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A Sensitive and Specific Procedure for Quantitation of ADR-529 in Biological Fluids by High-Performance Liquid Chromatography (HPLC) with Column Switching and Amperometric Detection[1]

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An HPLC method using electrochemical detection (ED) has been validated for the determination of ADR-529 in plasma and urine using ICRF-192 as an internal standard (IS). Prior to storage and quantitation, both plasma and urine samples require acid stabilization. Acidified plasma samples were prepared for HPLC using a two column solid-phase extraction (SPE). An aliquot of buffered plasma (i.e., pH 6–7) was first deproteinated and desalted on a C-18 SPE column. The analytes were then eluted onto a C-8 SPE column where retention and selective cleanup were achieved in the cation-exchange mode via silanol interactions. Acidified urine samples were diluted in acetonitrile prior to injection. The HPLC system for plasma and urine samples employed two narrow-bore silica columns used in the weak cation-exchange mode and separated by a switching valve. To prohibit late-eluting peaks from passivating the glassy carbon working electrode, a heart-cut containing ADR-529 and the IS was vented from the first silica column to the second using an automated switching valve. Amperometric detection at an oxidation potential of +1050 mV vs a $Ag/AgNO_3$ reference electrode was used. Linearity was validated between 5 and 500 ng/ml in plasma and between 2 and 100 μg/ml in urine. Imprecision and percentage bias were typically <10% for both plasma and urine controls throughout their respective dynamic ranges. The absolute recoveries for ADR-529 and the IS from plasma were >95%. This method is being successfully applied to the pharmacokinetic/dynamic evaluation of ADR-529 in animals and humans.

KEY WORDS: column switching; high-performance liquid chromatography; electrochemical detection; cardioprotectant (ADR-529); sensitive; validation; pharmacokinetics.

INTRODUCTION

ADR-529 (Fig. 1) [ICRF-187; (*S*)-(+)-bis-4,4′-(1-methyl-1,2-ethandiyl)2,6-piperazinedione] is undergoing development as a protective agent against anthracycline-induced cardiomyopathy. Several animal models have demonstrated reduced incidence and severity toward anthracycline-dependent heart lesions when pretreated with ADR-529 (1,2). Quantitation of this drug in biomatrices is necessary to support early Phase I pharmacokinetics/dynamics and preclinical ADME work. The following physicochemical properties were addressed prior to developing a sensitive and reproducible method. In water, ADR-529 degrades in a pH-dependent fashion to three hydrolytic products (3,4). At 25°C and at a constant ionic strength of 0.5, ADR-529 half-lives at pH 2.0 and 7.8 are 266 and 22.1 hr, respectively (3). The relatively high polarity complicates its extraction from plasma and subsequent separation by reverse-phase HPLC (RP-HPLC). In addition to these characteristics, it also possesses poor chromophoric properties (i.e., no UV maxima >210 nm), which severely limits the selective use of spectrophotometric detection.

Previous bioanalytical methods for this enantiomer or its racemic mixture have employed GLC (5), RP-HPLC with ultraviolet (UV) detection (6–9), and fluorescence detection after derivatization (3). Except for the HPLC-fluorescence method (3), the existing methodology lacks the required sensitivity for a thorough kinetic evaluation (5–9), lacks the ability to automate sample injection due to late-eluting peaks (6,9), and demonstrates a poor extraction efficiency (5–7). Simple ultrafiltration (7–9) has also been used for biological fluids because of the drug's low protein binding. These methods are limited in their quantitative ability to levels >0.1 μg/ml, primarily because of the nonselective ultrafiltration procedure. This prohibits the use of an autosampler (9) and results in a low throughput. Ultrafiltration methodologies are also affected by the variability in the free fraction of the drug in cancer patients.

Sadee *et al.* (5) extracted ADR-529 from plasma by protein precipitation followed by liquid/liquid extraction, derivatizing the resulting extract with diazomethane and separation by gas–liquid chromatography (GLC) using either flame ionization (5–50 μg/ml) or mass spectrometry (0.2–2.0 μg/ml) detection. Absolute recovery from plasma was 70%. Earhart *et al.* (6) used liquid/liquid extraction after protein precipitation. Quantitation was achieved using RP-HPLC with UV detection at 208 nm. A dynamic range of 0.5–50 μg/ml, an LOQ of 0.05 μg/ml, and an extraction efficiency of 58% from serum were reported. Although analyte retentions times were within 12 min, late eluters required staggered injections, permitting only two injections per hour. To improve recovery and sensitivity in plasma, Sisco (3) developed an extraction on a C-18 SPE column with subsequent derivatization using a proprietary fluorescent tag. The derivatized extract was quantitated by RP-HPLC with fluorescence detection. Linearity over 0.01–50 μg/ml was reported. Although this method demonstrated much improved sensitivity over existing procedures (5–9), acceptable precision estimates were reported only down to 50 ng/ml. Their method also lacked an internal standard to allow for sufficient control.

We have implemented the following to maximize sensitivity and ruggedness of this new procedure: (i) use of an amperometric detector to improve selectivity; (ii) use of a two-column SPE for selective extraction of ADR-529 and ICRF-192, the IS, from plasma; (iii) employment of ion-

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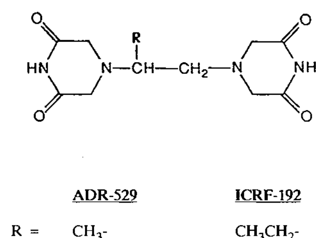

Fig. 1. Molecular structures for ADR-529 and ICRF-192.

exchange HPLC to enhance selectivity of the chromatographic windows and permit the use of acetonitrile as an injection solvent, thus improving analyte stability in the prepared sample; and (iv) use of an HPLC column switching configuration to accommodate automated sample injection and prevent passivation of the working electrode from the biofluid matrix. We present here a sensitive quantitation procedure and its validation and quality control performance for support of Phase I clinical pharmacology.

