

HPLC METHOD DEVELOPMENT AND VALIDATION FOR METFORMIN AND PHENYL BUTYRIC ACID AND *IN VITRO* EVALUATION OF THEIR PEGYLATED LIPOSOMAL FORMULATION AGAINST COLORECTAL CANCER

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ABSTRACT

Objective: This study aimed to develop a PEGylated liposomal formulation combining Metformin (MET) and Phenyl Butyric Acid (PBA) for colorectal cancer treatment. Current colorectal cancer therapies face limitations such as poor drug solubility, rapid clearance, and systemic toxicity. Combining MET and PBA in liposomes could potentially overcome these limitations by enhancing drug stability, prolonging circulation time, and improving targeted delivery.

Methods: A High-Performance Liquid Chromatography (HPLC) method was optimized and validated for the simultaneous quantification of MET and PBA, achieving calibration curve R^2 values above 0.99. Liposomal formulations were prepared, and encapsulation efficiencies were evaluated. Sustained drug release was studied over 72 h at 37 °C. Cytotoxicity assays were conducted using colorectal cancer cell lines HCT116 and HT29. Additionally, colony formation assays assessed the formulation's long-term effects on cell proliferation.

Results: The liposomal formulation showed encapsulation efficiencies of 48% for Lip-MET and 56.8% and 83.9% for Lip-MET-PBA. Sustained release profiles were observed, with 60% of MET and 55% of PBA released over 72 h. Cytotoxicity assays revealed that Lip-MET-PBA significantly reduced cell viability in HCT116 and HT29 cell lines, with IC50 values of 0.65 mmol and 0.82 mmol, respectively, compared to MET alone (1.1 mmol and 1.3 mmol). Colony formation assays demonstrated a concentration-dependent reduction in colony numbers, highlighting the enhanced anticancer efficacy of the combination. Statistical analysis showed significant differences ($p < 0.05$) in cell viability and colony formation between the liposomal formulation and the free drugs.

Conclusion: This is the first PEGylated liposomal formulation combining MET and PBA, offering a promising strategy for colorectal cancer therapy. The formulation combines sustained release, improved cytotoxicity, and a significant impact on colony formation, warranting further *in vivo* investigations.

Keywords: HPLC validation, Liposomes, Combination, Nanoparticles, Colorectal cancer

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INTRODUCTION

Cancer is a disease marked by uncontrolled cell growth, leading to tumor formation that harms the body. These cancerous cells can also spread to other parts of the body, a process known as metastasis, causing damage to new organs. The most common types of cancer include lung, breast, colorectal, prostate, stomach, and non-melanoma skin cancers, but over 100 types have been identified. Colorectal cancer is the third most common cancer worldwide, affecting both men and women, with more than 50,000 deaths reported in the U. S. in 2021 alone [1]. The most important sign of colorectal cancer is rectal bleeding. Other symptoms may include blood in the stool, changes in bowel movements, anemia, fatigue, and constipation. The most specific diagnostic tool for colorectal cancer is colonoscopy, which allows for direct visualization of the colon and rectum. Additional diagnostic tools include MRI and CT scans, which can help assess the extent of the disease and identify any metastasis [2].

Liposomes are a well-known drug delivery system, defined as colloidal, spherical nanoparticles with a membrane that ranges from 50 to 200 nm in size. They consist of a bilayer of lipids that encapsulate therapeutic agents. Liposomes offer several advantages: they are biocompatible and biodegradable, and they protect encapsulated drugs from degradation. Lipid-soluble drugs are incorporated into the bilayer, while water-soluble drugs are contained within the vesicle's core. This structure helps in delivering drugs effectively to targeted areas while minimizing side effects [3-6].

Recent advancements in PEGylated liposomes have shown significant promise in cancer therapy. PEGylation, the process of

attaching Polyethylene Glycol (PEG) chains to liposomes enhances their stability and prolongs their circulation time in the bloodstream. This modification reduces the recognition and clearance by the immune system, allowing for more effective drug delivery to tumor sites. PEGylated liposomes have been successfully used to deliver various anticancer drugs, improving their therapeutic efficacy and reducing systemic toxicity [7-9].

Liposomes can exploit the increased intercellular space found in tumors compared to normal tissues. The larger gaps between cancer cells, which range from 100 to 700 nm depending on the cancer type, allow liposomes to penetrate and accumulate within the tumor more effectively. This enables the targeted delivery and release of the encapsulated drug directly at the tumor site, enhancing therapeutic efficacy while minimizing off-target effects [10].

Metformin (MET) is one of the most widely prescribed medications globally, primarily used to manage Type 2 Diabetes Mellitus (T2DM). It is known for its affordability, effectiveness, and safety. Since its introduction in the 1950s, metformin has become a cornerstone in diabetes treatment due to its ability to lower blood glucose levels and improve insulin sensitivity [11-20]. This study aims to develop a PEGylated liposomal formulation combining MET and PBA for colorectal cancer treatment. The objectives are to optimize and validate a High-Performance Liquid Chromatography (HPLC) method for the simultaneous quantification of MET and PBA. Evaluate the encapsulation efficiencies and sustained drug release profiles of the liposomal formulations. Finally, Assess the cytotoxicity and long-term effects on cell proliferation using colorectal cancer cell lines HCT116 and HT29.

MATERIALS AND METHODS

Metformin HCl (MET) was obtained from Dar al-Dawa pharmaceutical company. Phenyl Butyric Acid (PBA) was obtained from ICT (Japan). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) and 1,2-Dipalmitoyl-Sn-Glycero-3-Phosphocholine (DPPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA) and Cholesterol (CHO) was obtained from Carbosynth (UK). Phosphate Buffer Saline (PBS) was from LONZA® (USA). HPLC grade methanol and ethanol were from Sigma-Aldrich (USA) and the carbon group-England, respectively. All chemicals and solvents were of high purity.

