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Simultaneous Concentration and Separation of Enantiomers with Chiral Temperature Gradient Focusing

Karin M. Balss, Wyatt N. Vreeland, Karen W. Phinney, and David Ross*

National Institute of Standards and Technology, Gaithersburg, Maryland 20899

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A new technique is demonstrated for the simultaneous concentration and high-resolution separation of chiral compounds. With temperature gradient focusing, a combination of a temperature gradient, an applied electric field, and a buffer with a temperature-dependent ionic strength is used to cause analytes to move to equilibrium, zero-velocity points along a microchannel or capillary. Different analytes are thus separated spatially and concentrated in a manner that resembles isoelectric focusing but that is applicable to a greater variety of analytes including small chiral drug molecules. Chiral separations are accomplished by the addition of a chiral selector, which causes the different enantiomers of an analyte to focus at different positions along a microchannel or capillary. This new technique is demonstrated to provide high performance in a number of areas desirable for chiral separations including rapid separation optimization and method development, facile reversal of peak order (desirable for analysis of trace enantiomeric impurities), and high resolving power (comparable to capillary electrophoresis) in combination with greater than 1000-fold concentration enhancement enabling improved detection limits. In addition, chiral temperature gradient focusing allows for real-time monitoring of the interaction of chiral analyte molecules with chiral selectors that could potentially be applied to the study of other molecular interactions. Finally, unlike CE, which requires long channels or capillaries for high-resolution separations, separations of equivalent resolution can be performed with TGF in very short microchannels (mm); thus, TGF is inherently much more suited to miniaturization and integration into lab-on-a-chip-devices.

Many of the pharmaceuticals used today and most currently under development are chiral,[1−3] occurring in two enantiomeric forms that are nonsuperimposable mirror images of each other. Because the two enantiomers are chemically and physically identical, differing only in the way they interact with other chiral molecules and their effect on plane-polarized light, their separation and quantitation continue to be challenging. In addition, for many classes of pharmaceuticals, only one enantiomer is efficacious as a drug, while the other enantiomer can often cause harmful side effects.[4] For these reasons, regulatory agencies, such as the U.S. Food and Drug Administration (FDA), are increasingly requiring pharmaceutical companies to understand the effects of each enantiomer separately and to produce drugs that contain only the pure, therapeutically active enantiomer.[5]

The increasingly stringent regulation of chiral drugs has created a corresponding need for robust, versatile, and sensitive methods of chiral separations on both analytical and preparative scales. FDA guidelines require the development of "...quantitative assays for individual enantiomers in in vivo samples..." as well as methods for assessing enantiomeric purity and stability.[5] Chiral high-performance liquid chromatography (HPLC)[6,7] is the most commonly used technique because it is easily automated and a wide variety of chiral stationary phases with differing enantioselectivity are commercially available.[8]

Chiral capillary electrophoresis (CE)[9] is an alternative technique that for some applications is gradually becoming a preferred method because of its low reagent and sample consumption and high efficiency. However, typical CE separations suffer from relatively poor detection limits, particularly when coupled with UV absorbance detection. Consequently, CE has not been widely adopted for the chiral analysis of drug molecules in biological fluids such as serum or urine in which the drug and its metabolites are usually at much lower concentrations than in a laboratory preparation.[2,10−13] Although sample extraction methods are often used to preconcentrate sample before analysis by CE, these methods increase analysis time and cost. Transient stacking, sweeping, or isotachophoresis techniques can also be used in conjunction with CE to improve detection limits.[14,15] With these

* Corresponding author: E-mail: david.ross@nist.gov.

(1) Lam, S.; Malikin, G. *Chirality* **1992**, *4*, 395−399.
(2) Scriba, G. K. E. *Electrophoresis* **2003**, *24*, 2409−2421.
(3) Verleysen, K.; Sandra, P. *Electrophoresis* **1998**, *19*, 2798−2833.
(4) Subramanian, G., Ed. *Chiral Separation Techniques A Practical Approach*, 2nd ed.; Wiley-VCH: Weinheim, 2001.
(5) FDA Policy Statement for the Development of New Stereoisomeric Drugs: Washington, DC, 1992.
(6) Pirkle, W. H.; Finn, J. M.; Schreiner, J. L.; Hamper, B. C. *J. Am. Chem. Soc.* **1981**, *103*, 3964−3966.
(7) Pirkle, W. H.; House, D. W. *J. Org. Chem.* **1979**, *44*, 1957−1960.
(8) Armstrong, D. W.; Zhang, B. *Anal. Chem.* **2001**, *73*, 557A−561A.
(9) Gassmann, E.; Kuo, J. E.; Zare, R. N. *Science* **1985**, *230*, 813−814.
(10) Bonato, P. S. *Electrophoresis* **2003**, *24*, 4078−4094.
(11) Hempel, G. *Electrophoresis* **2000**, *21*, 691−698.
(12) Chen, S. H.; Chen, Y. H. *Electrophoresis* **1999**, *20*, 3259−3268.
(13) Altria, K. D.; Elder, D. *J. Chromatogr., A* **2004**, *1023*, 1−14.
(14) Urbanek, M.; Krivankova, L.; Bocek, P. *Electrophoresis* **2003**, *24*, 466−485.
(15) Chien, R. L. *Electrophoresis* **2003**, *24*, 486-497.

