



Review

Analytical approaches for the detection of epoxides and hydroperoxides in active pharmaceutical ingredients, drug products and herbals

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ARTICLE INFO

Article history:

Received 16 July 2009

Received in revised form

23 November 2009

Accepted 23 November 2009

Available online 3 December 2009

Keywords:

Epoxide

Hydroperoxide

Genotoxic

Herbal

Chromatography

ABSTRACT

This review summarizes the analytical approaches reported in the literature relating to epoxide and hydroperoxide impurities. It is intended that it should provide guidance for analysts faced by the need to control such impurities, particularly where this is due to concerns relating to their potential genotoxicity.

An extensive search of the literature relating to this class of impurities revealed a large number of references relating to analysis of epoxides/hydroperoxides associated with herbal remedies. Given the general applicability of the analytical methodology and due to the widespread use of herbal products the authors decided to include herbal medicines in this review.

The review also reflects on the very different approaches taken in terms of the assessment/control of genotoxic impurities for such herbal remedies to that required for pharmaceutical products.

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1. Introduction

The EMEA guideline [1] relating to genotoxic impurities requires their control in new active pharmaceutical ingredients (APIs) and drug products. Paramount in the effective control of genotoxic impurities is the development of effective analytical methods. Such methods face major analytical challenges, which include sensitiv-

ity, selectivity, and matrix interference especially in the case of drug products. Previous reviews have explored how such issues have been addressed for sulfonate esters [2] and alkyl halides [3]. This review aims to summarize the analytical approaches reported in the literature relating to epoxide and hydroperoxide impurities, with a specific focus on those that are genotoxic (Table 1).

An extensive search of the literature relating to this class of impurities revealed a large number of references relating to analysis of epoxides/hydroperoxides associated with herbal remedies. Given the general applicability of the analytical methodology and due to the widespread use of herbal products the authors decided to include herbal medicines in this review. However, for herbal products it is often a moot point as to which components possess

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Table 1
Literature references and experimental details for analytical methods used to determine residual epoxides and hydroperoxides in API and herbal products.

Analyte	Impurities	Method details	Reference
Acebutolol	Impurity A: N-[3-acetyl]-4-[(2RS)-oxiran-2-ylmethoxy]phenyl butanamide	HPLC with a 5 µm octadecylsilyl stationary phase maintained at 40 °C, with a gradient mobile phase consisting of varying mixtures of A (phosphoric acid, triethylamine and distilled water) and B (acetonitrile (50%, v/v) and A (50%, v/v)). Flow rate 1.2 ml/min, λ 240 nm.	[39]
Almokalant	Epoxide	Residual volatile impurities concentrated LLE followed by on-column injection. GC with fused silica capillary (25 m × 0.32 mm) with a cross-linked methyl silicone (HP ultra-1) stationary phase with helium carrier gas (1.8 ml/min). The FID detector temperature was 290 °C, injector temperature varied between 180 and 230 °C. The oven was set at 40 °C for 3 min, then increased to 200 °C at 30 °C/min, then increased to 230 °C at 8 °C/min, then to 270 °C at 40 °C/min. The oven was held at 270 °C for 3 min.	[24,25]
Atenolol	Impurity C: 2-[4-[(2RS)-oxiran-2-yl]-methoxyphenyl] acetamide	HPLC with a 5 µm octadecylsilyl stationary phase, with a mobile phase consisting of sodium octane sulfonate (1 g) and tetrabutylammonium hydrogen sulfate (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.	[40]
Atorvastatin	Impurity 6: atorvastatin epoxy dihydroxy. Impurity 7: atorvastatin epoxy diketone	HPLC with a 1.8 µm octadecylsilyl stationary phase (Zorbax Eclipse XDB-C18), with a gradient mobile phase consisting of THF/acetonitrile/pH 3.5, 25 mM phosphate buffer. Flow rate 1.0 ml/min, λ 248 nm.	[13]
Benzyl and cumic alcohol	Hydroperoxide	HPLC with a 5 µm octadecylsilyl stationary phase (Waters Symmetry) and a gradient mobile phase consisting of varying concentrations of acetonitrile and water. λ 210 nm.	[5]
Betamethasone	Impurity E: 9,11-β-epoxy-17,21-dihydroxy-16β-methyl-9β-pregna-1,4-diene-3,20-dione	HPLC with a 5 µm octadecylsilyl stationary phase maintained at 45 °C, with a gradient mobile phase consisting of varying mixtures of A (acetonitrile and water) and B (acetonitrile). Flow rate 2.5 ml/min, λ 254 nm.	[41]
Betamethasone acetate	Impurity D: 9,11-β-epoxy-17-hydroxy-16β-methyl-9β-pregna-1,4-diene-3,20-dione-21-ylacetate	HPLC with a 5 µm octadecylsilyl stationary phase maintained at 45 °C, with a gradient mobile phase consisting of varying mixtures of A (acetonitrile and water) and B (acetonitrile). Flow rate 1.0 ml/min, λ 254 nm.	[42]
Betaxolol hydrochloride	Impurity C: 2-[[4-[2-(cyclopropylmethoxy)ethyl]phenoxy]methyl]oxirane Impurity D: 4-[2-(cyclopropylmethoxy)ethyl]phenol	HPLC with a 5 µm octadecylsilyl stationary phase maintained at 40 °C, with a mobile phase consisting of varying mixtures of acetonitrile (175 ml), methanol (175 ml), and pH 3.0 phosphate buffer (up to 1000 ml). Flow rate 1.5 ml/min, λ 273 nm.	[43]
Bicalutamide	Impurity VI: epoxide	HPLC with four different stationary phases. (1) 5 µm Symmetry C18, (2) 5 µm Inertsil ODS 3V, (3) 5 µm Kromasil KR100-5 C18 and (4) 5 µm BDS Hypersil C18. The mobile phase consisted of pH 3.0 phosphate buffer (10 mM) and acetonitrile (50/50, v/v). Flow rate 1.0 ml/min, λ 215 nm.	[32]
Carteolol	Impurity C: 5-[[2RS]-oxiranyl-2-yl]methoxy]3,4-dihydroquinolin-2-(1H)-one	HPLC with a 5 µm octadecylsilyl stationary phase, with a mobile phase consisting of methanol/acetonitrile/sodium hexane sulfonate (10/200/790, v/v/v). Flow rate 1.0 ml/min, λ 252 nm.	[44]
Celiprolol	Impurity G: 3-[3-acetyl]-4-[[RS]-oxiranyl]methoxy phenyl-1,1-diethylurea)	HPLC with a 5 µm octadecylsilyl stationary phase, with a mobile phase consisting of a mixture of tetrahydrofuran (91 ml), acetonitrile (63 ml), pentafluoropropanoic acid (0.6 ml), trifluoroacetic acid (0.2 ml) and distilled water (up to 1000 ml)). Flow rate 1.4 ml/min, λ 232 nm.	[45]
Chinese Herbal Medicine (<i>Angelica sinensis</i>) Danggui	Impurity 7: Z-6,7-epoxyligustilide	LLE extraction. GC with a fused silica (30 m × 0.25 mm) with 5% phenylmethylsiloxane stationary phase. The carrier gas was helium at a flow rate of 1.0 ml/min. The column was held at 50 °C, then programmed at 4 °C/min to 180 °C, then programmed at 20 °C/min to 300 °C. Detection by EI-MS in the scan range 50–550, with ionization energy of 70 eV. The inlet temperature was 320 °C and the ionization temperature was 300 °C.	[29]
Chinese Herbal Medicine (<i>Angelica sinensis</i>) Danggui	Impurity 7: Z-6,7-epoxyligustilide	LLE extraction. HPLC with 5 µm Symmetry with mobile phase consisting of pH 3.0 phosphate buffer (10 mM) and acetonitrile (50/50 v/v). Flow rate 1.0 ml/min, λ 215 nm.	[15]
Chinese Herbal Medicine (<i>Artemisia capillaries herba</i>)	<i>Trans</i> -Z-α-bisabolene epoxide and <i>cis</i> -Z-α-bisabolene epoxide	Residual volatile impurities concentrated by LLE followed by on-column injection. GC with a fused silica (30 m × 0.25 mm) with OV-101 stationary phase. The carrier gas was helium at a flow rate of 0.2 ml/min. The column was held at 50 °C for 6 min post-injection, then programmed at 8 °C/min to 250 °C, where the temperature was maintained for 5 min. Detection by EI-MS in the scan range 40–400, with ionization energy of 70 eV. The inlet temperature was 280 °C and the ionization temperature was 230 °C.	[26]

