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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ENANTIOMERIC RATIOS OF AMINO ACIDS WITHOUT CHIRAL SEPARATION

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SUMMARY

Two dual detector systems were studied for the determination of enantiomeric ratios of amino acids without chiral separation. Detection limits of 50 ng to 2 μg were observed for the four amino acids studied. These improvements are possible by using a micropolarimeter. The potential of the optical activity/refractive index (OA/RI) combination was limited by the sensitivity of the RI detector. Optical activity/ultraviolet (OA/UV) combination was determined to possess greater potential.

INTRODUCTION

The analysis of enantiomers by high-performance liquid chromatography (HPLC) of a wide variety of compounds is presently an area of active research. Efforts are being made to produce methods of racemate resolution which are less cumbersome than the traditional method of diastereomer preparation. The resulting methods involve a chiral stationary phase, a chiral mobile phase, or ligand exchange. Many reviews and papers have been published in this area[1–4]. The determination of the amount of each enantiomer present in a sample is important in many disciplines. For example, enantiomeric analysis is required in the pharmaceutical industry for quality control, in the synthesis of new drugs, and in the study of drugs in biological fluids[5]. The synthesis of biologically active peptides requires that the optical configuration be carefully monitored. A variety of columns for HPLC separations of enantiomers are presently being studied and some are commercially available. Unfortunately, one column is applicable to the enantiomeric separation of only a few compounds. Other methods require the preparation of diastereomers prior to chromatographic separation. This paper illustrates the determination of enantiomeric ratios of amino acids at low concentrations without derivatization or the physical separation of enantiomers.

The idea of using an ultraviolet detector in series with an optical activity detector (OA/UV) for the determination of enantiomeric ratios in HPLC was first demonstrated in 1982 by Boehme *et al.*[6], with their work on permethrinic acid pentafluorobenzyl ester (PBE). PBE exists in both the *cis* and *trans* configuration and

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each configuration has enantiomers: *R* and *S*. It is important to study the two pairs of enantiomers because of differing biological activities as insecticides. In this technique, chromatographic separation of the enantiomers is not necessary because the UV detector responds to the total amount of sample present and the OA response additionally depends on the ratio of the enantiomers. Using these two detectors in series allows the determination of the total amount of the sample as well as the D/L ratio of the enantiomers. Linear response was established up to 600 μg injected for both detectors. The limit of detection is 50 μg due to the limits of the Perkin-Elmer 241 LC polarimeter. A second application of this dual system for the determination of enantiomeric ratios was reported in 1985 by Scott and Dunn[7]. These workers were interested in the determination of the D/L ratio of epinephrine in opthalmic solutions used to treat glaucoma. This is important since the D isomer is inactive. Scott and Dunn[7] used a procedure similar to that of Boehme *et al.*[6]. L-Epinephrine solutions ranging from 6.0 to 14.0 mg/ml, with an injection volume of 50 μl, were used to check linearity. The authors note that this method, although adequate for opthalmic solutions containing 1% epinephrine, does not possess enough sensitivity to determine epinephrine in biological samples.

Besides the use of enantiomeric ratios for drug analysis, a second area of interest is the determination of amino acid ratios for use in dating. Geochronology involving amino acid racemization is based on the fact that nearly all amino acids present in living matter are in the L configuration. When an organism dies, racemization begins. Given enough time, a racemic mixture of the amino acids is formed. The increase in D/L ratio which occurs over time can be used to measure the amount of time that passed since the death of the organism. This method has been extensively studied and reviewed[8–10]. In theory, all amino acids can be used for dating purposes but a few have been favored. Aspartic acid is frequently used because it possesses a large racemization rate constant[11]. The conversion of L-isoleucine to D-alloisoleucine is also popular because these epimers are readily separated using conventional amino acid analyzers. In contrast, diastereomers of all other amino acids must be prepared prior to chromatographic separation.

A recent report[12] of interlaboratory comparison of amino acid enantiomeric ratios shows that there is an increase in interest in this dating method, but the methods of analysis have remained the same as when amino acid dating was first introduced. The most popular procedure was ion exchange for the determination of D-alloisoleucine/L-isoleucine. Gas chromatography after diasteromeric preparation was the other method used. Derivatization, however, is tedious and can introduce racemization. It is obvious that an ion-exchange separation of amino acids followed by optical activity/refractive index (OA/RI) or OA/UV detection would increase the amount of information available from a sample while simplifying the sample preparation.

