CHAPTER 2

DEVELOPMENT AND VALIDATION OF A CAPILLARY ELECTROPHORESIS METHOD FOR DETERMINING DIMETHYL SULFATE, A CARCINOGEN; AND CHLOROACETYL CHLORIDE A POTENTIAL GENOTOXIN AT TRACE LEVELS IN PHARMACEUTICALS
2.1 Introduction to genotoxic Dimethyl sulfate and Chloroacetyl chloride

In the pharmaceutical and chemical industries Dimethyl sulfate (DMS) has very wide applications as a reagent for methylation of phenols, amines and thiols and carboxylic acids. It is mainly used as an intermediate in many pharmaceutical and pesticides syntheses [1]. DMS also has sulfating properties and is therefore used in manufacturing dyes, fabric softeners etc. DMS has a lower cost and higher reactivity in comparison to other methylating agents and therefore is preferred over other reagents by the industry. DMS can affect the base-specific cleavage of guanine in DNA by rupturing the imidazole rings present in guanine [2]. This process has been successfully used to determine base sequencing, cleavage on the DNA chain and other applications.

The International Agency for Research on Cancer has classified DMS [3] as a Class 2 carcinogen based on the carcinogenicity test results. DMS is mutagenic, highly poisonous, corrosive and has a hazardous effect on the environment. Due to its volatile nature DMS also presents an inhalation hazard. It is directly absorbed through the skin, mucous membranes, and gastrointestinal tract. Since the toxicity of DMS is delayed, exposures to potentially fatal levels may occur before any warning symptoms [4] are actually developed. DMS has been tested for carcinogenicity in rats by inhalation, subcutaneous and intravenous injection, and following prenatal exposure and has yielded positive results. It is known to produce local
sarcomas and tumours of the nervous system [3] and therefore can be categorized as a carcinogenic chemical. Several studies have been performed for evaluating the mutagenicity of DMS on mammalian cells which yielded positive results [5-14].

In our case, DMS is used as a methylating agent in one of the manufacturing steps of an active pharmaceutical ingredient, Docetaxel Anhydrous (API-1). Owing to concerns rising from the known genotoxicity of DMS, it was necessary to demonstrate that DMS is controlled within safe limits in API-1 using a suitable analytical method. Based on the daily dosage of API-1, the acceptable limit for DMS derived by applying the threshold of toxicological concern (TTC) concept was “Not more than 50 ppm”.

Chloroacetyl chloride (CAC) is bifunctional; the acyl chloride easily forms esters and amides, while the other end of the molecule is able to form other linkages, e.g. with amines. CAC is a known reagent for acylation [15] and as a two carbon building block for cyclization reactions [16]. In our application it has been used for acylation followed by cyclization in an intermediate synthesis of EVT-201 (API-2). CAC decomposes on heating producing toxic and corrosive fumes including phosgene and hydrogen chloride. Though there is limited and insufficient information available regarding the carcinogenicity and genotoxicity of CAC, it can nevertheless be categorized as a structural alert for genotoxic potential (Class 3 category as per Muller classification [22]). Therefore a suitable analytical method was needed to be developed which could demonstrate that the content of CAC is
within acceptable limits in API-2. The acceptable limit of CAC calculated based on the maximum daily dosage of API-2 was “Not more than 75 ppm”.

The International Conference on Harmonization has provided guidelines on impurities in active substances (ICH Q3A (R) [17]) and medicinal products (ICH Q3B (R2) [18]). These guidelines describe a general concept of classification of impurities, rational for the reporting and control of impurities and qualification of the impurities. However the critical issues related with genotoxic impurities present in drug substances and drug products are beyond the scope of the current ICH Q3 guidelines and have not been addressed by these guidelines. To overcome the short comings of the ICH Q3 guidelines and to assist the pharmaceutical producers and regulators, the Committee for Medicinal Products for Human Use (CHMP) has subsequently issued a guideline on the limits of genotoxic impurities [19] in the year 2006. The CHMP guideline provides a general framework and a practical risk based approach to deal with providing limits to genotoxic impurities in drug substances. To define some acceptable limits for the genotoxic impurities, a prior knowledge of possible mechanism of action and of dose response relationship is a very valuable tool. As per this guideline, a limit for the genotoxic impurities with sufficient evidence for a threshold-related mechanism can be proposed using methods outlined for Class 2 solvents in ICH Q3C(R3)-Note for Guidance on Impurities: Residual Solvents. For genotoxic impurities without sufficient evidence for a threshold-related
mechanism, the guideline proposes a policy of controlling levels to “as low as reasonably practicable” (ALARP principle) if avoiding these impurities is altogether not possible.

