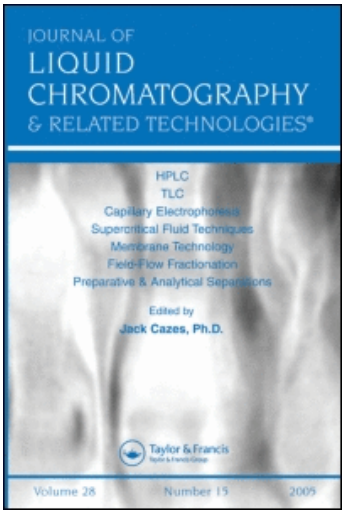

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APPLICATIONS OF A SENSITIVE LASER POLARIMETRIC HPLC DETECTOR: CONFIRMATION OF ENANTIOMERIC EXCESS FOR DANOFLOXACIN AND ANALYSIS OF A NOVEL MACROLIDE

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ABSTRACT

A commercially available laser-based polarimetric detector (Chiral Detector) was used to confirm the enantiomeric excess of danofloxacin and to analyze a novel macrolide. The detector is based on the work of Yeung et. al.[1] and uses a low noise laser diode which emits 675 nm radiation. Selectivity is obtained not through chromatographic resolution but through the sign and magnitude of detector response. An area ratio of the detector signals (Chiral/UV) was used to calculate Danofloxacin's optical purity. By calculating the ratio of the two detector signals an accurate measure of optical purity or enantiomeric excess can be calculated relative to standard responses without concern for potency, optical rotation of impurities, exact sample concentration or injection precision. The molecule is ideal for this type of determination since it absorbs UV radiation strongly, has a large optical rotation and the HPLC method resolves potential process impurities, synthetic byproducts and degradation products. The signal ratio was found to be linear with respect to % optical purity from 100% to 0% and is expected to be accurate to within 2%. Samples of danofloxacin mesylate were optically pure to within the 2% limit of the methodology.

A novel experimental macrolide was analyzed using a reversed phase HPLC system interfaced to both a UV detector and a laser-based polarimetric detector. Specific rotation of the novel macrolide was determined to be -23° in mobile phase using a bench top polarimeter. While polarimetric response for the experimental macrolide was shown to be reasonably linear from 0.1mg/mL to 1.5mg/mL, sensitivity was poor relative to UV detection at 210nm. The limit of quantitation was ~5000 ng for the laser-based polarimetric detector which was 50 times less sensitive than the UV detector.

INTRODUCTION

A commercially available laser-based polarimetric detector (Chiral Detector) was used to confirm the enantiomeric excess of danofloxacin and to analyze a novel macrolide. The detector is based on the work of Yeung et. Al.[1] and uses a low noise laser diode which emits 675 nm radiation. Both the magnitude and direction of optical rotation are detected. A thorough description and schematic of this type of detector is contained in the following reference[2]. Sensitivity of the detector has been reported as 25 micro-degrees of optical rotation with the 56 mL flow cell allowing detection limits of 10 to 100 ng for most optically active compounds by the manufacturer.

Confirmation of the Enantiomeric Excess of Commercial Danofloxacin Mesylate

Danofloxacin (Figure 1) is synthesized using a stereoselective synthesis, starting with a naturally occurring chiral amino acid to yield a single optical isomer[3,4]. Although two chiral carbons are contained within the molecule no diasteriomers are possible due to the steric hindrance.

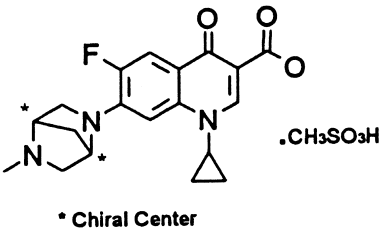

Figure 1. Structure of Danofloxacin mesylate.

To confirm that the chemistry was producing a single isomer specific rotation was monitored and both enantiomers were synthesized. Each isomer displayed the same magnitude of rotation, and as expected, with opposite sign (Danofloxacin mesylate [α] = -198°, Danofloxacin mesylate enantiomer [α] = +198°).

To further verify the optical purity of the synthetic route a reversed phase HPLC system was interfaced to both a UV detector and a laser-based polarimetric detector. A series of samples and standards were analyzed. An area ratio of the detector signals (Chiral/UV) was used to calculate optical purity. By calculating the ratio of the two detector signals for the same sample injection an accurate measure of optical purity or enantiomeric excess can be calculated relative to standard responses without concern for potency, optical rotation of impurities, exact sample concentration or injection precision. The molecule is ideal for this type of determination since it absorbs UV radiation strongly, has a large optical rotation and the HPLC method resolves potential process impurities, synthetic byproducts and degradation products.

