BECKMAN COULTER

P/ACE Setter

The worldwide newsletter for capillary electrophoresis

Volume 6, Issue 2 • July 2002

Table of Contents

BECKMAN COULTER

PACE Setter

Volume 6, Issue 2 • July 2002

The worldwide newsletter for capillary electrophoresis

Quantifying Counterions in Drug Discovery

Michael J. Little
Boehringer Ingelheim (Canada) Limited, Research and Development
Laval (Québec) Canada

The Issue

In the discovery phase of pharmaceutical development, concentration-dependent biological assays are used to test the efficacy of compounds to fight disease. One of the most important pieces of data when performing these assays is the correct molecular mass of the compound to be tested. The validity of the results from these assays cannot be certain without it.

During the lifetime of a project, thousands of organic compounds may be synthesized and tested for potency. These compounds are often isolated as a salt form with chloride or trifluoroacetate as the counterion. Although other counterions are possible, these are the most common salt forms that we encounter due to isolation of the compounds from either crystallization or preparative HPLC. As the formula weight of most orally active drugs on the market is less than 550,[1] the addition of a single molecule of hydrochloric acid would increase the formula weight by 6.6%. Adding one molecule of trifluoroacetic acid increases the formula weight by 21%. Clearly, the effect on the results of concentration-dependent assays can be dramatic, especially if there is more than one molecule of counterion present.

Until recently, we relied on an external laboratory to perform counterion quantitation as we did not have a method available in-house. With the advent of combinatorial chemistry, parallel synthesis, and high-throughput screening, the pharmaceutical industry has been striving to synthesize and test more compounds within smaller time periods. Ultimately, we had to develop in-house methods for these analyses in order to maintain our productivity as cost efficiently as possible.

The Methods

Many techniques exist for the analysis of small ions including titration and spectroscopic- and separation-based methods. We had the instrumentation in our laboratories to implement methods based on two techniques: ion chromatography (IC) and capillary electrophoresis (CE). With this paper, we compare our results using both these techniques and provide our rational for the selection of CE as the method of choice for counterion analysis in our laboratory.

The Results

Ion Chromatography

A summary of the IC methods developed is presented in Table 1. The Cl method was adapted from a method developed by Waters[2] while that of the TFA was adapted from a method published by Dionex.[3] For both of the methods, good limits of detection (LODs) were observed with limits of quantitation (LOQs) at the ppb level and reasonable linear dynamic ranges; however, the sample matrix was found to be crucial for obtaining good separations. Most of our chloride salts are soluble in the mobile phase; however, many of the trifluoroacetate salts are not because of the strong ion-pairing effect. Thus, alternative solvents had to be evaluated. A 1:1 mixture of acetonitrile and mobile phase was found to be quite useful for solubilizing many of these compounds; however, interfer-

ence from the solvent peak prevented quantitation of the analyte. Further method development would be required to alter the separation conditions in order to effect a separation between the solvent and the analyte peaks.

Capillary Electrophoresis

Table 2 presents a summary of the CE methods developed. Our method for TFA analysis was adapted from a method published by Altria, *et al.,* for the analysis of succinic and maleic acids.[4] Both of these methods were found to have ppm-level LODs and LOQs and linear dynamic ranges of almost 2 orders of magnitude. In contrast to IC, the solvent used for the sample and standard preparation was found to be less critical for the analysis. The ability to use different sample solvents is due in part to the fact that, with these methods, negatively charged species will reach the detector first, followed by neutral components such as solvents, and then positively charged species. Therefore, compounds with a wider range of solubility could be analyzed by CE without the need for further method development. Compounds prepared as trifluoroacetate salts tend to have poor water solubility and, although many of the chloride salt compounds we produce are water soluble, we wished to find a sample solvent that could dissolve virtually all of the TFA and Cl salt compounds produced at our research center. A sample solvent composed of 95% DMSO and 5% H_2O (v/v) was found to be suitable for this purpose. Figures 1 and 2 show a typical electropherogram for a chloride and TFA standard. An internal standard is employed in each method (see Table 1) to ensure good quantitation. The resulting calibration curves obtained with these methods are shown in Figure 3.

Table 1. IC Method Summaries

	Chloride[2]	Trifluoroacetate[3]
LOD	41 ppb	160 ppb
LOQ	500 ppb	500 ppb
LDR	500 ppb – 20 ppm	500 ppb – 50 ppm
Column	Waters IC-Pak* Anion HR, 4.6 x 75 mm	Dionex IonPac* AG14, 4 x 50 mm Dionex IonPac AS14, 4 x 250 mm
Mobile Phase	1.75 mM LiOH	3.5 mM Na_2CO_3, 0.8 mM $NaHCO_3$
Ion Suppressor	Alltech Anion Suppressor Cartridges	
Detector	Conductivity	

Table 2. CE Method Summaries

	Chloride	Trifluoroacetate
LOD	2 ppm	4 ppm
LOQ	5 ppm	11 ppm
LDR	5 – 20 ppm	11 – 800 ppm
Capillary	Fused silica, 75 µm i.d., 60 cm total length	
Temperature	20°C	
Electrolyte		5 mM Potassium Hydrogen Phthalate, 50 mM MES, 0.5 mM TTAB, pH 5.0
Internal Standard	Nitric Acid	Pentafluoropropionic acid (PFPA)
Detector	UV (indirect), 254 nm	
Field Strength	250 V/cm	333 V/cm
Typical Current Generated	10 µA	15 µA
Instrument Model	P/ACE MDQ Series	
Data System	32 Karat Software, Ver. 4.01	

The Costs

Finally, a cost comparison of the IC and CE methods was performed. To simplify the calculation, the costs for the instrumentation and the time to perform the analysis (salaries) were excluded as they were effectively identical for both techniques. Instead, we decided to focus on the main differences between the two methods by including only the column (or capillary) and all necessary reagents for the analysis of a single compound. All buffers and solvent were prepared in house except the electrolyte used for the Cl analysis by CE. In order to make the cost of the column/capillary reasonable, we amortized them both over the expected lifetime of the IC column at 2000 injections. By doing so, we estimated the cost of a trifluoroacetate analysis to be $12.00[a] for the IC method and $1.35[a] for the CE method. In the case of chloride analysis, we calculated $10.00[a] for the IC method and $29.00[a] for the CE method (commercial buffer). However, if an equivalent CE buffer is made in-house, the cost for analysis drops dramatically to $0.15* for the CE method.

The Decision

Overall, we chose CE as our method of choice for routine counterion analysis. While IC offered the best detection limits, CE provided the most flexibility when handling diverse sample matrices while meeting our sensitivity requirements. For trace analyses, we used 95% DMSO, 5% H2O (v/v) as the sample solvent allowing the compounds dissolved at a high enough concentration to be analyzed by CE. And, given that we can sample from 10 µL volumes, the amount of compound consumed in these cases is negligible. Of course, the most important factor for us was that the CE methods allowed the analysis of compounds covering a wide range of

polarities. In the drug discovery process compounds are not only synthesized individually, but also in batches by combinatorial chemistry or purchased as compound libraries from external sources. The number of compounds in these latter sources makes it difficult to obtain complete physical data on each compound. The fact that the CE methods can analyze these compounds for counterion content in the absence of solubility data is a major asset.

