ORIGINAL RESEARCH PAPER

Chemistry

KEY WORDS: LC Method; Genotoxic impurity; Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo-compound and Dibromo-compound, Salbutamol Sulpahte, Waters, ppm (parts per million); Threshold of Toxicological Concern (TTC).

DETERMINATION OF PROCESS RELATED GENOTOXIC IMPURITIES OF SALBUTAMOL SULPHATE BY LC METHOD

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ABSTRACT

The main aim of this research work is to develop a suitable LC method for the quantitative determination of genotoxic impurities contains in Salbutamol Sulphate which is coming from the chemicals used during the manufacturing process. In manufacturing process many unwanted chemical materials are being used and out that many are following under Genotoxic category. After screening and doing the assessment on the genotoxic predication in salbutamol sulphate. The possible genotoxic impurities identified and likely to present in salbutamol Sulphate as Salicylic acid,[1][2][3] Acetyl methyl Salicylate (AMS),[4][5][6] Benzyl methyl salicylate (BMS),[7] Bromo-compound[8] and Dibromo-compound[8]. The main challenge is to separate all impurities from each other to get better resolution and response. As genotoxic[19][24] impurities estimation limit in final molecule is very minute and low it is not easy to quantify at ppm level present in Salbutamol sulphate in Active Pharmaceutical Ingredients. Hence the LC method was developed on Waters HPLC system (Water's Ltd, USA) with 2995 UV detector at 273 nm as wavelength and 1.0 ml/min flow rate by using Spherical end-capped octylsilyl silica gel for chromatography (l = 0.15 m, Ø = 4.6 mm, 3µm) long with gradient system. The chromatographic and integrated data were recorded using Empower -3 data acquisition software. The limit of detection and the limit of quantitation for the impurity were established. Validation of the developed LC method was carried out as per ICH requirements and the data shows that the proposed method is specific, linear, accurate, precise and robust. This method has been tested in a number of Salbutamol Sulphate and used successfully for quantification of the reported impurities at ppm level. The developed LC method was found to be suitable to quantify the genotoxic impurities Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo-compound and Dibromo-compound at ppm level present Salbutamol Sulphate.

INTRODUCTION:

Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo-compound and Dibromo-compound are Process Impurities of salbutamol sulphate (Figure 1). All these impurities are shows presence of structural alert for genotoxic mutagenicity and carcinogenicity. QSTR models predict the compound positive for genotoxicity, mutagenicity and carcinogenicity the compounds is shown positive for mutagenicity in training set used for Ames mutagenicity model[9-13]. In genetics, Genotoxicity describes as property of chemical compounds which may damage the genetic information within a cell leading mutations, which can lead to different types of Cancers in Human body in any forms.

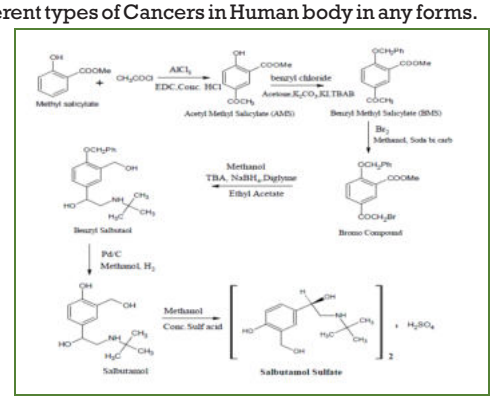
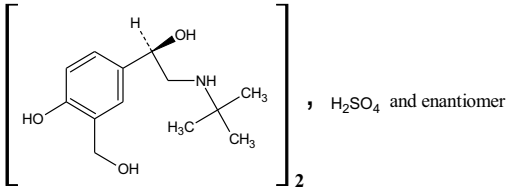


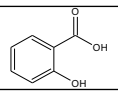
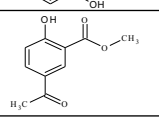
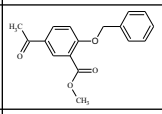
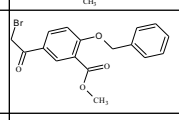
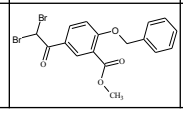
Figure 1: Reaction Scheme of Salbutamol Sulphate



Salbutamol Sulphate Chemical name: Bis[(*1RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3- (hydroxymethyl) phenyl]ethanol sulphate.

