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Separation and indirect detection of amino acids as acetylated derivatives

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ABSTRACT

Iron(II)–1,10-phenanthroline [$Fe(phen)_3^{2+}$] salts are used as mobile phase additives for the separation and indirect photometric detection (IPD) of N-acetylated amino acid (N-Ac-AA) derivatives on a reversed stationary phase. Mobile phase $Fe(phen)_3^{2+}$ concentration, pH, organic modifier concentration and counter anion affect retention and IPD. Disodium-1,5-naphthalene disulfonate and sodium benzoate as counter anions and buffer anion, respectively, are optimum because of their contribution to eluent strength, pH and location of their system peaks. The retention order of N-Ac-AA derivatives is influenced by AA side-chain structure. Mixtures of AA can also be separated, detected by IPD and determined after conversion of the AA to the N-Ac-AA derivatives via acetylation. The detection limit depends on the AA side-chain. For N-Ac-Val and derivatives of similar types of AA the detection limit was 0.5 nmol for a 10-μl injection. A similar detection limit was found for Val when the acetylation step was included in the procedure.

INTRODUCTION

Modern liquid chromatographic (LC) procedures for the separation and detection of complex mixtures of amino acids (AA) generally involve a derivatization step prior to or following the separation [1–5]. In the latter strategy free AA are separated on a cation or an anion exchanger [4], on a reversed stationary phase [1–3,5] or on a reversed stationary phase using an ion interaction (ion-pairing) reagent in the mobile phase [5–7]. The sole purpose of the derivatization step is to allow detection of the AA with low detection limits. If a precolumn derivatization strategy is used in the separation of AA the chromatographic mobile and stationary phase conditions are optimized according to the AA derivative structure. Thus, in this case, derivatization is used to influence both the separation and the detection. The more common derivatization reagents that are used are ninhydrin [1–5,8], *o*-phthalaldehyde [3,8,9] dansyl chloride [3,10] and phenyl isothiocyanate [3,11,12]. The former two can be employed with either absorbance or fluorescence detection while the third requires an absorbance detector.

We have previously shown that inorganic [13,14] and organic analyte anions can be separated and detected by an indirect detection strategy at favorable detection limits using iron(II)– or ruthenium(II)–1,10-phenanthroline or –2,2′-bipyridyl complexes as mobile phase additives. In a basic mobile phase AA are also anionic and

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they can be separated and indirectly detected without derivatization by using a Ru(phen)$_3^{2+}$ salt as the additive [15,16]. The Ru(phen)$_3^{2+}$ salts being chromophoric and fluorescent permit either an indirect photomeric (IPD) [13] or indirect fluorometric (IFD) detection [14] strategy to be used for detection of the resolved AA. A limitation in the separation from a basic mobile phase is that several AA analyte peaks overlap with the OH^- and HCO_3^-–CO_3^{2-} (due to dissolved CO_2) system peaks. As a basic mobile phase is required to convert the AA into anions, modifications of the mobile phase to move system peaks to a region of less interferences is not possible.

This paper describes a procedure for the separation of N-acetylated amino acids (N-Ac-AA) on a reversed stationary phase using an iron(II)–1,10-phenanthroline [Fe(phen)$_3^{2+}$] salt as a mobile phase additive. The contribution of the amine group to dissociation is significantly reduced when the amine group is converted into an acetylated derivative. Thus, the N-Ac-AA derivatives are converted into anions at a much lower pH than is possible for free AA. This allows the N-Ac-AA derivatives to be separated and detected by the IPD strategy at favorable detection limits without interference from OH^- and HCO_3^-–CO_3^{2-} system peaks as a basic mobile phase is no longer required. The procedure described here can be used for the separation and determination of naturally occurring N-Ac-AA. Furthermore, complex mixtures of AA can also be determined using this same separation and indirect detection strategy by first acetylating the AA mixture to form the N-Ac-AA derivatives, as by controlling the conditions it is possible to obtain a reproducible and quantitative acetylation.

