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Use of chromogenic and fluorescent oxycarbonyl chlorides as reagents for amino acid analysis by high-performance liquid chromatography

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Abstract

The aryloxycarbonyl chlorides 4-phenylazobenzyloxycarbonyl chloride (PAZ-Cl), *p*-nitrobenzyloxycarbonyl chloride (PNZ-Cl), *o*-nitrobenzyloxycarbonyl chloride (ONZ-Cl), 2-naphthoxycarbonyl chloride (NOC-Cl) and 2-(naphthylmethyl)oxycarbonyl chloride (NMOC-Cl) were used for the precolumn derivatization of amino acids. The separations of the derivatives thus formed by reversed-phase (C_{18} or C_8) high-performance liquid chromatography (HPLC) were investigated and compared with those with the established reagent 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl). A robotic autosampler enabled the fully automated mixing of samples with alkaline borate buffers, addition of excess of reagents (and of the scavenger 1-aminoadamantane if required), the final dilution of aliquots of the reaction mixtures with sodium acetate buffers and solvents and injection on to the HPLC column. Derivatives were eluted from the columns at 45°C using gradients made up from sodium acetate buffers and acetonitrile. Derivatives were detected by their UV absorbance (PAZ-, PNZ- and ONZ-amino acids) or by their fluorescence (NOC-, NMOC- and FMOC-amino acids). Hydrolysates of equine myoglobin, bovine β-lactoglobulin A and bovine serum albumin were quantitatively analysed with the reagents NOC-Cl and PNZ-Cl and the results were found to be in good agreement with those obtained by derivatization with FMOC-Cl and with the calculated amino acid composition of the proteins.

1. Introduction

For the precolumn derivatization of mixtures of amino acids (AAs) with UV-absorbing, chromogenic or fluorescent reagents, and separation of the resultant derivatives by reversed-phase high-performance liquid chromatography (HPLC), a relatively large number of reagents have been described [1,2] and, in part, compared with each other [3,4]. A survey of the literature (where possible, newer references or reviews are cited in this paper to provide entries to the respective literature) shows that the currently most frequently used reagents are *o*-phthaldialdehyde together with various thiols [5–7], phenyl isothiocyanate [8–10], 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) [11,12], 4-dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl chloride, DABS-Cl) [13,14], 5-(dimethylamino)-naphthalene-1-sulfonyl chloride (dansyl chloride) [15] and Sanger-type reagents [16,17]. Chiral variants of several of these reagents suitable for the HPLC separation of DL-AAs as diastereoisomers have also been described [18–23].

In analogy with FMOC-Cl, substituted aryloxycarbonyl chlorides ("chloroformates" or, more correctly "carbonochloridates") as derivatizing reagents for amines or imines in general deserve

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attention as a result of their high reactivity and structural diversity and the relative easy synthesis of these compounds. Use of an excess of oxycarbonyl chlorides under alkaline conditions for the precolumn derivatization of amino components should allow their quantitative derivatization within a short time and at ambient temperature. Consequently, 2-(9-anthryl)ethyl chloroformate [24] and 2-(1-pyrenyl)ethyl chloroformate [25] have been synthesized and used as fluorescent reagents for the derivatization and liquid chromatographic resolution of biogenic amines; these reagents are also considered to be suitable for the derivatization of AAs.

In this paper, we describe the use of PAZ-Cl, PNZ-Cl, ONZ-Cl, NOC-Cl and NMOC-Cl, which have not been employed before as analytical reagents for the precolumn derivatization of AAs, followed by the liquid chromatographic separation of the resultant derivatives, and compare their suitability with that of FMOC-Cl. The latter reagent [26], together with PAZ-Cl [27] and PNZ-Cl [28], were originally developed as selectively cleavable amino protecting groups in peptide chemistry and are variants of the classical reagent benzyloxycarbonyl chloride [29], usually designated Z-Cl in honour of one of its inventors, L. Zervas. NOC-Cl has been used for labelling amino acids in order to develop an assay for carboxy peptidase [30] and for the derivatization of drugs containing tertiary amino groups [31].

2. Experimental

2.1. Instruments

For HPLC, a LiChroGraph instrument consisting of an L-6200 pump, low-pressure gradient mixture, Model T-6300 thermostat, Model AS-4000 autosampler with freely programmable liquid handling functions and freely programmable sample and reagent racks, Mega D-2500 integrator and F-1050 fluorescence detector (with 12-μl cuvette) or SPD-6A variable-wavelength UV detector (with 8-μl cuvette) were used. Eluents were flushed with helium. Instruments were obtained from Merck–Hitachi (Darmstadt, Germany), with the exception of the UV detector, which was from Shimadzu (Kyoto, Japan).

For recording the absorption and fluorescence spectra shown in Fig. 2, a Hewlett-Packard (HP) LC 1090 Series L liquid chromatograph was used together with an HP 1046 fluorescence detector and an HP photodiode-array detector.

L-Alanine was derivatized by the AS 4000 autosampler, transferred to the HP LC 1090 liquid chromatograph and analysed using a Hypersil ODS column, particle size 5 μm, and gradient elution as described for the respective derivatives.

2.2. Columns

LiChroCART columns (250 mm × 4 mm I.D.) and precolumns (4 × 4 mm I.D.) were used. For analysis with PAZ-Cl the stationary phase was LiChrospher 100 RP-18 (5 μm) (guard column packed with the same material). For analyses with PNZ-Cl, ONZ-Cl and FMOC-Cl the stationary phase was Superspher 60 RP-8 (4 μm) and the respective guard columns were packed with LiChrospher 100 RP-8 (5 μm). For analyses with NOC-Cl and NMOC-Cl, the stationary phase was Superspher 100, RP-18, end-capped (4 μm), and the guard columns were packed with LiChrospher 100, RP-8 (5 μm).

2.3. Solvents, chemicals and reagents

Acetonitrile (MeCN) was of HPLC grade from Baker (Deventer, Netherlands). Tetrahydrofuran (THF) was of chromatography grade, acetic acid, boric acid and sodium hydroxide pellets were of analytical-reagent grade, toluene, dioxane, dichloromethane and dimethylformamide (DMF) were of synthetic grade, DMF being refluxed in the presence of ninhydrin and distilled before use; all these reagents were from Merck (Darmstadt, Germany). 2-Naphthol, 2-naphthylmethanol [(2-hydroxymethyl)napthalene], 2-nitrobenzyl alcohol (*o*-nitrobenzyl alcohol), quinoline and 1-aminoadamantane (1-adamantylamine, ADAM) were purchased from Aldrich (Steinheim, Germany) and 1.93 *M*

(20%) phosgene in toluene from Fluka (Buchs, Switzerland). FMOC-Cl was obtained from Merck.