Figure 2: Structure of Salbutamol Sulphate drug substance

Table 1: IUPAC & Structures of Genotoxic Impurities of Salbutamol Sulphate

Sr. No	Structure	Chemical Name	Kind of Impurity
1		salicylic acid	Process Genotoxic Impurity
2		methyl 5-acetyl-2-hydroxybenzoate	Intermediate - Process Genotoxic Impurity
3		methyl 5-acetyl-2-(benzyloxy)benzoate	Intermediate - Process Genotoxic Impurity
4		methyl 2-(benzyloxy)-5-(bromoacetyl)benzoate	Intermediate - Process Genotoxic Impurity
5		methyl 2-(benzyloxy)-5-(dibromoacetyl)benzoate	Intermediate - Process Genotoxic Impurity

The genotoxicity[19] is mostly confused with mutagenicity, all mutagens are genotoxic but however it's not necessarily all genotoxic substances are mutagenic. The alteration in body can have direct or indirect effects on DNA: the induction of mutations, mistimed event activation and direct DNA damage leading to mutations. The permanent, heritable changes can

affect either somatic cells of the organism or germ cells to be passed on to coming/future generations. Cells prevent expression of the genotoxic mutation by either DNA repair or apoptosis; however, the damage may not always be fixed leading to mutagenesis[11] Specifically, there is evidence that genotoxic substances may bind directly to DNA and may also act indirectly by affecting enzymes involved in DNA replication. There are three primary effects that Genotoxins can have on organisms by affecting their genetic information. Genotoxins can be carcinogens, or cancer-causing agents, mutagens, or mutation-causing agents, or teratogens, birth defect-causing agents [13]. The toxicological assessment of these genotoxic impurities and the determination of acceptable limits for such impurities in active substances is a difficult issue and not addressed in sufficient detail in the existing International Conference on Harmonization (ICH) Q3X guidelines[14]. The presence of trace level of the Genotoxic Impurity in drug substance or drug product is of genotoxicity concern and has been closely monitored by regulatory agencies and pharmaceutical industries[16]. The 'threshold of toxicological concern' (TTC) of 1.5 □g/person/day (exposure of genotoxic impurity in drugs that will be tested or dosed for longer than 12 months) has been suggested by the European Medicines Agency's (EMEA) "Guideline on the limits of genotoxic impurities"[14-16] and the Pharmaceutical Research and Manufacturers of America's (PhRMA) white paper [13]. Based on the TTC, the concentration limits of genotoxic impurity in drug substances or drug products can then be derived based on the maximum daily dose: concentration limit (ppm) = [1.5 □g /day)] / [dose (g/day)][16]. For a drug dosed at 1g per day, for example, 1.5 ppm would be the limit of a specific genotoxic impurity which would also be the 'target analyte level' (TAL) from an analytical perspective [14-16]. Given such a low ppm concentration limit, besides the control challenges in process chemistry, developing sensitive and robust methodology for their detection poses a tremendous analytical challenge for the pharmaceutical industry[17-22]. Therefore potential genotoxins must be minimized during the synthesis the compounds and where there is difficulty achieving this, the method of manufacture should preferably be changed. As Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo-compound and Dibromo-compound are genotoxic compounds, the regulators may require the toxin levels to be controlled below 31.5 ppm in the drug substance on the basis of Maximum Daily Dose of drug substance. Quantification at such low level can be possible only by using LC or LCMS/MS and also there is no method for the quantification of these impurities hence a high sensitive LC method developed for the quantification of these genotoxic impurities.

Experimental:

Chemicals and reagents:

Samples of Salbutamol Sulphate (Figure 2), AMS), Benzyl methyl salicylate (BMS), Bromo-compound and Dibromo-compound were collected from Supriya Lifescience Ltd., Maharashtra, India., and Salicylic acid from Sigma Aldrich, Mumbai, India.