It is clear that if the detectability of the polarimeter is lowered the above-mentioned studies can all be improved. The laser-based optical activity detector for HPLC developed in this research group offers many advantages over the commercially available polarimeters presently in use. Enhanced sensitivity and smaller cell volumes are two such advantages. The latter makes this system compatible with microbore chromatography. This is illustrated by the work of Bobbitt and Yeung[13] in which an OA/microbore LC system with a 1-μl flow cell was used. A detectability

of 11 ng for fructose was obtained. The most recent version allows 1 microdegree (1 ng fructose) to be detected[14]. Two adaptations of this technique for enantiomeric measurements were tried in the laboratory. OA/RI was used to study enantiomeric ratios of samples of threonine (Thr) and proline (Pro). Tyrosine (Tyr) and phenylalanine (Phe) were examined using OA/UV.

EXPERIMENTAL

Chromatography

The amino acids D-, L-proline, D-, L-threonine, D-, L-phenylalanine, and D-, L-tyrosine were obtained from Chemical Dynamics (South Plainfield, NJ, U.S.A.). Deionized water used in these experiments was further purified on a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Separation was performed on a 12 × 0.46 cm I.D., 6 μm cation-exchange column (Interaction Chemicals, Mountain View, CA, U.S.A., Model AA511) heated to 60°C with heat tape (Thermolyne Briskheat, Dubuque, IA, U.S.A.). Thr and Pro were eluted at a flow-rate of 0.5 ml/min with a 0.2 *M* sodium citrate (Pierce, Rockford, IL, U.S.A., pH buffer grade) buffer, adjusted to pH 3.25 with hydrochloric acid (Captree, Farmingdale, NY, U.S.A., electronic grade). A volume of 1 ml phenol (Fisher Scientific, Fair Lawn, NJ, U.S.A.) per liter of buffer was added as a preservative. Tyr and Phe were eluted with a buffer consisting of 0.2 *M* sodium citrate and 1.4 *M* sodium formate (Fisher Scientific, reagent grade). Formic acid (Fisher Scientific, reagent grade) was used to adjust the pH to 3.6. Flow-rate was 0.33 ml/min. All eluents were filtered through a 0.2-μm filter and degassed under vacuum using ultrasonic agitation to minimize degassing in the cell. To reduce any pressure fluctuations caused by the pump (Micrometrics, Norcross, GA, U.S.A., Model 750), we used a commercial pulse dampener (Handy & Harman, Norristown, PA, U.S.A., Model Li-Chroma-Damp III) in conjunction with a pressure gauge (Alltech, Deerfield, IL, U.S.A., Model 9299).

Injections were made through a conventional injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7010) with a 20-μl loop for the Thr/Pro analysis and 100-μl loop for Tyr/Phe. Since the OA flow cell was essentially at room temperature, the eluent must be cooled so that turbulence would not exist in the polarimeter cell. A 50-cm length of standard chromatographic stainless-steel tubing placed after the column was used for this purpose. For the Thr/Pro analysis, a refractive index detector (Waters Assoc., Milford, MA, U.S.A., Model R401) was used in series after the flow cell. UV detection (Rainin, Woburn, U.S.A., Model 153) was used prior to the flow cell in the Tyr/Phe experiments.

Optical activity detector

The arrangement of an OA detector for LC has been reported earlier[15,16]. For the Thr/Pro work, 20 mW of the 488 nm line from an argon ion laser was used with a photomultiplier tube (Amperex, Hicksville, NY, U.S.A., Model XP2020) operated at 1.7 kV. A 5 mW helium neon laser at 632.8 nm (Uniphase, Sunnyvale, CA, U.S.A., Model 1202-1) was used for the Tyr/Phe studies with a R928 phototube (Hamamatsu, Middlesex, NJ, U.S.A.) operated at 1.0 kV. The flow cell for the Thr/Pro work was 10 cm long with an internal volume of 200 μl. A second cell, 5 cm in length and 100 μl in volume was used for Tyr/Phe. The previously reported detection/modulation

