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Simultaneous determination of decitabine and vorinostat (Suberoylanalide hydroxamic acid, SAHA) by liquid chromatography tandem mass spectrometry for clinical studies

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Abstract

A reverse-phase high-performance liquid chromatography method with electrospray ionization and detection by tandem mass spectrometry is described for the simultaneous quantitative determination of decitabine (5-aza-2′-deoxycytidine) and vorinostat (Suberoylanalide hydroxamic acid, SAHA) in human plasma. The method involves a simple acetonitrile precipitation step and centrifugation followed by injection of the supernatant onto a C18 150 mm × 2.1 mm I.D., 3 μm HPLC column at 36 °C. Separation of decitabine, SAHA and their respective internal standards was achieved with a gradient elution and detection was via the mass spectrometer operated in selected reaction monitoring mode. The method was within the defined validation parameters for linearity, repeatability, reproducibility and stability. The limit of detection was determined as 1.0 and 0.125 ng ml^{-1} and lower limits of quantitation were 10 and 1 ng ml^{-1} for decitabine and SAHA, respectively. Effects of sample preparation on stability were also evaluated in human plasma. For clinical sample handling tetrahydrouridine, an inhibitor of cytidine deaminase was found to help prevent decitabine degradation. The method is currently being used in clinical pharmacokinetic studies for the evaluation of decitabine and SAHA combination therapies.

Keywords: Vorinostat; Decitabine; SAHA; Liquid chromatography; Mass spectrometry

1. Introduction

Epigenetic inactivation of key genes involved in control of normal cell growth have been identified as a fundamental characteristic of cancer cells [1,2]. Epigenetic mechanisms, including DNA methylation and histone deacetylation, interact to repress transcription of genes which may be important in the suppression of tumour growth and in sensitivity to anti-cancer drugs [3,4].

The potential anti-cancer activities of epigenetic drugs such as DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors have been studied in recent years [5–8]. DNMT inhibitors, such as decitabine (5-aza-2′-deoxycytidine) Fig. 1, are also widely studied because DNA hypomethylation induces the re-activation of tumour suppressor genes that are silenced by methylation-mediated mechanisms [9,10]. As a result genes that are silenced are switched back on to regulate differentiation, cell proliferation and apoptosis hindering cancer progression [11–13]. To give rise to differentiation-inducing activity decitabine has been shown to work optimally using low dose schedules [14,15].

HDAC inhibitors, such as vorinostat (Suberoylanalide hydroxamic acid, SAHA) Fig. 1, suppress the activities of multiple HDACs, leading to an increase in histone acetylation. This histone acetylation induces an enhancement of the expression of specific genes that elicit extensive cellular morphologic and metabolic changes, such as growth arrest, differentiation and apoptosis [7,8,16,17].

Recently, the combination of hypomethylating agents or HDAC inhibitors with other chemo-therapeutics has gained increasing interest as a possible molecularly targeted therapeutic strategy [18]. In particular, the combination of HDAC inhibitors with hypomethylating agents has become attractive since histones are connected to DNA by both physical and functional

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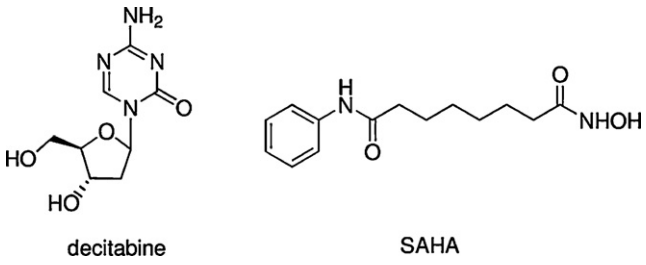

Fig. 1. Chemical structure of decitabine and SAHA.

interactions. To date, the accumulating evidence has confirmed the hypothesis that the combination of DNMT and HDAC inhibition is very effective (and synergistic) in inducing apoptosis, differentiation and/or cell growth arrest in human lung, breast, thoracic, leukemia and colon cancer cell lines [19–21].

Previous pharmacokinetic-pharmacodynamic (PK–PD) studies showed linear PK after intravenous [22] and oral administration of SAHA [23,24]. Significant variability of both C_{max} and AUC was observed between patients potentially related to the rate-limited absorption of the drug in the gastro-intestinal tract. Nonetheless, the persistence of acetylated histones paralleled the prolonged plasma concentrations observed after oral administration of SAHA [23]. Similarly, studies carried out with decitabine established a clear correlation between peak plasma levels of decitabine and demethylation in PBMC (pharmacodynamic endpoint) [12,25].

