Separation and Determination of Polymyxin B in Formulations

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A method for the separation and the determination of polymyxin B in formulations is described. It is based on the absorption of the antibiotic on a cation-exchange resin with subsequent elution with methanol–aqueous sodium chloride solution and the reaction of polypeptide with ninhydrin.

NUMEROUS microbiological methods (1, 2, 6–9) have been described for the determination of polymyxin B but little work has been carried out on the chemical assay of the antibiotic. In the fractionation of polymyxins, a spectrophotometric measurement at 259 mμ has been used (4). A gravimetric method has been described for the determination of polymyxin B by precipitation as phosphotungstate (3). The biuret reaction has been suggested for the determination of polymyxin in fermentation broths (5).

In formulations, in addition to polymyxin B, there frequently are substances present which interfere in a spectrophotometric or colorimetric determination of polypeptides. In these cases, it is essential to separate polymyxin B from impurities and other ingredients of a protein character. The proposed method is based on the separation of polymyxin B by means of a cation-exchange resin and the determination of the antibiotic with the ninhydrin reagent.

EXPERIMENTAL

Method

Reagents and Equipment—*Amberlite IRC-50 resin*, pH 7.0, sodium form; *methanolic aqueous sodium chloride solution*, 10% sodium chloride (10 Gm.) is dissolved in methanol–water (1:1); *Polymyxin B standard solution*, polymyxin B equivalent to about 100,000 units is dissolved in 25 ml. water; *Sodium acetate buffer*, pH 5.5 sodium acetate trihydrate (680 Gm.) is dissolved in 0.5 L. of distilled water and to the cooled solution 125 ml. of glacial acetic acid is added. The volume is made up to 1 L. with water. The reagent is stored at 5°; *Ninhydrin reagent*, ninhydrin (0.2 Gm.) and 25 mg. of hydrindantin are dissolved in 12 ml. of methyl cellosolve. Sodium acetate buffer 5.5 (2.5 ml.) is added and the reagent is stored in a low actinic flask. The reagent is prepared daily; *Chromaflex column*, size A-3, made by Kontes, catalog No. K-42082.

Procedure—A sample equivalent to 100,000 units of polymyxin B is weighed in a 100-ml. beaker. About 15 ml. of water is added and the solution is mixed. The solution is filtered through a sintered-glass filter of medium porosity having a diatomaceous earth[1] precoat of a 1/8 in. thickness. The beaker and filter are washed with 10 ml. of water and the wash is combined with the sample. Two columns are packed with wet IRC-50 resin until a height of 12.7 cm. is obtained. Water is withdrawn from the columns to about 0.6 cm. above the resin and the standard and sample solutions are transferred quantitatively to the corresponding columns. The flow of the effluent is regulated to 1 ml./min. After all of the solutions have been passed through the columns, the resin beds are washed with 100 ml. water at the rate of 5 ml./min. Polymyxin B is eluted with methanolic aqueous sodium chloride solution at the rate of 1 ml./min. until 100 ml. of the eluate is obtained.

Some samples may require the following purification: samples and standard solutions (25 ml.) are transferred to suitable flasks and methanol is evaporated *in vacuo* at about 5 mm. Hg and 60°. The remaining aqueous solutions are transferred quantitatively to separators and polymyxin B is extracted with 15 ml. and 2 × 5 ml. of butanol. One milliliter of butanol solution is taken for the ninhydrin reaction.

One milliliter of methanolic sodium chloride solution or 1 ml. of butanol solution (blank), 1 ml. of sample, and 1 ml. of standard eluates are transferred to 17.9 cm. test tubes. To each tube, 1 ml. of ninhydrin reagent is added and the tubes are incubated in a boiling water bath for exactly 15 min. To each tube 15 ml. of ethanol–water (1:1) is added and the solutions are mixed. The absorbance is measured at 570 mμ on a spectrophotometer using 1-cm. cells against the reagent blank.

The polymyxin B content is calculated as follows:

$$\frac{A \times C \times P \times S}{B \times D} = \text{polymyxin B, units/ml.}$$

where:

A = absorbance of sample
B = absorbance of standard
C = weight of standard, mg.
D = weight of sample, Gm.
P = potency of standard, units/mg.
S = specific gravity

DISCUSSION AND RESULTS

In formulations, in addition to polymyxin, other various organic and inorganic substances are usually present. For the quantitative determination of

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[1] Celite, Johns-Manville Products Corp., New York, N.Y.

TABLE I—ASSAY POLYMYXIN B IN EXPERIMENTAL FORMULATION

Run No.	Polymyxin B, units/ml. Method Biological	Chemical
1	4660	4880
2	4780	4890

polymyxin, it is essential to separate the antibiotic from impurities, especially from those of a proteinaceous nature. In this study, an experimental formulation consisting of neomycin, amphotericin B, polymyxin B, ethylenediamine, and other ingredients was assayed. No chemical methods for the determination of polymyxin B in the presence of neomycin and other amines have been found in the literature. Methods used in the isolation and the purification of polymyxin on a preparative or commercial scale have not been previously applied to analytical procedures.

Polymyxin B and neomycin are adsorbed on cation-exchange resin and their separation by means of ion exchange techniques was investigated. Various solvents were investigated for their efficiency in the elution of polymyxin from the resin. Aqueous hydrochloric acid solutions elute about 80% of the polymyxin, but also about 80% of the neomycin. With methanolic solution of hydrochloric acid, 100% elution of polymyxin and 85% of neomycin is obtained. With ammonium hydroxide only 60% of the polymyxin is eluted and 100% of the neomycin. With the 10% methanol aqueous sodium chloride solution, polymyxin B can be eluted quantitatively from a column while neomycin remains on the resin. With 5% methanol aqueous sodium chloride, only 40% of the polymyxin was eluted.

In the separation of polymyxin B and neomycin in an experimental suspension formulation stored at room temperature for 2 years, an incomplete purification of the polymyxin was obtained. A further purification was attained by the extraction of polymyxin from aqueous solutions with butanol.

Only a limited study has been carried out on the correlation of the proposed chemical assay and the standard microbiological assay. The results of this correlation are shown in Table I.

The correlation of the chemical method with the biological assay was studied by the degradation of polymyxin B in 0.1 *N* NaOH and 0.5 *N* NaOH at room temperature (25°). The samples were withdrawn periodically over 96 hr. and assayed by both the chemical and microbiological methods. About 50% of polymyxin B was degraded in 0.1 *N* NaOH in 24 hr. No further degradation was observed over the period of 72 hr. The degradation of polymyxin B in 0.5 *N* NaOH was rapid. After 24 hr. no polymyxin B activity was found by the microbiological method. About 45% polymyxin was found by the chemical method over the period of 72 hr. It appears that the chemical method does not correlate with the microbiological assay when polymyxin B is degraded in 0.5 *N* NaOH.

When a known amount of polymyxin B was added to the formulation samples, an average recovery of 98% was obtained.

This method is useful as a labeling assay of polymyxin in pharmaceutical formulations. The use of this assay as a stability method has not been explored.

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Keyphrases

Polymyxin B formulations—analysis
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Ninhydrin reagent