Induced Resistance in *Staphylococcus aureus* for Assay of Antibiotic Mixtures in Pharmaceutical Preparations

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Abstract □ A new microbiological method was developed for the assay of neomycin and spiramycin. These antibiotics usually cannot be quantitated individually when they are combined in a pharmaceutical preparation since both act on the same test organism. By inducing resistance to each antibiotic in *Staphylococcus aureus*, two new strains were obtained. Quantitation can be accomplished easily with these strains.

Keyphrases □ Neomycin—microbiological determination with spiramycin □ Spiramycin—microbiological determination with neomycin □ *Staphylococcus aureus*—microbiological determination of antibiotic mixtures, induced resistance to neomycin and spiramycin

The combination of two or more antibiotics in a pharmaceutical preparation is used widely in the therapy of several infections to permit reduction of the dose of a potentially toxic drug, to prevent the development of bacterial resistance, and to develop a synergistic effect. The last of these uses probably is the most important.

However, these mixtures frequently create problems in the quantitation of their components by microbiological procedures. Arret *et al.* (see Ref. 1) prepared tables with which approximate results may be obtained. Similar tables should be prepared in each laboratory for every antibiotic combination.

BACKGROUND

Arret *et al.* demonstrated that if the test organism used for the assay of one antibiotic (A) is not affected by a second antibiotic (B), no problem results. However, if the test organism is affected by both antibiotics, erroneous high or low values are obtained. Therefore, more specific methods must be developed to eliminate the effect of B. Such methods could involve: (*a*) inactivation of B, (*b*) use of a different test organism that is sensitive to A and relatively resistant to B, (*c*) separation of the antibiotics by differential solubility techniques, and (*d*) compensation for the presence of B by its addition to every solution of A used for the standard response curve.

Grove and Randall (2) presented numerous applications of these possibilities. For instance, they discussed the use of specific enzymes or chemical inactivators to eliminate certain antibiotics from action and the extraction of one antibiotic with a solvent in which the other is not soluble. However, the preferred technique is to choose as the test organism a microorganism that is very sensitive to one antibiotic and is not sensitive to the other antibiotic.

An organism can be made resistant to an antibiotic by forcing its growth in media containing increasing amounts of the antibiotic until the desired resistance is attained. This organism then can be used as a test organism. This paper describes a microbiological method to quantitate two antibiotics mixed in a pharmaceutical dosage form.

EXPERIMENTAL

Materials—The equipment used was that required by the USP XIX (3) cylinder-plate assay, and the assay plates were prepared (3) using antibiotic medium No. 11 for the base layer and No. 1 for the inoculum layer. Sample and standard solutions were diluted in phosphate buffer solution No. 3 (pH 8).

Neomycin sulfate USP was used as the standard. The test organisms were *Staphylococcus aureus* (ATCC 6538P) and *Bacillus subtilis* (ATCC 6633).

Table I—Precision and Accuracy of the Determination of Neomycin and Spiramycin

Antibiotic	Precision at 95% Confidence Interval, % ± *SD*	Accuracy at 95% Confidence Interval, % ± *SD*
Neomycin	105 ± 2.8	98.6 ± 2.0
Spiramycin	95.4 ± 3.07	102 ± 1.6

Table II—Results for Neomycin and Spiramycin in Stability Samples

Sample	Neomycin, %	Spiramycin, %
6 months at room temperature	107, 106	105, 100
	100, 107	105, 100
	93, 104	107, 105
	107, 116	102, 106
6 months at 37°	97, 94	71, 69
	94, 93	78, 78
	97, 98	76, 74
	93, 93	69, 70
6 months at 45°	89, 87	66, 60
	82, 82	65, 66
	84, 88	62, 62
	92, 88	64, 66

Resistance to Spiramycin[1]—The test organism was inoculated successively in a series of tubes of medium No. 1 containing increasing amounts of the antibiotic. From 1 unit of spiramycin base/ml in the first tube, the concentration was increased until a strain was obtained that was able to grow in a medium containing 20 units of spiramycin base/ml. Assays by the cylinder-plate method were made to confirm that the microorganism was resistant to this spiramycin concentration.

