Available online at www. scholarsresearchlibrary. com

Scholars Research Library

Der Pharmacia Lettre, 2020, 12 (5): 18-31
(http: //scholarsresearchlibrary. com/archive. html)

ISSN 0975-5071
USA CODEN: DPLEB4

Trace Level Determination of Residual Dimethyl Sulphate (PGI) in Tipiracil Hydrochloride Drug Substance by Gas Chromatography with Mass Spectrometry (GC/MS)

Srivalli Kasina[1*], Annapurna Nowduri[2], Raghu Babu K[2], Ramdas Chavakula[1], Hemant Kumar Sharma[1], Srinivas Nalla[1]

[1]*Aurobindo Pharma Limited Research Centre-II, Sangareddy, Telangana, India*

[2]*College of Engineering, Andhra University, Visakhapatnam, Andhra Pradesh, India*

[*]***Corresponding author:*** *Srivalli K, Aurobindo Pharma Limited Research Centre-II, Sangareddy, Telangana, India. E-mail: sivakrishna.sri2008@gmail.com*

ABSTRACT

Simple and sensitive Gas chromatography with mass spectrometry (GC/MS) method was developed, optimized and validated for the determination of Mutagenic impurity i.e. Dimethyl sulphate (DMS) in Tipiracil Hydrochloride drug substance. The lower level of detection was achieved on Capillary GC column (Rtx-624, Fused silica capillary column; 30m length; 0.32mm internal diameter, coated with 6% Cyanopropylphenyl and 94% dimethyl polysiloxane stationary phase of 1.8µm film thickness) with Electron Impact ionization (EI) in Selective Ion Monitoring (SIM) mode. The developed method was validated for specificity, linearity, accuracy and precision. The obtained detection and quantitation limits of DMS were 0.2µg/g and 0.6µg/g respectively. The method was found to be linear in the range between 0.6µg/g and 29.9µg/g with correlation coefficient 1.0000. The average recovery range obtained for this impurity was 97.1%. The detail experimental approach is explained in this research paper.

Keywords: Tipiracil Hydrochloride, Dimethyl sulphate (DMS), GC/MS, Genotoxic or Mutagenic impurity and Validation

---

INTRODUCTION

Tipiracil Hydrochloride is described chemically as 5-chloro-6-[(2-iminopyrrolidin-1-yl) methyl] pyrimidine-2,4-(1H,3H)-dione mono hydro chloride. It has a molecular formula $C_9H_{11}ClN_4O_2 \cdot HCl$ and the molecular weight is 279.12. Tipiracil is a Thymidine Phosphorylase Inhibitor. The mechanism of action of it is as a Thymidine Phosphorylase Inhibitor [1]. The chemical structure of Tipiracil Hydrochloride is shown in Figure 1.

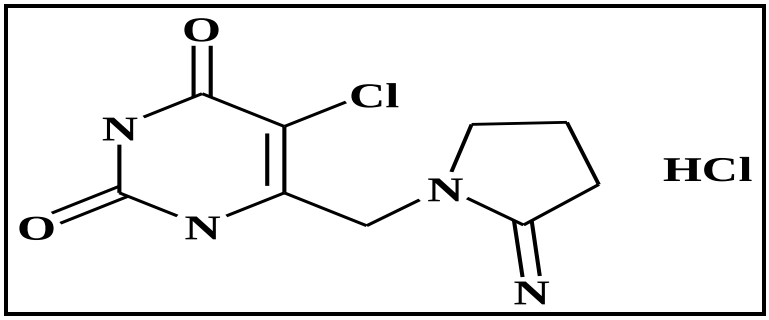


Figure 1: Chemical structure of Tipiracil Hydrochloride.

Tipiracil Hydrochloride is a drug used in the treatment of cancer. In Japan, it is approved for use in combination with Trifluridine (as the drug TAS-102 or Lonsurf) for the treatment of un-resectable advanced or recurrent colorectal cancer. Tipiracil Hydrochloride helps to maintain the blood concentration of Trifluridine by inhibiting the enzyme thymidine phosphorylase which metabolizes trifluridine.

Colorectal cancer (CRC) is the fourth most common cause of cancer-related mortality [2]. The main treatments for patients with advanced metastatic colorectal cancer (mCRC) include systemic combination chemotherapies [3-10]. Although the standard therapies are initially effective, many patients relapse due to the onset of drug resistance and are subsequently placed on salvage chemotherapy.

Lonsurf® is a novel oral nucleoside antitumor agent that consists of Trifluridine (TFD) and Tipiracil (TPR) at a molar ratio of 1:0.5 [11-14]. TFD is the antitumor component of Lonsurf®, whereas TPR prevents degradation of TFD through a first-pass effect as a thymidine phosphorylase inhibitor. Recently, Lonsurf® was found to significantly improve overall survival of mCRC patients in whom systemic chemotherapy is either ineffective or not tolerated.

Lonsurf® is a combination of Trifluridine, a nucleoside metabolic inhibitor, and Tipiracil, a thymidine phosphorylase inhibitor, indicated for the treatment of patients with metastatic colorectal cancer who have been previously treated with fluoropyrimidine, oxaliplatin and irinotecan based chemotherapy, an anti-VEGF biological therapy, and if RAS wild-type, an anti-EGFR therapy [15-18].

Based on animal studies and its mechanism of action, Lonsurf® can cause fetal harm when administered to a pregnant woman. Trifluridine/Tipiracil caused embryo-fetal lethality and embryo-fetal toxicity in pregnant rats when orally administered during gestation at dose levels resulting in exposures lower than those achieved at the recommended dose of 35 mg/m^2 twice daily. The other most common side effects of Lonsurf® include tiredness, vomiting, nausea, abdominal pain, decreased appetite, fever and diarrhea.

