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Fraction collection after an optimized micellar electrokinetic capillary chromatographic separation of nucleic acid constituents

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ABSTRACT

The possible use of capillary electrophoresis (CE) in micellar conditions with fast atom bombardment mass spectrometry (FAB-MS) for the characterization of DNA adducts with the ultimate goal of determining these compounds in biological matrices was explored. A method for fraction collection from an optimized and automated micellar electrokinetic capillary chromatographic (MECC) system is described. Parameters such as the reproducibility of migration times and injection and the maximum mass loadings are addressed. Fractions were collected directly in a small volume (5 μl) of buffer with sodium dodecyl sulphate (SDS) with recoveries of >75%. The fractions collected were further analysed using MECC and FAB-MS. Preliminary analysis by FAB-MS showed high background signals due to the presence of the SDS, demonstrating the difficulties that will be encountered with fractions deriving from a micellar separation and the need for more detailed investigations of the mass spectrometric conditions in this special case.

INTRODUCTION

The use of carcinogen–DNA adducts as biological dosimeters of exposure to chemical carcinogens is critically dependent on the development of analytical methods that allow their accurate identification and determination at very low (picomole) levels (*e.g.*, one adduct in 10^8–10^{10} normal nucleotides). Several techniques have been developed over the past few years to detect and measure DNA adducts. The most widely used is Reddy *et al.*'s ^{32}P-postlabelling technique [1], in which DNA is digested to 3'-monophosphates of normal and adducted nucleotides which are ^{32}P-labelled and detected after TLC or HPLC. However, despite its high level of sensitivity, this technique does not reveal any structural information for the identification of unknown adducts that are detected [2]. Identifications are based only on co-chromatography with known standards and, at best, only tentative identifications can be made for unknown adducts.

We therefore developed a capillary electrophoretic (CE) method to evaluate the possibility of its use for the separation, determination and the further identification of normal and modified

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nucleoside and nucleotide 3′-monophosphates (the form in which nucleotides are phosphorylated in the postlabelling technique). The advantages afforded by CE include multiple separation modes, high resolution, rapid separation times and automation. In a previous paper [3], we reported micellar electrokinetic capillary chromatographic (MECC) separations of normal and some modified nucleic acid bases, including deoxy- and ribonucleosides and deoxynucleotide 5′- and 3′-monophosphates. Together nucleosides and nucleotides are a mixture of neutral and charged species and thus the mechanism of their separation is a combination of both electrophoretic migration and partitioning. An interesting characteristic was that, because of their different behaviours in micellar conditions, deoxyribonucleosides were well separated from the ribonucleosides and from the nucleotides. This cannot be achieved in most chromatographic systems. We and others [4] have shown also that substituted nucleotides generally elute after their unmodified analogues as substitution decreases their polarity and thus increases their partition in sodium dodecyl sulphate (SDS) micelles.

The simultaneous separation of nucleosides and nucleotides was found to be useful because in the nuclease P1 enrichment procedure [5–8] normal nucleotides are preferentially 3′-dephosphorylated to nucleosides whereas bulky aromatic adducted nucleotides are not. In consequence, after enrichment we should find in the nucleotide's elution interval only the adducted compound. For bulky hydrophobic substitutes [*e.g.*, polycyclic aromatic hydrocarbons (PAHs)], the elution behaviour should be confirmed. As adduct standards are not widely available for the development of analytical methodology, we anticipate that fine tuning of the CE conditions will be required for each future system studied. This will require the further availability of synthetic DNA–carcinogen adduct standards that are representative of different chemical classes.

CE has been used for micropreparative applications [9–13] and collected fractions have in some instances been characterized and identified by mass spectrometry. In the future, both CE–MS and micropreparative CE will be utilized in the identification and confirmation of species. Continuous-flow fast atom bombardment (CF-FAB) has proved to be a useful technique for the low-level detection of FAB-amenable analytes, in particular microgram levels of purified nucleosides and carcinogen–nucleoside/nucleotide adducts [14–17], and has been found to lend itself well to interfacing with various separation methods, including in the last few years capillary zone electrophoresis (CZE) [18–21].

In this paper, we explore the use of CE in micellar conditions with FAB-MS for the characterization of DNA adducts. In order to evaluate the possible utilization of purified material obtained after an MECC separation, we collected the micellar separated fractions for preliminary off-line tests. As small-diameter capillaries limit the amount of material that one can inject (*e.g.*, sub-picomoles of material), fractions from multiple runs must be pooled in order to collect sufficient material for subsequent analyses. Automated multiple collection of fractions involves optimization of the separation [*i.e.*, good retention time repeatability and maximum resolution of the components(s) to be purified from the remaining species at the desired loading levels]. Separation conditions (buffer composition, concentration and pH, capillary treatments, temperature and operating conditions) were previously evaluated for their influence on the retention times, resolution and efficiency in the separation of standard deoxynucleoside and deoxynucleotide 3′-monophosphates [22]. The choice of a 50-μm capillary diameter was based on the resolution required and the high reproducibility of the migration (<1% R.S.D.) that permits collection of repeated separations into the same set of vials with no cross-contamination of adjacent peaks. Under the same conditions (buffer, voltage), a 75 μm I.D. capillary results in larger mass injection; however, the resolution and repeatability were not inadequate (data not shown). In this work, we evaluated the optimum conditions for the maximum sample loading capacity that maintained a sufficient baseline resolution to allow the collection of the different fractions. After verifying suitable conditions, multiple fraction collection runs were performed into a fraction vial containing 5 μl of running buffer without SDS. The fractions collected were

directly re-analysed by MECC and then by FAB-MS.

