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Quantitative analysis of mannitol polymorphs. FT-Raman spectroscopy

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Abstract

Mannitol is a polymorphic excipient which is usually used in pharmaceutical products as the beta form, although other polymorphs (alpha and delta) are common contaminants. Binary mixtures containing beta and delta mannitol were prepared to quantify the concentration of the beta form using FT-Raman spectroscopy. Spectral regions characteristic of each form were selected and peak intensity ratios of beta peaks to delta peaks were calculated. Using these ratios, a correlation curve was established which was then validated by analysing further samples of known composition. The results indicate that levels down to 2% beta could be quantified using this novel, non-destructive approach. Potential errors associated with quantitative studies using FT-Raman spectroscopy were also researched. The principal source of variability arose from inhomogeneities on mixing of the samples; a significant reduction of these errors was observed by reducing and controlling the particle size range. The results show that FT-Raman spectroscopy can be used to rapidly and accurately quantitate polymorphic mixtures.

Keywords: FT-Raman spectroscopy; Mannitol; Polymorphism; Quantitation

1. Introduction

Polymorphism describes different packing arrangements of the same molecular species [1]. The detection of polymorphs has become essential in the pharmaceutical industry since different modifications can have markedly different biopharmaceutical properties. By investigating the possible polymorphic modifications of a drug, costly repercussions can be avoided through the untimely appearance of new forms [2].

Large variation in the properties of some polymorphs may be exhibited which, leads to their exploitation to produce a polymorph more appropriate to the final dosage form [3]. Pharmaceutically desirable properties such as chemical stability may depend heavily on the polymorph selected and the polymorphic form of a drug may

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change on storage which necessitates the need for detection and quantitation of polymorphic mixtures [4]. The possibility of polymorphic transformations, therefore, imposes a potential risk to patients which is considered so important that the International Conference on Harmonisation (ICH) requires that proper investigations are conducted according to a decision tree [5,6].

Once elemental analysis has ascertained the same chemical composition of the various solid forms, then different physical methods are employed to verify the structural differences and hence confirm the presence of polymorphism. The most precise technique for providing this structural information is single-crystal X-ray crystallography. However, sample requirements include high-quality single crystals, which in practical terms can be difficult to produce [7].

X-ray powder diffractometry (XRPD) is a widely used technique in the identification of polymorphs since it is non-destructive in nature and requires relatively small amounts of sample. Analogous to single-crystal X-ray crystallography, XRPD provides information on long-range structure, however, XRPD can be used to characterise materials that are neither perfectly crystalline nor pure [7]. Solid-state nuclear magnetic resonance (SSNMR) has also proved useful in identifying polymorphs especially in cases where polymorphism has been mistaken for tautomerism as in the case of acetohexamide studied by Stephenson et al. [8]. Spectroscopic techniques such as infrared (IR) and Raman complement the diffraction techniques by supplying information on the short-range structure of polymorphs. The fundamental molecular vibrations are different for each polymorph providing a unique 'fingerprint' in the form of a spectrum [7].

Once the identification and characterisation of polymorphs has been accomplished, then the exploitation of some physical methods can allow the quantitation of polymorphic composition and degree of crystallinity. These methods include X-ray powder diffraction techniques where Takahashi et al. quantitatively analysed α- and β- forms of L-glutamic acid. Their work documented a lower limit of detection of approximately 2% for each form of L-glutamic acid although repetition of their experiment was discussed in order to improve accuracy of the results [9]. A thorough investigation of potential errors associated with quantitative analysis using XRPD was performed by Suryanarayanan in his work involving the various polymorphs of carbamazepine [10,11]. Surana and Suryanarayanan later applied this technique to the determination of crystallinity of sucrose, achieving a detection and quantitation limit of 0.9 and 1.8% (w/w), respectively, for the crystalline component [12]. Quantitation of hydrated forms of a given compound can also be achieved as demonstrated in the study performed by Bugay and his co-workers. An acetone slurry technique combined with XRPD was used to successfully quantitate dihydrate content in cefepime · 2HCl monohydrate samples. In addition to establishing a minimum quantifiable level of 2.5% (w/w) and a limit of detection of 0.75% (w/w) dihydrate in monohydrate material, their work included an in-depth evaluation of potential assay errors. Also recorded in their study was the quantitation of the dihydrate sample using IR spectroscopy. A lower minimum quantifiable level (1% w/w) and limit of detection (0.3% w/w) were noted in a working range of 1–8% (w/w) dihydrate in cefepime · 2HCl monohydrate [13].

A relatively uncommon quantitative method of solution calorimetry was used by Pikal et al. in their quantitative determination of crystallinity in β-lactam antibiotics. The observation that the amorphous forms of a compound are usually higher in energy than crystalline forms provides the basis for this calorimetric application. In the case of β-lactam antibiotics, quantitation using solution calorimetry proved more reliable than X-ray powder diffraction methods [14].