The Impact

With our in-house CE methods now in place, the total turnaround time for an analysis request is on average 1 to 2 days, while previously the time for the analysis to be done externally was one week. This change in operation allows our scientists to have access to the correct stoichiometry of the compounds in less time, which facilitates continued progress within our projects. Once a compound with desirable biological activity has been found, further compounds are synthesized based on structure activity relationships[5] (SAR). Performing SAR generates a series of molecules in which compounds generally have incremental changes in their structures. Prior to implementing the CE methods, we would only analyze a limited number of compounds from each series as the amount of counterion was expected to not vary greatly from compound to compound and the cost required to analyze each compound would have been prohibitive. With the CE methods in place, we now analyze many more compounds within each series in order to ensure that our assumptions of counterion levels are accurate. This gives us greater accuracy with our biological assay results.

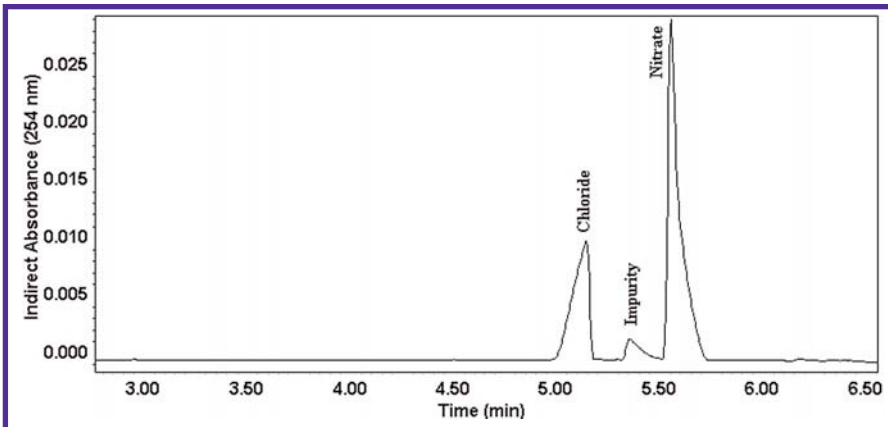


Figure 1. A 40-ppm HCl standard. Sulfate often appears as an impurity that does not interfere with the analysis.

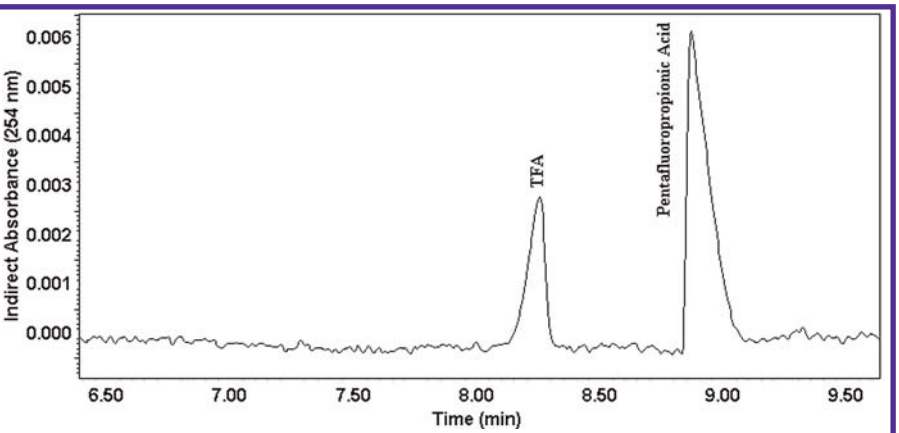


Figure 2. A 40-ppm TFA standard.

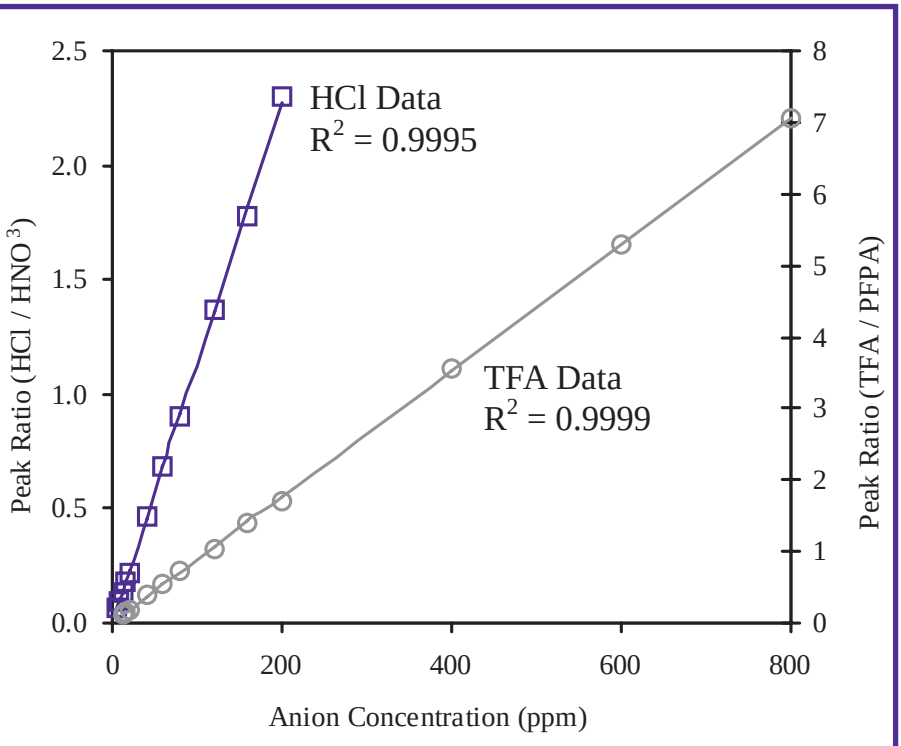


Figure 3: Typical calibration curves for HCl and TFA standards. Standards are prepared relative to the concentration of the acid by mass and then converted to ppm of Cl^- and TFA^-.

[a]All costs calculated in Canadian dollars.

Acknowledgements

The author wishes to acknowledge the contributions of Colette Boucher and Mathieu Guertin for the implementation of the ion chromatographic methods and also those colleagues who have provided chloride and trifluoroacetate salts for analysis.

References

1. Novia, M. A., Chaturvedi, P. R. Design principles for orally bioavailable drugs. *DDT 1 (5),* 179-186 (1996)
2. Krol, J., Benvenuti, M., Romano, J. *Ion Analysis Methods for IC and CIA* and Practical Aspects of Capillary Ion Analysis Theory.* Milford, MA: Waters Corporation, 508/478-2000, January 2000.
3. Determination of Trifluoroacetic Acid (TFA) in Peptides. *Dionex Application Note AN115,* Dionex Corporation, Sunnyvale, CA, LPN 0898 5M 4/97 (1997)
4. Altria, K. D., Assi, K. H., Bryant, S. M., Clark, B. J. Determination of organic acid drug counter-ions by capillary electrophoresis. *Chromatographia 44* (7/8), 367-371 (1997)
5. Patrick, G. L. *An Introduction to Medicinal Chemistry,* Chap. 7, p. 84. New York: Oxford University Press, 1995.

