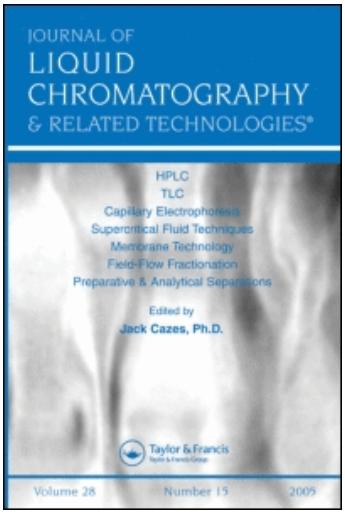

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A Direct Fractionation Procedure for the Isolation of Synthetic Products Utilizing Normal and Reversed Phase Liquid Chromatography

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A DIRECT FRACTIONATION PROCEDURE FOR THE ISOLATION OF SYNTHETIC PRODUCTS UTILIZING NORMAL AND REVERSED PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, one-step fractionation scheme was developed by utilizing liquid chromatography in order to provide new and improved isolation techniques for synthetic reactions, allowing elimination of tedious, multi-step processes presently in use. Thin-layer chromatography (TLC) data, used for monitoring and optimizing synthetic reactions, were directly extrapolated to high performance liquid chromatography (HPLC) systems. Inorganic reagents and highly polar side products were removed by a silica pre-column. A reversed phase column was used for clean-up process. The desired products were rapidly fractionated from the crude reaction mixture. General applicability of this procedure was demonstrated in the syntheses of various steroid derivatives.

INTRODUCTION

For optimization of reaction conditions in organic synthesis, monitoring of the reaction course by chromatographic techniques has been widely utilized. The chromatogram provides quick separation and semi-quantitative evaluation of reaction products. However, actual isolation is not carried out until much later, requiring a series of time con-

suming operations. A procedure for linearly extending the technique of the "development" chromatogram to the silica "elution" column has been attempted[1, 2]. Single-step isolation of synthetic products from the crude reaction mixtures via direct extrapolation of chromatographic parameters which were obtained by using thin-layer chromatography (TLC) as a pilot into high performance liquid chromatography (HPLC) has been elaborated[2].

In order to generalize this procedure, extensive investigation of more intricate systems is required. Inorganic reagents and their complexes are commonly associated with organic synthesis. The products are isolated via decomposition of the complex and several extraction steps. As inorganic reagents are usually retained hard and observed close to the starting point on TL chromatogram, a simple clean-up can be afforded by using a silica pre-column. Alternately, if a reversed phase column is employed, inorganic salts are usually passed through as non-retained components. Nevertheless, no general procedures have been postulated for the separation of crude synthetic reaction mixtures.

This paper presents a direct fractionation method for isolating products, including clean up of crude reaction mixtures and successive separation processes, utilizing normal and reversed phase columns. The steroid compounds, cholestane, androstane and estrane derivatives were adopted as substrates of typical organic reactions. Synthetic procedures were mainly taken from Organic Syntheses. Substrates and reactions examined in this report are listed in Table 1.

MATERIALS AND METHODS

Packing materials were irregularly shaped silica, with a pore size of 70 A, mean particle size of 10μ for semi-micro separation, 50μ for preparative use, 100 mesh for pre-column connected with preparative column and irregularly shaped ODS-silica, with a mean particle size of 10μ (Wakogel LC-10H, 50H, C-100 and LC-10ODS, respectively, Wako Pure Chemicals, Osaka).

Columns (CIG column system[2]) were 20 cm x 4 mm I.D. for analytical HPLC, 30 cm x 8 mm I.D. for semi-micro preparation with pre-column of 10 cm x 8 mm I.D., 15 cm x 15 mm I.D. for reversed phase clean-up and 40 cm x 15 mm I.D. for preparative purpose.

TLC was carried out by using micro plates[3].

HPLC was performed using an RI detector (RI-401, Waters Assoc., Milford, Mass.), UV detector operated at 254 nm and reciprocating piston pump (Kusano Scientific, Tokyo) according to the procedure described in an earlier paper[2].

Examples of Applications

The following reactions were run as examples of commonly performed organic synthesis. After each reaction, the crude reaction mixture was injected into the HPLC column and eluted, followed by evaporation of the solvent to give the purified products.

1) Oppenauer oxidation of cholesterol was carried out according to the procedure described in Organic Syntheses[6].

