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Article

Developed and validated stability indicating HPLC method for the determination of epirubicin in bulk drug, marketed injection and polymeric nanoparticles

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Present work is aimed to develop a simple, sensitive, robust and reliable HPLC method for routine quality control of epirubicin (EPI) in bulk drug, marketed injections and polymeric nanoparticles. Separation was carried out by C18 column. Isocratic elution was carried out using mobile phase A: 0.16% o-phosphoric acid solution, B: acetonitrile and methanol mixture (80:20, v/v) in the ratio of 60:40 (A: B) while the flow rate was maintained at 1mL/min. Analyses were performed at 233.5 nm using PDA detector. Excellent linear relationship was observed between peak-area versus drug concentration in the range of 1.0-100.0 µg/mL (r^2, 0.999). Developed method was found to be sensitive (Limits of detection and quantification were found to be ~8 ng/mL and ~25 ng/mL, respectively), precise (RSD <1.0%, for repeatability and <2.0% for intermediate precision, within acceptable ranges of precision), accurate (recovery in different dosage form, 94.65 -100.26%, within acceptable range, 80-120%), specific and robust (% RSD <2, for system suitability parameters). Stress-induced degradation studies demonstrated that method can suitability be applied in the presence of degradants. Developed method has been successfully applied for the determination of entrapment efficiency, drug loading, *in vitro* release profile, *in vitro* permeation studies as well as stability assessment of polymeric nanoparticles.

Keywords: Epirubicin. HPLC. Polymeric nanoparticles. Quality control. Forced degradation.

INTRODUCTION

Epirubicin (10-(4-amino-5-hydroxy-6-methyl-oxan-2-yl)oxy-6,8,11-trihydroxy-8-(2hydroxy acetyl)-1-methoxy-9,10-dihydro-7H-tetracene-5,12-dione) is an anthracycline anticancer agent which is used for the treatment of node positive breast cancer, ovarian cancer, gastric cancer, lung cancer and lymphomas (Neil, 2006). Epirubicin is obtained by chemical transformation of a substance produced by certain strains of *Streptomyces peucetius* (Neil, 2006). It acts by forming a complex with DNA by intercalation into nucleotide base pairs thus hindering DNA and RNA synthesis (Omrod *et al.,* 1999). It is favoured over doxorubicin as it is found to be less toxic at equimolar dose which may be due to the different spatial orientation of the hydroxyl group at the 4' carbon of the sugar moiety results in opposite chirality which leads to its faster elimination and reduced toxicity (Neil, 2006; Sweetman, Martindale, 2007). It is available as intravenous injection but due to sudden rise in the blood level and rapid elimination often to sub therapeutic levels necessitates frequent dosing leading to a serious adverse effect. Hence, we formulated polymeric nanoparticles delivery system, to achieve controlled drug release, which will not only limit the side effects but also help in reducing dose and dosing frequency. With the advancement in nanotechnology-based delivery system and their complex pharmacokinetics requires a stringent quality control of these dosage forms to maintain its safety and efficacy. At present, EPI is being used extensively in clinical practice and therapy. Therefore, accurate and validated analytical method is required for quantitative analyses of EPI in

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bulk drug, marketed injection, polymeric nanoparticles, *in-vitro* as well as in biological samples. Several analytical methods such as LC/MS-MS (Wall *et al.,* 2007; Sottani *et al.,* 2009), LC equipped with UV (Dodde *et al.,* 2003; Badea *et al.,* 2005), fluorescence (Dine *et al.,* 1990), and electrochemical detector (Ricciarello *et al.,* 1998) have been reported so far for analysis of EPI in biological fluids. These analytical methods (LCMS, Fluorescence detection) are more sensitive and accurate, however expensive, hence, limit their utility in the routine quality control of pharmaceutical dosage forms. In addition, no method has been reported so far for quantitative analysis of epirubicin in polymeric nanoparticles.

