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Review

Control and analysis of alkyl and benzyl halides and other related reactive organohalides as potential genotoxic impurities in active pharmaceutical ingredients (APIs)

D.P. Elder[a,1], A.M. Lipczynski[b,*,1], A. Teasdale[c,1]

[a] GlaxoSmithKline R&D, Park Road, Ware SG12 0DP, UK
[b] Pfizer Global R&D, Sandwich, Kent CT13 9NJ, UK
[c] AstraZeneca R&D, Charnwood, Bakewell Road, Loughborough LE11 5RH, UK

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ABSTRACT

This paper continues the review of the relevant scientific literature associated with the control and analysis of potential genotoxic impurities (PGIs) in active pharmaceutical ingredients (APIs). The initial review [D.P. Elder, A. Teasdale, A.M. Lipczynski, J. Pharm. Biomed. Anal. 46 (2008) 1–8.] focused on the specific class of sulfonate esters but in this instance reference is made to the analysis of alkyl and benzyl halides and other related reactive organohalide alkylating agents. Such reactive materials are commonly employed in pharmaceutical research and development as raw materials, reagents and intermediates in the chemical synthesis of new drug substances. Consequently a great deal of attention and effort is extended by the innovative and ethical pharmaceutical industry to ensure that appropriate and practicable control strategies are established during drug development to ensure residues of such agents, as potential impurities in new drug substances, are either eliminated or minimized to such an extent so as to not present a significant safety risk to volunteers and patients in clinical trials and beyond.

The reliable trace analysis of such reactive organohalides is central to such control strategies and invariably involves a state-of-the-art combination of high-resolution separation science techniques coupled to sensitive and selective modes of detection. This article reports on the most recent developments in the regulatory environment, overall strategies for the control of alkylating agents and the latest developments in analysis culminating in a literature review of analytical approaches. The literature is sub-categorized by separation technique (gas chromatography (GC), high-performance liquid chromatography (HPLC), thin layer chromatography (TLC) and capillary zone electrophoresis (CZE)) and further tabulated by API type and impurity with brief method details and references. As part of this exercise, a selection of relevant pharmacopoeial monographs was also reviewed. The continued reliance on relatively non-specific and insensitive TLC methodologies in several monographs was noteworthy.

Contents

* Corresponding author. Tel.: +44 1304 646327; fax: +44 1304 656726.
E-mail addresses: David.P.Elder@gsk.com (D.P. Elder), Andrew.Lipczynski@pfizer.com (A.M. Lipczynski), Andrew.Teasdale@astrazeneca.com (A. Teasdale).
[1] Members of the UK Pharmaceutical Analysis Science Group (PASG), Genotoxic Sub-group.

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1. Introduction

The issue of potential genotoxic impurities (PGIs) in active pharmaceutical ingredients (APIs) and drug products continues to receive considerable attention. Most recently, Jacobson-Kram and McGovern [2] at the Center for Drug Evaluation and Research, US Food and Drug Administration (FDA), while stating that impurities should always be reduced to the lowest levels that are reasonably practical, acknowledged that impurities cannot be reduced to zero and meaningful specifications for impurities need to be established. The authors advised that the presence of genotoxic impurities should be avoided however they also recognize that complete removal is often not possible. In these cases, the amounts of genotoxic impurity present should be limited to a level that represents an insignificant risk to clinical trial subjects or patients, for example, the threshold of toxicological concern (TTC).

Many of the issues surrounding the trace analysis of PGIs, particularly alkylating agents, are described in detail in a recent review by Elder et al. [1]. The paper reviewed the current regulatory guidelines and relevant scientific literature pertaining to the control and analysis of PGIs, with specific focus on the analysis of alkyl esters of alkyl and aryl sulfonic acids in novel APIs. This present paper continues to focus on the control of alkylating agents, but this time with an emphasis on a review of the scientific literature (including recent conference proceedings and relevant pharmacopoeial monographs) pertaining to the trace analysis of alkyl and benzyl halides and other reactive organohalides with chemical functional moieties correlated with mutagenicity (see Fig. 1 taken from Appendix 3 in Muller et al [3]). In an early study undertaken by the Associazione Farmaceutici Industria (AFI) mutagenesis study group (Friscia et al. [4]) a number of chemical reagents and intermediates employed in the synthesis of beta-lactam, quinolone, antiviral and other drugs were investigated for genotoxic activity. The alkylating agents bromomethanol acetate, chloromethanol acetate and 2,5-dibromopentyl acetate all tested positive, as expected based on their alerting structures.

This review aims to summarize the general analytical approaches in this area to provide technical guidance for analysts working in this rapidly evolving field. A brief summary of API and impurity type with analytical details are provided in Table 1 .

2. Overall strategies for the control of alkylating agents

Assessing the overall risks and developing robust strategies for the control of alkylating agents as potential impurities in new APIs invariably involves close collaboration between pharmaceutical process chemists, analytical scientists, toxicologists and regulatory specialists. During scale-up and development activities, process chemists will explore, where practicable, opportunities to avoid the use of reactive reagents, starting materials and intermediates. If impracticable, routes may be reordered and/or process steps added to increase the prospect of eliminating the impurity prior to final isolation of the API. Theoretical considerations may be employed to justify the approach however in many cases appropriate analytical methods will need to be developed to confirm that the synthetic strategy has been successful and is robust.

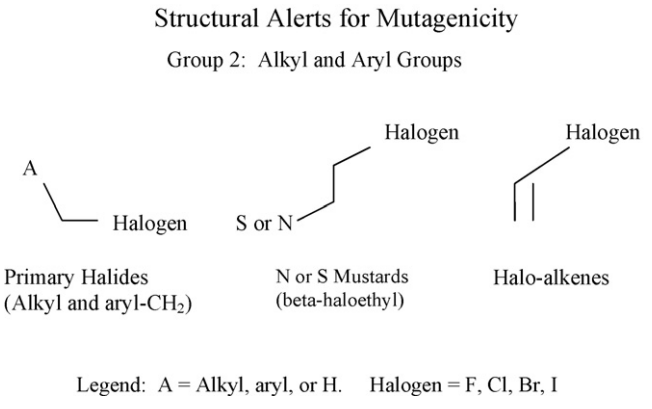

Fig. 1. Reactive organohalides with chemical functionalities correlated with mutagenicity [3].

McGovern and Jacobson-Kram [5] reviewed a number of international guidelines and regional guidance documents instructing drug developers and regulatory agencies on how to evaluate and control impurities in drug substances and products. These guidelines identify thresholds for reporting, identifying and qualifying impurities and also provide direction on the assays that should be used to determine if impurities are genotoxic. The authors described the FDA approach to the regulation of genotoxic impurities, including the use of computational toxicological approaches for the evaluation of impurities for structure-activity relationships for genotoxicity and carcinogenicity, followed by a discussion of practical and theoretical approaches for controlling levels of genotoxic impurities. From an analytical chemistry perspective, the authors recognized that while LC/GC coupled to mass spectrometry (MS) detection provide the technological platforms for method development, the separation, identification and quantification of impurities may be challenging in certain cases, e.g. where impurities of interest are degradants that form only in the presence of excipients, residual solvents or in contact with container closure components or in the separation of compounds of interest from the drug product matrix.

