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Original Article

Fluorescence Detection of Etoposide Encapsulated Mesoporous Silica Nanoparticles by Environmentally Benign Bioanalytical HPLC Method and its Application to Pharmacokinetic Study

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ABSTRACT

Introduction: Etoposide belongs to BCS Class IV suffering from solubility and permeability limitations. Thereby, hindering its bioavailability. Thereby, it is imperative to enhance its bioavailability by suitable formulation to achieve a desired therapeutic effect. **Objectives:** In present work, ETD was encapsulated into mesoporous silica nanoparticles (MSNs) with the aim of achieving bioavailability enhancement. Further, A simple, efficient, and environmentally benign HPLC method with highly sensitive fluorescence detection was developed for determination of Etoposide (ETD) in the mice plasma. **Materials and Methods:** The developed HPLC-FL method was sensitive enough to detect etoposide in the nano formulation in a plasma matrix with a high degree of accuracy and precision, taking Tapentadol as an internal standard. The chromatographic separation was conducted on a Waters symmetry C_{18} column with a mobile phase composition of methanol: formate buffer (20mM) pH 3.9 in ratio 51:49 at a flow rate of 1 mL/min with excitation wavelength fixed at 247 nm and emission measured at 323 nm. Plasma sample pre-treatment was done following protein precipitation method. The developed bioanalytical method was validated successfully. Green metric assessment was done and Eco-indicators were employed which suggested the Eco-friendliness of developed method as well as supremacy over those available so far. **Results:** The bioavailability was enhanced 4.35 times as compared to ETO alone. The pharmacokinetic parameters of orally administered MSNs formulation were $t_{1/2}$ (Half-life) 12.12 hr, Peak plasma concentration C_{max} 3.98, Area Under the Curve (AUC) 52.78 and Mean Residence Time 18.23 hr. **Conclusion:** It could be concluded that the developed mesoporous formulation played a major part in BA enhancement of ETD and the developed green method was highly efficient to serve the purpose of ETD determination from biological matrix.

Keywords: Etoposide, Mesoporous silica nanoparticles, Green metrics assessment, Pharmacokinetic study, Bioavailability enhancement.

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INTRODUCTION

Etoposide (ETD) is a widely used anticancer drug in treatment of cancers of prostate,[1] lung,[2,3] lymph[4] and ovary.[5,6] The mechanism of action is based on inhibition of topoisomerase-II and activation of redox reactions which will produce derivatives which will cause DNA damage.[7] However, ETD belongs to Biopharmaceutical Classification System (BCS) Class IV and suffers from both solubility and permeability limitations.[1,8] Hence, its efficacy in cancer treatment is marred by both these factors. Therefore, mesoporous silica nanoparticles were prepared with the aim of investigating whether the Bioavailability (BA) of ETD could be enhanced by encapsulation into Mesoporous Silica Nanoparticles (MSNs).

Although many nano formulations have been reported on ETD,[9-13] reports of functionalized and bare mesoporous formulation are scarce and oral pharmacokinetic evaluation for studying BA enhancement of ETD encapsulated MSNs has not been reported so far. Although, etoposide has been widely researched upon with several methods available for its intravenous pharmacokinetic and biodistribution estimation, facile and sensitive methods useful in determining its oral bioavailability in mice are not available. Therefore, a highly sensitive and simple isocratic bioanalytical method using HPLC equipped with a fluorescence detector

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and Tapentadol as internal standard was developed. Protein Precipitation Method (PPT) was used for extraction of drug from the plasma matrix. PPT is suitable for high protein matrices such as serum, plasma, and whole blood. Precipitating agents include acids, organic solvents, salts, and metals.[14] Precipitation happens when the pH or hydrophobicity of a protein affects interactions with the aqueous environment, or when salts or metals bind to protein functional groups, disrupting intramolecular connections and causing the protein to denature, clump, and fall out of solution. To obtain a particulate-free supernatant or filtrate, centrifugation or filtration are used.

Other methods reported are for iv determination of ETD in dog plasma.[15] UV methods in rat plasma,[16-17] and iv administered mice.[18] Fluorescence method is more efficient when we compare with methods based on the UV detection. Hence, we developed a highly efficient, simple and sensitive HPLC-FL method for detection and estimation of ETD in mice plasma even at low oral dose levels. This could be useful and handy at the same time as it can substitute the need of using LC-MS and other sophisticated instruments for detecting ETD in low level in plasma. The method was fully validated successfully. Additionally, a greenness assessment of the developed method was performed using HPLC-EAT (HPLC-Environment assessment tool).[19] The score was positive and the method was developed without employing Acetonitrile and found to be most environmentally benign than the other reported methods so far. The highlight of this study is that even though there are number of bioanalytical methods and extraction reported for ETD in plasma matrix, the present method is the most sensitive and simple isocratic one with detection and quantification of ETD possible at quite low levels up to ng using HPLC-FL system with Tapentadol as an IS. And most importantly, this is so far the greenest and most environmentally benign bioanalytical method reported for ETD till date.[15-17, 20-22] Yang and group[15] reported a gradient method, Pigatto *et al*[16] developed a method which involved use of Acetonitrile and non-green/lesser green. Method developed by Saadati *et al*[17] involved use of lesser green solvents like chloroform, Acetonitrile, and n-hexane. Patolla and group[22] developed a isocratic method but used Chloroform in sample preparation same as Raza *et al*[18] and Dhanaraju *et al*[21] Hence, altogether in present research a simple isocratic, green and most sensitive HPLC-FL method was developed for ETD estimation in plasma matrix. A comparison of all literature available methods and their detailed comparison with present developed method along with the efficiency is described in Table 1.

