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Simultaneous determination of all-*trans* and 13-*cis* retinoic acids and their 4-oxo metabolites by adsorption liquid chromatography after solid-phase extraction

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Abstract

All-*trans* retinoic acid (all-*trans* RA), the active metabolite of vitamin A, has been demonstrated to be an efficient alternative to chemotherapy in the treatment of acute promyelocytic leukemia (APL), the AML_3 subtype of the FAB cytological classification. Complete remission is obtained by inducing terminal granulocytic differentiation of the leukemic cells. To study all-*trans* RA pharmacokinetics in patients with APL, a rapid, precise and selective high-performance liquid chromatographic (HPLC) assay was developed. This method is easy and shows good repeatability (C.V. = 8.41–12.44%), reproducibility (C.V. = 9.19–14.73%), accuracy (C.V. = 3.5–11%) and sensitivity with a detection limit of 5 pmol/ml. The analysis is performed using normal-phase HPLC in an isocratic mode with UV detection after solid-phase extraction on octadecyl (C_{18}) columns. The mobile phase is hexane–dichloromethane–dioxane (78:18:4, v/v) containing 1% acetic acid.

1. Introduction

Retinoic acid (RA), a natural derivative of vitamin A, plays an important role in growth, differentiation and development of known normal tissues [1,2]. Direct antiproliferative effects of all-*trans* retinoic acid (all-*trans* RA) have been demonstrated in various malignant cells such as F9 teratocarcinoma cells and HL-60 or U-937 human myeloid leukemia cells [3]. In vitro, when tested on fresh human blast cells, this effect was restricted to the AML_3 subtype of myeloid leukemia in the FAB cytological classification [4]. The maturation of these promyelocytic leukemic cells is induced in vitro by all-*trans* RA with no possibility of leukemic cell renewal. In vivo, the transposition of these results shows that all-*trans* RA induced complete remission in AML_3 patients [5–8].

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Previously we studied the pharmacokinetics of all-*trans* RA in 15 patients treated with a single oral dose of 45 mg/m^2/day. Blood samples were drawn during a 24-h period after dosing [9], and plasma concentrations of all-*trans* RA and 13-*cis* RA were determined by reversed-phase liquid chromatography after liquid–liquid extraction according to the method described by McClean et al. [10]. Our results showed that the peak concentration of all-*trans* RA occurred between 60 and 210 min after ingestion and that maximum concentrations were between 0.03 and 2.5 μg/ml. The apparent elimination half-life was between 16.8 and 77.4 min [9].

To complete our work, we attempted to determine plasma concentrations of all-*trans* 4-oxo-RA and 13-*cis* 4-oxo-RA metabolites. The methods currently used for both retinoic acids and 4-oxo metabolites analysis are based on reversed-phase chromatography either in gradient (see e.g. Refs. [11–13]) or column-switching mode (see e.g. Refs. [13,14]). As these techniques are difficult with respect to work up, and are expensive and time-consuming, we developed a new isocratic normal-phase high-performance liquid chromatographic method using pure silica gel, which provides good separation of geometrical isomers.

2. Experimental

2.1. Reagents

All-*trans* RA, 13-*cis* RA, all-*trans* 4-oxo-RA, 13-*cis* 4-oxo-RA, and RO 13-6307 [internal standard (I.S.): (all-E)-3-methyl-7-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2,4,6-octanoic acid] were generously provided by Hoffmann-La Roche Laboratories (Basle, Switzerland). These compounds were individually dissolved in dimethylsulphoxide (DMSO) to obtain 10^{-3} *M* concentrations, and stock standards were stored at −20°C.

Methylene chloride, 1,4-dioxane, *n*-hexane, methanol and acetonitrile were of chromatographic grade; DMSO and glacial acetic acid of extra pure quality; and acetone of ACS quality (all Merck products, Nogent-sur-Marne, France).

2.2. Preparation of standards

From stock standards, a first series of standard solutions was prepared containing each acid at 10^{-4} *M* to 10^{-8} *M* concentrations by dilution in DMSO. These primary standards were then diluted in free serum to obtain 10^{-5} *M* to 10^{-9} *M* concentrations. This second series constituted the standard curve. A 10^{-5} *M* solution of the I.S. was prepared by diluting the stock solution with DMSO.

2.3. Instrumentation

The chromatographic system consisted of a L-6200A pump, a L-4250 UV-Vis detector connected to a D-2500 chromato-integrator and an AS-2000 autosampler (all from Merck). For chromatography, we used a 250 × 4 mm I.D. cartridge of 5 μm Lichrospher Si-60 placed in a Lichrocart 250-4 (Merck products).

