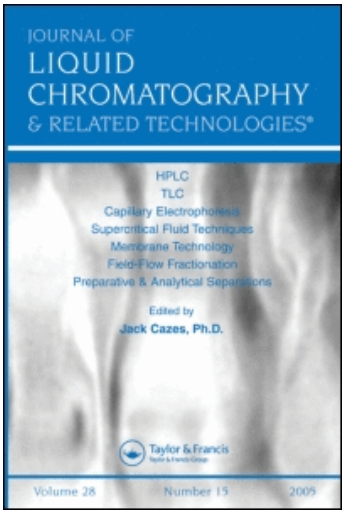


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# Application of Thin-Layer Chromatography to High-Performance Liquid Chromatographic Separation of Steroidal Hormones and Cephalosporin Antibiotics

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# APPLICATION OF THIN-LAYER CHROMATOGRAPHY TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF STEROIDAL HORMONES AND CEPHALOSPORIN ANTIBIOTICS

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## ABSTRACT

High-performance liquid chromatographic (HPLC) separation of steroidal hormones and cephalosporin antibiotics was investigated by adsorption chromatography and reversed-phase chromatography, respectively.

Prior to the HPLC separation of these pharmaceuticals, silica gel thin-layer adsorption chromatography of steroidal hormones and reversed-phase thin-layer partition chromatography of cephalosporin antibiotics with chemically bonded dimethylsilyl silica gel were performed in order to obtain suitable HPLC separation systems.

In the separation of steroidal hormones, the same binary mobile phase ratios of TLC did not give satisfactory results in HPLC. For the sharp separation in HPLC, solvent strength in the binary solvent mixture used for TLC had to be decreased.

The difference in solvent strength for efficient separation between TLC and HPLC might be

attributed to the fact that in HPLC the solvent elution power acts in an isocratic manner while in TLC it acts in a gradient manner.

On the other hand, a correlation of mobility between TLC and HPLC separation for cephalosporin antibiotics was obtained, and the possibility of direct transfer of chromatographic systems from TLC to HPLC for separation of these antibiotics was confirmed.

## INTRODUCTION

Several authors have reported the relationship between TLC and classical column chromatography of various kinds of compounds (1-4). Recently, similar studies between TLC and HPLC separations have been described (5-9).

This study used a TLC separation as a pilot study for HPLC analysis for steroidal hormones (adsorption chromatography) and cephalosporin antibiotics (reversed-phase partition chromatography). The HPLC separation of six hardly resoluble pairs of steroidal steric and geometrical isomers shows the parallelism with the TLC separation and the requirement of a lower solvent strength of the binary solvent mixture used for TLC. The analysis of eight kinds of cephalosporin antibiotics confirmed a correlation between separation by TLC and HPLC and the

possibility of direct transfer of chromatographic systems from TLC to HPLC.

## MATERIALS

Commercially available Merck silica gel 60 $F_{254}$ (Item No. 5715) and silica gel 60 $F_{254}$ silanised (Item No. 5747, laboratory-prepared) were used after activation at 110°C for 30 min. Waters Model 204 Liquid chromatograph with Models 6000 A Solvent Delivery System and U6K Universal Injector was used for HPLC analysis. An isocratic mode was employed and the eluent was monitored at 240 nm for steroidal hormones and 265 nm for cephalosporin antibiotics with Waters Model 440 ultraviolet absorbance detector or Shimadzu SPD-2A variable wavelength UV monitor in series with Waters Model R-401 Difference Refractometer. Ultraviolet and refractometry index chromatograms were recordered with Shimadzu Model C-R1A Chromatopac computing recorder and integrator. Chromatographic conditions are described in the figure legends. Merck LiChrosorb RP-2 [10 µm, 4 mm x 30 cm, laboratory-packed by balanced density slurry method (10-11)], LiChrosorb SI 100 (10 µm, 4 mm x 30 cm) and Waters µPorasil (7 µm, 4 mm x 30

cm) were used for analysis. The solvents used were filtered with Milipore membrane filter (0.45 μm) and degassed., As an ion-pair reagent, Waters PIC B-7 [1-heptane sulfonic acid (pH 3.5 with acetic acid)] and PIC A (tetrabutylammonium phosphate, pH 7.5) were used for the cephalosporin analysis.

## RESULTS AND DISCUSSION

### Chromatography of Steroidal Hormones (11)

As pairs of stereoisomers, 5:6-oxido-cholesteryl benzoates (α = 1, β = 2, 12), 5:10-oxido-19-norandrostane-3α,17β-diols (α = 3, β = 4, 12) and its 3,5-dinitrobenzoates (DNB), dexamethasone 21-acetate (16α-methyl = 5), betamethasone 21-acetate (16β-methyl = 6), 5-cyano-19-nortestosterone 3,5-DNB (α-cyano = 9, β-cyano = 10, 13) were used. As a pair of geometrical isomers, 3β-hydroxy-androsten-17-one oxime benzoate (syn = 7, anti = 8, 14) was used.

TLC separation of these steroidal isomers was performed with solvent systems having the same solvent strength (15) such as (A) benzene-i-propyl ether (1:1), (B) chloroform-ethyl acetate (9:1), (C) benzene-methanol (19:1) and (D) chloroform-acetone

TABLE 1

TLC of Steroidal Cyanoketone Stereoisomers[a)]

| Mobile phase | $hR_f$ value of stereoisomer α-Cyanoketone | α | β-Cyanoketone | Results |
|---|---|---|---|---|
| Benzene-isopropyl ether (1:1) | 51 | 1.21 | 42 | Separable |
| Chloroform-ethyl ether (9:1) | 46 | 1.15 | 40 | Separable |
| Benzene-methanol (19:1) | 38 | 1.00 | 38 | Inseparable |
| Chloroform-acetone (20:1) | 32 | 1.00 | 32 | Inseparable |

a) Stereoisomer: 5-Cyano-19-nortestosterone 3,5-dinitrobenzoate
TLC plate: Merck silica gel 60 $F_{254}$
Detection: Fluorescence quenching at 254 nm followed by sulfuric acid
α: Separation factor = larger $hR_f$/smaller $hR_f$

(20:1). For example, 5-cyano-19-nortestosterone isomers were separable with benzene-i-propyl ether (1:1) or chloroform-ethyl acetate (9:1) (Table 1 and Figure 1). Table 3 shows the favorable TLC condition for the six kinds of steroidal isomers.