EXPERIMENTAL

Materials

The Fe(phen)$_3^{2+}$ salts were synthesized and converted to a specific counter anion form by anion exchange [13,16]. Amino acids and N-Ac-AA derivatives were purchased from Sigma and disodium 1,5-naphthalenedisulfonate (1,5-NDS) from Eastman Kodak. Ionic strength and buffer salts were of analytical-reagent grade, organic solvents were of liquid chromatographic quality and distilled water was purified by a Sybron/Barnsted unit. Hamilton PRP-1 polystyrene–divinylbenzene (10-μm) columns (150 mm × 4.1 mm I.D.; and DuPont Zorbax ODS (5-μm) columns (150 mm × 4.6 mm I.D.) were used. The LC instrumentation used is described elsewhere [15,16].

Procedures

Aqueous solutions of N-Ac-AA derivative standards (1 mg/ml) and mixtures of standards were injected by syringe (10 μl). Preparations of acetylated derivatives were based on AA weight. Mobile phase solvent mixtures are expressed as percent by volume. Buffer, ionic strength and counter-anion salts, prepared as standard solutions, and Fe(phen)$_3^{2+}$ salts by weight were added to mobile phase solvent prior to dilution to volume. The procedure for column conditioning was described previously [13,16]. The flow-rate was 1.0 ml/min, the column temperature was 30°C, the inlet pressure was varied from 600 to 1000 p.s.i., depending on the column and mobile phase, the column void volume was 1.1–1.3 ml, IPD was at 510 nm, the background absorbance, which was always less than 0.7, was electronically offset to zero and the equilibrium amount of Fe(phen)$_3^{2+}$ salt maintained on the column (column and mo-

bile phase dependent), which was determined from column breakthrough data [13,16], was *ca.* 60–75 μmol per column. For an aqueous 0.10 m*M* $Fe(phen)_3$(1,5-NDS)–0.10 m*M* 1,5-NDS (pH 6) mobile phase and a Zorbax ODS column, the equilibrium amount of $Fe(phen)_3^{2+}$ maintained on the column was 71 μmol per column.

Amino acid N-acetylation

A weighed amount (about 0.1 mmol) of each AA was transferred into a 25 ml beaker. The AA were dissolved with stirring with small amounts of water and 0.50 *M* NaOH (pH 9.0). Excess of NaOH should be avoided because it increases the hydrolysis of the acetylating reagent and the total volume should be small so as to maintain an AA concentration as high as possible. The acetylating reagent was added so that the mole ratio of AA to acetic anhydride was in the range 1:1.5 to 1:5. The acetylating reagent was made by diluting 6.5 ml of acetic anhydride (Eastman Kodak) to 25.0 ml with acetonitrile. The solution, which was refrigerated when not in use, was stable for about 3 weeks. After 1 min of reaction the mixture was carefully heated at about 70°C until all the liquid had evaporated. (Excess of acetic anhydride should be removed in this step). The residue was dissolved in water, tranferred into a volumetric flask (10 ml) and diluted to volume with water. Aliquots (10 μl) of this solution were injected as samples.

RESULTS AND DISCUSSION

The $Fe(phen)_3^{2+}$ salt serves a dual role when used as a mobile phase additive. Its presence, depending on mobile phase composition, maintains an equilibrium amount of the $Fe(phen)_3C_2$, where C is a counter anion, on the reversed stationary phase surface as a double layer and provides the sites for interaction with analyte anions [14] such as the anionic form of an N-Ac-AA derivative. Second, the amount of Fe $(phen)_3^{2+}$ in the N-Ac-AA analyte band differs from the amount of $Fe(phen)_3^{2+}$ in the mobile phase background in proportion to the amount of analyte present, hence sensitive indirect detection of the analyte is possible by monitoring the effluent where $Fe(phen)_3^{2+}$ absorbs [13]. The peak can be either positive or negative depending on whether the amount of $Fe(phen)_3^{2+}$ increases or decreases in the analyte band relative to the amount of $Fe(phen)_3^{2+}$ in the background due to the mobile phase.

