Article

Chemometrics-assisted development of a validated LC method for simultaneous estimation of temozolomide and γ-linolenic acid: Greenness assessment and application to lipidic nanoparticles

Beg, Sarwar, Panda, Sagar S. and Singh, Kamalinder

Beg, Sarwar, Panda, Sagar S. and Singh, Kamalinder ORCID: 0000-0001-7325-0711 (2022) Chemometrics-assisted development of a validated LC method for simultaneous estimation of temozolomide and γ-linolenic acid: Greenness assessment and application to lipidic nanoparticles. Journal of Chromatography B, 1200 . p. 123261. ISSN 15700232

Journal of Chromatography B 1200 (2022) 123261

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Chemometrics-assisted development of a validated LC method for simultaneous estimation of temozolomide and γ-linolenic acid: Greenness assessment and application to lipidic nanoparticles

Sarwar Beg [a,c,*], Sagar S. Panda [b], Kamalinder K. Singh [a,d,*]

[a] School of Pharmacy and Biomedical Sciences, Faculty of Clinical and Biomedical Sciences, University of Central Lancashire, Preston PR1 2HE, United Kingdom
[b] Department of Pharmaceutical Analysis and Quality Assurance, Roland Institute of Pharmaceutical Sciences, Berhampur, Ganjam, Odisha 760010, India
[c] Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard University, New Delhi 110062, India
[d] UCLan Centre of Smart Materials, University of Central Lancshire, Preston PR1 2HE, United Kingdom

ARTICLE INFO

Keywords:
Liquid chromatography
Chemometrics
Lipid nanoparticles
Temozolamide
γ-Linolenic acid
Simultaneous estimation
Stability indicating
Green method
Box-Behnken design
Forced degradation

ABSTRACT

The described work entails the development of a simple, sensitive, green, and robust high-performance liquid chromatographic (HPLC) method for simultaneous estimation of temozolomide (TMZ) and γ-linolenic acid (GLA). The chemometric factor screening study helped identify the critical method parameters optimized using Box-Behnken design for improved understanding and enhancing the method performance. Chromatographic separation was performed on a Kinetex® C_{18} column (150 × 4.6 mm, 5 µm particle size) using methanol: water (pH adjusted to 3.5 using 0.5% v/v O-phosphoric acid) as the mobile phase at 0.5 mL/min flow rate and diode array detection between 210 and 360 nm. The linearity of the method was observed for concentrations of TMZ and GLA ranging between 1 and 100 µg.mL^{-1} ($R^2 = 0.999$, $p < 0.05$). Accuracy evaluation showed good percent recovery within 97.9–100%, while intra- and inter-day precision showed RSD values within 0.37%-1.01%. The limit of detection and quantification for TMZ was found to be 0.75 and 1.0 µg.mL^{-1}, respectively, while the values 0.55 and 1.0 µg.mL^{-1}, respectively, were observed for GLA. System suitability (96.9–102.8%), its limits, and robustness evaluation indicated good percent recovery within, while RSD values were found to be within the acceptable limit of less than 2%. The method was specific for its ability to detect the analytes and their degradation products during forced degradation studies, which also indicated that TMZ was highly prone to alkaline conditions while GLA showed mild degradation in all the studied conditions. The estimation of both the analytes from lipid nanoparticles formulation showed good values for total drug content (82.6–85.3%), entrapment efficiency (95.4 to 98.7%), and drug loading (25.2 to 38.4%). Overall, the results indicated that the developed method was reliable for its accuracy, precision, sensitivity, and specificity for simultaneous estimation of the analytes. The method was found to be stability-indicating in nature and suitable for simultaneous estimation of TMZ and GLA from the developed nanoparticles formulation. Further, employing a greenness assessment approach established the method greenness.

1. Introduction

Glioblastoma multiforme (GBM) is one of the most common and aggressive brain tumors with a high incidence in every two to three-persons in 1,00,000 adults per year. It accounts for 52% of all primary brain tumors and 17% of all brain tumors (including the primary and metastatic carcinomas) [1–3]. GBM tumors are highly proliferative and notorious (grade IV astrocytoma) tumors that originate from the glial cells with patient survival time of only 12 to 18 months. The use of radiotherapy provides symptomatic relief and chemotherapeutic drugs help in improving the quality of life in GBM patients with negligible impact on the mortality rate. As a first-line therapy, temozolomide (TMZ) is recommended for oral use by US Food and Drugs Administration since 1999 [4]. High chances of drug resistance to TMZ therapy and tumor recurrence due to gene mutation and activation of aberrant pathways are the current challenges in GBM treatment [4,5]. In this

* Corresponding authors at: School of Pharmacy and Biomedical Sciences, Faculty of Clinical and Biomedical Sciences, University of Central Lancashire, Preston PR1 2HE, United Kingdom (S. Beg and K.K. Singh).
E-mail addresses: sbeg4@uclan.ac.uk (S. Beg), KSingh1@uclan.ac.uk (K.K. Singh).

https://doi.org/10.1016/j.jchromb.2022.123261
Received 31 March 2022; Accepted 20 April 2022
Available online 25 April 2022

regard, the reports on the use of phytoconstituents as adjuvant therapeutics to TMZ can be helpful in the treatment of GBM. In order to surmount the challenges associated with the alkylating agent discussed above, the combined use of polyunsaturated fatty acids with standard TMZ therapy may provide better therapeutic outcomes, wherein both the molecules act by different mechanistic pathways for reducing tumor growth and thus may prevent frequent recurrence and avoid likely drug resistance encountered with the use of TMZ therapy when used alone [6].

Temozolomide (TMZ), an alkylating agent of imidazotetrazine derivatives, exhibits anticancer activity for the treatment of GBM. Chemically, it is 3-methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5] tetrazine-8-carboxamide which shows anticancer action by methylation of DNA at O^6 and N^7 positions of guanine residues, thus subsequently block the DNA synthesis and causes cell cycle arrest at G2/M phase leading to cell apoptosis [7,8]. TMZ exists as a pro-drug with stability only at acidic pH, but undergoes spontaneous nonenzymatic hydrolysis in blood plasma *in vivo,* and also at neutral or slightly basic pH under *in vitro* conditions to form the active metabolite 5-[2-(methylimino)hydrazinyl]-1H-imidazole-4-carboxamide (MTIC), a compound with very short half-life of 2 to 4 min in the body [9]. Further, MTIC reacts with water to produce 5-amino-imidazole-4-carboxamide (AIC) and methyl diazonium cation, a highly reactive alkylating species. TMZ has the ability to cross the blood-brain barrier (BBB) to reach GBM tumors while MTIC is unable to permeate BBB [8,10]. Several liquid chromatographic methods have been reported in the literature that employs ultraviolet (UV)/photodiode array (PDA)/diode array detector (DAD) for the estimation of TMZ under *in vitro* and *in vivo* conditions where mobile phase with acidic pH is used to protect the hydrolysis of drug [11–14]. The estimation of MTIC under *in vivo* conditions is quite difficult due to its very short half-life and rapid conversion further into AIC. However, some studies have reported liquid chromatographic methods specifically for the estimation of MTIC alone or in combination with TMZ, despite the limited stability of the active metabolite of the drug [12].

γ-Linolenic acid (GLA), a polyunsaturated fatty acid (PUFAs), has revealed a lot of promise in GBM treatment and has shown to decrease the anti-oxidant content of tumor cells, expression of oncogenes Ras and Bcl-2, enhanced the activity of p53, protected normal cells and tissues from the toxic actions of radiation and anti-cancer drugs, enhance the cytotoxic action of anti-cancer drugs and reversed tumor cell drug resistance [6,15,16]. Although GLA has proven to be useful in a variety of cancers, the analysis of GLA and other PUFAs is very challenging. A score of literature reports has discussed a number of challenges associated with the establishment of analytical methods for the PUFAs. Due to the absence of chromophores in GLA, the suitability of liquid chromatographic methods for its estimation is very unlikely with UV/DAD/PDA detections [17–19]. However, some literature reports have described the use of derivatization techniques (e.g., pre-and and post-column) for developing the liquid chromatographic (LC) methods where a number of variables involved during the derivatization process is difficult to monitor. Besides, such methods also require the use of specific columns (e.g., polysaccharide, silver ion-containing stationary phases) for the quantification of GLA and other polyunsaturated fatty acids [18,20,21], which altogether makes such methods as less reliable due to high variability and lack of robustness for routine applications. On the contrary, a few analytical methods have been published on the quantification of PUFAs by gas chromatography (GC). Although GC is considered quite effective for analytical estimation of GLA and other PUFAs, it has its own limitations like high running costs and maintenance of the equipment which makes it difficult to use in routine pharmaceutical applications [22,23]. On the contrary, the use of high-performance liquid chromatography (HPLC) techniques is considered the most sought after for analysis of a wide range of drug molecules due to numerous advantages, along with feasibility and cost-economy for routine applications [24].

Based on the extensive literature search, we found no analytical method for the simultaneous estimation of TMZ and GLA which can be used as a novel combination for the management of GBM tumors. The current research work, therefore, focused on developing a simple, reliable, robust, and cost-effective green high-performance liquid chromatographic (HPLC) method for the simultaneous estimation of TMZ and GLA using a C_{18} column and diode-array detector. In fact, green analytical chemistry is the most recent aspect for which researchers are seriously thriving to develop analytical methods that are eco-friendly [24,25]. Several theories and approaches are also proposed on tools that extensively assess the method greenness [26–28]. It is also essential that one develops a green method when optimizing a new analytical method. Hence in the present studies, we have implemented the analytical eco-scale (AES) approach and have assessed the greenness of the method. A detailed description and relevant applications are discussed elsewhere [25,26].

The developed method was validated as per the ICH Q2(R1) guideline. The robustness-cum-optimization was evaluated employing the principles of the analytical Quality by Design (QbD) approach, where critical factors influencing the method performance were optimized with the help of a suitable experimental design [27–30]. The developed method was applied to understand the degradation products of the drugs under forced degradation conditions, and to evaluate the specificity of the method for evaluating the drug content, entrapment efficiency, and loading efficiency of the drugs from the in-house developed lipidic nanoparticle formulations.

