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A HPLC method for the determination of 9-*cis* retinoic acid (ALRT1057) and its 4-Oxo metabolite in human plasma[1]

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Abstract

A HPLC method was developed and validated for the quantitation of 9-*cis*-retinoic acid (ALRT1057) and its major metabolite, 4-oxo-9-*cis*-retinoic acid (LG100182) in human plasma. Samples were buffered and extracted with methyl-tert-butyl-ether. The analytes and an I.S. were separated on a C_{18} HPLC column using a shallow gradient of 70–89% organic solvent. The analytes were quantitated by UV detection at 348 nm. Selectivity against endogenous compounds and potential metabolites (retinol, all *trans*-, 13-*cis*-, and 4-hydroxy-9-*cis*-retinoic acid) was demonstrated. The run time was 29 min. The standard curve was linear from 2.5 to 450 ng ml^{-1}. Interassay precision for both analytes in quality control samples was less than 5.0% RSD. Accuracy was within 11.0% RE for both compounds. Analyte stability during sample storage, extraction processing, and chromatography was established. Method ruggedness was tested by two analysts and on two HPLC systems. This method has been applied to the quantitation of clinical samples.

Keywords: 9-Cis-retinoic acid; 4-oxo metabolite; Retinoid; Gradient HPLC

1. Introduction

Retinoid analogs may reverse malignant processes and prevent carcinogenesis because of possible inhibition of cell proliferation and promotion of cell differentiation [1,2]. 9-*Cis*-retinoic acid has been shown to bind and activate nuclear receptors of retinoic acid (RARs) and retinoid *X* (RXRs) [3,4] and is under investigation for drug development in cancer treatment [5,6].

An HPLC method for 13-*cis*-, all-*trans* retinoic acid and their 4-oxo-metabolites was reported by Kraft et al. [7]. A similar HPLC method was used for the investigation of metabolites after oral administration of 9-*cis*-retinoic acid [8,9]. The major metabolites were identified to be 4-oxo-9-*cis*-retinoic acid and the glucuronides of 9-*cis*-retinoic acid and 4-oxo-9-*cis*-retinoic acid. Validation data on accuracy, precision, selectivity and stability of the analytes were lacking in these reports. An

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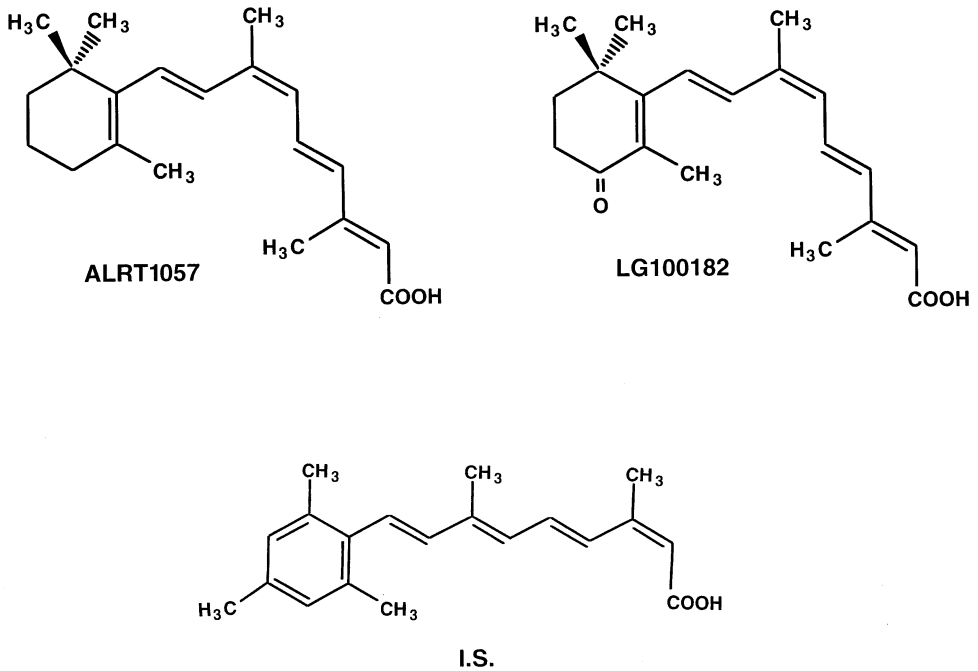

Fig. 1. Structures of ALRT1057 (A), LG100182 (B), and I.S. (C).

