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QUANTITATIVE ANALYSIS OF RETINOIDS IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING COLUMN SWITCHING

I. DETERMINATION OF ISOTRETINOIN AND TRETINOIN AND THEIR 4-OXO METABOLITES IN PLASMA

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SUMMARY

A fully automated gradient high-performance liquid chromatographic method for the determination of isotretinoin, tretinoin and their 4-oxo metabolites in plasma was developed, using the column-switching technique. After dilution with an internal standard solution containing 20% acetonitrile, 0.5 ml of the sample was injected onto a precolumn (17×4.6 mm I.D.), filled with C_{18} Corasil 37–53 μm. Proteins and polar plasma components were washed out using 1% ammonium acetate–acetonitrile (9:1, v/v) as mobile phase 1. After valve switching, the retained components were transferred to the analytical column in the backflush mode, separated by gradient elution and detected at 360 nm by UV detection. Using two coupled reversed-phase columns (125 mm long), the separation of *cis* and *trans* isomers was possible, and all four compounds could be quantified down to 2 ng/ml of plasma. The inter-assay precision in the concentration range 20–1000 ng/ml was between 1.0 and 4.7% for all compounds.

INTRODUCTION

The retinoids are a large group of compounds, structurally related to vitamin A (retinol), which can elicit specific biological responses by binding to and activating a specific receptor or set of receptors. The classical ligands are retinol and retinoic acid, but synthetic ligands may have a better molecular fit to these receptors [1]. Retinoids have been shown to possess selective activities in proliferation, differentiation, keratinization, sebum production, inflammation, immune reaction and tumor prevention and therapy [2].

Isotretinoin (13-*cis*-retinoic acid, Roaccutan®, I, Fig. 1) is orally effective in

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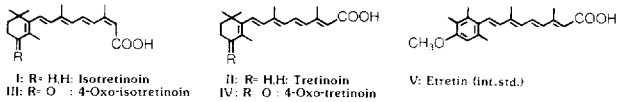

Fig. 1. Chemical structures of the compounds.

the treatment of severe cystic acne and related disorders [3]. Tretinoin (all-*trans*-retinoic acid, Airol®, II, Fig. 1), due to a low oral therapeutic index, is limited almost exclusively to topical application [4]. The main metabolites of these two drugs in blood are their 4-oxo compounds (see Fig. 1) [5, 6]. As I isomerizes in vivo to II and, therefore, IV is also a metabolite of I [7], the simultaneous determination of all four compounds is desirable. Together with retinol, I–IV can also be found as natural products in small amounts in human and animal blood [8–10]. The pharmacokinetics of I and II have been reviewed [11].

Several methods have been developed for the determination of I and/or II in blood, plasma or serum. These include normal-phase [8–10, 12] and reversed-phase [8, 9, 13–19] high-performance liquid chromatographic (HPLC) methods. For the simultaneous determination of I or II and its corresponding 4-oxo metabolite a gradient system is needed [20–26]. However, although three of these methods [21, 24, 26] describe the detection of both III and IV, none of them is suitable to quantify routinely both pairs of isomers simultaneously in the low nanogram range. In addition, all these methods are time-consuming because of the need of an extraction step, which has to be carried out in a darkened room to prevent isomerization of the light-sensitive retinoids. Therefore, a fully automated gradient HPLC method with direct injection of diluted plasma samples was developed, using a column-switching technique first described by Roth et al. [27], which enabled the simultaneous quantification of I–IV down to 2 ng/ml of plasma.

EXPERIMENTAL

Materials and reagents

Tetrahydrofuran (HPLC grade), methanol, 2-propanol, acetic acid and ammonium acetate (all puriss. p.a.) were obtained from Fluka (Buchs, Switzerland). Sodium hydroxide (Titrisol) was purchased from E. Merck (Darmstadt, F.R.G.) and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Water was distilled twice from all-glass apparatus. Argon and helium were obtained from Pan Gas (Lucerne, Switzerland). I–IV and the internal standard (etretin, Neotigason®, V, Fig. 1) were provided by F. Hoffmann-La Roche (Basle, Switzerland) and were kept under argon at 4 °C. Plasma standards were prepared using fresh frozen plasma from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland).

