Steroid Determination from Complex Mixtures by High Pressure Chromatographic Techniques

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Abstract □ The concepts described in this work emphasize the fabrication of components, the importance of partitioning supports, and an interesting ternary mobile phase system for high pressure liquid chromatography. The particular application of its utility as an analytical tool relates to quantitative determination of fluocinolone acetonide and fluocinonide from cream- or ointment-like formulations. Sensitivity of detection and resolution capability of the partitioning system for a number of related steroids are discussed in some detail.

Keyphrases □ Steroids in complex mixtures—analysis, high pressure liquid chromatography, ternary mobile phase system □ Fluocinolone acetonide cream, ointment—analysis, high pressure liquid chromatography □ Fluocinonide cream, ointment—analysis, high pressure liquid chromatography □ High pressure liquid chromatography—analysis, steroids in complex mixtures, ternary mobile phase system

The determination of specific steroids or other "active" components from their related degradation products in pharmaceutical preparations is a common problem (1). This requirement of specificity has frequently caused concern for quantitative measurements. As techniques in liquid chromatography have developed, the ability to resolve closely related components has improved to the extent that good quantitation at low concentration levels (2–4) is possible. To further this objective, a high pressure unit has been designed, built, and operated. This system permits efficient separation of a number of steroids from complex pharmaceutical preparations. The intent of this effort was to provide a rapid steroid analysis where problems of sensitivity and separation are simultaneously present. The nature of the substrate has proved to be an important factor in the development of this assay procedure.

EXPERIMENTAL

Instrument Design—Insofar as possible, the components fabricated into the system were acquired from commercial sources. The overall concept is depicted in Fig. 1. The majority of reciprocating pumps, as the one used here, is associated with cyclic pressure changes. Therefore, a surge attenuator is used in this system, and it is responsible for the reduction in cyclic pressure surges. The pump[1] employed (5) provides the pressurization of the mobile phase throughout the system. Upon leaving the pump, the fluid passes through a 10-μ fritted stainless steel filter prior to entering the surge attenuator[2] (6) operating at 3000 psi. This unit performs two functions: (*a*) it initially serves as a sump for the mobile phase, and (*b*) it efficiently dampens the pump surges which develop within the system. After leaving the surge attenuator, the fluid travels through narrow bore tubing (0.23 mm.) to the reference side of the differential UV detector and then to the sampling injector valve[3] (7). This loop injector valve permits varying the injection volume between 2 and 20 μl. Furthermore, this system can be continuously operated under high pressures while introducing samples. Upon exiting this valve, the mobile phase is carried through the chromatographic column to the sample side of the UV detector. The effluent material from the cell can be recovered for additional analyses.

The initial choice for a detector was a high pressure UV absorption unit[4]. This detector unit has an internal volume of 12 μl. and is capable of operating up to 2000 p.s.i. It normally operates at 254 nm. but has the capability of operating at 280 and 360 nm. with appropriate filtering. The 10-mv. output from the differential detector can be then applied to a recorder.

In addition to the UV detector described previously, a nondifferential UV detector[5] was employed. This detector provides a convenient alternative where desired wavelengths, other than those previously mentioned, should be employed. A 250-μl. cell is used in the current configuration; however, smaller cells have been found useful. No special fabrication is required to employ this model. Usually 100-mcg. samples are used where strong UV chromophores exist. Furthermore, the detector was utilized in preliminary preparative level work where milligram quantities of sample were employed. If one accepts the current limits of sensitivity for this UV detector, it provides an acceptable alternative to existing detectors. In addition, this detector has a wide range of wavelength utility, *i.e.*, ~200–~700 nm.

Perhaps the most important aspect of the total concept relates to the stationary support of this system. These materials consist of spherically shaped particles of silica, approximately 40–70 μ in size, having an effective surface area of 300–400 m.^{2}/g. (8–12) (Spherosil or Porasil particles). The selection of uniformly spherical particles is critical because the developed flow and pressure patterns reflect the homogeneity of the substrate's surface. Since the purpose of this effort was to achieve separation of relatively similar types of steroids, it was considered best to establish some degree of partitioning capability with the solid support. The "surface" of the solid support was, therefore, viewed as requiring partitioning between it and the mobile phase. A program for chemical modification of the surface was then developed.