The CHMP guideline and a more recent draft guidance document on limits of genotoxic and carcinogenic impurities [20] issued by the US Food and Drug Administration advocate the Threshold of toxicological concern (TTC) approach as the safest way to control the genotoxic impurities. These guidelines recommend that an exposure to a genotoxic impurity at a level of not more than 1.5 μg/person/day can be considered an acceptable qualification threshold [21]. A further safety qualification to address genotoxicity and carcinogenicity concerns is not deemed necessary for any impurity found at a level below this threshold. For a genotoxic impurity, there is an acceptable risk at an exposure level of 1.5 μg/day for a lifetime, which is an additional cancer risk of not greater than 1 in 10,00,000. Based on the existing rate of human cancer, and very conservative cancer risk assessments, this level of exposure may pose a negligible risk for carcinogenicity. The TTC approach is applied to impurities for which long-term carcinogenicity studies have not been performed or there is no data to establish a threshold mechanism of genotoxicity.

The CHMP also recommends that the limits for a genotoxic impurity can vary based on exposure period and these limits can be relatively relaxed in clinical trial studies. For high potency carcinogens such as N-nitrosoamines, aflatoxins and azyoxy compounds, 10-fold
lower values are recommended whereas for carcinogens with established dietary exposure and life threatening indication such as cancer chemotherapy the limits can be relatively relaxed. In accordance with this, Muller et al. [22] suggested 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 [23].

Dobo et al. have proposed an approach to control genotoxic impurities in the APIs during drug development based on understanding potential mutagenic and carcinogenic risks associated with compounds used for synthesis and the process capabilities in removal of these impurities [24]. The fact that many structural alerts may not realistically be genotoxic and biologically active has been explicitly reported by Snodin [25] who provides a qualification strategy based on a review of representative compounds from structurally alerting substances. Some structurally alerting compounds/reagents which are highly reactive in nature when introduced in the early stages of a multistep synthesis are unlikely to be carried over to the API. The regulatory assessors demand that carryover studies are performed to demonstrate that the potential genotoxic impurities in the drug substance are below the TTC levels, considering the potential threat of genotoxins to human health.

Among the techniques used for determining genotoxic impurities, GC-FID/MS and HPLC-UV/MS are the most favored techniques owing to their inherent high sensitivity and precision at
trace levels [26-28]. Irrespective of the analytical technique that is adopted, extractions are often an integral part of the sample preparation as it results in enhancement of sensitivity and also removes interferences from the sample matrix. Since the levels at which genotoxic impurities need to be quantified in the final API are very stringent, a higher limit the same impurities can be proposed when monitored at intermediate steps where these are actually introduced. An evidence of elimination of these impurities in the API due to several steps of purification in subsequent stages of synthesis or a scientific rationale that these impurities will involve in reactions which render them non-genotoxic should be provided to support higher than acceptable limits.

Capillary electrophoresis is a known technique which offers speed, simplicity, high resolution, low reagent consumption, cost effectiveness and is eco-friendly. However this technique is associated with some limitations such as low sensitivity and “not a very good precision” as compared to HPLC and GC. Therefore the application of CE in determining trace analytes is not as popular as the other techniques. The issue of low sensitivity can be addressed through utilizing sample stacking techniques and solid phase extractions (SPE) and liquid-liquid extractions (LLE) for the enrichment of analytes from sample matrices. Since the MS detector is selective and also more sensitive than other conventional detectors, the CE can be interfaced to an MS detector for enhancing sensitivity of trace analytes. The
second limitation of a “Not so good precision” obtained by CE can be overcome by using an internal standard for quantification needs.

A generic approach for determining alkylating agents which is based on derivatisation followed by headspace GC/MS analysis has been developed by Alzaga et al. [29]. This method utilizes an in situ derivatisation procedure with pentafluorothiophenol (PFTP) as the derivatisation agent. Methods for determining DMS by Head Space Gas Chromatography (HS-GC) using MS detection [30] and HS-GC using FID detection [31] in intermediates and drug substances have been reported. Five residual alkylating impurities including alkyl chloride and alkyl bromide have been determined in bromazepam API using capillary electrophoresis by Hansen and Sheribah [32] and the LOQ of this method has been reported to be 0.05%.

