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Simultaneous analysis of cyclophosphamide, doxorubicin and doxorubicinol by liquid chromatography coupled to tandem mass spectrometry

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Abstract

A method for the simultaneous determination of cyclophosphamide (CP), doxorubicin (dox), and doxorubicinol (dol) was developed and validated to analyze 400 μL of plasma from patients receiving chemotherapeutic treatment with CP and dox. Final calibration ranges for the analytes were 0.440–60.0 μg/mL for cyclophosphamide, 7.20–984 ng/mL for dox and 3.04–104 ng/mL for dol. The samples were prepared using solid phase extraction and analyzed using a gradient separation over a Waters Symmetry® C18, 2.1 by 30 mm (Milford, MA) column. Detection was achieved in positive mixed reaction monitoring mode on a triple quadrupole mass spectrometer.

Keywords: Anthracyclines; Pharmacokinetics; Obesity; Assay

1. Introduction

The combination of cyclophosphamide (CP) and doxorubicin (dox) is commonly used as adjuvant treatment of breast cancer in women with a high risk of recurrent disease [1,2]. Patients generally receive between four and six courses of these drugs, each course given every two to three weeks. Substantial variations in chemotherapy dosing in overweight and obese women indicate clinical uncertainty about how chemotherapy in heavy women should be dosed. [3–5] Although small studies have shown that clearance of doxorubicin and cyclophosphamide may be dependent on body weight, there is little clinical evidence to support dose reduction. [6,7] Obesity is a known risk factor for the development of breast cancer and a negative prognostic indicator in women; therefore, systematic undertreatment of breast cancer in overweight and obese women may contribute to a poorer prognosis. Individualization of drug doses has been proposed as a way to deliver the most effective and safe doses of these drugs [8–10]. Such an approach would require rapid and accurate assessment of drug and metabolite levels in order to tailor subsequent doses.

Chromatography methods have been published to measure each drug and its metabolites [11–18], but none have been developed to measure them within a single plasma sample.

While HPLC is a less expensive, more available approach, mass spectrometry can achieve more specificity often with a shorter analysis time. Desai et al. [11] found that using fluorescence to measure dox and dol nonspecificity due to the interference of several metabolites occurred. Since fluorescence is more specific than ultraviolet detection, they concluded that the specificity of tandem mass spectrometry (MS/MS) was needed to correctly identify and quantitate pure compound. Several methods cited analyze for CP and/or its metabolites. Sadagopan et al. [12] developed a liquid chromatography–tandem mass spectrometric assay for both CP and its hydroxyl metabolite (CPOH), using simple sample preparation of protein precipitation; however the average accuracy of the back-calculated standards in plasma was ±10% and higher

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in other matrices tested. Baumann et al. [13] developed a liquid chromatography assay for CP and its metabolite using MS in selected ion mode (SIM). The time per sample chromatogram was 18 min and the chromatography peak shape of cyclophosphamide was poor. Huitema et al.'s HPLC method for sample analysis required 35 min, analyzed for the mustard form only and required derivatization procedures [14]. Methods cited for dox and dol provide adequate sensitivity but variations reported are high in the Larchatre et al. method [15]. The method of Kummerle et al. does not assay for the doxorubicinol, and that of Arnold et al. is specific to the rat [16,17]. Overall, stability of all three analytes within various common laboratory conditions was not completely found in the literature and the method validation data presented in these articles varied greatly. None of these reports included any data proving the stablity of the method when samples were reanalyzed.

This report describes a method used to quantitate CP, dox and dol from a single human plasma sample of 0.4 mL. The method has been validated using the FDA guidelines [19] and applied to clinical plasma samples for a pharmacokinetic study. The data for reproducibility, accuracy, matrix effects, and stability is presented.

2. Experimental

2.1. Apparatus

Validation of this method was conducted using an Agilent high pressure liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA): Agilent Series 1100 Autosampler, Agilent Series 1100 Degasser, Agilent Series 1100 LC Pump. The HPLC system was coupled to an Applied Biosystems PE/Sciex API 3000 mass spectrometer (MS) (Applied Biosystems, Foster City, CA). All apparatus were controlled by Analyst Software, Version 1.4 (Applied Biosystems, Foster City, CA); all data was collected with the same. The chromatographic separation was conducted at ambient temperature using a Waters Symmetry® C18, 2.1 by 30 mm (Milford, MA) shielded by a guard column, 2.1 by 10 mm of the same material and manufacturer. The flow rate of the mobile phase was held at 250 μL/min. Mobile phase compositions are described in the chemicals and reagents section below. An 8 min gradient separation starting at 75% mobile phase A and 5% mobile phase B was utilized; final percentages were 25% mobile phase A, 75% mobile phase B, respectively. Re-equilibration to initial conditions was allowed for by adding 3 additional minutes.

