Application of Proton and ^{13}C-NMR Spectroscopy to Estimation of Diastereoisomer Ratio in Phenethicillin

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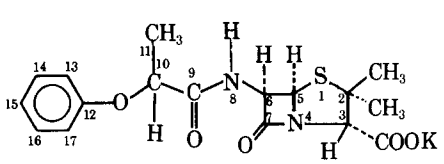
Abstract □ A procedure for the determination of the diastereoisomer ratio in phenethicillin potassium and its formulations is described. The method utilizes analyses of PMR and ^{13}C-NMR data. Assignments of ^{13}C-chemical shifts in D- and L-phenethicillin potassium also are included.

Keyphrases □ Phenethicillin—proton and ^{13}C-NMR spectral analysis of diastereoisomer ratio, bulk drug and commercial formulations □ NMR spectroscopy, proton and ^{13}C—analysis, diastereoisomer ratio of phenethicillin, bulk drug and commercial formulations □ Diastereoisomers—phenethicillin, proton and ^{13}C-NMR spectral analysis of ratio, bulk drug and commercial formulations □ Antibacterials—phenethicillin, proton and ^{13}C-NMR spectral analysis of diastereoisomer ratio, bulk drug and commercial formulations

The semisynthetic penicillin, phenethicillin potassium (I), is prepared by *N*-acylation of 6-aminopenicillanic acid with racemic α-phenoxypropionyl chloride (1). This condensation introduces another center of asymmetry into the molecule, and the resulting two diastereoisomers have different physical properties. The isomer composition of the product varies depending upon the solvent extraction and crystallization procedures. The two isomers show differences in their antibacterial spectra, thereby placing certain limitations on the interpretation of results of microbiological assay but also indicating that products from different manufacturers may vary in antibiotic effectiveness.

The L-phenethicillin potassium content of phenethicillin potassium NF is determined in the "Code of Federal Regulations" (2) by a microbiological assay. Limits of between 55 and 75% of this more active L-isomer are specified. The BP (3) allows optical rotation limits of $[\alpha]_D$ +215 to +240°, corresponding to isomer compositions of 100–34% of the L-isomer. This optical rotation measurement, however, sums the effect at all five asymmetric centers and is not as precise, although it is less tedious, than the microbiological procedure. In addition, none of the pharmacopeias specifies tests for monitoring this isomer ratio in pharmaceutical dosage forms, a control that would be useful from a regulatory standpoint.

In an earlier publication (4) on the NMR spectra of penicillins and cephalosporins, differences in the splitting patterns of the β-lactam protons in the D- and L-isomers of phenethicillin were noted. Since the area or intensity of an absorption band in an NMR spectrum is directly proportional to the number of nuclei that absorb energy at that particular frequency, an NMR assay should be possible provided that at least one resonance band from each component of a mixture is free from extensive overlap by other absorptions. Therefore, the applicability of proton (PMR) and ^{13}C- (CMR) magnetic resonance spectroscopy as a means of monitoring this isomer ratio was examined.



I

EXPERIMENTAL

Materials—Potassium 6-[L-(+)-α-phenoxypropionamido]penicillanate and potassium 6-[D-(+)-α-phenoxypropionamido]penicillinate were FDA reference standards. A sample of DL-phenethicillin potassium[1] contained 100% of the calculated amount of $C_{17}H_{19}KN_2O_5S$ with reference to the dried substance when determined by iodometric assay and had a microbiological potency of 880 μg/mg. The L-isomer content of this material as determined by the Code of Federal Regulations method (2) was 55.6%; by the BP (3) optical rotation method, it was 55.2%. A value of 55% was assigned to this material, which was used as a working standard. Phenethicillin potassium tablets were obtained locally.

Synthetic mixtures of varying L-isomer content were prepared by adding known amounts of the pure D- and L-isomers to a weighed amount of the DL-phenethicillin working standard. This approach was necessary because of the limited quantities of pure isomers available.

Sample Preparation—*Powders*—An amount of powder equivalent to about 50 mg of phenethicillin potassium was dissolved in 0.50 ml of deuterium oxide (99.8% D minimum isotopic purity) in a small glass vial. The solution was agitated for about 1 min and then transferred to a 5-mm diameter NMR tube.

Tablets—After grinding in a mortar and pestle, a weighed portion of the powder equivalent to about 250 mg of phenethicillin potassium was placed in a 15-ml centrifuge tube, and 2.5 ml of deuterium oxide was added. After mixing, the sample was centrifuged. Then 0.50 ml of the supernate was transferred to a 5-mm diameter NMR tube.

