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Use of direct injection precolumn techniques for the high-performance liquid chromatographic determination of the retinoids acitretin and 13-*cis*-acitretin in plasma

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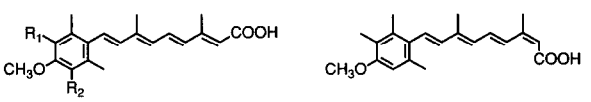
ABSTRACT

A previously developed highly sensitive high-performance liquid chromatographic method for the determination of retinoids, using direct injection of large plasma volumes, on-line solid-phase extraction and ultraviolet detection, was improved and fully validated for the determination of acitretin and 13-*cis*-acitretin in plasma samples. The addition of acetonitrile to improve the recovery was performed on-line by a T-piece, avoiding any *cis–trans* isomerization which could occur when acetonitrile was added prior to storage in the autosampler. About 30 injections could be made onto one precolumn despite the large injection volume (1 ml of plasma containing the internal standard). Full automation was attained by the use of automated precolumn replacement. In addition, forward- and back-flush purging of the precolumn enhanced the longevity of the analytical column. This consisted of three coupled C_{18} columns of 125 mm length each. The quantification limit was 0.3 ng/ml, using ultraviolet detection at 360 nm, and the mean inter-assay precision was 3.8% for the two compounds.

INTRODUCTION

Acitretin (Neotigason®, **1**, Fig. 1) is a synthetic retinoid which is used in the treatment of psoriasis [1]. Its ethyl ester, etretinate (Tigason®), which has been used for many years for the same indication, is now regarded as a pro-drug of acitretin [2]. Compared with etretinate, acitretin has a profound pharmacokinetic advantage because of its more rapid elimination from the body [3]. As acitretin is teratogenic [4], a more thorough investigation of its elimination characteristics is of great interest. For this purpose, highly sensitive methods are needed for the determination of acitretin and its main metabolite, 13-*cis*-acitretin (**2**) [3], after oral or topical administration.



1 : R_1=CH_3, R_2=H : Acitretin

2 : 13-*cis*-Acitretin

3 : R_1, R_2=CH_2CH_3 : Ro 11-6738 (int. standard)

Fig. 1. Structures of the studied compounds.

High-performance liquid chromatography (HPLC) is the method most often used for the determination of retinoids [5]. Gas chromatography is not suitable as a result of isomerization of the tetraene side-chain. On-line solid-phase extraction with automated column switching is especially useful for the pretreatment of biological fluids in HPLC to minimize photoisomerization and oxidation of the retinoids. In this way, liquid–liquid extraction under light protection and evaporation of the extraction solvents are avoided. On the other hand, on-line solid-phase extraction requires special injection conditions to overcome high and strong protein binding of the retinoids [6]. Plasma samples (two volumes) containing isotretinoin, tretinoin (13-*cis*- and all-*trans*-retinoic acid) and their 4-oxo metabolites were diluted either with three volumes of sodium hydroxide–acetonitrile (8:2) [7,8] or with *ca.* eight volumes of ammonium acetate–acetonitrile

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(5:1), followed by semi-automated on-line solid-phase extraction using the Varian AASP system [9]. As an alternative, protein precipitation was performed prior to injection, either with ethanol for the simultaneous determination of etretinate and its two acid metabolites **1** and **2** [8,10], or with propan-2-ol for the above-mentioned retinoic acids [11].

However, the most sensitive methods using HPLC with on-line solid-phase extraction require the injection of large plasma volumes with only minimal dilution. This has been achieved by adding 0.2 ml of acetonitrile to 1 ml of plasma, and the injection of 1 ml of sample by the autosampler. In addition, automated precolumn replacement, when a precolumn became clogged, was inserted into the column switching system for overnight injections [12]. This technique works well with arotinoids, and was successfully applied to sumarotene (Ro 14-9706), an arotinoid methyl sulphone [13]. However, during the routine analysis of acitretin samples, isomerization (mainly from **2** to **1**) was observed in some of the patient samples. This problem was not encountered during method validation with blood bank plasma from volunteers. A detailed investigation revealed that 10–20% of the blood bank plasmas did, in fact, show this effect, and, probably, an even higher proportion of the plasma samples from patients. However, the degree of isomerization varied from sample to sample from the same patient and was often completely absent. The isomerization only became relevant after the plasma samples had been stored for more than 10 h in the autosampler before injection.

