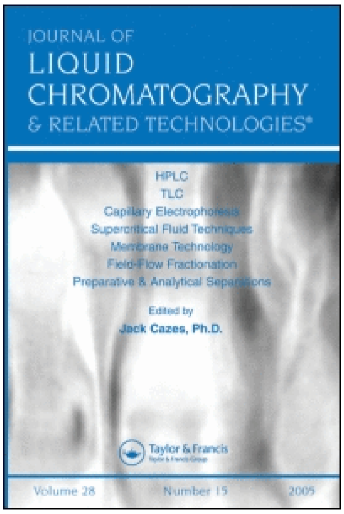

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CHARACTERIZATION OF AMINO-, CYANO-ALKYL BONDED SILICA COLUMNS IN NORMAL-PHASE LIQUID CHROMATOGRAPHY BY USING STEROIDS

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ABSTRACT

In order to characterize the chemically bonded phases in HPLC analysis, the retention behavior of fifteen steroids including estrogen, androgen, progestogen and corticoid were systematically examined using dioxan as the stronger component in an n-hexane-binary system. A linear relationship between the logarithm of the capacity ratio and logarithm of the molar concentration of the binary solvent was confirmed for amino- and cyano-type bonded as well as non-bonded silica gel columns. Based on the retention indices of these phases, the retentivity of the packing materials was determined as follows: the amino-type is similar to and the cyano-type is weaker (0.7 times) than bare silica gel when using dioxan as the stronger component. The specific retentivity of an amino column for polar steroids containing phenolic and alcoholic hydroxyl groups suggests a molecular interaction associated with hydrogen bonding between the polar packing surface and solute compounds. The selectivity of amino packing was found to be larger than cyano packing whose retention selectivity is similar to a bare silica gel.

INTRODUCTION

Applications of various chemically bonded silica gel columns have been developed recently and widely accepted by analytical laboratories probably because their favorable physical properties such as rigidity

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under high pressure, high speed performance and high efficiency. In various bonded phases, the octadecylsilylated silica gel has been used the most in recent HPLC analysis and involves a reversed-phase mode. However, aminopropyl- and cyanopropyl-silylated silica columns have also become important because of their wide range of selectivity in normal- and reversed-phase systems.

To clarify the retention characteristics of amino- and cyano-alkyl bonded phases, systematic studies using various solutes and binary solvents in the normal-phase mode have been carried out[1-5]. We examined the retention behavior of fifteen steroid hormones using binary solvents in liquid-solid chromatography. Medium polar compounds having hydroxyl, carbonyl and acyloxyl groups were selected as the solutes.

Dioxan was chosen in this paper as the stronger component in order to increase the polarity of the eluents to compete with active functional groups of sample molecules in the adsorption-desorption equilibrium process on the surface of the packing materials. Dioxan is an aprotic B-type solvent[6] and has no ultra-violet absorption in the detection region. It provides fairly large solvent strength for medium polar compounds[6,7] and thus has wide applicability as the stronger component in binary solvent systems.

Based on the linear relationship between the logarithm of the capacity ratio and the logarithm of solvent composition, as recently established by our cumulated experimental data[1-10], the retentivity of steroids on amino and cyano columns was determined and compared with the data obtained using a non-bonded silica gel column as the standard. The selectivity of three columns was evaluated on the basis of the systematic retention data for the steroid solutes.

EXPERIMENTAL

Apparatus The Liquid Chromatograph used was the 635 Hitachi model, Tokyo, equipped with a UV-detector, Uvilog 5 III, Oyo-Bunko, Tokyo.

Columns To examine the selectivity of the columns, we selected chemically bonded packing materials prepared from the same silica gel support. We used (A) silica gel, Nucleosil 100-5, (B) aminopropyl silica, Nucleosil 5NH2, (C) cyanopropyl silica, Nucleosil 5CN. The particle size of these gels made by Macherey-Nagel was 5 μm. The columns were prepared using stainless steel tubes, 250 mm x 4 mm i.d. and a slurry of the packing materials in dioxan, methanol and carbon tetrachloride. The slurry packed columns were finally purged by chloroform.

Reagents Fifteen steroid hormones were used as the samples. The molecular structures are presented in Table I. The steroids were obtained as follows: estradiol, ethynylestradiol (Uclaf); estriol, corticosterone, cortisone and prednisolone (Sigma); others (Tokyo Kasei). Sample purity was checked by thin-layer chromatography which afforded a single spot on the silica gel plate.

