Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 998 (2003) 109–117
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Assessment of purity and quantification of plasmid DNA in process solutions using high-performance hydrophobic interaction chromatography

M.M. Diogo[a], J.A. Queiroz[b], D.M.F. Prazeres[a,*]

[a]*Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal*
[b]*Departamento de Química, Universidade da Beira Interior, 6200-001 Covilhã, Portugal*

Received 22 January 2003; received in revised form 3 April 2003; accepted 8 April 2003

Abstract

A hydrophobic interaction HPLC method was developed for the quantification of plasmid DNA and assessment of its purity in crude *Escherichia coli* lysates and other process streams. A Phenyl Sepharose Source (Amersham Biosciences) column was used to separate the double-stranded plasmid DNA molecules from the more hydrophobic impurities present in the process streams. The method is rapid (each analysis takes 7 min), reproducible, easy to perform and does not require previous digestion of RNA in samples with RNase or other pre-treatment. Furthermore, it is capable of handling heavily contaminated samples, with less than 5% of plasmid DNA thus constituting a good alternative to other less robust analytical techniques currently in use.

Keywords: Hydrophobic interaction chromatography; DNA

1. Introduction

The monitoring of the performance of a plasmid DNA manufacturing process, as well as the assessment of final product quality in comparison with product specifications, is a key issue in process development, validation and product approval [1,2]. The development and set-up of reliable analytical methods for the quantification of plasmid DNA and assessment of its purity and homogeneity are especially important.

Although quantification of total plasmid DNA in pure solutions is relatively easy to accomplish (e.g., by spectrophotometry at 260 nm or fluorescence), mass estimation in impure process streams requires the separation of impurities such as RNA, genomic DNA (gDNA) fragments and proteins from plasmid molecules before quantitative detection. This separation of plasmid from impurities can be accomplished by electrophoresis carried out either in the conventional [3] or capillary operation modes [4–6]. Conventional agarose gel electrophoresis is not sufficiently reproducible or accurate for quantitative purposes and is time consuming. Furthermore, for purity analysis the method is only generally accepted for a qualitative assessment of RNA contamination [7]. Capillary electrophoresis (CE) on the other hand

*Corresponding author. Fax: +351-21-841-9062.
E-mail address: prazeres@alfa.ist.utl.pt (D.M.F. Prazeres).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00618-6

combines a high degree of resolution with sensitivity, reproducibility and a fast analysis time. This technique is becoming more and more used to quantify plasmid DNA and its topoisomers (supercoiled vs. open circular) [4–6]. Unfortunately, the superior performance of CE when compared with conventional gel electrophoresis has a high cost associated and its routine use in research laboratories is not likely to occur in the near future. Furthermore CE does not adequately handle process solutions that contain significant amounts of impurities.

High-performance liquid chromatography (HPLC) is a routine technique in many research and industrial laboratories, which has been used in many instances to measure plasmid mass. Whether based on anion-exchange [7–10], hydrophobic interaction [11] or reversed-phase binding modes [12–14], HPLC is a fast, reproducible and robust methodology. When using anion-exchange for the purpose of assessing the purity of plasmid in a solution, contaminating high-molecular-mass RNA usually co-elutes with the plasmid. In order to overcome this, a previous digestion with *RNase* is performed in order to obtain low-molecular-mass RNA that can then be resolved from the plasmid [8]. However, the procedure is time-consuming and costly [8]. Some HPLC columns can also selectively separate plasmid DNA topoisomers, thus providing a means to assess the heterogeneity of plasmid DNA preparations [9,11,12]. However, this higher degree of selectivity can only be achieved with longer analysis times.

Although the separation and quantification of plasmid isoforms in final plasmid DNA products is important, it may not be required for the purpose of a rapid process monitoring. Thus, a method that can rapidly measure total plasmid DNA in impure solutions is useful.