The mass spectrometer was operated in the mixed-reaction-monitoring (MRM) positive ion mode using a Turbo Ionspray® interface. The desolvation temperature of the interface was 350 °C and the ion current was set at 4000 V. Nitrogen was used as the desolvation, nebulizer and collision gas. Prior to elution of components from the column, the ion current voltage was set at 5 V to divert the solvent ions front from entering the mass spectrometer. Table 1 summarizes the assay characteristics. Fig. 1 displays the fragmentation pattern of each compound and their optimized MS setting for detection. Fragments for CP, dol, and the internal standard were previously reported in the literature [12,17]. All identification and fragmentation was optimized using the following procedure: Once prepared in 1.0 μg/mL solutions in optimum mobile Dox was infused using a syringe pump at a rate of 10 μL/min with a turbo ion spray source. A manual optimization is performed to show a mass spectrum of all precursor ions for the analyte using a full scan acquisition (*m/z* 200–7000 amu, step size 0.1 amu). This ionization generates few fragment ions, therefore an in-source induced fragmentation was used to obtain confirmation ions and optimised by modulating the collision energy through the orifice voltage. A quantification ion (corresponding to the most intense of the high mass ions in the spectrum) and a confirmation ion (free from interfering peak) were chosen for each analyte. The mass spectrophotometer was calibrated monthly by infusion of a commercial mixture of polyproplyene glycols and monitoring *m/z* ratios in the 100–1000 amu mass range.

2.2. Chemicals and reagents

Cyclophosphamide monohydrate, Doxorubicin HCl, and Daunorubicin HCl were purchased from Sigma Chemical Company (St. Louis, MO). A solution of doxorubicinol HCl was purchased from Quentas (Brandford, CT). All were corrected for purity and salt forms when weighed or diluted for standard stocks. Ammonium acetate, acetic acid, HPLC grade water, hydrochloric acid, and HPLC grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ).

The mobile phases consisted of 5 mM acetate buffer, pH 3.5 mixed with HPLC grade methanol. Mobile phase A was 95% buffer and 5% methanol; mobile phase B was 5% buffer and 95% HPLC grade methanol. Lots of heparinized human plasma was purchased from Valley Biomedical (Knoxville, TN).

2.3. Preparation of standard and quality control solutions

Stock solutions of analytes were prepared at 1 mg/mL in methanol. Calibrators were prepared by combining differing volumes of CP, dox and dol stocks to prepare the highest cali-

Table 1
Assay characteristics

Analyte	Calibration range	Ion rair *m/z*	Approximate retention time (min)
Cyclophosphamide	3.6–60.0 μg/mL	261.1/140.1	4.2
Doxorubicin	7.20–984 ng/mL	544.4/321.2	4.7
Doxorubicinol	3.04–104 ng/mL	546.2/363.2	4.3
Daunorubicin	Internal standard	528.5/321.00	5.9