Equipment:

The LC method development and validation were done on Waters HPLC system (Water's Ltd, USA) with 2995 UV detector at 273 nm as wavelength and 1.0 ml/min flow rate by using Spherical end-capped octylsilyl silica gel for chromatography (l = 0.15 m, Ø = 4.6 mm, 3µm) long with gradient sysytem. The chromatographic and integrated data were recorded using Empower -3 data acquisition software.

LC chromatographic conditions:

Column Size	l = 0.15 m, Ø = 4.6 mm
Flow rate	1.0 ml/min
Column temperature	30°C
Stationary Phase	Spherical end-capped octylsilyl silica gel for chromatography. (3µm)
Detector wavelength	Spectrophotometer at 273 nm
Injection volume	20µl of the test solution and reference solution
Run time	Time (Min) Mobile Phase A (%V/V Mobile Phase B (%V/V) 0-5 95 5 5-30 95 - 10 5 - 90

Preparation of genotoxic impurity standard and test sample Solution:

Dissolve each 10.0 mg of Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo Compound standard in mobile phase and make up with 100 ml mobile phase. Transfer 31 ml above stock solution in 100 ml mobile phase (mixture of 60 volumes of methanol, 40 volumes of Water, add 1.0 volumes of Acetic acid and 0.1 volumes of Triethylamine), with respect to test concentration. The testing API samples were typically prepared at approximately 10 mg/mL in mobile phase.

Method Validation:

The newly developed method was validated as per ICH guidelines.[15][23] The validation parameters include specificity, limit of detection and limit of quantification, accuracy, precision, linearity and robustness.

Specificity

Specificity was established by injecting samples of Salbutamol Sulphate drug substance spiked with its impurities 31.25 ppm with respect to Salbutamol Sulphate concentration. All the impurities were well resolved from one another and Salbutamol Sulphate indicating the specificity of the proposed method (Figure-4) alone with blank solution (Figure-3).

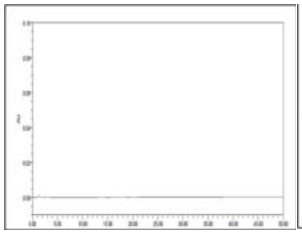


Figure 3: Blank Chromatogram

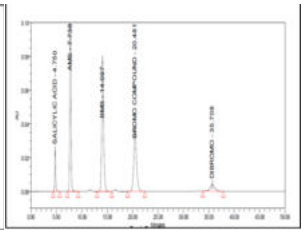


Figure 4: Chromatogram for all process impurities spiked

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The 10ppm stock solution of Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo Compound was prepared with respect to 0.4 mg mL^{-1} Salbutamol Sulphate, with this solution further series of dilutions of mixed concentrations was prepared of Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo compound and inject into the HPLC as under methodology. The basis of experimental LOD was 0.1 ppm and 0.3 ppm of LOQ was observed for all process impurities. Precision of LOD and LOQ are summarized in Table-2 and Table-3 also representing chromatographs are shown in Figure 5 and Figure 6 respectively.

Table 2: Precision of LOD for salbutamol sulphate process related impurities

LOD solution (ppm)	Area for 0.1 ppm – LOD solution				
	Salicylic acid	Acetyl methyl Salicylate (AMS)	Benzyl methyl salicylate (BMS)	Bromo compound	Dibromo Compound

LOD std. 1	1195	6565	6415	4103	1138
LOD std. 2	1269	6579	6295	4090	1090
LOD std. 3	1255	6486	6471	4088	1018
LOD std. 4	1247	6634	6343	4005	1054
LOD std. 5	1121	6588	6331	4005	1058
LOD std. 6	1231	6655	6422	4088	1145
Mean	**1220**	**6585**	**6380**	**4063**	**1084**
Std. Dev.	**54.52**	**59.09**	**66.55**	**45.21**	**50.31**
% RSD	**4.47**	**0.90**	**1.04**	**1.11**	**4.64**

