BECKMAN COULTER

PACE Setter

The worldwide newsletter for capillary electrophoresis

Volume 7, Issue 1 • January 2003

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The worldwide newsletter for capillary electrophoresis

Electrophoretically Mediated Microanalysis: Enzyme Analyses at a Nanoliter Scale

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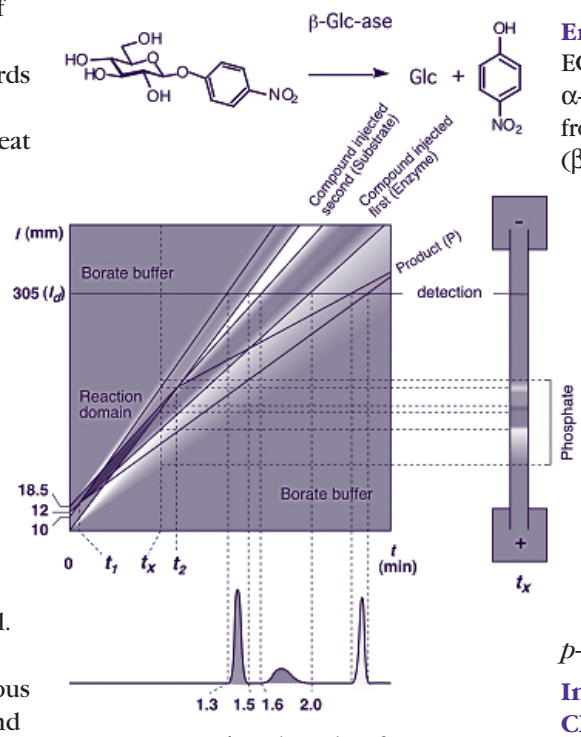
Introduction

Oligosaccharides existing on the cell surface and in the extracellular matrix as components of glycoproteins and glycolipids are involved in a variety of biological phenomena. Methodological investigation directed towards revealing the functions of oligosaccharides is, therefore, of great importance.

We have been studying carbohydrate-related enzymes, especially oligosaccharide processing enzymes, and have reported the usefulness of capillary zone electrophoresis (CZE) in analyzing the post-reaction products. To further scale down the quantity of enzyme required for analysis, we evaluated an approach that uses the capillary not only as a separation field but also as an enzymatic reaction vessel. This electrophoretically mediated microanalysis (EMMA) is advantageous because the amounts of enzyme and substrate can be minimized. The practical investigation of EMMA should prove valuable in future proteomics strategies.

There are basically two ways to mix reaction components in a capillary under electrophoretic conditions: 1) the continuous format and 2) the plug-plug format. In the continuous format, the capillary is filled with one of the reactants while the second component is introduced.

"The practical investigation of EMMA should prove valuable in future proteomics strategies."



In contrast, the plug-plug format is based on a plug-plug interaction. When two compounds forming individual plugs have different electrophoretic mobilities, the reaction proceeds while one of the components is passing through the other. The reaction product is then resolved based on the individual electromobilities in the electric field and passes by the detector along the way to the end of the capillary. In this study, we propose the use of the plug-plug method to obtain the Michaelis constant (Km) of various enzyme reactions.

Experimental

Materials

Enzymes: β-Glucosidase (β-Glc-ase, EC 3.2.1.21) from Sweet almond, α-glucosidase (α-Glc-ase, EC 3.2.1.20) from *Saccharomyces sp.,* β-galactosidase (β-Gal-ase, EC 3.2.1.23) from *Aspergillus oryzae,* β-N-acetylglucosaminidase (β-GlcNAc-ase, EC 3.2.1.52) from bovine kidney, α-mannosidase (α-Man-ase, EC 3.2.1.24) from jack bean, α-fucosidase (α-Fuc-ase, EC 3.2.1.38) from bovine kidney.

Substrates: *p*-Nitrophenyl-β-glucoside (PNP-β-Glc), *p*-nitrophenyl-α-glucoside (PNP-α-Glc), *p*-nitrophenyl-β-galactoside (PNP-β-Gal), *p*-nitrophenyl-β-N-acetylglucosaminide (PNP-β-GlcNAc), *p*-nitrophenyl-α-mannoside (PNP-α-Man), *p*-nitrophenyl-α-fucoside (PNP-α-Fuc).

Internal Standard: Uridine

CE Instrument: P/ACE™ System 5500 (Beckman Coulter)

CE Conditions:

Capillary: 75 µm (i.d.) x 37 cm total length, 30.5 cm to detector (bare fused-silica)
Capillary temperature: 37°C

Separation buffer: 40 mM Sodium borate buffer, pH 9.2
Field strength: 18 kV
Detection: PDA UV detector
Sample introduction mode: pressure

Sample introduction process:

1) Washing with 0.1 N NaOH (2 min), and regenerated with water (1 min)
2) Equilibration of the electrolyte buffer (2 min)
3) Injection of a solution containing enzyme in phosphate buffer (3 sec)
4) Introduction of phosphate buffer (1 sec)
5) Injection of a solution of substrate in phosphate buffer (5 sec)

Concentration and pH of phosphate buffer: see Table 1.

The stock solutions of enzyme, substrate, and buffers used in the EMMA were kept at 20°C on reservoir.

Estimation amounts:

Poiseuille equation:

$$V = \Delta P \pi r^4 t / 8 \eta L$$

where

- ΔP: the pressure drop across the capillary during injection
- r: capillary radius
- t: injection time
- η: the viscosity of the buffer
- L: total length of the capillary from inlet to outlet.

The length and volume (V) of the enzyme and substrate plugs are estimated to be 6.5 and 10 mm and 29 and 45 nL, respectively, which were isolated by a 2-mm (9-nL) phosphate buffer plug.

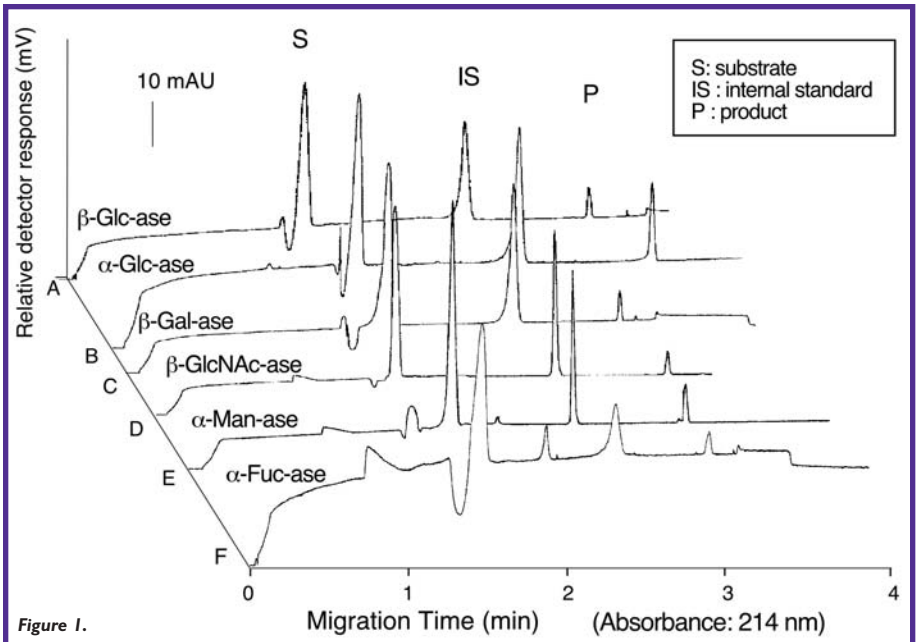


Figure 1.

