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GENOTOXICITY EVALUATION FOR THE STRESS INDUCED DEGRADANTS IN DIACEREIN

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ABSTRACT

Genotoxicity evaluation is an *in vitro* and *in vivo* test processes to decide the undesirable effects of chemical entity. It helps to evaluate the effect of chemical substance towards the induce genetic damage by various mechanisms. Regulatory authorities framed strict measures to control the genotoxic impurities in the active pharmaceutical ingredients. Monograph books have been already prescribed with certain related substances as an impurities raised from different level like last intermediates of synthesis, products of incomplete reactions, products of over reactions, impurities originating from starting materials, impurities originating from solvent materials, Impurities originating from catalysis. Product of side chain reactions, degradation products, enantiomeric excess, residual solvents, Inorganic impurities, and impurities in excipients. Active pharmaceutical ingredients undergo degradation with uncertain pathway hence the biosafety of those materials certainly could not be concluded in the initial stage. The present study aim to force the diacerein to different climatic condition to evaluate its different degrading pathway. The drug explored to different stress conditions, the stressed diacerein subjected to HPLC separation to isolate and separate the known and unknown impurities. The new impurities were isolated and structurally elucidated by evaluate their spectral data. Impurities were subjected to genotoxic effect using AMES test model. Based on the structural alert certain impurities were produced reverse mutation colony growth. The Ames model produced the revert colony formation in certain impurities. To conclude the bio-safety, the entire degradation product should be examined for their mutogenic character, all the impurities bio safety profile should be recorded by bulk drug manufacturers.

INTRODUCTION

Genotoxicity [1-7] tests can be defined as *in vitro* and *in vivo* tests designed to detect compounds that induce genetic damage by various mechanisms. These tests enable hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage or recombination is generally considered to be essential for heritable effects and in the multi-step process of malignancy, a complex process in which genetic changes might possibly play only a part. Numerical chromosome changes have also been associated with tumorogenesis and can indicate a potential for aneuploidy in germ cells. Compounds that are positive in tests that detect such kinds of damage have the potential to be human carcinogens and/or mutagens. Because the relationship between

