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CRITICAL REVIEW

GAS CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS IN PHYSIOLOGICAL FLUIDS: A CRITIQUE

D. LABADARIOS*, I.M. MOODIE and G.S. SHEPHARD

Metabolic Unit, Tygerberg Hospital, Department of Internal Medicine, University of Stellenbosch, and the M.R.C. National Research Institute for Nutritional Diseases, P.O. Box 63, Tygerberg 7505 (Republic of South Africa)

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1. INTRODUCTION

Amino acids are fundamental units in the living organism and the extent to which each is present affects the healthy existence of that living species. Because amino acid levels have such far-reaching effects, the ability to determine these levels accurately and precisely assumes paramount importance in many biochemical and clinical investigations.

Until recently it has been a widely held and accepted view, as judged by the number of publications, that such analyses are best accomplished by the classical ion-exchange technique using an automatic amino acid analyser. Despite the high degree of sophistication attained, however, this method has the inherent disadvantages of requiring high initial capital outlay, high running costs, and of being capable of a very limited application, mainly that of amino

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acid analysis. Furthermore, the actual time for analysis of one sample is approximately 2 to 2½ h, which when added to the time required for sample preparation, is a major limiting factor in sample throughput.

These considerations have prompted various research groups to adopt the technique of amino acid analysis based on gas chromatography (GC), despite the added time required in the preparation of volatile amino acid derivatives. In general, capital and running costs of commercially available equipment are considerably lower, elution time is shorter, and the precision and sensitivity achieved using GC are an improvement on those of the amino acid analyser. Thus relative standard deviations for peak areas in amino acid standard mixtures are frequently quoted to be ± 2% at the 5—10 ng level using the analyser, whereas equivalent figures of below 1% are routinely possible using GC. The sensitivity threshold using spectrophotometric detection of amino acid—ninhydrin products is claimed to be 100 pmol (about 10 ng) and this can be extended to 10 pmol (about 1 ng) using fluorescence detection of *o*-phthaldehyde (OPA) amino acid derivatives. These and lower levels (10—100 pg) can be obtained using GC with electron-capture detection and the sensitivity can be further increased by coupling up to a mass spectrometer. This combination of high-resolution and identification capability cannot be equalled by any other technique at present. In addition the gas chromatograph need not necessarily be dedicated to amino acid analysis but has the capability of being used in a wide range of analyses.

GC analysis of amino acids requires the synthesis of their volatile derivatives. Whilst an alternative [1] to the more widely used esterification—acylation procedure for preparing these derivatives offers the potential advantage of permitting analysis of glutamine and asparagine, the preparative procedure contains sample manipulations which do not lend themselves to a laboratory analytical routine. In addition, there is a major disadvantage in that it requires a second short column for the complete elution of tryptophan, cystine and histidine. Thus, it would appear that the most practicable derivatisation procedure is that described by MacKenzie and Tenaschuk [2], modified by Moodie and co-workers [3, 4] and applied to a micro-scale analysis by Labadarios et al. [5]. However, before embarking upon derivatisation steps, biological samples require some form of purification procedure, the one most widely used being that of ion-exchange, in which biological material, in acid medium, is applied to a small column of cation-exchange resin (see Tables 1—3).

The increasing use of GC in amino acid analysis of physiological fluids has resulted in many publications appearing in the literature and it is this area of interest which will now be reviewed.

2. METHODS

A retrospective literature search was conducted using a direct link to the Medline Search system, the National Interactive Retrieval Service, based at the National Library of Medicine, Bethesda, MD, U.S.A.

By using the interacting search terms (1) amino acid, (2) gas chromatography and (3) analysis in blood, plasma, cerebrospinal fluid, urine or any body tissue, applied only to publication titles, the scope of the search was purposely kept

broad so as to have the best chance of including all relevant publications. The literature was scanned from the beginning of 1971 up to June 1983 (English, French and German language).

A total of 941 references derived from 135 journals were captured of which 36 were found to be relevant to the present exercise; that is they contained details of the attempted purification procedure of all protein amino acids from biological fluids, in order to reduce or eliminate interfering substances prior to quantitative GC determination.

3. RESULTS AND DISCUSSION

For convenience in this discussion, the procedure for determining amino acids in physiological fluids and tissue extracts (Tables 1—3) has been divided into four sections (A—D), each containing stages essential to optimal quantitative analysis. The percentage of the authors, appropriately referenced, who perform these steps is also shown in Tables 1—3.

