CHAPTER 1V
EXPERIMENTAL

4.1 Instrumentation

All chiral separations were performed with a Jasco Model PU 980 pump (Jasco Inc., Easton, MD), a Jasco Model DG-980-50 on-line degasser, a Jasco Model UV 975 UV-Visible detector, a HP Model 1050 autosampler (Hewlett Packard, Little Falls, DE) and a HP Model 3396 digital electronic integrator or PE TurboChrom data station (Perkin Elmer Corp., Norwalk, CT). The chiral columns employed in this study were Chiralpak AD and Chiralcel OD (Daicel Chemical Industries Ltd., Tokyo, Japan; distributed by J.T. Baker, Phillipsburg, NJ).

Precise control of the mobile phase flow rate in the LC system was through the Jasco PU 890 ternary gradient pump. The dissolved gases in the mobile phase were removed by the Jasco DG-980-50 vacuum on-line degasser. Removal of dissolved gases from the mobile phase was necessary because they could affect flow stability and UV absorption causing either baseline drift and/or random noise. Degassing was made by passing the mobile phase through a special fluoropolymer membrane tube with the pressure outside the tube reduced. Degassing by membrane separation was carried out under mild conditions that had virtually no effect on composition of mobile phase.

Precise introduction of chiral analytes to the column was through the HP Model 1050 autosampler. UV detection of analytes was by the Jasco UV Model 975, variable UV-Vis dual beam spectrophotometric detector. The flowcell was of the taper design with a 13 μL volume and 10 mm pathlength.

All connections from the autosampler to column and column to detector were made with 0.005" I.D. Polyether ether ketone tubings and were made as short as possible to minimize extracolumn band broadening. All end fittings used were carefully chosen to contribute a zero dead volume. The connection tubes and fittings employed were

stainless steel and polyether ether ketone.

The column temperature was maintained by a 30-cm long plastic column jacket (Alltech Assoc. Inc., Deerfield, IL) attached to a Lauda Super Model RMS-6 constant temperature and circulating bath (Brinkmann Instruments, Westburg, NY) with a temperature stability of ± 0.05 °C. A mixture of 1/1 diethylene glycol/water served as the circulating heat transfer fluid for the column temperature. To test the performance of the heat exchanger, an Omega #HH-25TC digital thermometer (Omega Engineering Inc., Stamford, CT) with an Omega #CPSS-1160-12 thermocouple was used to monitor the bath temperature inside the reservoir. All tubings from the circulating bath to the column was covered with foam insulators to minimize temperature gradients.

For the collection of chromatographic data, a HP Model 3396 Inkjet electronic integrator or PE Turbochrom data station was used.

4.2 Chiral Stationary Phases

The chiral stationary phases (CSPs) used in this research were amylose tris(3,5-dimethylphenylcarbamate), Chiralpak AD, and cellulose tris(3,5-dimethylphenyl-carbamate), Chiralcel OD. These CSPs were adsorbed on a macroporous silica gel support that had been treated with 3-aminopropyl triethoxysilane in benzene. Because of its non-bonded nature and the solubility and morphological properties of cellulose, there were flow rate, pressure, mobile phase and temperature constraints upon the use of these chiral columns. The CSPs were received packed in a 4.6 x 250 mm stainless steel tube.

The J.T. Baker recommendations for the operating conditions of Chiralcel OD and Chiralpak AD limit the flow rate to 1 mL/min, maximum column pressure of 430 psi, and column temperature range from 0-40 °C. With respect to the mobile phase, pure hexane, 2-propanol, ethanol, or methanol could be used for Chiralpak AD. The use of ethylacetate or chloroform is limited up to 10%; water, acetone, dichloromethane, N,N-dimethylamine, trifluoroacetic acid, or acetic acid as 5% of the mobile phase.