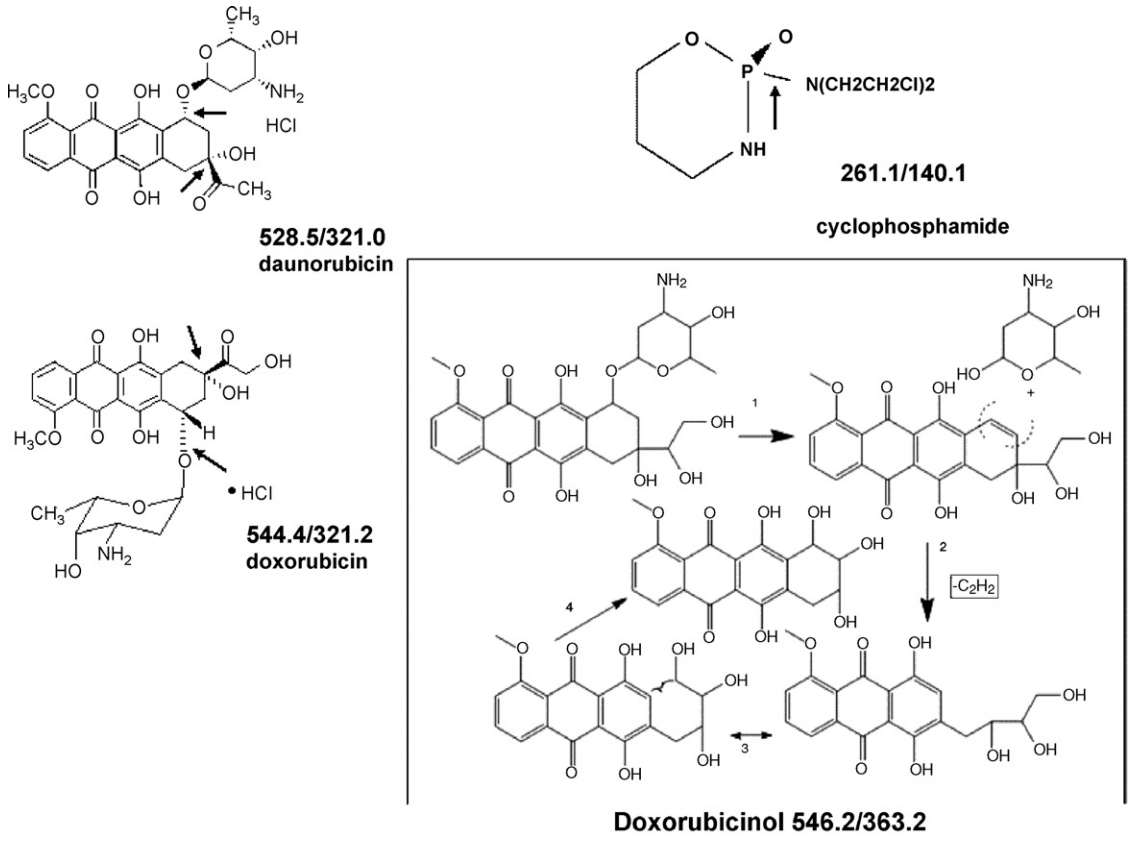

Fig. 1. Structures of analytes and their monitored ion pair for MS/MS determination; arrows mark fragmentation except for doxorubicinol where the conversion is shown.

brator at approximately 300.0 μg/mL CP, 5000 ng/mL dox, and 500.0 ng/mL dol. All dox concentrations in standard calibrators were 10-fold higher than dol.

Dilutions of the highest calibrator and a mid-range calibrator in methanol produced 12 calibration solutions. Calibrators were prepared by adding 80 μL of each stock solution into a 0.4 mL aliquot of blank heparinized plasma. Final calibration ranges for the analytes were as follows: 0.440–60.0 μg/mL for CP, 7.20–984 ng/mL for dox, and 3.04–104 ng/mL for dol. Internal standard stock was prepared at 1 mg/mL in methanol. To prepare a working dilution for use in the assay, the stock was diluted to 3.0 μg/mL in methanol.

Separate 1 mg/mL stocks were prepared for quality controls. Several quality controls were prepared by adding small volumes of stocks to heparinized plasma; six quality control levels were prepared and stored frozen in 1 mL aliquots at −70 °C. For CP 50.0, 20.0, 8.00, 3.20, and 1.60 μg/mL quality controls were utilized. For dox 800, 320, 128, 51.2, and 25.6, and 5.12 ng/mL quality controls were utilized. For dol 80, 32, 12.8, and 5.12 ng/mL quality controls were utilized.

All stocks, calibrators and controls were stored at −70 °C.

2.4. SPE extraction

Samples, standards, controls, and blanks were prepared for solid phase extraction by adding 80 μL of working internal standard solution to 0.4 mL of sample and 0.8 mL of 0.1 N HCL, vortexed well after each addition. The sample solutions were then centrifuged at 2800 × *g* for 10 min.

For each sample, a Waters Oasis HLB 1 mL extraction cartridge (Milford, MA) was conditioned using methanol and then HPLC grade water. 1 mL of each sample solution supernatant was loaded onto its cartridge and washed with 5% methanol in water. The final sample was eluted using two 1 mL rinses of HPLC grade methanol. The methanol was evaporated under house air at 35 °C. Each sample was reconstituted with 100 μL of reconstituting solution consisting of 75% mobile phase A: 25% acetonitrile solution. The reconstituted sample was transferred to a microcentrifuge and spun at moderately high speed to remove any particulates. The supernatants were transferred to amber glass autosampler vials with inserts for analysis. 20 μL were injected in the HPLC for LCMS/MS analysis.

2.5. Validation studies

To determine the validity of the method developed, the FDA guidelines were utilized [17]. Five days of calibration curves, with replicates of six quality controls were performed to determine intraassay and interassay variation and accuracy. Each day the lowest standard was prepared an additional six times and their results calculated as unknowns to determine the variation at the proposed lower limit on each day. In addition, duplicate control determination was performed on the day that recovery and

matrix studies were performed. Our laboratory chose to require 15% accuracy criteria at its lowest calibrator as opposed to the 20% required by the FDA. Within these validation study days, recovery, matrix effect, and stability issues were determined. These experiments are described below.

To determine recovery and matrix effects several lots of plasma ($n = 5$) containing sodium heparin anticoagulant were tested to determine whether endogenous interferences or matrix suppression or enhancement would occur and if matrices independent of the calibrators and validation samples would provide accurate results. To accomplish this, seven extracts of each matrix were prepared: three spiked with a mix of the three analytes and IS before extraction (pre-spike); three spiked with analytes and IS after extraction (post-spike); and one was left as blank. Actual analyte concentrations in the different sources of matrix were calculated as unknowns using the calibration curve. Percent recoveries were calculated by averaging the peak areas post- and pre-spike replicates for each matrix separately and dividing the mean pre-spike result by the mean post-spike result for each matrix. To determine the effect of matrix alone, mean responses from triplicate injection of analyte in mobile phase were compared to the mean response of the three plasma samples spiked after extraction.

Stability experiments included three freeze–thaw cycles for plasma samples (from −70 °C to room temperature) and room temperature stability in plasma for 24 h in light and dark. Due to stability issues found from the experiments, two freeze–thaw cycle studies and additional stability studies at room temperature were performed for 5 h and 1 h. An overnight thaw at 4 °C was also investigated. Control samples were thawed for 20 min. To test stability, triplicate sets of treated plasma controls were compared to the replicates of six plasma controls (untreated). Results for the treated group were compared to the untreated group using an unpaired *t*-test.

