Applications of FLEC - Derivatizing Agent for Chiral Separation with Fluorescence Detection.

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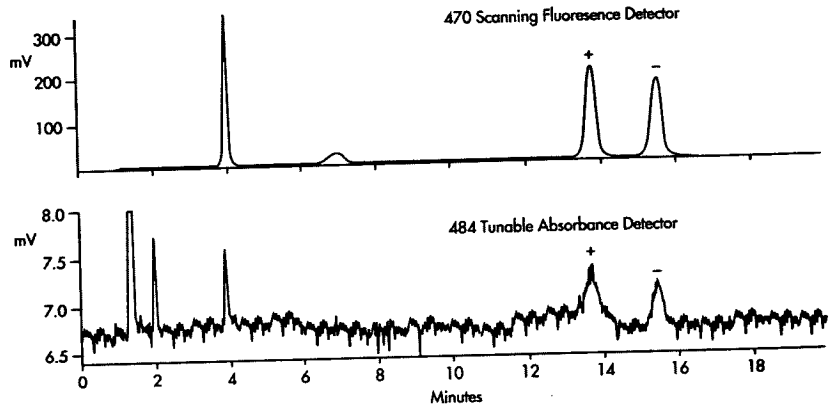
Waters 470 Scanning Fluoresence Detector Brings a New Dimension to Chiral Analysis.

Enantiomer specific analysis of optical isomers are needed in a variety of situations. These include the analysis of the enantiomeric composition of drugs, synthetic intermediates, monitoring stereospecific synthesis, or in pharmacological testing. In these situations, several approaches to the determination of enantiomers have been used. In recent work by J. Gal[1], derivatization was employed to form diastereomers from enantiomers. This indirect method separates the diastereomers using a typical reverse phase achiral HPLC column. The same indirect method can be used with capillary electrophoresis using non-chiral discriminating electrolytes. Work by I. Wainer[2] involves the direct analysis of enantiomers by a combination of chiral and achiral HPLC columns in pharmacokinetic studies. The total drug is measured by the achiral column and the enantiomeric ratio determined by the coupled chiral column. Another method being researched in Waters Pharmaceutical Laboratory is the use of capillary electrophoresis to separate the enantiomers without derivatization. An example of this work is presented elsewhere in this issue of pharmaceutical notes (page 7).

Each chiral separation technique has specific advantages depending on the sample matrix. The direct method employing chiral columns is often used for enantiomer analyses during chemical synthesis, stability studies or in the determination of a pharmacological agent before pharmacokinetic testing. In most cases the sample matrix is relatively clean (i.e., few interfering peaks are detected). Since chiral columns are custom designed for chiral discrimination, these are not as useful to separate non-chiral components, degradation products or drug metabolites.

Waters Pharmaceutical Laboratory has explored the analysis of chiral compounds by the indirect method to provide advantages over the current direct and indirect methods. The advantages of this new technique, using a fluorescent homochiral reagent and Waters 470 Scanning Fluoresence Detector, are enhanced sensitivity, selectivity and stability of the derivative. Although the choice of the homochiral derivatizing agent in the indirect method depends on the available enantiomer functional groups, this approach has been highly successful in a variety of stereospecific analysis problems.

Figure 1: Comparison of the UV and Fluorescence Response for the Derivatized Racemic Mixture of Ephedrine.



Waters 470 Fluorescence Detector provides over 2400 times more sensitivity for the derivatized sample compared to UV detection. The fluorescence detection limit for this derivative is 12 piccgrams.

Optimized Systems and Columns for Diastereomer Analysis.

The instrumentation used in this indirect method included a Waters 600E Multisolvent Delivery System, the 470 Scanning Fluorescence Detector, the 484 Tunable Absorbance Detector and the 715 Ultra WISP™ Sample Processor. All data was collected on a VAX™ based 845 Workstation. Derivatization was performed with (+) - 1 - (9-fluorenyl)ethylchloroformate (FLEC) as described by S. Einarsson[3]. The diastereomers were separated using the highly efficient 5 micron Nova-Pak® C_{18} columns in either steel or radial compression cartridge configurations. The mobile phase consisted of 20 mM monobasic ammonium phosphate buffer and various proportions of acetonitrile.

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Fluorescence Detection Increases Sensitivity.