To support further clinical studies combining SAHA and decitabine and determine their PK parameters, analytical methods that can quantify the analytes of interest reliably are essential. In addition the assay would need to be sufficiently sensitive for low dose drug monitoring. Previous analytical methods of quantification of decitabine have been achieved using HPLC-UV [26,27]. However these methods suffered from having a high limit of detection and a lack of specificity due to non-differentiation between decitabine and its breakdown products and matrix interferences which co-eluted with decitabine. Recently Liu et al. [28] reported a mass spectrometry method for the quantification of decitabine. The study found decitabine to be highly unstable in water and plasma and they subsequently characterised decitabine decomposition products. Due to its unstable nature the authors suggested processing samples quickly and at low temperatures (4 °C). However during clinical studies it may not always be possible to transfer and process plasma samples immediately from patients and at low temperature conditions. An alternative would be the use of cytidine deaminase inhibitors such as tetrahydrouridine that are known to prevent breakdown of decitabine [29]. This could potentially be applied to plasma or blood during the sample collection in clinical studies.

For SAHA there have also been a few published methods for quantitation [30,31]. SAHA, is not as polar as decitabine making a single method for both compounds chromatographically difficult to optimise. On the other hand in comparison to decitabine, SAHA was reported to be stable in serum through freeze-thaw stability studies [30].

This study presents a HPLC method for the simultaneous determination of decitabine and SAHA, validated in human plasma, allowing the quantification of the two analytes with a single LC–MS detection method. In order to preserve the stability of decitabine the use of tetrahydrouridine (THU) an inhibitor of cytidine deaminase has been evaluated. The method is currently being used in clinical pharmacokinetic studies for the evaluation of decitabine and SAHA when administered in combination.

2. Materials and methods

2.1. Chemicals and solutions

SAHA and deuterated-SAHA (SAHA-d5) were provided by Merck (West Point, PA, USA). Decitabine and 2′-azido-2′-deoxyuridine (2-ADU) were purchased from Sigma, (Sigma, Gillingham, UK). THU was purchased from Merck. Formic acid was from BDH (Poole, UK). HPLC grade acetonitrile (MeCN) and methanol was from Rathburn (Walkerburn, UK).

2.2. Plasma and standard solutions

Human plasma was obtained from healthy volunteers and aliquoted (1 ml) into tubes containing THU at 100 μg ml^{-1} unless otherwise stated. The THU doped plasma was used for analysis or stored in a −70 °C freezer until analysis.

All stock standard solutions of decitabine, 2-ADU, SAHA and SAHA-d5 (1 mg ml^{-1}) were prepared individually in methanol and stored at −70 °C conditions until analysis, the stock standard solutions were given a 1 month expiry date. A separate stock for quality controls (QCs) of decitabine and SAHA was also prepared.

Mixed working standard solutions (20 and 10 μg ml^{-1} of decitabine and SAHA, respectively from stock and then 1/10, 1/100 and 1/1000 dilutions) were made up fresh in water (1 ml final volume) on the day of analysis. For preparation of calibration curves concentrations of 0, 2, 10, 20, 40, 100, 200, 400, 1000 and 2000 ng ml^{-1} were used for decitabine and therefore SAHA at half decitabine concentrations. QC samples were prepared in plasma at 20, 100, 1000 and 10, 50 and 500 ng ml^{-1} for decitabine and SAHA, respectively. A mixed internal standard solution in water was freshly prepared on the day of analysis at 20 μg ml^{-1} for 2-ADU and 10 μg ml^{-1} for SAHA-d5.

2.3. Plasma preparation and extraction

Standard mixture working solutions of decitabine and SAHA were prepared by mixing and serially diluting the stock solutions (Section 2.2).

Various volumes of mixture solutions of decitabine and SAHA were added to 50 μl of plasma in 0.65 ml microcentrifuge tubes followed by 5 μl of internal standard to make a final concentration of 4000 ng ml^{-1} 2-ADU and 2000 ng ml^{-1} SAHA-d5. Next 150 μl of acetonitrile was added and the tubes vortexed for *ca.* 10 s and after centrifugation at 13,000 × *g* for 14 min at ambient temperature, the supernatant was transferred into a snap cap vial (300 μl) and samples analysed by LC–MS/MS.

