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Effect of the pH of the test solution on amino acid ion-exchange chromatography in a lithium cycle

The use of lithium citrate buffers as eluants of ion-exchange resin columns for the automatic chromatography of amino acids and related compounds found in biological fluids is becoming more widespread, particularly when glutamine and asparagine are present in the sample.

In our experience it has been found that when biological samples are chromatographed with this elution system, even slight variations of the pH of the amino acid solution applied to the long column give rise to large variations of both the elution times and the peak fraction volumes of some acidic amino acids, whose resolutions and peak shapes are consequently altered.

This phenomenon, which usually does not occur when sodium buffers are used, particularly affects peak evaluations carried out by measuring the peak height over the base-line. However, the evaluation by the $H \times W$ integration method can also become inaccurate if too great a displacement of a peak causes overlapping of different peaks.

It has been observed that, of acidic amino acids, the most sensitive to pH changes in the feed pulse is aspartic acid, whose peak can often undergo such great variations in elution time and shape that its digitization can no longer be carried out by the height method and other methods of evaluation must be used.

On the other hand, it must be stressed that the adjustment of the pH of a de-proteinated biological sample in order to match its pH with that of the calibration mixture is not always possible, or at least is not easy to accomplish.

In our laboratories, a method of sample de-proteination and conditioning that can overcome these difficulties is under investigation and in the present note we report some experimental results that illustrate the relation that exists between the pH of the test solution applied to the column and the elution times, peak heights and peak areas of some acidic amino acids eluted with a lithium buffer according to the method previously described by MONDINO[1].

Experimental

An automatic amino acid analyzer, previously described by MONDINO[2] and now manufactured by Optica Co., Milan, was used.

A long column with an I.D. of 10.4 mm was filled to a height of 37 cm with Amberlite IR-120 resin prepared as previously described[1] and operated in a lithium cycle at 1.2 ml/min using a lithium buffer of pH 2.8 whose composition has already been given[1].

The ninhydrin colour reagent and other conditions and parameters were similar to those previously described[1,2].

The optical path of the flow-cuvette in the colorimeter reading at 570 nm was 1 cm, as the sensitivity was set at an optical density of unity for a full-scale deflection of the recorder pen. The chart speed on the recorder was set at 30 in./h in order to permit a good evaluation of peak widths when the peak areas obtained by the $H \times W$ integration method were used.

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TABLE I

INFLUENCE OF THE pH OF THE TEST SOLUTION ON THE ELUTION TIME, PEAK HEIGHT AND PEAK AREA OF SOME ACIDIC AMINO ACIDS

The mean values were obtained from four determinations.

No.	Amino acid	Solution (a), pH 2.61					Solution (b), pH 2.19				
		t[a] (min, sec)	$\bar{H}$[b]	$VC_{\bar{H}}$[c]	$\bar{A}$[d]	$VC_{\bar{A}}$[e]	t (min, sec)	$\bar{H}$	$VC_{\bar{H}}$	$\bar{A}$	$VC_{\bar{A}}$
1	Taurine	27, 00	131.1	1.80	1437.1	2.62	27, 00	127.5	2.21	1441.2	2.19
2	Aspartic acid	62, 00	66.2	2.15	1715.9	1.78	63, 00	76.3	1.78	1839.4	2.38
3	Threonine	80, 00	55	2.13	1766.8	2.71	80, 00	55.1	1.76	1829.0	1.29
4	Serine	84, 00	58.7	2.09	1825.7	1.52	84, 00	58.7	2.01	1865.3	2.60
5	Glutamic acid	99, 00	40.4	2.01	1809	2.86	99, 00	40.6	1.67	1810	2.78

TABLE I (*continued*)

No.	Solution (c), pH 1.86					Solution (d), pH 0.91					Solution (e), pH 0.85				
	t (min, sec)	$\bar{H}$	$VC_{\bar{H}}$	$\bar{A}$	$VC_{\bar{A}}$	t (min, sec)	$\bar{H}$	$VC_{\bar{H}}$	$\bar{A}$	$VC_{\bar{A}}$	t (min, sec)	$\bar{H}$	$VC_{\bar{H}}$	$\bar{A}$	$VC_{\bar{A}}$
1	28, 00	133.8	0.43	1436.2	0.46	27, 45	128.5	0.87	1383.7	2.54	27, 30	130.4	2.29	1451.5	3.88
2	65, 00	81.6	1.13	1777.0	2.06	79, 30	176.7	4.87	1723.3	1.52	80, 00	155.8	6.14	1806	1.03
3	81, 30	56.8	1.26	1787.2	1.26	88, 00	59.9	1.83	1782.3	7.17	87. 30	60.6	2.80	1861	3.00
4	86, 00	60.6	0.47	1854.4	0.85	91, 50	61.03	1.17	1914.1	1.36	91, 45	61.8	2.31	1947.8	1.68
5	100, 40	41.5	1.95	1829.1	3.28	105, 20	41.9	0.85	1840.6	1.63	105, 00	42.9	2.58	1911.4	2.61

The elution times were not averaged, as they were constant in the four chromatograms run for each solution.

[a] t = elution time, min, sec.

[b] $\bar{H}$ = mean peak height, mm.

[c] $VC_{\bar{H}}$ = coefficient of variation of mean peak height.

[d] $\bar{A}$ ≡ mean peak area, mm².

[e] $VC_{\bar{A}}$ = of variation coefficient of mean peak area.

