Determination of α-Impurities in the β-Polymorph of Inosine Using Infrared Spectroscopy and X-ray Powder Diffraction

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An X-ray powder diffraction method for the quantitative determination of the α-inosine content of mixtures of the crystalline polymorphic forms of α- and β-inosine is described. Oriented sample discs are prepared by pressing with a cellulose binder and the α-inosine content is calculated from measurements of the 8.25 Å and 7.25 Å X-ray diffraction intensities of α- and β-inosine, respectively. The method has a relative deviation of 7% and a detection limit of 0.4% α-inosine. This represents a 35-fold improvement in sensitivity over conventional infrared spectroscopy and a 14-fold improvement over Fourier transform infrared spectroscopy.

Keywords: *α-Inosine determination; polymorphs; X-ray diffraction; infrared spectroscopy; pharmaceuticals*

There is a growing interest in the pharmaceutical applications of polymorphism[1] and hence in techniques for its identification and quantification. It is also required routinely by some national drug regulatory agencies that specific methods for detecting the absence of unwanted polymorphs be developed as routine quality control assays. Such unwanted polymorphs will not necessarily have any intrinsic toxicity but they must nevertheless be quantified.

Inosine, $C_{10}H_{12}N_4O_5$, crystallises in two polymorphic anhydrous forms, one of which is orthorhombic and one monoclinic.[2,3] Inosine also forms a dihydrate that is monoclinic. Suzuki and Nagashima[2] designated the orthorhombic form of anhydrous inosine the α-form and the monoclinic form of anhydrous inosine the β-form. In our work it was important to detect and quantify, with a detection limit of less than 5%, the presence of α-inosine in samples of the β-polymorphic form. Preliminary investigations were made using differential-scanning calorimetry (DSC) but this technique proved unsuitable because of the closeness of the melting-points of the two polymorphic forms. We therefore investigated the suitability of infrared spectroscopy and X-ray powder diffraction methods for this purpose.

The application of infrared spectroscopy to the analysis of pharmaceutical compounds is well known. However, no methods for the determination of inosine in the solid state appear to have been reported. Quantitative X-ray diffractometry is a technique that has considerable potential in the assay of pharmaceutical materials and was used by Christ *et al.*[4] in their determination of crystalline sodium penicillin G. Shell[5] pointed out that "published reports on quantitative diffraction applications to organic systems are almost non-existent," and described several methods of determining crystalline components in drug systems.

However X-ray powder diffraction has continued to find only occasional pharmaceutical application, such as in the analysis of intact tablets by Papariello *et al.*[6] and oral suspensions by Kuroda.[7] More recently Imaizumi *et al.*[8] reported a determination of the degree of crystallinity of indomethacin using lithium fluoride as an internal standard. A useful review is given by Zwell and Danko.[9] An X-ray diffraction analysis of mixtures of inosine polymorphs was described briefly by Suzuki[10] and this formed the basis of our method.

Experimental

Instrumentation

Conventional infrared spectra were obtained using a Pye Unicam SPS 300 instrument. Spectra were also recorded using a Nicolet 5MX Fourier transform infrared spectrometer using a continuous scan Michelson Interferometer, S/5 optics with spectral range 4600–400 cm^{-1}, laser-assisted sample alignment with a resolution of 4 cm^{-1} and a wavelength accuracy of 0.01 cm^{-1} and automatic gain. A standard glower source was used and a two-pen digital plotter with variable abscissa and ordinate expansion.

X-ray diffraction measurements were made using a Philips PW 1050 wide-range goniometer with a 1° dispersion slit, a 0.2-mm receiving slit and a 1° anti-scatter slit. The Cu anode X-ray tube was operated at 40 kV and 20 mA in combination with a Ni filter to give monochromatic Cu Kα X-rays of wavelength 1.5418 Å. The angular calibration of the goniometer was based on the (020) reflection of cholesterol[11] at 5.260 °2θ.

Materials

α-Inosine, crystalline. Ajinomoto Co., Inc., Japan.
β-Inosine, crystalline. Ajinomoto Co., Inc., Japan.
Cellulose powder, Whatman CF 11.

Sample Preparation

For the infrared spectroscopic studies potassium bromide discs containing 1% of inosine were prepared by compression under vacuum at a pressure of 10 ton.

