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# Quantification of 5-azacytidine in plasma by electrospray tandem mass spectrometry coupled with high-performance liquid chromatography

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**Abstract**

5-Azacytidine (5AC), a nucleoside analogue and hypomethylating agent, has anticancer properties and has been utilized in the treatment of various malignancies. 5AC is unstable and rapidly hydrolyzed to several by-products, including 5-azacytosine and 5-azauracil. A sensitive, reliable method was developed to quantitate 5AC using LC/MS/MS to perform pharmacokinetic and pharmacodynamic studies on 5AC combination therapy trials. Blood samples were collected in a heparinized tube and immediately processed for storage. To increase the stability of 5AC in plasma, 25 ng/mL tetrahydrouridine was added to the plasma and snap frozen. Plasma samples were extracted using acetonitrile then cleaned up by Oasis MCX ion exchange solid-phase extraction cartridges. 5AC was separated on an YMC Jsphr M80$^{TM}$ $C_{18}$ column with gradient elution of ammonium acetate (2 mM) with 0.1% formic acid and methanol mobile phase. 5AC elutes at $5.0 \pm 0.2$ min with a total run time of 30 min. Identification was through positive-ion mode and multiple reaction monitoring mode at $m/z+$ $244.9 \rightarrow 113.0$ for 5AC and $m/z+$ $242.0 \rightarrow 126.0$ for 5-methyl-2′-deoxycytidine, the internal standard. The lower limit of quantitation of 5AC was 5 ng/mL in human plasma, and linearity was observed from 5 to 500 ng/mL fitted by linear regression with $1/x$ weight. This method is 50 times more sensitive than previously published assays and successfully allows studies to characterize the pharmacokinetics and pharmacodynamics of 5AC.


*Keywords:* 5-Azacytidine; LC/MS/MS; Pharmacokinetics

## 1. Introduction

5-Azacytidine (5AC) is a nucleoside analogue and hypomethylating agent that has anticancer properties. As of May 2004, the United States FDA has approved 5AC in the treatment of all five myelodysplastic syndromes (MDS) [1]. Potential combinations include use of a hypomethylating agent with histone deacetylase inhibitors, such as phenylbutyrate [2,3]. 5AC at doses of 10–75 mg/m$^2$/day is being administered subcutaneously for 7–21 days every 28 or 35 days in combination with phenylbutyrate for either solid tumors or hematologic malignancies at our institution [4].

5AC is unstable and rapidly hydrolyzed to several by-products, including 5-azacytosine and 5-azauracil in aqueous solutions [5,6]. Quantitation of 5AC in plasma was originally performed using a microbiological assay that utilized the agent's cytostatic properties but was not specific since the degradation products had residual cytostatic properties [7]. Early HPLC assays were developed to quantitate 5AC in aqueous media but not plasma [5,8,9]. An ion pairing HPLC method was developed to quantitate 5AC in plasma with the lower limit of quantitation of 250 ng/mL [10]. 5AC is unstable in plasma with a 20% loss by 4.5 days when stored at −60 °C and with a 10% loss within 0.5 h when at room

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temperature [10]. To characterize the clinical pharmacology utilizing low-dose subcutaneous injections of 5AC, a reliable method for the quantitation was necessary. The method discussed in this paper utilizes reverse-phase liquid chromatography with electrospray tandem mass spectrometry detection (LC/MS/MS) to achieve a sensitive and specific assay method.

## 2. Experimental

### 2.1. Chemical and reagents

5AC and 5-methyl-2′-deoxycytidine, the internal standard, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tetrahydrouridine (THU) was purchased from Calbiochem (La Jolla, CA, USA). Methanol and acetonitrile were HPLC grade and obtained from EMD Chemicals (Gibbstown, NJ, USA). Ammonium acetate and formic acid (88%, v/v, in water) were purchased from J.T. Baker (Philipsburg, NJ, USA), and ammonium hydroxide and hydrochloric acid from Fisher Scientific (Fair Lawn, NJ, USA). Waters Oasis® MCX LP extraction cartridges were obtained from Waters Corporation (Milford, MA, USA). Distilled water was deionized from a Milli-Q Plus filtration system (Marlborough, MA, USA). Drug-free (blank) human plasma originated from Pittsburgh Blood Plasma Inc. (Pittsburgh, PA, USA).

