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Supercoiled plasmid quality assessment by analytical arginine-affinity chromatography

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ABSTRACT

Supercoiled plasmids are an important component of gene-based delivery vehicles, applied in new therapeutic strategies such as gene therapy or DNA vaccination. However, aiming at the general distribution of plasmid DNA (pDNA) therapeutics requires a procedure to easily and efficiently assess the purity and recovery yield of the supercoiled (sc) plasmid isoform. Based on affinity interactions between amino acids and nucleic acids, an arginine affinity methodology with UV detection was established to quantify and to control the quality of sc plasmid biopharmaceuticals. The fact that this new technique allows to distinguish between plasmid isoforms represents an advantage, since it allows the selective quantification of the biologically active pDNA topology, and a more accurate analysis of the quality of the isolated plasmid. The analytical experiments were performed in 12 min and the method was found to be accurate, precise, reproducible and linear for a sc plasmid concentration range between 2 and 150 μg/mL. In comparison with other established methods used in the quantification of native pDNA (oc + sc), the main advance introduced by this new method is the possibility to quantify the sc plasmid in a sample containing other plasmid topologies, ensuring the purity of plasmid products to be therapeutically applied.

1. Introduction

The clinical application of plasmid DNA (pDNA) is progressing and the commercial availability for immunotherapeutic and gene therapy purposes is approaching reality. Plasmid DNA is considered a new generation biotechnology product, and several clinical trials are currently in progress using these vectors to treat or to prevent severe pathologies including infectious diseases or cancer [1,2].

The production and application of pDNA usually involves several stages, starting with the design of the vector to ensure safety, efficacy and cost-efficient production. After completing the vector construction, the transformation of a bacterial cell, typically *Escherichia coli*, is performed with the plasmid encoding the gene of interest. The production stage is then developed by fermentation of the host cells in a bioreactor to achieve high yields of pDNA [3]. The stage of isolation and purification of pDNA consists in the disruption of *E. coli* cells to recover the pDNA, which is then purified using a suitable methodology [4]. Finally, the plasmid product is formulated and delivered to the eukaryotic cells to express the protein of interest.

In general, all the unit operations involved in the global process are adjusted in a way that pDNA can accomplish the properties to express the encoded information, preferably at the right place, time, and level [5]. On the other hand, the global process selected has to be safe and productive as recommended by the regulatory agencies [4]. In fact, the quality criteria of the final plasmid product focus on supercoiled percentage (>98%) and impurity levels, since these are the critical parameters influencing the safety and efficacy of cell transfection and protein expression [6].

The plasmids produced and purified from *E. coli* are mainly recovered in the supercoiled (sc) form, although the manipulation can induce some single- or double-stranded nicks producing the open circular (oc) or linear (ln) plasmid forms, respectively. For this reason, and considering the clinical application of pDNA, the pharmaceutical development of a functional, stable and sterile product for clinical trials depends heavily on the availability of an assay for the quantification and purity determination of active plasmid products [7]. Due to the particular interest in the bio-active plasmid conformation, it is crucial that this analytical methodology distinguishes between the main plasmid conformations, the sc and oc isoforms.

Several chromatographic techniques have been described to purify plasmids at a preparative level, but few analytical methods have been established. Supercoiled content in samples is typically assessed using agarose gel electrophoresis, gel staining and subsequent photography and densitometric scanning. Capillary electrophoresis and various chromatographic techniques can also be used for this purpose [8]. However, most of these methods are

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time consuming and labor intensive. Although the electrochemical methods can also provide an alternative, they are also restricted to laboratories equipped with voltammetric and chronopotentiometric analyzers [9]. With regard to the chromatographic methods, the hydrophobic interaction chromatography allied to an HPLC system was already applied to quantify total pDNA in different samples [10] and to separate plasmid isoforms [11]. The anion-exchange chromatography was also used by different authors to quantify or to determine the purity of plasmid samples [12] and, more recently, an anion-exchange LC–UV analytical method has been validated [7].