Results and Discussion

A micro-reaction in a capillary under electrophoretic conditions is presented schematically on the front page. The isolated plugs of enzyme and substrate are introduced into the capillary. The order of the introduction is determined based on the relative mobility. The reaction proceeds during plug-plug interaction *(t2-t1)*, and the individual plugs of substrate, enzyme, and products are subsequently separated.

After examination of the effect of the phosphate buffer concentration on the sensitivity of PNP-ol, we concluded that the substrate buffer concentration should be under 200 mM. This was based on our observation of a disturbance of the baseline, which is probably associated with the front and end boundaries of the phosphate buffer, that affected the substrate and PNP-ol peaks, respectively. The effect of the pH was then investigated. The optimal transformation was observed around the optimal pH of each enzyme.

Using optimized conditions for the kinetic analysis of each of the enzymes for the micro-reaction, we obtained the kinetic parameters of the reaction. Figure 1 shows the electropherograms of each reaction. The assay was performed at 37°C and detected at 214 nm. The Km values were obtained by a double-reciprocal plot where the relative area was used to indicate product concentrations which were used instead of the initial velocity of the enzyme reaction. Figure 2 represents an example of the double-reciprocal plot for α-Glc-ase

Table 1. Concentration and pH of Phosphate Buffer

Reaction	Phosphate buffer for enzyme Conc. (mM)	pH	Phosphate buffer for substrate Conc. (mM)	pH	Reported optimal pH
β-Glc-ase	40	4.5	200	6.0	5.5
α-Glc-ase	40	7.0	200	7.0	6.8-7.0
β-Gal-ase	40	4.5	200	4.2	4.5-5.0
β-GlcNAc-ase	40	4.5	80	4.4	4.0-5.0
α-Man-ase	40	4.5	40	4.5	4.5
α-Fuc-ase	40	5.0	200	6.0	5.3

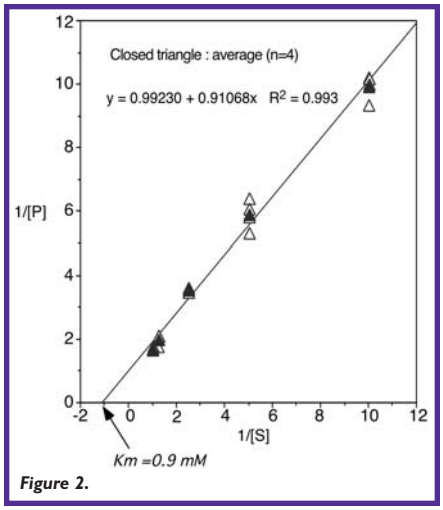


Figure 2.

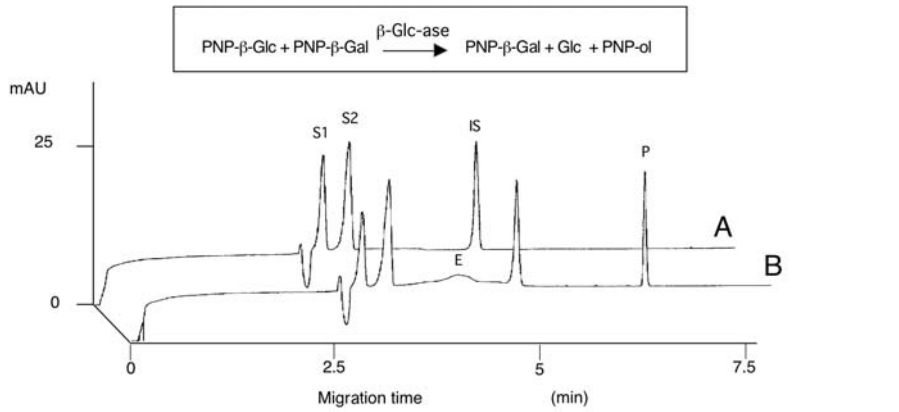


Figure 3. A: *Injection of 0.5 mM PNP-β-Glc (S1) and 0.5 mM PNP-β-Gal (S2) in 200 mM phosphate buffer containing internal standard (IS) after injection of 40 mM phosphate buffer (3s) and 200 mM phosphate buffer (1s).* ***B:*** *Injection of 0.5 mM PNP-β-Glc (S1) and 0.5 mM PNP-β-Gal (S2) in 200 mM phosphate buffer after injection of 0.5 mM β-Glc-ase (E) in 40 mM phosphate buffer (3s) and 200 mM phosphate buffer (1s). Capillary total length: 47 cm; Absorbance: 214 nm. S1: PNP-β-Glc; S2: PNP-β-Gal; IS: uridine; P: PNP-ol.*

reaction. The enzyme reaction is described by the Michaelis-Menten equation,

$$v = Vmax[S]/(Km+[S])$$

where

v is the initial rate of the given reaction

[S] is the substrate concentration

Km is Michaelis constant.

Although the reaction time is not given directly in EMMA because of the heterogeneous nature of the reaction that takes place during the plug-plug interaction, linearity was obtained for the relative area of the product PNP-ol over uridine during the experiment at a fixed concentration of the phosphate buffer. Therefore, we used the area as an equivalent value of v to obtain the constant. The Michaelis constants thus obtained are shown in Table 2 together with those obtained by conventional method.

Further examples of EMMA's potential utility in the functional analysis of carbohydrate-related enzymes and/or carbohydrates are shown in Figures 3 and 4. Figure 3 shows the electropherograms of the selective reaction which used a mixture of PNP-β-glycosides of glucose and galactose with β-Glc-ase. The structural difference of these glycosides is a single chirality at position C-4 of the sugar moiety. It is clearly shown in Figure 3A that these compounds were resolved using borate buffer as the separation electrolyte. Furthermore, in Figure 3B, it is evident that the β-Glc-ase catalyzed reaction proceeded where the formation of PNP-ol was observed and the area of PNP-β-Glc was decreased while PNP-β-Gal was unaffected. Figure 4 shows the electropherograms of the reaction using two enzymes and corresponding substrates (PNP-β-Glc and ONP-β-Gal) for the potential use of a mixture of functionally and/or structurally related enzymes. To confirm both reactions, *p*-nitro- and *o*-nitrophenyl glycosides were used. Figures 4A and B demonstrate that the two substrates and phenols were resolved well under EMMA condition without

Table 2. Michaelis Constants Obtained by EMMA and Photometric Assay

Reaction	EMMA (mM)	Photometric assay (mM)
β-Glc-ase	1.4	2.6
α-Glc-ase	0.9	0.7
β-Gal-ase	1.0	1.2
β-GlcNAc-ase	1.1	1.8
α-Man-ase	0.8	1.0
α-Fuc-ase	0.7	1.1