Finally. After evaluating the pharmacokinetic data using Excel add-in; pk solver and comparing the AUC for developed formulations with ETD and Marketed formulations (MF), it was found that BA was enhanced 4.25 times as compared to ETD alone and 1.52 times enhanced with respect to the MF. Thus, it could be concluded that the ETD mesoporous formulations were successful in enhancing the BA of ETD and the developed HPLC-FL method was green and sensitive enough for estimating the drug encapsulated mesoporous formulation in mice plasma.

MATERIALS AND METHODS

Chemicals and reagents

Pure Etoposide (ETD) was obtained as a sample gratis from Intas pharmaceuticals, Ahmedabad, Gujarat, India. Tapentadol (purity >99.99%) Internal standard was kindly gifted by Ami Life Sciences, Vadodara, Gujarat, India. Ingredients used in the synthesis of MSNs, Cetyl Trimethyl Ammonium Bromide (CTAB), Fumed Silica and (3-Aminopropyl) Triethoxysilane (APTES) were purchased from Sigma Aldrich. Methanol (HPLC grade) and Toulene was obtained from Fischer scientific, Mumbai. All the reagents used were of analytical grade. Deionized water was used unless mentioned otherwise. Double distilled water was used in HPLC-FL analysis.

Methods

Instrumental and chromatographic conditions

Shimadzu LC-20 AD HPLC equipped with binary pump and fluorescence detector was operated at an excitation wavelength of 247 nm and emission wavelength of 323 nm. LC solutions software was used for acquiring data and integration. The chromatographic separation was achieved on a Waters symmetry 300 column (250×4.6mm,5µ) with Phenomenex C-18 security guard column. Mobile phase consisted of Methanol: Ammonium formate buffer (20mM) in the ratio 49:51. The flow rate was 1.0 mL/min and pH of the buffer was 3.9. The injection volume of the sample was 20 µL. Butchi Rotary evaporator was used for Drug loading into the MSNs.

Preparation and functionalization of Nanoparticles with drug loading

Mesoporous silica nanoparticles (MCM-41 type) were prepared as per the methodology adapted[23] Fumed silica was used as a silica source and CTAB was used as a surfactant template in this template-based synthesis approach. Surfactant removal was done by calcination at a higher temperature. Thereafter, surface functionalization was done using APTES with toluene as a medium and the product obtained was tagged as MCM-41-NH_2.[23] Further, ETD was encapsulated using solvent evaporation method using rotavapor. Briefly, solution of drug was prepared in methanol and mesoporous carrier was added in D:C ratio 1:1.5. Drug loaded nanoparticles were labelled as ETD-MCM-41 and ETD-MCM-41-NH_2.

Preparation of standard and quality control samples

ETD and internal standard TAP stock solutions were prepared in Methanol at the concentration of 1000µg/mL and 100 µg/mL as primary and secondary stock solutions respectively. The

Table 1: Literature reported methods for Etoposide detection in Plasma and comparison with present developed method.

Sl. No.	Mobile phase	Ratio of solvents	pH	Run Time (min)	Sample preparation/ extraction	Method	Internal standard	Detection	Greenness comments	References
1	Methanol: water	49:51	3.9 (Formic acid)	7	Methanol Mobile phase	Simple Isocratic	Tapentadol	Fluorescence	Green method, isocratic, lesser runtime	Present method
2	Methanol: water	0-5min A=45-50% 5-10 min= 50-90%	-	10.5	Methanol (stock) 50:50 for dilution	Gradient	Teniposide	Fluorescence	Though green, run time is more and gradient method	Yang *et al.*
3	Water:ACN	70:30	4 (Formic Acid)	12.5	Tert-butyl methyl ether and mob phase for dilution	Gradient	Phenytoin	UV	Acetonitrile used. Non -green in comparison. Non-green sample preparation	Pigatto *et al.*
4	Methanol: water: Acetic acid	54:45:1	-	-	3mL chloroform Chloroform: Methanol Final mob phase	Isocratic	Phenytoin sodium	PDA	Chloroform used in sample preparation	Patlolla *et al.*
5	MeOH: ACN: Phosphate (0.007%TEA)	18:19:63	5.2 (Phosphor-ric Acid)	10	1ml mix chloroform: n-hexane	Isocratic	Lamotrigine	UV-vis	Chloroform, hexane, TEA	Saadati *et al*
6	ACN: Water	35:65	-	5	ACN, Chloroform	Isocratic	Teniposide	UV	Methanol 60%, Flow rate 1.5ml/min. 45°C temp.	Raza *et al*
7	MeOH:Phosphate buffer	80:20	6	6	LLE-$CHCl_3$ MeOH	Isocratic	-	UV	Methanol 80% in method, chloroform in sample preparation	Dhanaraju *et al.*

serial dilutions were made with mobile phase to obtain working standards of desired concentration. The calibration standards were prepared by spiking 100µL of blank plasma with proper working standard solutions of ETD and 5 µg/mL TAP (internal standard solution). The effective concentrations for calibration range were 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL for ETD. The QC samples were prepared in plasma matrix with low, mid and high concentration of 0.3, 15 and 40 µg/mL respectively. Protein precipitation method was used for extraction of drug from biological plasma matrix.