Although affinity chromatography presents high specificity and it has been used in other analytical methods, namely for protein quantification, its application for pDNA assessment has never been reported. Actually, the application of affinity chromatography can take advantage of the binding specificity and efficiency of pDNA or impurities to specific immobilized ligands [13,14]. In previous works, we have reported the implementation of a new affinity methodology to purify pDNA, at preparative scale, by using amino acids as specific ligands [14]. This method was developed based on the fact that biologically there are many different interactions occurring between proteins and nucleic acids, which mainly involve basic amino acids such as histidine or arginine [15]. In addition, several atomic studies have described preferential interactions between particular amino acids and nucleotide bases, and also the frequency at which they occur [16,17]. A particular chromatographic technique involving the arginine amino acid as immobilized ligand showed high efficiency to isolate sc and oc pDNA isoforms [18], using mild binding and elution conditions. Biorecognition of sc pDNA was verified [19], explained by the ability of arginine to interact in different conformations, by the length of its side chain and by the ability to produce good hydrogen bonding geometries [16].

Thus, the present work aims at developing a new analytical method, efficient on pDNA quantification and purity evaluation, by exploiting the specific interaction occurring between pDNA and the arginine ligands. The validation of the analytical method must be performed according to international guidelines [20]. In fact, the establishment of a new analytical method, able to monitor a biotechnological process in terms of controlling the sc pDNA quality, purity and yield, must guarantee the simplicity, reproducibility, linearity, accuracy and precision of the results.

2. Materials and methods

2.1. Materials

Arginine-Sepharose 4B gel and the Tricorn Empty High-Performance Column (5/20) were purchased from GE Healthcare (Uppsala, Sweden). All salts used were of analytical grade.

2.2. Plasmid and bacterial growth conditions

The analytical method to quantify pDNA was developed using the 6.05-kbp plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA). The plasmid was produced by fermentation of *Escherichia coli* DH5α after transformation. Growth was carried out at 37 °C in a shake flask with 250 mL of Terrific Broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with 30 μg/mL kanamycin. The cell growth was suspended at late log phase ($OD_{600} \approx 9$) and the cells were recovered by centrifugation and stored at −20 °C.

2.3. Lysis and primary isolation

Cells were lysed using a modification of the alkaline method [21] as previously described [22]. The recovered bacterial pellets were resuspended in 20 mL of 50 mM glucose, 25 mM Tris–Cl, 10 mM ethylene-diamine tetraacetic acid (EDTA), pH 8.0. Lysis was performed by adding 20 mL of a 200 mM NaOH, 1% (w/v) sodium dodecylsulfate solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated with 16 mL of pre-chilled 3 M potassium acetate, pH 5.0. The precipitate was removed by centrifuging twice at 20,000 × *g* for 30 min at 4 °C. The supernatant was concentrated by addition of 0.7 volumes of isopropanol and incubation on ice for 30 min. The pDNA was recovered by centrifugation at 16,000 × *g* for 30 min at 4 °C. The pellets were then re-dissolved in 2 mL of 10 mM Tris–Cl buffer, pH 8.0. Next, ammonium sulfate was dissolved in the pDNA solution up to a final concentration of 2.5 M, followed by 15-min incubation on ice. Precipitated proteins and RNA were removed by centrifugation at 16,000 × *g* for 20 min at 4 °C. The supernatant was desalted by passing through PD-10 desalting columns (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions, using 10 mM Tris–Cl buffer, pH 8.0 as the elution buffer.

2.4. Preparative chromatography to isolate sc and oc pDNA

The preparative chromatography was performed in a fast protein liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden). A 16 × 100-mm (about 20-mL) column was packed with the commercial arginine–Sepharose 4B gel. This support is characterized by the manufacturer as a cross-linked 4% beaded agarose matrix with a 12-carbon atom spacer and an extent of labeling between 14 and 20 μmol/mL. The experiments were performed at 5 °C, using a circulating water bath to maintain the adequate temperature. The column was equilibrated with 240 mM NaCl in 10 mM Tris buffer, pH 8.0 at a flow rate of 1.5 mL/min. Partially purified pDNA obtained after desalinization was loaded onto the column, using a 500 μL loop at the same flow rate. The absorbance of the eluate was continuously monitored at 280 nm. After elution of unbound species with 240 mM NaCl in 10 mM Tris buffer, pH 8.0, the ionic strength of the buffer was increased stepwise to 300 mM NaCl in 10 mM Tris buffer, pH 8.0. Fractions were pooled according to the chromatograms obtained and kept for further analysis as described bellow. After chromatographic runs, the column and FPLC system were cleaned with 3 volumes of 0.5 M NaOH.

