

Determination of the impurities in drug products containing montelukast and in silico/in vitro genotoxicological assessments of sulfoxide impurity



Esra Emerce^{a,*}, Ismet Cok^a, I.Tuncer Degim^b

^a Gazi University, Pharmacy Faculty, Toxicology Department, Ankara, Turkey

^b Gazi University, Pharmacy Faculty, Pharmaceutical Technology Department, Ankara, Turkey

HIGHLIGHTS

- Several impurities in commercial drugs containing montelukast were quantified.
- Sulfoxide impurity was higher than qualification threshold in some drugs.
- Qualification for sulfoxide impurity was done using in silico/in vitro tests.
- Sulfoxide impurity was dose-dependent cytotoxic in human peripheral lymphocytes.
- Sulfoxide impurity was concluded to be classified as a nonmutagenic impurity.

ARTICLE INFO

Article history:

Received 20 May 2015

Received in revised form 1 July 2015

Accepted 2 July 2015

Available online 20 July 2015

Keywords:

Genotoxic impurities

(Q)SAR analysis

Ames test

Chromosomal aberration test

Hplc

Regulations

ABSTRACT

Impurities affecting safety, efficacy, and quality of pharmaceuticals are of increasing concern for regulatory agencies and pharmaceutical industries, since genotoxic impurities are understood to play important role in carcinogenesis. The study aimed to analyse impurities of montelukast chronically used in asthma therapy and perform genotoxicological assessment considering regulatory approaches.

Impurities (sulfoxide, cis-isomer, Michael adducts-I&II, methylketone, methylstyrene) were quantified using RP-HPLC analysis on commercial products available in Turkish market. For sulfoxide impurity, having no toxicity data and found to be above the qualification limit, in silico mutagenicity prediction analysis, miniaturized bacterial gene mutation test, mitotic index determination and in vitro chromosomal aberration test w/o metabolic activation system were conducted.

In the analysis of different batches of 20 commercial drug products from 11 companies, only sulfoxide impurity exceeded qualification limit in pediatric tablets from 2 companies and in adult tablets from 7 companies. Leadscape and ToxTree programs predicted sulfoxide impurity as nonmutagenic. It was also found to be nonmutagenic in Ames MPF Penta I assay. Sulfoxide impurity was dose-dependent cytotoxic in human peripheral lymphocytes, however, it was found to be nongenotoxic. It was concluded that sulfoxide impurity should be considered as nonmutagenic and can be classified as ordinary impurity according to guidelines.

© 2015 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

An impurity is any component of drug substance or product that is not the chemical entity defined as drug substance or as an excipient of the drug product (ICH, 2006a,b). The presence of impurities in drugs may influence the efficacy and safety of

pharmaceutical products even in very small amounts and aside from no benefits; they only convey health risks (Pilaniya et al., 2010). Genotoxic impurities which can induce genetic mutations, chromosomal breaks and/or chromosomal rearrangements have a special place within the context due to the fact that they bear the potential to cause cancer in humans who are treated with pharmaceuticals that contain these impurities (Raman et al., 2011). It is estimated that in the intermediary steps within pharmaceutical development process drug product may have 20–25% potentially genotoxic intermediates (Delaney, 2007).

* Corresponding author.

E-mail address: esraemerce@gmail.com (E. Emerce).

In recent years, a number of reviews were published on impurities (Muller et al., 2006; Delaney, 2007; Giordani et al., 2011; Dobo et al., 2006) and several regulations related to impurities in drug applications have been introduced by international regulatory authorities such as EMA (European Medicines Agency), FDA (Food and Drug Administration) and ICH (International Conference on Harmonization) (EMA, 2006; FDA-CDER Guideline, 2008a; ICH, 2006a,b; ICH, 2011). Although the efforts focus on eliminating impurities from drug substance or drug products, technically it is often not possible to remove all impurities and the elimination processes also increase the costs. Therefore, in controlling impurities, an acceptable risk level concept or threshold of toxicological concern (TTC) approach is recommended (Bercu et al., 2008; EMA, 2006). Studies and debates on impurities are continued by regulatory bodies, specialists, stakeholders and likewise regulatory approaches are dynamically furthered as well. Although authorities concentrate on guidance on new drug substances and drug products during their clinical development and subsequent applications for marketing, they recommend relevant studies to be carried out in some circumstances on new marketing applications and post approval submissions for marketed products (ICH, 2014).

The montelukast from leukotrien antagonists is commonly used for controlling asthma that is one of the most common chronic respiratory disorders in people of all ages in all parts of the world (The Global Asthma Network's Report, 2014; U.S. NIH, Asthma Report, 2007) and its six organic impurities were mentioned in American and European Pharmacopeia in 2010. Since asthma typically begins much earlier in life than other chronic diseases and lasts quite a long time, use of medication in asthma requires more attention. Furthermore, probable chronic impurity exposures should be scrutinized thoroughly to avoid unnecessary exposures, particularly in children.

This study aimed to quantify the specified impurities in drug products containing montelukast as an active substance collected from pharmacies in Turkey and to assess mutagenic/genotoxic activity of the impurities which exceed the qualification threshold according to guidelines. In this context, the following objectives were set to be achieved; (a) to quantify six impurities which have been specified in USP (American Pharmacopeia) in montelukast containing drug products available in the Turkish market for adults and pediatric use, (b) to assess the impurities of each drug product according to guidelines on impurities in terms of reporting, identification and qualification thresholds, (c) to assess genotoxicological potential of the impurity which exceed the qualification threshold, respectively by performing *in silico* structure activity relationship analysis, bacterial reverse mutation test (Ames test), mitotic index determination and *in vitro* chromosomal aberration test, and (d) to classify these impurities.

2. Material and methods

2.1. The analysis of impurities in drug products using RP-HPLC

Quantitation of 6 impurities (sulfoxide, cis isomer, michael adducts I&II, methylketone, methylstyrene impurities) already indicated in USP was conducted using RP-HPLC analysis in two batches of 20 commercial drugs containing montelukast in Turkish market (4 mg for children; 10 mg for adults) from 11 pharmaceutical companies. Instead of providing generic names, drugs were coded with letters in lieu of the relevant pharmaceutical companies, and doses and drug forms of these drugs were also specified.

2.1.1. HPLC analysis

An analytical method for montelukast impurities was developed by modification of available methods in the literature (Saravanan et al., 2008; Sivri, 2009; Goverdhana et al., 2009) and it was validated according to ICH guideline (Table 1) (ICH, 2005). Montelukast sodium (CAS#:151767-02-1), sulfoxide impurity (CAS#:909849-96-3), cis isomer impurity (CAS#:774538-96-4), methylketone impurity (CAS#:937275-23-5), methylstyrene impurity (CAS#:918972-54-0), michael adducts I&II impurity (CAS#:1187586-61-3, 1187586-58-8) standards were supplied by Molcan Corp., Canada (Fig. 1). Acetonitrile, methanol, orthophosphoric acid, sodium dihydrogen phosphate, and water were purchased from Merck. 5-Methyl 2-nitrophenol (Sigma) was used as an internal standard. Michael adducts I & II impurities' standard was provided as a mixture of diastereomers. These impurities were not resolved entirely by the method; however analyses were done by considering total peak areas of two isomers as it has been referred in the USP.

Analyses were performed on a Shimadzu Prominence LC-20A HPLC system which consists of degasser (DGU-20A3), pump (LC-20AD), photodiode array detector (SPD-M20A), and the column oven (CTO-10AS). Chromatographic analyses were performed on a C18 analytical column (150 mm × 4.6 mm, 5 μm, Kromasil). The HPLC system was controlled using LC solutions, ver. 1.25 PC software (Shimadzu Corporation, Kyoto, Japan). The column temperature was maintained at 27 °C and the PDA detector was set at wavelength of 225 nm. The injection volume was 20 μl. Mobile phases consisted of solution A: 12.5 mM sodium dihydrogen phosphate (buffer pH 3.7 adjusted with diluted orthophosphoric acid) and acetonitrile in the ratio 63:37 (v/v), and solution B: a mixture of acetonitrile and water in the ratio 90:10 (v/v) at a flow rate of 1.1 ml/min. The gradient elution program was as follows: 50% solution A for 2 min, 13 min a linear decrease from 50% to 10% solution A, for 8 min 10% solution A maintained, then followed by a linear increase from 10% to 50% solution A in 3 min.

Preparation of montelukast samples from drug products: 10 tablets/chewing tablets/granules were weighed and grinded for

Table 1
Validation results for the RP-HPLC method.