For Chiralcel OD, 100 % of hexane, 2-propanol, or ethanol could be used as the mobile phase. However, the use of the following solvents is restricted: 40 % maximum of chloroform or ethyl acetate; 20% of acetone; 10% of tetrahydrofuran,

propionitrile, or dichloromethane; 5% of water; and 0.5% of N,N-diethylamine, trifluoroacetic acid, or acetic acid.

Furthermore, for both CSPs there is a restriction on the use of hexane/ethanol mixtures from 85/15 to 40/60 because of UV absorption. However, in this research, with the chiral separations using a homogeneous 80/20/0.15 mixture of hexane/ethanol/TFA there was no such problems. All chromatographic data were reproducible.

4.3 Chemical Reagents and Analyte Solutions

Analytical grade racemic profens: carprofen, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, and naproxen were purchased from Sigma Chemical Company (St. Louis, MO). Analytical grade *tert*-butyl alcohol, methanol, sodium bicarbonate, sodium hydroxide, ethyl ether, anhydrous sodium sulfate, heptafluorobutyric acid (HFBA), 1,3,5-tri-*tert*-butylbenzene, benzene, naphthalene, phenanthrene, and acetic acid (HOAc) were also purchased from Sigma Chemical Company. HPLC grade hexane, methanol, and isopropanol were obtained from Fisher Scientific Co. (Fairlawn, NJ). A 100% ethanol (EtOH) of USP grade was purchased from Aaper and Chemical Co. (Shelbyville, KY). Spectrophotometric grade trifluroacetic acid (TFA) was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI).

The esters of fenoprofen, flurbiprofen, and ketoprofen were synthesized in our lab because they were not commercially available.

Sample solution of each profen and their methyl esters was prepared by dissolving 10.0 mg in 80/20 v/v of either hexane/ethanol or hexane/isopropanol mixture. The concentration of analyte solutions used in the study ranged from 100 to 700 ppm.

4.4 **Esterification of Profens**

The methyl esters of fenoprofen, ibuprofen, and ketoprofen were prepared by the reaction of profens (RCOOH) with methanol in the presence of sulfuric acid that served as a catalyst (Fig. 13). This Fischer esterification reaction reached equilibrium after one hour of reaction.

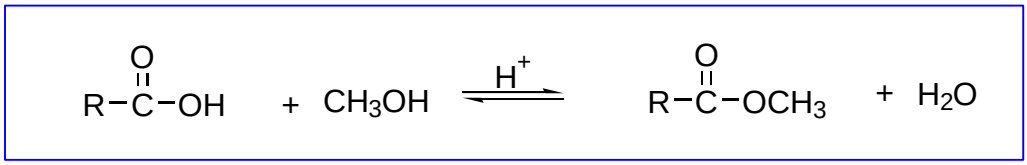

Figure 13. Fischer esterification reaction for a carboxylic acid and methanol with sulfuric acid as catalyst.

A simple distillation set up was used in the synthesis of the profen methyl esters. To facilitate mixing and heating of materials, the round-bottomed flask was mounted on a Corning Model PC-351 hot plate/stirrer (Fisher, Norcross, GA).

A 250 mg of the profen and 20 ml of methanol were mixed by a magnetic stirring rod in a 50-mL round bottomed flask. Subsequently, 2.0 mL of sulfuric acid was slowly added down to the walls of the flask to mix with the components. A condenser was then attached to the flask and the mixture was refluxed gently for one hour.

After the solution was cooled, it was adjusted to pH ≅ 9 with 40% NaOH and decanted to a 50-mL separatory funnel containing 15 mL of ethyl acetate. The flask was rinsed with ethyl ether and the rinse was added to the separatory funnel. The mixture was then shaken, with frequent release of pressure by inverting the separatory funnel and opening the stopcock, and draining off the aqueous layer. Afterwards, the ethyl acetate layer was washed three times with 15 mL of water followed by 15 mL of 5% NaHCO3. The ethyl ether layer was collected, dried over anhydrous sodium sulfate, filtered, and evaporated to dryness using a Buchi rotary evaporator (Brinkmann Instruments, Westburg, NY).

