Fluorometric Determination of Dihydrostreptomycin in a Complex Pharmaceutical Suspension After Separation by Alginic Acid

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Abstract □ A simple and sensitive method for the determination of dihydrostreptomycin has been developed. The method entails the recovery of dihydrostreptomycin by cation exchange with subsequent fluorometric determination.

Keyphrases □ Dihydrostreptomycin suspension—analysis □ Cation-exchange—dihydrostreptomycin separation □ Fluorometry—analysis

The separation of an antibiotic which is frequently dispensed together with a wide range of compounds is a difficult problem. The usual method of analysis is the biological assay. Because of the lack of rapid method for the routine control of complex preparations containing dihydrostreptomycin, the present paper describes a method of extracting dihydrostreptomycin from kaolin–pectin suspension and determining its concentration using a fluorometric procedure.[1] After the extraction, dihydrostreptomycin is further purified from the other interfering substances by column chromatography using alginic acid. Formaldehyde-treated alginic acid has been shown to act as a cation-exchange medium for the separation of organic bases from their solutions (1). The subsequent fluorometric procedure was selected as the method of choice for this assay because of its simplicity and brevity compared with other analytical methods (2–6). It allows the use of relatively small quantities of samples and is surprisingly free from interferences caused by various colorings and constituents of pharmaceutical preparations which often nullify the use of other chemical methods. A colorimetric method using oxidized nitroprusside is not applicable here because of NaCl formed with 0.1 *N* hydrochloric acid eluate. In this method NaCl reduces the intensity of color (7, 8). Had it been possible, dihydrostreptomycin and sulfaguanidine, total guanidines, would have been determined without separation, and sulfaguanidine then determined by Bratton-Marshall assay, the dihydrostreptomycin being calculated by difference. The Bratton-Marshall procedure (9) however, would have reacted with sulfaguanidine as well as sulfadiazine present in the suspension as a therapeutic ingredient (1.5 g./30 ml.). The fluorometric assay described below is based on the characteristic reaction between ninhydrin and guanidine groups reported by Conn and Davis (10). These authors found that ninhydrin in strongly alkaline media yields highly fluorescent products with guanidine and *N*-substituted guanidines. Since dihydrostreptomycin has two guanidino groups in the streptide portion of the molecule, the method was adaptable for assay. The same authors stated that the condensation compounds with ninhydrin produced two excitation peaks at 305 and 390 mμ. At 390 mμ the fluorescence was twice as intense as that at 305 mμ. Fluorescence was measured at 495 mμ, using the Farrand spectrofluorometer. Following the same principle of reaction, this method for dihydrostreptomycin was developed using a Beckman ratio fluorometer containing a sandwich excitation filter with a peak at 405 mμ and a sandwich emission filter with a peak at 515 mμ. The fluorescence reaches a maximum after 20–21 min. and is stable for 4 min.

EXPERIMENTAL

Procedure for Suspension Extraction and Chromatography—*Column*—Glass column 35 × 2 cm. with stem (5 cm.) fitted with buret key.

Reagents—Cation-exchange resin alginic acid, 40–100 mesh; pure ethanol; 40% ethanol v/v in water; 2 *N* hydrochloric acid in water; 0.1 *N* hydrochloric acid in water; 0.005 *N* hydrochloric acid in water. Except where otherwise specified, all reagents were of B.D.H. analar quality.

Column Preparation—A slurry of alginic acid, about 5 g., was made in water and allowed to soak 4 hr. The slurry was poured into a glass column fitted with a glass wool plug, and allowed to settle. The column was washed with 2 *N* HCl until the absorbance of the eluate (pathlength 1 cm.) was less than 0.005 at 258.5 mμ, and then washed with distilled water until the eluate was neutral to the litmus solution. Finally, 40 ml. of 40% ethanol in two portions of 20 ml. each was passed through the column. At this stage the material in the column contracted and it was compacted by pressure of the glass wool plug on top.

