*Journal of Chromatography*, 472 (1989) 256–260
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 494

## Note

# Altered retention of derivatives of *tele*-methylimidazoleacetic acid in acid-hydrolyzed samples of brain measured by capillary gas chromatography and mass spectrometry

GEORGE D. PRELL* and ALBERT M. MORRISHOW
*Department of Pharmacology, Mount Sinai School of Medicine, City University of New York, New York, NY 10029 (U.S.A.)*

(First received October 31st, 1988; revised manuscript received March 14th, 1989)

Classical chromatographic science holds that chemical substances, even those of similar structure, can be separated according to each compound's independent interaction with chromatographic media. In gas chromatography (GC), the hallmark of chromatographic separation of a substance is its characteristic retention, often expressed as a retention time, highly reproducible under defined conditions of column composition, size, gas flow, temperature, etc. With capillary GC columns, the range and variability of characteristic retention times are extremely narrow. Significant deviations from established retention times are uncommon when measured under identical chromatographic conditions[1,2]. Yet it has been observed, for instance, that if a trace constituent elutes after a much larger component[3], the latter acts as a transient stationary phase, increasing retention of the former. Absent such phenomena, in capillary GC analysis it is rare[1,2,4] to observe selective deviations from well characterized retentions of substances due to component(s) of the injectate under otherwise identical conditions. Under identical GC conditions we observed that the retention times of both derivatized *tele*-methylimidazoleacetic acid (t-MIAA) and its deuterated form ([$^2H_3$]t-MIAA) were consistently and reversibly increased. This occurred in samples prepared from homogenates of rat brains that were hydrolyzed but not in those prepared from aliquots of the same homogenates kept at room temperature or frozen. We present results of a typical experiment, extended briefly to study this effect.

## EXPERIMENTAL

Male Sprague-Dawley rats (200–400 g) were anesthetized with methohexital sodium (75 mg/kg, intraperitoneal), underwent cerebral perfusion[5] to remove blood, and were decapitated. Whole brains were homogenized with 4 vol. of distilled water. Aliquots were mixed with equal volumes of 0.2 *N* hydrochloric acid, vortexed, boiled for 10 min, cooled, then stored frozen at −80°C. Later, the acidified, boiled homogenates were thawed and vortexed, then several 1-ml aliquots were transferred to glass vials containing 1 ml 0.1 *N* hydrochloric acid and internal standards for t-MIAA and imidazoleacetic acid (IAA) (see below). Some samples were transferred to

0021-9673/89/$03.50 

hydrolysis tubes (Pierce) then heated at 150°C; others, from the same homogenates, were kept at room temperature. After 72 h, hydrolyzed and non-hydrolyzed samples were transferred to polypropylene tubes and centrifuged at 50 000 *g* for 20 min. Supernatants were retained for analysis.

t-MIAA was extracted, separated by ion-exchange chromatography, derivatized with boron trifluoride–butanol and measured by GC–mass spectrometry (MS) by the method of Khandelwal *et al.*[6] as modified[7]. In the same samples, IAA was extracted and its acidic side chain was derivatized along with t-MIAA. After mixing derivatized IAA with ethyl-chloroformate, *n*-butyl-(N-ethoxycarbonyl)imidazoleacetate was produced; two isomeric compounds are formed since IAA has two tautomeric forms[5]. Samples were injected onto a Durabond fused-silica capillary column [DB-WAX, polyethylene glycol phase with 0.25-$\mu$m phase film thickness; 15 m × 0.25 mm I.D. (J. & W Scientific)]. Derivatives of t-MIAA, its internal standard, [$^2H_3$]t-MIAA, and IAA and its internal standard, $^{15}N$,$^{15}N$-IAA, were analyzed by methane chemical ionization (CI) dual ion-group monitoring on a Hewlett-Packard combined gas chromatograph (HP 5890)–mass spectrometer (HP 5988A) interfaced with an HP 59970B workstation. Selected mass ions (*m/e*) were monitored from 7 to 9 min after injection of 3 $\mu$l of sample. Samples were chromatographed in the slitless mode with helium (10 p.s.i. head pressure) as carrier gas. The methane pressure was 1 Torr in the ionization source. The oven temperature was initially equilibrated at 100°C, then ramped at 30°C/min after sample injection, up to 200°C. Injection port, gas chromatograph–mass spectrometer transfer line and ion source were maintained at 250, 250 and 150°C, respectively. For IAA and $^{15}N$,$^{15}N$-IAA, the larger of each of the two peaks of the isomers was evaluated[5]. Authentic standards (0.1–150 ng of t-MIAA and IAA) in 0.1 *N* hydrochloric acid were processed in parallel. We compared retention times since all other aspects of the analyses were held constant. Differences among means of retention times, determined at maximal height, were evaluated by analysis of variance (ANOVA). Differences between groups were assessed using Dunnetts multiple range test.

