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Applications of automated amino acid analysis using 9-fluorenylmethyl chloroformate

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ABSTRACT

A rapid and sensitive fully automated method for the determination of primary and secondary amino acids in different matrices is described. Amino acids are derivatized with 9-fluorenylmethyl chloroformate using an automated precolumn derivatization technique. Data are presented to show that the technique is both reproducible and highly sensitive. Applications of the technique are presented, including the analysis of peptide and protein hydrolysates and the profiling of free amino acids in physiological fluids.

INTRODUCTION

In recent years, the analysis of amino acids using precolumn derivatization and reversed-phase high-performance liquid chromatography (RP-HPLC) separation of the derivatives has become widely accepted. This approach requires much shorter analysis times and gives greater sensitivity than the traditional methods using ion-exchange chromatography and postcolumn derivatization [1].

A number of reagents have been used for precolumn derivatization, including phenyl isothiocyanate (PITC) [2–4], *o*-phthaldialdehyde (OPA) [5–8], 1-dimethylaminophthalene-5-sulphonyl (dansyl) chloride [9–11] and 9-fluorenylmethyl chloroformate (Fmoc-Cl) [12–16]. The development of increasingly sophisticated autosamplers has allowed several of these derivatization methods to be automated, with both the derivatization and analysis performed by the autosampler. There are a number of automated amino acid analysis systems available based on precolumn derivatization, such as those using Fmoc [17,18], dansyl-Cl [19], PITC [20,21] and a combination of OPA and Fmoc [22]. Automated procedures are desirable in situations where large numbers of samples are to be analysed, such as routine quality control testing or the screening of biological samples in the study of amino acid metabolism or acidopathies.

The currently available automated Fmoc methods [17,18] suffer from the same problems as the manual derivatization procedure: a solvent extraction to remove excess of reagent can cause errors in the quantification of the hydrophobic derivatives, and when a solvent extraction is not used, quantification of histidine is still problematic as it forms

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multiple derivatives. Derivatization with dansyl-Cl [19] is slow, and the quantification of histidine is also difficult as it again forms multiple derivatives. The major difficulty in automating the PITC procedure is that it is necessary to remove all traces of derivatizing reagent using high vacuum [23], although recent efforts [21] have avoided this problem by using solvent extraction to remove the excess of reagent. An automated procedure using a combination of OPA and Fmoc overcomes the problem of the instability of the OPA derivatives, but sophisticated detection is required to determine the two different derivatives.

In a previous paper [24] we described a method for derivatization using Fmoc which does not require a solvent extraction and which gives single stable derivatives for the common protein amino acids. We report here details of an automated amino acid analysis system, using the Fmoc reaction chemistry described previously, which is highly sensitive and reproducible and carries out derivatization and analysis in 30 min. The HPLC separation uses a phosphate buffer eluent rather than an acetate buffer as was used in the manual derivatization procedure [24]. This change gave an improved separation and a considerable increase in effective column lifetime. The system has been used for the analysis of amino acids in different matrices including protein hydrolysates and physiological fluids.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of two LC1100 HPLC pumps controlled by a DP800 chromatography data station, a TC1900 column heater and an LC 1250 fluorescence detector (excitation wavelength 263 nm, emission wavelength 313 nm) or LC 1200 UV detector (absorbance wavelength 263 nm) (all from ICI Instruments). The column used was a 150 × 4.6 mm I.D. Spherisorb 3-μm ODS-2 coupled to a 15 × 3.2 mm I.D. Brownlee Newguard 7-μm ODS guard column. The DP800 chromatography data station was used to collect the data and to control an LC 1600 autosampler which was modified by ICI Instruments to prevent cross-contamination between samples and reagents. A continuous wash solution of 10% acetonitrile was drawn through the needle housing by a vacuum source, which was adjusted to give a flow-rate of the wash solution of *ca.* 10 ml/h.