Plasma sample preparation

The samples were centrifugated at 5000 rpm for 10 min at 4°C. The drug was extracted from plasma and to 150 µL plasma, 100 µL, aliquot of drug and 100µL internal standard TAP was mixed and vortexed for 2 min. 800 µL Methanol was added as a precipitating agent and centrifuged for 10 min. The supernatant was collected and ETD was quantified by HPLC equipped with a fluorescence detector with excitation and emission at 247 and 323 nm respectively. Similarly, 100 µL of 1, 5, 20, 40, 60, 80, 100, 200 and 500 µg/mL ETO solution was added and proceeded to have 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL plasma extracted ETO solution.

Bioanalytical method validation

Sensitivity

Sensitivity is defined by the lowest nonzero standard (LLOQ) on the calibration curve. The sensitivity study was carried out by injecting six replicates of ETD solution at LLOQ concentration and % RSD was calculated.

Linearity

The calibration curve was created by graphing the peak area ratio of analytes to internal standard vs the theoretical analytical concentration of spiking plasma standards using 9 points 0.1-50 µg/mL. The calibration curve's linearity was assessed using linear regression analysis, and the R^2 value was calculated.

Precision and accuracy

By comparing the predicted concentration derived from the linearity curve to the actual concentration of ETD spiked in blank plasma, the precision and accuracy of the devised approach were determined. Precision was calculated both intraday and interday. LLOQ, LQC, MQC, and HQC samples were analyzed on one day (n=6) for intraday precision, and repeated analysis was done on six days in a row for intraday precision (1 series per day). In accuracy and precision studies, freshly produced solutions were used.

Extraction Recovery

By comparing the mean response of QC samples at three different concentrations to the mean peak response of corresponding plain standards generated in the mobile phase, the absolute recovery of chemicals in plasma and tissue homogenates was established. The analyte or internal standard recovery does not have to be perfect, but it should be consistent and repeatable according to the guidelines. The formula that was employed was

$$\% \text{ Recovery} = \frac{\text{Area of extracted sample}}{\text{Area of unextracted sample}} \times 100 \ldots\ldots. (1)$$

Limit of detection and Limit of quantitation

The lowest detectable concentration of the analyte is known as the Limit of Detection (LOD), which is calculated using a signal-to-noise ratio of 3. The slope method and standard deviation of response were used to calculate both LOD and LOQ.

Stability

Three repetitions were used to determine the stability of ETD in biological matrix plasma at QC concentrations.

Freeze-thaw stability

The QC samples were subjected to three freeze-thaw cycles in a row. Three QC levels of standard solution were added to blank plasma for this experiment. The samples were frozen at -80°C and subsequently thawed at room temperature. The samples were analyzed by HPLC after refreezing and completing the procedure three times. The freeze-thaw stability of freeze-thawed QC samples was compared to that of freshly spiked calibration samples.

Bench top stability

The stability of the analyte of interest in the biological matrix plasma at RT, and more specifically at laboratory handling conditions, was assessed over a length of time that was longer than the standard sample preparation time. It was determined at RT over a duration of 6 hr. The stability of the calibration standards was determined by comparing them to freshly spiked calibration standards.

Long term stability

The stability of the QC solutions for 14 days was determined at -80°C. To determine stability, all of the observed concentrations were compared to nominal values.

Pharmacokinetic studies

All the animal experiments were performed as per the protocol approved by Institutional animal ethics committee guidelines (protocol number: MSU/IEAC/2017-18/1724). Four months old Healthy Female Swiss albino mice (weighing 22-28 g) were obtained from Zydus Research centre, Ahmedabad. All the

animals were kept under standard conditions in the laboratory with free access to food and water. They were allowed to get acclimatized to animal facility before beginning the experiments.

The mice were separated into five groups for the oral pharmacokinetic investigation, with one serving as a control and the others receiving ETD solution, ETD-MCM-41, ETD-MCM-41-NH2, and Marketed Formulation (MF) accordingly. Oral gavage was used to administer 10mg/kg formulations. At regular intervals, blood samples were collected in centrifuge vials containing EDTA. The plasma was separated and mixed with TAP after centrifugation at 4000 rpm for 10 min. As a precipitating agent, methanol was utilized, and the protein precipitation method was used for extraction.

From plasma concentration-time data, pharmacokinetic values were computed. For the calculations, the PK solver, an excel add-in, was used. The area under the curve of the respective formulations and the standard ETD solution were compared to get bioavailability increment data.

Green metrics assessment

Solvent selection guide

GlaxoSmithKline,[24] Astra Zeneca,[25] Pfizer,[25] Sanofi,[26] BMS,[27] and many others provide solvent selection instructions. All of these include a list of solvents and group them according to their environmental impact. For technique development and sample pre-treatment, an environmentally friendly reagent was chosen based on these guidelines. Methanol was discovered to be a green solvent, thus we used it in both sample preparation and method development. Another feature of this process was the reduced number of steps in protein precipitation compared to previous extraction methods, which is in line with one of the green chemistry concepts.