Each polymorphic system presents its' own problems with regard to the choice of quantitative technique and it is unlikely that any one method would be universally suitable. Previous quantitative work may have been compromised through workers selecting the technique that demonstrates the greatest measurable differences between the polymorphs [15]. This may no longer be the case since the recent successful application of FT-Raman spectroscopy as a potential tool for quantitative analysis. FT-Raman spectroscopy is a light

scattering technique whereby a monochromatic source (laser) illuminates the sample and the resulting scattered light is collected and analysed. Photon interaction with the molecular vibrations of a sample causes the light to be shifted to a wavelength away from the wavelength of the incident laser. This phenomenon is relatively weak with approximately 1 photon in 10 million scattering the light to a shifted wavelength [16]. Thus the Raman technique can be used to determine the chemical structure by the detection of molecular vibrations of characteristic frequency. The polymorphs of a given compound exhibit different molecular vibrations owing to their different packing arrangements and hence individual vibrational spectra are given.

Until recently, FT-Raman spectroscopy had largely been used when more established techniques such as XRPD, NMR, IR and differential scanning calorimetry (DSC) had failed to adequately discriminate between polymorphic forms [16]. However, FT-Raman spectroscopy is gaining a worthy reputation as an analytical tool for both qualitative and quantitative measurements and numerous compounds have now been examined by this spectroscopic technique. For example the concentration of vitamin A in sorbitan monooleate was quantified to a level of 0.005% (w/w) in the study performed by Hancewicz and Petty where quantitative analysis was achieved using FT-Raman spectroscopy and a partial least squares model [17]. Studies determining the degree of crystallinity have also been completed using polylactide and indomethacin [18,19]. In the latter case, Taylor and Zografi used FT-Raman spectroscopy to successfully quantitate mixtures of amorphous and crystalline indomethacin achieving a limit of detection of 0.6% and limit of quantitation (LOQ) of 2% for both components. In addition to this, they evaluated potential errors associated with the technique and attributed the largest source of variation in the measurements to inhomogeneous mixing of the amorphous and crystalline particles [19]. FT-Raman spectroscopy has also been utilised in a more diverse clinical application through the work of Kontoyannis et al. where a detection limit of 0.6 mol% was observed for calcium oxalate monohydrate, a mineral component found in urinary stones [20].

The nature of the polymorphic material can cause problems with regard to quantitative work, for example the possibility of polymorphic transitions during KBr disk formation opposes the use of IR spectroscopy for some polymorphic systems [15]. Also, crystals that exhibit needle-like morphologies are highly susceptible to preferred orientation effects which could have a significant influence on quantitative results collected from X-ray powder diffraction studies. This was observed by Bugay et al. in their work involving the cefepime · 2HCl hydrates [13]. Here, we have selected FT-Raman spectroscopy to examine its applicability as a quantitative method for measuring beta mannitol in binary mixtures of beta and delta mannitol.

Mannitol is used in the manufacture of chewable tablet formulations, granulated powders for oral use, in lyophilised products such as injections and commonly exists in three polymorphic forms: alpha, beta and delta. Recently Burger et al. addressed the confusion in the literature concerning the polymorphs in addition to investigating their compression behaviour [21]. Physico-chemical properties such as melting point and heat of fusion show very little difference between the alpha and beta forms of mannitol. For example, alpha melts at 166 °C and beta at 166.5 °C, this indicates there are small energetic differences between these two forms which have been observed to exhibit the same space group (orthorhombic $P2_12_12_1$) [21,22].

At temperatures of 20 °C and greater Burger et al. determined that beta is the most thermodynamically stable form. Conversely the delta form (exhibiting a monoclinic $P2_1$ space group and a melting point of approximately 155 °C) is thermodynamically unstable at ambient conditions although it was reported to exhibit significant kinetic stability [21].

In this work we used FT-Raman spectroscopy to quantify beta in binary mixtures of the beta and delta forms of mannitol. These particular forms were chosen because a delta to beta transition has been observed at elevated temperatures [21,23] and since beta is the most thermodynamically stable form, then this will be the form to which other forms will eventually convert [24].

This gives rise to the possibility of a delta to beta transition on storage at ambient temperatures which, could subsequently alter dosage form performance. In addition, delta mannitol has recently been shown to possess superior tabletting properties to the beta form [21] and there is commercial interest in using delta mannitol as an excipient.

Spectral peaks characteristic of each form were selected and their peak heights measured to give an intensity ratio of the beta to the delta component. Analysis of the subsequent correlation curve allowed the polymorphic composition of validation samples to be estimated from their spectra. Potential errors associated with the quantitative assay were also investigated.

2. Experimental section

2.1. Materials

Analar delta mannitol (1,2,3,4,5,6-hexanehexol, $>99\%$), from BDH Laboratory Supplies was used as obtained. The beta form was crystallised by adding approximately 13 g of commercial mannitol to 50 ml distilled water and stirring for 20 min at a temperature of 50 °C. The solution was allowed to cool to room temperature and the resulting crystals were removed and allowed to dry naturally. Generally, a sieve cut of <125 μm was used for both polymorphs although a sample of 125–500 μm was used to demonstrate problems associated with the FT-Raman analysis of larger sized particle mixtures.

2.2. X-ray powder diffractometry

To confirm the identity of the polymorphs, X-ray powder diffraction patterns of the polymorphs were obtained using a Siemens D5000 Powder Diffractometer (Siemens, Karlsruhe, Germany) equipped with a scintillation counter detector and a divergent beam. This beam employed a CuK_α source with a wavelength of 1.5418 Å containing 2 mm slits over the range of 9–40° 2θ at a scan rate of 3 s and a step size of 0.05° 2θ. The generator was set to 40 kV and 30 mA.