Development of method of analyst based on HPLC

Analyses were performed on HPLC system consisted of Shimadzu LC-2030 equipped with UV Detector (Shimadzu Corporation, Kyoto, Japan). Separation was carried out using Thermo Scientific™ Hypersil™ BDS C18 HPLC Column, 100Å, 5µm, 4.6 x 150 mm stationary phase utilizing mobile phase containing Methanol: water 70:30; v/v) (pH= 2.8).

Statistical acquisition, recording, and chromatographic integration were achieved using Lab Solutions version 5.92. The mobile phase was filtered through a nylon milli pore (0.2µm) membrane filter, degassed before use, and pumped at a flow rate of 1.0 ml/min, and the injection volume was 10 µl. The analytical column was kept at 40 °C. The chromatographic run time was set to 10 min. MET and PBA were detected at 210 nm [21].

Standard solution and limit of detection (LOD) and limit of quantification (LOQ)

Stock solutions of MET and PHA (1 mg/ml) were prepared by dissolving 1 mg/ml ethanol. Then, 8 serial dilutions were done. Finally, calibration curves were graphed composing of standard solutions with increasing concentrations that were used in the linearity assay. All solutions were filtered through a 0.45 µm cellulose membrane and injected into the HPLC system (n=3). Low limit of Detection (LOD) and low limit of Quantification (LOQ) were measured from the calibration plot based on the following equations (1, 2):

$$LOD = \frac{3.3 \sigma}{S} \dots \dots (1)$$

$$LOQ = \frac{10\sigma}{S} \dots \dots (2)$$

The statistical method used for comparing the LOD and LOQ involved calculating the standard deviation of the response (σ) and the slope of the calibration curve (S). To compare the calculated and measured values of LOD and LOQ, paired t-tests was used to assess any significant differences. This statistical approach ensures the reliability and accuracy of our method [22].

Precision and repeatability

The precision was evaluated by measuring samples with same concentrations for both drugs 0.13, 0.3 and 0.6 mg/ml (MET and PBA) at three different days (interday precision) and we expressed the precision as Relative Standard Deviation (RSD) (ICH Q8, 2009).

The repeatability was evaluated by measuring samples with same concentrations for both drugs 0.13, 0.3 and 0.6 mg/ml (MET and PBA) in a single day (intraday precision) and we expressed the precision as RSD as well.

Accuracy and recovery

Accuracy was done by triplet measuring of sample of known three different concentrations of PBA and MET and standard solutions (ICH Q8, 2009).

Robustness of the validated method

Robustness was done by measuring the samples on different parameters such as temperature, flow rate and wavelength. It was also done in triplicate for each parameter (ICH Q8, 2009).

Liposomes preparation

Liposomes were formed by ethanol injection method. As DPPC 8.5 mg, CHO, 2 mg, DSPE-PEG 0.8 mg and 1 mg of PBA were dissolved in 1 ml of ethanol, then 1 mg of metformin was dissolved in 3 ml of PBS and heated to 50 °C using hot plate. The ethanol was injected rapidly into the PBS by syringe with continuous stirring 500 rpm and left for 1 h to let the vesicle form [5]. During the ethanol injection method, temperature variations were managed by conducting the process on a hot plate set to 50 °C. This temperature was maintained consistently throughout the injection and stirring process to ensure uniform liposome formation. The temperature was monitored using a digital thermometer to prevent any deviations (fig. 1).

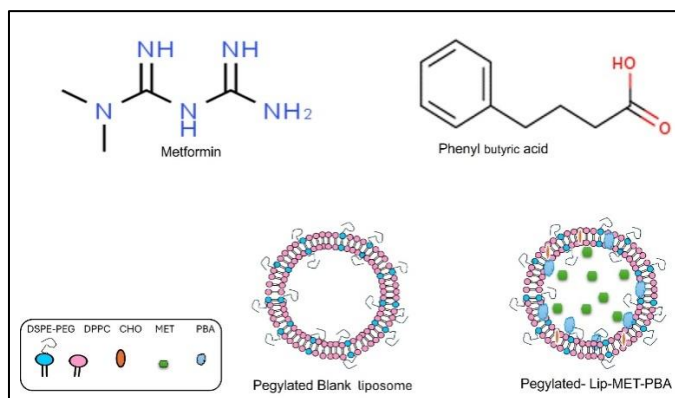


Fig. 1: Structure of metformin and phenyl butyric acid, images of pegylated blank liposomal and Lip-MET-PBA

Then, the formed suspension was passed through a polycarbonate membrane multiple times, if necessary, using a Mini-Extruder (Avanti Polar Lipids, Inc. USA) at 50 °C to obtain the proposed size (100 nm, Whatman®). This process was repeated 13 times to obtain liposomes with low polydispersity and the desired size. The newly formed liposomes were centrifuged to eliminate any free medications and stored at 4 °C [23]. The reproducibility of the extrusion process was tested by performing multiple extrusions of the same liposomal formulation and measuring the resulting particle

size and Polydispersity Index (PDI). Each extrusion was repeated 13 times, and the consistency of the results was evaluated to confirm the reproducibility of the process.

Characterization of formed liposomes

Zetasizer® device was used to measure the characteristics of the new formed vesicles; these measurements include hydrodynamic diameter, PDI and charge (ζ-Potential) by Dynamic Light Scattering (DLS) Technique. The average Particle Size, Zeta Potential (charge) and PDI

for liposomes were measured by DLS experiments using Zetasizer, Nano-ZS (Malvern Instruments Ltd., Malvern, UK). The liposomal samples were diluted with deionized water at a ratio of 20:980 μl (v/v) to achieve a suitable counting rate. Before the measurement, all samples were placed in the specimen holder of a Zetasizer and allowed to reach room temperature equilibrium for 60 sec.