To the best of our knowledge, no methods have been reported so far for determining DMS and CAC by capillary electrophoresis. In this chapter, the development and validation of a simple and fast method for determining DMS and CAC in drug substances at trace levels by capillary electrophoresis has been described. It has been demonstrated using this method that the levels of DMS and CAC were within acceptable regulatory limits in the APIs where they have been used in the manufacturing process.
2.2 Experimental

2.2.1 Chemicals and reagents

Sodium hydroxide (1.0N, 0.1N, NaOH, CE grade) and HPCE grade water were procured from Agilent Technologies, (Waldbronn, Germany). Pyridine 2,6-dicarboxilic acid was obtained from Merck (Hohenbrunn, Germany), hexadecyl trimethyl ammonium bromide (CTAB), pyromellitic acid and paratoulene sulfonic acid were procured from Sigma Aldrich (Steinheim, Germany). Benzoic acid and phthalic acid were obtained from Rankem (New Delhi, India). DMS and CAC were procured from Spectrochem (Mumbai, India). All reagents were of analytical grade or highest available purity. API-1 and API-2 were synthesized in Laurus Labs Private Limited (Hyderabad, India).

2.2.2 Equipment

An Agilent Technologies Capillary Electrophoresis system with a built-in diode-array detector was used for the separation studies. The system control, data acquisition and post-run processing of electropherograms was performed using Agilent ChemStation software. An uncoated, bare fused silica capillary of dimensions 64.5 cm (effective length 56 cm), 50-μm id, having an extended light path (bubble factor = 3) for enhanced sensitivity procured from Agilent Technologies (Waldbronn, Germany) was used for the development, validation and application of the method. An alignment interface, containing an optical slit matched to the internal diameter of 150-μm, was used.
**2.2.3 Preparation of solutions**

**Background electrolyte**

About 83.0±1.0 mg of pyridine 2,4-dicarboxylic acid was accurately weighed into a 100 mL volumetric flask. To this flask about 14.5±0.2 mg of CTAB was added and dissolved in HPCE grade water by ultra-sonication for about 10 min. The volume was made upto 100 mL with water and the pH of this solution was then adjusted to 5.6 ± 0.1 with 1.0N NaOH. Before use, the BGE was filtered through 0.2 μm nylon syringe filters.

**Standard and sample solutions**

Into separate 100 mL volumetric flasks, about 30±0.1 mg of standard compounds of DMS and CAC were accurately weighed. A 10 mL aliquot of methanol was added to each flask containing the DMS and CAC standards and ultra-sonication for 10 min. to dissolve. The volumetric flasks were allowed to cool to room temperature and the volumes of each flask were then made up to 100 mL mark with water to obtain stock solutions. A 100 fold dilution of each of these stock solutions was performed (1.0 mL - 100 mL) with the diluent (Methanol:Water 10:90 %v/v) to obtain the DMS and CAC standard solutions. The standard solutions were filtered through 0.2 μm nylon syringe filters before use.
Sample solutions

About 0.5 g of each API sample to be tested was accurately weighed into a 5 mL volumetric flask. To each of these flasks, a 0.5 mL aliquot of methanol was added and then sonicated for about 5 min. The flasks were allowed to cool to room temperature; the volume of each flask was made up to 5 mL mark with the diluent and then sonicated for 15 min. with intermittent shaking. The sample solutions thus obtained were filtered through 0.2 µm nylon syringe filters.

2.2.4 Electrophoretic conditions

The electrophoretic separation was achieved by applying a 30 kV potential (voltage) with negative polarity setting. The injection mode was hydrodynamic; in a programmed sequence of pressure applied at 50 mbar for 60 s at the sample vials, followed by application of a 50 mbar for 5 s pressure on the in-let BGE vial to plug the sample injection. A temperature of 20°C was maintained for the capillary cassette using the peltier-thermostat system. The diode-array detector (DAD) was tuned to wavelength of 350 nm (bandwidth 80 nm) using a reference wavelength of 200 nm (bandwidth 20 nm). DMS and CAC migrated at about 3.0 and 3.3 min respectively under these electrophoretic conditions and the total analysis was completed in 5 min. The BGE vials were replaced with fresh electrolyte after every 25 injections to maintain the reproducibility of separation.

Preconditioning of the new uncoated fused-silica capillaries was performed by flushing initially with CE grade water for 30 min.
followed by flushing with the BGE for 30 min. Prior to every analysis, the capillary was conditioned by flushing with water for 10 min. followed by the BGE for 15 min. Between analyses, the capillary was conditioned by flushing with the BGE for 2 min.

2.2.5 Method Validation

The developed CE method was validated with the assessment of the validation parameters namely specificity, precision, sensitivity (detection and quantitation limits), linearity and range, accuracy and stability of analyte solutions. The validation was performed in accordance with the ICH guidelines for analytical method validation [33].