2.6. Patient study and analysis of patient samples

The Research Subject Review Board at the University of Rochester approved this study and all subjects were required to provide informed consent before any study procedures were initiated. Nine samples were drawn from each of 23 patients (total samples = 206, one sample was not collected) receiving combination doxorubicin (Adriamycin; doxorubicin HCl) and cyclophosphamide (cytoxan; cyclophosphamide) therapy for breast cancer treatment. Samples were collected between at 1, 1.5, 2, 3, 4, 6, 8, 24 and 48 h after therapy was initiated. Whole blood collected into sodium heparinized tubes was processed to yield plasma within 30 min of collection. Plasma for this assay was aliquoted to cryovials and immediately frozen at −70 °C. Plasma for the assay of the metabolite was added to another cryovial containing derivatization reagent to capture and stabilize the hydroxylated form of CP as described by Belfayol [22]. After this method was successfully validated, a standard operating procedure was constructed based on the validation parameters and sample limitations derived from the validation process. All samples were subsequently analyzed for CP, dox, and dol concentrations.

2.7. Reproducibility of patient sample analyses

During the course of patient sample analyses, several samples required reanalysis for one or more analytes for various quality assurances reasons. Therefore, for some analytes, a second valid result was produced. The results were compared to determine the reproducibility of the method's measurements and further assure stability of analytes within true patient samples.

2.8. Calculations

Statistical tests for significance were performed using SYSTAT Version 11 (Systat Software, Inc, Richmond, CA). Two-sample *t*-tests were used to determine if any differences were affected during treatment of control samples under various environmental and handling conditions. The extreme studentized deviate test (Grubbs test) was used to determine if the matrix affected the accuracy of the analytes' results. Percent target was calculated as a percent of observed concentration divided by target concentration. Variability, or coefficient of variation (CV), was calculated as a percent of the relative standard deviation from the mean. Calibration curves and calculations of unknowns or controls were calculated using Analyst™, Version 1.4 (Applied Biosystems, Foster City, CA). Dox and dol calibration curves used a $1/x$ weighting with a linear fit. CP calibration curves utilized either $1/x$ or no weighting with a power fit.

Standard non-compartmental techniques were used to calculate pharmacokinetic parameters using WinNonlin™ Version 4.1 (Pharsight, Palo Alto, CA). The area under the concentration–time curve was determined using the trapezoidal rule and the maximum observed concentration during the dosing interval was determined by visual inspection.

3. Results

3.1. Precision and variability of calibration standards and quality controls [intraassay and interassay variation and accuracy]

Calibration curve performances over 6 days were well within acceptable parameters with mean % targets ranging from 91.3 to 109% and CV of <8%. Coefficients of determination (R^2) were greater than 0.992 over the course of validation. The final validated range of quantitation was 7.20–984 ng/mL for dox and 3.04–104 ng/mL for dol. An LLOQ of 3.6 could not consistently be achieved for dox due to omission of the lowest standard (outside ±15%) for 4 of the 6 days. Curves for dox and dol were linear and utilized a 1/concentration squared weighting. For CP, 0.440–60.0 μg/mL was the resulting validated quantitation range with 0.440 μg/mL as the lowest calibrator. For CP, linear curve fittings showed opposite biases at the middle and ends of the curves, due to the saturation of CP signal at high concentration. A power curve fit provided a better fit for CP than the linear fit. To achieve the wide range of nonlinear calibration for CP, two calibration curve ranges were utilized: 0.440 to 11.9 μg/mL and 3.60 to 60.0 μg/mL. For the

Table 2
Summary of accuracy and precision

Analyte	Concentration	Accuracy (%)	Precision (%)	
			Intraassay range (lowest day CV–highest day CV), $n=6$ each on 5 days	Interassay, $n=32$
CP (μg/mL)	50.0	91.4	0.85–6.70	12.4
	20.0	99.5	4.16–6.96	8.87
	8.00	96.3	1.07–13.3	13.2
	3.20	100	1.5–10.7	12.1
	1.60	96.1	1.9–14.4	12.1
Dox (ng/mL)	800	87.2	0.40–3.99	5.07
	320	90.2	1.02–2.53	4.35
	128	91.3	0.47–2.35	5.83
	51.2	89.7	1.24–8.03	6.97
	25.6	88.8	2.37–4.66	5.18
Dol (ng/mL)	80.0	112	1.34–3.88	2.69
	32.0	109	1.71–5.12	5.02
	12.8	107	1.40–5.28	5.86
	5.12	107	3.33–8.17	5.46

lower curve range no weighting was used and for the higher range curve a 1/concentration weighting was used; the coefficients of determination (R^2) achieved with the power fit were ≥0.992.

Variation at the lowest standard concentrations (LLOQ) ranged 1.7–7.2% for CP, 5.7–13.7 for dox, and 4.1–9.8 for dol over each of the 5 days. Across all days the interassay variation at the LLOQ were 4.4%, 16.7%, and 8.9% for CP, dox, and dol, respectively. Accuracy was within ±11% for all analytes.