Solutions and standards

The preparation of plasma standards and the dilution of the samples were performed in a darkened room.

A 10% (w/v) solution of ammonium acetate in bidistilled water was made up (100 g/l).

Four stock solutions were prepared in amberized volumetric flasks by dissolving 10 mg of I–IV in 10 ml of methanol. Appropriate amounts of each stock solution were combined and diluted with methanol to give solutions in the range 200–0.2 μg/ml I–IV. These solutions were used as plasma standards by diluting 0.1 ml with blank plasma to 10 ml, yielding concentrations of 2000, 1000, 500, 100, 50, 20, 5 and 2 ng/ml of plasma. The plasma standards were divided into portions of 1.2 ml and stored at −20°C.

A stock solution of the internal standard was prepared in an amberized volumetric flask by dissolving 10 mg of V in 5 ml of tetrahydrofuran and diluting to 100 ml with 2-propanol (100 μg/ml). This solution could be stored at 4°C for several months. An internal standard working solution was prepared by diluting 7.2 ml of 1 *M* sodium hydroxide to 800 ml with water and adding 200 ml of acetonitrile. Then 50 ml of this solution were spiked with 50 μl of the stock solution, yielding 100 ng of V per ml of 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v).

Chromatographic system

A schematic representation of the column-switching system is given in Fig. 2. Pump P1 [Model 414 (Kontron, Zurich, Switzerland) with pulse damper (Orlitta, Giessen, F.R.G.)] delivered mobile phase M1, which was used as purge solvent at a flow-rate of 1.5 ml/min. Aliquots (500 μl) of diluted plasma were injected by a WISP 712 automatic sample injector with cooling module (I1; Waters, Milford, MA, U.S.A.) onto the precolumn (PC). In order to inject larger sample

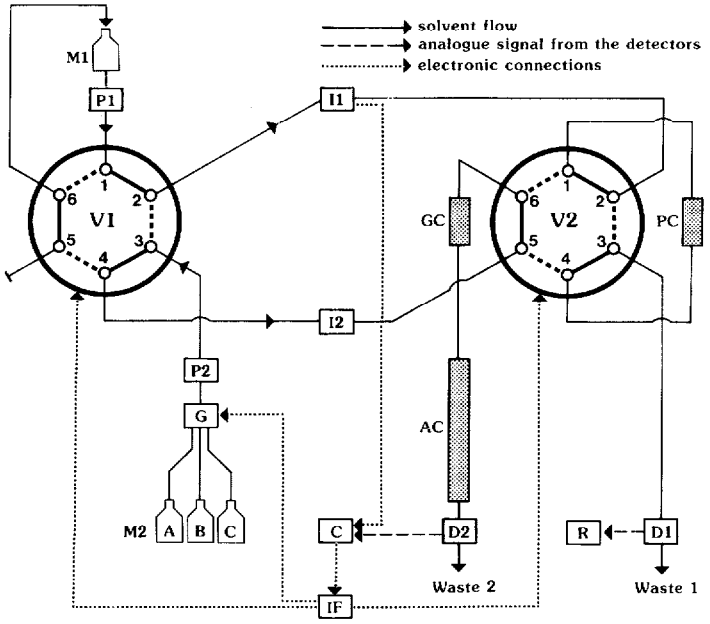

Fig. 2. Schematic representation of the column-switching system. The valves V1 and V2 are shown in position 0 (see text for further details).

volumes than 200 μl, the autoinjector was used with a 1-ml syringe, the auxiliary sample loop and a syringe motor rate of 1.85 μl/s. The UV detector D1 (Spectroflow 773, Kratos, Westwood, NJ, U.S.A.), operating at 240 nm, together with a W+W recorder 320 (Kontron; sensitivity 10 mV, chart speed 0.5 cm/min), were used to monitor the removal of plasma components from the precolumn during the purge step. Pump P2 with a low-pressure gradient system G (Spectroflow 400 solvent delivery system and 430 gradient former, Kratos) delivered the gradient mobile phase M2 (flow-rate 1.0 ml/min) for the elution of the retained components from the precolumn in the backflush mode onto the analytical column (AC).