	Sulfoxide	Michael adducts I&II	Cis isomer	Methylketone	Methylstyrene	Montelukast
Specificity (relative retention time, min.)	0.31	0.59/0.61	0.80	1.06	1.84	1
Linearity	$y = 0.881x - 0.047$ $R = 0.9996$, $r^2 = 0.9991$	$y = 0.790x + 0.036$ $R = 0.9995$, $r^2 = 0.9990$	$y = 1.427x - 0.018$ $R = 0.9993$, $r^2 = 0.9987$	$y = 1.155x - 0.065$ $R = 0.9990$, $r^2 = 0.9980$	$y = 0.676x - 0.024$ $R = 0.9990$, $r^2 = 0.9980$	$y = 1.315x + 0.195$ $R = 0.9995$, $r^2 = 0.9990$
Accuracy and recovery (mean recovery%, RSD%)	99.68, 2.40	101.31, 0.33	106.23, 2.69	106.56, 1.33	101.27, 2.82	106.93, 2.59
Intra-day precision (RSD%)	2.36	3.46	3.92	4.00	3.52	1.77
Inter-day precision (n = 3 day; RSD%)	0.74 – 2.23	0.37 – 3.10	0.72 – 1.00	0.55 – 2.50	1.72 – 3.41	0.20 – 1.40
Limit of detection (μg/ml)	0.04	0.08	0.06	0.09	0.07	0.03
Limit of quantification (μg/ml)	0.13	0.23	0.18	0.26	0.21	0.09

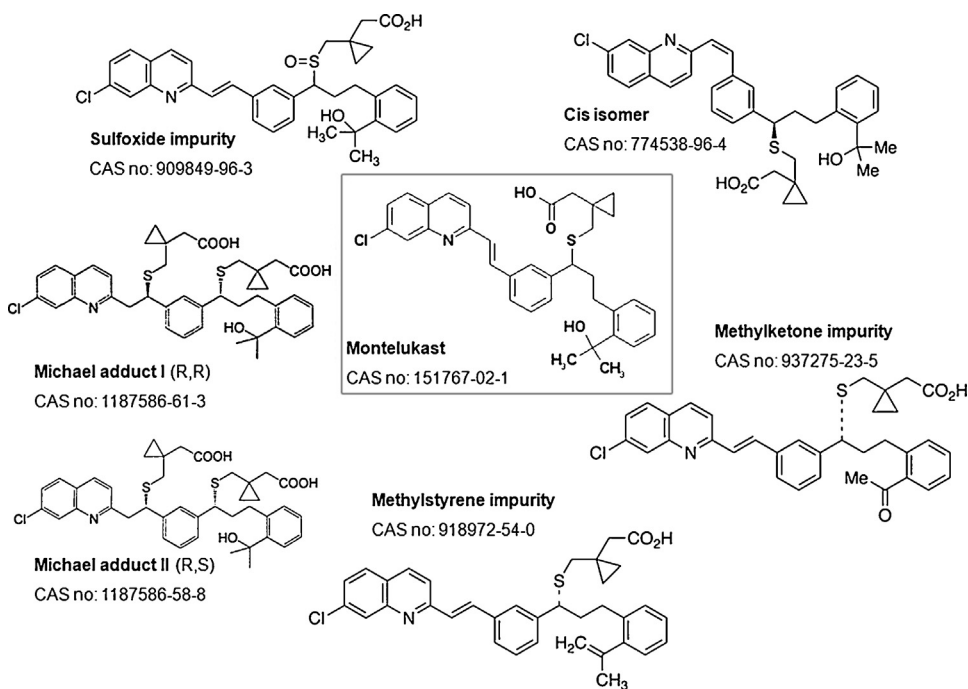


Fig. 1. Montelukast and its impurities.

each product. Accurately weighed samples equivalent to 4 mg or 10 mg of montelukast (0.5 mg/ml) were dissolved in methanol using an ultrasonic bath and kept at 4 °C. The internal standard was added to each sample (final concentration 25 µg/ml) for analysis. Each sample was analysed in triplicate (Fig. 2).

2.2. In silico prediction of mutagenicity

Leadscope Model Applier Suites, Genetic Toxicity, The gene mutation-microbial in vitro models - Salmonella Mut (v3) and *E. Coli* - Sal 102 A-T Mut (v1.), Model Applier Version: 1.7.3 (Leadscope, Inc.) was used for the prediction of mutagenic activity of the sulfoxide impurity as a statistical based system. The Genetic Toxicity model was developed through a Research Collaboration Agreement with the U.S. Food and Drug Administration. Binary

model shows 'prediction' information and a 'positive prediction probability' calculation. To predict mutagenicity for sulfoxide impurity, Toxtree® (v2.6.6) knowledge-based system was used as well. Toxtree® (toxic hazard estimation by decision tree approach) was developed by Ideacconsult Ltd. (Sofia, Bulgaria) under the terms of a contract with the European Commission Joint Research Centre. For the prediction of mutagenicity of impurities, ToxTree® programme conducts the Benigni/Bossa rulebase.

2.3. Bacterial reverse mutation test

Ames MPF™ Penta I kit (Microplate Format Mutagenicity Assay; Xenometrix, Switzerland) was used for mutagenicity assessment of the sulfoxide impurity. This kit enables studying 5 tester strains that carry different target sites for mutation,

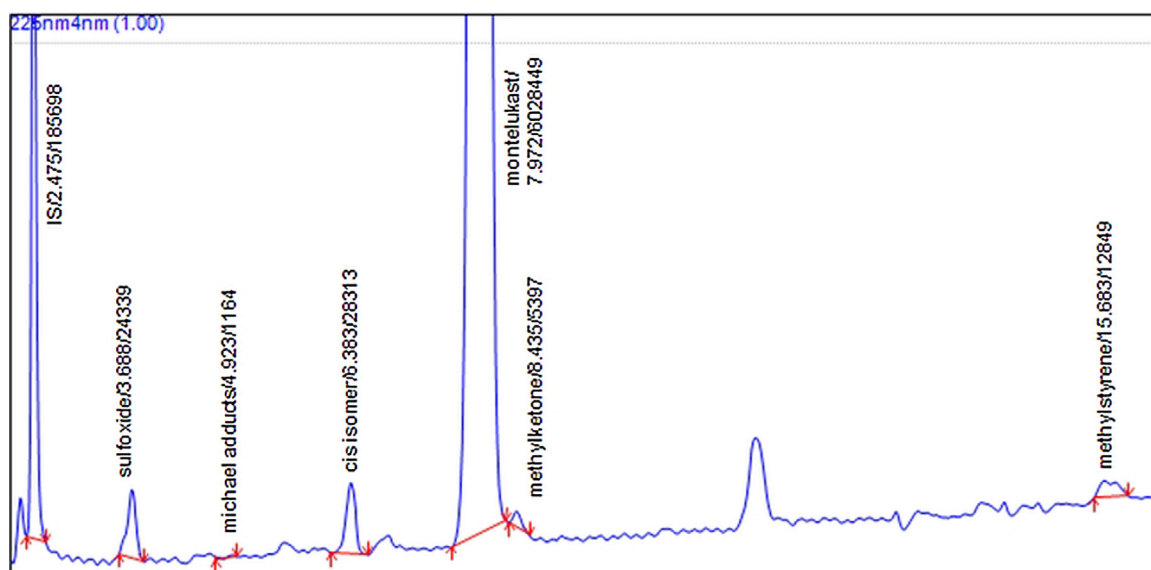


Fig. 2. Chromatogram sample of analysis of montelukast with the impurities in matrix of drug products (G chew. tab. 4 mg).

namely *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* wp2 [pKM101]+*E. coli* wp2 uvrA (EC Combo). The sulfoxide impurity was dissolved in DMSO. S9 mix was prepared by liver S9 fraction (30% v/v, Aroclor 1254-induced, rat, Xenometrix), MgCl₂ (0.25 M), KCl (1 M), NaH₂PO₄ buffer (0.2 M), glucose-6-phosphate (0.2 M), NADP (0.04 M).

Before the main test, pre-screening test was conducted to determine the optimal dose range of the impurity. As mentioned in the kit procedure, TA98 strain was treated with 7 concentrations (5.2–5000 µg/ml) of the impurity and was incubated 90 min in exposure medium. After incubation period, the turbidity of each well was compared visually and was also measured at 600 nm in a plate reader. In this test, cytotoxicity was not observed up to 5000 µg/ml dose.

In the main test, briefly, approximately 10⁷ bacteria were exposed to 6 concentrations (15.8–5000 µg/ml) of the sulfoxide impurity, as well as a strain specific positive and a negative control with/without metabolic activation system in triplicate, for 90 min in exposure medium containing trace amounts of the required histidine (*S. typhimurium*) or tryptophan (*E. coli*). After preincubation, the exposure cultures were diluted in pH indicator medium which lacked the required histidine or tryptophan and aliquoted into 48 wells of a 384-well plate. Within 2-days incubation, revertant bacteria grew and medium color of that well changed because of bacterial metabolism. The number of wells containing revertant colonies were counted for each dose and compared with the number of spontaneous revertants obtained with the negative control. The fold increases above the baseline (mean of negative control plus 1 SD) were determined for each concentration of the impurity. A test chemical showing a clear dose response and/or yielding multiple fold inductions greater than 2.0, was classified as a mutagen.

