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Rapid quantitation of cyclophosphamide metabolites in plasma by liquid chromatography–mass spectrometry

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Abstract

A method is described for the quantification of two metabolites of cyclophosphamide, specifically 4-hydroxycyclophosphamide (HCy), and carboxyethylphosphoramide mustard (CEPM). Plasma HCy is derivatized to the phenylhydrazone which is quantitated by LC–MS monitoring the chloride adduct of the derivative. The LLOQ based on material applied to the system is ∼20 fmol. Plasma CEPM concentration is determined using LC–MS with a deuterated internal standard. Both assays have 50-fold dynamic range and require less than 4 h to complete. The development of this rapid analytical method makes it feasible to adjust the dose of cyclophosphamide based on the pharmacokinetic disposition of HCy and CEPM in hopes of decreasing nonrelapse mortality in cancer patients.

Keywords: Hydroxycyclophosphamide; Carboxyethylphosphoramide mustard; CEPM; LCMS; Chloride adduct; Phenylhydrazine; Plasma analysis; Drug targeting

1. Introduction

The alkylating agent cyclophosphamide (Cy) is frequently used in patients undergoing myeloablative conditioning prior to a hematopoietic cell transplantation. There is substantial individual variability in Cy metabolism resulting in a wide range of the area under the concentration-time curve (AUC) of the parent drug, and an even greater interpatient variability in the AUC of its metabolites [1]. Cyclophosphamide is a prodrug, the majority of which undergoes hepatic oxidation to 4-hydroxycyclophosphamide (HCy) by multiple isozymes of cytochrome P450 (CYP) [2] Fig. 1. Hydroxycyclophosphamide tautomerizes to aldophosphamide, and the concentrations of these two metabolites have not been quantified independently [3]. HCy is a "transport" metabolite able to diffuse into target cells where it breaks down into acrolein and phosphoramide mustard (PM) – the active form of the drug. HCy itself is subject to further oxidation resulting in the nontoxic metabolites carboxyethylphosphoramide mustard (CEPM) and 4-oxocyclophosphamide (KetoCy). Several other metabolites are formed from HCy including reduction to the 3-hydroxypropylphosphoramide mustard, dehydration to the unsaturated imino cyclophosphamide, and conjugation with glutathione to form the 4-glutathionylcyclophosphamide (GS-Cy). These latter reactions are reversible and HCy can be regenerated by some combination of oxidation, elimination, and/or hydrolysis [2,4,5]. As such, they were considered "reservoir" compounds which did not contribute to the overall clearance of HCy. Recently, however, it has been determined that GS-Cy is a substrate for ABCC2, a member of the ATP binding cassette family of proteins, and is actively removed from the liver into the bile [6].

We recently completed a prospective study looking for a pharmacodynamic relationship between the concentrations of Cy, HCy, and CEPM with outcomes in patients receiving Cy in combination with total body irradiation as the conditioning regimen for hematopoietic cell transplant [1]. Increased AUC of CEPM was associated with a higher risk of liver toxicity and non-relapse mortality, leading to the hypothesis that rapid dose adjustment of Cy to a lower AUC of CEPM while maintaining a minimum AUC of HCY would lower non relapse mortality

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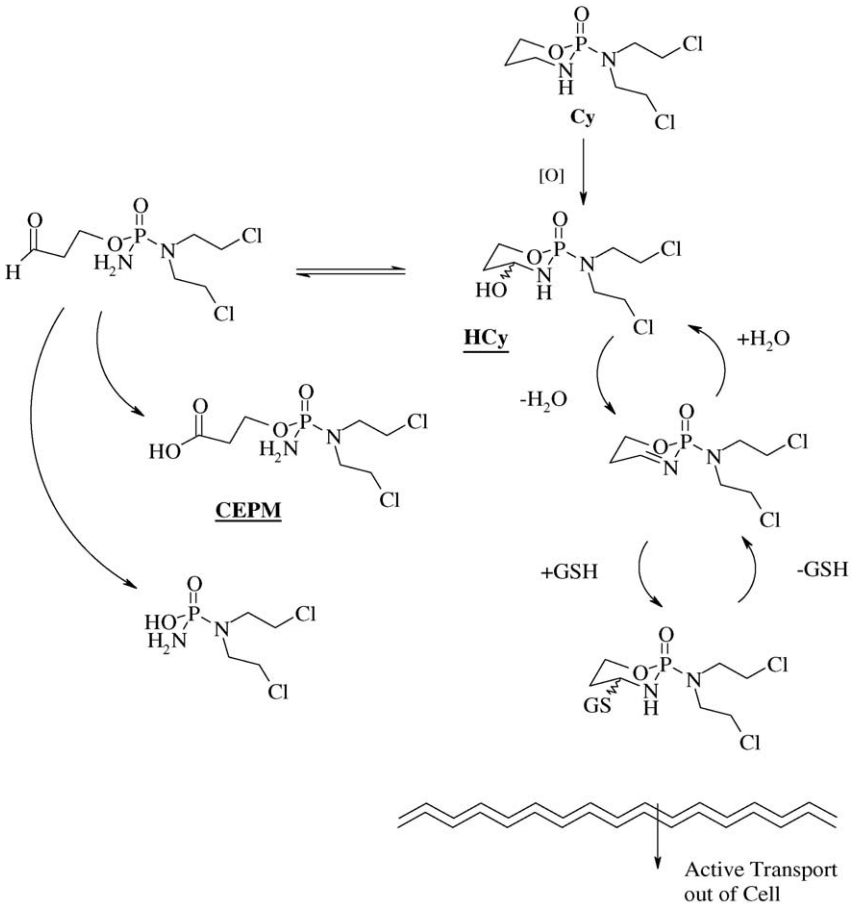

