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Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 1011–1040

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Review

Development of validated stability-indicating assay methods—critical review

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Received 11 October 2001; received in revised form 4 January 2002; accepted 19 January 2002

Abstract

This write-up provides a review on the development of validated stability-indicating assay methods (SIAMs) for drug substances and products. The shortcomings of reported methods with respect to regulatory requirements are highlighted. A systematic approach for the development of stability-indicating methods is discussed. Critical issues related to development of SIAMs, such as separation of all degradation products, establishment of mass balance, stress testing of formulations, development of SIAMs for combination products, etc. are also addressed. The applicability of pharmacopoeial methods for the analysis of stability samples is discussed. The requirements of SIAMs for stability study of biotechnological substances and products are also touched upon.

Keywords: Stability-indicating assay methods; Regulatory requirements; Development; Critical issues

1. Introduction

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. With the advent of International Conference on Harmonisation (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products.

A review of literature reveals a large number of methods reported over the period of last 3–4 decades under the nomenclature 'stability-indicating'. However, most of the reported methods fall short in meeting the current regulatory requirements.

Accordingly, the purpose of this write-up is to suggest a systematic approach for the development of validated SIAMs that should meet the current ICH and regulatory requirements. The discussion also touches upon various critical issues, such as the extent of separation of degrada-

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00047-X

tion products, establishment of mass balance, etc., which are important with respect to the development of stability-indicating assays, but are not yet fully resolved. Some other aspects like suitability of pharmacopoeial methods for the purpose and the role of SIAMs in stability evaluation of biological/biotechnological substances and products are also delved upon.

2. Regulatory status of stability-indicating assays

The ICH guidelines have been incorporated as law in the EU, Japan and in the US, but in reality, besides these other countries are also using them. As these guidelines reflect the current inspectional tendencies, they carry the de facto force of regulation. The ICH guideline Q1A on Stability Testing of New Drug Substances and Products [1] emphasizes that the testing of those features which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stability-indicating testing methods. It is also mentioned that forced decomposition studies (stress testing) at temperatures in 10 °C increments above the accelerated temperatures, extremes of pH and under oxidative and photolytic conditions should be carried out on the drug substance so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures. The ICH guideline Q3B entitled 'Impurities in New Drug Products' emphasizes on providing documented evidence that analytical procedures are validated and suitable for the detection and quantitation of degradation products [2]. It is also required that analytical methods should be validated to demonstrate that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the drug product. The ICH guideline Q6A, which provides note for guidance on specifications [3], also mentions the requirement of stability-indicating assays under Universal Tests/Criteria for both drug substances and drug products. The same is also a requirement in the guideline Q5C on Stability Testing of Biotechnological/Biological Products [4]. Since there is no single assay or parameter that profiles the stability characteristics of such products, the onus has been put on the manufacturer to propose a stability-indicating profile that provides assurance on detection of changes in identity, purity and potency of the product.

Unfortunately, none of the ICH guidelines provides an exact definition of a stability-indicating method. Elaborate definitions of stability-indicating methodology are, however, provided in the United States-Food and Drug Administration (US-FDA) stability guideline of 1987 [5] and the draft guideline of 1998 [6]. Stability-indicating methods according to 1987 guideline were defined as the '*quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.*' This definition in the draft guideline of 1998 reads as: '*validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference.*' The major changes brought in the new guideline are with respect to (i) introduction of the requirement of validation, and (ii) the requirement of analysis of degradation products and other components, apart from the active ingredients(s).

The requirement is also listed in World Health Organization (WHO), European Committee for Proprietary Medicinal Products and Canadian Therapeutic Products Directorate's guidelines on stability testing of well established or existing drug substances and products [7–9].

Even the United States Pharmacopoeia (USP) has a requirement listed under 'Stability Studies in Manufacturing', which says that samples of the products should be assayed for potency by the use of a stability-indicating assay [10]. The requirement in such explicit manner is, however, absent in other pharmacopoeias.

Current ICH guideline on Good Manufacturing Practices for Active Pharmaceutical Ingredients (Q7A), which is under adoption by WHO, also clearly mentions that the test procedures used in stability testing should be validated and be stability-indicating [11].

3. Review of the literature on stability-indicating assays

In absence of any guidance from regulatory agencies on practical steps to be followed for establishment of stability-indicating assays, a search was done on the available information in literature. The literature was found to be replete with publications on development of stability-indicating assays of specific drugs. A general review was published as early as 1971, and it gave general principles and discussed the methods developed till that period [12]. Kumar and Sunder also discussed the perspective of stability-indicating testing procedures [13]. Subsequently, Ho and Chen [14,15] reviewed stability-indicating high-performance liquid chromatography (HPLC) assay methods reported till 1996. A compilation of stability-indicating assays (>500) for various drugs was published in 1999 by Xu and Trissel [16]. A more recent publication is in the form of a chapter in the book 'Drug Stability: Principles and Practices' by Carstensen and Rhodes [17], which provides general discussion on HPLC method development and validation, with emphasis on stability-indicating assays. On whole, a critical guidance document on the topic, which encompasses current ICH requirements and discusses various critical issues, is still elusive.

4. An assessment of the extent to which the reported methods meet current regulatory requirements

A review of various literature reports shows that very few methods that are titled or claimed to be stability-indicating fit into the current definition of a stability-indicating assay in true sense. While the current requirement is of subjecting the drug substance to variety of stress conditions and then separation of drug from all degradation products, many studies have just shown the separation of drug from known synthetic impurities and/or potential degradation products without subjecting it to any type of stress (Table 1). There are also reports in which drug has been decomposed by exposing it to one (Table 2), two (Table 3), three (Table 4), four (Table 5) or more (Table 6) conditions among acidic, neutral or alkaline hydrolysis, photolysis, oxidation and thermal stress. Thus very few studies are truly stability-indicating, where drug has been exposed to all types of stress conditions and attempts have been made to separate the drug from degradation products and the latter among themselves. Different approaches have been employed in these cases, in absence of any defined requirements. There are some reports where directly the formulation, instead of the drug substance, has been subjected to stress studies for establishment of the stability-indicating behavior (Table 7). A few reports exist even on combinations of drugs (Table 8).

It may be pertinent to add here that the examples cited in Tables 1–8 are only representative and do not mean comprehensive coverage of all literature reports.

5. Techniques employed in literature reports

If one critically evaluates the literature reports, titrimetric, spectrophotometric and chromatographic techniques have been commonly employed in analysis of stability samples. There are also sporadic reports of the use of miscellaneous techniques.

5.1. Titrimetric and spectrophotometric

In these methods, usually the objective is the analysis of the drug of interest alone in the matrix of excipients, additives, degradation products, impurities, etc., and also other drugs in case of the combination products. Their advantage is the low cost and simplicity, though sometimes they are not sensitive. Due to limitation of specificity, there are hardly any reports these days on their

use for the assay of stability samples. However, a few reports involving derivative spectroscopy have been published lately [122–125].

5.2. Chromatographic

Because of the very nature of requirement of separation of multiple components during analysis of stability samples, chromatographic methods have taken precedence over the conventional methods of analysis. Other than separation of multiple components, the advantage of chromatographic methods is that these possess greater accuracy and sensitivity for even small quantities of degradation products produced. Various chromatographic methods that have been used are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), HPLC and newer technique like capillary electrophoresis (CE).

TLC is a simple technique that has been used in the past for developing SIAMs [126–128]. Its disadvantages, such as variability and non-quantitative nature, limit its use as a basic technique for SIAM development. However, it is very much used, especially during initial degradation [129] and stress studies to study the number of degradation products formed, to identify the products formed through matching studies using standards, and even for isolation where preparative TLC is employed.

A large number of publications have appeared in the last decade on the use of HPTLC for stability-indicating method development

Table 1
Selected reports of 'stability-indicating' methods where no stress testing has been done

	Drug	Methodology	Ref.
Separation from process impurities	Benazepril hydrochloride	HPLC	[18]
	Ribavirin	HPLC	[19]
Separation from known/potential degradation product(s)	Betamethasone 17-benzoate	HPLC	[20]
	Canrenone	HPLC	[21]
	Erythromycin estolate	HPLC	[22]
	Ethacrynic acid	HPLC	[23]
	Phenylbutazone	HPLC	[24]
	Sibutramine hydrochloride	HPLC	[25]
	Sulphacetamide	HPLC	[26]
	Homatropine methylbromide	UV spectrophotometry	[27]
	Cholesterol lowering drug	Micellar electrokinetic chromatography	[28]
	Felodipine	Supercritical-fluid chromatography	[29]
Separation from known/potential degradation products and process impurities	Benzodiazepines	HPLC	[30]
	Ranitidine	HPLC	[31]
	Temazepam	HPLC	[32]
	Melphalan	HPLC	[33]
	Piroxicam	HPTLC	[34]
	Tinidazole	HPTLC	[35]
	Fenclorac	GLC	[36]
	Azathioprine	CE	[37]

Table 2
Selected reports of 'stability-indicating' methods where only one stress condition has been employed

Stress condition	Drug	Methodology	Ref.
Acid	Dyclonine hydrochloride	HPLC	[38]
	Flunarizine dihydrochloride	HPLC	[39]
	Lisinopril	UV spectrophotometry	[40]
	Norfloxacin	UV spectrophotometry	[41]
	Lisinopril	Derivative UV spectrophotometry	[42]
Alkali	Allantoin	HPLC	[43]
	Meperidine hydrochloride	HPLC	[44]
	Metronidazole	HPLC	[45]
	Benazepril hydrochloride	UV spectrophotometry	[46]
	Carbachol	IR spectrophotometry	[47]
Neutral	Physostigmine salicylate	HPLC	[48]
Oxidation	Nortriptyline hydrochloride	UV spectrophotometry	[49]
Light	Atenolol	HPLC	[50]
	Danazol	HPLC	[51]
	Trifluoperazine hydrochloride	HPLC	[52]
	Nifedipine	HPTLC	[53]
	Ranitidine hydrochloride	Spectrodensitometric TLC	[54]
	Piroxicam	HPLC, HPTLC, CE	[55]

[34,35,53,63,69,74]. This technique overcomes the shortcomings of TLC, and is reliable, fast and accurate for quantitative drug analysis. Moreover, many samples can be run simultaneously using a small quantity of mobile phase, thus minimizing analysis time and cost per analysis. Unfortunately, its limitation is that the equipment is not routinely available in every laboratory.

