Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 900–910

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Review

Control and analysis of hydrazine, hydrazides and hydrazones—Genotoxic impurities in active pharmaceutical ingredients (APIs) and drug products

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ARTICLE INFO

Article history:
Received 4 August 2010
Received in revised form 3 November 2010
Accepted 5 November 2010
Available online 13 November 2010

Keywords:
Hydrazine
Genotoxic
Derivatisation
Chromatography
Spectroscopy

ABSTRACT

This is the latest of a series of reviews focused on the analysis of genotoxic impurities. This review summarises the analytical approaches reported in the literature relating to hydrazine, hydrazines, hydrazides and hydrazones. It is intended to provide guidance for analysts needing to develop procedures to control such impurities, particularly where this is due to concerns relating to their potential genotoxicity.

Of particular note is the wide variety of techniques employed, both chromatographic and spectroscopic, with most involving derivatisation. Such a wide variety of options allow the analyst a real choice in terms of selecting the most appropriate technique specific to their requirements. Several generic methodologies, covering the three main analytical approaches; i.e. HPLC (high performance liquid chromatography), GC (gas chromatography) and IC (ion chromatography), are also described.

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1. Introduction

Issues relating to guidance on safe levels of genotoxic impurities (GIs) in novel and generic active pharmaceutical ingredients (APIs) and drug products have received considerable attention in the recent past.

In the European Union (EU) the Committee for Medicinal Products for Human Use (CHMP) released a Guideline on the Limits of Genotoxic Impurities [1] which came into operation in January 2007. Similarly, the US Food and Drug Administration (FDA) also issued a draft guidance document in December 2008 [2] on limits for genotoxic and carcinogenic impurities. As a consequence of these guidelines and the need to control GIs to low, often to ppm levels, there has been considerable interest in relation to the analysis of such impurities.

This is the fourth in a series of ongoing reviews [3–5], aimed at summarising the analytical approaches utilized in the literature to analyze and control GIs. This particular review is focused on hydrazines, hydrazides and hydrazones.

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doi:10.1016/j.jpba.2010.11.007

The generic structures of hydrazines, hydrazides and hydrazones are shown in Fig. 1.

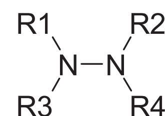
Hydrazine is a highly reactive base with reducing properties. It has been used as a synthetic intermediate to produce several different types of drugs, e.g. nifuroxazide, carbidopa, hydralazine, dihydralazine, isoniazid and iproniazid. Hydrazine itself, as its sulphate salt, has been used in the treatment of tuberculosis, sickle cell anaemia and in the treatment of various chronic illnesses [6].

There are limited reports of hydrazine occurring naturally. It has been reported in tobacco smoke [7], but this could be a thermal breakdown product or contamination, and recently as a by-product of metabolism of yeasts and some open-ocean bacteria e.g. *Brocadia anammoxidans* [8].

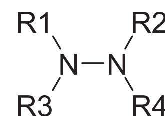
Metabolism of hydrazine is complex. Hydrazine is rapidly acetylated in most species. The initial mono-acetylation is too fast to measure for resultant metabolite identification and the excreted diacetyl metabolite has been reported to account for the majority of the administered dose [9]. However Huq [10] indicated that the acetylated metabolites accounted for <5% of the administered dose; 10% is excreted unchanged and 25% is excreted as nitrogen detectable in expired air. In addition, other metabolites are formed by conjugation with 2-oxoglutarate (1,4,5,6-tetrahydro-6-oxo-3-pyridazine carboxylic acid) and pyruvate (pyruvate hydrazone) and via ammonia to form urea.

Hydrazine has been reported as the principal hydrolytic degradation product of the anti-tuberculosis drug, isoniazid [11] and of structurally related analogues e.g. hydralazine, phenelzine and isocarboxazid [12,13].Hydrazine, methylated hydrazines and related hydrazines are thus fairly common synthetic intermediates and degradation products and consequently have been assayed by a variety of different analytical techniques.

Hydrazines



Hydrazides



Hydrazones

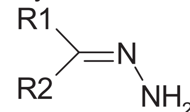


Fig. 1. Structural motifs of hydrazines, hydrazides and hydrazones.

2. Analytical approaches to trace analysis

2.1. Acceptable limits and general overview

Data from in vivo studies suggest that hydrazine and methylhydrazine are alkylating agents. The formation of methyl adducts with DNA bases in vivo may be one of the mechanisms by which hydrazines cause DNA damage and gene mutations [14,15]. Hydrazine, methyl hydrazine and related hydrazides are known human carcinogens [16] and hydrazine is reported to have an LD_{50} in mice of 57 mg/kg [17]. The maximum permitted exposure level in humans via the inhalation route is 1 ppm over an 8 h period [18]. The allowable level in pharmaceuticals has been suggested to be in the low ppm region [19].

Hydrazines, hydrazides and hydrazones (Fig. 1) all show conventional structural alerts for genotoxic potential [20]. Alerting structural elements are based mainly on the possession of electrophilic character (as such or following metabolic activation) and/or genotoxicity data from representative compounds. Evaluation of drug substances for genotoxic potential encompasses effects on both genes and chromosomes (i.e. clastogenicity), whereas for impurities testing is focused on the former in relation to the potential for interaction with DNA, the Ames bacterial reverse mutation test being the critical assay.

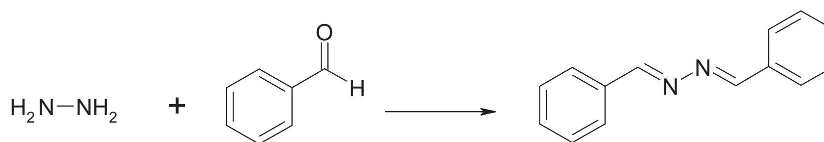
In the case of hydrazines and hydrazones Sawatari et al. [20] reported results on 55 and 25 compounds, respectively, approximately 40% of hydrazines and 50% of hydrazones being Ames-positive.

In the determination of the appropriate specification level of any potential GI, a limit based on compound-specific data should take precedence over any generic limit. The simplest situation arises when the impurity shows a structural alert for genotoxicity and no Ames-test or other relevant data are available. The default TTC limit of 1.5 μg/day would probably be appropriate in these circumstances, and the same limit might need to be applied if the impurity tested Ames-positive, although higher limits may be appropriate in certain circumstances, for example if the duration of patient exposure is less than one year. A major anomaly in relation to such low limits is provided by hydralazine hydrochloride (1-hydrazinylphthalazine) which, although Ames-positive and showing some evidence of tumorigenicity in rodent bioassays, is used in the treatment of hypertension at doses up to 200 mg/day – justified by the absence of any evidence for carcinogenicity over many years of clinical use.

Analysis of hydrazine and the related methylated hydrazines (methyl and dimethyl hydrazine) is challenging due to their volatility, high polarity, low molecular weight, absence of chromophore and in the case of hydrazine, the absence of any carbon atoms [21]. In addition, the potential for interference from the API matrix is significant and direct-injection GC of hydrazine derivatives is difficult due to the large amounts of API that can lead to cross-contamination, i.e. throughput is limited, allied with the necessity of frequent instrument cleaning [22]. In contrast, the analysis of aryl hydrazines (and related compounds) is fairly straight forward due to the presence of the aromatic ring system and presents fewer analytical challenges.