Silica surfaces were activated with either trichloro or similar silanes (13, 14) containing the desired substituted polar functional groups (*e.g.*, cyano) used in the reaction. When groups other than cyano were thought appropriate [*i.e.*, glycidoxy (15, 16)], they were substituted for the nitrile silanes. Glycidoxy groups could later be modified with other reagents. Approximately 15% of the composite weight of the treated beads was found to represent the increase in weight caused by the reactive silanes. To ensure complete removal of unreacted materials, the treated beads were extracted using a soxhlet extractor for varying lengths of time. In all cases, this was followed by appropriate oven drying. For a column of 100 cm. × 2 mm. i.d., about 3 ml. of this packing material was required. One normally obtains about 52 ml. of treated beads from 20 g. of the uncoated Porasil.

Packing of Columns—Steel columns (100 cm. × 2 mm. i.d.) are equipped with a single fritted steel plug (2-μ porosity) at one end. A small funnel is secured with Tygon tubing to the open end of the steel tube, and the packing material is introduced, 0.5 ml. at a time, into the column. To ensure uniform distribution of the material, the column is continuously tapped on a solid support. When the packing is within 5 mm. of the top of the steel tube, a glass wool plug is inserted securely into place to minimize support losses while handling. Each column is inserted into the system such that the

[1] Milton Ray D HBD1-30R.
[2] Robert Shaw hydropad.
[3] Valco.
[4] Nester/Faust model 250.
[5] Model 350 Turner spectrophotometer.