2) Anhydrous pyridine (4.92 g) in 78 ml of dichloromethane was cooled to approximately 15° C and anhydrous chromium

TABLE 1.
Silica-HPLC Data on Synthetic Reaction Products

Solvent system[*1]	Capacity factor of substrate and product	Usual isolation procedure[*2]	Reference
Remark (retention data on associated materials for isolation)			

1) Oppenauer oxidation

Cholesterol $\xrightarrow{(CH_2)_5CO,\ (2\text{-}PrO)_3Al}$ 4-Cholesten-3-one

O + B (4:1) 3.91, 1.45 (4, 8, 6, 5, 3, 7, 6, 9) Org. Syn.[6]

2-Cyclohex-1'-enylcyclohexanone[*3]: k'=0.16 (O+B, 4:1)
2-1'-Hydroxycyclohexylcyclohexanone[*3]: k'=0.55 (O+B, 4:1)

2) Collins oxidation

5α-Cholestan-3β-ol $\xrightarrow{CrO_3(C_5H_5N)_2}$ 5α-Cholestan-3-one

O + B (4:1) 3.47, 0.34 (2, 5, 8, 3, 7, 6, 9)

C_5H_5N: k'=2.35 (O+B, 4:1) tailing

3) Chromate oxidation

Cholesterol $\xrightarrow{Na_2Cr_2O_7,\ HOAc}$ 4-Cholestene-3, 6-dione

O + B (4:1) 3.90, 1.73 (5, 3, 4, 3, 4, 8, 6, 9) Org. Syn.[7]

HOAc: k'=2.24 (O+B, 4:1) tailing

4) Jones' oxidation

5α-Androstan-17β-ol $\xrightarrow{CrO_3,\ H_2SO_4}$ 5α-Androstan-17-one

O + B (9:1) 4.46, 0.71 (6, 5, 4, 3, 8, 6, 9)

5) Hydride reduction a

5α-Cholestan-3-one $\xrightarrow{NaBH_4,\ 2\text{-}PrOH}$ 5α-Cholestan-3α and 3β-ol

O + B (3:2) 0.12, 3α: 0.64 (6, 5, 4, 3, 8, 6, 9)
3β: 1.25

6) Hydride reduction b

5β-Cholestan-3-one $\xrightarrow[NaBH_4,\ 2\text{-PrOH}]{}$ 5β-Cholestan-3α and 3β-ol

O + B (17:3) 0.18, 3α: 3.45 (6, 5, 4, 3, 8, 6, 9)
3β: 3.25

7) Hydride reduction c

Estrone $\xrightarrow[NaBH_4,\ MeOH,\ H_2O]{}$ Estradiol

O + B (4:1) 4.38, 5.53 (6, 5, 4, 3, 8, 6, 9)

8) Grignard reaction

5α-Androstan-17-one $\xrightarrow[MeMgI,\ Et_2O,\ H_2SO_4]{}$ 17α-Methyl-5α-androstan-17β-ol

O + B (9:1) 0.71, 3.32 (4, 3, 8, 6, 9)

9) Hydrolysis

Testosterone propionate $\xrightarrow[NaOH,\ H_2O,\ MeOH]{}$ Testosterone

O + B (3:2) 0.92, 4.71 (6, 5, 4, 3, 8, 6, 9)

10) Oxime formation

4-Cholesten-3-one $\xrightarrow[NH_2OH\text{-}HCl,\ NaOAc]{}$ syn and anti-3-Oximino-4-cholestene

O + B (4:1) 1.44, syn: 3.36 (6, 5, 4, 3, 8, 6, 9)
anti: 1.97

11) Reduction

17β-Hydroxy-5α-androstan-3-one $\xrightarrow[TsNHNH_2,\ NaBH_4,\ MeOH]{}$ 5α-Androstan-17β-ol

O + B (4:1) 9.76, 1.38 (6, 5, 4, 3, 8, 6, 9) Org. Syn.[8]

12) Epoxidation

Cholesteryl acetate $\xrightarrow[H_2O_2,\ HCO_2H]{}$ 3β-Acetoxy-5α-formyloxy-cholestan-6β-ol

O + B (4:1) 0.14, 2.22 (1, 5, 4, 3, 8, 6, 9)

HCO_2H: k'=3.07 (O+B, 4:1)

(VI) oxide (3.10 g) was added over a 30-minute period. 5α-Cholestan-3β-ol (2.0 g) in 15 ml of dichloromethane was added at room temperature in one portion and stirred for 15 minutes.

