The 3′-Keto–Diol Equilibrium of Trospectomycin Sulfate Bulk Drug and Freeze-Dried Formulation: Solid-State Carbon-13 Cross-Polarization Magic Angle Spinning (CP/MAS) and High-Resolution Carbon-13 Nuclear Magnetic Resonance (NMR) Spectroscopy Studies

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Understanding how moisture interacts with a drug or formulation is a critical component of product development. This study demonstrates how water affects the 3′-*gem*-diol ↔ 3′-keto equilibrium in trospectomycin sulfate bulk drug and freeze-dried formulation, as probed by solid-state carbon-13 cross-polarization magic angle spinning (CP/MAS) and high-resolution nuclear magnetic resonance (NMR) spectroscopy. Drying the bulk drug or formulation to low water levels dehydrates trospectomycin sulfate from the diol to the keto form. Carbon-13 CP/MAS NMR spectroscopy measures the keto drug concentration in solid samples directly. The bulk drug, which contains approximately 16% water, is more than 90% in the 3′-diol form. Oven drying to <3% water converts approximately 75% of the drug to the 3′-keto form. The drug is formulated as a freeze-dried, sterile powder that can contain up to 12% water depending on the freeze-drying conditions. These studies show that the 3′-keto concentration rises uniformly (up to 75%) with decreasing residual water in the freeze-dried cake. The keto–diol equilibrium was also studied in solution by high-resolution carbon-13 NMR experiments, and it was found that raising the temperature or using dimethyl sulfoxide (DMSO) as a solvent also dehydrates the drug. For example, in aqueous solution at 25°C, nearly all (>95%) of the drug is in the 3′-diol form. After equilibration at 60°C, however, the 3′-keto content increases to 7%, and in d_6-DMSO solvent at 25°C the drug is mostly (60%) in the 3′-keto form.

KEY WORDS: nuclear magnetic resonance spectroscopy; carbon-13 cross-polarization magic angle spinning spectroscopy; water; antibiotic.

INTRODUCTION

Water generally affects the chemical, physical, and manufacturing properties of pharmaceutical solids including bulk drugs, excipients, and formulations. A significant effort has been made to understand how moisture interacts with these solids and how it alters drug and product stability, crystal structure, dissolution rate, tableting behavior, and solid-state equilibria (1,2). As an example of how water can affect an equilibrium and take part in a chemical reaction, it is known that the drug spectinomycin can exist as the diol or keto form, depending on the amount of water present, and that the keto form of the drug reacts to produce the ring-opened spectinoic acid under base hydrolysis conditions (2). In this paper, we examine how water affects the equilibrium between the keto and the diol forms of a related drug, trospectomycin sulfate, both in solution and in the solid state.

Trospectomycin sulfate (6′-*n*-propylspectinomycin) is an aminocyclitol antibiotic under development as a broad-spectrum antibacterial for the treatment of sexually transmitted diseases and anaerobic infections. The bulk drug is highly crystalline and contains approximately 16% water by weight. This is equivalent to five water molecules per drug molecule, with one molecule involved in specific hydration of the drug at the 3′ position (Fig. 1). This antibiotic will be marketed as a lyophilized, amorphous sterile powder that contains mostly drug and that will be reconstituted with aqueous diluents before use.

As Fig. 1 indicates, water is intimately involved in the equilibrium between the keto and the *gem*-diol forms of the drug. For example, dehydration results in conversion of the 3′-*gem*-diol form to the corresponding 3′-keto form; therefore, the equilibrium concentrations of these two forms are directly related to the amount of water present. We have used solid-state carbon-13 cross-polarization magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy to study this equilibrium in solid samples. In addition, high-resolution carbon-13 NMR measurements demonstrate how temperature and solvent influence this equilibrium in solution.

MATERIALS AND METHODS

Trospectomycin sulfate bulk drug, manufactured by The Upjohn Company, was used without further purification. The drug contains 16.1% water, as determined by Karl Fischer methods, and X-ray diffraction measurements indicate that the bulk drug is highly crystalline and exists predominately as one crystal form. Carbon-13 CP/MAS experiments analyze the solid bulk drug directly. For high-resolution NMR measurements, solutions were prepared by dissolving 25–30 mg of solid in 0.5 ml of D_2O or d_6-DMSO (dimethyl sulfoxide).