All N-Ac-AA derivatives, **I** where R is the AA side-chain, will dissociate as follows:

$$\underset{\mathbf{I}}{CH_3C(O)NHCHRCO_2H} + H_2O \rightleftarrows CH_3C(O)NHCHRCO_2^- + H_3O^+ \quad (1)$$

The acetyl group significantly diminishes the basicity of the amine group and its effects on dissociation. Thus, the N-Ac-AA derivatives have ionization constants (carboxyl group) at $pK_a \approx 5$ and will be appreciably anionic in a mobile phase with a pH larger than this value. The advantage of the intermediate mobile phase pH is twofold: the $Fe(phen)_3^{2+}$ salts are stable in this pH region [13] and interfering OH^- and HCO_3^-–CO_3^{2-} system peaks that are present when using a basic mobile phase are absent.

TABLE I

COMPARISON OF RETENTION OF NA-Ac-AA DERIVATIVES ON REVERSED STATIONARY PHASE

Mobile phase: 0.10 m*M* $Fe(phen)_3(ClO_4)_2$–0.10 m*M* 1,5-NDS–5:95 acetonitrile–water (pH 7.5).

Analyte	Column			
	Capacity factor, k'		Plate number, 10^3/m	
	PRP-1	Zorbax	PRP-1	Zorbax
N-Ac-Ser	2.23	1.09	14.8	29.6
N-Ac-Asn	2.23	1.12	14.0	45.2
N-Ac-Gln	2.41	1.31	12.0	45.2
N-Ac-Gly	2.41	1.23	10.9	29.0
N-Ac-Ala	2.77	1.65	11.4	29.6
Analyte pair	Selectivity, α		Resolution, R_s	
	PRP-1	Zorbax	PRP-1	Zorbax
N-Ac-Asn/N-Ac-Ser	1.00	1.03	0	0.82
N-Ac-Gly/N-Ac-Asn	1.08	1.10	1.37	2.13
N-Ac-Gln/N-Ac-Gly	1.00	1.07	0	1.97
N-Ac-Ala/N-Ac-Gln	1.15	1.26	2.56	5.53

Table I shows that N-Ac-AA derivatives are retained on reversed stationary phases from a mobile phase of pH 7.5 containing a $Fe(phen)_3^{2+}$ salt as a mobile phase additive. Further, the retention differs for the derivatives according to the structure of the AA side-chain. The more non-polar the side-chain, the higher is the retention. Retention is also higher for acidic side-chains which are dissociated at pH 7.5. In all instances the derivatives were detected as positive peaks by the IPD strategy. As the N-Ac-AA derivatives are readily retained at an intermediate pH, silica-based reversed phases can be used, unlike in the separation and detection of free AA [15,16] where a basic mobile phase is required to convert the AA into anions. Table I also compares the column efficiency, selectivity and resolution obtained for the retention of several N-Ac-AA derivatives on the polymeric PRP-1 and silica-based C_{18} Zorbax columns. Retention is higher on the PRP-1 because the equilibrium amount of the $Fe(phen)_3^{2+}$ maintained on PRP-1 at the given mobile phase condition is larger than on the Zorbax column. However, selectivity is more favorable on the Zorbax column, as is resolution, because of the improved efficiency. For these reasons all the studies reported here are for the Zorbax column.

The N-Ac-AA derivatives with their increased hydrophobic character and size will be more highly retained than the underiviatized AA. Thus, stronger eluents are required for the successful elution of the N-Ac-AA derivatives. Disodium 1,5-naphthalenedisulfonate was shown to be an eluent counter anion of high enough selectivity to elute the N-Ac-AA derivatives in a reasonable time while producing an eluent counter anion system peak that occurs at a high retention time that does not interfere with the analyte peaks. For example, for the mobile phase conditions used in Fig. 3 the 1,5-NDS system peak is observed at a retention time exceeding 3 h. Buffer

