Separation of Some Estrogen Sulfates from Their Oxidation Products

By A. R. HURWITZ, H. J. BURKE, and R. A. MARRA

Quantitative separations of sodium equilin sulfate from sodium equilenin sulfate and sodium 17β-dihydroequilin sulfate from sodium 17β-dihydroequilenin sulfate were obtained on dextran gel columns. It appears that these separations were the result of differences in adsorption character.

ALTHOUGH many quantitative methods are available for the determination of estrogens, it is generally necessary that the free form, the phenolic species, be used. Thus, naturally occurring sulfate or glucuronide esters have been submitted to hydrolysis of one form or another prior to analysis. In many instances direct separation of the conjugates would be preferred.

Steroid conjugates have been isolated from biological fluids by extraction with organic solvents (1), extraction as pyridinium salts into chloroform (3), gel filtration (4–7), and adsorption on charcoal or alumina–diatomaceous earth[1] (2, 8). Group separations of steroid sulfates and glucuronides have been accomplished by adsorption chromatography on alumina (9, 10) or diatomaceous earth (11), paper chromatography (12), thin-layer chromatography (13), and ion exchange (14). Steroid sulfates have been separated by partition chromatography on diatomaceous earth (15) or on methylated dextran gel (16), by ion exchange (14), and steroid glucuronides have been partially separated by gel filtration on dextran gel (7).

Procedures for the separation of estrogen conjugates have been generally concerned with estrone, estradiol, and estriol. Where other estrogens such as equilin, dihydroequilin, equilenin, and dihydroequilenin are also of interest, hydrolytic procedures have been employed prior to analysis.

The objective of this study was the separation of sodium equilin sulfate from sodium equilenin sulfate and sodium 17β-dihydroequilin sulfate from sodium 17β-dihydroequilenin sulfate. These separations were achieved on dextran gel[2] and were found to be quantitative and readily adaptable to ultraviolet determination. It was also possible to separate colored oxidation products of equilenin by this method. Observations made previously and independently elsewhere (17) have shown dextran gels to be useful in the qualitative separation of complex mixtures of estrogen conjugates. Observations on individual estrogens appear to indicate that quantitative separations of complex mixtures may now be possible. Apparently the separations are the result of differences in adsorption on the gel.

EXPERIMENTAL

Reagents—The sodium sulfate derivatives of the various estrogens used were prepared by D. Irvine, Ayerst Laboratories, Division of Ayerst, McKenna and Harrison Limited, Montreal, Canada. Dextran gel filtration media of various degrees of cross linking, G10, G15, and G25, were obtained from Pharmacia Fine Chemicals. Retention of sodium equilin sulfate on G10 and G15 was about the same and greater than that on G25. After these initial trials, G15 was used exclusively. The water used throughout the study was finally distilled from acid permanganate. All other reagents used were of analytical reagent grade and were used without further purification.

Preparation and Operation of Columns—The dextran gel was soaked in an excess of water for at least 4 hr. to allow for complete swelling. The slurried material was added to a 50-ml. buret, 70 cm. × 1.1 cm. i.d., containing a pledget of glass wool. While the water was allowed to drain out and as settling progressed, more slurry was added. Where salt or buffer solutions were used in elution, the column was prepared using these solutions. The columns were left to stand for several hours for completion of gel settling. The excess eluant above the gel surface was removed and the sample solution was carefully pipetted on to the top of the bed. After the sample had passed into the column bed, the eluant was added and maintained at an approximately steady level using a reservoir to decrease the chances of a change in flow rate, about 18 ml. per hour, during the course of the separation.

Eluate fractions of about 2 ml. were collected using an automatic siphon fraction collector[3] or a Volumeter collector.[4] For initial trials an ultraviolet absorbance spectrum, as shown in Fig. 1, was obtained for each fraction using a Cary 14 recording spectrophotometer, whereby the identity and concentration of the eluted materials could be determined. Chromatograms were obtained by plotting observed absorbance, corrected for dilution, against volume eluted.

Preparation of Samples—Fresh solutions of the sodium estrogen sulfate salts were prepared in a minimum amount of water prior to addition to the column. Samples of sodium equilin sulfate in phosphate buffer solution prepared for oxidation studies were applied to the column directly. Partially hydrolyzed samples were extracted with chloroform to remove free estrogens and the aqueous layer containing the conjugates was applied to the column.

