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# Micellar electrokinetic capillary chromatographic separation and laser-induced fluorescence detection of 2′-deoxynucleoside 5′-monophosphates of normal and modified bases

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ABSTRACT

Micellar electrokinetic chromatography is used to separate dansylated nucleotides, both normal and modified species. The high separation power allows detection of minor components present in less than 1 part per thousand of the major components. Laser-excited fluorescence is used to detect the separated components at the $6 \cdot 10^{-18}$ mol level or $10^{-9}$ *M* injected material. Combined with high-performance liquid chromatographic enrichment prior to labeling, this technique can be used to assess DNA damage in carcinogenesis studies.

## INTRODUCTION

In the past few years, capillary electrophoresis (CE) has emerged as a promising bioanalytical technique [1]. Separation is effected by the distinct electrophoretic mobilities of the analytes and, as a consequence, only charged species can be resolved [2]. Micellar electrokinetic capillary chromatography (MECC) is a major subdivision of CE [3]. A surfactant at concentrations greater than its critical micellar concentration is added to the buffer solutions. Neutral analytes can be resolved as separation is based upon differences in partitioning behavior between the aqueous and the micellar phases. However, the mechanism of separation for ionic species is a convolution of both electrophoretic migration and partitioning. Hence, MECC introduces additional selectivity in the separation of charged species with very similar electrophoretic mobilities.

The application of CE to the analysis of nucleosides and nucleotides is well documented [4–15]. Detection in CE can utilize modified UV–VIS high-performance liquid chromatography (HPLC) detectors, conductance, electrochemical

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(EC), $^{32}$P-radiochemical (RC) and fluorescence (FL) methods along with mass spectral interfacing. The more general detection techniques such as UV–VIS absorbance and conductivity method lack the sensitivity necessary to be broadly applicable to CE. EC detection offers much higher sensitivity but its application is limited by the fact that only electroactive species are amenable to detection. RC detection of nucleic acids has been restricted, thus far, to the $\alpha$-$^{32}$P-labeled triphosphates of A, C and T [11]. Detection by mass spectral interfacing involves costly instrumentation and lacks adequate sensitivity. An on-column FL detector is not yet commercially available. Kuhr and Yeung [13] reported detection of 5′-monophosphates of normal ribonucleosides by indirect laser-induced fluorescence (LIF) detection [limit of detection (LOD) 70 amol at a signal-to-noise ratio of 3]. Due to the lower background and higher signal-to-noise ratio, further improvement in detection sensitivity is expected using laser-induced direct detection of fluorescent-labeled nucleotides.

Kelman *et al.* [16] developed a novel assay for DNA damage by combining enzymatic digestion of DNA with fluorescence post-labeling. Briefly, DNA is digested enzymatically to 2′-deoxynucleoside-5′-monophosphates with normal bases (dNmp, N = A, C, G, T) and modified bases. The modified nucleotide is enriched from dNmp by HPLC and labeled with a fluorescent tag. The labeled nucleotides are analyzed by HPLC with fluorescence detection. The labeling procedure involves 5′-phosphoramidation with ethylenediamine followed by *in situ* conjugation of the free amino end with dansyl chloride. The efficiency of the labeling procedure is quantitative and has been found to work well with both normal as well as polar, alkylated and bulky aromatic modified nucleotides such as 8-hydroxydGmp (8-OHdGmp), 5-methyldCmp (5-MedCmp) and 8-(N-2-acetylaminofluorene)dGmp (8-AAFdGmp), respectively. 8-OHdGmp is one major modified nucleotide identified when DNA is exposed to ionizing radiation [17]. 5-MedCmp is the only naturally occurring modified nucleotide yet found in mammalian DNA. A large body of experimental data suggest the association of DNA methylation with gene activity [18]. 8-AAFdGmp is the major adduct of DNA modification by the chemical carcinogen N-acetoxy-N-2-acetylaminofluorene [19]. Using conventional HPLC with a conventional FL detector, the sensitivity of the fluorescence post-labeling assay allowed detection of one modified nucleotide per $10^6$ normal nucleotides from a 100-$\mu$g DNA sample [20]. In order to enhance the detection sensitivity, an FL detector with helium–cadium CW laser as an excitation source has been developed with the cell design of Kuhr and Yeung [21]. Analysis of the dansylated nucleotides by microbore HPLC coupled to the LIF detector is currently under investigation.

