High Performance Liquid Chromatographic Determination of Subpicomole Amounts of Amino Acids by Precolumn Fluorescence Derivatization with O-phthaldialdehyde

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High Performance Liquid Chromatographic Determination of Subpicomole Amounts of Amino Acids by Precolumn Fluorescence Derivatization with *o*-Phthaldialdehyde

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Using reversed-phase high performance liquid chromatography, 25 amino acids as *o*-phthaldialdehyde derivatives were determined with a 30-min sample turnover. The derivatization procedure is rapid, performed in aqueous medium, and has few transfer steps. The precision is ±0.5% at the 80-pmol level. Recoveries of standards added to urine samples were >98%. Intercalibration with an amino acid analyzer showed an agreement of >95%. The detection limit is about 50 fmol. The overall retention is controlled by the organic modifier, but second-order effects are ascribed to ion repulsion between anions in the eluent and the ionized carboxylate on the derivatives. Clinical, biochemical, and environmental applications are given. Amino acids and ammonia were determined in 25 μL of seawater.

A considerable number of investigations have appeared over the past few years dealing with high performance liquid chromatographic (HPLC) separation of amino acids derivatized to form strongly UV-absorbing and fluorescent compounds (*1–6*). While precolumn derivatization generally improves the detection and simplifies the chromatographic system, for a number of reasons many of these derivatization procedures are not well suited for the analysis of amino acids in environmental samples, e.g., lake and seawater, as well as biological samples, e.g., protein hydrolysates, urine, etc. For example, in the cases of Dansyl-Cl, Bansyl-Cl, and Mansyl-Cl (*1–3*), the reagents themselves and the reaction by-products fluoresce which we found to cause serious interference at the high sensitivity level needed for the direct analysis of, for example, seawater samples. Another reagent, fluorescamine (*1–3*), reacts rapidly and quantitatively with primary amines; however, the reaction products are rather bulky and, for amino acids, two fluorescent derivatives are formed (*7*).

It has been recently demonstrated that the *o*-phthaldialdehyde/2-mercaptoethanol reagent, abbreviated OPT, which has recently become popular for postcolumn fluorigenic detection of amino acids in conventional amino acid analyzers (*8, 9*) and as a reagent for the determination of total amino acids in natural waters (*10*), is also suitable for precolumn derivatization of amines. Davies et al. (*11*) derivatized various catecholamines in brain tissue extracts with OPT and the derivatives were transferred to an organic phase prior to reversed-phase HPLC separation. The recoveries were only 55–78%. Mell et al. (*12*) examined catecholamines in urine and Subden et al. (*13*) examined histamines in wine by means of OPT derivatization and reversed-phase HPLC. These investigators, however, performed the sample clean-up steps prior to derivatization; thus an aliquot of the reaction mixture could be injected directly onto the column and, therefore, much higher recoveries were attained.

In the present study we have examined the separation of OPT amino acid derivatives by reversed-phase HPLC. Some attention has been given to the qualitative interpretation of the retention mechanisms in light of the balance between the hydrophobic and hydrophilic parts of the OPT-amino acid derivatives. Furthermore, the repulsion between negatively charged OPT-amino acid derivatives and the anions in the moving aqueous–methanol eluent is considered. The application of the method to the rapid and sensitive analysis of amino acids in various environmental, biological, and clinical samples is demonstrated and discussed.

EXPERIMENTAL

Apparatus. The pumping system consisted of two LDC Constametric HPLC Pumps with a Gradient Master (Model 1601). Samples were loaded with a high-pressure sampling valve (Valco CV-6UHPa-N60) provided with a 25- or 250-μL sample loop.

The columns, 200 × 4.6 mm i.d., were packed by a slurry technique. A mixture of acetone and chloroform (50:50) was used for suspending the packing material (Nucleosil RP-18, 5 μm) and the column was packed in pure acetone. The column was packed downwards at a pressure of 600 atm with the aid of a Haskel pump (DST-150).

Column effluents were monitored with a FS 970 L.C. Fluorimeter (Schoeffel Instruments) equipped with a Corning 7-54 primary filter and a 418 nm cut-off secondary filter. The excitation wavelength was set to 330 nm. The cell volume was 5 μL. The recorder used was a two-channel Vitatron 2001 operated on the 10-mV scale.

Mobile Phases. The buffers were prepared from p.a. grade salts and double distilled water. The pH of the borate and citrate buffers was adjusted in the pH range of 6.0–7.5 with 12 M NaOH and 12 M HCl, respectively. For the phosphate buffer the pH was set by mixing equimolar solutions of Na_2HPO_4 and NaH_2PO_4 in different proportions. For gradient runs, the citrate buffer was made 20% (on a molar basis) in phosphate buffer to increase its buffer capacity in the pH range of 6.5–7.5. Methanol (HPLC grade, Rathburn Chemicals) was employed as the organic modifier in the aqueous eluent. All mobile phases were degassed daily with an ultrasonic bath and vacuum prior to use.

Reagent. *o*-Phthaldialdehyde solution: 270 mg of *o*-phthaldialdehyde (Merck, for fluorescence analysis) was dissolved in 5 mL of ethanol (99.5%).

Borate Buffer. Boric acid solution (0.4 M) was adjusted to pH 9.5 with 1 M NaOH.

Buffered Reagent Solution. Two hundred microliters of 2-mercaptoethanol and 5 mL of *o*-phthaldialdehyde solution were added to the borate buffer and the volume was adjusted to 50 mL with buffer. The reagent mixture was allowed to "age" for at least 24 h prior to use. The reagent strength was maintained by addition of 20 μL of 2-mercaptoethanol every 3–4 days.

Standard Solutions. Stock solutions: The amino acids (Mann Research Lab.) were dissolved to give 1.0 mM in water/methanol (50:50). For the ammonia standard, a 10.0 mM solution of ammonium chloride was used.

Derivatization Procedure. For mixed solutions of amino acid standards (approximately 40 pmol of each amino acid/μL) 20 μL were reacted with 100 μL of OPT reagent at room temperature. Then 5–10 μL were injected after 2 min.

For some natural samples, e.g., physiological fluids, varying amounts of phosphate buffer were added for dilution purposes prior to derivatization. Injection was made after a precise 2-min incubation period.

