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The Rapid Determination of Gemcitabine by Reversed-phase Ultra-Performance Liquid Chromatography

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Gemcitabine is an anticancer drug used to treat a variety of solid tumors. The drug is rapidly inactivated by cytidine deaminase in plasma and its hydrophilicity restricts the extent of quantification that is possible using reversed-phase liquid chromatography. In this paper, we report a rapid and precise method to analyze velocity and peak efficiency using ultra-performance liquid chromatography (UPLC) with a reversed-phase column. The retention periods of gemcitabine and 2′-deoxycytidine at 283 nm were 3.2 and 2.1 min, respectively. The assay provided highly linear results in the range of 0.1~20 μg/ml (r^2>0.999). The coefficients of variation of the intra-day and inter-day assays were less than 10.0%. We observed that the estimated average concentrations of the intra-day and inter-day assays ranged from 97.3 to 113.5% to verify the accuracy. These results suggest that this new reversed-phase UPLC method is a rapid and reliable way of determining gemcitabine levels.

Key words : Gemcitabine, ultra-performance liquid chromatography, anticancer drug

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine), a cytidine nucleosidic analogue, is an anticancer drug used to treat a variety of solid tumors with unusually high metabolisms. Gemcitabine is used to treat non-small cell lung, breast, ovarian, bladder, and pancreatic cancers, as well as head and neck squamous cell carcinoma [1,4]. Nucleoside analogues include therapeutically diverse families, such as cytotoxic compounds, antiviral agents, and immunosuppressive molecules. Anticancer nucleoside drugs are divided into pyrimidine analogues (gemcitabine and cytarabine) and purine analogues (fludarabine and cladribine), illustrated in Fig. 1. Gemcitabine has 2′,2′-difluoro groups in a ribose moiety as a pyrimidine analogue [6]. Because it is hydrophilic like other nucleoside analogues, gemcitabine requires specialized nucleoside transporter proteins to enter the cell. After entry, it is converted into 2′,2′-difluorodeoxycytidine triphosphate (dFdCTP) and exhibits cytotoxic activity through intracellular metabolic phosphorylation by deoxycytidine kinase in the salvage pathway. Through phosphorylation, gemcitabine retains the nucleotide residues in the cell and incorporates itself into the DNA of cancer cells undergoing DNA synthesis (S-phase). Finally, it interferes with various enzymes involved in the synthesis of nucleic acids, or modifies the metabolism of physiological nucleosides [3]. Standard doses of anticancer drugs manipulated by the body surface area (BSA) show that systemic exposure can vary up to 5.8- fold among patients [12]. Furthermore, previous studies have shown that individualized dosing by BSA, calculated using height and weight, was still inadequate [13]. To decrease inter-individual pharmacokinetic differences, each dosage should be pharmacokinetically tailored to the individual patients (individualized dosing). Although pharmacokinetic parameters for many chemotherapeutic drugs have been shown to be related with treatment outcomes or toxicity, high pharmacokinetic variability based on BSA can make it difficult to predict responses and can result in an increased risk of intoxication and subtherapeutic dosing. Like many anticancer agents, gemcitabine has high inter-individual pharmacokinetic variability and a narrow therapeutic index. In particular, gemcitabine exhibits a very short exposure duration because of its rapid catabolism to 2′-deoxydifluorouridine by cytidine deaminase in plasma and liver [12]. Although differences in individual genotypes among ethnic groups have been considered to be the cause of gemcitabine's pharmaockinetic variability, few studies have focused on the pharmacokinetics and pharmacodynamics of gemcitabine specifically with regard to the treatment of Korean patients.

