ORIGINAL ARTICLE

CONTENTS

Optimization and validation of an HPLC method for the quantification of Etodolac in liposomes and assessment of its pharmacokinetic profile

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ABSTRACT

Aim: To develop and evaluate high performance liquid chromatogram method to simultaneously quantify Etodolac in liposomes.

Materials and Methods: The chromatographic separation was carried out on the C18 column, mobile phase of methanol: acetonitrile: water at a ratio of (30:60:10) and a flow rate of (1ml.min^{-1}), with a detection wavelength of 225 nm. Liposomes were prepared by thin film hydrating method using Distearoylphosphatidylcholine and cholesterol.

Results: Linear concentration range of the assay was 2.5-25 µg.ml^{-1} with a regression coefficient factor $R^2 \geq 0.999$ with a retention time of Etodolac 4.11 ± 0.081 min. The method was rigorously validated for linearity, accuracy, precision, specificity, and stability, ensuring its reliability. The concentrations of Etodolac 2.5, 5, 7.5, and 10 µg.ml^{-1} were used, with the intraday n=6 and intraday n=6 precision and accuracy for Etodolac meeting the accepted criteria of the United States Food and Drug Administration guideline. The limited detection and limited quantification values for Etodolac were 0.42 µg.ml^{-1} and 1.3 µg.ml^{-1}, respectively. Conventional liposomes have characterization (PZ 88 ± 0.02, PDI 0.012 ± 0.01, %EE 89.5 ± 0.004, and Z-potential -10.2mV ± 0.001), while PEGylated liposomes (PZ 92 ± 0.003, PDI 0.01 ± 0.02, %EE 94.3% ± 0.01, and Z-potential -13.6mV ± 0.012).

Conclusions: The developed and validated method of HPLC was successfully applied to determine Etodolac in bulk and liposomes for routine quantitative analysis.

KEY WORDS: Etodolac, high performance liquid chromatogram, validation, analytical chemistry

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ABBREVIATIONS

HPLC: High Performance Liquid Chromatogram
BCS: Biopharmaceutical Classification System
DSPC: Distearoylphosphatidylcholine
FDA: Food and Drug Administration
LOQ: limit of quantification
LOD: Limit of Detection
RSD: Relative Standard Deviation
CCD: Central Composite Design
RBF: Round-Bottom Flask
PCS: Photon Correlation Spectroscopy
RSD: Injection Repeatability
k': Capacity Factor
T: Tailing Factor

INTRODUCTION

The bio-analytical method can be used to measure and identify the drug and its metabolite in biological matrices like blood, plasma, serum or urine, while the analytical method is used to determine the quantitative or qualitative of the drug in the sample. The chromatographic method represents a simple and accurate method that permits scientists to separate the closely related components in a complex mixture easily. The chromatographic process can be qualitative, and however, used to identify the active chemical substance, which establishes the amount of these given substances [1]. The Etodolac (ETO) drug belongs to the non-steroidal anti-inflammatory drug (NSAID) [2-3] with chemical formula 1, 8- diethyl-1, 3, 4, 9- tetrahydropyran (4, 4-b) –indole- 1- acetic acid [4-5]. Etodolac is widely used as an antipyretic [6], for patients with severe pain [7] or severe inflammation due to surgery, postoperative analgesia, rheumatoid arthritis, and osteoporosis [4, 8-9]. Etodolac is extensively metabolize in the liver to an inactive oxidative metabolite and a primary elimination renal route. Etodolac is a class II according to the biopharmaceutical classification system (BCS), with poor water solubility with limited dissolution, and poor absorption [10]. The recommended dose is 100-300 mg twice or three

times a day, which makes compliance and convincing for the patients difficult. Etodolac is a selective inhibitor for COX_2, and it has been reported that Etodolac is selective for COX_2 enzyme ten times as that for the COX_1 enzyme. Different methods were used to analyse Etodolac, either alone or in combination with other drugs, as HPLC with ultraviolet detection represents the most popular method used, but it is time-consuming and laborious [8], voltammetric [11], sequential injection analysis, other methods that can be used for the departure of Etodolac and its metabolite but not for determining the quantity like capillary electrophoresis [11-12] and spectrofluorimetric method [13-14] and high-performance thin-layer chromatography [15] furthermore was mention for identified Etodolac in bulk solution and in the pharmaceutical dosage form. In addition to that, several HPLC methods for Etodolac enantiomers and mechanism of depredate also have been reported [13, 16] but a fewer method to identify of Etodolac in liposome. Liposomes are artificial carriers with a spherical shape consisting of cholesterol and nontoxic phospholipid. Liposomes are biocompatible and biodegradable, having one or more phospholipid bilayers. Liposomes are an effective drug delivery system because they have the ability to load with hydrophilic or lipophilic drugs. In addition to that, liposomes can be targeted by a specific ligand to specific cells [17-20]. The aim of this study was a quantitative assay of Etodolac using the HPLC method. The method was developed and validated as a new, simple, sensitive, cost-effective, and accurate HPLC method for quantification of Etodolac as free and loaded in liposome. The method was developed using optimization parameters different from the literature. The analytical method was validated for sensitivity, selectivity, linearity, recovery, accuracy, precision, and stability according to the United States Food and Drug Administration (FDA) and the International Conference on Harmonization Guideline (ICH) [1, 21].

MATERIALS AND METHODS

MATERIALS

Etodolac and cholesterol 99%, were purchased from Shanghai Macklin Biochemical Co. Ltd/China. Phospholipid DSPC and DSPE-PEG were purchased from WEIHUA BIO. Acetonitrile 99%, methanol 99.8%, chloroform 99.8%, and water 100% are HPLC grade purchased from Hi Media Laboratories Pvt Ltd. in India. The used vials were purchased from CKlab in China. The C18 column and guard column were purchased from Novachrom and Wayeal in China.