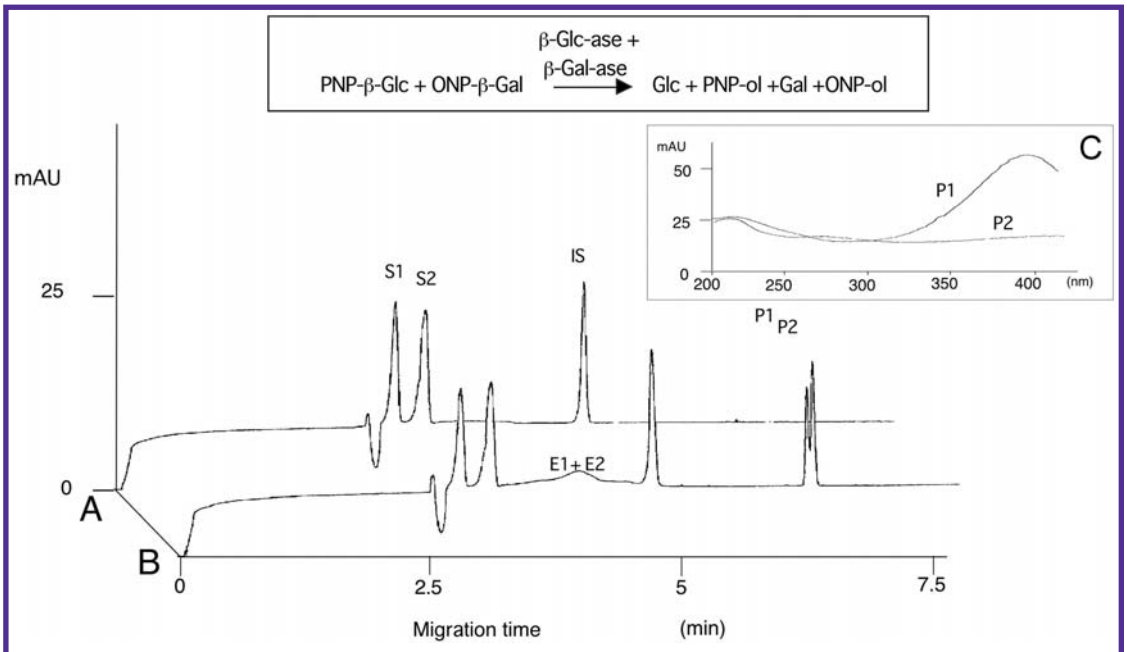


Figure 4. A: *Injection of 0.5 mM PNP-β-GLC(S1) and 0.5 mM ONP-β-Gal(S2) in 200 mM phosphate buffer after injection of 40 mM phosphate buffer (3s) and 200 mM phosphate buffer (1s).* ***B:*** *Electropherogram of enzyme reaction where both substrates, PNP-β-Glc and ONP-β-Gal, and products, PNP-ol and ONP-ol, are clearly isolated. Injection of 0.5 mM PNP-β-Glc and 0.5 mM ONP-β-Gal in 200 mM phosphate buffer after injection of 0.5 mg/mL β-Glc-ase(E1) and 0.26 mg/mL β-Gal-ase(E2) in 40 mM phosphate buffer (3s) and 200 mM phosphate buffer (1s). Capillary total length: 47 cm. Spectra in box: UV spectra of each peak in electropherogram B. S1: PNP-β-Glc; S2: ONP-β-Gal; IS: uridine; P1: PNP-ol; P2: ONP-ol.*

enzymes, respectively. Figure 4C shows that the mixed enzymatic reaction is possible, and corresponding product peaks could be differentiated and identified easily by PDA detection as shown in the box.

Conclusions

The native glycoenzyme-related reactions using a plug-plug format under EMMA conditions were achieved after investigation of the detailed reaction conditions. An evaluation of the kinetic constant was also carried out under the conditions. As a result, the scale of the enzymatic reaction was radically reduced (at least a 1,000-fold improvement compared to off-line analysis of the reaction carried out in a microcentrifuge tube). Thus, we were able to perform the enzymatic reaction at a nanoliter scale, whereas the limit in reaction volume for traditional methods is at the microliter scale level. We envision that this will be an important analytical tool to examine small amounts of enzyme mixture for their activities and specificities.

Reference

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An Ultrafast Method to Evaluate RNA Quality

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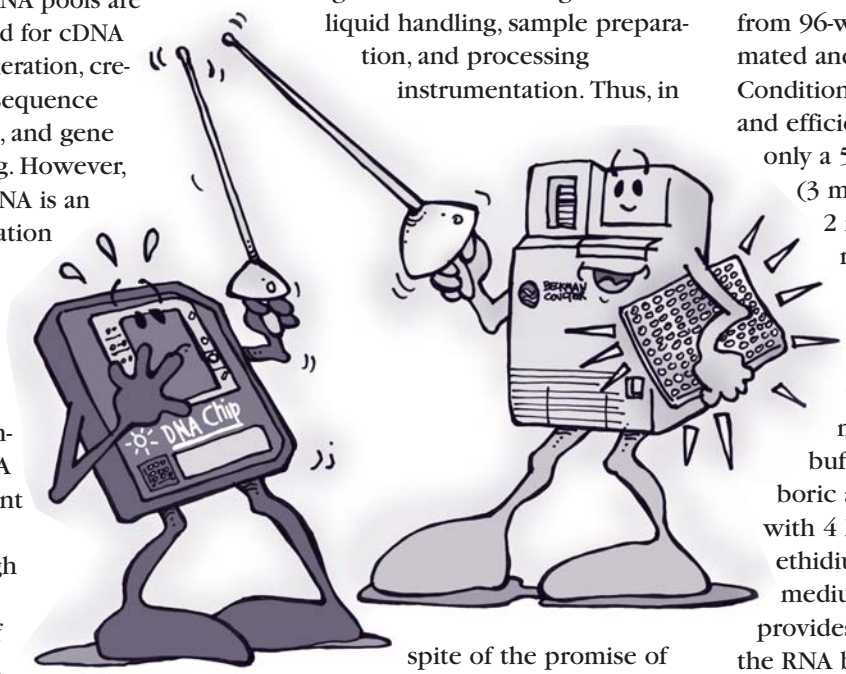
Introduction

Messenger RNA pools are widely used for cDNA library generation, creation of expressed sequence tag (EST) databases, and gene expression profiling. However, the quality of this RNA is an important consideration as these molecules are highly susceptible to degradation by naturally contaminating RNAses. Good methods to evaluate RNA purity are paramount in successfully implementing a high throughput genetic analysis process. Of course, advances in expression microarray technology have also led to the increased demand for techniques to assess the quality of RNA.

To implement such a process in our lab, we first evaluated an automated microfluidic RNA analysis system which can run 12 RNA samples sequentially in 20 minutes[1]. Unfortunately, for many RNA analyses, we found a need for much larger-scale, high-throughput separation techniques. Important to our work is compatibility with a 96-well plate format, enabling a link with existing robotic liquid handling, sample preparation, and processing instrumentation. Thus, in spite of the promise of the emerging microfabricated device technology for rapid analysis and small reagent consumption, we have turned to more established CE instrumentation for automated, large-scale, and low-cost assays.



Here we present a simple and effective RNA screening method using the P/ACE™ MDQ with laser-induced fluorescence detection. The P/ACE MDQ LIF detection was configured with a 488 nm argon ion laser with an emission filter of 605 ± 40 nm. This method allows the unattended processing of total RNA from 96-well plates, followed by automated and quantitative data analysis. Conditions were optimized for fast and efficient RNA separations with only a 5-minute cycle time per run (3 minutes separation and 2 minutes matrix replacement).

Methodology

In our experiments, we used 1% PVP (1.3 MDa; Sigma-Aldrich) in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) with 4 M urea and 0.5 µM ethidium bromide, as a separation medium. The PVP linear polymer provides a sieving matrix to resolve the RNA by size, while the use of urea as a denaturing agent has been reported to improve the resolution of RNA fragments by disrupting higher-order structures[2]. This low-viscosity, semi-dilute polymer solution enabled fast matrix replacement and resulted

in short analysis times. At the same time, the electroosmotic flow was suppressed by the dynamic PVP coating of the capillary walls[2]. Adding urea to the separation medium significantly improved resolution at the expense of slightly increased RNA fragment mobilities (data not shown). The latter was likely caused by changes in the matrix viscosity and analyte conformation.

