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Enantiomeric resolution of amino acid derivatives on molecularly imprinted polymers as monitored by potentiometric measurements

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ABSTRACT

Potentiometric measurements have been applied to the detection of enantiomeric separations on molecularly imprinted polymers. A flow-through column electrode, based on the use of polymers imprinted against L-phenylalanine anilide, is described. The electrode consisted of a glass column in which the polymer was packed and where the end frits constituted the electrodes. The flow stream potential across the column can be continuously recorded as solvent is pumped through the system. The column resolved the enantiomers of phenylalanine anilide as detected by both UV absorption and potentiometric measurements and the recorded signals could be correlated with the concentration of phenylalanine anilide. The calibration graphs obtained for the UV absorption of phenylalanine anilide were linear over the concentration range investigated, whereas the potentiometric signal was shown to be exponentially linear with concentration. The application of molecular imprints to the preparation of supports suitable for chromatographic separations of enantiomers and for the preparation of specific electrodes is discussed.

INTRODUCTION

Molecular imprinting, which is a technique for preparing synthetic polymers containing specific recognition sites and is particularly useful for enantiomeric separations, is gaining increasing acceptance and has been reviewed recently [1,2]. In principle the method involves (a) mixing the print molecule with selected monomers, chosen for their ability to interact in a specific manner with the print molecule, (b)

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polymerization in the presence of a suitable cross-linker and (c) removal of the print molecule from the resulting polymer. The result is a polymer possessing a "memory" for the print molecule. As molecularly imprinted polymers have the ability to bind the print molecule selectively in the presence of molecules of like structure, including the optical antipode, supports exhibiting excellent enantiomeric resolving capabilities have been reorted, especially for the separation of amino acid derivatives [2–4]. In many applications of chromatography, it is important not only to separate substances but also to determine them. In this paper, we demonstrate for the first time the use of molecularly imprinted polymers for quantitative analysis. In addition, potentiometry has been applied to molecular imprints.

Two detection systems were used: (i) UV absorption, which is the most common detection method in high-performance liquid chromatographic (HPLC) analyses, and (ii) potentiometric measurements. The latter detection technique is very interesting because it is a relatively new technique, simple to use and relies on a different mode of detection to UV absorption. Streaming potential measurements are based on recording the potential across a packed bed of, for example, a chromatographic material, placed in a continuous flow. The measurement of streaming potential can be regarded as a general method for recording binding reactions, as long as they involve a change in charge distribution on the surface at which they occur (see below). Several applications based on flow stream potential measurements have been reported, including quantification of the binding of proteins to ligands or antibodies on solid supports [5,6], flow-rate measurements [7] and in the construction of an HPLC detector [8].

We describe here the application of a flow-through column electrode for the separation and detection of small molecules in organic solvents. A polymer prepared against L-phenylalanine anilide, using methacrylic acid as the functional monomer, was used as this system has been well documented [3,4]. Polymer particles were packed into a glass column where the end frits were constructed as electrodes and connected directly to an electrometer. Solvent was pumped through the "electrode" and the flow stream potential was continuously recorded. The enantiomeric separation of a racemic mixture of phenylalanine anilide was detected by both UV absorption and potentiometric measurements and could be correlated with the concentration of solute.

THEORY

When a solid surface is brought into contact with a polar (most often aqueous) medium, an electric double layer is created. Ionization, ion adsorption and ion dissolution generate a net charge at the surface which attracts ions of the opposite charge from the bulk solvent. Also, the presence of a layer of oriented dipolar molecules at the surface may make a significant contribution to the electric double layer, especially in non-aqueous media. If the liquid phase surrounding the solid is forced to flow relative to the solid surface, a streaming potential will be developed. The flow of liquid carries a net charge, from the mobile portion of the electric double layer, which gives rise to a streaming current. A potential difference is thus created which causes a back flux of charge through ion migration, leak current. The measured streaming potential relates to an equilibrium condition when the streaming current and leak current cancel each other. When a liquid is forced through a porous "plug",

for example a column, the streaming potential measured between the ends of the plug, E_s, can be approximately described by the equation