METHOD

INSTRUMENTATION AND CHROMATOGRAPHIC CONDITIONS OF HPLC

A Knauer AZURA HPLC instrument from Germany was used with a UV-visible detector and a Clarity Chrom software version. The chromatography was performed isocratically at room temperature. The chromatographic separation was carried out using a C18 column, 250 mm × 4.6 mm, 5 µm particle size (Novachrom, Wayeal from China) with core-shell silica. The mobile phase used consisted of A methanol, B acetonitrile, and C water, which were mixed well and degassed by ultra-sonication in a water bath for 15 min using an HPLC pump that can be used to produce a mobile phase with a ratio of (60:30:10) delivered at a flow rate of 1 $ml.min^{-1}$ into the column, an opaque vial size 1.5 ml was used, and the sample size 100 µl put in the tray of the instrument and run. The sample size injection was 20µl, with a thermostat temperature of 30°C and a pressure of 15 MPa. The Clarity Chrome software was used to calculate the area under the peaks. Before each analysis, the column was rinsed with mobile phase at a flow rate of one millilitre per minute for 10 minutes, after each analysis, the column was cleaned with methanol for 5 minutes.

DETERMINATION WAVELENGTH OF DETECTION

In order to determine the wavelength of Etodolac, accurately weight 10 mg of Etodolac in 100 ml of methanol and dilute with a mobile phase to get to 10 µg/ml of Etodolac solution. The solution was scanned 200-400 nm by a UV spectrophotometer (Shimadzu 1700).

PREPARATION OF STOCK AND STANDARD SOLUTIONS

A stock solution of Etodolac was prepared by dissolving an accurate amount of Etodolac 10 mg in 100 of a mobile phases (methanol: acetonitrile: water) at ratio of (60:30:10). The working solution for calibration were prepared from serial dilution for stock solution with mobile phase to yield ten standard solutions 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5 and 25 $\mu g.ml^{-1}$, however, to ensure the accuracy of the processes, its repeated six times within a single day (intraday) and once over six days (intraday); for each repetition, a new stock solution was prepared.

METHOD VALIDATION

The HPLC validation process focus on accuracy, precision, recovery, linearity, stability studies, LOQ and LOD.

These parameters were assessed following guideline established by Food and Drug Administration FDA and the International Conference on Harmonization Guideline (ICH).

SUITABILITY OF HPLC FOR QUANTIFYING ETODOLAC

The HPLC suitability system was determined to study the accuracy and precision of the quant method. It was achieved by measuring the injection repeatability RSD, tailing factor T, capacity factor k', and theoretical plates N to analyse system suitability. The concentration 2.5 $\mu g.ml^{-1}$ of Etodolac dissolved in methanol was used. The optimized chromatogram system should pass the accepted limited value before being used for further studies with the development of the HPLC method.

PRECISION AND ACCURACY OF THE DEVELOPING METHOD OF ETODOLAC DETECTION

Precision is represented as closeness of agreement among a series of measurements. However, this involved running the analyses six times in the same day (intraday) and once day for six days (intraday) at four different concentrations of analyse: 2.5, 5, 7.5 and 10 micrograms per millilitre. It was determined by measuring the percentage of relative standard deviation (% RSD) using the following equation:

$$\%RSD = \frac{\text{Observed Concentration}}{\text{Nominal Concentration}}$$

While accuracy explained as the closeness of agreement among the theoretical and actual value, it was measured by using twelve repeated and determining the % RE (percentage of residual error), according to the following equation:

$$\%R = \frac{\text{Observed Concentration - Nominal Concentration}}{\text{Nominal Concentration}} \times 100$$

The percentage of residual error should not exceed 2%, four standard solutions were used for precision and accuracy analyses: 2.5, 5, 7.5 and 10 $\mu g.ml^{-1}$.

LINEARITY OF HPLC DEVELOPED PROCESS OF ETODOLAC DETECTION

Linearity can be detected depending on the least square method of the calibration curve (peak area as a function of Etodolac concentration) when adding regression. To prepare the calibration curve, ten standard solutions of Etodolac were used in the range 2.5-25 $\mu g.ml^{-1}$.

LOD AND LOQ SPECIFICITY

The LOD and LOQ were detected through HPLC validation to measure the lowest detectable and measurable concentration of Etodolac. The LOD and LOQ can be directly calculated from the calibration plot. The LOD was 3.3 σ/S, while LOQ was 10 σ/S, wherever (σ) represented the standard deviation of the intercept, (S) represented the slope of the calibration plot.

STABILITY AND RECOVERY STUDY FOR ETODOLAC

To study the stability of Etodolac during handling and lab formulation like liposome, the stability test was detected. Etodolac sock solution was tested under three scenarios, which include room temperature for one day (short term), -20 °C for ten days (long term), and freeze-thawing cycle (for 3 times), in which the solution was frozen at -20 °C for one day, thawed at room temperature for one day, and this cycle was repeated 3 times. For evaluation, the recovery of Etodolac from liposome, they compared the amount recovered with standard solution at four concentrations 2.5, 5, 7.5, and 10 $\mu g.ml^{-1}$, repeated six times.

PREPARATION OF LIPOSOME

Liposomes were prepared using the central composite design (CCD) (Design Expert version 13 software) by the thin film hydrating method. In this method, Etodolac (8 mg/ml), DSPC (20 mg), cholesterol (5 mg), or Etodolac (8 mg/ml), DSPC (20 mg), cholesterol (5 mg), and DPSE-PEG (1 mg) were dissolved in 4 ml at a 1:1 ratio of chloroform to methanol to prepare conventional and PEGylated liposomes, respectively. The component was put in a round-bottom flask (RBF) 1000 ml and put in a rotary evaporator (from Heidolph Instruments GmbH & Co. KG, Germany) and set at rotary evaporator 65°C and 100 rpm and allowed the solvent to evaporate, and a thin film was formed in the wall of the RBF and left aside for one hour in a vacuum desiccator to stabilize the film and remove the trace amount of organic solvent. Follow that the thin film was hydrated using phosphate buffer (pH 7.4), and the formation of liposomes was then retained in the rotary evaporator for 10-15 min at 65°C and 100 rpm [22]. The liposome suspension formed consists of multilayer vesicles that underwent three cycles of freezing and thawing. Consequently, liposome suspension extruder (10 times) through 0.2 µm polycarbonate membrane using hand extruder (Avanti Polar lipids Inc.). The polycarbonate filter was soaked in phosphate buffer at pH 7.4 prior to extrusion. The process was repeated with a smaller pore size of

0.1 µm and the final suspension of liposome appeared clear and transparent.