### 2.2. Preparation of stock solutions, calibration standards, and quality controls

Stock solutions of 5AC were prepared in duplicate by dissolving 10 mg accurately weighed in 10 mL of methanol:water (1:1, v/v) to a final concentration of 1 mg/mL. The area counts for each of the duplicated aliquots were checked in quintuplicate, and if the mean value for area counts was within 5%, the stock solutions were then stored in a glass vial at −20 °C for a week. The stock solution was serially diluted in methanol:water (1:9, v/v) on each day of analysis to prepare working standards of 100, 10, 1, and 0.1 μg/mL. Microliter amounts of 5AC stock solution were added into pooled human plasma containing 100 μM THU to prepare a calibration curve and quality controls (QC). The standards were prepared at 5, 10, 20, 50, 100, 200, and 500 ng/mL, and the QCs at 15, 75, and 400 ng/mL. An additional dilutional QC was prepared at 4000 ng/mL. The dilutional QC was diluted 1:10 with blank pooled human plasma containing 100 μM THU prior to sample preparation to ensure patient samples could be diluted. All standards and quality controls were prepared fresh daily. For long-term and freeze–thaw stability, quality controls were stored at −80 °C.

A stock solution of 5-methyl-2′-deoxycytidine was prepared by dissolving 10 mg, accurately weighed, in 100 mL of methanol:water (1:1, v/v), and stored in a glass vial at −20 °C. A 50 μL aliquot of the internal standard stock solution was added into 1 L of acetonitrile for a final concentration of 5 ng/mL at the time of analysis.

### 2.3. Sample preparation

Plasma samples (200 μL) were initially deproteinized with 1 mL of acetonitrile containing 5 ng/mL IS in a borosilicate glass tube. The mixture was then centrifuged at 2000 × *g* for 10 min at ambient temperature. The supernatant was dried with gentle nitrogen stream at 30 °C. The residue was dissolved in 0.5 mL of 0.1N HCl and transferred to an activated Oasis MCX 1cc (30 mg) ion exchange solid-phase extraction (SPE) cartridge. The SPE was conditioned and equilibrated by washing with 1 mL of methanol and then 1 mL of Milli-Q water. The samples were cleaned using two wash steps: first by 1 mL twice of 0.1N HCl and then with 1 mL of methanol. 5AC was eluted with 2 mL total (1 mL twice) of ammonium hydroxide in acetonitrile (5:95, v/v). The samples were evaporated to dryness under nitrogen at 30 °C. The residue was reconstituted in 100 μL of methanol:water (1:9, v/v) by vortex mixing (30 s). The sample was transferred to a 250 μL polypropylene autosampler vial, and a volume of 20 μL was injected onto LC/MS/MS instrument using a temperature-controlled autosampler device operating at 5 °C. Due to the instability of 5AC in plasma, all processing and handling of 5AC samples was performed on ice until the samples were dried and reconstituted.

### 2.4. Equipment

Analyses were performed utilizing a high performance liquid chromatograph (Agilent 1100 series, Agilent Technologies, Palo Alto, CA, USA) interfaced to a tandem mass spectrometer (API 3000, Applied Systems, Foster City, CA, USA). The chromatographic separation was performed on a 250 mm × 2.0 mm i.d., 4.0 μm, $C_{18}$ column (YMC J'sphere M80$^{TM}$, YMC Co. Ltd., Kyoto, Japan) and a 20 mm × 2.1 mm i.d., 3.5 μm, $C_{18}$ guard column (Waters Xterra RP18, Waters Corporation, Milford, MA, USA) kept at room temperature. The mobile phase used for chromatographic separation was composed of 2 mM ammonium acetate containing 0.1% formic acid and methanol, and was delivered using a gradient flow (see Table 1). The mass spectrometer was equipped with an electrospray interface, which was operated in a positive mode, and controlled by the Analyst version 1.2 software (Applied Biosystems). Identification was through selective reaction monitoring mode at *m*/*z*+

