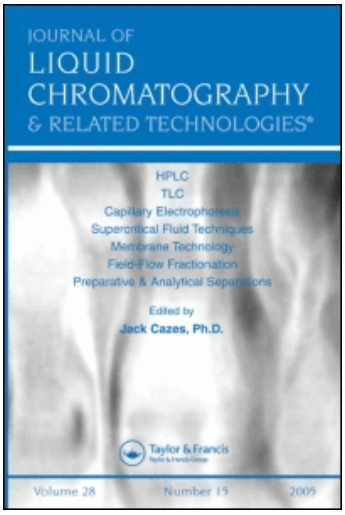

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Application of 3-(1,8-Naphthalimido) Propyl-Modified Silyl Silica Gel as a Stationary Phase In High Performanc Liquid Chromatography of Barbiturates and Diastereomeric Compounds

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APPLICATION OF 3-(1,8-NAPHTHALIMIDO) PROPYL-MODIFIED SILYL SILICA GEL AS A STATIONARY PHASE IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF BARBITURATES AND DIASTEREOMERIC COMPOUNDS

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ABSTRACT

The application of a column packing material, 3-(1,8-naphthalimido)propyl-modified silyl silica gel (NAIP) developed by us, for high performance liquid chromatography (HPLC) of barbiturates and diastereomeric compounds is described. The separation of barbiturates by using an NAIP column as a stationary phase was at first investigated. Six commercially available barbiturates (Barbital, Phenobarbital, Amobarbital, Pentobarbital, Secobarbital and Thiopental) were well separated on an NAIP column by an isocratic elution with borate buffer (pH 7.0)-acetonitrile (9:1, v/v). Further, the applicability of the HPLC system including an NAIP column to the determination of barbiturates in human blood plasma was examined. Barbiturates extracted from plasma sample using a solvent extraction (hexane-diethyl ether, 3:7, v/v) were subjected to the HPLC system and

determined by an internal standard method with UV detection at 220 nm. The recoveries of barbiturates spiked to human plasma were obtained in the range of 88.6 to 100.0%. The lower detection limits of barbiturates spiked ranged from 0.05 to 0.33 μg/mL, and the within-day and day-to-day precisions for plasma sample (n=5) gave relative standard deviations of less than 9.07% and 18.68%, respectively.

Next, the applicability of an NAIP column to the separation of diastereomeric derivatives was studied. Epinephrine (EP), norephedrine (NE) and α-phenylethylamine (PA) carrying an aromatic ring were used as representative targets for derivatization with three kinds of chiral reagents. Resolution behavior on NAIP was compared with that on a conventional reverse phase ODS column. Diastereomeric derivatives of targets were satisfactorily separated by an NAIP column as well as an ODS column. The best separation of S(+)-1-(1-naphthyl)ethyl isocyanate derivatives with PA was obtained on an NAIP column and the calibration curves showed good linearity in the concentration range examined.

INTRODUCTION

In recent years, we synthesized a series of 3-(*N*-substituted)aminopropyl-modified silyl silica gels immobilizing organic dyes or aromatic dicarboxylic anhydrides as new column packing materials with an expectation of a π-π interaction. Their ability to separate biologically important compounds related to nucleic acids, i.e., adenine derivatives,[1-3] was examined in HPLC. Among them, 3-(1,8-naphthalimido)propyl-modified silyl silica gel (NAIP, Figure 1) was found to be useful for the separation of purine derivatives, i.e., xanthine, hypoxanthine, uric acid, theobromine, theophylline and caffeine. The resolution behavior obtained with the gel suggested that an NAIP column had a reverse phase-like mode with some π-π interaction. By using this column, caffeine concentrations in commercially available medicinal drinks and pharmaceutical preparations were successfully determined. Furthermore, time curves of plasma caffeine and its demethylated metabolite, 1,7-dimethylxanthine, concentrations after an oral ingestion of caffeine could be also determined.[4]