HPLC method for the quantitation of 9-*cis*-retinoic acid in rabbit plasma was reported [10]. So far, no analytical method has been reported for simultaneous quantitation of 9-*cis*-retinoic acid and 4-oxo-9-*cis*-retinoic acid in biological fluids. This paper describes the method development and validation of an HPLC method for the quantitation of 9-*cis*-retinoic acid and its major metabolite, 4-oxo-9-*cis*-retinoic acid, in human plasma. Plasma samples were extracted by a simple liquid/liquid process. A shallow, linear gradient enabled the resolution of the analytes from endogenous matrix peaks and degradants of analytes.

2. Experimental

2.1. Instrumentation

Analytes were separated by an analytical column Microsorb Short One®, C_{18}, 4.6 × 100 mm 3 μ from Rainin (Woburn, MA) and a guard column of BDS Hypersil, C_{18}, 2 × 10 mm 5 μ from Keystone (Bellefonte, PA). Column temperature was maintained at 36°C by a Timberline column heater (Boulder, CO). Mobile phases were the following: (A) 5 mM NH_4OAc/HOAc pH 2.7, B) 1% HOAc in methanol. The HPLC system was comprised of a Model AS-100 autosampler from Bio-Rad (Richmond, CA), set at 23–4°C, model 484 detector and model 600E pumps from Waters (Milford, MA). Injection volume was 100 μl with UV detection at 348 nm. Flow rate was 1.0 ml min^{-1}. Run time was 29 min. The linear gradient program was as follows:

Time (min)	%A	%B
0.00	30	70
6.50	30	70
7.00	20	80
21.50	11	89
22.00	30	70
32.00	30	70

Table 1
Extraction recovery of analytes

	2.5 ng ml^{-1}		75 ng ml^{-1}		450 ng ml^{-1}		Overall
	% Rec	RSD%	% Rec	RSD%	% Rec	RSD%	Recovery
ALRT1057	82.5	1.5	75.4	2.0	72.1	1.8	76.7
LG100182	102	7.3	98.2	2.5	96.5	2.9	98.9
I.S.							75.6 (RSD 2.2%)

Recoveries over a range of 2.5–450 ng ml^{-1} of ALRT1057 and LG100182 were calculated by direct comparison of the peak height of extracted standards to unextracted test solutions prepared in an interference-free matrix at the same concentrations.

The mobile phase was sparged by He. Samples in the autosampler were protected from light. A second HPLC system consisted of an Eppendorf column heater (Madison, WI) and a model 783 detector from Applied Biosystems, (Foster City, CA) with other components identical to the first system. Two different analytical columns were tested between the two systems and the chromatographic performance was similar.

2.2. Materials

9-*Cis*-retinoic acid (ALRT1057), 4-oxo-9-*cis*-retinoic acid (LG100182), 4-hydroxy-9-*cis*-retinoic acid, were supplied by Allergan-Ligand Retinoid Therapeutics, San Diego, CA. The internal standard (I.S.), Ro 11-5036, was a gift from Hoffman-LaRoche. Structures of the two analytes and the I.S. are shown in Fig. 1. Retinol, 13-*cis*-retinoic acid, and all trans retinoic acid were purchased from Sigma (St. Louis, MO). All chemicals used were of analytical grade or HPLC grade. The organic solvents were from Fisher (Fair Lawn, NJ) with the exception of methyl-tert-butyl-ether which was purchased from Mallinckrodt (Paris, KY). Mobile phases were vacuum-filtered through 0.45 μm nylon membranes from Gelman Sciences (Ann Arbor, MI). Deionized water was prepared in-house using a NANOpure® Water Purification System from Barnstead (Dubuque, IA). Control human heparinized plasma was purchased from Nashville Biological (Cincinnati, OH).