The authors also discuss the challenges inherent in controlling individual impurities in a matrix of many structurally related impurities (e.g. polyaromatic hydrocarbons) and comment that this may be much more difficult proposition to achieve. However, their recommendation that the structurally related group of impurities should collectively meet the exposure limit “as if it were a single compound”, has profound implications and would (if enacted) have significant impact on the analytical chemist; as it would effectively drive down the LOQ of each individual impurity within that group. The joint challenge of increased selectivity (to resolve the myriad of related impurities) and increased sensitivity (lower LOQ requirements) would be a significant hurdle to existing analytical methodologies.

Dobo et al. [6] applied a combination of safety and chemistry considerations to support the development of such control strategies. Adopting the five impurity classification categories defined by Muller et al. [3], and using structure-based safety assessment tools, a retrospective analysis of 272 starting materials and intermediates previously tested in the Ames assay was performed. This exercise differentiated 75% of the starting materials and intermediates as mutagenic and non-mutagenic with high concordance (92%) when compared with Ames results. 26 API synthetic routes were then surveyed using the structure based assessment to identify genotoxic hazards and so focus attention on where the development of control strategies was required. A number of hypothetical case studies were explored and safety evaluations were integrated with process chemistry considerations regarding the probable fate of the alerting starting materials and intermediates leading to recommendations for impurity control strategies during the various stages of drug development.

In one example, in the penultimate step of a 4-stage synthesis, 2-trifluoromethyl-4-*tert*-butyl benzyl chloride is used in the alky-

Table 1
Literature references and experimental details for analytical methods used to determine alkyl and benzyl halides and other related reactive organohalides in API

API	Impurities/analytes	Method details	References
Azasetron	Impurity: methyl iodide	A GC head-space method with two capillary columns in series (HP1 column, 30 m × 0.53 mm dimethylpolysiloxane stationary phase together with an HP2 column, 10 m × 0.53 mm diphenyl/dimethylpolysiloxane stationary phase (5/95%, w/w)). The column temperature was initially held at 35 °C for 6 min, before being raised to 150 °C for a further 4 min, at a rate of 40 °C/min. The injector port was held at 200 °C and the detector temperature was maintained at 250 °C	[10]
β-Lactam API	Impurity: CCMTHP (carbonic acid chloromethyl tetrahydropyran-4-yl-ester)	GC/MS with a DB-5MS stationary phase (30 m × 0.25 mm), 1 μm film thickness. Carrier gas helium at a flow rate of 1.0 ml/min. A column temperature program of 40–310 °C at 10 °C/min and hold for 3 min at 310 °C. Inlet temperature 250 °C. Injection volume 1 μl with a split ratio of 10:1. Transfer line temperature 280 °C, MS source 230 °C; MS quadrupole 150 °C; ionization energy 70 eV. Full scan EI data acquired over the mass range 25–550 amu, scan rate 2.86 cycles/s. Selected ion monitoring (SIM) mode at single ion 49 *m*/*z*	[11]
API	Impurity: BC (benzyl chloride) Impurity: CEME (chloroethyl methyl ether)	GC/FID with a RTx-5 Amine stationary phase (30 m × 0.53 mm), 3 μm film thickness. The carrier gas was helium at a flow rate of 35 cm/s and the column held at an initial temperature of 85 °C with a linear ramp of 10 °C/min to 250 °C	[14]
Almokalant	Impurity chlorohydrin (4-cyano(3-chloro-2-hydroxypropyl)benzene)	GC with fused silica capillary column (25 m × 0.32 mm) coated with cross-linked methylsilicone (HP Ultra 1), carrier gas helium at a flow rate of 1.8 ml/min. A flame ionization detector (FID) was used with the column maintained at 150 °C, injection port at 200 °C and the detector at 280 °C. The oven was held at 40 °C for 2 min and increased at 12 °C/min to 270 °C and held there for a further 5 min	[16]
Amiodarone hydrochloride	Impurity H: 2-chloro-*N*,*N*′-diethylethanamine	TLC with a silica gel F_{254} TLC plate, mobile phase formic acid/methanol/methylene chloride (5/10/85, v/v/v). The analyte was visualized using potassium iodobismuthate followed by peroxide	BP 1 2007 127/128 (Ph Eur monograph 0803)
Amiodarone hydrochloride	Impurity H: 2-chloro-*N*,*N*′-diethylethanamine	HPLC with a 3-μm cyano/bonded octadecylsilyl stationary phase (Hypersil nitrile), mobile phase consisting of acetonitrile/pH 6.0 phosphate buffer (500/500, v/v). Flow rate 1.0 ml/min, λ 240 nm	[34]
Atenolol	Impurity D: 2-[4-[(2RS)-3-chloro-2-hydroxypropyl] phenyl] acetamide (and isomer)	HPLC with a 5-μm octadecylsilyl stationary phase mobile phase consisting of sodium octane sulphonate (1 g) and tetrabutylammonium hydrogen sulphate (0.4 g) in a mixture of tetrahydrofuran/methanol/water/pH 3.0 phosphate buffer (20/180/800 v/v/v). Flow rate 1.0 ml/min, λ 226 nm	BP 1 2007 187/189 (Ph Eur monograph 0703)
Bromazepam	Impurity B: *N*-[4-bromo-2-(pyridine-2-ylcarbonyl)phenyl]-2-chloroacetamide	TLC with a silica gel F_{254} TLC plate, mobile phase ethanol/triethylamine/methylene chloride/light petroleum (5/5/20/70 v/v/v/v). The analyte was visualized using UV/light exposure at 254 nm	BP 1 2007 288/289 (Ph Eur monograph 0879)
Bromazepam	Impurity B: *N*-[4-bromo-2-(pyridine-2-ylcarbonyl)phenyl]-2-chloroacetamide Impurity E: *N*-[4-bromo-2-(pyridine-2-ylcarbonyl)phenyl]-2-bromoacetamide	CZE with a fused silica capillary (40 cm × 50 μm) at 25 °C, mobile phase 100 mM formate and 1 mM trifluoroacetate for all aqueous buffers For MEKC, 2% lithium dodecyl sulphate and 6% butan-1-ol were added For MEEKC, 2% lithium dodecyl sulphate, 6.6% butan-1-ol and 1% octan-1-ol were added	[32]

Table 1 (*Continued*)

