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Sensitive Determination of Gemcitabine Molecule by Novel Magnetic Solid-Phase Extraction Coupled with HPLC-DAD

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ABSTRACT

Gemcitabine (GEM) is a widely employed antimetabolite with broad antineoplastic properties and is commonly administered in the treatment of pancreatic, breast, and ovarian cancers. In this study, a new sample preparation and quantification strategy was established to enable the trace-level determination of GEM. The approach integrates magnetic solid-phase extraction (MSPE) with subsequent HPLC–DAD analysis. Within the method, GEM is initially retained on a magnetic sorbent in a pH 5.0 buffer medium, followed by desorption into a reduced volume of ethanol to facilitate chromatographic measurement. Key experimental parameters particularly adsorption and desorption conditions as well as medium pH were systematically examined and optimized. Analytical performance characteristics, including the linear working range, enrichment factor, limit of detection, accuracy, and repeatability, were assessed using model solutions. Chromatographic detection of GEM was achieved at 277 nm under isocratic conditions composed of 60 % methanol, 25 % phosphate buffer at pH 6.0, and 15 % acetonitrile. Partial validation with model solutions demonstrated a detection limit of 3.43 ng mL^{-1} and an enrichment factor of 100. Overall, the developed method offers high sensitivity, strong reproducibility, and reliable applicability for monitoring GEM at trace concentration levels.

Keywords: Analytical method development, Gemcitabine, HPLC, Magnetic solid-phase extraction

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Introduction

Cancer is defined as the uncontrolled proliferation of abnormal cells in an organ or tissue, leading to over 100 different disease types. Although some cancers are hereditary, most cases result from DNA damage due to environmental factors, mutagen exposure, and replication errors. Early detection plays a crucial role in successful treatment[1]. All types of cancer originate from our body's fundamental units of life in the cells. Healthy cells possess the ability to divide when necessary; however, muscle and nerve cells lack this capacity. Cell division serves primarily to replace dead cells and repair damaged tissues. During the early years of life, cells divide more rapidly, whereas in adulthood, this rate gradually decreases. Nevertheless, this capacity is limited cells cannot divide indefinitely[2–4]. A healthy cell knows when to stop dividing and, when appropriate, undergoes programmed cell death, known as apoptosis[5]. Under normal physiological conditions, cell growth, division, and the production of new cells are tightly regulated processes essential for maintaining the body's proper function. Occasionally, however, this regulation fails, and cells begin to divide even when new cells are not needed. These malfunctioning cells lose control over their growth mechanisms, divide uncontrollably, and proliferate. The accumulation of these excess cells leads to the formation of a tumor[6,7]. Moreover, because cancer is a highly individualized disease and treatment responses vary significantly among patients, it is not possible to define a single, universally effective treatment strategy[8]. Chemotherapy, radiotherapy, and surgical interventions are among the most employed methods in cancer treatment. However, alternative approaches such as hormone therapy and biological treatments may also be used, either alone or in combination with conventional therapies, to enhance therapeutic outcomes. Each of these methods possesses its own advantages and limitations.

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is an important deoxycytidine analogue used as an anticancer drug. Its efficacy has been demonstrated in small-cell lung cancer, pancreatic cancer, breast cancer, ovarian cancer, and bladder cancer[9,10]. Gemcitabine functions as a prodrug that is phosphorylated by deoxycytidine kinase into its active metabolites, difluoro deoxycytidine diphosphate (dFdCDP) and triphosphate (dFdCTP). These metabolites inhibit DNA synthesis by incorporation into DNA, leading to masked chain termination. This unique mechanism, combined with self-potentiation effects, makes gemcitabine highly effective in cancer treatment[11,12]. Molecular structure of Gemcitabine HCl was given in Figure 1.

