HPLC–FLD for the Simultaneous Determination of Primary and Secondary Amino Acids from Complex Biological Sample by Pre-column Derivatization

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Abstract

A rapid, sensitive, and reproducible pre-column derivatisation procedure has been established for the simultaneous determination of 20 amino acids by high-performance liquid chromatography using fluorescence detection. The amino acids were derivatized using *o*-phthalaldehyde and 9-fluorenylmethyl-chloroformate reagents. The optimal conditions for simultaneous separation and detection of both primary and secondary amino acids were investigated. The developed method has several advantages, namely automated pre-column derivatization, short analysis time with optimal separation, a simple and economical mobile phase, high level of precision for peak area and retention time, and higher sensitivity with more reliability of peak identification. The biological media development is the key parameter for macromolecule drug discovery. Biological media amino acids in three consecutive discovery batches were determined and the results showed a good agreement with hypothetical value. The method appears suitable for application to measure biological media amino acids at various stages of macromolecule drug discovery.

Introduction

The amino acid is a key chemical in all branches of chemistry and life sciences. It has a long history in characterization during discovery and development of drug and therapeutic proteins. The amino acids form a large group of compounds of both biochemical and pharmaceutical interest and their determination necessitate the use of accurate and precise techniques (1).

The amino acids do not absorb UV–vis light significantly; however, to increase sensitivity, their analysis normally includes a derivatization step in which the amino acid react with a precursor to yield a strong fluorescent compound. Derivatization can be performed before the chromatographic separation or after the separation step.

The amino acid analysis was first introduced by Stein and Moore (2), which involved ion exchange chromatography for the separation of underivatized amino acid followed by the post column derivatization with ninhydrin. There are many other reagents such as ninhydrin (3,4), fluorescamine (4,5), dansyl chloride (6), 4-fluoro-7-nitrobenzo-2, 1, 3 oxadiazole (7), phenylisocynate (8), *o*-phthalaldehyde (9,10) that are commonly used in amino acid analysis. Birwe et al. (9) have used *o*-phthalaldehyde (OPA) for pre- and post-column derivatization of pri-

Table I. Structure of Primary and Secondary Amino Acids

Primary Amino acid $H_2N-CH(R)-C(=O)-OH$

Name	–R
Glycine	–H
Alanine	$–CH_3$
Serine	$–CH_2OH$
Threonine	$–CH (OH) CH_3$
Cystein*	$–CH_2SH$
Valine	$–CH (CH_3)_2$
Leucine	$–CH_2 CH (CH_3)_2$
Iso Leucine	$–CH (CH_3) CH_2 CH_3$
Methionine	$–CH_2CH_2SCH_3$
Phenyl Alanine	$–CH_2C_6H_5$
Tyrosine	$–CH_2C_6H_4OH$
Tryptophan	– CH_2 (indolyl, HN)
Aspartic Acid	$–CH_2COOH$
Glutamic Acid	$–CH_2CH_2COOH$
Asparagine	$–CH_2CONH_2$
Glutamine	$–CH_2CH_2CONH_2$
Histidine	$–CH_2$ (imidazolyl, HN, N)
Lysine*	$–(CH_2)_4NH_2$
Arginine	$–(CH_2)_3NH C(NH_2) = NH_2$

Secondary Amino acid (i.e., L-Proline*) — pyrrolidine (HN) with C(=O)–OH

*Not required to quantitate.

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mary amino acids in presence of 3-mercaptopropionic acid to form intensely fluorescent N-substituted-1-alkylthioisoindoles (11). Though several reagents are used with OPA for primary amino acids, but 3-mercaptopropionic acid has higher derivative stability with respect to time. The secondary amino acids do not react with OPA and remain as such in sample – biological media. This limitation is overcome by using 9-fluoronylmethyl chloroformate (FMOC) for derivatization of secondary amino acid [i.e., l-proline (12)].

The biological media for the discovery and production of therapeutic proteins and monitoring of amino acid during development is mandatory. The analysis of amino acid in complex biological mixture is challenging due to presence of many other components like fatty acids, lactic acid etc. Moreover, due to the significant differences in the functional groups of various components, ranging from non polar to highly polar, may interfere with the derivatization and detection of the amino acids.