Dimethyl sulphate has been highlighted as potential genotoxic and carcinogenic to humans so IARC classified DMS in Group 2A. It is a potent genotoxic chemical which can directly alkylate DNA both *in vitro* and *in vivo* [19,20]. In the manufacturing process of Tipiracil hydrochloride is the coupling reaction of 5-Chloro-6- (chloromethyl)-1H-pyrimidine-2,4-dione (Intermediate-1) (Figure 2 (a)) and pyrrolidin-2-amine Hydrochloride (Intermediate-2) (Figure 2 (b)). Pyrrolidin-2-amine is prepared from Pyrrolidin-2-one (Figure 2 (c)). In this synthesis of pyrrolidin-2-amine, Dimethyl sulphate (DMS) (Figure 2 (d)) is used as methylating reagent. It is used mainly for converting active-hydrogen compounds such as phenols, amines and thiols to the corresponding methyl derivatives. Because of the known carcinogenicity and genotoxicity, the presence of residual dimethyl sulphate in Tipiracil hydrochloride drug substance must be controlled as per European Medicines Agency (EMA), International Conference on Harmonization [21] and Food and Drug Administration (FDA) guidelines [22,23]. EMA and FDA guidelines proposed the use of the "threshold of toxicological concern" (TTC) concept for the limit of genotoxic/carcinogenic impurities. The concentration limit, in ppm, of genotoxic impurity in drug substance, is the ratio of TTC in µg per day to the expected dose of drug substance in a gram per day. Considering the recommended daily maximum dose of 75.36 mg, i.e. 0.07536 g Tipiracil per day [1], Hence the concentration limit in ppm of dimethyl sulphate must be limited to less than 19.9 µg/g in drug substance. So it is necessary to develop sensitive, accurate and robust analytical method.

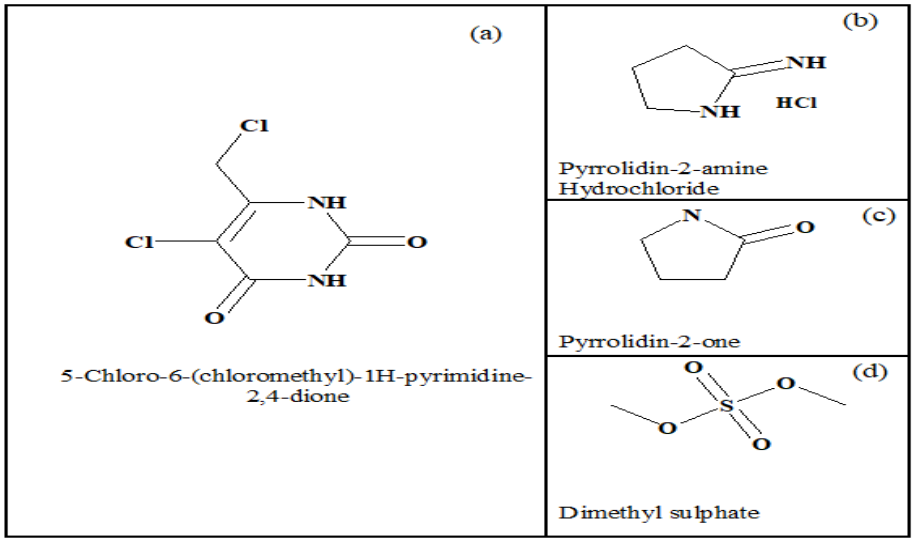


Figure 2: (a). 5-Chloro-6- (chloromethyl)-1H-pyrimidine-2,4-dione; (b). Pyrrolidin-2-amine Hydrochloride; (c). Pyrrolidin-2-one; (d). Dimethyl sulphate

Dimethyl sulphate is not controlled in the USP monograph of Tipiracil Hydrochloride [1]. During literature survey several analytical methods are found reported of estimation of dimethyl sulphate. Dimethyl sulphate is checked at workplace atmosphere by GC with electrolytic conductivity detector (sulphur mode) at trace level using stainless steel Chromosorb WHP column [24]. DMS is estimated at workplace atmosphere by thin layer chromatography (TLC) method derivatizing with 4-nitrophenol. The LOQ of DMS is reported 40 ppm which is very high because TLC technique has limitations in sensitivity point of view [25]. Quantification of DMS by GC/MS is reported by derivatizing with pentafluorobenzenethiol. This report suggests that solution stability and diluent study need to be optimized and mass detector is necessary to get DMS sensitivity at trace level [26]. Few more methods are reported by extracting the DMS in a solvent and tested by GC/MS [27-31]. DMS quantification by ion chromatography technique with conductivity detector is also reported by separating ionic compounds using All sep ™ anion anion exchange column [32]. Till date no method is available regarding determination of DMS in TPR in literature to the best of

our knowledge [33-35]. The present work deals with development, optimization and validation of the gas chromatography with mass detector method for the determination of DMS in TPR.

MATERIALS AND METHODS

Experimental

Chemicals, reagents, and samples: The investigated Tipiracil Hydrochloride drug substance was gifted from APL Research Centre laboratories (A division of Aurobindo Pharma Ltd., Hyderabad.), DMS obtained from AVRA Synthesis Private Ltd. with 99.56% purity and Methylene chloride (Analytical grade, used as diluent) and HPLC Grade water and Sodium hydroxide pellets procured from Rankem, India.

Instrumentation: The analysis was carried out on the Agilent GCMS-5977A and GCMS-5977B gas chromatograph equipped with 7890B GC System auto sampler and data handling system having Mass Hunter solution software (Make: Agilent Technologies, Santa Clora, CA, USA). The instrument was run in EI mode. Rtx-624, (30 m × 0.32 mm I.D, 1.8 µm film thickness, Restek Corporation, USA) column consists of 6% Cyanopropylphenyl and 94% dimethyl polysiloxane as a stationary phase. Chromatographic method conditions used were as follows (Tables 1-3).

Chromatographic method conditions:

Table 1: Gas chromatograph conditions for DMS analysis.