HPLC separation of these steroidal isomers was carried out under the same conditions used for TLC.



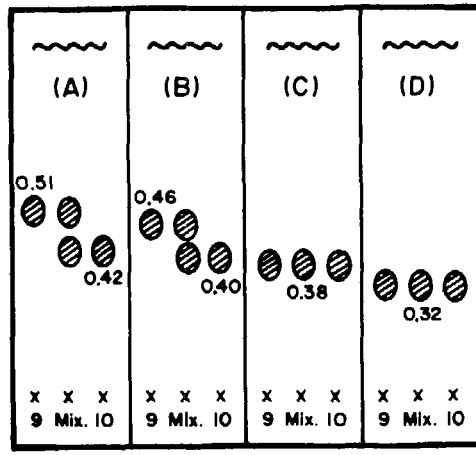


Figure 1. TLC of Steroidal Cyanoketone Stereoisomers (9) and (10)
Solvent : (A) benzene-i-propyl ether (1:1), (B) chloroform-acetone (9:1), (C) benzene-methanol (19:1), (D) chloroform-acetone (20:1) ; Plate : Merck silica gel 60 $F_{254}$ ; Detection : fluorescence quenching at 254 nm followed by $H_2SO_4$

## Relationship between Mobile Phase and Retention Times

A preliminary HPLC separation of 5:6-oxidocholesteryl 3-benzoates (1 and 2) was tried (Table 2). The chromatographic behaviors showed that the favorable TLC mobile phase ratios gave insufficient HPLC separation for the steroidal isomers. In order to obtain good HPLC separation of these stereoisomers, the $hR_f$ values had to be lowered by controlling the solvent strength in the mobile phases. Table 3 and Figure 2 show the results of favorable HPLC separation for the cholesteryl epoxides.

## HPLC Separations of Other Steroidal Isomers

Figures 3, 4, 5 and 6 show the HPLC separation of steroidal stereoisomers such as 3 and 4, 5 and 6, 9 and 10, and geometrical isomers (7 and 8). Table 4 summarize favorable TLC and HPLC conditions for these steroidal compounds.

## Chromatography of Cephalosporin Antibiotics (15)

To separate the cephalosporin antibiotics, 7-aminodesacetoxycephalosporanic acid (7-ADCA),

TABLE 2

HPLC of Cholesteryl Oxide Isomer[a)]

| Mobile phase[b)] | Flow rate (ml/min) | Pressure (psi) | $t_R$ (min) of stereoisomer α-Oxide | $t_o$ | β-Oxide | α | Result |
|---|---|---|---|---|---|---|---|
| n-$C_6H_{14}$-$CHCl_3$ (1:4) | 2.0 | 1100 | 1.5 | 1.2 | 1.5 | 1.00 | Inseparable |
| | 1.5 | 750 | 2.0 | 1.7 | 2.0 | 1.00 | " |
| | 1.0 | 550 | 3.2 | 2.7 | 3.2 | 1.00 | " |
| | 0.5 | 250 | 6.5 | 5.6 | 6.5 | 1.00 | " |
| n-$C_6H_{14}$-$CHCl_3$ (1:1) | 2.0 | 1000 | 2.0 | 1.6 | 1.8 | 2.00 | Separable |
| | 1.5 | 700 | 2.6 | 2.1 | 2.3 | 2.51 | " |
| | 1.0 | 480 | 3.7 | 3.1 | 3.6 | 1.20 | " |
| | 0.5 | 200 | 7.5 | 6.3 | 6.9 | 2.00 | " |

a) Stereoisomer: 5:6-Oxido-cholesteryl 3-benzoate
HPLC instruments: Waters 6000 A pump and U6K injector, Shimadzu SPD-2A variable wavelength UV monitor at 240 nm and 0.2 AUFS, Merck LiChrosorb SI 100 (10 μm, 4 mm x 30 cm), Shimadzu Chromatopak C-R1A computing recorder and integrator.

b) Not eluted with n-$C_6H_{14}$

TABLE 3

Favorable TLC $hR_f$ Value for HPLC Separation

| Compound | Mobile phase | TLC $hR_f$ [a] α | TLC $hR_f$ [a] β | TLC $hR_f$ [b] α | TLC $hR_f$ [b] β | HPLC $hR$ [c] α | HPLC $hR$ [c] β |
|---|---|---|---|---|---|---|---|
| 5:6-Oxido-cholesteryl 3-benzoate | $CHCl_3$-n-$C_6H_{14}$ (1:1) | 11 | 21 | 15 | 25 | 84 | 91 |
| 5:10-Oxido-19-nor-androstane-3α,17β-diol | $CHCl_3$-$CH_3OH$ (20:1) | 6 | 5 | 26 | 21 | 51 | 42 |
| Dexamethasone 21-Ac | " | 11 | | 11 | | 103 | |
| Betamethasone 21-Ac | | | 6 | | 8 | | 95 |
| 3β-Hydroxy-5-androsten-17-one 3-benzoate | Benzene-i-PrOH (4:1) | syn 22 | anti 11 | syn 27 | anti 18 | syn 55 | anti 43 |
| 5-Cyano-19-nor-testosterone 3,5-dinitrobenzoate | " | 19 | 16 | 28 | 24 | 39 | 34 |
| Cephalosporin stereoisomer [d] | $CHCl_3$-$CH_3OH$ (50:1) | A 6 | B 6 | A 18 | B 13 | A 118 | B 94 |

Merck TLC plate silica gel 60 $F_{254}$, a) solvent oversaturation, b) solvent normal saturation.

c) Merck LiChrosorb SI 100 (10 μm, 4 mm x 30 cm), $hR = 100 \times t_o/t_R$.

d) The chemical structures were not identified.

