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Quantification of a novel retinoic acid metabolism inhibitor, 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma by liquid chromatography with diode-array detection

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Abstract

A simple reversed phase high performance liquid chromatographic (HPLC) method was developed for the separation and quantification of a novel retinoic acid metabolism inhibitor, 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA), and other retinoids in rat plasma. VN/14-1RA, alone or in combination with ATRA, is effective at inhibiting the proliferation of prostate and breast cancer cell lines in vitro. Aliquots of rat plasma were spiked with the retinoids followed by addition of acetonitrile for precipitation of plasma proteins. The decanted supernatant was evaporated under a stream of nitrogen and reconstituted in acetonitrile. Analysis was accomplished by injection of an aliquot of the reconstituted sample into an HPLC system consisting of a Zorbax Rx-C18 column and a diode array detector. A mobile phase composed of ammonium acetate (0.1 M), acetic acid solution (2% (v/v)) and methanol at a flow rate of 1.0 mL/min was used for gradient elution. The recoveries for all compounds ranged from 65 to 85% regardless of the concentrations examined. The HPLC assay was linear over the range 0.10–5.0 μg/mL (CV < 10%) with a limit of quantification of 100 ng/mL for VN/14-1RA. A one-compartment model with apparent first-order elimination was used to describe the plasma concentration-time profile for VN/14-1RA after intravenous administration. The mean terminal elimination half-life ($t_{1/2}$) was 19.0 ± 3.2 min. This HPLC method is useful for the analysis and evaluation of the pharmacokinetics of VN/14-1RA in rats.

Keywords: All-*trans*-retinoic acid; Retinoic acid metabolism blocking agent; Reversed-phase HPLC

1. Introduction

Retinoids comprise a family of polyisoprenoid lipids, which include vitamin A (retinol) and its natural and synthetic analogues. All-*trans*-retinoic acid (ATRA) is a natural oxidative metabolite and biologically active form of retinol. Retinoids are currently the subject of intense biological interest stimulated by the discovery and characterization of retinoid receptors [1]. It is well established that retinoids such as ATRA control normal cell growth, differentiation, and apoptosis within epithelial tissues. The ability of this class of agents to function as differentiating agents provides a viable approach to cancer treatment [2]. However, current systemic therapy and clinical success with ATRA is limited due to toxicity and development of resistance. The development of resistance to retinoid therapy is thought to result from, at least in part, induction of oxidative metabolism mediated by cytochrome P450 enzymes. Consequently, it has been postulated that inhibition of oxidative metabolism of ATRA by retinoic acid metabolism blocking agents (RAMBAs) in vivo may increase circulating levels of ATRA due to a decrease in its clearance. RAMBAs elevate or maintain

Abbreviations: VN/14-1RA, 4-(1H-imidazol-1-yl)retinoic acid; RAMBA, retinoic acid metabolism blocking agent; ATRA, all-*trans*-retinoic acid; 4-OH-RA, 4-hydroxy all-*trans* retinoic acid; 4-oxo-RA, 4-oxo all-*trans* retinoic acid; HPLC, high performance liquid chromatography

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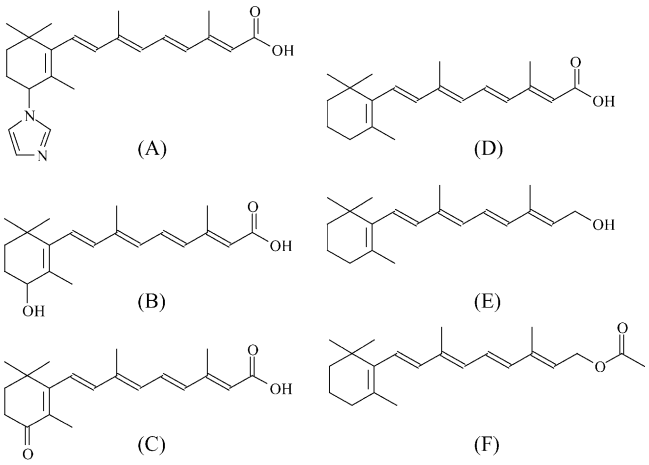