2.2. High performance liquid chromatography (HPLC)

Kean et al. [19] using a modified HPLC pharmacopoeial method involving derivatisation with benzaldehyde (see Scheme 1) combined with liquid–liquid extraction (LLE) demonstrated linearity over the range 0.04–2 ppm of residual hydrazine, with a limit of quantitation (LOQ) of 0.2 ppm. The extraction recovery was reported as 92%. The authors applied this quantitative HPLC method to the determination of residual hydrazine in samples of excipients e.g. povidone, copovidone, and API e.g. dihydralazine sulphate, hydralazine hydrochloride, nitrofurazone, nitrofural and nitro-



Scheme 1. Derivatisation with benzaldehyde to form benzalazine derivatives.

fuoroxazide. The data are summarised in Table 1 and showed good accord with the existing pharmacopoeial TLC method.

These authors also examined the levels of residual hydrazine in carbidopa API, using this HPLC (Table 2) method. They found elevated levels (10.4 ppm) versus the approved TLC method (3.2 ppm), but the sample was still in compliance with the monograph (20 ppm limit). Finally, these authors evaluated the residual hydrazine levels in isoniazid API. They found levels of 72.8 ppm. They considered that the current monograph limit for isoniazid was too high (125 ppm) and that the existing TLC monograph method was too insensitive for routine pharmacopoeial use.

Recently, an HPLC and HPLC–MS study of the decomposition of isoniazid was carried out by Bhutani et al. [23]. The authors reported that isoniazid was thermolytically stable, slightly unstable to photolysis and oxidation, but underwent extensive hydrolysis, to yield four primary degradation products that they designated I–IV: hydrazine (I), isonicotinic acid-*N*′-(pyridyl-4-carbonyl)hydrazide (II), isonicotinic acid-pyridine-4-ylmethylene hydrazide (III), isonicotinic acid ethylidene hydrazide (IV). Under photolytic conditions the API turned yellow to form the previously unreported degradation product (II). The method was validated for the API, but not for the degradation products, other than specificity assessments. The authors demonstrated resolution values in excess of 3 between the API and degradation products and high peak purity indexes for all peaks, i.e. greater than 0.9912.

The stability of the novel iron chelator, pyridoxal isonicotinoyl hydrazone (PIH) was studied by Kovaříková et al. [24]. The authors indicated that the principal degradation pathway was hydrolysis of the hydrazone bond to yield pyridoxal and the aryl hydrazine compound, isoniazid. A minor degradation product was isonicotinic acid. The method was fully validated for the PIH, but no details were provided for the degradation products.

A novel separation method for the impurities in mildronate API was recently reported by Hmelnickis et al. [25]. The authors used a hydrophilic interaction chromatography (HILIC) method with several different polar stationary phases (silica, cyano, amino and the zwitterionic sulfobetaine) to separate the six polar impurities, including the hydrazine impurity, 1,1,1-trimethylhydrazinium bromide (that they designated impurity II. Note that in cited examples the current authors have used the same designations that the original authors applied). They demonstrated that HILIC was a useful alternative to reverse phase or ion chromatography (IC). The impact of method separation conditions, including organic modifier content and pH were studied. Finally, a HILIC method using the zwitterionic sulfobetaine stationary phase (ZIC-HILIC) was developed and validated. The method showed good linearity for impurity II with a limit of detection (LOD) of 3 ppm and an LOQ of 10 ppm. The recoveries for impurity II over the range 0.05–0.125% were in the range 105.7–114.0% with RSDs of between 1.1 and 5.1%. The precision at the 0.1% level (designated specification limit) was 3.3%. The method was found to be robust and applied to two batches of mildronate API. In the technical batch the levels of residual impurity II were high (i.e. 3500 ppm), but in the commercial batch there were levels at the LOQ of the method; i.e. 3 ppm. This demonstrates a fairly universal truth that as a greater understanding of the process is gained throughout the development process that it typically leads to better control strategies and lowered levels of these reactive impurities at the point of commercialization.

A novel generic method for the direct determination of hydrazine and 1,1-dimethylhydrazine in a pharmaceutical intermediate was reported by Liu et al. [26]. The method also used a HILIC approach with ethanol as a weak eluent and interestingly chemiluminescent nitrogen detection (CLND). The method is simple and reasonably sensitive, i.e. 200 ppm. The method was linear. The precision of the standard solutions at 200 ppm was 5.0 and 3.9%, for hydrazine and 1,1-dimethylhydrazine, respectively. The recoveries were excellent: for 1,1-dimethylhydrazine at 200 ppm and 400 ppm they were 100.9 and 102.7%, respectively. However, in contrast, the recoveries for hydrazine were low (84.3%) at 200 ppm, but were satisfactory (102.7%) at 400 ppm.

Srinivasu et al. [27] assessed the residual levels of 4-hydrazine benzene sulphonamide (intermediate I) in the COX-2 inhibitor, celecoxib. The method was linear and accurate over the range 0.05–1.0%, with recoveries in the range of 96.7–100.2%. The method gave an LOQ of 97 ng/ml. The method was robust with respect to mobile phase compositions, pH and temperature. Satyanarayana et al. [28] also evaluated the residual levels of hydrazine and 4-hydrazine benzene sulphonamide in celecoxib, but there were no reported validation details.

Kirtley et al. [21] initially assessed GC methodologies for residual hydrazine, in various APIs, before evaluating HPLC with UV detection at 190 nm. They initially reported a 20 ppm LOD, which was too insensitive and resorted to derivatisation. They utilized the benzalazine derivative of the residual hydrazine (Scheme 1), but the method details were not reported. The authors observed that there was a gradual increase in reportable levels of hydrazine from this method over time giving artefactually high levels (8 ppm in API, 10 ppm in API spiked with 2 ppm of hydrazine and 12 ppm in API spiked with 5 ppm of hydrazine). They identified one source of the increase as the interference from another process impurity (bis-hydrazone), which was present at residual levels of 300 ppm and could also form the benzalazine derivative. They reported that this

Table 1
Comparative TLC versus HPLC residual hydrazine data for different batches of seven excipient and APIs batches. Summarised from Kean et al. [19].

Excipient/API	Lots	Residual hydrazine (ppm) by TLC	Residual hydrazine (ppm) by HPLC
Povidone	1	ND	0.32
Copovidone	1	1.73	1.47
	2	ND	ND
	3	1.26	1.03
	4	ND	ND
	5	ND	ND
Hydralazine	1	ND	0.25
Dihydralazine	1	ND	0.34
	2	ND	0.35
Nitrofural	1	5.0	10.1
Nitrofuroxazide	1	12.1	19.6
	2	28.7	62.4
	3	15.9	80.3
	4	ND	0.04
	5	ND	ND
Nitrofurazone	1	0.53	2.14
	2	19.1	12.6
	3	18.1	15.7
	4	7.1	9.9
	5	5.2	11.9

ND: none detected.

Table 2
Experimental details for analytical methods used to determine residual hydrazine (hydrazides and hydrazones) in API and drug products.