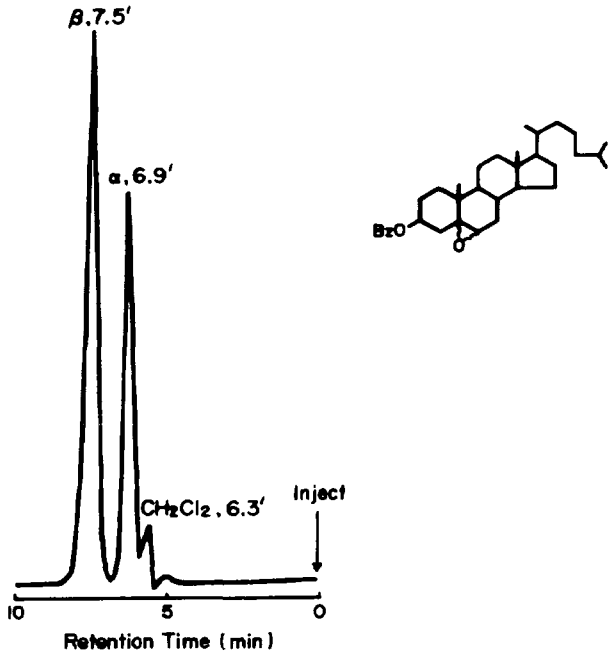


Figure 2. LC of cholesterol epoxides
Column, μPorasil (10μm, 4mm×30cm);
carrier, $CHCl_3$-n-hexane (1:1), isocratic;
flow rate, 2ml/min; pressure, 1100psi
detector, UV220 0.2 AUFS;
sample size, 10μg/μl in $CH_2Cl_2$
Temperature, ambient

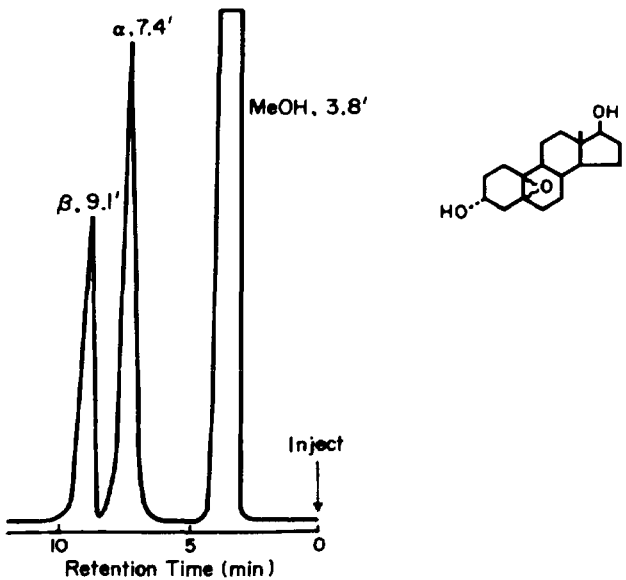


Figure 3. LC of steroidal 5,(10)-epoxides
Column, μPorasil (10μm, 4mm×30cm);
carrier, $CHCl_3$-MeOH (100:5);
flow rate, 1ml/min; pressure, 500 psi;
detector, RI 8X;
sample size, 100μg/10μl in MeOH

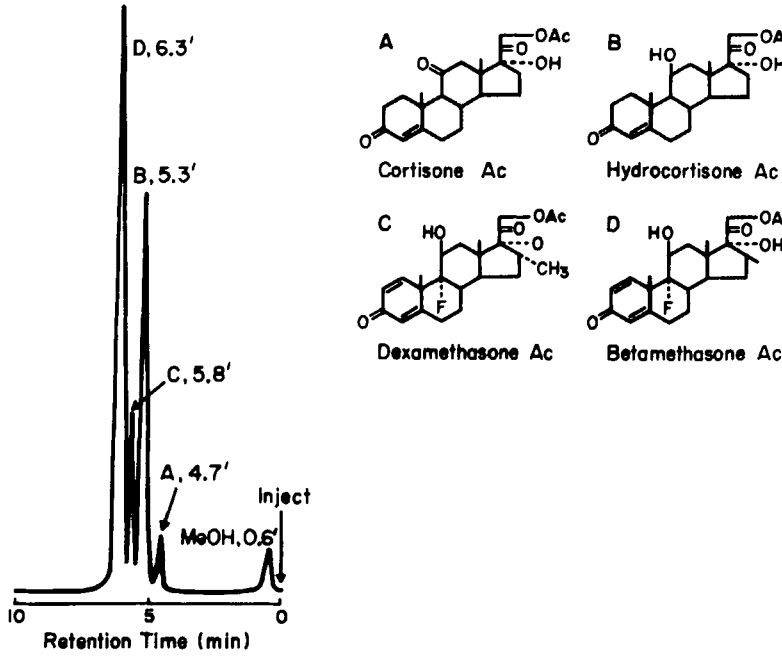


Figure 4. LC of steroidal hormones
Column, μPorasil (10μm, 4mm×30cm);
carrier, $CHCl_3$-MeOH (100:5);
flow rate; 0.6 ml/min; pressure, 500psi;
detector, UV240 0.2 AUFS;
sample size, A. 2μg B. 8μg C. 5μg
D. 10μg/μl in MeOH

cephalexin (CEX), 7-aminocephalosporanic acid (7-ACA), cephalothin (CET), cephaloglycine (CEG), desacetylcephalothin (DACET), ethoxycarbonyl DACET and cephaloridine (CER) were used.