Chromatography run Samples of 1 - 5 ng were dissolved in 10 μl of carrier solvent. Sample solutions of 0.2 - 2.0 μl were injected into the column. Flow rate was 1.2 ml/min. Column temperature was maintained at 40°C. The capacity ratio, k' was calculated by the formula: $k' = (t_R - t_0)/t_0$ where t_R is retention time, t_0, the hold up time measured by injecting n-nonane as the non-retained sample.

RESULTS AND DISCUSSION

1. Correlation between the Capacity Ratio and Solvent Composition

According to the mechanism involved in adsorption-desorption of the solute molecules and the silanol group as an active site on the silica gel surface, the mathematical relation between the capacity ratio logarithm for the silica gel column and molar ratio logarithm of the stronger solvent is proposed as follows:

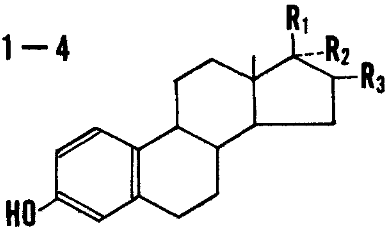
$$\log k' = c - n \log Xs \qquad (1)$$

where k' is capacity ratio, Xs, the molar fraction of the stronger component in the binary solvent and c and n are constants. This relation was confirmed by the experimental results obtained from various solutes and solvent systems for silica gel columns[6-10]. Equation (1) was also established for amino- and cyano-type columns in normal-phase operation using n-hexane binaries containing ethyl acetate, ethanol as the stronger components [1-5].

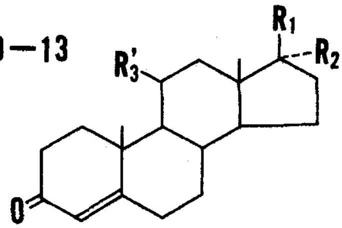
We examined systematically the retentivity of steroid samples using the dioxan-n-hexane binary solvent in this article. We classified the solutes into two groups, (a) four estrogens (1-4) and four androgens

TABLE I. Structural Formulas of the Steroid Hormones

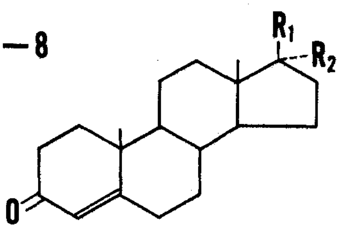
1—4



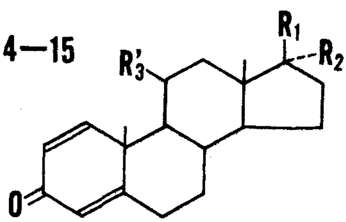
9—13



5—8



14—15



Sample No.	R_1	R_2	R_3	Name
1	=O		H	Estrone
2	OH	H	H	Estradiol
3	OH	C≡CH	H	Ethynylestradiol
4	OH	H	OH	Estriol
5	OH	H		Testosterone
6	OH	CH3		Methyltestosterone
7	OH	C≡CH		Ethynyltestosterone
8	OCOC2H5	H		Testosterone propionate
9	COCH3	H	H	Progesterone
10	COCH2OCOCH3	H	H	Deoxycorticosterone acetate
11	COCH2OH	H	OH	Corticosterone
12	COCH2OH	OH	=O	Cortisone
13	COCH2OCOCH3	OH	=O	Cortisone acetate
14	COCH2OH	OH	=O	Prednisone
15	COCH2OH	OH	OH	Prednisolone

(5-8) and (b) seven progestogens and corticosteroids (9-15). The retention indices were measured at various solvent compositions. The results obtained using silica, amino and cyano columns are presented in Figure 1 - 3, respectively. The linear relation between the logarithms of capacity ratio and molar fraction of dioxan was determined for three phase systems and a correlation coefficient value of 0.98 - 1.00 was obtained. The mathematical relation confirmed here suggests that the retention mechanism of the chemically bonded packings is similar to that of silica gel in a normal-phase operation. The adsorption-desorption process may occur by a hydrogen bonding interaction between the active functional groups and solute molecules on the surface of the adsorbents.