Increasing Oligonucleotide Analysis Throughput: Serial Injection before Analysis (SIBA)

D. W. Dyer[1], R. Shuburg[1], B. A. Williams[2], L. Maddera[1], and L. A. Lewis[1]

[1]Department of Microbiology and Immunology Laboratory for Microbial Functional Genomics and DNA Sequencing, The University of Oklahoma Health Sciences Center, Oklahoma City, OK (email address: david-dyer@ouhsc.edu)

[2]Beckman Coulter, Inc.

Introduction

Capillary electrophoresis (CE) has been used for oligonucleotide separations since the mid 1980s.[1,7] Subsequent analytical research led to improved gel matrices[2,11] and improved quantitation.[3-6,9,10] These advancements now allow the use of CE to assess accurately the quality of synthetic oligonucleotides.[8] However, current methodology for synthetic oligonucleotide quality control (QC) processes have limited throughput (approximately 25 samples per day per system). With the advent of multiplex oligonucleotide synthesizers, the requirement for high-throughput analysis of oligonucleotide integrity has become a necessity. To address this limitation, we developed a sequential injection before analysis (SIBA) protocol increasing the oligonucleotide sample throughput approximately four-fold over traditional CE separation. Using this protocol, the analysis of a 96-well microtiter tray of oligonucleotides can be completed in approximately 20 hours.

Materials and Methods

Preparation of Oligonucleotides.

Oligonucleotides were synthesized with the Bioautomation MerMade* multiplex oligonucleotide synthesizer using standard phosphoramidite chemistry. After deprotection, dehydration, and Sephadex* G50 cleanup, the oligonucleotides were suspended in 100 µL of deionized, ultra-filtered (DIUF) H_20, and the concentration of each was determined by UV spectroscopy using a SpectraMax* UV-Vis 96-well spectrophotometer. An aliquot of each oligo was then diluted to approximately 2 pmol/µL in DIUF H_20 in a 96-well microtiter tray and covered with 2 to 3 drops of mineral oil.

Preparation of CE Materials

For oligonucleotide separations, we used the eCAP™ ssDNA kit from Beckman Coulter which included capillaries (100 µm internal diameter), buffer (TRIS/borate/EDTA), urea, lyophilized sieving gel matrix, and Test mix pd (A) 40-60 (an oligonucleotide standard). The assay was prepared according to the manufacturer's instructions.

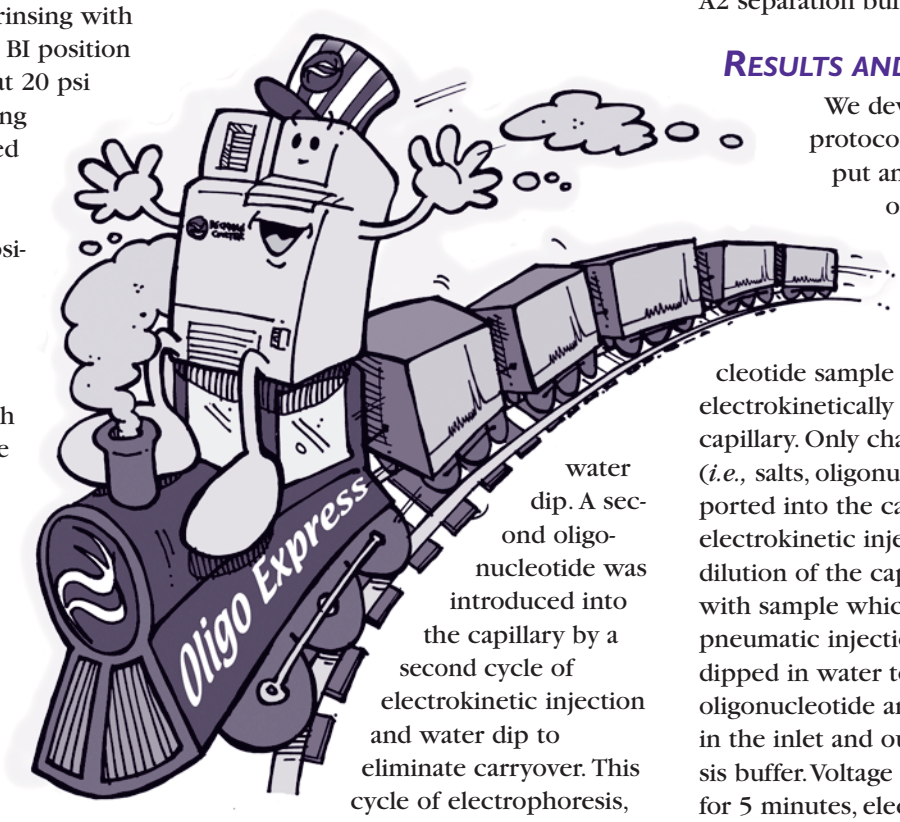
The SIBA Protocol

The P/ACE™ MDQ capillary electrophoresis instrument was used for all separations. The instrument was equipped with a Peltier-thermostatted sample chamber and ultraviolet (UV) detector at 254 nm. The sieving gel matrix was placed in position A1 of the Sample Outlet (SO) tray, and the oligonucleotide sample tray was placed in the Sample Inlet (SI) position. The A1 position of the Buffer Inlet (BI) tray and Buffer Outlet (BO) tray contained vials with 1.6 mL of DIUF water. Waste vials containing 800 µL of DIUF water were placed at BI tray F6 and BO tray B1 to catch waste purged from the capillary during the capillary rinsing and equilibration steps. BI tray and

BO tray A2 through A6 contained 1.5-mL vials of the TRIS/borate/urea separation buffer. A quantity of 100 µL test mix pd (A) 40-60 was placed in a micro vial and positioned in BI F1. The remaining positions of the BI and BO trays were empty.

