Cytarabine determination from urine for Toxicokinetic and Excretion studies by High- Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract: Cytarabine (Cyt) (also known as cytosine arabinoside (ara-C)) used in the treatment of acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL). CYT applied in high doses for treatment can cause renal failure. Monitoring excreted urine drug levels can help kidney failure. For this reason, a method was developed and validated by HPLC-MSMS for urine CYT analysis, which is not included in the literature. In this study, a liquid chromatography (HPLC) with triple quadrupole Mass Spectrometric (MS/MS) method developed for the determination of Cyt from urine for toxicokinetic evaluation. Positive MRM mode selected for the quantification of Cyt. The product and major fragment ion for Cyt 244.0 > 112.0 m/z, for IS 198.0 > 152.0 m/z. The optimal MS parameters for Cyt and IS are as follows Fragmentor 80 V, 70 V, Collision energy, 6, 9 respectively. A novel simple, high-throughput and highly sensitive HPLC-MS/MS method was successfully developed and validated for the determination of Cyt from urine. The developed method has a simple one-step extraction method and a short run time (2.0 minutes) for analysis. The proposed method could be practical and reliable for excretion and toxicokinetic studies and as well as the Therapeutic Drug Monitoring study in humans without an invasive route for Cyt.

Keywords: Cytarabine, therapeutic drug monitoring, toxicokinetics, mass spectrometry.

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1. INTRODUCTION

An average of one million new cancer patients are diagnosed on the world. According to the information obtained, a quarter of these patients treated with surgery and radiotherapy. Most remaining patients receive chemotherapy at any stage of this disease. It is pleasing that a certain number of cancer patients can be fully cured or a long recovery period with chemotherapy, depending on the type of cancer (1,2).

Cyt (3), used in the treatment of acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML) and non-hodgkin lymphoma. Cyt is a nucleotide analog and

its chemical name is 4-Amino-1-beta-D-arabinofuranosyl-2(1H)-pyrimidinone (Figure 1). Cyt metabolized intracellularly to the active triphosphate form (cytosine arabinoside triphosphate). Cyt maximum plasma level is 26.3 µg L-1. Less than 10% of the dose excreted in the urine as unchanged drug. Cyt is an important drug in the treatment of acute myeloid leukemia (AML) (4). High-dose cytarabine (2000 to 3000 mg per square meter of body surface area) is toxic but provides higher relapse-free survival rates than the conventional dose of 100 to 400 mg per square meter (5,6). Cyt has been one of the cornerstone drugs in the treatment of acute myeloid leukemia (AML) for over three decades. It initially used in remission induction therapy at a dose of 100 to 200 mg per square meter of body surface (6). The incidence of adverse reactions appears to be higher in patients with severe renal impairment, associated with an increase in adverse effects in patients (7). Absorption distribution metabolism excretory toxicity (ADMET) studies provide important information about the metabolism and urinary and fecal excretion of compounds.

In addition, ADMET studies are critical in modern drug discovery. The purpose of ADMET studies is not only to understand the metabolism but also how both parent and metabolites are eliminated. Drugs removed from the body by various elimination processes. Drug elimination refers to the irreversible removal of drugs from the body by all routes of elimination. Drug excretion is the removal of the intact drug. Toxicokinetics is essentially the study of how a substance gets into the body and what happens to it in the body. Toxicokinetics deals with what the body does with a drug when given a relatively high dose relative to the therapeutic dose. Monitoring excreted urine drug levels may help renal insufficiency (8). In addition, the information generated from these studies is extremely useful in determining whether the kidney or liver is an important organ in elimination and whether there is any safety concern in hepatic or renal impaired populations.

In this study, it is important to determine the excretion of high-dose CYT from the body at the first stage. For this reason, urine CYT analysis has gained importance in determining whether the drug administered in high doses is excreted from the body and its damage to vital organs such as kidneys and liver. In this study, a reliable and sensitive method was developed for urine analysis of CYT, which is not reported in the literature.

