Development and validation of a High Performance Liquid Chromatographic method for Isomer separation of industrial Toluidines established for their quality control

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Abstract

The development and validation of reversed phase liquid chromatographic method for the determination of positional Isomer contents in Toluidines used in bulk drug and active pharmaceutical ingredient is described. This method is simple isocratic for separation of Ortho, para and meta Toluidines from each other in a twenty minutes run time. To the best of our knowledge, no method described previously in the literature has demonstrated resolution of Toluidines from their isomer. The proposed method was validated with respect to accuracy, precision, linearity, and specificity. Also the method was determined to be robust with regards to the following parameters: mobile phase apparent pH; mobile phase organic content; detection wavelength and time dependence of sample and standard stability. This protocol has successfully provided a simple and feasible method for quality control of Toluidines for industrial use.

Keywords: o-Toluidine, p-Toluidine, m-Toluidine, Positional Isomers, HPLC, Fine chemical intermediates, bulk drug.

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INTRODUCTION

There are three Isomers of Toluidine, which are organic compounds. These isomers are o-Toluidine, m-toluidine, and p-toluidine. The o- stands for ortho-, m- stands for meta-, and p- stands for para-. All three are aryl amines whose chemical structures are similar to aniline except that a methyl group is substituted onto the benzene ring. The difference between these three isomers is the position where the methyl group (-CH₃) is bonded to the ring relative to the amino functional group (-NH₂). para-toluidine is a colorless solid. Ortho- and meta- toluatines are liquid. Typical end-use markets of toluatines and their derivatives are imaging products like pigment, dyestuffs and photographic chemicals. These compounds are also used in the production of antioxidants, agricultural, pharmaceutical and rubber chemicals [1].

para-toluidine used as an intermediate compound in making azo, sulfide and triphenylmethane dyes; starting material for preparation of aminotoluensulphonic acids; as an component of corrosion inhibitory compositions; in the production of antioxidants, agricultural, pharmaceutical and rubber chemicals, as a component of accelerators for cyanoacrylate glues; ortho-Toluidine, [2] the isomer produced in greatest volume, has found many commercial applications. By far the single largest use for ortho-toluidine is in the preparation of 6-ethyl ortho-toluidine, an intermediate in the manufacture of two very large-volume herbicides, metolachlor and acetochlor. Another important use for ortho-toluidine is in rubber chemicals, where it is used in the manufacture of a rubber antioxidant, and of di-ortho-tolylguanidine, a nonstaining rubber accelerator. Acetoacetortho-toluidine, 3-hydroxy-2-naphthoyl-ortho-toluidine, 2-toluidine-5-sulfonic acid, and ortho-aminoazotoluene are four of the more important dye and pigment intermediates manufactured from ortho-toluidine. In addition, ortho-toluidine is used to manufacture epoxy resin hardeners such as methylenebis-2-methylcyclohexylamine, fungicide intermediates such as 2-amino-4-methylbenzothiazole, and ortho-fluorobenzoyl chloride. Certain pharmaceutical intermediates are also prepared starting with ortho-toluidine (Bowers, 2000). Other minor uses of ortho-toluidine and its hydrochloride salt are as intermediates in organic synthesis and as ingredients in a clinical laboratory reagent for glucose analysis [3].

m-toluidine is used in many organic reactions and mainly used in dye stuff, it is also used in manufacturing process of pharmaceuticals. Although there are many ways to obtain these three chemicals, the preparation is mainly based on the reduction of o-, p- and m-nitro toluene respectively [4]. Therefore, the content of the relative isomeric substance in each toluatines have to be controlled during the manufacturing process [5]. Fig.1 shows the chemical structure of all three isomers of toluatines.

In this paper, an HPLC-UV method for the simultaneous determination of three isomer products specified has been developed. The optimization of experimental conditions was comprehensively investigated. On the basis of good separation and reliable determination, furthermore, we established a simple isocratic system employed and total elution time of 20 minutes is required. No unstable baseline is generated in this method therefore, this method is robust and by which quality assessment could be facilitated of industrial toluatines[6].

EXPERIMENTAL CONDITIONS

Reagents and chemicals

Sodium di Hydrogen phosphate monohydrate and ortho phosphoric acid were obtained from Rankem. HPLC-grade Methanol and water were obtained from Merck. All reagents were of HPLC grade unless otherwise specified [7].
Standards

The following standard synthesis grade like meta-toluidine, CAS [108-44-1], para-toluidine, CAS No.: 106-49-0, ortho-toluidine, CAS No.: 95-53-4, certified 99.9% pure is obtained from merck India Ltd.