In the given study, a simple, rapid, sensitive and accurate HPLC-UV method is developed which is capable of detecting and quantifying minute quantity of drug accurately and precisely with a short run time in bulk drug, marketed formulation, polymeric nanoparticles, and *in-vitro studies* sample which may be further extended to *in-vivo* biological fluids such as plasma, serum etc. Here, the method is validated for accuracy, precision, selectivity, sensitivity and various other validation parameters as per ICH guidelines.

MATERIAL AND METHODS

Instrumentation and chromatographic conditions

The liquid chromatographic system e2695 separation module (Waters Alliance, Milford, MA, USA) equipped with a gradient flow control pump, online solvent degasser, auto-sampler, PDA detector 2998, column manager and pre-column heater was used for the analysis. All the parameters of HPLC were controlled by LC solutions software Empower 2. Chromatographic separations were obtained by using Purosphere® C18 (5 µm, 250*4.6 mm) column, which was maintained at 30 ºC. Ten microliters of sample was injected into HPLC system. Isocratic elution was carried out using mobile phase which is the mixture of aqueous phase (A, 60 v/v) and organic phase (B, 40 v/v). Aqueous phase (A) comprising of 0.16% O-Phosphoric acid solution while organic phase (B) comprising of acetonitrile and methanol mixture (80:20). The flow rate was maintained at 1mL/min. The mobile phase was filtered through 0.45 µm filter (Sartorius, Germany) and deaerated for 15 min by sonication before injecting into the system and column was allowed to equilibrate for 20 min with the mobile phase. All samples were analyzed at wavelength of 233.5 nm for a total run time of 12 min. Data acquisition and data handling were performed by Empower Software v2.0. Developed method was validated for parameters like precision, accuracy, robustness, specificity, sensitivity, linearity as described in ICH guidelines (ICH, 2005).

Method development

Preparation of stock and standard solutions

Ten milligrams of EPI was accurately weighed and transferred to a 10 mL volumetric flask and dissolved in methanol followed by sonication and subsequently filtered through 0.45 µm filter to get a stock solution, 1 mg/mL. Stock solution was further diluted to obtain the required concentrations (1-100 µg/mL) before injecting into the system for analyses. Quality control samples at three levels, low (2 µg/mL), intermediate (10 µg/mL) and high (50 µg/mL) were also prepared in the same way.

Method validation

The developed method was validated for linearity range, precision, accuracy, robustness, limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines (ICH, 2005).

System suitability

For the assessment of system suitability, 10 µL of epirubicin solution (10 µg/mL, n=6) were injected and system suitability parameters like retention time, peak area, theoretical plates and tailing factor were calculated and compared with standard accepted values.

Linearity

A 7-point calibration curve was constructed in a concentration range of 1-100 µg/mL for linearity. The mean peak area of epirubicin was plotted against its concentration and linear least square regression analysis was performed.

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the signal to noise ratios (s/n) using analytical responses of three and ten times of the background noise, respectively using the following equation, s/n ratio was calculated through Empower software version 2 (Warsi *et al.,* 2011; Tariq *et al.,* 2015a).

$$LOD = 3.3x\frac{s}{n}$$

$$LOQ = 10x\frac{s}{n}$$

Precision

Precision of the methods was determined in terms of

intra-day and intermediate precision. Intra-day precision (Repeatability) of developed method was determined by analyzing quality control samples (2 µg/mL, 10 µg/mL, 50µg/mL, each 6 replicates) using identical test material, identical analytical method and similar experimental conditions over a short period. Results were analyzed by identical evaluation method. Intermediate precision was determined on different days, rest of the conditions was followed as described for intra-day precision. Intra-day and intermediate precision were expressed as relative standard deviation (RSD, %).

