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Articles

A Mass Spectrometry Method for the Determination of Enantiomeric Excess in Mixtures of D,L-Amino Acids

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A mass spectrometry method based on ion/molecule reactions for determining enantiomeric excess in mixtures of amino acids is illustrated. Protonated complexes of amino acids with *β*-cyclodextrin are produced in the gas phase using electrospray ionization. Under a background pressure of *N*-propylamine, the amino acid is replaced by the alkylamine. The rates of the guest exchange reaction vary with the chirality of the amino acid. A calibration curve employing varying mixtures of D- and L-amino acids is produced and the fractions of the D isomer in the test mixtures are determined. The quality of the curve is compared to the chiral selectivity of the reaction. In general, the best calibration curves are obtained in systems with the greatest selectivity.

Analytical methods for chiral differentiation are of immediate and general importance. Until now, separation science has led the way in the development of enantiospecific analytical tools. The most common methods employ cyclodextrins in conjunction with high-performance liquid chromatography (HPLC) to separate and quantify enantiomeric mixtures.[1−4] Similar systems have been extended to capillary electrophoresis (CE).[5] The small column dimensions and the fast time scale of the separation makes CE an extremely attractive method for chiral separation.[6−9]

Although a general method to analyze mixtures of small organic compounds based solely on mass spectrometry is not available, it does have several advantages and may complement the separation techniques. Analysis with mass spectrometry is rapid and provides structural as well as chemical information. Its detection limit is similar to those found in separation science. Mass spectrometry has the potential to achieve even higher detection limits, to the attomolar range, with ionization methods such as nanospray.[10] The current arsenal of ionization sources available in mass spectrometry also allows the analysis of nearly every type of compound. More importantly, mass spectrometry is potentially faster than chemical separations, which would be important in situations where a large number of samples must be screened as in, for example, combinatorial libraries.

There are several methods where mass spectrometry is used to determine enantiomeric excess (ee). The more traditional use of mass spectrometry is as an in-line detector for gas chromatography where the chiral separation is performed.[4,11−19] Similarly, liquid chromatography has been used with electrospray ionization to perform the chiral separation and detect the eluant with mass spectrometry.[11,13−16] More recently, mass spectrometry has been used to analyze products that are kinetically resolved by standard enantiomerically selective organic reactions.[20,21]

Methods that employ mass spectrometry specifically for enantiomeric analyses also employ a host molecule to complex with the analyte guest. The resulting complexes are diastereomeric with unique ionization efficiencies. The differences in ionization efficiencies in turn produce unique abundances that can be normalized to provide enantiomeric excess. Sawada and coworkers have used this method extensively with fast atom bombardment (FAB).[22−26] The analysis takes advantage of the excess energy during FAB ionization to produce metastable

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dissociation of the respective diastereomeric complexes. The rates of metastable decay differ among complexes to yield different ion intensities. The group has also synthesized a large number of different selector (host) molecules to analyze different types of chiral compounds.

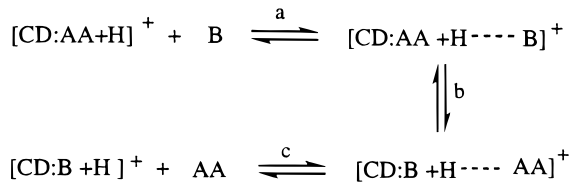
Tandem mass spectrometry has also been used to differentiate chiral compounds by forming diastereomers that have unique rates of dissociation. Sawada and co-workers extended their studies to host−guest complexes of crown ethers and some amino acid esters produced by electrospray ionization. The voltage differential between the nozzle and skimmer was used to dissociate the complex.[27] Leary and co-workers have used tandem MS to observe chiral recognition in diastereomeric lithium and cobalt complexes.[28,29] More recently, Vekéy and Czira investigated heterotrimers of amino acids and found the ratio of products depended on the chirality of the amino acids.[30] Cooks and co-workers have produced copper(II) complexes of amino acids and used competitive dissociation to determine enantiomeric excess.[31]

Chiral differentiation using gas-phase ion/molecule reactions have also been reported. Several groups have observed chiral specificity in protonated dimers and even trimers of tartrates. One of the earliest studies was reported by Fales on protonated dialkyltartrate dimers.[32] Other studies of the same or similar systems have followed, primarily by Nikolaev.[33,34] Chiral selectivity in the dimerization of host−guest complexes has been reported by Dearden.[35,36] Chiral specificity has been observed in this laboratory, as described earlier, in the deprotonation reactions of gas-phase cytochrome *c* ions.[37,38]

We recently reported a novel gas-phase guest exchange reaction involving complexes composed of cyclodextrins and protonated amino acids reacting with neutral alkylamines.[39] The amino acid (AA) is displaced by the amine (B) in a guest exchange reaction to produce a new cyclodextrin−amine complex [CD−B+H]$^+$ (Scheme 1). More importantly, we observe chiral selectivity as the magnitude of the rate constants varies according to the chirality (L or D) of the amino acids.

