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Electrokinetic Separation of Chiral Compounds

Abstract. *Femtomole amounts of racemic mixtures of derivatized amino acids were resolved and analyzed rapidly in about 10 minutes by means of high-voltage zone electrophoresis with laser-fluorescence detection. The electrophoresis was performed in capillary columns containing a chiral support electrolyte. A number of dansyl amino acids were resolved by the diastereomeric interaction between the* DL-*amino acid and the copper(II) complex of* L-*histidine present in the support electrolyte. A combination of electro-osmotic and electrophoretic action caused all species, positively charged, neutral, and negatively charged, to pass through the 0.5-nanoliter detection volume where they were subjected to laser excitation.*

E. Gassmann
J. E. Kuo
R. N. Zare
Department of Chemistry,
Stanford University,
Stanford, California 94305

As first pointed out by Pasteur (*1*), one approach to the resolution of a racemic mixture into its component enantiomers is the formation of diastereomeric pairs by the addition of some chiral agent, since diastereomers may have widely different physical properties. This approach is the basis for chromatographic separations of enantiomers by means of chiral stationary or mobile phases (*2*). We report an improvement in this technique in which rapid resolution of a mixture of optical isomers is achieved with unprecedented sensitivity. The improvement results from the combination of high-voltage electrophoresis in a capillary column containing a chiral support electrolyte with laser fluorescence detection. By this method we have resolved in less than 10 minutes a number of DL-amino acids that have been labeled with the highly fluorescent dansyl group.

The use of optically active copper(II) complexes for the chromatographic resolution of enantiomeric amino acids was first demonstrated by Davankov and his colleagues (*3*). For this purpose they immobilized L-proline on resins onto which Cu(II) metal ions were also loaded. Later it was shown by Hare and Gil-Av (*4*) as well as by Karger and coworkers (*5*) that optically active chelate additives in the mobile phase of high-pressure liquid chromatography (HPLC) resolved racemic mixtures into their constituent enantiomers. The optically active Cu(II) L-histidine complex used in our work was introduced by Lam *et al.* (*6*) for the separation of DL-dansyl amino acids by means of reversed-phase HPLC.

An alternative to liquid chromatography is high-voltage zone electrophoresis in capillary columns. As pointed out by Jorgensen *et al.* (*7*) and Terabe *et al.* (*8*), this new analytical tool combines high theoretical plate numbers—on the order of 1 million—with short analysis times.

We used a fused-silica capillary column (a gift from Hewlett-Packard) that was 75 cm in length and had a 75-μm inner diameter. The applied electric field was 300 V/cm, and the support electrolyte contained 5 m*M* L-histidine, 2.5 m*M* $CuSO_4 \cdot 5H_2O$, and 10 m*M* ammonium acetate adjusted to *p*H 7 to 8 by the addition of NH_4OH. The measured current was 30 to 33 μA.

The formation of a double layer on the inside surface of the capillary caused an electro-osmotic flow. Under our experimental conditions, the electrolyte solution moved toward the cathode. Therefore, cations, neutral species, and anions injected at the anode end of the column could be detected at the cathode end in a single run. The analysis was accomplished with an on-column fluorescence detector that had a helium-cadmium laser set at 325 nm (5 mW) as an excitation source. The filtered output of the laser was focused on an optical fiber that carried the excitation light to the on-column flow cell, which had a volume of ~0.5 nanoliter. The resulting fluorescence was collected at right angles to both the excitation direction and the capillary by a second optical fiber that led to a combination of a fast monochromator and a photomultiplier. The dansylated amino acids were either purchased (Sigma) or prepared by known methods (*9*).

Table 1. Migration times (t_D, t_L), Δt values (Eq. 1), and relative peak areas (A_D, A_L) for some DL-dansyl amino acids, measured under the conditions explained in the text, at *p*H 8.0. Abbreviation: DNS, dansyl.

Amino acid	t_D (minutes)	t_L (minutes)	Δt (× 100)	A_D	A_L
di-DNS-Tyr	6.30	6.36	−0.95	1.5	1.8
DNS-Met	6.75	6.71	0.63	1.6	1.6
DNS-αAB*	6.83	6.75	1.2	1.3	1.0
DNS-Phe	6.80	6.91	−1.6	0.18	0.36
DNS-Ser	7.0	7.0	0.0	0.46	
DNS-Val	7.40	7.32	1.1	2.1	1.8
di-DNS-cystine	7.90	8.00	−1.3	0.37	0.39
DNS-Asp	9.80	9.95	−1.5	0.18	0.24
DNS-Glu	10.30	10.10	1.9	1.71	1.38
*DNS-cysteic acid†	10.40	10.70	−2.9	0.15	0.29

*N-Dansyl-α-aminobutyric acid. †N-Dansyl-3-sulfoalanine.

Initially, the capillary was filled by syringe with the support electrolyte. Sample injection was accomplished by first dipping the anode end of the capillary into a small beaker containing the solutes ($\sim 10^{-4} M$) dissolved in the electrolyte solution and then turning on the voltage for a short period of time, typically 5 to 10 seconds at 6 kV. Finally, the anode end of the capillary was placed in a beaker containing the support electrolyte to start the electrokinetic separation. After each run the support electrolyte was renewed.