$$E_s = \frac{\varepsilon p \zeta}{\eta \left(k_0 + \frac{2k_s}{a} \right)}$$

where ε is the permittivity, p is the pressure difference applied to force the flow of the liquid, η is the viscosity of the solvent, k_0 is the conductivity of the solvent, k_s is the surface conductivity and a is a geometrical parameter related to the average pore radius. By changing the solvent from aqueous to organic, thereby decreasing the conductivity, an increased streaming potential is expected. In aqueous media, k_s/a is in most instances considered to be negligible and the conductivity term is simplified to be equal to k_0. ζ is the zeta potential, whose electrokinetic behaviour depends on the potential at the surface of shear between the charged surface and the bulk solvent. Adsorption of solute on the solid surface may lead to a change in the potential ζ, therefore streaming potential measurements can be used to record affinity interactions between solute and solid support. A more detailed analysis of the described phenomena is given in ref. 9.

EXPERIMENTAL

Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) were obtained from Aldrich Chemie (Steinheim, F.R.G.) and 2,2'-azobis(2-methylpropionitrile) (AIBN) from Janssen Chemica (Beerse, Belgium). D- and L-phenylalanine anilides were synthesized as described [3]. D- and L-leucine amides and D- and L-alanine amides were obtained from Nova Biochem (Läufelfingen, Switzerland). All solvents used were of the highest available grade. HPLC analyses were performed with an LKB (Bromma, Sweden) system consisting of a Model 2152 HPLC controller, two Model 2150 HPLC pumps and a Model 2151 variable-wavelength monitor.

Polymer preparation

The polymers were prepared as described [3] with EDMA as cross-linker and MAA as functional monomer. The crosslinker:monomer:print molecule molar ratio was 20:4:1. Polymerization in chloroform was initiated by AIBN and UV light at 0°C. The resulting bulk polymer was ground and sieved to particles of less than 25 μm.

Flow-through column electrode construction

The flow-through column electrode, shown schematically in Fig. 1, was constructed in a similar manner to one described previously [5,6]. The column consisted of a glass tube (50 mm × 5 mm I.D.) with PTFE adapters holding the nets of the electrodes in place. The electrodes were connected to a Keithley Instruments (Cleveland, OH, U.S.A.) Model 610 C solid-state electrometer. The nets (made of stainless steel) were welded to acid-proof stainless steel wires (diameter 0.3 mm) and the welding points were sealed with epoxy glue. All parts were carefully designed to be resistant to organic solvents. The solvent was pumped though the column at a constant flow-rate of 0.1 ml/min and a back-pressure of 15 bar.

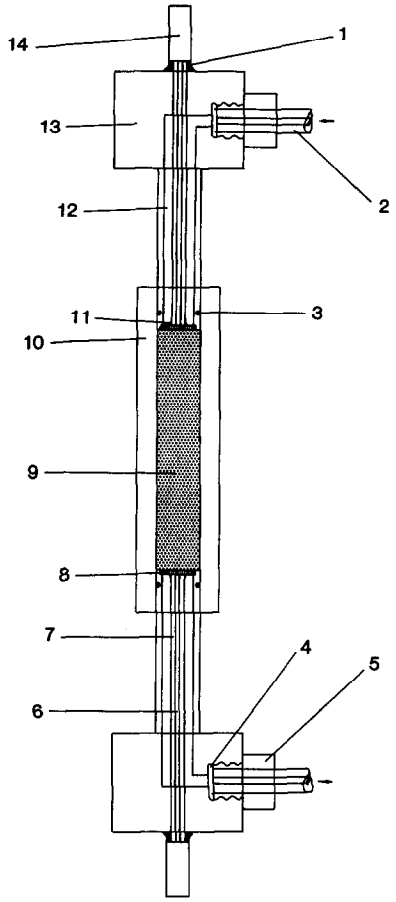

Fig. 1. Schematic diagram of the flow-through column electrode. 1 = Epoxy seal; 2 = PTFE tube; 3 = O-ring; 4 = rubber tube seal; 5 = PTFE tube support; 6 = stainless-steel wire (diameter 0.25 mm); 7 = PTFE tube; 8 = stainless-steel net electrode; 9 = imprinted polymer particles; 10 = glass tube 50 mm × 5 mm I.D., 3.5-mm thickness; 11 = epoxy seal; 12 = sample flow dome (diameter 3 mm); 13 = PTFE electrode support; 14 = lead wire contact.

