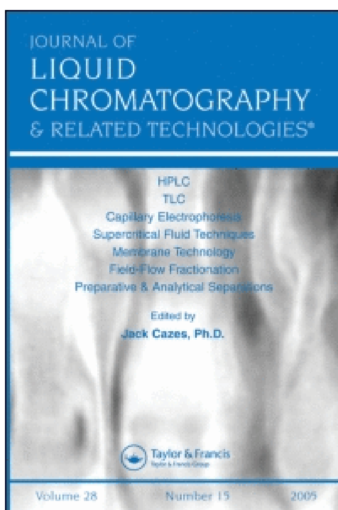
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Genotoxic Impurities: A Quantitative Approach

Zhanna Y. Yuabova[a]; David R. Holschlag[a]; Shirley A. Rodriguez[a]; Cindy Qin[a]; Vladimir V. Papov[a]; Fenghe Qiu[a]; John F. McCaffrey[a]; Daniel L. Norwood[a]
[a] Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut, USA

To cite this Article Yuabova, Zhanna Y. , Holschlag, David R. , Rodriguez, Shirley A. , Qin, Cindy , Papov, Vladimir V. , Qiu, Fenghe , McCaffrey, John F. and Norwood, Daniel L.(2008) 'Genotoxic Impurities: A Quantitative Approach', Journal of Liquid Chromatography & Related Technologies, 31: 15, 2318 — 2330

To link to this Article: DOI: 10.1080/10826070802281745

URL: http://dx.doi.org/10.1080/10826070802281745

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Journal of Liquid Chromatography & Related Technologies®, 31: 2318–2330, 2008
Copyright © Taylor & Francis Group, LLC
ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802281745

Downloaded At: 16:54 23 January 2011

Genotoxic Impurities: A Quantitative Approach

Zhanna Y. Yuabova, David R. Holschlag, Shirley A. Rodriguez, Cindy Qin, Vladimir V. Papov, Fenghe Qiu, John F. McCaffrey, and Daniel L. Norwood

Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut, USA

Abstract: Starting materials, intermediates and by-products are often found as impurities in active pharmaceutical ingredients (APIs). Some of these known impurities are potential mutagens or carcinogens but can be difficult or impossible to eliminate completely from the synthetic scheme. Based on current regulatory guidances for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5 μg/day daily intake of each individual impurity for new drug substances. During the early clinical development stages the Pharma Task group proposed a staged TTC concept, where greater daily intake can be allowed. The allowable daily intake thus calculated would then provide a basis for estimating the quantitation limit (QL) required for the development of an analytical method for determining genotoxic impurity levels. The approach for choosing an analytical technique will depend on many factors including availability of the instrumentation and physical and chemical properties of the analyte. Although UV absorbance is normally the first choice detection technique for HPLC analysis, many genotoxic impurities lack a UV chromophore and consequently are not suitable for quantitation via UV detection. The examples described in this report show different approaches for quantitation on a case-by-case basis using various detection techniques such as UV, ELSD, CAD and MS. Although in one case the required alert level of 30 ppm was easily achieved using standard UV detection, other examples highlight the need for using alternatives such as an LC-MS/MS method. The latter methodology was necessary to achieve a required QL level of 57 ppm for one particular impurity. Validation of this method as per ICH guidelines with respect to specificity, linearity, accuracy and QL is also discussed.

Correspondence: Zhanna Y. Yuabova, Boehringer Ingelheim Pharmaceuticals, Inc. 900 Ridgebury Road, P.O. Box 368, Ridgefield, CT 06877-0368, USA. E-mail: zhanna.yuabova@boehringer-ingelheim.com

Keywords: CAD, ELSD, Genotoxic impurities, LC-MS/MS, Quantitation, Validation

Downloaded At: 16:54 23 January 2011

INTRODUCTION

Genotoxic substances are chemicals that harm an organism by damaging its genetic material (DNA). The definition of genotoxicity is broad and includes both direct and indirect effects on DNA.[1] Specifically, there is evidence that genotoxic substances may bind directly to DNA and may also act indirectly by affecting enzymes involved in DNA replication. A major concern is that genotoxic substances could cause somatic mutations or be carcinogenic.