In this work, we at first examined the separation of barbiturates carrying a pyrimidine skeleton with a view to extend the applicability of the NAIP column. Barbiturates are widely used as sedative, hypnotic and anticonvulsant

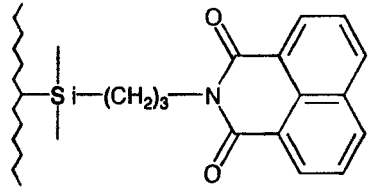

Figure 1. Proposed structure of NAIP.

drugs. Therefore, accurate, simple and rapid method for determining of these is important in pharmaceutical, clinical and toxicological studies. For this purpose, several methods including gas-liquid chromatography (GLC),[5,6] gas chromatography/mass spectrometry (GC/MS)[7] and HPLC[8-10] have been reported. In HPLC methods, a reverse phase ODS column is exclusively employed as a separation column. This paper describes an HPLC separation of barbiturates using an NAIP column and its preliminary application to the determination of these in human blood plasma. Furthermore, to evaluate the scope and limitations of an NAIP column, we examined the separation of diastereomeric compounds carrying an aromatic ring and compared the resolution behavior on an NAIP column with that on an ODS column. Epinephrine (EP), norephedrine (NE) and α-phenylethylamine (PA) were chosen as tentative targets for the labelling with three kinds of chiral reagents.

EXPERIMENTAL

Chemicals

NAIP prepared from 3-aminopropylsilyl silica gel (particle size, 5 μm; pore diameter, 1.2 μm) and 1,8-naphthalic anhydride was packed by a slurry method in a stainless-steel column (150x6 mm I.D.) as described previously.[2]

The sources of barbiturates employed were as follows: Barbital and Phenobarbital from Astra Japan (Osaka, Japan), Amobarbital from Nippon Sinyaku (Kyoto, Japan), Pentobarbital calcium and Thiopental sodium from Tanabe Seiyaku (Osaka, Japan) and Secobarbital sodium from Yoshitomi Pharmaceutical Industries (Osaka, Japan). 5-(2-Cyclohexen-1-yl)-1-phenyl barbituric acid and 5-(2-cyclohexen-1-yl)-5-propenyl barbituric acid as candidates for internal standards (I.S.) were prepared in our laboratory.

D-(+)-Norephedrine, L-(-)-norephedrine, 2,3,4-tri-*O*-acetyl-α-D-arabino-pyranosyl isothiocyanate (AITC) and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) were purchased from Aldrich (Milwaukee, WI, USA). D-(+)-α-Phenylethylamine and L-(-)-α-phenylethylamine were obtained from Nacalai Tesque (Kyoto, Japan). D-(+)-Epinephrine from Sigma (St. Louis, MO, USA), L-(-)-epinephrine from Merck (Darmstadt, Germany), and S(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) from Fluka (Buchs, Switzerland) were employed. An ODS column used was Hibar LiChrosorb 100 RP-18 (7 μm, 250x4.0 mm I.D., Kanto Chemical, Tokyo, Japan).

Borate buffer (Palitzsch buffer) was prepared as follows: H_3BO_4 (6.183 g) and NaCl (1.461 g) were dissolved in 500 mL water. The solution was brought to the appropriate pH by adding of $Na_2B_4O_7/10\ H_2O$ (4.767 g) in 250 mL water.

Water was deionized and passed through a water purification system (Pure Line WL21P, Yamato Kagaku, Tokyo, Japan). Other reagents and solvents used were of analytical reagent grade.

HPLC Apparatus and Conditions

The HPLC system for the separation of barbiturates consisted of a Tosoh CCPD pump (Tokyo, Japan), a Tosoh UV-8011 UV monitor (220 nm), a Rheodyne 7125 injector (Cotati, CA, USA) with a 20-μL sample loop, an NAIP column (5 μm, 150x6 mm I.D.) in a Tosoh CO 8010 column oven, and a Rikadenki R-01 recorder (Tokyo, Japan).