Chinese Herbal Medicine (<i>Attractylodes lancea</i>)	Caryophyllene oxide	Residual volatile impurities concentrated by both HS-SPME and MD-HS-SPME followed by on-column injection. GC with a fused silica (30 m × 0.25 mm) with HP-5MS stationary phase. The carrier gas was helium at a flow rate of 1.0 ml/min. The column was held at 40 °C for 1 min post-injection, then programmed at 8 °C/min to 160 °C, then programmed at 12 °C/min to 300 °C where the temperature was maintained for 5 min. Detection by EI-MS in the scan range 40–400, with ionization energy of 70 eV. The inlet temperature was 280 °C and the ionization temperature was 230 °C.	[28]
Chinese Herbal Medicine (<i>Atropa belladonna</i>)	Epoxide components: scopolamine	MEKC with fused silica capillary stationary phase (56 cm × 75 μm) at 25 °C and a mobile phase consisting of 30 mM borate/phosphate buffer (pH 8.7) with 40 mM sodium dodecyl sulfate and 16.5% acetonitrile. λ 195 nm. Samples injected hydrodynamically at a constant voltage of 30 kV (with an initial ramping at 0.5 kV/s).	[33]
Chinese Herbal Medicine (<i>Atropa belladonna</i>)	Epoxide components: scopolamine	CE with fused silica capillary stationary phase (85 cm × 50 μm) at 25 °C and a mobile phase consisting of 60 mM ammonium acetate buffer (pH 8.5) with 5% isopropanol. λ 195 nm. Samples injected hydrodynamically at a constant voltage of 30 kV. Detection by EI-ToF-MS in the scan range 50–800, with ionization energy of 70 eV.	[34]
Chinese Herbal Medicine (<i>Atropa belladonna</i> and <i>Datura innoxia</i>)	Epoxide components: scopolamine	SPE extraction. HPLC with 5 μm Phenomenex Luna C18 at 30 °C with a mobile phase consisting of acetonitrile/methanol/30 mM pH 6.0 phosphate buffer (12/7.9/80.1, v/v/v). Flow rate 1.0 ml/min, λ 190–400 nm.	[19]
Chinese Herbal Medicine (<i>Chan-Su</i>)	Epoxide components: resibufogenin, cinobufagin, and cinobufotalin	LLE extraction. HPLC with Zorbax Eclipse XDB-C18 with a gradient mobile phase consisting of 0.3% acetic acid in water/acetonitrile. Flow rate 0.7 ml/min, λ 190–400 nm and 296 nm.	[20]
Chinese Herbal Medicine (<i>Cimicifuga foetida</i> L)	Epoxide impurities: actein and 27-deoxyactein	SPE extraction. HPLC with 5 μm Hypersil ODS2 with a gradient mobile phase consisting of varying ratios methanol and water. Flow rate 0.8 ml/min, λ 200 nm, drift tube temperature of ELSD was 119 °C and the gas flow rate was 2.30 l/min.	[17]
Chinese Herbal Medicine (<i>Datura stramonium</i> L)	Epoxide components: scopolamine, scopolamine-N-oxide, scopolamine-N-methyl bromide	Residual volatile impurities concentrated by SPE. HPLC with 5 μm Waters Xterra C18 at 25 °C with a gradient mobile phase consisting of varying mixtures of acetonitrile and 15 mM ammonium hydroxide. Flow rate 0.8 ml/min, λ 205 nm.	[31]
Chinese Herbal Medicine (<i>Datura stramonium</i> L)	Epoxide components: scopolamine, scopolamine-N-oxide, scopolamine-N-methyl bromide	Residual volatile impurities concentrated by SPE. HPTLC silica gel 60 F ₂₅₄ plates with mobile phase (i) acetone/methanol/water/25% ammonia (82/5/5/8, v/v/v) for scopolamine and hyoscyamine and (ii) acetonitrile/methanol/85% formic acid (120/5/5, v/v/v) for scopolamine-N-oxide and scopolamine-N-methyl bromide.	[31]
Chinese Herbal Medicine (<i>Datura candida</i> and <i>aurea</i>)	Epoxide components: scopolamine	Residual volatile impurities concentrated by SPE. CZE with fused silica capillary stationary phase (22 cm × 50 μm) at 15 °C and a mobile phase consisting of pH 8.5 40 mM ammonium acetate buffer. λ 200 nm. Samples injected hydrodynamically at a constant voltage of 20 kV (with an initial ramping at 2.5 kV/s). Detection by EI-MS in the scan range 120–400, with ionization energy of 70 eV.	[33]
Chinese Herbal Medicine (<i>Evodia rutaecarpa</i> . Fructus <i>Evodiae</i> (Wuzhuyu))	Epoxide components: limonin	LLE extraction at 70 °C for 1 h in chloroform/ammonia. HPLC with Zorbax SB-C18 (3 μm) at 25 °C with a gradient mobile phase consisting of mixtures of acetonitrile and water. Flow rate between 0.6 and 1.1 ml/min, λ 215, 220, 225, 255 and 345 nm.	[22]
Chinese Herbal Medicine (<i>Hyoscyamus muticus</i> L)	Epoxide components: scopolamine	CZE with fused silica capillary stationary phase (60 cm × 75 μm) at 25 °C and a mobile phase consisting of 40 mM phosphate buffer (pH 7.8). λ 214 nm. Samples injected hydrodynamically at a constant voltage of 20 kV.	[36]
Chinese Herbal Medicine (<i>Hypericum perforatum</i> L)	Hydroperoxide impurities: furohyperforin isomer A hydroperoxide, furohyperforin isomer B hydroperoxide, oxepaferin hydroperoxide, furohyperin hydroperoxide and two un-named epoxide intermediates	Residual volatile impurities concentrated by SFC. HPLC with 5 μm Zorbax SB-C18 with a gradient mobile phase consisting of 0.01% TFA in water and 0.01% TFA in acetonitrile. Flow rate 1.0 ml/min, λ 190–400 nm.	[18]
Chinese Herbal Medicine (<i>Mercurialis perfoliata</i>)	Epoxide impurities: ledene oxide, caryophyllene oxide	Residual volatile impurities concentrated by LLE followed by on-column injection. GC with a fused silica (30 m × 0.25 mm) with HP-5 stationary phase. The carrier gas was helium at a flow rate of 1.0 ml/min. The column was held at 50 °C post-injection for 5 min, then programmed at 5 °C/min to 250 °C. Detection by EI-MS in the scan range 40–540, with ionization energy of 70 eV. Inlet temperature 250 °C, detector temperature 260 °C.	[9]