Different liquid chromatographic techniques have been reported in the literature dealing with the determination of Cyt in various samples except urine. For example, cytarabine and uracil-arabinoside from human plasma were determined by Burk et al. by using the ion couple liquid chromatography method (9). Hsieh and his team performed Cyt determinations in mouse plasma using different liquid chromatography-tandem mass spectrometric techniques such as ion-pairing, mixed-mode, and porous graphitic carbon (10-12). The high-performance liquid chromatography-tandem mass spectrometry method for the simultaneous determination of cytarabine and its valyl prodrug cytarabine in rat plasma by Sun et al. (13). There are also other HPLC studies conducted by different groups (14-16) and Cyt has excretion studies in Saliva (8). In toxicokinetic evaluations, it is important to analyze non-invasive urine samples instead of taking blood, and urine analysis of cytarabine, which causes renal failure in high doses. Cytarabine syndrome and acute toxicity may occur within 12 h after the start of drug infusion (17). So it's important to analyze Cytarabin level instead of metabolite. In this study, a liquid chromatography (HPLC) with triple quadrupole Mass Spectrometric method was developed for the determination of Cyt from urine. CYT urine analysis studies have not been found in the literature. Liquid chromatography (HPLC) types of equipment can provide more sensitive and reproducible results. Cyt excretion studies with urine will contribute and guide toxicokinetic studies. The urine analysis method of Cyt, which was developed and validated in this study, can be used in excretion studies. Cyt analysis method from urine has not been found in the literature search.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Cyt working standard was certified to contain 100.25% purity for Cyt. Levodopa was chosen as an Internal standard (IS) and certified to contain 99.82% purity for Levodopa. The water that obtained from a Milli-Q ultrapure water system (Millipore, Barnstead) used as the solvent. Ethanol obtained from Merck (Darmstadt, Germany) used for dilution. Formic acid and Acetonitrile obtained from Merck (Darmstadt, Germany) used for the mobile phase.

2.2. Chromatographic Conditions

Agilent 1260 Infinity Liquid Chromatography system and QQQ-6420 detector were used in this study. Separation was carried out on a Poroshell 120 EC-C18, (4.6 - 50 mm, 2.7 µm) column maintained at 30 °C. The LC mobile phase consisted of Acetonitrile: 0.5% Formic Acid (30:70% v/v). The flow rate was 0.600 mL min-1. The injection volume was 5.0 µL and the runtime was 2.0 minutes.

Figure 1: Chemical structure of 4-Amino-1-beta-D-arabinofuranosyl-2(1H)-pyrimidinone.

2.3. Mass Spectrometry Conditions

Detection was carried out by an Agilent 6420 triple quadrupole MS/MS fitted with Agilent Electrospray ionization (ESI) operated in the positive ion mode. Quantification was performed using multiple reaction monitoring (MRM) of the transition ions of m/z 244.0 > 112.0 for Cytarabine and m/z 198.0 > 152.0 for Levodopa, with the dwell time of 100 ms. Nitrogen (99.999% purity) was used as the collision gas. The optimized conditions were as follows: Nebulizer Pressure, 25 psi; Gas Temperature, 300 °C; Gas Flow, 10 (L min-1); Capillary, 5500 V. The MRM transitions and the related optimized Fragmentor Voltage were 80 V for Cyt and 70 V for Levodopa. Collision energy was 6 V for Cyt and 9 V for Levodopa.

2.4. Preparation of Stock and Working Solutions

Standard stock solutions of Cyt 1 mg mL-1 (w/v) and Levodopa (IS) 1 mg mL-1 (w/v) were separately prepared in 5 mL volumetric flasks with distilled water. Working solutions for calibration and quality control samples were prepared from the stock solution by adequate dilution using diluents ethanol. The Internal Standard (IS) working solution (100.0 µg mL-1) was prepared by diluting the stock solution with ethanol.

2.5. Sample Preparation

Working solutions were used to prepare increased volumes tubes that contain 100 µl urine to obtain calibration standards (0.1, 0.5, 5.0, 10.0, 20.0, 30.0, 40.0 µg mL-1) and quality control (15.0, 25.0, 35.0 µg mL-1) samples concentration. 50 µL Internal Standard (IS) working solution (100.0 µg mL-1) spiked tubes. The samples were vortexed for 30 seconds. Samples were extracted for a liquid-liquid extraction with Ethanol. To this, increased volumes ethanol were added to each tube to final volumes are 500 µL. The samples were vortexed for 30 seconds. 0.20 mL of supernatant layer was transferred and analyzed. The developed methods were used to determine urine levels of Cyt for excretion studies.