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exposure to particular chemicals and carcinogenesis is established for humans, whilst a similar relationship has been difficult to prove for heritable diseases, genotoxicity tests have been usedmainly for the prediction of carcinogenicity. Nevertheless, because germ line mutations are clearly associated with human disease, the suspicion that a compound might induce heritable effects is considered to be just as serious as the suspicion that a compound might induce cancer. Generally all degradants [8] are chemical breakdown compounds of the drug substance formed during storage. In rare cases, degradants are formed when the drug substance chemically interacts with other compounds or contaminants like acidic or basic primary packaging materials. In addition, degradants may also be obtained by abnormal physical conditions direct exposre to sunlight, heat, moisture etc., Commonly in the synthetic compounds last intermediates of synthesis, products of incomplete reactions, products of over reactions, impurities originating from starting materials, impurities originating from solvent materials, impurities originating from catalysis. product of side chain reactions, degradation products, enantiomeric excess, residual solvents, Inorganic impurities, and impurities in excipients considered as a impurities, the rate of degradation depends on the chemical nature of the drug substance. An understanding of the potential degradation pathways of the drug substance will lead to optimization of the storage conditions and will result in fewer impurities. The level of a degradation product [9] will increase with time; eventually the level of a degradation product present in a pharmaceutical agent may reach a level (typically 0.1% by FDA guidelines for drugs dosed at <2 g/day and 0.05% for drugs dosed at >2 g/day in the ICH guidelines) that triggers the decision to isolate and identify the component. the level of a degradation product will increase with time; eventually the level of a degradation product present in a pharmaceutical agent may reach a level (typically 0.1% by FDA guidelines [10] for drugs dosed at <2 g/day and 0.05% for drugs dosed at >2 g/day in the ICH guidelines) that triggers the decision to isolate and identify the component. The systematic approach in impurity identification [11] propos that, the entire degradation pathway should be scrutinized and individual impurities focused to observe, structurally characterized. Their prescribed limit should be highly specialized. In addition to that, identification of impurities and/or degradants in pharmaceuticals is critically important forreasons of both product efficacy and patient safety. In terms of efficacy and patient safety, it is important to isolate and identify impurities and/or degradants to ensure that their presence will not evoke any form of adverse response, either pharmacologic or toxicologic, in a patient taking the medication. All the impurities and/or degradants act as a potent genotoxcins although rationale for the registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. In the present status the monograph prescribes some impurities which are already proven for its bio safety. Another hand the undesired or new degradation product or impurities biosafety issues also should involve for extensive reviews. In present scenario the reviews reveals that many compounds that are mutagenic in the bacterial reverse mutation (Ames) test are rodent carcinogens. Addition of *in vitro* mammalian tests increases sensitivity for detection of rodent carcinogens and broadens the spectrum of genetic events detected. The OECD recommended the set of bacterial strains to predict the mutations. The Diacerein (DIA) is a familiar synthetic drug used to treat the arthritis. Very extensive literature enumerates that, the DIA assay have been performed by spectroscopy [12- 15], HPLC [16-25] Force degradation study were conducted for and two impurities were isolated and characterized[26] in DIA however the bio-safety of those impurities not yet proved. DIA estimated in rabit plasma by using LCMS[27] Aceclofenac and DIA were simultaneously separated and estimated from tablet dosage using HPTLC[28]. The literature information reveals that DIA were still not yet been approached in genotoxicity angle. The present assignment aim was to establish the entire degradation profile for DIA and individual degradation molecules was evaluated for its mutation capability followed by isolation and characterization. The mutation capacity of impurities examined by Ames test. Ames test been internationally demonstrated to prove reverse mutation mechanism. The genetically modified *E. coli AB1157* strain (auxotroph) used to respond for mutogens.

MATERIALS AND METHODS

Preparation of stock solution

DIA freely soluble in Di Methyl Sulfoxide (DMSO) and di methyl formamide (DMF). The 10µg/ml of solution was prepared in acetonitirile. The standard solutions were injected in to HPLC, the standard chromatogram shown in Figure. 1.

Forced degradation study

The stock solution of DIA was prepared by dissolving in one part of DMSO and 9 parts of acetonitrile. Stress degradation studies were performed by subjecting the standard drug solutions to different degradation conditions such as acidic, alkali, oxidative and photolytic degradation studies. The degradation studies were all performed at room temperature. The degradated solution was injected in to optimized chromatographic condition to isolate the different degradation products. The developed chromatographic method was validated as per ICH guidelines. The individual degraded products were collected by fraction collection method, subjected to identification and characterizations. The structures were elucidated by using different analytical tools like Fourier Transform Infra Red spectroscopy, Mass spectrometry, and ^{1}H Nuclear Magnetic Resonance spectroscopy. The

predicted structure shown in Table.1

Ames test

The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) has a remit to provide UK Government Departments and Agency with advice on the most suitable approaches to testing chemical substances for genotoxicity. The genotoxicity study performed by different levels. In the modern assessments, the genotoxicity demonstrated by computer based designing. The practical assessment may produce strong evidence in safety rather than the previous one. The *in vitro* models like gene mutation assay, chromosomal damage (nicking assay) produces promise results in the safety evaluation. *In vivo* studies like Investigation mutagenic end point, genotoxicity in tumour target tissue, genotoxicity in potential of germ cell, genotoxicity in site of control tissue and DNA sequence analysis are advance and very strong evidence based method.

Preparation of impurities solution

10 mg of the crystallized impurities weighed and transfer in to the trisodium citrate buffer the further dilutions were made by using sterile saline to obtain the concentration of 10 ng/ml.

