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Separation of acetylated amino acids

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ABSTRACT

Acetylated amino acids (NAc-AA) are separated as anions on a reversed stationary phase from a mobile phase containing a quaternary ammonium (R_4N^+) salt as a mobile phase additive. If the counteranion accompanying the R_4N^+ or ionic strength salt is detector active than the separated NAc-AA derivatives can be detected by an indirect detection strategy. Variables influencing the separation are NAc-AA side chain structure and the mobile phase parameters such as hydrophobicity of the alkyl groups in the R_4N^+ salt, R_4N^+ salt concentration, counteranion eluent strength, counteranion concentration, solvent composition, and pH. Indirect detection is influenced by these same mobile phase parameters as well as the properties of the detector active counteranion. The detection limit for indirect photometric detection at 287 nm using a tetrapentylammonium salt with a disodium 1,5-naphthalenedisulfonate–sodium benzoate counteranion mixture was about 70 pmol of NAc-AA depending on the amino acid injected as a 10-μl sample.

INTRODUCTION

The separation and determination of N-acyl amino acid (N-acyl-AA) derivatives in biological systems is of growing interest. Many of these derivatives, which have been identified as metabolites in urine, blood plasma, and other biological fluids and materials as well as in plant matter, have been correlated to exposure, dietary, and health disorders. For example, excretion of aromatic carboxylic acids that are produced endogenously or derived from exogenous sources such as drugs in normal human metabolism undergo reaction with glycine to yield an N-acyl glycine derivative, exposure to toluene leads to formation of N-*p*- and *m*-benzoylglycine [1,2], and the presence of aliphatic and aromatic N-acyl-AA have been correlated to organic acidurias and other disorders [3–6]. Other studies suggest N-acetylation is a pathway in the metabolism of amino acids and accounts for the presence of several different aliphatic N-acyl-AA in normal urine [7]. Typical N-acyl-AA that have been detected in physiological and plant samples, which is reviewed elsewhere [7,8], include N-benzoyl-, substituted N-benzoyl-, N-hydroxybenzoyl-, N-vanilloyl-, N-phenyl-, aliphatic-N-acyl, and N-acetyl-AA derivatives.

The N-acyl-AA derivatives have been separated by thin-layer chromatography [8–11], gas–liquid chromatography [3,4,6–8,10,12,13], and by high-performance liquid chromatography (HPLC) [1,2,5,8,14–19]. In general, HPLC separations have been carried out on either reversed stationary phases [1,2,5,8,14,16–19] or ion exchangers [15]. UV detection of N-acyl-AA derivatives is possible for derivatives that contain an aromatic ring attached to the acyl group. Aliphatic-N-acyl- and N-acetyl-amino acid (NAc-AA) derivatives, however, are not readily detected except at a very low wavelength where the acyl group absorbs. At this wavelength absorption interferences due to solvent, mobile phase components, and sample matrix can be significant.

It was recently demonstrated that NAc-AA mixtures are resolved on a reversed stationary phase using an iron(II)-1,10-phenanthroline, $Fe(phen)_3^{2+}$, salt as a mobile phase ion interaction reagent [19]. The additive enhances retention of the NAc-AA de-

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rivatives and allows an indirect detection of the derivatives with a detection limit of about 0.5 nmol in a 10-μl injection. It was also shown that free amino acids could be separated and detected at 0.5 nmol by first derivatizing the amino acids through an acetylation reaction.

This paper describes a procedure for the separation of NAc-AA derivatives on a reversed stationary phase by using a tetrapentylammonium ($TPeA^+$) salt and a detector active counteranion as mobile phase additives. The $TPeA^+$ salt is an ion interaction reagent that enhances retention of the anionic form of the NAc-AA derivatives while the counteranion provides eluting power and a means for indirect detection. Since the indirect detection is based on an anion-exchange equilibrium between the detector active counteranion and the NAc-AA anion, the mobile phase parameters influencing retention, elution, and indirect detection are fewer and more easily controlled than when using a $Fe(phen)_3^{2+}$ salt as the additive where the indirect detection is due to the shift of the equilibrium involving interactions between the $Fe(phen)_3^{2+}$ salt, counteranion, and stationary phase surface [19].