RNA samples were diluted in water, denatured for 5 minutes at 65ºC, and introduced at the cathode end of the matrix-filled fused-silica capillary (50 µm id, 30 cm total length, **10 cm** to the detection point). Samples were introduced either electrokinetically (5 s at 2 kV) or by vacuum (5 s at 3.44 kPa). Separations were performed at 25ºC for 3 minutes at 15 kV (500 V/cm). The same separation matrix was used in the running buffer reservoirs, and the polymer solution in the capillary was replaced by fresh matrix after each run (2 minutes at 551 kPa). Using this approach, good run-to-run reproducibility (RSD < 0.5%) was achieved over 600 runs. During the batch processing of multiple samples, the 96-well microplates were kept at 4ºC to minimize RNA degradation and renaturation.

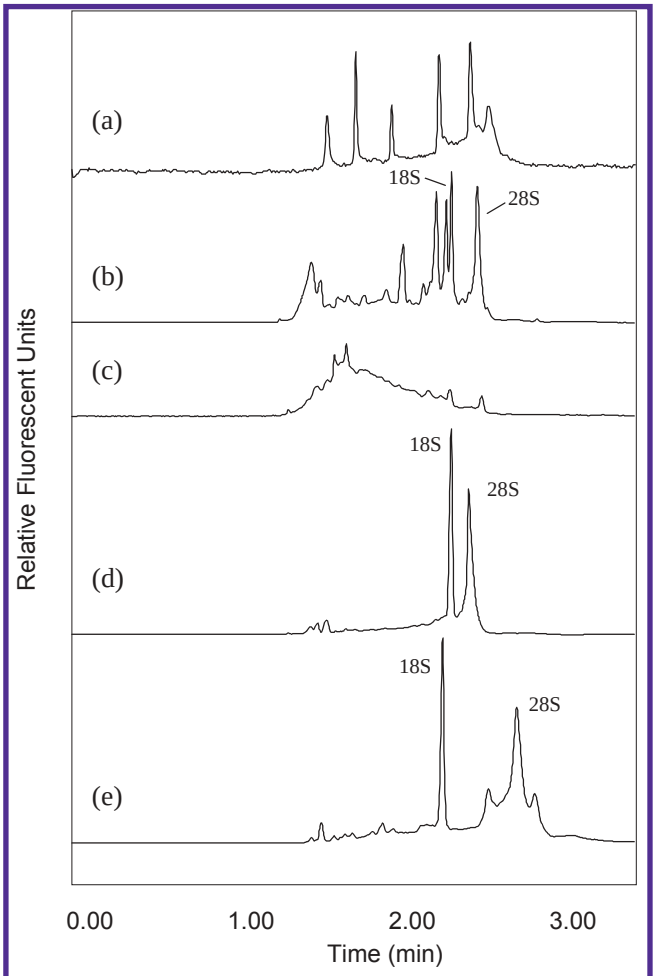


Figure 1. Electropherogram of RNA samples. Reproduced with permission from Biotechniques, Vol 32, No.6 (2002), p 1226. *Trace (a): RNA 6000 ladder size standard. Trace (b): Intact total RNA extracted from rice. Trace (c): Degraded total RNA from rice. Trace (d): Intact total RNA from yeast. Trace (e): Intact total RNA from mouse. Conditions—Capillary: 50 µm i.d., 30 cm total, 10 cm to detection, thermostatted 25°C. Sieving medium: 1% PVP (1.3 Mda), 4M urea, 1X TBE, 0.5 µM ethidium bromide. Field strength: 500 V/cm. Samples: diluted in deionized water and denatured at 65°C for 5 minutes prior to analysis. Sample introduction: 5 seconds at 2 kV.*

Results

Samples of total RNA (up to 5 kb size range) from *Arabidopsis,* rice, yeast, and mouse are routinely analyzed in our laboratory. Typical electropherograms of these samples are illustrated in Figure 1. Trace (a) in Figure 1 delineates the separation of the RNA sizing ladder that we used (RNA 6000, Ambion, Austin, TX, USA). This ladder consists of 0.2, 0.4, 1.0, 2.0 , 4.0, and 6.0 kb fragments diluted in water to 25 µg/mL. Intact total RNA is usually characterized by sharp, well-defined peaks in the 1 to 5 kb range, containing several representative major peaks referred to as, for example, 28 S and 18 S as seen in traces (b), (d), and (e). The actual size and migration rate of these fragments depend on the RNA source. In the case of degraded or poor-quality RNA, these major peaks disappear and a smearing profile prevails, shifted towards the smaller fragment lengths, as observed in trace (c) of Figure 1.

Another key criterion we use to assess RNA quality is the area ratio (corrected for velocity) of the 28 S and 18 S fragments which quantifies the extent of RNA degradation that has taken place. This method of quantitation is in good agreement with spectrophotometric data that we have generated (accuracy within 10 to 15%).

To meet our high throughput requirements, consistency across a large number of samples is important. We achieved excellent reproducibility and consistency with this method. We routinely generate results from 96 samples run in a continuous batch from a 96-well plate. Replacing the sieving matrix after each sample introduction/separation cycle resulted in reproducible performance with negligible standard deviation of the migration times. In large-scale batch processing, vacuum sample introduction was preferred.

Summary

In conclusion, a simple, automated, fast, economical, and quantitative RNA screening method was developed using standard CE instrumentation and reagents, enabling high throughput and large-scale evaluation of RNA samples.

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Use of PNA Probes for Detecting DNA Single-Base Mutations

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Introduction

Peptide nucleic acids (PNAs) are a class of compounds with potential use for diagnostic and therapeutic purposes. The PNA backbone consists of repeated units of N-(2-aminoethyl)-glycine with the secondary amino function acylated by a nucleobase acetic acid derivative[1]. The most striking feature of PNAs is their ability to bind, according to the Watson-Crick base-pairing rules, to complementary nucleic acids with higher affinity than their natural counterpart[2,3]. These features result from the PNA neutral backbone that provides a stronger binding and a greater specificity in the interaction with target molecules. In addition, the uncharged nature of these compounds allows binding reactions nearly independent from salt concentration, thus increasing the robustness and specificity of the pre-gel hybridization technique; moreover, the thermal stability of PNA/DNA duplexes usually exceeds that of the corresponding DNA/DNA or RNA/DNA duplexes[2,4,5].

We report here a method that simplifies the standard multistep Southern procedure[6]. Our approach consists of the hybridization of a PNA probe with a double-stranded DNA target. Denaturation and hybridization were carried out in a buffer of low ionic strength to increase specificity, and the hybrid was separated from the target DNA by CE. The neutral backbone of PNA imparts a slow electrophoretic mobility to the PNA/target DNA complex, ensuring a separation from both reannealed double-stranded DNA and single-stranded DNA. As a model system, we have selected the single-base mutations R553X[7] and R1162X[8] present in individuals affected with cystic fibrosis. Our data demonstrate the feasibility of the PNA pre-gel hybridization approach for diagnostic purposes.

Material and Methods

DNA fragments were obtained by polymerase chain reaction (PCR) using, as templates, genomic DNA from unaffected homozygous and heterozygous individuals for R553X and R1162X mutations. DNA primers were designed with the aid of the Primer 3 program (http://www-genome.wi.mit.edu/genome-software/other/primer3.html). DNA primers were obtained from MWG-Biotech AG (Ebersberg, Germany). The forward primers were labeled with a Cyanine-5 molecule.