Preparation liquid broth and buffer medium:

The liquid broth was prepared by dissolving 500mg of the LB mixture in to 20ml of double distilled water. The broth was sterilized by autoclaving technique. The autoclaving done in the controlled temperature to avoid the denaturing of LB. Around 5.25gm of di potassium hydrogen orthophosphate , 2.25 gm of potassium di hydrogen orthophosphate, 0.5gm of ammonium sulphate and 0.25 gm of tri sodium citrate was dissolve in 50ml of double distilled water to obtain the buffer solution.

Preparation of minimal agar plate

Approximately 50 ml of minimal agar broth was mixed with 7.5ml of buffer solution. 1.5ml of sterile glucose solution mixed with uniform parts (0.75ml) of Proline, Arginine, Threonine, vitamin B2 and megnisum sulphate solution. The solutions thoroughly mixed and then pour in sterile petri dishes at 55^{o}C.

The Host culture of *E.coli AB1157* processed for the culture revives. 0.5ml of host culture inoculated in 20ml of broth. The culture incubated for 37oC for overnight. 1.5 ml of overnight incubated culture was centrifuged at 10,000 rpm for 5 min at 4ºC the deposited pellet collected in sterile tube. The pellets washed 2-3 times with 0.85% sterile normal saline and re-suspended in equal volume of sterile normal saline. 10 ml of minimal soft agar broth taken in the three tubes the broth temperature was maintain up to 55º C±1^{o}C. 0.5 ml of culture re-suspended in all the tube. The first tube treated with 0.8 ml of freshly prepared Hydroxylamine (NH2OH). The hydroxylamine act as a positive control. The second test tube treated with 0.8 ml of sterile saline solution act as a negative control. The third group of test tubes added with 0.8 ml of impurity in solution form obtained from different stress conditions. All the test tubes were quickly mixed and the content was poured in to the petri dishes at 55^{o}C allow to solidify. The petri dishes were incubate the at 37ºc for 48- 72 hours. After 72 hours the incubated plate were observed for the colony formation. In certain impurity fractions the revert colonies growth were observed.

RESULTS AND DISCUSSION

Unidentified and potentially toxic impurities are health hazards in terms of quality, safety, efficacy and risk factors in drug therapy. These impurities may be Non-Genotoxic or Genotoxic in nature. Safety of the drugs necessitates screening of genotoxicity effect. Impurity profiling involves analytical activities aimed at detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities, as well as residual solvents in bulk drugs to establish their biological safety. The RI present in the Pharmaceutical product may hinder its pharmacological activity; sometime RI may act as a carcinogenic agent. It is also acceptable to bring down their concentration in the API and dosage forms to safe limits. Impurities can be identified and determined by using different analytical methods. The method should be validated and results will be documented as per standard guidelines. The present study aims to develop simple accurate, precise and sensitive method to estimate the amount of IM present in the selected bulk drugs and pharmaceutical dosage forms. The IM's present in the DIA drug were further screened for its GT effect by AMES test to prove its biosafety.

Chromatographic Conditions

The DIA and its impurities were sperated using Hibar® C18 (250 x 4.6 mm i.d., 5µ) as a stationary phase Acetonitrile and Water (pH 2.0) (60:40 % v/v) in isocratic flow mode used as a mobile Phase. The flow rate was fixed to 1 ml/min, the detection wavelength was 256nm. The Retention time was 4.7 min for DIA. The developed method validated as per ICH guidelines. The method validation parameters were satisfactory to adopt this method to separate the degrading product. The method was validated for certain parameters like accuracy, precision, selectivity, specificity, linearity and range, limit of detection and quantification robustness and regardless. The system suitability parameters were satisfactory.

