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Research Article

# Development and validation of TEMPO content in Saxagliptin by Gas chromatography

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**ABSTRACT**

A simple and sensitive gas chromatography method using a flame ionization detector has been developed, validated and applied for the determination of TEMPO in Saxagliptin monohydrate drug substance. The chromatography separation was achieved on capillary column [DB-FFAP (30m, 0.53mm, 1.5µm)] with fused silica coated with nitro terephthalic acid modified polyethylene glycol stationary phase. The developed GC method was validated in terms of specificity, Linearity, Precision (repeatability and reproducibility), Accuracy, Robustness, Limit of quantitation (LOQ) and Limit of detection (LOD). The obtained LOD and LOQ values were 2 µg/g and 6 µg/g respectively. The method was found to be linear in the range between 6 µg/g and 450 µg/g with correlation coefficient 0.9956. The average recovery was found to be 89%. The experimental results are discussed in this article.

**Keywords:** TEMPO, Saxagliptin monohydrate, GC-FID, Validation.

## INTRODUCTION

Saxagliptin is recently approved for treatment of type-II diabetes mellitus and significantly improves glycemic control vs placebo, as demonstrated by decreasing glycated hemoglobin, fasting plasma glucose, and postprandial plasma glucose levels when used as monotherapy;Saxagliptin also significantly improves β-cell function, is weight neutral, has a low risk for hypoglycemia, and has been shown to have cardiovascular safety[1]. Saxagliptin is one of a class of oral antidiabetic agents from a class of drugs that inhibits selectively and reversibly the enzyme, dipeptidyl peptidase-4 (DPP-4) that is involved in glucose homeostasis[2]. DPP-4 inhibitors represent a new therapeutic approach to the treatment of type-II diabetes that functions to stimulate glucose-dependent insulin release and reduce glucagon levels. This is done through inhibition of incretions inactivation, particularly glucagon-like peptide-1 and gastric inhibitory polypeptide, thereby improving glycemic control[3]. Saxagliptin monohydrate is marketed under Onglyza trade name[4] and USFDA has approved it combination tablet with metformin under trade name Kombiglyze XR. Saxagliptin monohydrate (SAX) is chemically known as (1S,3S,5S)-2[(2S)-2-Amino-2-(3-Hydroxytricyclo-[3.3.1.1$^{3,7}$]dec-1-yl)acetyl]-2-azabicyclo[3.1.0]hexane-3-carbonitrile monohydrate. Molecular formula is $C_{18}H_{27}N_3O_3$ and the chemical structure is given in Fig 1.

**Chemical structure of Saxagliptin monohydrate**

TEMPO is chemically known as 2,2,6,6-Tetramethyl-1-piperidinyloxy or 2,2,6,6-tetramethylpiperidine-1-oxyl. Molecular formula is $C_9H_{18}NO$. TEMPO is used as a catalyst for the oxidation of primary alcohols to aldehydes in organic synthesis[5].

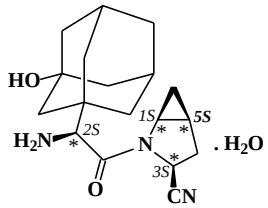


**Fig 1**

N-Boc 3-hydroxyadamantyl glycine, is one of the raw materials is used in the preparation of SAX. Further during the preparation of N-Boc 3-hydroxyadamantyl glycine, Adamantane 1-alcohol is used as starting material and which is converted into Adamantane 1-aldehyde where TEMPO is used as a catalyst. The inexpensive, high-potential TEMPO derivative, exhibits higher electrocatalytic activity than AZADO and ABNO for the oxidation of primary and secondary alcohols. Mechanistic studies provide insights into the origin of these unexpected reactivity trends[6].

The chemical structures of N-Boc 3-hydroxyadamantyl glycine, TEMPO and conversion phenomenon are given in Fig.2.

Hence control of TEMPO in SAX is essential as it is a potentially genotoxic impurity[7,8]. This impurity limit is considered as 300µg/g as per daily dose taken from European medicines Agency[9,10]As per literature on TEMPO[7,8], determination of TEMPO has been determined on GC-MS AND LC-MS. Since, GC with FID is mostly available at all labs, easy to handle and less expensive when compared with GC with mass detector and HPLC with mass detector the present work has been developed on GC-FID with good sensitivity. Till date no mention is available regarding determination of TEMPO in SAX in literature to the best of our knowledge. The present work deals with development, optimization and validation of the gas chromatography method for the determination of TEMPO in SAX.