Sample Treatment—An amount of suspension corresponding to approximately 50 mg. of dihydrostreptomycin was accurately weighed into a 50-ml. volumetric flask, 0.1 *N* HCl was added to the 50-ml. mark and the mixture mechanically shaken for 15 min. The mixture was centrifuged to obtain a clear solution and a 20-ml. aliquot was pipeted into a 50-ml. volumetric flask. Pure ethanol, 20 ml., was added and the pH of the solution adjusted to about 4 with dilute ammonia (1:50). The volume was brought to the 50-ml. mark with distilled water. An aliquot, 10 ml., was pipeted into the prepared alginic acid column and the solution was allowed to pass through the column at a rate of 4 ml./min. The column was then washed with 50 ml. 40% ethanol in two portions, also at the rate of 4 ml./min., and finally with 400 ml. of distilled water at as fast a rate as possible. The aminopentamide, contained in the products assayed and held by alginic acid together with dihydrostreptomycin, was eluted from the column with 0.005 *N* HCl. A total of 100 ml. was collected.[2] After this dihydrostreptomycin was eluted from the column with 0.1 *N* HCl at a rate of 2 ml./min. and the eluate collected into a 250-ml. volumetric flask. This is the sample solution containing approximately 16 mcg. of dihydrostreptomycin/ml. which was used in the fluorometric assay.

[1] The analysis was applied to products containing 300 and 500 mg. of dihydrostreptomycin/30 ml. and marketed, respectively, as Sulpec and Sulfaguanidine Cum Dihydrostreptomycin.

[2] If the determination of aminopentamide was required, the eluate was collected into a 100-ml. volumetric flask and the absorbance read at 258.5 mμ.

Table I—Dihydrostreptomycin Recovery

Mixture	Amount of Dihydrostreptomycin, mg./30 ml.	Recovery Using Water, %	Recovery Using 0.1 *N* HCl, %
1	300	89.20	100.22
2	300	86.40	101.02
3	300	83.10	98.72
4	300	84.50	100.22
5	300	86.80	98.68
6	500	89.20	99.77
7	500	88.40	100.99
8	500	85.60	100.68
9	500	88.80	98.66
10	500	88.20	100.96

Fluorescence Assay Procedure—*Apparatus*—A ratio fluorometer, Beckman, equipped with mercury lamp 101631 and Pyrex sample tubes (12 × 75 mm.). Two UG11 filters in the primary filter holder on the reference side of the lamp, a sandwich excitation filter containing CS5-58 + DS7-51 + CG13 filters in the primary filter holder on the sample side of the lamp, and a sandwich emission filter containing CS3-71 + CS4-72 filters in the secondary filter holder were used.

Reagents—Ninhydrin 1% in ethanol; potassium hydroxide 10% in ethanol.

Standard Solutions—The following solutions were prepared with suitable reference standard: (*a*) dihydrostreptomycin sulfate 20 p.p.m. in 0.1 *N* hydrochloric acid; (*b*) quinine sulfate 20 p.p.b. in 0.1 *N* sulfuric acid; (*c*) sodium fluorescein 50 p.p.b. in water.

Dihydrostreptomycin Determination—With quinine sulfate solution in the reference chamber, distilled water was used to adjust the meter to zero and fluorescein solution to set the instrument to read 70. One-milliliter aliquots of 10% potassium hydroxide were pipeted into 3- × 20-ml. glass-stoppered test tubes (low actinic glass). To each tube, 2-ml. aliquots of sample solution, dihydrostreptomycin standard solution, and 0.1 *N* HCl (the blank) were respectively added. One milliliter of 1% ninhydrin alcoholic solution was added to each tube, the contents gently mixed, and allowed to stand. The fluorescence of each solution was measured after exactly 21 min. and the concentration of the dihydrostreptomycin in the sample was calculated from the fluorescence of its standard solution.

RESULTS AND DISCUSSION

The liquid extraction-column chromatography method described here permits the isolation and quantitative determination of dihydrostreptomycin in kaolin–pectin suspension. Therefore, the first step of the method consists of a desorption procedure in which 0.1 *N* hydrochloric acid removes dihydrostreptomycin from kaolin. Water was ineffective to extract the antibiotic because of the strong adsorptive properties of kaolin at pH about 7 of the suspension.

