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Determination of orally administered all-*trans*-retinoic acid in human plasma by high-performance liquid chromatography

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Abstract

All-*trans*-retinoic acid (RA) is used successfully in the treatment of acute promyelocytic leukaemia (APL), although unexplained relapses occur in many of the patients. Pharmacokinetic studies may help in understanding the mechanism of resistance to RA and a simple and rapid procedure for its determination in biological samples may be advantageous. A high-performance liquid chromatographic procedure is described, involving one-step extraction of RA from plasma, isocratic elution from a reversed-phase column (LiChrosorb RP-18, 5-μm particle size) and UV detection at 340 nm. The calibration graph is linear over a wide range and the limit of detection is approximately 10 ng/ml, using 0.5 ml of human plasma. The method is selective for RA, accurate and robust and thus suitable for the routine analysis of plasma samples from patients undergoing RA therapy. Analysis of plasma in a patient on RA therapy (45 mg/m^2 per day) confirmed that during continuous treatment with RA the drug plasma concentrations are markedly lower at the time of relapse than on the first day of treatment.

1. Introduction

All-*trans*-retinoic acid (RA), a naturally occurring retinoid involved in various biological functions like its parent compound vitamin A, has selective *in vitro* and *in vivo* differentiating activity on abnormal promyelocytes in acute promyelocytic leukaemia (APL) [1,2]. It induces complete remission in a high proportion of newly diagnosed [2–5] and first-relapsed patients [2,3,6]. Unfortunately, most of the patients who achieve complete remission with RA and are maintained on this drug alone or on relatively mild chemotherapy rapidly relapse [7]. This has led to the suggestion that resistance to the antileukaemic action of RA is acquired during RA treatment, although the mechanism is not clear. Monitoring plasma concentrations of RA during drug therapy may help to elucidate the mechanism of resistance [7,8].

A number of sensitive and precise HPLC procedures have been reported for the determination of RA in biological fluids, but they are generally complex and time consuming [8–13]. Because RA is unstable in biological fluids at room temperature and may degrade under laboratory light, a simpler and faster procedure may be particularly advantageous in RA monitoring and pharmacokinetic studies. This paper describes a simple and rapid but selective pro-

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cedure for the determination of RA in human plasma by HPLC with UV detection at 340 nm. The procedure is sufficiently sensitive to measure the plasma concentrations that might be encountered in patients with APL under RA therapy.

2. Experimental

2.1. Chemicals and reagents

RA and the internal standard (I.S.), all-*trans*-retinyl acetate, were supplied by Fluka (Milan, Italy). The RA metabolites 4-oxo-RA, 5,6-epoxy-RA and 13-*cis*-RA were kindly supplied by Hoffmann-La Roche (Nutley, NJ, USA). A stock standard solution of RA and the I.S. was prepared in amber-coloured volumetric flasks by dissolving the compounds in methanol each at a concentration of 1 mg/ml. Working standard solutions were prepared from the stock standard solution by dilution with methanol [9]. These solutions were stable for at least 2 months if kept under nitrogen at −20°C.

Acetonitrile (HPLC grade) was obtained from Carlo Erba (Milan, Italy). Methanol and ammonium acetate (Merck, Darmstadt, Germany) were of analytical-reagent grade and were used as received.

2.2. Sample preparation

Sample preparation for the determination of RA in human plasma (0.5 ml) involved protein precipitation using 2 ml of acetonitrile after adding the I.S. (200 ng/ml). The samples were vortex mixed for 60 s and centrifuged for 2 min at 5000 *g*, then the supernatant was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.2 ml of acetonitrile–water–10% ammonium acetate (70:25:5, v/v) and transferred to autosampler vials, and volumes of 170 μl were injected into the HPLC system. All samples were prepared, stored and manipulated in the dark [12].

Recovery and precision studies were performed by overloading a pool of plasma samples from healthy blood donors (AVIS, Milan, Italy) with RA. All experimental points were obtained in quadruplicate.

