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Review

# Control and analysis of alkyl esters of alkyl and aryl sulfonic acids in novel active pharmaceutical ingredients (APIs)

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**Abstract**

This article reviews current regulatory guidelines and relevant scientific literature pertaining to the control and analysis of potential genotoxic impurities (PGIs) in new active pharmaceutical ingredients (APIs) with specific reference to a certain sub-class of PGIs, namely alkyl esters of alkyl and aryl sulfonic acids. Sulfonic acids are very important in pharmaceutical R&D employed both as counter-ions in the formation of acid-addition salts and also as reagents and catalysts in the synthesis of new drug substances. The article reviews the evolution of analytical methodology from early studies in the mid 1970s through development of direct injection GC and HPLC methods to liquid–liquid/solid phase extraction and headspace based techniques coupled to HPLC and GC methodologies employing UV and MS detection to new derivatisation-based techniques.

The paper also reflects on the significant challenges in developing robust analytical methodology capable of the trace determination of sulfonate esters, the challenges in transferring methodology from R&D to QC labs and on the cost of inappropriate limits for genotox impurities. In so doing, the authors seek to inform the debate that the control of genotoxic impurities should be driven primarily by safety and risk/benefit considerations rather than by state-of-the-art analytical and process chemistry capabilities that drive controls to levels 'as low as practicable' regardless of the risk/safety requirements.


*Keywords:* Sulfonate esters; Analysis; Regulatory guidelines

## Contents



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## 1. Introduction

The issue of genotoxic impurities has received considerable attention in the recent past. Focus of much of this attention has been on a specific class of genotoxins, that being alkyl esters of alkyl and aryl sulfonic acids. Outlined below is a historical perspective of this issue, the key events and outcomes and an exploration as to the implications this has for the pharmaceutical industry and in particular the impact on analytical methodology

The European Pharmacopoeia (Ph. Eur.) has been at the forefront of highlighting concerns in this area. The European Directorate for the Quality of Medicines (EDQM) first requested information on the need for pharmacopoeial limit tests for alkyl mesylate impurities in mesylate (mesilate) salts as long ago as January 2000 [1]. EDQM highlighted seven monographs of mesylate salts that were at that time included in the Ph. Eur. and requested information on analytical methods and the level of alkyl mesylate impurities found in practice.

Subsequently, the Ph. Eur. drafted a production statement for inclusion in the monographs of all mesylate containing drug substances [2]. This statement indicated that, "The production method must be evaluated to determine the potential for formation of alkyl mesylates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesylates are not detectable in the final product."

This approach, which is aligned with ICH Q9 [3], allows process knowledge concerning impurity formation [4] to be used to underpin a risk assessment of the likely formation of these reactive impurities, and obviates the need for routine testing.

In contrast, the United States Pharmacopoeia (USP) rarely includes tests for sulfonate esters in their monographs. The one obvious exception is for Atracurium Besylate (benzenesulfonic acid) [5], which includes a test for methyl besylate with an acceptance limit of 100 ppm (the limit in the Ph. Eur. is 10 ppm). The method's selectivity is provided by a fairly complex HPLC (high performance liquid chromatography) gradient profile, with three linear ramps and a 45 min analysis time. The method employs UV (ultra-violet) spectrophotometric detection at a wavelength of 217 nm.

Running concurrent with these events has been the drive from regulatory authorities to produce specific guidance covering this area. This has culminated in The Committee for Medicinal Products (CHMP) issuing their finalised Guideline on the Limits of Genotoxic Impurities [6], effective from 1st January 2007. The CHMP highlighted concerns that the existing ICH guidances (ICH Q3A (R) [7] and Q3B (R2) [8]) did not adequately address this issue. CHMP advocate a generally applicable approach for defining the acceptable risk, which is defined as an additional cancer risk of not greater than 1 in 100,000 based on a lifetime's exposure to a genotoxic impurity. This approach is defined by the threshold of toxicological concern (TTC) and equates to an exposure of 1.5 μg/day lifetime intake of a genotoxic impurity being associated with an acceptable risk [9]. Based on this defined threshold value, appropriate levels of the genotoxic impurity in the active substance can be then calculated based on the expected daily dose. The CHMP also accepts that higher limits may be justified based on extenuating circumstances, such as shorter exposure periods for medicines taken intermittently and for certain disease areas.

The pharmaceutical industry, in response to this greater regulatory focus on genotoxic impurities, has issued their own position paper [10]. Reflecting the CHMP's view that genotoxic impurity limits can vary based on differing periods of exposure, Muller et al. [10] advocate a *staged* TTC; whereby the acceptable daily intake values vary between 1.5 μg/day intake for lifetime exposure to 120 μg/day for 28 days (or less) exposure. This staged TTC ensures that human volunteers can be potentially exposed to virtually safe levels of genotoxic impurities during early clinical development with acceptable risk. At the same time, the higher TTC levels give chemists and analysts the time necessary to understand the mechanisms of formation of these genotoxic impurities and to elucidate approaches for either avoidance or control.