In this report, we illustrate the analytical application of chiral guest exchange reaction with various amino acids. Because the exchange reaction can be performed with all amino acids and other amine-containing compounds, the method is relatively general. Furthermore, it takes advantage of the speed and the detection limit of mass spectrometry.

Scheme 1. Proposed Mechanism for the Exchange Reaction



EXPERIMENTAL SECTION

Electrospray Analysis of the Complex. For the chiral exchange reactions, two different hosts were used: permethylated cyclodextrin (heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin or CD), purchased from Sigma Chemical Co. (St. Louis, MO) and permethylated maltoheptaose, synthesized via the method given by Ciucanu and Kerek.[40] The analyte guests used included the following amino acids: Ala, Val, Leu, Ile, and Phe, purchased from Sigma Chemical Co. and used without further purification. The alkylamine (B) used was *N*-propylamine, purchased from Aldrich Chemical Co. (Milwaukee, WI).

All experiments were performed on a home-built electrospray FTMS instrument described elsewhere.[41,42] The CD host solution was prepared by dissolving the methylated cyclodextrin in a water/methanol (50:50) solution, obtaining a concentration of 1 $\times$ 10^{-6} M. The concentration of maltoheptaose is approximately the same as that of CD. Analyte solutions were made by dissolving the amino acid in water/methanol (1 $\times$ 10^{-6} M). The electrospray solution was made by mixing a 100:1 ratio of analyte to host and was pumped at a rate of 15 μL/h. The electrospray needle was made of 50 μm fused-silica capillary. It was charged to ~2.5 kV to produce charged droplets that drifted downfield into the heated capillary. The resulting desolvated ions were transported into the analyzer cell of the FTMS by a single-stage quadrupole guide. The alkylamine was purified in the vacuum manifold with several freeze−thaw cycles. It was then introduced into the analyzer chamber, allowing the pressure to become stable. Depending on the amino acid analyte, the alkylamine pressure was maintained between 2 and 5 $\times$ 10^{-7} Torr.

The [CD:AA+H]$^+$ is observed in large abundance along with CD coordinated to Na^+ and K^+—retained in the mass spectrum for use as internal standards—and some product [CD:B+H]$^+$ which is entirely ejected at the beginning of the experiment. Identical conditions are employed for the enantiomeric pairs.