cell[16] was used. Modulation was driven by a waveform generator (Wavetek, San Diego, CA, U.S.A., Model 184) at a frequency of 500 Hz. Using an independent air-based Faraday rotator to provide a standard optical rotation, laser power drift and overall system performance was monitored. A 1-s time constant was used. The output of the OA detector and either the RI or the UV detector was connected to two voltmeters (Keithley, Cleveland, OH, U.S.A., Models 155 and 160B), the analogue outputs of which were connected to a computer (Digital Equipment, Maynard, MA, U.S.A., Model PDP 11/10 with a LPS-11 laboratory interface). The computer takes a reading every 0.1 s and averages a set of 10 before storing the information.

Procedure

In the first set of experiments, we used OA/RI detection to determine the enantiomeric ratio of samples of Thr and Pro. In a second experiment, a UV absorbance detector replaced the RI detector. Tyr and Phe which contain phenyl groups were detected at 254 nm. The samples examined ranged from 100% D, 0% L to 0% D, 100% L of each amino acid. Between 1 and 50 μg of each amino acid were injected.

Calculations

The area of each peak from the OA detector is determined by summation of adjusted values above a chosen baseline for each peak. These are then normalized against the signal obtained from the DC coil (which produces a constant 0.25 millidegree rotation) for each chromatographic trial. In this way, laser power fluctuations are properly accounted for. These normalized values were then divided by either the RI or UV area of the corresponding peak from the second detector. The values of (OA/DC)/UV and (OA/DC)/RI were then plotted *versus* the fraction of L-amino acid present in the sample.

RESULTS AND DISCUSSION

Figs. 1a and 2a show optical activity chromatograms of the amino acids examined in this experiment. Baseline separation is achieved in all cases. The limits of detection (LOD) for the pure enantiomers of Thr, Pro, Tyr and Phe were 0.12 μg, 50 ng, 2 μg and 2 μg injected, respectively (signal-to-noise ratio, S/N = 3). The LOD naturally depends on the specific rotation of the material. When this is taken into account, these LODs are 2–3 orders of magnitude better than the 50 μg LOD reported for the analysis of PBE[6]. The corresponding RI and UV chromatograms are shown in Figs. 1b and 2b. Specific rotation [α] is a function of wavelength, optical path length, temperature, concentration and solvent. Furthermore, the specific rotations of amino acids vary widely with pH[17], thus literature values for the conditions used were not available. Because of this it is interesting to calculate the specific rotation of these four amino acids under the conditions used in this study. The concentration at the apex of the peak can be calculated from the peak area, the area of a 1-s interval at the peak maximum, amount injected and flow-rate. Using

$$[\alpha] = \frac{\alpha 100}{cl} \tag{1}$$

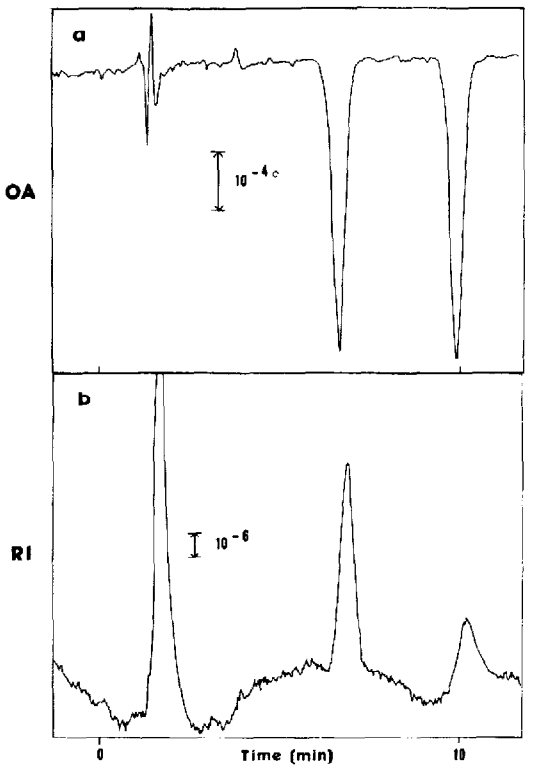

Fig. 1. Chromatograms of a mixture of 5.3 μg of L-threonine (7 min) and 2.3 μg of L-proline (10 min). (a) Optical activity, and (b) refractive index.

where α is the actual rotation measured, c is the concentration in grams solute per 100 ml solution, and l is the path length in decimeters, the specific rotation can be calculated. Results are given in Table I along with literature values for comparison. Solvent conditions are given in the Experimental section.