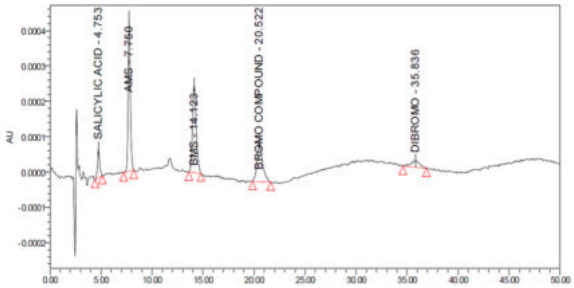


Figure 5: LOD chromatogram for all process impurities of Salbutamol Sulphate

Table 3: Precision of LOQ for salbutamol sulphate process related impurities

LOQ solution (ppm)	Area of 0.3 ppm- LOQ solution				
	Salicylic acid	Acetyl methyl Salicylate (AMS)	Benzyl methyl salicylate (BMS)	Bromo compound	Dibromo Compound
LOQ std. 1	3118	20024	19760	11614	3195
LOQ std. 2	3146	19828	19789	11739	3009
LOQ std. 3	3150	19886	19507	11507	2977
LOQ std. 4	3139	19879	19506	10843	3041
LOQ std. 5	3199	19855	19736	11572	2335
LOQ std. 6	3274	19891	19441	11425	3110
Mean	**3171**	**19894**	**19623**	**11450**	**3094**
Std. Dev.	**56.89**	**67.84**	**154.41**	**315.53**	**103.93**
% RSD	**1.79**	**0.34**	**0.79**	**2.76**	**3.36**

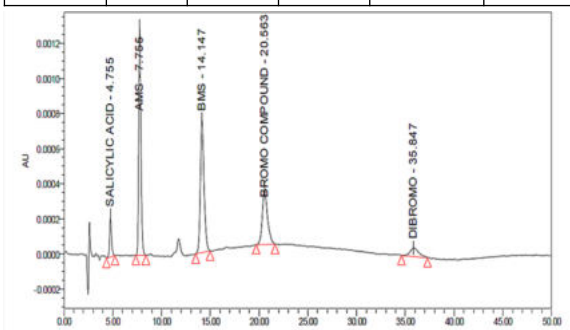


Figure 6: LOQ chromatogram for all process impurities of Salbutamol Sulphate

Precision

Precision was determined by six replicate injections of the Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo compound at specification level and inject into HPLC system.

%RSD values for precision at 100% level i.e 31.25 ppm for Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo compound were found to be 0.12%, 0.11%, 0.20%, 0.38% and 0.91% respectively. The precision was checked by injecting 31.25 ppm of impurities from individual preparations w.r.t. 500 ppm of salbutamol sulphate. The intermediate precision was verified on six different batches of salbutamol sulphate to see the presence of these process impurities. The observation were recorded Table 4 and Table 5 respectively.

Table 4: System Precision of process related impurities for salbutamol sulphate

Sr. No.	Salicylic acid	Acetyl methyl Salicylate (AMS)	Benzyl methyl salicylate (BMS	Bromo compound	Dibromo Compound
	Area	**Area**	**Area**	**Area**	**Area**
Injection 1	286653	2049508	2029737	1386814	262458
Injection 2	286966	2049892	2022472	1386145	267886
Injection 3	286939	2050189	2028616	1381256	264487
Injection 4	287078	2051432	2030251	1376008	265201
Injection 5	287125	2052547	2031672	1377392	266536
Injection 6	287705	2055469	2034696	1374500	269119
Mean	**287077**	**2051506**	**2029574**	**1380352**	**265948**
Std. Dev.	**348.66**	**2241.71**	**4061.49**	**5253.73**	**2409.50**
% RSD	**0.12**	**0.11**	**0.20**	**0.38**	**0.91**

Table 5: Method Precision of impurities for salbutamol sulphate

Sr. No.	Salicylic acid	AMS	BMS	Bromo compound	Dibromo compound
	%	%	%	%	%
SLL/SS/1117052	ND	ND	ND	ND	ND
SLL/SS/1117053	ND	ND	ND	ND	ND
SLL/SS/1117054	ND	ND	ND	ND	ND
SLL/SS/1117055	ND	ND	ND	ND	ND
SLL/SS/1117056	ND	ND	ND	ND	ND
Mean	ND	ND	ND	ND	ND