API	Impurities	Method details	Reference
Allopurinol	Hydrazine	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm cyanosilyl stationary phase (R type) at 30 °C. Mobile phase: 2-propanol/hexane (5/95, v/v). Flow rate 1.5 ml/min; detection at 310 nm.	[50] (Ph Eur Monograph 0576)
API (general method)	Hydrazine	Derivatisation using acetone or acetone d_6. Headspace conditions: incubation oven temperature 100 °C, incubation time 10 min, headspace syringe temperature 110 °C, agitation speed 500 rpm, agitation time 18 s, injection volume 500 μl. Agilent DB-624 GC stationary phase (25 m × 0.2 mm) at 100 °C for 6 min, then 50 °C/min to 220 °C, 220 °C for a further 2 min. Helium carrier gas flow rate: 1.2 ml/min. Injector temperature 200 °C. The MS operated in EI mode (70 eV). Source and quadrupole temperatures: 230 °C and 150 °C, respectively. The azine derivatives monitored using SIM mode at m/z 112, the azine-d_{12} derivatives at m/z 124 and 106.	[22]
API (general method)	Hydrazine	HPLC with (1) 5 μm ZIC HILIC (SeQuant), (2) 5 μm Develosil 100Diol-5 (Nomura), (3) 5 μm TSK-Gel Amide-80 (Tosoh Bioscience) and (4) 5 μm Zorbax NH_2 (Agilent) at different column temperatures (10–60 °C). Mobile phase: TFA/water/ethanol (0.1/30/70, v/v). Flow rate 0.4 ml/min; CLND detection.	[26]
API (general method)	Hydrazine	IC using a weak CEX stationary phase (CS12A). Mobile phase: 3.8 mM sulphuric acid. Detection by amperometry, with Pt working electrode and Ag/AgCl reference electrode.	[39]
API (general method)	Methyl hydrazine	IC using a weak CEX stationary phase (CS12A). Mobile phase: 7.5 mM sulphuric acid. Detection by amperometry, with Pt working electrode and Ag/AgCl reference electrode.	[39]
API (general method)	Hydrazine	(1) Derivatisation using benzaldehyde. HPLC with no operating conditions reported. (2) LSE, followed by derivatisation using benzaldehyde at lower temperatures. HPLC with no operating conditions reported. Detection at 190 nm.	[21]
API (general method)	Hydrazine	LSE using strong basic AEX resin. IC using a weak CEX stationary phase and detection by amperometry (no operating conditions reported).	[21]
Azelastine	Impurity A: benzohydrazide, impurity B: 1-benzoyl-2-[(4RS)-1-methyl-hexahydro-1H-azepin-4-yl]diazane	HPLC with a 10 μm cyanosilyl stationary phase (R) at 30 °C. Mobile phase: pH 3.0 phosphate buffer and sodium octane sulphonic acid in water/acetonitrile (740/260, v/v). Flow rate 2.0 ml/min; detection at 210 nm.	[52] (Ph Eur Monograph 1633)
Aryl hydrazones (API)	E-Arylhydrazones	HPLC with a 5 μm ODS stationary phase (Merck LiChrospher) at 25 °C. Mobile phase: 1 mM pH 6.0 phosphate buffer with 2 mM EDTA and methanol (40/60, v/v). Flow rate 1.0 ml/min; detection at 200–400 nm (DAD). HPLC with a 5 μm phenylhexyl stationary phase (Phenomenex Luna) at 25 °C. Mobile phase: water and acetonitrile (50/50, v/v). Flow rate 0.3 ml/min. Positive and negative ion mode ESI with ion trap analyzer in SIM mode (M+H ion). Range 50–1000 m/z. Voltage 4 kV, capillary temperature 250 °C.	[29]
Carbidopa	Hydrazine	LSE using strong basic AEX resin. Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel R TLC plate with a methanol/water (20/10, v/v) mobile phase; detection at 365 nm.	[51] (Ph Eur Monograph 0755)
Carbidopa	Hydrazine	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase: aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.	[17]
Carbidopa	Hydrazine	IC with a 8 μm CEX stationary phase (Dionex CS14) and a mobile phase consisting of 5 mM perchloric acid. Flow rate 1.0 ml/min, Amperometric detection, +80 V. Pt electrode (reference electrode not specified).	[17]
Celecoxib	Impurity III: 4-hydrazine benzene sulphonamide	MEKC with a 50 cm fused silica capillary at 35 °C, using a pH 9.3 25 mM sodium borate buffer, with 25 mM SDS, with an operating voltage of 25 kV and injected for 5 s at 10 mbar. Detection at 252 nm.	[44]
Celecoxib	Intermediate I: 4-hydrazine benzene sulphonamide	HPLC with a 4 μm ODS stationary phase (NovapaK C18). Mobile phase: pH 4.8 10 mM phosphate buffer and acetonitrile (450/550, v/v). Flow rate 1.0 ml/min; detection at 252 nm.	[27]
Celecoxib	Intermediate I: 4-hydrazine benzene sulphonamide	HPLC with a 5 μm ODS stationary phase (Hichrom C18). Mobile phase: pH 4.8 10 mM phosphate buffer and acetonitrile (450/550, v/v). Flow rate 1.0 ml/min; detection at 252 nm. HPLC with a 5 μm ODS stationary phase (Zorbax C18). Mobile phase: pH 4.8 10 mM phosphate buffer and acetonitrile 400/600, v/v). Flow rate 0.4 ml/min. Quadrupole detection, with He as nebuliser gas. Source manifold 250 °C; quadrapole 100 °C.	[28]
Copovidone	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel R TLC plate with a methanol/water (80/20, v/v) mobile phase. Examined at 365 nm.	[53] (Ph Eur Monograph 0891)
Copovidone	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel RP2 TLC plate with a methanol/water (20/10, v/v) mobile phase. Examined at 365 nm.	[17]
Copvidone	Hydrazine	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase: aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.	[17]
Dihydralazine sulphate	Hydrazine (impurity B)	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm ODS stationary phase (R type). Mobile phase: aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.	[49] (Ph Eur Monograph 1310)
Dihydralazine sulphate	Hydrazine	Derivatisation using salicylaldehyde, followed by lLLE. TLC with a silanised silica gel RP2 TLC plate with a methanol/water (20/10, v/v) mobile phase. Examined at 365 nm	[17]
Dihydralazine sulphate	Hydrazine	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase: aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.	[17]

Table 2 (*Continued*)

