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ESTIMATION OF GENOTOXIC IMPURITIES BY CHROMATOGRAPHIC TECHNIQUES

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Abstract

In addition to the challenge of setting a more pragmatic limit for GIs, the development of extremely sensitive and robust analytical methods that can adequately monitor GIs at very low levels is very difficult. Also, the pharmaceutical industry has no long-term experience in the use of these methodologies within the factory setting. Thus, analysts make attempts to determine a way for analyzing various GIs by using unique robust methods as far as possible. In this way, simple HPLC/UV or GC/FID methods are usually performed at the first stage, while more complicated LC/MS or LC/MS/MS methods are used as alternatives studied the formation of sulfonate esters as a mechanistic view, and showed that when a slight excess of base is present, there is no discernible reaction rate to form the sulfonate ester and no mechanistic pathway to their formation. From this point of view, the formation of GIs and suspicious substances in the API syntheses can be easily avoided, and therefore this is the preferred option (Robinson, 2010).Finally, it can be mentioned that in such a situation, *in silico* approaches can prove to be more effective solution in terms of time and cost for screening genotoxic compounds. As subjected by Luis and Valerio (2009), high-quality experimental data must be used. In addition, for non-genotoxic carcinogens, QSAR studies can provide a better understanding about the mechanism of carcinogenesis of these compounds. The in silico methods used in agencies have not been specified yet; however, by overcoming the limits these can become an innate part of regulatory systems.

1. Introduction[1, 5]

Genotoxic compounds induce genetic mutations and/or chromosomal rearrangements and can therefore act as carcinogenic compounds. These compounds cause damage to DNA by different mechanisms such as alkylation or other interactions that can lead to mutation of the genetic codes. In general, chemists employ the terms "genotoxic" and "mutagenic" synonymously; however, there is a subtle distinction. Genotoxicity pertains to all types of DNA damage (including mutagenicity), where as mutagenicity pertains specifically to mutation induction at the gene and chromosome levels. Thus, these compounds pose an additive concern to clinical subjects and patients. Considering the importance of this problem, the challenge for regulatory agencies is to form guidelines and standards for the identification and control of genotoxic compounds and their impurities especially in pharmaceuticals.

2. Genotoxic impurities (GIs)[3, 8]

2.1 Sulfonates[2]

- **Use of sulfonstes in pharmaceuticals:[15]**

1) Salt formation is a useful technique for optimizing the physic chemical processing (formulation), biopharmaceutical or therapeutic properties of active pharmaceutical ingredients (APIs), and sulfonate salts are widely used for this purpose. In addition to the advantages of processing, sulfonate salts possess some advantages over other salts such as producing higher melting point of the sulfonated API. This helps to enhance the stability and provide good solubility and may have certain *in vivo* advantages as well.

2) Another benefit of these salts is their high melting point because APIs with low melting points often exhibit plastic deformation during processing which can cause both caking and aggregation. Typically, an increase in the melting point has an adverse effect on aqueous solubility owing to an increase in the crystal lattice energies. Sulfonic acid salts tend to be an exception to this rule, since they exhibit both high melting points as well as good solubility. In addition, as mentioned in the literature, the high solubility and high

surface area of haloperidol mesylate result in enhanced dissolution rates (<2 min in pH 2 simulated gastric media), which are more rapid than the competing common ion formation.

3) **Reaction of sulfonic acids:** Sulfonic acids can react with low molecular weight alcohols such as methanol, ethanol, or isopropanol to form the corresponding sulfonate esters. In general sulfonic acid esters are considered as potential alkylating agents that may exert genotoxic effects in bacterial and mammalian cell systems and possibly carcinogenic effects *in vivo.*

Drugs containing Sulfonate impurities: [14,3]

Amlodipine besylate (Raman *et al.*, 2008), dimethyl sulfate (DMS) in pazopanib hydrochloride (Liu *et al.*, 2009), methyl methane sulfonate (MMS) in imatinib mesylate (Ramakrishna *et al.*, 2008), alkyl sulfonates in flouroaryl-amine (Cimarosti *et al.*, 2010)