EXPERIMENTAL

Reagents

All 2′-deoxynucleoside and 2′-deoxynucleotide 3′-monophosphate standards were obtained from Sigma (St. Louis, MO, USA) and were used without further purification. Stock standard solutions were prepared in water purified with a Milli-Q system (Millipore) at a concentration of 5 m*M* and stored at −20°C. The composition of the standard test mixture was 2′-deoxyadenosine (2′dAdo), 2′-deoxycytidine (2′dCyd), 2′-deoxyguanosine (2′dGuo), 2′-deoxythymidine (2′dThd), [2′-deoxyinosine (2′dIno)], 2′-deoxyadenosine 3′-monophosphate (2′dAdo3′mP), 2′-deoxycytidine 3′-monophosphate (2′dCyd3′mP), 2′-deoxythymidine 3′-monophosphate (2′dThd3′mP) and 2′-deoxyguanosine 3′-monophosphate (2′dGuo3′mP) dissolved in water.

All buffer components were obtained from Aldrich, Sigma or Merck and were of HPLC grade. The standard operating buffer 20 m*M* lithium phosphate–5 m*M* borate–100 m*M* SDS–5% acetonitrile prepared fresh each week from stock solutions of 0.2 *M* $Na_2B_4O_7$ and 0.2 *M* $LiOH–H_3PO_4$ (pH 7). The SDS was weighed and dissolved the mixture of buffer, water and acetonitrile and the pH was adjusted to 9.6 at 35°C. The solutions were filtered (0.22 μm), sonicated for 3 min and stored at room temperature.

Instrumental

MECC was performed with a Beckman P/ACE System 2100 controlled with an IBM PS/2 Model 55SX computer with Beckman P/ACE v. 1.1 software. The inlet was held at a positive voltage. The untreated fused-silica capillary (50 μM I.D. × 57 cm total length, 50 cm to the detector) was obtained from Beckman and fitted into a capillary cartridge. Unless mentioned differently, the temperature (strictly controlled by the refrigerating circuit of the P/ACE system) was held at 35°C, the samples were injected by pressure for 10 s and the detection was accomplished by on-column UV absorbance measurement at 254 nm. Data were collected at a rate of 20 points per second and processed with the GOLD software. Relative retention times, when used, were calculated relative to the first peak, 2′dC. FAB mass spectra were generated from the collected fractions by using a VG 70 SEQ high-resolution magnetic sector instrument in an EDqQ configuration. A xenon FAB gun operated at an accelerating voltage of 8 kV was used in all experiments. Spectra were recorded at a resolution of 500. FAB mass spectra were generated by dissolving the fraction in 10 μl of pure glycerol.

RESULTS AND DISCUSSION

Precision: migration time reproducibility

Effect of the buffer. We previously [22] evaluated all the parameters that had an influence on the reproducibility, efficiency and resolution for the separation of our test mixture, *i.e.*, buffer composition (cation type, concentration, organic modifier, surfactant), pH, capillary treatment, applied voltage and operating temperature. The best buffer conditions for our fraction collection purpose was found to be 20 m*M* lithium phosphate–5 m*M* borate containing 100 m*M* SDS and 5% acetonitrile at a pH of 9.6 determined at 35°C. Compared with sodium or potassium phosphate, the lithium phosphate buffer gave a shorter analysis time, better efficiency and good resolution of the peaks. The order of elution is deoxyribonucleosides 3′-monophosphates (C < A < T < G < I) before 2′-deoxyribonucleotide 3′-monophosphates (A < C < T < G < U).

Precise control of the pH of the mobile phase is very important. We previously demonstrated [3] its influence on the resolution of the nucleotide 3′-monophosphates: at pH 7 we were able to separate the nucleotide 5′-monophosphates but not the 3′-monophosphates. To achieve good separation of the 3′-monophosphates we had to increase the pH to >9. At pH 9.6 a good resolution of the peaks was obtained,

but it was observed that the last two eluting peaks of nucleotides, 2'dG3'mP and 2'dU3'mP, which are completely ionized at this pH, tend to have an asymmetric shape and sometimes even show peak splitting (Fig. 1a). This problem could be solved by decreasing the pH (Fig. 1b). As the pH was lowered from 9.6 to 8.8, the time of analysis greatly decreased because of the early elution of the nucleotides mostly as a consequence of the effects of the pH on their degree of ionization. As our purpose was fraction collection and we needed a sufficient resolution to be able to collect the peaks, we decided to continue to work at pH 9.6.