3) Chromate oxidation of cholesterol was carried out according to the procedure described in Organic Syntheses[7].

4) 5α-Androstan-17β-ol (500 mg) was dissolved in 10 ml of acetone and 1 ml of Jones' reagent was added at room temperature.

5) 5α-Cholestan-3-one (1.60 g) was dissolved in 462 ml of 2-propanol. Sodium borohydride (2.52 g) dissolved in 625 ml of 2-propanol was added. The mixture was warmed at 35° C for 15 minutes.

6) 5β-Cholestan-3-one (692 mg) was dissolved in 100 ml of 2-propanol. Sodium borohydride (1088 mg) in 120 ml of 2-propanol was added. The mixture was warmed at 35° C for 15 minutes.

7) Estrone (100 mg) was dissolved in 8 ml of methanol. Sodium borohydride (170 mg) in 2 ml of water was added. The mixture was warmed at 35° C for 15 minutes.

8) In a flask were placed 9 mg of magnesium ribbon and 5 ml of anhydrous ether. A solution of 52 mg of methyl iodide in 2 ml of ether was added and refluxed for 30 minutes. A solution of 100 mg of 5α-androstan-17-one in 5 ml of ether was added and refluxed for 1 hour.

9) Testosterone propionate (100 mg) and sodium hydroxide (22 mg) were dissolved in 2.0 ml of methanol and 0.5 ml of water. The mixture was refluxed for 1 hour.

10) 4-Cholesten-3-one (100 mg), hydroxylamine hydrochloride (36.1 mg) and sodium acetate (42.6 mg) were dissolved in 10

ml of methanol. The mixture was refluxed for 1 hour.

11) Reduction of 17-oxo-androstane derivative was carried out according to the procedure described in Organic Syntheses[8].

12) Cholesteryl acetate (74.3 mg) in 0.8 ml of 30 % hydrogen peroxide and 3.2 ml of formic acid was heated at 40 - 60° C for 10 hours.

RESULTS AND DISCUSSION

1. TLC Pre-test for the Clean-up Process and Optimization of the Solvent Strength

A given reaction mixture was directly applied to a TLC plate and developed by binary solvent systems which were prepared on the basis of the correlation between molecular structures of steroids being separated and their retention behaviors[2, 4]. It was found that the inorganic reagents which were included in the reaction mixtures described in this report were always retained near the origin of the TL chromatogram. Development of organic products was accompanied by the decomposition of complexes in the reaction mixture. The solvent strength of the eluent was controlled so that the spot of the object compound remained in an optimum retention range (Rf $\fallingdotseq$ 0.3) according to the correlation between TLC and HPLC mobilities[2, 4, 5].

2. Semi-micro Separation of the Products Utilizing HPLC Provided with Silica Pre-column

Some components in the crude reaction mixture such as the volatile materials, pyridine, acetic, formic acids, etc. in reactions 2, 3 and 12 are not detectable on TLC. The analytical HPLC pre-run of these components facilitates the se-

lection of a solvent system for preparative chromatography. The retention data of these compounds have been added to the remarks in Table 1.

The crude reaction mixture containing approximately 10 mg of the final product was directly injected into the top of the silica pre-column which was connected to the main column. Polar substances were removed by the pre-column, and the desired products were collected by isocratic elution. This procedure was successfully applied for the 12 synthetic reactions shown in Table 1. The capacity factors of the reaction products along with retention data on relevant substances are also listed in this table. The quantity and quality of the isolated materials were sufficient for IR, NMR and MS spectra to be taken. Products isolated by HPLC were identified with standard samples by spectral methods. A defect of this procedure is that the pre-column packing material usually deteriorates.

3. Clean-up by Using Reversed Phase Column

When a crude reaction mixture containing protic solvents such as alcohols or water are injected directly into the silica column in the clean-up process, the activity of the pre-column is lowered and the passing of a large amount of solution becomes extremely difficult. Reversed phase column and a hydrophilic mobile phase are preferred for the sample injection into the eluent stream and the frontal removal of the inorganic salts and protic solvents in the crude reaction mixture. As the retention data for reversed phase systems with regard to the reagents and the solvents which are encountered in organic synthesis are not known, the chromatographic behavior of these materials in the chemically bonded reversed phase col-

umn - methanol/water system were examined beforehand. Capacity factors of the main components in application examples 7, 9, 10 are listed in Table 2.