## MATERIALS AND METHODS

### Solvents, Chemicals and Samples:

TEMPO, Octadecane, Methanol, Ethanol, Ethyl acetate, Isopropyl alcohol, Acetonitrile, Methylene chloride, Benzene, N-Diisopropylethylamine, Methylmethanesulfonate, Ethylmethanesulfonate, Isopropylmethanesulfonate, Ethyl nicotinate, Isopropyl tert-butyl ether, t-butanol, Tetramethylethylenediamine were procured from Sigma Aldrich, Steinheim, Germany. Methylene chloride (Analytical grade, used as diluent), Formic acid (GR grade), HPLC water were procured from Merck chemicals, Mumbai, India. The investigated drug substance SAX was gifted from APL Research Centre Laboratories (A Division of Aurobindo Pharma Ltd., Hyderabad).

### Instrumentation:

A gas chromatograph 6890N equipped with flame ionization detector with CTC analytics auto sampler (Make: Agilent Technologies, Santa Clora, CA, USA) and gas chromatograph Shimadzu 2010 equipped with flame ionization detector with AOC-5000 auto sampler (Make: Shimadzu Corporation, Kyoto, Japan) with data acquisition and processing using Empower 3 Software Build 3471 were used in this research work.

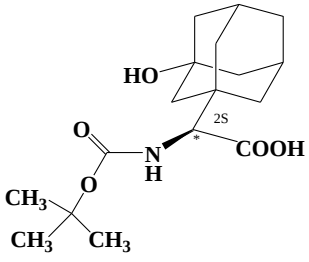


**Fig 2a**

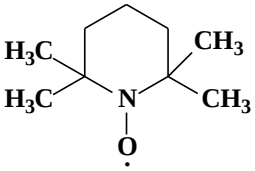


**Fig 2b**

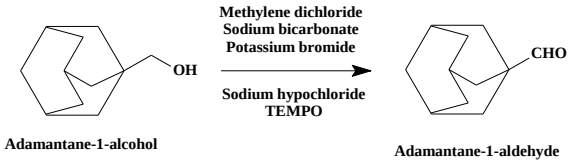


**Fig 2c**

**Fig 2.**
**(2a) N-Boc 3-hydroxyadamantyl glycine**
**(2b) TEMPO**
**(2c) Conversion phenomenon**

**Chromatographic conditions and methodology:**
The chromatographic separation was achieved on capillary column [DB-FFAP (30m, 0.53mm, 1.5µm)] with fused silica coated with nitro terephthalic acid modified polyethylene glycol stationary phase, Make: J&W Scientific. Nitrogen was used as carrier gas for entire experiments as it provides good base line. The other method parameters, which were used for this work has mentioned below.

Injector temperature: 220°C
Detector temperature : 260°C
Detector :Flame ionization detector (FID)
Carrier gas : Nitrogen
Spit ratio : 1:3
Run time : 30min
Injection volume : 2µL
Column Pressure programme : 60kPa

Column oven temperature programme:

120°C (5min) —20°C/min→ 220°C (20min)

**Preparation of solutions:**
***Formic acid solution***
Transfer 75 ml of Formic acid into a 100 ml clean dry volumetric flask containing about 10 ml of water, mix and make up to volume with water.

***Internal standard solution***
Accurately weigh and transfer about 155 mg of Octadecane into a 25 ml clean, dry volumetric flask containing about 15 ml of Methylene chloride, mix and make up to volume with Methylene chloride. Dilute 1.0 ml of this solution to 250 ml with Methylene chloride.

***Blank solution***
Into a clean, dry glass centrifuge tube, take 3 ml of Formic acid solution, add 3 ml of internal standard solution and shake vigorously for 1min. Allow the two phases to separate. Collect the lower layer (Methylene chloride layer) and inject into GC.

***Standard solution***
Accurately weigh and transfer about 62.5 mg of TEMPO into a 25 ml clean, dry volumetric flask containing about 10 ml of internal standard solution, dissolve and make up to volume with internal standard solution. Dilute 1.0 ml of this solution to 50 ml with internal standard solution.
Into a clean, dry glass centrifuge tube, take 3 ml of Formic acid solution, add 3 ml of standard solution and shake vigorously for 1min. Allow the two phases to separate. Collect the lower layer and inject into GC.

