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DETERMINATION OF AMINO ACIDS WITH 9-FLUORENYLMETHYL CHLOROFORMATE AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new method for the determination of primary and secondary amino acids is presented. The reaction of 9-fluorenylmethyl chloroformate with amino acids proceeds under mild conditions in aqueous solution and is complete in 30 sec. The derivatives are highly fluorescent and stable, only the histidine derivative showing any breakdown. Twenty amino acid derivatives were separated in 20 min. A linearity range of 0.1–50 μM was obtained for most of the amino acids. The between-analyses relative standard deviation ranged from 2.4 to 6.4%. The detection limit is in the low fmol range.

INTRODUCTION

The determination of amino acids is traditionally based on ion-exchange resin separation, followed by post-column derivatization for detection. More recently, high-performance liquid chromatography (HPLC) of amino acids has been developed and has become an efficient and versatile separation technique, which constitutes an alternative to amino acid analyzers. Many HPLC methods for amino acid determination have been proposed[1]. The methods that are based on pre-column fluorescence derivatization have shown promising results. They are simple and sensitive[2] and can be applied directly to complicated mixtures without time-consuming manipulations.

Two pre-column derivatizing reagents, Dns chloride[3–5] and *o*-phthaldehyde (OPA)–mercaptoethanol[6–10] have gained wide popularity. These reagents are added directly to buffered aqueous samples to convert the free amino acids into products which are then suitable for HPLC separation and detection. However, they have certain disadvantages. It is especially difficult to conduct the Dns derivatization reaction adequately when the amino acids are present in low concentrations in complex matrices[11]. The OPA derivatization is more selective and rapid but has other drawbacks. Its use is limited to primary amino acids, and the derivatives are not stable[12].

Various chloroformates are known to undergo very rapid condensation reactions with amino groups in buffered alkaline aqueous media to form carbamates in high yields[13,14]. Makita *et al.*[15] used isobutyl chloroformate to form N-isobutoxy-

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carbonyl amino acids. These were extracted into diethyl ether and esterified with diazomethane for gas chromatographic (GC) determination. Carpino and Han[16] introduced 9-fluorenylmethyl chloroformate (FMOC-Cl) as a protective reagent for the amino group during peptide synthesis. By this technique the derivatized amino acids can be recovered, and this may be useful for analytical purposes. Anson Moye and Boning[17] showed that this reagent was suitable for fluorescence labelling of primary and secondary amines. The reagent excess was removed with diethyl ether before the HPLC separation. They also reported the capacity factors (k') for five amino acids that were used as relative retention standards.

In contrast to the Dns chloride and OPA reagents, the FMOC-Cl reagent is fluorescent itself. However, this property need not be a limiting factor, provided that the reagent excess and fluorescent side products can be eliminated without loss of the amino acid derivatives. The labelled amino acids have fluorescence properties compatible with the fluorene moiety, which means a large quantum yield and minor influence by the solvent composition. The excitation and emission wavelengths (260 and 310 nm respectively) call for qualified detector arrangements.

The principal objective of this work was to develop an analytical method that could be used for primary and secondary amino acids in aqueous samples. The fluorescent derivatives should be stable, thereby facilitating quantitation and automation. The advantages of pre-column fluorescence derivatization should be retained.

EXPERIMENTAL

Apparatus

The chromatograph consisted of a Varian 5000 solvent delivery system, equipped with a Valco injector provided with a 10-μl sample loop. The recorder used was a Perkin-Elmer Model 56. A 125 × 4,6 mm I.D., 3-μm Shandon ODS Hypersil column was used. It was slurry-packed with 2-propanol by means of a Haskel pump.

Three different fluorescence detectors were used: (1a) Schoeffel FS 970: flow-cell, 5 μl; excitation, 260 nm; bandpass, 5 nm; time constant, 0.5 sec. On the emission side, a 2-mm Schott WG 295 was used as cut-off filter. (1b) The same detector equipped with a liquid excitation filter: 4-mm, 2,7-dimethyl-3,6-diaza-1,6-cycloheptadien perchlorate (Koch-Light), 400 mg/l in water[18] (Fig. 1). This filter suppressed the stray light. At the emission side, two filters were used. One was a 15-mm liquid filter containing a 4:11 (v/v) mixture of 0.5% (w/v) aqueous potassium hydrogen phthalate (BDH, p.a.) and 50% (w/v) aqueous $NiSO_4 \cdot 6H_2O$ (Merck, p.a.)[18] (Fig. 1). The other filter was a 1-mm Schott UG 11. This set-up was used to obtain the chromatograms in the illustrations. (2) Perkin-Elmer LS-4: flow-cell, 3 μl illuminated volume; excitation, 260 nm; bandpass , 10 nm; emission, 313 nm; bandpass, 10 nm; response 0 equivalent to 0.5 sec 98% full scale. (3) Shimadzu RF-530: flow-cell, 12 μl; excitation, 260 nm; bandpass, 18 nm; emission, 313 nm; bandpass, 22 nm; time constant, 1.5 sec.

