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Implementing a Generic Methods Development Strategy for Enantiomer Analysis



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Introduction

Whether one's approach to drug discovery involves natural products screening or the synthesis of innovative new compounds, the challenges presented to the analytical chemist are complicated when a product contains one or more chiral centres. Yet, these optically active compounds tend to make good drug candidates and, as such, require stereochemically selective assay methods in place for their analysis (1).

An important step towards separating enantiomers is to first create diastereomers. Diastereomers may be created through chemical derivatization with a chiral reagent or may be formed transiently through interactions with chiral selectors. The latter approach is the most desirable because it is the simplest to employ.

Using capillary electrophoresis (CE), a chiral environment may be easily created, by filling the capillary with an electrolyte solution containing a chiral additive. Although many types of chiral additives have been used in CE (2–4) the most comprehensive and effective separation strategies have been achieved with sulfated cyclodextrins (5–8). However, to implement an effective separation strategy, a clear definition and an understanding of

the chiral selector used are essential. Although used generically in many publications the term sulfated cyclodextrins actually represents several different types of selectors supplied from different manufacturers. Our experience has been that these selectors all behave differently so it is essential to know which selector you are working with when specifying it in a method.

In this article we describe an enantiomer methods development strategy using a family of highly sulfated cyclodextrins (HSCDs) to resolve a diverse array of compounds. Using this approach we successfully resolved 156/160 racemic mixtures tested (9) and propose this as a generic methods strategy to be considered as a first step in developing methods for separating enantiomers.

Highly Sulfated Cyclodextrins

In our experience, the most successful separations using HSCDs have been achieved at low pH. Under these conditions of suppressed electroosmotic flow (EOF), the negatively charged cyclodextrins have strong electrophoretic mobility towards the positive electrode (anode). If the enantiomers interact with these HSCDs

they will be swept towards the anode regardless of charge state. Neutral compounds interact hydrophobically with the cavity of the HSCDs, while basic compounds, being strong cations at low pH, interact with both the hydrophobic cavity and with the negatively charged sulfates. Many acidic compounds will be protonated at low pH and behave in a similar manner as neutral species, while zwitterionic analytes will be positively charged eliciting behaviour similar to basic compounds. Figure 1 illustrates the analysis of these four classes of enantiomers using a single analytical condition.

The methods development strategy we propose here uses a family of HSCDs developed in-house. These reagents include the BCI α , β and γ HSCDs and comprise a controlled distribution of product containing an average of 11, 12 and 13 sulfates/molecules, respectively. Detailed characterizations of these reagents have been described elsewhere (4–7).

Although there are trends developing that should allow future prediction of the best starting selector, we recommend implementing a strategy that uses the α , β and γ selectors independently to determine which one will provide the best resolution

of an analyte. At low pH the EOF in an untreated fused-silica capillary is minimal. In this instance the electrophoretic mobility of an analyte (μ) in the presence of HSCD can be represented by a weighted sum of the mobility of free analyte μ_F and that in the complexed state, μ_C , as shown in equation 1.

$$\mu = \mu_F + K_1(\text{HSCD})\mu_C / [1 + K_1(\text{HSCD})] \quad [1]$$

In this equation, the (HSCD) term is the concentration of free HSCD after interaction with the analyte. If this number is small, the mobility of the complex would be affected significantly by a change in analyte concentration. For this reason we recommend a starting concentration of the

HSCD at 5%, (25 mM), as this should be one to two orders of magnitude greater (typically 0.1 to 1 mM) than the concentration of the chiral species being separated. The excess amount of chiral selector over the chiral species makes the [free HSCD] virtually independent of variations in analyte concentration. As a greater population of analyte will be in the complexed state, a net higher mobility of analyte will result.

