamol was dissolved in 100 ml pure water to obtain a stock solution of 0.5 mg/ml. The working standard solution of paracetamol was prepared by diluting the first stock solution in water ten times (0.05 mg/ml). Then, standard curve samples were prepared by adding 25 μl of the I.S. working standard solution to 1 ml of the ISIS 2922 working standard samples.

All the aforementioned solutions were stored at 4°C.

2.3. Bovine serum albumin nanoparticles — sample preparation

ISIS 2922-loaded albumin nanoparticles were prepared by means of a coacervation technique and chemical cross-linking with glutaraldehyde. Briefly,

the different nanoparticle batches were prepared by the incubation of an aqueous albumin solution (2% w/v) with a variable amount of oligonucleotide. Then, the pH of the solution was adjusted to 5.5 with 1 *M* HCl prior to the dropwise addition of ethanol and the resulting coacervates were hardened with glutaraldehyde for 2 h. After ethanol elimination by evaporation under reduced pressure, ISIS 2922-loaded albumin nanoparticles were purified by centrifugation at 17 000 rpm for 10 min (Rotor SS-34, Sorvall RC5-plus). Finally, the recovered supernatants were spiked with I.S. and the resulting samples were assayed for ISIS 2922 content.

2.4. Instrumentation and electrophoretic conditions

HPCE separations were carried out by using a HPCE apparatus (Hewlett-Packard, Waldbronn, Germany) with a diode-array detection system set at 270 nm (reference 325 nm).

A fused-silica capillary of 48.5 cm (40 cm to the detector)×50 μm I.D., filled with buffer, was used for the separations. It was conditioned by rinsing with 1 *M* sodium hydroxide for 5 min. Then, it was cleaned with a 0.1 *M* sodium hydroxide solution and, finally, the capillary was filled with borate buffer. A potential of 30 kV was applied and the capillary temperature was 30°C. Before each analysis, the capillary was rinsed with borate buffer for 15 min. For analysis, samples were diluted to 1 ml with double-distilled water and injected by pressure at 50 mbar for 10 s.

2.5. Calibration procedures

It is widely known that oligonucleotide CZE quantification is greatly influenced by a number of factors such as the migration velocity and the injection volume [19]. In order to minimise these problems and to perform accurate drug quantifications, use of the 'normalized area' procedure has been proposed which is based on the following equation [19]

$$\mathrm{NA} = \frac{A_1 t_{M1}}{A_2 t_{M2}} \quad (1)$$

where NA represents the oligonucleotide 'normalized area', A_1 and A_2 are the peak areas for the drug and internal standard, respectively, and t_{M1} and t_{M2} are the ISIS 2922 and paracetamol migration times, respectively.

In our case, the quantification of ISIS 2922 was carried out by a 'corrected area' procedure in order to minimise both the effect of sample matrix characteristics and the variability resulting from the electrokinetic injection. For this purpose, the following mathematical equation was used

$$\mathrm{CA} = \frac{A_1}{A_2} \quad (2)$$

where CA represents the ISIS 2922 'corrected' area, and A_1 and A_2 are the oligonucleotide and paracetamol peak areas, respectively.

2.6. Application of the method

This HPCE method was applied to the determination of the ISIS 2922 (ISIS) content in new pharmaceutical dosage forms based on the use of albumin nanoparticles. For this purpose, different ISIS 2922-loaded albumin nanoparticle batches were prepared to study the influence of the oligonucleotide initial concentration on the drug content in the albumin nanoparticles. Therefore, the drug loading was calculated as follows

$$\text{Drug loading} = \frac{\text{amount of ISIS in nanoparticles (μg)}}{\text{albumin nanoparticles yield (mg)}} \cdot 100$$

The amount of ISIS 2922 in nanoparticles was determined as the difference between the initial added drug and the amount recovered in the supernatants after sample centrifugation.

On the other hand, the yield was determined by digesting the albumin nanoparticles with 1 *M* NaOH at room temperature for 24 h. The absorbance of this solution was then measured in a spectrophotometer at 280 nm (diode-array HP 8452, Hewlett-Packard, Germany) and compared with the absorbance value obtained after digestion of a control albumin solution.