PMR—Spectra were recorded on a 60-MHz NMR spectrometer[2] at ambient probe temperature (40 ± 2°) using a sweep width of 500 Hz and a 250-sec sweep time. For quantitative purposes, the portion of the spectrum between 5 and 6 ppm (β-lactam protons) was rerecorded at a sweep width of 250 Hz and carefully integrated five times. A radio frequency power of 0.25 milligauss (normal dial setting) gave maximum integral amplitude and was used for integrations. Tetramethylsilane in deuterochloroform was the external reference. Chemical shifts were measured in parts per million downfield from tetramethylsilane.

The percent of L-phenethicillin in a sample was calculated by dividing the peak areas attributed to the *AB* quartet of L-phenethicillin by the total integral value (combination of D- and L-phenethicillins).

CMR—Spectra were recorded on a 25.2-MHz spectrometer[3].

The "standard condition" spectra were recorded using a 5000-Hz sweep width, 8192 data points, a pulse angle of 60° (20-μsec pulse duration), and a pulse interval of 1 sec. Good quality spectra were obtained from samples of 50 mg/0.50 ml with about 20,000 transients (approximately 5-hr accumulation time). To remove the effects of ^{13}C–H coupling, complete noise decoupling conditions were used. For integration, the area near δ 32 ppm was expanded to a width of 200 Hz and was electronically integrated five times.

Peak height ratios were determined from the 2-β-methyl resonances on the standard condition spectra. Probe temperature was 32°, and the deuterium of the solvent was employed for a field-frequency lock. Chemical shifts quoted are in parts per million downfield from tetramethylsilane; a methanol capillary served as an internal reference point.

[1] Courtesy of Bristol Laboratories of Canada.
[2] Varian A-60A.
[3] Varian XL-100-12.