NEMI (National Environmental Method Index)

NEMI's evaluation wheel is made up of four primary pillars: PBT (persistent, bio accumulative, and toxic), Hazardous, Corrosive, and Waste. The list of compounds covered by the EPA's Toxic Release Inventory is included in PBT (TRI). Hazardous chemicals are those that are on the TRI or RCRA's D, F, P, or U lists. Corrosive procedures are those that have a pH range of less than or more than 2-12. If the amount of waste produced is larger than 50 g, the method is termed non-green. Furthermore, it was determined that safety, health, the environment, energy, and waste are all crucial factors to consider when evaluating green metrics.

Eco-scale

The eco-scale is a method for determining the greenness of analytical methods. It is based on the concept of deducting penalty points from a base score of 100 for non-green characteristics of a method. It's a nice semi-quantitative tool, as opposed to standard green metrics tools that incorporate Atom economy, E factor, and other factors that aren't as useful in analytical chemistry as they are in synthesis-based organic chemistry. Galuzka *et al* provide a detailed description of the complete analytical technique, including both green and non-green features. Direct approaches that save energy are considered to be the most environmentally beneficial. Off-line systems, as well as procedures that use time, energy, or reagents, are considered non-green. However, it is missing data on solvent usage and waste generation. There isn't a lot of data on the structure of hazards.

Analytical Eco-scale= 100- total penalty points……(2)

HPLC-EAT (Environment assessment tool)

Gaber *et al*[19] have created a highly useful and practical tool. Profiling the greenness of HPLC procedures is both straightforward and effective. It considers the method's solvents' environmental, health, and safety implications. Based on these three pillars of environmental, health, and safety estimation, a total score is calculated.

GAPI (Green Analytical Procedure Index)

GAPI, which is based on the NEMI and analytical eco-scale, provides both general and quantitative information about the greenness of analytical methods. It employs five pentagrams as method representative or greenness indicators. Green, yellow, and red color coding is used to represent low, medium, and high environmental impact, respectively. The more steps there are, the less green the methodology is. Various steps considered for evaluation include sample collection, preservation, transport, preparation, extraction, determination, and quantification of the analyte.

Ecosolvent tool: best waste treatment options

The use of an eco-solvent tool entails incorporating method data to determine the best treatment option for waste generated. The best option between incineration and distillation is chosen. This can aid in the effective treatment of waste, thereby reducing the negative impact on the environment. It is ideal for assessing the life cycle of waste disposal options.

AMVI (Analytical Method Volume Intensity)

Hartman and colleagues developed AMVI to calculate the total solvent consumption of an analytical method.[24] It can be very useful in determining ways to reduce the negative environmental impact of analytical methods. The environmental efficiency of various analytical methods can be compared using this AMVI score. AMVI is calculated using the formula shown below.

$$\text{Total solvent consumption} = \sum(\text{Sample prep solvent}) + \sum(\text{HPLC solvent}) \times \text{replicates} \ldots (3)$$

$$\text{AMVI} = \frac{\text{Total solvent consumption}}{\text{Number of chromatographic peaks of interest}} \ldots\ldots (4)$$

Table 2: Bioanalytical method validation of developed HPLC-FL method for ETO detection in mice plasma.

Parameters	Results obtained	
Wavelength (nm)	247 nm excitation and 323 nm emission	
Concentration range (µg/mL)	0.1-50	
Regression equation	y = 386781x + 133108	
Correlation coefficient (r^2)	0.998	
Retention time	5.9±0.2 min (ETO) and 4.5±0.1 min (TAP)	
Intra-day precision (%RSD)	LLOQ (0.1 µg/mL)	1.15%
	LQC (0.3 µg/mL)	0.85%
	MQC (15 µg/mL)	0.77%
	HQC (40 µg/mL)	0.56%
Inter-day precision (%RSD)	LLOQ (0.1 µg/mL)	0.99%
	LQC (0.3 µg/mL)	0.71%
	MQC (15 µg/mL)	0.57%
	HQC (40 µg/mL)	0.66%
Accuracy (%Recovery)	80%	98.66±0.70%
	100%	101.47±0.41%
	120%	99.56%±0.98%
LOD (µg/mL)	0.03	
LOQ (µg/mL)	0.09	
%Recovery	LQC (0.3µg/mL)	86.66±0.14%
	MQC (15µg/mL)	85.47%1.05%
	HQC (40µg/mL)	89.57%0.84%
Bench-top Stability	LQC (0.3µg/mL)	99.22%
	HQC (15µg/mL)	98.89%
Freeze-thaw stability	LQC (40µg/mL)	101.55%
	HQC (0.3µg/mL)	100.85%
Long-term stability	LQC (15µg/mL)	99.28%
	HQC (40µg/mL)	101.45%
System suitability	Theoretical plates	6945
	Area	3929648

RESULTS

Bioanalytical Method validation

The developed HPLC-FL method was validated successfully in accordance with USFDA guidelines. The method was specific, and no interference was observed in blank samples obtained from different sources of mouse plasma. In the assay range, the calibration curves were linear, with a regression coefficient greater than 0.998. In all QC samples, the percent Relative standard deviation was within limits for both accuracy and precision, both intraday and interday. A highly sensitive method with a limit of detection of 0.03 µg/mL. The LOQ was determined to be 0.09 µg/mL. In all three freeze-thaw cycles, ETD was found to be stable in plasma. Detailed results of Bioanalytical method validation are tabulated in Table 2.