Fig. 1. Metabolic disposition of HCy showing the different pathways resulting from the ring opened (aldehyde) vs. closed (alcohol) tautamers.

without impacting disease relapse or rejection. This hypothesis necessitated the development of an analytical method that has a rapid (or 'stat') quantitation of pharmacokinetic samples drawn over 16 h after administration of the first Cy dose. Essential characteristics of this stat analytical method include minimal sample workup, short chromatography runs, and maximal sensitivity. The development of such an analytic method provides for the ability to rapidly quantitate plasma HCy and CEPM, perform pharmacokinetic modeling and recommend a subsequent Cy dose to achieve the desired AUC of CEPM and HCy within 6 h of the last pharmacokinetic sample.

Both the continued interest in the compound and the difficulty in the analysis is indicated by the variety of methodologies developed over the years as recently reviewed [7,8]. Hydroxycyclophosphamide is particularly challenging. The metabolite is very unstable in plasma ($t_{1/2} \sim 4$ min) [9] and requires rapid derivatization to prevent hydrolysis to acrolein and PM. Various reagents have been used to trap the intermediate including aryl hydrazines [10,11], semicarbazide [12,13], sodium cyanide [14,15], or substituted hydroxylamines [16–18] forming the hydrazones, semicarbazone, cyanohydrin, or oximes, respectively. The resulting derivatives were then quantified by thin-layer chromatography, HPLC with U.V. or MS detection, or gas chromatography. These methods are useful for batch analysis, but the complex sample work up make them inappropriate for a stat assay. To this end, methodology has been developed to facilitate the analysis without sacrificing accuracy or precision. Phenylhydrazine is now used as the derivatizing reagent for HCy as it reacts at a higher pH and does not result in rapid hemolysis, which was problematic when using *p*-nitrophenylhydrazine. In addition the collection procedure uses vacutainer tubes containing the derivatizing reagent. The lab work-up involves a one step solid phase extraction (SPE) procedure of the centrifuged sample prior to analysis using negative ion ESI LC–MS. Sensitivity is such that sample concentration following SPE is not required. The analyte is subject to a variety of reactions in the ionization chamber and forms complexes with mobile phase constituents as well as simple loss of H^+. When a low concentration of ammonium chloride is added to the mobile phase, there is almost exclusive formation of the chloride adducts which is used for quantification. Although chloride adducts are well known in mass spectrometry, the chloride anions are usually generated using mobile phase modifiers such as chloroform [19] and methylene chloride [20] rather than from ammonium chloride. Using the salts allows one to optimize conditions to form the chloride adduct rather those needed to generate the ion from the solvent. The technique was first used in the qualitative analysis of polyfunctional neutral compounds that fragment under positive ion conditions. More recently it has been used for the quantitative analysis of nitrates [20,21] and glycones [22].

Although CEPM is not as widely studied as HCy, there have been a variety of methods developed for its quantification using LC–MS [17,23,24] or GC–MS [25]. The difficulties encountered with the analysis of CEPM have generally resulted from the chemical instability of the compound particularly at low pH and elevated temperature [17,26]. This problem has been minimized by keeping the samples at physiological pH, maintaining the samples at 4° throughout the procedure, and using a streamlined procedure with a deuterated internal standard. The sample work-up is a simple acetonitrile (ACN) deproteinization prior to chromatography using a short column and a rapid gradient. The efficient ionization of CEPM in the negative AP-ESI mode allows for a rapid "dilute and shoot" procedure. In addition, the method uses both the same mobile phases and column as the HCy assay. This allows both compounds to be analyzed using the same mobile phase and column with different gradient and data acquisition parameters.

2. Experimental

2.1. Materials

4-Hydroperoxy-cyclophosphamide (HOOCy), a chemical precursor of HCy, was the kind gift of Asta Medica (Frankfurt, Germany); CEPM and D_4-CEPM were prepared by our laboratory according to published methods [27]. The deuterated CEPM was made using D_4 mustard prepared by the method of Jardine et al. [15]. Ammonium chloride, sodium thiosulfate, methanol and acetonitrile were from Fisher (Pittsburgh, PA); phenylhydrazine hydrochloride was from Sigma (St. Louis, Mo), Ammonium hydroxide, sodium citrate dihydrate, and acetic acid were from Baker (Phillipsburg, NJ). All other chemicals were reagent grade or better.