In the manufacture of the Active Pharmaceutical Ingredient (API), starting materials, intermediates, and by-products are potentially present in the API as impurities. These API impurities are sometimes known or suspect mutagens and/or carcinogens. Genotoxic and carcinogenic substances may be acceptable for certain APIs (e.g., cancer chemotherapeutic agents)[2] but in most cases it is advisable to make every effort to remove the genotoxic impurities, or failing that to alter the synthetic scheme to avoid generation of genotoxic impurities. In either case, the presence of even trace levels of genotoxic impurities can be very problematic for the pharmaceutical manufacturer, which in turn presents another challenge–to develop a suitable analytical method with the necessary sensitivity, selectivity and ruggedness for controlling these trace impurities.

To date, the International Conference on Harmonization (ICH) has not provided any specific guidance on thresholds or limits for genotoxic impurities. The report published by The Joint Pharmaceutical Analysis Group in the United States has stated that "industry and regulators have attempted to meet the need".[3] On the other hand, a European proposal from the Committee for Medicinal Products for Human Use (CHMP) has provided recommendations based on two categories.[4] The first is that genotoxic impurities that act by a threshold mechanism (i.e., not involving direct damage to DNA) can be regulated in a similar manner to non-genotoxic carcinogens (for example, calculating Permitted Daily Exposure (PDE) similar to ICH procedure outlined for Q3C[5] on limits for residual solvents). The second category is applied to substances for which no thresholded mechanism is identified. In those cases, the CHMP has recommended a "modification of the synthesis and the use of the TTC (threshold of toxicological concern) concept".[4] Finally, a proposal from the Pharmaceutical Research and Manufacturers of America (PhRMA) group has suggested an additional step that requires alerts based on chemical structure.[3]

Downloaded At: 16:54 23 January 2011

A Threshold of Toxicological Concern (TTC) was originally developed at the FDA for food-contact materials.[6] It is a very conservative approach, described by Kroes, where a low level of exposure (permitted daily intake PDI) could be identified for many chemicals including those with unknown toxicity but no adverse effect.[7] A TTC allows a maximum intake of 1.5 μg/day of any one genotoxic impurity over a patient's lifetime and was established using a carcinogenic potency database originally contained 343 carcinogens (the number of carcinogens was later increased up to 700).[4]

The 1.5 μg/day level was derived from the dose (taken from the carcinogenic potency database) that resulted in a cancer risk of one in a million incidents for the most sensitive species and the most sensitive site.[6] From this threshold value, a permitted level of a genotoxic impurity in the API may be calculated based on the expected daily dose:[4]

$$\text{Concentration Limit (ppm)} = \text{TTC}[\mu\, g/\text{day}]/\text{Dose}[g/\text{day}]$$

The concentration of genotoxic impurities also has a direct correlation with the duration of the exposure. The Pharma Task group proposed a staged TTC concept where greater daily intake can be allowed for early clinical stages. Table 1 provides the relationship between daily intake levels and the daily dose of API.[8] As seen in Table 1 the maximum acceptable level of impurity in ppm for a given daily dose of API is significantly lower when duration of exposure is increased. This is an important piece of information to keep in mind during the development of analytical methods.

Table 1. Relationship between acceptable daily intake levels and daily dose of a pharmaceutical

	Concentration of impurity (ppm)				
	Acceptable (maximum) daily intake (ADI) and duration of exposure				
Daily dose of API (mg)	<4 weeks ADI = 120 μg	1–3 months ADI = 40 μg	3–6 months ADI = 20 μg	6–12 months ADI = 10 μg	>12 months ADI = 1.5 μg
1000.0	120	40	20	10	1.5
700.0	171	57	29	14	2.1
400.0	300	100	50	25	3.8
100.0	1200	400	200	100	15
70.0	1714	571	286	143	21
40.0	3000	1000	500	250	38
10.0	12000	4000	2000	1000	150