MATERIALS AND METHODS

Materials

ADR-529 (Fig. 1) reference standard with a purity of 99.5% was synthesized at Monsanto Co. (Dayton, OH) and recrystallized in-house. The IS, ICRF-192 (Fig. 1), was kindly provided by Dr. A. M. Creighton of the Imperial Cancer Research Fund (ICRF) Laboratories, Lincoln's Inn Fields, London, England.

Acetonitrile, hexane, and methanol were HPLC grade and purchased from either EM Science (Cincinnati, OH) or Baxter Burdick and Jackson (Muskegon, MI). Dibasic potassium phosphate, phosphoric acid (85%, v/v), and trifluoroacetic acid were procured from EM Science (Cincinnati, OH) and J. T. Baker (Phillipsburg, NJ). Deionized water was obtained in-house from a Milli-RO and Milli-Q purification system (Millipore Corporation, Milford, MA). All other chemicals were analytical reagent grade and used without further purification. Bond Elut C-18 and C-8 solid-phase extraction (SPE) columns (500 mg of sorbent/2.8 mL column) and Bond Elut "piggyback" column adapters were purchased from Analytichem International (Harbor City, CA). Two 12-port vacuum manifolds used for sample processing were obtained from Baxter Burdick and Jackson (Muskegon, MI). Blank human plasma used to prepare spiked standards and controls was purchased from the American Red Cross (Columbus, OH) and screened for the potential interferences in the assay.

Analytical Instrumentation and Conditions

The HPLC system (Fig. 2) consisted of two Spectroflow 400 pumps and a Spectroflow 783 absorbance detector (Applied Biosystems, Ramsey, NJ), an ISS-100 autosampler (Perkin-Elmer, Norwalk, CT), a Waters Automated Valve Station (WAVS) module (Millipore Corporation, Milford, MA), and an EG&G Model 400 electrochemical detector (EG&G Princeton Applied Research, Princeton, NJ). The analog signal from the detector was digitized by a Nelson 941 A/D Interface and acquired and processed by ACCESS*CHROM data acquisition software (PE Nelson, Cupertino, CA) on a MicroVAX 2000 (Digital, Maynard, MA).

The chromatographic separation was performed on two-5-μm narrow-bore Ultrasphere silica columns (column 1, 15 cm × 2-mm ID; column 2, 25 cm × 2-mm ID; Beckman, Fullerton, CA) which were separated by the WAVS switching valve. A precolumn filter equipped with a frit of 2-μm porosity (Upchurch Scientific, Inc., Oak Harbor, WA) and a silica NewGuard precolumn (15 × 3.2 mm; Brownlee, Santa Clara, CA) were connected in series prior to the first ana-

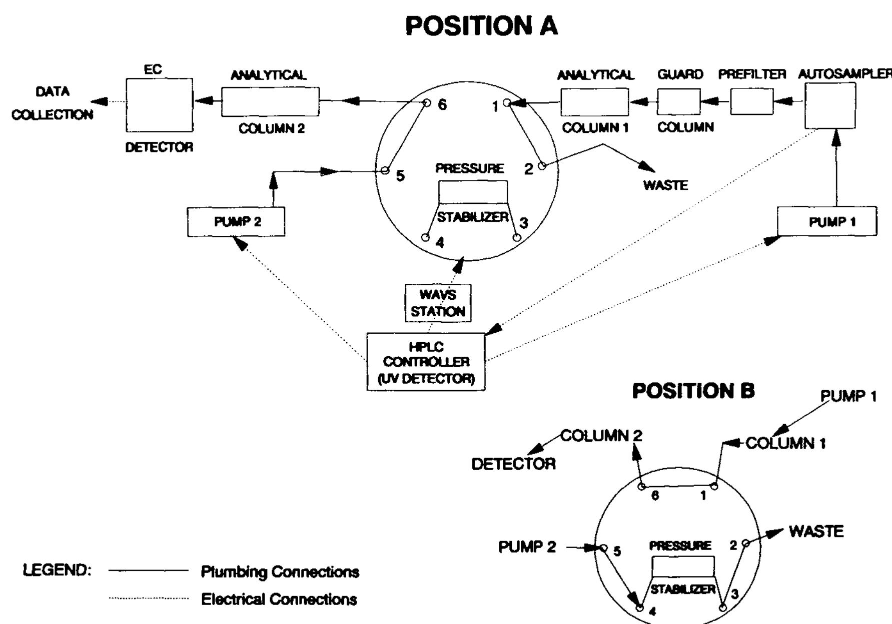

Fig. 2. Configuration of the HPLC with an exploded view of the column switching valve.

lytical column. The mobile phase for both plasma and urine methods was acetonitrile/water/phosphoric acid (90/10/20, v/v/m*M*). Both pumps were supplied by this mobile phase stored in an airtight reservoir (Omnifit, Ltd., Rainin Instruments Co., Woburn, MA) and continuously sparged with helium (i.e., <10 psi). The flow rate for each pump was 0.3 ml/min with a typical back pressure of ca. 70 bar when both analytical columns were in series. Column effluent was monitored amperometrically at a glassy carbon working electrode, operating at an oxidation potential of + 1050 mV vs a $Ag/AgNO_3$ reference electrode. The reference electrode filling solution was changed from aqueous 3 *M* KCl/saturated AgCl to 1 *M* $AgNO_3$ in acetonitrile (10).