PARTICLE SIZE, POLYDISPERSE INDEX, AND ZETA POTENTIAL FOR LIPOSOME PREPARATION

The average diameter and PDI were measured by photon correlation spectroscopy (PCS) using a nanosizer at a 25°C and 90° scattering angle; hence, 3 ml of liposome suspension was put in the glass cell of the instrument without dilution.

DRUG ENTRAPMENT EFFICIENCY (EE)

Entrapment efficiency refer to amount of drug that embedded in side liposome as compare to the entire amount of drug that applied in preparation of formula. Ultracentrifugation method was used to calculate %EE. Liposome suspension put in centrifuge at 20000 rpm for 25-30min.at 5C (remi cooling centrifuge), the supernatant withdrawal that contain a free drug by indirect method [23], calculated by following equation:

$$E = \frac{W_t - W_f}{W_t} \times 100\%$$

W_t : Total amount of drug use

W_f : Un-capsulated drug

MORPHOLOGY OF LIPOSOME BY FESEM

The morphology of surface liposome for liposomal Etodolac was observed by field emission electron microscope (Inspect TM F50 FEI Company). A few drops of the sample were deposited on a carbon-coated copper grid and then allowed to dry at room temperature and observed by microscope with different magnifications.

IN VITRO RELEASE OF ETODOLAC FROM LIPOSOME

The Etodolac release from liposome was measured by the dialysis membrane diffusion method with some modification. Sample 2 ml of pure Etodolac, liposome-loaded Etodolac, and conventional and PEGylated liposomes were put in a dialysis bag with a molecular weight cut (12000-14000 Da), the membrane soaking overnight in media before using. After that dissolution media consist from either 250ml of phosphate buffer saline pH7.4 at 37^{0}C at 100rpm, at predetermine time 3ml was taken and replace with fresh media to maintenance the volume of media constant. The amount of Etodolac released was assayed by HPLC, and the cumulative percentage of the drug release profile was recorded.

IN VIVO PHARMACOKINETICS

Pharmacokinetic studies were done on male rabbit weighting 2-2.5kg were randomly divided into three groups (each group n=6). The PK was done for free ETO, ETO loaded in conventional liposomes F1, and PEGylated liposome F2. The studies were done according to ethics approved by the Ethics Committee of the Experimental Animal Teaching and Research Center, University of Kufa. The rabbits were obtained from the College of Science, University of Kufa, Iraq. The rabbits were kept in standard conditions of temperature and humidity and a natural cycle of dark and light in a room at a house animal at a temperature of 20-25°C and RH of 60-68 for two weeks, with access to free water and food [24-25]. For three groups of rabbits: the first group (n=6) received free ETO solution, the second group (n=6) received ETO loaded in conventional liposomes, and the third group (n=6) received ETO loaded in PEGylated liposomes. The dose of 8 mg/kg of rabbit's body weight was given as a suspension by IV administration route through the jugular vein under anaesthesia. At predetermined times of 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hr. after dosing. The blood samples 500 µl were withdrawn from the jugular vein and rapidly centrifuged at 4000 rpm at 4°C for 10 min., and the plasma was collected in 2 ml. To prepare the sample for HPLC analysis, allow thawing at room temperature, add 500 µl of methanol, and shake well. Follow that with centrifugation at 10000 rpm for 10 min to precipitate protein, and the sample is ready to be analysed [25].

STATISTICAL ANALYSIS

The quantitative variables were present as average value mean ± SD, calculated using Microsoft Excel 2016 and SPSS ANOVA test $p<0.5$ was consider statistically significant. PK solver software for calculate the pharmacokinetic parameters.

RESULTS

REVERSED-PHASE HPLC METHOD DEVELOPMENT AND OPTIMISATION

The HPLC method was developed to quantify Etodolac, in which the optimization is based on the guidelines of both the ICH (International Conference of Harmonization) and FDA (Food and Drug Administration). A number of preliminary trials were conducted using various combinations of solvents and different ratios of methanol, acetonitrile, and water. The optimum resolution, the low retention time of the peak, the proper run time of the analytic, and good peak shape were achieved by

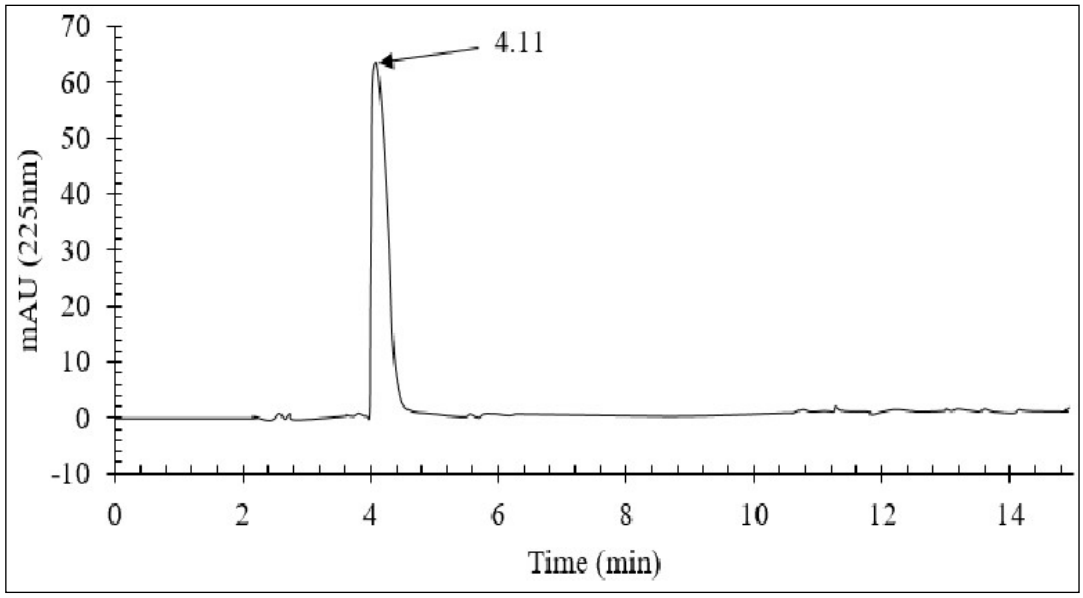

Fig. 1. HPLC Chromatographic of Etodolac (2.5 µg/ml) was analysed using a C18 column (250 mm × 4.6 mm, 5 µm particle size) at room temperature
Source: Own materials

Table 1. System suitability tests for Etodolac with accepted ranges.

