Journal of Chromatography, 369 (1986) 178–181
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 014

Note

Omission of aspartate values from amino acid analysis reports

LEWIS B. JAMES
Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra A.C.T. (Australia)

(First received July 8th, 1986; revised manuscript received August 12th, 1986)

It has been observed that, when using any chromatographic procedure which separates amino acids in a short period of time, even small variations in mobile phase pH will shift the relative retention time[1]. With the earlier models of Beckman amino acid analyzers, such as the 120B and 119CL, aspartate was retained on the ion-exchange column long enough so as to rid the sample of minor variations in pH due to acid contamination merely by the pumping of elution buffer. After continuous pumping of buffer, aspartate was eluted from the resin in 40 min with the Model 120B[2], and in 13 min with the 119CL analyzer[3,4]. The Beckman System 6300 analyzer, however, can elute aspartate in *ca.* 5–6 min, depending on buffer pumping rate, and this faster rate of analysis does not permit any variation in pH of the applied amino acid solutions.

This communication describes changes in procedures so as to overcome the problem of prolonged retention times for aspartate when using fast amino acid analyzers. It also discusses deficiences in the model of integrator recommended to be used with the analyzer.

EXPERIMENTAL

All analytical determinations were carried out with a Beckman System 6300 Analyzer, the analog output of which was monitored by a Hewlett-Packard 3390A reporting integrator. The amino acid analyzer used reagents supplied by Beckman (Palo Alto, CA, U.S.A.). Amino acids were reacted with ninhydrin reagent to develop colour and the total analysis time, including column regeneration, was 50 min. The instrument was calibrated with an amino acid concentration of 2.5 nmol. Taurine and γ-aminobutyric acid were added to the standard calibration mixture (Beckman P/N 338088) and retention times for taurine, aspartate, glutamate, glycine, alanine and γ-aminobutyric acid were determined with the Hewlett-Packard reporting integrator. The sample loop injection volume was 50 μl.

RESULTS AND DISCUSSION

Retention time delay for aspartate was first experienced after using a reversed-phase column to separate a mixture of peptides that had been obtained by cyanogen

0021-9673/86/$03.50

bromide cleavage of a protein. The peptides were separated by using a gradient beginning with 1% orthophosphoric acid solution against acetonitrile, on a Varian 5000 liquid chromatograph[3,4]. After hydrolysis of the collected peptides, the amino acid mixtures were taken up in Beckman Na-S dilution buffer and analysed. Some of the mixtures containing aspartic were found to contain residual orthophosphoric acid. Addition of a droplet of concentrated orthophosphoric acid to the standard calibration mixture used for re-calibrating the amino acid analyzer was sufficient to simulate the necessary delay in retention time for aspartate so that no further omission of aspartate value occurred when the hydrolyzed peptides were re-analysed.

When the integrator is initially calibrated it sets a time interval or window for each retention time that represents the presence of an amino acid. These windows are opened and closed automatically during the course of an analysis in a programmed sliding scale controlled by the integrator; narrow window openings at the start of an analysis where sharp peaks occur, and longer openings at the end of the chromatogram trace where wide peaks occur. Aspartate is eluted from the ion-exchange column near the start of the analysis and is covered by one of the shortest windows, 9.6 s, thus, any moderate shift in retention time can easily position the peak outside the window.

When the analysis of a series of physiological fluid extracts was attempted, it was discovered that the extracts contained varying amounts of perchloric acid which had been used to precipitate unwanted proteins during preparation of the samples for analysis. Upon analysis a wide variation in the retention time for aspartate was encountered; some samples provided retention times within the window, others provided retention times before the window opened and others after it had closed.

In Fig. 1 the retention time for aspartate is listed as 7.04 min. When the analyzer had been calibrated (with perchloric acid added to the calibration mixture), a retention time of 7.38 min had been established for aspartate. Thus, the small deviation from the ideal retention time proved to be of no consequence. In Fig. 2, which shows another chromatogram from the same series, the retention time for aspartate is recorded at 6.43 min. This retention time proved to be recorded before the window opened and although the peak was given a retention time, quantitation of the peak

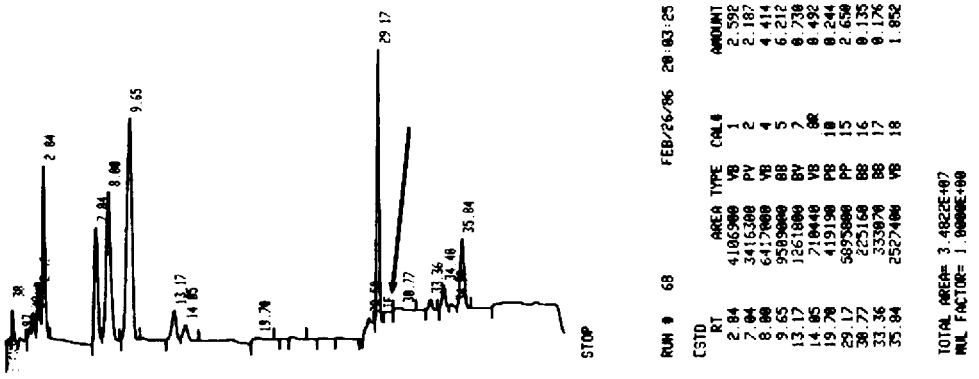

Fig. 1. Chromatogram of deproteinised nervous tissue extract showing the inclusion of aspartate in analysis report.

