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Quantification of Genotoxic Alkylating Impurity Butyl3-Methyl-3-(6-methoxy-2-naphtyl) Glycidate at ppm Level by LCMS/MS in Naproxen Drug Substance

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Abstract: ***The objective of present research work is to develop a suitable LCMS/MS method for the quantitative determination of suspected genotoxic impurity namely Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate present in Naproxen drug substance at ppm level. The LCMS/MS method was developed on Luna C18, 50 mm column using the mobile phase consists a mixture of 10 mM Ammoinium acetate and Acetonirile with isocratic composition of 10:90 at a flow rate of 0.8 mL/min respectively. Ion source is electospray ionization (ESI) positive mode, source temperature is 350°C, gas flow is 10 L/min, Nebuliser pressure is 50 psi, and capillary voltage is 4000 V. Under these LC and MS conditions Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate was quantified by selecting most stable MRM pair m/z of 315/213. The limit of detection and the limit of quantitation for the impurity were established. This method has been tested in a number of Naproxen samples and used successfully for quantification of the impurity at ppm level. Validation of the developed LCMS/MS method was carried out as per ICH requirements and the data shows that the proposed method is specific, linear, accurate, precise and robust. The developed LCMS/MS method was found to be suitable to quantify the genotoxic impurity at ppm level present in Naproxen drug substance.***

Keywords: Genotoxic impurity, Alkylating agent, Liquid chromatography mass spectrometer, Electro spray ionization

1. Introduction

The impurity present in Naproxen drug substance is Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate is a genotoxic alkylating agent. Alkylating agents has been defined and a detailed discussion made of mechanisms (Sn1 and Sn2) by which they interact with nucleophilic centers [1]. Alkylating agents are used in cancer for the treatment that attaches an alkyl group (C_nH2_{n+1}) to DNA [2]. Alkylating agents are used to treat several cancers, however they are toxic to normal cells (cytotoxic) leading to damage bone marrow testicals and ovaries and most of the alkylating agents are also carcinogic [3-5]. The most important is alkylation of DNA within the nucleus which leads to cell death. Alkylating agents alkylate within DNA at the N7 position of guanine which is the major cite [6] resulting in miscoding through abnormal base pairing with thymine leading to strong breakage [7]. Alkylating agents are two types elctrophilic and nucleophilic depends on the character and the two impurities present in Naproxen are nucleophilic alkylating agents.

Naproxen is a nonsteroidal anti-inflammatory drugs (NSAIDs). It works by reducing hormones that cause inflammation and pain in the body. Naproxen is used to treat pain or inflammation caused by conditions such as arthritis, ankylosing spondylitis, tendinitis, bursitis, gout, or menstrual cramps [8]. Naproxen generally sold as brand names like Anaprox , Naprelan, Naprosyn, Aleve.

During the synthesis of Naproxen drug substance Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate formed as an intermediate which is known to be alkylating agent. It was found to be genotoxic/Carcinogic [3-5] hence should be controlled at ppm level in the Naproxen drug substance. The toxicological assessment of this genotoxic impurity 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 ICH Q3X guidelines [9]. The presence of trace level of the impurity in drug substance or drug product is of genotoxicity concern and has been closely scrutinized by regulatory agencies and pharmaceutical industries [10].

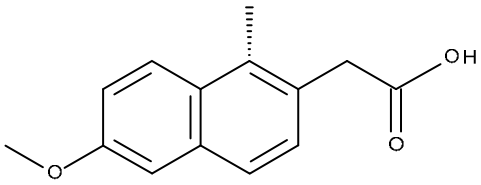
The 'threshold of toxicological concern' (TTC) of 1.5 µg/person/day (exposure of genotoxic impurity in drugs that will be tested or dosed for longer than 12 months) has been suggested by the European Medicines Agency's (EMEA) "Guideline on the limits of genotoxic impurities" [11-14] and the PhRMA's white paper [15-16]. 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 µg /day)] / [dose (g/day)]. 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. 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 [17-19].

Therefore it is required for the potential genotoxins to be controlled during the synthesis; where the levels cannot be controlled and no safety data yet exists it may be preferable for the pharmaceutical company to change the route of synthesis of the drug substances[20]. By considering the daily intake of Naproxen as 1.5 g /day the regulatory team decided the limit for the genotoxic impurity is 1 ppm. Quantification at such low level is possible by using triple quad LCMS hence in the present research work a high sensitive LCMS/MS was developed for quantification of genotoxic impurity at 1 ppm level present in Naproxen.