A guard column (GC) was used to protect the analytical column. A manual injector I2 (Model 7125 with a 500-μl loop, Rheodyne, Cotati, CA, U.S.A.), situated between pump 2 and valve V2, was used for direct injection onto the analytical column. Detection of the eluted compounds was carried out at 360 nm with UV detector D2 (Spectroflow 783, Kratos; rise time 2 s, range 0.02 a.u.f.s.), and data-handling was performed by means of the computing integrator C (Model SP 4200 with Kerr minifile 4100D, Spectra-Physics, San José, CA, U.S.A.; sensitivity 8 mV, chart speed 0.5 cm/min) and a special BASIC program (an improved version of that described previously for the integrator SP 4100 [28]). The gradient former G and the two air-actuated switching valves V1 and V2 (Model 7000A, Rheodyne; shown in position 0 in Fig. 2), the latter connected to two solenoid valves (Model 7163, Rheodyne), were controlled by the external time events of the computing integrator C. To achieve compatibility, an interface IF, designed by Timm [29] and produced in our electronic workshop, was placed between the integrator output and the solenoid valve input.

Columns and mobile phases

The precolumn PC (17×4.6 mm I.D.; Bischoff-Analysentechnik, Leonberg, F.R.G.) was dry-packed with Bondapak C_{18} Corasil, 37–53 μm (Waters) and used with sieves (3 μm) without fibre-glass filters to avoid column blocking.

The analytical column AC consisted of two columns (125×4 mm I.D., Hibar type; E. Merck) linked together by a sleeve-nut (Merck) and filled with Spherisorb ODS 1, 5 μm (Phase Separations, Norwalk, CT, U.S.A.). The guard column GC (30×4 mm I.D.; Merck) contained the same material and was also linked to the analytical column using another sleeve-nut. All columns were filled by a slurry technique. To obtain higher plate numbers, two linked columns, each 125 mm long, were preferred to a single 250-mm column.

Mobile phase 1 was prepared by mixing 90 ml of 10% ammonium acetate with 810 ml of water and 100 ml of acetonitrile. Mobile phase 2 consisted of two components: (A) 4 ml of 10% ammonium acetate, 396 ml of bidistilled water, 600 ml of acetonitrile and 30 ml of acetic acid; (B) 4 ml of 10% ammonium acetate, 146 ml of bidistilled water, 850 ml of acetonitrile and 10 ml of acetic acid. Component C (in Fig. 2) was not used. All mobile phases were degassed with helium prior to use.

Analytical procedure

Plasma (500 μl) was mixed with 750 μl of internal standard working solution. After centrifugation (1 min at 1500 *g*), 800 μl were transferred to the automatic

sample injector vial (microtubes 3810, Eppendorf Gerätebau, Hamburg, F.R.G.), and 500 µl were injected. The samples were kept at 10°C in the autosampler before injection.

The total sequence of automated sample analysis required 36 min and included the following four steps.

Step A (0–10 min, V1=0, V2=0). Injection of the diluted plasma samples onto PC. Polar constituents were washed out to waste 1. GC and AC were equilibrated with M2 (100% A).

Step B (10–29 min, V1=0, V2=1). M1 passed directly to waste 1. The retained components were transferred from PC to GC/AC in the backflush mode by the gradient M2: from 100% A to 70% A (10–16 min), 70% A to 0% A (16–21 min) and 0% A (21–29 min).

Step C (29–32 min, V1=1, V2=0). While M1 was running in a recycling mode, the capillaries between I1 and D1 were purged with M2 (100% B) to prevent any memory effects during the next injection. GC and AC were without any flow during this period.

Step D (32–36 min, V1=0, V2=0). M2 was changed from 0% A to 100% A in 10 s, and GC/AC and PC were re-equilibrated with M2 and M1, respectively.

Calibration and calculations

Together with the unknown and quality control samples, eight plasma standards, distributed over the whole set of samples, were processed as described above. The calibration curve ($y=a+bx$) was obtained by a weighted linear least-squares regression (weighting factor $1/y^2$) of the peak-height ratio y versus the concentration x. The calibration curve was used to interpolate unknown concentrations in the biological samples from measured peak heights. All data processing and calculations were carried out by the computing integrator.