Resistance to Neomycin—By following steps similar to those used for spiramycin, *S. aureus* was inoculated successively in tubes of medium No. 1 containing increasing amounts of the antibiotic, from 1 to 10 μg of neomycin base/ml of culture medium. Assays by the cylinder-plate method also were made to confirm the resistance of the strain obtained.

With this technique, two new strains were obtained. One strain was resistant to 20 IU of spiramycin base/ml of medium (for the determination of neomycin), and the other strain was resistant to 10 μg of neomycin base/ml of medium (for the determination of spiramycin).

Method—Analyses were performed as described in the USP under the cylinder-plate method (3). The basic steps of this method are: (*a*) inhibition of growth by different dilutions of the antibiotic placed in cylinders on agar plates inoculated with the test organism, (*b*) measurement of the zones of inhibition, and (*c*) quantitative estimation by interpolation of the values obtained in a standard curve.

Statistical Evaluation—To determine the precision of the method, repeated assays were carried out in a homogeneous sample of a topical solution for cattle containing, in 100 ml, 2.0 mg of neomycin base (as the sulfate), 2.0 mg of spiramycin base (as the adipate), and 2.5 mg of flumethasone with polyethylene glycol-water as the vehicle.

For accuracy, a placebo of the dosage form was dosed with known amounts of the antibiotics (5, 8, and 10 mg of neomycin base/g and 5, 8, and 10 IU of spiramycin/g), and recovery assays were done after homogenization. The results are given in Table I.

[1] The spiramycin was obtained from the World Health Organization.

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The specificity of the method was determined with dosage form samples maintained for 6 months at room temperature, 37°, and 45°. The results are given in Table II.

RESULTS AND DISCUSSION

A microbiological method for the determination of neomycin and spiramycin mixed in pharmaceutical preparations was developed. The initial plan was to develop a *Staphylococcus* strain that was resistant to spiramycin for the determination of neomycin and to develop a *Bacillus* strain that was resistant to neomycin for the determination of spiramycin.

Resistance to spiramycin in *S. aureus* was obtained easily. However, since the *B. subtilis* inoculum was a spore suspension, a strain resistant to neomycin was not obtained. Therefore, since *S. aureus* also is recommended for the evaluation of spiramycin (4), this microorganism was chosen for both antibiotics.

This method may be used as a quality control procedure for samples of production batches as well as in stability studies since substances produced by degradation products of excipients or other active ingredients do not interfere. The assay may be carried out without special preparation of the sample. This technique may be used in the assay of mixtures of other antibiotics by inducing resistance in the proper microorganisms.

REFERENCES

(1) F. Kavanagh, "Analytical Microbiology," Academic, New York, N.Y., 1963, pp. 252, 255.

(2) "Assay Methods of Antibiotics, a Laboratory Manual," Grove and Randall, Eds., Medical Encyclopedia, Inc., New York, N.Y., 1956, p. 124.

(3) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, pp. 596, 598.

(4) "Pharmacopee Francaise. Codex Medicamentarius," Gallicus ed. VIII, Paris, France, 1965, p. 1102.

Determination of Dextro- and Levomethorphan Mixtures Using Chiral Lanthanide NMR Shift Reagents

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Received August 20, 1979, from the *Division of Drug Chemistry, Food and Drug Administration, Washington, DC 20204.* Accepted for publication October 29, 1979.

Abstract □ Europium and praseodymium chiral NMR shift reagents were used to differentiate between dextro- and levomethorphan. The enantiomeric shift differences ($\Delta\Delta\delta$) demonstrated by the singlet associated with the methoxy protons were large enough to identify the *levo*- and *dextro*-isomers and to allow for the determination of as little as 10% of one enantiomer in the presence of the other in a prepared mixture. The analytical method is rapid and can analyze samples as small as 1 mg.