Chromatography

The particles were dry-packed into the flow-through column electrode. The column was then washed on-line with acetonitrile–acetic acid (9:1, v/v) until a stable baseline was obtained. HPLC analyses were performed isocratically with acetonitrile–acetic acid (9:1, v/v) at a flow-rate of 0.1 ml/min and detection at 250 nm. Weighed samples were dissolved in the mobile phase and diluted to the appropriate concentration before injection in a total volume of 30 μl.

RESULTS AND DISCUSSION

The solvent was pumped at constant flow-rate using an HPLC pump via the injector into the column. The flow stream potential across the column, between the net

electrodes (see Fig. 1), was recorded continuously by the electrometer. The column effluent also passed through a UV detector. As the flow stream potential is linearely proportional to the flow-rate [7], it is extremely important to maintain as constant a flow as possible. Early attempts at potentiometric measurements using a peristaltic pump resulted in severe noise and baseline drift. Using a high quality HPLC pump it was possible to obtain a stable baseline, but the piston strokes of the pump were still detected at regular interval in the baseline.

A polymer imprinted against L-phenylalanine anilide was used as the performance of this polymer has been well described [3]. Racemic mixtures and also the pure enantiomers of phenylalanine anilide were applied to the column via the injection valve. Fig. 2 shows typical chromatograms obtained for this system and shows both UV recordings and the potentiometric recordings (note the different time scales). As potentiometric detection is performed across the column, and not post-column as in UV detection, the potentiometric signal appears earlier than the UV signal but the capacity factors (k') for the peaks obtained were the same for both detection methods (see Fig. 2C–F). The peaks obtained with the potentiometric detector were broader than those for the UV detector, owing to the longer residence time in the detector cell, *e.g.*, the column. There is a small shift in the capacity factor for the L-form in the mixture compared with the pure enantiomer (see Fig. 2A–D), the cause of which is unknown. Injection of solvent alone gave no peak in either mode of detection. In agreement with previous findings [3], the enantiomers of phenylalanine anilide were resolved on this column, as shown by both detection methods (see Fig. 2A and B). It must be stressed that as the potentiometric signal is exponentially proportional to the concentration (see below), the chromatograms recorded with the potentiometric detector become distorted (flattened), and the resolution may appear to be worse than it truly is. Earlier reports showed that a polymer prepared against L-phenylalanine anilide was able to resolve the enantiomers of a number of amino acid amides and

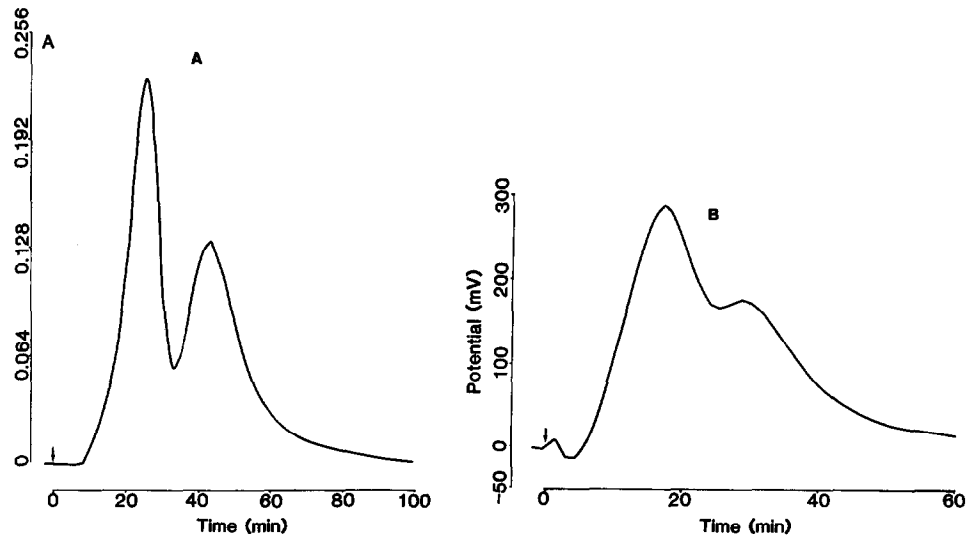

Fig. 2.