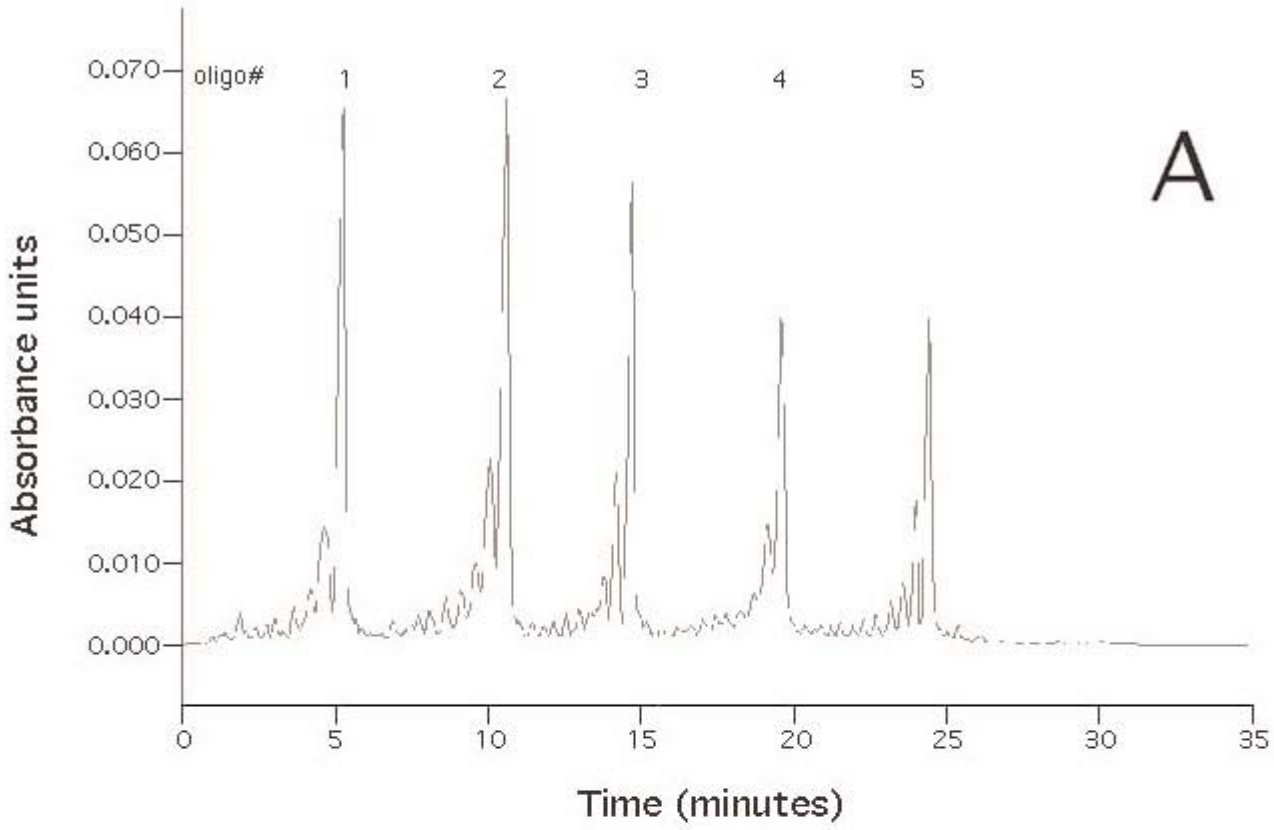
To start the electrophoresis run, the capillary was rinsed briefly with DIUF water from BI position A1 to BO position B1 (waste) at 20 psi for 1 minute followed by rinsing with separation buffer from BI position A2 to BO position B1 at 20 psi for 1 minute. The sieving gel was then introduced into the capillary by applying a reverse-pressure rinse from position SO A1 to position BI F6 (waste) at 50 psi for 10 minutes. The voltage was applied at 3 kV for 5 minutes with separation buffer at the inlet and outlet of the capillary (BI A2, BO A2). Then a 10-minute equilibration at 9 kV was applied using the same inlet and outlet separation buffer. After the equilibration step, the oligonucleotide standard was injected electrokinetically into the capillary at 10 kV for 2 sec. Pressure was applied simultaneously at both inlet and outlet using 20 psi during the separation at 9 kV for 45 min followed by a water dip of the capillary inlet and outlet.

During electrophoresis, the capillary temperature was kept constant at 30°C while the sample compartment was thermostat-controlled to 8°C. The temperature was kept low to preserve the integrity of the sieving gel. The first oligonucleotide was introduced into the capillary by electrokinetic injection at 10 kV for 2 seconds followed by a water dip of the capillary inlet and outlet. Pressure was applied at 20psi to both the inlet and outlet of the capillary during the separation at 9 kV to minimize loss of the capillary sieving gel to the buffer vials and also to minimize outgassing of the gel. The oligonucleotide was electrophoresed into the capillary at 9 kV for 5 minutes, whereupon the capillary was subjected to a second water dip. A second oligonucleotide was introduced into the capillary by a second cycle of electrokinetic injection and water dip to eliminate carryover. This cycle of electrophoresis, electrokinetic injection, and water dip of the capillary was then repeated an additional three times; five oligonucleotides were introduced into the capillary prior to the movement of the first oligo past the detector. This cyclic injection–electrophoresis– injection procedure was performed four times, allowing the injection and analysis of 20 oligonucleotide samples before fresh gel matrix was introduced into the capillary and equilibrated. To change gel matrices, reverse pressure was applied to SO A1 (containing the sieving gel) for 10 minutes at 50 psi, removing the used gel from the capillary to the waste vial at position BI F6. Following gel replacement, the new gel was equilibrated for 10 minutes at 9 kV. This "gel replacement" procedure was programmed as an individual method in the P/ACE MDQ Software. The next 20 oligonucleotides were electrophoresed using the next set of separation buffers (*i.e.,* BI A3 and BO A3 would be the next separation buffers following BI A2 and BO A2 separation buffers).



Results and Discussion

We developed the SIBA protocol for high-throughput analysis of oligonucleotide integrity. In brief, the SIBA approach is very simple. First, an oligonucleotide sample is injected electrokinetically into the inlet of the capillary. Only charged species (*i.e.,* salts, oligonucleotides) are transported into the capillary during electrokinetic injection, preventing dilution of the capillary gel matrix with sample which may occur with pneumatic injections. The capillary is dipped in water to remove residual oligonucleotide and then immersed in the inlet and outlet electrophoresis buffer. Voltage is applied at 9 kV for 5 minutes, electrophoresing this oligonucleotide toward the detector. After the first oligonucleotide travels a fraction of the capillary distance towards the detector, the cycle of electrokinetic injection of oligonucleotide, water dip, and 5-minute voltage application is repeated for a second oligonucleotide. This process continues until five oligonucleotides have been injected. The separation is allowed to proceed for 30 minutes after injection of the fifth oligonucleotide sample. We attempted to implement additional injections prior to the final 30-minute electrophoresis, but this resulted in a loss of resolution (data not shown). After 20