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types of methods, sample concentration is accomplished by causing the sample plug to move across a boundary between two buffers of different composition in a capillary or microchannel. The two buffer compositions are chosen such that the analytes of interest will move more slowly through one buffer than the other. As the sample plug moves from the fast side of the boundary to the slow side, it is compressed and made more concentrated. Concentration enhancements of the order of 100−1000-fold are typical,[14] though enhancements as high as 5000-fold have been reported for micellar sweeping with some strongly hydrophobic analytes.[16,17] However, these methods are not universally applicable and often require sample pretreatment or extraction to produce a sufficiently clean sample for stacking.[10−12]

Chiral microchip CE is another new technology area that has been the subject of several recent papers.[18] The change to a two-dimensional microchip geometry allows for increased injection efficiency (i.e., very narrow initial sample zones), enabling chiral separations to be performed more rapidly. However, detection sensitivity and resolution with microchip CE are typically less good than with traditional CE.[18]

Flow counterbalanced CE[19] is a modification of the CE method that has been used to achieve very high resolutions or to separate analytes with extremely small mobility differences. With this method, a controlled flow of buffer is applied to the capillary to counterbalance the electrophoretic motion of the analytes. The time that the analytes spend in the electric field in the capillary is thus greatly increased, resulting in a greater spacing between closely migrating analyte peaks. This method has more recently been applied to chiral separations[20] and can be used to greatly improve the resolution of CE separations. These improvements require longer analysis times, and higher detection limits than conventional CE, as the peaks diffusively broaden into wider and lower concentration bands.

Electric field gradient focusing (EFGF)[21−25] is an alternative technique for electrophoretic separations that also makes use of counterbalancing bulk buffer flow and electrophoretic motion. In this case, however, a series of electrodes and a semipermeable membrane along the length of the channel are used to create a nonuniform electric field in the separation channel. Consequently, the bulk flow rate can be adjusted so that the total velocity (electrophoretic + bulk) of an analyte is equal to zero at a unique point along the channel. Analytes will then move inward from both directions toward their respective zero-velocity points where they are focused. In this way, analytes with different electrophoretic mobilities are separated by being localized at spatially distinct points along the separation channel rather than by moving with different velocities along the channel. In this way, EFGF and related techniques resemble the more familiar technique of isoelectric focusing (IEF).[26]

Temperature gradient focusing (TGF) is another recently developed technique for spatially focusing and separating ionic analytes in microchannels or capillaries.[27] It is similar to EFGF but in some ways simpler in implementation. Instead of electrodes and semipermeable membranes, a combination of a buffer with a temperature-dependent ionic strength and the application of a temperature gradient are used to from the velocity gradient that is needed for focusing. TGF has been demonstrated with a wide variety of analytes including amino acids, DNA, proteins, and even colloidal particles and cells.

A major advantage of focusing separations such as IEF, EFGF, and TGF is that they do not suffer from diffusional band broadening. Analyte peaks get narrower and more concentrated as the separation proceeds rather than wider and more diffuse as with nonequilibrium techniques that separate based on differences in velocity such as CE and HPLC. Consequently, focusing methods can provide a combination of high resolution and low detection limits. Focusing methods are not as widely employed in analytical separations because there has been only one widely available technique, IEF, and that technique is only suitable for analytes with isoelectric points between 3 and 11, such as proteins and peptides. Although there are a few demonstrations of chiral separations of amino acids using IEF,[28−30] that technique requires the chiral analytes to be chemically derivatized so that they have a suitable isoelectric point which is dependent on the degree of interaction with a chiral selector. Most pharmaceutical molecules do not have appropriate isoelectric points and so cannot be focused or separated with IEF. However, with the advent of new focusing techniques including EFGF, TGF, and more recently, micellar affinity gradient focusing,[31] new methods are becoming available that can be used to focus and separate a wide variety of analytes.

Here we present the application of TGF to the chiral separation of amino acids and small pharmaceutical molecules. To achieve chiral separations with TGF, a chiral selector is added to the TGF buffer in much the same way as is done with chiral CE. The selector interacts with the analyte enantiomers, shifting their electrophoretic mobilities and therefore the position at which they focus. This interaction is stronger for one enantiomer than the other so that the two enantiomers are made to focus at different, spatially resolved positions.

As is shown below, chiral TGF shares many of the advantages of chiral CE including high resolving power, rapid method development, and low reagent consumption. In addition, it provides theoretically unlimited concentration enhancement allowing for greatly improved detection limits.