GC is stability-indicating but it is not very versatile, as the drug substance may be non-volatile or thermally unstable. Further any attempt to increase the volatility of the drug and components by increasing the temperature may lead to degradation or racemization. Therefore, there are very few reports on the use of GC [130–132] for the purpose of establishment of SIAMs.

In comparison, HPLC has been very widely employed. It has gained popularity in stability studies due to its high-resolution capacity, sensitivity and specificity. Non-volatile, thermally unstable or polar/ionic compounds can also be analyzed by this technique. Therefore, most of the SIAMs have been established using HPLC, which is evident from the lists given in Tables 1–8.

5.3. Miscellaneous

A few studies have also reported the use of proton nuclear magnetic resonance (NMR) spectroscopy for the development of SIAMs [133–135]. CE is the latest entry to the techniques for the development of SIAMs [37,136–138]. It has the advantage of high sensitivity, resolution and high efficiencies with minimal peak dispersion.

Table 3
Selected reports of 'stability-indicating' methods where two stress conditions have been employed

Stress conditions	Drug	Methodology	Ref.
Acid, alkali	Betaxolol hydrochloride	HPLC	[56]
	Captopril	HPLC	[57]
	Cephalexin	HPLC	[58]
	Ciprofloxacin	HPLC	[59]
	Indapamide	HPLC	[60]
	Omeprazole	HPLC	[61]
	Yohimbine hydrochloride	HPLC	[62]
	Nimesulide	HPTLC	[63]
Acid, light	Trimethoprim	HPLC	[64]

Table 4
Selected reports of 'stability-indicating' methods where three stress conditions have been employed

Stress condition	Drug	Methodology	Ref.
Acid, neutral, alkali	Diaziridinyl benzoquinone	HPLC	[65]
	Xilobam	UV assay supported by TLC and HPLC	[66]
Acid, alkali, light	Carprofen	HPLC	[67]
	Nitrendipine	HPLC, HPTLC and UV spectrophotometry	[68]
	Estradiol	HPTLC	[69]
Acid, alkali, oxidation	Mefenamic acid	HPLC	[70]
	Morphine	HPLC	[71]
Acid, alkali, dry heat	Amphotericin B	HPLC	[72]
	Hydrochlorthiazide	HPLC	[73]
	Timolol maleate	HPTLC	[74]

There are several publications involving use of hyphenated GC-MS [139], LC-MS [140–147], LC-MS-MS [141,142,145,148], LC-NMR [141,147] and CE-MS [149,150] techniques for identity confirmation of known and unknown degradation products and their selective determination.

6. Development of validated SIAMs that are likely to meet regulatory requirements

Though the requirements with respect to SIAM have been spelt out in regulatory documents, information on the basic steps to be followed for the development and validation of stability-indicating methods is neither provided in the regulatory guidelines nor in the pharmacopoeias. Therefore, the practical steps involved in the development of SIAMs are discussed below. It is expected that by following the steps, one should be in a position to develop a SIAM that would meet the regulatory requirements. Our discussion is typically oriented towards development of SIAMs by HPLC, as it is found that 85–90% of the methods reported in literature are by this technique.

6.1. Step I: critical study of the drug structure to assess the likely decomposition route(s)

This should be the first element whenever one takes up the project on establishment of a SIAM. Much information can simply be gained from the structure, by study of the functional groups and other key components. There are definite functional group categories, like amides, esters, lactams, lactones, etc. that undergo hydrolysis [151], others like thiols, thioethers, etc. undergo oxidation [152], and compounds like olefins, aryl halo derivatives, aryl acetic acids, and those with aromatic nitro groups, N-oxides undergo photodecomposition [153].

Most of the new drugs are congeners of existing drug molecules, and there are very few new drugs, which originate from absolutely new leads. For a new congener, its degradation chemistry can be easily postulated based on the reported behavior of other drugs in the series. For example, there are more than 40 penicillins in clinical practice today and almost all of them follow the same degradation behavior at the beta-lactam moiety. Most of them also follow similar subsequent reactions (Fig. 1). Similarly, studies in our laboratory have shown that three alpha-adrenergic blockers (prazosin, doxazosin and terazosin) that have similar parent structure follow the same hydrolysis route involving breakage of the amide bond (Fig. 2).

However, a word of caution is that in some congeners there might even be existence of a totally new degradation behavior, particularly when there is overwhelming influence of the substituent. A typical example here is that of aminopenicillins, which show formation of polymers [154]. Other example is that of 2-methyl-5-ni-

troimidazole series of drugs, including metronidazole, ornidazole, tinidazole and secnidazole. Fig. 3 shows the known decomposition behavior of these drugs. Tinidazole and secnidazole are reported to decompose in alkaline conditions to the parent nucleus 2-methyl-5-nitroimidazole [155,156], while metronidazole degrades to simple compounds like ammonia and acetic acid [157]. In contrast, ornidazole neither yields the nucleus nor undergoes complete decomposition. It yields ornidazole diol via an intermediate ornidazole epoxide in alkaline medium [158].

Thus one can have a good starting point from the study of degradation behavior of congeners, but critical requirement here is the conduct of an in-depth literature survey. For information on degradation chemistry of like drugs, one can look into the treatises like *Analytical Profiles of Drug Substances* [159] and the monographs provided by Connors et al. [151]. Specific searches can even be made through the use of abstracts, the internet search engines and the Chemweb.

6.2. Step II: collection of information on physicochemical properties

Before method development is taken up, it is generally important to know various physicochemical parameters like p*K*a, log *P*, solubility, absorptivity and wavelength maximum of the drug in question. The knowledge of p*K*a is important as most of the pH-related changes in retention occur at pH values within ± 1.5 units of the p*K*a value. The ionization value also helps in selecting the pH of the buffer to be used in the mobile phase [160]. The knowledge of log *P* for the drug and the identified degradation products provides good insight into the separation behavior likely to be obtained on a particular stationary phase. p*K*a and log *P* can be practically determined or even theoretically calculated using the commercial software, such as Pallas (CompuDrug Chemistry Ltd., Budapest, Hungary), CLOGP (Pamona College, Pamona, USA), etc.

The analysis of the drug or degradation products requires that they are soluble in HPLC compatible solvents in the first place. The availability of the solubility data in aqueous, organic and commonly used HPLC solvents and their combinations can thus prove to be very useful in the selection of the sample solvent and the mobile phase.

As the HPLC analysis employing a UV detector is usually carried out at the wavelength maximum or at a wavelength where all components show good absorbance, therefore, the necessity to know the wavelength maxima and extinction of the drug and degradation products in different solvents and at different pH becomes an absolute requirement. This may be an easy exercise when the degradation products are known and available in the pure form. But when it is a new drug for which degradation pattern has not yet been estab-

Table 5
Selected reports of 'stability-indicating' methods where four stress conditions have been employed

Stress conditions	Drug	Methodology	Ref.
Acid, neutral, alkali, oxidation	Clonazepam	HPLC	[75]
	Dipyridamole	HPLC	[76]
	Esmolol hydrochloride	HPLC	[77]
Acid, alkali, oxidation, dry heat	Suprofen	HPLC	[78]
Acid, neutral, alkali, light	Guanabenz	UV spectrophotometry	[79]
	Tolmetin sodium	UV spectrophotometry	[80]
Acid, alkali, light, thermal (methanolic solution)	Retinoic acid	HPLC	[81]
Acid, alkali, oxidation, light	Trimetazidine	HPLC	[82]
	Trimetazidine	HPTLC	[83]
Acid, oxidation, light, dry heat	Fentanyl	HPLC	[84]

Table 6
Selected reports of 'stability-indicating' methods where five (and additional) stress conditions have been employed

Stress conditions	Drug	Methodology	Ref.
Acid, alkali, oxidation, dry heat, light	Sodium levothyroxine	HPLC	[85]
	Enalapril maleate	HPLC	[86]
Acid, alkali, oxidation, dry heat, light (separation from synthetic impurities also seen)	Sildenafil citrate	HPLC	[87]
Acid, neutral, alkali, oxidation, light	Nicardipine hydrochloride	HPLC	[88]
Acid, alkali, oxidation, dry heat, wet heat, light dry, light wet	Paroxetine	HPLC	[89]
Acid, alkali, oxidation, dry heat, light, reduction	Cyproterone acetate	HPLC	[90]
Acid, alkali, light, oxidation, dry heat, moisture, sonication	Buspirone hydrochloride	HPLC	[91]

lished, the same might prove to be a difficult exercise. In the latter case, the suggested way is to subject the drug to stress studies (see Section 6.3 for details) and to observe changes in the spectrum [161], first individually in each reaction solution and then in a mixture of all the solutions. This gives a fair idea (though not absolutely) on the shifts in wavelength spectra during the reaction and also guides on the best wavelength for analysis. If necessary, more than one wavelength can be selected for analysis, but taking the benefit of the same requires a dual or multi-wavelength detector. The best choice thence is using a photodiode array (PDA) detector, which allows recording of UV–visible spectrum of the components, as they get resolved on the stationary phase. Later necessary inferences can be made based on the records.