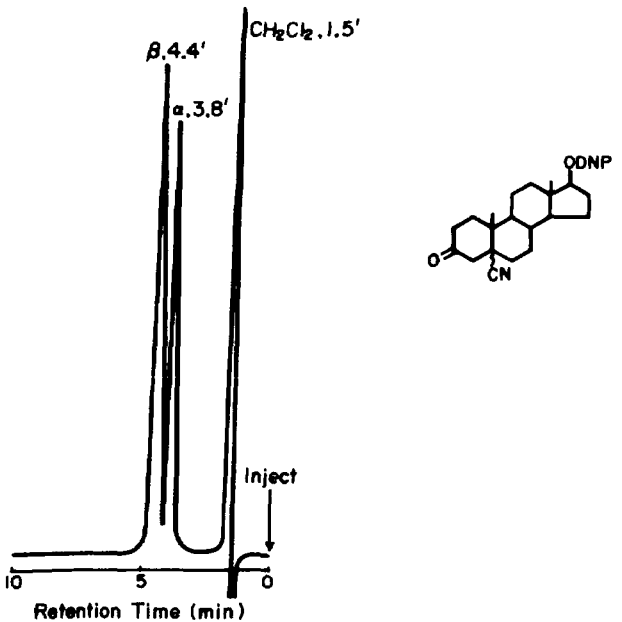


Figure 5. LC of steroidal 17-ketoximes
Column, μPorasil (10μm, 4mm×30cm);
carrier, benzene-IPE (4:1);
flow rate, 1ml/min; pressure, 600 psi;
detector, RI 8X;
sample size, 100μg/5μl in $CH_2Cl_2$

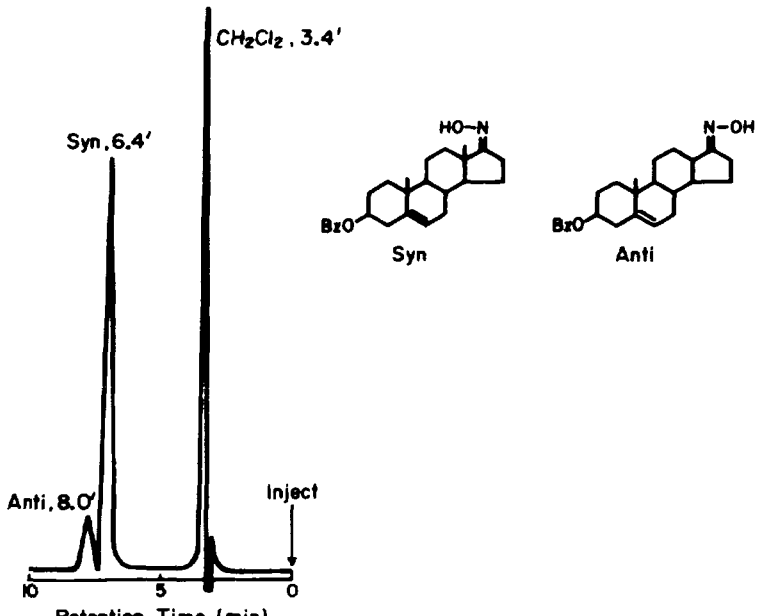


Figure 6. LC of steroidal cyanoketone
Column, μPorasil (10μm, 4mm×30cm);
carrier, benzene-IPE (4:1);
flow rate, 2ml/min; pressure, 1200 psi;
detector, RI 8X;
sample size, 100μg/10μl in $CH_2Cl_2$

TABLE 4

Favorable TLC and HPLC Condition for Steroid Compounds[a)]

| Steroid | Mobile phase | Solvent ratio HPLC | TLC |
|---|---|---|---|
| 5:6-Oxido-cholesteryl 3-benzoate | $CHCl_3$-n-$C_6H_{14}$ | (1:1) | (4:1) |
| 5:6-Oxido-19-nor-androstane-3α,17β-diol | $CHCl_3$-$CH_3OH$ | (20:1) | (10:1) |
| Dexamethasone 21-acetate<br>Betamethasone 21-acetate | $CHCl_3$-$CH_3OH$ | (20:1) | (10:1) |
| 3β-Hydroxy-5-androsten-17-one oxime 3-benzoate | Benzene-isopropyl ether | (4:1) | (2:1) |
| 5-Cyano-19-nor-testosterone 3,5-dinitrobenzoate | Benzene-isopropyl ether | (4:1) | (2:1) |
| Cephalosporin stereoisomer | $CHCl_3$-$CH_3OH$ | (50:1) | (20:1) |

a) TLC and HPLC condition as in TABLES 1-3.

Reversed-Phase TLC (RPTLC) of Cephalosporin Antibiotics

The author devised an eluotropic series for reversed-phase chromatography prior to RPTLC (Table 5). From this series, methanol-water, acetone-water, dioxane-water, acetonitrile-water and tetrahydrofuran-water were selected for RPTLC of the cephalosporins, 7-aminocephalosporanic acid, cephalothin,



TABLE 5

Eluotropic Series for Reversed-Phase Chromatography

| Solvent | Polarity index |
|---|---|
| Water | 9.0 |
| Methanol | 6.6 |
| Acetic acid | 6.2 |
| Ethanol | 5.2 |
| Acetonitrile | 6.2 |
| 2-Propanol | 4.3 |
| N,N-Dimethylformamide | 6.4 |
| Acetone | 5.4 |
| n-Propanol | 3.9 |
| Dioxane | 4.8 |

cephalexin, cephaloglycine and cephaloridine were carried out (Table 6). Comparison of the mean separation factor $\bar{\alpha}$ (larger $hR_f$ value/smaller $hR_f$ value) gave methanol-water (1:4) as the optimum condition. Among the mixing ratios of methanol-water of 1:1, 1:2 and 1:4, the last was the most effective (Table 7). Eight kinds of cephalosporin were separable using this mobile phase (Figure 7).



