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Determination of retinoids by packed-capillary liquid chromatography with large-volume on-column focusing and temperature optimization

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Abstract

An isocratic reversed-phase packed capillary high-performance liquid chromatography method for the determination of all-*trans*-retinol, all-*trans*-retinoic acid and 13-*cis*-retinoic acid has been developed, utilizing on-column focusing large volume injection and operation at 50°C. The focusing mobile phase consisted of acetonitrile–ammonium acetate (0.5% in water)–water (45:5:50). The eluting mobile phase consisted of acetonitrile–ammonium acetate (0.5% in water)–acetic acid (94.9:5:0.075). In addition to high water content, pH contributed to additional selective focusing of the retinoic acids when using the non-eluting mobile phase. Injection volumes up to 100 μl were successfully applied. Operation at elevated temperature provided reduced column backpressure, and allowed higher volumetric flow-rates to be used during sample introduction. Thus the overall analysis time was reduced from 45 to 25 min. Furthermore, increasing the temperature from 25 to 70°C gave a 48% reduction in the reduced plate height. A mass limit of detection of 0.5 ng of the retinoic acids, corresponding to a concentration limit of detection of 5 ng ml^{-1}, was found using on-column UV detection at 360 nm.

Keywords: Large-volume injections; On-column focusing; Retinoids; Retinoic acid; Vitamins

1. Introduction

Vitamin A is a generic term reserved to designate any compound possessing the biological activity of retinol, whereas the term retinoids includes both naturally occurring forms of vitamin A and the many synthetic analogs of retinol, with or without biological activity [1].

Many procedures have been devised for determining the quantities of naturally occurring and synthetic retinoids in biological samples. The majority of these involve reversed-phase HPLC. Retinoids are a class of highly unstable compounds. To preserve the geometric orientations of the retinoids, precautions must be taken to protect the sample from light, heat and air exposure during sample work-up and analysis. The fact that retinoic acids are present at the sub-ng g^{-1} concentrations or lower [2,3], in combination with their labile nature, has led to the development of mild and sensitive on-line solid-phase extraction HPLC systems with UV detection [4,5]. The mass limit of detection (mLOD) obtained with these systems was about 150–330 pg, corresponding to a concentration limit of detection (cLOD) of 0.3–1 ng ml^{-1}, when 0.5 ml plasma samples were analyzed. Gundersen and Blomhoff showed that the use of microbore column switching systems with UV detection improved the detection limits substantially,

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from 333 pg to 60 pg, when compared to normal-bore HPLC systems with UV detection [6]. With a similar system utilizing coulometric electrochemical detection and normal-bore columns, Sakhi et al. obtained a mLOD of 10 pg for all-*trans*-retinoic acid (all-*trans*-RA) [7].

Recently, the use of packed capillary columns in chromatography has increased, and columns with inner diameter ranging from 100 to 350 μm I.D. packed with 3–10 μm mean diameter sorbent particles have been evaluated in LC [8]. Packed capillary columns advantageously combine many of the valuable properties of both packed and open tubular columns [9], and are characterized by low mobile phase and stationary phase consumption, high resolution of complex mixtures when using long columns, increased mass sensitivity due to reduced dilution and excellent suitability of direct connection to mass spectrometers and Fourier transform infrared spectrometers [8].

In the last few years there has been an increasing interest in using the temperature for retention control and efficiency optimization in LC [10–21]. LC is known for relatively high column backpressure and low diffusion coefficients compared to gas chromatography and supercritical fluid chromatography, and thus poorer efficiency is observed. By reducing the viscosity of the mobile phase in LC, the column backpressure will be reduced and the diffusion increased. This can easily be achieved by increasing the operating temperature, resulting in improved efficiency [10–16]. Temperature programming has been used as a successful tool to adjust the elution strength of the mobile phase, as an alternative to more commonly used gradient elution [20,21]. Packed capillary columns with small inner diameters are more suitable for temperature programming as compared to conventional sized columns, due to their quick response to temperature changes and minimized radial temperature gradients [8]. The mLOD for late eluting peaks can be lowered substantially by operating at elevated temperatures, because of improved efficiency and reduced retention time [21].