During early development phases, clinical doses tend to vary considerably (often over several orders of magnitude), and this has resulted in limits based on relative levels, i.e. parts per million (ppm) becoming the norm; rather than an absolute TTC value (e.g. 1.5 μg/day). For ease of comparison, the relationship between ppm and percentage is summarized: 10,000 ppm = 1%, 1000 ppm = 0.1% and 1 ppm = 0.0001%. However, the relationship between dose, maximum permitted daily intake of an impurity and concentration of the impurity is complex (see Table 1 and Fig. 1) and as indicated does change rapidly during early development. The FDA reinforced this trend by requesting pharmaceutical companies (at time of IND) for limits based on ppm levels, particularly for alkyl methanesulfonates (mesylates) and benzenesulfonates (besylates) potential impurities. This is unfortunate as exposure is typically expressed in terms of an absolute amount/unit time. However, recent communiqués from the agency have indicated that their thinking may now be aligned with both the CHMP and the pharmaceutical industry views. FDA/CDER is in the process of drafting guidance for genotoxic impurities and it is anticipated that this will be issued shortly.

In the absence of clear guidance from US/EU regulatory authorities, this has led, in many cases, to impurity limits having been driven by analytical and process capabilities rather than by safety and risk/benefit considerations and it is in this context that mention is made of the concept of as low as reasonably practical (ALARP). ALARP has significant implications for both the analytical and process chemist. In some ways the control of genotoxic impurities has historical parallels with the con-

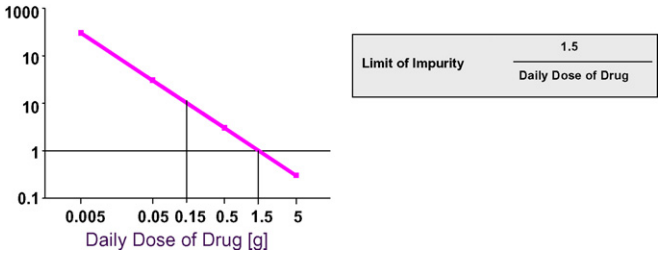



Fig. 1. Schematic representation of relationship between limit of impurity (ppm) and dose of drug (g).

Table 1
Relationship between dose and maximum permitted daily intake (MDI) of an impurity and concentration of the impurity

| Daily Dose of API (mg) | Concentration of Impurity (%) Maximum Daily Intake (MDI) and Duration of Exposure | | | | |
|---|---|---|---|---|---|
| | < 4 weeks | 1-3 months | 3-6 months | 6-12 months | > 12 months |
| | MDI =120 ug | MDI=40 mcg | MDI=20 mcg | MDI=10 mcg | MDI=1.5 mcg |
| 3000.0 | 0.004 | 0.001 | 0.0007 | 0.0003 | 0.00005 |
| 1500.0 | 0.008 | 0.003 | 0.0013 | 0.0007 | 0.00010 |
| 1200.0 | 0.010 | 0.003 | 0.0017 | 0.0008 | 0.00013 |
| 1000.0 | 0.012 | 0.004 | 0.0020 | 0.0010 | 0.00015 |
| 900.0 | 0.013 | 0.004 | 0.0022 | 0.0011 | 0.00017 |
| 500.0 | 0.024 | 0.008 | 0.0040 | 0.0020 | 0.00030 |
| 400.0 | 0.030 | 0.010 | 0.0050 | 0.0025 | 0.00038 |
| 300.0 | 0.040 | 0.013 | 0.0067 | 0.0033 | 0.00050 |
| 200.0 | 0.060 | 0.020 | 0.0100 | 0.0050 | 0.00075 |
| 100.0 | 0.120 | 0.040 | 0.020 | 0.0100 | 0.00150 |
| 90.0 | 0.133 | 0.044 | 0.022 | 0.011 | 0.00167 |
| 40.0 | 0.300 | 0.100 | 0.050 | 0.025 | 0.00375 |
| 30.0 | 0.400 | 0.133 | 0.067 | 0.033 | 0.00500 |
| 25.0 | 0.480 | 0.160 | 0.080 | 0.040 | 0.00600 |
| 20.0 | 0.600 | 0.200 | 0.100 | 0.050 | 0.00750 |
| 10.0 | 1.20 | 0.400 | 0.200 | 0.100 | 0.015 |
| 9.0 | 1.33 | 0.444 | 0.222 | 0.111 | 0.017 |
| 8.0 | 1.50 | 0.500 | 0.250 | 0.125 | 0.019 |
| 7.0 | 1.71 | 0.571 | 0.286 | 0.143 | 0.021 |
| 6.0 | 2.00 | 0.667 | 0.333 | 0.167 | 0.025 |
| 5.0 | 2.40 | 0.800 | 0.400 | 0.200 | 0.030 |
| 4.0 | 3.00 | 1.00 | 0.500 | 0.250 | 0.038 |
| 3.0 | 4.00 | 1.33 | 0.667 | 0.333 | 0.050 |
| 2.0 | 6.00 | 2.00 | 1.00 | 0.500 | 0.075 |
| 1.0 | 12.00 | 4.00 | 2.00 | 1.00 | 0.150 |
| 0.5 | 24.00 | 8.00 | 4.00 | 2.00 | 0.300 |
| 0.4 | 30.00 | 10.00 | 5.00 | 2.50 | 0.375 |
| 0.3 | 40.00 | 13.33 | 6.67 | 3.33 | 0.500 |
| 0.2 | 60.00 | 20.00 | 10.00 | 5.00 | 0.750 |
| 0.1 | >100% | 40.00 | 20.00 | 10.00 | 1.50 |