PAZ-Cl and PNZ-Cl were purchased from Bachem (Bubendorf, Switzerland) and were recrystallized from light petroleum (b.p. 50–70°C). PAZ-Cl had m.p. 78–79.5°C (lit. [27] m.p. 82°C); PNZ-Cl had m.p. 28–30°C (lit. [28] m.p. 33.5–34°C).

NOC-Cl was synthesized in our laboratory from 30 mmol of 2-naphthol and 30 mmol of quinoline in a mixture of 18 g of toluene and 5 g of dichloromethane by treatment with 47 ml of 1.93 *M* phosgene in toluene at 0°C according to the literature [30]; yield 5.1 g (84%); m.p. 45–48°C (lit. [30] m.p. 65–67°C). NMOC-Cl was synthesized analogously from 10 mmol of 2-naphthylmethanol in 15 ml of toluene by treatment with 16 ml of 1.93 *M* phosgene in toluene at 0°C; yield 1.9 g (85%); m.p. 59–62°C (crystals). ONZ-Cl was synthesized from 10 mmol of 2-nitrobenzyl alcohol in 4 ml of dioxane and treatment with 15.5 ml of 1.93 *M* phosgene in toluene; yield 1.7 g (80%) (yellow oil). The oxycarbonyl chloride reagents used were of acceptable purity with regard of the aim of this work but were not of analytical quality.

2.4. Sources and hydrolyses of proteins

Bovine serum albumin (product No. 11920) and lyophilized equine myoglobin from skeletal muscle (product No. 29895) were purchased from Serva (Heidelberg, Germany). The myoglobin had, according to manufacturer's specification, a purity of >95% and consisted mainly of metymyoglobin. β-Lactoglobulin A from bovine milk (product No. L-7880) was obtained from Sigma (St. Louis, MO, USA). For AA determination, amounts of 3–5 mg were hydrolysed in 0.7 ml of 6 *M* HCl at 110°C for 41 h in 1-ml Reacti-vials (Wheaton, Millville, NJ, USA). Samples were evaporated to dryness in a stream of nitrogen (80°C at the beginning and ambient temperature at the end of the evaporation). The residues were dissolved in 700 μl of 0.1 *M* HCl and the solutions were filtered using disposable 0.2-μm ANOTOP membrane filters (Merck). Aliquots were used for analyses by HPLC with NOC-Cl, PNZ-Cl or FMOC-Cl as reagents (see below).

2.5. Amino acid standards

The AA standard mixtures were purchased from Sigma (product No. AA-S-18) or from Pierce (Product No. 20088). The standards were fortified (if required; see chromatograms) by addition of cysteic acid (CyA), Gln, 5-hydroxylysine (Hyl) (all from Sigma), L-*homo*-Arg, γ-aminobutyric acid (GABA) (from Serva), Asn (from Fluka) and ornithine (Orn) (from Merck). The hydrolysis-sensitive amino acids Asn and Gln were freshly added to standards before use.

2.6. Derivatization with oxycarbonyl chloride reagents and elution conditions for amino acid derivatives

Preparation of sodium acetate and sodium borate buffers

A 0.5 *M* sodium acetate (NaOAc) buffer was prepared from 0.5 mol of acetic acid in 950 ml of doubly distilled water, adjusted to pH 4.0 by addition of 20% (w/w) NaOH and made up to a final volume of 1 l by addition of water. We prepared 100 m*M* NaOAc eluent buffers of pH 4.4, 4.6 and 7.0 analogously, and 0.4 and 0.5 *M* sodium borate buffers were prepared similarly from solutions of boric acid, with adjustment of the pH to 8.0, 9.0 and 10.5 by addition of 20% NaOH and final dilution to 1 l.

Derivatization procedures for amino acids with the various reagents, performed by the robotic autosampler at ambient temperature, are described below. The column temperature was 45°C in all instances and the flow-rate of the mobile phases was 1.25 ml min^{-1} for all analyses with the exceptions of derivatization with NOC-Cl and NMOC-Cl, where it was 1.0 ml min^{-1} for analyses and 1.25 ml min^{-1} for equilibration.

PAZ-Cl

Amounts of 100 μl of 0.5 *M* sodium borate buffer (pH 9.0), 20 μl of AA standard mixture or analyte solution and 250 μl of PAZ-Cl solu-

tion (5 m*M* in MeCN) were mixed by the autosampler. After 5 min, 250 μl of ADAM solution [40 m*M* in acetone–water (3:1, v/v)] were added and, after a further 5 min, 320 μl of a mixture of 0.5 *M* NaOAc buffer (pH 4.0) and MeCN (1:1, v/v) were added to 80 μl of the reaction mixture and 20-μl aliquots were injected on to the HPLC column. For gradient elution of the derivatives, eluent A (100 m*M* NaOAc of pH 7.0) and eluent B (100% MeCN) were used. The gradient programme was 22 to 50% B (0–40 min), 50 to 80% B (40–45 min), 80 to 100% B (45–46 min), 100% B (46–55 min). The column was then equilibrated for 12 min with 22% B.

PNZ-Cl and ONZ-Cl

Amounts of 150 μl of 0.5 *M* sodium borate buffer (pH 10.5), 30 μl of AA standard mixture or analyte solution in 0.1 *M* HCl and 300 μl of PNZ-Cl (or ONZ-Cl) solution (50 m*M* in MeCN) were mixed by the autosampler. Then, in the case of derivatization with ONZ-Cl, after 2 min amounts of 300 μl of 40 m*M* ADAM in acetone–water (3:1, v/v) were added. After 2 min, 320 μl of a mixture of 0.5 *M* NaOAc (pH 4.0) and MeCN (1:1, v/v) were added to 80 μl of the reaction mixture and 20-μl aliquots were injected on to the HPLC column. For elution of the PNZ or ONZ derivatives, gradients from eluent A (100 m*M* NaOAc of pH 4.4 for PNZ and of pH 4.6 for ONZ) and eluent B (100% MeCN) were used. The gradient programme was 10 to 25% B (0–10 min), 25 to 30% B (10–20 min), 30 to 45% B (20–30 min), 45 to 100% B (30–35 min) and 100% B (35–40 min). The column was then equilibrated for 14 min with 10% B.

