Comments on ICH M2 Step 2

Christopher R. Lee

A decade after regulatory authorities began taking a systematic approach to genotoxic impurities, this ICH draft document can still not be considered mature. One positive point, however, is that attention is now focussed exclusively on DNA-reactive impurities. This opens the way for research on general and inexpensive methods for screening and determination, and reduces the uncertainty about whether one has done enough to ensure that a product complies with regulatory requirements.

Genotoxic compounds that don't react with DNA do exist, of course, but searching for trace impurities of this type can never reach a definitive conclusion. In any case, such impurities would be expected to have a classical sigmoid dose-response and, therefore, to present a risk that is low unless the daily dose of drug substance is at the high end of the usual range.

One surprising aspect of the draft is the continued exclusion of excipients and formulated products from regulatory control, although these are obvious potential sources of genotoxic impurities. In the absence of an explanation for this selectivity, a reader of inquiring mind might ask whether the underlying reason for the genotoxic impurities initiative is that stated, and may wish to know more, not only about the nature of the safety concerns and technical difficulties, but also of any pressures that may have contributed to shaping regulatory documents.

Definition of genotoxic risks

The following statement appears, perhaps strangely, under 'General Principles' (section 3):

> *'The use of a numerical cancer risk value (1 in 100,000) and its translation into risk-based doses (TTC) is a highly hypothetical concept...'*

One would not normally expect that a regulatory document that has an impact on the safety of medicines, and on the cost and duration of their development, be founded upon a 'highly hypothetical concept'. The statement recalls the general tenor of an article by an industry lobby group that was published in 2006 in a sponsored journal, and which I will not honour with a citation. For the benefit of its readers unable to calculate a simple formula, that article presented a large table of acceptable impurity concentrations according to daily dose and duration of administration. For added utility, the same set of concentrations was presented, separately, in percent and parts per million.

Given the doubts that have been expressed about the pertinence of genotoxic risk estimations, as well as sometimes cynical industrial responses to official policy, the ICH guideline could, perhaps, include a statement to the effect that the incidence of cancer has been increasing, and that hypotheses related to a concomitant increase in low exposures to genotoxins have not yet been refuted.

The statistical basis of the kind of risk evaluation involved was not established by or for the pharmaceutical establishment. The following quotation is from the WHO guideline on drinking water (World Health Organization, 2011), section 8.2.3:

> '*These models compute an estimate of risk at a particular level of exposure, along with*

upper and lower bounds of confidence on the calculation, which may include zero at the lower bound. Guideline values are conservatively presented as the concentrations in drinking-water associated with an estimated upper-bound excess lifetime cancer risk of 10^{-5} (or one additional case of cancer per 100 000 of the population ingesting drinking-water containing the substance at the guideline value for 70 years). This value does not equate to the number of cases of cancer that will be caused by exposure to the substance at this level. It is the maximum potential risk, taking into account large uncertainties. It is highly probable that the actual level of risk is less than this, even approaching zero, but risks at low levels of exposure cannot be experimentally verified.'

Here, the ('default') Threshold of Toxicological Concern (TTC) is not mentioned, because the carcinogenic potencies of the relatively few carcinogens that are commonly found in drinking water are known, and the calculations are (as allowed in M7) compound-specific. The estimated upper-bound risk is in fact precisely defined, and the admitted uncertainty concerns only its value. The conservative nature of the definition is due in part to the use of the upper bound of risk estimates.

The WHO also deals adequately with short-term exposures exceeding the daily limit for lifetime exposure to carcinogens in drinking water, a situation that arises frequently. There is no need for special pleading or special vocabulary. Fortunately, the term 'staged TTC' proposed by the industry lobby has disappeared.

Treatment of negative results when the lower bound of the physical model is zero
Concerning the lower bound, the WHO, without going into details, implies that at a relevant level of exposure, the dispersion of experimental results may be such that the lower part of the measured or calculated probability distribution may go below zero cancer risk. One reason for this may be statistical, as the incidence of cancer or mutation in both control and experimental groups is subject to random variation. When this happens the lower bound is set to zero, effectively truncating the distribution.

Physically impossible negative results should be familiar to analysts, for example with the sulfated ash test on a substance for which the expected result is close to zero. We know how to report negative results internally and for the client, though the statistical analysis of data sets that have to be truncated for reporting purposes is difficult. Analytical methods (for example chromatographic) that involve the measurement of a peak can't usually produce negative results, but similar problems of censored data do arise if one operates too close to the limit of detection. Genotoxic risks have to be calculated, inevitably, at or below the level of spontaneous biological "noise".