2.4. Mitotic index determination and in vitro chromosomal aberration test

The in vitro mammalian chromosome aberration test was performed according to the OECD test guideline No. 473, with and without metabolic activation (S9-mix). Before starting the study, approval of the Clinical Research Ethics Committee of Gazi University was obtained (date and the number of the committee approval: 13.04.2011/90). Peripheral blood lymphocytes were collected from two healthy donors (one female, one male, non smokers, 28 year

olds). Sulfoxide impurity was dissolved in methanol. Methanol was used as a negative control; mitomycin C (MMC; 0.2 µg/ml for 24 h exposure period, 0.6 µg/ml for 3 h exposure period) and cyclophosphamide (CP; 15 µg/ml) were used as positive controls for assays without metabolic activation system and with metabolic activation system, respectively. S9 mix was prepared by liver S9 fraction (10% v/v, pooled Sprague Dawley rat liver S9, BD Bioscience), KCl (1 M), MgCl₂·6H₂O (0.25 M), NaH₂PO₄ buffer (0.2 M; pH 7.4), glucose-6-phosphate (0.2 M), NADP (0.04 M).

Whole blood of 0.2 ml was added to 2.5 ml Chromosome Medium-B (Biochrom, F5023). For the cytotoxicity assessment, 6 different concentrations (15.7–500 µg/ml) of sulfoxide impurity were added for 3 h (S9-/+) and 24 h (S9-) exposure duration after 48 h incubation. Then cultures which were treated for 3 h were washed twice at the end of the treatment period and reincubated with fresh medium for a further 21 h. All cultures were incubated in duplicate at 37 °C for 72 h in 2.7 ml total culture volume. 0.2 µg/ml colcemid was added to all cultures 3 h before the end of the incubation period. Cells were treated with hypotonic KCl (0.075 M) for 20 min at 37 °C and then fixed, stained with 4% Giemsa's solution and scored according to standard procedure. The cytotoxicity assessment was investigated by determining the mitotic index of each culture via analyzing number of metaphases per 1000 cells under the light microscope with 40× magnification. Based on the result of the mitotic index assessments, three analysable concentrations which have 50% or higher level of mitotic index in comparison to the mitotic index of the solvent control were selected for chromosomal aberration test. Test concentrations of sulfoxide impurity were 15.7, 31.3, 62.5 µg/ml for 3 h and 20, 30, 40 µg/ml for 24 h. In vitro chromosomal aberration test was performed by the same procedure described above. Chromosomal aberrations were counted blindly in 200 metaphase cells (2n=46±2) per concentration by the same researcher under the light microscope with 100× magnification. The structural CAs (chromatid/chromosome gaps, breaks and exchanges) and numerical CAs (polyploidy, endoreduplication) were evaluated in metaphase cells. Concurrently, mitotic index was also determined in chromosomal aberration test.

2.5. Statistical analysis

The statistical analysis was performed using GraphPad Prism software (version 5.00; demo; GraphPad Software Inc, California,

Table 2

The amounts of the impurities in paediatric drug products containing montelukast (µg; mean ± SD; n = 3).

Drugs (4 mg)	Sulfoxide	Michael Adducts I and II	Cis isomer ^c	Methylketone	Methylstyrene
A Chew. Tab.	34.53 ± 1.07	5.06 ± 2.86	36.43	14.82 ± 3.62	23.74 ± 2.66
	24.94 ± 0.78	<LOD	34.82	17.17 ± 1.70	18.76 ± 2.44
B Chew. Tab.	18.33 ± 0.69	<LOD	17.91	14.74 ± 0.80	18.20 ± 2.62
	21.83 ± 1.12	<LOD	9.86	11.66 ± 0.07	20.67 ± 3.08
E Chew. Tab.	20.46 ± 1.32	<LOD	13.56	11.67 ± 0.18	11.55 ± 2.58
	34.84 ± 0.72	<LOD	38.64	17.59 ± 1.21	15.24 ± 2.49
F ^d Chew. Tab.	35.52 ± 1.15	<LOD	27.42	14.44 ± 2.54	27.96 ± 5.48
G Chew. Tab.	43.10 ± 2.65*	<LOD	23.89	16.69 ± 1.70	30.82 ± 4.53
	42.06 ± 1.03*	8.07 ± 3.15	23.65	12.73 ± 1.25	28.02 ± 4.41
H Chew. Tab.	27.36 ± 0.09	<LOD	6.23	12.14 ± 0.42	24.14 ± 3.11
	27.73 ± 0.44	14.02 ± 2.00	19.26	18.23 ± 0.51	20.17 ± 4.43
J Chew. Tab.	43.39 ± 0.27*	<LOD	32.70	11.61 ± 0.16	18.78 ± 1.60
	45.43 ± 3.84*	<LOD	9.78	11.47 ± 0.15	21.03 ± 3.79
J Granules	26.71 ± 2.45	<LOD	12.06	16.01 ± 1.41	19.51 ± 3.09
	24.93 ± 1.19	<LOD	38.12	13.52 ± 1.58	21.82 ± 0.62
K Chew. Tab.	25.62 ± 0.57	<LOD	11.28	13.32 ± 1.47	24.70 ± 3.22
	14.74 ± 0.87	<LOD	33.0	13.35 ± 0.60	20.31 ± 1.25

*Qualification tests required; a. MA I&II LOD = 0.075 µg; b. MA I and II LOQ = 0.228 µg; c. First measurement results were used for cis isomer impurity; d. One batch of F coded chewable tablet was provided from market; Each batch of all drugs was shown individually.

USA). The Kolmogorov–Smirnov test was used to determine whether the continuous variables were normally distributed or not. Descriptive statistic results were calculated as mean $\pm$ standard deviation. The Student's *t*-test (one-sided, unpaired) was used to determine statistical significance for Ames test. For chromosomal aberration analysis, differences between treated cells and controls were tested with Kruskal–Wallis and Dunn's multiple comparison tests or Chi-square test. Dose–response relationships were determined from the correlation and regression coefficients for the mitotic index. Results were considered statistically significant, when the *p*-value was <0.05 .

3. Results

3.1. Quantification of the impurities in drug products

According to the ICH guideline, the reporting, identification and qualification thresholds of the impurities for pediatric (4 mg) montelukast products are 0.1% (4 μ g), 0.5% (20 μ g) and 1% (40 μ g), respectively. In the case of adult (10 mg) montelukast products, impurity thresholds for reporting, identification and qualification are 0.1% (10 μ g), 0.5% (50 μ g), and 0.5% (50 μ g), respectively. It was found that amounts of montelukast impurities except sulfoxide were less than qualification limits as stated in the guidelines. The sulfoxide exceeded the limits in pediatric chewable tablets from 2 companies (G and J) and in tablets for adults from 7 companies (A, D, F, G, H, I, and J) (Tables 2 and 3). Importantly, it was observed that the amounts of methylstyrene and methylketone impurities were close to qualification thresholds. However, quantities of Michael adduct I & II impurities were found remarkably low. In HPLC analysis, only first measurement values of cis isomer impurity in drug sample solution were taken into consideration due to the fact that the amount of cis isomer impurity rapidly increased in second and third injections in the solution. In Fig. 3, impurity means calculated for drug products with two batches were illustrated with the purpose of assessing the reporting, identification and qualification limits. For the sulfoxide impurity which was determined to be above the threshold according to the obtained results, qualification was needed.

3.2. Mutagenicity prediction of sulfoxide impurity with (Q)SAR models

In this study, the *E. coli*–Sal 102 A-T Mut model was used to predict A-T base pair mutations but the sulfoxide impurity was not within the domain of applicability for the Leadscape model therefore prediction could not be calculated. On the other hand, Salmonella Mut model predicted mutagenicity as a negative (Positive Prediction Probability = 0.131) in Leadscape. In parallel, ToxTree SAR model reported no alerts for *S. typhimurium* mutagenicity for the impurity.

3.3. Mutagenicity assessment of sulfoxide impurity using bacterial reverse mutation test

The sulfoxide impurity did not increase the number of wells containing revertant colonies in presence and absence of metabolic activation system in TA98, TA1535 and *E. coli* combo strains. Although cytotoxicity was observed at 1581 and 5000 μ g/ml doses, it was also nonmutagenic at lower doses in TA1537. In TA100 strain in presence of metabolic activation system, sulfoxide impurity did not cause mutagenic effect up to 1581 μ g/ml concentration. For the TA100 strain without metabolic activation system, while high doses of the impurity caused cytotoxicity, bacteria, though less, were still observed at 158 and 500 μ g/ml doses as well. However, the impurity did not show sign of mutagenicity at non-toxic doses in TA100 (Table 4). Considering overall test, the results showed that the sulfoxide impurity was not mutagenic (Fig. 4).