Reagents and materials

All aqueous solutions were prepared with water purified with a Milli-Q purification system (Millipore). Fmoc-Cl (Sigma, St. Louis, MO, USA) was dissolved in acetonitrile (Mallinckrodt Australia, HPLC grade) as a 4.16 mg/ml solution (16 m*M*). Borate buffer was prepared from 200 m*M* boric acid (Ajax Chemicals, Sydney, Australia) solution adjusted to pH 8.5 with 5 *M* sodium hydroxide solution prepared from sodium hydroxide pellets (BDH, Poole, UK). The alkaline cleavage reagent was prepared daily in 250-μl batches by mixing 170 μl of 850 m*M* sodium hydroxide solution with 75 μl of 500 m*M* hydroxylamine hydrochloride (Aldrich, Milwaukee, WI, USA) solution and 5 μl of 2-(methylthio)ethanol (Aldrich). The quenching reagent was acetonitrile–water–acetic acid (20:3:2).

Ammonium dihydrogenorthophosphate (Merck, Darmstadt, Germany) stock solution (2.67 *M*), used for preparation of HPLC eluents, was adjusted to pH 6.5 with ammonia solution (Ajax AR Select). Sepramar amino acid calibration standard A was purchased from BDH and individual amino acid standards from Sigma, Angiotensin-II was from Auspep (South Melbourne, Australia), neurotensin, pepsin and chicken egg white lysozyme from Sigma and chymotrypsinogen-A from Boehringer (Mannheim, Germany).

Chromatographic separation

Separation of the Fmoc amino acid derivatives was carried out using a binary gradient. Eluent A was 20 m*M* ammonium dihydrogenorthophosphate (pH 6.5)–methanol (85:15) and eluent B was acetonitrile–water (90:10). The flow-rate was constant at 1.0 ml/min and the column was maintained at 35°C. The gradients used for protein hydrolysates and physiological samples are shown in Table I.

Hydrolysis procedure

Samples were placed in glass autosampler vials (ICI Instruments), dried in a Speed Vac vacuum centrifuge (Savant Instrument, Hicksville, NY, USA) for 1 h and then placed in a hydrolysis vessel (Pierce, Rockford, IL, USA). A 500-μl volume of 6 *M* hydrochloric acid (Pierce) was added to the

TABLE I

CHROMATOGRAPHIC GRADIENT CONDITIONS FOR Fmoc AMINO ACID ANALYSIS

Eluent A–B. A = 20 m*M* ammonium dihydrogenorthophosphate–methanol (85:15) (pH 6.5); B = acetonitrile–water (90:1). Flow-rate = 1.0 ml/min.

Protein hydrolysates		Physiological samples	
Time (min)	B (%)	Time (min)	B (%)
0	18	0	18
2	18	2	18
3	23	3	23
6	23	10	23
16	40	20	36
17	45	21	48
20	45	26	48
22	55	28	55
23	99	29	99

bottom of the vessel, which was then flushed with helium and evacuated. The hydrolysis was carried out at 110°C for 24 h, and the samples were then dried in the vacuum centrifuge. A 10-μl volume of triethylamine–ethanol–water (2:2:1) was added to each sample and evaporated to remove residual hydrochloric acid and the residues were then dissolved in 5 μl of the derivatization buffer.

Physiological sample preparation

Plasma samples were deproteinized by mixing vigorously with acetonitrile (1:3, v/v) and centrifuging at 12 000 *g* for 3 min [25]. Aliquots of the supernatant were evaporated to dryness in the vacuum centrifuge and reconstituted in derivatization buffer.

Derivatization procedure

The derivatization of amino acid samples dissolved in borate buffer (5 μl) was performed in the sample vial using an autosampler programme. An aliquot (5 μl) of each of the three derivatization reagents [24] was transferred to the sample vial in turn and mixed with the sample using an effective mixing procedure developed for the LC 1600 autosampler specifically for this application. A description of the full autosampler program is shown in Table II. The amounts of reagents used and sample injected can be adjusted as required.