An alternative reason for observed negative risks in studies of toxins and infectious organisms is that a suitable dosage schedule might lead to stimulation of inducible protective mechanisms. ICH M7 does refer to inducible DNA repair mechanisms, though as I argue below, it may be too soon for such reasoning to be included in a regulatory document. If DNA repair and other protective mechanisms are taken into account, it would no longer be possible to maintain the present regulatory assumption that a single fairly large dose carries the same risk as the same dose divided over a certain period.

Need for background documentation
Documents such as ICH M7 should be accompanied by background documentation that gives the reasoning behind each statement or decision, and the sources of outside information. Such documentation should explain why it was necessary to draft new wording for those parts of Section 3 that could have been aligned with long-standing practice in other fields. As it stands, this section

of M7 may give the impression that the authors either do not understand what is at stake or else that they are still looking for a 'pharmaceutical exception' to rules that apply everywhere else in this field of toxicology.

Considerations for 'marketed products'

'Marketed products' is a pair of ordinary words, redefined in Section 4 of M7 to denote products that have been or will be introduced before the guideline finally comes into effect. This special pharmaceutical vocabulary should have no place in a regulatory document. Readers familiar with business buzzword terminology may suspect some influence on the part of the industry side, which may perhaps have preferred 'marketed products' to be totally excluded from new guidelines or regulations. The trouble is that those older products are precisely the ones that were developed without consideration for possible genotoxic hazards.

The draft guideline does require new investigations of existing products when there are changes affecting chemistry, manufacturing, controls, or clinical use. This may seem reasonable, except that the wording might tempt sponsors to take care to avoid excessive diligence during periodic product reviews. Also, since an out-of patent product may have several manufacturers, details of the manufacturing process may vary. We know from the nelfinavir affair of 2007 that details can be important. Section 4.4 (Alternative Considerations) covers the action to be taken if new information becomes available, but in my opinion it isn't too clear how and when such information might arise. There should be a requirement to study all existing products to a defined depth within a defined time-frame. Quite likely, many such studies would amount to no more than a brainstorming session, particularly where the daily dose is low. A few could be embarrassing.

One point that ought to be addressed is the dissemination of CMC information on out-of-patent products; one has no way of knowing if the multiple dossiers that may exist for a generic product have been updated in this respect. The pharmacopeias tend to be conservative in their approach to drafting monographs, but for products likely to be on the generics market, these authorities should be capable of indicating in each monograph whether a genotoxicity study has been performed, and whether specific precautions are required at critical manufacturing steps (a well-rehearsed example is the avoidance of alcohols or ethers in the presence of certain anions). I am unable to comment further on the current situation because pharmacopeial compendia are not readily available to the general public.

DNA repair mechanisms

In modern life, one is exposed to numerous established and potential sources of exposure to DNA-reactive genotoxins and to ionising radiation. An unknown proportion of these exposures existed before the industrial era. According to the standard model, the cancer risk is a linear function of the lifetime dose, and this leads to the conclusion that exceedingly low doses fall within the confidence limits of significant risk. Consequently, there is considerable interest, not only in industrial circles, in finding out whether an exposure can have, in reality, a threshold below which the cancer risk is essentially zero (classical sigmoid dose-toxicity response). DNA repair mechanisms are ubiquitous, numerous, inducible and essential for life to exist. Much ongoing research is aimed at demonstrating a threshold response when DNA adducts are of kinds that are repairable, and a linear response when they are not. "Repairability" is linked to regiospecificity among the numerous nucleophilic sites on DNA. Regiospecificity is thought to be correlated with reaction mechanism and, leaving aside a certain amount of ineptitude manifested in the literature, it seems possible that

the majority of electrophilic drug impurities will indeed cause mainly repairable lesions, if ever they reach the cell nucleus.

Research on DNA repair is promising in many respects, but I don't think it's ready to be mentioned in an official guideline. Since low levels of mutagenicity and carcinogenicity can't be demonstrated in intact animals or humans (except for the very large populations that were exposed to radiation from atomic bombs), artificial systems such as cell culture have to be used. We know only too well from drug R&D that laboratory models don't reliably predict the results of clinical trials; there is no reason to suppose that the same argument doesn't apply to genotoxicity studies.

Cell culture is a difficult art and culture media don't always model physiological conditions. In one study (Doak *et al.*, 2007), cited in the aftermath of the nelfinavir affair of the same year, the medium contained 1% (68 mmol/L) of the nucleophile glutamine. Selective reactivity of endogenous nucleophiles (or those in artificial media) with certain test electrophiles could provide a possible though partial explanation for the reported results (see *Scavenging* below). Studies really need to be supported by direct measurements of DNA adducts in the experimental system that is being used; that may not yet be economically practicable, and managing such joint projects can be difficult.