The main drawback of packed capillary columns is their sensitivity to dead volumes, and extra attention must be paid to extra-column band-broadening processes. These processes are mainly initiated from dead volumes in the injection valve, column end-fittings, connecting tubing and the detector cell, and have to be minimized to utilize the potential of capillary LC. However, the extra-column band broadening introduced prior to the column inlet can to some extent be eliminated when on-column focusing techniques are used. Such techniques are also used to introduce large volumes of analytes dissolved in a non-eluting solvent, and thereby decrease the cLOD of the method [22–33]. According to the theoretical down-scale factor, the sensitivity can be increased approximately 200 times by reducing the column inner diameter from conventional size (4.6 mm) to 320 μm capillary size, given an optimized on-column capillary detector cell and the same amount of analyte injected [34]. This illustrates the benefits of using capillary dimensions and large volume injections, when limited sample volumes of low concentrations are to be determined.

Hagen et al. used a packed capillary LC system equipped with a specially designed amperometric electrochemical detector, and an injection volume of 2 μl, for the determination of retinoic acids. They obtained a mLOD and a cLOD of 142 fg and 64.1 pg ml^{-1}, respectively [35]. Increasing the injection volume to 100 μl or more would improve the cLOD substantially. However, because of the low volumetric flow-rates in capillary LC, typically 1–5 μl min^{-1}, the sample introduction would have taken at least 20 min, resulting in an unacceptable long analysis time.

In this paper we describe an approach to a packed capillary LC–UV method for determination of low concentrations of retinoids, by means of on-column focusing large volume injection, temperature optimization and flow programming during sample introduction, to optimize the cLOD and time of analysis.

2. Experimental

2.1. Materials and reagents

Acetonitrile and methanol of HPLC grade were obtained from Rathburn (Walkerburn, UK). Ammonium acetate, sodium hydroxide and glacial acetic acid of analytical-reagent quality were obtained from Merck (Darmstadt, Germany). Absolute ethanol was purchased from Arcus (Oslo, Norway). Water was

de-ionized and glass distilled. The stationary phase material was Suplex pKb-100 (Supelco, Bellefonte, PA, USA). All-*trans*-retinol (all-*trans*-ROH), all-*trans*-RA and 13-*cis*-retinoic acid (13-*cis*-RA) were supplied by Sigma (St. Louis, MO, USA). All fused-silica capillaries, except the detection capillary, were obtained from Composite Metal Services (UK). Helium and argon (99.998%) were purchased from Aga (Oslo, Norway).

2.2. Column preparation

The packed capillary columns were prepared according to a procedure previously described, using supercritical CO_2 as the slurry medium [21]. According to this method, the start pressure is normally 100 bar and the end pressure 550 bar, with a pressure increment of 10 bar min^{-1}. However, the start pressure had to be 300 bar to prepare the columns with the packing material Suplex pKb-100 (particle size 5 μm). This is possible due to the fact that this packing material had been removed from a well-used, discarded 4.6 mm I.D. HPLC column. Only the packing material from the middle part of the analytical column was used. The material was resuspended in methanol and dried before packing. Valco ZU1C unions with 2 μm Valco 2SR1 steel screens served as column end fittings, and the columns were connected to the end fittings by Valco FS1.4 polyimide ferrules and steel nuts (Valco Instruments, Houston, TX, USA). The column body was of fused-silica (320 μm I.D.×450 μm O.D.) with a polyimide protection layer, and the columns were prepared in lengths of 25 cm.

2.3. Preparation of standard solutions

Stock solutions of the retinoids were prepared under red light and stored in inert Argon atmosphere. Twenty-five mg of each retinoid was dissolved in 10 ml absolute ethanol. The stock solutions were stored in the dark at −10°C. Standard mixtures of the retinoids were prepared from the stock solutions. In the preliminary experiments a test mixture of approximately 180 μg of each retinoid per ml mobile phase solution was used. When working with the large volume injection system, the retinoids were dissolved in the non-eluting mobile phase, and the concentration of each retinoid in the standard mixtures was between 500 and 1 ng ml^{-1}.

