SESSION 2

Analytical Chemistry

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The ICH guidance in practice: stress degradation studies on Satranidazole and development of a validated stability-indicating HPTLC assay method

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Satranidazole, 3-(1-Methyl-5-nitroimidazol-2-yl)-1-(methylsulfonyl) imidazolidin-2-one is used as Intestinal and hepatic amoebiasis, giardiasis, trichomoniasis, anaerobic infections (Pargal 1993). Satranidazole is also available in combination with ofloxacin. Satranidazole is not official in any pharmacopoeia. Few reports for analysis of Satranidazole are available in literature, Various spectrophotometric methods have been reported for Satranidazole in bulk drug and pharmaceutical formulation (Mruthyunjayaswamy 2001). To our knowledge, no article related to the stability indicating HPTLC determination of satranidazole has ever been mentioned in literature. All 5 –nitroinidazoles are sensitive to alkali hydrolysis and photo degradation (Pfoertner 1987). The International Conference on Harmonization (ICH) guideline entitled Stability Testing of New Drug Substances and Products requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance. Susceptibility to hydrolysis is one of the required tests. Also the oxidation and the photolytic stability are required. The aim of the present work is to develop an accurate, specific, repeatable and stability-indicating HPTLC method for the determination of satranidazole in the presence of its degradation products and related impurities for assessment of purity of bulk drug and stability of its bulk dosage forms. A sensitive, selective, precise and stability-indicating high-performance thin layer chromatography (HPTLC) method for analysis of satranidazole both as a bulk drug and in formulations was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene/acetonitrile (60:40, v/v). Densitometric analysis of satranidazole was carried out in the absorbance mode at 314 nm. This system was found to give compact spots for satranidazole (Rf value of 0.53 ± 0.02, for six replicates). Satranidazole was subjected to acid and alkali hydrolysis, oxidation, and photo degradation. The drug undergoes degradation under acidic and basic conditions, oxidation, and photo degradation. Also the degraded products were well resolved from the pure drug with significantly different Rf values. The method was validated for linearity, precision, limit of detection (LOD), limit of quantitation (LOQ), and accuracy. Linearity was found to be in the range of 100–500 ng/spot with significantly high value of correlation coefficient $r2 = 0.9979 \pm 0.66$. The LOD and LOQ were 50 and 85 ng/spot, respectively. Statistical analysis proves that the method is repeatable and specific for the estimation of the said drug. As the method could effectively separate the drug from its degradation products, it can be employed as a stability-indicating one. Moreover, the proposed HPTLC method was utilized to investigate the kinetics of alkali degradation process.

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Identification and quantitation of intact β-endorphin by LC-MS-MS

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Several LC-MS-MS methods have been developed for the quantitation of β-endorphin (BE) although the majority of these were not true hyphenated LC-MS, but relied on the collection of fractions by HPLC and their subsequent introduction into MS as a stand-alone spectrometer (Dass et al 1991). A report by Desiderio (1999) has been the only analytical method where on-line LC-MS-MS has been used for quantitation of BE. However, this method was lengthy since the peptide was digested with trypsin to obtain

Table 1 Fragmentation of the BE molecular ion (694.4)

m/z	Fragment	N-terminus	C-terminus
136	Y(1–1)	0	a1
193	YG(1–2)	0	a2
221	YG(1–2)	0	b2
278	YGG(1–3)	0	b3
397	YGGF(1–4)	0	a4
425	YGGF(1–4)	0	b4
556	YGGFM(1–5)	0	b5
691	MTSEKS(5–10)	a4	b10

'0' at an N-terminus denotes no apparent cut to this end; fragment includes all atoms from the N-terminus to the nearest cut. Fragment represented by the letter 'a' indicates fragmentation on the N-terminus side of the peptide carbonyl. Fragment represented by 'b' indicates fragmentation on the C-terminus side of the peptide carbonyl.

smaller fragments before LC-MS-MS analysis. In the work reported herein we investigated the possibility of quantifying intact BE by LC-MS-MS. The separation of rat and human BE was carried out using an Agilent binary HPLC system and a Zorbax 300 SB-C18 (2.1×50 mm, $3.5\,\mu$m) HPLC column (Agilent Technologies, Palo Alto, CA). An API 3000 tandem mass spectrometer equipped with a turbo ion spray interface (Applied Biosystems, Foster City, CA) was used to detect the separated compounds. Isocratic elution using 23% v/v acetonitrile in water containing 0.1% v/v formic acid separated both human and rat BE within 10 min. It is therefore possible for human BE to act as the internal standard for the determination of rat BE and vice versa. The use of a large pore size (300Å) stationary phase was critical in obtaining narrow peaks and baseline resolution. The direct infusion of a 10 μM BE standard in about 20% acetonitrile in water containing 0.1% formic acid showed four protonated molecular ions of different charges: 1734.4 (2^+), 1156.7 (3^+), 867.9 (4^+), 694.4 (5^+). The abundance increased with the charge of the ion. The same ions and similar abundances were observed for both human and rat BE. The singly charged protonated molecular ion (3470) was not observed as it was beyond the mass/charge range of the spectrometer (3000 m/z). The 694.4 ion was chosen for further fragmentation as it was the most abundant. Collision induced dissociation (CID) using the MS-MS option of the instrument produced several fragments (Table 1). The fragmentation pattern was also identical with human and rat BE. The multiple reaction monitoring (MRM) mode of MS was used for quantitation as it provides the highest specificity and sensitivity to the analyte of interest. The most abundant parent/fragment pair of the protonated molecule, 694.4/136, was used as the MRM pair for both endorphins. The MS parameters were optimized for this pair so that BE was detected at the highest possible sensitivity. The on-column limit of detection for BE was estimated at 3 × baseline noise to be 50 fmol. The method is simple, sensitive and can easily be used for accurate quantitation of either rat or human BE.

