*Journal of Chromatography*, 456 (1988) 33–43
Elsevier Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 839

# DETERMINATION OF HIGHLY PROTEIN BOUND DRUGS IN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND COLUMN SWITCHING, EXEMPLIFIED BY THE RETINOIDS

R. WYSS* and F. BUCHELI
*Pharmaceutical Research, Preclinical Development, F. Hoffmann-La Roche & Co. Ltd., CH-4002 Basle (Switzerland)*

## SUMMARY

During method development for the determination of either isotretinoin, tretinoin and their 4-oxo-metabolites, or etretinate, acitretin and 13-*cis*-acitretin in plasma using high-performance liquid chromatography and column switching, recovery problems arose, when undiluted plasma samples were injected directly onto the precolumn. These recovery problems may be due to the strong binding of the retinoids to different plasma proteins. Measures to overcome this strong protein binding, such as variation of the injection solution composition and the purge mobile phase, were systematically investigated. Best recoveries were obtained by diluting of plasma with 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v) and protein precipitation with ethanol for the isotretinoin and etretinate series, respectively, in combination with the use of a purge mobile phase containing ammonium acetate and 10–20% acetonitrile. Less effective was the use of a longer precolumn or heating of the precolumn.

## INTRODUCTION

The term retinoids is a generic name for a large group of compounds which is structurally related to vitamin A (retinol). According to the definition of Sporn and Roberts[1], a retinoid is a substance that 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. Isotretinoin (13-*cis*-retinoic acid, Roaccutan®, 3, Fig. 1) and tretinoin (all-*trans*-retinoic acid, Airol®, 4) are used in the treatment of severe cystic acne[2,3], whereas etretinate (Tigason®, 7) and acitretin (Neotigason®, 6) are effective against psoriasis and other keratinizing disorders[4,5]. However, retinoids also show interesting selective activities in other fields of medicine, such as oncology, inflammation, rheumatism or immune reactions[6].

Among the many analytical methods for retinoids which have been developed within the last ten years, nearly all involve high-performance liquid chromatography (HPLC), including two methods from the authors' laboratory with automated column switching (refs. 7 and 8 and references cited therein). This technique, which allows the

0021-9673/88/$03.50 

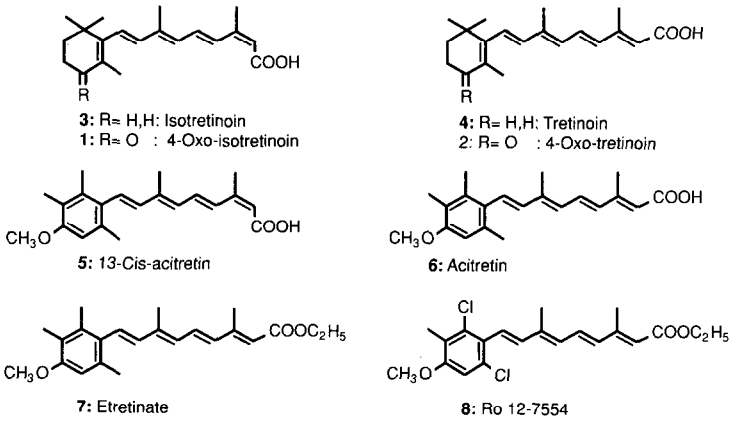


Fig. 1. Chemical structures of the compounds.

direct injection of biological fluids, appeared to be especially suitable for retinoids because of their sensitivity to light and their lipophilicity. Thus, cumbersome extraction in a darkened room can be avoided and preconcentration on a hydrophobic stationary phase should be no problem.

Recently, Westerlund[9] reviewed the possibilities of direct injection of plasma into HPLC systems. Apart from micellar mobile phases, different precolumns can be used for direct injection, including internal surface reversed-phase supports, protein-coated silica and polymer or silica gel based phases for size-exclusion, ion-exchange or affinity chromatography. However, silica bonded hydrophobic phases ($C_{18}$, $C_8$, $C_2$, etc.) are most often used today[9]. In particular, the backflush technique introduced by Roth *et al.*[10] allows the injection of large volumes of plasma without decrease of efficiency of the analytical column. As observed by several authors, polar drugs are the most difficult to handle because of low adsorption on the precolumn, which can lead to breakthrough during the purge step[11,12]. Strategies using different packing materials for precolumns and analytical columns to obtain reconcentration on the second column, depending on the polarity of the drug, have been described[12].