Column Switching Routine. Chromatography of plasma and urine samples was isocratically achieved using a column switching configuration (Fig. 2). With the WAVS switching valve in position A, the sample was injected onto the first silica column (15 cm × 2-mm ID), which was vented to waste. Three minutes after injection, the WAVS switching valve rotated to position B, which vented the eluent from the first to the second silica column (25 cm × 2-mm ID) for 6 min. This heart-cut contained ADR-529 and the IS without most of the plasma constituents. At 9 min, the WAVS switching valve returned to position A; the late eluters from the first column were vented to waste, while the analytes from the second column were vented to the electrochemical detector. The microprocessor-controlled UV detector supplied timed contact closures to the WAVS switching valve.

Preparation of Stock and Matrix Standards and Quality Controls

Plasma Standards and Quality Controls. An ADR-529 stock standard was prepared at 125 μg/ml in acetonitrile and was serially diluted with the same to provide spiking solutions (working standards) for preparation of plasma standards. The acetonitrile working standards were stored in leakproof high-density polyethylene bottles (Nalge Co., Rochester, NY) at 4°C until use. Aliquots of these working standards (20.0 μl) were spiked fresh daily into blank acidified plasma (1 ml) to provide plasma standards in the range of 5–500 ng/ml. Blank acidified plasma, pH 2, was prepared by adding 50 μl of diluted phosphoric acid (42.5%) per ml of plasma.

Pooled quality control samples were prepared at 7.21, 72.1, and 721 ng/ml. These QC samples were aliquoted into polyallomer vials (Perfector Scientific, Atascadero, CA) and stored at −20°C until assayed. For the plasma assay, an ICRF-192 standard (5 μg/ml in acetonitrile) was spiked (20.0 μL) into 1 ml of acidified plasma to a concentration of 105 ng/ml.

Urine Standards and Quality Control Samples. Acidified urine standards and controls were prepared as pooled samples. From a stock acidified urine standard (100 μg/ml), standards ranging from 2 to 100 μg/ml and controls at 3 and 80 μg/ml were transferred to polyallomer vials and stored at −20°C until assayed. Blank acidified urine, pH 2, was prepared by adding 50 μl of diluted phosphoric acid (42.5%) per ml of urine. For the urine assay, an ICRF-192 standard (100 μg/ml in acetonitrile) was spiked (20.0 μl) into 0.1 ml of acidified urine to a concentration of 21 μg/ml.

Sample Workup Procedure

Plasma Samples. To respective 15-ml polypropylene tubes (Becton Dickinson and Co., Lincoln Park, NJ), the following aliquots were added: 20.0 μl of the IS working standard, 20.0 μl of the ADR-529 working standard (for plasma standards), 1.0 ml of acidified plasma (i.e., blank, control, or study sample), and 1.0 ml of 0.5 *M* K_2HPO_4 (pH 7). This mixture was loaded onto the C-18 SPE column, which was preconditioned with 2 column vol each of methanol and water. The buffered plasma was aspirated through the SPE column (5 in. Hg), then rinsed with 2 × 2.5 ml water (5–10 in. Hg), dried for 5 min (15 in. Hg), rinsed with 1 ml of hexane (15 in. Hg), and dried for an additional 5 min (15 in. Hg). On the second 12-port vacuum manifold, C-8 SPE columns were preconditioned with 2 ml of 2% TFA in acetonitrile followed by 1 and 2 column vol of methanol and acetonitrile, respectively. Using a "piggyback" configuration, the C-18 SPE column was then fitted on top of the C-8 column via a column adapter. The drug and IS were eluted by gravity flow for 1–2 min from the C-18 to the C-8 SPE column with 2 ml of acetonitrile and then under mild vacuum (<5 in. Hg) to ensure complete elution. After discarding the C-18 SPE column, the C-8 column was rinsed with an additional 2 ml of acetonitrile (<5 in. Hg). The analytes were eluted with 2 ml of 2% TFA in acetonitrile (<5 in. Hg), evaporated to dryness under vacuum at 40°C (Buchler Vortex-Evaporator, Buchler Instruments, Inc., Fort Lee, NJ), and then reconstituted with 400 μl of acetonitrile. Depending on the sample concentration, 20 or 100 μl was injected into the HPLC system.

Plasma samples containing ADR-529 levels above the standard curve range were first diluted volumetrically with acidified water. The acidified water diluent was prepared by adding 10 μl of 42.5% phosphoric acid per ml of water. Finally, a 0.5-ml aliquot of the diluted sample was further diluted with 0.5 ml of blank acidified plasma for extraction by the procedure described above.

Urine Samples. To respective 15-ml polypropylene tubes the following aliquots were added: 20.0 μl of the IS, 100 μl of the urine standard, control, or study sample, and 10 ml of acetonitrile. The tube was capped and inverted to mix. Depending on the sample concentration, 20 or 100 μl was injected onto the HPLC system. Urine samples with ADR-529 levels above the dynamic range were first diluted volumetrically with acidified water and then prepared as described above.