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Profiling acetaminophen metabolites in mouse urine by LC-MS-MS

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The metabolism of acetaminophen (A) produces two major metabolites that are detected in urine; acetaminophen glucuronide (AG) and acetaminophen sulfate (AS). Many HPLC methods exist for the determination of acetaminophen and its metabolites in biological fluids. The detection method used in all of these studies was UV spectrophotometry which is non-selective towards analytes in the mixture. Vertzoni et al (2003) used 242 nm as an optimum wavelength of detection for A, AG and AS. With non-selective detection there exists the danger of over-estimation of the analyte as many other substances in the biological fluid which may co-elute with the analyte will also absorb at that wavelength. Mass spectrometric (MS) detection overcomes this problem as it specifically detects the assigned mass of the analyte. Furthermore, when tandem mass spectrometry, MS-MS (multiple reaction monitoring, MRM) is used as the detection methodology it yields enhanced selectivity as it will only provide a signal when both the

Table 1 Urinary concentrations (mM) of A, AS and AG for 4 mice, 2 hours after i.p. injection of 250 mg /kg acetaminophen

Mouse #	A	AS	AG
1	2.02	0.09	2.19
2	2.68	0.13	2.48
3	1.05	0.09	1.75
4	1.49	0.05	1.74

mass of the analyte and its specific fragment are detected. Co-elution will not affect the accuracy of the quantitation. Although there are some recently published methods available for the determination of acetaminophen by LC-MS-MS (Yin et al 2005), there are no LC-MS-MS methods available for the determination of its metabolites. In this work LC-MS-MS has been used, for the first time, for the quantitative analysis of acetaminophen (paracetamol) and its metabolites in mouse urine. The separation of compounds was carried out using an Agilent binary HPLC system (Agilent Technologies, Wilmington, DE) and a Cogent phenyl 100Å (2.1×50 mm, 5 μm particle size) HPLC column (Microsolv Technology Corporation, Long Branch, NJ). An API 3000 tandem mass spectrometer equipped with a turbo ion spray interface (Applied Biosystems, Foster City, CA) was used to detect the separated analytes. Isocratic elution using 3% v/v acetonitrile in water containing 0.1% v/v formic acid separated A, AG, AS and the internal standard (3-acetamidophenol, IS) in less than 10 min. The analytes were detected using electrospray mass spectrometry in multiple reaction monitoring (MRM) mode using the following parent/fragment pairs of the protonated molecules, 152/110 (A), 328/152 (AG), 232/152 (AS) and 152/110 (IS). Optimized MS parameters were used for each compound, whereby all analytes were detected at the highest possible sensitivity. Before LC-MS-MS analysis the internal standard (to a final concentration of 0.1 mM) was added to urine samples, which were diluted ten-fold with the aqueous mobile phase and centrifuged to remove particulate matter. The standard solutions were prepared by spiking the urine of the untreated animals with known amounts of standard A, AG, AS and IS. Data obtained for 4 animals (mean values of duplicate analysis) are shown in Table 1. The on-column limits of detection for A, AG and AS were 2, 1 and 1 pmol, respectively. In summary, this method was used to determine the concentration of acetaminophen and its metabolites in mouse urine: the method may be easily adapted to biological fluids of other animals.

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Setting meaningful dissolution specifications for MR Products

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The aim of establishing an IVIVC is to use the in vitro dissolution method as a surrogate for bioavailability. However, this is seldom a straightforward exercise. The objective of this work was to develop robust and meaningful dissolution specifications for an MR product. Individual dissolution profiles of the 4 different tablet strengths (1–4) drawn from batches produced at 2 different manufacturing sites (A and B), were compared using the graphical techniques of Sathe et al (1996). Dissolution profiles of 12 individual tablets (USP stage II) were generated using 37 batches (including 10 clinical or biobatches) of the MR product. Data were generated every 30 min for a 7-h period. Individual dissolution profiles showed excellent correlation demonstrating a very robust manufacturing process. For each batch, of each tablet strength, from each manufacturing site, a plot of slope versus intercept was derived, together with an associated 90% confidence ellipse. Graphical comparison of these ellipses was undertaken across different manufacturing sites for a given tablet strength and for a given tablet strength and across tablet strengths for any site. A Box and Whisker statistical evaluation was used to set the target specification at each time point based on the average of the individual means from the 8 site and tablet strength combinations, for both biobatches and all batches. Tablet and batch effects were assumed to be random, whereas site and tablet strength were considered fixed effects. For each site and tablet strength combination, a 95% confidence interval for a single tablet was constructed around the mean value, based on variance comprised of tablet-to-tablet and a batch-to-batch variation. This graphical and numerical comparison of data sets (Tables 1 and 2) gave good assurance that the specification limits derived from the restricted dataset (clinical and biobatches only) are equally applicable to the full dataset of this MR product. The GSK proposed specification and the FDA agreed specification (Table 3) were in good accord demonstrating the effectiveness of this approach.

Table 1 Specification limits for MR Product (based on clinical/bio batches only)

Time point (h)	Mean target release (% label)	Low specification limit (% label)	High specification limit (% label)
1	10	0	21
2	33	18	46
3	55	39	68
4	72	54	91
5	86	66	100
6	98	88	100

Table 2 Specification limits for MR product (based on all batches)

Time point (h)	Mean target release (% label)	Low specification limit (% label)	High specification limit (% label)
1	9	0	20
2	32	19	45
3	54	41	68
4	71	56	89
5	88	69	100
6	98	89	100

Table 3 Specification limits for MR product (GSK vs FDA)

Time point (h)	GSK low specific^n limit (% label)	GSK high specific^n limit (% label)	FDA low specific^n limit (% label)	FDA high specific^n limit (% label)
1	0	20	0	20
2	20	45	20	45
4	55	90	60	90
6		Q = 85		Q = 85

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Genotoxic impurities: strategies for balanced approach