Hydrophobic interaction chromatography (HIC) has been used in the preparative purification of plasmid DNA for therapeutic applications. This method has been described using a Sepharose CL-6B gel derivatized with 1,4-butanediol diglycidyl ether [15–17]. The technique takes advantage of the more hydrophobic character of nucleic acid impurities (RNA, denatured genomic DNA, oligonucleotides and denatured plasmid forms) and lipopolyssaccharides when compared with double-stranded plasmid DNA. In the presence of an eluent with a high concentration of ammonium sulphate, the hydrophilic double-stranded plasmid DNA molecules are rapidly eluted from the column. The single-stranded hydrophobic impurities can then be eluted by decreasing the ionic strength of the eluent. It has also been proved that the gel filtration properties of the Sepharose CL-6B porous matrix are partially responsible for the separation between the high-molecular-mass plasmid DNA and the lower-molecular-mass RNA and gDNA, proteins, oligonucleotides and others [17]. The preparative HIC separation is reproducible, it provides baseline resolution of plasmid DNA and the impurities and yields a pure, biologically active, plasmid DNA product [16]. Furthermore, it does not require any pre-treatment of the feed other than an adequate conditioning with salt. These results prompted us to adapt the HIC method for analytical purposes. A HPLC HIC column (Source 15PHE PE 4.6/100) from Amersham Biosciences was chosen. The Source media, originally developed for the separation of proteins and peptides, consist of 15 μm, monosized, rigid, polystyrene–divinyl benzene beads with an optimised pore size distribution, derivatized with hydrophobic phenyl groups. In order to optimise the analytical method, pure and impure solutions of a 7383 base pair (bp) plasmid DNA vaccine developed for the immunisation against the ovine Maedi-visna virus were used as model.

2. Materials and methods

2.1. Analytical chromatography

HPLC analysis was used to measure plasmid concentration and purity in several process samples as described next. A 10×4.6 cm HIC Source 15 PHE PE column from Amersham Biosciences (Uppsala, Sweden) was connected to a HPLC system (Merck–Hitachi, Darmstadt, Germany). Unless otherwise stated, the column was initially equilibrated with 1.5 *M* ammonium sulphate in Tris–Cl 10 m*M*, pH 8.0. In the case of the experiments described by the chromatograms presented in Fig. 1a–c, the column was initially equilibrated, respectively, with Tris–Cl buffer (pH 8.0) with no ammonium sulphate and with 450 and 1050 m*M* ammonium sulphate in