To test the utility of the dual detector scheme for determining enantiomeric ratios, one can compare the measured values of [α] for a series of samples of known D/L-ratios with the expected values. This is done for each of the four amino acids in Figs. 3–6. Each data point is the average of 2–4 chromatograms. The fraction of the L enantiomer in each sample is used as the abscissa. Since the UV and the RI detectors respond linearly with concentration (at these low concentration levels), eqn. 1 shows that the OA/RI and the OA/UV responses should be proportional to [α]. These responses then represent the ordinate. Each set of responses should fall on a straight line with intercepts (at 1.0 and 0.0 fractional concentrations) of equal magnitudes but opposite signs. Also, the response at 0.5 fractional concentration (a racemic mixture) should be zero. The ordinates are in arbitrary units, so that the conversion between the chromatographic peak areas and actual concentrations need not be separately determined. For routine applications, Figs. 3–6 can be used directly as calibration curves for determining enantiomeric ratios without chiral separation. In this

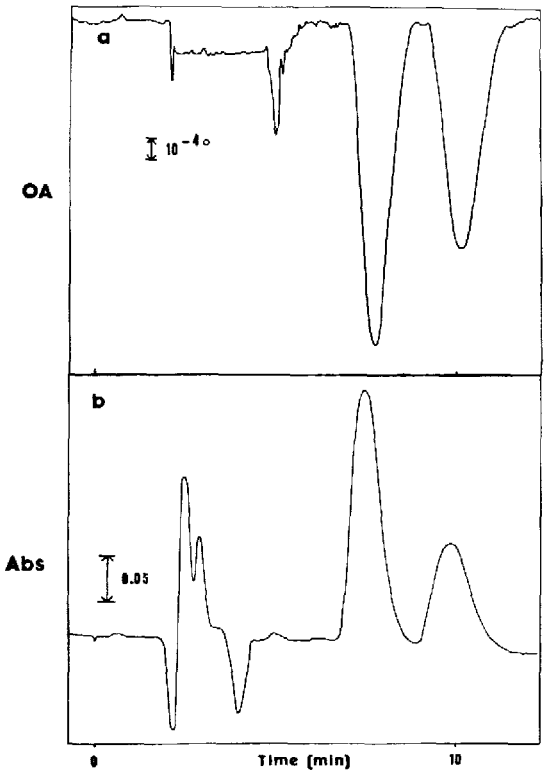

Fig. 2. Chromatograms of a mixture of 50 μg of L-tyrosine (7 min) and 50 μg of L-phenylalanine (10 min). (a) Optical activity, and (b) absorption at 254 nm.

mode of operation, the LOD for the OA detector depends on the enantiomeric ratio, but the LODs for the UV or RI detectors do not. So, the useful concentration range for this mode of operation is limited by either the usual LODs of the UV or RI detector, or the LOD of the OA detector, whichever is higher.

TABLE I

SPECIFIC ROTATIONS OF AMINO ACIDS

L-amino acid	$[\alpha]^*$	*Concentration (mg/100 ml)*	$[\alpha]_D$ *in water***	$[\alpha]_D$ *in 5 M hydrochloric acid***	*Concentration (g/100 ml)*
Thr****	−20.6	2	−28.5	−15.0	1–2
Pro****	−53.3	1	−86.2	−60.4	1–2
Tyr§	−19.5	16		−10.0	2
Phe§	−17.4	13	−34.5	−4.5	1–2

* This work 25°.
** Ref. 18.
*** At 488 nm; solvent see text.
§ At 632.8 nm; solvent see text.