Freeze-dried powders containing 3–12% residual water were prepared by varying shelf temperatures and drying times during freeze-drying (3). The resulting cakes are amorphous, giving nearly featureless X-ray diffraction patterns. The amount of water in each sample was determined by thermogravimetric analysis (TGA). In these measurements, the samples were heated from room temperature to 120°C and the total weight loss was recorded. This procedure measures the total amount of water in the sample, including water obtained from dehydration of the drug to the keto form. To reduce sample heterogeneities, the freeze-dried cakes were crushed and mixed. The solid-state NMR rotors were quickly packed (within 90 sec) to minimize changes in sample water content; loss of water during data collection was insignificant. For solution state measurements, the freeze-

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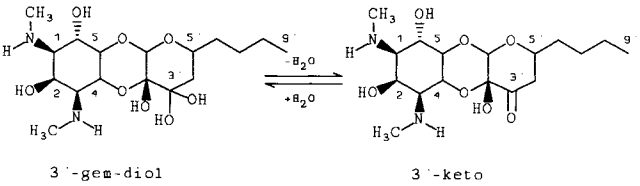

Fig. 1. The 3′ keto–diol equilibrium of trospectomycin sulfate.

dried samples were dissolved in d_6-DMSO under a dry nitrogen atmosphere to exclude extraneous moisture.

Carbon-13 CP/MAS experiments yield high-resolution NMR spectra of solids directly. This technique has been extensively used to study bulk synthetic polymers (4) and to probe tautomerism and hydrogen bonding in organic solids (5). Recently, carbon-13 CP/MAS spectroscopy has been used to study polymorphism, to probe conformational states of drugs, and to distinguish crystalline hydrates of drugs (6). In these experiments, the carbon signal is enhanced by transferring magnetization from the proton spins to the carbons, a process known as cross-polarization. The time during which the two spin reservoirs are in magnetic contact is called the contact time. Spectral resolution is enhanced by magic angle spinning, which reduces line broadening from chemical shift anisotropies. Spectral resolution is further increased by high-power proton decoupling, which reduces broadening arising from proton–carbon dipolar interactions. The solid-state carbon-13 CP/MAS spectra were collected using a Bruker MSL-200 NMR spectrometer. The 4.7-T Oxford magnet gives a proton resonance frequency of 200 MHz and a carbon-13 resonance frequency of 50 MHz. A Doty Scientific CP/MAS probe was used, and the angle between the sample rotation axis and the magnetic field was adjusted to 54.7° (the "magic angle") using KBr (7). The samples were spun about the magic angle at 4.7 kHz in 7-mm standard sapphire rotors. The proton 90° pulse time was 5.8 μsec, and the contact time was 3.0 msec. The applied field strength for protons, B_1(H), was 10 G and that for carbon was 40 G.[4] The spectral width and acquisition time used for these experiments were 20 kHz and 51 msec, respectively, and each free induction decay was digitized into 2048 points. The recycle delay between scans was 4.0 sec.

When processing the free induction decay from the sterile powders, a Lorentzian multiplication of 50 Hz was used to improve the signal-to-noise ratio; the free induction decay signals from the bulk drug samples, however, were Fourier transformed directly, without line broadening. Chemical shifts are relative to tetramethylsilane, using adamantane as an external standard.

High-resolution carbon-13 NMR spectra were recorded using a Varian XL-400 spectrometer with a 9.4-T Oxford magnet operating at 100 MHz for carbon-13. Data were acquired using a Varian 5-mm broadband probe with proton decoupling into 32,000 or 16,000 points, and a Lorentzian multiplication of 2 Hz was applied prior to Fourier transformation. The pulse width was set to 8.7 μsec, corresponding to a flip angle of 45°. The interpulse delay was 2 sec, and the acquisition time 0.64 sec. Probe temperature was maintained using a Varian variable-temperature unit. Chemical shifts were referenced to the DMSO solvent resonance at 39.5 ppm or to an external 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt reference at 0 ppm.

[4] The effective magnetic field strength, B_1(H), is related to the tip angle (θ = 90°) and the 90° pulse time (τ = 5.8 μsec) by $B_1(H) = \theta/(\gamma\tau)$. The magnetic field B_1(C) is calculated as $B_1(C) = B_1(H)[\gamma(H)/\gamma(C)] \approx 3.98B_1(H)$, where γ(H) and γ(C) are the magnetogyric ratios for proton and carbon nuclei, respectively.