RESULTS AND DISCUSSION

Stability of derivatives

The stability of the Fmoc derivatives at room temperature (21°C) was investigated by repeated injection of constant-volume aliquots, containing 100 pmol of each amino acid, from a single derivatization of an amino acid standard hourly for 24 h. All the amino acid derivatives were stable over this time, with relative standard deviations for normalized peak areas varying between 0.8 and 5.0% as shown in Table III. The monosubstituted histidine derivative gave a relative standard deviation of 0.9%, which indicates that it is far more stable than the disubstituted derivative, which has been reported previously [17] to show breakdown of 49.4% in

TABLE II

SEQUENCE OF STEPS INVOLVED IN AUTOMATED DERIVATIZATION PROCEDURE

Step	Description	Step	Description
1	Wash needle	9	Mix in sample vial
2	Collect Fmoc reagent	10	Wait for reaction time 2
3	Eject to sample vial	11	Collect quenching reagent
4	Mix in sample vial	12	Eject to sample vial
5	Wait for reaction time 1	13	Mix in sample vial
6	Wash needle	14	Wash needle
7	Collect cleavage reagent	15	Collect required quantity of derivatized sample
9	Eject to sample vial	16	Inject onto column

TABLE III

STABILITY OF Fmoc AMINO ACID DERIVATIVES

Values shown are relative standard deviations (R.S.D.) of normalized peak areas of Fmoc amino acids calculated from repeated injection of aliquots from a single derivatization of an amino acid standard hourly for 24 hours.

Amino acid	Peak-area R.S.D. (%) (n = 24)	Amino acid	Peak-area R.S.D. (%) (n = 24)
Asp	2.9	Tyr	1.0
Glu	2.4	Arg	0.9
Ser	1.9	Val	2.4
His	0.9	Met	1.8
Gly	2.1	Ile	3.5
Thr	1.7	Leu	4.1
Ala	0.8	Phe	5.0
Pro	0.8	Lys	3.1

similar stability trials. These results show that the amino acid derivatives are stable enough to allow for reanalysis of previously derivatized samples at any time within 24 h.

Limit of detection

The Fmoc derivatives of amino acids are highly fluorescent, and can be detected at very low levels [13,17,22]. The detection limit for hydroxyproline, chosen for this study as it was not present in reagent blank derivatizations, was 50 fmol at a signal-to-noise ratio of 3:1. In practice, the limit of detection for routine analysis is governed by the background levels of amino acids in both reagents and samples.

Reproducibility and linearity of derivatization

The reproducibility of the automated derivatization procedure was established at two different

TABLE IV

RELATIVE STANDARD DEVIATIONS FOR PEAK AREAS AND RETENTION TIMES OF Fmoc AMINO ACIDS PREPARED USING MANUAL AND AUTOMATED PROCEDURES

Values for peak areas and retention times for automated and manual derivatization procedures. Values for peak areas for manual procedure from Haynes *et al.* [24].

Amino acid	Peak-area R.S.D. (%), 100 pmol (n = 20)	Retention-time R.S.D. (%) (n = 20)	Peak-area R.S.D. (%), 5 pmol (n = 10)	Peak-area R.S.D. (%) (manual procedure), 500 pmol (n = 10)
Asp	0.6	1.1	2.5	1.5
Glu	0.8	1.0	1.5	1.7
Ser	1.8	0.5	2.3	1.5
His	1.5	0.5	3.8	1.1
Gly	1.3	0.5	2.6	0.5
Thr	2.2	0.5	1.6	1.5
Ala	1.0	0.5	3.3	0.8
Pro	0.5	0.5	2.2	0.3
Tyr	1.1	0.5	4.0	1.4
Arg	2.0	0.5	2.5	1.6
Val	0.9	0.5	2.1	1.0
Met	0.8	0.5	4.1	0.9
Ile	1.8	0.4	1.8	0.6
Leu	1.6	0.4	1.9	0.6
Phe	0.8	0.3	2.0	0.6
Lys	1.9	0.1	3.2	1.4