In this paper a procedure which consists of pre-column derivatization with *o*-phthalaldehyde (OPA) and 9-fluoronylmethyl chloroformate (FMOC) in a borate buffer (alkaline medium) is described, giving fluorescent active iso-indoles that can be separated by reversed phase chromatography. A total of 20 amino acids were identified, out of which 17 amino acids, were added in cell culture and quantified simultaneously using the described procedure. The amino acids studied with structure are given in Table I. Cysteine, lysine, and proline were not monitored in the process and thus were not quantified.

The method shows excellent linearity, accuracy, precision, and very short analysis time (< 19 min) for the baseline resolution of 20 amino acids. OPA used in the present study does not interfere as it is non fluorescent, though its derivatives give fluorescent compounds. The method was accurately validated for specificity, linearity, precision, recovery, limit of detection (LOD), and limit of quantification (LOQ), according to approved guideline (13) and other reports (14–15).

Table II. Autosampler Programming Instruction

Position of Vials
Position 10: Borate Buffer
Position 11: OPA reagent
Position 12: HPLC grade water
Position 14: FMOC reagent
Position X: Sample (Amino acid standard, biological media)
X is any other position in autosampler tray

Derivatization/Injection routine

1	Draw 5 µL from Vial 10 (Borate buffer)
2	Draw 1 µL from Vial 11 (OPA reagent)
3	Draw 0 µL from Vial 12 (Water)
4	Draw 1 µL from X Vial (Sample or Standard Vial)
5	Draw 0 µL from Vial 12 (Water)
6	Mix 8.0 µL in air, maximum speed, six times
7	Draw 1.0 µL from Vial 14 (FMOC)
8	Draw 1.0 µL from Vial 12 (Water)
9	Mix 9 µL in air, maximum speed, 3 times
10	Inject
11	Wait 0.1 min
12	Valve Bypass

Experimental

Chemicals and materials

AR grade sodium acetate trihydrate, triethyl amine, acetic acid glacial, while high-performance liquid chromatography grade methanol and acetonitrile were purchased from S.D. Fine-Chem limited (Mumbai, India). High-performance liquid chromatography (HPLC) grade tetrahydrofuran and AR grade hydrochloric acid (35%, w/w) were procured from Merck (Mumbai, India). The amino acid kit was purchased from Sigma (St. Louis, MO). Biological samples or media were received from Department of Biotechnology, Zydus Research Centre (Zydus Cadila, India), The OPA reagent, FMOC reagent and borate buffer were purchased from Agilent (Waldbronn, Germany). Ultra pure water was obtained from a NANO pure Diamond water purification system (Barnstead, Iowa) with conductivity lower than 0.055 µs/cm. Solutions to be analyzed were filtered through 0.45-µm mdi Nylon syringe filters from Advanced Microdevices Pvt. Ltd., (Ambala Cantt, India). Hypersil-AA ODS stainless steel column, amber wide-opening HPLC vials, glass conical inserts with polymer feet and screw caps were purchased from Agilent (Waldbronn, Germany), while syringes were purchased from Becton Dickinson Indian (P) Ltd., (Haryana, India).

Equipment

HPLC measurement was performed on Agilent 1200 series HPLC–DAD and Agilent 1100 series fluorescence detector (FLD), (Agilent Technologies, Germany). Automated derivatization was

Table III. Gradient Composition of Mobile Phase and Flow Rate*

Time (min)	% A	% B	Flow rate (mL/min)
0.00	100	0	0.45
10.00	78	22	0.45
17.00	40	60	0.45
18.00	0	100	0.45
18.10	0	100	0.45
18.50	0	100	0.80
23.90	0	100	0.80
24.00	0	100	0.45
25.00	100	0	0.45

*Post time: 5.00 min.

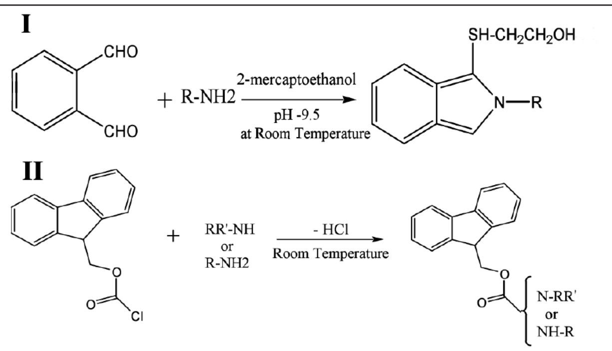

Figure 1. Derivatization reaction for (I) primary amino acids with OPA in 3-mercaptopropionic acid using borate buffer and (II) secondary amino acid using FMOC reagent.

carried out using an Agilent 1200 series autosampler (Agilent, Böblingen, Germany). The data were collected and processed on PC equipped with ChemStation software. Sonication was performed on Sonicator 8891 (Cole-Parmer, Vernon Hills, IL), while the vortexer was purchased from Tarson - 3020 (Kolkata, India). Weighing was done on Analytical balance (AG245) and Microbalance (UMT2) from Mettler Toledo (Greifensee, Switzerland). Autopipettes were procured from Eppendorf (Hauppauge, NY).