Quantitation Procedure

The 3′-keto drug concentrations are calculated as follows: Since both the 3′-diol and the 3′-keto forms of the drug contain the 9′ methyl group, the ratio of the 3′-keto intensity to 9′ methyl intensity gives the fraction of drug in the 3′-keto form. For this quantitation procedure to be valid, however, the cross-polarization efficiencies of the 3′-keto and 9′-methyl carbons must be identical. Since protonated carbons tend to cross-polarize faster and to a greater extent for short contact times, the 3′-keto and 9′ methyl carbons are not expected *a priori* to have similar cross-polarization rates. Grant and co-workers, however, have examined the quantitative reliability of CP/MAS, and have found that carbons with protons within two or three bonds will give relative signal intensities that agree with atomic ratios (8). Since the 4′ carbon of the drug is protonated, quantitative measurements based on the 3′ carbon signal might not be unreasonable. For example, it was found that the sum of the signal intensities from the 1′, 2′, and 3′ (in both 3′-diol and 3′-keto forms) carbons was 2.5 to 3.1 times that from the 9′ carbon; this figure is in reasonable agreement with the 3:1 ratio expected from the number of nuclei present. (Because of spectral overlap, it is necessary to measure the total signal from these three carbons.) Furthermore, if cross-polarization rates were vastly different, one would expect signal intensities to vary greatly with contact time. It was found, how-

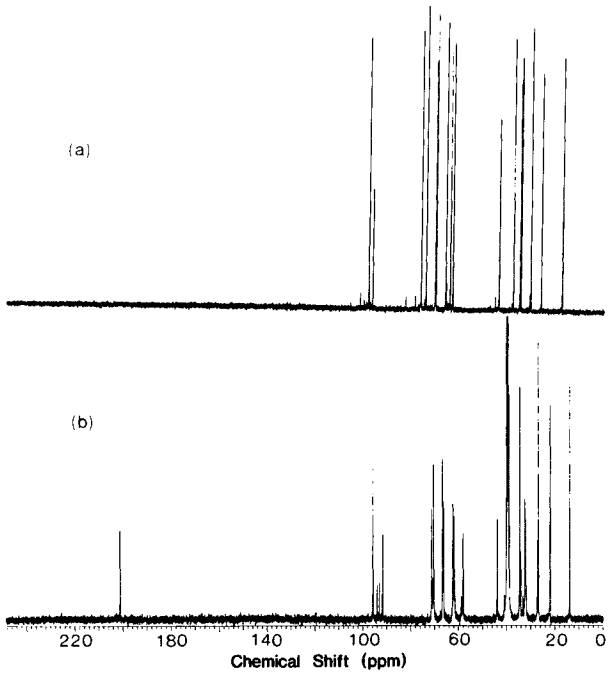

Fig. 2. Carbon-13 NMR spectrum of trospectomycin sulfate bulk drug dissolved (a) in D_2O at 25°C and (b) in d_6-DMSO.

Table I. Trospectomycin Sulfate Carbon-13 Chemical Shifts (ppm) for the (R)-cis Diol and (R)-cis Keto Anomers in Aqueous and DMSO Solvents

Carbon position	In D_2O[a]		In DMSO[b]	
	Diol	Keto	Diol	Keto
9′ CH_3	15.88	N/R[c]	13.85	13.79
8′ CH_2	24.63	24.81	21.97	21.85
7′ CH_2	29.01	29.26	27.21	26.74
N(3)-CH_3	33.01	34.27	31.83	32.15
N(1)-CH_3	33.45	34.63	33.22	32.45
6′ CH_2	36.14	37.14	34.14	34.52
4′ CH_2	42.10	46.13	40.98	43.85
3 CH	61.33	61.76	58.81	58.04
2 CH	62.41	63.48	N/R	61.83
1 CH	64.27	64.78	N/R	62.33
4 CH	68.29	68.91	N/R	66.24
6 CH	68.63	N/R	N/R	66.63
5 CH	72.49	74.92	N/R	70.36
5′ CH	74.55	73.61	70.68	71.16
2′ C	94.64	94.29	91.80	91.56
3′ C	96.28	205.69	93.08	201.11
1′ CH	96.33	98.92	93.94	95.69

[a] Determined in aqueous solution (pH 4.5) at 25°C.
[b] Determined in d_6-DMSO at 25°C.
[c] Not resolved.

ever, that the intensities and calculated keto drug concentrations remain relatively constant, and within the experimental error of 10%, for contact times between 0.5 and 6.0 msec. Therefore, an intermediate contact time of 3.0 msec was chosen for quantitative measurements.

