Rapid and Precise Determination of Cellular Amino Acid Flux Rates Using HPLC with Automated Derivatization with Absorbance Detection

Application Note

Pharmaceutical, Food Industries

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Abstract

A method is presented for analyzing primary amino acids in complete cell media using an Agilent 1100 HPLC with a ZORBAX Eclipse Plus C18 column with absorbance detection. Amino acids are derivatized with orthophthaldildehyde (OPA) using an online injector program, which decreases preparation time while increasing reproducibility over traditional offline methods. The method is rapid, with an injection-to-injection time of 26 minutes, and highly reproducible, with %RSD of peak area of 0.9 to 4.1 for all amino acids, with most between 1 and 2 percent. Furthermore, this technique is applied to analysis of amino acid flux in the hepatocyte cell line AML12. The flux rate is quantifiable for 18 of 19 amino acids.

Agilent Technologies

Introduction

Determination of amino acid flux in cultured cells is an important indicator of the metabolic rate and health of those cells. It can also be used as an indicator of the remaining carbon and nitrogenous fuel available. This is especially true in hepatocyte and hepatoma cell lines where extensive gluconeogenesis, urea production, and protein synthesis may consume larger quantities of amino acids than other cell types.

HPLC with precolumn derivatization is a standard technique in the analysis of amino acids. Precolumn derivatization of free amino acids in solution for HPLC separations with UV or fluorescence detection is at times done offline, manually. Some immediate drawbacks to offline derivatization are sources of error due to operator skill, competence, and laboratory technique; extra sample manipulation; additional time required; and increased risk of contamination. Automated online derivatization minimizes these error sources, immediately improves precision, and saves time. A rugged high-resolution HPLC method including online derivatization, therefore, can increase productivity compared to offline methods.

Consistent automated OPA-derivatization using injector programming of the HPLC's autosampler, and short, highly efficient columns generate a rapid, reproducible amino acid method ideal for cell culture media. This method is convenient because the cell media samples are simply transferred to autosampler vials and analyzed. The selectivity of the Eclipse Plus C18 and the gradient mobile phase provide high resolution of 19 primary amino acids.

Experimental

HPLC

The ZORBAX Eclipse Plus amino acid analysis (AAA) method was performed using an Agilent 1100 HPLC system and is an adaptation of previously used methods. [1, 2]

- G1312A binary pump, flow rate: 1.5 mL/min

Gradient timetable:

Time (min)	%B
0.0	2.0
0.5	2.0
16.0	47
16.1	100.0
19.5	100.0
19.6	2.0
Stop time 21.0	

- G1329A autosampler, with injection program:
  1. Draw 2.5 µL from borate vial (0.4 M pH = 10.2).
  2. Draw 1.0 µL from sample vial.
  3. Mix 3.5 µL "in air," max speed, 5x.
  4. Draw 0.0 µL from water vial (needle wash).
  5. Draw 0.5 µL from OPA vial (10 mg/mL orthophthaldildehyde, 10 mg/mL 3-mercaptopropionic acid).
  6. Mix 4.0 µL "in air," max speed, 10x.
  7. Draw 0.0 µL from water vial (needle wash).
  8. Draw 32 µL from injection diluent vial (mobile phase A with 1.5% v/v conc. H_3PO_4).
  9. Mix 20 µL "in air," max speed, 8x.
  10. Inject.
  11. Wait 0.1 min.
  12. Valve bypass.
- G1314A Variable Wavelength Detector (VWD) 338 nm wavelength 13.74 Hz

Column

- ZORBAX Eclipse Plus, C18 4.6 x 150 mm, 3.5 µm, p/n 959963-902, with Eclipse Plus C18, 4.6 x 12.5 mm, 5 µm Guard Cartridge, p/n 820950-936, in Guard Column Hardware Kit, p/n 820888-901
- The column temperature was maintained at 40 °C

Mobile Phase and Injection Diluent

Mobile Phase A: 2.8 g anhydrous Na_2HPO_4 + 7.6 g $Na_2B_4O_7.10\ H_2O$ + 16 mg NaN_3 in 2 L H_20. This makes a solution 10 mM in phosphate and 10 mM in borate. Adjust pH with 2.7 mL concentrated HCl, then add 100 µL aliquots until pH 8.15 (approx. 300 µL). Sodium azide (8 ppm) is usually added to prevent microbial growth, but was not added because the mobile phase was only used for a brief time.