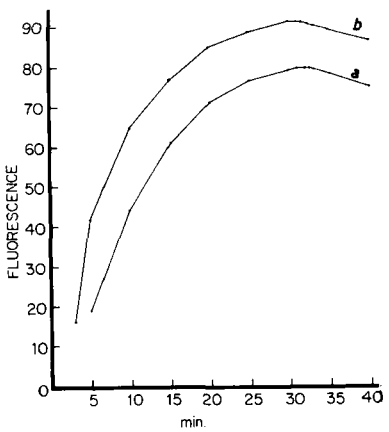

Figure 1—*Development and stability of fluorophor. Key:* a, *dihydrostreptomycin sulfate (12.25 mcg./ml.);* b, *sulfaguanidine (2 mcg./ml.).*

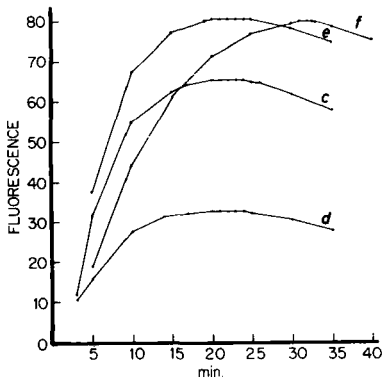

Figure 2—*Development and stability of fluorophor. Key:* d, *dihydrostreptomycin sulfate, 5 mcg./mg., total volume of reaction, 4 ml.;* c, *dihydrostreptomycin sulfate, 10 mcg./ml., total volume of reaction, 4 ml.:* e, *dihydrostreptomycin sulfate,* 12.25 *mcg./ml., total volume of reaction,* 4 *ml;* f, *dihydrostreptomycin sulfate,* 12.25 *mcg./ml., total volume of reaction,* 7 *ml.*

Differences in recovery of dihydrostreptomycin from 10 mixtures, prepared in the laboratory and containing dihydrostreptomycin sulfate, sulfaguanidine, sulfadiazine, aminopentamide sulfate, kaolin, and pectin, can clearly be seen in Table I when dissolved in water and when treated with 0.1 *N* hydrochloric acid.

Dihydrostreptomycin was further purified by alginic acid column chromatography from sulfaguanidine, which exhibits fluorescence 10 times as intense as that of dihydrostreptomycin in the same condition of reaction. Figure 1, where the fluorescence was plotted against time, shows the results of the first series of experiments designed to evaluate the reliability of the fluorometric procedure when 5 ml. of dihydrostreptomycin standard solution in 0.1 *N* HCl and containing 17.15 mcg./ml. was mixed with 1 ml. of 1% ninhydrin and 1 ml. 10% potassium hydroxide solutions, respectively. The fluorescence was found to reach its maximum after 31 min. with a stability of 1.5 min. Figure 1 also shows when, for comparison, the same procedure was applied to a solution of sulfaguanidine in 0.1 *N* HCl containing 2 mcg./ml. In applying the procedure using different volumes of dihydrostreptomycin standard solution, it was found that a correlation existed between the time required for the fluorescence to reach its maximum and the volume taken. The procedure was, therefore, critically investigated to find the cause of these variations. To prove that real influence occurs between the volume of standard dihydrostreptomycin solution taken and the time-fluorescence evolution, a set of measurements was carried out. The results of this investigation can be observed in Fig. 2.

The fluorescence-time curves of solutions containing in the same volume of reaction different amounts of dihydrostreptomycin show similar behavior. This is the case of Curves C and D (Fig. 2) which represent the time-fluorescence evolution of solutions containing 10 and 6 mcg./ml. of dihydrostreptomycin sulfate, respectively. Instead the time-fluorescence curves of solutions containing the same amounts of dihydrostreptomycin/ml., dissolved in different volumes, appear to be dissimilar. Here it is the case of the Curves E and F (Fig. 2) which represent the behavior of solutions both containing 12.25 mcg./ml. of dihydrostreptomycin sulfate.