2. Experimental

2.1. Chemicals and reagents

TMZ was provided by Ind-Swift Laboratories Limited, Chandigarh, India, while GLA was purchased from Sigma-Aldrich Co., Gillingham, UK. HPLC grade organic solvents (methanol and acetonitrile), and analytical grade reagents (ethanol, hydrochloric acid, sodium hydroxide, hydrogen peroxide, orthophosphoric acid) were purchased from Fisher Scientific Ltd., Leicestershire, UK. Disposable syringe filters of 0.22 μm were purchased from Fisher Scientific, Cheshire, UK, for filtration of all the samples.

2.2. Instrumental details and analytical conditions

The Agilent 1200 series HPLC system (Agilent Technologies, Cheshire, UK) equipped with Agilent diode array detector G1315D and auto-sampler G1329A was used for the chromatographic estimation. The instrument was monitored with the help of ChemStation LC 3D system software (Agilent Technologies, Cheshire, UK) and also used for sample management, data acquisition, and data processing. Chromatographic separation was performed on Kinetex® biphenyl C_{18} column (Phenomenex Limited, Cheshire, UK) with trimethylsilyl endcapping (150 × 4.6 mm, 5 μm particle size). The isocratic flow was employed where a mobile phase mixture containing methanol as the organic phase and water containing 0.5% v/v O-phosphoric acid (pH 3.5) was used as the aqueous phase at a flow rate of 0.5 $mL.min^{-1}$, while injection volume was fixed at 10 μL and column oven thermostat was maintained at 40 °C. The quantification was performed using DAD detector at a wavelength ranging between 210 and 360 nm was used for the detection of multiple analytes. The aforementioned range covered absorption maxima (λ_{max}) of TMZ at 350 nm and GLA at 220 nm.

2.3. Solutions and reagents preparation

2.3.1. Buffer solutions

The buffer solution (i.e., 0.5% v/v orthophosphoric acid) was prepared by adding 2.5 mL of orthophosphoric acid to 500 mL of water to obtain the desired buffer solution. The pH of the buffer solution was

measured using a calibrated digital pH meter.

2.3.2. Standard stock solutions and serial dilutions

The stock solution of TMZ (1000 μg.mL^{-1}) was prepared in acetonitrile, while the stock solution of GLA (1000 μg.mL^{-1}) was prepared with ethanol. Aliquot weight quantities (5 mg) were taken separately in 5 mL volumetric flasks and the aforementioned solvents were added to prepare the stock solutions. Various serial dilutions of TMZ and GLA were prepared from the stock solutions in volumetric flasks (5 mL) to obtain the serial dilutions of 1, 3, 5, 10, 20, 40, 60, 80, and 100 μg.mL^{-1} for both the drugs. The prepared samples were filtered through 0.22 μm syringe filters before HPLC analysis.

2.4. Preliminary method development

The initial method development exercise involved quantification of the individual analytes (TMZ and GLA) separately by HPLC. Chromatographic analysis was performed individually on both the drugs where peak area, retention time, and peak symmetry were evaluated. Based on the observed results, the chromatographic conditions were set for simultaneous detection of both the drugs. The published literature reports on TMZ indicated the use of a mobile phase mixture containing 0.5% v/v glacial acetic acid in water and methanol in ratio of 90:10, which indicated a highly polar mobile phase or the elution of the drug. On the contrary, the literature reports on GLA indicated the use of mobile phase mixture containing a higher fraction of organic solvents like acetonitrile, methanol, dichloromethane, n-hexane (90–99%), and water with pH adjusted using acetic acid, ammonium acetate as the aqueous phase. Based on these observations, the mobile phase mixture containing methanol and water (pH adjusted with O-phosphoric acid) was explored for simultaneous estimation of TMZ and GLA on a C_{18} column with the help of a DAD detector at a wavelength ranging between 210 and 360 nm.

2.4.1. Chemometrics-assisted method optimization

The systematic method development practice employing a chemometric approach is considered a highly effective way for method optimization. As per the ICH Q14 guideline, the analytical method development using chemometric principles provides high method flexibility and method robustness for continuous improvement [31]. The quality target method profile (QTMP) was framed which included a summary of method development goals and suitable justifications for them. From the QTMP elements, critical analytical attributes (CAAs) were earmarked.

2.4.1.1. Factors screening by risk assessment. The factor screening was performed by formal risk assessment analysis where all the possible factors with their direct and indirect influence on the method parameters were assigned with low, medium, and high-risk rankings. The medium to high-risk factors was optimized with the help of experimental design. As TMZ (no pKa and pH-independant solubility) [32] and GLA (pKa 4.92 and insoluble in water) [33] exhibit different physiochemical properties, the method development involved challenges. For this, all chromatographic parameters such as mobile phase composition, buffer pH and strength, flow rate, column temperature, and injection volume were considered for their critical influence on CAAs of the developed method.

2.4.1.2. Factor optimization by response surface design. The highly influential method parameters were systematically optimized by employing a response surface design i.e., Box-Behnken design (BBD) at three different levels such as low (−1), medium (0), and high (+1) values. The obtained data were subjected to mathematical modeling to fit with a suitable model where main and interaction effects were taken into consideration. The model statistical validity was evaluated from parameters like the coefficient of correlation, lack of fit, and predicted residual error sum of squares. The optimum chromatographic solution was identified by numerical optimization and desirability function, while graphical optimization was useful to demarcate the analytical design space.

2.4.2. Validation studies

Method validation was carried out as per the ICH guideline, where parameters like specificity, linearity, accuracy, precision, detection (LOD) and quantification (LOQ) limits, robustness and system suitability etc. were evaluated [34–36].

2.4.2.1. Specificity. The specificity was measured by evaluating the ability of the method to differentiate analyte from other components. For this, the estimation of both TMZ and GLA was carried out from the lipidic nanoparticles. The mean percent recovery of analytes (n = 3) and %RSD were calculated for this purpose.

2.4.2.2. Linearity range. The linearity range was evaluated for TMZ and GLA at different concentration levels ranging between 1 and 100 μg.mL^{-1}. These serial were dilutions (n = 3) of the drugs in combination were prepared from the individual standard stock solutions using a mobile phase mixture as the diluent. The samples were filtered through 0.22 μm nylon syringe filters. The peak areas obtained for both the drugs were noted and calibration plots were drawn between the peak area versus drug concentration for both TMZ and GLA. Linear regression was performed and the values of correlation coefficients were calculated for the calibration curves of both the drugs.

2.4.2.3. Accuracy and precision. The accuracy was determined as the agreement between the accepted values with reference values of the recovered concentration of the drug, while precision (inter and intraday) was determined (n = 3) from the agreement between the results of recovered concentration of the drug on the same day at different time points and the other day. The accuracy of the developed method was determined from the net recovered concentration (n = 3) of the drug from the known theoretical quality control (QC) drug concentrations 5, 10, and 15 μg.mL^{-1} as low, mid, and high, respectively. The acceptance criterion for precision was made for %RSD of the recovered drug concentration less than 2%.

2.4.2.4. Limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined for both TMZ and GLA from the slope of the linearity plot (S) and standard deviation of the response (σ) to the blank samples (i.e., mobile phase) using Equation (1) and (2).

$$\mathrm{LOD} = 3.3 \times \frac{\sigma}{\mathrm{S}} \tag{1}$$

and

$$\mathrm{LOD} = 10 \times \frac{\sigma}{\mathrm{S}} \tag{2}$$

However, the obtained values were subjected to practical verification for suitability and a visual inspection of signal-to-noise (S/N) ratio was evaluated (3:1 for LOD and 10:1 for LOQ) to confirm the final limits.

2.4.2.5. Robustness. Apart from pre-validation QbD based robustness-cum-optimization studies, the robustness was separately evaluated by analyzing the system suitability parameters from standard drug concentrations of TMZ and GLA after alteration in the volume of the organic phase (±2% v/v) and flow rate (±0.2 mL.min^{-1}), followed by estimation of %RSD for peak area and retention time.

2.4.2.6. System suitability. The system suitability was evaluated by analyzing three replicate injections of the selected standard

concentration (10 μg.mL^{-1}) of both TMZ and GLA, followed by estimation of %RSD for retention time, theoretical plate, and tailing factor. The system suitability test (SST) limit was calculated for the aforementioned parameters using the below-mentioned Equations (3) and (4), respectively [31].

$$\{\overline{Z} - t\alpha, n-1.(s/\sqrt{n})\} \quad (3)$$

$$\{\overline{Z} + t\alpha, n-1.(s/\sqrt{n})\} \quad (4)$$

Where, $\overline{Z}$ is the average of three observations $t\alpha, n-1 = t_{\text{critical})(0.05,n\text{-}1}$ =degrees of freedom, s = standard deviation of three observations and *n* the square root of number of observations.

2.4.3. Forced degradation studies

The forced degradation studies were performed for evaluating the degradation products formed for both the investigated drugs under various ICH recommended conditions [37]. The key degradation conditions explored include acidic and alkaline hydrolysis, neutral hydrolysis, oxidative hydrolysis, and photolysis.

2.4.3.1. Acid and alkaline hydrolysis. The acid and alkaline hydrolysis was performed for TMZ and GLA exposed to 0.1 M HCl and 0.1 M NaOH as the degradants. The studies were performed by refluxing standard solutions (0.5 mL solution of 100 μg.mL^{-1} concentrations) of both drugs with degradants (0.5 mL) taken in 5 mL volumetric flasks for 1 h at 80 °C. After reflux, the samples were neutralized by adding an equal volume of alkali and acid, and the final volume was adjusted up to 5 mL by adding the mobile phase. The samples were filtered through 0.22 μm nylon syringe filters before analysis. The blank sample was also prepared without drugs to obtain the chromatograms for comparing the peaks.

