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Review

Chromatography of retinoids

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CONTENTS

LIST OF ABBREVIATIONS

BHT	Butylated hydroxytoluene
DL	Detection limit
EDTA	Ethylenediaminetetraacetate
EI	Electron impact
4-EPR	4-Ethoxyphenyl retinamide
FFAP	Free fatty acid phase
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectrometry
2-HER	2-Hydroxyethylretinamide
HPLC	High-performance liquid chromatography

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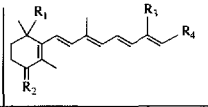
HPLC–MS	High-performance liquid chromatography–mass spectrometry
4-HPR	4-Hydroxyphenylretinamide
I.S.	Internal standard
LC	Liquid chromatography
4-MPR	4-Methoxyphenylretinamide
MS	Mass spectrometry
NARP	Non-aqueous reversed phase
NICI	Negative-ion chemical ionization
PICI	Positive-ion chemical ionization
QL	Quantification limit
RA	Retinoic acid (all-*trans* if not otherwise specified)
SFC–MS	Supercritical fluid chromatography–mass spectrometry
SIM	Selected-ion monitoring
TLC	Thin-layer chromatography
UV	Ultraviolet

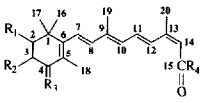
1 INTRODUCTION

The term "retinoid" includes both the naturally occurring compounds with vitamin A activity and synthetic analogues of retinol, with or without biological activity [1]. Vitamin A (retinol) is essential for normal growth, vision, reproduction and epithelial differentiation. Whereas retinol itself is required for reproduction, its metabolite retinaldehyde (retinal) is the active form in the vision process. Retinoic acid (RA), another metabolite of retinol, is now seen as a fundamental mediator of cell differentiation and cell proliferation. Retinoids as drugs have been shown to possess selective activity in proliferation, differentiation, keratinization, sebum production, inflammation, immune reaction and tumour prevention and therapy [2]. A comprehensive two-volume monograph on the chemistry and biology of the retinoids has been published [1].

The rather slow progress in the investigation of the metabolism of vitamin A is due primarily to the lack of suitable techniques to deal with these extremely labile compounds. The development of high-performance liquid chromatography (HPLC) in the past 15 years revolutionized the isolation and separation of retinoids, including their isomers. This resulted in the identification of many new metabolites of retinol. In addition, the use of retinoids as drugs in dermatology and cancer research has led to a number of drug assays during the past decade. A comprehensive review on the extraction, separation and chemical analysis of the retinoids by Frolik and Olson [3] can be found in the above-mentioned monograph on retinoids. Chromatography of retinoids was also discussed by Thompson [4] and De Leenheer *et al.* [5] in publications concerned with the determination of fat-soluble vitamins.

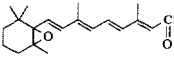
This review deals with modern chromatographic methods (mainly HPLC) for the determination of retinoids in biological samples. Retinol, retinyl esters and retinal, as well as multivitamin determinations, are not discussed, as this was the



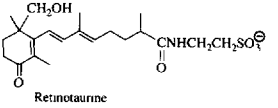


	R_1	R_2	R_3	R_4
RA (tretinoin)	CH_3	H H	CH_3	COOH
4 Oxo-RA	CH_3	O	CH_3	COOH
4-Hydroxy-RA	CH_3	H OH	CH_3	COOH
4-Oxo-16-hydroxy-RA	CH_2OH	O	CH_3	COOH
1-Carboxy-4-oxo-RA	COOH	O	CH_3	COOH
13 Demethyl-RA	CH_3	H,H	H	COOH
Retinoyl β-glucuronide	CH_3	H H	CH_3	CO-β-glucuronide
4-Oxoretinoyl β-glucuronide	CH_3	O	CH_3	CO β-glucuronide
Ethyl retinoate	CH_3	H,H	CH_3	$COOCH_2CH_3$
4-HPR	CH_3	H,H	CH_3	CONH C_6H_4-OH
4 MPR	CH_3	H H	CH_3	CONH-C_6H_4-OCH_3
4-EPR	CH_3	H,H	CH_3	CONH-C_6H_4-OCH_2CH_3
2-HER	CH_3	H,H	CH_3	$CONHCH_2CH_2OH$
N-Acetyl retinyl amine	CH_3	H,H	CH_3	$CH_2NHCOCH_3$
Retinal acetylhydrazone	CH_3	H,H	CH_3	$CH{=}NNHCOCH_3$