## Ion-Pair RPTLC of Cephalosporin Antibiotics

In order to decrease the $hR_f$ values of 7-aminocephalosporanic acid, cephalothin and cephaloglycine, ion-pair chromatography was applied to RPTLC of the cephalosporins (Tables 8 and 9). The ion-pair chromatographic (16) effect was recognized in the separation of cephaloglycine, cephalexin and cephaloridine [mobile phase; methanol-water (1:1) with PIC B-7], 7-aminocephalosporanic acid and cephalothin [methanol-water (1:4) with PIC B-7] (Table 10), and also in the separation of 7-aminocephalosporanic acid, cephaloglycine, cephalexin and cephalothin [methanol-water (1:2) with PIC A] (Table 11). Thus, ion-pair chromatography was found to be useful for cephalosporin compounds and amino acid.

TABLE 6

Solvent Effect in Reversed-Phase TLC of Cephalosporin Antibiotics[a)]

| Compound | MeOH-$H_2O$ (1:4) | | Dioxane-$H_2O$ (1:4) | | AN-$H_2O$ (1:4) | | $CH_3CN$-$H_2O$ (1:4) | | THF-$H_2O$ (1:4) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $hR_f$ | α | $hR_f$ | α | $hR_f$ | α | $hR_f$ | α | $hR_f$ | α |
| 7-Aminocephalosporanic acid | 93 | | 96 | | 94 | | 89 | | 92 | |
| | | 1.33 | | 1.37 | | 1.38 | | 1.31 | | 1.37 |
| Cephalothin | 70 | | 49 | | 54 | | 53 | | 49 | |
| | | 1.95 | | 1.21 | | 1.26 | | 1.19 | | 1.08 |
| Cephalexin | 61 | | 70 | | 68 | | 68 | | 67 | |
| | | 1.17 | | 1.18 | | 1.23 | | 1.08 | | 1.27 |
| Cephaloglycine | 52 | | 58 | | 42 | | 57 | | 62 | |
| | | 1.49 | | 1.23 | | 1.05 | | 1.26 | | 1.11 |
| Cephaloridine | 35 | | 40 | | 44 | | 42 | | 44 | |
| Mean separation factor $\bar{\alpha}$ | | 1.28 | > | 1.25 | > | 1.25 | > | 1.21 | > | 1.21 |
| Developing rate (min/10 cm) | 55 | | 50 | | 50 | | 45 | | 75 | |

a) TLC plate: Merck silica gel 60 $HF_{254}$ silanised
Detection: Fluorescence quenching followed by $I_2$ vapor
$hR_f = 100 \times R_f$, α = larger $hR_f$/smaller $hR_f$
AN: acetone, THF: tetrahydrofuran

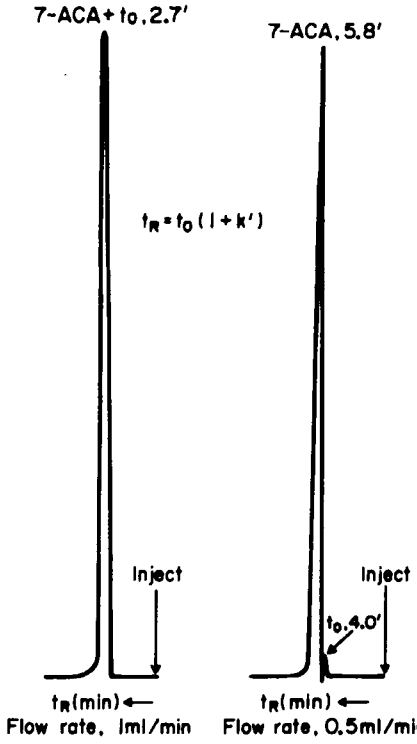


Figure 7. Simultaneous Reversed Phase TLC of Cephalosporins Solvent, MeOH-$H_2O$ (1:4); Plate, Silica gel 60 $HF_{254}$ silanised; Detection, $UV_{254}$-quenching followed by $I_2$ vapor.

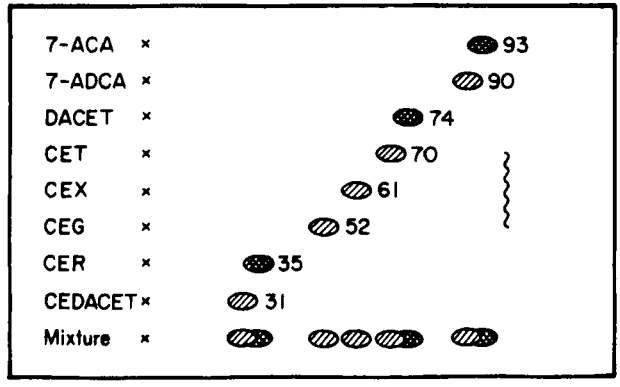


Figure 8. Influence of Flow rate on Separation of Cephalosporin Column, RP-2 (4x30cm); Mobile Phase, MeOH-$H_2O$ (1:4)

TABLE 7

Reversed-Phase TLC of Cephalosporin Antibiotics[a)]

| Compound | $hR_f$ values of cephalosporins | | | |
|---|---|---|---|---|
| | MW11 | MW12 | MW14 | $H_2O$[b)] |
| 7-Aminocephalosporanic acid | 93 | 92 | 93 | 55 |
| Cephaloglycine | 81 | 64 | 52 | 15 |
| Cephalexin | 81 | 71 | 61 | 34 |
| Cephaloridine | 68 | 51 | 35 | 6 |
| Cephalothin | 83 | 76 | 70 | 31 |
| Developing rate (min/10 cm) | 40 | 35 | 53 | 65/7 cm |

a) TLC plate: Merck silica gel $HF_{254}$ silanised (laboratory-prepared)
Detection: Fluorescence quenching, $I_2$ vapor, Fluorescamine and Ninhydrin reagents
MW = MeOH-$H_2O$

b) Abnormal development due to the lipophilic adsorbent



## Reversed-Phase HPLC (RPHPLC) of Cephalosporin Antibiotics

Four kinds of mobile phases used in RPTLC separation, methanol-water, tetrahydrofuran-water, acetonitrile-water and dioxane-water, were applied