Fig. 1. Chemical structure of the analytes: (A) 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA); (B) 4-hydroxy all-*trans* retinoic acid (OH-RA); (C) 4-oxo all-*trans* retinoic acid (oxo-RA); (D) all-*trans*-retinoic acid (ATRA); (E) retinol; (F) internal standard (all-*trans*-retinol acetate).

endogenous ATRA levels by inhibiting ATRA 4-hydroxylase mediated catabolism and may help overcome the problem of resistance [3,4]. It has been argued that the action of RAMBAs may improve the control of differentiation and growth and possibly enhance the antitumor activity of ATRA. This rationale has been extensively tested with liarozole fumarate (Liazal^{TM}) in vitro and in vivo [4–8]. Unfortunately, liarozole is not selective and has been shown to inhibit other cytochrome P450 enzymes. Thus, there is a need to identify more selective inhibitors of retinoic acid metabolism and evaluate them as potential treatments for prostate and breast cancers. Recently, novel (±)-4-azoyl retinoic acid analogues have been synthesized and shown to be potent inhibitors of microsomal ATRA 4-hydroxylase(s). It was demonstrated that 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) (Fig. 1) is significantly more potent than liarozole in blocking the activity of ATRA 4-hydroxylase(s) [7]. In addition, VN/14-1RA, alone or in combination with ATRA, was effective at inhibiting the proliferation of prostate and breast cancer cell lines in vitro. VN/14-1RA could be more selective and effective than current inhibitors of retinoic acid metabolism [8].

Although several HPLC methods have been reported in the literature for the measurement of retinoids [9–11], there are no reports that described the recovery, separation and quantification of 4-azolyl retinoids such as VN/14-1RA in biological matrices. The present work describes a rapid and facile reversed phase HPLC method with diode-array detection (DAD) for the simultaneous separation and quantification of VN/14-1RA, retinol, ATRA and its metabolites; 4-hydroxy all-*trans* retinoic acid (4-OH-RA) and 4-oxo all-*trans* retinoic acid (4-oxo-RA); in rat plasma and some aspects of the pharmacokinetic properties of VN/14-1RA after intravenous administration in rats. This HPLC method may have general application in the analysis and pharmackinetic evaluation of other 4-azolyl retinoic acids.

2. Experimental

2.1. Materials and reagents

All-*trans*-retinoic acid (ATRA), all-*trans*-retinol, all-*trans*-retinol acetate (internal standard), and hydroxypropyl-β-cyclodextrin (HPβCD) were purchased from Sigma–Aldrich (St. Louis, MO, USA). VN/14-1RA, 4-OH-RA and 4-oxo-RA were provided by Drs Brodie and Njar of the University of Maryland, School of Medicine, Baltimore. Distilled water was obtained from an in-house Barnstead NANOpure® apparatus (GenTech Scientific Inc., Arcade, NY, USA). Methanol, acetonitrile, ammonium acetate and glacial acetic acid were obtained from VWR Scientific Products (Bridgeport, NJ, USA). All chemicals and solvents were of analytical or HPLC grade.

2.2. Preparation of standard solutions and formulations for dosing

Stock solutions of all analytes were prepared with acetonitrile (1.0 mg/mL). Solutions were kept in capped test tubes, wrapped with aluminium foil, and stored at −70 °C. Appropriate working standard solutions of all compounds were obtained by sequential dilutions of the respective stock solutions with acetonitrile.

Formulations of ATRA (5 mg/mL) and VN/14-1RA (10 mg/mL) were prepared in HPβCD (5 mg/mL in saline), sonicated until completely dissolved, and stored at 0 °C. These formulations were diluted with saline to obtain required concentrations for animal treatment. The dosing solutions were placed in a water bath and allowed to reach a temperature of 37 °C before dosing.