Procedure. The column dead volume was measured from the leading edge of the least retained solute, OPT-cysteic acid, eluted

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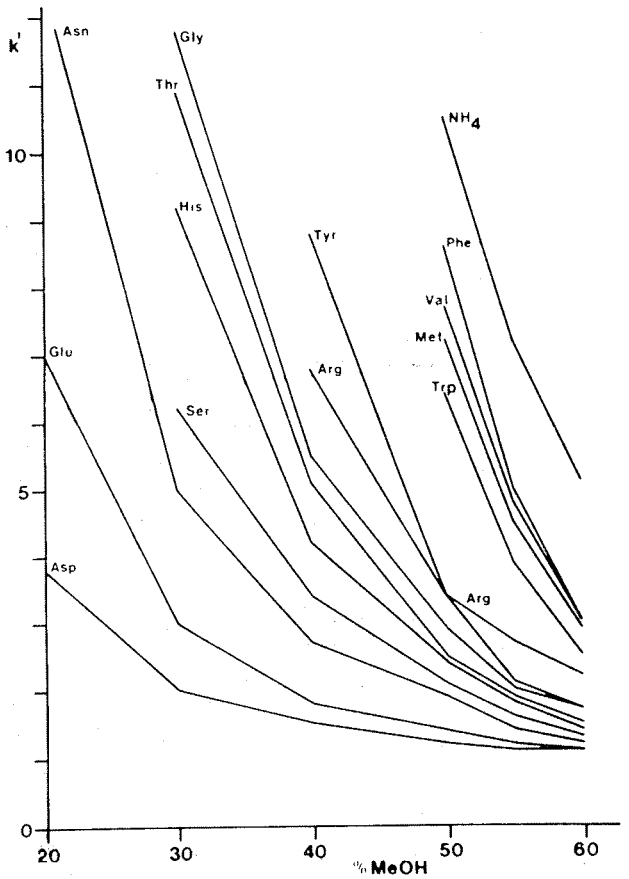


Figure 1. k' vs. the methanol concentration; isocratic 0.1 M phosphate buffer, pH 7.3

with 100 % methanol. All runs were performed at ambient temperature.

For gradient runs the following mobile phases were usually employed: (i) 0.1 M phosphate buffer (pH 6.8) + methanol gradient starting at 35% methanol and increasing by 2%/min for 20 min; and (ii) 0.1 M sodium citrate + 0.1 M phosphate buffer (4:1, pH 6.8) + methanol gradient starting at 25% methanol and increasing by 3%/min for 15 min. The flow rate was 1 mL/min. At the end of the run, initial conditions were restored by running a reversed methanol gradient of max. 10%/min. The use of a mild reversed gradient was found essential for maintaining optimal column performance. A delay period of about 10 min is required for equilibration. The above gradients functioned well for the separation of the common protein amino acids. The gradient could be tailored to any particular separation problem. For example, by starting at a lower methanol concentration and using a slightly convex gradient (e.g., exponent 0.5 on the LDC Gradient Master), the first peaks could be better resolved and thereby accommodating other solutes in the first part of the run.

Shut-Down and Conditioning Procedures. The following overnight shut-down procedure was employed. After the final gradient run, the methanol content of the mobile phase was increased to 100% at the rate of 10%/min. The column was flushed with methanol for 10–15 min. The column was then disconnected and sealed. The buffer pump and tubings before and after the column were flushed with water. This last step was found particularly essential when phosphate buffer was employed, which otherwise tended to precipitate in the tubing.

During the initial conditioning of a newly packed column with phosphate buffer, swelling of the packed bed occurred. To avoid this effect the column was first gradually conditioned in a borate or citrate buffer (0.1 M, pH ~7) for several hours and then gradually reconditioned in the phosphate buffer.

RESULTS

Optimization of Separations. *Effect of Methanol Concentration.* A series of isocratic runs with 20, 30, 40, 50, and 60% methanol were performed. In each case a phosphate buffer (0.1 M, pH 7.3) was employed. As expected the capacity factors (k') of all amino acid derivatives tested decreased exponentially in the interval of 20–60% methanol, Figure 1. With the exception of arginine and tyrosine, capacity factor lines of the amino acids do not cross in this interval. However, at methanol concentrations less than 50%, arginine elutes prior to tyrosine and, at concentrations greater than 50%, arginine elutes after tyrosine. The optimal methanol concentration range for isocratic runs is 40 to 55% and in this range the analysis time varies from about 120 to 30 min. Figure 2 shows a typical isocratic run. As a result of the wide range of polarities of the amino acid derivatives, gradient elution is preferred over isocratic elution. Figure 3 shows a typical gradient elution run in which 25 OPT-amino acid derivatives are separated.

Effect of the Nature of the Ionic Medium. Figure 4 illustrates the effect of changing the ionic medium on the capacity factors of the derivatives of 13 amino acids plus ammonia. The runs were performed isocratically with 45% methanol, and the pH and concentration of the ionic media were always 7.3 and 0.1 M, respectively. From Figure 4 it is evident that the nature of the salt has a very strong effect on the capacity factors. Phosphate and citrate media are pre-

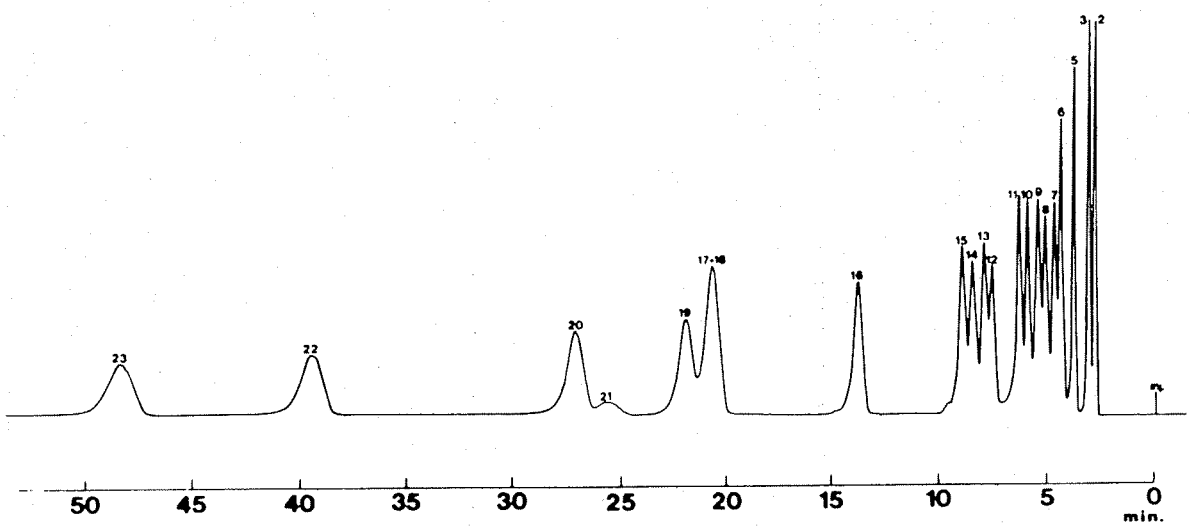


Figure 2. Isocratic run: Column: 200 × 4.6 mm i.d., Nucleosil RP-18, 5 μm. Mobile phase: phosphate buffer, 0.1 M, pH 6.8: methanol (57/43). Peaks as in Figure 3