Quality control performances within each day were acceptable with all mean values for all analytes within 85–115% of target and CV usually well below 10%. Across all 6 days, the interassay variation and accuracy achieved was successful.

For CP, accuracy ranged 91–100% and the CV range across control levels was 4–7%. For dox, accuracy ranged 87–90% and the CV range across control levels was 9–12%. Lastly, for dol, accuracy ranged 107–112% and the CV range across control levels was 3–6%. Table 2 summarizes the accuracy and precision data.

3.2. Recovery and accuracy from independent matrices

Recoveries from the six independent matrices ranged 78–86% for CP, 68–71% for dox, and 69–72% for dol. Accuracies displayed the same range characteristics, 93–101% for CP, 100–105% for dox, and 100–105% for dol; all accuracies were well within 7% of the target or better. Table 3 summarizes the matrices data.

3.3. Matrix effects

Cyclophosphamide exhibited the slight matrix suppression effect, with 92–99% responses in the matrix containing samples as compared to samples without matrix. Dox exhibited no matrix effect with 96–102% responses within the six matrices tested. Dol exhibited a slight enhancement effect, with 101–108% responses measured. None of the values from each matrices were considered statistically significant from others and overall the effects seen were <5% from 100% target (no matrix effect).

3.4. Stability

Results for the stability of the analytes in plasma samples under various laboratory conditions are shown in Table 4. Experimental investigation during method validation provided evidence of stability issues for both freeze–thaw (CP only) and prolonged room temperature exposure (all analytes). CP concentration increased 10% in the high control and 20% in the medium control after three freeze–thaw cycles were conducted ($p=0.016$ and 0.002, respectively). An additional study after two freeze–thaw cycles showed no change in CP concentration ($p>0.05$). Dox and dol exhibited changes from control 1–6%

Table 3
Recovery and matrix effects

Analyte	Matrix #	Recovery (%)	Matrix measurements	
			Accuracy (%)	Suppression (enhancement) (%)
CP	1	80	92.9	7
	2	81	94.0	8
	3	78	94.4	1
	4	80	99.3	3
	5	86	101	7
Dox	1	70	102	3
	2	71	104	3
	3	71	105	(2)
	4	71	102	4
	5	68	100	1
Dol	1	71	105	(4)
	2	72	106	(4)
	3	70	104	(8)
	4	71	103	(1)
	5	69	100	(3)

Table 4
Stability of analytes in plasma

Condition	% Change from control (control level)		
	CP	Dox	Dol
Three-times freeze–thaw (−70 °C)	+10% (H)*	−2% (H)	−6% (H)*
	+20% (M)*	−2% (M)	−1% (M)
	+10% (L)*	−1% (L)	−1% (L)
Two-times freeze–thaw (−70 °C)	−2% (H)	−2% (H)	−4% (H)
	−5% (L)	−2% (L)	+2% (L)
24 h room temperature–light	+17% (H)*	−59% (H)*	−23% (H)*
	+4% (M)	−65% (M)*	−29% (M)*
	+14% (L)	−61% (L)*	−16% (L)*
24 h room temperature–dark	+19% (H)*	−51% (H)*	−4% (H)*
	+10% (M)*	−53% (M)*	−5% (M)*
	+1% (L)	−56% (L)*	−9% (L)*
5 h room temperature	−4% (H)	−16% (H)*	−2% (H)
1 h room temperature	−2% (H)	−3% (H)	+4% (H)
Refrigeration overnight (4 °C)	+6% (H)	−2% (H)	−2% (H)
	H = 50 μg/mL	H = 800 ng/mL	H = 88.8 ng/mL
	M = 20 μg/mL	M = 320 ng/mL	M = 32.0 ng/mL
	L = 3.20 μg/mL	L = 51.2 ng/mL	L = 5.12 ng/mL

* denotes $p < 0.05$.

in both the three-times and two-times freeze–thaw experiments but overall the change was not statistically significant. All analytes demonstrate instability when held at room temperature in the light for 24 h ($p \leq 0.05$ for all tests). CP high control increased in concentration by 17%. Dox and dol decreased in concentration by 59–61% and 16–29%, respectively. Samples held for 24 h in the dark at ambient gave similar results, except dol decrease was slightly lower. Samples were again tested for stability by thawing and holding at room temperature for 1 h or 5 h or thawing overnight at 4 °C. The control groups of samples were thawed for 20 min. This further investigation proved that a 5 h exposure to room temperature was acceptable for CP and dol, but not for dox, which still dropped 16% ($p < 0.05$). However, both 1 h exposure and overnight 4 °C thaws exhibited no change in analyte responses ($p > 0.05$). All tests were performed in replicates ($n = 3$–6) and all replicate CVs were less than 15%.

The stability of the analytes in prepared sample (reconstitution buffer) at ambient temperature was acceptable. Overall analytes showed <4% change from the original results, except for the lowest concentration of dol, 5 ng/mL, which showed a drop of 11%.