Furthermore, unlike CE, which requires long channels or capillaries for high-resolution separations, TGF can be performed in very short microchannels or capillaries. The major limitation of microchip CE is that it requires long channels for high-resolution separations, and long channels are difficult to fit into the small area of a microfluidic chip without introducing dispersion that degrades the resolution. High-resolution TGF does not

(16) Quirino, J. P.; Terabe, S. *Anal. Chem.* **2000**, *72*, 1023−1030.
(17) Quirino, J. P.; Terabe, S. *Science* **1998**, *282*, 465−468.
(18) Belder, D.; Ludwig, M. *Electrophoresis* **2003**, *24*, 2422−2430.
(19) Culbertson, C. T.; Jorgenson, J. W. *Anal. Chem.* **1994**, *66*, 955−962.
(20) Chankvetadze, B.; Burjanadze, N.; Bergenthal, D.; Blaschke, G. *Electrophoresis* **1999**, *20*, 2680−2685.
(21) Koegler, W. S.; Ivory, C. F. *Biotechnol. Prog.* **1996**, *12*, 822−836.
(22) Koegler, W. S.; Ivory, C. F. *J. Chromatogr., A* **1996**, *726*, 229−236.
(23) Ivory, C. F. *Sep. Sci. Technol.* **2000**, *35*, 1777−1793.
(24) Huang, Z.; Ivory, C. F. *Abstr. Pap. Am. Chem. Soc.* **2000**, *219*, 208-BIOT.
(25) Huang, Z.; Ivory, C. F. *Anal. Chem.* **1999**, *71*, 1628−1632.
(26) Kolin, A. *J. Chem. Phys.* **1954**, *22*, 1628−1629.
(27) Ross, D.; Locascio, L. E. *Anal. Chem.* **2002**, *74*, 2556−2564.
(28) Ogle, D.; Ho, A.; Gibson, T.; Rylatt, D.; Shave, E.; Lim, P.; Vigh, G. *J. Chromatogr., A* **2002**, *979*, 155−161.
(29) Rizzi, A. M.; Kremser, L. *Electrophoresis* **1999**, *20*, 3410−3416.
(30) Righetti, P. G.; Ettori, C.; Chafey, P.; Wahrmann, J. P. *Electrophoresis* **1990**, *11*, 1−4.
(31) Balss, K. M.; Vreeland, W. N.; Howell, P. B.; Henry, A. C.; Ross, D. *J. Am. Chem. Soc.* **2004**, *126*, 1936−1937.

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require long channels; the separations presented in this report used relatively short 30-mm capillaries, with the separation occurring in only 2 mm of the column (most of the length of the capillaries was required for attachment to the sample and waste reservoirs). High-resolution microfluidic chip-based TGF separations could conceivably be performed in microchannels only a few millimeters long—many of which could easily fit into a small, planar chip format. We therefore believe TGF is intrinsically compatible with miniaturization and integration into a lab-on-a-chip platform.

EXPERIMENTAL SECTION

Materials.[32] All reagents were used as received, and all aqueous solutions were prepared using deionized ultrafiltered water (Fisher Scientific, Fair Lawn, NJ). D-Glutamic acid, L-glutamic acid, dansyl-DL-glutamic acid, dansyl-L-glutamic acid, dansyl chloride, (*R*)(+)-baclofen hydrochloride, (*S*)(−)-baclofen hydrochloride, tris(hydroxymethyl)aminomethane (Tris), boric acid, α-cyclodextrin, and γ-cyclodextrin hydrate were purchased from Sigma (St. Louis, MO). Acetone was purchased from J.T. Baker (Pillipsburg, NJ). Dimethyl sulfoxide (DMSO) was obtained from Matheson, Coleman and Bell Co. (Norwood, OH). Potassium cyanide, and 3-(4-carboxybenzoyl)quinoline-2-carboxyaldehyde (CBQCA) were obtained from Molecular Probes (Eugene, OR). Urine was reconstituted from NIST Standard Reference Material 1511 (freeze-dried urine) and filtered before use.

All TGF separations were performed in buffers composed of 1 mol/L Tris and 1 mol/L boric acid (1 mol/L Tris−borate), pH 8.3.

Fluorescent Labeling Reactions. For fluorescence detection, glutamic acid was labeled with dansyl chloride to produce pure solutions of dansyl-D-glutamic acid and dansyl-L-glutamic acid according to the following procedure:[33] Amino acid solutions were prepared (1 mg/mL) in 200 mmol/L carbonate buffer (pH 9.4) and reacted with 1 mmol/L dansyl chloride in acetone in a 1:1 volume ratio for 60 min. The initial yellow solution turns clear upon reaction after which the acetone was removed under nitrogen. These solutions were used for the TGF separations shown in Figure 6b,d as well as other separations (data not shown) of pure enantiomer and spiked solutions to facilitate peak identification. For all concentrations referenced, it was assumed the labeling reaction yield was 100%.

For the separations of dansyl-DL-glutamic acid shown in Figures 3, 4, and 6a,c, dansyl-DL-glutamic acid and dansyl-L-glutamic acid were used as received from Sigma.[32]

Baclofen (Figure 5) was labeled with CBQCA according to the following procedure:[34] (*R*)(+)- and (*S*)(−)-baclofen were each dissolved at a concentration of 10 mmol/L in 100 mmol/L carbonate buffer, pH 9.4. Each enantiomer was (separately) labeled by combining 20 μL of 10 mmol/L KCN (in water), 20 μL of 10 mmol/L CBQCA (in DMSO), and 20 μL of baclofen solution. The mixtures were then protected from light exposure and vortexed at room temperature for 1−2 h. For all concentrations referenced, it was assumed the labeling reaction yield was 100%.