Instrument	Agilent 7890B		
Column	Rtx-624, 30 m × 0.32 mm I.D. × 1.8 µm Film thickness		
Carrier gas	Helium		
Injector temperature (°C)	220°C		
Injection type	Auto sampler		
Column oven program	**Heating rate (°C/min)**	**Initial temperature (°C)**	**Hold time (min)**
		60	2
	20	220	10
Flow rate (ml/min)	2		
Injection volume (µL)	2		
Split ratio	01:10		
Run time (min)	20 min		

Table 2: Gas chromatography mass spectrometer conditions for DMS analysis.

Instrument	Agilent GCMS-5977A and GCMS-5977B Single Quad MS
MS transfer line temperature (°C)	250°C
MS source temperature (°C)	230°C
Function type	SIM (selective ion monitoring)
Gain factor	5

Table 3: SIM Time segments.

Solvent delay time	Group name	Resolution	Mass (m/z)	Dwell time (ms)
3	Dimethyl sulfate	Low	95*, 96**	100, 100
*Quantification ion; ** Qualifier ion				
Timed MS Detector: The MS must be Detector Off after 8.5 min.				

Preparation of standard solution

1N Sodium Hydroxide Solution: Accurately weigh and transfer about 4 g of Sodium hydroxide pellets into a 100 ml, volumetric flask containing about 50 ml water, dissolve, and then dilute to volume with water.

Standard Stock solution: Accurately weigh and transfer about 62.6 mg of Dimethyl sulphate standard into a 25 ml clean, dry volumetric flask containing about 10 ml of Methylene chloride, dissolve and make up to volume with Methylene chloride. Dilute 1.0 ml of this solution to 50 ml with Methylene chloride. Further transfer 1.0 ml of above solution into a 50 ml volumetric flask and dilute to volume with Methylene chloride and mix.

Transfer 2.0 ml of 1N Sodium Hydroxide solution into a clean glass centrifuge tube and add 2.0 ml of standard solution and vortex the centrifuge tube for 1min. Allow the two phases to separate. Collect the lower organic layer (Methylene chloride layer) and transfer it into a 2 ml vial use for analysis.

Blank solution: Transfer 2.0 ml of 1N Sodium Hydroxide solution into a clean dry glass centrifuge tube, add 2.0 ml of Methylene chloride and vortex the centrifuge tube for 1min. Allow the two phases to separate. Collect the lower organic layer (Methylene chloride layer) and transfer it into a 2 ml vial for injection.

Sample solution: Accurately weigh and transfer about 100 mg of test sample into a clean dry glass test tube and add 2.0 ml of 1N Sodium Hydroxide to dissolve the sample. Then add 2.0 ml of Methylene chloride and vortex the centrifuge tube for 1min. Allow the two phases to separate. Collect the lower organic layer (Methylene chloride layer) and transfer it to a 2 ml vial for injection.

RESULTS AND DISCUSSION

Method development and optimization

The objective of this work is to determine trace level of Dimethyl sulphate in Tipiracil Hydrochloride drug substance**.** Dimethyl sulphate is a volatile compound of boiling point 188°C and has no chromophores. For analysis of such a ultra-violet (UV) inactive volatile compound gas chromatography (GC) is a suitable technology.

Initially, trails were performed on GC FID detector for the trace level determination. However, due to low response of DMS peak in FID detector, GC/MS technique has been chosen, as DMS peak is not detected at specification level in scan mode. However, the response is observed at higher levels only. It is evident that mass spectroscopy detectors including electron impact (EI) or Chemical ionization (CI) operating in the SIM mode offer the more sensitive and selective detection (compound specific) in most of the GC methods. The GC/MS analysis for DMS is performed on an Agilent (Model No: 7890B/5977A) by using a Rtx-624 capillary column with a dimension of 30 m × 0.32 mm ID × 1.8 µm film thickness. Due to its mid-polar stationary phase (6% Cyanopropylphenyl and 94% dimethyl polysiloxane), this column has been chosen, since it is better to retain DMS with

good peak shape and resolves with other peaks. Further hard ionization technique (EI) mode is selected; because of selective ion monitoring (SIM) is employed. Trails were performed with low boiler solvents like Methanol, Methylene chloride, Methyl tert-butyl ether, Cyclohexane, Ethyl acetate and Acetone to get the maximum response for DMS. But, Tipiracil Hydrochloride drug substance was not dissolved in any of these solvents. Tipiracil Hydrochloride drug substance soluble in 1N Sodium hydroxide, 1N Potassium hydroxide and Water. Hence to overcome this solubility issue Liquid-Liquid extraction technique has been chosen. Moreover, in this extraction technique sample matrix interference is less and no need to clean inlet port often. In extraction technique, Sample was dissolve in 1N Sodium hydroxide solution and extracted with Methylene chloride has given satisfactory results. DMS standard solution is prepared by diluting DMS with Methylene chloride (approximately 2.5 mg/ml) is injected through the auto-injector into GC/MS (in SCAN mode). After taking acquisition target analyte DMS was extracted by Software [(mass to charge ratio (m/z)]. The GC/MS spectrum of DMS was shown in Figure 3.