API	Impurities	Method details	Reference
Ebifuramin	Impurity III: (+)-5-morpholinomethyl-3-(5-nitrofurfurylidene amino)-oxazolidin-2-one	HPLC with a 5 μm ODS stationary phase (Hypersil ODS). Mobile phase: acetonitrile/THF/pH 2.6 10 mM dibutylamine phosphate (15/5/80, v/v/v). Flow rate 1.5 ml/min; detection at 254 nm.	[33]
Hydralazine	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel G R TLC plate with a ethanol/toluene (10/90, v/v) mobile phase. Examined at 365 nm.	[54] (Ph Eur Monograph 0829)
Hydralazine	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel RP2 TLC plate with a methanol/water (20/10, v/v) mobile phase. Examined at 365 nm with Camag Reprostar 3.	[17]
Hydralazine	Hydrazine	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.	[17]
Hydralazine tablets	Hydralazine hydrazone	HPLC with a 10 μm ODS stationary phase (Waters μBondapak) at room temperature. Mobile phase: acetonitrile/5 mM SDS/phosphoric acid (150/850/0.45, v/v/v). Flow rate 2.0 ml/min; detection at 220 nm.	[32]
Hydralazine Tablets	Hydrazine	Derivatisation using benzaldehyde, followed by LLE with IS. SE-54 fused silica GC stationary phase (25 m × 0.32;mm) with nitrogen selective detection. Column 100 °C for 60 s, then 15 °C/min to 285 °C. Injector 280 °C, detector 300 °C. Flow rate of nitrogen carrier gas 20 ml/min.	[37]
Hydralazine and isoniazid API and formulations	Hydrazine	Derivatisation using benzaldehyde, followed by LLE with IS. OV-101 GC stationary phase with nitrogen selective detection.	[36]
Isoniazid, iproniazid	Hydrazine	Derivatisation using 2-hydroxy-1-naphthaldehyde, followed by LLE. Spectrofluorimetric analysis with excitation at 416 nm and emission at 512 nm.	[48]
Isoniazid	Hydrazine	Derivatisation using benzaldehyde, followed by liquid-LLE. DB-5 fused silica GC stationary phase (15 m × 0.32 mm) at 65 °C for 30 s, then 12 °C/min until the benzalazine derivative eluted. Total run time 10 min. Detection using EC with a Ni^{63} source. Detector and injection port 315 °C.	[18]
Isoniazid	Hydrazine and methylhydrazine	CE with a 52 cm uncoated fused silica capillary using pH 7.2 phosphate buffer, with an high operating voltage (not specified) and EC using a Pd modified carbon micro-disk array electrode.	[41]
Isoniazid	Hydrazine and methylhydrazine	CE with a 37 cm uncoated fused silica capillary using pH 7.2 phosphate buffer, with an operating voltage of 15 kV and EC using a 4-pyridylhydroquinone micro-disk Pt array electrode.	[42]
Isoniazid	Impurity I: 1-nicotinyl-2-lactosylhydrazine	Derivatisation with ammoniacal 2,3-dichloro-1,4-naphthaquinone in ethanol. Spectrophotometry at 640 nm.	[45]
Isoniazid	Impurity I: 1-nicotinyl-2-lactosylhydrazine	HPLC with a 10 μm cyanopropyl stationary phase and a mobile phase consisting of a mixture of pH 3.5 10 mM acetate buffer and acetonitrile (95/5, v/v). Flow rate and detection wavelength not specified.	[31]
Isoniazid	Hydrazine (I), isonictonic acid-N′-(pyridyl-4-carbonyl)hydrazide (II), isonictonic acid-pyridine-4-ylmethylene hydrazide (III), isonictonic acid ethylidene hydrazide) (IV)	HPLC with a 5 μm ODS stationary phase (Zorbax XDB Eclipse C18). Mobile phase water and acetonitrile (960/40, v/v). Flow rate 0.5 ml/min; detection at 252 nm. HPLC-MS using negative electrospray ionization ESI with a Bruker Daltonics ToF.	[23]
Isoniazid	Hydrazine	TLC with a silica gel F_{254} TLC plate with a water/acetone/methanol/ethyl acetate (10/20/20/50, v/v) mobile phase. Visualization using dimethylaminobenzaldehyde solution; examination under daylight.	[55] (Ph Eur Monograph 0146)
Isoniazid	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel RP2 TLC plate with a methanol/water (20/10, v/v) mobile phase.	[17]
Isoniazid tablets	Hydrazine	Solid phase spectroscopy based on fixation of 4-dimethylbenzaldehyde on a CEX resin (Dowex 50 W ×8).Spectra accumulated at a scan rate of 250 nm/min and the absorbance read at 464 nm.	[47]
Mildronate	Impurity 2: 1,1,1-trimethylhydrazinium bromide	HILIC with a 3 μm silica stationary phases (Atlantis HILIC silica, Alltima HP silica and Spherisorb silica), 5 μm cyano stationary phase (Discovery cyano), 3 μm amino stationary phase (Hypersil APS-1) and 5 μm sulfobetaine stationary phase (ZIC-HILIC) at 30 °C. Mobile phase acetonitrile and 0.1% formic acid in water. Flow rate 0.2 ml/min with positive ion mode ESI detection at 20–35 kV using a triple quadrapole MS.	[25]
Nitrofural, nitrofurazone and nitrofuroxazide	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel RP2 TLC plate with a methanol/water (20/10, v/v) mobile phase.	[17]
Nitrofural, nitrofurazone and nitrofuroxazide	Hydrazine	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm ODS stationary phase (Altima C18 or Hypersil ODS). Mobile phase aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.	[17]
Nitrofurazone	Impurity A: Bis-[(5-nitrofuran-2-yl)methylene]diazane	HPLC with a 5 μm ODS stationary phase (R type). Mobile phase acetonitrile/water (400/600, v/v). Flow rate 1.0 ml/min; detection at 310 nm.	[56] (Ph Eur Monograph 1135)
Povidone	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel RP2 TLC plate with a methanol/water (20/10, v/v) mobile phase.	[17]
Povidone	Hydrazine	Derivatisation using benzaldehyde, followed by LLE. HPLC with a 5 μm ODS stationary phase (Altima C18, Hypersil ODS). Mobile phase aqueous 0.03% EDTA and acetonitrile (300/700, v/v). Flow rate 1.0 ml/min; detection at 305 nm.	[17]

Table 2 (*Continued*)

API	Impurities	Method details	Reference
Povidone	Hydrazine	Derivatisation using salicylaldehyde, followed by LLE. TLC with a silanised silica gel R TLC plate with a methanol/water (20/10, v/v) mobile phase. Examined at 365 nm.	[57] (Ph Eur Monograph 0685)
Pyridoxal isonicotinoyl hydrazone	Hydrazine, isoniazid	HPLC with 5 μm ODS (Nucleosil C18) and an isocratic mobile phase consisting of a mixture of methanol (A) and pH 3.0 10 mM phosphate buffer containing 5 mM 1-heptane sulphonic acid and 2 mM EDTA (B) in a ratio of 49/51, v/v. Flow rate 0.9 ml/min; detection at 297 and 254 nm.	[24]
Rifampicin	Hydrazones: rifampicin quinone and 25-desacetyl rifampicin	HPTLC with a silica gel 60 TLC plates (Merck) with a chloroform/methanol/water (80/20/2.5, v/v/v) mobile phase. Examined using Scanner II (Camag) at 330 nm for 25-desacetyl rifampicin and 490 nm for rifampicin quinone.	[40]
Rifampicin	Hydrazones: rifampicin quinone	HPLC with 10 μm silyl and 10 μm nitrile stationary phases (MicroPak Si-10 and MicroPak CN, respectively) and an isocratic mobile phase consisting of a mixture of chloroform and methanol of varying proportions. Flow rate 0.2–0.7 ml/min; detection at 334 nm.	[58]
Rifampicin	Hydrazones: rifampicin quinone, 25-desacetyl-21-acetyl-rifampicin, 25-desacetyl-23-acetyl-rifampicin	HPLC with direction injection (DI) onto a 3 μm ODS stationary phase (Hypersil ODS) at 25 °C and an isocratic mobile phase consisting of a mixture of pH 7.4 50 mM phosphate buffer and acetonitrile (64/36, v/v). Flow rate 1.4 ml/min; detection at 240 nm. Alternatively, a 10 μm ODS stationary phase (Hypersil ODS) pre-column (PC) to pre-concentrate the sample	[34]
Rifampicin, isoniazid, pyrazinamide FDC	Hydrazones: rifampicin quinone, desacetyl rifampicin, isonicotinyl hydrazone	HPLC with a 5 μm L1 ODS stationary phase at 25 °C and a gradient mobile phase consisting of a varying mixtures of mobile phase A (pH 6.8 phosphate buffer/acetonitrile, 96/4, v/v) and mobile phase B (pH 6.8 phosphate buffer/acetonitrile, 45/55, v/v or 55/45, v/v). Flow rate 1.5 or 1.0 ml/min; detection at 238 nm. Three L1 columns were evaluated: 1: Zorbax XDB, 2: Shim-pak CLC ODS and 3. Nucleosil EC 120-5.	[35]
Rizatriptan	Impurity I: 1-(4-hydrazinophenyl) methyl-1,2,3-triazole	HPLC with a 5 μm nitrile stationary phase (Zorbax SB-CN) at 25 °C and a gradient mobile phase consisting of varying mixtures of pH 3.4 10 mM phosphate buffer, acetonitrile and methanol. Flow rate 1.0 ml/min; detection at 225 nm.	[30]
Rizatriptan	Hydrazone degradation product	MEEKC with a 32 cm uncoated fused silica capillary at 40 °C, using 0.81% octane, 6.61% butan-1-ol, 3.31% SDS and 89.27% pH 9.2 10 mM sodium borate buffer, with an operating voltage of 13 kV and injected for 3 s at 20 mbar. Detection at 230 nm using a ×3 bubble cell, with a detection window of 23 cm.	[43]
SQ-84251	Hydrazine	IC with CS-14 CEX polymeric stationary phase. Mobile phase 10 mM perchloric acid. Flow rate 1.0 ml/min. EC in the direct amperometry (DC) mode with the electrode potential set at +0.8 V. Using a Pt working electrode and an Ag/AgCl reference electrode.	[38]
Vindesine sulphate	Impurity C (desacetylvinblastine hydrazide)	HPLC with a 5 μm ODS stationary phase (R type) and a gradient mobile phase consisting of varying mixtures of pH 7.5 diethylamine phosphate buffer and methanol. Flow rate 2.0 ml/min; detection at 270 nm.	[59] (Ph Eur Monograph 1276)