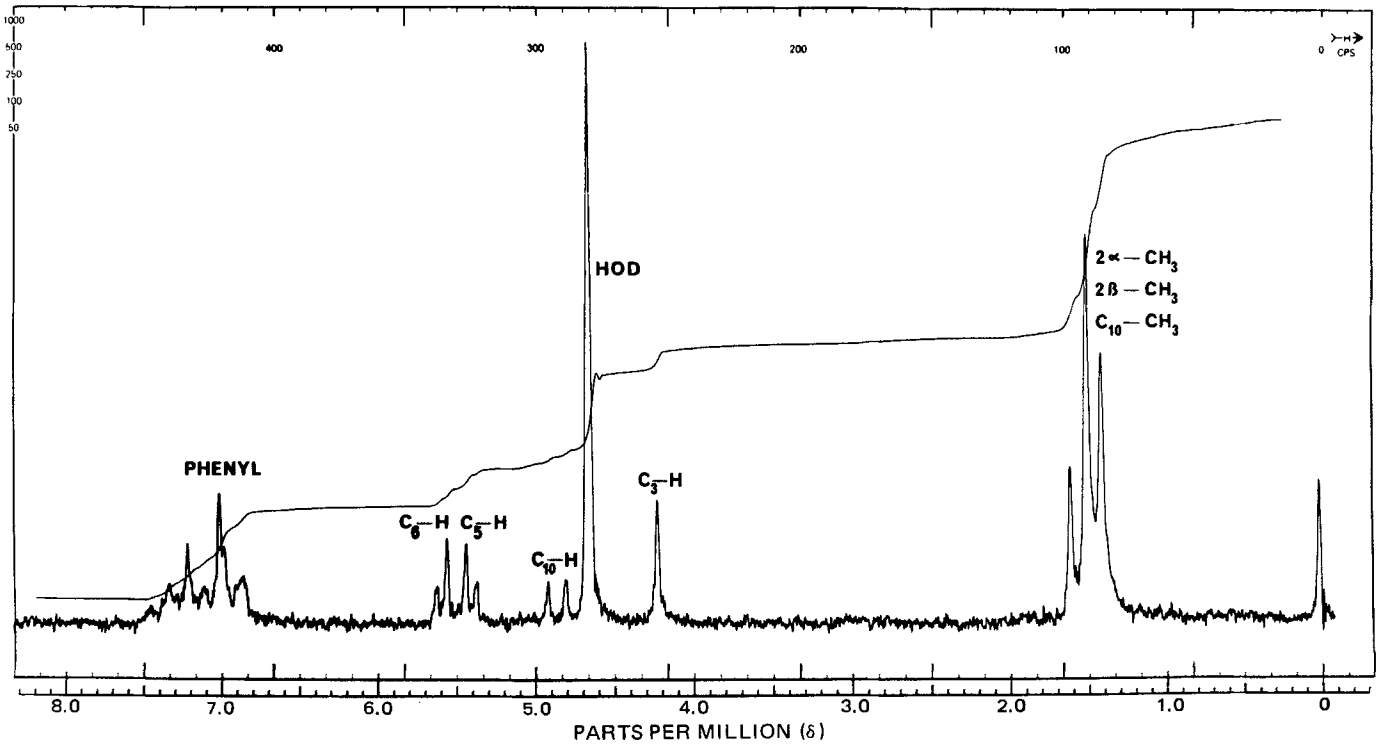


Figure 1—*PMR spectrum of* L-*phenethicillin potassium in deuterium oxide.*

The T_1 relaxation experiments, employing the kinetically inert paramagnetic metal complex tris(acetylacetonato)chromium, were conducted in the presence of 2×10^{-2} molar equivalents of the relaxation reagent. In the gated decoupling experiments, the ^{1}H-decoupler was "gated on" only during data acquisition periods, which were typically 0.5 sec. For the off-resonance decoupling experiments, the proton decoupling frequency was offset by 500 Hz from its optimum value. The selective decoupling experiments were conducted using low decoupler power in the absence of noise modulation.

RESULTS AND DISCUSSION

PMR—The PMR spectra of penicillins would be expected to have a characteristic single proton doublet arising from the C-5 hydrogen and a single proton doublet of doublets at the lower field for hydrogen at C-6. In spectra obtained from deuterium oxide solutions, these β-lactam proton signals should appear as an *AB* quartet (5).

Figure 1 illustrates the PMR spectrum of L-phenethicillin with the expected *AB* splitting pattern for the β-lactam proton signals ($J_{5,6}$ = 4 Hz; ν_5 = 5.4 and ν_6 = 5.6 ppm). The assignments of the other resonances were reported earlier (4). In the PMR spectrum of D-phenethicillin (Fig. 2), these β-lactam protons appeared as a singlet at 5.5 ppm. The β-lactam protons of ampicillin sodium also have given a two-proton singlet in deuterium oxide, possibly due to accidental equivalence of chemical shifts (6).

These spectra were recorded using a concentration of approximately 100 mg/ml. Under more dilute conditions (<60 mg/ml), broadening of the singlet in D-phenethicillin occurred, indicating that it too was splitting into an *AB* pattern.

The PMR spectrum of a typical sample of DL-phenethicillin is shown in Fig. 3. The β-lactam region was expanded and integrated (Fig. 3, inset). PMR spectra of mixtures containing varying amounts of D- and L-phenethicillins as well as commercial bulk drug powder and tablet formulations were analyzed by this method, and the percentage of the L-isomer was calculated from an average of five integrations. The results (Table I) indicated that the accuracy of this PMR quantitative method decreased as the proportion of D- and L-isomers deviated from a near equal mixture. As the relative amount of the L-isomer increased, the method led to an underestimation of its content. However, excellent correlation with calculated values was obtained in the region normally expected with commercial samples, *i.e.*, 55–75% L-isomer. Sample 9 (Table I) illustrates that results can also be obtained directly from tablets, which is particularly useful from a quality control and regulatory standpoint.

The high precision of this PMR assay method is indicated by the standard deviation and coefficient of variation of 0.6 and 1.1%, respectively, found for Sample 9 (Table I) when two separate determinations were each integrated five times. Because of broadening of the β-lactam proton signal in D-phenethicillin with dilute solutions (as mentioned previously) and the need for concentrated solutions to obtain large and,

Table I—Percent L-Phenethicillin Potassium Content by PMR and CMR Assay Methods

Sample	L-Phenethicillin Potassium, %			
	Actual[a]	By PMR[b]	By CMR	
			Integration[b]	Peak Height
1. Synthetic mixture	87	—	85.8 (±0.9)[c]	83
2. Synthetic mixture	78	73.3 (±0.7)	—	—
3. Synthetic mixture	64	63.5 (±0.6)	—	—
4. Working standard	55[d,e]	55.2 (±0.7)	57.0 (±0.6)	54
5. Synthetic mixture	53	53.8 (±0.1)	—	—
6. Synthetic mixture	44	48.7 (±0.3)	—	—
7. Synthetic mixture	28	—	25.6 (±0.2)	27
8. Tablet	64[d]	63.7 (±0.6)	—	—
9. Tablet	55[d]	57.6 (±0.6, 1.1%)[f]	54.9 (±0.2)	54

[a] Calculated for synthetic mixtures by adding known amounts of pure D- and L-isomers to working standard. [b] Average of five integrations. [c] Standard deviation from five integrations of one weighing. [d] Value from microbiological assay. [e] Value from optical rotation assay. [f] Standard deviation and coefficient of variation from five integrations of each of two weighings.

