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Optimization of the determination of amino acids in parenteral solutions by high-performance liquid chromatography with precolumn derivatization using 9-fluorenylmethyl chloroformate

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Abstract

An automatic precolumn derivatization procedure is presented for the determination of amino acids in parenteral solutions by reversed-phase high-performance liquid chromatography. The fluorenylmethyl chloroformate was used as the reagent for derivatization. The optimum conditions for the derivatization reaction, separation and detection were investigated from reproducibility and linearity data. This method has the advantage of automatic precolumn derivatization, a shorter analysis time with optimum separation and high reproducibility of retention times and peak areas for all amino acids with both UV and fluorescence detection.

1. Introduction

Amino acids have traditionally been determined by methods based on ion-exchange chromatography followed by postcolumn derivatization with ninhydrin [1,2]. In recent years, the determination of amino acids using precolumn derivatization and reversed-phase high-performance liquid chromatography (RP-HPLC) separation of the derivatives has become widely accepted and recognized as a powerful method. It requires much shorter analysis time and yields greater sensitivity.

The most common reagents used for amino acid derivatization are *o*-phthalaldehyde (OPA), phenyl isothiocyanate (PITC), 1-dimethylamino-naphthalene-5-sulphonyl (dansyl) chloride and 9-fluorenylmethyl chloroformate (FMOC-Cl) [3–15]. FMOC-Cl has several advantages: it reacts very quickly with both primary and secondary amino acids under alkaline conditions in a buffered aqueous solution, the derivatives are fairly stable and either UV absorbance or fluorescence detection can be applied [16–27].

We report here a procedure for precolumn derivatization with FMOC-Cl and RP-HPLC that we have successfully used for the determination of amino acids in parenteral solutions approved by the Farmacopea Ufficiale Italiana. These aqueous solutions contain 3–19 synthetic L-amino acids (essential and non-essential) with concentrations ranging from 4% to 10%; in order to assess the procedure, we purposely prepared a 6% solution of eighteen L-amino

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acids. The method of Pernigo et al. [28] was adapted and modified to optimize the separation of amino acids. Differences in detection between fluorescence and UV methods, reproducibility and linearity data are presented.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of a Varian Model 9010 HPLC pump, a Perkin-Elmer Model 101 column thermostat (column temperature 32°C), a Varian Aminotag (5 μm) column (150 × 4.6 mm I.D.), a Varian Model 9090 autosampler with a 20-μl loop, a Perkin-Elmer Model LS-1 spectrofluorimeter (excitation at 265 nm, emission at 340 nm) and a Perkin-Elmer Model LC-95 UV detector (265 nm). The data were collected using a Hewlett–Packard Model 3396A integrator.

2.2. Materials

Boric acid, sodium hydroxide, acetonitrile, isopropyl alcohol, methyl alcohol, sodium acetate and glacial acetic acid were purchased from Carlo Erba, 9-fluorenylmethyl chloroformate from Fluka, tetrahydrofuran from BDH and 1-amino-amantadine and amino acids from Sigma.

2.3. Preparation of standard solutions

A 6% amino acid stock standard solution was prepared from each amino acid solution (stored at −30°C) and diluted in 0.5 *M* borate buffer (adjusted to pH 8 with 30% NaOH) to have the following composition (m*M*): isoleucine 3.68, leucine 5.71, lysine 3.28, phenylalanine 1.87, threonine 2.26, tryptophan 0.62, valine 4.30, histidine 2.0, methionine 1.45, arginine 4.43, glycine 3.25, serine 2.45, alanine 3.82, proline 3.86, tyrosine 0.24, taurine 0.16, glutamic acid 2.21 and aspartic acid 1.06.

Norvaline (20 m*M* in borate buffer) was used as an internal standard. All aqueous solutions were prepared with water purified with a Milli-Q water system (Millipore). Solvents were of HPLC grade and chemicals were of the highest purity available.

2.4. Derivatization procedure

A 50-μl volume of test solution and 50 μl of norvaline solution were added to 100 μl of borate buffer in glass sample vials. Subsequent steps followed the autosampler injector programme: 200 μl of FMOC-Cl reagent (30 m*M* in dry acetone) were added, the mixture was quickly shaken and the reaction was allowed to proceed for 10 min at room temperature.

Excess FMOC-Cl was destroyed by addition of 200 μl of 1-amino-amantadine solution (25 m*M* in methyl alcohol) and after 2 min the solution was ready to be injected for HPLC analysis.