Forced degradation study

The standard solution of DIA was subjected to various stress conditions like acid, alkali, oxidative and

photo degradation. The DIA has undergone 100% degradation at the end of 48 hours of acid degradation, the degradation peaks eluted at the retention times 2.13 and 2.70 min. the chromatogram were shown in Figure 2. In case of basic degradation study it was observed that the DIA has undergone 100% degradation after 48 hours. The degradation peaks eluted at the retention times of 2.18, 2.73 and 6.34 min. The chromatogram shown in Figure 3. The oxidative degradation study was performed by using hydrogen peroxide as a oxidizing agent. The drug undergone 100% degradation after 48 hours and the degradation peaks eluted at the retention times 2.73, 5.04, 5.78 and 6.28 min. The respective study chromatogram shown in Figure No. 4. Photo degradation was performed under ultra violet light and direct sun light. When the sample was exposed to UV light it was observed that DIA concentration has decreased to 7.65 % at the end of 48 hours and the degradation peaks eluted at the retention times 2.73, 3.52, 5.65, 6.53 min. When the sample was exposed to sunlight it was observed that DIA concentration has decreased to 59.83% at the end of 48 hours and the degradation peaks eluted at the retention times 2.72 and 3.52 min respectively. The UV light and sunlight degradation chromatogram were shown in Figure No. 5 and 6 respectively. The isolated fractions evaporated and purified by re crystallization process. The purified substance subjected to structural prediction analysis, based on the molecular mass and their structures were interpreted the results were shown in Table 1.

Genotoxicity evaluation

The *E. coli* bacteria treated with structurally predicted new impurities to examine their mutogenic nature. The Ames kit *E.coli* is genetically modified microorganism to produce counter action against the mutogen within 48 hours incubation. The host culture converted to revive culture before 24 hours of Ames test. The stimulated *E. coli* culture converted to pellets and re-suspended in the minimal agar plate where the *E. coli* explored to impurities and incubated for 48 hours. The auxotroph *E. coli AB1157* (HIS-) is reverse mutated to prototroph *E.coli AB1157* (HIS+). The degradation products formed under various stress conditions such as acid, base and UV light were found to be genotoxic. The colony growth was observed for the compound retained at 2.21 min in acid degradation. The compound retained at 2.73 min, 4.4 min, 5.7 min and 6.46 min. The result was interpreted based on the positive colony growth in the Ames test. The revert colony growth shown in Figure 9-13. Based on the genotoxicity structural alerts guidelines and predicted structures of certain unknown compounds proved that, the compound posses with carbonyl functional groups credited with more chance for mutation. However, those suspected impurities were not yet included in the official monograph book. If the synthetic chemist stumble on the degradation route it can be officially included in the official location, it develops heedful in the synthetic chemist, manufacturers, analysts and drug regulatory authorities to upgrade the safety guidelines.

Table. 1. The predicted impurity structures

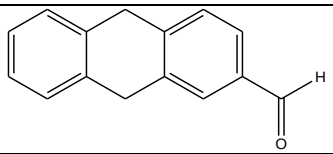
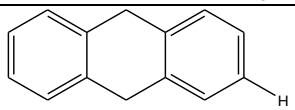
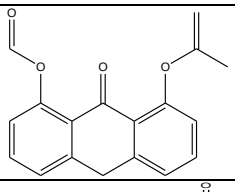
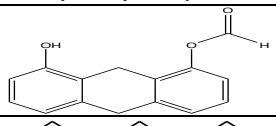
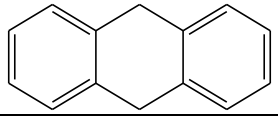
S.No	Stress Type	Retention time	Predicted structure
1.	**Acid**	**2.21 Min**	
2.	**Base**	**2.71 Min**	
3.	**Base**	**4.1 Min**	
4.	**Base**	**5.71 Min**	
5.	**Base**	**6.46 Min**	

Figure 1. Typical chromatogram of standard DIA (100µg/ml)