trols of residual solvents or volatile organic impurities (VOCs). Although, ICH Q3C [11] gave guidance for allowable levels of VOCs based on appropriate safety considerations (acceptable exposure levels); industry soon realised that the regulators were viewing the same issue from a process capability perspective and requiring industry to control VOCs at levels significantly lower than those established in ICH Q3C.

Synthetic starting materials and intermediates are reactive by design and may occur as impurities in the final API. Unfortunately, the nature of this chemical reactivity often translates into biological reactivity, and these materials can often be mutagens and/or carcinogens. Consequently, pre-clinical development scientists need to understand both the potential risks associated with these suspected genotoxic impurities deriving from the proposed synthetic route; as well as understanding the capabilities of the selected synthetic route to control these genotoxins at low ppm levels [12]. It needs to be clearly understood that default ppm limits will drive sacrificial purifications and/or alternative route development work, which are costly in terms of time and money, impacting on the timelines for bringing novel medicinal products to patients.

Interestingly, the chemical reactivity of these intermediates can be often utilized to develop strategies for control. Dobo et al. [12] reported on the impurity fate mapping of several alkylating agents and showed that the high reactivity generally precluded their retention within the final API (especially if their formation was separated from the final stage API by several synthetic steps).

Similar conclusions were reported by Miller et al. [13], where kinetic experiments using an API spiked with ethyl methanesulfonate (EMS), demonstrated that this ester readily hydrolyses in water. Hydrolysis rates in the API appeared to be dependant on the relative amounts of residual ethanol, water and methanesulfonic acid (MSA). They used a capillary GC/MS method capable of determining EMS within the range 50–200 ppm.

One of the biggest scientific challenges facing the pharmaceutical analyst has been the need for rapid development of extremely sensitive and robust analytical methodologies that can adequately monitor potentially genotoxic impurities at these very low levels. The major issues are sensitivity, selectivity and the related problem of overcoming matrix interference in APIs. The issue of selectivity cannot be overstated, as basic understanding of chemistry at the ppm level is limited. As with environmental analysis, technique is everything, and even the best analytical methodologies will struggle to overcome the problems of transient cross-contamination, i.e. between the sam-

ple under test and the standards employed resulting in false positives. Similar problems may also arise, for instance in GC, sample pyrolysis and in HPLC, reaction between alcoholic solvents and residual reagents, which can generate trace levels of the analyte of interest. Hence appropriate controls need to be built into the analytical procedures to ensure confidence in the results generated. The analyst may then be faced with the significant additional challenge of transferring methodologies developed in specialist R&D laboratories equipped with latest (expensive) state-of-the-art instrumentation requiring highly trained specialist staff to global production/QC environments and regulatory laboratories. It is therefore imperative that the analytical methodology is robust.

This review aims to summarise the general analytical approaches in this area and to provide guidance for analysts working in this developing field. Given the high level of interest pertaining to alkyl esters of alkyl and aryl sulfonic acids much of the analytical work in this field has focused on such analytes. Thus this review concentrates on this specific class of analyte.

## 2. Analysis of sulfonic acid and sulfate esters

### 2.1. Introduction

As stated, there has been a high degree of concern from a regulatory perspective in relation to the potential formation of sulfonic acid esters, e.g. mesylate and besylate esters. These compound classes may arise from chemical reaction between alcoholic solvents (e.g. methanol, ethanol, propanol and isopropanol) and strong acids (sulfuric and sulfonic acids) commonly employed as reagents and counter-ions in the preparation of pharmaceutical salts. Consequently, there has been a general renaissance and increased number of publications in the trace analysis of these analytes through the use of both gas chromatography (GC) and high performance liquid chromatography (HPLC), coupled to selective detection techniques, e.g. mass spectrometry (MS).

