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

II. SIMULTANEOUS DETERMINATION OF ETRETINATE, ACITRETIN AND 13-*cis*-ACITRETIN IN PLASMA

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SUMMARY

An automated gradient high-performance liquid chromatographic method for the determination of etretinate, acitretin and 13-*cis*-acitretin in plasma was developed, using a column-switching technique. After protein precipitation with ethanol, 0.5 ml of the supernatant was injected onto a precolumn (17 mm × 4.6 mm I.D.), filled with 37–53 μm C_{18} Corasil. Polar plasma components were washed out using 1% ammonium acetate and 1% acetic acid–acetonitrile (8:2, v/v); the retained retinoids were then transferred to the analytical column (125 mm × 4 mm I.D., filled with 5-μm ODS material) in the backflush mode, separated by gradient elution and detected at 360 nm by UV detection. The limit of quantification was 2 ng/ml and the inter-assay precision in the concentration range 20–1000 ng/ml was between 0.9 and 4.0% for all three compounds. To optimize the recovery for etretinate (> 60%), protein was precipitated from plasma with ethanol before injection, instead of direct injection of plasma samples, and a mobile phase containing 20% acetonitrile, instead of pure water or buffer, was used.

INTRODUCTION

Etretinate (Tigason®, I, Fig. 1), a second-generation aromatic retinoid ethyl ester, is an oral dermatological drug which is effective in the treatment of psoriasis and other keratinizing disorders [1,2]. Pharmacokinetic [3] and metabolism [4] studies have shown that it is hydrolysed to the corresponding acid (acitretin, II, Fig. 1) in vivo. A disadvantage of etretinate is its long elimination half-life (84–168 days) after multiple-dose administration [3,5], which is due, at least in part, to storage in adipose tissue [6,7]. Acitretin showed the same activity

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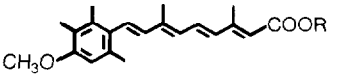
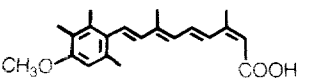
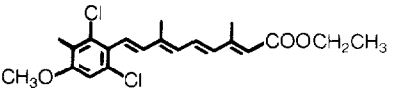


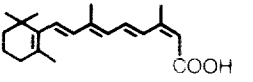
I: R = CH_2CH_3 : Etretinate
II: R = H : Acitretin



III: 13-cis-acitretin



IV: Ro 12-7554 (int. std.)



V: 13-cis-retinoic acid (int. std.)

Fig. 1. Chemical structures of the compounds.

in clinical studies [8,9] but has a considerably shorter elimination half-life (33–60 h) [10]. 13-*cis*-Acitretin (III, Fig. 1) is formed by isomerization in vivo from acitretin and has been identified as the major metabolite in blood after multiple dosing of etretinate [3].

Several methods for the determination of etretinate and acitretin have been reported, including normal-phase [11–13] and reversed-phase [6,14–17] high-performance liquid chromatography (HPLC). Two additional assays [18,19] are suitable for simultaneous quantification of I–III, whereas two other methods [20,21] are limited to the determination of II and III only. The latter two are appropriate after the administration of acitretin (Neotigason®), the successor of etretinate. However, all these methods are either not sensitive enough or make no mention of precision data at the quantification limit or use too much plasma (> 1 ml) for routine analysis. In addition, all these methods are time-consuming because of the need for an extraction step, which has to be carried out in a darkened room to prevent isomerization of these light-sensitive retinoids.

We planned to use the same HPLC method for I–III that we developed for the determination of the retinoids isotretinoin, tretinoin and their 4-oxo metabolites. In this method, using direct injection of diluted plasma samples and a column-switching technique [22], acitretin served as an internal standard, and, in addition, etretinate was separated under the same conditions. However, the recovery problems associated with the high protein binding of the retinoids, described in the previous paper [22], were only solved for the isotretinoin series. Etretinate required new injection conditions, which are presented in this paper. A systematic investigation of the influence of the injection solution composition, the precolumn and the purge mobile phase on the recovery of several retinoid drugs and metabolites will be discussed elsewhere [23].