Once an appropriate separation was developed, each peak was scanned using the unique scanning capabilities of the Waters 470 detector to optimize the excitation and emission wavelengths. Enhanced sensitivity is a major advantage of the fluorescent FLEC derivative. In Figure 1, a derivatized racemic mixture of Ephedrine was injected and the separation monitored by both UV and fluorescence. The enhanced sensitivity provided by the FLEC derivative coupled with the high sensitivity of the 470 detector provides a 2400 fold increase in sensitivity over UV detection. The detection limit (signal to noise ratio of 3:1) for ephedrine using the UV detector was 5 nanograms while the detection limit was 12 picograms with the fluorescence detector. Not only does the FLEC derivative provide different selectivity, chiral discrimination and increased sensitivity, it imparts fluorescent activity to compounds not possessing native fluorescence, expanding the applicability of the fluorescent detector.

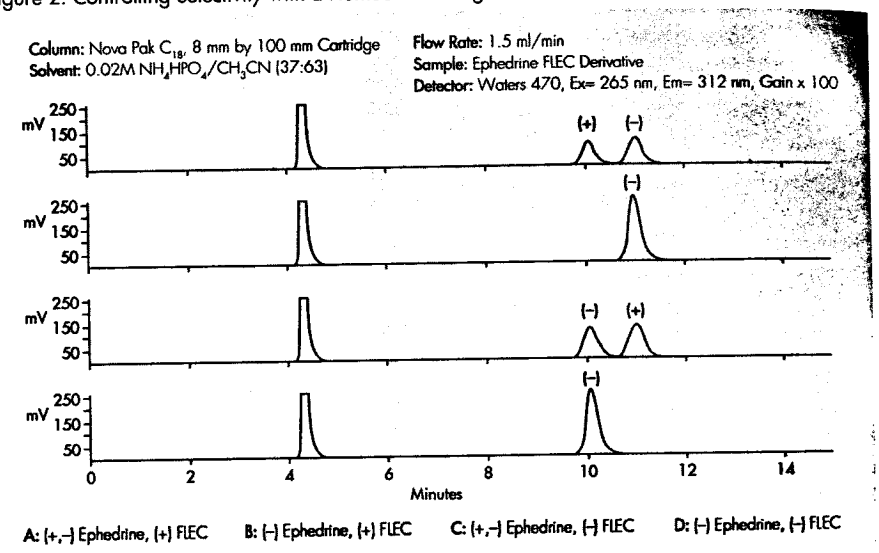
Diastersomer Stability Allows Overnight Analysis.

In order to study the stability of the FLEC derivative, derivatized samples were stored at room temperature, and chromatographed every hour during a 17 hour period. The peak area's relative standard deviation was 0.6% over this time period. No significant degradation of the derivatives was observed nor did the area response change under these conditions.

Minor Component Analysis Made Easy by Controlling Elution Order.

Another major advantage of the indirect method is that the elution order of the compound of interest can be easily altered. Figure 2 shows the separation of the ephedrine-FLEC diastereomers. Since FLEC is available in both the (+) and (–) forms the diastereomer elution order can be reversed by using the appropriate form of the reagent. This is important when assaying non-racemic enantiomer mixtures. When the enantiomer ratio differs by 100:1 or greater as occurs in pharmacokinetic studies or in chiral specific synthesis, the minor enantiomer quantitation can be difficult. Using the appropriate homochiral reagent the trace level enantiomer can always elute before the major enantiomer providing better integration and quantitation. This ensures good accuracy and precision even if the enantiomer ratio is 100:1 or greater. With the selectivity available for the FLEC derivative, it is easy to customize a method which provides excellent precision and accuracy.

Figure 2: Controlling Selectivity with a Homochiral Reagent.



Selectivity is easily controlled by judiciously selecting the homochiral derivatizing reagent. The derivatizing reagent is chosen to elute the least concentrated diastereomer first.

Manual or Automated Derivatization Offered with Waters Sample Processor.

Whether to form the derivative manually or with the WISP 1700 Sample Processor is dictated by other sample matrix requirements. Based on Waters MilliLab™ Workstation, the WISP 1700 Sample Processor performs all operations involving handling liquids including automated filtration and solid phase extraction with a unique, streamlined probe on a rugged XYZ Transport Module. If biological samples need to be extracted, concentrated or cleaned prior to derivatization, then the WISP 1700 is the appropriate sample preparation tool.

Summary.

The use of FLEC derivatives with high sensitivity fluorescence detection brings a new dimension to chiral analyses. The demonstrated ease of use, versatility, enhanced sensitivity, precision and reliability provide the researcher with a new tool for enantiomer separations.

References:

1. J. Gal, J. of Chromatography, 307 220 (1987)
2. F. Gimenez, I. Wainer, Pharmaceutical Notes Vol.1, No. 3 (Fall 1990).
3. S. Einarsson, Analytical Chemistry, 59 1191 (1987)

For more information on Waters 470 Scanning Fluorescence Detector please check box 6 on the attached reply card. For more information on Waters WISP 1700 Sample Processor please check box 7 on the attached reply card.