The derivatization and the extractions were carried out in silanized 3-ml reaction vials.

Reagents and materials

9-Fluorenylmethyl chloroformate and fluorenylmethyl amino acid derivatives

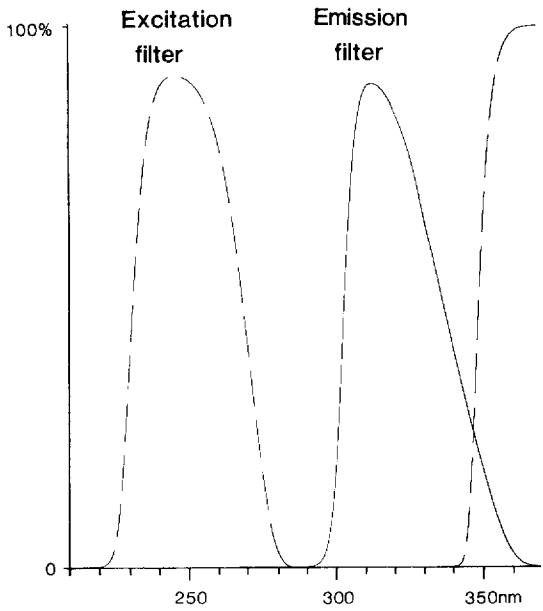


Fig. 1. Transmission characteristics of liquid filters used in the Schoeffel detector. Pathlength 1 cm. For concentrations see Apparatus.

were obtained from Chemical Dynamics (South Plainfield, NJ, U.S.A.). The amino acid standards were purchased from Sigma (St. Louis, MO, U.S.A.) and Mann Labs. (New York, NY, U.S.A.). Methanol (HPLC grade) and acetonitrile (HPLC grade S) were obtained from Rathburn Chemicals (Walkerburn, U.K.).

Borate buffer was prepared from Boric acid (Merck, p.a.) solution (1 *M*) adjusted with sodium hydroxide (Merck, suprapur) solution to pH 6.2. This solution, diluted five times in water (sample), gives pH 7.7. The reagent was prepared by dissolving 155 mg of fluorenylmethyl chloroformate in 40 ml acetone (Merck, p.a.) to give a concentration of 15 m*M*. The acetic acid buffer was obtained by adding to 1 l of distilled water 3 ml glacial acetic acid (Merck, p.a.) and 1 ml triethylamine (Merck). The desired pH was achieved by adding sodium hydroxide solution.

Derivatization

To 0.4 ml of sample were added 0.1 ml borate buffer and 0.5 ml of the reagent. After about 40 sec, the mixture was extracted with 2 ml pentane. The extraction was repeated twice. The aqueous solution with the amino acid derivatives was then ready for injection. If necessary, samples were diluted to suitable concentrations prior to derivatization.

Separation and quantitation

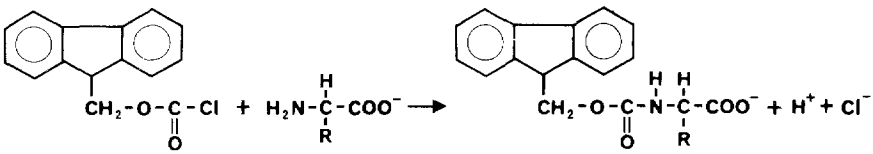
The separation was carried out by gradient elution. The eluent was varied linearly from acetonitrile–methanol–acetic acid buffer (10:40:50) to acetonitrile–acetic acid buffer (50:50) over 9 min. The gradient was started 3 min after injection. A flow-rate of 1.3 ml/min was used in the beginning. It was increased to 2 ml/min in

half a minute at the end of the gradient. The amino acids were quantified by peak-height measurements.

RESULTS AND DISCUSSION

Derivatization

The reaction of FMOC-Cl with the amino acids proceeds as follows:



The chloroformates also react with water to yield the corresponding alcohol as a hydrolysis product[13].