Accuracy

Accuracy of the developed method was assessed by assaying 9 samples. Test samples (100 µg/mL claimed amount) were spiked with 3 levels of claimed amount i.e. low (LQC, 50 µg/mL), medium (MQC, 100 µg/mL) and high (HQC, 150 µg/mL). Accuracy was determined as following and expressed in terms of % recovered;

$$Recovery\ (\%) = \frac{Experimentally\ determined\ concentration}{Theoretical\ concentration} \times 100$$

Robustness

Robustness of method was determined by changing pH of mobile phase, column temperature and column manufacturer. The robustness of methods was expressed as RSD (%) for system suitability parameters like peak areas, RT, and peak tailing.

Forced degradation studies

The specificity of the method was determined by exposing drug solution of known concentration (100 µg/mL) to different conditions like acidic (0.1 M HCl for 24 h), basic (0.1 M NaOH for 24 h), oxidizing (30% H_2O_2 v/v for 24 h), photolytic degradation (for 24 h), and thermal stress at 50 ºC for 24 h (Tariq *et al*., 2014). The resulting solutions were diluted and processed accordingly and analyzed for the drug peak as well as degradation product.

Applications of method

Epirubicin loaded poly-lactide-co-glycolic acid nanoparticles (EPI-NPs) were prepared by double emulsion solvent evaporation as described in our previous report (Tariq *et al.,* 2014).

Determination of entrapment efficiency and drug loading

Entrapment efficiency and drug loading was calculated as following;

$$Entrapment\ efficiency\ (\%) = \frac{Amount\ of\ drug\ loaded\ into\ the\ NPs}{Total\ amount\ of\ drug\ taken} \times 100$$

$$Drug\ loading\ (\%) = \frac{Amount\ of\ drug\ loaded\ into\ the\ NPs}{Total\ amount\ of\ formulation} \times 100$$

In vitro Release Profile

In vitro release study was performed by dialysis bag method and compared with free drug solution. EPI (2 mg) and EPI-NPs (equivalent to 2 mg EPI) were suspended into 5 mL dissolution media and poured into dialysis bag ((MWcut off of 8–10 kDa, Spectra/Por®) and subsequently dialysis bag was dipped into 50 mL of dissolution media. Study was carried at 37 ºC and 100 rpm under magnetic stirrer. Samples were withdrawn at regular time interval and analyzed by HPLC.

In vitro permeation study

EPI is a substrate for both P-glycoprotein (P-gp) and cytochrome P450 (CYP 450), and also highly hydrophilic in nature hence elicits poor oral bioavailability. Therefore, epirubicin loaded PLGA NPs were developed to improve oral bioavailability. Developed formulation was evaluated for *in-vitro* permeation profile by using Caco-2 cell line model and intestinal gut sac method as previously reported (Tariq *et al.,* 2015b).

For cellular transport study, cells (10^5/insert) were grown on polycarbonate membrane filters with 0.4 µm pore size and 1.12 cm2 growth area. Only cell monolayers with TEER values over 300 Ω cm^2 were included in the study. Transport of EPI-S and EPI-NPs was studied in the apical to basal direction. For the purpose, 0.5 mL of test solutions (diluted with serum free growth media) were added to apical side whereas 1.5 mL of serum free growth media was added to basolateral side. At each time point, 0.2 mL samples from basolateral side were withdrawn and stores at -20 °C.

For intestinal permeation study, Ileum (5-7 cm) was washed with saline followed by ligation of on end while other end was mounted on assembly use to conduct the study. Ileum sac was filled with EPI-S solution (1 mL, 100 µg/mL) or EPI-NPs suspension (equivalent to 100 µg of EPI in 1 mL) and suspended into pre-warmed (37 ± 0.5 °C) and pre-oxygenated Tyrode's buffer (10 mL). Samples (0.5 mL) were withdrawn defined time intervals,15, 30, 45, 60, 75, 90 min and stored at -20 °C.

Samples of cellular and intestinal transport studies were analyzed by HPLC and permeation data was expressed as flux (Jss, µg/cm2/h).