ND – Not detected

Accuracy

The study conducted for recovery/accuracy of salbutamol sulphate process impurities for quantification was carried out in triplicate at 50%, 100% and 150% w.r.t. specification level i.e 31.25 ppm (Table-6). The average percentage recovery was calculated and found to be within the range and tabulated in Table -7

Table 6: % Recovery of all impurities

Sample No.	Salicylic acid	AMS	BMS	Bromo compound	Dibromo compound
	% Recovery	**% Recovery**	**% Recovery**	**% Recovery**	**% Recovery**
Acc. 50% -1	103.00	102.30	102.74	102.74	99.30
Acc. 50% -2	100.70	103.20	104.20	104.20	105.60
Acc. 50% -3	101.32	105.90	103.06	103.06	104.40
Acc. 100% -1	101.80	104.70	100.80	100.80	104.90
Acc. 100% -2	102.43	97.50	100.39	100.39	97.80
Acc. 100% -3	100.40	104.70	100.80	100.80	97.40
Acc. 150% -1	101.50	103.40	104.90	104.90	97.70
Acc. 150% -2	99.00	100.90	101.50	101.50	100.90
Acc. 150% -3	103.80	101.70	100.23	100.23	99.80
Mean	**101.55**	**102.70**	**102.07**	**101.48**	**100.87**
SD	**1.463**	**2.44**	**1.758**	**3.078**	**3.25**
% RSD	**1.463**	**2.44**	**1.758**	**3.078**	**3.25**

Table 7: Average %Recovery of Salbutamol Sulphate process impurities

Parameter	% Recovery Salicylic acid	% Recovery Acetyl methyl Salicylate	% Recovery Benzyl methyl salicylate	% Recovery Bromo compound	% Recovery Dibromo compound
50% level	101.67	103.80	103.33	101.39	103.10
100% level	101.54	102.30	100.66	103.44	100.03
150% level	101.43	102.00	102.21	99.62	99.47

Linearity

Linearity was performed over a wide range of analytes which ensured that calculations could be performed using a single working standard rather than an equation of a calibration line. Solutions were prepared by diluting stock solutions at six concentration levels Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo compound concentration values at LOQ level, 50 %, 80 %, 100 % 120 % and 150 % of the specification levels. Prepared concentration at each level should be analyzed in duplicate, from the responses obtained for each conc. Level, (y value) should be plotted against conc. (X value) using a least squares of test results versus analyte conc. %RSD value for slope, Y-intercept and correlation coefficient of calibration curve were calculated and the results are summarized in Table-8. The linearity plots areas shown in Figure-7, Figure-8, Figure-9, Figure-10 and Figure-11 for Salbutamol Sulphate process impurities of Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo compound respectively.

Table 8: Linearity summary for Salbutamol Sulphate related substances

Sr. No.	Salicylic acid	Acetyl methyl Salicylate (AMS)	Benzyl methyl salicylate (BMS)	Bromo compound	DibromoCompound
	Area	Area	Area	Area	Area
Lin -LOQ	2660.0	18704.0	19224.0	13210.0	2499.0
Lin - 50%	148494.0	1005945.0	1000916.0	677784.0	123225.0
Lin - 80%	221662.0	1642038.0	1501978.0	1100806.0	213229.0
Lin - 100%	267077.0	2252547.0	2002472.0	1376008.0	276536.0
Lin - 120%	322492.0	2563056.0	2402966.0	1751210.0	329843.0
Lin - 150%	425571.0	3378492.0	3103388.0	2063792.0	389761.0
Correlation coefficient	**0.997**	**0.995**	**0.997**	**0.997**	**0.997**