The pH of the buffer used in the derivatization is kept so low that tyrosine can be measured as the mono derivative. This probably also reduces the yield of derivatives of other phenolic substances that may be present. Low pH also has a favourable effect on hydrolysis, the rate of which increases with pH. However, the buffer capacity of the borate buffer at pH 7.7 is low, and this has to be taken into consideration when dealing with highly buffered samples. When a phosphate buffer is substituted for the borate buffer, a broad peak at the beginning of the chromatogram results.

After 30 sec, the derivatization of amino acids is complete. The extraction is carried out immediately in order to minimize the formation of the hydrolysis product. The reagent consumption due to hydrolysis during the reaction is negligible. The extraction is performed with pentane. The use of solvents of higher polarity, such as diethyl ether, causes losses of the more hydrophobic amino acid derivatives, especially those forming doubly labelled derivatives, such as histidine, ornithine and lysine. The extraction removes the reagent efficiently and each extraction lowers the concentration of the hydrolysis product about seven times.

The solubility of the reagent in water is low, necessitating the presence of acetone in the reaction mixture. The extractions reduce the acetone content by a factor of four. This allows larger volumes to be injected into the column.

Yields of 88–89% have been reported in the preparative derivatization of amino acids with FMOC-Cl[15]. Very high yields (113–131%) were obtained when nine commercially available amino acid derivatives were used as reference substances. Since no attempt had been made to purify these, the excessive recoveries are probably due to low purity.

Stability of the derivatives

The stability of the derivatives, after normal derivatization and extraction of a 10-μM standard mixture of twenty amino acids, was studied. The derivatives were stored at 4°C and at room temperature in darkness as well as in daylight for a period of 13 days, during which time they were analyzed three times. The only derivative showing any significant breakdown was the doubly labelled histidine. This resulted

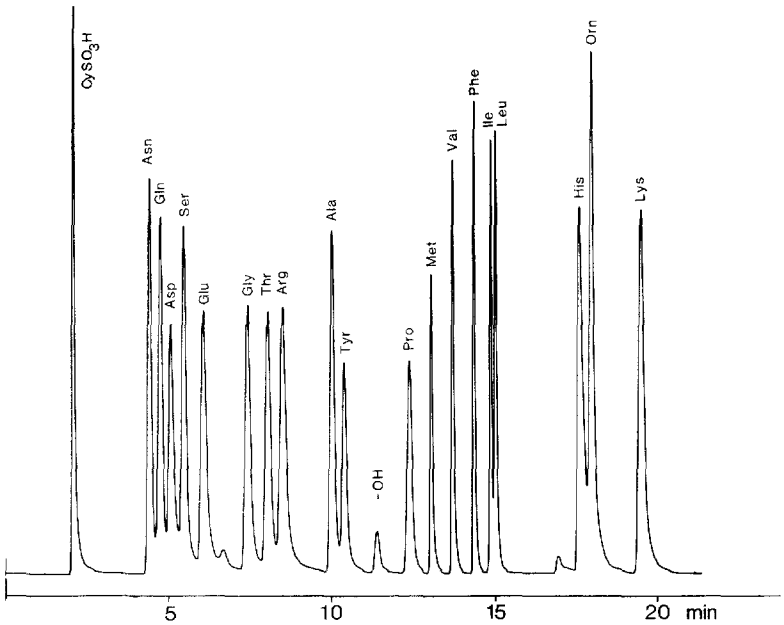


Fig. 2. Separation of amino acid standards. Concentration of each amino acid, 10 μM (His, 50 μM). For chromatographic conditions, see Experimental. Buffer pH 4.20. Detector range 0.2.

in an increase of the monoderivative. Assuming first-order kinetics, the half-life of the doubly labelled histidine is in the order of 5–6 days at room temperature and about 4 weeks in a refrigerator. No attempt was made to increase the stability. As expected, daylight had no effect on stability. The mono derivative of histidine showed up as a well-separated peak in the chromatogram and gave a far greater response than the double derivative. It is not yet known if the conversion to the mono derivative is quantitative. If that were so, one could take advantage of the instability and work only with the mono derivative. At the same time, the disappearance of the double derivative would simplify separation near the end of the chromatogram, allowing more rapid analysis.