The mobility of two enantiomers (racemates), 1 and 2, can be compared according to equation 2.

$$\mu_1 - \mu_2 = \{\mu_{F1} + K_1(\text{HSCD})\mu_{C1} / [1 + K_1(\text{HSCD})]\} - \{\mu_{F2} + K_2(\text{HSCD})\mu_{C2} / [1 + K_2(\text{HSCD})]\} \quad [2]$$

Because HSCDs are highly negatively charged with substantially greater molecular weight than that of the analytes being separated, the mobility of the complex can be approximated as $\mu_{C1} = \mu_{C2}$ and nearly equal to μ_{HSCD} . Neutral enantiomers should satisfy the condition of $\mu_{F1} = \mu_{F2}$. Thus, equation 2 can be modified as equation 3:

$$\mu_1 - \mu_2 = (\mu_{C1} - \mu_{F1}) (K_1 - K_2) (\text{HSCD}) / [1 + K_1(\text{HSCD})] [1 + K_2(\text{HSCD})] \quad [3]$$

For a neutral species, $\mu_{F1} = \mu_{F2} = 0$, and for basic compounds, $\mu_{C1} \gg \mu_{F1}$

Thus, as in equation 4

$$\mu_1 - \mu_2 = \mu_{\text{HSCD}} (K_1 - K_2) (\text{HSCD}) / [1 + K_1(\text{HSCD})] [1 + K_2(\text{HSCD})] \quad [4]$$