To prepare samples for X-ray diffraction analysis the following method is used. Grind together by hand 1.00 g of inosine sample and 1.00 g of cellulose powder using an agate pestle and mortar. Transfer the mixture into a glass vial, add two 6 mm diameter polystyrene balls, stopper and further homogenise the mixture by shaking for 5 min in a Glen Creston ball mill. Transfer the resulting powder into a 32 mm diameter stainless-steel die and press into a disc at a pressure of 10 ton. The reasons for adopting this method of sample

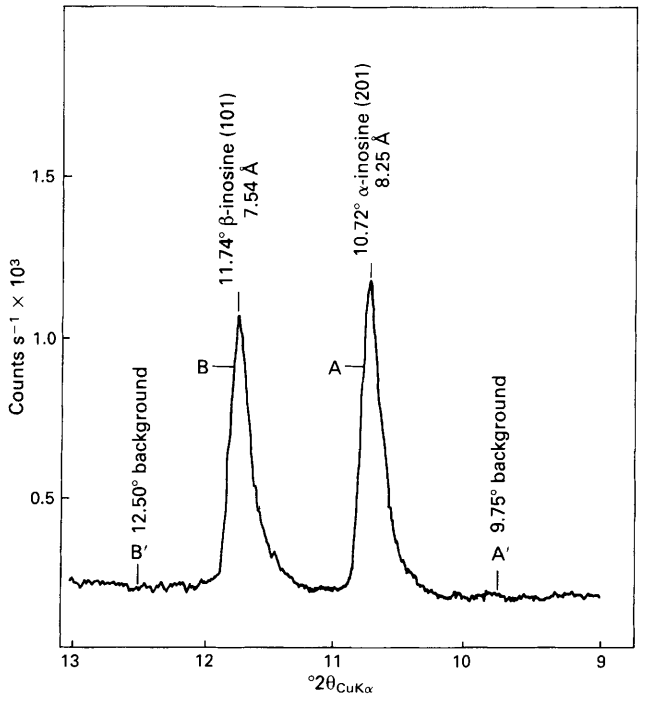

Fig. 1. Portion of X-ray diffraction chart of a mixture of α-inosine (30%) and β-inosine (70%) showing the measurements used to derive the intensity values I_α and I_β. Pressed disc, cellulose binder. $I_\alpha = A - A'$; $I_B = B - B'$. Indexing of peaks from Suzuki and Nagashima[2]

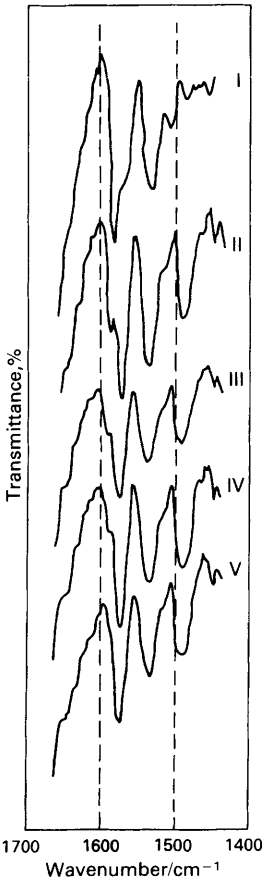

Fig. 2. Infrared transmittance spectra of inosine mixtures. I, 50%; II, 25%; III, 20%; IV, 15%; and V, 10% α-inosine

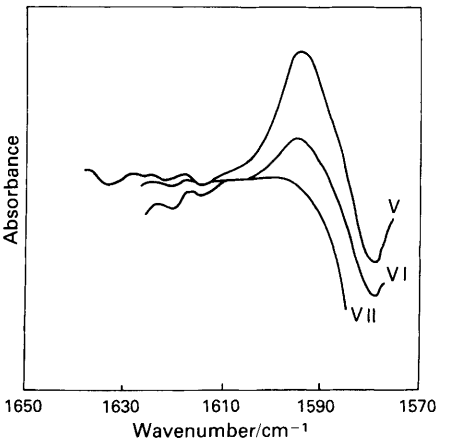

Fig. 3. Absorbance spectra of inosine mixtures acquired using the Fourier transform IR spectrometer. V, 10%; VI; 5% and VII, 2.5% α-inosine

preparation as opposed to the conventional cavity mounts are discussed in detail below.

Procedure for X-Ray Diffraction Analysis

Insert the inosine - cellulose disc into the diffractometer and record the number of counts obtained in 60 s at 9.75 °2θ (α-inosine background), 10.72 °2θ (α-inosine peak), 11.74 °2θ (β-inosine peak) and 12.50 °2θ (β-inosine background). Subtract the appropriate background counts from the peak counts to give the diffracted intensity values I_α and I_β for α- and β-inosine, respectively (Fig. 1). Calculate the α-inosine concentration, C_α from the following equation:

$$C_\alpha = \frac{100 I_\alpha}{(I_\alpha + 2.52 I_\beta)} \%$$

Determine also the blank using a sample of pure β-inosine reference standard.