Solid-phase extraction was performed on Bakerbond SPE Octadecyl columns using a Baker SPE-21 system (Baker, Noisy-le-Sec, France).

2.4. HPLC analysis

The mobile phase was prepared by mixing 780 ml of hexane, 180 ml of methylene chloride and 40 ml of 1,4-dioxane. The solvents used contained 1% acetic acid. The mobile phase was filtered under vacuum, then degassed and maintained under helium during use. Chromatography was performed on a Lichrocart Si-60 5 μm column, at a flow-rate of 0.8 ml/min, which gave a pressure of approximately 50 bar. Prior to analysis, the column was reconditioned with the new mobile phase for 1 h.

UV detection was performed at 360 nm and a sensitivity of 0.002 AUFS. The autosampler was set at an injection volume of 50 μl and a run time of 35 min.

2.5. Sample collection

Blood samples were collected in Vacutainers without additive and kept in the dark at +4°C before being sent to the laboratory. After centrifugation at 1500 *g*, serum was decanted, divided into aliquots of 1.2 ml and frozen at −20°C. All these operations were performed in the dark under subdued light. Before analysis, samples were thawed at room temperature in the dark.

2.6. Extraction

All operations between extraction and chromatography were conducted in the dark under subdued light. The standard curve, quality controls and samples were processed simultaneously.

Before extraction, the columns were conditioned by successive aspirations of methanol (2 ml) and acetic acid 1 *M* (2 ml). Acetic acid 1 *M* (3 ml) and 50 μl of I.S. solution were added to 1 ml of serum and then mixed. The sample was then quantitatively transferred to the column. Then, the column was washed with 2 ml of acetone–1 *M* acetic acid (1:1, v/v) and dried under vacuum for 15 min. The analytes were eluted with 500 μl of acetonitrile and the solvent evaporated. Prior to injection, the dry residue was dissolved in 200 μl of mobile phase and the extract poured into the microvials of the autosampler. Extraction recovery was calculated by comparing the peak area of the extracted retinoid standards to that of retinoid standards injected directly onto the column.

3. Results and discussion

Dilution of standards in DMSO was used to prevent protein precipitation with other solvents. Addition of 1 *M* acetic acid to serum ensures a good pH for extraction and gives a lower viscosity. Although the standards in DMSO and the sample stored at −20°C in the dark showed no deterioration after 4 months, we preferred to prepare small quantities of the standards and renew the stock solutions every month. The extracts remain stable for more than 24 h at room temperature, which is sufficient for the analysis, provided that the extracts are kept in the dark or in amber tubes.

Chromatography was performed on silica gel columns since the five analytes could not be separated in normal- or reversed-phase isocratic mode on C_{18} columns. Typical chromatograms for separation of the 5 retinoids studied are shown in Figs. 1–4.

Fig. 1 shows a chromatogram from blank serum showing that the extraction gave good sample clean-up. The peaks at 6–7 min corresponded to endogenous 13-*cis* RA and all-*trans* RA and represented 1.3 and 2.9 ng/ml, respectively. Fig. 2 represents a chromatogram obtained with pure standards for determination of the peak identities. Fig. 3 shows a chromatogram of standards in serum after extraction. Com-

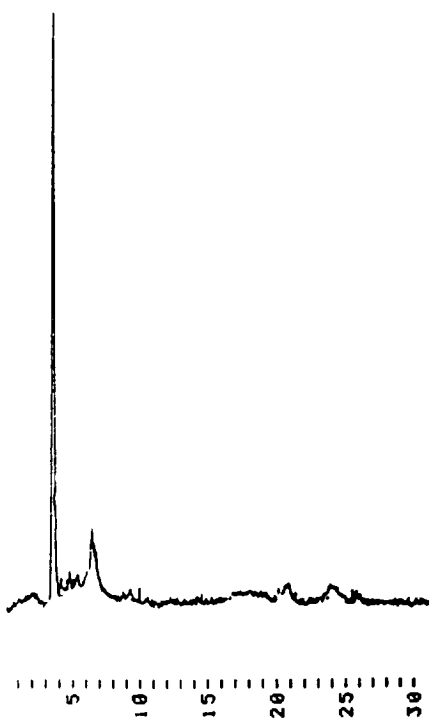


Fig. 1. Chromatogram of blank serum after extraction.