Shih *et al.* [14] investigated the isomerization of 13-*cis*- and all-*trans*-retinoic acid by thiol-containing compounds in a non-enzymatic chemical reaction. Catalytic activity was found for glutathione, mercaptoethanol, L-cysteine methyl ester, apoferritin (a thiol-containing protein) and native, and even (to a lesser extent) boiled, microsomes. The ability of the ubiquitous glutathione to catalyse the *cis–trans* isomerization of retinoids was also confirmed by Jewell and McNamara [15] for **1** and **2**. To test the hypothesis that glutathione or a thiol-containing enzyme could be responsible for the observed isomerization in the plasma samples in the acitretin method [12], several inhibitors of this interconversion, proposed previously [14,15], were investigated for plasma stabilization. Sodium *p*-hydroxymercuribenzoate [15], L-cysteine and iodoacetate [14], as well as the addition of sodium hydroxide [7], inhibited the isomerization. Surprisingly, this was also true for glutathione, which was reported to be a catalyst [14,15]. However, all these substances only partially prevented the isomerization of **2** to **1** when they were added to the plasma sample (containing *ca.* 17% acetonitrile). All substances produced accelerated clogging of the precolumn, which excluded overnight injections. Finally, it was found that the isomerization only occurred in the presence of acetonitrile, and that undiluted plasma samples were completely stable for more than 20 h in the autosampler. Therefore, in this work, a modified HPLC method for **1** and **2** was developed, using a direct injection technique with acetonitrile addition by a T-piece, to avoid long contact of the retinoids with this solvent. This resulted in good recoveries without isomerization.

EXPERIMENTAL

The preparation of plasma standards, handling of samples and the addition of the internal standard were performed under diffuse light conditions. The materials and solvents, preparation of calibration standards and other general conditions were as described by Wyss and Bucheli [10,12].

Chromatographic system and conditions

A schematic representation of the modular HPLC column-switching system is given in Fig. 2. A 420 LC pump (P1A; Kontron, Zurich, Switzerland) delivered mobile phase M1A (or alternatively M3) at a flow-rate of 1.4 ml/min. Plasma samples (1.0 ml) were injected by a WISP 712 automatic sample injector with cooling module (I1; Waters, Milford, MA, USA; 10°C) onto one of the precolumns (PC). To inject sample volumes larger than 200 μl, the autosampler was used with a 1-ml syringe, the 2-ml auxiliary sample loop and a syringe motor rate of 1.85 μl/s. In addition, a 2 m × 0.18 mm I.D. capillary was used as a restrictor. The injected sample plug was diluted on-line with a mobile phase containing acetonitrile (M1B) by the HPLC pump P1B (Spectroflow 400 solvent delivery system, Kratos, Westwood, NJ, USA; flow-rate 0.7 ml/min) and a T-piece (T; Valco Instruments, Hous-