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Genotoxic impurities are currently receiving huge world-wide regulatory focus. ICH Q3A and ICH Q3 B limits do not apply when the impurity presents a probable potent risk, either based on knowledge of toxic or genotoxic risk, or based on class association, or known reactive potential. The objective of this work is to provide an understanding of the nature and impact of genotoxic (DNA reactive mutagens) impurities. In particular, the structural motifs of concern, the current regulatory environments, the analytical impacts and the risk/benefits of controlling genotoxic impurities to the ppm level. A genotoxic agent is any agent that causes a heritable genetic change. Genotoxins may be categorised as threshold or non-threshold mediated. The former do not directly interfere with DNA, but induce DNA damage via interference with a cellular activity (e.g. topoisomerase inhibitors, spindle poisons), whereas the latter directly interfere with DNA (e.g. alkylating agents). The structural motifs that give greatest concern are: hydroperoxides; epoxides (and precursors); aziridines (and precursors); hydrazines; aromatic nitro; N-nitroso/nitrosamines; aryl azo; alkylating agents (aryl and alkyl halides); and alkylating agents (alkyl sulphonate, sulphinate and sulphate esters). The CHMP draft guidance on Genotoxic Impurities (July 2004) divided genotoxins into threshold and non-threshold classes. The former would be addressed using a PDE approach (ICH Q3C), whereas the latter would be addressed using the Toxicological Threshold of Concern (TTC). The risk is based on exposure, dose and probability. CHMP proposed that a TTC of 1.5 micrograms/day for a particular genotoxin represented a 1×10^{-5} increased risk of cancer following lifetime exposure (70 years). FDA is advocating a similar 'risk based' approach, with similar limits for life time exposure, but slightly elevated levels for shorter duration of exposure (that will provide meaningful cover for single and repeat dose early human clinical studies). The analytical implications of these ppm impurities are profound. There has been a renaissance of GC methodologies for volatile alky and aryl halides. As well as the more conventional approaches offered by LC-MS and

LC-MS-MS. Sensitivity, as well as selectivity, becomes hugely important and the increased sensitivity equates to decreased robustness and has implications on method development, validation and transfer. The regulations are evolving, but there is often inadequate guidance on ppm versus total exposure (amount of exposure), absolute versus relative risk (duration of exposure) and potential versus known genotoxic impurities. The typical western lifestyle results in the daily uptake of 1.5 g of genotoxic materials per day. Most of these agents deemed to be genotoxic in vitro either do not have adverse effects in vivo or are present in too small a quantity to have an in vivo effect. The formation of genotoxins (direct or indirectly acting electrophiles) is commonplace in nature. The impact on human health is mitigated by antimutagens in food and the Electrophile Counterattack Response (induction of metabolic responses; e.g. epoxide hydrolase, glutathione conjugation). In conclusion, does the current focus on ppm levels of genotoxic impurities materially improve patient safety?

Table 1 Summary of validation parameters

Parameters	Data
Linearity range	10–60 $\mu g\ ml^{-1}$
Correlation coefficient	0.997
Limit of detection	0.10 $\mu g\ ml^{-1}$
Limit of quantification	0.40 $\mu g\ ml^{-1}$
Recovery (n = 6)	99.88% ± 0.31%
Precision (% RSD)	
Inter-day (n = 6)	0.13
Intra-day (n = 6)	0.94

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An initial assessment of the use of process conductivity profiles in pharmaceutical manufacturing

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The manufacture of pharmaceutical products is classically achieved using batch processing, followed by off-line quality testing performed on individual and/or bulk samples. For aqueous nasal spray suspensions (ANS), analytical testing is performed on samples taken from manufactured product. A process analytical technologies (PAT) approach encourages the use of continuous real time quality assurance, by constant monitoring of the process rather than by end product testing. This approach has the advantage that analyses are often performed on-line (or in-, and/or at-line), in real time (or near real time) and are completed within a similar timeframe to the manufacturing process, thereby improving process cycle times. We report on the investigation of point conductivity measurements, taken during manufacture of the small scale (2 kg) manufacture of an aqueous nasal spray suspension. These conductance measurements using Knick Portamess 910 conductivity meter (Knick GmbH, Berlin, Germany) reflect the underlying conditions prevailing within the manufacturing process allowing a continuous process signature to be derived. Experiments were designed to evaluate the use of this type of process signature in providing a real time measurement of the quality of the manufacture and ultimately the quality of the product. To achieve this objective, a standard manufacturing process was defined, and then the effects of variation of quality critical parameters, on the process conductivity profile were investigated. Process conductivity measurements gave reproducible profiles for the standard manufacturing process for aqueous nasal spray suspensions. Good reproducibility (RSD < 1% for EDTA, Polysorbate and Avicel) and a linear response ($R^2 = 0.9987$) were shown across a broad dynamic range (4-fold increase in concentration) indicating that process conductivity profiles would be a valuable addition to the suite of techniques used in this type of PAT approach. Moreover it has the potential to be used as a profile signature for this type of manufacturing process. In support of this contention, deviations from the standard process could be detected using the conductivity profiles. The experiments that evaluated order of addition showed that unique profiles for each sub-process were obtained. Investigation of the temperature of components utilised showed minor, but detectable changes in the process conductivity profiles. This enhanced process understanding is in line with FDA's 'Risk-based Approach'; where there is an expectation that enhanced process understanding should reduce the risk of manufacturing an inferior quality product. These investigations show good progress towards this ultimate aim. However, it should be noted that the variation in key quality critical parameters used, were substantial and in practise we may need to be able to measure more subtle variations, and further work would be required to achieve this goal. This additional work could also investigate the correlation of product performance with the process conductivity profiles.