TABLE 8

Ion-Pair Reversed-Phase TLC[a] of Cephalosporin Antibiotics[a]

| Compound | $hR_f$ values of cephalosporins[b] | | | |
|---|---|---|---|---|
| | MW11B | MW12B | MW14B | $H_2O$B |
| 7-Aminocephalosporanic acid | 89 | 85 | 81 | 42 |
| Cephaloglycine | 71 | 56 | 49 | 17 |
| Cephalexin | 70 | 65 | 55 | 23 |
| Cephaloridine | 58 | 48 | 37 | 15 |
| Cephalothin | 75 | 57 | 46 | 18 |
| Developing rate (min/10 cm) | 35 | 35 | 35 | 90 |

a) To each mobile phase, 0.005 M 1-heptanesulfonic acid-AcOH (pH 3.5, B) was added.

b) TLC plate and detection as in TABLE 7.



to RPHPLC of cephalosporin antibiotics (Table 12). Among these mobile phases, metanol-water (1:4) was the most effective, as for RPTLC separation (Figure 9). The 1:1 and 1:2 ratios of methanol-water gave three peaks [7-aminocephalosporanic acid and cephalothin, cephalexin and cephaloglycine, and cephaloridine]. The 1:4 ratio gave four peaks [7-

TABLE 9

Ion-Pair Reversed-Phase TLC of Cephalosporin Antibiotics[a)]

| Compound | $hR_f$ values of cephalosporins[b)] | | | |
|---|---|---|---|---|
| | MW11A | MW12A | MW14A | $H_2$OA |
| 7-Aminocephalosporanic acid | 81 | 80 | 77 | 75 |
| Cephaloglycine | 78 | 50 | 43 | 17 |
| Cephalexin | 79 | 61 | 64 | 29 |
| Cephaloridine | 60 | 46 | 32 | 10 |
| Cephalothin | 78 | 59 | 49 | 22 |
| Developing rate (min/10 cm) | 40 | 50 | 40 | 60 |

a) To each mobile phase, 0.005 M tetrabutylammonium phosphate (pH 7.5, A) was added.

b) TLC plate and detection as in TABLE 7.

TABLE 10

Ion-Pair TLC Effect on Cephalosporin Antibiotics

| Compound | $\Delta hR_f$ values of cephalosporins[a)] | | | |
|---|---|---|---|---|
| | MW11B | MW12B | MW14B | $H_2$OB |
| 7-Aminocephalosporanic acid | 4 | 7 | 12 | 13 |
| Cephaloglycine | 10 | 8 | 3 | -2 |
| Cephalexin | 11 | 6 | 6 | 11 |
| Cephaloridine | 10 | 3 | -2 | -9 |
| Cephalothin | 8 | 21 | 24 | 13 |

a) $\Delta hR_f = hR_f$ in TABLE 8 - $hR_f$ in TABLE 7.

TABLE 11

Ion-Pair TLC Effect on Cephalosporin Antibiotics

| Compound | $\Delta hR_f$ values of cephalosporins[a)] | | | |
|---|---|---|---|---|
| | MW11A | MW12A | MW14A | $H_2OA$ |
| 7-Aminocephalosporanic acid | 12 | 12 | 16 | -20 |
| Cephaloglycine | 3 | 14 | 8 | -2 |
| Cephalexin | 2 | 10 | -3 | 5 |
| Cephaloridine | 8 | 5 | 3 | -4 |
| Cephalothin | 5 | 17 | 21 | 9 |

a) $\Delta hR_f = hR_f$ in TABLE 9 - $hR_f$ in TABLE 7.



aminocephalosporanic acid and cephalothin, cephalexin, cephaloglycine, and cephaloridine (Table 9).

Influence of Flow Rate

Three flow rates, 1 ml/min, 0.5 ml/min and 0.3 ml/min were used for the HPLC separation [stationary phase, Merck RP-2 home-packed column; mobile phase, methanol-water (1:4)] (Figure 8). A flow rate of 0.5 ml/min resulted in separation of dead volume ($t_o$) and 7-aminocephalosporanic acid.

TABLE 12

Solvent Effect in Reversed-Phase HPLC of Cephalosporin Antibiotics[a)]

| Compound | MeOH-$H_2O$(1:4) | | | THF-$H_2O$(1:4) | | | $CH_3CN$-$H_2O$(1:4) | | | Dioxane-$H_2O$(1:4) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $t_R$ | k' | α | $t_R$ | k' | α | $t_R$ | k' | α | $t_R$ | k' | α |
| 7-Aminocephalosporanic acid | 5.8 | 0.07 | | 5.2 | 0.04 | | 4.6 | -0.04 | | 5.6 | 0.14 | |
| | | | 1.45 | | | 3.00 | | | | | | 1.57 |
| Cephalothin | 6.0 | 0.10 | | 5.6 | 0.12 | | 4.8 | 0.00 | | 6.0 | 0.23 | |
| | | | 6.67 | | | 2.17 | | | | | | 1.72 |
| Cephaloglycine | 9.0 | 0.67 | | 6.3 | 0.26 | | 6.0 | 0.25 | | 6.8 | 0.39 | |
| | | | 1.30 | | | 1.69 | | | 1.17 | | | 1.21 |
| Cephalexin | 8.2 | 0.52 | | 7.2 | 0.44 | | 6.2 | 0.29 | | 7.2 | 0.47 | |
| | | | 3.07 | | | 1.55 | | | 3.35 | | | 1.79 |
| Cephaloridine | 14.0 | 1.60 | | 8.4 | 0.63 | | 9.5 | 0.98 | | 9.0 | 0.84 | |
| $t_o$ | 5.4 | | | 5.0 | | | 4.8 | | | 4.9 | | |
| Column pressure (psi) | 1600 | | | 2500 | | | 1600 | | | 2100 | | |
| Separation | 4 peaks | | > | 4 peaks | | > | 3 peaks | | > | 3 peaks | | |