The column temperature was maintained at 30 °C. The elution was performed with borate buffer (pH 7.0)-acetonitrile (9:1, v/v) at a flow rate of 1.0 mL/min.

The HPLC system for the separation of diastereomeric compounds was consisted of two HPLC pumps (LC-9A) with a system controller (SCL-6B, Shimadzu, Kyoto, Japan), a 7125 injector with a 20-μL loop (Rheodyne, Cotati, CA, USA) and a Shimadzu SPD-6A UV-VIS detector (290 nm) for absorbance measurement.

The mobile phases for HPLC separation were as follows: acetonitrile-water for NEIC derivatives, and acetonitrile-10 mM phosphate buffer (pH 3.0) for AITC and GITC derivatives. The flow rate was set at 1 mL/min at an ambient temperature.

Extraction of Barbiturates from Human Plasma

To 100 μL of plasma were added 500 μL of 0.1 M phosphate buffer (pH 7.0) and 10 μL of ethanolic solution of barbiturates. After addition of 5.0 mL of hexane-diethyl ether (3:7, v/v), the mixture was vortex-mixed for 10 s and allowed to stand for 3 min. The organic layer (4.5 mL) was evaporated to dryness under a stream of nitrogen gas, and the resultant residue was dissolved in 100 μL of methanol. The solution was passed through a membrane filter (0.45 μm) and injected onto the HPLC system.

Procedure for Diastereomeric Derivatization

NEIC derivatives: to 200 μL of 5 mM PA or NE in chloroform, or EP in *N,N*-dimethylformamide (DMF) was added 200 μL of 5 mM NEIC in chloroform. After mixing, the mixture was evaporated to dryness under a stream of nitrogen gas and the resultant residue was dissolved in 2 mL of methanol. The solution was passed through a membrane filter (0.45 μm) and injected onto the HPLC system. For the preparation of calibration curves, 25 mM NEIC was used.

AITC and GITC derivatives were prepared according to the previously reported method[11] with minor modifications: 500 μL of 0.2 mM PA, NE or EP in 0.25 M acetic acid was evaporated by a centrifugal evaporator (Yamato Kagaku) and to the resultant residue was added 500 μL of 1 mM AITC or GITC in DMF. After standing at room temperature for 30 min, the solution was passed through a membrane filter and injected onto the HPLC system.

RESULTS AND DISCUSSION

HPLC Separation of Barbiturates by an NAIP Column

In this study, six commercially available barbiturates (Barbital, Phenobarbital, Amobarbital, Pentobarbital, Secobarbital and Thiopental) and two barbiturates as candidates for I.S., 5-(2-cyclohexen-1-yl)-1-phenyl and 5-(2-cyclohexen-1-yl)-5-propenyl barbituric acids, were tested.

Separation of barbiturates by an NAIP column was examined using the borate buffer and 10 mM phosphate buffer with various pHs as mobile phases, and the former providing better results was selected for further experiments.

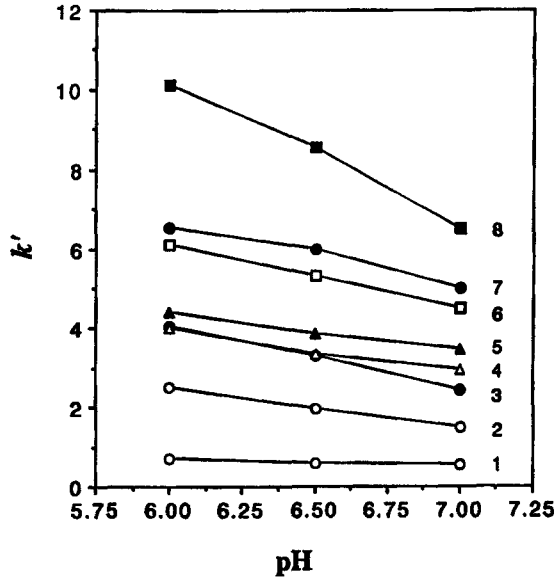

Figure 2. Effect of pH of borate buffer used in a mobile phase on separation of barbiturates by an NAIP column.