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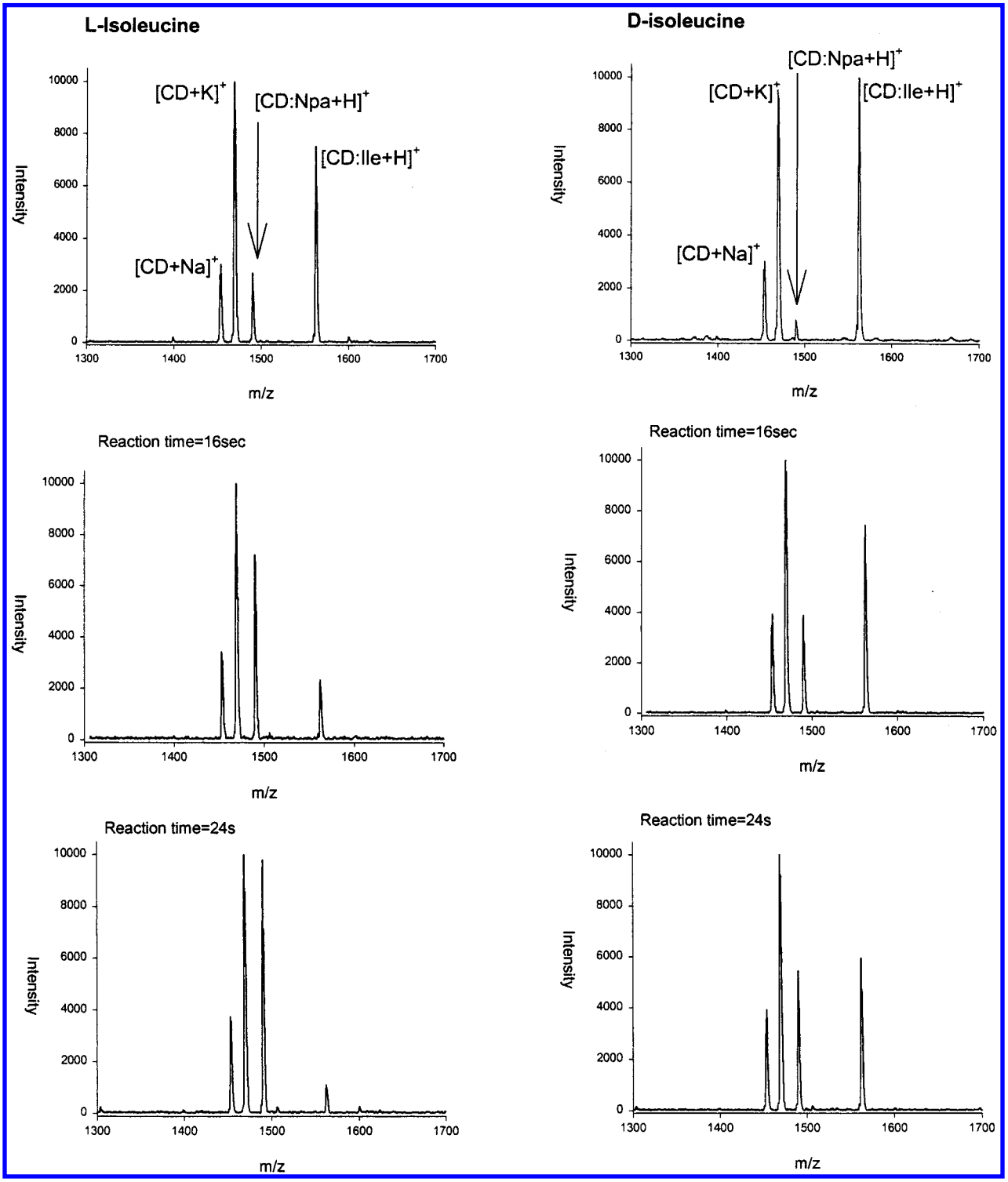

Figure 1. FTMS spectra of enantiomers of protonated isoleucine complexed to β-cyclodextrin (CD) and allowed to react with *n*-propylamine (Npa, 3.4 $\times$ 10^{-7} Torr) for 5 s. The L enantiomer is more reactive than the D as illustrated by the intensity of the product peak $[CD:Npa+H]^+$ relative to the reactant peak $[CD:Ile+H]^+$.

Preparation of the Calibration Curve. The calibration curve was prepared by directly comparing the relative peak heights of the standard (or known) and "unknown" mixtures. The steps involved follow: (1) The proper conditions for the exchange reaction are chosen. The conditions include the selection of a proper alkylamine reagent, its pressure in the analyzer chamber, and a reaction time that provides the optimum between the shortest analysis time and largest difference in relative intensities. (2) At a chosen reaction time, various known enantiomeric mixtures are electrosprayed into the FTMS. To construct one calibration curve, six or seven analyte solutions are made including the pure D form (0:100) and the pure L form (100:0).

Between each mixture analysis, the electrospray syringe setup is flushed with methanol for several minutes and the capillary needle is discarded to ensure that there is no memory effect carried onto the next mixture. Inside the analyzer chamber, the

alkylamine pressure is kept constant. Using linear regression, a calibration curve is obtained by plotting the ratio of the intensity of the product (I) and the sum of the product and reactant intensities (I_0) versus mole fraction of D. The equation that fits the calibration curve is determined. Unknown enantiomeric mixtures are analyzed at the same reaction time that the calibration curve was made. The analysis of "unknown" mixtures can take place even several months after the curve was made and at an alkylamine pressure that differs from the one used in making the calibration curve.

