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Evaluation of D-amino acid levels in human urine and in commercial L-amino acid samples

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Summary. Detectable levels of four free D-amino acids (phenylalanine, tyrosine, tryptophan and leucine) were found in human urine. This was accomplished using a liquid chromatographic coupled column (i.e., achiral-chiral) method that was developed for the rapid and sensitive analysis of these compounds. The technique was tested on a series of commercial L-amino acids. Care was taken to minimize racemization. Trace to percent levels of contaminating D-enantiomers were found in all commercial samples. In urine the D-amino acids ranged from several hundredths of a percent up to percent levels of the corresponding excreted natural L-amino acids. There were no apparent correlations between the amount of amino acids excreted and the relative amount of D-enantiomers present. Currently this study is being expanded to include additional amino acids and a variety of other physiological fluids.

Keywords: D-amino acids – Separation of racemic amino acids

Introduction

Both free, oligomeric and polymeric amino acids are found in all known living organisms. Literally hundreds of different amino acids have been identified (Hunt, 1985). However, there are only twenty primary protein constituents which consist of 19 L-α-amino acids and one cyclic L-α-imino acid, proline (Hardy, 1985). Over 50 years ago Kögl and Erxleben (1939) reported the presence of D-amino acids in the hydrosylates of tumor proteins. Although most other investigators believed that the D-amino acids were produced via racemization during the acid hydrolysis step, the controversy went on for over a decade (Miller, 1950; Kögl, 1949). Currently, there is not a lot of conclusive evidence for

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the occurrence of D-amino acids in the proteins of man. However, there is undisputed evidence for the occurrence of both free and bound D-amino acids in a variety of micro-organisms (Meister, 1957). Given the central importance of amino acids in all living systems and their use in the diagnosis of certain disorders or diseases in man, it seems logical to investigate all possible aspects of these compounds.

In the last few years there have been tremendous developments in the chromatographic analysis of enantiomers (Armstrong and Han, 1988; Krstulovic', 1989). Current liquid and gas chromatographic methods utilize chiral stationary phases (CSPs) for the efficient and sensitive determination of enantiomeric purities. Several of these methods can be used for the evaluation of trace levels of D-amino acids in the presence of large amounts of the corresponding L-enantiomers. In developing methods for this study, particular attention was given to maintaining mild separation conditions and short analysis times so as to minimize any possible racemization. The techniques were then tested on a number of "enantiomerically pure" L-amino acid standards to determine the limit of detection for D-amino acids. The technique was then used to evaluate the levels of several free D-amino acids in human urine. There were no hydrolysis steps and bound or linked amino acids (in peptides or proteins) were not considered.

Experimental

Materials

The LC system used for the analysis of commercial sources of L-amino acids consisted of a Shimadzu LC-6A pump and CR3A chromatopac recorder, an ESA (Bedford, MA) Coulochem model 5100A detector and analytical cell model 5011, and a Rheodyne (Cotati, CA) model 7125 injector. Two different chromatographic systems were used for the urine study. The electrochemical (LCEC) system consisted of two Rheodyne injectors (models 7125 and 7010), two Shimadzu LC-6A pumps, a Perkin Elmer (Norwalk, CT) LC-85B spectrophotometric detector, the above-mentioned ESA electrochemical detection system, a Perkin Elmer R100A recorder and a Shimadzu CR3A Chromatopac recorder. The LC system which utilized fluorescence detection was made up of the following Shimadzu devices: two LC-6A pumps, a SCL-6B system controller, a CR601 chromatopac recorder, SPD-6AV UV/VIS spectrophotometric detector, and a RF-535 fluorescence HPLC monitor. The "fluorescence" chromatographic system also included a Rainin (San Carlos, CA) model A-30-S pump, a Scientific Systems, Inc. (State College, PA) pulse dampener model LP-21 and two Rheodyne injectors (models 7125 and 7010) for post column derivatization.

A Labconco (Kansas City, MO) Freeze Dryer model 75018 was used to concentrate some samples and a Precision Clinical Centricone manufactured by Precision Scientific Company (Chicago, IL) was used for centrifugation.

The chromatographic columns used in these studies included the Crownpak CR(+) columns supplied by Michael Henry at J. T. Baker (Phillipsburg, NJ) and 5 μ octadecylsilane (C_{18}) columns obtained from Advanced Separation Technology (Whippany, NJ).