2.6. Method Validation

The developed method was validated according to the requirements for European Medicine Agency (EMEA) Gıidline on Bioanalytical Method Validation, which includes specificity, linearity, precision, accuracy, limit of detection and limit of quantitation and Matrix effect (18).

2.7. Matrix Effect

Liquid chromatography coupled to mass spectrometry (LC–MS) is a powerful tool for bioanalysis. Reliable bioanalysis requires the characterization of the matrix effect, i.e. influence of the endogenous or exogenous compounds on the analyte signal intensity.

Matrix effects are major concerns in quantitative LC - MS analysis because they detrimentally affect the accuracy, precision, and sensitivity of a method. Therefore, the presence of matrix effects needs to be evaluated during the method development to achieve reliable analytical results.

The matrix effect is evaluated using pool urine from different sources to prepare samples. The matrix factor (MF) is calculated, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with analyte at a concentration of quality control samples after extraction), to the peak area in absence of matrix (pure solution of the analyte). The IS normalized MF is calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalised MF calculated from the six lots of matrix should not be greater than 15%. Extracted and aqueous samples were compared to determine the MF for the analyte and IS. IS normalized MF for individual series are also determined. The calculated MF of all LQC (low quantity concentration) and HQC (high quantity concentration) samples must be within 0.85 - 1.15 of their nominals concentrations.

Table 1: Accuracy and Precision results of Calibration standards.

	C (μg mL^{-1})	Mean	Error %	Standard Deviation (SD)	Coefficient of variation %CV
ST1	0.1000	0.1028	102.8333	0.0054	5.2027
ST2	0.5000	0.4840	96.8067	0.0326	6.7371
ST3	5.000	5.0408	100.8160	0.0559	1.1090
ST4	10.000	9.9319	99.3187	0.1406	1.4154
ST5	20.000	20.0612	100.3058	0.1681	0.8377
ST6	30.000	29.9903	99.9676	0.3387	1.1295
ST7	40.000	39.9893	99.9733	0.2355	0.5890

2.8. System Suitability

System suitability tests are an integral part of GC and LC methods. These tests are used to verify that the chromatographic system is adequate for the intended analysis. The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such. Resolution is a function of the number of theoretical plates, N (also referred to as efficiency), a, and k.

3. RESULTS AND DISCUSSION

3.1. Results

The analysis was performed in the mass spectrometer using an electrospray (ESI) in the positive ionization mode. The reliability of the method was assessed on the basis of linearity, sensitivity, selectivity, precision, and accuracy. The results obtained for the above parameters, the combination of mobile phase, flow rate, and detector parameters were selected for validation. Out of several tried combinations, a liquid chromatography (HPLC) method with MSMS detection was developed for the determination of Cyt from urine. The analysis was carried out using a Poroshell 120 EC-C18, (4.6 - 50 mm, 2.7 μm) column with a mobile phase consisting of ACN and 0.5% formic acid at a flow rate of 0.5 mL min^{-1}. Levodopa was used as an Internal standard (IS). The mass spectrometer was tuned initially in both positive and negative ionization modes for Cyt. It was observed that the signal intensity of positive ion was much higher than that of a negative ion. Parameters, such as capillary and nozzle voltage, desolvation temperature, ESI source temperature, and flow rate of desolvation gas and cone gas, were optimized to obtain the optimum intensity of molecules of Cyt and IS for quantification. Multiple reaction monitoring (MRM) is a highly specific and sensitive mass spectrometry technique that can selectively quantify compounds within complex mixtures. This technique uses a triple quadrupole MS that firstly targets the ion corresponding to the compound of interest with subsequent fragmentation of that target ion to produce a range of productions. One or more of these fragment product ions can be selected for quantification purposes. Only compounds that meet both these criteria, i.e. specific precursor ion and specific productions corresponding to the mass of the molecule of interest are isolated within the mass spectrometer. Mass spectrometry MRM mode also provides us to analyze compounds with the same retention time. Positive MRM mode was selected for the quantification of Cyt. The product and major fragment ion for from Cyt 244.0 to 112.0 m/z, for IS from 198.0 to 152.0 m/z. The optimal MS parameters for Cyt and IS as follows Fragmentor 80 V, 70 V, Collision energy, 6, 9 respectively.