During the development of methods for the determination of either isotretinoin (3), tretinoin (4) and their 4-oxo-metabolites (2 and 1, see Fig. 1)[7], or etretinate (7), acitretin (6) and 13-*cis*-acitretin (5)[8], special measures had to be taken to solve the recovery problems of these highly protein bound drugs (>98%[13]). These problems showed that, after the injection of plasma, not only polar drugs are difficult to pre-concentrate using column switching, but also very lipophilic and highly protein bound drugs. This finding may not be new, but was underestimated by the pioneers of this technique[10,14]. Lecaillon *et al.*[12] recommended $C_2$ or even CN material in the precolumn for very lipophilic compounds such as retinoids, although he too had not considered the problem of highly protein bound lipophilic drugs. Several measures to overcome strong protein binding in the extraction process, using off-line column techniques, have been described in the literature and comprise dilution[15], change of pH, addition of buffers or salts, addition of ion-pairing agents, heating[16], addition of displacers or change of the chemical structure of the protein or the drug[15]. This paper describes a systematic investigation of some of these measures on the recovery of the retinoid drugs and metabolites 1–8.

## 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). Ethanol (HPLC grade) and sodium hydroxide (Titrisol) were obtained from E. Merck (Darmstadt, F.R.G.) and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Water was distilled twice from an all glass apparatus. Argon and helium were obtained from Pan Gas (Lucerne, Switzerland). Substances 1–8 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 dilution or deproteination of the samples were performed in a darkened room. Stock solutions of compounds 1–4 were prepared in amberized volumetric flasks by dissolving 10 mg in 10 ml of methanol. Stock solutions of compounds 5–8 were prepared in the same manner by dissolving 10 mg in 1 ml of tetrahydrofuran and making up to 10 ml with 2-propanol. A 0.1-ml volume of each stock solution was taken and diluted in ethanol to 10 ml. This solution was diluted 1:100 in blank plasma, yielding a plasma standard containing 100 ng/ml each of compounds 1–8.

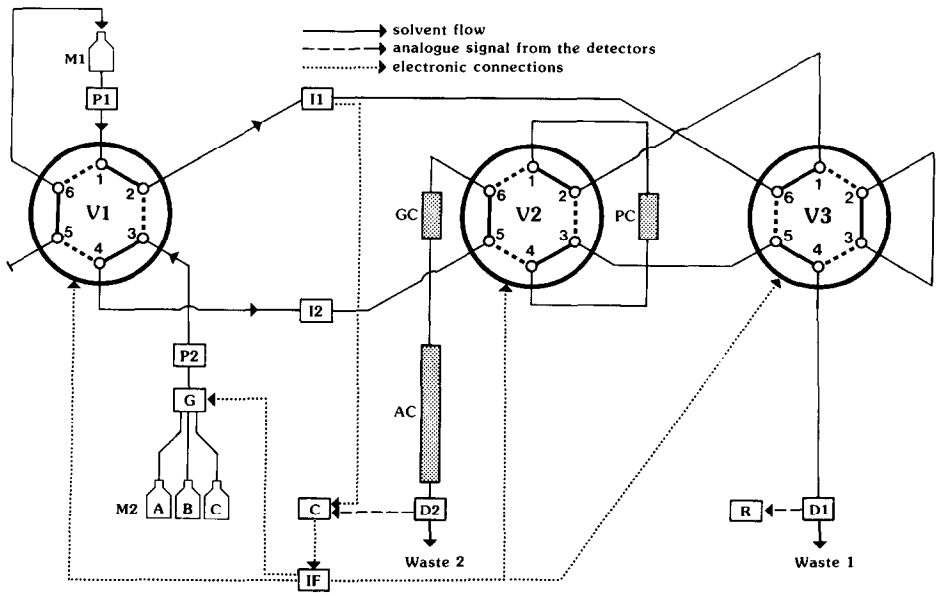


Fig. 2. Schematic representation of the column switching system. The valves V1–V3 are shown in position 0 (see text for further details).

*Chromatographic system*

The column switching system was similar to that already described[7,8], consisting, in addition, of a third valve for purging the precolumn in the forwardflush and the backflush mode. A schematic representation is given in Fig. 2.