EXPERIMENTAL

Materials and reagents

Tetrahydrofuran (HPLC grade), 2-propanol, acetic acid and ammonium acetate (all puriss. p.a.) were obtained from Fluka (Buchs, Switzerland). Ethanol

(HPLC grade) 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). Compounds I–III and the internal standards IV (Ro 12-7554, Fig. 1) and V (13-*cis*-retinoic acid, isotretinoin) were provided by F. Hoffmann-La Roche (Basle, Switzerland) and were kept under argon at −20°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 deproteination of the samples were performed in a darkened room.

A 10% (w/v) solution of ammonium acetate was made up in bidistilled water (100 g/l). Three stock solutions were prepared in amberized volumetric flasks by dissolving 10 mg of I–III in 1 ml of tetrahydrofuran and making up to 10 ml with 2-propanol. Appropriate amounts of each stock solution were combined and diluted with ethanol to give solutions in the range 100–0.2 μg/ml I–III. These solutions were used as plasma standards by diluting 0.2 ml with blank plasma to 20 ml, yielding concentrations of 1000, 500, 100, 50, 20, 5 and 2 ng/ml of plasma. The plasma standards were divided into aliquots of 1.2 ml and stored at −20°C.

Two stock solutions of the internal standards were prepared in amberized volumetric flasks by dissolving 10 mg of V in 100 ml of ethanol and 10 mg of IV in 5 ml of tetrahydrofuran and diluting to 100 ml with 2-propanol (100 μg/ml). An internal standard working solution was prepared by diluting 50 μl each of the two stock solutions with ethanol to 50 ml (100 ng/ml). This solution was freshly prepared prior to use. The two stock solutions could be stored at 4°C for several months.

Chromatographic system

The HPLC column-switching system was as described for isotretinoin, tretinoin and their 4-oxo metabolites [22]. The precolumn (17 mm×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 (125 mm×4 mm I.D.) and the guard column (30 mm×4 mm I.D., both Hibar type; E. Merck) were filled with Spherisorb ODS 1, 5 μm (Phase Separations, Queensferry, U.K.), using a slurry technique.

Mobile phase 1 (M1) was prepared by mixing 80 ml of 10% ammonium acetate with 720 ml of water, 200 ml of acetonitrile and 8 ml of acetic acid. Mobile phase 2 (M2) consisted of two components: (A) 4 ml of 10% ammonium acetate, 296 ml of bidistilled water, 700 ml of acetonitrile and 3 ml of acetic acid; (B) 4 ml of 10% ammonium acetate, 146 ml of bidistilled water, 850 ml of acetonitrile and 1 ml of acetic acid. All mobile phases were degassed with helium prior to use.

Analytical procedure

To 0.5 ml plasma, 1 ml of the ethanolic internal standard working solution (containing 100 ng/ml IV and V) was added for protein precipitation. After vortexing and standing for 15 min in the refrigerator at 4 °C, the vial was centrifuged (5 min at 1800 *g*), the supernatant was transferred to the automatic sample injector vial (microtubes 3810, Eppendorf Gerätebau, Hamburg, F.R.G.), and 0.5 ml was injected. The samples were kept at 20 °C in the autosampler before injection.

The total sequence of automated sample analysis required 32 min and included the following four steps:

Step A (0–10 min, V1 = 0, V2 = 0). Injection of the deproteinized plasma samples onto the precolumn. Polar components were washed out to waste 1. Guard column and analytical column were equilibrated with M2 (100% A).

Step B (10–25 min, V1 = 0, V2 = 1). M1 passed directly to waste 1. The retained components were transferred from the precolumn to the guard column/analytical column in the backflush mode by the gradient M1: from 100% A to 0% A (10–18 min) and 100% B (18–25 min).

Step C (25–27.8 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. There was no flow through the guard column and analytical column during this period.

Step D (27.8–32 min, V1 = 0, V2 = 0). M2 was changed from 0% A to 100% A in 0.2 min, and the guard column/analytical column and the precolumn were re-equilibrated with M2 and M1, respectively.

