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VALIDATION OF A SPECTROPHOTOMETRIC METHOD TO ESTIMATE THE ADSORPTION ON NANOEMULSIONS OF AN ANTIMALARIAL OLIGONUCLEOTIDE

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This study describes the validation of a spectrophotometric method to estimate oligonucleotides association with cationic nanoemulsions. Phosphodiester and phosphorothioate oligonucleotides targeting *Plasmodium falciparum* topoisomerase II were analyzed at 262 nm. Linear response ($r > 0.998$) was observed from 0.4 to 1.0 nmol/mL, the relative standard deviation values for the intra- and inter-days precision were lower than 2.6% and the recovery ranged from 98.8 to 103.6% for both oligonucleotides. The association efficiency was estimated based on an ultrafiltration/centrifugation method. Oligonucleotides recovery through 30 kDa-membranes was higher than 92%. The extent of oligonucleotides association (42 to 98%) varied with the composition of nanoemulsions.

Keywords: antimalarial oligonucleotides; nanoemulsions; UV analysis.

INTRODUCTION

Malaria is a disease that affects approximately 300 million people every year. Over the last 2 decades, the situation of malaria has become worse due to the resistance of parasites (gender *Plasmodium*) to the main antimalarial drugs, as well as that of mosquitoes to the insecticides.[1]

The use of oligonucleotides has been considered a new strategy for the treatment of malaria given the fact that these single strand nucleic acids can interfere in protein synthesis, thus inhibiting parasite growth.[2,3] However, the use of oligonucleotides is hampered *in vivo* due to their very low penetration ability[4,5] and susceptibility against nuclease degradation.[6,7] In addition to chemical modifications, oligonucleotides association with positively-charged colloidal carriers has been extensively investigated to circumvent such drawbacks.[8]

For this reason, cationic oil-in-water (o/w) nanoemulsions have been investigated.[9-13] Nucleic acids interact spontaneously with the oppositely charged cationic lipids to form polyionic complexes. Such complexes are able to increase the oligonucleotides cell uptake and protect them against nuclease degradation.[10,11] Recently, the cationic lipid composition of nanoemulsions has been optimized in order to obtain the best conditions for the adsorption and release of a model oligonucleotide (oligothymidilate) from emulsions. Both electrostatic and hydrophobic interactions were found to play important roles in those complexes.[13]

However, the estimation of oligonucleotides association rate with colloidal carriers, such as o/w cationic nanoemulsions, is difficult due to the physical obstacles associated with the small size of the oil droplets.[14,15] In most cases, drug association efficiency is estimated indirectly, by calculating the difference between the amount of the drug added and the amount of free drug found in the external phase of colloidal carriers, obtained after separation of part of the external phase (water phase) on ultrafiltration membranes, using centrifugation or pressure.[16,17]

The design of intravenous nanoemulsions for antimalarial oligonucleotides delivery is currently under study by our research group. In the present study, we describe the validation of a spectrophotometric method, aimed at estimating the oligonucleotides association efficiency with cationic nanoemulsions, using an ultrafiltration/centrifugation procedure. Phosphodiester and chemically modified phosphorothioate oligonucleotides sequences targeting *P. falciparum* topoisomerase II have been previously described in the literature.[2,3]

EXPERIMENTAL

Chemical and reagents

Medium-chain triglycerides and egg lecithin (Lipoid E-80®) were kindly donated by Lipoid GmbH, Germany. The cationic lipid dioleoyl trimethylammonium propane (DOTAP) was obtained from Sigma (USA), oleylamine from Aldrich (USA), and glycerol from Merck (Brazil). Oligonucleotides were purchased from Invitrogen Brazil Ltda. The antisense sequence (5' ATG TAA TAT TCT TTT GAA CCA TAC GAT TCT 3') was obtained as phosphodiester and phosphorothioate oligonucleotides. Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). Ethanol was obtained from Quimex (Brazil).