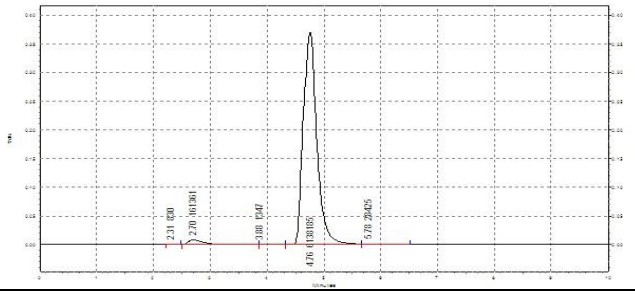


Figure 2. Typical chromatogram of Acid stressed DIA

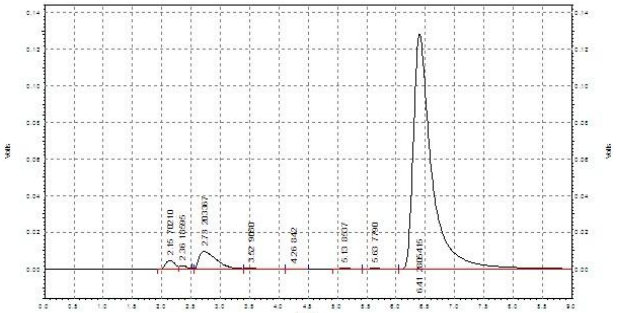


Figure 3. Typical Chromatogram of basic degradation

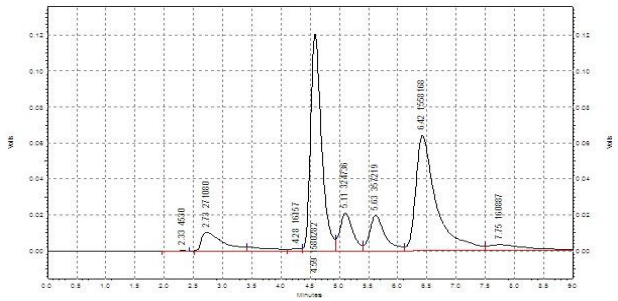


Figure 4. Typical chromatogram for oxidative degradation

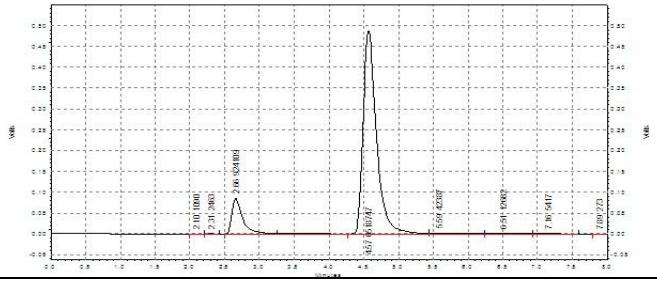


Figure 5. Typical chromatogram of Photolytic degradation (UV light)

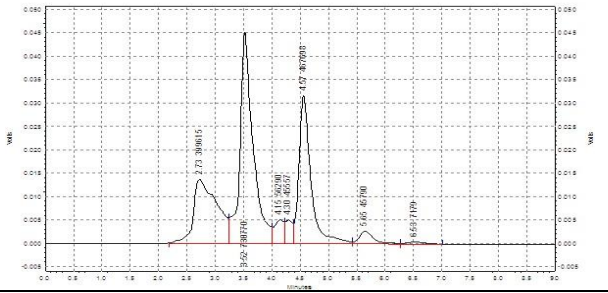


Figure 6. Typical chromatogram for photolytic degradation (Sunlight)

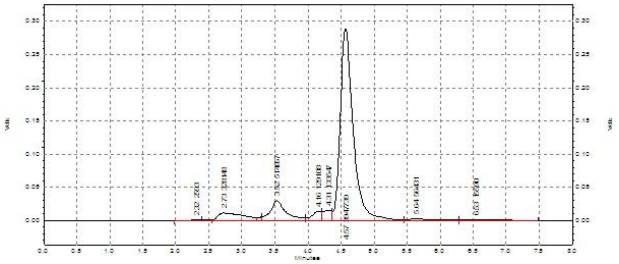


Figure 7. Positive control (Hydroxylamine 8% v/v)