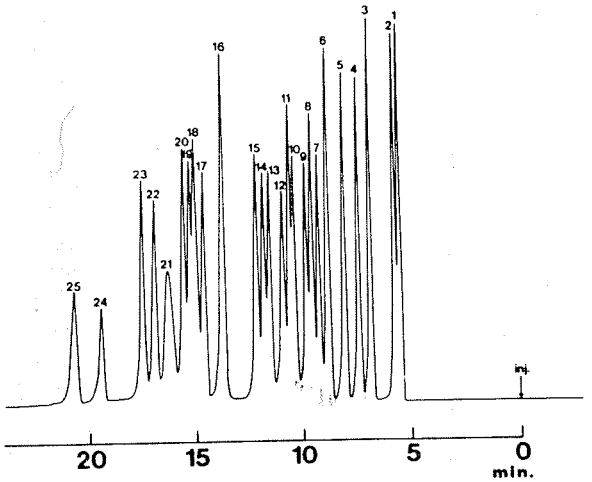


Figure 3. Gradient run: Column: as in Figure 2. Mobile phase: (A) citrate/phosphate buffer, pH 7.7. (B) methanol. Convex solvent gradient (0.5) from 20% B to 70% B in 15 min. Peaks: (1) Cysteic acid, (2) Asp, (3) Glu, (4) *S*-carboxymethyl cysteine, (5) Asn, (6) Ser, (7) Gln, (8) His, (9) methionine sulfone, (10) Thr, (11) Gly, (12) Arg, (13) β-Ala, (14) Tyr, (15) Ala, (16) α-aminobutyric acid, (17) Trp, (18) Met, (19) Val, (20) Phe, (21) NH_4^+, (22) Ile, (23) Leu, (24) Orn, (25) Lys

ferred over borate and water. Reasonably good separations are achievable even with borate (mostly boric acid at pH 7.3), but at lower methanol concentrations (20 to 35%). The elution positions of the arginine and ammonia derivatives appear to be nearly independent of the ionic medium chosen. The same is true for ornithine and lysine (not shown in Figure 4).

Effect of Ionic Medium pH. The effect of pH was examined over the interval 5.9 to 7.9 with a series of isocratic runs; for each run 45% methanol and 0.1 M sodium phosphate buffer was used. Thus the sodium concentration will vary between 0.1 and 0.2 M. As shown in Figure 5, the k' values generally decrease with increasing pH. In contrast to the other amino acid derivatives, the k' of arginine increases and the k' of ammonia remains approximately constant in this pH interval. While using gradient elution, it was observed that the retention of all amino acids could be changed relative to ammonia and arginine by altering the ionic medium pH. The pH was normally adjusted so that ammonia eluted between phenylalanine and isoleucine and arginine eluted between glycine and β-alanine as shown in Figure 3. For a newly packed column, this optimal pH is 7.5–7.6. For the phosphate and citrate buffers, the optimal pH changed with time in the acidic direction and stabilized at about 6.3–6.8 after several weeks of operation. For borate buffers the optimal pH actually increased with time; therefore, the use of borate was discontinued. Since some erosion of the stationary phase occurred at pH values >7, the top of the column was checked daily and filled when necessary. The initial shift in the optimal pH thus seems to be related to surface erosion which decreases as the eluent pH decreases.

Effect of Ionic Medium Concentration. A series of isocratic runs with water, 0.05, 0.1, and 0.2 M phosphate buffer in 45% methanol were performed. The pH of the buffers was 7.3. As shown in Figure 6, k' values increase markedly with increasing phosphate concentration, especially at low concentrations. However, the k' values of arginine and ammonia are relatively constant in this interval. The same is true for ornithine and lysine (not shown in Figure 6). For gradient elutions with increasing percentage of methanol, the use of phosphate concentrations greater than 0.1 M occasionally gave rise to precipitation problems (most likely disodium hydrogen phosphate); thus a concentration in the range of 0.05 to 0.1 M was usually employed. For citrate buffers a working range

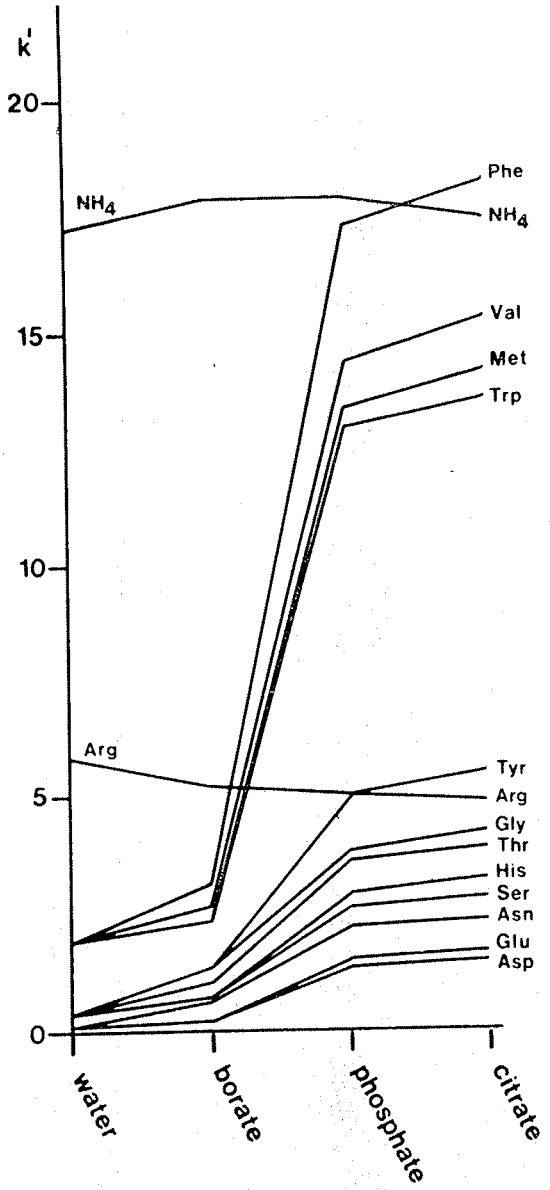


Figure 4. k' vs. different buffer ions, 0.1 M, pH 7.3; isocratic 45% methanol

of 0.05 to 0.14 M was feasible.