API	Impurities/analytes	Method details	References
		For non/aqueous CE a mobile phase consisting of 25 mM acetate and 100 mM TFA in methanol/acetonitrile (50/50, v/v) was used CE with 1% polyvinyl sulfonated coated fused silica capillary (40 cm × 50 μm) at 25 °C was also used Samples were injected at a pressure of 50 mbar for 2 s, with a voltage of 15 kV, λ 230 nm	
Carmustine	Impurity A: 1,3-bis (2-chloroethyl)urea	TLC with a silica gel F_{254} TLC plate, mobile phase methanol/methylene chloride (10/90, v/v). The analyte was visualized using diethylamine, heating the plate to 125 °C for 10 min, cooling the plate and spraying with silver nitrate followed by UV/light exposure at 365 nm until brown/black spots appear	BP 1 2007 387 (Ph Eur monograph 1187)
Clindamycin hydrochloride	Impurity: clindamycin B	HPLC with a 5-μm octadecylsilyl stationary phase (Hypersil ODS) at 45 °C, mobile phase consisting of acetonitrile/pH 7.5 phosphate buffer (450/550, v/v). Flow rate 1.0 ml/min, λ 210 nm	BP 1 2007 530/531 (Ph Eur monograph 0582)
Clindamycin	Impurity: clindamycin B	HPLC with a 5-μm octadecylsilyl stationary phase (Hypersil ODS) at 45 °C, mobile phase consisting of acetonitrile/pH 6.0 phosphate buffer/water (350/400/250, v/v/v). Flow rate 1.0 ml/min, λ 210 nm	[21]
Diltiazem hydrochloride	Impurity: DMC (*N*,*N*-dimethylaminoethyl chloride)	IC with a 5-μm cation exchange column (Hypersil SCX), mobile phase consisting of acetonitrile/pH 2.9 aqueous formic acid (300/100, v/v). Flow rate 0.6 ml/min. EMI operated in positive mode, SIM at *m*/*z* 108	[17]
Famotidine	Impurity: CPN (1-chloroethyl nitrile, Impurity: DACT (diaminomethyl-enamino(chloromethyl)thiazole), Impurity CAPA (1-chloro-*N*-(aminosulphonyl)propanimidoamine)	HPLC with a 10-μm octylsilyl stationary phase (Spherisorb C8), mobile phase consisting of acetonitrile/pH 3.15 phosphate buffer/water (250/750, v/v). Flow rate 1.2 ml/min, λ 254 nm	[22]
Famotidine	Impurity: CAPA (1-chloro-*N*-(aminosulphonyl)propanimidoamine)	Normal phase HPLC with a Lichrosorb stationary phase, mobile phase consisting of chloroform/methanol/acetic acid/ammonia solution (90/15/6/2.4 v/v/v/v). Flow rate 1.0 ml/min, λ 287 nm	[23]
Famotidine (API and formulations)	Impurity: chloromethyl derivative (FPI). 2-[[Diaminomethylene]amino]-thiazol-4-yl]methylchloride	HPLC with an octadecylsilyl stationary phase (Supelcosil LC18), mobile phase consisting of acetonitrile (130 ml) and pH 3.0 phosphate buffer containing 0.2% triethylamine (870 ml). Flow rate 1.0 ml/min, λ 265 nm	[24]
Fentanyl citrate injectable	Impurity: 2-bromoethylbenzene	HPLC with a 5-μm octaylsilyl stationary phase (Inertsil C8), mobile phase consisting of 0.23% aqueous perchloric acid (650 ml) and acetonitrile (350 ml). Flow rate 1.0 ml/min, λ 206 nm	[38]
Folic acid	Impurity F: 2-amino-7-(chloromethyl)pteridin-4(1*H*)-one	HPLC with a 5-μm octadecylsilyl stationary phase, mobile phase consisting of methanol/phosphate buffer (120/880, v/v). Flow rate 0.6 ml/min, λ 280 nm	BP 1 2007 922–923 (Ph Eur monograph 0067)
Halothane	Impurity A: (*E*)-1,1,1,4,4,4-hexafluorobut-2-ene, Impurity B: (*EZ*)-2-chloro-1,1,1,4,4,4-hexafluorobut-2-(BP, 2007) trans), Impurity D: (*EZ*)-2-bromo-1,1,1,4,4,4/hexafluorobut-2-ene (cis and trans), Impurity E: 2-chloro-1,1,1-trifluoroethane, Impurity F: 1,1,2-trichlorchloro-1,2,2-trifluoroethane, Impurity G: 1-bromo-1-chloro-2,2-difluoroethane, Impurity H: 2,2-dichloro-1,1,1-trifluoroethane, Impurity I: 1-bromo-1,1-dichloro-2,2,2-trifluoroethane, Impurity J: 1,2-dichloro-1,1-difluoroethane	GC with a fused silica stationary phase (15 m × 0.53 mm) coated with poly(dimethyl)siloxane. The carrier gas was nitrogen at a flow rate of 15 ml/min. A flame ionization detector (FID) was used with the column maintained at 150 °C, injection port at 170 °C and the detector at 200 °C	BP 1 2007 1005–1006 (Ph Eur monograph 0393)

Table 1 (*Continued*)

API	Impurities/analytes	Method details	References
Ifosfamide	Impurity A: 3-[(2-chloroethyl)amino]propyl dihydrogen phosphate, Impurity B: bis [3-[(2-chloroethyl)amino]propyl] dihydrogen phosphate, Impurity C: 2-chloroethanamine, Impurity E: 3-chloro-*N*-(2-chloroethyl)propan-1-amine, Impurity F: (*RS*)-2-chloro-3(2-chloroethyl)-1,3,2-oxazaphospinane-2-oxide	TLC with a silica gel F_{254} TLC plate with a mobile phase comprising of water/methanol/acetic acid/methylene chloride (10/15/25/50, v/v/v/v). The analyte was visualized using a mixture of acidified potassium permanganate, followed by spraying with alcoholic tetramethybenzidine solution	BP 1 2007 1074–1076 (Ph Eur monograph (1529)
Isoflurane	Impurity A: 2-(chlorodifluoromethoxy)-1,1,1-trifluoroethane, Impurity B: 2-(difluoromethoxy)-1,1,1-trifluoroethane, Impurity C: (2*RS*)-2-chloro-2-(chlorodifluoromethoxy)-1,1,1-trifluoroethane, Impurity D: 1,1-dichloro-1-(difluoromethoxy)-2,2,2-trifluoroethane, Impurity E: 1,1-dichloro-1-(chlorodifluoromethoxy)-2,2,2-trifluoroethane	GC with a fused silica stationary phase (30 m × 0.32 mm) with macrogol 20,000. The carrier gas was helium at a flow rate of 1.0 ml/min. A flame ionization detector (FID) was used with the column maintained at 35 °C, injection port at 150 °C and detector at 250 °C	BP 1 2007 1123–1124 (Ph Eur monograph 1673)
Indoramin hydrochloride	Impurity A: 3-(2-bromoethyl)indole	TLC with a silica gel F_{254} TLC plate, mobile phase ammonia/ethanol/toluene (1/20/79, v/v/v). The analyte was visualized using UV/light exposure at 254 nm	BP 1 2007 1081
Lofepramine hydrochloride	Impurity H: 2-bromo-4′-chloroacetophenone	HPLC with a 5-μm base deactivated octylsilyl stationary phase (Lichrospher 60/RP Select B) at 50 °C, mobile phase consisting of a 0.09% solution of sodium dodecyl sulphate in a mixture of acetonitrile/water/pH 1.0 glycine buffer (550/325/125, v/v/v). Flow rate 0.9 ml/min, λ 254 nm	BP 2 2007 1258/1259
Lomustine	Impurity A: 1,3-bis (2-chloroethyl)urea, Impurity B: 1-(2-chloroethyl)-3-cyclohexylurea	HPLC with a 5-μm octadecysilyl silica stationary phase, mobile phase consisting of methanol/water (50/50, v/v). Flow rate 2.0 ml/min, λ 230 nm	BP 2 2007 1260–1261 (Ph Eur monograph 0928)
Loteprednol etabonate	Impurity LE-11-keto, Photolytic impurities I and II and III	HPLC with a 5-μm phenyl silica stationary phase (Alltima Phenyl), mobile phase consisting of water/acetonitrile/acetic acid (570/425/5, v/v/v). Flow rate 1.8 ml/min λ 244 nm	[25]
Melphalan	9 Alkylating impurities	HPLC with a 5-μm octadecysilyl silica stationary phase (Hypersil BDS) at ambient temperatures, complex gradient mobile phase consisting of varying amounts of acetate buffer, acetonitrile and water. Flow rate 1.5 ml/min, λ 260 nm	[26]
Metrifonate	Impurity A: methyl (*R,S*)-(2,2,2-trichloro-1-hydroxyethyl)phosphonate acid Impurity B: 2,2-dichloroethenyl dimethyl phosphate	HPLC with a 10-μm octadecysilyl silica stationary phase at 40 °C, gradient mobile phase consisting of pH 2.9 phosphate buffer and acetonitrile. Flow rate 1.0 ml/min, λ 210 nm	BP 2 2007 1387–1389 (Ph Eur monograph 1133)
Nefadazone hydrochloride	Impurity III: 1-(3-chlorophenyl)-4-(3-chloropropyl)piperazine hydrochloride	HPLC with a 5-μm octadecysilyl silica stationary phase (Inertsil ODS3V) at ambient temperature, mobile phase consisting of pH 3.0 phosphate buffer, acetonitrile and methanol (50/40/10, v/v/v). Flow rate 1.0 ml/min, λ 220 nm	[27]
Tolnaftate	Impurity C: *ortho*-naphathalene-2-yl chlorothioformate	TLC with a silica gel F_{254} TLC plate, mobile phase toluene (100%). The analyte was visualized using UV/light exposure at 254 nm.	BP 2 2007 2073–2074 (Ph Eur monograph 1158)
Tolnaftate	Impurity C: *ortho*-naphathalene-2-yl chlorothioformate	HPLC with a 5-(m phenyl substituted octadecylsilyl stationary phase (Hypersil phenyl), mobile phase consisting of a mixture of water and methanol (400/600, v/v). Flow rate not reported, λ 220 nm	[35]