The short chain alkyl esters (where the alkyl chain length is between 1 and 3) of methanesulfonic acid (MSA) are known DNA reactive genotoxins, and possibly carcinogenic alkylating agents. Regulatory concerns over the potential formation of these esters during salt formation (in alcoholic solvents), has prompted requests to demonstrate that these esters are not present in the API, at low ppm levels. However, Snoddin [14] has argued that these concerns are misplaced and bases this contention on the very low nucleophilicity of the mesylate ion, which suggests that mechanistically the alkyl mesylates should not be formed.

### 2.2. Early analytical studies

Filby et al. [15] assessed the relative molar response (RMR) of a GC/FID (gas chromatography-flame ionization detector) of 13 organo-sulfur compounds. These included methyl methanesulfonic acid (MMS), methyl ethanesulfonic (MES), methyl *n*-propanesulfonic (MPS), methyl *i*-propanesulfonic (MIPS), methyl *n*-butanesulfonic acid (MBS) and methyl *t*-butanesulfonic acid (MTBS).

The authors utilized a 10% diethylene glycol succinate on chromosorb stationary phase with a mixture of hydrogen and air (30 ml/min) as the mobile phase; the inlet temperature was 80 °C with a temperature increase of 2 °C/min, up to 190 °C. The authors found that, with the exception of compounds containing quaternary carbon atoms, that the RMR values were roughly proportional to the numbers of carbon atoms in the molecule.

As the chemical reactivity of volatile alkyl sulfonic acid esters diminishes with increasing alkyl side chains; many research groups have tried to develop generic methodology capable of determining ppm levels of these esters in the acid starting materials (alkyl sulfonic acids) and API.

### 2.3. Direct injection techniques (HPLC and GC)

The first authors to report on the typical levels of methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) within starting materials and API were Ramjit et al. [16]. They developed a GC/MS method to identify and quantify levels of these sulfonic acid esters in the bismesylate salt of the positive inotrope agent, DPI 201-106. They utilized a DB-WAX (0.25 μm film thickness) fused silica capillary GC column (30 m × 0.25 mm i.d.). Analyses were performed using splitless injection mode with an injection port temperature of 200 °C. The column oven temperature was programmed to start at 80 °C and held for 2 min and then to increase by 16 °C/min to 160 °C, where it was held for a further 10 min. The carrier gas was helium and the flow rate was 1.2 ml/min.

Quantitative GC/MS was performed in the single ion-monitoring mode (SIM) using *m/z* of 78.9853 as the common mass peak for MMS and EMS. One microliters of a 100 ng/μl stock solution of MMS and EMS was used as the reference standard. Using these analytical conditions, Ramjit et al. showed that the free base of DPI 201-106 did not contain any detectable levels of these esters; in contrast the bismesylate salt contained 0.51 and 1.31 ppm (μg/g) of MMS and EMS, respectively. Levels of residual alcoholic solvents in the API were typically 10× the levels of the sulfonic acid esters observed (methanol 9 ppm and ethanol 10.8 ppm).

The authors could not unequivocally demonstrate that MMS was formed during the preparation of the bismesylate salt, as the levels of MMS in the acid starting material (methanesulfonic acid) were similar (0.8 ppm) to that observed in the API (0.51 ppm). Snoddin [14] did subsequently argue based on molar equivalencies that based on the bismesylate salt containing approximately 32% of MSA, that if MMS were carried over in directly proportional levels to the MSA levels, that the concentration in the API should be 0.26 ppm.

However, Ramjit et al. [16] could state that EMS formation was as a result of salt formation as there was no EMS detected in the acid starting material and there was 1.31 ppm of EMS in the API. The authors also confirmed that these esters were not formed in situ, even with aged analytical solutions (up to 30 days).

Miller et al. [13] developed a GC/MS method for residual levels of EMS in a mesylate salt of an API crystallized from ethanol. The method was capable of detecting EMS down to levels of 50–200 ppb. The authors found that EMS did not form on storage of the API.

Li [17] utilized a stationary phase comprised of a cross-linked polyethylene glycol; DB-WAX (1 μm film thickness) capillary (30 m × 0.53 mm i.d.) with FID detection for the determination of residual methyl (MMS), ethyl (EMS) and *i*-propyl methanesulfonic acids (IMS) in the mesylate salt of an API. The mobile phase was helium with a flow rate of 5 ml/min. The column oven temperature was programmed to start at 80 °C and held for 1 min and then to increase by 16 °C/min to 200 °C, where it was held for a further 1 min. Samples were introduced using splitless injection techniques (5 μl), with an inlet temperature of 120 °C.