RESULTS AND DISCUSSION

The nature of chiral selectivity will be the subject of future reports, but a complete understanding is not crucial to the analytical application of the guest exchange reaction. The gas-phase complex is readily formed from solution but whether the gas-phase structure is akin to the solution phase, which is known to involve inclusion, is not known.[43−47] We will provide in an upcoming report evidence that supports gas-phase inclusion and that chiral selectivity is a consequence of gas-phase inclusion complexes.[48]

In this report, we chose several systems whose chiral selectivity (defined as the ratio of the rate constants k_L/k_D) varies between 1 and 5. A method for determining enantiomeric excess needs to be fast and to require only small amounts of material. We explored at least two methods for determining enantiomeric excess (or rather a fraction of D isomer) while employing the guest exchange reaction. One method is to obtain apparent rate constants and deconvolution to individual rate constants. This method was dismissed for several reasons. It requires a large amount of material and a considerable amount of time to construct the rate curves. Any advantages that mass spectrometry offers would be lost by having to perform the gas-phase reactions at various reaction times. It is also difficult to deconvolute exponential functions particularly when the rate constants are similar.

A more convenient method is to fix the reaction time and simply compare the height of the reactant and the product peaks. Once the calibration curve has been produced, the analyses would require only a single spectrum to determine the unknown mixture.

Figure 1 shows the mass spectra of L- and D-isoleucine complexed to β-CD and reacted with *n*-propylamine (3.4×10^{-7} Torr) for reaction periods of 5, 16, and 24 s. The reactant complexes $[CD:Ile+H]^+$ are composed of a protonated amino acid and cyclodextrin. Their intensities decrease with time while the intensities of the respective product ions $[CD:Npa+H]^+$ increase. In both series, nonreacting, alkali metal-coordinated species $[CD+K]^+$ and $[CD+Na]^+$ are retained to show that the reactant complexes are converted directly to the product complexes. Note that the L isomer reacts faster than the D isomer. With 24 s of reaction time, the product of the L is ~80% of the total complex intensities, while for the D the reactant is ~50% of the total complex intensities. From the rate plots (not shown), the rate constants are obtained using standard methods and an average selectivity (k_L/k_D) of 3.8 is obtained for isoleucine.

Table 1. Reaction Selectivity (Defined by the Ratio k_L/k_D) of Various Amino Acid Complexes Reacting with *n*-Propylamine and Ethylenediamine (Italics)[a]

amino acid	β-CD	Mhep	amino acid	β-CD	Mhep
Ala	1.6[a]	1.1[c]	Met	0.37 (2.70)	
Asn	*0.93*		Phe	0.82[a]	4.6[c]
Asp	2.2[c]		Pro	1.5	
Cys	2.2		Ser	1.2[c]	
Glu	1.9[c]		Thr	0.63 (1.6)[b]	
His	*2.3*		Trp	*2.1*	
Ile	3.8[b]	2.3[c]	Tyr	0.67[b]	4.9[c]
Leu	3.6[b]	1.9[c]	Val	3.1[a]	2.1[c]

[a] Permethylated β-CD and maltoheptaose (Mhep) were used as hosts. Inverses of some values are in parentheses. The list was compiled from references new data and refs 39 (a), 49 (b), and 48 (c).

Table 1 lists the selectivity of a number of amino acids with two different hosts, permethylated (β-cyclodextrin (β-CD) and maltoheptaose (Mhep). For this study, five amino acids were selected to provide varying ratios of k_L/k_D (Table 1). Alanine complexed to β-CD was chosen to illustrate a reaction with low selectivity ($k_L/k_D = 1.6$). Valine, isoleucine, and leucine were chosen as examples with moderate selectivity. Phenylalanine was chosen as an example with high selectivity. Note that the selectivity of Phe is only 0.8 when complexed to β-CD but is 4.6 when complexed to maltoheptaose.

The behavior of phenylalanine is a consequence of its large size.[48] Its selectivity is low and even reversed. Tyrosine behaves in a similar manner.[48] Its selectivity with β-CD is 0.66, which increases to 5.2 with maltoheptaose. We have performed extensive molecular dynamics calculations of the complexes involving Tyr, Phe, Val, and Leu with β-CD and maltoheptaose.[49] The results predict that both β-CD and maltoheptaose produce inclusion complexes. In maltoheptaose, the host wraps around the guest and solvates it to produce a quasi-inclusion complex. However, in the β-CD complex, the relatively rigid cavity constrains the phenyl group, which in turn forces the ammonium and the carboxylic group to interact with the cyclodextrin rims in only one way. The maltoheptaose host has an adjustable cavity that allows the phenyl group to find its preferred orientation for both isomers. This in turn allows the ammonium and carboxylic groups in the L and D isomers to have distinct interactions with the cyclodextrin.