Calibration and calculations

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

RESULTS AND DISCUSSION

Sample pretreatment

The original objective was to use exactly the same conditions as in the isotretinoin assay [22]. However, owing to recovery problems with etretinate, the injection of diluted plasma samples had to be abandoned and replaced by protein precipitation with ethanol. This procedure is not much more time-consuming, and has the advantage of improving the recovery of etretinate from 15% to more than 60%. Nevertheless, the column-switching technique was retained, because pre-concentration on a precolumn allowed the injection of a large volume, and column deterioration was prevented by additional protein precipitation. This de-

terioration would have occurred if the injection had been made directly onto the analytical column because of the high acetonitrile content of M2. The recovery, for one of these substances, could be improved when methanol or 2-propanol was used for protein precipitation, but ethanol gave the best results for all three analytes.

Although the use of an internal standard in a column-switching technique is not absolutely necessary as far as precision is concerned, it may be helpful for correcting the following problems that could arise: autosampler malfunction, premature blocking of the precolumn, incomplete recovery and instability. Two internal standards (V and IV) were used in this assay because of the different chemical properties of the acids II and III, and the ester I, respectively. The internal standards were added with the ethanol for protein precipitation to keep sample handling as simple as possible, although this procedure may not compensate for every loss, owing to incomplete separation from the binding protein.

After subsequent vortex-mixing, the samples were put in the refrigerator (4 °C) for 15 min. This prevented additional protein precipitation in the autosampler if the latter was kept at 20 °C. If the autosampler was not cooled, degradation of the analyses was observed.

Chromatography and column switching

The HPLC column-switching system was as described for isotretinoin [22], with the following differences. The purge mobile phase (M1) contained 20% ace-

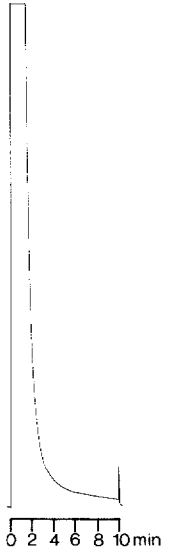


Fig. 2. Control of the purge process on the precolumn by detector D1.

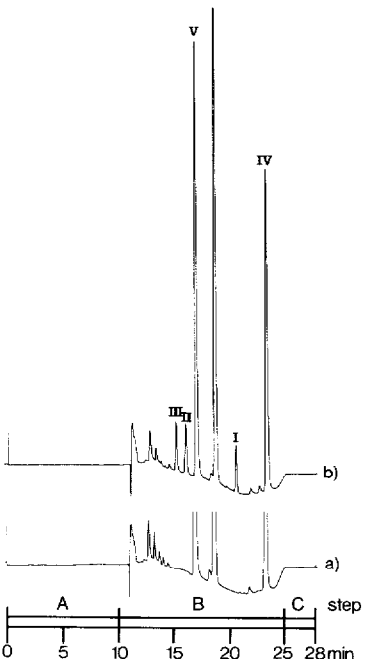


Fig. 3. Chromatograms of human plasma. (a) Blank plasma sample containing the internal standards IV and V; (b) blank plasma sample spiked with 20 ng/ml I–III and 200 ng/ml IV and V.

tonitrile instead of 10%, which improved the recovery of the analysed compounds. The addition of acetic acid to M1 prevented, by ion suppression, the elution of II and III during the purge process. The chromatogram, showing the clean-up process on the precolumn (Fig. 2, detector D1 at 240 nm), was very similar to that obtained after the direct injection of diluted plasma samples [22]. The composition of M2, the gradient steps and the valve-switching times were adapted to obtain optimal separation of the compounds for the minimal analysis time. A typical chromatogram of the separation of I–V is shown in Fig. 3. No interferences were visible, and although only one column (125 mm × 4 mm I.D.) was used, good separation of *cis* and *trans* isomers was obtained. For the more difficult separation of 4-oxoisotretinoin from 4-oxotretinoin, two coupled columns (125 mm × 4 mm I.D.) were needed [22].

Memory effects produced by adsorption of the retinoids on the steel capillaries between I1 and the precolumn were even more critical with etretinate than with isotretinoin because of its greater lipophilicity (ethyl ester instead of acid). However, using V1 and the purge process during step C, no memory effect could be observed, even after the injection of samples containing more than 1000 ng/ml I.

As precaution, the precolumn was replaced every day, even when no deterioration was observed. The guard column was changed every week and the analytical column could be used for ca. two months.