Table 1
HPLC gradient conditions

| Time (min) | A (%)[a] | B (%)[b] | Flow rate (mL/min) |
|---|---|---|---|
| 0 | 85 | 15 | 0.15 |
| 8 | 85 | 15 | 0.15 |
| 9 | 10 | 90 | 0.25 |
| 20 | 10 | 90 | 0.25 |
| 21 | 85 | 15 | 0.25 |
| 25 | 85 | 15 | 0.25 |
| 30 | 85 | 15 | 0.15 |

[a] A: 2 mM ammonium acetate with 0.1% formic acid.
[b] B: MeOH.

244.9 → 113.0 for 5AC and *m/z*+ 242.0 → 126.0 for the internal standard.

### 2.5. Validation procedures

5AC working standards were added to human plasma containing 100 μM THU, a cytidine deaminase inhibitor, to prepare calibration samples over the range of 5–500 ng/mL [11]. Calibration curves were computed using the ratio of the peak area of 5AC and internal standard by using a weighted (1/[nominal 5AC concentration]) linear regression analysis. The assay lower limit of quantitation (LLOQ) was determined to be 5 ng/mL for 5AC. The LLOQ was determined by meeting the following two criterion: a signal to noise ratio of the peak areas larger than 20 and the values for precision and accuracy less than 20%.

Method validation runs were performed on eleven days. Each analytical run consisted of a calibration curve using single samples with duplicate samples at the LOQ and upper limit of quantitation (ULQ) and QC samples in duplicate. QC samples were prepared independently in blank plasma at 5AC concentrations of 15, 75, 400, and 4000 ng/mL. The 4000 ng/mL dilutional QC was assayed once it was diluted 1:10 (v/v) with blank plasma containing 100 μM THU prior to sample preparation. The dilutional QC was used to determine the accuracy and precision of diluted patient samples and was performed on seven days. The accuracy and precision of the assay was assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$\mathrm{DEV}_{(5\mathrm{AC})} = 100 \times \left\{ \frac{([5\mathrm{AC}]_{\mathrm{mean}} - [5\mathrm{AC}]_{\mathrm{nominal}})}{[5\mathrm{AC}]_{\mathrm{nominal}}} \right\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square ($\mathrm{MS_{bet}}$), the within-groups mean square ($\mathrm{MS_{wit}}$), and the grand mean (GM) of the observed concentrations across runs were calculated using the JMP™ statistical discovery software version 4 (SAS Institute, Cary, NC, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$\mathrm{BRP} = 100 \times \left( \frac{\sqrt{(\mathrm{MS_{bet}} - \mathrm{MS_{wit}})/n}}{\mathrm{GM}} \right)$$

where $n$ represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = 100 \times \left( \frac{\sqrt{\mathrm{MS_{wit}}}}{\mathrm{GM}} \right)$$

The mean and standard deviation were calculated on the slope and intercept of the linear regression line. The minimum and maximum correlation coefficient is reported. The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from six different donors for the presence of endogenous or exogenous interfering peaks. The extraction efficiency of the assay was measured by comparison of extracted plasma samples and aqueous samples of 5AC in triplicate at concentrations of 15, 75 and 400 ng/mL. The mean values of the triplicate samples were compared. The short-term stabilities of 5AC in plasma were also assessed in triplicate on the benchtop for 1, 2, 4 and 6 h on ice. The long-term stability test of 5AC was assessed in triplicate at −80 °C. The mean values of the triplicate samples were compared to the initial condition for both short-term and long-term stability.

### 2.6. Pharmacokinetic analysis

The patients analyzed participated in one of two clinical Phase I studies of 5AC. Patients were administered 25 mg/m$^2$/day subcutaneously for 7–21 days every 28 or 35 days in combination with phenylbutyrate [9,10]. The protocols were approved by the Institutional Review Board of The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (Baltimore, MD, USA), and the patients provided written informed consent.