TABLE 1

PERCENTAGE OF REFERENCED AUTHORS PERFORMING THE APPROPRIATE STEPS IN THE PURIFICATION PROCEDURE OF PLASMA/SERUM PRIOR TO GC ANALYSIS OF AMINO ACIDS

Procedure	Percentage of authors	References
A. Deproteinisation	81	13, 16, 18, 19, 20, 22, 25, 26, 29, 33, 34, 37, 40, 41, 44, 45, 46
B. Ion-exchange "clean-up" step	90	7, 13, 16, 18, 19, 20, 26, 29, 33, 34, 35, 37, 38, 39, 40, 41, 44, 45, 46
Details of:		
1. Resin type, column and resin bed size	52	7, 18, 29, 34, 35, 37, 38, 39, 40, 41, 46
2. Resin equilibration/ regeneration	43	7, 13, 16, 18, 38, 39, 40, 44, 45
3. Sample amount applied to resin	57	7, 13, 18, 20, 29, 34, 38, 39, 40, 41, 44, 45
4. Washing step	57	7, 13, 18, 26, 29, 34, 37, 38, 39, 40, 41, 44
5. Elution rate	33	7, 18, 29, 34, 38, 39, 40
6. Eluent volume	43	7, 18, 29, 34, 37, 38, 39, 40, 41
7. Eluate collected	24	13, 18, 26, 34, 35
C. Recoveries of amino acids:		
1. From column	29	13, 26, 38, 39, 41 46
2. From plasma	33	13, 18, 20, 25, 38, 39, 45
3. Amount used in recovery	43	13, 18, 20, 25, 26, 38, 39, 41, 46
4. Reproducibility of recovery	33	13, 18, 20, 25, 38, 39, 46
D. Reproducibility of derivatisation and chromatography	43	20, 25, 29, 38, 39, 41, 44, 45, 46

TABLE 2

PERCENTAGE OF REFERENCED AUTHORS PERFORMING THE APPROPRIATE STEPS IN THE PURIFICATION PROCEDURE OF URINE PRIOR TO GC ANALYSIS OF AMINO ACIDS

Procedure	Percentage of authors	References
A. Deproteinisation/reflux	67	12, 15, 16, 18, 19, 22, 26, 33, 34, 46
B. Ion-exchange "clean-up" step	93	7, 15, 16, 18, 19, 22, 23, 26, 33, 34, 36, 38, 39, 46
Details of:		
1. Resin type, column and resin bed size	60	7, 18, 22, 23, 26, 34, 38, 39, 46
2. Resin equilibration/regeneration	33	7, 16, 18, 38, 39
3. Sample amount applied to resin	60	7, 15, 18, 22, 23, 34, 36, 38, 39
4. Washing step	53	7, 18, 22, 23, 34, 36, 38, 39
5. Elution rate	40	7, 18, 23, 34, 38, 39
6. Eluent volume	53	7, 18, 22, 23, 34, 36, 38, 39
7. Eluate collected	27	15, 18, 26, 34
C. Recoveries of amino acids:		
1. From column	40	15, 22, 26, 38, 39, 46
2. From urine	27	15, 18, 38, 39
3. Amount used in recoveries	47	15, 18, 22, 26, 38, 39, 46
4. Reproducibility of recoveries	27	18, 38, 39, 46
D. Reproducibility of derivatisation and chromatography	40	12, 15, 23, 38, 39, 46

A careful study of these procedural steps, described in these cited publications, reveals a wide diversity both in reagents used and also in the degree of descriptive experimental detail.

A. Deproteinisation

Deproteinisation has been carried out using a variety of reagents, the most common by far being picric acid, followed by sulphosalicylic acid and trichloroacetic acid, both of which have been used to a similar extent. This is in line with the findings described by Ohara and Ariyoshi [6], who compared the performance of a number of plasma protein precipitating reagents. They conclude that (1) no single precipitant leads to good results for all amino acids, (2) picric acid gave the most reliable results except for the basic amino acids and (3) sulphosalicylic acid should be used when dealing with the latter. The problem of variable recoveries associated with the precipitation step has led various workers [7, 13, 38, 39] to adopt a procedure involving sample dilution with acetic acid rather than deproteinisation. Low yields of some plasma amino acid with this procedure made it unattractive, although the use of a reversed-