Construction of the Calibration Curve. The construction of a calibration curve involves the analyses of several mixtures and the corresponding pure enantiomers. The exchange reaction (Scheme 1) is performed for a specific period, and the ratio of the reactant and the product complexes are measured after that period.

A series of spectra is shown in Figure 2 for mixtures of D- and L-phenylalanine after a reaction time of 15 s with an *N*-propylamine (Npa) pressure of 3.4×10^{-7} Torr. The reactant ion $[CD:Phe+H]^+$ and the product ion $[CD:Npa+H]^+$ are labeled. As the fraction of the D isomer is increased in the mixture, the combined reaction

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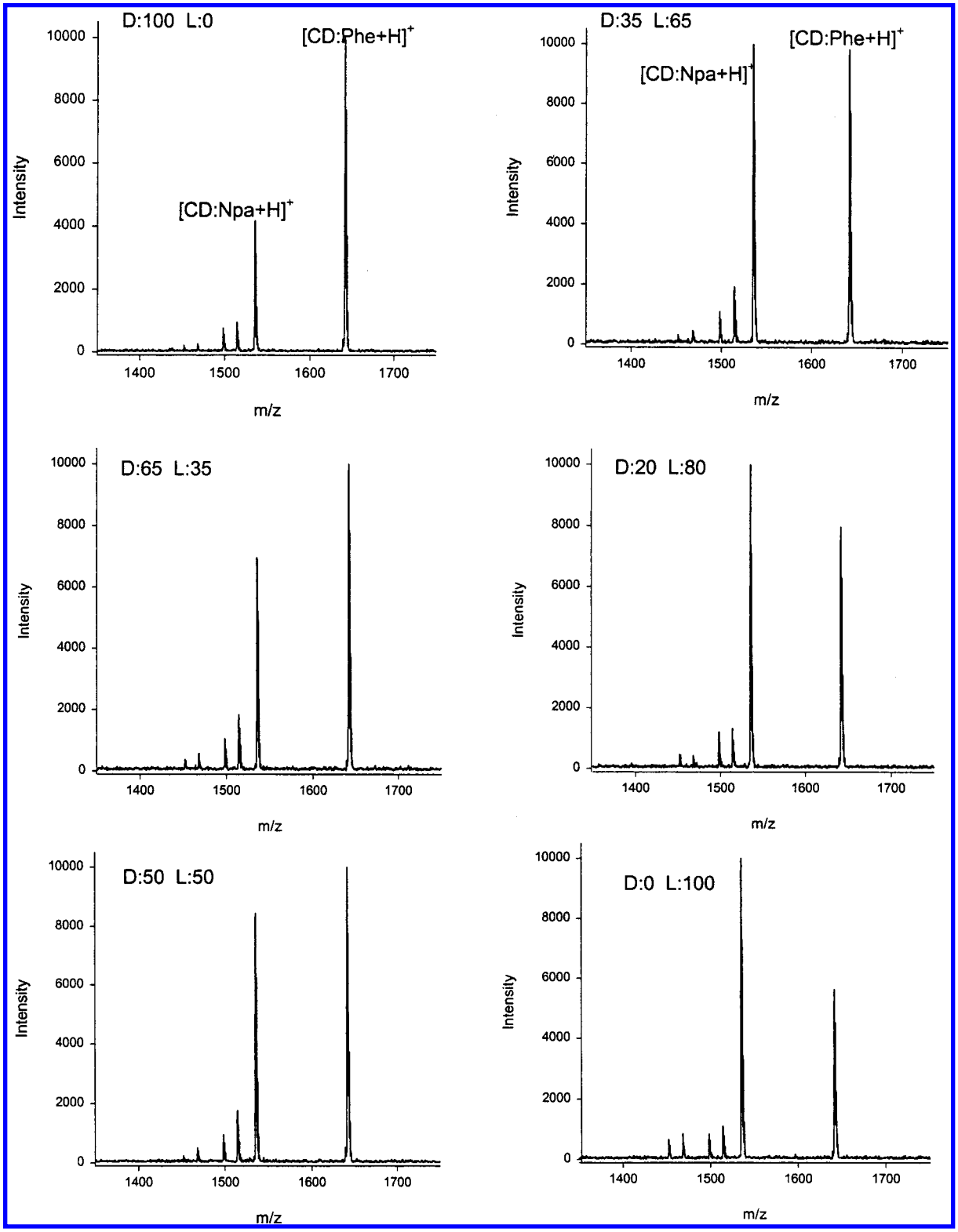