Downloaded At: 16:54 23 January 2011

While it is always prudent to take a conservative approach toward method development for genotoxic trace level impurity quantitation, this does not mean that these methods always require the most sensitive analytical instrumentation such as LC-MS/MS (Liquid Chromatography/Mass Spectrometry/Mass Spectrometry). Instead, the analytical technique should be chosen based on the detector's capabilities and the required quantitation level (QL) of the method, which in turn is derived from the daily dose of the API, the concentration level of the impurity, and finally the duration of exposure as provided in Table 1.

The low level of tolerance for potential genotoxic impurities, as required by regulatory agencies, necessitates highly sensitive methods for monitoring and quantitation. The need for sensitivity is challenging analytical scientists to utilize different techniques and detectors to provide a lower limit of detection. Even though there are many different detection techniques that can be applied for trace level quantitation, this paper describes an approach adapted for routine testing of genotoxic impurities by HPLC based methods. Although widely used, UV detection does not always provide the necessary sensitivity and furthermore, detection is limited to analytes containing chromophores which absorb light at appropriate wavelengths. On the other hand, the use of UV detection is widespread because of its excellent precision, wide dynamic range and compatibility with mobile phase gradient liquid chromatography methods. While Evaporative Light Scattering Detectors (ELSD) can detect compounds that lack chromophores, this detection technique tends to be limited in sensitivity and dynamic range. The response is highly dependent upon eluent composition and analyte volatility and because of this, ELSD is not as routinely used as UV detection. A newly developed technology, Charged Aerosol Detection (CAD), has similar properties to ELSD (response is dependent upon eluent composition) and can also detect compounds that do not contain a chromophore. CAD, which relies on the charging of the aerosol particles, is similar in principle to the Atmospheric Pressure Chemical Ionization (APCI) source used for LC-MS.[9] However, CAD response is also highly dependent on the analyte volatility. Liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) have been established as some of the most sensitive and selective analytical techniques. Methods using MS detection are also gradient compatible and have good precision and dynamic range.

A decision tree for choosing a detection technique described previously is shown in Figure 1. The decision for choosing the detection technique should be primarily based on the level of the genotoxic impurity and also the physical and chemical properties of the analyte.

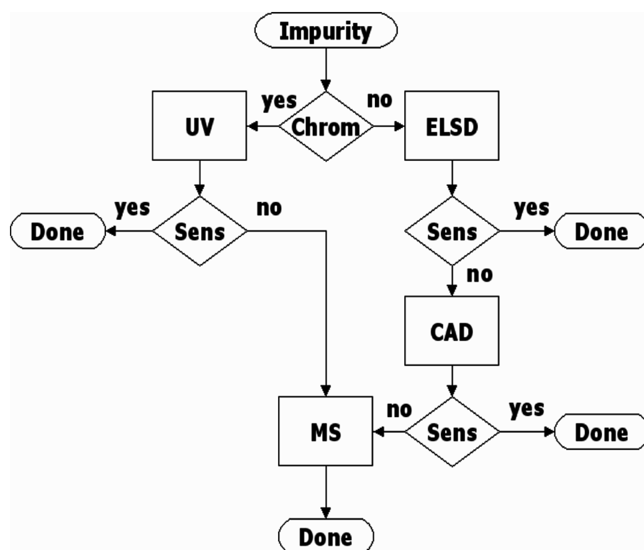


Figure 1. Decision tree for selecting detection techniques; Where: "Chrom" – Chromophore; "Sens" – Sensitivity.

Downloaded At: 16:54 23 January 2011

EXPERIMENTAL

Experiments described in Case Study 1 were performed on an Agilent HP 1100 System (Agilent Technologies, Waldbronn, Germany) which consisted of an HP 1100 pump, variable UV detector, Diode Array Detector (DAD), autosampler and column oven. A SYNERGI MAX-RP, 4 μm, 150 × 4.6 (Phenomenex, Torrance, USA) column was used in this case. The separation was achieved using water and acetonitrile with a linear gradient for 17 min.