Equation (1) should be quite useful for optimizing the phase system and thus the micro-computer program in equation (1) was compiled at our laboratory for data filing and a systematic approach to the design of an optimum phase system. The computer-assisted solvent selection is such that solvent composition output can be made by using at least two retention data values for input. The two constants of equation (1) and the correlation coefficient of the experimental data can also be calculated by computer. An example of filing data for estriol is shown in Figure 4. On the basis of six experimental data, solvent compositions corresponding to given capacity ratio values were determined.

2. Molecular Structures and the Retentivity

In Figures 1-3, the correlation lines representing the logarithm of capacity ratios for steroids versus the logarithm of solvent composition are distributed in a fan-shaped manner except for a few lines which cross over each other. The retention sequence of the steroids did not change with respect to solvent composition. This means that not only k', but also the increment of logarithm k' (logarithm of α, the separation factor) increases with concentration of the stronger component which decreased for nearly all solvent pairs. The two constants of equation (1) as the retention parameters for a class of compounds such as estrogens, androgens, progestogens and corticosteroids, vary in a similar manner for the three columns.

Retention sequence of the solutes in the silica gel column used as the standard is as follows: $1 < 3 < 2 \ll 4$ for estrogens, $8 \ll 6 < 7 < 5$ for androgens as shown in Figure 1a and $9 \ll 10 \ll 13 < 11 < 12 < 14 < 15$ for

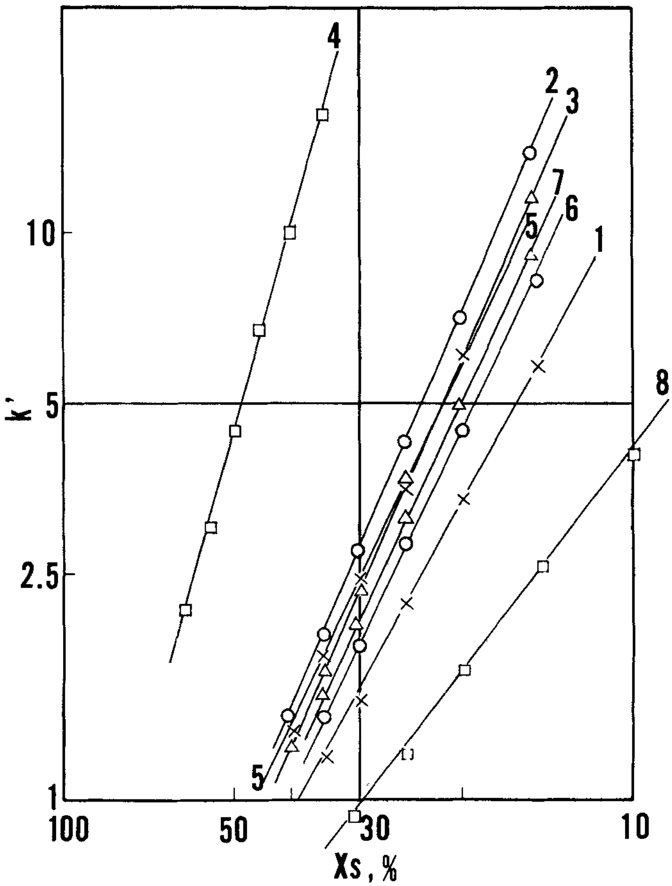


Fig. 1a

Fig. 1 - 3. Logarithm of Capacity Ratio of Silica Gel and Chemically Bonded Silica Columns as a Function of the Logarithm of Dioxan Concentration in n-Hexane

Packing: silica gel (Fig. 1); amino-bonded silica (Fig. 2); cyano-bonded silica (Fig. 3).

Samples in Table I: a, estrogen, androgen; b, progestogen, corticosteroid.

