

Quantitative high-performance liquid chromatographic determination of retinoids in human serum using on-line solid-phase extraction and column switching

Determination of 9-*cis*-retinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinoic acid, 4-oxo-all-*trans*-retinoic acid and 4-oxo-13-*cis*-retinoic acid

Th.E. Gundersen^a, E. Lundanes^{a,*}, R. Blomhoff^b

^aDepartment of Chemistry, University of Oslo, P.O. Box 1033 Blindern, N-0315 Oslo, Norway

^bInstitute for Nutrition Research, University of Oslo, P.O. Box 1046 Blindern, N-0316 Oslo, Norway

Received 20 December 1995; revised 27 August 1996; accepted 10 September 1996

Abstract

A fully automated isocratic high-performance liquid chromatographic method for the determination of 9-*cis*-retinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinoic acid, 4-oxo-13-*cis*-retinoic acid and 4-oxo-all-*trans*-retinoic acid, has been developed using on-line solid-phase extraction and a column switching technique allowing clean-up and pre-concentration in a single step. A 500- μ l sample of serum was diluted with 750 μ l of a solution containing 20% acetonitrile and the internal standard 9,10-dimethylanthracene. About 1000 μ l of this mixture was injected on a 20 \times 4.6 mm I.D. poly ether ether ketone (PEEK) pre-column with titanium frits packed with Bondapak C₁₈, 37–53 μ m, 300 Å particles. Proteins and very polar compounds were washed out to waste, from the pre-column, with 0.05% trifluoroacetic acid (TFA)–acetonitrile (8.5:1.5, v/v). More than 200 aliquots of diluted serum could be injected on this pre-column before elevated back-pressure enforces replacement. Components retained on the pre-column were backflushed to the analytical column for separation and detection at 360 nm. Baseline separation was achieved using a single 250 \times 4.6 mm I.D. Suplex pKb-100 column and a mobile phase containing 69:10:2:16:3 (v/v) of acetonitrile–methanol–*n*-butanol–2% ammonium acetate–glacial acetic acid. A total time of analysis of less than 30 min, including sample preparation, was achieved. Recoveries were in the range of 79–86%. The limit of detection was 1–7 ng/ml serum and the precision, in the concentration range 20–1000 ng/ml, was between 1.3 and 4.5% for all five compounds. The method was applied for the analysis of human serum after oral administration of 60 mg Roaccutan. The method is well suited for pharmacological studies, while the endogenous levels of some retinoic acid isomers are below the limit of quantitation.

Keywords: Retinoids; Retinoic acid; Oxoretinoic acid

1. Introduction

It is well known that the retinol (ROH) (vitamin

*Corresponding author.

A) metabolite 11-*cis*-retinal covalently bound to opsin proteins is the chromophore in the visual process. Early research also showed that vitamin A deficiency and vitamin A excess dramatically change the differentiation of epithelial cells. Today, vitamin A is known to be involved in the regulation of proliferation and differentiation of many cell types during fetal development as well as for most cells throughout life [1,2]. The extravisual mechanism of action of vitamin A was unknown until late 1987 when researchers discovered nuclear retinoic acid (RA) receptors in the cell nucleus [3,4]. These receptors (called RAR α , RAR β , RAR γ , RXR α , RXR β and RXR γ) are turned on to various degrees by retinoic acid isomers and metabolites such as all-*trans*-retinoic acid, 9-*cis*-retinoic acid, all-*trans*-3,4-didehydro-retinoic acid and all-*trans*-4-*oxo*-retinoic acid [3–5]. The holo receptor complex regulates gene expression by binding to short DNA sequences in the vicinity of target genes. In addition, many other retinoids, such as 13-*cis*-retinoic acid, the retinoyl glucuronides, 4-*oxo* and 4-hydroxy-retinoic acid, do seem to be important as intermediate products in retinoid catabolism [6,7]. However, at present we have little insight into the actual pathways by which the different retinoids are produced and degraded *in vivo*, and the actual concentrations of the active retinoids in cells and extracellular fluids during different conditions. Studies dealing with these questions have been hampered since the available analytical methods have been characterized by poor sensitivity and selectivity.

Many procedures have been devised for measuring the quantities of naturally occurring and synthetic retinoids in biological samples. Among the many analytical methods for determination of retinoids which have been developed within the last years, nearly all involve reversed-phase high-performance liquid chromatography (HPLC) [8–16].

Naturally occurring retinoic acids are present at the sub nanogram/g level [1,2]. In determinations of trace amounts of analytes in complex matrices like serum by high-performance liquid chromatography with UV detection, two main types of problems are encountered. Firstly, if the analyte concentration is too low, enrichment is necessary to improve detection limits. This step usually involves cumbersome and time-consuming liquid–liquid extractions

in darkened environments. Secondly, removal of proteins and macromolecular constituents are necessary to avoid clogging and deterioration of the analytical column (AC) [16].

An elegant and time-saving approach to these problems is the use of on-line solid-phase extraction (SPE) and column switching [17–23]. The technique allows trace enrichment and clean-up in a single step. Minimal loss due to light induced isomerisation, air exposure and adsorption to glassware during pre-treatment, is achieved. This makes the technique especially suited for retinoids because of their photo- and thermo-sensitive nature [23]. The main obstacle to overcome is the high degree and very strong protein binding of the retinoids (99.9%) [24]. This problem can to some extent be solved by diluting the sample with an organic modifier prior to injection [25–27]. Sufficiently high and consistent recoveries of all the RAs are difficult to achieve under only one set of conditions, and gradient elution is often necessary to obtain adequate resolution of the different types of isomers simultaneously [23].

In order to achieve automation a sufficiently large number of injections must be possible. Denaturation and adsorption of proteins, when frits and other steel parts come in contact with the biological sample, will shorten the pre-column's life, and the number of injections will therefore mainly be limited by clogging of the pre-column (PC) [16,18,28,29]. In addition, low recoveries of analytes due to breakthrough on the pre-column caused by poor mass transfer of the drug from the protein to the stationary phase together with adsorption of the retinoids to steel components in the system, is often a major problem [19,23].

In 1992, Wyss and Bucheli introduced an automated gradient HPLC method for the determination of all-*trans*-RA and 13-*cis*-RA, and their 4-*oxo* metabolites [21]. The column switching technique combines a clean-up and pre-concentration step with a separation step. To our knowledge, an isocratic separation of the *cis/trans* isomers of RA and its 4-*oxo* metabolites in the same analysis has not yet been performed. This statement is supported by the unsuccessful attempt to apply this alternative procedure performed by Lefebvre et al. [30] in 1995.

In this paper we present a fully automated isocratic reversed-phase (RP)-HPLC method with on-