2.4. Instrumentation

The HPLC system comprised of a Dionex (Sunnyvale, CA, USA) 3000 Ultimate series LC connected to a 4000 Q Trap LC–MS/MS system (Applied Biosystems, Foster City, CA, USA) mass spectrometer, equipped with an orthogonal electrospray ion source. Data were acquired and processed with Chromeleon 6.1 and Analyst 1.4 chromatography manager software.

Compounds were separated on a Gemini C18 (150 mm × 2.0 mm I.D.) and 3 μm particle size (Phenomenex, Torrance, CA, USA) protected by a Phenomenex Gemini C18 (4.0 mm × 2.0 mm I.D.) and 3 μm particle size guard cartridge. The HPLC method used gradient elution; mobile phase solvent A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The initial mobile phase composition of 92% solvent A and 8% solvent B was maintained for 2 min. Between 2 and 9 min the percentage of mobile phase B was increased to 35% and then back to initial the mobile phase composition within 0.1 min, with a total time of 14 min. The column was set at a flow rate of 0.2 ml min^{-1} and a temperature of 36 °C. Sample volume of 6 μl was used for all LC–MS experiments.

The mass spectrometer was operated in electrospray mode. The source temperature was 450 °C and the spray voltage 3 kV was used. The collision gas pressure was 1.5 mTorr. All analytes were optimised using the Analyst software auto tune facility for SRM transitions (Table 1) with dwell times set at 75 ms.

2.5. Quantification of decitabine and SAHA

The peak area ratios of decitabine/2-ADU and SAHA/SAHA-d5 were used for all quantitation and to construct calibration curves using a regression analysis with $1/x$ weighting. Quality control concentrations were calculated from the regressed equation.

The limit of detection (LOD) was defined as three times the signal-to-noise ratio. The lowest limit of quantitation (LLOQ) was defined as the lowest level of analyte that could be reliably detected and reproducible with a precision of ≤20% and accuracy of 80–120%.

2.6. Determination of recovery, accuracy and precision

The absolute recovery of decitabine and SAHA was determined by comparison of the peak areas from non-extracted and extracted samples at two levels of concentrations in duplicate. The intra-day accuracy and precision were determined at three different levels of concentrations from six replicate QC samples. The inter-day accuracy and precision were determined at three levels of concentrations from six replicate QC samples on three independent occasions.

The accuracy was calculated as the relative mean error (RME) with RME (%) = [(mean − theoretical concentration)/theoretical concentration] × 100. The precision was calculated as the relative standard deviation (RSD) of the mean with RSD (%) = (standard deviation of the mean/mean) × 100.

2.7. Stability

2.7.1. Short-term stability

Two aliquots of mid concentration QC were left at room temperature and analysed at intervals of 0, 1, 2, 3, 4, and 5 h. The study was repeated using the same intervals and a 24 h time-point with and without THU. Freeze-thaw stability: six aliquots of low, mid and high QCs were left in light and ambient conditions until thawed and then frozen for 12 h. The process was repeated a further two times before analysis. Post-preparation stability: post-preparative stability was determined by re-running QC samples ($n = 6$) at three concentrations after a period of 24 h in the HPLC autosampler. Long-term freezer stability: three aliquots at each of the QC concentration were stored at −70 °C for 5 months and analysed compared to fresh QC.

3. Results and discussion

The method was validated in terms of selectivity, limits of quantification, recovery, accuracy and precision, and stability.