Stability studies of EPI-NPs as per ICH guidelines

Stability study of (EPI-NPS) was carried out as per ICH Q1A (R2) guidelines (ICH, 2003). Formulation was

subjected to 5±3 ºC (long term study) and 25±2 ºC and 60±5% RH (accelerated storage condition) for a period of six months. Samples were analyzed at regular time intervals 0, 30, 60, 90, and 180 days for the change in their drug content by HPLC.

RESULTS AND DISCUSSIONS

Method development

In the present study, various parameters like column, mobile phase composition, mobile phase ratio, flow rate and its pH were optimized to obtain a good peak shape with high resolution, symmetry and reproducibility. Columns such as Supelco® and Purosphere® were used and the best peak was obtained with Purosphere®. Different mobile phases were tried such as acetonitrile-water, methanol-water, and mixture of acetonitrile: methanol and water. As the epirubicin hydrochloride is an ionizable compound hence best peak was expected at lower pH therefore pH adjusted (pH 3) with phosphoric acid which resulted in best peak with good symmetry and high resolution. Thus, best peak and low retention time was obtained with 0.16% aqueous o-phosphoric acid solution (A) and mixture of acetonitrile: methanol, 80:20 v/v (B) when isocratic elution was carried out at the mobile phase ratio of 60:40 (A:B). The flow rate was maintained at 1mL/min. All the above optimized parameters produced a peak with good shape and very short retention time, 4.52±0.3 min (Figure 1) which is the lowest retention time of all the methods developed so far in our belief. However, the total run time was 12 min, giving an additional time for complete elution of excipients and degradation products to avoid carry over peak in the next injections.

Method validation

System suitability

System suitability was evaluated by six replicated injections at a concentration of 10 µg/mL and system suitability parameters were summarized in Table I. All the parameters met the acceptance criteria (USP) and relative standard deviation for individual parameters was found to be less than 1%.

Linearity

The linearity of an analytical method is its capability (within a given range) to achieve test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity of method was determined by calibration plot constructed between peak area and analyte concentration (Table II and Figure 2). The linear regression data over the concentration range of 1-100 µg/mL was found to be within 95% confidence limit with a correlation coefficient (r^2), 0.999 which confirmed the excellent linearity of developed method in the range (Table II).