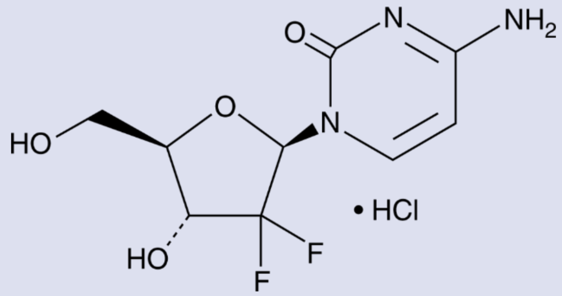

Figure 1. Molecular formula of Gemcitabine Hydrochloride

Several studies have demonstrated highly sensitive HPLC-based methods for gemcitabine quantification, often combining sample extraction techniques to achieve low detection limits. For example, Lafazanis et al. developed a straightforward HPLC-UV protocol in mouse serum using a single protein precipitation step; they reported a limit of detection (LOD) of 0.166 µM (approximately 45 ng/mL), with recovery around 96.5 % [13]. In a different approach, Soni et al. validated an RP-HPLC assay for gemcitabine loaded in nanosized solid lipid nanoparticles. Their extraction utilized methanol, achieving a linear range from 100 to 5000 ng/mL [14]. Stability-indicating methods have also been reported: Singh et al. described an RP-HPLC method for injectable formulations using a C18 column, with an LOD of 0.1498 µg/mL (≈150 ng/mL) and LOQ of 0.4541 µg/mL [15]. Additionally, Shaik et al. proposed a gradient RP-HPLC method to assess impurities in gemcitabine API, using a C18 phase and achieving acceptable LOD/LOQ values during validation[16]. These methods have been successfully applied to various sample types, including plasma, serum, nanoparticle formulations, and raw drug, illustrating both the versatility and high analytical sensitivity of HPLC for gemcitabine detection.

The quantification of drug molecules at trace levels in biological matrices or pharmaceutical formulations has become a central component of contemporary analytical science. Many therapeutic agents exert significant pharmacological effects even at very low concentrations, making high sensitivity and selectivity essential for any analytical approach. Biological matrices such as plasma, whole blood, or urine contain numerous endogenous substances that may interfere with detection[17,18]. Additionally, metabolic transformations and protein binding can alter the measurable fraction of the drug, creating further challenges for accurate quantification. For these reasons, the development of analytical methods capable of reliably detecting trace concentrations is critical for obtaining meaningful pharmacokinetic data[19].

From a therapeutic standpoint, monitoring drugs at very low levels contributes directly to patient safety. Medications with narrow therapeutic windows may show clinically relevant effects when their concentrations shift even slightly[20]. Detecting sub-therapeutic or residual concentrations helps clinicians evaluate adherence, adjust dosing regimens, and prevent cumulative toxicity. Advanced chromatographic systems combined with mass spectrometric detection have therefore become indispensable tools in clinical and pharmaceutical laboratories[21–23]. In the context of pharmaceutical products, trace-level analysis serves as a fundamental quality control requirement. Degradation products, impurities arising from synthesis, or contaminants introduced during manufacturing may appear in extremely low amounts yet still influence the overall stability or safety of the final formulation[24]. Monitoring these trace compounds not only supports regulatory compliance but also ensures that good manufacturing practices are consistently upheld. In summary, the ability to measure drug molecules at trace levels is vital across multiple domains, including therapeutic monitoring, toxicological evaluation, pharmacokinetic research, and product quality assurance. Each improvement in sensitivity and specificity enhances both patient welfare and the scientific reliability of pharmaceutical analysis[17,25]. Solid phase extraction (SPE) based methods are useful for sensitive analysis of organic molecules thank to functional materials used as adsorbent[26,27]. The materials in SPE can be modified by considering molecular structure of target molecules. Thus, these methods are preferred for sample preparation before analysis step. The aim of this study was to develop and optimize a sensitive, reliable, and cost-effective method for the determination of gemcitabine molecule using magnetic solid-phase extraction (MSPE) combined with HPLC-DAD.