Table 1 (*Continued*)

API	Impurities/analytes	Method details	References
Trazodone hydrochloride	Impurity F: 1-(3-chloropropyl)-3-chlorophenylpiperazine)	HPLC with a 5-μm Supelco Suplex pKb 100 stationary phase, mobile phase consisting of diethylamine/water/acetonitrile (0.4/320/680, v/v/v). Flow rate 1.7 ml/min, λ 254 nm	BP 2 2007 2086–2087
Verapamil hydrochloride	Impurity D: 3-chloro-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-methylpropan-1-amine	HPLC with a 5-μm end-capped palmit-amidopropylsilica gel stationary phase and a gradient (double isocratic) mobile phase consisting of pH 7.2 phosphate buffer and acetonitrile. Flow rate 1.5 ml/min, λ 278 nm	BP 2 2007 2145–2147 (Ph Eur monograph 0573)
Verapamil	Impurity VI: (3,4-dimethoxy-*N*-(3-chloropropyl)-*N*-methyl benzene ethanamine)	HPLC with a 3-μm Spherisorb ODS-2 stationary phase, isocratic mobile phase consisting of a mixture of acetate buffer/acetonitrile/2/aminoheptane (550/450/5, v/v/v). Flow rate 0.9 ml/min, λ 278 nm	[28]
Verapamil	Impurity VI: (3,4-dimethoxy-*N*-(3-chloropropyl)-*N*-methyl benzene ethanamine)	HPLC with a 5-μm Supelco Suplex pKb 100 stationary phase, gradient mobile phase consisting of varying mixture acetonitrile and pH 7.2 phosphate buffer. Flow rate 1.5 ml/min, λ 278 nm	[29]
Ziprasidone hydrochloride	Impurity 4: 5-(2-chloroethyl)-6-chlorooxindole	HPLC with a 5-μm YMC Pack Pro C18 stationary phase at 40 °C, gradient mobile phase consisting of varying mixtures of acetonitrile and 0.05% phosphoric acid. Flow rate 1.0 ml/min, λ 220 nm	[30]
Various substrates	Impurities: various sulfur and nitrogen mustards	TLC with a silica gel G_{254} TLC plate, mobile phase dichloromethane (100%). The analyte was visualized using NBP	[31]

Abbreviations: GC (gas chromatography); HPLC (high performance liquid chromatography); TLC (thin layer chromatography); IC (ion chromatography); CZE (capillary zone electrophoresis); MEKC (micellar electrokinetic chromatography) and MEEKC (microemulsion micellar electrokinetic chromatography). λ detection wavelength. NBP (4-(4-nitrobenzoyl)pyridine). BP = British Pharmacopoeia (2007), The Stationery Office on behalf of the Medicines and Healthcare products Regulatory Agency (MHRA), London. Ph Eur = European Pharmacopoeia (2005) 5th ed., Council of Europe, Strasbourg.

lation of an amide prior to the hydrolysis of an ester to a carboxylic acid that is the final API. Any unreacted benzyl chloride is an obvious potential genotoxic impurity in the API. In the final stage of synthesis it was reasoned that any residual benzyl chloride would be hydrolysed to the corresponding alcohol, but at a slower rate than the ester. The benzyl chloride is a liquid and therefore would be removed during final crystallization. However, as the benzyl chloride is used late in the reaction sequence and the subsequent work-up procedures are relatively mild it may be appropriate to assess the risk using an Ames bacterial mutagenicity test, and then, based on the outcome, to develop an appropriate analytical strategy. There are then two potential outcomes. If the benzyl chloride is Ames positive, then there is an increased risk and the need to develop a specific analytical method, e.g. HPLC/MS with a limit of quantitation (LOQ) of 10 ppm. Alternatively, if the benzyl chloride is Ames negative, it would be treated as a normal impurity and a default ICH analytical method, e.g. HPLC/ultra-violet (UV) detection with LOQ of 0.05% or 500 ppm.

This paper highlights that within any specific synthetic route and process, there may be a number of potentially genotoxic impurities however, in reality, the risk associated with each impurity varies considerably in terms of the probability of being present in the final drug substance at levels of concern. This is not surprising as many genotoxic compounds are genotoxic as a direct result of their intrinsic reactivity. It is this intrinsic reactivity that explains their use in the synthetic process and also it is this reactivity that is reason in many cases for the destruction/elimination of the potential impurity concerned. In order to avoid an unnecessary over-proliferation of analytical methods developed to essentially 'prove a negative' the appropriate use of risk assessment should be permitted, as aligned with current regulatory considerations described in ICH Q9 [7]. This would allow analytical investigations to be concentrated on those impurities of real concern thus ensuring efforts to exercise control are themselves properly focused.

3. Analytical approaches for trace analysis

Not surprisingly, most of the analytical attention has been focused on volatile alkyl halides as they are potent alkylating agents and hence of safety concern as potential impurities in APIs. This has seen a general renascence in the area of high-resolution gas chromatography (GC) linked with tandem techniques, e.g. MS or electron capture detection (ECD) to enable selective and sensitive detection. While direct injection can be used, headspace or solid phase extraction (SPE) techniques are usually employed to concentrate the analytes and/or overcome matrix effects.