2.3. Preparation of samples

Triplicate mixtures (0.5 g) of known composition (w/w%) of the beta and delta polymorphs were prepared gravimetrically (Sartorius Research balance, model R160P, Gottingen, Germany). All samples were tumble mixed (Wad Turbula T2C, System Schatz, Switzerland) for 100 revolutions before spectroscopic analysis. Other than the validation samples, each of the triplicate known composition mixtures were also analysed in triplicate (i.e. three sub samples of each composition mixture were assayed giving nine analyses per composition).

2.4. FT-Raman spectroscopy

Samples were placed into stainless steel cups and triplicate FT-Raman spectra of each sample mixture were obtained using a Bruker FRA Raman module on a Bruker IFS 66 Optics system. The Nd:YAG laser operated at 1.064 μm and the laser was focussed on the sample as an approximately 100 micron spot. A range of laser powers and scan accumulations were investigated before determining that a laser power of 450 mW and 100 scans provided optimum sampling conditions. The scattered radiation was detected by a liquid-nitrogen-cooled germanium detector, which gave a spectral range of approximately $50-3600$ cm^{-1}. Spectral analysis using peaks at 1037 and 1052 cm^{-1} for beta and delta polymorphs, respectively, was completed using GRAMS 32 version 5 software (Galactic Industries Corporation, USA).

2.5. Estimation of assay errors

Potential sources of error associated with the assay were investigated. Various sources of error have previously been studied by Taylor and Zografi, and Bugay et al. in their quantitation work involving FT-Raman spectroscopy, and diffuse reflectance IR spectroscopy and XRPD, respectively, [13,19]. The variation of detector response during a typical day is a further potential source of error and so was included in our investigation. Estimation of assay errors was completed using a beta:delta mixture of mid-calibration range.

The following potential error categories were examined.

2.5.1. Instrument and day-to-day reproducibility

Reproducibility of the instrument was investigated by acquiring six consecutive spectra of a single sample. Variability in instrument response was also investigated over a time period of 5 days. A single sample was placed in the instrument and a spectrum was recorded each day.

2.5.2. Intra-day reproducibility

Variability of the detector response (liquid-nitrogen-cooled) over a typical working day was investigated by using a single sample and acquiring ten data sets over an 8 h period.

2.5.3. Sample mixing

To assess the variation due to sample mixing, the spectra of ten sub-samples were obtained from the whole sample mixture.

2.5.4. Sample packing

Variations due to crystal orientation were assessed by re-packing the same sample ten times and acquiring a single spectrum after each packing.

2.5.5. Sample positioning

Effects of variation in position of the sample holder within the instrument were examined by using a single sample and randomly repositioning the holder ten times.

2.5.6. Laser power

Effects of laser power variation were examined by obtaining three spectra at laser powers of approximately 225, 450 and 900 mW.

2.5.7. Particle size

Effects of particle size were examined by manually sieving samples to give particle size ranges of below 125 and 125–500 μm. The homogeneity of the mixture of each particle size range was examined by recording the spectra of ten sub-samples from the whole sample mixture.

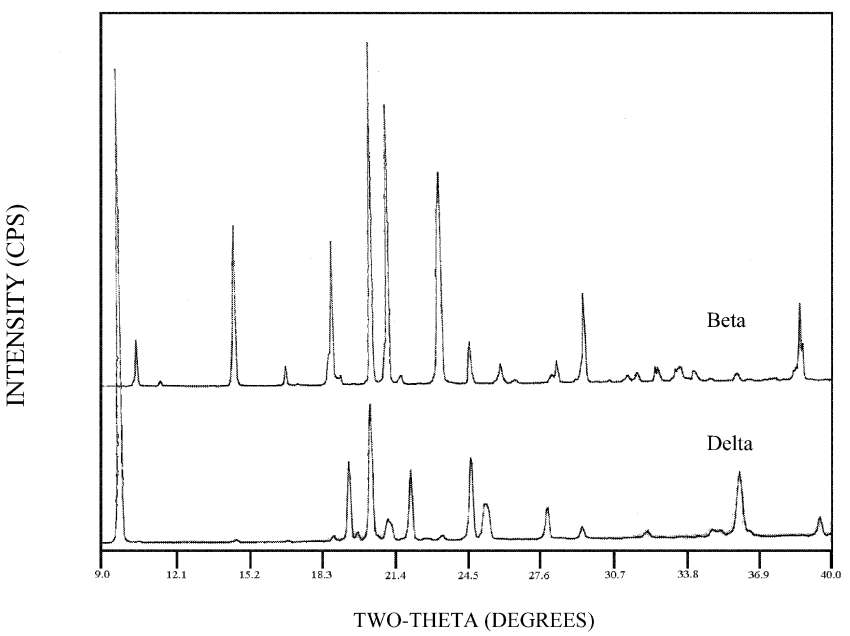

Fig. 1. XRPD patterns of the beta and delta polymorphs of mannitol.