NOC-Cl and NMOC-Cl

Amounts of 100 μl of 0.4 *M* sodium borate buffer (pH 9.0), 20 μl AA standard mixture or analyte solution and 100 μl of NOC-Cl (or NMOC-Cl) solution (10 m*M* in MeCN) were mixed by the autosampler. After 2 min, 250 μl of ADAM solution [40 m*M* in acetone–water (3:1, v/v)] were added. After a further 2 min, 360 μl of a mixture of 0.5 *M* NaOAc (pH 4.0) and MeCN (1:1, v/v) were added to 40 μl of the reaction mixture and 20-μl aliquots were injected on to the HPLC column.

For the elution of the derivatives a ternary gradient from eluent A (100 m*M* NaOAc, pH 7.0), eluent B (100 m*M* NaOAc, pH 4.6) and eluent C (100% MeCN) was used. The gradient programme started with a mixture of 75% A, 15% B and 10% C, changing to 0% A, 70% B and 30% C (0–15 min), 0% A, 40% B and 60% C (15–30 min) and 0% A, 0% B and 100% C (30–35 min), followed by isocratic elution with 100% C (35–43 min). The column was then equilibrated for 13 min. The flow-rate was 1.0 ml min^{-1} during analyses and 1.25 ml min^{-1} for equilibration.

FMOC-Cl

Amounts of 100 μl of 0.4 *M* sodium borate buffer (pH 8.0), 20 μl of AA standard solution and 100 μl of FMOC-Cl solution (3 m*M* in acetone; MeCN is recommended as the solvent with UV detection) were mixed by the autosampler. After 2 min, 100 μl of ADAM solution [40 m*M* in acetone–water (3:1, v/v)] were added. After a further 2 min, 380 μl of a mixture of 0.5 *M* NaOAc (pH 4.0) and MeCN (1:1, v/v) were added to 20 μl of the reaction mixture and 20-μl aliquots were injected on to the HPLC column. For elution of the derivatives, gradients from eluent A [100 m*M* NaOAc of pH 4.6–THF–DMF (90:5:5, w/w/w)] and eluent B (100% MeCN) were used. The gradient programme was 7 to 15% B (0–10 min), 15 to 50% B (10–35 min), 50 to 100% B (35–40 min), isocratic elution with 100% B (40–45 min) and equilibration with 7% B for 10 min.

3. Results and discussion

The structures of the oxycarbonyl chloride reagents are illustrated in Fig. 1. The absorption spectra of L-Ala derivatized with PAZ-Cl and PNZ-Cl are shown in Fig. 2a and b, respectively, and the absorption and fluorescence spectra of L-Ala derivatized with NOC-Cl are shown in Fig. 2c and d, respectively. The spectra were taken under gradient elution conditions using a photo-

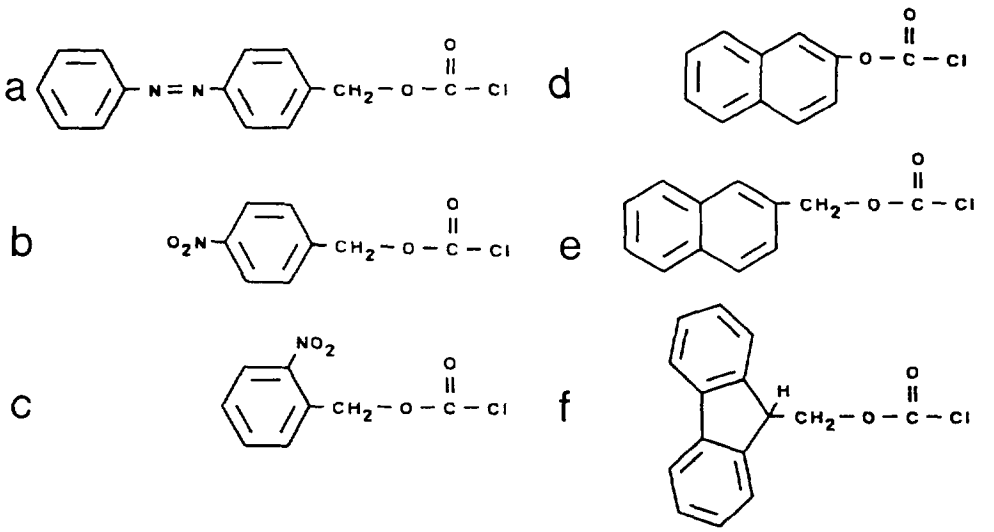

Fig. 1. Structures of (a) *p*-phenylazobenzyloxycarbonyl chloride (PAZ-Cl), (b) *p*-nitrobenzyloxycarbonyl chloride (PNZ-Cl), (c) *o*-nitrobenzyloxycarbonyl chloride (ONZ-Cl), (d) 2-naphthoxycarbonyl chloride (NOC-Cl), (e) 2-(naphthylmethyl)oxycarbonyl chloride (NMOC-Cl) and (f) 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl).

diode-array detector or a fluorescence detector with the stopped-flow method (see Experimental).

The elution profiles of AA standard mixtures derivatized with PAZ-Cl, PNZ-Cl, ONZ-Cl NOC-Cl, NMOC-Cl and FMOC-Cl are shown in figs. 3–8. Chromatograms of analyses of hydrolysates of equine myoglobin with PNZ-Cl are shown in Fig. 4b and c and of bovine serum albumin with NOC-Cl in Fig. 6b.

Data for the analyses of hydrolysates of myoglobin, lactoglobulin and albumin using PNZ-Cl, NOC-Cl and FMOC-Cl are listed in Table 1 and agree satisfactorily with values calculated from the amino acid composition of these proteins.

Derivatization conditions as described under Experimental were investigated and optimized with respect to the pH of derivatization (tested range pH 7–11), derivatization temperature (tested range 4–60°C) and reaction time (tested range 2–20 min). The optimum pH for derivatization was 9.0 for NOC-Cl and PAZ-Cl, 10.5 for PNZ-Cl and 8.0 for FMOC-Cl. As a result of the fast reactions of the reagents, very little influence of temperature on derivatization yields was observed. The derivatization was completed within 2 min at room temperature, with the exception of PAZ-Cl, which needed 5 min. The conditions are a compromise, as the derivatization kinetics of the various AAs are slightly different and the use of larger excesses of reagents is limited by their solubilities. Intensive efforts were undertaken in order to optimize the gradient elution conditions with regard to the separation of AA derivatives on the stationary phases used. It is expected, however, that testing of other stationary phases and eluents will further improve those resolutions which are not completely satisfactory.

