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Column Chromatographic Determination of Polymyxin B Sulfate

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Abstract □ A column chromatographic method for the quantitative determination of polymyxin B sulfate in bulk samples and pharmaceutical formulations is presented. The method is based on the absorption of polymyxin B on a weak cation-exchange resin and elution with an ionic strength gradient. Polymyxin B is determined in the eluate with ninhydrin by means of an Auto-Analyzer. The results are in good agreement with those obtained by the microbiological method.

Keyphrases □ Polymyxin B sulfate—analysis, column chromatography and ninhydrin, compared to microbiological method □ Column chromatography—analysis, polymyxin B sulfate □ Ninhydrin—analysis, polymyxin B sulfate after column chromatography

Polymyxin B sulfate is a cyclic heteropeptide antibiotic, valuable in the treatment of infections caused by Gram-negative bacteria. It is frequently combined with other antibiotics to extend the antimicrobial spectrum.

The microbiological diffusion method (1) is the common method for assaying polymyxin B sulfate bulk samples and pharmaceutical formulations. Several attempts have been made to develop chemical assay methods. The procedures that can be used include: colorimetric determination with ninhydrin reagent (2), biuret reagent (3), and Folin reagent (4); gravimetric determination with phosphotungstic acid (5); UV spectrophotometric determination (6); amino acid (7) or fatty acid (8) analyses; and methods based on optical rotation (9).

Assay methods used in the pharmaceutical control of drugs must be specific and stability indicating. In the case of antibiotics, a chemical method is valueless if the results differ from those obtained by the microbiological method. The above-mentioned chemical assay methods were tested (10). Results from these methods were found to be in poor agreement with microbiological data, especially with samples containing relatively large amounts of degraded polymyxin B.

Polymyxin B with low potency was prepared by heating active samples. A decrease in activity of up to 30% was obtained by heating polymyxin B sulfate powder in a closed bottle at 100° for 3–21 days. A stronger fall in activity (up to 80%) was obtained by heating a 2% aqueous solution of polymyxin B sulfate in a sealed tube at 100° for from 6 hr. to 7 days.

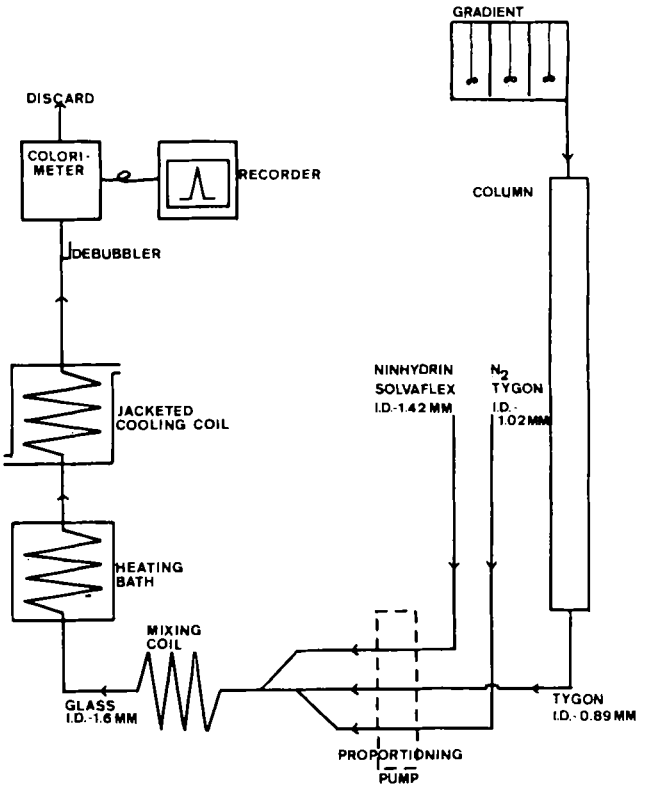

Figure 1—*Flow diagram of apparatus for automatic determination of polymyxin B sulfate.*

Only optical rotation methods had a certain degree of stability-indicating value. The Cotton-curve obtained with polymyxin B as a nickel complex showed a change in shape if degraded polymyxin B was present. Nevertheless, this method could not be used as an assay method. Ivashkiv (11) described a chromatographic method based on the separation of polymyxin B and impurities by means of a cation exchanger. The antibiotic was eluted with a sodium chloride, methanol, and water mixture and determined with ninhydrin. Although in some samples this method appeared

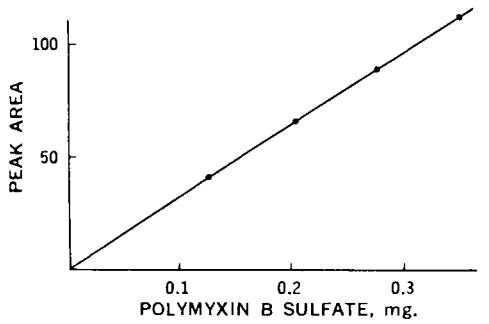

Figure 2—*Plot showing the linear relationship between peak area and amount of polymyxin B sulfate.*

stability indicating, no agreement was obtained between biological and chemical results in many cases. Moreover, the accuracy and the reproducibility of this method were very poor.

