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HSPiP, Computational Modeling, and QbD-Assisted Optimized Method Validation of 5-Fluorouracil for Transdermal Products

Afzal Hussain, Mohhammad Ramzan,* Mohammad A. Altamimi, Tahir Khuroo, Mohd Usman Mohd Siddique, and Osamah Abdulrahman Alnemer

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ABSTRACT: This study addressed the simplest and most efficient HPLC (high-performance liquid chromatography) method for the estimation of 5-fluorouracil (5-FU) from rat blood plasma by implementing the Hansen solubility parameters (HSP), computation prediction program, and QbD (quality by design) tool. The mobile phase selection was based on the HSP predictions and experimental data. The Taguchi model identified seven variables (preoptimization) to screen two factors (mobile phase ratio as A and column temperature as B) at three levels as input parameters in "CCD (central composite design)" optimization (retention time as Y_1 and peak area as Y_2). The stability study (freeze−thaw cycle and short- and long-term stability) was conducted in the rat plasma. Results showed that HSPiP-based HSP values and computational model-based predictions were well simulated with the experimental solubility data. Acetonitrile (ACN) was relatively suitable over methanol as evidenced by the experimental solubility value, HSP predicted parameters (δ_h of 5-FU − δ_h of ACN = 8.3−8.3 = 0 as high interactive solvent whereas δ_h of 5-FU − δ_h of methanol = 8.3−21.7 = −13.4), and instrumental conditions. CCD-based dependent variables (Y_1 and Y_2) exhibited the best fit of the model as evidenced by a high value of combined desirability (0.978). The most robust method was adopted at A = 96:4 and B = 40 °C to get earlier Y_1 and high Y_2 as evidenced by high desirability (D) = 0.978 (quadratic model with $p < 0.0023$). The estimated values of LLOD and LLOQ were found to be 0.11 and 0.36 μg/mL, respectively with an accuracy range of 94.4−98.7%. Thus, the adopted method was the most robust, reliable, and reproducible methodology for pharmacokinetic parameters after the transdermal application of formulations in the rat.
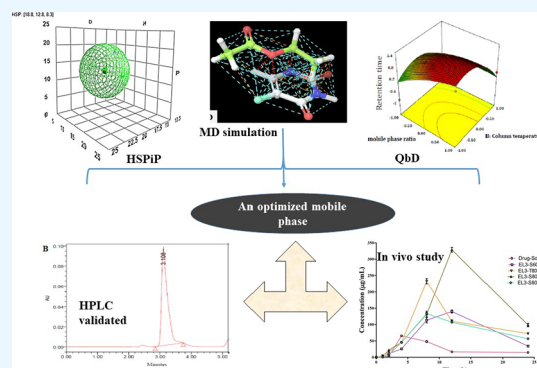
INTRODUCTION

Chromatographic techniques are applied to a quantity of pharmaceutical ingredients (PIs) in the blood plasma, urine, and skin tissue.[1] High-performance liquid chromatography (HPLC) is an advanced technique of chromatography applied in biological chemistry for identification and quantification of active compounds from biological samples (human plasma).[2,3] Moreover, high sensitivity and accuracy are the quality control parameters in HPLC method development as compared to conventional analytical techniques.[4] In order to understand the significance of delivering 5-fluorouracil (5-FU) in the skin, the quantification of the drug from biocomponents, the HPLC method was extensively developed, optimized, and validated to get reliable results for the analysis of 5-FU from human plasma and predicting various bioparameters of various drugs.[5−7]

Chemically, 5-FU is 5-fluoro-1,3-diazinane-2,4-dione extensively used in a variety of diseases, particularly in colorectal, breast, head, and neck. It is rapidly metabolized to produce cytotoxic fluoronucleotides with established anticancer effects.[8] Furthermore, 5-FU is a drug of choice clinically for skin cancer,

vitiligo, and psoriasis.[9] It has a short plasma half-life (15−20 min), and a high dose is required for maintaining a therapeutic level in the blood.[10] Several analytical approaches have been reported for the quantification of 5-FU from the biological samples such as solid phase extraction (SPE), gas chromatography (GC), and LC−MS/MS.[11−13] These methods require highly sophisticated equipment and invasive methods (degraded in high temperature) that are expensive, tedious, time-consuming, and slow for routine clinical assay.[8] The reported techniques required a relatively large plasma volume (>2 mL) involving complex extraction procedures, low sensitivity, poor reproducibility, and high expenses, and
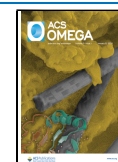
validation was not properly performed as per ICH guidelines (Hanif et al.).[14]

The Hansen HSPiP program is fundamentally based on the cohesive energy of materials (solvents and solute) to screen the right combination of solvents. The total energy of the material was distributed over three prime energies, such as dispersion energy (δ_d), hydrogen bonding ability energy (δ_h), and polarity energy (δ_p). The program is quite reliable and reproducible to confirm with the experimental data. Therefore, the program was applied to select solvents and ratios for developing the most robust mobile phase for the analysis of the drug using the physicochemical properties of solvents, and the SMILE file. Moreover, the Quality by Design (QbD) tool has been frequently used for optimizing the process variables to get a robust and reliable bioanalytical technique through the product life cycle.[15] Analytical QbD (AQbD) is a systematical approach for analytical method development to classify the critical and noncritical variables directly affecting HPLC method performance.[16] The major objective for applying AQbD is to identify nonperformance modes. Risk factors will be minimized and ascertain analytical method will be robust within the design space.[17] Validation parameters were estimated followed by freeze–thaw cycles and short- and long-term stability studies. Finally, the developed method was successfully implemented to investigate pharmacokinetic parameters after transdermal application of 5-FU formulations as compared to the control drug solution.

The main objective of the present study was to develop an HSP and AQbD-assisted bioanalytical method for the quantification of 5-FU using HPLC. The analytical method should be free from any error and exhibit some quality characteristics such as robustness, stability, and high precision. Moreover, the proposed analytical HPLC method is advantageous over the conventional approach of method validation due to HSPiP-based solvent selection in a suitable ratio. The program predicted solvents for the mobile phase based on Hansen parameters of the drug and solvents. This reduced the long-term developmental period and excess waste of organic solvents generally consumed in the hit-and-trial method. An analytical method fundamentally operates on relative polarity-based interaction existing between the solute and mobile phase.[18] QbD-based optimization helped to identify critical factors having an impact on the analytical method of the investigated drug from the biological sample. Furthermore, both programs were implemented to get low optimal rum time to save organic solvents and analytical time with high reproducibility, accuracy, sensitivity, and precision following the method reported before.[18]

■ MATERIALS

5-Fluorouracil (5-FU, 99.1%) was procured from Spectrochem Pvt. Ltd. (Mumbai, India). Soya phosphatidylcholine (SPC, ≥ 98.0%) was a kind gift sample from Lipoid, Germany. Chloroform and buffer chemicals (dipotassium hydrogen phosphate, potassium dihydrogen orthophosphate, and sodium hydroxide), all were analytical grade purchased from SD. Fine Chemicals Ltd. (Mumbai, India). Surfactants (span 60, span 80, and tween 80) were purchased from Himedia (Mumbai, India). Methanol and water were procured from Sigma-Aldrich (Mumbai, India).

Hansen Solubility Parameters As Predicted: HSPiP Software. HSP values are estimated using the well-known software "HSPiP" (version 5.0.2). The principle of the program

is the total energy present in the compound or solvent. The energy is theoretically considered as the total cohesive energy (δ_t) present in solvents or solutes. Mathematically, it is the sum of square of the prime three energy which are termed as "dispersive energy (van der Waals) as δ_d", "polarity energy as δ_p", and "hydrogen bonding energy as δ_h" as shown in eq 1.

$$[\delta_t]^2 = 4[\delta_d]^2 + [\delta_p]^2 + [\delta_h]^2 \qquad (1)$$

These terms (HSP) are used to screen a suitable solvent for drug solubility/miscibility at constant temperature. These parameters are the workhorse technique in diverse domains of medical science, chemistry, polymer science, material science, food science, dentistry, and coating.[19] There has been remarkable interest among scientists in using the program for the selection of green solvents for extraction, formulation development, and analytical methods in the last two decades. Notably, a solvent possessing HSP values close to the HSP value of the targeted drug is considered to be the most ideal solvent as predicted in the program. However, it can be further justified and simulated with the experimental value. Other parameters are (a) RED (relative energy difference) and (b) MVol (molar volume). A solvent possessing RED value <1 is considered as a solvent expected to solubilize the drug (good solvent, miscible) and vice versa (bad solvent >1, immiscible).