2.2 ALKYL HALIDES AND ESTERS[12,7]

Owing to their electrophilic nature, alkylating agents can introduce lesions at nucleophilic centers of DNA. Drug salt formation includes strong acid/base interactions in the presenceof alcohols, and can form impurities such as alkyl halides. As salt formation is a common method in drug formulation processes, alkyl halides exist as impurities in several drugs(Sobol *et al.*, 2007; Elder *et al.*, 2008a).The nucleophilic attack mechanisms of alkylating compounds determine their reactivity against DNA. The SN1 mechanism leads to *O*-alkylation (*O-6*-methylguanine) which is mutagenic but not clastogenic, whereas the SN2 mechanism leads to N-methylation which is clastogenic and not mutagenic. In this group, it seems that bromo compounds are more reactive as compared to chloro compounds (Sobol *et al.*, 2007; Snodin, 2010).

HYDRAZINES:[11,4]

Hydrazine is used as a medicine or as a starting compound for synthesizing some medicines. Hydrazine and some of its *N*-alkyl, *N*-aryl, and *N*-acyl analogues have beensubjected to extensive toxicological evaluations. Hydrazines, hydrazides, and hydrazoneshave structural alerts for genotoxic potential and the metabolism increases their effects.Hydrazines adduct with DNA and the mechanism of adduction

could include the formation of methyl diazanium ions or methyl free radicals. In addition, it seems that hydrazine reacts with endogenous formaldehyde to produce formaldehyde hydrazone. Subsequent to some other reactions, alkylating compounds like diazomethane as the genotoxic moiety are produced (Bercu *et al.*, 2009; Snodin, 2010).

- **Genotoxicity profile**[10,9]

In vitro studies have shown genotoxic effects for three hydrazine derivatives (hydrazines, hydrazides, and hydrazones). These compounds induce gene mutations in human teratomacells, mouse lymphoma cells, and in several strains of bacteria. Hydralazine (1-hydrazinylphthalazine) and its hydrochloride salt are Ames-positive. In another study, 20hydrazine-derivatives were found to induce a direct DNA damage in *Escherichia coli* and of them (80%) were Ames positive as well (Flora *et al.*, 1984; Agency for Toxic Substance and Disease Registry, 1997; Snodin, 2010).The non-carcinogenic effects of hydrazine were also evaluated; however, it was found that hydrazine, methyl hydrazine, 1,1- and 1,2-dimethylhydrazine, and other analogues are carcinogenic in rodents and possibly in human. In addition, it was seen that hydrazine derivatives like hydralazine and its hydrochloride salt were tumorigenic in rodents.

3.4 EPOXIDES[5]

Epoxides are considered as electrophilic compounds owing to the strained epoxide ring. These alkylating agents directly react with DNA. Alkene oxides are more reactive than are oxides and symmetrically substituted epoxides are less reactive than asymmetrically substituted compounds. Some examples for APIs with epoxide impurities are betamethasone acetate, atenolol, and some herbal remedies. Carbamazepine, cyproheptadine, and protriptyline have stable epoxide metabolites.

Drugs containing epoxides:

Lamotrigine,

Amitryptiline

Diclofenac

The metabolism of epoxides mainly involves epoxide hydrolase (EH) and glutathione *S*transferase(GST), which leads to either

detoxification or production of epoxides. These pathways play a key role in the genotoxic action of epoxides (Snodin, 2010).

3.4.1 Genotoxicity profile[12,1]

As indicated in *in vitro* studies, epoxides are genotoxic in bacterial reverse mutation assays, however, other studies have shown different results. Hude *et al.* (1990) reported that 12/51epoxides were non genotoxic in the Ames *Salmonella* assay. In this study, 51 epoxides were assessed with the SOS-Chromo test using *Escherichia coli* PQ37 followed by a comparison with the results of the Ames test. All compounds were tested with and without S9 mixture up to cytotoxicity. In tests without S9 mixture the SOS-repair induction of each experiment was controlled by the response to 4-nitroquinoline-N-oxide, and in tests with S9 mixture, it was controlled with benzo[a]pyrene. In the Ames test, 20 epoxides were tested for mutagenic activity with the *Salmonella typhimurium* strains TA100, TA1535, TA98, andTA1537. By comparing the results of the Ames test and the SOS-Chromo test, it was found that among 51 epoxide-bearing chemicals 39 induced base-pair mutations in at least one Salmonella strain.