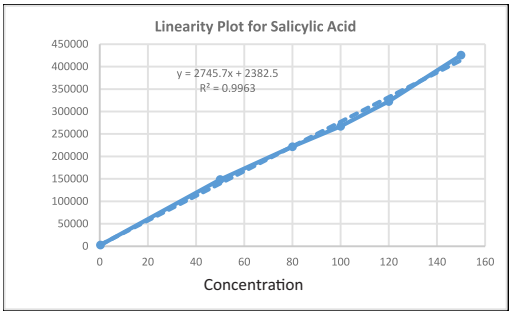


Figure 7: Linearity plot for Salicylic Acid

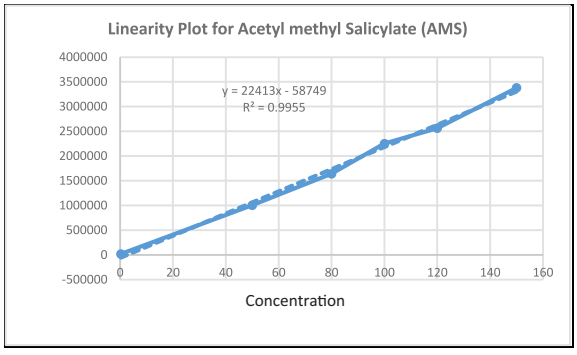


Figure 8: Linearity plot for Acetyl Methyl Salicylate (AMS)

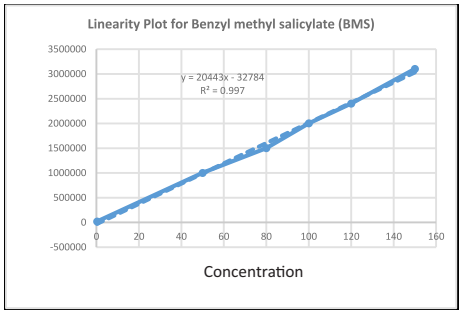


Figure 9: Linearity plot for Benzyl Methyl Salicylate (BMS)

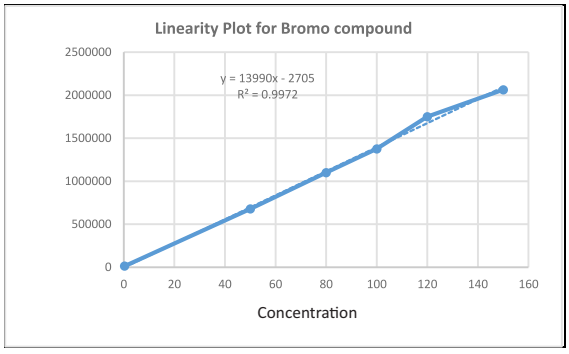


Figure 10: Linearity plot for Bromo Compound

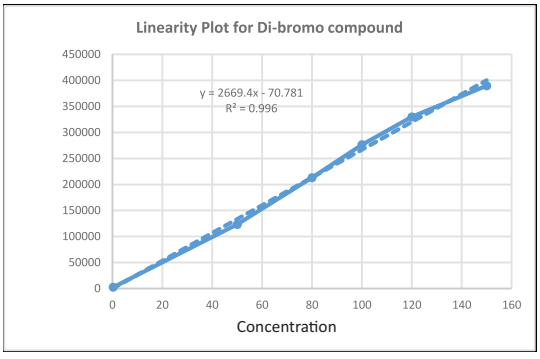


Figure 11: Linearity plot for di-Bromo Compound

CONCLUSION:

The study conclusion is, reported method is very sensitive specific, accurate, validated and well defined LC method for the Quantification of genotoxic impurities - Salicylic acid, Acetyl methyl Salicylate (AMS), Benzyl methyl salicylate (BMS), Bromo compound and Dibromo compound at ppm level in Salbutamol Sulphate. The detection limit and quantification limit found to be 0.1 ppm and 0.3 ppm respectively each impurity. The described method is highly reliable technique for the quantification of the genotoxic impurities present in the Salbutamol Sulphate during routine analysis.