Solution and Sample preparation

Mobile phase consist of a mixture of Methanol and aqueous sodium dihydrogen phosphate buffer in the ratio (40:60v/v). Mobile phase contain 0.05 M Sodium dihydrogen phosphate buffer pH 3.0 adjust with H$_3$PO$_4$ dilute solution. The sample solution containing all three isomers at a concentration of 500 ppm allowed accurate and precise quantification. Mobile phase was added to flask and shaken vigorously until the product clumps were dissolved. The flask was then sonicated at 25$^\circ$C for 10 min. The content of the flask was diluted to volume with mobile phase. A toluidines working standard test solution are prepared directly without centrifuging and was utilized for quantification.

Apparatus

Sample analysis were performed on an chromatographic system of waters alliance series chromatograph equipped with an in-built solvent degasser, quaternary pump and Waters-2996 Photo diode array detector with variable injector and auto sampler. The chromatographic column utilized in these studies was a Zorbex SB-Phenyl column (250 X 4.6 X 5.0 µm). The detection wavelength was 254 nm. Mobile phase flow rate was 0.8 ml/min. Twenty micro liters of sample was injected in to the HPLC for each analysis. A waters column heater module was used to maintain a constant column temperature of 25$^\circ$C. Photodiode array spectra were obtained from waters separation module equipped with a model 2696 photo diode array detector. Peak purity analysis was carried out over a wavelength range 208-350 nm through the use of the EmpowerTm-2 Build-2154 software. The stability chamber utilized during forced degradation studies was a controlled by temperature controller. All measurements were carried out at a temperature of (25±2.0$^\circ$C). The pH metric studies were carried out on Decibel, Db-1011 digital pH meter fitted with a glass electrode as an indicator and saturated calomel electrode as reference electrode.

RESULT AND DISCUSSION

Method development

Optimal separation of isomers from each other was achieved with an buffer/methanol isocratic mobile phase. A mobile phase temperature of 25$^\circ$C was employed for the separation. No significant degradation of toluidines was observed at 25$^\circ$C temperature during its elution time. Typical chromatogram with retention time and elution order observed for all three isomers are presented in Fig 2.

Effect of Ionic strength in Mobile phase

When methanol was fixed 40% respectively, we also observed the effect of the buffer concentration in mobile phase on separation by adding different quantities of sodium dihydrogen phosphate (0.05 M) solution. It was found that the buffer concentration in the range of 40-80% has little influence on the retention times of all compound involved, but their retention time highly increased when the proportion was blow 20%. Accordingly, we choose 60% as the buffer proportion.

Effect of organic content in Mobile phase

In this experiment, we fixed the proportion [8] of buffer (60% of 0.05 M) and adjusted the contents of methanol ranging from 20-60% in mobile phase. By comparing with separation phenomenon under various methanol contents, it indicated that all compounds could be separated perfectly when methanol proportion was 40%.
Effect of organic modifiers in Mobile phase

Although as mentioned above during the experiment, we had found that there are many peaks that needed to be separated in the sample, initial focus of the mobile phase development activity was to separate each toluidines from each other at trace level. This was because the initial strategy for any new mobile phase development activity should be to identify the pairs of known peaks that would pose the most challenge in achieving baseline separation. Methanol is commonly used organic modifier. We optimized 40% volume ratio of Methanol in the mobile phase for best baseline resolution between all peaks.

Table 1 shows Quantitation of all isomer in each other was conducted as an area/area basis individual peak area of toluidines in each injection. An external standard was utilized for the quantitation [9].

Method Validation
The method was validated according to ICH guidelines [10]

Selectivity
A separate selectivity [11] test was performed by applying the proposed method to the determination of toluidines synthetic mixture consisting of all three isomer. Synthetic mixture diluted up to the final volume with diluents. It was found that assay results were not changed.

Specificity
Specificity is the ability of the method to measure the analytical response in the presence of all potential isomers. For the specificity test, chromatogram of the standard solution of each standard was recorded under selected conditions. The response of the analyte in this mixture was compared with the response of pure individual analyte. It was found that assay results were not changed.

Accuracy
The accuracy of the method for quantification of each isomer was evaluated by analysis of solution of individual sample of standard. The sample solutions were prepared individually at target concentration of 500 ppm and were spiked with related isomers at following percent (w/w) levels. i.e. 10.0, 8.0, 5.0, 2.0, 0.01 and 0.05. For each strength study, four sets of accuracy analysis were conducted by each of two analysts. Accuracy results are presented in table 2 and 3. In review of the data it was noted that mean recovery value at the 0.05% spiking level differs significantly from the mean recovery value at higher levels. It is important to note that 0.05 % level represent the LOQ for related substance in this method [12].

Precision
Method repeatability
A composite sample of meta-toluidine was prepared by mixing two lots. Using the proposed method two analysts performed the related substance determination on five samples from the composite on two different days. The mean p-toluidine and ortho-toluidine content of the fix composite was 9.55% and 8.99% respectively. Data for the assessment of method repeatability are presented in table 3.