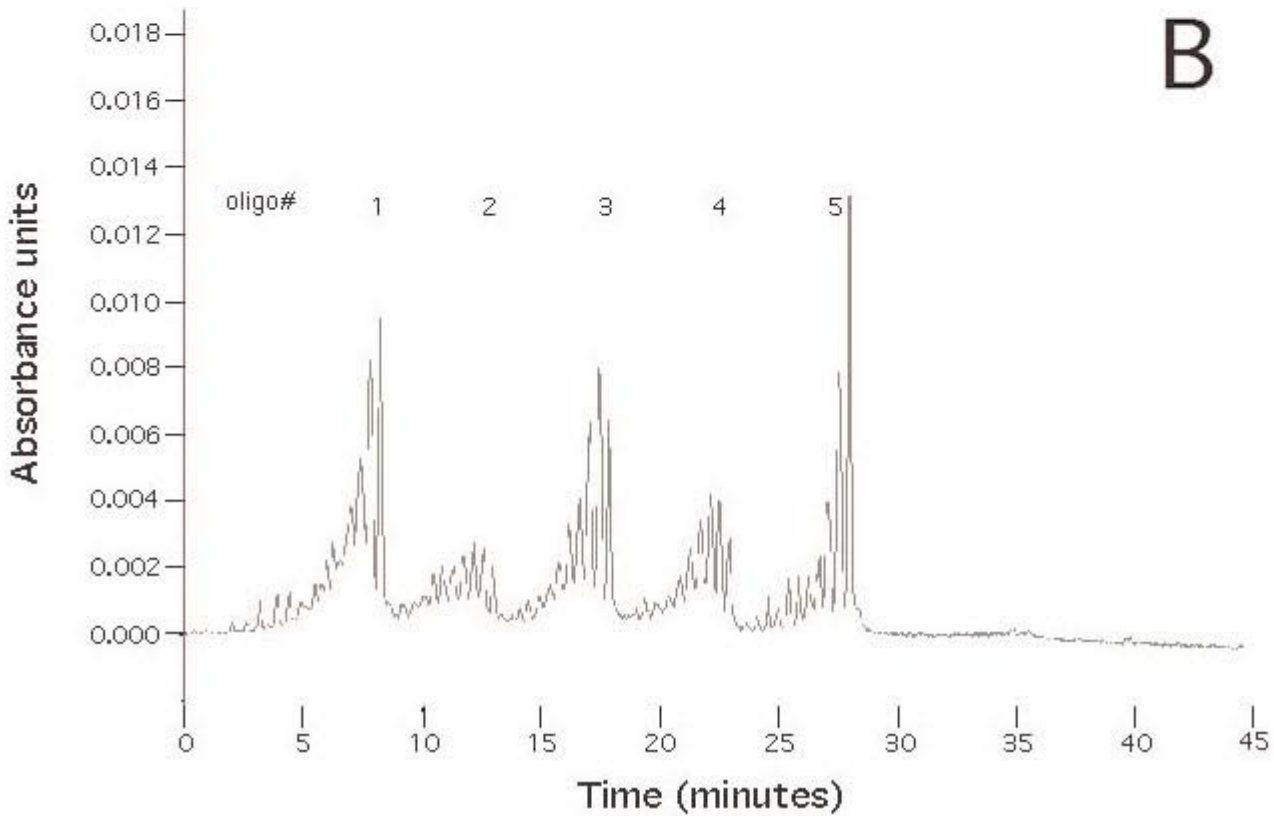


Figure 1: Capillary electrophoresis of oligonucleotides using the SIBA protocol. Panel A: high quality 25-mer oligonucleotides. Panel B: low-quality 25-mer oligonucleotides.

oligonucleotides (4 runs) have been analyzed, the gel matrix is replaced.

We mixed oligonucleotides differing by one base pair in length in equal molar amounts and analyzed these using the SIBA protocol. The P/ACE MDQ software was used to integrate the major and minor oligonucleotide peaks to estimate percent purity of the major product. This demonstrated that the peaks were resolved with single-base resolution, allowing for proper peak integration and subsequent analysis of oligonucleotide purity (data not shown).

We tested the SIBA protocol using 96 oligonucleotides synthesized using a Bioautomation MerMade oligonucleotide synthesizer. Analysis of 96 25-mer oligonucleotides took approximately 22 hours. By contrast, using standard CE protocols, analysis of 96 oligonucleotides would have taken almost 90 hours. Typical electropherograms are shown in Figure 1. Panel A shows the electropherogram of five 25-mer oligonucleotides. Based on peak area analysis, the full-length oligonucleotide products were present at approximately 65% of the total (mean 65.4%, std. dev. 3.4%; range of 60.4-70.9%). This is the expected abundance of the full-length product for oligonucleotide synthesis at >98-99% coupling efficiency. These oligonucleotides were shown subsequently to be successful in PCR® amplification (data not shown). By contrast, Panel B shows the results of an electropherogram of low-quality 25-mers; full-length products were only a mean of 16.4% of the total products seen on the electropherogram (std. dev. 11.8%; range of 0.1–21.5%). These PCR primers subsequently failed to amplify successfully in a standard PCR reaction. The SIBA protocol is time-, labor-, and cost-effective and allows the quality control analysis of a complete 96-well plate of oligonucleotides in fewer than 24 hours.

References

1. Cohen, A. S., Terabe, S., Smith, J. A., Karger, B. L. High-performance capillary electrophoretic separation of bases, nucleosides, and oligonucleotides: retention manipulation via micellar solutions and metal additives. *Anal. Chem. 59(7):*1021-7 (1987)
2. Heller, C., Viovy, J. L. Brief report: electrophoretic separation of oligonucleotides in replenishable polyacrylamide-filled capillaries. *Appl. Theor. Electrophor. 4(1):*39-41 (1994)
3. Khan, K., Liekens, K., Van Aerschot, A., Van Schepdael, A., Hoogmartens, J. Stability measurement of oligonucleotides in serum samples using capillary electrophoresis. *J. Chromatogr. B Biomed. Sci. Appl. 702(1-2):*69-76 (1997)
4. Leeds, J. M., Graham, M. J., Truong, L., Cummins, L. L. Quantitation of phosphorothioate oligonucleotides in human plasma. *Anal. Biochem. 235(1):*36-43 (1996)
5. Lowery, J. D., Ugozzoli, L., Wallace, R. B. Application of capillary electrophoresis to the measurement of oligonucleotide concentration and purity over a wide dynamic range. *Anal. Biochem. 254(2):*236-9 (1997)
6. Naqvi, N. A., Rede, T., Bonifacio, M., Ma, D. D. Quantitative analysis of porphyrin-linked oligonucleotides and their biodegraded products by capillary gel electrophoresis using simultaneous multiple wavelengths. *Anal. Biochem. 269(2):*426-7 (1999)
7. Paulus, A., Ohms, J. I. Analysis of oligonucleotides by capillary gel electrophoresis. *J. Chromatogr. 507:*113-23 (1990)
8. Pietta, P. G., Mangano, M. F., Battaglia, C., Salani, G., Bernardi, L. R., De Bellis, G. Application of capillary electrophoresis at low pH to oligonucleotides quality control. *J. Chromatogr. A. 853(1-2):*355-8 (1999)
9. Reyderman, L., Stavchansky, S. 1997. Quantitative determination of short single-stranded oligonucleotides from blood plasma using capillary electrophoresis with laser-induced fluorescence. *Anal. Chem. 69(16):*3218-22.
10. Srivatsa, G. S., Batt, M., Schuette, J., Carlson, R. H., Fitchett, J., Lee, C., Cole, D. L. Quantitative capillary gel electrophoresis assay of phosphorothioate oligonucleotides in pharmaceutical formulations. *J. Chromatogr. A. 680(2):*469-77 (1994)
11. Sudor, J., Foret, F., Bocek, P. Pressure refilled polyacrylamide columns for the separation of oligonucleotides by capillary electrophoresis. *Electrophoresis 12(12):*1056-8 (1991)

Acknowledgements

The authors thank Allison Gillaspy, Ph.D., and Amanda Hendrickson for critical reading of the manuscript. This work was supported in part by USPHS grant P20-RR15564 from the National Center for Research Resources, National Institutes of Health.