2.3. Instrumentation and chromatographic conditions

A Waters (Milford, MA, USA) HPLC system was used, consisting of a Model 6000A pump, a Model 712 automatic sample injector (WISP) and a Model 440 UV detector at 340 nm. The reversed-phase analytical column (LiChrosorb RP-18, 5 μm; 250 × 4.6 mm I.D.) (Merck) was fitted with an MPLC New Guard RP-18 precolumn (15 × 3.2 mm I.D.) (Brownlee Labs., DuPont, Santa Clara, CA, USA) and was maintained at room temperature. Chromatograms were recorded with a Model C-R6A Chromatopac integrator (Shimadzu, Kyoto, Japan).

The mobile phase was acetonitrile–methanol–water–10% ammonium acetate (60:16:20:4, v/v); it was degassed before use and delivered isocratically at a flow-rate of 1.2 ml/min.

2.4. Validation

The precision and reproducibility of the extraction procedure and chromatography were established by analysis of quality control (QC) samples with each calibration graph. The intra-assay precision over the working range (10–1000 ng/ml) was checked by replicate analyses ($n = 4$) of QC samples on the same day. The inter-assay precision and accuracy ($n = 8$) were evaluated by preparing QC at the start of the validation study: plasma samples containing small, medium and large amounts of RA were prepared and immediately frozen at −20°C with no significant instability for up to 1 month, as established by comparison with samples prepared on the day of analysis. During each day's analysis, these QC samples were assayed with standard samples, and the calculated concentrations were compared.

Daily calibration graphs with six concentrations across the working range were measured in duplicate, with QC samples injected between the two sets of standards. The lowest concentration used for these calibration graphs was equal to

the lower limit of quantification (LLOQ), *i.e.*, the lowest concentration of RA that can be measured with acceptable accuracy and precision, as determined in separate studies.

Calibration graphs were constructed by linear least-squares regression analysis of the plot of the peak-height ratios between the compound and the I.S. against the concentration of the former in biological samples (internal standard method). The concentrations of RA in the QC and biological samples were determined by interpolation from the calibration graphs using peak-height ratios obtained from the samples.

2.5. Application

Plasma samples were obtained from four male APL patients on RA therapy. On day 1 of therapy they received a single oral dose of 45 mg/m^2 and a venous blood sample was drawn before (time zero) and at various times after dosing. These analyses were repeated at the time of third relapse in a patient who had received remission induction treatment with RA at the same dose. The clinical and molecular data for this APL patient have been reported previously [14].

The drug was kindly provided by Istituto delle Vitamine (Segrate, Italy) and prepared as capsules containing 10–50 mg of RA by Monza Hospital Pharmacy. No significant alterations of hepatic and renal functions were recorded and no other drugs were co-administered with RA.

Blood samples were collected in heparinized polyethylene tubes and wrapped in aluminium. Each sample was processed under low-light conditions to produce plasma, which was stored at −20°C until assay after 1–3 weeks.

The peak time (t_{max}) was defined as the sampling time when the mean maximum concentration (C_{max}) was found. The area under the curve from zero to the last measurement time (6 h) was calculated by the trapezoidal rule (AUC_t). Then the value was extrapolated to infinity (AUC), using the terminal slope (β), the last plasma concentration (C_t) and the time where C_t occurred. The elimination half-life ($t_{1/2}$) was determined from β by the usual equation [15].

Results and discussion

3.1. Sample preparation and chromatography

RA was selectively and reproducibly isolated from human plasma constituents by extraction with acetonitrile. The overall mean recovery, determined by comparing the peak heights measured in extracts of human plasma spiked with the compound with the peak height for acetonitrile-diluted compound standards, was 85 ± 7%, with no significant dependence on concentration over the range investigated (10–1000 ng/ml).

The isocratic chromatographic system was selected by varying the composition of common mobile phase components and the best separation was obtained with acetonitrile–methanol–water–10% ammonium acetate (60:16:20:4, v/v). This gave optimum resolution between RA and the I.S. (all-*trans*-retinyl acetate) [16], and also between these compounds and endogenous constituents (*i.e.*, retinol, which eluted with a retention time of about 11 min).