6.3. Step III: stress (forced decomposition) studies

The next step in the development of SIAM is the conduct of forced decomposition studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed: (i) 10 °C increments above the accelerated temperatures (e.g. 50 °C, 60 °C, etc.), (ii) humidity where appropriate (e.g. 75% or greater), (iii) hydrolysis across a wide range of pH values, (iv) oxidation and (v) photolysis. However, the guideline provides no details on how hydrolytic, photolytic and oxidative studies have to be actually performed. On the other hand, the information is available in literature but in a staggered way, with suggested approaches differing a lot from one another [162,163]. A comprehensive document providing guidance on the practical conduct and issues related to stress testing under variety of ICH prescribed conditions has been published lately [164]. This report from the authors proposes a classification scheme and offers decision trees to help in the selection of the right type of stress condition in a minimum number of attempts.

The hydrolytic degradation of a new drug in acidic and alkaline conditions can be studied by refluxing the drug in 0.1 N HCl/NaOH for 8 h. If reasonable degradation is seen, testing can be stopped at this point. However, in case no degradation is seen under these conditions, the drug should be refluxed in acid/alkali of higher strengths and for longer duration. Alternatively, if total degradation is seen after subjecting the drug to initial conditions, acid/alkali strength can be decreased along with decrease in the reaction temperature. In a similar manner, degradation under neutral conditions can be started by refluxing the drug in water for 12 h. Reflux time should be increased if no degradation is seen. If the drug is found to degrade completely, both time and temperature of study can be decreased.

To test for oxidation, it is suggested to use hydrogen peroxide in the concentration range of 3–30%. The photolytic studies should be carried out by exposure to light, using either a combination of cool white and ultraviolet fluorescent lamps, or one among the xenon and metal halide lamps. Exposure energy should be minimum of 1.2 million lux h fluorescent light and 200 W h/m^2 UV and if decomposition is not seen, the intensity should be increased by five times. In case still no decomposition takes place, the drug can be declared photostable.

A minimum of four samples should be generated for every stress condition, viz. the blank solution stored under normal conditions, the blank subjected to stress in the same manner as the drug solution, zero time sample containing the drug which is stored under normal conditions and the drug solution subjected to stress treatment. The comparison of the results of these provides real assessment of the changes. Furthermore, it is advised to withdraw samples at different time periods for each reaction condition. By doing so, one can get a clear idea on the number of products formed, their relative strengths and whether they are stable or unstable, resulting further in newer products. This information is essential in establishment of SIAMs.

The studies should be initiated at a concentration of 1 mg/ml. If solubility is a limitation, varying amounts of methanol may be used to get a clear solution or even the testing can be done on a suspension [165]. By using drug concentration of 1 mg/ml, it is usually possible to get even minor decomposition products in the range of detection. If several degradation products are formed in different conditions, the establishment of SIAM may involve a lot of development work. For this, repeat injections of reaction solutions might be required. Therefore, the volume of samples subjected to stress studies should be in sufficient quantity and also enough sample volume should be drawn at each period. The withdrawn samples can be stored in cold cabinets to stop further reaction. The aliquots might be diluted or neutralized before injecting into HPLC.

Table 7
Reports of 'stability-indicating' methods on drug formulations

Stress conditions	Drug	Dosage form	Methodology	Ref.
Acid	Fluconazole	Admixtures	GC	[92]
	Flucytosine	Extemporaneous solutions	HPLC	[93]
	Levothyroxine sodium	Tablets	HPLC	[94]
	Ipratropium bromide	Metered dose inhalers and inhalation solutions	HPLC	[95]
Acid, alkali	Ganciclovir	Capsules	HPLC	[96]
Light, thermal	Sodium levothyroxine	Tablets	HPLC	[85]
Acid, alkali, oxidation	Pentoxifylline	Suspension	HPLC	[97]
	Granisetron hydrochloride	Injection	HPLC	[98]
Acid, alkali, oxidation, thermal	Chlorobutanol	Ointment	HPLC	[99]
Acid, alkali, oxidation, light	Fotemustine	5% dextrose	HPLC	[100]
	Efavirenz	Capsules	HPLC	[101]
Acid, oxidation, light, thermal	Fentanyl	Injection	HPLC	[84]
Acid, alkali, oxidation, thermal, light	Cyclosporine	Oral solution	HPLC	[102]
Acid, alkali, thermal, light, 45 °C/75% RH for 2 weeks	Aspirin and warfarin sodium	Tablets	HPLC	[103]
Aged samples (3 years at 40 °C and 75% RH)	Losartan	Tablets	HPLC and LC-MS	[104]

Table 8
Selected reports of 'stability-indicating' methods for combination of different drugs

Stress condition(s)	Drug	Dosage form	Methodology	Type of study	Ref.
No stress	Otilonium bromide and diazepam	Finished pharmaceutical dosage forms	HPLC	Separation from related compounds and potential degradation products of both drugs shown	[105]
	Naphazoline and tetrahydrozoline	Ophthalmic preparations	HPLC	Separation seen using potential degradation products of both drugs	[106]
	Captopril and hydrochlorthiazide	Tablets	HPLC	Separation seen using potential degradation products of both drugs	[107]
	Sulfonamides and erythromycin ethyl succinate	Oral suspension	HPLC	Separation seen using potential degradation products of both drugs	[108]
	Hydroxyzine hydrochloride and benzyl alcohol	Injection	HPLC	Separation seen using potential degradation products of both drugs	[109]
Oxidation	Oxycodone and lidocaine	Rectal gel	HPLC	Stress studies done on individual drugs	[110]
Acid, alkali	Ramipril and hydrochlorthiazide	Different dosage forms	HPLC	Only ramipril exposed to stress conditions. Both drugs analyzed in presence of ramipril degradation products	[111]
Thermal	Minocycline hydrochloride and rifampicin	Intravenous solutions	HPLC	Stress studies done on individual drugs. Samples analyzed individually using different stability-indicating methods for each drug	[112]
Acid, neutral, alkali	Hydrochlorthiazide and triamterene	Capsules	HPLC	Capsules subjected to stress studies	[113]
Acid, alkali, oxidation	Pseudoephedrine and cetrizine	Pharmaceutical dosage forms	HPLC	Stress studies done on individual drugs	[114]
	Cisatracurium besylate and propofol	Mixtures	HPLC	Mixtures subjected to stress studies	[115]
Acid, alkali, thermal, light, 45 °C/75% RH for 2 weeks	Aspirin and warfarin sodium	Tablets	HPLC	Tablets subjected to stress studies and spiking with warfarin sodium related substances done	[103]

Table 8 (*Continued*)

Stress condition(s)	Drug	Dosage form	Methodology	Type of study	Ref.
Acid, alkali, thermal, light	Phenylepherine hydrochloride, phenylpropanolamine hydrochloride and guaifenesin	Capsules	HPLC	Well-mixed capsule material exposed to acidic and alkaline hydrolysis, thermal degradation and photolysis	[116]
Acid, alkali, oxidation, light	Paclitaxel with ondansetron hydrochloride or ranitidine hydrochloride	Admixtures in 5% dextrose	HPLC	Only paclitaxel subjected stress studies. Drugs analyzed by individual assays	[117]
Acid, alkali, thermal, oxidation, light	Acetazolamide, allopurinol, azathioprine, clonazepam, flucytosine	Extemporaneous oral solution	HPLC	Stress studies done on individual drugs and composite chromatogram after degradation in all conditions shown. Samples analyzed individually using different stability-indicating methods for each drug	[118]
	Baclofen, captopril, diltiazem hydrochloride, dipyridamole and flecainide acetate	Extemporaneous oral solution	HPLC	Stress studies done on individual drugs and composite chromatogram after degradation in all conditions shown. Samples analyzed individually using different stability-indicating methods for each drug	[119]
	Hydrocodone bitartarate and acetaminophen	Tablets	HPLC	Stress studies performed only on hydrocodone	[120]
Acid, neutral, alkali, thermal, light and/or high humidity	Norgestimate and ethinyl estradiol	Tablets	HPLC	Norgestimate subjected to acid, neutral, alkali, thermal and light degradation. Ethinyl estradiol subjected to thermal and high humidity stress. Separation shown for each drug in the presence of degradation products of other	[121]

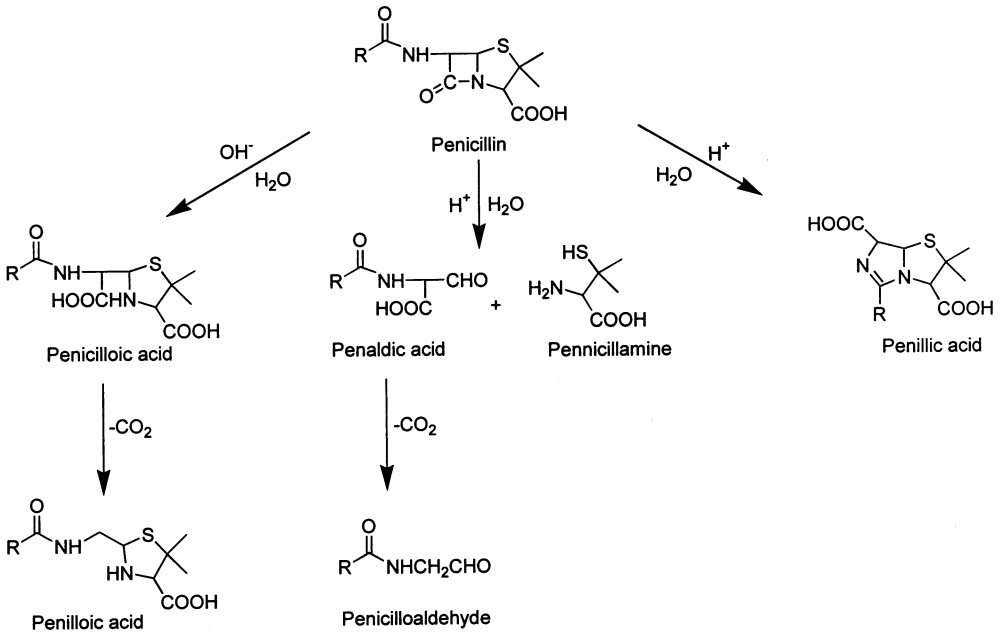

Fig. 1. Known degradation behavior of penicillins under different hydrolytic conditions.

