Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 783-790

Quantitative Determination of Amino Acids in Tissue Culture Cells by High Performance Liquid Chromatography

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Received August 12, 1982

Amino acids derivatized with o-phthaldialdehyde were separated by high performance liquid chromatography on a reverse phase C8 column with a ternary gradient. Multilevel calibration permits the analytical quantitation within the range of 50 to 3500 pMol of the individual amino acids in one run. The reproducibility of the analysis in consecutive runs is ± 3%. HeLa cells grown in suspension cultures were harvested by either centrifugation in the cold or by centrifugation through dibutylphthalate. Amino acids were extracted with methanol-water. Cells not exposed to aqueous media prior to extraction show an up to 3 fold higher level of amino acids in the intracellular pool.

INTRODUCTION

The classic method of amino acid analysis based on the ninhydrin reaction (1), has been complemented recently by HPLC (high performance liquid chromatography) methods based on derivatization of amino acids with fluorescamine (2), dansyl chloride (5-dimethylaminonaphthalene-1-sulphonylchloride) (3), dabsyl chloride (dimethylaminoazobenzene sulphonic acid) (4) and OPA (o-phthaldialdehyde) (5, 6). Several problems have arisen in cell biology which require rapid multisample analysis of the intracellular levels of amino acids in tissue culture cells. The application of HPLC techniques shortens the time required for analysis and increases the sensitivity for quantitation of amino acids (7, 8). Derivatization by OPA combines the advantages of increased sensitivity and short reaction time. The use of a ternary gradient allows the clean separation of all derivatized amino acids except for proline and cysteine, which are not clearly detectable (9). For derivatization we use the pre-column procedure described by JONES et al. (9). One major problem in analysis of amino acid pools in tissue cells derives from the fact that intracellular amino acids exchange with external amino acids and rapidly exit from cells in medium or buffers without amino acids. In the past amino acid pools have been

0006-291X/82/180783-08$01.00/0

determined after washing of cells (10, 11). It was reasonable to assume that washing may lead to loss of intracellular amino acids and therefore to estimates of amino acid content which are too low. This suspicion was verified in the experiments described below where we used centrifugation through dibutylphthalate to harvest cells. The concentrations of individual amino acids in tissue culture cells (HeLa) vary over a 50-fold range. All previous published methods do not allow an accurate estimate of all amino acids within this range in one analysis.

METHODS

The studies were performed with a high performance liquid chromatograph from Spectra Physics (Model SP 8700 with a ternary solvent delivery system) consisting of a microprocessor computing pump and a Rheodyne injection valve (Model 7161 with 10 µL loop). For peak detection we use a filter fluorimeter from Amino Inc. (Model J 4-7439) with a 18 µL flow cell and exitation filter for 330nm. The emission was measured with a 418nm cut-off filter. Chromatographic separations were performed with a Merck Hibar HPLC-column (250x4mm RP-8, 5 µm particle size) fitted with a guard column (Chrompack Inc., 100x2.1mm, 40 µm particle size, spherically RP-8). Integration of peaks from chromatographic separations was achieved using a Spectra Physics Computing Integrator (Model SP 4100). This integrator permits a multilevel calibration based on several consecutive HPLC separations of all 19 amino acid concentrations ranging from 50 to 3500 pMol. The calibration curves of each amino acid were stored in the computer memory. For the determination of unknown amino acid concentrations of a sample the calibration curves , e. g. calibration equations were recalled and exact values of the amino acid concentrations interpolated by the computer. We have compared the quantitative analysis of amino acids by the described HPLC technique to that achieved by use of the classic ninhydrin method. These experiments were performed with the Beckman analyser. 100 pMol of each reference amino acid was used in each assay. Reagents: Phosphate buffered saline (PBS) used for washing cells prior to analysis contains: 0.14 M (8 gr) NaCl, 0.027 M (0.2 gr) KCl, 0.008 M (1.15 gr) Na_2HPO_4, 0.0015 M (0.2 gr) KH_2PO_4 per liter solution. The reagents o-phthaldialdehyde and 2-mercaptoethanol required for derivatization of amino acids were of analytical grade and purchased from Sigma. The other reagents, all of analytical grade were purchased from Merck. Sodium dodecylsulfate was obtained from Fluka. Distilled water was purified by reverse phase chromatography on a RP-8-column. Amino acid standards were diluted with purified distilled water from a Beckman amino acid analysis standard (2.5 µM/mL) to a concentration of 1nM/mL. Aliquots were stored at -20°C. Reagent for derivatization: 27 mg of OPA was dissolved completely in 500 µL ethanol, then 4.5 mL 0.4 M sodium borate buffer (pH 9.5) was added. The solution was mixed with 25 µL mercaptoethanol and allowed to stand for 24 hrs before use. This was done to reduce the level of the baseline. The stability of the reagent was maintained by application of 20 µL mercaptoethanol (per 5 mL), every two days. Derivatization of Amino Acids: 50 µL of diluted amino acid standard or of an unknown sample were mixed with 50 µL SDS-buffer (2% in 0.4 M borate buffer, pH 9.5) and 50 µL o-phthaldialdehyde reagent-solution. After exactly 1 min the reaction was stopped by addition of 50 µL phosphate buffer (0.1 M potassium phosphate, pH 4.0). 10 µL of the mixture was injected into the reverse phase column. Elution Gradient: For the separation of amino acid derivatives we chose a continous ternary gradient which was run according to the following table:

Time	Methanol	KAc, 7 mM, pH 5,4	Acetonitrile
0 min	22.0%	78.0%	0.0%
8 min	22.0%	78.0%	0.0%
27 min	50.8%	44.2%	5.0%
32 min	5.0%	44.2%	50.8%

The flow rate was 0.8mL/min and column temperature was maintained at a constant 32°C. Cells: HeLa cells were grown in suspension cultures in Joklik's modified MEM and 10% new born calf serum as described by Kalvelage & Koch (10). Isolation of Amino Acids from Cells: Amino acid containing methanol extracts were prepared by harvesting the cells by one of the following procedures. a) Centrifugation and washing in the cold: 1.5x 10^7 (15mL) cells were sedimented (5 Min., 200 g) in the cold and washed twice by suspension in 10 mL of ice-cold PBS and centrifugation. b) Centrifugation and washing with sucrose: 5 x 10^6 cells/mL (5 mL) cells were collected by centrifugation and washed twice at 35° C with 0.25 M sucrose. c) Centrifugation through dibutylphthalate: 5x10^6 cells in 5 mL medium, were layered on top of 0,5 mL dibutylphthalate in centrifuge tubes and collected by centrifugation (1800 g) for 5 min. The cells in the pellet were frozen at -20° C and thawed after 0.5 to 4 hrs. 500 µL ice-cold methanol-water, 6 : 4, was added to the cells which were thoroughly mixed and incubated at -20°C overnight. The extract was separated from cell debris by centrifugation (1800 g) for 20 min. Solvent was removed from the extracts by evaporation at 20 torr and at room temperature. The amino acids were dissolved in 600 µL 0.004N NaOH or directly in the borate buffer.

RESULTS AND DISCUSSION

Quantitative Analysis of amino acids

C-8 modified silica-gel resins from several manufacturers were used with different gradients. We obtained a clear separation of derivatized standard amino acids only with a Hibar-column, filled with Lichrosorb RP-8. Fig. 1 shows an example of the degree of separation achieved for an amino acid mixture. The gradient described in the Methods section was set up for the separation of amino acids from small peptides and primary amines present in the methanol extract. The change from ethanol to acetonitrile in the last gradient step yields higher resolution of the OPA derivative of lysine. Furthermore, we found a better regeneration of the column upon treatment with acetonitrile than with methanol.

The intracellular concentration of individual amino acids in HeLa-cells varies over a 50-fold range of concentration (Fig. 2) as determined by OPA-HPLC analysis. In order to quantitate amino acids in this range accurately in one run, a multilevel calibration (see methods) is necessary. In this mode, results obtained with the procedure described in the Methods section have deviations of less than $^+$-5 % in the mid range of concentration and deviations of $\pm$ 10% in the extreme concentration range.