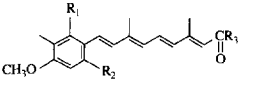
	R_1	R_2	R_3	R_4
13-cis-RA (isotretinoin)	H	H	H H	OH
13-cis-4-Oxo RA	H	H	O	OH
13-cis-2-Hydroxy-4-oxo-RA	OH	H	O	OH
13-cis-3-Hydroxy-4-oxo-RA	H	OH	O	OH
13-cis-Retinoyl β-glucuronide	H	H	H,H	β glucuronide
13-cis-4 Oxoretinoyl β-glucuronide	H	H	O	β-glucuronide
13 cis-Ethylretinamide	H	H	H H	$NHCH_2CH_3$
13-cis 4-HPR	H	H	H H	NH-C_6H_4OH



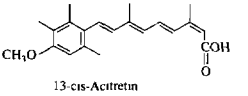
	R
5,6-Epoxy-RA	OH
5,6-Epoxyretinoyl β-glucuronide	β-glucuronide



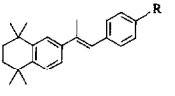
Retinotaurine



	R_1	R_2	R_3
Acitretin	CH_3	CH_3	OH
Etretinate	CH_3	CH_3	OCH_2CH_3
Motretinide	CH_3	CH_3	$NHCH_2CH_3$
Ro 12-7554	Cl	Cl	OCH_2CH_3



13-cis-Acitretin



	R
Temarotene	H
Ro 14-6113	OH
Ro 13-9272	CH_3
Ro 13-6298 (arotenoid ethyl ester)	$COOCH_2CH_3$
Ro 13-7410 (arotenoid free acid)	COOH

Fig 1. Structures of first-, second- and third-generation retinoids discussed in this review Abbreviations without a prefix are all-*trans*.

topic of the excellent review by De Leenheer *et al* [5], which appeared quite recently. Retinoid researchers distinguish between first-generation retinoids, namely retinoic acids (tretinoin and isotretinoin) and their metabolites, aromatic retinoids (second generation), such as etretinate and acitretin, and the so-called arotinoids (third generation, see Fig 1). In this article, the emphasis is on drug assays for these retinoids Papers describing the isolation and identification of metabolites are also mentioned, but without claim to completeness. For a more comprehensive description of these assays and the literature before 1980, the reader is referred to the article by Frolik and Olson [3].

2. SAMPLE COLLECTION AND STORAGE

Retinoids are very sensitive to light and oxidation Therefore, special care has to be taken from the beginning of the sample collection, throughout storage, thawing, extraction, till the final separation step. It is well known, now, that retinoids isomerize under physiological conditions [6–8] To study these metabolic processes, further isomerization must be prevented after sample collection.

Photoisomerization of retinoids was investigated by several authors. After exposure to fluorescent light for 2 h, only 77% of 13-*cis*-RA and 34% of etretinate, respectively, could be recovered in serum [9]; even more degradation was observed in an organic solvent [9,10] Goodman *et al.* [11] reported instability of 13-*cis*-RA in ethyl acetate and plasma at 23–26°C under standard fluorescent lighting. Equilibrium was reached in 1–2 h with a final 13-*cis*-RA/RA ratio of 75:20. Bugge *et al.* [12] exposed a pooled patient blood sample to white fluorescent light for 1 h. From the observation that etretinate, acitretin and 13-*cis*-acitretin concentrations were not significantly different from pre-exposure values, they concluded that whole blood provides better protection against photodecomposition than serum.

In some cases, special storage conditions were used for biological samples to prevent oxidation of retinoids. These precautions included storage under nitrogen or argon at −70 or −80°C [13,14], storage in liquid nitrogen [15,16], use of ascorbate and ethylenediaminetetraacetate (EDTA) in the Vacutainer tubes and storage of the plasma at −35°C [17], or addition of butylated hydroxytoluene (BHT) to blood [18]. Zile *et al.* [19] collected bile under nitrogen over ice in vials containing *n*-propyl gallate and BHT before storage at −20°C. In most cases, the following precautions should be sufficient: blood samples should be protected from light and immediately centrifuged; the headspace above the plasma or serum in the tubes should be as small as possible, or the tubes could be flushed with nitrogen, or even better, with argon; tissue samples should be wrapped in aluminium foil to prevent dehydration.