EXPERIMENTAL

Materials

Tetraalkylammonium chloride or bromide salts were obtained from Eastman Kodak while the sodium sulfonates and carboxylates or free acids were obtained from Eastman Kodak, Fisher Scientific, and Sigma. NAc-AA derivatives of L-AA were purchased from Sigma. Ionic strength salts were analytical reagent grade, organic solvents were LC quality, and LC water was obtained by passing inhouse distilled water through a Millipore Milli-Q Water System. PRP-1 (Hamilton) polystyrenedivinylbenzene (10 μm, 150 mm × 4.1 mm I.D.), Zorbax-ODS (Mac Mod Anal.) (5 μm, 150 mm × 4.6 mm I.D.), and Supelco ODS (Supelco Sep. Tech.) (250 mm × 4.6 mm I.D.) columns were used. LC instrumentation consisted of a Spectra Physics 8800 gradient pump, a Waters U6K or Rheodyne 7125 fixed loop injector, and a Spectra-Physics 770 variable-wavelength, Kratos 900 fluorescence, or a EG and G Princeton Applied Research 400 electrochemical detector. Column temperature was controlled with a Bioanalytical LC-22A controller and data analysis was done on a Spectra-Physics 4270 recording integrator and manipulated with an Epson Equity I+ computer equipped with Spectra-Physics WINner software.

Procedures

R_4N^+ salts were converted into a specific counteranion form by anion exchange (Amberlite IRA-400). Mobile phases were prepared by combining aliquots of standard solutions of R_4N^+, ionic strength, and buffer salts that provided the desired counteranion, solvent, and pH. The pH was adjusted with dilute NaOH prior to dilution to volume when necessary. Solvent mixtures were percent by volume and basic mobile phases were protected from atmospheric CO_2. Column peformance was monitored during the study and compared to manufacture certification using a phenol, benzene, toluene mixture and an acetonitrile–water (85:15) mobile phase.

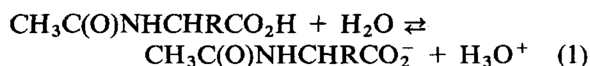
Columns were conditioned by passing the R_4N^+ salt mobile phase through the column beyond the column breakthrough until the detector background signal remained constant. Column breakthrough volume was determined by monitoring column effluent at a detector setting that responds to the appearance of the counteranion. The equilibrium amount of R_4N^+ salt maintained on the column for a given mobile phase condition was calculated from the breakthrough volume and the R_4N^+ mobile phase concentration. The R_4N^+ salt was removed from the column by elution with acetonitrile–water (4:1). Aqueous solutions of NAc-AA (0.1 to 1.0 mg/ml) derivatives were prepared and injected by syringe in 1–10 μl aliquots. NAc-AA derivatives were detected by indirect detection at detector settings where the counteranion benzoate (228 nm), 1,5-naphthalenedisulfonate (NDS) (287 nm), naphthalenesulfonate (NS) (287 nm), and salicylate (298 nm) absorb, where salicylate (λ_{ex} 300 nm, λ_{em} 410 nm) and NDS (λ_{ex} 320 nm, λ_{em} 450 nm) fluoresces, or where I^- (500–800 mV *versus* SCE) and salicylate (850–1050 mV *versus* SCE) are oxidized. The background absorbance, fluorescence, or electrochemical signal due to the counteranion was compensated for by the detector offset capability. For indirect photometric detection the background absorbance should not exceed about 0.7 AU. Mobile phase flow was usually 1.0 ml/min, in-

let pressure was under $6.9 \cdot 10^6$ Pa, column temperature was 25°C, and column void volume was 1.1–1.3 ml. Usually, three or more measurements were made to establish the effects of mobile phase and indirect detection parameters, while for the determination of the calibration curve, data points were established with five or more measurements.