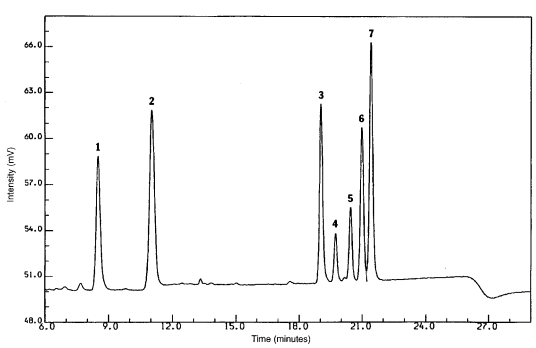

Fig. 2. Chromatogram of: LG100182 167 ng ml^{-1} (1), 4-hydroxy-9-*cis*-retinoic acid 193 ng ml^{-1} (2), I.S. 167 ng ml^{-1} (3), 13-*cis*-retinoic acid 217 ng ml^{-1} (4), retinol 222 ng ml^{-1} (5), ALRT1057 167 ng ml^{-1} (6), and all-trans-retinoic acid ng ml^{-1} (7). Of each solution, 100 μl, was injected onto the HPLC column.

2.3. Preparation of solutions

All weighings and solution preparations of retinoid analogs were performed under yellow lighting. Primary stocks for standards and quality control samples (QCs) were prepared from separate weighings. The primary stocks of ALRT1057

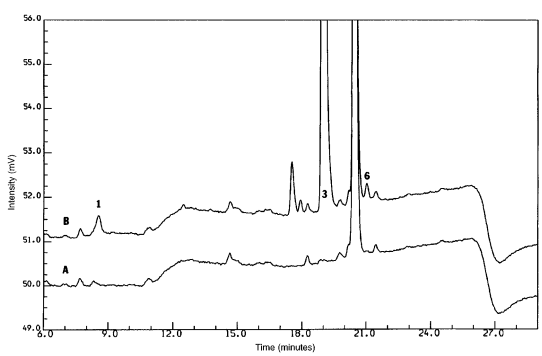

Fig. 3. Typical chromatograms of an extracted control blank plasma (A) and an extracted standard in plasma at 2.5 ng ml^{-1} (B).

Table 2
Light sensitivity of ALRT1057, LG100182, and I.S. in methanol

	As a percent of control		
	ALRT1057	LG100182	I.S.
Yellow lighting	90.6	91.2	99.2
White lighting	36.2	49.1	104

and LG100182 at 500 μg ml^{-1} in methanol were diluted to form a combined secondary stock of both compounds at 50 μg ml^{-1}. These solutions were prepared in silanized volumetric flasks. Ten-fold spiking standards were prepared in methanol and stored at −70°C in foil-wrapped polypropylene tubes for up to 1 month. Working standards were prepared daily by adding 100 μl of the spiking standards to 1.0 ml of control plasma. The standard concentrations were 2.5, 5, 10, 25, 75, 200, 400 and 450 ng ml^{-1}. QCs were prepared in bulk in silanized volumetric flasks, aliquoted into foil-wrapped polypropylene tubes, and stored at −70°C with the clinical samples until assayed. Another group of QCs was set aside and stored separately at −70°C for long-term storage stability tests.

2.4. Sample processing procedures

A 1 ml aliquot of QC or analytical sample was added to a 16 × 100 mm glass screw-cap tube. After adding 50 μl of I.S. (0.5 mg ml^{-1} of 1:1 methanol/acetonitrile, v/v) and 1.0 ml of 1 M phosphate buffer, pH 6.0, and mixing, 6 ml of methyl-tert-butyl ether was introduced. The tube was capped and shaken on a horizontal shaker for 10 min. The aqueous layer was frozen in a dry-ice-acetone bath. The organic layer was decanted into a silanized 13 × 100 mm culture tube and evaporated to dryness under N_2 in a 25°C water bath. After reconstituting with 200 μl methanol, 100 μl 5 mM ammonium acetate was added. The extract was transferred into a 1.5 ml polypropylene microcentrifuge tube and centrifuged for 3 min at 13 000 × *g*. The supernatant was transferred into an autosampler vial.