2.4.3.2. Neutral hydrolysis. The neutral hydrolysis was performed for TMZ and GLA by refluxing standard solutions (0.5 mL solution of 100 μg.mL^{-1} concentrations) of both drugs in a 5 mL volumetric flask for 1 h at 80 °C. After reflux, the volume was adjusted upto5 mL by adding the mobile phase. The samples were filtered through 0.22 μm nylon syringe filters before analysis. The blank sample was also prepared without drugs to obtain the chromatograms for comparing the peaks.

2.4.3.3. Oxidative hydrolysis. The oxidative hydrolysis was performed for TMZ and GLA exposed to 0.5 mL of H_2O_2 (30% v/v) as the degradant. The studies were performed by refluxing standard solution (0.5 mL solution of 100 μg.mL^{-1} concentrations) of both drugs with degradant (0.5 mL) taken in 5 mL volumetric flask for 1 h at 80 °C. After reflux, the final volume was adjusted up to 5 mL by adding the mobile phase. The samples were filtered through 0.22 μm nylon syringe filters before analysis. The blank sample was also prepared without drugs to obtain the chromatograms for comparing the peaks.

2.4.3.4. Photolysis. The photodegradation was performed for TMZ and GLA exposed to day sunlight as the degradant. The standard solutions (0.5 mL of 100 μg.mL^{-1} concentrations) of both the drugs were volume adjusted up to 5 mL with the mobile phase and exposed to direct sunlight for 6 h. The samples were filtered through 0.22 μm nylon syringe filters before analysis. The blank sample was also prepared without drugs to obtain the chromatograms for comparing the peaks.

2.5. Greenness assessment: a semi-quantitative approach

In the current studies, we employed the AES approach. This semi-quantitative approach assigns Penalty Points (PPs) to different method parameters such as the amount of reagents and electricity consumed waste generated and overall hazards considering reagents, instruments, and during the process. Finally, this approach awards an overall greenness score to the studied analytical method.

2.6. Estimation of analytes in the nanoparticles

The developed HPLC method was used for the estimation of drug content, entrapment efficiency and *in vitro* drug release profiles from different nanoparticle formulations of TMZ and GLA. The dual drug-loaded lipid nanoparticles were prepared using the melt-emulsification technique followed by probe sonication using the procedure previously published by our group [38,39]. Briefly, an accurately weighed amount (20 mg) of TMZ was added to the aqueous phase containing Tween 80 (4% v/v) and sodium deoxycholate (1% w/v) dissolved in double-distilled water (pH adjusted at 5.5 with diluted glacial acetic acid), mixed on a magnetic stirrer and heated at 85 °C in a water bath. On the other hand, an accurately weighed amount (20 mg) of GLA was added to the lipid phase containing trimyristin (1% w/v), propylene glycol monocaprylate (0.3% w/v), soyabean phospholipids and egg phospholipids (0.5% w/v). The lipid phase was also allowed to melt in a water bath maintained at 85 °C. The aqueous phase was added to the organic phase, followed by continuous stirring at 600 rpm for 20 min. The dispersion was subjected to probe sonication to obtain lipid nanoparticles. The obtained nanoparticles dispersion was then stored in refrigerated condition until further analysis.

2.6.1. Drug content, entrapment and loading efficiency

Total drug content, entrapment and loading efficiency of drug in the nanoparticles were determined using the developed HPLC method. For drug content determination, an aliquot (1 mL) of the nanoparticles dispersion was taken and dissolved in 4 mL acetonitrile, sonicated, and filtered for analysis. Entrapment efficiency was determined by adding 0.5 mL of nanoparticles dispersion to Amicon® centrifugal filter (100 kDa, Merck Millipore, Waltford, UK) and the obtained filtrate was analyzed by HPLC to quantify the amount of unentrapped drug. Moreover, the loading efficiency was determined from the nanoparticles pellet collected after centrifugation of the dispersion (1 mL) in an eppendorf tube at 13,000 rpm (1136 × g) for 15 min. The pellet was redispersed in 1 mL of tetrahydrofuran and acetonitrile (1:1) mixture and analyzed by HPLC. For all the mentioned parameters, the drug concentrations were measured with respect to the blank nanoparticles formulation to avoid any interference of analytes with the formulation excipients. All the studies were performed in triplicate and values were reported as mean ± S.D. The characterization parameters (drug content, entrapment and loading efficiency) of the developed nanoparticles were analyzed as per the formulae reported in the literature [38,39].

3. Results and discussion

3.1. Defining the QTMP and CAAs

As per the principles of chemometrics employed for method development, QTMP elements were defined (as enlisted in Supporting Information Table S1) for simultaneous estimation of TMZ and GLA. Among the various elements defined, the attributes such as specificity, sensitivity, and robustness were selected as the key quality parameters which describe the method's efficiency. From the QTMP elements, some of the key analytical attributes were earmarked as the CAAs in Supporting Information Table S2. A detailed justification for each of the QTMP and CAAs selected for the target method for simultaneous estimation of TMZ and GLA is given in the Supporting Information (Tables S1 and S2).

3.2. Preliminary method development

In the preliminary method development exercise, attempts were made for developing the HPLC method individually for each of the selected drugs (i.e., TMZ and GLA). The analysis of TMZ was performed with varying mobile phase combinations of methanol and water, where the pH of the aqueous phase was varied between 2.5 and 3.5 with the help of O-phosphoric acid. The initial chromatography results suggested

methanol and water (pH adjusted to 3.5 with 0.5% v/v O-phosphoric acid) mixture in the ratio 30:70, flow rate 1 mL.min^{-1}, injection volume 10 µL revealed a single peak for TMZ at retention time (Rt) of 4.55 min with good peak shape and symmetry for DAD detection in the range 210 to 360 nm. Using the aforementioned mobile phase mixture and flow rate, the analysis of GLA was attempted. However, there was no peak observed for the GLA. This could be due to the reason that a low fraction of the organic phase involved in the conditions observed to be suitable for the analysis. Hence, a modified mobile phase mixture containing a higher fraction (90%) of the organic phase was selected for the estimation of GLA, where both the analytes were simultaneously estimated. Based on the understanding obtained from the preliminary method development exercise, the factor screening and optimization studies were performed to identify the critical independent variables and optimize them for enhanced method performance.

3.3. Chemometrics-assisted method development

Based on the understanding developed on the basis of preliminary method development, the knowledge space was established which helped in designing the right strategy for systematic optimization of the developed method for simultaneous estimation of the target analytes. A detailed process map helped (Fig. 1) in understanding the knowledge space highlighting the critical steps involved in systematized development of the chromatographic method for simultaneous estimation of TMZ and GLA.

3.3.1. Factor screening study

The risk estimation matrix was established as shown in Table 1 for identifying the factors with possible influence on the chromatographic estimation by the target HPLC method. Various parameters and steps involved during the entire process of method development were mapped into the matrix, followed by assigning the low, medium, high-risk demarcation with green, yellow, and red-color coding, respectively. Based on prior knowledge and experience, it was observed that factors like mobile phase composition, pH and flow rate, diluent composition, column chemistry, and particle size were categorized as high-risk parameters due to the likelihood and occurrence of the impact of these variables on the CAAs. However, other parameters like column oven temperature, injection volume, and washing cycles were considered as the medium risk variables of concern for the CAAs. Besides, the parameters such as mobile phase sonication, sample preparation, and sample filtration were considered as the low risk variables. Only the aforementioned high-risk factors selected as the critical method parameters (CMPs) were considered for factor optimization where experimental design was applied forunearthing the chemometric information from the study.

3.3.2. Factor optimization study

The high-risk factors such as organic phase (%), flow rate (mL.min^{-1}), and pH of the mobile phase identified from the risk assessment exercise were optimized with the help of BBD experimental design. Table 2 enlists the design matrix indicating the experimental trials performed for a 3-factor and 3-level containing BBD along with the values of CAAs as the responses. The mathematical modeling of the data indicated the suitability of the quadratic model which was decided on the basis of a higher correlation coefficient (close to 1), statistically significant model p-value ($p < 0.05$), and insignificant lack of fit ($p > 0.05$). Supporting Information Table S3 enlists the coefficients of the polynomial equations generated for each of the response variables (CAAs) along with the values of the statistical parameters.

The 3D-response surface analysis was performed to understand the interactions among the studied CMPs and their influence on the CAAs. Figs. 2 to 4 illustrate the influence of studied factors on the peak area of TMZ and GLA. In Fig. 2(a-f) the effect of the organic phase (%) was highly pronounced on the peak area of both the drugs. Upon an increase in the percentage of the organic fraction in the mobile phase, a sharp increase in the peak area of TMZ and GLA was observed. Higher sensitivity could be attributed to the better separation efficiency of the analytes in the mobile phase containing a higher organic fraction. The effect of mobile phase pH on the peak area was not very pronounced for TMZ. An increase in pH of the mobile phase from 3 to 4 showed only a mild increase in the peak area for TMZ followed by no further effect for an increase in pH up to 5. This could be attributed to the influence of pH on the ionization of the drug molecules based on their pKa values [10,13]. The observed results could be further supported by the fact that TMZ has no pKa value as it exists in unionized form, thus pH change has no direct effect on the peak area. The acidic pH of the mobile phase, however, was found to be helpful for the stability of TMZ from protecting against hydrolysis in alkaline pH. Similarly, the increase in pH of the mobile phase showed a reduction in the peak area of GLA beyond pH 4. Hence, the observations indicated lower pH values between 3 and 3.5 were seems to be suitable for the estimation of analytes. Unlike organic phase (%) and pH, the factor flow rate showed no significant influence on the peak areas of TMZ and GLA.