Separation

A separation of an amino acid standard is shown in Fig. 2. A good separation of the bands eluted late was achieved by using acetonitrile in the mobile phase. Attempts to use acetonitrile for the first part of the chromatogram were not successful, as serine and aspartic acid were eluted together. However, this pair is easily separated with methanol. Thus, a good separation of all the amino acids is obtained with a gradient from methanol–acetonitrile to acetonitrile, while a constant percentage of buffer is maintained.

The effect of pH on the separation was studied (Fig. 3). A decrease in retention for all the amino acids, except for monohistidine, was observed at higher pH. A loss in resolution of bands eluted early was also seen. The pH has no effect on the retention of the hydrolysis product, and by appropriate choice of pH it is easily separated from the amino acids.

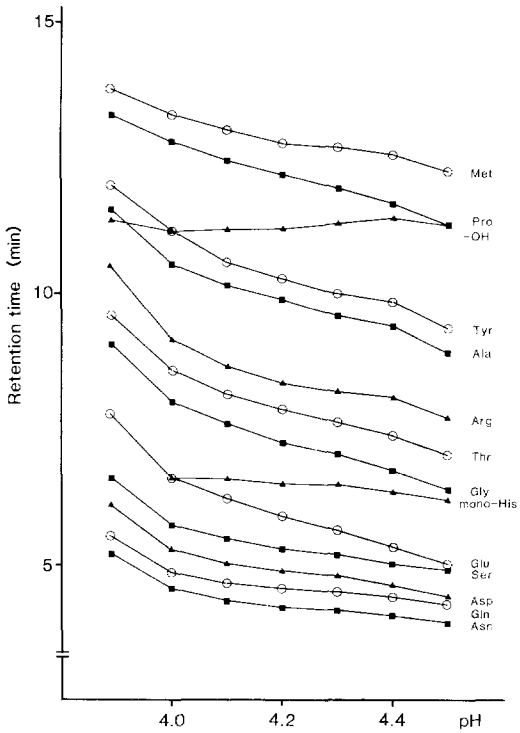


Fig. 3. Retention time *vs.* buffer pH. For chromatographic conditions, see Experimental.

TABLE I

REPRODUCIBILITY OF THE METHOD

Relative standard deviation (%) for peak height/concentration of amino acid. Three separate derivatizations of amino acid standards were performed at each concentration level (0.1, 1.0, 10, 50 μM).

Amino acid	*Concentration range*	
	0.1–50 μM (n = 12)	*1–50 μM (n = 9)*
Cysteic acid	5.9	5.0
Asparagine	3.1	2.7
Glutamine	3.3	2.5
Aspartic acid	4.3	3.8
Serine	2.9	2.4
Glutamic acid	4.6	2.7
Glycine	6.4	6.0
Threonine	5.1	4.8
Arginine	4.4	3.3
Alanine	2.6	2.5
Tyrosine	2.9	2.6
Proline	3.1	2.9
Methionine	4.1	4.5
Valine	3.2	3.2
Phenylalanine	4.0	4.3
Isoleucine	3.0	2.5
Leucine	3.1	2.7
Ornithine	6.0	4.2
Lysine		3.3

TABLE II

DETECTION LIMITS

Signal-to-noise ratios for the alanine peak. Derivatization of 1 μM amino acid standard. 10 μl injected. For detector settings, see Apparatus.

Detector	*Signal-to-noise ratio*
1a Schoeffel FS 970, glass filter	55
1b Schoeffel FS 970, liquid filters	400
2 Perkin-Elmer LS-4	1000
3 Shimadzu RF-530	2200

Addition of triethylamine to the mobile phase reduces the tailing of arginine and causes it to be eluted faster. Arginine can also be manipulated with the buffer concentration, a higher concentration resulting in faster elution.

Linearity and reproducibility

The linearity of the method was tested for amino acid standards of 0.1, 1.0, 10 and 50 μM. Three separate derivatizations were performed at each concentration level. Peak heights were proportional to concentration in this range, with two exceptions. The response of histidine was almost doubled from the lowest to the highest concentration level. The response of lysine was linear from 1 to 50 μM but was *ca.* 15% lower at the 0.1-μM level. This might indicate a loss in the extraction step.

Information on the reproducibility was obained from the same experimental data. The results are presented as relative standard deviations for peak height/concentration, using only points within the linear range (Table I). Reproducibility was

TABLE III

COMPARISON WITH OTHER METHODS

Concentration of amino acids (μM) as found by different methods. FMOC = present method. AAA = Beckman amino acid analyzer; OPA = OPA–mercaptoethanol as precolumn reagent, followed by HPLC separation.