2.3. Sample preparation

Whole blood (250 μL) was collected via the jugular vein from male Sprague-Dawley rats into heparinized Eppendorf centrifuge tubes. The blood was immediately centrifuged at 3200 g for 5 min using an Abbott laboratories centrifuge (model 3531) min) and plasma was transferred into clean Eppendorf centrifuge tubes with snap cap for storage at −20 °C. Aliquots (100 μL) of thawed plasma sample were transferred into a 1.5 mL centrifuge tubes using Eppendorf® pipette (Hamburg, Germany). A mixture (200 μL) of acetonitrile containing acetic acid (1% (v/v)) and the internal standard (0.5 mg/mL) was added to the samples to denature plasma proteins and minimize variability, respectively. The samples were vortexed for 10 s and centrifuged at 3200 g for 10 min using an Abbott laboratories centrifuge (model 3531); the supernatant (290 μL) was transferred into clean 5 mL glass test tubes and evaporated to dryness using nitrogen at room temperature. The residue was dissolved in acetonitrile (15 μL), transferred into amber colored HPLC vials and injected into the HPLC for analysis. All handling of retinoids and biological samples was performed in a room

with dim yellow light. Sample preparation was performed in duplicate on three occasions ($n = 3$).

2.4. Stability studies

Retinoids are known to be sensitive to light, heat and oxygen. Therefore, the stability of the analytes was monitored. A set of standard solution in plasma was stored at room temperature (20 °C) and another in a refrigerator (0 °C). Each sample ($n = 3$) was analyzed periodically at day 1, 2, 7 and 14 after preparation of the standard solution and compared to freshly prepared standards. Before HPLC analysis, a mixture (200 μL) of acetonitrile containing acetic acid (1% (v/v)) and the internal standard (0.5 mg/mL) was added to each sample and prepared as described above.

2.5. Chromatographic conditions

The analysis was performed on an HP-1100 HPLC system equipped with a vacuum degasser, quaternary pump, autosampler, column heater, and a diode array detector (DAD). HPLC analysis was conducted on a 5 μm Zorbax-Rx C_{18} column (25 cm × 4.6 mm i.d., Agilent Technologies, Wilmington, DE) with a C_{18} μBondapak guard cartridge (Waters, Milford, MA). Agilent ChemStation® (revision A.06.01) was used for data collection and integration. The mobile phase consisted of three solvent: solvent A: ammonium-acetate solution in water (0.1 M); solvent B: glacial acetic acid solution in water (2% (v/v)); solvent C: methanol (100%). Elution was started at a composition of 25% of solvent A, 15% of solvent B and 60% of solvent C. The mobile phase composition was changed linearly to 15% of solvent A, 0% of solvent B, and 85% of solvent C from the initial time to 12 min. The mobile phase composition was changed linearly to 0% of solvent A, 0% of solvent B, and 100% of methanol from 12 to 22 min and kept at 100% methanol for 4 min. The column was then equilibrated in a mobile phase composed of 25% of solvent A, 15% of solvent B, and 60% of solvent C for 8 min before the next sample was injected. The temperature was maintained at 25 °C. The diode array detector (DAD) was set at wavelengths of 324 nm (retinol, and internal standard), 346 nm (VN/14-1RA, 4-OH-RA, and ATRA) and 358 nm (4-oxo-RA) to monitor all the compounds simultaneously. The optimum wavelengths of all the compounds were determined by Agilent ChemStation® three-dimension spectral analysis module. Quantitation was based on peak areas.