A limit of detection (LOD) of about 0.04 μg/ml, which is equivalent to 1 μg/g (1 ppm) and a limit of quantitation (LOQ) of 0.2 μg/ml, equivalent to 5 μg/g (5 ppm) of each of the sulfonic esters was achievable. Linearity was demonstrated over the range 0.04–4 μg/ml (1–100 ppm), with a regression coefficient of $R^2 > 0.9999$. Accuracy was acceptable at these very low levels, with recoveries in the range 78–123% (0.2 μg/ml), 82–105% (0.4 μg/ml) and 80–104% (1.6 μg/ml). Standard precision at 0.2 μg/ml was again acceptable (<3.8%). Replacement of the injection liner was necessary after 20 injections, due to build up of residual API.

### 2.4. Extraction based studies

As highlighted in the work described above matrix related interference can adversely affect the robustness of the analytical procedure employed. To eliminate/reduce such affects there has been considerable focus on developing appropriate extraction techniques aimed a cleaning up the analyte to remove such interference.

A liquid–liquid extraction (LLE) pre-concentration procedure with GC/MS detection was utilized by Colon and Richoll [18] to measure levels of residual alkyl sulfonic acid esters in methanesulfonic acid (MSA) starting material. Three esters could be rapidly quantitated at the ppm level; methyl methanesulfonic acid (MMS), ethyl methanesulfonic (EMS) and propyl methanesulfonic (PMS).

However, LLE methodologies are labour-intensive and can be prone to interference from other solvents utilized in the process, as well as problematical emulsion formation. Recent advances in the field of continuous extraction technologies; particularly, liquid phase micro-extraction (LPME), solid phase extraction (SPE) and solid phase micro-extraction (SPME) offer an elegant way forward.

A comparative assessment of these approaches was conducted by Colon and Richoll [18] who developed and validated SPME, LPME and SPE methodologies, coupled with GC/MS and single ion-monitoring (SIM). Using these approaches they developed limit tests (5 ppm) for the methyl (MMS), ethyl (EMS) and isopropyl esters (IMS) of methanesulfonic acid (MSA), the methyl (MBS) and ethyl esters (EBS) of benzenesulfonic acid (BSA) and the methyl (MTS) and ethyl esters (ETS) of *p*-toluenesulfonic acid (TSA) in APIs.

Based on the polarity of the analytes a DB-1701 GC column was selected to optimise selectivity of the 7 esters and thereafter three selective *m/z* ions were used for each ester in the MS/SIM determinations. The authors optimised the extraction stationary phase, extraction times and assessed the impact of the pH of the mobile phase on the SPME procedure. Analytical interference from the sample matrix were minimised by ionization at acidic pH.

Four model API molecules with p$K_a$'s in the range 7.1–10.9 and with good solubility in phosphate buffer (>10 mg/ml) were assessed. Each API was spiked with the mixture of the 7 esters at the 5 ppm level and validation data for API 1 was reported. The method reproducibility was good with all seven esters having R.S.D.s of less than 6%. The method showed good linearity in the range of 80–120% of analyte concentration (5 ppm) with $R^2$ values in excess of 0.9 for all analytes (both with and without API present). Accuracy data was again good and ranged from 92.0% (MTS) to 104.0% (EMS) recovery, and was able to distinguish between negative outcomes of the limit test (<5 ppm) and positive outcomes (>5 ppm).

The SPE method was also optimised and gave good linearity in the analyte range 2.5–50 ppm ($R^2$ values in excess of 0.9). The authors suggested that this approach offered utility when the aqueous solubility of the API is limited. The LPME method was more problematical as low polarity solvents were required which effectively extracted the less polar esters, but caused analytical challenges for their more polar cousins. Dichloromethane was the best solvent with concentration factors in the range 189–247%.

### 2.5. Comparative studies GC versus HPLC

Taylor et al. [19] evaluated both GC with flame ionization detection (GC/FID) and HPLC/MS for the detection of residual sulfonic acid esters in API. The former method was discontinued as hydrolysis of the esters in the injection liner was observed when aqueous acetonitrile solvent was used as solvent for the API. As the impurities of interest were aryl sulfonic esters: methyl (MTS), ethyl (ETS) and isopropyl tosylates (ITS), methyl (MBS), ethyl (EBS), butyl (BBS) and isopropyl besylates (IBS), all containing a phenyl moiety, reversed-phase HPLC was applicable and HPLC/MS/SIM methods were developed. Zorbax RX C8 and Prodigy ODS stationary phases were evaluated and the former was selected. A range of mobile phase conditions were employed. These were based on either aqueous ammonium acetate or formic acid (with a trace of ammonia). Both methanol and acetonitrile were studied as organic modifiers. In all cases ammonium adducts were measured.

Two APIs were evaluated; the first (1) was a carboxylate salt, which used *p*-toluenesulfonic acid and methanol, ethanol and propan-2-ol in its synthesis, whilst the second (2) was a besylate salt. API (1) was spiked with tosylate esters (MTS, ETS and ITS) at 0.5, 1.0 and 1.5 ppm; whereas API (2) was spiked with the besylate esters (MBS, EBS, BBS and IBS) at the same levels.