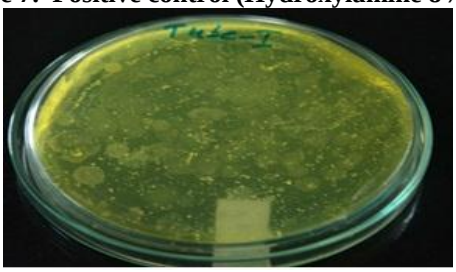


Figure 8. Negative control (without Hydroxylamine 8% v/v)

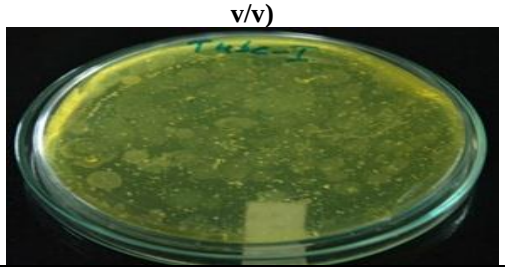


Figure 9. Genotoxicity study for the compound retention time at 2,20 min (Base degradation)

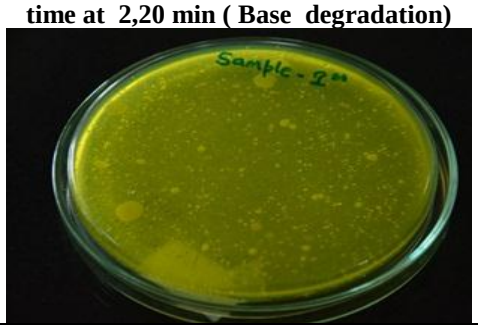


Figure 10. Genotoxicity study for the compound retention time at 5.70 min (Base degradation)

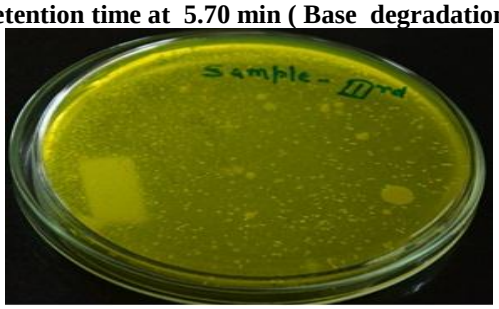


Figure 11. Genotoxicity study for the compound retention time at 4.10 min (Base degradation)

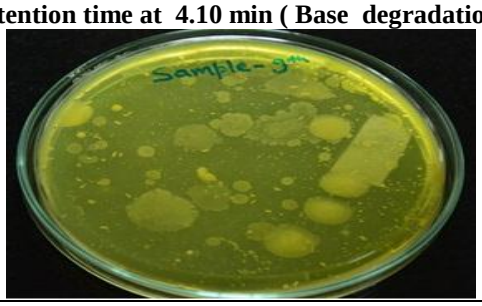


Figure 12. Genotoxicity study for the compound retention time at 6.46 min (Base degradation)

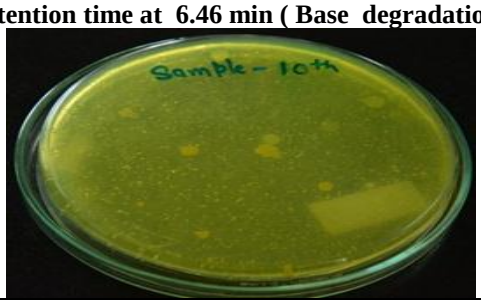
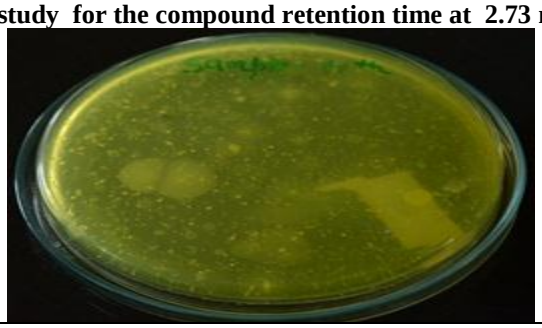