3.1. Optimisation

The chromatographic conditions were optimised to give adequate separation of decitabine from the column void volume ($t_0 = 1.4$ min) due to its high polarity when using common reversed phase columns such as the Phenomenex® Gemini® C18. Elution of analytes of interest is preferential after the column void volume due to matrix components that do not interact with the column eluting at the same time. This can lead to suppression of the signal of interest and the effect was observed for decitabine in the optimisation experiments for this method. Liu et al. [28] noted that reversed phase columns with hydrophilic end-capping provide better retention of highly polar molecules. Although the use of these columns resulted in better retention for decitabine, SAHA was also very highly

Table 1
Ion transitions and optimised parameters for detection of decitabine, 2′-azido-2′-deoxyuridine (2-ADU—internal standard for decitabine), SAHA, deuterated-SAHA (SAHA-d5) and THU

Analyte	Decitabine	2-ADU	SAHA	SAHA-d5	THU
Transition	229.0 → 113.0	270.1 → 113.0	265.2 → 232.1	270.3 → 237.1	249.3 → 117.0
Collision energy (CE)	17	13	19	19	17
Declustering potential (DP)	21	36	51	51	51
Entrance potential (EP)	10	10	10	10	10
Cell exit potential (CXP)	18	6	18	18	18

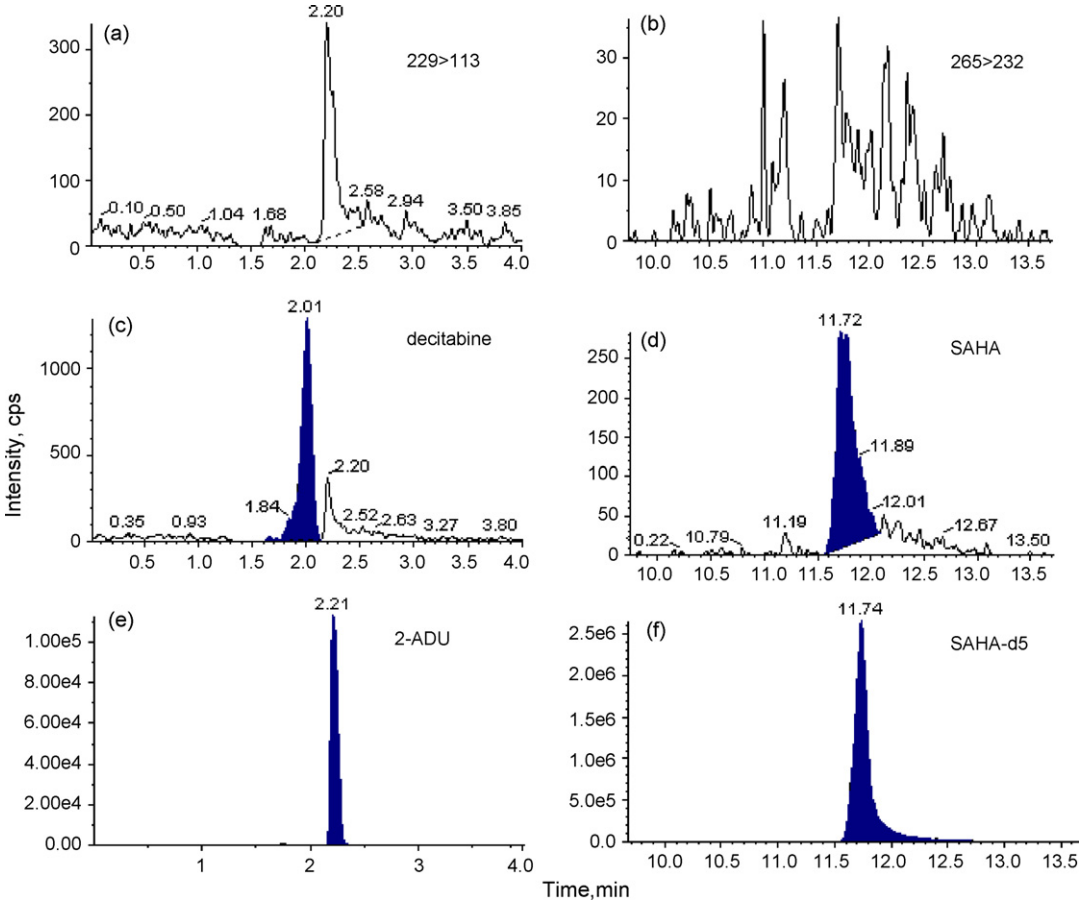

Fig. 2. LC–MS/MS extracted ion chromatogram of (a) plasma blank using transition 229 > 113, (b) plasma blank using transition 265 > 232, (c) decitabine at LLOQ (10 ng ml^{-1}), (d) SAHA at LLOQ (1 ng ml^{-1}), (e) 2′-azido-2′-deoxyuridine (2-ADU as decitabine internal standard) and (f) SAHA-d5.

retained leading to longer chromatographic run times and a lower response for SAHA due to a broader peak shape even whilst using various gradient elution profiles. To solve these problems, a gradient was optimised using the Phenomenex® C18 column where the initial gradient condition started with a high aqueous percentage (92% aqueous 0.1% formic acid and 8% acetonitrile).

