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ORIGINAL ARTICLE

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A liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of actinomycin-D and vincristine in children with cancer

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Abstract Actinomycin-D (Act-D) and vincristine (VCR) are cytotoxic agents commonly used in the treatment of pediatric cancers. To date, there are few published methods on quantifying Act-D or VCR and no published methods on quantifying the two drugs together. We present a methodology for the simultaneous quantification of Act-D and VCR in human plasma using liquid chromatography-tandem mass spectrometry (LC/MS/MS) detection. Following solid phase extraction, plasma samples were separated and analyzed using electrospray ionization (ESI). The lower limit of quantitation (LLOQ) for both Act-D and VCR was 0.5 ng/ml. The analytical accuracy for detection of both Act-D and VCR was ≥ 90%. The analytical precision, as estimated by the coefficient of variation was ≤ 6% for Act-D and ≤ 11% for VCR. Given the prevalence of the use of the two drugs as combination therapy in a variety of pediatric oncological indications, the small sample volume requirements and the assay sensitivity, this methodology is expected to support several ongoing and future pediatric trials.

Keywords Actinomycin-D · Vincristine · Method · Validation · Pediatric · Pharmacokinetics

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Introduction

The combination of actinomycin-D (Act-D, molecular weight 1254.6 Da; Fig. 1a) and vincristine (VCR, molecular weight 825.4 Da; Fig. 1b) is used for the treatment of a spectrum of childhood solid tumors including Wilms tumor [1] and rhabdomyosarcoma [2, 3]. Although these drugs have been used for more than 40 years, our knowledge of their clinical pharmacology is limited. For vincristine, a remarkably wide interpatient variability in half-life, volume of distribution, and subsequent plasma drug exposure following intravenous dosing has been reported [4, 5]. The basis for this remains an enigma. Significantly less is known about the disposition of Act-D for which, until recently, there has not been a suitable method available to accurately quantify drug in plasma.

Critical gaps in our knowledge for such widely used anti-cancer agents have resulted in sub-optimal dosing, most notably for infants and young children who experience an increased risk of morbidity, mortality, and treatment failures [6, 7]. When treated with this combination of drugs, young children with Wilms tumor and rhabdomyosarcoma have an increased risk of liver toxicity [8], but precise risk factors remain elusive.

In order to gain a better understanding of the clinical pharmacology of Act-D and VCR, we have developed and validated a LC/MS/MS method based on the method of Veal et al. [9] for the simultaneous quantification of these two drugs in plasma.

Materials and methods

Chemicals

Act-D and VCR and their internal standards, 7-amino-actinomycin-D and vinblastine (VBL), were obtained from Sigma-Aldrich (St. Louis, MO). Optima water, methanol, glacial acetic acid, and ammonium hydroxide