Figure 13. Genotoxicity study for the compound retention time at 2.73 min (Base degradation)



CONCLUSION

The impurity present in the DIA was analyzed by newly developed analytical method and the level was monitored in the API and dosage forms as per ICH guidelines Q3B (R2). The developed method was simple accurate, precise and sensitive. It can be useful to quantify the impurity present in API. The through forced degradation studies were performed and the DIA shows makeable changes in case all the stress study. The new fractions were subjected to structural interpretation and the impurity structures the unknown structures were predicted and it was harmonized with the regulatory structural alerts. The study also reveals that, certain impurity molecular mass was not similar to the recommended impurities present in monograph. It suspected that, those compounds are new impurities which was not been mentioned in the officially. The carcinogenicity of the all the fractions were screened by AMES test, the test reports also responded positively for AMES test. The API manufactures have to reveal the effect of impurity in biological system not only in quantitative level but also genotoxicity has to be filed individually for all the impurities to prove its bio safety.

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REFERENCES

1. ICH Harmonised Tripartite Guideline (1995). Guideline on the need for Carcinogenicity Studies Of Pharmaceuticals S1A.
2. ICH Harmonised Tripartite Guideline (1997). Testing for Carcinogenicity of Pharmaceuticals S1B.
3. ICH Harmonised Tripartite Guideline (2011). Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals intended for Human Use S2(R1).
4. ICH Harmonised Tripartite Guideline (1994). Note for Guidance on Toxicokinetics: The Assessment of Systemic Exposure in Toxicity studies S3A.
5. ICH Harmonised Tripartite Guideline(2013). Assessment and Control of DNA reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic risk.
6. OECD (2002). Chapter 2 Existing genotoxicity assay Pg.no1-36.
7. ICH Harmonised Tripartite Guideline (2003). Stability Testing of New Drug Substances and Products Q1A(R2).
8. ICH Harmonised Tripartite Guideline (1996). Stability Testing: Photostability testing of New Drug Substances and Products Q1B.
9. ICH Harmonised Tripartite Guideline (2003). Evaluation for Stability data Q1E.
10. Richard J Smith. (2007), Analysis of drug impurities, 11-12, Pg.no. 124-130
11. Selvam P, Mahalingam U, Sridharan D, A.Thenmozhi, (2011). A simple UV Spectrophotometric determination of Diacerein in pure and in Pharmaceutical dosage form, *Asian.J.Biochem.Pharm.Res*, 3(1). Pg.no. 429-434.