The synthesized profen methyl esters were analyzed and identified by HP Model 5970 gas chromatograph / mass spectrometer (Hewlett Packard, Little Falls, DE).

4.5 Data Treatment

Chromatographic data of retention time, peak area, and peak width of each injection from the integrator report were collected and manually entered into a spreadsheet of Microsoft Excel Version 5 (Microsoft Corp., Redmond WA) of a personal computer. The raw data consisted of four values for each injection: t_{R1}, t_{R2}, w_1, and w_2, where the subscripts 1 and 2 represents the first and second eluted enantiomer. Subsequently. a subprogram in Microsoft Excel was used to calculate the adjusted retention time t'_R, retention factor k, enantioselectivity α, and resolution R for each peak.

The following were the equations used to calculate V'_R, t'_R, k, α, and R:

$$V'_R = V_R - V_o \qquad (4.5a)$$

where V_R and V_0 are the peak retention volume and void volume, respectively;

$$t'_R = t_R - t_o \qquad (4.5b)$$

where t_R is the peak retention time and t_0 is the hold-up or dead time;

$$k = (t_R - t_o) / t_o \qquad (4.5c)$$

$$\alpha = k_2 / k_1 \qquad (4.5d)$$

where k_2 is the capacity factor of the late eluting peak;

$$R = 2 (t_2 - t_1) / (w_1 + w_2) \qquad (4.5e)$$

where w_1 and w_2 are width at base of peaks 1 and 2.

The reported peak width value was the ratio of peak area divided by height (A/H). Thus, resolution R was calculated from

$$R = (t_2 - t_1) / (w_1 + w_2) \qquad (4.5f)$$

The values for the thermodynamic parameters: Gibbs free energy difference $\Delta(\Delta G)$, enthalpy change $\Delta(\Delta H)$, and entropy change $\Delta(\Delta S)$ for the separation of enantiomers were obtained from the plot of the natural log of selectivity ($\ln \alpha$) against the reciprocal of the column absolute temperature ($1/T$). Plots of $\ln \alpha$ vs. $1/T$ were used to calculate $\Delta(\Delta G)$, $\Delta(\Delta H)$, and $\Delta(\Delta S)$ based on the equation:

$$\ln \alpha = \Delta(\Delta G)/RT \qquad (4.5g)$$

where R is the gas constant. Applying the Gibbs-Helmholtz equation, $\Delta G = \Delta H - T\Delta S$, equation (6) can be expressed as

$$\ln \alpha = \Delta(\Delta H)/RT + \Delta(\Delta S)/R \qquad (4.5h)$$

Thus, $\Delta(\Delta H)$ and $\Delta(\Delta S)$ between enantiomers were calculated from the slope and intercept of the line with the Y-axis, respectively. Determination of the $\Delta(\Delta G)$ values were from equation (6) and from the expression

$$\Delta(\Delta G) = \Delta(\Delta H) - T\Delta(\Delta S) \qquad (4.5i)$$

The linearity of plots from the regression coefficient r^2 , as well as the uncertainties of all experimental and calculated values, were obtained from the Microsoft Excel program itself.

4.6 Experimental Procedure

4.6.1. Preliminary Optimization Studies

Initial chiral separations were made in the isocratic mode using a pre-mixed mobile phase of 80/20 and 90/10 v/v hexane/ethanol at a flow rate of 1.0 mL/min. Ethanol was chosen as the polar modifier because it gives a short analysis time. Preliminary results showed no separation of enantiomers. Hence minute amounts (0.1-0.4%) of trifluoroacetic acid (TFA) was added to the premixed 80/20 hexane/ethanol mixture. The mobile phase composition of hexane/ethanol/TFA was then adjusted to obtain the optimized separation of enantiomers, a maximum enantioselectivity value.