3.4. Cytotoxicity and genotoxicity assessments of sulfoxide impurity

Treated dose of the impurity was defined as cytotoxic if it caused over 50% decrease in mitotic index compared to the control. Short (3 h) and long (24 h) treatments of cells with sulfoxide impurity resulted in cytotoxicity at $125 \leq \mu$ g/ml and $62.5 \leq \mu$ g/ml concentrations, respectively (Fig. 5). Analysis of the MI after treatments of lymphocytes with sulfoxide impurity showed that there was concentration-dependent reduction in MI in 3 h (S9–), 3 h (S9+) and 24 h (S9–) conditions (respectively, Pearson correlation, $r = -0.893$, $r = -0.826$, $r = -0.762$, $p < 0.05$).

Table 3
The amounts of the impurities in adult drug products containing montelukast (μ g; mean $\pm$ SD; $n = 3$).

Drugs (10 mg)	Sulfoxide	Michael Adducts I and II	Cis isomer ^c	Methylketone	Methylstyrene
A Tab.	54.85 $\pm$ 3.89*	<LOD ^a	24.36	36.26 $\pm$ 2.53	39.35 $\pm$ 1.85
	39.13 $\pm$ 6.38	<LOD	45.19	42.69 $\pm$ 2.77	47.29 $\pm$ 5.64
B Tab.	37.94 $\pm$ 0.26	<LOD	42.50	43.13 $\pm$ 1.07	49.86 $\pm$ 0.94
	33.53 $\pm$ 2.77	<LOD	29.13	45.34 $\pm$ 2.47	46.38 $\pm$ 5.48
C Tab.	48.86 $\pm$ 2.45	<LOD	26.98	34.89 $\pm$ 1.62	40.59 $\pm$ 3.45
	45.70 $\pm$ 1.10	<LOD	29.45	40.61 $\pm$ 1.20	30.26 $\pm$ 5.29
D Tab.	59.88 $\pm$ 5.34*	<LOD	45.70	38.81 $\pm$ 4.29	48.87 $\pm$ 8.49
	64.48 $\pm$ 0.83*	<LOD	30.23	46.93 $\pm$ 0.48	48.44 $\pm$ 3.25
E Tab.	49.86 $\pm$ 1.91	<LOD	45.20	42.19 $\pm$ 2.35	45.31 $\pm$ 4.07
	39.48 $\pm$ 2.56	<LOD	23.65	43.99 $\pm$ 2.74	33.72 $\pm$ 1.28
F Tab.	99.01 $\pm$ 4.20*	<LOD	40.62	32.49 $\pm$ 1.58	49.20 $\pm$ 0.24
	63.05 $\pm$ 9.46*	<LOQ ^b	16.84	47.37 $\pm$ 3.50	49.96 $\pm$ 1.26
G Tab.	53.02 $\pm$ 6.71*	<LOD	49.03	38.66 $\pm$ 2.87	49.26 $\pm$ 1.94
	49.18 $\pm$ 5.42	<LOD	35.72	44.23 $\pm$ 1.88	47.21 $\pm$ 7.78
H Tab.	73.03 $\pm$ 3.35*	<LOD	26.67	35.71 $\pm$ 6.10	48.84 $\pm$ 7.50
	58.41 $\pm$ 2.77*	5.03 $\pm$ 1.38	48.74	35.51 $\pm$ 1.69	48.64 $\pm$ 1.84
I Tab.	78.85 $\pm$ 2.46*	<LOD	48.41	48.64 $\pm$ 5.68	49.94 $\pm$ 1.55
	73.49 $\pm$ 7.88*	<LOD	47.39	36.28 $\pm$ 1.23	40.99 $\pm$ 0.07
J Tab.	55.99 $\pm$ 3.03*	<LOD	35.11	40.29 $\pm$ 4.14	42.85 $\pm$ 3.17
	52.17 $\pm$ 2.27*	<LOD	25.50	49.60 $\pm$ 5.79	45.36 $\pm$ 9.75
K Tab.	45.43 $\pm$ 2.45	<LOD	47.32	50.59 $\pm$ 1.11	45.93 $\pm$ 5.39
	45.96 $\pm$ 3.99	<LOD	49.60	42.76 $\pm$ 3.67	42.05 $\pm$ 6.51

*Qualification tests required; a. MA I&II LOD = 0.075 μ g; b. MA I and II LOQ = 0.228 μ g; c. First measurement results were used for cis isomer impurity; Each batch of all drugs was shown individually.

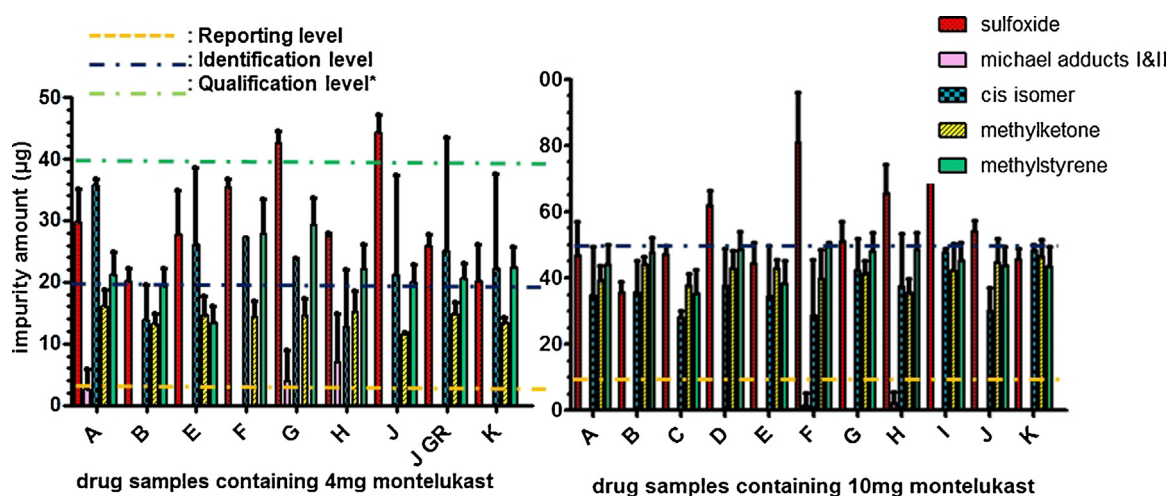


Fig. 3. Status of the impurities quantities in drug products containing montelukast according to ICHQ3B thresholds levels. (*: identification and qualifications level are same for 10 mg drug samples).

In in vitro chromosomal aberration test, the numbers of background chromosomal aberrations of each donor were determined to be high, particularly in terms of chromatid exchanges. In any case, since the statistical analyses were performed by comparing the dose groups with the control; it would be possible to determine clearly the damage related to impurity. In comparison to solvent control, no biologically relevant increases in the number of structural chromosome aberrations were observed in

the absence and presence of metabolic activation. Sulfoxide impurity also did not cause numerical aberration in any of the experiments and test conditions (Table 5).

4. Discussion

The investigated impurities in this study were chosen particularly considering the clinical importance of the drug which

Table 4

Sulfoxide impurity-induced mutagenicity in the absence and presence of S9 mix (means of number of wells containing revertant colonies $\pm$ SD; $n = 3$).