12. Rele RV, Warkar CB. (2011), Estimation of Lornoxicam and Diacerein in dosage form by simultaneous equation and Q-Analysis method using UV Spectroscopic technique, *Int. J. Pharm. Biosci.* 2(2), Pg.no. 124-129.
13. Ramchandra Pandey, Pravin O. Patil, Manohar U. Patil, Prashant K. Deshmukh, and Sanjay B. Bari. (2012), Quantitative estimation of Diacerein in bulk and in capsule formulation using hydrotropic solubilizing agents by UV-Spectrophotometry and the first order derivative using the area under curve method, *Pharm.Methods*, 3(1), Pg.no. 4-8.
14. Sivakumar R, Nallasivan PK, Saranya KC, Solomon WD Sam, Akelesh T, Venkatnarayanan R. (2010), Visible Spectrophotometric estimation of Diacerein in bulk and pharmaceutical dosage form, *J. Young. Pharm*, 2(4), Pg.no. 414-416.
15. Sharma MC, Sharma S. (2011), Application to Dissolution studies and HPLC assay method for the determination of Diacerein and Aceclofenac in tablet dosage form, Am-Euras. *J. Sci. Res*, 6(3), Pg.no.149-154.
16. Kabeer Ahmed Shaik, Ashish Tanaji Patil, Audumber Bhagwan Ingole. (2012), Sensitive LC method for the simultaneous determination of Diacerein and Aceclofenac in tablet dosage form, *Int. J. Ind. Chem*, 3(3).
17. Kannappan N, Madhukar.A, Srinivasan.R, Srinivas.R,.L.A, Kumar CH, Naveen, Mannavalan.R. (2010), Analytical method development and validation of Diacerein tablets by RPHPLC, *Int. J. Chem. Tech. Res*, 2(1), Pg.no.143-148.
18. Nouruddin W. Ali, Nada S. Abdelwahab, M. Abdelkawy, Am l A. Em am. (2012), Validated Stability Indicating TLC-Densitometric method for the determination of Diacerein. *J.Chromatogr.Sci*. 2(1), Pg.no.1093-97
19. Purnima Hamrapukar, Priti Patil, Masti Desai, Mitesh Phale, Sandeep Pawar. (2011), Stress degradation studies and development of a validated Stability-Indicating-Assay method for the determination of Diacerein in presence of degradation products, Pharmaceutical Methods, 2(1), Pg.no.30-35.
20. Hanimi Reddy Bapatu, Ravi Kumar Maram, R.Satyanarayana Murthy. (2014), Stability Indicating HPLC method for Quantification of Celecoxib and Diacerein along with impurities in capsule dosage form, *J Chromatogr Sci*.10. Pg.no. 1093.
21. Chaudhari Ashok, Maikap Golak, Deo Adwait, Vivek Krishna, Agrawal Himani, Peshawe Umesh,Gawande Amol, Sompalli Srinivas, Mane Sharad, Jadhav Deepali, Chaudhari Atul. (2000), Isolation and structural elucidation of two impurities from a Diacerein bulk drugs, Pg.no. 525-528.
22. Janhavi Rao, Kanchan Chauhan, KR Mahadik, SS Kadam. (2009), A Stability-indicating high performance liquid chromatographic method for the determination of Diacerein in capsules, *Indian J Pharm. Sci*. 71(1), 24-29.
23. Suganthi A, Ravi TK. (2010), Stability Indicating HPLC method for simultaneous determination of Diacerein and Aceclofenac, *Res. J. Pharm. Tech*. 3(2), Pg.no. 600-603.
24. Gandhi Santosh V, Sadaf Syed. (2013), Development and Validation of RP-HPLC method for estimation of Diacerein in human plasma, *Int. J. Pharma. Res. Pharma. Biomed. sci*. 4(4), Pg.no. 1089-1094.
25. Chaudhari Ashok, Maikap Golak, Deo Adwait, Vivek Krishna, Agrawal Himani, Peshawe Umesh, Gawande Amol, Sompalli Srinivas, Mane Sharad, Jadhav Deepali, Chaudhari Atul. Isolation and structural elucidation of two impurities from a Diacerein bulk drug, *J. Pharma. Biomed. Ana*. 2009; 49, Pg.no. 525-528
26. Arun Shirwaikar, A.Sarala Devi, K.Premalatha , KR.Sreejith. (2012), Determination of Diacerein in Rabbit plasma by Liquid Chromatography-Mass Spectroscopy and its application to Bioavailability study, *Int. J. Pharma. Res. Pharma. biomed.sci.* 3(4), Pg.no. 1738-1744.
27. Kumar R. Siva, Srisutherson N., Nallasivan P.Kumar, Arulraj P., Venkatnarayanan R. (2010). HPTLC method for the simultaneous estimation of Aceclofenac and Diacerein in tablets dosage forms, *Res. J. Pharm. Tech.* 3(3), Pg.no. 825-827.