2.5. Chromatographic separation

Separation of the FMOC-Cl amino acid derivatives was carried out using a binary gradient. Eluent A was acetonitrile–isopropyl alcohol (90:10) and eluent B was 50 m*M* sodium acetate buffer containing 4% tetrahydrofuran adjusted to pH 4.03 with glacial acetic acid.

The stop time was 75 min after injection; the flow-rate was constant at 1.5 ml/min. The gradient programme used is given in Table 1.

2.6. Quantitative analysis

Amino acid peaks were identified from reference relative retention times of the standard amino acid solution and by adding appropriate amounts of amino acids to the sample. The

Table 1
Eluent gradient programme

Step	Time (min)	A (%)	B (%)
0	15	20	80
1	25	25	75
2	10	30	70
3	10	40	60
4	10	50	50
5	15	80	20
6	5	100	0

external standard method was used for quantification.

The reproducibility of the retention times and peak areas was tested by a series of ten derivatizations of 46.65 m*M* standard solution. The linearity was established by triplicate determinations of five different concentrations of amino acids ranging from 9.36 to 46.65 m*M*.

3. Results and discussion

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent and stable derivatives; the derivatization agent is fluorescent itself and therefore it is essential to remove excess FMOC-Cl with an appropriate reagent. The present procedure uses 1-aminoadamantadine (ADAM) because it is stable and particularly soluble in the borate buffer so that the final volume of the solution is more reproducible [17,21,23]. Most workers have used pentane extraction to remove the excess of the reagent from the derivatized sample; this solvent has the advantage of concentrating the sample, but the transfer of acetone can cause the undesirable extraction of non-polar amino acids [8,17,19].

Further critical aspects of the derivatization procedure are the concentration of the FMOC-Cl solution and the reaction time. To study the effect of these factors on the formation of amino acid derivatives, the reproducibility was determined by a series of ten derivatizations of 46.65 m*M* standard solution with 30 and 15 m*M* FMOC-Cl with reaction times of 3 and 10 min followed by UV and fluorescence detection. The standard solution tested can be considered to be equivalent to a parental solution because of the similar concentration and composition.

Table 2 shows the reproducibility of retention times and normalized peak areas for each amino acid. The results for normalized peak areas show that aspartic acid, glutamic acid and tyrosine

Table 2
Relative standard deviations (%) ($n = 10$) of retention times and peak areas

Amino acid	15 m*M* FMOC-Cl								30 m*M* FMOC-Cl							
	Retention time				Peak area				Retention time				Peak area			
	3 min		10 min		3 min		10 min		3 min		10 min		3 min		10 min	
	UV	FL	UV	FL	UV	FL	UV	FL	UV	FL	UV	FL	UV	FL	UV	FL
Arg	0.29	0.29	0.33	0.29	2.0	2.3	6.3	3.9	0.28	0.27	0.30	0.16	5.7	4.0	1.0	1.5
Tau	0.51	0.32	0.37	0.38	2.0	3.2	4.4	11.4	0.43	0.41	0.21	0.18	7.4	3.5	0.1	3.0
Ser	0.34	0.34	0.39	0.36	1.8	1.8	5.9	3.6	0.37	0.37	0.33	0.32	5.4	4.4	1.1	0.9
Asp	0.38	0.39	0.39	0.40	15.0	13.7	16.4	24.7	0.42	0.42	0.33	0.35	5.0	6.0	1.3	1.4
Glu	0.42	0.45	0.47	0.38	10.0	10.0	14.3	13.8	0.46	0.46	0.35	0.50	5.7	4.7	1.2	2.1
Thr	0.38	0.39	0.43	0.38	3.4	3.5	6.4	4.9	0.42	0.43	0.35	0.33	5.6	4.4	1.1	1.1
Gly	0.43	0.42	0.51	0.39	3.5	2.6	3.9	3.0	0.40	0.40	0.36	0.33	5.7	5.7	0.8	1.4
Pro	0.47	0.51	0.39	0.40	3.2	2.5	6.5	4.8	0.33	0.35	0.26	0.24	5.4	7.9	0.4	1.4
Ala	0.22	0.24	0.35	0.31	2.6	2.2	4.2	2.7	0.30	0.39	0.45	0.45	5.7	6.6	1.0	1.6
Met	0.18	0.18	0.27	0.22	1.8	2.2	4.9	2.6	0.28	0.23	0.22	0.22	5.7	4.1	0.9	1.3
Val	0.15	0.35	0.22	0.18	1.7	1.6	4.3	1.5	0.19	0.20	0.17	0.17	5.6	4.4	0.9	1.7
Phe	0.14	0.13	0.20	0.32	2.3	2.0	3.8	2.5	0.19	0.18	0.11	0.11	6.4	5.4	1.0	1.6
Trp	0.13	–	0.21		3.2	–	2.3	–	0.19	–	0.10	–	5.3	–	1.3	–
Ile	0.12	0.12	0.18	0.15	1.7	2.0	4.1	1.4	0.18	0.19	0.11	0.11	5.5	4.2	1.1	1.7
Leu	0.20	0.18	0.18	0.15	1.4	3.0	1.1	1.9	0.18	0.19	0.10	0.10	5.0	3.0	1.0	1.2
His	0.09	0.09	0.11	0.10	9.7	9.3	15.4	16.9	0.19	0.19	0.06	0.06	5.5	13.0	3.1	3.7
Lys	0.09	0.09	0.09	0.10	2.4	3.4	2.1	4.3	0.14	0.15	0.04	0.05	6.8	2.6	0.7	1.6
Tyr	–	–	–	–		–	–	–	0.10	0.10	0.02	0.02	9.7	4.7	1.2	2.2
Nor	0.15	0.13	0.21	0.23	1.8	5.2	4.1	1.9	0.19	0.25	0.15	0.21	3.3	1.0	2.0	1.3