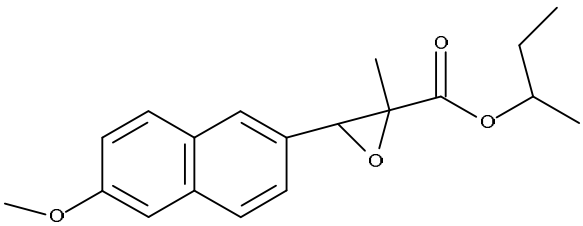
2. Experimental

2.1. Chemicals and reagents

Samples of Naproxen, 2-butyl p-toluene sulfonate and Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate (Fig. 1) were received from Bulk Actives, Unit-II of Symax Laboratories, Hyderabad, India. HPLC grade Acetonitrile was purchased from J T Baker, Mumbai, India. Ammoinium acetate was purchased from Sigma Aldrich, Mumbai, India. High pure water was prepared by using Millipore Milli Q plus purification system (Millipore, USA).



Naproxen: 2-(6-methoxy-1-methyl-2-naphthyl)acetic Acid
Figure 1: Chemical structure and chemical names of Naproxen.



Impurity: Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate
Figure 2: Chemical structures and chemical names of Naproxen and its genotoxic impurity.

2.2 Equipment

The LCMS method development and validation was carried out using Agilent 1200 series HPLC system Connected with Agilent mass spectrometer LCMS/MS-QqQ system (Agilent technologies, Germany) equipped with Electro spray ionization probe. The data were collected using Agilent mass haunter work station software.

2.3 LCMS Chromatographic Conditions

Luna C18 column 50 mm length X 2 mm ID with 3 µm particle size using the isocratic mobile phase of mixture of 10 mM ammonium acetate and Acetonirile of 10:90(v/v) at a flow rate of 1.0 mL/min. Mass spectrometer (MS) was operated in electospray ionization (ESI) positive ion mode with a capillary volatage of 4000V. The fragmentor was set at 70 V, the drying gas gas flow was 10 L/min with a temperature of 350°C and nebulizer pressure was 50 psi. Impurity was quantified by selecting most stable MRM pair 315/213.The test concentration was about 2.5 mg mL^{-1} and the injection volume was 10 µL. Water and Acetonitrile (50:50) was used as diluent during the standards and test samples preparations

2.4. Preparation of impurity Standard and test sample Solution

The stock solutions of impurity standard are prepared at approximately 1 mg mL^{-1} in pure diluent. For linearity, the stock solution impurity was diluted using diluent to give standards at 0.2, 0.4, 0.7, 1.0, 1.2, 1.5 ppm with respect to test concentration. The testing Naproxen samples were typically prepared at 2.5 mg/mL in respective diluent and sonicated about 10 minutes and filtered through 0.45µ poly tetrafluoroethylene (PTFE) filter.

3. Results and Discussion

3.1 Optimization of Chromatographic Conditions

The main target of LC-MS/MS method was to quantify the Impurity present in the Naproxen drug substance. As volatile buffers required for analysis in LCMS the mobile phase was restricted to volatile buffers like Formic acid, trifluoro acetic acid, ammonium acetate. Different trails were made by using these mobile phases and C18 column; the results are showing that ammoinium acetate mobile phase shows good sensitivity and separation impurity from Naproxen. Various proportions of Acetonitrile and Ammonium acetate and Different concentrations of Ammonium acetate tried the impurities shows good sensitivity at 10mM of ammonium acetate. Using these mobile phases, different columns C8 and C18 trails were made; C18 column shows good peak shape for the impurity. Ammonium acetate is showing most suitable buffer to get more sensitivity. The optimized conditions impurity Luna C18 column 50 mm length X 2 mm ID with 3µm particle size using the isocratic mobile phase of mixture of 10 mM ammonium acetate and Acetonirile of 10:90(v/v), diluent is water:acetonitrile (50:50) with a run time of 2 minute.

3.2 Optimization of MS/MS parameters

By using the developed LC conditions the two impurities were injected in Elctrospray ionization (ESI) and Atmospheric pressure chemical ionization (APCI) of triple quad mass spectrometer. The data reveals that, in ESI positive mode the two impurities are showing good ionization compared to APCI. The ion for the impurity shows a m/z 315$[M+H]^+$ in ESI . The ion fragmented using collision energy; it shows a stable fragment of 213 hence impurity was quantified by multi reaction monitoring (MRM) of 315/213. Mass parameters optimized using MRM pair of 315/213 is fragmentor voltage 70 V, the drying gas flow was 10 L/min with a drying temperature of 350°C and nebulizer pressure was 50 psi.