3. Results and discussion

3.1. Optimisation of HPCE conditions

For the optimisation of the HPCE method, the

influence of the buffer physicochemical characteristics on the resolution and selectivity of ISIS 2922 separation was elucidated. In this context, the influence of the borate buffer concentration on the resolution of ISIS 2922 was studied in the range 0.125–25 m*M*. The results obtained clearly show that a decrease in the buffer concentration induced an increase in the electroosmotic flow and, therefore, the analysis time was shorter (data not shown). However, it was observed that the current value fell from 50 to 3 μA when the buffer concentration decreased from 25 to 1.25 m*M* (data not shown). These experimental conditions did not provide good analytical conditions for the ISIS separation. Therefore, the ideal buffer concentration for adequate buffering capacity, minimising solute adsorption on the capillary wall and short analysis time was calculated to be 12.5 m*M*.

The influence of pH conditions on the ISIS 2922 electrophoretic mobility was also studied. For this purpose, borate buffers with pH values ranging from 10.4 to 8.8 were prepared. It is interesting to note that a decrease in the pH value increased the oligonucleotide migration time and broke the electrophoretic peak symmetry (data not shown). Therefore, the sensitivity of the method decreased. In conclusion, the electrolyte chosen for ISIS 2922 separations was 12.5 m*M* buffer, pH 9.5.

The oligonucleotide ISIS 2922 exhibited one absorption peak in the UV–visible spectrum at 254 nm. However, at this wavelength, albumin also showed a high spectrophotometric response producing wide electrophoretic signals, which interfered with the ISIS 2922 peak. This problem could be solved by performing the analysis detection at 270 nm. On the other hand, we tried to improve the technique sensitivity using an extended light path capillary (‘bubble cell’), because it is well known that a higher absorption can be obtained by increasing the pathlength [21]. The ‘bubble cell’ capillaries enabled us to improve the method sensitivity 3–5 times over that of standard capillaries.

3.2. Use of internal standard

A number of electrophoretic conditions have a significant impact on the migration time and the detector response of the analyte signals in CZE [15]. These conditions include temperature, sample matrix effects and electrokinetic injection effects, which may be dealt with adequately by the use of a suitable I.S. in combination with strict control of the experimental conditions. Moreover, it is known that, in CE, suitable I.S.s should have a similar mobility to the analytes to avoid changes on the electrosmotic flow (EOF) during the run [21]. In this work, the suitability of different substances was evaluated such as sodium diclofenac, metrochlopramide, ketorolac trometamine, benzocaine, tenoxicam and paracetamol. The best results were obtained with paracetamol, a molecule with similar electrophoretic mobility and light-absortion properties to those of ISIS 2922.

3.3. Validation

General guidelines for the validation of CE methods are already proposed and include linearity, accuracy, precision (repeatibility and reproducibility), limit of detection (LOD), limit of quantitation (LOQ) and robustness [21].

3.3.1. Migration time

The analytical peaks of ISIS 2922 and paracetamol were well resolved with migration times of 2.85 ± 0.043 and 1.57 ± 0.011 min, respectively. They were also well resolved from other components used during the preparation of the nanoparticles.

3.3.2. Selectivity of the assay

The selectivity of the assay was determined by the individual analysis of blank samples containing bovine serum albumin (BSA) and glutaraldehyde, with and without I.S. Under these chromatographic conditions, no sources of interference were observed and the resolution between ISIS 2922 and paracetamol was satisfactory (Fig. 1).

3.3.3. Sensitivity of the assay

Detection and quantification limits of the CZE method were determined by the analysis of the peak baseline noise in ten blank samples. LOD, determined as three times the variation in the measured response, was calculated to be 0.38 μg. Similarly the LOQ, estimated as ten times the variation in the measured response, was calculated to be 1.27 μg. This LOQ was confirmed, in separate experiment,