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Stability indicating HPLC method for the determination of levocetirizine dihydrochloride as bulk and in pharmaceutical dosage forms

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Levocetirizine dihydrochloride (2-(4-[(R)-*p*-chloro-α-phenylbenzyl]-1-piperazinyl)ethoxy)acetic acid is a second-generation H$_1$-receptor antagonist which is the active (R) enantiomer of cetirizine (Day et al 2004). Levocetirizine acts by competitive antagonism of H$_1$ receptors. Levocetirizine is a new single isomer antihistamine with a

proven efficacy on chronic urticaria as documented in two recent clinical studies, which have included effectiveness and quality of life assessments (Kapp & Wedi 2004). To our knowledge, no article related to the stability indicating chromatographic determination of levocetirizine dihydrochloride in pharmaceutical dosage forms has been reported in the literature. The International Conference on Harmonization (ICH) guideline entitled "Stability testing of new drug substances and products" requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance (Q1A ICH guidelines October, 1993). The aim of this work was to develop a stability indicating HPLC method for the determination of levocetirizine dihydrochloride in the presence of its degradation products and related impurities for assessment of purity of the bulk drug and stability of its dosage forms. A sensitive and reproducible method is described for the quantitative determination of levocetirizine dihydrochloride in the presence of its degradation products. The method was based on high performance liquid chromatographic separation of the drug from its degradation products on a Hypersil column (C$_{18}$ (5-micron, 25 cm × 4.6mm, i.d.)) at ambient temperature using a mobile phase consisting of methanol and water (85:15, v/v). Flow rate was 1.0 ml min^{-1} with an average operating pressure of 180 kg cm^{-2} and t$_r$ was found to be 3.80 ± 0.05 min. Quantification was achieved with UV detection at 232 nm based on peak area with linear calibration curves in the concentration range 10–60 $\mu g\ ml^{-1}$. This method has been successively applied to pharmaceutical dosage formulations. The method was validated in terms of precision, robustness, recovery and limits of detection and quantification (Table 1). The drug was subjected to acid, alkali and neutral hydrolysis, oxidation, dry heat, wet heat treatment and photo and UV degradation. Degradation studies concluded that the levocetirizine gets rapidly degraded under alkaline condition and by oxidation, while it is more stable in an acidic medium. As the proposed method could effectively separate the drug from its degradation products at different temperatures, their respective apparent pseudo-first order rate constant, half-life and activation energy were calculated with the help of an Arrhenius plot. In addition, the pH rate profile of degradation of levocetirizine dihydrochloride in constant ionic strength buffer solutions with in the pH range 2–10 was also studied. The pH rate profile study shows that levocetirizine dihydrochloride was found to be most stable at pH 5. The study is an example of the development of a stability-indicating assay, and hence can be suitable for routine analysis in quality control laboratories.

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Bioequivalence evaluation of cilostazol 50-mg tablets (Pletoz® and Steloz®) in healthy human volunteers

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Cilostazol, 6[4–9,1-cyclohexyl-1H-tetrazole-5-yl]-butoxyl]-3,4-dihydro-2-(1H-quinolinone), is a cAMP phosphodiesterase III inhibitor that suppresses phosphodiesterase activity and cAMP degradation with resultant increase in cAMP in platelets and blood vessels, resulting in inhibition of platelet aggregation and vasodilation. To monitor intra- and inter-individual variability for the drug it is desirable to determine plasma concentrations of the drug in clinical practice (Shargel & Andrew 1992). Mean peak plasma concentration (C$_{max}$) of 1500 ng ml^{-1} was reported after single oral dose of 50 mg of cilostazol (Bramer et al 2001). This communication describes a simple, sensitive and highly selective, reversed phase, isocratic high-pressure liquid chromatographic method, for the estimation of cilostazol in human plasma and its application to bioequivalence studies in healthy human volunteers. Bioequivalence of two cilostazol 50 mg tablets was determined in healthy human volunteers after a single dose in

a randomized cross-over study. Formulations were administered to volunteers after overnight fasting. 24 volunteers were given a single dose of either formulation of cilostazol 50 mg with 240 ml of water in morning; no food was allowed until 4 h after dose administration. Water intake was allowed after 2 h of the dose; water, lunch and dinner were given to all volunteers according to the time schedule. The volunteers were continuously monitored by qualified physicians. Approximately 4 ml of blood sample was drawn in heparinised tubes after definite time intervals. The blood samples were centrifuged at 2400 rpm for 15 min. at 4°C and plasma was separated and analyzed for drug using an in-house developed and validated high performance liquid chromatographic method. Sample preparation for the developed method employed liquid-liquid extraction with ethyl acetate. The technique employed a C_{18} column (250 × 4.6 mm i.d; 5 μ particle size) for separation and acetonitrile:water:acetate buffer (50 mM) of pH 5.0 adjusted with glacial acetic acid as mobile phase at a flow rate of 1.5 ml min^{-1}. Detection was accomplished at 257 nm using diode array detection. The chromatograms showed good resolution and sensitivity with no interference from plasma. The direct method was found to be linear in the range of 50–2000 ng ml^{-1} (r^2 = 0.998). Results of the sample analysis showed a relative standard deviation of ± 2.87%; the limit of detection was found to be 20 ng ml^{-1}. The pharmacokinetic parameters $AUC_{0-\infty}$, C_{max}, T_{max}, $t_{1/2}$, and elimination rate constant were determined from plasma concentration-time profiles of both formulations. $AUC_{0-\infty}$ was found to be 2225 ng ml^{-1} h^{-1} and 2672.5 ng ml^{-1} h^{-1}, respectively, for Pletoz and Steloz, C_{max} was found to be 800 ng for both formulation 1 and 2, T_{max} was found to be 30 min for Pletoz and 40 min for Steloz while $t_{1/2}$ and elimination rate constant values were found to be 50 min and 0.831 ng ml^{-1}, respectively, for both formulations. All values were found to be in good agreement with previously reported values. The calculated pharmacokinetic parameters were compared statistically to evaluate bioequivalence between two brands, using the statistical modules recommended by the FDA. The analysis of variance (ANOVA) did not show any significant difference between the two formulations and 90% confidence interval fell within the acceptable range (80–120%) for bioequivalence. Based on these statistical inferences it was concluded that the two formulations exhibited comparable pharmacokinetic profiles and the formulations are bioequivalent.