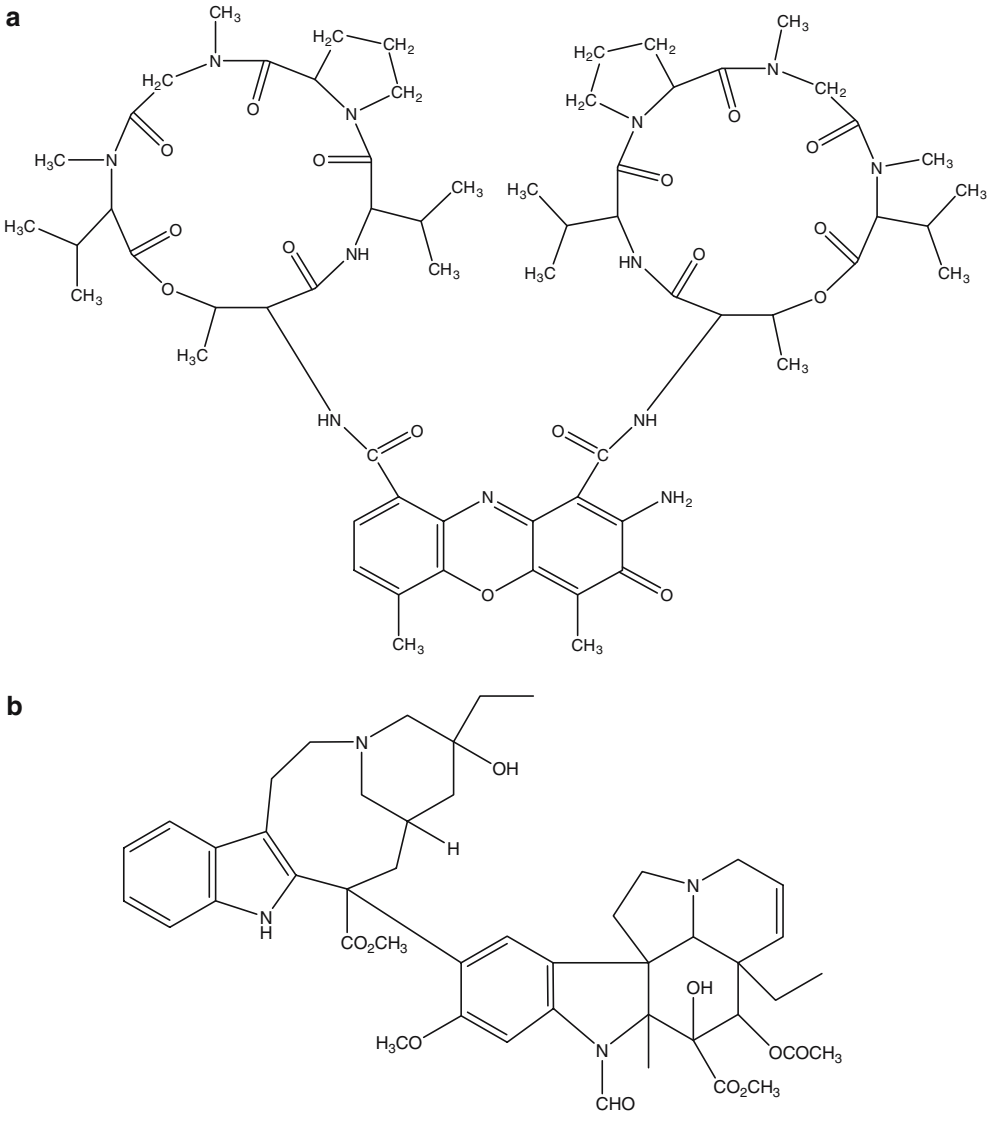

Fig. 1 Chemical structures of Act-D (**a**) and VCR (**b**)

were obtained from Fisher Scientific (Pittsburg, PA). All solvents used were HPLC grade.

Quality control, standard preparation, sample extraction, and quantification

Act-D stock solution, prepared by mixing 1 mg/10 ml drug in 50% methanol/50% water, was stored at 4°C. VCR stock solution, prepared by mixing 1 mg/10 ml drug in water, was stored at −20°C. Stock solutions were replenished every 12 months. Working standard solutions of Act-D and VCR were prepared by diluting the stock solutions to 10 μg/ml in 50% methanol/50% water. These were further diluted into 50% methanol/50% water, and then spiked into aliquots of human plasma. Separate stock solutions were used to create quality control and standard working solutions. Standard curve samples were spiked with 50 μl of Act-D/VCR working standard at each of eight concentrations and diluted into 450 μl of thawed plasma on the day of extraction, whereas quality control samples were spiked with 50 μl of Act-D/VCR working standard at each of three concentrations, aliquotted into 500 μl samples, and frozen at −80°C at 3 month intervals. Plasma was obtained from the general blood bank inventory of The Children's Hospital of Philadelphia. The standard curve consisted of 0.5, 1, 2, 5, 10, 20, 50, and 100 ng/ml concentrations (corresponding to 0.40, 0.80, 1.6, 4.0, 8.0, 15.9, 39.9, and 79.7 nM for Act-D, and 0.6, 1.2, 2.4, 6.1, 12.1, 24.2, 60.6, and 121.2 nM for VCR, respectively). Quality control concentrations included 4 ng/ml (low), 40 ng/ml (mid-range), and 80 ng/ml (high). Internal standard stock solutions were similarly prepared, and 20 μl of each internal standard (10 μg/ml) were mixed with 960 μl of 50% methanol/50% water, yielding a final

concentration of 200 ng/ml internal standard. For each analytical run, a standard curve together with a blank plasma sample, a solvent blank, and at least two quality control (QC) specimens at each of three concentrations were included.