Blood samples were collected in heparin-containing tubes before drug administration and at 15, 30 min and at 1, 2, 4, 8, and 24 h after administration of the dose. Samples were processed immediately by centrifugation at 3000 × *g* for 5 min in a refrigerated centrifuge. THU was added to the plasma supernatant at a final concentration of 100 μM. Plasma (300 μL) was then aliquoted into two tubes with the remaining plasma in a third tube. Plasma that did not contain 100 μM THU was discarded. The plasma was frozen at −80 °C until analysis.

## 3. Results and discussion

### 3.1. Chromatographic separation and detection

A LC/MS/MS method to determine 5AC concentrations in human plasma containing 100 μM THU was developed, validated, and utilized to quantitate drug in plasma from patients receiving treatment with 5AC. Over the development of the assay, the mobile phase changed several times to allow for better separation of 5AC. Initially, an isocratic run of 5mM ammonium acetate and methanol (75:25, v/v) with a total run time of 7 min was utilized [12]. The ammonium acetate concentration was decreased to 2 mM with the addition of 0.1% formic acid when the gradient method was finalized. Due to its hydrophilic nature (Fig. 1), 5AC was not retained on typical mass spectrum columns, which are 50 mm in length. Instead, the chromatographic separation was performed on a longer, narrow bore (250 mm × 2.0 mm i.d., 4.0 μm) $C_{18}$ column using 85:15 (v/v), 2 mM ammonium acetate containing 0.1% formic acid and methanol. This resulted in a retention time of approximately 5.4 min which was sufficient to separate 5AC from interfering peaks, including THU, that were observed with the shorter columns.

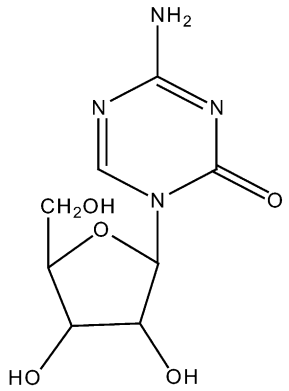


Fig. 1. Chemical structure of 5AC.

The mass spectrum of 5AC showed a protonated molecular ion ([$MH^+$]) at *m*/*z* 244.9 (Fig. 2A). The high collision energy fragmented 5AC into several fragments. The major fragment observed was at *m*/*z* 113.0, which was selected for subsequent monitoring in the third quadrupole (Fig. 2B).

Chromatograms of blank and spiked human plasma samples are shown in Fig. 3 for 5AC and Fig. 4 for the internal standard. The selectivity for analyte is shown by the sharp and symmetrical resolution of the peak, with no significant interfering peaks for both 5AC and the internal standard in drug-free specimens, obtained from six different individuals. The retention times for 5AC and internal standard under current HPLC conditions were $5.4 \pm 0.2$ min and $4.6 \pm 0.2$ min, respectively. The overall chromatographic run time was 30 min. After 5AC was eluted, multiple compounds were retained on the column, which were removed using a higher organic solvent and a higher flow rate. In order to re-equilibrate the column to the original condition prior to another injection, a run time of 30 min was necessary.

### 3.2. Standards, quality controls, and calibration curves

Calibration curves for 5AC standards were constructed from the peak area ratio of 5AC to the internal standard. An excellent linear relationship ($r > 0.98$, range 0.9870–0.9975) was observed using a linear standard curve over the entire range of 5–500 ng/mL with a weight factor (1/[nominal concentration]) to correct the heteroscedasticity observed using no weight factor. The lower limit of quantitation was established at 5 ng/mL for 5AC with a signal to noise ratio of 20 or larger. At this concentration, the values for accuracy and within-run and between-run precision were 100.4%, 17.6%, and 1.0%, respectively (see Table 2). The slope ($0.151 \pm 0.120$, mean ± standard deviation; $n = 11$) and *y*-intercept ($0.007 \pm 0.006$, mean ± standard deviation; $n = 11$) were calculated for each calibration curve. Back-calculated errors were obtained for each point in the calibration curve and the quality controls. Individual analytical runs were accepted if greater than 75% of the calibrators were within 15% of the nominal concentration or 20% at the LOQ, and greater than 66% of the quality controls were within 15% of the nominal concentration.