Limit of quantification

The limit of quantification of I–III in plasma samples was 2 ng/ml, injecting 0.5 ml of deproteinized plasma (0.167 ml plasma equivalents). A chromatogram of a spiked plasma sample at this concentration is shown in Fig.4. The intra-assay coefficients of variation (C.V.) of spiked plasma samples at this concentration were 8.4% ($n=7$), 9.0% ($n=6$) and 9.1% ($n=6$), with deviations from the nominal value of 4.1%, −6.2% and −9.3% for I, II, and III, respectively. Real detection limits, defined by a signal-to-noise ratio of 3:1, were ca. 1 ng/ml.

Linearity

The correlation of peak-height ratios of I–III and the concentrations of I–III, respectively, was linear in the range 2–1000 ng/ml, at least. The coefficients of determination (r^2) were better than 0.99, using the weighting factor $1/y^2$.

Recovery

When a classical column-switching technique, that is direct injection of plasma and using water as M1, as described by Roth et al. [24], was applied to the determination of I–III, recoveries of only 2, 57 and 22%, respectively, were obtained. The large difference between II and III under these conditions is very interesting and seems to be due to different protein binding of *cis* and *trans* isomers, whereas for I and the internal standard IV, which is not recovered at all, the greater lipophilicity may be the reason. Similar results, although less marked, had already been obtained with isotretinoin and its metabolites [22]. Under the conditions used there, i.e. dilution of the plasma samples with 20% acetonitrile and using a

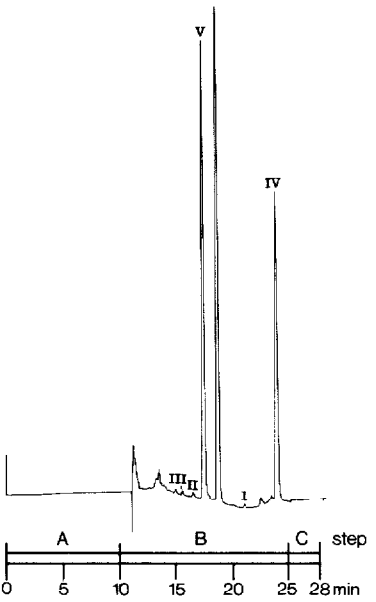


Fig. 4. Chromatogram showing the quantification limit: human blank plasma sample spiked with 2 ng/ml I–III.

TABLE I

RECOVERIES OF I–III ($n=5$)

Concentration (ng/ml)	I		II		III	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
20	66.7	2.3	80.5	2.6	75.2	3.2
100	60.0	3.4	79.0	2.8	74.4	2.8
1000	60.8	8.0	80.6	5.5	76.9	4.7

buffered M1 with 10% acetonitrile, the recoveries of II and III could be improved to over 90%, but were still not acceptable for I (15%) and IV (5%). Therefore, other measures were investigated. An overview of these systematic investigations of the influence of the injection solution composition, the precolumn and the purge mobile phase on the recovery of several retinoid drugs and metabolites, showing the limits of the column-switching technique with highly protein bound drugs in plasma, will be presented elsewhere [23].

The best results for I–V were obtained with protein precipitation with ethanol before injection, and with the use of ammonium acetate buffer containing acetic acid and 20% acetonitrile as M1. Under these conditions, acceptable recoveries were obtained (see Table I). The 100% value was established by replicate injection of 0.2 ml of a 50% solution of I–III in ethanol directly onto the analytical column.

TABLE II

INTRA-ASSAY REPRODUCIBILITY ($n=5$)

Substance	Concentration (ng/ml)		Coefficient of variation (%)	Difference between found and added (%)
	Added	Found		
I	20	21.0	2.5	+5.0
	100	100	1.3	0.0
	1000	1020	0.9	+2.0
II	20	20.7	2.1	+3.5
	100	101	1.1	+1.0
	1000	1042	0.3	+4.2
III	20	21.0	3.3	+5.0
	100	102	1.7	+2.0
	1000	1059	0.8	+5.9

TABLE III

INTER-ASSAY REPRODUCIBILITY ($n=9$)

Substance	Concentration (ng/ml)		Coefficient of variation (%)	Difference between found and added (%)
	Added	Found		
I	20	20.5	4.0	+2.5
	100	102	1.6	+2.0
	1000	1035	2.4	+3.5
II	20	20.5	2.1	+2.5
	100	101	0.9	+1.0
	1000	1027	1.0	+2.7
III	20	20.6	2.2	+3.0
	100	101	1.3	+1.0
	1000	1031	2.1	+3.1

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 for I–III were evaluated over the concentration range 20–1000 ng/ml of 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 nine days over a period of three weeks. The results are compiled in Tables II and III.