The primer sequences for the R553X mutations are: Cy-5 labeled forward primer 5´-CAG ATT GAG CAT ACT AAA AGT G-3´ and reverse primer 5´-CAT TTA CAG CAA ATG CTT GCT AG-3´; for R1162X mutation: Cy-5 labeled forward primer 5´-TGT GAA ATT GTC TGC CAT TC-3´ and reverse primer 5´-CGA GAG TTG GCC ATT CTT GT-3´. The lengths of the PCR fragments are, respectively, 186 (R553X) and 149 bp (R1162X). The amplification reactions were carried out using 200 ng of genomic DNA from peripheral blood as the template in a 50 µL reaction. Amplified products were obtained by 40 cycles of PCR using a 52°C annealing temperature. We used PE AmpliTaq Gold* DNA Polymerase (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

The PCR products were separated on a 1.8% agarose gel and purified by using a QIAquick* Gel Extraction kit (QIAGEN GmbH, Hilden, Germany).

PNA oligomers were synthesized by Primm s.r.l. (Milan, Italy). The antisense oligomers synthesized were (N-> C terminus): -O-O- CTT GCT CGT TGA CCT R553 (wild type); -O-O-CTT GCT CAT TGA CCT R553X (mutant); -O-O- AGA CTC GGC TCA CAG R1162 (wild type); -O-O- AGA CTC AGC TCA CAG R1162X (mutant) [O stands for a 6-carbon spacer unit].

In a typical experiment, 0.1-0.2 pmoles of a Cy-5 purified PCR fragment were transferred into a 0.2 mL Eppendorf tube; 1-2 pmol of complementary PNA and 1 µL of 10 mM Tris/HCl, pH 8.0, were added and the total volume was adjusted to 10 µL with MilliQ* water. The tube was placed in a heating block set at 95°C for 10 minutes and then cooled to room temperature over a 30-minute period.

CE Analysis

Separations were performed using a P/ACE™ MDQ System (Beckman Coulter, Inc, Fullerton, CA). A 75 µm i.d. 48 cm (38 cm to the detection window) capillary was flushed with 0.1 N NaOH for 10 minutes, rinsed with MilliQ water, and coated with poly(dimethylacrylamide-*co*-allyl glycidyl ether) EPDMA 0.1% (w/v)[9]. The sample was injected by electromigration by applying 10 Kv for 15 seconds (cathode at sample injection end). Separations were performed with the cathode at the injection side by applying a voltage of 250 V/cm. A 2% solution of a copolymer of

dimethylacrylamide and allyl glycidyl ether in which the epoxy groups had been hydrolyzed was used as the sieving matrix (HYDRO EPDMA). The background electrolyte was 100 mM TAPS-Tris, pH 8.3, 2 mM EDTA, and 4 M or 6 M Urea. Laser-induced fluorescence detection was performed by a P/ACE™ MDQ single λ LIF detection module with excitation at 635 nm and emission at 655 nm.

Results and Discussion

This article reports a fast method to detect single-base variations in a DNA sequence by CE. The electrophoretic mobility of a DNA/PNA hybrid is slower than that of a double- or single-stranded DNA fragment due to the neutral character of the PNA backbone. This characteristic provides a simple tool to identify PNA/DNA hybrids by CE without the need for labeling the PNA oligomers used in the pre-gel hybridization. Moreover, the CE system allows the stringency of the analysis to be accurately tuned by adjusting the temperature of the capillary cartridge and by selecting the appropriate concentrations of denaturants (such as urea) in the sieving matrix. By carrying out electrophoresis under conditions of stringency, it is possible to obtain the melting of PNA/DNA hybrids with a mismatch whereas those perfectly complementary are stable. This phenomenon provides information on the sequence of the PNA target.

Figures 1A and 1B show electropherograms of wild-type and mutant DNA, where the latter is a single-base substitution in the target region hybridized with PNA, run at two different temperatures. The mutation under investigation is the R553X. A 15-mer PNA probe was hybridized with a 186 base pair PCR fragment obtained by amplifying the region carrying the mutation. The PNA probe sequence was complementary to the DNA strand bearing the mutation which was labeled with Cy-5. The PNA/DNA hybrid, indicated in Figure 1 by an arrow, was therefore detectable as a peak migrating with slower mobility than that of the double-stranded DNA target. The separation was carried out in a sieving matrix containing 4 M urea. Addition of urea was necessary to keep the melting temperature of the hybrid within the operating temperature range of the CE instrument. At 37°C, both the mutant and wild type samples migrated as a single peak corresponding to the PNA/DNA hybrid. As shown in Figure 1A, the mobility of the perfectly matched hybrid is slightly faster than that of the mismatched one. This might be due to conformational differences. An increase of the CE running temperature, up to 42°C, caused the hybrid to melt, thus generating a new molecular species with a slightly higher mobility than that of the PNA/DNA hybrid. The new peak presumably corresponds to the Cy-5-labeled single-stranded DNA. At this temperature, the perfectly matched hybrid is not completely melted, and its electrophoretic profile results in a double peak as reported in Figure 1B. This different behavior allowed us to discriminate the hybrid with single-base mismatch from the one with a perfect match.

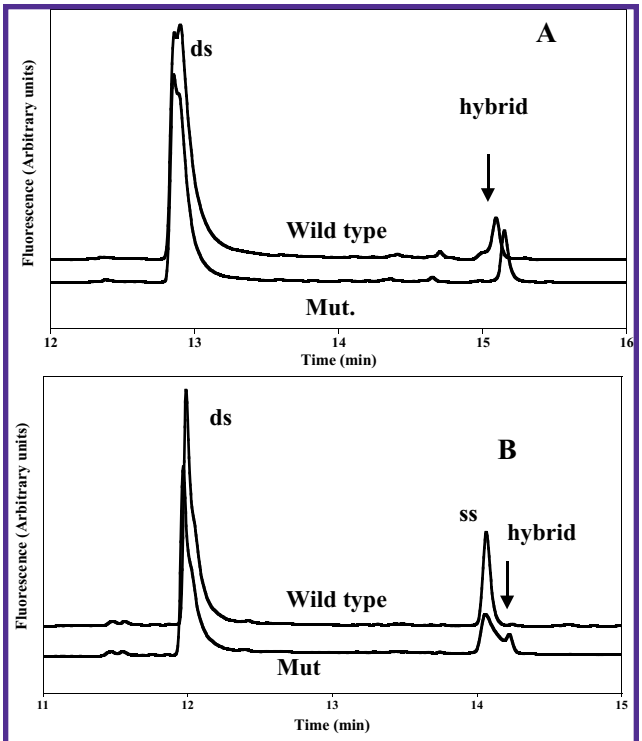

Figure 1. Electropherograms of wild-type and R553X homozygous DNA run at A) 37°C, B) 42°C. The R553X PNA probe (15 mer) was hybridized with a 186-bp PCR fragment labeled with Cy-5. The PNA/DNA hybrid is indicated by the arrow. Separation conditions: capillary: 75 µm i.d., 48 cm (38 cm to the detection window), dynamically coated with EPDMA, filled with a 2% solution of HYDRO EPDMA in 100 mM TAPS-Tris, pH 8.3, 2 mM EDTA, 4 M urea. Sample: injected by electromigration (-10 kV, 15 s); running conditions: 250 V/cm; cathode at the injection side. Laser-induced fluorescence detection: excitation at 635 nm and emission at 655 nm.