2.6. Calibration curves and assay validation

Aliquots (10 μL) of appropriate dilutions of standard solutions of ATRA and VN/14-1RA were spiked into clean test tubes, evaporated to dryness and reconstituted into blank plasma (100 μL) in order to generate concentrations in the range of 50 ng/mL to 10 μg/mL. The following concentrations were used for the construction of calibration curves for ATRA and VN/14-1RA: 0.0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0, 5.0 μg/mL). Similarly, aliquots (10 μL) of appropriate dilutions of standard solutions of the other retinoids were spiked into clean test tubes, evaporated to dryness and reconstituted into blank plasma (100 μL) in order to generate concentrations in the range of 20 ng/mL to 3 μg/mL. The following concentrations were used for the construction of calibration curves for 4-OH-RA, 4-oxo-RA and retinol: 0.0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0 μg/mL). Before HPLC analysis, a mixture (200 μL) of acetonitrile containing acetic acid (1% (v/v)) and the internal standard (0.5 mg/mL) was added to each sample and prepared as described above. The reconstituted extract was injected into the HPLC system. Calibration curves for all the compounds were calculated from the least-squares linear regression analysis of peak area ratio (analyte/internal standard) versus sample concentrations using Microsoft® Excel. The ratios of the peak areas for VN-14/1RA, ATRA, 4-OH-RA, 4-oxo-RA, and retinol to the peak area for the internal standards were determined from the chromatograms. The area under the peak of each analyte was normalized for the area under the peak of the internal standard to minimize variability of the assay. The concentrations of the analytes in the samples were computed from the regression parameters. The limit of detection was defined as the smallest concentration of the analyte that produced a peak size that was three times greater than the standard deviation of the HPLC detector noise level (LOD = 3 × S/N). The limit of quantification was defined as the concentration of the analyte that produced a peak size that was ten times greater than the standard deviation of the HPLC detector noise level (LOQ = 10 × S/N). Retinol and its active metabolite (ATRA) are normally present in rat plasma. Although physiologic levels of ATRA in plasma were not detected by this HPLC assay, endogenous retinol was routinely detected in rat plasma. The endogenous concentration of retinol was determined from the mean intercept of the calibration curve for retinol. The recovery of each analyte was determined from the ratio of chromatographic peak area after extraction from plasma to that of an equivalent working standard. Each sample was evaluated in duplicate on three occasions ($n = 3$).

To determine the precision and accuracy of the HPLC assay, known concentrations of the analytes, ranging from 50 to 5000 ng/mL, were spiked in blank plasma samples and analyzed as described above. The validation study was performed in duplicate on three occasions ($n = 3$) over a 2-week period. The coefficient of variation (a measure of precision) and the relative error (a measure of accuracy) were calculated from the spiked concentrations, measured concentrations and the standard deviations of the measured concentrations of the analytes in plasma.

2.7. Preliminary pharmacokinetics of VN/14-1RA

Male Sprague Dawley rats (230–250 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were maintained in a controlled environment of constant temperature (20 °C), 50% relative humidity and

12 h light/dark cycles for at least 4 days prior to use. The rats were surgically prepared, under ketamine (90 mg/kg) and xylazine (10 mg/kg) anaesthesia, by implanting indwelling cannula into the jugular vein 24 h prior to drug administration and blood sampling. A single dose of VN/14-1RA (5 mg/kg) combined with ATRA (0.5 mg/kg) was administered through the jugular vein to the rats (n = 3). Blood samples (200 μL) were collected in heparinized tubes before treatment and at 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300 min, and 24 h after drug administration. Plasma levels of ATRA and VN/14-1RA were determined by HPLC with UV detection as described above. The resulting plasma level data for VN/14-1RA were used to define the pharmacokinetic model and to calculate the basic pharmacokinetic parameters of VN/14-1RA in rats using WinNonlin (Pharsight Corporation Inc., Mountain View, CA).