## RESULTS AND DISCUSSION

The retention times of derivatized t-MIAA (*m/e* 197) and IAA (*m/e* 255) and their respective internal standards (*m/e* 200 and 257) from the various sample groups are shown in Table I. In samples that had been hydrolyzed, the retention times of derivatives of t-MIAA and [$^2H_3$]t-MIAA were altered, each to the same extent; the retention times of derivatives of IAA and $^{15}N$,$^{15}N$-IAA in the same injectates were unchanged. Although the mean retention time of derivatized t-MIAA in hydrolyzed samples was increased only 3% compared to retention of non-hydrolyzed samples, this shift was highly significant (ANOVA: $p < 0.0001$), representing a mean peak prolongation of about 10 s. The 95% confidence interval range about the mean retention times of hydrolyzed and non-hydrolyzed samples did not overlap and none of the retention times of derivatized t-MIAA from hydrolyzed samples was within the confidence range of samples kept at room temperature; the latter were almost identical to values for authentic standards (Table I). In each hydrolyzed sample, the retention time of derivatized t-MIAA always exceeded that of its non-hydrolyzed sample pair. Changing the order of injections or alternating between samples prepared from

TABLE I

RETENTION TIMES OF DERIVATIVES OF t-MIAA (*m/e* 197), [$^2H_3$]t-MIAA (*m/e* 200), IAA (*m/e* 255) AND $^{15}N$,$^{15}N$-IAA (*m/e* 257) OF HYDROLYZED AND NON-HYDROLYZED SAMPLES MEASURED BY GC–MS

Arithmetic means ± S.E.M. of 11–20 independent replicates.

| | *Retention time (min) (mean ± S.E.M.)* | | | |
|---|---|---|---|---|
| | *m/e 197* | *m/e 200* | *m/e 255* | *m/e 257* |
| Authentic | 7.290 ± 0.010 | 7.285 ± 0.010 | 8.337 ± 0.003 | 8.337 ± 0.003 |
| Non-hydrolyzed | 7.270 ± 0.005 | 7.264 ± 0.005 | 8.328 ± 0.007 | 8.328 ± 0.007 |
| Hydrolyzed | 7.439 ± 0.027[a] | 7.424 ± 0.027[a] | 8.352 ± 0.014 | 8.345 ± 0.012 |
| Hydrolyzed and dried authentic | 7.378 ± 0.017[b] | 7.368 ± 0.018[b] | 8.357 ± 0.003 | 8.364 ± 0.004 |
| Hydrolyzed mixed with authentic | 7.347 ± 0.008[c,d] | 7.338 ± 0.011[c,d] | 8.310 ± 0.002 | 8.310 ± 0.002 |
| ANOVA | | | | |
| *F* | 18.27 | 14.98 | 1.59 | 2.16 |
| *p* | <0.0001 | <0.0001 | Not significant | Not significant |

[a] $p < 0.001$ *versus* authentic and non-hydrolyzed.
[b] $p < 0.01$ *versus* authentic and non-hydrolyzed.
[c] $p < 0.05$ *versus* authentic and non-hydrolyzed.
[d] $p < 0.05$ *versus* hydrolyzed only.

hydrolyzed or non-hydrolyzed material or authentic compounds did not influence the retention time or area counts of analytes within each group. The aqueous standards were unaltered after acid hydrolysis. There was no correlation between the retention time and the magnitude of the elevation[8] of the levels of t-MIAA or IAA in hydrolyzed homogenates. There was no evidence for extra peaks or tailing in any of the preparations. Mass fragmentography confirmed the identity[5,6] of the derivatives of t-MIAA (evaluated at *m/e* 95), IAA (evaluated at *m/e* 81, 154 and 254) and their internal standards at the various retention times.