2.2. Sample collection and work up

2.2.1. HCy

The derivatizing reagent for HCy was prepared as a 4.0% (w/v) of phenylhydrazine hydrochloride in 0.05 M sodium citrate buffer adjusted to pH 6.0 with 1.0 M NaOH. Aliquots (4.0 mL) were transferred to vented 7.0 mL plastic vacutainer tubes. The tubes were lightly capped without sealing and placed in a vacuum chamber. A vacuum was applied and maintained at −20 psi for 5 min at which times the caps were pressed firmly down forming an airtight seal. The tubes were stored at −20 °C until use, at which time they were thawed and kept on ice or refrigerated at the patient's bedside. At the time of pharmacokinetic blood sampling, 2 ml of whole blood was collected into the vacutainer tubes, inverted to complete mixing, and stored at 4 °C until processed. All blood samples were transported to the laboratory within 17 h of the start of Cy infusion at which time they were centrifuged for 5 min at 4 °C at 3000 rpm, 2.0 mL of the supernatant removed and left at room temperature for 45 min to complete derivatization. Subsequently, 1.0 mL of the sample was applied to a 200 mg C-18 Bondelute SPE column (Varian, Lake Forest, CA) which had been washed with 3.0 mL MeOH and 6.0 mL water. Impurities were washed through with 2.0 mL water followed by 2.0 mL 77% 0.1M AcOH; 23% MeOH, the column allowed to dry under vacuum for 2.0 min, and the analytes eluted with 1.0 mL ACN. Two microliters of the resulting solution was applied to the LC–MS system without further processing.

2.2.2. CEPM

A second sample of blood was collected over EDTA and stored at 4 °C until CEPM analysis. It was then centrifuged at 1500 × *g* for 5.0 min at 4 °C. One hundred microliters of the supernatant was transferred to a 0.5 microfuge tube and 20 μL internal standard solution (D_4CEPM; 25 ug/mL) added followed by 120 μL ACN. The suspension was vortexed for 30 s and centrifuged at 14,000 rpm for 5 min; supernatant (50 μL) transferred to an injection vial, and 1.0 μL applied to the LC system for quantification.

2.3. Mass spectrometry

An Agilent Series 1100 MSD (Agilent, Palo Alto, CA) single quadrapole mass spectrometer configured with an electrospray ionization (ESI) source capable of both positive and negative modes of ionization was used in these studies. Salient features of the Agilent ChemStations software (Agilent, Palo Alto, CA) were used to optimize MS parameters for the analytes using flow injection analysis (FIA) and for subsequent integration and data processing.

2.4. HPLC

The HCY-phenylhydrazone derivative (HCyPH) and CEPM were separated on an Agilent Series 1100 HPLC system equipped with a cooled autosampler and directly interfaced with the mass spectrometer. Separation of both analytes was achieved on a Zorbax Extend 50 mm × 2.1 mm, 5 μ C-18 column (Agilent, Palo Alto, CA) using a water:methanol gradient. The aqueous phase consisted of 1.0 mM ammonium chloride, 10 mM ammonium acetate adjusted to pH 8.5.

In the analysis of HCyPH, 2.0 μL of the sample were injected and the following gradient was used. The initial MeOH content of 40% was increased to 90% over 1.0 min, maintained there for an additional minute, and returned to initial conditions at 2.5 min The column was then reequilibrated for 2.5 min prior to the next injection. The flow was maintained at 250 μL/min throughout and the column temperature was unregulated; column eluent prior to 2.0 min and after 4.0 min was diverted to waste. The mass spectrometer was operated in the negative ion ESI mode with fragmentor and capillary voltages held at 30 and 3000 V, respectively, with a nitrogen desolvation gas flow of 10 L/min. Quantification was based on the integrated peak area values obtained from the *m/z* 401 selected ion current chromatogram, corresponding to the $[^{35}Cl_2HCyPH + {}^{35}Cl]^-$ abundance.

The same mobile phase and column were used in the analysis of CEPM, but the HPLC gradient conditions were altered to improve chromatography and response. The initial MeOH fraction was 4% and increased to 35% over 1.0 min. It was maintained at 35% for an additional minute before a linear decrease to

the initial conditions at 3.0 min. The column was reequilibrated for 3 min prior to the next injection. The mass spectrometer was operated as before. The ions monitored were *m/z* 291, 293, and 297 consistent with the $[^{35}Cl_2CEPM\text{-}H]^-$, $[^{35}Cl^{37}ClCEPM\text{-}H]^-$, and $[D_4{}^{35}Cl^{37}ClCEPM\text{-}H]^-$, respectively. Quantitation was based upon the peak area ratio of *m/z* 293 to *m/z* 297 ion chromatograms.

2.5. Standard curves and control

2.5.1. HCy

An HCy stock solution (1.0 mg/mL) was prepared by the reduction of a 10.58 mg HOOCy in 10 mL sodium thiosulfate solution (1.2 mg/mL) at room temperature for 30 min. The stock solution (3.61 mM) was then diluted with blank plasma: derivatizing reagent (1:2) solution producing five standards ranging in concentration from 0.5 to 25 μM in plasma. Aliquots (1.5 mL) were transferred to individual vials and kept at −70 °C until use.

2.5.2. CEPM

Stock solutions of CEPM are prepared in water ranging in concentration from 341 to 5.3 μM. Ten microliters aliquots are diluted with 90 μL blank plasma sealed and stored at −70 °C until use.

2.6. Validation

2.6.1. Derivatization parameters

The kinetics of HCy derivatization over 2 h was studied in terms of reaction rate and maximum response of the derivative observed at 0 °C, 25 °C and 40 °C. The stability of the isolated derivative under analytical conditions in various solvents at several pH values was tested over 10 h, more than twice the actual run time.