2.5. Agarose gel electrophoresis

The identification of the biomolecules recovered from the preparative chromatography was performed by agarose gel electrophoresis. Briefly, the pooled fractions were analyzed by horizontal electrophoresis using 15 cm 0.8% agarose gels (Hoefer, San Francisco, USA), stained with ethidium bromide (0.5 μg/mL). Electrophoresis was carried out at 60 V with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gels were visualized in a Vilber Lourmat system (ILC Lda, Lisbon, Portugal).

2.6. Analytical chromatography

Analytical chromatography was performed in an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden). The Tricorn Empty High-Performance Column (5/20) was packed with the commercial arginine-Sepharose 4B gel, completing a bed volume of 0.5 mL. Briefly, the system was prepared with a 200 mM NaCl in Tris–HCl 10 mM buffer pH 8.0 in the pump A, and 500 mM in Tris–HCl 10 mM buffer pH 8.0 in the pump B. The column was initially equilibrated with 15% of buffer B. Supercoiled plasmid sam-

ples recovered from the preparative chromatography (20 μL) were injected and eluted at a flow rate of 0.6 mL/min. After injection, the elution occurred with 15% of buffer B for 0.7 column volumes, and then the elution buffer was instantaneously changed to 100% of buffer B. This elution condition was maintained for 1.5 column volumes. Next, the column was re-equilibrated for 10 min with the equilibration buffer in order to prepare the column for the next run. The absorbance of the eluate at 254 nm was continuously recorded.

3. Results and discussion

According to the FDA guidelines, the quantification of pDNA and the analysis of its purity must be evaluated using suitable analytical methods [23], and the high performance chromatographic techniques are usually applied to ensure the plasmid quality considering the clinical application. In this work, the two key plasmid isoforms (oc and sc) were first isolated by arginine-affinity chromatography at preparative scale, in accordance to what was previously described [18]. Afterwards, an analytical method was developed also using the arginine-affinity matrix. The development of the method was based on the binding and elution conditions already described for this affinity chromatographic matrix. Briefly, a NaCl concentration of approximately 200 mM can be used to recover the oc isoform and by increasing the ionic strength to 300 mM the bound sc isoform is recovered [18].

The accurate validation of an analytical procedure involves the control of several characteristics. Accordingly, in this study, the selectivity, the linearity, the accuracy, the reproducibility, the repeatability, and the lower quantification and detection limits were evaluated.

3.1. Specificity

The specificity of an analytical method is described [20] as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present, which in this case can be considered as the ability of the method to distinguish between both plasmid isoforms. Fig. 1 presents an agarose electrophoresis where it is observed in lane 1 the plasmid DNA sample containing the oc and sc plasmid isoforms. This sample was applied to the analytic arginine column and two peaks were obtained when using the gradient represented by the dashed line (Fig. 2a). Briefly, the buffer A consists of a 200 mM NaCl solution at pH 8.0 in Tris–HCl buffer and the buffer B is a 500 mM NaCl solution in the same Tris–HCl buffer. The elution of the first plasmid component was verified at 1.43 min using a gradient of 15% of buffer B (Fig. 2a). After 5 min of the chromatographic run, a stepwise gradient was applied, by adjusting the elution conditions to 100% of buffer B, and the elution of the second component was observed with a retention time of 8.55 min (Fig. 2a).