Fig. 3(a-f) portrays the 3D-response surface plots indicating the effect of change in the organic phase (%), pH, and flow rate on Rt of TMZ and GLA. As clearly observed from the figure, an increase in the organic phase (%) showed a sharp decline in Rt values which could be attributed to the better separation efficiency for both the drugs. Similarly, an increase in the flow rate also showed a sharp reduction in Rt values due to the faster elution of the analytes. However, pH showed a moderate effect on the Rt of TMZ and GLA, where lower values of retention were observed at lower pH for both the drugs. As TMZ is unstable in alkaline pH, thus mobile phase pH increasing from the highly acidic value towards alkaline pH causes the formation of metabolites (i.e., MTIC and

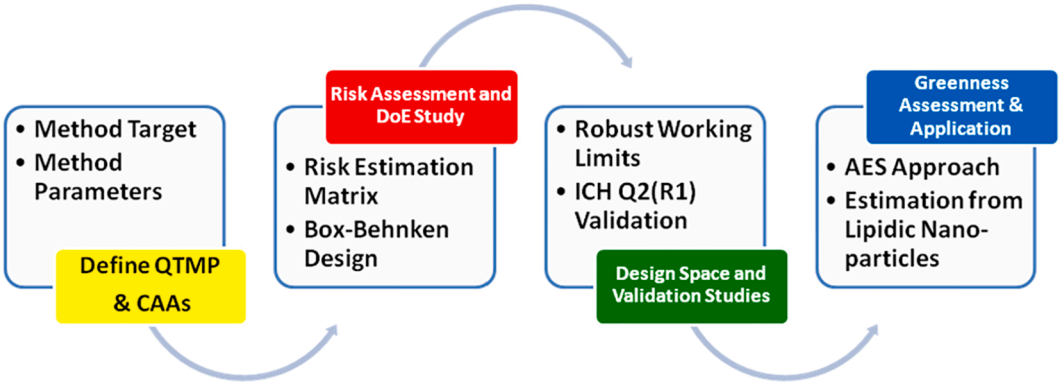

Fig. 1. Typical process map depicting development of a systematized liquid chromatographic method; QTMP: Quality target method profile, CAAs: Critical quality attributes, AES: Analytical eco-scale.

Table 1
Risk assessment matrix for the simultaneous estimation method.

CAAs	Mobile phase (Composition/ flow rate)	Diluents (pH/Organic modifier)	Column (chemistry/ particle size	Buffer pH	Injection volume	Column/sample temperature	Filtration/ Sonication
Linearity							
Accuracy							
Precision							
Sensitivity							
Specificity							
Robustness							

Table 2
Experimental trials performed for method optimization using BBD.

Runs	Factor 1 Organic phase (%)	Factor 2 Flow rate (ml/ min)	Factor 3 Mobile phase pH	Response 1 Peak area TMZ	Response 2 Peak area GLA	Response 3 Rt of TMZ	Response 4 Rt of GLA	Response 5 Tailing factor of TMZ	Response 6 Tailing factor of GLA
13	90	0.75	4	961.3	514.5	3.32	4.57	0.33	0.23
15	90	0.75	4	977.8	522.1	3.33	4.59	0.37	0.24
6	95	0.75	3	1218.6	1346.7	3.83	5.22	0.13	0.23
11	90	0.5	5	817.1	257.5	3.19	4.41	0.65	0.64
12	90	1	5	971.3	176.5	3.12	4.31	0.34	0.29
4	95	1	4	2453.5	887.2	2.98	3.78	0.17	0.15
5	85	0.75	3	81.7	182.7	3.34	5.94	0.45	0.49
10	90	1	3	1093.4	432.1	3.03	4.21	0.27	0.33
14	90	0.75	4	957.8	554.5	3.34	4.52	0.31	0.29
8	95	0.75	5	1786.5	757.1	3.12	4.45	0.14	0.19
2	95	0.5	4	2875.4	1311.3	3.44	4.81	0.17	0.23
7	85	0.75	5	101.1	87.6	3.52	4.15	0.21	0.29
9	90	0.5	3	1878.9	340.9	3.46	6.42	0.61	0.34
3	85	1	4	100.3	123.5	3.11	5.67	0.45	0.29
1	85	0.5	4	96.2	89.4	3.98	6.12	0.32	0.37

BBD: Box-Behnken design.

AIC). Although at pH 5 of the mobile phase, no such metabolites were observed in the samples and in the chromatograms. At higher pH of the mobile phase, the retention time and peak symmetry of TMZ was not very good. Hence, the mobile phase with a higher organic fraction, and lower pH and flow rate suggested a better estimation of the analytes.

Like the results observed for the peak area and Rt, the studied factors also showed quite analogous observations for their influence on the tailing factor of TMZ and GLA. As shown in 3D plots portrayed in Fig. 4 (a-f), the organic phase (%) in the mobile phase showed a major influence on the tailing factor. With a higher fraction of organic phase (%), minimal tailing was observed with the peaks of TMZ and GLA which could be attributed owing to better retention and elution of the analytes. The effect of flow rate on the tailing factor was quite negligible, where no drastic change was observed on peak tailing upon changes in the flow rate. The effect of pH showed a moderate influence on peak tailing of TMZ and GLA. An increase in the pH of the mobile phase indicated a curvilinear trend, where an initial increase in pH from low to mid-levels showed lower values of tailing factors followed by an inclining trend afterward. Hence, high organic phase (%), low flow rate, and pH were suitable for the tailing factor of TMZ and GLA.

3.3.3. *Search for the optimum chromatographic solution*

The optimum chromatographic solution for simultaneous estimation of TMZ and GLA was identified by numerical optimization. During numerical optimization, the desired goals of CAAs such as higher peak area, lower retention time, and tailing factor were provided. The suggested numerical solution with a desirability value 1 or closer to 1 was selected from the experimental design as the optimum chromatographic conditions. The optimum solution was further demarcated in the overlay plot of the design space as shown in Supporting Information Figure S1. As per the suggested optimum condition with mobile phase containing methanol and water (pH 3.8) in the ratio of 92:8 at a flow rate of 0.5 mL. min^{-1}, column oven temperature at 40 °C. Fig. 5(a-d) portrays the HPLC chromatogram for blank (mobile phase), individual peaks for TMZ and GLA, and a combination of both drugs in the optimized chromatographic conditions. The chromatogram for simultaneous estimation of both the analytes indicated two clear, well separtaed, sharp peaks with good peak symmetry for TMZ and GLA at Rt 3.44 min and 4.81 min respectively without any peak tailing. Upon comparison of the Rt, it was observed that there is no change in the Rt for both the drugs and does not interfere with each other when the analysis was performed alone versus

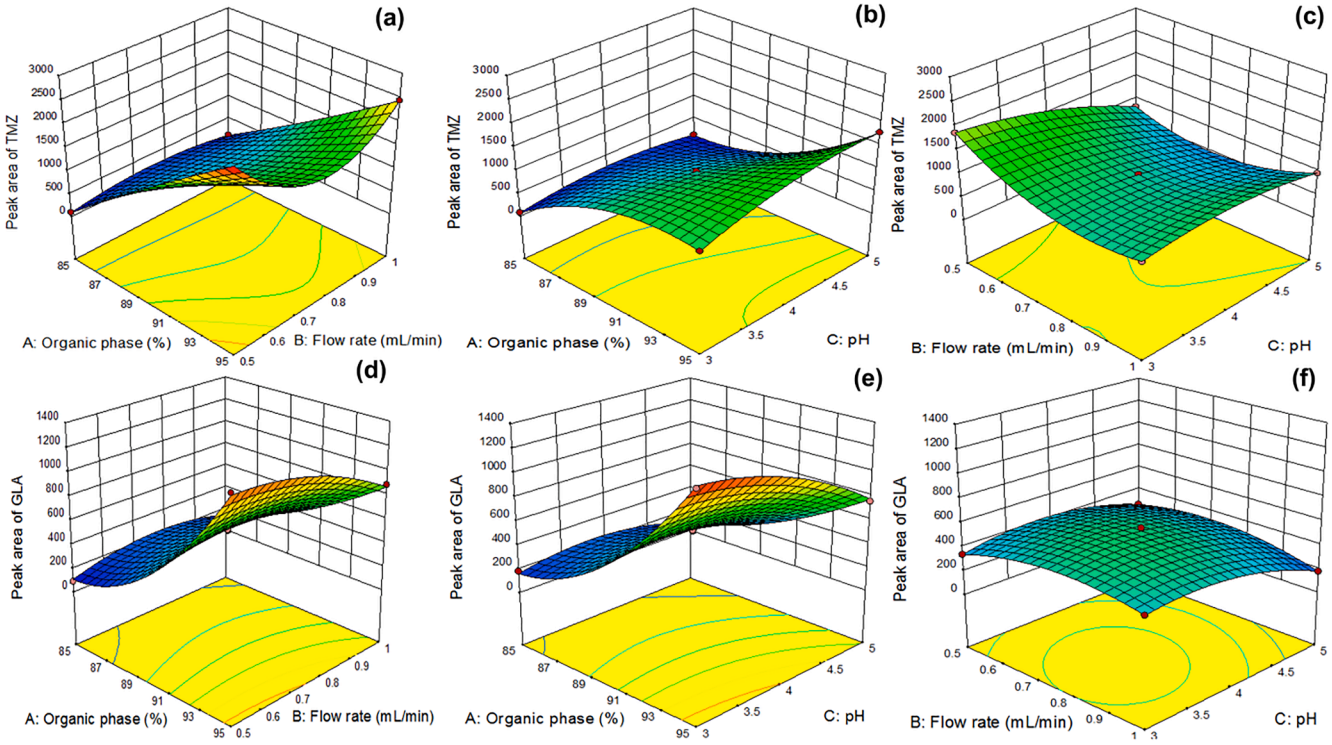

Fig. 2. 3-Dimensional response surfaces depicting effect of method parameters on peak area of TZM and GLA.