In the following we discuss the chromatograms in more detail.

Derivatization of a seventeen-component AA standard with PAZ-Cl (Fig. 3) leads to a satisfactory resolution of the derivatives, with the exception of Pro and Arg, which elute together under the chromatographic conditions used. PAZ-Cl is to some extent comparable to the structurally related DABS-Cl [13,14], which requires, however, derivatization of AA at 70°C for ca. 15 min. The PAZ-AAs, which at high concentration were intensely red, were detected by their absorption at 320 nm; a second absorption maximum was found in the visible range, in agreement with the literature [27] at 440 nm (cf., Fig.

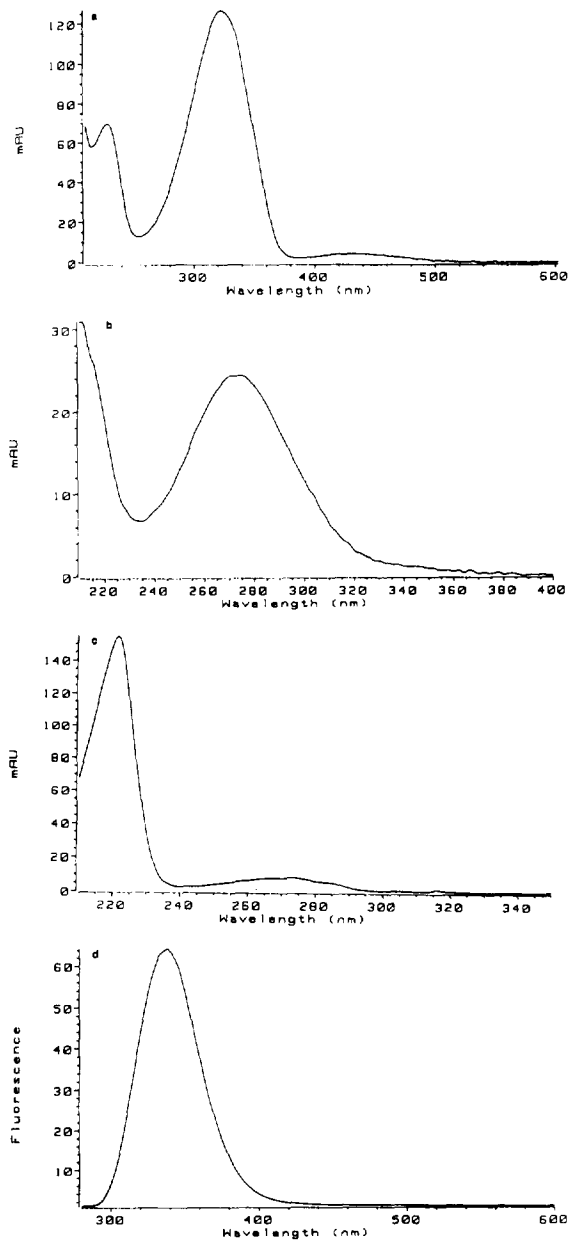

Fig. 2. Absorption spectra of L-Ala derivatized with (a) PAZ-Cl, (b) PNZ-Cl and (c) NOC-Cl, and (d) fluorescence spectrum of NOC-Ala when excited at 274 nm. For conditions see Experimental; mAU = milli-absorption units.

2a). It is worth noting that peaks arising from the hydrolysed reagent (designated A in the chromatograms) and the ammonolysis product of the reagent (designated B) elute at positions in the chromatogram which are not occupied by AAs, with the exception of cystine (designated Cys–Cys), which elutes close to the hydrolysed reagent. The peak designated C in this and the other chromatograms is assumed to be the carbonate formed by reaction of the respective oxycarbonyl chloride with its alcohol, the latter formed by partial hydrolysis of the chloride. Tyr gives rise to both the mono- and the disubstituted product under the derivatization conditions used. Four additional peaks, probably originating from impurities of the reagent (designated X in chromatograms), can be seen in the chromatograms but do not interfere with the AAs eluted. These peaks, and also those arising from impurities of the other oxycarbonyl chlorides used, might disappear if analytical-reagent grade reagents are prepared. The reaction product with the scavenger ADAM elutes with 100% MeCN and is not shown in the chromatograms.

The retention times of AAs measured on one column ($n = 5$) had a reproducibility of 0.11–0.32 min, and relative standard deviation (R.S.D.) of the retention times was 0.18–2.35%, with the exceptions of Glu and Asp, R.S.D.s of 6.16% and 7.21%, respectively; the R.S.D. of peak areas was 1–4% with respect to Val (Tyr >4%); linearity was found in the range 1.3–130 pmol per AA injected, and the correlation coefficient of linearity ($n = 3$ for each concentration, 7–9 calibration points were used in all experiments) was 0.993–0.999 for all AAs; the detection limit was approximately 0.5 pmol per AA.

We found that the resolution of PAZ-AA decreased after injection of a relatively small number (ca. 40) of samples. It appears that the PAZ derivatives are partly irreversibly bonded to the C_{18} stationary phase. It has not yet been investigated whether this phenomenon (which was not observed with the other oxycarbonyl chlorides investigated) can be overcome by changing the stationary phase.

The elution profile of AAs from a seventeen-component AA standard derivatized as described under Experimental with PNZ-Cl is shown in Fig. 4a. For quantification of the AAs in a total hydrolysate of equine myoglobin, the

Table 1
Amino acid (AA) composition of total hydrolysates[a] of proteins determined by HPLC ($n = 5$) and derivatization with NOC-Cl, PNZ-Cl and FMOC-Cl[b] in comparison with calculated (calc.) values [32–35][c]