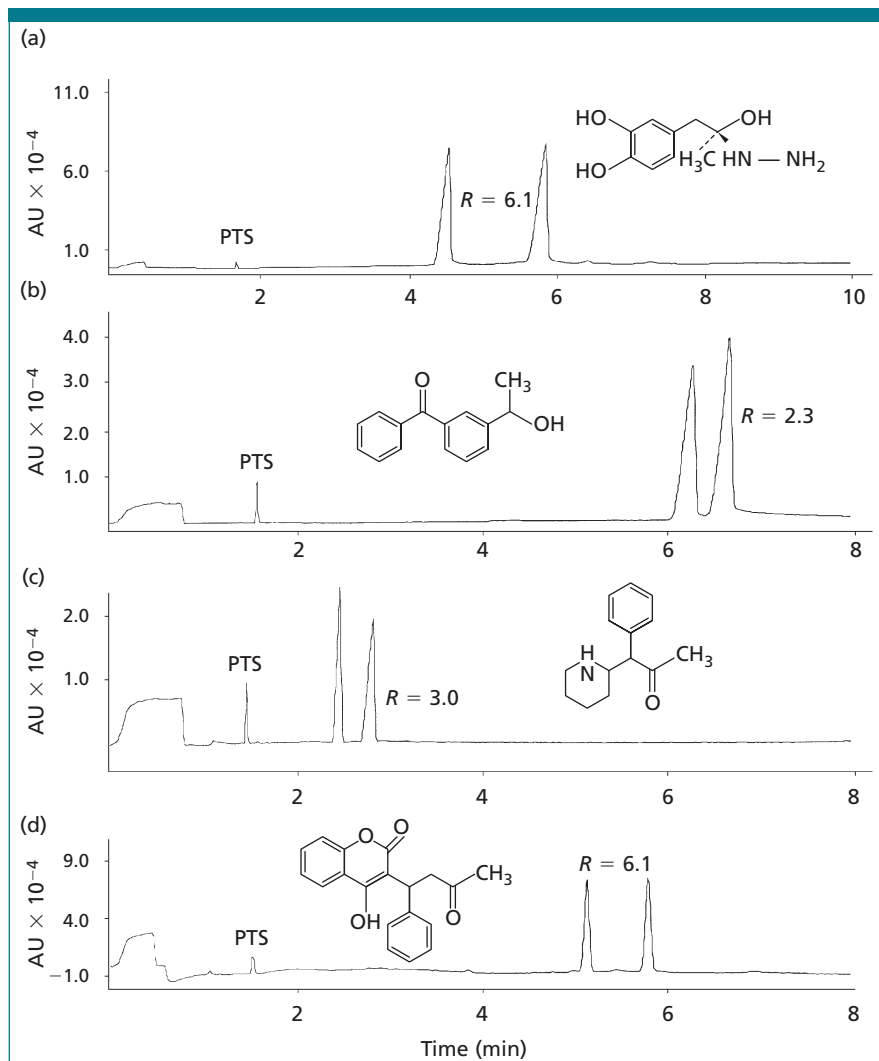


Figure 1: Electropherograms of zwitterionic, acidic, basic and neutral chiral compounds using 5% β -HSCD in 25 mM phosphate buffer pH 2.5 — (a) carbidopa (zwitterionic drug), (b) ketoprofen (acidic drug), (c) ritalin (basic drug) and (d) warfarin (neutral drug). Conditions: untreated fused-silica capillary 50 $\mu\text{m} \times 30 \text{ cm}$ (20 cm to detector); capillary temperature: 22 $^{\circ}\text{C}$ (recirculating liquid); field strength: 500 V/cm; detection: PDA 200 nm; sample introduction: pressure 1.2 psi*s; current generated: 156 μA .

In principle, the mobility difference $\Delta\mu$ is intrinsically related to K_1 and K_2 and when [HSCD] is substantially large, a small variation of racemate concentration would not affect the total free (HSCD), thus the $\Delta\mu$ (resolution term) will remain essentially constant. This is what we have seen in practice, when maintaining a high concentration of chiral selector. In such instances the resolution term and migration time remain relatively constant even with changing analyte concentration.

Although racemic mixtures may be baseline resolved at a calculated resolution greater than 1.0, detection of low-level enantiomers can be easily masked when the concentration of the eutomer (major enantiomer) is increased to the level necessary to detect the lower concentration enantiomer. However, as resolution values of 5 or greater are commonly obtained using the HSCD approach, low-level enantiomer quantification below 0.1% levels can be readily achieved. Figure 2 illustrates the detection of (–) phenylpropranolamine enantiomer at 0.1%. The good resolution afforded by this assay makes this formerly difficult task routine.

Analyte Detection

During the methods development phase, photodiode array (PDA) detection can prove to be a valuable tool in removing the ambiguity of enantiomer assignment. As enantiomers are physicochemically identical, they will have matching spectra, allowing discrimination of enantiomer pairs from contaminating analytes.

Although most drug candidates are ultraviolet (UV)-absorbing, there may be instances in which the chiral compound of

interest does not have significant UV absorbance. In these examples, one can employ a pre-separation derivatization to render either a UV-absorbing or a

fluorescing product. An example is shown in Figure 3 with the analysis of homoproline. Shown is the separation of dansylated homoproline enantiomers, both

as a racemic mixture and the single enantiomer preparation.

With the determination of low-level enantiomers, the linearity of detection is also an important consideration. Figure 4 illustrates the detection response linearity for the phenylpropanolamine enantiomers described above. This plot represents the percent impurity (lower level enantiomer) as a function of the ratio of velocity corrected peak area of the impurity/total. A good linear response is generated allowing an accurate determination of enantiomer impurity even at levels as low as 0.1%.

Capillary Selection and Conditioning

Within this methods development strategy, we recommend using a 50 μm bare fused-silica capillary with a relatively short effective length (10 or 20 cm to detector), allowing you to operate under high field strengths, while still maximizing mass load. Both the speed and resolution of this approach are highlighted in Figure 5 where dansylated phenylalanine enantiomers are well resolved in less than 1 min. It is notable that a resolution value of 3 could be generated in such a rapid manner. However, this really has become a trademark for this type of analysis.