Modeling-Based Prediction of Interactions between 5-FU with Different Solvents. HSPiP predicted four different solvents (methanol, ethyl acetate, acetonitrile, and water) in which 5-FU was expected to be soluble. To validate this, these solvents and 5-FU were subjected to investigate interaction behavior using an in-silico molecular modeling software (MD simulation) study. The structures of these solvents with water molecules were drawn in the 2D sketcher module of Mastro utility. These molecules were processed with the ligprep utility module to generate the possible conformers, tautomers, or stereoisomers, and their energy minimization was done by using the OPLS2005 force field. These energy-minimized structures were subjected to a molecular modeling study against 5-FU with a Van der scaling of 0.8. Maximum 5 poses were generated and analyzed using the XP visualizer. The different contacts and interactions were seen, and the solubility of 5-FU was predicted in different solvents. This prediction or generated in-silico data established a supportive and predictive conformation for the maximized solubility as predicted in the HSPiP program. However, experimental data in these solvents were imperative to confirm the best fit of the model.

Experimental Solubility of 5-FU in Various Predicted Solvents. The solubility of 5-FU was studied in various solvents, as predicted in HSPiP at 40 °C. For this, a weighed amount of the drug was transferred to a glass vial containing 5 mL of solvent. The drug was added in excess to obtain saturated solubility. Each glass vial containing the drug in the excipient/solvent was tightened, closed, and labeled. Then, these were placed in a water shaker bath previously set at a fixed temperature (40 °C) and rotation (100 rpm). The study was conducted for 72 h to achieve equilibrium. The supernatant (after centrifugation) was removed and dissolved in methanol to assay the dissolved drug using a UV-vis spectrophotometer at 257 nm.[20] The study was replicated (n = 3) to get an average value.

HPLC Instrumentation Conditions. The chromatographic (HPLC system; Alliance e2695, Waters Corporation,

Table 1. Factor Screening Affecting 5-FU Method Development (Taguchi Design)

run	flow rate (A)	injection volume (B)	pH of buffer solution (C)	mobile phase ratio (D)	column temperature (E)	volume of protein precipitant (F)	autosampler temperature (G)
1	−1	−1	−1	−1	−1	−1	−1
2	+1	+1	−1	+1	−1	−1	+1
3	+1	−1	+1	+1	−1	+1	−1
4	+1	+1	−1	−1	+1	+1	−1
5	−1	+1	+1	+1	+1	−1	−1
6	−1	+1	+1	−1	−1	+1	+1
7	+1	−1	+1	−1	+1	−1	+1
8	−1	−1	+1	+1	+1	−1	−1

		Levels	
HPLC method variables		low (−1)	high (+1)
flow rate (mL/min)		1.0	2.0
injection volume (µL)		10.0	20.0
pH of buffer solution		5.5	6.5
mobile phase		92:8	96:4
column temperature (°C)		30.0	40.0
protein precipitant volume (µL)		50.0	80.0
autosampler temperature (°C)		5.0	10.0

Table 2. Optimization of the HPLC Method for the Bioanalysis of 5-FU Using CCD

	factors and their levels			
design run	A	B	responses	goal
1	0.0	−1.0	peak retention time (min)	<5
2	−1.0	1.0		
3	0.0	0.0	peak area (mAU)	>10,000
4	0.0	0.0		
5	1.0	0.0		
6	−1.0	−1.0		
7	1.0	−1.0		
8	−1.0	0.0		
9	0.0	1.0		
10	0.0	0.0		
11	1.0	1.0		
12	0.0	1.0		
13	0.0	0.0		

statistical analysis						
evaluation parameter	low (−1)	middle (0)	high (+1)	model	p-value	R^2
A: mobile phase ratio	92:8	94.6	96:4	quadratic	0.0023	0.9784**
B: column temperature (°C)	30*	35	40**	quadratic	0.0019	0.9597*

* and ** indicated the lower and the higher values, respectively.

34 Maple Street, Milford, MA, 01757, USA) system consists of a 2998 diode array detector (DAD), a column system in which temperature can be varied, and a pump system injecting the mobile phase into the HPLC pipeline. The bioanalytical method was developed and validated using a Waters column (C$_{18}$; 250 × 4.6 mm, 5.0 µm) as predicted in the HSPiP. The mobile phase consists of acetonitrile and phosphate buffer (pH 6.5, 96:4). The composition of the mobile phase was fixed throughout the process (isocratic mode). The 5-FU sample was scanned on a broad range of wavelengths (200−400 nm) using a DAD detector. Furthermore, data were processed and analyzed using HPLC software Empower (Waters, USA) at 257 nm. The acetonitrile selection was based on RED, computational-based comparative predictive interaction, and experimental solubility data. Moreover, other advantageous roles of acetonitrile over methanol were taken into account to run the isocratic mode of HPLC for successful method

validation for the investigated transdermal product in the present study. The column pressure was relatively low with the acetonitrile−water combination at the studied column temperature as compared to methanol−water.

Drug Extraction Method. The study was conducted using male Wistar rats (250−300 g) approved by Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh (KSU-SE-20-64). Blood (1 mL) was withdrawn from the eye (retro-orbital plexus) using a fine glass capillary tube. The plasma was separated by centrifugation (rpm: 10,000; time: 10 min).

The proteins present in the supernatant were precipitated with methanol and the blank plasma was stored in a deep refrigerator (−30 °C).[21] The plasma samples of 5-FU were prepared following the stock solution of 5-FU in the mobile phase. This stock solution was used to spike 5-FU solution (30 µL) in the blank plasma for each appropriate working dilution

of 5-FU (concentration range of 0.1−1 μg/mL). Prepared concentrations were subject to a liquid chromatography system with an injection volume of 10 μL with an autosampler for further analysis. A standard plot of 5-FU in plasma was prepared by plotting the area under the curve (AUC) on the y-axis and concentration (μg/mL) on the x-axis for each peak. Furthermore, validation was carried out by three consecutive quality control (QC) samples of 5-FU (0.4, 0.6, and 0.8 μg/mL) using the mobile phase.

Preoptimization Studies (Taguchi Design). Preoptimization or screening of various factors was carried out using Taguchi design (a systematic tool of AQbD) employed in optimization. Taguchi's design employed seven factors at two levels (Table 1) using Design Expert (Minneapolis, MN 55413, USA). In design, factors were selected at two levels such as minimum (−1) and high (+1) which affect peak intensity (peak area) and retention time (RT) in HPLC-based analysis. After analyzing all of the factors, we obtained Pareto charts and half-normal charts, which revealed the most significant factors influencing the method's performance.[22] 5-FU is a pyrimidine nucleoside analogue having a Log P value of −0.89 suggesting slight solubility in an aqueous media and also soluble in lower alcohol.[23] Thus, we have identified important factors such as (a) flow rate, (b) injection volume, (c) pH of buffer solution, (d) organic phase ratio, (e) column temperature, (f) volume of protein precipitant, and (g) autosampler temperature. These factors (Table 1) mainly exhibited some interaction to impact directly on the significant responses of HPLC methodology.

Optimization Studies. The results from the screening study revealed that two significant factors (organic phase ratio and column temperature) were selected for the optimization of the HPLC method. Central composite design (CCD) was employed with the aforementioned factors at varying levels to select the most robust parameters affecting AUC and RT. Thirteen experiments were designed in the CCD analysis, which was performed at various levels (−1, 0, + 1) of significant factors (Table 2).[24]

HPLC Method Validation. After completing the method development and optimization processes, the validation was carried out as per ICH Q2 (R1) guidelines for the analysis of 5-FU blood plasma by estimating validation parameters. The validation was carried out on the basis of different validation parameters such as system linearity, inter- and intraday accuracy and precision, method sensitivity (lower limit of detection: LLOD and lower limit of quantification: LLOQ), and system suitability.[25]

Linearity and Range. A linearity curve was prepared by spiking the drug in the rat plasma to establish the relationship between the concentrations of the drug and the responses. The simple method was adopted for the determination of linearity by preparing the different dilutions from stock solution added into the clear plasma (protein-free) resulting final concentration range from 0.1 to 1.0 μg/mL within the mobile phase. The drug-spiked samples were filtered through nylon membrane filters of 0.2 μm pore size (Fintech, research and innovation, Taiwan) followed by injection into the column system. The standard curve was prepared by plotting the AUC vs concentration. A Microsoft Excel program was used to analyze the linear data.

System Suitability Studies. The suitability testing was carried out using the developed HPLC method by measuring AUC following injected middle-quality control sample (0.6

μg/mL) (without spiking). There was a comparison between the peak area of the blank sample and the peak area of the spiked sample with known concentrations in the plasma (n = 3).