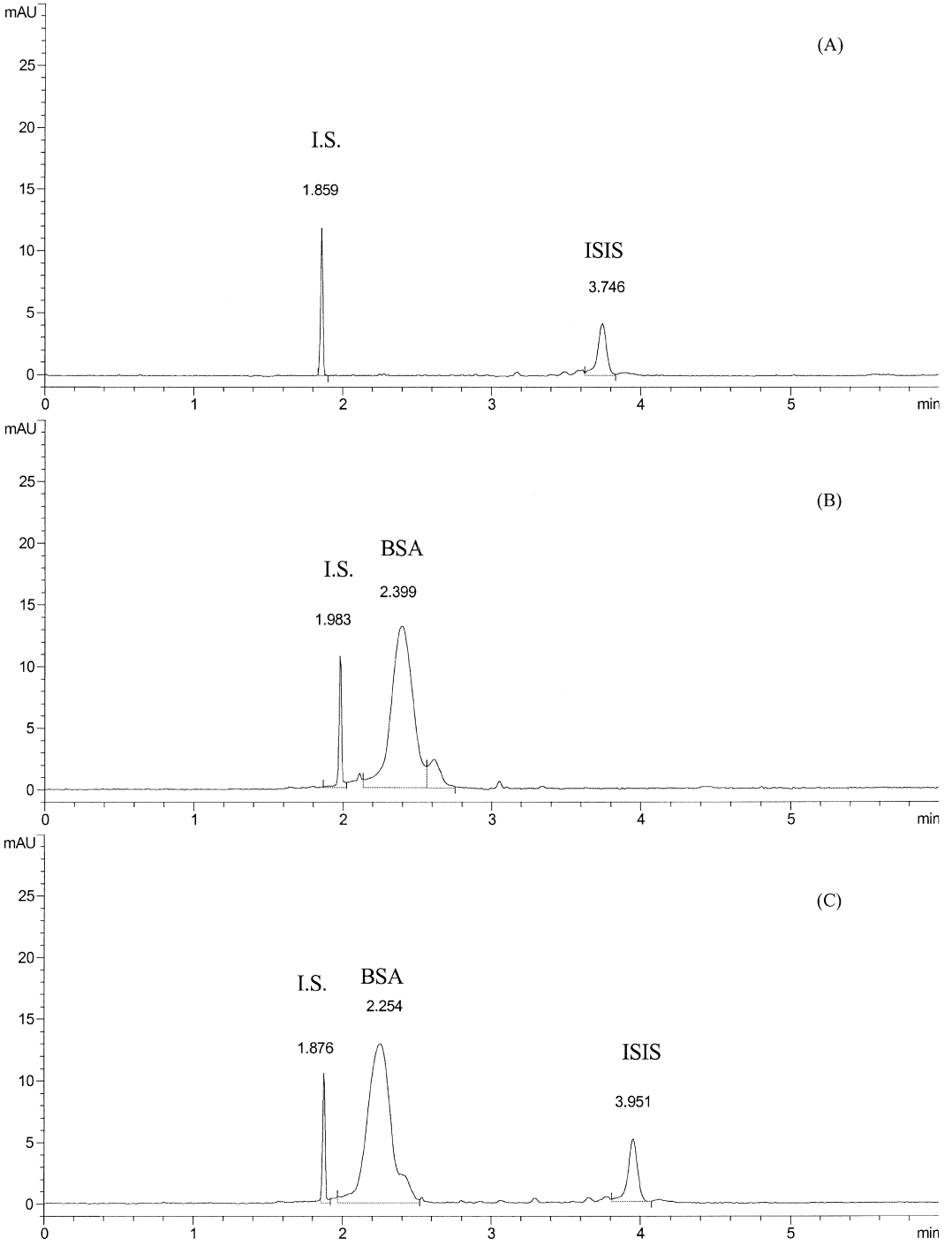

Fig. 1. Electropherograms resulting from the analysis in water of (A) a standard solution (11.07 μg ISIS), (B) a supernatant obtained during the purification step of unloaded nanoparticles and (C) a supernatant obtained during the purification step of ISIS 2922 loaded-albumin nanoparticles. I.S., paracetamol, BSA, bovine serum albumin.

Table 1
Comparison of different quantification procedures by determining the predicted values (expressed as the mean±SD, $n=6$) for different ISIS 2922 standards of the calibration curves; procedure I: uncorrected area (ISIS area), procedure II: normalized area, procedure III: corrected area

Quantity added (μg)	Uncorrected area		Normalized area		Corrected area	
	Quantity predicted (μg)	RSD[a] (%)	Quantity predicted (μg)	RSD[a] (%)	Quantity predicted (μg)	RSD[a] (%)
1.27	1.27±0.16	0.21	1.27±0.14	0.03	1.27±0.15	0.03
60.99	59.82±1.20	1.92	57.69±1.05	5.42	57.43±1.52	5.83
152.49	154.06±3.89	1.01	159.23±4.50	4.42	159.82±5.00	4.80

[a] RSD, relative standard deviation (expresed as absolute value).

using calibrators with 1.27 μg of ISIS oligonucleotide. Finally, the mean assay result was 1.39 μg ($n=6$), with a variation coefficient of <5%.