Stability investigations showed no decrease when 13-*cis*-RA was stored in plasma at −20°C for 9 weeks [20] or for 3 months [21] In one case, instability was found (−20% to −34% after 22 weeks at −17°C) [22]. This could have been

caused by the use of a 10-ml tube containing only 1.5 ml of plasma (*i.e.* too great a headspace of air). In addition, stability investigations involving analysis of only one sample after a defined storage time cannot be significant Etretinate was reported to be stable in plasma for 8 weeks at −20°C [23], and acitretin and 13-*cis*-acitretin for 90 days at −20°C [24], at least. Third-generation retinoids are more stable than first- and second-generation retinoids No decrease for temarotene could be found in human, dog or rat plasma at −17°C after 60–70 days [25], or for Ro 13-7410 at −20°C for 7.5 months [26]. The stability of first- and second-generation retinoids in plasma can be a problem at room temperature or 37°C. Investigations by Cullum and Zile [8] with RA revealed, mainly, the generation of more polar compounds, rather than isomerization. The author's own observation with RAs, etretinate or acitretin plasma samples indicates considerable isomerization potential in the plasma from a few patients or volunteers. This isomerization is probably caused by thiol-containing compounds [27].

Finally, care should also be taken when calibration standards are prepared because of possible solubility problems with the retinoids Another important pitfall would be the occurrence of memory effects in the glassware or chromatographic system [21,28].

3 SAMPLE PREPARATION

3.1. General

Before the chromatographic separation, the analyte has to be extracted from the biological matrix, first to achieve a clean-up of the sample and second to be able to prepare an injection solution that is compatible with the chromatographic system. The second point is obvious for protein-containing or tissue samples. As previously mentioned, precautions have to be taken during sample preparation to prevent photoisomerization and oxidation of the retinoids. This can be done by working under subdued or yellow light The tetraene system of the first- and second-generation retinoids is much more sensitive to light than that of the third-generation retinoids; however, the latter can also isomerize in solution. The need for antioxidants and/or flushing of the sample with nitrogen or argon is dependent on the complexity of the sample work-up As mentioned by Thompson [4], evaporation should be avoided whenever possible, and then addition of antioxidants is not absolutely necessary. However, several methods describe the addition of BHT, ascorbic acid or *n*-propyl gallate before extraction, and the benefit of this measure should be investigated for every analytical method. Another difficulty in dealing with retinoid extraction is to overcome the high and strong protein binding and the low solubility of these compounds in aqueous solution, which hamper the transfer from the protein to a water-immiscible extraction solvent through a hydrophilic phase.

In the recent past, the determination of retinoids in plasma, serum or blood for

pharmacokinetic studies or therapeutic drug monitoring has assumed increasing importance. Other biological fluids analysed were bile, urine and, less frequently, cerebrospinal fluid [11], amniotic fluid [29], or lacrimal gland fluid or tears [30]. Different tissues, such as liver, kidney, small intestine, etc., as well as different cell cultures, have been analysed for metabolic studies. A topic of great interest is the determination of retinoids in skin, which has gained more and more importance in connection with the use of retinoids in dermatology.

3.2. Biological fluids

The simplest way to analyse biological fluids would be direct injection into the HPLC system. Using conventional columns, this is only possible for protein-free fluids (or fluids with rather low content of proteins), as reported for bile [31–33], cerebrospinal fluid [11] or lacrimal gland fluid and tears [30]. For plasma, serum or whole blood, a simple protein precipitation can be performed. Methanol [34] and acetonitrile [35] were used, but did not produce very sensitive methods (100 ng/ml) owing to the lack of a concentration step and a clean-up step. An improvement was obtained by Verweij *et al.* [36], using acetonitrile–tetrahydrofuran–acetic acid (780:80:5) with ammonium acetate for deproteination, followed by addition of water before injection. However, this method is only briefly described, and no chromatograms are shown. Addition of butanol–acetonitrile (1.1) to serum (McClean *et al.* [9]) or to blood [12,18], followed by dipotassium hydrogenphosphate, resulted in a phase separation and, therefore, a concentration effect. Kerr *et al.* [17] used protein precipitation with methanol, followed by evaporation of the solvent