***Sample solution***
Accurately weigh and transfer about 500 mg of SAX sample into a clean, dry glass centrifuge tube, Take 3 ml of Formic acid solution and shake to dissolve the sample. Add 3 ml of internal standard solution and shake vigorously for 1min. Allow the two phases to separate. Collect the lower layer and inject into GC.

**RESULTS AND DISCUSSION**
**Method development and optimization:**
The objective of this work is to determine low level concentration of TEMPO in SAX drug substance by using GC-FID which is easily available instrument, good separation and desired sensitivity. Some of analytical methods were available in literature for quantification of TEMPO by GCMS and LCMS techniques [7-8]. The present investigation was initiated for the quantification of TEMPO by GC-FID technique in SAX, as GC instrument is mostly available at all laboratories and easy to handle.
There was no option for UV or Fluorescence detection to quantify TEMPO as no chromopore present in this analyte. Hence, gas chromatography was chosen as analytical technique. Further, the method development trails were carried out based on SAX and TEMPO solubilities. Initially, DB-1 column (30m long with 0.53mm ID, 3.0 µm Particle diameter) with 100% dimethyl polysiloxane as stationary phase, direct injection technique with methanol has been chosen with by setting the following temperature programme at constant pressure 40 kpa.

Column oven temperature programme:

60°C (5min) —20°C/min→ 220°C (17min).

Standard solution (300µg/g) concentration has been prepared with respect to sample concentration and injected in to GC by using all the above method parameters. In this trail, interference from sample matrix and low response of analyte was observed. During method optimization, various solvents were used to avoid interference and response issues in direct injection technique. Hence, the extraction procedure was chosen with formic acid and methylene chloride and internal standard as octadecane as mentioned in chromatographic conditions and methodology section. In this trail, sample interference was resolved but analyte peak shape and response was not good. Finally, DB-FFAP column (30m, 0.53mm, 1.5µm) was used and

programme was changed by keeping pressure as 60 kpa constantly.

Column oven temperature programme:

$$120°C\ (5min) \xrightarrow{20°C/min} 220°C\ (20min).$$

Finally satisfactory separation with better peak shapes and response were achieved on chromatographic conditions. The optimized chromatographic conditions and sample preparations were given at chromatographic conditions and methodology section.

**Method validation:**

The optimized gas chromatography method was validated according to ICH guideline Q2 (R1)[11] in terms of Precision (System precision, Method precision and intermediate precision), Specificity, Limit of detection (LOD), Limit of quantification (LOQ), linearity, accuracy and Robustness.

***Specificity***

Specificity is the ability of the method to measure the analyte response in presence of all residual solvents (Methanol, Ethanol, Isopropyl alcohol, Methylene chloride, Acetonitrile, N, N-Diisopropylethylamine, Ethyl acetate) which are used in the synthesis process of SAX drug substance.

In specificity experiment, blank solution, all residual solvents which are used in synthesis process of SAX (Methanol, Ethanol, Isopropyl alcohol, Methylene chloride, Acetonitrile, N, N-Diisopropylethylamine, Ethyl acetate) including TEMPO and Octadecane solutions were prepared individually and injected into GC to confirm the retention times. Benzene was also injected, since benzene can be considered as possible contaminant. Solutions of SAX (control sample), SAX spiked with TEMPO at 300µg/g level (spiked sample) and SAX spiked with all residual solvents including TEMPO at 300µg/g (all spiked sample) solutions were prepared and injected into GC. From the obtained data, TEMPO peak was well separated from all other solvents and indicating that the test method is selective and specific for the determination of TEMPO in SAX. Retention times of all solvents are given in Table 1, and Spiked sample data is reported in Table 2. Typical GC chromatograms of Blank solution, Standard solution, Control sample (As such sample), Spiked sample and all spiked sample solutions are given in Fig 3.