3. Results

3.1. Chromatography

The maximum UV absorption for the analytes in the mobile phase were as follows; retinol acetate and retinol at 324 nm; VN/14-1RA, 4-OH-RA and ATRA at 346 nm; and 4-oxo-RA at 358 nm. The chromatographic conditions used for the assay provided a well-defined separation (R_s > 1.5) between the compounds and the internal standard. Under the conditions described, retention times for VN/14-1RA, 4-oxo-RA, 4-OH-RA, ATRA, retinol and retinol acetate were approximately 12.51, 13.36, 13.82, 19.58, 22.47 and 25.27 min, respectively. The standard deviations of the retention times were within 0.05 min. The chromatograms for blank plasma and blank plasma spiked with VN/14-1RA, 4-OH-RA, 4-oxo-RA, ATRA, and retinol acetate are shown in Fig. 2. A chromatogram of an extract of plasma obtained from an animal treated with VN/14-1RA (5.0 mg/kg) in combination with of ATRA (0.5 mg/kg) is shown in Fig. 3.

3.2. Stability

When the analytes (1000 ng/mL in plasma), placed in ambler HPLC vials, were stored at room temperature with normal lighting, no detectable degradation occurred within 2 days. However, on day 7 and 14, significant degradation of the analytes occurred based on measurements of the chromatographic peak size (Table 1). The degradation was less than 20% after 14 days for all the compounds. In order to minimize degradation of analytes and to obtain accurate results, HPLC analysis of all samples was completed within 2 days of sample collection.

3.3. Calibration curves and assay validation

The recovery for each analyte was obtained by comparing the peak area from plasma with peak area from standard solutions at three different concentrations on three occasions. The recoveries for all compounds ranged from 65 to 85% regardless of the concentrations examined (Table 2).

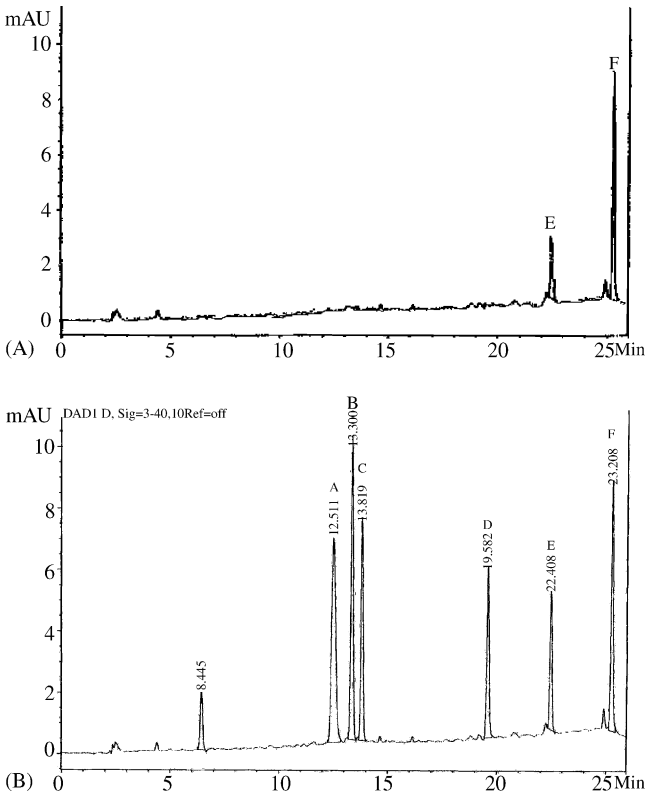

Fig. 2. Typical chromatogram of (a) blank plasma spiked with internal standard; (b) plasma spiked with standards (1.0 μg/mL). (A) VN/14-1RA; (B) oxo-RA; (C) OH-RA; (D) ATRA; (E) retinol; (F) internal standard.

Linear calibration curves were obtained over the range of 0.10–5.0 μg/mL for VN/14-1RA ($y = 0.1081x - 10.176$, $r^2 = 0.9976$). Similarly, linear calibration curves were obtained over the range of 0.05–5.0 μg/mL for ATRA ($y = 0.1877x + 1.2047$, $r^2 = 0.9962$). Linear calibration curves were also