Parameters	Etodolac	Accepted ranges
Injection repeatability (RSD)	0.04	<1
Capacity factor (K')	2.422	>2
Tailing factor (T)	1.09	~ 1
Number of theoretical plates (N)	4793	>2000

using a mobile phase (methanol: acetonitrile: water, at a ratio of 60:30:10). The wavelength adjustment was investigated for optimum response; 225 nm showed the optimum response [26]. The retention time was detected at 4.11 ± 0.081 min, appearing as a sharp peak with no tailing observed, meaning a good peak symmetry, figure (1); the run time was totally 15 min.

VALIDATION OF HPLC METHOD FOR ETODOLAC QUANTIFICATION

SUITABILITY TESTS OF SYSTEM

To ensure that the system is sensitive, specific, and reproducible for the analyses, a system suitability of the chromatographic method was conducted. An injection of 2.5 µg/ml of Etodolac was used to study the suitability of system parameters for the HPLC method. Repeatability, column efficiency, and resolution were determined for HPLC detection of Etodolac. The result, shown in Table 1, revealed acceptable parameters

The HPLC instrument performance was evaluated by measuring how well it could detect small amounts of Etodolac (sensitivity) and how consistent its results were (precision). The % RSD (relative standard deviation) was calculated to determine the limitations of the analysis.

THE LINEARITY, LIMITED QUANTIFICATION, AND LIMIT OF DETECTION OF THE HPLC METHOD

The linearity of the analytical method can be defined as the ability of the technique to get test results in which the proportion is directly related to the concentration of the material to be analysed in the sample; the range can be expressed as an interval between the lower and upper concentrations of the substance to be analysed, for which the method can detect a better level of linearity, precision, and accuracy. The standard curve of ETO was used to establish the linearity (LOD) representing the lowest Etodolac concentration that can be measured. In contrast, (LOQ) represents the lowest Etodolac amount that can be measured with suitable accuracy and precision. The Etodolac calibration curve was determined by plotting the peak of its area against the known concentration of the sample, figure (2).

The calibration curve n = 12, with a correlation coefficient R^2 (0.999 ± 0.0007), indicates a highly linear relationship between peak area and concentration within the tested range (2.5 - 25 µg/ml). The LOD and LOQ were measured according to the coefficient of slope and standard error of intercept; the HPLC method was used to measure them. They were found to be 0.42 µg/ml and 1.3 µg/ml, respectively.

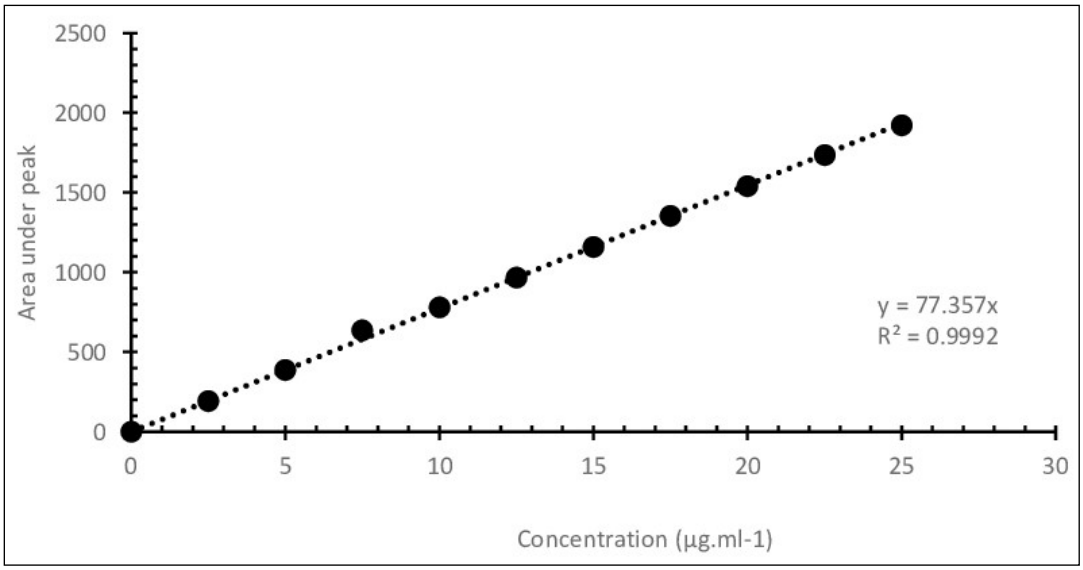

Fig 2. Linearity plot of Etodolac over conc.2.5-25 µg.ml^{-1} (n=12)
Source: Own materials

Table 2. Accuracy and precision within single day and between days

Within-day (n=6)			
Etodolac concentration in sample (µg.ml^{-1})	**HPLC detection (µg.ml^{-1})**	**Accuracy %RE**	**Precision %RSD**
2.5	2.50±0.0052	99.87-100.13	0.2
5	5.01±0.0440	99.77-100.23	0.87
7.5	7.49±0.0103	99.91-100.09	0.1
10	10.01±0.0427	99.87-100.13	0.04
Between-day (n=6)			
Etodolac concentration in sample (µg.ml^{-1})	**HPLC detection (µg.ml^{-1})**	**Accuracy %RE**	**Precision %RSD**
2.5	2.50 ± 0.0137	99.87-100.13	0.5
5	5.01 ± 0.0427	99.73-100.27	0.8
7.5	7.51 ± 0.0456	99.87-100.13	0.6
10	10.06 ± 0.0852	99.37-100.63	0.847

Source: Own material

ACCURACY AND PRECISION OF DEVELOPED HPLC METHOD

Intraday and intraday HPLC analysis methods were used for measuring the repeatability of the developed method and if the investigative method was appropriate for additional studies to determine the accuracy and precision. The accuracy and precision of the HPLC method were assessed both within a single day (intraday) and between days (Intraday) to determine if the developed method was a reliable result and if the analytical method was suitable for future studies. The accuracy was measured as a percentage of residual error (RE), while precision was represented as a percentage of residual standard error (RSD), as shown in Table 2

STABILITY ANALYSES OF ETODOLAC

Three conditions were used to assay the stability of Etodolac: short-term storage, long-term storage and freeze-thawing cycle. These studies are critical in determining the ability of Etodolac to withstand during preparation and in plasma. The results obtained from these studies are in three situations, as shown in Table 3.