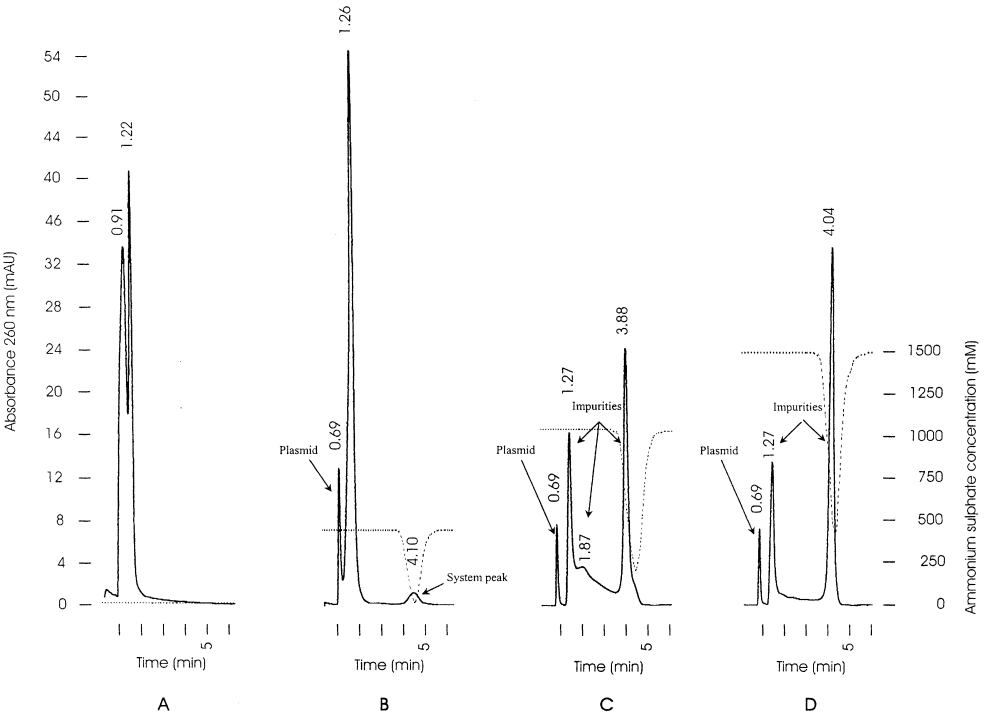

Fig. 1. HPLC analysis of a clarified *E. coli* lysate (1:10 diluted) containing 44.2±0.32 μg/ml of plasmid DNA with a purity of 4.8±0.42% using a Source 15PHE column equilibrated with an initial concentration of 0 *M* (A), 450 m*M* (B), 1050 m*M* (C) and 1.5 *M* (D) of ammonium sulphate in 10 m*M* Tris–Cl, pH 8.0. After injecting the sample, the concentration of ammonium sulphate was maintained at the initial value during the first 0.8 min. The eluent was then instantaneously changed to 10 m*M* Tris–Cl, pH 8.0. After 0.7 min the ammonium sulphate concentration in the eluent was instantaneously increased back to its initial value. This was maintained for 5.5 min. The ammonium sulphate concentration gradients obtained at the outlet (dashed line) are plotted in the figures together with the absorbance (continuous line) readings.

Tris–Cl, pH 8.0. Samples (30 μl) were injected and eluted at 1 ml/min. After injecting the sample, the column was eluted for 0.8 min with the same buffer used for equilibration. The concentration of ammonium sulphate was then instantaneously decreased to zero (no ammonium sulphate) by eluting the column with Tris–Cl. This condition was maintained during the next 0.7 min in order to elute bound species. After this period, the concentration of ammonium sulphate was instantaneously increased to its initial value by performing elution with the equilibration buffer. This condition was maintained during the next 5.5 min in order to re-equilibrate the column. The absorbance at 260 nm and the conductivity (which is directly related to the ammonium sulphate concentration) of the eluate were continuously recorded. The plasmid amount was quantified using a calibration curve constructed with standards of the model plasmid prepared in the 0 to 20 μg/ml concentration range. A HPLC purity degree was defined as the percentage of the plasmid peak area when compared with the total area of all peaks on the chromatogram.

2.2. Preparation of process samples

Plasmid DNA containing samples with different degrees of purity were prepared by carrying out a preparative purification process which consists of the sequential use of the following steps: alkaline lysis, isopropanol precipitation, ammonium sulphate precipitation and HIC [15,16]. The process is briefly described next.

2.3. Bacterial growth and lysis

Escherichia coli DH5α strain harbouring the plasmid pVAX1*lacZ*gag3951 (7383 bp) was grown overnight at 37 °C in a 500-ml shake-flask containing 200 ml of LB (Sigma, St. Louis, MO, USA) medium with 30 μg kanamicin/ml. Cells were harvested at late log phase (A_{600}≈3.7) and centrifuged at 14 300 *g* (15 min, 4 °C) in a Beckman (Fullerton, CA, USA) J2-21 centrifuge equipped with a JA-10 rotor. Supernatants were discarded and the cell pellets were resuspended in 8 ml of 50 m*M* glucose, 25 m*M* Tris–Cl and 10 m*M* EDTA (pH 8.0). Alkaline lysis was performed by adding 8 ml of a 200 m*M* NaOH, 1% (w/v) sodium dodecyl sulphate solution. Cellular debris, genomic DNA (gDNA) and proteins were precipitated by adding 8 ml of pre-chilled (on ice) 3 *M* potassium acetate (pH 5.0). The precipitate was removed by centrifugation at 39 200 *g* (30 min at 4 °C) using the same centrifuge but with a JA-20 rotor. A second centrifugation step was carried out under the same conditions to separate the remaining suspended material. Samples of this clarified lysate where then kept for further analysis.

The same procedure was used to lyse a plasmid DNA-free cell culture.

2.4. Isopropanol and ammonium sulphate precipitation

The plasmid DNA present in 24 ml of the clarified lysate was precipitated with 0.7 volumes of isopropanol. A plasmid containing pellet was obtained by centrifugation at 39 200 *g* (30 min, 4 °C), followed by washing with 2 ml room temperature 70% ethanol and subsequent centrifugation for 10 min under the same conditions (39 200 *g*, 4 °C). After discarding the supernatant, the pellet was dried under air for 5–10 min and re-dissolved in 500 μl 10 m*M* Tris–Cl buffer (pH 8.0). Samples of this solution where kept for further analysis. Next, solid ammonium sulphate was dissolved in the plasmid solution up to a concentration of 2.5 *M*. After 15-min of incubation on ice, precipitated impurities were removed by centrifugation at 20 000 *g* (15 min, 4 °C) in a Sigma 1K 15 centrifuge equipped with a 12024-H rotor. Samples of this clarified lysate where then kept for further analysis. The plasmid containing supernatant was then loaded directly onto the HIC column.