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RP-HPLC based determination for alkaline hydrolysis of pioglitazone hydrochloride

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The objective of this work was to develop a selective, accurate, precise and stability indicatory RP-HPLC method for determination of pioglitazone HCl in the presence of its degradation product with respect to alkaline hydrolysis for assessment of purity of the bulk drug and stability of dosage form according to the ICH guidelines (Singh & Bakshi 2000). Oral anti hyperglycemic agent pioglitazone HCl is less stable in alkaline medium (ICH guidelines 2002). HPLC analytical measurement and separation were performed using Shimadzu liquid chromatograph LC-10ATVp instrument with SPD- M10 AVP Shimadzu UV/Vis Diode Array Detector (λ = 226 nm) and Phenomenex, (250 × 4.60 mm) Luna 5μ C_{18} Column. Mobile phase consisted of acetonitrile:10 mM potassium dihydrogen phosphate buffer (pH 6) (50:50v/v), flow rate was maintained at 1.5 ml/min and column temperature at 25–30°C. The samples were diluted with water:acetonitrile (50:50, v/v). The hydrolysis of pioglitazone HCl was studied at three variable conditions, different concentration of sodium hydroxide, temperature and time. It was found that degradation rate vary with the temperature and strength of alkali. After several trials it was observed that pioglitazone HCl gets extensively degraded in 1 N NaOH at the temperature of 80°C, and shows complete degradation after 30 min but in 0.1 N NaOH, it degrades gradually therefore degradation was observed at 0.1 N NaOH. The accurately weighed pioglitazone HCl was dissolved in 0.1 NaOH and volume was made up to get solution of 1000 μg/ml with 0.1 NaOH. The above solution mixture was kept at 80°C for 210 min. The aliquote were withdrawn at different time interval, allowed to cool, neutralized with 0.1 N HCl and diluted with water:acetonitrile (50:50, v/v) to get a final concentration of 50 μg/ml. Before injection, the solution was filtered through 0.45μ syringe filter, and were analysed for degradation pattern in aforementioned mobile phase. Chromatogram of degraded sample shows well-resolved peak of pioglitazone HCl and its various degradation products at different time intervals. The analysis of reaction mixture showed that the degradation starts immediately, the primary degradation product eluated at

2.4 ± 0.1 min having a resolution of 4.6 ± 0.1 with pioglitazone HCl. As time increases successively, area under the curve for primary degradant increases and for pioglitazone HCl decreases. After 120 min a secondary degradation product was observed having retention time 8.2 ± 0.1 min. It gets practically completely degraded in 0.1 N NaOH at 80°C after 4 h. At 1.4 ± 0.1 min, a peak corresponding to diluent was observed in all analysis. The HPLC method was found to be selective, accurate, precise and stability indicatory for pioglitazone HCl and capable of resolving the degradation product of pioglitazone HCl in bulk drug as it resolve in its alkaline hydrolysis of piolitazone HCl. The method can be used for the routine industrial analysis of pioglitazone HCl in bulk as well as tablet dosage form.

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Generic, multi-component analysis of aqueous nasal spray formulations

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Aqueous Nasal Spray formulations for current development compounds within GlaxoSmithKline use a preservative system of Ethylenediaminetetraacetic acid (EDTA) and Benzalkonium Chloride (BKC), each that are currently monitored by single component methodologies. However, with the current speculation around the long term safety effects of BKC (alleged problems with inhibition of mucocilliary clearance), new preservative systems are being developed. The objective of this work was to develop and validate a generic, multi-component High Performance Liquid Chromatography (HPLC) method that would be able to separate and quantify three preservatives in new formulations. These preservatives are EDTA, Potassium Sorbate and the anti-oxidant Butylated Hydroxyanisole (BHA). To separate and quantify three preservatives in an acceptable time in one method with one sample preparation would provide incredible time-saving and cost benefits for the analysis of aqueous nasal sprays when transferred to a manufacturing environment. Separation in an acceptable time proved challenging due to the nature of the three components for analysis. EDTA is UV transparent without the use of metal complexation, and is very polar resulting in little or no retention on generic reverse phase C18 HPLC columns. Conversely, BHA is a bulky molecule and much less polar resulting in longer retention times, whilst Potassium Sorbate gives a huge UV response over all of the 220–300nm range due to a significantly greater chromophore. Each analyte is present at different concentrations (Potassium Sorbate high and EDTA low), which became problematic due to loading and sensitivity issues. Sample preparation required the extraction of all three components into solution without interferences from the Active Pharmaceutical Ingredient (API), related impurities, or other excipients in the formulation. A suitable sample preparation was found that achieved all this whilst giving an acceptable response for EDTA, Potassium Sorbate and BHA. Isocratic RP HPLC separation was achieved in under 15 min (the same time it currently takes to analyse EDTA by a single component method) that can be adapted to all future Aqueous Nasal Spray formulations containing different API's. An Agilent 1100 binary pump HPLC system was used with a mobile phase mix of tetrabutylammonium phosphate/methanol on a SB-C8 150 × 4.6mm i.d., 3.5um column. A flow rate of 1.0 mL/min and UV detection wavelength of 300nm gave acceptable response for the three preservatives. Validation has been performed based upon criteria specified in the ICH guidelines. Linearity is given over the 50–150% range for all analytes and solution stability of 48 h for stock standards has been shown. Analytes are specific from one another, repeatability and range are also acceptable. All validation to date has been performed on standard solutions since the composition for the final formulation has yet to be decided. Further validation for suspensions themselves is scheduled once this has been finalised. Multi-component methodologies that support generic formulation platforms have the potential to increase through-put of samples, to the benefit of both Research & Development and factory environments. Likely time and cost savings of robust assay methods will hopefully ensure that these methods are utilised more frequently in future years.