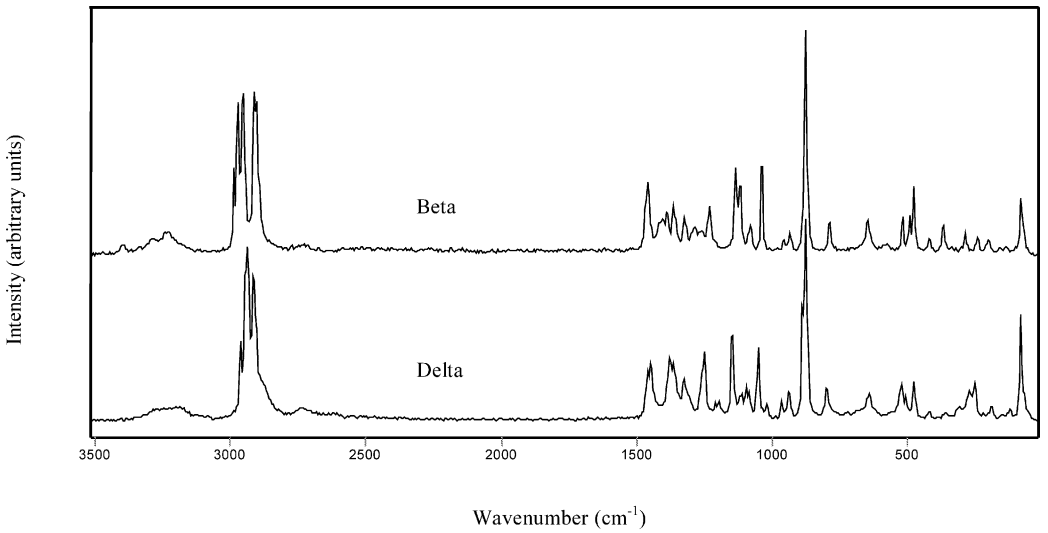

Fig. 2. FT-Raman spectra of the beta and delta forms of mannitol assumed to be 100% pure.

3. Results and discussion

3.1. Characterisation of the reference polymorphs

Figs. 1 and 2, respectively, show the X-ray powder diffraction patterns and FT-Raman spectra of the beta and delta polymorphs. Each sample was considered 100% pure polymorph. As can be seen from the diffraction patterns, the beta and delta forms of mannitol are highly crystalline since no broad halo pattern synonymous with amorphous materials can be observed. The diffraction patterns are unique to each form and are comparable with those found in the literature [21].

Generally, for most polymorphic systems the FT-Raman spectral patterns below 200 cm^{-1} show significant differences allowing differentiation of the various modifications. This region contains information concerning the lattice vibrations of a compound, and so the different packing arrangement of molecules found in different polymorphs leads to observable spectral variations [16].

Thus, for polymorphic analyses, FT-Raman spectroscopy may be preferable to IR spectroscopy since information on lattice modes is not typically collected by mid-IR spectroscopy [16]. However, the relative intensities of these lattice modes are weak compared with the stronger FT-Raman peaks visible at higher wavenumbers and thus quantitation would be difficult using this spectral region for analysis. Selecting a suitable region for quantitative analysis would initially appear difficult since the FT-Raman spectra have very similar patterns. This is expected since the material is essentially the same with only relatively minor differences in the molecular vibrations reflecting the different packing arrangements of the molecules.

The FT-Raman spectrometer allows good quality data to be collected with little interference from background noise and this allows one to observe subtle differences between the spectra of the two modifications. These patterns were compared with those recorded by other workers and were found to be in good agreement [21].

3.2. Preparation of the correlation curve

The peaks contained within a Raman spectrum can be used to quantify materials in several ways e.g. using peak height or peak height times half width measurements. The measured intensity of a Raman line is dependent upon a number of factors, the relationship of which is given in Eq. (1):

$$I(v) = I_o K(v) C \quad (1)$$

where $I(v)$ is the measured intensity of the Raman line, I_o is the intensity of the excitation laser line, v is the Raman shift, C is the concentration of the scattering species and $K(v)$ describes the overall spectrometer response, the self absorption and molecular scattering properties of the medium. The K value can be difficult to determine since all spectrometers differ slightly. However, as long as the instrumental conditions (laser power, resolution and number the scans) do not change, then it can be assumed that the only factor changing will be the concentration of the polymorphs and it is this that will ultimately be responsible for the observed change in peak intensities [25,26].

In order to analyse the spectra quantitatively, characteristic peaks of each form were selected; 1037 cm^{-1} for the beta form and 1052 cm^{-1} for the delta form (Fig. 3). The literature does not contain comprehensive assignments for all the vibrational modes of mannitol, but these peaks are consistent with a CCO stretching vibration [27,28].

From Fig. 3 it can be seen that the peaks at 1037 cm^{-1} (β) and 1052 cm^{-1} (δ) are not completely resolved. Therefore, the contribution of the beta peak to the intensity of the delta peak

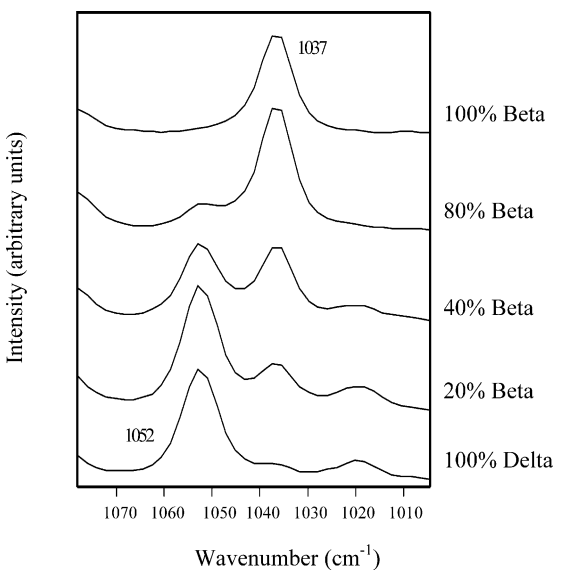

Fig. 3. FT-Raman spectra indicating the spectral regions associated with the quantitative analysis.