Plasma samples were kept frozen at −80°C and were thawed on the day of extraction. The solid phase extraction column consisted of a Waters Oasis HLB 1 ml/30 mg extraction cartridge, conditioned with 1 ml of methanol (1,800*g* for 2 min) followed by 1 ml of water (1,800*g* for 2 min). Five hundred microliter of spiked plasma was mixed with 30 µl of 200 ng/ml internal standard solution, vortexed for 30 s, loaded onto the column and centrifuged (2,600*g* for 10 min). The column was washed with 5% methanol in water (1,800*g* for 5 min) and drugs were eluted with 100% methanol (2,600*g* for 10 min). The extracted samples were evaporated to dryness using a Zymark TurboVap LV evaporator (Caliper, Hopkinton, MA) and reconstituted in 200 µl of 65% mobile phase A (described below)/35% methanol. Twenty-five microliter was injected onto the column for analysis.

Table 1 Elution gradient for HPLC conditions

Time (min)	Percent A (%)	Percent B (%)	Gradient
0	90	10	None
6	20	80	Linear
7	0	100	Linear
10	0	100	None
10.1	80	20	None
18.1	90	10	None

Instruments and HPLC operating conditions

High-performance liquid chromatography (HPLC) was performed on a Waters 2690 separation module equip-

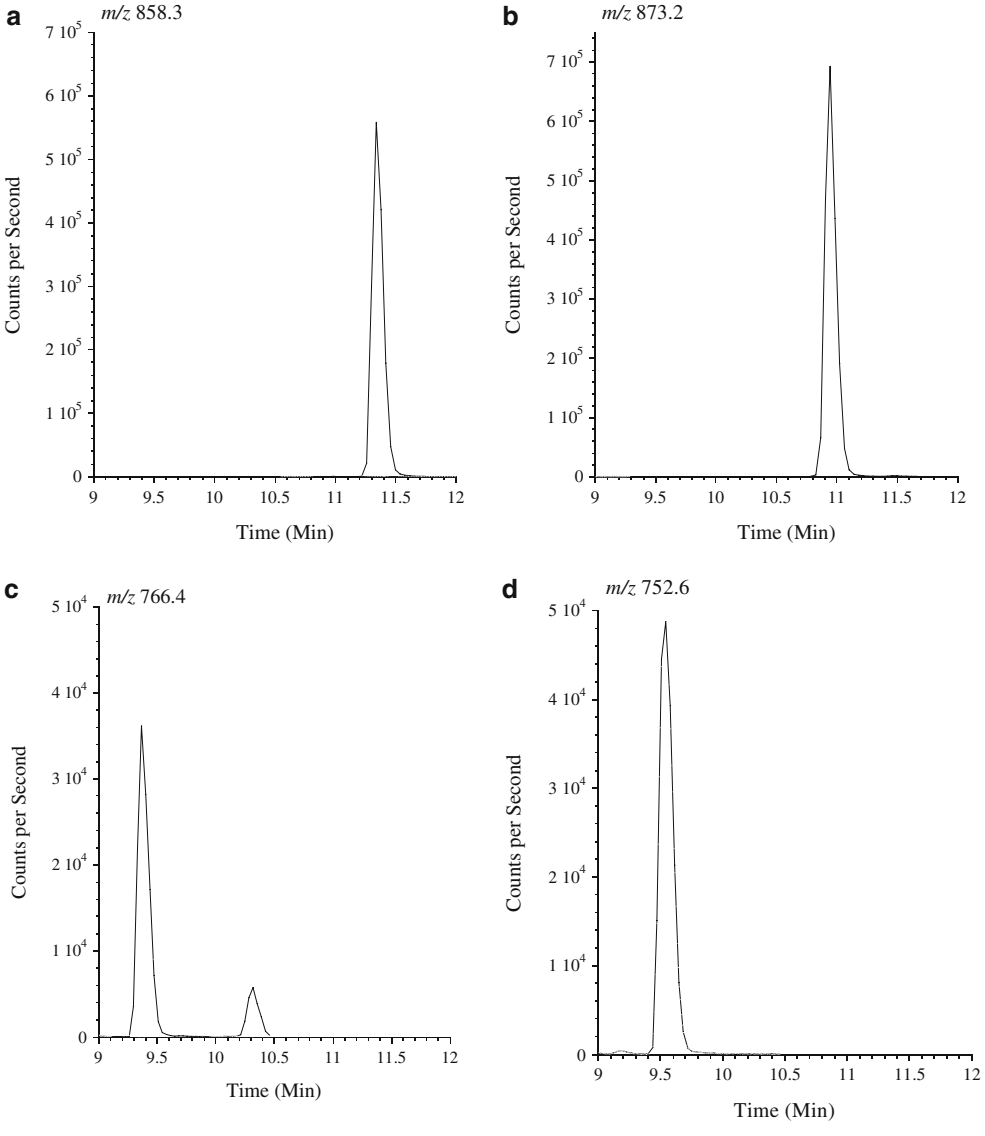

Fig. 2 Sample chromatograms of Act-D (**a**), 7-Amino Act-D (**b**), VCR (**c**) and VBL (**d**) for a 100 ng/ml injection. The Q3 monitoring ion is shown