Calculations

Peak height ratios (PHRs) were calculated for ADR-529/ICRF-192. Parameters of a linear model were obtained by employing an unweighted least-squares regression of PHR versus analyte concentration in the biomatrix calibrators. To accommodate the large dynamic range in plasma, a split-curve approach was used for improving accuracy at the low levels relative to a single-curve approach. A single curve was employed for the urine standards. The PHRs for the unknown and QCs were converted to concentration using the regression parameters. Relative weight responses (RWR's) were also calculated for each standard in order to validate linear regression. All plasma and urine concentrations are

expressed in terms of mass per milliliter of *un*acidified matrix.

Absolute Recovery from Plasma

Plasma Assay. For ADR-529, absolute recovery was determined by spiking human plasma at three concentrations in replicates of six. The spiked samples were extracted as described previously but ICRF-192 was added just prior to injection. The peak height ratios (PHRs) derived from the extracted samples were compared to unextracted solution standards. Similarly, for ICRF-192, absolute recovery was determined at 100 ng/ml, with ADR-529 added just prior to injection.

Specificity

Solutions of doxorubicin, epirubicin, idarubicin, methotrexate, and cyclophosphamide were prepared in acetonitrile. At least 5 ng on column was injected to assess any coelution with analytes of interest.

Stability

ADR-529 working standards prepared in acetonitrile and stored in leakproof high-density polyethylene bottles at 4°C for 12 weeks were assessed for stability. ADR-529 acidified dog plasma controls stored in polypropylene tubes at −20°C for 1 year were used to assess stability in plasma matrix.

RESULTS AND DISCUSSION

Chromatography

The ion-exchange separation for ADR-529 and ICRF-192 on silica is shown in chromatograms (Fig. 3). Using the described mobile phase and HPLC parameters, typical retention times for ICRF-192 and ADR-529 were 14 and 19 min, respectively. Typical run time for plasma and urine samples was 22 min. The rigorous plasma extraction procedure provided chromatographic windows free from endogenous matrix interferences. The adoption of a heart-cut column switching technique prevented electrode passivation by venting the late eluters to waste. Because of the added ruggedness afforded by column switching, it was also used for urine samples.

Recent reports (11–14) have described the use of silica as a cation exchange support for the separation of basic compounds from biological fluids. For strongly basic amines (i.e., $pK_a > 7$), Flanagan *et al.* (11–13) successfully employed bare silica using nonaqueous mobile phases composed of methanol, with ammonium perchlorate and sodium hydroxide as ionic and pH modifiers, respectively. Retention and peak shape were controlled by adjustment of pH and ionic strength. These investigators have reported that addition up to 10% water appeared to have little effect on analyte capacity factors. For weaker amines, methanolic mobile phases were acidified with either perchloric acid or camphorsulfonic acid, which would protonate the amine group to create a stronger interaction with the acidic silanol sites. Acetoni-

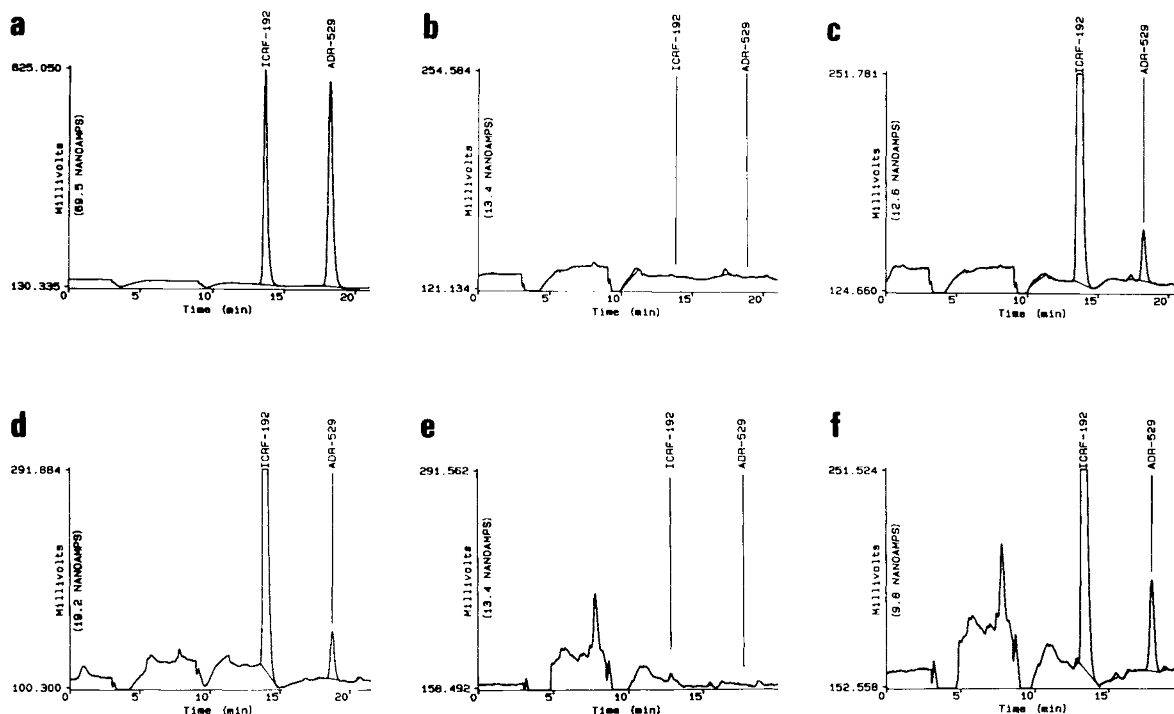

Fig. 3. (a) System suitability test mixture: ICRF-192 and ADR-529 (each at 250 ng/ml in acetonitrile). (b) Human plasma blank. (c) Low human plasma standard: ICRF-192 (103 ng/ml) and ADR-529 (5.22 ng/ml). (d) Dog plasma sample 12 hr post 10 mg/kg i.v. infusion (ADR-529 quantified at 6.27 ng/ml). (e) Human urine blank. (f) Low human urine standard: ICRF-192 (21.2 μg/ml) and ADR-529 (2.04 μg/ml).