In this context, MECC appears to be the ideal method for the analysis of dansyl-labeled nucleotides and their structurally similar derivatives. In the present work, the extremely high sensitivity of LIF detection is combined with mixed-mode MECC separation to allow the determination of modified nucleotides in the presence of normal nucleotides at the attomole level.

## EXPERIMENTAL

### *Chemicals*

dNmp and 5-MedCmp were obtained from Sigma (St. Louis, MO, U.S.A.). 8-OHdGmp and 8-AAFdGmp were synthesized as reported earlier [19,20]. Dansylation of the monophosphates and the calf thymus DNA digest were carried out as described previously [16]. Sodium dodecyl sulfate (SDS) was purchased from Gallard-Schesinger Industries (Carle Place, NY, U.S.A.) and was recrystallized twice from 95% ethanol before use.

### *Capillary electrophoresis*

The CE instrument used has been described previously [21]. A 75-cm fused-silica capillary (20 μm I.D., 150 μm O.D.; PolyMicro Technologies, Phoenix, AZ, U.S.A.) was rinsed with a 50:50 (v/v) methanol–water mixture for 30 min followed by a 0.05 *M* aqueous sodium hydroxide solution for the same period of time. After equilibrating for 24 h with the buffer solution (0.010 *M* $Na_2HPO_4$, 0.007 *M* $Na_2B_4O_7$, 0.045 *M* SDS, pH 9.0), the capillary was ready for use. All separations were performed at 30 kV and samples were injected hydrodynamically by raising the level of the sample vial 32 cm above the exit end for 6.0 min (injected volume, 6 nl).

### *Fluorescence detection*

Several important differences exist between the previous and present set-ups [21]. An argon ion laser (Model 2045, Spectra-Physics, Mountain View, CA, U.S.A.) operating at 350 nm was used to excite on-column fluorescence. The beam passes through a laser power stabilizer (Cambridge Research and Instrumentation, Cambridge, MA, U.S.A.) and then through a band-pass filter (Type UG-1; Schott Glass Technologies, Duryea, PA, U.S.A.) to remove plasma emission lines before it was focused on the capillary. Three Schott long-pass filters (one CG435 and two CG455) were employed to remove scattered light and the detection region was located at 15 cm from the cathodic end of the capillary.

## RESULTS AND DISCUSSION

Fig. 1 is an electropherogram showing the separation of a mixture of dansyl dNmp, 8-OHdGmp, 5-MedCmp and 8-AAFdGmp prepared from HPLC-purified samples. Separation of the first six components took less than 10 min while the whole separation was completed in approximately 16 min. The peak at 7.6 min resulted from 5-dimethylaminonaphthalene sulfonate (deprotonated form of dansyl hydroxide) whereas the broad, short peak at about 15.8 min was probably due to polymers. Both are derivatized forms of impurities that were still present in the HPLC-purified samples. Other than this, baseline separation of all the components was achieved. The unusually long migration time of dansyl 8-AAFdGmp

