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A VALIDATED RP-HPLC ASSAY METHOD FOR DETERMINATION OF GEMCITABINE LOADED NANOSIZED SOLID LIPID NANOPARTICLES

Namrata Soni, Neetu Soni, Pramode W. Ramteke, Himanshu Pandey

Department of Pharmaceutical Sciences, Faculty of Health Sciences, Sam Higginbottom Institute of Technology and Sciences, Naini, Allahabad, (U.P.) 211007, India.

ABSTRACT

A novel reverse phase HPLC assay method has been developed and validated for the simultaneous determination of Gemcitabine Hydrochloride (dFdCH) along with solid lipid nanoparticles SLNs and conjugated with mannose to targeting the lungs for chemotherapy. Methanol was used as the extracting solvent for preparation of tissue sample. Methanol: Ammonium acetate buffer; 10:90 v/v (pH 5) was the mobile phase at flow rate 1.5 mL/min at pressure of 102/101 bars using Luna Phenomenex, C18 (4.6mm×250 mm; 5 µm bead size) at wavelength 269 nm. The column oven temperature was optimized at 35^0C. The biodistribution studies were conducted to evaluate the target potential at the sites of interest in liver, spleen, lung and kidney respectively, the calibration curve was found to be linear over the concentration range of 100-5000 ng/mL(r^2=0.9980, r^2=0.9980, r^2=0.9990, r^2=1 respectively). Plain drug have greatest access to liver and secondarily to spleen and then kidney but on the contrary the concentration of drug was greatest in lung when treated with mannosylated SLNs, data suggested that the mannose attachment has provided the access for the drug in the lungs via the formulation in greater quantity than free drug due to interaction of mannose with mannose receptors present on lung macrophages. The developed method was validated in accordance to ICH guidelines.

Keywords: High Performance Liquid Chromatography, Mannosylated SLNs, Macrophage Mannose, Receptor (MMR), Lung Cancer, Gemcitabine Hydrochloride.

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***Address for Correspondence:**

Mrs. Namrata Soni, Assistant Prof., Department of Pharmaceutical Sciences, Faculty of Health Sciences, Sam Higginbottom Institute of Technology and Sciences, Naini, Allahabad (U.P.)

Abbreviations: dFdCH: 2'-2'-difluorodeoxycitidine hydrochloride (Gemcitabine), SLNs: Solid lipid nanoparticles, dFdC-SLNs: gemcitabine loaded SLNs, dFdC-MSLNs: gemcitabine loaded mannosylated SLNs.

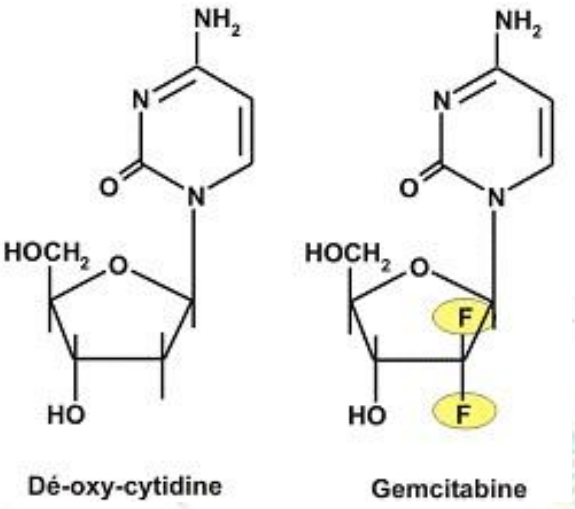
INTRODUCTION

Gemcitabine is a pyrimidine antimetabolite. Chemically it is 2'-2'-difluorodeoxycitidine (dFdC). This molecule bearing fluorine atom replaces the hydroxyl group and the hydrogen atom at the 2'-position of cytidine. After its anabolism to diphosphate and triphosphate metabolites, gemcitabine inhibits ribonucleotide reductase and competes with 2' – deoxycytidine triphosphate for incorporation into DNA. These effects produce cell-cycle-specific cytotoxicity. [1-2]