Equipment

The UV apparatus consists of a UV-Vis scanning spectrophotometer, model Hewlett-Packard 8452A, with the software HP Chemstation for data processing. For the ultrafiltration/centrifugation process, an Eppendorf centrifuge 5417 R was used (Eppendorf, Germany).

Method validation

The method used in the present work was validated according to the "International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use" (ICH)[18] parameters required for assaying dosage forms: specificity, linearity, accuracy, and precision (repeatability and intermediate precision).

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The specificity of the method was assessed by comparing the absorption spectra of a water solution of oligonucleotides in comparison with a solution containing glycerol at 2.25%. This solution was prepared so as to reproduce the ultrafiltrate of the nanoemulsions (water phase) after the ultrafiltration/centrifugation process used to estimate the oligonucleotides association with cationic nanoemulsions.

To assess the linearity of the method, oligonucleotide solutions were analyzed in 3 different days. Each day, 7 concentrations within the range of 0.4 to 1.0 nmol/mL were prepared in triplicate and determined three times in the spectrophotometer. The detection limit and the quantification limit were also calculated, based on the deviation of the response and the slope, using the linearity curve data.

The precision of the UV method was analyzed in two different ways. First, precision was evaluated by analyzing the linearity data. The repeatability (intra-day precision) and intermediate precision (inter-day precision) were evaluated according to the relative standard deviation of the analysis on the same day and on the 3 different days, respectively. After that, 6 independent samples at 100% of the concentration (0.7 nmol/mL) of oligonucleotides in glycerol solution were prepared on three different days. The absorbance was determined 3 times for each sample and a mean was calculated. The relative standard deviation was calculated for the intra-day and inter-day precision.

The accuracy was evaluated by means of the recovery method, using 3 concentration levels (low, medium, and high) prepared in triplicate and determined 3 times, corresponding to 0.5, 0.7, and 0.9 nmol/mL. Water solution containing 2.25% of glycerol were spiked with known amounts of oligonucleotides and analyzed. The results are expressed as the mean of recovery for three independent samples.

The recovery of the oligonucleotides throughout the ultrafiltration membranes was also tested. Solutions (0.5 to 20 nmol/mL) were ultrafiltrated (10 mins at 5,000 × g through a porous membrane, 30 kDa Ultrafree Millipore, USA) and the concentration was determined. Results were expressed as oligonucleotides recovery (%).

Preparation of nanoemulsions

Nanoemulsions were obtained through the spontaneous emulsification procedure, as previously described.[12,13] The organic phase contained lipid components and ethanol as a solvent. This solution was injected into the aqueous phase (glycerol 2.25%) under magnetic stirring for 15 min. Subsequently, the organic solvent was removed by evaporation under reduced pressure at 50 ^{o}C. The final composition of emulsions (%, w/w) was medium chain triglycerides 8.0, egg-lecithin 2.0, glycerol 2.25, either oleylamine 0.05 (oleylamine-emulsion) or DOTAP 0.132 (DOTAP-emulsion), and MilliQ water up to 100. The cationic lipid concentration in the formulations corresponds to 2 mM of oleylamine or DOTAP cationic lipids. A control emulsion obtained in the absence of cationic lipids (lecithin-emulsion) was also prepared.

The physicochemical properties of formulations used for the oligonucleotides association study were previously characterized using a Malvern Zetasizer Nano ZS (Malvern Instrument, UK). Nanoemulsions exhibit a mean droplet size of approximately 220-250 nm, showing a unimodal distribution (polydispersity index < 0.2). The ζ-potential of oleylamine and DOTAP emulsions were approximately +55 mV, whereas lecithin formulation presents a negative value of -23 mV. These results are in agreement with previously reported data for formulations obtained at a similar quali-quantitative composition and under the same emulsification conditions.[13]

Oligonucleotides association efficiency

Oligonucleotides association efficiency was performed at the end of the manufacturing process. Water solutions at the concentration of 5 nmol/mL were combined with nanoemulsions and incubated for 30 min. Free oligonucleotides (in the external water phase) were determined in the clear ultrafiltrate, obtained following a 10 min centrifugation (at 5,000 × g through a porous membrane 30 kDa Ultrafree Millipore, USA). The amount of oligonucleotides was determined by specrophotometry at 262 nm. Their association efficiency could be obtained as follows:

$$\text{Association efficiency (\%)} = [(ON_{added}-ON_{free})/ON_{added}] \times 100$$

where, ON_{added} = amount of oligonucleotide added (nmol), and ON_{free} = amount of free oligonucleotide determined in the ultrafiltrate (nmol).