The extensive and fascinating literature on DNA repair mechanisms, including the publication cited above (Doak *et al.,* 2007) is concerned largely with methyl and lower alkyl adducts. Some of these modifications, which occur naturally, are repaired by specific de-alkylating enzymes, in addition to more 'general-purpose' systems such as base excision repair (BER). However, exogenous electrophiles are much more varied in type (for example acylating agents and the metabolic activation products of aromatic amines). They are also more bulky; it would be difficult to predict how the mechanisms for damage recognition and repair might operate in a particular real case.

Scavenging

Endogenous nucleophiles might 'scavenge' small concentrations of certain reactive electrophilic impurities, depending on their reaction mechanism (see Lee, 2012). I insist on this, because if scavenging can be demonstrated for common kinds of genotoxic impurities, it ought to be possible to obtain direct experimental support for a threshold mechanism that would withstand regulatory scrutiny. The arguments would be based more on physical parameters (rate constants and diffusion coefficients) than on biological systems that are too complex to be fully understood at present. Nevertheless, we must be aware that likely scavenging nucleophiles such as glutathione and thiosulfate may be depleted in certain disease states.

The main but not the only cytoplasmic nucleophile to be considered is glutathione, for which a review written in the context of food science is freely available (Wu *et al.*, 2004). Intracellular concentrations are in the range 0.5 - 10 mmol/L. Glutathione is involved in enzymatic detoxification reactions and, like all thiol reagents it is an antioxidant. Here, we are concerned with the less studied aspect of spontaneous reactions with reactive electrophiles such as alkylating and acylating agents.

Perhaps this approach has been relatively neglected for some good reason of which I am not aware; a less good reason would be related to long-standing confusion over the reaction mechanisms of the experimental systems that have been used. Administration of dimethylsulfate vapour directly to the respiratory tract of animals leads to quite localised alkylation (and cancer) of the DNA of respiratory epithelial cells, because the compound reacts so rapidly with cellular nucleophiles that it gets no further. Dimethylsulfate is a genotoxin that reacts by the classical SN2 mechanism without preliminary formation of a reactive species. Here, we are concerned with low doses that would not

be detectably mutagenic but that, by extrapolation, are considered hazardous in humans. The question is whether the kinetics of spontaneous reaction with (for example cytoplasmic glutathione) might be sufficiently rapid to eliminate small amounts of DNA-reactive molecules as they diffuse through the short distance between the cell wall and the chromosomes.

Prospects for future development

The new guideline is about DNA-reactive impurities; impurities that are genotoxic for other reasons are no longer being discussed. Mutagenic impurities are *'usually detected in a bacterial reverse mutation (mutagenicity) assay'* (Section 3). Compounds that have genotoxic metabolites are included (implicitly) because metabolic activation forms part of standard assay protocols. I understand that the *in vitro* metabolic activation methods of the currently-approved battery of tests are considered sufficient, and according to the literature they are usable in conjunction with different kinds of assay indicative of mutagenicity, in particular purely chemical ones.

A gap in the reasoning is that, while reverse mutation assays currently provide the test criterion, they are in fact unsuitable for impurity screening purposes because the detection limit for an unsuspected genotoxic impurity present at relevant concentrations in a drug substance is much too high. "Ordinary" impurities, which have higher reporting levels, are detected fairly reliably by chromatographic methods (subject to one's opinion on that). As is well known to those who have worked in the field, not all of these will have been predicted by the development chemist. By contrast, searching for genotoxic impurities has to depend almost entirely on reasoning and speculation about what might occur during the manufacturing process; consequently, current procedures leave a commercially unacceptable amount of uncertainty.

Analytical reagents for electrophilically-reactive compounds

The DNA-reactive impurities and metabolites covered by M7 are electrophilic reagents. Reagents and sensors for these reactive compounds have been available for many decades, and they are sensitive enough. The available reagents could be described as nearly but not entirely satisfactory for present purposes. However, designing some improved reagents should not be a serious challenge.

Some research has been done on sensors in which immobilised fragments of DNA are coupled to an electrochemical system; one of the aims is to develop economical screening methods for the safety testing of industrial chemicals in accordance with safety legislation (REACH in Europe). For the presentation that follows, we will focus on 'traditional' analytical chemical reagents, some of which can be considered as approximate surrogates for DNA. Later, we will mention methods based on the use of tritium-labelled DNA or surrogates.