RESULTS AND DISCUSSION

Retention and detection of NAc-AA derivatives

Acetylation of the amino group in an amino acid reduces its basic property and dissociation occurs only at the carboxyl group according to eqn. 1.



$$CH_3C(O)NHCHRCO_2H + H_2O \rightleftarrows CH_3C(O)NHCHRCO_2^- + H_3O^+ \quad (1)$$

Thus, NAc-AA derivatives are anions at an intermediate pH and should be retained on a reversed stationary phase, S, when the mobile phase contains a quaternary ammonium (R_4N^+) salt additive. This occurs because an equilibrium amount of the R_4N^+ salt, assuming the R groups are hydrophobic and micelle formation is absent, is maintained on the stationary phase's surface for a given mobile phase condition as shown in eqn. 2.

$$S + R_4N^+ + C^- \rightleftarrows S \cdots R_4N^+C^- \quad (2)$$

The formation of the double layer on the surface provides the sites for interaction with the NAc-AA anions as shown in eqn. 3.

$$S \cdots R_4N^+C^- + \text{NAc-AA}^- \rightleftarrows S \cdots R_4N^+\text{NAc-AA}^- + C^- \quad (3)$$

The number of sites is determined by the R_4N^+, its concentration, solvent composition, the counteranion C^-, and the reversed stationary phase [20–22]. For a given R_4N^+ salt and concentration manipulation of the other variables, for example C^- concentration, allows the elution of the NAc-AA as shown in the reverse of eqn. 3. If C^- is also detector active then indirect detection is possible when the detector is adjusted to respond to C^-. If C^- absorbs, fluoresces, or is electrochemically active indirect photometric (IPD), indirect fluorescence (IFD), and indirect electrochemical (IED) detection is possible. Because of the equilibrium in eqn. 3 and the detector's response to the detector active counteranion, the chromatographic peak for the eluted NAc-AA derivatives will be negative.

Column optimization

Two reversed stationary phases, PRP-1, which is more stable in a basic mobile phase, and Zorbax ODS, were compared. The amount of R_4N^+ salt maintained on the PRP-1 was found to be higher than on the Zorbax column of the same dimensions. For example, breakthrough measurements indicated 210 μequiv. of TPeA salicylate is maintained on the PRP-1 for an aqueous 0.25 m*M* tetrapentylammonium ($TPeA^+$) salicylate, pH 7.5 mobile phase while for the Zorbax column the equilibrium amount is 150 μequiv./column. If the R chain length or the R_4N^+ salt concentration is increased the equilibrium amount on the stationary phase is increased. Adding organic modifier or switching to a counteranion that is weaker in eluent power (see eqn. 3) decreases the equilibrium amount of R_4N^+ salt on the stationary phase surface.

Table I compares the retention of several NAc-AA derivatives on the PRP-1 and Zorbax columns for a given TPeA salicylate mobile phase. Even though retention is higher on the PRP-1 the Zorbax column was used in additional studies because resolution on the latter column is greater due to a higher column efficiency (by a factor of 1.5–2 depending on the NAc-AA) and a modest enhancement in se-

TABLE I

RETENTION OF NAc-AA DERIVATIVES ON TWO REVERSED STATIONARY PHASES

Amino acid derivative	Capacity factor, k' [a]	
	PRP-1	Zorbax ODS
NAc-L-Asn	6.91	4.39
NAc-L-Ser	7.04	4.55
NAc-Gly	7.65	4.75
NAc-L-Glu	7.72	4.70
NAc-L-His	8.39	
NAc-L-Ala	8.43	5.34
NAc-L-Pro [b]	10.6/12.7	6.55/7.67
NAc-L-Val	16.4	14.1
NAc-L-Met		15.2
NAc-L-Ile	34	34
NAc-L-Leu	39	41

[a] Mobile phase, aqueous 0.25 m*M* TPeA salicylate, pH 7.5; column, Zorbax ODS; detection, IPD at 298 nm.

[b] Double peak is probably due to acetylation at both N in proline.

lectivity. Table I also demonstrates that retention, which is significant, correlates to the structural properties of the AA side chain group; the more hydrophobic or acidic the side chain is, the higher the retention becomes. Furthermore, indirect detection is sensitive since each NAc-AA derivative was readily located by monitoring column effluent at a wavelength where the salicylate counteranion absorbs.