Mobile Phase B: methanol:acetonitrile:water (9:9:2, v:v:v)

See **Ordering Information** for descriptions and part numbers of all supplies available for the Eclipse Plus C18 AAA method.

Cell Media and Amino Acid Standards

Complete Cell Media: 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 media with 10% fetal bovine serum, 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL dexamethasone.

Amino Acid Standard: Amino acid standard from Thermo Scientific (p/n 20088) with additions of L-glutamine, L-asparagine, and L-tryptophan. Standard was diluted to make the concentration of each amino acid 800 µM except cystine, which was 400 µM.

Internal Standards: Internal standards were made using 80% cell media with 20% internal amino acid standard. Dilutions of this standard were used to prepare a calibration curve with five equally spaced points. The most concentrated internal standard increased the concentration of the standard relative to the cell media by 160 µM for most amino acids; exceptions were 320 µM each of L-threonine, L-leucine, L-isoleucine, L-valine, and L-lysine, 1,600 µM of L-glutamine, and 32 µM of tryptophan.

Cell Culture

The nontransformed hepatocyte cell line AML12[3] was cultured in five wells of a 12-well plate at a density of 1×10^5 cells per well in 1 mL cell media as described above. After 3 hours, the media was aspirated, the cells were rinsed with Hank's buffered salt solution (HBSS) to remove any dead or unattached cells, and 1 mL fresh media was applied. In addition, five wells of the sample plate were filled with 1 mL cell media without any cells. Each day for three days, 50 µL media was collected from each well and 50 µL fresh media replaced. Collected media was stored at –20 °C until analysis. After the final collection, the media was aspirated from all wells and cells were rinsed three times with HBSS. Plates were stored dry at –20°C for DNA assay.

DNA Quantification

DNA quantification was performed using a picogreen assay (Invitrogen) according to the supplied directions. Cells were lysed with Cell Culture Lysis Buffer (Promega) using four freeze-thaw cycles. The calibration was performed using a linear range of DNA standard from 0 to 4 µg/mL DNA. All calibration standards were run in duplicate, and all samples were run in triplicate. Fluorescence was measured on a CytoFluor II plate reader at 485/530 nm. DNA concentrations from all samples were averaged and subtracted from the averaged media controls to obtain the final concentration of DNA.

Results and Discussion

Complete cell culture media was analyzed on an Agilent 1100 HPLC using the conditions described in the experimental section. The chromatogram was compared to an amino acid standard solution containing 800 µM of each of the 20 standard protein amino acids (shown in Figure 1). The retention time of each amino acid in the culture media closely matches that of the amino acid standard. The concentration of each amino acid in the media is also labeled on the figure. Peak 19, lysine, contains two primary amine groups, thus doubling the concentration of fluorophores and doubling the relative peak intensity.

The reproducibility of the technique for complete cell media was demonstrated by testing five media samples collected after three days in a cell culture incubator. The relative standard deviation for the peak areas and retention time are shown in Table 1. The samples were collected in one continu-