Before exploring pharmacokinetically guided dosing, we

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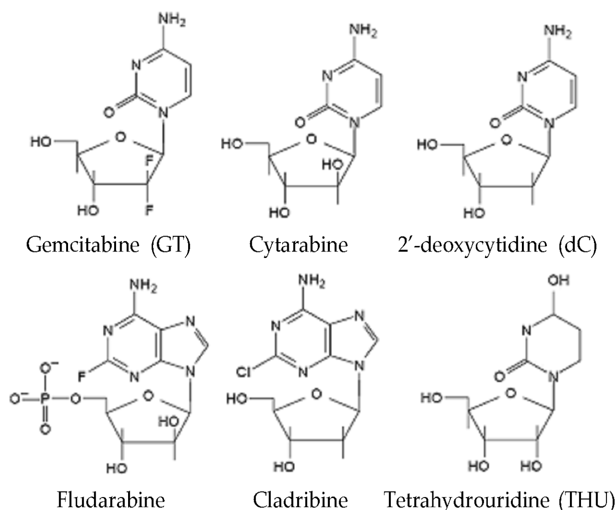

Fig. 1. Structures of active nucleoside analogues.

explain the method of analyzing gemcitabine levels in human blood or tissues. Until recently, gemcitabine levels have been determined by high-pressure liquid chromatography (HPLC) [2,8,10,11,16]. However, HPLC's major limitation lies in its low efficiency, especially when compared to gas chromatography and capillary electrophoresis [15,18]. Recently, highly sensitive and precise tandem mass spectrometry coupled with HPLC (LC/MS/MS) is increasingly used to determine gemcitabine levels [5,14]. However, LC/MS/MS requires very expensive equipment and highly skilled operators. More recently, new commercial HPLC systems, such as the ultra-performance liquid chromatography (UPLC) system, have been developed that are capable of generating pressures of up to 1000 bar (14,500 psi); these systems are more efficient and precise than conventional HPLC systems [17,18]. A typical UPLC system employs particles with diameters of 1.7 μm under pressures of up to 1,000 bar. In comparison, conventional liquid chromatography (LC) only uses particles with diameters of 3.5 or 5.0 μm under pressures of up to 400 bar. In addition, several reports demonstrate the application of UPLC coupled with mass spectrometry in pharmaceutical analysis [17]. Although UPLC has been shown to be fast and highly efficient, there are very few reports demonstrating UPLC as an alternative to conventional HPLC analysis. Therefore, this study aims to develop a quick and more sensitive UPLC method that is capable of determining gemcitabine levels in plasma samples and that is applicable to pharmacokinetic study, including therapeutic drug monitoring (TDM).

Methods

Chemicals and reagents

Gemcitabine was supplied by Eli Lilly and Company (Indianapolis, IN, USA) in a vial that was composed of 1.14 g gemcitabine hydrochloride and other additives (mannitol, sodium acetate, etc.). 2′-deoxycytidine, used as an internal standard, was purchased from Fluka (Buchs, Switzerland), tetrahydrouridine and triflic acid (trifluoromethanesulfonic acid) was obtained from Sigma (St. Louis, MO, USA), and acetonitrile, methanol, ethyl acetate, isopropanol, and all other reagents were obtained from Merck (KGaA, Darmstadt, Germany). All solvents were of high analytical grade and suitable for HPLC. Human blank plasma was obtained from healthy male volunteers. Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Instruments and chromatography

Gemcitabine levels were determined with high separating efficiency at high linear velocities using the new UPLC system, known as the Waters Acquity ultra-performance^{TM} LC. The system was equipped with a binary solvent delivery manager, an auto-sample management system, a column-heating compartment, and a tunable UV detector. A column packed with small-diameter particles was used with the Waters Acquity UPLC BEH C18 column (100 mm long, ID 2.1 mm, 1.7 μm particle size, Milford, NJ, USA). This system was controlled by Waters Empower soft-ware (version 2.0). Column and sample temperatures were maintained at 25°C and the operating pressure of the UPLC system was set at 6,800±20 psi (469 bar). UV absorbance data were collected at 283 nm. The mobile phase was eluted in an isocratic manner, which consisted of 5% methanol and was adjusted to pH 2.9 with triflic acid. The flow rate was set at 0.25 ml/min.