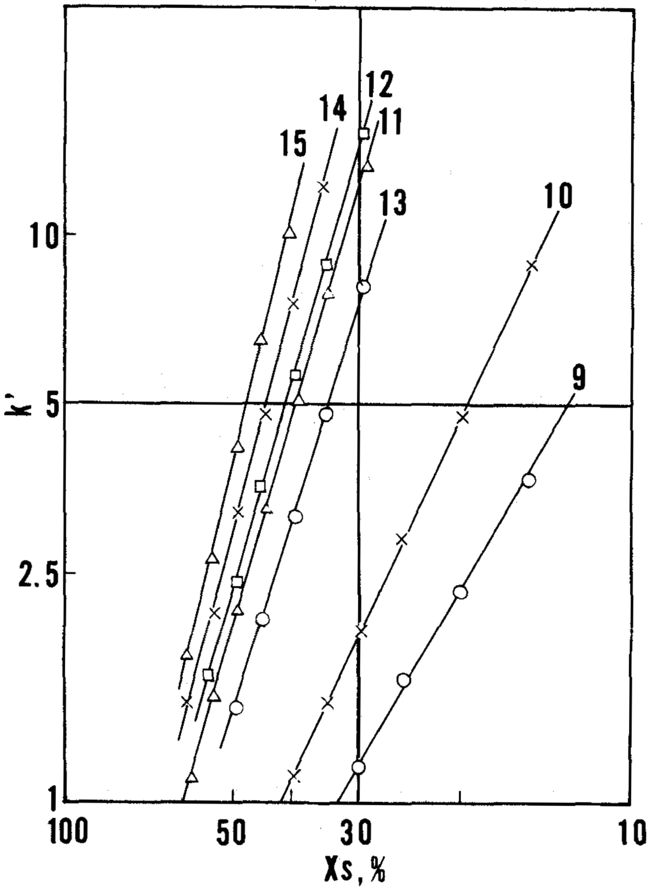


Fig. 1b

progestogens and corticosteroids as shown in Figure 1b. It was found that the capacity ratio increased when different functional groups were used in the following manner: the carbonyl group at 12 or 17 position was hydrogenated to a hydroxyl group, the O-acyl group was hydrolyzed to a hydroxyl group, the 4-en-3-one was dehydrogenated to 1,4-dien-3-one functional groups and additional groups accumulated about this molecule. These findings are in agreement with the retention mechanism involved in the hydrogen bonding association between polar groups in the solute and silanol group as the active site on the silica gel surface.

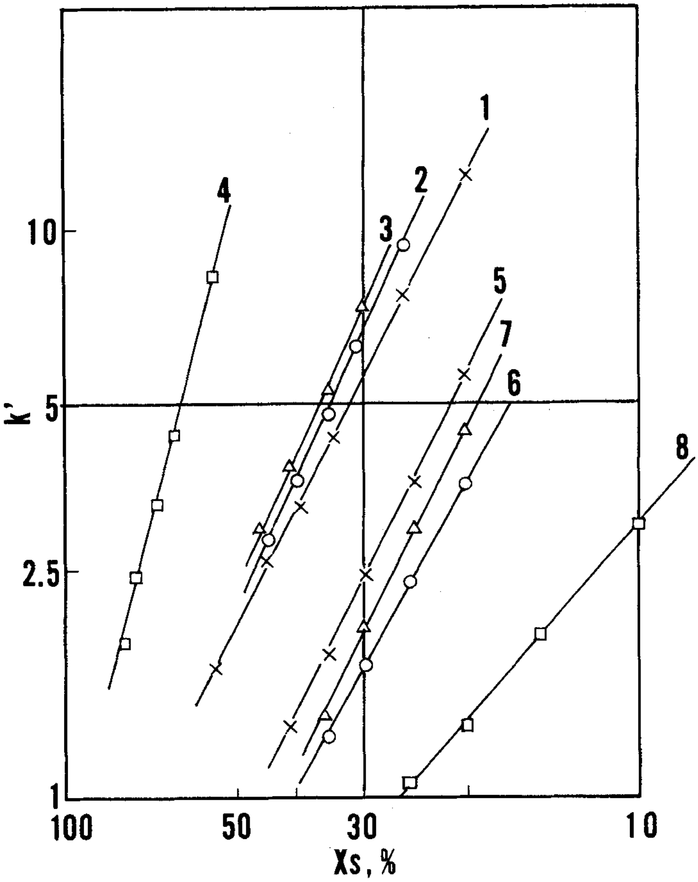


Fig. 2a

The retentivity of amino column increased in the following order: 1 < 2 < 3 ≪ 4 for estrogens, 8 ≪ 6 < 7 < 5 for androgens as shown in Figure 2a and 9 ≪ 10 ≪ 13 < 11 < 12 < 15 < 14 for progestogens and corticosteroids as shown in Figure 2b. Sequence of steroid retentivity for the amino column was very close to that of the silica column used as the standard. Inversion of the retention order for the two columns was found only in the case of a few samples: 2, 3 and 14, 15.