Sample (0.1 mM): 1 = Barbital, 2 = 5-(2-cyclohexen-1-yl)-1-phenyl barbituric acid, 3 = Phenobarbital, 4 = Amobarbital, 5 = Pentobarbital, 6 = Secobarbital, 7 = 5-(2-cyclohexen-1-yl)-5-propenyl barbituric acid, 8 = Thiopental.

As shown in Figure 2, the best separation was observed at pH 7.0 of the borate buffer, and thus was selected in this study. The content of acetonitrile in the mobile phase also affected the separation of barbiturates.

The capacity factor *(k')* for each barbiturate decreased with an increase in acetonitrile contents from 5 to 10%. The best peak separation with a proper retention time was obtained with 10 % acetonitrile.

Figure 3 shows a typical chromatogram of eight barbiturates. Under the experimental conditions, all barbiturates were separated from each other within 30 min.

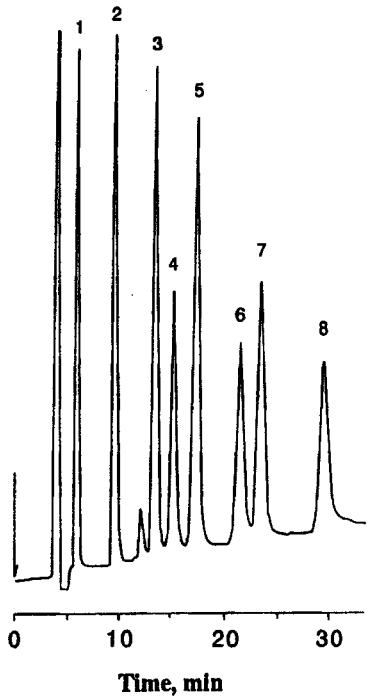

Figure 3. A typical chromatogram of barbiturates by an NAIP column.
Sample (0.1 mM): 1 = Barbital, 2 = 5-(2-cyclohexene-1-yl)-1-phenyl barbituric acid, 3 = Phenobarbital, 4 = Amobarbital, 5 = Pentobarbital, 6 = Secobarbital, 7 = 5-(2-cyclohexen-1-yl)-5-propenyl barbituric acid, 8 = Thiopental.

Preliminary Study on the HPLC Analysis of Barbiturates in Human Plasma

A solvent extraction method was examined for the sample pretreatment using plasma spiked with six commercially available barbiturates. Recoveries of barbiturates spiked to plasma were affected by the pH of 0.1 M phosphate buffer added to the plasma sample before extraction. The ratio of hexane and diethyl ether in a solvent for extraction of barbiturates also affected the recoveries. As shown in Figure 4, the combination of the buffer of pH 7.0 and the extraction with hexane-diethyl ether (3:7, v/v) gave the best results; the recoveries obtained for six barbiturates were in the ranged of 88.6 to 100.0 %.