Specificity of the Method. The plasma samples (without drug) were diluted with an optimized quantity of mobile phase, phosphate buffer (pH 6.5), and acetonitrile. The samples were subjected to filtering via a nylon-based membrane syringe filter (0.2 μm, Finetech Research and Innovation, Taiwan) into small glass vials. The samples (vials) were placed in an HPLC autosampler tray. They were injected into the column system to analyze any corresponding peaks near the characteristics peak of 5-FU.[26,8]

Drug Recovery Study. 5-FU recovery from the spiked plasma was measured by injecting three different concentrations at three levels (0.4, 0.6, and 0.8 μg/mL) in the HPLC system. Recovery of 5-FU was measured by comparison between the actual concentration spiked and the concentration obtained after analysis by the HPLC method. Extraction of 5-FU was measured using the optimized analytical method of HPLC for recording the drug concentrations (n = 3).[27]

Sensitivity of the Method. The sensitivity of the method was determined in terms of LLOQ (lower limit of quantification) and LLOD (lower limit of quantification). These parameters were obtained by measuring the signal-to-noise ratio (S/N) from the HPLC system. The various concentrations of the drug were injected into the plasma in series. Equations 2 and 3 are given to determine LLOD and LLOQ:

$$LLOD = 3.3 \times \sigma/SD \tag{2}$$

$$LLOQ = 10 \times \sigma/SD \tag{3}$$

σ denotes the standard deviation (SD) of the intercept in the linear regression line.

Precision and Accuracy. Interday and intraday precision and accuracy were measured by a known spiked 5-FU concentration in the plasma. The %RSD (relative standard deviation) from the mean was determined to use as a parameter of precision of the developed HPLC method. Three different level concentrations were prepared (0.4, 0.6, and 0.8 μg/mL). The precision and accuracy were determined on three consecutive days (interday) and on the same day at an interval of 4 h (intraday). The %RSD value >2 was an acceptance limit for precision.[15]

Robustness of the Method. The bioanalytical method shows its robust properties by controlled process variables used as various optimization parameters such as the flow rate from the column line, mobile phase composition, and temperature of the column. The deviation was expected for each set response (AUC and RT) when different levels of concentrations were injected (0.4, 0.6, and 0.8 μg/mL).[28]

Stability Study. Short-term stability was performed in the plasma. It was determined that 5-FU was spiked at two levels (0.6 and 0.8 μg/mL). Briefly, the stability of the drug in plasma samples was assessed under room conditions (30 ± 2 °C) for 24 h (short term). For this, each sample was properly packed in a glass vial, followed by storage in a stability chamber. Three samples are incubated to obtain the mean and standard deviation. At the end of 24 h, each sample was visually inspected for any physical sign of variation, such as precipitation, creaming, coloration, turbidity, and drug content (%).

Table 3. Composition and Pharmaceutical Characterization of 5-FU (5 wt %) Vesicular Formulations[a]

parameters	EL3-S60	EL3-S80	EL3-T80	EL3-S80 gel
soya phosphatidylcholine (%)	70	80	85	80
surfactant (%)	30	20	15	20
particle size (nm)	173 ± 8.0	154 ± 7.6	221 ± 11.4	-
zeta potential (mV)	−11.3 ± 0.7	−9.5 ± 0.3	−2.4 ± 0.2	−27.9 ± 0.2
viscosity (cP)	2966 ± 12.61	3554 ± 11.22	3787 ± 12.05	4743 ± 13.05
elasticity	32.5	44.5	40.8	-
drug entrapment efficiency (%)	81.2 ± 1.8	85.7 ± 2.9	79.4 ± 1.2	92.73 ± 5.76
pH	7.3	7.5	7.4	7.4

[a]Mean ± SD (n = 3), EL3-S60 = elastic liposomes based on span 60, EL3-S80 = elastic liposomes based on span 80, EL3-T80 = elastic liposomes based on tween 80.

Table 4. Hansen Solubility Parameters Estimated for the Drug and the Targeted Solvents[a]

	HSP values predicted in HSPiP software					
name	δ_d	δ_p	δ_h	δ_t	RED	MVol
5-fluorouracil	18.8	12.8	8.3	24.2		80.7
methanol	16.4	12.3	21.7	29.9		41.1
ethanol	15.6	9.3	17.2	25.0		58.2
acetonitrile	15.6	16.6	8.3	24.3		53.4
ethyl acetate	18.2	5.1	7.1	18.2		97.6
water	15.6	16.0	42.0	47.6		18
methanol + water (99:1)	14.7	12.3	22.5		1.8	
methanol + water (90:10)	14.8	12.76	24.3		1.9	
methanol + water (50:50)	15.1	14.2	31.5		2.4	
methanol + water (40:60)	15.2	14.52	34.88		2.9	
methanol + water (10:90)	15.4	15.6	40.97		3.5	
acetonitrile + water (96:04)	15.3	17.8	8.7		0.97	
acetonitrile + water (50:50)	15.4	17.0	24.2		1.92	
acetonitrile + water (40:60)	15.4	16.8	27.4		2.3	
acetonitrile + water (10:90)	15.5	16.2	38.7		3.4	
acetonitrile + water (75:25)	15.4	17.5	15.2		0.97	
acetonitrile + water (93:07)	15.3	17.9	8.6		0.9	
acetonitrile + water (60:40)	15.4	17.2	20.6		1.6	
acetonitrile + methanol (81:19)	15.2	16.9	9.2		0.9	
acetonitrile + methanol (70:30)	15.1	16.3	11.0		0.9	
acetonitrile + methanol (60:40)	15.1	15.7	12.6		1.0	
acetonitrile + methanol + water (10:65:25)	15.0	13.8	25.4		2.0	
acetonitrile + methanol + water (25:65:10)	14.1	14.9	20.3		1.5	
acetonitrile + methanol + water (65:25:10)	15.2	16.4	13.8		1.1	
acetonitrile + methanol + water (75:15:10)	15.2	16.9	12.2		1.0	
name	SMILE					
5-fluorouracil	c1c(c(=O)[nH]c(=O)[nH]1)F					
methanol	CO					
ethanol	CCO					
acetonitrile	CC#N					
ethyl acetate	CCOC(=O)C					
water	OH					

[a]RED: relative energy difference, MVol: molecular volume (as by-default value estimated in HSPiP software).

In the freeze−thaw cycle, the samples were passed through cyclic steps of cooling (at 5 °C) and freezing (−20 °C) with intermittent room temperature (25 °C). Initially, the sample was placed at a freeze temperature for 24 h. After incubation for 24 h, the sample was removed to place at room temperature to resume its original physical form. The same was kept at a cooling temperature for 24 h. Then, it was removed after incubation to get the original state (at room temperature) without the sign of any physical changes. The process was repeated for each sample.

In the case of long-term stability, the samples were stored for 30 days at −20 °C. The procedure followed was the same as before.