Encapsulation efficiency (EE %) and loading efficiency (LO%)

The EE % and LO % of MET and PBA into liposomes was expressed as the percentage of drug encapsulated inside liposomes and calculated using the following equations (3, 4):

$$\text{Encapsulation Efficiency (EE\%)} = \frac{[\text{Entrapped drug}]}{[\text{Total drug}]} \times 100 \dots \dots (3)$$

$$\text{Loading Efficiency (LO\%)} = \frac{[\text{Entrapped drug}]}{[\text{Total formula}]} \times 100 \dots \dots (4)$$

In vitro stability of loaded liposomes

The stability test for the liposomes was done at 4°C for one month. Liposomes were collected (10-20 μl) every day, diluted with deionized water and entered Zetasizer®. The mean hydrodynamic diameter, Zeta Potential and PDI of the loaded liposomes were determined every day using a similar DLS method described before.

Release of the medications

Liposomes suspension of 1 ml PBS were added in a dialysis bag (molecular weight cut off [MWCO] 100 kDa, Thermo Fisher Scientific). The dialysis system that contains 1 ml of sample was inserted in a release volume of 15 ml PBS, pH 7.4, at 37 °C with continuous shaking. At scheduled intervals, 500 μl of the release medium was collected for the HPLC assay. To keep the release volume stable, the same amount of PBA was added after each withdrawal. The dialysis bag length was kept constants for all samples to keep the release surface area constant for all [23].

Lyophilization of liposomal formulations

After the liposomes were prepared, we kept them at -70 °C for 24 h, underwent freeze-drying for an additional 24 h, and moved for refrigerating for one week at 4 °C. Then we reconstituted the liposomes in deionized water and evaluated the stability by using the DLS and scanned them by full IR spectrum [23].

Fourier transform-infrared (FTIR) spectroscopy.

FTIR spectroscopy for the lyophilized liposome characterization was performed using Perkin-Elmer Spectrum Two with universal ATR FT-IR Spectrometer over a spectrum range 4000 to 650 cm^{-1} [24].

Cell viability assay (MTT assay)

To determine the IC₅₀ of Free MET–PBA mix (MET-PB), MET (MET), Lip-MET and Lip-MET-PBA were added on cell lines, an MTT Assay was done by using 200 mmol concentration of each treatment. HCT116 and HT29 cell Lines were seeded at a density of approximately 9×10^3 cells/well in a 96-well plate (Corning, USA). A variety of concentrations of treatment ranging from 0.001 to 400 μM were added to all cell lines. Then, the cells were incubated at 37 °C in a 5% CO₂ incubator for 72 h. After incubation, the old media was removed, and 100 μl of new fresh media containing MTT Assay salt (Bioworld, USA) was added to each well. The plates were then incubated at 37 °C for an additional 3 h. After that, 50 μl of solubilization solution Dimethyl Sulfoxide (DMSO) was added to each well to assess cell viability. The absorbance of the solution was measured at 560 nm using a Glomax plate reader (Promega, USA). For the cytotoxicity assays, three biological replicates for each treatment condition were performed. This ensures the reliability and reproducibility of the results [23].

Colony formation assay

Both colorectal cell lines, HCT16 and HT29 were seeded into 6-well plates at 5000 cells in each well and kept in a 5% CO₂ 37 °C in a 2 ml media for 24 h. Following the first day of seeding, the media was removed, and cells were washed with PBS. Then all wells were treated of two dilutions (1 mmol) and (2 μM) of MET, PBA and MET-PBA-free drugs, and their liposomes. The media was replaced continuously as needed for two weeks. After two weeks, cells were fixed with formalin and crystal violet dye was added. Then cell colonies were observed and counted [25].

Statistical analysis

Dose-Response Analysis: Non-linear regression was used to fit the dose-response curves and determine the IC₅₀ values for each treatment. The data were analyzed using GraphPad Prism software, which provides a robust platform for fitting non-linear models and calculating the corresponding parameters.

RESULTS

Characterization of formed liposomes

Our study showed that the prepared liposomes by ethanol injection method then performing the extrusion process helped achieving the desirable size of the liposomes, which was between 100-200 nm. It also contributed to having the desirable PDI as it ranging from 0.07 to 0.2. The zeta potential is in the optimum range as well. The measurements for the formulas were different depending on the loaded drugs. The stability was evaluated for a period of 30 days and the formulas gave excellent stability in terms of hydrodynamic diameter, PDI and charge (fig. 2A, 2B, 2C, D).

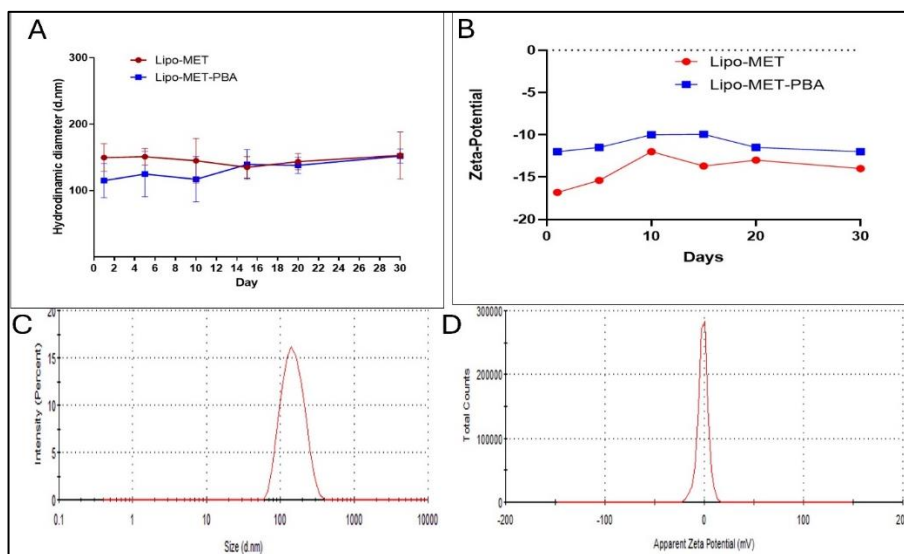


Fig. 2: Stability size distribution and zeta potential over one month of Lip-MET and Lip-MET-PBA (each experiment was done three times, mean \pm SD n=3)