3.1.1. Selectivity

The selectivity of the method against urinary matrix components was evaluated against human blank urine. Among the analyzed batch, the urine batch showing any interferences at the retention times of analyte and IS. All samples were processed and analyzed using the proposed extraction method (Figure 2).

3.1.2. Linearity

Calibration curves were constructed using matrix-matched calibration standard solutions by plotting the peak area of the quantitative ion of each analyte versus concentrations. For the quantification of the samples, a calibration curve was constructed by spiking blank plasma at seven concentration levels (100 - 40000 ng mL^{-1}). For this purpose, urine samples spiked at seven concentration levels (100 - 40000 ng mL^{-1}) were extracted. The calibration curve regression for Cyt was linear regression. This gave the best fit and coefficient of determination (r^2) for validation and was greater than 0.99 which was in the acceptable range. The average value for r^2 was found to be 0.9926. Calibration curve's equation is $y = 6.8755x - 0.2479$. The concentration range of Cyt was found to be accurate and precise from 100 to 40000 ng mL^{-1} (Table 1). The LOQ of the method 100 ng mL^{-1}, Signal to noise ration of LOQ was found that S/N: 14.1, LOD of the method 50 ng mL^{-1} (S/N: 6).

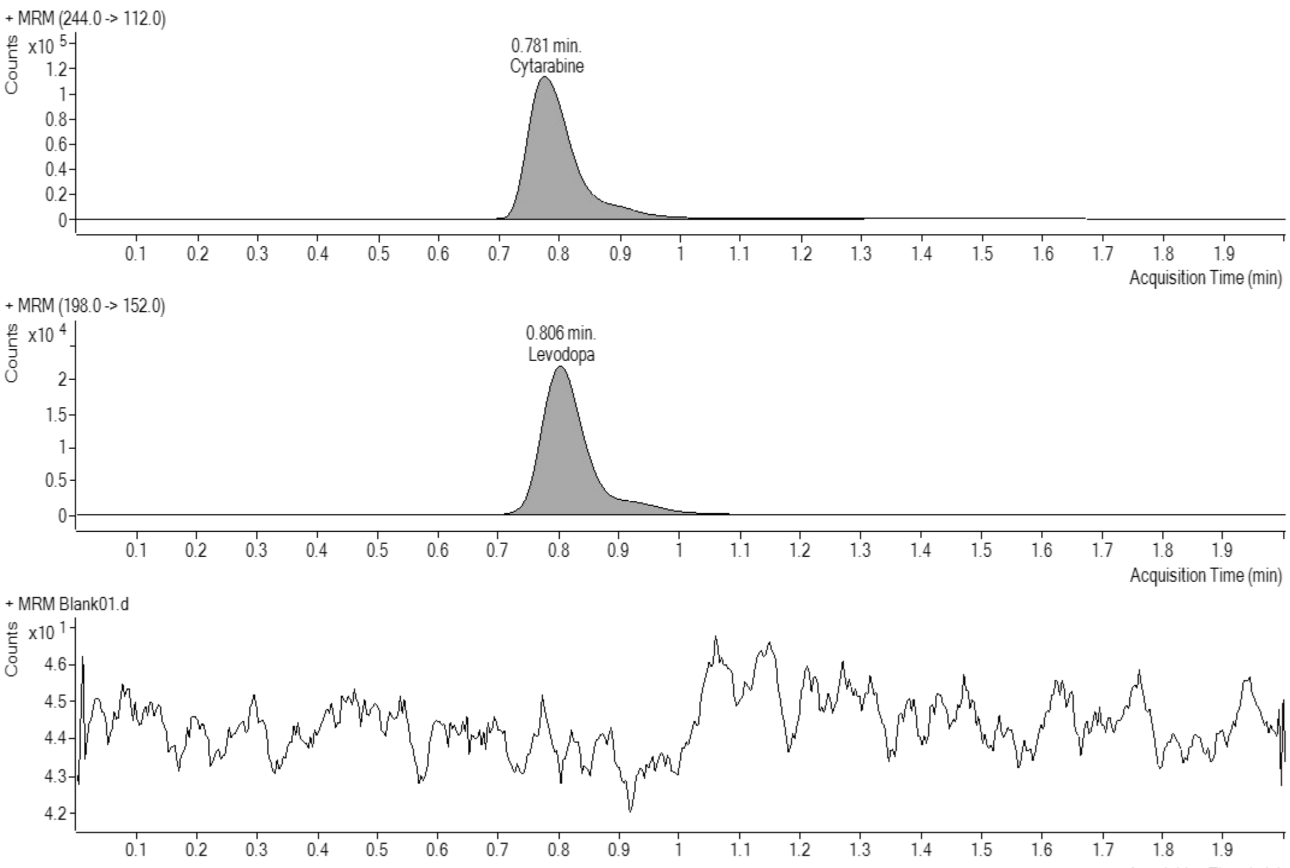

Figure 2: The MRM chromatograms of Cyt (15 μg mL^{-1}, QC1) **(a)**, IS (Levodopa) **(b)** and blank **(c)**

3.1.3. Accuracy and precision

Accuracy and precision of Cyt were calculated at 15000 ng mL^{-1}, 25000 ng mL^{-1} and 350000 ng mL^{-1} levels for the six replicates results are shown in Table 2. The coefficients of variation results were 3.23%, 2.86%, and 2.70% respectively whereas intra-day accuracies were 103.0247 ± 3.0246, 99.06268 ± 0.9373, and 101.0055 ± 1.0055 respectively in urine samples. These results indicate that the method has good precision and accuracy are within the acceptance limit of < 15% and ± < 15% for precision and accuracy respectively.

3.1.4. Matrix effect

The Matrix effect calculated was 2.16 for low concentration and 0.79 for high concentration. The overall CV calculated for the concentration should not be greater than 15%. Therefore ion suppression or enhancement from urine was negligible under current conditions. The formula about matrix effect was given below.

$$\text{Normalized Matrix Factor} = \frac{(\text{Peak area of analyte in the matrix})/(\text{peak area of analyte})}{(\text{Peak area of IS in the matrix})/(\text{Peak area of IS})}$$