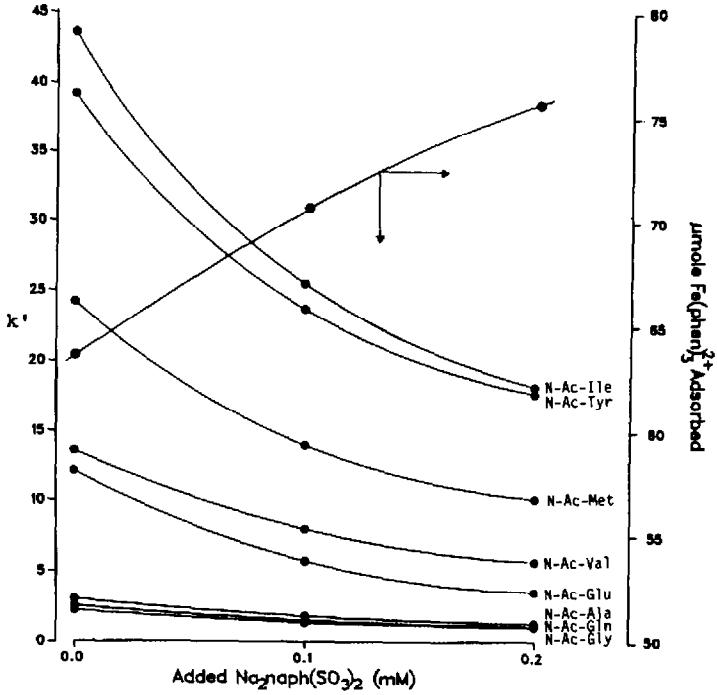

Fig. 1. Retention of N-Ac-AA and equilibrium amount of $Fe(phen)_3^{2+}$ salt on the stationary phase as a function of 1,5-NDS mobile phase concentration. Mobile phase: aqueous 0.10 m*M* $Fe(phen)_3$(1,5-NDS)–1,5-NDS (pH 6).

anions, such as citrate, phthalate and benzoate, which are weaker counter anions than 1,5-NDS, were evaluated. The benzoate salt was selected because its system peak, which occurs within the range of the N-Ac-AA derivative chromatographic peaks, does not overlap with any of the N-Ac-AA derivative peaks.

Increasing the amount of organic solvent in the mobile phase decreases the equilibrium amount of $Fe(phen)_3^{2+}$ salt maintained on the stationary phase surface, which results in a decreased retention of the N-Ac-AA derivatives. When the ionic strength of the 1,5-NDS mobile phase concentration is increased, the equilibrium amount of $Fe(phen)_3^{2+}$ maintained on the stationary phase increases; however, the analyte retention decreases because of the mass action effect of 1,5-NDS as a counter anion. This is illustrated in Fig. 1 where both the retention data for several N-Ac-AA derivatives and the stationary phase surface loading of $Fe(phen)_3^{2+}$ are plotted as a function of mobile phase 1,5-NDS concentration. When the mobile phase pH is increased the retention of the N-Ac-AA derivatives increases as dissociation increases. This is illustrated in Fig. 2 for several derivatives and indicates that a mobile phase pH of about 5 is optimum. The presence of an acidic side-chain in the AA will also influence retention because of additional dissociation as pH is increased. For example, at pH < 5.0 the elution order is N-Ac-Asp $<$ N-Ac-Glu $<$ N-Ac-Val, whereas at pH > 5.4 N-Ac-Val is eluted first. The higher pH value increases the mole ratio of N-Ac-AA as a divalent anion because of additional dissociation at the side-chain