Doses (μ g/ml)	S9 -/+	TA98			TA100			TA1535			TA1537			E.Coli Combo		
		Mean $\pm$ SD	^a Fold increase	<i>p</i>	Mean $\pm$ SD	Fold increase	<i>p</i>	Mean $\pm$ SD	Fold increase	<i>p</i>	Mean $\pm$ SD	Fold increase	<i>p</i>	Mean $\pm$ SD	Fold increase	<i>p</i>
0	–	4.00 $\pm$ 1.00			5.33 $\pm$ 2.08			2.33 $\pm$ 0.58			2.33 $\pm$ 1.53			4.67 $\pm$ 2.08		
	+	3.33 $\pm$ 0.58			5.33 $\pm$ 3.21			3.00 $\pm$ 1.73			1.67 $\pm$ 0.58			2.00 $\pm$ 1.00		
15.8	–	3.00 $\pm$ 1.73	0.60	0.22	6.00 $\pm$ 4.00	0.81	0.41	1.33 $\pm$ 0.58	0.46	0.05	2.33 $\pm$ 1.15	0.60	0.50	3.67 $\pm$ 2.52	0.54	0.31
	+	3.33 $\pm$ 0.58	0.85	0.50	4.67 $\pm$ 3.51	0.55	0.41	3.67 $\pm$ 2.31	0.77	0.35	3.00 $\pm$ 1.00	1.34	0.06	2.33 $\pm$ 2.52	0.78	0.42
50	–	3.33 $\pm$ 1.15	0.67	0.25	4.33 $\pm$ 2.08	0.58	0.29	3.00 $\pm$ 2.65	1.03	0.35	1.67 $\pm$ 0.58	0.43	0.26	2.33 $\pm$ 0.58	0.35	0.07
	+	2.00 $\pm$ 2.00	0.51	0.16	7.00 $\pm$ 2.00	0.82	0.24	2.00 $\pm$ 0.0	0.42	0.19	1.33 $\pm$ 1.15	0.59	0.34	3.00 $\pm$ 1.73	1.00	0.22
158	–	5.00 $\pm$ 3.61	1.00	0.33	1.67 $\pm$ 1.15	0.22	0.03	2.67 $\pm$ 1.15	0.92	0.34	0.67 $\pm$ 0.58	0.17	0.08	4.00 $\pm$ 2.65	0.59	0.37
	+	3.00 $\pm$ 2.65	0.77	0.42	4.33 $\pm$ 3.21	0.51	0.36	2.67 $\pm$ 1.53	0.56	0.41	2.00 $\pm$ 2.00	0.89	0.40	4.33 $\pm$ 1.53	1.44	0.05
500	–	3.67 $\pm$ 1.15	0.73	0.36	1.33 $\pm$ 2.31	0.18	0.05	1.00 $\pm$ 1.00	0.34	0.06	0.67 $\pm$ 1.15	0.17	0.10	2.33 $\pm$ 1.53	0.35	0.10
	+	4.00 $\pm$ 2.00	1.02	0.30	5.00 $\pm$ 3.61	0.58	0.46	2.00 $\pm$ 1.00	0.42	0.22	1.67 $\pm$ 1.53	0.74	0.50	1.00 $\pm$ 1.00	0.33	0.14
1581	–	3.67 $\pm$ 1.53	0.73	0.38	0.67 $\pm$ 0.58	0.09	0.01	1.33 $\pm$ 1.53	0.46	0.17	0.00	0.0	0.03	2.33 $\pm$ 3.21	0.35	0.18
	+	2.67 $\pm$ 1.15	0.68	0.21	2.00 $\pm$ 2.65	0.23	0.12	1.00 $\pm$ 0.0	0.21	0.06	0.33 $\pm$ 0.58	0.15	0.02	2.67 $\pm$ 1.15	0.89	0.25
5000	–	2.67 $\pm$ 0.58	0.53	0.06	0.00	0.00	0.01	1.33 $\pm$ 1.15	0.46	0.13	0.00	0.00	0.03	4.67 $\pm$ 2.08	0.69	0.50
	+	2.33 $\pm$ 1.53	0.60	0.17	0.33 $\pm$ 0.58	0.04	0.03	1.00 $\pm$ 1.73	0.21	0.12	0.33 $\pm$ 0.58	0.15	0.02	2.33 $\pm$ 0.58	0.78	0.32
2-NF (2)	–	25.67 $\pm$ 0.58														
2-AA (1)	+	46.00 $\pm$ 2.00														
4-NQO (0.1)	–				47.67 $\pm$ 0.58											
2-AA (2.5)	+				48.00 $\pm$ 0											
N ⁴ ACT (100)	–							48.00 $\pm$ 0.0								
2-AA (5)	+							29.67 $\pm$ 4.51			44.00 $\pm$ 2.65					
9-AA (15)	–										48.00 $\pm$ 0.0					
4-NQO (2)	–													41.33 $\pm$ 1.15		
2-AA (50)	+													18.33 $\pm$ 3.06		

a. Over baseline; 2-NF: 2-nitrofluorene, 2-AA: 2-aminoanthracene, 4-NQO: 4-nitroquinolone-*N*-oxide, N⁴ACT: N⁴-aminocytidine; 9-AA: 9-aminoacridine; Statistical significant *p*-values were displayed in bold.

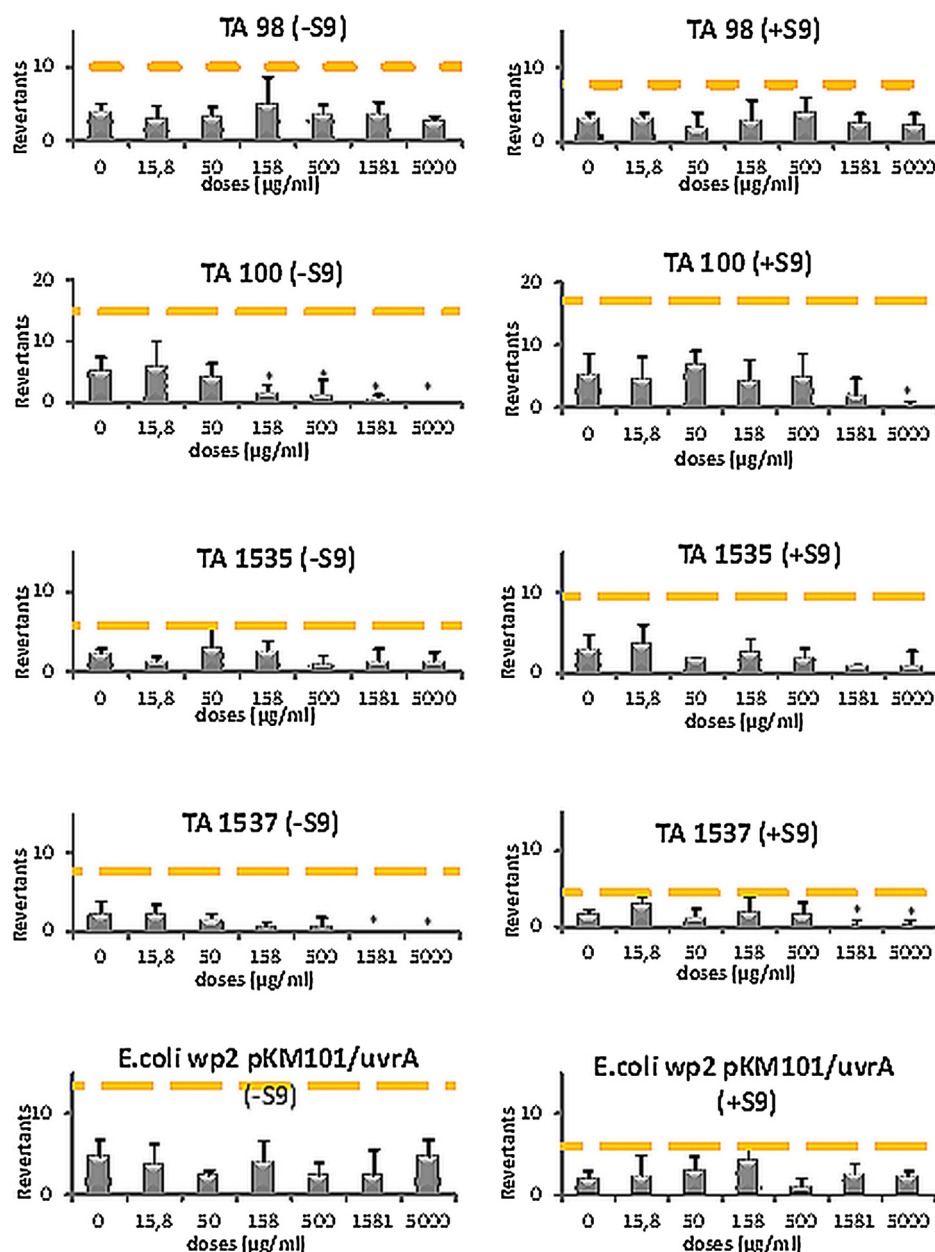


Fig. 4. Mutagenicity results of sulfoxide impurity in *S. typhimurium* and *E. coli* strains (*: $p \leq 0.05$; —: 2 folds increase over baseline).

includes these impurities in addition to its target population and its usage duration. Long-term control medications including montelukast are used daily and regularly to achieve and maintain control of asthma (U.S. NIH, Asthma Report, 2007). Moreover, because of better long term safety profile, montelukast is also preferred as add-on treatment to corticosteroids and sometimes it is preferred to replace corticosteroids in cases of experienced adverse reactions or corticophobia in patients (Aran and Karakaya, 2005; Joos et al., 2008). Toxicities of montelukast impurities should be carefully assessed because montelukast is chronically used in patients, particularly in children who are treated with montelukast almost their entire lifetime. From toxicological perspective, it is important to note that children are more vulnerable than adults to xenobiotics and that they might respond with different health

effects related to xenobiotics (Fernandez et al., 2011; Scheuplein et al., 2002). It has also been suggested that cancer risks are generally higher in early-life exposure than from similar exposure durations later in life (U.S. EPA, 2005).