6.4. Step IV: preliminary separation studies on stressed samples

The stress samples so obtained are subjected to preliminary analyses to study the number and types of degradation products formed under various conditions. For doing so, the simplest way is to start with a reversed-phase octadecyl column, preferably a new or the one in a healthy condition. Well-separated and good quality peaks at the outset provide better confidence because of the unknown nature of products formed during stressing. It should be preferred to use water–methanol or water–acetonitrile as the mobile phase in the initial stages. The use of buffers is not suggested at this stage because as is normally required, one can extend the buffer-free mobile phase to preparative LC or LC-MS studies. Between methanol and acetonitrile, the former should be preferred due to its low cost. The wisdom from previous studies on the development of assay method for the drug can also be applied here and the organic modifier can be chosen accordingly. The solvent can be changed, if the peak shape or separation problems are seen.

Initially, water:organic modifier ratio can be fixed at 50:50 or can be suitably modified so as to obtain the capacity factor of around 5–10 for the drug. As degradation products from drugs are generally polar in nature (of course with exceptions), pushing the drug peak to say $\sim$15 min or somewhat more in a 25-cm column, can result in separation of even several degradation products, when formed. The retention time can be brought earlier or pushed further by changing the mobile phase but it should not be pushed very far, as though it might lead to an overall increase in resolution (and ruggedness), but oppositely the peaks flatten out resulting in a decrease in sensitivity. Normally, the total run time should be 2.5 times more than the drug peak, at least in initial studies, and this long period is to show up any peak that would elute later to the drug peak.

The detection wavelength can be set, based on the study of spectral behavior of degraded samples, as discussed earlier. The injection volume and the flow rate can be suitably adjusted based on the length of the column.

Using these chromatographic conditions, one should follow the changes in all the stress sam-

ples, at various time periods. The results should be critically compared with the blank solutions injected in a similar manner. It should be observed whether the fall in drug peak is quantitatively followed by a corresponding rise in the degradation product peaks. It should not be taken as a surprise if the peak rise is not in correspondence to fall of the drug. This is because the drug and its products can have very different extinction values. Even there can be situations where no additional peak appears in the chromatogram, other than the drug. A typical example is given in Fig. 4 where the drug fall is clearly seen, but with no additional peak rise. Such a situation can either arise due to the formation of non-chromophoric products or due to decomposition of drug to low molecular weight fractions. In such situations, the detection at multiple wavelengths or the use of LC-MS becomes necessary. Sometimes the absence of simultaneous rise in degradation product peak might also be due to total insolubility of the product in the reaction solutions, which can be confirmed through physical observation of the reaction mixture. In such case the product can be separated and can be injected separately using the solvent in which it is soluble to find out its retention time (RT) in the chromatogram. Later, during the final method development changes can be made in mobile phase or the sample solvent to have the product shown up in the chromatogram. Even the absence of degradation peak can happen when the product is colored and shows no UV absorption at a particular wavelength at which the analysis has been conducted. This can be verified by simple observation whether any color has developed in the reaction solution. Here also suitable adjustment in the wavelength of analysis can be made for the product to appear in the chromatogram.

6.5. Step V: final method development and optimization

Subsequent to preliminary chromatographic studies, the RT and relative retention times (RRT) of all products formed should be tabulated for each reaction condition. Special attention is then paid to those components whose RT or RRT is very close. PDA spectra or LC-MS profile of such components are obtained and critically evaluated to ascertain whether the products are same or different. It has happened with us once that what we were considering as a drug peak, proved rather to be due to the degradation product. The drug peak appeared at a particular RT in acid conditions, but when the reaction was done in alkali, again a peak appeared at almost same RT. However, the LC-MS studies indicated it to be a different product. It was later established that the drug was almost instantly converted when brought in contact with the alkali and the product was formed quantitatively. Therefore, if PDA or

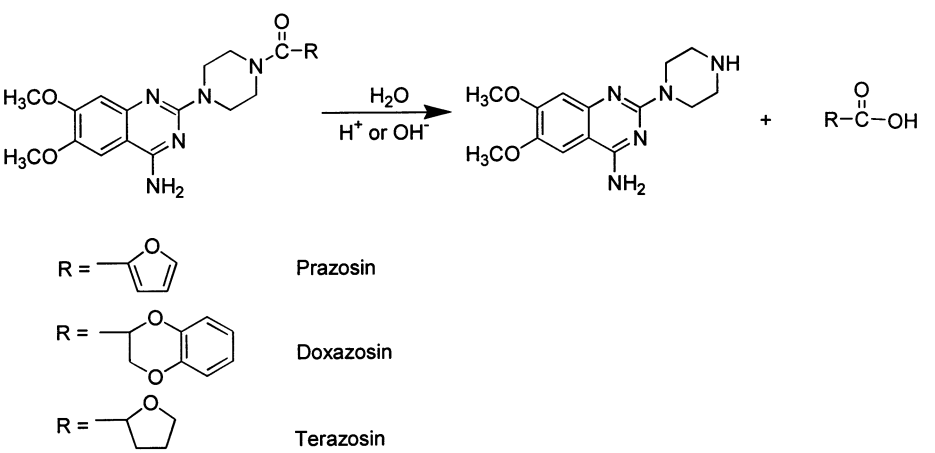

Fig. 2. Hydrolysis of different alpha-adrenergic blockers.

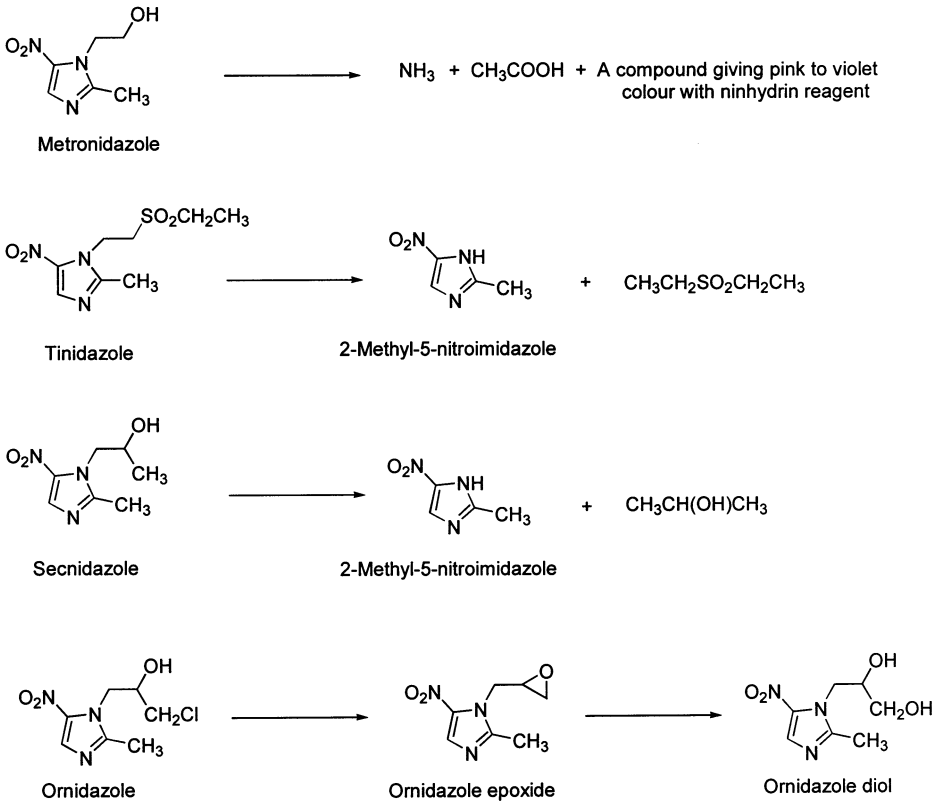

Fig. 3. Reported degradation products of different 2-methyl-5-nitroimidazoles.

LC-MS results suggest that any of the products are different but are co-eluting, then suitable modification should be done in the chromatographic method to achieve a satisfactory resolution.

In the final step, a mixture of the reaction solutions is prepared, and subjected again to resolution behavior study. While making this mixture, it is not always necessary to add all reaction solutions withdrawn at different time for all conditions. That would make the situation too complex. Rather, only those solutions are mixed where different products are formed in sufficient quantity. Resolution in the mixture is studied closely, to see whether the resolution is similar to that obtained in individual samples. This is important to rule out any changes that can happen when reaction solutions of different pH and media (3–30% hydrogen peroxide solution) are mixed. There might be a situation where products show different chromatographic behavior in a mixture.

To separate close or co-eluting peaks, the method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type. Details of the basic issues in method development are not discussed here, as they are covered elsewhere [17].