Mobile phase and indirect detection optimization

Several mobile phase parameters influence retention of the NAc-AA derivatives and their optimization requires a compromise. As the R_4N^+ salt concentration increases the equilibrium amount of R_4N^+ salt on the stationary phase surface increases (see eqn. 2) which increases the number of ion interaction sites. However, NAc-AA retention does not continue to increase but eventually decreases [21,22] because of the increase in the counteranion concentration that accompanies the R_4N^+ salt, thus, reducing retention according to eqn. 3. Elution of NAc-AA derivatives requires a strong eluent counteranion or one that has a large anion-exchange selectivity to elute the NAc-AA derivatives in a reasonable time. This type of counteranion also produces a system peak that does not interfere with the analyte peaks [19,23]. In addition, the counteranion should be detector active. Several counteranions, which have favorable molar absorptivities, were evaluated and their effect on IPD and NAc-AA retention is shown in Table II. Increasing counteranion concentration decreases NAc-AA retention. The eluent strength for several counteranions on Zorbax ODS follows the order: I^- < benzoate < salicylate < naphthalenesulfonate (NS) < naphthalene disulfonate (NDS), which is consistent with their anion-exchange selectivities towards strong base quaternary ammonium type anion exchangers. In each case IPD was used at a wavelength where the counteranion absorbs. IFD is an option since salicylate fluoresces while IED is possible by using I^- as the counteranion. However, I^- is not a strong enough eluent counteranion to resolve NAc-AA derivatives particularly those with very non-polar or acidic amino acid side chains. Thus, if I^- is used, eluent power must be increased through an additional factor. Decreasing counteranion concentration, for example NDS, sharply decreases NAc-AA retention as shown in Fig. 1. In the absence of NDS in Fig. 1 eluent strength is due solely to benzoate.

For a given counteranion NAc-AA retention decreases as organic modifier is increased with aceto-

TABLE II

EFFECT OF COUNTERANION ON RETENTION

Amino acid	Capacity factor, k'		
	Counteranion[a]		
	Benzoate	NMS	NDS
NAc-L-Ser	8.23	3.68	1.69
NAc-L-Ala	11.5	5.05	2.25
NAc-L-Pro	16.6	8.90	3.60
NAc-L-Val	23	13.4	6.86
NAc-L-Met	29	16.2	7.91
NAc-L-Ile	81	45	18.5
NAc-L-Leu	95	52	21
NAc-L-Phe			47

[a] Mobile phase, aqueous 0.10 m*M* TPeA benzoate–0.10 m*M* Na salt of benzoate (NMS) or NDS, pH 7.5; column, Zorbax ODS; detection, IPD at 287 nm.

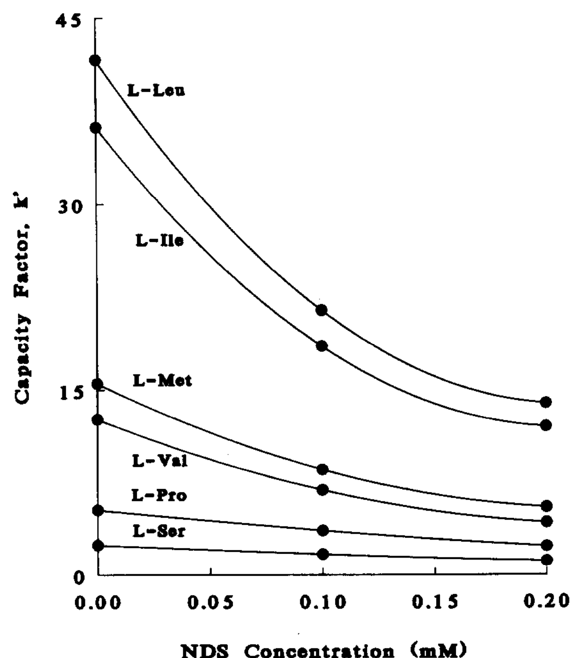


Fig. 1. Effect of NDS mobile phase concentration on the retention of NAc-AA derivatives. Conditions are the same as in Table II with varying NDS concentration.