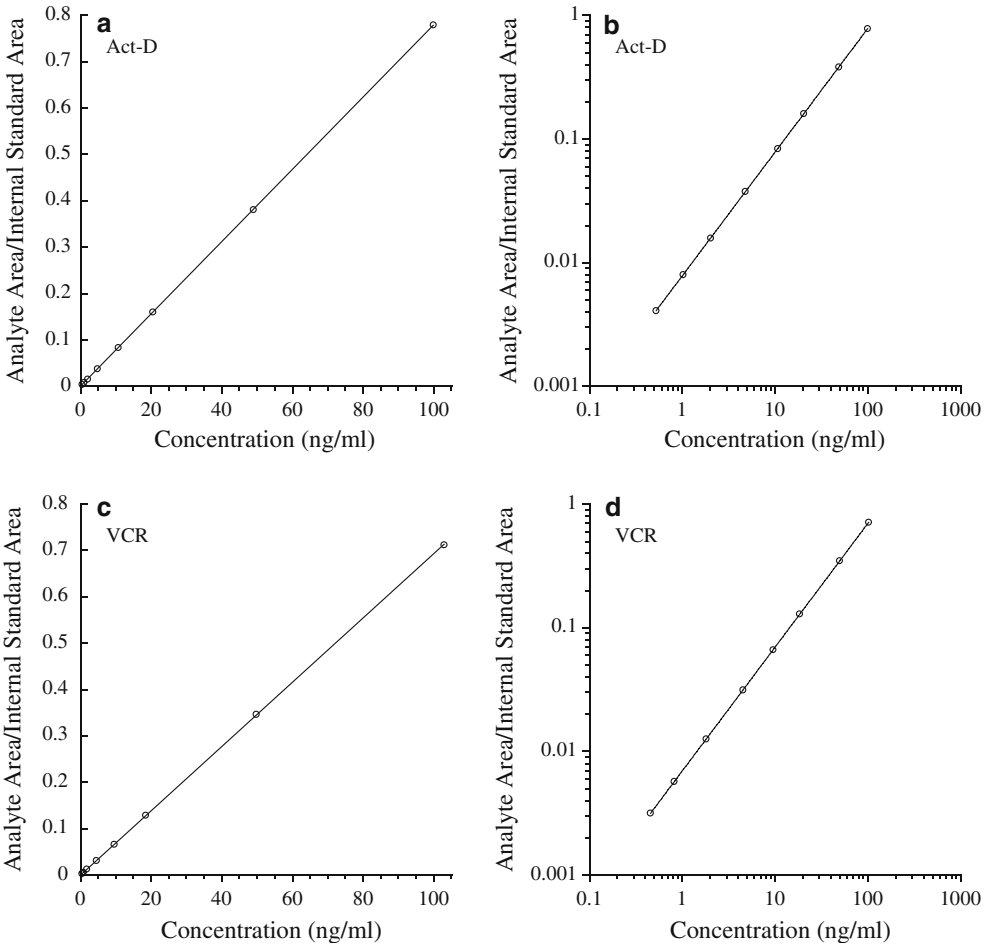

Fig. 3 Linear (**a**, **c**) and logarithmic (**b**, **d**) standard curves for Act-D and VCR in spiked human plasma ($r^2 > 0.999$ for all curves)

Table 2 Precision determined at 6 concentrations of Act-D and VCR, including the LLOQ

