Note

Peak identification of amino acids in liquid chromatography by optical activity detection

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Detection in liquid chromatography (LC) has made rapid advances in the last decade[1,2]. In addition to improvements in detectability, a high degree of selectivity is offered by some types of detection schemes. The latter is useful when the separation efficiency is not sufficient to resolve all the components in a complex mixture. Information-rich detectors include infrared spectroscopy[3] and mass spectrometry[4].

A relatively new detection scheme for LC is the measurement of optical rotation[5]. As little as 1 ng of an analyte with a specific rotation ($[\alpha]$) of 100° is now detectable[6]. Much of our earlier work was centered around its selectivity, so that clinically interesting species can be monitored in biological matrices[7,8]. In the course of our work, we realized that the information content of $[\alpha]$ is actually quite high. For a group of related compounds, such as the dansylated amino acids[9], $[\alpha]$ can range from $-250°$ to $+250°$. Since one can expect to be able to determine $[\alpha]$ to the $\pm 1\%$ level, the number of resolution elements available should be better than 200. This then opens up the possibility for analyte identification based on $[\alpha]$. We note that the dansylated amino acids are spectrally very similar, and other optical detectors cannot be used for identification.

Sequential identification of phenylthiohydantoin (PTH) amino acids resulting from Edman degradation[10] in proteins or peptides sequencing requires a rapid and sensitive method. Although various methods have been used, high-performance liquid chromatography in the reversed-phase mode (RP-HPLC) has become the standard system for the separation and detection of PTH-amino acids in most of the commercial automated gas- or liquid-phase Edman protein sequencers[11–13]. A lot of papers have been published for the separation and identification of PTH-amino acids using RP-HPLC. In general, the PTH-amino acids eluted from a chromatographic column are monitored by UV absorption and are identified by their corresponding retention times. In order to have all the common PTH-amino acids well resolved in time for identification, extensive manipulation of the mobile phases (eluents) or the stationary phases or both are usually required. Furthermore, this manipulation step frequently involves some undesirable conditions for the separation system; for example, elevated temperature, high flow-rate, long analysis time and baseline drift. In addition, it is not uncommon to have some PTH-amino acids still unresolved in time, even after a lengthly manipulation of the various conditions.

The purpose of this study is to detect and identify PTH-amino acids after

chromatographic separation using polarimetric and UV absorption detection. By monitoring both the optical activity and the UV absorption, the PTH-amino acids that are not identified by retention times will readily be identified by their optical rotations or *vice versa*. This detection scheme will allow rapid identification of PTH-amino acids under simple and favorable chromatographic conditions based on isocratic elution.

EXPERIMENTAL

Chemicals

L-Phenylthiohydantoin amino acids were obtained from Fluka (Ronkonkoma, NY, U.S.A.) or Pierce (Rockford, IL, U.S.A.). Reagent-grade sodium monophosphate, phosphoric acid, sodium acetate, and HPLC-grade acetonitrile were obtained from Fisher (Fair Lawn, NJ, U.S.A.). Analytical-grade glacial acetic acid was obtained from Baker (Phillipsburg, NJ, U.S.A.). Deionized water was prepared by passing distilled water through a Nanopure water-purification system (Barnstead, Boston, MA, U.S.A.). 0.02 M Phosphate buffer (pH 3.2) was prepared by dissolving 3 g sodium monophosphate in 1 l deionized water–acetonitrile (3:2, v/v). The pH of the resulting solution was adjusted to 3.2 by adding phosphoric acid. 0.02 M Acetate buffer (pH 5.0) was prepared by dissolving 2 g sodium acetate in 1 l water–acetonitrile (3:2, v/v). The pH of the resulting solution was adjusted to 5.0 by adding glacial acetic acid.

Chromatography

A Model 2600 syringe pump (Isco, Lincoln, NE, U.S.A.) was used for eluent delivery. Injections of samples were made through a Model 7010 six-port injector (Rheodyne, Berkeley, CA, U.S.A.), equipped with a 20-μl sample injection loop. Separations of the PTH-amino acids were performed on a 100 × 4.6 mm I.D., 3-μm, C_8 Econosphere column (Alltech, Deerfield, IL, U.S.A.). All eluents were filtered through a 0.2-μm nylon membrane filter, and were degassed under vacuum using ultrasonic agitation before use. All experiments were performed at room temperature with a flow-rate of 0.67 ml/min. All the PTH-amino acid samples were freshly prepared in the eluents before use.

