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Determination of Glucocorticoids by Liquid Chromatography. III. Application to Ointments and a Cream Containing Cortisone Acetate, Dexamethasone Acetate, Fluorometholone, and Betamethasone Valerate

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In order to separate cortisone acetate, dexamethasone acetate, fluorometholone, and betamethasone valerate in ointments and a cream from the base and excipients, a silica gel column pretreatment was carried out (pre-screening procedure) and then glucocorticoids were successfully determined by high-performance liquid chromatography on a LiChrosorb RP-18 column. By the use of the pre-screening procedure, the base, excipients, and other active ingredients (except glycyrrhetinic acid) in commercial preparations were almost completely removed, and hence a lowering of the efficiency of the LiChrosorb RP-18 column and blocking of the flow of the mobile phase in column caused by the base remaining on the top of the column could be avoided. The contents of glucocorticoids in various commercial preparations were found to be 95.7 to 102.5% of the labeled amounts.

Keywords——cortisone acetate; dexamethasone acetate; fluorometholone; betamethasone valerate; silica gel column; HPLC; LiChrosorb RP-18 column

Colorimetric[1] and high-performance liquid chromatographic[2] (HPLC) methods have been used as official methods for the determination of glucocorticoids in ointments and creams. The keto-alcohol side chain of glucocorticoids possesses a reductive property and in the case of the official colorimetric method, triphenyltetrazolium chloride as the color reagent is reduced by glucocorticoid and hence colored. Since commercial ointments and creams often contain active ingredients such as anti-histaminics and antibiotics as well as glucocorticoids, it is difficult to analyze them colorimetrically. In the case of the official HPLC method, glucocorticoids are extracted from the ointment with methanol. The complete extraction of a small amount of glucocorticoid (*e.g.* 0.0125%) is not easy, and small amounts of ointment base and excipients are also dissolved in methanol. When glucocorticoid is determined by the HPLC method, the base excipients remain on top of the HPLC column, reducing the efficiency of the HPLC column and blocking the flow of the mobile phase in the column.

We reported that prednisolone, hydrocortisone, fluocinolone acetonide, prednisolone acetate, and hydrocortisone acetate in ointments, creams, and supositories could be determined by the HPLC method after a pre-screening procedure on a silica gel column.[3] By this method, we were able to separate glucocorticoids from the base, excipients, and other active ingredients.

This paper describes an application of the method for the determination of cortisone acetate (CA), dexamethasone acetate (DA), fluorometholone (FM), and betamethasone valerate (BV) in commercial ointments and a cream.

Experimental

Apparatus and Chromatographic Conditions——The liquid chromatograph consisted of a Hitachi 655 liquid

TABLE I. Claimed Contents of Glucocorticoid and Active Ingredients in One Gram of Commercial Ointments and a Cream

	Glucocorticoid and active ingredient
A[a]	Cortisone acetate (15 mg)
B[b]	Dexamethasone acetate (0.25 mg), chlorhexidine hydrochloride (1 mg), diphenhydramine hydrochloride (10 mg), glycyrrhetinic acid (3 mg)
C[a]	Dexamethasone acetate (0.25 mg), glycyrrhetinic acid (3 mg)
D[a]	Dexamethasone acetate (0.25 mg), chlorhexidine hydrochloride (1 mg), glycyrrhetinic acid (5 mg)
E[a]	Fluorometholone (0.125 mg), isothiopendyl hydrochloride (7.5 mg)
F[a]	Betamethasone valerate (0.6 mg)
G[a]	Betamethasone valerate (1.2 mg), fradiomycin sulfate (3.5 mg)

a) Ointment. *b*) Cream.

chromatograph with a Hitachi variable-wavelength ultraviolet monitor (set at 254 nm) and a Shimadzu C-R1B Chromatopac. A stainless steel column (250 × 4 mm i.d.) was packed with LiChrosorb RP-18 (particle size, 5 μm; Merck Co.) and the column temperature was maintained at 35 °C. The flow rate was 1 ml/min. A mixture of acetonitrile and water was used for CA and FM at a ratio of 45 : 55 and for DA and BV at a ratio of 55 : 45.

Reagents and Solution——CA, DA, and 17α-hydroxyprogesterone (HP) were the products of Sigma Co. FM and BV were gifts from Sumitomo Chemical Co. and Glaxo Laboratories Co., respectively. The ointments and cream were purchased locally and their claimed contents are listed in Table I. All other reagents were of reagent grade.

Internal Standard (IS) Solution: Ten mg of HP and 20 mg of DA were dissolved in chloroform to give concentrations of 10 and 20 μg/ml, respectively (IS-I and IS-II solution).

Standard Stock Solution: About 20 mg of each glucocorticoid (dried beforehand at 105 °C for 3 h) was weighed accurately and dissolved in chloroform to make 100 ml.

Standard Solution: Ten ml of standard stock solution was diluted with chloroform to a total volume of 100 ml. Five ml of this solution for CA and FM was mixed and the mixture was evaporated *in vacuo*. The residue was dissolved in 5 ml of the mobile phase.