Wade *et al.* (1978) studied the mutagenicity of 17 aliphatic epoxides using the speciallyconstructed mutants of *Salmonella typhimurium* that were developed by Ames. It was foundthat all the compounds in the study, with the exception of 2-methyl-3,3,3-trichloropropyleneoxide, *cis-stilbene* oxide, and cyclohexene oxide that were mutagenic in strain TA100 were also mutagenic, but-with reduced sensitivity, in the second strain TA1535. However, none of the epoxides in this study were found to be mutagenic in strains TA1537 and TA98 whichdetect frame-shift mutagens. The results indicate that the mono substituted epoxides are the most potent mutagens and that the addition of a single methyl group to the oxirane ringcould reduce or eliminate mutagenicity.

Glatt *et al.* (1983) investigated 35 epoxides for mutagenicity, using reversion of his-*Salmonella typhimurium* TA98 and TA100 as the biological end-point. The results obtained were negative with the antibiotics oleandomycin, anticapsin and asperlin, the cardiotonic drug resibufogenin, the widely

used para sympatholytic drugs butyl scopolamine and scopolamine, the sedatives valtratum, didovaltratum and acevaltratum, the tranquilizer oxanamide as well as the drug metabolites carbamazepine 10,11-oxide and diethylstilbestrol and oxide. It was found that among the drugs and drug metabolites, only the cytostatic ethoglucide was markedly mutagenic. Three barbiturate epoxides showed very weak mutagenicity only at extremely high concentrations such that the effects were probably of low practical relevance.

3.5 Aromatic compounds[8, 9]

Aromatic compounds involve various impurities; some impurities, such as fentanyl impurities, tremogenic impurities, p-nitrophenol (PNP) that have aromatic structure and aromatic amines will be discussed in this section.

3.5.1 AROMATIC AMINES[3,4]

Primary and secondary aromatic amines (generally after metabolism) generate an electrophilic species and thus produce a positive result in the Ames test when S9 mixture exists. 2, 4-Diaminotoluene, 2, 4-diaminoethylbenzene and a few amines containing a nitro group are direct mutagens. According to the *in vivo* carcinogenicity test, Ames positive compounds produce positive results, although *p*-anisidine and *p*-chloroaniline arenoncarcinogenic in rodent bioassays (Snodin, 2010).

3.5.2 p-Nitrophenol[9]

This synthetic chemical possesses fungicidal activity and is used as a starting material forthe synthesis of some drugs. PNP and other substituted nitro benzenes after reduction produce aryl hydroxylamines or hydroxamic esters which contain electrophilic nitrogenatoms. Thus, the electrophilic atoms might show genotoxic property for these compounds(Eichenbaum *et al.*, 2009).It should be mentioned that negative results were obtained for Ames tests with the various

strains of *Salmonella typhimurium* in the absence and presence of metabolic activation withrat liver S9. Another *in vitro* test, the hprt mutation test in Chinese hamster ovary (CHO)cells presented the same result as the Ames test for PNP. However, it was seen that PNPcould induce chromosomal aberrations in mammalian cells, particularly in the presence of

metabolic activation. Also, PNP was negative in the bone marrow micronucleus assay in mice at doses ranging from little toxicity to the maximum tolerated dose. In addition, PNP was cytotoxic to the bone marrow of male mice at tested doses (Eichenbaum *et al.*, 2009).

3.5.3 FENTANYL IMPURITIES[1,3]

The forced degradation of fentanyl produced seven aromatic degradants. Among these, propionanilide (PRP), N-phenyl-1-(2-phenylethyl)-piperidin-4-amine (PPA), 1-phenethyl-1H-pyridin-2-one (1-PPO), fentanyl N-oxide, and 1-styryl-1H-pyridin-2-one (1-SPO)possibly indicate safety concerns. PPA was suggested as a potential genotoxic compoundand the DNA damage in unscheduled DNA synthesis (UDS); the results were positive forPRP when *in vitro* rat hepatocytes were checked. In the ACD/Tox suite, 1-PPO and 1-SPOwere identified as Ames hazards. These compounds were also predicted to have higherprobabilities of being Ames positive (Garg *et al.*, 2010).