(and vice versa) must be taken into consideration. A method developed by Kontoyannis et al. and practised by Taylor and Zografi allows this contribution to be calculated in cases where the peaks are not completely resolved [19,20].

The intensity at 1037 cm^{-1} is the sum of the intensities of the beta ($I_\beta^{1037} = I_o\ K_\beta^{1037}\ x_\beta$) and delta ($I_\delta^{1037} = I_o\ K_\delta^{1037}\ x_\delta$) constituents where x_β and x_δ are the molar fractions of the beta and delta components, respectively. The intensity at 1052 cm^{-1} is, therefore, the sum of the intensities of the beta ($I_\beta^{1052} = I_o K_\beta^{1052}\ x_\beta$) and delta ($I_\delta^{1052} = I_o K_\delta^{1052}\ x_\delta$) components. The ratio of intensity of the 1037 cm^{-1} peak to the intensity of the 1052 cm^{-1} peak (I_r) can be determined using Eq. (2)

$$I_r = \frac{I^{1037}}{I^{1052}} = \frac{x_\beta K_\beta^{1037} + x_\delta K_\delta^{1037}}{x_\beta K_\beta^{1052} + x_\delta K_\delta^{1052}} \quad (2)$$

I^{1037} and I^{1052} represent the measured intensities of the 1037 and 1052 cm^{-1} bands, respectively. The ratios $K_\beta^{1052}/K_\beta^{1037}$ and $K_\delta^{1037}/K_\delta^{1052}$ can be determined from the 'pure' (assumed to be 100%) spectra of the beta and delta polymorphs, respectively. The ratios of K values were obtained by calculating the intensity ratio of the peak heights at 1052 and 1037 cm^{-1} for nine samples of each 'pure' polymorph and obtaining the mean value. The $K_\beta^{1052}/K_\beta^{1037}$ ratio was calculated as 0.0472 ($\pm$0.0083, S.D.) and $K_\delta^{1037}/K_\delta^{1052}$ as 0.0586 ($\pm$ 0.0136).

The above ratios indicate that the contribution of the beta component to the spectral region where the intensity of the delta peak is measured is larger than the contribution of the delta component to the region where the intensity of the beta peak is measured. The K ratio values may then be substituted into Eq. (3), which is derived from Eq. (2):

$$\frac{K_\delta^{1037}}{K_\delta^{1037}} \frac{(I_r 0.0472 - 1)}{(I_r - 0.0586)} = \left(1 - \frac{1}{x_\beta}\right) \quad (3)$$

The ratio of peak intensities at 1037 cm^{-1} to that at 1052 cm^{-1} was then calculated in triplicate to give the intensity ratio (I_r) of mixtures containing 0.5 – 99.5% of the beta polymorph. $(I_r - 0.0586)(x_\beta - 1)$ was then plotted against $x_\beta(I_r\ 0.0472 - 1)$, the slope of which equalled the value of $K_\beta^{1037}/K_\delta^{1052}$ (Fig. 4). The slope of this plot is

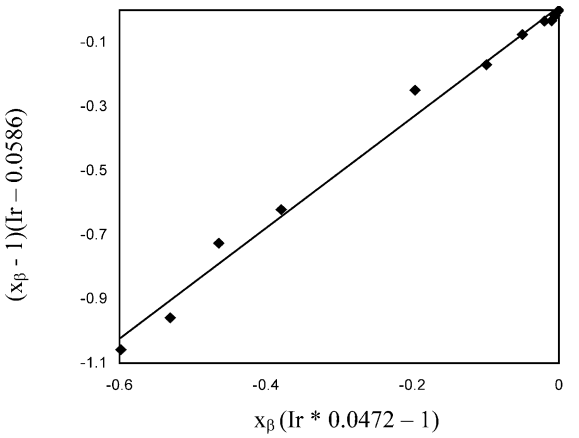

Fig. 4. Plot of $(x_\beta - 1)(I_r - 0.0586)$ against $x_\beta(0.0472^* I_r - 1)$ where x_β represents the molar fraction of the beta constituent and I_r is the intensity ratio of the peak at 1037 cm^{-1} to that at 1052 cm^{-1} using 0.5–80% beta samples.

