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The identification of polymyxin B sulphate

The chemistry and the structure of the polymyxin antibiotics have been elucidated by the studies of VOGLER AND STUDER[1], who published a complete list of the known polymyxins. The list shows that polymyxins are peptides with very similar structure, containing, in addition to amino acids, a fatty acid. They are always found as a mixture of two peptides, differing only in their fatty acid composition. Table I shows the amino acid and fatty acid components of the different polymyxin antibiotics.

TABLE I

AMINO ACID AND FATTY ACID COMPONENTS OF THE POLYMYXIN ANTIBIOTICS

Sample	L-*DAB*	L-*THR*	L-*LEU*	D-*LEU*	L-*ILE*	D-*PHE*	D-*SER*	D-*VAL*	*Fatty acid*
Polymyxin A	+	+	−	+	−	−	−	−	6 MOA
Polymyxin B_1	+	+	+	−	−	+	−	−	6 MOA
Polymyxin B_2	+	+	+	−	−	+	−	−	IOA
Polymyxin C	+	+	−	−	−	+	−	−	6 MOA
Polymyxin D_1	+	+	−	+	−	−	+	−	6 MOA
Polymyxin D_2	+	+	−	+	−	−	+	−	IOA
Polymyxin E_1	+	+	+	+	−	−	−	−	6 MOA
Polymyxin E_2	+	+	+	+	−	−	−	−	IOA
Polymyxin M	+	+	−	+	−	−	−	−	6 MOA
Circulin A	+	+	−	+	+	−	−	−	6 MOA
Circulin B	+	+	−	+	+	−	−	−	IOA
Polypeptin	+	+	+	−	+	+	−	+	?

Polymyxins B and E are the least toxic and at present are the only ones used in medicine. For that reason the pharmaceutical control of these antibiotics requires a specific identification, which is able to distinguish these polymyxins from the others. In this paper, a complete method for the identification of polymyxin B sulphate, specifically, as a pure substance and in pharmaceutical preparations, is given.

Material and methods

All chemicals used, except (+)-6-methyloctanoic acid (MOA) and isooctanoic acid (IOA), are commercially available. The two acids were synthetised from (−)-2-methylbutanol-1 and 2-methylpropanol-1 as described by VOGLER AND CHOPPARD-DIT-JEAN[2]. Cellulose layers for thin-layer chromatography were prepared by coating in the usual way with Cellulose G.

Amberlite IR C50 (sodium form) was prepared by stirring the resin (acid form) for 10 min with 1 *N* sodium hydroxide. The mixture is washed with water by decantation and transferred into a chromatographic column (1 cm in diameter) until a height of 20 cm is obtained.

Amino acid analysis

5 mg polymyxin B sulphate is dissolved in a tube in 0.5 ml of 5.6 *N* hydrochloric acid. The tube is sealed and heated for 22 h at 110°. The liquid is evaporated to dryness

in a warm air stream, the residue dissolved in 0.5 ml water and reevaporated. The amino acids are dissolved in 0.5 ml water. 2 μl of this solution are used for the thin-layer chromatographic analysis on Cellulose G. The following solvent system is used (non-saturated chamber): propanol-2–water–formic acid–pyridine (80:20:4:1). 1 μl of 1 mg/ml solutions of L-leucine, D-phenylalanine, L-threonine and L-α,γ-diamino-butyric acid are used as standards. Two-dimensional development with the above solvent and with methyl ethyl ketone–pyridine–acetic acid–water (70:15:2:15) can be used to check the absence of serine, valine and isoleucine.

After development and drying the plate is sprayed with a solution of 100 mg ninhydrin in a mixture of 70 ml ethanol, 21 ml acetic acid and 2.9 ml collidine. The spots appear after heating for 5 min at 60°. This staining reagent is reported as being the most convenient for the detection of amino acids on cellulose[3].

Fatty acid analysis

30 mg polymyxin B sulphate is hydrolysed in 1 ml 5.6 *N* hydrochloric acid at 120° in a sealed tube for 6 h. The hydrolysate is diluted with water and extracted three times with ether. After drying over anhydrous sodium sulphate the ether is evaporated and the residue dissolved in 1 ml carbon disulphide. 1 μl of this solution is injected into a gas chromatograph on a 20% DEGA–3% phosphoric acid column (on acid washed Chromosorb G, column length 1.5 m, carrier gas N_2 regulated at 30 ml/min, flame ionisation detector) at 175°. The retention times are compared with those of 6-methyl octanoic acid and isooctanoic acid.

The usefulness of phosphoric acid treated polyester columns for the separation of free fatty acids has been proved by METCALFE[4].

Analysis of pharmaceutical preparations

We identified polymyxin B sulphate in three preparations: an ointment, containing 30 mg oxytetracycline and 1 mg polymyxin B sulphate in 1 g vaseline base; an ointment containing 5 mg dexamethasone, 20 mg polymyxin B sulphate, 15 mg tyrothrycin, 100 mg neomycin sulphate, 4 mg 5-chloro-*p*-(diethylaminoethoxyphenyl) benzthiazole in a base of glycerol triacetate, barium sulphate and silicone oil; and an aerosol preparation containing 625 mg neomycin sulphate, 625 mg zinc bacitracin and 20 mg polymyxin B sulphate in a chlorofluorohydrocarbon propellent.