Selective and site-specific administration of anticancer drugs may help in overcoming low survival rate and cytotoxic effects on normal tissues. Even highly toxic agents could be rendered safer and more effective, if it could be possible to direct them specifically into the tumor cells. This is because of accomplishment of higher drug concentration within the tumor cells, with fewer or no distribution towards normal tissues. To achieve the above mentioned goal we planned to construct mannose anchored SLNs nano-constructs loaded with anticancer bioactive for efficiently targeting the lung cancer cells. [3-7]

SLNs are typically spherical with an average diameter between 10 and 1000 nanometers. Solid lipid

nanoparticles possess a solid lipid core matrix that can solubilize lipophilic molecules. The lipid core is stabilized by surfactants (emulsifiers). The term lipid is used here in a broader sense and includes triglycerides (e.g. tristearin), diglycerides (e.g. glycerol bahenate), monoglycerides (e.g. glycerol monostearate), fatty acids (e.g. stearic acid), steroids (e.g. cholesterol), and waxes (e.g. cetyl palmitate). All classes of emulsifiers (with respect to charge and molecular weight) have been used to stabilize the lipid dispersion. It has been found that the combination of emulsifiers might prevent particle agglomeration more efficiently [8-9]



SLNs offer additional advantages as drug carrier for cancer targeting due to their nanoscopic size range and high drug loading propensity. High performance liquid chromatography (HPLC) is the most commonly used method for determining the dFdC concentration in plasma and tissues.[10-17]

2 . MATERIAL AND METHODS

2.1 Chemical and reagents

(dFdCH) was obtained as gift sample from Sun Pharma Laboratories Limited, Vadodara, India, HPLC grade acetonitrile and methanol (Sigma Aldrich, Mumbai, India) were used. Analytical reagent grade (AR) potassium dihydrogen orthophosphate and disodium phosphate (SD Fine Chemicals, Mumbai, India) (99.5% purity) were used. Water was purified by Millipore Synergy (Millipore France). The SLNs were synthesized by using specifically the solvent injection method. Mannose anchoring in SLNs was done by ring opening reactions followed by reaction of aldehyde groups of mannose in 0.1M sodium acetate buffer (pH 4.0) with the amines groups of SLNs to form M-SLNs[18-21]

2.2. Instruments

For chromatography a SIL 10A auto injector HPLC system comprising of SCL 10A system controller, SPD 10A prominence UV/VIS detector, and Shimadzu LC 10 AT pump with LC Solutions software was used (all from Shimadzu Japan) . Separation was performed on a Luna Phenomenex ODS C18 HPLC column, (4.6×250 mm; 5 µm bead size) maintained at 35°C. The pH measurement was carried out by Elico, model LI 120, pH meter equipped with a combined glass-calomel electrode.

2.3. Chromatographic conditions

The HPLC analysis was carried out at 35^0C. The compound was chromatographed isocratically with a mobile phase consisting of methanol (HPLC grade): Acetate Buffer 0.1M (Disodium hydrogen phosphate): (10:90 v/v) with the apparent pH adjusted if required to 5 ± 0.1 using Acetic Acid 0.1 M. The mobile phase was filtered by passing through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA). The flow rate was 1.5 mL/ min, and the injected volume was 20 µL. The effluent was monitored spectrophotometrically at wavelength of 269 nm [22].

2.4. Preparation of standard stock and working solutions.

Heparin rinsed 1 mL syringe was loaded with 0.1 mL of heparin solution, and the anaesthetized rats were selected for the standard curve preparation. Organs as liver, spleen, lung and kidney were removed using scissors and forceps, and weighed accurately. Later they were placed in a separate well of a tissue culture plate containing HEPES buffer in freezer for further studies.