Derivatization procedure

The primary and secondary amino acids were derivatized with OPA and FMOC reagent by programming of robotic autosampler (Table II). The N-substituted-1-alkylthioisoindole derivatives formed and their structures are described in Table I and Figure 1.

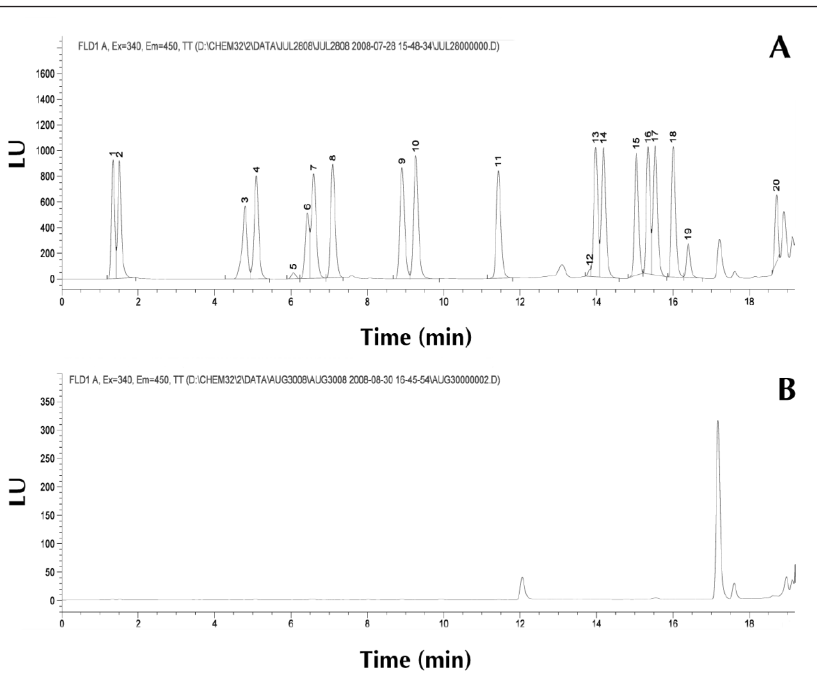

Figure 2. Chromatograms for amino acids (A), and blank solution (B), using 17 mM sodium acetate in mobile phase A and 85 mM sodium acetate in mobile phase B.

Table IV. Order of Elution, Retention Time and Resolution for Amino Acids

Peak No.	Amino acid	Sequence Name	Derivative Type	Retention time (min)	% RSD*	R_s†
1.	Aspartic Acid (ASP)	D	OPA	1.47	0.24	–
2.	Glutamic Acid (GLU)	E	OPA	1.65	0.35	1.08
3.	Asparagine (ASP)	N	OPA	5.06	0.45	19.25
4.	Serine (SER)	S	OPA	5.37	0.33	1.70
5.	Glutamine (GLU)	Q	OPA	6.43	0.17	5.19
6.	Histidine (HIS)	H	OPA	6.78	0.19	1.62
7.	Glycine (GLY)	G	OPA	7.02	0.01	1.12
8.	Threonine (THR)	T	OPA	7.54	0.15	2.52
9.	Alanine (ALA)	A	OPA	9.46	0.15	9.15
10.	Arginine (ARG)	R	OPA	9.81	0.12	1.66
11.	Tyrosine (TYR)	Y	OPA	2.06	0.15	10.81
12.	Cysteine (CYS)‡	C	OPA	14.21	0.15	9.38
13.	Valine (VAL)	V	OPA	14.51	0.11	1.43
14.	Methionine (MET)	M	OPA	14.73	0.12	1.12
15.	Tryptophan (TRP)	W	OPA	15.57	0.11	4.31
16.	Phenylalanine (PHE)	F	OPA	15.90	0.10	1.72
17.	Iso Leucine (ILE)	I	OPA	16.08	0.10	0.87
18.	Leucine (LEU)	L	OPA	16.58	0.10	2.46
19.	Lysine (LYS)‡	K	OPA	6.93	0.09	1.88
20.	Proline (PRO)‡	P	FMOC	19.17	0.04	13.25

* Relative standard deviation
† Resolution factor.
‡ Not for quantification only for identification in this experiment or study.