Specificity

Standard solutions of DMS and CAC were injected individually in the developed method. The corresponding APIs were spiked with DMS and CAC and also with all other known process related impurities and solvents at their specification levels. These spiked solutions were also injected and the resultant electropherograms of standards and spiked samples were evaluated for any potential interferences arising due to impurities and sample matrices at the migration times of DMS and CAC.
**Precision**

The method’s precision was determined in terms of repeatability and intermediate precision. Replicate preparations \((n = 6)\) of API-1 containing DMS and API-2 containing CAC at LOQ levels, 50, 100, 150 and 300% of nominal analyte concentrations were injected to measure the repeatability of the method. The intermediate precision was assessed by injecting six replicate samples of the APIs containing DMS and CAC at LOQ, 50, 100, 150 and 300% levels, every day, on three different days. The %RSD for migration times and content of DMS and CAC were determined as a measurement of method’s precision.

**Sensitivity**

The sensitivity of the method for DMS and CAC was determined by establishing the limits of detection (LOD) and limits of quantitation (LOQ). A series of dilute solutions containing known concentrations of DMS and CAC were prepared and injected to obtain the detector response. The concentrations which yielded signal-to-noise ratios of about 3:1 and 10:1 were considered as detection limits and quantitation limits respectively for these two analytes. At the level of quantification, the method’s precision was assessed in terms of %RSD.
**Linearity and Range**

Stock solutions of DMS and CAC were prepared and then diluted to obtain the linearity solutions. Triplicate solutions were prepared from the stock solutions at nine concentration levels – LOQ, 50, 75, 100, 125, 150, 175, 200 and 300% and each solution was injected once. The linearity data for both analytes was subjected to linear regression analysis with the least squares method. The range of the method was determined from the minimum and maximum concentrations of DMS and CAC analyzed in the linearity, precision and accuracy studies.

**Accuracy**

The accuracy samples were prepared by spiking DMS to API-1 and CAC to API-2 at levels of quantitation (LOQ), 50, 100, 150 and 300% of the nominal analyte concentrations. At each concentration level three spiking samples were prepared which were then analyzed as per the method. The response from fresh prepared standards was used to calculate the recoveries for DMS and CAC in the spiked samples. The accuracy of the method was determined in terms of the mean percentage recoveries of the analytes at each level. An ANOVA test was performed to confirm that the recoveries were independent of the spiked concentrations.
Stability in Analytical Solution

Standard solutions of DMS and CAC were prepared at analyte concentrations in the diluent. Each solution was analyzed immediately after preparation and divided into two parts. One part of the solution was stored at 2–8 °C in a refrigerator and the other part at bench top in well-sealed volumetric flasks. These solutions were injected after 24 and 48 hours. The recoveries of DMS and CAC were calculated in these stored samples against the zero hour samples.

2.3 Results and Discussion

2.3.1 Method development and optimization

Dimethyl sulfate rapidly decomposes on contact with water to yield methanol and methyl sulfate [34] as shown in Fig. 2.1a. In a similar manner, when chloroacetyl chloride reacts with water the resultant products are chloroacetic acid and hydrochloric acid (Fig. 2.1b). At the working pH of 5.6 both DMS and CAC are anionic in nature carrying a uni-negative charge. When an electrical field is applied, these ions tend to migrate towards the anode terminal due to electromigration.

![Chemical Reaction](image)

**Fig. 2.1a – Reactivity of Dimethyl sulfate with water**
The development of a CE method with indirect photometric detection often requires skillful manipulation of the background electrolyte to suit the applications based on the charge and mobility of the analytes. Some literature methods are available for determining the anionic analytes by CE using indirect photometric detection [35-44].

In the development and optimization trials of this method, different anionic probes or visualizing agents were evaluated at 2, 5 and 10 mM concentrations such that maximum sensitivity good peak symmetry and high selectivity is obtained for DMS and CAC through indirect photometric detection. In all BGE systems, an EOF modifier CTAB was used at a sub-micellar concentration (0.4 mM) for reversing the EOF and the separations were performed by applying potential in negative polarity mode (detector end towards anode terminal). This ensures that both the EOF and the analyte migration are directed towards the detector thereby speeding up the analysis.

When the separation trials were made with the BGE containing 5 mM pyromellitate+0.4 mM CTAB (pH 7.7±0.1) the peaks of both DMS and CAC were found to be fronting. The separation was not adequate and rather poor in the BGE containing 5 mM p-toluene
sulfonic acid+0.4 mM CTAB (pH 6.0±0.1). In another BGE system that consisted of 5 mM benzoic acid + 0.4 mM CTAB (pH 6.0±0.1), the DMS peak was found to be splitting. A high level of background noise at the operational wavelength and poor and unacceptable symmetries for the DMS and CAC was obtained when the separation was attempted in the background electrolyte having a different probe namely 5 mM phthalate + 0.4 mM CTAB, (pH 6.5±0.1). The structures of the anionic visualizing reagents are provided in Fig. 2.2.

