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Advance Reserve Phase High Performance Liquid Chromatography for Determination of Methyl Camphor Sulfonate by Derivatization with 2, 4- Dnph in Clopidogral Hydgrogen Sulphate

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ABSTRACT:

The purpose of this research study to develop a novel, simple, precise, accurate and economical method for determination of methyl camphor sulfonate by derivatization with 2,4- DNPH in clopidogrel hydrogen sulphate. Chromatographic analysis was performed on Phenomenex Synergi Hydro (250 × 4.6 mm, 5 µm) Column. Mobile phase (a) was 5mL perchloric acid in 1000 mL water and mobile phase (b) was a mixture of acetonitrile and methanol in the ratio of 75:25. A initial linear gradient proportion of mobile phase (a) and mobile phase (b) was in 50:50 for 8 min, 20:80 for 12 min then 5:95 for 18 min followed by 20:80 for 20 min and finally in original composition 50:50 for 25min and 30 min. The Flow rate kept for throughout analysis was 1.0 mL min-1 and chromatographic elution was monitored by waters 2489 UV/visible detector. The peak detection was done at 360 nm wavelength. This method allows for detection of methyl camphor sulfonate at 0.315 µg/mL and 0.954 µg/mL for quantification respectively. The linear response was found in a concentration range of 0.97-30.27 µg/mL with a squared correlation coefficient was 1.0000. The mean recovery was found to be 101.9%. Hence the developed method was simple, fast, linear, accurate and reproducible. The same method was validated by following ICH guidelines.

KEY WORDS: HPLC; Methyl camphor sulfonate; 2, 4-Dinitrophenylhydrazine (2, 4- DNPH); C18, Synergi Hydro.

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INTRODUCTION:

Clopidogrel hydrogen sulphate is a thienopyridine class inhibitor of P2Y12 ADP platelet receptors. Chemically it is methyl (+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetate sulfate. Clopidogrel hydrogen sulphate is a white to off-white powder and its empirical formula of Clopidogrel hydrogen sulphate is $C_{16}H_{16}ClNO_2S.H_2SO_4$. The molecular weight is 419.9 and CAS Number was 113665-84-2.The formulation brands name is Plavix. Plavix is a potent antiplatelet and anti-thrombotic drug. It is a dihydro thieno pyridine derivative pro-drug which is inactive in vitro, in vivo, it is selectively and irreversibly inhibits the binding of adenosine diphosphate (ADP) to its platelet receptors [1].

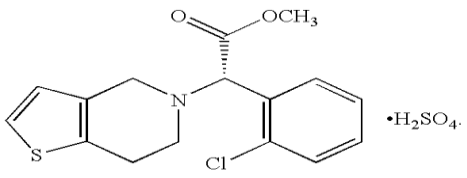


Figure 1 : Structure of Clopidogrel hydrogen sulphate

IUPAC name: – (S)-(+)-Methyl2-(4,5,6,7-tetrahydrothieno[3,2-c]pyridin-5-yl)-2-(2- chlorophenyl) acetate hydrogen sulfate