3.3.4. Linearity of the assay

Calibration curves were determined by least squares linear regression analysis (weighting $1/X^2$). Linearity was determined by plotting a standard curve from the ratio between ISIS 2922 to the paracetamol peak areas versus the corresponding drug amount in water. Standard curves were found to be linear on three different days over the range 1.27–152.49 μg (typical equation: $y=0.166x-0.0152$). Furthermore, linear regression analysis showed correlation coefficients greater than 0.997 in all cases.

The influence of different calibration procedures on the oligonucleotide ISIS 2922 quantitation by CE was examined. Table 1 summarises the predicted quantity, and RSD values, of calculated standard points determined by the three following procedures: (i) procedure I based on the use of ISIS peak areas (uncorrected areas); (ii) procedure II based on the use of normalized areas (Eq. (1)) and, (iii) procedure III based on the determination of corrected areas (Eq. (2)). It is interesting to note that the three different procedures enabled us to obtain similar results (Table 1).

3.3.5. Accuracy

The accuracy of the assay was defined as the percentage of the systematic error, which was calculated as the agreement between the measured and the true values. In principle, measures should be within ±10% at all amount levels to be acceptable [22].

Table 2 shows that the calculated accuracy values, in intra-day variation studies at low, medium and high oligonucleotide amounts in samples, were always within the acceptable limits.

3.3.6. Precision of the method

The precision of the method was tested by determining both the within- and the between-day variabilities. These assay parameters were determined by repeated analysis of quality control samples at low, medium and high amounts of oligonucleotide on the same day (within-day variability) and on different days (between-day). Moreover, precision was expressed as the RSD (%) from the replicate measurements. In principle, this RSD value should be within a range of ±10% at all concentrations to be acceptable [22]. The results are

Table 2
Accuracy of the HPCE method, expressed as the relative error, for the ISIS 2922 quantification

Quantity added (μg)	Quantity found (mean±SD) (μg)	Accuracy (%) ($n=5$)
30.50	30.43±0.68	−0.22
101.66	99.71±0.68	−1.91
152.49	163.04±2.92	6.92

Table 3
Between- and within-day variability of the HPCE method for determining ISIS 2922

Quantity added (μg)	Between-day variability ($n=15$)		Within-day variability ($n=5$)	
	Quantity found (mean±SD) (μg)	RSD (%)	Quantity found (mean±SD) (μg)	RSD (%)
30.498	30.46±0.74	2.44	30.43±0.68	2.24
101.66	101.01±2.02	2.00	99.71±1.53	1.53
152.49	160.08±3.91	2.44	163.04±2.92	1.79

summarised in Table 3. From these data, it is clear that the assay method was reproducible within the same day and during different days, using identical (i) buffer conditions, (ii) capillary and (iii) sample preparation.

3.4. Stability of ISIS 2922

ISIS 2922 appeared to be stable when stored as freeze-dried powders at −20°C. On the other hand, this oligonucleotide was less stable in solution, although its standard solutions can be successfully stored at −20°C for several months. Similarly, ISIS 2922 concentrations in processed samples were stable when left at room temperature (20±3°C) for 24 h.

3.5. Ruggedness

Ruggedness has been evaluated between 3 months using different capillary batches and sample mediums.

In order to evaluate the influence of sample composition on oligonucleotide quantification, a number of working-standard solutions were prepared in phosphate buffer (154 m*M*, pH 7.4) prior injection in the CZE apparatus. In our case, as is shown in Fig. 2, effective mobilities decreased when samples were prepared in phosphate buffer. These modifications altered a number of electrophoretic parameters such as symmetry (1.49±0.14 in water and 1.76±0.35 in phospate buffer) and resolution (8.9±0.51 in water and 4.3±2.2 in phospate buffer), which obviously influenced the method selectivity and linearity. These changes clearly confirm that the ionic strength and the pH conditions play an important role in the ISIS 2922 quantification.