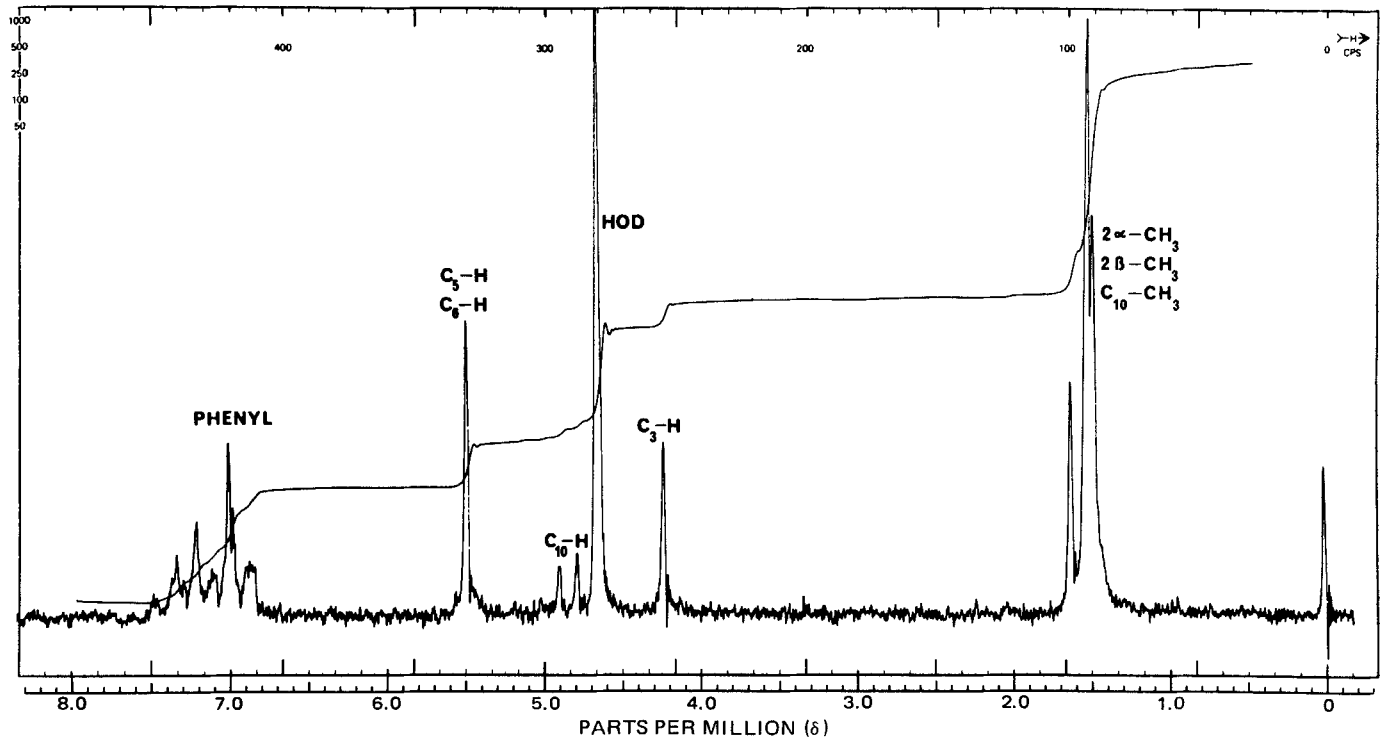


Figure 2—*PMR spectrum of* D*-phenethicillin potassium in deuterium oxide.*

therefore, more precise integrals, DL-phenethicillin potassium concentrations of 80–120 mg/ml were optimum for reproducible and precise quantitative results. Higher concentrations led to ill-defined separation of the *AB* quartet because of the concentration dependence of the chemical shift of protons at C-5 and C-6 (7).