Silica Gel Column: About 2 g of silica gel (Silica gel 60 extra pure, diameter 0.063 to 0.2 mm; Merck Co.) was placed in a vessel and 10 ml of chloroform was added. This slurry was transferred to a glass column (20 × 1 cm i.d.).

Assay——A portion of ointment or cream containing about 1 mg of glucocorticoid was accurately weighed in a vessel and dissolved in 25 ml of chloroform. The solution was then transferred to a 50-ml volumetric flask. After rinsing of the vessel with 15 ml of chloroform, the solution was added to the volumetric flask. The flask was made up to a total volume of 50 ml with chloroform. A 5 ml aliquot was charged onto the silica gel column. The column was washed with 20 ml of chloroform and the eluate was discarded. Ten ml of a mixture of dichloromethane and methanol (4 : 1) was then added on the column and the eluate was collected in a vacuum flask. Five ml of IS-I solution for DA and BV or IS-II solution for CA and FM was added to the vacuum flask and then the mixture was evaporated *in vacuo* (pre-screening procedure). The residue was dissolved in 5 ml of the mobile phase and then a 20 μl aliquot was injected into the HPLC column. The ratio of the peak area of glucocorticoid was calculated against that of IS-I or IS-II.

$$\text{Amount of glucocorticoid (mg)} = \text{amount of standard (mg)} \times A_T/A_S \times 1/20$$

A_T and A_S are the ratios of the peak areas obtained from the sample and standard solution.

Results and Discussion

Selection of HPLC Conditions

Column Temperature——The effect of the column temperature on the retention time (t_R) was examined in the range of 30 to 50 °C. An increase of the column temperature slightly shortened the t_R of glucocorticoids. For instance, the t_R of DA was 9.6 min at 30 °C and 7.7 min at 50 °C when 45% acetonitrile was used as a mobile phase. The column temperature was set at 35 °C for convenience.

Concentration of Acetonitrile——As shown in Fig. 1, an increase of the concentration of acetonitrile brought about a large reduction in the t_R of glucocorticoids. The concentration of

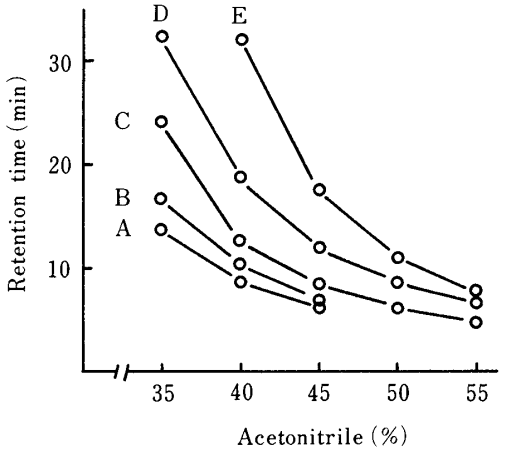

Fig. 1. Effect of Concentration of Acetonitrile on Retention Behavior

A, FM; B, CA; C, DA; D, HP; E, BV.

acetonitrile was set at 45% for CA and FM, and 55% for DA and BV to obtain appropriate separation times.

Working Curves and Reproducibility——The working curves for each glucocorticoid were linear from 0 to 24 μg/ml and passed through the origin.

At the concentration of 20 μg/ml of CA, FM, DA, and BV, the average values of the ratios of peak area were 0.995, 1.175, 1.459, and 1.196 ($n=6$), respectively, and the coefficients of variation were 0.78, 1.13, 0.14, and 0.18%, respectively.

Effect of the Pre-screening Procedure

When glucocorticoid in ointment was extracted with methanol,[2] small amounts of the base and excipients were also dissolved in the methanol. The base and excipients in methanol remained on top of the HPLC column, lowering the efficiency of the HPLC column and blocking the flow of the mobile phase in the column. In order to eliminate the base and excipients, we ensured that glucocorticoid was separated from the base and excipients by passing the solution obtained from the ointment through a small column of silica gel as a pre-screening procedure. We prepared 0.02 mg/ml of DA, CA, FM, and BV in chloroform and the extent of adsorption of each glucocorticoid on the silica gel column was examined. After charging a 5 ml aliquot onto the column, the eluate was discarded. Glucocorticoid on the column was then eluted with several solvents or mixtures as listed in Table II. The eluate was collected in 5 ml fractions (fractions 1—4). The content of glucocorticoid in each fraction was determined by the HPLC method. For instance, as shown in Table II, DA could not be eluted with chloroform or dichloromethane alone, but the addition of methanol resulted in the elution of DA. Similar results were also obtained in the case of CA, FM, and BV, the base and excipients should not be adsorbed on the silica gel column and should pass through the column with chloroform. Therefore, after adsorption of glucocorticoid on the silica gel column, the column was washed with 20 ml of chloroform and glucocorticoid was separated from the base and excipients. Glucocorticoid on the column was then eluted with 10 ml of a mixture of dichloromethane and methanol (4 : 1) and determined by the HPLC method. When 5 ml of glucocorticoid solution containing 0.02 mg/ml was used, the recovery of glucocorticoid from the silica gel column was examined. DA, BV, CA, and FM were recovered completely and the coefficients of variation of their recoveries were less than $\pm 0.6\%$ ($n=5$). In addition, to examine the influence of the base, excipients, and antiseptics on the elution of glucocorticoids from the silica gel column, we prepared a test solution containing 1 mg of glucocorticoid, 3.45 g of white petrolatum, 1 g of liquid paraffin, 0.35 g of paraffin, 0.2 g of propylene glycol, 4 mg of methylparaben, and 4 mg of butylparaben per 50 ml of chloroform. A 5 ml aliquot was charged on the silica gel column, and the recovery of glucocorticoid was