Polymyxin B is extracted from the ointments by stirring 3 g ointment with 15 ml water in a beaker for 5 min on a hot plate. The cold solution is filtered and transferred to an Amberlite IR C50 column. The flow rate is regulated at 1 ml/min. The resin is washed with 100 ml water at a rate of 5 ml/min. The polymyxin B is eluted from the resin with 100 ml 10% solution of sodium chloride in methanol–water (50:50) at a rate of 1 ml/min. The methanol in the eluate is evaporated on a water bath and the remaining aqueous solution is extracted three times with 10 ml *n*-butanol. The butanol is washed with 10 ml water to remove the sodium chloride and evaporated in a hot air stream. The residue is dissolved in 0.5 ml 5.6 *N* hydrochloric acid and analysed as described.

The aerosol preparation is analysed in a similar way: after the propellents have been removed, polymyxin B sulphate is extracted from the mixture by stirring with 10 ml water. The mixture is filtered and the clear solution analysed on the Amberlite IR C50 column as described above.

Discussion

As polymyxin antibiotics essentially have the same antibacterial spectra and are active in similar concentrations[5], microbiological methods cannot be used for the specific identification of polymyxin B sulphate. Similarly direct colour reactions on peptides, as described by ROETS AND VANDERHAEGHE[6], cannot be used, all polymycins give the same colour reactions for the polymyxins. No convenient thin-layer chromatographic methods for the separation of the different polymyxins have been found in the literature. Only polymyxin B, D and M are separated by the method described by IGLOY AND MISZEI[7,8]. We also were unable to resolve the polymyxins B and E by thin-layer chromatography.

An amino acid analysis appears to be the only method of specifically identifying polymyxin B. Table I shows clearly how a qualitative amino acid analysis can distinguish polymyxin B from the other polymyxin antibiotics. This method is used by the British Pharmacopoeia[9] and by HOWLETT AND SELZER[10]. The former method consists in hydrolysis at 135° for 5 h and a paper chromatographic amino acid identification. The second method gave good results with a hydrolysis at 120° for 6 h and amino acid identification on silica gel. To avoid the appearance of ghost spots we prefer a hydrolysis at 110° for 22 h. The thin-layer chromatographic method on Cellulose G gives a very good separation of the four polymyxin B amino acids. The R_F values are: leucine 0.82, phenylalanine 0.68, threonine 0.36, α,γ-diaminobutyric acid 0.12.

A fatty acid analysis is necessary to prove the presence of the polymyxins B_1 and B_2. We found it impossible to separate the C_8–C_9 acids as ammonium salts by thin-layer chromatography and, as an identification as the dinitrophenylhydrazide[11] or hydroxamate[12] requires a lot of time, we prefer to use a gas chromatographic identification of the methyl esters. The retention time is 5 min for isooctanoic acid and 14 min for 6-methyloctanoic acid. We observed also the presence of a third acid, only present in low concentration (5% of the total acid), with the same retention time as *n*-octanoic acid (9 min).

In pharmaceutical preparations polymyxin B sulphate can be separated from neomycin sulphate and zinc bacitracin[13] or from tetracyclin[14] by thin-layer chromatography or electrophoresis. These methods are unable to distinguish polymyxin B from the other polymyxins. For a specific identification of polymyxin B sulphate an amino acid analysis is required. Therefore polymyxin B must be separated from the other ingredients. Elutions from thin-layer plates or electrophoresis strips gave unsatisfactory results. A good separation is obtained by using the weakly acid cation exchanger Amberlite IR C50 (sodium form). Polymyxin is isolated by eluting the resin with a 10% solution of sodium chloride in a methanol–water mixture[15]. After removal of the methanol, polymyxin B is further purified by extraction with butanol and analysed as described.

Some difficulties, however, can be encountered in analysing ointments containing polymyxin B sulphate and tyrothricin. Probably owing to the solubilising effect of glycerol triacetate tyrothricin is partly extracted from the ointment with water. Some faint ghost spots, belonging to hydrolysis products of tyrothricin, interfere with the polymyxin amino acid pattern.

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1 K. VOGLER AND R. O. STUDER, *Experientia*, 22 (1966) 345.
2 K. VOGLER AND L. H. CHOPPARD-DIT-JEAN, *Helv. Chim. Acta*, 43 (1960) 574.
3 E. VON ARX AND R. NEHER, *J. Chromatog.*, 12 (1963) 329.
4 L. D. METCALFE, *J. Gas Chromatog.*, 1, No. 1 (1963) 7.
5 O. K. SEBEK, in D. GOTTLIEB AND P. D. SHAW (Editors), *Antibiotics I—Mechanism of action*, Springer Verlag, Berlin-Amsterdam-New York, 1967, p. 142.
6 E. ROETS AND H. VANDERHAEGHE, *Pharm. Tijdschr. Belg.*, 44 (1967) 57.
7 M. IGLÓY AND A. MIZSEI, *J. Chromatog.*, 28 (1967) 456.
8 M. IGLÓY AND A. MIZSEI, *J. Chromatog.*, 34 (1968) 546.
9 *British Pharmacopoeia, 1968*, p. 786.
10 M. R. HOWLETT AND G. B. SELZER, *J. Chromatog.*, 30 (1967) 630.
11 A. C. THOMPSON AND P. A. HEDIN, *J. Chromatog.*, 21 (1966) 13.
12 J. BLOCK, E. L. DURRUM AND G. ZWEIG, *Paper Chromatography and Paper Electrophoresis*, Academic Press, New York, 1955, p. 161.
13 R. J. STRETTON, J. P. CARR AND J. WATSON-WALKER, *J. Chromatog.*, 45 (1969) 155.
14 J. KEINER, R. HÜTTENRAUCH AND W. POETHKE, *Pharm. Zentralhalle*, 108 (1969) 525.
15 E. IVASHKIV, *J. Pharm. Sci.*, 57 (1968) 642.

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