RESULTS AND DISCUSSION

High-Resolution Carbon-13 NMR Studies

The 9.4-T (100-MHz) carbon-13 NMR spectrum of trospectomycin sulfate bulk drug dissolved in D_2O at 25°C is shown in Fig. 2a. From this spectrum, we conclude that >95% of the drug is in the diol form in aqueous solution. The 3′-ketonic resonance at 205 ppm is not visible. The resonances in the spectrum arise mainly from the (R)-cis-3′-diol anomer of the drug (chemical shifts are listed in Table I), but the weaker resonances arise from the other mutarotational anomers of the drug, mainly the (S)-trans isomer (see Fig. 3).[5] The equilibrium concentrations of the four anomers, attained after 18 hr at room temperature and as determined by NMR, are 90% (R)-cis, 6% (S)-trans, 1% (R)-trans, and 1% (S)-cis. The 3′-keto anomers account for the remaining 2% of drug. The chemical shifts of the (S)-trans anomer relative to the parent (R)-cis anomer are listed in Table II.

The carbon-13 spectrum of trospectomycin sulfate freeze-dried formulation dissolved in D_2O is nearly identical to that of the bulk drug in aqueous solution, except that resonances from the citrate buffer of the formulation are present as well. Because only a few weak resonances from the 3′-keto drug are present in this spectrum, the drug appears to exist predominately in the diol form in the freeze-dried formulation. However, solid-state NMR measurements, discussed below, demonstrate that the 3′-keto drug concentration is high in these powders. In fact, the keto drug is quickly converted to the diol form in aqueous solution.

In addition to solvent effects, temperature influences this equilibrium as well. For example, the keto drug concentration increases to 7% when the aqueous solution is warmed to 60°C.

The equilibrium can be further shifted in favor of the keto form by using a "dehydrating" solvent such as DMSO. The resulting carbon-13 NMR spectrum of the bulk drug dissolved in d_6-DMSO is given in Fig. 2b, with chemical shifts listed in Table I. Unlike the case in D_2O, most of the drug (65%) is in the keto form when dissolved in this solvent. This indicates that DMSO effectively dehydrates the molecule.

Solvent effects, therefore, cause keto drug concentrations calculated from high-resolution NMR measurements to

[5] Y. Hiyama, R. J. Taylor, R. H. Robins, and K. S. Manning (to be published). A single-crystal X-ray diffraction study of spectinomycin dihydrobromide pentahydrate (9) has shown that the spectinomycin moiety takes the (*R*)-cis configuration of the diol. Freshly prepared spectinomycin and trospectomycin samples yield identical carbon-13 spectra except, as expected, for alkyl (6′–9′) side-chain resonances. This leads us to conclude that the major anomer for trospectomycin sulfate in solution is (R)-cis as in the case for spectinomycin.

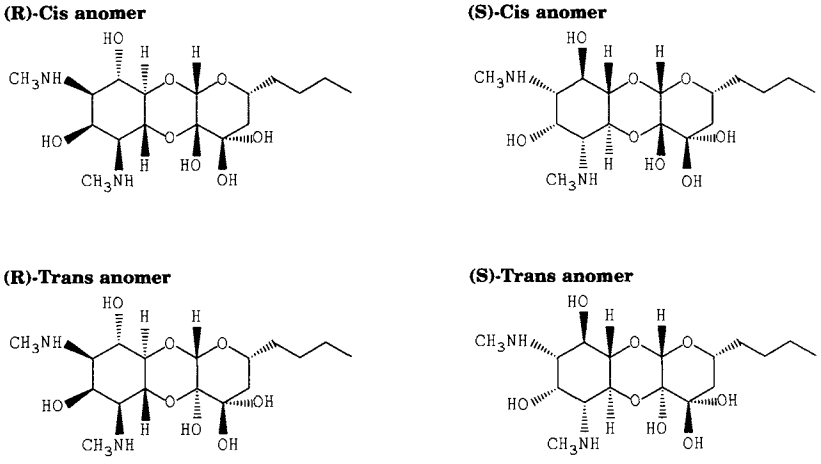

Fig. 3. The mutarotational anomers of trospectomycin.