Even in a uniform liquid phase synthetic reaction, some products are often precipitated from the solution, and the solution is sometimes divided into two phases in the midst of the reaction progress. If a two phase sample solution is acceptable for injection into the reversed phase column with no loss of its function, a crude reaction mixture with precipitates or two layers can be directly applied to HPLC separation.

Precipitated materials in the product solution of example 10, for instance, were dissolved by addition of water and chloroform. The two layer solution was injected into the column. The peak of the target compound appeared normally as in a standard pure sample. Application of two phase sample solutions to the reversed phase column - methanol/

TABLE 2.
ODS-HPLC Data of the Synthetic Products

Reaction No.	Solvent system	Capacity factor of the product	Starting material, mg	Yield mg	%
Remark (retention data on associated materials for isolation)					
7)	MeOH:H_2O (v/v) 1 : 1	2.50	100.0	78.0	77
Decomposed products from $NaBH_4$: k'=0.25 (MeOH:H_2O, 1:1 v/v)					
9)	MeOH:H_2O (v/v) 3 : 1	2.43	40.9	33.0	93
MeOH, H_2O, NaOH: k'=0.00 - 0.36 (MeOH:H_2O, 3:1 v/v)					
10)	MeOH:H_2O (v/v) 20 : 1	4.46	100.0	73.0	70
NH_2OH-HCl: k'=0.00; NaOAc: k'=0.16 (MeOH:H_2O, 20:1 v/v)					

water system can be widely utilized as a technique for the separation of multi-component samples with no pre-treatment.

4. Preparative Separation of Crude Reaction Mixtures

Some of the experiments described above were then scaled up to a few grams of starting materials. A large amount of sample needs a large pre-column. As the silica pre-column for this experiment is irreversibly deteriorated, an economical silica gel having a larger particle size was used as packing material. When the crude reaction mixture forms a suspension or precipitates, glass powder is added to the pre-column packing as a filter aid, and an open column was preferably used for pre-chromatography. A large volume of sample involved a few extra steps, consisting of pre-chromatography, followed by evaporation of the eluent, and subsequent re-injection into the separation column. For preparative purposes, an excess of sample amount far over the loading capacity can be applied because the compounds in a synthetic reaction mixture usually show marked retention behavior relative to each other and peak-shape deformation caused by sample over-loading, and slight change of capacity factors are tolerable. A few application examples are listed in Table 3.

If elution conditions are properly optimized, direct fractionation of the products in a crude reaction mixture using normal and reversed phase columns as described above can generally be used as a simple isolation technique in place of the standard method. For comparing these methods, currently used multi-step procedures are listed in Table 1.

It should be noticed that this procedure allows the isolation of various side products which are not isolable by the

TABLE 3.
Preparative HPLC of the Synthetic Products

Reaction No.	Starting material, g	(Quantity injected of each, g)	Yield g	%
Remark (pre-column and eluent*1)				
1)	5.00	(1.0)	4.00	81
i Open column: 3.5 cm×11.5 cm I.D. with silica 100 mesh. Eluent: O+B (13:7) 300 ml. ii Pre-column: 10 cm×15 mm I.D. with silica 100 mesh and glass powder (2:1 w/w). Eluent: O+B (13:7) 80 ml.				
2)	2.00	(2.0)	1.62	81
Pre-column: 10 cm×10 mm I.D. with silica 100 mesh. Eluent: O+B (4:1) 300 ml.				
5)	1.60	(0.4)	3β: 1.45 3α: 0.12	98
Open column: 25 cm×24 mm I.D. with silica 100 mesh. Eluent: 2-propanol 300 ml. Concentrated to 100 ml. Repeated injection into the same column. Eluent: O+B (3:2) 350 ml.				

standard methods. The procedures even allowed a pair of diastereomers in examples 5 and 6 and geometric isomers in example 10 having close characteristics to be quantitatively fractionated.

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*1 Abbreviations of the solvents systematically classified[2]: O = n-hexane, B = ethyl acetate. Solvent ratio: w/w.

*2 (1) Addition of water (2) Filtration (3) Washing with water (4) Washing with acidic and basic aqueous solutions (5) Extraction by organic solvent (6) Evaporation-condensation (7) Drying (8) Funnel separation (9) Recrystallization.

*3 Products derived from cyclohexanone.