Materials and Methods

Chemicals

All chemicals employed in this study were of analytical grade and obtained from Sigma-Aldrich or Merck. Ultrapure water (18.2 MΩ·cm resistivity), produced by an MP Minipure Destup purification system (Türkiye), was used to prepare all solutions immediately prior to use. A Britton–Robinson buffer (0.05 M), composed of H_3BO_3, H_3PO_4, and CH_3COOH, was utilized to adjust the pH within the range of 2.0–11.0. A gemcitabine stock solution (1000 mg L^{-1}) was prepared by dissolving 50 mg of GEM in 50 mL of methanol and stored at +4 °C in amber glass containers to prevent photodegradation. A model gemcitabine solution with a concentration of 50 mg L^{-1} was used as the working standard throughout the experiments.

Instruments

Chromatographic analyses were performed on a Shimadzu Prominence HPLC system equipped with an LC-20AD quaternary pump, an SPD-M20A photodiode array detector, a DGU-20A degasser, and a CTO-10AS VP column oven. Separation was achieved using a reversed-phase C18 column (Inertsil ODS-3, 250 × 4.6 mm, 5 µm). Data acquisition and processing were conducted with LC Solution 2.0 software. Additional laboratory instrumentation included a Mettler Toledo pH-2005 pH meter, a Jeotech vortex mixer (Korea), an SK5210HP ultrasonic bath, and a WhyShake SHO-2D orbital shaker.

HPLC determination conditions

Optimization of direct determination for Gemcitabine molecules was studied and determined before magnetic solid phase extraction determinations. The best results were obtained with a mobile phase composed of methanol, trifluoroacetic acid, and acetonitrile. A conventional C-18 HPLC column was preferred with easy accessibility. Calibration standards were prepared in the range of 2.0–50.0 µg mL^{-1}. The optimized HPLC parameters were shown in Table 1. The chromatogram obtained from model solutions of Gemcitabine molecule were shown in Figure 2.

Table 1. HPLC conditions for direct determination of GEM

Parameter	Value
Mode	Isocratic
Eluent	Methanol: TFA (0.1 %): Acetonitrile (20:40:20)
Flow rate	1.0 mL min^{-1}
Run time	12 min
Column	Inertsil ODS-3, 250 × 4.6 mm, 5 µm
Column temperature	45°C
Injection volume	10 µL

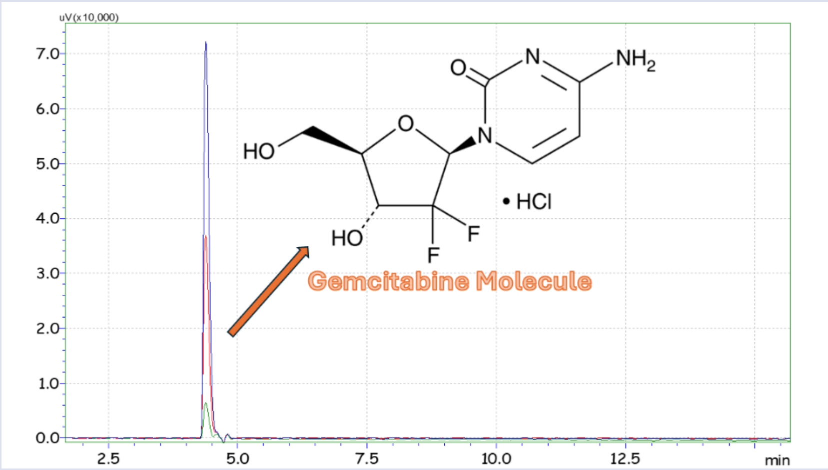

Figure 2. Chromatogram of GEM obtained via HPLC-DAD system

Synthesis of the Solid-Phase Support Material

A well-established synthesis procedure, previously applied in our earlier studies and widely reported in literature[28–30], was employed for the preparation of the magnetic support material. The magnetic material used was characterized with all details in our previous study[31].