The starting composition of ethanol as the modifier was 20% because above this percentage a heterogeneous mobile phase of hexane and ethanol would be generated. A heterogeneous mobile phase would give irreproducible results.

For the determination of the chiral column's dead volume, 1,3,5-tri-*tert*-butylbenzene (a very nonpolar analyte) was used.

Prior to every change of mobile phase, both chiral columns were flushed with 90/10 of hexane/isopropanol (column storage solvent) five times their column. This was to ensure that the previous mobile phase had no influence on the later chromatographic runs.

With the change to a new mobile phase, both chiral columns were flushed ten times their volume so as to remove traces of the storage solvent, as well as to obtain good equilibration. At least two replicate injections were made for each analyte during the initial optimization chromatographic studies.

For the chromatographic runs at optimized mobile phase composition, six replicate injections were made at a column temperature of 25°C.

Column performance was monitored daily by injecting ketoprofen. Retention factors and selectivity were compared to those obtained when the column was first used. This procedure checked that there was no variation in column performance over the period of this study.

At the end of a day's chromatography, the chiral columns were flushed with five column volumes of the recommended 90/10 hexane/isopropanol storage solvent. Flushing preserved the lifetime of chiral columns and minimized any possible irreversible interaction of the chiral stationary phase with components in the mobile phase.

4.62. Temperature Dependence Studies

The optimized mobile phase condition (see Section 4.6.1) for each analyte was employed for the thermodynamic study of chiral separations on Chiralpak AD and Chiralcel OD. Six replicate injections were made for each analyte at a flow rate of 1.0 mL/min and at temperatures of 5, 10, 15, 20, and 25 °C. For every temperature change the column, with the mobile phase flowing through it, was equilibrated for thirty minutes at the desired temperature. Data was processed right after each run in case any anomalous results would be produced due to inconsistent column temperature.

Chromatographic runs below 0 °C was made initially with ketoprofen. However, this study at low temperatures was aborted because equilibration of the column temperature took too long. Furthermore, the recommended working temperature range for both Chiralpak AD and Chiralcel OD column was from 0 °C to 40 °C.

4.6.3 Mobile Phase Studies (Polar Modifiers)

The influence of mobile phase modifier was explored by varying the nature and concentration of the modifiers. This study consists of two parts: (a) the influence of acidic mobile phase modifiers on retention and enantioselectivity of profens and profen methyl esters; and (b) the effects of alcoholic mobile phase modifiers.

The acidic mobile phase modifiers studied were acetic acid (HOAc), trifluoroacetic acid (TFA), and heptafluorobutyric acid (HFBA). The choice of organic acids as modifiers was restricted to the compatibility of the acids with the chiral stationary phases and the mobile phase of hexane/ethanol. The analytes employed in the study were carprofen, fenoprofen, flurbiprofen, ketoprofen, and the methyl esters of fenoprofen, ibuprofen, and ketoprofen.

Ethanol, isopropanol, and *tert*-butyl alcohol were the alcoholic mobile phase modifiers examined. The same set of analytes as in the study of the influence of the acidic mobile phase modifiers were also used. To understand further the chiral separation mechanism aromatic hydrocarbons of benzene, naphthalene, and phenanthrene were included in the study.

The mobile phase conditions (see Section 4.6.1) for all the chiral separation of each analyte was seen to it it would give a retention factor greater than 1.0. Molar concentrations of the mobile phases were used instead of the v/v% composition of hexane/ethanol/trifluoroacetic acid. For example, the mobile phase composition of 80/20 of hexane/ethanol is 3.43 M ethanol in hexane, and 0.15 % of trifluoroacetic acid in the mobile phase is 1.95×10^{-6} M. The rationale behind this was to insure that the same number of molecules of the acidic modifier or alcoholic modifier was used throughout

the study.

In all chiral chromatographic studies, the solvents used came from the same production lot to insure that the solvent assays and impurities were the same, as well as to obtain reproducible results. Moreover, after each chromatographic run with a particular mobile phase, the chiral column was flushed five times of its volume with the recommended storage solvent of 90/10 hexane/isopropanol.