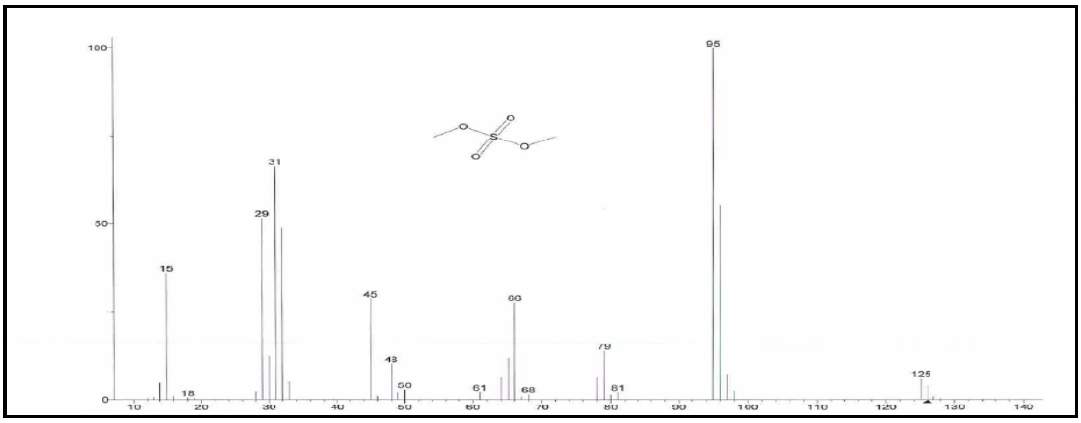


Figure 3: Mass Spectrum of Dimethyl sulphate

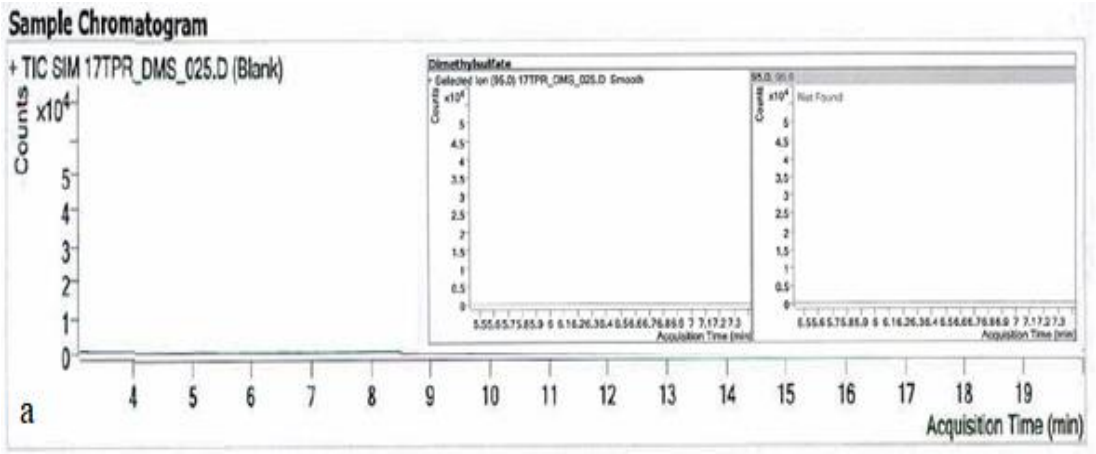
The Quantification and Qualifier ions were selected m/z-95 and m/z-96 respectively. Finally, the standard solution consists approximately 1.0 µg/ml was prepared and transferred 2 ml into a clean dry glass centrifuge tube added 2.0 ml of 1N Sodium hydroxide and vortex the centrifuge tube for 1min to separate for two phases in Liquid-Liquid extraction. Collected the lower organic layer (Methylene chloride layer), transfer it into a 2 ml vial, and then allowed it for acquisition. In the same manner, sample and recovery have been attained at desired specification level. Column temperatures & split modes were modified to get better DMS peak shape & optimized based on boiling point of DMS and its response. Finally, the optimized method was validated as per International Council for Harmonisation (ICH) guidelines [35].