Examples of chromatograms of extracts from drug-free plasma and plasma from a patient given 45 mg/m^2 per day of RA orally and containing 76 ng/ml of unchanged compound are shown in Fig. 1. Approximate retention times were 6.8 min for RA and 25 min for the I.S., as shown by injection of non-extracted standard solutions of the compounds. We observed no interfering peaks in several samples of drug-free plasma from patients.

Resolution of RA from its known metabolites was established by chromatographing authentic standards of each compound (Fig. 2A). None of the available RA metabolites (4-oxo-RA, retention time 2.8 min; 5,6-epoxy-RA, 3.7 min; 13-*cis*-RA, 5.4 min) interfered with the parent compound or the I.S. under these conditions. Trace amounts of metabolite 13-*cis*-RA, but not 5,6-epoxy-RA, were detectable in plasma of all patients at the start of RA therapy (4-oxo-RA eluted with the solvent front). However, the

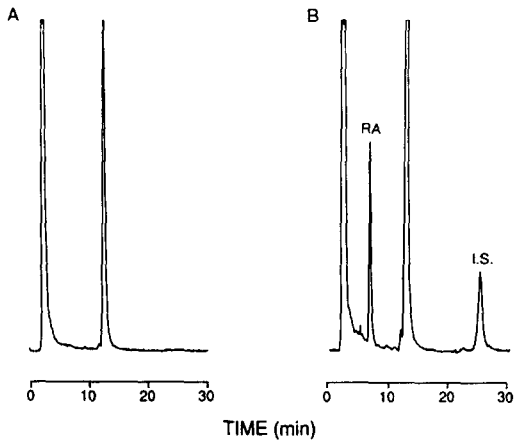

Fig. 1. Chromatograms of extracts of (A) drug-free plasma and (B) plasma from a patient with APL on RA therapy (45 mg/m^2) (1 h after the first dose), containing 76 ng/ml of unchanged compound. I.S. = internal standard (200 ng/ml).

metabolite 5,6-epoxy-RA appeared in plasma of one APL patient at the time of relapse (Fig. 2B).

No interference was seen when drug-free plasma spiked with RA and the I.S. and some antibiotics, chemotherapeutic agents and other drugs often administered to APL patients on RA therapy (adriamycin, allopurinol, amikacin, aracytin, ceftazidime, ciprofloxacin, fluconazole and prednisone) were processed according to this procedure at concentrations typically encountered in clinical samples.

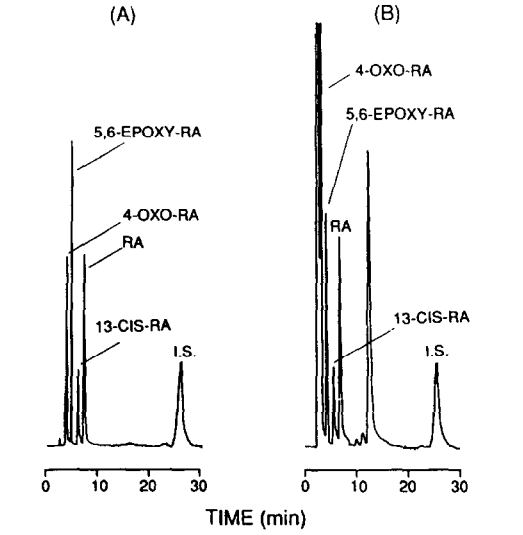

Fig. 2. Separation of RA, its metabolites 4-oxo-RA, 5,6-epoxy-RA and 13-*cis*-RA and all-*trans*-retinyl acetate (I.S.) on a LiChrosorb RP-18 (5-μm) column (250 × 4.6 mm I.D.). Mobile phase, acetonitrile–methanol–water–10% ammonium acetate (60:16:20:4, v/v). Chromatograms of (A) a methanolic solution of the five compounds, and (B) a plasma extract (1 h after dosing) from one patient with APL on RA therapy (45 mg/m^2 per day), at the time of relapse.