As the capillaries are operated at low pH it is best not to use NaOH rinses between runs. In fact, we typically only rinse with run buffer prior to each analysis. To reduce the daily equilibration cycle we recommend rinsing at least once a day with a polyethylene oxide solution (25 mM lithium acetate, 0.4% PEO (MW 300 000), 10% ethylene glycol, adjusted to pH 4.75). This polymeric solution acts to coat the capillary surface and in our experience has reduced the need for the traditional daily start-up equilibration runs. In this manner, reproducible migration times are established quickly. However, should a capillary become excessively fouled, treatment with a strong acid, followed by the polymer solution, followed by a run buffer is recommended. Dedicating capillaries for this assay will also be of benefit; although a capillary may be quickly exchanged with different buffers, the surfaces do take time to become uniform, especially when different buffer ions are used.

Electrophoresis Parameters

For the purposes of comparative analysis we recommend that the electrophoresis conditions used for the initial screening runs are standardized. Once the best cyclodextrin type and concentration are determined the process of optimization

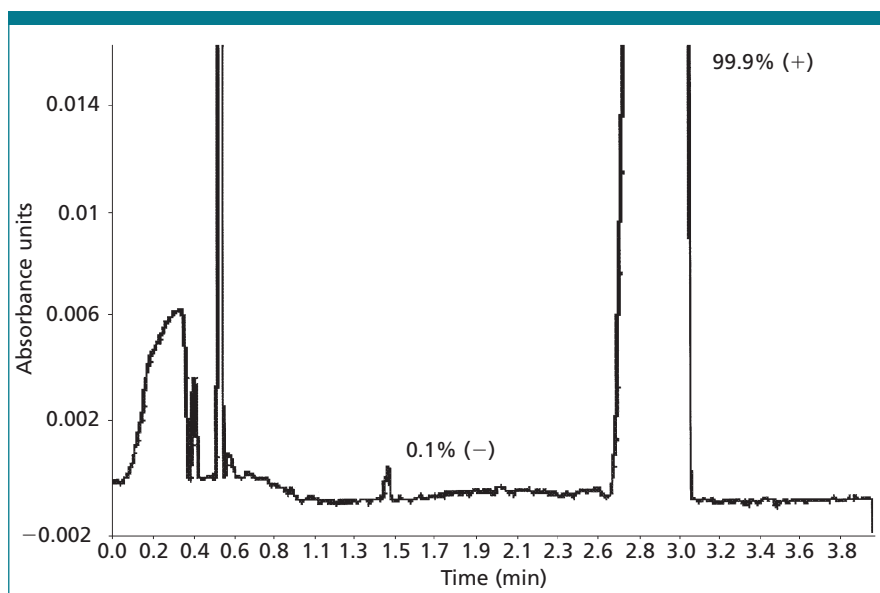


Figure 2: Electropherogram of phenylpropanolamine enantiomers, with the (-) form readily detected at 0.1%. Conditions: buffer: 5% γ -HSCD in 25 mM phosphate, pH 2.5; capillary: untreated fused-silica capillary 50 μm \times 30 cm (20 cm to detector); capillary temperature: 22 $^{\circ}\text{C}$ (recirculating liquid); field strength: (-)500 V/cm (anode at outlet); detection: PDA 200 nm; sample introduction: pressure 1.2 psi* s ; current generated: 152 μA .