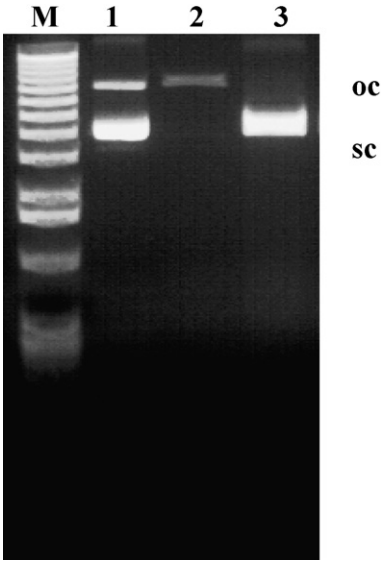

Fig. 1. Agarose gel electrophoresis analysis of the samples purified with the arginine-chromatography at preparative scale and used in the analytic method. Lane M – molecular weight marker; lane 1 – pDNA sample containing open circular and supercoiled isoforms; lane 2 – open circular sample; lane 3 – supercoiled pDNA sample.

The identification of the plasmid components eluting in both differentiated peaks was investigated by performing two experiments of injecting each isolated plasmid isoform, recovered from the preparative assay, into the arginine analytic column. Fig. 1 presents the isolated isoforms, the oc conformation in lane 2 and the sc conformation in lane 3, which were used in the analytic method to compare with the elution profiles and retention time achieved in Fig. 2a. Thus, in Fig. 2b it can be observed the chromatographic profile obtained by the injection of the oc plasmid conformation. As it is visible, a single peak was obtained with a retention time of 1.45 min, being coincident with the elution of the first peak in Fig. 2a. Furthermore, the application of the isolated

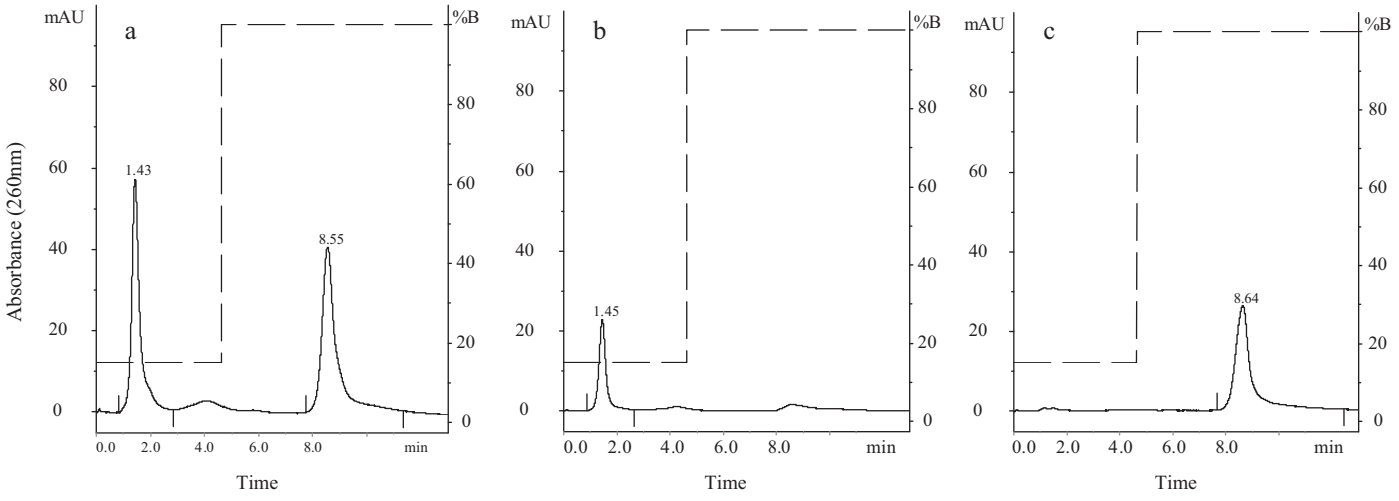

Fig. 2. Analysis of pDNA using the analytical method based on arginine-affinity chromatography. (a) Chromatographic profile of a pDNA sample containing the open circular and supercoiled isoforms. (b) Chromatographic profile of open circular pDNA isoform. (c) Chromatographic profile of supercoiled pDNA isoform.