Preparation of standard solutions and quality controls

Stock solutions of gemcitabine and 2′-deoxycytidine (2dC) at a concentration of 1 mg/ml were prepared with 50% methanol and were kept at -70°C. Working solutions (10-fold standard solutions) were obtained by serial dilutions of stock solutions at the following concentrations: 1, 2, 10, 50, 100, and 200 μg/ ml. A 10-fold working solution of 2dC (30 μg /ml), used as an internal standard, was also prepared with 50% methanol, of which a 10 μl volume was spiked to 100

μl standard solutions. Standard solutions of 100 μl volume were prepared by mixing 90 μl plasma with each 10 μl working solutions to achieve final concentrations of 0.1, 0.2, 1, 5, 10, and 20 μg/ml. Each 5 ml human plasma was pretreated by mixing with 40 μl of 10 mg/ml tetrahydrouridine (THU), an inhibitor of cytidine deaminase, prior to the addition of gemcitabine. After the addition of 1.5 ml extraction solvent (5:1, v/v, ethyl acetate:isopropanol) to the standard solutions in a 2 ml mini-Eppendorf tube, the mixture was vortex-mixed for 5 min and centrifuged at 13,000 rpm for 10 min. A 1.4 ml volume of the supernatant was transferred to another tube and evaporated to dryness in a Centrivap mobile system (Labconco, Kansas, MO, USA). The residue was reconstituted in a 100 μl mobile phase, filtered through a 0.22 μm PVDF filter in a 1 ml syringe (Norm-ject) with a Luer-lock tip, and a 3 μl volume of the filtrate was injected into the autosample management system of the UPLC system. The 0.2, 5, and 20 μg/ml concentrations of the quality control (QC) samples were prepared using the same method described above.

Validation of the method

According to the guidelines of the Korean Food and Drug Administration (KFDA), the method was validated for selectivity, limit of quantification, linearity, accuracy, precision, sensitivity, extraction recovery and stability [7]. All test samples were prepared with human plasma treated with THU, which is an inhibitor of cytidine deaminase. All concentrations were calculated from the response obtained with the peak area ratio of gemcitabine to 2′-deoxycytidine per sample. Selectivity was assessed by analyzing six blank plasma samples obtained from healthy volunteers and the signal-to-noise (S/N) ratios for the retention times of analytes were analyzed. For the lower limit of quantification (LLOQ), which is the lowest standard concentration among the quantifiable concentrations of the calibration curve, we selected more than five values of the S/N ratio. Linearity was analyzed as the correlation coefficient (r^2) after the calibration curves were plotted with five copies of standard solutions in the following concentrations: 0.1, 0.2, 1, 5, 10, and 20 μg/ml. Accuracy and precision were determined by obtaining five copies of QC sample concentrations (0.2, 5, and 20 μg/ml) for the intra-day assay and five copies over 5 consecutive days for the inter-day assay. The accuracy of the method was described by the percentage of mean concentration of gemcitabine [$(C_{Mean\ estimated}/C_{nominal})\times100\%$]. The precision was described using the coefficient of variation (CV, %), or the percentage of the standard deviation to the mean concentration of each QC sample. Recovery experiments were performed with five copies of the QC samples by comparing the mean concentration of gemcitabine that passed through the extraction preparation from human plasma with the concentration of directly injected aqueous standard solution. As a control, unextracted standards represented 100% recovery.

In addition to the above methods of validation, the short-term temperature stability and post-preparative stability of gemcitabine in human plasma were tested. All stability determinations used three copies of the low- and high- concentration QC samples (0.2, and 20 μg/ml, respectively) prepared from freshly made stock solution in the previous analytes-free, interference-free, and THU-pretreated human plasma. The short-term temperature stability was determined after three aliquots of each of the low and high concentrations had been kept at room temperature for 6 h. The post-preparative stability was determined after extraction-processed samples had been kept in the auto-sampler for the resident time of 24 hr. The short-term temperature stability and the post-preparative stability were defined as the percentage of determined mean concentration to the nominal concentration (known amounts) of gemcitabine.