Effect of the capillary treatment. For good operation of the capillary and repeatability of the separations, several factors are very important: the initial treatment, the after-run treatment, the storage treatment and the age of the capillary [23]. A systematic study of these treatments was not performed, but the following procedures gave the best reproducibility among those tested. A new capillary was first rinsed for 20 min with water followed by the storage treatment, then equilibrated with the running buffer for 15 min. Rinsing between runs was found to be necessary to return the system to the initial conditions, keeping the current constant from run to run, and consequently reducing the fluctuations in retention times. Rinsing with 0.1 *M* NaOH, followed by the running buffer without SDS at a pH slightly higher than the running buffer, was found to give better reproducibility, re-establishing the working pH gradually. Before a separation the capillary was equilibrated with the running buffer. At the end of a set of separations or for storage, the capillary was treated for 10 min with 0.1 *M* NaOH, 1 min with a first vial of water to remove the residue of NaOH from the outside capillary wall, 20 min with a second vial of water and finally dried with air under pressure from an empty vial. Under these conditions, we were able to perform more than 400 separations with a decrease in resolution. In our case, the loss of resolution occurred for the 2'dA and 2'dT peaks, which were found to be the peaks most sensitive to the operating conditions.

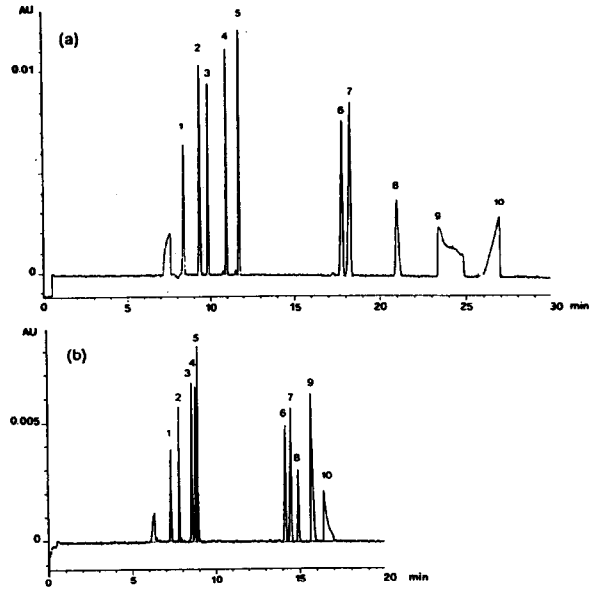

Fig. 1. Electropherograms of the standard mixture. Constant-current mode at 38 μA. (a) pH 9.6; (b) pH 8.8. Peaks: 1 = 2'dCyd; 2 = 2'dAdo; 3 = 2'dThd; 4 = 2'dGuo; 5 = 2'dIno; 6 = 2'dAdo3'mP; 7 = 2'dCyd3'mP; 8 = 2'dThd3'mP; 9 = 2'dGuo3'mP; 10 = 2'dUrd3'mP.

Effect of instrumental operating conditions. The best operating temperature was found to be 35°C, giving both good resolution and sufficient efficiency for our purposes [22]. At lower temperatures, the 2'dA and 2'dT peaks were not sufficiently resolved to allow fraction collection. The Ohm's law plot (current *versus* applied voltage) at 35°C exhibited good linearity below an applied voltage of 15 kV, then it began to deviate from linearity owing to less efficient heat dissipation because of the high voltage (Fig. 2). Therefore, for reproducible retention times one should operate below 15 kV. If the voltage is increased, the retention times decreases proportionally for all the solutes but the resolution is unaffected. The efficiency is also similar at all voltages for the nucleosides whereas for the nucleotides the efficiency increased as the voltage increased. This is in agreement with the fact that their separation is mainly due to their electrophoretic mobility and that an increase in the applied voltage leads to a decrease in their longitudinal diffusion [22]. A standard constant voltage of 12 kV or a constant current of 38 μA was generally used throughout this work because

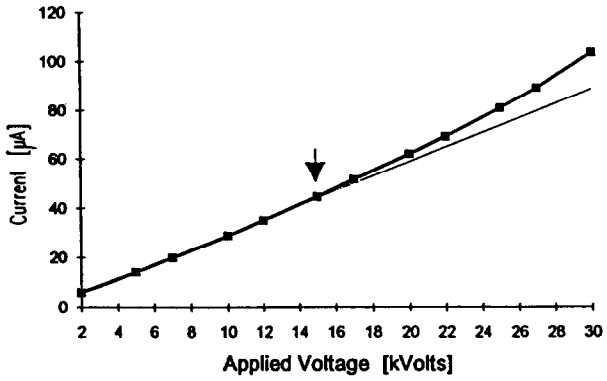

Fig. 2. Plot of current *versus* applied voltage at 35°C. Constant-voltage mode from 2 to 30 kV.

the time of analysis was found to be adequate for our fraction collection purposes.

Run-to-run repeatability and day-to-day reproducibility. Run-to-run values of R.S.D. range from 0.2 to 1.5% for the retention time and from 0.1 to 0.8% for the retention time relative to 2'dC. The day-to-day reproducibility of different series of separations, some also with different capillaries and preparations of running buffer, ranges from 0.5 to 4.2% for the retention time relative to 2'dC. It was noted that different preparations of running buffers lead to slight differences in current with consequent slight variations of the retention time. This is probably the most important factor that influences the reproducibility.

Efficiency and resolution. For the 57 cm × 50 μm I.D. under our standard operating conditions (electrical field of 21.05 kV/m) and 1 pmol of each standard loaded, the efficiency for the nucleosides is of 350 000 plates/m, greater than that of the nucleotides, 237 000 plates/m. This reflects the greater band broadening that occurs for the later eluting peaks [22]. With regard to the resolution, except for 2'dA3'mP and 2'dC3'mP, it is sufficient to allow the collection of each single peak.