**Fig. 2.2: Structures of anionic visualizing reagents**

In CE, the Kohlraush regulating function determines the probe displacement by the analyte [45] and the probe’s mobility and optical properties must be considered. The separation performance of the method is guided by the selection of the right probe as they influence the peak shapes and efficiency. Thus the mobility and concentration of the probe play a very critical role in developing a selective and
sensitive method. The mobilities of the probes that were evaluated are in the order: pyromellitate > phthalate > pyridine dicarboxylate > benzoate > p-toluene sulfonate [46]. Pyromellitic acid has a high mobility and is a good probe suitable for analyzing smaller fast moving anions. The mobility of the benzoate and p-toluene sulfonate probes is low and they are more appropriate for analyzing compounds having a lower mobility such as short chain (C4-C8) carboxylic acids. Phthalate and pyridine 2,6-dicarboxylate probes have similar mobilities and are most suitable and tailor made for analyzing medium mobility species. The absorptivity of the probe at the detection wavelength is a key parameter which determines the sensitivity of the method. Higher the absorptivity of the probe, greater is the sensitivity of the method. The benzoate and pyridine 2,6-dicarboxylate probes have higher molar absorptivities when compared to the other probes that were tested [47].

Pyridine 2,6-dicarboxylate has a medium mobility that is well suited for the targeted analytes and has a very high molar absorptivity; therefore it was the reagent of choice and the preferred probe for analyzing the DMS and CAC species. BGEs containing 2-10 mM pyridine 2,6-dicarboxylic acid + 0.4 mM CTAB (pH 5.6±0.1) were evaluated for optimum separation and sensitivity. At lower concentrations of pyridine 2,6-dicarboxylic acid broad peaks of DMS and CAC were observed due to electromigration dispersion. On the other hand higher concentrations of the probe resulted in higher noise levels, decreased sensitivity and an adverse effect on the linearity of
detection [48]. The best separation yielding symmetric peaks, high sensitivity and high resolution between DMS and CAC could be achieved using the background electrolyte system containing 5 mM pyridine 2,6-dicarboxylic acid + 0.4 mM CTAB (pH 5.6±0.1) which was used for in the optimized method.

The effect of applied voltage on the separation was evaluated at 20, 25 and 30 kV. As expected, with an increase in the applied voltage there was a proportional decrease in the migration times of the analytes. At higher voltage the separation proceeds rapidly minimizing the effects of diffusion and peak broadening. Therefore the highest available voltage on the equipment (30 kV) was chosen as it provided higher theoretical plates and a very good resolution.

The temperature effects on separation were evaluated at 15, 20, 25, 30 and 35°C. The migration times decreased with the increase in temperature due to a decrease in BGE viscosity. However the temperature was optimized to 20°C as the theoretical plates decreased and the noise increased significantly at elevated temperatures.

The indirect photometric detection is applied to visualize transparent analytes that lack chromophores. A high background absorbance that is continuous is created by the probe ions and when the transparent analyte species displace these probe ions, negative peaks corresponding to these analytes are generated. The negative signals can be made to positive by choosing the sample wavelength at which the sample has minimum or no absorbance and the reference wavelength at which the probe has maximum absorbance. Utilizing
the known knowledge of probe’s absorbance, several combinations of
the signal (sample wavelength) and reference channels were evaluated
in Diode Array Detection (DAD) mode such that the sensitivity for the
analytes was maximized. The highest sensitivities (signal-to-noise)
ratios were obtained with the signal (sample wavelength) acquired at
350 nm (with a bandwidth of 80 nm) using a reference wavelength of
200 nm (with a bandwidth of 20 nm).

A well-known limitation of CE is decreased sensitivity due to
injection of very small sample volumes and a very short optical path-
length. However these limitations can be overcome by sample stacking
and injection of higher volumes of the analyte by modified injection
techniques [49-53]. To maximize the sensitivities of analytes present
at trace levels, high volumes of the sample solutions were injected
through hydrodynamic mode of injection. To induce field amplified
stacking of the analyte species, samples were prepared in a buffer free
diluent (water:methanol (9:1)). The stacking ensured that there was no
significant electrodispersion and broadening of the peaks even with
longer injection plugs. Thus the sensitivity could be increased
significantly without any detrimental effects on the separation
efficiencies.