Amino acid	*Protein hydrolysate*		*Cerebrospinal fluid*	
	FMOC	*AAA*	*FMOC*	*OPA*
Glutamine			430	572
Aspartic acid	902	880		
Serine	1380	1150		
Glutamic acid	612	603	1.9	1.8
Glycine	1030	1010		
Threonine	378	363	28.4	19.2
Arginine	83	72		
Alanine	634	613	29.2	31.2
Tyrosine	429	410		
Proline	309	464		
Methionine	88	91	3.0	12.5*
Valine	599	566	12.9	12.7
Phenylalanine	127	126	7.4	7.4
Isoleucine	577	549	3.3	3.4
Leucine	601	603	7.5	7.3
Lysine	570	573	18.6	19.3

* Ammonia interferes.

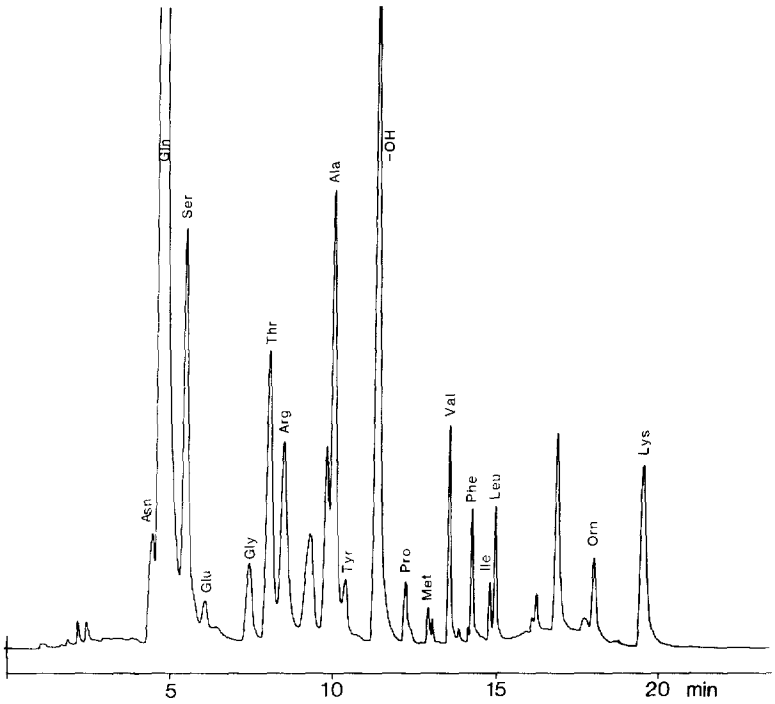


Fig. 4. Chromatogram of cerebrospinal fluid diluted 1:10. For chromatographic conditions, see Experimental. Buffer pH 4.20. Detector range 0.05.

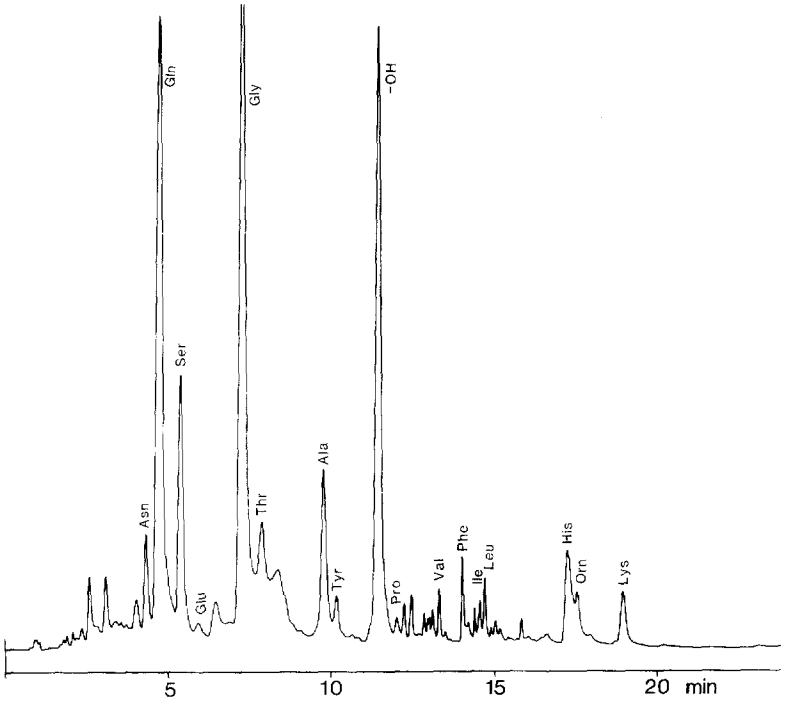


Fig. 5. Chromatogram of urine diluted 1:200. For chromatographic conditions, see Experimental. Buffer pH 4.27. Detector range 0.05.

in general better than 5%. Better results may possibly be obtained by improving the reproducibility of the gradient and retention times (s = 0.04–0.09 min) *e.g.*, by premixing the solvents.