Results derived from multilevel calibration were compared to those of the standard ninhydrin technique. In the mid range of amino acid concentration, the data obtained with the HPLC ninhydrin methods agree to within $\pm$10 %. However, for extreme concentrations the deviation between the two methods is up to $\pm$50 %. This difference is probably due to the lack of a multilevel calibration in the ninhydrin method, since it is also observed in

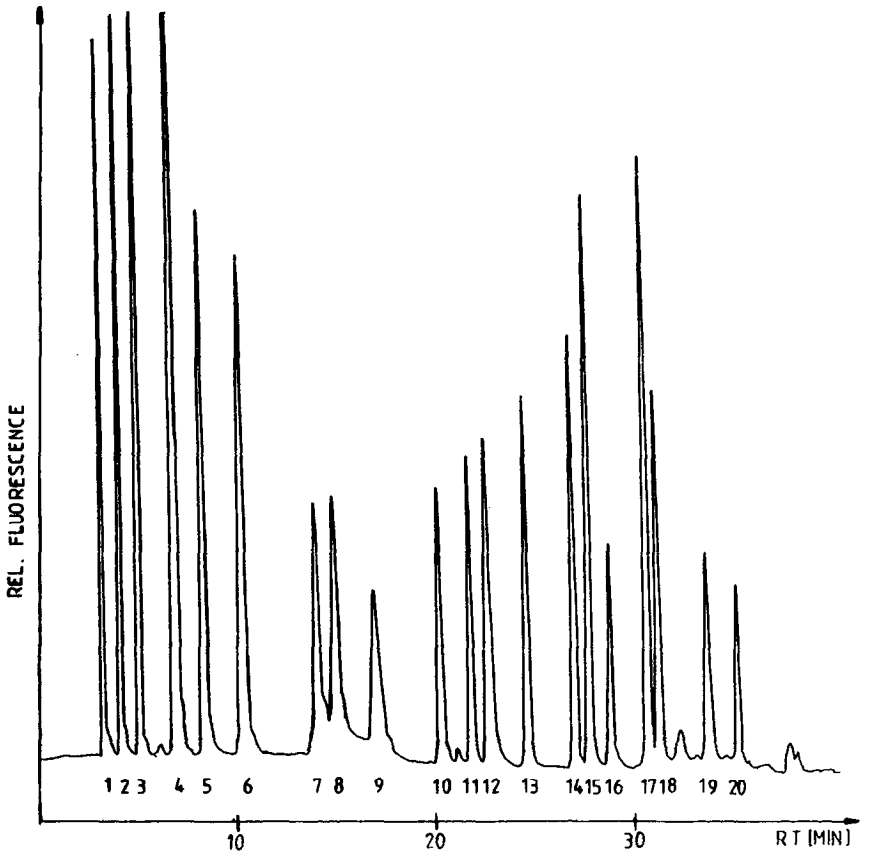

Figure 1

HPLC-Chromatogram of 18 standard amino acids and a reference amino acid. Amino acids in the sample amounts to 175 pMol for each per 10 µL injection volume. The chromatographic conditions and the derivatization are described in the methods section. Peak numbers refer to 1-Cys-SO_3H, 2-Asp, 3-Glu, 4-Asn, 5-Ser, 6-Gln, 7-Gly, 8-Thr, 9-His, 10-Ala, 11-Arg, 12-Tyr, 13-α-aminobutyric acid, 14-Met, 15-Val, 16-Phe, 17-Ile, 18-Leu, 19-Ammonia, 20-Lys.

HPLC analysis of OPA derivatized amino acids without the extended calibration. In contrast to amino acid analysis by the standard ninhydrin technique, the method described here allows clear separation of all intracellular amino acids without interference by other primary amines and small peptides, which are present in the methanol extract of HeLa cells.

Concentrations of amino acids in tissue culture medium and in HeLa cells

In order to determine the difference between the intra- and extracellular concentrations of amino acids in HeLa cells, we measured the amino acid concentration of the medium used for the growth of our cells. Table 1 shows our determination of amino acid