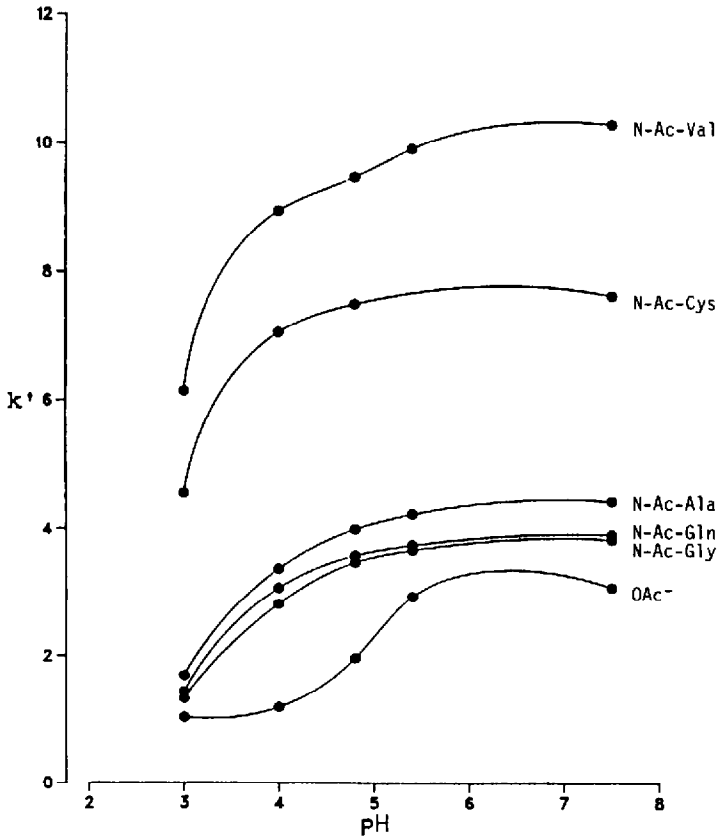

Fig. 2. Effect of pH on N-Ac-AA retention. Mobile phase: 0.10 m*M* $Fe(phen)_3(ClO_4)_2$–0.10 m*M* $NaClO_4$–5:95 acetonitrile–water (pH 3–7.5).

carboxyl group for the Asp and Glu derivatives compared with the monovalent anion that is present when the sample is the Val derivative.

Fig. 3 illustrates a typical separation of a mixture of N-Ac-AA standards using isocratic elution and indirect detection at 510 nm. All analyte peaks are positive peaks for the mobile phase conditions used. The Phe and Trp derivatives, which are not included in Fig. 3, are more highly retained and a stronger eluent is required to elute these derivatives. The benzoate system peak occurs at about 38 min and does ot interfere with the N-Ac-AA derivative peaks while the 1,5-NDS system peak occurs at >3 h. A double peak was found for the N-Ac-Pro standard that was used, which may be due to a sample that contained derivatives where acetylation has occurred at the ring or terminal amine group or both. For the separation of simpler mixtures of N-Ac-AA derivatives the analysis time can be adjusted by altering the mobile phase organic solvent and/or 1,5-NDS concentration.

Fig. 4 shows a calibration graph that was obtained when using N-Ac-Val as a standard. A fixed-loop 10-μl aliquot was used with indirect detection at 510 nm. No attempt was made to determine the upper limit of linearity. For the conditions and instrumentation used the detection limit using a 3:1 signal-to-noise ratio was *ca.* 0.5

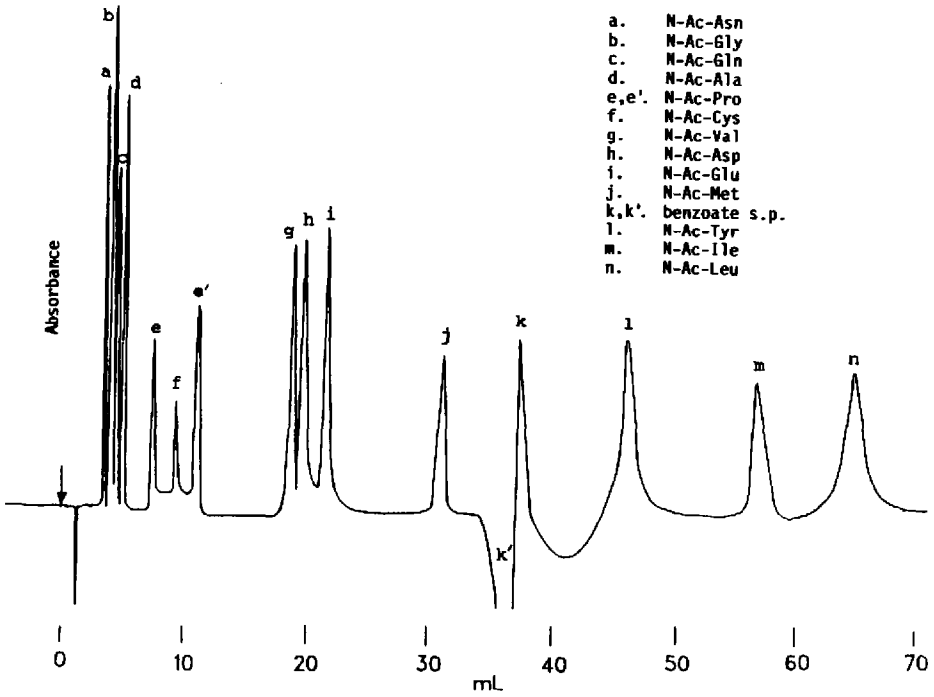

Fig. 3. Separation of a mixture of N-Ac-AA standards. Mobile phase: aqueous 0.050 m*M* $Fe(phen)_3$(1,5-NDS)–0.050 m*M* $Fe(phen)_3$ $(benzoate)_2$ (pH 5.4). s.p. = System peak.

nmol of N-Ac-Val injected as a 10-μl sample. The equation describing the calibration graph is nmol N-Ac-Val (peak area $\times$ 10^5) = -0.325 + 1.89 (nmol), with a linear correlation coefficient of 1.00.