3.1.5. System suitability

System suitability parameters found as specified in the guideline. Theorical plate number (N) was calculated as 15700, capacity factor (k') 9.8, resolution (Rs) 14, tailing factor (T) T ≤ 2 for Cyt. Detailed parameters are given in Table 3

Table 2: Accuracy and precision of Cyt in urine (in three validation days, six replicates at different concentration levels per day).

	Intra-day				Inter-day			
QC samples µg mL^{-1}	**Measu-red Concen-tration (µg mL^{-1})**	**Error %**	**SD**	**% CV**	**Measu-red Concent-ration (µg mL^{-1})**	**Error %**	**SD**	**% CV**
15	14.8107	98.7379	0.4796	3.2385	15.2713	101.8087	0.5567	3.6455
25	25.3978	101.5912	0.7275	2.8644	24.7053	98.8213	0.4148	1.6790
35	34.7578	99.3079	0.9386	2.7005	35.1486	100.4245	1.7106	4.8667

Table 3: System suitability results and acceptability limits*.

Parameter	**Value (Cyt)**	**Limit (FDA guidline)**
Retention time (min)	0.78	-
Peak Width (W)	0.28	-
Tailing (T)	2	T ≤ 2
Theoretical Plates (N)	15700	N > 2000
Resolution (R)	14	Rs > 2
Capacity Factor (k)	9.8	k' > 2

*FDA: Center for Drug Evaluation and Research

3.2. Discussion

A novel simple, high-throughput, and highly sensitive LC-MS/MS method was successfully developed and validated for the determination of Cyt from urine. The developed method has a simple one-step extraction method and a short run time (2.0 minutes) for analysis. With the developed method, the toxic effect of CYT applied at high doses can be determined by obtaining information about its excretion by urine analysis. The proposed method could be practical and reliable for excretion studies as well as the Therapeutic Drug Monitoring study in humans without an invasive route for Cyt. The normal dose of Cyt is set at 100 - 400 mg / body surface area, however, high doses of 2000 - 3000 mg / body surface area are given for the treatment of the disease. In this case, determining how much Cyt is excreted from the urine per hour will be effective in determining its toxic level in the body. With the developed method, toxicokinetic studies can be carried out easily and in a short time.

4. CONCLUSION

It is important to determine whether the drug given in high doses is excreted from the body and the damage it causes to vital organs such as kidney and liver. Cyt is metabolized in the body, but less than 10% is excreted without being metabolized. With the developed method, CYT urine analysis will help to determine the harmful effects of the treatment and selection of the therapeutic dose.

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