Homogenates of various organs were prepared by homogenizing various organs in methanol. To 1g tissues of organs the corresponding quantity of stock solution of dFdCH ranging from 100-5000 ng/mL was added. The contents were vortexed for 30 sec., and kept aside for 30 min. The contents were then treated with 100 µl of acetonitrile to precipitate, again contents were vortexed for 1 min and 5 mL methanol was added to it. The mixture was extracted (Cole multipulse vortexer, Glas-Col, USA) for 10 min and centrifuged at 3000 rpm for 10 min. The supernatant was decanted into another vial and evaporated to dryness at 60±2 °C. The dried residue was reconstituted with 1 mL methanol and centrifuged at 15000 rpm for 10 min. Clear supernatant was collected in vials and loaded onto the HPLC system (**Figure.1).**

The standard curve data was obtained in the range of 100 ng/mL to 5000 ng/mL(**Table.1)** The HPLC column ($C_{18,}$ Shimadzu, Japan) was washed thoroughly with methanol and then with mobile phase (Methanol/Ammonium acetate buffer; 10:90 v/v; pH 5). The samples starting from blank to increasing concentration were injected and run through the column.

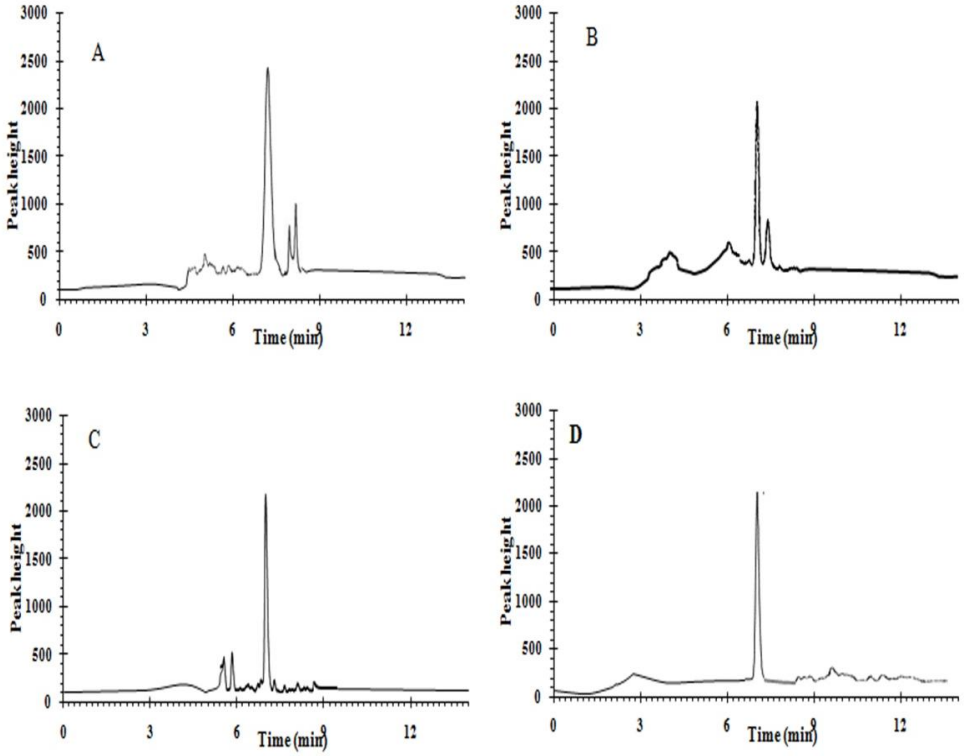


Figure 1: The HPLC chromatograms of dFdCH in (A) kidney, (B) liver, (C), lungs, (D), spleen, in the tissue extracts of Albino rats, 40 min after infusion of 1000ng/mL of dFdCH