RESULTS AND DISCUSSION

Sample pretreatment

In the first papers describing column-switching methods [27, 30], the plasma sample was injected without dilution or addition of an internal standard, using water or buffer as mobile phase 1. Subsequently, the usefulness of an internal standard and of the dilution of the plasma to circumvent premature column blockage has been demonstrated several times [29, 31, 32]. It has been found that the addition of acetonitrile to the injection solution may be important for optimization of the recovery [32, 33], and we confirmed this. This may be attributed to a "loosening" of the protein binding of the retinoids, and an improved mass transfer to the stationary phase. The addition of sodium hydroxide to the injection solution improved the stability of the retinoids during overnight injection. In a preliminary method, a buffer with 1 *M* sodium acetate (adjusted to pH 9 with sodium hydroxide) was used, but this resulted in precipitation of plasma proteins and clogging of the precolumn when the injection solution was cooled below 20°C.

Chromatography and column switching

The start signal for an analytical run was given by the automatic sample injector. The switching of the valves and the start of the gradient was controlled by the integrator and the interface. The composition of M1 (together with the composition of the injection solution) proved to be crucial for obtaining a good recovery, especially for the less polar retinoids I and II. As observed with the injection solution, 10% of acetonitrile in M1 gave the best results. More organic modifier could lead to protein precipitation or elution of the retinoids from the precolumn. Using a flow-rate of 1.5 ml/min, the clean-up process was completed in less than 10 min (see Fig. 3).

A typical chromatogram of the separation of the analysed retinoids is shown in Fig. 4. Because of the big difference in polarity, a gradient was necessary for the simultaneous determination of I and II and their metabolites III and IV. In addition, the gradient was useful for purging highly non-polar compounds from the analytical column. A further benefit from gradient elution are the resulting

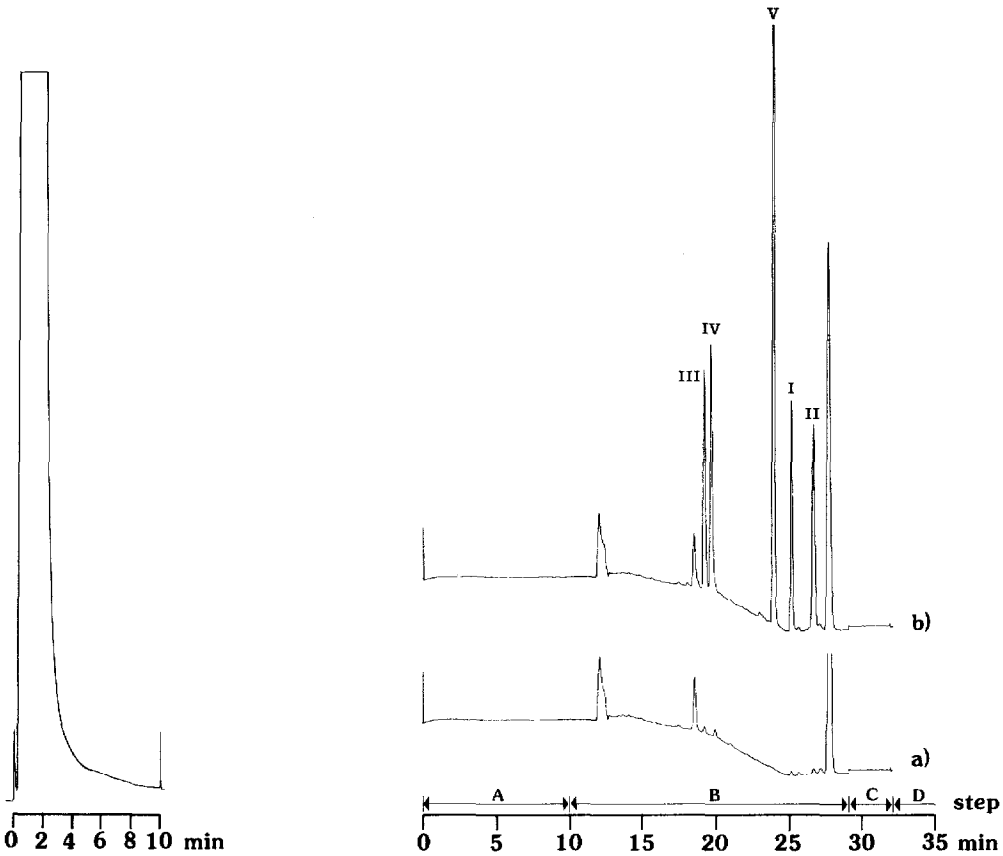

Fig. 3. Control of the purge process on the precolumn by detector D1.