Temperature Gradient Focusing Apparatus. The capillary device used in these experiments was a 3-cm-long, 30-μm-i.d. 360-μm-o.d. fused-silica capillary embedded between polycarbonate sheets. The device was prepared by sandwiching the capillary between two polycarbonate sheets (McMaster Carr, Atlanta, GA), placed in a hydraulic press at 180 °C at 1000 lb for 10 s and cooled to 120 °C before releasing the pressure. Metal shims were used in the press to define the final thickness of the device and to prevent crushing of the capillary. The capillary device was thermally and mechanically anchored to two copper blocks as shown in Figure 1. T_1 was regulated using a thermoelectric module and T_2 (as well as the temperature of the sample reservoir) was set by the temperature of a recirculating water bath. The capillary was connected at one end to a polypropylene sample reservoir (150-μL volume) via a 360-μm hole drilled into the reservoir and on the other end to the waste reservoir via a silicone rubber septum. The waste reservoir was connected via nylon tubing to another reservoir anchored to a vertical translation stage (not shown in Figure 1). The waste reservoir, nylon tube, and reservoir on the translation stage were all filled with buffer, and the pressure applied to the waste reservoir was precisely controlled by varying the height of the translation stage.

Fluorescence Microscopy. The experiments were performed with a fluorescence microscope equipped with a long-working-distance 10× objective and appropriate filter sets for the fluorescent labels. A color CCD camera with frame grabbing software was used to acquire all images. Step 1 of movie M1 (Supporting Information) was acquired digitally. Step 2 of movie M1 and all of movie M2 (Supporting Information) were recorded on VHS videotape and then digitized to avi file format.

Chiral Capillary Electrophoresis. Capillary electrophoretic separations of dansyl-DL-glutamic acid were performed on an HP^{3D} capillary electrophoresis system (Hewlett-Packard, Wilmington, DE[32]) with a photodiode array detector. Separations were performed in unmodified fused-silica capillaries (66 cm × 50 μm i.d., effective length 57.5 cm) from Polymicro Technologies[32] (Phoenix, AZ). The cartridge temperature was maintained at 30 °C, and injections were performed by pressure (2.5 Pa, 5 s). The applied voltage was 20 kV, and detection was performed at 214 nm.

Capillary electrophoretic separations of CBQCA-labeled (*R*)(+)- and (*S*)(−)- baclofen were performed on a Beckman P/ACE 5510[32] system (Fullerton, CA) with laser-induced fluorescence detection. Separations were performed in unmodified fused-silica capillaries (57 cm × 75 μm i.d., effective length 50 cm) from Polymicro Technologies.[32] The cartridge temperature was maintained at 25 °C, and injections were performed in the electrokinetic mode with an applied voltage of 10 kV. The separation voltage was 15 kV. Detection was performed with an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

CE run buffers (pH 9.1) were prepared by dissolving sodium tetraborate decahydrate at a concentration of 15 mmol/L and γ-cyclodextrin at a concentration of 10 mmol/L in water. Samples for CE were dissolved in water at a concentration of ∼0.2 mg/mL.

At the beginning of each day, the capillary was conditioned with 0.1 M NaOH (5 min), water (2 min), and run buffer (20 min).

(32) Certain commercial equipment or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

(33) Airhart, J.; Sibiga, S.; Sanders, H.; Khairallah, E. A. *Anal. Biochem.* **1973**, *53*, 132−140.

(34) Molecular Probes. Product Information Sheet MP 02333, ATTO-TAG CBQCA, 2001.

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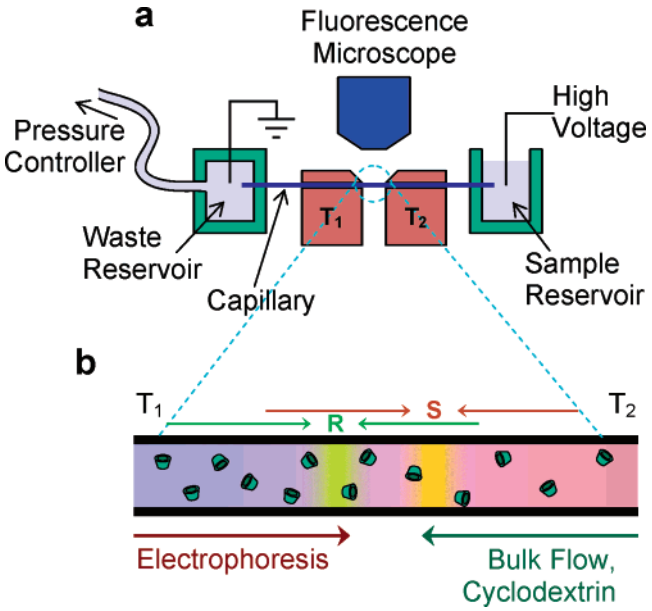

Figure 1. (a) Schematic illustration of the TGF apparatus. The capillary is mounted to span two controlled temperature blocks. A linear temperature gradient is formed along the capillary in the 2-mm space between the copper blocks regulated at temperatures T_1 and T_2. Sample is loaded into the sample reservoir, and an applied high voltage is used to drive the electrophoretic motion of the ionic analytes. The counterbalancing bulk flow is primarily due to electroosmosis in the capillary but is adjusted by the application of a controlled pressure to the waste reservoir. Separations are observed via fluorescence microscopy. (b) Schematic of chiral TGF separations. The electrophoretic motion is counterbalanced by the bulk flow. The application of a temperature gradient gives rise to a corresponding gradient in the electrophoretic velocity: on the low-temperature side of the gradient, the electrophoretic velocity is greater than the bulk velocity. On the high-temperature side, the bulk velocity is greater. Analytes move to and are focused at the point along the gradient where the electrophoretic and bulk velocities sum to zero. Chiral separations are accomplished by adding a chiral selector (represented by truncated cones in the figure) to the buffer. The chiral selector interacts preferentially with one enantiomer, shifting its focusing location so that it is resolved from the other enantiomer.