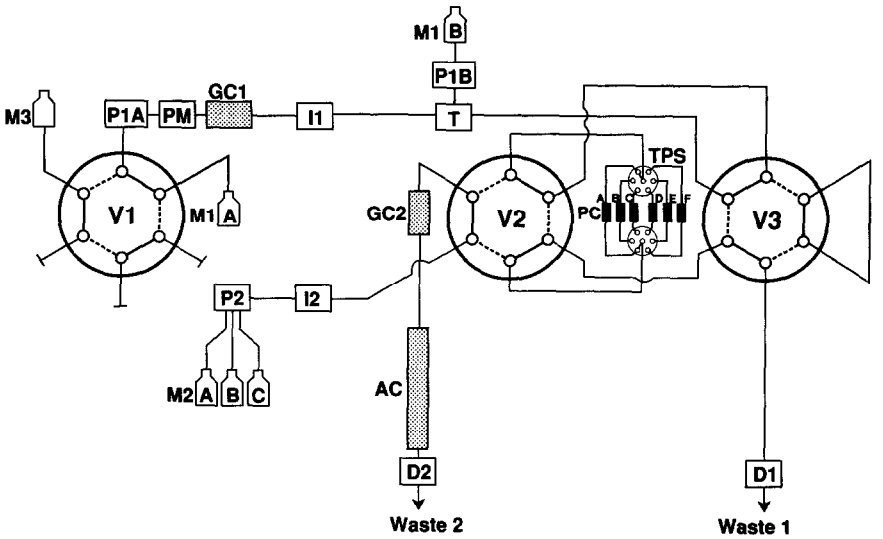

Fig. 2. Schematic representation of the HPLC column-switching system. Position of the valves: V1 = T5, V2 = T4 and V3 = T8. After valve switching the positions are defined as V1 = T6, V2 = T3 and V3 = T7 (see text for further details).

ton, TX, USA; 1/16 in., bore 0.25 mm). The UV detector D1 (Spectroflow 773, Kratos), operating at 230 nm, together with a W+W recorder 600 (Kontron) were used to monitor the removal of plasma components from the precolumn during the purge step; they were not needed for routine analysis. The gradient pump P2 (Model 480, Gynkotek, Germering b. München, Germany) delivered mobile phase M2 (flow-rate 1 ml/min), which was degassed online (Shodex DEGAS KT-35M degassing device, Showa Denko, Tokyo, Japan). A manual injector (I2; Model 7125 with a 200-μl loop, 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 a UV detector (D2; Spectroflow 783, Kratos; rise time 1 s, range 0.01 a.u.f.s.), and integration was performed by a computing integrator (Model SP 4200, Spectra-Physics, San Jose, CA, USA; sensitivity 8 mV, chart speed 0.5 cm/min).

The low-pressure three-way rotary valve (V1; Model 5032P, Rheodyne) and the two high-pressure switching valves (V2 and V3; Model 7000P, Rheodyne), all pneumatically operated and connected to three solenoid valves (Model 7163, Rheodyne), were controlled by the external time events (T3–T8) of the integrator. To achieve compatibility, an interface, produced in the electronic workshop at F. 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, Germany). When a pressure of 80 bar was reached, indicating PC clogging during the following injections, a signal was sent to a second interface which, after obtaining an end-of-run signal from the gradient pump, effected replacement of the PC by the tandem precolumn selector (TPS; Model 7066, Rheodyne).

Columns and mobile phases

The guard column GC1 and the PC (all 14 mm × 4.6 mm I.D.; Bischoff) were packed with Bondapak C_{18} Corasil, 37–50 μm (Waters). The analytical column (AC; three coupled columns, 125 mm × 4 mm I.D. each) and the guard column GC2 (30 mm × 4 mm I.D., all from E. Merck, Darmstadt, Germany) were packed with Spherisorb ODS 1, 5 μm (Phase Separations, Queensferry, UK), using a slurry technique.

Mobile phase M1A consisted of 1% ammonium acetate–acetonitrile (100:2, v/v), M1B of 1% ammonium acetate–acetonitrile (6:4, v/v) and M3 of acetonitrile–water (8:2, v/v). The gradient mobile phase 2 (M2) contained three components: (A) 0.1% ammonium acetate–acetonitrile–acetic acid (40:60:3, v/v/v); (B) 0.8% ammonium acetate–acetonitrile–acetic acid (5:95:1, v/v/v); (C) water–acetonitrile–acetic acid (20:980:1, v/v/v).

Procedure

A 5-μl aliquot of internal standard solution [5 μg/ml **3** in acetonitrile–ethanol (95:5, v/v)] was added to 1.2 ml of plasma. After vortex-mixing and centrifugation (6 min at 3400 *g*), 1 ml was injected. The total sequence of automated analysis required 31 min, and included the following steps.

