STUDIES OF RADIATION EFFECTS ON ALLOPHATHIC FORMULATIONS FOR CANCER MANAGEMENT

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Abstract

In the present study, two anticancer drugs, Cyclophosphamide and Doxorubucin Hydrochloride have been investigated. The results of various physico-chemical tests on unirradiated and irradiated drugs indicate possibility of use of lower radiation doses and cryo-irradiation in case of sterilization of Cyclophosphamide. Doxorubcin Hydrochloride could be sterilized at 25 kGy without any significant changes in its physico-chemical properties. HPLC studies reveal formation of several trace level degradation products in irradiated cyclophosphamide. HPLC/MS studies revealed that higher and lower molecular weight products of the original molecules are formed on irradiation. Although, no significant changes are observed in absolute purity values, a little discolouration and formation of degradation products in Cyclophosphamide are the main impediments in acceptability of radiation sterilization. On the other hand, orange-red coloured Doxorubicin Hydrochloride did not show any such changes and could be radiation sterilized at normal sterilization dose of 25 kGy.

1. INTRODUCTION

Radiation processing for sterilization is now well established technique for large number of products in medical field. However, radiation sterilization of pharmaceutical products is one grey area where full potential of the technology is yet to be realized. The reason being , adequate data is not available on such products, especially on parenteral drugs whose solutions are not stable and which are dispensed as sterile powder. Formation of low concentration radiolytic products, discolouration and safety of the irradiated drug are the main points of concern. For sterilization of pharmaceutical powders, aseptic filtration, lyophilization, hot air sterilization, EtO etc. are used where ever applicable.

Aseptic handling, using lower radiation doses and higher dose rates, use of inert atmosphere and low temperature (cryoirradiation) could be some of the approaches to minimize undesirable changes which could be produced by radiation sterilization. Availability of modern techniques now enables to probe into chemical structure and quantities of radiation degradation products, knowing which might help to promote use of radiation processing for sterilization of pharmaceutical products.

In the present study, two anticancer drugs namely (1) cyclophosphamide(CP) and (2) Doxorubicin Hydrochloride (DOXO) have been selected for investigation. Both are normally dispensed as dry powders along with sodium chloride (CP) and sodium chloride or lactose (DOXO). These drugs are quite expensive and are presently sterilized by micro-filtration and lyophilization.. In India, these drugs are imported presently. A local manufacturer, who has indigenously developed these molecules is interested in radiation sterilization of these drugs because of the problems encountered by other methods of sterilization [1-5].

The study has been divided in two sections. The first section describes results of investigations on cyclophosphamide and the other on Doxorubicin Hydrochloride.

2. CYCLOPHOSPHAMIDE

The basic structure of Cyclophosphamide is:

- 2-[Bis(2-chloroethyl)amino]perhydro-2H-1,3,2-oxazaphosphorine 2-oxide monohydrate

- Molecular Weight = 279
- Melting Point = 50-53 ^{0}C

Cyclophosphamide is used for treatment of Lymphosarcoma, Hodgkin Disease, Multiple Myeloma, Lymphatic Leukemia, Ovarian and Other Carcinomas, Tumors of Neck and Head, Retinoblastoma, Carcinoma of the Breast etc. It is used in the form of tablets and injections.

Considering the above structure of the molecule, irradiation could result in formation of chloride ions, hydrogen ions and free radicals and possibly some radiolytic products. DSC and TGA profiles of CP were recorded on Mettler DSC-30 and TG-50. It was found that the melting point was about 50 ^{0}C, followed by thermal decomposition at around 80 ^{o}C. Significant weight loss is only observed at about 210 ^{0}C. The profiles clearly showed that CP cannot be heat sterilized (dry or wet). Some of the DSC results of our investigations to check overall integrity of CP before and after irradiation are give in the Table I. The results show that irradiation does not significantly affect melting points, heat of fusion and mole percentage purity values.

TABLE I. HEAT OF FUSION, MELTING POINTS AND PURITY VALUES OF CYCLOPHOSPHAMIDE AT GRADED GAMMA RADIATION DOSES

Property	Control	10 kGy	15 kGy	30 kGy
H fusion (J/g)	136	132	134	132
Tf (^{0}C)	50.9	50.2	50.7	50.5
Purity mole %	99.9	99.96	99.82	99.78

Tf = fusion temperature
Dose Rate = 10 kGy/Hour, GC-5000
Temperature of Irradiation = -196 ^{0}C

The results of various pharmacopoeia (IP) tests conducted on unirradiated and irradiated CP are given in Table II [6]. As shown in Table II, irradiated CP does not pass all pharmacopoeia tests. High assay value is however retained in all the irradiated samples. The irradiated samples do not pass chloride test and show drop in pH values. Discolouration on irradiation is prominent at dose of 10 kGy and above. These results indicate that CP can not be radiation sterilized at a lower of dose of 10 kGy even at liquid nitrogen temperature.

Since the drug acts by alkylating diseased cells, the alkyl chloride part of the molecule is very important. Considering insignificant change in assay value of the irradiated drug, the efficacy of the drug is not expected to change.

TABLE II. SOME PHARMACOPOEIA TESTS CONDUCTED ON CYCLOPHOSPHAMIDE(IP)

Sample Description(I.P.):	pH	Chloride	M.P(^{0} C)	Water (K.F.)	Assay (HPLC, USP)
White or almost white powder	4 to 6	Meets I.P	49.5 – 53	5.8-7.0	98-102%
Control White Powder	5.40	Complies	50-53	6.12	100.08
10 kGy Yellowish-off white powder	4.28	Does not comply	50-54	6.17	98.86
15 kGy Yellowish white powder	4.40	Does not comply	50-54	6.01	99.5
30 kGy Pale yellow powder	4.20	Does not comply	50-53	6.07	99.03

Microbiological studies

Cyclophosphamide is a cytotoxic drug and does not support growth of microorganisms. Aseptically filled 100 vials (vials, rubber bunk were presterilized by heat) containing 200 mg of CP were tested for sterility (IP). Only 2 vials failed the test (sterility tests were performed by the manufacturer). Irradiation between 5-50 kGy gave all sterile samples.

Electron Beam Irradiation

Samples of CP were irradiated at different radiation dose using ILU-6 Electron Beam Machine at liquid Nitrogen Temperature as well at room temperature (30 ^{0}). Table III gives the results of DSC analysis for samples irradiated at liquid nitrogen temperature. Results of samples irradiated at room temperature are given in Table IV.