Accuracy and the within-run and between-run precision at the LOQ and three different quality controls were calculated during replicate assays ($n = 2$ per day, for 11 days) using one-way ANOVA (see Table 2). Accuracy and the within-run and between-run precision at the dilutional quality control were calculated during replicate assays ($n = 2$ per day, for 7 days) using one-way ANOVA (see Table 2). Both within-run and between-run precision was less than 15% over a wide range of 5AC concentrations [Table 2]. The accuracy of QC samples for 5AC was between 95.9% and 102.3%, which meet the FDA requirement well. The good precision and accuracy indicated that this assay is reproducible and accurate.

### 3.3. Extraction, recovery, and stability

Various methods of extracting 5AC were attempted over a period of several years including protein precipitation, liquid–liquid extraction, and SPE. The extraction of 5AC from plasma was challenging alone since it is an unstable, hydrophilic compound. The addition of THU, which stabilizes 5AC for long-term freezer stability caused interferences at the retention time for 5AC. Straight methanol or acetonitrile protein precipitation of human plasma resulted in no detectable 5AC peaks, most likely because the resultant reconstitute was dirty and caused ion suppression, which interferes with MS detection. Drug extraction with 10% perchloric acid, a strong oxidation agent, degraded 5AC and resulted in a low recovery (20–30%).

Utilizing standard C-18 SPE cartridges, 5AC was not retained on the SPE sorbent and therefore offered no improvement in separation from the plasma matrix. However, the Oasis® MCX SPE was utilized for the initial cation-exchange to retain ionized 5AC. The interaction allowed for retention of 5AC by first washing the SPE with 0.1N hydrochloric acid to remove residual proteins and water soluble compounds and lock the 5AC ions to the sorbent. A second wash involved methanol to remove lipid soluble components. 5AC was eluted with 5% ammonium hydroxide in acetonitrile, which replaced the ions from the sorbent with the ammonium hydroxide. The eluant was then concentrated for injection. Despite better selectivity with a 3 mL (60 mg) SPE cartridge, a smaller 1 mL (30 mg) cartridge was selected from an economical standpoint. Several manipulations to determine the optimal extraction procedure included precipitation with 10% perchloric acid, washing and eluting with a single 2 mL step versus the separation into two separate 1 mL steps, and eluting with 5% ammonium hydroxide in methanol. The final procedure resulted in maximal reproducibility and decreased interferences in the samples. Despite the variability of 77–95% noted in the recovery of 5AC from human plasma (Table 2), overall accuracy and precision were within 17.6% and 4.1%, respectively. Improving the extraction technique and changing the instrumentation from a Micromass Quatro

