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Short Communication

Ultra-sensitive coupled-column liquid chromatographic determination of retinoids by direct injection of large plasma volumes and ultraviolet detection*

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Introduction

The retinoids are a class of compounds which includes both naturally occurring substances with vitamin A activity and synthetic analogues used as drugs in dermatology and oncology [1]. First generation retinoids [tretinoin or all-*trans*-retinoic acid (**4**, see Fig. 1) and isotretinoin or 13-*cis*-retinoic acid (**3**)] are effective against acne and photodamaged skin, second generation retinoids, such as acitretin (**6**), are used in the treatment of psoriasis and other keratinizing disorders, whereas the third generation retinoid Ro 14-9706 (**9**), an arotinoid methyl sulphone, is in clinical development for reversal of photodamage.

Liquid chromatography (LC) with ultraviolet (UV) detection is the method of choice for the determination of retinoids in biological samples, because it is rapid, sensitive and allows separation of geometrical isomers and metabolites within a wide polarity range. The chromatography of retinoids in biological samples has recently been reviewed [2]. Automated coupled-column LC systems, using direct injection of plasma samples and on-line solid-phase extraction, are especially useful for the determination of photosensitive retinoids due to total protection from light and simplified sample work-up. This was demonstrated by the development of sensitive methods (limits of quantification 2–5 ng ml^{-1}) for first generation [3, 4] and second generation [5] retinoids. However, there is still a need for the determination of even lower retinoid levels in plasma, both for pharmacokinetic studies, and for plasma-level monitoring after cessation of long term systemic treatment or after topical application.

The objective of this investigation was to improve the sensitivity of the coupled-column technique by direct injection of high plasma volumes (1 ml). Measures are presented for performing these analyses under routine conditions, i.e. allowing overnight injections.

Experimental

In the following section specific conditions for acitretin and its 13-*cis* metabolite are described; for other general conditions see refs 3 and 5.

Instrumentation

A schematic representation of the modified column-switching system is given in Fig. 2. An LC pump 420 (P1; Kontron, Zurich, Switzerland) delivered mobile phase M1, which was used as the purge solvent at a flow-rate of 1.5 ml min^{-1}. Aliquots (1.0 ml) were injected

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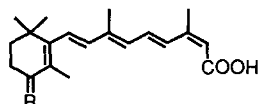

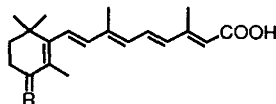

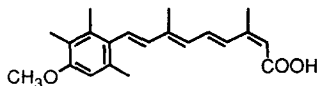

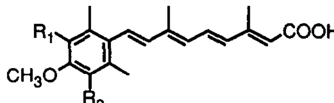

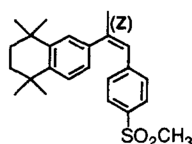

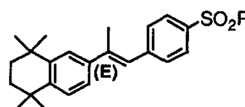

Figure 1
Structures of the compounds.

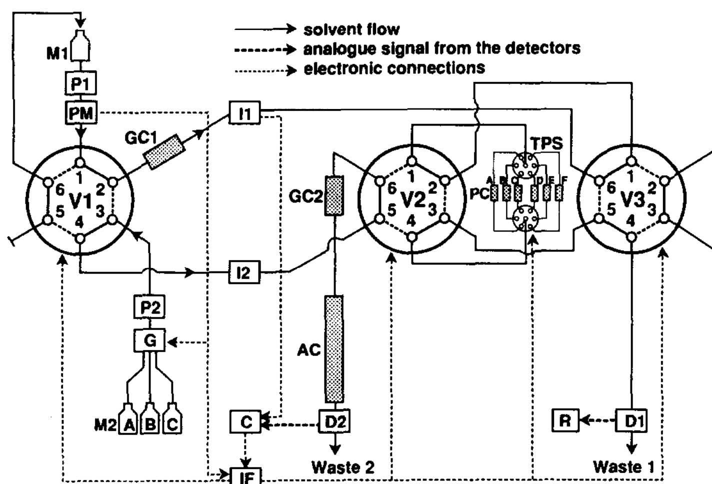

Figure 2
Schematic representation of the LC column-switching system. Position of the valves: V1 = T5, V2 = T4 and V3 = T8. After valve switching the positions are defined as V1 = T6, V2 = T3 and V3 = T7 (see text for further details).