Table 1: HPLC standard curve data of dFdCH in different organs

S.N	Organ	Conc. (ng/mL)	Peak area(mm^2)	Regressed values	Retention Time
1	In liver	100	5979	75056	7 min
2		500	10987	24372	
3		1000	61941	54846	
4		2000	113882	115795	
5		3000	180238	176744	
6		4000	239461	23694	
7		5000	297014	298643	
1	In spleen	100	4987	1721	7 min
2		500	11998	24466	
3		1000	67898	57201	
4		2000	126793	122671	
5		3000	193496	188141	
6		4000	259592	252611	
7		5000	309490	319081	
1	In Lungs	100	5578	7852	7min
2		500	11567	26198	
3		1000	65687	59928	
4		2000	131074	127387	
5		3000	195061	194847	
6		4000	262448	262306	
7		5000	328235	329746	
1	In Kidney	100	5765	4169	7min
2		500	18907	31897	
3		1000	70946	66558	
4		2000	141995	135878	
5		3000	209938	205199	
6		4000	279784	274520	
7		5000	335769	331785	

2.5. Biodistribution studies [23], (Figure. 2 a &b)

For biodistribution studies Albino rats were divided into four groups with three rats in each group. Each group was administered with same intravenous dose of the formulation through tail vein (10mg/g body weight). All rats from each group were sacrificed at 2, 8 and 24 hr. The mice organs viz. spleen, kidney, liver and lungs were carefully removed and weighed. Each of these specimens was stored under freeze condition. Weighed tissue samples were immediately homogenized to separate the tissues, vortexed for 30 sec., and kept aside for 30 min. The contents were then treated with 100 µL of acetonitrile, vortexed for 1 min and then added with 5 mL methanol. The mixture was extracted (Superfit vortexer, India) and centrifuged at 3000 rpm for 10 min. The supernatant was decanted into another vial and evaporated to dryness at 60°C. The dried residue was reconstituted with 1 mL methanol then centrifuged at 15000 rpm for 10 min. clear supernatant was collected in vials and analyzed for dFdCH. (**Table. 2)**

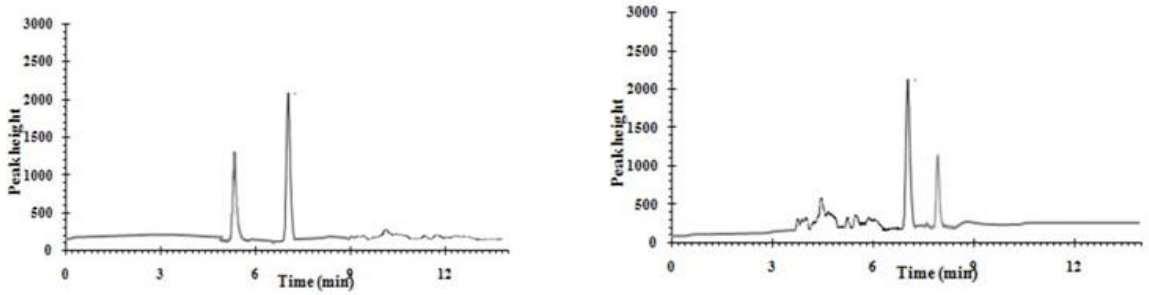


Figure 2a & 2b: Chromatogram of dFdC-M-SLNs and dFdC-SLNs at10 mg/g body weight of rats at 269nm in lungs.

Table 2: Comparision of Calibration Standards of (free Gemcitabine) dFdCH, dFdC-PPI and dFdC –M-PPID using HPLC at λ_{max} 269 nm. in kidney, lungs; Spleen; Liver. At 10 mg/kg body weight of albino rat

	Time (hr)	dFdCH concentration (µg/g tissue)		
Tissues		Free dFdCH (Mean±SD)	dFdCH-SLNs (Mean±SD)	dFdCH- M-SLNs (Mean±SD)
Liver	2	335.5±4.6	204.4±4.61	156.2±4.1
	8	229.6±4.1	104.6±3.2	68.3±4.5
	24	156.2±4.3	43.8±4.1	12.5±3.8
Spleen	2	278.9±3.1	119.8±3.5	90.95±2.1
	8	202.7±3.3	110.6±4.2	87.4±5.7
	24	169.1±3.9	59.6±2.6	79.6±3.1
Lung	2	123.8±4.44	144.2±4.6	378.1±5.7
	8	78.6±5.2	93.2±4.32	245.2±4.5
	24	44.1±3.5	65.3±4.94	155.2±3.3
Kidney	2	193.9±1.2	97.2±5.3	79.3±5.9
	8	78.6±3.2	58.5±4.5	22.7±4.2
	24	54.2±2.6	11.5±1.8	2.7±0.9