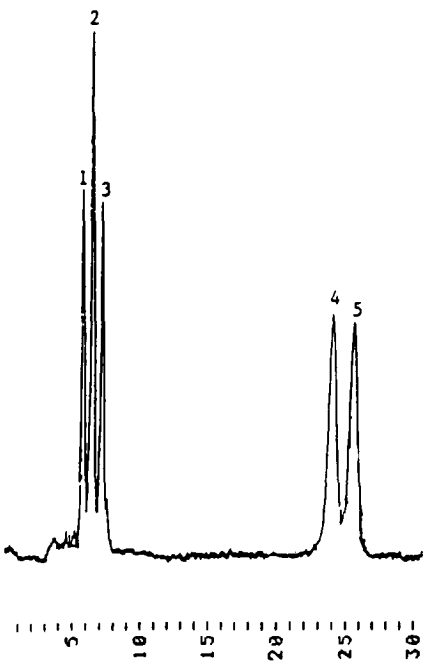
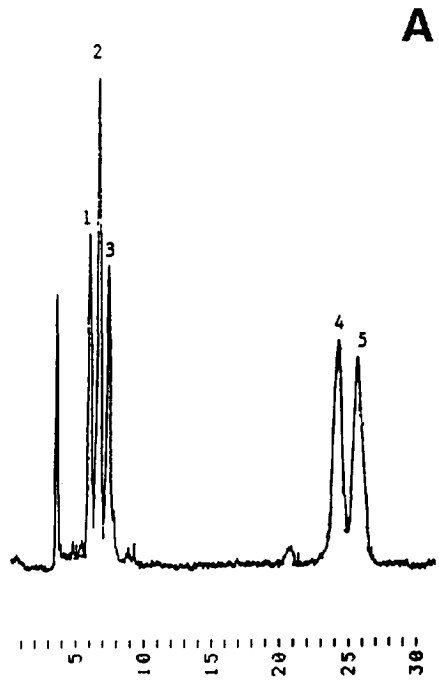


Fig. 2. Chromatogram of pure standards without extraction, each at 10^{-7} *M*. Peaks: 1 = 13-*cis* RA, 2 = all-*trans* RA, 3 = internal standard (I.S.), 4 = all-*trans* 4-oxo-RA, 5 = 13-*cis* 4-oxo-RA.

parison of the chromatograms shown in Figs. 2 and 3 indicates that the peaks from serum remaining after extraction did not interfere with the analysis of the retinoic acids. Extraction also allowed 5-fold sample enrichment. Fig. 4 shows a chromatogram of retinoic acids in a patient treated by all-*trans* RA.

With regard to the quantitative results, the standard curve was linear from 10^{-5} *M* to $5 \cdot 10^{-9}$ *M* (10 000 pmol/ml to 5 pmol/ml or 3000 ng/ml to 1.5 ng/ml), with a detection limit of $5 \cdot 10^{-9}$ *M* (5 pmol/ml or 1.5 ng/ml). Analytic recoveries of standards added to serum samples before extraction ranged from 92 to 95%.

Day-to-day repeatability, reproducibility, precision and accuracy were studied on serum samples overloaded with different concentrations (high, medium and low) for each analyte. The results are reported in Tables 1–3, respectively.



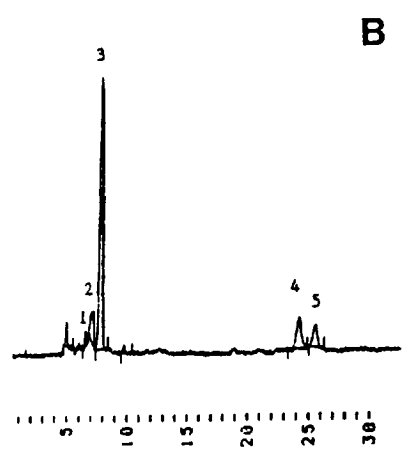


Fig. 3. Chromatograms of standards in serum after extraction. (A) Each at 10^{-7} *M*, (B) each at $5 \cdot 10^{-9}$ *M*.

These results are satisfactory for this type of method and sufficient for pharmacokinetic studies.

Fig. 5 shows plasma retinoid concentration–

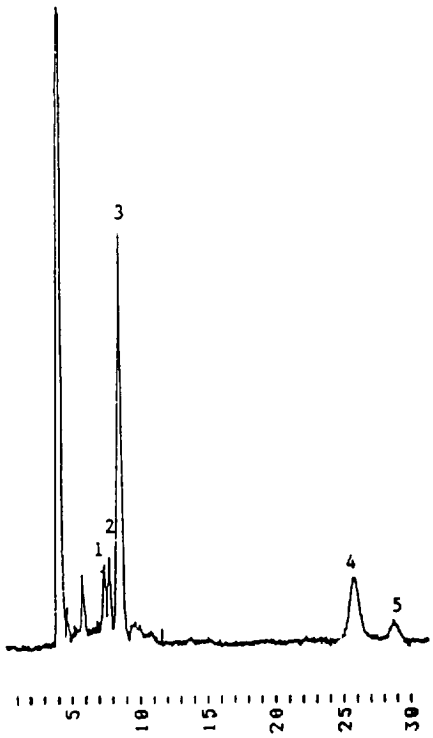


Fig. 4. Chromatogram of patient serum after an oral dose of 25 mg/m^2/day of all-*trans* RA.