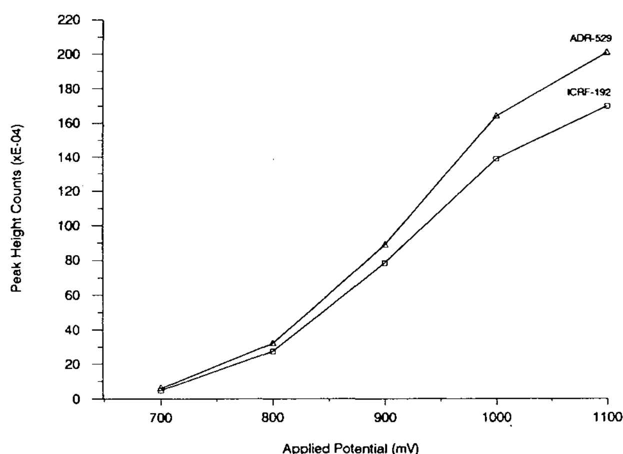

Fig. 4. ADR-529 and ICRF-192 peak height counts versus applied oxidation potential.

trile-based mobile phases with ionic modifiers were successfully used on strong cation-exchange (13) and bare silica (14) packings.

The pK_a of ADR-529's most basic nitrogen is 2.5 (3), which suggests that the compound is a weak base. Initial attempts to ion-exchange on silica with methanolic mobile phases resulted in low capacity factors. Acetonitrile-based mobile phases exhibited higher capacity factors and more control over the separation. Both camphorsulfonic acid and phosphoric acid were used successfully as pH and ionic modifiers. Phosphoric acid was selected because of improved resolution between the drug and the IS. Contrary to results reported for stronger amines (11,13), water content up to 10% in the mobile phase progressively reduced the capacity factors for these weaker amines. Therefore, the combination of water and phosphoric acid was used to control analyte peak shape and retention.

Traditional chromatography on silica with nonaqueous mobile phases, without ionic modifiers has now been replaced by more rugged RP-HPLC systems for biomatrices. Contamination of the mobile phase by water has been shown to result in poor analyte peak shape and variable retention times for normal phase systems (15). Ion-exchange chromatography on silica appears to be immune from these classical normal phase problems (11–14). In our laboratory, nearly a thousand extracts have been injected without any evidence of column deterioration. No special column conditioning or cleaning has been necessary.

Modification of the column switching times also allows the detection of the two monoacid, monoamide hydrolysis products of ADR-529 (3,4) with this HPLC system. Retention times of approximately 35 and 36 min were observed for the minor and major hydrolysis products (each with a single open ring), respectively. This procedure is also capable of assaying the hydrolysis products in urine.

During the assay development, it was necessary to replace the metal needles attached to the vacuum manifold lid with polypropylene tips to avoid a large late eluting peak seen in most chromatograms. This late eluter was probably due to the corrosive activity of the 2% TFA in acetonitrile used for elution of the analytes from the C-8 SPE columns.

Electrochemical Detection

Electrochemical detection with nonaqueous or high-organic strength mobile phases has been documented (12,13,16,17). These systems have been used successfully to detect basic compounds (e.g., tertiary amines) requiring high oxidation potentials (i.e., >0.9 V vs Ag/AgCl). For ADR-529 and the IS, the tertiary piperazine nitrogen apparently undergoes oxidation at high anodic potentials. The responses, as peak height, for ADR-529 and the IS vs applied potential are plotted in Fig. 4. The selected potential of +1050 mV vs the $Ag/AgNO_3$ reference electrode was a compromise between desired analyte sensitivity (signal) and background noise. The detector was operated at 100 nA full scale, with a typical offset of 70 nA. Although the electrochemical detector was operated at a relatively high potential, it was substantially more selective than UV detection at 208–210 nm for biological samples containing ADR-529.

Linearity

The intraday mean (±SD) RWR estimates for the low curve ranged from 0.883 (±0.046) to 0.937 (±0.042), while those for the high curve ranged between 0.880 (±0.015) and 0.959 (±0.029). The percentage RSD associated with the intraday RWRs for the low and high plasma and the urine curves ranged between 1.4 and 8.6 and between 2.0 and 2.5%, respectively, and hence suggests linearity in the system. Linearity estimator, the coefficient of determination, for the low and high plasma curve ranged between 0.9974 and 0.9999 and between 0.9955 and 0.9999, respectively. Random distribution of the actual data about the fitted regression line further supported the adequacy of a linear

Table Ia. Reproducibility and Precision Estimates from Six Low and High Standard Curves in Plasma