Our objective could be to develop one or a small number of reagents and simple test procedures for:

1. Limiting the total amount of DNA-reactive impurities in development and production batches. The discredited general heavy metals test is quite rightly destined to disappear from the pharmacopeias, but this does not invalidate the principle of using limit tests of broad specificity.
2. Determining the concentrations of already-identified impurities without the use of expensive or time-consuming techniques. This is the same as 1 above, except that calibration is performed using the target impurity (or a surrogate). Since specificity with respect to other DNA-reactive impurities should not normally be required, methods need not involve chromatographic or other separations.
3. Identifying the electrophilic impurity responsible for a positive result obtained using a non-

specific method. This would usually involve the identification of a product of reaction between the impurity and a test reagent.

4. Investigating the reaction mechanisms of identified impurities, with the aim of predicting likely reaction sites on DNA and consequently the mutagenic risk. This approach may be out of favour (see Lee, 2012), but a re-evaluation by researchers who have some knowledge of organic chemistry could still provide valuable information.

One could envisage the development of a single analytical reagent suitable for all of these purposes, but a small battery of reagents would be a more likely outcome.

Chromogenic and fluorogenic reagents

Although there are some practical difficulties with the available reagents, I think that some further development would enable the requirements of the guideline to be met, simply and at little expense. Techniques for detecting reactive electrophiles can be traced back to the detection of the chemical weapons used in World War I. The reagent 4-(4-nitrobenzylpyridine) (NBP), which I describe in the main article (Lee, 2012) and discuss below, was introduced in 1925 and is still the most usual choice. A recent review on the use of NBP to study alkylation mechanisms (Gómez-Bombarelli *et al.*, 2012) is broader than its title suggests, though it doesn't mention *all* the traps for the unwary I note in my main article. Also, the review may give an analytical chemist or pharmacist the impression that one accepts too willingly a somewhat unsatisfactory reagent for reasons of compatibility with the extensive earlier literature.

NBP is one of a huge number of chromogenic and other kinds of reagents that were available in the days before instrumental techniques became generally available. These were used, not only for the detection and determination of compound classes, but also for structural analysis. Libraries had shelves full of compendia of methods, but these almost disappeared from the pharmaceutical and fine chemical fields when instrumental methods began to dominate. When I used NBP for a genotoxic impurity in the early 1990s, my method was decried in some quarters as "colorimetric" and therefore out of date. In fact, the only thing that has changed is the vocabulary; the term refers to the use of filter photometers and visual assessment in the days when spectrophotometers were too expensive for routine analyses. The extensive current literature refers, for example, to derivatisation and to chromogenic and fluorogenic reagents, sometimes coupled to physical sensors or used in conjunction with chromatographic techniques, and often as simple testing sticks.

In the two cases using NBP I was able to study in detail, developing a limit test with a limit of 1 μg for a 1 g test sample of drug substance was not particularly difficult. Some batches of commercial reagent need to be recrystallised as described in the literature, as reagent impurities or degradation products contribute to the blank and degrade the reaction product. The other practical difficulty was finding solvents that would dissolve both the reagent and the drug substances under test, and that would give low blank readings. We should consider developing at least two new reagents: one that is soluble in water, and one that is more lipophilic.

Currently, for target-compound determinations (second requirement in the list above), analysts may cause their impurity to react with an amine or other nucleophile and determine the product by LC/MS, an expensive technique requiring method development for each kind of impurity. Probably, the small number of publications does not reflect the number of (confidential) applications. We developed a more general method for alkylating agents with small R-groups, in which volatile thiocyanate/isothiocyanate derivatives are determined by headspace gas chromatography (Lee *et al*., 2003). This method works with a variety of headspace solvents (unpublished information), and so is adaptable with little or no specific development for determining a limited range of alkylating agents

in a wide variety of substances to be examined, with no sample preparation.

Various chromogenic alternatives to NBP have been proposed, but none is in widespread use. It isn't certain whether this situation is due to the need for compatibility with earlier data, or because the new reagents have been found unsatisfactory in practice. Since most drug substances don't absorb in the visible, we can retain the old approach of developing colourless reagents that give coloured products. This provides specificity without the need for a separation method, provided the blank is low enough. A review of current research on the detection of chemical weapons (with the focus on nerve gases) was published fairly recently (Royo *et al.*, 2007). The emphasis is on visible fluorescence detection, which is a suitable choice for battle-field deployment because the matrices are simple (air or drinking water). However, this review does provide up-to date information that may help to define possibilities and general requirements for new reagents for the detection and quantitation of DNA-reactive impurities in the context of pharmaceutical and fine chemical analysis.