Detection limit

The detection limits for four different detector set-ups were compared. As shown in Table II, the signal-to-noise ratio for the Schoeffel detector is increased seven times by use of the liquid filters. This is due to a more efficient reduction of stray light, a sharper cut-off of the excitation light at about 300 nm and a lower fluorescence background from these filters. The emission filter combination gives a narrow bandpass (Fig. 1), suitable for the emission of the FMOC derivatives. The detector is then also more selective. Details will be reported later. The other detectors are equipped with double monochromators. The Shimadzu detector showed the largest signal-to-noise ratio. However, this detector has the largest cell volume and the largest time constant, resulting in loss of chromatographic resolution.

In summary, very high instrumental sensitivity is attainable, and the detection limit of the method is rather restricted by the level of amino acid contamination. As the method includes removal of the reagent, the contamination risk is limited to the reaction step.

Comparison with other methods

Determination of amino acids in a protein hydrolysate was performed both with the present method and a Beckman amino acid analyzer. The results (Table III)

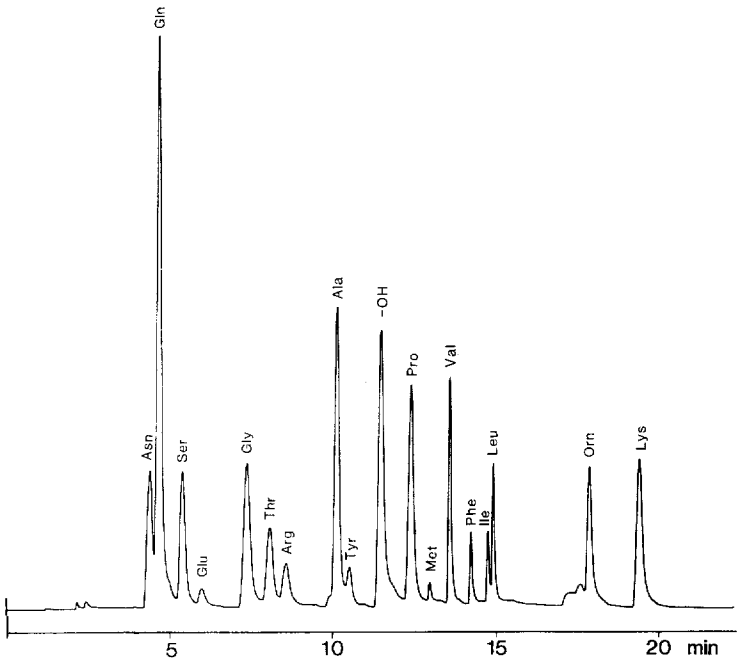


Fig. 6. Chromatogram of blood serum diluted 1:50. For chromatographic conditions, see Experimental. Buffer pH 4.20. Detector range 0.1.

showed good agreement, within 6%, except for three amino acids. Evaluation was uncertain for proline on the amino acid analyzer and for arginine in the present method. Possible explanations for the disagreement in the serine values are: the use of different standards, contamination and interfering substances. The mean recovery, tested by adding known amounts of amino acids to the hydrolysate, was 98% ($s = 3$) for twenty amino acids.

Analysis of cerebrospinal fluid with OPA–mercaptoethanol as a precolumn reagent was also compared with the present method (Fig. 4). Results for peaks that were well separated in both methods are presented in Table III. The results of the analysis were generally in agreement. In the case where three amino acids gave different results the probable explanation is that high values are due to interfering substances. Chromatograms from the analysis of urine and serum are illustrated in Figs. 5 and 6. The specimens were not pretreated except for appropriate dilution prior to derivatization.

CONCLUSIONS

Pre-column derivatization in aqueous samples, followed by pentane extraction is a simple procedure for obtaining stable fluorescent amino acid derivatives. Stability implies that derivatization could be used as a means of preserving unstable samples and permits automation of the method.

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