Historically, the issue of impurities was stressed by stakeholders in the mid 1990s which roughly corresponds with the period when montelukast was launched in the market in 1998. This was followed by references of regulatory agencies via guidelines about how impurity issues would be handled which also included the analytical and toxicological approaches related to impurities. In developed countries, while guidelines for residual solvents had been put into effect in 1998, guidelines for non genotoxic and for genotoxic impurities were introduced respectively in 2002/2003 and 2007. Therefore, these guidelines did not cover already

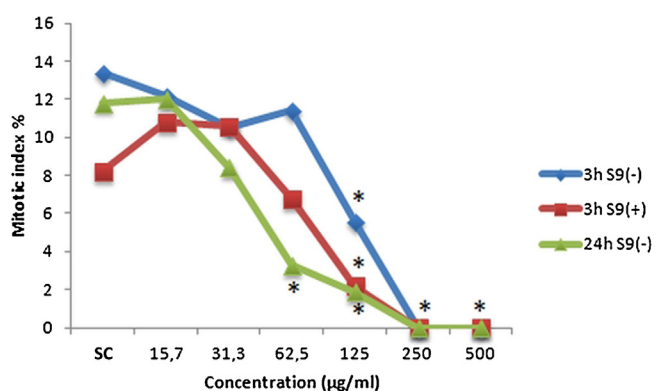


Fig. 5. Mitotic index in human lymphocytes following incubation with sulfoxide impurity (*: cytotoxic doses).

marketed montelukast and similarly genotoxicity evaluation of impurities belonging to montelukast was not done yet (ICH, 2006 a,b; ICH, 2011 ICH, 2011; EMA, 2006).

In addition to the original drug production, there are plenty of pharmaceutical companies that manufacture generic drugs which might contain different impurity compositions and different amounts of impurities than original drug due to various reasons such as source of materials, environmental conditions, changes in process etc. (Kovaleski et al., 2007; Schneidera and Wessjohann, 2010; Taylor et al., 2010; Gasser et al., 2013). There are also studies indicating isolation and identification of various kinds of montelukast impurities by generic drugs manufacturing companies (Chandra Sekhar et al., 2011; Bouskova et al., 2010; Overeem and Ma Van, 2005; Haidar et al., 2012). In this aspect considering the studies for comparison, it may be observed that quality in terms of impurity might change from company to company. In this study, it was found that generic drugs produced by Turkish pharmaceutical companies did not provide drastically negative outputs compared to innovator drug in terms of investigated impurities. While these results present a positive aspect of Turkish pharmaceutical industry, given the limits of this study in which only certain impurities were investigated instead of impurity profiling, the study cannot provide a view of the entire situation and understanding for other potential impurities in drug products.

It is acknowledged that the effective therapy with montelukast taken by a patient is reduced due to generation of sulfoxide during manufacturing and storage, as it is considered an inactive compound pharmacologically (Hrakovsky et al., 2010). In the analyses, it was detected that sulfoxide impurity exceeded the qualification limits more frequently and occurred with high quantities in drug products for adults compared to pediatric products. Although presence of more sulfoxide impurities in drug products for adults may be suggested to occur due to having more ingredients in their formulation compared to pediatric drug products and/or differences in tablet form, it is not possible to deduce with certainty since many factors play a role in generation of impurities. In previous studies conducting thermal stress testing study of montelukast, sulfoxide impurity was observed as a major degradation product (Al Omari et al., 2007). Furthermore in a separate research, it was stressed that the presence of microcrystalline cellulose in the formulation may induce peroxide oxidation of montelukast and presence of aspartame as a sweetener might cause higher amount of sulfoxide impurity (Hrakovsky et al., 2010).

Sulfoxide is also one of the metabolites of montelukast (Filppula et al., 2011). The guidelines suggest that the impurity is considered qualified if exposure to the metabolite in appropriate nonclinical studies of the drug substance is higher than it would be achieved from the impurity in the administered drug substance (EMA, 2006; ICH, 2014; ICH, 2006a,b). In safety testing of drug metabolites, the metabolite is of interest; if it accounts for plasma levels greater than 10% of parent drug systemic exposure at steady state (FDA-CDER Guideline, 2008b). However, taking into account sulfoxide metabolite which is identified as a minor metabolite (Balani et al., 1997) safety testing is hardly expected to be done.

There are a number of studies on identification, quantification and characterization of impurities in drugs. However, there are limited scientific literature on investigating toxicities of the impurities (Agarwal et al., 2004; Eichenbaum et al., 2009; Ellis et al., 2013; Luo et al., 2014; Meng et al., 2013; Paim et al., 2011; Vijayan et al., 2007; Zhu et al., 2012, 2014). In this study, among researched impurities, sulfoxide impurity exceeded qualification thresholds in some drug products, thereby it was systematically qualified. In silico and in vitro genotoxicological assessments of the impurity was handled considering regulatory approaches. Firstly, a computational mutagenicity assessment was performed by two

Table 5
Chromosome aberrations in cultured human lymphocytes treated with sulfoxide impurity.

Treatment	Concentration (µg/ml)	No. of cells scored	No. of structural chromosome aberrations						No. of numerical chromosome aberrations		% of cells with structural aberration(s) ^a	No. of structural aberration ^a per cell (mean ± SD)	Mitotic index (%)
			ctg	ctb	cte	csg	csb	cse	PP	ER			
3h (S9 -)	MeOH	200	5	10	31	15	1	0	0	0	18.0	0.21 ± 0.48	12.0
	15.7	200	5	18	45	26	2	0	0	0	24.0	0.33 ± 0.66	11.9
	31.3	200	5	20	41	26	1	0	0	0	23.0	0.31 ± 0.65	9.8
	62.5	200	6	31	34	12	8	0	0	0	28.5	0.37 ± 0.67	9.2
	MMC (0.6)	200	45	117	101	31	90	0	1	0	66.5*	1.54 ± 1.68*	8.5
3h (S9 +)	MeOH	200	10	25	46	21	2	0	0	0	25.5	0.37 ± 0.73	8.3
	15.7	200	2	31	43	15	6	0	0	0	30.5	0.40 ± 0.68	9.6
	31.3	200	11	35	66	19	4	0	0	0	36.5	0.53 ± 0.79	9.3
	62.5	200	15	31	67	27	4	0	0	0	36.5	0.51 ± 0.77	7.8
	CP (15)	200	11	50	96	32	16	0	0	0	51.5*	0.81 ± 0.97*	8.2
24h (S9 -)	MeOH	200	5	11	48	7	2	0	0	0	23.5	0.31 ± 0.60	6.2
	20	200	4	20	66	4	1	0	0	0	30.0	0.44 ± 0.78	5.9
	30	200	3	20	58	8	6	0	0	0	29.0	0.42 ± 0.75	4.7
	40	200	5	20	40	3	0	0	0	0	20.5	0.30 ± 0.68	5.5
	MMC (0.2)	200	50	108	135	23	40	1	0	0	61.5*	1.42 ± 1.69*	5.4

ctg: chromatid gap, ctb: chromatid break, cte: chromatid exchange, csg: chromosome gap, csb: chromosome break,

cse: chromosome exchange, PP: polyploidy, ER: endoreduplication, MMC: mitomycin-C, CP: cyclophosphamide, a: gaps excluded

* Statistically significant compared to solvent control ($p < 0.05$).

prediction methodologies in line with ICH M7. The ICH M7 guidance suggests that in silico assessment of bacterial mutagenicity for the qualification of impurities in pharmaceuticals be conducted using a knowledge-based methodology and statistical-based methodology complementing each other (ICH, 2014). Leadscape and ToxTree programs which were used in the study have shown a high sensitivity and negative predictivity using an external test set in validation studies (Contrera, 2013; Valerio and Cross, 2012). Obtained data showed that sulfoxide impurity has no structural alert for mutagenicity. According to the ICH M7 guideline, in the absence of structural alerts from the two complementary methodologies, it is sufficient to conclude that the impurity is of no concern and that further testing is not required. On the other hand, as it is applied in this study as well, ICH Q3A/B and EMA guidelines recommend “minimum screen” consisting of bacterial gene mutation test and in vitro chromosome aberration test for evaluation of genotoxic potential of impurities that exceed qualification limits.