(1) Step A (0–8 min, V1 = T5, V2 = T4, V3 = T8). Injection and pre-concentration of the sample on PC. Proteins and polar compounds were washed out to waste 1 with M1. AC was equilibrated with M2 (100% A).

(2) Step B (8–10 min, V1 = T5, V2 = T4, V3 = T7). PC was purged in the back-flush mode by M1.

(3) Step C (10–14 min, V1 = T6, V2 = T3, V3 = T7; 14–16 min, V1 = T6, V2 = T4, V3 = T7; 16–17 min, V1 = T6, V2 = T4, V3 = T8). Transfer of the retained components from PC to AC in the back-flush mode by a gradient of 100% A to 100% B (10–26 min). In the meantime, the capillaries between V1 and D1 were purged with M3.

(4) Step D (17–31 min, V1 = T5, V2 = T4, V3 = T8). Gradient of 100% B to 100% C (26 27 min), 100% C (27–30 min), 100% C to 100% A (30–31 min). Meanwhile, PC was re-equilibrated with M1.

RESULTS AND DISCUSSION

Analytical system and chromatography

The addition of acetonitrile or another water-miscible organic solvent was found to be necessary for a good recovery in the on-line solid-phase extraction of retinoids [6,7,9]. For very lipophilic compounds (*e.g.* etretinate), protein precipitation with an organic solvent may be indispensable [6]. However, for **1** and **2**, protein precipitation was not necessary, and the addition of acetonitrile alone (0.2 ml to 1 ml of plasma) was sufficient to obtain a good recovery. The omission of additional dilution with water or buffer resulted in a higher sensitivity [12]. As acetonitrile addition prior to storage in the autosampler induced isomerization, on-line dilution with an acetonitrile-containing mobile phase (M1B) via a T-piece was used. Table I shows the absence of a time-dependent *cis–trans* isomerization of **2** using this technique, compared with the acetonitrile addition used previously.

The addition of an internal standard is recommended for the control of the autosampler injection volume and possible decrease in the peak height on the analytical column after many injections onto the same precolumn. Ro 11-6738 (**3**) showed better results in this respect compared with the previously used isotretinoin (13-*cis*-retinoic acid) [10]. The wash-out time of 8 min (step A of the procedure) needed careful investigation to keep the total run time as short as possible, and to exclude any transfer of plasma proteins to the analytical column, which would reduce the efficiency and the longevity of the analytical column.

The number of injections possible (1 ml of plasma per injection) onto one precolumn was about 30, resulting in a pressure increase to about 80 bar. This was the limit set at the pressure monitor to give a signal for precolumn replacement. During sample injection onto a new precolumn, the pressure was constant (about 30 bar) over many injections. It then increased relatively sharply to >100 bar. The

TABLE I

COMPARISON OF TIME DEPENDENT *cis–trans* ISOMERIZATION

A plasma sample spiked with 100 ng/ml 13-*cis*-acitretin (**2**) was stored in the autosampler at 10°C. Acitretin (**1**) was formed by isomerization in the same sample. Conditions: (A) 1 ml of plasma injected, analytical conditions as described in the text. (B) 1 ml of plasma was diluted with 0.2 ml of acetonitrile prior to storage in the autosampler, and 1 ml of the mixture was injected as under (A).

Condition	Storage time (h)	Peak heights	
		1	**2**
A	2.9	2567	73 169
	26.0	3045	74 667
B	3.6	4323	50 059
	26.7	17 147	38 285

injection of 30 ml of plasma onto one precolumn is about twice the volume which is normally possible. This is, however, still considerably less than the 64.5 ml which could be injected after acetonitrile addition to the plasma sample prior to injection [12]. The immediate addition of acetonitrile appears to be better for the dissolution of plasma components. This could also be observed from stored samples without acetonitrile, which often contained lipid layers or suspended particles. In addition, large numbers of injections could only be obtained by using sieves instead of frits [16].