Elastic Lipid Vesicular Formulations (Elastic Liposomes and Elastic Liposomal Gel of 5-FU) for Transdermal Applications. Our previous research emphasized the preparation of different types of vesicular formulations loaded with 5-FU by different surface active agents. These vesicular formulations were extensively studied for their release behavior in vitro and in vivo.[10] These reported formulations were EL3-S60 (elastic liposomes with span 60),
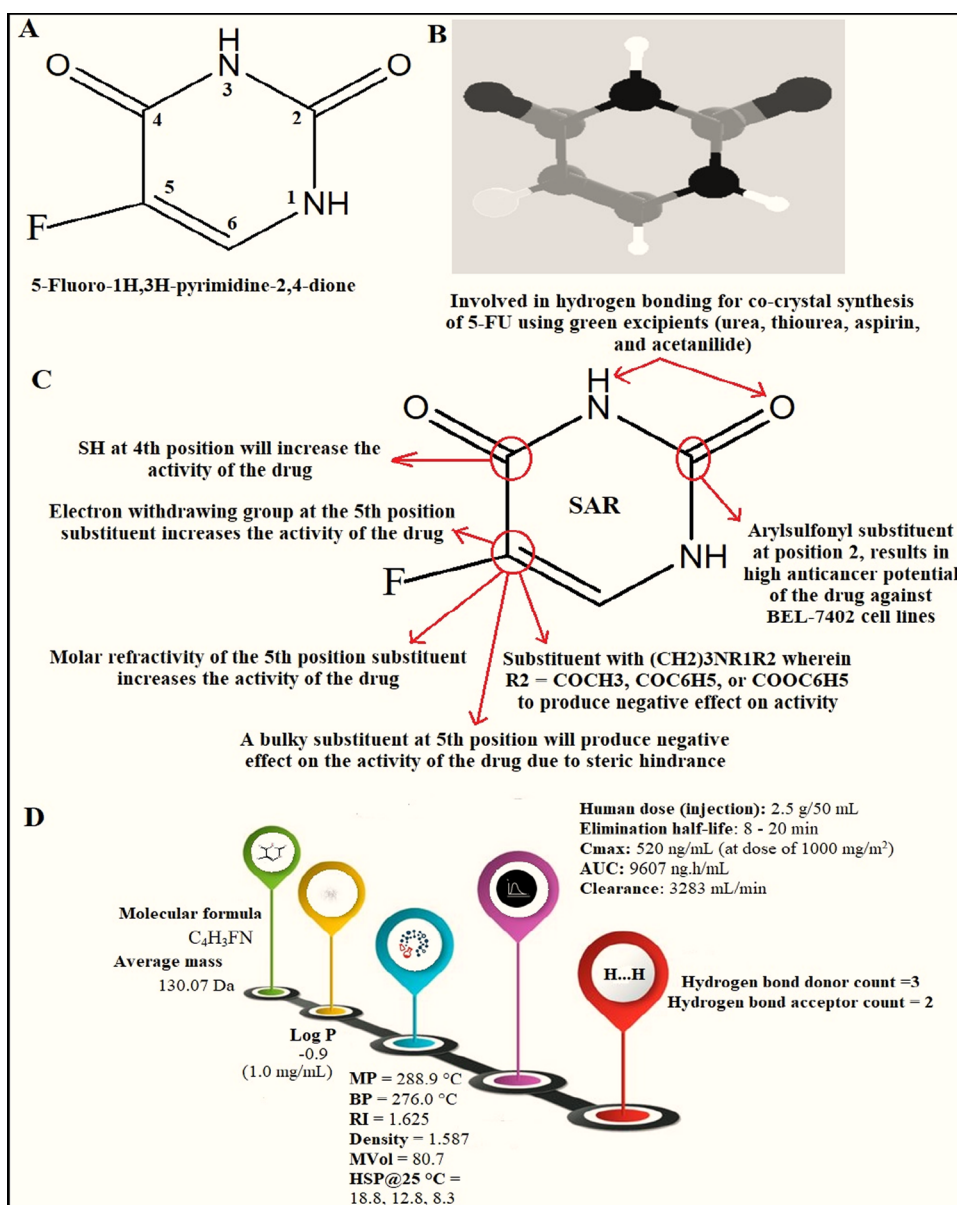
Figure 1. (A) Chemical structure of 5-fluorouracil, (B) three-dimensional structure of 5-FU, (C) structure−activity relationship (SAR), and (D) physicochemical and pharmacokinetic profile of the drug. MP: melting point, BP: boiling point, RI: refractive index, MVol: molecular volume, HSP: Hansen solubility parameters (δ_d, δ_p, and δ_H), AUC: area under the curve, and C_{max}: the maximum drug concentration reached in the blood for BEL-7402 cell line statement.

EL4-S80 (elastic liposomes with span 80), EL5-T80 (elastic liposomes with tween 80), EL3-S80 gel (1% Carbopol gel with elastic liposomes), and drug solution (5-FU drug solution as control). The composition and evaluated parameters are summarized in Table 3. Elastic vesicular systems were composed of soya phosphatidylcholine and nonionic surfactants (span 80 and tween 80).

Quantification of 5-FU in Rat Plasma (an In Vivo Study). The rationale to develop a bioanalytical method of 5-FU was to perform quantitative estimation in vivo (pharmacokinetic study) using HPLC. The pharmacokinetic experiment was performed on male rats (SD/Wistar) weighing 250−300 g. The in vivo experiment was carried out after being approved by the Institute Ethics Committee. The animals were randomly distributed into five groups ($n = 3$). The rats were thoroughly examined for any potential abnormalities at the application site. The backside part of the body was chosen to apply 5-FU formulations over a circle (1 cm^2) and hairs were removed with an electric trimmer 24 h before the experiment. After 24 h, different vesicular formulations (EL3-S60, EL3-S80, EL3-T80, EL3-S8 gel, and drug solution) containing 5-FU (5% w/v) were applied in equivalent dose strength (10.0 mg/0.2 mL).[29]

A blood volume of 0.5 mL was withdrawn from the retro-orbital plexus at varied periods of intervals (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h) followed by a cold centrifugation process. These processed plasma samples were injected into HPLC for further quantification by a validated bioanalytical method. The drug concentration versus time profile is presented as a graph and various pharmacokinetics parameters such as the area under the moment curve (AUC$_{0−t}$ and AUMC$_{0−t}$), the highest concentration reached in the blood (C_{max}), and the time period
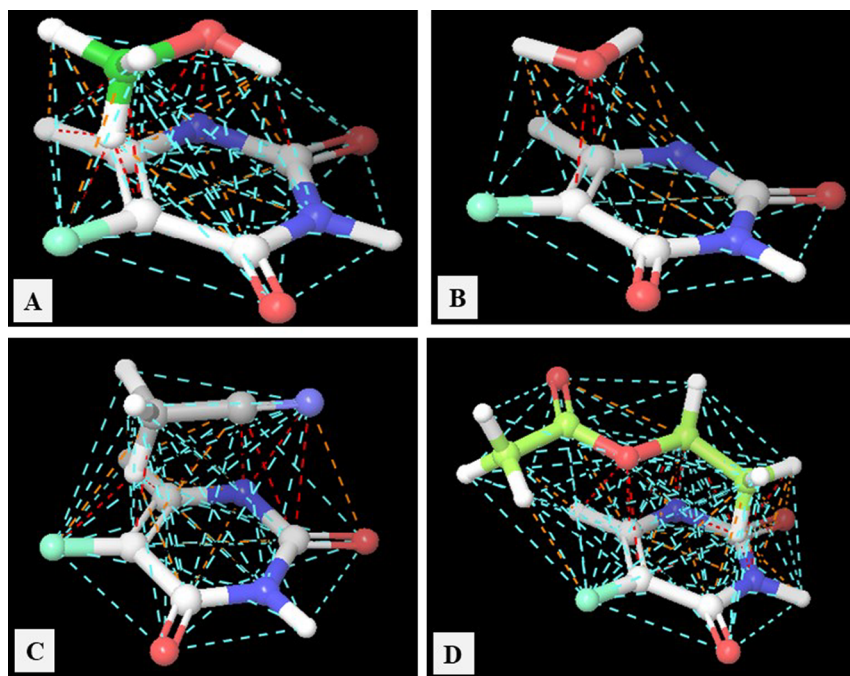
Figure 2. Depiction of different interactions and physicochemical contacts between 5-FU and solvents used, such as (A) 5-FU + methanol, (B) 5-FU + water, (C) 5-FU + acetonitrile, and (D) 5-FU + ethyl acetate.

(T_{max}) to reach C_{max}. These were assessed by using a PK solver (version 1.1). The experiment was replicated for mean and standard deviation ($n = 6$).

RESULTS AND DISCUSSION

Hansen Solubility Parameters As Predicted: HSPiP Software. The results of HSP for the drug and the targeted solvents are summarized in Table 4. The program predicted HSP and RED values for the drug and solvents. Moreover, these were estimated for the combination of selected solvents ratio to get the most suitable binary solvent mixture with HSP values close to the drug. The HSP values of the drug were estimated as 18.8, 12.8, and 8.3 for δ_d, δ_p, and δ_h, respectively. Based on the HSP values, methanol and acetonitrile are competitive to the drug for maximum solubilization, as predicted in the program (Table 4). However, the hydrogen solubility parameter of acetonitrile is quite close to the drug which differentiates it from methanol. Moreover, the acetonitrile–water combination reduced the column pressure. These are the reasonable factors in the selection of acetonitrile over methanol. Notably, the hydrogen bonding parameter is the main determining factor for the solubility of the drug in both combinations (acetonitrile–water and methanol–water). Increasing the relative content of water in both combinations, polarity and dispersion parameters are approximately similar (Table 4), whereas the hydrogen bonding parameter is significantly increased. Thus, the value "δ_h" was the deciding parameter. In all combinations of methanol + water, the RED values are >1 whereas this is 0.97 for acetonitrile + water (96:04). This may be prudent to correlate with the hydrogen bond donor counts and hydrogen bond acceptor counts of the drug with solvent (as shown in Figure 1A–D). Figure 1A,B depicts the chemical structure of the drug (the site of hydrogen bond interaction) and the reported cocrystal formation of the drug with urea, aspirin, and acetanilide.[30,31] Figure 1C depicts a detailed SAR (structure–activity relationship), whereas