Table 1 (Continued)

Analyte	Impurities	Method details	Reference
Chinese Herbal Medicine (<i>Notopterygium incium</i>)	<i>Trans</i> -2 α -bisabolene epoxide and <i>trans</i> -Z- α -bisabolene epoxide	Residual volatile impurities concentrated by LLE followed by on-column injection. GC with a fused silica (30 m \times 0.25 mm) with OV-101 stationary phase. The carrier gas was helium at a flow rate of 0.2 ml/min. The column was held at 50 °C post-injection, then programmed at 8 °C/min to 250 °C, where the temperature was maintained for 5 min. Detection by EI-MS in the scan range 40–400, with ionization energy of 70 eV. The inlet temperature was 280 °C and the ionization temperature was 230 °C.	[27]
Chinese Herbal Medicine (<i>Tripterygium wilfordii</i>)	Epoxide impurity: triptolide	SPE extraction. HPLC with 5 μ m Phenomenex Curosil PFP (Pentafluorophenyl) with a gradient mobile phase consisting of water and acetonitrile. Flow rate 1.0 ml/min, λ 219 nm.	[16]
Dexamethasone	Impurity E: DOB-MN	HPLC with a 4 μ m octadecylsilyl stationary phase (Waters YMC ODS H-80) maintained at 25 °C, with a gradient mobile phase consisting of varying mixtures of mobile phase A (acetonitrile/water/phosphoric acid; 100/900/0.5, v/v/v) and mobile phase B (acetonitrile/water/phosphoric acid; 900/100/0.5, v/v/v). Flow rate 1.4 ml/min increasing to 2.0 ml/min after 70 min, λ 240 nm.	[7]
Fluocinolone acetonide	Impurity E: 9,11- β -epoxy-6 α -fluoro-21-hydroxy-16 α ,17-(1-methylethylidenedioxy)-9 β -pregna-1,4-diene-3,20-dione	HPLC with a 5 μ m base deactivated, end capped octadecylsilyl stationary phase, with a mobile phase consisting of a mixture of acetonitrile (450 ml) and water (made up to 1000 ml). Flow rate 1.0 ml/min, λ 238 nm.	[46]
Loteprednol etabonate	Chloromethyl-17 α -ethoxycarbonyloxy-1 β ,11 β -epoxy-2-oxo-10 α -androsta-4-ene-17 β -carboxylate	HPLC with 5 μ m phenyl silica stationary phase (Altima Phenyl), with a mobile phase consisting of water/acetonitrile/acetic acid (570/425/5, v/v/v). Flow rate 1.8 ml/min, λ 244 nm.	[8]
16 α -Methylepoxyde in corticosteroids	16 β -Methylepoxyde in corticosteroids	HPLC with a octadecylsilyl stationary phase (YMC Hydrosphere) at 30 °C with a gradient mobile phase consisting of varying mixtures of A (water) and B (acetonitrile/i-propanol (50/50, v/v). Run time 10 min, λ 254 nm.	[9]
Mometasone furoate	Impurity D: 21-chloro-9,11- β -epoxy-16 α -methylpregna-1,4-diene-17-yl-3,20-dione furan-2-carboxylate	HPLC with a 5 μ m octadecylsilyl stationary phase with a mobile phase consisting of a mixture of acetonitrile (500 ml) and water (500 ml). Flow rate 1.0 ml/min, λ 254 nm.	[47]
Mupirocin	Impurities A: pseudomonic acid B, C: pseudomonic acid D and F: 7-[[2E]-4-[(2S,3R,4R,5S)-5-[(2S,3S,4S,5S)-2,3-epoxy-5-hydroxy-4-methylhexyl]-3,4-dihydroxy-3,4,5,6-tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]heptanoic acid).	HPLC with a 5 μ m octylsilyl stationary phase, and a mobile phase consisting of a mixture of distilled water (190 ml), tetrahydrofuran (320 ml) and pH 5.7 acetate buffer (490 ml). Flow rate 1.0 ml/min, λ 230 nm.	[48]
Nadalol	Impurity III: epoxide	HPLC with a 3 μ m nitrile stationary phase (Hypersil ODS), with a mobile phase consisting of a mixture of pH 3.4 acetate buffer and acetonitrile (900/100, v/v). Flow rate 1.5 ml/min, λ 219 nm.	[10]
Propafenone	Impurity C: 1-2-[[RS]-oxiranyl]methoxy phenyl-3-phenylpropan-1-one.	HPLC with a 5 μ m end capped octadecylsilyl stationary phase, with a gradient mobile phase consisting of varying mixtures of pH 2.5 phosphate buffer and acetonitrile. Flow rate 1.0 ml/min, λ 220 nm.	[49]