Pump P1 [Model 414 (Kontron, Zurich, Switzerland) with pulse damper (Orlita, Giessen, F.R.G.)] delivered mobile phase M1, which was used as the purge solvent at a flow-rate of 1.5 ml/min. Aliquots (0.5 ml) 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 sample volumes larger 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. For heating of the precolumn, an HPLC column block heater (Model 7930; Jones Chromatography, Hengoed, U.K.) was used. The UV detector (D1) (Spectroflow 773; Kratos, Westwood, NJ, U.S.A.), operating at 240 nm, together with a W + W recorder 320 (R) (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 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 an UV detector (D2) (Spectroflow 783, Kratos; rise time 1 s, range 0.02 a.u.f.s.) and integration was performed by means of a 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).

The gradient former (G) and the three air-actuated switching valves (V1–V3) (Model 7000A, Rheodyne; shown in position 0 in Fig. 2), the latter connected to three solenoid valves (Model 7163, Rheodyne), were controlled by the external time events of the computing integrator C. To achieve compatibility, a laboratory-made interface (IF) was placed between the integrator output and the solenoid valve input.

*Columns and mobile phases*

The precolumn (PC) (17 or 40 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 (AC) consisted of two columns (125 mm × 4 mm I.D.; Hibar type; Merck), linked by a sleeve-nut (Merck) and packed with Spherisorb ODS-1, 5 μm (Phase Separations, Queensferry, U.K.). The guard column (GC) (30 mm × 4 mm I.D.; Merck) contained the same material and was also linked to the analytical column using another sleeve-nut. All columns were packed by a slurry technique. To obtain higher plate numbers, two linked columns, each 125 mm long, were preferred to a single 250-mm column.

Five different variants for mobile phase 1 were used: (a) water, (b) 1% ammonium acetate, (c) 1% ammonium acetate–acetonitrile (9:1, v/v), (d) 1% ammonium acetate and acetic acid–acetonitrile (9:1, v/v) and (e) 1% ammonium

acetate and acetic acid–acetonitrile (8:2, v/v). Mobile phase 2 consisted of three components: (A) 400 ml of 0.1% ammonium acetate, 600 ml of acetonitrile and 30 ml of acetic acid; (B) 150 ml of 0.27% ammonium acetate, 850 ml of acetonitrile and 10 ml of acetic acid; (C) 10 ml of water, 980 ml of acetonitrile and 10 ml of acetic acid. All mobile phases were degassed with helium prior to use.

*Analytical procedure*

Plasma (0.5 ml) was either injected directly or diluted in 0.75 ml of 7.2 m*M* sodium hydroxide or 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v), or deproteinated by adding 1 ml of ethanol or 2-propanol, then left to stand for 15 min in a refrigerator at 4°C. After centrifugation (5 min at 1800 *g*), 0.8 ml of the supernatant were transferred to the autosampler vial (microtubes 3810; Eppendorf Gerätebau, Hamburg, F.R.G.), and 0.5 ml were injected. The samples were kept at 10°C in the autosampler before injection.

The complete sequence of automated sample analysis took 44 min and included the following five steps.

*Step A (0–7 min, V1 = 0, V2 = 0, V3 = 0).* Injection of the sample onto PC. Polar components were washed out to waste 1. GC and AC were equilibrated with M2 (100% A).

*Step B (7–10 min, V1 = 0, V2 = 0, V3 = 1).* PC was purged in the backflush mode by M1.

*Step C (10–37 min, V1 = 0, V2 = 1, V3 = 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–30% B (10–16 min), 70% A–30% B to 100% B (16–21 min), 100% B (21–32 min), 100% B to 100% C (32–32.1 min) and 100% C (32.1–37 min).

*Step D (37–38 min, V1 = 1, V2 = 1, V3 = 1; 38–39.9 min, V1 = 1, V2 = 0, V3 = 0).* While M1 was running in a recycling mode, the capillaries between I1 and D1 were purged with M2 (100% C) to prevent any memory effects during the next injection. There was no flow through GC and AC during this period.

*Step E (39.9–44 min, V1 = 0, V2 = 0, V3 = 0).* M2 was changed from 100% C to 100% A in 0.2 min, and GC/AC and PC were re-equilibrated with M2 and M1, respectively.

*Determination of recoveries*

Spiked plasma (100 ng/ml of compounds 1–8), treated in different ways as described below, was injected and analysed using five different variants of mobile phase 1. The percentage recoveries were determined by comparing the mean of the peak heights of replicate analyses under the same conditions ($n$ = 2–4) with the mean of the peak heights of compounds 1–8 in 50% ethanol, injected directly onto GC/AC (using I2). A 200-$\mu$l volume of 50% ethanol, containing the same amount of substance, was injected, representing the 100% reference value.