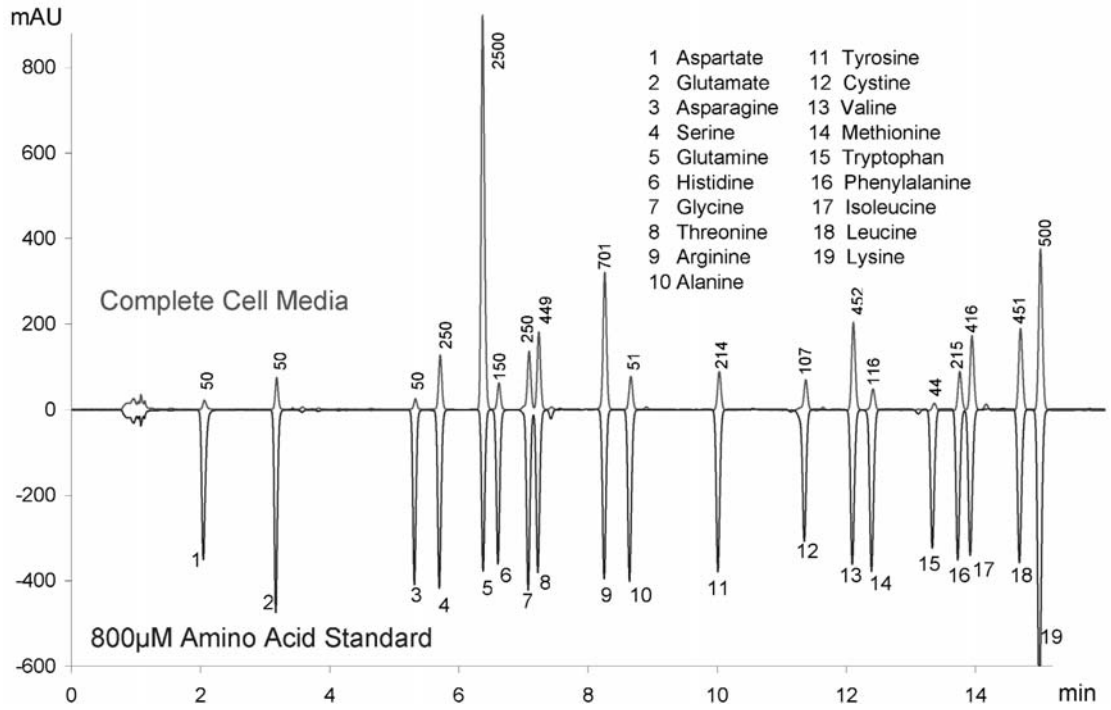

Figure 1. Separation of complete DMEM:F12 cell media (positive axis), 800 µM amino acid standard (negative axis) on Agilent Eclipse Plus C18 column on Agilent 1100 HPLC. Concentration of amino acids in media (µM) shown above each peak and the number of the amino acid in the key is shown below.

ous sequence, which included 39 samples. The individual replicates were spaced evenly throughout the sequence. Therefore, the standard deviations shown do not represent five samples that were run sequentially, but rather were spread throughout the 39-sample sequence. The method is very reproducible. All but two amino acids demonstrated a relative percent standard deviation of less than 2% in peak area, and all amino acids demonstrated a less than 1% deviation in retention time, with 12 of the 10 being less than 0.1%.

AML12 hepatocytes were cultured for three days in a 12-well plate. In addition, media without cells were also placed in the same plate as a control. Five replicates were cultured and aliquots were taken from each well each day for three days. Shown in Figure 2 is an example series of partial chromatograms from each of the three days, including a media control. As can be seen, glutamine decreased in concentration while alanine increased in concentration due to flux from the cells. Other amino acids shown did not change appreciably.

Table 1. Relative Percent Standard Deviations of Peak Areas and Retention Time of Five Samples of Online OPA Derivatized Amino Acids in Complete DMEM:F12 Cell Culture Media (Peak numbers correspond to the numbers in Figure 1.)

Amino acid	Peak no.	Peak area %RSD*	Retention time %RSD
Aspartate	1	1.9	0.44
Glutamate	2	1.0	0.22
Asparagine	3	1.3	0.21
Serine	4	1.2	0.19
Glutamine	5	1.1	0.14
Histidine	6	1.2	0.13
Glycine	7	0.87	0.077
Threonine	8	4.1	0.062
Arginine	9	1.0	0.11
Alanine	10	1.1	0.052
Tyrosine	11	1.0	0.070
Cystine	12	2.7	0.048
Valine	13	1.4	0.045
Methionine	14	0.64	0.036
Tryptophan	15	1.9	0.041
Phenylalanine	16	1.3	0.040
Isoleucine	17	1.3	0.039
Leucine	18	1.0	0.061
Lysine	19	0.90	0.037

*Relative percent standard deviation

Since the cultured cells were replicating, the magnitude of the change increased for each successive day as the number of cells increased.