TABLE V

CORRELATION COEFFICIENTS FOR DERIVATIZATION OF AMINO ACID STANDARDS OVER A 200-FOLD RANGE

Calculated using five data points from amino acid standards containing 5, 100, 200, 500 and 100 pmol

Amino acid	r (n = 5)	Amino acid	r (n = 5)
Asp	0.9989	Tyr	0.9987
Glu	0.9984	Arg	0.9986
Ser	0.9989	Val	0.9987
His	0.9986	Met	0.9987
Gly	0.9989	Ile	0.9988
Thr	0.9990	Leu	0.9987
Ala	0.9988	Phe	0.9989
Pro	0.9989	Lys	0.9978

concentrations by analysing a series of twenty consecutive amino acid standards containing 100 pmol of each amino acid and a hydroxyproline internal standard, and a further series of ten amino acid standards containing 5 pmol of each amino acid and the internal standard. The relative standard deviations of normalized peak area for both series are shown in Table IV, together with retention time reproducibility data and reproducibility data for the manual derivatization procedure, from Haynes *et al.* [24]. The results show that the reproducibility of the automated derivatization is comparable to that of the manual procedure, with relative standard deviations for peak areas at the 100-pmol level varying between 0.5 and 2.2%. The relative standard deviations for peak areas at the 5-pmol level are higher, between 1.5 and 4.1%, but this is expected as the background levels of amino acids are a far more significant source of error.

The linearity of the automated derivatization procedure was established over a 200-fold concentration range, with the analysis of amino acid standards containing 5, 100, 200, 500 and 1000 pmol. All of the amino acids were found to give linear derivatization over this range, with the correlation coefficients greater than 0.997 as shown in Table V.

Analysis of peptide and protein hydrolysates

The chromatograms from the analysis of a typical protein hydrolysate amino acid standard by both fluorescence (10 pmol) and ultraviolet detection (500 pmol) are shown in Fig. 1. Ultraviolet detection is typically 25 times less sensitive than fluorescence detection, but it is useful for the analysis of tryptophan and cystine, which form non-fluorescent Fmoc derivatives.

A number of different peptide and protein hydrolysates have been successfully analysed using this procedure. A comparison of experimental results