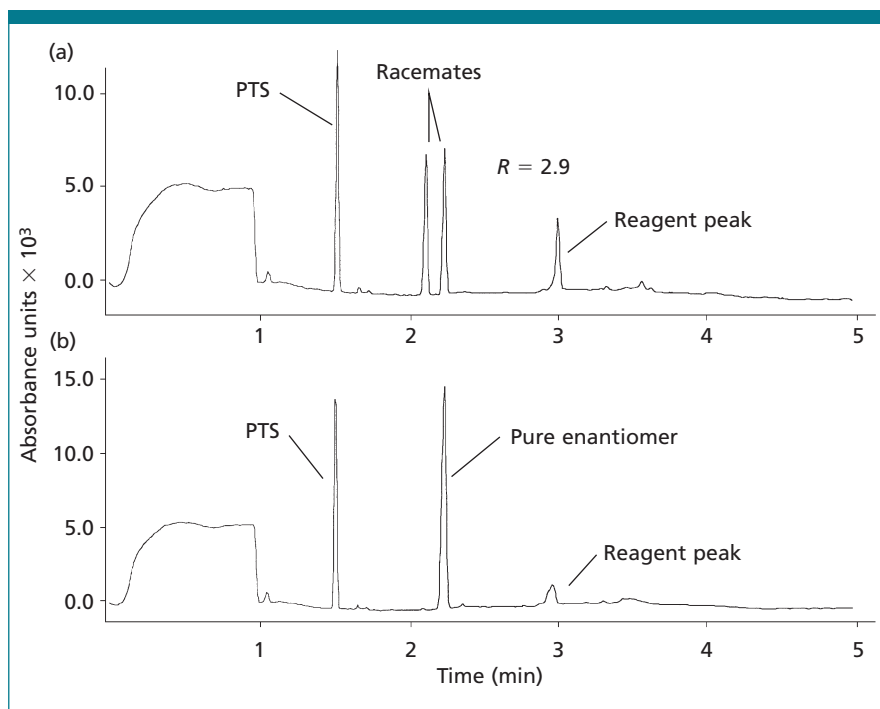


Figure 3: Electropherogram of dansylated derivatives of homoproline. (a) Racemic mixture of homoproline, (b) L-homoproline enantiomer. Conditions: buffer: 5% γ -HSCD in 25 mM phosphate, pH 2.5; capillary: untreated fused-silica capillary 50 μm \times 30 cm (20 cm to detector); capillary temperature: 22 $^{\circ}\text{C}$ (recirculating liquid); field strength: (-)500 V/cm (anode at outlet); detection: PDA 200 nm; sample introduction: pressure 1.2 psi* s ; current generated: 155 μA .

can be started. The starting conditions that we have chosen are as follows:

- Capillary temperature = 22 °C**
- Field strength = 500 V/cm (generating currents of approximately 150 μ A)
- Detection: PDA = UV 200 nm (Scanning 190–320 nm)

- Sample introduction = 1.2 psi*s (4 s at 0.3 psi).
**The capillary temperature represents that of the environment used to thermoregulate the capillary. When transferring methodology between instruments using different

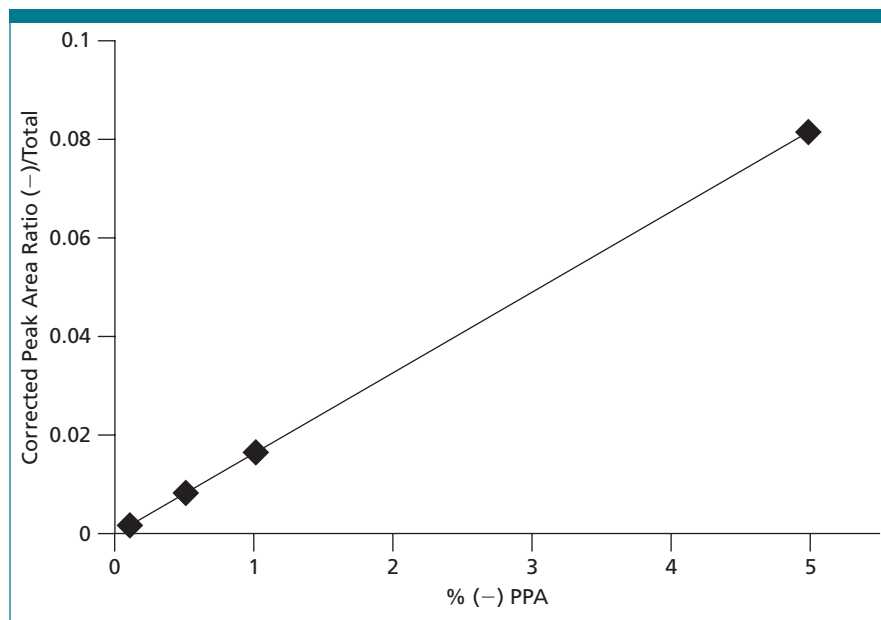


Figure 4: Plot of the ratio of the impurity velocity corrected area/total velocity corrected peak area versus the % contaminating enantiomer (impurity) for phenylpropanolamine. A linear response from 0.1% to 5% is readily attained, allowing effective quantification of the lower level enantiomer to levels as low as 0.1%.