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The development of a MS compatible HPLC method for the analysis of 2,4-DNPH derivatised aldehydes present as secondary oxidative degradants in non ionic excipients

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Excipients play a key role in oxidation; either as a primary source of oxidants, trace amounts of metals, or other contaminants. Aldehydes are a very common impurity in many excipients, particularly polymeric excipients and non aqueous solvents such as Polysorbates, Polyethylene glycols (PEG) and polyvinyl pyrrolidones. They are used as initiators in polymerization reactions, but are difficult to remove. Aldehydes and other related impurities (hydroperoxides, peroxy, hydroperoxy radicals, peroxides, etc.) form in polymeric excipients and vegetable oils as a result of auto-oxidative processes involving initiation, propagation and termination. The levels of these oxidative impurities are dependant on a variety of factors, for example heat, light, time and concentration; as well as being batch, sub-lot and even container dependant (top, middle and bottom). Several authors recommended testing affected excipient batches for oxidative impurity levels prior to use, the use of inert atmospheres for storage, and single use containers. The use of specific processing to reduce or remove oxidative impurities was recommended by some authors, especially for formulations designed for topical application to sensitive organs, such as the eye. Formulations optimized for excipients from single sources could well be sub-optimal if the vendor source is changed and hence, dual sourcing of key excipients is recommended. The development of a generic method for the analysis of aldehyde levels in key excipients is required to quantify the level of these impurities and the suitability of the batch for formulation purposes. Also, a generic method capable of assessing the stability of formulations prepared using these excipients will assist in assessing the potential formation of such degradation products in pharmaceutical products and in setting an appropriate shelf life based upon this data. Analysis has been performed using Reversed phase High Performance Liquid Chromatography (RP HPLC). Samples are derivatised using 2,4-Dinitrophenylhydrazine (2,4-DNPH) presented in acidic solution and react with the carbonyl functional group to form the respective 2,4-Dinitrophenylhydrazone. Analysis is performed using a Pheny Hexyl stationary phase and is selective for 15 commonly available small carbonyl compounds presented as their respective 2,4-Dinitrophenylhydrazone. Each compound has been qualitatively analysed using Atmospheric Pressure Chemical Ionisation (APCI) negative mode. Quantitative analysis of the level of Formaldehyde and Acetaldehyde by external standardisation has been determined against appropriate reference standards. A series of polymeric excipients and non aqueous solvents (including Polysorbates, Polyethylene glycols (PEG) and polyvinyl pyrrolidones) have been analysed and the level of Formaldehyde and Acetaldehyde present in each sample has been quantified. Method validation in accordance with ICH has been performed for these two analytes and the method is applicable to support relevant stability studies. This HPLC method is suitable for quantifying the level of Formaldehyde and Acetaldehyde present in many excipients, particularly polymeric excipients and non aqueous solvents. The information this provides will allow formulation scientists to make reasoned decisions regarding excipient quality, compatability with their pharmaceutical active, and assess the long term stability of products containing these excipients and set appropriate shelf lives.

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The application of the Ferric oxide (FOX) assay for the analysis of peroxides present as primary oxidative degradants in non ionic excipients

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Excipients play a key role in oxidation; either as a primary source of oxidants, trace amounts of metals, or other contaminants. Peroxides are a very common impurity in many excipients, particularly polymeric excipients and non aqueous solvents such as Polysorbates, Polyethylene glycols (PEG) and polyvinyl pyrrolidones. They are used as initiators in polymerization reactions, but are difficult to remove. Peroxides and other related impurities (hydroperoxides, peroxy, hydroperoxy radicals, aldehydes, etc.) form in polymeric excipients and vegetable oils as a result of auto-oxidative processes involving initiation, propagation and termination. The levels of these oxidative impurities are dependant on a variety of factors, for example heat, light, time and concentration; as well as being batch, sub-lot and even container dependant (top, middle and bottom). Several authors recommended testing affected excipient batches for oxidative impurity levels prior to use, the use of inert atmospheres for storage, and single use containers. The use of specific processing to reduce or remove oxidative impurities was recommended by some authors, especially for formulations designed for topical application to sensitive organs, such as the eye. Formulations optimized for excipients from single sources could well be sub-optimal if the vendor source is changed and hence, dual sourcing of key excipients is recommended. The development of a generic method for the analysis of peroxides levels in key excipients is required in order to quantify the level of these impurities and the suitability of the batch for formulation purposes. Also, a generic method capable of assessing

the stability of formulations prepared using these excipients will assist in assessing the potential formation of such degradation products in pharmaceutical products and in setting an appropriate shelf life based upon this data. Analysis has been performed using the Ferric Oxide (FOX) Assay. This method has advantages over other analytical approaches presented by some authors. This method is commonly used for the detection of peroxides in detergents, lipids; monitoring cellular activity and determining protein glycation Pre-prepared assay kits are available from commercial suppliers. The basis of the assay is that hydroperoxides convert Fe(II) to Fe(III) at acidic pH. The converted Fe(III) complexes with the xylenol orange dye to yield a purple product with a maximum absorbance at 560nm. The molar extinction coefficient of the xylenol orange-Fe complex is $1.5 \times 10^{-4} \text{ M}^{-1} \text{ cm}^{-1}$ in 25 mM H_2SO_4 at room temperature. The composition of the reagents vary based upon whether the test material is aqueous or lipid compatible. A series of aqueous and lipid compatible excipients (including Polysorbates, Polyethylene glycols (PEG) and polyvinyl pyrrolidones) have been analysed and the level of peroxide present in each sample has been quantified. Method validation has been performed and the method is applicable to support relevant stability studies. The FOX assay is a suitable method for quantifying the level of peroxides present in many excipients, particularly polymeric excipients and non aqueous solvents. The information this provides will allow formulation scientists to make reasoned decisions regarding excipient quality, compatability with their pharmaceutical active, and assess the long term stability of products containing these excipients and set appropriate shelf lives.