A single run, carried out at the temperature at which the two hybrids have different melting behavior, allows one to distinguish a R553X homozygote from an unaffected individual (Figure 1B). An R553X homozygous one could be distinguished from a heterozygous one by carrying out a second hybridization

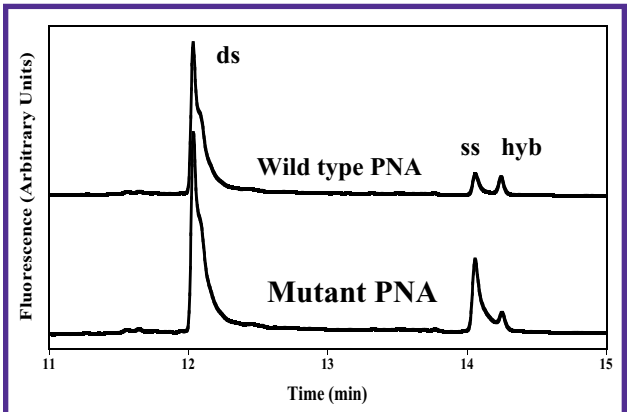

Figure 2. Electropherograms of R553X heterozygous DNA hybridized with R553 and R553X PNA probes. Run temperature: 42°C. All other separation conditions as in Figure 1.

experiment using a wild-type PNA probe. Only R553X heterozygous carriers present at 42°C the PNA/DNA hybrid peaks with both R553 and R553X PNA probes since the amplified DNA contains a mixture of fragments whose sequence is perfectly complementary to both mutated and wild type PNA probes. Figure 2 shows the electrophoretic profile of the wild-type and mutant PNA probes hybridized with an R553X heterozygous DNA sample. At 37°C, the two hybrids migrate as a single peak (data not shown), whereas at 42°C both peaks of hybrid and single-stranded DNA are present (Figure 2).

Similar experiments were also performed to detect another single-base CFTR mutation, the R1162X. Figures 3A and 3B depict the results obtained with a 149-bp PCR fragment from wild-type and R1162X-affected individuals that were hybridized with R1162X PNA probes.

Concluding Remarks

The data reported here show the feasibility of using peptide nucleic acid pre-gel hybridization and CE–LIF detection for the discrimination of DNA single-base mutations. CE allows real-time detection of the melting

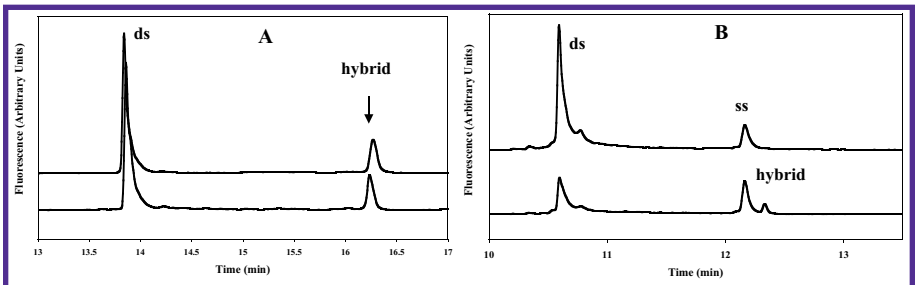

Figure 3. Electropherograms of wild type and R1162X homozygous DNA run at A) 37°C, B) 47°C. The R1162X PNA probe (15 mer) was hybridized with a 149-bp PCR fragment labeled with Cy-5. All other separation conditions as in Figure 1.

equilibrium, while the sequence specificity of PNA probes provides an efficient tool to detect sequence variations in the target DNA. The method does not require the use of labeled PNAs, thus enabling a more convenient and less expensive analysis. The use of multiple fluorophores will further enhance the number of mutations analyzed by a single CE run.

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Generating Stable and High EOF for CE-MS

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Introduction

Mass spectrometry (MS) represents an ideal detection principle because of its universality, sensitivity, and selectivity. The combination of both capillary electrophoresis and MS creates a very powerful analytical tool for evaluating complex charged solutes. As the external extension of the capillary to an MS interface creates longer migration times, mechanisms to upregulate electroosmotic flow (EOF) while maintaining good reproducibility of migration are highly desirable. This is especially significant at low pH where the EOF generated in bare fused-silica capillaries is very low.

The aim of this study was to develop an optimal volatile buffer system at low pH for use with CE-MS with an electrospray ionization interface. The major criteria for this buffer system were that it:

- Be compatible with MS
- Be reproducible
- Give a short analysis time
- Provide a low detection limit

The approach that we propose here is to use a low pH volatile buffer which includes a dynamic capillary double coating reagents, to obtain a very fast and stable EOF. A test sample of basic drugs is used to evaluate the approach.

Experimental

Materials

The test samples (basic drugs) were dissolved in methanol (stock solution, 1000 ppm). These stock solutions were diluted in water prior to injection. The test mix comprised Amphetamine, Ephedrine, Codeine, Salbutamol, and Trazodone and was diluted to 2 µg/mL.

All solvents used were HPLC grade. Buffers and dynamic coating reagents were obtained from Analis, Belgium. Fused-silica capillaries were obtained from Polymicro Technologies and were cut in-house to the appropriate length.

Instrumentation

CE was performed using a P/ACE™ MDQ capillary electrophoresis system (Beckman Coulter). MS was performed on an LCQ ion trap (IT) mass spectrometer equipped with an ESI source (ThermoFinnigan).

All experiments were carried out in the positive ionization mode. The CE was adjusted in height in such a way that the inlet of the capillary was at the same height as the outlet (ESI-needle) to avoid siphoning effects.

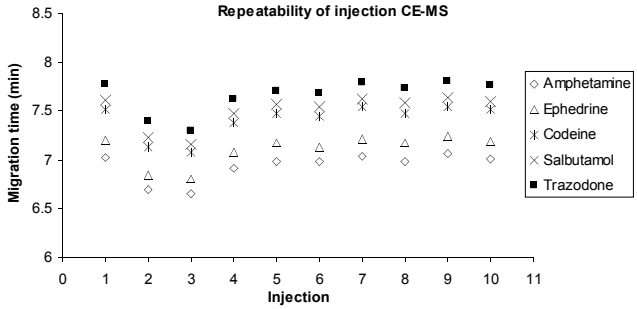


Figure 2. Migration time vs. injection number for 10 consecutive injections of a standard mixture of basic drugs.

CE-MS

The buffer system is based on a dynamic double coating of the capillary in order to condition the EOF. It is composed of two buffers: one containing a polycation ("intiator" buffer), which will put a positive charge on the inside capillary wall. The other is composed of the separation buffer but contains a polyanion ("accelerator" buffer). After rinsing with this second buffer, the inside wall will bear a negative charge. To be able to condition the capillary for CE-MS, the capillary is first rinsed with 0.1 N NaOH, water, and initiator solution with the ESI source opened. Then the ESI source is closed and, before each analysis, the capillary is rinsed with the accelerator solution.