A typical example of the study in author's laboratory where the desired separation was achieved in a mixture of various reaction solutions is shown in Fig. 5. The steps undertaken in optimization of the developed method can be found in a published report [166].

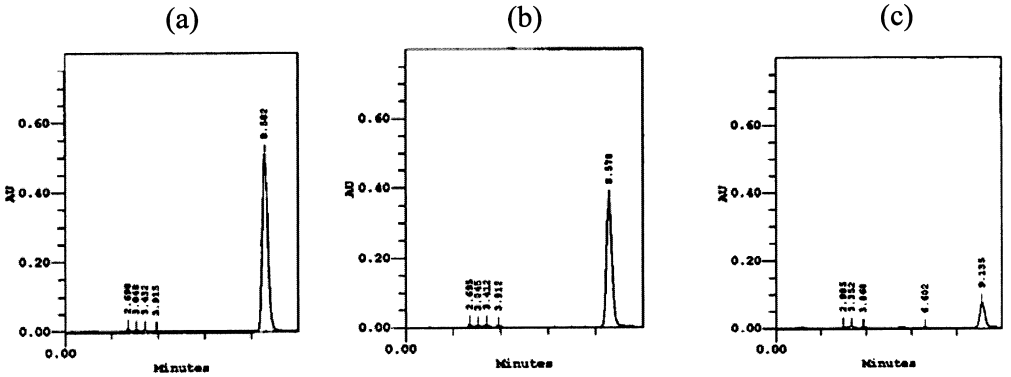

Fig. 4. Degradation of metronidazole in acidic conditions under light: (a) initial sample (b) 3 day sample (c) 12 day sample.

6.6. Step VI: identification and characterization of degradation products, and preparation of standards

Before moving to the validation of a SIAM, it is necessary to identify the drug degradation products and arrange for their standards. These are required to establish specificity/selectivity of the method. The work on this aspect can even be initiated once an idea on the nature and number of degradation products formed under different degradation conditions is obtained from preliminary separation studies.

To identify the resolved products, a conventional way is to isolate them and determine the structure through spectral (MS, NMR, IR, etc.) and elemental analysis. However, this approach is tedious and time consuming when multiple degradation products are formed. Against it, the modern approach is to use hyphenated LC techniques coupled with mass spectrometry. This strategy integrates in a single instrument approach, analytical HPLC, UV detection, full scan mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS-MS) and provides a fair idea on identity of resolving components. These days a further integrated approach is becoming popular wherein LC-MS or LC-MS-MS is employed to obtain molecular weight and fragmentation information, and further detailed structural information is obtained through LC-NMR analysis. The integrated approach provides rapid and unambiguous identification of several degradation products at one time.

Regarding the product standards, a direct way is to procure them from commercial sources (Section 8). However, in case they are not available commercially, they have to be either isolated from the degradation reaction solutions or synthesized in the laboratory. To isolate a product, the best way is to identify a reaction condition where it is formed selectively. If the product precipitates or

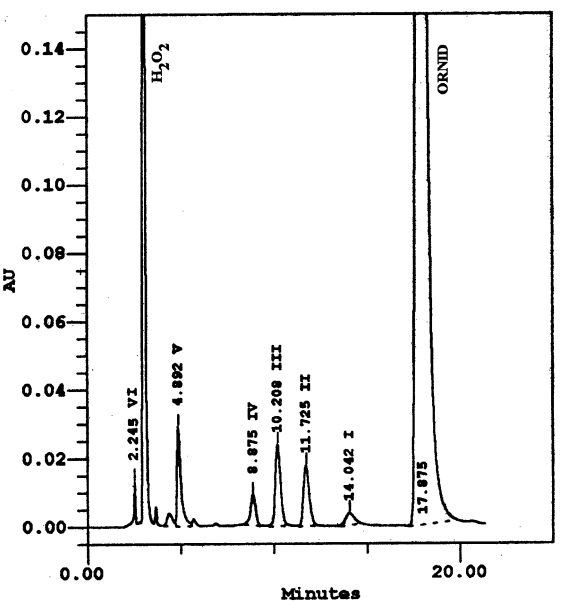

Fig. 5. Example of a 'Selective SIAM' showing separation of different degradation products of ornidazole in a mixture of reaction solutions. (Reprinted from Ref. [166] with permission from authors and Elsevier Science).

crystallizes on its own on completion of the reaction, it can be recovered simply. Otherwise, the reaction mixture can be lyophilized directly. If freeze-drying is done after neutralization of the reaction mixture, the product can be recovered by extraction with dry methanol or any other suitable dry solvent. The recovery can also be made by selective extraction with an organic solvent after acidification, neutralization or basification of the solution, depending upon the initial pH. Subsequently the extraction solvent can be evaporated to recover the product. A note of caution here is that one must check whether the product of interest decomposes further on change of pH, as it so happens frequently, as experienced by the authors.

When no condition is identified where the product is formed quantitatively into single entity, then the product can be isolated from the mixture by selective solubility based extraction, preparative TLC or preparative HPLC. Use can also be made here of normal column chromatography, medium-pressure liquid chromatography, chromatotron, flash chromatography, etc.

If the identity of the products has been previously established through sophisticated LC-MS and/or LC-NMR studies, the envisaged molecules can be synthesized, characterized and the presence confirmed through spiking in the degraded sample. The synthesis route has the advantage that it results in a much neater product than can be obtained through isolation.

6.7. Step VII: validation of SIAMs

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2A and Q2B [167,168], in the FDA guidance [169] and by USP [170]. There are several other reports in literature, which have reviewed the concept, either in general [171,172], or specifically the validation of spectroscopic [173], non-chromatographic [174] and chromatographic [175] methods. Numerous other investigations on development of SIAMs on different drugs also encompass validation steps, and a critical study of these reports give a fair idea on how validation can be carried out practically.

Overall, there are two stages in the validation of a SIAM. First stage is early in the development cycle when drug substance is subjected to forced decomposition studies and the SIAM is established based on the knowledge of drug degradation behavior. The main focus of validation at this stage is on establishment of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness, etc. The limits of detection and quantitation are also determined for degradation products to help in establishment of the mass balance. This validated method finds application in the analysis of stability samples of bulk drug for determination of its retest or expiry period. In the second stage, when the SIAM so developed is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents. Here only parameters of critical importance like specificity/selectivity, accuracy and precision are revalidated. If the SIAM is being developed directly for a formulation, without involving the bulk drug route, then all validation parameters are necessary to be established.

The specificity/selectivity of a SIAM can be established very simply if degradation chemistry of the drug is known and the standards of the products are available. The only effort involved then is the development of a method that separates components from a physical mixture of drug and the degradation products. At this stage, only peak purity becomes crucial. The peak purity can be established by a variety of techniques, like PDA detection, absorbance ratio method, dual wavelength ratio chromatography, second order derivative spectroscopy, spectral suppression, spectral overlay, etc. [176]. However, not all these are applicable for on-line peak purity testing. The most popular technique is the PDA analysis, the principle of which is the comparison of the spectra of the analyte peak, taken upslope, at the apex and on the downslope. If these spectra do not match then the peak is non-homogeneous. A limitation of the PDA detection for peak homogeneity testing is that this technique is not very sensitive and hence it is unlikely to detect $< 1\%$

of an interfering component in an analyte peak. Further limitation is the high cost of the detector. The normal UV HPLC detectors these days allow for simultaneous measurement at multiple wavelengths, and some of them even give output of ratio plots at two wavelengths. This technique has also been promoted for peak purity testing during development of SIAMs [177]. The technique requires critical selection of measuring wavelengths and is of limited use where the UV spectrum of the co-eluting component is unknown. The second derivative spectroscopy can also be employed to assess peak non-homogeneity, as it amplifies slight deviations from Gaussian peak shape caused by overlapping peaks. Another approach that can be employed is the collection of fractions from the peak and comparing the results with a significantly different chromatographic technique or mass spectrometry. The construction of kinetic plots during drug decomposition [177] is an additional validation step that can be used to confirm specific analysis during establishment of SIAMs.

The accuracy is usually determined by spiking the known amount of drug to either the placeboes or the formulations, and determination of percent recovery of the drug. However, a better method of determining accuracy of a SIAM is by spiking the drug in a mixture of degraded solutions [166]. As far as the precision is concerned, there are no special requirements for stability-indicating methods and the same procedure as advocated for normal assay methods can be applied.

The linearity for SIAMs should be established initially in the range of 0–100%, as the drug may fall to very low concentrations during forced decomposition studies. The final validation range, however, can be narrowed based upon the form in which the drug substance or formulation is dispensed. For example, it may vary from 80 to 120% for solid bulk drug and stable solid formulations. The range may be 50–120% in case of injections or other formulations where the drug is more prone to degradation. Validation range for the degradation products during stability studies usually should vary from 0 to 20% [171].

The detection and quantitation limits are not important for active drug substances, as their concentration is not expected to fall to such a low level in different formulations during their shelf life. However, these limits should be established for the degradation products.

Robustness can also be established for SIAMs in a similar manner as it is done for conventional methods.

7. Some critical issues concerning development of SIAMs and their validation

There are several other issues concerning development of SIAMs on which routinely the questions are asked and clarifications are sought. The important ones are discussed below.