2.6.2. Linearity, precision, limits of detection

The linearity of the assay was established over the described range of concentrations used in the standards described above. Intra- and inter-day variability was tested by running controls on a single day and at least 2 controls per day over several months. The limits of detection are described both as the lower limit of quantification (LLOQ) of HCY or CEPM in plasma and the minimum amount of the derivative applied to the column assuming 100% derivatization and recovery.

3. Results and discussion

3.1. Sample collection and preparation

3.1.1. HCy

The initial studies demonstrated that the system described is both precise and accurate. In tests of 51 samples the average sample drawn was 1.99 ± 0.06 mL (3.0%). Although the method is more robust when using glass vacutainer tubes, plastic collection tubes are now predominant in clinical settings, and we adapted the method to these tubes. The vacuum is patent and the derivatizing reagent effective for 3 days at −20 °C. When using glass tubes, the system is stable for 10 days at −20 °C. Routinely, the tubes are prepared the day of use. Several other derivatizing reagents were tried including semicarbazide, hydroxylamine, and nitrated phenylhydrazines. The derivatizing reagent and solution were chosen to cause minimal hemolysis during sample collection and thus eliminate the need for immediate work up in the clinical setting. In addition, at pH 6, chosen to optimize the rapid formation of the desired hydrazone, the rate of HCy breakdown is dramatically decreased.

3.1.2. CEPM

Tests done on the CEPM breakdown occurring in whole blood kept at 4 °C or on ice demonstrated virtually no breakdown over 24 h. This was true both in blank plasma and patients' blood samples, suggesting there was no detectable interaction of CEPM with other Cy metabolites at low temperatures.

3.2. Mass spectrometry and derivatization

3.2.1. HCy

Initial studies were carried out on synthesized derivative in the positive ion ESI mode yielded spectra exhibiting ions at *m/z* 367, 389, and 405, corresponding to the monoisotopic protonated species $[HCyPH + H]^+$, as well as the sodium and potassium adducts, respectively (data not shown). The ionization of the derivative in the negative mode appeared to be substantially more efficient based on total ion abundance. The spectra was quite complicated as shown in Fig. 2A. Peaks were observed for monisotopic masses of the deprotonated derivative, $[M - H]^-$ at *m/z* 366, and adducts of chloride $[M + Cl]^-$, acetate $[M + AcO]^-$, and trifluouroacetate $[M + TFA]^-$ at *m/z* 401, 425, and 479, respectively. When 1.0 mM NH_4Cl was added to the mobile phase and acetate eliminated the $[M + Cl]^-$ ions predominated and all other decreased to 5% of the total (Fig. 2B). The superimposed extracted ion chromatograms of derivatized HCy analyzed with and without ammonium chloride dramatically illustrate the effect of this modification on ion abundance (Fig. 3). In an attempt to gain further sensitivity by the reduction of the number of charged species in the isotopic envelope of the adduct, ammonium fluoride instead of ammonium chloride was added to the mobile phase. Unfortunately, the adduct was not formed efficiently and the chloride adduct was still evident. All of these ionic species observed with or without NH_4Cl in the mobile phase elute at the same time. Therefore we assume that they result from gas-phase cyclization or elimination reactions that release chloride ions in the ionization chamber which subsequently form an adduct with unfragmented analyte. A possible pathway for the formation of these ions are summarized shown in Fig. 4.

As would be expected, the chloride adduct formed varies with the concentration of ammonium chloride in the mobile phase. This relationship is shown in Fig. 5. Although the optimal response occurs at 0.3 mM, the response is more reproducible at 1.0 mM ammonium chloride. Despite the 18% loss of sensitivity, we use the higher concentration to increase the precision of the assay.