Chromatographic condition

After automatic derivatization step, an amount equivalent to 1 µL of each sample and standard was injected on a pre equilibrated Hypersil AA ODS, 5 µm (250 mm, length × 4.6 mm, internal diameter) stainless steel column operated at 40°C ± 2°C. The detection of amino acid derivatives was performed using FLD at excitation wavelength (λ Ex) 340 nm and emission wavelength (λ Em) 450 nm from 0 min to 18.0 min. Further, from 18.0 to 25.0 min at the excitation and emission wavelengths were maintained at 266 nm and 305 nm respectively. Mobile phase A was 3% (v/v) tetrahydrofuran in 17 mM sodium acetate trihydrate [pH 7.2 adjusted using 2% (v/v) glacial acetic acid], while mobile phase B was a mixture of 85 mM sodium acetate trihydrate [pH 7.2 adjusted using 2% (v/v) glacial acetic acid]–acetonitrile–methanol (1:2:2, v/v/v). The separation was obtained with gradient program of mobile phase and flow rate (Table III). The total analysis time was 25.0 min.

Standard amino acid preparation

The combined stock solution of amino acids (containing 50 µg/mL of each) was prepared by dissolving their requisite amounts in 0.1N HCl. Calibration standards of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL concentration were prepared by taking 0.2, 0.4, 0.6, 0.8, and 1.0 mL aliquots, respectively from stock solution in 20 mL volumetric flask and diluting with 0.1N HCl. The solutions were filtered through 0.45-µm mdi nylon filter before measurement.

Analysis of biological media

Sample preparation

Sample solution was prepared by mixing 10 µL of biological sample with 950 µL of water, sonicated and vortex mix to get a homogeneous sample. The sample solution was filtered through 0.45-µm nylon mdi filter and used for further study.

Assay procedure

A 1 µL aliquot of all calibration standard solutions was derivatized with OPA and FMOC prior to injection in the HPLC system, equipped with

FLD. Likewise, the sample solutions were derivatized and the amino acid content in each sample was determined from linearity graph.

Validation procedures

Specificity of the method was checked by analyzing blank biological media, diluent (0.1N HCl) and mobile phase (initial ratio) following the assay procedure. Similarly, linearity of the method was ascertained by analyzing the calibration standards. The accuracy of the method was determined as mean recovery of two solutions containing known amount of amino acids comparable with the amount in cell culture feed. Precision, expressed as % relative standard deviation (%RSD) was evaluated by analyzing the highest calibration standard (2.5 µg/mL) in triplicate. Similarly, the biological media from three different batches was analyzed in duplicate.

Results and Discussion

Derivatization

The derivatization process of primary and secondary amino acids with OPA and FMOC reagent in the presence of 3-mercaptopropionic acid in borate buffer was carried out by automated injector program. The derivatization of complex biological media was also performed in the same manner after dilution, however, the complexity of the matrix makes it difficult to derivatize. The stability of the reagent was also crucial during derivatization, the OPA reagent can be used upto seven days (stored at 5°C ± 3°C) from the date of opening the ampoule. After seven days, there was a gradual reduction in resolution factor and intensity of the peak. It may be due to variation in pH of the buffer, concentration of the OPA and its degradation. The FMOC reagent is also useable up to 7 days when stored at 5°C ± 3°C.

Chromatography

During development, a resolution factor of 1.0 was considered optimum for the separation of analytes (i.e., derivatives of amino acids), based on the series of in-house experiments. Except isoleucine (ILE), which had 0.87 as the resolution factor, all other analytes were separated with much higher factor values (Table IV). However, this did not effect the quantitation of iso lucine or other analytes. The elution order for N-substituted-1-alkylthioisoindole derivatives of primary amino acids and N^{α}-(9-Fluorenylmethoxycarbonyl)-L-proline is also mentioned in (Table IV). The method was suitably optimized to have short chromatographic analysis time including derivatization for routine monitoring of large number of samples at industrial level.