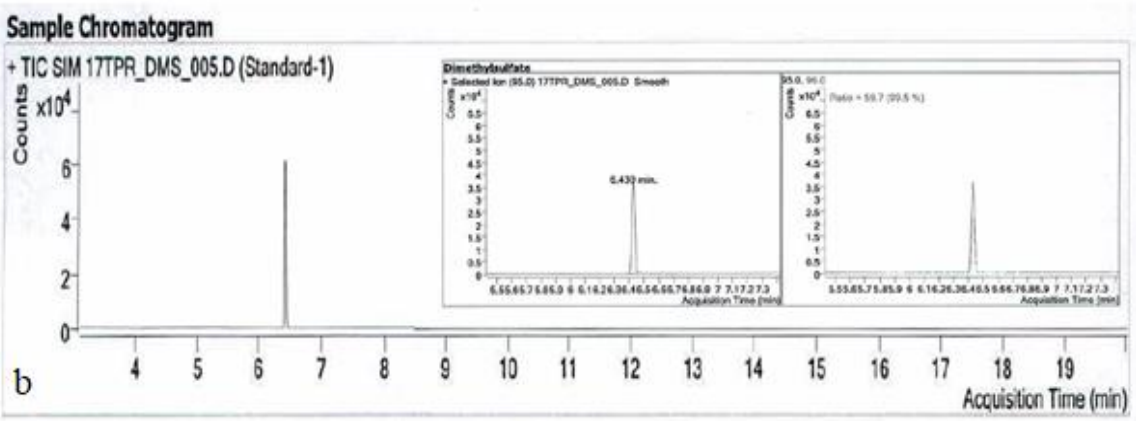
Method validation

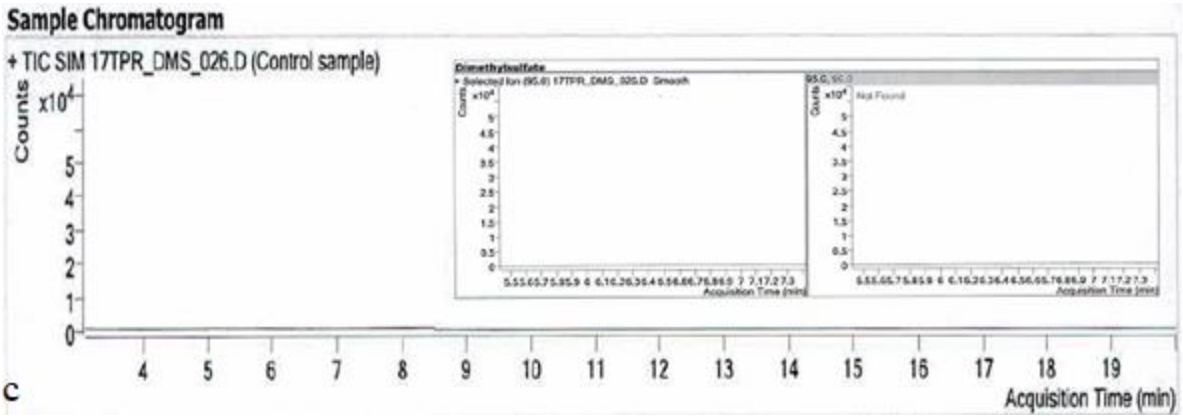
Specificity: As per ICH guidelines, specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the developed GC/MS method was verified in presence of residual solvents like Methanol, Acetone, Methylene chloride, Triethylamine, N,N-Dimethylformamide and Acetic acid which were used in the Tipiracil Hydrochloride synthesis process. Tipiracil Hydrochloride sample solution (Control sample), Tipiracil Hydrochloride drug substance spiked with DMS at specification level (Spiked Sample) and Tipiracil Hydrochloride drug substance spiked with DMS and all other known residual solvents at specification level (All Spiked Sample) were injected into GC/MS to confirm any co-elution of DMS and with any other known residual solvents. Typical GC/MS chromatograms of a control sample, spiked sample, and all spiked sample are shown in Figure 4. Specificity results are shown in Table 4 and these experimental results indicating that DMS peak is homogeneous from all other known residual solvents.

Table 4: Specificity experiments results

Sample	DMS Response (counts/area)	DMS content (µg/g)
Control sample	Not detected	Not detected
Spiked sample	55419	19.31
All spiked sample	55210	19.26







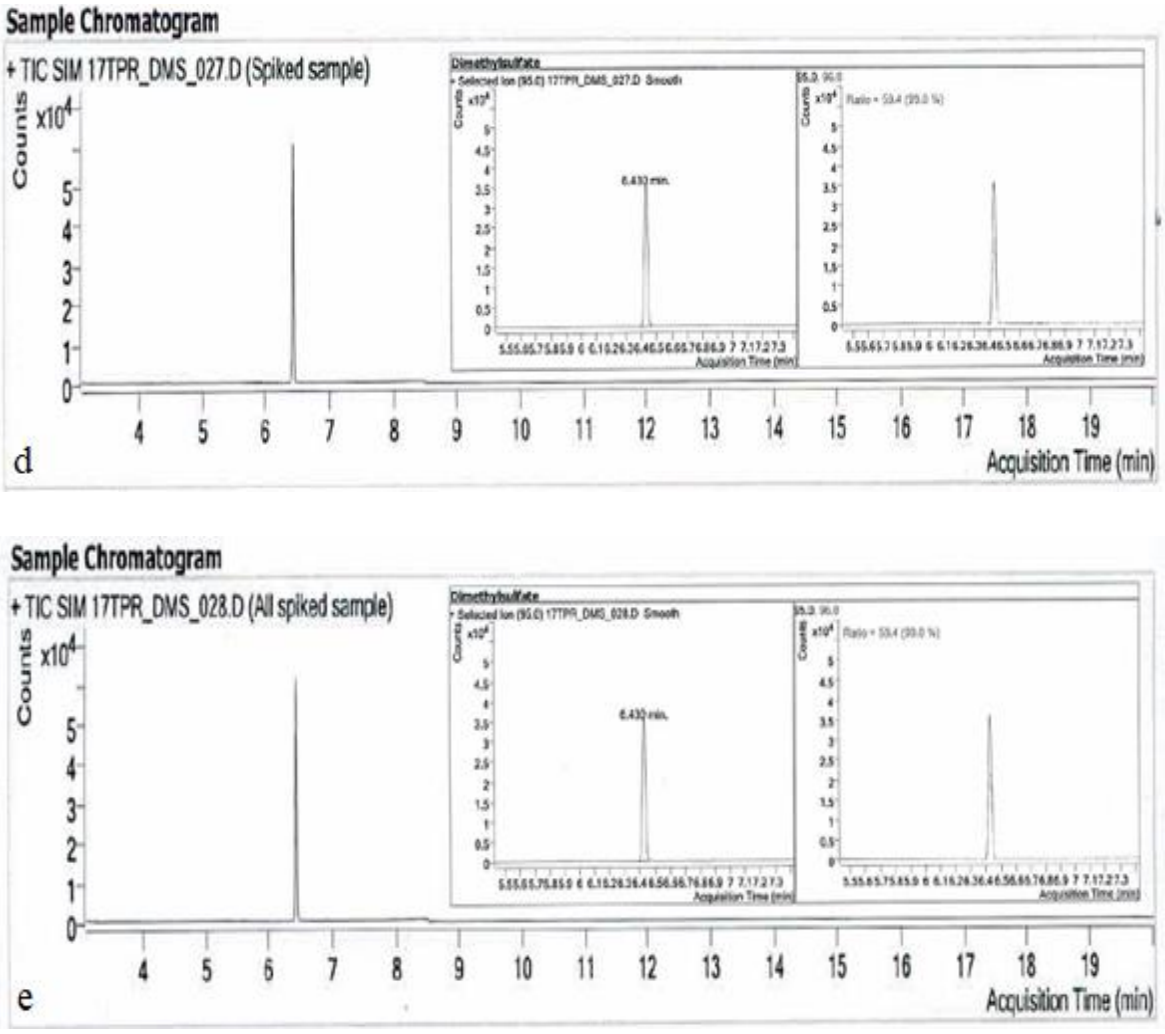


Figure 4: Typical GC/MS chromatograms of the (a). Blank, (b). Standard, (c). Control sample, (d). Spiked sample, (e). All spiked sample.