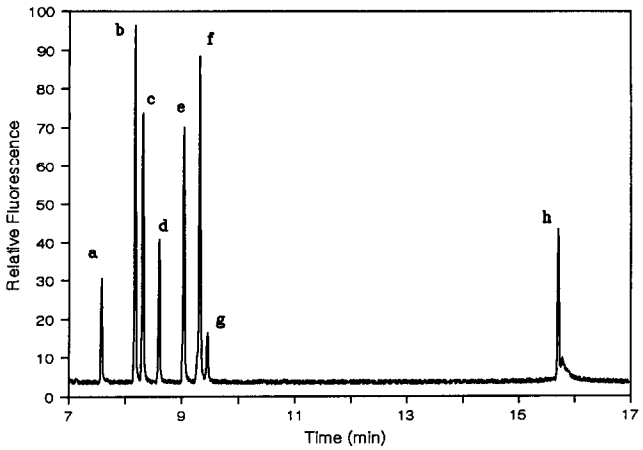


Fig. 1. MECC–LIF analysis of deprotonated dansyl hydroxide (a), dansyl dTmp (b), dansyl dCmp (c), dansyl 5-MedCmp (d), dansyl dAmp (e), dansyl dGmp (f), dansyl 8-OHdGmp (g), and dansyl 8-AAFdGmp (h).

relative to the other components can be understood in terms of the more hydrophobic nature of the former arising from its fluorenyl moiety, which favorably enhances the partitioning of dansyl 8-AAFdGmp into the slow-moving micellar phase. The fact that the pyrimidine derivatives (dansyl dCmp and dTmp) as well as the purine derivatives (dansyl dAmp and dGmp) possessed similar migration times is not surprising in light of the strong resemblances in their structures. The same reasoning applies to the close proximity of the peaks representing dansyl dGmp and its 8-hydroxy derivative. In fact, dansyl dCmp and dTmp could not be resolved at a wide range of solution acidities based solely upon differences in their electrophoretic mobilities when micelles were not used (data not shown). This illustrates the ability of MECC in resolving components with very similar electrophoretic mobilities through the introduction of an additional parameter to the separation mechanism, namely, selective partitioning between the aqueous and micellar phases. The high efficiency of MECC can readily be appreciated by comparing the theoretical plate number of 120 000 for dansyl dTmp obtained in the present work with that of 5000 in an HPLC separation where dansyl dCmp and dGmp were not totally resolved. This is despite the shorter analysis time of 9.5 min with MECC relative to the 19 min required for the HPLC procedure in the determination of the dansyl dNmp [16].

Depending on the sample preparation steps, it may be desirable to use larger capillaries so that the injection volume can be increased. The same separation was attempted using a 50 $\mu$m I.D. capillary, but adequate resolution of the components was not possible at 30 kV. In addition, it was noticed that as the applied potential decreased, a corresponding improvement in the separation was realized. Baseline resolution of all the components was achieved at 10 kV (data not shown) with an analysis time three times that with the 20-$\mu$m capillary at 30 kV. The fact

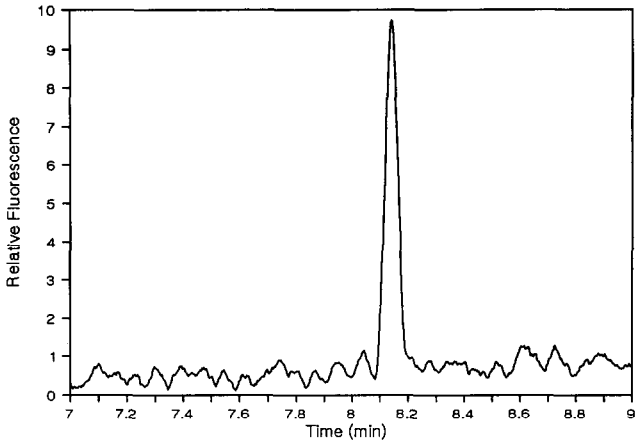


Fig. 2. Electropherogram showing detection of 6 amol of dansyl dTmp (data smoothed with a 31-point Golay-Savitsky procedure).

that higher separation efficiencies at 30 kV were possible with the 20-$\mu$m capillary than with the 50-$\mu$m one can be explained by the reduced heating of the 20-$\mu$m capillary due to its smaller I.D. This is because roughly six times more Joule heat is generated in a 50-$\mu$m capillary than a 20-$\mu$m one, resulting in serious zone broadening due to turbulent mixing in the former. In a theoretical study of MECC, the efficiency of analytes which partition poorly into the micellar phase is predicted to increase with applied potential [22]. Indeed, this is the case for the 20-$\mu$m separation in the present study, where the same separation carried out at