High-performance liquid chromatography (HPLC) also has a key role to play in the analysis of alkylating impurities, especially in those cases where analytes are insufficiently volatile and/or thermally labile and so preclude reliable GC analysis. The selection of MS as a detection mode is usually necessitated by the requirement for additional sensitivity and selectivity, driven no doubt by the recent greater regulator focus on alkylating agents, which in turn has driven lower limits of detection and quantitation. In contrast the previously lower level of regulatory concern over trace levels of such impurities allied with the lack of MS instrumentation in certain laboratories may explain the many older literature references to the use of single wavelength UV detection.

Perhaps, somewhat surprisingly, there are few reports in the literature of the use of supercritical fluid chromatography (SFC) for the analysis of alkylating agents. Similarly very few examples of capillary zone electrophoresis (CZE) or the related electro-chromatography techniques have been reported, presum-

ably attributable to the perceived lack of sensitivity of this particular analytical approach. Some pharmacopoeial monographs still apply the older chromatographic techniques such as thin layer chromatography (TLC).

3.1. Gas chromatography

Many recent examples of the application of GC relate to the development of methodology that attempts to minimize and/or eliminate the affect of the analytical matrix in which the alkylating agent is present. These approaches are often 'generic' in nature and can be used to analyse a series of related analytes. This strategy is particularly attractive in the context of establishing high throughput screens. Several approaches are described below. These have in common the fact that they all utilize a specific physical property of the analyte not shared by the matrix, e.g. low boiling point and/or the presence of halide atom.

Skett [8] utilized ECD allied to GC to determine the presence of alkyl halides. He reported that the sensitivity of the alkyl halides using the ECD detector increases in the order, chloride < bromide < iodide. He evaluated a series of short chain alkyl halides (methyl, ethyl, propyl and butyl) at analyte concentrations of 10 ppm relative to a 10 mg/ml API solution, and showed that the alkyl chlorides gave very weak responses, whereas the iodides all saturated the detector.

Ellison [9] reported on the development of a generic headspace GC/ECD method for the determination of 23 genotoxic alkyl/aryl halides as potential impurities in API (see Fig. 2). She reported that headspace GC had great applicability for volatile compounds and is based on partitioning of the volatile analyte into the gas phase from the liquid phase; until the headspace is saturated and equilibrium is attained. The liquid phase was optimized as a mixture of water and dimethylsulphoxide (70/30, v/v). The headspace was optimized at a ratio of 20/1 (v/v) gas/liquid phase. The method was generically validated with two different APIs, and is then validated for every additional API using spiked batches. Specificity was demonstrated by lack of interference from the blank, solvents, impurities and other analytes. The accuracy of the method was good with recoveries at the 1000 ng/ml level in the range 80–120%. The detection limits for the 23 analytes ranged from 2.5 to 290 ng/ml; iodo alkylating agents gave the best analytical responses and the bromo alkylating agents the worst responses. These findings largely match those of Skett [8].

A GC head-space (HS) method for the determination of volatile alkylating impurities in the antiemetic, azasetron hydrochloride was reported by Wan [10]. The method's linearity was good, with a correlation coefficient of greater than 0.99. The precision was acceptable (R.S.D. < 2%), as were the recoveries (>96%). The detection limit of methyl iodide was reported as 41.3 ng.

Li and Slugget [11] developed a direct injection GC/MS method utilizing selected ion monitoring (SIM) for assessing trace levels of an alkylating agent; carbonic acid chloromethyl tetrahydropyran-4-yl ester (CCMTHP) in a β-lactam API. The authors observed that some of the standard approaches of environmental analysis, which are heavily reliant on SPE and enrichment (or sample pre-concentration) were not applicable as the genotoxic impurity (CCMTHP) and the API had very similar physicochemical properties. They discounted chemical derivatisation, which is often used to enhance sensitivity, because of concerns over low derivative yields [12] or worse, multiple analyte formation [13].

During their development activities they assessed a variety of analytical techniques; including HPLC/MS, GC/flame ionization detection (FID), GC/ECD, as well as GC/MS. However, GC/MS with SIM at m/z of 49 amu provided the best sensitivity at the 10 ppm level (50 ng/ml) using external standard quantitation. They demonstrated linearity over the range 10–1000 ppm, with a correlation coefficient of 0.9999, acceptable accuracy over the range 0.05–5 μg/ml and that the method was highly reproducible (R.S.D. < 3.0%). They indicated that method which required no extraction, derivatisation or cleanup procedures could be readily adapted to other halide containing alkylating agents.

Frost et al. [14] reported on a GC/FID method for the determination of trace levels of pharmaceutical process impurities of toxicological concern, including the volatile alkylating agents, benzyl chloride (BC) and chloroethyl methyl ether (CEME). They utilized a powerful combination of automated headspace and solid phase micro-extraction (with carboxen/polysiloxane/divinylbenzene fibres) to concentrate the analytes. The effects of extraction parameters, e.g. HS volume and agitation rate, extraction temperature, extraction time, as well as the impact of competitive displacement using inorganic salts on extraction efficiency, were all assessed. The method was linear over the range 0.1–0.5 μg/ml for CEME (correlation coefficient 0.9999) and over the range 0.01–10.0 μg/ml for BC (correlation coefficient 0.9979). The method was sensitive demonstrating LOQs of 10.0 and 0.9 ng/ml and LODs of 4.0 and 0.3 ng/ml for CEME and BC respectively. The method was reasonably accurate, but did show low recoveries for both analytes. The recoveries for CEME over the range 0.1–10.0 μg/ml were in the range 84.8–90.8%; whereas, recoveries for BC over the range 0.1–10.0 μg/ml were in the range 74.1–77.2%. The low recoveries were attributed to matrix interference from the API. This was demonstrated by reducing the concentration of API from 10 to 1 mg/ml, which increased the recoveries of BC at levels of 10.0 μg/ml from 77.2% to in excess of 85%. The authors commented

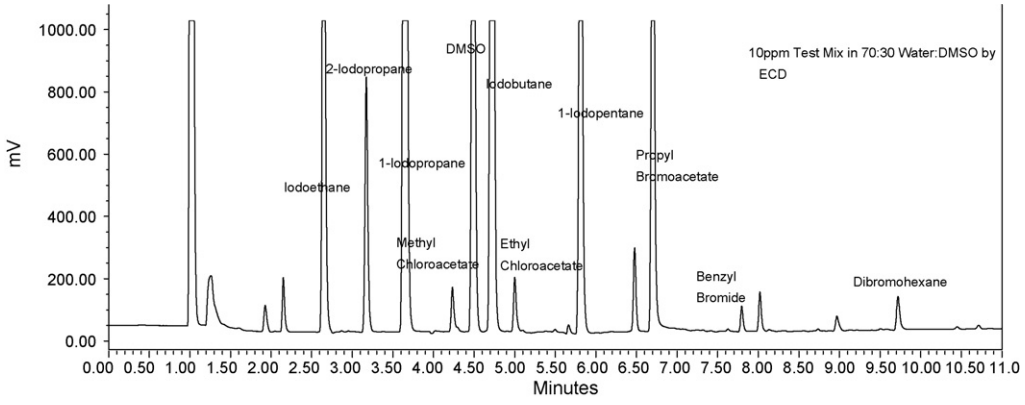

Fig. 2. Headspace GC/ECD separation of organohalides [9].

that the sensitivity of the method could be further increased by using mass or element-specific detectors.