Fig. 4. Chromatograms of human plasma. (a) Blank plasma sample; (b) blank plasma sample spiked with 50 ng/ml I–IV.

sharp peaks, which give lower sensitivity limits compared with isocratic operation. Retention times were highly reproducible. Since two groups of peaks had to be separated, a non-linear gradient was used. To achieve a good separation of *cis* and *trans* isomers, two columns, each 125 mm long, were coupled. Spherisorb ODS 1 gave the best results, probably because it is not an end-capped material and has residual silanol groups, which are known to improve the separation of *cis* and *trans* isomers [24]. The high proportion of acetic acid in M2 was necessary to separate II from endogenous retinol (the large peak with a retention time of 28 min in Fig. 4). I–IV are oxidation products of natural retinol and were found in every human blank plasma, as shown in Fig. 4a. The usual endogenous levels were 0.5–4 ng/ml. This is in good agreement with other observations [8–10]. To prepare plasma standards, blank plasma containing less than 1 ng/ml I–IV was used.

Because of the low solubility of retinoids in aqueous solution, memory effects could occur by adsorption on the steel capillaries. This problem was solved by purging the capillaries between I1 and D1 with M2 during step C. An advantage of the fully automated analytical process is that the samples are not exposed to light. It was therefore unnecessary to work in a darkened room except for the dilution of the plasma samples. Thus, significant light-induced isomerization of I–IV was avoided, which was proved by the analysis of plasma samples with pure isomers.

The precolumn was replaced every day, namely after 40–50 injections. This had the additional advantage of preventing problems due to repeated injections of alkaline solution. The guard column was changed every week, or after 200–250 injections. The analytical column showed no decrease in performance after more than 200 injections.

Limit of quantification

The limit of quantification of I–IV in plasma samples was 2 ng/ml, injecting 0.5 ml of diluted plasma (0.2 ml plasma equivalents). A chromatogram of a spiked plasma sample at this concentration is shown in Fig. 5. The intra-assay ($n=5$) coefficients of variation (C.V.) of spiked plasma samples at this concentration were 6.0, 9.5, 2.1 and 1.8%, with deviations from the nominal value of 6.2, −10.6, −4.5 and −4.2% for I–IV, respectively. Real detection limits, defined by a signal-to-noise ratio of ca. 3:1, were in the range 0.5–1 ng/ml.

Linearity

The correlation of peak-height ratios of I–IV and the concentrations of I–IV, respectively, was linear in the range 2–2000 ng/ml, at least. The coefficients of determination (r^2) were better than 0.999, using the weighting factor $1/y^2$.

Recovery

The first injections of undiluted spiked plasma samples resulted in a low recovery for I (ca. 40–50%). These observations were in conflict with an earlier report concerning this technique [30], which claimed that there was no recovery problem using the column-switching technique for various drugs, although reti-

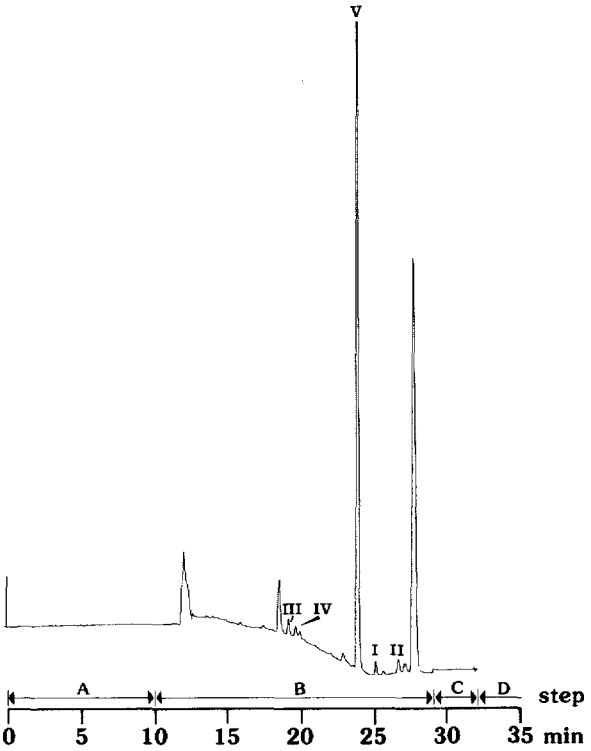

Fig. 5. Chromatogram showing the quantification limit: human blank plasma sample spiked with 2 ng/ml I-IV.

