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AN ACCELERATED OPERATION OF A THREE-SAMPLE AMINO ACID ANALYZER*

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SUMMARY

An operational procedure of a three-sample amino acid analyzer is described which facilitates the simultaneous analysis of three samples in slightly more than 6 h. The procedure features the washing and regeneration of the ion-exchange columns with lithium ions followed by the use of an eluent buffer gradient of sodium ions. This accelerated procedure has been found well suited for the analysis of protein hydrolysates, parotid fluid, and protein-free filtrates of plasma and cerebrospinal fluid. When the 6-h procedure is used in conjunction with a previously reported overnight (16-h) operation of the three-sample analyzer, six samples can be readily analyzed within 24 h with instrumentation designed to analyze only one sample per day.

INTRODUCTION

We recently described[1] an automated procedure for quantifying amino acids, imino acids, and other ninhydrin-positive compounds which featured the simultaneous analysis of three samples with an automatic amino acid analyzer designed to analyze only one sample at a time. An added feature of that procedure was the convenience of starting a set of three analyses during the final hour of one work day and terminating the analyses during the first hour of the following work day. This overnight operational procedure has been found especially useful for the analysis of such complex fluids as urine, in which more than 100 ninhydrin-positive compounds have been detected[2].

The present report describes an operational procedure which complements the overnight procedure by utilizing the 7-h interval during the normal work day when the three-sample analyzer would otherwise be idle. This accelerated procedure requires an instrument time of slightly more than 6 h and has been found well suited for the analysis of less complex fluids such as protein hydrolysates, parotid fluid, and protein-free filtrates of plasma and cerebrospinal fluid. As noted below, a novel

* Research reported in this paper was conducted by personnel of the above-named activity of the United States Air Force at Brooks Air Force Base, Texas. Further reproduction is authorized to satisfy the needs of the U.S. Government.

procedural step emerged from this methodologic endeavor which can be advantageously used to enhance the resolving power of the aforementioned overnight procedure.

MATERIALS AND METHODS

Instrument

The three-sample amino acid analyzer used for the 6-h procedure described below differed from the one used for the previously reported overnight procedure[1] in three respects. First, a set of three 90 × 0.636 cm columns packed to a height of 80 cm with Technicon "Chromobeads Type B" (Technicon, Ardsley, N.Y., U.S.A.) was used instead of a set of three 140 × 0.636 cm columns packed to a height of 130 cm with the same resin. Secondly, a Technicon positive displacement pump of the type available in 1972 was used; earlier models of this pump could not deliver eluent buffer to the three columns at the combined rate of 3.00 ml/min. Thirdly, a shaft encoder was used to interface the output of the three-point recorder with a punch-tape teletypewriter which facilitated the computerized analysis of chromatograms. The instrumentational and operational features of this computerized capability are given elsewhere[3].

Reagent, buffers, and buffer gradient

The ninhydrin reagent and sodium citrate buffers of pH 2.88 and 5.00 were prepared in accordance with instructions furnished with the analyzer[4]. These buffers were used to produce the buffer gradient in the following manner: (i) nine volumes of the pH 2.88 buffer were mixed with one volume of reagent-grade ethylene glycol immediately prior to use, (ii) 120 ml of this buffer mixture were added to each of the first five compartments of a Technicon nine-chambered Autograd, and (iii) the same volume of the pH 5.00 buffer was added to each of the four remaining compartments of the Autograd. A lithium citrate buffer was prepared as described by Kedenburg[5], with two exceptions, *i.e.*, the pH was adjusted to 2.60 instead of 2.80 and one volume of ethylene glycol was added to nine volumes of this buffer prior to its use as the regeneration buffer. A 0.3 *N* lithium hydroxide solution was used for washing the columns at the conclusion of each run.

Procedure

The columns were subjected to a 20-min wash cycle and a 60-min regeneration cycle prior to each run. The columns were maintained at 50° throughout the analysis period of 6 h as well as during the wash and regeneration cycles. After the sample had entered into each column, the space above the surface of each column was filled with regeneration buffer. This buffer was also present in the tubing between the Autograd and the three columns. As customary, the run was started by activating the eluent pump and immediately adjusting the four-way stopcock so as to withdraw buffer from the Autograd. The reagent baselines were adjusted after the recording of "breakthru" effluent which occurred approximately 25 min after the run had been started. The analyzer was then left unattended for the next 5½ h, at which time the first chamber of the nearly evacuated Autograd was isolated by closing the second valve and 50 ml of the pH 5.00 buffer were added therein. After 15 min the run was

terminated and the wash cycle was simultaneously started by an appropriate turn of the four-way stopcock.