Effect of Hydrophobic Support. The influence of the length of the bonded alkyl chain on the separations was examined with C_8 and C_{18} stationary phases. Column bed dimensions, particle sizes, and mobile phase gradients were identical in these trials. As expected, the retention times were shorter (by about 25%) for the C_8 column. The order of elution for the two supports was identical. Most analyses, however, were performed with the C_{18} phase since it is more resistant to buffer erosion and has a greater column capacity (*14*).

Optimization of the Derivatization Procedure. *Reaction Composition.* For a fixed reaction time, the fluorescence responses of the amino acid derivatives were linear and nearly independent of the reagent composition as long as at least a 200 times excess of reagent was maintained. When the excess was allowed to fall below this limit, nonlinear responses were obtained. This limit plays an especially important role for the analysis of various physiological fluids, e.g., urine and cerebrospinal fluid, since high concentrations of non-amino acid, reagent consuming amines are generally present. In this case, the sample should be excessively diluted

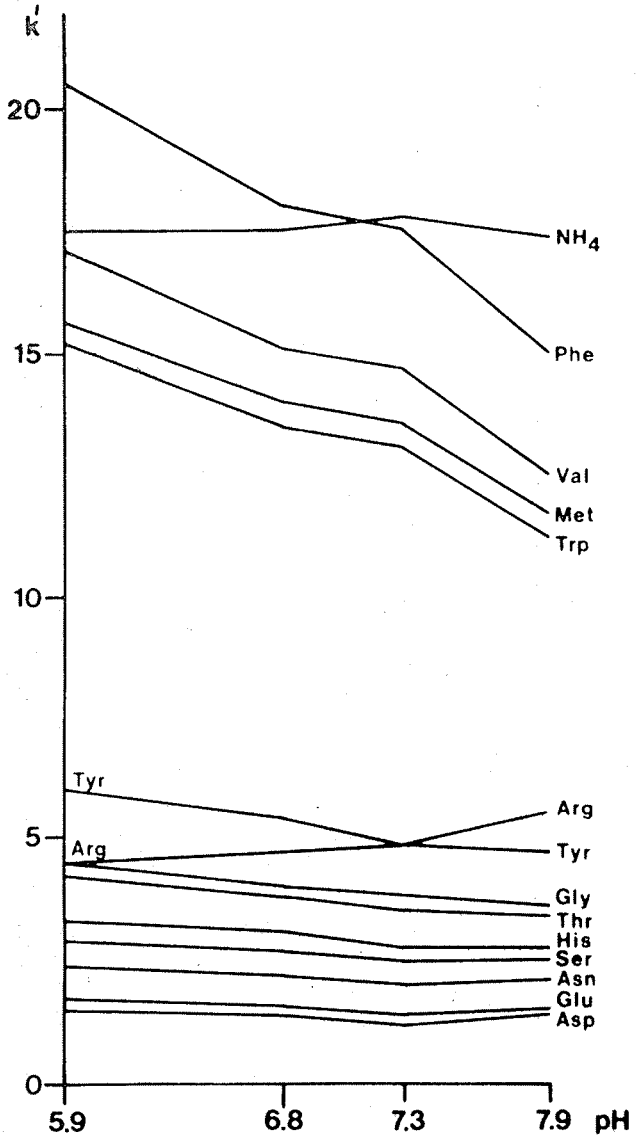


Figure 5. k' vs. pH of a 0.1 M phosphate buffer; isocratic 45% methanol

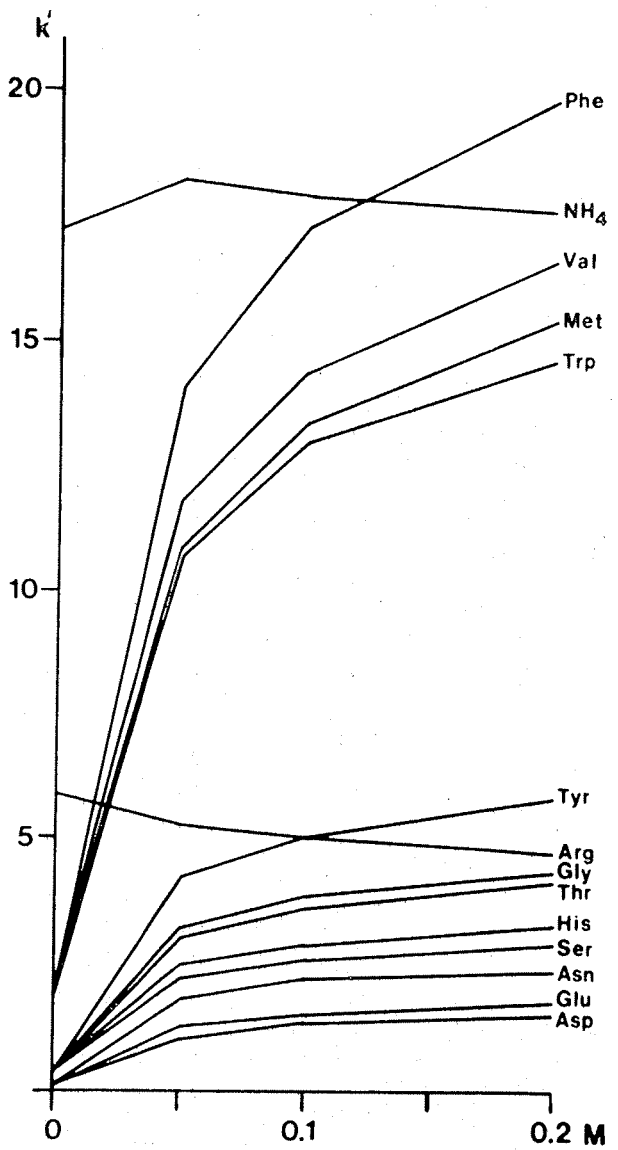


Figure 6. k' vs. the phosphate buffer concentration at pH 7.3; isocratic 45% methanol

prior to derivatization and then analyzed at a high detector sensitivity. The linearity of the responses should also be examined in cases of uncertainty.