Most methods consist of a classical liquid–liquid extraction. However, to optimize the recovery of the retinoids, special measures are needed. One way is to liberate the retinoid from the plasma proteins by protein precipitation with a water-miscible solvent. For example, after deproteination with methanol, 0.2 *M* acetate buffer is added, followed by hexane–dichloromethane–2-propanol (80:19:1) for extraction [37]. Alternatively, acidic methanol may be used in combination with hexane [14,38]. Other laboratories performed the protein precipitation with ethanol followed by extraction with ethyl acetate [39] or hexane [40,41]. After ethanol addition, De Leenheer and co-workers performed a hexane extraction at acidic pH, which was either preceded by a clean-up extraction at basic pH (discarding the organic phase) [42], or followed by a Sephadex LH-20 purification step for final determination by gas chromatography–mass spectrometry (GC–MS) [43]. In his GC–MS method, Chiang [44] used petroleum ether after acidification of plasma and deproteination with ethanol. Variations of this hexane extraction procedure were used by Napoli and co-workers, either by addition of 0.1 *M* phosphate buffer pH 5.4 to the plasma sample, which had previously been deproteinated with ethanol [14,38], or by addition of sodium chloride and potassium hydroxide in ethanol for a first clean-up hexane extraction step, fol-

lowed by acidification of the aqueous phase and subsequent extraction of the retinoids with hexane [14]. Puglisi *et al.* [25] used acetonitrile for protein precipitation and *tert.*-butyl methyl ether for extraction of temarotene from plasma.

Instead of an initial protein precipitation with an organic solvent, direct extraction methods are also used. In most cases, salt or buffer is added to improve the recovery of the retinoids. In some of these procedures the extraction was performed three times for an acceptable recovery Citrate buffer (pH 6) [45] as well as addition of ethanol and phosphate buffer (pH 3.5) [46] have been used together with subsequent hexane extraction. In the latter example, this liquid–liquid extraction was followed by a reversed-phase HPLC clean-up step, and final determination by normal-phase micro HPLC and MS detection [46] Puglisi and de Silva [47] used phosphate buffer (pH 6 or 7) or borate buffer (pH 9) and diethyl ether or ethyl acetate for the extraction of different first- and second-generation retinoids from blood, plasma or urine. Phosphate buffer and diethyl ether [13,22,48–50] or diethyl ether–ethyl acetate mixtures [24,51,52] were also used by others. In one case, ammonium acetate was used instead of phosphate buffer before the diethyl ether extraction [10]. Another approach used citrate buffer (pH 6) [53] or addition of methyl acetate and sodium sulphate [23] in combination with ethyl acetate extraction Finally, the use of plasma pH adjustment with 5% perchloric acid, followed by ethyl acetate extraction [11,54,55] should be mentioned. However, extraction under acidic conditions could cleave glucuronides and, as a consequence, result in too high a concentration of RA.

Surprisingly, only a few publications deal with solid-phase extraction, which has become popular in other fields in recent years The difficulty of obtaining good recoveries for the retinoids when undiluted plasma samples are applied to a C_{18} bonded phase could be a reason for this lack of reported methods. In an off-line solid-phase extraction, McPhillips *et al.* [16] performed a protein precipitation with acetonitrile and diluted the supernatant with 40 m*M* acetic acid before applying it to a Bond Elut C_{18} cartridge. A rather complicated sample work-up was carried out by Egger *et al.* [26] for a highly sensitive GC–MS method for the arotinoid Ro 13-7410. Plasma samples were extracted with *tert* -butyl methyl ether using Extrelut columns, followed by an ion-exchange chromatography step with Bond Elut NH_2 cartridges.

Solid-phase extraction can also be performed on-line, using a precolumn coupled to the analytical column and automated column switching. Such a system is shown in Fig. 2 [56,57]. The rather complex valve-switching system allows direct injection of biological fluids (such as plasma) and tissue homogenates with full automation and light protection. In principle, one valve (V2) is sufficient for coupling one precolumn to an analytical column. Valve V3 permits forward- and backflush washing of the precolumn, which increases the number of injections onto the precolumn, and valve V1 is used for purging of the capillaries to prevent memory effects in routine analysis. To improve the stability and the recovery of 13-*cis*-RA and its metabolites, plasma samples were diluted with 9 m*M* sodium