3.5.4 TREMOGENIC IMPURITIES[1]

Tremogenic impurities comprise another sub-class of highly toxic impurities in APIs. Two pharmacopoeial APIs are known to have the potential to be contaminated with tremogenic impurities; pethidine and paroxetine (3-[(1, 3-benzodioxol-5-yloxy) methyl]-4-(4-fluorophenyl) piperidine). Pethidine can contain trace amounts of 1-methyl-4- phenyl-1, 2, 3,6-tetrahydropyridine (MPTP) derived from the hydrolytic degradation of side chain. 4-(4-Fluorophenyl)-1-methyl-1,2,3,6-tetrahydropyridine (FMTP) can be a potential reactant/intermediate in the synthesis of paroxetine. Owing to their toxicity to cells in the *Substantia nigra*, these highly potent impurities can induce Parkinsonism in humans. Thus, these compounds are known toxic impurities; however their genotoxicity remains unclear(Borman *et al.*, 2008).

3.6 β-LACTAM RELATED IMPURITIES[11]

The following two impurities relate to the well known antibiotics cefotaxime and piperacillin.

Piperacillin impurity-A

The piperacillin impurity-A is a prominent degradation product of piperacillin that appearsduring manufacturing and storage processes. In all the strains of *S.*

typhimurium; TA 97a, TA 98, TA 100, TA 102, and TA 1535, piperacillin impurity in the presence and absence of metabolic activation was found to be non mutagenic. Also, *in vitro* chromosomal aberration assay did not reveal any significant alterations. It is found that piperacillin impurity-A up to 5 mg/ml is non clastogenic to CHO cell lines in the presence and absence of metabolic activation (Vijayan *et al.*, 2007).

4. ANALYTICAL APPROACHES[13]

As discussed above, GIs possess unwanted effects and their contamination levels should be controlled. To achieve this, pharmaceutical R&D should employ robust and sensitive analytical methods for supporting drug development and monitoring the levels of GIs. In addition, analytical methods that are capable of measuring trace GIs must be employed to monitor the outcome of GIs during chemical synthesis. In recent years, manufacturers have developed sensitive methods for analyzing various GIs. In this context, conventional HPLC/UV methods are the first option for GIs analysis; however, these methods are often inadequate for the accurate determination of analytes at trace levels, depending on the properties of the analytes and sample matrices. Some of the challenges in the analytical determination of GIs in pharmaceuticals at trace levels include the diverse structural types of GIs, the unstable or chemically reactive nature of GIs, and an extremely high level of APIas contaminant (Bai *et al.*, 2010; Liu *et al.*, 2010).

4.1 HPLC methods[3,9]

In general, non-volatile GIs are analyzed by HPLC separation techniques, among which reversed phase HPLC (RPLC) is the most widely used separation mode (Elder *et al.*, 2008a; Liu *et al.*, 2010). A simple isocratic RPLC method has been employed for the determinationof four genotoxic alkyl benzenesulfonates (ABSs) viz. methyl, ethyl, *n*-propyl, and *iso*propylbenzenesulfonates (MBS, EBS, NPBS, and IPBS) in amlodipine besylate (ADB). The RPLC is

also applicable for sulfonate impurities with phenyl moiety such as methyl (MTs), ethylETs) and *iso*propyl tosylates (ITs), methyl (MBs), ethyl (EBs), butyl (BBs) and isopropylbesylates (IBs) (Raman *et al.*, 2008).

Epoxides/hydroperoxides were analyzed using HPLC, and simple RPLC methods employing direct analysis (no sample preparation) were used for some of them. Yasueda *etal.* (2004) described an HPLC method for the determination of loteprednol impurities including a minor photolytic epoxide degradation product. Lacroix *et al.* (1992) reported anHPLC method for the determination of related substances, including the epoxide impurityof nadalol. A rapid resolution HPLC method was used for separating and quantifying the related impurities of atorvastatin, including two epoxide impurities atorvastatin epoxy

A more common method for the analysis of alkylating impurities is by RPLC and MSdetection; however, HPLC/UV methods are also carried out successfully for alkylating impurities. Valvo *et al.* (1997) reported an HPLC/UV method for the separation of 13impurities of verapamil; this method is claimed to be superior to both the existing pharmacopoeial methods for verapamil. Using this method, the LOD and LOQ were found to be 0.01% (0.05 □g/ml) and 0.02% (1.0 □g/ml), respectively. Also, the method was found to be sensitive to pH and mobile phase composition; however, it was in contrast to the findings of previous studies insensitive to stationary phase changes.