The perchloric acid, mercaptoethanol, boric acid, o-phthalaldehyde, and DL-amino acids were obtained from Aldrich (Milwaukee, WI). Methanol, ethanol and potassium hydroxide were supplied by Fisher (St. Louis, MO). The L-amino acids were provided by

the following companies: Kodak (Rochester, NY), Lancaster Synthesis (Windham, NJ), Fluka (Ronkonkoma, NY), Sigma (St. Louis, MO), Spectrum (Gardena, CA) and Research Plus, Inc. (Bayonne, NJ). All of the samples and mobile phase solutions were filtered using 0.2 μ filters supplied by Alltech Associates, Inc. (Deerfield, IL).

Methods

Prior to using the LCEC systems, voltage scans were run on several essential amino acids. The following amino acids were chosen for the LCEC commercial study: cysteine, methionine, ornithine, tryptophan and tyrosine. The applied voltage for the analysis of cysteine, methionine, tyrosine, and tryptophan was +0.5 volts. A somewhat better response was noted for these compounds if the voltage was greater than +0.5 and less than +0.8 volts but the background current was considered to be too high (greater than 1 μ amp). A high background current can have detrimental effects on the electrodes. Ornithine was analyzed at −0.10 volts.

Before beginning this study, it was necessary to optimize the separation of enantiomers by modifying the mobile phase conditions, the flow rate and the column temperature. Cysteine and ornithine elute rather quickly so the optimum temperature was determined to be 0°C and 10°C respectively. The optimum flow rate for these two amino acids was 0.1 ml/min. The mobile phase composition was aqueous perchloric acid (pH = 1.5 or 2.0). Under the stated conditions, it was possible to quantitate the amount of D-amino acid present without interference from the void volume peak. Methionine, tryptophan, phenylalanine, and tyrosine had longer retention times so it was possible to analyze these amino acids at room temperature. The flow rate for methionine and tyrosine was 0.3 ml/min; for phenylalanine, 0.5 ml/min; and the flow rate for tryptophan was 0.8 ml/min. The mobile phase composition was 97.5/2.5 aqueous perchloric acid/methanol (v/v) or 100% pH 2 $HClO_4$ solution for these three compounds.

All samples were dissolved in the mobile phase solution immediately prior to analysis. The results of this study were reproducible if the column and the detector current were allowed to stabilize before beginning the analysis. The average concentration of the L-amino acids was 10 mM. Despite the large amount of sample injected, it was still possible to attain baseline resolution with a single column. When using fluorescence detection the average concentration of the L-amino acid standard solution was 0.2 mM.

Urine samples were collected from young healthy adults. The mobile phase solution and the urine samples were filtered with 0.2 μ filters. If the urine appeared cloudy, the sample was centrifuged at 1550 rpm for 5 minutes. The supernatent was filtered and analyzed. All remaining urine samples were refrigerated at 4°C.

A C_{18} column was used to isolate the individual amino acids. The mobile phase consisted either of 95:5 water:methanol (v:v) or 95:5, 5×10^{-3} M $HClO_4$:methanol (v:v). The flow rate was 1 or 1.5 ml/min. The column was at room temperature and the UV detector setting was at 200 nm.

A column switching or coupled column method was used for the analysis of tyrosine, phenylalanine, tryptophan and leucine in urine (Fig. 1). Specimens taken in the early morning were prefered as they contained higher overall amino acid concentrations. The optimum separation conditions for the chiral separation are as listed above and in the individual figures.

In one set of experiments the samples were concentrated by freeze drying. The freeze-dried samples were dissolved in the mobile phase solution immediately prior to analysis.