Figure 1D illustrates various physicochemical properties and pharmacokinetic parameters of the drug.[30,31] Conclusively, the maximum content of water in the "acetonitrile + water" combination to achieve RED < 1.0 could be 25% as predicted in the program. Thus, the optimized composition of the mixture for maximum solubility of the drug could be acetonitrile with 75−96%. The prediction is in good agreement with the published report where Iqubal et al. employed a 75:25 ratio of acetonitrile and water for HPLC-based method validation of analysis.[30] However, the predicted ratio needs to be revalidated to corroborate the tangible interaction-based solubilization in the predicted combination by comparison with the experimental solubility data. The anticancer potential of the drug depends upon the ability of the drug capable of being taken up by the targeted cells. Therefore, few authors found that the arylsulfony substituent at position 2 resulted in high anticancer potential as investigated in BEL 7402 cell lines.[31]

Modeling-Based Prediction of Interactions between 5-FU with Different Solvents. Molecular modeling studies of 5-FU and HSPiP-based predicted organic solvents (methanol, acetonitrile, ethyl acetate, and water) were conducted. The results obtained are displayed in Figure 2A−D. The different dotted lines indicated the good or bad contacts between intraligand or interligand (5-FU + solvent molecules). Figure 2A,B illustrates the interactions (hydrogen bonding, donor−acceptor interaction, electrostatic interaction, and $\pi-\pi$ polarity interaction) of the drug with methanol and water, respectively. Figure 2C,D displays the good interactions between 5-FU and solvents, in which C is an acetonitrile solvent and D represents an ethyl acetate solvent. Comparing the polarity of HSPiP-based predicted organic solvents, acetonitrile is a relatively more polar aprotic organic solvent than methanol. Water is an inorganic polar solvent with a relatively high polarity parameter (Table 4). Moreover, by considering the fact that 5-FU is a polar and nonionic

compound, it would execute good solubility in polar aprotic organic solvents. Here, we found acetonitrile as the most polar aprotic organic solvent as predicted in the HSPiP and their combination with water. The results obtained in modeling studies also supported that the 5-FU + acetonitrile complex showed a better and maximum number of contacts (Figure 2C) than methanol. The geometry and the stability of 5-FU (in keto and enol form) with five molecules of polar solvents (water, methanol, and acetonitrile) are rendered by intermolecular hydrogen bonding (−OH).[32] The greater number of contacts indicates the greater stability of the complex, and it leads to good solubility of 5-FU. Thus, we can suggest that 5-FU will be more soluble in acetonitrile among the four different solvents as evident from a molecular modeling study. From the separation point of view, temperature and water content play a great role in chromatography. Increased column temperature reduces the retention time of 5-FU due to the reduced polarity of water at high temperatures. This reduced polarity further strengthens water's eluent strength.[33] Thus, water content in the mixture can be recommended as low to reduce the water effect on acetonitrile and subsequently reduce retention time using a column operating at ambient temperature.

Experimental Solubility. The result of the experimental solubility values of the drug in various suggested solvents is illustrated in Figure 3. The solubilities of the drug in DMSO
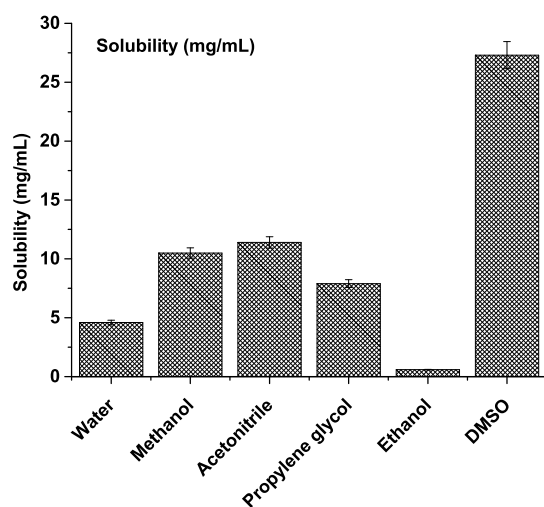

Figure 3. Experimental solubility of 5-FU in various solvents (as suggested in the HSPiP program). Data are expressed as mean ± standard deviation ($n = 3$).

and ethanol were found to be maximum and minimum, respectively. The solubility values were obtained as 4.7 ± 0.2, 10.5 ± 0.39, 11.4 ± 0.6, 7.9 ± 0.25, 0.6 ± 0.02, and 27.1 ± 1.2 mg/mL, in water, methanol, acetonitrile, propylene glycol, ethanol, and DMSO (dimethyl sulfoxide), respectively, at 40 °C. Thus, the drug was sparingly soluble in water due to the high polarity dielectric constant of water (≈ 80 units). Methanol and acetonitrile are class II solvents, and the drug was found to be maximally soluble as predicted in the program and MD simulation (computational prediction). The high solubility of the drug methanol can be correlated to hydrogen bonding through the functional hydroxyl group interacting with the molecule. It is said that a molecule of 5-FU (keto and enol forms) is stabilized for maximum solubility in methanol

by surrounding five molecules of methanol through intermolecular hydrogen bonding.[32] Similarly, acetonitrile might have executed a similar molecular phenomenon (possessing the N atom as the most electronegative atom for the site of intermolecular hydrogen bonding) for maximum solubility of the drug. Thus, HSPiP and computational program-based predicted solvents are well simulated with the experimental data.

HPLC Method Conditions. Based on the result obtained from HSPiP, computational prediction, and experimental data, acetonitrile and water-containing phosphate buffer components were selected as the mobile phase. To avoid any drug precipitate or crystal formation during storage at a low temperature (4 °C), water was replaced with a buffer solution.[34,35] The selection of acetonitrile over methanol is based on experimental and theoretical background for the analysis of 5-FU from the transdermally applied formulations. Hansen parameters, computational interaction, and the experimental drug solubility dictated screening acetonitrile in the binary mixture. Moreover, other factors were also considered for the solvent selection such as the "acetonitrile + water" combination. These were column temperature based column low pressure, low viscosity, high separation selectivity due to $\pi-\pi$ interaction with 5-FU (no $\pi-\pi$ interaction observed in methanol), high resolution at low retention time, probable chance drug precipitation with "methanol+water" combination (phosphate buffer >10% causes drug precipitation), and degassing effect of methanol.[36] The bioanalytical method was developed after optimizing the chromatographic conditions and assisted with the design of expert (DoE) process. The optimized method was validated with reliability, sensitivity, accuracy, and precision. The validated analytical method can be transferred and reproducible easily with a highly robust property. All selected attributes related to HPLC conditions are summarized in Table 1 (screening) and Table 4. The maximum absorbance (λ_{max}) for quantification of 5-FU in rat plasma showed at 257 nm.[35] The method was carried out using a C_{18} stationary phase (5.0 μm, 250 × 4.7 mm). The optimized concentration of mobile phase acetonitrile: phosphate buffer pH 6.5 (96:4). Finally, the variables that could significantly influence respective responses were selected.

Screening Studies (Taguchi Design). The screening is the selection of different chromatographic processes and material factors that directly affect the outcome of HPLC responses (RT and mAU) during bioanalytical method development.[37] Pareto and half-normal charts of the preoptimization study revealed two primary factors (mobile phase ratio and column temperature) as desired evaluation parameters. Figure 4A−D reveals the significant values of important parameters obtained for each response after statistical analysis (Table 1). Figure 4A,B represents the normal plot and Pareto chart for retention time (dependent variable), respectively, whereas Figure 4C,D represents peak area, respectively.