The linearity of the tosylate esters was determined in the range 0.05–2.0 ppm, with a $R^2$ of greater than 0.998 for all analytes. The recoveries of the tosylate esters were good at all three spiked levels: giving values in the range of 82–92% (0.5 ppm, $n = 3$), 94–95% (1.0 ppm, $n = 6$) and 99–104% (1.5 ppm, $n = 3$), respectively. The limit of quantitation (LOQ) was determined at between 0.02 and 0.05 ppm for all of the tosylate esters. There was slight interference from API (1) with the earliest eluting analyte (MTS) and this increased the LOQ from 0.05 to 0.11 ppm; however, the remaining esters were all well resolved from the API and their LOQs were not affected. The stability of the esters in the presence of API (1) was evaluated. At the 2 ppm level, the least stable ester (ITS) was only stable for 30 min at ambient temperature (less than 5% hydrolysis), whereas, the other esters were stable for 3 h.

The linearity of the besylate esters was determined in the range 0.1–2.0 ppm, with a $R^2$ of greater than 0.998 for all analytes. The recoveries of the besylate esters were good at all three spiked levels: giving values in the range of 94–100% (0.5 ppm, $n = 3$), 86–102% (1.0 ppm, $n = 6$) and 86–105% (1.5 ppm, $n = 3$), respectively. The recoveries were worst for MBS and hydrolysis and/or ion suppression were attributed as the cause. The limit of quantitation (LOQ) was determined at between 0.02 and 0.20 ppm for all of the besylate esters, with the MBS giving the poorest response. As with API I, there was slight interference of API II with the earliest eluting analyte (MBS) and this decreased the LOQ from 0.20 to 0.33 ppm; however, the remaining esters were all well resolved from the API and their LOQs were again unaffected.

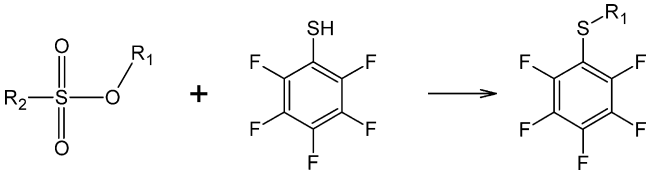
This method employed the use of a switching valve in order to avoid the introduction into the MS detector of non-volatile API. A drawback of this approach is the lack of stability of the analyte in aqueous acetonitrile. At the 2 ppm level, solutions were only found to be stable for 3 h at ambient temperature, i.e. less than 5% hydrolysis. This may impact upon the practicality of the method concerned.

When formic acid was employed sensitivity was found to be poorer and a series of adducts were observed. For API (2), both ammonia and acetonitrile adducts were formed. This was resolved through the deliberate introduction of ammonia, leading to the formation of a single predominant ion of $M+NH_4^+$.

### 2.6. Derivatisation

The other principal approach to the determination of alkylating agents is via analyte derivatisation. Such an approach has several potential advantages, e.g. by stabilizing reactive analytes and by facilitating extraction, separation or detection of analytes by judicious selection of the appropriate derivative. Lee et al. [20] determined MMS, EMS, IMS and dimethyl sulfate (DMS; another potent genotoxin) using derivatisation with aqueous sodium thiosulfate, giving mixtures of the corresponding alkylthiocyanates and alkylisothiocyanates. These derivatives can be directly analysed by headspace GC–MS and the less favoured derivatives (alkylisothiocyanates) are formed in only minor amounts. Under the optimum derivatisation conditions (20 min at 85 °C) reaction yields were optimum for the methyl methanesulfonate (100%) and 74% for ethyl methanesulfonate.

Table 2
Derivatisation reaction, retention time and selected ions for the determination of alkylating agents as their pentafluorothiophenol derivatives[a]