RG12915	Impurity III: hydroperoxide of RG12915	HPLC with a 10 μ m octadecylsilyl stationary phase (Waters μ Bondapak) with a mobile phase consisting of acetonitrile/water/trifluoroacetic acid (400/600/1, v/v/v) Flow rate 1.0 ml/min, λ 216 nm (600 μ g/ml) then λ 230 nm (800 μ g/ml).	[12]
Stavudine	Cyclic hydroperoxide (1-(3',5'-anhydro-2'-deoxy- β -D-threo-pentafuranosyl) thymine	HPLC with a 5 μ m octadecylsilyl stationary phase (R) with a gradient mobile phase consisting of A: acetonitrile/acetate buffer (0.77 g/l) (35/965, v/v) and B: acetonitrile/acetate buffer (0.77 g/l) (250/750, v/v). Flow rate 2.0 ml/min, λ 254 nm.	[50]
Stavudine	Cyclic hydroperoxide (1-(3',5'-anhydro-2'-deoxy- β -D-threo-pentafuranosyl) thymine	HPLC with a 5 μ m silyl stationary phase (Zorbax Rx-Sil) with a mobile phase consisting of acetonitrile/pH 8.0 acetate buffer (160/840, v/v). Flow rate 0.3 ml/min, λ 225, 275 and 350 nm.	[14]
Traditional Herbal Medicine (<i>Carapa guianensis</i> seed oil)	Epoxides: gedunin, 7-oxo-7-deacetoxy gedunin and 6 α -acetoxygedunin	HPLC with a 5 μ m octadecylsilyl stationary phase (Hibar Lichrospher) with a gradient mobile phase consisting of acetonitrile/water/methanol (35/35/30, v/v/v). Flow rate 1.0 ml/min, λ 210 nm.	[23]
Vincristine sulfate	Impurity F: leurosine, G: formylleurosine	HPLC with a 5 μ m octadecylsilyl (Zorbax Rx-Sil) stationary phase and a gradient mobile phase consisting of varying mixtures of pH 7.5 phosphate buffer and methanol. Flow rate 2.0 ml/min, λ 297 nm.	[51]

Vitamin A (retinoic acid)	All- <i>trans</i> -5,6-epoxy retinoic acid, 13- <i>cis</i> -5,6-epoxy retinoic acid	HPLC with a 5 μm silyl (Zorbax Rx-Sil) stationary phase and a mobile phase consisting of heptane/THF/acetic acid (96.5/3.5/0.015, v/v/v). Flow rate 1.4 ml/min. EI-MS at 77 eV, scanning 200–350 eV (+ve ion), 1 s/scan, 1200 V, multiplier voltage and 300 °C, ion source temperature.	[38]
Vitamin A (retinoic acid)	All- <i>trans</i> -5,6-epoxy retinoic acid, 13- <i>cis</i> -5,6-epoxy retinoic acid	CZE with fused silica capillary stationary phase (50 cm × 50 μm) at 30 °C and a mobile phase consisting of (1) acetonitrile modified pH 8.5 borate buffer or (2) acetonitrile and α-cyclodextrin modified pH 8.5 borate buffer. λ, 345 nm. Samples injected hydrodynamically at a constant voltage of 30 kV.	[32]
Vitamin A (retinoic acid)	All- <i>trans</i> -5,6-epoxy retinoic acid, 13- <i>cis</i> -5,6-epoxy retinoic acid	MEKC with fused silica capillary stationary phase (100 cm × 50 μm) at 30 °C and a mobile phase consisting of SDS modified pH 8.5 borate buffer. λ, 345 nm. Samples injected hydrodynamically at a constant voltage of 30 kV.	[32]
Vitamin D3	Hydroperoxide	HPLC with a 5 μm octadecylsilyl stationary phase (Nertsil ODS-3) and a mobile phase consisting of 100% acetonitrile. Flow rate 1.0 ml/min, λ, 265 nm.	[5]

Abbreviations: GC (gas chromatography), HPLC (liquid chromatography), HPTLC (high performance thin layer chromatography) and MEKC (micellar electro kinetic chromatography), MS (mass spectrometry), ELSA (evaporative light scattering detection), APCJ (atmospheric pressure chemical ionization), SRM (selective reaction monitoring), NSAD (non-steroidal anti-inflammatory), λ (detection wavelength), LLE (liquid liquid extraction), SPE (solid-phase extraction), HS-SPME (headspace solid-phase microextraction), HSME (headspace solvent microextraction)

relevant pharmacological activity, and which could be considered as impurities that possess, for example, inappropriate pharmacological activity, no activity or even toxic potential. In the case of epoxide and hydroperoxide impurities this may even relate to potential genotoxicity.