A specific identification method of polymyxin B, developed in this laboratory, was published previously (8). This report presents a column chromatographic assay. Polymyxin B is taken up by a weakly acid polysaccharide ion-exchange resin with carboxyl groups[1] in citrate buffer, pH 6.2, and eluted by a continuous ionic strength gradient at the same pH. The antibiotic is determined in the eluate with ninhydrin by means of an AutoAnalyzer[2]. The ninhydrin reaction was chosen because it is the most sensitive color reaction for polymyxin B sulfate.

EXPERIMENTAL

Instrumentation—An AutoAnalyzer[2] consisting of an autograd, a proportioning pump, a heating bath, a colorimeter (with a 570-nm. interference filter and 15-mm. flow cell), and a recorder was used. The column (Pharmacia) was 60 cm. in length and 0.9 cm. in internal diameter. This system is illustrated in Fig. 1.

Materials—Cation-exchange resin[1] was used. All chemicals used were reagent grade.

The ninhydrin reagent consisted of: ninhydrin, 4 g.; hydrindantin, 0.3 g.; methoxyethanol (peroxide free), 280 ml.; 4.0 *N* sodium acetate buffer, pH 5.51, 70 ml.; and distilled water, 150 ml. This reagent is made with the following precautions (12). Ninhydrin and hydrindantin are dissolved in 130 ml. methoxyethanol in a dark bottle, and the air is displaced by bubbling oxygen-free nitrogen through it for 15 min. Then 70 ml. of 4 *N* acetate buffer are added and bubbling of nitrogen is continued for 30 min. The solution is then diluted with 300 ml. of a 50% (v/v) aqueous solution of methoxyethanol. Nitrogen is bubbled through for about 30 min. more. This reagent is always kept under nitrogen.

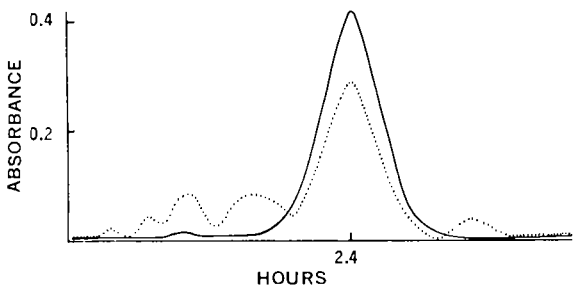

Figure 3—*Chromatogram of polymyxin B sulfate (0.25 mg.). Key: ——, 8400 I.U./mg.; and . . . , 5700 I.U./mg.*

[1] Sephadex CM-C 25, Phamacia Fine Chemicals.
[2] Technicon Chromatography Corp.

Table I—Polymyxin B Sulfate (Bulk Samples)

Sample Number	Assay, % —Potency against Standard— Chemical Method	Microbiological Method
Standard	100	100
1	91	90
2	86	88
3	97	97
4	93	94
5	104	104
6	79	80
7	71	70
8	54	57
9	35	33
10	<20	<20

Buffer Solutions—*4.0 N Acetate Buffer, pH 5.51*—This consisted of sodium acetate (164 g.) and glacial acetic acid (50 ml.) diluted to 500 ml. with distilled water.

Citrate Buffer, pH 6.2—Four citrate buffer solutions were used: A (0.05 *M* sodium chloride), B (0.75 *M* sodium chloride), C (1 *M* sodium chloride), and D (1.25 *M* sodium chloride). They consisted of 7.2 ml. of 0.1 *M* citric acid, 42.8 ml. of 0.1 *M* sodium citrate, and 0.292, 4.383, 5.845, or 7.307 g. sodium chloride, respectively, diluted to 100 ml. with distilled water.

Sample Preparation—The sample must contain 0.2–0.3 mg. polymyxin B sulfate in a maximum volume of 10 ml.

For the determination of polymyxin B sulfate bulk samples, 1.0 ml. of a 0.02% aqueous solution is transferred on the column. If polymyxin B sulfate occurs in solution (*e.g.*, ear drops), a suitable volume of the solution is transferred on the column without further treatment. For the determination in tablets, hydrophilic ointments, or aerosol powders, the antibiotic is extracted with water. Ointments with a paraffin base are dissolved in chloroform. The chloroform is transferred into a separator, and the polymyxin B sulfate is extracted with water.