Experiments described in Case Study 2 were carried out on an Agilent HP 1100 System (Agilent Technologies, Waldbronn, Germany) which consisted of a quaternary pump, diode array detector, an autosampler, and a temperature controller. An Atlantis T3 column 3 μm, 2.1 × 100 mm (Waters) was used in this study. The separation was attained using 0.1% formic acid in water and acetonitrile (EMD Chemicals Inc., an affiliate of Merck KGaA, Gibbstown, NJ) with a linear gradient for 15 min at a flow rate of 0.3 mL/min. Mass spectrometry experiments were done on a triple stage quadruple mass spectrometer (Micromass Quatro Ultima, Waters/Micromass, Manchester, UK) equipped with an electrospray (ES) interface operating in positive ionization mode.

All mass spectrometry experiments in Case Study 3 were performed on a triple stage quadruple mass spectrometer (Quattro Micro, Waters/Micromass, Manchester, UK) equipped with an APCI interface operating in positive ionization mode, connected to an HP 1100 system. Experiments described in Case Study 3 were performed on two different HPLC systems. System 1 consisted of an HP 1100 pump, column oven, and autosampler (Agilent Technologies, Waldbronn, Germany) connected to a Sedex 75 ELSD (Sedere, Alfortville, France). The second system consisted also of an Agilent HP 1100 pump, column oven, autosampler, and DAD connected to a Corona Charged Aerosol Detection (CAD)*Plus* (ESA, Chelmsford, MA, USA). A Gemini C18 4.6×100 5 µm (Phenomenex, Torrance, USA) column was used in LC-MS/MS and ELSD experiments. For CAD experiments, the separation was evaluated using a Phenomenex Inertsil C8, 5 µm, 250×4.6 mm column (Phenomenex, Torrance, USA). 0.1% heptafluorobutyric acid in water (Fluka, Sigma-Aldrich) and acetonitrile (EMD Chemicals Inc., an affiliate of Merck KGaA, Gibbstown, NJ) were used to prepare mobile phase for all experiments described in Case Study 3. Dimethylsulfoxide was supplied by Sigma- Aldrich (St. Louis, MO).

Downloaded At: 16:54 23 January 2011

RESULTS AND DISCUSSION

The case studies in this report are examples of using the decision tree shown in Figure 1 to choose a detection method for quantitation of a genotoxic impurity.

Case Study 1

In this study, the potential genotoxic impurity was an intermediate with a molecular weight of less than 300 Da, and contained a UV chromophore. The alert level calculated using a staged TTC concept was 30 ppm. Based on the available information, HPLC coupled with UV detection was chosen for the initial assessment. A Synergi MAX-RP column was utilized and a linear mobile phase gradient was applied. The QL level of this method was achieved at 3 ppm using the standard addition technique, and was significantly lower than the required alert level. The standard addition method was employed to compensate for matrix effects which were demonstrated to contribute to loss of sensitivity. The difference in response from the two sample solutions (spiked and unspiked) was used to determine a calibration factor for the added standard amount. The amount of impurity XX (the potential genotoxic impurity) present in unspiked sample was calculated using the calibration

factor. The chromatogram (Figure 2) shows impurity XX at the 10 ppm level well separated from the parent compound and all other impurities.

Case Study 2

In this second case study, a polar, small molecule starting material containing chromophore was the potential genotoxic impurity. HPLC/UV was determined to have inadequate sensitivity. According to the decision tree shown in Figure 1 an LC/MS method was applied. Gradient separation on an Atlantis T3 column with multiple reaction monitoring (MRM) and positive electrospray ionization provided a quantitation limit of 0.5 ng/mL, well below the required alert level calculated using staged TTC approach. An average percent recovery obtained for 6 spiked samples was 101.4% with 3.9% RSD. Linear response for the method was established from 0.5 to 10 ng/mL with a coefficient of determination greater than 0.99. Figure 3 shows a chromatogram of this starting material impurity at the 15 ppm level.