Table II. Carbon-13 Chemical Shift Differences in D_2O Between the 3′-Diol (S)-trans and the 3′-Diol (R)-cis Anomers

Carbon position	Chemical shift difference[a] (ppm)
1′	+3.95
2′	+1.36
5′	+2.80
5	+8.88
1	−0.10
3	+0.55
4′	+2.10
7′	+0.68

[a] Defined as δ[(S)-trans] − δ[(R)-cis], where δ is the chemical shift of each carbon resonance (ppm).

be unreliable. Because of this, we have used carbon-13 CP/MAS NMR to probe the keto–diol equilibrium of this antibiotic in the solid state.

Solid-State Carbon-13 CP/MAS NMR Studies

The carbon-13 CP/MAS spectrum of trospectomycin sulfate bulk drug (Fig. 4a) is similar to the high-resolution spectrum of the drug in solution. Using the assignments determined in solution, some of the resonances shown in Fig. 4a can be assigned. The solid-state chemical shifts of the 9′-methyl, 8′-methylene, 4′-methylene, and 2′ carbons are 16.3, 23.7, 40.4, and 92.8 ppm, respectively. The N-methyl carbons absorb at 30.7 and 29.2 ppm. The 1′- and 3′-diol carbons are isochronous at 94.0 ppm, as in solution. Resonances from the mutarotational isomers of the drug are not obvious in the spectrum, because they are either unresolved or too weak to be detected. The resonances are narrow (the 9′-methyl resonance has a line width of 40 Hz), due to the highly crystalline character of the sample. The weak methyl resonance at 14.0 ppm presumably arises from drug molecules in noncrystalline regions of the sample.[6]

[6] M. S. Bergren, R. S. Chao, and M. D. Likar (to be published).

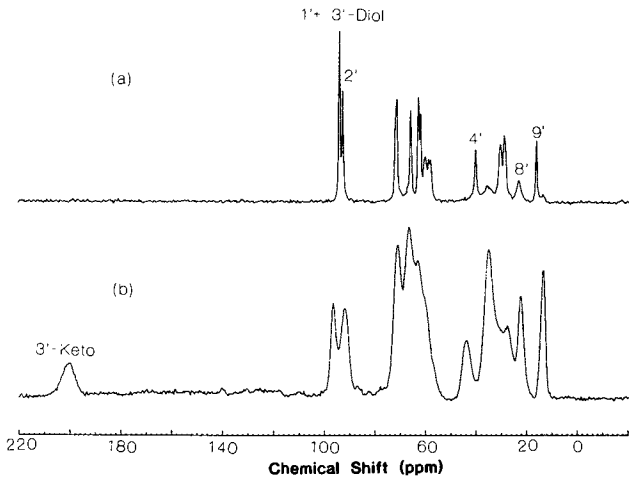

Fig. 4. Solid-state carbon-13 CP/MAS spectra of (a) fully hydrated (16.1% water) and (b) dried (<2.8% water) trospectomycin sulfate bulk drug.

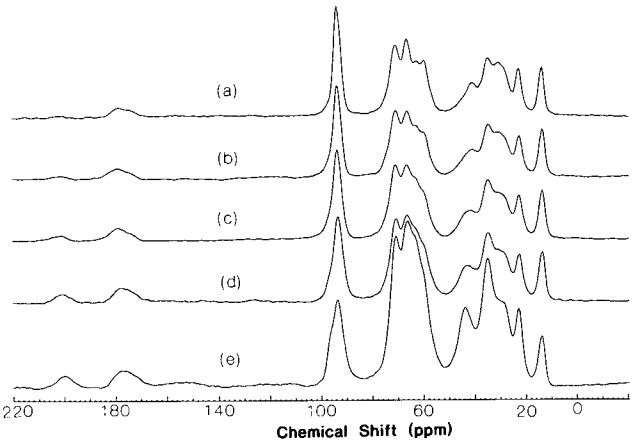

Fig. 5. Solid-state carbon-13 CP/MAS spectra of freeze-dried powders containing (a) 12.2% (7200 scans), (b) 9.9% (16,100 scans), (c) 6.5% (12,600 scans), (d) 4.7% (5200 scans), and (e) 3.0% (7100 scans) water. The spectra are compared by normalizing to the 9′-methyl intensity.