Influence of tetrahydrouridine on the stability: Incubation study

For the sample not pretreated with THU, the stabilities of gemcitabine and 2′-deoxycytidine in human plasma were tested. The human plasma samples with gemcitabine (20 μg/ml) and 2′-deoxycytidine (3 μg/ml) were kept at room temperature in the same condition as the short-term stability samples and analyzed at 0, 2, 4, and 6 hr after incubation. The samples were then compared with peak areas.

Results and Discussion

Specificity and linearity

Fig. 2 shows the chromatograms of blank, plasma spiked with 2dC (3 μg/ml), calibration standard plasma spiked with 2dC (3 μg/ml), and gemcitabine (5 μg/ml). The retention times of 2dC and gemcitabine were about 2.1 and 3.2 min, respectively. No interfering peaks of endogenous plasma components in blank plasma samples were detected at the retention times for 2dC and gemcitabine. Furthermore, the

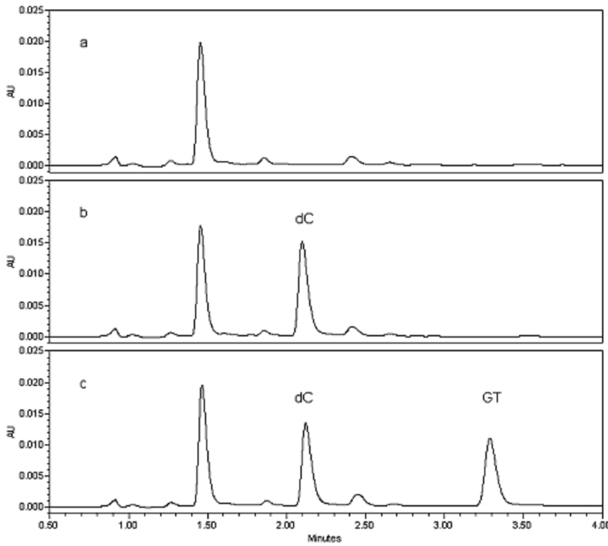

Fig. 2. Chromatogram for specificity. (a) Blank; (b) Blank spiked with 2′-deoxycytidine; (c) Blank spiked with 2′-deoxycytidine and gemcitabine. GT: gemcitabine (5 μg/ml), dC: 2′-deoxycytidine (3 μg/ml).

assay was linear over the concentration range (0.1, 0.2, 1, 5, 10, and 20 μg/ml). Excellent linear regressions were obtained for gemcitabine with correlation coefficients (r^2) of more than 0.999 (n=5). The regression equation for gemcitabine was y=0.4986 x+0.02454 ($r^2>0.999$, $p<0.01$).

Studies using the conventional HPLC method have reported a total running time of 40 [2,11], 30 [16], 15 [8], and 17 min [10]. However, the new UPLC method allows one to perform the same analysis in 10 min with a high separation efficiency. A reversed-phase HPLC analysis of gemcitabine is generally not effective because gemcitabine has hydrophilic characteristics. As a result, many endogenous hydrophilic components in human plasma elute together with gemcitabine during the early phase and hinder separation analysis. Therefore, Freeman and Yilmaze [2,19] adopted a normal phase column as opposed to the reverse phase column, frequently used for current pharmaceutical analysis. Freeman allowed an additional 20 min of elution time to obtain more strongly retained endogenous interferences. The validated UPLC method that we report in the current publication employs additional time for gradient elution (from 5% to 80% methanol for 2 min) and equilibrium (5% methanol for 2 min) to protect the column from contamination by hydrophobic endogenous interferences. According to previous reports, the retention times of gemcitabine were 18 [2], 24 [16], 16.5 [11], 9.1 [8] and 5.2 min [10]. However, in the current study, gemcitabine and 2dC are eluted more