The signal ratio was found to be linear with respect to % optical purity from 100% to 0% and is expected to be accurate to within 2%. Commercial samples of danofloxacin mesylate contained no enantiomer to within the 2% limit of the methodology.

Analysis of a Novel Macrolide

Quantitative analysis of macrolides has historically been challenging and has required the use of electrochemical detection and/or extremes of pH or temperature to obtain appropriate sensitivity, peak shape and resolution[5-9]. These difficulties are largely due to the lack of a strong UV chromophore and the basic nature of the amine(s) (pKa 8.8 for erythromycin[10]) which contribute to the poor peak shape at elevated pH values and the low k' (capacity factor) at reduced pH values. A method for sensitive analysis of erythromycin using a laser-based polarimetric detector has recently been reported[11] with a limit of quantitation of ~ 10 ng.

A novel experimental macrolide was analyzed using a reversed phase HPLC system interfaced to both a UV detector and a laser-based polarimetric detector. Specific rotation of the novel macrolide was determined to be -23° in mobile phase using a bench top polarimeter. While polarimetric response for the experimental macrolide was shown to be reasonably linear from 0.1mg/mL to 1.5mg/mL sensitivity was poor relative to UV detection at 210nm. The limit of quantitation was ~5000 ng for the laser-based polarimetric detector which was 50 times less sensitive than the UV detector.

EXPERIMENTAL

Equipment

A model HP1100 (Hewlett-Packard, Avondale, PA) HPLC with autosampler, was used. A model G1315A (Hewlett-Packard, Avondale, PA) UV detector and a Model CH96.001 (PDR-Chiral, Palm Beach, FL) Laser-based polarimetric detector were interfaced to the HPLC. Data were collected and processed using a ChemStation (Hewlett-Packard, Avondale, PA) data system. A bench top polarimeter Model DIP-370 with a 10cm cell (Jasco, Easton, MD) was used to measure specific rotation.

Confirmation of the Enantiomeric Excess of Commercial Danofloxacin Mesylate

Standard Preparation

Standards of danofloxacin mesylate and danofloxacin mesylate enantiomer (obtained from Pfizer Central Research, Groton, CT) were prepared in mobile phase containing 8.84:1.00:0.14:0.02 (v/v) water (HPLC Grade): acetonitrile (HPLC Grade): triethylamine (reagent grade): acetic acid (reagent grade). The working standards were prepared to contain a total of 0.13 mg/mL danofloxacin and/or danofloxacin enantiomer in various ratios.

Sample Preparation

Samples of danofloxacin mesylate (obtained from Pfizer Central Research, Groton, CT) were prepared in mobile phase at 0.13 mg/mL.

Chromatographic System

A Hamilton 5mm RPR-1 column (4.1 x 150 mm) was used with a flow rate of 1 mL/min at ambient temperature. An injection volume of 20 mL was used and the UV detection wavelength was set at 278 nm.

Analysis of a Novel Macrolide

Sample Preparation

Samples of an experimental macrolide (obtained from Pfizer Central Research, Groton, CT) were prepared in mobile phase containing 2.5:3.5:4.0 (v/v) 40 mM pH8.5 phosphate buffer (prepared from reagent grade potassium phosphate dibasic and phosphoric acid): methanol (HPLC Grade): acetonitrile: (HPLC Grade). The range of concentrations were from 0.1 to 1.5 mg/mL for HPLC analysis and 5mg/mL for specific rotation determination.

Chromatographic System

An Asahipak ODP-50 cartridge column (5um, 4.0x250mm) was used with a flow rate of 0.7 mL/min at ambient temperature. An injection volume of 20 μL and UV detection at 210 nm were used for this portion of the work.

RESULTS AND DISCUSSION

Confirmation of the Enantiomeric Excess of Commercial Danofloxacin Mesylate

Selectivity

Selectivity is obtained not through chromatographic resolution but through the sign and magnitude of detector response. This is illustrated by the overlayed chromatograms in Figure 2.