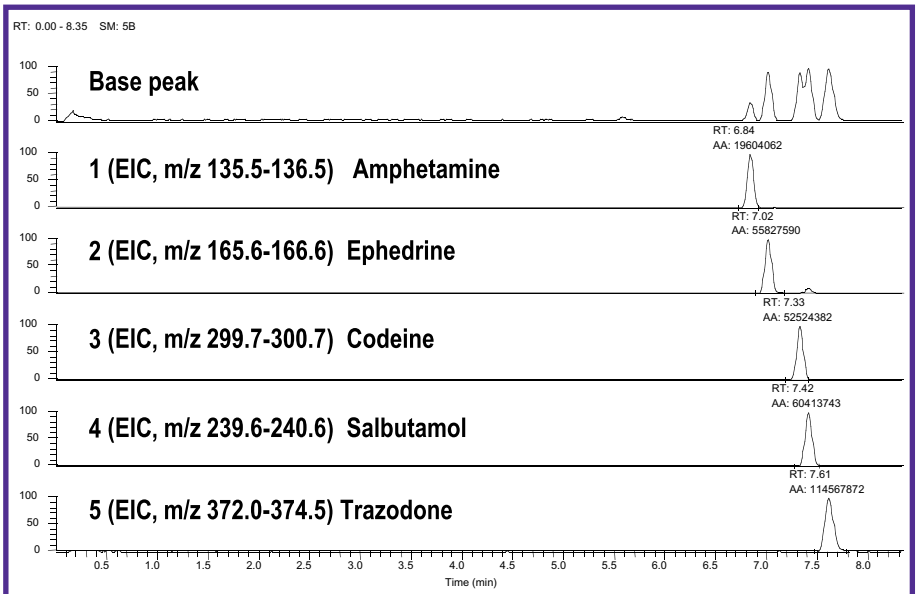


Figure 1. CEMS analysis of a standard mixture of 5 basic drugs (2 µg/mL each) with a 100 mM formic acid + accelerator + 1 mM TFA solution. Capillary: 75 µm ID x 93.5 cm L_{tot}, CE current: 25 µA, injection: 5 s at 2 psi, ESI spray current: 19 µA, N_2 gas flow: sheath 20 units/auxiliary 0 units.

Analytical Results

All parameters were optimized and following method was used:

- Pre-run rinse: 1 min at 20 psi with buffer
- Separation electrolyte: 100 mM formic acid + accelerator + 1 mM TFA
- Capillary: 75 µm x 93.5 cm
- CE voltage: 30 kV (- to +), built up in 1 min ramp
- CE current: 25 µA
- Make-up flow: 2 µL/min, methanol/water (80/20 v/v) with 0.5% formic acid, degassed daily in an ultrasonic bath, provided by a syringe pump installed on the mass spectrometer
- ESI needle voltage: 5 kV (0 kV during injection and voltage build-up)
- ESI spray current: 19 µA
- ESI heated capillary temperature: 160°C
- Sheath gas: 20 units; auxiliary gas: 0 units
- Scan range: 100-400 amu
- Position CE-capillary outlet: equal to ESI needle

Migration time repeatability was less than 2.3% RSD for all compounds. The first few injections appeared to be part of the equilibration process. When discarding the first three injections, the RSD was 0.85% or lower for all compounds (Table.1). The capillary appears to need some time to condition: after three injections, the system is stable and the migration time is highly reproducible (Table 1).

Please note the very short analysis time when using the EOF-conditioner. When performing the same analysis (data not shown) without EOF-conditioner, we found the following migration times: Amphetamine: 15.46 min, Ephedrine: 16.48 min, Codeine: 17.95 min, Salbutamol: 18.95 min, and Trazodone: 19.69 min.

Table 1. Repeatability of Injection for CE-MS of a Standard Mixture of Basic Drugs (2 ppm each), n=7 (run 4-10)

Migration Time (min)		
Compound (peak)	Average	RSD (%)
Amphetamine (1)	6.996	0.69
Ephedrine (2)	7.171	0.72
Codeine (3)	7.481	0.78
Salbutamol (4)	7.576	0.77
Trazodone (5)	7.729	0.85

Conclusion

This study demonstrates the feasibility of using a buffer containing EOF-conditioner for CE-MS analysis without interference. The result was a highly reproducible, sensitive, and generally a fast separation.

Note

Analis s.a. produces different buffers systems for use with capillary electrophoresis for research, QC, and clinical laboratories. The products are sold under the trade mark CEofix. More information can be obtained at www.analis.com or send an e-mail to ceofix@analis.be.

Analyzing Enantiomers of Aliphatic Amines

Albert F.-T. Chen
Beckman Coulter, Inc.

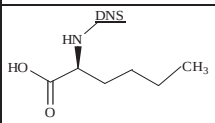
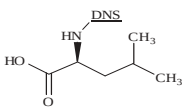
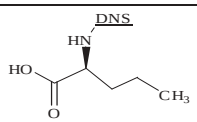
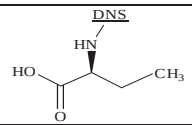
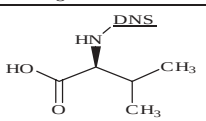
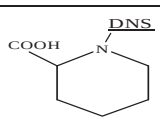
Aliphatic amines and amino acids are transparent to UV. Derivatization with either 4-dimethylaminoazobenzene-4´-sulfonyl chloride (dabsyl chloride) or 5-dimethylamino-1-naphthalene-sulfonyl chloride (dansyl chloride) is the most common practice to provide the resulting adducts for UV or fluorescent analysis.

Most racemic drugs and their precursors are basic in nature, containing primary or secondary amines, most likely at or near the asymmetric carbon. Natural amino acids and peptides, for example, are routinely derivatized with dansyl chloride (DNS) to yield DNS derivatives for quantitative analysis by LC or CE. Variation in the side chain of α-amino acids or their derivatives is the ideal model for the analysis of their interactions with chiral selectors.

Using a class of highly sulfated cyclodextrins (HS-CDs) as the chiral selector for CE analysis revealed that HS-γCD (average of 13 sulfates per γCD) provided rapid and high resolution for most DNS aliphatic α-amino acids shown in the first six rows of Table 1. CE-based chiral analysis of a series of DNS-aliphatic amino acids in phosphate buffer containing 5% HS-γCD at pH 2.5 is shown in Figure 1.

Each enantiomer pair was resolved with an effective separation length of 10 cm within 2.6 minutes except that of the DNS-valine that was partially resolved. The DNS group is the common denominator in all DNS-aliphatic amino acids with a positively

Table 1. Relative Migration Time and Resolution of Racemates of DNS-Aliphatic Amino Acids in HS-CDs

	DNS-derivatives	Structure	HS-αCD	MT	HS-βCD	MT	HS-γCD	MT
1	DNS-norleucine		0.90	*5.04*	0.0	*5.92*	6.5	*1.91*
2	DNS-leu		3.30	*5.46*	0.4	*5.21*	3.8	*2.06*
3	DNS-norvaline		0.00	*6.40*	0.0	*7.00*	2.1	*2.13*
4	DNS-α-aminobutyric acid		0.00	*7.64*	1.0	*6.12*	3.7	*2.26*
5	DNS-valine		0.00	*8.06*	0.2	*6.94*	0.5	*2.51*
6	DNS-homoproline			*ND*		*ND*	2.9	*2.12*