Quantitative flux rates of amino acids could be obtained for the AML12 hepatocytes as shown in Table 2. This was done by subtracting the media over cells from the control media without cells. To obtain accurate concentration changes, a five-point calibration curve, using internal standards as described in the experimental section, was constructed. The slope of this calibration was used to determine the change in concentration. Data were normalized to DNA. The error represents the 95% confidence interval and was calculated by propagating the error from the media control, media over cells, and the calibration. A change in the amino acid concentration, demonstrated by having the confidence interval less than the calculated flux rate, was demonstrated for all but one. The high reproducibility is demonstrated given that the

Table 2. Flux Rates for Cultured AML12 Hepatocytes in Complete Cell Media (Data is ± 95% confidence interval.)

Amino acid	Flux rate (μmol day^{-1} mgDNA^{-1})		
Aspartate	–7	±	0.8
Gluamate	13	±	1.7
Serine	–38	±	3.9
Glutamine	–306	±	21.0
Histidine	–8	±	2.2
Glycine	18	±	6.5
Threonine	–8	±	10.1
Arginine	–21	±	10.8
Alanine	87	±	5.1
Tyrosine	–10	±	2.8
Cystine	–8	±	2.3
Valine	–25	±	6.6
Methionine	–14	±	2.8
Tryptophan	–3	±	2.2
Phenylalanine	–14	±	3.0
Isoleucine	–19	±	5.7
Leucine	–30	±	5.9
Lysine	–31	±	6.8

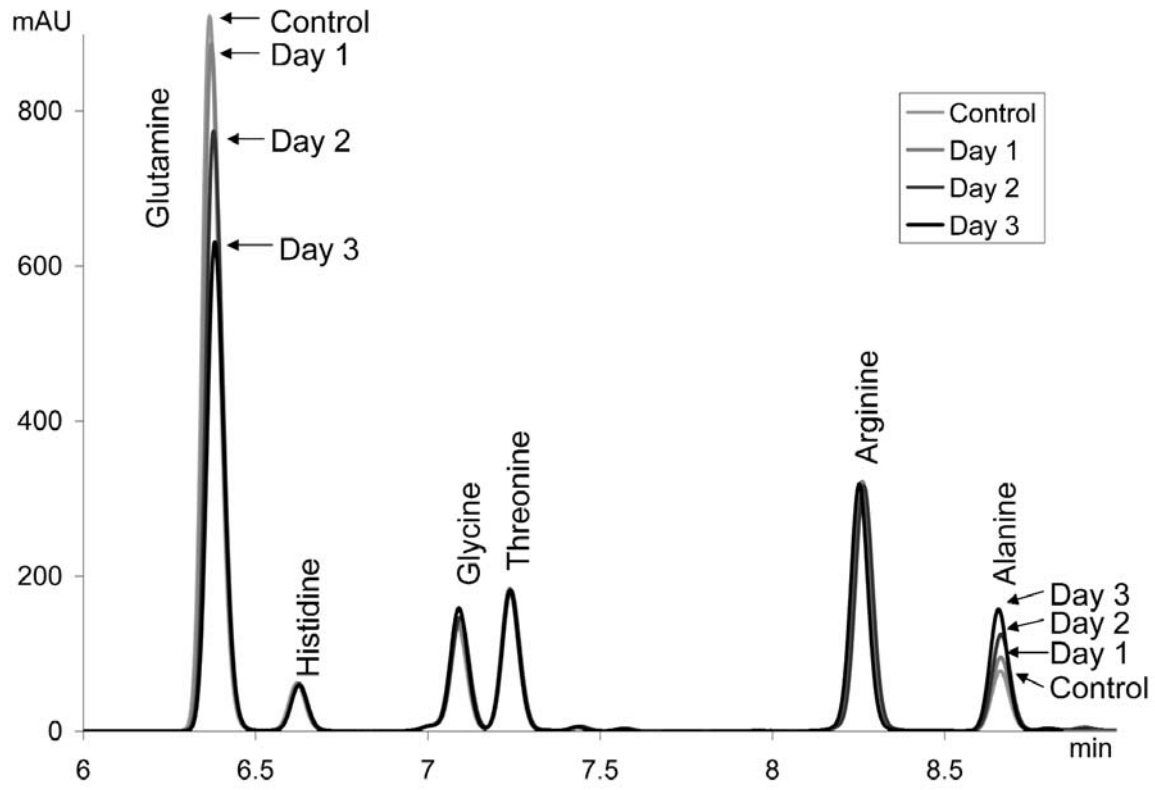

Figure 2. Time series of amino acids in cell media in contact with AML12 hepatocytes. Chromatograms show amino acids glutamine to alanine.

flux rate could be observed even though the percent change in amino acid concentration caused by the cells was as low as 5%.