## RESULTS AND DISCUSSION

*Column-switching system*

In principle, one switching valve is sufficient for direct injection of plasma onto

a precolumn and subsequent separation on an analytical column[14]. However, in the two methods developed for the determination of compounds 1–4 and 5–7[7,8], two valves have been used, enabling the purge of the steel capillaries between the automatic sample injector and detector 1. The installation of a third valve allows forward- and backflush purging of the precolumn[17]. Proteins and solid particles, which would be partly adsorbed on the sieves on the top of the precolumn, are transferred to waste instead of to the analytical column. This technique proved to be very effective after the injection of tissue samples, which were homogenized with ethanol–water and directly injected after centrifugation. Many more samples could be injected than when using forwardflush purging only[18]. However, more experience is needed to assess this technique regarding its general application, especially with more polar compounds.

*Variation of injection solution composition and mobile phase 1*

Variation of the injection solution composition and mobile phase 1 appeared to have the greatest influence on the recoveries of compounds 1–8 from plasma in preliminary experiments. In the first investigation, five different mobile phases 1 were

TABLE I

INFLUENCE OF THE COMPOSITION OF THE INJECTION SOLUTION AND MOBILE PHASE 1 ON THE RECOVERIES OF COMPOUNDS 1–8

| *Injection solution (0.5 ml)* | *Mobile phase 1* | *% Recovery of 100 ng/ml plasma standards* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| Plasma | Water | 88 | 86 | 50 | 40 | 22 | 57 | 2 | 0 |
| | 1% $CH_3COONH_4$ | 100 | 100 | 59 | 42 | 29 | 62 | 2 | 1 |
| | 1% $CH_3COONH_4$–$CH_3CN$ (9:1) | 97 | 94 | 62 | 47 | 56 | 66 | 3 | 1 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (9:1) | 97 | 93 | 44 | 25 | 62 | 50 | 4 | 2 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (8:2) | 93 | 92 | 56 | 38 | 75 | 65 | 11 | 6 |
| Plasma (2 vol.) + 7.2 m*M* NaOH (3 vol.) | Water | 81 | 81 | 51 | 42 | 7 | 57 | 4 | 0 |
| | 1% $CH_3COONH_4$ | 100 | 100 | 63 | 48 | 13 | 65 | 3 | 0 |
| | 1% $CH_3COONH_4$–$CH_3CN$ (9:1) | 95 | 93 | 66 | 54 | 35 | 70 | 5 | 2 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (9:1) | 94 | 90 | 47 | 28 | 62 | 53 | 5 | 3 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (8:2) (8:2) | 88 | 88 | 57 | 39 | 72 | 67 | 12 | 7 |
| Plasma (2 vol.) + 9 m*M* NaOH–$CH_3CN$ (8:2) (3 vol.) | Water | 91 | 89 | 82 | 84 | 76 | 91 | 11 | 0 |
| | 1% $CH_3COONH_4$ | 100 | 100 | 91 | 88 | 93 | 96 | 12 | 4 |
| | 1% $CH_3COONH_4$–$CH_3CN$ (9:1) | 97 | 96 | 91 | 88 | 93 | 92 | 15 | 5 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (9:1) | 94 | 95 | 83 | 71 | 88 | 86 | 17 | 6 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (8:2) | 86 | 89 | 86 | 78 | 89 | 90 | 25 | 13 |
| Plasma (1 vol.) + ethanol (2 vol.) | Water | 26 | 24 | 50 | 53 | 40 | 41 | 57 | 58 |
| | 1% $CH_3COONH_4$ | 34 | 32 | 53 | 55 | 42 | 43 | 70 | 62 |
| | 1% $CH_3COONH_4$–$CH_3CN$ (9:1) | 23 | 22 | 41 | 43 | 35 | 36 | 66 | 59 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (9:1) | 35 | 35 | 77 | 81 | 66 | 71 | 73 | 68 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (8:2) | 32 | 31 | 76 | 81 | 62 | 68 | 71 | 63 |
| Plasma (1 vol.) + 2-propanol (2 vol.) | Water | 18 | 16 | 26 | 27 | 24 | 24 | 58 | 66 |
| | 1% $CH_3COONH_4$ | 24 | 23 | 25 | 26 | 24 | 24 | 76 | 78 |
| | 1% $CH_3COONH_4$–$CH_3CN$ (9:1) | 17 | 16 | 25 | 25 | 24 | 24 | 61 | 64 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (9:1) | 26 | 25 | 40 | 45 | 34 | 38 | 65 | 71 |
| | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (8:2) | 22 | 21 | 39 | 42 | 34 | 36 | 55 | 59 |

compared by replicate injection of five different injection solutions. The results are compiled in Table I.