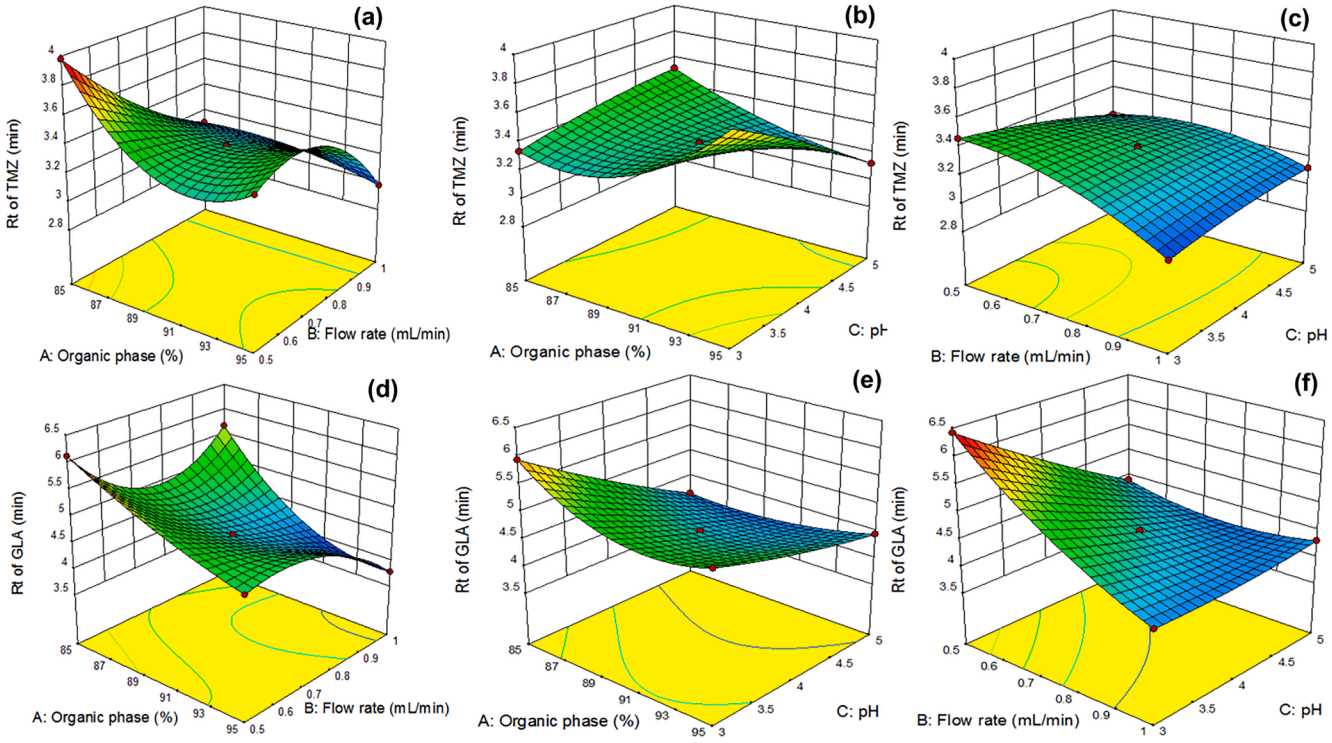

Fig. 3. 3-Dimensional response surfaces depicting effect of method parameters on retention of TZM and GLA.

simultaneous estimation.

3.4. Method validation studies

The method validation studies were performed on the developed method under the optimized chromatographic conditions. The data corresponding to all the validation parameters indicated excellent reliability and applicability of the developed method for the intended purpose.

3.4.1. Specificity

The specificity evaluation of the developed method indicated good

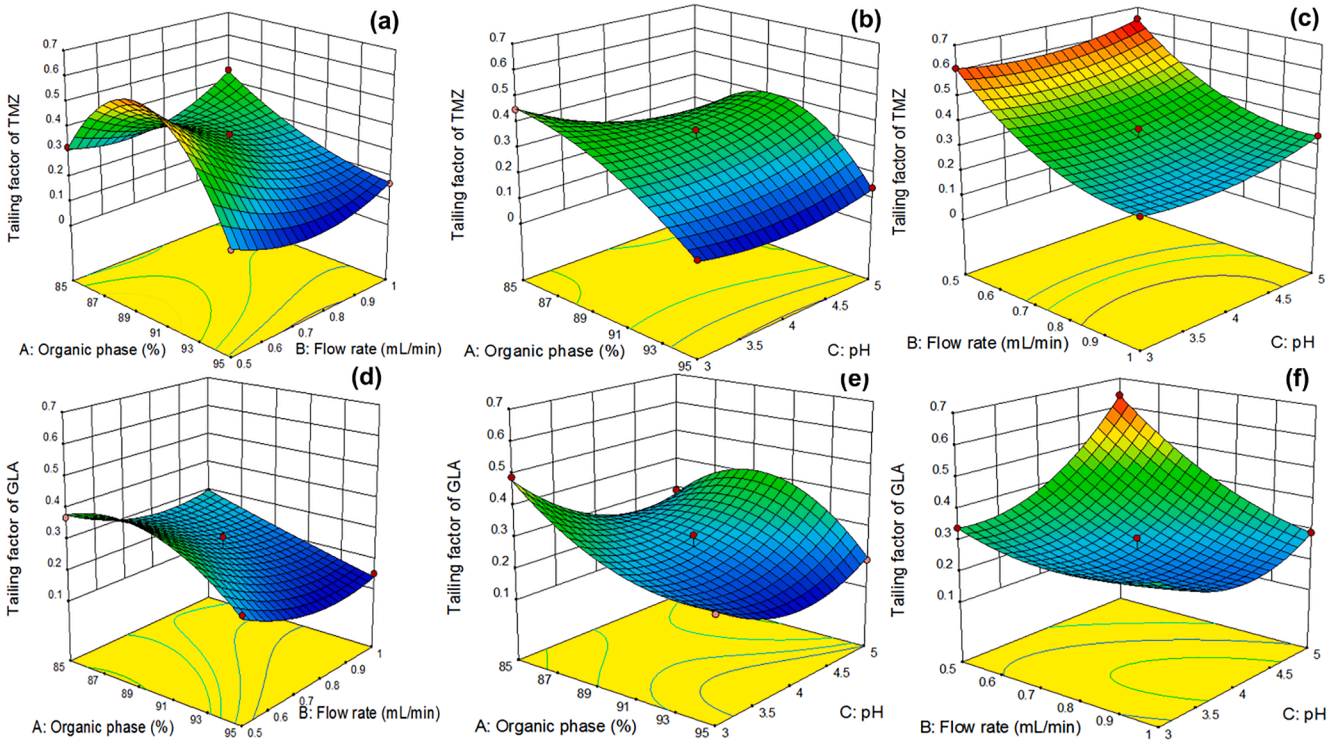

Fig. 4. 3-Dimensional response surfaces depicting effect of method parameters on peak tailing of TZM and GLA.

recovery of the drugs from the lipid nanoparticles. The chromatograms for the blank lipid nanoparticles and estimation of the drug from the lipid nanoparticles are shown in Fig. 6(a-b), which indicated a lack of interference of the formulation components with the peaks of TMZ and GLA during simultaneous estimation. The peak purity was calculated for the peaks of analytes during simultaneous estimation of both the analytes from the nanoparticles which suggested a value close to 1, indicating no interference by the formulation components during analysis.

3.4.2. Linearity and range

The developed method for simultaneous estimation of TMZ and GLA showed linearity for the drug concentrations ranging between 1 and 100 $\mu g.mL^{-1}$. The linear calibration plot for TMZ and GLA depicted (Supporting Information Figure S2) a high value of correlation coefficient ($R^2 = 0.999$, $p < 0.001$), while the figure inset depicts the residual plot indicating the concentrations observed as the outliers for the developed method.

3.4.3. Accuracy

The accuracy of the developed method was evaluated using the standard drug concentrations, i.e., LQC (5 $\mu g.mL^{-1}$), MQC (20 $\mu g.mL^{-1}$), and HQC (60 $\mu g.mL^{-1}$). Table 3 enlists the data corresponding to quality control samples studied for accuracy evaluation of the developed method which indicates good percent recovery observed for TMZ (98.6 to 100%) and GLA (97.9 to 100%), and %RSD was less than 2%. These results confirmed the high accuracy of the developed method.

3.4.4. Precision

The intra- and inter-day precision of the developed method was evaluated using the standard drug concentrations, i.e., LQC (5 $\mu g.mL^{-1}$), MQC (20 $\mu g.mL^{-1}$), and HQC (60 $\mu g.mL^{-1}$). Table 3 enlists the data corresponding to quality control samples studied for intra- and inter-day precision evaluation of the developed method where good overall results for TMZ (0.05–0.99%) and GLA (0.1–0.9%) was observed with RSD less than 2%. These results confirmed a high precision of the developed method.

3.4.5. LOD and LOQ

The derivation of LOD and LOQ considering the linearity graph and practical visual inspection of the S/N ratio helped in identifying the correct concentrations for both analytes. The values of LOD and LOQ calculated for TMZ were found to be 0.75 and 1.0 $\mu g.mL^{-1}$, while the values for GLA were found to be 0.55 and 1.0 $\mu g.mL^{-1}$, respectively. The observed results matched the calculated concentrations and indicated good sensitivity of the developed method for simultaneous estimation of TMZ and GLA.

3.4.6. Robustness

The robustness evaluation was performed by varying the optimized chromatographic conditions (±2% v/v for organic phase, ±0.1 mL. min^{-1} for flow rate) indicated a lack of significant difference ($p < 0.05$) in the peak area, retention time, and peak tailing. Also, RSD was found to be less than 2% (0.55 and 1.82% for deliberate changes in flow rate and acetonitrile percentage, respectively) and the method was found to be having good robustness.

3.4.7. System suitability

The system suitability analysis of the developed method indicated no significant difference ($p < 0.001$) in the peak area and retention time of TMZ and GLA observed after three replicate injections. The calculated percent recovery for both analytes was found to be 96.9 and 102.8% respectively, and %RSD was also found to be less than 2%. The observed results indicated good repeatability of the chromatographic instrument for the developed method for simultaneous estimation of TMZ and GLA. Moreover, SST limits derived for the system suitability data are shown in Table 4. The results indicated that a lower limit can be chosen for retention time and plate number, while for the tailing factor it would be the upper limit.