2. Epoxides, hydroperoxides

Epoxide-containing substances from both natural and man-made sources are ubiquitous in the environment and food supply, as well as being generated endogenously. Epoxides are electrophilic, this property being related to the chemical reactivity of the strained epoxide ring. The reactivity of the epoxide and hydroperoxide groups makes them particularly useful as alkylating reagents. Short chain aliphatic epoxides, in particular, are commonly used in synthetic processes, e.g., ethylene oxide, propylene oxide, epoxybutane, styrene oxide, and epichlorohydrin. A consequence of this chemical reactivity is that many aliphatic and aromatic epoxides are mutagenic.

Oxidation is a common degradation pathway in liquid and solid formulations. Peroxyl radicals can add to alkenes either intra- or inter-molecularly, leading to the formation of hydroperoxides. However, these degradation investigations are often mechanistically focused, with limited analytical information available.

2.1. Impurity fate mapping and risk assessment

As described, epoxides and hydroperoxides are highly reactive, and this intrinsic reactivity can aid in their decomposition and elimination in API manufacturing process. However, little is reported in the literature relating to this. Dobo et al. [4] reported on the impurity fate mapping of an epoxide impurity, demonstrating that the chemical reactivity of these intermediates can be often utilized to develop control strategies.

The authors described a seven-stage synthesis in which 1.5 equiv. of an epoxide, ((*R*)-(–)glycidyl methyl ether), was reacted with an amine to form the free base of the API, which was then reacted with a sulfonic acid to form the mesylate salt. The epoxide was Ames-positive and hence human exposure needed to be minimized. Concerns existed over the potential carryover of the epoxide into the API, particularly as there was only one workup stage after the above reaction. Although this work up utilized slightly acidic conditions, there was concern that this may not fully hydrolyze any residual epoxide. The paper reported that appropriate control was achieved through the process described.

3. Analytical approaches to trace analysis

Unlike alkyl halides where there has been a general renaissance in the area of high resolution gas chromatography (GC), high performance liquid chromatography (HPLC) appears to be the most prevalent analytical technique utilized for epoxides and hydroperoxides. This is seen for new chemical entities (NCEs), for commercial drugs with established pharmacopoeial monographs and for herbal medicines.

In terms of detection, the need to control levels of genotoxic impurities to low (ppm) levels has generally necessitated the selection of MS as the detection mode of choice. 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 why the many older literature references (and pharmacopoeial monographs in particular) generally used single wavelength UV detection.

Perhaps, somewhat unsurprisingly, there are few reports in the literature of the use of supercritical fluid chromatogra-

phy (SFC) or capillary zone electrophoresis (CZE) or the related electro-chromatography techniques. Notable exceptions are herbal products where CZE, often coupled with MS detection, is used. Given our earlier findings for alkylating agents [3], it is somewhat surprising that none of the pharmacopoeial monographs reviewed used older chromatographic techniques such as thin layer chromatography (TLC).

3.1. High performance liquid chromatography (HPLC)

A number of applications relating to analysis of epoxides/hydroperoxides by HPLC are described in the literature.

Researchers from Merck used a novel peroxy based radical system to assess the oxidative stability of 7 known and 10 developmental compounds [5]. The authors monitored levels of hydroperoxide formation in their novel radical system (a mixture of 10% Tween 80 and 10 mM Fe (III)) using a previously reported HPLC method [6]. They monitored the formation of triphenyl phosphine oxide from the starting material, triphenyl phosphine. The limit of quantitation for the hydroperoxide was about 1 μ M.

Interestingly, Harmon et al. [5] described an HPLC-related substances method for the determination of a hydroperoxide degradation product. In this case rather than a degradation product of the API it was instead a degradation product of vitamin D3 (present as an excipient in the formulated product). The same authors also described a related substances method for the determination of hydroperoxide degradation products of benzyl and cumic alcohol with drug product (they being present as excipients in the drug product).

Spangler and Mularz [7] reported on the development of a stability-indicating method for dexamethasone and resolution of some of its related products, including 9,11-epoxy dexamethasone (impurity E: DOB-MN). The method employed was a simple HPLC method with no sample pre-treatment. The recovery of the epoxide was reported to be 82% at the LOQ value (0.05%, w/w in API and 0.1%, w/w in drug product). Equivalent recoveries were achieved from various matrices, including API, powder blend and an intravenous solution.

Yasueda et al. [8] described an HPLC method for the determination of loteprednol impurities, including that of a minor photolytic epoxide degradation product in loteprednol etabonate API.

Xiao et al. [9] recently reported on the separation of an epimeric pair of steroidal epoxides (16 α -methyl epoxide and 16 β -methyl epoxide), which are key intermediates for the corticosteroids, betamethasone and dexamethasone. They developed a fast HPLC method that separated the epimers with a resolution factor >3.0 and which had an LOQ of 0.0001% w/w (1 ppm) for the unwanted epimer.

An HPLC method for the determination of related substances, including the epoxide impurity (III), of the β -blocking drug, nadalol, was reported by Lacroix et al. [10]. They found that the method was very sensitive to changes in pH of the mobile phase, at pH 3.6 the epoxide impurity (III) co-eluted with nadalol.

Rao et al. [11] recently reported the isolation and characterization of process-related impurities and degradation products of the non-steroidal anti-androgen compound, bicalutamide. The HPLC method they developed was shown to resolve six related compounds of bicalutamide, including an epoxide synthetic intermediate. The resolution of the epoxide was found to be quality critical in terms of method system suitability and thus it was defined as a system suitability test for the method. The LOD was 0.138 μ g/ml and the LOQ was 0.41 μ g/ml.

Won et al. [12] reported on the photolytic and oxidative degradation of the antiemetic agent, RG12915. Using HPLC with diode array detection, they followed the degradation kinetics of RG12915,

focusing on the initial generation and subsequent disappearance of a hydroperoxide impurity.

A rapid resolution HPLC method was used to separate and quantify related impurities of atorvastatin, including two epoxide impurities; atorvastatin epoxy dihydroxy and atorvastatin epoxy diketone [13]. The authors used an extra densely bonded, double end capped stationary phase (Eclipse XDB-C18) to provide high chromatographic resolution in a short time frame. The two epoxide impurities were well resolved from each other and from atorvastatin. The LOD and LOQ for atorvastatin epoxy dihydroxy were 0.025 and 0.075 μ g/ml, respectively; whereas, the LOD and LOQ for atorvastatin epoxy diketone were 0.026 and 0.077 μ g/ml, respectively. The method was determined to be robust towards reasonable changes in chromatographic parameters using a chemometric-based central composite design (response surface methodology).