7.1. Definition of 'Specific' and 'Selective' stability-indicating assay methods

The foremost issue is the lack of clarity on the terms used for differentiating the methods that measure quantitatively the component of interest in the sample matrix without separation, and the ones where separation is done of the drug as well all other degradation products. Hong and Shah [17] describe the former as '*stability-specific*', while the discriminating nature of the latter is described as being the combination of '*stability-indicating*' and '*stability-specific*'. Unfortunately, the term *stability-indicating* has been invariably used in the vast number of publications in literature to describe even the so-called 'stability-specific' methods. Here we would rather suggest the use of terms '*Specific stability-indicating*' and '*Selective stability-indicating*' for defining, respectively, the two types of assays. Thus 'Specific stability-indicating assay method (Specific SIAM)' can be defined as 'a method that is able to measure unequivocally the drug(s) in the presence of all degradation products, excipients and additives, expected to be present in the formulation.' The 'Selective stability-indicating assay method (Selective SIAM)' on the other hand can be defined as 'a method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in the formulation.' By this definition, it means that a 'Selective SIAM' is a procedure

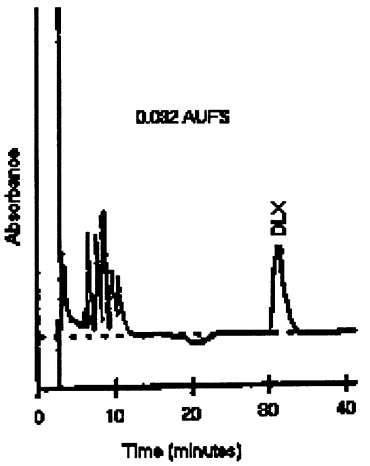

Fig. 6. Example of a 'Specific SIAM' showing degradation of dicloxacillin (DLX) to cluster of products in acid conditions. (Reprinted from Ref. [177] with permission from authors and Elsevier Science).

that is selective to the drug as well its degradation products (separates all of them qualitatively) and is also specific to all the components (measures them quantitatively).

Thus all titrimetric methods employed and reported in literature for the purpose can be classified under 'Specific SIAMs'. The UV methods also fall in this category. In these methods, despite the absence of separation, the analyte of interest is determined quantitatively and specifically.

The chromatographic methods, however, can be of both types—'Specific SIAM' as well as 'Selective SIAM'. In case of the former, the method is not fully separative to all components, but does separate the drug equivocally. This normally is a situation where efforts fail to separate degradation products when they are large in number. Two typical examples of 'Specific SIAM' are shown in Figs. 6 and 7. One of the examples is of dicloxacillin (Fig. 6), and most other penicillins show similar type of behavior [177,178]. Penicillins, as discussed earlier, are very facile undergoing degradation through a complex route (Fig. 1) resulting in multiple products under every condition. The other example (Fig. 7) is of photolytic decomposition of alpha-adrenergic receptor blocking agents where seemingly a free radical mediated reaction results in a series of products. The chromatograph shown in Fig. 5 is a typical example of a 'Selective SIAM' where all degradation products formed under all conditions are well-separated from each other and hence there is possibility of simultaneous and quantitative estimation of the drug as well as the degradation products.

7.2. Does 'Specific SIAM' also has a purpose and is acceptable?

The question thence arises, as to which of the method among the 'Specific' and 'Selective' meets the requirements of ICH and other regulatory guidelines. Certainly, it is the 'Selective SIAM',

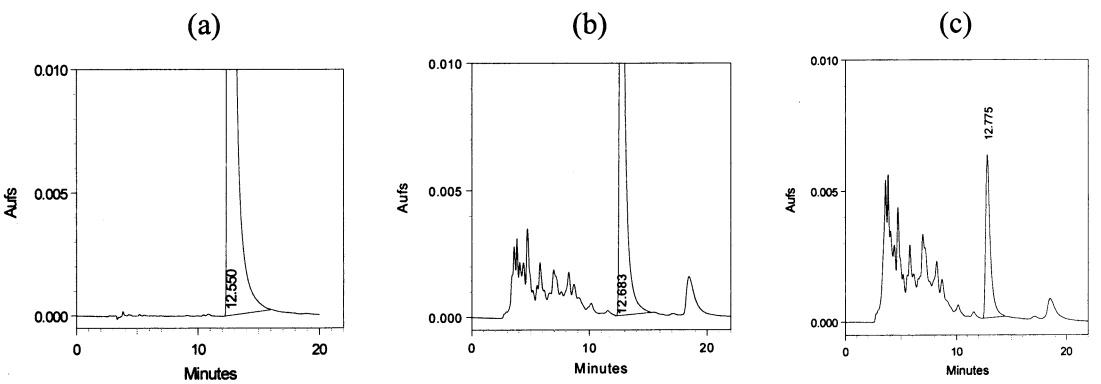

Fig. 7. Another example of 'Specific SIAM' showing formation of cluster of degradation products during photolytic decomposition of prazosin under acid conditions: (a) initial sample (b) 4 day sample (c) 8 day sample.

which the regulatory officers would love to see in the drug registration dossiers. In the opinion of the authors, the 'Selective SIAM' is that of more importance with respect to new drugs, but for old and established drugs, where significant body of information exists; the use of 'Specific SIAM' can do an equally good job during stability sample analysis. This is the reason that the pharmacopoeias, other than USP, have a policy to continue with the titrimetric and spectrophotometric analysis for the assay of drugs, while having control on important degradation products through related substance or impurity tests [179].

The authors suggest that even in the case of new drugs, the use of a 'Selective SIAM' may not be absolutely necessary in all situations. A 'Specific SIAM', if it is less cumbersome and costly, and is proved to give the same results as a 'Selective SIAM' can be used for post-approval follow-up stability testing and also for analysis of market surveillance and returned samples. This can spare chromatographic instrumentation that can be used for other routine and important applications.

The published or pharmacopoeial 'Specific SIAMs' also find use in countries where there is no regulatory requirement of a 'Selective SIAM'. However, preference should be given to those published methods where the specific method has been compared with a selective one [180].

7.3. Is it really necessary to follow the stress-testing route to develop a SIAM?

This is another dilemma that has often been expressed, particularly by the practitioners in industry. Even to the experience of the authors, there are several instances, like shown in Figs. 6 and 7, where large number of degradation products is formed during forced decomposition of drug even in one stress condition. In such situation, it might be truly difficult or impossible to develop a 'Selective SIAM' if degradation products formed under all conditions are simultaneously taken into consideration. Moreover, it has been expressed that some degradation products formed during forced decomposition are never developed in the stability samples. A typical example again is that of ornidazole where several

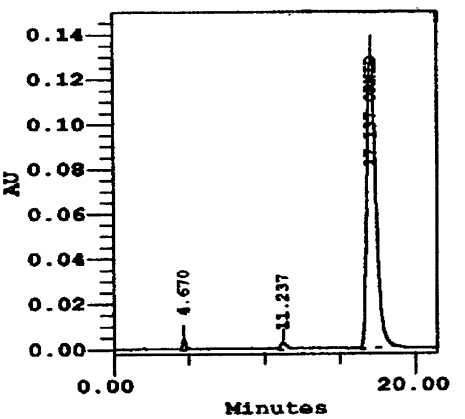

Fig. 8. Chromatogram showing the products formed after subjecting ornidazole infusion to accelerated testing.

products are formed when stressed samples in different stress conditions are mixed together (Fig. 5). Against this, the infusion samples of the drug subjected to stability tests showed only two products (Fig. 8), which are previously identified major degradation products [158]. So a tagged question is 'Should only major degradation products be targeted while developing a SIAM, instead of all degradation products formed under the ICH suggested test conditions?' Interestingly, the dilemma is well answered in the ICH guideline itself [165] where the clarification is provided in the statement '*However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions.*'

Therefore, it emerges that a SIAM separating all types of possible degradation products should normally be developed through stress testing under different ICH suggested conditions. In case, however, it is not possible to develop a 'Selective SIAM' due to the complex nature of degradation, one can target for a method that takes into account degradation products only formed under accelerated and long-term storage conditions. In any case, here it would be needed to be proved through sufficient trials that separation of degradation products formed under various conditions is a difficult proposition, and that only a few or major products are found actually in stability samples of long-term and accelerated studies.

The authors at a personal level are also convinced that the regulatory approach of development of a SIAM through stress testing under variety of conditions is a sound approach. Once such a method is established and is validated, its distinct advantage would be that it could be applied to specific determination of drug and degradation products in a broad range of situations. It can eliminate the necessity of modification and revalidation each time when the method is extended from bulk drug analysis to formulation or one formulation to another. The exception might be interference due to excipients and additives, where modification and revalidation may be unavoidable.

7.4. Can formulations instead of drug substance be subjected to stress (forced decomposition) studies for development of a SIAM?

This is another interesting aspect, tagged with the above point. It is whether formulations instead of drug substance can be directly subjected to stress conditions for the development of a SIAM. In true sense, ICH guideline Q1AR and the ICH's Common Technical Document [181] suggest stress testing only of the drug substance. For drug products, however, a definition of 'Stress Testing (drug product)' is provided in Q1AR, which reads as '*Studies undertaken to assess the effect of severe conditions on the drug product. Such studies include photostability testing (see ICH Q1B) and specific testing on certain products, (e.g. metered dose inhalers, creams, emulsions, refrigerated aqueous liquid products).*' [182]. This means there is no suggestion on conduct of stress studies directly on formulations, other than photostability testing.

However, Table 7 shows that there exist a few literature reports where stability-indicating assay has been established by carrying out stress tests directly on pharmaceutical formulations. Looking into it more objectively, this approach seems to be rational for use by the manufacturers involved in production of formulations alone, albeit in specific situations. For new drugs, the information on intrinsic stability behavior of the drug substance and the stability-assay method is usually kept secret by the innovators to protect even this element from exploitation. However, there can be situations where companies in countries where innovator has not sought patent protection manufacture formulations of these drugs, by arranging the drug substance from varied sources. This is practically happening today. Therefore, in such cases if the formulation manufacturer does some sort of stress testing directly on the drug product and uses an analytical method developed on that basis, it can better cover the consumer's risk. The same can even be a case with formulations containing existing and pharmacopoeial drugs, where a bit of stress tests followed by method development can be carried out by the generic formulation manufacturers. This way at least the influence of excipients, additives and package on the degradation behavior can be encompassed directly in the analytical method.