Stability

The stability of I–III in spiked plasma samples at ambient temperature and $-20°C$ has already been reported by other authors [13,20]. No significant instability was observed under these conditions. However, the retinoids are known to

be very sensitive to isomerization under the influence of light. As a consequence, all the methods developed so far have to be used under yellow light or in a darkened room. Exposed to normal light, I–III showed severe degradation after 15 min, not only in transparent but also in amberized glass tubes [19]. The automated column–switching methods described in this and the previous paper [22] have the advantage that nearly all analytical steps are carried out in an enclosed apparatus and were, therefore, protected from light. Only for the simple and rapid sample preparation (dilution of plasma samples [22] or protein precipitation) and for the occasional preparation of calibration standards was working in semi-darkness necessary. Under these conditions, less than 0.5% isomerization due to the analytical process was observed.

The stability of the samples in the autosampler was improved by protein precipitation compared with direct injection of diluted plasma samples. However, it was still insufficient under normal operating conditions (ca. 29°C) of the autosampler. Cooling of the autosampler to 20°C improved the stability, but differences of up to 10% in the decrease of peak heights between plasma samples from various volunteers, even using the same anticoagulant, were observed. Because of subsequent further protein precipitation, which could result in column or capillary clogging, cooling below 20°C was not possible. However, it was observed that these stability differences between different plasma samples were the same for all the analysed substances including the internal standards, and that the ratios between analytes and internal standards were constant during a 24-h period within the C.V. presented for the intra-assay reproducibility in Table II.

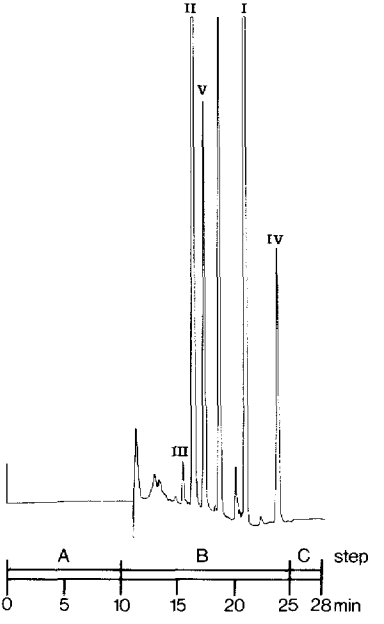


Fig. 5. Chromatogram of a volunteer's plasma sample taken 3 h after a single oral dose of 100 mg of I. Measured concentrations: 935 ng/ml for I, 379 ng/ml for II and 18.5 ng/ml for III.

Application of the method to biological samples

The method described was successfully applied to the analysis of plasma samples from man, rat and mouse. Fig. 5 shows the chromatogram of a volunteer plasma sample taken 3 h after a single oral dose of 100 mg of etretinate. As described earlier, the major metabolite detected in plasma following a single dose of etretinate is II; however, following multiple dosing, III becomes the major component [3].

Although not yet fully validated, the method is also suitable for the determination of I–III in seminal fluid or tissue samples, using protein precipitation with ethanol or homogenization with ethanol–water, respectively, and subsequent direct injection of the supernatants.

CONCLUSION

An automated gradient HPLC method was developed using a column-switching technique. As a result of pre-concentration on a precolumn, 0.5 ml of the supernatant, after plasma protein precipitation, could be injected. As a consequence, high sensitivity was achieved despite the small sample volume, and minimal sample handling (no extraction or evaporation of solvents) prevented isomerization of the light-sensitive retinoids or the need to work in a darkened room all the time. Although the highly protein bound drugs I–III showed the limits of the column-switching technique using direct injection of plasma samples, sufficient recoveries were obtained by protein precipitation with ethanol and addition of 20% acetonitrile to M1.

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