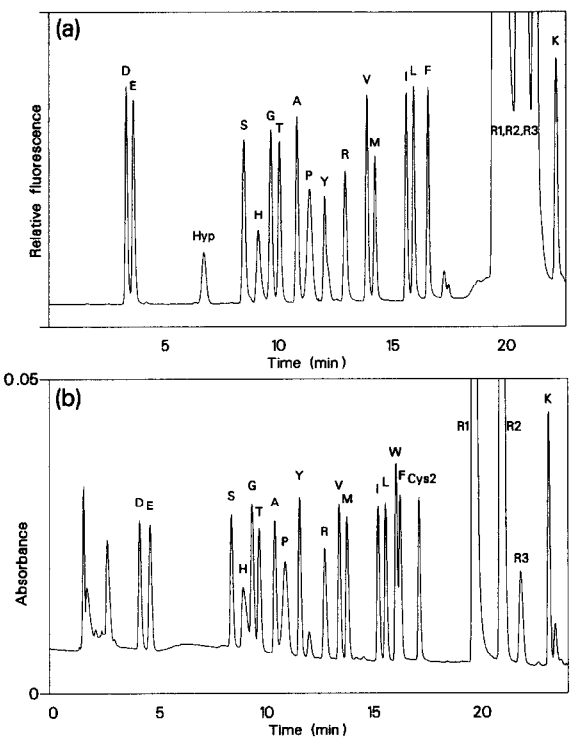

Fig. 1. (a) Chromatogram of a 10-pmol amino acid standard derivatized with Fmoc using automated protocol as in Experimental and detected by fluorescence (excitation wavelength 263 nm, emission wavelength 313 nm). Peaks are labelled with one-letter abbreviations for protein amino acids, and Hyp = hydroxyproline, R1 = Fmoc-hydroxylamine; R2 = Fmoc-OH, R3 = present in reagent blank derivatizations. Chromatographic conditions: column, 150 × 4.6 mm I.D. Spherisorb 3-μm ODS-2 with 15 × 3.2 mm I.D. Brownlee Newguard 7-μm ODS guard column; eluent A = 20 m*M* ammonium dihydrogenorthophosphate (pH 6.5)–methanol (85:15); eluent B = acetonitrile–water (90:10); flow-rate, 1.0 ml/min; column temperature, 35°C; gradient as for protein hydrolysates in Table I. (b) Chromatogram of a 500-pmol amino acid standard derivatized with Fmoc using automated protocol as in Experimental and detected by ultraviolet absorbance at 263 nm. Peaks are labelled as in (a), and W = tryptophan, Cys2 = cystine. Chromatographic conditions as in (a).

TABLE VI

AMINO ACID COMPOSITIONS OF ANGIOTENSIN-II, NEUROTENSIN, LYSOZYME, CHYMOTRYPSINOGEN-A AND PEPSIN

Comparison of automated Fmoc analysis data and literature values: peptide sequences from suppliers' data and protein compositions from Swiss protein data bank. Expected values given in parentheses. The molecular weights of the samples are angiotensin-II = 1047, neurotensin = 1673, lysozyme = 14 300, chymotrypsinogen-A = 26 400 and pepsin = 34 700.

Amino acid	Angiotensin-II	Neurotensin	Lysozyme	Chymotrypsinogen-A	Pepsin
Asx[a]	1.2 (1)	1.1 (1)	21.4 (21)	23.1 (23)	44.1 (42)
Glx[b]		2.0 (2)	5.2 (5)	14.4 (15)	26.9 (26)
Ser			9.3 (10)	23.2 (28)	40.7 (44)
His	0.8 (1)		0.8 (1)	2.0 (2)	1.0 (1)
Gly			11.8 (12)	21.7 (23)	34.9 (35)
Thr			7.0 (7)	21.9 (22)	26.4 (26)
Ala			12.6 (13)	22.3 (22)	17.2 (16)
Pro	1.1 (1)	2.0 (2)	2.1 (2)	8.9 (9)	14.8 (15)
Tyr	1.0 (1)	2.0 (2)	2.9 (3)	4.0 (4)	13.7 (16)
Arg	1.0 (1)	2.0 (2)	11.0 (11)	4.3 (4)	2.3 (2)
Val	0.9 (1)		5.6 (6)	20.4 (23)	19.9 (22)
Met			1.7 (2)	1.9 (2)	3.6 (4)
Ile	0.9 (1)	1.0 (1)	5.5 (6)	9.0 (10)	21.9 (26)
Leu		2.0 (2)	8.0 (8)	19.8 (19)	25.8 (26)
Phe	1.0 (1)		3.1 (3)	6.4 (6)	13.8 (14)
Lys		1.0 (1)	5.9 (6)	13.8 (14)	1.3 (1)
Trp			N.D.[c] (6)	N.D. (8)	N.D. (5)
Cys			N.D. (8)	N.D. (10)	N.D. (6)

[a] Aspartic acid + asparagine.
[b] Glutamic acid + glutamine.
[c] Not detected.

and literature values for the analysis of two synthetic peptides, angiotensin-II and neurotensin, and three proteins, lysozyme, chymotrypsinogen-A and pepsin, is presented in Table VI. Each derivatization was performed on 10 pmol of hydrolysate and 5 pmol of the derivatized samples were analysed. The results are in very good agreement with the expected values, including the determination of histidine at low levels in complex mixtures of amino acids. A chromatogram of the analysis of chymotrypsinogen-A is shown in Fig. 2.