TABLE II. Content of DA in Fractions Eluted from the Silica Gel Column with Several Solvents and Mixtures

	Fraction[a]			
	1	2	3	4
$CHCl_3$	0	0	0	0
CH_2Cl_2	0	0	0	0
CH_2Cl_2–MeOH (4:1)	99.7[b]	0	0	0
CH_2Cl_2–MeOH (9:1)	81.7[b]	19.3[b]	0	0
$CHCl_3$–MeOH (4:1)	101.7[b]	0	0	0
CH_2Cl_2–EtOH (4:1)	101.2[b]	0	0	0

a) After charging of 5 ml of DA in $CHCl_3$ (0.02 mg/ml) on the silica gel column, 5 ml fractions of eluate with solvents or mixtures were collected. The content of DA in each fraction was expressed as a percentage of the charged amount.
b) Average of the contents of DA found in two experiments.

TABLE III. Contents of Glucocorticoids in Pharmaceutical Preparations against Labeled Amount

	Content (%)	Average
A	101.0, 102.8, 100.3	101.4
B	94.9, 93.6, 98.7	95.7
C	102.5, 101.0, 102.6	102.0
D	101.1, 100.4, 100.8	100.8
E	97.1, 97.9, 96.7	97.2
F	102.7, 101.7, 101.7	102.0
G	101.2, 104.1, 102.1	102.5

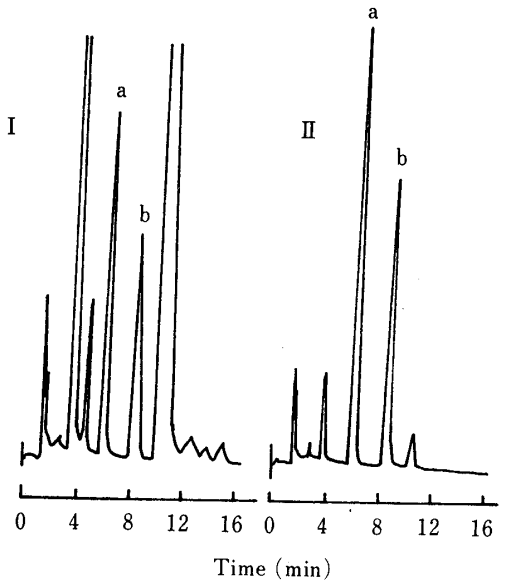

Fig. 2. Chromatograms for FM, Active Ingredients, and Excipients in Ointment E before and after the Pre-screening Procedure

I: Before pre-screening procedure.
II: After pre-screening procedure.
a, FM; b, IS.

examined. DA, BV, CA, and FM were recovered completely and their coefficients of variation were less than $\pm 0.8\%$ ($n=5$). After the pre-screening procedure, no greasy products (base and excipients) could be observed in the evaporated residue. These results suggested that DA, BV, CA, and FM were effectively separated from the base and excipients by the pre-screening procedure and determined successfully by the HPLC method.

Application to Ointments and a Cream

Typical high-performance liquid chromatograms for FM, active ingredient, and excipients in ointment E before and after the pre-screening procedure are shown in Fig. 2.

The results suggest that the active ingredient and excipients (except FM) were almost completely eliminated. Similar results were obtained for the other ointments and a cream, except glycyrrhetinic acid. Glycyrrhetinic acid showed the same behavior as DA on the silica gel column. Since its peak on the chromatogram had a t_R of 15.6 min, it did not influence the peak of DA, which had a t_R of 4.9 min.

Glucocorticoids in commercial preparations were determined by the proposed assay method and their contents are compared with the labeled amount in Table III. The results suggest that the method should be generally satisfactory for the determination of CA, DA, FM, and BV in ointments and a cream.

References and Notes

1) British Pharmacopeia, British Pharmacopeia Commission, London Her Majesty's Stationery Office, Cambridge, 1980, p. 699.
2) United States Pharmacopeia, 20th edition, United States Pharmacopeial Convention, Inc., Rockville, Maryland, 1980, p. 172.
3) H. Tokunaga, T. Kimura, and J. Kawamura, *Iyakuhin Kenkyu*, **14**, 31 (1983); *idem, ibid.*, **15**, 87 (1984).