by a WISP 712 automatic sample injector with cooling module (I1; Waters, Milford, MA, USA; 5°C) onto one of the pre-columns (PC). In order to inject sample volumes larger than 200 μl, the autoinjector was used with a 1 ml syringe. The UV detector D1 (Spectroflow 773, Kratos, Ramsey, NJ, USA), operating at 240 nm, together with a W + W recorder 320 (R; Kontron) were used to monitor the removal of plasma components from the PC during the purge step; they were not needed for routine analysis. Pump P2 with a low-

pressure gradient system (G; Spectroflow 400 solvent delivery system and 430 gradient former, Kratos) delivered mobile phase M2 (flow-rate 1 ml min^{-1}). A manual injector (I2; Model 7125, Rheodyne, Cotati, CA, USA) was used for direct injection onto the analytical column (e.g. for recovery experiments). Detection of the eluted compounds was carried out at 360 nm with an UV detector (D2; Spectroflow 783, Kratos, range 0.01 a.u.f.s.), and integration was performed by means of a computing integrator (C; Model SP 4200, Spectra-Physics, San Jose, CA, USA).

The gradient former (G) and the three air-actuated switching valves (V1–V3; Model 7000P, Rheodyne), the latter connected to three solenoid valves (Model 7163, Rheodyne), were controlled by the external time events (T3–T8) of the computing integrator (C). To achieve compatibility, an interface (IF), produced in the electronic workshop at Hoffmann-La Roche, was placed between the integrator output and the solenoid valve input. The positions of the valves in Fig. 2 are V1 = T5 (alternative flow T6), V2 = T4 (T3) and V3 = T8 (T7). During injection and purging of the PC the pressure was measured by a pressure monitor (PM; Bischoff-Analysentechnik, Leonberg, FRG). When a pressure of 120 bar was reached, indicating PC clogging during the next few injections, a signal was sent to a second interface (also represented as IF in Fig. 2) which, after obtaining an end-of-run-signal from the gradient former, effected replacement of the PC by the tandem pre-column selector (TPS; Model 7066, Rheodyne).

Columns and mobile phases

The guard column GC1 and the PC (14 × 4.6 mm i.d.; Bischoff) were packed with Bondapak C18 Corasil, 37–50 μm (Waters). The analytical column (AC; 250 × 4 mm i.d.) and the guard column GC2 (30 × 4 mm i.d., both E. Merck, Darmstadt, FRG) were packed with Spherisorb ODS 1 (5 μm) (Phase Separations, Queensferry, UK) using a slurry technique.

Mobile phase 1 (M1) consisted of 1% ammonium acetate–acetonitrile (9:1, v/v), and the gradient mobile phase 2 (M2) contained three components: (A) 0.2% ammonium acetate–acetonitrile–methanol–acetic acid (200:400:400:3, v/v/v/v); (B) 0.33% ammonium acetate–acetonitrile–acetic acid (120:850:3, v/v/v); (C) water–acetonitrile–acetic acid (10:990:1, v/v/v).

Procedure

To 1 ml of plasma, 0.2 ml of acetonitrile (containing the internal standard) were added. After vortex mixing and centrifugation, 1 ml was injected by the autosampler.

Step A. (0–7 min, V1 = T5, V2 = T4, V3 = T8.) Injection of the sample onto PC. Proteins and polar compounds were washed out to waste 1. AC was equilibrated with M2 (100% A).

Step B. (7–10 min, V1 = T5, V2 = T4, V3 = T7.) PC was purged in the backflush mode by M1.

Step C. (10–23 min, V1 = T5, V2 = T3, V3 = T7.) M1 passed directly to waste 1. The retained components were transferred from PC to AC in the backflush mode by the gradient M2: from 100% A to 100% B (10–20 min), 100% B to 100% C (20–20.1 min), 100% C (20.1–23 min).