TABLE I

STATE-OF-THE-ART LIMITS OF DETECTION WITH HPLC AND CE

| Detection | HPLC | | CE | |
|---|---|---|---|---|
| | Amount (mol) | Concentration ($M$) | Amount (mol) | Concentration ($M$) |
| Absorption | $2 \cdot 10^{-9}$[a] | $2 \cdot 10^{-4}$[a] | $7 \cdot 10^{-14}$[b] | $3 \cdot 10^{-5}$[b] |
| Electrochemical | $2 \cdot 10^{-13}$[c] | $2 \cdot 10^{-8}$[c] | – | – |
| Fluorescence | $2 \cdot 10^{-13}$[d] | $2 \cdot 10^{-8}$[d] | $6 \cdot 10^{-18}$[e] | $1 \cdot 10^{-9}$[e] |
| Radiochemical | – | – | $7 \cdot 10^{-18}$[f] | $1 \cdot 10^{-10}$[f] |

[a] From ref. 16 for dansyl dNmp.
[b] From ref. 15 for dAmp, dCmp, dGmp, dUmp.
[c] From ref. 24 and 26 for 8-OHdG.
[d] From ref. 16 for dansyl dNmp.
[e] This study.
[f] From ref. 11 for Atp, Ctp, Ttp.

15 kV showed poorer efficiency (data not shown) than at 30 kV. The discussion above points to the importance of LIF in MECC because the separation power does not have to be compromised to maintain useful sensitivity and speed of analysis, which would have been the case when wider capillaries, necessary for conventional FL, UV and RC detection schemes, were used.

The remarkably high sensitivity of LIF is demonstrated in Fig. 2 where the peak represents dansyl dTmp. As expected from the more hydrophobic environment, the fluorescence signal increased in the presence of micelles. However, the background signal also increased (scattering and impurity fluorescence). So, net detectability was not influenced by the micelles. According to the method of Knoll [23], the LOD for dansyl dTmp here is estimated to be 6 amol or roughly 4 million molecules. From Fig. 1, the variations in sensitivity among the dansyl dNmp are all within 26% of that for dansyl dTmp. It is, therefore, reasonable to expect similar LODs from the remaining components. Table I contains a summary of the state-of-the-art LODs with various detection schemes coupled to HPLC or CE in the determination of derivatized or native nucleosides (N), nucleoside-5′-monophosphates (Nmp), nucleoside-5′-diphosphates (Ndp), nucleoside-5′-triphosphates (Ntp), 2′-deoxynucleoside (dN), dNmp, 2′-deoxynucleoside-5′-diphosphates (dNdp) and 2′-deoxynucleoside-5′-triphosphates (dNtp). Several interesting points are noted. First, the mass LODs obtained with CE are typically five orders of magnitude lower than those with HPLC when the same type of detection scheme is employed. This is primarily a consequence of the smaller sample size and peak volumes of CE *versus* conventional HPLC. A comparison of the concentration LODs shows only a difference of one order of magnitude. In the case of the concentration LODs with FL detection, the ten-time improvement in CE over HPLC is probably due to the employment of LIF and the sharper peaks in the former *versus* a conventional FL detector in the latter. Secondly, the LODs of FL or RC are often 1000 times lower than that of UV detection. The explanation for this is that FL and RC measurements are, in theory, shot-noise-limited whereas UV detection is frequently hindered by flicker noise. Thirdly, the mass LOD in the present study is better than those with HPLC–FL by $3 \cdot 10^4$ times, HPLC–UV by $3 \cdot 10^8$ times, CE–UV by $1 \cdot 10^4$ times and matches the 7-amol level obtained for adenosine-5′-triphosphate (Atp), cytidine-5′-triphosphate (Ctp) and thymidine-5′-triphosphate (Ttp) labeled with $\alpha$-$^{32}$P in a CE–RC scheme [11]. Fourth, CE–RC can separate and detect $^{32}$P-labeled Atp, Ctp and Ttp present at a concentration of $10^{-10}$ *M*, an order of magnitude lower than that for CE–LIF which, in turn, is still 20 times better than HPLC–FL, $3 \cdot 10^4$ times CE–UV and $2 \cdot 10^5$ times HPLC–UV. However, a procedure has not been devised to assay DNA damage in the form of $^{32}$P-labeled dNmp, dNdp or dNtp using CE–RC at the present moment, even though separation of dAmp, dGmp, dTmp and dCmp with CE [5] and enzymatic labeling of 8-OHdG with $\gamma$-$^{32}$P-tagged Atp [2] have already been accomplished. Furthermore, in our studies using 50-$\mu$m capillaries, a concentration LOD of $10^{-10}$ *M*

