Review Article

RAMAN SPECTROSCOPY: A VERSATILE TOOL IN PHARMACEUTICAL ANALYSIS

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ABSTRACT

Raman spectroscopy is a simplest form of spectroscopy where a photon of light interacts with a sample to produce scattered radiation of different wavelengths. Raman spectroscopy has become an important analytical and research tool. Raman spectroscopy provides extreme information useful for identification and characterization of molecular structures, effects of polymorphism and effects of electronic environments, effects of bonding and stress on a sample. The method of analysis is non-destructive. It has wide range of applications such as in pharmaceuticals, forensic science, polymers, thin films, papers, surface technology, semiconductors, analysis of fullerene structures and carbon nano-materials. This review gives coverage to principle of Raman spectroscopy, brief discussion of instrumentation and applications in Pharmaceutical field.

Keywords: Raman Spectroscopy, electronic environments, polymorphism, pharmaceuticals.

INTRODUCTION

Theory of Raman Spectroscopy

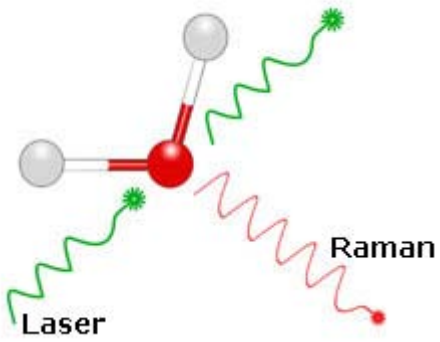

Figure 1: scattering of light from a molecule

When monochromatic radiation is incident upon a sample then this light will interact with the sample in some fashion. It may be reflected, absorbed or scattered in some manner. It is the scattering of the radiation which gives information about molecular structure of sample.

If the frequency or wavelength of the scattered radiation is analyzed, the incident radiation wavelength (Rayleigh scattering) along with a small amount of radiation that is scattered at some different wavelength (Stokes and Anti-Stokes Raman scattering) is seen. (Approx. only 1 x 10^{-7} of the scattered light is Raman). It is the change in wavelength of the scattered photon which provides the chemical and structural information. Light scattered from a molecule has several components - the Rayleigh scatter and the Stokes and Anti-Stokes Raman scatter.

In molecular systems, these frequencies are principally in the ranges associated with rotational, vibrational and electronic level transitions. The scattered radiation occurs in over all directions and may also have observable changes in its polarization along with its wavelength.

The scattering process without a change of frequency is called Rayleigh scattering.

A change in the frequency (or wavelength) of the light is called Raman scattering. Raman shifted photons of light can be either of higher or lower energy, depending upon the vibrational state of the molecule.

By far the stronger of the two processes is the Stokes scattering, whereby the photon is scattered at lower energy (shifted wavelength towards the red end of the spectrum). Since at room temperature the population state of a molecule is principally in its ground vibrational state this is the larger Raman scattering effect. (See the figure 1)

A small number of molecules will be in a higher vibrational level, and hence the scattered photon can actually be scattered at a higher energy, (a gain in energy and a shift to higher energy and a blue shifted wavelength). This is the much weaker Anti-Stokes Raman scattering.

The incident photons will thus interact with the present molecule, and the amount of energy change (either lost or gained) by a photon is characteristic of the nature of each bond (vibration) present. Not all vibrations will be observable with Raman spectroscopy (depending upon the symmetry of the molecule.) but sufficient information is usually present to enable a very precise characterization of the molecular structure.

Hence, the amount of energy shift for a C-H bond is different to that seen with a C-O bond, and different again to that seen with a Metal-O bond. By looking at all these various wavelengths of scattered light, one can

detect a range of wavelengths associated with the different bonds and vibrations.

Raman scattering is a relatively weak process. The number of photons Raman scattered is quite small. However, there are several processes which can be used to enhance the sensitivity of a Raman measurement.[1]

Techniques to enhance the sensitivity of a Raman measurement

Resonance Raman

It can be used when the laser wavelength utilized is close to the absorption wavelength of the molecule. By irradiating the sample with a wavelength close to this wavelength an order of magnitude greater detectivity may be achieved. Not all samples will show resonance enhancement with common Raman lasers, but generally species such as porphyrins and those with a heavy central atom can show such an enhancement.