The effects of injection plug length on the signal-to-noise ratios
(S/N), peak symmetry and plate-counts (N) were evaluated and are
presented below (Table-2.1):
Table-2.1: Effects of injection volume on System Suitability

<table>
<thead>
<tr>
<th>Injection plug length</th>
<th>Dimethyl sulfate</th>
<th>Chloroacetyl chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S/N</td>
<td>Peak Sym.</td>
</tr>
<tr>
<td>50 mbar x 15 s</td>
<td>25</td>
<td>0.94</td>
</tr>
<tr>
<td>50 mbar x 30 s</td>
<td>53</td>
<td>0.91</td>
</tr>
<tr>
<td>50 mbar x 45 s</td>
<td>58</td>
<td>0.93</td>
</tr>
<tr>
<td>50 mbar x 60 s</td>
<td>78</td>
<td>0.90</td>
</tr>
<tr>
<td>50 mbar x 75 s</td>
<td>85</td>
<td>0.85</td>
</tr>
<tr>
<td>50 mbar x 90 s</td>
<td>88</td>
<td>0.80</td>
</tr>
</tbody>
</table>

It is evident from the results presented above that the improvement in sensitivity (S/N) was not very significant with plug lengths of more than 60s. Even though there was a decrease in the efficiency, this did not have any serious impact on quantification. An overlay of the electropherograms with different injection plug lengths is presented below in Fig.2.3.

Individual electropherograms in Fig. 2.4-2.6 indicate that the desired separation has still been achieved with an increased plug length.
Fig. 2.3: Overlay of injections with different injection plug lengths

It can be noted that the shifting in migration times is due to the delay in sample acquisition due to different injection plug lengths.

Fig. 2.4: Injection plug length = 50 mbar x 90s
The buffering capacity was determined by performing overnight 50 injections of the standard solution from the same set of background electrolyte vials. The injections were found to be precise.
and the %RSD in migration times and peak areas of both dimethyl sulfate and chloroacetyl chloride from 50 injections is presented below (Table-2.2).

**Table-2.2: Buffering Capacity of BGE**

<table>
<thead>
<tr>
<th>Dimethyl sulfate (%RSD)*</th>
<th>Chloroacetyl chloride (%RSD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migration time</td>
<td>0.7</td>
</tr>
<tr>
<td>Peak Area</td>
<td>1.9</td>
</tr>
<tr>
<td>Migration time</td>
<td>0.9</td>
</tr>
<tr>
<td>Peak Area</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*n = 50 injections

A drift in the baseline was observed after the migration of both analytes which became significant after about 35 injections. However this drift did not have any detrimental effects on the analysis and integration. Based on this data on the buffering capacity, the BGE vials were replaced after every 25 injections. An overlay of electropherograms 1, 10, 25, 40 and 50 are provided below in Fig. 2.7.
Specificity

Specificity often termed as selectivity is the ability of the method to measure the analyte response in the presence of its potential impurities and other interferences. In the spiked API sample analyses, the DMS and CAC peaks were free from any interference from all other process related impurities, residual solvents or sample matrices. A specimen electropherogram of the DMS and CAC standards is presented in Fig. 2.8.
In case of API-1, acetic acid is a process related solvent. The residual acetic acid being negatively charged can possibly interfere with DMS which carries the same charge. However, it is worthy to appreciate when compared to chromatographic techniques such as HPLC and GC, CE is highly selective as the separation between analytes is achieved due to differences in the charge-to-mass ratios. The acetic acid was well separated from DMS (Fig. 2.9) in the real time sample analysis of API-1.

In API-2, a major anionic impurity is chloride impurity which is formed as a byproduct from CAC and is also introduced from the manufacturing process of API-2. The chloride peak was very well resolved from the CAC peak (Fig. 2.10) in the real time sample analysis. Thus the method was found to be highly specific and
selective for its intended application of determining DMS and CAC in presence of potential interferences from sample matrices.

**Fig. 2.9** - Specimen electropherogram of API-1 spiked with dimethylsulfate
Fig. 2.10 – Electropherogram of API-2 spiked with Chloroacetyl chloride

**Precision**

The precision of the developed method was assessed in terms of repeatability and inter-day precision. The %RSD for migration times and recoveries of DMS and CAC in the spiked samples during repeatability and inter-day precision experiments was calculated. It was observed that the overall RSD of migration times for DMS was not more than 2.2% and for CAC was not more than 2.0%. In the precision studies, the overall RSD of recoveries were not more than 6.5% and 6.2% for DMS and CAC respectively (Table-2.3). Thus the method was found to be precise and reproducible.
Table-2.3: Precision results of DMS and CAC

<table>
<thead>
<tr>
<th>Level</th>
<th>DMS (%RSD)</th>
<th>CAC (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Repeatability&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Inter-day precision&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LOQ</td>
<td>3.8</td>
<td>5.3</td>
</tr>
<tr>
<td>50%</td>
<td>4.2</td>
<td>6.5</td>
</tr>
<tr>
<td>100%</td>
<td>2.2</td>
<td>4.2</td>
</tr>
<tr>
<td>150%</td>
<td>3.7</td>
<td>4.9</td>
</tr>
<tr>
<td>300%</td>
<td>2.5</td>
<td>3.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>n = 6 determinations, <sup>b</sup>n = 6 determinations each on three different days