| Compound | Abbrev. | $R_1$ (analyte) | $R_1$ (IS) | Retention time (min) | | Diagnostic ions ($m/z^+$) | |
|---|---|---|---|---|---|---|---|
| | | | | Analyte | IS | Analyte | IS |
| Mesylates $R_2 = –CH_3$ | | | | | | | |
| Methyl methanesulfonate | MeMS | $–CH_3$ | $–CD_3$ | 4.34 | 4.33 | 214 | 217 |
| Ethyl methanesulfonate | EtMS | $–CH_2CH_3$ | $–CD_2CD_3$ | 4.45 | 4.44 | 228 | 233 |
| Isopropyl methanesulfonate | *i*PrMS | $–CH(CH_3)_2$ | $–CD(CD_3)_2$ | 4.64 | 4.62 | 242 | 249 |
| Besylates ($R_2 = –C_6H_5$) | | | | | | | |
| Methyl benzenesulfonate | MeBS | $–CH_3$ | $–CD_3$ | 4.34 | 4.33 | 214 | 217 |
| Ethyl benzenesulfonate | EtBS | $–CH_2CH_3$ | $–CD_2CD_3$ | 4.45 | 4.44 | 228 | 233 |
| Isopropyl benzenesulfonate | *i*PrBS | $–CH(CH_3)_2$ | $–CD(CD_3)_2$ | 4.64 | 4.62 | 242 | 249 |
| Tosylates ($R_2 = –C_6H_4CH_3$) | | | | | | | |
| Methyl *p*-toluenesulfonate | MepTS | $–CH_3$ | $–CD_3$ | 4.34 | 4.33 | 214 | 217 |
| Ethyl *p*-toluenesulfonate | EtpTS | $–CH_2CH_3$ | $–CD_2CD_3$ | 4.45 | 4.44 | 228 | 233 |
| Isopropyl *p*-toluenesulfonate | *i*PrpTS | $–CH(CH_3)_2$ | $–CD(CD_3)_2$ | 4.64 | 4.62 | 242 | 249 |
| Sulfates ($R_2 = –OR_1$) | | | | | | | |
| Dimethyl sulfate | DMeS | $–CH_3$ | $–CD_3$ | 4.34 | 4.33 | 214 | 217 |
| Diethyl sulfate | DEtS | $–CH_2CH_3$ | $–CD_2CD_3$ | 4.45 | 4.44 | 228 | 233 |
| Diisopropyl sulfate | D*i*PrS | $–CH(CH_3)_2$ | $–CD(CD_3)_2$ | 4.64 | 4.62 | 242 | 249 |

[a] Reprinted from Ref. [21], copyright 2007, with permission from Elsevier.

The linearity of the esters were determined in the range 0.02–50.0 ppm, and no deviations from linearity were apparent for the analytes; however, the authors did concede that it was difficult testing for this statistically, in the absence of an internal standard. The recoveries of the methyl and ethyl esters at the 1 ppm level were acceptable, with values of 108 and 94%, respectively. Although, the API was crystallized from both methanol and ethanol, only small levels of MMS were observed, at a value of less than 10 ppm.

The precision of the esters at the 0.1 ppm level, were assessed using triplicate assays, by inverse regression analysis utilising a 5-point regression line. The worst values were obtained from the IMS ester, but the R.S.D. of ±15.6% was still considered to be acceptable at this very low residual level (2× LOD).

The limit of detection (LOD) was determined at between 0.02 ppm for the MMS and EMS esters and DMS, and 0.05 ppm for the IMS ester. The authors also assessed the LODs for the same method using FID detection; however detection levels were much higher (5–10 ppm). They speculated that additional method optimisation (e.g. volume injected, split ratio, reduced transfer line flow rate and use of wide bore columns, etc.) could have improved both sensitivity and precision, as there were no interfering peaks.

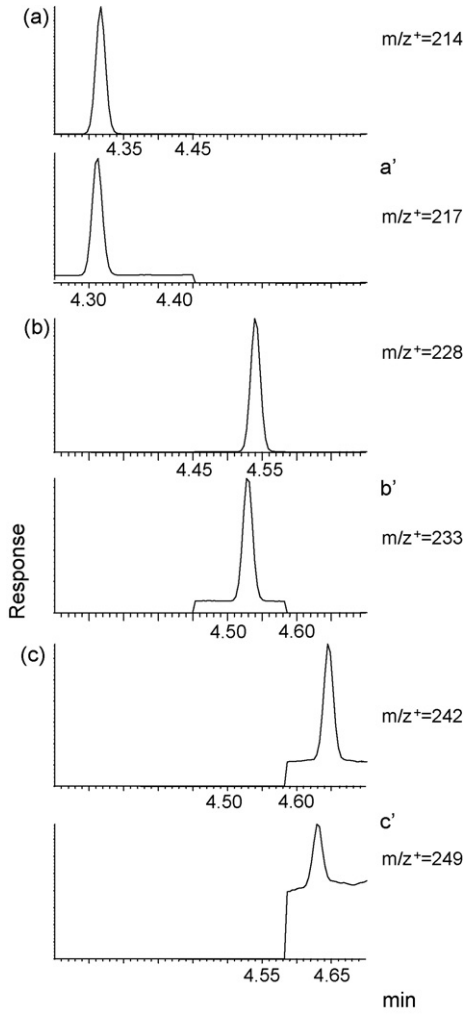



Fig. 2. HS–GC–MS selected ion chromatograms showing the derivative (a) methyl ($m/z^+ = 214$), (b) ethyl ($m/z^+ = 228$), (c) isopropyl ($m/z^+ = 242$), alkylating agent and their corresponding deuterated IS (a′) $m/z^+ = 217$, (b′) $m/z^+ = 233$, (c′) $m/z^+ = 249$ (analyte = alkyl tosylate at 1.0 μg $g^{-1}$, 1 ppm relative to 50 mg of sample).