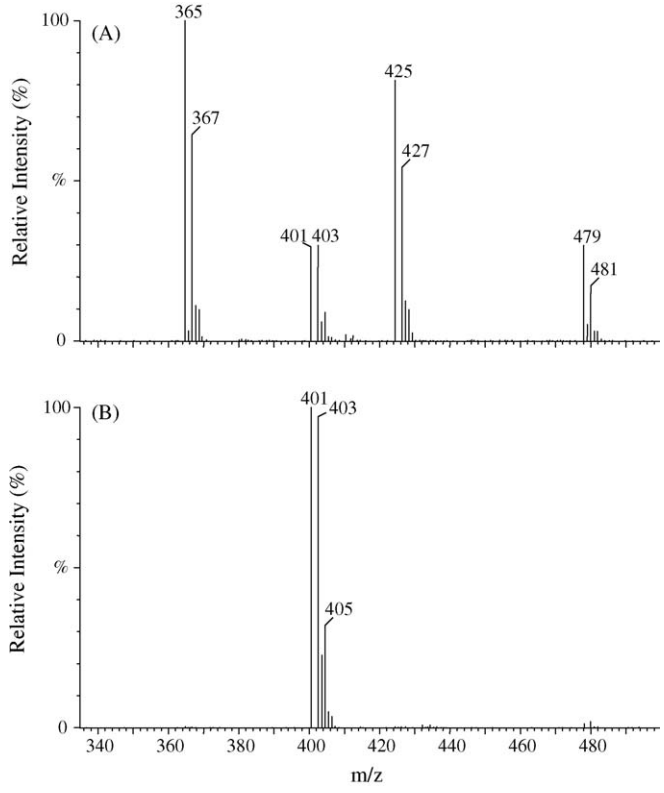

Fig. 2. Mass spectrometry of the ionization of the HCy-PH derivative in the negative mode. (A) (upper) Spectrum taken in acetate buffer/MeOH showing signals at monisotopic mass *m/z* 366 $(M-H)^-$, 401 $(M+Cl)^-$, 425 $(M+AcO)^-$ and 473 $(M+TFA)^-$. (B) (lower) Spectrum taken in 1.0 mM NH_4Cl/MeOH with one signal at the monoisotopic mass *m/z* 401 $(M+Cl)^-$.

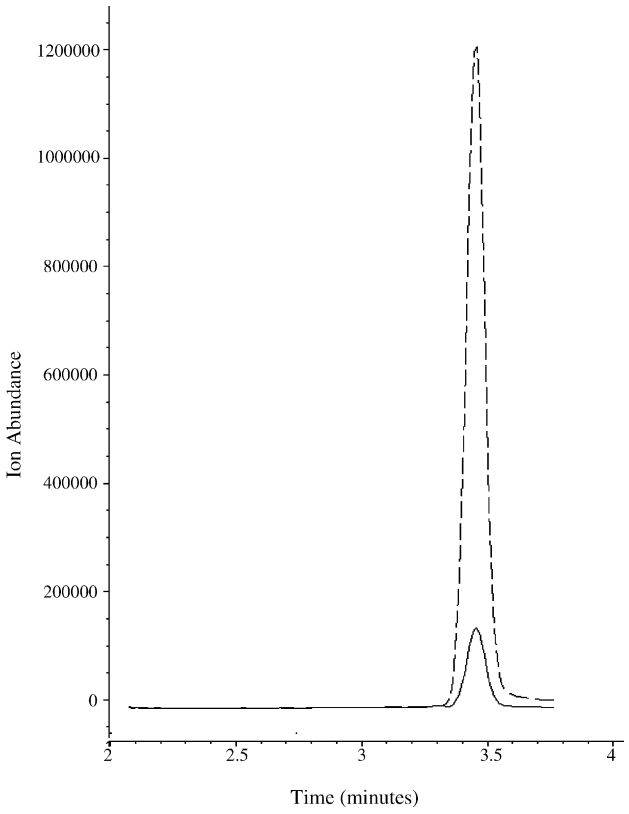

Fig. 3. The effect on the intensity of the $(HCyPH+Cl)^-$ ion at *m/z* 401 with (dashed line) and without (solid line) NH_4Cl. Approximately 500 fmol of the derivative were applied to the column.

The kinetics of the HCy derivatization showed that at room temperature the product reached its maximum concentration in plasma after 45 min with less than 5% change up to 60 min and a slow degradation thereafter ($t_{1/2} \sim 10$ h). The reaction at 40 °C reached its maximum after 15 min, but substantially less derivative was observed and subsequent breakdown more rapid. The reaction rate at 4 °C was prohibitively slow; the maximum had not been reached after 4 h.

3.2.2. CEPM

Mass spectra of CEPM and D_4CEPM show the expected ions at *m/z* 291 and 295, respectively, corresponding to the deprotonated molecular species. Under the described conditions, there is virtually no fragmentation of the $[M-H]^-$ ion and no effect was observed when ammonium chloride was added to the mobile phase. The isotopic envelopes for these compounds contained the expected ion overlap of the $^{37}Cl_2$ species of CEPM to the $^{35}Cl_2$ of the D_4CEPM at *m/z* 295. For this reason the $^{35}Cl^{37}Cl$ isotopic species, *m/z* 297 of the internal standard was used for quantification.

3.3. Chromatography

3.3.1. HCy

Chromatograms from a plasma blank and patient plasma 8 h post infusion of 45 mg/kg cyclophosphamide are shown in

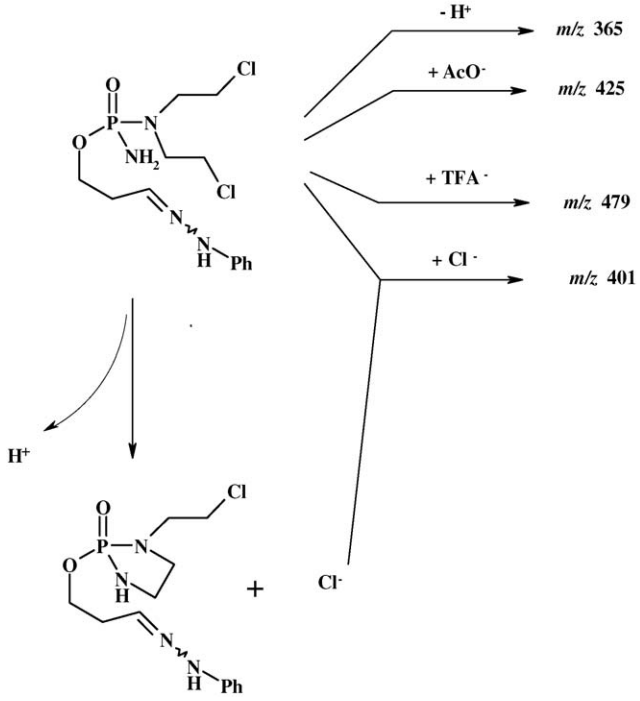

Fig. 4. A possible series of reactions which would account for the different ionic species observed in the MS scans. Initial fragmentation from thermal and/or ionization catalysis results in chloride anions that can then complex with the parent molecule. Other ions result from deprotonization and complex formation with acetate or trifluoroacetate anions.