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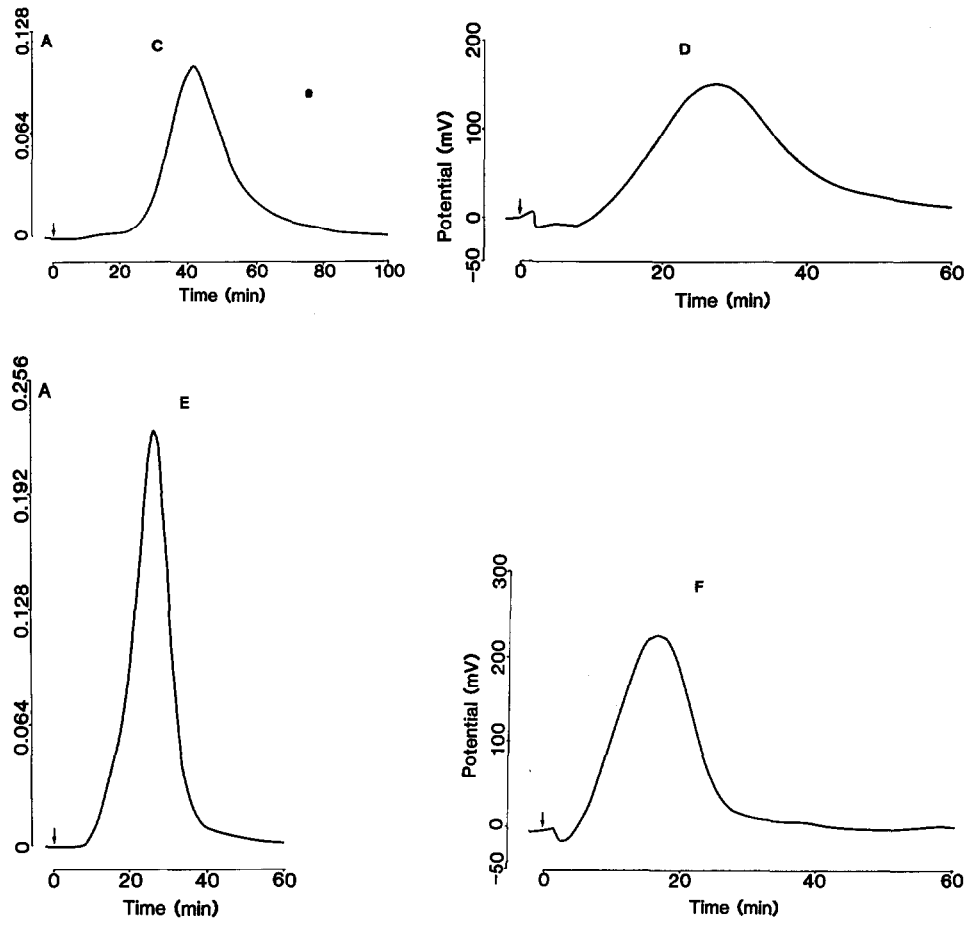

Fig. 2. Representative elution profiles of phenylalanine anilide in the flow-through column electrode. Note the different time scales. Particles of <25 μm, prepared from a polymer imprinted against L-phenylalanine anilide were dry-packed into a 50 mm × 5 mm I.D. glass column. Analyses were performed under isocratic conditons using acetonitrile–acetic acid (9:1, v/v) as the eluent at a flow-rate of 0.1 ml/min (15 bar). Samples consisted of a mixture of 15 μg of each of the enantiomers (A and B), 15 μg of the L-enantiomer (C and D) or 15 μg of the D-enantiomer (E and F). UV detection (A, C and E) was at 250 nm. The potentiometric recordings (B, D and F) were drawn by connecting the peaks of the noise, which were constant both in frequency and amplitude. The void volumes, calculated using acetic acid, were 1.11 ml on the UV recordings and 0.725 ml on the potentiometric recordings. Capacity factors, k', and peak widths at half peak height, $t_{1/2}$, were calculated to be (A) $k'_L = 2.86$ and $k'_D = 1.32$; (B) $k'_L = 2.93$ and $k'_D = 1.34$; (C) $k'_L = 2.73$, $t_{1/2} = 1.74$ ml (D) $k'_L = 2.79$, $t_{\frac{1}{2}} = 1.90$ ml; (E) $k'_D = 1.35$, $t_{\frac{1}{2}} = 1.02$ ml; (F) $k'_D = 1.31$, $t_{1/2} = 1.35$ ml. Separation factors, α, were calculated to be (A) 2.17 and (B) 2.19.