The active compound clopidogrel hydrogen sulphate is the S-enantiomer. This implies that the unwanted R-enantiomer must be carefully controlled in therapeutically active substance. In Clopidogrel hydrogen sulphate four related compounds are specified as impurities i.e (R)-(2-chlorophenyl)-(6,7-dihydro-4H-thieno[3,2-c]pyridin-5-yl)-acetic acid methyl ester R-enantiomer);(R,S)-(2-chlorophenyl)-(5,7-dihydro-4H-thieno[2,3-c]pyridin-6-yl)-aceticacid methyl ester (Imp.1) and (S)-(2-chlorophenyl)-(6,7-dihydro-4H-thieno[3,2-c]py-ridin-6-yl)-acetic acid (Imp.2). Imp. 1 is a process-related impurity and it may be present in the drug substance as racemic mixture. Imp.2 is the main degradation impurity obtained by hydrolysis of the ester group due the action of combined moisture and temperature. Till date a number of works has been done on Clopidogrel hydrogen sulphate and its related compounds/impurities. Some previous literature survey also reveal that the study was done on oxidation impurity of Clopidogrel hydrogen sulphate but no official study has been reported so far on genotoxic impurities of Clopidogrel hydrogen sulphate. The European Agency for the Evaluation of Medicinal Products (EMEA) provided guidance on genotoxic impurities. According to the guidelines genotoxic impurities exposure limit was set 1.5 ^g daily. In most of pharmaceuticals based on a precedent application of the threshold of toxicological concern (TTC) concept to food additives and food contact materials. Genotoxic impurities (GTIs) in pharmaceuticals at trace levels are of increasing concerns to both pharmaceutical industries and regulatory agencies due to their potentials for human carcinogenesis. Pharmaceutical genotoxic impurities (GTIs) may induce genetic mutations, chromosomal breaks, or chromosomal rearrangements, and have the potential to cause cancer in human. Therefore, exposure to even low levels of such impurities present in final active pharmaceutical ingredient (API) may be of significant toxicological concern. Hence determination of these impurities at microgram levels requires highly sensitive analytical methodologies,

This manuscript describes the development and validation of a rapid, simple, specific, robust precise a HPLC method for determination of methyl camphor sulfonate by derivatization with 2, 4- DNPH in clopidogrel hydrogen sulphate.

MATERIALS AND METHODS

Chemical and Reagents:

Table.1: Chemical and Reagents

Name	**Make**	**Batch no.**
Perchloric Acid	Merck	**A17A570523**
Acetonitrile	ASHONUJ CHEM	**186H1012**
O-phosphoric acid	Merck	**AC1A610123**
Water, Milli Q	Millipore	**Not**
2,4 dinitro phenyl	**Merck**	**QB2Q620434**

Equipment:

The HPLC analysis was carried out with a Waters e2695 Pump, Waters PDA 2996 and water 2489UV/Vis detector with dynamic automatic sample Injector. The whole data integration was done with waters empower 2.0 software and it complies 21CFR part 11.

Preparation of mobile phase:

Mobile Phase-A :-5 mL Perchloric acid in 1000 mL water

Mobile Phase-B : - Acetonitrile: methanol (75:25)

Preparation of solutions:

2, 4, DINITRO PHENYL HYDRAZINE (2, 4 DNP) REAGENT PREPARATION:

Transfer 1.0 g of 2, 4, dinitro phenyl hydrazine(2,4 DNP) reagent in 100 mL volumetric flask add 50 mL of acetonitrile , sonicate up to dissolves and make up to volume 100 with Acetonitrile, if turbid then filter through whatman filter paper 1.

Ortho phosphuric acid solution (0.1%):

Transfer 1 mL of ortho phosphoric acid in 1000 mL volumetric flask and make up to volume with water.

STANDARD STOCK SOLUTION (CONC.4000 PPM W.R.T. SAMPLE):

Transfer 55.00 mg of Methyl Camphor Sulfonate into 100 mL volumetric flask containing 10 mL Acetonitrile sonicate to dissolve, then make up to volume with acetonitrile.

Standard solution (conc.20 ppm w.r.t sample):

Transfer 0.25 mL of standard stock solution into 50.0 mL volumetric flask add 10 mL Acetonitrile, 5 mL water, 1.0 mL 2, 4 DNP reagent then 5 mL 0.1% OPA Solution. Heat at 70° to 80° c for 1 hrs in water bath then cool solution and make up to volume up to mark with acetonitrile, shake well.

Test solution (Conc., 10000 ppm):

Weigh accurately 500.0 mg of Clopidogrel Hydrogen Sulfate test sample into a 50 mL volumetric flask, dissolved it 10mL Acetonitrile,5 mL water, 1.0 mL 2,4 DNP Reagent then 5 mL 0.1% OPA solution. Heat at 70° to 80° c for 1 hrs in water bath then cool solution and make up to volume up to mark with acetonitrile, shake well.

Blank solution:

Prepare blank same as test solution without adding sample in it.