Figure 2. FTMS spectra of the [CD:Phe+H]$^+$ complex reacting with *n*-propylamine for various mixtures of L and D with a reaction time of 15 s. The L is more reactive than the D; mixtures with greater amounts of L produce more product complexes at a fixed reaction time.

rate of the mixture is also increased. Calibration curves for phenylalanine at two reaction times are shown in Figure 3. On the ordinate is the ratio of the intensity of the reactant complex (I) to the sum of the product and reactant complexes (I_o), while on the abscissa is the mole fraction of the D isomer (D/[D+L]). For this calibration curve, six points were used including pure D (100:0), 20:80, 35:65, 50:50, 65:35, and pure L (0:100). The coefficient of determination (r^2) is at least 0.995 for both curves.

Complexes that exhibit poorer selectivity have slightly smaller values of r^2. We find a direct correlation between r^2 and the selectivity; that is, as the selectivity decreases, the linear correlation also decreases. For the Ala complex with β-CD where the selectivity is only 1.6, the best calibration curve has $r^2 = 0.961$. For Ile with $k_L/k_D = 3.8$, the best calibration curve yields $r^2 =$

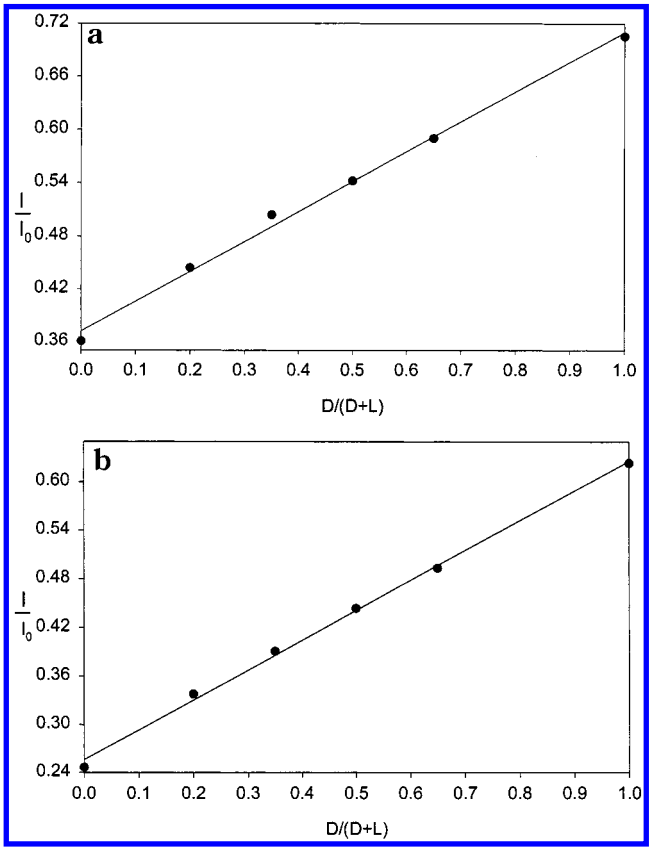

Figure 3. Calibration curve for mixtures of L- and D-phenylalanine complexes reacting with *n*-propylamine (3.4×10^{-7} Torr) for 15 (a) and 20 s (b). The term I/I_o is the ratio of the intensity of the reactant complex and the sum of the intensities of the product and reactant complexes. Because of the high selectivity ($k_L/k_D = 4.6$), the values for r^2 do not vary significantly from unity with different reaction times (0.995 and 0.999, respectively).