3.2. Specificity

The specificity of the extraction and chromatographic method tested the ability of the method to differentiate and quantitate the analyte in the presence of other endogenous constituents in the sample and to detect potential interferences. No interfering peaks were observed and no significant peaks were found at the retention times of the analytes using the simple acetonitrile precipitation step for both analytes. A peak was observed in the decitabine plasma blank chromatogram at 2.2 min and was attributable to 2-ADU as without 2-ADU (results not shown) there was no peak at this retention time. Nevertheless there is baseline separation between decitabine and the peak at 2.2 min. The chromatographic separation of plasma blank extract whilst using SRM transitions for decitabine and SAHA are presented in Fig. 2.

Ion suppression was monitored by constant infusion of a mixed decitabine and SAHA standard at mid-QC level through a T-piece into the out-coming HPLC column eluent (i.e. bypassing the HPLC column). This was followed by subsequent injections through the HPLC column of MeCN (i.e. extraction solvent) and extracted blank plasma. Ion suppression was observed by any negative peaks that appeared in the elevated baseline. Negative peaks were observed at 1.4 min for both decitabine and SAHA in both solvent and blank plasma injections and at 4 min in for decitabine in blank plasma only. At the retention times of decitabine and SAHA there was no difference in baseline response between solvent and blank plasma injections suggesting ion suppression is minimal or absent.

3.3. Linearity

Standard curves were performed in duplicate for each analyte in plasma. In all cases the regression coefficient was >0.99. Decitabine was linear over the range of 0–2000 ng ml^{-1} and SAHA over a range of 0–1000 ng ml^{-1} with a weighting of 1/x. Typical regression parameters (r^2, slope and intercept) for calibration curves were 0.9998, 0.004 and 0.012 for decitabine and 0.9994, 0.001 and −0.001 for SAHA.

Table 2
Intra- and inter-day accuracy and precision for decitabine and SAHA in human plasma with THU

Analyte	Nominal concentration (ng ml^{-1})	Intra-day accuracy and precision[a]		Inter-day accuracy and precision[b]	
		RME (%)	RSD (%)	RME (%)	RSD (%)
Decitabine	20	7.4	7.0	3.6	7.1
	100	3.2	3.0	3.2	5.5
	1000	−8.4	6.9	−8.3	7.6
SAHA	10	3.6	8.6	4.3	10.0
	50	−7.0	10.5	−5.1	6.9
	500	−11.7	6.8	−12.1	4.4

[a] $n = 6$.
[b] $n = 18$.

3.4. LOD and LLOQ

The LOD determined experimentally was 1.0 and 0.125 ng ml^{-1} for decitabine and SAHA, respectively. The LLOQ was 10 and 1 ng ml^{-1} for decitabine and SAHA, respectively. Results for accuracy and precision (RSD) at the LLOQ for six replicates were 102 ± 13.2% and 92.3 ± 10.4% for decitabine and SAHA, respectively.

3.5. Recovery

The recovery was determined by calculating the concentration of the low and high QCs processed in duplicate using a non-extracted calibration line. The recovery was low but consistent for decitabine: 55.0 ± 11.6% and 56.4 ± 3.6% for the low and high QC, respectively. The recovery in the plasma was excellent for SAHA with values from 90.1 ± 3.8% and 94.3 ± 4.1% for the low and high QC, respectively.

3.6. Intra-day accuracy and precision

The accuracy and precision were calculated from six QC replicates at three levels of concentrations for each compound. The intra-day results are summarised in Table 2. The accuracy as determined by the relative mean error was comparable for both analytes with values between 7.4% and −8.4% for decitabine and 3.6% and −11.7% for SAHA. The precision, evaluated by the RSD, ranged from the lower value of 3.0% for decitabine to maximum 10.5% for SAHA.

3.7. Inter-day accuracy and precision

The accuracy and precision were calculated from six QC replicates at three levels of concentrations for each compound on three independent occasions. The inter-day results are summarised in Table 2. The accuracy was satisfactory and was ≤12.1% for both analytes. The precision (RSD) was also consistent ranging from 5.5% to 7.6% for decitabine and 4.4% to 10.0% for SAHA.