Limit of detection and Limit of quantification/linearity: The limit of detection (LOD) and limit of quantification (LOQ) values of DMS was determined using S/N ratio evaluation method. The predicted concentrations of LOD and LOQ of DMS was verified for precision by preparing the solutions containing at about predicted concentrations and injected each six times into GC/MS and calculating the % RSD of peak areas. The series of solutions were prepared using DMS at concentration levels from LOQ to 150% of specification level (19.9 µg/g) and each solution was injected and calculating the statistical values like slope, intercept and correlation coefficient from linearity plot drawn for concentration versus area. The statistical experimental values are shown in Table 5, typical GC/MS chromatograms of a LOD and LOQ are shown in Figure 5 and Linearity plot has been presented in Figure 6.

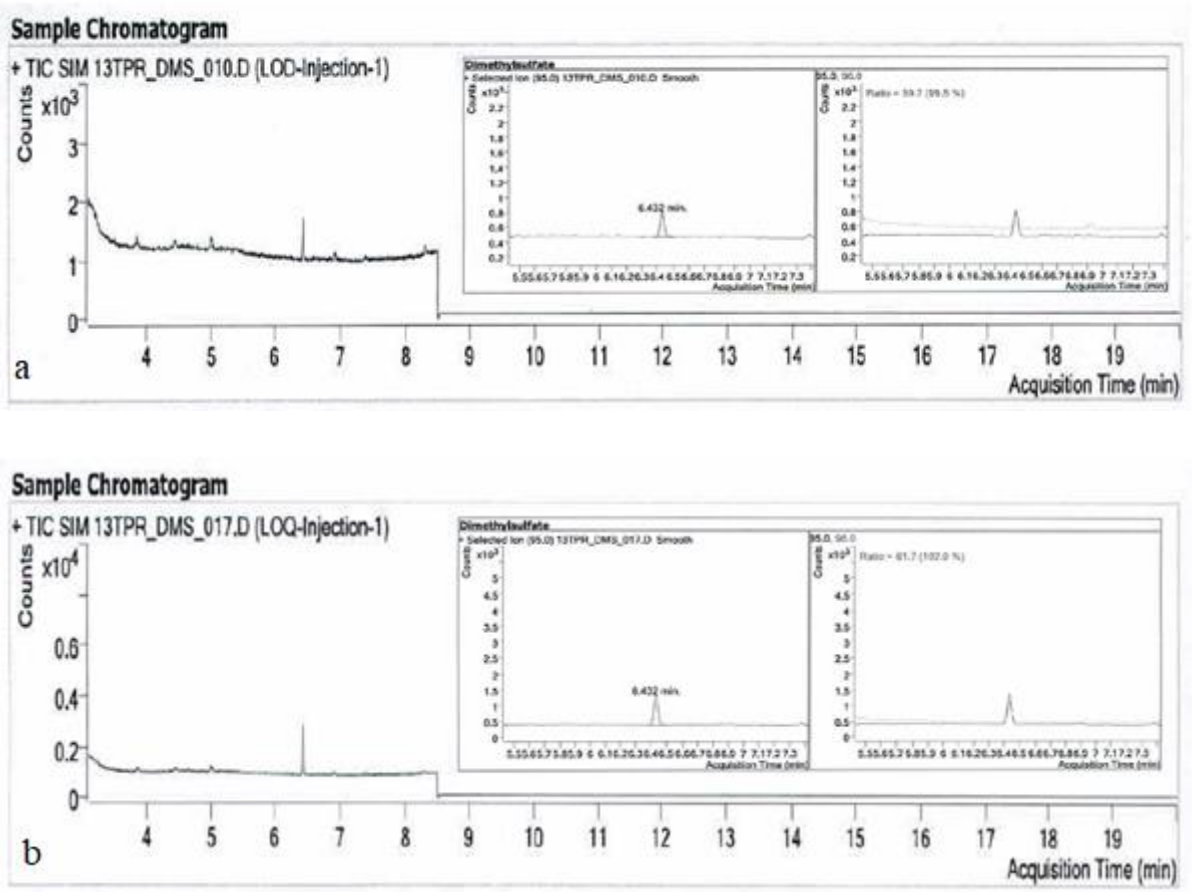


Figure 5: Typical GC/MS chromatograms of the a. LOD and b. LOQ.

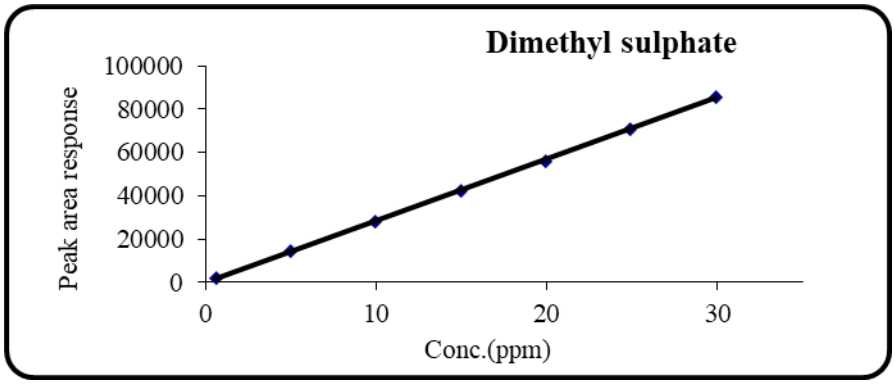


Figure 6: Linearity plot between concentration and area.

Table 5: LOD/LOQ and Linearity experiments results.