4. Method Validation

4.1. Linearity

The linearity of an analytical test procedure is its ability to obtain test results (within a given range), which is directly proportional to the concentration of the analyte in the sample [21]. A series of solutions were prepared separately using impurity at a concentration levels from around detection level to 150% and the concentration levels are 0.2, 0.4, 0.7 1.0, 1.2, 1.5 ppm respectively. The peak area versus concentration data was done by linearity plot slop, intercept, and residual sum of squares analysis. The calibration curve was given based on response over the concentration range for the impurity. The correlation coefficient for impurity was 0.997 and the Linearity results are tabulated in Table 1.

Table 1: Results of Linearity

Slope and Intercept Equation	*Correlation coefficient*	*Range (ppm)*	*LOD (ppm)*	*LOQ (ppm)*
y = 1.6326x+3.345	0.9997	0.2-1.5	0.06	0.2

4.2 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [21]. The LOD and LOQ values are predicted from the linearity data. Each predicted concentration was verified for precision by preparing the solutions at about predicted concentration and injecting each solution six times for LC-MS/MS study and the concentration of LOQ was 0.2 ppm and LOD was 0.06 ppm for impurity (Figure 3&4) respectively.

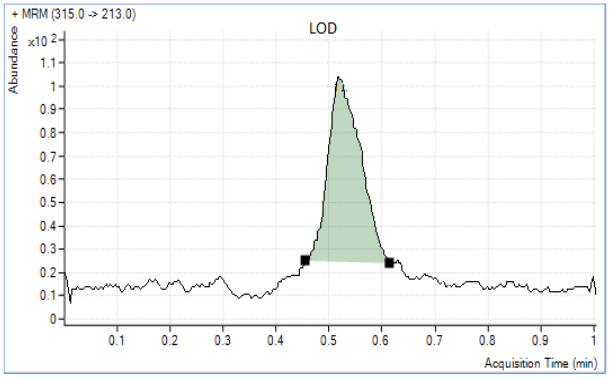


Figure 3: Typical Mass spectrogram of LOD Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate

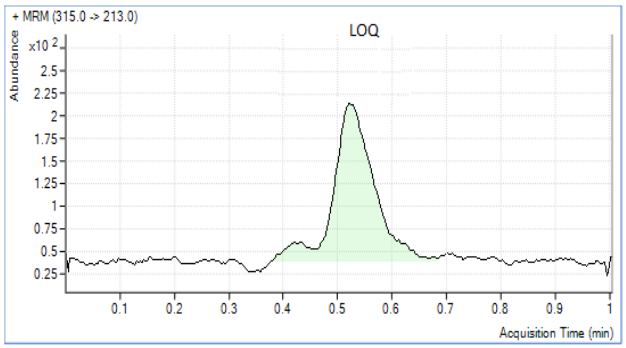


Figure 3: Typical Mass spectrograms of LOQ of Sec. 4utyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate

4.3 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the homogeneous sample under prescribed conditions [21]. The precision of the method was checked by preparing test solutions by spiking the impurities at LOQ, 50%, 75%, 100%, 125% and 150% level with the drug substance for six times and injected each once also injected 100% spiked solution for 6 times to show the system precision. The % RSD of the areas are within 1.2% and 3.8% confirming the good precision of the developed method.

Table 2: Results LOQ Precision

%RSD			
LOQ level	*Limit Level*	*Analyst-1*	*Analyst-2*
3.5	1.8	1.2	2.7

4.4 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the expected value found [21]. The accuracy of the method was evaluated in sample solutions were prepared in triplicate by spiking impurity at LOQ level, 50%, 75%, 100%, 125% and 150% with Naproxen drug substance and injected each solution in to LCMS as per methodology. The percentage of recovery was calculated and the values are within 93.9% to 104.4% for impurity. At ppm levels these recoveries were satisfactory and the results are tabulated in Table 3.

Table 3: Results of Accuracy Study

% Recovery					
LOQ Level	*50% Level*	*75% Level*	*100% Level*	*125% Level*	*150% Level*
94.5%	98.2%	104.4%	102.2%	93.9%	102.3%

4.5 Robustness

To evaluate the robustness of the developed LCMS method, the LC conditions like flow rate, ratio of the composition of the mobile phase, and mass parameters like gas flow, drying gas temperature, collision energy were slightly altered and Injected 100% impurity spiked solution of naproxen. In all the varied condition of LC and MS the method shows required sensitivity to quantify the impurity. The method was repeated in different days with different columns,

analysts and tested the number of quality control batches of Naproxen samples and the results revealed that robustness of the method.

5. Conclusion

In this paper a sensitive specific, accurate, validated and well-defined LCMS/MS method for the Quantification of genotoxic alkylating agent Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate at 1 ppm level in Naproxen drug substance was described and the limit of quantification found to be 0.2 ppm. The developed method is highly specific and sensitive reliable technique for the quantification of the gentoxic impuritiy Sec. Butyl 3-methyl-3-(6-methoxy-2-naphtyl) glycidate in the Naproxen drug substance. The method is well suited for the quantitation of the alkylating genotoxic impuritiy present in Naproxen during quality control testing.