Green metrics assessment

Various solvent selection guides available lists or categorize the solvent according to their Environment, Health and Safety Impact (EHS) value and aid in selecting a environmentally benign solvent for use. Herein Methanol falls under a comparatively green category and hence has been employed here in bioanalytical method development and validation. As per the NEMI assessment, the waste generated for method is less than 50 g and pH range in which this method works is under non-corrosive criteria (pH 2-12) giving a green label rating to the method (Figure 1). The penalty points were deducted to calculate analytical Eco-Scale. The results of calculation are ranked on a scale with scores ranked accordingly.>75 score represents excellent green analysis; >50 represents excellent green analysis and <50 represents inadequate green analysis. The analytical Eco-scale rating obtained for

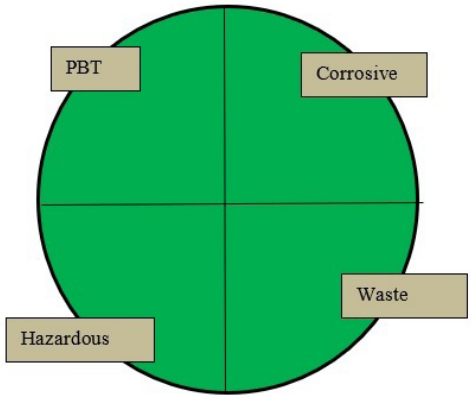

Figure 1: NEMI Assessment of the developed method.

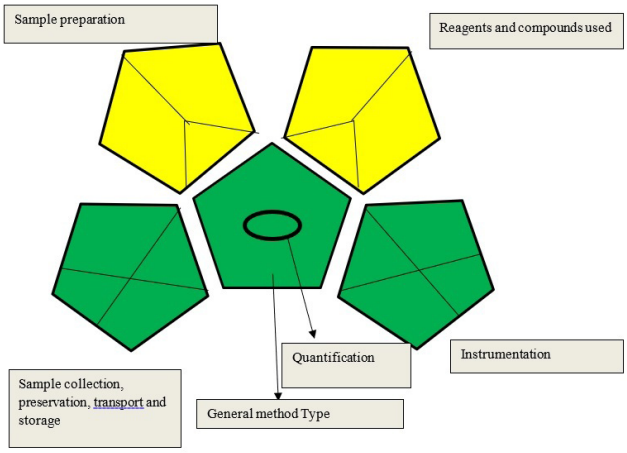

Figure 3: GAPI Index of the developed Bioanalytical method.

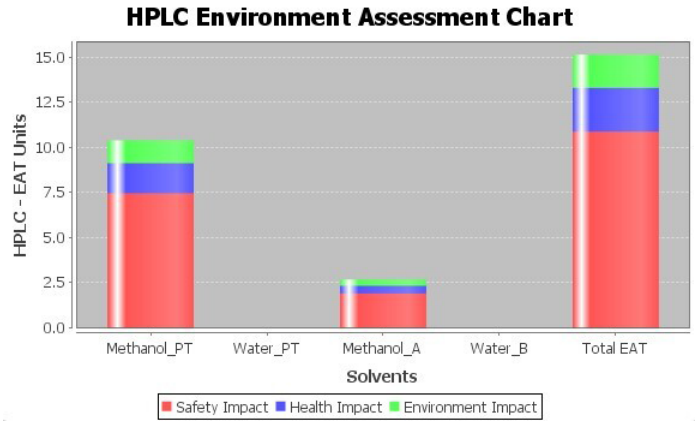

Figure 2: HPLC-EAT score of the developed Bioanalytical method.

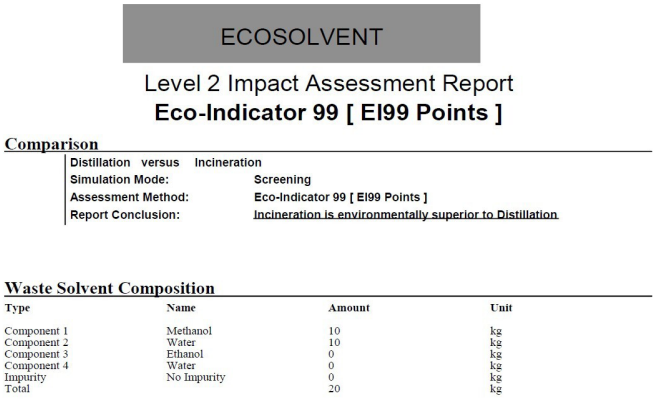

Figure 4: Ecosolvent Impact assessment report.

developed method is 88 which falls in category 1 (>75) i.e., the method represents excellent green analysis. The Analytical Eco-scale calculation and ratings are given in Table 3. HPLC-EAT tool was used to obtain EHS impact of developed method. The figure depicts the least impact of method on environment which precludes any non-greenness of the method. Further, the total EAT score is also near to 15 which is not considered high as compared to high scores obtained with use of non-green solvents like n-Hexane or lesser green like Acetonitrile (Figure 2). GAPI index was also designed and greenness depiction of method was done. Sample preparation in-line or at-line has intermediate impact and denoted by yellow color. Preservation, transport, and storage requirements are precluded in this case and hence denoted by green (least environment impact). Reagents and solvents; methanol has a slight impact and is flammable and hence denoted by yellow color. Energy consumption by Instrument used (HPLC-Fl) is less than 0.1kWh per sample and hence green impact. A circle in the middle pentagon denotes whether process is for qualitative purpose or quantitative purpose (absence of circle). Here the method is used for quantification and hence a small circle in center symbolizes the same (Figure 3). Eco solvent tool based on E-199 Points served as the indicator for best waste treatment option which was found to be incineration rather than distillation (Figure 4). The AMVI for developed chromatographic method was found to be 135 based on triplicates.