HPCE 2002—Small but Mighty!

The 15th International Symposium on Microscale Separations and Analysis was held in the Aula Magna of the University of Stockholm in Stockholm, Sweden, April 13-18, 2002. More than 500 scientists from Europe, North America, and Asia participated in six plenary, 19 keynote, and 56 lectures in parallel sessions. As usual, the poster sessions did not disappoint with more than 250 novel presentations.

Scientific Programs

Although many areas of application were represented, the utilization of CE technology in proteomics was most highly visible. There were three proteomics sessions, two protein analysis sessions, and a large number of posters on this topic. There was a good sampling of graduate student and post-doctoral submissions—all of very high caliber, boding well for the future implementation and advance of this technology. The scientific program also included a rather unique session with a format tailor-made for industry. The Pharmaceutical Analysis session began with short presentations from representatives of twelve different pharmaceutical/biotechnology companies. These presentations summarized where each organization had implemented CE technology effectively and also where they had struggled. The goal was to share successful separation strategies and provide feedback where future development will be warranted. This was a very successful format—one which will undoubtedly be built upon at future conferences. Perhaps it should be called "Putting CE to Work" in the pharmaceutical industry? This format was also very useful for companies like Beckman Coulter as it provided direct feedback from scientists like yourselves—an important element that drives future product development.

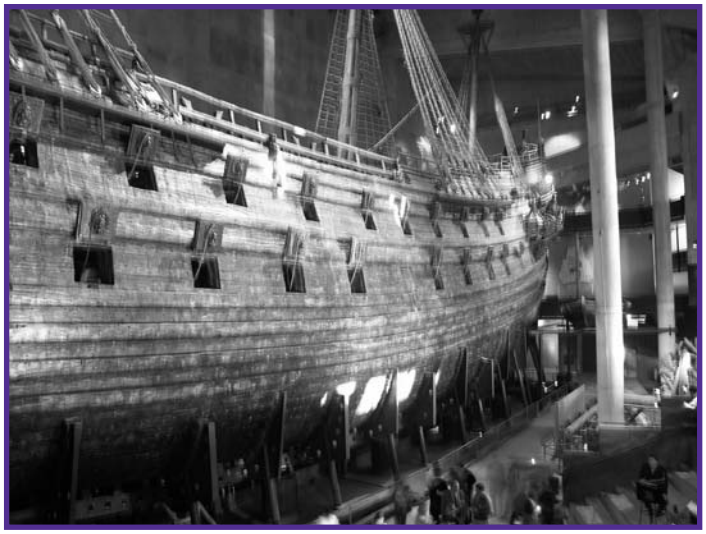
The social program also was of high quality—treating delegates for lunch within the famous Gold and Blue rooms at Stockholm City Hall. This is, of course, home to the prestigious Nobel Prize ceremony—one could feel the sense of inspiration while walking the same steps as those who have made such great contributions to science.

Exhibition

The conference exhibition included representation from 17 different companies. The Beckman Coulter exhibition featured a 20-foot booth with the central theme of "Putting CE to Work." One section of this booth featured large-screen projection of a selection of scientist interviews describing how they have routinely Put CE to Work in their analytical processes. Although diverse in application, all these examples held a common theme: the routine implementation of CE. The second half of the booth featured a 10-foot timeline that highlighted Beckman Coulter's advances in CE technology since the first introduction of the P/ACE 2000 in 1989. Of course, this time line extended beyond the present time and into the future. Our message here was straightforward: Beckman Coulter is committed to CE as a core technology and will continue to advance it with an objective of developing comprehensive analytical solutions toward genomics, proteomics, and drug discovery. These developments will include well-integrated and comprehensive assays in a format that we like to call Smart Solutions™.

P/ACE User Event

The Beckman Coulter P/ACE User Event continues to be a highlight of the HPCE symposium. This venue provides us with an opportunity to thank you for your investment in P/ACE technology and your work in propelling this technology into applications key to the advancement of science. This year's event was titled "Dive into History" featuring a venue of the famous Vasa Museum in Stockholm. The evening included tours of



the impressive Vasa exhibit, a four-course gourmet dinner, and entertainment by fire-eaters and jugglers whose comedy certainly kept us all entertained.

This event also included the presentation of the 2002 "Putting CE to Work" Award which was given this year to Professor G. Blaschke from the Institute of Pharmaceutical and Medicinal Chemistry, Muenster, Germany, for the submission titled "Determination of Dinucleoside Polyphosphates in Human Platelets by Capillary Electrophoresis." This award is peer-selected by all of those attending the P/ACE User Event and, as this event is open to all P/ACE Users and does not require attendance to participate, you may want to consider preparing now for next year's event to be held in San Diego in conjunction with HPCE 2003. As an incentive, the winner receives the coveted "Putting CE to Work" trophy, a very nice gift and bragging rights for a full year. Start now and ensure that your entry is ready for submission to this event.

Meeting Reminder

CASSS California Separation Science Society

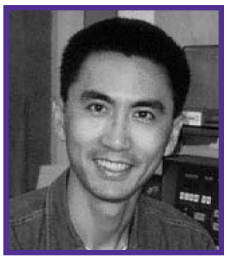
CE in the Biotechnology & Pharmaceutical Industries: Practical Applications for the Analysis of Proteins, Nucleotides and Small Molecules

August 17 – 19, 2002, Renaissance Mayflower Hotel, Washington, DC

The goal of this symposium is to provide a forum for the discussion of recent developments in CE analysis of protein, nucleotide and small molecule pharmaceuticals. The presentations and workshops will be devoted completely to practical concerns to strengthen the use of CE within the biotechnology and pharmaceutical industries. The symposium will include two full days of seminars, practical workshops, technical seminars on CE applications, a networking reception, and poster session.

For more information please visit the symposium web site at www.casss.org.

Congratulations to Dr. David Chen, Recipient of the 2002 Royal Society of Chemistry Award in Analytical Separation Methods



"Distinguished for his significant contributions to the development of the relatively new separation technique of capillary electrophoresis (CE). In particular, he has provided new insights into the fundamental understanding of the phenomena observed in CE, proposing as a result of these studies, a unified theory to describe analyte migration and peak resolution in a range of separation procedures."[1]

David Chen is an Associate Professor in the Department of Chemistry, and an Associate Member in the Department of Pharmacology and Therapeutics, University of British Columbia. He has worked in the CE area for over a decade and has published extensively on the principles of CE. More information on this research can be found at http://www.chem.ubc.ca/personnel/faculty/chen/

1. Quotation from the President of the Royal Society of Chemistry, Professor Steven V. Ley.

Quantifying Carbohydrate-Deficient Transferrin (CDT) in Serum

François de l'Escaille,
Francis Pourignaux,
Michel Brohet
ANALIS s.a./n.v.
Namur Belgium —
http://www.analis.com -
E-mail : ceofix@analis.be

Introduction

Transferrin is an iron-transport glycoprotein which consists of a polypeptide chain with two binding sites for iron and two N-linked oligosaccharide chains. The oligosaccharide chains are micro-heterogeneous and carry sialic acid residues.Transferrin can be separated into several isoforms based on this structure. The isoforms of transferrin have isoelectric points (pI) that range from 5.2 to 5.9, with the predominant isoform (tetrasialo) having a pI of 5.4. Asialo- and disialotransferrin, which result from an impaired glycosylation mechanism, are commonly referred to as carbohydrate-deficient transferrin (CDT), which is an important marker of chronic alcohol abuse.