	Standard concentration (ng/ml)										
	Low curve					High curve					
Label	5.22	7.32	10.4	20.9	41.8	20.9	41.8	62.7	104	261	522
Mean	5.18	7.41	10.5	20.5	41.9	20.9	43.2	62.9	99.4	264	520
% bias	−0.8	+1.2	+1.0	−1.9	+0.2	0.0	+3.3	+0.3	−4.4	+1.1	−0.4
SD	0.38	0.34	0.37	0.73	0.34	3.94	2.86	3.10	2.90	18.1	8.04
% RSD	7.34	4.58	3.49	3.54	0.82	18.8	6.62	4.94	2.92	6.87	1.55

Table Ib. Reproducibility and Precision Estimates from Three Standard Curves in Urine

Label	Standard concentration (μg/ml)						
	2.04	5.10	10.2	20.4	40.8	61.2	102
Mean	1.98	5.10	10.3	20.6	40.8	60.6	102
% bias	−2.9	0.0	+1.0	+1.0	0.0	−1.0	0.0
SD	0.24	0.12	0.21	0.51	0.75	1.10	0.58
% RSD	12.4	2.3	2.0	2.5	1.8	1.8	0.6

model. The reproducibility and precision estimates obtained from the backcalculated standard values for the low and high plasma ($n = 6$) and the urine ($n = 3$) curves are reported in Tables Ia and b, respectively. Further, the slope of the regression line for the low and the high plasma curve showed a percentage RSD of <7.

Plasma Assay. For the split-curve approach, the low (LC) and high (HC) standard curve dynamic ranges were 5–40 and 20–500 ng/ml, respectively. The consistency of RWRs over the entire dynamic range supported the use of the linear regression algorithm. However, the use of a single curve for the 100-fold dynamic range produced unacceptable bias (i.e., >±20%) for the low standards. Acceptable accuracy and precision for the back-calculated standard values were obtained by employing a split curve approach (Table Ia). The mean estimate of percentage bias did not exceed 5% in magnitude for the LC or HC. The coefficient of variation did not exceed 8% on the LC. The % RSD for the mean slope estimate for the LC and the HC did not exceed 7%. The coefficients of determination for the regression were always greater than 0.99.

Urine Assay. The dynamic range for the standard curve in urine was 2–100 μg/ml. The RWRs were consistent over the entire concentration range (range, 0.815 to 1.08). Acceptable accuracy (<±3% bias) and precision (<13% RSD) were obtained for the back-calculated standard values at the LLOQ (Table Ib). The linearity in the urine matrix was adequately demonstrated by the high r^2 (>0.999) and a percentage RSD of <12% in the slope of the regression curve.

Accuracy and Precision

Pooled quality control samples prepared in plasma and urine were used for assessing accuracy, and within (intraday) and between (interday) precision. Samples stored at −20°C were assayed in replicates on separate days.

Plasma. As shown in Table IIa, the within- and between-run imprecision estimates for ADR-529, from replicates of six, were <9% RSD from all QC samples ranging between 7 and 700 ng/ml. Accuracy, expressed as percentage bias, was <±10% for the low (7.21 ng/ml) and <±8% for all other controls. The extraction performed equally well for both 0.5- and 1.0-ml plasma aliquots, with no loss in precision or accuracy.

Urine Assay. The intra- and interday imprecisions were generally <10% RSD (Table IIb) and the experimentally found analyte levels were within ±12% of the label claim.

Sensitivity

Instrumental Limit of Detection (ILOD). A series of ADR-529 solution standards injected into the HPLC system showed that a mass of analyte producing a s/n ratio of 3 in the absence of biomatrix was 0.1 ng on column, which corresponds to a concentration of 0.4 ng/ml.

Lower Limit of Quantification (LLOQ). For the plasma assay, the defined LLOQ was 5 ng/ml when 25% of the volume was injected. At this level, the s/n ratio was >20, the accuracy and imprecision estimates were <10%, and the RWRs were consistent throughout the dynamic range. Increasing the injection volume twofold and redefining the low standard curve range, an LLOQ of 1–2 ng/ml is possible.

For urine, the LLOQ was 2 μg/ml. Performance of the low standards generally equaled the above criteria used for plasma.

Table IIa. Within- and Between-Run Estimates of Accuracy and Precision for ADR-529 in Plasma from Quality Controls

Label concn. of control sample (ng/ml)	Day	n	Accuracy		Precision		
			Mean found (ng/ml)	% bias	SD	% RSD (intraday)	Pooled % RSD (interday)
7.21	1	6	7.54	+4.6	0.13	1.8	
	2	6	6.96	−3.5	0.07	1.1	
	3	6	6.79	−5.8	0.22	3.3	
	4	5[a]	7.91	+9.7	0.71	8.9	
	5	6	7.13	−1.1	0.09	1.3	6.87
72.1	1	5[a]	70.5	−2.2	6.1	8.6	
	2	6	70.2	−2.6	1.5	2.2	
	3	6	77.8	+7.9	5.5	7.0	
	5	6	71.7	−0.6	5.5	7.7	7.68
721[b]	3	6	766	+6.2	31.2	4.1	
	5	6	720	−0.1	46.4	6.4	
	6	6	760	+5.4	38.2	5.0	5.64

[a] One replicate omitted due to contamination of the sample.
[b] Only 0.5-ml aliquots of the 721 ng/ml control assayed.