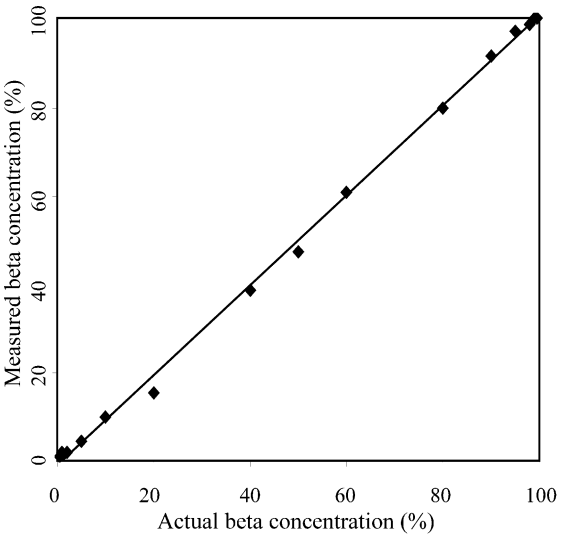

Fig. 5. Correlation plot for samples containing 0.5–99.5% of the beta component.

1.7227, i.e. $K_\beta^{1037}/K_\delta^{1052} = 1.7227$. This could then be included into Eq. (3), which was rewritten to incorporate the intensity ratio and the K ratio contribution factors Eq. (4).

$$x_\beta = \frac{(I_r - 0.0586)}{(0.9187 \times I_r + 1.6641)} \quad (4)$$

Once the intensity ratio of a particular sample is known, then the percentage of β can be determined using Eq. (4). The calibration data may then be plotted to demonstrate the relationship between the actual percentage of the beta component and the calculated amount (Fig. 5). The slope of this plot is 1.0219 and the correlation coefficient (R^2) is 0.9986 illustrating the validity of Eq. (4).

3.3. Preparation of the validation curve

In order to demonstrate the validity of the correlation data, samples of known composition were prepared and analysed (Fig. 6). Nine validation samples were used in the construction of the validation plot, the concentrations of which were selected to differ from those of the calibration samples. The validation samples covered a large proportion of the composition range: 0.75, 3, 8, 30, 55, 70, 92, 97 and 99.25% of the beta component. Measurement of these compositions gave linear data with a slope of 1.0137 and a correlation coefficient of 0.9989. This indicates that the measured concentration of beta in a sample is in good agreement with the actual concentration.

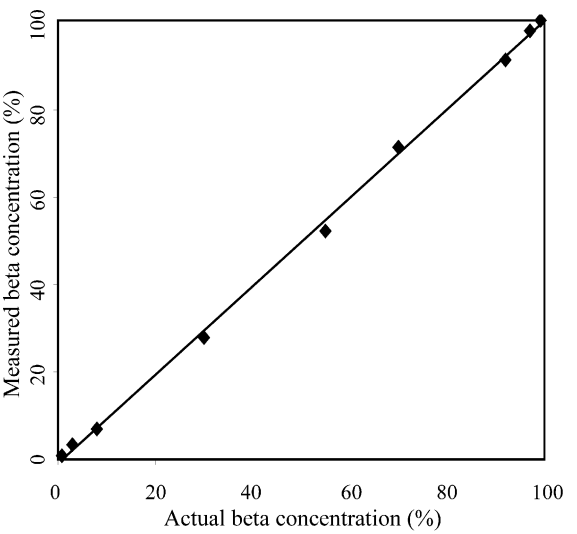

Fig. 6. Validation plot for samples containing the beta and delta forms of mannitol.

Table 1
Measured beta concentrations (%) with standard deviation in parentheses

Actual beta concentration (%)	Measured beta concentration (%)		
	Sample 1	Sample 2	Sample 3
0.5	1.6 (0.2)	0.4 (0.3)	0.8 (1.0)
0.75	0.9 (0.2)		
1	1.1 (0.7)	2.4 (0.6)	2.2 (1.0)
2	2.3 (0.3)	1.0 (0.4)	2.5 (0.6)
3	3.4 (0.2)		
5	4.7 (0.9)	4.2 (0.2)	4.4 (1.3)
8	6.9 (0.7)		
10	10.2 (1.1)	11.3 (1.7)	8.2 (0.3)
20	17.1 (1.1)	15.2 (2.4)	14.2 (1.2)
30	27.9 (0.1)		
40	38.0 (2.6)	39.9 (0.6)	37.6 (3.8)
50	45.0 (1.4)	46.6 (1.2)	50.4 (2.2)
55	52.4 (0.8)		
60	61.0 (0.8)	59.3 (2.5)	62.9 (1.7)
70	71.5 (2.5)		
80	81.8 (3.4)	78.1 (2.6)	80.6 (1.7)
90	92.5 (1.3)	93.3 (4.0)	91.3 (1.7)
92	91.6 (0.2)		
95	96.7 (1.2)	98.5 (1.8)	98.5 (1.3)
97	98.2 (0.1)		
98	99.4 (1.0)	99.6 (0.9)	100.3 (1.4)
99	100.5 (1.4)	100.9 (1.3)	101.0 (0.5)
99.25	100.6 (0.8)		
99.5	101.0 (1.1)	101.2 (0.8)	100.3 (1.5)

3.4. Evaluation of limit of detection and limit of quantitation

The limit of detection (LOD) of the β form in a mixture of the beta and delta polymorphs can be estimated by calculating three standard deviations (S.D.) of the measurement of the delta concentration, which can be derived using Eq. (4) for the pure delta sample. The S.D. for eight measurements of pure delta mannitol was 0.57% resulting in a lower limit of detection of 1.7%. The LOQ can be determined by multiplying ten S.D.s, which results in a quantitation limit of 5.7%. These values appear overly cautious since the experimental data demonstrate that approximately 2% beta could be quantified using this method (Table 1).