Minimum detectable concentration. The minimum detectable concentration (MDC) was calculated using the equation $MDC = 3C(N/S)$ where C = sample concentration, N = detector noise and S = peak signal; the factor 3 represents an arbitrarily chosen minimum signal-to-noise level. The minimum detectable mass (MDM) was calculated as the product of MDC and the injection volume. With on-column UV detection at 254 nm the MDM with a signal-to-noise of 3 was *ca.* 100 fmol. However, given the small injection volumes typically required for good CE performance, the MDC was only from 1–6 μg/ml for a 10-nl injection volume. This is not sufficient to allow the detection of adducts in DNA samples. Moreover, the UV detector does not allow the characterization of the molecules detected unless standards are available.

Sample injection reproducibility

Previous comparison of injection methods have generally shown hydrodynamic injections to be more reproducible and linear than electrokinetic injections, primarily because of fluctuating local electric fields at the capillary inlet during electrokinetic injection. It has also been demonstrated that electrokinetic injection induces a sample discrimination that occurs as a consequence of the different mobilities of the sample species [24]. Both procedures depend on the viscosity of the sample solution and therefore care should be exercized with respect to the temperature control of the sample solution for good quantitative reproducibility. Injection volumes can be calculated using the Poiseuille equation: for a capillary with an I.D. of 50 μm and a length to the detector (L_d) of 50 cm (6.9 cm less than the total capillary length), the volume of water (viscosity at 30°C = 79.8 P) injected per second is 1.2 nl [25].

To examine the precision of the sample size injected, the peak areas and the peak heights of each solute were calculated under constant injection conditions with five repetitions. The sample injection was performed by pressure for 20 s. The results are shown in Fig. 3a and b. The peak-area and peak-height values were reasonably constant, increasing slightly with successive repetitions because of the evaporation occurring in the sample vial (a 30-μl vial with 10 μl of sample). This can be limited by using a 400-μl vial instead of the 30-μl vial. Other variations can be attributed to errors in the calculation of the area by the Gold software because of the fluctuations of the baseline.

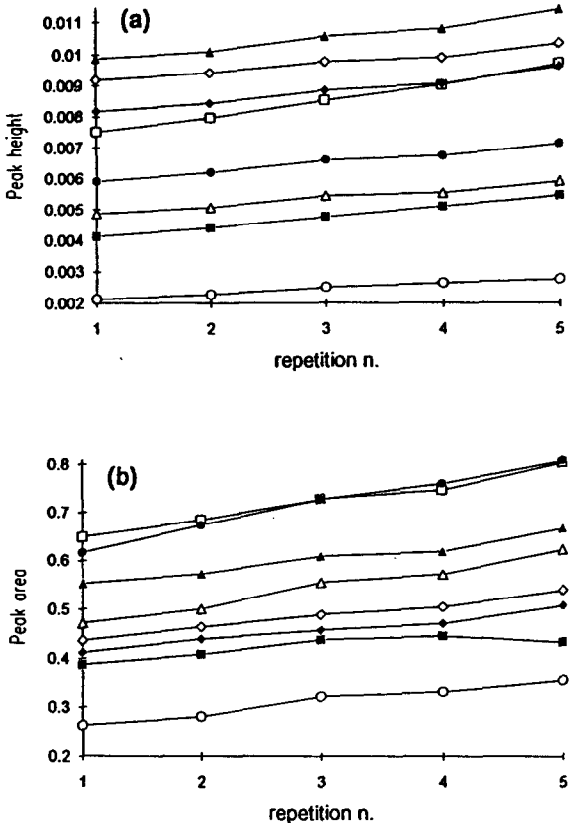

Fig. 3. Plots of (a) peak height and (b) peak area for repeated injections (in arbitrary units as reported by the Beckman Gold peak integrating software). Standard conditions, sample concentration at 0.1 pmol/nl, 20-s pressure injection. ■ = 2′dC; □ = 2′dA; ◆ = 2′dT; ◇ = 2′dG; ▲ = 2′dl; △ = 2′dA3′mP; ● = 2′dC3′mP; ○ = 2′dT3′mP.

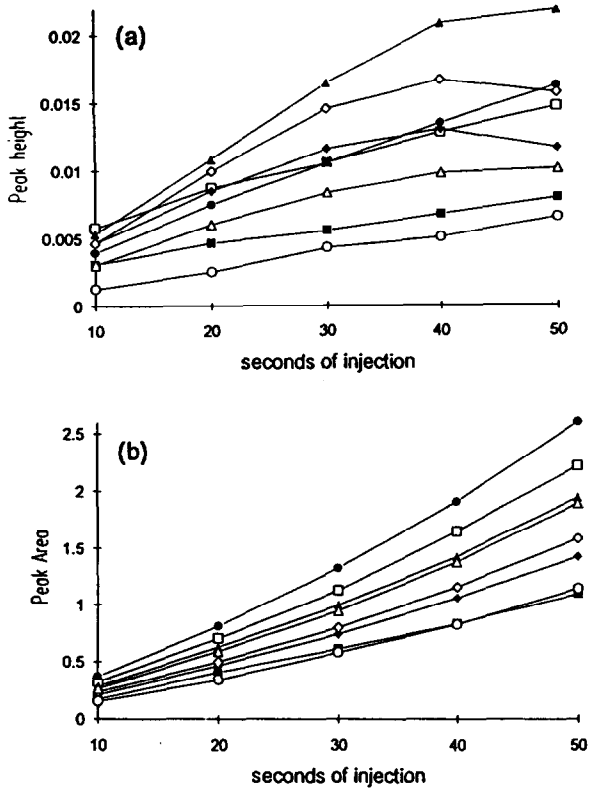

Fig. 4. Plots of (a) peak height and (b) peak area for increasing times of pressure injection (in arbitrary units as reported by the Beckman Gold peak integrating software). Standard conditions, sample concentration at 0.1 pmol/nl. Symbols as in Fig. 3.