3.5. Forced degradation studies

The forced degradation was attempted for TMZ and GLA exposed to various stress conditions (acid and alkaline hydrolysis, neutral

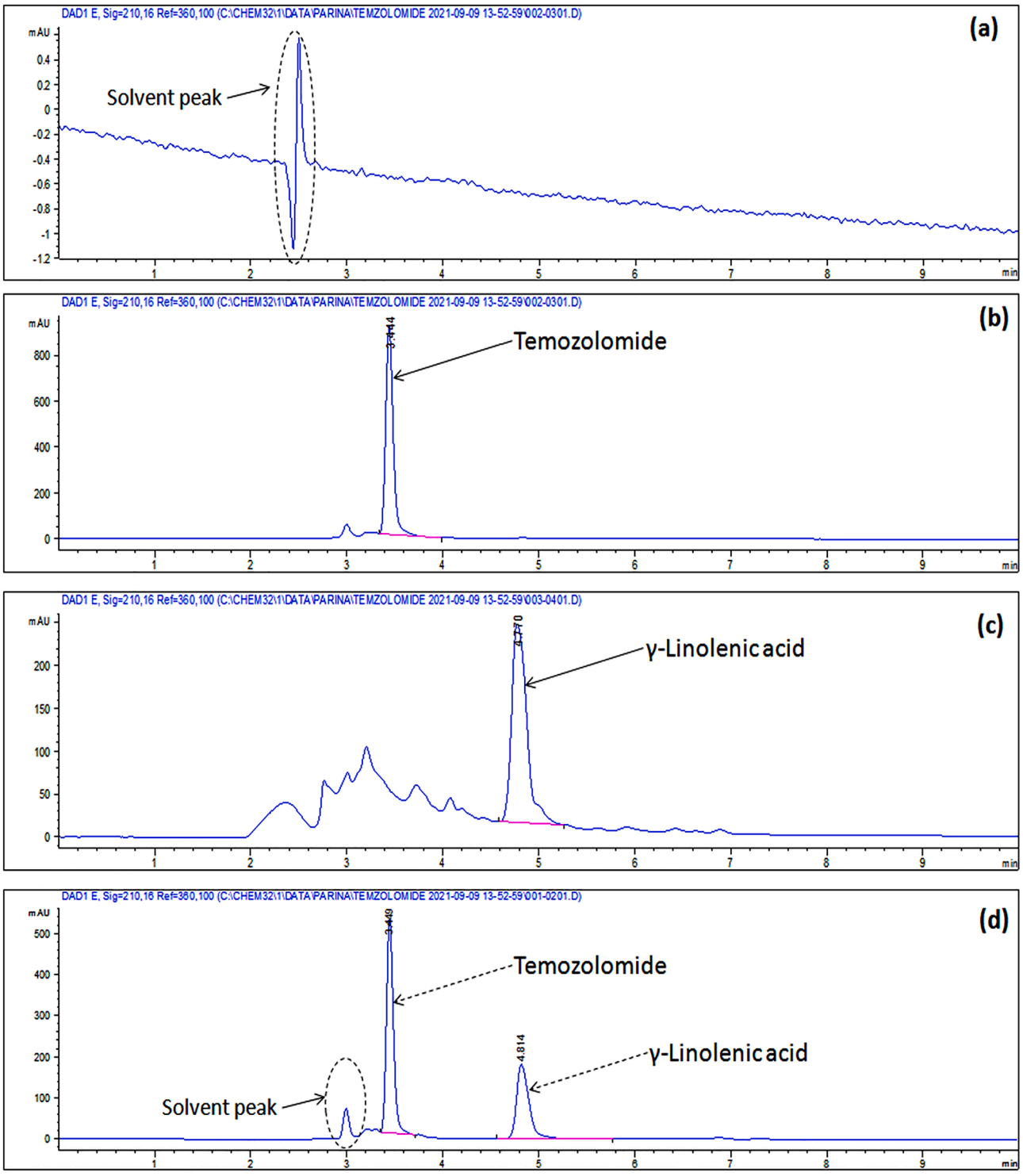

Fig. 5. Typical chromatograms of blank solvent (a), TMZ (b), γ-linolenic acid (c) and combined standards of TMZ and γ-linolenic acid.

hydrolysis, and photostablity) and percent degradation of the parent compound was estimated (Fig. 7). Among all the studied conditions, TMZ was highly prone to alkali hydrolysis where a complete degradation of the parent compound was observed to produce the metabolite (i.e., AIC) upon 1 h exposure to 0.1 M NaOH (Supporting Information Figure S3). Hence, the stress conditions were optimized by reducing the strength to 0.01 M, 0.001 M, and 0.0001 M of NaOH, along with a reduction in exposure time to 15 min. Upon reducing the stress conditions, the degradation of TMZ was found to be reduced from 99.08% (with 0.1 M NaOH) to 65% (with 0.0001 M NaOH). Under other stress conditions, however, only a minor fraction (<10%) of TMZ was found to be degraded, as is clear from the enlisted values in Table 5. On the contrary, GLA was found to be moderately influenced by the studied forced degradation conditions, except alkaline hydrolysis where the degradation was up to 76% using 0.0001 M NaOH. Over 10–25% degradation of the parent compound (i.e., GLA) was observed in all the other studied conditions. The forced degradation performed at all the studied conditions, revealed satisfactory peaks for TMZ and GLA. The observed results for TMZ degradation supported the results reported in the literature [12,13], while degradation results obtained for GLA are

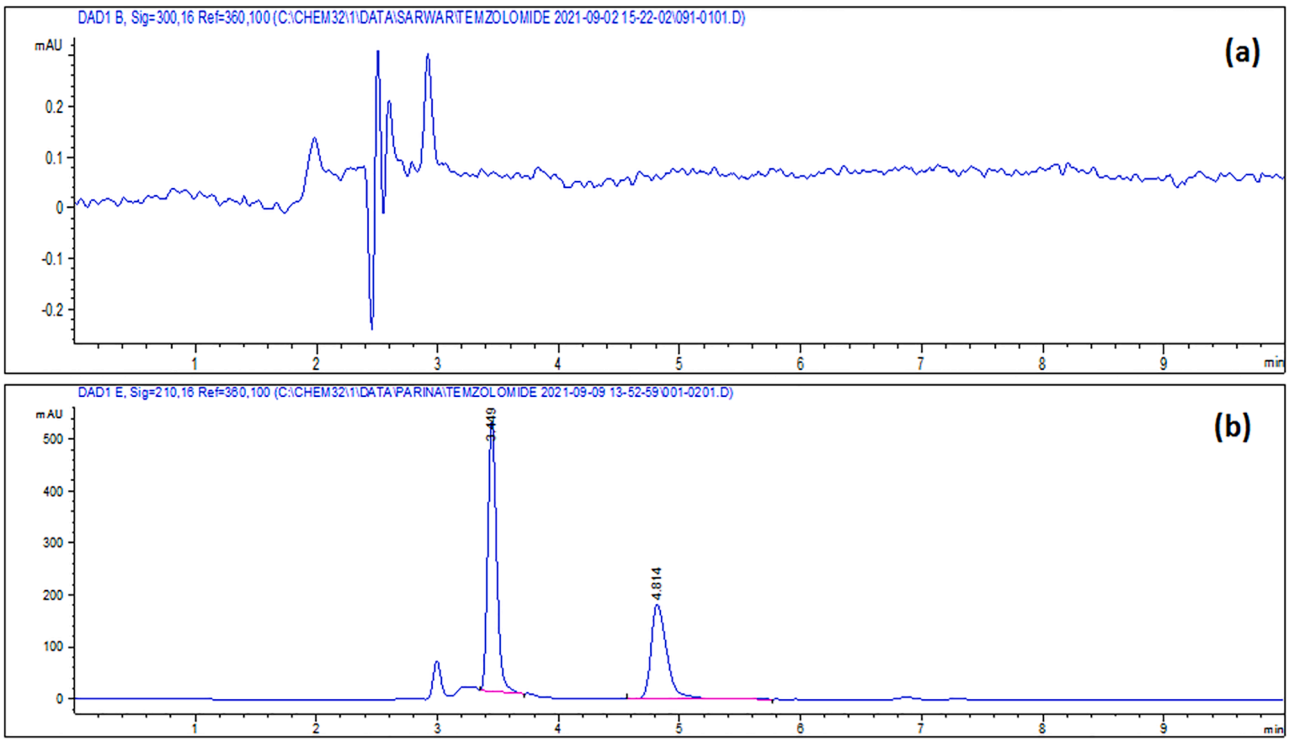

Fig. 6. Typical chromatograms of blank lipidic nanoparticles (a), estimation of TMZ and GLA in lipidic nanoparticles (b).

Table 3
Accuracy and precision data of the method.

Analyte	Nominal Concentration ($ng.mL^{-1}$)	Accuracy (n = 3) ± S.D.	Precision (%RSD)	
			Intraday (n = 3)	Interday (n = 3)
TMZ	5 (LQC)	98.6 ± 0.8	0.8	0.9
	20 (MQC)	99.6 ± 0.2	0.1	0.1
	60 (HQC)	100 ± 0.3	0.05	0.11
GLA	5 (LQC)	97.9 ± 1.0	0.99	0.63
	20 (MQC)	100 ± 0.4	0.6	0.72
	60 (HQC)	99.1 ± 0.2	0.11	0.11

%RE: Percent recovery, RSD: Relative standard deviation.

not reported in any of the literature reports.

3.6. Method greenness assessment

From the results obtained from the AES approach, we found the present method to score an excellent greenness score (greater than 75 are considered excellent green procedures) of 80 (Table 6). Therefore, comparing both the results, we can conclude that the first developed method for the said novel combination of TMZ and GLA is green and is environment friendly.