***Limit of detection (LOD) and Limit of quantification (LOQ)***

Standard solution (300µg/g concentration) of TEMPO was prepared and injected into gas chromatograph. The Limit of detection (LOD) and Limit of quantification (LOQ) values for TEMPO was determined by signal to noise ratio (s/n) method. The minimum concentration at 3:1 s/n was considered as LOD and the concentration at 10:1 s/n was established as LOQ. The predicted LOD and LOQ values obtained for TEMPO were 2 µg/g and 6 µg/g respectively with respect to sample concentration. Precision was verified by preparing the solutions at about LOD and LOQ concentrations and injected each solution six times in to GC and the achieved précised values are given in Table 3.

***Linearity***

The linearity was evaluated by measuring area ratio for TEMPO with respective internal standard (Octadecane) over concentration range of 6 µg/g to 450µg/g (LOQ to 150% of specification level) with respect to sample concentration and the obtained data was subjected to statistical analysis and the statistical results are reported in Table 3.

***Accuracy***

Accuracy of the method was verified through recovery experiments by spiking known amount of TEMPO at four levels i.e. LOQ level , 50%, 100% and 150% of specification level i.e.300 µg/g) in to SAX. Each preparation was analyzed in triplicate (n=3) and percent recovery was calculated. The obtained recovery results are tabulated in Table 4.

***Precision***

The precision of the method was studied using repeatability and reproducibility (ruggedness). The System precision was evaluated by injecting six replicates of standard solution for checking the performance of the gas chromatograph under the chromatographic conditions on the day tested (system precision) and calculated the area ratios of TEMPO and Octadecane from obtained areas.

Repeatability and reproducibility of the method was studied by analyzing six sample solutions separately. Repeatability was the intra-day variation (method precision) demonstrated by preparing six sample solutions individually using a single batch of SAX spiked with TEMPO at about 300µg/g concentration level and content was determined.

The intermediate precision was the inter-day variation (ruggedness) defined as the degree of reproducibility obtained by following the same procedure as mentioned for method precision experiment. Ruggedness of the method was evaluated by preparing six individual sample preparations (same sample which was used in method precision

experiment) by spiking TEMPO to SAX and injected into different column, different instrument and different analyst on different days. The achieved precision experiment results are given in Table 5.

***Robustness***

This study was performed by making deliberate variations in the method parameters. The study was carried out with respect to flow/pressure variation of carrier gas initial pressure and ramp temperature ±10% and column oven initial temperature and ramp temperature ± 2°C as follow.

Conditions: In each robustness conditions remaining gas chromatography conditions are same as per test method.

(i) Column flow/Pressure (-10%): 54kPa.

(ii) Column flow/Pressure (+10%): 66kPa.

(iii) Column oven temperature (-2°C):

$$118°C\ (5min) \xrightarrow{18°\ C/min} 220°C\ (20min).$$

(iv) Column oven temperature (+2°C):

$$122°C\ (5min) \xrightarrow{22°\ C/min} 220°C\ (20min).$$

**Table 1**
**Individual injections of all residual solvents**

| Solvent Name | RT(min) |
|---|---|
| TEMPO | 5.578 |
| Octadecane | 7.828 |
| Methanol | Not Detected |
| Methylene chloride | 0.842 |
| Ethyl acetate | Not Detected |
| Isopropyl alcohol | Not Detected |
| N,N-Diisopropylethylamine | Not Detected |
| Acetonitrile | 1.056 |
| Isopropyl tert-butyl ether | 0.783 |
| tert-butanol | 1.010 |
| Benzene | 0.977 |
| Ethyl nicotinate | 8.484 |
| Methyl methanesulfonate | 7.013 |
| Ethyl methanesulfonate | 7.190 |
| Isopropyl methanesulfonate | 6.745 |
| Tetramethylethylenediamine | 1.096 |
| Ethanol | Not Detected |

**Table 2**
**All spiked sample (Saxagliptin monohydrate drug substance spiked with TEMPO including all residual solvents)**

| Solvent Name | RT(min) | RRT |
|---|---|---|
| Isopropyl tert-butyl ether | 0.789 | 0.10 |
| Methylene chloride | 0.848 | 0.11 |
| Acetonitrile | 1.013 | 0.13 |
| TEMPO | 5.700 | 0.73 |
| Isopropyl methanesulfonate | 6.727 | 0.86 |
| Methyl methanesulfonate | 6.991 | 0.90 |
| Ethyl methanesulfonate | 7.169 | 0.92 |
| Octadecane | 7.800 | 1.00 |
| Ethyl nicotinate | 8.475 | 1.09 |