2.4. μ-HPLC instrumentation

The μ-HPLC instrument consisted of a Merck-Hitachi L-7100 piston pump. Manual injections were performed with a Valco C14W injection valve with an internal loop of 60 nl. The large volume injection system is described in detail later in this chapter. A HP 5700A gas chromatograph served as column oven (Hewlett-Packard, Amsterdam, Netherlands), and the ultraviolet detector was a Thermo Separation System UV 2000, operated at 360 nm, with an on-column capillary detector-cell device (9550-0155) (Fremont, CA, USA), in which a fused-silica capillary detection cell was mounted: a 20 cm long (75 μm I.D.) Hewlett-Packard capillary electrophoresis capillary (G1600-60332) with an extra light path (200 μm) at the detection spot. This capillary was directly mounted to the outlet of the column inside the oven. A fused-silica linear restrictor (20 cm×20 μm I.D.) was connected to the end of the detection capillary, when operating at elevated temperatures beyond 50°C, to suppress bubbling of the mobile phase. The packed capillary column was connected to the injection valve by a fused-silica capillary of 15 cm×50 μm I.D.×375 μm O.D. A C-R6A integrator (Shimadzu, Kyoto, Japan) was used to record the chromatograms. The mobile phase was helium degassed for 15 min each day.

2.5. Large volume injections

Two valves in series were used to perform large volume injections. The first valve, a Model 7010 sample injection valve (Rheodyne, Cotati, CA, USA), was used as the carrier for the non-eluting mobile phase. The external loop had a volume of 100 μl. The second valve, Rheodyne Syringe Loading Sample Injector Model 7725, was used to introduce the sample, which was dissolved in the non-eluting mobile phase. The sample was transferred from the loop during injection in the opposite direction of that used during loading. Three different sample loop volumes were utilized: 20, 50 and 100 μl. The sample loops were made of 250 μm I.D. fused-silica, and were connected to the injection valve by poly-

ether ether ketone (PEEK) tubing sleeves and Rheodyne steel ferrules. The eluting mobile phase was delivered continuously by the pump. The non-eluting focusing mobile phase consisted of acetonitrile–ammonium acetate (0.5% in water)–water (45:5:50). The eluting mobile phase consisted of acetonitrile–ammonium acetate (0.5% in water)–acetic acid (94.9:5:0.075).

Before each large volume injection the column was preconditioned for an optimized period of time with the non-eluting mobile phase, introduced by the first valve, before the sample was introduced and focused at the inlet of the column. After a predetermined period of time, the eluting mobile phase was introduced, by switching both valves back to the load position. To decrease the time needed for preconditioning and sample introduction, the flow-rate was increased from 5 $\mu l\,min^{-1}$ to 20 $\mu l\,min^{-1}$. The flow-rate was reduced to 5 $\mu l\,min^{-1}$ prior to introduction of the eluting mobile phase. The operating temperature was 50°C. The valve switching and flow-rate settings were controlled manually.

2.6. Calculations

The efficiency was calculated using Eqs. (1), (2) and (3).

$$N = 5.54\,(t_R/t_{W1/2})^2 \quad (1)$$

where t_R and $t_{W1/2}$ are the retention time and the peak width at half the peak height, respectively.

$$H = L/N \quad (2)$$

where L is the column length.

$$h = H/d_p \quad (3)$$

where d_p is the particle size.