Two different methods of detection were used in the urine study: electrochemical and fluorescence. The applied voltages for the LCEC experiments were the same as those listed above. Post column OPA derivatization of the amino acids was required for fluorescence detection. The excitation wavelength was 340 nm and the emission wavelength was 450

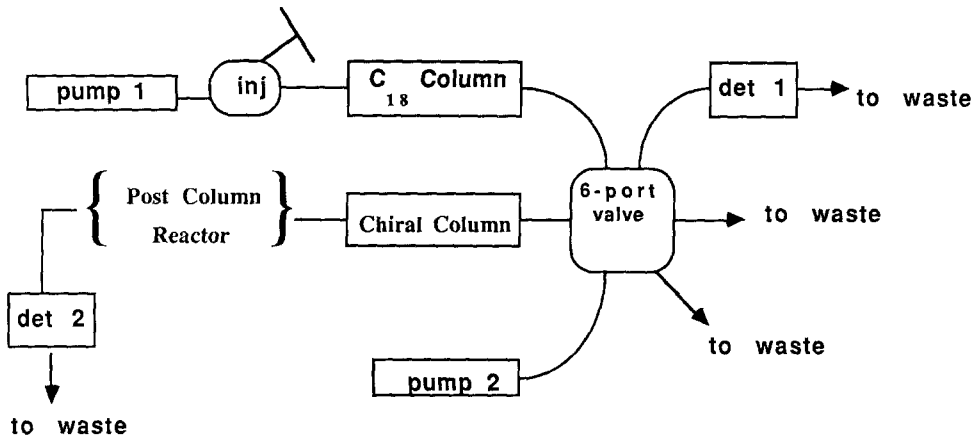

Fig. 1. Schematic showing the coupled column (i.e., achiral-chiral) system used for the determination of the enantiomeric purity of amino acids in urine. Note, when electrochemical detection was used (detector 2) there was no post-column derivatization. When fluorescence detection was used (detector 2), post-column OPA derivatives of the amino acids were made

nm. The Rainin pump was the post column pump for this system. The OPA solution was prepared as follows. The OPA reagent was prepared by dissolving 700 mg of o-phthalaldehyde in 15 ml of ethanol. Three hundred milliliters of mercaptoethanol was added to this solution. The solution was then added to 1 liter of 3% boric acid solution which had been adjusted to pH = 10.0 with KOH. Teflon tubing (0.5 mm i.d.) was used with the post column apparatus and the post column reactor was 3 M long.

Results and discussion

The general racemization profile versus pH for many free amino acids is illustrated in Fig. 2. Typically there are minima between pHs of 0 to 3 and a plateau

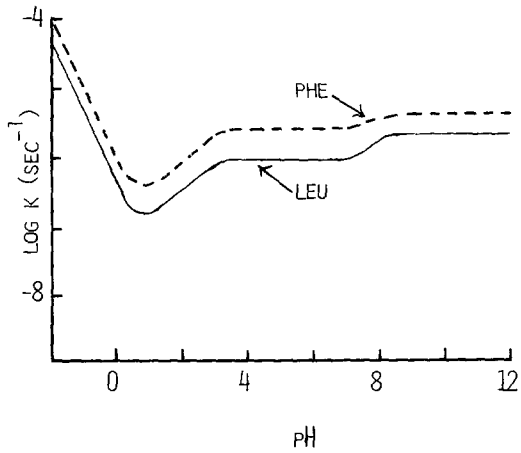

Fig. 2. Plot showing the rate of L:D interconversion versus pH for phenylalanine and leucine at 142°C. Data redrawn from Bada (1985)

Table 1. Data showing the amount of contaminating D-enantiomers in commerical samples of L-amino acids

Sample Source	Cysteine % D[a]	Methionine % D[a]	Ornithine % D[a]	Tyrosine % D[a]	Tryptophan % D[a]
A. Fluka	2.9×10^{-1}	4.2×10^{-3}	1.7×10^{-3}	3.6×10^{-2}	1.9×10^{-3}
B. Kodak	8.0×10^{-1}	6.0×10^{-3}	7.6×10^{-2}	2.1×10^{-2}	5.7×10^{-3}
C. Lancaster Synthesis	3.9×10^{-1}	5.0×10^{-3}	3.9×10^{-1}	6.5×10^{-3}	8.9×10^{-4}
D. Sigma	4.1×10^{-1}	3.3	3.5×10^{-2}	2.9×10^{-2}	7.3×10^{-3}
E. Spectrum	6.5×10^{-1}	3.8×10^{-1}	3.0×10^{-2}	1.5×10^{-3}	1.0×10^{-3}

[a] % D denotes the % D-amino acid enantiomer present in the commerically available L-amino acid samples. All samples were prepared immediately prior to analysis. Conditions for separation are indicated in the experimental section.