Detection

The system consisted of an UV absorption detector in series with a laser-based polarimeter. UV detection at 330 nm was performed on a Model 100-10 variable-wavelength spectrophotometer (Hitachi, Tokyo, Japan) equipped with a Model 155-00 analytical flow cell (Altex, Berkeley, CA, U.S.A.). The waste line of the UV detector was connected to the inlet of the flow cell of the polarimeter via a 0.007-in. I.D. PTFE tubing. The length of this tubing was kept short to minimize the extra-column band broadening. The basic arrangement of the laser-based polarimeter was reported before[5,8]. The flow cell had a volume of 100 μl and was 5 cm in length. An independent air-based Faraday rotator (d.c. coil) was placed between the polarizer and the flow cell to produce a standard optical rotation. Modulation with square wave was driven by a Model SG-1271 wave generator (Heath, Benton Harbor, MI, U.S.A.) at a voltage of 25 V and a frequency of 500 Hz. A 1-s time constant was used

at the lock-in amplifier. The outputs of the lock-in amplifier and the UV detector were displayed on a Model CR 452 dual-channel recorder (Measurement Technology, Denver, CO, U.S.A.). The two outputs were also connected to a Model 5150 personal computer (IBM/PC, Armonk, NJ, U.S.A.) for data acquisition through the Adalab interface system (Interactive Microware, State College, PA, U.S.A.). The computer took a reading every 0.1 s to allow signal averaging.

Calculations

All raw data were subjected to a 25-point moving window smooth. The peak areas from both the UV and optical activity detectors were determined by summation of the baseline corrected measurements. The optical activity peak areas and responses were normalized against the signal obtained from the d.c. coil to account for the drift in laser intensity. The d.c. coil produced a constant 0.25-millidegree rotation. These normalized peak areas were then divided by their corresponding UV peak areas to obtain the optical activity-to-UV ratios. The specific rotations [α] of the PTH-amino acids were calculated using the following formula:

$$[\alpha] = \frac{\alpha}{Cl}$$

where α is the actual rotation measured, C is the concentration of analytes at the peak maximum in g/ml solution, and l is the path length in dm. The concentration at the peak maximum can be calculated from the peak area, the area of a 1-s interval at the peak maximum, the amount of analytes injected, and the flow-rate. The retention times of the PTH-amino acids were taken as the times for the peak maximum to appear.

RESULTS AND DISCUSSION

Identification of analytes in chromatographic separations is generally based on retention times (t_R). The t_R values of the twenty PTH-amino acids for separations at pH 3.2 are shown in Table I. The conditions were chosen so that all PTH-amino acids were eluted under 10 min at a flow-rate of 0.67 ml/min. Also, we required that all PTH-amino acids were retained so that the optical activity measurement will not be affected by the refractive index disturbance present at the void volume. It is obvious that the identification of some of the PTH-amino acids based on t_R are difficult or impossible as they were poorly resolved or unresolved. For example, the PTH-amino acids within the pairs of Ser–Gln, Thr–Asp, Gly–Cmc, Val–Met, and Trp–Ile are not resolved. In addition, Asn–Ser, Glu–Thr, Asp–Glu, Glu–Gly, His–Ala, and Ile–Phe are poorly resolved. Therefore, only a few of the twenty PTH-amino acids can be identified based on t_R alone. These poor results are expected since the chromatographic conditions used were not designed for the complete separation of the PTH-amino acids.

The measured specific rotations [α] of the PTH-amino acids are also shown in Table I. Most of the PTH-amino acids show negative rotations and have small values. The [α] of some of the PTH-amino acids in methanol[14] and ethanol[15] obtained at 590 nm have been reported. Those results are similar to ours, but strict comparison can-

not be made since [α] is solvent dependent. Since the optical activity-to-UV ratios are proportional to [α][9], if the molar absorptivities are identical it will be easier in practice to monitor the optical activity-to-UV ratios rather than to calculate [α]. The optical activity-to-UV ratios of the PTH-amino acids at pH 3.2 are also shown in Table I. These ratios are approximately, but not exactly, proportional to [α]. However, they still vary substantially among the amino acids. Most of the unresolved and poorly resolved peaks mentioned above are now readily identified by their optical activity-to-UV ratios. As a result, Glu–Gly–Cmc is the only group in which the PTH-amino acids could not be identified either by the t_R or the optical activity-to-UV ratios at pH 3.2.