Liquid/liquid extraction (LLE) has also been used successfully. Klick [15] reported on the development of a GC method for the determination of residual levels of a chlorohydrin and the corresponding epoxide impurities in the potassium channel blocker, almokalant. The method concentrated the residual volatile impurities by liquid/liquid extraction followed by on-column injection. The chlorohydrin gave good precision for repeat injections (R.S.D. 0.69%). Klick [16] separately reported that on-column injection gave the best results in terms of both precision and sensitivity. She reported that the LOD and LOQ for the chlorohydrin were 0.09 and 0.31 ppm (μg/g), respectively. The author mentioned the on-column formation of the epoxide from the chlorohydrin was slightly ameliorated by the use of high initial column pressure.

3.2. High-performance liquid chromatography

In the most recent literature references to HPLC analysis of alkylating impurities, reversed phase modes of separation and MS detection predominates. Selectivity can be enhanced by single SIM on a single quadrapole or selective reaction monitoring (SRM) on MS/MS instruments [8]. The ionization mode is important in HPLC/MS; important modes include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and more latterly, atmospheric pressure photoionisation (APPI). Ionic compounds tend to give excellent responses on ESI.

Lee et al. [17] demonstrated that HPLC/ESI/MS gave better responses than APCI and was used to measure *N*,*N*-dimethylaminoethyl chloride (DMC), in diltiazem hydrochloride using SIM, with *m*/*z* 108. The interfering API was removed on a reversed phase HPLC column and the polar analyte was determined using ion exchange chromatography. The analyte response was linear over the range 0.2–10 ppm, with a correlation coefficient in excess of 0.999. The detection limit is in the range 0.05–1 ppm. The repeatability of the method at the 1 ppm level is 7% R.S.D.

Clarke [18] used HPLC/MS to determine three residual alkyl bromides (A, B and C) in a secondary amine API. The stationary phase used was a 3-μm Phenomenex Luna C18 at 40 °C. The method used a linear gradient of 0–95% mobile phase B over 18 min, where mobile phase A was 0.05% trifluoroacetic acid in water and mobile phase B was 0.05% trifluoroacetic acid in acetonitrile. The flow rate was 1.0 ml/min and detection employed electrospray positive ionization (ESI) MS. Standard solutions of the three alkylators were run to establish sensitivity at 0.05 mg/ml. Although, impurity C gave acceptable recoveries (88%), those for impurities A and B were not acceptable (0%). This was attributed to the high reactivity of the latter two impurities. It was established that in the presence of spiked API, impurity A was rapidly converted to the non-genotoxic impurity, product B. An analogous reaction occurred with impurity B. The method conditions were therefore modified to reduce reactivity using a chilled autosampler (Waters 2695 Alliance-ZQ) at 15 °C and by mixing the samples using vortexing, rather than sonication. Using these modified conditions recoveries of these impurities in spiked API was increased to 90% and solution stability was acceptable (24 h). The method gave acceptable detection limits (LODs) for all three impurities with LODs of 10 μg/g (10 ppm) or better. The method was validated and used to analyse and release API for clinical use.

Hamilton et al. [19] reported the use of negative ion APCI coupled with SIM at *m*/*z* 127, 79 or 35 (for the appropriate halide) to determine residual alkyl halides. The best responses were given for alkyl iodides aligned with the findings of Skett [8] and it was proposed that this approach could be a suitable approach for determination of these analytes in API.

In a very recent example, Huybrechts [20] described D013197, an *N*-chloro impurity in a novel API of undisclosed chemical structure which was determined to have a TTC of 0.67 ppm. A powerful combination of orthogonal HPLC and SFC methods both with MS detection were developed to determine D013197 at sub-ppm levels.

The first procedure was an HPLC/MS/SIM method which monitored the impurity at an *m*/*z* of 689.6. The method comprised of a 3.5 μm XBridge C18 stationary phase with a gradient mobile phase comprising of varying mixtures of mobile phase A (10 mM ammonium acetate with 0.25% (v/v) ammonium hydroxide solution) and mobile phase B (acetonitrile) at 60 °C. The flow rate was 1.0 ml/min with a 15-μl injection volume and it gave a peak that eluted at RRT 1.21. The concentration of the API was 10 mg/ml.

The HPLC method was determined to be linear (correlation coefficient >0.996) over the operating range 0.2–18 ppm D013197 and had a limit of detection (LOD) of 0.03 ppm and an LOQ of 0.1 ppm. The accuracy was acceptable and gave recoveries in the range of 93.4–104.2% over the range 0.2–25 ppm (limits 70.0–130.0%). In addition, three different samples were spiked with 0.6 and 6 ppm, of D013197 and the recoveries were in the range 92.6–111.4% at 0.6 ppm, and 83.5–126.7 ppm at 6 ppm (limits 70.0–130.0%), respectively. The repeatability of the method at the 0.5 ppm limit ($n = 6$) was assessed and found to be acceptable (range 0.45–0.52 ppm, mean 0.48 ppm, % R.S.D. 4.6% (limits % R.S.D. less than or equal to 15.0%). The reproducibility of the method was determined by analysis of the same sample in a second laboratory and found to be acceptable (limits % R.S.D. less than or equal to 15.0% and relative difference between the mean values was less than or equal to 30.0%). The analyte was found to be stable in the dimethyl formamide (DMF) solvent for at least 48 h at room temperature.

In parallel, a second SFC/MS/SIM method was developed which monitored the impurity at an *m*/*z* of 689.6. The stationary phase utilized a 2-ethylpyridine column at 35 °C and a mobile phase of SFC carbon dioxide/10% methanol and 1 mM ammonium acetate. The flow rate was 2.0 ml/min, with a back pressure of 150 bar and it gave a peak that eluted at RRT 0.71. The concentration of the API was 10 mg/ml. Both methods were claimed to be quality control (QC) friendly at the same reporting threshold of 0.5 ppm, although no validation details were reported for the SFC procedure. The authors concluded that the analysis of genotoxic impurities is neither straightforward nor trivial. The complexity is due to the large number of critical parameters, e.g. sample preparation, chromatography, MS, etc.

There are numerous references in the literature, to HPLC methodology utilizing single wavelength UV detection. The impurity profile of clindamycin was studied by Orwa et al. [21]. They developed an HPLC/UV method for the determination of an alkylating agent, clindamycin B. The robustness of the method was assessed with respect to stationary phase, mobile phase, temperature and pH using a full factorial design and found to be acceptable. The method was linear over the range 20–60 μg on-column (correlation coefficient 0.9999), the LOQ was 0.12% of the nominal value (40 μg ml on-column); whilst the LOD was half of this. The precision was acceptable (R.S.D. 0.55%). The method was applied to commercial clindamycin capsule (Antirobe™) samples and levels of clindamycin B were found between the LOQ and LOD of the method (0.37–0.44%) with acceptable precision (R.S.D. < 3.1%).