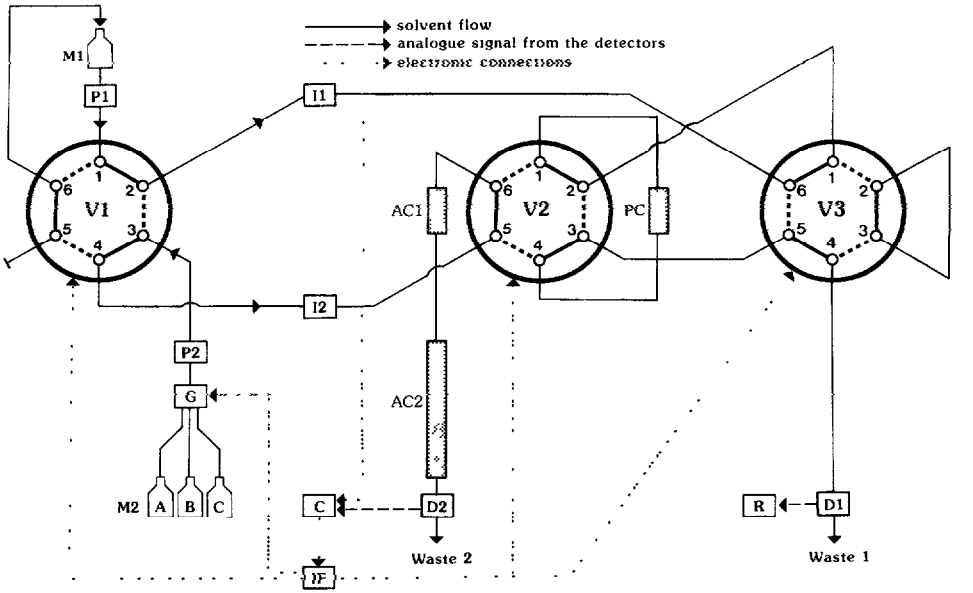


Fig 2 Schematic representation of an HPLC column-switching system M1 = mobile phase 1, P1 = HPLC pump, flow-rate 1 5 ml/min, V1–V3 = switching valves, I1 = automatic sample injector with cooling module, PC = precolumn (14 mm × 4 6 mm I D , Bondapak C_{18} Corasil, 37–50 μm), D1 = UV detector, 240 nm; R = recorder, M2 = mobile phase 2, G = low-pressure-gradient former, P2 = HPLC pump, flow-rate 1 0 ml/min; I2 = manual injector, AC1 = guard column (30 mm × 4 mm I D , Spherisorb ODS 1, 5 μm), AC2 = analytical column (two 125 mm × 4 mm I D , Spherisorb ODS 1, 5 μm), D2 = UV detector, 360 nm, C = computing integrator, IF = interface (Reproduced with permission from ref 56)

hydroxide–acetonitrile (8:2) before injection [21,57]. For very lipophilic and highly protein-bound retinoids such as etretinate, a protein precipitation with ethanol was performed before injection [28,57] The precolumn was washed with 1% ammonium acetate containing 10–20% acetonitrile. Recovery problems with lipophilic retinoids, using this column-switching technique, were discussed [56]. In a method recently developed for acitretin, the plasma sample was simply diluted with acetonitrile (final content 17%) allowing sufficient recovery (more than 80%) and high sensitivity (0.3 ng/ml with a 1-ml injection solution) [58]. A similar column-switching technique, consisting of two precolumns for alternating injections, was used by Creech Kraft *et al.* [29], who performed a protein precipitation with 2-propanol, with subsequent overnight freezing of the sample in liquid nitrogen. A 0.1–0.2 ml volume was injected onto the first precolumn, whereas the second, coupled to the analytical column, was eluted by the gradient mobile phase.

In parallel with tissue extraction, several groups also used lyophilization for biological fluids This time-consuming technique is now no longer the method of choice for plasma samples. After lyophilization, usually in the presence of antiox-

idants and sometimes of buffer, the extraction was performed with methanol [20,31,59–61], methanol followed by hexane [8], or chloroform–methanol (1:1) followed by methanol [62,63]. Another approach, which was adapted for serum, consists of storage of the samples in liquid nitrogen, followed by homogenization in a solution containing EDTA and ascorbic acid and extraction with butanol–methanol (95:5) [15,64]. The direct injection of this solvent is preferred by these authors because of better stability of the retinoids compared with protein precipitation with methanol [34]. Rollman and Vahlquist [65] performed alkaline hydrolysis not only with skin samples but also with serum, followed by pH adjustment and hexane extraction.

As well as direct injection of small volumes [31–33], bile was also deproteinated with methanol [66] or ethanol [9], or, after sodium acetate and Glusulase addition and incubation, extracted with ethyl acetate in the presence of phosphate buffer (pH 7) [50]. Other bile extraction methods are very complicated and consist of Sephadex LH-20 chromatography and semipreparative HPLC clean-up [19,67]. The retinoids discussed in this review are normally not excreted in urine. However, a few groups analysed urine samples for confirmation of the absence of these retinoids. The same extraction methods were used as for plasma or blood [11,24,47,50,68].