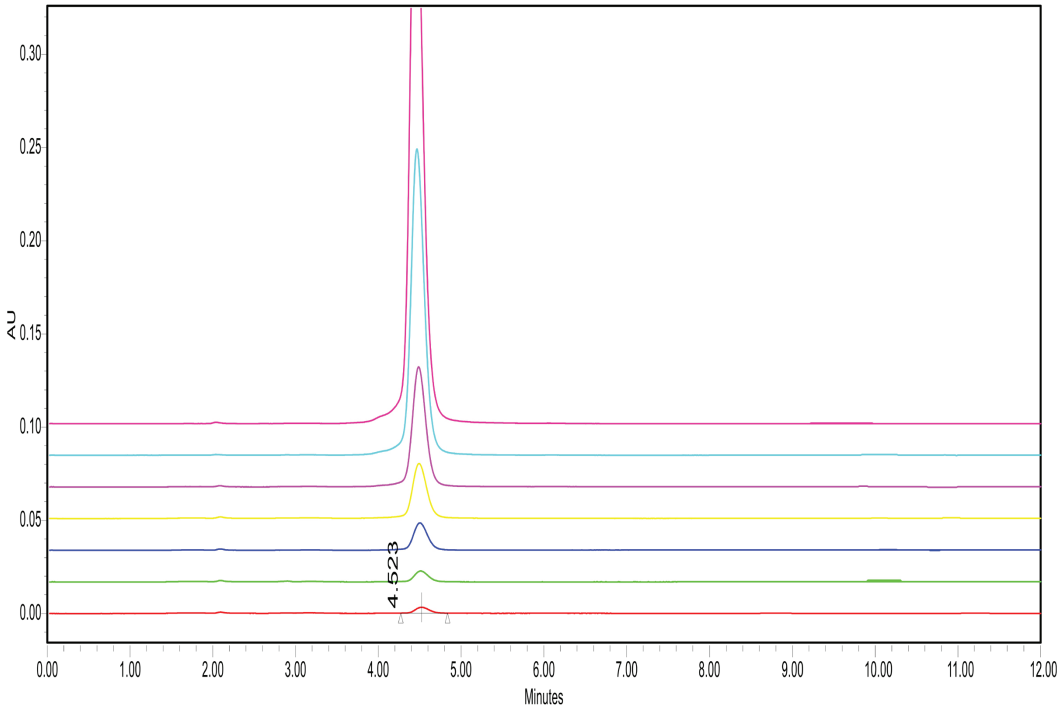

FIGURE 1 - Overlay of representative chromatograms of epirubicin.

TABLE I - System suitability (n=6)

	Peak area	RT	UPS tailing	HETP
	348533	4.548	0.94	3223
	350123	4.503	0.92	3312
	352437	4.528	0.93	3245
	345673	4.539	0.94	3284
	349896	4.511	0.92	3305
	351001	4.55	0.92	3213
Mean	349611	4.53	0.93	3263.7
SD	2320.85	0.02	0.01	42.49
RSD	0.66	0.43	1.06	1.30

TABLE II - Linear regression data for calibration plots

Parameters	Regression Values
Linearity range	1-100 µg/mL
Regression equation	Y=37712 X -27028
Correlation coefficient (r^2)	0.999
Slope	37712
Intercept	-27028

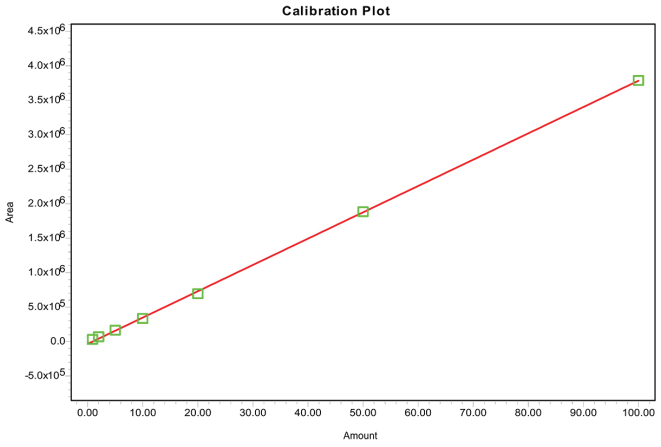

FIGURE 2 - Calibration plot of epirubicin hydrochloride (concentration range 1-100 µg/mL).

Limit of detection and limit of quantification

Kurbanoglu et al. (2013) reported LOQ for epirubicin 27 ng/mL and LOD and LOQ were found to be 8ng/mLand 25 ng/mL for our developed method which indicated that the developed method is more sensitive than previous one. Sensitivity of any analytical method is expressed in term of limit of detection and limit of quantification (LOD and LOQ). LOD represents the lowest amount of analyte that can be detected but not quantified precisely, while LOQ represents the quantity of analyte which can be quantified accurately and precisely. Low values of LOD (8 ng/mL) and LOQ (25 ng/mL) reflect that the developed method is sensitive.

Precision

Table III summarizes repeatability and intermediated precision which were found to be <1% and <2%, respectively. Both repeatability and intermediated precision were within the acceptance limit (ICH, 2005), thus developed method is said to be precise. Precision represents random error i.e. errors which occur at any point of analysis from sampling to calculation. It can be determined at 3 levels i.e. repeatability (Intra-day precision 1st level), intermediate precision (2nd level) and reproducibility (3rd level). Repeatability is determined over a short period of time under similar test concentration, materials, method and experimental conditions while intermediate precision is determined on different days, by different analysts using different equipment. Reproducibility represents inter-laboratory precision, usually not considered as a part of analytical validation until unless developed method is meant for analytical transfers. Hence, precision was determined in terms of repeatability and intermediate precision only.