Reaction Time. The fluorescence responses (peak heights) of OPT-amino acid derivatives as a function of reaction time prior to injection are shown in Figure 7. With the exception of cysteic acid, glycine, β-alanine, ammonia, lysine, and ornithine, the fluorescence responses of the amino acid derivatives show only minor variations with reaction time as exemplified by phenylalanine and tryptophane in Figure 7. These results contrast with those of Mell et al. (*12*) who found that the fluorescence of catecholamine derivatives reached a maximum after 2–3 min and decreased rapidly thereafter. From our results it therefore appears that the presence of an electron donating group, in this case the carboxyl group, α to the isoindole structure has a stabilizing effect on the latter. The reason for the exceptional instability of glycine, lysine, and ornithine, Figure 7, is presently unknown. The long-term instability of OPT derivatives (over a period of several hours to days) was examined by Simons and Johnson (*15*). These investigators found that the fluorescent product was a simple thioalkyl-substituted isoindole (*16*) and that the instability of this adduct was due to a slow, spontaneous intermolecular rearrangement, with sulfur being displaced by oxygen from the ethanolic portion (*16*). These investigators found that by replacing 2-mercaptoethanol in the reagent with ethanethiol, the stability of the fluorescent derivative is greatly enhanced (*15*). We tested ethanethiol and found, in contrast to Simons and Johnson, that glycine and β-alanine were still very unstable. Thus, some form of hydrolysis effect must be active. Lysine and ornithine were, however, stabilized, although they chromatographed as double peaks. The ammonia derivative with ethanethiol was considerably more unstable than with mercaptoethanol. Furthermore, for this derivative the peak form was very broad and unsymmetric. No increase in sensitivity was gained with ethanethiol. For the above reasons the use of ethanethiol was discontinued.

As previously mentioned the fluorescence responses of most amino acid derivatives were nearly independent of the reaction time prior to injection, cf. phenylalanine in Figure 7. However, to maximize the reproducibility of the method, a precise reaction time should be used and, in regard to the plateau responses of cysteic acid and ammonia (Figure 7), an exact 2-min reaction period is recommended.

Examination of Fluorescence Response. *Relative Responses.* A calibration mixture was diluted, derivatized, and analyzed by gradient elution. The peak areas were

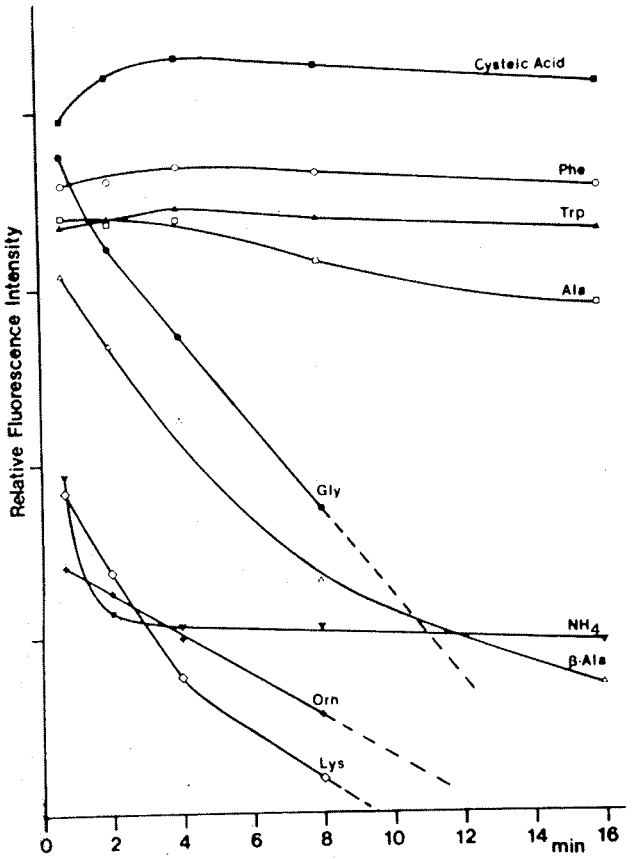


Figure 7. Influence of reaction time before injection on the fluorescence intensity of eight amino acids and ammonia

Table I. Response Factors, Normalized to Glycine

Asp	1.30	Ala	1.00
Glu	1.10	Met	1.00
Ser	1.20	Val	1.10
His	1.00	Phe	0.90
Thr	1.10	Ile	1.00
Gly	1.00	NH_4	0.05
Arg	1.00	Leu	0.90
Tyr	1.10	Lys	0.45

measured and normalized to glycine. The results are presented in Table I. It should be noted that only approximate response factors are given, since changes in, for example, eluent pH and composition and gradient program (e.g., % methanol) can slightly alter these values (*17*). Therefore, response factors should be calculated for each particular chromatographic system. In general the relative responses are rather uniform with the exception of ammonia and lysine which show only 5 and 45% relative responses, respectively. Interestingly the response of lysine in the present system is considerably higher than that found by Roth (*18*) and Josefsson et al. (*10*).

Excitation Wavelength. The excitation wavelength was varied in steps from 320–360 nm and within this range the fluorescence responses of the individual OPT amino acid derivatives varied by less than 10%. Thus 330 nm was found to be satisfactory.

Precision. *Derivatization.* A series of 10 identical gradient runs were performed with a 24-component amino acid standard mixture. The derivatization step was performed prior to each injection and the reaction time was 1 min. Peak height was used as a measure of the fluorescence response. The average deviation at the 80-pmol level was ±0.5%. From Figure 7 it is seen that ammonia, lysine, glycine, and β-alanine are very sensitive to variations in the reaction time. The overall deviations for these derivatives were: ±1.2, ±1.5, ±0.6 and ±0.5%, respectively.

Table II. Number of Amino Acid Residues[a] per Molecule of Human Erythrocyte Carbonic Anhydrase B after Hydrolysis[b] as Determined by the Present Method and by a Conventional Ion-Exchange Amino Acid Analyzer

amino acid	present method	ion-exchange	actual composition[c]
Glu	23	22	22
Ser	29[d]	26	30
His	10	10	11
Thr	13	12	14
Gly	15	16	16
Arg	7	7	7
Ala	18	19	19
Val	14	15	17
Phe	11	11	11
Leu	21	20	20
Lys	18	18	18

[a] Only those residues which could be accurately measured from the chromatogram were selected. [b] Performic acid, ca. 80%, 4 h, 100 °C. [c] Prior to hydrolysis. [d] Slight contamination suspected.