SERS Raman (surface enhanced resonance Raman spectroscopy)

SERS is arguably a less well understood enhancement technique. It requires a further moiety to be present (e.g. A SERS prepared surface or colloid). The presence of such an agent can provide quite dramatic enhancements and has been used successfully in the study of biological samples such as DNA, peptides and proteins.

Active Substrates

Here a specialized coating can be used to enhance the sampling sensitivity of a liquid or solution sample. The sample does not 'wet out' the surface remaining in a concentrated micro-droplet. Enhancements for such applications as assay screening have been shown to be a likely candidate for such technology.

Raman Instrumentation

Best suited Laser wavelength - The correct selection of the laser wavelength can be an important consideration for Raman spectroscopy. With modern equipment, often several laser wavelengths may be employed so as to achieve the best detection of the Raman signal.

For instance, many samples, especially those of an 'organic' or 'biological' nature will be quite fluorescent species. Exciting these samples with a laser in the green (532 nm) may promote this fluorescence, and may swamp any underlying Raman spectrum to such an extent that it is no longer detectable.

In this instance, the use of a laser in the red (633 nm) or NIR (785 nm) may provide a solution. With the lower photon energy, a red or NIR laser may not promote the electronic transition (and hence the fluorescence) and so the Raman scatter may be far easier to detect. Conversely, as one increases the wavelength, from green to red to NIR, the scattering efficiency will decrease, so longer integration times or higher power lasers may be required. Thus, it is often most practical to have a number of laser wavelengths available to match the various sample properties one may encounter, be it resonance enhancements, penetration depth of fluorescence.[2]

High Performance Laser Sources

Critical for efficient Raman measurements, the use of high performance lasers sources is important within the Raman spectrometer.

Laser sources should follow strict criteria for:

- High beam quality
- High Stability
- Extended lifetime
- True Confocal microscope operation

Raman Microscope

The Raman microscope is by far one of the best instrumentation enhancements one can make. The new generation of Raman microscope can offer a powerful non-destructive and non-contact method of sample analysis.

The micro Raman system can open up a whole new dimension of spectroscopic analysis. They are now far easier to operate, and laser adjustment and alignment are virtually eradicated. It becomes a simple operation to use the micro Raman instrument with even computer control of laser switching and grating selection now possible. One of the greatest benefits is the use of a true CONFOCAL Raman microscope design. This enables a very small sample area or volume to be analyzed – down to the micron scale. Combine this micro Raman analysis with automated focusing, XYZ movement, and it becomes possible to produce 'chemical' images of a sample.

A Raman microscope allows non-destructive chemical micro-analysis, and automated high definition Raman mapped images can be obtained with ease. Transmission Raman analysis allows bulk analysis and screening of opaque materials including powders and tablets.

Analytical Raman

Analytical Raman microscopes and analyzers ideally suited for routine Raman analysis and screening. Ease of use is assured through intelligent automation, GO! ™ (Guided Operation) Wizards and auto calibration/auto validation protocols.

Examples of Raman microscopy systems:

XploRA™

LabRAM ARAMIS

LabRAM HR

AccuRA

Raman images

The Raman microscopes have true Confocal mapping performance. The Raman images produced are of the

highest definition and resolution. The sophisticated software and hardware enables fast and accurate Raman images to be obtained routinely. Followings are the examples of Raman imaging systems:

- Lines can Raman Imaging
- SWIFT™ Ultra fast Raman Imaging
- Duoscan™ Macro/Sub-Micron Imaging
- Nanoscale Raman Imaging

Raman imaging is a powerful technique for generating detailed chemical images based on a sample's Raman spectrum. A complete spectrum is acquired at each and every pixel of the image, and then interrogated to generate false colour images based on material composition and structure:

- Raman peak intensity yields images of material concentration and distribution
- Raman peak position yields images of molecular structure and phase, and material stress/strain
- Raman peak width yields images of crystallinity and phase

Thus with a single data set a wide variety of Raman images can be created which take the researcher well beyond what the eye can see.[1-3]

APPLICATIONS OF RAMAN SPECTROSCOPY

Raman spectroscopy is used in many varied fields – in fact, any application where non-destructive, microscopic, chemical analysis and imaging is required. Whether the goal is qualitative or quantitative data, Raman analysis can provide key information easily and quickly. It can be used to rapidly characterize the chemical composition and structure of a sample, whether solid, liquid, gas, gel, slurry or powder.

Applications of Raman other than pharmaceuticals:

- Art and archaeology : characterization of pigments, ceramics and gemstones
- Carbon materials : structure and purity of nanotubes, defect/disorder Characterization
- Chemistry : structure, purity, and reaction monitoring
- Geology : mineral identification and distribution, fluid inclusions and Phase transitions
- Life sciences : single cells and tissue, drug interactions, disease diagnosis
- Semiconductors : purity, alloy composition, intrinsic stress/strain

Pharmaceutical Applications

Raman spectroscopy fulfils a critical analytical role at many stages of the pharmaceutical product design and production process. Applications range from monitoring and controlling large scale manufacturing processes, to profiling the distribution of active pharmaceutical ingredients (API) and excipients at different stages in a formulation cycle.

- High throughput screening
- Compound distribution in tablets
- Blend uniformity
- API concentration
- Powder content and purity
- Raw material verification
- Polymorphic forms
- Crystallinity
- Contaminant identification
- Combinatorial chemistry
- *In vivo* analysis and skin depth profiling

High throughput screening (HTS)

Crystallization experiments are an integral part of high throughput screening (HTS) studies in the pharmaceutical industry. Raman spectroscopy is ideally suited for such studies, where polymorphic variants are created. This is due to a combination of the inherent vibrational information gained from polymorphic states and the recent advancement in instrument automation. Further aiding this application is the speed of analysis and the complementary information gained to supplement X-ray diffraction and thermal analysis screening techniques.[3]

Flufenamic acid polymorphs were analyzed using a series of crystallization reactions from solvents. Flufenamic acid is an anthranilic acid derivative with analgesic, anti-inflammatory and antipyretic properties.[4]

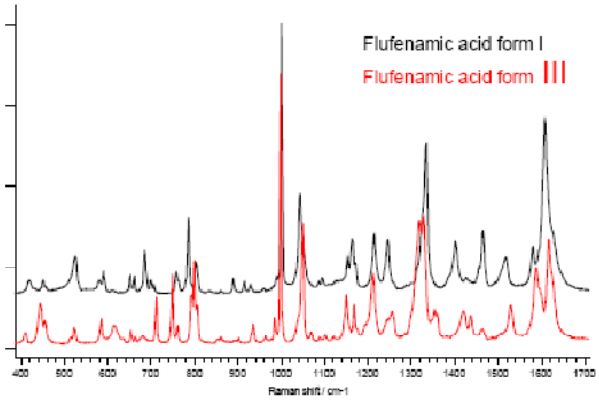

Figure 2: Well plate spectra of different flufenamic acid polymorphs

Carbamazepine polymorphs are used to demonstrate the power of an inVia Raman system equipped with NExT filter technology for direct analysis of polymorphic information and how this information can be used for differentiation purposes.[5]

Polymorph distribution analysis within formulations

The distribution of an API within a formulation is thought to affect the effectiveness of the drug, primarily, by modifying the release rate. The need to collect quality Raman information, representative of the whole tablet, is therefore particularly important for the pharmaceutical industry.[6]