Between runs, the capillary was flushed with 0.1 M NaOH (1 min) and run buffer (3 min).

RESULTS AND DISCUSSION

Sequential Concentration and Separation of Amino Acids. To demonstrate the chiral separation of small chiral molecules, a fluorescent-labeled amino acid was first concentrated via standard TGF, and then a chiral selector was introduced to the separation channel to produce a chiral separation as follows: Low-concentration sample was continuously injected into the capillary from the sample reservoir (see Figure 1a) for 30 min, during which time the dansyl-DL-glutamic acid was focused to a peak concentration 600-fold greater than the sample input concentration (Figure 2). The solution in the sample reservoir was then replaced with buffer containing the chiral selector, γ−cyclodextrin, but no dansyl-DL-glutamic acid. The neutral cyclodextrin (CD) was then carried toward the temperature gradient zone by the bulk buffer flow where its effect on the focused sample was observed with fluorescence video microscopy (Figure 3 and movie M1 in Supporting Information). The time at which the cyclodextrin first

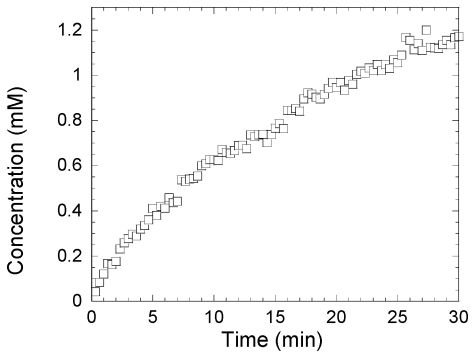

Figure 2. Peak concentration vs time for TGF of dansyl-DL-glutamic acid without chiral selector. The focusing conditions were T_1 = 13 °C, T_2 = 40 °C, +1000 V/cm (high voltage applied on the right end of the channel, ground on the left; see Figure 1a). Input sample: 2 μmol/L dansyl-DL-Glu in 1 mol/L Tris−borate, pH 8.3.

entered the gradient zone and interacted with the focused sample was clearly visible as a marked increase in the intensity of the peak and a blue shift of the fluorescence emission as well as a sharp movement of the peak to the left.[35] In less than 30 s, the focused peak split into two baseline-resolved peaks corresponding to the D and L enantiomers. Furthermore, with the addition of the cyclodextrin, the enantiomers were focused into narrower and more concentrated peaks so that the final peak concentrations were ~1200-fold greater than the initial sample input concentrations.

Resolution can be further increased by reducing the steepness of the temperature gradient as shown in Figure 4. In addition, because of the temperature dependence of the interaction between the dansylglutamic acid and the cyclodextrin, the resolution can also be improved by reducing the average temperature of the separation channel, so that in an optimized TGF separation, the resolution is comparable to that obtained with CE. For these measurements, the primary limit to the resolution was due to the requirement that both peaks be visible at the same time in the 2-mm-wide field of view of the microscope. An advantage of chiral TGF is that this optimization can be done quickly, by simply adjusting the temperature gradient or other parameters, while maintaining the same focused sample peaks and monitoring the results.

Chiral Selector Selection and Optimization in a Single Analysis. This approach can be employed for the rapid screening of various chiral selectors and selector concentrations to optimize the separation. Only a small fraction of chiral molecules are resolvable with any given chiral selector or stationary phase,[36] and with conventional chiral separation techniques, the screening of chiral selectors can be an expensive and time-consuming process. Although strategies for the selection of a chiral selector are reported, empirical data must still be collected for each new analyte to identify the selector and other separation conditions that will provide adequate resolution.[36,37] With chiral TGF, this screening can be done in a relatively short time using a single injection of sample. As in the amino acid experiment described

(35) A comparison of fluorescence emission spectra in spectrofluorometry measurements indicated a 10-nm blue shift upon addition of γ-CD to dansyl-DL-glutamic acid solutions.
(36) Blanco, M.; Valverde, I. *TrAC, Trends Anal. Chem.* **2003**, *22*, 428−439.
(37) Guttman, A.; Brunet, S.; Cooke, N. *LC GC Mag. Sep. Sci.* **1996**, *14*, 32−42.