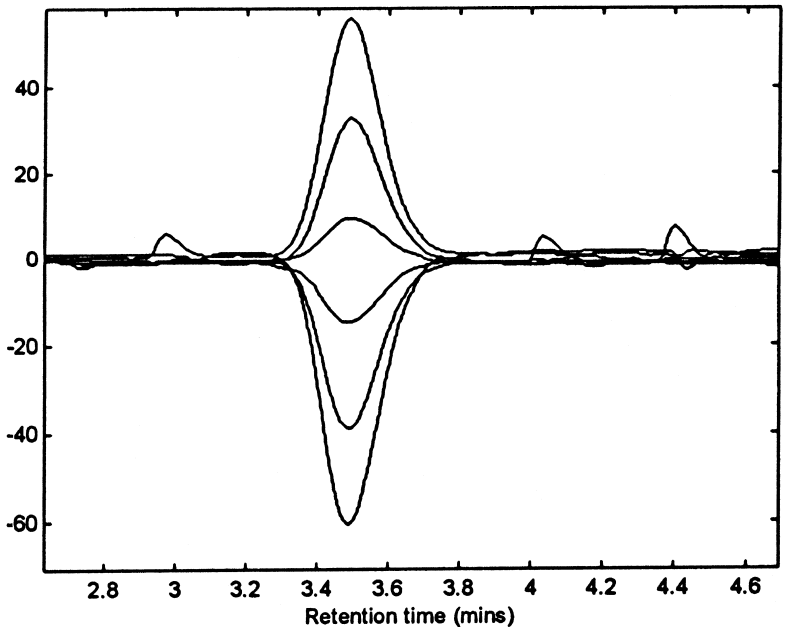

Figure 2. Overlaid chromatograms showing detector selectivity. (From top to bottom: 100% enantiomer; 80%/20% enantiomer/Danofloxacin; 60%/40% enantiomer/ Danofloxacin; 40%/ 60% enantiomer/Danofloxacin; 20%/ 80% enantiomer/ Danofloxacin; 100% Danofloxacin).

A sample containing pure danofloxacin mesylate exhibits a large negative signal. A racemic mix would exhibit no signal and pure danofloxacin mesylate enantiomer exhibits a large positive signal. Magnitude and sign of the signal should be interpretable to within a 2% optical purity as discussed in the following section.

Linearity

Linearity for the area ratio of laser-based polarimetric signal to UV signal was determined from 0% to 100% danofloxacin mesylate enantiomer in 20% increments and from 0% to 16% in 4% increments. The ratio was found to be ideally linear from 0% to 100% with a correlation coefficient squared (R^2) >0.999 and an intercept <1% of the nominal response (a plot containing the line equation is contained in the Figure 3). Note that the plot passes through the origin at 50% optical purity.

Linearity from 0% to 16% Danofloxacin mesylate enantiomer is less than ideal and R^2 is 0.995. Detecting small differences in a large polarimetric signal would seem to be a likely cause for the non-ideal linearity and this experiment

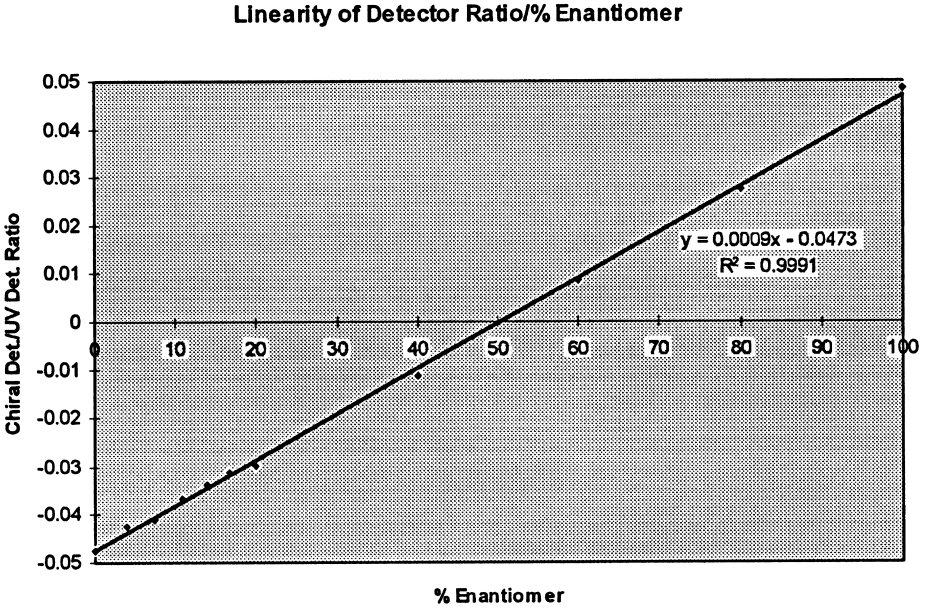

Figure 3. Linearity of the detector ratio with respect to sample composition.

was used to establish an accuracy of ~ ±2% for the determination of optical purity. Data used to calculate linearity are contained in Table 1.