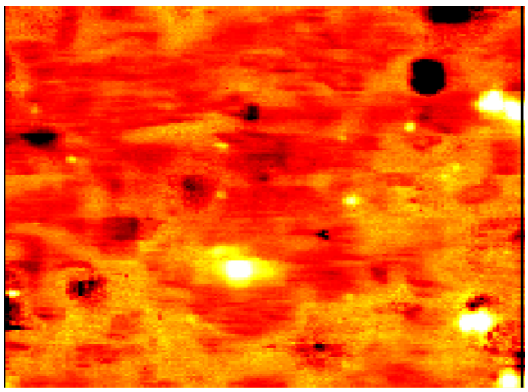

Figure 3: Distribution image of gatifloxacin sesquihydrate

Low concentration polymorph formulation example using multivariate analysis

Tablets are usually sectioned to gain representative component distribution information. Although, careful consideration is necessary regarding the sensitivity of the API to polymorphic change when presenting the sample in this way.

The distribution of a low concentration API within a tablet was investigated, the major component of which is a polymorphic form. The ability to resolve the mixed spectra of different polymorphs is now crucial for successful analysis. When analyzing low concentration components, where the initial experimental conditions are crucial, the following should be considered:

• Sufficient signal-to-noise ratio required to allow spectral information to be extracted

• The map size. Small maps will be less likely to contain an API component

• The spatial resolution required is highly dependent on the particle size and distribution (percentage API by volume)

The data was collected using Renishaw's rapid line focus imaging technique. A direct classical least squares (DCLS) method was used to identify and locate chemical components. Pre-loaded reference spectra were used to identify spectral proportions to discrete sample locations. Figure 3 shows the DCLS image created from the low concentration (mass fraction of 2%) gatifloxacin sesquihydrate component.

The light areas represent the sesquihydrate form (API), red represents anhydrous, and black the excipients. Spectra that are highly similar to the sesquihydrate (such as the anhydrous) are emphasized within the image by adjusting the contrast. Black regions within the image represent spectra of highly dissimilar materials (excipients)[4-6].

Polymorphy

Polymorphic form can influence solubility and efficacy of an active drug as well as provide patent protection. Yet it may be altered during processing. Dispersion of a drug through a tablet ensures correct dosage, yet aggregation can occur even though there were only subtle changes in raw material or processing conditions. Raman spectroscopy can give detailed information on these most important properties. Fructose and anhydrous dextrose share the same chemical formula, but are different chemical isomers. Anhydrous and hydrous dextrose differ in the water of hydration in the crystal of the hydrous form - in the pharmaceutical nomenclature, it is a pseudo polymorph. For clarity the spectra have been split into the fingerprint regions (100-1750cm^{-1}) and CH/OH regions (2500-3700cm^{-1}).

Inspection of the spectra shows clear differences between these species that would enable rapid identification of them. Such spectroscopic differences could easily be used to establish presence and indeed distribution of the different compounds.

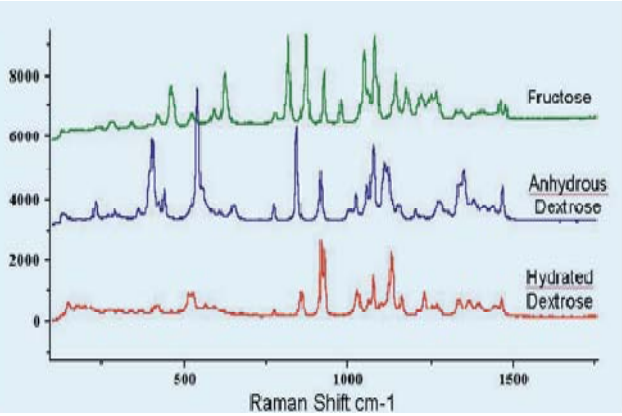

Figure 4: Raman spectra of Fructose, Anhydrous dextrose and hydrated dextrose

The spectra shown in the following figure 5 were generated from material that had been dissolved in hexane, and then precipitated during evaporation. Two phases were observed with optical microscopy - a grey, fairly planar material, and a white 'fluffier' material of very fine crystals.