TABLE III. HEAT OF FUSION, MELTING POINTS AND PURITY VALUES OF CYCLOPHOSPHAMIDE AT GRADED ELECTRON BEAM RADIATION DOSES

Property	Control	10 kGy	15 kGy	30 kGy
H fusion (J/g)	137	136	133	128
Tf (^{0}C)	50.4	49.7	49.9	48.9
Purity mole %	99.9	99.84	99.89	99.75

Tf = fusion temperature; Temperature of Irradiation = -196 ^{0}C

TABLE IV. HEAT OF FUSION, MELTING POINTS AND PURITY VALUES OF CYCLOPHOSPHAMIDE AT GRADED ELECTRON BEAM RADIATION DOSES (ROOM TEMPERATURE)

Property	Control	10 kGy	15 kGy	30 kGy
H fusion (J/g)	137	139	128	118
Tf (^{0}C)	50.4	49.6	49.1	48.8
Purity mole %	99.9	99.76	99.78	98.49

Tf = fusion temperature ; Temperature of Irradiation = 30 ^{0}C

Irradiation in air results in slightly more radiation degradation. This could be due to temperature rise during rapid irradiation (irradiation time=5min.). At low temperature (liq.N2) no significant differences were observed between samples irradiated by either Gamma or Electron Beam.

Free Radicals

Formation and decay of free radicals in CP was studied using Bruker ESR spectrometer. The concentration of the free radical was estimated using standard KBr and DPPH mixture. It was observed that free radical concentration increased linearly with increasing radiation dose.. Saturation starts at about 50 kGy dose (plateau). The free radicals decayed by 25% in a month and 40% in three months time as observed by ESR. The values of concentrations of free radicals/gm are given below in Table V.

TABLE V. FREE RADICAL CONCENTRATION IN CYCLOPHASPHAMIDE

DOSE (kGy)	SPINS/g 10^{16}	CP+NaCl (30%)
0	0	0
10	1.44	0.94
15	2.00	1.49
30	3.54	2.49
50	1.82	3.40

The "g" value of about 2.007 indicated free radicals to be of free electrons type. Inclusion of NaCl in the formulation, reduced the free radical concentration in proportion to its weight (Table V) showing, NaCl and CP in dry state act independently.

Discolouration:

Cyclophosphamide discolours on irradiation either by gamma or electron beam. The colour changes from white to off-white at less than 10 kGy and to brown at higher doses such as 50 kGy. The colour changes were monitored by diffuse reflectance spectroscopy using Shimadzu spectrophotometer. Unirradiated sample was used as reference in the integrating sphere of the instrument. While unirradiated samples did not show any absorbance, irradiated ones showed absorption between 450 to 550 nm. The absorption increased with increasing radiation dose as shown in Fig.1. Irradiation at lower temperature (liq. Nitrogen) does result in lower absorption (less discolouration) as shown in Fig.2. In this case, it is observed that the colour produced at Liq. N2 is half of that produced at the same dose when irradiated at 40 ^{o}C.

Discolouration in the samples could be due to free radicals, chemical impurities and surface defects. Temperature of irradiation does affect their concentrations. Lower thermal stability, as that of cyclophosphamide could be the reason for increased degradation/discolouration in electron beam irradiation. Although, these changes are quantitatively small but pose aesthetic problems. Present study reveals that irradiation at low temperature reduces these changes.

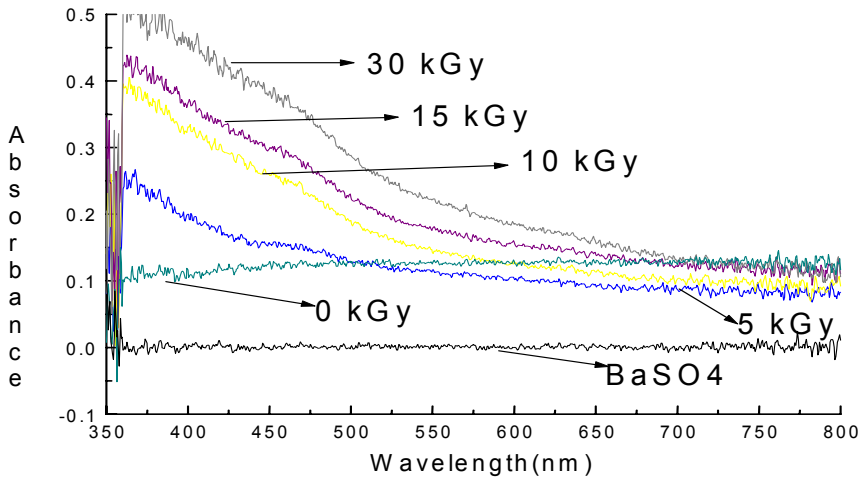

FIG. 1. Absorption increased with increasing radiation dose

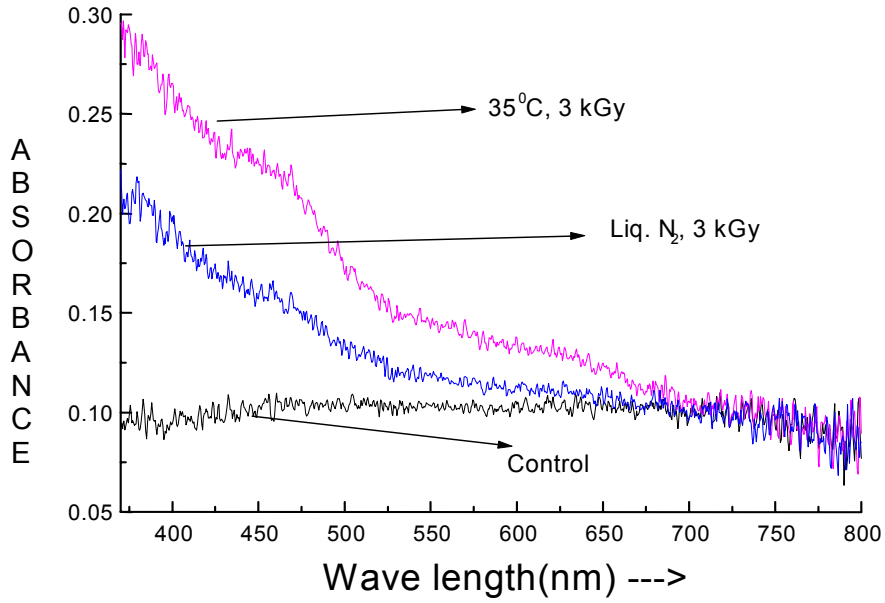

FIG. 2. Cyclophosphamide, Temperature on Irradiation Effect

Chemical Degradation

To investigate extent of chemical degradation, unirradiated and irradiated samples were analysed using UV/VIS spectrophotometer, model M-350 (CAMSPEC, U.K). The spectra of the unirradiated and irradiated samples are given Fig.3. The spectra of irradiated samples shows a strong absorption at 280 nm. Since the high purity level is maintained (DSC&HPLC purity values), it indicates that the impurity formed must be that of high extinction coefficient.