Hydrophilic interaction liquid chromatography (HILIC) seems complementary to RPLC for the retention and separation of small molecule polar analytes, and has thus gained increasing attention recently. Good retention can be achieved for more polar analytes, which is notpossible on RPLC columns. In the hydrazine group, the HILIC method was used in addition tothe HPLC/UV and HPLC/MS methods (Elder *et al.*, 2010c; Liu *et al.*, 2010). An Indian research group reported 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 (Rao *et al.*, 2006). Hmelnickis *et al.* (2008) used an HILICmethod with different polar stationary phases (silica, cyano, amino, and the zwitterionicsulfobetaine) to separate six polar impurities, including 1,1,1-trimethylhydrazinium bromide,and demonstrated that HILIC was a useful alternative to reverse phase or ion

chromatography(IC). Elder *et al.* (2010c) reported a table summarizing the various HPLC methods that were used in the literature for a wide range of drugs (Table 4).

4.2 GC methods[3,9]

GC methods are commonly used for the analysis of several volatile small molecule GIs. Some examples include the liquid injection technique and the headspace sampling technique. Liquid injection is prone to contamination in which injection of a large amount of non-volatile API can accumulate in the injector liner or on the head of the GC column, which can cause a sudden deterioration in method performance. Headspace injection, on the other hand, is desirable because it minimizes potential contamination of the injector or column by avoiding the introduction of a large quantity of API (Liu *et al.*, 2010).David *et al.* (2010) proposed a method selection chart (Figure 4) containing GC or LCmethods, both in combination with a single quadrupole mass spectrometer as detector. These methods applied for a wide range of analytes including sulphonates, alkyl halides and epoxides.

Nassar *et al.* (2009) developed a GC/MS method for residual levels of EMS in a mesylate saltof an API crystallized from ethanol. The method was capable of detecting EMS down to levels of 50-200 ppb. Subsequently, extraction techniques were developed for eliminating or reducing matrix related interference. Thus, Colon and Richoll (2005) surveyed liquid–liquidextraction (LLE), liquid phase micro-extraction (LPME), solid phase extraction (SPE), and solid phase micro-extraction (SPME) coupled with GC/MS and single ion-monitoring (SIM).Using these approaches, they developed limit tests (5 ppm) for some alkyl aryl esters of sulfonic acids. Similar attempts were made for reducing or eliminating the matrix effect for alkylatingagents as well. In all these procedures, a specific physical property of the analyte not shared by the matrix was utilized, e.g. low boiling point and/or in the presence of halide atom(Elder *et al.*, 2008a).

GENOTOXIC IMPURITIES IN PHARMACEUTICALS[13]

GC methods were rarely used for the analysis of epoxides/hydroperoxides, as compared to other impurities, owing to the size of molecule and the volatility properties within this group (Elder et al., 2010b). Klick (1995) used a GC method for the determination of residual levels of a chlorohydrin and the corresponding epoxide impurities in almokalant. Other literatures give an account of GC–MS methods for the analysis of volatile components in traditional Chinese herbal medicines (Yu *et al.*, 2007; Guo *et al.*, 2003).Fig. 4. Method selection chart for analyzing genotoxic impurities with GC/LC; 1APES/APCI: atmospheric pressure electrospray ionization/ atmospheric pressure chemical ionization; 2 If the analyte has sufficient vapor pressure in water or other low volatile solvent; 3 SHS: static headspace; 4 SPME: solid-phase micro-extraction; 5 DHS: dynamic head space; 6 HILIC: hydrophobic interaction liquid chromatography; 7 derivatization-RPLC:reversed phase HPLC with precolumn derivatization; 8 Back-flush (CFT): capillary flowtechnology based back-flushing; 9 Deans 2DGC (CFT): capillary flow technology based two dimensional GC (Figure is reproduced from David *et al.*, 2010).