In addition, the different calibration functions obtained during 3 months have been compared (Table 4). The results obtained showed a great variability. Relatively small differences in analytical conditions (buffer concentration, pH) lead to dramatic differences in the oligonucleotide quantitation by CZE. Consequently, it appears to be necessary to perform a daily recalibration in order to achieve a correct quantification. In this way, it is possible to avoid changes in the electrophoretic parameters due to small variations in the buffer physicochemical characteristics.

The influence of the different calibration procedures on the method accuracy was also evaluated. The predicted values with each calibration function and the RSD values of calibration samples (evaluated between 3 months using different capillaries and sample mediums), with the uncorrected, normalized or corrected area procedures were compared. These results are reported in Table 5. In this case, the data calculated with the uncorrected area procedure clearly shown a high variability (Table 5). Nevertheless, no significant differences were found when compared the RSD values obtained with the normalized and the corrected area procedures ($P<0.05$). Thus, the drug quantification based on the determination of the relative relation between the oligonucleotide and the I.S. electrophoretic responses may attenuate the method's irreproducibility in the same way as other calibration procedures (i.e.: normalized area).

3.6. Application of methods

The reported method was used for the determination of the ISIS 2922 content of albumin nanoparticles. For this purpose, the drug loading was calculated by means of Eq. (1) and plotted against the oligonucleotide initial amount added to the albumin solution for preparing the nanoparticulate