For short-term stability, the percentage of accuracy ranges from (99.72-100.33%); for long-term stability, % the accuracy is (97.92-99.42%) and for freezing-thawing (98.93-99.93%).

INTERFERENCE STUDY OF ETODOLAC

It's essential to detect Etodolac in liposomes to study if there is any effect or interference of components of liposomes

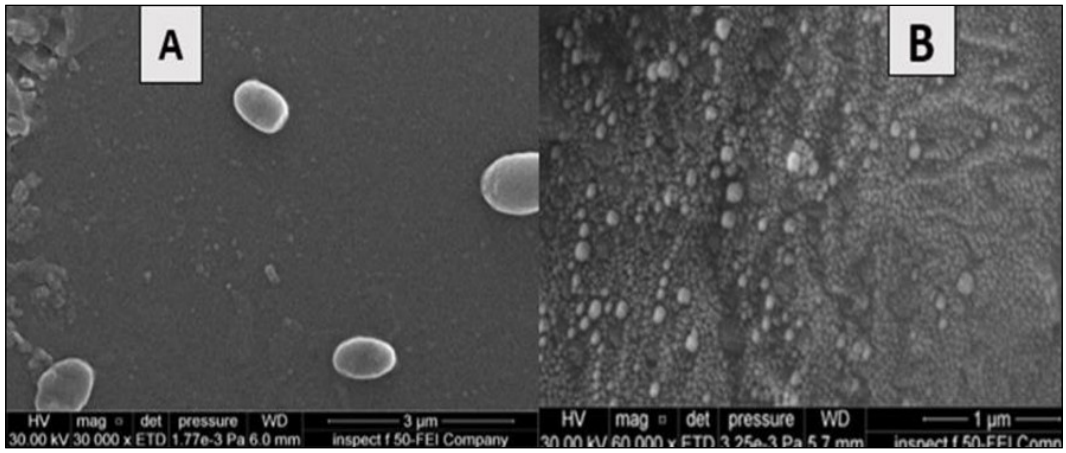

Fig. 3. FESEM photograph for A: Etodolac loaded conventional liposome, B: Etodolac loaded PEGylated liposome
Source: Own materials

Table 3. Short-term storage, long- term storage and freezing-thawing cycle stability of Etodolac (n=6)

Experimental conditions	actual concentration (µg.ml^{-1})			
	2.5	5	7.5	10
Short term				
Average conc. (µg.ml^{-1})	2.49±0.03	5.02±0.065	7.51±0.046	9.97±0.042
Accuracy %	99.73	100.33	100.11	99.72
Long term				
Average conc. (µg.ml^{-1})	2.48±0.018	4.92±0.037	7.46±0.036	9.79±0.24
Accuracy %	99.33	98.47	99.42	97.92
Freezing-thawing				
Average conc. (µg.ml^{-1})	2.47±0.016	4.99±0.056	7.48±0.02	9.99±0.018
Accuracy %	98.93	99.77	99.76	99.93

Source: Own material

Table 4. Recovery of Etodolac from liposome (n=6)

Etodolac	Liposome	
µg.ml^{-1}	HPLC detection	Recovery (%)
2.5	2.48±0.038	99.33
5	4.92±0.037	98.47
7.5	7.52±0.045	100.22
10	9.99±0.029	99.85

Source: Own material

(lipid and cholesterol) on the detection of Etodolac by HPLC. Liposome formulas consist of four concentrations of stock Etodolac prepared (2.5, 5, 7.5, and 10 µg.ml^{-1}) separately mixed with DSPC and cholesterol and analysed by HPLC. The result shown in Table 4 is that retention time was 4.1 min. The recovery percentage was in the range (98.47-100.22%).

CHARACTERIZATION OF LIPOSOME LOADED WITH ETODOLAC

Liposomal preparation containing Etodolac was developed using the thin film hydrated method with extrusion. Conventional liposome that prepared using DSPC and cholesterol, the PZ diameter 88 nm±0.02, with narrow size of distribution 0.012±0.01, while PEGylated liposome prepared using DSPC-Chol-DSPE-PEG 2000 has PZ 92 nm ± 0.003 and a PDI 0.01±0.02,. PDI was less than 0.1 mean particle size well controlled and narrow disperse. The Z-potential was (-10.2mV±0.001, -13.6mV±0.012) for conventional liposome and PEGylated liposome, respectively. The entrapment efficiency %EE (89.5±0.04, 94.3%±0.01) for conventional liposome and PEGylated liposome, respectively. The FESEM analysis explain the spherical shape of vesicles and confirm diameter of liposome, figure (3).

The *in vitro* drug release kinetic profile shows there is a significant difference between the release of drug from free Etodolac and liposomal Etodolac ($p < 0.05$) as shown in Fig 4. As seen from the figure, the % release of