RESULTS

The chromatograms depicted in Fig. 1 illustrate the resolving power of the herein described accelerated operation of the three-sample amino acid analyzer. Although the three baselines are usually adjusted so as to overlap one another at an approximate optical density of 0.010, the baselines were widely separated for these purely illustrative chromatograms. The perfect synchrony of the three elution patterns attests to the equal distribution of flow attainable from using a single eluent pump for the simultaneous elution of three chromatograms. In this connection it was found that the flow-rates for the three columns varied by less than 1 % when pumping at a total rate of 3.00 ml/min. Whereas this demonstrated ability of the three columns to yield identical elution patterns is requisite for a qualitatively and quantitatively sound procedure, it is obvious that considerable difficulty would be encountered in quantifying these chromatograms by the conventional dot-counting technique. Although not specifically acknowledged in our earlier report[1], this difficulty was inherent in the overnight operation of the three-sample analyzer. It was primarily for this reason that efforts were made to quantify chromatograms by a computerized technique; the time-saving feature of using such a technique was of secondary importance.

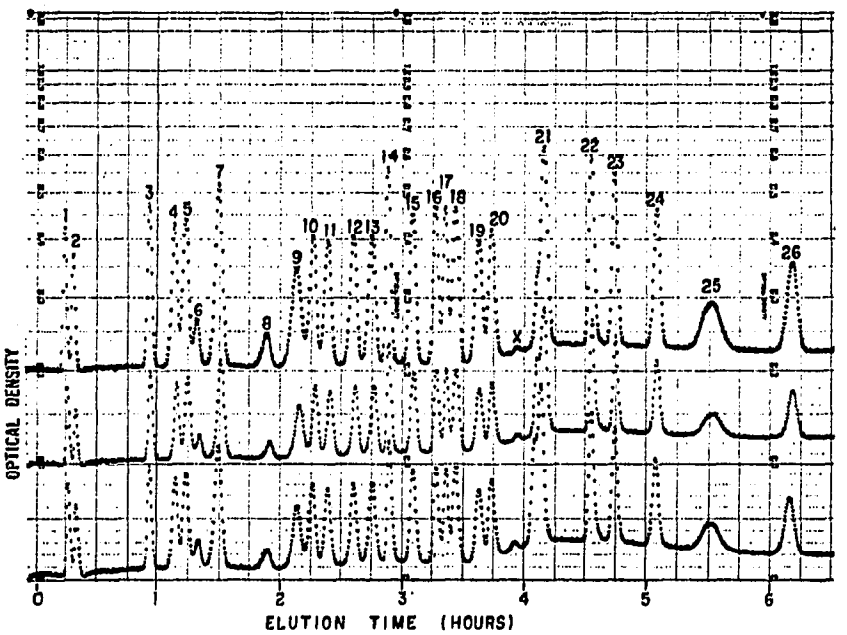

Fig. 1. Chromatograms of standard amino acid mixture. The numbering of peaks corresponds with the numbering of the amino acids in Table I. X denotes contaminant from regeneration buffer. The quantity analyzed for the lower and middle chromatograms was 0.025 μmoles for each constituent, except for urea, which was 1.00 μmoles; the upper chromatogram was based on 0.075 μmoles for each constituent, except for urea, which was 3.00 μmoles.

That the present accelerated procedure is quantitatively sound is reflected by the data shown in Table I. As an overall appraisal, the coefficients of variation found for columns 1, 2, and 3 averaged 2.1, 1.6, and 2.0%, respectively. This relatively high degree of reproducibility is virtually the same as that reported for the overnight operation of the three-sample analyzer[1]. However, a striking difference between the data obtained from the two procedures is in the area constants. In this connection the constants obtained from the overnight procedure were, in most instances, precisely twice as high as those shown in Table I. This apparent loss in sensitivity of the present procedure is attributed primarily, if not solely, to the necessity of eluting the columns at twice the flow-rate, *i.e.*, 3.00 ml/min *versus* 1.50 ml/min.