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Liquid chromatographic separation of phenobarbitone, ethosuximide, phenytoin and carbamazepine on a polystyrene-divinyl benzene column

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Epilepsy is a common cause of morbidity and social stigmatization in Kenya. Phenobarbitone, ethosuximide, phenytoin and carbamazepine are the most commonly used drugs for epilepsy management in Kenya. Liquid chromatography is recognized as the most rapid, specific, precise, sensitive and cost effective method of analysis in multiple drugs. There is no compendial method for the simultaneous analysis of the four drugs. However, several silica based reversed-phase liquid chromatographic methods have been reported for their analysis (Szabo & Browne 1982; Torra et al 2000). Although such reversed phase stationary phases still dominate the liquid chromatographic field, polymeric columns are increasing in popularity as their retention characteristics and applications become better understood. One such material, polystyrene-divinylbenzene, is stable in a wide pH range (pH 1–13). A validated liquid chromatographic method for the simultaneous separation of phenobarbitone, ethosuximide, phenytoin and carbamazepine on a polystyrene-divinyl benzene column in 15 min is described. The method was developed by the systematic study of different types of co-polymer materials, type and concentration of organic modifiers, buffer pH and concentration and column temperature. A PLRP-S 100 Å 8-μm column maintained at 60°C and a mobile phase consisting of acetonitrile-tertiary butanol-phosphate buffer (pH 7.6, 0.2 M)-water (25:5:10:60, v/v) were used. The flow rate was 1 ml/min with ultraviolet detection at 220 nm. The detector response was linear for all the compounds (R > 0.999) in the concentration ranges of 0.005–3.1, 0.05–25, 0.005–3.1 and 0.003–1.6 μg for phenobarbitone, ethosuximide, phenytoin and carbamazepine, respectively. The within-day and between-day coefficients of variation ranged from 1.1 to 2.0 and 1.4 to 6.9%, respectively. The limits of detection were 2.0, 15.6, 6.4 and 2.0 ng while the limits of quantitation values were 7.5, 62.5, 32.5 and 15.6 ng for phenobarbitone, ethosuximide, phenytoin and carbamazepine, respectively. By studying the effect of small changes in acetonitrile concentration, pH and column temperature, the method was found to be robust. Satisfactory peak symmetry, selectivity and column stability was maintained over the 7 months study period at the high column temperature without any aggressive column solvent clean up. This method has been used for analysis of pharmaceutical raw materials, finished products and dissolution studies of the drugs in commercial samples on the Kenyan market (Orwa et al 2004). This paper therefore reports the development of a cost effective liquid chromatographic method on a polymer column for the analysis of the four commonly used anticonvulsant drugs in resource-poor countries.

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Development and validation of a simple HPLC determination of ketotifen fumarate for quality control purposes

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Ketotifen fumarate (KF) has been widely used as an anti-allergic and antianaphylactic agent in adults and children. (Yagi et al 2002). Different chromatographic and spectrometric method have been reported for determination of KF in pharmaceutical dosage forms and biological preparations (Abounassif et al 2004; El-Obeid et al 2005), but there isn't any simple HPLC method with a convenient sample preparation procedure for determination of KF applicable to assay, content uniformity and dissolution tests. In this study an HPLC-UV method for the assay, content uniformity, and dissolution rate determination of ketotifen fumarate in tablet has been developed and validated. Chromatography was carried out on a Novapack C18 (5.0 μm, 300 mm × 4.0 mm) column using acetonitrile:water (45:55) adjusted to pH 6.5 with acetic acid as mobile phase at a flow rate of 0.8 ml/min and a 299 nm UV detection with an analysis time of 8.5 min. The method was validated for linearity, intra- and inter-day precision, recovery, matrix interference, LOD and LOQ. Determination of content uniformity was carried out on 10 tablets separately, by the validated method, also assay and dissolution test of the tablets were performed by the method. Also a dissolution test based on USP apparatus II, with a rotating speed of 50 rpm in 500 mL water has been developed and validated. Sampling was done up to 30 min. The method was linear over the concentration range of 0.5–12 μg/ml (R $\approx$ 0.999). RSD% for both intra and inter day precision were lower than 3%. Recovery of the method was > 98% in the mentioned concentration range. Tablet matrix materials including lactose, microcrystalline cellulose and PVP had not any interference with the main component analysis. Results showed that LOD and LOQ were 0.1 and 0.3 μg/ml, respectively. Analysis of real samples manufactured at Zahravi Pharmaceutical Co. and Zaditen tablet as a reference product showed all tested parameters were in predefined range. As indicated by results, we can conclude that the method is fast, precise, selective and accurate for determination of ketotifen fumarate in its tablet dosage form. Because there isn't any monograph for quality control of it in its dosage form, this method can easily and reliably be applied for content uniformity, assay and dissolution testing of ketotifen tablet.

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Quantifying dissociation of pharmaceutical salts using FTIR and multivariate analysis

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Active pharmaceutical ingredients (APIs) are commonly formulated as salts in order to increase solubility and/or bio-availability in the formulated product. Monitoring dissociation of salt to free-base is critical to understanding formulation behaviour. This analysis must be performed on the solid material, although this is difficult to achieve, often requiring XRPD, which may not have the required sensitivity, or solid state NMR, which is expensive and time consuming. The objective of this investigation was to evaluate Fourier Transform infrared spectroscopy (FTIR) for detection and quantification of dissociated API in the solid state, both in drug substance and in formulated product, in order to replace solid state NMR. Multivariate analysis was used to help develop a robust model for predicting free base concentration. Samples of API, formulated as a salt, were spiked with known amounts of dissociated API. FTIR spectra were collected over the range 4000–400 cm^{-1}. DRIFTS (diffuse reflectance infra-red Fourier transform spectroscopy), KBr discs and golden gate ATR (attenuated total reflectance) sampling techniques were all evaluated. As the salt form was found to dissociate when subjected to pressure or grinding, as when collecting golden gate ATR or KBr disc spectra, the DRIFTS sampling technique was taken forward. Initial evaluation showed significant differences in peak shape and intensity, between salt and free-base, in several regions of the spectra. The region 3120–3060 cm^{-1} contains a peak that is indicative of the concentration of free-base and was modelled using multivariate analysis. A successful three component PLS (partial least squares) model was generated using mean centered, second derivative data. This model was validated using "leave one out validation," in which each sample is left out of the model in turn and its value predicted. The predicted data correlated well with the actual data giving a linear regression with a correlation coefficient of R^{2} = 0.9951 and a root mean square error of cross validation of 1.15% (Brereton 2003). This PLS model may be used to give quantitative predictions of free-base concentration in drug substance. A semi-quantitative model was developed for use with drug substance/excipient binary mixtures. This semi-quantitative model focuses on the 3100–3030 cm^{-1} region of the spectrum and uses a quadratic regression, calculated by taking a ratio of two peaks that are indicative of free-base concentration. This ratio is corrected for the effect of the excipient by subtracting of the excipient spectrum. This model was validated using mixtures of 20% drug and 80% excipient and found to give good agreement with solid state NMR data. This investigation has proved that FTIR may be used quantitatively for determining salt forms. Two techniques have been developed to evaluate free-base concentration in samples of salt: a quantitative chemometric model for drug substance and a semi-quantitative method for drug substance/excipient binary mixtures. This method is at least as quantitative as solid state NMR and allows much more rapid analysis of samples, thus permitting more efficient evaluation of API and formulated product, which will aid the drug development process.