Analysis of physiological fluids

The analysis of amino acids in physiological fluids is important in the studies of disorders of amino acid metabolism and transport [26–34]. There are about 170 different physiological amino acids and at least 66 known disorders of amino acid metabolism

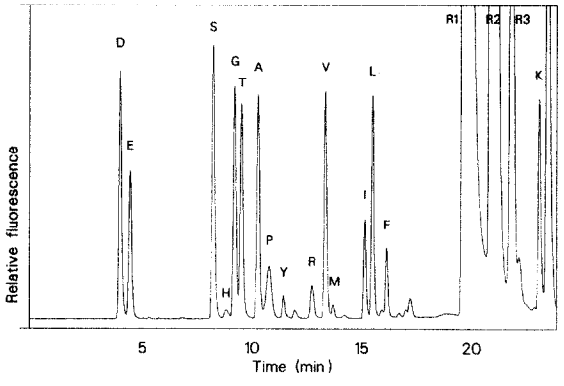

Fig. 2. Chromatogram of 5 pmol of chymotrypsinogen-A hydrolysate derivatized with Fmoc using automated protocol as in Experimental and detected by fluorescence (excitation wavelength 263 nm, emission wavelength 313 nm). Peaks are labelled as in Fig. 1a. Chromatographic conditions as in Fig. 1.

[26,27], most of which are detected by the accumulation of one or more amino acids in plasma, urine or cerebrospinal fluid [22]. The seven most frequently occurring amino acidopathies involve the protein amino acids Phe, Leu, Ile, Met, Val, Tyr and His, but even the most common disorder, phenylketonuria, occurs only in 1 in 10 000 people [28]. The number of samples involved in a screening programme for such disorders requires an analysis system that is both cost efficient and capable of detecting more than one disorder in a single analysis, unlike bacterial inhibition assays which were used prior to the advent of current HPLC technology [28].

The analysis of a standard containing 30 amino acids, including the major components of plasma and urine, is shown in Fig. 3. This is both considerably faster and more sensitive than analysis by conventional ion-exchange chromatography, and has the advantage of being able to detect secondary amino acids, which do not react in OPA derivatization systems [25,29].

Fig. 4 shows the amino acid profile of three deproteinized plasma samples: (a) normal adult human plasma; (b) from a subject with phenylalanine hydroxylase deficiency (phenylketonuria); and (c) from a 4-year-old male subject with tyrosine aminotransferase deficiency (tyrosinaemia type II).

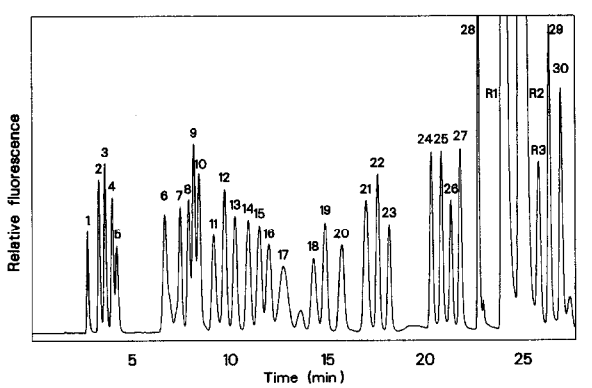

Fig. 3. Chromatogram of 100 pmol of an amino acid standard prepared with 30 amino acids, derivatized with Fmoc using automated protocol as in Experimental and detected by fluorescence (excitation wavelength 263 nm, emission wavelength 313 nm). Peaks: 1 = phosphoserine; 2 = aspartic acid; 3 = glutamic acid; 4 = α-aminoadipic acid; 5 = S-carboxymethylcysteine; 6 = hydroxyproline; 7 = asparagine; 8 = glutamine; 9 = citrulline; 10 = serine; 11 = histidine; 12 = glycine; 13 = threonine; 14 = β-alanine; 15 = alanine; 16 = taurine; 17 = proline; 18 = tyrosine; 19 = α-aminobutyric acid; 20 = arginine; 21 = homoarginine; 22 = valine; 23 = methionine; 24 = isoleucine; 25 = leucine; 26 = norleucine; 27 = phenylalanine; 28 = cystathionine; 29 = ornithine; 30 = lysine. Chromatographic conditions as in Fig. 1; gradient as for physiological samples in Table I.