nitrile being more effective than ethanol at comparable percent water–solvent mixtures. Retention of the more polar NAc-AA is already low for benzoate, NS, or NDS counteranion and adding organic modifier is required only when separating the more highly retained non-polar and acidic side chain NAc-AA. Mobile phase pH influences retention as shown in Fig. 2. Over the pH range studied the non-polar side chain NAc-AA are dissociated (see eqn. 1) and retention gradually increases as pH increases. For derivatives with acidic side chains retention increases significantly as pH increases because of additional dissociation at the acidic side chain. Thus, the elution order of L-Glu and L-Asp derivatives is more dependent on pH relative to the other NAc-AA. For low pH dissociation is suppressed and retention is sharply reduced. A mobile phase pH >8.0 is not recommended because this contributes to the formation of OH^- and HCO_3^-/CO_3^{2-} system peaks [19,23] both of which will interfere with several of of the NAc-AA chromatographic peaks. As hydroxide concentration increases the OH^- system peak area increases and shifts to a lower retention time while at the same time preventing CO_2 absorption in the mobile phase becomes more difficult. Also, the higher mobile phase pH limits the use of the silica based ODS column.

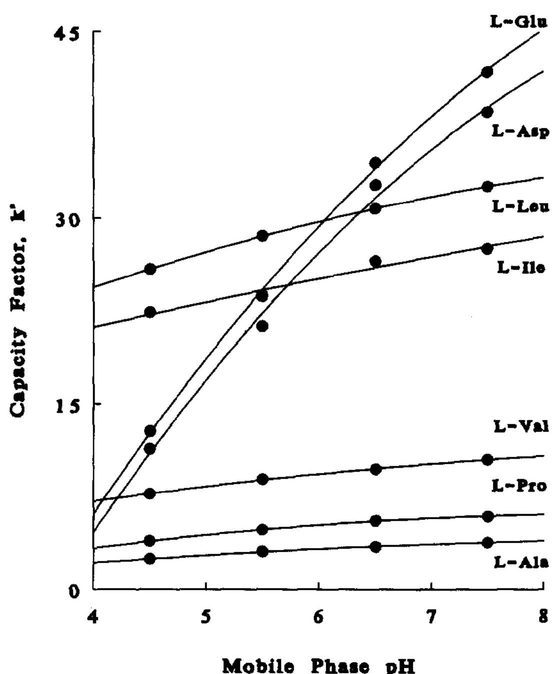


Fig. 2. Effect of mobile phase pH on the retention of NAc-AA derivatives. Mobile phase: 0.10 m*M* TPeA salicylate–0.10 m*M* Na salicylate, pH 4.5–7.5; column, Zorbax ODS; detection, IPD at 298 nm.

Separations

Fig. 3 illustrates a typical separation of several NAc-AA derivatives using IPD where salicylate absorbs. Chromatographic peaks, which are negative and inverted in Fig. 3 for convenience, are well defined, free of tailing, and yield a favorable detection limit. A double peak was observed for the NAc-L-Pro standard and is probably due to derivatization at both Pro nitrogens. If salicylate or organic modifier concentration in Fig. 3 is increased retention decreases. While both conditions favor elution of the highly retained NAc-AA resolution is significantly reduced for the low retained, polar derivatives. A salicylate system peak occurs at a high retention time and does not interfere with the NAc-AA peaks. If the pH is lowered from pH 7.5 (Fig. 3) to pH 4.5 with all other conditions being the same, NAc-AA retention decreases and resolution of the early eluting derivatives is still maintained. The major advantage of the lower pH is that elution times for the acidic side chain L-Asp and L-Glu derivatives are reduced significantly; their peaks at the lower pH follow L-Val (about 10 min) at 12 and 14.5 min, respectively. At pH 4.5 the L-Phe derivative is eluted at about 60 min in contrast to >100 min at pH 7.5. Elution of the L-Tyr derivative is also pH sensitive because of the acidic side chain and its peak occurs between the L-Glu and L-Ile at lower pH and greater than L-Glu at higher pH (see Fig. 3). The L-Trp derivative, which contains a non-polar side chain, elutes in the L-Phe derivative region. Although not shown peaks in Fig. 3 can be detected by IFD since salicylate fluoresces.