Abbreviations: ODS, octadecylsilyl; LLE, liquid liquid extraction; LSE, liquid solid extraction; SDS, sodium dodecyl sulphate; FDC, Fixed Dose Combination; CEX, cation exchange; AEX, anion exchange; EC, electrochemical detection; IS, internal standard; SIM, single ion monitoring; ToF, time of flight; EI, electron impact; ESI, electrospray ionization; MS, mass spectroscopy; DAD, diode array detection; CLND, chemiluminescent nitrogen detection.

method was suitable for samples of API containing greater than 50 ppm of hydrazine, where the interference was negligible.

They introduced a clean up procedure (API precipitated out of solution as free acid and they used the supernatant for assessing levels of residual water soluble analyte) and modified the initial derivatisation procedure, using a lower temperature to limit any unwanted side-reactions. The method gave recoveries in the range 95–105% and was suitable for determining residual hydrazine levels <10 ppm during in-process testing of reaction liquors and crude API (LOD 1 ppm).

A Czech research group [29] recently reported on aryl hydrazone isomerisation resulting in formation of residual geometrical *E*-isomer impurities determined by HPLC with diode array and MS–MS detection. Their chromatographic investigation was supported by complementary NMR data, but no validation data was reported.

An Indian research group [30] reported on the development and validation of a stability indicating HPLC method for the determination of the anti-tuberculosis drug, rizatriptan and its degradation products, including a hydrazone impurity (impurity I: 1-(4-hydrazinophenyl)methyl-1,2,3-triazole). The method was linear over the range from the LOQ to 3000 ppm (the specification limit proposed was 1500 ppm). The LOD and LOQ values for impurity I were 400 ppm and 1300 ppm, respectively. The recoveries of impurity I at typical levels in the bulk API were in the range 91–107%. The authors examined the impact of flow rate, pH of mobile phase and the affect of column temperature (20–30 °C). The method was shown to be robust with resolution of API from all impurities greater than 4.0.

Butterfield et al. [31] investigated the formation of aldose reaction products of isoniazid and lactose. They developed an HPLC method for the simultaneous determination of isoniazid and 1-*iso*-nicotinyl-2-lactosylhydrazine (impurity I) in tablet formulations. The authors reported sensitivity at the 5000 ppm level, with a RSD of 0.7%. They assayed seven commercial batches of isoniazid tablets and showed levels of impurity I in the range 3000–58,000 ppm.

Lessen and Zhao [32] performed similar HPLC and fluorescence investigations on aldose reaction products of hydralazine and starch, identifying the corresponding hydrazone (impurity IIa) as a key intermediate in the overall degradation pathway. They theorized that the hydrazone subsequently cyclises to the triazolo phthalazine (impurity IIIc). They used these investigations to provide explanations for the lack of mass balance in ongoing hydralazine tablet stability studies. No method validation details were provided.

The hydrolysis of the cytotoxic agent ebifuramin to the hydrazone impurity III [(+)-5-morpholinomethyl-3-(5-nitrofurfurylidene amino)-oxazolidin-2-one] was studied by Prankerd and Stella [33]. They determined that the hydrolysis kinetics was initially concentration dependant (not first order), followed by apparent first order degradation of at least one of the initial hydrolysis products. No method validation details were provided.

The validation of a rapid and selective HPLC method for the determination of rifampicin and its hydrazone degradation products was reported by Bain et al. [34]. The major decomposition pathways of rifampicin are basic oxidation [rifampicin quinone (RQU)] and acidic hydrolysis [3-formylrifampicin (RSV) and 1-amino-4-methyl piperazine]. In addition, under anaerobic basic conditions, 25-desactylrifampicin (DAR) is formed, which then in turn decomposes to 25-desactyl-21-rifampicin (25-21) and 25-desactyl-23-rifampicin (25-23). Specificity of the method was assured by measuring peak purity. The method did not resolve impurities 25-21 and 25-23, as there is an acetyl migration occurring in solution between positions 21 and 23, and they are reported as a composite value (25/21-23). 1-Amino-4-methyl piperazine has no chromophore and was not detected in this method. The authors evaluated both direct injection (DI) and column switching (CS) onto a pre-column to pre-concentrate the analytes and then back-flushing onto the analytical column.

The method was linear for all of the hydrazone impurities (RQU, DAR and 25-21/23). The LOQ of each of these analytes was significantly enhanced by column switching. The LOQs were 400, 215, and 275 ng/ml, respectively, using direct injection compared to 39, 20 and 36 ng/ml, respectively, using column switching to remove the API. The accuracy of the method was appropriate. The within-day precision for these three analytes at 1 μg/ml were significantly improved using pre-concentration approach (RSD 0.25–0.65%) compared with direct injection (RSD 1.75–3.56%).

An Indian research group [35] evaluated the USP monograph method for fixed-dose combinations of anti-tuberculosis drugs (isoniazid, pyrazinamide and rifampicin) and in particular they assessed the method for its ability to resolve rifampicin from its hydrazone degradation products (RQU, DAR and isonicotinoyl hydrazone; the latter an interaction product formed between RSV and isoniazid). The authors found that the resolution of the method was highly dependant on the stationary phase selected. Three columns representative of USP stationary phase L1 were evaluated. Columns 1 and 2 were able to resolve the three different APIs and rifampicin from its hydrazone degradation products (with the exception of DAR and rifampicin *N*-oxide which co-eluted); whereas, column 3 failed to resolve these analytes. Satisfactory resolution could be achieved by decreasing the organic component and reducing the flow rate.