3.5. Determination and quantitation of sources of error

Acquiring six consecutive spectra of a mid-range composition sample gave a beta concentration of $44.9 \pm 0.5\%$ (Table 2). Thus the variation introduced to the method through the reproducibility of the instrument was 1.1%. This variation designated the relative standard deviation (R.S.D.) can be determined using Eq. (5) where m represents the mean measured beta concentration and is the S.D. of the measured beta concentration.

$$\text{R.S.D.} = \frac{\text{S.D.} \times 100}{m} \tag{5}$$

A R.S.D. of 0.4% was calculated for the variation involving alteration of laser power. This suggests that relatively little error is introduced to the method through these two categories, although it is clearly preferable to maintain constant sampling conditions.

Table 2
Summary of estimation of potential assay errors

Source of error	Mean beta concentration ($\pm$S.D.) %	R.S.D. (%)
Day–day reproducibility ($n = 5$)	49.3 ($\pm$1.2)	2.4
Instrument reproducibility ($n = 6$)	44.9 ($\pm$0.5)	1.1
Intra-day reproducibility ($n = 10$)	49.3 ($\pm$0.6)	1.2
Laser power ($n = 3$)	50.0 ($\pm$0.2)	0.4
Sample mixing <125 μm ($n = 10$)	49.0 ($\pm$1.5)	3.1
{125–500 μm ($n = 10$)}	{54.2 ($\pm$16.1)}	{29.7}
Sample packing ($n = 10$)	48.3 ($\pm$3.2)	6.6
Sample positioning ($n = 10$)	49.7 ($\pm$0.6)	1.2

The assessment of variation of instrument response inter-day and intra-day gave identical mean measured beta concentrations of 49.3% with R.S.D. values of 2.4 and 1.2%, respectively. This indicates that the response of the instrument is consistent and demonstrates the robustness of the method. Examination of the sample positioning error resulted in a R.S.D. of 1.2%, again indicating that relatively little error is introduced to the method through the random positioning of the sample cup in the FT-Raman instrument.

One of the most important sources of error to be considered in any quantitative study is the homogeneity of the sample mixtures. This is of considerable importance in quantitation involving FT-Raman spectroscopy since the laser beam focuses on a sample spot size with a diameter of approximately 100 microns. The density of the two individual components may influence mixing, however in this case, the polymorphs show little difference in density (β is 1.490 g cm^{-3}, δ is 1.499 g cm^{-3}) [21] and so was not considered as a significant source of error. Likewise, particle morphology can affect mixing; as both morphologies exist as needles, this factor is also of minor importance in this study. Two particle size ranges were investigated to estimate the contribution of error involving sample homogeneity. In order to control the particle size, the samples were sieved several times, however, the beta and delta forms exist as needle-like crystals and their morphology may allow relatively long needles to pass through the sieve. Although this is rare, it would inevitably alter the particle size range and consideration must be given when quantifying samples with needle-like morphologies [29].

From Table 2 it can be seen that the larger particle size range (125–500 μm) gave a R.S.D. that was almost ten-fold that obtained with the smaller range for the sample mixing category. Thus the results indicate that by reducing the particle size to below 125 μm FT-Raman spectra more representative of the overall sample mixture were obtained.

Previously it has been shown that changes in the intensity of the Raman signal occur as a consequence of alterations in particle size. Generally, an increase in the overall intensity of a Raman spectrum is observed as the particle size is decreased [29] presumably as particulate packing efficiency increases. Therefore, by controlling the particle size range of each sample to below 125 μm, any intensity change should have been relative and, therefore, should not have contributed to the errors associated with the method.

An earlier FT-Raman quantitative study completed by Taylor and Zografi found that inhomogeneities in mixing of the samples contributed the largest source of error [19]. In this study, however, the largest source of error appeared to involve the packing of the sample into the sample cup (6.6%). The estimation of this source of error involved repacking the same sample ten times and acquiring a single data set after each packing. This would have inevitably led to some of the sample being lost at each packing which may account for the greater degree of variation, although one would expect to lose the same proportion of beta and delta. A more likely explanation for the variation is that the needle-like crystals may be susceptible to preferred orientation effects. The morphology of the crystal would favour its' horizontal position in the sample cup with regard to the laser beam. This may allow greater detection of some vibrational modes resulting in an increase in the intensity of the corresponding peak. Further work regarding presentation of the sample to the laser beam would be required to establish whether preferred orientation effects have a significant effect on quantitative studies involving FT-Raman spectroscopy.

Analysis of the samples involved measurement of peak heights and it is appreciated that ideally, peak area measurements (which are invariant to peak distortion) should be employed. However, the FT-Raman spectra of beta and delta mannitol are very similar and since the majority of peaks were found to overlap only peak height measurement was permitted. The measurement of peak heights may contribute to the errors discussed in this paper. The overall error for the method was calculated by assaying one sample ten times and using Eq. (5) where R.S.D. is the relative standard deviation of the overall method.

The overall error from the FT-Raman quantitation of the mannitol polymorphs was calculated as 3.1% for 10 data sets obtained using a 50% (w/w) beta:delta composition sample.