Pharmacokinetic study

The applicability of the currently available environmentally friendly method for studying the pharmacokinetics of oral ETD nanoparticles in male swiss albino mice was investigated. The Figure 5 depicts the plasma concentration time profiles for a 10 mg/kg oral ETD dose. ETD concentrations in plasma were easily measurable up to 24 hr after ETD administration. C_{max} for ETD was reached earlier, around 2 hr. Peak plasma concentrations of ETD-MCM-41 and ETD-MCM-41-NH_2 were reached after approximately 3.98 hr and 2.51 hr, respectively. ETD loaded nanoparticles, both functionalized and bare, cleared at a slower rate than ETD solution until 24 hr. As a result, when compared to ETD alone, both coated and uncoated ETD loaded MSNs had higher AUC and improved bioavailability. When compared

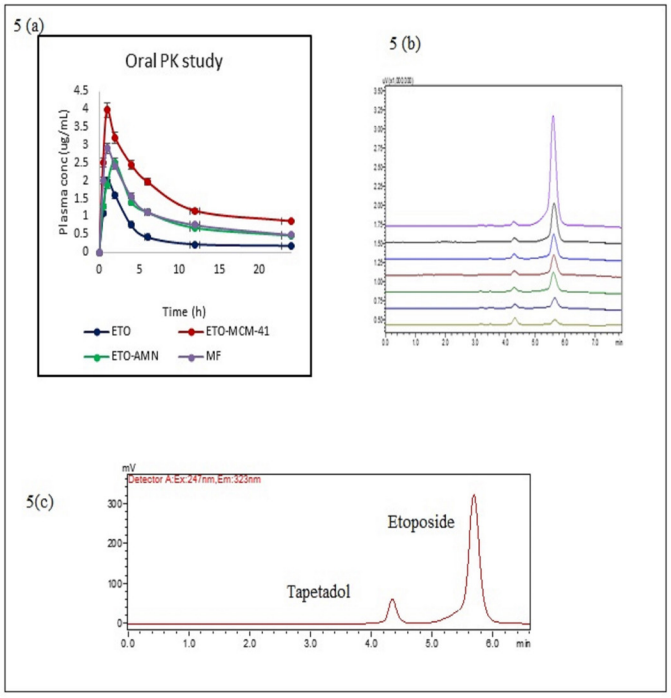

Figure 5: (a) Plasma concentration-time profile of developed formulations (b) and (c) Chromatgrams of Drug Etoposide and internal standard Tapentadol.

Table 3: Analytical Eco-scale score obtained for the developed method after deduction of penalty points.

The Penalty points (PPs) for the developed bioanalytical method for ETD estimation	
Reagents	
	Penalty Points
Methanol	
Water	0
Amount (>100mL)	3
Hazard (less severe)	1
Reagents (total)	4
Instruments	
	Penalty Points
Energy (HPLC= 60.1 kWh per sample)	0
Ocuupational hazard	0
Waste (>10 mL)	5
Waste (No treatment)	3
Instruments (total)	8
Overall PPs	8+4= 12
Analytical Eco-scale total score =100-PPs= 100-12= 88	

Table 4: Pharmacokinetic parameters and Bioavailability enhancement of developed Nanoparticles with drug and Marketed formulation.

Parameters / Sample	$t_{1/2}$	T_{max}	C_{max}	AUC	MRT	BA Enhancement
ETD	6.88	1	2.06	12.44	10.61	4.35 (Compared to ETD)
ETD-MCM-41	12.12	1	3.98	52.78	18.23	1.52 (Compared to MF)
ETD-MCM-41-NH_2	13.01	2	2.51	30.0	17.97	2.47 (Compared to ETD)
MARKETED FORMULATION	14.98	1	2.91	34.59	19.49	2.78 (Compared to ETD)

to ETD, MCM-41 MSNs increased ETD bioavailability by 4.35 times and MCM-41-NH_2 increased ETD bioavailability by up to 2.47 times. The BA enhancement of ETD-MCM-41 over the marketed formulation was 1.52 times. Both synthesized nano formulations had maximum retention times that were very close to and comparable to the marketed formulation. HPLC-FL chromatograms are depicted in Figure 5 (b) and (c). Table 4 details the pharmacokinetic parameters and bioavailability enhancement details for the developed formulations.

CONCLUSION

The current study sought to synthesize and investigate MSN formulations for BA enhancement of ETD, as well as to develop a simple, isocratic, highly sensitive, and environmentally benign rapid HPLC-FL method for ETD estimation in plasma matrix. The simplest Protein precipitation method yielded consistent and reproducible analyte recoveries. The quantitation limit was adequate and sensitive enough to monitor ETD presence intraday and inter-day at all QC levels. The developed method was also very environmentally friendly, with low EAT scores and a high greenness profile across all green metrics assessment results. Furthermore, it was established that the BA of ETD was significantly increased, up to more than four times, providing a ray of hope for the application of MSNs in the enhancement of BA of various BCS class II and IV drugs suffering from solubility and permeability limitations, which affect BA.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ETD: Etoposide; **MSNs:** Mesoporous Silica Nanoparticles; **QC:** Quality Control; **BA:** Bioavailability; **HPLC-FL:** High performance Liquid Chromatography-Fluorescence; **MCM:** Mobile Composition of Matter; **AUC:** Area under curve; **EHS:** Environment Health Safety; **EAT:** Environment Assessment Tool; **AMVI:** Analytical Method Volume Intensity; **GAPI:** Green Analytical Procedure Index.