The widths of peaks at half-height were measured in millimeters with a pin-point caliper[3]. In our system, the peak evaluation can usually be achieved with a precision greater than that obtained by the $H \times W$ integration method by simply measuring the heights of the peaks over the base-line[1-3].

Five solutions containing 125 nmole/ml of taurine, aspartic acid, threonine, serine and glutamic acid were prepared by dissolving them in the following media:

(*a*) 1 volume of 0.01 *N* hydrochloric acid and 3 volumes of lithium citrate buffer of pH 2.80. The pH of this solution was 2.61.

(*b*) 1 volume of a 1% aqueous solution of sulphosalicylic acid and 3 volumes of lithium citrate buffer of pH 2.80. The pH of this solution was 2.19.

(*c*) 1 volume of 0.1 *N* hydrochloric acid and 3 volumes of lithium citrate buffer of pH 2.80. The pH of this solution was 1.86.

(*d*) 1 volume of a 1% aqueous solution of sulphosalicylic acid and 3 volumes of 0.25 *N* hydrochloric acid. The pH of this solution was 0.91.

(*e*) 1 volume of a 2% aqueous solution of sulphosalicylic acid and 3 volumes of 0.25 *N* hydrochloric acid. The pH of this solution was 0.85.

A 0.4-ml volume of each solution was loaded on to the drained surface of the resin bed and then forced in by air pressure, followed by 0.4 ml of lithium citrate buffer of pH 2.80, which was forced into the resin column in the same manner for washing purposes. Four chromatograms were run for each solution.

Results

The elution times, the average peak heights and their coefficients of variation, together with the average peak areas and their coefficients of variation for each amino acid and for each solution are reported in Table I. It can be seen that the elution times of each amino acid in the analyses of a particular solution, *i.e.*, at the same pH, are constant, as reflected by the low coefficient of variation on the heights of the corresponding peaks. These times are taken corresponding with the tops of the peaks.

Passing from solution (*a*) through solution (*e*), as the pH of the feeding pulse diminishes, the elution times of aspartic acid, threonine, serine and glutamic acid increase and, in apparent contradiction, the heights of their peaks also increase. This effect can be explained by assuming that at low pH, during the loading operation, the first layers of the resin bed and the acidic amino acids present in the feed pulse become more reactive and consequently these are bound to the resin in narrower distribution zones.

Greater delays in elution times and greater increases in peak height occur with aspartic acid; this particular sensitivity may be related to the fact that the pI of aspartic acid (2.77) is quite near to the pH value of the first lithium buffer.

The large coefficients of variation of the heights of the aspartic acid peaks obtained in the chromatograms of solutions (*d*) and (*e*) are probably due to small and uncontrollable operative variations of the column loading, which, for this amino acid at the low pH values of the feed pulse, can affect the reproducibility of the peak shape from chromatogram to chromatogram.

The coefficients of variation of the peak areas of all the amino acid peaks are constantly low with the exception of those of the threonine peaks in the chromatograms of solutions (*d*) and (*e*). This is due to the overlapping of the threonine peak

with that of aspartic acid, which, in the chromatograms of these solutions, has undergone a large delay. Consequently, the evaluation of threonine peak by the $H \times W$ integration method is less precise and accurate.

TABLE II

COMPARISON BETWEEN EVALUATION BY HEIGHT AND EVALUATION BY AREA OF THE AMINO ACID PEAKS REPORTED IN TABLE I

Amino acid	$\overline{H}$[a]	$SD_{\overline{H}}$[b]	$VC_{\overline{H}}$[c]	$\overline{A}$[d]	$SD_{\overline{A}}$[b]	$VC_{\overline{A}}$[c]
Taurine	130.2	2.44	1.87	1429.9	26.55	1.85
Aspartic acid	111.3	50.98	45.79	1772.2	53.08	2.99
Threonine	57.4	2.63	4.57	1807.2	42.42	2.34
Serine	60.16	1.40	2.32	1881.4	48.91	2.59
Glutamic acid	41.4	1.01	2.43	1840.0	42.06	2.28

[a] $\overline{H}$ = mean value of peak heights of solutions(*a*)–(*e*).
[b] SD = standard deviation.
[c] VC = coefficient of variation.
[d] $\overline{A}$ = mean value of peak areas of solutions(*a*)–(*e*).

In Table II, the means and coefficients of variation of the heights and areas of the amino acid peaks obtained in the chromatograms of all the solutions (four values for each solution) are reported.

It can be seen that the coefficient of variation of the peak heights of taurine, serine and glutamic acid are very similar to those obtained for the areas of the same amino acids. On the contrary, the coefficient of variation of the heights of aspartic acid peaks is considerably higher than that of the areas. The same effect is observed to a lesser extent with the threonine peaks.

From these results, as far as the digitization of the first part of the chromatogram of biological fluids in a lithium cycle is concerned, it can be stated that the evaluation by the height method of the aspartic acid peak can be made only when the pH of the de-proteinated sample solution is the same as or very near to that of the calibration solution; in other words, when the elution times of this amino acid are the same in both the sample and the standard chromatograms. When a difference in the elution time is noticed, the evaluation with the $H \times W$ integration method is preferred as it may give more accurate results. In fact, it has been repeatedly observed in preceding work by MONDINO[1-3] that the condition required for evaluating the amino acid peaks simply in terms of peak heights is a strict repeatibility in the analytical system, when analyses both of the calibration solution and of the sample are carried out.

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