Case Study 3

Case Study 3 describes a more complex method developed for quantitation of three impurities classified as intermediates: A (suspect

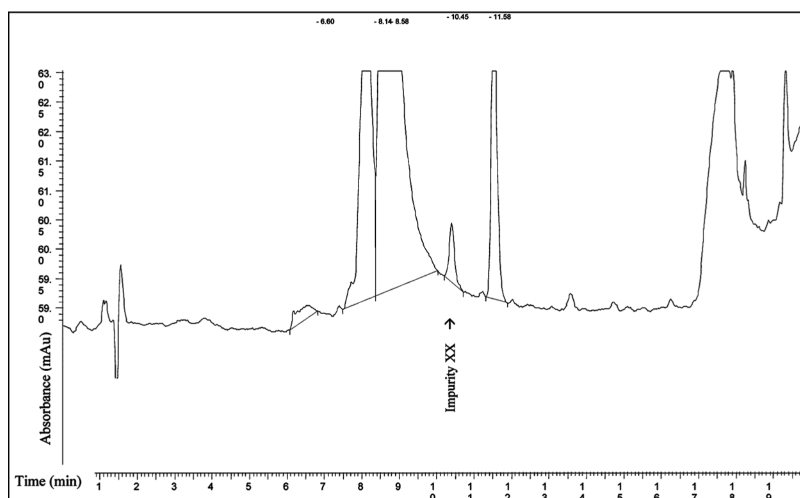


Figure 2. Chromatogram of impurity XX using HPLC/UV detection (Case Study 1).

Downloaded At: 16:54 23 January 2011

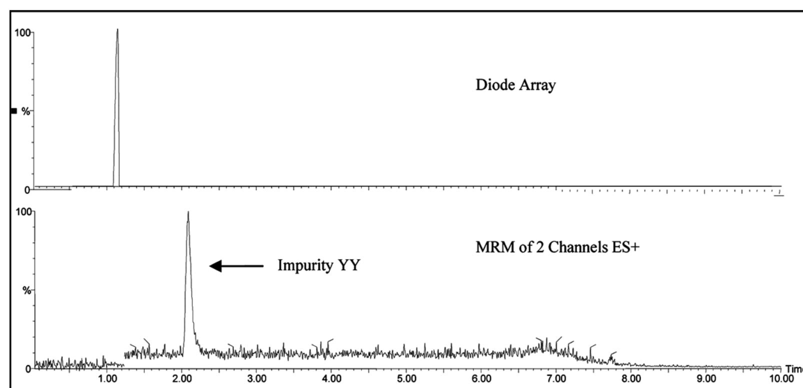


Figure 3. Chromatogram of impurity YY using LC/MS/MS detection (Case Study 2).

Downloaded At: 16:54 23 January 2011

genotoxic), B and C. All impurities were small molecules with molecular weights less than 300 Da. Unlike the analytes in Case Studies 1 and 2, they all lack a UV chromophore and were also moderately stable in protic solvents (except impurity C). While the API is highly soluble in aqueous solution, impurity C significantly degrades in the presence of water (after 2 hours) and partially converts into impurity B. The alert level for impurity A based on TTC calculations was 57 ppm (0.0057%). The approach using the decision tree in Figure 1 suggests the use of ELSD detection. Since the impurity C is unstable in protic solvents, dimethylsulfoxide (DMSO) was chosen as a dissolving solvent. In terms of the observed solubility and stability of the impurities, DMSO proved to be a good choice of solvent. Unfortunately, the desired sensitivity was not achieved using ELSD detection, and consequently further development efforts focused on CAD and LC-MS. The method was modified for MS use by substituting water for dimethylsulfoxide as the dissolving solvent and therefore, the concentration of the impurity C was calculated using the relative response factor due to relatively poor stability in aqueous solution. In addition, a different approach using the standard addition technique was evaluated to minimize the matrix effects. Both single ion monitoring (SIM) and multiple reaction monitoring (MRM) LC-MS experiments were compared. MRM was chosen due to better selectivity and sensitivity. Four separate MRM transitions were monitored using atmospheric pressure chemical ionization in positive mode (APCI+) for impurities A, B, C and an internal standard (IS). A chromatogram of 4 MRM channels is shown in Figure 4. An analog of impurity B was used as the internal standard, which mimics the analyte in chromatography and mass spectrometric