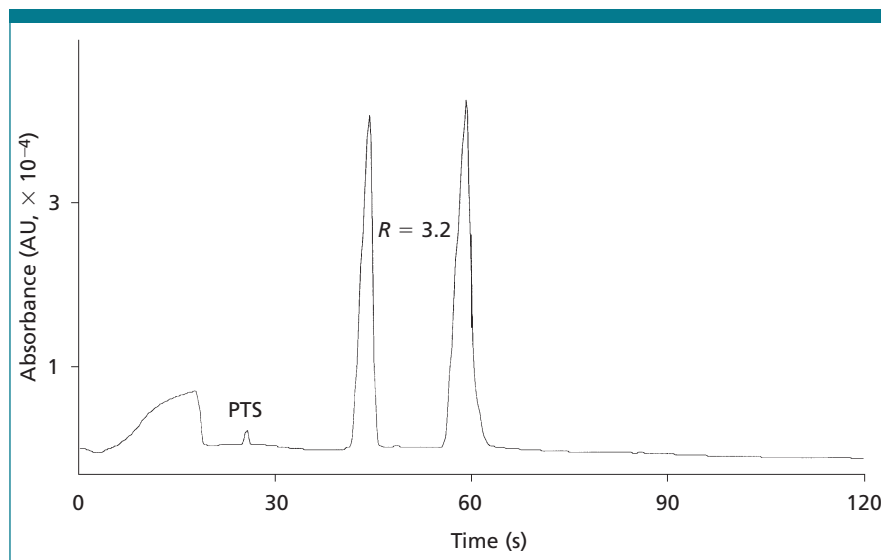


Figure 5: Electropherogram of dansylated-phenylalanine enantiomers separated using a short capillary (10 cm – sample introduction from the outlet side). Conditions: buffer: 5% γ -HSCD in 25 mM phosphate, pH 2.5; capillary: untreated fused-silica capillary 50 μ m \times 30 cm (10 cm to detector); capillary temperature: 22 °C (recirculating liquid); field strength: (+)500 V/cm (cathode at outlet); detection: PDA 200 nm; sample introduction: pressure 1.2 psi*s — sample introduced from outlet side; current generated: 157 μ A.

thermoregulatory mechanisms it is useful to use the run current as a diagnostic to assess differences in actual capillary temperature. In this way you can fine-tune your capillary temperature by lowering or elevating your system temperature until a similar current is achieved.

As these reagents are viscous, simple steps, such as water dips after both sample introduction and the end of the run, can be used to prevent sample carryover/contamination.

Another important variable to pay attention to is the total run time programmed. As the resolution of enantiomer pairs can be quite high, a failed separation attempt can easily be mistaken by simply not allowing enough separation time to resolve the two enantiomers. In Figure 6 we illustrate the analysis of amphetamine enantiomers for which such an oversight could easily be made. Notice the elapsed run time from the first enantiomer to the second. If the run were aborted too soon, the assumption could have been made that no separation was achieved. A good experiment to consider is to run your sample with and without the chiral selector. If complete resolution is achieved, the peak heights will be approximately half that of the control where no selectors were present. Alternatively, by keeping the capillary as short (10 cm) as possible the total run time required is significantly reduced.

Methods Optimization

Once the primary three runs, evaluating the α , β and γ HSCDs, have been performed, an optimization process may be implemented.