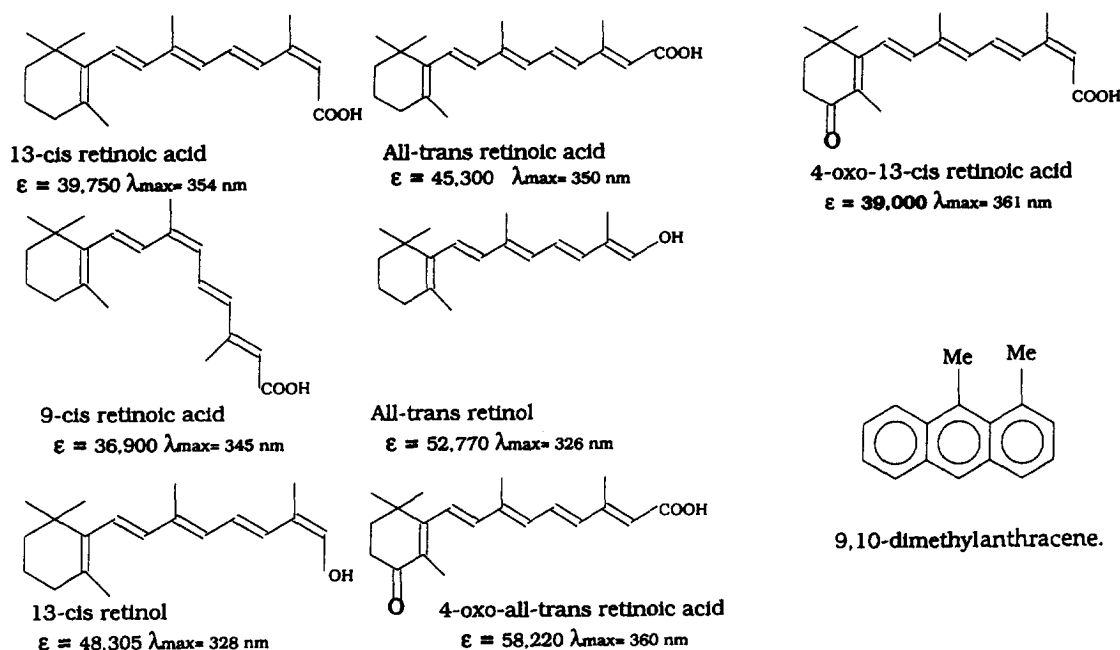


Fig. 1. Chemical structure, ϵ and λ_{\max} (in ethanol) of the retinoids, naturally occurring retinol, its 13-*cis* isomer as well as the structure of the internal standard 9,10-dimethylanthracene.

line solid-phase extraction and column switching, for the quantitative determination of all-*trans*-RA, 13-*cis*-RA and their 4-oxo metabolites together with 9-*cis*-RA (Fig. 1) in serum samples. Since the method should be applicable for analysis of blood samples from children as well, a method based upon a sample size of 500 μ l was chosen.

Furthermore, a substantial reduction in analysis time, compared to a gradient method is achieved, yielding the same signal resolution by isocratic separation. For minimisation of the problems arising in particular from protein precipitation, all crucial connections and fittings are made of titanium or poly ether ether ketone (PEEK).

2. Experimental

2.1. Materials and reagents

Acetonitrile, tetrahydrofuran and methanol of HPLC grade, were obtained from Rathburn (Walkburn, UK). Ethanol, absolute, was obtained from

A/S Vinmonopolet (Oslo, Norway). Acetic acid was purchased from The Norwegian Medicinal Depot (Oslo, Norway), 1-butanol, ammonium acetate, toluene, trifluoroacetic acid, formic acid, 2-propanol, acetone and sodium hydroxide, all p.a., from E. Merck (Darmstadt, Germany). Water was de-ionized and glass distilled. All-*trans*-retinol, 13-*cis*-retinol, all-*trans*-RA, 13-*cis*-RA, sodium dodecyl sulphate (SDS) and dimethyl-dichlorosilane were supplied by Sigma (St. Louis, MO, USA). The 9-*cis* isomer of retinoic acid, 4-oxo-all-*trans*-RA and 4-oxo-13-*cis*-RA, were kindly provided by F. Hoffman-La Roche (Basle, Switzerland). Roaccutan 20 mg, was provided by Roche (Basle, Switzerland). Tritium labelled 9-*cis*-RA and all-*trans*-RA were purchased from Amersham International (Bucks, UK). Helium and argon both of grade 4.8 were obtained from Hydro Gas (Oslo, Norway). The internal standard 9,10-dimethylanthracene was purchased from Tokyo Casei Industry (Tokyo, Japan). Human serum, obtained from a blood bank (Ullevaal Hospital, Oslo, Norway), and all standards were kept under argon at -18°C and protected from light at all times. The

scintillation liquid used for radioisotope measurements was INSTA-GEL II Plus (Packard Instruments, Groningen, Netherlands).

2.2. Preparation of standard solutions

Stock solutions of the retinoids were prepared, under red light, by dissolving 10 mg of all-*trans*-RA and 13-*cis*-RA in 10 ml absolute ethanol. Due to limited availability of the three remaining isomers, stock solutions of each isomer were prepared and their concentrations determined spectrophotometrically using literature ϵ values (Fig. 1). These solutions were combined and further diluted with methanol yielding concentrations in the range 0.2–100 $\mu\text{g/ml}$. An aliquot of 100 μl of these solutions was diluted with 10 ml of blank human serum to give concentrations of 2, 5, 20, 50, 100, 500 and 1000 ng of each retinoid/ml of serum. All solutions were flushed with argon and frozen in amber glass containers at -18°C .

A stock solution (10 $\mu\text{g/ml}$) of the internal standard, 9,10-dimethylanthracene, was prepared by dissolving 1 mg in 5 ml of tetrahydrofuran and diluting to 100 ml with isopropanol. This solution, when stored cold and dark, was stable for at least seven months. Fresh working solutions of 25 ng/ml were made every week by diluting 2.5 ml with 200 ml of acetonitrile, 7.2 ml of 1 M sodium hydroxide

and distilled water to 1000 ml. This solution was used for dilution of the serum samples prior to injection.

Silanisation of the amber glassware was performed with a 5% (w/v) solution of dichloro-dimethylsilane in toluene. Subsequent washing of the treated glassware was performed with a 1:1 (v/v) mixture of methanol–acetone.

2.3. Chromatographic system

All components of the HPLC system were delivered by Shimadzu Europe GmbH (Duisburg, Germany) except the personal computer and the single channel UV detector which was delivered by Compaq Computers (Wilmington, DE, USA) and Waters (Milford, MA, USA), respectively. To accommodate the special requirement of the highly sensitive and fragile retinoids, a bio-compatible system was chosen. A schematic drawing of the HPLC system can be seen in Fig. 2.

For on-line degassing of the mobile phases, a Shimadzu DGU-3A apparatus was used. The two HPLC pumps needed were of the type Shimadzu LC-10AD, including Shimadzu's automatic washing kit for LC-10AD, together with a FCV-10AL low pressure mixer and a bio-compatible mixing chamber. The auto injector, a Shimadzu SIL-10 A apparatus with 2 ml titanium loop (Rheodyne, valve

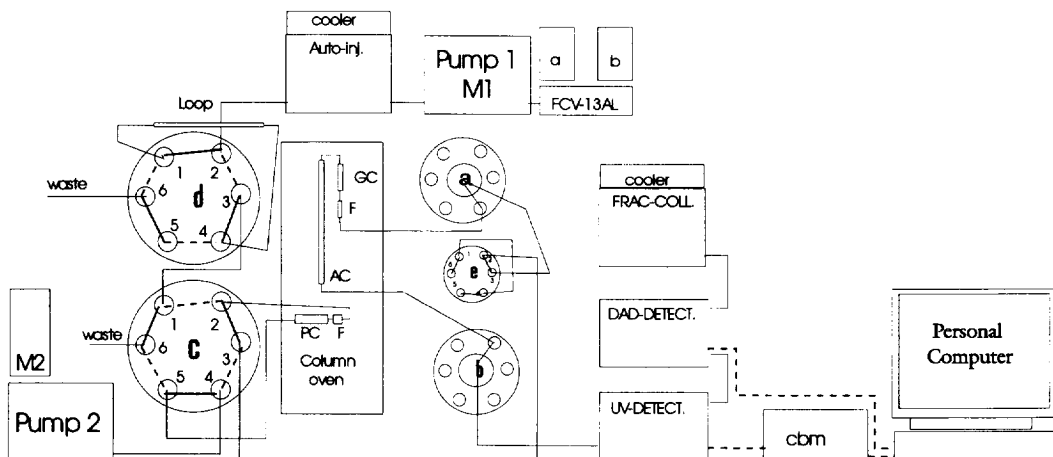


Fig. 2. Schematic representation of the final automatic column switching system operated in the "for-flush" mode. See Section 2.3 for detailed description.

d in Fig. 2), came equipped with a Peltier sample cooler L (Shimadzu) and 3.7 ml, 15 mm O.D. vials were used. In order to achieve compability of 1.5 ml, 12 mm O.D. vials with the rack, sleeves in alumina, were prepared at the institute's workshop. An additional Peltier element was used for cooling of the column oven, Shimadzu CTO-10AC, and the fraction collector, Shimadzu FRC-10A. The set of valves needed for the column switching system were the following: one PEEK Rheodyne injector 7125 (e), two PEEK Rheodyne 9060 flow selection valves (a,b) and one Shimadzu FCV-12AH with a six-port automatically driven Rheodyne valve (c). In addition, a type FCV-13AL reservoir selection valve was used for selecting mobile phase to pump 1. For recovery tests and non-automated HPLC analyses, the manual injection valve (e) was placed in front of the flow selection valve used to select the analytical column.