We wish to detect any electrophile that will react to form a covalent bond with a nucleophilic nitrogen or oxygen of DNA under physiological conditions. Leaving aside controversial aspects, a pyridine nitrogen (as in NBP), perhaps in the presence of a ketone, is thought to meet this requirement. Other nucleophilic reagents such as hydroxyoximes have been used or proposed, though we should avoid functions that are much more reactive than those of DNA (see below).

The colour reaction may have one or more steps (2 in the examples shown here). The two steps of the NBP method are shown in Figure 1 for a carbonyl compound and an alkylating agent.

With a two-step system, it may be convenient to perform any chromatographic separation on the initially-formed product, followed by a post-column reaction. Post-column detection can be useful if the dye finally produced is (as with NBP and many other dyes) photosensitive, susceptible to oxidation, and perhaps difficult to chromatograph. The added specificity provided by chromatography makes it relatively easy to obtain a low blank (which otherwise is a problem with impure NBP and reaction solvents). Some pharmaceutical readers may perhaps be unfamiliar with post-column reaction detection; it became routine in other domains with the introduction of the Moore and Stein amino acid analyser in 1958, which is still in use after half a century and a Nobel prize.

The alkyl, acyl or other functional group of the initial adduct or the final dye can be identified by liquid chromatography - mass spectrometry. We note that the initial pyridinium adduct with NBP is "ready-ionised", facilitating selective mass spectrometric detection.

Nowadays, it should be possible to design a reagent (perhaps an analogue of NBP) that is more suitable for both chromatographic and non-chromatographic applications, and that yields a stable dye. One could choose a fairly large molecule that, as it were, dominates the chromatographic properties of the adducts, facilitating the development of "generic" methods.

The reaction products of classical reagents like NBP are adducts whose spectra and specific absorbances depend on the functional group resulting from the reaction with the electrophile. Therefore, the response should, ideally, be determined for each electrophilic impurity being determined. Some researchers make the approximation of applying to all test electrophiles the specific absorbance given by a convenient but otherwise arbitrarily-chosen standard; this should be acceptable for a pharmaceutical limit test, bearing in mind all the uncertainties. After all, the TTC is defined in terms of mass not moles, and limits for existing tests such as residue on ignition do not take account of the nature of the residue. As proposed earlier, this is one case for which an exception could be made to the current tendency to replace general tests with specific ones.

Some current research on chemical weapons is aimed primarily at developing fluorogenic reagents, although a few of the approaches are suitable for spectrophotometry (Royo *et al.*, 2007). As

mentioned, fluorescence detection is fine for matrices such as air or drinking water, and the extra sensitivity provided by fluorescence detection is needed in this field. With pharmaceuticals and fine chemicals there would be concerns about quenching by the sample matrix (drug substance or product); furthermore, not all electrophiles we encounter would give fluorescent derivatives.