4. Discussion

The comparison of actual and measured values of the beta concentration found in the samples is given in Table 1. The measured beta values of the triplicate correlation samples are represented by the mean value of three measurements whereas those of the validation samples are represented by the mean of three measurements from a single sample.

Data in Table 1 and Figs. 5 and 6 show that the actual and measured beta concentration values compare well, particularly beyond a lower limit of 2% beta mannitol. The sample mixtures were prepared gravimetrically by weighing appropriate amounts of beta and delta mannitol onto glass slides and transferring them to glass vials. This method of preparation may have introduced an error to the quantitation technique which, is possibly reflected in the discrepancies between some of the expected and measured beta values.

The S.D. (in parentheses) of the measured beta concentrations are relatively small, the largest deviation being around 4%. The consistency of peak height ratios gives rise to the relatively small S.D.s observed and is indicative of reasonable homogeneity of the samples. This is also reflected in the observation that sample homogeneity (mixing), whilst giving a significant error (3.1%) was less influential than the error from sample packing (6.6%).

In cases where homogeneity of the sample was problematic, previous methods have been developed to improve this. Bugay et al. used an acetone slurry technique to improve the homogeneity of the calibration and validation samples. A sample and acetone mixture was agitated before being filtered and dried in a vacuum desiccator. Subsequent scanning electron microscopic (SEM) photographs of the slurry mixture convincingly show that homogeneous mixing was achieved [13]. However, the possibility of phase transformation may limit the use of this acetone slurry technique with some polymorphic systems.

A rotating sample holder was developed by Langkilde et al. in their study involving the quantitation of a pharmaceutically active compound [30]. The main objective of this holder was to increase the volume of sample studied thus compensating for the possibility of sub-sampling. They estimated that when the rotating holder is used with an additional up-down motion, the volume of sample studied per spectrum collected is approximately 10 mm^3. Rotating the sample holder served to avoid undesirable sample degradation through laser heating and was also designed so that the sample was easily positioned thus reducing the possibility of phase transformations through pressing the sample into the holder. The introduction of such a rotating sample holder resulted in spectra more representative of the overall sample mixture however, a rotating holder was not used in this study and methods were employed to ensure representative measurements of the samples. These included the collection of triplicate measurements of each sample and controlling the particle size. In the latter case, the relatively small S.D. observed indicates that representative spectra of each sample were recorded.

It appeared that during the construction of the correlation curve (Fig. 4), only samples whose beta concentration was in the range 0.5–80% produced a linear relationship. The relationship of $(x_\beta - 1)(I_r - 0.0586)$ against $x_\beta(I_r^* 0.0472 - 1)$ is not linear for beta concentrations of 90% and greater. The reason for this, is at this time unclear and this observation may only hold for the data presented in this study or it may occur in every case where the molar fraction of a component is 0.9 and above. Using only samples containing $0.5 - 80\%$ of the beta component may not have significantly influenced the results since this study was concerned with quantifying low concentrations of beta which have been accommodated in the correlation plot.

The results observed in this assay complement suggestions made by previous workers that FT-Raman spectroscopy would be an effective technique in the quantitation of pharmaceutical compounds [19,30]. The choice of quantitative technique is no longer limited to that which demonstrates the greatest measurable differences between the polymorphs. As illustrated in this study, subtle differences like those exhibited by the FT-Raman spectra of the beta and delta mannitol can be used to successfully identify and quantitate polymorphic mixtures.

FT-Raman spectroscopy offers a distinct advantage over XRPD in that quantitation can be extended to both amorphous and crystalline materials. The broad halo XRPD pattern given by amorphous compounds can be difficult to analyse quantitatively although this appears to have been overcome in the recent study by Chen et al. where 0.37% amorphous lactose was detected using XRPD [31]. FT-Raman instruments with near-IR radiation operate at wavelengths of approximately 1 μm. Since glass is transparent in this region, samples can be directly measured in their original glass bottles or vials. This allows the preferential use of FT-Raman over IR spectroscopy in situations where the material is cytotoxic or hazardous because direct handling of the sample is avoided. Again this advantage extends to polymorphic compounds that are sensitive on exposure to moisture or atmospheric conditions [16]. Minimal sample preparation is probably the greatest advantage offered by FT-Raman spectroscopy when dealing with polymorphic compounds. Samples containing the neat drug can be analysed directly, no pressure or grinding is required to prepare a disk or otherwise.

Future quantitative studies involving FT-Raman spectroscopy should consider adopting a rotating sample holder and look to control particle size as this would improve results by minimising preferred orientation effects, avoiding sample degradation through laser heating and ensuring true representative measurements of the sample.

5. Conclusion

A quantitative method employing FT-Raman spectroscopy has been utilised to successfully analyse mixtures of varying proportions of the beta and delta mannitol polymorphs. The experimental data indicated that levels down to approximately 2% beta could be accurately quantified using this method.

A thorough investigation of the potential errors associated with the method was also completed. The results indicated that by controlling the particle size range to below 125 μm, spectra more representative of the overall sample mixture were obtained. Employing a rotating sample holder in addition to reducing and controlling the particle size range could further reduce potential errors associated with this method.

The rapid rate of data acquisition and non-destructive, non-intrusive nature of the method, coupled with a rotating sample holder and control of particle size of the sample make FT-Raman spectroscopy an attractive choice as an accurate quantitative technique.

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