sc plasmid isoform (Fig. 2c) resulted in the elution of a single peak with a retention time of 8.64 min, corresponding to the second peak obtained in the chromatographic analysis of total pDNA. Thus, with these experiments it was possible to verify the selectivity of the analytical method to efficiently distinguish between both major plasmid conformations. Although this is considered the development of a new analytical approach, these results could be expected because, as already described in previous works, the arginine-based chromatography was efficient on plasmid isoforms separation by exploiting an affinity interaction [18]. As previously described, this biorecognition may involve different elementary interactions such as general electrostatic forces between the ligand and the pDNA backbone or other specific interactions involving the bases. In this respect, the ability of arginine to produce good hydrogen bonding geometries [24] and to induce multiple-contact complex interactions [16] are the main supporting considerations. In fact, this can explain the favored binding of sc pDNA to arginine ligands, due to the exposure of bases brought about by superhelicity.

The applicability of the analytical method was also tested for the quantification of plasmids purified with other chromatographic techniques. Thus, the plasmid samples were purified by hydrophobic interaction chromatography [22] and anion-exchange chromatography, using a commercial kit. The analytical chromatographic profiles obtained in these assays were similar to the chromatographic results presented in Fig. 2a, the same elution time for the open circular and supercoiled plasmid DNA isoforms being achieved. Thus, it was concluded that the technique used to purify the plasmid samples, at preparative level, does not influence the analytical result. In addition, two plasmids with different size and nucleotides composition were also analyzed, in order to understand the influence of these parameters on the interaction with the analytical arginine-affinity matrix. The plasmids pUC19 (2.7-kpb) and pVAX1-*LacZ*gag (7.4-kpb) were chosen. The plasmid pVAX1-*LacZ*gag was obtained from pVAX1-*LacZ* by addition of a 1.3-kpb insert, rich in adenine (37%) and guanine (27%) bases. The plasmid pUC19 presents an equal distribution of 25% of each base, while the complete sequence of pVAX1-*LacZ* and pVAX1-*LacZ*gag revealed a presence of 22% and 25% of adenine, respectively, and 28% of guanine for both plasmids [25]. The retention of each isoform of the different plasmids was also identical to the pVAX1 *LacZ*. This fact can be explained by the elution strategy used, because the first step allows the immediate elution of the oc isoform and the second step presents a significantly higher ionic strength that is suitable for the total recovery of the sc isoform. In this way, it is concluded that the method is adequate to elute different plasmids, irrespective of their size or composition.

In this context, the selectivity found is extremely relevant as it is required for the implementation of a new analytical technology able to ensure the quality and purity criteria of a bioproduct like pDNA. However, other parameters have to be established to validate an analytical method.

3.2. Linearity, accuracy, limit of detection and limit of quantification

The study of the linearity of this method was performed with 8 standards in the range of 2–150 μg/mL (2, 5, 10, 20, 50, 75, 100 and 150 μg/mL) using four replicates for each sc plasmid concentration. The standards were prepared with the sc pDNA fraction recovered from the preparative assay, by measuring the initial absorbance of the sample in UV at 260 nm and considering that 1 unit of absorbance corresponds to a concentration of 50 μg/mL. Each standard was obtained by dilution of the sc plasmid solution with the mobile phase used in the beginning of the chromatographic experiment.