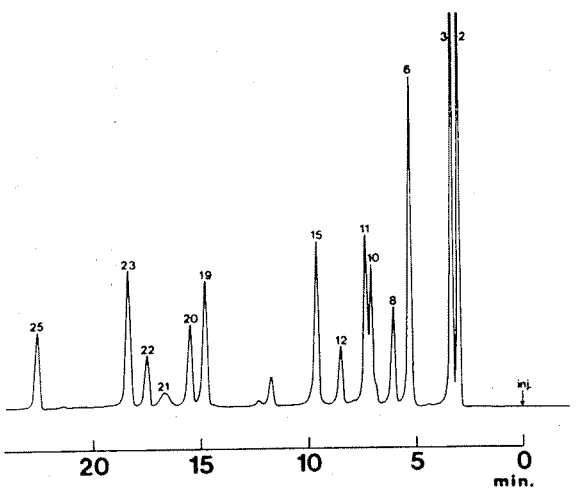


Figure 8. Protein hydrolysate: Column as in Figure 2. Mobile phase: (A) phosphate buffer, 0.1 M, pH 7.5. (B) methanol. Linear solvent gradient from 40% B to 75% B in 23 min. Peaks as in Figure 3

Retention Times. From the same series of 10 runs above, the retention times were measured. The average deviation for the 24 amino acids was ±0.3% with the largest deviations occurring closest to the manually marked injection point. Reproducibility of retention times from column to column was about ±3%.

Accuracy and Intercalibration. To determine the accuracy of the method, an intercalibration was performed. Aliquots of a protein hydrolysate were analyzed with the present method and with a conventional Stein and Moore-type (*19*) amino acid analyzer. The latter was a 2-column Beckman instrument employing ninhydrin as the locating agent. The total analysis time for acid/neutral and basic amino acids was 4–5 h. Table II shows the results of the intercalibration in terms of amino acid residues per protein molecule. In general, the agreement is quite good. Figure 8 shows the actual HPLC chromatogram upon which the measurements were based.

Recovery. When a reagent excess of greater than 200 times was used, recoveries of standards added to a urine sample averaged close to 100%. However, if the reagent excess was allowed to fall below 200 times, the recoveries decreased significantly. With a <100 times excess, recoveries ranged from 70–95%. The worst recoveries were for those amino acids

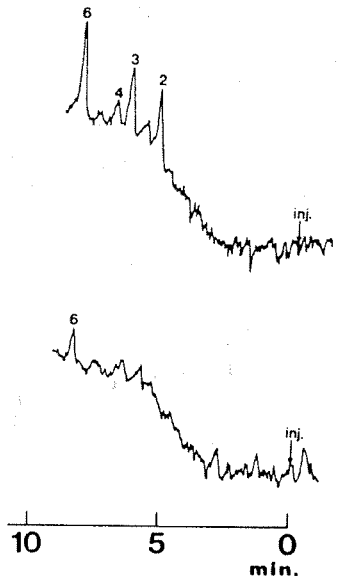


Figure 9. Upper: Chromatogram of 60 fmol Asp (2), Glu (3), Ser (6) and 50 fmol *S*-carboxymethyl cysteine (4). Lower: Chromatogram of a blank at the same sensitivity. Run condition as in Figure 3

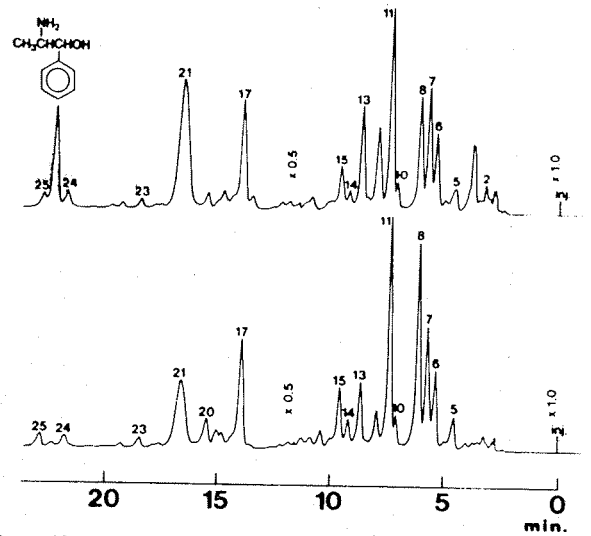


Figure 10. Upper: Urine sample from patient taken 2 h after ingestion of an α-receptor stimulator, 2-amino-1-phenylpropanol; injected without prior cleanup, diluted 1:200. Lower: Normal urine sample injected without prior cleanup, diluted 1:200. Run condition as in Figure 8. Peaks as in Figure 3

which were also dominant in the urine.

Detection Limit. Figure 9 shows the analysis of a standard containing 60 fmol of aspartic acid, glutamic acid, and serine derivatives and 50 fmol of *S*-carboxymethylcysteine derivative. The blank reveals a minor serine contamination. At this high sensitivity level, it was necessary to use freshly distilled water, a previously unopened bottle of glass distilled methanol, and a previously unused >24-h-old reagent in order to minimize contamination peaks, e.g., ammonia, glycine, serine.

Applications of the Method. One of the major advantages of the method is its extremely high sensitivity. In many instances, samples can be analyzed without clean-up steps if they are sufficiently diluted prior to derivatization. However, the dilution step itself may introduce contaminants which would interfere at subpicomole sensitivities. To avoid the dilution step, an injector/spitter system can be used to advantage. Figures 10, 11 and 12 illustrate a series of clinical, biochemical, and environmental applications of the method.

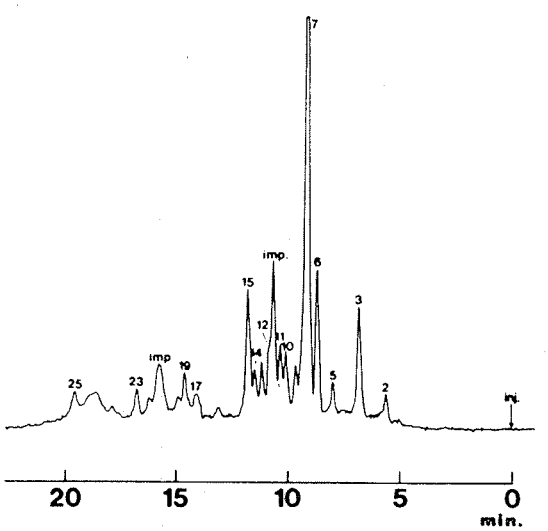


Figure 11. Cerebrospinal fluid injected without prior cleanup, diluted 1:200; imp = impurity. Run condition and peaks as in Figure 3. Peak 15: Ala is not resolved from γ-aminobutyric acid