was also obtained. This is due to the larger signals and improved stray-light rejection. So, if a longer analysis time is acceptable (see above), FL is competitive with RC detection. Fifth, assuming the modified forms of the nucleotides possess similar sensitivities as the unmodified versions, what was mentioned previously regarding the LODs of the latter is also applicable to the former. Sixth, HPLC–EC offers a mass and concentration LOD for 8-OHdG [24] which are inferior to those of CE–LIF by $3 \cdot 10^4$ and 20 times, respectively.

It has been claimed that $^{32}$P-postlabeling [25] and HPLC–FL [20] each allows the detection of one residue of 8-OHdG in $10^6$ normal nucleotides from a 100 $\mu$g size DNA sample. Using the same argument, CE–LIF is able to detect one residue in $10^9$ normal nucleotides from a 3 $\mu$g size DNA sample. In practice, this kind of sensitivity is difficult, if not impossible, to demonstrate because a procedure capable of selectively removing a large portion of the components excluding the

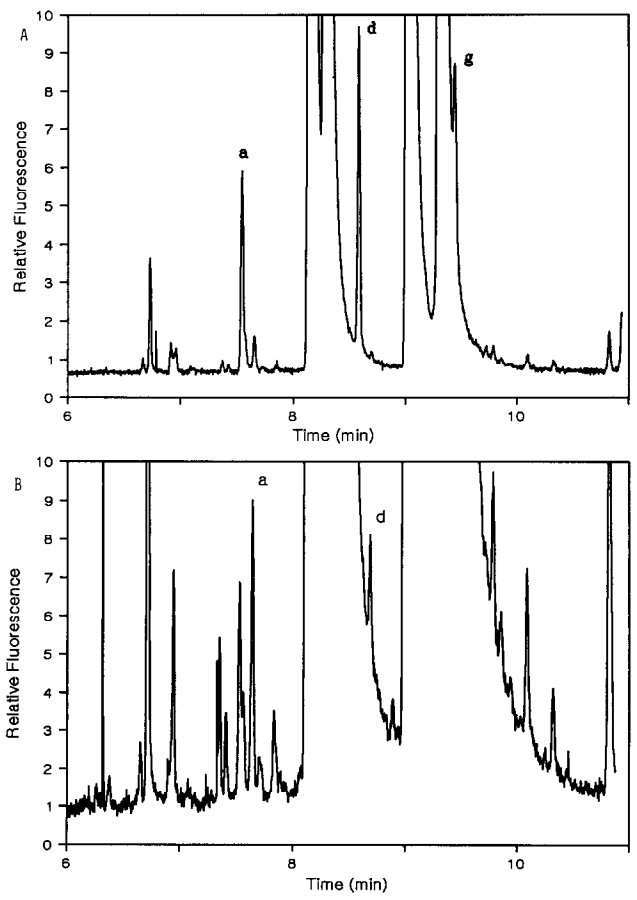


Fig. 3. (A) Electropherogram of dansyl dNmp ($10^3$ excess), dansyl 8-OHdGmp (g) and dansyl 5-MedCmp (d). (B) Electropherogram of dansyl dNmp ($10^4$ excess), dansyl 8-OHdGmp and dansyl 5-MedCmp.