3.5. Analysis of patient samples and noncompartmental pharmacokinetics

Twenty-three women completed the study. The mean (SD) age, total body weight, ideal body weight, body mass index (BMI) and body surface area were 52.7 years (8.8), 77.3 kg (19.6), 55.1 kg (5.2), 30.0 (7.6), and 1.86 m^2 (0.24), respectively. Based on BMI category, 4.3% were underweight, 21.7% healthy weight, 43.5% overweight, 8.7% obese and 21.8% severely obese.

This method was successfully used to measure CP, dox, and dol in plasma from patients receiving dual chemotherapy treatment [20]. Fig. 2(a and b) displays the overlaid MRM chromatographic responses. Fig. 3 illustrates a typical patient pharmacokinetic profile1–48 h post IV-bolus doses. CP concentrations ranged from 4 to 100 μg/mL, eliminating the need use of the lower curve range. Dox concentrations ranged from 7.21 to 162 ng/mL; dol concentrations ranged from 3.66 to 78.6 ng/mL. Of 206 results for each analyte, 21%, 7% and <1% of CP, dox, and dol concentrations were below the limit of quantitation, respectively. Of those below the limit for CP, nearly all were 24 h and 48 h observations. For dox, approximately 50% of the 48 h values were below the limit of quantification. Pharmacokinetic parameters estimated from plasma sample results are listed in Table 5.

3.6. Reproducibility of patient sample analyses

The reproducibility of results for the samples reanalyzed supports the reproducibility of the assay as well as the stability of the patient samples under the sample handling conditions defined. All concentrations ($n = 15$) were within ±20% of their original measured value, except one whose value upon another repeat analysis agreed well with the second determination. There appeared to be no apparent transformation of dox to dol or vice versa as there was no bias seen on the trend of the changes in values. For CP, samples without CP measured were reanalyzed to determine if a degradant was causing the increase in CP concentrations after freeze–thaws and we saw no false positives in these samples when subjected to another CP analysis. One sample with measurable CP was repeated for valid analyses of dox and dol and the first value of 32.8 was reproduced as 32.5 on the second analysis.

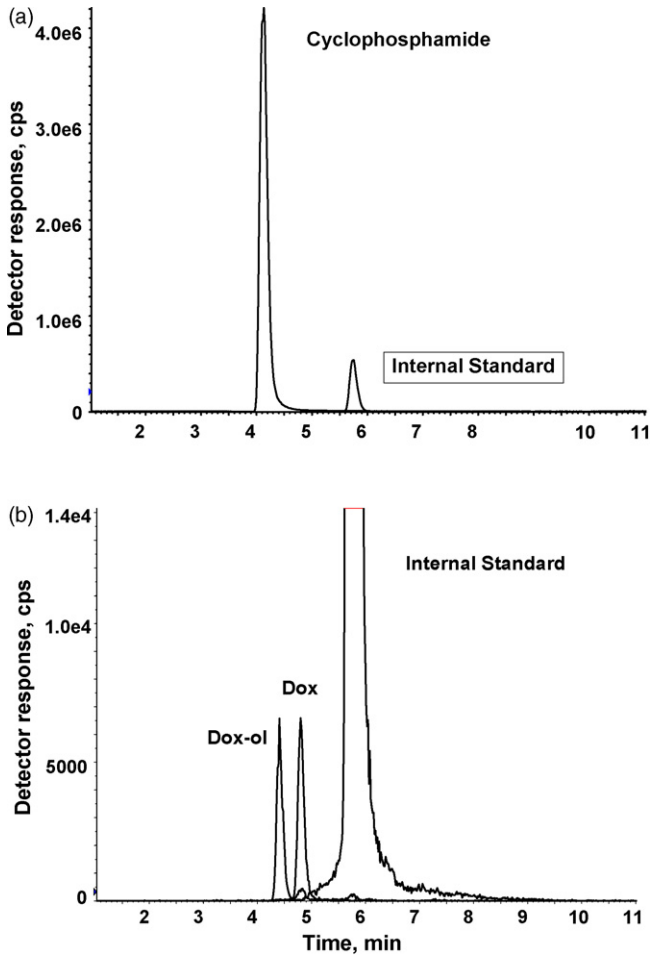

Fig. 2. (a) Cyclophosphamide in patient sample with internal standard; (b) doxorubicinol (dol), doxorubicin (dox) and internal standard in patient sample.

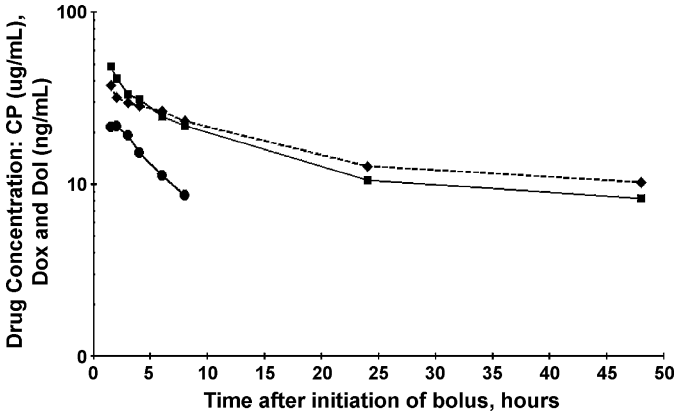

Fig. 3. Patient concentrations measured over 48 h post-dose using the method: (●) cyclophosphamide (CP), (■) doxorubicin (dox), (♦) doxorubicinol (dol).

