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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF NEAMINE, NEOMYCIN B AND NEOMYCIN C IN NEOMYCIN SULPHATE

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SUMMARY

A fast and reliable isocratic high-performance liquid chromatographic method suitable for simultaneous determination of the main component and impurities in neomycin sulphate is elaborated. Neomycin B and the related substances are separated as their 2,4-dinitrophenyl derivatives on a Zorbax SIL column using 1,2-dichloroethane–heptane–methanol–water–diethylamine (790:150:55:3.6:1.5) as the eluent. The method is compared to the commonly used microbiological method for quantitating neomycin.

INTRODUCTION

Pharmacopoeias, even modern ones, contain specifications for purity and assay of neomycin sulphate that are based on a combination of various chromatographic tests for related substances, including thin-layer chromatography (TLC) and ion-exchange column chromatography, and a microbiological assay[1,2]. These procedures are time consuming and as far as the microbiological assay is concerned, not very precise. The speed of analysis using the ion-exchange method may be increased by automation and refractive index (RI) detection[3], but due to poor column performance the method is still unsuitable for the detection of impurities present in low concentrations, *i.e.*, neamine. Recently Tsuji *et al.*[4] described a high-performance liquid chromatographic (HPLC) method to separate neomycin B and C and neamine as their 2,4-dinitrophenyl (DNP) derivatives. The method did not, however, allow simultaneous determination by isocratic elution of these components, but two slightly different isocratic systems or, alternatively, a gradient system were used.

The major feature of an HPLC method suitable for pharmacopoeial control of neomycin sulphate would be that an isocratic method should allow simultaneous determination of the contents of neomycin B and C and those of related substances, *i.e.*, neamine. The present paper describes a method which meets these demands.

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EXPERIMENTAL

Chemicals

All reagents were of analytical grade from E. Merck (Darmstadt, G.F.R.). Samples of neomycin sulphate of pharmacopoeial grade were investigated. Neamine hydrochloride was the European Pharmacopoeia Chemical Reference Substance. Pure neomycin B sulphate and enriched neomycin C sulphate (containing 95% of neomycin C and 5% of neomycin B) were supplied by H. Lundbeck (Copenhagen, Denmark). The 1st International Reference Preparation for neomycin B was used as the reference material in quantitations.

Chromatography

The liquid chromatograph used consisted of a Kontron Model 410 LC pump, a Cecil 2012 spectrophotometer detector operated at 350 nm and a Rheodyne Model 7120 injection valve with a 20-μl loop. Chromatograms were recorded on a Kipp and Zonen Model BD-8 recorder, and peak areas were measured and processed by means of a Hewlett-Packard Model A laboratory data system.

A prepacked silica column, Zorbax SIL (250 × 4.6 mm) (DuPont, Hitchin, Great Britain), was used. The eluent was 1,2-dichloroethane–heptane–methanol–water–diethylamine (790:150:55:3.6:1.5).

Derivatization

The derivatization reagent was prepared by dissolving 0.5 ml of 2,4-dinitrofluorobenzene (DNFB) in 25 ml methanol. It was freshly prepared daily. A 0.02 *M* borate buffer (pH 9.0) was prepared by dissolving 4.02 g of anhydrous sodium borate in 1000 ml water.

A 500-μl volume of test or standard solution (1 mg/ml) of neomycin sulphate in 0.02 *M* borate buffer (pH 9.0) was placed in a 25-ml volumetric flask. 1.5 ml of DNFB were added and the mixture was heated in a water-bath at 60°C for 60 min. After cooling, 15 ml of 1,2-dichloroethane were added and the mixture was shaken in a mechanical shaker for 5 min. 1,2-Dichloroethane was added until the lower phase reached the 25-ml mark, and following centrifugation 20 μl of the lower phase were injected.

RESULTS AND DISCUSSION

Derivatization

Preliminary investigations were carried out using the procedure proposed by Tsuji *et al.*[4], *i.e.*, heating a mixture of neomycin and DNFB solution in an oil-bath at 100 C. Problems were encountered, however, through the formation of insoluble lumps which adhered to the walls of the flask when mixing with eluent. This might be due to evaporation of water from the reaction mixture; no difficulties occurred when performing the derivatization at lower temperatures. Fig. 1 shows the formation of the derivatives of neomycin B and C and neamine as a function of reaction time at 60°C, and it appears that the derivatization is complete within about 1 h.