All sample contacting parts, including tubings, were made of PEEK or titanium.

Detection was performed using a Shimadzu SPD-M10A diode-array detector containing a titanium flow cell and a Waters tuneable single-wavelength UV detector connected in series.

An interface and communication module, Shimadzu CBM-10, was needed for control of the system by the HPLC manager CLASS LC-10 software installed on a Compaq Prolinea MT 4/66. For printout a Tally MT904 plus laser printer (Mannesmann Technology, Workingham, Berkshire, UK) was used. The entire system is controlled by the personal computer with the HPLC software based on Microsoft Windows and MS-DOS.

2.4. Columns and mobile phases

The analytical column (AC) was a Suplex pKb-100 (250×4.6 mm I.D., 5 μm particles) obtained from Supelco (Bellafonte, PA, USA), with a 20×4.6 mm Pelliguard C₈ guard column (GC) (Supelco) in an integrated guard column housing. The 20×4.0 mm I.D. PEEK cartridge with a steel body and 2 μm titanium frits (Valco) used as pre-column, was dry packed with Bondapak dimethyl-octadecylsilyl bulk packing material, 37–53 μm (Waters). A 2 μm PEEK interchangeable filter (Upchurch Scientific) was located in front of both the guard column and the pre-column (F in Fig. 2). In addition, the

following commercial pre-columns were examined: Pelliguard LC-18 20×4.6 mm, Supelguard LC-18 20×4.6 mm, Hisep shielded hydrophobic phase (SHP) 20×4.6 mm (all from Supelco) and a RP-18 30×4.0 mm from Brown Lee Labs.

Mobile phase M1a consisted of 0.05 % trifluoroacetic acid–acetonitrile (85:15, v/v), mobile phase M1b of 100% methanol and mobile phase M2 of acetonitrile–1-butanol–methanol–2% ammonium acetate–glacial acetic acid, (69:2:10:16:3, v/v). A 10% ammonium acetate solution was made up by solving 100 g in 1000 ml distilled water and filtering it through a 0.45-μm filter HA type (Millipore). This solution was further diluted to give the 2% solution used in M2.

The automated injector utilised a liquid in the Teflon tubing between a motorised syringe and the sample needle. This liquid consisted of 40% acetonitrile in distilled water.

2.5. Analytical procedure

A 500-μl serum sample was transferred to a 1.5-ml silanized amber vial, flushed with argon, sealed with a 11-mm Teflon/alumina crimp cap and placed in the cooled sample rack (5°C). Dilution of the sample with 750 μl of the internal standard working solution was performed prior to every new injection by the auto sampler. The two liquids were mixed by aspirating the mixture in and out of the auto sampler needle three times. A sample of 1000 μl was injected on the pre-column (PC) while the start signal to the integrator was delayed for 10 min. The time sequence of the following column-switching procedure is also shown in Table 1. First, the flow of mobile phase M1a was 1 ml/min, which was reduced to 0.5 ml/min after 2 min. The flow of M1a was kept at 0.5 ml/min for 1 min and then increased linearly to 1.5 ml/min in 1 min. The flow of M1a was kept at 1.5 ml/min until a total time of 10 min, from sample injection, had elapsed. The position of switching valve c (Fig. 2) was changed, and M2 introduced to the PC in the back-flush mode transferring the analytes to the analytical column while time monitoring of the signal was simultaneously initialised.

While M2 (1 ml/min) was purging the pre-column in a back-flush mode, the flow of pump 1 was raised to 3 ml/min and the six port selection valve (FCV-

Table 1
Time sequence of column-switching procedure

Time (min)	Valve c	Pump 1	Pump 2 M2 1 ml/min	Comment
0–2	Position 1	M1a 1.0 ml/min	Through AC	
2–3	Position 1	M1a 0.5 ml/min	Through AC	Linear increase
3–4	Position 1	M1a 1.5 ml/min	Through AC	from 0.5 to 1.5 ml/min
4–10	Position 1	M1a 1.5 ml/min	Through AC	
Monitoring of detector signal is executed				
10–13	Position 2	M1b 3.0 ml/min	Through PC and AC	Back-flush PC
13–16	Position 1	M1b 3.0 ml/min	Through AC	Fore-flush PC
16–29	Position 1	M1a 3.0 ml/min	Through AC	PC is cleaned
29–30	Position 1	M1a 1.0 ml/min	Through AC	Conditioning

Pump 1 introduces the sample onto the precolumn. Pump 1 delivers M1a or M1b. Pump 2 always delivers M2, but the position of valve c decides whether the flow is directed only through the analytical column (AC) or through the precolumn (PC) as well. When the latter is the case, the flow direction of M2 on the PC is opposite of M1a.

13AL) was switched to mobile phase M1b consisting of pure methanol. Thus, the flow lines carrying the sample and the auto injector sample loop, were purged with methanol. In order to avoid any memory effects from the sample needle and the Teflon tubing connected to it, 1100 μ l of methanol was taken up from a separate container in the auto injector and dispensed into the washing port. Three min after the analytes have been transferred to the analytical column by M2, valve c was switched to its original position and the methanol (M1b) also cleaned the pre-column. The pump 1 mobile phase selection valve (FCV-13AL) was then, after another 3 min, set to position 1 again and the flow line and pre-column conditioned with M1a. In this way no precipitation took place when the next sample was injected. Shortly before the next injection, the flow of pump 1 was reset to 1 ml/min.

Detection of the retinoids was carried out at 360 nm with a tuneable single channel UV detector. Qualitative information together with peak purity information were provided by the diode array detector. For injections directly onto the analytical column, a manual injector (Rheodyne 9125, e in Fig. 2), placed in front of the AC, was used. (The six-port selection valves a and b can be used for changing between different columns.)

All the integration and calculations were carried out by the CLASS LC-10 software.

2.6. Recovery

Recoveries of the retinoids were examined by spiking human serum samples with known amounts of the different isomers and comparing the resulting peak area with the peak areas obtained by injecting the same amount of retinoids, dissolved in 20 ml ethanol, directly onto the AC using the manual injection valve.

An additional recovery study using the tritium labelled isomers of all-*trans*-RA and 9-*cis*-RA were also performed. Two 7-ml aliquots of serum were each spiked with one of the two radiolabeled retinoids and vortex mixed thoroughly. From both batches twelve samples of 500 μ l were selected. Scintillation liquid was added to six of the samples from each of the two batches and their activity was counted on a Packard 1900 TR liquid scintillation analyser (Packard Instrument, Meriden, CT, USA).

The twelve remaining samples were diluted and analysed by the HPLC system in the manner described earlier (Section 2.5). The eluate from the pre-column was collected for 10 min after sample injection. As the system was changed to back-flush mode, collection at the pre-column outlet was terminated and collection in a different vessel was started for the AC effluent. The eluates were concentrated to a volume of about 500 μ l, 8 ml of scintillation liquid was added and the activity counted. By comparing

the counts (dpm) before and after the chromatographic separation, recovery for the two isomers was established.