Fourier transform-infrared (FTIR) spectroscopy

Lyophilized liposomes encapsulating with MET and PBA were analyzed utilizing FTIR spectroscopy (fig. 3). The liposomes spectrum showed abroad band between 3700 and 3100 cm^{-1} for O-H stretching of phosphate and cholesterol phenol groups. Additionally, notable bands were found at 2857 and 2927 cm^{-1} for CH_3 and CH_2 , respectively, that corresponded to intermolecular interactions between phospholipids hydrocarbon chain via van der Waals interactions. Furthermore, lip-

MET-PBA spectrum showed a 1169 cm^{-1} band corresponding to the ether linkage of polyethylene groups (PEG). A spectrum of a low-intensity stretching bands for $\text{C}=\text{O}$ and $\text{C}=\text{C}$ that may be attributed to the aromatic and carbonyl groups of the encapsulated PBA. Lip-MET-PBA spectrum may act as a good indication for liposomes stability before and after lyophilization. Spectrums of MET and PBA displayed N-H band and for O-H stretching in the 3700 and 3100 cm^{-1} , respectively. Furthermore, stretching bands for $\text{C}=\text{O}$ and $\text{C}=\text{C}$ may be attributed to the aromatic and carbonyl group in PBA in its powder spectrum.

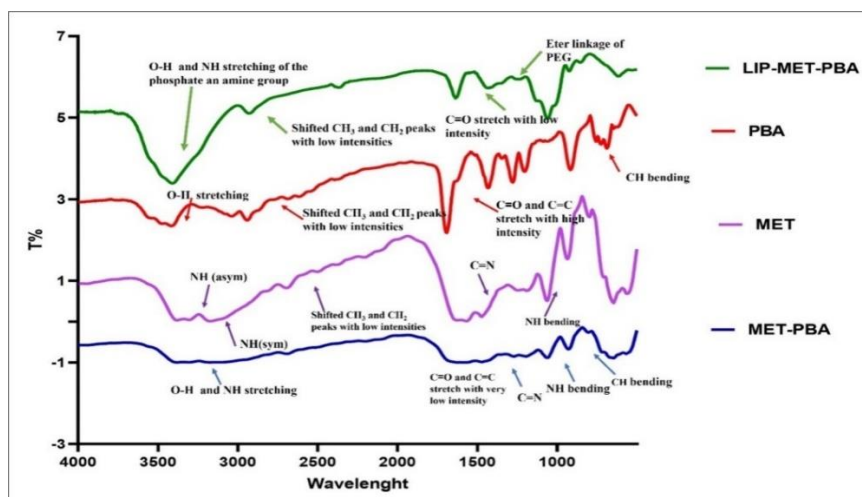


Fig. 2: IR spectroscopy for MET, PBA, MET-PBA and LIP-MET-PBA

In vitro drug release

HPLC analyses were done to find the calibration curve for MET, PBA and to validate our method. The EE% and LE% were also determined using this analysis. The results of the EE were 48.4% for Lip-MET and 56.8% and 83.9% for Lip-MET-PBA (fig. 4A). In vitro release showed Lip-MET-PBA had a slower release rate than the free mix, this is expected due to the encapsulation inside the bilayer

system. It took 6 h for the release of 30% and 70% of PBA and MET from the liposomes, respectively. While 25% and 55% of PBA and MET were released from the free mix within the first hour (fig. 4B). Encapsulation within liposomes can lead to a controlled and sustained release of the drugs, reducing the initial burst effect and potentially lowering the immediate cytotoxic impact. Additionally, the liposomal delivery system may alter the intracellular uptake and distribution of the drugs, affecting their cytotoxicity.

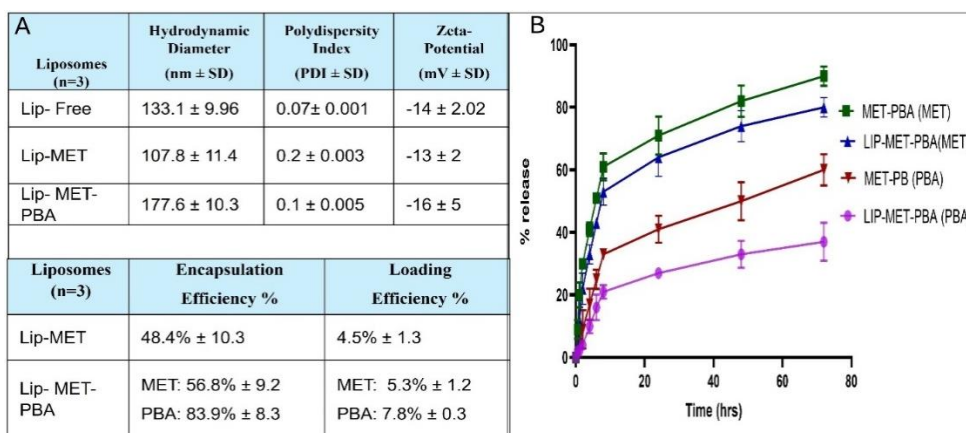


Fig. 4: (A) Summary table for the average hydrodynamic diameter, zeta potential, PDI, Encapsulation efficiency (EE%) and Loading Efficiency (LE%) (B) Release profile of both MET and PBA from Lip-MET-PBA and MET-PBA at 37 °C (each experiment was done three times, mean ± SD n=3)

HPLC analysis and validation of developed method

Our method was validated according to the International Conference on Harmonization guidelines regarding the following parameters: linearity, precision, accuracy, and robustness. Fig. 5C illustrates chromatogram of MET and PBA in which simultaneous detection of the two drugs at the same HPLC conditions.