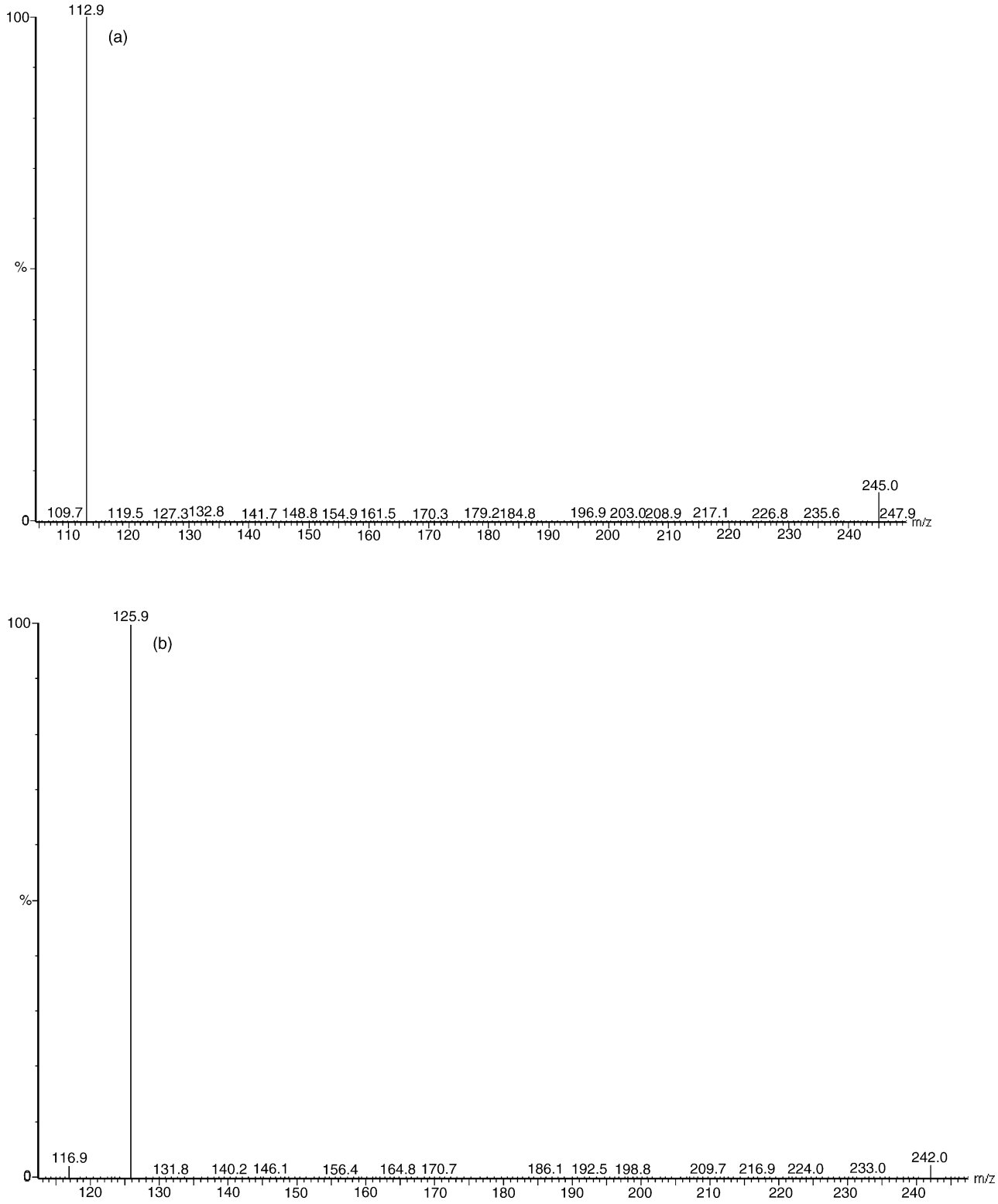


Fig. 2. Mass spectra of 5AC (a) and the internal standard (b).