In both experiments the recovery was defined as:

$$\text{Recovery(\%)} = \frac{\text{measured amount} \times 100\%}{\text{added amount} + \text{amount in sample}}$$

2.7. Quantitative determinations

Replicates of six different concentrations [2 ($n=6$), 5 ($n=1$), 20 ($n=6$), 100 ($n=1$), 500 ($n=6$), 1000 ($n=1$) ng/ml] of the retinoids with the internal standard added were prepared and analysed in the manner described above (Section 2.5). One determination of each of the concentrations was selected by random, and loaded into the Class LC-10 software as demanded by the calibration program. The program only allows for one replicate of y for each x . The ratios of the peak height of the retinoids and the internal standard were plotted against the concentration of the retinoids. The concentration of the analytes were determined by interpolation obtained by linear least-squares regression. Because of the severe precautions that have to be taken to obtain adequate accuracy when preparing the standards and the poor availability of standard retinoids, it was decided to use the same calibration curve until the daily control showed significant deviation from the initial value.

This control sample was from a 250 ml batch of serum, stored at -80°C in darkness. When a new batch of the control sample had to be made or significant deviation from the initial concentrations (20 ng/ml) were found, a new standard curve was established. Calculations were automatically done by the software and the results printed out as concentrations in the report together with other desired parameters. Additional calculations were performed in Microsoft Excel 5.0.

2.8. Application of the method

After oral administration of 60 mg Roaccutan, blood samples were collected and allowed to clot in the dark. Serum was isolated by centrifugation and 500 μl was transferred to an amber glass vial flushed

with argon and loaded into the cooled automatic injector. The samples were analyzed as described above (Section 2.5).

3. Results and discussion

3.1. Clean-up and pre-concentration

Extractions of retinoids by use of the SPE techniques have been known to give low recoveries which are most probably caused by their highly protein-binding nature and their poor solubility in aqueous media [19,20]. The normal approach to overcome the low recoveries associated with protein-bound drugs is to precipitate the proteins. Precipitation of proteins in serum is normally performed with an alcohol. This will liberate the drug, but care must be taken to avoid coprecipitation of the drug. When a highly lipophilic drug is liberated from its carrier protein it must have the possibility to be completely extracted into an organic solvent. Otherwise, adsorption onto the vial surface, and as a consequence losses might be the result. The use of a solution with a high percentage of organic modifier on a RP based pre-column, will however increase the risk of breakthrough of the analyte during the extraction step.

Another approach to liberate a drug from proteins in serum is to add, in high concentration, a substance having a mutual binding site with the drug in question. However, the search for an efficient displacer for a drug from its binding site on a protein is very difficult. In many applications addition of trichloro acetic acid (TCA) is a successful approach [31], but had no significant effect in this study. The use of SDS in the injection solution, however, did have a large effect on the recovery of 9-*cis*-RA and the recovery increased from 22 to 100% on the commercial C_{18} pre-columns. On the other hand, all-*trans*- and 13-*cis*-RA isomers revealed a slight reduction in recovery. An explanation for this observation could be that SDS function as a selective displacer for 9-*cis*-RA or that it releases all three isomers and the losses in the yield of all-*trans*- and 13-*cis*-RA are attributable to a different mechanism. Retinoic acid does form micelles [23] which are

rather similar to those formed by SDS. The 9-*cis* isomer of RA has an alkene chain being present in a structural arrangement by which the distance between the hydrophilic and hydrophobic end is shortened compared with all-*trans*- and 13-*cis*-RA. For this reason it is probable that the all-*trans* and the 13-*cis* isomer will be able to form micelles with SDS while 9-*cis* will not. If this is the case, the loss of all-*trans* and 13-*cis* could be explained by breakthrough, on the pre-column, of all-*trans* and 13-*cis*-RA incorporated in SDS micelles. The use of SDS in an RP column-switching system will unfortunately result in a concentration of SDS on the pre-column with subsequent transfer to the analytical column in the back-flush step. This will lead to deterioration of the column and loss of efficiency. Therefore, this approach was not further investigated. A time saving alternative procedure would be the direct injection of serum on the analytical column. However, since retinoic acid is present in the sub nanogram/g level in biological materials [8], fairly large samples would have to be injected if detection with UV should be achievable. However, this would cause a severe increase in back pressure or clogging of the column after only few injections [16] and thus was not applied.

3.1.1. Choice of SPE material

The pre-column must fulfil the requirements of both an efficient extraction yield and negligible interactions towards unwanted components, such as proteins and other highly polar sample constituents [14].

The results from the preliminary tests for choice of an appropriate pre-column, performed with four commercially available columns, showed that the column based on the restricted access media (RAM) principle was not very well suited for extraction of the retinoids. The small particle and pore size made it impossible to inject more than 100 μl of serum without causing total clogging of the column, but most importantly and surprisingly the recoveries obtained on this column were very low. When using different additional measures to weaken the protein binding, like addition of TCA, SDS or an organic modifier, recoveries of approximately only 40% at most could be obtained (data not shown). The low recoveries with this type of column material can

most likely be explained by the restricted contact to the hydrophobic stationary phase.

Ordinary C_{18} material seemed to be the best choice. Using particles with 300 \AA pore size gave somewhat better recovery than 100 \AA pores and the maximum loading capacity was much higher on the material with the larger particle size; typically 500 μl for the packing with 5 μm particle size and 2000 μl or more with the 45 μm particle size. Despite the fact that some of the column materials gave adequate recovery for virtually all of the isomers, none of these commercial pre-columns gave satisfactory recovery for the 9-*cis* isomer. A remarkable increase in recovery for the 9-*cis* isomer was observed when an injection solution containing 50 mM SDS was used as discussed above.

All the commercial columns were equipped with steel bodies and steel frits. These steel components were suspected to be the cause of observed memory effects (data not shown). Since none of the tested materials gave satisfactory results, a column housing for self-packing was purchased. Guided by the recovery results on the commercial columns, we decided to use a 20 \times 4.6 mm PEEK column packed with a C_{18} material having 30–50 μm particle size and 300 \AA pores. This column gave the same high recovery for the 13-*cis* and all-*trans* isomers as the commercially available columns. In addition, high recoveries were also obtained for the 9-*cis* isomer. The column had titanium frits and gave one-fifth the memory effects of the columns with the steel frits (measured by injecting 1000 μl absolute ethanol after the injection of a standard solution, results not shown). The reason for this is probably attributable to titanium's properties as a harder acid than iron and therefore less susceptible to complex with the π -electrons in the polyene chain of the retinoids, which can be regarded as typical soft bases [32].

A pre-column showing high recoveries even with 2000 μl of diluted serum allows injection of the whole amount of sample. However, the injection volume is limited by the dead volume in the injector, the shape of the container bottom and the excess volume taken up by the automatic injector. Nevertheless, with the available amount of 500 μl of serum, flat bottom amber vials and 100 μl excess volume, the final injection volume was 1000 μl .

3.1.2. Effect of organic modifier and pH on SPE

Addition of acetonitrile or an alcohol to the sample prior to the injection on the pre-column, in combination with the most suitable RP packing material and washing mobile phase, seemed to be the most efficient way to obtain a high recovery. Centrifugation of the sample, after dilution, was not necessary when only 20% of acetonitrile was added, and the dilution performed on-line by a robot. Furthermore, this procedure avoids the storage problems associated with isomerisation of the retinoids in the auto sampler after addition of acetonitrile, experienced by Wyss and Bucheli [19].

Due to the acidic character of retinoic acid, careful pH adjustment will be necessary to ensure control of both retention and the extraction efficiency. One of the problems encountered in the extraction of retinoids with the SPE technique is the poor mass transfer of the analyte from the protein through the highly hydrophilic eluent to the stationary phase during the clean-up step [18]. The initial pH of the mobile phases M1 and M2 were 7.4 and 3.0, respectively. The initial physiological pH of M1 was chosen to ensure no precipitation of the biological matter leading to faster clogging of the pre-column. However, at this pH the retinoic acid will have small retention on the pre-column. When the pH of M1 was lowered to 2–3, by exchanging ammonium acetate with an acid, the recovery of RA became significantly higher. The high recovery may partly be caused by an increase in hydrophobicity and thus in retention by ion-suppression, but also due to a more efficient release from the proteins at this pH. A low pH will also ensure that the polar sample containing amino groups most likely are washed out of the PC in the clean-up step.