TABLE 3

PERCENTAGE OF REFERENCED AUTHORS PERFORMING THE APPROPRIATE STEPS IN THE PURIFICATION PROCEDURE OF CEREBROSPINAL FLUID, AMNIOTIC FLUID, TISSUES AND TISSUE EXTRACTS PRIOR TO GC ANALYSIS OF AMINO ACIDS

Procedure	Percentage of authors	References
A. Deproteinisation/hydrolysis	80	11, 14, 16, 17, 21, 24, 27, 28, 31, 32, 41, 42
B. Ion-exchange "clean-up" step	80	9, 11, 16, 17, 24, 27, 28, 30, 31, 32, 41, 42
Details of:		
1. Resin type, column and resin bed type	73	9, 11, 17, 24, 27, 28, 30, 31, 32, 41, 42,
2. Resin equilibration/regeneration	40	9, 11, 16, 24, 28, 32
3. Sample amount applied to resin	47	9, 27, 28, 30, 31, 41, 42
4. Washing step	53	9, 27, 28, 30, 31, 32, 41, 42
5. Elution rate	27	17, 24, 31, 32
6. Eluent volume	60	9, 11, 27, 28, 30, 31, 32, 41, 42
7. Eluate collected	47	9, 17, 24, 28, 30, 32, 42
C. Recoveries of amino acids:		
1. From column	13	9, 41
2. From tissue extract/fluid	7	31
3. Amount used in recovery	7	41
4. Reproducibility of recovery	7	9
D. Reproducibility of derivatisation and chromatography	33	14, 21, 31, 41, 43

flow technique has recently been claimed to greatly improve quantitative recoveries [7].

B. Ion-exchange clean-up

The use of a single cation-exchange step involving Dowex 50W resin was the most commonly encountered clean-up procedure (23 papers). The degree of cross-linking varied from X-2 to X-12 and the resin mesh sizes from 50—100 to 200—400. Both these parameters may be expected to impart different characteristics upon a resin and may further adversely influence quantitative analysis. The importance of standardisation in the type of resin used is substantiated by experimental work [7] showing the extent to which frequency of divinylbenzene cross-links in the ion-exchange polystyrene matrix influences the recovery of some basic amino acids from the resin bed. Some clearly have not recognised this important aspect and even omit to indicate details related to these parameters. Yet others, in addition to a single-stage clean-up, describe the use of both cation- and anion-exchange resins in a dual-step process.

Details in respect of the amount of resin and column dimensions used are widely divergent and do not appear to be related to the quantity of material subjected to clean-up. Furthermore, it is often not clear whether dimensions, when quoted at all, refer to a column containing the resin or to the resin bed itself. The most frequently used resin bed size varied from 5 to 10 cm in length and about 0.5 cm diameter to which deproteinised, centrifuge sample aliquots of 0.5—5 ml or even as large as 15 ml in the case of tissue extracts are applied. Moreover, columns as large as 15 × 1.5 cm diameter, and as small as 50 mg resin "in a pasteur pipette" are also described though the dimensions of the resultant resin bed are frequently not defined. It is noteworthy that the largest and smallest volumes of material for "clean-up" are not applied to the largest and smallest columns respectively.

Procedures for the preparatory washing and equilibration of resins also vary widely. For example, 7 *M* ammonia solution for a 3-h period followed by washing to neutral with water and regeneration with 3 *M* hydrochloric acid has been used on the one hand whereas other workers do not describe any special washing or equilibration steps.

The present data show that only just over one half of the publications indicate the sample volume or the deproteinised aliquot thereof which is applied to the ion-exchange clean-up column. Moreover, scant, if any, reference is made to the exchange capacity of a particular resin bed, surely another important parameter if quantitative recoveries are considered important.

Washing of unwanted material from the column whilst retaining amino acids on the resin is a step described again in about half the publications though the nature and quantity of washing reagent vary considerably and do not appear to relate to either resin bed or sample volume. It is not stated for instance, or even implied, that experiments have been conducted to establish the optimum column operating parameters.

Details of amino acid elution appear in approximately one third of all the papers. This is somewhat surprising as the final stage of recovering amino acids from the resin is reasonably expected to be the most important. Elution rates vary from 1 drop per 5—10 sec to 16 ml/min.