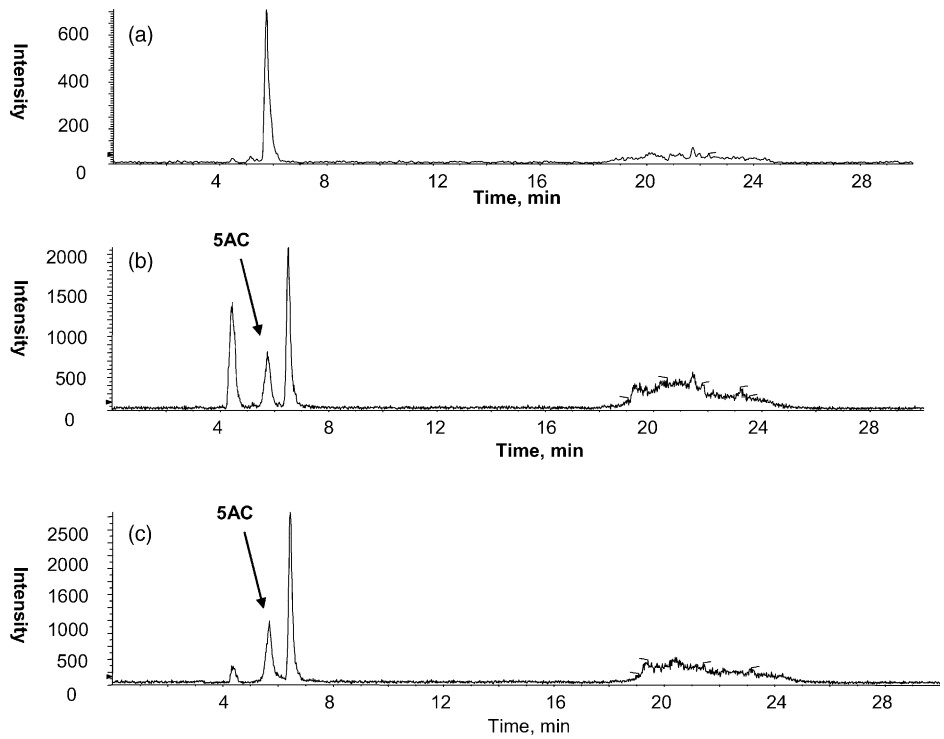


Fig. 3. Chromatograms of blank plasma spiked with 100 μM THU (a), blank plasma spiked with 100 μM THU and 5 ng/mL 5AC (b), and a patient sample (c). The selective reaction monitoring mode at $m/z$+ 244.9 → 113 for 5AC was monitored.

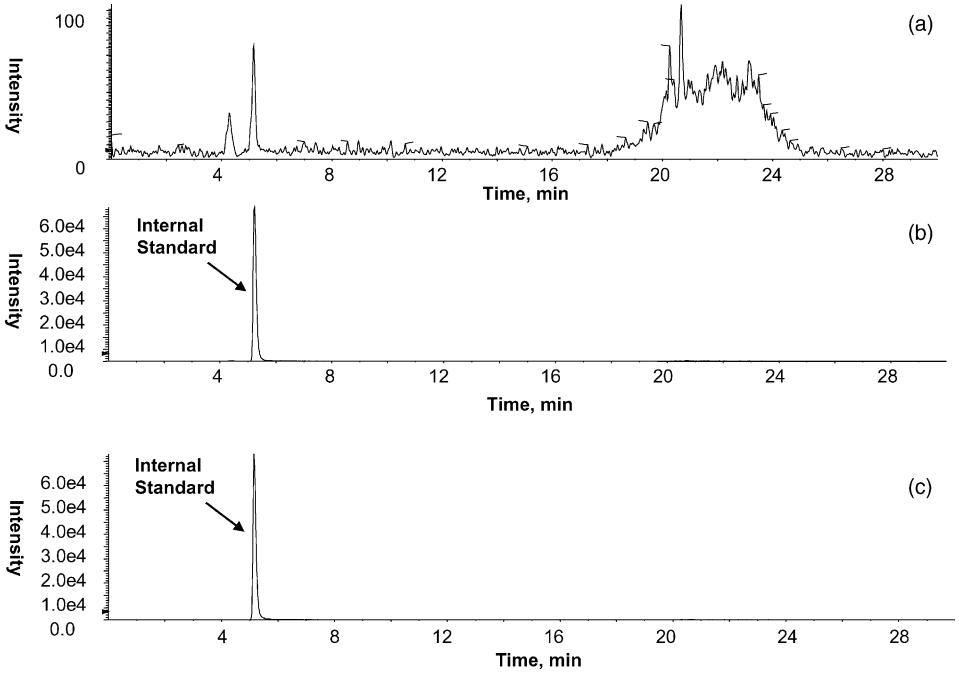


Fig. 4. Chromatograms of blank plasma spiked with 100 μM THU (a), blank plasma spiked with 100 μM THU and 5 ng/mL 5AC (b), and a patient sample (c). The selective reaction monitoring mode at $m/z$+ 242 → 126 for the internal standard was monitored.