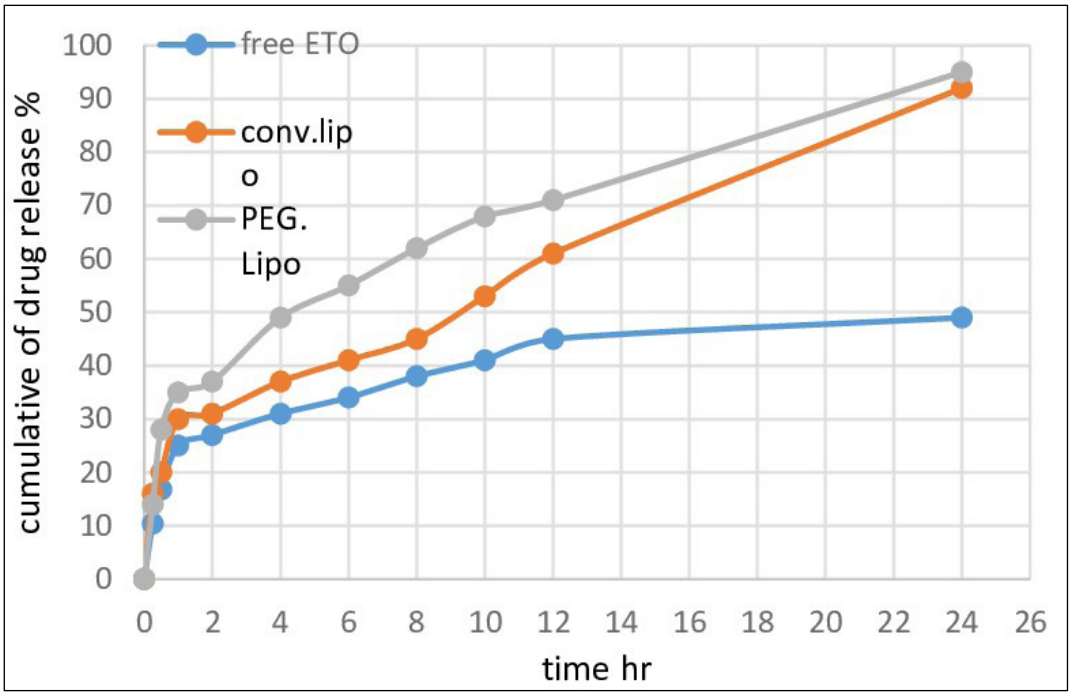

Fig. 4. *In vitro* release of free Etodolac, Etodolac loaded conventional liposome and Etodolac loaded PEGylated liposome
Source: Own materials

Table 5. Pharmacokinetic parameter for pure ETO, conventional liposome and PEGlytated liposome follow IV administration (n=6)

Parameters	pure ETO	Conventional liposome	PEGylated liposome
T_{max} hr.	0.25	0.25	0.25
C_{max} µg/ml	10.49	15.32	19.43
K_{el} hr^{-1}	0.138	0.103	0.084
$t_{½}$ hr.	6.5	8.12	10.74
$AUC_{0-\infty}$ µg/ml*h	69.12	111.17	162.03
$AUMC_{0-\infty}$ µg/ml*h^2	743.04	1172.54	2447.91
MRT hr.	4.37	7.04	7.82
CL (mg)/(µg/ml)/h	0.173	0.107	0.07
Vd (mg)/(µg/ml)	2.034	1.29	1.14

Source: Own material

Etodolac occurs in two patterns from conventional lip and PEGylated liposome as a burst release within (2 hr.) as 31.4% and 37.8% for conventional lip and PEGylated liposome, respectively, followed by a sustained release until 24 hr., in which it reaches 92.1% and 96.3% for conventional lip and PEGylated liposome, respectively, while pure Etodolac is 49%.

IN VIVO PHARMACOKINETIC STUDIES

In vivo pharmacokinetics was done for pure drugs, conventional and PEGylated liposomes using PK Solver software, and a non-compartment model was used to fit the data. All formulas were subject to a sterilization process by filtration method using 0.22 µm PVDF filters. The free ETO is rapidly removed from the blood as compared with ETO loaded with liposome; these results might be due to metabolism or distribution to other organs. The result shows the pharmacokinetic profile of liposomes loaded with ETO significant differences ($p<0.05$) from free ETO; the PEGylated formula shows the higher plasma concentration as compared with the conventional and free drug after IV administration; the pharmacokinetic parameters obtained from the plasma-time profile are summarized in Table (5) and figure (5).

AUC, which involves AUC_{0-24}, $AUC_{24-\infty}$, and total $AUC_{0-\infty}$ measured by the trapezoidal method for each animal of these data, in the group (n=6), and take the average of these data as shown in Table 6 and Fig 6. The total AUC for PEGylated liposome shows a significant difference from both pure ETO and conventional liposome ($p < 0.05$). PEGylated liposome had a higher AUC_{0-24} and

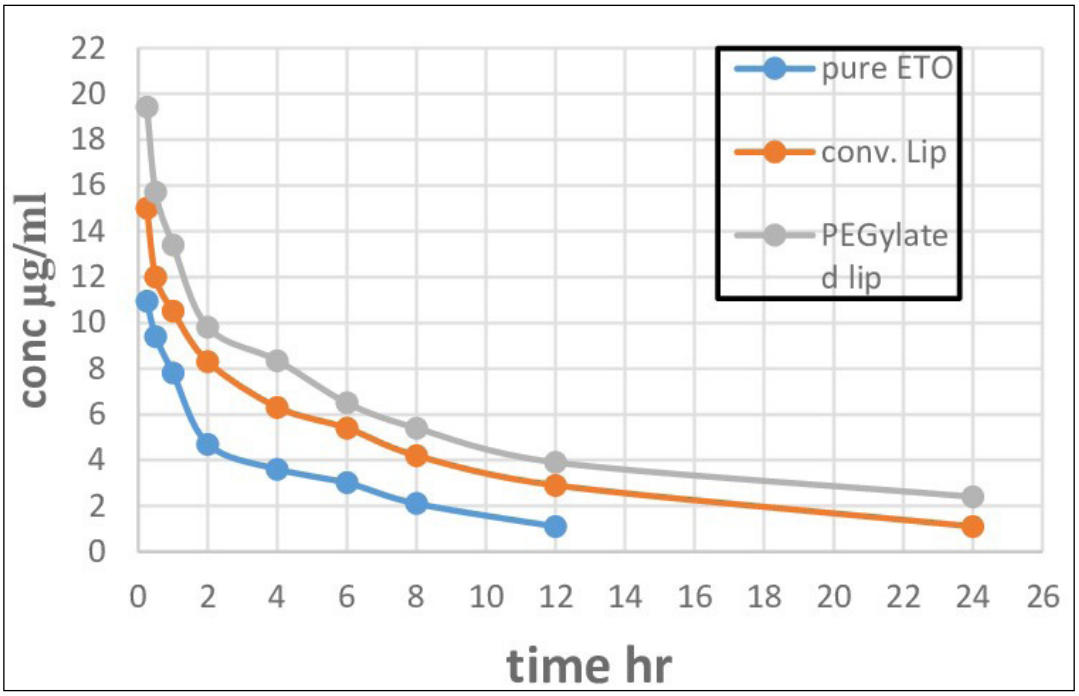

Fig. 5. *In vivo* pharmacokinetic profile for pure ETO, conventional liposome (F1) and PEGylated liposome (F2) follow IV administration (8mg/kg per rabbit). The data show mean± SD (n=6). The ETO loaded in liposome profile has a significant difference from pure ETO ($P<0.05$)
Source: Own materials