Most frequently ammonia solutions are used as an eluent, the concentration of which varies considerably (1—9 *M*). Only two publications, by the same group, refer to eluting with 1 *M* ammonia solution. The findings of Boila and Milligan [8], which are in agreement with our experience, that the use of ammonia at concentrations in excess of 2 *M* leads to increasingly variable recoveries of amino acids, must therefore throw considerable doubt upon results obtained from procedures using ammonia in excess of 2 *M*. Moreover, Cancalon and Klingman [9] have shown that major losses occur with methionine, phenylalanine, tyrosine and tryptophan and James [10] has reported major losses of arginine not only during the ion-exchange "clean-up" but also during a simple evaporation of a mixture of arginine in ammonia solution even at a concentration of 1 *M*.

C. Recovery of amino acids

It was expected that laboratories would test the recovery of amino acids

from an ion-exchange column by adding known quantities of standards to the column. Comparison of the eluent composition with that of the original standard would give a measure of column recovery efficiency. Moreover, "spiking" of the original sample with a known quantity of standards and passing it through the column would, by comparison with a similarly treated "unspiked" sample, permit an assessment of the recovery of amino acids from the sample. It was surprising to find that, overall, less than half of the publications carried any account of recovery or of analytical precision.

D. Reproducibility of derivatisation

It was also expected that laboratories would report on the reproducibility of the recoveries together with an assessment of the precision of their derivatisation and chromatographic steps. This is considered to be a fundamentally important omission, in view of the importance of sample preparation and its role in accurate quantitative analysis.

4. CONCLUSION

It is concluded that, despite the important advantages offered by GC in amino acid analysis, the existing sample purification procedure by ion-exchange of biological fluids and tissue extracts is far less than optimal. There appears to be an urgent need for standardisation of the steps involved in such purification, especially in view of the increasing interest in amino acid determination in a variety of scientific disciplines and clinical investigations in particular. The impression should not, however, be gained that amino acid analysis is more precise when performed by amino acid analysers, as recent evidence [47] indicates that even in this area "there is clearly room for improvement". Presumably the authors are referring to their collaborative study of amino acid analysis in which a statistical evaluation, involving the interaction of laboratories, sample preparations and determinations was performed. Coefficient of variation for a given laboratory performing a single analysis on a single preparation varied from 5.6% to 151.2%. In this respect the cited conclusion of Gerritsen and Niederwieser [48], that the accuracy of determination of better than 3% claimed for commercial amino acid analysers is "utopian", may be considered more generally appropriate.

5. SUMMARY

The available methodology for sample purification prior to gas chromatographic analysis of amino acids in physiological fluids and tissue extracts is analysed. It would appear that over the past ten years the method of choice is that of an ion-exchange purification step, and little, if any, progress has been achieved in sample purification procedures. The inherent disadvantages of such a methodology are not only perpetuated but also cast some considerable doubt on the accuracy of the quantitative analysis of amino acids.