4.3 TLC/HPTLC METHODS[4,7]

In general practice, thin layer chromatography (TLC) is not preferred for the accurate determination of very low residual analyte level. However, this technique is still used for the determination of related substances in the pharmacopoeial monographs for amiodarone,bromazepam, carmustine, ifosamide, indoramin, and tolnaftate (Elder *et al.*, 2008).Nevertheless, there are several examples of its use in association with determining levels of the epoxyl alkaloid, including scopolamine in extracts of *Datura stramonium*. Sass and Stutz(1981) used TLC to determine residual sulfur and nitrogen mustards (beta haloethyl compounds) in a variety of substrates in which the sensitivities in the microgram range were typically achievable. High performance thin layer chromatography (HPTLC) was used for monitoring the degradation products of rifampicin, including the hydrazones (25-desacetylrifampicin (DAR)) and rifampicin quinone (RQU). Finally, it was concluded that the method is suitable for routine

quality control and stability analyses, especially in the developing world (Jindal *et al.* 1994).

4.4 CAPILLARY ELECTROPHORESIS METHODS[14]

Jouyban and Kenndler (2008) reviewed the applicability of capillary electrophoresis (CE) methods for the analysis of pharmaceutical impurities. In addition, they discussed the applications of these methods in various groups of compounds such as chemo therapeutic agents, central nervous system (CNS) drugs, histamine receptor and cardiovascular drugs. The main advantage of CE techniques is their selectivity; thus, they are suitable for the analysis of complex herbal products. Bempong *et al.* (1993) reported the separation of 13-cisand all-trans retinoic acid and their photo-degradation products (including all-trans-5, 6-epoxy retinoic acid, 13-cis-5, 6-epoxy retinoic acid) using both capillary zone electrophoresis(CZE) and micellar electro kinetic chromatography (MEKC) methods. A Chinese research group reported the development of CE methods for the simultaneous determination of some hydrazine related impurities (Liu *et al.*, 1996).

Hansen and Sheribah (2005) evaluated a series of electrically driven separation techniques: CZE, MEKC, and microemulsion electrokinetic chromatography (MEEKC) for the determination of residual alkylating impurities in bromazepam API. However, the poor sensitivity of the techniques posed a problem even when specialized detection cells (e.g.bubble or Z-cells) were used. Mahuzier *et al.* (2001) demonstrated the poor sensitivity of CE.

4.5 Enhancing methods

Alternatively, the structure of the molecule as well as its properties can be altered to enhance detectability which in turn will help to achieve the desired sensitivity. This is especially true for GIs that lack structural features for sensitive detection (Bai *et al.*, 2010; Liu *et al.*, 2010). A number of general approaches could be considered, some of which are explained below.

4.5.1 CHEMICAL DERIVATIZATION

This method is generally used for stabilizing reactive GIs and for introducing a detection specific moiety for enhanced detection, i.e.

chromophore for UV. Also, this method sometimes produces a single compound for several GIs; thus, it becomes non-specific which can be considered as an advantage in determining a group of structurally related compounds (Liu *et al.*, 2010). Bai *et al.* (2010) introduced a chemical derivatization method for analyzing two alkyl halides and one epoxide. The objective of the three derivatization reactions is to generate a strong basic center by introducing an amine functional group. All three derivatization products are good candidates for electrospray ionization (ESI)-MS owing to the high proton affinity or the permanent charge.

4.5.2 COORDINATION ION SPRAY-MS

Owing to their structural features, several analytes are not amenable to atmospheric pressure ionization methods, such as the ESI method. Alkali metal ions such as Li+, Na+, andK+ can form complexes with some organic molecules in the gas phase; this fact could be used as a solution for the analytes subjected previously (Liu *et al.*, 2010).