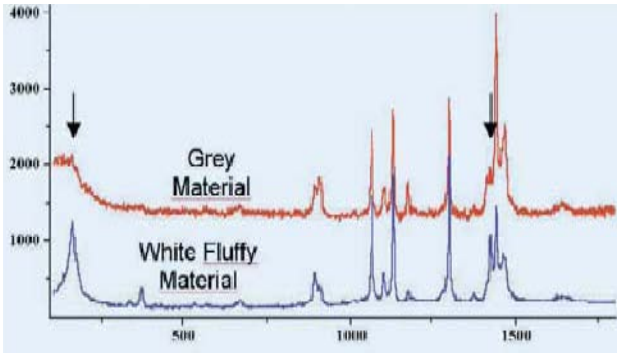

Figure 5: Raman spectra of grey and white material

Although the spectra in the accompanying figure look fairly similar there are some observable differences. The bottom spectrum, recorded from the white phase, shows a well-defined feature at about 165cm^{-1}, and differences in relative intensities in the 1400-1500cm^{-1} region when compared to the second form. An explanation of the origin of these differences is that the grey material represents a less crystalline and more amorphous material than the bottom spectrum of the white fine crystals. Evidence for this is shown by the presence of the well-defined band at about 165 cm^{-1} which is consistent with a lattice vibration (only present in a crystalline phase), and also in the behavior of the bands in the region of the CH_2 bending motion (1400-1500cm^{-1}). The spectral differences in this region have a strong precedent in the comparison with the behavior of polyethylene where, crystallinity also affects the spectral features in this region. Systematic studies of polyethylene, which can have different states of crystallinity, indicate that when crystalline there is a fairly strong band at about 1420 cm-1 which is absent in the amorphous phase[7,8].

Simple Raman measurements of even 'off-the shelf' organic materials illustrate that it is possible to differentiate between materials that differ by:

- Stereo isomer,
- Polymorphy,
- Pseudo Polymorphy (due to water of hydration)

The potential of analytical Raman instrumentation in qualifying pharmaceutical products in this area are only now beginning to be exploited.

Identification of the different ingredients

Several point measurements have been carried out in different areas of the sample and two typical spectra have been obtained. The main Raman bands have been pointed out and a spectral search using an electronic library enables to identify the components characterized by these two spectra (Figure 6) as being Lactose and Starch.

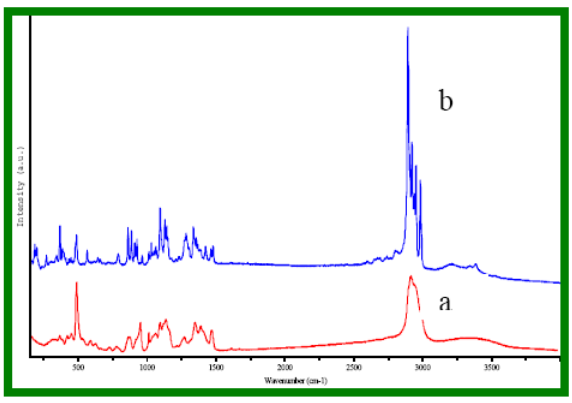

Figure 6: Spectrum recorded on tablet assigned to starch (a) and lactose (b)

The different components of this pharmaceutical sample can be characterized easily by Raman spectroscopy and the comparison with spectra from libraries allowed identifying some of the molecules. A good spectral discrimination could be obtained as the characteristic spectral fingerprints have exclusive features. The Raman imaging technique is a very powerful tool as shown here, as it also enables to characterize the spatial distribution of the components with a high spatial resolution.

The complementary results obtained by FTIR spectroscopy on the sample confirm the presence of lactose and allows the identification of the third component also observed by Raman spectroscopy. The combination of FTIR and Raman analysis on the same spectrometer represents a powerful analytical tool to readily record both IR and Raman vibrational information from a same sample position.

Raman Microscopy in Pharmaceutical Salt Analysis

Pharmaceutical and crystallographic samples typically require detailed characterization and analysis to optimize a samples stability, physical properties and indeed general efficacy where an active drug substance is involved.

Demand precise characterization of a solid salt form drug candidate and the possible influence of environmental conditions. The hydration and structure of such samples under varying conditions is important in this full characterization.