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean ± SD	CV (%)
Act-D (ng/ml)							
0.5	0.5	0.5	0.5	0.5	0.5	0.5 ± 0.0	4.2
1	1.0	1.1	0.9	1.1	1.1	1.1 ± 0.0	5.7
5	5.0	4.8	5.0	5.2	5.2	5.0 ± 0.1	3.3
10	9.9	9.8	10.2	10.1	10.1	10.0 ± 0.1	1.6
50	52.1	51.5	51.2	49.8	50.9	51.1 ± 0.8	1.7
100	96.5	99.3	96.9	98.6	99.0	98.1 ± 1.3	1.3
VCR (ng/ml)							
0.5	0.4	0.4	0.5	0.4	0.4	0.4 ± 0.0	10.8
1	0.9	1.0	0.8	0.8	0.9	0.9 ± 0.1	6.1
5	5.6	4.6	4.7	4.5	4.7	4.8 ± 0.4	9.2
10	10.2	8.8	9.7	9.0	9.9	9.5 ± 0.6	6.3
50	49.3	50.1	51.0	53.6	51.1	51.0 ± 1.6	3.2
100	102.0	102.0	101.0	99.0	101.0	101.0 ± 1.2	1.2

ped with an autosampler and an electronic degasser (Waters/Alliance Systems, Milford, MA). This was coupled to an API 4000 LC/MS/MS spectrometer (Applied Biosystems/MDS SciEx, Ontario, Canada). A Luna 3 μm C8 50×2 mm Phenomenex reverse-phase analytical column (Phenomenex, Torrance, CA) was used for separation. Mobile phase A consisted of water with 1% acetic acid titrated to pH 4.0 with ammonia, and mobile phase B was 100% methanol. The elution gradient is shown in Table 1. Total run time was 18 min.

Mass spectrometry operating conditions

Mass spectrometry operating conditions were established to detect Act-D and VCR. Tandem mass spectroscopy was carried out under positive electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. Nitrogen was used as a nebulizer gas. For Act-D, nitrogen was used as a curtain gas at 10 psi, and ion source gases of 25 psi and 50 psi. Voltages were as follows: declustering potential 150 V, entrance po-

Table 3 Accuracy determined by replicate analysis of quality control samples containing known amounts of Act-D and VCR, and calculated for both intra-day and inter-day variability

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean ± SD	% deviation
Intra-day measurements							
Act-D (ng/ml)							
4	4.1	4.1	3.3	3.7	4.0	3.8 ± 0.4	5
40	37.8	42.2	37.1	37.2	37.0	38.3 ± 2.2	4.3
80	78.2	75.1	75.6	79.5	85.0	78.7 ± 4.0	1.6
VCR (ng/ml)							
4	4.2	4.1	4.0	4.5	4.3	4.2 ± 0.2	5.5
40	42.5	45.0	43.0	43.9	40.4	43.0 ± 1.7	7.5
80	81.4	83.8	85.2	88.5	86.0	85.0 ± 2.6	6.3
	Day 1	Day 2	Day 3	Day 4	Day 5	Mean ± SD	% deviation
Inter-day measurements							
Act-D (ng/ml)							
4	4.3	4.3	4.1	4.5	3.8	4.2 ± 0.3	5
40	44.4	39.8	37.8	46.7	40.7	41.9 ± 3.6	4.8
80	86.0	81.4	78.2	80.4	75.5	80.3 ± 3.9	0.4
VCR (ng/ml)							
4	4.1	4.8	4.1	4.5	4.3	4.4 ± 0.3	10
40	48.2	42.4	42.5	45.0	39.8	43.6 ± 3.2	9
80	94.1	85.8	81.4	82.6	82.1	85.2 ± 5.3	6.5

Table 4 Six-hour stability determined by replicate analysis of quality control samples containing known amounts of Act-D and VCR

Conc (ng/ml)	Mean ± SD calculated (ng/ml)	% mean deviation
Act-D		
4	3.9 ± 0.0	2.4
40	35.6 ± 2.1	11.0
80	73.5 ± 2.3	8.1
VCR		
4	3.9 ± 0.1	3.5
40	41.4 ± 1.9	3.5
80	77.1 ± 5.5	5.9

tential 10 V, collision energy 55 V, collision energy exit potential 10 V, at a temperature of 450°C. Dwell time was 400 ms. Ion recording used the Q1 (parent ion) of Act-D and the internal standard at m/z 1255.6 and 1270.6, respectively, with the Q3 (daughter) ion of Act-D and the internal standard at m/z 858.3 and 873.2, respectively. For VCR, the nitrogen curtain gas was at 10 psi, with ion source gases of 10 psi and 0 psi. Voltages were as follows: declustering potential 160 V, entrance potential 10 V, collision energy 50 V, collision energy exit potential 5 V, at a temperature of 275°C. Dwell time was 250 ms. Ion recording used the Q1 (parent ion) of VCR and the internal standard at m/z 825.4 and 811.4, respectively, with the Q3 (daughter) ion of VCR and the internal standard at m/z 766.4 and 752.6, respectively.