Sample injection linearity

To determine the sample injection linearity for peak height and peak area we made successive runs with increasing injection times (from 10 to 50 s). Each injection was performed in triplicate. Plots are shown in Fig 4. For a sample concentration of 0.1 pmol/nl the peak-height values reached a plateau at 40 s of injection (4 pmol injected), then no further gain in detectability was obtained for any of the peaks. In contrast, for the peak area, the plateau was not already reached even with a 50-s injection. The slight deviation from the linearity in the plot of peak area *versus* time of injection is due to the evaporation that occurs during the successive runs. To avoid band broadening problems, the injection time must be kept as short as possible (<40 s), otherwise the peak shape and efficiency and consequently the resolution will be compromised. Peak areas were used for quantitative analysis.

Maximum loadability

In comparison with HPLC, CE is more limited with respect to the maximum mass load. Consequently, the amounts of material collected from capillaries are relatively small, which constrains further manipulation and analysis. To alleviate this problem, the mass load may be increased by increasing the capillary diameter (in our case ideally >50 μm). However, eventually the generation of Joule heat caused by the passage of electric current through the capillary will limit the separation efficiency and may impair the

integrity of biological samples. Another approach to solve the mass load problem in micropreparative work is to perform multiple runs with the same collection vial. Thus, the concentration of analyte in the collection vial should increase in proportion to the number of runs.

Peak shape and efficiency deteriorate with increasing injection time and/or sample concentration, owing to the increase in peak width. To establish the limit of overloading with reasonable resolution, we made successive runs with increasing solute concentrations (from 0.125 to 4 pmol/nl) with an injection time of 10 s (*ca.* 10 nl injected). Chromatograms are shown in Fig. 5. At the lowest concentration injected (1.25 pmol injected), the efficiency was at the maximum observed. Apart from 2'dT, which showed peak tailing when overloaded (>10 pmol), the efficiency and the resolution of the other nucleosides remained very good, allowing even higher loadability. The resolution of 2'dA3'mP and 2'dC3'mP began to be degraded above 10 pmol injected, and at 40 pmol we observed the appearance of a third, unidentified, peak eluting before 2'dA3'mP. These two peaks, however, could not be easily collected separately. The efficiency of 2'dT3'mP deteriorated above 20 pmol injected and the peak shape for 2'dG3'mP worsened considerably with overloading. In conclusion, for overloaded runs, concentrated samples should be used, when available, with the time of injection kept as short as possible to main good efficiency.

Fraction collection

Fraction collection with CE is technically different from that with HPLC. With electroelution, the end of the capillary must remain in contact with a solution containing buffer and an electrode during the collection of the fraction in order to maintain the electric field that drives the separation. Immediately prior to this step, the field is temporarily interrupted while the outlet buffer vial is replaced with a vial in which the fraction is to be collected. The volume in the latter vial must be kept small (<15 μl) to minimize analyte dilution. As an alternative to electroelution, pressure-driven mobilization can be used to collect fractions [26].