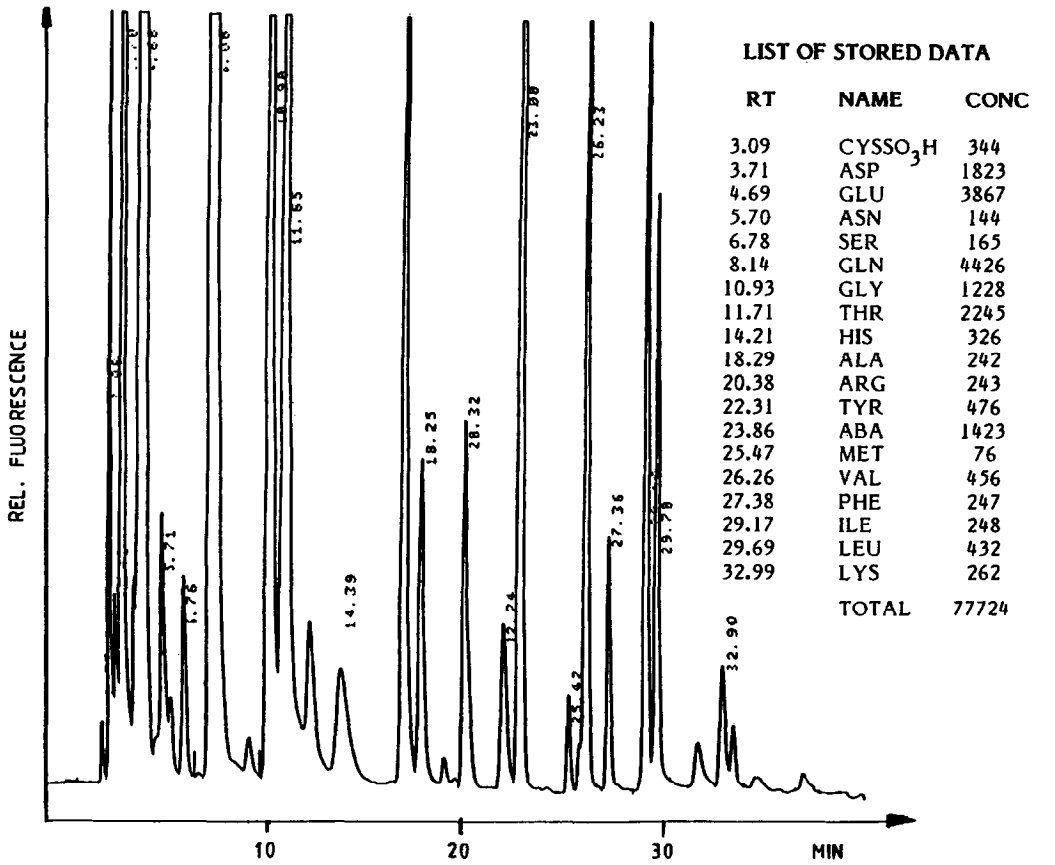

Figure 2

Separation of amino acids found in HeLa-cell extract. HeLa cells were isolated by centrifugation and washing in the cold. Concentrations are listed in pMol for each amino acid.

concentration by the OPA/HPLC procedure. The data are compared to the amino acid concentration of the medium as described by the manufacturer. The differences seen in this comparison are small in relation to the differences in the extra- and intracellular concentrations of amino acids. Table 2 described experiments where three methods were used to harvest cells prior to extraction with methanol - water. Centrifugation through dibutylphthalate prior to analysis permitted detection of high levels of all amino acids present intracellularly. Most amino acids are concentrated approximately 20 fold above the level found in the original growth medium. The degree of concentration of specific amino acids appears to be a property related to both the type and physiological state of the cells (data not shown). It is none the less clear that it is important to remove the cells from aqueous mediums as soon as possible, since washing with 0.25 mM sucrose or washing

Table 1

Quantitative Determination of Amino Acid in Medium containing 10% New Born Calf Serum and Data from the Manufacturer