Step D. (23–26 min, V1 = T6, V2 = T3, V3 = T7; 26–26.9 min, V1 = T6, V2 = T4, V3 = T8.) While M1 was running in a recycling mode, the capillaries between V1 and D1 were purged with M2 (100% C) to prevent any memory effects during the next injection.

Step E. (26.9–31 min, V1 = T5, V2 = T4, V3 = T8.) After 28 min, M2 was changed from 100% C to 100% A within 0.1 min. AC and PC were re-equilibrated with M2 and M1, respectively.

Results and Discussion

The principle of the new technique was to inject high plasma volumes to improve the sensitivity. 1 ml of plasma is the maximum volume which is usually available for routine determinations and which can be injected by the WISP autosampler. The injection of untreated plasma samples was not possible because of low recoveries arising from strong protein binding of the retinoids [6]. To minimize dilution of the sample, only acetonitrile (final content *ca* 17%) was added. This amount of acetonitrile was sufficient for a good recovery (as long as no lipophilic retinoid esters

were analysed, which appear to need protein precipitation [6]) and was not too high to produce precipitation of proteins in the autosampler vial and blockage of the PC or the capillaries during routine injections.

About 15 ml of plasma can usually be injected onto one PC before the PC is clogged. This would have allowed only 15–20 injections of 1 ml under the conditions described. Therefore, a tandem pre-column selector (TPS) and a pressure monitor (PM) were incorporated in the column-switching system, allowing automated switching to a new PC, either after a defined number of injections, or when a certain pressure (100–120 bar) was reached. Surprisingly, 77.5 ± 24.5 injections (mean ±SD, n = 12) could be made onto one PC. This corresponded to 64.5 ml of plasma, and was much more than the expected 15 ml. The reason for this high volume could be the use of valve V3, which allowed purging of the PC in the forward- and back-flush mode. In this way, proteins and solid particles which could have been adsorbed on the sieves on the top of the PC were transferred to waste 1 instead of to the analytical column. In addition, simple addition of acetonitrile to the plasma sample may be preferred to dilution with water or sodium hydroxide; the latter would certainly cause deterioration of the stationary phase of the PC. Forward- and back-flush purging of the PC may be generally useful for lipophilic compounds [6, 7], but has to be carefully investigated for more polar analytes because of possible breakthrough during the purge process.

Chromatograms of a plasma standard and the corresponding blank plasma for the second generation retinoids acitretin (**6**) and its metabolite 13-*cis*-acitretin (**5**) are shown in Fig. 3. The method was linear in the range 0.3–1000 ng ml^{-1}. The mean recoveries were 76.1 ± 1.8% and 78.4 ± 1.7% for **5** and **6**, respectively. The inter-assay precision (RSD, n = 7) was 3.0–3.7% in the range 1–500 ng ml^{-1} and 11.9 and 9.2% at the limit of quantification (0.3 ng ml^{-1}) for **5** and **6**, respectively. The inaccuracy was 2.4–6.2% in the range 0.3–500 ng ml^{-1}. The limit of quantification of 0.3 ng ml^{-1}, using a 1 ml sample, was a considerable improvement on the previous method [5] which allowed a quantification limit of 2 ng ml^{-1}. However, in this first method, the ethyl ester of acitretin, etretinate, could also be determined. The replacement of

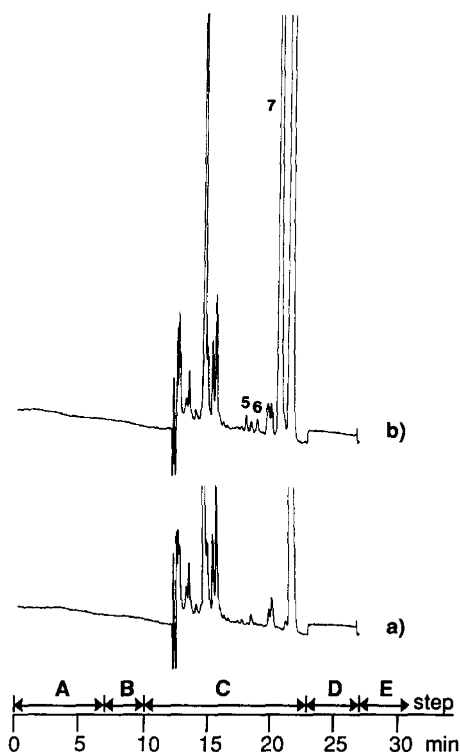

Figure 3
Chromatograms of second generation retinoids: (a) human blank plasma sample; (b) blank plasma sample spiked with 0.5 ng ml^{-1} of acitretin (**6**) and 13-*cis*-acitretin (**5**) and 50 ng ml^{-1} of the internal standard **7**. For conditions see text.