3. Results and discussion

3.1. Effect of pH upon retention on the pKb-100 material

In serum and in most tissues, all-*trans*-ROH is the most abundant retinoid. The ratio between retinol and retinoic acid and other retinoids can be as high as 1000/1. This fact often precludes separation of various retinoids present at low concentrations from retinol itself. In reversed-phase systems retinol normally elutes later than retinoic acid, and gradient elution will usually be needed for the separation of the geometrical isomers of retinoic acid. Technical difficulties in generating gradients for liquid capillary systems have encouraged the development of an isocratic separation. The pKb-100 material has shown to be remarkably well suited for the isocratic separation of *cis*/*trans* isomers of retinoids [6,7,36]. In addition to hydrophobic interactions, there are pronounced secondary interactions, especially for the retinoic acids on this material. This is clearly demonstrated by the elution of all-*trans*-RA several minutes later than the methylated analogue [6]. The result is that changes in pH have minimal effects on retinol whereas the retinoic acids are strongly affected. In this manner, separation of retinoic acids from retinol can elegantly be manipulated through changes in the pH only. This is especially convenient because the number of endogenous retinoids is large, and continuous method improvements make detection of an increasing number of retinoids possible. In every class of retinoids there are *cis*/*trans* isomers, thus the demand for high resolution and selectivity. In Fig. 1 the retention factor k is plotted against the pH for all-*trans*-ROH, 13-*cis*-RA and all-*trans*-RA. Fig. 1 demonstrates that increasing the pH from 4.1 to 5.0 increases the separation of all-*trans*-ROH and 13-*cis*-RA. The composition of the mobile phase at pH 5.0 was acetonitrile–ammonium acetate (0.5% in water)–glacial acetic acid (94.9:5:0.075). The same effect was observed when operating at 60°C. This solvent composition was subsequently used as the eluting mobile phase in the large volume injection system.

3.2. Temperature as elution parameter

Many newly developed stationary phase materials require accurate temperature control at sub- or super-ambient temperatures. In some cases, a slight change in the operating temperature may have a pronounced effect on the selectivity of a separation [37]. The influence of temperature is a function of free energy changes in the interaction involving the analyte and the stationary phase [18,37,38]. Enthalpy changes

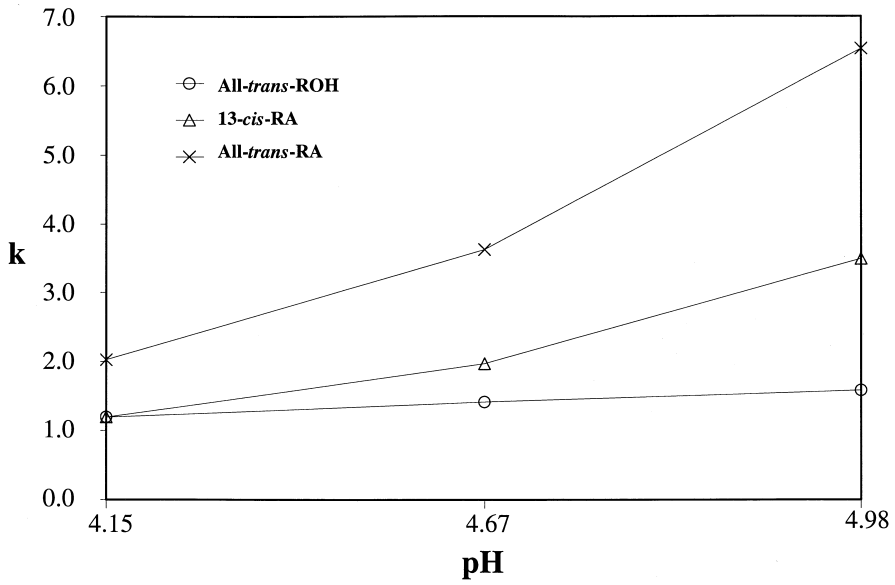

Fig. 1. Effect of mobile phase pH on retention factor (k) for the retinoids at ambient temperature. The mobile phases consisted of 94.9% acetonitrile–5% ammonium acetate (5% in water) and various amounts of acetic acid.

dominate the retention mechanism in most chromatographic separations, and the reaction is regularly exothermic regarding reversed-phase systems, typically -17 kJ mol^{-1} [39]. A Van't Hoff plot of ln of the retention factor (k) vs. the reciprocal temperature will normally fit into a linear curve, with the slope representing the enthalpy change for the retention mechanism. Non-linear behavior indicates a shift in the retention mechanism, and therefore it is often valuable to perform a Van't Hoff plot when investigating the temperature effect upon a separation. This is important when operating at elevated temperature with highly unstable compounds, such as retinoids.