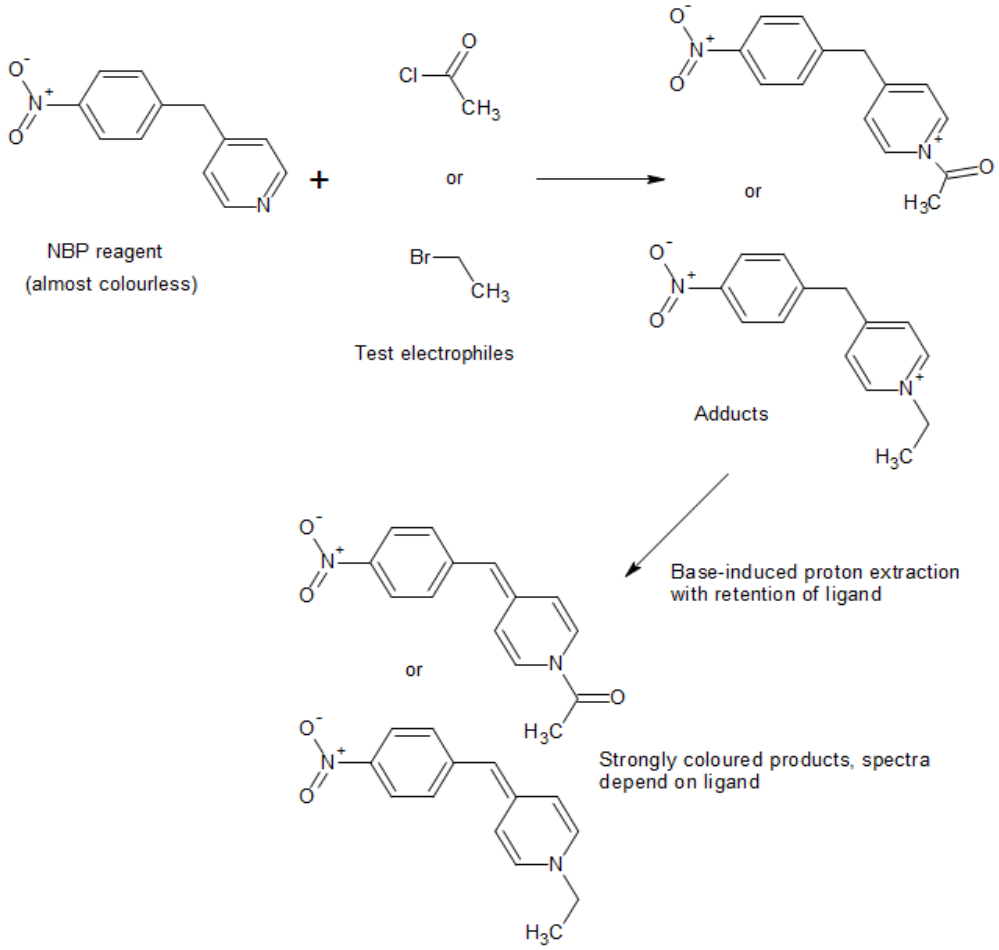

Figure 1: Reaction of chromogenic reagent 4-(4-nitrobenzyl)pyridine (NBP) with an acylating and an alkylating reagent

An attractive feature of some of the latest reagents is that once an electrophile has reacted, it is eliminated during the subsequent step that yields the coloured or fluorescent derivative. In other words, all electrophiles for which the reagent is a substrate give the same derivative and consequently the same response factor (assuming quantitative reaction). Clearly, identification of the R-group of the impurity responsible for a positive response would require analysis of the initial adduct. As with NBP, the leaving group of the impurity can not be identified. The approach is illustrated below (Figure 2) for the simplest of the hydroxy oxime reagents introduced by Dale and Rebek (2009).

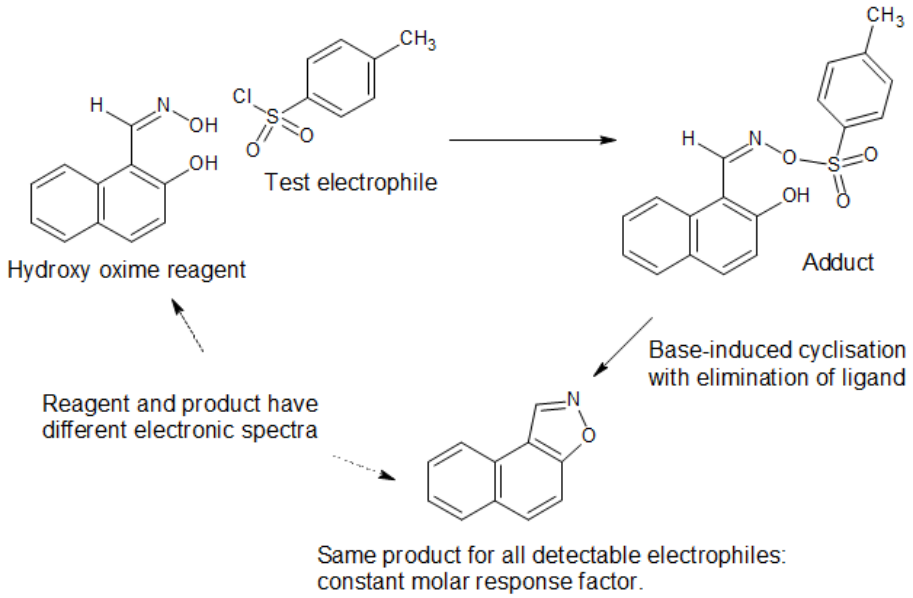

Figure 2: Reaction of a hydroxy oxime chromogenic/fluorogenic reagent with an arylsulfonyl chloride.

The authors used *p*-toluenesulfonyl chloride as test electrophile; they did not discuss alkylating agents, for which the ligand may not be sufficiently electron-withdrawing. Reagents with limited ranges of applicability can have their uses; we recall the ways in which closely-related functional groups were identified in the past.

Here, we are concerned with the principle of a reagent that yields the same final product whatever the ligand. In present circumstances it may be appropriate to propose that field test kits for chemical weapons should give some information on the identity of the compound detected, to provide 1) multiple independent sources of evidence and 2) means of detecting fraudulent use of common and relatively innocuous reagents to obtain false positive results.