Values for derivatization procedure carried out with 15 and 30 m*M* FMOC-Cl with reaction times of 3 and 10 min and with UV and fluorescence (FL) detection.

were the amino acids most sensitive to the reaction conditions. The relative standard deviations (R.S.D.) for aspartic acid and glutamic acid with FMOC-Cl 15 m*M* at reaction times of 3 and 10 min were elevated, whereas tyrosine was not detectable. When the concentration of FMOC-Cl was increased to 30 m*M* with a 3-min reaction time, tyrosine became detectable and the R.S.D. of aspartic acid and glutamic acid decreased to 6.0%, but worsened for the other amino acids.

To avoid carrying out two different analyses for the determination of all amino acids, the reaction time was extended to 10 min and the FMOC-Cl concentration was kept at 30 m*M*. Under these conditions, there was a clear improvement in the R.S.D., which ranged from 0.1 to 3.0% except for histidine (3.7%) because of its low relative fluorescence response [8,29].

The linearity of response was estimated by analysing five different amino acid solutions with total concentrations ranging from 9.36 to 46.65 m*M* obtained by serial dilution. Table 3 shows the correlation coefficients for the analyses carried out with 15 m*M* FMOC-Cl at 3 min and 30 m*M* FMOC-Cl at 10 min with both UV and fluorescence detection; these reaction conditions were selected on the basis of reproducibility data. A regression equation for each amino acid was constructed. Fig. 1 shows the calibration graphs for aspartic acid, glutamic acid and tyrosine under the two different reaction conditions.

In view of the good agreement between the reproducibility and linearity data of the analyses carried out with 30 m*M* FMOC-Cl and a 10-min reaction time, these conditions may be regarded as the most favourable. Fig. 2 shows the amino acid profile for a 46.65 m*M* standard solution.

The results obtained by fluorescence detection were similar to those with UV detection except for tryptophan, which was not detectable by fluorimetry because the fluorescence of the adduct is quenched [30].

Table 3
Correlation coefficients of the linearity test ($n = 3$)

Amino acid	15 m*M* FMOC-Cl, 3 min		30 m*M* FMOC-Cl, 10 min	
	UV	FL	UV	FL
Arg	0.9985	0.9976	0.9991	0.9989
Tau	0.9995	0.9988	0.9999	0.9996
Ser	0.9998	0.9993	0.9994	0.9992
Asp	0.9769	0.9840	0.9995	0.9993
Glu	0.9529	0.9700	0.9993	0.9997
Thr	0.9982	0.9953	0.9996	0.9998
Gly	0.9994	0.9999	0.9991	0.9989
Pro	0.9984	0.9913	0.9993	0.9989
Ala	0.9953	0.9886	0.9992	0.9997
Met	0.9991	0.9987	0.9900	0.9974
Val	0.9994	0.9981	0.9991	0.9990
Phe	0.9993	0.9994	0.9990	0.9983
Trp	0.9986	–	0.9989	–
Ile	0.9996	0.9995	0.9990	0.9990
Leu	0.9990	0.9924	0.9990	0.9990
His	0.9911	0.9860	0.9978	0.9964
Lys	0.9992	0.9983	0.9968	0.9958
Tyr	–	–	0.9999	0.9960

Values were calculated using five data points from amino acid solutions containing 9.36, 18.72, 28.07, 37.50 and 46.65 m*M* of total amino acids.