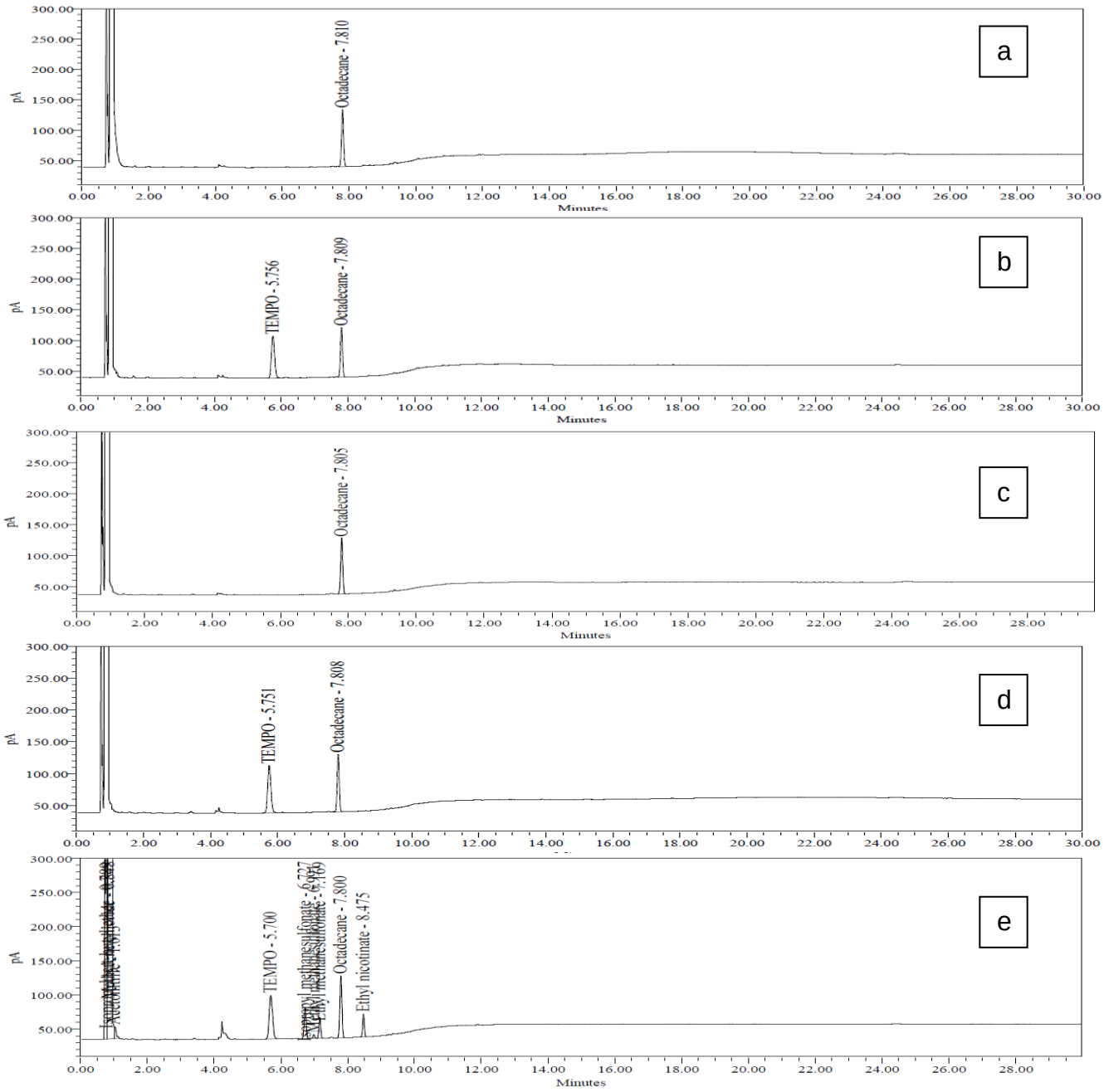


**Fig 3**
**Typical GC chromatograms of a) Blank solution, (b) Standard solution, (c) Saxagliptin monohydrate drug substance (as such sample), (d) Saxagliptin monohydrate drug substance spiked with TEMPO(spiked sample) and (e) Saxagliptin monohydrate drug substance spiked with TEMPO including all residual solvents (all spiked sample)**