Accuracy

Accuracy of a method is defined as closeness between the experimentally measured value and the true value. Difference between experimentally measured value and true value represents the systematic error or bias of a method in the presence of excipients/formulation components. Accuracy of method was determined as % recovered

TABLE III - Intra-day and inter-day precision (n=6)

Theoretical Conc. (µg/mL)	Intra-day precision		Inter-day precision	
	Mean Conc. (µg/mL)	% RSD	Mean Conc. (µg/mL)	% RSD
2 (LQS)	2.094±0.01	0.48	2.08±0.03	1.63
10 (MQS)	9.87± 0.05	0.55	9.88± 0.135	1.36
50 (HQS)	49.11±0.215	0.43	49.28±0.50	1.0

TABLE IV - Accuracy as % recovery (n=3)

Dosage form	Label claimed (µg)	Amount added (µg)	% recovered	% CV	Bias
	100	50	99.45±.16	0.156	-0.55
Bulk drug	100	100	99.89±0.08	0.087	-0.11
	100	150	100.026±0.29	0.29	0.027
	100	50	95.32±2.83	2.97	-4.68
Marketed Injection	100	100	96.15±1.62	1.69	-3.85
	100	150	96.68±2.9	3.0	-3.32
	100	50	92.22±2.8	2.80	-7.77
PLGA NPs	100	100	92.89±1.93	1.93	-7.11
	100	150	94.65±1.93	1.93	-5.35

amount after spiking 50%, 100%, and 150% of claimed concentration of bulk drug, the marketed injection and PLGA NPs sample. These samples were analyzed by the proposed method and results were expressed in term of % recovered, % CV and bias (Table IV). The % recovery of epirubicin was obtained in the range of 94.65 -100.26% which was within the acceptance range 80-120% (ICH, 2005). Thus, method can be said accurate.

Robustness

Method was also evaluated for its robustness by measuring its capacity to remain unchanged through putting little but intentional variations in method's parameters. Robustness of the method was determined by calculating RSD (%) of system suitability parameters (Table V) after making deliberate changes in column, column temperature and mobile phase pH, no substantial change was observed in system suitability parameters (% RSD< 2) which confirmed the consistency of developed method during normal usage.

Forced degradation studies

Forced degradation studies were performed to determine the specificity of the analytical method. In this study, the developed method was found able to clearly separate the analyte peak and the degradation products (Table VI). Chromatograms obtained after forced degradation study (acid, base, photo, oxidative, thermal stress) demonstrated separate peaks of analyte and degradants, which indicated the specificity of the developed method. Epirubicin was found to be most resistant to acid treatment while highly sensitive to alkali treatment (Figure 3).

Applications of method

For the development and optimization of epirubicin loaded PLGA NPs, various formulation parameters like polymer type, polymer concentration, drug-polymer ratio, sonication time, stabilizer type, stabilizer concentration,

TABLE V - Robustness (n=3)

		Parameter evaluated		
		Peak area (% RSD)	RT (%RSD)	Tailing (% RSD)
Column	Purosphere® Supelco®	1.26	0.209	1.26
Temperature	25 ºC 30 ºC	1.37	0.53	0.76
pH	3.0 3.5	1.55	0.51	0.65

TABLE VI - Stress induced degradation studies

Stress conditions	% Mean Recovered ± SD	Figure
Untreated Sample	100	4a
HCl (0.1M) 24 h	98.32±0.87	4b
NaOH (0.1 M) 24 h	0.0%	4c
Daylight, 24 h	90.23±0.96	4d
H_2O_2 (30%, v/v), 24 h	87.36±0.91	4e
Thermal treatment (50 °C, 24 h)	82.16±1.01	4f

volume of internal aqueous phase etc. were studied to see their effect on the entrapment efficiency and drug loading of NPs. Entrapment efficiency was found to be in the range of 11.5-44.12% while the drug loading was found to be in the range of 1.13-4.34%.