The only hitch in implementation of this approach perhaps is that no stress conditions, except the photostability testing, have been defined for stress testing of formulations in any current guidelines. This might be due to the reason that formulations do not withstand stringent stress conditions. But the positive aspect of this approach is that the formulator can at least consider degradation products formed 'realistically' in the formulation environment during the method development. To the authors, a scientifically sound step in this regard would be to extend ICH recommended stress test conditions for drug substance to the formulations. For example, the recommended [165] dry heat stress condition of 10 °C increments above the accelerated temperature (e.g. 50, 60 °C) can be extended easily to the drug formulations. For those dosage forms, which show severe physical instability at high temperatures, the increments can be reduced to 5 °C or even lower, as applicable. The liquid formulations can be easily tested after changing the pH from low to high. Oxidative stress testing can also be done by purging oxygen or through the addition of hydrogen peroxide. Another stress condition can be 40 °C/90% RH, which can be applied to observe the effect of high humidity in case of solids, semisolids, etc.

It is suggested that scientists involved in drug stability testing research and regulatory authorities should give a serious thought to this approach, and if merit is found, then the above suggested stress conditions can be a starting point for discussions. The approach can be of specific and practical use to manufacturers outside the developed world, where there is a need to provide simple stability test solutions to help improve the quality of drug products in circulation, than available at present.

7.5. The trauma of establishment of SIAMs for combination drug formulations

A large number of formulations around the world are sold as combination preparations of two or more drugs. As the number of drugs in a formulation increases, the complexity per se increases. Therefore, the development of SIAM for formulations that contain more than one drug becomes a real tedious exercise. The effort is compounded, first due to separation of multiple drugs from one another and then from the degradation products, which might be any number. The latter are also supposed to be separated among themselves. This extent of complexity is same whether the combination formulation is a manufactured product or an extemporaneously produced preparation. The development of a 'Selective SIAM' certainly can be a nightmarish experience if it is expected that every drug substance in the formulation (some combinations may contain 6–10 drugs) is subjected to forced decomposition studies in a variety of conditions, and all solutions for all the drugs are pooled and separated on a HPLC column.

Thus for combination formulations, the approaches suggested above of targeting separation of degradation products formed only in long-term and accelerated stability test conditions or limited stressing of formulations, seem to be the best option. This issue again requires thorough discussions and should be settled among the scientists and regulatory agencies for the benefit of manufacturers.

7.6. The aspect of mass balance in development of SIAMs

The mass balance is a process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of the initial value, with due consideration of the margin of analytical error. This is the definition of mass balance given in the ICH parent drug stability guideline [1]. Its establishment hence is a regulatory requirement. The mass balance is very closely linked to development of a SIAM as it acts as an approach to establish its validity. The balance would not be achieved unless all degradation products are separated well.

If a few specified and stable degradation products are formed, which can be separated easily and for which the standards are available, then the establishment of mass balance becomes an easy affair. By the use of the standards, one can easily determine the exact response factors and hence the levels of the products. However, there might be many situations where the mass balance may be difficult to establish. This can happen due to one or more of the following situations [183]:

- Formation of multiple degradation products, involving complex reaction pathways and drug-excipient interaction products
- Incomplete detection due to loss of UV chromophore or lack of universal detection
- Loss of drug/degradation products as volatiles
- Diffusive losses into or through containers
- Elution/resolution problems
- Inappropriate or unknown response factors due to lack of standards
- Errors and variability in the drug content assay

It is common for the drugs to degrade into multiple degradation products. This may be so in a single reaction condition (Figs. 6 and 7) or when samples of different stress conditions are mixed (Fig. 5). Multiple products can be formed through parallel, consecutive or chain reactions, which may occur even together. A typical example is of penicillins, where several complex reactions go together (Fig. 1). Similarly, there can be development of new products due to interaction of drugs with the excipients. Achieving separation and accounting for level of multiple degradation prod-

ucts can be an involved exercise, becoming more tedious, if there are any unstable intermediary products and when the standards of one or more are not available.

A similar complexity is added when the intermediates or final products are not shown up due to their non-UV absorbing nature. The authors observed this during acid decomposition of ornidazole, where there was no corresponding rise of peak with the loss of the drug peak [166]. It has been estimated that $>7\%$ of drugs degrade with the formation of products devoid of or with reduced UV response. At times, the lack of UV transparency of solvents and buffers also limits the detection of compounds with absorptivity only at <220 nm. In all such situations, the use of alternate refractive index (RI), evaporative light scattering, MS, NMR and IR detectors is advocated. However, their disadvantage is that they restrict the types of chromatography, for example, use of gradient elution is incompatible with RI detector, and non-volatile buffers are incompatible with evaporative light scattering and MS detectors. In exactness, no detector is available which is universally applicable. Furthermore, the response factor differences of unknowns as compared to the drug are an issue with absorbance and fluorescence detectors, though it is a smaller problem in case MS, NMR or IR detection is used.

The problems in establishment of mass balance are also encountered if the products are volatile and are lost before completion of analysis. For example, metronidazole decomposes to acetic acid and ammonia, both volatile components, on hydrolysis in alkali [157]. As a matter of fact, almost more than 20% of drugs degrade with production of volatile components. Then there might be physical losses like diffusion into plastic containers e.g. nitroglycerin, diazepam, diltiazem, benzyl alcohol, etc. are all lost to PVC bags. Even there can be loss of volatile component through glass bottles due to exchange of compounds via the closures.

There can even be situations where some of the products are strongly bound to stationary phase and do not elute or elute after very long periods. The other situation can be those where despite best efforts, the products fail to resolve completely. A typical example of the latter is given in Fig. 7. In such situations again, there can be difficulties in achieving the mass balance.

As discussed briefly earlier, the availability of reference standards of degradation products is a very important factor in establishment of mass balance. Even at the global level, the acquisition of reference standards of impurities and degradation products is a difficult proposition. Only a few and major ones are generally available, but in that case too, the costs are high and there is also a problem of long delivery periods. Making one's own standards is again a difficult exercise, requiring facilities for confirmation of structure and purity. This is a time-consuming task, and also a costly affair, requiring expert manpower and sophisticated analytical instrumentation. The lack of standards results in inappropriate or unknown response factors, acting again as a bottleneck in the establishment of mass balance.

Finally, the mass balance may not also be established due to reasons of errors and variability in the drug content assay. The design of analytical method, the calculation approaches, etc. have to be absolute to get the correct results. There might be other issues, like area percent methods have lower sensitivity and overestimate minor components. On the other hand, external standard methods have higher sensitivity and lower errors, but are much more complex to perform.

So there might be a number of situations where one may not be able to attain a mass balance. Keeping all these difficulties into view, a change in stance is taking place at the regulatory level on the mass balance requirements. The changes that have been brought in are as follows:

(i) The original ICH guideline Q1A contained along with the definition of mass balance (already given above), an additional paragraph '*This concept is a useful scientific guide for evaluating data but it is not achievable in all circumstances. The focus may instead be on assuring the specificity of the assay, the completeness of the investigation of routes of degradation, and the use, if necessary, of identified degradants as indicators of the extent of degradation via particular mechanisms.*' [184]. This paragraph has been removed in the revision.

(ii) The original statement in the text of ICH guideline Q1A [185] reads under both 'drug substance' and 'drug product' as '*Any evaluation should consider not only the assay*, ***but the levels of degradation products*** *and other appropriate attributes*.' Under drug product, the following additional statement exists: '*Where appropriate, attention should be paid to reviewing the adequacy of the mass balance, different stability and degradation performance*'. The first sentence has been changed in the revised guideline Q1AR under the drug product to '*Any evaluation should consider not only the assay* ***but also the degradation products*** *and other appropriate attributes*.' [186]. Evidently, there is exclusion of emphasis on 'levels' of the degradation products in the revision for 'drug product'.

(iii) Changes have also been made with respect to mass balance in ICH guideline Q3A entitled 'Impurities in New Drug Substances' where the whole paragraph '*A summation of assay value and impurity levels generally may be used to obtain mass balance for the test sample. The mass balance need not add to exactly 100% because of the analytical error associated with each analytical procedure. The summation of the impurity level plus the assay value may be misleading, e.g. when the assay procedure is non-specific, (e.g. potentiometric titrimetry) and the impurity level is relatively high*.' has been removed [187].

Therefore, the authors can only suggest that if the establishment of mass balance becomes possible during development of a SIAM, it is very fine. Otherwise, it must be kept in mind that a method may be valid for other parameters even when the mass balance is not observed. Hence any efforts towards compulsory establishment of mass balance should not be at the altar of sacrificing basic characteristics, like specificity, precision, ruggedness, etc. The problems encountered in establishment of mass balance must be clearly defined and indicated in the pertinent part in the registration application. The truthful projection of the difficulty can be helpful, as the possible failures in meeting the mass balance requirement have been increasingly realized by the regulators.