noids were not investigated. The reason for the low recoveries in our case may be the lipophilicity of the retinoids; these compounds are insoluble in water, and, therefore, nearly completely bound to plasma proteins (99.9%) [11]. To overcome the recovery problems, the plasma was diluted with 20% acetonitrile, and the aqueous mobile phase 1 was replaced by a buffer with 10% acetonitrile. The influence of these two measures is shown in Table I. A limited investigation of the influence of the length of the precolumn on the recovery was also carried out. However, the effect was less pronounced than the improvement obtained from the addition of acetonitrile to the injection solution and to the mobile phase.

The recovery from plasma was determined by replicate analysis of spiked plasma samples, followed by replicate injection of 500 μl of a standard solution directly onto the analytical column (using I2), providing the 100% value. However, it was not easy to find the ideal solvent for this solution because of the insolubility of the retinoids in water and peak-broadening when only organic solvents were used. A methanol content of 50% gave the highest peaks for all four compounds and was used in this experiment. The results are shown in Table II.

Reproducibility

The precision (defined as the C.V. of replicate analyses) and the accuracy (defined as the deviation between found and added concentration) of the method

TABLE I

INFLUENCE OF THE COMPOSITION OF THE INJECTION SOLUTION AND MOBILE PHASE 1 ON THE RECOVERY (PEAK HEIGHTS OF I-IV)

Injection solution	Mobile phase 1	Peak height			
		I	II	III	IV
Plasma (200 μl)	1% Ammonium acetate-acetonitrile (9:1, v/v)	3091	2221	3683	4112
Plasma (200 μl) + 7.2 m*M* sodium hydroxide (300 μl)	1% Ammonium acetate-acetonitrile (9:1, v/v)	2800	2004	3517	3952
Plasma (200 μl) + 9 m*M* sodium hydroxide-acetonitrile (8:2, v/v) (300 μl)	Water	3099	2209	3491	3904
Plasma (200 μl) + 9 m*M* sodium hydroxide-acetonitrile (8:2, v/v) (300 μl)	1% Ammonium acetate	2891	2168	3647	4030
Plasma (200 μl) + 9 m*M* sodium hydroxide-acetonitrile (8:2, v/v) (300 μl)	1% Ammonium acetate-acetonitrile (9:1, v/v)	3817	3346	3674	4133

TABLE II

RECOVERIES OF I-IV ($n=5$)

Concentration (ng/ml)	I		II		III		IV	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
20	98.3	3.5	93.6	3.5	102.9	3.2	98.1	3.5
100	98.2	1.1	91.0	1.5	100.3	1.3	100.3	1.1
1000	89.1	1.1	84.4	0.7	93.2	0.5	94.4	0.4

for I–IV were evaluated over the concentration range 20–1000 ng/ml plasma. The intra-assay reproducibility was determined by analysing five specimens of spiked plasma samples on the same day. The inter-assay reproducibility was obtained by analysing one specimen of a spiked plasma sample on eight days over a period of three weeks. The results are compiled in Tables III and IV.