Retention of the solutes in the cyano column also increased in a similar manner to the silica and amino columns, with the following se-

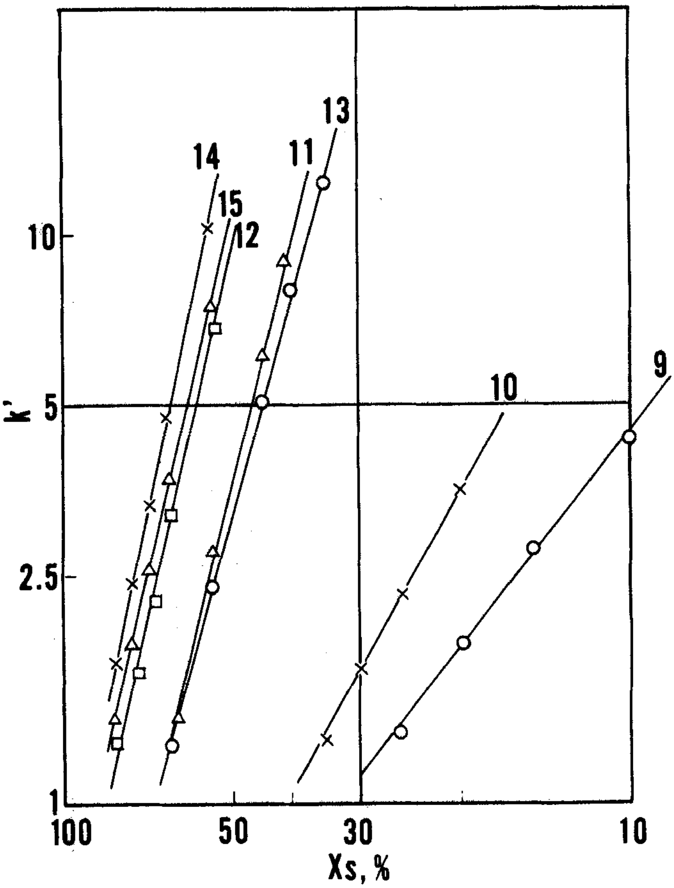


Fig. 2b

quence: 1 < 3 ≤ 2 ≪ 4 for estrogens, 8 < 6 < 5 < 7 for androgens as shown in Figure 3a and 9 ≪ 10 ≪ 11 < 13 < 12 < 14 < 15 for progestogens and corticosteroids as shown in Figure 3b. The inversion of the retention order for the cyano and silica gel columns was observed for two pairs of samples: 5, 7 and 11, 13. Such chromatographic behavior of two packing materials was generally given and so retention mechanism for any of these solute samples in the three columns should be quite nearly the same.