REFERENCES

1 P. Hušek, J. Chromatogr., 234 (1982) 381.
2 S.L. MacKenzie and D. Tenaschuk, J. Chromatogr., 97 (1974) 19.
3 I.M. Moodie and J.A. Burger, in R.E. Kaiser (Editor), 4th Int. Symp. Capillary Chromatography, Hindelang, May 3—7, 1981, Hüthig, Bad Dürkheim, 1981, p. 523.
4 I.M. Moodie, G.S. Collier, J.A. Burger and B.C. Werb, J. Sci. Food Agric., 33 (1982) 345.
5 D. Labadarios, G.S. Shephard, I.M. Moodie and E. Botha, S. Afr. J. Sci., 80 (1984) 240.
6 I. Ohara and S. Ariyoshi, Agric. Biol. Chem., 43 (1979) 1473.
7 P. Hušek, G. Herzogová and V. Felt, J. Chromatogr., 236 (1982) 493.
8 R.J. Boila and L.P. Milligan, J. Chromatogr., 202 (1980) 283.
9 P. Cancalon and J.D. Klingman, J. Chromatogr. Sci., 12 (1974) 349.
10 L.B. James, J. Chromatogr., 166 (1978) 333.
11 J.D. Schulman, J.T. Queenan and L. Doores, Amer. J. Obstet. Gynecol., 114 (1972) 243.
12 T.W. Larsen and R.F. Thornton, Anal. Biochem., 109 (1980) 137.
13 E.D. Pellizzari, C. Rising, J.H. Brown, R.W. Farmer and L.F. Fabre, Anal. Biochem., 44 (1971) 312.
14 N. Lachovitzki and B. Björklund, Anal. Biochem., 38 (1970) 446.
15 R.F. McGregor, G.M. Brittin and M.S. Sharon, Clin. Chim. Acta, 48 (1973) 65.
16 H.N. Tucker and S.V. Molinary, Clin. Chem., 19 (1973) 1040.
17 M. Butler, A. Darbre and H.R.V. Arnstein, Biochem. J., 150 (1975) 419.
18 R.W. Zumwalt, D. Roach and C.W. Gehrke, J. Chromatogr., 53 (1970) 171.
19 R.W. Zumwalt, K.C. Kuo and C.W. Gehrke, J. Chromatogr., 55 (1971) 267.
20 E.D. Pellizzari, J.H. Brown, P. Talbot, R.W. Farmer and L.F. Fabre, Jr., J. Chromatogr., 55 (1971) 281.
21 M.S. Greaves and J.M.H. Moll, Clin. Chem. 22 (1976) 1608.
22 K.M. Williams and B. Halpern, Aust. J. Biol. Sci., 26 (1973) 831.
23 J. Chauhan, A. Darbre and R.F. Carlyle, J. Chromatogr., 227 (1982) 305.
24 J. Gabrýs and J. Koneckie, J. Chromatogr., 182 (1980) 147.
25 S. Yamamoto, S. Kiyama, Y. Watanabe and M. Makita, J. Chromatogr., 233 (1982) 39.
26 A. Doussin and R. Bourdon, Ann. Biol. Clin., 30 (1972) 417.
27 T.P. Stein, G.P. Buzby, M.J. Leskin and J.L. Mullen, Cancer, 49 (1982) 1476.
28 Y. Okano, M. Kataoka, T. Miyata, H. Marimoto, K. Takahama, T. Hitoshi, Y. Kase, I. Matsumoto and T. Shinka, Anal. Biochem., 117 (1981) 196.
29 H. Frank, A. Rettenmeier, H. Welcker, G.J. Nicholson and E. Bayer, Clin. Chim. Acta, 105 (1980) 201.
30 K.F. Faull, J.R. DoAmaral, P.A. Berger and J.D. Barchas, J. Neurochem., 31 (1978) 1119.
31 W.P. Leighton, S. Rosenblatt and J.D. Chanley, J. Chromatogr., 164 (1979) 427.
32 J. Gabrýs and J. Koneckie, J. Chromatogr., 222 (1981) 345.
33 F.S. Collins and G.K. Summer, J. Chromatogr., 145 (1978) 456.
34 J. Desgres, D. Boisson and P. Padieu, J. Chromatogr., 162 (1979) 133.
35 J. Sanchez and L. Enjuanes, Oncology, 25 (1971) 44.
36 P.J. Finlayson, R.K. Christopher and A.M. Duffield, Biomed. Mass Spectrom., 7 (1980) 450.
37 E.E. Kingston and A.M. Duffield, Biomed. Mass Spectrom., 5 (1978) 621.
38 R.F. Adams, J. Chromatogr., 95 (1974) 189.
39 R.F. Adams, F.L. Vandermark and G.J. Schmidt, J. Chromatogr. Sci., 15 (1977) 63.
40 F.E. Kaiser, C.W. Gehrke, R.W. Zumwalt and K.C. Kuo, J. Chromatogr., 94 (1974) 113.
41 A.M. Lewis, C. Waterhouse and L.S. Jacobs, Clin. Chem., 26 (1980) 271.
42 T. Tepper, G.K. van der Hem, G.J. Tuma, L. Arisz and A.J.M. Donker, Clin. Nephrol., 10 (1978) 16.
43 M. Wolfensberger and U. Amsler, J. Neurochem., 38 (1982) 451.
44 H. Frank, A. Rettenmeier, H. Welcker, G.J. Nicholson and E. Bayer, Anal. Chem., 54 (1982) 715.

45 D.E. Matthews, E. Ben-Galim and D.M. Bier, Anal. Chem., 51 (1979) 80.
46 C.W. Gehrke, R.W. Zumwalt and K.C. Kuo, J. Agric. Food Chem., 19 (1971) 605.
47 A.P. Williams, D. Hewitt, J.E. Cockburn, D.A. Harris, R.A. Moore and M.G. Davis, J. Sci. Food Agric., 31 (1980) 474.
48 T. Gerritsen and A. Niederwieser, in H.E. Curtius and M. Roth (Editors), Clinical Biochemistry, Principles and Methods, Vol. 2, De Gruyter, Berlin, New York, 1974, p. 1062.