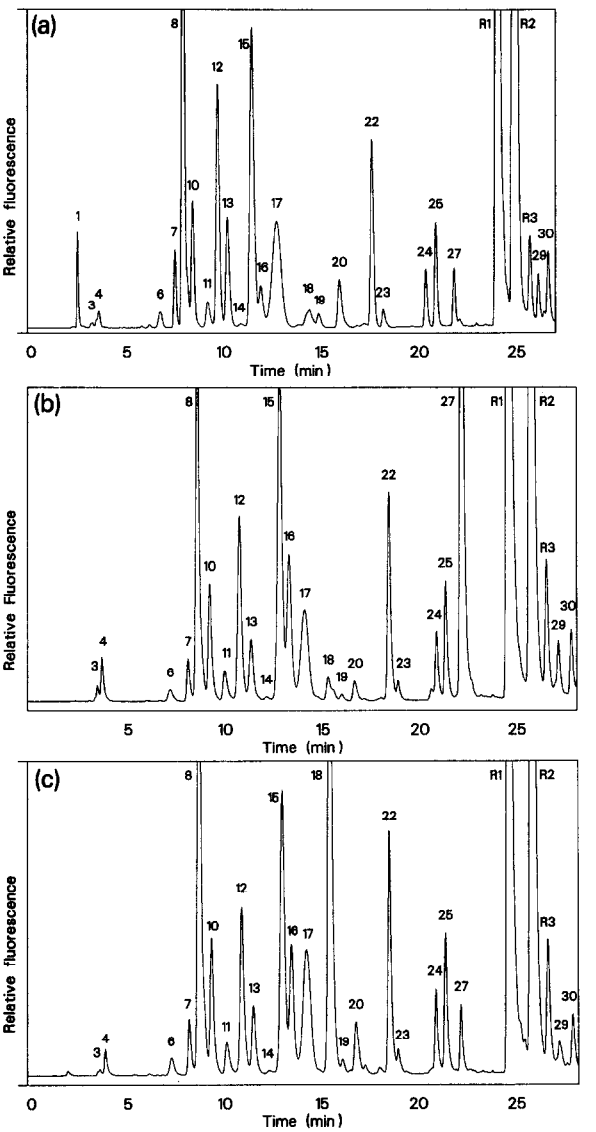

Fig. 4. Chromatograms of three deproteinized human plasma samples derivatized with Fmoc using automated protocol as in Experimental and detected by fluorescence (excitation wavelength 263 nm, emission wavelength 313 nm). (a) Normal adult human plasma; (b) Plasma from an adult patient with phenylketonuria; (c) Plasma from a 4-year-old male patient with tyrosinaemia type II. Peaks are labelled as in Fig. 3. Chromatographic conditions as in Fig. 3.

The greatly elevated levels of phenylalanine (Fig. 4b) and tyrosine (Fig. 4c) are immediately apparent, and show that the method could be used in a screening programme for the detection of the major amino acid metabolism disorders.

CONCLUSION

A method has been developed for the analysis of amino acids using an automated version of the reaction chemistry developed previously [24], which forms single stable derivatives of the protein amino acids. The method is highly sensitive and reproducible, and applicable to the analysis of amino acids in different matrices. These include the analysis of peptide and protein hydrolysates at low levels and the profiling of free amino acids in physiological fluids.

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