The peak heights of **1** and **2** were relatively stable during the injection of plasma standards onto the same precolumn. The decrease of less than 10% (until the pressure had reached 80 bar) was compensated sufficiently by the internal standard. After automatic precolumn replacement, the initial peak heights were again obtained. This indicates that the plasma injections resulted in an alteration of the precolumn, probably by coating with lipids. The gradient mobile phase (M2) and the purge mobile phase (M3) eluted part of these lipids. A higher acetonitrile content (100%) in M3 would probably enhance the number of injections onto one precolumn. However, a change from the ammonium acetate containing M1 to 100% acetonitrile in M3 was not possible due to solubility problems, and would have required an intermediate purge step which was not used in this method. In the previous method without a T-piece [12], purging of the capillaries was performed with M2, and not with M3. In that configuration, the precolumn was cleaned for 4 min with M2 containing 99% acetonitrile. This may have resulted in the elution of a higher amount of lipids and, therefore, could be another explanation of the higher number of injections possible onto one precolumn under these conditions. The use of the low-pressure valve V1 resulted in a *ca.* 8 min shorter run time.

The benefits of back-flush purging of the precolumn was also investigated under the experimental conditions described. Whereas the number of injections onto two precolumns tested without back-flush purging (step B of the procedure) was high (>40), an increase in the pressure on the analytical column of 5–10 bar was observed. This confirms the advantage of this additional purge step [6,12] which prevents a pressure increase on the analytical column.

Selectivity

The injection of large plasma volumes makes high demands on the selectivity of the chromatographic system. These requirements were adequately fulfilled by the use of three coupled 125-mm columns and gradient elution, allowing improved separation, compared with the method of Wyss and Bucheli [12], of the internal standard from endogenous interferences. The pressure was about 200 bar using acetonitrile as an organic modifier in the mobile phase (M2). Typical chromatograms of spiked plasma samples are presented in Fig. 3. Fig. 4 shows a plasma sample from a patient before and 24 h after receiving the drug.

Recovery

Recoveries without the addition of acetonitrile (on- or off-line) were low, independent of the amount of acetonitrile in M1A. Therefore, the addition of acetonitrile by M1B was used. Mixing of the

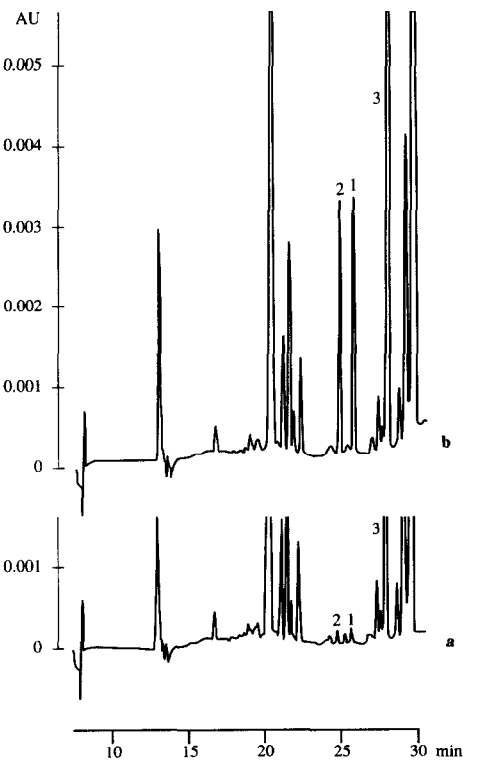

Fig. 3. Chromatograms of human plasma samples. Spiked with (a) 0.3 ng/ml and (b) 5 ng/ml acitretin (**1**) and 13-*cis*-acitretin (**2**); **3** is the internal standard.