region between pHs 3 to 7 (Bada, 1985). At very high (>12) and low (<0) pHs, the rate of racemization increases substantially. In addition to pH, racemization rates also are influenced by temperature, ionic strength, presences of metal ions and so forth. Each amino acid has a small maximum at pH ~ 3.8 and another minima at pH ~ 5.0 (Bada, 1985). One goal of the analytical methodologies developed for this work was to minimize the effect of racemization on the measured enantiomeric purities. Hence most samples were analyzed immediately after they were obtained. The analyses were complete <45 minutes from sample introduction. No pH or temperature extremes that could lead to high racemization rates were used at any time. No precolumn reaction or derivatization of the amino acids were done (see Materials and methods).

Each analytical system was "tested" with commercial L-amino acid preparations. Typical results for five amino acids are shown in Table 1. D-amino acids were found in all commercial samples although some were at very low levels. Clearly, analytical methodologies for determining enantiomeric purities are reaching high levels of sophistication and sensitivity. In many cases the sensitivity is such that an enantiomeric impurity is almost always found (Table 1). Of the L-amino acids evaluated, tryptophan usually had the lowest levels of the "unnatural" D-amino acid. It was not unusual to see tenths of a percent and occasionally percent levels of the D-enantiomers. There were significant batch to batch differences in the enantiomeric purity of individual amino acids from the same company. These variations often were as great at the variations between companies (Table 1). It might be interesting to consider the effect that the varying levels of enantiomeric impurities could have on some of the numerous biological, biochemical and medicinal experiments that utilize these amino acids. Fig. 3 shows the chromatographic separation of D-cysteine and D-tryptophan from the dominant L-enantiomer in two commercial samples.

Four amino acids (phenylalanine, tryptophan, tyrosine, and leucine) were analyzed in human urine. Because of the large number of contaminating com-

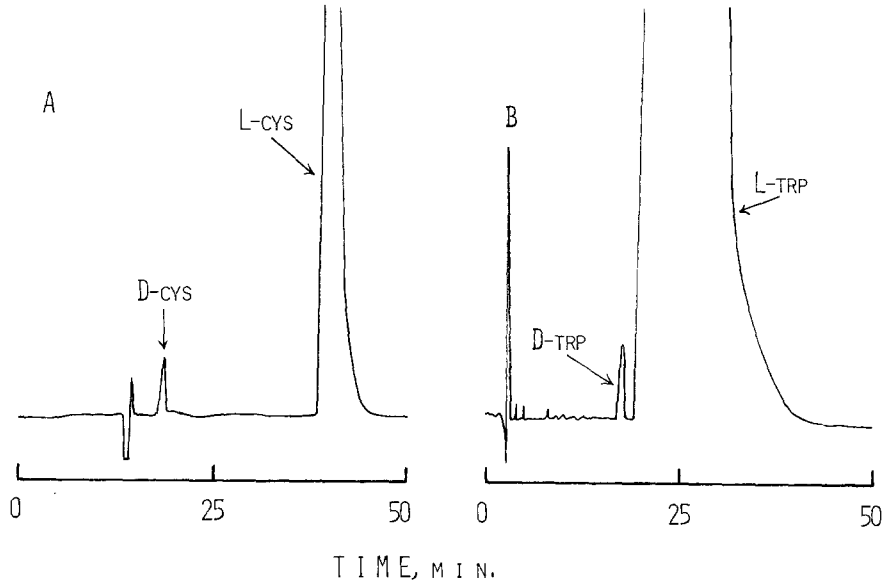

Fig. 3. LC chromatograms used to evaluate the enantiomeric purity of L-cysteine from Fluka and L-tryptophan from Sigma. Electrochemical detection was used in both cases. For chromatogram **A**, the mobile phase was pH 1.5 aqueous $HClO_4$; the flow rate was 0.1 ml/min; the voltage was +0.50v and the gain was 1 × 50. For chromatogram **B**, the mobile phase was pH 1.5 aqueous $HClO_4$; the flow rate was 0.8 ml/mm; the voltage was +0.50v and the gain was 10 × 5

pounds and high ionic strength, it is not advisable to directly inject biological samples onto a chromatographic chiral stationary phase (CSP). A column switching procedure (see experimental section) is essential for accurate results and increasing the longevity of the CSP. The fresh sample is directly injected onto a reversed phase C_{18} column which separates the amino acids from one other as well as from the other compounds in the urine. Fig. 4 shows a typical C_{18} chromatogram. Upon elution, the amino acid of interest is then switched onto the CSP for enantiomeric resolution and quantitation. Fig. 5 shows the enantiomeric separation of phenylalanine from human urine on a chiral crown ether column (Hilton and Armstrong, 1991). Some amino acids can be detected directly by electrochemical detection, while others must undergo postcolumn derivatization to form a fluorescent analogue (see Experimental Section).

