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CHROMATOGRAPHY OF ADRENOCORTICAL STEROIDS ON SILICIC ACID COLUMNS

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SUMMARY

The effect of variable parameters, such as water content and activation, on the separation of adrenocortical steroids on silicic acid columns was studied. Screening of nine commercial silicic acid samples, using a standard technique of partition chromatography, showed wide variations in chromatographic behavior. Data is presented on the effects of varying stationary phase (water) and activation on separation and recovery of eight adrenocortical steroids. A standardized technique for preparation of an efficient and reproducible partition column for the separation of steroid mixtures on one of the commercially available silicic acids is described.

INTRODUCTION

A method developed in this laboratory[1,2] for the separation of adrenocortical steroids by partition chromatography on a silicic acid column utilized Merck silicic acid as the column support. Due to an apparent change in commercial preparation of the silicic acid, the method suffered from poor and inconsistent flow rate, separation, and yield, despite changes in activation and water added as stationary phase. Consequently, nine commercially available silicic acids were screened to find a suitable replacement for the Merck silicic acid. In this initial survey activation and water content were the same as described in the original method[2]. Following the screening process, the effects of changes in the stationary phase (water) and activation (time and temperature) of the silicic acid on peak resolution and recovery of steroids were investigated.

EXPERIMENTAL

Survey of commercial silicic acids

Table I lists the product information, numerical designation, and percent water content of the nine silicic acids obtained commercially. The percent water content was determined by heating 5 + g amounts in an oven for 3 h at 105°, and measuring the weight loss. The values in the fourth column of the table represent the average of

TABLE I

PRODUCT INFORMATION, NUMERICAL DESIGNATION AND WATER CONTENT OF SILICIC ACIDS

Product	*Numerical designation*	*Mesh size*	*Water (%)*
Sigma-SIL-R	1	100	10.14
Bio-Rad-72315	2	200–325	4.78
Bio-Rad-723101	3	100–200	10.30
Nutritional Biochemicals-9606	4	none given	3.00
Mallinckrodt-2847	5	100	5.96
Fisher-A-288	6	none given	2.95
Fisher-A-945	7	325	5.16
Matheson, Coleman and Bell CB-699	8	none given	3.20
Baker-0324	9	none given	2.36

three such determinations. It can be seen that there is a wide variation in the water content of the silicic acids as originally obtained.

Each silicic acid sample used for preparation of a column was heated in an oven at 105° for five h with frequent stirring to insure uniform activation. The activated silicic acid was immediately transferred to a screw-capped brown bottle, which was taped air tight and stored in a desiccator. For the initial screening procedure, a 40% water impregnated column was used. The column was prepared by adding 20 ml of distilled water in two 10 ml portions to 50 g of the activated silicic acid, while quickly grinding the mixture in a glass mortar. A mobile slurry with water saturated petroleum ether (PE) was prepared and poured into the column in small portions while tamping with moderate pressure to liberate trapped air bubbles. The slurry and column were kept constantly covered with PE to prevent alteration of the 1 to 2.5 ratio by weight of water to silicic acid. The packed column was then washed successively with 200 ml portions of water-saturated dichloromethane (DCM) and PE to insure the removal of impurities with UV absorption at 240 mμ. Ethanol (95%) aliquots containing

TABLE II

SURVEY OF SILICIC ACIDS USING 40% WATER-IMPREGNATED COLUMNS

Silicic acid	*Flow rate (min/fraction)*	*Steroids eluted*	*Yield*	*Quality of separation*
1	2.5–15.0 erratic	P,Q	P, 50%; Q, 50%	good
2	3.0–7.5	P,Q,A	P and Q, 100%; A, 50%	good
3	3.0–7.0	P,Q,A	P and Q, 100%; A, 50%	poor
4	9.0–12.0	P,Q,A,S,B,E	90% (P,Q,A,S,B,E)	poor
5	5.0–23.0 erratic	P,Q	P and Q, 100%	poor
6	9.0–11.0	P,Q,A	P and Q, 100%	poor
7	51.0–65.0	Silicic acid formed gel; no column		
8	2.0–11.0 erratic	P,Q	P, 100%; Q, 50%	good
9	8.5–10.0	P,Q	P and Q, 50%	poor

300 μg of each steroid standard* were combined in a round-bottomed flask and concentrated *in vacuo* at 40°. The sample mixture was then transferred to a $\frac{1}{2}$ in. diameter filter paper disc with ethanol (95%). The air-dried disc was placed on the column and covered with a $\frac{1}{4}$ in. layer of sea sand. The column was connected to the gradient elution analyzer[3] for separation. From previous work[2] definite percent mixtures had been determined, which were capable of eluting individual steroids in the range between zero and one hundred percent DCM. Each fraction was evaporated *in vacuo*, and the residues dissolved in 5.0 ml of ethanol (95%). The absorbance was read at 240 mμ and plotted against tube number for each column. Quantitative amounts were calculated by referring to a linear plot of reference standards, and the peak fractions were pooled separately for identification by thin-layer chromatography[4].