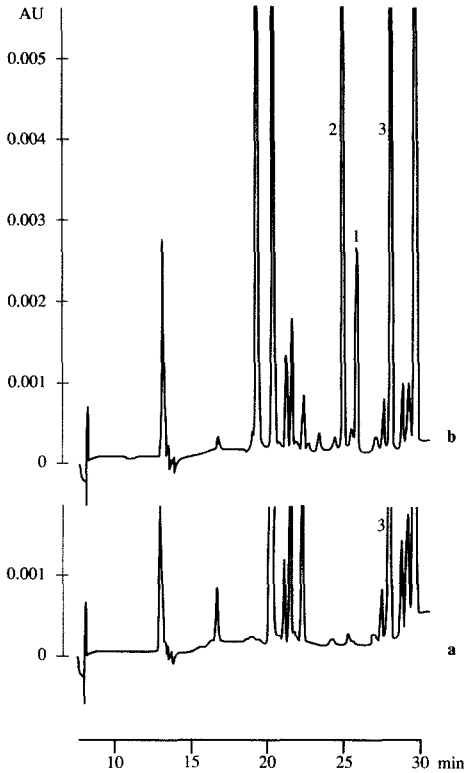

Fig. 4. Chromatograms of patient plasma samples. (a) Pre-dose sample; (b) sample taken 24 h after an oral daily dose of 50 mg acitretin over three weeks. Measured concentrations: acitretin (**1**) 5.77 ng/ml, 13-*cis*-acitretin (**2**) 56.6 ng/ml; **3** is the internal standard.

injected plasma sample, delivered by P1A, and M1B in the 50-cm-long steel capillary (0.5 mm I.D.) between the T-piece and V3 was efficient. Only a minimal improvement was obtained when a coiled, 2-m-long capillary of the same diameter was used. However, the amount of acetonitrile added by the T-piece was crucial for good recoveries of the individual compounds. Table II shows the influence of the acetonitrile content in M1B on the recoveries of **1–3**. More lipophilic compounds (*e.g.* the internal standard **3**) needed a higher proportion of acetonitrile in M1B to achieve maximum recovery. The actual conditions of the method were 40% acetonitrile. The 100% values for recovery experiments were established by replicate injections of 100 µl of **1–3** in M2A directly onto the analytical column (using I2). The recoveries under the conditions used are shown in Table III.

TABLE II

INFLUENCE OF THE ACETONITRILE PORTION OF MOBILE PHASE M1B (ADDED BY THE T-PIECE) ON THE RECOVERIES OF **1**, **2** AND **3**

Plasma samples spiked with 100 ng/ml acitretin (**1**) and 13-*cis*-acitretin (**2**) and 20.8 ng/ml internal standard (**3**) (n = 3).

Acetonitrile (%)	Recovery (%)		
	1	**2**	**3**
2	58.4	38.3	28.3
10	69.3	74.1	40.6
20	82.6	91.3	64.0
30	84.7	94.8	78.1
40	88.4	99.0	83.3
50	80.7	92.5	88.8
60	68.8	83.3	87.3

Linearity

The method was linear in at least the range 0.3–1000 ng/ml. Standard curves (0.3–500 ng/ml) were

TABLE III

RECOVERIES OF **1**, **2** AND **3** (n = 5–8)

Concentration (ng/ml)	**1**		**2**		**3**	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
0.3	86.3	10.3	91.4	2.2	84.2	1.1
1	88.5	2.1	104	2.4	85.7	1.3
100	88.4	1.9	100	1.5	86.5	2.4
500	87.9	0.8	95.9	1.5	86.0	0.7

calculated by weighted least-squares regression, using $1/y^2$ as the weighting factor.

Limit of quantification

The limit of quantification of **1** and **2** was 0.3 ng/ml. This high sensitivity was obtained by the injection of 1-ml plasma volumes. In contrast to off-line extraction methods, this technique prevents any loss of analyte by the re-injection of aliquots of dissolved extracts. Full automation of the method was realized using precolumn replacement by a tandem precolumn selector. A chromatogram of a spiked plasma sample at the quantification limit of 0.3 ng/ml is shown in Fig. 3a. The inter-assay (n = 6) relative standard deviations (R.S.D.) at this concentration were 7.9 and 5.9% for **1** and **2**, respectively (see Table IV). The detection limit, defined by a signal-to-noise ratio of 3:1, was *ca.* 0.1 ng/ml. Even though simple UV detection was used, this is the highest sensitivity attained for these compounds, even considering liquid chromatography–mass spectrometry [17].