Since the retention and the specific rotation are expected to be pH dependent, the experiment was repeated at pH 5.0 (acetate buffer) to find out if better identification of the peaks could be achieved. The t_R, [α], and the optical activity-to-UV ratios of the PTH-amino acids separated at pH 5.0 are shown in Table II. The elution times and elution orders of most of the PTH-amino acids at pH 5.0 are similar to those at pH 3.2, except Asp and Cmc. At pH 5.0, these two compounds were not retained in the column and were eluted with the solvent front. The dependence of the rotatory power of amino acids on pH has been discussed in detail[16]. In general, the rotatory power of L-amino acids becomes more positive when the concentration of acid, [H^+], increases. This trend for the PTH-amino acids is not observed here, i.e., the [α] at pH 3.2 and pH 5.0 are not significantly different. This may be because PTH derivatization changes the composition of most free amino acids to such an extent that, unlike

TABLE I

SEPARATION AND DETECTION OF PTH-AMINO ACIDS AT pH 3.2

PTH-Amino acid	Abbreviation	t_R(min)	Capacity factor k'	[α]	Optical activity-to-UV ratio
L-Asaparagine	Asn	2.48 ± 0.02	0.36	−31.2 ± 0.7	−8.4 ± 0.3
L-Serine	Ser	2.67 ± 0.00	0.46	−42.1 ± 0.6	−7.0 ± 0.4
L-Glutamine	Gln	2.66 ± 0.01	0.46	−25.2 ± 0.4	−5.3 ± 0.2
L-Threonine	Thr	2.84 ± 0.01	0.56	−71.7 ± 1.8	−11.6 ± 0.5
L-Aspartic acid	Asp	2.92 ± 0.00	0.60	−14.6 ± 1.1	−3.4 ± 0.2
L-Glutamic acid	Glu	3.18 ± 0.00	0.75	≈0	≈0
Glycine	Gly	3.32 ± 0.00	0.82	≈0	≈0
Carboxymethyl-L-Cysteine	Cmc	3.36 ± 0.01	0.85	≈0	≈0
L-Histidine	His	3.86 ± 0.01	1.12	≈0	≈0
L-Alanine	Ala	4.00 ± 0.00	1.20	−13.5 ± 0.5	−2.3 ± 0.1
L-Tyrosine	Tyr	4.43 ± 0.00	1.43	−24.3 ± 1.0	−6.1 ± 0.2
L-Arginine	Arg	4.85 ± 0.02	1.66	≈0	≈0
L-Valine	Val	5.98 ± 0.01	2.29	−10.4 ± 0.6	−1.6 ± 0.1
L-Methionine	Met	5.98 ± 0.01	2.29	+14.7 ± 0.6	+2.9 ± 0.1
L-Proline	Pro	6.33 ± 0.00	2.48	−65.8 ± 1.9	−10.1 ± 0.4
L-Tryptophan	Trp	7.65 ± 0.01	3.20	≈0	≈0
L-Isoleucine	Ile	7.71 ± 0.01	3.24	−22.4 ± 0.9	−3.5 ± 0.2
L-Phenylalanine	Phe	7.84 ± 0.01	3.31	−14.2 ± 1.3	−2.8 ± 0.2
L-Leucine	Leu	8.46 ± 0.01	3.65	−34.2 ± 1.6	−7.2 ± 0.2
ε-PTC-L-lysine	Lys	8.88 ± 0.00	3.88	≈0	≈0