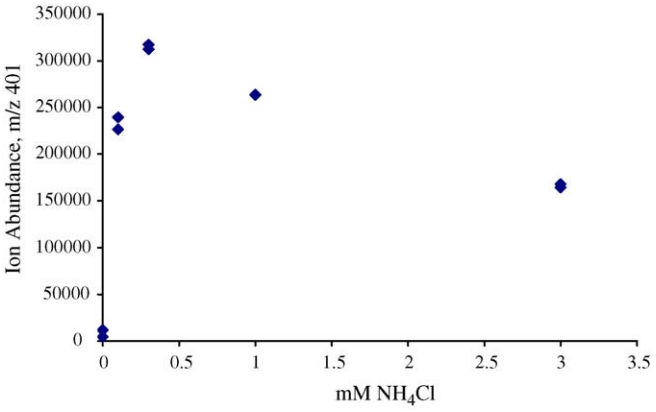

Fig. 5. The effect of the NH_4Cl concentration in the aqueous mobile phase on the intensity of the $[M + Cl]^-$ ion. The data was obtained in triplicate in the SIM mode under the chromatographic conditions described in the text. The optimum response was observed at 0.3 mM salt when no buffer was present.

Fig. 6A and B, respectively. The separation system as described does not separate the *cis* and *trans* isomers of the phenyl hydrazone, thus avoiding the loss of sensitivity that would occur if the two components were resolved. Theoretically, this could result in a loss of precision if the response factor for the cis isomer was different than the trans and distribution of the stereoisomers varied from sample to sample. Empirically, we have found the precision of the assay suggests this factor is not a significant problem.

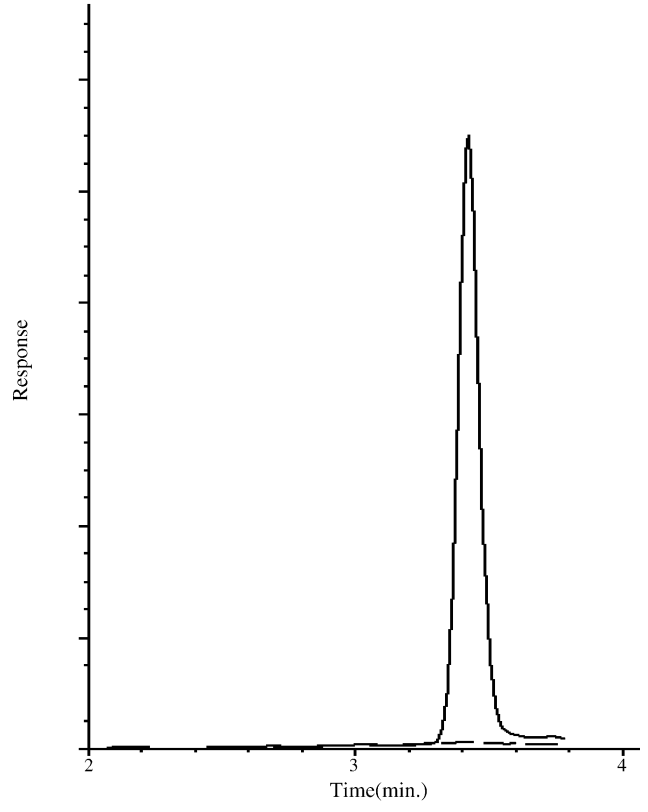

Fig. 6. SIM chromatograms at *m/z* 401 of HCyPH from patient plasma before (dashed line) and 8 h after an infusion of 45 mg/mL cyclophosphamide over 1 h. The observed signal is equivalent to an HCy concentration of 18 μM. There is virtually no interference observed in the blank samples under clinical conditions.

3.3.2. CEPM

The chromatograms of CEPM and D_4CEPM from patient plasma at $T = 0$ and $T = 8$ h are shown in Fig. 7A and B. As one can see there is no interference from the internal standard in the analyte chromatogram. The patient's sample drawn 8 h after infusion demonstrates an easily quantifiable signal.

3.4. Assay validation

3.4.1. Linearity, precision, and limits of detection

The method described has excellent linearity for both analytes. Routinely, the r^2 values for both compounds are >0.995 and the calculated intercept less than 20% of the lowest standard. Back calculated values for the standards accumulated over 12 months are shown in Table 1 for HCy and Table 2 for CEPM. Controls were prepared at 7 μM for both components and were analyzed with every run. Calculated concentrations had to be within 10% of the known amounts or the assay was rerun. Over a 12 month period, this has never been required.