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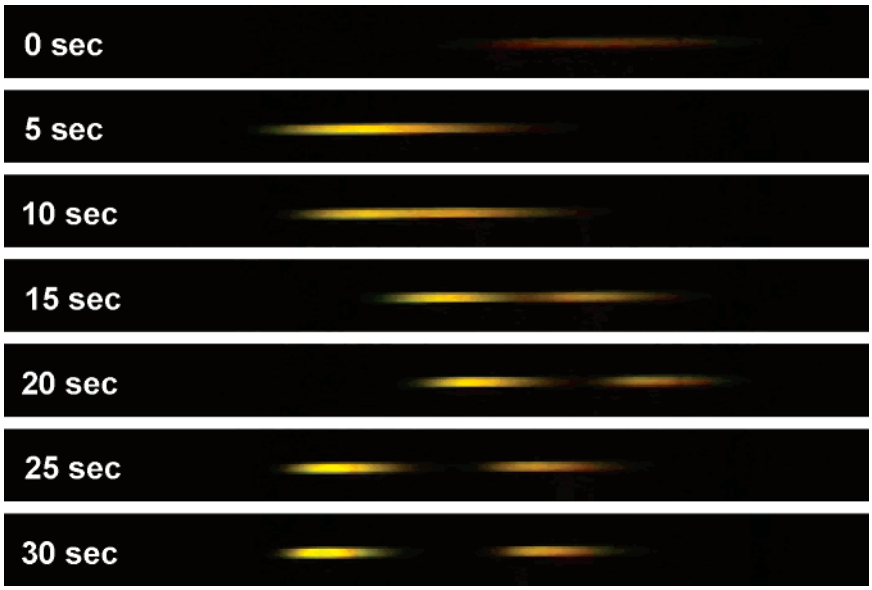

Figure 3. Real-time chiral TGF of dansyl-DL-glutamic acid. The input sample (1 μmol/L dansyl-D-glu, 1 μmol/L dansyl-L-glu in 1 mol/L Tris−borate) was initially focused without chiral selector for 30 min to a peak concentration of 1.2 mmol/L (See Figure 2.). The focusing conditions were $T_1 = 13$ °C (left side in image), $T_2 = 40$ °C, +1000 V/cm. The sample solution was then replaced with a solution of 10 mmol/L γ-cyclodextrin (γ-CD) in 1 mol/L Tris−borate, pH 8.3. The sequence of images was taken in 5-s intervals starting just as the γ-CD entered the gradient zone. The fluorescence quantum yield increased sharply upon complexation with the cyclodextrin, and the emission color changed from orange to yellow. The D enantiomer peak is to the left in the figure; the L enantiomer is to the right. A real-time video sequence corresponding to this figure is available as Supporting Information (movie M1). A similar movie (movie M2) is available showing the separation of the (*R*)(+)- and (*S*)(−)-enantiomers of the drug baclofen. For scale, each image is 2 mm long.

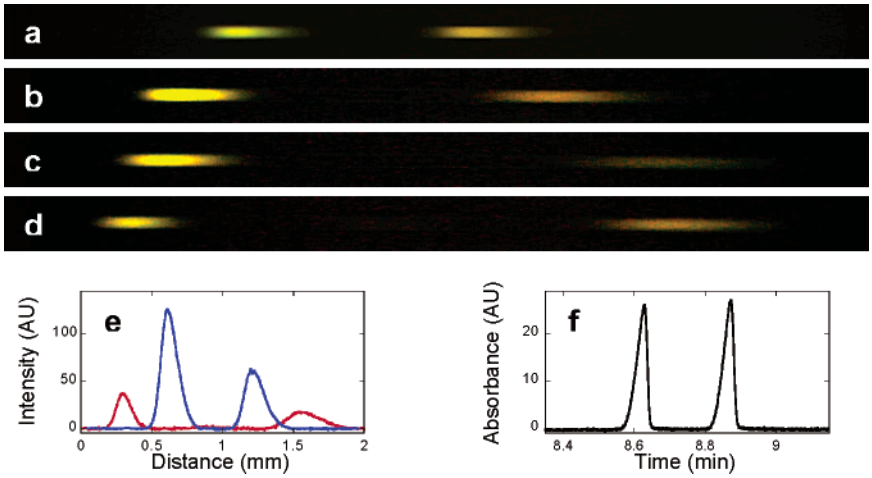

Figure 4. Optimizationof chiral TGF separation of dansyl-DL-glutamic acid. (a) $T_1 = 13$ °C, $T_2 = 40$ °C. (b) $T_1 = 11$ °C, $T_2 = 30$ °C. (c) $T_1 = 15$ °C, $T_2 = 30$ °C. (d) $T_1 = 2.5$ °C, $T_2 = 15$ °C. (e) Intensity vs distance plots for chiral TGF separations of dansyl-DL-glutamic acid; the blue curve, resolution 2.4, is from (a); the red curve, resolution 3.8) is from the optimized separation (d). (f) Chiral CE electropherogram of the same analyte, resolution 4.1. CE conditions: 10 mmol/L γ-CD in 15 mmol/L sodium tetraborate, 20 kV, capillary length 66 cm.

above, the chiral analyte of interest is first focused without a chiral selector. Various chiral selector solutions are then, in turn, placed in the sample reservoir. Each chiral selector is carried by bulk flow to the gradient zone where it can interact with the focused analyte.[38] At each step, the original, focused sample is retained and reused, reducing reagent consumption and analysis time. In one example, chiral TGF of the drug baclofen, a γ-aminobutyric B receptor agonist,[39,40] was tested with four different CDs each at eight different concentrations (32 total separation conditions) in $\sim 2\,^1/_2$ h—with completely manual sample handling. The resulting optimized separation is shown in Figure 5 (see also movie M2 in the Supporting Information) along with a chiral CE separation of the same analyte for comparison. As was the case for dansyl-glutamic acid, the resolution obtained with TGF is comparable to that obtained with CE.