3.7. Estimation of analytes from the nanoparticles

The developed method was employed for the estimation of analytes from the lipid nanoparticles. Both TMZ and GLA exhibited good entrapment efficiency ranging between 95.4 and 98.7%, and loading efficiency ranging between 25.2 and 38.4%. The total drug content evaluation from nanoparticles indicated values ranging between 82.6 and 85.3% for both the studied drugs.

4. Discussion

The present research work endeavored to establish a simple, sensitive, and robust HPLC method for simultaneous estimation of the analytes, TMZ and GLA. As both the molecules exhibit very different physiochemical properties, it was challenging to develop a liquid chromatographic method for their detection using the C_{18} column on the DAD detector. Although TMZ has many reported HPLC methods, yet a few methods have documented estimation of GLA by HPLC. As fatty acids have no chromophores in their chemical structure, the derivatization approach was used in some of the published reports. However, derivatization is often challenging, time-consuming, and involves variability in optimizing the conditions. In an attempt, we rationally developed an assay method for simultaneous estimation of both the analytes on Kinetex® C_{18} column (biphenyl with trimethylsilyl end-capping) and the DAD detector in the wavelength ranging between 210

Table 4
Derivation of system suitability limits for the method.

Run	Retention Time (min)		Theoretical Plate		Tailing Factor	
	TMZ	GLA	TMZ	GLA	TMZ	GLA
Mean	3.458	4.829	5192.2	5381.26	0.257	0.129
S.D.	0.0112	0.0536	14.03	129.32	0.057	0.0155
(n)	3	3	3	3	3	3
SST-Limit	3.458–2.92 (0.0112/√3) = 3.439	4.829–2.92 (0.0536/√3) = 4.738	5192.2–2.92 (14.03/√3) = 5168.607	5381.26–2.92 (6.64/√3) = 5163.23	0.257 + 2.92 (0.057/√3) = 0.353	0.129 + 2.92 (0.02/√3) = 0.155

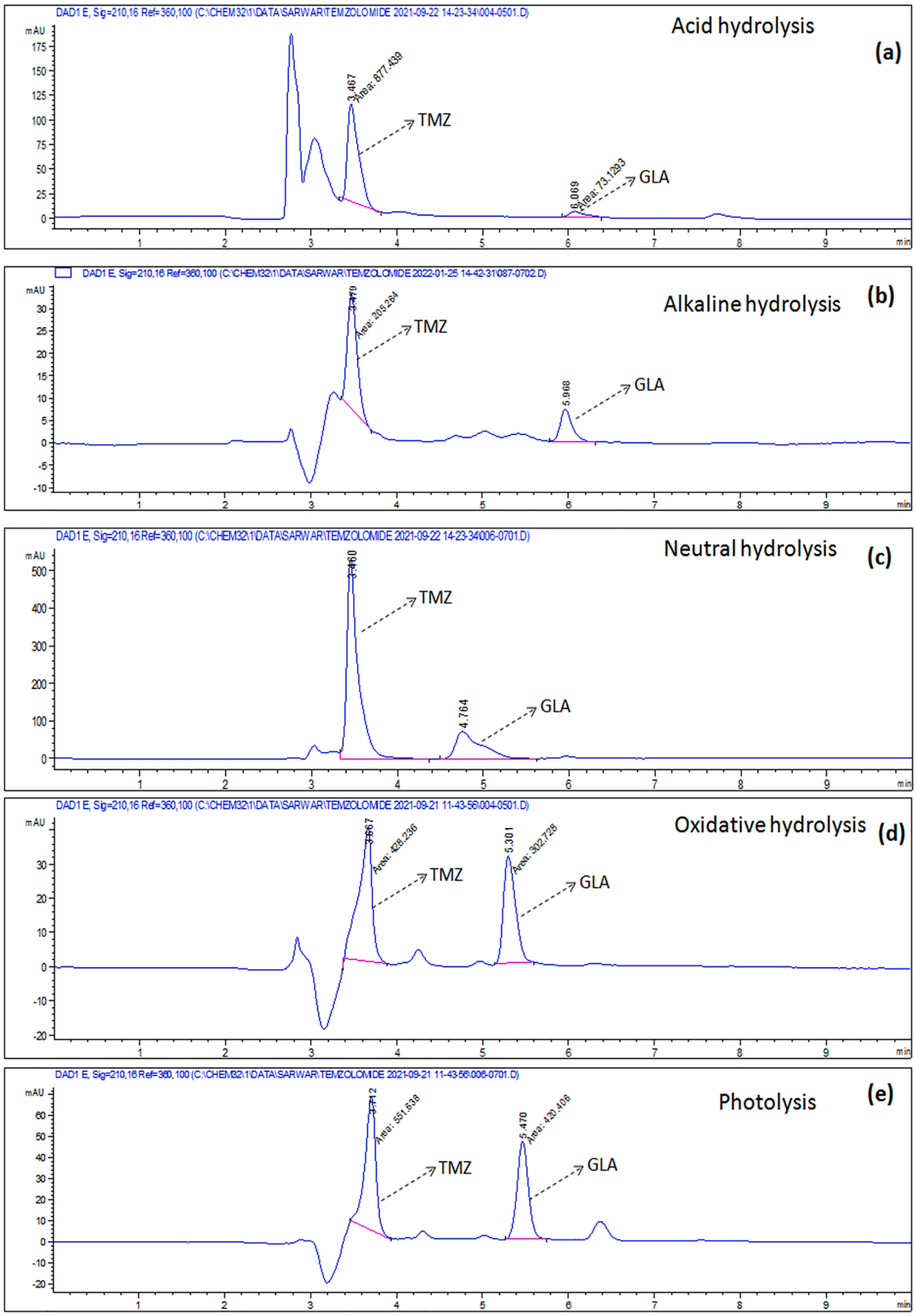

Fig. 7. Typical chromatograms of degraded samples of TMZ and γ-linolenic acid: acidic hydrolysis (a), alkaline hydrolysis (b), neutral hydrolysis (c), oxidative hydrolysis (d) and photolysis (e).

Table 5
Results of forced degradation study performed on TMZ and GLA.

Stress Conditions	Rt of TMZ	Rt of GLA	% Degradation of TMZ	% Degradation of GLA	Peak Purity
Acid hydrolysis	3.46	6.06	7.31	24.63	1.0
Alkaline hydrolysis	3.47	5.96	65.58	76.18	1.0
Neutral hydrolysis	3.46	4.76	8.18	11.14	1.0
Oxidative hydrolysis	3.56	5.30	9.14	21.07	1.0
UV light exposure	3.71	5.47	ND	15.76	1.0

Rt: Retention time; ND: No degradation.

Table 6
Penalty points and greenness score of the method.

Reagents	Amount	Amount PP	Hazard	Hazard PP	Total PPs
(1)Acetonitrile	<10 mL	1	Yes	4	4
(2)Ethanol	<10 mL	1	Yes	4	4
(2)Methanol	<10 mL	1	Yes	6	6
(3)Orthophosphoric acid	<10 mL	1	None	4	4
(3)Water	<10 mL	1	None	0	0
Instrument	**Energy**				
(1) HPLC	≤1.5 kWh per sample				1
(2) Sonicator	<0.1 kWh per sample				0
(3) Vaccum Filter	<0.1 kWh per sample				0
(4) Occupational hazards	Process Hermetization				0
(5) Waste	<10 mL				1
Overall PPs: 20					
Greenness score: 100 – 20 = 80					
Remark: Excellent greenness					

PP: Penalty points; Total PP: Amount PP × Hazard PP.

and 360 nm. Further, chemometric principles facilitated the identification of the critical method variables by risk assessment and experimental design was used for systematic optimization of the method variables for enhanced method robustness and performance. The mobile phase mixture of methanol and water (pH adjusted to 3.5 by 0.5% v/v O-phosphoric acid) in the ratio of 92:8 at a flow rate of 0.5 $mL.min^{-1}$ yielded the best chromatographic separation and retention of TMZ and GLA at 3.44 min and 4.81 min respectively. The optimum solution was demarcated in the experimental design space and the control strategy for controlling the critical method variables was postulated for continuous improvement of method performance.

The validation studies performed as per the ICH Q2(R1) guideline indicated various parameters were within the limits. Regression analysis of linearity data showed overall goodness of fit. The values of recovery show high levels of accuracy of the method. The method was found to be precise as the %RSD for intraday and interday precision studies were within the acceptable limits. The forced degradation study additionally indicated that the method was specific to the detection of analytes and their degradation products. Though the present method utilized methanol as a mobile phase component, the assessment indicated its green nature for routine quality control use. The method was applied for evaluating drug content, entrapment, and loading efficiency from the lipid nanoparticles where observed results indicated satisfactory values and no interference was observed for detection of the drugs with the formulation excipients. This could be attributed to the efficient extraction of drugs from the nanoparticles and also due to the high degree of specificity of the developed method for the target analytes [40,41].

5. Conclusions

The work attempted to develop an HPLC method for simultaneous estimation of TMZ and GLA was successful. As there are no analytical methods available in the literature, the reported method has unique importance with respect to its applicability for simultaneous estimation of both the drugs in bulk as well as in pharmaceutical formulation. Besides, the method also confirmed that analysis of GLA was possible with HPLC having DAD detection without any incorporation of any derivatization techniques. The systematic chemometric approach employed was highly useful for a science and risk-based understanding of the analytical variables for enhanced method performance. The validation results were highly satisfactory and found to be in agreement with the recommended ICH guidance. Moreover, the method was found green in nature and suitable for the estimation of analytes from the in-house developed lipid nanoparticles. In nutshell, the study recommended the suitability of the developed analytical method for simultaneous estimation of TMZ and GLA for other nanopharmaceutical formulations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The financial support provided by The Academy of Medical Sciences (UK) and Dept. of Biotechnology, Ministry of Science & Technology (India) as Newton International Fellowship (NIFR8\1038) to authors (SB) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123261.