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Determination of lysolecithin in propofol injection by high performance liquid chromatography

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In developing a British Pharmacopoeia monograph for a finished dosage form it is necessary to control not only the content of the active constituents but also the levels of potentially toxic degradation products arising from the formulation. Propofol Injection is a short-acting intravenous anaesthetic agent used for the induction of general anaesthesia in adult patients and paediatric patients older than 3 years of age. Several products (Diprivan and equivalents) are marketed in the UK, for which the BP is currently developing a monograph. These products contain egg yoke lecithin (phosphatidylcholine) as an emulsifier. Lysolecithin (lysophosphatidylcholine) is produced by hydrolysis of lecithin and may be present in Propofol Injection. Lysolecithin is known to cause membrane damage and haemolysis (Martin et al 1992). It is therefore important to be able to quantify and limit this impurity in Propofol Injection. Accordingly, an HPLC method for the determination of lysolecithin in Propofol Injection has been developed. A method for the determination of bile acids (Nieder 1985) was used as a starting point and modified to achieve satisfactory separation and limit of detection. The optimised method used a Nucleosil C18 column (15 × 0.46 cm) and a mobile phase comprising acetonitrile:water:orthophosphoric acid in the ratios 300:200:7. Ultraviolet detection was used at a wavelength of 210 nm and injection volumes of 250 μl were used. The peak due to lysolecithin was satisfactorily resolved from the propofol peak and other peaks in the chromatogram. The method was shown to be able to accurately recover lysolecithin from a spiked sample of Propofol Injection (recovery of lysolecithin was 109.6%). The response of lysolecithin using the chosen detection method was found to be linear over the expected concentration range of 0.0005% w/v to 0.1% w/v (r = 0.999995575) and to pass through the origin (confidence interval for the intercept was −3262 to 1209) and the limit of detection was found to be 0.0005% w/v of lysolecithin. Several samples from the marketplace were analysed using the method and lysolecithin was found in all batches tested at levels ranging from 0.064% w/v to 0.127% w/v. The method was therefore considered to be suitable for the quantitative determination of lysolecithin in Propofol Injection. The method was specific, linear and accurate.

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Development of XRPD methodology for recrystallisation studies of pharmaceuticals

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Secondary processing of pharmaceutical materials can sometimes induce disorder onto crystal surfaces. Process-induced disorder can be problematic since the physical characteristics of the material may be changed. This may result in handling difficulties in subsequent processing operations and possible variation in the final product performance. Additionally, since regions of disorder are metastable to crystalline

regions, the disordered regions may convert to the crystalline state given the appropriate environmental conditions. The recrystallisation may result in further changes to the physical characteristics of the material. During the Drug Development Process, scientists perform diligent studies to investigate the propensity for the generation of disorder in materials, develop methods for its detection and quantification, and thereby generate knowledge of the recrystallisation kinetics. This knowledge enables scientists to gain a thorough understanding of their materials and evaluate if the chosen manufacturing process will induce disorder. Manufacturing processes that induce disorder can remain viable options if the quantity of disorder produced can be controlled and does not affect product performance, or if the amorphous material recrystallises during a quarantine period following the manufacturing process. The purpose of this study was to develop XRPD methodology and data handling for investigating the recrystallisation process of several pharmaceutical materials. A Phillips X'pert Pro (Phillips Analytical, Holland) with a Cu anode and an X'celerator detector was used. The diffractometer was fitted with a variable divergence slit on the incident beam side and the samples were mounted in a stainless-steel front-filled holder or on a zero-background silicon wafer. Phillips Data Viewer and Highscore software packages were used for data handling operations. Amorphous and partially amorphous samples were prepared by ball-milling or spray-drying and monitored during storage under ambient and stressed conditions. The samples were analysed over the range of 3–40° 2θ at various timepoints and details kept of the X-ray tube deterioration. For the materials studied, the diffractograms obtained showed a clear decrease in background height with a corresponding increase in peak height demonstrating that recrystallisation was occurring. For each material, several peaks that were well resolved at the initial timepoint were chosen and parameters measured using manual peak limits. Measurements on the chosen peaks were made in the same way at all successive timepoints. Parameters of background height, peak height, peak area and full-width-half maximum (FWHM) were recorded along with the peak limits. The repeatability (n = 5) of the peak measurements was also recorded. These parameters were trended along with peak:background ratio measures to remove the effect of X-ray tube deterioration. The trending showed that the parameter of FWHM was not effective at demonstrating the recrystallisation process observed in the successive diffractograms. Peak area values were not repeatable owing to the appearance of small peak shoulders in certain data sets. The peak height, peak background and peak:background ratio measures demonstrated the recrystallisation process tending towards a plateau over time and were representative of the observations from overlays of the diffractograms. Significantly, the materials studied were shown to recrystallise to the thermodynamically stable form of the material. The data obtained in this study demonstrate that appropriately chosen peak parameters can provide a numerical measure of the recrystallisation process.