Most of those methods described have been simple reversed phase methods employing direct analysis (no sample preparation). However not all attempts to apply such approaches to the analysis of epoxides/hydroperoxides have been successful. Kažoka and Madre [14] were unsuccessful in their attempts to resolve the antiviral agent stavudine from four of its process impurities, including a cyclic hydroperoxide using reversed phase liquid chromatography. However, using mixed partition/adsorption chromatography under normal-phase conditions they achieved adequate resolution (α) between the cyclic hydroperoxide and stavudine ($\alpha = 1.62$ – 1.90) and the cyclic hydroperoxide and another impurity ($\alpha = 1.80$ – 2.10) using various silica stationary phases (Lichrospher Si-60, Kromasil 60-5-Si, Silasorb 600, Lichrosorb Si-60, Zorbax SIL, Zorbax Rx-SIL and Supelcosil LC-Si). The authors commented that the main difference from classical normal-phase chromatography was the use of a mixed phase where there was limited solubility of the two mobile phase components (ethyl acetate partially saturated with ethylene glycol).

The following examples highlight the use of sample pre-treatment in the analysis of herbal medicines, due to the complexity of the matrix.

Brinker and Raskin [16] employed sample pre-treatment when they assessed the residual levels of the epoxide diterpenoid (triptolide) in root extracts from Thunder God vine (*Tripterygium wilfordii*), a popular Chinese herbal medicine. They used solid-phase extraction (SPE) from an aminopropyl column followed by HPLC with UV detection. Triptolide was well resolved from 10 other analytes. The sensitivity of the method was good with LOD and LOQ of 0.028 and 0.094 ng, respectively. The levels of triptolide were dependent on the age of the roots. Levels ranged from 2 ng triptolide/g to 152 ng triptolide/g on a dry weight basis for the root samples.

Kong et al. [17] reported on the determination of two epoxide terpenoid impurities (actein and 27-deoxyactein) in a traditional Chinese herbal preparation (*Cimicifuga foetida* L). They compared HPLC with evaporative light scattering detection (ELSD) with UV detection and found that the ELSD was significantly more sensitive. Using UV detection the on-column sensitivity for the two epoxides was 606 and 880 ng, respectively; in contrast the sensitivity using ELSD was 40 and 33 ng, respectively. Using the optimized extraction procedure (methanol/water, 80/20, v/v) they detected levels of the two analytes to be $3.44 \pm 0.02\%$ and $1.42 \pm 0.01\%$, respectively, in herbal medicines obtained from Huan province in China.

An Austrian research group [18] used supercritical fluid extraction techniques to concentrate lipophilic plant constituents from St. John's wort (*Hypericum perforatum* L) that were subsequently identified by HPLC-MS and GC-MS. In addition to the principal phloroglucinols, hyperforin (36.5%) and adhyperforin (4.6%), there was a proliferation of oxidation products. These oxidative impurities were furohyperforin isomer A hydroperoxide, furohyperforin

isomer B hydroperoxide, oxepaferin hydroperoxide, furohyperin hydroperoxide and two un-named epoxide intermediates.

Kursinszki et al. [19] used SPE and HPLC to determine levels of the epoxy-alkaloid scopolamine and its related tropane alkaloids; hyoscyamine, 6 β -hydroxyhyoscyamine and apoatropine in Solanaceous plant species (*Atropa belladonna* and *Duboisia innoxia*). They indicated that the advent of the newer high purity (type B) stationary phases (e.g. Phenomenex Luna) had resulted in excellent peak shapes compared to the older stationary phases and that the simplicity and improved selectivity made the developed method a preferred alternative to the previously published ion-pair chromatographic methods. The method gave excellent LODs of 0.8 ng for scopolamine and 0.6 ng for the other analytes. The method showed very disparate levels of scopolamine in various plant extracts. Three different samples of *Duboisia innoxia* gave levels between 1440 and 1890 $\mu\text{g/g}$; whereas, *A. belladonna* gave much lower levels in the range 590–675 $\mu\text{g/g}$. The implications of these differences were not articulated.

In those cases where there is awareness of the toxicity of traditional Chinese medicines, the pharmacopoeia tends to control their quality. Chan-Su (toad venom), which is used for the treatment of serious liver and gastric cancers, is known to contain bufadienolides, which are potent cytotoxic compounds. Ye et al. [20] used HPLC–MS–MS to unambiguously characterize methanol extracts of Chan-Su for 12 bufadienolides, of which 6 were epoxides (resibufogenin, cinobufagin, bufotalin, 3-oxo-cinobufotalin, cinobufotalin and 19-oxo-cinobufagin). In parallel, they used the HPLC method with diode array detection to quantify eight bufadienolides, of which three were epoxides (resibufogenin, cinobufagin, and cinobufotalin) over a 30-min analysis period. LODs in the range 0.78–1.75 ng and LOQs in the range 3.68–4.96 ng were reported.

The authors used the method to quantify three samples of Chan-Su from Beijing and Zhejiang as well as an official reference sample from the China Institute for Control of Pharmaceutical and Biological Products (CICBP). The inter-sample variability of individual epoxide constituents was marked. Resibufogenin ranged from 15.1 to 22.1% (versus CICBP reference standard of 24.6%), that of cinobufagin from 32.0 to 34.7% (versus CICBP reference standard of 27.8%) and of cinobufotalin from 10.4 to 14.0% (versus CICBP reference standard of 11.6%). The only official guidance is a monograph in the Chinese Pharmacopoeia, which states total levels of resibufogenin and cinobufagin to be >6% [21]. Another Chinese research group [22] also evaluated levels of the epoxide component, limonin (as well as four other signature analytes: wuchuyamide, evodiamine and rutaecarpine [alkaloids] and quercetin [flavonoid]) in 36 batches of *Evodia rutaecarpa*, collected at different times or from different regions of China. They used reversed phase HPLC and diode array detection at five wavelengths to assay these analytes. The LOD for limonin was 1.98 ng and the LOQ was 6.20 ng.