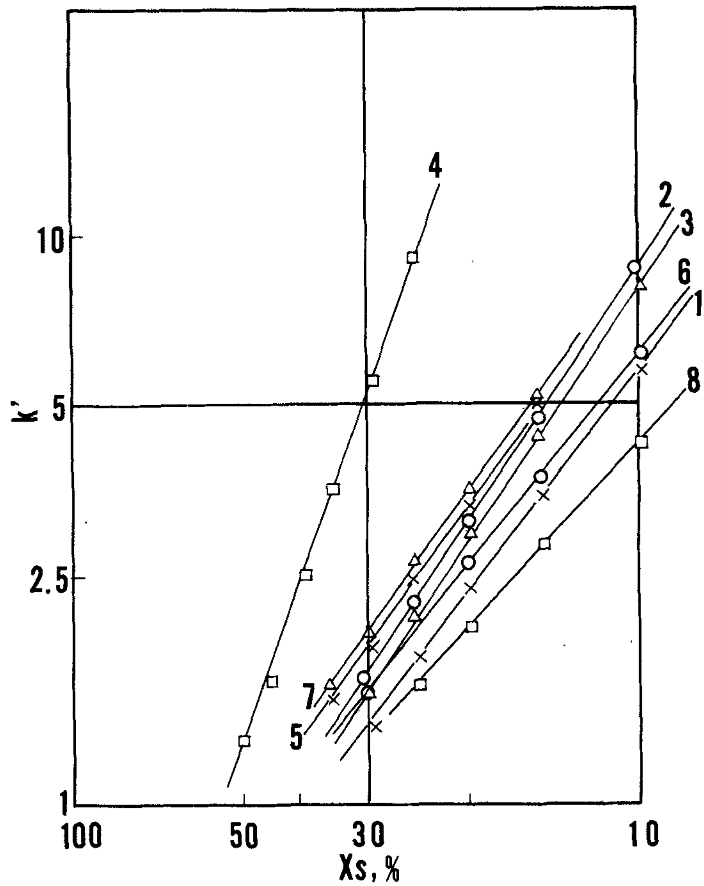


Fig. 3a

3. Selectivity of the Column Packing

Up to this point, observation has been made of different retentivities in three columns through use of binary mobile phases of the same composition and quantitative evaluation of column selectivity is examined in this section. The constants of equation (1) for a specific solute are considered to depend on the characteristics of the column and thus the quotients of the constants for a pair of columns by a using silica gel column as the standard were calculated as follows: the c-ratio and n-ratio for amino/silica and cyano/silica are the quotients of the two constants in equation (1) for the amino column/silica gel column

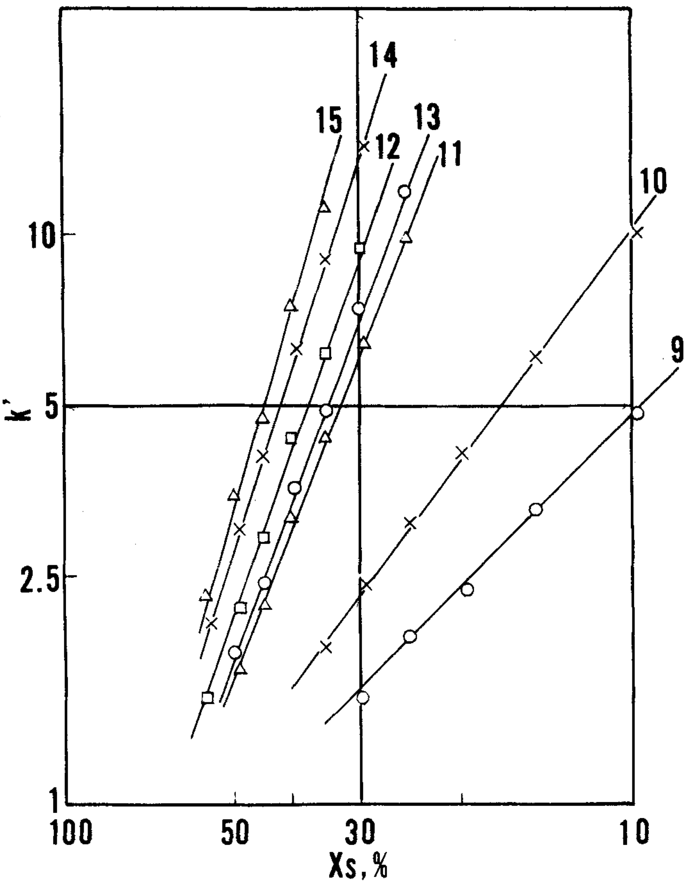


Fig. 3b

and cyano column/silica gel column, respectively. The constants c and n for the silica gel column and the quotients for the two chemically bonded columns to the silica column as the standard are tabulated in Table II. The mean values and standard deviation are also given in the table.

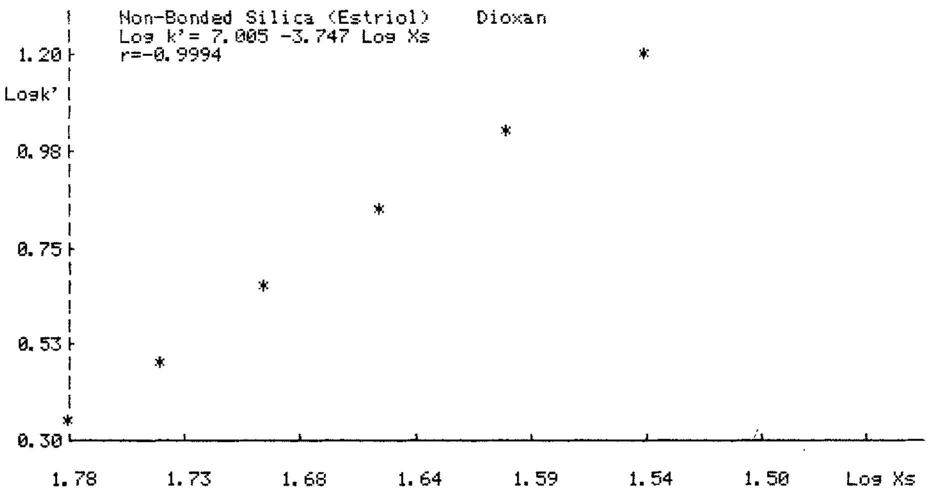
The mean values of the two constant ratios in Table II indicate that the relative retentivity of amino column is similar to that of the silica gel column and the retentivity of cyano column about thirty percent less than that of the silica column. As far as the column selectivities are concerned it was found that the retention indices of