3.3. Tissues

Analysis of tissues was historically important to identify and quantify retinoids in the target organs. Following early successes with the direct extraction of tissue homogenates, difficulties arose with retinoic acids and their metabolites. Therefore, Ito *et al.* [62] introduced the lyophilization of tissue samples, followed by extraction with chloroform–methanol (1:1) and methanol [61–63] or petroleum ether [69]. Various other extraction solvents were also used, such as chloroform–methanol (2:1) [70], diethyl ether [71], methanol [6], or methanol followed by hexane [8,40], or methanol followed by Sephadex chromatography or semipreparative HPLC clean-up [7,72–75]. Instead of evaporation of the extraction solvent, direct injection of the methanolic extraction solution was also performed [60,76,77] Another way of removing water from the tissue is to grind the sample in the presence of anhydrous sodium sulphate, followed by extraction with diethyl ether [71], dichloromethane [78], or chloroform (for 24 h) and methanol [40].

Direct extraction of tissue samples still remains an important alternative. Advantages (less oxidation) and disadvantages (lower recoveries for polar metabolites) have to be investigated for each analyte. Extraction solvents used were chloroform–methanol (1:1) [79] or (2:1) [80], and methanol followed by methanol–water (95:5) [81]. In the last method, an additional hexane wash and solid-phase clean-up using C_{18} bonded phases were incorporated. Simpler sample handling was realized by direct injection of the extraction solvent into HPLC systems, *e.g.* with acetonitrile–tetrahydrofuran–acetic acid (780:80·5) in the pres-

ence of ammonium acetate and sodium sulphate [36], or with methanol–water (5:4) [34], or butanol–methanol (95:5) [15,82].

Alkaline hydrolysis of skin samples was used by Rollman and Vahlquist [65,69,83] for the determination of various retinoids. In the last version of these similar procedures, skin biopsies or subcutaneous fat in 80% ethanolic potassium hydroxide and internal standard (I.S.) solution (ethanol containing BHT) were flushed with nitrogen and heated at 80°C for 15 min. After the addition of water, neutral retinoids were extracted with hexane, followed by acidification and extraction of acidic retinoids; the solvent was then evaporated [83]. An advantage of this procedure is the hydrolysis of retinyl esters which allows, therefore, the simultaneous determination of total retinol. However, these drastic conditions have several disadvantages. Etretinate could only be determined as its free acid, acitretin, which is also a metabolite of etretinate [65]. In addition, extensive *cis–trans* isomerization (25–60%) occurred [83].

In contrast, considerably milder conditions could be attained with the column-switching method. After homogenization in ethanol–water (3:2) and centrifugation, the supernatant could be directly injected onto the precolumn for automated solid-phase extraction. This approach could be used for the determination of first-, second- and third-generation retinoids in skin and other tissue samples [84]. A similar on-line extraction was performed after freezing mouse embryos in ethanol–2-propanol (1:2) in liquid nitrogen overnight, followed by sonication [29].

4 CHROMATOGRAPHIC TECHNIQUES

4.1. General

In this review only HPLC and GC–MS of retinoids will be discussed. For older, conventional LC techniques (such as alumina, silicic acid or liquid-gel chromatography) the reader is referred to the excellent article by Frolik and Olson [3] Thin-layer chromatography (TLC), which was also used in the past (see ref. 3), was applied in one example for the determination of RAs, etretinate or the arotinoid Ro 13-6298 in mouse skin. Silica gel plates were developed with hexane–diethylether–acetic acid (90:10:1) or methanol–chloroform (1:1), and zones were scraped for radioactivity measurement [79]. Stability problems on the TLC plate prevented multi-dimensional development In a further investigation TLC was replaced by HPLC [85].

HPLC and GC–MS will be discussed in the following paragraphs. A few general comments should be made first. Whereas the use of an I.S. in GC–MS is unavoidable, in HPLC methods it can sometimes be omitted, especially in column-switching methods, where the recovery during the on-line solid-phase extraction is often quantitative [86]. However, this is often not the case with retinoids [56] and, therefore, the use of an I.S. is also strongly recommended for

classical liquid–liquid extraction methods. In addition, a correctly chosen I.S. may also compensate for possible photoisomerization or oxidation of the analyte. For the simultaneous determination of an ester and its corresponding acid, two I.S. (an ester and a carboxylic acid) may be appropriate [28]. It could even be relevant to consider the right geometrical isomer as I.S.