Table 2 summarizes the analytical data for four of the amino acids found in human urine. The total levels of these amino acids were within ranges previously reported for human urine (Soupart, 1962). Trace levels of the "unnatural" D-amino acids were found in all cases. The relative amounts of D-amino acids present in these samples ranged from several hundredths of a percent to percent levels (Table 2). In general there were greater amounts of D-amino acids found in human urine than were found in commercial standards for L-amino acids (Table 1). The range of total amino acid concentrations as well as the percent

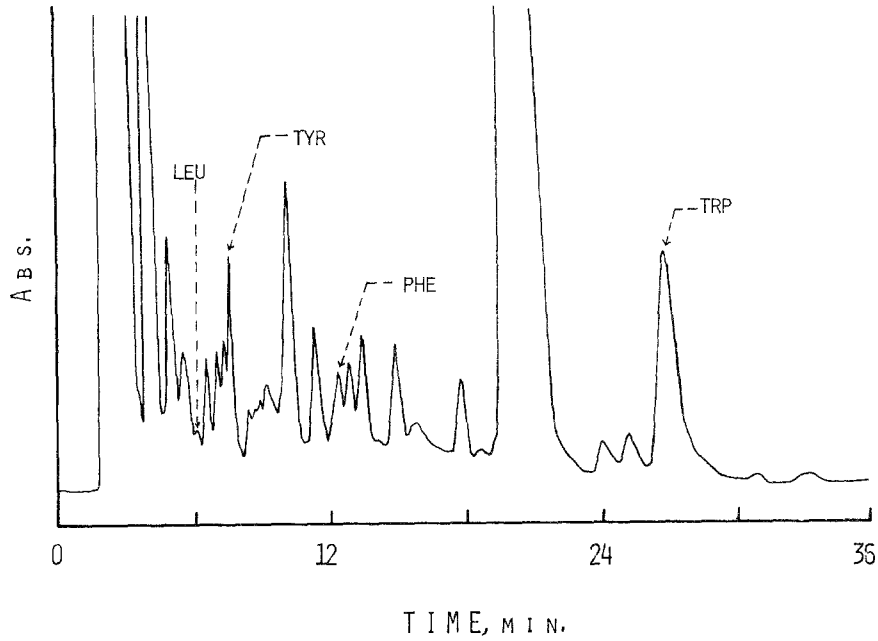

Fig. 4. A C_{18} chromatogram for the direct injection of urine. Peaks for four amino acids (Leucine, tyrosine, phenylalanine and tryptophan) are indicated. UV detection at 200 nm was used. The injection volume was 20 μl, the flow rate was 1.5 ml/min and the mobile phase consisted of 5:95 (v:v) methanol:5×10^{-3}M $HClO_4$. Note: in this chromatogram the peak labeled tryptophan actually consists of tryptophan plus another impurity. However, these two components are resolved when using a mobile phase consisting of 5:95 (v:v) methanol:water

D-enantiomer content varied not only from person to person but also for one person from day to day. For example, in the case of the phenylalanine data in Table I, both the high (5.1%) and low (0.3%) values came from two different specimens provided by the same individual two weeks apart. In general, the relative amount of D-phenylalanine was greater than that found for D-tyrosine and D-tryptophan. There did not seem to be a correlation between the total amino acid concentration in urine and the relative amount of the D-enantiomer.