CMR—The variation in carbon shieldings in neutral organic compounds is approximately 20-fold greater than that of protons, *i.e.*, approximately 200 ppm. These ^{13}C-shifts are also sensitive to steric, conformational, and electronic changes in a molecule. Because of the greater peak resolution of CMR, its application to this phenethicillin diastereoisomer study also was investigated. The CMR spectrum of DL-phenethicillin potassium is illustrated in Fig. 4. Since peaks were not assigned previously, they are listed in Table II.

Spectra of pure D- and pure L-phenethicillin potassium salts were recorded, which greatly facilitated the assignments for the diastereoisomeric mixture. To date, the only CMR data available for related systems are those for the methyl- and phenoxymethylpenicillins (8). Only assignment procedures for the L-isomer will be discussed, since completely

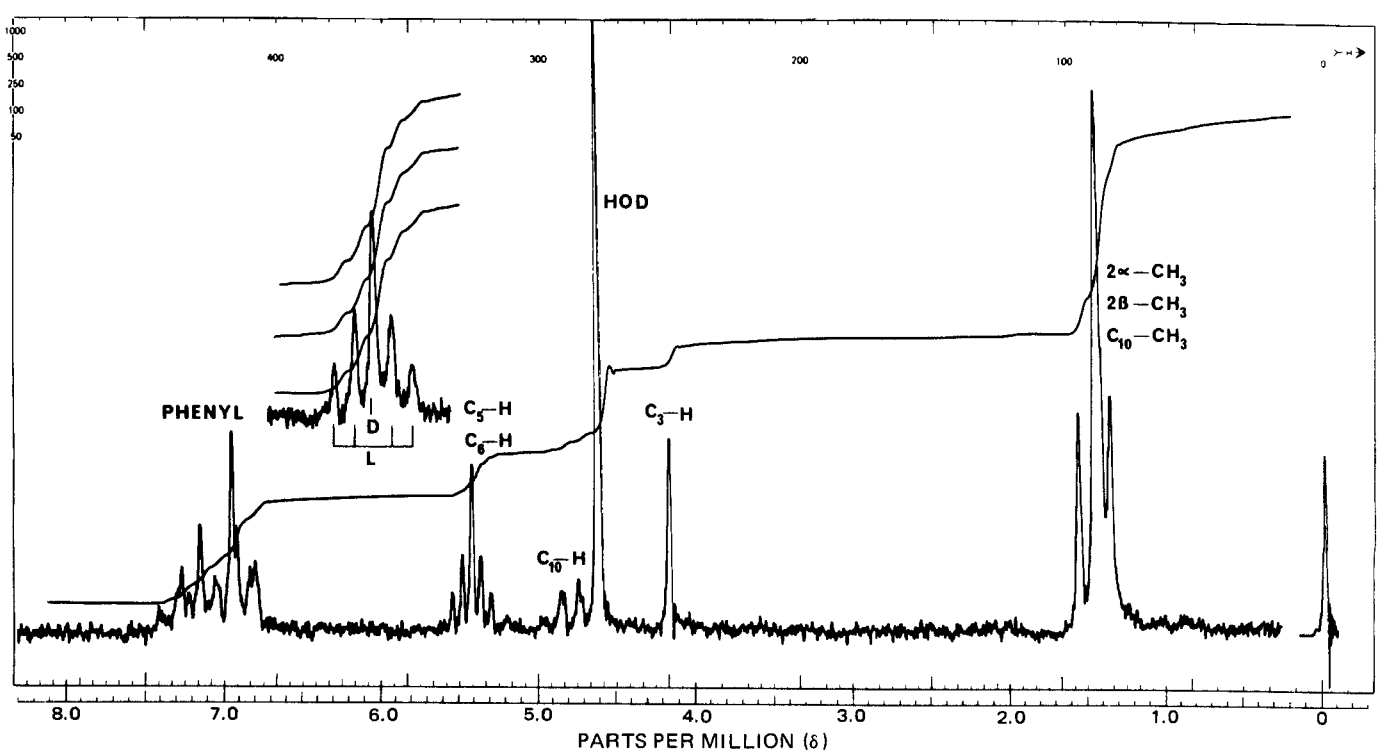


Figure 3—*PMR spectrum of commercial* DL*-phenethicillin potassium in deuterium oxide. Inset shows the β-lactam proton resonances recorded with a 250-Hz sweep width. Peaks labeled D and L refer to β-lactam protons of* D*- and* L*-phenethicillins, respectively.*