RESULTS AND DISCUSSION

The column chromatographic procedure described permits the separation, isolation, and direct quantitative determination of two estrogen conjugates and their oxidation products. Initially, on

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[1] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

[2] Marketed as Sephadex G15 by Pharmacia Fine Chemicals, Piscataway, N. J.

[3] Marketed by G. M. Instrument Co., Greenville, Ill.

[4] Marketed as model V Volumeter with model 270 collector by Instrumentation Specialties Co., Lincoln, Nebr.

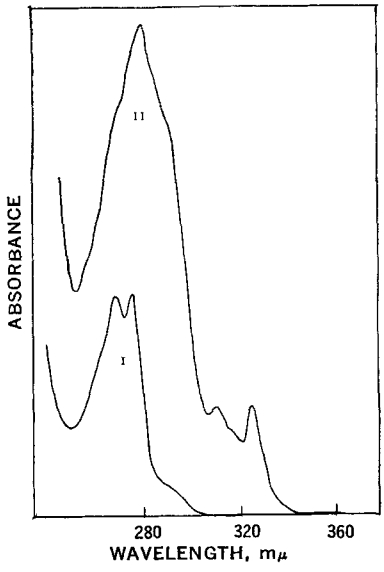


Fig. 1—Comparison of ultraviolet spectra of the sodium salts of equilin sulfate, dihydroequilin sulfate, equilenin sulfate, and dihydroequilenin sulfate. Key: I, equilin and dihydroequilin sulfates; II, equilenin and dihydroequilenin sulfates.

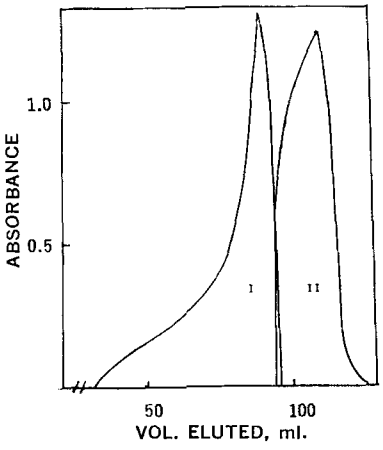


Fig. 3—Separation of sodium 17β-dihydroequilin sulfate from sodium 17β-dihydroequilenin sulfate on dextran gel (14 Gm.) with water as eluant. Key: I, sodium 17β-dihydroequilin, absorbance 275 mμ; II, sodium 17β-dihydroequilenin sulfate, absorbance 325 mμ. Recovery data shown in Table I, 2.

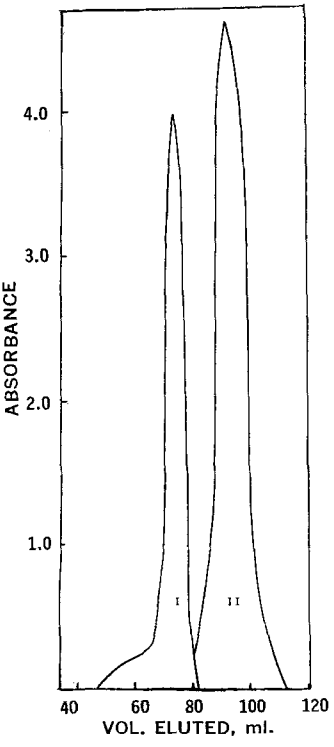


Fig. 2—Separation of sodium equilin sulfate from sodium equilenin sulfate on dextran gel (14 Gm.) with water as eluant. Key: I, sodium equilin sulfate, absorbance at 275 mμ; II, sodium equilenin sulfate, absorbance at 325 mμ. Recovery data shown in Table I, 1.

Table I—Per Cent Recovered for Several Standard Sodium Estrogen Sulfate Samples (Eluant: Water)

Estrogen Moiety	Added, mg.	Found, mg.	Recovery, %
1, Equilin	19.2	19.1	99.5
Equilenin	18.9	18.1	95.8
2, 17β-Dihydroequilin	10.8	10.4	96.3
17β-Dihydroequilenin	5.7	6.0	105
3, Equilenin	3.9	3.9	100
4, Estrone	27.3	28.2	103
5, 17β-Dihydroequilin	17.3	17.1	98.9
6, Equilin	940	873	92.9
Equilenin (trace impurity)		11.9	1.3
7, Equilin	235	219	93.2
Equilenin (trace impurity)		3.0	1.3

a 7-Gm. column with water as the eluant, after the elution of the traces of inorganic salts present in the samples, sodium equilin sulfate and sodium equilenin sulfate were eluted with only partial separation. By increasing the column length with twice the amount of dextran gel, these two compounds were separated, as shown in Fig. 2. Samples subjected to accelerated oxidation gave elution patterns in which the appearance of equilin and equilenin was preceded by the elution of colored oxidation products of equilenin. Similar chromatograms were obtained for sodium 17β-dihydroequilin sulfate and sodium 17β-dihydroequilenin sulfate, as shown in Fig. 3.