a) HPLC instruments: Waters ALC/GPC 204, Shimadzu SPD-2A variable wavelength UV monitor at 265 nm and 0.1 AUFS
Column: Merck RP-2 home-packed by balanced density slurry method (4 mm x 30 cm)
Chart spped: 0.5 cm/min
Sample size: 0.25 μg/μl in $NaHCO_3$-HCl buffer (pH 6.6)

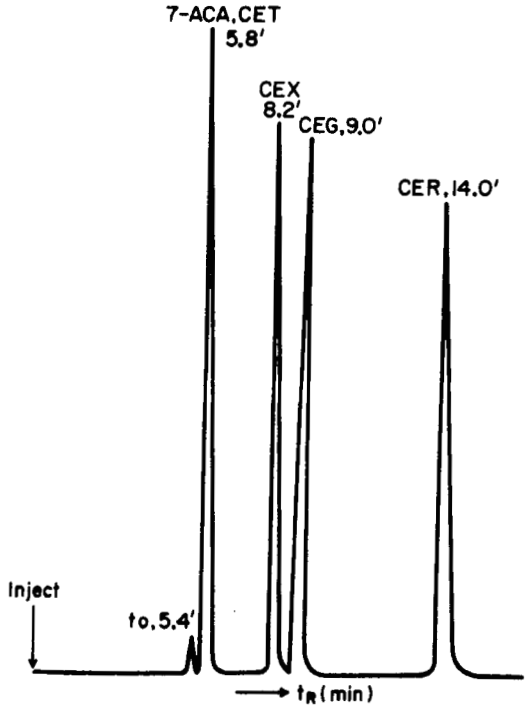


Figure 9. LC of Cephalosporins
Column, RP-2(4×30cm): Mobile Phase, MeOH-$H_2O$(1:4): Flow Rate, 0.5ml/min: Column Pressure, 1600psi.



Ion-Pair RPHPLC of Cephalosporin Antibiotics

7-Aminocephalosporanic acid and cephalothin could not be separated without addition of PIC reagent (Table 12). By using methanol-water (1:2) with PIC B-7 and A as for the RPTLC separation, five cephalosporins, 7-aminocephalosporanic acid, cephaloglycine, cephaloridine, cephalothin and cephalexin, could be separated from each other (Figures 10, 11 and 12).

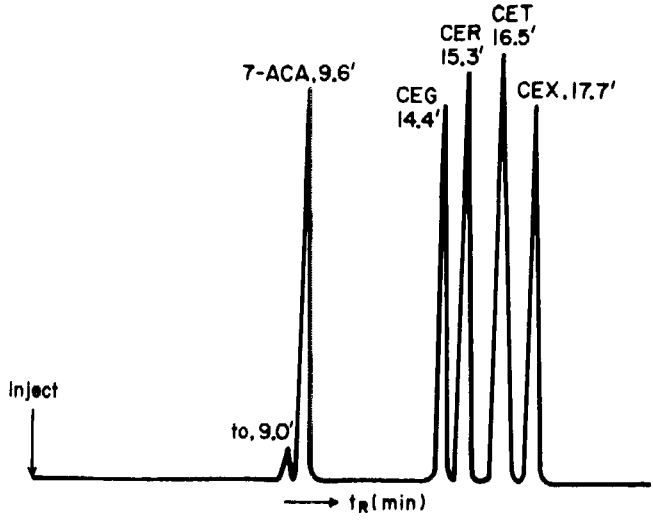


Figure 10. LC of Cephalosporins
Column, RP-2(4x30cm); Mobile Phase, MeOH-$H_2O$(1:2) with PIC B-7; Flow Rate, 0.3ml/min; Column Pressure, 1000 psi.



## Correlation of Mobilities of Cephalosporin Antibiotics in RPTLC and RPHPLC

Table 13 shows compares the separation behavior on RPTLC and RPHPLC described above. After correlation analysis between TLC $hR_f$ values and HPLC R ($R = t_o/t_R$), a correlation between the two separations was obtained, and the possibility of direct transfer of chromatographic systems from TLC to HPLC for separation of these antibiotics was confirmed.

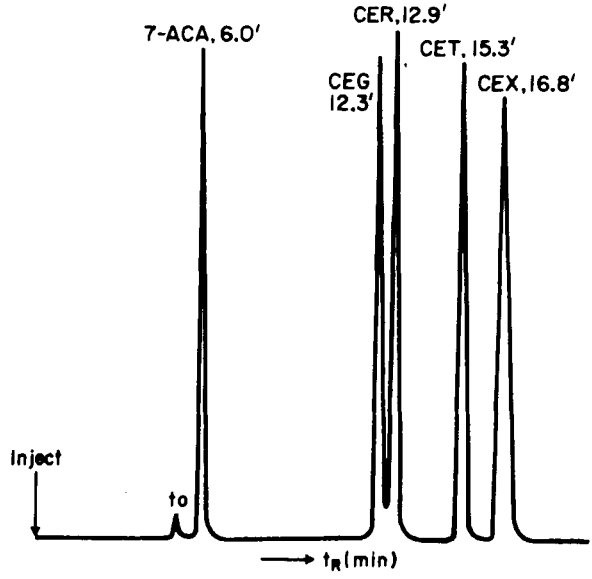


Figure 11. LC of Cephalosporins
Column, RP-2 (4x30cm); Mobile Phase, MeOH-$H_2O$ (1:4) with PIC B-7; Flow Rate, 0.5ml/min; Column Pressure, 1600 psi.



