Technical Note

Application of Monolithic Silica Column for HPLC Separation of Estrogens

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Abstract

A monolithic silica column was used for the HPLC separation of estrogens, and its chromatographic behavior was compared to that of three particle−packed columns (conventional columns). Generally speaking, the monolithic silica column provided a satisfactory separation with a smaller retention factor than those obtained from the conventional columns. The application of the monolithic silica column for separation of the formed products during the *in vitro* and photocatalytic degradation experiments using estrogen as a substrate was described.

Keywords: Monolithic Silica Column/ HPLC/ Fast Separation/ Estrogen/ In Vitro Experiment/ Photocatalytic Degradation Experiment

1. Introduction

A monolithic silica column has recently been widely used for HPLC separation analysis. Compared with the particle−packed column (conventional column), the monolithic silica column eluted the analyte with a smaller retention factor (k), lower pressure and satisfactory separation [1]. However, the monolithic silica column has not been widely used for the HPLC or LC/MS analysis of steroids. These results prompted us to compare a monolithic silica column, Chromolith Performance RP−18e, with the representative conventional columns, J'sphere ODS−H80, −M80, −L80, regarding their chromatographic behavior during the separation of estrogens as model steroids (Fig. 1). The application of the monolithic silica column for the separation of products formed *in vitro* and the photocatalytic degradation experiments using estrogen as a substrate is also described.

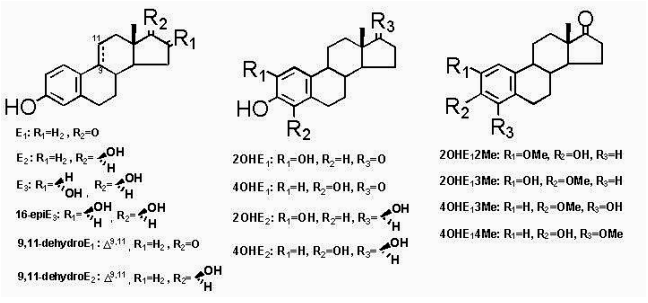

Figure 1. Structures of estrogens

2. Experimental

2.1 Instruments and Materials

The HPLC was performed using a JASCO PU−980 chromatograph (JASCO, Tokyo, Japan) equipped with a Shimadzu SPD−10 A UV−Visible detector (Shimadzu, Kyoto, Japan). A Chromolith Performance RP−18e (100 × 4.6 mm i.d.; Merck, Darmstadt, Germany) and J'sphere ODS−H80, −M80 or −L80 (each 4 μm, 150 × 4.6 mm i.d.; YMC, Kyoto) columns were used at a flow rate of 1.0 ml/min and 40 ℃. $NaNO_3$ was used for measurement of the dead time (t_0) using 220 nm. 9,11−Dehydroestrone (9,11−dehydroE$_1$) and 9,11−dehydroestradiol (9,11−dehydroE$_2$) were synthesized and provided by Dr. Kohtani (Kanazawa University) [2].

The photocatalyst, BL 2.5 DX (diameter, 2.5 mm; membrane thickness, 1.0 μm: TiO_2 immobilized on glass beads: Lot No. 34040526), was purchased from Photo−Catalytic Materials (Komaki, Japan). A Strata−X cartridge (60 mg, 3 ml) (Shimadzu,

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Kyoto) was successively conditioned with MeOH (2 ml) and water (2 ml) prior to its use.

All other reagents and steroids were of analytical grade and commercially available.

2.2 Formation of Catechol Estrogen from Estradiol

The liver homogenate of a Wistar strain rat (male, 7 weeks old; Japan S.L.C., Hamamatsu, Japan) was used as the enzyme source as described in a previous paper [3]. A rat liver homogenate (2 mg protein), NADPH (8 μmole), estradiol (E_2, 800 nmole) in MeOH (20 μl) and sufficient 0.05 M Tris–HCl buffer (pH 7.4) were used to make a total volume of 0.8 ml. The incubation was carried out at 37 ℃ for 3 hr. After the addition of 5% HCl (1 ml) to terminate the reaction, the reaction mixture was treated as described in a previous paper [3].