Ruggedness

The ruggedness of the system was investigated by making small modifications in the mobile phase composition and observing any differences in the resultant chiral and UV profiles for Danofloxacin mesylate. The peak area information for three replicate injections is tabulated in Tables 2 and 3 for slight modifications to the buffer concentrations. The tables also show the calculated % RSD for the replicate injections as well as the % RSD between the 3 buffer concentrations. Comparing the results in these two tables it is observed that the uncertainty in the chiral detector replicate area responses is higher compared to the UV detector generated areas. However, the two detection modes are comparable in terms of % RSD induced by the buffer concentration changes.

The second portion of the ruggedness investigation was to compare the performance of the chiral and UV detectors when small changes are made to the organic modifier content in the mobile phase. Tables 4 and 5 show peak area and % RSD tabulations for changes in the content of acetonitrile, which was changed from 6 to 10%. It is observed that the % RSD for the replicate injections is comparable between the chiral and UV detectors. However, the high % RSD values obtained between the modifier concentrations indicate that the chiral detector is affected more significantly by these types of changes.

The UV response is not significantly affected by the changes in acetonitrile content in the mobile phase. This is a somewhat expected result since the chiral detector will be affected by the refractive index changes that accompany changes in the organic modifier content.

In summary, the chiral detector is not as rugged as the UV detector that has been used in this comparison. This translates into a larger degree of precision necessary in controlling all the variable method parameters as well as any other parameters likely to induce a change in the refractive index of the mobile phase. With due care and diligence, it is possible to use the chiral detector and obtain meaningful information.

Sample Analysis and Discussion

A total of 5 Danofloxacin Mesylate drug substance samples were analyzed. Results of the study are contained in Table 6 and demonstrate that a pure single isomer of Danofloxacin mesylate is produced to within the limits of the methodology (±2%).

Table 1

Data Collected to Demonstrate Linear Detector Response

Sample	mg/mL Danofloxacin Mesylate	mg/mL Enantiomer	%Enantiomer	Area Optical Rotation Detector	Area UV Detector	Ratio
1	0.1260	0	0	-745.563	15752.2	-0.047
2	0.1260	0.00504	3.8	-602.309	14181.5	-0.043
3	0.1260	0.01008	7.4	-600.367	14725.7	-0.042
4	0.1260	0.01512	10.7	-560.189	15305.5	-0.037
5	0.1260	0.02016	13.8	-518.396	15409.8	-0.034
6	0.1260	0.02520	16.7	-504.518	16143.3	-0.031
7	0.1008	0.02520	20	-457.799	15485.6	-0.030
8	0.0756	0.05040	40	-173.704	15298.4	-0.011
9	0.0504	0.07560	60	132.088	14976.2	0.009
10	0.0252	0.10080	80	408.503	14763.9	0.028
11	0	0.12600	100	660.808	13646.2	0.048

Table 2

Chiral Detector Response to Changes in the Buffer Concentration

	Replicate 1	Replicate 2	Replicate 3	Mean	% RSD (Replicate)	% RSD (concs)
1.5%TEA/0.1% AcOH	13543.1	13419.6	13368.5	13443.7	0.67	1.13
1.4%TEA/0.2% AcOH	13459.2	13791.7	13728.9	13659.9	1.29	1.07
1.3%TEA/0.3% AcOH	13742.0	13887.1	13974.5	13867.9	0.85	

Table 3

UV Detector Response @ 278 nm for Changes in Buffer Concentration

	Replicate 1	Replicate 2	Replicate 3	Mean	% RSD (Replicate)	% RSD (concs)
1.5%TEA/0.1% AcOH	15501.7	15492.2	15470.3	15488.1	0.10	
						0.78
1.4%TEA/0.2% AcOH	15650.1	15675.8	15656.0	15660.6	0.09	
						0.94
1.3%TEA/0.3% AcOH	15874.3	15849.6	15885.4	15869.8	0.12	

Table 4

Chiral Detector Response to Changes in the Organic Modifier in the Mobile Phase

	Replicate 1	Replicate 2	Replicate 3	Mean	% RSD (Replicate)	% RSD (concs)
10% Acetonitrile	14636.1	14575.8	14668.1	14626.7	0.32	2.98
8% Acetonitrile	14009.2	14163.6	14004.9	14059.2	0.64	1.51
6% Acetonitrile	13887.2	13704.0	13693.7	13761.6	0.79	