Figure 1a shows the separation and resolution of a 1:1 mixture of D- and L-dansyl amino acids. The excellent signal-to-noise ratio indicates that dansyl amino acids can be detected at femtomole levels by the present simple experimental arrangement. Baseline resolution is possible when the absolute magnitude of the quantity

$$\Delta t = (t_D - t_L) / [\tfrac{1}{2}(t_D + t_L)] \quad (1)$$

exceeds 0.01, where t_D and t_L are the migration times of the D and L optical isomers. Replacement of L-histidine by D-histidine in the support electrolyte reverses the migration order of the DL-amino acids, that is, changes the sign of Δt. And when a 1:1 mixture of D- and L-histidine is used, no resolution of the D- and L-amino acids is observed (Fig. 1b). Figure 1a also shows that the fluorescence signals differ for the two optical isomers of the same dansyl amino acid.

We have listed in Table 1 the migration times, Δt values, and relative peak areas (referred to L-arginine as an internal standard) for ten DL-dansyl amino acids under slightly different conditions than in Fig. 1a. In all cases except DL-serine, resolution is achieved, but the sign of Δt varies with the amino acid. Although the migration order of the amino acids in our work differs from that found in HPLC (*6*), the migration order of the enantiomers—the sign of Δt—is the same. HPLC has distinctly superior resolution with Δt values an order of magnitude larger than in the present work. The detection limit of the present technique, however, is much lower.

Chiral recognition with the Cu(II) L-histidine support electrolyte can be explained by mixed chelate complexation to form two diastereomeric, ternary complexes (*10–12*). Amino acids bound to Cu(II) L-histidine migrate faster than free amino acids because the Cu(II) L-histidine carries positive charge under our *p*H conditions. However, amino acids bound more strongly to Cu(II) L-histidine show a weaker fluorescence signal, which is the result of quenching from association with the copper ion. Thus, the more strongly bound enantiomer migrates faster but shows a lower fluorescence signal (Fig. 1a and Table 1). Complex formation constants have been determined by Brookes and Pettit (*13*) for Cu(II) DL-histidine with various L-amino acids, and our conclusions are in agreement with their findings for those cases where comparisons can be made. Differences in the formation and dissociation rates of the ternary diastereomer pairs with Cu(II) L-histidine thus appear to play a crucial role in effecting resolution. When Cu(II) is replaced by Co(II), no resolution of D and L isomers results, which appears to be a consequence of the fact that Co(II) forms kinetically inert (stable) ternary complexes (*3*).

At present there is much interest in resolving and quantitating DL-amino acids for geological dating (*14,15*) and in the diagnosis of certain diseases (*11,15*). For these purposes, additional work will be required to establish analytical protocols that make use of the present electrokinetic separation method. However, the present results suggest that this technique may find wide application in the resolution of racemic mixtures.

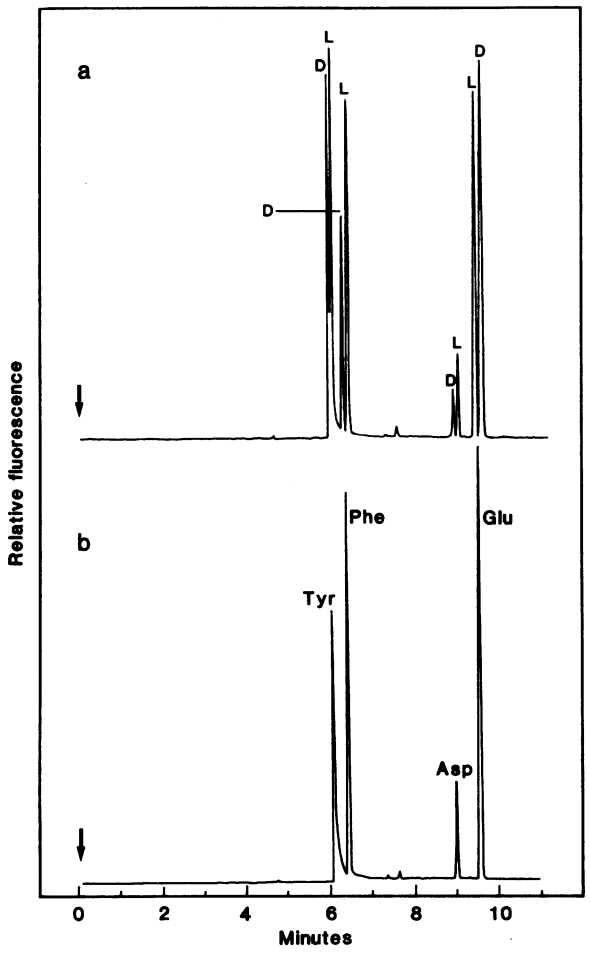

Fig. 1. Electropherograms of DL-dansyl amino acids with (a) Cu(II) L-histidine electrolyte at *p*H 7.0 and (b) 1:1 Cu(II) D- and L-histidine electrolyte at *p*H 7.0. The concentration of each amino acid is approximately $10^{-4} M$. For other conditions refer to the text. The relative standard deviation (R.S.D.) for migration times is less than 0.03, and the R.S.D. for relative peak areas is 0.05.

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