2.5. HIC

A HIC stationary phase was prepared by coupling 1,4-butanediol diglycidyl ether (Aldrich. St. Louis, MO, USA) to Sepharose CL-6B (Amersham Biosciences) according to the protocol of Sundberg and Porath [18]. A 20-ml Econo-Pac disposable chromatography column (Bio-Rad, Hercules, CA, USA) was packed with 10 ml of the HIC gel. The column was equilibrated with 20 ml of 1.5 *M* ammonium sulphate in 10 m*M* Tris–HCl (pH 8.0) by gravity flow. Plasmid-containing supernatant, which resulted from the ammonium sulphate precipitation (500 μl), was directly loaded onto the column. After allowing this feed solution to enter the column, isocratic elution was carried out with 1.5 *M* ammonium sulphate in 10 m*M* Tris–HCl (pH 8.0). The purified plasmid was collected in a 2-ml fraction after discarding the first 3.5 ml of eluent. After elution of the unbound species (plasmid DNA), the ionic strength of the buffer was reduced in a step mode (10 m*M* Tris–Cl, pH 8.0) in order to elute bound and weakly retained species.

3. Results and discussion

3.1. Method optimisation

The HIC Source 15 PHE HPLC column described was tested for the quantification and quality control of plasmid DNA in different process samples withdrawn at different stages of an established purification process used to manufacture therapeutic plasmid DNA [15,16]. The process is based on hydrophobic interaction chromatography as the main step. As mentioned before, the HIC preparative column was used with success for the separation of plasmid DNA from the impurities presented in clarified lysis solution from *E. coli*. In the presence of 1.5 *M* of ammonium sulphate, the plasmid was eluted from the column without being retained, whereas the impurities presented an higher retention due to the fact that they are more hydrophobic and that they have a lower molecular mass. In order to reproduce

and optimise this behaviour on the analytical column, a clarified lysate from *E. coli* diluted in 1.5 *M* ammonium sulphate in 10 m*M* Tris–Cl was injected into the HIC–HPLC column equilibrated with a mobile phase with different ammonium sulphate concentrations (Fig. 1). After an 0.8-min elution with the mobile phase used for equilibration, the eluent was changed to 10 m*M* Tris–Cl (pH 8.0) with no ammonium sulphate. With no ammonium sulphate on the mobile phase (Fig. 1a) only two non-resolved peaks eluted. This profile reflects the gel filtration properties of the porous column that enables a partial separation of solutes on the basis of their molecular masses [17]. In this way, this separation is not due to the hydrophobic properties of the column. One of the characteristic features of the other analytical chromatograms from Fig. 1 is the first peak of non-retained species at 0.69 min. This peak corresponds to plasmid DNA, as confirmed by injecting a pure plasmid DNA standard in the column at 1.5 *M* ammonium sulphate (Fig. 2a). The chromatographic profile of the plasmid standard also shows a small peak at 4.15 min. This system peak results from the changes on the ammonium sulphate concentration in