Amines can be determined using either acid- [17] or base-catalyzed [18,19] acetylation with acetic anhydride as the acetylating reagent. In general, water is avoided in these procedures because acetylating reagents such as acetic anhydride or acetyl chloride hydrolyze quickly in water, particularly in the presence of the catalyst.

When the water concentration was kept low, the AA concentration was high and the acetylating reagent-to-AA ratio was 1:1.5 to 1:5 in the acetylating mixture, it was shown that AA N-acetylation will occur at a faster rate than hydrolysis of acetic anhydride. The effect of each of these parameters on acetylation *versus* hydrolysis was established by systematically changing each parameter while the others were held constant. Thus, N-acetylation of an aqueous AA sample is not only possible but is also a simple and rapid method that provides excellent conversion to the acetylated derivative.

In the presence of OH^-, acetylation of Val:

$$H_2NCHCH_3CO_2H + (CH_3CO)_2O \overset{OH^-}{\rightleftharpoons} CH_3C(O)NHCHCH_3CO_2^- + CH_3CO_2^- \quad (2)$$

yields 98% N-Ac-Val. The reaction is complete in seconds, and the following step of

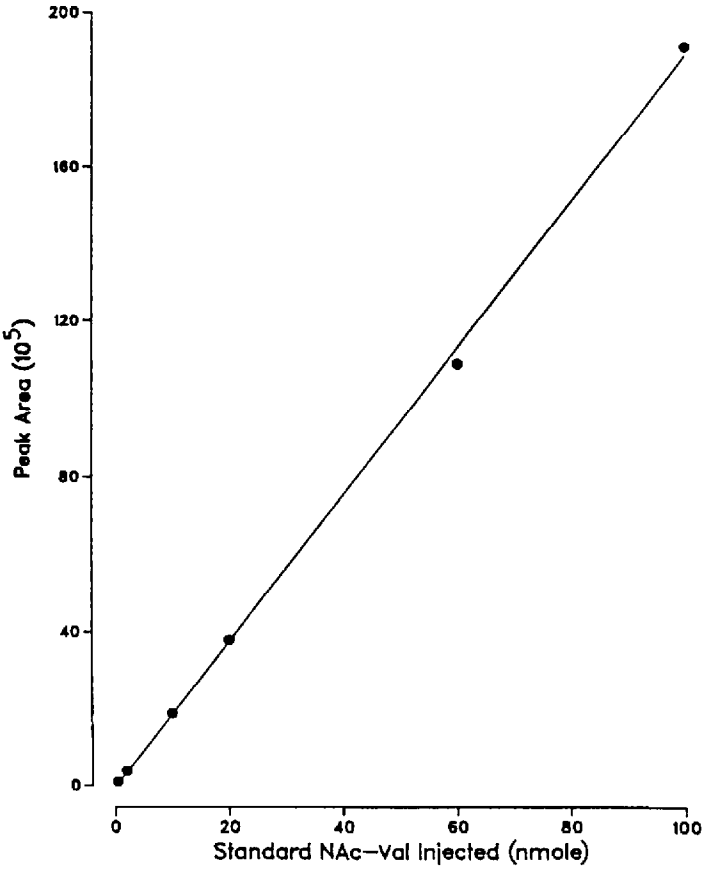

Fig. 4. Calibration graph for N-Ac-AA as a standard. Mobile phase as in Fig. 3 except with acetate in place of benzoate.