At this stage it seems sound to suppose that the variability of the time of maximum fluorescence and its constancy, reflects the effect of reduced catalytic properties of 10% KOH solution, in part neutralized by different volumes of 0.1 *N* HCl used to prepare standard dihydrostreptomycin solutions. Further attention was focused on the possibility of finding the most suitable dilution which would give more fluorescence stability, in addition to more accurate and reproducible results. The most favorable system was found when 2 ml. of standard and sample dihydrostreptomycin solutions were used.

It is reasonable to assume that the interaction between 0.1 *N* HCl and 10% KOH solution does not affect the reliability of the fluorometric assay as long as standard, sample, and blank solutions contain hydrochloric acid of the same strength, and the reaction takes place in the same volume. To determine these optimum conditions, analyses were carried out using the following supplementary stan-

Table II—Dihydrostreptomycin Recovery Data Using Fluorometric and Microbiological Methods

Product	Claim, mg./30 ml.	Found, Fluorometric Assay	Found, Microbiological Method
A	300	310	307
B	300	311	309
C	300	340	338
D	300	305	310
E	500	525	526
F	500	550	563
G	500	524	521
H	500	541	538

dard solutions containing 10, 17.15, and 24.50 mcg./ml. of dihydrostreptomycin sulfate in 0.1 *N* HCl.

This assay as well as the above-mentioned one regarding the guanidino groups may be applied to dihydrostreptomycin as well as to streptomycin. It does not differentiate between the biologically active product and the degradation product. Although the assay is not specific, it may be carried out in such laboratories where there are no facilities for bacteriological assay and where the potency of the dihydrostreptomycin sulfate used is known. It might also be useful for a rapid control of the suspensions during their production. Apart from these considerations the method has the advantage of being adaptable for routine assay work, whereas the biological assay for this preparation is lengthy and may yield inaccurate results in inexperienced hands.

The method besides, it not restricted to the products examined, but might be used in modified pharmaceutical preparations of a similar nature while a microbiological method is not usually the best procedure to follow in all circumstances. A number of cations and anions change the reactivity of dihydrostreptomycin and streptomycin in a microbiological test. The confidence in this assay is based upon agreement between results found chemically and biologically. The results of this fluorometric assay applied to fresh commercial products compared to those obtained by microbiological method (11) using *B. subtilis* as test organism, are shown in Table II. Finally the fluorescence was found to be linearly proportional to the concentrations of dihydrostreptomycin sulfate between 5 and 12 mcg./ml. The standard deviation of standard was ± 1.0%.

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Oximino Ethers: Dialkylaminoalkyl Derivatives

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Abstract □ Dialkylaminoalkylether derivatives of a variety of aromatic, heterocyclic, and steroidal ketone oximes were prepared and examined for their antiandrogenic effects. The compounds were prepared by two general methods. The diethylaminoethyl ether of 2-benzoylpyridine oxime exhibits interesting antiandrogenic activity in rats but the effective dose is very close to the toxic dose. In the course of this investigation other pharmacological properties of the compounds were noted.

Keyphrases □ Oximino ethers—dialkylaminoalkyl derivatives □ Dialkylaminoalkyl derivatives, oximino ethers—synthesis □ Antiandrogenic activity—oximino ether derivatives □ Pharmacological screening—oximino ether derivatives

In the routine screening of compounds for their endocrine effects, it was noted that compounds containing the grouping $=N—O—(CH_2)_n—N—(R)_2$ lowered the weights of the sex organs of male rats, indicating a possible antiandrogenic effect.[1] To explore this interesting lead, a series of dialkylaminoalkyl oximino ethers was prepared from a variety of aromatic, heterocyclic, and steroidal ketones and their effects on the preputial glands, seminal vesicles, and prostate gland were examined. Other biological parameters of the oximino ethers were examined as described below.

The compounds listed in Tables I and II were prepared by two general methods as shown in Scheme I.[2] Method A was used in those cases where the ketone oxime was readily available (1–4) and did not contain a reactive functional group. In case of steroidal ketones containing the reactive hydroxyl group, Method B was employed. The *O*-alkylated hydroxylamine derivative

[1] The biological data herein reported was obtained by Drs. S. Tolksdorf, R. Neri, and R. Taber of the Biological Division of the Schering Corp.

[2] After this work was completed, similar procedures have recently been described. See *Reference 1.*