Response Surface Methodology (Optimization). CCD-assisted optimization was carried out by taking two significant factors (mobile phase ratio: D; column temperature: E) obtained from the Taguchi design (Figure 4). The CCD design of 13 experimental runs provided various combinations and identified the impact of factors on the investigated responses (Table 2). A generalized mathematical model was generated as quadratic, and quadratic eq 4 was obtained which establishes the interaction among significant attributes.
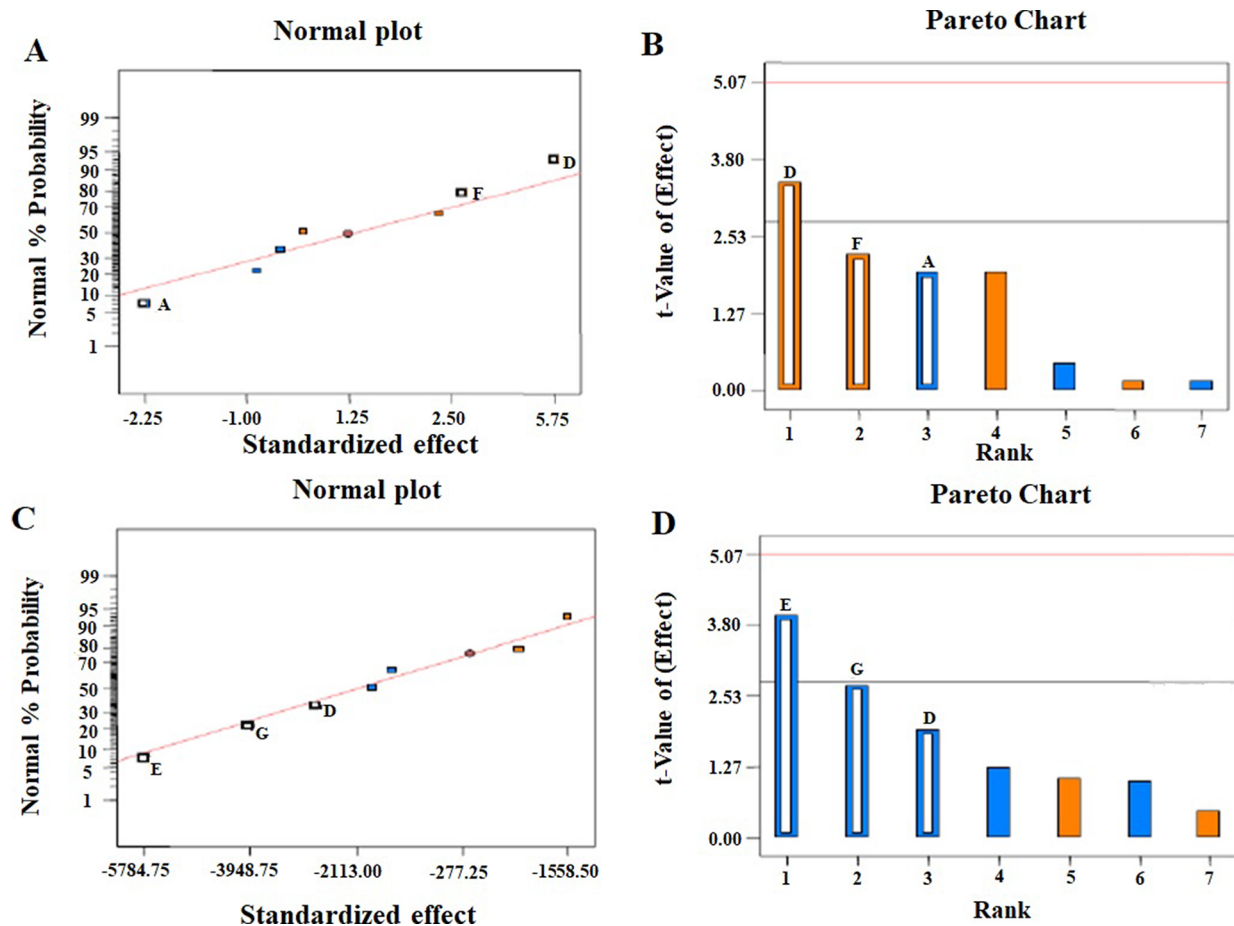
Figure 4. Normal graph and Pareto charts showing the important factors effecting bioanalytical attributes: (A, B) RT (min) and (C, D) peak area (mAU).

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_1 X_2 + \alpha_4 X_1^2 + \alpha_5 X_2^2 \quad (4)$$

Y: dependent variables of α_1 and α_2
α_0: an intercept
α_3: interaction constants involved in factors X_1 and X_2
α_4 and α_5: quadratic coefficients of the corresponding X_1 and X_2

Optimization generated two polynomial equations for both responses. Positive and negative signs of terms indicate synergistic and antagonistic relationships with the responses, respectively. The following equations are the generated polynomial equations with the actual factors:

$$\text{RT} = + 9.45385 + 1.5 \, (\text{mobile phase ratio}) + 1.03333$$
$$(\text{column temperature}) + 3.2 \, (\text{mobile phase ratio})$$
$$\times (\text{column temperature}) \quad (5)$$

$$\text{Peak area} = + 48801.6 + 41353.65 \, (\text{mobile phase ratio})$$
$$+ 3773.03 \, (\text{column temperature})$$
$$- 2786.875 \, (\text{mobile phase ratio})$$
$$\times (\text{column temperature}) + 61768.55$$
$$(\text{mobile phase ratio})^2 - 52658.6$$
$$(\text{column temperature})^2 \quad (6)$$

It is apparent that RT and peak area are proportionally related to the mobile phase ratio and the column temperature. Thus, a low retention time can be achieved by reducing the mobile phase ratio by reducing the buffer content. Similarly, the column temperature can be set at an optimal value to avoid drug degradation and the column stationary phase. Peak area is quadratic with each term as shown in eq 6 wherein high mobile phase ratio (increased relative content of acetonitrile as compared to buffer) and optimal column temperature could be a promising optimized analytical condition with high reproducibility, accuracy, sensitivity, and precision. The program exhibited the impact of the explored factors on responses (Figure 5A,B).

From Figure 5 (Figure 5A,B), it is obvious that with the rise in the mobile phase ratio, the RT value of the peak was increasing followed by a reduction after a certain time period. Furthermore, this increment resulted in no significant ($p < 0.05$) change in RT value. Moreover, column temperature has also a positive effect on peak symmetry. As a result, the mobile phase ratio was optimized as 96:4 (ACN: Phosphate buffer) for the robust method. Similarly, the contour plot and three-dimensional plots in Figure 5C,D portray that peak area sharply increases with an increase in column temperature. Moreover, a consistent increase in AUC was seen with a relative increase of acetonitrile in the mobile phase (96:4). Furthermore, a statistical test was applied as an analysis of variance (ANOVA) to analyze the suitability of the quadratic model. Table 2 summarizes the statistical analysis of the
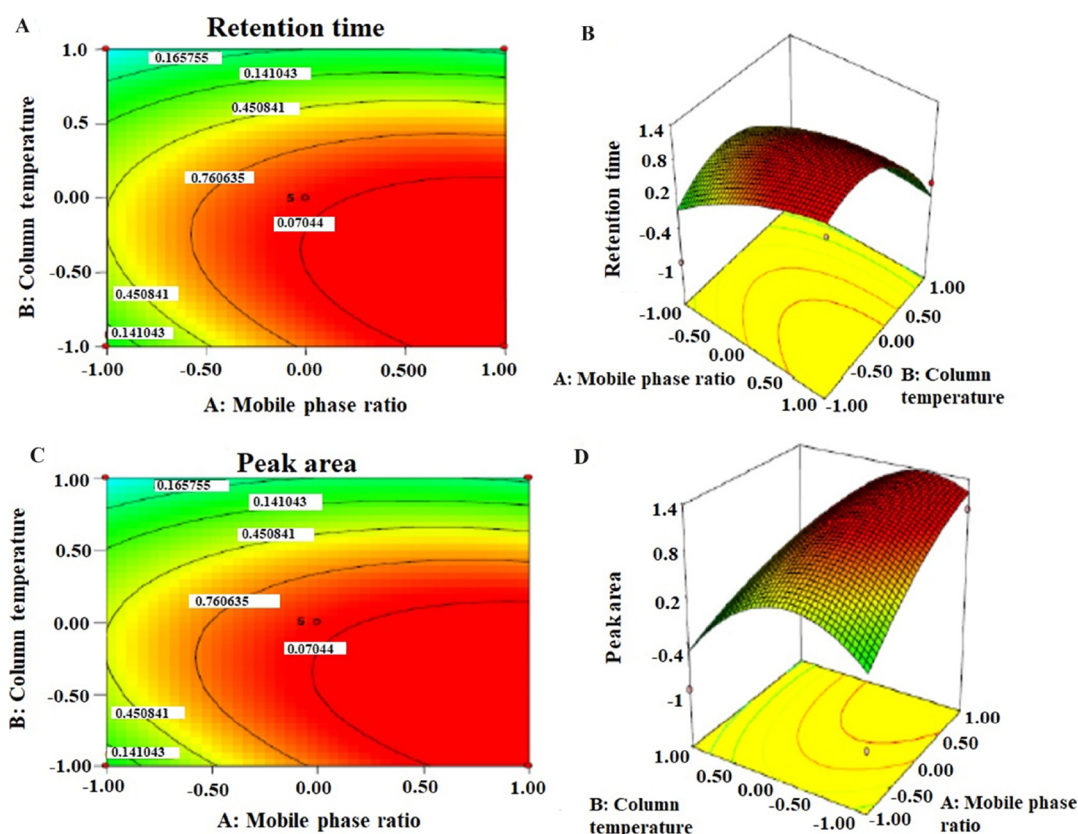
Figure 5. Two-dimensional and three-dimensional response plots depicting the effect of significant factors on chosen responses, (A, B) RT and (C, D) peak area.

optimization process. The low probability (p) value, 0.917 for mobile phase ratio (X_1) and 0.881 for column temperature (X_2) revealed the establishment of the most suitably chosen statistical model for RT and peak area (Table 2). To set the optimization criteria were to increase the peak area (Y_2) and decrease RT (Y_1). The desirability value varied variable from 0 to 1 for respective responses and the overall desirability is 0.978 (Figure 6).[38]
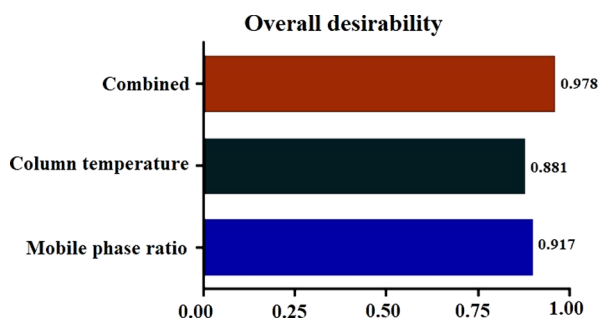

Figure 6. Desirability function graph of critical bioanalytical attributes (mobile phase ratio and column temperature).