Precision and accuracy

The inter-assay precision and accuracy of the method were evaluated by analysing one series of calibration standards over six days against an independent calibration set. The results are given in Table IV. The overall precision was 3.8% for the two compounds.

TABLE IV

INTER-ASSAY PRECISION AND ACCURACY (n = 6)

Concentration (ng/ml)		R.S.D. (%)	Difference between found and added (%)
Added	Found		
Compound 1			
0.3	0.30	7.9	−1.7
0.5	0.51	6.9	+1.3
1	1.01	4.2	+1.0
5	5.06	3.1	+1.1
20	20.3	2.0	+1.6
100	101	3.7	+0.6
200	197	1.2	−1.7
500	499	1.3	−0.1
Compound 2			
0.3	0.29	5.9	−2.7
0.5	0.50	8.4	−0.6
1	1.00	5.1	+0.3
5	5.13	2.4	+2.5
20	20.6	1.2	+2.9
100	101	4.2	+1.4
200	198	1.8	−1.1
500	505	1.4	+1.0

Stability

The stability of retinoids in general and of **1** and **2** in particular have been discussed [5,10]. Plasma samples stored in the autosampler at 10°C were stable for at least 24 h. Therefore, after having prevented any *cis–trans* isomerization, fully automated routine analyses could be performed for 20–22 h per day.

CONCLUSIONS

A previously developed highly sensitive HPLC method for retinoids, using the direct injection of large plasma volumes, on-line solid-phase extraction and UV detection, was improved and fully validated for the determination of acitretin (**1**) and 13-*cis*-acitretin (**2**) in plasma samples. The addition of acetonitrile, which is necessary for good recovery in the on-line solid-phase extraction of retinoids, was performed on-line by a T-piece. In this way, long contact of the retinoids with acetonitrile in the autosampler could be avoided; this was shown to be responsible for the unexpected *cis–trans* isomerization in some of the plasma samples. About 30 injections could be made onto one precolumn, despite the large injection volume (1 ml of plasma). Full automation was attained with automated precolumn replacement. In addition, forward- and back-flush purging of the precolumn enhanced the number of injections possible onto the analytical column. The potential *cis–trans* isomerization, using the conditions described previously [12], also exists for other first- and second-generation retinoids (with a tetraene side-chain), but apparently not for third-generation retinoids [13]. Other ways to prevent the isomerization other than that proposed here would either only allow the analysis of short sample series, or, alternatively, result in less sensitive methods lacking full automation. Two examples of the latter are protein precipitation with ethanol [10] and solid-phase extraction using the Varian AASP system [9].

As a possible alternative to the on-line addition

of acetonitrile to an aqueous plasma sample, large injection volumes containing organic solvent could be diluted on-line with water (instead of acetonitrile) to prevent elution from the precolumn. This technique was used successfully in *in vitro* studies with incubated liver homogenates, where up to 20 ml of ethanol-containing solutions of acitretin and etretinate were injected [18]. The same procedure can also be applied to routine injections of relatively large volumes (> 1 ml) obtained after deproteinization of plasma samples with ethanol. Until now this has not been possible due to the breakthrough of polar compounds. Which of the two precolumn techniques, fully automated direct injection of large plasma volumes, or the more robust injection of deproteinized supernatants (the latter being more flexible with respect to recoveries), is superior will depend on the analyte, the matrix and the preference of the analyst.

HPLC methods with on-line solid-phase extraction and automated column switching are very useful for the determination of retinoids in biological samples because of their high automation potential, high precision and sensitivity. In this respect, this HPLC precolumn technique is superior to liquid chromatography–mass spectrometry, which has been introduced recently for the determination of retinoids in plasma samples [17, 19]. Whereas this latter technique is more specific, simplicity, economics, absence of manual extraction and derivatization, and even higher sensitivity are the advantages of HPLC with on-line solid-phase extraction and UV detection, making it the more direct of these two less travelled roads.

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