In-vitro release study of free epirubicin solution showed a very fast release i.e. drug diffused out completely within an hour while epirubicin loaded PLGA NPs demonstrated sustained biphasic release pattern, initially ~20% of total loaded drug was diffused out in first 2 h and remaining thereafter i.e. ~85% in 48 h. Burst effect can be explained by high drug solubility in dissolution media and availability of drug molecules at the surface which leached out rapidly while sustained release can be attributed to existence of drug molecules at the core of hydrophobic polymer matrix.

Epirubicin loaded PLGA NPs demonstrated better permeation profile (Jss 0.63 µg/cm^2.h) over free drug solution (Jss 0.23 µg/cm^2.h) across Caco-2 cell line. Similarly, better permeation profile of epirubicin across rat ileum through NPs (Jss 1.0 µg/cm^2.h) was observed when compared with free solution (Jss, 0.223 µg/cm^2.h). Significantly high flux across the Caco-2 cell line and rat ileum ($p<0.001$) through NPs can be attributed to encapsulation of drug into NPs provided protection to it from P-gp, and CYP-450. In addition, endocytic uptake of particles improved the permeation.

Figure 4 summarizes stability data. Six-month stability profile demonstrated no significant change in assay at both long-term condition, 5±3 °C (~1.8%) and accelerated storage condition, 25±2 °C, 60±5% RH (~2.5%). As per the ICH guidelines (Q1A R2), more than