Table 5

UV Detector Response @ 278nm to Changes in Organic Modifier Content in the Mobile Phase

	Replicate 1	Replicate 2	Replicate 3	Mean	% RSD (Replicate)	% RSD (concs)
10% Acetonitrile	15737.9	15752.0	15714.8	15734.9	0.30	
						0.07
8% Acetonitrile	15749.2	15750.2	15756.9	15752.1	0.57	
						0.01
6% Acetonitrile	15737.3	15739.9	15791.6	15756.3	0.69	

Table 6

Sample Analysis Results

mg/mL Sample	Area Chiral	Area UV	Ratio Chi/UV	% Optical Purity
0.126	-686.653	14404.2	-0.04767	100.7%
0.126	-707.104	14558.6	-0.04857	101.6%
0.126	-698.857	14199.0	-0.04922	102.2%
0.126	-688.861	14144.1	-0.0487	101.7%
0.126	-685.594	14012.7	-0.04893	101.9%

Analysis of a Novel Macrolide

Sensitivity

Serial dilutions were used to probe sensitivity for both UV and laser-based polarimetric detectors. The chiral detector was found to be less sensitive than the UV detector for determination of an experimental macrolide by a factor of ~50. The limit of detection and limit of quantitation are tabulated below and were determined where signal to noise ratios were approximately 3 and 10 respectively. A representative chromatogram near the limit of detection is included as Figure 4. Specific rotation was determined to be -23° (35°C, sodium line) using a bench top instrument and sensitivity is likely to be proportional to specific rotation.

	UV at 210 nm	**Optical Rotation**
Limit of Quantitation (LOQ)	100 ng	5000ng
Limit of Detection (LOD)	20 ng	1500 ng

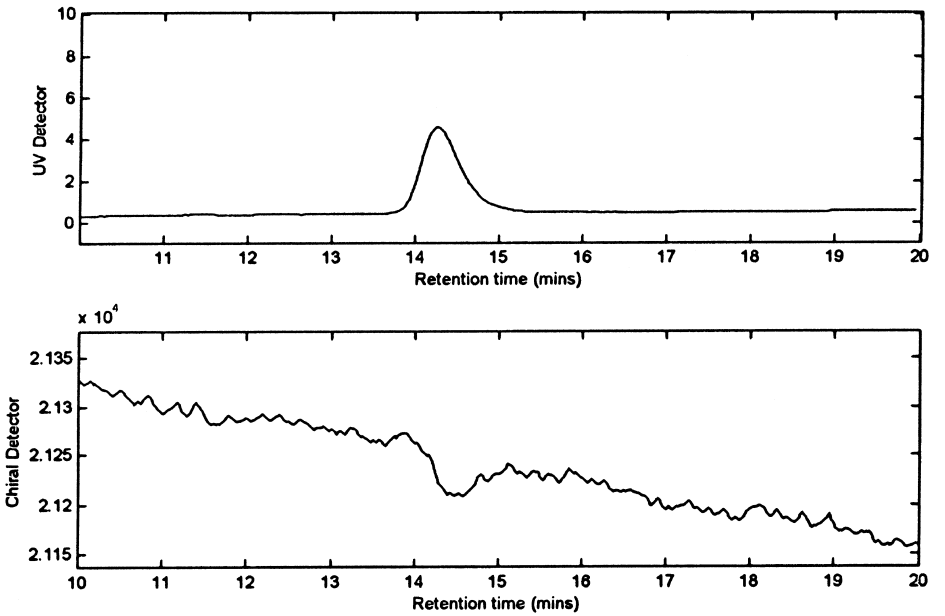

Figure 4. Chromatogram of a novel macrolide sample near the limit of detection (LOD) for the polarimetric detector.

Table 7

Linearity of Novel Macrolide Response

Concentration	UV Area Response	Polarimetric Area Response
1.5 mg/mL	41406	3198
1.1 mg/mL	29954	2289
0.75 mg/mL	20587	1621
0.50 mg/mL	12799	1033
0.25 mg/mL	6215	504
0.10 mg/mL	2278	200
Slope	28424	2166
Intercept	-787	-24.2
R^2	0.999	0.999

Linearity

Linearity of response for both the UV and the polarimetric detector were determined from 0.10 to 1.5 mg/mL as shown in Table 7. The response was linear for both detectors over this range, however, significance of the intercept may indicate that column loading is too high. This level of column loading was necessary to achieve appropriate sensitivity for the polarimetric detector.

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