Stability

The stability of I and III in spiked plasma samples was investigated. Storage at $-20\,^{\circ}C$ for three months showed no significant instability, and storage at $20\,^{\circ}C$

TABLE III

INTRA-ASSAY REPRODUCIBILITY ($n=5$)

Substance	Concentration (ng/ml)		C.V. (%)	Difference between found and added (%)
	Added	Found		
I	20	20.5	3.9	+2.5
	100	101	0.3	+1.0
	1000	1002	0.1	+0.2
II	20	20.5	3.9	+2.5
	100	103	0.8	+3.0
	1000	1020	0.7	+2.0
III	20	20.5	3.7	+2.5
	100	104	1.0	+4.0
	1000	1039	0.6	+3.9
IV	20	21.0	3.8	+5.0
	100	106	0.9	+6.0
	1000	1053	0.7	+5.3

TABLE IV

INTER-ASSAY REPRODUCIBILITY ($n=8$)

Substance	Concentration (ng/ml)		C.V. (%)	Difference between found and added (%)
	Added	Found		
I	20	20.2	3.6	+1.0
	100	103	2.3	+3.0
	1000	1019	1.0	+1.9
II	20	20.2	3.4	+1.0
	100	104	2.6	+4.0
	1000	1030	1.5	+3.0
III	20	20.0	3.8	0.0
	100	102	2.7	+2.0
	1000	1008	1.8	+0.8
IV	20	20.2	4.7	+1.0
	100	102	2.7	+2.0
	1000	1004	2.1	+0.4

for 24 h indicated a decrease of ca. 10% for I in the concentration range 20–1000 ng/ml. The stability of plasma samples in the autosampler, a potential problem in the column-switching technique, was improved by adjusting the pH of the injection solution to ca. 8.5–9 (depending on the plasma) with sodium hydroxide and cooling of the autosampler (10°C). Under these conditions, I and III were

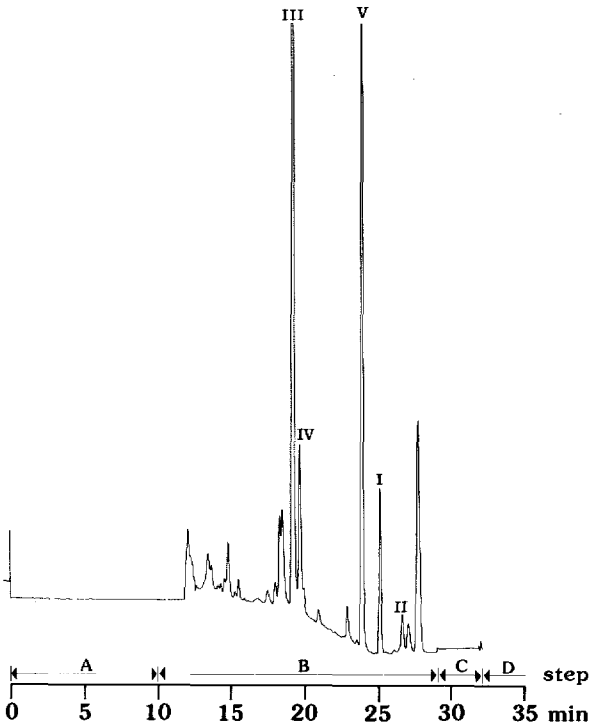

Fig. 6. Chromatogram of a patient plasma sample taken 24 h after a daily dose of 0.5 mg/kg isotretinoin over four months. Measured concentrations: 38.1 ng/ml for I, 8.63 ng/ml for II, 219 ng/ml for III and 37.8 ng/ml for IV.

injected over a period of 19 h. The slight decrease of the peak heights of 2–7% was fully compensated by the internal standard.

Application of the method to biological samples

The method described was successfully applied to the analysis of more than 200 human and monkey plasma samples. Fig. 6 shows the chromatogram of a patient plasma sample taken 24 h after a daily dose of 0.5 mg/kg isotretinoin over four months. As described earlier [11, 24], III becomes the major component after multiple dosing. However, the concentration of IV was almost as high as that of I and was found to be even higher in other samples. This, and the occurrence of even more polar metabolites, confirms the importance of a good separation in this region of the chromatogram.

CONCLUSION

A fully automated gradient HPLC method with direct injection of diluted plasma samples was developed, using the column-switching technique. Time-consuming extraction steps were avoided, and therefore practically no isomerization of the extremely light-sensitive retinoids was observed. As both pairs of isomers could

be separated, quantification of I–IV down to 2 ng/ml of plasma could be achieved routinely.