7.7. Are pharmacopoeial methods stability-indicating?

This is again a general dilemma. The authors also found a lot of question–answers taking place on this issue in the discussion groups spread over the web. To get an answer to this question, one has to really understand the structure of a pharmacopoeial monograph. As has been discussed briefly above also, the compendial monographs usually control critical decomposition products through separate tests for related substances and impurities, and the purity tests. It is for this reason that assay methods prescribed in the monographs have classically and primarily been designed to be stability-specific ('Specific SIAM') by nature, and not meant to be selective to each decomposition product and other constituents in the drug substance or formulation. The whole class of titrimetric methods is a simple example here as in these methods the target is the drug alone. Specific examples, cited during discussion on the web, are the assay for calcium gluconate that is based on the determination of calcium by complexation with EDTA. This method does not tell anything about the possible chemical transformations of gluconate, hence can not be used for the analysis of stability samples. The principle behind the polarimetric assay of dextrose is based on proportionality of optical rotation to the potency. This assay can not be considered stability-indicating, as the optical rotation being an additive property, remains equal to the original value even when dextrose has degraded to its constituent sugars.

Therefore, it can be said, that pharmacopoeial methods historically were not 'Selective SIAMs' and perhaps, as exemplified above, there are several, which are still not. However, with the advent of technology allowing resolution of an article into its components and introduction of ICH guideline Q1A, in which there is a clear mandate for simultaneous analysis of degradation products, the situation has changed over

the period. The ICH guideline was printed in USP 23 and made official, though it has been removed in USP 24 due to restraint on printed pages. The USP contains a large number (there were > 2000 in USP 23) of assays and tests based on HPLC and several of them supposedly are 'selective' by nature. USP also defines Category II analytical methods that are meant for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products and provides data elements required for validation of these. Thus USP fully recognizes the necessity of compendial methods to be 'Selective'.

Interestingly, the 'Monograph Development: Guidance to Manufacturers' in British Pharmacopoeia, prescribes that '*For bulk drug substances, it has been BP policy generally to use a robust and precise method of assay (such as titration) rather than a specific, but sometimes less precise, stability-indicating method (such as liquid chromatography). Wherever possible, control of potential impurities is provided separately by means of specific impurity tests. It is appreciated, however, that a manufacturer may use, and therefore propose, a chromatographic method for both related substances and assay. In such circumstances, each case is judged on its merits on the basis of the data provided, which must relate to validated methods.*' Separately it is stated that '*The method of assay will not necessarily be that used for the bulk drug substance. For formulations a specific, stability-indicating method is preferred*' [188]. Evidently, there is a slightly different approach in BP as compared to USP, although there is an endeavor for shifting to 'Selective' methods.

The authors, however, feel that even the compendial chromatographic methods should be validated for their 'selectivity' by the end user, as there can be cases where a potentially stability-indicating pharmacopoeial assay does not prove to be so when applied to actual formulations. This is in line with the validation requirements given in the ICH/WHO guideline on GMP of APIs, which clearly states that '*The suitability of all testing methods used should nonetheless be verified under actual conditions of use and documented*' [189].

8. Commercial availability of standards of degradation products

The success of establishment of validated SIAM and also the mass balance depends much on the availability of standards of degradation products. Therefore, a brief discussion on the commercial sources from where one can acquire them would be pertinent here. The standards for old and established degradation products controlled by pharmacopoeial monographs can be procured from the respective pharmacopoeial authorities (www.pheur.org; www.usp.org; www.promochem.com). Also, there are other national and international organizations that supply the standards, and help can be taken in this regard from a comprehensive list of globally available standards published annually by the WHO (WHO/EDM/QSM/2001.2). Apart from this, there are independent agencies that also supply these standards, and information on such sources can be assessed through internet, using search engines like, Altavista, Yahoo, Google, etc. It may be pertinent to add here that the author's lab at NIPER specializes in separation, synthesis and supply of degradation products and those interested can check the institute's web site (www.niper.nic.in) for an updated list.

9. The emerging techniques for analysis of stability samples

As discussed under the instrumental methods employed in literature reports and elsewhere in the text, there is an increasing trend in recent times on involvement of hyphenated techniques (GC-MS, LC-MS or LC-MS-MS, CE-MS, LC-NMR, etc.) at various stages in development of SIAMs. Their use is picking up due to easy availability of bench-top instrumentation and their distinct advantages, like versatility; sensitivity; possibility of profiling, substructural analysis and rapid selective quantitative determination of targeted products even in mixtures. The only limitation yet is the heavy cost of instrumentation, due to which their use is not common and spread

worldwide, like simple GC, HPLC, CE, or NMR systems. These sophisticated techniques as of today are being used mainly for the purpose of monitoring, characterization and identification of impurities, degradation products, metabolites, etc. However, there is a good scope of their use in routine quantitative analysis of stability samples, as their cost per analysis tends to be much lower than conventional techniques. Therefore, developments in the applications and quantitative use of these techniques must be followed and watched with interest.

Other than hyphenated techniques, Fourier-transform near-infrared (FT-NIR) spectroscopy is another emerging technique, which holds lot of promise. The instrument works on the principle of Kubelka–Munk function, and determines the fragment of light reflected from the sample depending upon scattering and absorption of light [190]. It has a distinct advantage that it allows analysis of drugs directly in the dosage forms, without even the need of sample preparation, thus eliminating the use of extraction solvents and hassles involved in their disposal. Being a non-destructive technique, it has capability to analyze drug in tablets, powders, solids, liquids or pastes. Even non-homogenous samples such as multi-layered, coated or cored tablets can be analyzed reliably. It allows measurement in sealed glass containers, and even of sterilized samples without opening. As such, only a small amount of sample is needed for obtaining useful test results. The reproducibility of the test result is ensured. The technique is very fast, with sample time reduced to around 5 s, allowing large number of samples to be analyzed within a short time. Thus the major benefit is that quality standards are maintained while costs and efforts are reduced. It is envisaged that this technique will be very fruitfully employed for the analysis of stability samples, when there are sufficient spectral differences between the drug and the degradation products. Though technically, the assay using FT-NIR would only be 'Specific', but it can be employed selectively if there are only few degradation products formed on storage, which differ structurally among themselves and also from the drug.

10. Making use of computer simulation in development and optimization of SIAMs

As must be realized from the above discussion, the process of development of SIAMs by HPLC is a time consuming and difficult exercise. In general also, there are a large number of interdependent parameters, which exist in the practice of HPLC and the consequent requirement to study these parameters during method development through multiple chromatographic runs makes the situation very difficult overall [191].

A good strategy for development of a SIAM, like any other HPLC method, should require only as many experimental runs as are necessary to achieve the desired result. The manual approach, involving manipulation of experimental variables until the desired separation has been achieved, provides a good understanding of the principles and theory involved and the interaction of the various variables. But unfortunately it is a slow, time consuming and a potentially expensive exercise. These limitations of the manual HPLC method development approach have led to an increased use of computers-based expert systems. These can be used to automate various phases of HPLC process or fit the retention data to various models in order to find the best conditions for a particular separation. The advantages of computer simulation over manual method development are: (i) the computer simulation of chromatographic separations avoids most of the experimental work to be done in chromatographic method development and optimization. Consequently, the cost and time spent in optimization process are dramatically reduced, (ii) once the simulation process begins, it can continue in an unattended manner, and (iii) only the computer is blocked during the optimization process and not the chromatograph, which can be used for other purposes.

Table 9 lists various available expert systems based on their capabilities. More information can be obtained from numerous reports in literature on the subject [192–195].

There are yet not many publications, which have indicated the use of expert systems in the development of SIAMs. However, there are all

the chances that they are being used in the development laboratories in the industry. Due to their distinct advantages, it is worth giving a try and those who are not exploiting them at present, must explore the utility in their set-ups.

11. The SIAM requirements for stability study of biotechnological products

Biotechnological products also undergo degradation during storage. A variety of degradation products arise resulting from deamidation, oxidation, sulfoxidation, aggregation or fragmentation. No single stability-indicating assay or parameter is available that profiles the stability characteristics of biotechnological products, unlike those of chemical drugs. Hence it is a requirement in the ICH guideline Q5C on Stability Testing of Biotechnological Products that the manufacturer should propose a 'stability-indicating profile' that provides assurance that the changes in identity, purity and potency of the product will be detected [4]. Tests for stability should cover those features, which are likely to change during storage and it is required that the tests employed should be product-specific. There are a large number of publications where the use of stability-indicating methodology to determine the shelf life of different biotechnological products has been reported [196–198].

12. Conclusions

As can be seen from the plenty of the examples given in the tables in the text above, the stability-indicating assays have been developed for a large number of drugs for last several decades, starting almost from 1960s. But most of them unfortunately fail to meet the current regulatory requirements of separation and analysis of individual degradation products. Furthermore, there is little guidance provided in the literature on how to establish true 'Selective' stability-indicating methods. In that respect it is hoped that the discussion provided above on development and validation of SIAMs and on several connected issues would be of general and wide interest. It is, however, cautioned that the opinions expressed are purely personal to the authors and do not represent thinking of the regulatory agencies.

Table 9
Selected examples of software employed in HPLC method development

Software	Properties
DryLab	Allows change of one retention variable at a time and predicts separation as a function of that variable. Also predicts separation for any gradient conditions, based upon data for change in gradients
DryLab, ENHANCER	Predicts separation for different chromatographic conditions (column dimensions, particle size, flow rate, etc.)
ICOS, DIAMOND	Allows change of one or more variables at a time and predicts separation as a function of those variables
PESOS	Based on change of one or more conditions, examines experimental chromatograms for best separation
PRISMA model	Software based on correlation of solvent strength and mobile phase selectivity
ELUEX, CHROMSWORD	Expert systems to predict best initial separation conditions on the basis of molecular structure of the sample components
HPLC-METABOLEXPERT, ProDigest-LC, CHROMDREAM	Special purpose programs

The list is only representative and not comprehensive.

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