The simplest approach, injection of undiluted plasma and use of water as M1, was tried first when developing a method for compounds 1–4[7]. Whereas the recovery was sufficient for 1 and 2 (>80%), only 50% could be obtained for 3 and 40% for 4. For compounds 5–8, the recoveries were even lower, as shown in Table I. Improved recoveries for oxazepam metabolites were reported by Roth and Beschke[11], when 1% ammonium acetate was used as M1. This was also observed in this investigation, but the results were still only acceptable for compounds 1 and 2. Dilution of the plasma sample (0.2 ml) with 7.2 m*M* sodium hydroxide (0.3 ml) did not improve the recoveries significantly. Sodium hydroxide was used instead of water, because a basic pH (8–9, depending on the plasma sample) improved the stability of the samples in the autosampler during overnight injection. The big difference between compounds 5 and 6 (7 and 57% recovery using water, 13 and 65% recovery using 1% ammonium acetate as M1, respectively) is surprising, and may be explained by different protein binding of the *cis* and *trans* isomers of acitretin.

The addition of acetonitrile (or an alcohol) to the injection solution resulted in a significant improvement, as observed by other authors[17,19,20]. However, the maximum amount was limited to 30%, otherwise protein precipitation occurred. Good recoveries for compounds 1–6 were obtained, when 0.2 ml of plasma were diluted in 0.3 ml of 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v). Addition of sodium hydroxide was preferred to a buffer such as sodium acetate, since use of the latter

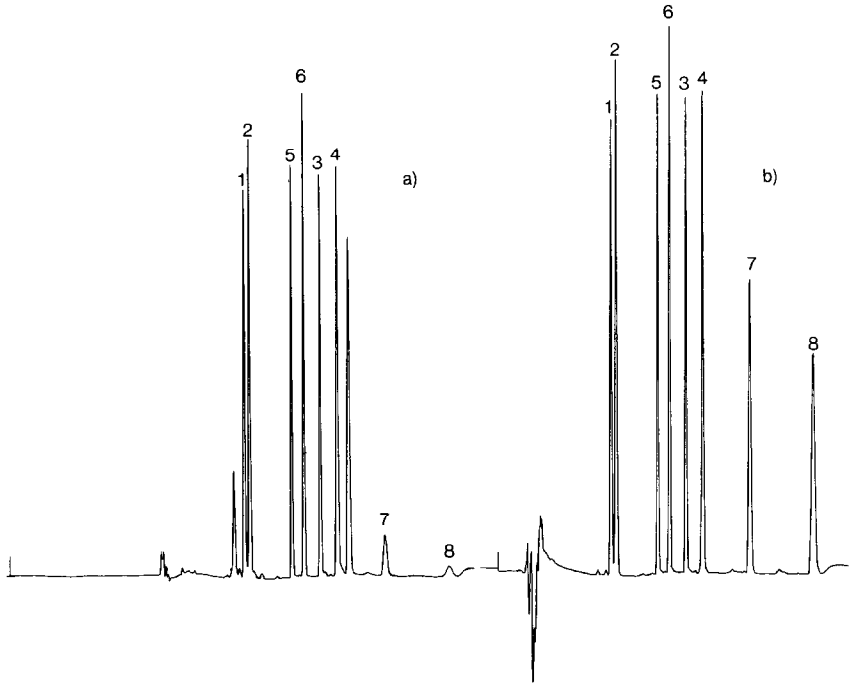


Fig. 3. Chromatograms of compounds 1–8. (a) Plasma standard 100 ng/ml; (b) 100% reference value in 50% ethanol, directly injected onto the analytical column.

resulted in protein precipitation when the autosampler was cooled to 10°C during overnight injection. This injection solution and 1% ammonium acetate–acetonitrile (9:1, v/v) as M1 were the conditions used in a method developed for the determination of compounds 1–4[7]. Compounds 5 and 6 could also be determined under these conditions; the latter served as an internal standard in the above mentioned method[7]. However, when 7 and 8 were injected, the recoveries were still very low (15 and 5%, respectively). Chromatograms of a plasma standard containing compounds 1–8, injected under these conditions, as well as the corresponding 100% reference value are shown in Fig. 3. Addition of acetic acid, and increasing the acetonitrile content of M1 to 20%, resulted in a improvement, which was however still insufficient.