All the values we got were expressed as RSD. Precision and repeatability values were expressed as RSD and since it was below 2

this means our method was precise. Same goes for linearity, accuracy and recovery and robustness.

Linearity and limit of detection and limit of quantification

The Calibration Curves of MET, PBA and mixed MET-PBA were established using standard solution (1-0.0165 mg/ml) with an excellent determination coefficient more than 0.997 for both drugs (fig. 5A, 5B). The ICH showed that the lowest concentration for detection (LOD) that can be detected is important to be evaluated

and quantitated. However, the lowest quantity of analysts in the sample that can be quantified is defined as the limit of quantification (LOQ). The concentrations that were calculated from linear regression statistics according to equations that represent LOD and LOQ were (0.016 mg/ml and 0.059 mg/ml for MET and were (0.03 mg/ml and 0.12 mg/ml) for PBA, respectively. The estimated values

meet performance requirements for the method. The calculated LOD and LOQ were evaluated using multiple injections of the estimated values. The measured LOD and LOQ of both MET and PBA were found and were (0.0156 mg/ml and 0.08 mg/ml) and (0.017 mg/ml and 0.12), respectively. No significant difference was found when comparing the calculated and measured values ($p < 0.005$) [26].

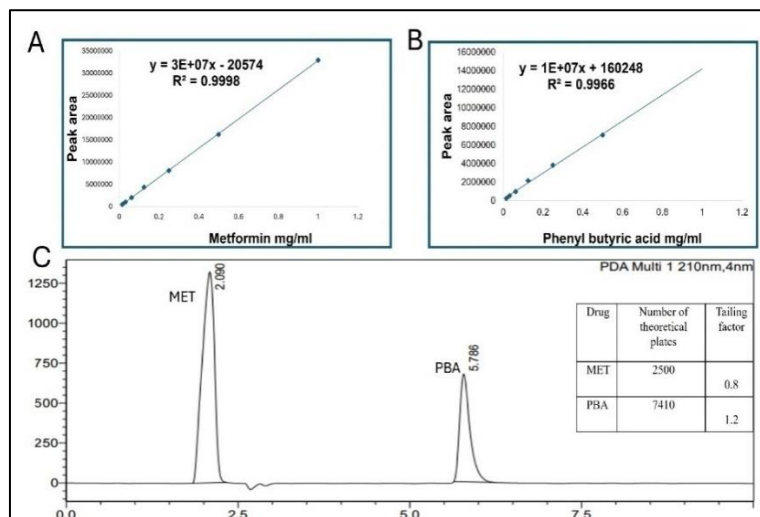


Fig. 5: HPLC Calibration of metformin (A) Phenyl butyric acid (B) HPLC chromatogram (C) (each experiment was done three times, mean \pm SD n=3)

Precision and repeatability

The inter-day was done by measuring each concentration 3 times on the same day, while the inter-day was done by measuring on 3 different days. It was found out the values of the RSD for MET were

as follows 0.17 = 0.59%, 0.3 = 0.58% and 0.6 = 0.33%. While for the PBA it was as follows 0.17 = 0.83%, 0.3 = 0.58% and 0.6 = 0.86%.

Inter-day precision (repeatability) for MET was as follows 0.17 = 0.59%, 0.3 = 1.3% and 0.6 = 0.5% (table 1).

Table 1: Precision and repeatability

Sample	Injected (mg/ml)	Intra-day measured (n=3, mean \pm SD mg/ml)	RSD%	Inter-day measured (n=3, mean \pm SD mg/ml)	RSD%
MET	0.6	0.612 \pm 0.002	0.33%	0.611 \pm 0.003	0.5%
	0.3	0.34 \pm 0.002	0.58%	0.33 \pm 0.004	1.3%
	0.17	0.171 \pm 0.001	0.59%	0.170 \pm 0.001	0.59%
PBA	0.6	0.58 \pm 0.005	0.86%	0.53 \pm 0.0041	0.77%
	0.3	0.34 \pm 0.002	0.58%	0.33 \pm 0.0019	0.58%
	0.17	0.193 \pm 0.0016	0.83%	0.19 \pm 0.003	1.5%

*(each experiment was done three times mean \pm SD)

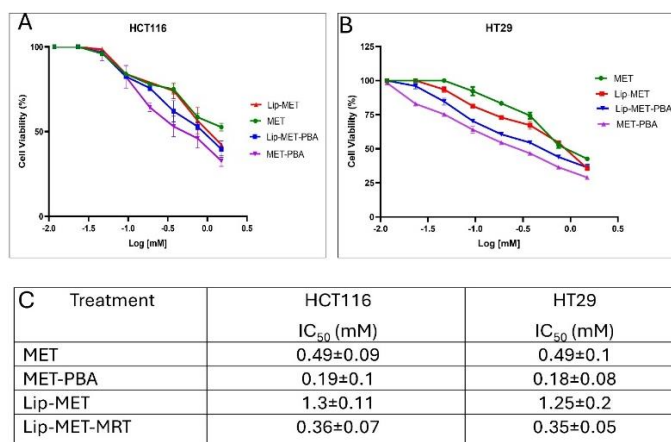


Fig. 6: The dose-response curve for HCT116 (A) HT29 (B) colorectal cancer cells treated with MET, MET-PBA, Lip-MET, and Lip-MET-PBA (0.4-1.5 mmol, n=3) (C) Summary table of IC₅₀ for all formulations (each experiment was done three times, mean \pm SD, n=3)