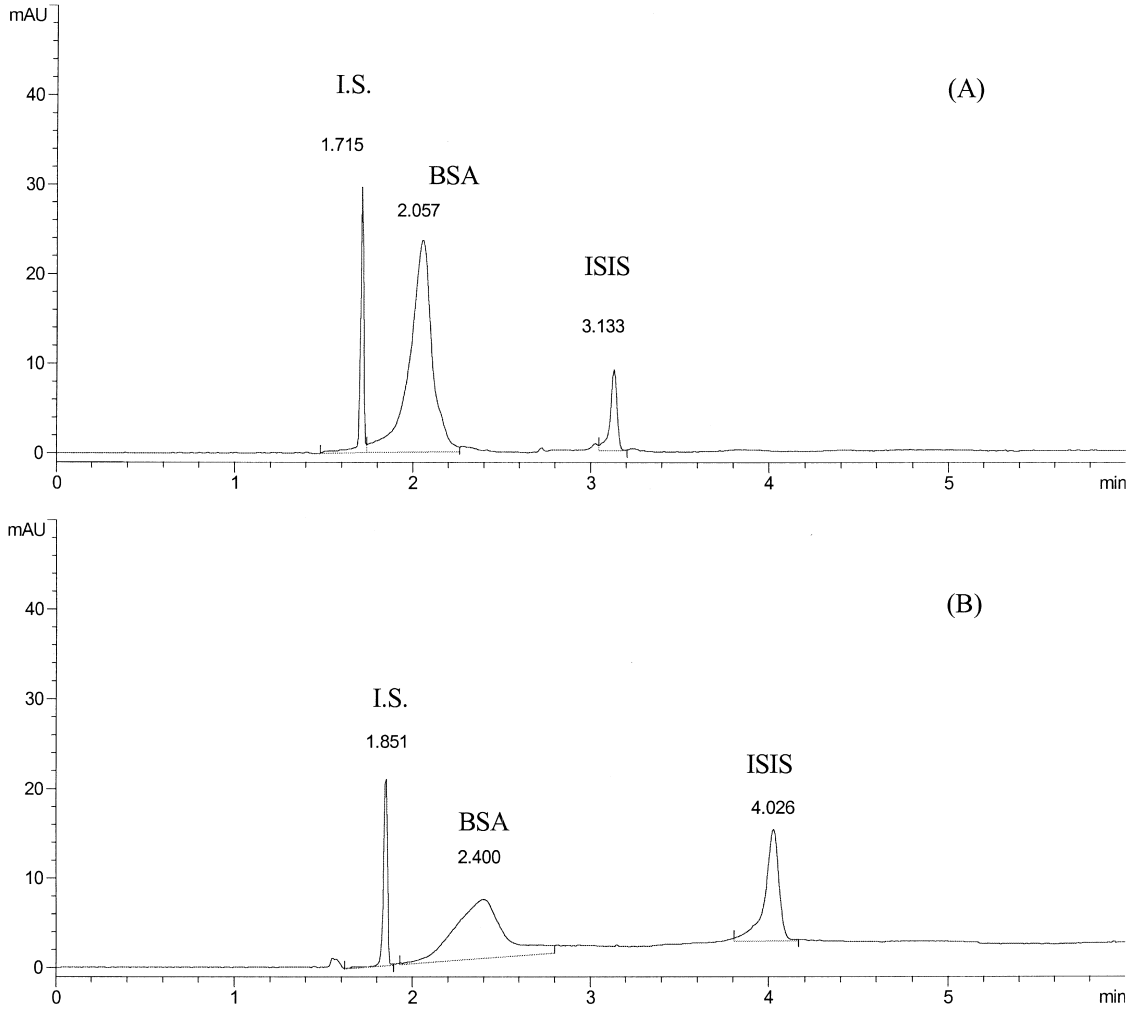

Fig. 2. Influence of the sample composition on the oligonucleotide quantification. Electropherograms obtained from samples either in water (A) or in PBS (B), obtained during the purification step of ISIS 2922 loaded-albumin nanoparticles. I.S., paracetamol, BSA, bovine serum albumin.

carriers (see Experimental). The results are reported in Fig. 3. It is interesting to note that the coacervation technique used here for preparing ISIS 2922-loading albumin nanoparticles, enabled us to obtain an oligonucleotide entrapment efficiency into the nanoparticles of about 40%.

Table 4
Calibration curve reproducibility during 3 months; ISIS 2922 quantifications were performed by means of the 'corrected area' procedure

Month	Slope	Intercept
1	−0.0967	0.3900
2	−0.0625	−0.0654
3	−0.0595	−0.1780

4. Conclusion

In summary, the CZE method reported here is a selective, sensitive, cheap and rapid method for the separation and accurate determination of an antisense oligonucleotide such as ISIS 2922. Moreover, the use of an internal standard (i.e., paracetamol) and quanti-

Table 5
Comparison of different quantification procedures by determining the predicted values (expressed as the mean±SD, $n=3$) for different ISIS 2922 standards of the dairy calibration curves. Procedure I: uncorrected area (ISIS area); Procedure II: normalized area; Procedure III: Corrected area

Day	Quantity added (μg)	Uncorrected area		Normalized area		Corrected area	
		Quantity predicted (μg)	RSD[a] (%)	Quantity predicted (μg)	RSD[a] (%)	Quantity predicted (μg)	RSD[a] (%)
1	25.45	23.65±0.87	7	25.43±0.21	2	24.66±0.27	3
2	22.70	22.70±4.07	0	22.70±0.37	0	22.70±0.21	0
3	22.70	22.70±0.18	0	22.70±0.84	0	22.70±0.39	0
1	50.90	52.55±1.36	3	51.38±0.18	0	51.62±0.01	1
2	45.40	45.40±1.64	0	45.40±2.33	0	45.40±2.78	0
3	45.40	45.40±2.88	0	45.39±0.50	0	45.40±0.39	0

[a] RSD, relative standard deviation (expressed as absolute value).

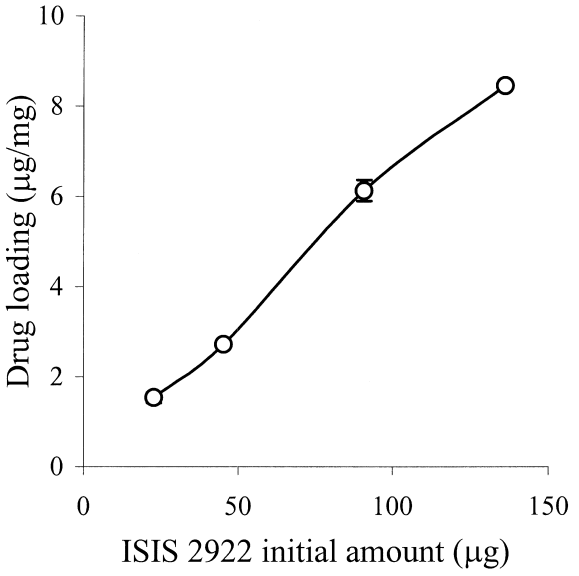

Fig. 3. Application of the method: influence of the ISIS 2922 initial amount on its drug loading (μg drug/mg nanoparticles).

fication by means of the use of a 'corrected area' procedure enabled us to reduce the peak variability. Finally, this technique can be successfully applied to the calculation of the ISIS 2922 loaded into albumin nanoparticles.

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