Although the lowest standard of HCy is 0.5 μM, the method can be used to quantify substantially lower concentrations. Fig. 8A shows a chromatogram of a sample of 0.02 μM HCy superimposed on an appropriate blank. Assuming 100% derivatization and recovery, this is equivalent to approximately 15 fmol on column. The LLOQ for CEPM is 0.4 μM and is shown in Fig. 8B. This translates to 180 fmol on column.

3.4.2. Stability of assay products

After the HCyPH has been isolated as described, there is approximately 5% breakdown over 6 h when left in the HPLC injector at 4 °C. The stability is highly solvent dependant making the SPE elution media critical. When methanol or a solvent with aqueous constituents is used, the breakdown is much faster, apparently from hydrolysis of the hydrazone. By partially drying the SPE column after rinsing off impurities and eluting with pure acetonitrile, the problem from hydrolysis is minimized. Six hours is over twice the time needed to complete the assay. As has been reported, CEPM is most stable at neutral pH and the stability enhanced by maintaining the samples at 4 °C. This precludes the use of acid ($HClO_4$, TCA) or base ($ZnSO_4$) deproteinization. We have found that treatment with one volume of acetonitrile results in a suitable injection sample with no difficulty from analyte breakdown over the time required. In addition, the deuterated internal standard used in the assay compensates for any variability in MS response.

4. Discussion

Negative ion MS in Cy metabolite analysis has, to the best of our knowledge, not been used before. For both of the components of interest it provides a major increase in sensitivity relative to the positive mode. The lower background noise routinely seen in negative ion MS provides better signal to noise ratios and increased precision.

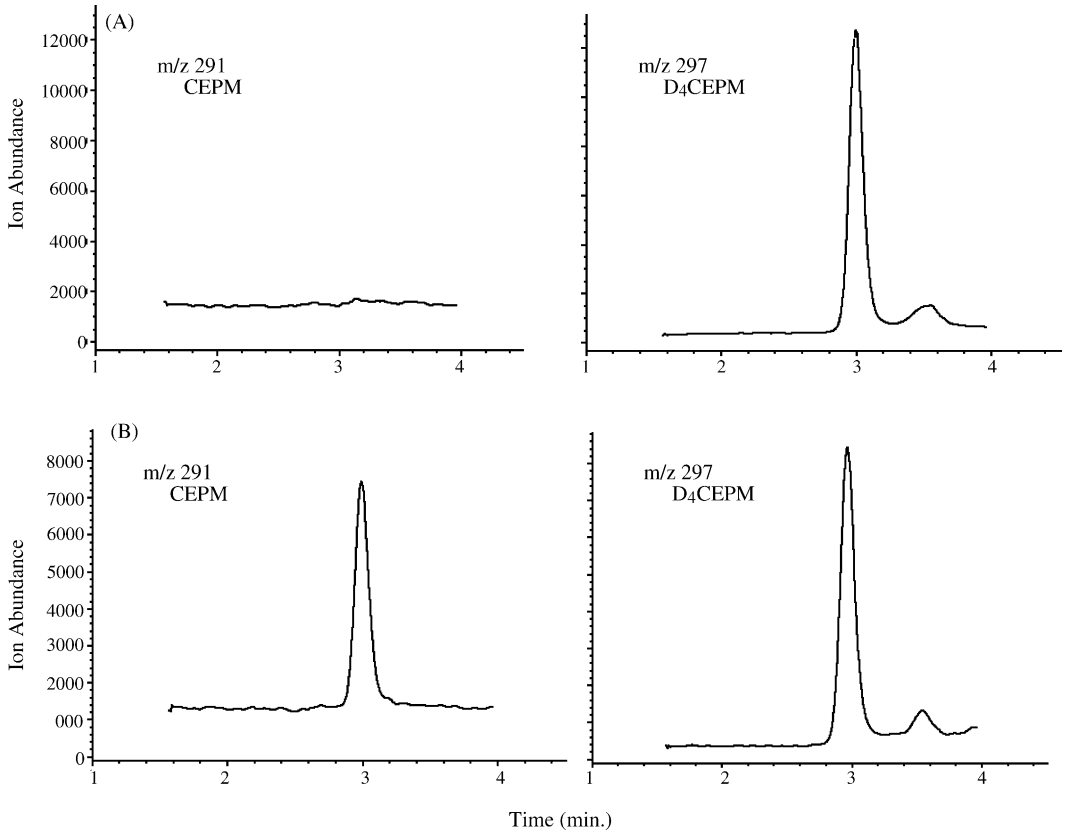

Fig. 7. Chromatograms of CEPM before (A); and 8 h after (B) cyclophosphamide infusion. Generally, there is no significant interference from endogenous compounds in patient plasma. The signal represent approximately 3 μM CEPM concentration or 1.5 pMol applied to the system.