In summary, 0.745 g of $FeCl_3 \cdot 6H_2O$ and 0.383 g of $FeSO_4 \cdot 4H_2O$ were first dissolved in 50 mL of 0.001 M HCl to obtain a clear precursor solution. This solution was subsequently transferred into 100 mL of 50% methanol under vigorous stirring at 600 rpm and heated to 85 °C under a nitrogen blanket to maintain an oxygen-free environment. While stirring continued, 20 mL of ammonia solution was introduced dropwise, triggering the coprecipitation of magnetite (Fe_3O_4) nanoparticles. The formation of a dark suspension signaled successful nanoparticle production. The magnetite particles were separated using an external magnet, rinsed several times with a water–alcohol mixture to remove residual ions, and then dried in an oven at 60 °C for 6 hours. For subsequent surface functionalization, 1 g of the dried Fe_3O_4 powder was dispersed in 80% ethanol, to which approximately 1 mL of concentrated ammonia was added. Tetraethyl orthosilicate (TEOS) was then introduced to generate a silica shell around the magnetic cores. Building on previous laboratory experience and supporting literature, an additional modification step was performed by adding 3 mL of Tergitol to improve the interaction of the magnetic adsorbent with the target analytes. The mixture was stirred at room temperature for 6 hours, after which the functionalized particles were rinsed repeatedly with an ethanol–water mixture and allowed to dry under ambient conditions.

Proposed Method

The experimental conditions were optimized with respect to pH, vortex time, solvent type, and solvent volume to achieve maximum extraction efficiency. For the extraction procedure, 50 mg of the solid-phase support material was accurately weighed and transferred into a 50 mL Falcon tube. Subsequently, 20 mL of deionized water and 1 mL of pH 5.0 buffer were added to tubes. A 1 mL aliquot of the sample solution containing gemcitabine (10.0–600.0 ng mL^{-1}) was then introduced, and the volume was adjusted to 50 mL with deionized water. The tube was tightly sealed and rotated at 50 rpm for 40 min to ensure effective adsorption of the analyte onto the sorbent surface. After magnetic separation, the aqueous phase was removed, and 500 µL of ethanol was added to desorb the retained analyte. The mixture was vortexed for 50 s, and the magnetic sorbent was collected via an external magnet. The separated ethanol phase was filtered through a 0.45 µm syringe filter, transferred into HPLC vials, and analyzed under the optimized chromatographic conditions. This procedure enabled the efficient enrichment and reliable quantification of gemcitabine using the proposed HPLC method.

Results and Discussion

The primary objective of the prosed method is to achieve maximum retention of the target analyte on the solid phase to ensure effective separation from other matrix components, followed by complete desorption of the analyte onto magnetic sorbent phase. Preliminary experiments were conducted to identify the optimal conditions required to achieve these outcomes. The method was designed to enable rapid and straightforward separation while minimizing the use of organic solvents and maximizing analyte enrichment. This approach effectively concentrated gemcitabine to levels detectable by the HPLC system. By systematically optimizing all critical parameters, a robust and efficient chromatographic method was established for the accurate quantification of gemcitabine.

Optimization of the Developed Method

Effect of pH

The pH of the medium plays a crucial role in the extraction process, as it directly influences the interaction between the analyte and the solid phase by affecting the ionization state of the analyte and the surface chemistry of the sorbent. To determine the optimal pH, enrichment experiments were conducted using Britton–Robinson (BR) buffer solutions in the pH range of 2.0–10.0. According to the results, the most efficient retention of the analyte on the solid phase was achieved at pH 5.0, indicating favorable electrostatic and hydrophobic interactions under these conditions. The corresponding results are presented in Figure 3.

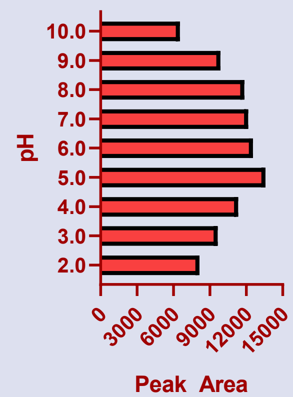

Figure 3. The effect of pH on MSPE of Gemcitabine molecule

Effect of Adsorption Time

The adsorption time is a key parameter influencing the transfer of the analyte from the sample matrix to the solid phase. Efficient enrichment requires sufficient contact between the sorbent and all components of the solution to ensure maximum analyte retention. In this study, nine identical sample solutions were prepared, keeping all experimental conditions constant except for the shaking time. The adsorption period varied between 0 and 90 minutes. The results showed that the highest peak intensities were obtained when the shaking time was set to 40 minutes, indicating complete adsorption equilibrium under these conditions. The corresponding results are presented in Figure 4.