While the recovery was improved using a low pH in M1, the bulk peak of unretained components was much larger when M1 contained acetic acid. However, the front signal was less broad when trifluoroacetic acid was used instead of acetic acid, probably because less material was retained on the pre-column during the pre-concentration step.

If ammonium acetate is used in M1a, the retinoids present as their anions at a pH of 7.4 will be immediately protonated when back-flushed onto the AC with mobile phase M2. Peak broadening observed using a neutral M1, was probably caused by a

too slow transfer from high to low pH during back-flush, and narrower peaks were obtained by increasing the amount of glacial acetic acid in M2 from 1 to 3%. With an acidic M1 this problem was not present at all and thus, this alternative was chosen.

3.1.3. Manipulation of secondary interactions on the pre-column

Initially, problems with peak broadening and change in retention sequence, when back-flushing the retinoids from the pre-column to the AC, were observed. It was suspected that these problems were caused by secondary interactions between oxygen atoms in the retinoids and rest silanol groups on the uncapped RP material in the pre-column. We suppose that these secondary interactions are also responsible for the high recovery obtained using this type of pre-column material. It was the 4-oxo metabolites of RA that showed a higher affinity for the pre-column packing material (Bondapak) and were eluted later compared to a direct injection on the AC. An increase of the methanol content in M2, however, ensured an efficient and rapid cleavage of these hydrogen-bonding mediated interactions. In addition, when the percentage of ammonium acetate was increased, the observed problem was eliminated due to the masking of residual silanols with NH_4 counter-ions. For this reason, addition of 36 mM ammonium acetate to M2 results in (i) a smaller elution volume from the pre-column, (ii) narrower peaks and (iii) improved sensitivity.

There was no measurable extra band broadening when a 20 μl sample was analysed by the column switching system as compared with direct injection onto the AC. When volumes larger than 20 μl were used, narrower peaks were obtained by back-flushing from the pre-column as compared to direct injection (data not shown).

3.1.4. Duration of clean-up step

It is important to ascertain that most of the proteins have been eluted before the flow direction is changed. This will lengthen the lifetime of both the analytical column and the pre-column and thereby allow the analysis of even longer series of samples. An optimal clean-up time was obtained by monitoring the elution of the proteins from the pre-column with an additional UV detector at 240 nm. It

was found that most of the proteins were eluted at 10 min which was chosen as the final time for the clean-up step.

The diluted serum sample was introduced onto the pre-column with a flow of 1 ml/min. When the sample reached the column, the flow was reduced to 0.5 ml/min and then slowly increased to 1.5 ml/min. This was done in order to give the retinoids sufficient time to be extracted from the protein. The technique is commonly used in off-line SPE when extracting protein-bound drugs [33].

3.2. Chromatographic separation

Despite the relatively large difference in polarity between 4-oxo-RA and RA, adequate resolution of their *cis* and *trans* isomers was obtained on the pKb-100 column using isocratic elution. The Suplex pKb-100 stationary phase represents a C₁₈ non-encapped column of the ABZ type showing excellent separation properties of acidic and basic components. The pKb-100 column had recently been reported to separate seven geometric isomers of RA [34]. The reported mobile phase consisted of methanol–acetonitrile–water–acetic acid. Substitution of a small amount of the acetonitrile with butanol, however, resulted in a significant reduction in analysis time and improved separation. As a result, baseline separation of the *cis*–*trans* isomers of ROH, RA and RA's 4-oxo metabolites could be obtained in less than 18 min with one single 250 mm column (Fig. 3).

With a slight modification of M2, baseline separation of 11,13-di-*cis*, 9,13-di-*cis* and 11-*cis*-retinoic acid was also obtained (results not shown), but in contrast extended retention times (up to 30 min) were observed.

When the purchased serum was spiked with the retinoids and the internal standard, interferences from an endogenous component were observed. This substance had a UV maximum at 440 nm and presumably represents a structure similar to a carotenoid derivative. However, the interference disappeared when the column temperature was raised from 25°C to 40°C and baseline separation between the internal standard and the unidentified substance

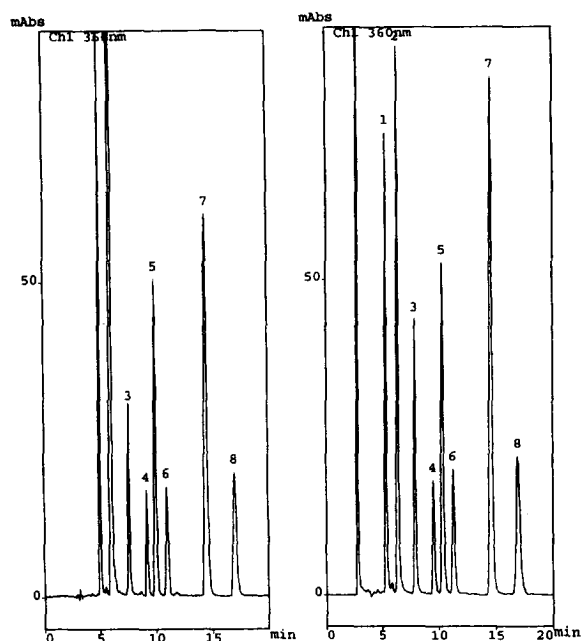


Fig. 3. Chromatogram showing separation, on a 250×4.6 mm Suplex pKb-100 column using mobile phase M2, of the seven isomers: 4-oxo-13-*cis*-RA(1), 4-oxo-all-*trans*-RA(2), 13-*cis*-ROH(4), all-*trans*-ROH(5), 13-*cis*-RA(6), 9-*cis*-RA(7), all-*trans*-RA (8) and the internal standard, 9,10-dimethylanthracene(3), obtained from 20 µl of serum directly injected onto the AC (left) as well as 20 µl serum injected to PC extraction and back-flushed onto the AC (right). Column oven temperature was 40°C and detection performed at 360 nm. The concentrations of the isomers are between 20 ng/ml (lowest peaks) and 100 ng/ml (highest peaks).

was observed. Chromatograms of blank and spiked human serum are shown in Fig. 4.

3.3. Validation

3.3.1. Linearity

Replicates of six different concentrations [2 (*n* = 6), 5 (*n* = 1), 20 (*n* = 6), 100 (*n* = 1), 500 (*n* = 6), 1000 (*n* = 1) ng/ml] for each of the five retinoids were prepared and the detector response (peak height) plotted against concentration. Their correlation was then calculated mathematically by least square regression using Microsoft Excel. The correlation was found to be linear in the region 2–1000

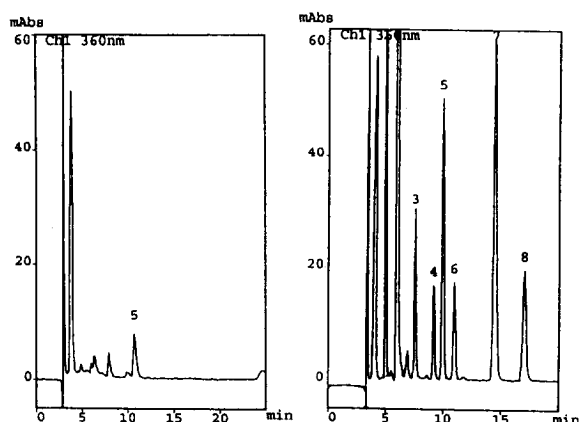


Fig. 4. Chromatograms of a blank 500 μ l serum sample (left), and serum spiked with the same compounds as used in Fig. 3 including the I.S. (right). Separation is accomplished on the 250 \times 4.6 mm pKb-100 column with M2 as mobile phase at a column oven temperature of 40°C. Detection was performed at 360 nm.

ng/ml serum, at least. The coefficient of correlation (r) was better than 0.999 for all five retinoids. The equations for the linear regression line and the

coefficient of correlation (r) for each retinoid are shown in Table 2. All experiments were performed by the same analyst and on the same day.