In the temperature interval between 25 and 70°C, the retention factor for each retinoid was measured as a function of temperature. The Van't Hoff plots (Fig. 2) showed linear behavior for all the retinoids,

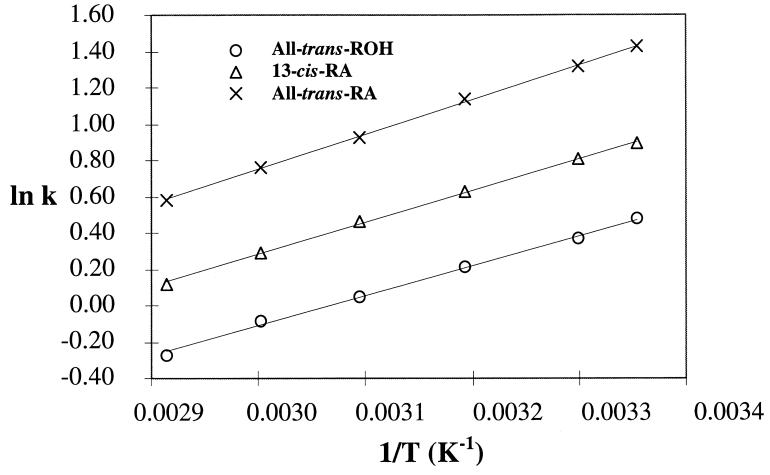

Fig. 2. Van't Hoff plots of the retinoids, illustrating effect of temperature on retention factor (k).

Table 1
Enthalpies of the retinoids, using the eluting mobile phase

Compound	ΔH (kJ mol^{-1})
All-*trans*-ROH	−13.7
13-*cis*-RA	−14.5
All-*trans*-RA	−15.9

indicating the same retention mechanisms and enthalpy changes throughout the whole temperature interval. The enthalpy changes for the retention mechanisms are shown in Table 1.

It is well known that a temperature increment in LC results in increased diffusion rates, due to reduced viscosity. Consequently, the mass transfer rate between the mobile and stationary phase is enhanced, and better efficiency is expected [40]. However, different research groups have investigated the efficiency as a function of temperature in LC, with variable results. Some studies have been able to establish a link between temperature increment and enhanced efficiency [12–16], while others have reported effects only in means of reduced analysis time [20,21]. Differences between the LC systems, such as dead volumes, the temperature intervals investigated and choice of analyte and stationary and mobile phase, seems to be important. Consequently, it was of interest to investigate the effect of operating at elevated temperature upon efficiency in our chromatographic system. The investigated temperature interval was 25–70°C, due to the labile character of the retinoids and the risk of hydrolysis of the stationary phase [11]. No degradation of the stationary phase material or the retinoids induced by the elevated temperature was observed during these experiments. The isothermal chromatograms of the retinoids at different temperatures are shown in Fig. 3. The data in Table 2 illustrate the efficiency, as reduced plate height (h), as a function of temperature, for the last eluting peak, all-*trans*-RA. As illustrated, enhanced efficiency was observed when the temperature was increased. When elevating the temperature from 25 to 70°C, and operating at a flow-rate of 5 μl min^{-1}, the reduced plate height decreased from 17.8 to 9.1. This is equivalent to a reduction of 48%. However, the obtained efficiency was rather poor. Regarding packed capillary columns, reduced plate heights as low as 2 have been reported [41–43]. The rather poor efficiency in our experiment is possibly due to the fact that the stationary phase material was removed from a well-used and discarded analytical column, and repacked in the capillary columns. A start pressure of 100 bar is found to be superior with regard to efficiency, using the same column preparation instrumentation [44]. However, the columns had to be prepared with a start pressure of 300 bar to obtain a uniform