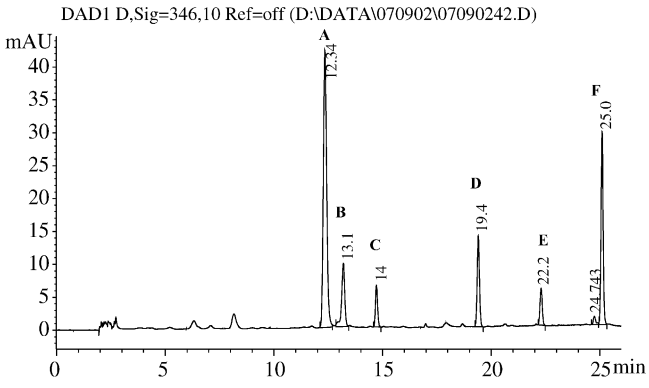

Fig. 3. Typical chromatogram of an extract of a plasma sample obtained 20 min after intravenous administration of VN/14-1RA (5 mg/kg) in combination with ATRA (0.5 mg/kg). The concentrations of VN/14-1, ATRA, OH-RA, oxo-RA and retinol in this sample were 5000, 3200, 775, 1125, and 600 ng/mL, respectively. (A) VN/14-1RA; (B) oxo-RA; (C) OH-RA; (D) ATRA; (E) retinol; (F) internal standard.

Table 1
Stability profile of 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma

Analytes (1.0 μg/mL)	Refrigerator (0 °C)			Room temperature (20 °C)		
	% Loss			% Loss		
	2nd day	7th day	14th day	2nd day	7th day	14th day
VN/14-1RA	0.4	0.9	2.2	1.1	12.4	17.4
ATRA	−0.3	−0.2	1.2	0.4	7.1	11.9
Retinol	−1.5	0.1	0.9	−0.4	4.1	4.5
OH-RA	1.3	2.2	3.0	0.9	8.3	12.2
Oxo-RA	0.5	2.0	1.9	0.7	13.5	17.9
IS	0.4	−0.3	2.5	0.5	10.0	14.6

The values represent the percentage of loss for each analytes after 2, 7 and 14 days of storage. Loss (%) = ((1000 ng/mL − determined concentration (ng/mL))/1000 ng/mL) × 100. Negative values indicated that the concentrations calculated from calibration curve were higher than 1000 ng/mL.

Table 2
The recovery of the 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids from rat plasma

Analyte	Recovery (%)
VN/14-1RA	70.1 ± 6.5
ATRA	78.9 ± 7.2
4-OH-ATRA	68.2 ± 7.8
4-oxo-RA	69.2 ± 8.2
Retinol	79.1 ± 5.7

Table 3
The accuracy and precision of the HPLC measurement of 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma

Analyte	Spiked concentration (ng/mL)	Measured concentration (ng/mL)	CV (%)	Relative error (%)
VN/14-1RA	100	106 ± 3.0	2.8	6.0
	200	206.4 ± 8.1	3.9	3.2
	350	361.2 ± 15.2	4.2	3.2
	1000	1050 ± 26.3	2.5	5.0
	2300	2378 ± 78.5	3.3	3.8
	5000	5305 ± 281	5.3	6.1
ATRA	50	51.4 ± 2.0	3.9	2.8
	100	103 ± 2.6	2.5	3.0
	200	202.2 ± 3.8	1.9	1.1
	350	360.5 ± 7.9	2.2	3.0
	1000	1021 ± 11.2	1.1	2.1
	2300	2318 ± 34.8	1.5	0.8
	5000	5160 ± 216.7	4.2	3.2
4-OH-RA	100	103 ± 3.0	2.9	3,0
	200	204.6 ± 10	4.9	2.3
	350	362.3 ± 8.7	2.4	3.5
	1000	1039 ± 33.3	3.2	3.9
	2300	2364 ± 54.4	2.3	2.8
4-oxo-RA	100	101 ± 4.0	3.9	1.0
	200	203 ± 6.9	3.4	1.4
	350	360.2 ± 3.6	1.0	2.9
	1000	1019 ± 22.4	2.2	1.9
	2300	2325 ± 113.9	4.9	1.1
Retinol	200	202.6 ± 2.0	1.0	1.3
	350	354.6 ± 3.9	1.1	1.3
	1000	1007 ± 3.0	0.3	0.7
	2300	2314 ± 11.6	0.5	0.6