dipeptides [10] and a mechanism for the molecular recognition by these polymers was proposed based on the reported findings [10,11]. The detection of simple amino acid amides was not possible because of the absence of suitable UV absorbance. With the present potentiometric detection system, such analyses were possible. Racemic mixtures of leucine amide and alanine amide were therefore applied to the column and

followed potentiometrically. However, no enantiomeric resolution of these simple amides was observed. It is worth noting that separate injections of the pure enantiomers of these amides were clearly detectable and produced distinct peak maxima (leucine amide, $k'_L = 0.34$, $k'_D = 0.17$; alanine amide, $k'_L = 0.28$, $k'_D = 0.10$).

Known concentrations of the pure D- and L-enantiomers of phenylalanine anilide were applied to the column and UV and potentiometric signals were recorded. The calibration graphs obtained from these analyses are shown in Fig. 3. The UV signals were linear over the concentration range measured (1–100 μg) whereas the potentiometric signals were shown to be exponentially proportional to concentration. In potentiometric detection, irreversible "immobilization" or binding of the analyte to the solid support gives rise to a linear relationship between the signal obtained and the concentration applied. This relationship is exemplified by recording the change in streaming potential as protein is adsorbed on an affinity column [5,6]. The present system is in a state of dynamic equilibrium, where the binding of the analyte to the affinity support is reversible, in which case the potentiometric signal may not necessarily be linear with respect to concentration. Also, the effect(s) of the analyte in the moving liquid on the electric double layer, at the surface of the solid support, have to be considered. Consequently, the potentiometric signal will not bear a simple relationship to concentration but, as in the present instance, may be exponentially proportional to concentration. This is in agreement with other reports [8,12].

The signal response is also intimately related to the shape of the peaks observed in the present system, which indeed differ from those described previously for systems in which the analyte was irreversibly bound to the support. In the irreversible system, when analyte was applied to the column a stepwise change in the potential was recorded, which was constant until another aliquot of analyte was applied [5,6]. Introduction of analyte onto the column in the present system gave rise to a peak in the potentiometric signal (not a square peak as in the irreversible system), which then returned to the baseline. This may be attributed to a number of effects, of which only a few are well documented in the literature [9]. Further studies need to be performed before the characteristics of the potentiometric detector in organic media can be fully evaluated. As can be seen in Fig. 3B, the slope of the calibration graph for L-phenylalanine anilide is greater than that for D-phenylalanine anilide in potentiometric measurements. As the flow stream potential is a surface phenomenon, the concentration of the L-form will have a stronger influence on the signal than for the D-form, because the L-form has a higher affinity for the polymer. The lower limit of linearity of the potentiometric signal was calculated to be 10^{-5} g, but it must be stressed that as the system was not fully optimized this by no means reflects the true detection limit of this system.

The results presented above clearly show the utility of potentiometric detection in organic solvents, as applied to separations on molecularly imprinted polymers. Potentiometric detection systems should therefore be considered as an alternative or adjunct to the more common systems such as UV detectors, refractometers and polarimeters. The successful detection of phenylalanine anilide (and other compounds) in the flow-through column electrode suggests that molecularly imprinted polymers may be useful in the preparation of substrate-selective electrodes. By combining the general detection principle of potentiometric measurements and the selectivity achievable with molecular imprinting it may be possible to develop such an