Sensitivity

The sensitivity of the method was derived from the signal-to-noise ratios obtained for DMS and CAC at very dilute known concentrations. The detection limits (LOD) for DMS and CAC were found to be 0.3µg/mL which corresponds to 3 ppm with respect to the sample concentration. Similarly the quantification limits (LOQ) were found to be 1.0µg/mL for both DMS and CAC corresponding to 10 ppm with respect to the sample concentration. These low limits of detection and quantification emphasize on the method’s high sensitivity and the suitability for analyzing DMS and CAC at trace levels in the real time samples. As discussed in the introduction of this chapter, it is noteworthy that owing to the known genotoxicity, the permissible levels of these impurities were very stringent. An overlay of electropherograms of the blank, LOD and LOQ solutions is presented in (Fig.-2.11).
Fig. 2.11 – Overlaid electropherograms of Blank, LOD and LOQ solutions

**Linearity and range**

Standard solutions containing DMS and CAC at nine different concentrations ranging from LOQ to 300% of nominal analyte concentration were analyzed to evaluate the linearity of the response obtained from the detector for different analyte concentrations. Linearity curves (Area vs Conc.) were plotted for DMS and CAC and the data was subjected to regression analysis. Linear relationships were obtained which confirm that the test results are directly proportional to the concentrations.

The linear equation of regression for DMS was $y = 9.9398x - 0.8980$ with a correlation coefficient ($R$) of 0.9990 (Fig. 2.12). Similarly
the regression equation for CAC was $y = 15.9874x - 0.9366$ with a correlation coefficient ($R$) of $0.9992$ (Fig. 2.13).

The range of a method is the interval in which it has a suitable level of precision, accuracy and linearity. From the results of validation tests that were performed, the range for this method was LOQ to 300% of the nominal analyte concentration.

![DMS-Linearity](image)

**Fig. 2.12: Linearity plot of Dimethylsulfate**
Fig. 2.13: Linearity plot of Chloroacetyl chloride

Accuracy

The accuracy of a method expresses the closeness of results that are determined experimentally with those of the theoretically computed values. The accuracy of the method was revealed from the good recoveries that were obtained for DMS and CAC from the spiked samples. At LOQ level the recoveries for DMS and CAC were 97.4% and 92.8% respectively and at other concentration levels (50, 100, 150 and 300%) recoveries in the range of 95.3-97.8% and 90.7-97.0% were obtained for DMS and CAC respectively (Table-2.4).
An important aspect of the method is its ability to demonstrate linearity in determining accuracy at different concentrations. To separate out the systematic variability due to the sample preparation, injection and integration and demonstrate linear accuracy, a one-way hierarchical analysis of variation (ANOVA) within the range was performed. For both the target analytes (DMS and CAC), the recoveries were found to be independent of the spiked concentrations (ANOVA, p> 0.05) as presented in Table-2.5 and Table-2.6. Thus it can be concluded that this method has a very handy application in determining DMS and CAC in pharmaceutical drug substances with a high degree of accuracy distributed over a wide range of concentrations.
### Table-2.5: ANOVA Results for Dimethylsulfate

Anova: Single Factor  
DMS

<table>
<thead>
<tr>
<th>Groups</th>
<th>Count</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ</td>
<td>3</td>
<td>292.28</td>
<td>97.42667</td>
<td>61.89163</td>
</tr>
<tr>
<td>50%</td>
<td>3</td>
<td>285.99</td>
<td>95.33000</td>
<td>50.02830</td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
<td>290.66</td>
<td>96.88667</td>
<td>40.46173</td>
</tr>
<tr>
<td>150%</td>
<td>3</td>
<td>293.33</td>
<td>97.77667</td>
<td>4.28323</td>
</tr>
<tr>
<td>300%</td>
<td>3</td>
<td>288.65</td>
<td>96.21667</td>
<td>12.95703</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>11.48663</td>
<td>4</td>
<td>2.871657</td>
<td>0.084649</td>
<td>0.985265</td>
<td>3.47805</td>
</tr>
<tr>
<td>Within Groups</td>
<td>339.2439</td>
<td>10</td>
<td>33.92439</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>350.7305</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table-2.6: ANOVA Results for Chloroacetyl chloride