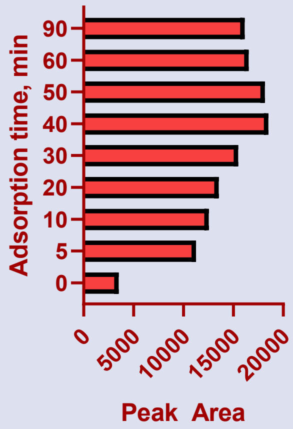

Figure 4. The effect of adsorption time on MSPE of Gemcitabine

Selection of Desorption Solvent and Volume Optimization

The choice and amount of solvent are critical parameters for the efficient description of analytes retained on the solid phase, enabling their transfer into the liquid phase for HPLC analysis. The selected solvent should provide distinct and well-resolved chromatographic peaks, effectively elute the analyte from the sorbent surface, and ensure high solubility of the target compound. The results obtained are presented in Figure 4. For this purpose, a range of commonly available laboratory solvents—methanol, ethanol, 2-propanol, acetonitrile, distilled water, acetone, n-hexane, acetonitrile: methanol (1:1), pH 5.0 buffer, and 50% methanol—were evaluated under identical experimental conditions, with solvent type as the only variable. Among the tested solvents, ethanol yielded the most intense and well-defined chromatographic peaks, indicating superior desorption efficiency under the optimized conditions. The corresponding results are shown in Figure 5.

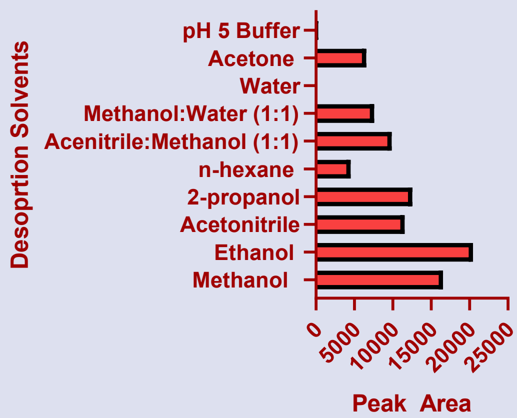

Figure 5. The effect of desorption solvent on MSPE of Gemcitabine

After determining the most suitable solvent, the solvent volume was optimized to obtain the highest and most well-defined chromatographic peaks. Enrichment experiments were performed in nine separate tubes using ethanol volumes ranging from 200 to 1250 µL, while keeping all other parameters constant.

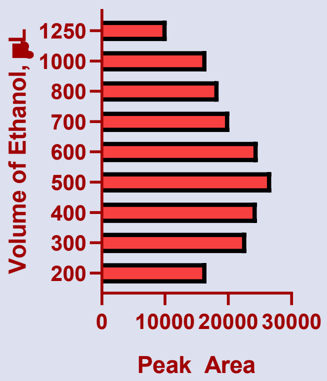

Figure 6. Optimization for volume of desorption solvent

The best peak intensity was observed with 500 µL of ethanol, indicating optimal desorption efficiency under these conditions. It was also noted that increasing the solvent volume led to dilution of the analyte and consequently reduced signal intensity. Therefore, the optimal solvent type and volume were determined as 500 µL of ethanol. The corresponding results are presented in Figure 6.

Effect of Desorption Time

To determine the influence of vortex time on the dissolution of the analyte in the solvent phase, nine identical enrichment experiments were conducted, varying only the vortex duration between 0 and 90 seconds. All other experimental parameters were kept constant. The results indicated that the most intense and well-defined chromatographic peaks were obtained with a vortex time of 50 seconds, suggesting that this duration was sufficient to achieve complete desorption of the analyte from the solid phase. The corresponding results are presented in Figure 7.