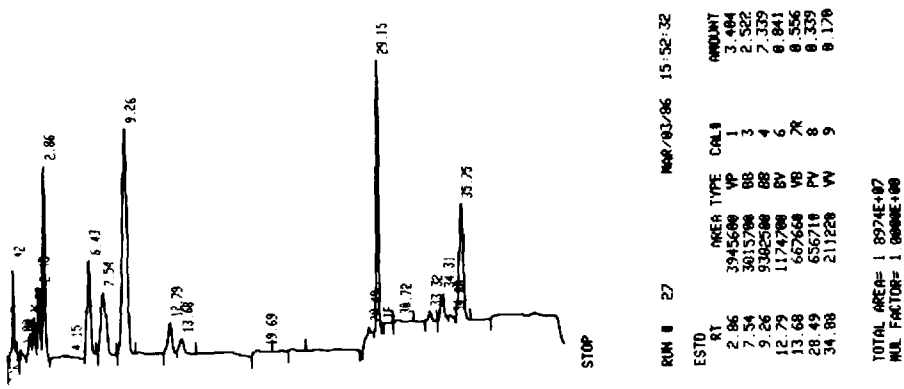

Fig. 2. Chromatogram of deproteinised nervous tissue extract showing the omission of aspartate from the analysis report.

did not take place. For those extracts in which aspartate had been delayed beyond the duration of the window a simple procedure was adopted; one of the reagents supplied with the System 6300 Analyzer was an alkali solution labelled Na-R, and a droplet of this reagent was added to the extracts before re-analysis. Addition of the alkali permitted the retention time to be shortened and the aspartate peak was now within the window. However, for those extracts in which aspartate had been prematurely eluted from the column, *i.e.*, before the window opened, the procedure initially adopted of adding further amounts of perchloric acid to the extract (so as to prolong the retention time of aspartate) proved to be unreliable. Thus, it was decided to utilize the integrator's capacity to store methodologies; the amino acid analyzer was recalibrated with standard mixtures having a variation in pH and each stored as a separate methodology. These methodologies provided an overlap of the aspartate window and extended the open time duration in both directions. If the initial analysis did not provide a value for aspartate, the alternative methodology was used for a repeat analysis of the extract.

In the cases where minute additions of reagents had been used to adjust the pH of the samples, no significant effect on the accuracy of the levels of amino acid amounts reported was discovered. The presence of varying amounts of perchloric acid in the extract did not have any significant effect on the retention times for taurine. Although taurine is eluted before aspartate, at 2.84 min in the chromatogram shown in Fig. 1 and at 2.86 min in Fig. 2, no omission from the final reports was observed for this amino acid. It is concluded, therefore, that taurine is unimpeded in its passage through the ion-exchange column.

The omission of the amount of amino acid from a final report can occur even when a peak is accurately located with the window allocated to it. Interference to the integrator's monitoring of a peak can occur if a forced baseline correction has been programmed to take place as the integrator is about to print out a retention time for a peak. Such is the case in Fig. 2 where the γ-aminobutyric acid value has been left out although a retention time of 29.15 min has been assigned to the peak. The occurrence of an automatic baseline correction has been marked by an arrow in Fig. 1. The purpose of this function is to compensate for the entry of a more concentrated buffer into the colorimeter of the analyzer.

The linking of the Hewlett-Packard Model 3390A reporting integrator to the Beckman System 6300 analyzer has proved to be successful for the routine, rapid analysis of proteins. It is when analytical samples have been specially treated during their preparation for analysis that the integrator's deficiency becomes demonstrable. The rigid fixed range of window openings utilized prevents modest adjustments to a methodology to be made, and once a methodology has been prepared, the storing of this methodology into the memory of the integrator precludes any further attempts to change any parameter contained in that methodology. To make even a minor change in operating conditions the entire methodology has to be deleted and the analyzer recalibrated.

REFERENCES

1 D. C. Olson, G. J. Schmidt and W. Slavin, *Chromatogr. Newsl.*, 7 (1979) 22.
2 L. B. James, *J. Chromatogr.*, 68 (1972) 123.
3 A. L. Fimmel, D. A. Jans, L. Langman, L. B. James, G. R. Ash, J. A. Downie, A. E. Senir, F. Gibson and G. B. Cox, *Biochem. J.*, 213 (1983) 451.
4 A. L. Fimmel, D. A. Jans, L. Hatch, L. B. James, F. Gibson and G. B. Cox, *Biochim. Biophys. Acta*, 808 (1985) 252.