4.5.3 MATRIX DEACTIVATION

The matrix deactivation approach is a chemical approach to stabilize unstable/reactive analytes. It is based upon the hypothesis that the instability of certain GIs at trace level iscaused by the reaction between the analytes and reactive species in the sample matrix. Thus, controlling the reactivity of the reactive species in the sample matrix would stabilize the unstable/reactive GI analytes (Liu *et al.*, 2010).As an example the alkylators are reactive unknown impurities which possess mainly nucleophilic characteristics. Their reactivity can be attenuated by either protonation or scavenging approaches. Sun *et al.* (2010) reported a matrix deactivation methodology for improving the stability of unstable and reactive GIs for their trace analysis. This approach appears to be commonly applicable to techniques like direct GC–MS and LC–MS analyses, or coupled with chemical derivatization as well.

5. Genotoxicity prediction[3,8]

The concept of using structural alerts to predict potential genotoxic activity for identified impurities is now well established; however, the concordance between such alerts and biologically relevant genotoxic potential (in the context

of genotoxic impurities) could behighly imperfect. Structural alerts are defined as molecular functionalities (structuralfeatures) that are known to cause toxicity, and their presence in a molecular structure alerts the investigator to the potential toxicities of the test chemical. Nevertheless, the assumption that any impurity with a structural alert is potentially DNA-reactive and thus subject to the default TTC limit may often lead to unnecessary restrictive limits. From a resource and timetable viewpoint of a new drug production, the experimental determination of genotoxicity isnot feasible for millions of drug candidates in the pharmaceutical industry. Thus, compounds identified as potential hazards by *in silico* methods would be high priority candidates for confirmatory laboratory testing (Kruhlak *et al.*, 2007; Snodin, 2010).

In silico toxicology is the application of computer technologies to analyze existing data,model, and predict the toxicological activity of a substance. In sequence, toxicologically based QSARs are mathematical equations used as a predictive technique to estimate the toxicity of new chemicals based upon a model of a training set of chemicals with known activity and a defined chemical space (Valerio, 2009).Ashby and Tennant (1991) reported some correlations of electrophilicity with DNA reactivity (assessed by Ames-testing data) for about 300 chemicals and elucidated theconcept of structural alerts for genotoxic activity in the 1980s/1990s. Using a database of>4000 compounds, Sawatari *et al.* (2001) determined correlations between 44 substructures and bacterial mutagenicity data. A high proportion of genotoxic compounds were found for electrophilic reagents such as epoxides (63 %), aromatic nitro compounds (49 %), and primary alkyl monohalides (46 %). In a retrospective analysis of starting materials and intermediates involved in API syntheses, the most common structurally alerting groups were found to be aromatic amines, aromatic nitros, alkylating agents and Michael acceptors(Snodin, 2010).

One of the strengths of QSAR models is that they contribute to a mechanistic understanding of the activity, and, at the same time, they constitute practical tools to predict the activity of further, untested chemicals solely based on chemical

structure (Benigni *et al.*, 2005). Another strength of QSAR models is that they are strictly data-driven, and are not based on a prior hypotheses. On the other hand, high-quality experimental data must be used to build the training data set. As error (e.g. incorrect molecular structure or erroneous data from toxicology studies of a chemical) is introduced into the model, amplification of that error is generated and represented in the prediction (Benigni *et al.*, 2005; Valerio, 2009).

Cunningham *et al.* (1998) investigated a SAR analysis of the mouse subset of the carcinogenic potency database (CPDB) which also included chemicals tested by the USnational toxicology program (NTP). This database consisted of 627 chemicals tested in micefor carcinogenic activity with the tumor genicity data being standardized and reported asTD50 values. In addition, MULTICASE software (www.multicase.com) was used to identify several structural features that are not explained by an electrophilic mechanism and which may be indicative of non-genotoxic chemicals or mechanisms involved in carcinogenesis other than mutations. The prediction capabilities of the system for identifying carcinogens and non carcinogens were 70 % and 78 % for a modified validation set.

Tafazoli *et al.* (1998) used the micronucleus (MN) test and the alkaline single cell gelelectrophoresis (Comet) assay for analyzing potential mutagenicity, genotoxicty, and cytotoxicity of five chlorinated hydrocarbons. Using the generated data as well as the dataof another five related chemicals that were investigated previously, a QSAR analysis was performed and the results indicated that LBC_C1 (longest carbon-chlorine bond length), MR (molar refractivity), and ELUM0 (energy of the lowest unoccupied molecular orbital, indicating electrophilicity) were the most significant factors to be considered for discriminating between genotoxins and nongenotoxins.