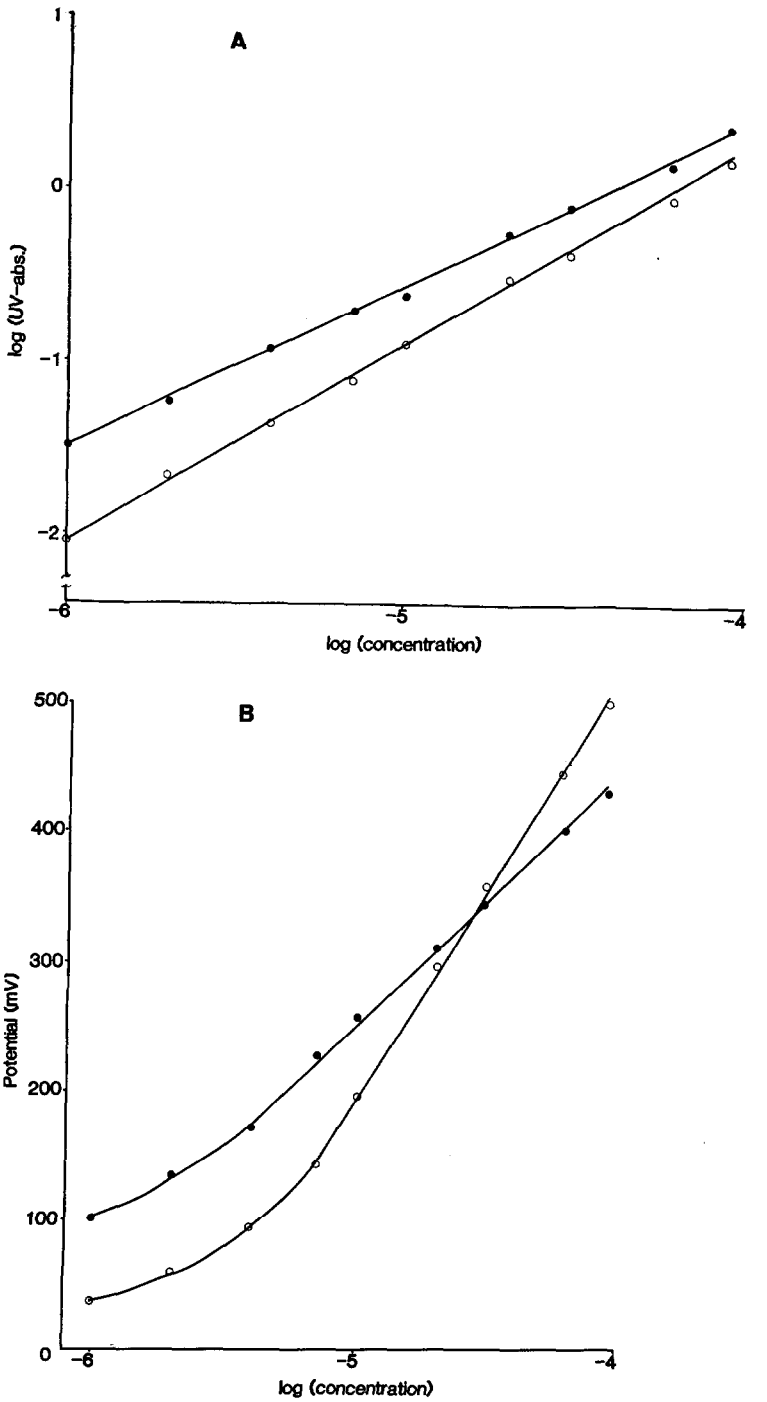

Fig. 3. Calibration graphs for (A) UV detection and (B) potentiometric detection. Analyses were performed with acetonitrile–acetic acid (9:1, v/v) as the eluent at 0.1 ml/min (15 bar). Weighed samples of (●) D- and (○) L-phenylalanine anilides were dissolved in the mobile phase and diluted to the appropriate concentration (1–100 μg) before injection in a total volume of 30 μl. UV detection was at 250 nm.

analytical device. Such electrodes may be prepared in the conventional format, as a miniature device or as an integrated system, where the active surface of the electrode is coated with the selective polymer.

It is apparent from the results presented that molecularly imprinted polymers can be used for the separation and quantification of amino acid derivatives. Although the resolution of the enantiomers of phenylalanine anilide was reasonable ($\alpha = 2.2$), peak broadening was evident. This has been described previously for molecular imprints and may result from the poor packing characteristics of the extremely irregular and poorly defined particles [10]. The preparation of beaded polymers, or more uniform particles, may improve the peak shapes obtained and therefore the resolution. Also, polymers obtained using print molecules allowing multiple interactions may lead to improved separations.

In conclusion, we have reported the use of potentiometric measurements in organic solvents as applied to enantiomeric separations of amino acid amides on molecularly imprinted polymers. Potentiometry measures changes in the charge distribution on the surface of the solid support and can therefore be used to show an affinity binding event, either reversible or irreversible in nature. The demonstration that the binding of compounds to these highly specific molecularly imprinted polymers can be followed by potentiometry, or UV analysis, suggests that it may be possible to prepare substrate-specific electrodes using the technique of molecular imprinting. Moreover, such polymers may be useful in the quantitative analysis of amino acid derivatives or other organic compounds.

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