Keyphrases □ Dextromethorphan—NMR determination with levomethorphan □ Levomethorphan—NMR determination with dextromethorphan □ Enantiomers—NMR determination of dextromethorphan and levomethorphan mixtures, chiral lanthanide shift reagents □ NMR spectroscopy—determination of dextro- and levomethorphan mixtures, chiral lanthanide shift reagents □ Analgesics, narcotic—levomethorphan, NMR identification with dextromethorphan □ Antitussives, nonnarcotic—dextromethorphan, NMR identification with levomethorphan

The determination of the enantiomeric purity of a substance is an important aspect of the regulatory process. This determination is especially important in cases such as methorphan where the *levo*- and racemic forms of the drug are regulated narcotics while the *dextro*-isomer is a widely used nonnarcotic antitussive.

BACKGROUND

Specific rotation (1) is the standard method used to distinguish dextromethorphan from levomethorphan. This method is simple and accurate if there is an adequate amount of sample and if one can be reasonably certain that there are no interfering compounds. The hanging microdrop technique (2), which has a sensitivity of 0.2 μg, also is used. However, these approaches cannot serve as identification tests and they cannot accurately detect one enantiomer in the presence of the other.

Chiral lanthanide nuclear magnetic shift reagents may be utilized in the development of rapid, sensitive, and accurate assays of enantiomeric purity. Shaath and Soine (3) recently demonstrated the utility of this approach in the determination of the enantiomeric purity of isoquinoline alkaloids. By using a europium chiral shift reagent, they differentiated rapidly between the components of five enantiomeric pairs and accurately detected an enantiomeric mixture of 95:5.

This paper reports the application of the chiral lanthanide shift reagents to the identification of the methorphan enantiomers and the determination of one isomer in the presence of the other.

EXPERIMENTAL

Instrumentation—A 60-MHz NMR spectrometer[1] equipped with a Fourier transform system[2] was used for all spectra.

Reagents and Chemicals—Deuterated chloroform was used as purchased[3].

Dextro- and levomethorphan were supplied as their hydrobromide salts[4], and the free bases were prepared according to the NF procedure (1). Stock solutions (0.16 *M*) of the free bases were prepared by dissolving 220 mg of the respective enantiomer in 5.00 ml of deuterated chloroform. Enantiomeric mixtures were prepared from the two stock solutions.

The europium and praseodymium chiral shift reagents were used as purchased[5]. Stock solutions (0.2 *M* in deuterated chloroform) were prepared in a dry box under a nitrogen atmosphere before the start of each series of experiments. Each solution was stored in a sealed vial equipped with a septum and sampled using a microliter syringe.

Procedure—The appropriate methorphan solution (0.4 ml) was placed in an NMR tube equipped with a rubber septum. The shift reagent then was added sequentially in 20-μl increments. An NMR spectrum with 64 additive pulses was run after each addition, and the peak chemical shifts were computer recorded.

The peak height ratios associated with the various molar ratios of levo- and dextromethorphan were measured after the addition of 300 μl of the europium shift reagent or 260 μl of the praseodymium shift reagent.

RESULTS AND DISCUSSION

The effect of the europium shift reagent on the NMR spectrum of an equimolar mixture of dextro- and levomethorphan is shown in Fig. 1. The

[1] Model R12B, Perkin-Elmer Corp., Norwalk, CT 06856.
[2] TT7, Nicolet Instrument Corp., Madison, WI 53711.
[3] Aldrich Chemical Co., Milwaukee, WI 53233.
[4] Hoffmann-La Roche, Nutley, NJ 07110.
[5] Eu-Opt and Pra-Opt, Alfa Products, Ventron Corp., Danvers, MA 01923.

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