Another difficulty is the calibration with samples that already contain the added analyte as an endogenous compound (*e.g.* retinoic acids). This is mainly a problem for very sensitive methods [quantification limit (QL) < 5–10 ng/ml]. Plasma from animals depleted in vitamin A could be used for the preparation of calibration samples, or the endogenous retinoids could be destroyed by ultraviolet (UV) irradiation before spiking [14,38]. However, this latter method can produce erratic results [46] and, therefore, plasma may be replaced by phosphate-buffered saline [46] or serum albumin solution [29]. Since this albumin solution might also contain some retinoic acid [43] and because the calibration matrix should be as similar as possible to the matrix of the analyte, the standard addition of known amounts to the matrix, containing the endogenous levels of the analyte, may be more appropriate. In this way, the calibration curves and the concentration intercept can be calculated and the added concentration corrected [57].

4.2. Gas chromatography–mass spectrometry

Older publications have shown the difficulty of using GC for the determination of vitamin A [3]. This is due to the instability of the polyene system at elevated temperatures Despite these problems, GC has been used in combination with MS for the determination of RAs and, with more success, for third-generation retinoids. De Ruyter *et al.* [43] used GC–MS with selected-ion monitoring (SIM) for RA. After extensive sample clean-up and derivatization with diazomethane, methyl retinoate was separated on a glass-silanized column filled with 1% FFAP on Gas Chrom Q. Electron-impact (EI) MS at *m/z* 314 showed a peak for endogenous RA, but without separation of *cis* and *trans* isomers Chiang [44] used dimethylformamide dimethylacetal for conversion of retinoic acids to methyl retinoate, followed by separation on a glass column packed with 3% SE-30 on Chromosorb W HP. Although a separation between all-*trans* and 13-*cis* isomers of methyl retinoate was reported, and a detection limit of 2 ng/ml was claimed, no endogenous levels could be found in human plasma.

Napoli's group [14,38] also investigated the use of GC–MS for the determination of physiological levels of RAs. They found that negative-ion chemical ionization (NICI) resulted in about ten-fold greater sensitivity than EI or positive-ion chemical ionization (PICI). A detection limit of 75 pg could be achieved using a deuterated RA as I.S., methylation with diazomethane, and a glass column packed with 3% SP100-DOH on Supelcoport [14,38]. This was the most sensitive method reported for a retinoid at that time. However, it has to be noted that the all-*trans* and 13-*cis* isomers of the methyl retinoates could not be resolved, or, at

best, only partially resolved, or that a HPLC pre-separation had to be performed.

Lambert *et al.* [41] briefly reported a GC–MS method for the arotinoid Ro 13-6298. After extraction and HPLC purification, separation was performed on a chemically bonded methyl silicone FSOT capillary, followed by SIM at *m/z* 361 The corresponding methyl ester was used as I.S., and a limit of detection or quantification of 100 pg/ml was obtained. The most sensitive method reported so far was published by Egger *et al.* [26] for the arotinoid free acid Ro 13-7410. After two solid-phase extraction steps and derivatization with pentafluorobenzyl bromide, two-dimensional capillary GC using zone-cutting was performed. The two fused-silica capillary columns consisted of a cross-linked coating of SE54 and OV240. NICI and SIM resulted in a quantification limit of 50 pg/ml. A chromatogram obtained by this highly sensitive method is shown in Fig. 3. However, to avoid time-consuming and cumbersome work-up and analysis under subdued light, the *E* isomer was converted into the *Z* isomer by UV irradiation. Although the authors did not find any *Z* isomer in human and dog plasma samples analysed in the dark and without irradiation, this procedure may not be ideal and demonstrates the problems of an extensive sample clean-up

In conclusion, GC–MS is not the method of choice for the determination of retinoids because of the extensive sample work-up needed and the problems associated with distinguishing between geometric isomers of first- and second-generation retinoids. However, very sensitive and specific methods can be developed for third-generation retinoids [26,41].