3.2. Validation studies

The calibration graphs obtained by plotting the peak-height ratios (RA/I.S.) (y) against the concentrations (x) of RA added to the human plasma were always linear over the range 0.01–1 μg/ml. The correlation coefficient for each calibration graph plotted invariably exceeded 0.997. The slopes of five graphs, prepared throughout the validation study, had a coefficient of variation (C.V.) of 5% (average regression equation $y = 0.0361x - 0.1552$). The lower limit of quantification was approximately 10 ng/ml, using 0.5 ml of plasma.

Plasma QC samples (0.5 ml) containing the equivalent of 5, 50 and 500 ng of RA were assayed with each of the HPLC chromatographic runs in support of this study. The results are summarized in Table 1. At the limit of quantification of the analytical procedure, the within-day and between-day C.V.s were 10% and 8.3%, respectively, and for all higher concentrations the C.V. was always less than 10%. The overall mean accuracy of the method calculated from the deviation of the mean concentrations found from the nominal value of the compound indicated an intra-assay variation in the range 2 to −4%. Hence the precision and accuracy of the method appear to be acceptable over the concentration range investigated.

2.2. Clinical application

The utility of the procedure was established by analysing plasma samples from APL patients ($n = 4$) at the start of RA therapy. The plasma specimens were obtained from 15 min to 6 h after drug administration (45 mg/m^2 body surface). The observed t_{max} was 2 h in all instances.

Table 1
Summary of quality control results for RA in human plasma from the within-day and day-to-day validation study (results are means ± S.D.)

Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)	Accuracy (%)
Within-day (n = 4)			
10	10.2 ± 1.0	10.0	2.0
100	98.9 ± 2.4	2.0	−1.1
1000	1012.1 ± 12.9	1.0	1.2
Day-to-day (n = 8)			
10	9.6 ± 0.8	8.3	−4.0
100	96.7 ± 3.1	3.2	−3.3
1000	984.8 ± 43.0	4.4	−1.5

The observed C_{max} averaged 298 ng/ml, ranging from 166 to 458 ng/ml, and the plasma *AUC* averaged 732 ng h/ml, ranging from 407 to 991 ng h/ml. The plasma concentration then fell close to the limits of the analytical procedure at 6 h. Therefore, the method reported here has sufficient sensitivity to monitor the plasma levels of unchanged drug that might be encountered in pharmacokinetic studies, considering its short half-life in these patients (mean 62 min, range 48–88 min). These values agree well with those reported by other workers [7].

The plasma concentration–time curve of the compound on the first day of treatment (45 mg/m^2 per day) and at the time of relapse in an APL patient is shown in Fig. 3. On day 1 the plasma C_{max} was 359 ng/ml and the plasma AUC_t (t = 0–6 h) was 949 ng h/ml. However, C_{max} dropped to 172 ng/ml and AUC_t to 451 ng h/ml after seventeen daily doses, in agreement with previous findings that clinical relapse and resistance to continued treatment with RA are associated with much lower drug plasma concentrations than on the first day of treatment [12]. Whether this effect is due to decreased drug bioavailability or increased clearance through autoinduction of one or more metabolic pathways, or to other mechanics during repeated RA dosing in some patients, merits more detailed studies.

Table 2
Mean kinetic parameters of RA in patients with APL after a single oral dose of 45 mg/m^2 (n = 4)

Parameter[a]	Mean ± S.D.
Time to peak (h)	2
C_{max} (ng/ml)	298 ± 135
AUC (ng h/ml)	732 ± 285
Elimination $t_{1/2}$[b] (min)	62 ± 23

[a] C_{max} = maximum concentration; *AUC* = area under the curve.
[b] Mean for only three patients.