AA	Equine myoglobin				Bovine β-lactoglobulin A				Bovine serum albumin[c]				
	NOC-Cl	PNZ-Cl	FMOC-Cl	Calc. [32]	NOC-Cl	PNZ-Cl	FMOC-Cl	Calc. [32]	NOC-Cl	PNZ-Cl	FMOC-Cl	Calc.	[33–35]
Asx	10.1	11.5	10.6	10.0	17.0	17.7	16.0	16.0	54.4	58.5	52.3	54.0	54.0
Glx	19.9	21.6	22.5	19.0	27.0	27.0	28.4	25.0	81.2	86.0	87.1	79.0	79.0
Ser	4.6	5.2	5.3	5.0	6.2	6.9	6.3	7.0	24.8	26.5	27.1	28.0	28.0
Gly	14.2	16.9	16.1	15.0	3.0	3.4	3.3	3.0	16.5	17.8	18.9	16.0	16.0
Arg	2.2	1.9	2.5	2.0	3.1	2.7	3.4	3.0	23.4	22.9	25.6	23.0	23.0
Thr	6.9	7.2	7.1	7.0	7.8	7.1	7.7	8.0	32.2	30.9	32.3	34.0	33.0
Ala	15.2	14.0	14.7	15.0	14.8	12.4	13.4	14.0	46.1	42.2	44.0	46.0	47.0
Pro	4.1	4.3	4.5	4.0	8.3	8.5	8.3	8.0	27.6	29.2	28.7	28.0	28.0
Val	7.0	7.4	7.4	7.0	10.5	10.5	10.1	10.0	36.3	37.2	36.5	36.0	36.0
Phe	7.2	7.6	7.8	7.0	4.3	4.4	4.2	4.0	27.7	29.2	27.7	27.0	27.0
Ile	8.4	9.0	9.1	9.0	9.8	9.6	9.7	10.0	13.6	14.6	14.2	14.0	14.0
Leu	17.4	17.6	18.5	17.0	23.8	22.2	23.0	22.0	61.2	61.3	62.6	61.0	61.0
His	10.6	14.5	10.6	11.0	1.7	2.3	1.7	2.0	15.6	19.4	13.7	17.0	17.0
Lys	19.5	20.1	20.0	19.0	15.1	14.4	14.3	15.0	61.3	61.0	58.7	59.0	59.0
Tyr	1.9	1.8	1.7	2.0	3.8	3.5	3.7	4.0	19.3	17.6	18.3	19.0	20.0

[a] Cys, Met and Trp (if present) not determined; Asx = Asp + Glu; Glx = Glu + Gln
[b] For abbreviations of reagents and derivatization conditions, see text.
[c] Suppliers do not provide or guarantee amino acid composition of proteins; in bovine serum albumin a difference is found for classical and DNA-deduced sequences.

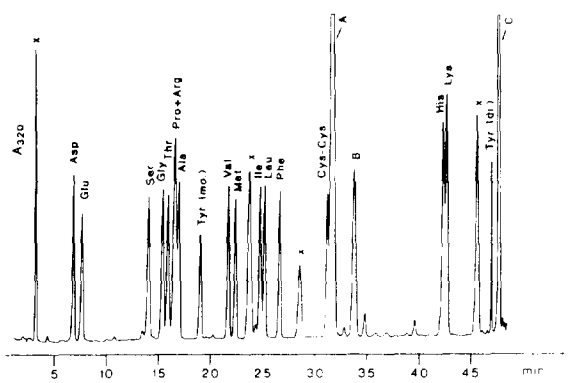

Fig. 3. Chromatogram of an AA standard (injected amounts 65 pmol per AA, cystine 32.5 pmol) derivatized with PAZ-Cl. A_{320} = absorption at 320 nm; peaks designated A, B, C and X in all chromatograms arise from reagents (see text); Cys–Cys = cystine; mo. and di. refer to mono- and disubstituted AA. For stationary phases, derivatization and elution conditions for all chromatograms, see Experimental.

absorption maxima at 265 nm of the weakly yellow derivatives were used (Fig. 4b). The ratios of those AAs that could be determined in the protein hydrolysate are given in Table 1. The AAs are satisfactorily resolved in these chromatograms. It was observed that, using a new stationary phase, Arg and Ala are completely resolved. During use, owing to the ageing of the column, Arg shows a shift in retention time and, finally, elutes together with Ala (Fig. 4c). This age-dependent shift of the retention time on reversed phases is characteristic of Arg and has also been reported for FMOC-Arg [11].

Asp and Ser are acceptably resolved on the column using the chromatographic conditions described for Fig. 4a and c. These AAs are baseline resolved on a new stationary phase using an eluent of pH 3.4, but this resolution occurs at the expense of that of other AAs. His and Tyr form disubstituted derivatives with an excess of reagent (cf., Fig. 4a and b); disubstituted His shows a time-dependent decay forming the monosubstituted derivative (ca. 50% in 24 h). The chromatograms in Fig. 4a and b were recorded directly after derivatization with PNZ-Cl, and therefore no monosubstituted His can be seen. The chromatogram shown in Fig. 4c was recorded ca. 2.5 h after derivatization; the monosubstituted His released at this time elutes close to Thr. Cystine elutes close to the hydrolysed reagent (A), and the ammonolysis product of the reagent (B) is satisfactorily resolved from Val.

The reproducibility of retention times of AA derivatives ($n = 5$), measured on one column, was 0.02–0.08 min, with an R.S.D. of 0.8–1.20% for Glu, Ser and Asp and 0.08–0.34% for the other AAs; The R.S.D. of peak areas ($n = 5$) was 1.5–4.0% (His >4%); linearity was found in the range 6–600 pmol per AA injected, the correlation coefficient of linearity ($n = 3$) was 0.9997–0.9998 for the AAs with the exceptions of cystine (0.9996), His (0.9994) and Tyr (0.9990); the detection limit was ca. 1 pmol per AA, with the exceptions of Met (6 pmol) and cystine (12 pmol).

The elution profile of a seventeen-component AA standard derivatized with ONZ-Cl in the presence of ADAM (which was not used for PNZ-Cl) as scavenger is shown in Fig. 5. The elution conditions are almost identical with those for the derivatives of PNZ-Cl (see Experimental) and the chromatograms look very similar. The position of the nitro group in these derivatives (*para* in PNZ-AAs and *ortho* in ONZ-AAs) therefore has little influence on retention times. Arg and Ala are resolved, but, cystine elutes together with an unknown component (designated X), Val together with X and the ammonolysis product of the reagent and disubstituted Tyr together with peak C. Peaks arising from ADAM and its derivatization product are eluted from the column when eluent B is applied and are not seen in the chromatograms. Acetone, eluting at the beginning of the chromatogram, had been used as the solvent for ADAM, but the use of MeCN is recommended when derivatives are determined by measuring their UV absorbance. As ONZ-Cl has no noticeable advantage over PNZ-Cl, the former reagent was not investigated further.