Anova: Single Factor  
CAC

<table>
<thead>
<tr>
<th>Groups</th>
<th>Count</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ</td>
<td>3</td>
<td>278.51</td>
<td>92.83667</td>
<td>6.452133</td>
</tr>
<tr>
<td>50%</td>
<td>3</td>
<td>281.26</td>
<td>93.75333</td>
<td>7.233033</td>
</tr>
<tr>
<td>100%</td>
<td>3</td>
<td>284.69</td>
<td>94.89667</td>
<td>12.37343</td>
</tr>
<tr>
<td>150%</td>
<td>3</td>
<td>271.98</td>
<td>90.66000</td>
<td>1.8453</td>
</tr>
<tr>
<td>300%</td>
<td>3</td>
<td>291.05</td>
<td>97.01667</td>
<td>2.816133</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>67.00223</td>
<td>4</td>
<td>16.75056</td>
<td>2.726325</td>
<td>0.090368</td>
<td>3.47805</td>
</tr>
<tr>
<td>Within Groups</td>
<td>61.44007</td>
<td>10</td>
<td>6.144007</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>128.4423</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Stability in Analytical solution**

The integrity of the analyte in the sample solutions is a very important aspect of an analytical method which is often optimized during the method development itself. If the analyte is unstable in the diluent then non-reproducible and misleading results may be obtained. The DMS and CAC samples, after initial analysis were stored at 2-8°C and at room temperature and reanalyzed after 24 and 48 hours. The percentage recoveries of DMS and CAC did not show any remarkable variation in samples of initial time point and those that were reanalyzed after 24 and 48 hours. It is obvious from these findings that the analytes were stable in the diluent for at least 48 hours at 2-8°C or at room temperature.

**Analysis of real time samples**

The appropriateness and applicability of this CE method was utilized by determining DMS in API-1 and CAC in API-2. Using this validated method, six consecutive commercial scale batches of each API were analyzed, with each batch involving a triplicate determination. The results of analyses confirmed that the content of DMS in API-1 and the content of CAC in API-2 were within the limits that can be considered acceptable and safe. The content of DMS and CAC in the real time samples is presented in Table-2.7.
Table-2.7: Content of DMS in API-1 and CAC in API-2

<table>
<thead>
<tr>
<th>Lot # of API-1</th>
<th>DMS Content (ppm)* (Limit = 50 ppm)</th>
<th>Lot # of API-2</th>
<th>CAC content (ppm)* (Limit = 75 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>&lt; 10</td>
<td>001</td>
<td>17</td>
</tr>
<tr>
<td>002</td>
<td>11</td>
<td>002</td>
<td>ND</td>
</tr>
<tr>
<td>003</td>
<td>15</td>
<td>003</td>
<td>ND</td>
</tr>
<tr>
<td>004</td>
<td>10</td>
<td>004</td>
<td>13</td>
</tr>
<tr>
<td>005</td>
<td>&lt;10</td>
<td>005</td>
<td>ND</td>
</tr>
<tr>
<td>006</td>
<td>ND</td>
<td>006</td>
<td>22</td>
</tr>
</tbody>
</table>

*n = 3 determinations; ND = Not detected

2.4 Conclusion

In the recent past there has been a growing concern in controlling genotoxic impurities in the drug substances and drug products at very low levels (ppm range) such that their maximum daily intake does not pose any adverse health effects. Analysts across the globe rely on HPLC-UV/MS and GC-FID/MS techniques for determining genotoxic impurities at trace levels. Analysts encounter challenges in the form of matrix interference and low sensitivity when dealing with determination of impurities at ppm levels. A lack of chromophore in the analyte makes it even more complicated and challenging task.

A CE method was developed for determining DMS (a known genotoxin) and CAC (a potentially genotoxic impurity) in drug substances through indirect photometric detection. The development of the method involved a systematic screening of various anionic visualizing reagents such as pyromellitate, phthalate, pyridine dicarboxylate, benzoate and p-toluene sulfonate. The electrophoretic
mobility and the concentration of the visualizing reagent were found to be critical in obtaining the best peak symmetry and sensitivity. The effects of applied violated and capillary cassette temperature on the separation were evaluated. A high sensitivity was obtained by hydrodynamic injection of the samples through field amplified sample stacking.

The method is sensitive, offers simplicity and does not include laborious steps of derivatization involved for analyzing compounds lacking chromophores by HPLC and GC. The method is very specific, cost effective and eco-friendly too as there is a minimal usage of chemicals and solvents. The CE method, after validation has been successfully applied for analyses of real time samples. Though genotoxins are mostly determined using HPLC-UV/MS and GS-MS techniques for their proven ruggedness and sensitivity, the use of Capillary electrophoresis for estimating genotoxins can complement the existing approaches for analysis and also open new horizons.
2.5 References


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103

Chapter-2


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