8-OHdG residue has to be devised, such that the peak of dansyl 8-OHdGmp at its LOD can still be discerned in the presence of peaks due to all the other components in the same electropherogram. Fig. 3A shows the detection of dansyl 8-OHdGmp and 5-MedCmp, each present at a concentration $10^3$ times smaller than those of dansyl dNmp. One can clearly distinguish the peaks corresponding to dansyl 8-OHdGmp and 5-MedCmp albeit peaks resulting from impurities in the dansyl dNmp samples can be seen. However, the same cannot be said about Fig. 3B which depicts an electropherogram of dansyl 8-OHdGmp and 5-MedCmp with $10^4$ times more dansyl dNmp present. Hence, the practical LOD for both 8-OHdGmp and 5-MedCmp in the presence of an excess of dNmp that can be attained by fluorescence post-labeling [16], as proposed by Sharma *et al.* [20], with CE–LIF lies between a normal to modified nucleotide ratio of $10^3$ and $10^4$. Therefore, prior to labeling, the enrichment of the modified nucleotide from the normal nucleotides in the digest is critical to the success of DNA damage studies by CE at its detection limit.

Because of the large quantity of excess reagents relative to analytes, it is crucial that the dansylated impurities elute far from the analytes of interest in the analysis of real samples which have not been purified with HPLC. Otherwise, the analyte baseline would be obscured by peaks from the impurities. With capillary zone electrophoresis (CZE) (*i.e.* in the absence of micelles, data not shown), the major impurity peak not only eluted before those of the analytes, but the impurities also appeared to adhere strongly to the inner wall of the capillary, resulting in severe tailing. This renders the identification and quantitation of the analytes impossible. Fig. 4 shows the elecropherogram of a dansylated mixture of dNmp from a digested calf thymus DNA sample, using the same labeling scheme and analyzed with MECC–LIF. Prominent in the electropherogram is the large peak due to the ionized form of dansyl hydroxide (a), which has been identified as a

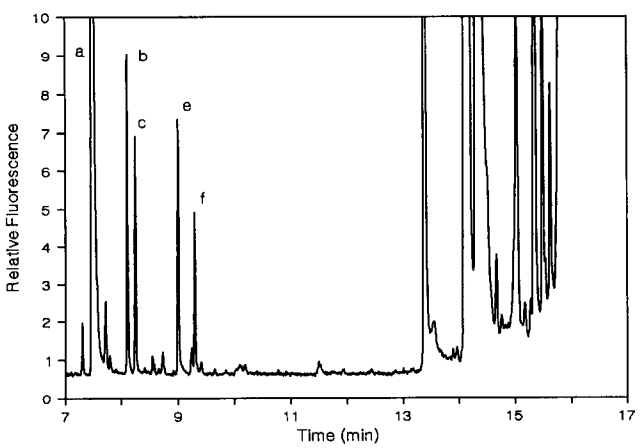


Fig. 4. MECC–LIF analysis of dansylated calf thymus DNA digest.

major impurity in the derivatization process [5]. In contrast to CZE, MECC was able to slow down the rate of migration of most of the major impurities (with the exception of the ionized from of dansyl hydroxide) and allowed resolution of all the dansylated analytes. Although tailing of the late-eluting impurity peaks still persisted, the extent of tailing was dramatically reduced compared to HPLC. This is probably the result of the stronger partitioning of the impurities into the micellar rather than the aqueous phase. The relative migration times of the impurities in MECC and CZE suggest that the impurities are more hydrophobic in nature than the dansylated analytes due to the fact that hydrophobic compounds partition well into the slow-moving micelles. This, in turn, lends support to the polymeric character of the late-eluting impurities. Naturally, the size of these impurity peaks can be substantially reduced if one limits the amount of excess reagents during the derivatization step. The long migration time of the major impurities can be circumvented by flushing out the column (hydrodynamically) 17 min into each run to speed up the analysis.

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