Amino acids	medium + 10% calf serum via HPLC mol/L	medium data from manufacturer mol/L	deviation in %
Asp	$5{,}2 \times 10^{-6}$	- [2]	
Glu	$7{,}3 \times 10^{-4}$	-	
Ser	$2{,}5 \times 10^{-5}$	-	
Gln [1]	$5{,}6 \times 10^{-4}$	$2{,}0 \times 10^{-3}$	-72 [3]
Gly	$7{,}0 \times 10^{-5}$	-	
Thr	$3{,}5 \times 10^{-4}$	$4{,}0 \times 10^{-4}$	-13
His	$1{,}7 \times 10^{-4}$	$2{,}0 \times 10^{-4}$	-15
Ala	$5{,}9 \times 10^{-5}$	-	
Arg	$4{,}3 \times 10^{-4}$	$6{,}0 \times 10^{-4}$	-28
Tyr	$1{,}7 \times 10^{-4}$	$2{,}0 \times 10^{-4}$	-15
Met	$7{,}8 \times 10^{-5}$	$1{,}0 \times 10^{-4}$	-22 [3]
Val	$3{,}9 \times 10^{-4}$	$3{,}9 \times 10^{-4}$	± 0
Phe	$1{,}7 \times 10^{-4}$	$1{,}9 \times 10^{-4}$	-11
Ile	$3{,}6 \times 10^{-4}$	$4{,}0 \times 10^{-4}$	-10
Leu	$3{,}8 \times 10^{-4}$	$4{,}0 \times 10^{-4}$	-5
Lys	$4{,}4 \times 10^{-4}$	$6{,}0 \times 10^{-4}$	-27

1) Not available in the standard reference amino acid mix. Gln was added to the standard mix at the same concentration as the other reference amino acids.

2) Non essential amino acids are not present in Joklik medium.

3) Losses are possible by breakdown or oxidation.

in the cold with PBS resulted in substantially lower yields of amino acids when compared to cells harvested by centrifugation through dibutylphthalate. The most likely source of this difference between the described preparations is efflux of certain amino acids during exposure of cells to aqueous media. That is obviously the reason why the size of the amino acid pools of HeLa cells as measured by Piez and Eagle (11), correlates rather well with the size of the amino acid pool, which we determined in cells after washing with a cold aqueous solution (see table 2). Certain amino acids which are rapidly exchanged i.e. Asp and Ala, appear to be preferentially lost after centrifugation and washing with aqueous

Table 2

Contents of Amino Acids in HeLa Tissue Culture Cells and Distribution Factors Related to Medium with 10 % New Born Calf Serum

amino acids	amino acids in medium + 10 % serum used for cell experiments	Influence of preparation: cells washed with cold PBS,[1] concentration-factor, relative to medium	cells, washed with sucrose,[1] concentration-factor, relative to medium	cells, washed shortly with salt solution-concentration factor, relative to medium PIEZ and EAGLE (11)	centrifugation through dibutyl-phthalate[1]-concentration factor, relative to medium[3]
	mol/L				
Asp	6.5×10^{-6}	528	68	106	2,500
Glu	1.5×10^{-4}	25	160	14	160
Asn	3.0×10^{-6}	-[2]	210	30	470
Ser	1.5×10^{-5}	14	37	10	80
Gln	1.5×10^{-3}	29	9	11	25
Gly	7.0×10^{-5}	33	56	158	81
Thr	4.1×10^{-4}	11	13	5	29
His	2.1×10^{-4}	9	11	8	21
Ala	4.7×10^{-5}	19	64	18	100
Arg	1.8×10^{-4}	2	2	2	6
Tyr	4.3×10^{-4}	8	10	9	21
Met	6.9×10^{-5}	11	8	5	21
Val	3.3×10^{-4}	5	6	7	17
Phe	1.6×10^{-4}	7	6	8	21
Ile	2.9×10^{-4}	5	6	8	18
Leu	3.1×10^{-4}	6	6	8	18
Lys	3.9×10^{-4}	2	1	3	7

1) See methods section

2) Not determined.

3) After centrifugation through dibutylphthalate about 30% of the medium remains cell associated. However, the concentrations of amino acids in the medium are small in relation to the content of amino acids of HeLa cells.

medium, while amino acids such as Met which do not readily exchange are retained (12). The use of dibutylphthalate centrifugation coupled with HPLC of OPA derivatized amino acids provides a rapid accurate method to determine amino acid pools without artifacts due to efflux of specific amino acids from cells during centrifugation and washing.

ACKNOWLEDGEMENT

We thank Dr. S. Stein, Roche Institute for Molecular Biology, and his associates for stimulating discussions at the onset of this study and for providing us with their unpublished observations.
We are grateful to Dr. John Bilello, Johns Hopkins University, School of Medicine,

Biochemical Virology Laboratorium, 600 North Wolfe Street, Baltimore, Maryland 21205, for helpful support in the preparation of the manuscript.
Supported by Deutsche Forschungsgemeinschaft and Stiftung Volkswagenwerk.

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