Limitations: impurities that react with the drug substance

For a chromogenic method, solutions of the substance being examined and of the reagent are mixed and allowed to react under prescribed conditions. The method of standard additions is used during the validation exercise and/or calibration. Sometimes, an electrophilic impurity being determined will give a low or zero response in the presence of the substance under test. This is likely to be due to reaction of the impurity with a nucleophilic function of the substance being examined, such as a basic nitrogen or perhaps a counter-anion. With trace analyses at least, these reactions may still occur if the base is largely protonated, and we are reminded that nucleophilic reactivity is not always correlated with proton affinity.

Such observations support the presumption by generations of development chemists that certain reagents could never persist in the presence of certain drug substances, at least in solution. In the past, regulatory authorities would sometimes accept a well-worded argument in that sense. Unfortunately one heard relatively recently of analysts being instructed to determine residues of reactive electrophiles in amino compounds that had been prepared as the free bases. Sulfonyl chlorides react vigorously with water, and the amounts in dilute solutions used for calibration would most certainly be destroyed by traces of moisture. Analysts have been given the impossible task of

determining such compounds in drug substances on the grounds, apparently, that they have tested positive in a reverse-mutation test. The false positives are due, it seems, to reaction products formed when the compounds are dissolved in DMSO, which is the usual vehicle for *in vitro* toxicological testing.

The subject of highly reactive residues is referred to in the M7 draft (Section 8.2) which, naturally, would not reflect the sometimes acrimonious nature of discussions between scientists and those who have to make sure that all the tickboxes have been ticked. The draft indicates that analytical data may be required to support an assertion that an impurity is too reactive to be present in a drug substance. Whether or not this is required in a given case seems to be left to the discretion of the regulatory agency, which (as explained above) may not be aware of or take into account the expense, futility and difficulty of the study. A simple, rapid and general chromogenic test using perhaps an existing reagent such as NBP could provide an approach requiring little or no specific development for each case. For this approach to be accepted by all parties, it will be necessary to accept the (apparently retrograde) argument that a specific chromatographic method is not needed to demonstrate the absence of a compound or class of compounds. On the contrary, a general test of broad specificity provides considerable assurance that a product is safe with respect to reactive genotoxic impurities.

Limitations: drug substances that are electrophilic reagents

A general chromogenic or other reagent for the detection of electrophilic reagent may give similar responses to a small amount of a recognised reagent like dimethylsulfate and a large amount of a weaker electrophile such as S-adenosylmethionine. The two situations can be distinguished by varying the reaction time.

With our thiocyanate method for lower alkylating agents (Lee *et al*., 2003), we obtained a weak response for methanol, a residual solvent. We had chosen acidic conditions, under which alcohols and ethers have some alkylating reactivity. The project was set up to deal with a particular urgent question, and readers familiar with the R&D scene will understand that time was lacking to vary all the analytical conditions.

As discussed in my main article (Lee, 2012), various already-marketed drug substances or their metabolites have fairly obvious electrophilic reactivities, though the authorities don't seem too much bothered by that. These compounds would presumably give a positive response to a general reagent for electrophiles. Quantitative studies of weakly-reactive electrophiles under physiological (or surrogate) conditions seem to be rare. Endogenous compounds that might be worth investigating would include S-adenosylmethionine and perhaps (I speculate) acetylcholine. Such studies might help provide some perspective for the current limits for DNA-reactive exogenous compounds.

One point of difference between a small exposure to a highly reactive electrophile and a high exposure to a weak one is that in the latter case, alkylation of DNA would be relatively less attenuated by the presence of other endogenous nucleophiles such as glutathione.

Compendial general method for ethylene oxide

Ethylene oxide is a highly genotoxic (liquefied) gas of which residues may occur in numerous products. The pharmacopeias have a standard method for the determination, by headspace gas chromatography. This is inconvenient, because chromatographic methods must always be calibrated on each occasion using the analyte being determined, and in this case elaborate and expensive safety precautions are required. A general reagent for DNA-reactive electrophiles would provide an

alternative method.

Methods using NBP for the determination of ethylene oxide have been described in the literature. Since they are not necessarily chromatographic, system suitability testing and calibration (if this is required) could be carried out using a surrogate electrophile that is less hazardous. For cost-conscious readers unfamiliar with these old tests, I recall that they amount to mixing two solutions, heating the mixture, adding a solution of a base, and then measuring the absorbance of the solution.