4. Discussion

CP is present in millimolar (μg/mL) concentrations after dosing, whereas dox and its metabolite are present in micromolar (ng/mL). This creates a challenge for the laboratory scientist when using mass spectrometry to accurately detect all the analyte concentrations in a single injection. In seeking accurate response calibrations, the response of the highly concentrated analyte must be addressed when the response of another lower concentration analyte is optimized to gain the most sensitivity for itself. Since nonlinear relationship with concentration is typically seen when the mass detector approaches saturation, the more concentrated analyte's calibration curve, CP, produced a typical asymptote prior to detector over-saturation. Therefore during validation, a nonlinear curve fit over the defined calibration ranges was used for CP to provide an accurate calibration relationship from which unknown concentrations were determined. It was necessary to create two curve ranges to eliminate bias in the curve fit and it was also necessary to overlap the upper and lower limits of the curves to allow for the required 6 or more calibration standard points. This approach produced adequate variability and accuracy as shown in Table 3, with parameters achieving values of <15% and ±10%, respectively. During method application, the upper calibration curve range was well suited for CP and no values were observed in the lower calibration curve range, so there was no need to apply the second calibration curve. For dox and its metabolite, dol, efforts were made to obtain the best sensitivity and specificity through traditional chromatographic separation and MS/MS optimization of the ion pair of interest while balancing sample preparation procedures such as extracted volume, reconstitution volume, and mass injection amounts. The upper and lower limits of quantitation achieved were adequate in detecting dol through 48 h post-infusion in patient samples (LLOQ of dol = 3.6 ng/mL). Whereas for dox, a concentration range of 7.21–162 ng/mL was measured, indicating little need for an upper limit near 1000 ng/mL, as was also utilized by both Lachatre and Kummerle methods. Since approximately 50% of the 48 h values for dox were LLOQ (LLOQ dox = 7.2 ng/mL), a lower limit of dox would have been preferable.

Another challenge faced in the development of this method was the stability of the analytes of interest within the confines of handling and processing procedures. Publications provided enough information to provide guidance on the proper collection and processing of the immediate whole blood sample to obtain

Table 5
Median (interquartile range) PK parameters

	AUC	CL	Vss	C_{max}
CP	275.88 (244.74–308.54)	4.13 (3.57–5.27)	26.84 (21.30–34.95)	36.80 (32.60–45.80)
Dox	1116.62 (818.54–1289.44)	99.18 (86.37–131.02)	2573.44 (2205.20–3271.70)	66.50 (50.90–80.10)
Dol	1232.71 (902.30–1437.00)	NA	NA	42.70 (28.70–57.60)

AUC is h × μg/mL for CP and h × ng/mL for dox and dol; CL is L/h; Vss is L; and C_{max} is μg/mL for CP, ng/mL for dox and dol; and NA = not applicable in noncompartmental analysis since dol is a metabolite.

plasma. [14,16,18,23]. However, publications did not provide a comprehensive view of longer term storage and handling issues for all three analytes. For instance, Kümmerle et al. reported freeze–thaw stability of dox only at −20 °C after three cycles and long-term stability of when samples are stored at −70 °C for up to 1 month; dol was not assayed for. Kümmerle also reported loss of dox when sodium heparin was utilized, however this was only found to be significant at high concentrations of dox, and therefore not applicable to plasma concentrations lower than 3000 ng/mL. For CP, de Jonge et al. reported stability of CP in plasma after three freeze–thaw cycles and after seven months storage at −70 °C [23]. Andersen et al. [24] reported on the stability of dox and dol in plasma at 37 °C, 22 °C and 4 °C up to 48 h and stated that dox and dol were stable at 4 °C for at least 24 h, but failed to discuss the length of stability at 22 °C. Since sample preparation methods are typically carried out at room temperature, the length of these compounds stability in plasma at room temperature required characterization. Since dol is present at approximately 10-fold lower concentrations than dox, it was important to characterize its accurate measurement with this assay. Even small conversions of dox to dol would result in higher dol concentrations. Therefore, validation required this experiment be accomplished. Whereas many investigators choose to accept up to a 15% change in concentration as "stable", we chose a more stringent approach using the student's *t*-test. The *t*-test was used to determine if the results of the treated versus untreated samples were significantly different.

Traditional tests such as three freeze–thaw cycles and 24 h room temperature stability displayed unacceptable ($p < 0.05$ between treated and untreated samples) and varying responses (concentration drops 10% to 59%,) from the analytes resulting in defined limitations for each analyte contained in the sample. Defining these limitations required further experimentation, such as single and duplicate freeze–thaw tests, and varying room temperature exposure times in order to pinpoint an acceptable window when samples could be processed and still obtain accurate results. The latter was indeed challenging as even the thaw of samples at 4 °C overnight was investigated for stability purposes. Our final sample handling procedure required that we thaw samples either at 4 °C overnight or for 1 h or less to maintain stability of all analytes. If reanalysis was required for CP alone, the sample could be thawed again only one more time. If reanalysis was required for dol and dox, the sample could be thawed and frozen up to three times, but room temperature exposure required limitation to less than 5 h for dol and less than 1 h for dox. These stability issues required that careful records be kept on the handling of samples.