Chromatography

The eluent mixture prescribed by Tsuji *et al.*[4] was found to be unsuitable as the

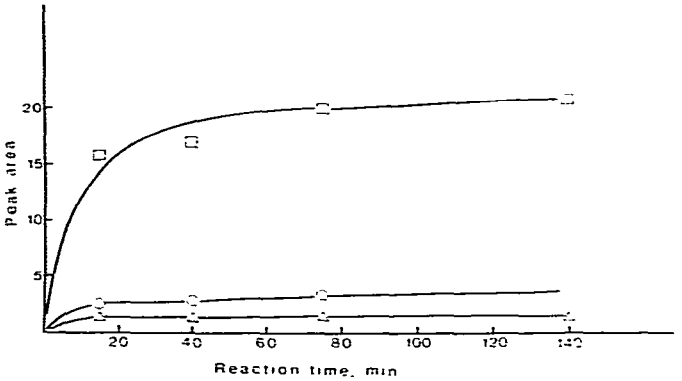

Fig. 1. Peak area (in arbitrary units) plotted as a function of reaction time. For conditions see Experimental. △, Neamine; ○, neomycin C; □, neomycin B.

basis for an isocratic system for the separation of neamine and neomycin B and C within a reasonable time, even when changing the ratio between the components. More promising results were obtained when using mixtures of 1,2-dichloroethane, methanol, water and diethylamine; such mixtures had previously proven valuable for several separations, and are a modification of a system originally proposed by Hansen and Madsen[5].

The main problem in optimizing the eluent composition appeared to be that small amounts of impurities originating from neomycin and from the reagents disturbed the peak corresponding to neamine. Extraction of the derivatization mixture with pure 1,2-dichloroethane rather than with the eluent gave an obvious improvement in the shape of the neamine peak and in its separation from disturbing peaks. This is probably due to the fact that the eluent which contains an appreciable amount of methanol is able to extract some water from the reaction mixture, thereby leading to a change in polarity relative to the eluent proper and consequently to solvent induced anomalies in the peak shapes. Furthermore, it was found that the addition of heptane to the eluent also improved the separation.

Fig. 2a–d shows the results of further investigations into the influence of the eluent composition on the retention of the compounds of interest. The influence of the concentrations of water and methanol are evident, but also the concentrations of heptane and diethylamine are of importance for the separation of the neamine peak from other impurity peaks and from a small, negative solvent peak. The optimal composition of the eluent was found to be 1,2-dichloroethane–heptane–methanol–water–diethylamine (790:150:55:3.6:1.5). Fig. 3 shows a chromatogram of a neomycin sample and a chromatogram exhibiting the peaks resulting from the reagents. The peak identities in Fig. 3a were established by chromatographing derivatized samples of pure neamine and neomycin B and a sample of enriched neomycin C.

Linearity and precision

For the quantitative evaluation of the chromatograms the method of external standardization was used, with the International Reference Preparation for neomycin B as the external standard. The linearity of the method, including the derivatization procedure and the detector response, was checked by analyzing samples in concentra-

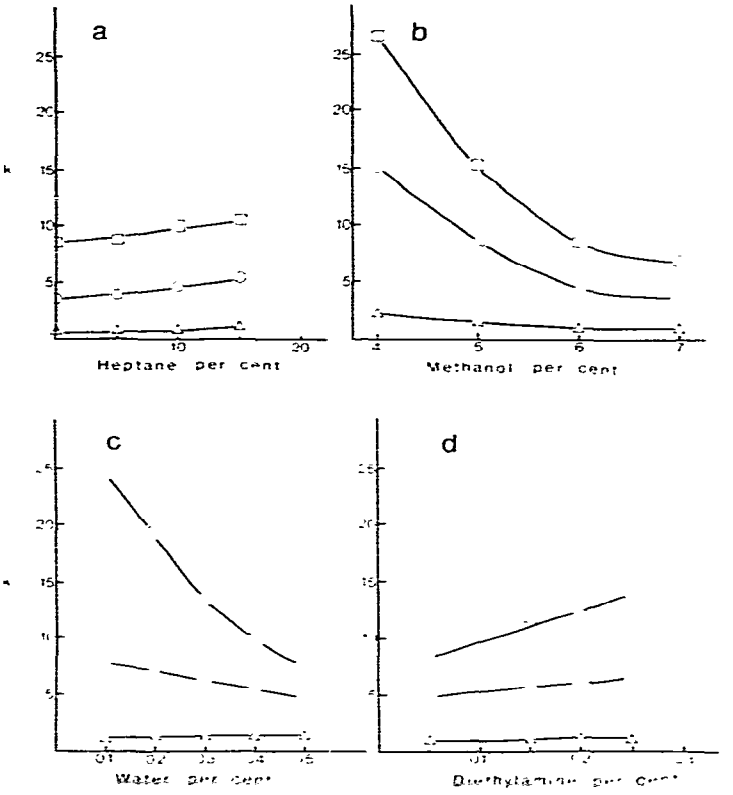

Fig. 2. Influence of the concentrations of the eluent components on retention. △, Neamine; ○, neomycin C; □, neomycin B.

tions up to 1 mg/ml of neomycin B, 0.15 mg/ml of neomycin C and 0.03 mg/ml of neamine, respectively. Plots of detector responses against the concentrations gave straight lines through the origin for all three compounds, thus establishing the linearity of the method in the concentration range of interest.