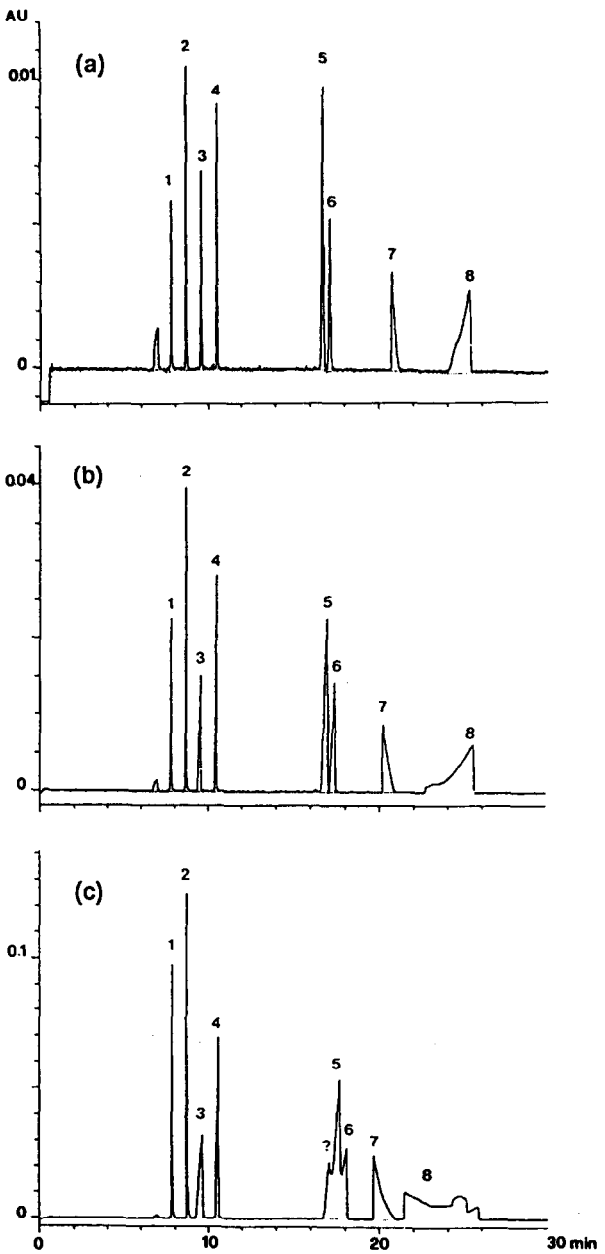

Fig. 5. Electropherograms of increasing concentration of standard mixture with 10-s pressure injection: (a) 0.25; (b) 1; (c) 4 pmol/nl. Peaks: 1 = 2'dCyd; 2 = 2'dAdo; 3 = 2'dThd; 4 = 2'dGuo; 5 = 2'dAdo3'mP; 6 = 2'dCyd3'mP; 7 = 2'dThd3'mP; 8 = 2'dGuo3'mP.

We chose to collect two fractions of nucleosides (2'dT and 2'dG) and one of nucleotides (2'dA3'mP–2'dC3'mP). Initial runs were performed to establish the migration time

precision under our standard conditions (see Figs. 8a and 9a). After verifying adequate precision, the retention time of the leading edge (t_{R1}) of the peak and the peak width was determined. The retention times are those on the appearance of the peak in the detector window. The time that it takes to reach the end of the capillary (t'_{R1}) is calculated as follows: $t'_{R1} = (L/l)t_{R1} =$ (56.9 cm/50 cm)t_{R1}, where L is the total length of the capillary and l the length from the inlet to the detector window. During the collection period the field strength is reduced to 10 μA and the autosampler positions the collection vial containing 5 μl of buffer without SDS at the outlet end of the capillary. The start of the peak collection period is calculated as follows: $t'_{R1} - 0.1 - 0.2 = t_{R1s}$ (0.2 min for safety margin and 0.1 min to compensate for peak migration during the ramp-down period from 38 to 10 μA). The duration of the collection period at 10 μA is calculated as (0.2 + peak with + 0.2) · 38 μA/10 μA. When two successive peaks were collected the current was maintained at 10 μA and the corresponding times for collection were calculated in the same way. For multiple fraction collection, several micropreparative separations in an automated sequence were run successively into the same collection vial.

Recovery

After the micropreparative sequence was completed, the pooled fractions were re-injected to determine the recovery. For the first fraction collected (2'dT overloaded at a concentration of 4 pmol/nl in a mixture of only 2'dC and 2'dT injected for 40 s in nine successive runs) and re-injected for 60 s, we obtained a peak with a very poor shape (Fig. 6). Using the standard 2'dT in a mixture of 5 μl of running buffer without SDS plus 1 μl with SDS (final concentration 0.5 pmol/nl, similar to that of the collected fraction), we tried increasing the time of injection from 10 to 60 s (Fig. 7). Up to a 20-s injection time (*ca.* 10 pmol loaded) the peak had a good shape. For injection times ≥30 s the peak height decreased whereas the peak width increased, leading to a very poor shape of the peak. This is a typical matrix injection-related effect: the high salt concentration and the presence of SDS in the fraction vial lead to zone spreading at the injection end of the capillary when the high voltage is applied. Therefore, we observed peak broadening and slight variations in the retention times of the re-injected fractions (Figs. 8b and 9b). For this reason, the time of re-injection of the fractions collected must be kept as short as possible (<30 s). These effects could be reduced by using a lower salt concentration in the buffer placed in the collection vial.

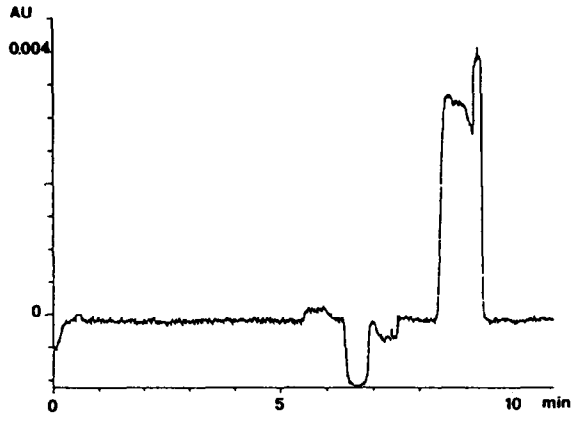

Fig. 6. Electropherogram of the re-injected pooled fraction (nine separations with 40-s pressure injection and a sample concentration of 4 pmol/nl) of 2'dT: 60-s pressure injection, approximate concentration 0.34 pmol/nl.

To determine the mass contained in the collected peak, we first evaluated the amount of solute loaded on the capillary in each run (concentration in pmol/nl × injection volume at 1.2 nl/s). The total amount loaded was deduced by multiplying by the number of successive runs. As this amount was collected in a fraction vial containing 5 μl of buffer, we estimated the concentration that should be obtained in an hypothetical 100% recovery, and consequently the amount that should be found in the re-injected fraction. The effective amount of the re-injected fraction was calculated either by the external or internal standard method and the recovery was then deduced. The values were always >75% (Figs. 8b and 9b).