2.3 Formation of Guaiacol Estrogen from 2–Hydroxyestrone

The liver homogenate of a Wistar strain rat (male, 7 weeks old; Japan S.L.C.) was used as the enzyme source as described in a previous paper [3]. The assay medium (2 ml) contained 2–hydroxyestrone ($2OHE_1$, 35 nmole) in MeOH (100 μl), *S*–adenosyl–L–methionine (0.16 μmole), $MgCl_2$ (3.2 μmole) and 0.07 M phosphate buffer (pH 7.5). The reaction mixture was incubated at 37 ℃ for 2 hr. The mixture was deproteinized with heat at 80 ℃ and then centrifuged (1,500*g* × 5 min). The obtained supernatant was treated as described in a previous paper [4].

2.4 Photocatalytic Degradation of Estradiol

The ethanolic solution of E_2 was diluted with water to 1 μM (the ethanol concentration was less than 0.3%, v/v). The photocatalytic glass beads (*ca.* 13.6 g) were spread so as to cover the bottom of a glass petri dish (diameter, 8 cm), and the E_2 solution (1 μM, 15 ml) was placed in the dish. The petri dish was placed in a light shielded box [22.5 (wide) × 13.5 (depth) × 8 cm (height)], and irradiated by a black light lamp (365 nm, 4 W) (Vilber Lourmat, Cedex, France) at a distance of 8 cm for 3 hr. All the solutions were subjected to a Strata–X cartridge and washed with water (2 ml), the estrogen was eluted with ethyl acetate (1.5 ml) and evaporated under a N_2 gas stream. The residue was dissolved in MeOH (50 μl) and an aliquot (10 μl) of which was used for the HPLC analysis [5].

3. Results and Discussion

We usually used three types of conventional columns, J'sphere ODS–H80, –M80 or –L80, for analysis of the steroids [6]. These columns are different in their carbon contents, that is, J'sphere ODS–H80 (22%), –M80 (14%) and –L80 (9%), respectively, according to the supplier. The column length of these was 150 mm, but a monolithic silica column having a 150 mm length has not been commercially available. Although the column lengths of the conventional columns (J'sphere ODS–H80, –M80, –L80; 150 mm) and the monolithic silica one (Chromolith Performance RP–18e; 100 mm) are different, the following experiments were done for the practical use of these columns.

In order to compare the chromatographic behavior of estrogens using three conventional columns and the monolithic silica one, we measured t_0, the theoretical plate number, height equivalent to a theoretical plate number (HETP) and symmetry factor using estrone (E_1) as the model compound (Table 1). Both the J'sphere ODS–L80 and the monolithic silica column showed the best HETP (0.014 mm). The symmetry factor was measured according to the definition of JPXIV [7] and the best one (1.1) was obtained using the J'sphere ODS–M80 column. The column pressure of the monolithic silica column (*ca.* 16 kg/cm^2) was much less than those of the conventional columns (each *ca.* 66 kg/cm^2) using MeCN–H_2O (1:1) as the mobile phase. Although the column length of the monolithic silica column is three quarters that of the three types of conventional ones, almost the same t_0 (1.5 and 1.4–1.7 min, respectively) was obtained from these columns, therefore, we used the *k* in the following experiments.

We next used a pair of estrogens including the positional isomers as model compounds for observation of the chromatographic behavior. The data obtained from the three types of conventional columns and monolithic silica column are summarized in Tables 2

Table 1. Comparison of properties of conventional columns with monolithic silica column using E_1

	t_0 (min)[a)]	theoretical plate number	HETP	symmetry factor
J'sphere ODS–H80	1.4	9600[b)]	0.016 mm	1.6
J'sphere ODS–M80	1.5	9800[c)]	0.015 mm	1.1
J'sphere ODS–L80	1.7	11000[d)]	0.014 mm	1.4
Chromolith Performance RP–18e	1.5	7100[e)]	0.014 mm	1.4

a) $NaNO_3$ (220 nm); b) MeCN–H_2O (1:1), t_R 6.7 min; c) t_R 6.6 min; d) t_R 6.1 min;
e) MeCN–H_2O (19:31), t_R 6.4 min