Table IIb. Within- and Between-Run Estimates of Accuracy and Precision for ADR-529 in Urine from Quality Controls

Label concn. of control sample (μg/ml)	Day	*n*	Accuracy		Precision		
			Mean found (μg/ml)	% bias	SD	% RSD (intraday)	Pooled % RSD (interday)
3.06	1	3	3.44	+12.4	0.16	4.6	
	2	3	2.82	−7.8	0.025	0.89	
	3	3	3.31	+8.2	0.12	3.5	9.4
81.6	1	3	87.1	+6.7	2.11	2.4	
	2	3	81.4	−0.2	0.53	0.65	
	3	3	84.3	+3.3	3.78	4.5	3.9

Absolute Recovery from Plasma

The absolute recoveries for ADR-529 in the range of 5–500 ng/ml were >95%, with an imprecision of <4% RSD. The internal standard demonstrated a similar extraction efficiency at 100 ng/ml.

The two-column SPE procedure is based on multimodal retention mechanisms which selectively isolate the drug and IS from plasma (18,19). A reverse-phase retention mechanism seems to dominate on the first C-18 SPE column, as acetonitrile quantitatively elutes both analytes. However, acetonitrile apparently behaves only as a weak eluent toward retention on the second C-8 column. Addition of TFA probably protonates the cation-exchange sites (i.e., $SiO^- \rightarrow SiOH$) and thus assists in eluting basic analytes. The primary interaction is suspected to be between the analytes' protonated basic nitrogens and the SPE column's acidic silanol sites. This ion-exchange process is minimal on the first SPE column because of endogenous plasma amines saturating the available silanol sites. Once the sample is desalted and deproteinated on the first column, the ionic interaction between the analytes and the silanol sites dominate on the second column. Multimodal retention properties have been successfully used for the selective isolation of other organic amines and xenobiotics from biological fluids (20–23). A variety of SPE column types has been exploited for their silanol interaction including octadecyl (20), cyano (21), and bare silica (22,23). Throughout our method development efforts, the C-2, C-8, C-18, and phenyl Bond Elut SPE columns were evaluated for their weak ion-exchange retention of ADR-529 and the IS. The C-8 column was selected because of its lot to lot consistency.

Specificity

Doxorubicin, epirubicin, idarubicin, methotrexate, and cyclophosphamide, which are likely to be administered concomitantly with ADR-529, were injected into the HPLC system and either were not detected by the electrochemical detector or eluted outside of the analytes' chromatographic windows and hence did not interfere with the quantification of ADR-529.

Stability

Acetonitrile Solutions. No degradation was observed for ADR-529 acetonitrile working standards (i.e., 0.25–25 μg/ml) when stored in leakproof polyethylene bottles at 4°C for 12 weeks.

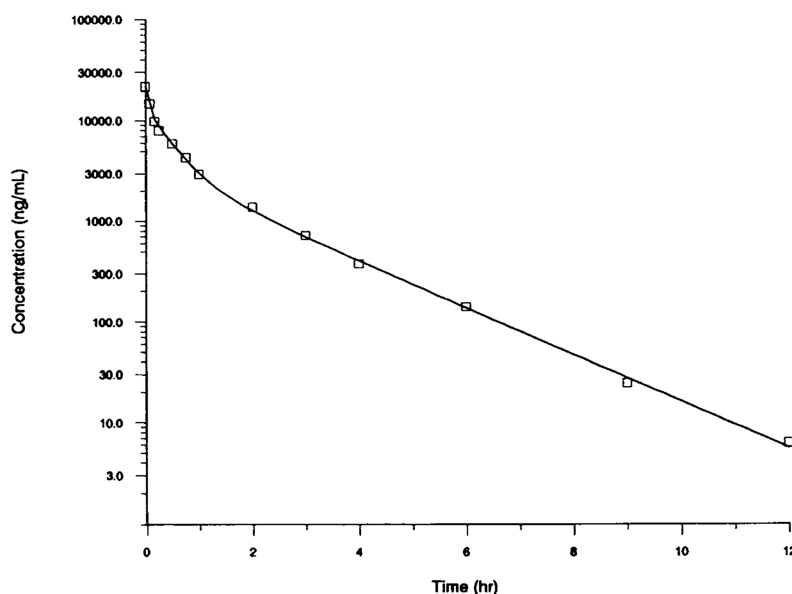

Fig. 5. A representative plot of plasma ADR 529 concentration vs time profile following i.v. administration of a 10 mg/kg dose to dog.

Although not rigorously monitored, degradation of ADR-529 and ICRF-192 stocks (i.e., 125 and 100 μg/ml, respectively) in acetonitrile was observed after storage at 4°C. Therefore, the stock standard was serially diluted to the working standards within a day of its preparation.

Plasma. Acidified controls of ADR-529 prepared in dog plasma at 21.6, 104, and 542 ng/ml exhibited no quantifiable degradation over 1 year when stored at −20°C.

Method Application

Figure 5 shows the kinetic profile of ADR-529 in dog plasma following a single iv dose of 10 mg/kg. Drug concentrations declined in a biphasic manner with an apparent terminal half-life of 1.3 hr.

CONCLUSIONS

A specific and sensitive HPLC-ED methodology has been developed and validated for quantitation of ADR-529 in biological fluids. The use of multimodal solid-phase extraction, ion-exchange HPLC, and amperometric detection provides a selective system to quantify ADR-529 over a dynamic range of 5–500 ng/ml and 2–100 μg/ml in plasma and urine, respectively. The rigorous two column solid-phase extraction from plasma affords excellent accuracy and precision and assures consistent and clean chromatographic windows. Imprecision as a result of such an extensive extraction was controlled further by the addition of an IS.

This methodology is currently being employed for studying clinical pharmacology and pharmacokinetics of ADR-529 in cancer patients, in whom it is being given as a cardioprotectant.