Table 6. Area Under Curve AUC_{0-24}, $AUC_{24-\infty}$ and total AUC (n=6)

Formula type	AUC_{0-24} µg/ml*h	$AUC_{24-\infty}$ µg/ml*h	AUC_{total} µg/ml*h
Pure ETO	46.85	22.26	69.12
Conventional lip	97.93	13.24	111.17
PEGylated lip	124.82	37.21	162.03

Source: Own material

AUC_0-∞ (124.82, 162.03 µg/ml*h), respectively, while (97.93, 111.17 µg/ml*h) for the conventional liposome formula and (46.85, 69.12 µg/ml*h) for pure Etodolac.

The maximum plasma concentration Cmax and Tmax were obtained from the plasma concentration versus time curve. Cmax is equal to 10.84, 15.32, and 19.34 µg/ml for pure ETO, conventional liposome, and PEGylated liposome, respectively, while t_{max} 0.25 hr. The results show Cmax of PEGylated liposome significant differences as compared with conventional liposome and pure ETO at any time. AUMC0-∞ for pure ETO, conventional lip, and PEGylated lip were found to be 743.04 µg/ml*h^2, 1172.54 µg/ml*h^2, and 2447.91 µg/ml*h^2, respectively. The result shows the AUMC0-∞ for pure ETO the lowest while AUMC0-∞ for PEGylated lip is the highest, there is a significant difference p< 0.05) between conv. lip and PEGylated lip as compare with pure ETO. The t1/2 was found to be 6.5 hr., 8.12 hr., and 10.74 hr. for pure ETO, conventional liposome, and PEGylated liposome, respectively. The MRT (mean resident time) equal to 7.82 hr., for PEGylated liposome as compare with 7.04 hr. for conventional liposome and 4.37hr for pure ETO.

DISCUSSION

The HPLC method was developed to quantify Etodolac, in which the optimization is based on the guidelines of both the ICH (International Conference of Harmonization) and FDA (Food and Drug Administration). The mobile phase, which consists of methanol, acetonitrile, and water in a ratio of 60:30:10, was pumped through the column at a flow rate of 1 millilitre per minute. The sample was injected at a volume of 20 µl, and Etodolac was detected at a wavelength of 225 nm. The calibration curve was validated over the range of 2.5 - 25 µg/ml. The flow rate was, and the investigation was conducted in isocratic mode, with a flow rate of 1 ml/min, the thermostat temperature is 30°C, and the injection volume is 20 µl. The results are consistent with the result found by Akman et al. (2019), who show the retention time of Etodolac 4.21 min when using a mobile phase containing acetonitrile: water at a ratio of (80:20), a flow rate of 1 ml/min, and a wavelength of 272 nm [27], also, these results were compatible with Mohan *et al.* , 2011, who stated a retention time for Etodolac was 4.8 min using a mobile phase containing phosphate buffer 5.5 and acetonitrile

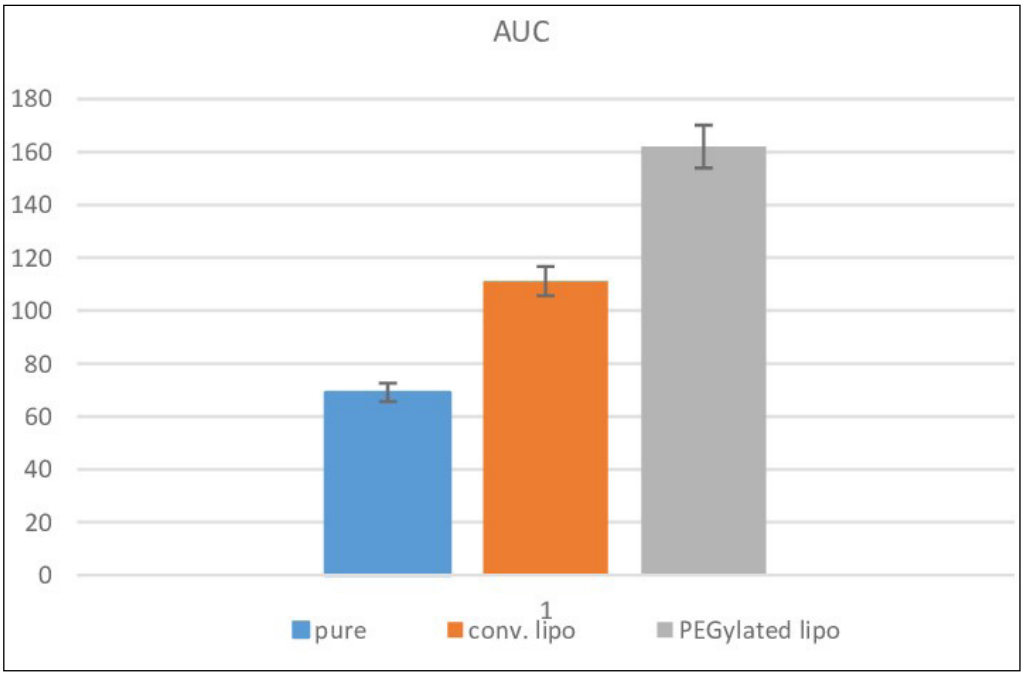

Fig. 6. $AUC_{0-\infty}$ for pure ETO, conventional liposome and PEGylated liposome by trapezoidal method
Source: Own materials