The elution profile of a 24-component AA standard, including hydrolysate AAs and also CyA, Asn, Gln, GABA, Hyl, Orn and homo-Arg, derivatized with NOC-Cl and followed by addition of the scavenger ADAM, is shown in

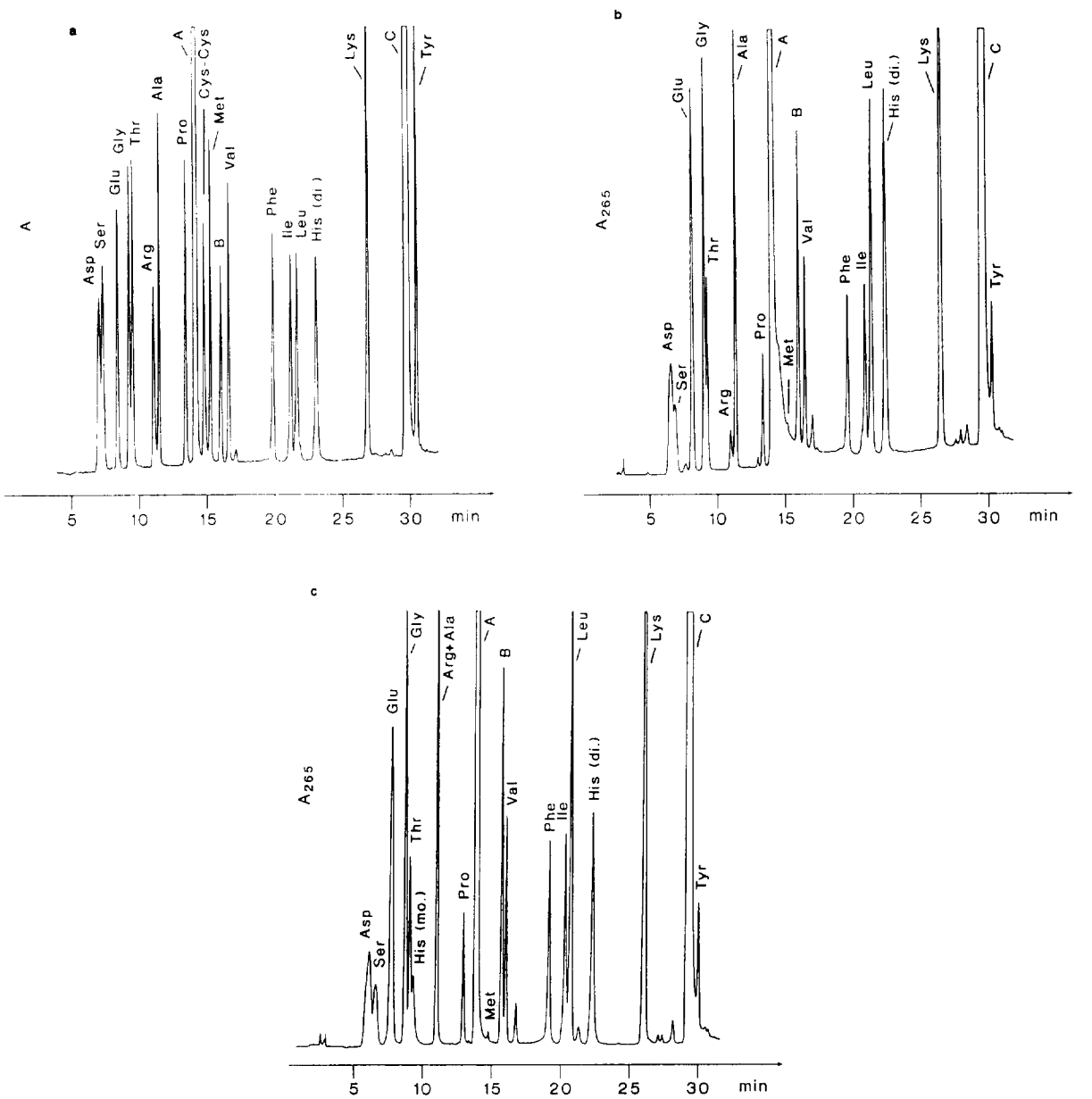

Fig. 4. Chromatograms of (a) AA standard on a new column (injected amounts 5 nmol per AA, cystine 2.5 nmol), (b) total hydrolysate of equine myoglobin (1.5 μg) derivatized with PNZ-Cl and immediately injected on to the column and (c) total hydrolysate of equine myoglobin injected 2.5 h after derivatization on to an aged column A_{265} = absorbance at 265 nm; for details, see text.

Fig. 6a. The fluorescence of the derivatives was measured at an emission of 336 nm with excitation at 274 nm. The AAs of the standard are satisfactorily resolved. Ser and Gln in the standard elute together; this might be a problem in physiological samples; Gln in proteins, however, is hydrolysed to Glu under the conditions of total hydrolysis (cf., Fig. 6b and Table 1). Tyr and His form mono- and disubstituted derivatives under the derivatization conditions used; monosubsti-

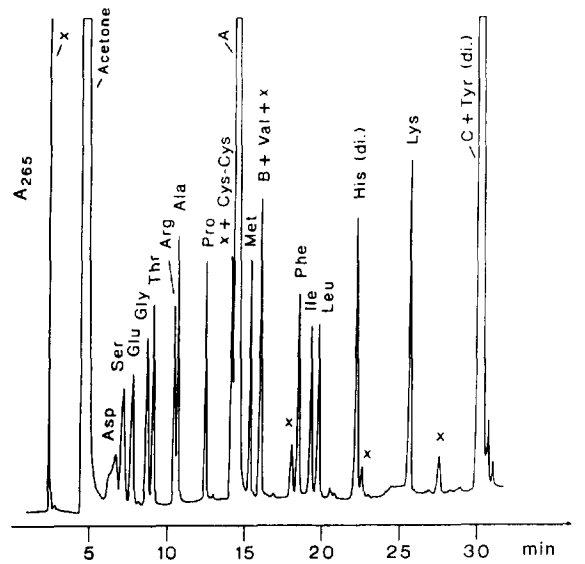

Fig. 5. Chromatogram of an AA standard derivatized with ONZ-Cl (injected amounts 0.4 nmol per AA, cystine 0.2 nmol). The same standard and column were used as in Fig. 4a; for other conditions, see Experimental.