Table 2
Validation characteristics of 5AC in plasma with 100 μM THU

| | 5 ng/mL (LOQ) | 15 ng/mL (low QC) | 75 ng/mL (medium QC) | 400 ng/mL (high QC) | 4000 ng/mL (AULQ QC) |
|---|---|---|---|---|---|
| *n* | 21 | 22 | 22 | 22 | 14 |
| Accuracy (%) | 100.4 | 98.5 | 102.3 | 98.8 | 95.9 |
| Precision (%) | | | | | |
| Within-day | 17.6 | 16.0 | 11.0 | 11.7 | 10.7 |
| Between-day | 1.0 | 11.3 | [a] | 10.4 | 9.6 |
| Extraction recovery (%) | | 96.0 | 79.6 | 77.1 | |
| Stability (% of initial) | | | | | |
| 1 h at 4 °C | | 61 | | 57 | |
| 2 weeks at –80 °C | | 100 | | 102 | |
| Autosampler stability (21 h at 5 °C) | | 95 | | 99 | |

[a] No additional information was observed as a result of performing the assay in different runs.

MS/MS system to a PE Sciex 3000 MS/MS system, allowed for a lower limit of quantitation of 5 ng/mL where initially, the LLOQ was at 100 ng/mL for this assay [12].

5AC was reported to be unstable in plasma with a 20% loss by 4.5 days when stored at −60 °C and a 10% loss within 30 min when at room temperature [8]. However, we found that 5AC is much more stable in plasma with 100 μM THU [11]. No significant degradation of 5AC was detected in human plasma after the addition of 100 μM THU and storage at −80 °C for 2 weeks. There was no significant decomposition observed after the reconstituted extracts of 5AC were stored in the auto-injector at 5 °C for 21 h [Table 2]. However, 5AC was unstable in plasma with 100 μM THU at room temperature, by 1 h approximately only 60% 5AC remained.

### 3.4. Plasma concentration–time profile

This LC/MS/MS method was applied to plasma samples from patients who have received 5AC in combination with phenylbutyrate [4]. At doses of 25 mg/m$^2$ ($n = 3$) administered subcutaneously, a maximum concentration ($C_{max}$) of 374.3 ± 140.9 ng/mL occured between 0.25 and 1.0 h. The half-life was 1.82 ± 1.51 h with no detectable concentrations after 8 h at any dosage level (see Fig. 5).

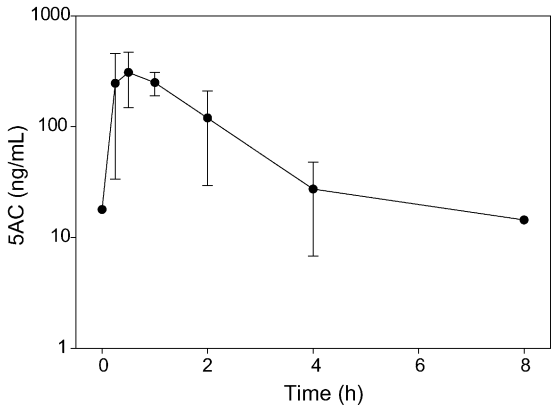


Fig. 5. Mean plasma concentration–time profile for three patients who were administed 5AC subcutaneously at a dose of 25 mg/m$^2$.

## 4. Conclusion

Over several years, we developed and validated a novel assay for quantitation 5AC levels in human plasma. Several modifications were necessary to maintain sensitivity, specificity, and reproducibility. However, our final modifications resulted in a method that was rugged and stable for over 6 months. In addition, between analytical runs for the quantitation of 5AC human plasma, the assay on the LC/MS/MS was changed for the quantitation of another structurally unrelated compound on three separate occasions without a loss in specificity or sensitivity when the assay was switched back to 5AC. The method was shown to meet the current requirements as to validation of bioanalytical methodologies, providing good accuracy and precision. The described method permits the analysis of patient samples and quantitation of 5AC at a concentration as low as 5 ng/mL, which is 50 times more sensitive than previously published assays. The method is currently being used to measure 5AC concentrations to fully characterize the clinical pharmacology of this agent. This method may be utilized in part or whole as a quantitative method for decitabine, a structurally-related anticancer agent that is also unstable and which the pharmacokinetics have not been adequately characterized due to the lack of a sensitive and reliable analytical method [13].

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