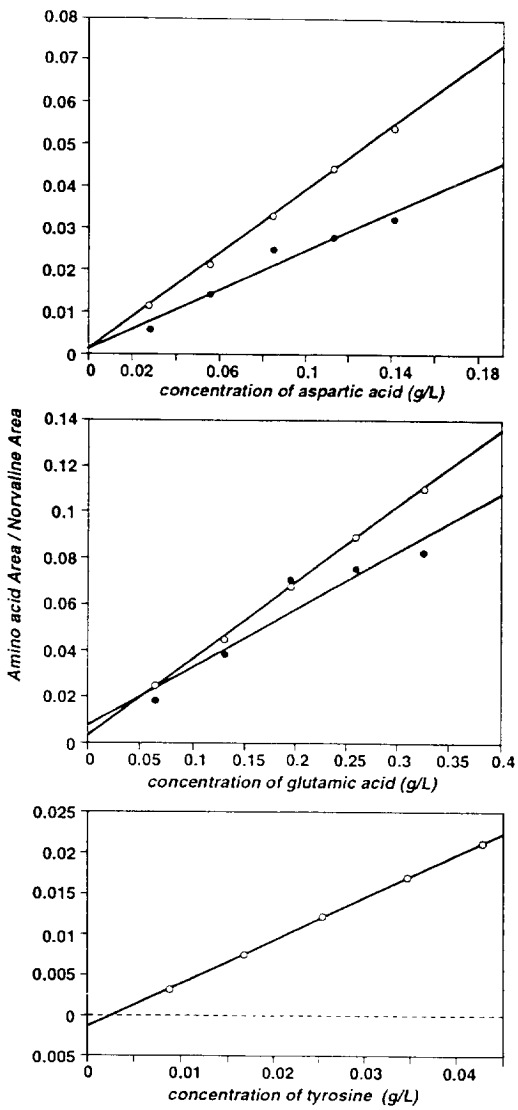

Fig. 1. Linearity for aspartic acid, glutamic acid and tyrosine (UV detection). ● = 15 m*M* FMOC with a 3-min reaction time; ○ = 30 m*M* FMOC with a 10-min reaction time.

The determination of free cystine is not practicable with this method; it is advisable to carry out a second run after oxidation to cysteic acid with performic acid [22,30].

4. Conclusion

The proposed method offers numerous advantages: easy sample preparation, room temperature for the derivatization reaction, high stability of derivatives, simultaneous determination of primary and secondary amino acids, good reproducibility and linearity for solutions over a wide range of concentrations. The analysis requires no specialized equipment other than standard HPLC equipment; to automate the derivatization procedure fully an autosampler is also needed.

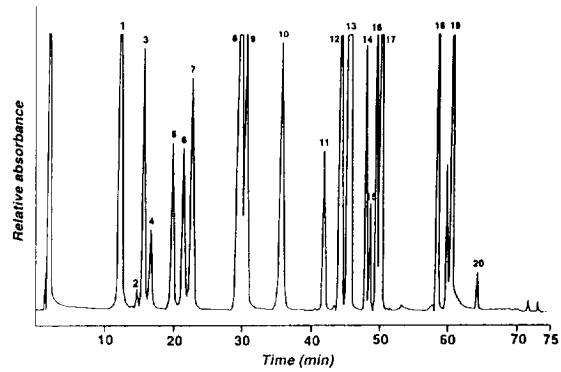

Fig. 2. Chromatogram of 46.65 m*M* amino acid standard solution derivatizated with 30 m*M* FMOC-Cl with a 10-min reaction time and with UV detection (265 nm). The analytical conditions are described in the text. Peaks: 1 = arginine; 2 = taurine; 3 = serine; 4 = aspartic acid; 5 = glutamic acid; 6 = threonine; 7 = glycine; 8 = FMOC-OH; 9 = proline; 10 = alanine; 11 = methionine; 12 = valine; 13 = norvaline; 14 = phenylalanine; 15 = tryptophan; 16 = isoleucine; 17 = leucine; 18 = histidine; 19 = lysine; 20 = tyrosine.

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