Statistical parameters	Results
Correlation coefficient	1
Concentration range (µg/g)	0.60 – 29.92
Calibration points	7

Intercept	-106.5313
Slope (S)	2849.1807
Limit of detection (µg/g)	0.2
Limit of quantification (µg/g)	0.6
Precision for Limit of Detection (%RSD)	1.3
Precision for Limit of Quantification (%RSD)	2.1

Precision: The system precision of the method was checked by injecting standard solution for six replicates and method precision was checked by preparing the six individual sample solutions by spiking the DMS at specification level (19.9µg/g) to the drug substance and injected into GC/MS. The results of system precision experiment and method precision experiment are shown in Table 6.

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 GC/MS under the chromatographic conditions on the day tested (system precision) and calculated the area of DMS from obtained areas. Repeatability and Reproducibility of the method was studied by analysing six sample solutions separately. Repeatability was the intra-day variation (Method precision) demonstrated by preparing six sample solutions individually using a single batch of Tipiracil Hydrochloride drug substance spiked with DMS at about 19.9µg/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 DMS to Tipiracil Hydrochloride drug substance and injected into different column, different instrument and different analyst on different day. The achieved precision experiment results are given in Table 6.

Table 6: Precision experiments results.

Injection ID	System Precision	Method Precision DMS content, µg/g	Ruggedness DMS content, µg/g
1	59365	20.51	18.9
2	59462	19.92	18.86
3	60914	19.64	18.97
4	60078	20.57	18.87
5	57941	19.76	18.41
6	60976	20.05	18.46
Mean	59789	20.08	18.75
SD	1137	0.39	0.24
RSD (%)	1.9	1.9	1.3
95%Cl (±)	1193	0.41	0.25
Overall statistical data (n=12)	Mean	19.41	
SD		0.76	
RSD (%)		3.92	

Accuracy: To prove the recovery for developed GCMS method, standard addition experiments were conducted in triplicate preparations (Tipiracil Hydrochloride drug substance sample solutions were prepared in by spiking with DMS) at LOQ, 50%,

100% and 150% of specification level and recoveries of DMS was determined. The obtained recovery values lie between 102.0 and 109.1 shows method is accurate. The accuracy experiment results are reported in Table 7.

Table 7: Accuracy experiments results.

Level	Amount added (µg/g)	Amount found (µg/g)	%Recovery	Mean	SD	%RSD
LOQ-1	0.6	0.58	96.7	98.4	2.9	2.9
LOQ-2	0.6	0.58	96.7			
LOQ-3	0.6	0.61	101.7			
50% Level-1	9.97	9.53	95.6	96.5	1.03	1.07
50% Level-2	9.97	9.73	97.6			
50% Level-3	9.97	9.59	96.2			
100% Level-1	19.94	19.52	97.9	96.9	1	1.03
100% Level-2	19.94	19.35	97			
100% Level-3	19.94	19.12	95.9			
150% Level-1	29.92	29.5	98.6	98	0.71	0.72
150% Level-2	29.92	29.35	98.1			
150% Level-3	29.92	29.09	97.2			

Robustness: Robustness of the method was evaluated by deliberately altering the method conditions from original method parameters and verifying compliance to the system suitability parameters. The impact of variation of column oven temperature and flow rate of carrier gas on system suitability was conducted. In robustness verification of test method, one parameter changed while keeping the other unchanged from actual parameter. The study was carried out with respect to Column flow variation of carrier gas initial flow rate ±10% and column oven initial temperature and ramp temperature ± 2°C as follow. Results of peak areas for Dimethyl sulphate is summarized in Table 8.

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

i. Column flow (-10%): 1.8 ml/min.
ii. Column flow/ (+10%): 2.2 ml/min
iii. Column oven and ramp temperature (-2°C):

$$58℃\ (2min) \xrightarrow{18℃/min} 220℃\ (10min)$$

iv. Column oven and ramp temperature (+2°C):

$$62℃\ (2min) \xrightarrow{22℃/min} 220℃\ (10min)$$

Test method conditions: Column flow: 2.0 ml/min

Column oven temperature:

$$60℃\ (2min) \xrightarrow{20℃/min} 220℃\ (10min)$$

Table 8: Robustness experiments results

Robustness condition	Variation	DMS Results
		System suitability criteria (% RSD)
As per methodology	-	1.1
Flow variation	-10%	0.7
	10%	1.1
Temperature variation	-2°C & -2°C/min	1.5
Initial Oven and Ramps	+2°C & +2°C/min	1.8

Solution stability: The standard solution and sample solutions were prepared by spiking DMS at known concentration level to Tipiracil Hydrochloride 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

A reliable and sensitive validated GC/MS method for the determination of Dimethyl sulphate in Tipiracil Hydrochloride drug substance is presented. Based on validation data, it is concluded that method is Specific, Sensitive, Linear, Precise, Accurate and Suitable. Hence the validated GC-MS method can be employed in to the routine analysis for the determination of Dimethyl sulphate in Tipiracil Hydrochloride drug substance.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the management of APL Research Centre-II (A Division of Aurobindo Pharma Ltd.) for allowing us the opportunity to carry out the present research work. The authors are also thankful to the colleagues of Analytical Research Department and Chemical Research Department for their co-operation.

REFERENCES

[1]. https://www.drugs.com/monograph/trifluridine-and-tipiracil-hydrochloride.html

[2]. Costin, D., et al., Trifluridine therapy in herpetic in keratitis. *Rev Med Chir Soc Med Nat Iasi*, **2004.** 108 (2):409-412.

[3]. Hong, D.S., et al., Phase I study to determine the safety and pharmacokinetics of oral administration of TAS-102 in patients with solid tumors. *Cancer*, **2006.**107 (6):1383-1390.

[4]. Temmink, O.H., et al., The Hollow Fibre Assay as a model for *in vivo* pharmacodynamics of fluoropyrimidines in colon cancer cells. *Br J Cancer*, **2007.** 96 (1): 61-66.

[5]. Tsunoda, N., Yoshimura, H., Metabolic fate of noscapine. III. Further studies on dentification and determination of the metabolites. *Xenobiotica*, **1981**. 11 (1): 23-32.