2.5. Data regression

Chromatograms were measured using a VG® Multichrom data system for VAX®/VMS. The raw data output was acquired on a VG® Chromserver and then transferred to the VAX®/VMS. Response in peak height ratio of each analyte to the I.S. (y) was plotted against analyte concentration (x). A linear regression with $1/y$ weighting was used to determine slopes, intercepts and correlation coefficients. Unknowns were calculated by the equation: $x = [y - (y - \text{intercept})]/\text{slope}$.

3. Results and discussion

3.1. Method development

The initial methodology employed was developed for ALRT1057 only and used an unbuffered extraction into methyl-tert-butyl ether. The recovery was 64%. Recovery linearity was improved post extraction when the dried extracts were first reconstituted with methanol followed by the addition of ammonium acetate buffer rather than reconstituting with a pre-mixed solution. Under these conditions, the recovery was appproximately 75% for ALRT1057, and approximately 50% for LG100182. The metabolite recovery varied from 42 to 55% between six plasma lots tested at both the low and high standard levels and was not tracked by the I.S. Extraction recovery and precision between and within different plasma lots was subsequently improved by adding a pH 6.0 buffer to the plasma samples. Extraction recovery of the final method was consistent, with RSD of ≤2, 7.3, and 2.2 for ALRT1057, LG100182, and I.S., respectively (Table 1).

Resolution of the 4-oxo metabolite from matrix peaks and 4-hydroxy-9-*cis*-retinoic acid was greatly improved by changing the organic modifier of the mobile phase from acetonitrile to methanol. This change also reversed the elution order of retinol and 9-*cis*-retinoic acid. Fig. 2 and Fig. 3 show that the following compounds were adequately resolved from matrix peaks and analytes of interest: 4-hydroxy-9-*cis*-, all-*trans*- and 13-*cis*-retinoic acids, and retinol. Fig. 3 shows

Table 3
Calibration standards interday variation ($n = 7$)

	ng ml^{-1}							
	2.50	5.00	10.0	25.0	75.0	200	400	450
ALRT1057								
Mean	2.53	5.05	10.1	24.8	73.0	200	411	450
RSD%	4.5	1.3	1.5	1.0	2.1	1.9	1.6	2.1
RE%	+1.0	+0.9	+0.8	−0.6	−2.7	+0.1	+2.8	−1.9
LG100182								
Mean	2.51	5.01	10.1	25.0	73.9	200	407	444
RSD%	5.0	5.8	1.9	3.0	2.2	1.3	2.1	1.7
RE%	+0.5	+0.3	+1.0	−0.2	−1.4	−0.1	+1.9	−1.3

that degradants generated in-house by light/heat exposures of 4-oxo-9-*cis*-retinoic acid did not interfere with the compounds of interest.

Retinoids are known to be light sensitive. Light sensitivity of standard solutions was investigated. A methanolic solution containing I.S. and 4500 ng ml^{-1} of ALRT1057 and LG100182 was placed under white light, yellow light, and in the dark (as control) for 4 h. The solutions were then injected onto the HPLC. Peak heights were compared. Table 2 shows that ALRT1057 and LG100182 in methanol are very sensitive to white lighting; as only about one third or half of the compounds remained after the 4 h exposure. Under yellow lighting, about 10% degradation was observed after 4 h. Therefore, samples were processed as quickly as possible under yellow lighting to avoid degradation from light exposure.

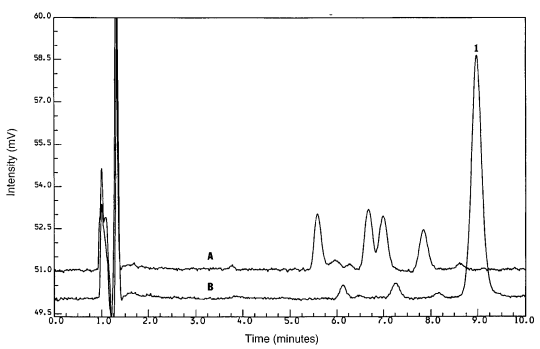

Fig. 4. Chromatogram of degradants of LG100182 (1) after white light exposure for 5 h, in glass, at 40–45°C. Trace A: after exposure; B: before exposure.