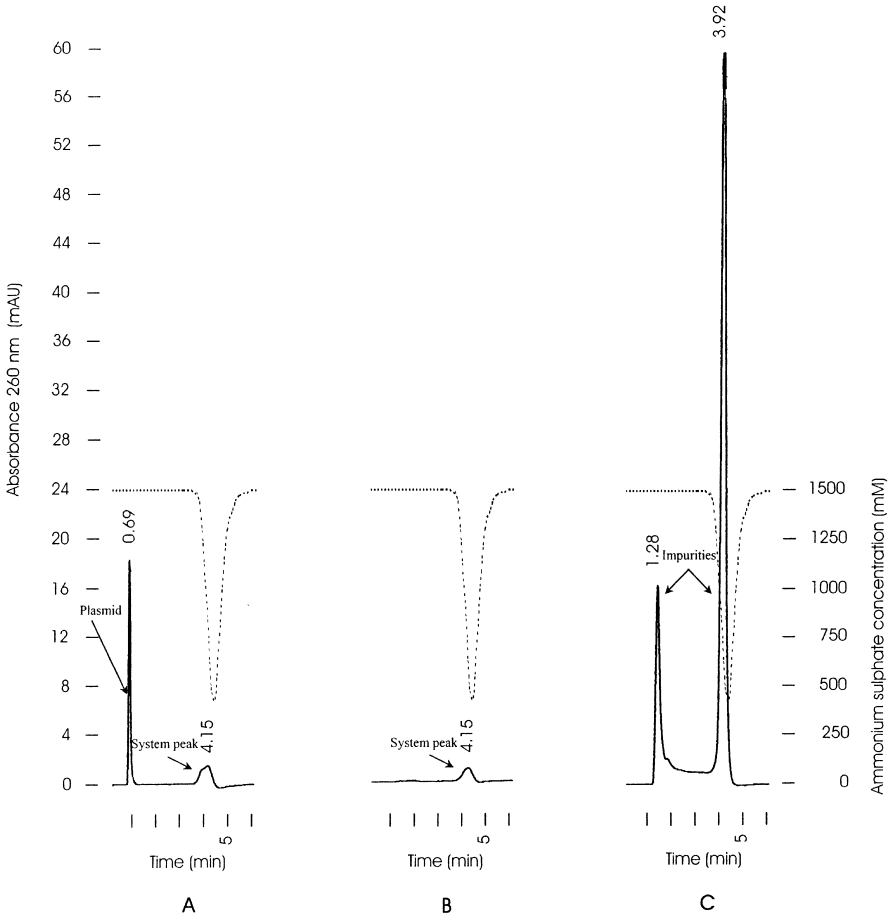

Fig. 2. HPLC analysis of 10 μg/ml pure plasmid DNA standard (A), 1.5 *M* ammonium sulphate in 10 m*M* Tris–Cl solution (B) and clarified *E. coli* lysate with no plasmid DNA (1:10 diluted) (C), using a Source 15PHE column equilibrated with 1.5 *M* of ammonium sulphate in 10 m*M* Tris–Cl. After injecting the sample, the concentration of ammonium sulphate was maintained at the initial value during the first 0.8 min. The eluent was then instantaneously changed to 10 m*M* Tris–Cl, pH 8.0. After 0.7 min the ammonium sulphate concentration in the eluent was instantaneously increased back to its initial value. This was maintained for 5.5 min. The ammonium sulphate concentration gradients obtained at the outlet (dashed line) are plotted in the figures together with the absorbance (continuous line) readings.

the eluent (a decrease followed by an increase) (Fig. 2b). The other peaks in the chromatograms of Fig. 1 were attributed to the impurities present in the lysis solution. In fact, control studies carried out at 1.5 *M* ammonium sulphate using a clarified lysis solution without plasmid DNA (Fig. 2c) proved that all impurities eluted with higher retention times (≥1.27 min) when compared with the plasmid DNA (0.69 min). The less hydrophobic impurities are only retarded in the column and elute at 1.28 min. In fact, this peak is eluted even in the absence of the salt gradient (results not shown). On the other hand, the more hydrophobic impurities are retained in the column and elute at 4.04 min due to the decrease of the ionic strength of the mobile phase. This means that when using a salt concentration of 1.5 *M* ammonium sulphate the plasmid peak is not overlapping with *E. coli* impurities.

As observed in Fig. 1, and as expected for HIC, the strength of the interaction between the impurities and the column increases when increasing the concentration of ammonium sulphate. This result indicates the hydrophobic nature of the interaction of the impurities with the column. Due to these results the injection of plasmid containing samples was carried out at 1.5 *M* ammonium sulphate in 10 m*M* Tris–Cl in order to fully resolve the plasmid from the impurities.

The chromatograms in Fig. 1 show the ammonium sulphate concentration gradients recorded at the column outlet. The shape of these gradients reflects the existence of some axial dispersion effects in the column and associated tubing. For this reason the ammonium sulphate concentration does not reach zero in the case of Fig. 1c and b. Nevertheless, the elution pattern used was appropriate to elute all bound impurities. In fact, when using longer elution periods with no ammonium sulphate, very similar elution profiles were obtained (results not shown).