3.8. Sample stability

Drug stability in plasma or serum is a function of the storage conditions, the chemical properties of the analyte, the matrix and the container system. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling from being taken from the patient to final analysis. Therefore, we evaluated the short-term stability where two aliquots of mid concentration QC were left at room temperature and analysed at intervals of 0, 1, 2, 3, 4, and 5 h. This short-term stability experiment revealed no significant degradation for SAHA in human plasma when left at room temperature over a period of 5 h. In contrast decitabine showed significant degradation (∼60%) under the same conditions. A subsequent time-course was taken of plasma spiked with decitabine and SAHA and completed at the same time intervals as above and with a 24 h time point with and without THU, an inhibitor of cytidine deaminase. Included in the time-course study (results not shown) was monitoring of decitabine in THU doped plasma blank at various concentrations (25, 50, 100,

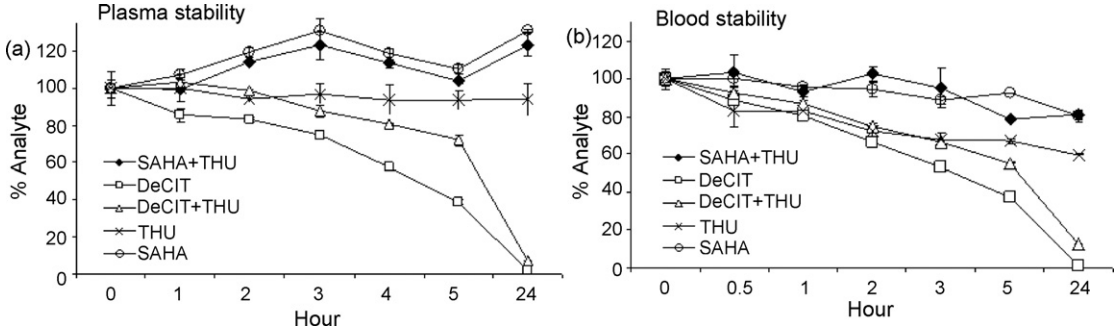

Fig. 3. Stability time profile of decitabine and SAHA ± THU and THU in human plasma at intervals over a period of 24 h at room temperature. Decitabine and SAHA at mid QC concentration and THU at 100 μg ml^{-1} ($n = 2$). SRM transitions used as specified in Table 1.

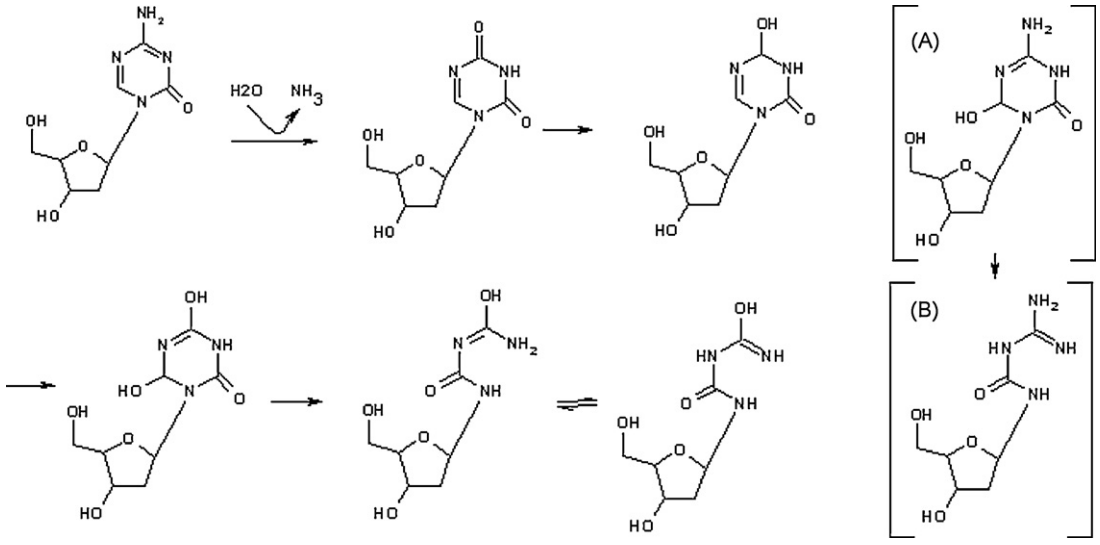

Fig. 4. Proposed mechanism of deamination and decomposition of decitabine in human plasma. The undeaminated hydrated product of decitabine is shown as (A) and its decomposition product is shown as (B).