FAB–MS of the collected fractions

The further analysis of the collected 5-μl fractions by FAB-MS posed several problems. The extremely high SDS concentration, com-

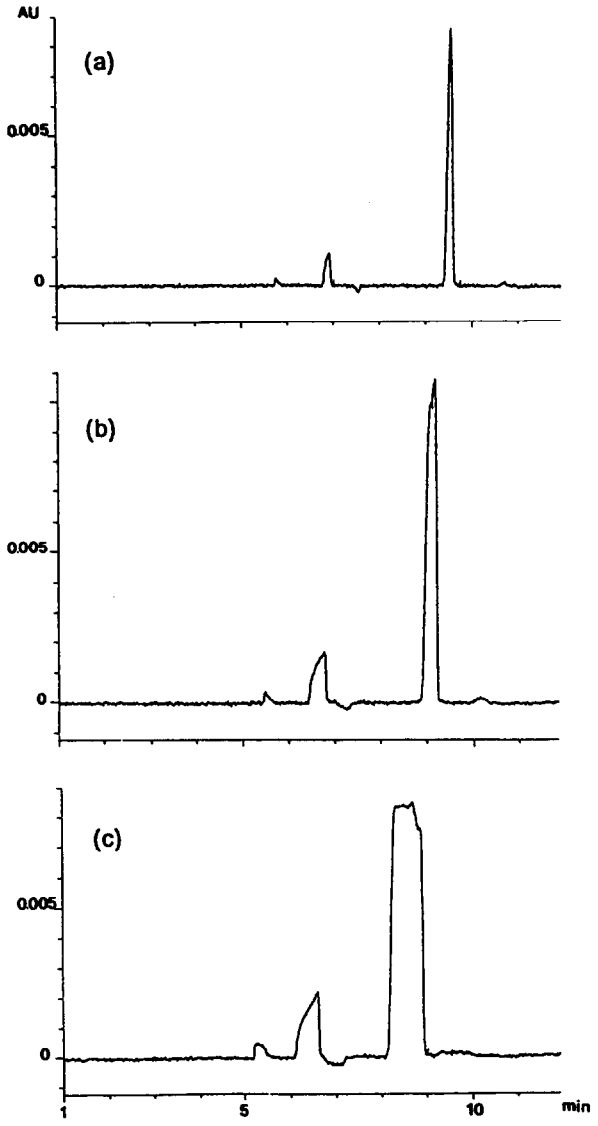

Fig. 7. Electropherograms of standard 2′dT (final concentration 0.5 pmol/nl) in a mixture of 4 μl of buffer without SDS + 1 μl of buffer with SDS injected for increasing times of pressure injection: (a) 10 s; (b) 30 s; (c) 60 s.

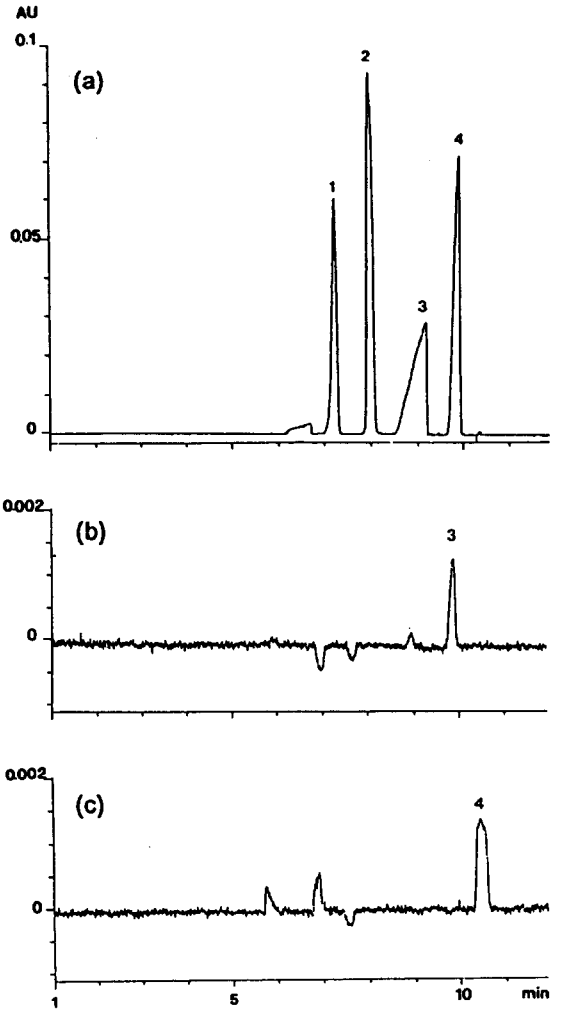

Fig. 8. Nucleoside fraction collection. (a) Electropherogram of the mircopreparative separation: 30-s pressure injection, sample concentration 2 pmol/nl. Peaks: 1 = 2′dCyd; 2 = 2′dAdo; 3 = 2′dThd; 4 = 2′dGuo. (b) Electropherogram of the re-injected pooled fraction (six repetitions) of 2′dT: 20-s pressure injection. Fraction concentration *ca.* 0.05 pmol/nl, recovery 70%. (c) Electropherogram of the re-injected pooled fraction (five repetitions) of 2′dG: 20-s pressure injection. Fraction concentration *ca.* 0.06 pmol/nl, recovery 83%.