Effect of buffer concentration

The effect of buffer concentration was investigated to attain sharp peak shape as well as estabilsh the order of elution with optimum resolution. Initially, 20 mM of sodium acetate in mobile phase A and 100 mM of sodium acetate in mobile phase B were tested, however, the resolution between aspartic acid and glutamic acid was less than 0.5 with a broad peak. Furthermore, different combinations of mobile phase A and B were tried by varying the concentration of sodium acetate, but the resolution between the peaks 6 and 7; 16 and 17 was not adequate. Finally, 17 mM sodium acetate in mobile phase A and 85 mM sodium acetate in mobile phase B gave the best resolution and peak shape for all the analytes. The resolution factor obtained between aspartic acid and glutamic acid was 1.08 (Figure 2A).

Effect of column oven temperature

The effect of column oven temperature was also investigated to achieve optimal resolution. At 25°C, the separation of peaks 1 and 2 (aspartic acid and glutamic acid); peaks 6 and 7 (phenyl alanine and iso leucine) and peaks 16 and 17 (histidine and lycine) were insufficient with resolution factor < 0.5. Raising the temperature to 30°C helped in resolving peak 6 and 7; 16 and 17 with

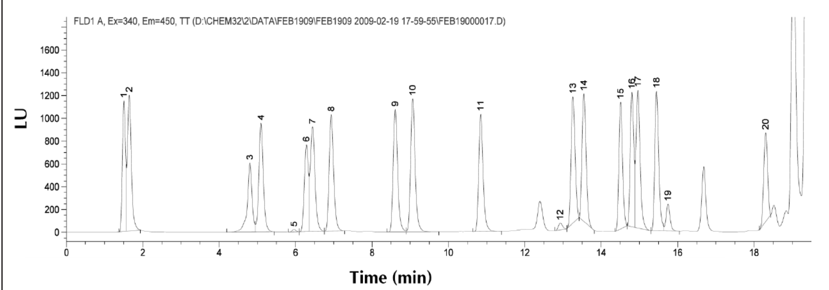

Figure 3. Chromatogram at column oven temperature 40°C, showing resolution between co-eluting amino acid, peak 1 and 2 (aspartic acid and glutamic acid), peak 6 and 7 (histidine and glycine) and peak 16 and 17 (phenylalanine and iso Leucine).

Table V. Linearity, Precision, Recovery, LOD, and LOQ for 17 Amino Acids

Amino acid	FLD coefficient of linearity* (r^2)	Precision† (%RSD)	Recovery (%)	LOD (ng/mL)	LOQ (ng/mL)
Aspartic Acid	0.99979	2.23	101.32	70.02	212.18
Glutamic Acid	0.99715	0.26	89.28	46.58	141.17
Aspargine	0.99820	3.50	97.63	53.28	161.45
Serine	0.99942	3.96	102.22	196.05	594.08
Glutamine	0.99953	4.48	100.51	40.27	122.02
Histidine	0.99095	1.29	96.27	102.34	310.10
Glycine	0.99969	3.68	91.51	182.95	554.40
Threonine	0.99851	2.59	97.64	89.36	270.80
Alanine	0.99792	3.25	96.70	40.52	122.77
Arginine	0.99142	3.99	109.50	34.17	103.57
Tyrosine	0.99462	1.68	90.26	64.111	94.27
Valine	0.99617	1.06	99.50	63.03	191.01
Methionine	0.99740	3.63	112.07	49.28	149.32
Tryptophan	0.99978	0.07	100.61	45.57	138.08
Phenylalanine	0.99610	2.32	95.13	58.78	178.13
Isoleucine	0.99364	4.36	97.45	96.96	293.82
Leucine	0.99765	2.77	103.62	36.61	110.95

* Linearity from 0.5 to 2.5 µg/mL.
† Precision (RSD) of peak area for three replicates.

a factor greater than 1.0. Finally, optimizing the temperature at 40°C resulted in baseline separation of all twenty amino acids (Figure 3).

Specificity

The specificity of the method was demonstrated by analyzing the blank and matrix following the proposed derivatization procedure. No interference was found for any of the amino acid peak in blank and matrix sample (Figure 2A and 2B). The method is specific for simultaneous identification (20) and quantification (17) of amino acids.