[6]. Overman, M.J., et al., Phase I clinical study of three times a day oral administration of TAS-102 in patients with solid tumors. *Cancer Investigaton,* **2008**.26 (8):794-799.

[7]. Hong, D.S., et al., Phase I study to determine the safety and pharmacokinetics of oral administration of TAS-102 in patients with solid tumors. *Cancer Investigaton*, **2006**.107 (6):1383-1390.

[8]. Lenz, E.M., et al., HPLC-NMR with severe column overloading: fast-track metabolite identification in urine and bile samples from rat and dog treated with [14C]-ZD6126. *J Pharm Biomed Anal*, **2007**.43 (3):1065-1077.

[9]. Sweetman, S.C., Martindale: the complete drug reference. 36th ed. London: The Pharmaceutical Press; **2009**.

[10]. Tanaka, N., et al., Repeated oral dosing of TAS-102 confers high trifluridine incorporation into DNA and sustained antitumor activity in mouse models. *Oncology reports*, **2014**.12:01.

[11]. VA Pharmacy Benefits Management Services, Medical Advisory Panel, and VISN Pharmacist Executives: Trifluridine - tipiracil (LONSURF®). National Drug Monograph February 2016.

[12]. FDA approves new oral medication to treat patients with advanced colorectal cancer. *Fiercebiotech*. **2015**.

[13]. Burness, C.D., Duggan, S.T., Trifluridine/Tipiracil: A Review in Metastatic Colorectal Cancer. *Drugs*, **2016**.76 (14):1393-1402.

[14]. Lonsurf EPAR- Summary for the Public. *European Medicines Agency*. **2016**.

[15]. Tomohiro, E., A novel combination antimetabolite, TAS-102, exhibits antitumor activity in FU-resistant human cancer cells through a mechanism involving FTD incorporation in DNA. *Int J Oncol*, **2004**.25 (3): 571-578.

[16]. Matsushita, S., The Effect of a Thymidine Phosphorylase Inhibitor on Angiogenesis and Apoptosis in Tumors. *Cancer Research*, **1999**.59 (8):1911-1916.

[17]. Godefridus, J.P., TAS-102: more than an antimetabolite. *Lancet Oncol*, **2012**. 13 (12): e518-519.

[18]. FDA Professional Drug Information on Lonsurf.

[19]. IARC., Some pharmaceutical drugs. *IARC Monogr Eval Carcinog Risk Chem Hum*, **1980**. 24: 1-337.

[20]. IARC., Genetic and related effects. An updating of selected IARC monographs from Volumes 1 to 42. *IARC Monogr Eval Carcinog Risks Hum Suppl*, **1987**. 6b: 1-729.

[21]. ICH. M7 (R1) Addendum to ICH M7. Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk; Application of the Principles of the ICH M7 Guidance to Calculation of Compound-Specific Acceptable Intakes. *ICH Harmon Guid*, **2017**.

[22]. Guideline, I.H.T., Impurities in new drug products. Q3B (R2), *current step*,**2006**. 4:1-5.

[23]. Guidance for Industry ANDAs: Impurities in Drug Substances. Dept of Health and Human Services Food and Drug Adm. FDA, **2009**. US.

[24]. Dimethyl sulfate: Method 2524. NIOSH. Manual of Analytical Methods (NMAM). Fourth Edition. **1994**.

[25]. Federation of the Employment Accidents Insurance Institutions of Germany. Centre for Accident Prevention and Occu Med. Method for the determination of dimethyl sulfate (DMS). *Ana Met Air Monit Met*, **1997**.5: 75-105.

[26]. Prabhu, P., Trace level determination of residues of dimethyl sulfate and diethyl sulfate in pharmaceutical products by headspace gas chromatography and mass spectrometry following derivatization with pentafluorobenzenethiol *Experimental Notes Perkin Elmer*, **2015**.

[27]. J. Zheng, J., et al., Determination of low ppm levels of dimethyl sulfate in an aqueous soluble API intermediate using liquid- liquid extraction and GC- MS. *J Pharm Biomed Anal*, **2009**. 50:1054-1059.

[28]. Nalavade, A., et al., Development and evaluation of class-1 solvent 1, 2-dichloroethane and dimethyl sulphate as a enotoxic impurity in sildenafil citrate drug substance by using GC-MS technique. *Int J Pharm Pharma Sci*, **2014**. 6: 552-555.

[29]. Schäfer, C., Zöllner, P., Determination of trace levels of dimethyl sulfate in the presence of monomethyl sulfate by gas chromatography-mass spectrometry. *J Chrom A*, **2013**. 4:10.

[30]. Rahman, M.H.A., et al., Determination of genotoxic impurities monomethyl sulfate and dimethyl sulfate in active pharmaceutical ingredients. *Anal Methods*, **2017**. 7:1112-1118.

[31] Chromatographia, N.V., et al., Validated chromatographic methods for the determination of process related toxic impurities in pantoprazole sodium. *Chromatographia*, **2008**. 68: 481-484.

[32]. Narendrakumar, M., et al., Simple and sensitive ion chromatography method for the simultaneous determination of dimethyl sulfate and diethyl sulfate contents in metoprolol tartrate drug substance. *Chem Sin*, **2011**. 2:164-172.

[33]. Kusuma, J., et al., An effective and sensitive stability indicating chromatographic approach based on RP-HPLC for Trifluridine and Tipiracil in bulk and pharmaceutical dosage form ; *IJRPC*, **2017**. 7 (1):63-70.

[34] Swapna, G., et al., Development and Validation of stability indicating RP-HPLC method for the simultaneous estimation of combination drugs Trifluridine and Tipiracil in bulk and pharmaceutical dosage forms. *IMPACT: IJRANSS*, **2017**. 5:93-104.

[35]. ICH, I., Q2 (R1): Validation of analytical procedures: text and methodology. *In International Conference on Harmonization*, **2005**. Geneva.