TABLE I

ANALYSIS OF STANDARD AMINO ACID MIXTURE*

No.	*Amino acid*	*Area constant (H × W/μmole)***		
		Column 1	*Column 2*	*Column 3*
1	Taurine	19.05 (2.5)	16.79 (2.5)	18.45 (2.5)
2	Urea	13.06 (3.6)	10.52 (2.8)	12.43 (2.5)
3	Aspartic acid	28.69 (1.3)	26.00 (0.7)	25.13 (0.9)
4	Threonine	28.85 (0.8)	26.57 (0.9)	25.66 (0.3)
5	Serine	29.43 (2.1)	26.96 (1.4)	26.49 (0.9)
6	Glutamine	13.26 (4.0)	12.21 (3.4)	12.21 (2.6)
7	Glutamic acid	49.28 (1.6)	43.81 (0.7)	43.76 (1.8)
8	Proline	7.65 (2.1)	6.89 (2.0)	6.76 (1.6)
9	Citrulline	29.12 (1.5)	26.72 (1.0)	26.62 (3.5)
10	Glycine	29.98 (1.7)	27.27 (1.3)	26.94 (2.0)
11	Alanine	28.19 (2.1)	25.23 (1.0)	24.82 (1.9)
12	α-Amino-*n*-butyric acid	28.17 (1.8)	25.31 (1.1)	24.59 (2.7)
13	Valine	27.31 (1.4)	24.50 (1.8)	23.91 (1.8)
14	Cystine	35.22 (2.5)	31.49 (1.6)	30.31 (1.9)
15	Methionine	29.71 (2.0)	26.84 (1.7)	25.57 (2.7)
16	Isoleucine	27.41 (2.2)	24.55 (2.3)	24.04 (3.0)
17	Leucine	29.11 (2.4)	26.34 (2.2)	25.38 (3.4)
18	Norleucine (internal standard)	29.32 (2.5)	26.26 (2.0)	25.53 (3.2)
19	Tyrosine	26.84 (3.0)	24.37 (2.8)	22.79 (3.0)
20	Phenylalanine	27.07 (0.9)	24.68 (0.7)	25.19 (0.9)
21	$(NH_4)_2SO_4$	42.18 (4.6)	37.22 (2.8)	39.95 (3.9)
22	Ornithine	39.06 (1.7)	34.95 (0.6)	33.23 (2.1)
23	Lysine	36.84 (1.5)	32.83 (1.1)	31.44 (1.2)
24	Histidine	33.29 (1.8)	29.88 (0.9)	28.40 (1.3)
25	Tryptophan	27.98 (1.2)	25.55 (1.4)	23.76 (0.9)
26	Arginine	30.03 (2.2)	27.04 (0.3)	25.70 (1.0)

* The quantity analyzed was 0.125 μmoles for each constituent, except for urea, which was 5.00 μmoles.

** The values are mean ($n = 5$); the coefficient of variation (as %) is given in parentheses.

In comparing the color constants obtained from the three sets of data shown in Table I, it is apparent that those obtained from columns 2 and 3 were very similar but were approximately 10% lower than those obtained from column 1. A preliminary inquiry into the reason for the greater sensitivity of column 1 revealed that the set of interference filters used in colorimeter 1 differed in optical properties from those used

in colorimeters 2 and 3. This difference in sensitivity is of no consequence in the routine analysis of biologic specimens since chromatograms obtained from a given system are quantified by color constants periodically determined for that particular system.

DISCUSSION

In undertaking this endeavor to establish an accelerated procedure that would complement the overnight operation of a three-sample amino acid analyzer, initial attention was focused on separating and quantifying the amino acids commonly found in protein hydrolysates. For this reason, a standard mixture containing eighteen amino acids was used for experimental testing. Very little difficulty was encountered in establishing what appeared to be a suitable procedure. The resulting procedure differed from the overnight procedure[1] in only three respects, *i.e.*, the columns were 50 cm shorter, the flow-rate of eluent buffer was doubled, and the total volume making up the buffer gradient was reduced by 25%.