Analysis of Raw Biological Fluids. An additional advantage shared by chiral TGF and chiral CE is that biological fluid samples such as urine can be used directly, with no sample extraction or other pretreatment. For the analysis of urine spiked with the drug baclofen, the urine was simply mixed with an equal volume of chiral TGF buffer, and the mixture was placed in the sample

(38) This method could also be adapted for use with charged selectors.
(39) Ali, I.; Aboul-Enein, H. Y. *Electrophoresis* **2003**, *24*, 2064−2069.
(40) Chiang, M. T.; Chang, S. Y.; Whang, C. W. *Electrophoresis* **2001**, *22*, 123−127.

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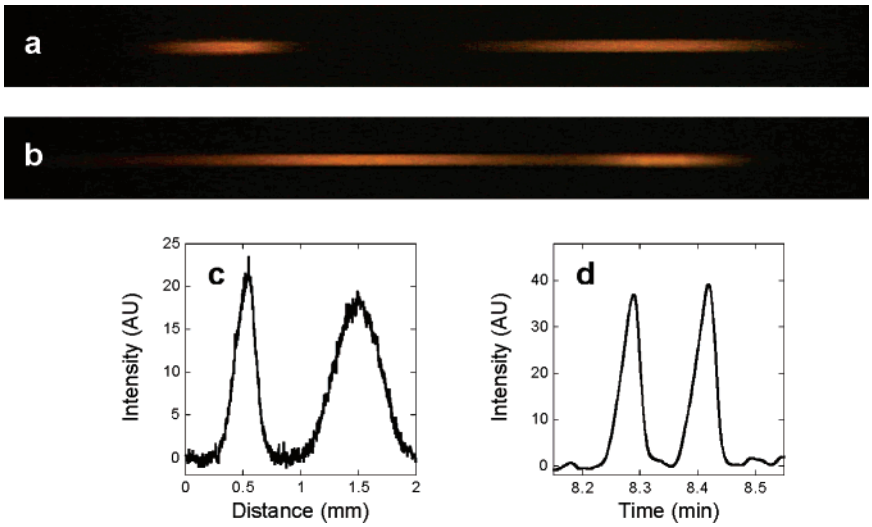

Figure 5. Chiral TGF of CBQCA-labeled (*R*)(+)- and (*S*)(−)-baclofen. (a) Focusing of baclofen from in buffer. Sample: 24 μmol/L (*S*)(−)-baclofen, 54 μmol/L (*R*)(−)-baclofen in 1 mol/L Tris−borate, pH 8.3 with 10 mmol/L α-CD. The (*S*)-enantiomer peak is to the left in the figure; the (*R*)-enantiomer is to the right. Focusing conditions: $T_1 = 12$ °C, $T_2 = 40$ °C, +1000 V/cm (see Figure 1a). (b) Focusing of baclofen from human urine. Sample: 3 μmol/L (*S*)(−)-baclofen, 3 μmol/L (*R*)(−)-baclofen in 50% v/v 1 mol/L Tris−borate, urine with 20 mmol/L α-CD; focusing conditions: $T_1 = 30$ °C, $T_2 = 10$ °C, −1000 V/cm. The direction of the temperature gradient and the bulk flow were reversed, so that the urine in the sample reservoir would not flow into the capillary (also note the reversal of the peak order). The waste reservoir was filled with 1 mol/L Tris−borate with 20 mmol/L α-CD. (c) Fluorescence intensity vs distance plot for optimized chiral TGF separation (a), resolution 1.7. (d) Chiral CE separation of CBQCA-labeled (*R*)(+)- and (*S*)(−)-baclofen, resolution 2.0. Separation conditions: 10 mmol/L γ-CD in 15 mmol/L sodium tetraborate, pH 9.1, 15 kV, capillary length 57 cm.