3.2. Calibration curve

A plasmid DNA standard sample was quantified by UV at 260 nm, considering that 1 unit of absorbance corresponds to 50 μg/ml of plasmid DNA. The standard sample was diluted in 1.5 *M* ammonium sulphate in Tris–Cl 10 m*M* in order to obtain plasmid DNA standards with concentrations of 20, 15, 10, 5 and 2.5 μg/ml. The standard samples were then injected into the HPLC column using the procedure described above. Four replicate analyses were performed with each of the five standards. The average plasmid peak area and associated standard deviation were calculated and a calibration curve constructed. A linear correlation between peak area and plasmid concentration was obtained (correlation coefficient 0.9997). The standard deviation was found to be lower than 2% for all the standards analysed, providing a good indication of the method reproducibility. The detection limit of the method was determined to be 1 μg/ml. In fact, the standard deviation of the plasmid peak areas was less than 2% for all the samples analysed with a concentration higher than 1 μg/ml (results not shown).

3.3. Validation of the analytical method

In order to validate the method developed, the concentration obtained when analysing a pure plasmid solution in the column was compared with the one obtained when analysing a clarified lysis solution obtained from a cell culture without plasmid spiked with known amounts of a pure plasmid standard (Fig. 3). Four replicate analysis were performed with each one of the samples. The differences between the measured concentrations in the plasmid standards and spiked samples is lower than 5.6%. These differences may be attributed to the reproducibility of the analysis but essentially to the preparation of different sample solutions. Overall, these results confirm the validity of the HIC method for the quantification of plasmid DNA in impure solutions.

3.4. Process monitoring

Plasmid DNA containing samples collected throughout the preparative purification process described in the Materials and methods section were analysed by the HIC method (Fig. 4). Again, the chromatograms show the plasmid eluting in the flow through, followed by the impurities. As already refereed, the less hydrophobic impurities are retarded in the column and elute at 1.28 min as seen for the clarified lysis solution (Fig. 4a). The more hydrophobic impurities are retained in the column and