A rapid HPLC/UV method for the determination of the process impurities in famotidine API and drug product including the alkylating agents; 1-chloroethyl nitrile (CPN), diaminomethylenamino(chloromethyl)thiazole (DACT) and 1-chloro-*N*-(aminosulphonyl)propanimidoamine (CAPA), was described by Hussain et al. [22]. The method gave good recoveries for DACT and CAPA, with values of 103.7% and 104.2%, respectively. The method demonstrated linearity for DACT over the range

0.3–61.5 ng (correlation coefficient 0.987) and CAPA over the range 20.5–35.0 ng (correlation coefficient 0.968). The limit of detection of DACT was reported as being 3.7 ng. The method was used to analyse residual process impurities in a typical batch of famotodine. Residual levels of DACT (0.07%) and CAPA (0.43%) were reported. Görög et al. [23] reported on the use of normal phase HPLC/UV methodology for the determination of CAPA in famotidine. A simple HPLC/UV method was developed for determination of famotidine and its impurities, including a chloromethyl alkylating impurity (FPI), in pharmaceutical formulations by Helali et al. [24]. The method was shown to be linear over the range 3–50 μg/ml for FPI (correlation coefficient >0.9997). Method repeatability and reproducibility for FPI were good (R.S.D. 1.94%, $n = 6$), with recoveries of 98.1%. The detection limit for FPI was reported at 0.12 μg/ml.

Yasueda et al. [25] described an HPLC/UV method for the determination of related substances, including the alkylating impurity, LE-11-keto (chloromethyl-3,11-dioxo-17α-ethoxycarbonyloxy-androsta-1,4-diene-17β-caroboxylate), two principal photolytic alkylating impurities; chloromethyl-17α-ethoxycarbonyloxy-11β-hydroxy-5α-methyl-2-oxo-19-norandrosta-1(10)3-diene-17β-caroboxylate and chloromethyl-17α-ethoxycarbonyloxy-11β-hydroxy-1-methyl-3-oxo-6(5–10α)–*abeo*-19-norandrosta-1,4-diene-17β-caroboxylate, and a minor photolytic degradation product, chloromethyl-17α-ethoxycarbonyloxy-1β,11β-epoxy-2-oxo-10α-androsta-4-ene-17β-caroboxylate in loteprednol etabonate. Linearity of LE-11-keto over the range 0.05–2.0% was determined and found to have an acceptable correlation coefficient (0.999), the relative response factor was 0.97, and the LOD was 0.004%, whereas the LOQ was 0.013%. The accuracy over the same concentration range for LE-11-keto was in the range 91.2–100.3% (R.S.D.s < 2.4%), the repeatability was in the range 92.4–100.5% (R.S.D.s < 2.2%) and the intermediate precision was 99.5% (R.S.D. 0.9%).

Brightman et al. [26] described an HPLC/UV method for the determination of related substances of the cytotoxic drug, melphalan. The method resolved 9 alkylating impurities out of a total 13 impurities that were observed. Linearity of the most abundant alkylating impurity (monohydroxymelphalan) was determined over the range of 0–3.6% and was found to be acceptable. The precision of this impurity was determined and found to have an R.S.D. 4.2%. The LOD of the method for all impurities was found to be 0.05%. The authors determined reproducibility of the method using three different batches of API, two different sites and using different column loadings. In all cases the correlation between the data for all 13 related impurities was good; with the exception of melphalan dimer (0.2%, site 1 vs. 0.5%, site 2). However, the authors contended that this was a late eluting impurity and the peak shape was poor.

The HPLC/UV determination of process related impurities including an alkylating agent (impurity III) in the antidepressant drug nefadazone hydrochloride was described by Rao et al. [27]. The LOD and LOQ of impurity III were found to be 79 and 240 ng/ml, respectively. The precision of the method was acceptable (<1.2% R.S.D.) when used for the analysis of the medicinal product, Serzone-R™. The recoveries of impurity III over the range 0.5–1.3 μg was acceptable (101.2–104.7%). The method was found to be rugged and robust and was used for ongoing stability studies.

Lacroix et al. [28] reported on an HPLC/UV method for the separation of 17 related impurities of the calcium channel blocker, verapamil. 3,4-Dimethoxy–*N*-(3-chloropropyl)-*N*-methyl benzene ethanamine (Impurity VI) an alkylating agent is resolved using this method. The LOD and LOQ were 0.008 and 0.02%, respectively for impurity VI. The method was linear over the range from the LOD to 0.08% of analyte concentration, with a correlation coefficient of 0.999. The ruggedness of the method was evaluated. It was found to be sensitive to stationary phase changes; the order of elution of impurities VI, VII and XII, changing with changes in stationary phase. The method probably under estimates residual levels of impurity VI (relative response factor was 0.74 compared with verapamil).

Valvo et al. [29] similarly reported on an HPLC/UV method for the separation of 13 related impurities of verapamil. It was claimed to be superior to both of the existing pharmacopoeial methods for verapamil. Impurity VI is also resolved using this method. The LOD and LOQ were 0.01% (0.05 μg/ml) and 0.02% (1.0 μg/ml), respectively. The accuracy of the method over the range 0.02–0.5% of impurity VI was in the range 86.9–109.2%, with R.S.D.s decreasing as the analyte concentration increased (<6.2%). The method was linear over this same concentration with a correlation coefficient of 0.9964. The ruggedness of the method was evaluated. It was found to be sensitive to pH and mobile phase composition, but in contrast to the findings of Lacroix et al. [28] insensitive to stationary phase changes.

The gradient HPLC/UV determination of a residual alkylating agent (5-(2-chloro-ethyl)-6-chlorooxindole) in the antipsychotic, ziprasidone hydrochloride was reported by Singh et al. [30]. The LOQ was 0.06%, the precision at this level was < 10% R.S.D., whereas the intermediate precision was < 11% R.S.D. The alkylating impurity gave a linear response over the range LOQ to 0.3%, and the correlation coefficient was greater than 0.990. The accuracy of the method was in the range 91.7–106.8%.

3.3. Thin layer chromatography

There are a few publications from several decades ago describing the use of TLC to determine residual alkylating agents at low levels. In principle, this is a powerful generic technique; the entire sample is utilized, minimum clean up procedures are required and a wide range of specific derivatisation techniques are available [8]. In practice the need to accurately determine very low residual analyte levels does not support the general use of TLC. However, this technique is still used for the determination of related substances in the pharmacopoeial monographs for amiodarone, bromazepam, carmustine, ifosamide, indoramin and tolnaftate (see Table 1).

Sass and Stutz [31] used TLC to determine residual sulfur and nitrogen mustards (beta haloethyl compounds) in a variety of substrates. The authors demonstrated that sensitivities in the low microgram range were typically achievable.

3.4. Capillary electrophoresis (CE)

Hansen and Sheribah [32] evaluated a series of electrically driven separation techniques: capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and microemulsion micellar electrokinetic chromatography (MEEKC) for the determination of residual alkylating impurities in bromazepam API. Of the five named impurities (A–E); two were alkyl halides (impurities B: alkyl chloride and E: alkyl bromide). The focus of the investigations was primarily on selectivity, rather than sensitivity. The LOQ for all five impurities was 0.05%. Linearity was observed for all five impurities with correlation coefficients in excess of 0.985. The wider application of CE in relation to trace analysis is undoubtedly hindered by the poor sensitivity of the technique even where specialized detection cells (e.g. bubble or Z-cells) are used.