The values represent the mean from three separate determinations (n = 3).

obtained over the range of 0.10–3.0 μg/mL for 4-OH-RA (y = 0.0893x + 0.1455, r^2 = 0.9847), 4-oxo-RA (y = 0.1709x + 1.5987, r^2 = 0.9983) and retinol (y = 0.0799x + 0.002, r^2 = 0.9915). The limit of quantification for VN/14-1RA was estimated to be about 100 ng/mL. The limit of quantification of ATRA and its metabolites were estimated to be about 50 and 100 ng/mL, respectively.

The precision and accuracy of the assay were satisfactory; the coefficient of variations (CV (%)) and relative error were less than 10% for all analytes (Table 3).

3.4. Preliminary pharmacokinetics

This HPLC method was used to characterize the pharmacokinetics of VN/14-1RA in rats after co-administration with ATRA. A single dose of VN/14-1RA (5 mg/kg) in combination with ATRA (0.5 mg/kg) was administered intravenously to male Sprague-Dawley rats (n = 3). The plasma concentrations of VN/14-1RA (5.0 mg/kg) showed a monoexponential decline following intravenous administration (Fig. 4) in rats. VN/14-1RA was rapidly eliminated with a mean terminal elimination half-life ($t_{1/2}$) of about 19.0 ± 3.2 min. The lev-

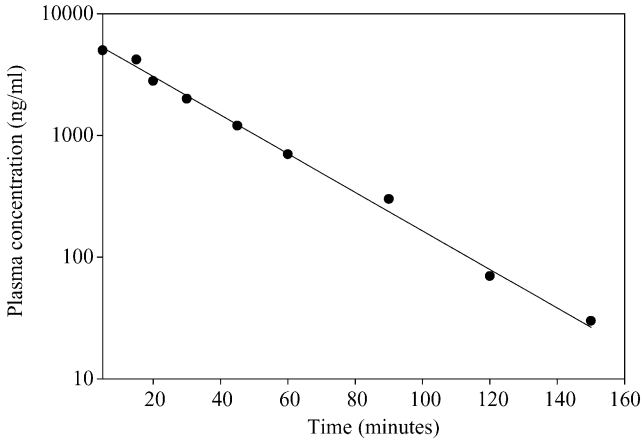

Fig. 4. Pharmacokinetics profile of VN/14-1RA (5 mg/kg) after intravenous administration (in combination with ATRA) in male Sprague-Dawley rats. The values represent the mean plasma concentration from three rats. The terminal elimination half-life = 19.0 ± 0.2 min.

els of 4-OH-RA in rat plasma were very low as a result of rapid conversion of 4-OH-RA to 4-oxo-RA.

4. Discussion

The HPLC method achieved good separation of VN/14-1RA, ATRA, 4-oxo-RA, and 4-OH-RA, with sharp and symmetrical peaks and was useful to monitor the plasma levels of the retinoids at multiple wavelengths simultaneously. Protein precipitation using acetonitrile with acetic acid enhanced the recovery of the analytes from rat plasma. Although ATRA and its metabolites are normally present in rat plasma, physiologic levels of these retinoids in blank plasma samples were not detected by this method. On the other hand, endogenous retinol was routinely detected in blank rat plasma samples. The calibration curves for all analytes were linear in the concentration range examined and the assays were reproducible and allowed accurate determination of the retinoids in rat plasma.

This HPLC method is suitable for the study of the chemical stability, enzymatic metabolism and pharmacokinetics of VN/14-1RA and ATRA in rats. It appears that VN/14-1RA has a relatively short half-life in rats. The findings that have arisen from the preliminary pharmacokinetic studies have been helpful in understanding the disposition of VN/14-1RA and other 4-azolyl retinoids in rats.

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