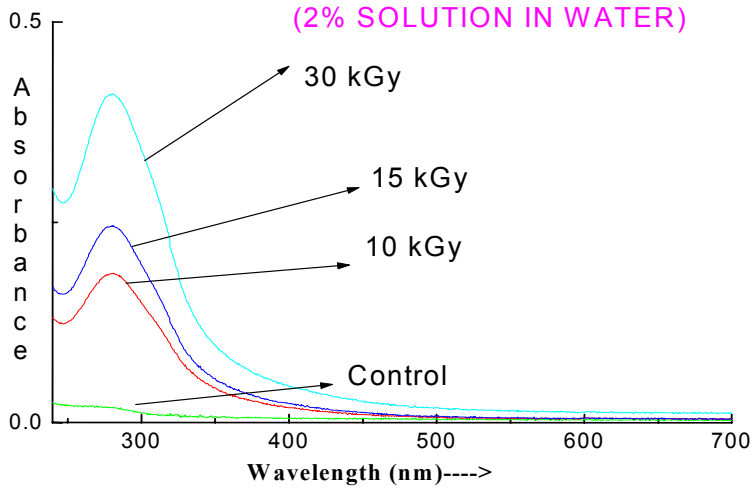

FIG. 3. UV/ VIS Spectra of Cyclophosphamide

Fig. 4 shows linear increase in absorbance with increasing radiation dose indicating radiation degradation with increase in dose

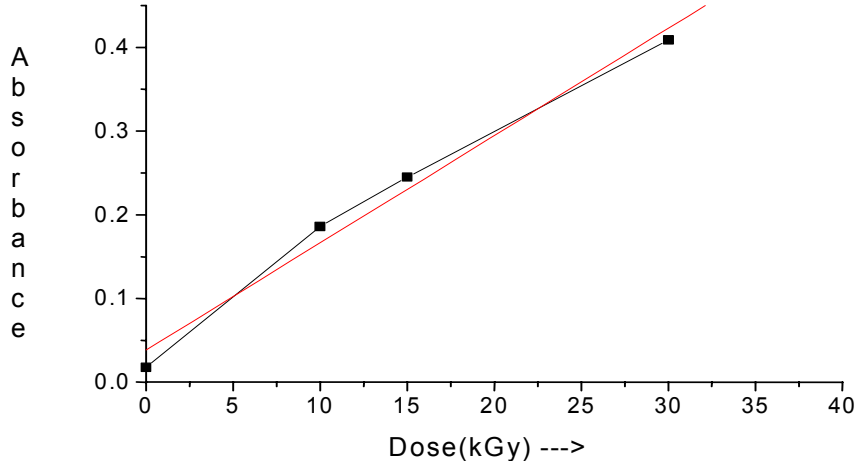

FIG. 4. Dose Vs. Absorbance at 280 nm

On checking absorbance values after a gap of four months no significant changes were observed in control and irradiated samples.

Lower Dose Irradiation

Considering the observation of low microbial contamination in aseptically processed CP vials, 10 vials each containing 200 mg of CP were exposed to 5 kGy at liquid Nitrogen temperature in Gamma chamber 5000. The samples were tested for sterility and chemical stability. These samples passed pharmacopoeia as well as sterility tests. This study implies that lower doses of irradiation may be used for sterilization of aseptically processed product.

The samples of CP irradiated in plant for 5 kGy actually received 6-7 kGy due to overdose ratio. The results of pharmacopeia tests are given in Table VI. Here also, chloride test does not comply.

TABLE VI. PHARMACOPOEIA TEST ON CP IRRADIATED IN PLANT AT 5.0 KGY

TEST	SPECIFICATION	RESULT
Description	White powder	cream coloured powder
pH(2%) Sol.	4.0 to 4.6	3.88
Chloride	Meets test	Does not comply
Reconstituted Solution free from particles	Clear, colourless	Slight opalescent solution
Sterility	Meets test	Complies

Due to over dose ratio and requirement of Liq. Nitrogen, it could not be feasible to deliver exactly 5 kGy dose in commercial gamma sterlization plant.. The actual dose received was 7 kGy . Therefore, such factors should be considered while carrying out feasibility studies.

3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES(HPLC)

The insignificant changes in assay values determined by HPLC and DSC indicate that the drug molecule is quite stable to radiation. The degradation products formed at trace level could be separated and detected by this technique. The effect of temperature of irradiation and high dose rate on degradation profile (number and concentration of degradation products) of the drug was studied using E. Merck-Hitachi HPLC system with Diode Array Detector (DAD). About 20 microgram of the samples were injected for assay and about 150-200 microgram for recording degradation profiles of unirradiated and irradiated samples. Reverse phase, C-18 analytical column, Acetonitrile: Water (30:70) as mobile phase and 200 nm as detection wavelength were employed. The spectra of various impurities were recorded using capabilities of DAD .

Figure 5 shows HPLC profile of control and irradiated samples. The main peaks of both the profiles are overlapping and normalized peak areas show insignificant change in over all purity of the samples. The retention time (Rt) of the main peak is about 8 minutes. Fig. 6 shows degradation profile of the control and irradiated samples (Rt upto 30 minutes). The concentration of several degradation products eluting at different (Rt) increased at higher doses. A few degradation products are observed after main peak retention time.

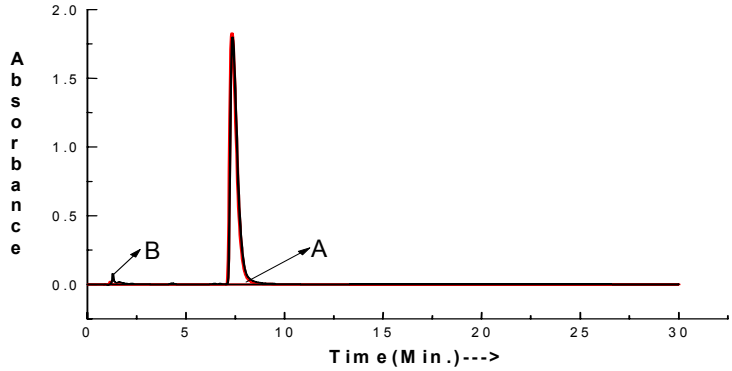

FIG. 5. HPLC profile of control and irradiated samples

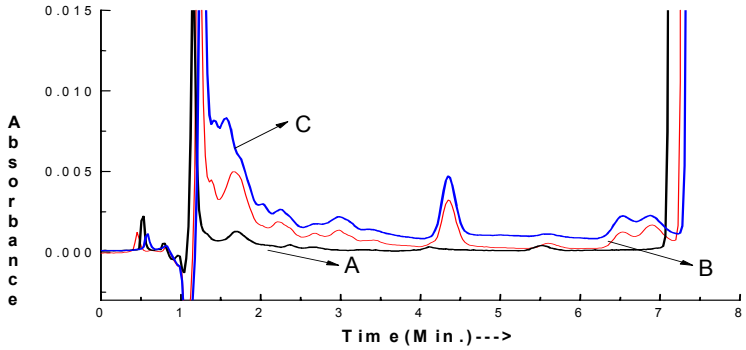

FIG. 6. Degradation profile of the control and irradiated samples

Purity values of samples irradiated to 30 kGy by gamma radiation at 40 ^{o}C, Liquid nitrogen temperature (-196 ^{o}C) and EB irradiated samples did not show significant differences. The degradation profiles of the samples however showed differences as shown in Figure 7. The higher concentration and number of degradation products are more in EB irradiated samples. While, low temperature of irradiation does reduce concentration of impurities in the degradation profile of the irradiated samples. High dose rate, as in the case of cyclophophamide is not suitable as indicated by the results of present study.