We have developed a simple and rapid method to quantify the percentage of CDT from serum. With this method, we are able to show a difference of migration between transferrin carrying two sialic acids after desialylation and the disialotransferrin from serum of alcoholic patients. Conversely, the transferrin carrying three sialic acids after desialylation and the trisialotransferrin co-migrate. This suggests that the disialotransferrin fraction not only carries less sialic acid, but also lacks one of the entire carbohydrate chains. Reproducibility, total imprecision, and the influence of variants were determined. A commercial kit to assay for CDT has been developed from this work and is available through Analis s.a./n.v., Belgium.

Materials and Methods

Capillary zone electrophoresis was performed on both a P/ACE™ 5000 system equipped with a UV detector and on a P/ACE MDQ system equipped with UV detector, sample refrigeration and large buffer reservoir (all from Beckman Coulter). Buffers and diluents were from the CEofix* CDT (Analis s.a., Namur, Belgium) kit. The borate buffer at pH 8.5 uses a patented double coating of the capillary to improve resolution and reproducibility. The kit contains also an Fe solution for sample preparation.

The capillary (Polymicro, Phoenix, AZ, USA) has an internal diameter of 50 µm and a length of 50 cm to the detector. Both the P/ACE 5000 and P/ACE MDQ gave similar results, varying only by migration time. Sample preparation entails mixing volume-by-volume 50 µL serum and the Fe solution in a microvial for the P/ACE 5000 system or 60 µL for the P/ACE MDQ. First the fused-silica capillary is rinsed with the "initiator" solution which coats the capillary wall with a polycation. This is followed by a second rinse with a "buffer" solution, containing a polyanion, which adds a second layer of coating. The "serum-solution" is loaded by pressure injection for P/ACE 5000 system and by vacuum for P/ACE MDQ. Separation is performed at a voltage of 28 kV and detection is at 200 nm. The corrected peak areas are

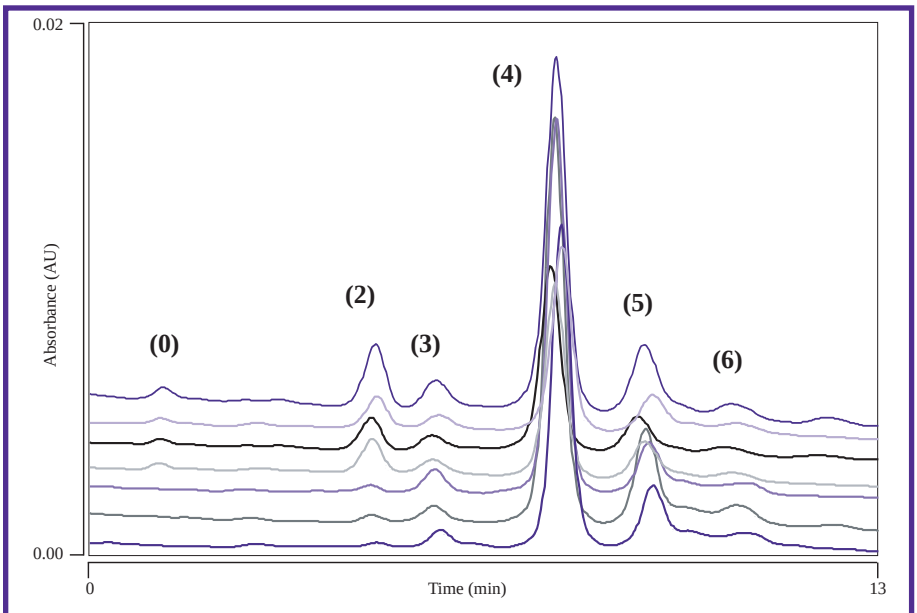


Figure 1. Example of separation of transferrin isoforms obtained for 4 heavy drinkers and 3 social drinkers. Instrument used: P/ACE 5000. Peaks detected: (0) asialotransferrin, (2) disialotransferrin, (3) trisialotransferrin, (4) tetrasialotransferrin, (5) pentasialotransferrin, (6) hexasialotransferrin.

calculated by the integration software and expressed in area percent of the total transferrin. After separation, the coating is cleared by a rinse with NaOH, and the capillary is ready for the next analysis.

Results

Separation

Sera were obtained from both men and women with well-documented drinking habits. These subjects were classified as heavy drinkers or social drinkers and analyzed with the above method. For social drinkers, the separation and quantification of disialo-, trisialo-, tetrasialo-, pentasialo-, and hexasialo-transferrin is shown in Figure 1. For heavy drinkers, an increase of disialo-transferrin and the presence of asialotransferrin were noted.

Reproducibility and Imprecision

Evaluation of the precision performance was conducted in the Department of Clinical Pharmacology, University of Bern, Switzerland, according to "Evaluation of Precision Performance of Clinical Chemistry Devices; Approved Guideline. NCCLS document EP5-A" [ISBN 1-56238-368-X]. This evaluation was carried out on a P/ACE MDQ system.

Samples and controls (sera) were run in duplicate, two runs per day, over 20 days. The results are reported in Table 1. (Professor Wolfgang Thormann, Ph.D., Professor and Head of the Analytical Laboratory Dept. of Clinical Pharmacology, e-mail: wolfgang.thormann@ikp.unibe.ch; and Christian Lanz, University of Bern, Switzerland.)

Effect of Desialylation on the Various Isoforms

To demonstrate the ability of the proposed method to resolve all the CDT isoforms, serum of a heavy drinker was incubated with neuraminidase for increasing time to obtain sequential cleavage of the sialic acid residue. These results are shown in Figure 2. This electropherogram clearly indicates that after desialylation pentasialo-, tetrasialo-, and trisialotransferrin produce an asialotransferrin called 0a, whereas disialotransferrin produces another asialotransferrin called 0b. Both are different from the asialotransferrin found in sera from heavy drinkers.