at a ratio (60:40) [28]. The %RSD was less than 1%; indicate that the chromatographic conditions were optimal and that the injections were highly repeatable. To measure resolution, the capacity factor k' was detected, and the result revealed $k' > 2$, which indicates a good resolution. The tailing factor T also measures the asymmetry of a peak; the result appears T = 1.09 close to one, reproducing a good symmetry of the peak. The theoretical plate number N > 2000 indicates a good efficiency of column, according to the Centre for Drug Research and Evaluation. The results of the system suitability test were comparable to those reported by Patel et al. (2011), which included theoretical plates N greater than 2000, capacity factor and resolution k' greater than 2, and asymmetry 1.03, all factors within accepted and limiting ranges [29]. Additionally, the finding was consistent with those of Siddiraju et al. (2013), who confirmed the system suitability of Etodolac using a tailing factor (T=1.04) and theoretical plates N greater than 2000, capacity factor and resolution k' greater than 2 within accepted ranges [30]. The calibration curve n = 12, with a correlation coefficient R^2 (0.999 ± 0.0007), indicates a highly linear relationship between peak area and concentration within the tested range (2.5 - 25 µg/ml). However, these results demonstrate a good fit between the data and linear regression model, confirming a strong relationship across the chosen range of concentration, and this finding aligns with previous research by Lee et al. (2008), who reported the linear calibration curve of Etodolac across a range of concentrations (0.1-25 µg/ml), with an R^2 correlation coefficient (0.9975 ± 0.0018) [31]. So this indicate the method was sensitive and easy quantify method for Etodolac. The results obtained are consistent with the finding of Thomas et al. (2023), who determined Etodolac succinic acid co-crystals in spiked rabbit plasma to be 0.370 µg/ml and 1.121 µg/ml, for LOD and LOQ, respectively [32]. LOD and LOQ were measured according to the coefficient of slope and standard error of intercept; the HPLC method was used to measure them. They were found to be 0.42 µg/ml and 1.3 µg/ml, respectively, refer the method was sensitive to determine a minimal quantity of Etodolac in a bulk sample. For precision and accuracy, the outcomes of the method show that accuracy and precision, are within the accepted limited range (85–115%) as mention via the analytical method of the FDA, while for % RSD less than 2. The results are consistent with the findings of Biswal et al. (2019); that finding appeared, the % RSD for a precision of less than 2% [33]. Results were also consistent with Patel *et al.,* 2011, who informed an %RSD of < 2%, Results were also consistent with Patel et al. (2011), who reported an %RSD of < 2% , and also results were reliable with the result of Dhiware et al. (2012), who reported a calculated % RSD of less than 2% [34]. The results obtained from the stability test study proved that the sample that contains Etodolac can be handled under standard laboratory conditions and stored in the refrigerator at -20°C. The experiment's results are compatible with Patel *et al.*, 2011, who reported that Etodolac can be stable for two days at ambient temperature [29]. The result confirmed no interferences

between Etodolac and a component of liposome in detection by HPLC, which is compatible with the result of Shaikh and his colleagues, 2017, that informed there is no interference was observed between Etodolac and excipient present in tablet dosage form [21]. the liposomes successful formulation by thin film method, the results show, PZ of PEGylated liposome higher PZ than conventional liposomes due to the PEG formed a thick layer at the surface of liposome make it large PZ. The Z-potential of PEGylated liposome more than conventional liposome because of present of PEG give a charge on the surface due to the DSPE-PEG2000 has a negative charge and prevent the aggregation of particles by electric repulsive effect. The PEGylated liposome shows a higher %EE than the conventional liposome, and this may be result from that the PEG enhancing the solubility of drug lead to improve its %EE, the pH of the liposome a round 7.4, so the addition of PEG does not affect the pH of liposome make it suitable for IV administration [35]. This result consistence with the finding of Sara Pereira et al. (2016), who reported that the increase of %EE of Docetaxel in DSPC-Chol-DSPE-PEG 2000 liposome than DSPS or DPPC base formulation [36]. Etodolac is a hydrophobic drug with poor water solubility 0.016mg/ml and lg_{P} 2.5, so can be partition between lipid layers and aqueous phase. The in-vitro study show, the fast release of the drug result can be explained as the drug being present near the head of the phosphate group, followed by a slow or sustained release due to the entrapment of the drug in the lipid by layer [37]. Effect of PEG2000 which prolongs the release of Etodolac from liposomes and also improves solubility and stability of Etodolac. The results show the PEGylated liposome shows a higher % release reach 95% within 24hr.The release of Etodolac from liposomes shows a biphasic pattern, and these findings are in agreement with the finding of Vivek et al. 2019, who reported that the release of celecoxib from PEGylated liposomes was a biphasic. for in-vivo study, the lowest half-life of pure ETO and conventional liposome due to the opsonization process by Kupffer and RES was responsible for elimination from systemic circulation. In addition to that, the volume of distribution (Vd) and elimination rate constant (Kel) were found to be less for PEGylated liposomes as compared to conventional and pure ETO, as listed in Table 5, reflecting the long circulation time of liposomes due to the PEGylated liposome containing PEG 2000 with stearic properties, making it a stealth liposome that has a long circulation time. The modification the surface of liposomes with polyethylene glycol (PEG) has emerged as a prominent strategy for enhancing their stability and extending circulation time. The inherent hydrophobicity of PEG molecules, upon grafting onto the liposomal surface, facilitates the formation of a robust hydration layer. This hydration shell effectively mitigates nonspecific interactions between the liposome and plasma components, consequently diminishing the formation of the protein corona—the adsorption of plasma proteins onto the liposome surface. A reduced protein corona minimizes recognition and clearance by the reticuloendothelial system (RES). Furthermore, PEG's steric hindrance properties impede particle aggregation and confer protein-repellent characteristics, further contributing to decreased RES uptake and a prolonged drug half-life. These attributes have earned PEG the designation of a "stealth polymer" in liposomal drug delivery, leading to its widespread adoption in liposome modification [38].

CONCLUSIONS

The HPLC process was developed and evaluated for its ability to rapidly measure and quantify Etodolac in a pure bulk sample and liposomes from the outcomes that obtain from the validation parameter, it has been established that the HPLC method was simple, reliable, sensitive, rapid, and reproducible with a short runtime. Etodolac was stable during handling and formulation. The method fully meets FDA guidelines. It can be used for quality control assays of Etodolac in test laboratory samples and formulation of pharmaceutical industries, and in vivo pharmacokinetic. The liposome loaded with Etodolac was successful prepared using thin film hydrate technique

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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