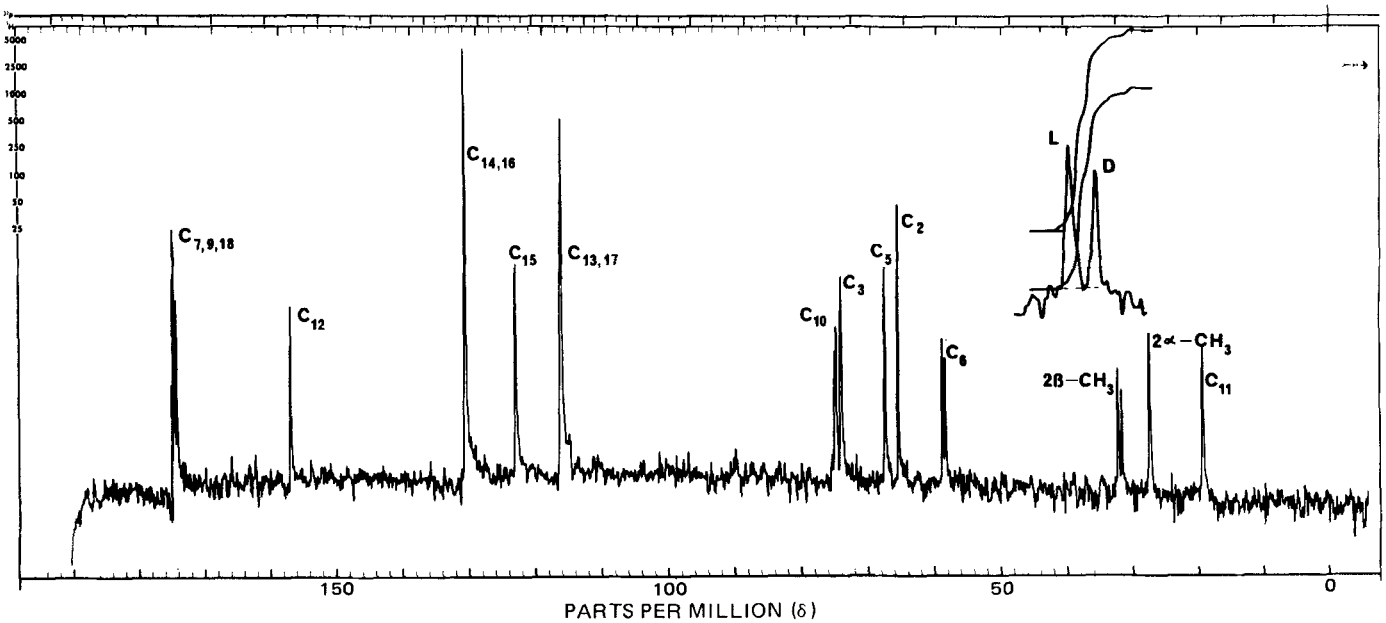


Figure 4—*CMR spectrum of commercial* DL*-phenethicillin potassium in deuterium oxide. Inset shows the 2-β-methyl carbon of* D*- and* L*-phenethicillins recorded with a 200-Hz sweep width.*

analogous techniques were used for the D-spectrum.

Initially, nonprotonated carbons were identified by their retention of singlet structure in the off-resonance decoupling experiment (9), whereas CH and CH_3 carbons yielded quartets and doublets, respectively. The three high field (lowest δ) resonances were due to the methyl groups, with that designated as C-11 being most shielded at δ 19.6 ppm. The 2-α and 2-β-methyl carbons were less shielded, which was consistent with their *gem*-dimethyl character. The 2-α group, being *cis* to the CO_2^- K^+ function, appeared 4.8 ppm upfield from its 2-β-counterpart. This shielding phenomenon at the 2-α-position presumably resulted from the γ steric interaction (10) between this function and the carboxylate moiety. The shift difference between signals from the epimeric methyls was very similar to that found in phenoxymethylpenicillin (8), although a strict comparison was not possible since the present data were recorded in deuterium oxide solutions as opposed to dimethyl sulfoxide-d_6 in the earlier work (8).

The C-2 resonance at δ 65.8 was similar to the C-2 of phenoxymethylpenicillin (δ 64.0). Distinction between the CH resonances at δ 74.3 and 75.1 was accomplished using selective ^1H-decoupling, since the corresponding proton shifts were known. Accordingly, C-3 was associated with the resonance at δ 74.3, and C-10 was associated with the δ 75.1 absorption. For the lactam ring carbons C-5 and C-6, phenoxymethylpenicillin again was a useful model compound, since the C-5 and C-6 resonances were at δ 67.4 and 58.4 ppm, respectively (8).