Table 2. Separation pf pair of estrogens using J'sphere ODS−H80, −M80 and −L80

	J'sphere ODS−H80		J'sphere ODS−M80		J'sphere ODS−L80		
Compound	k	R_S	k	R_S	k	R_S	Mobile phase
E_2 E_1	3.6 4.8	10.1	3.4 4.4	7.1	2.6 3.6	6.6	MeCN−H_2O (1:1)
E_3 16−epiE_3	3.7 6.6	11.5	4.3 7.3	10.1	3.8 6.1	10.2	MeOH−H_2O (1:1)
9.11−dehydroE_1 E_1	10.2 10.9	1.7	9.2 9.9	2.0	7.5 8.1	1.7	MeOH−H_2O (11:9)
9.11−dehydroE_2 E_2	5.3 6.4	4.1	5.5 6.5	4.0	4.6 5.4	3.4	MeOH−H_2O (3:2)
2OHE_1 4OHE_1	10.7 11.4	1.8	11.4 12.3	2.5	9.5 10.2	2.1	MeCN−MeOH−0.1% KH_2PO_4 (pH 3.05 adjusted with H_3PO_4) (2:1:5)
4OHE_2 2OHE_2	7.6 8.4	2.8	7.5 9.6	4.5	7.5 7.9	1.3	
2OH$E_1$2Me 2OH$E_1$3Me	7.6 9.2	3.4	7.3 8.1	2.8	5.7 6.4	2.4	MeOH−H_2O (3:2)
4OH$E_1$4Me 4OH$E_1$3Me	7.1 11.6	12.5	7.0 9.7	8.8	5.5 7.5	6.9	

Table 3. Separation of pair of estrogens using Chromolith Performance RP−18e

	A			B		
Compound	k	R_S	Mobile phase	k	R_S	Mobile phase
E_2 E_1	1.7 2.0	3.2	MeCN−H_2O (1:1)			
E_3 16−epiE_3	1.8 2.6	4.7	MeOH−H_2O (1:1)			
9.11−dehydroE_1 E_1	3.8 4.0	1.0	MeOH−H_2O (11:9)	9.3 10.0	1.5	MeOH−H_2O (9:11)
9.11−dehydroE_2 E_2	2.2 2.5	1.7	MeOH−H_2O (3:2)			
2OHE_1 4OHE_1	1.5 4.7	1.0	MeCN−MeOH−0.1% KH_2PO_4 (pH 3.05 adjusted with H_3PO_4) (2:1:5)	9.5 10.0	1.5	MeCN−0.1% KH_2PO_4 (pH 3.05 adjusted with H_3PO_4) (2:1:5)
4OHE_2 2OHE_2	3.3 3.7	2.1				
2OH$E_1$2Me 2OH$E_1$3Me	3.0 3.3	0.9	MeOH−H_2O (3:2)	4.1 4.6	2.0	MeOH−H_2O (4:3)
4OH$E_1$4Me 4OH$E_1$3Me	2.7 3.8	5.7				