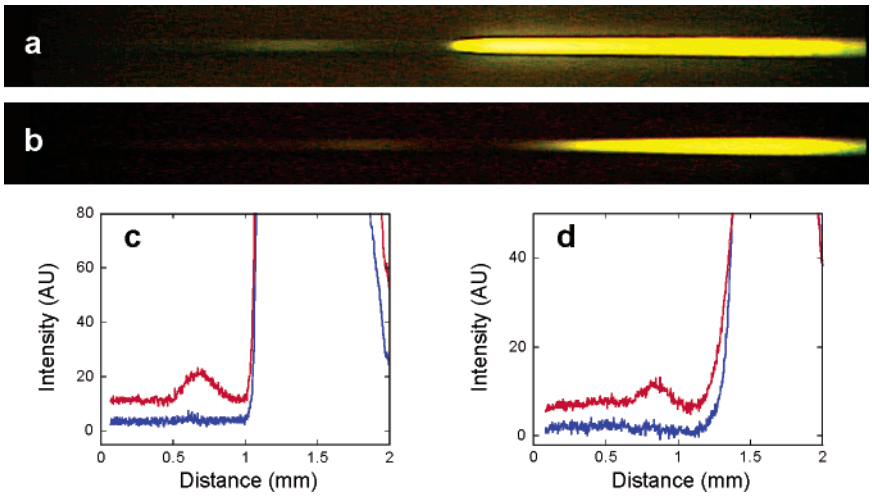

Figure 6. Trace analysis of enantiomeric purity by chiral TGF. (a) Dansyl-L-glutamic acid with 0.05 mol % dansyl-D-glu. Focusing conditions: $T_1 = 10$ °C, $T_2 = 30$ °C, +1000 V/cm (see Figure 1A), 1 mol/L Tris-borate, pH 8.3 with 20 mmol/L γ-CD. (b) Dansyl-D-glu with 0.1 mol % dansyl-L-glu. Focusing conditions: $T_1 = 40$ °C, $T_2 = 10$ °C, −1000 V/cm, 1 mol/L Tris−borate, pH 8.3 with 20 mmol/L γ-CD. (c) Intensity vs distance plots for (a, red curve) and similar results with no impurity (blue curve). (d) Intensity vs distance plots for (b, red curve) and similar results with no impurity (blue curve). For clarity, the red curves in (c,d) have been offset.

reservoir (see Figure 1a) for focusing. The resulting TGF separation is shown in Figure 5b. Although there was some interference with the chiral selector due to the urine (the peaks were closer together than in a similar separation without urine), the two enantiomer peaks were still well resolved. In addition, the higher resolution of the two enantiomer peaks could be recovered by replacing the urine in the sample well with blank buffer once the drug from the urine had been focused to the desired concentration (data not shown).

Analysis of Low-Level Enatiomeric Impurities. An important variable in the pharmaceutical industry is the enantiomeric purity of drugs. To comply with regulatory guidelines, techniques should be able to detect trace amounts (0.1−1%) of an impurity enantiomer in the presence of a large excess of the desired enantiomer. A demonstration of the capabilities of chiral TGF for trace impurity analysis is shown in Figure 6. Comparison of the results of chiral TGF separations of a pure sample of dansyl-L-glutamic acid (Figure 6c blue curve) and a similar sample with 0.05% dansyl-D-glutamic acid impurity (Figure 6a, Figure 6c red curve) indicates that enantiomeric impurities well below the 0.1% level can be detected.

Further, for the determination of enantiomeric purity, it is also important to be able to control the peak order so that the small impurity peak eluting first is not obscured by tailing of the much larger major enantiomer peak. Consequently, it is often necessary to reverse the elution order of the two enantiomers. With HPLC,

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this is can be challenging because the chiral stationary phases are often biologically derived, and it is not possible to simply use a stationary phase of the opposite chirality. In chiral CE, this challenge in some cases can be overcome by changing the chiral selector (if there is more than one that will provide resolution), changing the concentration of the chiral selector, or both.[37,41] Chiral TGF, however, allows the peak order to be reversed simply by changing the direction of the temperature gradient, the sign of the applied voltage, and the sign of the applied pressure. As an example, in addition to impurity analysis of the L enantiomer discussed in the previous paragraph, chiral TGF could also be used to assay the purity of the D enantiomer using the same chiral selector and virtually identical separation conditions simply by reversing the above-mentioned experimental parameters (Figure 6b,d). The results show that an impurity of either enatiomer in a large excess of the other can be readily detected. Note that, with both "forward" (a,c) and "reversed" (b,d) temperature gradients, tailing of the major enantiomer peak is in the direction of the sample input (to the right in the figures).

CONCLUSIONS

These results demonstrate that chiral TGF provides an alternative to currently available chiral separation techniques. The combination of resolutions equivalent to chiral CE with concentration enhancement and minimal sample preparation should be amenable to the chiral analysis of drugs in biological fluids (i.e., serum, urine) to monitor how the drug is metabolized. In addition, the capability for real-time monitoring of analytes/selector interactions could be applied to other drug/biomolecule interactions or rapid separation optimization for rare or extremely valuable samples where sample availability may limit separation optimization.

To date, TGF has only been performed with high-pH buffers such as the Tris borate (pH 8.3) shown in this work. The chiral electrophoretic separation of many drug compounds, particularly basic pharmaceuticals, is best performed at low pH. Therefore, future efforts will be aimed at the identification of low-pH TGF buffers. In addition, the peak capacity of chiral TGF as presented in this work is limited because only a few peaks can be simultaneously focused within the current apparatus (2-mm gap, 3-cm capillary, etc.). One strategy to increase the peak capacity is to operate the method in "scanning mode" in which the bulk velocity would be varied as a function of time, and the focused peaks would be detected as they move past a detection point near the end of the temperature gradient.[42] Further studies to explore these issues are currently ongoing in our laboratory as well as work to investigate other classes of chiral analytes such as neutral molecules[31] and analytes with multiple chiral centers.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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(41) Chankvetadze, B.; Schulte, G.; Blaschke, G. *Enantiomer* **1997**, *2*, 157−179.

(42) Balss, K. M.; Ross, D.; Begley, H.; Olsen, K. G.; Tarlov, M. J., *J. Am. Chem. Soc.* **2004**, *126*, 13474−13479.

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