2.3. Gas chromatography (GC)

As already outlined in Section 1 hydrazine itself possesses no carbon atoms, thus the normal FID (Flame Ionization Detection) mode employed in GC analysis is unsuitable. Therefore alternative detection techniques are typically employed, as exemplified in the examples described.

A GC procedure involving the formation of a benzalazine derivative (Scheme 1) was developed to monitor the residual levels of hydrazine in hydralazine and isoniazid API, tablets, combination tablets, syrups and injectable products [36]. This method utilized nitrogen selective detection. The LOD of the method when applied to API was found to be ≤3 ppm of hydrazine. No hydrazine (<LOD) was found in hydralazine API, 3 ppm was seen in some tablet lots and 200 ppm was observed in the injectable product. Similarly, traces (3 ppm) of hydrazine were found in one lot of isoniazid API, 12 and 29 ppm, respectively, were seen in two tablet lots and 2000 ppm were observed in the syrup product.

Gyllenhaal et al. [37] modified a previously published method [36] again involving the formation of a benzalazine derivative and monitoring this derivative using capillary gas chromatography with nitrogen selective detection. They utilized 5-chloromethyl-2-amonibenzophenone as the internal standard. The method gave recoveries of between 86 and 94% and the precision was acceptable (RSD 7.7%). The authors noted artefactually elevated levels of the derivative after a 20 min reaction period or where the hydralazine solutions were allowed to stand. They therefore utilized fresh solutions of hydralazine and a 5 min reaction period. The method was applied to hydralazine API that had been stored for between 1 and 2 years and the levels were in the range of 0.2–0.4 ppm, which is near the LOQ of this method.

Carlin et al. [11] also adapted this previously published method [36], again using a benzalazine derivative and monitoring this using GC with electron capture (EC) detection. The authors commented that although EC did not produce increased sensitivity over FID, the increased selectivity would reduce potential interference due to the matrix when applied to commercial formulations. The LOQ was 10 ppm and the method was linear over the range 10–100 ppm. The inter-day residual standard deviation (RSD) based on six measurements at analyte levels of 10 ppm was 15%. However, this improved slightly at increased analyte concentrations of 25 and 100 ppm, to 9.5% and 11.3%, respectively.

Carlin et al. [11] also found elevated levels of hydrazine in isoniazid syrup, which ranged from 1.6 to 12.6 ppm, when stored at ambient temperatures for 4-months. In contrast, the levels at 0 °C, over the same storage period were <1 ppm. The authors recommended that the product labelling was changed to a recommended storage at sub-ambient temperatures, until the safety of the product could be better addressed.

Researchers from the Canadian Health Protection Board reported on levels of residual hydrazine in formulations of isoniazid, hydralazine and phenelzine over a 2-year period [12]. Although isoniazid tablet formulations appeared to be stable the level of hydrazine in the corresponding paediatric elixir and in a pyridoxine combination tablet doubled to 44 μg/ml and 19 μg/tablet, respectively.

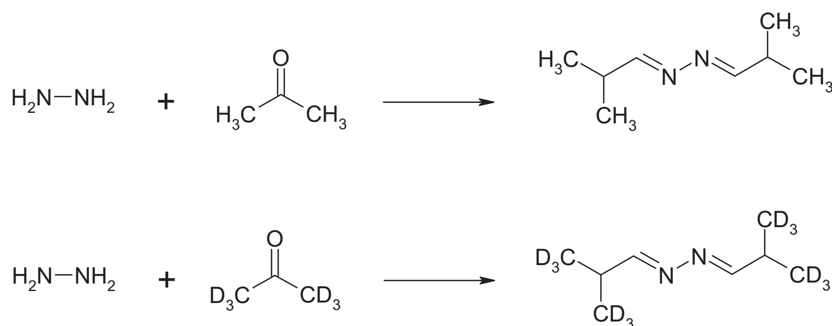
This research group also evaluated the levels of residual hydrazine and benzyl hydrazine in isocarboxazid [13]. The LODs for the two analytes were 20 ppm for hydrazine and 200 ppm for benzyl hydrazine. Levels of hydrazine in phenelzine tablets remained unchanged over the assessment period, but the levels were high (60 μg/tablet) and showed significant tablet to tablet variability.

Similarly, the levels of hydrazine in hydralazine tablets at ambient storage conditions remained unchanged, but a corresponding injectable formulation doubled from 4.5 μg/ml to 10.0 μg/ml.

Kirtley et al. [21] assessed several different approaches to the challenge of residual hydrazine determination. They evaluated GC-FID, but observed no signal due to the absence of carbon atoms. For GC-NPD (nitrogen phosphorus detector) and GC–MS they observed poor sensitivity and the LODs were inadequate for the routine monitoring of low levels of these analytes.

Sun et al. [22] recently reported on a generic in situ derivatisation headspace GC–MS method for the determination of hydrazine in API at low ppm levels. The method utilizes either acetone or the deuterium labelled analogue, acetone-d_6 as the derivatising agent (see Scheme 2), yielding the corresponding azine (acetone-azine or acetone-azine-d_{12}). This volatile derivative can be analysed by headspace GC–MS. The method is highly sensitive and is capable of detecting levels of hydrazine at 0.1 ppm in the presence of 10 mg of API. The method is linear. The recoveries at the 1 ppm level were in the range 79–117%, and the precision at this level were ≤5.6% RSD. The recoveries were slightly impacted by the presence of interfering functional groups on the different APIs (e.g. ketone, primary amine, etc.) that could react with acetone or a weak Michael acceptor that could react with hydrazine. However, these interferences were fairly insignificant considering the very low level of analyte i.e. 0.1 ppm.

The generic method was successfully applied to the determination of residual hydrazine at 1 ppm in 5 different APIs and using the stable isotope alternative (acetone-d_6) for another 2 APIs where



Scheme 2. Derivatisation with acetone (or acetone-d_6) to form acetone-azine or corresponding acetone-azine-d_{12} derivatives.

acetone was present as a residual solvent. The method was validated at slightly higher residual analyte levels (25 and 100 ppm hydrazine) for a further 2 APIs due to the higher control limits afforded by these projects. The authors commented that instrument contamination was minimized and that the method could be automated by using autosamplers with heating, stirring and reagent addition facilities.

2.4. Ion chromatography (IC)

Residual levels of hydrazine in the penultimate intermediate (SQ-84251) of a novel anti-infective API, were determined by an ion chromatographic method with electrochemical detection [38]. The method was selective, with a LOD of 50 ppm and a LOQ at twice this level (100 ppm). The method was linear over the range 100–400. The method's precision at concentrations of 100 ppm of hydrazine was good (RSD 1.1%). The method was accurate over the range 100–300 ppm, with recoveries in the range of 90–105%. The method also demonstrated good solution stability over a 3-day period (RSD 2.0%). The method required no pre-treatment, the API could be dissolved in methanol/water (50/50, v/v) and injected directly onto the column.

Residual levels of genotoxic impurities in high-dose drugs are problematical as the increased dose drives down safety-based residue limits. Kirtley et al. [21] reported on the determination of residual hydrazine, which was used in the penultimate stage in the synthesis of a highly water-soluble sodium salt and needed to be controlled in the final API at levels below 1 ppm. They assessed several different approaches including GC, HPLC using benzalazine derivatives and IC with conventional conductivity detection, but observed interference from sodium ions. They pre-treated the sample matrix by dissolving in water and passing it through a strong anion exchange column before using IC with a weak anion exchange stationary phase with amperometric detection. The interference from sodium ions precluded conventional conductivity detection. However, when they utilized amperometry detection they showed significant improvements with an LOD of 0.05 ppm in API, with a linear range between 0.05 ppm and above 200 ppm. The recoveries at 5 ppm were good (90%). The authors claimed that this IC method was superior to standard derivatisation HPLC methods because of increased selectivity and reduced matrix interference. Amperometric detection is extremely selective for analytes able to undergo redox reactions, such as hydrazine.