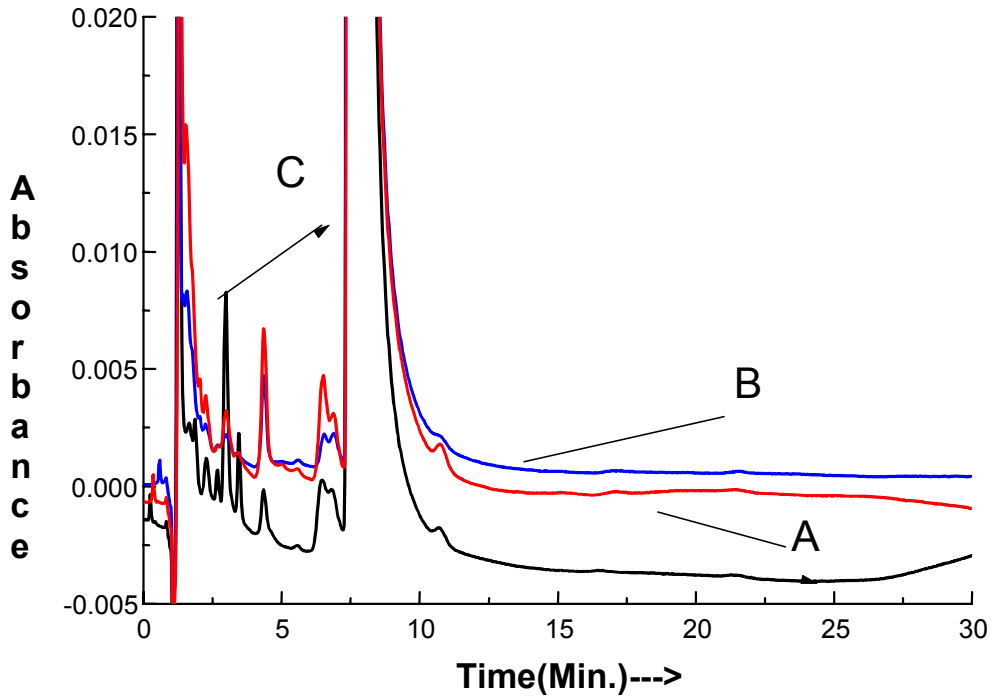

FIG. 7. HPLC Degradiation profiles of Cyclophosphamide

A – Gamma 30 kGy RT; B- Gamma 30 kGy LIQ N_2, C- EB 30 kGy, LQ N_2

To enquire into nature of these degradation products, the DAD spectra of degradation products is given in Fig. 8. Degradation peaks at Rt 1.28 and Rt 6.45 have absorption maximum at about 280 nm. Peak at Rt 1.80 and 2.24 have contribution near 310 nm. These compounds could be responsible for extra absorption arising in irradiated samples in normal uv/visible spectrum. Other peaks absorb near 200 nm region.

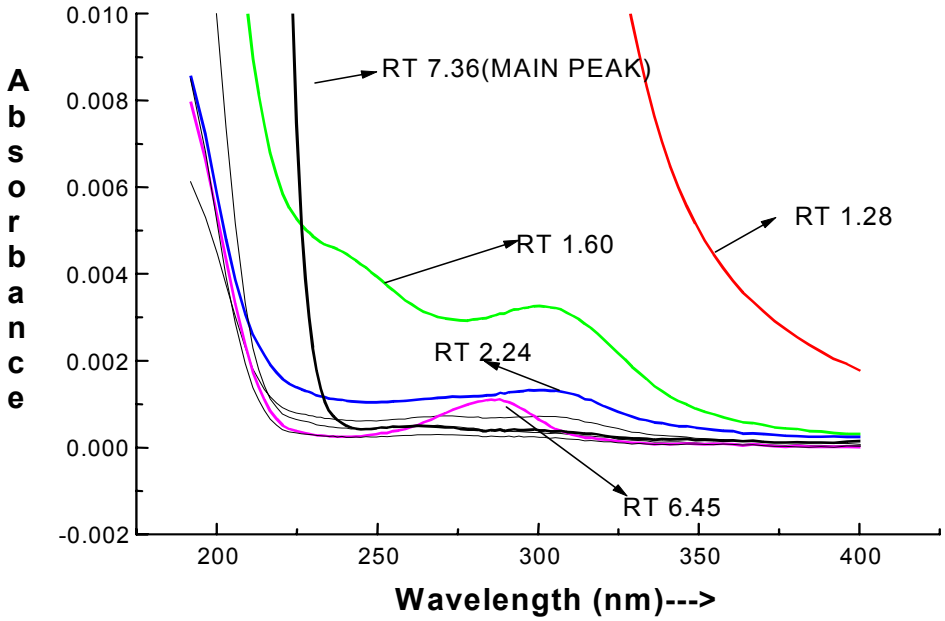

FIG. 8. DAD spectra of degradation products

4. LC/MS STUDIES

Figs. 9&10 show Total Ion Current(TIC) mass spectra of control and irradiated (30 kGy) samples. Spectra were recorded on E. Merck – Hitachi HPLC-MS system(M-8000, ion trap based, ESI interface, mass range upto 1000 amu, 200 microgram sample injected) using mobile phase containing (A) 20 mM Amonium acetate in water, pH 4.6, adjusted with acetic acid and (B) Methanol containing acetic acid(0.1%). Reverse phase, RP-18 column and a flow rate of 0.2 ml was employed for separation. Table VII gives the summary of results obtained from the mass spectra of control and irradiated samples.