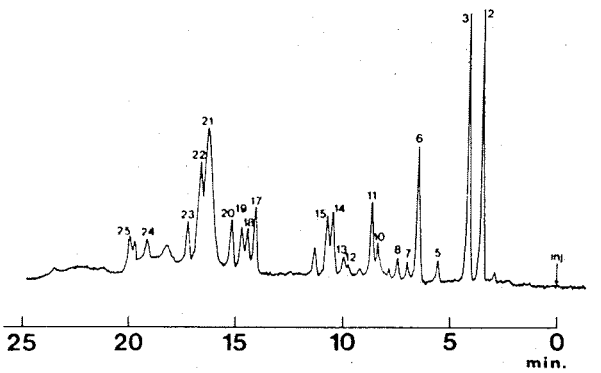


Figure 12. Seawater injected without prior cleanup; 10 parts seawater to 1 part reagent. Mobile phase: (A) phosphate buffer, 0.1 M, pH 7.5. (B) methanol. Linear solvent gradient from 35% B to 80% B in 20 min

No clean-up step was performed prior to analysis. Most biological samples, e.g., urine, plasma, cerebrospinal fluid, are rich in amines, therefore a dilution step was necessary prior to derivatization. In many instances <1 nL of the original sample was injected. For some types of samples, such as cerebrospinal fluid (Figure 11), a clean-up step or a guard column may be advisable since it was found that strongly adsorbed components accumulate at the top of the column and eventually lead to a loss of column efficiency. For the above samples the column efficiency was completely restored by replacing the upper 1 to 2 mm of the column bed after every 5 to 6 samples. In contrast to biochemical and clinical samples, natural water samples such as seawater, Figure 12, often contain extremely low levels of free amino acids. For these samples a small amount of a stronger reagent is directly added, e.g., 10 μL of reagent to 100 μL of sample. Since aqueous salt solutions are considerably poorer eluents than the mobile phase, a preconcentration of the solutes occurs at the top of the column, thus permitting the injection of several hundred microliters without noticeable band spreading (*20*). Applications of the method to natural and polluted water samples are presently being studied.

DISCUSSION

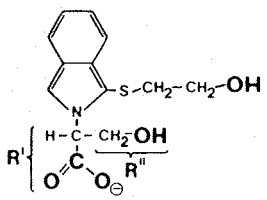
General. The elution order of the various amino acid derivatives of *o*-phthaldialdehyde (OPT-amino acids) on a hydrophobic (or lipophilic) stationary phase appears to be

Table III. OPT-Amino Acids Listed According to Increasing Retention[a]

amino acid	symbol	k'	R''(R')
cysteic acid (1)[b]	CysA	0.9	$^{-}O_3SCH_2$-
aspartic acid (2)	Asp	1.0	$^{-}OOCCH_2$-
glutamic acid (3)	Glu	1.0	$^{-}OOCCH_2CH_2$-
S-carboxymethylcysteine (4)	CM Cys	1.1	$^{-}OOCCH_2SCH_2$-
asparagine (5)	Asn	1.4	$CO(NH_2)CH_2$-
serine (6)	Ser	1.6	$HOCH_2$-
glutamine (7)	Glu	1.6	$HOCH_2CH_2$-
histidine (8)	His	1.7	$C_3H_3N_2CH_2$-
methioninesulfone (9)	Met-SO	1.8	$CH_3SO_2CH_2CH_2$-
threonine (10)	Thr	1.9	$CH_3CH(OH)$-
glycine (11)	Gly	2.1	H-
β-alanine (13)	β-Ala	2.6	R' = $^{-}OOCCH_2CH_2$-
tyrosine (14)	Tyr	2.6	p-$OHC_6H_4CH_2$-
arginine (12)	Arg	2.7	$^{+}(NH_2)_2CNHCH_2CH_2CH_2$-
alanine (15)	Ala	2.8	CH_3-(R' = CH_3CHCOO^{-})-
α-aminobutyric acid (16)	α-Abu	4.6	CH_3CH_2-
tryptophan (17)	Trp	5.5	(3-indol)CH_2-
methionine (18)	Met	6.2	$CH_3SCH_2CH_2$-
valine (19)	Val	6.6	$(CH_3)_2CH$-
phenylalanine (20)	Phe	7.4	$C_6H_5CH_2$-
ammonia (21)	NH_3	8.8	(R' = H)
isoleucine (22)	Ile	10.3	$CH_3CH_2CH(CH_3)$
ornithine (24)	Orn	25.8	$^{+}NH_3CH_2CH_2CH_2$-
lysine (25)	Lys	38.8	$^{+}NH_3CH_2CH_2CH_2CH_2$-

[a] The α-residue R'' or N-bonded residue R' (see structure in text) is given together with the capacity factor (k') for an isocratic run with 50% methanol and 0.1 M phosphate buffer, pH 7.3. [b] Peak numbers as in Figures 2 and 3.

governed mainly by the balance between the hydrophobic and hydrophilic groups of these derivatives. The hydrophobic (lipophilic) parts of the molecules are forced (squeezed) out of the mobile aqueous–methanolic phase onto the hydrocarbon stationary phase mainly as a result of the energy needed to make a cavity in the network of hydrogen bonds in the associated mobile phase (*21*, *22*). This energy corresponds to about 3.4 kJ at 25 °C per mole CH_2 transferred from pure water onto (or into) a hydrocarbon phase. The energy decreases with increasing percentage of methanol. The hydrophilic groups on the other hand link the derivatized amino acids to the mobile phase (25–75% methanol). The elution order (or retention time) is thus dependent on both the volume size of the hydrophobic parts and the number and nature of the hydrophilic groups. In order to elucidate this, the formula of OPT-serine is given below. In this formula the hydrophilic groups are denoted by enlarged print:



For the other amino acids, R'' is given in Table III where the OPT-amino acids are listed in order of increasing retention times or capacity factors (k'). For ammonia R' = H. The ionization constant of the carboxylic acid group in OPT-amino acids has not been measured, but it is most probably closer to a carboxylic acid pK_a than to an amino acid pK_{a1}. Carboxylate group protonization is probably an important equilibrium with regards to retention since the carboxylic acid (COOH) group most likely is less hydrophilic than the carboxylate group (COO^-). On the other hand, protonization of nitrogen-containing R'' groups, as in OPT-histidine (pH <6), OPT-arginine (pH <12.5) and OPT-ornithine and -lysine (pH <10.5), will set the net charge of the OPT-amino acid to zero because of zwitterion formation. These are expected to behave as neutral molecules, such as OPT-ammonia. (Note that in the case of Arg, Orn, and Lys, the OPT reagent is unreactive toward the ionized amino group.)