Table 5 Freeze-thaw stability determined by replicate analysis of quality control samples containing known amounts of Act-D and VCR

Conc (ng/ml)	Mean ± SD calculated (ng/ml)	% mean deviation
Act-D		
4	3.9 ± 0.1	3.8
40	39.8 ± 0.1	10.3
80	79.4 ± 0.1	5.5
VCR		
4	4.4 ± 0.1	11.1
40	44.4 ± 0.2	14.2
80	91.4 ± 0.1	14.3

Method validation

The lower limit of quantitation was calculated as the lowest concentration that could be detected at a level of at least five times the average baseline noise in a blank plasma sample, with adequate precision. Accuracy was determined by calculating the percent deviation from expected concentration of five repeat measurements of the QC samples on 3 days. Precision was determined by calculating the coefficients of variation of five different measurements of the standard curve samples at each of six concentrations. Freeze-thaw analysis was performed on three QC samples at each concentration, thawed and frozen a minimum of three times. Six-hour room temperature stability was evaluated on three QC samples at each concentration.

Extraction recovery

To assess extraction recovery, standards were prepared by spiking blank plasma specimens that had been ex-

tracted as described above. Plasma spiked with 0.5, 1, 5, 10, 50 and 100 ng/ml was then extracted and quantified using this blank plasma standard curve.

Pharmacokinetic study

A protocol to study the pharmacokinetics of Act-D and VCR was approved by the institutional review board of The Children's Hospital of Philadelphia. As part of this study, following parental permission, two patients with rhabdomyosarcoma, age 2.5 and 2.75 years (11.2 kg and 11.6 kg, respectively), scheduled to receive Act-D and VCR as part of routine chemotherapy, were evaluated following an Act-D dose of 0.05 mg/kg and a VCR dose of 0.05 mg/kg. A total of 12 blood samples were obtained over a 48 h period. Samples were placed on ice, centrifuged, and plasma separated within 30 min of collection. Plasma was frozen at −80°C until analysis. A three-compartment model was fit to the plasma concentration data using WinNonlin Professional version 4.1 (Pharsight Corporation, Mountain View, CA).

Results

A single LC/MS/MS method was developed and validated to identify and quantify Act-D and VCR in human plasma. Using the method described above, VCR eluted at approximately 9 min, and its internal standard eluted at 9.5 min. Act-D eluted at approximately 11.5 min, and its internal standard at 11 min (Fig. 2). Standard calibration curves are presented in Fig. 3. The lower limit of detection for Act-D was 0.1 ng/ml and for VCR was 0.2 ng/ml, and the lower limit of quantitation for both Act-D and VCR was 0.5 ng/ml. Precision determination found that the coefficient of variation (CV) ranged from 1.3 to 5.7% for Act-D and from 1.2 to 10.8% for VCR (Table 2). The relative error (accuracy) ranged from 0.4 to 5% for Act-D and 5.5 to 10.0% for VCR (Table 3). The mean value was within 10% of the actual value for all concentrations. Drug in plasma was stable for at least 6 h at room temperature (Table 4) and during freeze-thaw cycling (Table 5). The mean extraction recovery of Act-D was 80.2 ± 4.9% and for VCR 50.7 ± 8.3%.

Plasma Pharmacokinetics

The plasma concentration time curves for two patients are shown in Fig. 4 and the pharmacokinetic parameters are given in Table 6. The mean terminal half-life of Act-D was 39 h and of VCR was 14 h. The mean clearance of Act-D was 3.9 ml/min/kg and of VCR was 15.4 ml/min/kg.