Chromatographic conditions:

Table.2: Chromatographic conditions

Column	Phenomenox Synergi Hydro , 250 x
Column	40°C ± 2°C
Flow Rate	1.0 mL/min
Injection Volume	10 µL
Detector	360 nm
Run Time	30 minutes
Retention Time	Methyl Camphor Sulfonate at about 15.7 min

Table.3: Gradient program

No.	Flow (mL)	TIME (Min)	%A	%B
1	1.0	0	50	50
2	1.0	8	50	50
3	1.0	12	20	80
4	1.0	18	5	95
5	1.0	20	20	80
6	1.0	25	50	50
7	1.0	30	50	50

System Suitability Acceptance Criteria:

1. % RSD: The percent relative standard deviation should not be more than 2.0 for peak area of Methyl Camphor Sulfonate injections of standard solution.
2. Tailing Factor should not be more than 2.0.
3. Number of Theoretical plates should be more than 2000.

Specificity

The specificity of the analytical method was determined by injecting 2,4, dinitro phenyl hydrazine(2,4 DNP) reagent blank ; Ortho phosphoric acid Solution (0.1%); Standard solution; Test solution and then spike standard solution in test solution under the above chromatographic conditions. The individual retention times of standard, blank and test were noted (Table 4 & fig no. 3, 4, 5 and 6). No peak was observed at the retention time of Methyl Camphor Sulfonate standard and peak purity of Methyl Camphor Sulfonate standard is greater than peak threshold hence method was specific.

System Suitability (System Precision):

Six replicate standard solution of Methyl Camphor Sulfonate was injected and check the precision and system suitability of the system. %RSD of the six replicate injections for all the standards was found below 5% (Table 5 and Table 6).

Limit of detection (LOD) and limit of quantitation (LOQ):

The LOD and the LOQ were determined based on styex method. The LOQ and the LOD for the Methyl Camphor Sulfonate derivatives peak was found to be 0.315 µg/ mL and 0.954 µg /mL (Table 7).

Precision and Batches Analysis

The repeatability or precision of the method was evaluated by the determination of peak area %RSD of derivatized Methyl Camphor Sulfonate peak for six replicate injections from different preparation. The precision was calculated at the LOQ level concentration and the 100 % level concentration (Table.8)

Linearity

Under the optimized working conditions, standard calibration curve for Methyl Camphor Sulfonate derivatized peak was plotted over the range of LOQ to 150%. The squared correlation co efficient was found to

be 1.000 and linearity curve was shown in (Table.7 & Fig.2)

Accuracy/ Recovery Study

Accuracy of method can be determined by doping the respective concentration solution of Methyl Camphor Sulfonate in test preparation and find out the content of Methyl Camphor Sulfonate recover from test preparation. Recovery studies were carried at standard concentration 50%, 100%, and 150%. The %recovery was determined at three concentration ranges chromatograms were shown in figure no. 5 & 6 respectively and total data was shown in (Table.9)

Table.4: specificity (Individual RT of Methyl Camphor Sulfonate)

Sample ID	Retention time of MCS	Purity angle	Purity threshold
Blank	No peak	-	-
Standard	15.756	0.50	1.013
Test solution	Not detected	-	-
Test spike with STD	15.754	0.51	1.018

Table.5: System suitaiability parameter

Parameters	Tailing Factor	Theoretical Plate
Specificity	0.79	183047.44
Linearity ,LOD,LOQ	1.12	129797.43
Accuracy	1.12	128922.95
Batch Analysis	1.12	128922.95

Table.6: % RSD of area for standard solution of Methyl Camphor Sulfonate

Sr. No	Sample ID	Peak area of Methyl Camphor Sulfonate
	Blank	00
1	Standard solution-1	20582
2	Standard solution-2	20582
3	Standard solution-3	20565
4	Standard solution-4	20528
5	Standard solution-5	20540
6	Standard solution-6	20534
Average area		**20555**
Standard deviation		**24.30**
% RSD		**0.12**