*** Non-Bonded Silica (Estriol) ***

Dioxan(%)	k'	Log k'	Log Xs
35.00	16.05	1.2055	1.5441
40.00	10.16	1.0069	1.6021
45.00	6.74	0.8287	1.6532
50.00	4.42	0.6454	1.6990
55.00	3.00	0.4771	1.7404
60.00	2.16	0.3345	1.7782

Data N= 6
Log k'=c-nLog Xs

n= -3.7469
c= 7.0051
r= -0.9994



k'	Xs Dioxan(%)
1.0	74.05
2.0	61.55
3.0	55.23
4.0	51.15
5.0	48.20
6.0	45.91
7.0	44.06
8.0	42.51
9.0	41.20
10.0	40.06

Fig. 4. Filing Example of Retention Data Obtained by a Micro-Computer

r is the correlation coefficient.

TABLE II. Constants and Constant Ratios of the Linear Relationship between the Retention Index and Solvent Composition of Dioxan in n-Hexane for a Silica Gel and Chemically Bonded Silica Columns

	constant		constant ratio			
Column	silica gel		amino/silica		cyano/silica	
No. steroid	c	n	c-ratio	n-ratio	c-ratio	n-ratio
1	2.71	1.69	1.29	1.10	0.72	0.72
2	3.56	2.11	1.13	1.02	0.70	0.74
3	3.38	2.03	1.25	1.13	0.72	0.74
4	7.01	3.75	1.13	1.07	0.71	0.76
5	3.66	2.21	0.94	0.93	0.62	0.61
6	3.39	2.10	0.83	0.84	0.55	0.53
7	3.44	2.11	0.90	0.90	0.66	0.63
8	2.05	1.41	0.87	0.91	0.74	0.65
9	2.56	1.68	0.71	0.72	0.61	0.55
10	3.43	2.11	0.85	0.86	0.70	0.64
11	6.48	3.61	1.06	1.03	0.69	0.69
12	6.63	3.68	1.32	1.24	0.83	0.82
13	5.81	3.32	1.17	1.11	0.83	0.81
14	7.18	3.94	1.26	1.17	0.81	0.80
15	8.03	4.36	1.08	1.03	0.80	0.80
mean value	4.62	2.67	1.05	1.00	0.71	0.70
standard deviation	1.99	0.98	1.19	0.14	0.08	0.09

c and n: intercept and slope of equation (1) in the text. c-ratio and n-ratio: quotients of constants c and n for a pair of columns.

estrogens (1 - 4) and corticosteroids (11 - 15) except for acetate (10) were larger and those of androgens (5 - 8) and progestogen (9) were smaller for the amino column than for the silica column. This suggests that a specific affinity between the surface of the amino packing and phenolic hydroxyl groups in the estrogens or alcoholic hydroxyl groups in the androgens and corticosteroids.

In Table II, the constants of the cyano column are always smaller than those of the silica column. The standard deviation of the constant ratios for the cyano and silica columns is fairly small. Thus the selectivity of the cyano column is approximately the same as that of the silica column. Consequently, the cyano column can be used as one similar to but having a retentivity weaker than the silica gel column.

CONCLUSIONS

The linear correlation between the logarithm of the capacity ratios for the chemically bonded amino- and cyano-propylsilylated silica and non-bonded silica gel columns versus the logarithm of the molar concentration of dioxan as the stronger component in an n-hexane-binary solvent was confirmed experimentally by the normal-phase liquid-solid chromatography of fifteen steroid hormones.

On the basis of the average retention indices of the steroids in these phase systems, column retentivity was evaluated quantitatively as follows: the retentivity of the amino column was similar to and that of the cyano column approximately 0.7 times more than that of the silica column as the standard. However, from the selectivity of the amino column, it was found that the retention of phenolic and alcoholic solutes was greater and that of acylated samples was smaller than retention of a silica gel column.

These results facilitate the design of an optimum phase system in binary solvent liquid-solid chromatography for samples of known molecular structure.

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