TABLE II

SEPARATION AND DETECTION OF PTH-AMINO ACIDS AT pH 5.0

PTH-AA	t_R (min)	k'	[α]	Optical activity-to-UV ratio
L-Aspartic acid	1.82 ± 0.04	0	*	−4.6 ± 0.2
Carboxylmethyl-L-cysteine	1.82 ± 0.04	0	≈0	≈0
L-Glutamic acid	2.46 ± 0.01	0.35	≈0	≈0
L-Asparagine	2.50 ± 0.00	0.37	−39.1 ± 1.0	−9.9 ± 0.3
L-Glutamine	2.61 ± 0.00	0.43	−29.1 ± 1.0	−6.9 ± 0.2
L-Serine	2.66 ± 0.03	0.46	−43.0 ± 1.5	−8.9 ± 0.3
L-Threonine	2.85 ± 0.01	0.57	−66.5 ± 3.2	−15.5 ± 0.8
Glycine	3.25 ± 0.00	0.79	≈0	≈0
L-Histidine	3.87 ± 0.01	1.13	≈0	≈0
L-Alanine	3.95 ± 0.02	1.17	−9.4 ± 0.2	−2.4 ± 0.1
L-Tyrosine	4.34 ± 0.01	1.38	−19.4 ± 0.7	−6.4 ± 0.3
L-Arginine	5.16 ± 0.06	1.84	≈0	≈0
L-Valine	6.03 ± 0.03	2.31	−8.2 ± 0.4	−1.8 ± 0.1
L-Methionine	6.25 ± 0.02	2.43	+17.5 ± 0.7	+4.8 ± 0.1
L-Proline	6.68 ± 0.00	2.67	−72.8 ± 2.8	−14.2 ± 0.6
L-Tryptophan	7.81 ± 0.01	3.29	≈0	≈0
L-Isoleucine	8.08 ± 0.03	3.44	−16.7 ± 0.1	−4.2 ± 0.1
L-Phenylalanine	8.21 ± 0.04	3.51	−8.28 ± 0.4	−2.4 ± 0.2
L-Leucine	8.70 ± 0.00	3.78	−38.3 ± 1.2	−10.3 ± 0.1
ε-PTC-L-Lysine	9.27 ± 0.00	4.09	≈0	≈0

* Cannot be determined due to interference of refractive index disturbance at the void volume.

dansylation[9], they no longer behave as amino acids with respect to this acid rule. It may also be the case that a plateau has been reached such that any change in the $[H^+]$ will not affect the rotatory power[17]. Another possibility is that the change in pH in this study is not large enough to cause any noticeable changes in [α].

At pH 5.0, the PTH-amino acids within the pairs of Asp–Cmc, Glu–Asn–Gln–Ser, Ser–Thr, His–Ala, Val–Met, Trp–Ile–Phe are poorly resolved or unresolved in t_R. However, all of the PTH-amino acids within these pairs can be readily identified by their optical activity-to-UV ratios. Asp and Cmc were co-eluted with the solvent front. The solvent did not affect the UV peaks much, but it produced a small disturbance in the optical activity chromatograms. This small disturbance within the peaks did not allow [α] to be calculated accurately. In fact, this is why the original conditions were chosen (at pH 3.2) to avoid interference from this disturbance. However, the irregular peak shapes for Asp and for Cmc are reproducible. An optical activity-to-UV ratio can still be derived from the chromatograms to distinguish the two. Naturally, the injection volume and the solvent for the analyte must be fixed to allow such inferences. Another way to confirm the Cmc peak is to look for the presence of an extra UV peak which is due to the degradation product of Cmc. Finally, we note that Cmc is not a naturally occurring amino acid and is produced only via certain protein degradation schemes. The implication of Table II is that, using pH 5.0 acetate eluent, all of the twenty PTH-amino acids can be identified either by the retention times or the optical activity-to-UV.

So, the combination of optical activity and UV detection has allowed simplifi-

cation of the chromatographic procedure necessary for the identification of the PTH-amino acids. A 10-min isocratic run is a substantial improvement over the normal 50–60 min gradient run, which also requires cycling the column back to the original solvent strength prior to each run. The detection limits for optical activity–UV here is in the low ng range depending on the species, which is comparable to the 10-pmol levels in the standard protein sequencing systems. Naturally, other amino acid derivatives, such as dansyl[9], can be expected to allow the same simplification. There, the $[\alpha]$ values are larger and detectability should be even better. other optical activity monitors, such as circular dichroism[18] and fluorescence detected circular dichroism[19] should also be applicable. In fact, since fluorescence–circular dichroism detectors can be miniaturized substantially to become compatible with open tubular capillary columns, one may have identification at very low mass quantities for each component. Finally, actual application to protein sequencing by Edman degradation is more complicated. This is because the coupling and cleavage steps are not 100% efficient and not 100% synchronized. It is probably possible to account for these effects by background subtraction based on the amino acids identified in the previous steps. For the worst case of Glu–Ser in Table II, one can expect to be able to tolerate up to 30% contamination before identification becomes ambiguous.

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