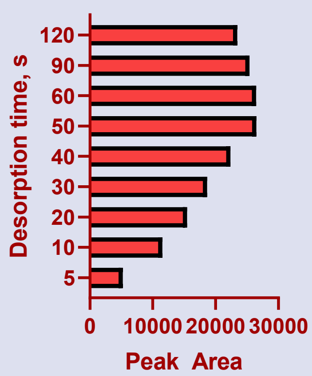

Figure 7. The effect of desorption time on MSPE of Gemcitabine

Analytical Performance Properties of the Developed Method

After optimizing the experimental conditions for the solid-phase extraction procedure, enrichment studies were performed using gemcitabine solutions at various concentrations to establish the linear working range of the method. The results demonstrated that the analytical response exhibited a linear correlation with gemcitabine concentration between 10 and 600 ng mL^{-1} at 277 nm. The calibration curves generated from these measurements using the proposed method are illustrated in Figure 8. In addition, the enrichment step produced a clear proportional increase in signal intensity with rising analyte concentration. Enrichment factor; was derived from the ratio of the initial aqueous phase volume (50 mL) to the final volume after enrichment (0.5 mL). The enhancement factor was obtained by comparing the slopes of the calibration curves before and after the enrichment process. A comprehensive summary of all analytical performance characteristics of the developed method is provided in Table 2.

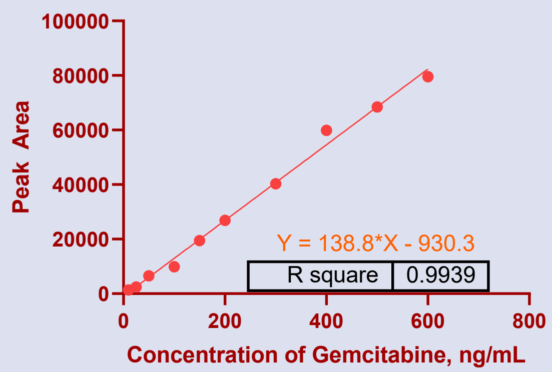

Figure 8. Calibration plot for gemcitabine molecules after MSPE

Table 2. Analytical performance parameters of the proposed method

Parameter	After MSPE	Direct HPLC Determination
Linear range	10.0–600.0 ng mL^{-1}	1.0–20.0 µg mL^{-1}
LOD	3.43 ng mL^{-1}	0.35 µg mL^{-1}
LOQ	9.71 ng mL^{-1}	0.89 µg mL^{-1}
RSD (%)	3.2	4.5
Enrichment factor	100	-
Enhancement factor	86	-

Conclusion

A novel MSPE-HPLC-DAD method was successfully developed for the sensitive determination of gemcitabine. The method offers significant advantages such as high sensitivity, low solvent consumption, and rapid sample preparation compared to conventional techniques. The enrichment factor of 100 and low detection limit (3.43 ng mL^{-1}) highlight its potential for trace-level analysis. This method can be applied for pharmaceutical quality control studies. Solid-phase extraction (SPE), a separation and purification technique that emerged in the 1970s, is a widely used, rapid, simple, and sensitive sample preparation method. Owing to these advantages, it has become a frequently preferred technique, and hundreds of studies encompassing a wide range of applications are published in the literature each year. In analytical procedures, it is generally desired to minimize the use of organic solvents, achieve rapid and cost-effective analysis, simplify sample preparation, reduce time consumption, and obtain efficient results while employing environmentally friendly solvents. The main objective of the solid-phase extraction method, which meets these criteria, is to extract analytes—originally dissolved in a solvent medium—onto a solid phase, and subsequently transfer them into a smaller volume of solvent phase, thereby achieving preconcentration. In this way, the concentration of analytes that are initially present below the detection limits of the analytical instruments can be increased to measurable levels, enhancing the sensitivity and detectability of the analysis.

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Conflict of Interest

The authors stated that they did not have conflict of interest.

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