2.6. Method validation [24]

The method validation was performed in following ICH guideline according to which the assay validation was performed via various procedures including specificity, linearity, range, accuracy, intra-day and inter-day precision etc. Concentrations of dFdCH entrapped into SLNs and M-SLNs were determined by three calibration curves run over a three week of period to compare the retention of drug .To determine within –run variation, triplicate samples at seven different concentrations of dFdCH were prepared in different tissue extracts and injected on the same day. Between –run variation was determined by injecting triplicate sample prepared at three different concentrations on three separate occasions.

3. RESULT AND DISCUSSION

3.1. High performance Liquid Chromatography

dFdCH, monitored at their optimal UV wavelength at 269 nm based on the UV absorption spectra .To obtain the best chromatographic conditions ,different column, mobile phase with different pH values were tested to provide sufficient selectivity and sensitivity. Chromatographic separation was performed on a C18 reversed phase column with a mobile phase consisting methanol and ammonium acetate buffer (10:90v/v). The homogenized samples of various tissues were deproteinized with acetonitrile. The results were reproducible and the chromatogram showed a good resolution.

3.2. Linearity, selectivity and sensitivity

Calibration curves were plotted between peak area ratios and concentrations of dFdC in tissues extracts of liver, spleen, lung and kidney respectively, the calibration curves were found to be linear over the concentration range of 100-5000 ng/mL(r^2=0.9980, r^2=0.9980, r^2=0.9990, r^2=1 respectively). The calibration curves were obtained by weighed linear regression (weighing factor $1/x^2$) using the Microsoft Excel 2008 software. The limit of quantification (LOQ), the lowest

concentration of the standard curve can be measured with acceptable accuracy and precision for the analyte was 200ng/mL. Limit of quantification (LOD), three times of the value of background noice signals was 60 ng/ml **(Figure. 3).**

Selectivity of the method was established by blank tissues extract spiked at 1:1 of dFdC. No significant interference observed at the retention time in spiked and blank tissue extracts.