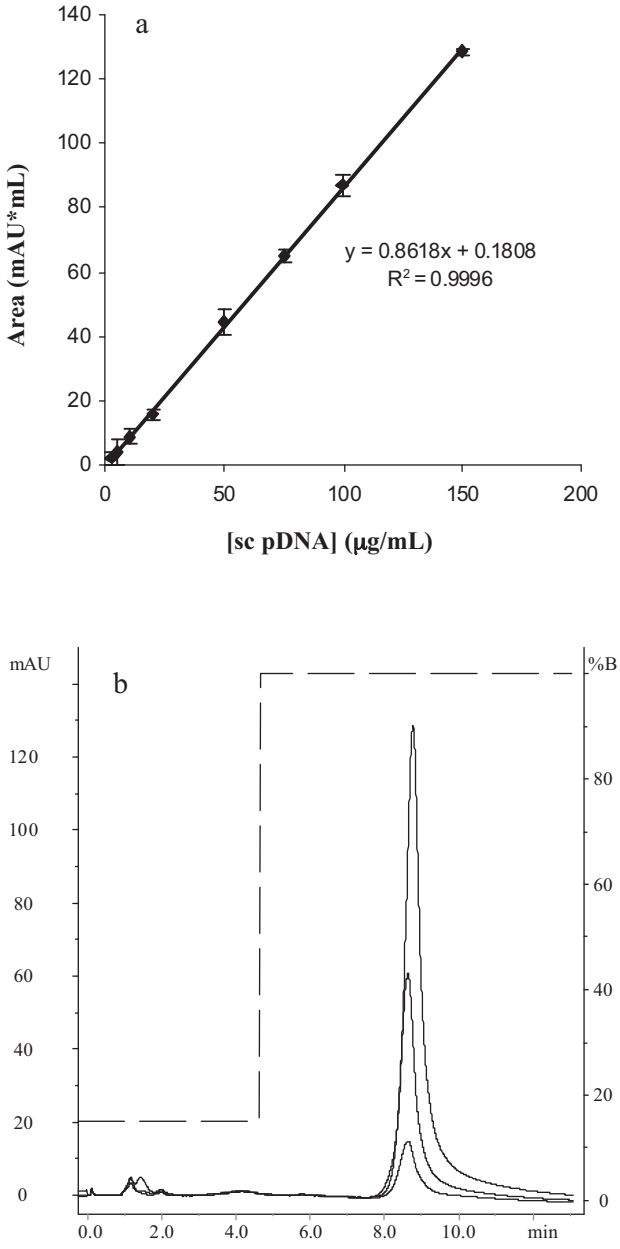

Fig. 3. (a) Calibration curve obtained from the correlation between the supercoiled pDNA peak area and the concentration. (b) Chromatographic profile obtained for 3 sc plasmid standards under study, with the concentration of 10, 50 and 100 μg/mL.

With this study it was verified that the method is linear within the range studied (2–150 μg/mL). A linear correlation was found for this sc pDNA concentration range considering either the height or area of the sc pDNA peak. In fact, the correlation coefficients were of about 0.9992 and 0.9996, respectively. The correlation found for the calibration curve relating the sc pDNA concentration range with the peak area is presented in Fig. 3. In this figure it is also possible to analyze an example of the chromatographic profiles obtained for three standards, where it is observed the consistency of the elution of the sc pDNA at 8.64 min. The total run time of the analytical experiment was of 12 min, which represents an advantage because the separation of pDNA isoforms and the elution of sc pDNA occurs faster than the described for other methods, for example the anion exchange-based method that requires 35 min to complete the run [7].

Table 1
Intra-day and inter-day precision for the assessment of sc pDNA ($n = 4$).

Intra-day precision				Inter-day precision			
Nominal concentration (mg/mL)	Mean concentration ± SD (mg/mL)	CV (%)	Mean relative error (%)	Nominal concentration (mg/mL)	Mean concentration ± SD (mg/mL)	CV (%)	Mean relative error (%)
10	10.51 ± 0.26	2.78	5.08	10	10.30 ± 0.37	4.05	2.98
60	64.11 ± 1.59	3.03	6.86	60	61.93 ± 0.26	0.48	3.22
100	98.37 ± 2.93	3.45	−1.63	100	95.97 ± 4.25	5.13	−4.03

In relation to the accuracy of the method, it was assessed by back-calculated concentration for all the standards and for an intermediate sc pDNA concentration of 60 μg/mL. Thus, a comparison was made between the nominal concentration of the samples and the sc pDNA concentration obtained from the calibration curve. Furthermore, the coefficient of variation and the mean relative error were determined to conclude about the accuracy of this method. With regard to these results, it was possible to conclude that the accuracy is adequate for all the standards of the calibration curve and for a pDNA intermediate concentration of 60 μg/mL, because both parameters (the coefficient of variation and the mean relative error) were within the range of ±15% of the nominal concentration, including the lower limit of quantification (LLOQ), which shows a coefficient of variation of 14% that is bellow the value of 20% accepted by the guidelines [20].