Furthermore, the statistical regression value of adjusted R^2 was very close to the observed R^2 value, thus revealing the suitability of the model. However, Table 5 depicts the optimized conditions and their corresponding values for the robust bioanalytical technique of 5-FU. Sinha et al. reported that the estimation of 5-FU in an alkaline medium is quite challenging due to self-degradation. This causes a tangible interference in the result for the biological sample.[39] Therefore,

Table 5. Optimum HPLC Experimental Conditions for Analysis of 5-FU in Rat Plasma[a]

analytical conditions (HPLC)	value
composition of mobile phase	ACN: phosphate buffer (96:4) v/v
pH	6.5
column temperature	40 °C
column dimension	250 × 4.6 mm
packing particle size	5 μm
injection volume	10 μL
flow rate	1.0 mL/min
run time	5 min
detection wavelength	257 nm

[a]ACN = acetonitrile.

our developed method was quite sensitive and reproducible to estimate the drug at optimized pH 6.8 shown in Table 5. Therefore, the program predicted a relatively high ratio of acetonitrile (96%) as compared to buffer (4%) to reduce the total run time (5 min) at the same wavelength of detection (257 nm).

Bioanalytical Method Validation. *Linearity of the Method.* The samples for linearity validation were prepared from a stock solution to make it easy, simple, and faster. The study tested the linearity of various freshly prepared samples in the rat plasma to avoid any interference in the result due to possible self-degradation.[39] To report method linearity validation, the data were analyzed to obtain validation parameters (coefficient of correlation, slope, and intercept) keeping ICH guidelines in mind (typically $r^2 \geq 0.999$). The linearity of the method is expressed from the calibration graph.

A minimum of six concentrations are required to plot the linearity graph. The calibration graph of 5-FU in rat plasma was linear over the concentration range of 0.1−1 μg/mL with an r^2 value of 0.999 (Figure 7A−C) and a slope of 108.8. Equation 5 obtained from linear regression is

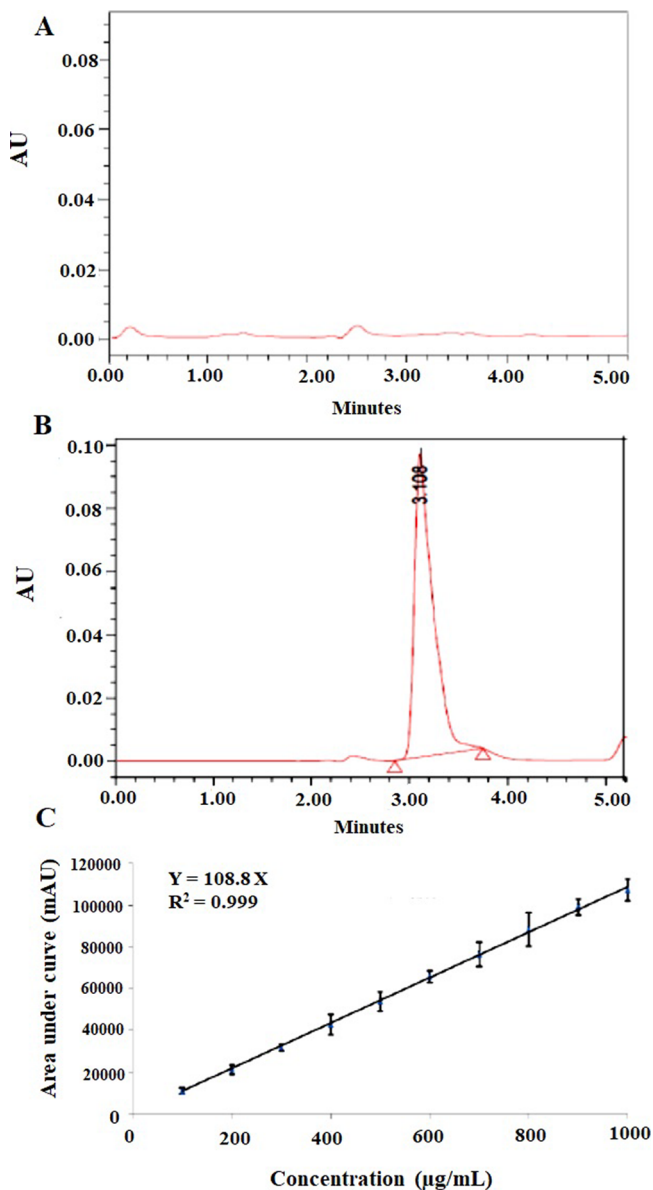$$Y = 108.8x \qquad (7)$$

Figure 7. Chromatogram of (A) blank plasma, (B) drug spiked plasma, and (C) calibration curve of 5-FU.

This finding was compared with a published report on 5-FU method development. Sinha et al. reported linearity over the concentration range of 10−100 μg/mL and r^2 was found to be 0.999. Moreover, the authors established an analytical method with a high flow rate (1.2 mL/min), high retention time (6.0 min), and high content of buffer in the mobile phase.[39] Thus, the previous method cannot be considered economic and reproducible as compared to our explored method, as evidenced by high sensitivity, low run time (5 min), low retention time (3.1 min), and low mobile phase flow rate. Similarly, the drug was estimated from the plasma at a low

detection limit and high extraction yield using the solid phase extraction method. However, it was reported with a high run time (11 min).[21]

Determination of LLOD and LLOQ. The objective behind the determination of LLOD and LLOQ was to assess the sensitivity of the AQbD-assisted validated bioanalytical method. The computed value of LLOD was 0.11 and LLOQ was 0.36 μg/mL for 5-FU. It is clearly evident from LLOD and LLOQ that the HPLC method was highly reproducible and sensitive particularly for 5-FU in plasma samples, even at low concentrations. The developed HPLC method could be further utilized for the quantification of 5-FU in vivo bioavailability studies.

Drug Recovery From Plasma. Table 6 provides a summary of the plasma recovery values. The results depict that the average drug recovery from plasma was 97.3 to 99.1% for 5-FU. The high drug content recovery was due to the adoption of an optimization approach for the developed HPLC method which is in compliance with ICH Q2 (R1) guidelines.

Inter- and Intraday Accuracy and Precision. Inter- and intraday accuracy and precision were measured at consecutive days (interday) and different time points on the same day (intraday) for the developed analytical method. All the concentrations exhibited >2% RSD values. According to observations obtained, accuracy values ranged from 94.4 to 98.7%, with RSD values of less than 2% (Table 6). The findings show that the bioanalytical HPLC method is appropriate, precise, and most suitable for quantitative analysis of 5-FU in rat plasma samples.

Drug Stability in Plasma. In order to evaluate the different types of stability exhibited by 5-FU in biological fluid, plasma samples were spiked with 5-FU and these were kept under variable conditions of storage. The values obtained from the stability study are summarized in Table 6. The findings clearly demonstrate that 5-FU moiety has stability properties in plasma even under freeze conditions.

In Vivo Pharmacokinetic Study. In vivo dermal bioavailability study was a requisite for 5-FU after transdermal application using the optimized bioanalytical technique. In a previously published research study, we elaborated on the development and optimization of different 5-FU formulations. Moreover, some extensive in vitro, ex vivo (permeation and deposition), and the proof of concept studies were carried out.[10] Here, a bioavailability study was conducted to quantify 5-FU in rat skin to confirm the fate of developed formulations (EL3-S60, EL3-S80, EL3-T80, EL3-S80 gel, and drug solution). The results of various pharmacokinetic parameters are depicted in Figure 8 and Table 7.