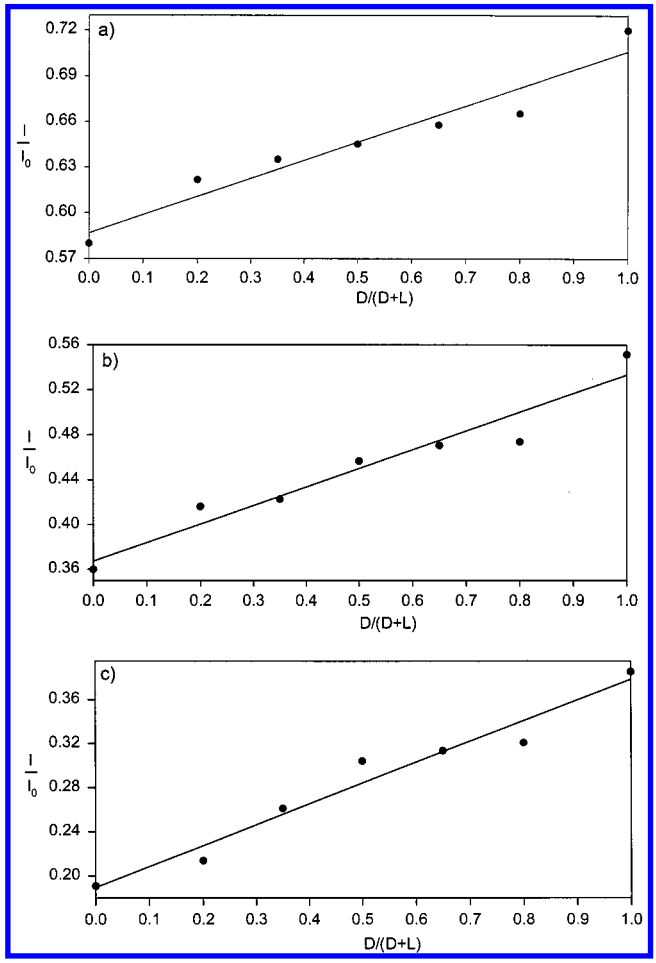

Figure 4. Calibration curve for mixtures of L- and D-alanine at reaction times of (a) 4, (b) 5.5, and (c) 7.7 s. The corresponding complexes are reacted with *n*-propylamine (3.0×10^{-7} Torr). The points become more linear at longer reaction times.

0.988. Val and Leu produce similar r^2 values. It should be emphasized that the poor selectivity for Ala was purposely chosen to illustrate the behavior of the curve for a compound with poor selectivity. Better selectivity is obtained for alanine when a suitable host is used. For example, selectivities greater than 2.0 are obtained when partially methylated β-cyclodextrins are employed as hosts.[48]

When the selectivity is low as in Ala, the selection of an optimal reaction time is more important for obtaining a calibration curve with good linear correlation. The calibration curves generally get better with the use of longer reaction time. For example, in Figure 4a, the shorter reaction period for Ala of 4 s yields an r^2 of 0.933. There is a greater degree of scatter compared to the reaction time of 7.5 s. The r^2 increases to 0.935 at 5.5 s (Figure 4b) and 0.961 for 7.5 s (Figure 4c). In a reaction period that is too short, ions are in a preequilibrium state and do not have thermal energies. As the system is allowed to react for longer times, thermal energy is achieved.

For compounds with large selectivities (>2), the reaction times do not strongly affect the quality of the curve. For example, panels a and b of Figure 3 show the calibration curves for Phe with reaction periods of 15 and 20 s, respectively. The r^2 values for both times are 0.995 and 0.998, respectively. Longer reaction times, for example, 24 s, improve the quality of the calibration curve slightly (0.999). Although the optimal reaction time differs for various amino acids, the best calibration curve is generally obtained when 50% of the reactant ion is consumed. Waiting longer yields no noticeable improvement in the quality of the calibration curve.

Analysis of Test Mixtures. Test mixtures were performed to determine the general utility of the method with samples of "unknown" ee. The mixture would be added to the cyclodextrin solution and injected into the ionization source. To determine ee, the ions are produced, trapped, and allowed to react for the same reaction period as the mixtures in the calibration curve. In this way, the analysis of the mixture requires only a single spectrum yielding detection limits normally available to ESI/FTMS.

Test mixtures were prepared with a known ee but were performed several days after the calibration curves were constructed. Figure 5 shows three test mixtures each for (a) alanine, (b) leucine, and (c) phenylalanine. The pressures used to determine the test samples were corrected using the equation

$$\ln(I/I_o)_1 = P_1/P_2 \ln(I/I_o)_2$$

where $(I/I_o)_2$ is the ratio of the intensities of the reactant complex and the sum of reactant and product complexes at a new pressure P_2.