A compilation of some theoretical and recovered weight data for various trials is given in Tables I and II. From the data presented in Tables I and II it can be seen that the procedure should also offer a means of purifying large amounts of individual estrogen conjugates. Purification methods would necessitate modification of column dimensions, bed volume, and eluent composition in order to improve capacity. Data of this type will be reported at a later date.

TABLE II—PER CENT RECOVERED FOR STANDARD SODIUM ESTROGEN SULFATE SAMPLES (ELUANT: SALT OR BUFFER SOLUTION)

Estrogen Moiety	Eluant	Added, mg.	Found, mg.	Recovery, %
1, Equilin	5×10^{-4} *M*	10.4	10.7	103
Equilenin	NaCl	2.0	2.2	109
2, Equilin	0.001 *M* pH 4.3 Phosphate	14.4	14.0	97.2
3, Equilin	0.001 *M* pH 10.0 Phosphate	22.8	21.7	95.2
4, Equilin	0.01 *M* NaCl	94.3	59.8	63.4[a]
Equilenin		19.3	...	...

[a] Elution incomplete after 150 ml. of eluate collected.

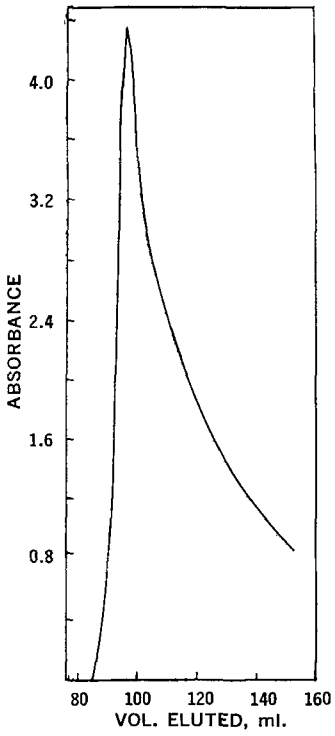


Fig. 4—Elution of sodium equilin sulfate from dextran gel (14 Gm.) with 0.01 M *sodium chloride as eluant. Recovery data shown in Table II,* 4.

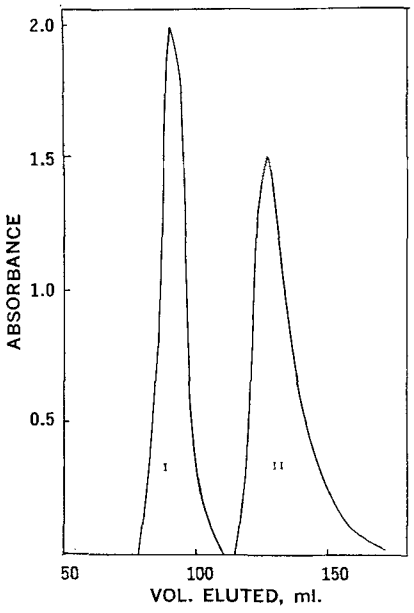


Fig. 5—Separation of sodium equilin sulfate from sodium equilenin sulfate on dextran gel (14 Gm.) with 5×10^{-4} M *sodium chloride as the eluant. Key:* I, *sodium equilin sulfate, absorbance 275 mμ;* II, *sodium equilenin sulfate, absorbance 280 mμ. Recovery data shown in Table II,* 1.

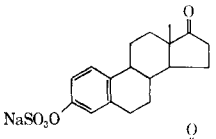
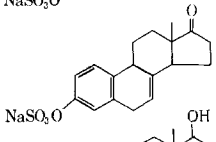
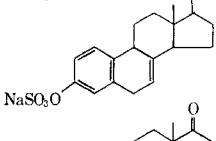
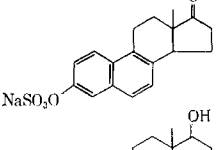
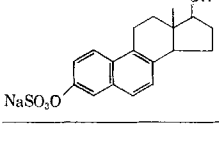
There are three possible factors to be considered when interpreting elution patterns on dextran gels: gel filtration, adsorption, and ion exchange. Simple gel filtration depends on the molecular size with the larger molecules being eluted first. Aromatic, heterocyclic, or basic compounds have been observed to adsorb reversibly with the degree of adsorption increasing with the amount of cross linking in the gel. Dextran gels also exhibit a weak ion-exchange property where the active functional groups are available carboxylic acid groups.