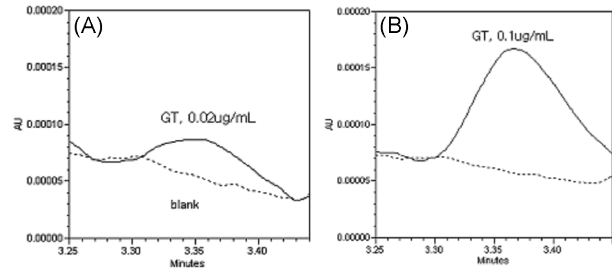

Fig. 3. LOD (A) and LLOQ (B) of gemcitabine. GT: gemcitabine.

quickly without other components overlapping. In this UPLC system, the retention times for gemcitabine and 2dC were found to be around 3.3 and 2.1 min, respectively.

Limit of detection (LOD) and lower limit of quantification (LLOQ)

The limit of detection (LOD) was defined as the concentration with a peak height of more than three times that of the blank plasma at the same retention time. The concentration level of the LOD was 0.02 μg/ml because the S/N ratio (the peak height ratio) was 5.8 (Fig. 3). The LLOQ was defined as the lowest standard on the calibration curve that was at least five times the peak height of the blank plasma with a precision of below 20% (CV, %) and an accuracy interval between 80 and 120%. The LLOQ was 0.1 μg/ml for an S/N ratio of 21 and a CV of less than 5%.

Recovery

As summarized in Table 1, the mean recovery ratios of gemcitabine at concentrations of 0.2, 5, and 20 μg/ml were 73.5, 75.9 and 74.2%, respectively. The internal standard (2dC) had a 62.7% recovery ratio. For all the concentrations studied, the CV values for the QC plasma samples were <10%.

Precision and accuracy

Table 2 summarizes the precision and accuracy data of

Table 1. Recovery ratios of gemcitabine and 2′-deoxycytidine after sample preparation

	QC* nominal concentration (μg/ml)		Mean±S.D.† (%)	CV‡ (%)
Gemcitabine (Standard)	Low	0.2	73.5±4.0	5.5
	Middle	5	75.9±3.8	4.9
	High	20	74.2±0.8	1.0
2′-deoxycytidine (IS)§	3		62.7±0.7	1.1

*Quality control samples, †Standard deviation.
‡Coefficient of variation, §Internal standard.

Table 2. Intra-day accuracy and precision from the determination of gemcitabine

QC* nominal concentration (μg/ml)		Mean±S.D.† (*n*=5)	Accuracy‡ (%)	Precision (CV, %)§
Low	0.2	0.202±0.004	101.1	2.0
Middle	5	5.677±0.049	113.5	0.9
High	20	21.779±0.233	108.9	1.1

*Quality control samples, †Standard deviation.
‡(Estimated mean concentration/QC nominal concentration)×100.
§Coefficient of variation, (S.D/mean)×100.

Table 3. Inter-day accuracy and precision from the determination of gemcitabine

QC* nominal concentration (μg/ml)		Mean±S.D.† (*n*=5)	Accuracy‡ (%)	Precision (CV, %)§
Low	0.2	0.195±0.006	97.3	3.3
Middle	5	5.147±0.462	102.9	9.0
High	20	20.696±0.890	103.5	4.3

*Quality control samples, †Standard deviation.
‡(Estimated mean concentration/QC nominal concentration)×100.
§Coefficient of variation, SD/mean×100.

the intra-day assay and Table 3 shows the corresponding data for the inter-day assay. The concentrations of tested QC samples were 0.2, 5, and 20 μg/ml. The CV of the intra-day assay ranged from 0.9 to 2.0%, and that in the inter-day assay ranged from 3.3 to 9.0%. With regard to the accuracy, the concentrations for the intra-day assay are in the range of 101.1~113.5%, while those for the inter-day assay are in the range of 97.3~103.5%.