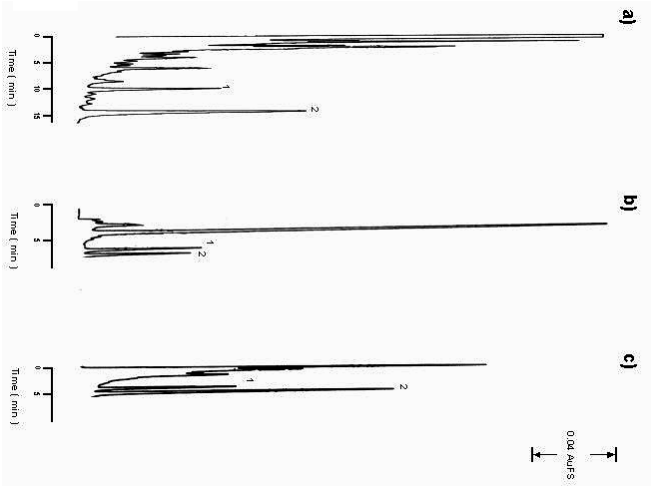

Figure 2. Application of monolithic silica column for separation of products formed *in vitro* and photocatalytic degradation experiments using estrogen as a substrate
a) Formation of catechol estrogen from E_2 using rat liver homogenate; mobile phase, MeCN–0.1% KH_2PO_4 (pH 3.05 adjusted with H_3PO_4) (1:3), UV 280 nm; 1. $2OHE_2$ 2. $2OHE_1$
b) Formation of guaiacol estrogen from $2OHE_1$ using rat liver homogenate; mobile phase, MeOH–H_2O (4:3), UV 280 nm; 1. $2OHE_12Me$, 2. $2OHE_13Me$
c) Photocatalytic degradation of E_2; mobile phase, MeOH–H_2O (3:2), UV 262 nm; 1. 9,11–dehydroE_2, 2. E_2

and 3, respectively. E_2 and E_1 are clearly separated by all these columns using MeCN–H_2O (1:1) and the monolithic silica column gave a satisfactory separation (resolution; R_S 3.2) with a smaller k (E_2, 1.7; E_1, 2.0) compared with those (2.6–4.8) obtained from the three types of conventional columns (Table 3 A, Table 2). An almost satisfactory results (R_S >1.3) have been obtained for the separation of estriol (E_3) and 16–epiE_3, 9,11–dehydroE_1 (or 9,11–dehydroE_2) and E_1 (or E_2), a pair of catechol estrogens [$2OHE_1$ and 4–hydroxyE_1 ($4OHE_1$), 4–hydroxyE_2 ($4OHE_2$) and 2–hydroxyE_2 ($2OHE_2$)], and a pair of guaiacol estrogens [2–methoxyE_1 ($2OHE_12Me$) and $2OHE_13$–methyl ether ($2OHE_13Me$), 4–methoxyE_1 ($4OHE_14Me$) and 4–hydroxy$E_1$3–methyl ether ($4OHE_13Me$)], respectively, using the three types of conventional columns (Table 2). The J'sphere ODS–L80 gave an almost satisfactory separation (R_S >1.3) together with the smaller k among these columns. The satisfactory separations (R_S >1.7) of the following pairs of estrogens (E_2 and E_1, E_3 and 16–epiE_3, 9,11–dehydroE_2 and E_2, $4OHE_2$ and $2OHE_2$, $4OHE_14Me$ and $4OHE_13Me$) were obtained with smaller ks by using the monolithic silica column and the same solvent system used for the conventional columns, but the separations of the following pairs of estrogens (9,11–dehydroE_1 and E_1, $2OHE_1$ and $4OHE_1$, $2OHE_12Me$ and $2OHE_13Me$) are not satisfactory (R_S 1.0 or 0.9) (Table 3 A). The satisfactory separations (R_S >1.5) were obtained by using the other mobile phases as shown in Table 3 B.

The application of the monolithic silica column for the separation of products obtained from the *in vitro* and photocatalytic degradation experiments using estrogen as a substrate has been completed. The formation of catechol estrogen from E_2 [3] and that of guaiacol estrogen from $2OHE_1$ [4], which were produced by the rat liver homogenate, are shown in Figs. 2 a and 2 b, respectively. The photocatalytic degradation of E_2 using TiO_2 as a catalyst gave 9,11–dehydroE_2 as a by–product, which was reported by Kohtani *et al.* as shown in Fig. 2 c [8].

4. Conclusion

Generally speaking, the monolithic silica column eluted estrogens, including the positional isomers, with a smaller k, lower column pressure and satisfactory separation. The column pressure of the monolithic silica column is much less than that of the conventional one, from which we can use the monolithic silica column at a higher flow rate to give a smaller k. However, attention should be focused on the phenomenon that the higher flow rate decreases the response of the compound to the detector using a flow cell.

The application of the monolithic silica column for steroid analysis is now in progress in our laboratory.

5. Acknowledgment

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6. References

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