The recoveries of compounds 7 and 8 were significantly improved only when plasma proteins were precipitated with an organic solvent before injection. However, this was not an ideal solution for all eight compounds, as the more polar 1–4 were already partly eluted from the precolumn because of the relatively high content of organic solvent in the injection solution. Ammonium acetate (1%) and acetic acid–acetonitrile (8:2, v/v) as M1 and protein precipitation with ethanol were the conditions chosen for the determination of compounds 5–7 in plasma (using 3 and 8 as internal standards)[8]. Protein precipitation with 2-propanol (see Table I) or methanol (data not shown) did not further improve the recoveries of the compounds investigated.

*Variation of precolumn length and temperature*

Neither decreasing the flow-rate nor the use of porous ODS materials in the precolumn, as observed by several authors[21,22], had any effect on the recoveries of compounds 1–8. At first, we used a 5 mm long cartridge, packed with $C_{18}$ Nucleosil, 30 μm. Longer precolumns (10 or 20 mm) improved the recovery. However, $C_{18}$ Corasil, 37–53 μm (in a 17 mm long precolumn), gave the same results as $C_{18}$ Nucleosil regarding the recovery, but resulted in less clogging problems, and was therefore chosen for further investigations. In view of the very difficult recovery problems with compound 7 (and 8), a 40 mm long precolumn (packed with $C_{18}$ Corasil) as well as heating of the precolumn was tried, as described for lipoprotein bound steroids, which are known to be difficult to recover[16]. The results of these investigations are summarized in Table II.

The 40-mm column, used at room temperature, did not significantly improve the recoveries of compounds 1–8. Heating of the 17-mm precolumn gave positive results above 60°C only. At 80°C, recoveries from diluted plasma were improved, but the effect was less distinct than with protein precipitation. This latter measure in combination with heating resulted in no further improvement. This is reasonable, because the separation of the drug from plasma proteins has already been effected before injection and heating. Heating of the precolumn to 80°C together with the injection of sodium hydroxide containing solutions resulted in deterioration of the analytical column after only a few injections, probably due to degradation of the silica gel, and was, therefore, not suitable for routine determinations.

*Possible explanation of the recovery problems*

The retinoids investigated are very lipophilic drugs, with decreasing polarity from compound 1 to 8. When injected in an aqueous solution into a chromatographic

TABLE II

INFLUENCE OF THE PRECOLUMN LENGTH AND TEMPERATURE ON THE RECOVERIES OF COMPOUNDS 1–8

| *Injection solution (0.5 ml)* | *Mobile phase 1* | *Column* | | *% Recovery of 100 ng/ml plasma standards* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Length (mm)* | *Temperature (°C)* | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| Plasma | Water | 17 | 22 | 88 | 86 | 50 | 40 | 22 | 57 | 2 | 0 |
| | | 40 | 22 | 81 | 78 | 55 | 42 | 24 | 62 | 2 | 1 |
| | | 17 | 80 | 82 | 79 | 76 | 69 | 29 | 77 | 4 | 2 |
| Plasma (2 vol.) + 9 m*M* $NaOH$–$CH_3CN$ (8:2) (3 vol.) | 1% $CH_3COONH_4$–$CH_3CN$ (9:1) | 17 | 22 | 97 | 96 | 91 | 88 | 93 | 92 | 15 | 5 |
| | | 40 | 22 | 84 | 85 | 87 | 84 | 88 | 90 | 17 | 4 |
| | | 17 | 80 | 81 | 87 | 79 | 89 | 83 | 87 | 36 | 18 |
| Plasma (1 vol.) + ethanol (2 vol.) | 1% $CH_3COONH_4$/$CH_3COOH$–$CH_3CN$ (8:2) | 17 | 22 | 32 | 31 | 76 | 81 | 62 | 68 | 71 | 63 |
| | | 40 | 22 | 32 | 33 | 71 | 72 | 62 | 68 | 78 | 70 |
| | | 17 | 80 | 25 | 25 | 62 | 71 | 45 | 51 | 68 | 61 |

system consisting of an aqueous mobile phase and a $C_{18}$ column, the highest adsorption is expected for 8, and the lowest for 1 and 2. In the experiments described the opposite was observed. The greater the lipophilicity of the drug, the lower was the adsorption on the precolumn. This can be explained only by the strength of the binding of the drug to plasma proteins, or the difficulty of lipophilic drug transfer from protein to hydrophobic stationary phase through an hydrophilic mobile phase.