TABLE VII.MASS AND BASE PEAKS OF DEGRADATION PRODUCTS IN TIC SPECTRA OF CONTROL AND IRRADIATED SAMPLES

Rt(Control)	Mass Peak	Base Peak	Rt(30 kGy)	Mass peak	Base Peak
1.89	257	257	1.93	298.5	261
			3.88	199	199
			7.07	351	239
			8.00	375	140
9.09	247	247	9.33	297	247
			10.26	380	158
			11.46	227	227
12.73 *	261	261	13*	261	261
			16.71	309	309
* = Main Peak					

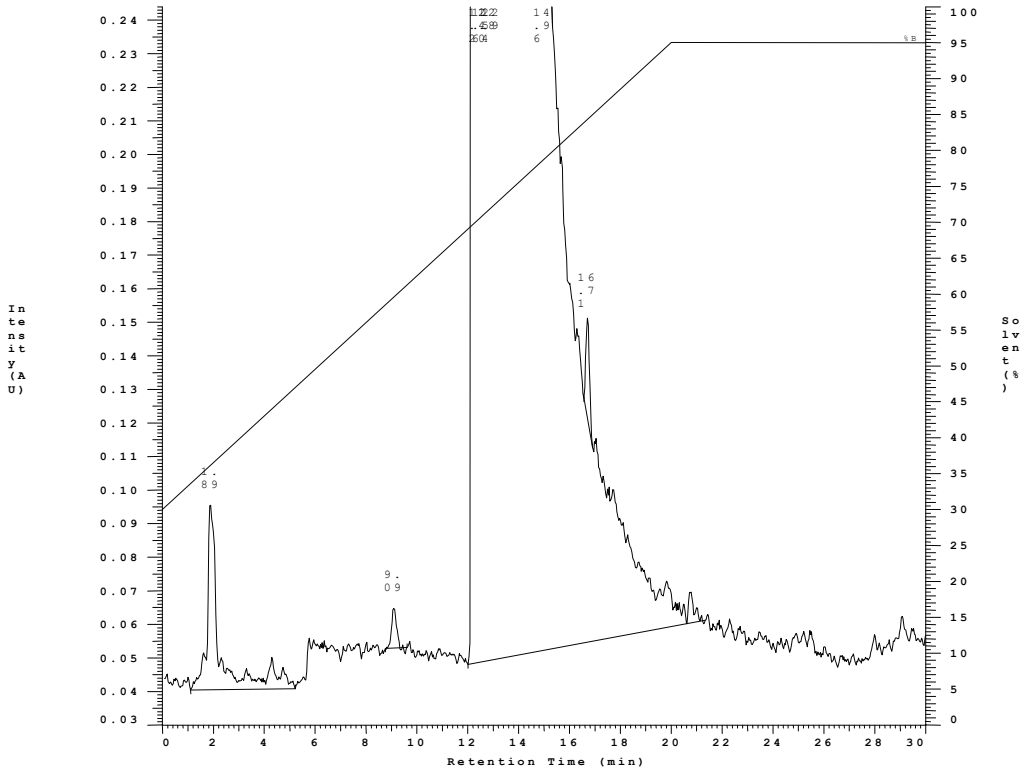

FIG. 9. LC/MS TIC Spectrum of unirradiated sample

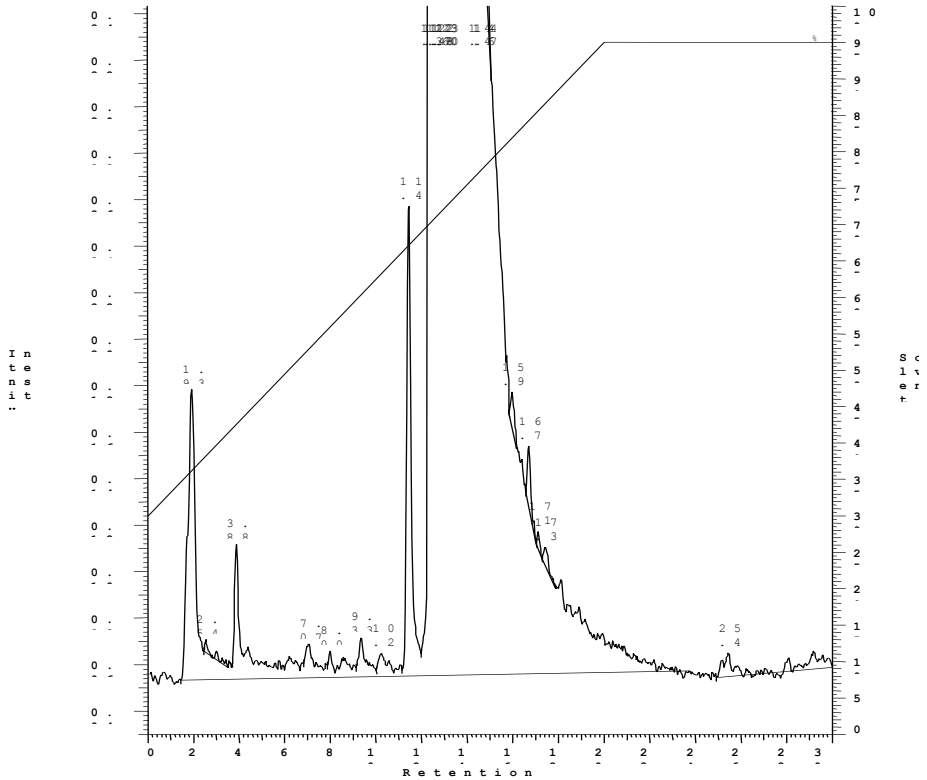

FIG. 10. LC/MS TIC spectrum of irradiated Cyclophosphamide sample

TIC spectrum of irradiated samples shows three prominent peaks at Retention times 1.93, 3.88 and 11.46. Other peaks are relatively small. From fragmentation pattern of the peaks the probable structures of the main degradation products are following.

The origin of formation of higher mass molecules could be due to inter molecule reactions probably occuring during dissolution(free radicals) or in amorphous regions of the crystalline powder during irradiation. No multiples of original mass were detected, indicating fragments of the original molecules getting attached (Table VII). Residual solvents could also form/associate some of the impurities. Quantitation of the impurities could not be done due inherent limitation of small concentration.

Conclusion: Cyclophosphamide has been observed to be radiation sensitive. It is possible to sterilize it at 5 kGy using lower irradiation temperature (-196oC) .