The highly specific spectral information provided by Raman spectroscopy can elucidate polymorphic form, crystallinity and hydration of a sample -giving a far better characterization and understanding of drug candidate's physical state. For this reason, the LabRAM series of instruments has become an important analytical tool for many pharmaceutical researchers and corporation.

Salt hydration activity

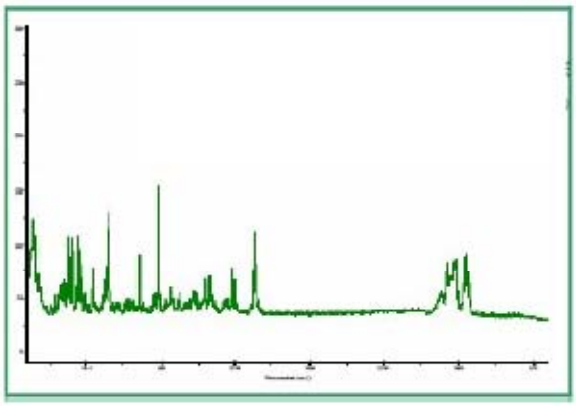

Figure 7: Raman spectrum of a Morphine Sulphate pentahydrate salt

Morphine $C_{17}H_{19}NO_3$, (the principle alkaloid of opium) and its various salts are a relatively well know group of analgesic drugs. They form different crystalline structures depending upon conditions and composition. In Figure 7

the spectrum is rich in spectroscopic information to study the effects of water vapor upon the states of a particular Sulphate salt, the VGI 2000 vapor pressure stage was attached to the LabRam Confocal Raman microscope system.

It is possible to see various spectroscopic differences in the sample when the relative humidity was changed from 0 to 50 % (RH) relative humidity; this corresponds to the known change in hydration of the sample which has been characterized in the past by XRay and thermo-gravimetric techniques.

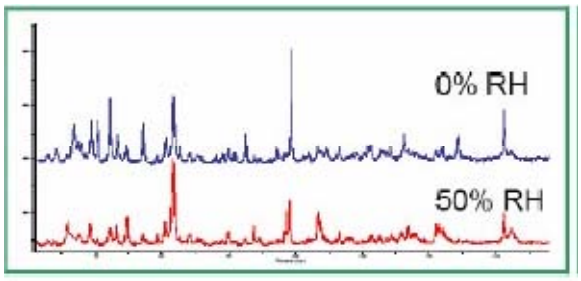

Figure 8: Raman spectrum of a Morphine Sulphate at different relative humidity

Temperature dependent Raman scattering study of l-ascorbic acid

Temperature dependent Raman study of l-ascorbic acid has been performed from 15 to 418 K. Changes in the wave number vs. temperature plots for some internal modes were interpreted as conformational molecular change and the discontinuity in the wave number vs. temperature plots along with the appearance of a new vibrational mode in the temperature range 200–270K suggests that l-ascorbic acid undergoes a structural phase transition. For temperatures higher than 300 K, no relevant modification was observed on the Raman spectra thus indicating a stable structure at high temperatures. Additionally, a correlation between OH stretching wave number and the behavior of hydrogen bond is also made.[9]

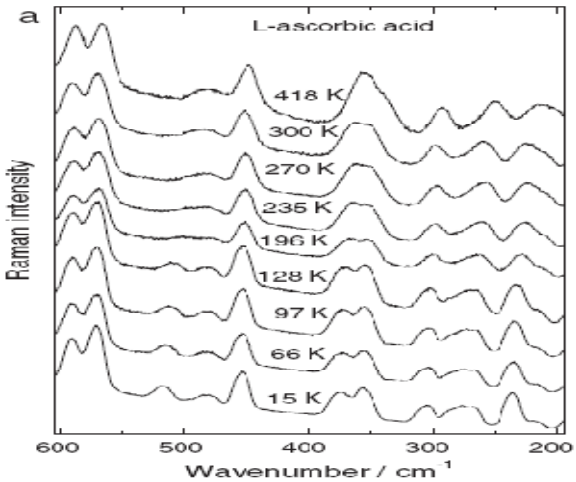

Figure 9: Raman spectra of ascorbic acid in the 50–200cm−1 spectral range recorded at several temperatures values.