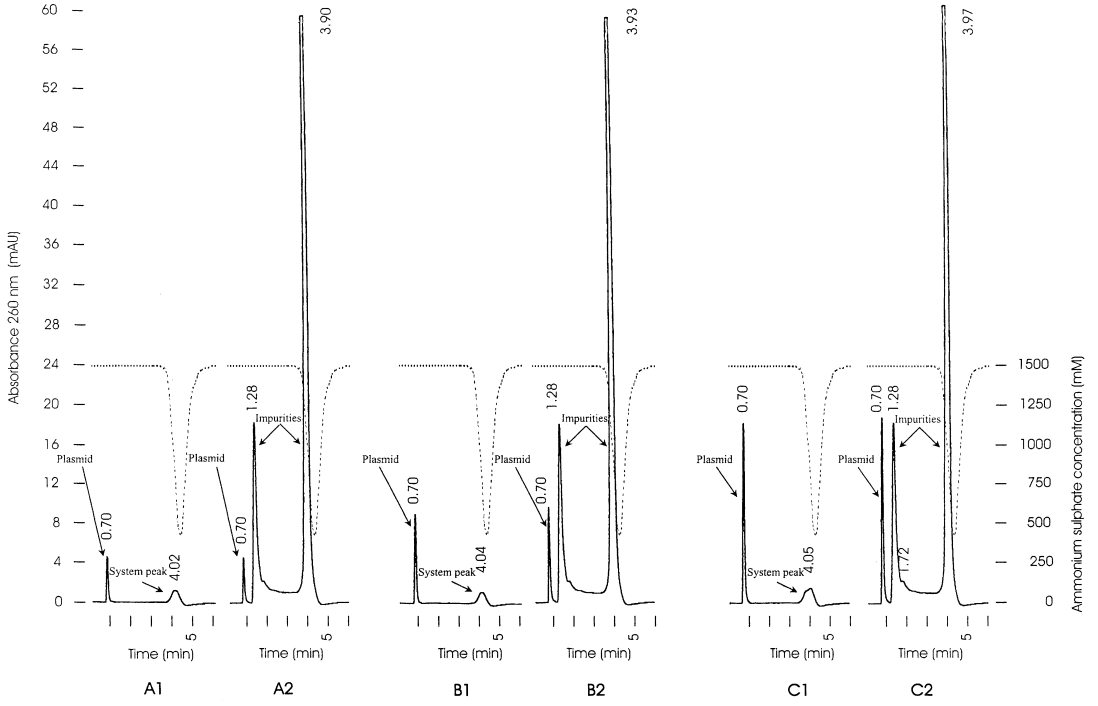

Fig. 3. HPLC analysis of 2.5 (A1), 5.0 (B1) and 10.0 (B3) μg/ml pure plasmid DNA standards and of clarified plasmid free lysates spiked with 2.5 (A2), 5.0 (B2) and 10.0 (C2) μg/ml of plasmid DNA, using a Source 15PHE column equilibrated with 1.5 *M* of ammonium sulphate in 10 m*M* Tris–Cl, pH 8.0. After injecting the sample, the concentration of ammonium sulphate was maintained at the initial value during the first 0.8 min. The eluent was then instantaneously changed to 10 m*M* Tris–Cl. After 0.7 min the ammonium sulphate concentration in the eluent was instantaneously increased back to its initial value. This was maintained for 5.5 min. The ammonium sulphate concentration gradients obtained at the outlet (dashed line) are plotted in the figures together with the absorbance (continuous line) readings.

elute at 4.03 min due to the decrease of the ionic strength of the mobile phase.

The plasmid concentration in all the samples collected throughout the process was determined using the standard calibration curve. Four replicate analyses were performed for each sample. The standard deviation was lower than 2% for all the samples, confirming the reproducibility of the method. The analytical chromatograms also bear information about the purity degree of the samples, which can be estimated as the percentage of the plasmid peak area in the chromatogram.

An analysis of Fig. 4A and B show that precipitation with isopropanol efficiently separates the less hydrophobic impurities, such as low-molecular-mass RNA. On the other hand, the precipitation with ammonium sulphate results in a significant, 8.8-fold increase in the purity degree. After preparative HIC, the plasmid pool is almost 100% HPLC pure (Fig. 4D). The peak that elutes at 4.11 min is a system peak that results from changes in the concentration of ammonium sulphate.

4. Conclusions

The results obtained show that HIC can be successfully used for the quantification of total plasmid DNA in impure process solutions and for the control of plasmid DNA purity in process solutions. The method developed enabled an easy and rapid (7 min long) determination of the concentration of plasmid DNA in samples collected throughout a preparative purification process. Replicate analysis of pure and impure samples containing plasmid DNA yielded standard deviation values for the concentration of plasmid lower than 2%, indicating that the method is reproducible. A relevant feature of the method is the