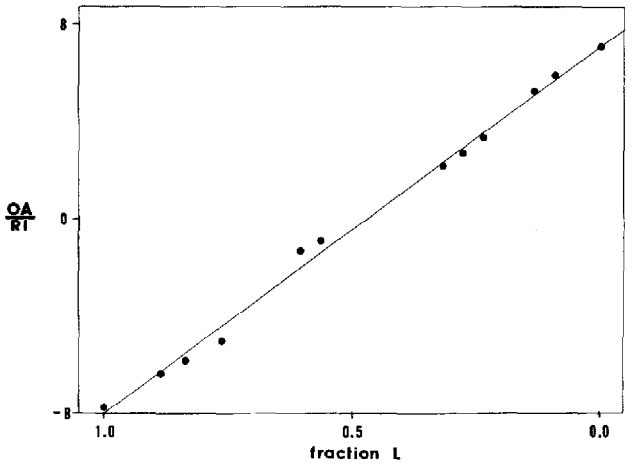

Fig. 3. Enantiomeric ratio calibration curve for threonine. Total amount of threonine injected is about 5 μg. The exact amount is not needed for calculations, since the ratio of responses is plotted.

Table II summarizes the results in Figs. 3–6. The racemic response (RR) indicates the overall accuracy for determining enantiomeric ratios. Any deviation from zero is a measure of the bias of the method. Since the ordinates are in arbitrary units, it is useful to use the value of the slope (S) for normalization. So, the last column in Table II (RR/S) reflects the percentage accuracy that is achieved. The precision of any individual measurement naturally depends on the number of replicate injections and on the noise level in the chromatograms. The correlation coefficients in Table II and the scatter of the data points in Figs. 3–6 show the precision that can be expected for triplicate injections and at the noise levels in Figs. 1 and 2.

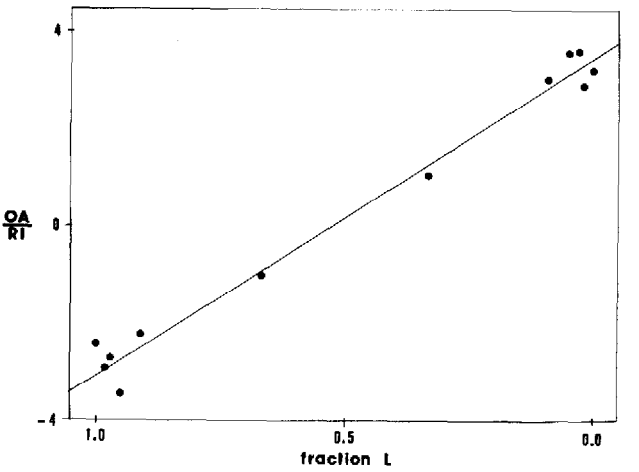

Fig. 4. Enantiomeric ratio calibration curve for proline. Total amount of proline injected is about 2 μg. The exact amount is not needed for calculations, since the ratio of responses is plotted.

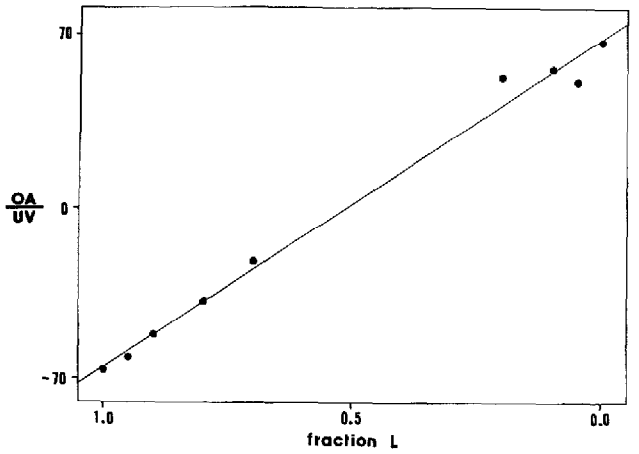

Fig. 5. Enantiomeric ratio calibration curve for tyrosine. Total amount of tyrosine injected is about 50 μg. The exact amount is not needed for calculations, since the ratio of response is plotted.