Methods involving radioisotopes

This topic is presented separately because "small-molecule" CMC pharmaceutical analytical laboratories are not usually equipped for working with radio-labelled compounds. Specialists in metabolism and pharmacokinetic laboratories could handle this; after all, drug metabolism is involved. However, they may be reluctant to use tritium, which tends to get everywhere and may interfere with low-level measurements of carbon-14.

When a guanosine residue is alkylated at N-7, the hydrogen at C-8 becomes acidic and exchanges rapidly with the hydrogen of water (Figure 3). Hydrogen exchange is very slow in unsubstituted guanosine at physiological pH. The increased acidity is due to the positive charge that is shared between the two nitrogens, as shown in the figure using a conventional representation of delocalised bonding. If the DNA is tritium-labelled, the amount of tritiated water formed is stoichiometrically equivalent to the degree of substitution. Determining this is a standard procedure.

The subject was covered in a review by Gates *et al*. (2004), which also discusses the consequent depurination and strand breakage reactions. An analytical method was described by Mattes (1992) whose article provides, incidentally, a particularly explicit example of the sort of confusion about reaction mechanisms that has led to widespread misunderstanding of chemical mutagenicity; this confusion has even led to uncertainty about the mode of action of the classical nitrogen mustard anti-cancer agents.

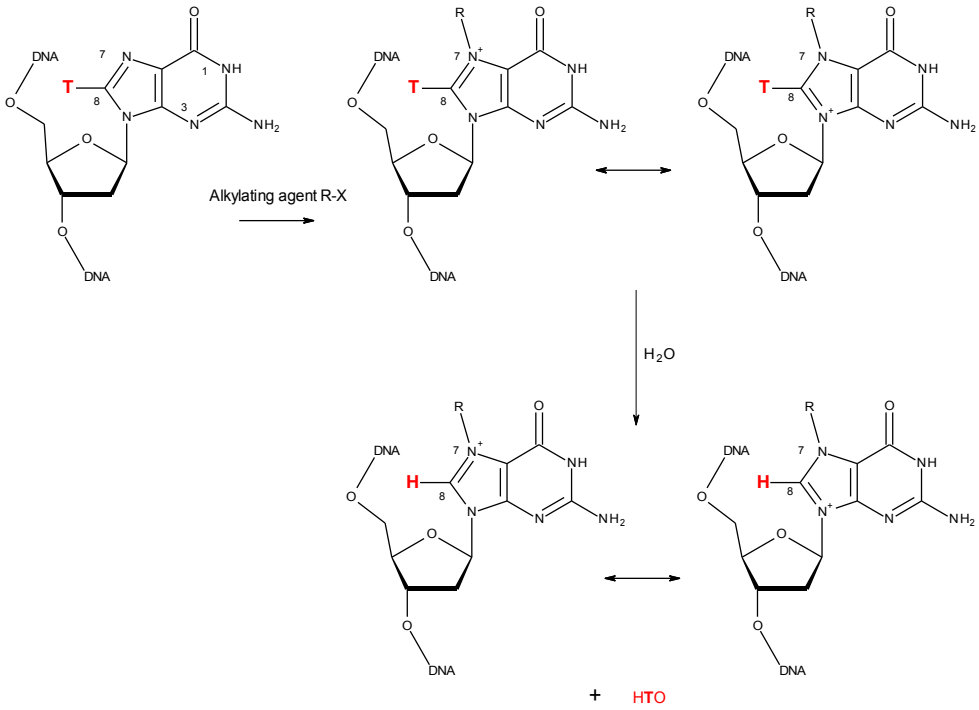

Figure 3: Hydrogen exchange of guanosine upon alkylation at N-7

As explained earlier, labelled DNA in aqueous media would not cover all requirements for an analytical reagent to be used for screening purposes. Clearly, the same principle could be applied using synthetic surrogate reagents. My main objective here is to show that several ways of screening for electrophiles that react with DNA are used in applications other than CMC pharmaceutical analysis.

Who should be charged with developing new reagents and methods?

I propose here that one or more new analytical reagents be developed for the detection and determination of DNA-reactive impurities in pharmaceuticals. The primary objective is to develop a simple and inexpensive limit test whereby substances that are found to comply may be deemed to require no further examination. Other objectives are to be able to identify impurities that are responsible for a positive response, and to determine identified reactive impurities.

These objectives appear to attainable because reagents that fall only slightly short of requirements exist already, and research is currently being carried out in closely related fields. Nevertheless, some additional research work will be required, and one may ask whose responsibility that should be. Any project should involve the collaboration of experts in reagents and sensors who possess the necessary knowledge and experience.

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