The precision of the method was examined by analyzing ten solutions, made from ten individual weighings of one sample of neomycin sulphate, and the results are shown in Table I. It appears that the relative standard deviation is fairly small for the two main components neomycin B and C, whereas it is larger for neamine which is present in only small amounts. The limit for neamine stated in pharmacopoeias is usually 1 or 2%.

Chromatographic versus microbiological assay

Quantitation of neomycin sulphate by both chromatographic and microbiological methods is complicated by the fact that both neomycin B and C exhibit an antimicrobial effect, but not to the same extent.

In the microbiological assay the potency ratio varies with the microorganism used and with the experimental conditions[6], which means that the most reliable results are obtained when the same B/C ratio is present in the test and the reference material.

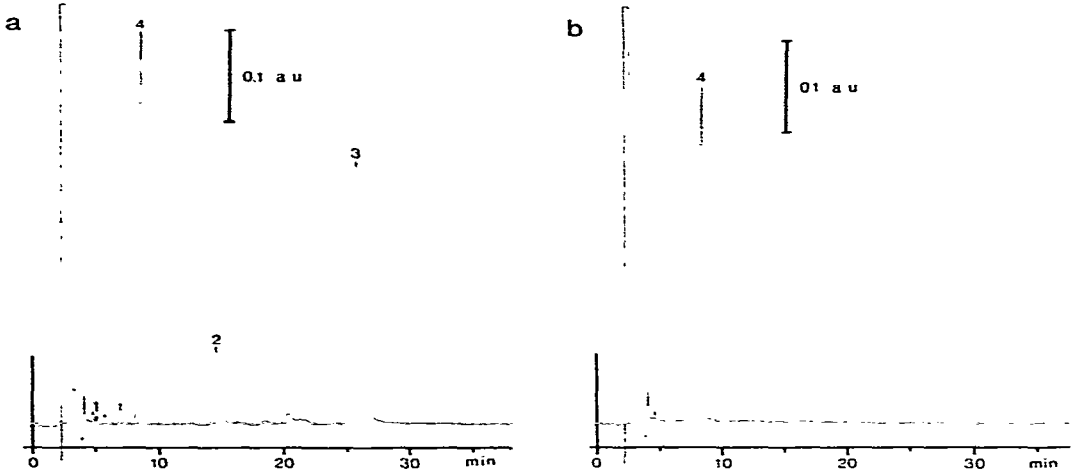

Fig. 3. Separation of components from a derivatized sample of neomycin sulphate (a) and components originating from the reagents (b). Support: Zorbax SIL. Eluent: 1,2-dichloroethane–heptane–methanol–water–diethylamine (790:150:55:3.6:1.5). Solvent velocity: 1.5 mm/sec. Pressure: 7 MPa. Detection wavelength: 350 nm. Peaks: 1 = neamine; 2 = neomycin C; 3 = neomycin B; 4 = 2,4-dinitrophenol.

When using the International Reference Preparation for neomycin as the external standard in the chromatographic assay, in order to be able to compare the results with the microbiological assay, it is necessary to know its exact content of neomycin C (*ca.* 10%) as well as the relative antimicrobial activity of neomycin B and C. By assaying an enriched neomycin C sample (containing 95% of neomycin C and 5% of neomycin B) using the microbiological method[1] it was found that neomycin C exhibits one third of the potency of neomycin B. The chromatographic standardization against the International Reference Preparation for neomycin, however, is still complicated owing to the fact that the standard is a mixture. Hence the standardization against the International Reference Preparation for neomycin B, which contains no neomycin C, was found more convenient.

In Table II the results of determinations using the two different methods are shown. It appears that after correcting for the difference in the potency of neomycin B and C the HPLC method and the microbiological method show concordant results.

TABLE I

PRECISION OF THE HPLC METHOD

Means and relative standard deviations were determined by derivatizing and analyzing ten individually prepared solutions of the same sample of neomycin sulphate. Each solution was chromatographed in triplicate.

Compound	$\bar{x}_{10}$ (%)	s_r (%)
Neamine	0.43	11.0
Neomycin C	15.6	4.0
Neomycin B	84.0	2.9

TABLE II

COMPARISON OF THE HPLC AND MICROBIOLOGICAL METHODS

In both methods the 1st International Reference Preparation for neomycin B was used as the reference material.

Sample	*Potency (in % of neomycin B standard)*				
	HPLC			*Microbiological*	
	B	*C*	*B + 1/3C*	*Potency*	*Fiducial lim.*
1	95	10.7	99	95	90–110
2	108	1	108	101	92–109
3	80.8	15	86	84	90–111

CONCLUSION

An isocratic HPLC method has been elaborated for the simultaneous determination of active ingredients and impurities in neomycin sulphate. The method is deemed suitable for pharmacopoeial purposes, and could replace the various chromatographic methods for determination of neamine and neomycin C commonly used and also the microbiological assay.

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