solvent anhydride removal and product drying takes only a few minutes. The AA N-acetylation method provides a rapid, simple, and convenient way to convert free AA to N-Ac-AA derivatives which can then be separated: (1) at an intermediate mobile phase pH without interference from the OH^- and CO_3^{2-}-HCO_3^- system peaks and (2) with a high-efficiency silica-bonded stationary phase. The effects of pH, AA concentration, acetylating reagent concentration and buffer concentration were established [16] using Val as a standard by determining the N-Ac-Val chromatographic peak area as a function of the conditions. Using the procedure outlined under Experimental the acetylation was shown to be reproducible, quantitative for the AA tested and provided chromatography identical with N-Ac-AA standards. A calibration graph was prepared for synthesized N-Ac-Val based on Val weight as the sample followed by the acetylation step. The calibration graph, which was almost identical with Fig. 4, corresponded to the equation nmol N-Ac-Val (or Val) (peak area x 10^5) = 1.05 = 1.86 nmol, with a linear correlation coefficient of 1.00. The linear range, like the standard N-Ac-Val calibration graph shown in Fig. 3, is over two orders of magnitude and the detection limit using a 3:1 signal-to-noise ratio is *ca.* 0.5 nmol

calculated as Val. When repeated five times the yield for the derivatization step was reproducible at about 98% acetylation compared with a standard N-Ac-Val calibration graph. Similar results were found when using Ala, Pro and Ile. Hence, it should be possible also to determine free AA after conversion to their acetylated derivatives. Only for AA with reactive side-chains was acetylation found to be low. For example, Glu yielded about 70% acetylation when using the outlined acetylation procedure. Also, AA containing alcoholic or side-chain amine groups will be acetylated at these groups [17–19].

A 0.2 *M* AA solution containing 0.1 nmol each of twelve AA was taken and its pH was adjusted to 9.0 with NaOH solution. A 2-ml volume of acetylating reagent was added and the acetylation and work-up were carried out as outlined under Experimental. The chromatogram for this mixture of synthesized N-Ac-AA derivatives is shown in Fig. 5 using the same mobile phase conditions as shown in Fig. 3 except that the mobile phase pH was 6.0. This mobile phase pH was used in order to improve the separation of N-Ac–N′-Ac-Lys, N-Ac-Asp, N-Ac-Glu. As indicated in Fig. 5, side-chain acetylation can occur in those cases where AA contain an amine or hydroxyl side-chain. The acetateO^-, which is formed in the acetylation (and hydrolysis of acetic anhydride) (see eqn. 2) is not highly retained for the chromatographic conditions used and only partially interferes with the chromatographic peak for the least retained derivative, N-Ac-Gly. Residues of the acetylating reagent do not cause any other interferences of the N-Ac-AA derivatives. If acid catalysis is used, which also

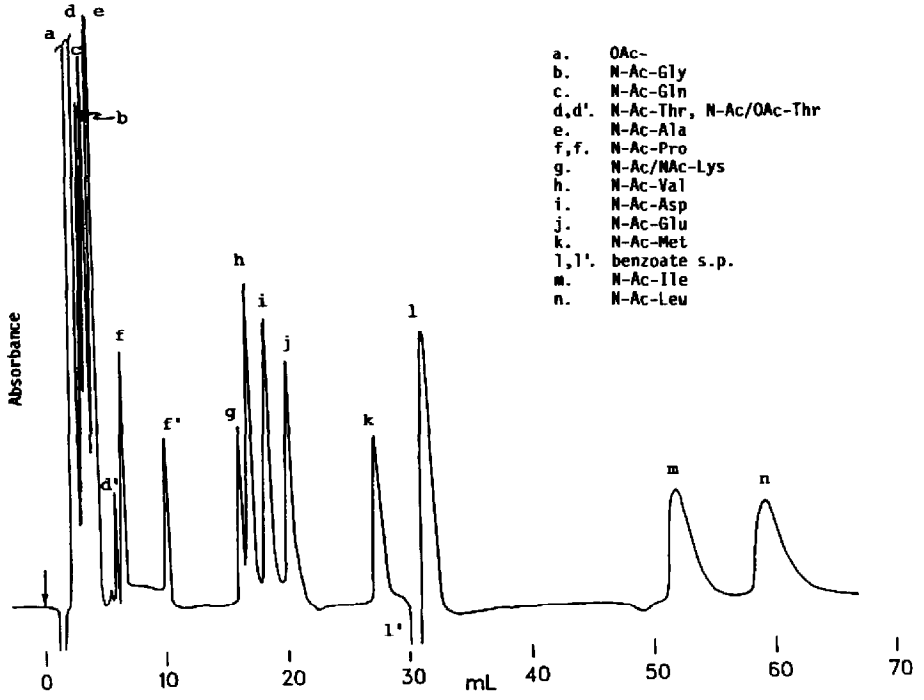

Fig. 5. Separation of an amino acid mixture after acetylation. Mobile phase as in Fig. 3 except pH = 6.0.