It is assumed that a preliminary diffractogram of the sample will have been recorded in order to check the peak positions and to verify that interfering peaks are absent. All our samples were known to contain only anhydrous inosine.

Results and Discussion

Infrared Methods

Conventional infrared spectra provided a rapid means of identifying the form of inosine present. Visual examination of the spectra suggested that the α-band at 1593 cm^{-1} and the β-band at 1577 cm^{-1} were the most suitable bands for quantification by the base-line method.[12] Transmittance spectra illustrating these bands for inosine mixtures containing 10, 15, 20, 25 and 50% of the α-polymorph are shown in Fig. 2. The peak at 1593 cm^{-1} is visible in spectra of samples containing 15% or greater of the α-form, but was absent in samples containing 10% or less of α-inosine. The detection limit for α-inosine was improved by using the Fourier transform instrument. Absorbance spectra (with subtraction) of inosine mixtures containing 10, 5 and 2.5% α-inosine are shown in Fig. 3 and indicate that α-inosine may be detected down to about 5% by this method.

X-Ray Diffraction Method

Peak height versus *peak area*

On many diffractometers it is easier to measure peak heights rather than peak area and for the sake of simplicity we have adopted this practice here. In some instances peak-height measurement also has the added advantage of avoiding interference from adjacent peaks. On the other hand,

peak-area measurement can result in improved precision and compensates for the loss of intensity owing to crystal defects and small particle size.

Preferred orientation effects

In our preliminary investigation of an X-ray diffraction assay for inosine polymorphs we used conventional cavity mounts. However, satisfactory precision could not be obtained and this was attributed to the crystal morphology of the two phases. α-Inosine forms long needle-like crystals while β-inosine crystals have a platy habit (shape), and it is very difficult to make a randomly oriented cavity mount of either of these materials because of the tendency for the crystals to adopt preferred orientations. Consequently, the reproducibility of X-ray diffraction intensities from cavity mounts is very poor even when careful packing procedures are employed. It is worth pointing out that preferred orientation affects (enhances or diminishes) all X-ray reflections from a crystalline powder and not just those arising from lattice planes parallel to the orientation. The problem cannot, therefore, be approached by choosing to measure a different reflection. An attempt was made to overcome these orientation effects by grinding the sample with a small amount (2%) of carbon black, as recommended for this purpose by Christ *et al.*[4] However, no improvement in precision could be obtained by this means and scanning-electron micrographs of the resulting powders showed the characteristic morphology of the inosine crystals to be unchanged. The use of cavity mounts was then abandoned and instead *highly oriented* mounts were made in the form of pressed discs with a cellulose binder. These discs are self supporting, easy to handle and can be inserted directly into the diffractometer. A comparison of the diffraction patterns of both α-inosine and β-inosine showed little difference in relative peak intensities between the cavity mounts and the pressed discs, confirming that a high degree of preferred orientation was already present in the cavity mounts. The reproducibility of X-ray intensities from the pressed discs was, however, greatly improved and this method of sample preparation was therefore adopted. No deleterious effects on crystal structure owing to pressure were observed.

It is possible that different crystallisation regimes may give rise to crystals of different habit and hence different orientation behaviour. The grinding step is therefore of considerable importance as grinding alters the original crystal habit by producing cleavage flakes, thus ensuring that the morphology of the grains in the resulting powder is uniform for any given phase. It also ensures that the habits present in both standard and sample are essentially the same.

Accuracy

The accuracy of the X-ray method was assessed by analysing inosine mixtures containing 0, 0.5, 1, 10, 20, 30, 40 and 50% of the α-polymorph. The results are shown in Table 1. The relationship between the α-inosine concentration taken and the α-inosine concentration found is described by a linear regression equation with slope equal to 0.997 and an intercept of 0.20% of α-inosine. This closely approximates to the ideal line of unit slope and zero intercept.

Table 1. Results of X-ray diffraction analysis of mixtures containing known masses of inosine polymorphs

Amount of α-inosine, %			
Taken	Found*	n	σ_{n-1}
0.00	0.002	8	0.10
0.50	0.54	8	0.14
1.00	0.90	8	0.13
10.00	11.45	2	—
20.00	19.31	8	1.47
30.00	30.24	2	—
40.00	40.49	2	—
50.00	49.72	2	—

* Mean of n replicate analyses of the same disc.