## CONCLUSION

### Adsorption Chromatography of Steroidal Hormones

HPLC separation was investigated by adsorption chromatography of hardly separable stereoisomers such as 5:6-oxido-cholesteryl benzoates, 5:10-oxido-19-nor-androstane-3α,17β-diols and their 3,5-dinitrobenzoates, dexamethasone 21-acetate and betamethasone 21-acetate, and 5-cyano-19-nor-testosterones and their 3,5-dinitrobenzoates, and syn-,

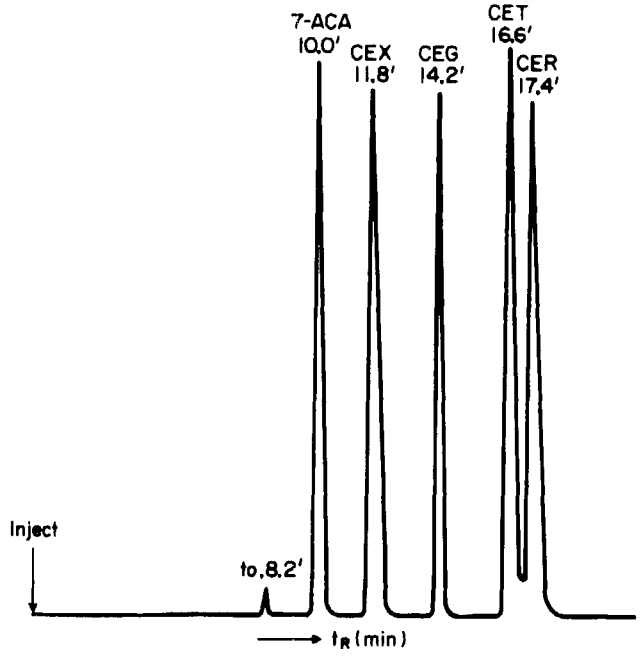


Figure 12. LC of Cephalosporins
Column, RP-2 (4×30cm); Mobile Phase, MeOH-$H_2O$ (1:2) with PIC A; Flow Rate, 0.3ml/min; Column Pressure, 1200 psi.



anti-geometrical isomers such as $\Delta^5$-3β-hydroxyandrostane-17-one oxime benzoates. Prior to HPLC with silica gel columns, silica gel TLC was prformed to obtain suitable HPLC systems for separating of these compounds. Seven solvent systems for TLC did not give satisfactory separation in HPLC when used in the same ratios. As retention times ($t_R$) and capacity factors (k) of these isomers were small, the separation factors (α) were insufficient for

TABLE 13

Relationship of Mobility between Reversed-Phase TLC and Reversed-Phase HPLC with Cephalosporin Antibiotics

| Compound | MeOH-$H_2O$ (1:4) TLC, $R_f$ [a] | HPLC, R [b] |
|---|---|---|
| 7-Aminocephalosporanic acid | 0.93 | 0.90 |
| 7-Aminodesacetoxycephalosporanic acid | 0.90 | 0.94 |
| Cephalothin | 0.70 | 0.90 |
| Desacetylcephalothin | 0.74 | 0.94 |
| Cephalexin | 0.61 | 0.63 |
| Cephaloglycine | 0.52 | 0.58 |
| Cephaloridine | 0.35 | 0.35 |

a) Merck silica gel 60 $F_{254}$ silanised (laboratory-prepared), Developing rate; 2 mm/min

b) Column: Merck RP-2 (10 μm, 4 mm x 30 cm, home-packed)

Flow rate: 0.3 ml/min

Linear Velocity: 2.38 mm/min

$R = t_o/t_R$

Regression analysis between TLC $R_f$ and HPLC R:

$R = 1.0275R_f + 0.05$, $\gamma = 0.915^{**}$, $n = 7$

good separation. For the sharp separation in HPLC, it was necessary to decrease the solvent strength of the same binary solvent mixture used for TLC had to be decreased, for instance from a 4:1 to a 1:1 ratio

in chloroform-n-hexane systems to separate 5:6-oxido-cholesteryl benzoates. The difference in solvent strength for efficient separation between TLC and HPLC might be attributed to the fact that in HPLC the solvent elution power acts in an isocratic manner while in TLC it acts in a gradient manner.

## Reversed-Phase Chromatography of Cephalosporin Antibiotics

HPLC separation was investigated by reversed-phase partition chromatography of eight kinds of cephalosporin compounds, 7-ACA, 7-ADCA, CEX, CET, CEG, CER, DACET and ECDACET. Prior to RPHPLC with dimethylsilanised silica gel columns packed by balanced density slurry method RPTLC with the same stationary phase was performed to obtain the suitable HPLC conditions for separating these compounds. Five solvent systems and five kinds of HPLC packings were tested: methanol-water, acetonitrile-water, acetone-water, dioxane-water and tetrahydrofuran-water; and aminopropyl-, cyanopropyl-, dimethyl-, octyl- and octadecyl-silanised silica gels. Among these, the combination of methanol-water and a di-

methylsilanised silica gel column gave satisfactory HPLC separation when the solvent ratio was 1:4.

The ion-pair chromatographic method was effective for sharp separation of CET from 7-ACA in HPLC. Correlation of mobility between TLC ($hR_f$) and HPLC (R) was obtained [$R = 1.05\ R_f$ (n = 7), correlation analysis: y = 1.0275x + 0.0513, $\gamma = 0.9153^{**}$, n = 7], and the possibility of direct transfer of chromatographic systems from TLC to HPLC to separate cephalosporin antibiotics was confirmed.



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