Bacterial reverse mutation test (OECD Test Guideline; No:471) is the most widely used and well validated short-term test to identify genetic damage that leads to gene mutations. However, a major problem in impurity studies is acquiring sufficient compound quantity. Achieving the necessary test concentrations for impurities that are not feasible to isolate or synthesize can be quite costly, difficult and limited. Therefore, as an alternative to conventional bacterial reverse mutation test, some miniaturized tests were developed enabling mutagenicity assessment with less quantity of impurity (Flamand et al., 2001; Flückiger-Isler and Kamber, 2012; Gatehouse, 1978; Gatehouse and Delow, 1979; Kamber et al., 2009). In OECD Test Guideline No:471, it is recommended that at least 5 tester strains carrying different target sites for mutation should be used in order to achieve sensitivity for a broad range of chemicals. In line with this recommendation the Ames MPF Penta I being among the miniaturized format tests was used in this study enabling to work on *S. typhimurium* TA98, TA100, TA1537, TA1535 ve *E. coli* WP2 uvrA + *E. coli* WP2 [pKM101] strains. Furthermore, in another study in which concordance of results between Ames MPF and conventional Ames method was tested with a set of 15 equivocal to weakly positive mutagenic chemicals selected from the National Toxicology Program (NTP) database, 87% concordance was determined (Flückiger-Isler and Kamber, 2012).

Before mutagenicity analysis, prescreening assay was performed in TA98 strain in order to determine cytotoxicity in line with kit protocol mentioned and no cytotoxicity was observed. However, in determining cytotoxicity, it was observed that this prescreening assay had some limitations. These limitations are thought to arise from the short exposure period and the principle of the test which defines that the assay can only identify compounds that lyse bacteria within 90 min. Although TA100 strain being more sensitive to killing effects of chemicals than that of TA98, the TA98 strain was used in cytotoxicity test as mentioned in the protocol, hence this might affect determining cytotoxicity precisely as well (Jurado et al., 1993). However, Kenyon et al. (2007) demonstrated that in Ames test, most mutagenic impurities can be detected in concentrations of 250 µg/plate or less. In this study, even though cytotoxic signs are observed at high doses in some strains, overall results showed that sulfoxide impurity has no mutagenic activity in absence and presence of metabolic activation.

In human peripheral lymphocytes, it was found that sulfoxide impurity significantly inhibited mitotic index in a concentration and time dependent manner. However, in in vitro human lymphocyte chromosomal aberration test, sulfoxide impurity did not show clastogenic activity with and without metabolic activation and no numerical aberrations were observed as well.

In this study, it has also been noted that results obtained from computational based mutagenicity analysis concurred with that of bacterial gene mutation test and in vitro chromosomal aberration test. All results indicate that sulfoxide impurity is nonmutagenic/nongenotoxic and that it should be classified as ordinary impurity according to the guidelines.

In light of the results achieved which showed cytotoxicities at high concentrations, in vivo acute toxicity tests may further be considered to be important for the impurity in the future. In the guidelines, while there is mentioning of in vivo acute toxicity test in some cases, there are no particular references to in vitro cytotoxicity assessment at qualification. Nevertheless, one would opt to argue that in vitro cytotoxicity would only be influential if it was extremely cytotoxic and that such an impurity had to be limited at a low level (personal communication with L.Mueller).

In this study, qualification was only needed for the sulfoxide impurity above the limit. In fact, not only investigated impurities but also all impurities in drugs may affect organism as a mixture. Recommendations on multiple impurities are provided regarding genotoxic impurities (Bercu et al., 2008; EMA Q&A, 2010; ICH, 2014). However, risks which can occur in organism in case of interaction between any types of impurities are unknown. Furthermore, there are also combination montelukast products with desloratadine or levocetirizine in market. So, interaction with impurities coming from other active substance might also be thought.

5. Conclusion

In drugs containing montelukast for adults and children in the Turkish market, only the sulfoxide impurity which was among the six impurities investigated, required qualification tests according to the regulatory guidelines. Impurity levels of generic drugs and innovator drug were not observed to be significantly different. The sulfoxide impurity for which no prior data of genotoxicity was available was concluded to be an ordinary (nonmutagenic) impurity in the studies conducted for hazard identification. While this is a positive result for adults and children who use these drugs regularly, in countries like Turkey where regulations on genotoxic impurities are not in effect, it is of essential importance for public health that guidelines of global regulatory agencies are taken into consideration to guide the pharmaceutical industry.

Acknowledgment

This study was funded by the Scientific and Technological Research Council of Turkey (TUBITAK Project No: 111T506).

References

- Agarwal, S.K., Bhatnagar, U., Rajesh, N., 2004. Acute and genotoxic profile of a dimeric impurity of cefotaxime. *Int. J. Toxicol.* 23 (1), 41–45.
- Al Omari, M.M., Zoubi, R.M., Hasan, E.I., Khader, T.Z., Badwan, A.A., 2007. Effect of light and heat on the stability of montelukast in solution and in its solid state. *J. Pharm. Biomed. Anal.* 45 (3), 465–471.
- Aran, M.R., Karakaya, G., 2005. Bronş astımı tedavisi. *Dahili Tıp Bilim Derg* 12 (3), 130–137.
- Balani, S.K., Xu, X., Pratha, V., Koss, M.A., Amin, R.D., Dufresne, C., Miller, R.R., Arison, B.H., Doss, G.A., Chiba, M., Freeman, A., Holland, S.D., Schwartz, J.I., Lasseter, K.C., Gertz, B.J., Isenberg, J.I., Rogers, J.D., Lin, J.H., Baillie, T.A., 1997. Metabolic profiles of montelukast sodium (Singulair), a potent cysteinyl leukotriene1 receptor antagonist, in human plasma and bile. *Drug Metab. Dispos.* 25 (11), 1282–1287.
- Bercu, J.P., Hoffman, W.P., Lee, C., Ness, D.K., 2008. Quantitative assessment of cumulative carcinogenic risk for multiple genotoxic impurities in a new drug substance. *Regul. Toxicol. Pharmacol.* 51 (3), 270–277.
- Bouskova, O., Gibala, P., Halama, A., Jirman, J., 2010. Specific impurities of montelukast. European Patent, EP2260025 A2.
- Chandra Sekhar, B.V.V.N., Ramesh Kumar, N., Kalyan Chakravarthy, A., Mukkanti, K., 2011. Synthesis of montelukast sodium via a nitrile intermediate; synthesis and characterization of impurities. *Int. J. Pharma. Bio. Sci.* 2 (3), 417–425.