Kean et al. [19] examined the levels of residual hydrazine in carbidopa API, using an IC method again with amperometric detection. They demonstrated concordance (3.9 ppm) with an approved TLC method (3.2 ppm), but different levels to the HPLC method (10.4 ppm).

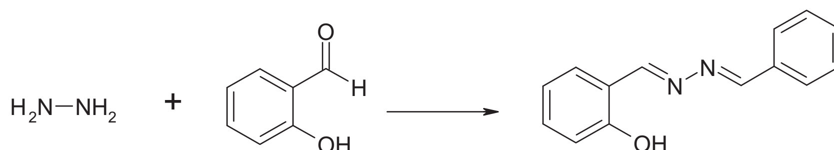
Wang et al. [39] reported on generic IC methods for hydrazine and methyl hydrazine in API utilizing electrochemical/amperometric detection methods. Limited validation was provided. The method was appropriately sensitive and the LOQs for the two analytes were 0.5 and 1.2 ppm, respectively.

2.5. Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC)

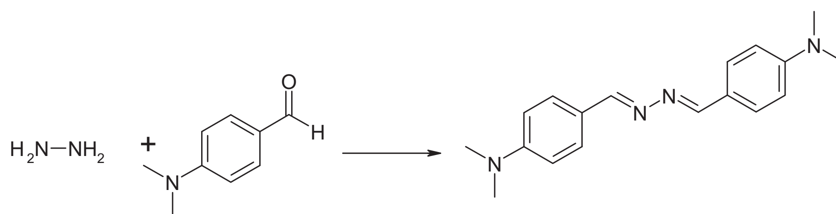
Kean et al. [19] used a modified pharmacopoeial method involving derivatisation with salicylaldehyde (Scheme 3), extraction into toluene and fluorescence detection at 366 nm, and the authors were able to demonstrate adequate sensitivity of the method. The area of the hydrazine derivative was photographed and scanned using Videoscan system, and was linear over the range 0–20 ppm with an LOQ of 1 ppm. The authors applied this quantitative TLC method to the determination of residual hydrazine in samples of excipients e.g. povidone, copovidone and API e.g. dihydralazine sulphate, hydralazine hydrochloride, nitrofurazone, nitrofural and nitrofuoroxazide.

There were no residual levels of hydrazine reported in the batches of povidone analysed, but in contrast, 2 out of 5 batches of copovidone showed some levels of the analyte (see Table 1). For those APIs which have pharmacopoeial monographs with limit tests for residual hydrazine (hydralazine and dihydralazine) there were no reportable levels of hydrazine, but those APIs whose monographs did not feature a limit test showed high and variable batch-to-batch levels. For nitrofural 5.0 ppm hydrazine was determined in the single batch tested; for nitrofuroxazide, residual hydrazine was reported in 3 out of 5 batches tested with levels ranging from 12.1 to 28.7 ppm and finally in nitrofurazone, hydrazine was reported in all 5 batches, with levels failing a proposed 1 ppm limit in 4 out of the 5 batches tested. Residual levels in nitrofurazone ranged from 0.53 to 19.1 ppm.

The levels of residual hydrazine in the seven excipients and APIs were broadly similar to those reported by the HPLC method (see



Scheme 3. Derivatisation with salicylaldehyde to form salicylazine derivatives.



Scheme 4. Derivatisation with 4-dimethylaminobenzaldehyde to form 4-dimethylaminobenzalazine derivatives.

Table 1). The only major differences being that the TLC method appeared to underestimate the residual hydrazine levels in nitrofuroxazide and nitrofural, which was attributable to fluorescence quenching seen with those APIs and therefore that the residual levels were out with the linear range of the method. The other differences were attributable to the enhanced sensitivity of the HPLC method.

The authors [19] also examined the pharmacopoeial monograph methodology for residual hydrazine in carbidopa API, which has a current limit of 20 ppm. They compared the existing method utilizing salicylaldehyde derivatisation versus a similar method using benzaldehyde derivatisation. The two methods were in good accord, the former showing levels of 3.2 ppm whereas, the latter method showed levels of 3.0 ppm of hydrazine.

Finally, these authors [19] evaluated the residual hydrazine levels in isoniazid API, using the salicylaldehyde derivatisation method. They found levels of 50.6 ppmand commented that the existing monograph method, which involves spraying with dimethylaminobenzaldehyde, was too insensitive and the 50 ppm spot was barely visible.

Researchers at an Indian laboratory [40] used HPTLC to monitor degradation products of rifampicin, including the hydrazones (25-desacetyl rifampicin (DAR) and rifampicin quinone (RQU)). The peaks were quantified by densitometry with an LOD of 10 ng per band. The recoveries in all cases were excellent (99.6% for RQU and 100.3% for DAR). Similarly, linearity was demonstrated in the range 50–300 ng/band for DAR and 100–300 ng/band for RQU. The reproducibility of the method was good with RSDs of ≤1.86% for DAR and ≤2.58% for RQU. The authors indicated that the high sensitivity, precision, simplicity and throughput (50 samples/4 h) of the method made it suitable for routine QC and stability analyses, especially in the developing world.

2.6. Capillary electrophoresis (CE), micellar electrokinetic chromatography (MEKC) and micro-emulsion electrokinetic chromatography (MEEKC)

A Chinese research group [41] reported on the development of a palladium-modified carbon fibre array microdisk electrode for the simultaneous determination of hydrazine, methylhydrazine and isoniazid by capillary electrophoresis (CE). There was a need to modify the electrode because hydrazine is difficult to analyze owing to its high over-potential towards electro-oxidation at ordinary solid electrodes. The method gave a linear response for hydrazine and the LODs were 1.0, 5.0 and 5.0 ppm, respectively, for the three analytes.

The same research group also reported on the simultaneous determination of hydrazine, methylhydrazine and isoniazid by CE [42]. In this case the authors utilized a 4-pyridyl hydroquinone self-assembled microdisk platinum electrode for the detection of the three analytes. The linearity of the method for all three analytes over three orders of magnitude was good. The method was linear for hydrazine, methylhydrazine and isoniazid in the ranges: 0.2–400 ppm, 0.2–400 ppm and 0.5–2000 ppm, respectively. The LODs were 0.1, 0.1 and 0.2 ppm, respectively.

Mahuzier et al. [43] reported on a MEEKC method for the determination of degradation products of rizatriptan. The method was shown to be stability-indicating and semi-quantitative with an LOD of 1000 ppm and an LOQ of 2000 ppm (0.2%).

Srinivasu et al. [44] developed a MEKC method for the assay of celecoxib and residual levels of 4-hydrazine benzene sulphonamide (impurity III). The LOD and LOQ for impurity III were 3500 ppm and 12,000 ppm, respectively. The recoveries for impurity III were good (95.5–97.5%) and presence of impurity III and the related impurity IV, had no affect on recoveries of celecoxib. These latter two methods [43,44] illustrate the general disadvantages of poor sensitivity of CE based methods, in comparison to other separation methods.