Stability

In the case of the sample plasma pretreated with THU, gemcitabine and 2dC were determined to be highly stable in the short-term temperature stability and post-preparative stability tests. After three aliquots of each concentrations had been kept at room temperature for 6 hr and extracted, the concentration levels in the short-term temperature stability test were determined to be 95.2% (0.178±0.002 μg/ml) for the low concentration (0.2 μg/ml) and 97.1% (19.389±0.223 μg/ml) for the high concentration (20 μg/ml). The concentration levels in the post-preparative stability test were determined to be 112.1% (0.213±0.014 μg/ml) for the low concentration and 98.5% (19.827±0.134 μg/ml) for the high concentration, after 24 hr in the auto-sampler following extraction.

Influence of tetrahydrouridine on the stability

Fig. 4 showed the stability of the gemcitabine and 2′-deoxycytidine samples that were, not pretreated with THU, according to the different incubation time (0, 2, and 4 hr). The peak areas of gemcitabine (20 μg/ml) were reduced from 215868 (100%) to 14510 (67%) and 99969 (46%) at 2 and 4 hr, respectively. For 2′-deoxycytidine, the peak areas were reduced from 61728 (100%) to 3305 (5%) after 4. However, P2, an unknown peak, increased from 7187 (100%) to 18953 (264%) and 20166 (281%) at 2 and 4 hr, respectively.

The gemcitabine and 2dC samples were detected at 200-500 nm by examining the UV spectra of the analytes dissolved in 5% methanol (pH 2.9) (Fig. 5). No THU absorption maximum is observed because its structure does not include a chromophore. Solvent absorbance was confounded at near 200 nm (data not shown). The absorbance maxima of gemcitabine and 2dC are close to 274 nm. However, this method employs 283 nm excitation because the background absorbance of human plasma is low at this wavelength and the separation efficiency is high.

The newly developed UPLC system, which uses particles

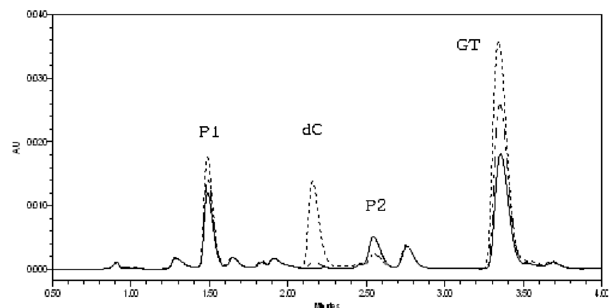

Fig. 4. Comparative stability according to the different incubation time without the treatment of THU. The short-dashed line gives the incubation for 0 hr, long-dashed line gives the incubationfor 2 hr, and solid line gives the incubation for 4 hr. P1, P2 refer to two unknowm peaks, GT indicates the gemcitabine (5 μg/ml) peak, and dC indicates the peak due to 2′-deoxycytidine (3 μg/ml).

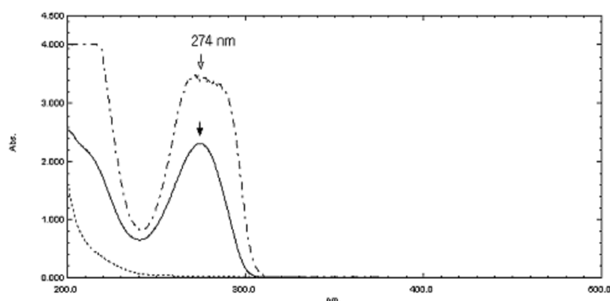

Fig. 5. Absorbance spectra of gemcitabine, 2′-deoxycytidine and tetrahydrouridine. Gemcitabine (solid line); 2′-deoxycytidine (dotted-dashed line); tetrahydrouridine (dotted line).