etretinate by acitretin as a drug makes this superfluous. In order to be able to inject more plasma equivalents, the selectivity had to be improved to separate the analytes from plasma interferences. As the UV detection wavelength of 360 nm was relatively selective, further improvement was achieved by use of a longer analytical column (250 instead of 125 mm) and an optimized gradient elution.

Figure 4 shows chromatograms of retinoic acids and their 4-oxo metabolites, analysed using three coupled columns, each 125 mm long, and otherwise similar conditions. Figure 4(a) shows a blank plasma containing about 1.5 ng ml^{-1} of endogenous isotretinoin (**3**) and tretinoin (**4**), whereas Fig. 4(b) shows the same plasma sample spiked with an additional 2 ng ml^{-1} of **1**–**4**. These chromatograms demonstrate the usefulness of this method for the determination of endogenous plasma levels of retinoic acids and their 4-oxo metabolites which had not so far been possible under routine conditions. The same plasma injection technique could also be adapted to the third generation retinoid Ro 14-9706 (**9**), an aro-

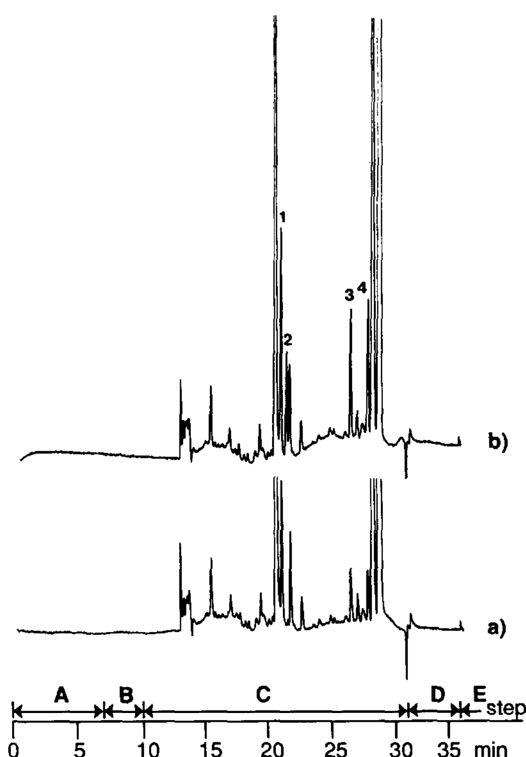

Figure 4
Chromatograms of first generation retinoids: (a) human blank plasma sample containing endogenous levels of **1–4**; (b) blank plasma sample spiked with an additional 2 ng ml^{-1} of **1–4**. Conditions different from those described in the text: AC, three columns 125 × 4 mm; M2, A: 0.1% ammonium acetate–acetonitrile–acetic acid (400: 600:30), B: 1% ammonium acetate–acetonitrile–acetic acid (40:960:10), C: water–acetonitrile–acetic acid (10:990:1).

tinoid methyl sulphone, and its Z-isomer. Despite the less favourable detection wavelength (303 nm) and extinction coefficient (25,000), concentrations of 0.5 ng ml^{-1} could be determined. This is comparable to a GC–MS method developed for the arotinoid ethyl sulphone Ro 15-1570 (**10**) (H.-J. Egger, W. Philipp, U.B. Ranalder, M.C. Hundsbuchler and C. Albrecht, personal communication).

In conclusion, a fully automated HPLC system was developed for the determination of first, second and third generation retinoids using direct injection of plasma samples and on-line solid-phase extraction. The injection of high plasma volumes under routine conditions resulted in ultra-sensitive methods (limits of quantification 0.3–0.5 ng ml^{-1}) even with simple UV detection.

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