Table II summarizes the results of the survey using 40% water impregnated columns.

The flow rates of the columns varied considerably (from 2.0–65.0 min), and in some cases were erratic (columns 1, 5, and 8) due to expansion of the silicic acid during chromatography. With all the columns (except 4) only the first two or three steroids in the series (P, Q, and A) were eluted with yields of 50–100%. In fact, the combination of poor separation and low yield (qualitative and quantitative) eliminated silicic acids 3, 5, 6, and 9 from further consideration. Silicic acid 7 was excluded because of its slow flow rate. Of the silicic acids that gave good separations (1, 2, and 8), 2 was selected for further study because it alone of the three eluted 100% of P and Q, and 50% of A. Silicic acid 4 was also selected because it alone eluted six of the eight steroids.

Variation of stationary phase (water)

The weight ratio of stationary phase (water) to support (silicic acid), expressed as the percent water content of the column, was varied in several columns using silicic acid No. 4 in an attempt to improve the quality of the separation and/or the yield. In this series of experiments the fractions were evaporated and read immediately after collection to determine the elution pattern of the steroids, and to enable the subsequent adjustment of the gradient program. Generally, the percent DCM in PE was increased in 5 or 10 percent increments until a steroid peak appeared as an absorbance reading at 240 mμ. The percent DCM in PE was then dropped by 10% to hold back the next steroid in the series, until the absorbance reading decreased to background. The percent DCM in PE was then increased again to elute the next steroid, or until 100% DCM was attained. With silicic acid No. 4, as with all the others tested, the eight steroids elute in the following sequence: P, Q, A, S, B, E, Aldo and F.

Table III summarizes the results of varying the stationary phase on columns using silicic acid No. 4.

As can be seen by comparing "Water content" and "Steroids eluted", increasing the water content of the column from 40 to 60% did not cause a consistent increase in the number of steroids eluted. Although the increase in water content did allow the elution of B, the fifth steroid in the series, the separation pattern deteriorated so

* Stock solutions of 100 μg per ml of absolute ethanol were prepared from the following steroids: Δ^4-pregnene-3,20-dione (P), Δ^4-pregnen-21-ol-3,20-dione (Q), Δ^4-pregnen-21-ol-3,11,20-trione (A), Δ^4-pregnene-17α,21-diol-3,20-dione (S), Δ^4-pregnene-11β,21-diol-3,20-dione (B), Δ^4-pregnene-17α,21-diol-3,11,20-trione (E), and Δ^4-pregnene-11β,17α,21-triol-3,20-dione (F). Preweighed 100 μg ampoules from Calbiochem used for Δ^4-pregnen-18-al-11β,21-diol-3,20-dione (Aldo.) solution.

TABLE III

RESULTS OF VARYING STATIONARY PHASE

Water content (%)	*Flow rate (min/fraction)*	*Steroids eluted*	*Yield*[a]	*Quality of separation*
40	6.0 to 9.0	P,Q,A,S,B	93%	poor
40	22.0	P,Q,A	82%	good
50	1.5–30.0 erratic	P,Q,A,S,B	86%	poor
50	2.5–20.0 erratic	P,Q,A,S	90%	good
50	15.0–20.0	P,Q,A,S	95%	good
55	9.0–11.0	not determined	not determined	very poor
60	3.0–11.0	P,Q	99%	very poor
60	9.0–26.0 erratic	not determined	not determined	very poor
47 + 3	5.0–25.0 erratic	P,Q,A,S,B	100%	very poor

[a] The % yield expressed is based on the total μg's of the steroids eluted.

badly that individual peak identification was impossible. With the 55% and 60% columns the steroids actually eluted in a badly overlapped zone, despite the use of hold back percents. The flow rates varied from 1.5–30.0 min, and were sometimes erratic, because silicic acid No. 4 was difficult to pack consistently. Packing the No. 4 slurry tightly resulted in some improvement in the separating ability of the column, but its flow rate was a slow 15+ min per fraction. The last column in this series was an attempt to improve the separation, and/or yield by using unactivated silicic acid. A 50% water slurry was prepared by adding 23.5 ml of water to the 50 g of silicic acid and 1.5 ml of water already present in the silicic acid (see Table I). The smeared elution pattern obtained indicated that activation of the silicic acid was necessary to improve its separating ability.