RESULTS AND DISCUSSION

Figure 1a shows the UV spectra of oligonucleotides water solutions. As can be seen, phosphodiester and phosphorothioate oligonucleotides exhibit typical nucleic acid UV spectra, with a maximum absorption wavelength found at 262 nm. This absorption occurs because of the conjugated system in their nitrogenated bases. Considering that phosphorothioate differs from phosphodiester only as regards the modification in the phosphodiester linkage, where non-bonding phosphate oxygen is replaced by sulphur, the conjugated system and the extinction coefficient remained unmodified. Thus, absorption spectra obtained for both oligonucleotides were quite similar, which is in accordance with previous literature.[19]

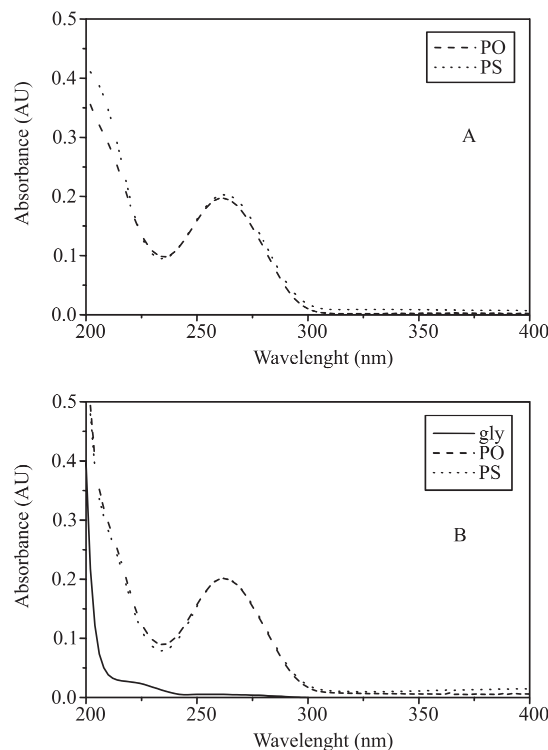

Figure 1. *UV spectra of phosphodiester and phosphorothioate oligonucleotides in water (A) or in glycerol solution at 2.25% (B). Key: Phosphodiester-PO (dash), Phosphorothioate-PS (dot), and control glycerol solution (straight)*

To assess whether excipients could interfere in the determination of the oligonucleotides, the first validation procedure was aimed to determine the specificity of the method. This parameter was evaluated using a water solution containing glycerol 2.25% (external phase of nanoemulsions) due to the fact that oligonucleotides were determined

in the presence of this excipient after having performed the ultrafiltration/centrifugation procedure. No interference of glycerol was noticed since no significant changes in the oligonucleotides spectra were detected in the wavelengths tested, as shown in Figure 1b.

The linearity of the method (Table 1) was satisfactory over the concentration range of 0.4-1.0 nmol/mL and the linear equations were similar for phosphodiester and phosphorothioate oligonucleotides ($p < 0.05$), with a correlation coefficient higher than 0.998. Least square regression equations were highly significant for the method ($p < 0.05$). The confidence intervals for the intercept included zero, which confirms the absence of a constant systematic error. From linearity data, the detection and the quantification limit were also calculated based on the standard deviation of the response and slopes of the calibration curves. The results remained quite similar for both oligonucleotides.