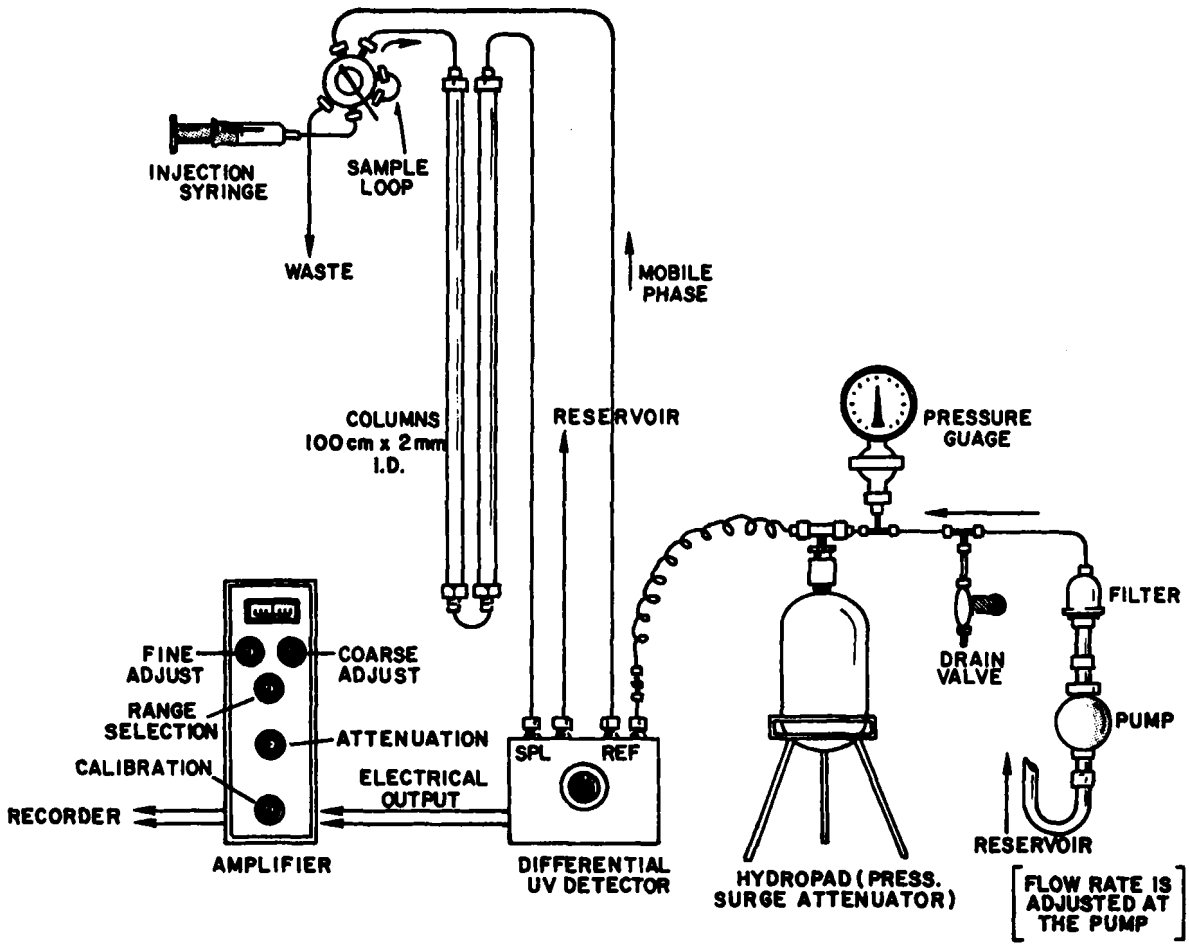


Figure 1—*Schematic layout of high pressure liquid chromatographic system.*

glass plug faces the incoming solvents. This practice tends to minimize the plugging of the fine bore tubing. In a normal exchange of, or addition to, the columns in the system, about 10–15 min. is required.

Analytical Procedure—A 10-ml. disposable syringe is filled with the ointment or cream sample, and the weight is noted to the nearest milligram. A sample weight of 5 g., determined by differential weighings, is then transferred into a 250-ml. separator. Fifty milliliters of an 80% methanol–water mixture is added to 50 ml. of cyclohexane in the separator. Total mixing of the phases is completed by vigorous shaking; subsequently the two layers are permitted to separate and the lower aqueous methanol layer is removed. Repeated extraction of the remaining cyclohexane is completed with portions of a methanol–10% aqueous salt solution (4:1). The latter extract is added to the first methanol extract. About 100 ml. of a 5% potassium alum sulfate solution is added to the methanol phase. This solution is then extracted with 15-ml. portions of chloroform. The latter volumes are dried by pouring the solution through anhydrous desiccant (sodium sulfate). These combined extracts are reduced in volume under nitrogen to a final volume of about 4 ml. The remaining volume is transferred quantitatively to a 10-ml. volumetric flask for a final 10-ml. dilution using chloroform.

The final transfer of this diluted volume of extracted sample is achieved using a 1-ml. gas-tight glass syringe[6] using a Luer lock fitting. It is connected to the hub fitting of the injection valve described in Fig. 1. The sample is transferred to the loop and thereafter injected onto the chromatographic column. This loop has a variable volume ranging from 10 μl. to 1 ml. For much of the current work, three 100-cm. columns were connected in series using the treated Porasil beads. Flow rates varied from 2 to 5 ml./min., and the usual pressure was about 1500 p.s.i. at room temperature with the following mobile phase:

2,2,4-trimethylpentane	69.0% (v/v)
isopropanol	18.5%
acetonitrile	12.5%

Sensitivity ranges most frequently used for the UV detector were about 0.05 × 1 for fullscale absorbance. In all determinations, two or more samples were examined. These values were compared areawise (mm.²) with those peaks of the standard solutions or internal standards used for the comparison. Peak height and half height widths were used in these comparisons.

DISCUSSION

Calibration Data—The data shown in Table I were obtained with a solution of fluocinonide in chloroform by dilution of a

Table I—Calibration Data Using Fluocinonide in Chloroform[a]

Concentration, mg./ml.	Retention Time, min.	Peak Area, mm.² (Sensitivity = 0.05 × 1)	mcg./mm.² × 10^{-3}
0.134	18.8	210	6.38
0.134	18.5	255	5.25
0.267	18.5	465	5.75
0.267	18.5	440	6.07
0.400	18.5	700	5.73
0.400	18.5	670	5.98
0.534	18.0	910	5.87
0.534	18.3	890	6.00

[6] Hamilton 101.