The stability results for CP are perplexing since a 15–20% increase in CP concentration was observed after three freeze–thaws and the control samples contained only CP (no metabolites). During validation the recovery of CP was found to be 80 ± 3% across matrices and the accuracy 96 ± 4%. Control data was 97% ± 3% accurate. Two possible explanations are given. Degradants in the sample after three freeze–thaws may have introduced substances which chromatographically co-eluted with CP and caused ion enhancement at the source. Or protein binding of CP was affected by repeated freeze–thaws causing an increase in recovery of the analyte. The former seems more likely as de Jonge et al. reported freeze–thaw stability but used differing methodology to assay for CP. However, after subjecting patient samples with unmeasurable CP to freeze–thaw cycles, we saw no false positives. Regardless, the experiments provided support that the samples should only be frozen and thawed twice to provide accurate CP concentrations using this method of analysis.

Dox and dol declined in concentrations during prolonged exposure to room temperature conditions and exposure to light enhanced the decline of concentration. A slight bias in dol and dox control concentrations was observed as the overall accuracy was 109 ± 2% for dol and 89 ± 2% for dox. An inter-conversion of dox to dol was considered, since the quality controls utilized contained 10-fold higher dox concentration than dol. Therefore, 1% conversion of dox to dol could have mathematically accounted for an approximate 10% increase in dol as seen. However, dox was biased by −11% not −1%. Futhermore, the reanalysis of patient samples did not show the same results. When samples that were reanalyzed for both dox and dol were compared, such biases were not apparent to suggest such an inter-conversion. Another possible cause may be a biased preparation of the controls themselves since separate stock solutions were prepared to make these controls. Despite these biases, the accuracy for the data are still within the 15% accuracy requirements. Further investigation may be warranted when new lots of controls and calibrators are prepared should the same trend continue. Overall, careful control of handling samples was warranted due to the data achieved during validation.

Two shortcomings of this method were its failure to measure the active metabolite, 4-hydroxycyclophosphamide (CPOH) and its need to employ CP nonlinear standardization. The sample preparation included stabilizing this metabolite by derivatization with semicarbazide [21]. Incubation for derivatization was accomplished over 5 h holding the processed plasma at room temperature. Since it became apparent that dox was not stable for this length of time at room temperature, including this analyte in the assay would have resulted in inaccurate dox determination. In addition, there were other major chemical incompatibilities with the methodology. First, the derivatized form of CPOH did not recover in the extraction well and attempts to include it resulted in loss of the recovery of other analytes. Next, the presence of the derivatization reagent resulted in conversion of the internal standard. Furthermore, the derivatized form of CPOH was not retained well under any chromatographic conditions developed for the other analytes. It was therefore decided that the CPOH should be analyzed separately. Regarding the nonlinear standardization of CP, two other detection changes for CP might provide more linear standardization: detection of a minor product ion and/or employment of a collection window of narrower atomic mass unit.

The pharmacokinetic results of this study are reasonable compared to previous studies. Although some of the doxorubicin 48 h samples were less than the LLOQ, this will only have a minor impact on the estimated area under the concentration–time curve since the area between 24 and 48 h after the dose is only a small fraction of the area under the curve. However, this will affect

estimates of the terminal elimination rate which is why we are not reporting half-lives. One way to correct this would be to add more sampling time points between 24 and 48 h after the dose. Powis and colleagues reported a mean (SD) cyclophosphamide clearance and volume of distribution of 4.17 (38.8) L/h and 36.1 (12.7) L in 16 women with advanced breast cancer. When studying the pharmacokinetics of doxorubicin and doxorubicinol in 21 cancer patients receiving their first course of doxorubicin, Rodvold et al. reported a doxorubicin mean (SD) clearance of 73.7 (23.1) L/h and volume of distribution at steady state of 1049 (432) L and a doxorubicinol area under the concentration–time curve (AUC) of 730 (362) h × ng/mL. One possible explanation for the difference in reported doxorubicin volume of distribution and doxorubicinol AUC is the difference in inclusion criteria. Rodvold and colleagues included any cancer patient receiving their first course of doxorubicin regardless of their type of cancer and included both male (eight male) and female subjects.

5. Conclusion

The combination of dox and CP is commonly used in the adjuvant treatment of high risk breast cancer. The ability to quantitate the level of these drugs will allow for more precise and effective dosing. The analytical method we describe provides a sensitive, reproducible, and accurate technique to quantitate CP, dox, and dol using 400 μL of plasma. It requires a liquid chromatography tandem mass spectrometry operation to accomplish, which appears to be becoming a more common approach for clinical operations which cater to therapeutic drug monitoring programs [24]. This trend is most likely due to the lack of specificity of immunoassay methods in the presence of metabolites. Lastly, care should be taken in sample processing and use due to instabilities of these compounds under various common laboratory conditions. Such care should be taken regardless of the method used.

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