In order to see and quantitate the low levels of D-amino acids, relatively large samples (20–50 μl) had to be injected. If amino acid levels in urine are too low, a preconcentration step may be necessary. This was the case for some of the amino acids not reported in this work. Although preconcentration increases the level of the trace analyte in a given volume of sample, it also increases the level of interfering contaminants. This could lead to overlapping peaks or column overloading and poor resolution. Lypholization seemed to be the most effective way to concentrate excreted amino acids. Solid phase extraction of amino acids from this matrix proved to be ineffective in our hands. Table 3 compares the measured levels of D-tryptophan and D-tyrosine in lypholized samples versus untreated samples that were analyzed directly. The lypholization step did not affect the measurement of D-tryptophan in urine. However, the

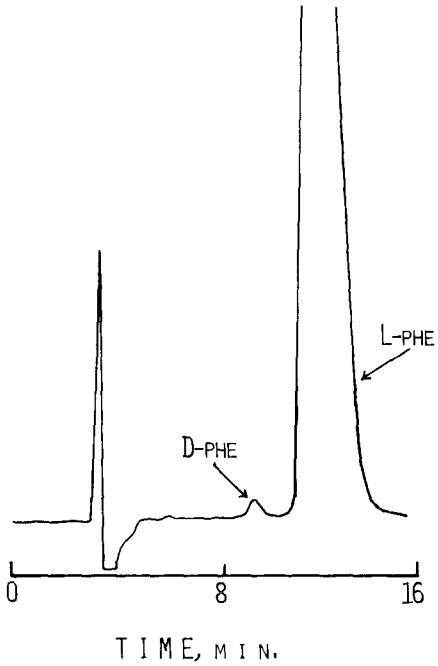

Fig. 5. Chromatogram showing the enantiomeric purity of phenylalanine from the direct injection of a urine sample onto the coupled column system (Fig. 1). The conditions for the C_{18} separation were the same as in Fig. 4. Note the absence of interfering peaks. A chiral crown ether column was used at room temperature. The mobile phase consisted of 0.02 M $HClO_4$, and the flow rate was 0.5 ml/min. Fluorescence detection was used after post column OPA derivatization (see experimental)

Table 2. Summary of free amino acid data from human urine specimens

Amino acid	Number of urine samples analyzed	Total range of AA concentration μM/L	% D-amino acid, range	Average % D-amino acid
Phenylalanine	11	35–130	0.3–5.1	1.9
Tyrosine	9	45–210	0.1–0.7[a]	0.3[a]
Tryptophan	8	46–280	0.06–0.4	0.2
Leucine	3	b	b	b

[a] One result (i.e., 6.6% D-tyrosine) was excluded from this data as it was approximately an order of magnitude higher than the next closest value. Four different individuals provided all samples for this study. However, the high tyrosine value (vide supra) was the sole sample provided by one individual. Currently, it is not known if this high value is legitimate or is due to an impurity or some other error.

[b] Peaks were obtained for both the D- and L-leucine. However, neither peak was baseline resolved from other contaminate peaks. This made accurate quantitation difficult. Although we are reasonably confident that some D-leucine is present, we would prefer to improve the analytical methodology somewhat before reporting any numbers.

Table 3. Data showing the effect of a freeze-drying preconcentration step on the measurement of D-amino acid levels

	Range of D-amino acids		Average % D-amino acid concentration	
Amino acid	Direct injection	Preconcentration by lypholization	Direct injection	Preconcentration by lypholization
Tryptophan	0.1–0.36	0.06–0.6	0.18	0.21
Tyrosine	0.1 to 0.70	3.0 to 9.2	0.3	4.8

apparent amount of D-tyrosine in lypholized urine was anomalously high (Table 3). This was thought to be the result of overlapping impurity peaks rather than to racemization during lypholization. However, further investigations must be completed before racemization can be completely disregarded.

This study provides evidence for the presence of free D-amino acids in human urine. In some cases the "unnatural" D-enantiomer was present at percent levels relative to the corresponding L-amino acid concentration. However, somewhat lower levels were more common. Care must be taken to resolve the amino acids of interest from other similarly retained components so as not to overestimate the amount of the D-enantiomer. Any biochemical, physiological or clinical significance of these enantiomers is yet to be established. Certainly there are a number of interesting possibilities. Many past studies on the efficiency of free amino acid reabsorption by the kidney have not taken into account possible differences in the rates or mechanism of D-versus L-amino acid absorption for example.

In subsequent work a comparison will be made between the levels of D-amino acids in the blood versus the urine. In addition we are attempting to further improve analytical methodologies so that a greater variety of amino acids and other chiral molecules can be examined.

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