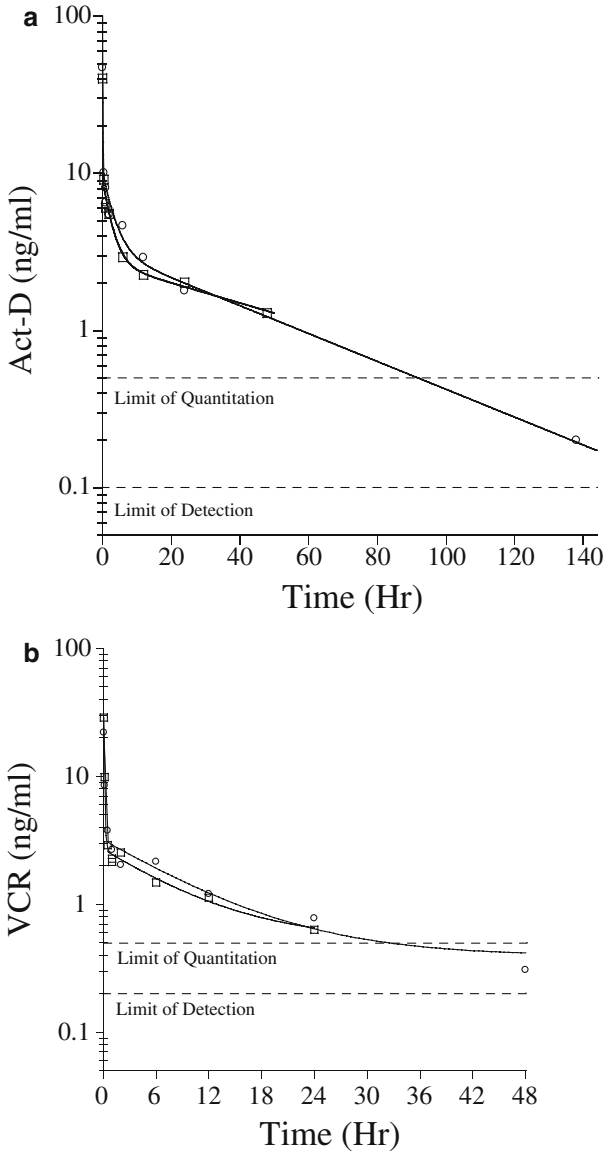

Fig. 4 Plasma pharmacokinetics of Act-D (**a**) and VCR (**b**) in two patients after 0.05 mg/kg of each drug. *Open circles* represent patient 1 and *open squares* represent patient 2

Table 6 Plasma pharmacokinetics of 0.05 mg/kg Act-D and 0.05 mg/kg VCR in two pediatric patients

Drug	Parameter	Units	Patient 1	Patient 2
Act-D	Half-life	h	43.2	35.6
	C_{max}	ng/ml	113	102
	AUC_{last}	min ng/l	7.7	12.9
	AUC_{inf}	min ng/l	12.5	13.5
	Cl	ml/min/kg	4	3.7
	V_{ss}	l/kg	12.5	6.5
VCR	Half-life	h	18.2	11.8
	C_{max}	ng/ml	21.9	28.6
	AUC_{last}	min ng/l	3.1	1.9
	AUC_{inf}	min ng/l	3.6	2.6
	Cl	ml/min/kg	11.2	19.5
	V_{ss}	l/kg	14.9	16.6

Discussion

An analytical method to quantify Act-D and VCR in human plasma has been validated and found suitable for subsequent clinical pharmacokinetic evaluation. The method described quantifies Act-D and VCR simultaneously and is expected to provide clinical utility to upcoming clinical trials as these agents are frequently co-administered. Despite the limited sampling available at this time, our measured pharmacokinetic parameters are in agreement with the previously reported pharmacokinetics for these agents [9, 10].

Although Act-D and VCR have been used for many years, human pharmacokinetic information for these drugs has been limited. This has lead to empiric dosing of these agents in children, with subsequent infrequent but severe toxicities including liver failure [11]. Despite these many decades of use, there are still no dosing guidelines by which one can safely administer these drugs without concerns of overdose; infants and young children are specifically at increased risk. Equally concerning is the absence of pharmacokinetic profiling useful in estimating efficacy of these agents.

This method is sensitive, with an LLOQ of 0.5 ng/ml for each drug, accurate, with a deviation of less than or equal to 10%, and reproducible, with a CV of less than or equal to 10%. It is also well-suited to a pediatric population because it employs a small volume of plasma. Previous experience with electrochemical detection of VCR has demonstrated similar sensitivity but with a lower signal to noise ratio [12].

Our preliminary, single-center pharmacokinetic study will permit an initial examination of *in vivo* performance of our method prior to an upcoming multi-institutional study involving over 100 children with cancer to study dose-exposure and exposure-toxicity profiles of these agents. It is anticipated that this improved combined method will allow rapid quantification of both drugs in the pediatric population.

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