SUMMARY

Etoposide loaded Mesoporous silica Nanoparticles were synthesized successfully and *in vivo* pharmacokinetic studies were conducted in Swiss albino mice, data of which fulfilled the target of oral Bioavailability enhancement. The enhancement in bioavailability can be attributed to the unique properties of mesoporous silica nanoparticles like ease of surface functionalization, higher pore volume to surface area ratio, biocompatibility and zero premature leakage leading to higher stability. Low Bioavailability is a major barrier in effectiveness of Therapeutics and many potent drugs. Entrapment of drug in the mesoporous cavity might lead to change in the form from crystalline to amorphous. This may have further lead to overcoming the solubility barriers leading to achieving the goal of Bioavailability enhancement. It is visible from results that ETD loaded into Bare MCM-41 was exhibiting lesser Bioavailability enhancement than ETD loaded into functionalized MCM-41 which are amine coated. This could be attributed to the characteristic of amine group capable of imparting a sustained release profile to nanocarriers. A significant increase was obtained even when compared to Marketed formulation.

Further, focus of this work was also on developing a suitable bioanalytical method, which was environment friendly and highly sensitive at the same time, including the but obvious simple and effective. Hence, an isocratic HPLC-FL method was developed which utilized greener solvents and was highly sensitive based on the Florescence detection. The green metrics assessment was done for the developed method and owing to use of greener solvents, the developed method passed the green metrics assessment with flying colors. The extraction was also based on the simple Protein precipitation method. Further, environmentally benign assessment also involved giving a negative point or penalty points for any non-green aspect of the developed method. The AMVI assessment gave a indication of total solvents consumed and further, Eco solvent E-199 scale includes the disposal part which is incineration or distillation which method would be favorable for disposal of the waste generated. Thus, an overall assessment including from initial beginning stages of method to the final steps like the disposal of solvent. Such assessment should be encouraged and a database needs to be maintained which would prove to be useful for future assessments. This could also aid in incorporation of sensitive and green methods in routine pharmacopeial monographs as well. Which could lead to an overall positive impact on the environment if followed by commercials and industry scale as well.

REFERENCES

1. Saroj S, Rajput SJ. Etoposide encapsulated functionalized mesoporous silica nanoparticles: Synthesis, characterization and effect of functionalization on dissolution kinetics in simulated and biorelevant media. J Drug Deliv Sci Technol. 2018; 44:27-40. doi: 10.1016/j.jddst.2017.11.020.
2. Zatloukal P, Cardenal F, Szczesna A, Gorbunova V, Moiseyenko V, Zhang X, *et al*. A multicenter international randomized phase III study comparing cisplatin in combination with irinotecan or etoposide in previously untreated small-cell lung cancer patients with extensive disease. Ann Oncol. 2010;21(9):1810-6. doi: 10.1093/annonc/mdq036, PMID 20231298.
3. Lawson MH, Cummings NM, Rassl DM, Russell R, Brenton JD, Rintoul RC, *et al*. Two novel determinants of etoposide resistance in small cell lung cancer. Cancer Res. 2011;71(14):4877-87. doi: 10.1158/0008-5472.CAN-11-0080, PMID 21642373:canres.0080.2011.
4. Koga S, Hamazoe R, Maeta M, Shimizu N, Murakami A, Wakatsuki T. Prophylactic therapy for peritoneal recurrence of gastric cancer by continuous hyperthermic peritoneal perfusion with Mitomycin C. Cancer. 1988;61(2):232-7. doi: 10.1002/1097-0142(19880115)61:2<232::aid-cncr2820610205>3.0.co;2-u, PMID 3121165.
5. de La Motte Rouge T, Pautier P, Duvillard P, Rey A, Morice P, Haie-Meder C, *et al*. Survival and reproductive function of 52 women treated with surgery and bleomycin, etoposide, cisplatin (BEP) chemotherapy for ovarian yolk sac tumor. Ann Oncol. 2008;19(8):1435-41. doi: 10.1093/annonc/mdn162, PMID 18408223.
6. Bruzzone M, Centurioni MG, Giglione P, Gualco M, Merlo DF, Miglietta L, *et al*. Second-line treatment with intravenous gemcitabine and oral etoposide in platinum-resistant advanced ovarian cancer patients: results of a phase II study. Oncology. 2011;80(3-4):238-46. doi: 10.1159/000328451, PMID 21734415.
7. Van Maanen JM, Retèl J, De Vries J, Pinedo HM. Mechanism of action of antitumor drug etoposide: A review. J Natl Cancer Inst. 1988;80(19):1526-33. doi: 10.1093/jnci/80.19.1526, PMID 2848132.
8. Dahan A, Miller JM, Amidon GL. Prediction of solubility and permeability class membership: Provisional BCS classification of the world's top oral drugs. AAPS J. 2009;11(4):740-6. doi: 10.1208/s12248-009-9144-x, PMID 19876745.
9. Reddy LH, Sharma RK, Chuttani K, Mishra AK, Murthy RR. Etoposide-incorporated tripalmitin nanoparticles with different surface charge: formulation, characterization, radiolabeling, and biodistribution studies. AAPS J. 2004;6(3): e23. doi: 10.1208/aapsj060323, PMID 15760108.
10. Reddy LH, Murthy RS. Etoposide-loaded nanoparticles made from glyceride lipids: Formulation, characterization, *in vitro* drug release, and stability evaluation. AAPS PharmSciTech. 2005;6(2): E158-66. doi: 10.1208/pt060224, PMID 16353973.
11. Mehrabi M, Esmaeilpour P, Akbarzadeh A, Saffari Z, Farahnak M, Farhangi A, et al. Efficacy of pegylated liposomal etoposide nanoparticles on breast cancer cell lines. Turk J Med Sci. 2016;46(2):567-71. doi: 10.3906/sag-1412-67, PMID 27511525.
12. Yadav KS, Sawant KK. Formulation optimization of etoposide loaded PLGA nanoparticles by double factorial design and their evaluation. Curr Drug Deliv. 2010;7(1):51-64. doi: 10.2174/156720110790396517, PMID 20044908.
13. Qian WY, Sun DM, Zhu RR, Du XL, Liu H, Wang SL. pH-sensitive strontium carbonate nanoparticles as new anticancer vehicles for controlled etoposide release. Int J Nanomedicine. 2012; 7:5781-92. doi: 10.2147/IJN.S34773, PMID 23185118.
14. Stone J. Sample preparation techniques for mass spectrometry in the clinical laboratory. Mass Spectrometry for the Clinical Laboratory. Elsevier; 2017. p. 37-62.
15. Yang HZ, Liu L, Xu X. Fluorescence detection of etoposide-loaded nanoparticles by HPLC and pharmacokinetics study. Arzneim Forsch. 2012;62(12):661-5. doi: 10.1055/s-0032-1330019, PMID 23192689.