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REFERENCES

1 J. Nugent and S. Clark (Editors), Retinoids, Differentiation and Disease, Ciba Foundation Symposium 113, Pitman, London, 1985, p. 3.
2 W. Bollag, in J.H. Saurat (Editor), Retinoids: New Trends in Research and Therapy, Karger, Basle, 1985, p. 274.
3 G.L. Peck, T.G.Olsen, F.W. Yoder, J.S. Strauss, D.T. Downing, M. Pandya, D. Butkus and J. Arnaud-Battanier, N. Engl. J. Med., 300 (1979) 329.
4 W. Bollag, in C.E. Orfanos, O. Braun-Falco, E.M. Farber, Ch. Grupper, M.K. Polano and R. Schuppli (Editors), Retinoids. Advances in Basic Research and Therapy, Springer-Verlag, Berlin, 1981, p. 7.
5 C.A. Frolik, Ann. N.Y. Acad. Sci., 359 (1981) 37.
6 F.M. Vane and C.J.L. Buggé, Drug Metab. Dispos., 9 (1981) 515.
7 R.K. Brazzell and W.A. Colburn, J. Am. Acad. Dermatol., 6 (1982) 643.
8 M.G. De Ruyter, W.E. Lambert and A.P. De Leenheer, Anal. Biochem., 98 (1979) 402.
9 A.P. De Leenheer, W.E. Lambert and I. Claeys, J. Lipid Res., 23 (1982) 1362.
10 J.L. Napoli, Methods Enzymol., 123 (1986) 112.
11 R.W. Lucek and W.A. Colburn, Clin. Pharmacokin., 10 (1985) 38.
12 C.V. Puglisi and J.A.F. de Silva, J. Chromatogr., 152 (1978) 421.
13 C.A. Frolik, T.E. Tavela, G.L. Peck and M.B. Sporn, Anal. Biochem., 86 (1978) 743.
14 C.-C. Wang, S. Campbell, R.L. Furner and D.L. Hill, Drug Metab. Dispos., 8 (1980) 8.
15 J.-G. Besner, R. Leclaire and P.R. Band, J. Chromatogr., 183 (1980) 346.
16 G.E. Goodman, J.G. Einspahr, D.S. Alberts, T.P. Davis, S.A. Leigh, H.S.G. Chen and F.L. Meyskens, Cancer Res., 42 (1982) 2087.
17 S.W. McClean, M.E. Ruddel, E.G. Gross, J.J. DeGiovanna and G.L. Peck, Clin. Chem., 28 (1982) 693.
18 R. Shelley, J.C. Price, H. Won Jun, D.E. Cadwallader and A.C. Capomacchia, J. Pharm. Sci., 71 (1982) 262.
19 P.V. Bhat and A. Lacroix, Methods Enzymol., 123 (1986) 75.
20 I.G. Kerr, M.E. Lippman, J. Jenkins and C.E. Myers, Cancer Res., 42 (1982) 2069.
21 F.M. Vane, J.K. Stoltenborg and C.J.L. Buggé, J. Chromatogr., 227 (1982) 471.
22 T. Annesley, D. Giacherio and K. Wilkerson, J. Chromatogr., 305 (1984) 199.
23 H.C. Furr, O. Amédée-Manesme and J.A. Olson, J. Chromatogr., 309 (1984) 299.
24 C.J.L. Buggé, L.C. Rodriguez and F.M. Vane, J. Pharm. Biomed. Anal., 3 (1985) 269.
25 M.E. Cullum and M.H. Zile, Anal. Biochem., 153 (1986) 23.
26 O. Rollman and A. Vahlquist, J. Invest. Dermatol., 86 (1986) 384.
27 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F.W. Koss, J. Chromatogr., 222 (1981) 13.
28 U. Timm, D. Dell and D.A. Ord, Instrum. Comput., 3 (1985) 29.
29 *U. Timm and A. Saner, J. Chromatogr., 378 (1986) 25.*
30 W. Voelter, T. Kronbach, K. Zech and R. Huber, J. Chromatogr., 239 (1982) 475.
31 J.-B. Lecaillon, N. Febvre and C. Souppart, J. Chromatogr., 317 (1984) 493.
32 J. Dow, M. Lemar, A. Frydman and J. Gaillot, J. Chromatogr., 344 (1985) 275.
33 H. Nielsen, J. Chromatogr., 381 (1986) 63.