Linearity
The linearity relationship of detector response was measured as peak area versus concentration. Solutions were prepared so that the concentration analyzed for Toluidines were at the 250, 150, 100, 60, 20, 5, 1.5 , 0.05 and 0.02%(w/w) levels relative to the 500 ppm target concentration of m-toluidine. The relationship for detector response measured as peak area versus concentration was linear over the range of 0.05-250% of target concentration of 500 ppm of meta-toluidine in the sample solution. The product-moment correlation coefficient, R, was 0.99996. The slope was 15656.4 and the value of y-intercept was 3.998.

Quantification limit
The accuracy results are presented in table 3 and 4. The means of recovery of ortho and para isomers were 102% and 101 % respectively. In review of data, it was noted
that 0.05% spiking level represent the quantification limit [13] for the method and that this spiking level is 0.05% of 500 ppm meta-toluidine so the LOQ was found to be 2.5 ppm.

**Ruggedness**

This parameter evaluates the consistency of the results when external factors such as analyst, instruments, laboratories, reagents, days are varied deliberately [13,14]. Ruggedness of the proposed method was estimated by changing, Days, Analyst, each analyst run two sets, each set on a different HPLC system using a different column with same make. Waters and Merck two different HPLC systems were utilized to conduct the analysis.

**Robustness**

This parameter evaluates the consistency of the results when internal factors such as column, flow rate mobile phase composition, temperature, injection volume or any other variable inherent to the method of analysis [14] are varied deliberately. Robustness of the proposed method was estimated by changing the mobile phase buffer concentration ±0.002 M, mobile phase organic content ±3.0%, flow rate from 0.8 ml to 1.2 ml /min, mobile phase temperature ±5.0 °C , detection wavelength setting ±2.0 nm, changing column brand and mobile phase composition. System suitability parameters were found to be within acceptable limits [15].

**Stability**

Two sets of samples with a low and a high concentration of toluidines were analyzed and left in the auto-sampler at ambient temperature. The samples were analyzed using a freshly prepared calibration sample 5 days later the results meet the guideline of the international conference on Harmonization (ICH) for validation of pharmaceutical assays of drug products [16-18].

**CONCLUSION**

The above-mentioned data demonstrates that the proposed method is accurate, precise, linear, specific and robust for the determination of related substances in each individual toluidines used in industries for different purpose. Also the method was determined to be robust with regards to the following parameters: mobile phase buffer composition, mobile phase organic content; detection wavelength and time dependence of sample and standard stability.

Fig.1 Chemical structure ortho, para and meta Toluidines.

Fig.2 Chromatogram shows the separation and elution order of o-toluidine, m-toluidine, p-toluidine.
Table – 1: Typical retention times observed for all three Isomers of Toluidines using the condition of proposed method

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention time range(min)</th>
<th>Relative retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ortho-toluidine</td>
<td>6.10-6.30</td>
<td>1.07 – 1.11</td>
</tr>
<tr>
<td>2. meta-toluidine</td>
<td>5.50-5.70</td>
<td>1.0</td>
</tr>
<tr>
<td>3. p-toluidine</td>
<td>4.90-5.10</td>
<td>0.86 – 0.90</td>
</tr>
</tbody>
</table>

Table – 2: Results obtained for the determination ortho-Isomer in meta-toluidine prepared and analyzed as specified in the proposed method.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>% Ortho-Isomer</th>
<th>Analyst-I</th>
<th>Analyst-II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day-1</td>
<td>Day-2</td>
<td>Day-1</td>
</tr>
<tr>
<td>01</td>
<td>9.55</td>
<td>9.66</td>
<td>9.66</td>
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<tr>
<td>02</td>
<td>9.45</td>
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<td>9.59</td>
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<tr>
<td>05</td>
<td>9.61</td>
<td>9.59</td>
<td>9.68</td>
</tr>
<tr>
<td>Mean</td>
<td>9.54</td>
<td>9.58</td>
<td>9.66</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.67</td>
<td>0.86</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Table 3: Results obtained for the determination para-isomer in meta-toluidine prepared and analyzed as specified in the proposed method.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>% para-Isomer</th>
<th>Analyst-I</th>
<th>Analyst-II</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day-1</td>
<td>Day-2</td>
<td>Day-1</td>
</tr>
<tr>
<td>01</td>
<td>8.99</td>
<td>8.66</td>
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<td>02</td>
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<tr>
<td>Mean</td>
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<td>8.58</td>
<td>8.66</td>
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<tr>
<td>% RSD</td>
<td>1.26</td>
<td>0.96</td>
<td>0.34</td>
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References