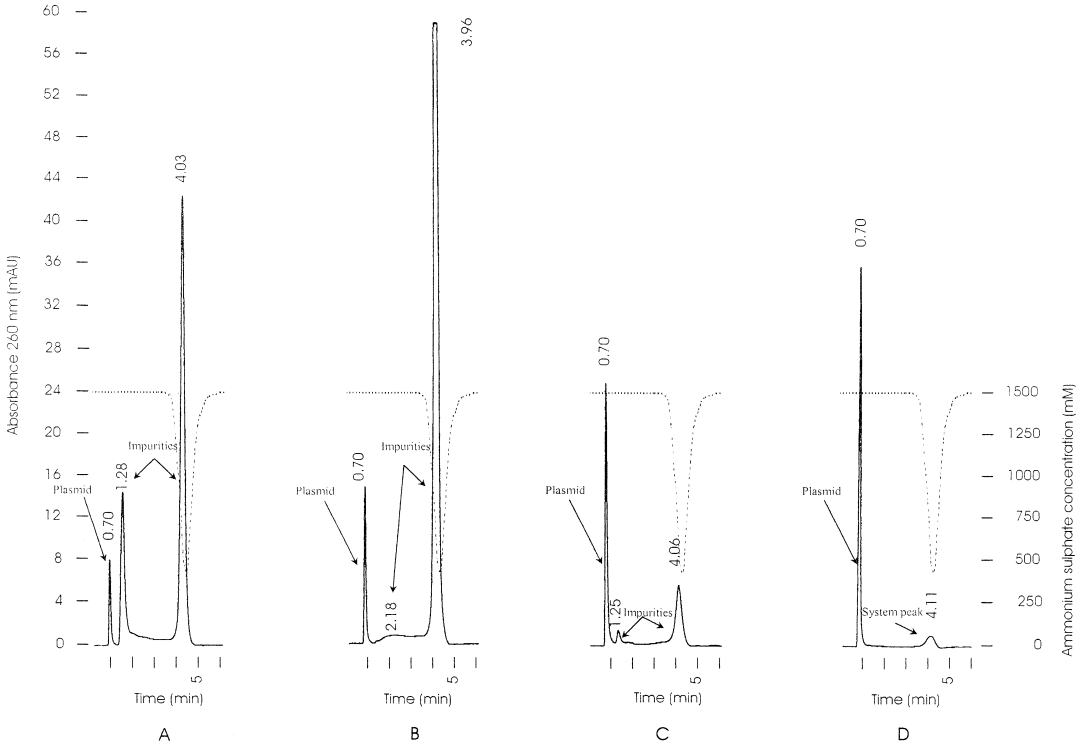

Fig. 4. HPLC analysis of plasmid DNA containing solutions collected throughout the preparative purification process: clarified *E. coli* lysate (A) and samples collected after isopropanol precipitation (B), ammonium sulphate precipitation (C) and preparative HIC (D), using the Source 15PHE column equilibrated with 1.5 *M* of ammonium sulphate in 10 m*M* Tris–Cl, pH 8.0. The dilution factors of the samples injected were (1:10), (1:200), (1:100) and (1:10), respectively. The samples analysed contained 44.2±0.32, 1608.0±24, 1461.0±13 and 212.0±2.7 μg/ml of plasmid DNA with purities of 4.8±0.42, 5.90±0.91, 51.9±3.4 and 100%, respectively. After injecting the sample, the concentration of ammonium sulphate was maintained at the initial value during the first 0.8 min. The eluent was then instantaneously changed to 10 m*M* Tris–Cl. After 0.7 min the ammonium sulphate concentration in the eluent was instantaneously increased back to its initial value. This was maintained for 5.5 min. The ammonium sulphate concentration gradients obtained at the outlet (dashed line) are plotted in the figures together with the absorbance (continuous line) readings.

ability to handle heavily contaminated samples (<5% of plasmid DNA) without the need for any sample pre-treatment such as digestion of high-molecular-mass RNA with RNase as occurs with anion-exchange HPLC. The detection limit of the method, near to 1 μg/ml, is quite satisfactory from a monitoring point of view since plasmid DNA concentration in process streams is usually higher than 10 μg/ml. Overall the method constitutes a good alternative to other less robust analytical techniques currently used.

Acknowledgements

This work was supported by the Portuguese Ministery of Science and Technology (POCTI/BIO/43620/2000 and Ph.D. grant BD/21241/99 to M.M.D.).

References

[1] M. Marquet, N.A. Horn, J.A. Meek, BioPharm May (1997) 42.
[2] M. Schleef, in: A. Mountain, U. Ney, D. Schomburg (Eds.), Recombinant Proteins, Monoclonal Antibodies and Therapeutic Genes, Wiley–VCH, Weinheim, 1999, p. 443.
[3] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.
[4] T. Schmidt, K. Friehs, E. Flaschel, J. Biotechnol. 49 (1996) 219.

[5] T. Schmidt, K. Friehs, M. Schleef, C. Voss, E. Flaschel, Anal. Biochem. 274 (1999) 235.
[6] T. Schmidt, K. Friehs, M. Schleef, C. Voss, E. Flaschel, PACE Setter 4 (2000) 1.
[7] N. Horn, J. Meek, G. Budahazi, M. Marquet, Hum. Gene Ther. 6 (1995) 565.
[8] G. Ferreira, J. Cabral, D. Prazeres, Pharm. Pharmacol. Commun. 5 (1999) 57.
[9] Y. Onishi, Y. Azuma, H. Kizaki, Anal. Biochem. 210 (1993) 63.
[10] D. Prazeres, T. Schluep, C. Cooney, J. Chromatogr. A 806 (1998) 31.
[11] S. Iuliano, J.R. Fisher, M. Chen, W.J. Kelly, J. Chromatogr. A 972 (2002) 77.
[12] J.A. Thompson, Biochromatography 1 (1986) 68.
[13] S. Colote, C. Ferraz, J.P. Liautard, Anal. Biochem. 154 (1986) 15.
[14] A.N. Best, D.P. Allison, G.D. Novelli, Anal. Biochem. 114 (1981) 235.
[15] M. Diogo, J. Queiroz, G. Monteiro, G. Ferreira, S. Martins, D. Prazeres, Biotechnol. Bioeng. 68 (2000) 576.
[16] M. Diogo, S. Ribeiro, J. Queiroz, G. Monteiro, N. Tordo, P. Perrin, D. Prazeres, J. Gene Med. 3 (2001) 577.
[17] M. Diogo, J. Queiroz, D. Prazeres, Bioseparation 10 (2002) 211.
[18] L. Sundberg, J. Porath, J. Chromatogr. 90 (1974) 87.