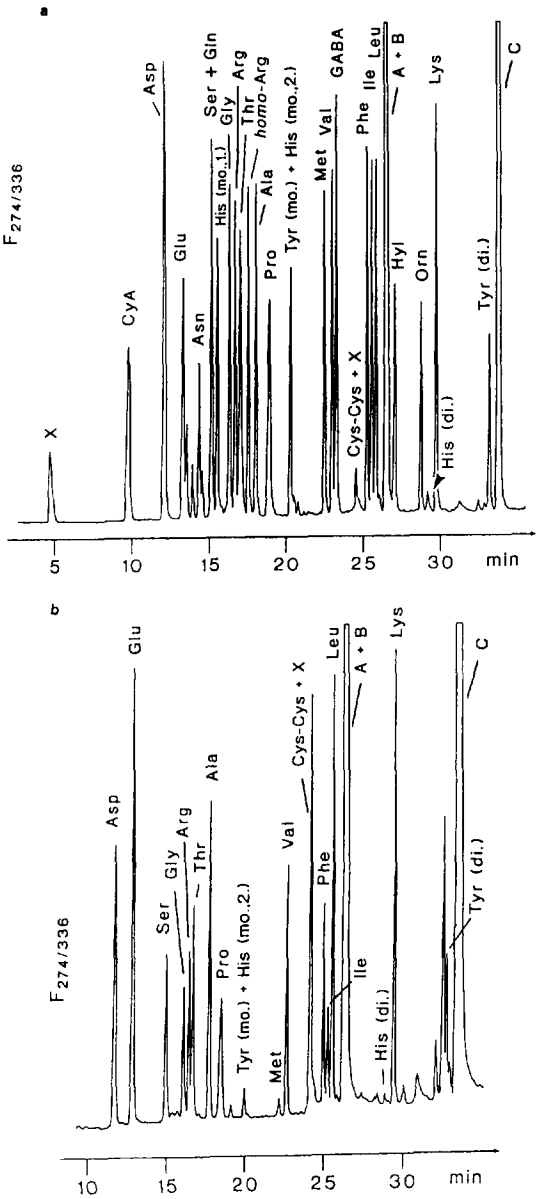

Fig. 6. Chromatograms of (a) an AA standard (injected amounts 10 pmol per AA, His 40 pmol, GABA and Asp 20 pmol, cystine 5 pmol) and (b) bovine serum albumin (3.5 ng total hydrolysate), both derivatized with NOC-Cl. $F_{274/336}$ = relative fluorescence at 274 nm (excitation) and 336 nm (emission).

tuted Tyr and His elute together. The disubstituted derivative of His forms ca. 50% monosubstituted His in 5 h. The phenomenon that His apparently forms two monosubstituted derivatives (designated mo. 1 and mo. 2), in addition to the disubstituted derivative (designated di.), requires further investigation (cf., Fig. 6a). For quantification of NOC-AA in Table 1 the disubstituted derivatives of Tyr and His were used.

The reproducibility of the retention times (n = 5) of the derivatives was 0.03–0.07 min; the R.S.D. of peak areas was 3.5–4.5% (monosubstituted His >4.5%); linearity was found in the range 0.9–45 pmol per AA injected, and the correlation coefficients of linearity of AA were 0.9995–0.9998 (Tyr, His and cystine <0.9995); the detection limit was ca. 25 fmol per AA, with the exceptions of mono- and disubstituted His (0.1 pmol) and cystine (0.5 pmol). The hydrolysed reagent (A) and the ammonolysis product (B) of the reagent elute together and do not overlap with the AA. Two additional peaks (X) arise from the reagent.

Investigation of protein hydrolysates by use of NOC-Cl gave satisfactory ratios for AAs with respect to calculated data (cf., Table 1). The chromatogram of AAs from a total hydrolysate

of bovine serum albumin derivatized with NOC-Cl is shown in Fig. 6b. Met and Cys are partly oxidized and Trp is destroyed under the hydrolysis conditions used and therefore cannot be determined. Cystine elutes together with an unknown component and cannot be determined under the chromatographic conditions used; further, Cys also shows strong fluorescence quenching. For the determination of Tyr and His, the disubstituted derivatives were used.

The elution profile of a seventeen-component AA standard derivatized with NMOC-Cl is shown in Fig. 7. Under the chromatographic conditions used, this standard shows an unsatisfactory resolution of AA. Ser and the monosubstituted derivative of His and Pro and the monosubstituted derivative of Tyr are not resolved. Further, neither Val and the hydrolysed reagent (A), nor Phe, cystine and the ammonolysis product (B), nor Phe, cystine and the ammonolysis product (B), nor the disubstituted Tyr and peak C are separated. As the chromatographic conditions, in particular with respect to the selectivity of the stationary phase, can certainly be improved upon, no statistical data are given.

The elution profile of a 24-component AA standard, derivatized with FMOC-Cl, followed by addition of ADAM is shown in Fig. 8. The fluorescence of the derivatives was measured at an emission of 313 nm with excitation at 263 nm. All hydrolysate AAs and CyA are satisfactorily resolved from Asn and Gln and from the unusual AAs *homo*-Arg, GABA, Hyl and Orn, with the exceptions of the pair Val and GABA, which elute almost together. Tyr forms the mono- and disubstituted derivatives and His the disubstituted derivative. The latter is relatively stable and forms ca. 10% of the monosubstituted derivative in 24 h at room temperature.

For the determination of AAs in protein hydrolysates (Table 1), the monosubstituted derivative of Tyr and the disubstituted derivative of His were used. Cysteine, although a component of the standard, cannot be seen in the chromatogram, probably as a result of fluorescence quenching. Cysteine and cystine can be determined, however, by UV detection of derivatives, or after oxidation with formic acid

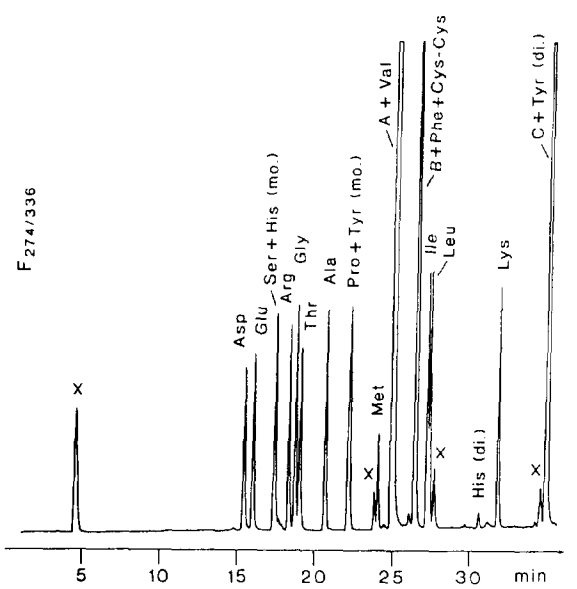

Fig. 7. Chromatogram of AA standard derivatized with NMOC-Cl (injected amounts 20 pmol per AA, cystine 10 pmol). $F_{274/336}$ = fluorescence at excitation 274 nm and emission of 336 nm.