[a] Average response ± *SD* = (5.88 ± 0.33) × 10^{-3} mcg./mm².

Table II—Analytical Data for Fluocinonide Ointment

Sample Weight, g.	Peak Area, mm.²	Fluocinonide, mg. Found	Theory	Percent Recovery
4.930	405	2.38	2.47	96.5
	395	2.32		94.3
			Average	95.4
5.436	480	2.82	2.72	103.5
	476	2.80		103.0
			Average	103.3

g.	mm.²	Found	Theory	Recovery
2.324	344	0.572	0.581	98.4
	348	0.578		99.5
2.509	368	0.612	0.627	97.8
	376	0.608		97.2
			Average	98.2
			$SD = \pm$	0.98

standard stock solution (2.67 mg./ml.) of the steroid in methanol–chloroform (4:1).

Figure 2 illustrates a typical separation using the current column-partitioning system.

Assay data for a fluocinonide ointment preparation (0.05%) is displayed in Table II. In comparison, the assay data relating to a typical cream formulation containing 0.025% fluocinolone acetonide are contained in Table III.

Mobile Phase System—The use of a multiple-component mobile phase system permits wider flexibility in the choice of operating parameters (17). From an inspection of the ternary phase diagram (Fig. 3), the operating range of mutual solubilities of trimethylpentane, isopropanol, and acetonitrile can be seen. The composition of this three-component system that is most frequently used is 10–15% acetonitrile, 15–30% isopropanol, and the remainder trimethylpentane. To a limited extent, the use of the ternary mobile phase permits modification of the retention times of the solutes. These types of correlations are under continued study. If one operates the ternary system in the region of two phases, the problem of light scattering by the second phase becomes a serious source of noise. Therefore, one should select those concentrations of mobile phases that will not lead to phase separation.

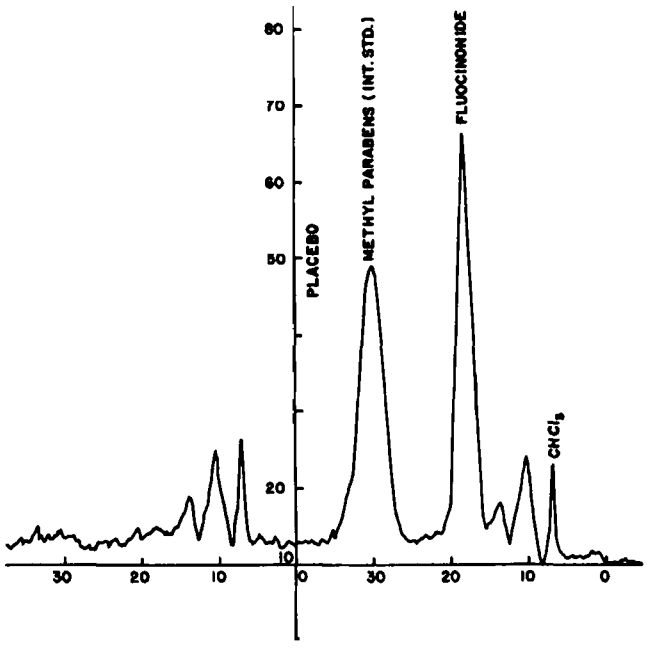


Figure 2—*High pressure liquid chromatogram of fluocinonide* versus *placebo separation.*

Retention Times of Various Steroids—Examination of Table IV illustrates the relative retention times of a number of steroids examined during the present work. By using progesterone (I) as the reference, the data were tabulated (Table V) for several steroids where specific types of substitution were made, and the differences in the retention times are shown.