Fig. 4 illustrates the separation of NAc-AA derivatives using a longer ODS column (250 mm) and a stronger eluent. Detection is by IPD at 287 nm where NDS absorbs. IFD is also possible since NDS fluoresces. Since the pH is 5.0 the L-Asp and L-Glu peaks elute early following the L-Val peak. The weakly retained NAc-AA are still well resolved because of the column length while the more highly retained peaks are eluted as sharp, well resolved peaks at a reasonable time because the mobile phase

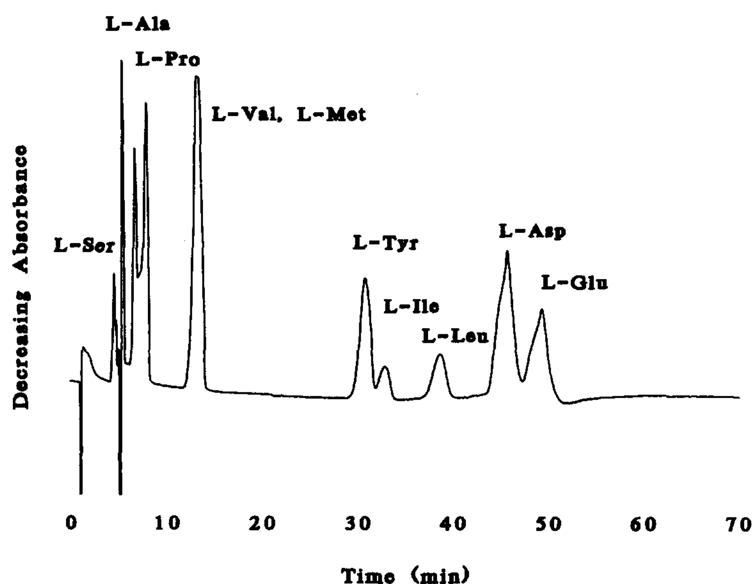


Fig. 3. Separation of NAc-AA derivatives. Conditions are the same as in Fig. 2 with pH = 7.5.

contains the strong eluent counteranion NDS. The benzoate system peak is due to the TPeA benzoate which was employed as the ion interaction reagent.

Detection limits

The IPD detection limit for NAc-AA derivatives depending on the mobile phase and detector was about 70 pmol injected as a 10-μl sample for a signal to noise ratio of 3:1. The calibration curve linear range covered about three orders of magnitude. For example, for IPD of NAc-L-Ser the calibration curve using the conditions in Fig. 4 was shown to follow the equation peak area = 673 (ng NAc-L-Ser) + 58.8 with a correlation coefficient of

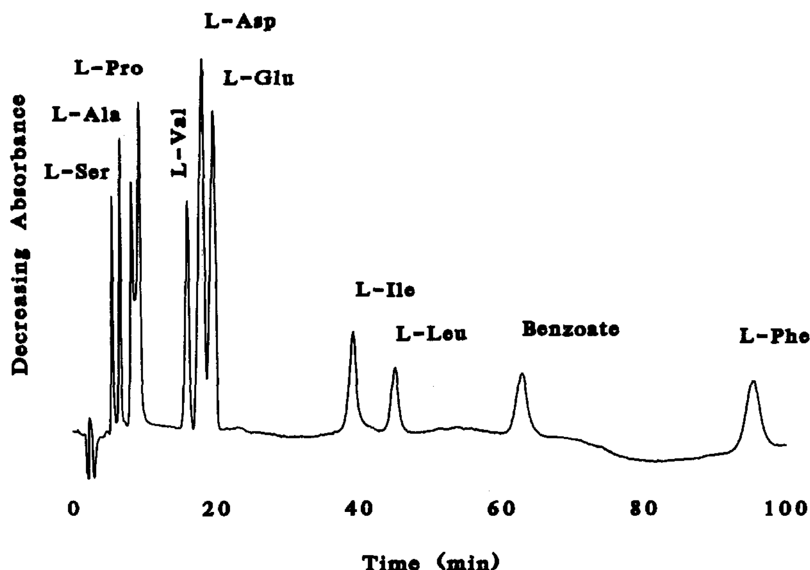


Fig. 4. Separation of NAc-AA derivatives on a longer column. Mobile phase: aqueous 0.10 mM TPeA benzoate, 0.10 mM Na_2NDS, pH 5.0; column, Supelco ODS; detection, IPD at 287 nm.