Table.7: Linearity; LOD and LOQ values

Linearity Level	Sample ID	Conc. of Methyl Camphor Sulfonate	Peak area of Methyl		
			Inj-1	Inj-2	Average Area
LOQ	Linearity	0.97	1063	1090	1077
20%	Linearity	4.04	3966	4088	4027
50%	Linearity	10.09	10301	10203	10252
100%	Linearity	20.18	20517	20624	20571
150 %	Linearity	30.27	30990	31056	31023
Slope				1024.04	
Intercept				-33.00	
Standard deviation				97.64	
Regression coefficient (R2)				1.0000	
Limit of detection (ppm w.r.t				0.315	
Limit of quantitation (ppm w.r.t				0.954	

Table.8: Precision and batches analysis

Batch NO.	Injection	Peak area Methyl Camphor Sulfonate	Concentration of Methyl Camphor Sulfonate in ppm (w.r.t sample)
BLANK	1	00	--
	2	00	--
305E12001	1	00	00
	2	00	00
305E12002	1	00	00
	2	00	00
305E12003	1	00	00
	2	00	00

Figure.2: Linearity of Methyl Camphor Sulfonate

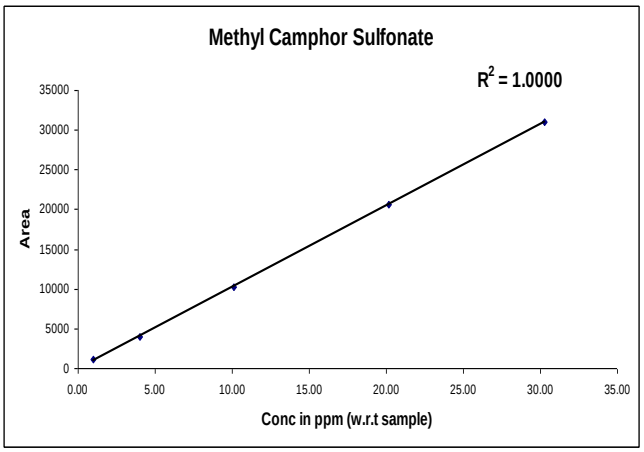


Table.9: % Recovery or accuracy of Methyl Camphor Sulfonate

Accuracy Level	Sample ID	Injection	Wt of sample(mg)	Peak area of MCA	Actual Conc. in ppm	Recovered Conc. in ppm	% Recovery
LOQ	Accuracy Level-1	1	500.06	1048	0.97	1.03	106.21
	Accuracy Level-1	2	500.06	1047	0.97	1.03	106.10
	Accuracy Level-1	3	500.04	1078	0.97	1.06	109.25
80%	Accuracy Level-2	1	500.12	16465	16.12	16.14	100.10
	Accuracy Level-2	2	500.12	16284	16.12	15.96	99.00
	Accuracy Level-2	3	500.13	16495	16.12	16.17	100.28
100%	Accuracy Level-3	1	500.13	20573	20.15	20.16	100.06
	Accuracy Level-3	2	500.03	20759	20.15	20.35	100.99
	Accuracy Level-3	3	500.02	20104	20.15	19.71	97.80
120 %	Accuracy Level-4	1	500.11	24961	24.18	24.47	101.17
	Accuracy Level-4	2	499.99	24872	24.18	24.39	100.84
	Accuracy Level-4	3	500.06	24888	24.18	24.40	100.89
Batch analysis	305E12003	1	500.02		Below detection limit		
		2					
						% Mean Recovery	101.9