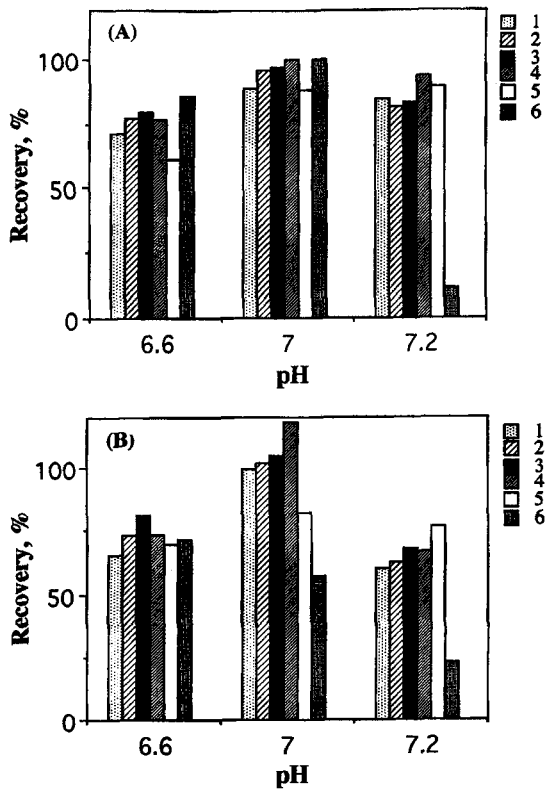

Figure 4. Recoveries of barbiturates spiked to human plasma by extraction with solvent (A) hexane-diethyl ether (3:7, v/v) and (B) hexane-diethyl ether (5:5, v/v). Spiked sample (μg/mL): 1 = Barbital (18.4), 2 =Phenobarbital (23.2), 3 = Amobarbital (22.6), 4 = Pentobarbital (22.5), 5 = Secobarbital (23.7), 6 = Thiopental (24.1).

The calibration curves, in which 5-(2-cyclohexen-1-yl)-5-propenyl barbituric acid was used as an I.S., were linear over the range of 1.11-27.63 μg/mL for Barbital, 1.39-34.84 μg/mL for Phenobarbital, 1.36-33.94 μg/mL for Amobarbital, 1.35-33.79 μg/mL for Pentobarbital, 1.42-35.59 μg/mL for Secobarbital and 1.45-36.20 μg/mL for Thiopental (r=0.982-1.000). The lower limits of detection obtained ranged from 0.05 to 0.33 μg/mL at a signal-to-noise ratio of 3, which are comparable with those of the HPLC method using an ODS column with UV detection[8] (Table 1). These values suggest that the proposed method can be applied to the blood monitoring of barbiturates, because the blood levels of most barbiturates after an oral ingestion of therapeutic doses are generally more than 1 μg/mL.[8]

Table 1

Precision Data and Detection Limits with the Proposed Method

Compound	Spiked (μg/mL)	Precision (R.S.D., %) Within-day	Day-to-day	Detection Limit (μg/mL)
Barbital	5.53	3.48	6.83	0.10
Phenobarbital	6.97	3.87	4.72	0.05
Amobarbital	6.97	4.82	4.68	0.12
Pentobarbital	6.76	4.60	4.99	0.05
Secobarbital	7.12	4.61	6.55	0.13
Thiopental	7.24	9.07	18.68	0.33

The precision of the proposed method was examined using plasma samples spiked with known concentrations of barbiturates (5.53-7.24 μg/mL). The within-day and day-to-day precision with five replicate determinations gave relative standard deviations (R.S.D.) of 3.48-9.07% and 4.72-18.68%, respectively (Table 1). A typical chromatogram of a plasma spiked with six commercially available barbiturates and I.S. is presented in Figure 5.

Resolution Behavior for Diastereomeric Derivatives

Since an NAIP column behaved like a reverse phase column with some π-π interaction as reported previously,[4] it was expected to separate diastereomeric compounds carrying an aromatic ring. Consequently, we examined a separation of these compounds. EP, NE and PA were selected as targets for the labelling with chiral reagents.

NEIC Derivatives

The separation properties of NEIC derivatives of PA, NE and EP on an NAIP packed column were examined using acetonitrile-water as a mobile phase. As shown in Figure 6, the capacity factor (k') and separation factor (α) of NEIC derivatives of PA decreased with increasing acetonitrile contents in the mobile phase; it seemed to be a reverse phase-like separation. NE showed the similar tendency, but in the case of EP, diastereomeric derivatives could not be well separated by a peak tailing. This might be caused by a strong interaction between a catechol moiety of EP derivatives and the stationary phase under the conditions tested.