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REFERENCES:

1. Madan RK; Levitt J, (2014), A review of toxicity from topical salicylic acid preparations, J Am Acad Dermatol. 70 (4): 788–92.
2. Péc J, Strmenová, M, Palencárová E, Pullmann R, Funiaková S, Visnovský P, et al, (1992), Salicylate intoxication after use of topical salicylic acid ointment by a patient with psoriasis, Cutis. 50 (4): 307–309.
3. Goldberg DR, (2009), Aspirin: Turn of the Century Miracle Drug, Chemical Heritage Magazine. 27 (2): 26–30.
4. Scully FE, Hoigné J, (1987), Rate constants for reactions of singlet oxygen with phenols and other compounds in water, Chemosphere. 16 (4): 681–694.
5. Shulaev V, Silverman P, Raskin I, (1997), Airborne signalling by methyl salicylate in plant pathogen resistance, Nature 385, 718–721.
6. Hoffman R, (2015), Goldfrank's Toxicologic Emergencies, 10th edition, New York, 915–922.
7. Toxicologic and Dermatologic Assessments for Three Groups of Fragrance Ingredients, (2007), Food and Chemical Toxicology. 45
8. https://pubchem.ncbi.nlm.nih.gov/compound/Methyl-2-_benzyloxy_-5-_2-bromoacetyl_benzoate.
9. EMEA, (2010), Safety Working Group, Questions and Answers on the Guideline on the Limits of Genotoxic Impurities, EMA, (2008 &2009) ,21. EMA/CHMP/ SWP/431994/2007.
10. ICH M7, (2010): Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk, Business Plan.
11. Gold, LS., Sawyer, CB., Magaw, R., Backman, GM., de Veciana, M., Levinson, R., (1984), A carcinogenic potency database of the standardized results of animal bioassays, Environ Health Perspect, 58, 9-319.
12. Dobo, KL., Greene, N., Cyr, MO., Caron, S., Ku, WW., (2006), The application of structure-based assessment to support safety and chemistry diligence to manage genotoxic impurities in active pharmaceutical ingredients during drug development, RegTox Pharm., 44, 282-293.
13. Cheeseman, MA.,Machuga, EJ., Bailey AB., (1999), A tiered approach to threshold of regulation, Food ChemToxicol. 37, 387-412.
14. Genotoxicity, (2011 & 2013): Validated Non-animal Alternatives.
15. ICH Q2 (R1) (2005), Validation of Analytical Procedures: Definitions and Methodology.
16. US Food and Drug Administration (FDA), Food additives, (1995): Threshold of regulation for substances used in food-contact articles (final rule), Fed, Regist, 60, 36582-36596.
17. Narayana, MBV, Chandrasekhar, KB., Rao, BM., (2012), Quantification of Genotoxic Impurity 4-Chloro-1-Hydroxy Butane Sulfonic Acid Sodium Salt by LCMS/ MS in Sumatriptan Succinate. J Bioanal Biomed, 4:104-107. doi:10.4172/1948-593X.1000072
18. Kroes, R., Renwick, AG., Cheeseman, M., Kleiner, J., Mangelsdorf, I., Piersma, A., (2004), Structure-based threshold of toxicological concern (TTC): guidance for application to substances present at low levels in the diet, Food ChemToxicol, 42, 65-83.
19. Zate PB, Kothari S, Lokhande MV. Confirmation and Quantification of Genotoxic Impurity 2-Dimethylaminoethyl chloride hydrochloride (DMC HCl) by GCMS in Chlorpheniramine/ Chlorphenamine Maleate. J Applied Chem.2017; 10(7): 21-26
20. Müller, L., Mauthe, RJ., Riley, CM., Andino, MM., De Antonis, D., Beels, C., (2006), A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity, RegTox Pharm, 44, 198-211.
21. N.G. Rathod, M.V. Lokhande, Development and Characterisation of process related impurity in Hydralazine Hydrochloride by some analytical technique, J Applicable Chem.2014; 3 (5): 2011-2019.
22. Delaney EJ (2007), An impact analysis of the application of the threshold of toxicological concern concept to pharmaceuticals. Regul Toxicol Pharmacol 49: 107-124.
23. ICH Q3A and Q3B (R2), (2006), Impurities in New Drug Substances
24. Zate PB, Kothari S, Lokhande MV., Determination and Quantification of Carryover Genotoxic Impurities 2-Chloropyridine (2CP) and 4-Bromobenzyl Cyanide (PBBCN) by GCHS in Brompheniramine Maleate API, Ijppr Human, 2017;Vol. 10 (2): 13-24.