Assignment of the aromatic ring carbons C-12 to C-17 was straightforward, since it is well known that conjugative electron release by the ethereal oxygen shields the *ortho*- and *para*-carbons relative to the *meta*-position in anisoles and diphenyl ethers (11). Finally, the three carbonyl carbons were the most deshielded; however, unambiguous assignments were not possible.

For quantitative analysis of the diastereoisomeric mixtures, the 2-β-methyl resonances proved to be most useful since the chemical shift difference between them was the largest (0.6 ppm) and was essentially independent of the ratio of D- and L-phenethicillin potassium salts present in solution. In ^{13}C-Fourier transform NMR spectroscopy (12), great care must be taken in quantitative interpretation of data due to possible differences in spin lattice relaxation time (T_1's) and nuclear Overhauser enhancements between carbons of interest. In this study, the ratios of D- to L-forms as determined from two types of experiments were compared to verify that valid quantitative measurements were made.

Initially, the relaxation reagent tris(acetylacetonato)chromium (13) was used; no change was noted in the relative intensity of the 2-β-methyl resonances as compared to the standard condition spectrum, indicating that similar Overhauser enhancements and T_1's existed for the diastereomeric 2-β-methyl resonances. Subsequently, spectra were recorded using a gated decoupling technique (14), and again no alteration in the relative intensities of these resonances was noted. This result verified that essentially identical Overhauser enhancements operate for the diastereomeric 2-β-methyl resonances.

These experiments support the claim that the quantitative assessments are valid, and the close agreement between the CMR findings and the results from other techniques substantiates this claim. The CMR method is as accurate and as precise as the PMR assay but over a wider range of D to L ratios (Table I). Peak height measurement can be used instead without sacrificing accuracy. If more concentrated samples are used in the analysis, data accumulation time by the CMR method can be further reduced, giving reliable spectra within 2 hr.

Table II—^{13}C-Shift Assignments for D- and L-Phenethicillin Potassium in Parts per Million Downfield from Tetramethylsilane

L-Isomer	Assignment	D-Isomer
175.8	$C_{7,9,18}$	175.7
175.4	$C_{7,9,18}$	175.4 (2)
175.9	$C_{7,9,18}$	—
157.7	C_{12}	157.8
131.3	$C_{14,16}$	131.3
123.7	C_{15}	123.7
116.7	$C_{13,17}$	116.8
75.1	C_{10}	75.3
74.3	C_3	74.5
67.8	C_5	67.7
65.8	C_2	65.7
59.2	C_6	58.7
32.4	2-β-CH_3	31.8
27.6	2-α-CH_3	27.7
19.6	C_{11}	19.7

REFERENCES

(1) Y. G. Perron, L. B. Crast, W. J. Gottstein, W. F. Minor, and L. C. Cheney, "Antibiotics Annual 1959–1960," Antibiotica, Inc., New York, N.Y., 1960, p. 107.

(2) "Code of Federal Regulations," Title 21 440.82, U.S. Government Printing Office, Washington, D.C., 1975.

(3) "British Pharmacopoeia 1973," Her Majesty's Stationery Office, London, England, 1973, p. 353.

(4) W. L. Wilson, H. W. Avdovich, and D. W. Hughes, *J. Assoc. Offic. Anal. Chem.*, **57**, 1300 (1974).

(5) G. F. H. Green, J. E. Page, and S. Staniforth, *J. Chem. Soc.*, **1965**, 1595.

(6) J. M. Padfield and I. W. Kellaway, *J. Pharm. Sci.*, **63**, 143 (1974).

(7) A. L. Thakkar and W. L. Wilham, *Chem. Commun.*, **1971**, 320.

(8) R. A. Archer, R. D. G. Cooper, P. V. Demarco, and L. F. Johnson, *ibid.*, **1970**, 1291.
(9) J. B. Stothers, "Carbon-13 N.M.R. Spectroscopy," Academic, New York, N.Y., 1972, p. 38.
(10) W. R. Woolfenden and D. M. Grant, *J. Am. Chem. Soc.*, **88**, 1496 (1966).
(11) G. W. Buchanan, G. Montaudo, and P. Finocchiaro, *Can. J. Chem.*, **52**, 767 (1974).
(12) G. C. Levy and U. Edlund, *J. Am. Chem. Soc.*, **97**, 4482 (1975).
(13) O. A. Gansow, A. R. Burke, and W. D. Vernon, *ibid.*, **94**, 2550 (1972).
(14) G. C. Levy, "Topics in Carbon-13 NMR Spectroscopy," vol. 1, Wiley, New York, N.Y., 1974, p. 105.