pared with the analyte concentration in the fraction, produced a very intense background signal with strong chemical noise at the lower masses. The concentration of the collected nucleosides and nucleotides was too low for an appreciable signal in the presence of this high SDS background. This is probably due to an ion suppression effect of the SDS against the analytes present in the glycerol matrix. The ion suppression effect is a well studied phenomenon [27] in which a compound present in a sample will be recorded at an unusually low intensity or not at all. This has been found to be the result, in large part, of the tendency of the hydrophilic compounds to migrate to the interior of the sample droplet, away from the liquid/vacuum

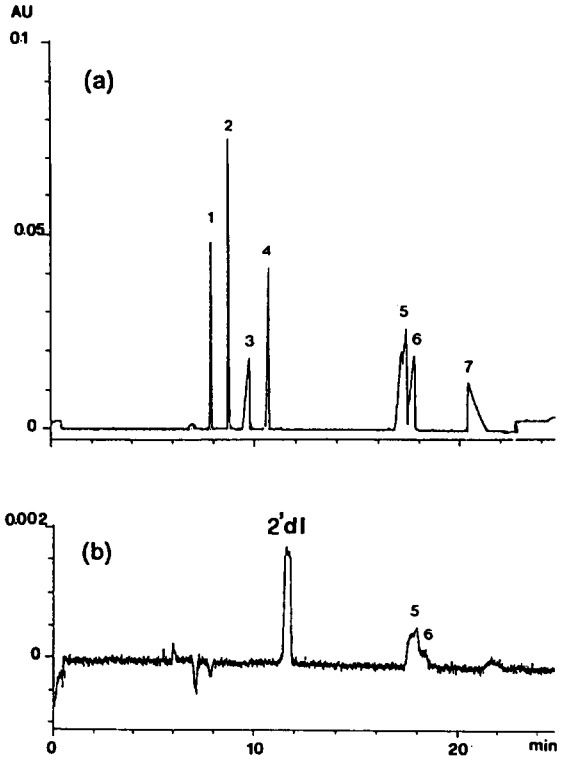

Fig. 9. Nucleotide fraction collection. (a) Electropherogram of the micropreparative separation: 10-s pressure injection, sample concentration 2 pmol/nl. Peaks: 1 = 2'dCyd; 2 = 2'dAdo; 3 = 2'dThd; 4 = 2'dGuo; 5 = 2'dAdo3'mP; 6 = 2'dCyd3'mP; 7 = 2'dTh3'mP. (b) Electropherogram of the re-injected pooled fraction (six repetitions) of 2'dA3'mP–2'dC3'mP: 20-s pressure injection. Internal standard, 2'dI. Fraction concentration *ca.* 0.22 pmol/nl, recovery 93%.

interface. At the same time, hydrophobic compounds tend to migrate to the surface layers of the droplet, suppressing the ionization of other compounds. With SDS we expect it to form a layer on the surface of the glycerol droplet, suppressing completely the ionization of the more hydrophilic nucleosides and nucleotides that migrate to the interior of the droplet (Fig. 10). Indeed, nucleosides in a pure glycerol matrix produced good spectra compared with those from fractions in the presence of SDS, presenting no signal at all for the collected nucleoside or even for the glycerol matrix.

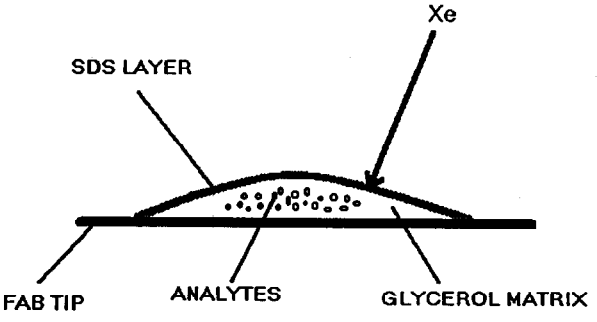

Fig. 10. Schematic representation of the situation at the FAB tip in the presence of SDS.

CONCLUSIONS

Fraction collection after an optimized MECC separation of nucleic acid constituents has been successfully performed. The recoveries were relatively high (>75%). Nevertheless, the presence of high concentrations of SDS in the collected fractions posed a problem in FAB-MS owing to a strong ion suppression effect. It seems that these types of fractions cannot be directly analysed, at least by FAB-MS, without an intermediate step that could drastically reduce the presence of SDS. This may, however, have the serious disadvantage of leading to sample loss and/or eventual modification in an already very reduced sample volume and concentration. We nevertheless expect an improvement by using a dynamic FAB system, such as CF-FAB, coupled to tandem MS. This will be the subject of future work.

This type of separation with UV detection could still be extremely useful for many purposes, *e.g.*, for the rapid control of the DNA digestion to nucleotides, for the degree of its degradation or digestion in nucleosides, for the determination of RNA contamination and for the detection and determination of normal and some modified deoxy- and ribonucleosides or nucleotides. Moreover, fraction collection after MECC separations could be of interest for other spectroscopic investigations less sensitive to SDS interference.

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