It is evident from the plasma concentration−time profile that pharmacokinetic parameters such as C_{max} (510%), AUC_{0-t} (211%), and $AUMC_{0-t}$ (218%) for EL3-S80 were significantly ($p < 0.05$) high as compared to drug-Sol. Similarly, these parameters for EL3-S80 gel were remarkably increased many times C_{max} (208%), AUC_{0-t} (195%), and $AUMC_{0-t}$ (188%) as compared to the drug solution. The high values of various parameters of EL3-S80 were due to nano size and squeezing nature of vesicles which increase the penetration. Lag time (36 min) was exhibited by the drug-sol which may be attributed to its poor water solubility. EL3-S80 and EL3-S80 gel showed lag time values of 18 and 21 min, respectively, for 5-FU to permeate through rat skin due to the nano-sized particle and aqueous solubility, which help the high content of the drug to penetrate beyond the dermis region.[40] Regulatory bodies

Table 6. % Recovery Parameters Obtained from the Optimized HPLC Technique of 5-FU[a]

concentration (μg/mL)	levels (%)	theoretical concentration (μg/mL)	recovery concentration (μg/mL)	recovery (%)	CV (%)
0.4	92	0.37	0.36	97.3	1.3
	97	0.38	0.37	98.6	1.7
	99	0.39	0.38	99.5	1.6
0.6	92	0.55	0.54	97.3	1.5
	97	0.58	0.57	98.2	1.9
	99	0.59	0.58	99.1	2.1
0.8	92	0.74	0.74	99.5	1.4
	97	0.77	0.76	98.3	1.6
	99	0.79	0.77	97.5	0.9

inter- and intraday accuracy and precision of 5-FU			
concentration (μg/mL)	observed concentration (μg/mL)	% accuracy	precision (%RSD)
interday			
0.4 (LQC)	0.39	97.5	1.2
0.6 (MQC)	0.58	98.3	1.4
0.8 (HQC)	0.78	98.7	1.5
interday			
0.4 (LQC)	0.36	94.4	1.7
0.6 (MQC)	0.57	96.2	1.3
0.8 (HQC)	0.77	97.1	1.6

plasma stability parameters of 5-FU under different environmental conditions				
	average value (μg/mL)	coefficient of variance (%)	average value (μg/mL)	coefficient of variance (%)
nominal concentration	0.6		0.8	
freeze−thaw cycle	0.54 ± 0.03	1.51	0.72 ± 0.05	1.62
short-term	0.55 ± 0.02	1.43	0.74 ± 0.06	1.34
long-term	0.59 ± 0.02	1.56	0.78 ± 0.02	1.86
physical changes	no signs of any changes (color or precipitation)		no signs of any changes (color or precipitation)	

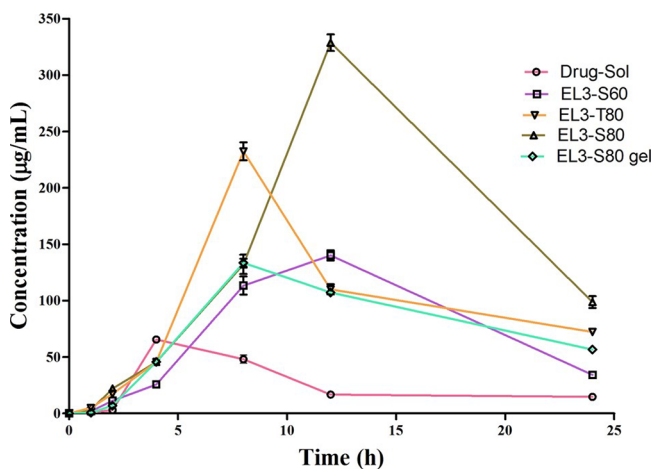[a]CV = coefficient of variance, RSD = relative standard deviation.

Figure 8. In vivo pharmacokinetic behavior in rat plasma of various vesicular formulations (Drug sol, EL3-S60, EL3-T80, EL3-S80, and EL3-S80 gel) following transdermal application.

investigate various critical dermato-pharmacokinetic parameters (C_{max}, AUC$_{0−t}$, and T_{max}) for topical/transdermal products in appraising the products' safety and efficacy. However, various deceptive considerations such as diseased skin conditions, the presence of furrows, and follicular drug delivery are significant limitations for the tape stripping method to study dermato-pharmacokinetics.[41] Therefore, plasma drug concentration−time profile assessment was relatively convincing and reliable compared to the tape stripping technique.

■ CONCLUSIONS

The analytical method of 5-FU estimation from biological samples is challenging in terms of reproducibility, simplicity, accuracy, and simplicity. No report has been published for the Hansen solubility and computationally predicted parameter-based method development for the drug so far. Both programs were found to be fit for the prediction and selection of the suitable solvent (ACN) and the ratio (96:4). HSP values helped to screen solvents which were confirmed and simulated with the experimental solubility data in the predicted solvents. Furthermore, the Taguchi model identified various factors affecting the drug analysis. Two factors (column temperature and mobile phase ratio) were of prime importance to recruit as the input parameters for CCD. The high value of the overall desirability numerical parameter (0.97) suggested that these two parameters had a substantial impact on the peak area and retention time. Quadratic polynomial models suggested that a robust, reliable, reproducible, and simplest analysis method was established by setting the column temperature at 40 °C (reduced viscosity) and mobile phase ratio of 96:4 (reduced aqueous phase effect to avoid precipitation). There may be a probable chance of drug precipitation if the aqueous content is >25% due to the high value of RED (>1). The validation parameters indicated the reliability and reproducibility of the method to estimate the drug content from rat plasma samples for the studied formulation (after transdermal application). The developed sensitive method was reproducible, robust (optimized from QbD), accurate, and high precision. The developed method was suitably implemented to estimate

Table 7. In Vivo Pharmacokinetic Parameters of Various 5-FU Formulations Following Transdermal Application on Rat Skin (Mean ± Standard)[a]

pharmacokinetic parameters	drug-sol	EL3-S60	EL3-S80	EL3-T80	EL3-S80 gel
C_{max} (μg/mL)	65.56 ± 2.4	140.09 ± 4.5	328.70 ± 7.3	110.05 ± 4.7	133.56 ± 2.4
T_{max} (h)	4.3	12.1	12.4	8.2	8.1
$AUC_{0-\infty}$ (μg h/mL)	78.77 ± 2.1	125.44 ± 5.1	166.32 ± 3.7	146.18 ± 3.2	153.21 ± 3.1
AUMC (μg h^2/mL)	724.15 ± 7.5	1174.42 ± 12.3	1578.53 ± 11.5	1243.44 ± 8.3	1365.91 ± 12.1
MRT (h)	9.19	9.36	9.49	8.51	8.91
$T_{1/2}$ (h)	5.78	13.86	23.1	17.32	34.64
Ke (h^{-1})	0.12 ± 0.02	0.05 ± 0.01	0.03 ± 0.02	0.04 ± 0.03	0.02 ± 0.03

[a]AUC = area under the curve, AUMC = area under first moment curve, Ke = elimination rate constant, MRT = mean residence time.

pharmacokinetic parameters as presented in Table 4 wherein it was observed that span 80 elastic liposomes improved C_{max} and AUC after transdermal application as compared to the drug solution. Moreover, ELP3-S80 gel added permeation behavior of vesicles across the SC layer for maximized drug permeation and sustained delivery as evidenced with relatively low C_{max} and high AUC as compared to ELP3-S80 colloidal suspension. This may be due to gel-mediated hydration provided to the skin and the slow rate of vesicle migration from the gel matrix toward the skin surface and subsequently slow drug diffusion from the vesicle to the outside across the lipid bilayer. Conclusively, span 80-based elastic liposomes were a promising approach for transdermal delivery of 5-FU to treat cutaneous and dermal skin cancer with high patient compliance and low systemic toxicity.

■ AUTHOR INFORMATION

Corresponding Author

Mohhammad Ramzan − *School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab 144411, India;* ⊙ orcid.org/0000-0001-9029-9752; Email: ramzan.pharm@gmail.com

Authors

Afzal Hussain − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia;* ⊙ orcid.org/0000-0002-6275-5375

Mohammad A. Altamimi − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Tahir Khuroo − *Department of Pharmaceutics, PGx Global Foundation, Houston, Texas 77035, United States*

Mohd Usman Mohd Siddique − *Department of Pharmaceutical Chemistry, Shri Vile Parle Kelavani Mandal's Institute of Pharmacy, Dhule 424001 Maharashtra, India;* ⊙ orcid.org/0000-0001-9503-1771

Osamah Abdulrahman Alnemer − *Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.3c07061

Author Contributions

A.H.: drafting, methodology, and conceptualization; M.R.: data curation and software; M.A.A.: validation and software (HSPiP); T.K.: review and visualization; M.U.M.S.: methodology, analysis, and editing; O.A.A.: data curation and analysis.

Notes

The authors declare no competing financial interest.

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