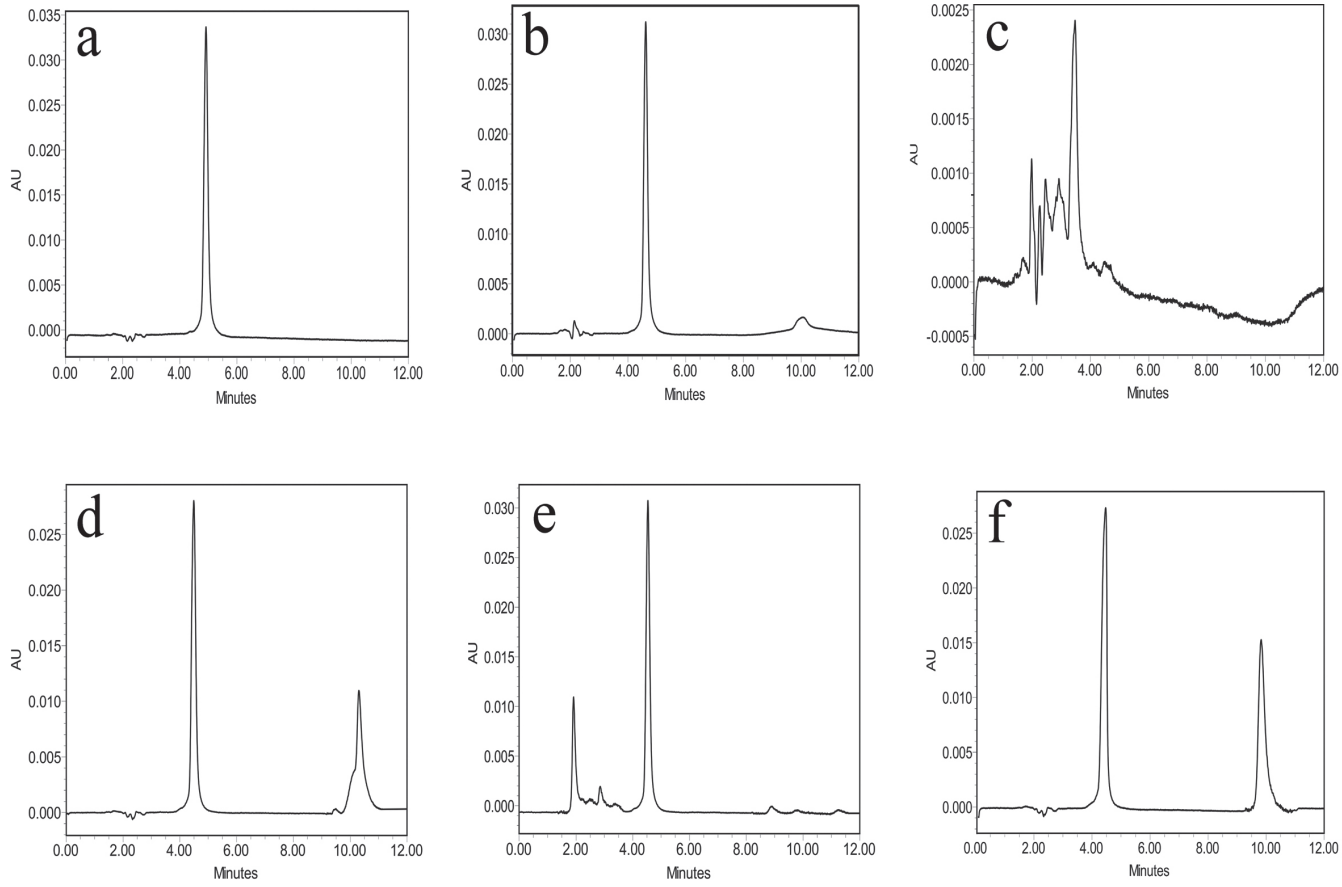

FIGURE 3 - Chromatograms obtained from forced degradation studies; **a)** Pure epirubicin hydrochloride; **b)** acid treatment; **c)** alkali treatment; **d)** Photo treatment; **e)** Oxidative treatment; **f)** Thermal treatment.

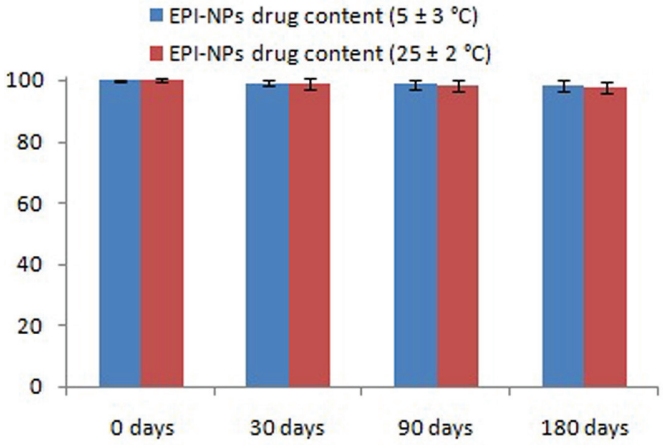

FIGURE 4 - Change in drug content into the EPI-NPs after storage at normal condition (5±3 °C) and accelerated condition (25±2 °C).

5% change in assay is considered "significant" at any sampling point during 6 months at accelerated conditions (ICH, 2003). Thus, formulation can be considered stable.

CONCLUSION

A simple and sensitive HPLC method for the quantitative estimation of epirubicin was successfully developed. Statistical assessment of methods proved that the developed method is specific, precise, and accurate and can be used successfully for the quantitative estimation in bulk drug, marketed formulation and polymeric nanoparticles for routine quality control of said dosage form. Stress induced degradation studies disclose the suitability of method for the quantification of drugs in the presence of degradants. The developed method has been successfully applied for the estimation of drug loading, entrapment efficiency, *in vitro* release profile, *in vitro* permeation profile and stability of the developed polymeric nanoparticles.

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