Table 1
Precision and accuracy of HCy assay from plasma

Nominal [HCy] (μM)	*N*	Found [HCy] (μM)	S.D.	Precision (% of nominal)	Accuracy (% of nominal)
25.0	30	24.13	0.68	2.83	96.5
15.00	30	15.60	0.45	2.86	104.0
5.00	30	5.17	0.35	6.72	103.4
2.00	30	1.98	0.17	8.84	99.0
0.50	30	0.48	0.06	11.78	96.0

Chloride adducts are well documented and have been described particularly when using APCI where the chloride anion is generated from the reduction of small amounts of chloroform or carbon tetrachloride which are included in the mobile phase. This technique is typically used for qualitative analysis and is particularly effective for the analysis of polyfunctional compounds subject to elimination and/or fragmentation in the positive mode. Using ammonium chloride in the mobile phase has a major advantage in that all ionization parameters can be adjusted to maximize the formation of the desired adduct rather than a compromise between Cl^- generation and adduct formation. While there is the statistical loss of sensitivity resulting

Table 2
Precision and accuracy of CEPM analysis from plasma

Nominal [CEPM] (μM)	*N*	Found [CEPM] (μM)	S.D.	Precision (% of nominal)	Accuracy (% of nominal)
34.13	20	34.49	1.25	3.62	101.5
17.06	20	15.31	1.56	10.22	89.7
8.53	20	8.52	0.42	4.93	99.9
4.27	20	4.32.	0.23	5.35	101.2
2.13	20	2.19	0.12	5.53	102.8
1.07	20	1.05	0.12	11.53	98.1
0.53	20	0.55	0.13	23.72	103.8

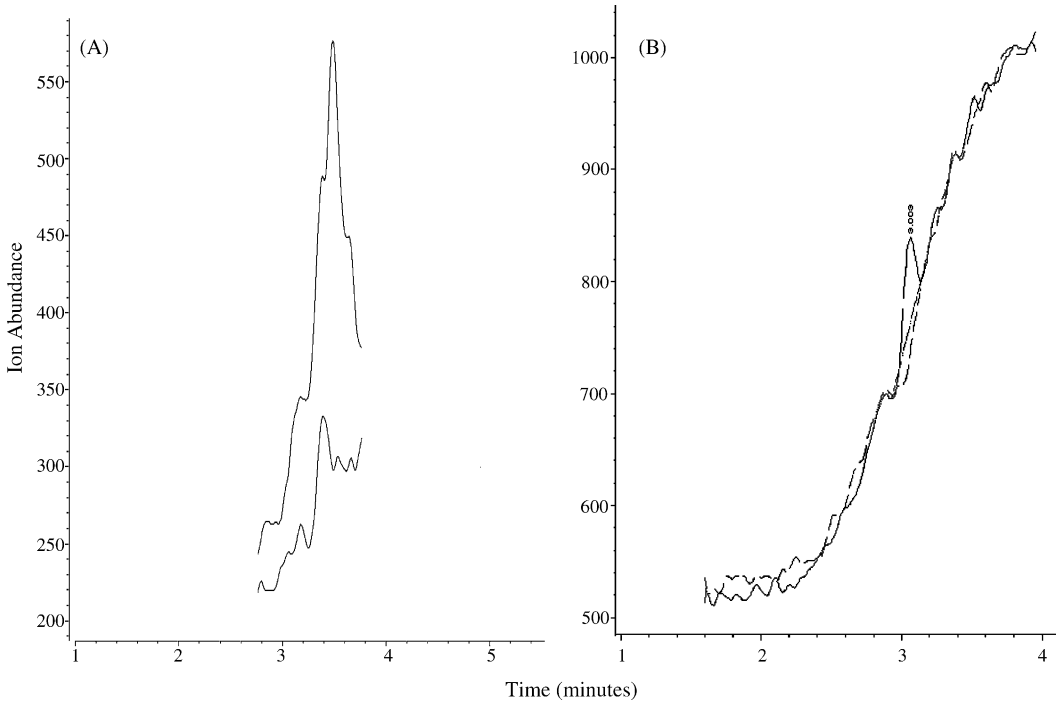

Fig. 8. The lower limits of detection of HCy (A); and CEPM (B). The HCy signal results from 25 fmol applied to the column assuming 100% recovery from the sample workup. The LLOQ of CEPM equivalent to 100 fmol applied to the column. Relevant blanks are shown with both chromatograms.

from the charge distribution among the 4 possible combinations of chloride isotopes, this is more than compensated by the increased intensity of the ion. If, however, better sensitivity is necessary, it is possible to quantitate based on the sum of signals at *m*/*z* 401 and 403 which are almost equal in intensity. With the direct addition of chloride into the mobile phase, the concentration dependant variations of different adduct formation are eliminated. An additional advantage of the use of chloride this method is that it successfully competes with trifluoroacetate in adduct formation. Since TFA is a common component in HPLC separations and difficult to completely wash out of a system, this characteristic increases the ruggedness of the system.

The assay presented has met the clinical demands of a stat procedure. The method, from the time the samples are delivered from the hospital through derivatization, sample work-up and chromatography of the two different metabolites of interest takes ∼4 h for an individual patient including standard curves and controls. This allows time for pharmacokinetic analysis and calculations of any Cy dose adjustments based on HCy and CEPM AUCs to be made within 6 h of the last sample. This method was used in a phase 1 study in HCy patients in which the CEPM and HCy target AUCs were achieved [28]. Successful completion of this study was facilitated by communication with the clinical study staff which gave the laboratory at least 7 days notice of when the Cy dose adjustments were necessary. The LC–MS utilized is not dedicated solely to Cy dose adjustments, although priority is given to these studies and there is a second machine available if the first instrument is non-operational. Further clinical trials are needed to demonstrate the decrease non-relapse mortality and/or increased overall survival with real-time targeting of HCy and CEPM AUC.

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