Column Preparation—The cation-exchange resin (acid form) is slurried in citrate buffer A, heated on a waterbath for 2 hr., and washed with citrate buffer A until the pH of the ion exchanger is 6.2. The column is packed with the slurry until a height of 55 cm. is obtained. Then 50 ml. of citrate buffer A is run through the column. Excess buffer is withdrawn from the column to about 0.2 cm. above the gel.

Determination—The sample solution is transferred on the column without disturbing the surface. When all of the sample has entered the ion exchanger, the top of the column is washed with small aliquots of citrate buffer A. About 5 cm. of this buffer is left above the bed surface. The column is connected with the autograd; three chambers are used. The first is filled with 50 ml. of citrate buffer B, the second with 50 ml. of citrate buffer C, and the third with 50 ml. of citrate buffer D. The eluate is mixed by means of a proportioning pump with oxygen-free nitrogen and ninhydrin reagent. The color

Table II—Polymyxin B Sulfate (Pharmaceutical Formulations)

Sample Number[a]	—Assay, % of Theoretical Value— Chemical Method	Microbiological Method
1	105	102
2A	110	106
2B	94	90
2C	110	105
2D	112	113
2E	80	78
2F	102	101
2G	37	37
3	99	102
4A	100	103
4B	59	55
4C	100	98
4D	110	115

[a] 1 = aerosol, 2 = solutions, 3 = tablet, and 4 = ointments.

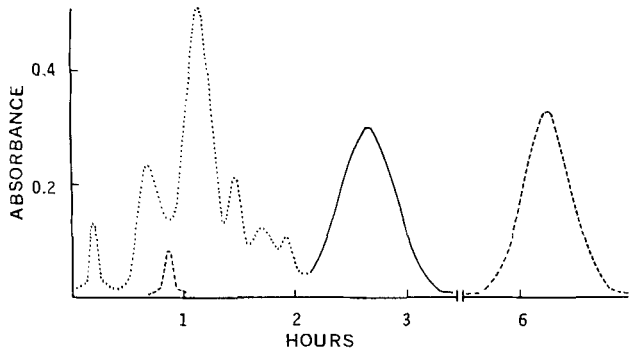

Figure 4—*Chromatogram of: —, polymyxin B sulfate (0.2 mg.); - - -, neomycin sulfate (0.2 mg.); and . . ., bacitracin (2.0 mg.).*

is developed in a heating bath at 95°. The speed of the pump is adjusted to allow a color development time of 15 min. After cooling and removal of the nitrogen, the liquid finally passes through the colorimeter and the ninhydrin color value is recorded. After the run, the ion exchanger can be regenerated by washing with 0.1 *N* hydrochloric acid and water.

Calculation—The concentration can be determined by an area calculation. The area obtained with the sample to be analyzed is compared with the area obtained with a standard sample. The standard and the unknown must be determined after each other with the same ninhydrin reagent. The concentration of polymyxin B sulfate plotted against the peak area shows a straight line going through the origin (Fig. 2).

RESULTS AND DISCUSSION

Chromatography of polymyxin B sulfate under the experimental conditions yields the chromatogram showed in Fig. 3. Chromatogram 1 was obtained with polymyxin B sulfate international standard (potency 8400 I.U./mg.); chromatogram 2 was obtained with a sample of polymyxin B sulfate (potency 5700 I.U./mg.). For each chromatogram, 0.25 mg. antibiotic was analyzed. The approximate retention time was 2.4 hr. Assay values of polymyxin B sulfate bulk samples and of polymyxin B sulfate in pharmaceutical formulations were determined, and the results were compared with those obtained by the microbiological methods (Table I). Good agreement was obtained also with the samples containing a large amount of degraded polymyxin B. The samples were determined against the Belgian National Standard (relative potency 100%). In the formulations, polymyxin B was combined with other antibiotics (neomycin, bacitracin, oxytetracycline, virgimycine, and tyrothricin), antiseptics, local anesthetics, steroids, *etc.* The method is very specific. Other antibiotics (neomycin, bacitracin, and oxytetracycline) that interfere with the ninhydrin reaction do not interfere with the assay. Figure 4 shows the separation of bacitracin, polymyxin B, and neomycin. Polymyxin E, however, yields the same chromatographic pattern as polymyxin B. Table II shows the good correlation between chemical and biological results in preparations where polymyxin B was partially degraded.

This column chromatographic method can be considered as a specific and stability-indicating assay for polymyxin B sulfate and accepted as a valuable substitute for the microbiological method. The procedure presented offers greater accuracy and reproducibility (standard deviation 2.8%) than the microbiological method.

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