Table 3: Robustness of developed method

Conditions (n=3)	Injected (mg/ml)	Measured MET (mg/ml)*	RSD%	Recovery %	Measured PBA (mg/ml)*	Recovery %	RSD%
Oven temperature 35 °C	0.6	0.62±0.003	0.5%	103%	0.60±0.004	100%	0.66%
	0.3	0.34±0.004	1.1%	113%	0.34±0.001	113%	0.29%
	0.17	0.17±0.002	1.7%	100%	0.195±0.0003	115%	0.15
Oven temperature 40 °C	0.6	0.62±0.001	1.1%	103%	0.57±0.004	95%	0.7
	0.3	0.34±0.002	0.5%	113%	0.35±0.001	116%	0.2%
	0.17	0.17±0.0001	0.5%	100%	0.19±0.003	112%	1.5%
Oven temperature 45 °C	0.6	0.61±0.002	0.3%	102%	0.57±0.002	113%	0.4%
	0.3	0.34±0.003	0.9%	113%	0.34±0.001	113%	0.3%
	0.17	0.171±0.002	1.1%	101%	0.194±0.004	102%	2%
Flow rate 0.8 ml/min	0.6	0.77±0.002	0.3%	128%	0.73±0.005	121%	0.7%
	0.3	0.41±0.005	1.2%	136%	0.42±0.003	140%	0.7%
	0.17	0.21±0.004	1.9%	124%	0.24±0.004	141%	1.6%
Flow rate 1 ml/min	0.6	0.62±0.006	0.9%	103%	0.57±0.005	95%	0.9%
	0.3	0.34±0.002	0.5%	113%	0.35±0.003	116%	0.8%
	0.17	0.17±0.003	1.7%	100%	0.19±0.002	112%	1%
Flow rate 1.2 ml/min	0.6	0.508±0.001	1.7%	85%	0.496±0.002	83%	0.4%
	0.3	0.28±0.0006	0.2%	93%	0.285±0.003	95%	1%
	0.17	0.143±0.001	0.7%	84%	0.164±0.001	96%	0.6%
Wavelength 205	0.6	0.698±0.003	0.4%	116%	0.864±0.009	144%	1%
	0.3	0.405±0.005	1.2%	135%	0.478±0.006	160%	1.2%
	0.17	0.202±0.0008	0.4%	119%	0.276±0.002	163%	0.7%
Wavelength 210	0.6	0.62±0.004	0.6%	103%	0.57±0.001	95%	0.2%
	0.3	0.34±0.002	0.5%	113%	0.35±0.003	116%	0.8%
	0.17	0.17±0.003	1.7%	100%	0.19±0.001	112%	0.53%
Wavelength 215	0.6	0.662±0.001	0.2%	110%	0.495±0.002	83%	0.4%
	0.3	0.364±0.005	1.3%	121%	0.289±0.001	97%	0.35
	0.17	0.181±0.0009	0.5%	107%	0.158±0.0007	93%	0.4%

(*each experiment was done three times; mean±SD)

Accuracy and recovery

Since the value of the RSD is less than 2, this means the method was accurate. The recovery of 0.17, 0.3 and 0.6 MET was 100%, 110% and 102%, respectively. While for PBA it was 112%, 113% and 97%, respectively (table 2).

Robustness of the validated method

Robustness is used to see if this method can detect any small variations in different analytical conditions. In this method temperature was changed ± 5 degrees Celsius, flow rate with 0.2 points and the wavelength with 5 points (table 3).