Determination of Active constituent from different dosage form

Table 1: Determination of Active constituent from different dosage form

Active constituent	Formulation
Ambroxol[10,11]	Capsules
Budesonide[12]	Bulk
Clotrimazole, Ketoprofen[13]	Extruded formulations
Diclofenac[14]	Tablets
Hydrogen peroxide[15]	Solution
Mebendazole[16]	Tablets
Theophylline[17]	Bulk
Ticlopidine[18]	tablets

On-site inspection of excipients

One application in this industry is compelled by the need for on-site inspection of excipients e.g., diluents, binders, lubricants, disintegrants, colors, and sweeteners and active pharmaceutical ingredients. A more recent application is field testing for the active ingredient artesunate in anti-malaria tablets; 30 analysis through blister packs allows identification without compromising the packaging. The small size of commercially available Raman systems allows inspectors to easily transport the instruments long distances and conceal them for use in undercover operation.[19]

Process Optimization of a Complex Pharmaceutical Polymorphic System via In Situ Raman Spectroscopy

In situ Raman spectroscopy was used to determine the rate of polymorph turnover for MK-A, a multipolymorphic compound in development at Merck Research Laboratories. The known crystal forms of MK-A include four anhydrous polymorphs, two hydrates, and numerous solvates. The penultimate and pure steps of this process involve a coupling reaction to generate a mixture of crystal forms followed by turnover to the desired polymorph, form A. Experiments carried out to measure the kinetics of polymorph turnover from all relevant MK-A crystal forms to form A. Additionally, the turnover kinetics for polymorph reversion from form A to undesired forms were measured under simulated process upset conditions. The use of thermodynamic data to establish process boundaries and kinetic data to establish process time cycles resulted in the definition of a highly robust, cycle time efficient slurry turnover process to produce form A from any combination of other MK-A crystal forms.

Raman spectroscopy is a useful tool for in situ characterization of complex polymorphic slurry systems, particularly for the determination of turnover rates and elucidation of turnover pathways. As a tool for pharmaceutical process development, Raman kinetic studies were most useful in defining process time cycles and investigating process upsets but only after the

thermodynamic boundaries of the process were well-defined.

From these studies, 65 °C was chosen as the optimal temperature for slurry turnover of form C/hemihydrates to form A in the chosen solvent system (dry isopropyl acetate). Picking a temperature well above the enantiotropic crossover temperature of the two anhydrous forms capitalized not only on the increase in absolute solubility of both forms (thereby affording a greater percentage of the MK-A in solution) but also on the increase in relative solubility between desired and undesired forms (thereby increasing the driving force to crystallize form A). From a practical standpoint, these kinetic studies also indicated that while deleterious polymorph reversion from form A is slow below the enantiotropic crossover temperature, the risk of form C contamination in the final product is not worth the 1-2% increase in yield.[20]

Online Estimation and Monitoring of Diastereomeric Resolution Raman Spectroscopy

The estimation of fractional solid composition of two diastereomers is possible by incorporating Raman spectra, slurry density, and temperature into partial least squares (PLS) model.

Since slurry density measurement is not readily available, the online estimation is obtained through another PLS model that utilizes online Raman spectroscopy, focus beam reflectance measurement (FBRM), attenuated total reflection Fourier transform infrared (ATR-FTIR), and/or additional process information such as temperature and agitation rate to infer slurry density online. It is argued that the model, which infers slurry density from IR spectra, gives more accurate estimation of the fractional composition of two diastereomers.