Precision

The precision of the method was determined from the analysis of eight replicate discs made from an approximately 1 + 3 mixture of α-inosine and β-inosine by mass. The mean α-inosine concentration found was 24.18% *m/m* with a standard deviation of 1.68% *m/m*. This represents a relative deviation of 6.95%. Counting statistics alone account for a relative deviation of about 2%.

Detection limit

In this work it was anticipated that most of the inosine samples that would be assayed would not contain any detectable amount of α-inosine. An accurate determination of the detection limit of the method was therefore of paramount importance. Accordingly, two approaches were adopted for the estimation of the detection limit. The first approach was based on the standard deviation of the blank. A blank disc was prepared using a pure β-inosine reference standard. This standard was considered to be pure because no α-inosine peak was present in the X-ray diffraction pattern and measurement of the parameter I_α gave a value identical to that of pure cellulose. Eight replicate analyses of this disc had a standard deviation of 0.095% *m/m*. The detection limit can therefore be taken to be three times this value, *i.e.*, 0.29% α-inosine. However, we consider this to be an over-optimistic estimate of the detection limit because it assumes that the noise characteristics of the background are similar to the noise characteristics of the signal.

In X-ray diffraction methods this is not a valid assumption as a significant component of the signal noise is likely to be represented by non-reproducibility in the sample preparation technique. The second approach is an extension of the first and is more realistic in that it incorporates information about the analytical precision into the definition of detection limit. Following the theoretical model of the relationship between precision and concentration developed by Thompson and Howarth[13] the detection limit is defined as the concentration at which the precision is 100%. For a 3σ detection limit this value can be found from the following equation:

$$C_d = \frac{300\sigma_0}{(100 - 3k)}$$

where C_d is the detection limit, σ_0 is the standard deviation at zero concentration and k is the precision of the method at high concentrations expressed as a relative deviation. Substituting the values $\sigma_0 = 0.095$ and $k = 6.95$ leads to a detection limit of 0.4% α-inosine.

Calculation of α-Inosine Concentration from I_α and I_β Values

The equation already given for the calculation of α-inosine concentrations can be derived from the fundamental equation for quantitative X-ray diffraction stated by Klug and Alexander.[14] The diffracted X-ray intensity (I) arising from a component of a mixture is given by the equation

$$I = \frac{kx}{\rho\,[x(\mu - \mu_m) + \mu_m]} \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where k is a constant depending on the nature of the component, x the mass fraction of the component, ρ the density, μ the mass absorption coefficient and μ_m the mass absorption coefficient of the matrix. In the special case of

mixtures of polymorphic forms of a substance, as is the case with the inosine system, we have

$$\mu = \mu_m$$

and therefore equation (1) can be simplified to

$$I = \left(\frac{k}{\rho\mu}\right) x \text{ or } I = Kx$$

where the intensity is directly proportional to the concentration and the constant K is a measure of the sensitivity.

For a binary mixture of two components, A and B, we obtain the two equations:

$$I_A = K_A x_A \quad \ldots \quad (2)$$

and

$$I_B = K_B x_B \quad \ldots \quad (3)$$

As A and B are the only components (the cellulose binder can be neglected),

$$x_B = 1 - x_A$$

Substituting this value of x_B in equation (3) and combining equations (2) and (3) leads to the equation

$$x_A = \frac{I_A}{I_A + [(K_A/K_B)\, I_B]} \quad \ldots \quad (4)$$

The ratio K_A/K_B is the ratio of the sensitivities of the two components A and B.

The value of this ratio was determined by measuring the diffraction intensities of the α- and β-diffraction peaks using a disc pressed from a mixture of equal masses of the two components and was found to be 2.52. It would be advisable for other workers to determine their own values for this ratio as it may vary slightly between different instruments and different laboratory procedures.

Interestingly, Chung[15] has shown that relationships similar to equation (4) are applicable to any binary system and not only those consisting of two polymorphs. This means that the method of calculation described above can be used in a wide range of pharmaceutical assays.

Conclusion

X-ray powder diffractometry, in combination with a pressed-disc sample-mounting technique, provides an accurate and sensitive method for determining the polymorphic composition of crystalline inosine samples. The sensitivity is 35-fold greater than that obtainable using conventional infrared spectroscopy and 14-fold greater than that using Fourier transform infrared spectroscopy. X-ray diffraction methods such as this are capable of application to a wide variety of pharmaceutical assays.

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