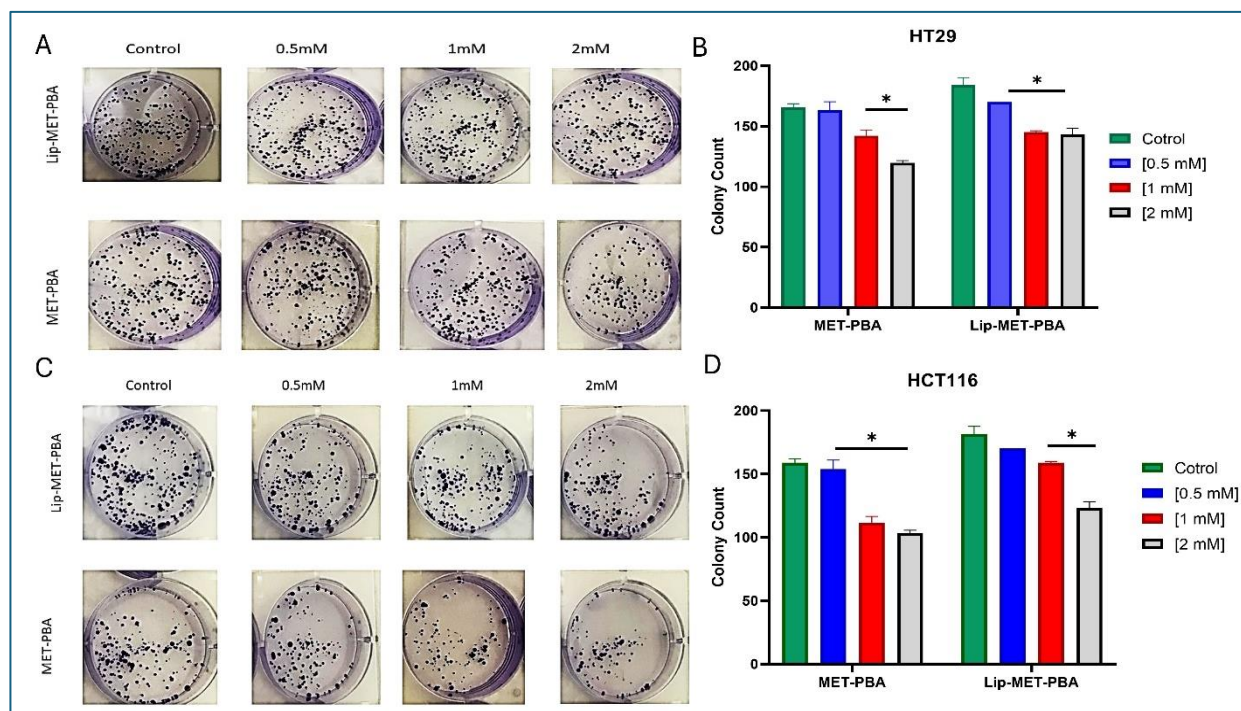


Fig. 7: Colony formation assay of MET-PBA combination and Lip-MET-PBA shows a concentration dependent decrease in the number of HT29 colonies (A) and (B) of HCT116 colonies (C) and (D). (*p-value<0.05) (Each experiment was done three times, mean±SD, n=3)

Table 2: Accuracy and recovery

Injected mg/ml (n=3)	Measured 1 mg/ml	Measured 2 mg/ml	Measured 3 mg/ml	Mean±SD	Recovery	RSD%
MET	0.6	0.610444	0.614884	0.612±0.002	102%	0.33%
	0.3	0.337378	0.336643	0.33±0.004	110%	1.2%
	0.17	0.171116	0.170779	0.17±0.001	100%	0.59%
PBA	0.6	0.573314	0.588376	0.580494	97%	0.86%
	0.3	0.345292	0.33735	0.34±0.002	113%	0.58%
	0.17	0.192186	0.193117	0.19±0.003	112%	1.5%

*each experiment was done three times

In vitro cell viability assay (MTT)

Cell viability assay (MTT) was done to measure the effect of the drugs and formulas on cancer cells. It is obvious how the MET-PBA had a greater effect on the cells compared to MET alone, this highly indicates a synergistic effect between these medications (fig. 6A, 6B, 6C). Interestingly the effect of the liposomal medications was lower than the free medications, yet still Lip-MET-PBA has a better effect than Lip-MET.

Colony formation assay

The colony assay is in line with the MTT assay as it shows free MET-PBA mixture has a better effect than Lip-MET-PBA. In both cell lines it is noticed that by increasing concentration, the inhibitory effect increases. In HCT116 the free double IC₅₀ (2Mm) has lowered the colony count by nearly 40% (fig. 7C, 7D). In HT29 the MET-PBA double IC₅₀ (2Mm) concentration has lowered the colony count by nearly 30% (fig. 7A, 7B).

DISCUSSION

The characterization of PEGylated liposomes using a zeta sizer provides essential information about the physicochemical properties of the liposomal formulations. The extrusion process was crucial in achieving the desired liposomal characteristics by effectively reducing size variation, as indicated by the PDI, while maintaining an optimal hydrodynamic diameter. This optimization is vital for cancer treatment as it ensures consistent and uniform distribution of liposomes, enhancing their ability to deliver therapeutic agents to tumor sites. Additionally, maintaining liposome size below 200 nm leverages their ability to penetrate tumors through the intercellular spaces, facilitating targeted drug delivery and improving the overall effectiveness of cancer therapy.

The choice of lipid composition greatly influences the properties and behavior of liposomes. In this study, incorporating anionic lipid (CHO) alongside PEG resulted in liposomes with a negative zeta potential ranging from -14 to -16 mV. In contrast, Shukla *et al.* (2019) used a cationic lipid, DOTAP, in their metformin liposomal formulation, leading to a positive zeta potential due to DOTAP's positively charged nature. This difference in zeta potential reflects the impact of lipid choice on the surface charge and stability of liposomes [27].

Encapsulation and loading efficiency were assessed using HPLC analysis, revealing EE% of 48.4% and LE% of 4.5% for metformin-loaded liposomes. In mixed-loaded liposomes, the encapsulation efficiency improved, reaching 56.8% for metformin and 83.9% for PBA. These results are comparable to those reported by Vozgirdaite *et al.* (2024), where metformin-loaded liposomes demonstrated a good entrapment efficiency (~65%). Additionally, the loading efficiency for PBA in the mixed-loaded liposomes was notably higher at 7.8%, compared to metformin's 5.3%. These findings suggest that both drugs were efficiently incorporated into the liposomes, with PBA showing a higher loading efficiency, which may enhance its delivery to the target site [28].

Both formulations of liposomes demonstrated good storage stability, maintaining their integrity when stored at 4 °C for one month. Minimal changes were observed in their size, PDI and charge during this period, with no significant impact on their therapeutic properties. These findings indicate that the liposomal formulations can be reliably stored without compromising their efficacy, ensuring their suitability for long-term storage and potential clinical applications [27].

The data showed an initial rapid release within the first half hour. After 1 hour, the release levels from the mixed liposome Lip-MET-PBA reached approximately 25% for PBA and 55% for MET. The increased release of MET can be attributed to its hydrophilic nature and low molecular weight, which likely facilitate its easier release from the liposomal matrix compared to PBA. [23, 29]. Additionally, the lower glass transition temperature (*T_m*) of DPPC (~39–41 °C), which is close to the physiological temperature of 37 °C, may lead to liposomal disruption. This disruption can enhance drug release from the liposomes, as the temperature closely matches the body's temperature, promoting increased permeability and release of the encapsulated drugs [30].