Table 4. Post- and pre-preparative stability from the determination of gemcitabine (n=5 for each concentration)

QC* nominal concentration (μg/ml)	Estimated concentration (μg/ml)				
	QC references	Post-preparative (for 24 hr)		Pre-prepatative (for 6 hr)	
		Mean±S.D.†	Stability‡ (%)	Mean±S.D	Stability (%)
0.2	0.190	0.213±0.014	112.1	0.178±0.002	95.2
20	20.132	19.827±0.134	98.5	19.389±0.223	97.1

*Quality control samples, †Standard deviation, ‡Estimated mean concentration/QC nominal concentrtion

with diameters of 1.7 μm, is much quicker than previous methods and does not sacrifice separation efficiency. Generally, the efficiency of HPLC increases in proportion to the number of theoretical plates per column. As the height equivalent to a theoretical plate (HETP), the height of a slice as a part of a column is smaller, while the number of theoretical plates per column and the interaction of analytes with packing materials increase. Therefore, HPLC is a highly efficient method in terms of separation and sensitivity.

As described in the van Deemter equation (Eq. 1), the introduction of smaller-diameter particles is required to diminish the HETP, because the HETP increases in proportion to the square of the particle diameter. However, using smaller-diameter packing materials results in ultra-high pressure through the column when pumping the mobile phase to keep an appropriate linear velocity (u). In addition to high efficiency, high linear velocity (u) is required to increase the speed of the analysis. Recently, instruments and columns have been introduced that can operate the speed of the analysis. Reducing the column length to 100 mm and the particle size to 1.7 μm resulted in shorter detection times while still maintaining separation efficiency. Ito verify the bioanalytical method of the UPLC system, we used samples pretreated with THU. CD-mediated deamination is considered the main cause for the short half-life of these drugs, Ki of 28-240 nM [9]. Therefore, samples not treated with THU did not ensure the stability of gemcitabine and 2dC, nor the reproducibility of the method. From these results, we recommended that THU be added to plasma samples

$$H = A \cdot d_p + B\frac{1}{\mu} + C \cdot d_p^2 \cdot \mu$$

Eq. 1. The simplified van Deemter equation. H is the HETP, d_p is the particle size of the column packing material, u is the linear velocity of the mobile phase, and A, B, C are related with the analyte diffusion coefficient in the mobile phase, a packing constant, an obstruction factor for diffusion in a packed bed and a function of the retention factor.

prior to TDM.

In conclusion, we claim that the new UPLC system is extremely efficient with regard to separation and results in shorter detection times. These results are largely due to the use of smaller particles (1.7 μm diameter). Furthermore, the system is capable of operating at ultra-high pressures (up to 1,000 bar or 14,500 psi). Therefore, gemcitabine and the internal standard are well separated in less than 5 min without background interference. The speed, sensitivity, and linearity over a wide range make this method applicable to large-scale clinical pharmacokinetic studies and TDM of gemcitabine levels.

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초록 : 역상 초고속액체크로마토그라피에 의한 gemcitabine의 빠른 농도 분석법

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Gemcitabine은 다양한 고형암 치료에 사용되는 항암제이다. 혈장내에서 cytidine deaminase에 의해 빠르게 대사되며, 친수성 성질로 인해 역상 액체크로마토그라피(reversed-phase liquid chromatography)를 이용한 농도 분석이 어렵다. 본 연구에서는 역상칼럼(reversed-phase column)을 이용한 초고속 액체크로마토그라피(ultra-performance liquid chromatography, UPLC) 방법에 의해 빠르고 정확한 속력(velocity)과 최고효능(peak efficiency)를 갖춘 분석법을 개발하고자 하였다. Gemcitabine과 2′-deoxycytidine의 머무름 시간(retension time)은 293 nm에서 각각 3.2분과 2.1분이었다. 검량선의 직선성 검정은 0.1~20 μg/ml 의 농도범위에서 높은 직선성을 나타내었다(r^2>0.999). 일내(intra-day) 변이계수(coefficients of variation)와 일간(inter-day) 변이계수는 모두 10% 이하였다. 정확성(accuracy) 검정을 위한 일내 및 일간 평균농도 측정치가 97.3~113.5% 범위로 나타났다. 이러한 결과를 토대로, gemcitabine 농도를 측정하기 위한 새로운 분석법으로 빠르고 정확한 역상 UPLC 방법을 제안하고자 한다.