4. Discussion

The determination of very low levels of reactive organohalides and related alkylating agents as potential impurities in APIs presents very real analytical challenges. Historically, analysts have

tended to rely on the volatility of the alkylating agents and developed GC methodologies with FID but now are more routinely using sensitive and selective detection techniques such as GC/MS or ECD. HPLC, almost exclusively in reversed phase mode, remains a key separation technique for those analytes that are insufficiently volatile and/or too thermally labile for reliable GC analysis. While many historical references employ HPLC with single wavelength UV detection, the more recent reports employ a variety of MS techniques [8] due to the greater regulator focus on alkylating agents driving the need for lower limits of detection and quantitation. There are single literature references to the use of normal phase HPLC [23], SFC [20], ion chromatography (IC) [17] and CE techniques [32].

The need for specific and sensitive trace analytical methodologies combined with the chemical reactivities of the analytes concerned present analytical challenges not only in the selection of robust separation and detection techniques but also in sample preparation. This challenge will almost certainly be greatly enhanced should multiple, structurally related impurities need to be analysed and controlled.

Substrate interference, principally from the API, is often a restricting factor and extraction and/or pre-concentration techniques, such as HS and to a lesser extent liquid/liquid extraction, are used to reduce matrix interference in GC methodologies. The elegant and powerful combination of such techniques is routinely employed in many pharmaceutical Research and Development (R&D) laboratories as it offers the potential for generic methodologies capable of the trace analysis of a wide range of volatile analytes, as demonstrated by Skett [8] and Ellison [9]. Alzaga et al. [33] on the development of a generic approach to the trace determination of alkylating agents by *in situ* derivatisation (using pentafluorothiophenol) and HS/GC/MS, while exemplified by studies of a range of alkyl esters of sulfonic and sulphuric acids, should theoretically be applicable to reactive organohalides. RP HPLC methodologies also present challenges for sample preparation due instability of reactive analytes aqueous-based mobile phase systems. The application [18] of reduced energy mixing (vortexing vs. sonication) and reduced temperature storage of the analytes to provide appropriate stability to ensure analysis could be completed in a reasonable time frame. Ironically, the same chemical reactivity issue that bedevils the analytical procedures may also ensure that many of the PGIs are unlikely to survive intact the chemical synthetic clean up procedures used in API manufacture and even less likely to survive the biological environment.

Methodologies based on thin layer planar chromatography (TLC) separations followed by visualization either by UV or via use of an eclectic variety of specific chemical agents, would appear to be now restricted to use in pharmacopoeial monographs, is surely the legacy of a technologically simpler time and may be due to be superseded by more selective and sensitive methodologies. For instance the most recent 2007 BP/Ph Eur monograph (see Table 1) for amiodarone hydrochloride still describes a TLC method for the determination of the alkylating agent 2-chloro-*N*,*N*′-diethylethanamine (Impurity H) while the Lacroix et al. reversed phase HPLC/UV procedure [34] was published in 1994. Similarly the latest BP/Ph Eur monograph for tolnaftate (Table 1) describes a TLC procedure for the determination of *ortho*-napthalene-2-yl chlorothioformate (Impurity C), whereas a reversed phase HPLC procedures was published in 2000 by Vaidya et al. [35]. Similarly, the BP/Ph Eur monograph for bromazepam (Table 1) describes the TLC determination of the alkylating impurity B (a chloroacetamide) while Hansen and Sheribah [32] describe the CZE determination of impurity B plus its bromo analogue, impurity E, however the limited sensitivity of this methodology may have precluded its adoption.

Even in cases where pharmacopoeial monographs and literature references employ the same analytical techniques, subtle differences can exist. For instance, the BP/Ph Eur monograph for verapamil (Table 1) describes the reversed phase HPLC-UV determination of 16 process impurities including the alkylating agent 3-chloro-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-methylpropan-1-amine (Impurity D). Both Lacroix et al. [28] and Valvo et al. [29] describe similar RP HPLC-UV methodologies for the determination of 17 and 13 process impurities, respectively, plus the determination of the alkylating agent 2,4-dimethoxy-*N*-(3-chloropropyl)-*N*-methyl benzene ethanamine (Impurity VI). All three methods employ different column chemistries although at least they agree on the selection of 278 nm as detection wavelength.

In a few cases, differences exist in the spectrum of impurities determined. For instance, the BP/Ph Eur [36] and USP [37] monographs for famotidine API and medicinal drug products list, respectively, 7 and 4 related substances while the literature [22–24] describes the HPLC-UV determination of four impurities with alkylating functionality (Table 1) which are not listed in the monographs. Similarly, Lambropoulis et al. [38] describe the development and validation of an HPLC assay for fentanyl and its related substances in fentanyl citrate injection (USP) plus the determination of 8 related substances including the alkylating agent 2-bromoethylbenzene. 2-Bromoethylbenzene is not included in the lists of related substances/ordinary impurities cited in the relevant BP/Ph Eur [39] and USP monographs [40] for both the API and medicinal drug product and Lambropoulis et al. is silent on the reason for its inclusion in their studies.

In mitigation, the pharmacopoeias are legally constrained in their abilities to react and respond to changing scenarios impacting on the monographs (including newly published data). There is a well-established process for updating individual monographs which involves interactions with the Licensing Authorities. The latter defines the approvable levels of individual impurities, both established and new (including toxic impurities) based on clinical and pre-clinical data that is used to qualify each impurity. In addition, the Pharmacopoeias have to assess the robustness and general applicability of the proposed novel analytical methods, as new does not always mean better in routine use.

Following issuance of the Committee for Medicinal Products (CHMP) Guideline on the Limits of Genotoxic Impurities [41] it was not entirely clear as to how the guideline would influence the future development of pharmacopoeial monographs, in particular to the acceptable limits of those listed related substances that possess alkylating functionality. However, the European Directorate for the Quality of Medicines and Healthcare (EDQM) have recently identified the need to develop a policy for dealing with potentially genotoxic impurities (PGIs) that can be applied during elaboration and revision of monographs [42]. The group have stated that the European Pharmacopoeia needs to derive a pragmatic approach and that for existing monographs, in the absence of new study data demonstrating genotoxicity of an impurity; the existence of structural alerts alone is considered insufficient justification to trigger follow-up measures. The USP [43] have held similar discussions to discuss the issues of genotoxic impurities.

Industry also has had significant concerns with respect to the practical details of implementation and how national regulatory agencies are interpreting the CHMP guidelines (often demonstrating widely different perspectives) and have cautiously welcomed a Q&A discussion process as a mechanism which attempts to build common understanding and alignment [44]. In particular Industry has welcomed the clarification that TTC considerations have primacy over the somewhat ill-defined concept of ALARP (as low as reasonably practicable), as this will be invaluable in guiding analytical method development and impurity control strategies.

At this point it useful to reflect on the complex nature of many of the analyses described for the trace analysis of reactive organohalides. This has in many cases required the application of techniques that although common in an R&D environment are not well established in quality control laboratories and, possibly, in the laboratories of the worldwide regulatory agencies. Moreover, the techniques often require not only specialised equipment but also specialised expert analytical scientists with extensive knowledge of the techniques and procedures concerned. While most/all literature references include extensive method validation data, there are only a few reports [20,26] of inter-laboratory studies, the true test of the method robustness. It is therefore the view of the authors that the pharmaceutical industry will continue to face a significant challenge associated with the establishment of such approaches in a quality control environment and suggests that further research is required in order to increase our understanding of the issues associated with the transfer of such methods.

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