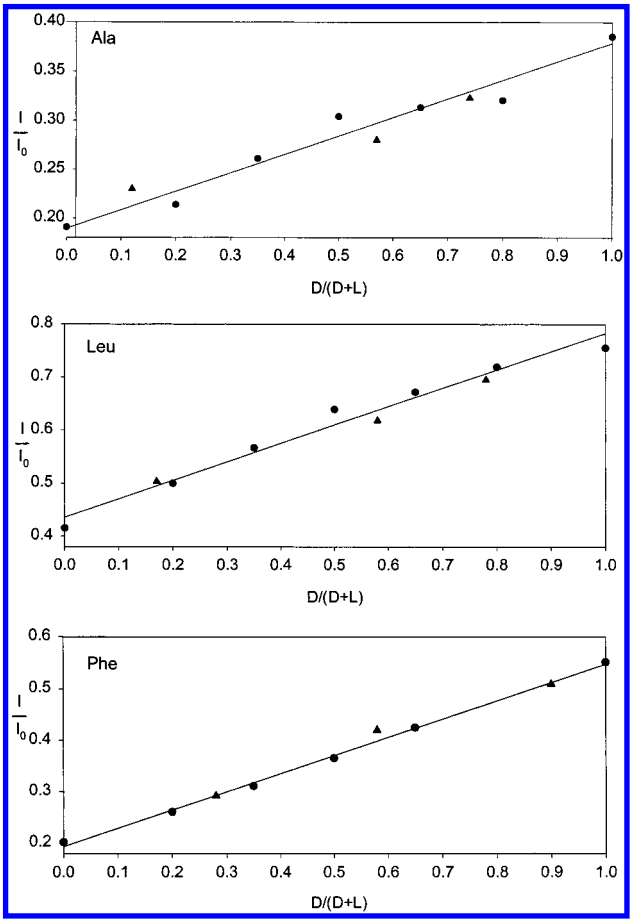

Figure 5. Calibration curves with test mixtures (solid triangles) for Ala, Leu, and Phe. The r^2 values for the calibration curves, without the text mixtures, are 0.961, 0.975, and 0.998, respectively. The test mixtures were analyzed with fresh solutions several days, weeks, and in some cases months after the calibration curves were produced.

A visual inspection shows that the there is more scatter for the amino acid with the lowest selectivity (Ala), while the least scatter is found with the amino acid with the highest selectivity (Phe). Table 2 lists the composition of the mixtures and the values obtained through the calibration curves. For the system with the lowest selectivity (Ala, 1.6), the average difference between the actual and determined values is 7.4. As the selectivity is increased to 3.2 (Ile), the average difference decreases to 3.8, while for a selectivity of 4.6 (Phe), the average absolute difference is only 2.3.

CONCLUSIONS

We illustrate a new method for the rapid determination of enantiomeric excess based on ion/molecule reactions. This method has several features that make it particularly amenable to process monitoring and combinatorial libraries. It takes advantage of the speed and sensitivity of mass spectrometry. Furthermore, this method is general and can be applied to other chiral organic compounds. Although this method is limited in this application to amino acids, it can readily be expanded for use with other chiral amines. We are currently developing similar applications for pharmaceutical compounds.

The analysis of an unknown mixture requires a single spectrum. This method is illustrated with single mixtures. We are currently exploring its application to more complex mixtures involving several amino acids to determine whether the analyses can be performed simultaneously. As in every method for determining ee, calibrations must be performed. The calibration curve is produced from a series of more abundant standards. However, we find once a calibration curve is produced, it can be used indefinitely. We are still using one calibration curve created over a year ago.

To make the method widely available, we are currently mapping the selectivities of all amino acids with a number of oligosaccharide hosts to provide the optimum selectivity for the analyses. The selectivity map of all amino acids with numerous guest compounds will be the subject of future publications.

Table 2. Enantiomeric Excess and the Determined Values of Mixtures of L- and D-Amino Acids[a]

	D/[D+L](× 100)		
	actual	determined	difference
Ala			
test 1	12.0	22.2	10.2
est 2	57.0	48.1	8.9
test 3	74.0	70.9	3.1
av absolute diff			7.4
Leu			
test 1	17.0	19.3	2.3
test 2	58.0	52.4	5.6
test 3	78.0	74.6	3.4
av absolute diff			3.8
Phe			
test 1	28.0	27.9	0.1
test 2	58.0	64.2	6.2
test 3	90.0	89.3	0.7
av absolute diff			2.3

[a]The determined values were obtained directly from the calibration curves.

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