**Table 3**
**Statistical data of LOD, LOQ and Linearity experiments**

| Statistical Parameters | Experimental Results |
|---|---|
| Limit of Detection (LOD) (µg/g) | 2 |
| Limit of Quantification (LOQ) (µg/g) | 6 |
| Precision for LOD (RSD%) (n=6) | 1.6 |
| Precision for LOQ (RSD%) (n=6) | 2.8 |
| Correlation coefficient | 0.9956 |
| Concentration range (µg/g) | 6 – 454 |
| Intercept | -0.0068 |
| Slope | 0.0031 |
| STEYX | 0.055 |
| No. of points covered | 7 |

**Table 4**
**Accuracy data of TEMPO**

| Accuracy | Level-I | Level-II | Level-III | Level-IV |
|---|---|---|---|---|
| Added(µg/g) | 6.1 | 151 | 303 | 453 |
| Found(µg/g) | 5.6 | 134 | 270 | 385 |
| Recovery(%) | 92.9 | 88.7 | 89.3 | 84.9 |
| RSD(%) | 2.0 | 1.5 | 0.4 | 1.1 |
| Overall Recovery (%) | | | 88.9 | |

**Table 5**
**Statistical data of precision experiments**

| Injection ID | System Precision<br>Ratios of area counts<br>[TEMPO/Octadecane] | Method Precision<br>TEMPO content, µg/g | Ruggedness<br>TEMPO content, µg/g |
|---|---|---|---|
| 1 | 1.3006 | 290 | 289 |
| 2 | 1.3006 | 287 | 288 |
| 3 | 1.3033 | 290 | 288 |
| 4 | 1.2977 | 291 | 287 |
| 5 | 1.3005 | 291 | 285 |
| 6 | 1.3007 | 292 | 283 |
| Mean | 1.3006 | 290 | 287 |
| SD | 0.0018 | 1.7 | 2.3 |
| RSD(%) | 0.1 | 0.6 | 0.8 |
| 95%Cl(±) | 0.0019 | 2 | 2 |
| Overall statistical data(n=12) | Mean | 288 | |
| | SD | 2.6 | |
| | RSD(%) | 0.9 | |
| | 95%Cl(±) | 2 | |

**Table 6**
**Robustness data of TEMPO**

| Robustness condition | Variation | TEMPO | | Octadecane | |
|---|---|---|---|---|---|
| | | RT, min | RRT | RT, min | RRT |
| As per methodology | - | 5.751 | 0.74 | 7.808 | 1.00 |
| Flow Pressure variation – Initial Pressure and Ramp | -10% &-10%/min | 6.148 | 0.76 | 8.092 | 1.00 |
| | +10% & +10%/min | 5.338 | 0.71 | 7.557 | 1.00 |
| Temperature variation – Initial Oven and Ramps | -2°C & -2°C/min | 6.000 | 0.74 | 8.115 | 1.00 |
| | +2°C & +2°C/min | 5.510 | 0.73 | 7.532 | 1.00 |

**Test method conditions**

Column flow/Pressure: 60kPa

Column oven temperature:

120°C (5min) $\xrightarrow{20°\ C/min}$ 220°C (20min).

In each robustness condition, solutions of Blank, Standard and SAX spiked with TEMPO at about 300µg/g concentration level were prepared per methodology and injected in to GC to confirm the retention times. There is no much variation in the relative retention time (RRT) of TEMPO of different deliberately varied robustness conditions from the developed methodology. Hence the test method is robust for all varied conditions. All experiments system suitability results (resolution between TEMPO and Octadecane) are given in Table 6.

***Solution stability***

The standard solution and sample solutions were prepared by spiking TEMPO at known concentration level to SAX drug substance and stability of the solution was tested as freshly prepared and at different intervals with the gap of every one hour and up to 24hrs at ambient conditions. The stability of solution was determined by comparing results with freshly prepared standard and sample solutions. The results indicating that standard and sample solutions were stable for 24hrs at ambient conditions.

**CONCLUSION**

The method validation data demonstrated that the developed GC method is almost equally sensitive, less expensive when compared with GCMS method referred in the literature and also specific, precise and robust. The range of validated method is from LOQ level to 450µg/g concluded from linearity, method precision and accuracy experiments data. Hence the validated GC method can be employed in to the routine analysis for the determination of TEMPO in SAX drug substance.

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