Table 1. Linearity data, detection limit and quantification limit of UV assay for oligonucleotides

	Phosphodiester	Phosphorothioate
Concentration range (nmol/mL)	0.4 - 1.0	0.4 - 1.0
Equation	y = 0.2794x -0.0032	y = 0.2767x -0.0053
R	0.9993	0.9981
Intercept	[-0.007 to +0.001]	[-0.011 to +0.001]
Detection Limit (nmol/mL)	0.0278	0.0233
Quantification Limit (nmol/mL)	0.0927	0.0706

Considering 3 days with three individual experiments each one.

The precision of the method was first assessed considering repeatability (intra-day analysis) and intermediate precision (inter-day analysis) from linearity data (Table 2).

The intra-day and inter-day precision presented relative standard deviation values which were lower than 2.60% (intra-day) and 1.34% (inter-day) for phosphodiester oligonucleotides and lower than 2.42% (intra-day) and 2.38% (inter-day) for phosphorothioate oligonucleotides. Concerning intra-day and inter-day experiments, performed in glycerol solutions (Table 3), the relative standard deviation values found were lower than 2.03 and 1.40% for phosphodiester and phosphorothioate oligonucleotides, respectively. Based on these results (Tables 2 and 3), the analytical methodology was considered precise.

The results of accuracy were expressed as recovery of oligonucleotides (%) added to the glycerol solution (2.25%). The recovery for the three concentration levels remained comprised between 98.8 and 100.3% for phosphodiester oligonucleotides, and between 99.5 and 103.6% for phosphorothioate oligonucleotides, indicating a good agreement between the amount added and the amount experimentally found (Table 4).

Before estimating the oligonucleotides association efficiency, the membrane recovery was evaluated to verify whether any adsorption or interaction mechanisms between oligonucleotides and the ultrafiltration units had occurred[20] (Figure 2). Irrespective of oligonucleotides concentration (0.5 to 20 nmol/mL) and type (phosphodiester or phosphorothioate), the recovery proved to be higher than 92%. Such results showed a limited binding of oligonucleotides across the regenerated cellulose membranes, presenting a molecular weight cut-off of 30 kDa. Therefore, it can be assumed that free oligonucleotides were allowed to pass through the pores into the filtrate.

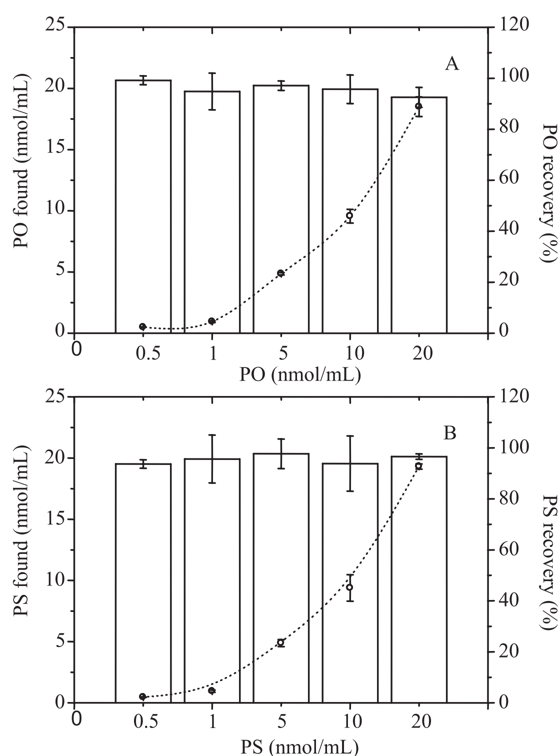

Figure 2. Membrane recovery of phosphodiester (A) and phosphorothioate (B) oligonucleotides. Key: Phosphodiester-PO and phosphorothioate-PS oligonucleotides found (symbols) and recovered (bars) on 30 kD cut off membranes

Table 2. Intra-day and inter-day precision of UV assay of oligonucleotides in water solution