5. DOXORUBICIN HYDROCHLORIDE

Doxorubicin

Hydrochloride is an anticancer drug used for treatment of various types of cancers [7]. The cytotoxicity of the drug is due to its ability to interact with DNA, plasma membrane and various oxidation, reduction reactions. It is available in the form of dry mixtures along with either lactose or NaCl in glass vials for injection. In the present study Doxorubicin with lactose and without lactose were investigated. The molecular structure is given Figure 11. It has a ring structure attached to an amino sugar. The drug is quite heat stable and decomposes only at about 200 ^{o}C. Mol. Weight = 579.99, $C_{27}H_{29}NO_{11}$.HCl

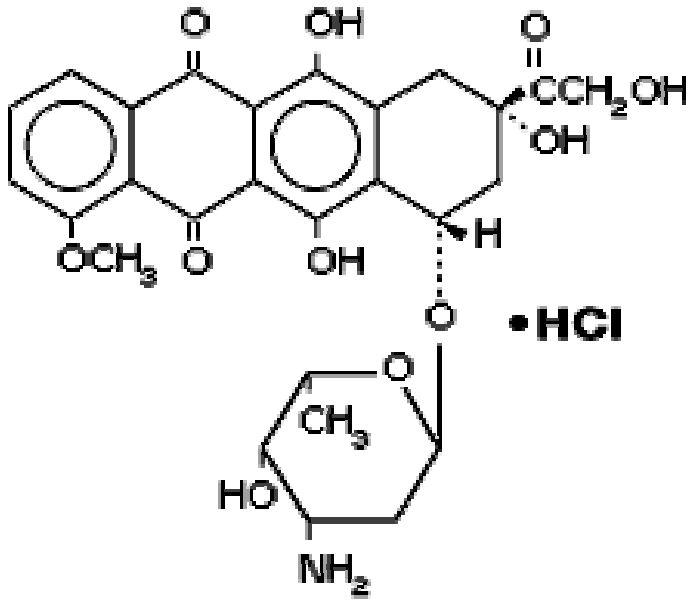

FIG. 11. Structure of Doxorubicin Hydrochloride

Doxorubicin HCl contained about 1.2×10^{16} spins/gm in 25 kGy irradiated sample. Unirradiated sample did not show ESR signal. The free radical detected in irradiated sample have free electron characteristics having a " g" value of 2.004.

The drug vials were irradiated at graded radiation doses of 0, 15, 30 kGy in radiation sterilization plant, ISOMED. The 50 mg vial contained 40 mg of lactose and 10 mg of Doxorubicin.HCl. The non irradiated and irradiated drugs (0,15, 30 kGy) comply with the USP pharmacopoeia tests. Radiation degradation studies were investigated using HPLC of E.Mecrck-Hitachi. Mobile phase contained equal volumes of acetonitrile and 0.01 M Sodium Lauryl Sulphate in 0.02 M phosphoric acid. For monitoring, wavelength 487 nm was used. About 40 µm sample was injected for separation, purity evaluation and recording and degradation profile of DOXO.RP-18 reverse phase column was employed for separation.

The UV-VIS spectra and pH values of the control and irradiated samples did not show significant differences. The comparison of average peak area of five injections for each of control, 15 and 30 kGy irradiated samples did not show significant differences indicating stability of the drug at 30 kGy (Fig.12). The degradation profiles of the drug extracted at 487 nm (Fig. 13) and 200 nm revealed that even unirradiated drug contains number of impurities and irradiation produces one new impurity at retention time 3.77 minute in the HPLC profile. The DAD spectrum of most of the impurities was similar. Therefore, the new impurity has structure similar to that of DOXO. To identify the source of degradation product in Lactose mixture and to evaluate Doxorubicin and NaCl mixture, high purity (>99.5%) standard sample (DOXOR)was exposed to 30 kGy radiation dose

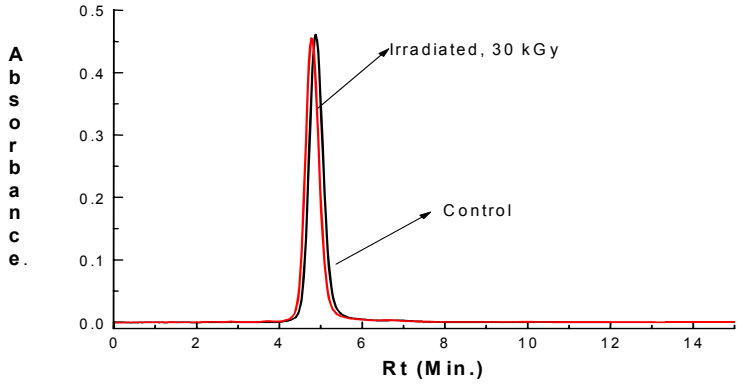

FIG. 12. HPLC PROFILES OF DOXORUBICIN.HCl (lactose) AT 487 nm

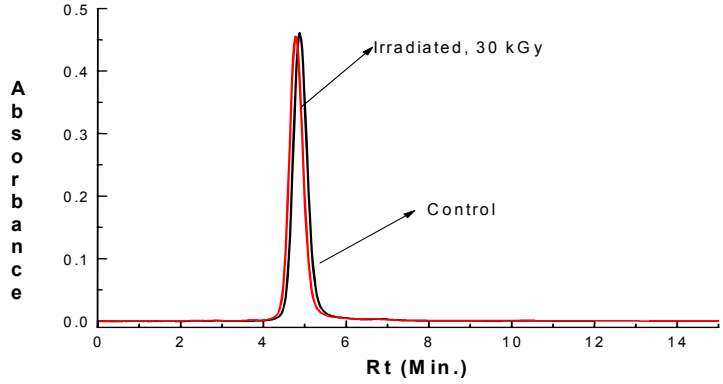

FIG. 13. HPLC PROFILES OF DOXORUBICIN.HCl AT 487 nm

No significant changes were observed in 30 kGy irradiated DOXOR with respect to its original orange-red colour (Fig. 14). The HPLC profile of standard Doxorubicin shows formation of one impurity at Rt 2.45 detectable at 487 nm. At 200 nm another impurity was detected at Rt. 1.45 (Fig. 15). The DAD spectrum of degradation product at Rt 2.5 is similar to Doxorubicin.HCl (Fig. 16) indicating similar structure. Absorption spectrum of the impurity detected at 200 nm is shown in Fig. 17. The impurity at Rt 2.45 is also formed on heating the sample at 130 oC for two hours as shown in Fig. 18. The peak also increases on addition of acid to the injection solution indicating it to be hydrolysis product. From the observations, the product at Rt 2.45 appears to be Adriamycinone and Daunosamine(amino sugar, Rt 1.45).

Considering extinction coefficient of degrdation product at Rt 2.45 similar to that of DOXOR, as eveidenced by DAD spectrum, the concentration of the impurity in irradiated sample (30 kGy) is 0.05% in DOXOR and 0.62% in DOXO (lactose). This indicates that the presence of lactose increases the degradation of DOXOR. Therefore, DOXOR mixture with NaCl should be preferred for injections.