The limit of quantification (LOQ) was defined as the lowest concentration of sc pDNA that could be assessed with suitable precision (a coefficient of variation lower than 20%) and adequate accuracy, i.e. within ±20% of the nominal concentration. The limit was 2 μg/mL, which also corresponds to the lower limit of detection (LOD). The study of lower sc pDNA concentrations revealed an increased signal-to-noise that restricts the precise and accurate detection of the sc isoform peak, justifying the establishment of the same value for the LOQ and LOD. Although some works describe limits of detection of 1 μg/mL [10], the limit found for this new analytical affinity technique is in accordance with the limit described by the authors who have developed the anion-exchange method [7]. In addition, the linearity was found for the concentration range of 2-150 μg/mL, the same range used in the anion-exchange method [7]. Other authors have already described alternative methods based on hydrophobic interaction chromatography [10,11] that were not efficient on plasmid isoforms separation and assessment, and the linearity was only studied for a concentration range between 2.5 and 20 μg/mL [10]. Hence, this new method presents a great advantage over the hydrophobic-based methods because of the possibility to quantify specifically the supercoiled plasmid isoform. The assessment of this conformation is a major concern for the industry focused on the production and purification of pDNA, since a higher than 98% of sc pDNA homogeneity is mandatory for this bio-product to be applied in gene therapy [6]. For this reason, this methodology proved to be simple, selective, linear and accurate in a concentration range adequate to measure sc pDNA content and to assess its purity as a pharmacological product [7].

3.3. Precision

According to the guidelines, the precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the analytical conditions [20]. In the validation protocols, precision may be considered at two levels, the repeatability and the reproducibility, that are usually expressed as the variance, standard deviation, mean relative error or coefficient of variation of a series of measurements.

In this work, the precision of the method was evaluated on the basis of repeatability and reproducibility, also termed as intra-day and inter-day precision, respectively. The intra-day repeatability of the method was assessed by performing four consecutive injections of three sc pDNA samples at three different levels of concentration (low, medium and high) under selected conditions. The same samples were also analyzed over a period of four consecutive days to determine the inter-day precision. The data presented in Table 1 show the coefficient of variation and mean relative error [(measured concentration − nominal concentration)/nominal concentration × 100] for the three sc pDNA concentrations (10, 60 and 100 μg/mL).

The values of intra-day precision were lower than 4% and for the inter-day precision a value close to 5% was achieved. The mean relative error for the intra-day experiments was between – 1.63 and 6.86%, and the inter-day values did not deviate more than 4% from the nominal concentration (Table 1), which also confirms the accuracy of this new analytical method.

4. Conclusions

The development of new and optimized methods to quantify pDNA and to assess its purity is extremely important regarding the evolving technology to produce plasmids and the increased number of clinical trials using plasmids as a biopharmaceutical product. The primary methods were not efficient on pDNA isoforms differentiation; however, because of the improved relevance attributed to the sc pDNA isoform, new requirements were described, and the new methods must be able to isolate plasmid isoforms.

In this work, an analytical method based on affinity chromatography to quantify and monitor the sc pDNA quality is described for the first time. The performed experiments showed that the novel technique is robust, specific and selective. Indeed, the validation of the method was performed according to the guidelines, and it was proved that the method is precise and accurate in a suitable concentration range (2–150 μg/mL) for the monitorization of sc pDNA as a pharmaceutical product. In addition, the linearity was also achieved for this concentration range, and suitable lower limits of detection and quantification were obtained. The method was also assessed in relation to the reproducibility and repeatability, it being concluded that it is precise and accurate. Moreover, this new method allows a rapid analysis of the sample, completing the run in 12 min, which is an advantage for application in the quality control of a biotechnological process.

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