3.3.2. Limit of quantification (LOQ) and limit of detection (LOD)

From the linear regression analysis used for validation of linearity, the standard deviation of the y -values around the regression line was calculated ($S_{x/y}$) and used as SB in the formula $Y_{LOQ} = YB + 10 \cdot SB$ and $Y_{LOD} = YB + 3 \cdot SB$. The value YB was estimated from the regression line as the y -axis intercept value [35]. The corresponding LOQ and LOD was calculated using the regression equation (Table 3). Fig. 5 shows the chromatogram obtained after injection of a 2 ng/ml serum sample. LODs and LOQs based on a signal to noise ratio of 3:1 and 10:1, respectively were somewhat lower than those obtained from statistical calculations.

Verification of the repeatability of the LOQ was performed by analysis of ten actual samples ($n = 10$), with approximate LOQ values, in one day. The mean and R.S.D. are shown in Table 4.

Table 2

Equations for the linear regression lines and the coefficients of correlation for each retinoid where y represents the peak-height ratio and x the analyte concentration in ng/ml serum

Isomer	Equation	Coefficient of correlation (r)
4-Oxo-13- <i>cis</i> -RA	$y = 0.0120x + 0.0171$	0.99994
4-Oxo-all- <i>trans</i> -RA	$y = 0.0257x + 0.0076$	0.99949
13- <i>cis</i> -RA	$y = 0.0054x + 0.0119$	0.99990
9- <i>cis</i> -RA	$y = 0.0054x + 0.0119$	0.99995
All- <i>trans</i> -RA	$y = 0.0059x + 0.0033$	0.99941

Table 3

Values of $S_{x/y}$, LOD and LOQ of the retinoic acids

Isomer	$S_{x/y}$	LOD (ng/ml)	LOQ (ng/ml)
4-Oxo-13- <i>cis</i> -RA	0.0041805	1.05	3.48
4-Oxo-all- <i>trans</i> -RA	0.0278812	3.25	10.9
13- <i>cis</i> -RA	0.0130756	1.85	6.17
9- <i>cis</i> -RA	0.0028902	2.10	6.89
All- <i>trans</i> -RA	0.0162581	7.28	24.3

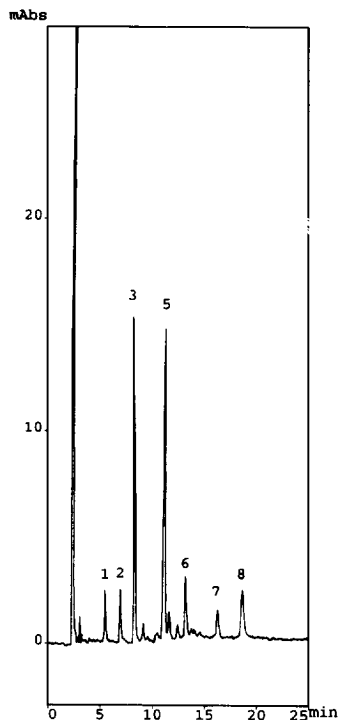


Fig. 5. Chromatogram showing serum spiked with 2 ng of each retinoid/ml of serum. Chromatographic conditions as in Figs. 3 and 4.

3.3.3. Recovery and accuracy

When applying SPE techniques to protein-bound substances, the most challenging task is to obtain a high recovery. A high recovery is necessary because of the very low concentration of the retinoids in human serum and to obtain a high accuracy of the method. In on-line SPE-HPLC sample pre-treatment, prior to extraction, is rapid and no, or at least, only small losses in recovery occurred. Loss in

recovery is often observed when using off-line sample preparation methods. Nevertheless, the critical factor for optimal recovery will be the extraction of the drug from the protein when the sample passes the pre-column packing material. Little is known with respect to the mechanism of RA transport in the blood stream, but earlier observations of different recoveries for the different geometrical isomers, can be interpreted by the presence of different binding sites and/or binding affinity for the various isomers [18]. The 9-*cis* isomer, for example, has been shown to be extracted with difficulty (see Section 3.1). A *cis* or *trans* conformation being the only difference between most of the compounds to be determined, makes it impossible to use a retinoid as the internal standard that is representative for the whole entity of the isomers. The extent of discrimination, if present, will be difficult to predict. An internal standard is, however, desirable to compensate for variations in the injection volume and for peak broadening caused by deterioration of the analytical column. An aromatic retinoid such as acitretin or 13-*cis*-acitretin or a demethyl analog of retinoic acid is a natural choice as internal standard. However, due to difficulties in obtaining these compounds, we chose to use the commercially available methylated anthracene as an internal standard. This internal standard will compensate for variations in injection volumes and chromatographic performance. Lack of acidic moiety of this internal standard is not a factor which will influence the performance of the method. Small changes in the pH of the mobile phases will not affect the retention of the analytes since the pH in the mobile phases is far below the pK_a (6–8) of the retinoic acids [36] and the silanol groups. The required lipophilicity of the internal standard, to match the retention of the analytes, will likely give rise to protein-binding. However, this protein-binding will mainly be at albumin which is presumed to be the protein carrying retinoic acid (in the blood stream). Since the internal standard is added in a 20% acetonitrile solution, protein-binding is probably low and weakened. In addition, because the same procedure is used for both calibration standards and samples, any effect resulting from differences in protein-binding will be negligible. 9-Methylanthracene has been used as an internal standard by others [8].

Table 4
Repeatability of the LOQ of the different retinoic acids as R.S.D. (%) of ten replicate samples with approximate LOQ concentrations

Isomer	Level (ng/ml)	Mean	R.S.D. (n = 10)
4-Oxo-13- <i>cis</i> -RA	5	5.01	4.4
4-Oxo-all- <i>trans</i> -RA	10	10.17	3.7
13- <i>cis</i> -RA	5	5.08	3.4
9- <i>cis</i> -RA	5	5.06	4.7
All- <i>trans</i> -RA	20	20.57	5.6

In addition to the physiological binding sites on a protein there are also low affinity sites on the outer surface of the protein [37]. When spiking a serum or plasma sample with a standard solution, some of the isomers will undoubtedly attach to these low affinity sites because they are more accessible. An essential question will therefore be whether the recovery data obtained by SPE of spiked samples are representative for real samples or not. A reasonable explanation is very difficult and a better insight into the problem requires determinations of physiologically carried RA. Injection of radiolabeled RA into the intestine of rats is possible, but the rapid, and poorly characterised in vivo isomerisation and metabolism of RA makes it difficult to draw conclusions about the recovery of the different isomers. This fact also raises the question of whether or not calibration curves based on spiked serum samples will give a sufficiently accurate result. However, so far no other method seems to give better information.

Significantly different results may also be found depending on the way recovery tests on spiked serum samples are performed. For a 20- μ l injection volume, we found a 3–5% higher recovery ($n=6$) when the peak height was used instead of the peak area. Diluting the 20- μ l ethanol solution to 200 μ l with 50:50 methanol–water and injecting the 200 μ l directly on to the AC, resulted in a 25% decrease in peak height (results not shown). This is probably caused by a combination of the peak broadening effect from the increased volume, but also due to adsorption of the retinoids to the walls in the glass container. Since the peak height thus will be lower for the sample injected directly, representing the

Table 6

Recoveries and losses due to breakthrough on the precolumn ($n=6$) using tritium labelled isomers

Isomer	Recovery (%)	R.S.D.	To waste (%)	R.S.D.
All- <i>trans</i> -RA	90	1.4	7	3.2
9- <i>cis</i> -RA	85	2.2	10	1.8

Chromatographic conditions as described in Section 2.6 with 0.05% TFA in M1.