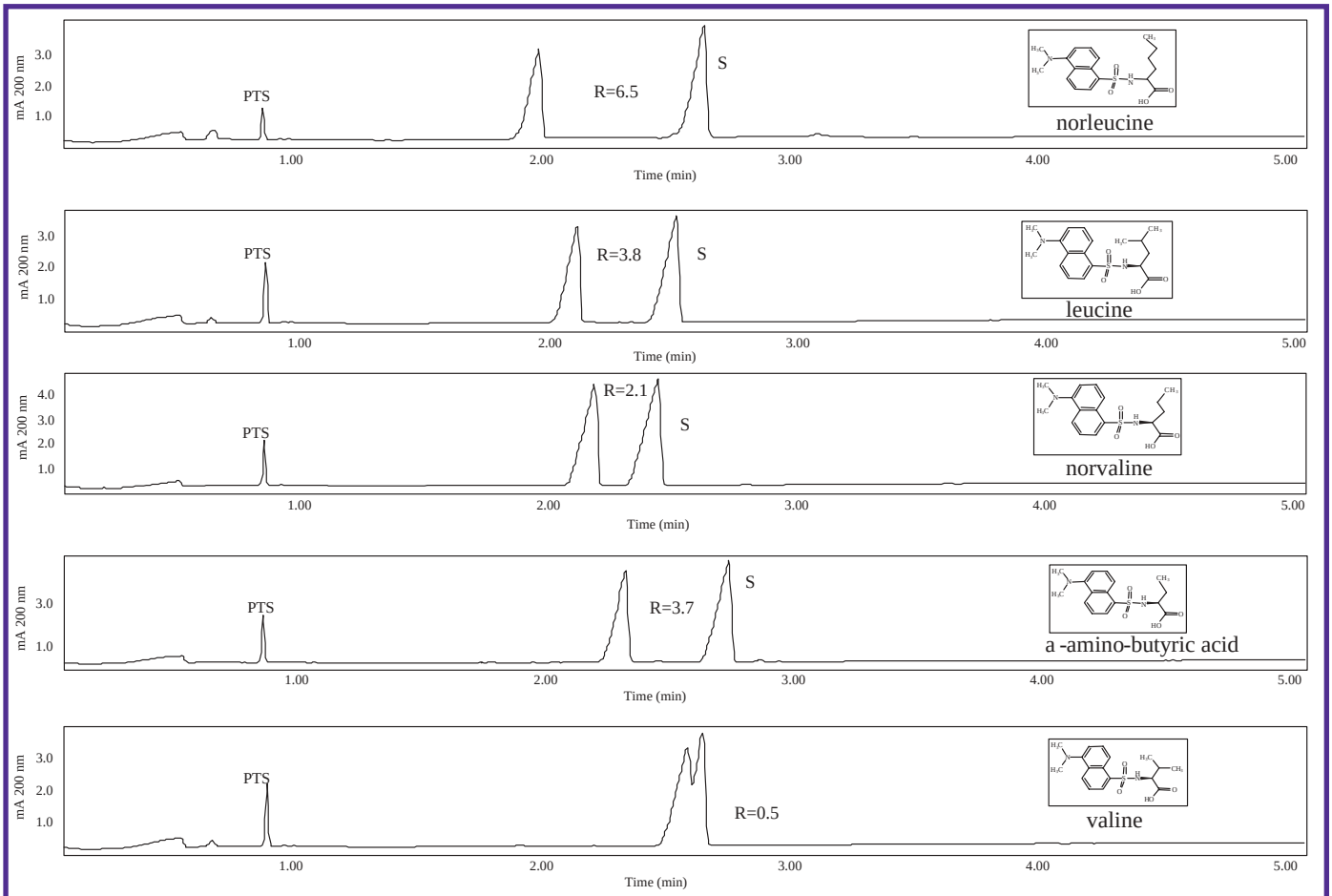


Figure 1. DNS-Alyl α-Amino acids in HS-γCD. MDQ system / 50 µm x 31.5 cm; 5% HS-γCD in 25 mM TEA phosphate, pH 2.5. Effective separation: 10.5 cm; injection: 0.3 psi/4s; voltage: 15 kV/146 µA to anode; 200 nm.

charged tertiary amino group as the initial contact with sulfate ester of HS-γCD. The α-carboxylic acid group of the DNS-amino acids is essentially non-ionized at pH 2.5 and provides a hydrogen bonding site with the 3-hydroxyl around the rim of HS-γCD next to the sulfate moieties. This leaves the remainder of the side chain of the DNS-aliphatic amino acids to roam into the hydrophobic cavity of HS-γCD. All racemates of DNS-aliphatic amino acids in Table 1 were resolved in HS-γCD with relatively short migration time.

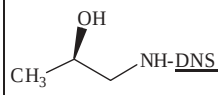
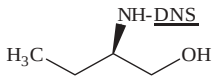
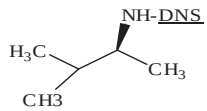
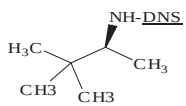
This supports the hypothesis that the larger cavity in HS-γCD not only accommodates the side chain but also interacts significantly with portions of the naphthylsulfonamide moiety of the DNS-aliphatic amino acids.

As a logical extension of the results obtained in Table 1 and Figure 1, the DNS-derivatized aliphatic amines are arranged spatially similarly to those of the DNS-aliphatic amino acids. The α-carboxylic acid moiety in DNS-α-amino acids is substituted with an aliphatic moiety in DNS-amine. The resulting racemic DNS-aliphatic amines provide two discretely different aliphatic moieties, both extended from the α-carbon, and they would have to compete for the spatially situated hydrophobic cavity in the cyclodextrin of HS-CDs. A few racemates of alkylamines were derivatized with dansyl chloride by a standard procedure[1] for testing the utility of enantiomeric separation by the proposed strategy. Adducts of enantiomers of dansyl alkylamine without purification were introduced to the CE system using HS-γCD as the chiral selector (conditions: same as those of Table 1 and Figure 1). The results of a few enantiomeric separations of racemic DNS-alkyl amines are summarized in Table 2. Rapid resolution of two DNS-alkylamines enantiomers of 3-methyl-2-butylamine and 3,3-dimethylbutylamine was achieved with significant resolution above 3.0. Similarly the enantiomers of DNS-α-hydroxyamine of 1-amino-2-propanol and 2-amino-1-butanol were also well resolved. The addition of the hydroxyl moiety in that of the α-hydroxyamine had significant effect in its affinity to HS-γCD. The DNS-2-amino-1-butanol is structurally similar to DNS-α-amino-butyric acid, both exhibiting relatively high resolutions of 5.1 and 3.7, but with significant difference in migration times of 7.1 and 2.3 minutes, respectively, in HS-γCD. The large differences in migration

time are presumably due to the favorable hydrogen bonding of the carboxylic moiety in DNS-α-amino-butyric acid to the hydroxylmethyl moiety in DNS-2-amino-1-butanol with the 3-hydroxyl group of the HS-γCD. Thus, the mechanism of enantiomeric separation of DNS-alkylamine racemates and DNS-aliphatic amino acids in HS-γCD appears to be similar, and the proposed method would be applicable as a general procedure for chiral analysis of racemic aliphatic amines.

1) Alkyl amine was dissolved in methanol/0.2 M $NaHCO_3$ (75/25:v/v) at a final concentration of 5-10 mM. An equal volume of DNS-Cl reagent (2.5 mg/mL in acetone) was mixed well with the above solution at RT/2 hr. The resulting mixture was diluted in 0.1 N HCl at a final concentration of 0.5-1.0 mM in aqueous solution containing 0.25 mM of 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt (PTS) (Acros Chemicals, Pittsburgh, PA) as the internal migration marker.
2) Highly sulfated cyclodextrins were obtained from Beckman Coulter, Inc., as 20% (w/v) aqueous solutions. The CE-based enantiomeric separations were performed in a fused-silica capillary of 50 µm I.D. x 31.5 cm (10 cm effective length). The analyses were performed on a P/ACE™ MDQ capillary electrophoresis system (Beckman Coulter, Inc.) equipped with a photodiode array detector. For all chiral separations, the running buffer was 5% HS-CDs in 25 mM phosphate pH 2.5 (triethylammonium salt) with an applied voltage of 15 kV.

Table 2. Relative Migration Time and Resolution of Racemates of DNS-Aliphatic Amino Acids in HS-γCD

	DNS-derivatives	Structure	HS-αCD	MT	HS-βCD	MT	HS-γCD	MT
1	1-amino-2-propanol			ND		ND	5.7	1.80
2	2-amino-1-butanol			ND		ND	5.1	7.20
3	3-methyl-2-butylamine			ND		ND	3.3	2.60
4	3,3-dimethyl-2-butylamine			ND		ND	3.1	4.50

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