One of the consequences of their inherent reactivity is that alkyl mesylate esters tend to readily hydrolyse in aqueous environments to form the corresponding alcohol. The authors [20] showed that there was slight interference from the residual methyl alcohol (equivalent to a level of about 0.1 ppm); but there was no detectable interference from the corresponding ethyl and isopropyl alcohol.

Recently the development of a rapid and generic approach for the determination of alkylating agents by derivatisation followed by headspace GC/MS has been reported by Alzaga et al. [21]. The method utilises an in situ derivatisation procedure with pentafluorothiophenol (PFTP) as the derivatisation agent (see Table 2).

The authors highlighted a number of advantages inherent in this approach. These include improved separation and detectability. The derivatives have increased volatility/decreased polarity and hence are more amenable to headspace sampling leading to greatly reduced interference from the matrix, thereby enhancing selectivity. The derivatives are amenable to sensitive analysis using GC/MS/SIM (see Fig. 2) The authors have applied this generic approach to methyl, ethyl and isopropyl mesylates, besylates, tosylates and sulfates in a range of matrices with R.S.D.s in the range of 2.8–10% at analyte concentrations of 1 μg/g (1 ppm). Recoveries in the range of 85–100% were determined however some matrix-dependant effects were observed reducing recoveries (e.g. 40% for methyl besylate) necessitating the use of deuterated internal standards.

## 3. Conclusion

The pharmaceutical industry has risen to the significant challenge of controlling genotoxic and potential genotoxic impurities in new APIs. Synthetic starting materials, reagents and intermediates are reactive by design and may occur as impurities into the final API. However, this chemical reactivity often translates into subsequent biological reactivity, and hence these genotoxic impurities are often mutagenic and/or carcinogenic by nature.

Some commentators have expressed concerns that some of this regulatory focus is misplaced [14] and that mechanistically the alkyl mesylates in particular, should not be formed by reaction between alkyl alcohols and methane sulfonic acid. Further experimental studies are planned through the Product Quality Research Institute (PQRI) to investigate this.

Impurity fate mapping [12] has been utilized to assess the capabilities of the selected synthetic route to either eliminate or control these genotoxins. The pharmaceutical industry has advocated a *staged* TTC approach; whereby the acceptable daily intake values vary between 1.5 μg/day intake for lifetime exposure to 120 μg/day for 28 days (or less) exposure [10].

However, these very low impurity levels (μg/day) do present very real analytical challenges. Initially, analysts relied on the volatility of the sulfonic acid esters and developed GC (usually

with FID) and GC/MS methodologies. However, substrate interference, principally from the API, was often a restricting factor. The use of a variety of extraction techniques (LLE, LPME, SPE and SPME) to reduce matrix interference was an elegant alternative approach and allowed reproducible GC/MS methodologies to be developed [18].

Lee et al. [20] determined residual MMS, EMS, IMS and dimethyl sulfate (DMS; another potent genotoxin) levels using alkylthiocyanate derivatives; which could be directly analysed by headspace GC/MS. The limit of detection (LOD) was determined at between 0.02 ppm for the MMS and EMS esters and DMS, and 0.05 ppm for the IMS ester. The authors also assessed the LODs for the same method using FID detection; however detected levels were much higher (5–10 ppm).

More recently, Alzaga et al. [21] also adopted a derivatisation approach using pentafluorothiophenol (PFTP) as the derivatisation agent with claims to have developed rapid and generic methodology for the trace analysis of alkylating agents. The derivatives are amenable to sensitive analysis using GC/MS/SIM. The authors analysed methyl, ethyl and isopropyl mesylates, besylates, tosylates and sulfates in a range of matrices with R.S.D.s in the range of 3–10% at analyte concentrations of 1 μg/g (1 ppm). Matrix-dependant effects were observed to lead to reduced recoveries and necessitating the issue of deuterated internal standards.

However, there are still practical issues inherent with both GC/FID and GC/MS approaches which has prompted development of LC/MS methodologies. Direct analysis, using HPLC/MS in the single ion mode was favoured by Taylor et al. [19]. They reported a limit of detection (LOD) of 0.02 ppm for the MMS and EMS esters and DMS, and 0.05 ppm for the IMS ester.

The intrinsic robustness (or lack thereof) of these extremely sensitive analytical methodologies capable of determining very low levels (ppm) of alkyl sulfonic acids should not be underestimated. The challenges of developing, validating and transferring these methods into a routine, factory environment are significant. In addition, the pharmaceutical industry has no long-term experience in the use of these methodologies within the factory setting, and whether this is a viable option, without significant investment in technology and analytical skillsets.

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