Downloaded At: 16:54 23 January 2011

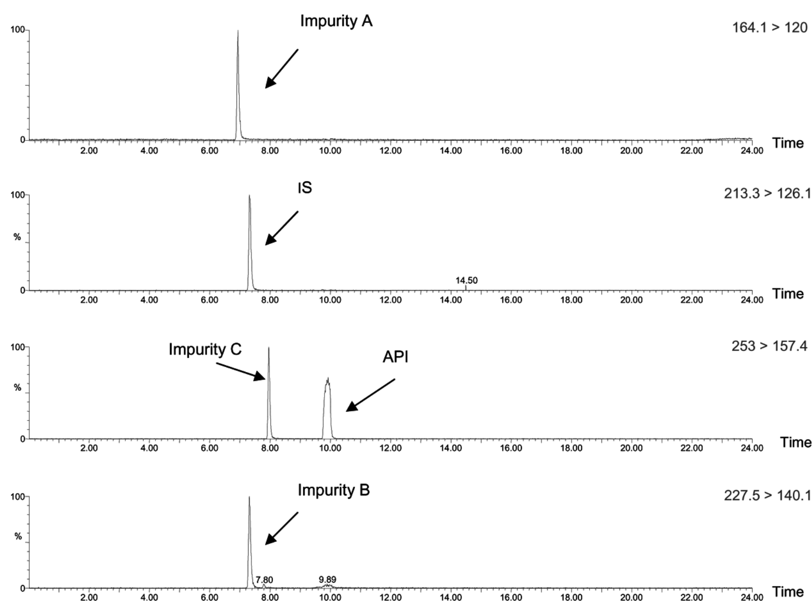


Figure 4. MRM chromatogram (Impurities A, B, C and Internal standard, Case Study 3). Note the MRM transitions m/z 164.1 > m/z 120, m/z 213.3 > m/z 126.1, m/z 227.5 > m/z 140.1, m/z 253 > m/z 157.4.

detection. A Gemini C18 column and a linear gradient with 0.1% heptafluorobutyric acid in water and acetonitrile was applied. As mentioned above the concentration of impurity C was calculated using a relative response factor versus impurity A. A stability study for impurities A, B and C was conducted to establish a safe margin for the run time.

The latter method was fully validated as per ICH guidelines. The linearity experiment conducted for impurities A and B showed that MS responses (peak area analyte/peak area IS) are proportional to their concentrations within the ranges of 0.0052% to 0.3% (equivalent to 0.0003 mg/mL to 0.0152 mg/mL) and 0.05% to 0.5% (equivalent to 0.0025 mg/mL to 0.0256 mg/mL) respectively. Both linearity plots were obtained by analyzing samples prepared in the presence of API. Figure 5 shows a linearity plot for impurity A.

Similarly, Figure 6 shows the linearity plot for impurity B (note that the line in this plot does not go through the origin due to the standard addition method for the calculation of the unknown concentrations). The linearity experiment for impurity C was not done due to a short stability time in aqueous solution.

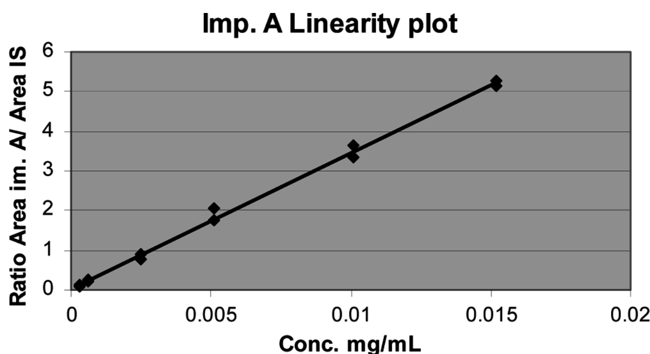


Figure 5. Linearity plot for impurity A from Case Study 3.