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References

[1] G. P. Warwick, The Mechanism of Action of Alkylating Agents, *Cancer Res* 1963; 23: 1315-1333.
[2] http://faculty.swosG. P. Warwick, The Mechanism of Action of Alkylating Agents, *Cancer Res* 1963; 23: 1315-1333.
[3] Wiedemann GJ, Robins HI, Gutsche S, Mentzel M, Deeken M, Katschinski DM,et al. "Ifosfamide, carboplatin and etoposide (ICE) combined with 41.8 degrees C whole body hyperthermia in patients with refractory sarcoma". *European Journal of Cancer(2006)* 32A (5): 888–92.
[4] Doll, R. & Peto, R. J. (1981) J. Natl. Cancer Inst. 66, 1192-1308.
[5] Bartsch, H. & Montesano, R. (1984) Carcinogenesis 5,1381-1393
[6] Charles C. Price, G. M. Gaucher, P. Koneru, R. Shibakawa, J. R. Sowa, M. Yamaguchi, Article first published online: 16 DEC 2006 DOI: 10.1111/j.1749-6632.1969.tb24877.x
[7] Kondo N, Takahashi A, Ono K, Ohnishi T. DNA damage induced by alkylating agents and repair pathways J Nucleic Acids(2010):543531.
[8] Todd PA, Clissold SP.,Naproxen. A reappraisal of its pharmacology, and therapeutic use in rheumatic diseases and pain states, Drugs (1990).40(1):91-137
[9] ICH, Q3A (R2) Guideline on Impurities in New Drug Substances (2006). IFPMA, Geneva
[10] Lutz Muller, Robert J. Mauthe, Christopher M. Riley, Marta M. Andino, David De Antonisd, Chris Beels et al, A Rationale for Determining, Testing and Controlling Specific Impurities in Pharmaceuticals that Possess Potential for Genotoxicity. Regulatory Toxicology and Pharmacology (2006); 44:198- 211.
[11] Guidelines on the Limits of Genotoxic Impurities. Committee for Medicinal Products for Human Use [CHMP]. European Medicines Agency [EMEA] 2006:28.(CPMP/SWP/5199/02,EMEA/CHMP/QWP/ 251344/2006).
[12] Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, et al. The Threshold of Toxicological Concern Concept in Risk Assessment. Food Chem Toxicol (2004) ;42:65–83.
[13] Timothy McGovern, David Jacobson-Krma, Regulation of genotoxic and carcinogenic impurities in drug saubstances and drug products, TrAC Trends in Analytical Chemistry (2006). 25(8):790-795.
[14] European Medicines Evaluation Agency, Committee for Medicinal Products for Human Use (CHMP), Guideline on the limits of genotoxic impurities (2004), CPMP/SWP/5199/02, London, UK.
[15] PhRMA GTI Task Force in 2005- Muller L et al, A Rationale for Determining, Testing and Controlling Specific Impurities in Pharmaceuticals that Possess Potential for Genotoxicity. Regulatory Toxicology and Pharmacology (2006); 44, 198- 211.
[16] E.J.Delaney,An impact analysis of the application of the threshold of toxicological concern concept to pharmaceuticals. Regulatory Toxicology and Pharmacology (2007); 49(2):107-124.
[17] K.L. Dobo, N. Greene, M.O. Cyr, S. Caron, W.W. Ku, The application of structure-based assessment to support safety and chemistry diligence to manage genotoxic impurities in active pharmaceutical ingredients during drug development, Regulatory Toxicology and Pharmacology (2006); 44:282-293.
[18] Colon I, Richoll SM. Determination of methyl and ethyl esters of methanesulfonic, benzenesulfonic and *p*-toluenesulfonic acids in active pharmaceutical ingredients by solid-phase microextraction (SPME) coupled to GC/SIM-MS. Journal of Pharmaceutical and Biomedical Analysis (2005); 39:477–85 .
[19] Development and validation of a sensitive GC-MS method for the determination of trace levels of an alkylating reagent in a beta-lactam active pharmaceutical ingredient.J Pharm Biomed Anal (2005):39(3-4):486-94
[20] D.P. Elder, A.M. Lipczynski, A. Teasdale, Control and analysis of alkyl and benzyl halides and other related reactive organohalides as potential genotoxic impurities in active pharmaceutical ingredients (APIs), Journal of Pharmaceutical and Biomedical Analysis 48 (2008) 497–507
[21] International Conference on Harmonization (ICH) guideline, Text on validation of analytical procedures, Q2A (R1), 1996. IFPMA, Geneva.

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