The anti-inflammatory properties of the traditional herbal remedy, andiroba oil extracted from the seeds of *Carapa guianensis*, have long been known about in Brazil. Tappin et al. [23] recently reported on the analysis of some of the constituent tetranortriterpenoids, including the three epoxide analytes (gedunin, 7-oxo-7-deacetoxy gedunin and 6 α -acetoxygedunin). The constituent tetranortriterpenoids were concentrated by SPE using a silica gel 60 column and eluted with chloroform/acetonitrile (95/5, v/v) and analyzed using reversed phase liquid chromatography. The method was optimized for selectivity using a statistically based approach. The LOD and LOQ values were based on standard error of intercept determined by regression analysis and were 0.3 g/ml and 0.93 g/ml, respectively. The total amounts of tetranortriterpenoids in the oil were 7.07 mg/g (0.71%), with the individual components have the following levels: gedunin (1.62 mg/g; 0.16%), 7-oxo-7-deacetoxy gedunin (2.48 mg/g; 0.25%), 6 α -acetoxygedunin (1.82 mg/g; 0.18%) and methyl angolensate (1.15 mg/g; 0.12%).

3.2. Gas liquid chromatography (GC)

As outlined in Section 1, the number of literature references relating to the application of GC to the analysis of epoxides/hydroperoxides is significantly lower than that seen for other genotoxic impurity classes, e.g., sulfonate esters/alkyl halides. This is perhaps indicative of the wide variability seen within this class from the perspective of size of molecule and hence volatility, with only a moderate proportion of such compounds being amenable to analysis by GC.

Klick [24,25] reported on the development of a GC method for the determination of residual levels of a chlorohydrin and the corresponding epoxide impurities in almokalant. In another report, the same author observed that on-column injection gave the best results in terms of both precision and sensitivity. The LOD and LOQ for the epoxide were 0.09 and 0.29 ppm ($\mu\text{g/g}$), respectively. On-column formation of the epoxide from the chlorohydrin was slightly ameliorated by the use of high initial column pressure.

Guo et al. [26,27] used GC–MS and correlative chemometric resolution methods to analyze the volatile components in traditional Chinese herbal medicines (*Notoptergium incium* and *Artesima capillaris herba*). For *Notoptergium* [26], 65 of the 98 analytes that were resolved could be identified by spectral similarity searches. Two of the identified analytes were epoxides; *trans*-2- α -bisabolene epoxide and *trans*-Z- α -bisabolene epoxide, and their relative contents were 1.9 and 0.8%, respectively. Similarly, for *Artesima* [27], 42 of the 75 analytes that were resolved could be identified by spectral similarity searches. Two of the identified analytes were epoxides; *trans*-Z- α -bisabolene epoxide and *cis*-Z- α -bisabolene epoxide, and their relative contents were 4.4 and 0.5%, respectively.

Li et al. [28] used GC–MS following microwave distillation and headspace solid-phase microextraction (MD–HS–SPME) for the fast analysis of essential oils present in a traditional Chinese medicine (*Attractylodes lancea*). The authors compared the recoveries of different analytes extracted from *Attractylodes lancea* using the standard HS–SPME method (headspace solid-phase microextraction) and the novel MD–HS–SPME method. The levels of the epoxide, caryophyllene oxide was similar using the two methods (0.56% versus 0.86% for MD–HS–SPME and HS–SPME methods, respectively). The method was partly validated, but only for the five major analytes, not including caryophyllene oxide.

A Chinese research group [29] also analyzed the volatile components in the traditional Chinese herbal medicine, Danggui (*Angelica sinensis*) by GC–EI–MS. They addressed the instability of Z-ligustilide by performing the analysis within a 12-h period. The method was validated for the principal components. The levels of the various phthalides including Z-6,7-epoxyligustilide in Danggui from various sources were reported. *Angelica acutiloba* and *Angelica gigas* are commonly used as “substitutes” in Danggui products due to the shortages of *Angelica sinensis* in southeast Asian markets. The levels of the various phthalides including Z-6,7-epoxyligustilide in Danggui from these various alternative sources were also reported. Levels of the epoxide impurity (Z-6,7-epoxyligustilide) were fairly consistent in the Chinese Danggui samples (2.06–3.24%), but were either absent or present at 2–3-fold higher levels (6.83%) in alternative Danggui samples. The authors indicated that the clinical efficacy of these different Danggui medicines had not been ascertained.

3.3. High performance thin layer chromatography (HPTLC) and thin layer chromatography (TLC)

Although, quantitative TLC is not usually utilized there are several examples of its use in association with determining levels of the epoxy alkaloid, scopolamine in extracts of *Datura stramonium*. The LOD was reported to be between 35 and 50 $\mu\text{g/ml}$ [30].

Mroczek et al. [31] used SPE and cation-exchange solid-phase extraction (CESPE) to extract the epoxide scopolamine (and related tropane alkaloids) from *D. stramonium* and related species. The optimum extraction procedure was using hot 1% tartaric acid solution in methanol, followed by purification with SPE on Oasis MCX cartridges. The authors tested extracts from a broad range of different *Datura* species and varieties (14 leaf and seed samples). They showed good correlations between the HPTLC (preferred for its versatility, simplicity and low cost) and reference HPLC method. Not unexpectedly the HPLC method was more sensitive than the corresponding HPTLC method.

3.4. Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC)

To date there are no reported applications based on CZE/MEKC relating to pharmaceutical analysis of epoxides/hydroperoxides, however the power of these techniques in terms of selectivity has been utilized in the analysis of complex herbal products. Bempong et al. [32] reported on the separation of 13-*cis* and all-*trans*-retinoic acid and their photo-degradation products (including all-*trans*-5,6-epoxy retinoic acid, 13-*cis*-5,6-epoxy retinoic acid) using both capillary zone electrophoresis (CZE) and electrokinetic chromatography (MEKC). The authors described the effect of buffer concentration, pH, modifier type, modifier concentration and capillary length on the resolution of the degradation products.