The release of PBA and metformin from the mixed liposome (Lip-MET-PBA) was slower compared to their release from the free MET-PBA mixture (fig. 14). This slower release is expected due to the encapsulation of the drugs within the liposomes, which acts as a barrier and limits the diffusion of the drug molecules into the surrounding medium. Additionally, interactions between the drugs and the liposomal components can affect release kinetics, with some drugs binding more strongly to the liposome and resulting in slower release rates. After 6 h, the Lip-MET-PBA formulation showed a release of 30% for PBA and 70% for MET [5, 31]. Encapsulation within liposomes can lead to a controlled and sustained release of the drugs, reducing the initial burst effect and potentially lowering the immediate cytotoxic impact. Additionally, the liposomal delivery system may alter the intracellular uptake and distribution of the drugs, affecting their cytotoxicity.

In line with our study, Divakar *et al.* formulated metformin liposomes and observed a similar release pattern in their *in vitro* drug release studies. They reported a rapid initial release within the first 0.5 h, followed by a slower and more sustained release over a 12-hour period. At 6 h, their study showed a release of 75.7% for the formulation. (DPPC: CHO, 8:2) [32].

The study conducted a cell viability assay (MTT) to evaluate the effects of various drug formulations on HT29 and HCT116 colorectal cancer cell lines. The cells were treated with different concentrations of MET, a combination of MET and PBA, and their corresponding liposomal formulations. Lip-MET-PBA showed more pronounced inhibition compared to Lip-MET in both cell lines, likely due to the synergistic effect of MET and PBA combined. This result aligns with the study's primary goal of assessing the impact of combining MET with PBA. Interestingly, the IC₅₀ values for each liposome were higher than those for their free drug counterparts, indicating that encapsulating the drugs within liposomes reduced their cytotoxic effects. This finding contrasts with Shukla *et al.*, who reported approximately a two-fold reduction in IC₅₀ values with Lip-MET treatment, showing IC₅₀ values of 2.5±1.0 mmol (*p*<0.05) compared to 5.1±0.5 mmol for free MET. The decrease in IC₅₀ values for Lip-MET in their study may be attributed to the hypothesis that enhanced intracellular localization in cells leads to increased cytotoxic potential. The study found that liposomal encapsulation of MET led to enhanced inhibition of cancer cell migration compared to free MET, which could help in preventing tumor metastasis. Specifically, migration was significantly inhibited in the treatment groups, with free MET showing 63.1±3.0% wound closure and Lip-MET demonstrating a further reduced wound closure of 41.7±4.9% [27]. The observed enhanced effects of MET and PBA combination can be explained by their complementary mechanisms of action. Metformin activates the AMP-Activated Protein Kinase (AMPK) pathway, which inhibits tumor growth and induces apoptosis [12, 33]. On the other hand, PBA acts as a Histone Deacetylase (HDAC)

inhibitor, promoting chemosensitivity and inducing cellular differentiation in cancer cells. The combination of these two agents in a PEGylated liposomal formulation enhances their individual effects, leading to improved cytotoxicity and reduced colony formation in colorectal cancer cells [34].

The clonogenic assay is an *in vitro* technique used to evaluate a single cell's ability to proliferate indefinitely and form colonies of at least 50 cells, known as clonal expansion. This assay is valuable for assessing the impact of different chemotherapeutic agents on cell survival and proliferation. It determines the fraction of cells that maintain reproductive integrity after treatment, which could potentially evolve into tumors [35]. In both cell lines, the colony count was higher in the Lip-MET-PBA group compared to the MET-PBA group across various concentrations. This observation aligns with the MTT results, which suggested that the liposomal delivery system might reduce the toxicity or efficacy of the free drugs, thereby increasing the cancer cells' ability to form colonies. This finding contrasts with the results reported by Shukla *et al.* (2019), who found that encapsulating metformin in liposomes led to a significant reduction in colony numbers. This reduction may inhibit the stemness of cancer cells and potentially impede metastasis [27, 36]. Several considerations should be addressed in future work. Firstly, the absence of *in vivo* validation means that the therapeutic efficacy and safety of the PEGylated liposomal formulation need to be confirmed in animal models. Secondly, there may be variability in drug encapsulation efficiency, which could affect the reproducibility and consistency of the formulation. Future studies should address these limitations by conducting *in vivo* experiments and optimizing the encapsulation process to minimize variability.

CONCLUSION

To further validate the therapeutic potential of the PEGylated liposomal formulation, we suggest conducting *in vivo* studies to assess its efficacy and safety in animal models. Additionally, exploring the combination of MET and PBA with other anticancer agents could provide insights into potential synergistic effects and broaden the scope of this formulation in cancer therapy. While the primary focus of this study is on colorectal cancer therapy, the combination of MET and PBA in a PEGylated liposomal formulation holds promise for other applications. MET improves metabolic function and reduces inflammation through AMPK activation, while PBA alleviates endoplasmic reticulum stress and inflammation. Together, they could be effective in treating conditions such as metabolic disorders, neurodegenerative diseases, fibrotic diseases, cardiovascular conditions, and chronic inflammatory or autoimmune disorders. Future work should explore these potential non-cancer applications in dedicated studies.

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Nil

AUTHORS CONTRIBUTIONS

Hussein S. Janabi: Synthesizing and characterizing of the formulations (e.g., Lip), Performing preliminary optimization of the synthesis process, contributing to data collection and analysis and writing draft, Zainab Lafi (*Corresponding Author*): Conceptualizing and designing the experiment. Supervising the overall progress of the project and coordinating among team members and revising the manuscript for publication, Sina Matalqah: Supervising the overall progress of the project and coordinating among team members and revising the manuscript for publication.

CONFLICT OF INTERESTS

Declared none

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