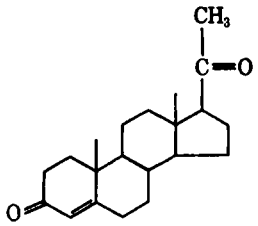
Since the work is only preliminary in nature, claims cannot be made for unambiguous incremental changes due to specific substitution. However, the development of this preliminary work suggests that further efforts to secure possible correlations are desirable.

SUMMARY

In the present paper the utility of employing chemically treated, spherically shaped particles for chromatographic purposes has been established. In particular, partitioning of various steroids between the mobile phase and the solid phase supporting system has been demonstrated. The mechanism for such a partitioning appears to arise from the polar interactions of the solid support with the solute

Table IV—Retention Times of Various Steroids

Number	Compound	Retention Time, min.
	17-acetal with acetone)	18.5
4	Desoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione)	20.4
5	Flurandrenolide (6α-fluoro-11β,16α,17,21-tetrahydroxypregn-4-ene-3,20-dione, cyclic 16,17-acetal with acetone)	22.0
6	Paramethasone acetate (6α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-acetate)	22.0
7	11-Deoxycortisol (17α,21-dihydroxy-4-pregnene-3,20-dione)	23.2
8	Corticosterone (11β,21-dihydroxy-pregn-4-ene-3,20-dione)	26.0
9	Cortisone acetate (17,21-dihydroxypregn-4-ene-3,11,20-trione 21-acetate)	28.2
10	Fluocinolone acetonide (6α,9α-difluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione, cyclic acetal with acetone)	27.6
11	Lactone from Compound 10 (6α,9α-difluoro-11β,16ξ,20ξ-trihydroxypregna-1,4-diene-3-one-21-oic-16-lactone)	64.0
12	21-Aldehyde from Compound 10 (6α,9α-difluoro-11β,16α,17α-trihydroxypregna-1,4-diene-3,20-dione-21-al-16,17-acetonide)	66.0
13	21-Acid from Compound 10 (6α,9α-difluoro-11β,16α,17α,20-tetrahydroxypregna-1,4-diene-3-one-21-carboxy-16,17-acetonide)	>60.0



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molecules (18). The desire to employ chemically bound polar phases was initiated by the inability to obtain satisfactory results from commercially available products. In general, these commercial materials were found to degrade rather quickly and to be of lower overall performance for the present work.

In comparison to other methods for extraction of steroids from various formulations, the single method alluded to in the *Experimental* section was the most satisfying and, therefore, was used exclusively in the present work. In attempting to quantitate the method, internal and external standards have been used with success. At present, the use of internal standards has been found to be more acceptable since smaller intrinsic errors are involved with their use.

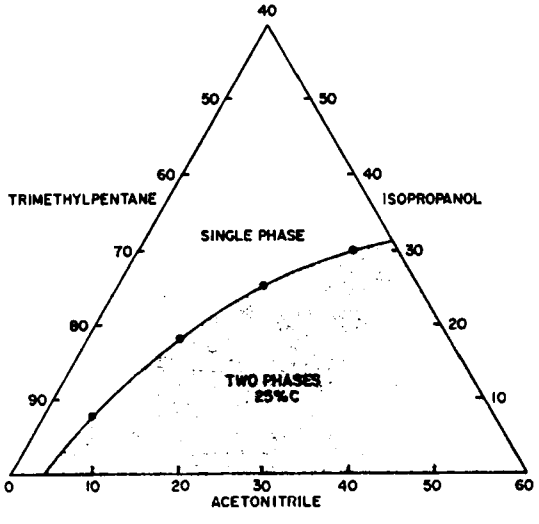

Figure 3—*Ternary phase diagram for mutual solubilities of trimethylpentane, isopropanol, and acetonitrile.*

Table V—Steroid Retention Times

15.8 min., Room Temperature, Progesterone Substitution	Retention Time Increment, Δ_{time}		
	21-OH	11-OH, 17-OH	11-one
21-OH	4.6	—	—
17α-OH, 21-OH	4.6	2.8	—
11β-OH, 21-OH	4.6	5.6	—
11-one, 17α-OH, 21-OH	4.6	2.8	4.0

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