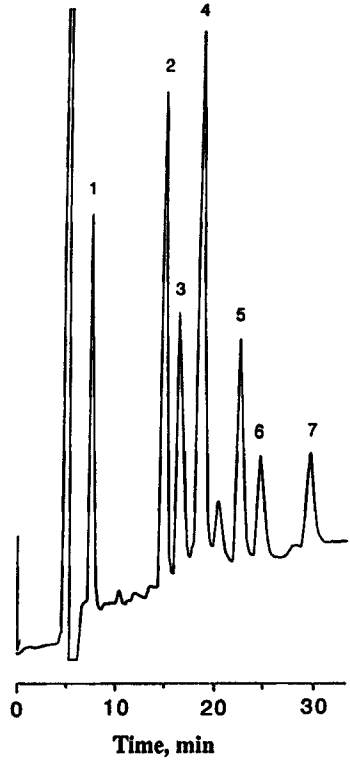

Figure 5. A typical chromatogram of plasma sample spiked with barbiturates by an NAIP column. Spiked sample (μg/mL): 1 = Barbital (5.53), 2 =Phenobarbital (6.97), 3 = Amobarbital (6.79), 4 = Pentobarbital (6.76), 5 = Secobarbital (7.12), 6 = 5-(2-cyclohexen-1-yl)-5-propenyl barbituric acid (7.41), 7 = Thiopental (7.24).

A comparison of diastereomeric separation data for NE and PA on NAIP and ODS columns are given in Table 2. The ratio of acetonitrile and water in the mobile phase was individually optimized for each derivative. The values for α and resolution *(Rs)* were calculated by the following equations:

$$\alpha = k'2 / k'1$$
$$Rs = 2 (t_{R2} - t_{R1}) / 1.70 (W1+W2)$$

where t_R = retention time and W = peak width.

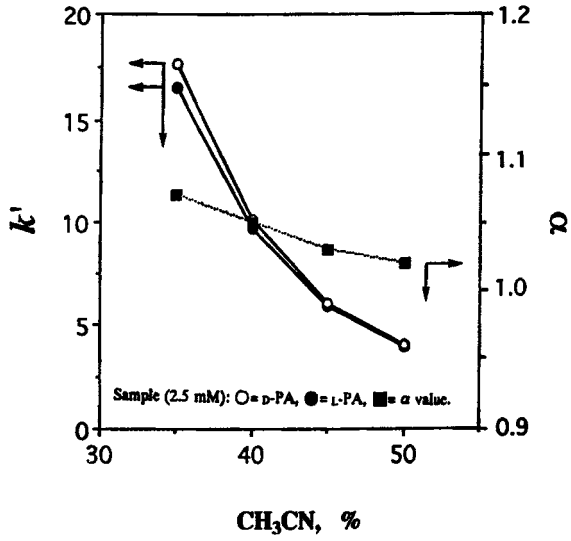

Figure 6. Effect of acetonitrile contents in the mobile phase on separation of NEIC derivatives of PA by an NAIP column.
Sample (2.5 mM): O = D-PA, ● = L-PA, ■ = α value.

Table 2

Comparison of Separation Properties of NAIP and ODS Column for NEIC Diastereomers

Compound	**NE**		**PA**	
Column	**NAIP**	**ODS**	**NAIP**	**ODS**
CH_3CN/Water*	**30/70**	**33/67**	**35/65**	**40/60**
First eluent	**D**	**D**	**L**	**D**
k'	17.99	22.55	16.51	20.30
α	1.05	1.06	1.07	1.05
Rs	1.14	0.94	1.59	1.06

* The ratio of acetonitrile and water in the mobile phase.