100% value, collection of recovery data by this method can lead to an overestimation of the recovery. The recovery data presented in this work (Table 5) are therefore based on injection of a 20- μ l aliquot directly on to the AC and comparing the peak areas obtained with those obtained by spiking the blank serum with the same amount of retinoids (as described under Section 2.6). Table 6 shows the recoveries and losses due to breakthrough on the pre-column using tritium labelled isomers.

3.3.4. Repeatability and reproducibility

A series of sequential determinations ($n=6$), was performed in 2 h and the mean values, absolute (S.D.) (the contribution of $S_{x/y}$ is not included) and the relative (R.S.D.) standard deviations calculated. The results for the within-day repeatability are shown in Table 7.

The between-day reproducibility was estimated by performing the same determination on the same batch of sample every third day for a period of one month ($n=10$). The mean, S.D. and R.S.D. are shown in Table 7.

Table 5

Recovery (%) and R.S.D. (%) values obtained for different levels of spiked serum samples based on comparing the mean of the peak areas ($n=6$) with those obtained for a 20- μ l standard solution of the different isomers when injected directly on to the AC

Isomer	20 ng/ml				100 ng/ml				1000 ng/ml			
	Recovery		R.S.D.		Recovery		R.S.D.		Recovery		R.S.D.	
	A	B	A	B	A	B	A	B	A	B	A	B
4-Oxo-all- <i>trans</i> -RA	89	85	2.9	3.4	90	86	1.4	2.5	88	81	0.5	1.5
4-Oxo-13- <i>cis</i> -RA	85	82	3.0	3.2	87	83	0.9	2.3	86	83	0.7	1.6
9- <i>cis</i> -RA	89	80	3.1	3.4	88	79	1.2	2.1	87	82	0.3	2.0
13- <i>cis</i> -RA	104	82	2.8	2.9	99	82	1.2	2.5	98	84	0.9	1.4
All- <i>trans</i> -RA	89	79	3.4	3.5	90	80	1.3	1.9	88	84	1.1	1.8

Washing solutions were 0.05% TFA in M1 (A) and 1% ammonium acetate in M1 (B).

Table 7
Validation results

Isomer	Level		Accuracy	Precision						
	Added (ng/ml)	Found (ng/ml)		Repeatability			Reproducibility (see text)			
				S.D. (ng/ml)	<i>n</i>	R.S.D. (%)	Found (ng/ml)	S.D. (ng/ml)	<i>n</i>	R.S.D. (%)
1	20	20.4	2.0	0.58	6	2.5	–	–	–	–
	100	106	6.0	2.4	6	2.3	–	–	–	–
	500	507	1.4	11	6	2.0	506	7.1	10	1.42
2	20	21.0	0.5	0.61	6	2.9	–	–	–	–
	100	107	7.0	3.3	6	3.2	–	–	–	–
	500	508	1.6	11	6	2.2	502	3.4	10	0.68
3	20	20.3	1.5	0.62	6	3.1	–	–	–	–
	100	104	4.0	2.4	6	2.3	–	–	–	–
	500	516	3.2	6.7	6	1.3	502	4.4	10	0.87
4	20	20.6	3.0	0.58	6	2.8	–	–	–	–
	100	102	2.0	2.3	6	2.2	–	–	–	–
	500	511	2.2	7.3	6	1.4	503	4.6	10	0.91
5	20	20.3	1.5	0.92	6	4.5	–	–	–	–
	100	104	4.0	2.7	6	2.6	–	–	–	–
	500	510	2.0	7.4	6	1.5	504	3.9	10	0.77

(1) 4-Oxo-13-*cis*-RA, (2) 4-oxo-all-*trans*-RA, (3) 13-*cis*-RA, (4) 9-*cis*-RA, (5) all-*trans*-RA. Accuracy is defined as % deviation between found and added.

3.3.5. Stability and robustness

When protected from light at all times, flushed with argon and kept at maximum -20°C , the retinoids were stable for one year in a serum matrix. Standards stored in absolute ethanol were stable for six months at high concentrations and when the amber glass containers were silanized. When compared with the values from the initial determinations, the results obtained a few weeks later are substantially lower for small concentrations of RAs. The internal standard stock solution was found to be stable for at least four months when stored cold (-20°C) and dark in amber silanized glass containers. Making up fresh working solutions every second week ensured sufficient consistency of the concentration of the internal standard working solution.

The pre-column was repacked every 200 injections and the pre-column cartridge changed every 1000 injections. The PEEK filters were changed every 100 injections or when large series of samples were to be analysed. The guard-column was changed every 200 injections to ensure no bleeding of highly non-polar endogenous components onto the analytical column.

Both the auto-injector and the column oven were equipped with a thermostat and thus variations in ambient temperature do not need to be taken into consideration. A systematic investigation of small variations in mobile phase composition on the results was not performed. However, highly reproducible retention times were observed.

3.4. Application

The method was applied for the determination of retinoids found in human serum after oral administration of Roaccutan (13-*cis*-RA).

Fig. 6a and Fig. 6b show the chromatograms of serum samples taken 30 min and 60 min after a single oral dose of 60 mg Roaccutan. The concentrations of 13-*cis*-retinoic acid and 4-oxo-13-*cis*-retinoic acid were 26 ng/ml and 8.3 ng/ml, respectively, 60 min after ingestion. The chromatogram in Fig. 6c illustrates the change in retinoid levels that are taking place during 30–60 min. Fig. 6c shows that the major change is an increase of 13-*cis*-RA and its metabolite 4-oxo-13-*cis*-RA. The depletion of the internal standard peak illustrates the excellent