> 0.995. Three replicate injections of seven different standards were used to establish the calibration curve. In general, the more weakly retained NAc-AA have lower detection limits than the more highly retained NAc-AA derivatives, because of sharper chromatographic peaks. IPD sensitivity and detection limits are directly related to molar absorptivity of the counteranion [19,23] and detection limits by IPD can be lowered by using a counteranion with a higher absorptivity. While IFD and IED are also options by using counteranions that fluoresce or are electrochemically active (salicylate and NDS and iodide, respectively, were studied), IPD is more versatile because more counteranions are readily available that have both high absorptivity values and suitable counteranion elution characteristics. Also, measurement of a fluorescence or electrochemical change on a significant fluorescent or electrochemical background signal, respectively, is more susceptible to background noise depending on the detector and the quality of its offset capabilities [19].

CONCLUSIONS

NAc-AA are quantitatively separated using a R_4N^+ salt and a chromophoric anion in the mobile phase to enhance retention and provide indirect detection, respectively. The detection limit is about 70 pmol depending on the NAc-AA. Retention and resolution are altered by adjusting mobile phase solvent composition, R_4N^+ and counteranion concentration, and pH. IPD, which is sensitive to these parameters, is largely affected by counteranion absorptivity. Free AA can also be separated and determined by this procedure following an acetylation derivatization step [19].

REFERENCES

1 M. Matsui, M. Kasao and S. Imamura, *Br. J. Ind. Med.*, 34 (1977) 310–313.
2 R. Tardif, J. Brodeur and G. L. Plaa, *J. Anal. Toxicol.*, 13 (1989) 313–316.
3 E. C. Horning and M. J. Horning, *Clin. Chem.*, 17 (1971) 802–809.
4 O. Stokke, L. Eldjam, E. Jellum, H. Pande and P. E. Waller, *Pediatrics*, 49 (1972) 726–735.
5 L. Zimmerman, H. Jörnvall and J. Bergström, *Nephron*, 55 (1990) 265–271.
6 H. M. Liebich and C. Först, *J. Chromatogr.*, 338 (1985) 187–191.
7 H. S. Ramsdell and K. Tanaka, *J. Chromatogr.*, 181 (1980) 90–94.
8 C. F. Van Sumere, K. Vande Casteele, R. Hanselaer, M. Martens, H. Geiger and L. Van Rompaey, *J. Chromatogr.*, 234 (1982) 141–155.
9 H. Van Den Berg and F. A. Hommes, *J. Chromatogr.*, 104 (1975) 219–222.
10 J. Gartzke and H. J. Weigmann, *J. Chromatogr.*, 162 (1979) 234–236.
11 M. S. Dubra, D. M. Alperin, A. Sagedahl, V. P. Idoyaga-Vargas, and H. Carminatti, *J. Chromatogr.*, 250 (1982) 124–128.
12 J. P. Kamerling, M. Brouwer, D. Ketting and S. K. Wadman, *J. Chromatogr.*, 164 (1979) 217–221.
13 H. S. Ramsdell and K. Tanaka, *Clin. Chim. Acta*, 74 (1977) 109–114.
14 S. Tsunasawa and K. Narita, *J. Biochem. (Tokyo)*, 92 (1982) 607–613.
15 K. Narita, *Methods Enzymol.*, 91 (1983) 84–92.
16 E. Busker and J. Martens, *Fresenius' Z. Anal. Chem.*, 559 (1986) 325–326.
17 A. Astier and A. M. Deutsch, *J. Chromtogr.*, 182 (1980) 88–93.
18 H. J. C. F. Nelis, M. C. Lefevere, E. Baert, W. D'Hoore and A. P. De Leenheer, *J. Chromatogr.*, 333 (1985) 381–387.
19 D. Yuan and D. J. Pietrzyk, *J. Chromatogr.*, 557 (1991) 315–324.
20 Z. Iskandarani and D. J. Pietrzyk, *Anal. Chem.*, 54 (1982) 1065–1071.
21 Z. Iskandarani and D. J. Pietrzyk, *Anal. Chem.*, 54 (1982) 2427–2431.
22 R. H. A. Sorel and A. Hulshoff, *Adv. Chromatogr.*, 21 (1983) 87–129.
23 P. G. Rigas and D. J. Pietrzyk, *Anal. Chem.*, 60 (1988) 454–459.