9-*Cis*-retinoic acids are variably sensitive to light, heat, oxygen, acidity, and silica. The extraction method was designed to avoid temperature and pH extremes. Liquid/liquid extraction was chosen over solid-phase extraction, and chromatography was performed on a high-carbon load, end-capped column.

3.2. Linearity, accuracy, and precision

The standard curve for plasma samples was linear over the concentration range of 2.5–450 ng ml^{-1} with a correlation coefficient of $r^2 > 0.999$. Fig. 5 shows a typical standard curve plot. The RSD from seven validation analytical runs over 9 days was ≤ 4.5 and 5.8% for spiked plasma standards of ALRT1057 and LG100182, respectively (Table 3). The low limit of quantitation (LOQ) was 2.5 ng ml^{-1}. The signal-to-noise ratio at this concentration was 7–18 for LDG100182, and 13–42 for ALRT1057 estimated from performance of two HPLC systems. Interday variation of the LOQ from seven analytical runs was 4.5% RSD. Fig. 4 shows a typical chromtagram of the LOQ compared with the blank control. Interday variation from plasma QCs determined from assaying six replicates each of low, medium, and high concentrations the RSD was less than 3.7 and 5.0% for ALRT1057 and LG100182, respectively (Table 4) Fig. 5. The within-day variation of control samples was 2.9% or less.

Table 4
Quality control samples interday and intraday variation

	ng ml^{-1}					
	7.50	50.0	375	7.50	50.0	375
Interday ($n=42$)						
Mean	7.38	45.7	351	8.13	50.5	388
RSD%	3.4	3.6	3.7	4.5	4.5	5.0
RE%	−1.6	−8.6	−6.4	+8.4	+0.9	+3.5
Intraday ($n=6$)						
Mean	7.43	44.5	341	7.70	48.7	371
RSD%	2.0	1.6	0.7	1.9	1.4	2.9
RE%	−1.0	−11.0	−9.2	+2.6	−2.6	−1.0

3.3. Stability

Stability testing of the analytes was performed on plasma samples before and after extraction (Table 5). When protected from light, the analytes were stable in the plasma matrix at room temperature for up to 26.5 h, and at −70°C for up to 20 weeks. There was no effect of three cycles of freezing and thawing. The analytes in the reconstituted sample extract were stable on the refrigerated autosampler tray for 66 h. Samples could be reinjected after 62 h to quantitate ALRT1057. However, reinjection data for LG10082 were inaccurate because of problems of evaporation. The I.S. appeared to have a different solubility after evaporation and did not track the analyte as well as it did for ALRT1057.

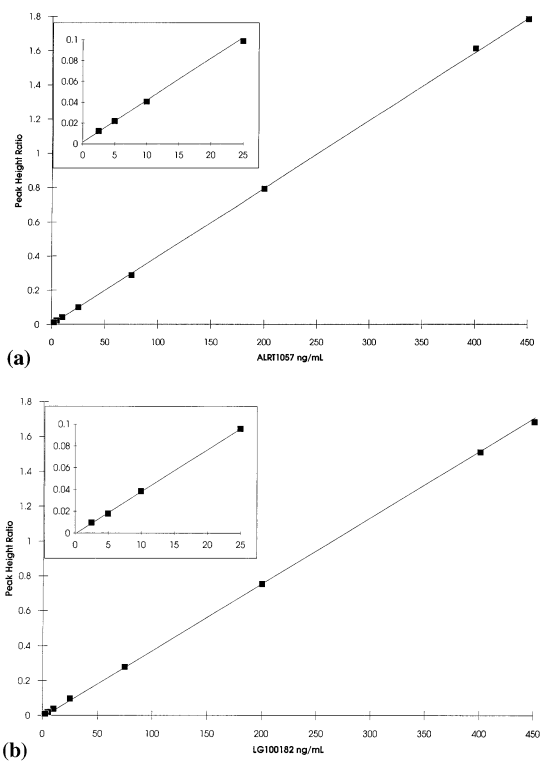

Fig. 5. A typical standard curve, A. ALRT1057, B. LG100182. The insets show details of low concentrations.