Because a 40% water column of silicic acid No. 2 eluted 100% of P and Q, and 50% of A with excellent separation, the percent water was increased by 10% increments in a series of experiments using silicic acid No. 2 to see if the number of steroids eluted would also increase. As with the silicic acid No. 4 study, a variable gradient elution program was used; that is, the % DCM in PE was increased in 5 or 10% increments, and the percent solvent mixture was held constant whenever a steroid was being eluted. The silicic acid No. 2 for these columns was heated for 5 h at 120°. The number of steroids eluted proved to be directly related to the water content used as stationary phase. A 50% water column eluted 90–100% of P, Q, A, S, and B; whereas, a 60% water column eluted 90–100% of P, Q, A, S, B, and E. Both columns gave efficient separation of the peaks. A 70% water column, although yielding 100% of all eight steroids, lacked the resolution of the columns with lower percent water. The 70% silicic acid had apparently reached its physical limit of adsorbed water phase, as evidenced by the marked change in the slurry prepared, and the extremely fast running rate of the column. Consequently, a 65% water column was tried, and did elute 100% of all eight steroids with excellent separation, except for the last two steroids in the series (Aldo and F) which eluted together at 100% DCM. Unfortunately,

the results of this 65% water column could not be reproduced, because the activation parameters (time and temperature) of the silicic acid preparation had been inadvertently altered from 120° for 5 h during the study. This difficulty, coupled with the unactivated silicic acid No. 4 experiment (Table III), suggested that the method of silicic acid activation might be of importance.

A study of the activation parameters was then undertaken with silicic acid No. 2. In these experiments, the percent water content was kept at 65%, the variable gradient elution program was used, and the columns were packed as consistently as possible. Table IV summarizes the results of these experiments.

TABLE IV

RESULTS OF VARYING ACTIVATION PARAMETERS

Activation	*Steroids eluted*	*Quality of separation*
5½ h, 130°	P,Q,A,S,B,E	poor
5 h, 124°	?	very poor
7 h, 114°	all	poor
5 h, 114°	?	very poor
5 h, 110°	?	very poor
3 h, 110°	[a]A,S,B	good
3½ h, 102°	[a]A,S,B	good

[a] Only A, S, and B were applied to these columns, since their separation is always the most difficult.

The activation time was varied from 7 h to 3 h, and the temperature from 130° to 102°. The selection of the parameter combinations was random because no relationship between them and the separating quality of the columns was known. The shorter heating time of 3–3½ h did produce the best resolution of peaks, indicating that the longer heating periods, used to insure removal of all the water, undoubtedly alters the structure of the support itself. Despite the varied activation conditions, all the columns had consistent flow rates between 5.0 and 8.0 min per fraction. The yield of the columns, with the exception of the 3 and 3½ h columns, was not determined because the separations were so poor. For the two exceptions, the yield of A, S and B was 100%. In those columns where the separation was very poor, thin-layer identification of the fractions was impractical; hence, the question marks in the "Steroids eluted" column. Of the two procedures that yielded good resolution and recovery, the activation for 3½ h at 102° was selected because A, S, and B were eluted at different percents of DCM; whereas, with activation for 3 h at 110°, A and S were eluted at the same percent DCM. When the separation of all eight steroids was tried with this 102°, 3½ h activation, the result was a poor separation pattern similar to the one obtained with the 70% water column (activation assumed to be 120° for 5 h). Apparently the decrease in time (from 5 to 3 h) and temperature (from 120° to 102°) of activation had a decreased drying effect on the silicic acid and thereby increased its inherent water content. Therefore, when the 32.5 ml of water was added to the 50 g of silicic acid, it produced a slurry of greater than the 65% water content expected.

It was then decided to decrease the water content by 1% increments to de-

termine the precise water content that would give the best resolution of peaks. The water added was measured by a burette to insure accuracy. As the percent water added decreased from 64 to 59 the separation gradually improved. A 58% column did give excellent separation of the first six steroids in the series, but did not elute the last two. In fact, its elution pattern was exactly the same as the 60% water column (activation assumed to be 120° for 5 h) in the initial percent water study of silicic acid No. 2. Since that study proved a direct relationship between water content and number of eluted steroids, but an indirect relationship between water content and quality of separation (as the support's water capacity is reached), it was decided that a slight increase in the percent water added (from 58 to 60) to increase the number of eluted steroids, coupled with a slight increase in activation temperature (from 102° to 110°) to avoid overloading the support, might achieve the correct balance. The good separation of A, S, and B achieved with the 65% column (activation 110° for 3 h, Table IV) indicated that this combination might work. Consequently, a column was prepared with silicic acid No. 2, which was activated for 3 h at 110°, and made 60% water by the burette addition of 30.0 ml of water. A variable gradient elution program