Downloaded At: 16:54 23 January 2011

The precision of the MS response was established by making 6 injections of the linearity standards at low and middle concentrations of the linearity plot for both impurities A and B. The % Relative Standard Deviation (RSD) was calculated and estimated to be less than 5% for the potentially genotoxic impurity A and 2% for impurity B. Table 2 presents a summary of the validation experiments.

The accuracy of the method was determined during the linearity experiment. Triplicate samples were prepared by spiking impurities into API at 0.05%. Although, no replicate samples were prepared for impurity C, two different concentrations (0.05% and 0.1%) of impurity C were analyzed. The overall average % recovery for all individual samples prepared for the accuracy experiment was 90% ($n = 21$) for impurity A with an RSD of 16%. This included % recoveries back calculated for each

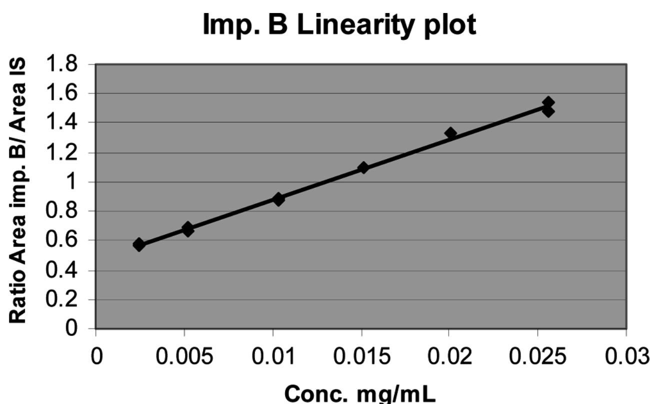


Figure 6. Linearity plot for impurity B from Case Study 3.

Table 2. Summary of the validation experiments from Case Study 3

	Impurity A	Impurity B
Linearity (R^2)	0.9964	0.9968
Range		
Concentration mg/mL	0.0003–0.0152	0.0025–0.0256
Precision ($n = 6$)		
% RSD	4.7	2.4
Accuracy		
Average % Recovery	90 $n = 21$	103 $n = 14$

Downloaded At: 16:54 23 January 2011

individual point of the linearity plot and QL samples using the regression line observed for the combined plot. Similarly, the average % recovery calculated for impurity B was 103% with an RSD of 9% ($n = 14$). In addition, a recovery experiment conducted for impurity C at 0.05% and 0.1% showed individual results at 114% and 95% respectively. The QL level of the method for impurity A was less than 0.0057% (the alert level). The QL levels for impurities B and C were determined to be 0.05%.

As mentioned above the CAD detector was also evaluated and found to be sensitive enough to monitor some of the process impurities in the API, including impurity B. However, the genotoxic impurity A was found to be too volatile for detection by CAD. Figure 7 shows

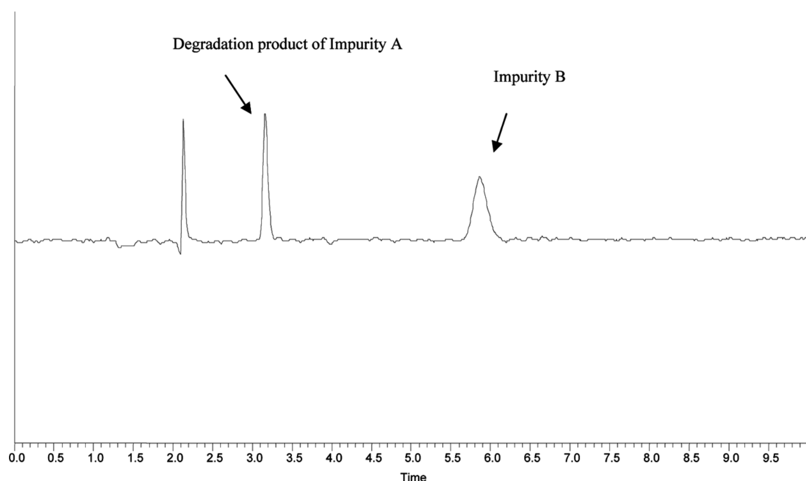


Figure 7. Chromatogram of the mixture of impurities A and B using HPLC/CAD (Case Study 3).