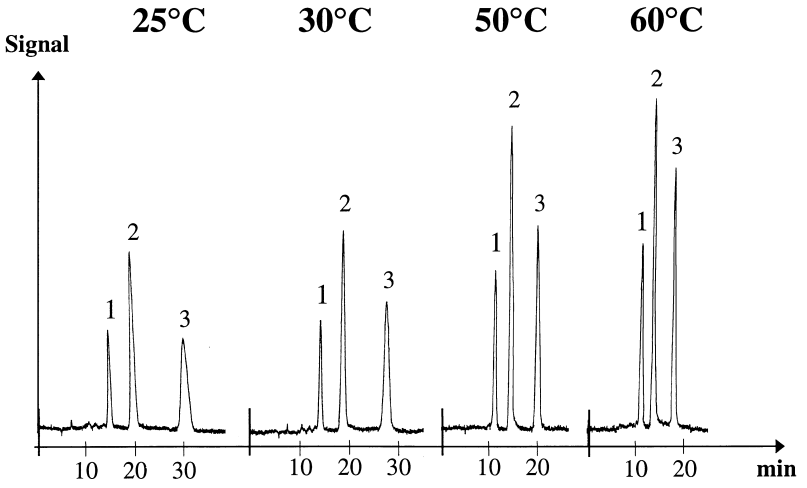

Fig. 3. Effect of temperature on the capillary LC–UV separation of the retinoids. The injection volume was 60 nl: (1) all-*trans*-ROH; (2) 13-*cis*-RA; (3) all-*trans*-RA.

packing structure, possibly due to agglomeration of the stationary phase material. In addition, the column end fittings was not optimized with regard to dead volumes. Finally, the volumetric flow-rate during these experiments was 5 $\mu l\,min^{-1}$, resulting in a linear velocity of 0.14 and 0.17 $cm\,s^{-1}$ when operating at 25 and 70°C, respectively. When the flow-rate was reduced from 5 to 2 $\mu l\,min^{-1}$ at 70°C, a decrement of the reduced plate height from 9.1 to 6.0 resulted, equivalent to 34%. The linear velocity was reduced to 0.06 $cm\,s^{-1}$, which is obviously closer to the optimal linear velocity with regard to efficiency.

The retention time for the last eluting peak, all-*trans*-RA, when increasing the temperature from 25 to 70°C, was reduced from 27.0 to 17.9 min, which is equivalent to a reduction of 34% on the overall analysis time. In combination with the enhancement of the efficiency in the same temperature interval, an increased peak height is observed. As illustrated in Fig. 3, the peak height for all-*trans*-RA increased from 2.1 cm when operating at 25°C to 5.9 cm when operating at 60°C. Accordingly, the LOD was increased with a factor of 2.8, since the baseline noise was constant ($S/N=3$). Thus, operation at elevated temperature was beneficial with regard to efficiency, time of analysis and LOD.

3.3. Large volume injection

In the preliminary experiments, an injection volume of 60 nl was used, and the cLOD was found to be approximately 30 $\mu g\,ml^{-1}$ for all-*trans*-RA, when operating at room temperature. Since naturally occurring retinoids are present in sub-$ng\,g^{-1}$ level in real samples, larger volumes must be injected. Large volume injections were performed by utilizing on-column focusing at the inlet of the column. The retinoids were dissolved in a non-eluting mobile phase and introduced onto the capillary column, which had been preconditioned with the same non-eluting mobile phase. The increased retention of RAs when a liquid mobile phase with low acid content is used contributes to the focusing effect during large volume injections. The additional focusing is more selective, eluting non-acidic compounds in the front. The final composition of the non-eluting mobile phase was acetonitrile–ammonium acetate (0.5% in water)–water (45:5:50). The composition of the non-eluting mobile phase was established from the data shown in Fig. 4. In addition, the pH effect was utilized. The eluting mobile phase was introduced immediately after the end of the sample introduction. Three different injection volumes were investigated:

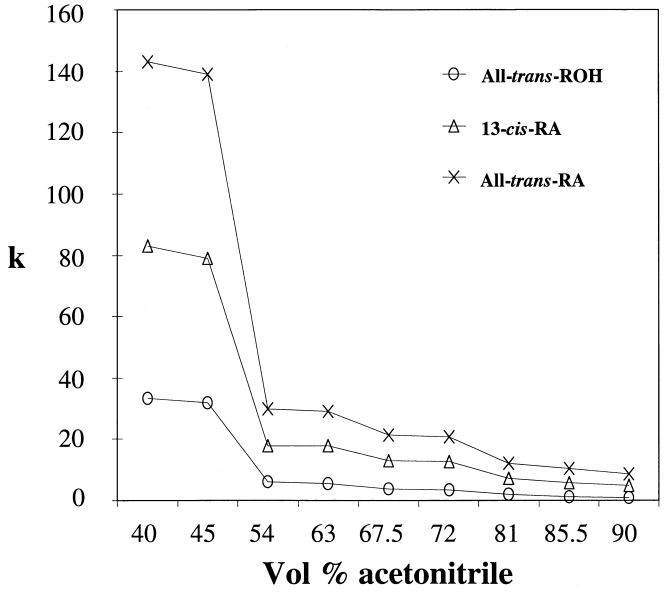

Fig. 4. Effect of amount organic modifier (acetonitrile) in the mobile phase on retention factor (k) for the retinoids at ambient temperature. The mobile phases consisted of 5% ammonium acetate (0.5% in water) and various amounts of water and acetonitrile.

Table 2
Effect of temperature on reduced plate height (h) for all-*trans*-RA at a flow-rate of 5 μl min^{-1}

Temperature (°C)	25	30	40	50	60	70
h	17.8	14.1	12.8	10.7	10.0	9.1

20, 50 and 100 μl, and the absolute amount of each retinoid were in all cases 20 ng. The chromatographic performance was excellent for all the investigated injection volumes. As shown in Table 2, improvement in efficiency was marginal at temperatures higher than 50°C as compared to the lower temperatures. The column backpressure decreases with increasing temperature. However, at temperatures above 50°C the reduction of the column backpressure did level out. In addition, the risk for degradation of the retinoids or the stationary phase material is more critical at such high temperatures, although this was not observed when operating at 70°C for a limited period of time. Consequently, an operating temperature of 50°C was chosen when performing the large volume injections. The data in Table 3 illustrate the effect of different injection volumes upon efficiency and resolution. The retention times, used in the equation for efficiency calculations, were measured from the point where the eluting mobile phase was introduced onto the column. Consequently, the retention times did not vary with the different injection volumes. The efficiency measurements were carried out, despite the fact that a pseudo step gradient initially occurs when the eluting mobile phase is introduced. As shown in Table 3, the reduced plate height for all-*trans*-RA decreases when the injection volume is increased, due to improved solute focusing in a narrow band at the column inlet in the more dilute samples. The resolution of the two retinoic acids did not vary much when increasing the injection volume from 60 nl to 100 μl.

Table 3
Effect of injection volume on reduced plate height (h) and resolution (R_s) for the retinoids at a flow-rate of 5 μl min^{-1} and an operating temperature of 50°C

	Injection volume (μl)			
	0.06	20	50	100
R_s (all-*trans*-ROH/13-*cis*-RA)	20.9	11.3	9.5	6.0
R_s (13-*cis*-RA/all-*trans*-RA)	7.4	9.0	9.5	7.5
h (all-*trans*-RA)	10.7	8.0	3.5	2.5

When large volume injections are performed, the overall analysis time is dramatically increased. An injection volume of 100 μl resulted in a time of analysis of 45 min, compared to 23 min when the 60 nl injection loop was used. As mentioned earlier, the column backpressure decreases when operating at elevated temperature. This can be exploited during the sample introduction by increasing the volumetric flow-rate, and thereby decrease the analysis time. The volumetric flow-rate was set to 20 μl min^{-1} when operating at 50°C. Further increment of the flow was not possible without exceeding the pressure limitations of the pump. The column was preconditioned for 2 min before the injection took place. The flow was set back to 5 μl min^{-1} prior to the introduction of the eluting mobile phase, to operate closer to the optimal linear velocity during the separation. This led to an overall analysis time of 25 min, equivalent to a reduction of 44%, without significantly loss of efficiency or resolution. The flow-rate was measured immediately after the flow was reset to 5 μl min^{-1}, and afterwards every minute. The chromatographic system needed an equilibrium time of 7 min, before the selected flow-rate was reached. Houdiere et al. recently performed simultaneous temperature programming and flow programming in a capillary LC system [45]. When the mobile phase flow-rate and the column temperature were changed simultaneously during the separation run, the analysis time was reduced by 50%, while the separation efficiency was preserved. This is due to the fact that the optimal linear velocity in LC is increased when elevating the temperature [19,20]. Consequently, it is not of great importance that the system needs an equilibrium time when reducing the flow-rate.