Table 5
Stability of quality control samples

	% Of control		
	Time period	ALRT1057	LG100182
Plasma Sample			
Benchtop ($n=4$)	26.5 h	84.9–87.2	94.7–96.8
Freeze/thaw	3 cycles ($n=4$)	94.3–96.5	95.2–96.5
Sample storage −70°C	44 weeks	99.9–115	—
$n=6$	20 weeks	—	101–110
Sample extract			
Refrigeration ($n=6$)	40.5 h	99.8–103	102–103
On-system ($n=4$)	66 h	98.7–99.3	119–124
Reinjection ($n=6$)	62 h	97.4–99.1	131–138

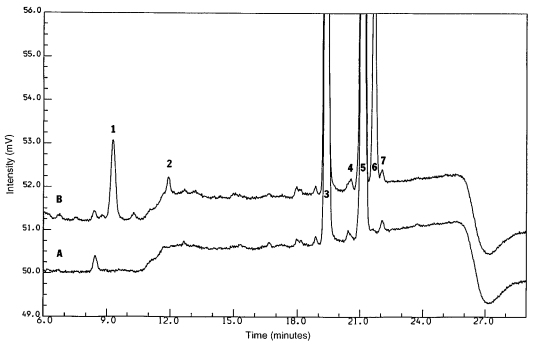

Fig. 6. Chromatograms of plasma samples from a patient dosed with ALRT1057. Trace A: predosed; B: postdosed. Peak identification is the same as Fig. 2.

4. Application

Patient samples from a clinical trial were analyzed for ALRT1057 and LG100182 using the validated HPLC method. Chromatograms of a predose and a postdose patient sample were shown in Fig. 6. The analytes peaks were resolved from endogenous matrix peaks.

5. Conclusion

An HPLC method was developed and validated for the determination of ALRT1057 and its 4-oxo metabolite in human plasma. Method ruggedness was tested by two analysts and on two HPLC systems during method validation. The method was shown to be robust through patient sample analysis by a third analyst using two HPLC systems.

References

[1] M.S. Tallman, P.H. Wiernik, J. Clin. Pharmacol. 32 (1992) 868–888.
[2] M.E. Huang, U.C. Yu, S.R. Chen, J.R. Chai, J.X. Lu, L. Zhoal, L.J. Gu, Z.Y. Wang, Blood 72 (1988) 567–572.
[3] R.A. Heyman, D.F. Mangelsdorf, J.A. Dyck, R.B. Stein, G. Eichele, R.M. Evans, C. Thaller, Cell 68 (1992) 397–406.
[4] A.A. Levin, L.J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovery, J.F. Grippo, Nature 355 (1992) 359–361.
[5] V.A. Miller, J.R. Rigas, F.M. Benedetti, A.L. Verret, W.P. Tong, M.G. Kris, G.M. Gill, G.R. Loewen, J.A. Truglia, E.H. Ulm, R.P. Warrell Jr., Clin. Cancer Res. 2 (1996) 471–475.
[6] J.M. Kurie, J.S. Lee, T. Griffin, S.M. lippman, P. Drum, M.P. Thomas, C. Weber, M. Bader, G. Massimini, W.K. Hong, Clin. Cancer Res. 2 (1996) 287–293.
[7] J.C. Kraft, W. Slikker Jr., J.R. Bailey, L.G. Roberts, B. Fischer, W. Wittfoht, H. Nau, Drug Metab. Dispos. 19 (1990) 317–324.
[8] C. Eckhoff, H. Nau, J. Lipid Res. 31 (1990) 1445–1454.
[9] J.O. Sass, E. Masgrau, J.-H. Saurat, H. Nau, Drug Metab. Dispos. 23 (1995) 887–891.
[10] M.N. Marchetti, H. Bun, J.M. Geiger, A. Durand, Anal. Lett. 27 (1994) 1847–1862.