250 and 500 μg ml^{-1}). THU at a concentration of 100 μg ml^{-1} provided optimum inhibitory effect (competitive inhibition) at room temperature, above this concentration there was no gain in stability.

Fig. 3 indicates that SAHA as a percentage of analyte remaining from time = 0 h was unaffected over a period of 24 h (100–130%), but there was a significant loss of response for decitabine: 20% after 2 h, 65% after 5 h and 95% after 24 h. Fig. 3 also shows the percentage of analyte remaining from time = 0 h decitabine in plasma doped with THU. Clearly THU increased the stability of decitabine: from 35% to 65% without and with THU, respectively at 5 h. As degradation of decitabine is a continuous process in plasma, the effect of THU on the stability of decitabine was also tested in blood. The results display a similar pattern to that of plasma with increased stability of decitabine in the presence of THU. However, unlike in plasma, THU concentrations decreased over time and might be explained by a redistribution of both analytes in blood. Therefore it is recommended that blood is immediately spun down to get plasma. Degradation of decitabine was also prevented by transferring spiked plasma samples without THU doping, immediately at −70 °C. There was no difference in stability in the preparation of spiked samples between THU doped plasma at room temperature and THU doped plasma on ice, this eliminates having to carry out extractions at 4 °C as suggested by Liu et al. [28].

Overall, the results suggest that storage of decitabine plasma samples in presence of THU would increase the stability of decitabine. Moreover, the extraction processes would not have to be carried out under and <5 °C. Previously Liu et al. [28] suggested a possible breakdown mechanism for decitabine in water and plasma via a different hydration mechanism. The results from these experiments with THU suggest that the mechanism of decomposition of decitabine follows both hydration and deamination pathways independently (Fig. 4) in which the latter can be delayed by competitive inhibition using THU.

The freeze-thaw and long-term stability results are presented in Table 3 and did not reveal any degradation problems for both analytes, using the optimal storage conditions for decitabine. Samples stored at −70 °C doped with THU at 100 μg ml^{-1} for 5 months were stable for both analytes. Post-preparative stability where samples were re-injected after being left in the HPLC autosampler for 24 h showed an average accuracy for decitabine six replicates at 20, 100 and 1000 ng ml^{-1} was 113.0 ± 7.3%, 107.6 ± 4.3% and 91.8 ± 4.2% and for SAHA at 10, 50 and 500 ng ml^{-1} was 107.3 ± 5.4%, 95.4 ± 9.7% and 84.8 ± 4.3%.

Table 3
Freeze-thaw and long-term stability accuracy and precision for decitabine and SAHA in human plasma with THU

Analyte	Nominal concentration (ng ml^{-1})	Freeze-thaw[a]		Long-term stability[b]	
		Mean	RSD (%)	Mean	RSD (%)
Decitabine	20	110.2	4.9	100.2	10.0
	100	111.7	1.6	86.7	7.7
	1000	100.6	8.3	87.6	4.5
SAHA	10	105.9	8.1	99.6	3.3
	50	93.0	3.6	95.7	3.3
	500	87.8	2.3	92.0	6.3

[a] $n = 6$, samples analysed on third freeze-thaw cycle.
[b] $n = 3$, samples stored at −70 °C for 5 months.

4. Conclusion

A few HPLC methods have been developed in the last few years for the quantification of decitabine in plasma and SAHA in serum separately for clinical application. The present method was validated for the simultaneous quantification of decitabine and SAHA in human plasma.

The limit of quantification was similar to the previous published method for decitabine [28]. For SAHA, the limits were better than previous studies [25,26]. The method should lend itself to low dose scheduling studies involving decitabine and SAHA.

The enhancement of stability for decitabine can be gained by adding THU an inhibitor of cytidine deaminase to plasma sample prior to storage at −70 °C. This should allow sufficient time to process samples after blood sampling from patients without substantially affecting the sample integrity.

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