Retinoid drugs are known to be almost completely bound to plasma proteins (99.9 and >98% for compounds 3 and 7, respectively[13]). However, it seems to be not only the extent, but also the strength of protein binding which may be responsible for the observed recovery problems. Retinol is transported in blood bound to retinol binding protein (RBP)[23]. Retinoic acids (compounds 3 and 4) and acitretin (6) are bound to albumin, whereas etretinate (7) is bound to lipoproteins[13]. It is well known that lipoprotein bound drugs are difficult to extract with column techniques[16]. However, it appears that so far these types of drugs have not been analysed using on-line solid-phase extraction with automated column switching.

## CONCLUSION

During method development for the determination of compounds 1–4 and 5–7 in plasma using HPLC with column switching, recovery problems arose, which may probably be correlated to binding to different plasma proteins. Injection of undiluted plasma samples and the use of a purge mobile phase of water or 1% ammonium acetate, the "classical" approach[10,11,14], resulted in very low recoveries for most of the retinoids investigated. Recovery problems were overcome by dilution of plasma in 9 m*M* sodium hydroxide–acetonitrile (8:2, v/v) and protein precipitation with ethanol for compounds 1–4 and 5–7, respectively, and use of a purge mobile phase containing ammonium acetate and 10 or 20% acetonitrile.

## ACKNOWLEDGEMENTS

The authors thank Mr. H. Suter for the drawings, Drs. D. Dell and J. Burckhardt for correction and Mrs. Th. Itin for typing of the manuscript.

## REFERENCES

1 M. B. Sporn and A. B. Roberts, in J. Nugent and S. Clark (Editors), *Retinoids, Differentiation and Disease*, Ciba Foundation Symposium 113, Pitman, London, 1985, p. 1.
2 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.
3 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, Berlin, 1981, p. 7.
4 W. Bollag and A. Hanck, *Acta Vitamin. Enzymol.*, 31 (1977) 113.
5 C. W. Ehmann and J. J. Vorrhees, *J. Am. Acad. Derm.*, 6 (1982) 692.
6 W. Bollag, in J. H. Saurat (Editor), *Retinoids: New Trends in Research and Therapy*, Karger, Basle, 1985, p. 274.
7 R. Wyss and F. Bucheli, *J. Chromatogr.*, 424 (1988) 303.
8 R. Wyss and F. Bucheli, *J. Chromatogr.*, 431 (1988) 297.
9 D. Westerlund, *Chromatographia*, 24 (1987) 155.
10 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F. W. Koss, *J. Chromatogr.*, 222 (1981) 13.
11 W. Roth and K. Beschke, *J. Pharm. Biomed. Anal.*, 2 (1984) 289.

12 J.-B. Lecaillon, N. Febvre and C. Souppart, *J. Chromatogr.*, 317 (1984) 493.
13 R. W. Lucek and W. A. Colburn, *Clin. Pharmacokin.*, 10 (1985) 38.
14 W. Voelter, T. Kronbach, K. Zech and R. Huber, *J. Chromatogr.*, 239 (1982) 475.
15 K.-G. Wahlund and T. Arvidsson, *J. Chromatogr.*, 282 (1983) 527.
16 J. Sjövall and M. Axelson, *J. Pharm. Biomed. Anal.*, 2 (1984) 265.
17 U. Timm, G. Hopfgartner and R. Erdin, *J. Chromatogr.*, 456 (1988) 21.
18 R. Wyss and F. Bucheli, unpublished results.
19 H. Nielsen, *J. Chromatogr.*, 381 (1986) 63.
20 W. S. Powell, *Anal. Biochem.*, 164 (1987) 117.
21 A. Nazareth, L. Jaramillo, B. L. Karger, R. W. Giese and L. R. Snyder, *J. Chromatogr.*, 309 (1984) 357.
22 U. Juergens, *J. Chromatogr.*, 310 (1984) 97.
23 D. S. Goodman, in M. B. Sporn, A. B. Roberts and D. S. Goodman (Editors), *The Retinoids*, Vol. 2, Academic Press, New York, 1984, p. 41.