Table 3

Comparison of Separation Properties of NAIP and ODS Column for AITC Diastereomers

Compound	NE		PA		EP	
Column	NAIP	ODS	NAIP	ODS	NAIP	ODS
CH_3CN/Buffer*	15/85	30/70	20/80	30/70	10/90	15/85
First eluent	D	D	L	L	D	D
k'	15.73	9.09	10.76	16.65	13.61	18.00
α	1.10	1.20	1.10	1.06	1.08	1.22
Rs	1.43	3.46	1.25	1.25	1.02	3.22

* The ratio of acetonitrile and 10 mM phosphate buffer (pH 3.0) in the mobile phase.

Table 4

Comparison of Separation Properties of NAIP and ODS Column for GITC Diastereomers

Compound	NE		PA		EP	
Column	NAIP	ODS	NAIP	ODS	NAIP	ODS
CH_3CN/Buffer*	20/80	33/67	20/80	33/67	15/85	20/80
First Eluent	L	L	D	D	L	L
k'	10.10	18.68	12.08	18.46	17.20	17.98
α	1.05	1.19	1.10	1.07	1.09	1.22
Rs	0.74	3.85	1.38	1.39	1.40	3.46

* The ratio of acetonitrile and 10mM phosphate buffer (pH 3.0) in the mobile phase.

As shown in Table 2, the Rs values obtained from NE and PA derivatives on an NAIP column was found to be even or relatively better than those on an ODS column. The elution order for PA derivatives on an NAIP column was observed to be reversed on an ODS column.

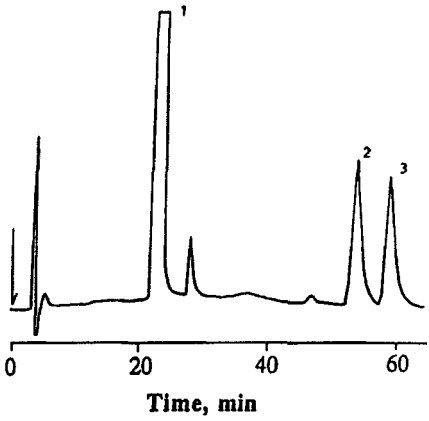

Figure 7. A typical chromatogram of NEIC derivatives of L- and D-PA by an NAIP column.
Sample (2.5 mM): 1 = Blank, 2 = L-PA, 3 = D-PA; mobile phase, CH_3CN-water (30:70, v/v).

AITC and GITC Derivatives

The separation data for AITC and GITC derivatives are given in Table 3 and 4, respectively. Acetonitrile-10 mM phosphate buffer (pH 3.0) system was used as a mobile phase. AITC and GITC derivatives of NE, PA and EP on an NAIP column behaved like those on a reverse phase column; the retention time for each derivative was shortened with an increase in acetonitrile contents in the mobile phase. AITC and GITC diastereomers were satisfactorily separated on both columns. However, the *Rs* values for NE and EP derivatives on an ODS column were better than those on an NAIP column for both AITC and GITC diastereomers.

As a preliminary study on the quantitation of the diastereomers, the linearity of the calibration curves obtained by an NAIP column was examined by using NEIC derivatives of L-and D-PA, which showed the best separation in the derivatives examined on this column (a typical chromatogram was shown in Figure 7). The linear relationships were obtained between the peak-heights and the concentrations of each derivative over the range of 3.14 μM to 0.25 mM ($r=1.000$ for both derivatives). The lower limits of detection were 0.83 μM for NEIC-L-PA and 1.07 μM for NEIC-D-PA at a signal-to-noise ratio of 3.

CONCLUSION

An NAIP column was found to be very useful for the separation of pyrimidine derivatives e.g., barbiturates as well as purine derivatives.[2,4] The sensitivity for barbiturates obtained by an NAIP column with UV detection was comparable with that by an ODS column. Thus, an NAIP column should be useful for the determination of barbiturates in human blood plasma.

In addition, it has become apparent that an NAIP column was also applicable to the separation of diastereomeric derivatives of NE, AP and EP like an ODS column. An NAIP column might be usable for the separation and determination of these compounds in pharmaceutical preparations and biological materials. It will be worthwhile to study further on the separation of other diastereomeric derivatives.

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