First, choose the HSCD that gave the best resolution. You may wish to vary the concentration between 2% and 10% to evaluate whether any enhancement of resolution is achieved. Capillary temperature, length and field strength may all be used to fine-tune the resultant method. However, it is preferable that the field strength be optimized last, once you have decided upon the optimum capillary length and temperature.

If resolution of enantiomers is not achieved with any of the three cyclodextrins, attention should be focused first on the peak shape. Did any of the runs illustrate a broadening of peak shape?

If the answer is yes, there is a good chance that the HSCD concentration is too high, forcing a stronger interaction than desired. In this instance, lowering the concentration is advised. An HSCD dilution

series of 1 to 5% can rapidly provide you with a standard curve to estimate the best concentration of selector to choose.

If the answer to the above question was no (i.e., that there was no change in peak shape), the HSCD concentration should be increased in a series from 5 to 10%.

Automation of this optimization process can be readily achieved with the introduction of a buffer array.

Results

Using only the primary screen of α , β and γ HSCD, and the standard method described above, we have been able to resolve ($R > 1.0$) 156/160 drugs tested, with no optimization process employed.

The compounds tested included

- 56/58 neutral drugs
- 92/92 basic drugs
- 8/10 acidic drugs.

A library of these drugs, their structures and the separations achieved have been published and can be reviewed on the Internet at www.beckmancoulter.com/chiral38

In summary, we have presented a powerful chiral analysis strategy using a combination of capillary electrophoresis and a family of highly sulfated cyclodextrins. The result is, in our opinion, a sound enantiomer analysis strategy that uses a single generic method approach to resolve a broad array of compounds. An approach we suggest you try first, when

developing methodology for enantiomer separations.

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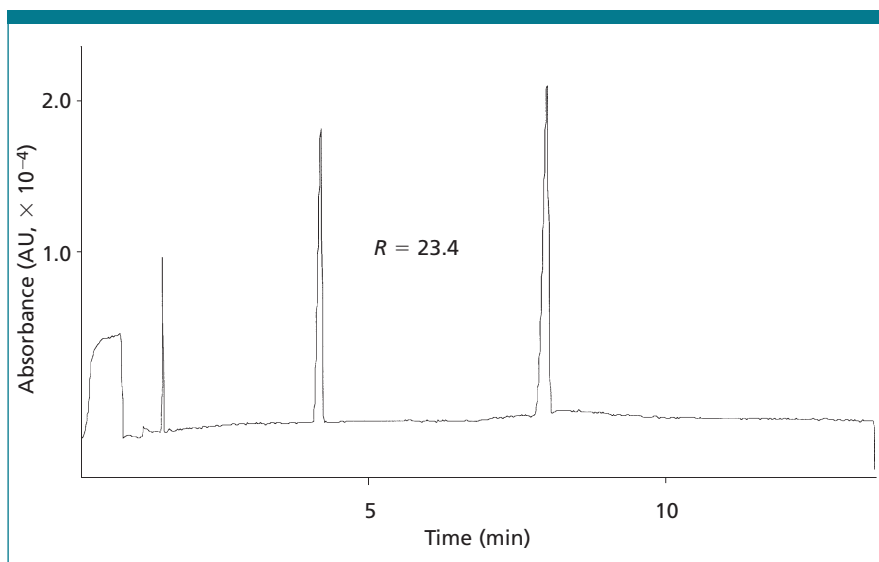


Figure 6: Electropherogram of amphetamine enantiomers demonstrating the high resolution achieved with highly sulfated cyclodextrins. Conditions: buffer: 5% γ -HSCD in 25 mM phosphate, pH 2.5; capillary: untreated fused-silica capillary 50 $\mu\text{m} \times 30$ cm (20 cm to detector); capillary temperature: 22 $^{\circ}\text{C}$ (recirculating liquid); field strength: (–) 500 V/cm (anode at outlet); detection: PDA 200 nm; sample introduction: pressure 1.2 psi*s; current generated: 156 μA .

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