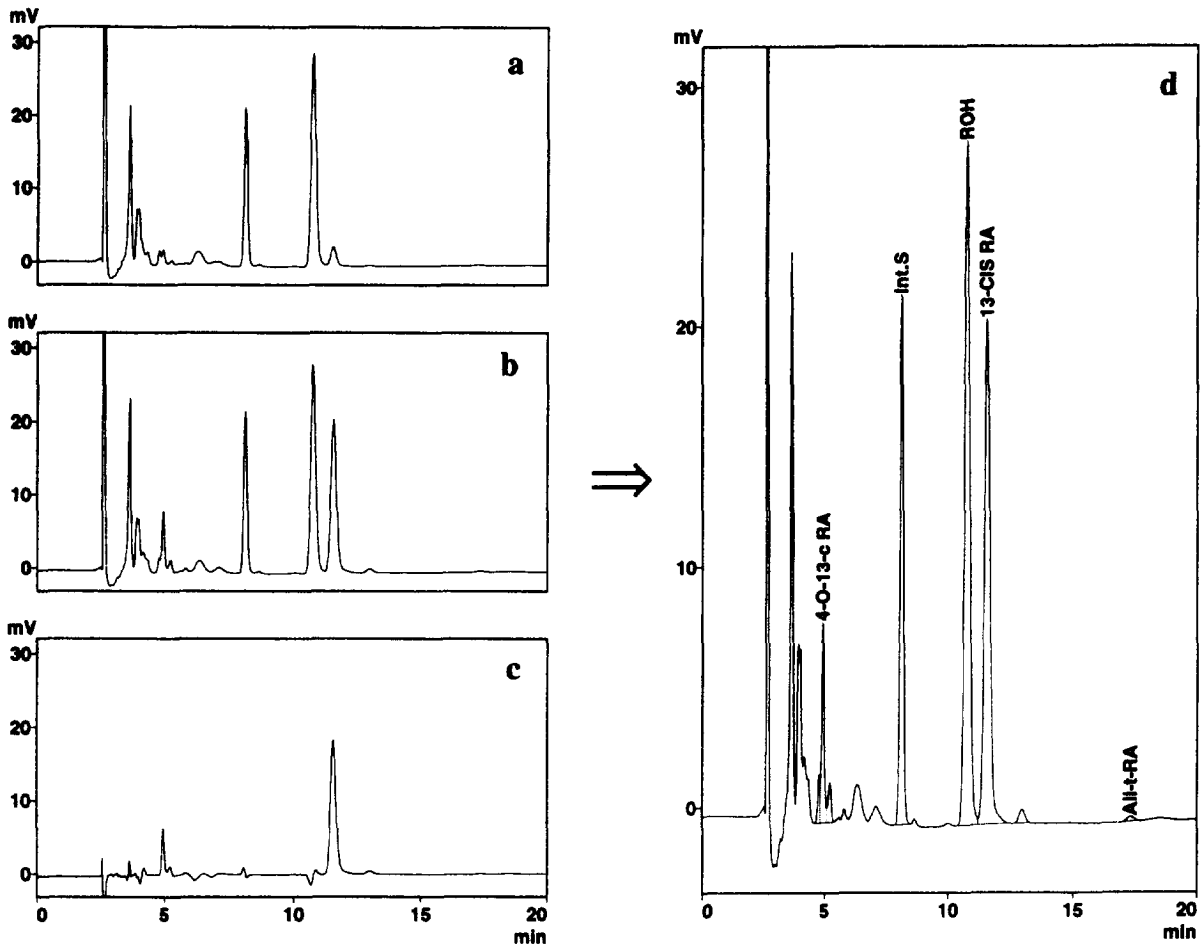


Fig. 6. Chromatograms of serum samples after a single oral dose of 60 mg Roaccutan, (a) 30 min after administration, (b) and (d) 60 min after administration. Chromatogram (c) which is a subtraction of (a) from (b) illustrates the change in retinoid concentration from 30 to 60 min after ingestion. Chromatographic conditions as in Figs. 3 and 4. For identification of the peaks, see chromatogram (d), where ROH is retinol.

repeatability of both injection volume, recovery and retention times.

4. Conclusion

A fully automated RP-HPLC method for the determination of 9-*cis*-, 13-*cis*- and all-*trans*-RA together with their main metabolites 4-oxo-13-*cis*- and 4-oxo-all-*trans*-RA in human serum has been developed. The method involves injection of serum, diluted by a robot integrated with the Peltier cooled automatic injector, onto a RP pre-column. Minimal

exposure to organic solvents, total protection from light, use of silanised amber vials, titanium frits, PEEK tubings and connections, and several purge and washing procedures of flow lines and injector parts between injections, eliminated both memory effects and isomerisation. Combination of SPE and isocratic separation is accomplished with a column switching technique. Optimum conditions for pre-column extraction as well as chromatographic separation giving high recovery and high sample loading capacity have been established. Baseline separation of seven components, including 9-*cis*-RA, was achieved by isocratic elution on a single RP-HPLC

column in less than 18 min. The method allows quantitative routine determination of 40 samples containing >2 ng of retinoic acids/ml human serum in 20 h. The method is somewhat less sensitive for late eluting peaks compared to some methods utilising gradient elution [21] due to peak broadening. However, the time consumption is significantly reduced, and the LOQ is sufficient for clinical investigations. The method was validated and found to be highly reproducible.

References

- [1] R. Blomhoff, M.H. Green, T. Berg and K.R. Norum, *Science*, 250 (1990) 399–404.
- [2] R. Blomhoff, M.H. Green, J.B. Green, T. Berg and K.R. Norum, *Physiol. Rev.*, 71 (1991) 951–90.
- [3] P. Kastner, P. Leid and P. Chambon, in R. Blomhoff (Editor), *Vitamin A in Health and Disease*, Marcel Dekker, New York, 1994, p. 189.
- [4] S.A. Kliewer, K. Umesono, D.J. Mangelsdorf and R.M. Evans, in R. Blomhoff (Editor), *Vitamin A in Health and Disease*, Marcel Dekker, New York, 1994, p. 239.
- [5] C. Thaller and G. Eichele, *Nature*, 345 (1990) 815.
- [6] M.A. Leo and C.S. Lieber, *J. Biol. Chem.*, 260 (1985) 5228.
- [7] A.B. Barua and J.A. Olson, *Am. J. Clin. Nutr.*, 43 (1986) 481.
- [8] R.R. Gadde and F.W. Burton, *J. Chromatogr.*, 593 (1992) 41.
- [9] F. Vane, K. Stollenborg and C. Bugge, *J. Chromatogr.*, 227 (1982) 471.
- [10] N.R. Al-Mallah, H. Bun, P. Coassolo, C. Aubert and J.P. Cano, *J. Chromatogr.*, 421 (1987) 177.
- [11] Ch. Eckhoff and H. Nau, *J. Lip. Res.*, 31 (1990) 1445.
- [12] C.A. Frolic, T.E. Tavela, G.L. Peck and M.B. Sporn, *Anal. Biochem.*, 86 (1978) 743.
- [13] N. Takeda and A. Yamamoto, *J. Chromatogr. B*, 657 (1994) 53.
- [14] J.G. Besner, S. Meloche, S. Leclaire, P. Band and S. Mailhot, *J. Chromatogr.*, 231 (1982) 467.
- [15] C.J.L. Bugge, L.C. Rodriguez and F.M. Vane, *J. Pharmacol. Biomed. Anal.*, 3 (1985) 26.
- [16] P. Campins-Falco, R. Herraez-Hernandez and A. Sevillano-Cabeza, *J. Chromatogr.*, 619 (1993) 177.
- [17] D.C. Turnell and J.D. Cooper, *J. Chromatogr.*, 492 (1989) 59.
- [18] R. Wyss and F. Bucheli, *J. Chromatogr.*, 456 (1988) 33.
- [19] R. Wyss and F. Bucheli, *J. Chromatogr.*, 424 (1988) 303.
- [20] R. Wyss, *J. Chromatogr.*, 531 (1990) 481.
- [21] R. Wyss and F. Bucheli, *J. Chromatogr.*, 593 (1992) 55.
- [22] J.C. Craft, Ch. Eckhoff and W. Kuhn, *J. Liq. Chromatogr.*, 11 (1989) 2051.
- [23] H.C. Furr, A.B. Barua, *The Retinoids*, Raven Press, Ltd., New York, 2nd ed., 1994, p. 179.
- [24] R.W. Lucek and W.A. Colburn, *Clin. Pharmacokin.*, 10 (1985) 38L.
- [25] U. Timm, G. Hofgartner and R. Erdin, *J. Chromatogr.*, 456 (1988) 21.
- [26] H. Nielsen, *J. Chromatogr.*, 381 (1986) 63.
- [27] W.S. Powell, *Anal. Biochem.*, 164 (1987) 117.
- [28] U.A.Th. Brinkman, *J. Chromatogr. A*, 665 (1994) 217.
- [29] R.D. McDowall, *J. Chromatogr.*, 492 (1989) 3.
- [30] P. Lefebvre, A. Agadir, M. Cornic, B. Gourmel, B. Hue, C. Dreux, L. Degos and C. Chomienne, *J. Chromatogr. B*, 666 (1995) 55.
- [31] J.J. MacKichan, *Clin. Pharmacokin.*, 16 (1989) 65.
- [32] J. March, *Advanced Organic Chemistry*, Wiley Interscience, 4th ed., 1992, p. 248.
- [33] K.C. Van Horne (Editor), *Sorbent Extraction Technology*, Analytichem International Varian, 1990.
- [34] A.R. Sundquist and W. Stahl, *J. Chromatogr.*, 637 (1993) 201.
- [35] J.C. Miller and J.N. Miller, *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester, 2nd ed., 1992.
- [36] N. Noy, *Biochim. Biophys. Acta*, 1106 (1992) 159.
- [37] W.W. Lindup, *Prog. Drug Metab.*, 10 (1987) 141.