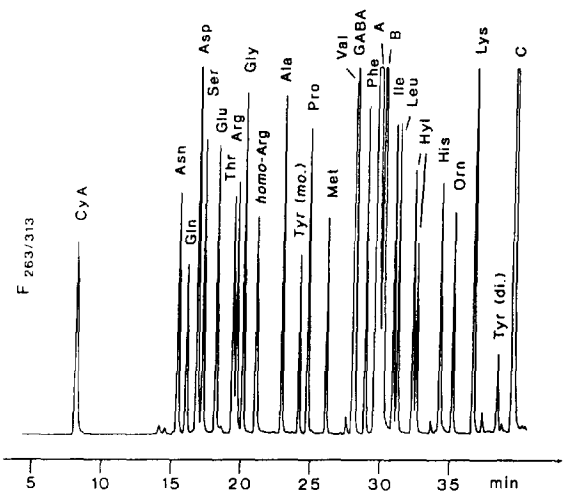

Fig. 8. Chromatogram of standard of AA derivatized with FMOC-Cl (injected amounts 6 pmol per AA, Asp 12 pmol, GABA 15 pmol, His 30 pmol). $F_{263/313}$ = relative fluorescence at 263 nm (excitation) and 313 nm (emission). CyA = Cysteic acid.

peroxide as cysteic acid (CyA) by measuring the fluorescence of the derivative. The hydrolysed reagent (A) and the ammonolysis product (B) of the reagent elute at a position not occupied by the AAs of the standard. Data of the analysis of the hydrolysed test proteins are given in Table 1.

The reproducibility of retention times ($n = 5$) was 0.02–0.10 min; the R.S.D. of peak areas ($n = 5$) was 2.0–5.0%; linearity of the detector response ($n = 3$) for each concentration was found in the range 0.06–6 pmol per AA injected; the correlation coefficient of linearity was 0.9995–0.9998 per AA (Tyr <0.9995). Under the chromatographic conditions used the detection limit is ca. 5 fmol per AA, with the exceptions of Ile and Leu (ca. 30 fmol), and Lys and disubstituted His (ca. 150 fmol). The excellent resolution of the AAs of the standard (which is among the best reported up to now) by derivatization with FMOC-Cl does not necessarily imply that this reagent is far superior to the others, in particular NOC-Cl. The chromatogram shown in Fig. 8 rather reflects the most intensive efforts with respect to the selection of an appropriate stationary phase together with optimum gradient elution conditions.

3.1. General aspects of the use of oxycarbonyl chlorides as derivatizing reagents

Ideally, reagents used for the precolumn derivatization of AAs, followed by the liquid-chromatographic separation of the derivatives formed, should react rapidly and quantitatively with the AAs at room temperature and yield stable derivatives. These should be detectable with high sensitivity by employing UV–visible or fluorescence detectors. The AA derivatives should follow the Beer–Lambert law over several orders of magnitude, and trifunctional AAs such as His, Tyr and Lys should yield uniform derivatives. The derivatization procedure should advantageously be carried out in a fully automated device [36] at room temperature, and the derivatives formed should be completely resolved within a short time on employing standard reverse-phase columns and simple linear gradient elution conditions. The stationary phase should also be stable for a large number of analyses. The method should at least allow the complete resolution of hydrolysate AAs including an internal standard or, ideally, of a mixture of so-called physiological AAs. Peaks necessarily arising from the unreacted or hydrolysed reagents, the carbonate formed therefrom, the ammonolysis product of the oxycarbonyl chloride and the reaction product formed from the scavenger and the excess of reagents should elute at positions away from those of the AAs to be determined, or be eluted last, and completely, from the column. Further, as secondary AAs such as Pro and Hyp, and also cysteine and cystine, are important protein AAs, the reagent should also allow their derivatization and determination. Finally, the reagent should be stable and of high purity and be commercially available at a moderate price. A critical survey of the literature reveals, however, that none of the reagents used for the precolumn derivatization and liquid chromatographic resolution of AAs fulfils all of the above requirements. Analysts agree, therefore, that the method that is finally chosen for AA analysis has to be a compromise depending on the aim of the work.

In comparison with other methods for the precolumn derivatization of AAs, the new reagents show the inherent advantages and disadvantages of oxycarbonyl chlorides, including FMOC-Cl: the reaction proceeds rapidly and quantitatively at room temperature with primary and secondary AAs, thus making derivatization chemistry fully automatable. Derivatives of the new reagents are stable for at least 3 days when stored at room temperature, with the exception of disubstituted His, which forms increasing amounts of monosubstituted His during storage. The necessary use of an excess of reagents and side-reactions lead to the formation of byproducts that interfere in the chromatography of AA derivatives. The ratio of mono- and disubstituted derivatives formed from His and Tyr depends on the structure and excess of reagents used. The latter is difficult to control in real samples and is limited by the solubility of reagents. In practice, an advantage of the oxycarbonyl chlorides de-

scribed is that an instrument designed for automated AA analysis employing automated pre-column derivatization chemistry can be easily used with the new reagents. Thus a change in the elution positions of AAs in chromatograms is possible where no interference by other AAs or certain absorbing components of the analyte occurs. This is of importance when AAs are to be determined in biomatrices, physiological samples or foodstuffs. In these cases the choice among reagents with different spectrophysical properties might also be of advantage. With respect to the overall resolution of derivatives, NOC-AAs are approximately comparable to FMOC-AAs; the simple eluent used for the separation of NOC-AAs, however, does not require addition of DMF and THF. Shortcomings of the chromatography of the derivatives of the other reagents should certainly be circumventable by testing other stationary phases. In conclusion, the availability of a set of highly reactive reagents and the favourable chromatographic properties of the derivatives formed therefrom should contribute to the advancement of AA analysis by HPLC.

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