As it is known aqueous solution are highly radiation sensitive and degrade extensively even at lower doses and also observed in the present study (Fig. 19)

Conclusion: Purity values, colour and degradation profile of irradiated (30kGy) Doxorubicin.HCl (powder) did not show significant differences with respect to unirradiated samples. Irradiated drug also passes Pharmacopoeia tests. Therefore Doxorubicin.HCl could be radiation sterilized at normal sterilizing dose of 25

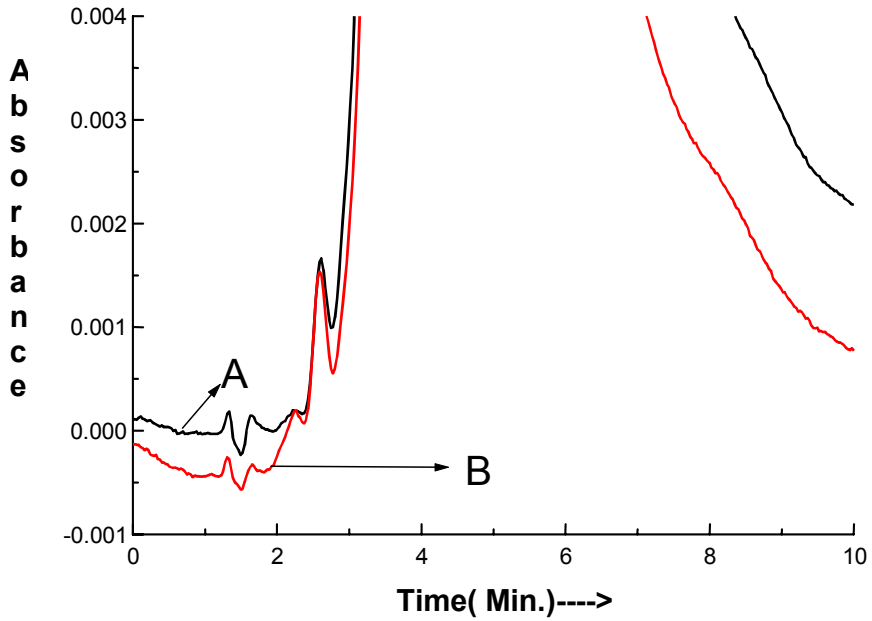

FIG. 14. HPLC DEGRADATION PROFILES OF DOXORUBICIN HYDROCHLORIDE

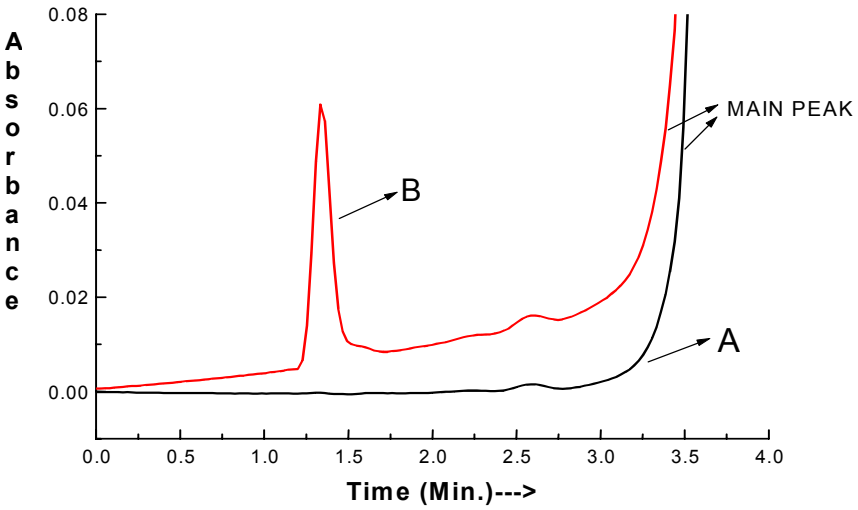

FIG. 15. HPLC DEGRADATION PROFILES OF DOXORUBICIN.HCl

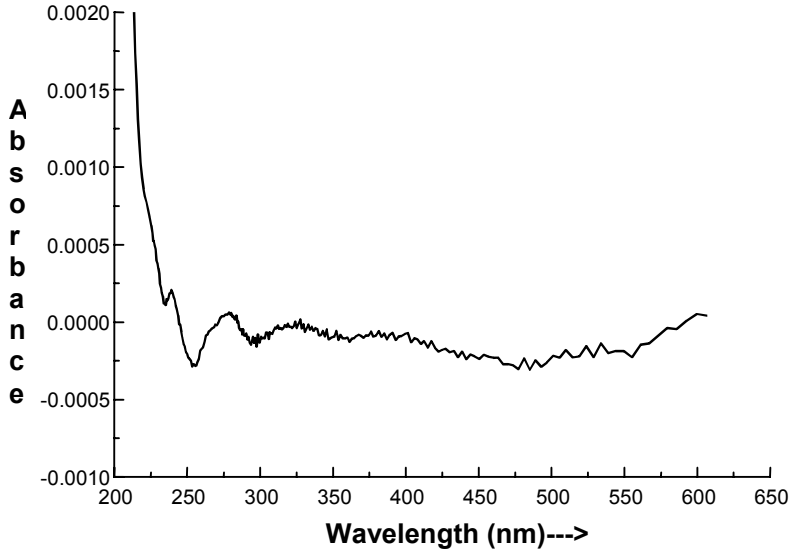

FIG. 16. SPECTRUM OF IMPURITY IN DOXORUBICIN.HCl (27 kGy)