4. Conclusions

An HPLC method has been developed and validated to measure the plasma concentrations of RA in clinical studies. The sample treatment

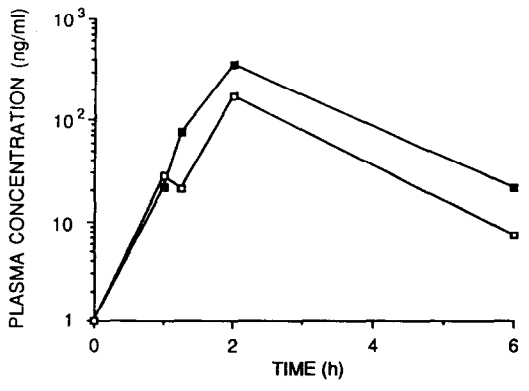

Fig. 3. Plasma concentration–time plots for RA in an APL patient (■) on the first day of treatment, and (□) at the time of relapse. The daily dose of RA was 45 mg/m^2 body surface.

procedure involving one-step extraction with acetonitrile is simple, selective and rapid and thus suitable for large-scale routine analysis, using a relatively small plasma volume. The sensitivity, linearity, precision and reproducibility of the overall procedure are adequate for pharmacokinetic studies. This method is therefore suitable for determining the concentrations of RA in APL patients, and should be useful for gaining a better knowledge of the mechanism(s) involved in the clinical relapse and resistance to repeated drug treatment in some APL patients.

References

[1] C. Chomienne, P. Ballerini, N. Balitrand, M.T. Daniel, P. Fenaux, S. Castaigne and I. Degos, *Blood*, 76 (1990) 1710.

[2] R.P. Warrel, M.D. Stanley, R. Frankel, M.D. Wilson, H. Miller, D. Scheinberg, L. Itri, W. Hitlelman, R. Vyas, M. Andreen, A. Tafori, A. Jakubowski, J. Gabrilove and E. Dmitrowsky, *N. Engl. J. Med.*, 324 (1991) 1385.

[3] S. Castaigne, C. Chomienne, M.T. Daniel, R. Berger, P. Fenaux and L. Degos, *Blood*, 76 (1990) 1704.

[4] Z.X. Chen, Y.Q. Xue, R. Zhang, T. Rei Fan, L. Chun, W. Wei, Z. Wan Zing, Y. Xue-Zhen and I. Bao Jue, *Blood*, 78 (1991) 1413.

[5] M.E. Huang, Y.C. Ye, S.R. Chen, J.R. Chai, J.X. Lu, L. Zhoa, L.J. Gu and Z.Y. Wang, *Blood*, 72 (1988) 567.

[6] L. Degos, C. Chomienne, M.T. Daniel, L. Berger, H. Dombret, P. Fenaux and S. Castaigne, *Lancet*, 2 (1990) 1440.

[7] J. Muindi, S.R. Frankel, W.H. Miller, Jr., A. Jakubowski, D.A. Scheinberg, C.W. Young, E. Dmitrovsky and R.P. Warrel, Jr., *Blood*, 79 (1992) 299.

[8] P.C. Adamson, F.M. Balis, M.A. Smith, R.F. Murphy, K.A. Godwin and D.G. Poplack, *J. Natl. Cancer Inst.*, 84 (1992) 1332.

[9] R. Wyss and F. Bucheli, *J. Chromatogr.*, 424 (1988) 303.

[10] E.A. Duell, A. Astrom, C.E.M. Griffiths, P. Chambon and J.J. Voorhees, *J. Clin. Invest.*, 90 (1992) 1269.

[11] D.M. McPhillips, J.R. Kalin and D.L. Hill, *Drug Metab. Dispos.*, 15 (1987) 207.

[12] P. Lefebvre, G. Thomas, B. Gourmel, A. Agadir, S. Castaigne, C. Dreux, L. Degos and C. Chomienne, *Leukemia*, 5 (1991) 1054.

[13] C.J.L. Buggé, L.C. Rodriguez and F.M. Vane, *J. Pharm. Biomed. Anal.*, 3 (1985) 269.

[14] A. Biondi, A. Rambaldi, P.P. Pandolfi, V. Rossi, G. Giudici, M. Alcalay, F. Lo Coco, D. Diverio, E.M. Pogliani, E.M. Lanzi, F. Mandelli, G. Masera, T. Barbui and P.G. Pelicci, *Blood*, 80 (1992) 492.

[15] M. Gibaldi and D. Perrier, *Pharmacokinetics*, Marcel Dekker, New York, 2nd ed., 1982.

[16] J.G. Besner and R. Leclaire, *J. Chromatogr.*, 183 (1980) 346.