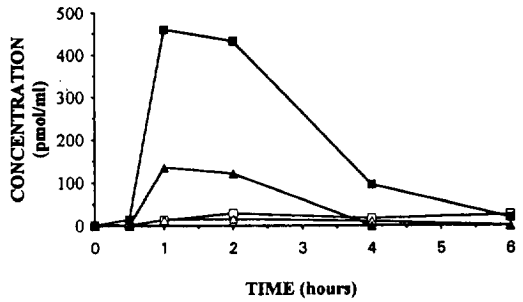


Fig. 5. Plasma concentration–time curves in APL patient after a single oral dose of 25 mg/m^2/day. (■) All-*trans* RA, (▲) 13-*cis* RA, (□) all-*trans* 4-oxo-RA, (△) 13-*cis* 4-oxo-RA.

time curves in APL patient serum after a single oral dose of 25 mg/m^2/day.

4. Conclusions

The described isocratic HPLC method after solid-phase extraction is currently used in our laboratory for pharmacokinetic studies of patients treated with all-*trans* RA. Complete phar-

Table 1
Repeatability studies (n = 16)

Values	13-*cis* 4-oxo-RA	All-*trans* 4-oxo-RA	13-*cis* RA	All-*trans* RA
High				
Mean (pmol/ml)	4838.28	4798.27	2250.18	5841.06
S.D.	438.46	462.94	219.47	515.32
C.V. (%)	9.06	9.65	9.75	8.82
Medium				
Mean	536.88	504.72	262.61	327.58
S.D.	50.74	52.71	18.84	31.50
C.V. (%)	9.45	10.44	7.17	9.61
Low				
Mean	48.91	45.58	20.72	25.52
S.D.	5.40	5.67	1.74	2.97
C.V. (%)	11.04	12.44	8.41	11.64

Table 2
Day-to-day reproducibility studies ($n = 16$)

Values	13-*cis* 4-oxo-RA	All-*trans* 4-oxo-RA	13-*cis* RA	All-*trans* RA
High				
Mean (pmol/ml)	5931.29	3608.03	2590.83	3111.65
S.D.	544.95	411.20	312.02	371.67
C.V. (%)	9.19	11.40	12.04	11.94
Medium				
Mean	525.71	336.78	244.47	242.56
S.D.	62.06	39.14	30.22	23.78
C.V. (%)	11.80	11.62	12.36	9.81
Low				
Mean	53.54	32.06	20.39	19.75
S.D.	7.03	4.61	2.21	2.91
C.V. (%)	13.13	14.38	10.82	14.73

Table 3
Precision and accuracy data ($n = 10$)

Concentration added (pmol/ml)	*Concentration calculated (pmol/ml)*	*Concordance (%)*	*S.D.*	*C.V. (%)*	*Minimum calculated (pmol/ml)*	*Maximum calculated (pmol/ml)*
13-cis 4-oxo-RA						
5000	4979	99.5	161.1	3.2	4625	5250
500	483.40	96.6	24.0	4.9	448	512
50	50.39	100.8	4.9	8.7	42.5	54.5
50	4.885	97.7	0.5	10.9	4.1	5.6
all-trans 4-oxo-RA						
5000	4965.20	99.3	259.0	5.2	4532	5260
500	501.10	100.2	26.7	5.3	456	535
50	51.44	102.9	5.1	9.9	41.8	57.1
5	5.10	102.0	0.5	9.9	4.3	5.9
13-cis RA						
5000	5108.70	102.2	288.6	5.6	4515	5440
500	522.30	104.5	26.5	5.0	483	562
50	51.86	103.7	4.8	9.4	42.7	58.2
5	5.24	104.8	0.5	8.8	4.4	5.8
all-trans RA						
5000	5138	102.2	316.3	6.1	4575	5380
500	520.80	104.2	24.7	4.7	478	552
50	51.67	103.3	5.0	9.8	43.1	57.9
5	5.13	102.6	0.4	8.2	4.2	5.6

macokinetic analysis requires 1.5 days for 20 samples and 10 standards form the time of extraction until interpretation. This method is not time-consuming and is easy to perform in any laboratory using an HPLC system with an automated sampler.

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