Benigni *et al.* (2005) showed that the QSAR models could correctly predict— based only on the knowledge of the chemical structure—–the genotoxicity of simple and unsaturated aldehydes. The active and inactive compounds were separated based on the hydro phobicity(log P) and bulkiness (MR) properties.

Bercu *et al.* (2010) used *in silico* tools to predict the cancer potency (TD50) of a compound based on its structure. SAR models (classification/regression) were developed from the carcinogenicity potency database using MULTICASE and VISDOM (a Lilly Inc. in-housesoftware).

It is commonly accepted that the carcinogenicity of chemicals is owing to their genotoxicity and, in fact, the mutation and carcinogenesis data are practically coincident. Thus, the two endpoints were collapsed into one ''genotoxicity'' classification, in which QSAR analysis was applied. Now the question remains as to how to predict non-genotoxic carcinogenicity. In fact, it cannot be well approached until some mechanistic understanding of non genotoxic carcinogenesis is achieved. At this time, this approach is unable to grasp the structural features of non-genotoxic carcinogens (Ashby, 1990; Cunningham *et al.*, 1998;Benigni *et al.*, 2005).

The other limitation to currently available QSARs is the lack of models for organo metallics, complex mixtures (e.g. herbal extracts), and high molecular weight compounds such as polymers (Valerio, 2009). However, the QSAR predictive software offers a rapid, reliable, and cost effective method of identifying the potential risk of chemicals that are well represented in QSAR training data sets, even when experimental data are limited or lacking(Kruhlak *et al.*, 2007). These models should be further developed/validated by employing new mechanistic findings and using newly reported experimental data.

6. Conclusion[1,2]

Since 2007, following the EMEA suspension of the marketing authorization of viracept (nelfinavir mesylate), genotoxic impurities have become a common issue for health concerns. Thus, regulatory agencies have made several attempts to construct a systematic method for controlling and analyzing GIs. However, several points must be considered for achieving a general view on the regulation of GIs.

One of the main problems is the very conservative limit regulated by agencies (1.5 µg/day).Bercu *et al.* (2009) calculated the permissible daily exposure (PDE) for EMS, which was the first GI of concern in 2007, as 0.104 mg/day. This value was

found to be about 70-fold higher than the TTC level of 1.5 µg/day currently applied to EMS based on the generic linear backextrapolation model for genotoxins acting via non-threshold mechanisms. Other literatureshighlighted this conservative limit as well (Gocke *et al.*, 2009b; Elder *et al.*, 2010a; Snodin,2010). In addition, Gocke *et al.* (2009b) reported that the accidental exposure of viracept patients did not result in an increased likelihood for adverse genotoxic, teratogenic or cancerogenic effects. In addition to the challenge of setting a more pragmatic limit for GIs, the development of extremely sensitive and robust analytical methods that can adequately monitor GIs at very low levels is very difficult. Also, the pharmaceutical industry has no long-term experience in the use of these methodologies within the factory setting. Thus, analysts make attempts to determine a way for analyzing various GIs by using unique robust methods as far as possible. In this way, simple HPLC/UV or GC/FID methods are usually performed at thefirst stage, while more complicated LC/MS or LC/MS/MS methods are used as alternatives(Dobo *et al.*, 2006; Elder *et al.*, 2008b; Liu *et al.*, 2010).Teasdale *et al.* (2009) studied the formation of sulfonate esters as a mechanistic view, and showed that when a slight excess of base is present, there is no discernible reaction rate to form the sulfonate ester and no mechanistic pathway to their formation. From this point of view, the formation of GIs and suspicious substances in the API syntheses can be easily avoided, and therefore this is the preferred option (Robinson, 2010).Finally, it can be mentioned that in such a situation, *in silico* approaches can prove to be more effective solution in terms of time and cost for screening genotoxic compounds. As subjected by Luis and Valerio (2009), high-quality experimental data must be used. Inaddition, for non-genotoxic carcinogens, QSAR studies can provide a better understanding about the mechanism of carcinogenesis of these compounds. The in silico methods used in agencies have not been specified yet; however, by overcoming the limits these can become an innate part of regulatory systems.

7. References

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