- Contrera, J.F., 2013. Validation of toxtree and SciQSAR in silico predictive software using a publicly available benchmark mutagenicity database and their applicability for the qualification of impurities in pharmaceuticals. *Regul. Toxicol. Pharmacol.* 67 (2), 285–293.
- Delaney, E.J., 2007. An impact analysis of the application of the threshold of toxicological concern concept to pharmaceuticals. *Regul. Toxicol. Pharmacol.* 49 (2), 107–124.
- Dobo, K.L., Greene, N., Cyr, M.O., Caron, S., Ku, W.W., 2006. The application of structure-based assessment to support safety and chemistry diligence to manage genotoxic impurities in active pharmaceutical ingredients during drug development. *Regul. Toxicol. Pharmacol.* 44, 282–293.
- Eichenbaum, G., Johnson, M., Kirkland, D., O'Neill, P., Stellar, S., Bielawne, J., DeWire, R., Areia, D., Bryant, S., Weiner, S., Desai-Krieger, D., Guzzie-Peck, P., Evans, D.C., Tonelli, A., 2009. Assessment of the genotoxic and carcinogenic risks of p-nitrophenol when it is present as an impurity in a drug product. *Regul. Toxicol. Pharmacol.* 55 (1), 33–42.
- Ellis, P., Kenyon, M., Dobo, K., 2013. Determination of compound-specific acceptable daily intakes for 11 mutagenic carcinogens used in pharmaceutical synthesis. *Regul. Toxicol. Pharmacol.* 65 (2), 201–213.
- European Medicines Agency (EMA), Committee for Medicinal Products for Human Use, 2006. Guideline on the Limits of Genotoxic Impurities.
- European Medicines Agency (EMA), Committee for Medicinal Products for Human Use, Safety Working Party, 2010. Question & Answers on the CHMP Guideline on the Limits of Genotoxic Impurities (Rev. 3).
- Fernandez, E., Perez, R., Hernandez, A., Tejada, P., Arteta, M., Ramos, J.T., 2011. Factors and mechanisms for pharmacokinetic differences between pediatric population and adults. *Pharmaceutics* 3 (1), 53–72.
- Filppula, A.M., Laitila, J., Neuvonen, P.J., Backman, J.T., 2011. Reevaluation of the microsomal metabolism of montelukast: major contribution by CYP2C8 at clinically relevant concentrations. *Drug Metab. Dispos.* 39 (5), 904–911.
- Flandam, N., Meunier, J., Meunier, P., Agapakis-Caussé, C., 2001. Mini mutagenicity test: a miniaturized version of the Ames test used in a prescreening assay for point mutagenesis assessment. *Toxicol. In Vitro* 15 (2), 105–114.
- Flückiger-Isler, S., Kamber, M., 2012. Direct comparison of the Ames microplate format (MPF) test in liquid medium with the standard Ames pre-incubation assay on agar plates by use of equivocal to weakly positive test compounds. *Mutat. Res.* 747, 36–45.
- Food and Drug Administration (FDA), Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2008a. Guidance for Industry, Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches (draft).
- Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), 2008b. Guidance for Industry, Safety Testing of Drug Metabolites.
- Gasser, U.E., Fischer, A., Timmermans, J.P., Arnet, I., 2013. Pharmaceutical quality of seven generic Levodopa/Benserazide products compared with original Madopar/Prolopa. *BMC Pharmacol. Toxicol.* 14 (1), 24.
- Gatehouse, D., 1978. Detection of mutagenic derivatives of cyclophosphamide and a variety of other mutagens in a microtitre fluctuation test, without microsomal activation. *Mutat. Res.* 53 (3), 289–296.
- Gatehouse, D.G., Delow, G.F., 1979. The development of a microtitre fluctuation test for the detection of indirect mutagens, and its use in the evaluation of mixed enzyme induction of the liver. *Mutat. Res.* 60 (3), 239–252.
- Giordani, A., Kobel, W., Gally, H.U., 2011. Overall impact of the regulatory requirements for genotoxic impurities on the drug development process. *Eur. J. Pharm. Sci.* 43, 1–15.
- Goverdhana, G., Reddy, A.R., Srinivas, K., Himabindub, V., Reddy, G.M., 2009. Identification, characterization and synthesis of impurities of zafirlukast. *J. Pharm. Biomed. Anal.* 49, 895–900.
- Haidar, S., Boettcher, S., Al Mardini, M.A., Hartmann, R.W., 2012. Purity of five generic bulk montelukast sodium using liquid chromatography/mass spectrometry. *Int. J. Pharm. Pharm. Sci.* 4 (3), 686–690.
- Hrakovsky, J., Tenengauzer, R., Bogomolny, G., Dolitsky, Y., 2010. Stable pharmaceutical formulations of montelukast sodium, European Patent, EP 1818057B1.
- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2014. Multidisciplinary Guidelines, M7, Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk (Step 4).
- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2005. Quality Guidelines, Q2(R1), Validation of Analytical Procedures: Text and Methodology (Step 4).
- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2006a. Quality Guidelines, Q3A(R2), Impurities in New Drug Substances (Step 4).
- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2006b. Quality Guidelines, Q3B(R2), Impurities in New Drug Products (Step 4).
- International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2011. Quality Guidelines, Q3C(R5), Impurities: Guideline For Residual Solvents (Step 4).
- Joos, S., Miksch, A., Szecsenyi, J., Wieseler, B., Grouven, U., Kaiser, T., Schneider, A., 2008. Montelukast as add-on therapy to inhaled corticosteroids in the treatment of mild to moderate asthma: a systematic review. *Thorax* 63, 453–462.
- Jurado, J., Alejandro-Durán, E., Pueyo, C., 1993. Genetic differences between the standard Ames tester strains TA100 and TA98. *Mutagenesis* 8 (6), 527–532.
- Kamber, M., Flückiger-Isler, S., Engelhardt, G., Jaekch, R., Zeiger, E., 2009. Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity. *Mutagenesis* 24 (4), 359–366.
- Kenyon, M.O., Cheung, J.R., Dobo, K.L., Ku, W.W., 2007. An evaluation of the sensitivity of the Ames assay to discern low-level mutagenic impurities. *Regul. Toxicol. Pharmacol.* 48 (1), 75–86.
- Kovaleski, J., Kraut, B., Mattiuz, A., Giangiulio, M., Brobst, G., Cagno, W., Kulkarni, P., Rauch, T., 2007. Impurities in generic pharmaceutical development. *Adv. Drug Deliv. Rev.* 59 (1), 56–63.
- Luo, Q., Li, Y., Zhang, Z., 2014. A systematic assessment of genotoxicity on pivaloylacylation-7ADCA-a wide existing antibiotic impurity. *Int. J. Clin. Exp. Med.* 7 (11), 4260–4271.
- Meng, F., Yan, J., Li, Y., Fu, P.P., Fossom, L.H., Sood, R.K., Mans, D.J., Chu, P.I., Moore, M.M., Chen, T., 2013. Genotoxicity of 2-bromo-3'-chloropropiophenone. *Toxicol. Appl. Pharmacol.* 270 (2), 158–163.
- Muller, L., Mauthe, R.J., Riley, C.M., Andino, M.M., De Antonis, D., Beels, C., DeGeorge, J., De Knaep, A.G.M., Ellison, D., Fagerland, J.A., Frank, R., Fritschel, B., Galloway, S., Harpur, E., Humfrey, C.D.N., Jacks, A.S., Jagota, N., Mackinnon, J., Mohan, G., Ness, D.K., O'Donovan, M.R., Smith, M.D., Vudathala, G., Yotti, L., 2006. A rationale for determining testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity. *Regul. Toxicol. Pharm.* 44, 198–211.
- Overeem, A., Ma Van, D.H.D.J., 2005. Solid-state montelukast. *PCT Patent*, 2005/040123.
- Paim, C.S., Führ, F., Barth, A.B., Gonçalves, C.E., Nardi, N., Steppe, M., Schapoval, E.E.S., 2011. Gemifloxacin mesylate (GFM) stability evaluation applying a validated bioassay method and in vitro cytotoxic study. *Talanta* 83 (5), 1774–1779.
- Pilaniya, K., Chandrawanshi, H.K., Pilaniya, U., Manchandani, P., Jain, P., Singh, N., 2010. Recent trends in the impurity profile of pharmaceuticals. *J. Adv. Pharm. Technol. Res.* 1 (3), 302–310.
- Raman, N.V.V.S.S., Prasad, A.V.S.S., Ratnakar Reddy, K., 2011. Strategies for the identification, control and determination of genotoxic impurities in drug substances: a pharmaceutical industry perspective. *J. Pharm. Biomed. Anal.* 55 (4), 662–667.
- Saravanan, M., Siva Kumari, K., Pratap Reddy, P., Naidu, M.N., Moses Babu, J., Srivastava, A.K., Lakshmi Kumar, T., Chandra Sekhar, B.V., Satyanarayana, B., 2008. Identification, synthesis, isolation and spectral characterization of potential impurities of montelukast sodium. *J. Pharm. Biomed. Anal.* 48 (3), 708–715.
- Scheuplein, R., Charnley, G., Dourson, M., 2002. Differential sensitivity of children and adults to chemical toxicity I biological basis. *Regul. Toxicol. Pharmacol.* 35 (3), 429–447.
- Schneider, A., Wessjohann, L.A., 2010. Comparison of impurity profiles of Orlistat pharmaceutical products using HPLC tandem mass spectrometry. *J. Pharm. Biomed. Anal.* 53 (3), 767–772.
- Sivri, E.G., 2009. Montelukast Sodyum İçeren Tabletlerde Safsızlık Tayini Ve Yöntem Validasyonu. Yüksek Lisans. Gazi Üniversitesi, Ankara.
- Taylor, P.W., Arnet, I., Fischer, A., Simpson, I.N., 2010. Pharmaceutical quality of nine generic orlistat products compared with Xenical®. *Obes. Facts* 3 (4), 231–237.
- The Global Asthma Network, 2014. The Global Asthma Report, Auckland, New Zealand: <http://www.globalasthmareport.org/index.php> (last accessed date: 15.04.2015).
- U.S. National Institutes of Health (NIH), National Heart, Lung, and Blood Institute, National Asthma Education and Prevention Program. Expert Panel Report 3 Guidelines for the Diagnosis and Management of Asthma Full Report. 2007; U.S. Environmental Protection Agency (EPA), 2005. Risk Assessment Forum. Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens, EPA/630/R-03/003F.
- Valerio Jr., L.G., Cross, K.P., 2012. Characterization and validation of an in silico toxicology model to predict the mutagenic potential of drug impurities. *Toxicol. Appl. Pharmacol.* 260, 209–221.
- Vijayan, M., Deecaraman, M., Pudupalayam, K.T., 2007. In vitro genotoxicity of piperacillin impurity-A. *Afr. J. Biotechnol.* 6 (18), 2074–2077.
- Zhu, Q., Li, T., Wei, X., Li, J., Wang, W., 2014. In silico and in vitro genotoxicity evaluation of decarboxyl levofloxacin, an impurity in levofloxacin. *Drug Chem. Toxicol.* 37 (3), 311–315.
- Zhu, Q., Li, T., Li, J., Guo, M., Wang, W., Zhang, X., 2012. In silico and in vitro genotoxicity evaluation of levofloxacin n-oxide, an impurity in levofloxacin. *Toxicol. Mech. Methods* 22 (3), 225–230.