leads to quantitative acetylation, the counter anion from the strong acid catalysts that are usually used in acid-catalyzed acetylation [17], such as Cl^- or SO_4^{2-}, will provide a peak that interferes with several AA derivative peaks depending on the counter anion. For this reason base-catalyzed acetylation is preferred.

CONCLUSIONS

Naturally occurring N-acetylated amino acids can be separated and determined with a favorable detection limit using an indirect photometric detection strategy in the visible region where the iron(II)–1,10-phenanthroline complex absorbs. Indirect photometric and fluorimetric detection are also possible if the ruthenium(II) complex [13] is used. It is possible to determine amino acids after acetylation to form the N-acetylated amino acid derivative. Formation of the derivative is not used to enhance the detection limit but rather to block the contribution of the amine group to dissociation, thus allowing the derivatives to be separated as anions at a lower pH. The advantage of the low pH is that system peaks due to OH^- and CO_3^{2-}–HCO_3^- are eliminated. Acetylation of amino acids under a based-catalyzed controlled condition is rapid and quantitative and does not contribute system peaks to the separation.

REFERENCES

1 S. Blackburn, in G. Zweig and J. Sherman (Editors), *CRC Handbook of Chromatography: Amino Acids and Amines*, Vols. I and II CRC Press, Boca Raton, FL, 1983.
2 W. S. Hancock, *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins*, Vol. I, CRC Press, Boca Raton, FL, 1984.
3 Z. Deyl, J. Hyanek and M. Horakova, *J. Chromatogr.*, 379 (1986) 177–250.
4 R. F. Pfeifer and D. W. Hill, *Adv. Chromatogr.*, 22 (1983) 37.
5 M. T. W. Hearn and W. S. Hancock, in G. L. Hawk (Editor), *Biological/Biochemical Applications of Liquid Chromatography*, Vol. 2, Marcel Dekker, New York, 1979, p. 243.
6 Z. Iskandarani, R. L. Smith and D. J. Pietrzyk, *J. Liq. Chromatogr.*, 7 (1984) 111–150.
7 T. A. Walker and D. J. Pietrzyk, *J. Liq. Chromatogr.*, 10 (1987) 161–174.
8 R. L. Cunico and T. Schlabach, *J. Chromatogr.*, 266 (1983) 461–470.
9 J. D. Cooper, M. T. Lewis and D. C. Turnell, *J. Chromatogr.*, 285 (1984) 484–489.
10 C. DeJong, G. J. Hughes, E. Van Wieringen and K. J. Wilson, *J. Chromatogr.*, 241 (1982) 345–359.
11 S. M. Kim, *J. Chromatogr.*, 247 (1982) 103–110.
12 B. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin, *J. Chromatogr.*, 336 (1984) 93–104.
13 P. G. Rigas and D. J. Pietrzyk, *Anal. Chem.*, 60 (1988) 454–459.
14 P. G. Rigas and D. J. Pietrzyk, *Anal. Chem.*, 60 (1988) 1650–1654.
15 D. J. Pietrzyk, D. Yuan and P. G. Simonson, presented at the *Tenth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Wiesbaden (Germany), October 29–31, 1990*, paper no. 621.
16 D. Yuan, *Ph. D. Thesis*, University of Iowa, 1988.
17 G. H. Schenk and J. S. Fritz, *Anal. Chem.*, 32 (1960) 987–990.
18 C. L. Ogg, W. L. Porter and C. O. Willits, *Ind. Eng. Chem., Anal. Ed.*, 17 (1945) 394–397.
19 G. H. Schenk, P. Wines and P. Mojzis, *Anal. Chem.*, 36 (1964) 914–919.