Prior to formally reporting the accelerated procedure mentioned above, an effort was made to determine if the procedure might be equally as useful for analyzing the free amino acids in plasma. Known amino acid composition of plasma dictated the incorporation of seven additional compounds in the standard amino acid mixture that would be used for this further testing. As shown in Table I, those additional compounds were taurine, urea, glutamine, citrulline, α-amino-n-butyric acid, ornithine, and tryptophan. Upon analyzing this mixture, the procedure was found inadequate in the following respects: (i) taurine and urea were poorly resolved, (ii) threonine, glutamine, and serine appeared as a single peak, and (iii) proline and citrulline appeared as a single peak on the descending side of the glutamic acid peak. Subsequent experimentation revealed that adequate separation between urea and taurine could be achieved by merely regenerating the columns at a pH of 2.60 instead of 2.88. It was apparent after further testing, however, that a rather drastic departure from the overnight procedure would be needed to correct the other two problematic regions of the chromatogram.

From the recent literature it seemed likely that an existing accelerated procedure which utilized a lithium buffer gradient might accomplish the desired objective. The procedure initially selected for trial was the one reported by Kedenburg[5]. It was promptly found that the threonine–glutamine–serine complex was resolved into three discrete peaks by the selective delay in the elution of glutamine. Moreover, it was found that the glutamic acid–citrulline–proline complex was resolved into three discrete peaks by the selective and disproportionate delay in the elution of proline and citrulline. Unfortunately, these highly desirable findings were accompanied by two undesirable features. First, inadequate separation was found for the compounds eluted between α-amino-n-butyric acid and leucine. Secondly, almost 8 h were required for the elution of arginine, which, in view of the added time needed to wash and regenerate the columns, rendered the procedure too time-consuming to be compatible with the overnight procedure.

Before examining other published lithium gradients, it was reasoned that the presence of lithium ions at the start of the run might resolve the threonine–glutamine–serine and glutamic acid–citrulline–proline regions of the chromatogram and that the

subsequent use of sodium ions in the buffer gradient might improve the separation in the α-amino-*n*-butyric acid–leucine region and, at the same time, hasten the elution of the basic amino acids. Accordingly, the columns were washed with 0.3 *N* LiOH and regenerated with a pH 2.60 lithium citrate buffer, and the columns were then eluted with the same sodium citrate gradient that had previously proved useful for protein hydrolysates. The results obtained from this rather unorthodox procedure were most gratifying as good resolution was achieved throughout the entire chromatogram and arginine was eluted in only slightly more than 6 h. That procedure was adopted without further modification and is the one that forms the basis of this report.

In addition to forming a vital part of the present 6-h procedure, the use of lithium ions for washing and regenerating columns in conjunction with an elution gradient of sodium ions has been found to increase the resolving power of the previously reported overnight procedure[1]. With respect to the latter application, it was initially found that a 30-min wash cycle with 0.3 *N* LiOH followed by a 90-min regeneration cycle with the pH 2.60 lithium citrate buffer resulted in an almost complete overlap of the valine and cystine peaks. Prior experience with this sodium gradient had shown that the elution of cystine could be selectively delayed by placing a greater volume of starting buffer (pH 2.88) in the Autograd. However, as all compartments of the Autograd were already filled to capacity according to previous specifications[1], the only way to increase the volume of that buffer was to add it to the front six compartments of the Autograd instead of the first five. This change led to two other procedural modifications. First, as a sufficient volume of the pH 5.00 buffer could not be added to the three remaining compartments of the Autograd, an additional volume of 425 ml of this buffer was added to the Autograd 12 h into the run. As the latter step had to be taken shortly after 4:00 a.m., a means was devised to add this buffer automatically from a reservoir. The second modification was that of increasing the eluent pump rate from 1.50 to 1.94 ml/min; this step was taken in order to keep the operation time for the overnight run at 16 h.

REFERENCES

1 J. P. Ellis, Jr. and J. B. Garcia, Jr., *J. Chromatogr.*, 59 (1971) 321.
2 P. B. Hamilton, *Clin. Chem.*, 14 (1968) 535.
3 R. Garcia and J. B. Garcia, Jr., *Technical Report*, U.S. Air Force School of Aerospace Medicine, Brooks Air Force Base, Texas, in press.
4 *Amino Acid Analyzer Instruction Manual AAA-1*, Technicon Corp., Ardsley, N.Y., 1967.
5 C.-P. Kedenburg, *Anal. Biochem.*, 40 (1971) 35.