In separations that were performed with small amounts of sodium chloride or buffer present using water as the eluant, the electrolyte was eluted prior to the elution of the estrogen conjugate. Varying the pH (4.3 and 10.0, 0.001 *M* phosphate) of the eluant had little or no effect on the elution pattern. When 0.01 *M* sodium chloride was used as the eluant, the retention of sodium equilin sulfate on the gel was increased, as shown in Fig. 4, while higher salt concentrations resulted in nearly complete retention. From these observations, it would appear that the major factor operative in the observed separations was adsorption.

Proper selection of eluant electrolyte concentration slightly improves the separation of sodium equilin sulfate from sodium equilenin sulfate, as shown in Fig. 5. A sample of sodium equilin sulfate was prepared and subjected to accelerated oxidation conditions until only sodium equilenin sulfate and further oxidation products remained as determined by equilin and equilenin color tests. To this sample, 33.8 mg. of sodium equilin sulfate was added and the mixture placed on a dextran gel column for separation using 5×10^{-4} *M* sodium chloride as the eluant. Colored oxidation products were either irreversibly adsorbed on the gel or were eluted initially. Sodium equilin sulfate was recovered at 33.5 mg. or 99%.

The difference in the adsorption and elution be-

TABLE III—APPROXIMATE ELUATE VOLUME AT PEAK APPEARANCE IN THE CHROMATOGRAMS OF SEVERAL SODIUM ESTROGEN 3-SULFATE SALTS (14-GM. COLUMN, BED VOLUME, 45. ML.)

Estrogen	Vol. at Peak (ml.) Eluant: H_2O	Eluant: 5×10^{-4} M NaCl
 Estrone	70	73
 Equilin	75	90
 17β-Dihydroequilin	90	100
 Equilenin	95	125
 17β-Dihydroequilenin	110	160

havior of the individual estrogen salts and the effect of dilute aqueous sodium chloride eluant can be seen from the comparison of eluate volumes at peak appearance, as shown in Table III.

With due consideration of experimental conditions, it should be possible to modify this chromatographic procedure for the quantitative separation of complex mixtures of sodium estrogen sulfate salts. Preliminary work toward this end is already under way (17).

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Nigerian Plants III. Phytochemical Screening for Alkaloids, Saponins, and Tannins

By GEORGIA J. PERSINOS* and MAYNARD W. QUIMBY

Fifty species of Nigerian plants were evaluated for the presence of alkaloids, saponins, and tannins. Of these, 24 contained alkaloids, 43 contained tannins, and 16 contained saponins.

THIS WORK is a continuation of the investigation carried out on plants used as medicinals by a pagan tribe, the Anagutas, living on the Jos Plateau in northern Nigeria, and the Hausas, living throughout northern Nigeria. Since plants occupy an important and sometimes central role in the religious beliefs and social practices of these peoples, an investigation of certain of these plants[1] has been undertaken

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[1] The plant specimens were authenticated by Dr. Quimby and were made a part of the botanical collections of the Massachusetts College of Pharmacy.

EXPERIMENTAL

Preparation of Extracts—The screening procedures for alkaloids, saponins, and tannins were adapted from those described by Wall *et al.*(1, 2). An extract of each plant was prepared by refluxing 10 Gm. of the air-dried milled plant sample with 100 ml. of 80% ethanol for 1 hr. Each extract was then cooled to room temperature, suction-filtered, and washed with sufficient 80% ethanol to bring the volume of filtrate to 100 ml.

Alkaloids—Twenty milliliters of each extract, equivalent to 2 Gm. of dried plant material, was evaporated to dryness using a steam bath and the residue was stirred with 5 ml. of 1% aqueous hydrochloric acid. One milliliter of the filtrate was treated with a few drops of Mayer's reagent and a separate 1-ml. portion was treated similarly with silicotungstic acid reagent (12% aqueous). Precipitation or turbidity with either of these reagents was taken as preliminary evidence for the presence of alkaloids in the extract being evaluated. A confirmatory test designed to remove nonalkaloidal compounds capable of eliciting "false-positive" reactions with either of these reagents was conducted