The 3′-diol drug concentration cannot be quantitated directly because of spectral overlap, including the overlap of the 3′ carbon resonance of the diol with the 1′ carbon signals of both the diol and the keto forms. Instead, the amount of 3′-keto drug in the sample is quantitated by integrating the carbonyl resonance at 202 ppm. The bulk drug spectrum (Fig. 4a) shows no evidence of this resonance; based on an estimate of the detection limit, we conclude that more than 90% of the bulk drug exists as the 3′-diol form. This is reasonable due to the 5:1 molar excess of water in the bulk drug sample.

Unlike fully hydrated samples, oven-dried samples with <2.8% water contain mostly 3′-keto drug, as shown in Fig. 4b. The 3′-carbonyl resonance is clearly visible in the spectrum of the dried sample as a broad resonance centered at 202 ppm. As explained previously, the ratio of the keto-to-methyl intensities in this spectrum allows us to estimate that 70–75% of the drug in the dried sample is in the 3′-keto form. The resonances for the dried sample are also broader than those in the fully hydrated drug spectrum, suggesting that the dried sample is less crystalline.[6] Evidently, heating drives water from the sample and significantly reduces crystallinity.

Freeze-dried powders of trospectomycin sulfate also contain significant quantities of keto drug. The spectra from lyophilized powders containing 3.0, 4.7, 6.5, 9.9, and 12.2% water are shown in Fig. 5. As expected, the carbonyl resonance clearly increases in intensity as the water content of the freeze-dried cake decreases. The acidic carbons of the citrate buffer yield the broad feature centered at 175 ppm. The resonances in these spectra are inhomogeneously broadened[7] compared to those of the crystalline bulk drug (see Fig. 4a). For example, the methyl resonance at 13.7 ppm has a measured line width of about 110 Hz before line broaden-

[7] A refocusing pulse applied 5 msec after the cross-polarization step of the experiment produces an echo, which is indicative of inhomogeneous broadening.

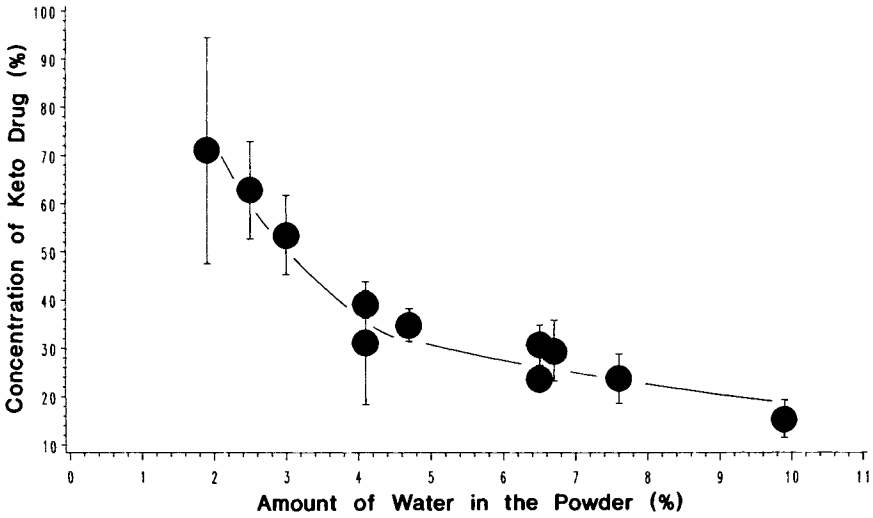

Fig. 6. The fraction (percentage) of drug in the keto form for freeze-dried cakes with 2 to 10% water. The solid line simply connects the data points.

ing, compared to the 40-Hz width measured for this resonance in the bulk drug spectrum.

Figure 6 illustrates how the 3′-keto drug concentration decreases monotonically with increasing cake water content. For the powder with 2.5% water, 63 ± 10%[8] of the drug is present as the 3′-keto form, while 15% of the drug is in the 3′-keto form for a sample with 10% water. This study shows that a significant fraction of the drug in the freeze-dried powder exists in the 3′-keto form at all water contents, indicating that the marketed product contains both forms of the drug. However, since the sterile powder is reconstituted with aqueous diluents, the drug is effectively converted to the diol form before use.

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[8] The estimate of the uncertainty (±10%) is based on replicate data processing. Difficulties in phasing the spectrum and correcting for baseline roll led to the largest uncertainties in this measurement.