the chromatogram of the mixture of the impurities A and B. Only the degradation product of the impurity A and impurity B were observed.

CONCLUSION

The overview of the recent proposals and guidelines by regulators and the pharmaceutical industry regarding thresholds and limits for potentially genotoxic impurities has been discussed. The required low tolerance of these impurities presents a major challenge for the pharmaceutical industry. Although there are a number of different detection techniques available, these have to be chosen carefully on a case-by-case basis. Results presented in this report demonstrate that in some cases quantitation limits in the low-nanogram level (3 ppm) can be achieved by using routine UV detection. However, the Case Study 2 illustrated that UV detection was not successful for the low level quantitation of the analyte containing chromophore and an LC/MS/MS method was required. In another experiment the QL level of 56 ppm was achieved only by the use of tandem mass spectrometry coupled to an HPLC. Although, ELSD detection was initially attempted, it was determined to have low sensitivity for the particular impurities in this example.

While the relatively new CAD detection technology exhibited higher sensitivity compared to ELSD, CAD ended up being a poor choice due to the extreme volatility of one of the impurities. Finally, the chosen method in Case Study 3, LC-MS, was fully validated as per ICH guideline.

Downloaded At: 16:54 23 January 2011

REFERENCES

1. http://www.ilo.org/encyclopaedia/?d&nd=857400278 – Sorsa, M. Genotoxic chemicals. The International Labour Organization, Geneva, Switzerland.
2. McGovern, T.; Jacobson-Kram, D. Regulation of genotoxic and carcinogenic impurities in drug substances and products. TrAC September **2006**, *25*(8), 790–795.
3. The meeting of the joint pharmaceutical analysis group in association with King's College London. The Pharm. Jour. **2006**, *276*(7395), 422–423.
4. Guideline on the limits of genotoxic impurities. Committee for Medical Products for Human Use (CHMP). European Medicines Agency. London, 28 June, 2006, p. 28.
5. http://www.ich.org/cache/compo/363-272-1.html–International Conference on Harmonization.
6. Munro, I.C. Safety assessment procedures for indirect food additives: An overview. Report of a workshop. Regul. Toxicol. and Pharmacol. **1990**, *12*, 2–12.

7. Kroes, R.; Kleiner, J.; Renwick, A. The threshold of toxicological concern concept in risk assessment. Toxicol. Sci. **2005**, *86*(2), 226–230.
8. Muller, L.; Mauthe, R.J.; Riley, C.M.; Andino, M.M.; De Antonis, D.; Beels, C.; DeGeorge, J.; De Knaep, A.G.M.; Ellison, D.; Fegerland, J.A.; Frank, R.; Fitschel, B.; Galloway, S.; Harpur, E.; Humfrey, C.D.N.; Jacks, A.S.; Jagota, N.; Mackinnon, J.; Mohan, G.; Ness, D.K.; O'Donovan, M.R.; Smith, M.D.; Vudathala, G.; Yotti, L. A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. Regul. Toxicol. Pharmacol. **2006**, *44*(3), 198–211.
9. Gamache, P.H.; McCarthy, R.S.; Freeto, S.M.; Asa, D.J.; Woodcock, M.J.; Laws, K.; Cole, R.O. HPLC analysis of non-volatile analytes using charged aerosol detection. LC-GC Europe **2005**, *18*(6), 341–354.

Manuscript 6312H

Downloaded At: 16:54 23 January 2011