Several studies have described large volume injections in capillary LC [22–33]. Injection volumes of 100 μl or more on capillary columns are rare, but Brunmark et al. reported a linear peak area response of injection volumes up to 200 μl [26]. However, the injection was time demanding. As previously described, this can be overcome by operation at elevated temperature and increased flow-rate during sample introduction. For this reason the 100 μl

injection loop was chosen for the rest of the experiments, to reduce the cLOD. Fig. 5 shows a chromatogram of the retinoids, where an injection volume of 100 μl was utilized. The concentration of each retinoid was 50 ng ml^{-1}. The cLOD was found to be 5 ng ml^{-1} for the last eluting peak, all-*trans*-RA, equivalent to an absolute mass of 0.5 ng ($S/N=3$).

Limited detectability, in means of UV and fluorescence detection, has been considered to be a problem in capillary LC. In our case an on-column flow cell with a light path of 200 μm was used. Recently, low dispersion 'Z'-shaped flow cells designed for capillary LC, with a light path of 8 mm, has become commercially available. Unfortunately, such a flow cell was not available in this experiment. If an optimized flow cell with an 8 mm light path had been utilized in this experiment, the sensitivity could, according to Beer-Lambert's law, theoretically be increased with a factor of 40, resulting in a mLOD of 12 pg, corresponding to a cLOD of 0.12 ng ml^{-1}. This is approximately the same mLOD that Sakhi et al. obtained in the normal bore column switching system with electrochemical detection [7]. Utilizing large volume injection capillary LC in combination with miniaturized electrochemical detection would enhance the mLOD and cLOD substantially.

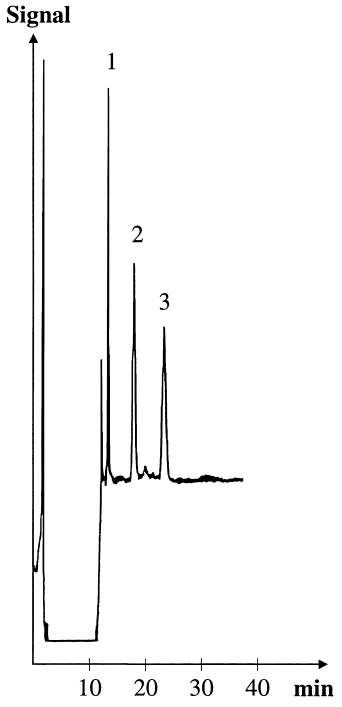

Fig. 5. On-column focusing large volume injection capillary LC–UV separation of the retinoids. The injection volume was 100 μl, the operating temperature 50°C and the volumetric flow-rate 20 μl min^{-1} during sample introduction. The concentration of each retinoid was 50 ng ml^{-1}: (1) all-*trans*-ROH; (2) 13-*cis*-RA; (3) all-*trans*-RA.

4. Conclusions

This work has demonstrated that large volume injection capillary LC can be a powerful technique to improve the cLOD of retinoids with UV detection. The use of elevated temperature enhanced the efficiency of the separation and the time of analysis. Further improvements can be done with regard to detection, and thereby reduce the mLOD. Based on the results of Hagen et al. [35], electrochemical detection in combination with the large volume injection capillary LC method described here, could theoretically result in a mLOD as low as 3 fg. We intend to investigate the use of this instrumental combination of large volume injection capillary LC and electrochemical detection in our laboratory in the search for retinoids during early development of mouse embryos.

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