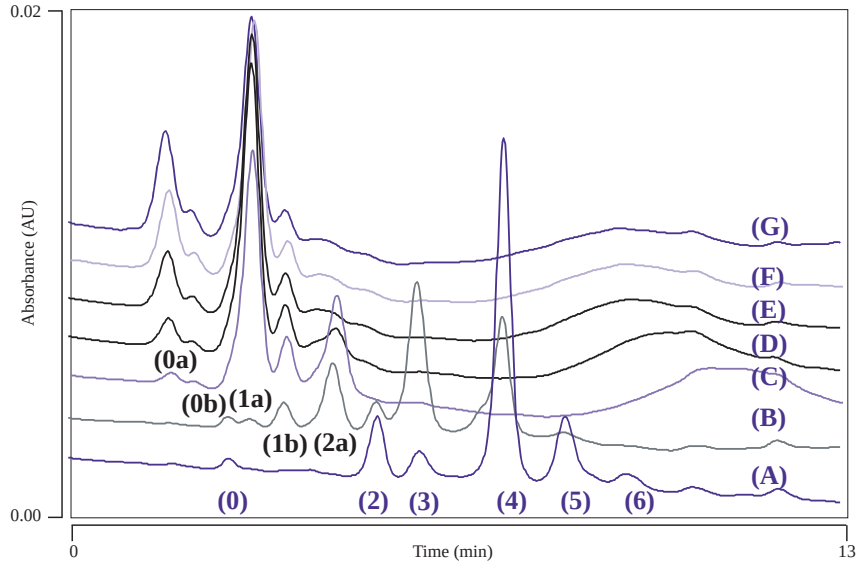


Figure 2. Desialylation obtained, by incubation with neuraminidase of serum from a heavy drinker. From electropherogram (A) to (G), each time a 20-minute longer incubation time. Instrument used: P/ACE 5000. Peaks detected: (0) asialotransferrin, (2) disialotransferrin, (3) trisialotransferrin, (4) tetrasialotransferrin, (5) pentsialotransferrin, (6) hexasialotransferrin. Desialylation of pentasialo-, tetrasialo-, and trisialotransferrin will result in fraction (2a), (1a), and (0a). Desialylation of disialotransferrin will result in fractions (1b) and (0b).

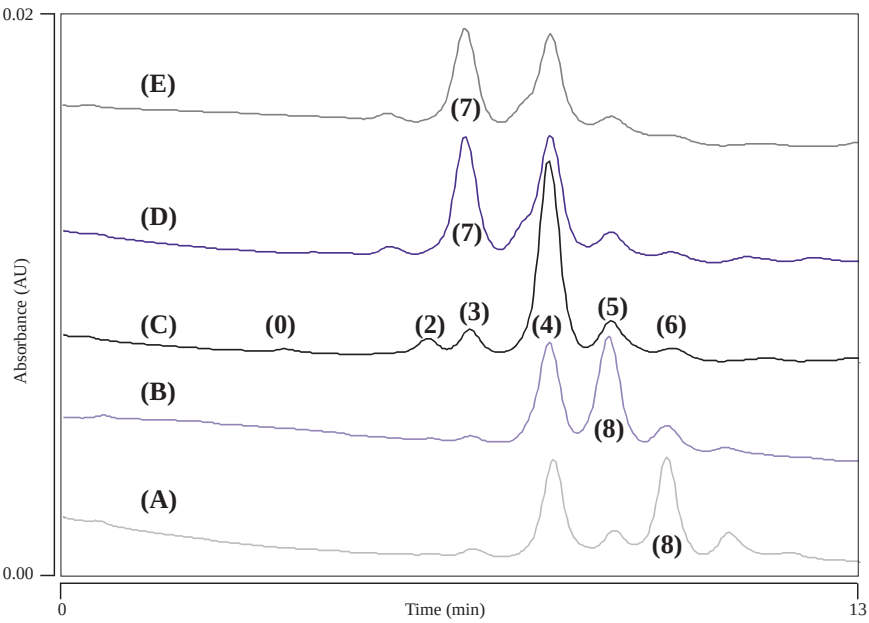


Figure 3. Separation of genetic variants of transferrin. Trace (C) is from heavy drinkers, Traces (A) and (B) are CB phenotypes, while Traces (D) and (E) are CD phenotypes. Instrument used: P/ACE 5000. Peaks detected: (0) asialotransferrin, (2) disialotransferrin, (3) trisialotransferrin, (4) tetrasialotransferrin of C phenotype, (5) pentasialotransferrin, (6) hexasialotransferrin, (7) tetrasialotransferrin of D phenotype, (8) tetrasialotransferrin of B phenotype.

Interference of Variants

Genetic variants, attributable to substitutions of amino acids in the

Table I. Evaluation of Precision Performance

Samples and controls (sera) were run in duplicate, 2 runs per day, during 20 days (N=80). Instrument used: P/ACE™ MDQ.

	Sample				Control			
	Low		High		Low		High	
	Asialo	Disialo	Asialo	Disialo	Asialo	Disialo	Asialo	Disialo
Mean	—	0.89	0.68	3.20	—	0.94	0.75	3.59
SD	—	0.06	0.12	0.12	—	0.06	0.07	0.13
CV	—	7.2	17.7	3.9	—	6.6	9.0	3.6

polypeptide chain of human serum can occur. At least 38 transferrin variants have been described. In addition to the common (C) transferrin type, anodal (B) and cathodal (D) variants have been reported in different human populations. It is important to be able to identify the heterozygous CB and CD phenotypes to avoid false positive or false negative results. The resolution of these variants is highlighted in Figure 3.

Conclusion

This capillary electrophoresis method has proven to be effective to quantify CDT which is a valuable marker in alcohol abuse research. This method can be automated easily with the P/ACE capillary electrophoresis system from Beckman Coulter. The high resolution and reproducibility of the method make it possible to identify potential variants while avoiding false results.Commercial kits are available through Analis for research purposes: Each kit contains: rinse, conditioner, initiator, buffer, Fe-solution.

CEofix CDT kit for P/ACE 5000 (40 tests)
Analis PN: 10-004240/844111046

CEofix CDT kit for P/ACE MDQ (100 tests)
Analis PN: 10-004740/844111036

**FluorInert is a registered trademark of Minnesota Mining and Manufacturing Company. CEofix is a trademark of Analis s.a. Belgium. All other trademarks are the property of their respective owners.*

NSAI I.S. EN ISO 9001

BECKMAN COULTER™

Developing innovative solutions in genetic analysis, drug discovery, and instrument systems.

Innovate SIMPLIFY Automate

Beckman Coulter, Inc. • 4300 N. Harbor Boulevard, Box 3100 • Fullerton, California 92834-3100
Sales: 1-800-742-2345 • Service: 1-800-551-1150 • Telex: 678413 • Fax: 1-800-643-4366 • www.beckmancoulter.com

Worldwide Life Science Research Division Offices:
Australia (61) 2 9844-6000 **Canada** (905) 819-1234 **China** (86) 10 6515 6028 **Eastern Europe, Middle East, North Africa** (41) 22 994 07 07 **France** 01 49 90 90 00 **Germany** (89) 35870-0 **Hong Kong** (852) 2814 7431 / 2814 0481 **Italy** 02-953921 **Japan** 03-5404-8359 **Mexico** 525-605-77-70 **Netherlands** 0297-230630 **Singapore** (65) 6339 3633 **South Africa/Sub-Saharan Africa** (27) 11-805-2014/5 **Spain** (34) 91 3836080 **Sweden** 08-564 85 900 **Switzerland** 0800 850 810 **Taiwan** (886) 2 2378 3456 **Turkey** 90 216 309 1900 **U.K.** 01494 441181 **U.S.A.** 1-800-742-2345

NL-9198A B2002-5107 CB-8

Printed in U.S.A. on recycled paper.