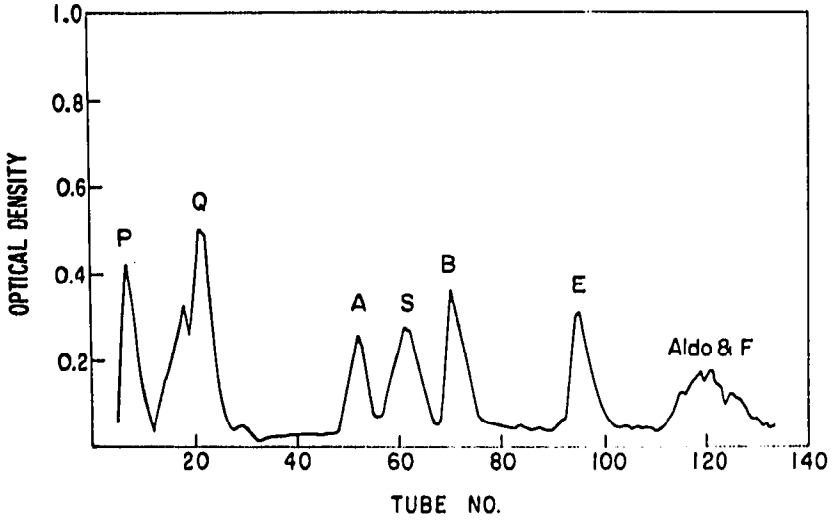

Fig. 1. Separation of eight adrenal steroids on a 60% water-impregnated column of silicic acid No. 2.

was used, and a consistent flow rate of 7.0–8.0 min per fraction was obtained. The eight steroids were eluted with excellent separation, except for Aldo and F which elute together at 100% DCM (Fig. 1). The results of this column were reproducible when the parameters of activation, and the ratio of stationary phase (water) to support (silicic acid) were kept constant.

DISCUSSION

Based on the type of elution peaks obtained from separation of adrenocortical steroids on water-impregnated silicic acid, the method is generally recognized as partition rather than adsorption chromatography[5,6]. In a similar study, LINFORD[7] concluded that the properties of silicic acid as an adsorbent can be modified by changing

its free water content. He reported that up to 17% of free water, adsorption at the solid surface predominates; whereas, at 32% of free water, partition effects appear with the steroids used. Our studies further indicate that the performance of a silicic acid column is very dependent, not only on the water content of the support, but also its commercial source and method of activation. Both the recovery of the steroids, and the resolution of the peaks can be adjusted by a slight change in one of these variables. It was found that commercial preparations of silicic acid vary considerably in their chromatographic behavior (ease of packing, flow rate, yield, and separating ability), and that the selection of the best silicic acid must be done by trial and error since the catalogue information does not give sufficient data.

Our results also indicate that the method of activation plays an important role in the performance of silicic acid columns. The temperature and time used for activation influence the choice of water content to be used to achieve the best separation and recovery of steroids. It was found that the time of heating should be limited to under 4 h since longer times seem to cause alterations in the structure of the silicic acid. The temperature of activation must then be balanced with the water added to the silicic acid to give the best resulting percent water. For the adrenocortical steroids in these studies, a 60% water column prepared with Bio-Rad 200–325 mesh silicic acid, heated for 3 h at 110° proved to be the best combination. There is no doubt that the choice of these parameters will vary with the type of compound mixtures to be chromatographed.

A direct relationship appears to exist between the percent water of the silicic acid column, the number of steroids eluted, and the quantitative recovery from the column; but an indirect relationship between percent water and resolution of peaks. In practice it was found advisable to run a preliminary separation using an arbitrary water content (40% in these studies), and then to increase the percent water of the column, which results in a corresponding increase in the yield, until the separation pattern begins to smear. The selection of a percent water slightly less than that point will give the best combination of yield and peak resolution.

Although the effect of temperature changes in column performance was not studied, it is known that varying the operating temperature of silicic acid and silica gel columns can also be a parameter affecting the efficiency and resolution of the column[8,9]. To insure a constant temperature of 19.5° during these studies, a water jacketed column system was used.

Preliminary investigations with the above combination of parameters selected for chromatography of adrenocortical steroids indicate that the ketosteroids may also be separated on the same column. Studies are in progress in the hope of developing a method for separation of adrenocortical steroid and ketosteroid mixtures on a single column. A similar approach to the chromatography of 17-OH-corticosteroids and 17-ketosteroids has been done by SEKI[10,11], who used a support of Sephadex LH-20 to separate the 17-OH-corticosteroids from the 17-ketosteroids. The latter were separated into two groups of $C_{19}O_2$-17-ketosteroids and $C_{19}O_3$-17-ketosteroids.

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