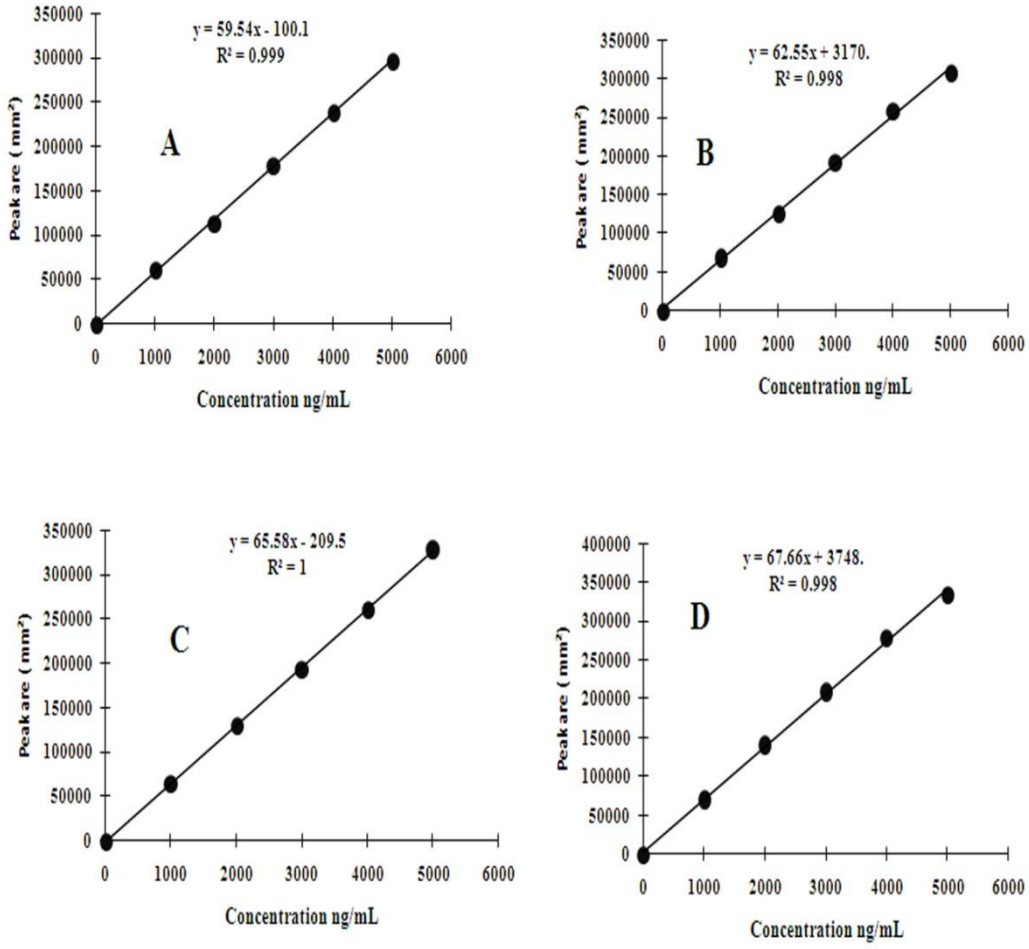


Figure 3: Calibration curves of dFdCH using HPLC at λ_{max} 269 nm. A-lungs; B-Spleen; C-Kidney; D-Liver

3.3. Recovery:

The recovery of dFdCH (pure drug) and dFdCH entrapped SLNs (dFdCH –SLNs, dFdCH -M-SLNs) in various tissue extracts (liver, spleen, lung and kidney respectively) at different concentrations was compared to the same concentration in methanol and ammonium acetate buffer (90:10 v/v). The recoveries of dFdCH, dFdCH-SLNs and dFdCH-M- SLNs were consistent. The overall recovery of all twelve extracts was 76.3%, 78.2%, 76.3%. 78.9% for dFdC, 68.2%, 70.1%, 66.4%, 69%, for dFdC-SLNs, 82.4%, 86.4%,85%, 83.1%, for dFdCH-M-SLNs.

3.4. Stability:

Stock solutions of 100-5000 ng/ml dFdC is stable at -30^{o}C for one month in tissue extracts but stock solutions of formulations at same concentrations in same medium is less stable. They are stable for two weeks at - 40^{o}C.

3.5. Accuracy and Precision

Intra – day and inter – day precision and accuracy were determined at seven concentrations for pure drug and formulations. Intra -day precision was determined by comparing peak heights of each sample on same day (*n=3*). Inter –day precision was determined over a 3-week period.

4. CONCLUSION

In this study a simple and rapid bioanalytical assay method has been developed and validated in various tissue extracts. The validated method was demonstrated to be accurate, precise, selective and sensitive. The method was found to be linear (r^2=0.9980, r^2=0.9980, r^2=0.9990, r^2=1 respectively) within the analytical range of 100-5000 ng/mL. A maximum recovery of the drug from the tissue extracts resulted. The present assay method was carried out with extensive validation parameters as per ICH guidelines. The drug was stable in extracts and developed method was used in biodistribution studies. The present work establishes suitability of mannosylated SLNs to target dFdcH in lungs. Encapsulation of dFdCH in mannosylated SLNs enhances the residence time as well as concentration of drug in lung which could be useful in reducing the dosing frequency as well as dose. This could help in reduction of toxicity associated with this anticancer bioactive.

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