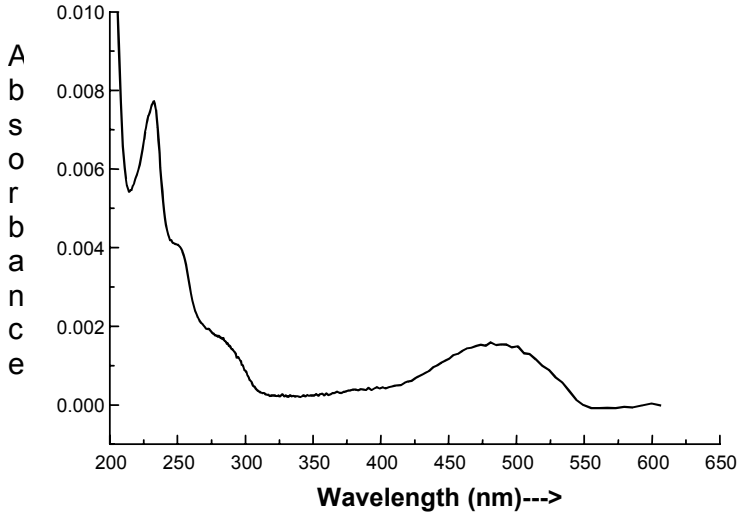

FIG. 17. SPECTRUM (DAD) OF IMPURITY IN IRRADIATED DOXORUBICIN.HCl OBSERVED IN HPLC PROFILE AT 487 nm

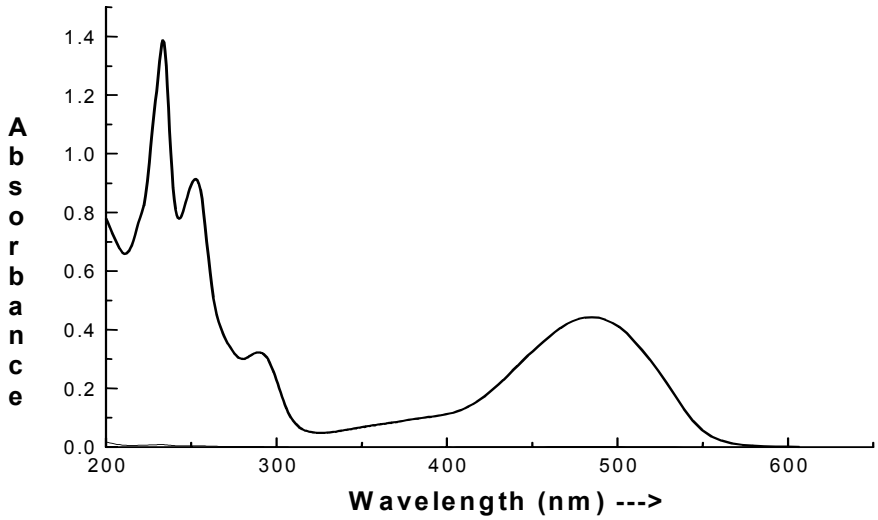

FIG. 18. SPECTRUM OF DOXORUBICIN.HCl, MAIN PEAK IN HPLC PROFILE, RECORDED USING DAD

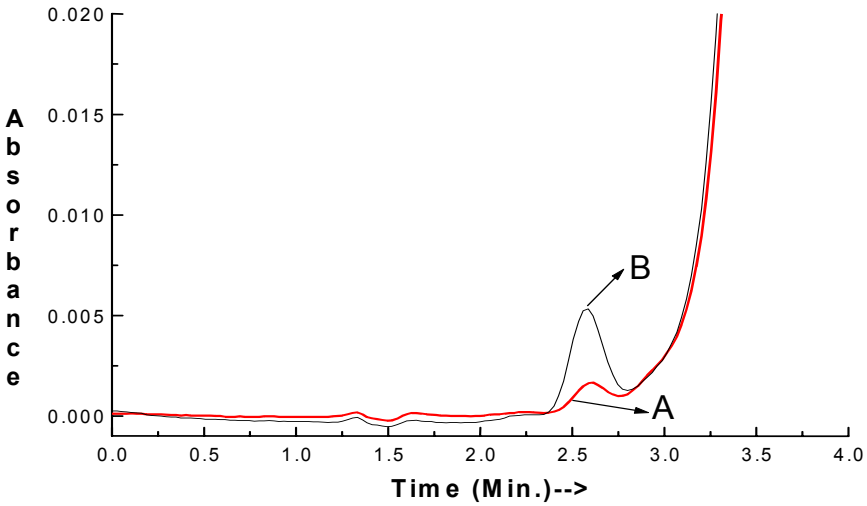

FIG. 19. HPLC DEGRADATION PROFILES OF (CONTROL,A, 80 MICRO gm) AND (B) HEATED AT 130 OC ,DOXORUBICIN.HCl

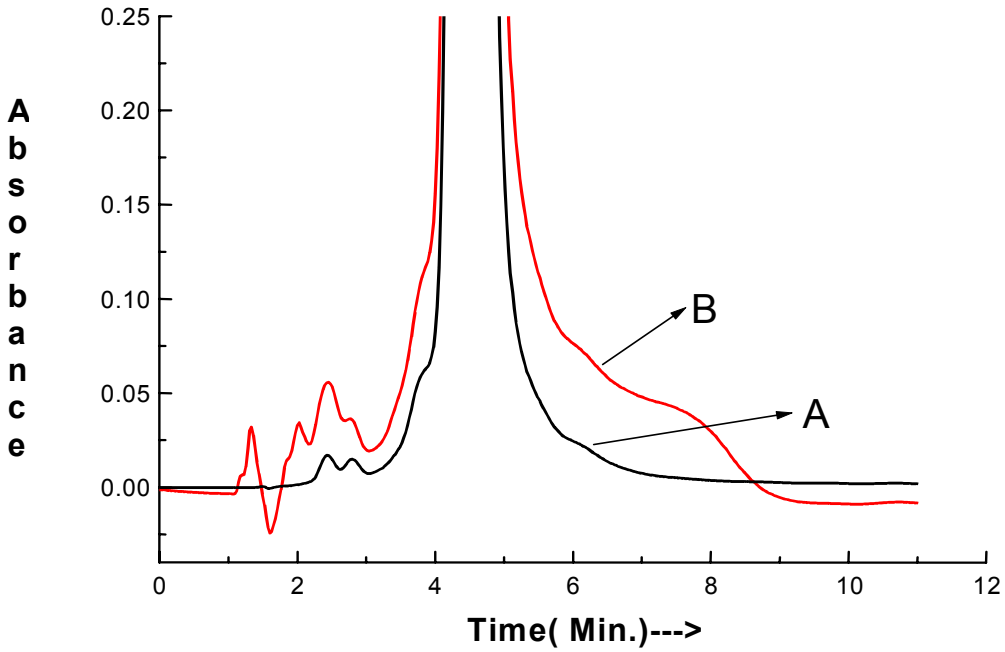

FIG. 20. HPLC DEGRADATION PROFILES OF DOXORUBICIN.HCl

6. CONCLUSIONS

From the results of the studies carried out on Cyclophophamide and Doxorubicin Hydrochloride, the following inferences could be drawn:

- Irradiated pharmaceutical powders, inspite of maintaining high chemical purity value might fail in some of the pharmacopoeia tests. These tests are formulated considering the production processes only. Some minor changes that could arise due to irradiation without affecting the overall efficacy of the drug are not considered. Considering the advantages of radiation sterilization, these points should be in the monographs. It would be desirable to consider radiation sterilization right at development stage of New Drug.
- HPLC degradation profiles should be used to evaluate quality of irradiated drugs. Because other techniques may not be able to resolve the differences.
- HPLC/MS analysis should be preferred for separation and identification of the degradation products. Total Ion Current spectrum can give information about the major degradation products which could be identified with the help of fragmentation pattern.
- Lower irradiation temperature could reduce some of the undesirable changes in the irradiated products.
- High dose rates Electron Beam Irradiation may not be desirable for thermal sensitive drugs like Cyclophosphamide.
- ICH guidelines could be useful in evaluating irradiated products with respect to presence of degradation products.
- The question of toxicity of small concentration of degradation product is debatable. It would be difficult to resolve toxicity status of unirradiated and irradiated drugs. Cyclophosphamide and Doxorubicin.HCl are themselves known cytotoxic drugs. Still it would be preferable to carry out some biological tests to rule out undue toxicity and histamine like substances. Considering an irradiated drug as NEW DRUG is not in line with scientific data available so far.
- Undesirable radiation effects can always be reduced by using aseptic conditions and lower irradiation doses .
- Residual solvents also could form/ associate new degradation products in the irradiated drugs[8]

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