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Parameter for Assessing Parenteral Cleanliness Based on Particle-Size Distributions

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Abstract □ A new parameter for assessing the particulate matter content of large-volume parenteral solutions was developed and tested. Some problems and shortcomings associated with previously proposed standards are discussed, together with the potential advantages of employing the proposed parameter. Cleanliness factors were compared with another parameter and were less susceptible to changes resulting from the method of measurement utilized and the premeasurement conditions encountered by the solution. The use of these cleanliness factors in conjunction with an automatic particle counter is proposed as a worthwhile supplement to the USP–NF standard for monitoring the quality of large-volume parenteral solutions.

Keyphrases □ Particle-size distributions—characterized in large-volume parenteral solutions, cleanliness factor developed □ Parenterals, large volume—particle-size distributions characterized, cleanliness factor developed □ Dosage forms—large-volume parenterals, particle-size distributions characterized, cleanliness factor developed

Particulate matter is defined as "extraneous, mobile, undissolved substances other than gas bubbles, unintentionally present in parenteral solutions" (1) and has been long recognized as a problem. Recently, official standards specified the allowable levels of particulate contamination (1, 2).

To aid in the evaluation of the cleanliness[1] of a parenteral solution, an auxiliary parameter that would describe accurately the contamination level is needed. Several desirable characteristics of this parameter were described previously (3, 4); it should provide a true measurement of particulate cleanliness that is not affected by the premeasurement handling conditions, and it should indicate the correct degree of particulate cleanliness using various instrumental techniques. The method also should be rapid, nondestructive, nonsubjective, simple, inexpensive, easy to standardize, and, preferably, not require the use of a clean room.

The present study was undertaken to develop such a parameter for the objective assessment of the relative particulate cleanliness of parenteral solutions.

[1] Throughout this article, the term "cleanliness" is used to denote the level of particulate matter.

BACKGROUND

Single-Point Standards—One early standard was proposed in 1966 (5). The initial proposal was revised and formed the basis of the first provisional Australian standard (6), which stated that a parenteral solution should not contain more than 250 particles/ml exceeding 3.5 μm in diameter. In the same year, another suggested single-point standard stated that a parenteral solution should not contain more than 50 particles/ml exceeding 5 μm in diameter (7).

Single-point standards are validly subject to criticism on the basis that the particles counted exceeding a given diameter are not necessarily indicative of the number of particles exceeding another diameter. Single-point standards were based on the observation that log–log plots of $N_{>D}$ (the particle concentration exceeding the diameter, D) *versus* D (the particle diameter) were essentially linear and parallel to one another among the individual solutions examined (5). However the slopes of the log–log plots vary among individual solutions[2] (6, 8), thereby invalidating the use of single-point standards.

Multiple-Point Standards—Recent attempts to establish standards focused on multiple-point determinations. As Kendall (9) noted, a standard should ideally be based upon the determination of the particle-size distribution over a broad size range. This consideration formed the basis for an Australian standard proposed in 1966 (5), which limited the allowable levels of particles exceeding four particle diameters. This and other recently proposed multiple-point standards are shown in Table I.

Multiple-point standards (as well as single-point standards) can be criticized on the grounds that the particle-size distribution may vary with the degree of agitation to which the solution is subjected (8, 10–12). Another potential criticism is that the measurement of particle concentrations may not be obtainable using a single technique (*i.e.*, instrumental, microscopic, *etc.*). If the particle diameters specified in the standard require measurement by more than one technique, the counts provided by each technique would have to correlate extremely well with one another. Besides introducing unnecessary uncertainty, this requirement would necessitate proficiency in more than one technique.

The USP–NF standard (Table I) suggests the use of a membrane filtration and microscopic examination technique. In a practical sense, it is only suitable for counting particles larger than 10 μm in diameter (3, 6). The Australian and British Pharmacopoeia standards do not prescribe a specific measuring technique. However, an electrical resistance counter or a light-scattering or light-blocking device is required, since these standards specify the counting of particles smaller than 10 μm in diameter. A recent version of the Australian standard (Table I), soon to become effective[3], specifies the use of an instrumental particle counter operating

[2] J. Blanchard, J. A. Schwartz, and D. M. Byrne, *J. Pharm. Sci.*, in press.
[3] C. E. Kendall, National Biological Standards Laboratory, Canberra, Australia, personal communication.