16. Pigatto MC, Mossmann DL, Dalla Costa T. HPLC-UV method for quantifying etoposide in plasma and tumor interstitial fluid by microdialysis: Application to pharmacokinetic studies. Biomed Chromatogr. 2015;29(4):529-36. doi: 10.1002/bmc.3308, PMID 137530.
17. Saadati R, Dadashzadeh S. S. Simple and efficient HPLC-UV quantitation of etoposide and its cis-isomer in rat micro-volume plasma and tissue samples: Application to pharmacokinetic and biodistribution studies. J Liq Chromatogr Relat Technol. 2011;34(18):2130-48. doi: 10.1080/10826076.2011.585483.
18. Raza A, Aman A, Bashir S, Ahmad B, Irfan J, Mehta JD, *et al*. Rapid and sensitive liquid chromatographic method for determination of etoposide in plasma and biological samples. J Liq Chromatogr Relat Technol. 2013;36(19):2796-813. doi: 10.1080/10826076.2012.725698.
19. Gaber Y, Törnvall U, Kumar MA, Amin MA, Hatti-Kaul R, HPLC-EAT. HPLC-EAT(Environmental Assessment Tool): A tool for profiling safety, health and environmental impacts of liquid chromatography methods. Green Chem. 2011;13(8):2021-5. doi: 10.1039/c0gc00667j.
20. Strife RJ, Jardine I, Colvin M. Analysis of the anticancer drugs etoposide (VP 16-213) and teniposide (VM 26) by high-performance liquid chromatography with fluorescence detection. J Chromatogr. 1981;224(1):168-74. doi: 10.1016/s0378-4347(00)80153-x, PMID 7263816.
21. Dhanaraju MD, Sundar VD, Vijayalakshmi R, Naveena VSH. Estimation and validation of etoposide by RP-HPLC method in rat plasma by liquid-liquid extraction. Am J Phytomed Clin Ther. 2013; 2:201-7.
22. Patlolla RR, Vobalaboina V. Pharmacokinetics and tissue distribution of etoposide delivered in parenteral emulsion. J Pharm Sci. 2005;94(2):437-45. doi: 10.1002/jps.20249, PMID 15614812.
23. Saroj S, Rajput SJ. Tailor-made pH-sensitive polyacrylic acid functionalized mesoporous silica nanoparticles for efficient and controlled delivery of anti-cancer drug etoposide. Drug Dev Ind Pharm. 2018;44(7):1198-211. doi: 10.1080/03639045.2018.1438467, PMID 29412022.
24. Henderson RK, Jiménez-González C, Constable DJC, Alston SR, Inglis GGA, Fisher G, *et al*. Expanding GSK's solvent selection guide–embedding sustainability into solvent selection starting at medicinal chemistry. Green Chem. 2011;13(4):854-62. doi: 10.1039/c0gc00918k.
25. Prat D, Hayler J, Wells AJGC. A survey of solvent selection guides. Green Chem. 2014;16(10):4546-51. doi: 10.1039/C4GC01149J.
26. Byrne FP, Jin S, Paggiola G, Petchey THM, Clark JH, Farmer TJ, *et al*. Tools and techniques for solvent selection: green solvent selection guides. Sustain Chem Process. 2016;4(1):1-24. doi: 10.1186/s40508-016-0051-z.
27. Leahy DK, Simmons EM, Hung V, Sweeney JT, Fleming WF, Miller MJGC. Design and evolution of the BMS process greenness scorecard. Green Chem. 2017;19(21):5163-71. doi: 10.1039/C7GC02190A.

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