Six replicate chromatographic runs for the individual racemic profens with a particular mobile phase were made at a flow rate of 1.0 ml/min and at 25 °C. Chromatographic data for the calculation of enantioselectivity and resolution were processed daily to establish a trend in the study.

4.6.4 Retention and Chiral Recognition Mechanisms

Data from the optimization studies (4.6.1), temperature dependence (Section 4.6.2) and mobile phase studies (polar modifiers) (Section 4.6.3) were analyzed to evaluate the chiral recognition mechanisms for the enantioseparation of 2-methlyarylpropionic acids on Chiralpak AD and Chiralcel OD.

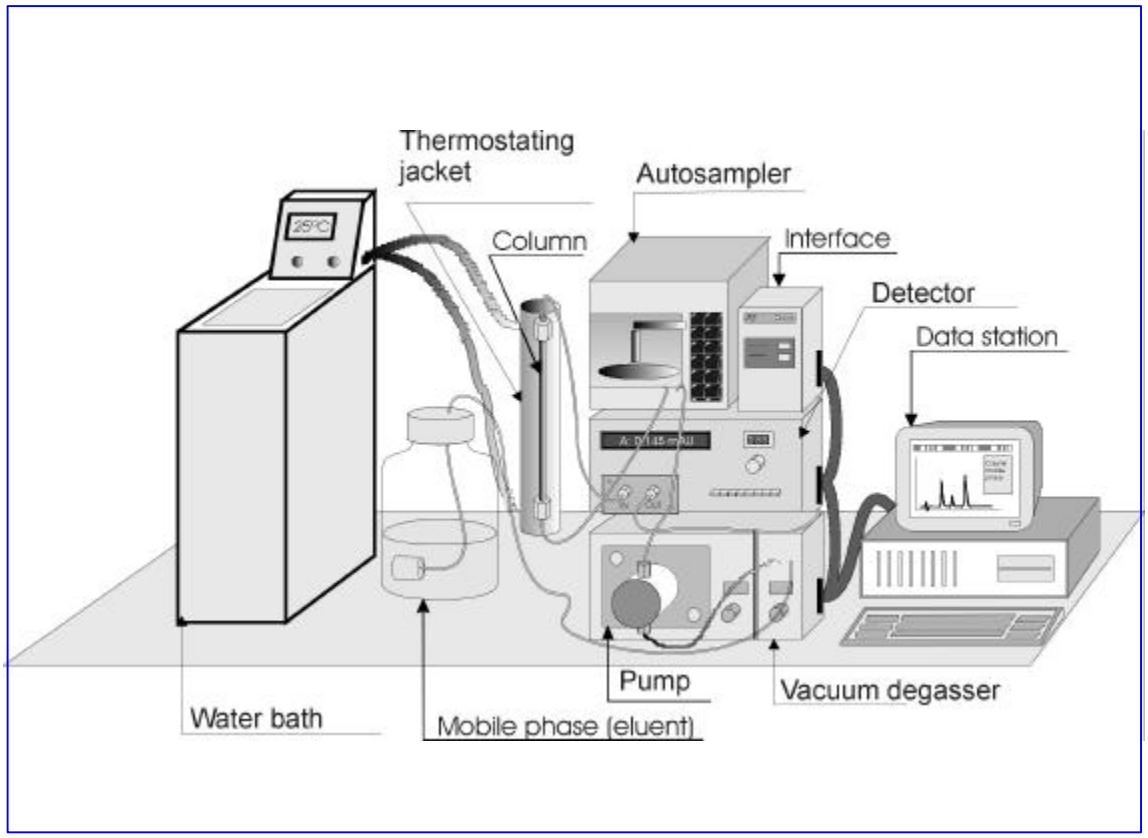

Figure 14. HPLC system with temperature control equipment.