Prolonged retention of a constituent due to the components of a sample has been known to occur with column overloading (*e.g.* ref. 3). Retention of trace substances may be retarded when eluted after a major component. This produces broadening of the major peak but not of the minor peak; the latter is unsymmetrical, and elutes earlier as sample size increases[3]. However in our samples, total ion scanning of hydrolyzed and non-hydrolyzed material during the first 10 min after sample injection indicated that no major peaks (*i.e.* those greater than 10% of area counts of t-MIAA) eluted within 2 min before the peak for t-MIAA. Injection of larger samples slightly increased the retention times for the t-MIAA derivatives. Moreover in the hydrolyzed samples, peaks of the derivatives for t-MIAA and [$^2H_3$]t-MIAA were symmetrical. The peaks ($n = 19$) for derivatized t-MIAA in hydrolyzed samples, evaluated by valley-to-valley baseline analysis on the system's software, were an average of 3.78 s broader ($p < 0.001$) than peaks from samples kept at room temperature, whose average peak width was 5.12 ± 0.29 (S.E.M.) s. Thus, there was no evidence for a single hydrolysis component acting as a stationary phase in our study. This suggested a different mechanism that

affected the elution properties of the derivatives of t-MIAA, but not those of IAA, from Durabond columns.

To probe this phenomenon further, hydrolyzed samples that had been analyzed were diluted with chloroform and spiked with aliquots prepared from either 30 or 100 ng of authentic t-MIAA and IAA. Authentic material was transferred to silanized glass vials and either (a) evaporated to dryness then resuspended with 5 $\mu$l of hydrolyzed sample, or (b) mixed with an equal volume of hydrolyzed sample. Under both circumstances, the retention times of derivatives of t-MIAA and [$^2H_3$]t-MIAA, but not those of IAA or its internal standard, were significantly and consistently increased compared to non-hydrolyzed samples or standards in aqueous media (Table I). The increased retention was independent of the quantity of authentic compound. The mean retention time of dried standards resuspended with hydrolyzed samples did not differ significantly from mean values of the latter. The retention times of standards mixed in equal volume with hydrolyzed samples, which diluted the latter, were midway between, and differed significantly (each $p < 0.05$) from, the retention times of both non-hydrolyzed and hydrolyzed samples (Table I). Therefore, as the fraction of hydrolyzed material in the injectate decreased, so did the retention time for t-MIAA. A similar but lesser shift of the retention time was observed in hydrolyzed samples of cerebrospinal fluid[9]; the latter samples presumably contain fewer components than tissue homogenates contain. Since it is likely that amounts of chloroform in sample vials vary to some degree, this may partially account for the greater variation in retention values from hydrolyzed samples. Consonant with this hypothesis, the coefficient of variation from the latter was about five-fold higher than that of non-hydrolyzed samples.

The precise reason(s) for this selective change in the retention time is unknown. Non-specific factors such as increased ionic strength and reduced lipid composition in these hydrolyzed samples probably have little bearing since the retention of the IAA derivatives was unchanged. Nevertheless, it is apparent that components in the injectate prepared from hydrolyzed homogenates of rat brain have a significant influence on the elution characteristics of t-MIAA derivatives but not on IAA derivatives on this and other Durabond-wax columns (obtained from the same manufacturer) that we have used. Since the side chains of derivatized t-MIAA and IAA are identical[5,6], the column–analyte interaction(s) responsible for these changes most likely rest with the substituted nitrogen of the imidazole moiety of the t-MIAA molecule. Such an interaction(s) is not immediately obvious since the rings for t-MIAA and derivatized IAA have no reactive nitrogens, the former is methylated, the latter is linked to a carboxyethyl group.

ACKNOWLEDGEMENTS

This work was supported by grant MH-31805 from the National Institute of Mental Health and by the NIH Stable Isotope Program (PR02231) USDOE/OHER at Los Alamos. We thank Dr. Zbigniew Cichon for technical assistance and Dr. Jack Peter Green for helpful comments in the preparation of this manuscript.

REFERENCES

1 W. H. McFadden, *Techniques of Combined Gas Chromatography–Mass Spectrometry: Applications to Organic Analysis*, Wiley-Interscience, New York, 1973, p. 79.
2 L. S. Ettre, in L. S. Ettre and A. Zlatkis (Editor), *The Practice of Gas Chromatography*, Wiley-Interscience, New York, 1967, p. 3.
3 D. R. Deans, *Anal. Chem.*, 43 (1971) 2026.
4 W. Jennings, *Gas Chromatography with Glass Capillary Columns*, Academic Press, New York, 2nd ed., 1980, Ch. 7, 12, 16, pp. 119, 183, 273.
5 J. K. Khandelwal, G. D. Prell, A. M. Morrishow and J. P. Green, *J. Neurochem.*, 52 (1989) 1107.
6 J. K. Khandelwal, L. B. Hough, B. Pazhenchevsky, A. M. Morrishow and J. P. Green, *J. Biol. Chem.*, 257 (1982) 12815.
7 J. P. Green and J. K. Khandelwal, *Adv. Biosci.*, 51 (1985) 185.
8 G. D. Prell *et al.*, in preparation.
9 G. D. Prell and A. M. Morrishow, unpublished results.