Furthermore, it was successfully demonstrated through a third and a more general PLS model the ability of real time monitoring several crystallization process variables by the use of the aforementioned online measurements. Besides monitoring the changing diastereomeric composition and slurry density, one can also monitor the solute concentration of each diastereomer in a complex crystallization system.[21]

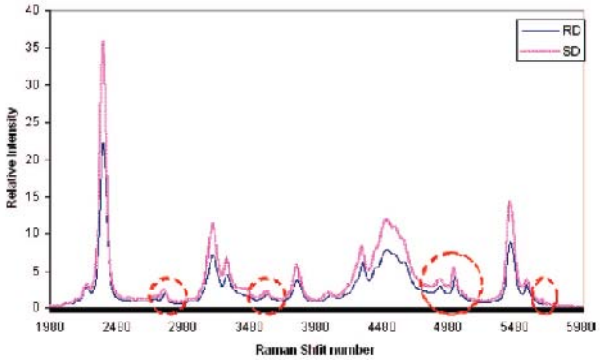

Figure 10: Raman spectra of pure R-D and S-D at the same temperature and slurry density to illustrate the baseline offset. The circles indicate different regions of slight peak shifts between the diastereomers.

Use of Raman Spectroscopy to Characterize Hydrogenation Reactions

Raman spectroscopy was used to characterize hydrogenation reactions involving single-step and two-step processes. The Raman technique was shown to be well-suited for endpoint determination as well as process optimization. In this investigation, hydrogenation of cyclohexene to produce cyclohexane was used as a model system. Conditions were varied to determine the effect of catalyst loading, solvent ratios, and reactant concentrations. Four catalysts were evaluated. The kinetic profiles of each reaction process were determined for each of the catalysts. In one case, a side reaction leading to an intermediate was observed for the hydrogenation reaction when run under hydrogen-starved conditions. After these cyclohexene hydrogenations were characterized, Raman spectroscopy was applied to the conversion of carvone to tetrahydrocarvone and the hydrogenation of 2-(4-hydroxyphenyl) propionate. Raman was used to characterize the kinetics of these reactions and was also used to prove that two-step hydrogenation mechanisms occurred in each. Raman was shown to be useful for process understanding, process optimization, process monitoring, and endpoint determination. Accomplishment of these goals leads to better process controls upon transfer of the procedure to a process environment. This ultimately leads, in turn, to the mitigation of risk of making out-of-specification product in manufacturing.[22]

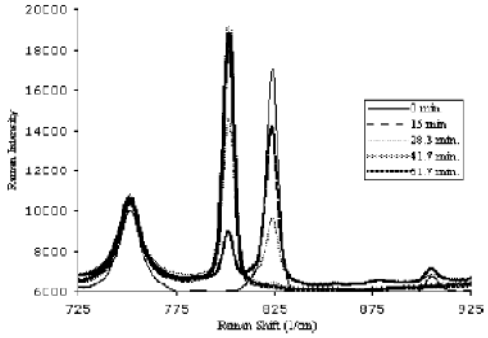

Figure 11: Raman spectra extracted from the process of cyclohexene reduction to cyclohexane

The resolved band for cyclohexane at 802 cm^{-1} clearly increases with time, while the resolved band for cyclohexene at 823 cm^{-1} clearly decreases with time. The band at 802 cm^{-1} corresponds to the CH_2 deformation plus ring breathing vibration of cyclohexane, and the one at 823 cm^{-1} corresponds to a similar vibrational mode of cyclohexene.

The trend of cyclohexene disappearance and cyclohexane formation can easily be followed qualitatively using the areas under the curves of the unique peaks previously noted (Figure 11). The advantage of using a band area ratio method for two unique peaks is that any baseline

fluctuation will be effectively accounted for in the analysis. In the example shown, the reaction is completed between 1.5 and 2 h. It is noteworthy that if endpoint determination is all that is desired, this simple analysis is sufficient. Based on the initial concentration of cyclohexene, the intensity of the 802 cm^{-1} band was used to construct a quantitative model for the kinetic calculations.

CONCLUSION

Raman spectroscopy continues to grow in popularity both among analytical chemists and in Pharmaceutical fields. It has become a standard method for materials characterization and is finding greater acceptance in industrial process monitoring. In addition, applications to determine content in various dosage forms look promising.

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