Conclusions

Cell culture media was easily analyzed for primary amino acids with the Agilent 1100 HPLC equipped with a Rapid Resolution Eclipse Plus C18 column. Samples were measured using an automated online OPA-derivatization method. Reproducibility was exceptional (0.90 to 4.1 %RSD), and likely better than offline precolumn derivatization methods due to minimizing several sources of error. The method is also rapid, with an injection-to-injection time of 26 minutes.

The method was used to measure quantitative flux rates of amino acids from AML12 hepatocytes. Flux rates could be measured in 18 of 19 amino acids, despite having changes in concentration of amino acids in the media of only 5% or more.

References

1. J.W. Henderson, R.D. Ricker, B.A. Bidlingmeyer, and C. Woodward, "Rapid, Accurate, Sensitive, and Reproducible HPLC Analysis of Amino Acids," Agilent Technologies, 2007
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3. J.C. Wu, G. Merlino, and N. Fausto, "Establishment and Characterization of Differentiated, Nontransformed Hepatocyte Cell-Lines Derived From Mice Transgenic for Transforming Growth-Factor-Alpha," *PNAS* (US), 91, 674–678, 1994

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Ordering Information

ZORBAX Eclipse Plus-C18 HPLC Columns			
Description	Size (mm)	Particle Size (µm)	Agilent Part No.
Eclipse Plus-C18	4.6 × 150	3.5	959963-902
Eclipse Plus-C18 Guard Cartridge	4.6 × 12.5	5	820950-936
Eclipse Plus-C18 Guard Cartridge Hardware Klt			820888-901

Derivatization Reagents	
Description	Agilent Part No.
Borate Buffer: 0.4 M in water, pH 10.2, 100 mL	5061-3399
FMOC Reagent, 2.5 mg/mL in ACN, 10 × 1 mL ampoules	5061-3337
OPA Reagent, 10 mg/mL in 0.4M borate buffer and 3-mercaptoproprionic acid, 6 × 1 mL ampoules	5061-3335
DTDPA Reagent for analysis of cysteine, 5 g	5061-2479

Mobile Phase and Injection Diluent Components			
Description	Mfgr.'s	Manufacturer	Mfgr.'s Part No.
Na_2HPO_4, Sodium Phosphate, Dibasic		Sigma	S 7907
$Na_2B_4O_7 \cdot 10H_2O$, Sodium Tetrborate Decahydrate		Sigma	S 9640
NaN_3, Sodium Azide		Sigma	S 2002
H_3PO_4, ortho Phosphoric Acid		Sigma	79617

Vials	
Description	Agilent Part No.
100 µL Conical insert with polymer feet, 100/pk	5181-1270
Amber, wide-opening, write-on, screw-top vial, 2 mL,100/pk	5182-0716
Blue polypropylene cap, PTFE/silicone septum, 100/pk	5182-0721
Clear glass screw cap vial, 6 mL,16 mm cap size, 100/pk	9301-1377
Screw caps, 16 mm, 100/pk	9301-1379
PTFE/silicone septa, 16 mm, 100/pk	9301-1378

Standards	
Description	Agilent Part No.
Amino Acid Standards in 0.1 M HCl, 10 × 1mL ampoules	
1 nmol /mL	5061-3330
250 pmol /mL	5061-3331
100 pmol /mL	5061-3332
25 pmol /mL	5061-3333
10 pmol /mL	5061-3334
Supplemental Amino Acids:	
Nva, Sar, Asn, Gln, Trp, Hyp, 1g each	5062-2478

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