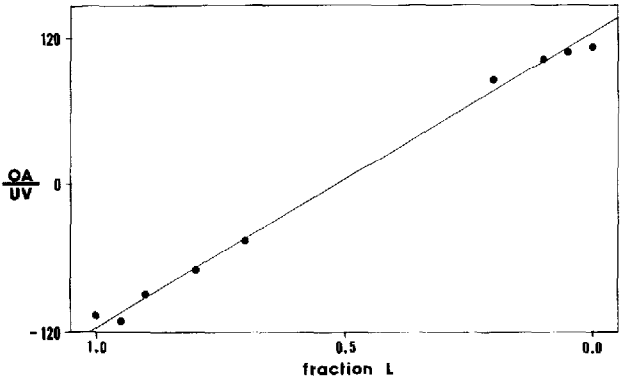

Fig. 6. Enantiomeric ratio calibration curve for phenylalanine. Total amount of phenylalanine injected is about 50 μg. The exact amount is not needed for calculations, since the ratio of responses is plotted.

TABLE II

DETERMINATION OF ENANTIOMERIC RATIOS USING DUAL DETECTORS

Species	*Mode*	*No. points*	*Correlation coefficient*	*Slope (S)*	*Racemic* response (RR)*	*RR/S (%)*
Thr	OA/RI	12	0.998	15.0	−0.40	−2.7
Pro	OA/RI	12	0.991	6.54	0.17	2.6
Tyr	OA/UV	9	0.995	135	1.0	0.7
Phe	OA/UV	9	0.998	241	3.5	1.5

* Racemic response is defined as the value for a sample with a fractional concentration of 0.5 l.

The implications of Table II are as follows. First, there is no obvious bias (last column) in the results in all cases, so that good quantitative accuracy can be achieved in either detector combination. This is in fact a confirmation that the micropolarimeter used provides reliable quantitative data at these low concentrations. Second, the precision of the dual detector method is good, judging from the correlation coefficients and the scatter of the data in Figs. 3–6. Third, for the OA/RI combination, the LOD for the OA is better than for the RI detector. Precision is therefore limited by the RI chromatogram. We note in Fig. 1 that the concentration of Pro is smaller than that of Thr for about the same response in the OA detector. This means that the S/N for the RI peak of Pro is worse than that for Thr. The precision is thus also worse for Pro (Fig. 4) than for Thr (Fig. 3). If an alternate method is used to determine their individual concentrations, then the precision for Pro should be better than that for Thr by a factor of 2.6, which is the ratio of their respective [α] values. Unfortunately, there are no convenient absorption bands for these two amino acids to allow the use of an absorption detector. Fourth, for the OA/UV combination, the LOD for the OA detector is worse than the UV. Precision is therefore limited by the OA chromatogram, particularly since the [α] values are small for these two amino acids. The high salt content of the buffer required for the separation in Fig. 2 also degrades the S/N of the OA chromatograms by a factor of 2 compared to Fig. 1. Bacterial contamination may have been another source of uncertainty because of the omission of the preservative, which is UV absorbing. Still, Table II shows that the UV results are better than the RI results. Naturally, UV works well only for the aromatic amino acids. Recently, the LOD of the OA detector has been improved to 1 ng for materials with [α] = 100° in conjunction with microbore columns[14]. The OA/UV combination is thus expected to be useful at the 1-ng level for ideal cases.

Even though the results here are for amino acids, one can expect similar accuracy when other species (*e.g.*, pharmaceuticals) are of interest. The best results are obtained for species that are highly absorbing and have high specific rotations. Phenylthiohydantoin derivatives of amino acids are frequently used for reversed-phase separation followed by UV detection[19,20]. This procedure renders all amino acids UV detectable and makes OA/UV a viable technique for the determination of enantiomeric ratios of all amino acids. An added bonus is that these derivatives all show a larger [α] than the amino acids themselves to further enhance S/N. It can also be expected that the other common derivatization methods for amino acids (*e.g.*, dansyl or *o*-phthalaldehyde derivatives) will enhance detection for both OA and UV to extend the scope of optical purity determinations. Naturally, the important consideration is whether racemization occurs in the derivatization reaction.

In summary, we have evaluated two dual detector schemes for determining enantiomeric ratios without derivatization and without chiral separation. The recent improvements in LOD in the micropolarimeter allowed an improvement of a factor of 10–100 over previous attempts.

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