Concentration (nmol/mL)	Phosphodiester					Phosphorothioate				
	Absorbance Day 1	Absorbance Day 2	Absorbance Day 3	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Absorbance Day 1	Absorbance Day 2	Absorbance Day 3	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)
0.4	0.111	0.108	0.110	1.39 - 2.60	1.16	0.107	0.109	0.112	1.07 - 2.42	2.02
0.5	0.136	0.137	0.137	0.59 - 1.78	0.49	0.134	0.131	0.130	0.78 - 1.34	1.61
0.6	0.165	0.163	0.166	1.23 - 1.86	0.82	0.158	0.160	0.158	0.37 - 1.63	0.68
0.7	0.193	0.188	0.192	0.44 - 1.82	1.34	0.190	0.190	0.183	0.87 - 1.51	2.20
0.8	0.223	0.220	0.221	0.30 - 1.15	0.79	0.219	0.214	0.209	0.74 - 1.70	2.38
0.9	0.243	0.246	0.248	0.89 - 1.98	1.02	0.244	0.242	0.246	0.42 - 1.64	0.70
1.0	0.277	0.280	0.279	0.75 - 1.03	0.57	0.276	0.277	0.271	0.24 - 1.29	1.05

Range of relative standard deviation (R.S.D.) considering 3 days of validation.

Table 3. Intra-day and inter-day precision of UV assay of oligonucleotides in glycerol solution

Days of Analysis	Phosphodiester		Phosphorothioate	
	Mean concentration determined	R.S.D. (%)	Mean concentration determined	R.S.D. (%)
Day 1	0.703	2.03	0.706	1.40
Day 2	0.698	1.80	0.702	1.21
Day 3	0.687	1.39	0.702	1.07
Inter-day	0.696	1.16	0.703	0.34

Range of relative standard deviation (R.S.D.) considering three individual experiments and 3 days of analysis.

Table 4. Accuracy of UV assay for oligonucleotides

Oligonucleotide added (nmol/mL)	Phosphodiester		Phosphorothioate	
	Found (nmol/mL)	Recovered (%)	Found (nmol/mL)	Recovered (%)
0.50	0.50	100.3	0.52	103.6
0.70	0.69	99.0	0.70	99.5
0.90	0.89	98.8	0.90	99.8

The values represent the mean of three individual experiments.

Table 5. Association efficiency of oligonucleotides to nanoemulsions

Formulation	Association efficiency (%)	
	Phosphodiester	Phosphorothioate
Lecithin	41.6±2.0	44.0±1.9
Oleylamine	74.6±2.4	78.3±3.9
DOTAP	90.6±13.3	97.8±3.2

Phosphodiester and phosphorothioate oligonucleotides inicial concentration of 5 nmol/mL.

The present method was also used to estimate the association of oligonucleotides with nanoemulsions, using the conditions described above. The data (Table 5) clearly show that oligonucleotides association efficiency with lecithin-emulsion (control) was significantly lower ($p < 0.05$) than with oleylamine- and DOTAP-emulsions, regardless of the type of oligonucleotide. As expected, because of their positive charge, oleylamine and DOTAP allow to engage electrostatic interactions between oligonucleotides and the oil droplets, which have favored the association.[9-11,13] In addition, no significant difference ($p > 0.05$) between phosphodiester and phosphorothioate oligonucleotides association efficiency was found, for a given formulation. However, despite the negative surface charge (related to the presence of negatively-charged phospholipids of egg-lecithin), oligonucleotides were able to associate with lecithin-nanoemulsions. Considering that phospholipids located at the emulsion interface are zwitterionic molecules, it can be suggested that due to the existence of positive charges in their polar head group, they are able to attract nucleic acids, even if the global surface charge is negative.[9,10]

CONCLUSIONS

In conclusion, the proposed spectrophotometric method can be used to determine either phosphodiester or phosphorothioate oligonucleotides in the water phase of nanoemulsions, aimed at estimating their association efficiency. The method was found to be linear, specific, precise, and accurate. The results remained quite similar for both forms of oligonucleotides.

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