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REFERENCES

1. E. H. Herman and V. J. Ferrans. Pretreatment with ICRF-187 provides long-lasting protection against chronic daunorubicin cardiotoxicity in rabbits. *Cancer Chemother. Pharmacol.* **16**:102–106 (1986).
2. E. H. Herman, V. J. Ferrans, R. S. K. Young, and R. L. Hamlin. Effect of pretreatment with ICRF-187 on the total cumulative dose of doxorubicin tolerated by beagle dogs. *Cancer Res.* **48**:6918–6925 (1988).
3. J. Sisco. *The Physicochemical, Analytical and Pharmacokinetic Properties of the Antineoplastic Agent, ICRF-187*, Ph.D. dissertation, Department of Pharmaceutical Chemistry, University of Kansas, August 1989.
4. D. T. Rossi. *Structural elucidation of ADR-529 hydrolysis degradation products*. Adria analytical research report 88-9 (1989).
5. W. Sadee, J. Staroscik, C. Finn, and J. Cohen. (±)-1,2-Bis(3,5-dioxopiperazinyl)propane plasma levels in rats, rabbits, and humans by GLC and mass fragmentography. *J. Pharm. Sci.* **64**(6):998–1001 (1975).
6. R. Earhart, K. Tutsch, J. Koeller, R. Rodriguez, I. Robins, C. Vogel, H. Davis, and D. Tormey. Pharmacokinetics of (+)-1,2-di(3,5-dioxopiperazin-1-yl)propane intravenous infusions in adult cancer patients. *Cancer Res.* **42**:5255–5261 (1982).
7. M. Collins, X. Fu, N. Greig, and K. Hellmann. Determination of razoxane by high-performance liquid chromatography. *J. Chromatogr.* **274**:434–440 (1983).
8. C. Vogel, E. Gorowski, E. Davila, M. Eisenberger, J. Kosinski, R. Agarwal, and N. Savaraj. Phase I clinical trial and pharmacokinetics of weekly ICRF-187 (NSC 169780) infusion in patients with solid tumors. *Invest. New Drugs* **5**:187–198 (1987).
9. J. R. Baldwin, B. A. Uhrig, and P. K. Narang. *Validation of the ultrafiltration assay procedure for the quantitation of ADR-529 in rat plasma.* Adria report PK/BAR-3154-89-01 (1989).
10. B. Bailey. *Personal communication.*
11. R. Flanagan and I. Jane. High-performance liquid chromatographic analysis of basic drugs on silica columns using non-aqueous ionic eluents. I. Factors influencing retention, peak shape and detector response. *J. Chromatogr.* **323**:173–189 (1985).
12. I. Jane, A. McKinnon, and R. Flanagan. High-performance liquid chromatographic analysis of basic drugs on silica columns using non-aqueous ionic eluents. II. Application of UV, fluorescence and electrochemical oxidation detection. *J. Chromatogr.* **323**:191–225 (1985).
13. R. Flanagan, R. Bhamra, S. Walker, S. Monkman, and D. Holt. Measurement of cardioactive drugs in biological samples by HPLC. *J. Liq. Chromatogr.* **11**(5):1015–1040 (1988).
14. D. Vendrig, J. Teeuwsen, and J. Holthuis. Determination of vinca alkaloids in plasma and urine using ion-exchange chromatography on silica gel and fluorescence detection. *J. Chromatogr.* **434**:145–155 (1988).
15. L. Snyder and J. Kirkland. *Introduction to Modern Liquid Chromatography,* 2nd ed., John Wiley & Sons, New York, 1979, pp. 374–383, 391–398.
16. M. Thomas and T. Last. Voltammetric electrochemical detection for normal-phase high-performance liquid chromatography. *Anal. Chem.* **60**:2158–2161 (1988).
17. J. Cooper, E. Hawes, J. Hubbard, G. McKay, and K. Midha. An ultrasensitive method for the measurement of fluphenazine in plasma by high-performance liquid chromatography with coulometric detection. *Ther. Drug Monit.* **11**(3):354–360 (1989).
18. D. Blevins. Sample preparation involving multimodal bonded phase extraction. *Sample Preparation and Isolation Using Bonded Silicas; Proceedings from the Third Annual International Symposium,* Analytichem International, 1986, pp. 301–327.
19. R. Lewis and F. Schwende. HPLC determination of nicorandil in plasma using a double solid phase extraction clean-up. *Sample Preparation and Isolation Using Bonded Silicas; Proceedings from the Third Annual International Symposium,* Analytichem International, 1986, pp. 197–222.
20. F. Schwende and R. Lewis. Determination of nicorandil in plasma using high-performance liquid chromatography with photoconductivity and ultraviolet detection; Application to preclinical pharmacokinetics in beagle dogs. *J. Chromatogr.* **525**:151–160 (1990).
21. D. Lakings, J. Friis, and M. Bruns. High-performance liquid chromatographic method for the determination of eclanamine and its N-desmethyl and N,N-didesmethyl metabolites in urine. *J. Chromatogr.* **428**:131–139 (1988).
22. R. Kupferschmidt and R. Schmid. Specific routine determination of 3′-azido-3′-deoxythymidine (AZT) in plasma by partly automated liquid chromatography. *Clin. Chem.* **35**(7):1313–1317 (1989).
23. J. Cox and R. Pullen. Isomodal column switching high-performance liquid chromatographic technique for the analysis of ciglitazone and its metabolites in human serum. *J. Chromatogr.* **307**:155–171 (1984).