Figure 3: Typical chromatogram of Reagent Blank

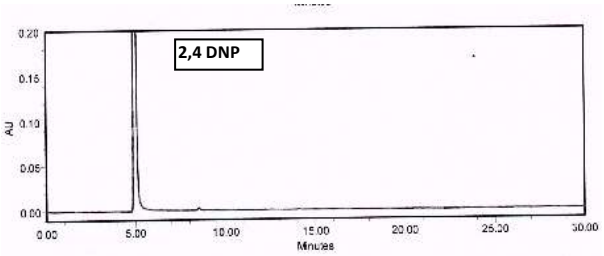


Figure 4: Typical chromatogram of Standard Methyl Camphor Sulfonate

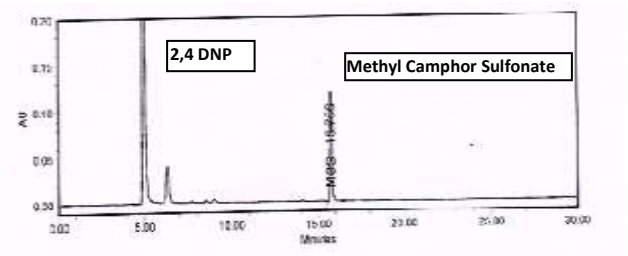


Figure 5 Typical chromatogram of Test spike with standard Solution

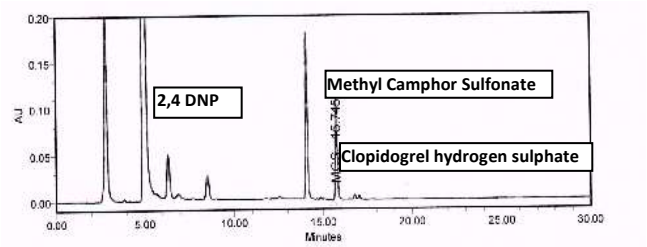
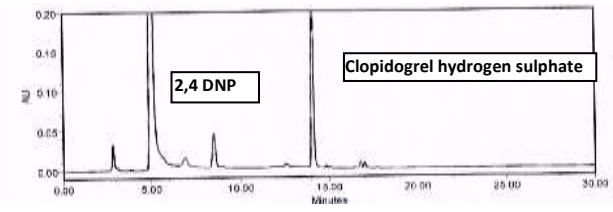


Figure 6: Typical chromatogram of Test solution



RESULTS AND DISCUSSION

Sulfonate salts are the most frequently used compounds in pharmaceutical developments. Salt formation is a useful technique for optimizing the physicochemical processing, biopharmaceutical or therapeutic properties of active pharmaceutical ingredients (APIs), and sulfonate salts are widely used for this purpose. Sulfonate group is a genotoxic capable of inducing mutagenesis changes hence Methyl Camphor Sulfonate is a genotoxic and it is used for resolve R-enantiomer in mixture RS enantiomer. Methyl Camphor Sulfonate has no chromophoric group hence the approach towards derivatization pre column leading to chromophoric evolution of the methyl camphor sulfonate using UV- detector in HPLC or such compound can detect by gas chromatograph technique. The most popular measurement method for sulfonate group determination by using derivatising reagents like 2,4-dinitrophenylhydrazine, converted to stable hydrazones. This hydrazones is detected by HPLC coupled with UV or diode array detection. The DNPH-method exploits acceptable sensitivity and reproducibility as well as stability of reagent and products. Therefore for development we have been adopted such technique.

A Phenomenex Synergi Hydro, 250 x 4.6 mm, 5 µm column was used to determine the Methyl Camphor Sulfonate content. The derivatized hydrozone has maximum intensity at a wavelength of 360nm. The absence of interference between the derivatized product and other impurities of Clopidogrel hydrogen sulphate was confirmed by Diode array detector having peak purity is less than peak threshold in water instrument. All unknown impurities peaks are well separated using a gradient program technique. In the course of development we observed that the areas of the standard peaks are not reproducible, it was thus inferred that 2, 4-DNPH stock being added was not sufficient for proper derivatization hence changes in sample preparations were made. The concentration of 2, 4-Dinitro phenyl hydrazine being added was increased, reproducible areas were then observed. The blank and the optimized chromatogram were shown as above.

CONCLUSION

The developed reserve phase high performance liquid chromatography for determination of methyl camphor sulfonate by derivatization with 2, 4-DNPH in clopidogrel hydrogen sulphate was found to be simple and useful with high accuracy, precision, reproducible and linear method. The same method has been validated as per ICH guideline Q2 (R1). This method can be used for routine quality control sample analysis or we can use for control monitor for genotoxic impurities in the process formation of active pharmaceutical ingredient such as methyl camphor sulfonate in clopidogrel hydrogen sulphate.

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