Detailed. The order of amino acids in Table III demonstrates the order of hydrophilic linking to the mobile phase: $SO_3^- > COO^- > CO(NH_2) > OH >$ imidazol $> CH_2SO_2CH_3 > H$. The position of the end group in arginine ($NHC(NH_2)_2^+$) in this series is most likely before $CO(NH_2)$ group in asparagine. The positions in Table III can be affected by protonization. For example, protonization of the imidazol group ($pK_a \approx 6$) in histidine will make it considerably more hydrophilic while the protonization of the carboxylate groups in aspartic and glutamic acids ($pK_{a1} \approx 4.2$, $pK_{a2} \approx 5.5$ as for succinic and glutaric acids) will make them less hydrophilic. Deprotonization of tyrosine (pK_a of the phenol group ≈ 10) makes it more hydrophilic while deprotonization of the guanidinium group on arginine, which only would occur in very basic solutions ($pK_a \approx 12.5$), makes it less hydrophilic. However, in this case, deprotonization eliminates half of the arginine zwitterion which would probably cause a decrease in retention. This also applies to the deprotonization of the respective δ and ε amino groups of ornithine and lysine ($pK_a \approx 10.5$). The group of derivatives that do not contain hydrophilic groups in R'' starts with glycine and ends with leucine. The following hydrophobic series is displayed in Table III:

$$H < CH_3 < C_2H_5 < CH_2(3\text{-indol}) < C_3H_7 < CH_2C_6H_5 < C_4H_9$$

The series demonstrates that the phenyl group corresponds to something in between a C_2H_5 and a C_3H_7 group (No. 19 valine and No. 22 isoleucine). The effect of introducing a CH_2 group is demonstrated in this hydrophobic series as well as for those R'' groups that contain hydrophilic parts, i.e., the pairs aspartic and glutamic acids, serine and threonine. Since the transfer of the molecules from the mobile phase to the stationary phase involves van der Waals' forces in both phases, intramolecular packing (in order to minimize the cavity), binding to unreacted silanol groups (nonbonded Si–OH groups) on the silica gel surfaces, intramolecular bonding (e.g., hydrogen bonding with the thioglycol part), etc., there is likely to be position effects among isomers. Thus the partition coefficient (capacity factor) for alanine is larger than for

β-alanine; and that for leucine is larger than for isoleucine. Since most of the OPT-amino acids are present at pH 7 in the form of bulky carboxylate ions, the capacity factors should increase upon addition of anions in the aqueous methanol phase because of ion repulsion between the added anions and the ionized carboxylate on the OPT amino acid derivatives. This is clearly brought out in Figure 4. Note that most of the 0.1 M $B(OH)_3$–$B(OH)_4^-$ buffer is in the form of boric acid at pH 7.3. The 0.1 M phosphate buffer is dominated by HPO_4^{2-} and the 0.1 M citrate buffer by $C_6H_5O_7^{3-}$. Four derivatives do not respond to ion repulsion, namely arginine, ornithine, lysine, and ammonia; however these molecules are neutral even if R′ in arginine, ornithine, and lysine are zwitterions. The increase in k' (Figure 4) between borate and phosphate is about 5.5 to 5.6 times for the top group (No. 17 tryptophan through No. 20 phenylalanine), but only 3.5 to 3.6 times for the lower group (No. 2 aspartic acid through No. 14 tyrosine). A similar behavior is demonstrated in Figure 6, which shows the increase of k' with increasing phosphate concentration.

The sodium ion concentrations are approximately 0, 0.002 M (borate), 0.17 M (phosphate), and 0.3 M (citrate) in Figure 4. Likewise the sodium concentration increases in Figure 6. It may therefore be thought that the increase in k' would be due to ion-pairing according to the following equilibrium

$$Na^+(\text{aq. mob.}) + A^-(\text{aq. mob.}) \rightleftharpoons NaA(\text{org. stat.})$$

However, we prefer to look upon the sodium ion as a medium ion providing electroneutrality in both the aqueous mobile phase (aq. mob.) and the organic stationary phase (org. stat.). The increase in k' with decreasing pH shown in Figure 5 can thus not be an effect of the sodium ion concentration since it is higher at pH 7.9 (≈0.19 M) than at pH 5.9 (≈0.11 M). The increase when lowering the pH is more likely due to ion suppression as expressed by the following equilibrium

$$H^+(\text{aq. mob.}) + A^-(\text{aq. mob.}) \rightleftharpoons HA(\text{org. stat.})$$

Thus protonization of the carboxylate group of the OPT-amino acid followed by adsorption onto the stationary phase explains these results. A rough value of the equilibrium constant could be obtained from a study of the two-phase equilibrium

$$H^+(\text{aq}) + A^-(\text{aq}) \rightleftharpoons HA(\text{liq. hydrocarbon})$$

Hexane and dodecane may be suitable choices for the liquid organic phase. Similarly, the effect of ion repulsion on the equilibrium constant can be quantitatively studied in such two-phase systems. These studies are presently being conducted.

Ion-pair formation may be used to increase the retention. This was confirmed in a run with C^+ = *N*-cetyl-*N*,*N*,*N*-trimethyl ammonium ion (0.1–0.3 w/v %)

$$C^+(\text{aq. mob.}) + A^-(\text{aq. mob.}) \rightleftharpoons CA(\text{org. stat.})$$

The results are shown in Figure 13. The loss of selectivity is considerable as can be seen when compared with the results in Figure 3. Of course less bulky (hydrophobic) cations should be tested before one draws the conclusion that ion repulsion is to be preferred over ion-pairing to achieve good retention and selectivity.

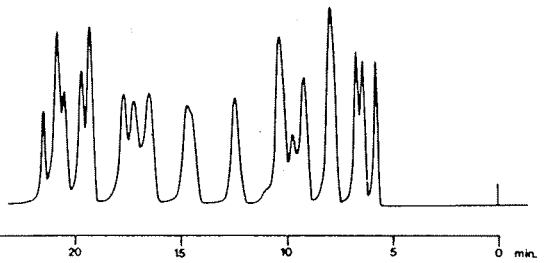


Figure 13. Separation of 24 amino acid OPT-derivatives by ion pair chromatography using same eluent as in Figure 3 containing 3 g/L *N*-cetyl-*N*,*N*,*N*-trimethyl ammonium bromide. Linear solvent gradient from 55% B to 85% B in 15 min. Peak elution order not determined

The main drawback of the present method is the lack of reaction with secondary amines. A simple system for the simultaneous fluorescence detection of primary and secondary amines based on reversed-phase HPLC separation is near completion and will be presented shortly.

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