Table VI. Results of Three Batches of Biological Media

Amino acid	Actual amount	Amino acid obtained (µg/mL)		
		B 1	B 2	B 3
ASP	13.00	13.17	13.25	13.03
GLU	14.70	13.12	12.96	13.31
ASN	15.00	14.64	14.73	14.79
SER	10.50	10.73	10.55	10.63
GLN	146.00	146.74	145.61	146.30
HIS	20.96	20.17	19.96	20.58
GLY	7.50	6.86	6.90	6.80
THR	11.90	11.62	11.69	11.31
ALA	9.00	8.70	8.57	8.52
ARG	211.00	231.05	221.55	224.15
TYR	7.78	7.22	7.18	7.34
VAL	11.70	11.64	11.84	11.14
MET	4.48	5.02	4.90	4.98
TRP	1.04	1.05	1.06	1.02
PHE	4.96	4.72	4.58	4.72
ILE	3.94	3.84	3.87	3.84
LEU	13.00	13.47	13.16	12.78

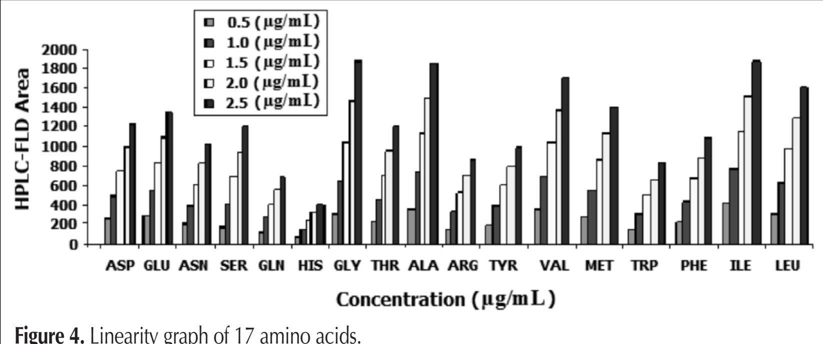

Figure 4. Linearity graph of 17 amino acids.

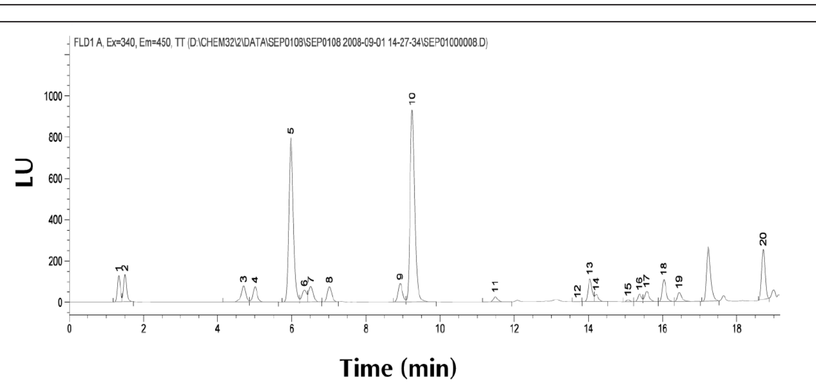

Figure 5. Typical chromatogram of a biological sample.

Linearity, LOD, and LOQ

The linearity was determined by linear least square regression on five spiked standard solutions at 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL concentration as shown in Figure 4. Correlation coefficient found for seventeen amino acids was greater than 0.99 (Table V) which is within the acceptance criteria. The LOD and LOQ for all amino acids is also summarized in the same table.

Precision and accuracy

The precision of the method was determined by three test solutions of 2.5 µg/mL concentration in matrix. The peak area calculated was very wide due to complexity of media, thus the precision (%RSD) was kept at 5.0% (Table V). The precision obtained for retention time was less than 0.50% for five determinations as shown in Table IV.

The accuracy or recovery was done by spiking amino acids to complex biological media, corresponding to the amount fed to the cell line. The average recovery for different amino acids obtained was between 90% and 110% except methionine and glutamic acid, which 112.07% and 89.28% respectively. Hence the limit was set between 87% and 113% for all the amino acids.

Sample analysis

The three consecutive batches of biological media samples were analyzed as per the described procedure. The required quantity of seventeen amino acids were weighed and diluted with biological media to feed up cell line for growth of therapeutic protein. The chromatographic separation of each amino acids in the biological media (Figure 5) was similar to the composite standard solution. The individual amounts of amino acid found in three different batches are presented in Table VI. The results show good agreement of accuracy and reproducibility compared to added amount.

Conclusion

The analytical method described is suitable for rapid and highly sensitive determination of primary and secondary amino acid present in complex biological media. The major advantage of this method is the short analysis time (< 19 min) required for separation of 20 amino acids. The method offers excellent linear and quantitation range, as well recovery for simultaneous identification of twenty amino acid and quantification of seventeen baseline resolved amino acid using HPLC-Fluorescence. The automated pre-column derivatization procedure together with a simple and economical mobile phase makes this method highly attractive for routine high throughput analysis.

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