References

[1] K. Urbanska, J. SokoÅ, owska, M. Szmidt, P. Sysa, Glioblastoma multiforme - An overview, Contemp. Oncol. 18 (2014) 307–312.
[2] W. Wick, M. Platten, M. Weller, New (alternative) temozolomide regimens for the treatment of glioma, Neuro-Oncol. 11 (2009) 69–79.
[3] E.K. Noch, R. Ramakrishna, R. Magge, Challenges in the treatment of glioblastoma: Multisystem mechanisms of therapeutic resistance, World Neurosurg. 116 (2018) 505–517.
[4] M.H. Cohen, J.R. Johnson, R. Pazdur, Food and drug administration drug approval summary: temozolomide plus radiation therapy for the treatment of newly diagnosed glioblastoma multiforme, Clin. Cancer Res. 11 (2005) 6767–6771.
[5] S.Y. Lee, Temozolomide resistance in glioblastoma multiforme, Genes Dis. 3 (2016) 198–210.
[6] A. Bakshi, D. Mukherjee, A. Bakshi, A.K. Banerji, U.N. Das, γ-linolenic acid therapy of human gliomas, Nutrition 19 (2003) 305–309.
[7] S.D. Baker, M. Wirth, P. Statkevich, P. Reidenberg, K. Alton, S.E. Sartorius, M. Dugan, D. Cutler, V. Batra, L.B. Grochow, R.C. Donehower, E.K. Rowinsky, Absorption, metabolism, and excretion of C_{18}-temozolomide following oral administration to patients with advanced cancer, Clin. Cancer Res. 5 (1999) 309–317.
[8] J. Portnow, B. Badie, M. Chen, A. Liu, S. Blanchard, T.W. Synold, The neuropharmacokinetics of temozolomide in patients with resectable brain tumors: potential implications for the current approach to chemoradiation, Clin. Cancer Res. 15 (2009) 7092–7098.
[9] M.A. Rudek, R.C. Donehower, P. Statkevich, V.K. Batra, D.L. Cutler, S.D. Baker, Temozolomide in patients with advanced cancer: Phase I and pharmacokinetic study, Pharmacotherapy 24 (2004) 16–25.
[10] L. Reyderman, P. Statkevich, C.M. Thonoor, J. Patrick, V.K. Batra, M. Wirth, Disposition and pharmacokinetics of temozolomide in rat, Xenobiotica 34 (2004) 487–500.
[11] A. Khan, S.S. Imam, M. Aqil, Y. Sultana, A. Ali, K. Khan, Design of experiment based validated stability indicating RP-HPLC method of temozolomide in bulk and pharmaceutical dosage forms, Beni-Suef Univ, J. Basic Appl. Sci. 5 (2016) 402–408.

[12] H. Kim, P. Likhari, D. Parker, P. Statkevich, A. Marco, C.-C. Lin, A.A. Nomeir, High-performance liquid chromatographic analysis and stability of anti-tumor agent temozolomide in human plasma, J. Pharm. Biomed. Anal. 24 (2001) 461–468.
[13] L.R. Michels, F.v.N.S. Fachel, J.H. Azambuja, N.E. Gelsleichter, E. Braganhol, H.F. Teixeira, HPLC-UV method for temozolomide determination in complex biological matrices: Application for in vitro, ex vivo and in vivo studies, Biomed. Chromatogr. 33 (2019) e4615.
[14] A. Khosa, K.V. Krishna, R.N. Saha, S.K. Dubey, S. Reddi, A simplified and sensitive validated RP-HPLC method for determination of temozolomide in rat plasma and its application to a pharmacokinetic study, J. Liq. Chromatogr. Rel. Technol. 41 (2018) 692–697.
[15] U.N. Das, Gamma-linolenic acid therapy of human glioma-a review of in vitro, in vivo, and clinical studies, Med. Sci. Monit. 13 (2007) RA119-131.
[16] J.A. Miyake, M. Benadiba, A. Colquhoun, Gamma-linolenic acid inhibits both tumour cell cycle progression and angiogenesis in the orthotopic C6 glioma model through changes in VEGF, Flt1, ERK1/2, MMP2, cyclin D1, pRb, p53 and p27 protein expression, Lipids Health Dis. 8 (2009) 8–18.
[17] S. Chung, S. Kong, K. Seong, Y. Cho, γ-Linolenic acid in borage oil reverses epidermal hyperproliferation in guinea pigs, J. Nutr. 132 (2002) 3090–3097.
[18] B.l. Bravo, G. ChÃ¡vez, N. PiÃ±a, F. Ysambertt, N. MÃ¡rquez, A. CÃ¡ceres, Developing an on-line derivatization of FAs by microwave irradiation coupled to HPLC separation with UV detection, Talanta 64 (2004) 1329–1334.
[19] V. Guarrasi, M.R. Mangione, V. Sanfratello, V. Martorana, D. Bulone, Quantification of underivatized fatty acids from vegetable oils by HPLC with UV detection, J. Chromatogr. Sci. 48 (2010) 663–668.
[20] A.C. Mysłek, U. Siekierko, M. Gajewska, Application of silver ion high-performance liquid chromatography for quantitative analysis of selected n-3 and n-6 PUFA in oil supplements, Lipids 51 (2016) 413–421.
[21] F. Ianni, F. Blasi, D. Giusepponi, A. Coletti, F. Galli, B. Chankvetadze, R. Galarini, R. Sardella, Liquid chromatography separation of α- and γ-linolenic acid positional isomers with a stationary phase based on covalently immobilized cellulose tris(3,5-dichlorophenylcarbamate), J Chromatogr. A 1609 (2020) 460461.
[22] S. Bullock, Analysis of Complex Triglycerides in Starflower Oil from Borage by HPLC using ELSD, https://www.agilent.com/cs/library/applications/SI-01233.pdf, 1-2.
[23] A. Kotani, T. Fuse, F. Kusu, Determination of plasma free fatty acids by high-performance liquid chromatography with electrochemical detection, Anal. Biochem. 284 (2000) 65–69.
[24] T. Tome, N. Žigart, Z. Časar, A. Obreza, Development and optimization of liquid chromatography analytical methods by using AQbD principles: Overview and recent advances, Org. Process Res. Dev. 23 (2019) 1784–1802.
[25] F. Pena-Pereira, W. Wojnowski, M. Tobiszewski, AGREE-Analytical GREEnness metric approach and software, Anal. Chem. 92 (2020) 10076–10082.
[26] S.S. Saleh, H.M. Lotfy, G. Tiris, N. Erk, Y. Rostom, Analytical tools for greenness assessment of chromatographic approaches: Application to pharmaceutical combinations of indapamide, perindopril and amlodipine, Microchem. J. 159 (2020) 105557.
[27] S. Beg, M. Rahman, M.S. Hasnain, W.H. Almalki, Chapter 11 - Design of experiments application for analytical method development, Handbook of Analytical Quality by Design, Academic Press, New York, USA, 2021, pp. 191–197.
[28] S. Beg, M.S. Hasnain, M. Rahman, S. Swain, Chapter 1 - Introduction to Quality by Design (QbD): Fundamentals, Principles, and Applications, Pharmaceutical Quality by Design, Academic Press, New York, USA, 2019, pp. 1–17.
[29] S. Beg, J. Haneef, M. Rahman, R. Peraman, M. Taleuzzaman, W.H. Almalki, M. S. Hasnain, Chapter 1 - Introduction to analytical quality by design, Handbook of Analytical Quality by Design, Academic Press, New York, USA, 2021, pp. 1–14.
[30] S. Beg, M. Rahman, S.S. Panda, Pharmaceutical QbD: Omnipresence in the product development lifecycle, Eur. Pharm. Rev. 22 (2017) 58–64.
[31] Final Concept Paper - ICH Q14: Analytical Procedure Development and Revision of Q2(R1) Analytical Validation. https://database.ich.org/sites/default/files/Q2R2-Q14_EWG_Concept_ Paper.pdf.
[32] Temomedac, INN-Temozolomide. https://www.ema.europa.eu/en/documents/assessment-report/temomedac-epar-public-assessment-report_en.pdf.
[33] National Center for Biotechnology Information. PubChem Compound Summary for CID 5280933, gamma-Linolenic acid. https://pubchem.ncbi.nlm.nih.gov/compound/gamma-Linolenic-acid. Accessed Mar. 24, 2022.
[34] The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), Validation of analytical procedures: text and methodology Q2 (R1), International conference on harmonization, Geneva, Switzerland, 2005, pp. 11–12.
[35] The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), Stability testing of new drug substances and products, Q1A (R2), current step, 4 (2003) 1–24.
[36] U.S.P. Convention, The United States Pharmacopeia: The National Formulary, United States pharmacopeial convention, 1980.
[37] ICH Harmonised Tripartite Guideline Stability Testing of New Drug Substances and Products Q1A(R2). https://database.ich.org/sites/default/files/Q1A%28R2%29%20Guideline.pdf.
[38] T. Zwain, J.E. Alder, B. Sabagh, A. Shaw, A.J. Burrow, K.K. Singh, Tailoring functional nanostructured lipid carriers for glioblastoma treatment with enhanced permeability through in-vitro 3D BBB/BBTB models, Mater. Sci. Eng. C 121 (2021) 111774.
[39] C. Houacine, D. Adams, K.K. Singh, Impact of liquid lipid on development and stability of trimyristin nanostructured lipid carriers for oral delivery of resveratrol, J. Mol. Liq. 316 (2020) 113734.
[40] S. Beg, G. Sharma, O.P. Katare, S. Lohan, B. Singh, Development and validation of a stability-indicating liquid chromatographic method for estimating olmesartan medoxomil using Quality by Design, J. Chromatogr. Sci. 53 (2015) 1048–1059.
[41] S. Beg, K. Kohli, S. Swain, M.S. Hasnain, Development and validation of rp-hplc method for quantitation of amoxicillin trihydrate in bulk and pharmaceutical formulations using Box-Behnken experimental design, J. Liq. Chromatogr. Rel. Technol. 35 (2012) 393–406.