CZE with UV detection and interfaced with EI-MS in the single ion mode (SIM) was developed to determine residual scopolamine, and the related tropane alkaloid hyoscyamine in *Datura candida* and *Datura aurea* root extracts [33]. Using UV detection, the LOD for the tropane alkaloids was established as 1 µg/ml; however, using SIM the sensitivity could be increased by a factor of between 3- and 4-fold and the LOD of scopolamine was 1 ng/ml and the LOD for hyoscyamine was 0.1 ng/ml. The authors used this optimized method to quantify levels of both analytes in root extracts.

CZE with UV detection and interfaced with EI-MS in the single ion mode (SIM) was developed to determine residual scopolamine, and the related tropane alkaloid hyoscyamine in *A. belladonna* leaf extracts [33]. Using UV detection, the LOD for the tropane alkaloids was established as 1 µg/ml; however, using SIM the sensitivity could be increased by a factor of between 3- and 4-fold and the LOD of scopolamine was 1 ng/ml and the LOD for hyoscyamine was 0.1 ng/ml. The authors used this optimized method to quantify levels of both analytes in root extracts.

A modification to this original method was proposed by Arráez-Román et al. [34]. The authors added organic modifiers to the separation buffer and used CZE–EI-ToF-MS (ToF, Time of Flight) to characterize the tropane alkaloids in *A. belladonna* leaf extracts. MEKC with UV detection was developed to determine residual scopolamine, and the related tropane alkaloid hyoscyamine in *A. belladonna* leaf extracts [35]. The method was optimized using a design of experiment approach (Doehlert). Using UV detection, the LOD for the tropane alkaloids was 1 µg/ml and the LOQ was 3-fold higher (3 µg/ml). CZE with UV detection [36] was also used to determine residual scopolamine in transgenic Egyptian henbane (*Hyoscyamus muticus*). The LOD was ca. 1.5 µg/ml. Both samples of transgenic Egyptian henbane gave levels of scopolamine at or slightly above the LOD.

4. Conclusion

HPLC, particularly 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. It is clear that in terms of epoxides and hydroperoxides many of the analytes described in the literature fall into such categories. Interestingly, many of literature reports employed HPLC with either single or dual wavelength UV detection, or diode array UV detection. There were surprisingly few reported usages of HPLC–MS. This is despite the recently increased regulatory focus on residual alkylating agents driving the need for lower limits of detection and quantitation.

A variety of extraction and/or pre-concentration techniques, which are designed to improve matrix interference prior to HPLC analysis, were routinely used for traditional Chinese herbal medicines.

GC, whilst not seeing the same scale of renaissance as was observed in our recent review of alkyl halides [3], is still well represented. GC-FID [24,25], and GC–EI-MS [9,26–29] are the typical analytical approaches. As with HPLC, a variety of extraction and/or pre-concentration techniques, which are designed to improve matrix interference prior to GC analysis, were routinely used for traditional Chinese herbal medicines. CZE was also represented in this survey. The typical sensitivity deficiencies of CE have been addressed by hyphenated MS approaches [32,35,36], including CE–EI-ToF-MS [34]. Extraction and/or pre-concentration techniques were less routinely applied [35], presumably because of enhanced selectivity of CE compared to other separation techniques. There were also examples of the related technique of MEKC–EI-MS [32,33] used for both traditional Chinese herbal medicines and standard medicinal products.

Methodologies based on thin layer and high performance thin layer chromatography (TLC and HPTLC) followed by visualization, were not used in any of the Pharmacopoeial monographs reviewed. In contrast, their use as an adjunct to other chromatography techniques was reported in conjunction with the quantitative analysis of traditional Chinese herbal medicines [30,31].

As we remarked in our earlier review [3], some differences were seen between Pharmacopoeial monographs and literature references. For instance, the latest Pharmacopoeial monographs for stavudine does not control the cyclic hydroperoxide reported by Kažoka and Madre [14].

One striking observation is that unlike the alkyl halides and sulfonate esters, there are no reports of generic methods applicable to a range of epoxides/hydroperoxides having been developed. This is certainly an area, particularly in relation to low molecular weight epoxides commonly used in synthetic processes that the authors believe should be addressed.

A fairly common observation is the variability of levels of both active constituents and impurities in traditional Chinese medicines, often predicated by the source of the herbal extract. The inter-sample variability of individual constituents is often marked. In the traditional Chinese medicine, Chan-Su, the levels of the epoxide components were very different from the reference standard.

Regulatory authorities seem to view the risk of genotoxic impurities from medicinal and herbal products from very different perspectives. The Herbal Medicinal Products Committee (HPMC) within the EMEA has issued a draft guideline [37] on the assessment of genotoxic constituents of herbal medicines. However, in contrast to the CHMP guidance, HPMC have highlighted that the growth in use of herbal medicines for self-treatment is unlikely to be impacted by this guidance and cautioned that regulatory authorities should not be “over zealous” in banning such products based on “extrapolated suspicions”. HPMC stressed the need to develop robust risk-benefit assessments for herbal products. They conceded that the complex and variable (season to season, geographical origin or mode of preparation) nature of herbal products presents additional challenges compared to standard medicinal products. The HPMC indicated that “the complete composition is very difficult to unravel, so one can argue that there are always many unknown constituents and thus there may be hidden dangers”.

The committee cautioned that even for a herbal product that had been established as being genotoxic, the complexity of the herbal medicine may make it difficult, if not impossible, to establish a TTC. In addition, they conceded that the herbal product could also contain variable levels of radical scavengers (antioxidants) and even anti-carcinogens, making the assessment even more complicated.

This highlights two very real issues. Firstly the differing approach taken in respect to pharmaceuticals to that proposed for herbal medicines. The guideline covering medicinal products is predicated on the very same extrapolated suspicions the herbals guideline cautions against and furthermore it specifically prohibits

the holistic approach taken towards safety promoted within the herbals guideline.

The second issue relates to the lack of context associated with the EMEA guideline covering pharmaceuticals in terms of the level of exposure experienced through other sources including the diet and as exemplified here through the use of herbal remedies. If genotoxic impurities are truly seen as an issue in terms of actual patient risk then it could be argued that far more impact would be achieved through tackling the levels of exposure through these other sources.

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