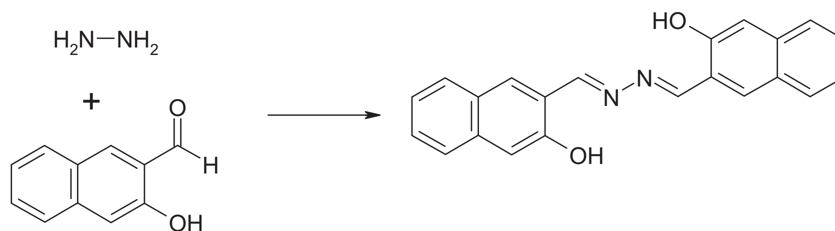
2.7. Spectrophotometry

Devani et al. [45] investigated the formation of aldose reaction products of isoniazid and lactose using UV/visible spectrophotometry. The authors derivatised the isoniazid with 2,3-dichloro-1,4-naphthaquinone and monitored the resultant coloured derivative at 640 nm. The quantitation stage of the method obeyed Beer–Lambert law in the range 1–14 μg/ml. The method was applied to commercial isoniazid tablets and the authors showed significantly lowered recoveries (10–22%) compared to the official compendial method, which was ascribed to the formation of 1-nicotinyl-2-lactosylhydrazine adducts.

A rapid, spectrophotometric determination of residual hydrazine in certain isoniazid formulations was described by Davidson [46]. The method involved in situ formation of the 4-dimethylaminobenzalazine (see Scheme 4) derivative which is unaffected by large excesses of isoniazid. Experimental conditions impacting on selectivity, sensitivity and accuracy were discussed.

A sensitive (sub-ppm) solid-phase spectroscopy method was developed for determining residual hydrazine in a wide variety of sample matrices including isoniazid tablets [47]. The method involves in situ formation of an azine derivative (using 4-dimethyl aminobenzaldehyde), which is fixed onto a cation exchange resin and the derivative is assayed at 464 nm. The method was linear in the range 1–12 ppm, with an LOD of 0.26 ppm and an LOQ of 0.87 ppm. The authors compared this method with other spectrophotometric methods and found it to be significantly more sensitive (10,000× greater than conventional solution methods).

A fluorescence method for the determination of residual hydrazine in isoniazid formulations was developed by Mañes et al. [48]. The method is based on the use of the derivatising agent 2-hydroxy-1-naphthaldehyde which forms the 2,2′-hydroxy-1-naphthaldazine (DHND) derivative (see Scheme 5), which is extracted into chloroform before direct analysis. This derivatisation reaction is specific to the analyte and interference is minimal, as isoniazid rapidly forms the very stable non-fluorescent isonicotinylhydrazone of 2-hydroxy-1-naphthaldehyde. There was no reported interference from any common excipients. The method is linear over the range 10–160 ppb of hydrazine in the presence of 500 ppm of isoniazid. This equates to residual hydrazine levels of 2–32 ppm in isoniazid formulations. The LOD of the method is 3.2 ppb. The method is accurate and precise at the 80 ppb level with



Scheme 5. Derivatisation with 2-hydroxy-1-naphthaldehyde to form 2,2′-hydroxy-1-naphthalazine derivatives.

a standard deviation of 1.9 ppb, the RSD was 2.2% and the standard error was 1.6%. The recoveries from isoniazid preparations were in the range 98.7–102.5%.

The method can also be applied to iproniazid formulations with similar performance. In the case of hydralazine a yellow reaction product strongly interferes. For nialamide and phenelzine there are interfering fluorescent reaction products, but if the APIs are diluted to 250 ppm, then the method has an LOD of 80 ppm of residual hydrazine in these formulations.

3. Conclusion

Considering the huge challenges inherent in the determination of very low levels of volatile, reactive hydrazines and related compounds in APIs, the breadth of analytical techniques utilized is striking. Derivatisation has been utilized as one of the main analytical strategies and this has ensured that spectrophotometric, as well as chromatographic (HPLC, GC, TLC, and IC) and electrochromatographic methods (CE, MEKC and MEEKC) have been widely applied.

HPLC, almost exclusively in reversed phase mode, remains a key separation technique and the favoured derivative using this approach has been benzalazine (see Scheme 1). There are, however two very recent examples of non-derivatised hydrophilic interaction chromatography (HILIC) and this may become the favoured analytical approach in the future [25,26].

The majority of references employed HPLC with single-wavelength UV/visible detection, the derivatisation approach precluding the need for more sensitive detection approaches.

Similarly, the literature shows the same emphasis when using GC as the separation technique again using the benzalazine derivative [11,36,37]. There are though recent reports of acetone-azine derivatives [22] being used, which may see greater utilization in the future.

There are also several literature references to the use of CE and related electrokinetic chromatography techniques (MEKC and MEEKC). For instance there are two examples of CE being used for the determination of hydrazine and methylhydrazine in isoniazid [41,42]. In both cases the authors utilized electrochemical detection to overcome the intrinsic issue of the high over-potential of these analytes towards oxidation using standard electrodes. There was one reported example of MEKC for the determination of residual levels of 4-hydrazine benzenes sulphonamide in celecoxib. However, many literature reports continue to highlight the perennial issue of lack of detector sensitivity.

There are several reports of the use of ion chromatography [19,21,38,39], exclusively in the non-derivatised mode with amperometric detection. Kirtley et al. [21] reported that the advantages over the standard derivative HPLC method were increased selectivity and reduced matrix interference.

Considering the sensitivity requirements (ca. 1 ppm, but dose-dependent), TLC methods surprisingly, still feature heavily in this review and can generate equivalent data compared to more sensitive techniques e.g. HPLC [19] (see Table 1). All of the TLC methods reviewed used derivatisation, but here the favoured derivatising reagent was salicylaldehyde, rather than benzaldehyde.

There were two examples of spectroscopy methods, which assessed residual hydrazine levels in isoniazid/iponiazid [48] and isoniazid [47]. The former method utilized fluorescence spectroscopy and used 2-hydroxy-1-naphthaldehyde derivatisation; whereas, the latter utilized UV/visible spectroscopy and used 4-dimethyl aminobenzaldehyde (DMAB) derivatisation. Interestingly, by fixing the DMAB derivatising agent onto a cation exchange resin, the sensitivity of the UV/visible method was markedly increased.

Substrate interference appears to be less of a restricting factor when derivative analysis is employed with HPLC techniques. However, there are still some reported examples of liquid–liquid extraction (LLE) techniques utilized for HPLC methods [19,21,50,51] and one reported example of liquid solid extraction (LSE) techniques [21]. In contrast, extraction and/or pre-concentration techniques, such as static head-space (HS) analysis [22] and more particularly liquid–liquid extraction (LLE) are used routinely to reduce matrix interference in GC methodologies [11,36,37]. For ion chromatography, one of the research groups utilized an ion exchange clean-up procedures [21]. TLC methods used extraction techniques almost exclusively. Finally, none of the CE or electrokinetic methods used extraction techniques, perhaps a testament to the greater selectivity of these techniques.

Importantly, for the practicing analyst there have been several generic methods developed covering the three main analytical approaches; i.e. HPLC [26], GC [22] and IC [19].

In conclusion, a variety of approaches have been reported whereby hydrazine and its related species can be analysed encompassing both chromatographic, spectroscopic and electro-chemical techniques. This case is due in large part to the use of derivatisation coupled with very sensitive detection methods.

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