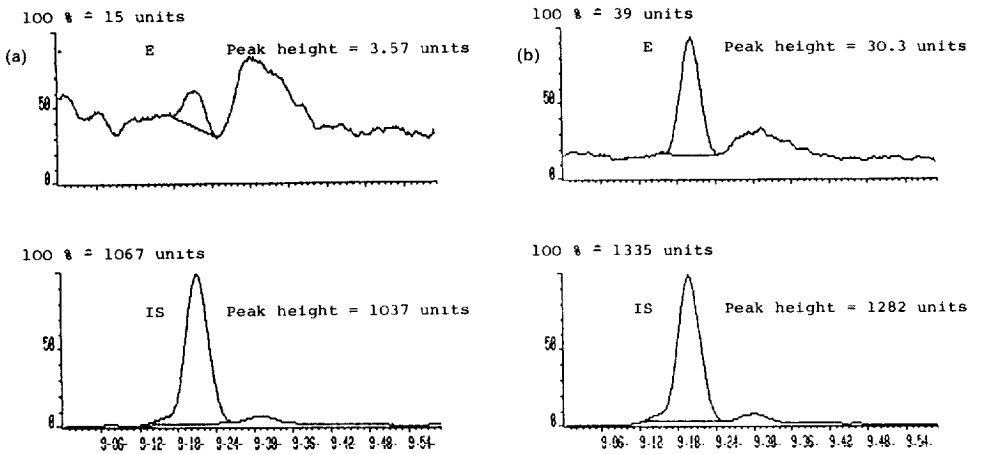


Fig 3. Two-dimensional capillary GC–MS of the arotinoid free acid Ro 13-7410, using NICI and SIM after derivatization with 2,3,4,5,6-pentafluorobenzyl bromide and UV irradiation to form the *Z* isomer Two fused-silica capillary columns (6 5 m × 0.32 mm I D) with a cross-linked coating of SE54 and OV240 (film thickness 0 4 μm) were used in series (a) Human blank plasma sample, (b) human blank plasma sample spiked with 50 pg/ml Ro 13-7410 Upper traces: pentafluorobenzyl derivative of *Z* isomer (*m/z* 347). Lower traces pentafluorobenzyl derivative of I.S (tetradeuterated analogue, *m/z* 351) Different scale factors are used. (Reproduced with permission from ref 26)

TABLE I

NORMAL-PHASE HPLC METHODS FOR FIRST- AND SECOND-GENERATION RETINOIDS

Analyte	Sample (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref
RA, 13-*cis*-RA	Blood, urine (1 ml)	1 *M* Phosphate buffer (pH 6), diethyl ether	Partisil	Dichloromethane–acetic acid (99 5 0 5)	365	DL 10–20 ng/ml	47
Acitretin, etretinate	Blood (1 ml), plasma (0 5 ml)	1 *M* Phosphate buffer (pH 7), ethyl acetate	Partisil	Dichloromethane acetic acid (99 1)	365	DL 10–20 ng/ml	47
Motretinide	Blood (1 ml)	1 *M* Borate buffer (pH 9), ethyl acetate	Partisil	Dichloromethane methanol acetic acid (97 5·1 5·1)	365	DL 10–20 ng/ml	47
Acitretin, etretinate	Plasma (0 5 ml)	0 1 *M* Citrate buffer (pH 6), hexane	LiChrosorb Si 60	Hexane–tetrahydrofuran–acetic acid (98·1 5·0 6)	360	DL 10–20 ng/ml	45
RA	Serum (3 5 ml)	Ethanol, 2 *M* NaOH and HCl, hexane	RSIL	Petroleum ether–acetonitrile acetic acid (99 5·0 2 0 3)	350	DL 0 3 ng/ml, QL 1 ng/ml	42
Acitretin, etretinate	Plasma (0 5 ml)	Methyl acetate, sodium sulphate, ethyl acetate	LiChrosorb Si 60	Hexane–tetrahydrofuran–acetic acid (200 3 1 2)	360	DL 4 ng/ml	23
RA, 13-*cis*-RA	Plasma, serum, cultured cells	NaCl, 0.025 *M* KOH and 4 *M* HCl, hexane	?	0 2% Acetic acid in hexane–dichloromethane (95 5) and others	340	DL 20 pmol	14
13-*cis*-RA, 13-*cis*-4-oxo-RA	Serum (0 1–0.5 ml), skin (10–50 mg)	80% KOH in ethanol, incubation, acidified hexane	Nucleosil	Gradient hexane–ethyl acetate (93 7 to 85 15) containing 0 5% ethanol and 0 05% acetic acid	365	DL 5 ng/ml, 20 ng/g	83

4.3 Normal-phase high-performance liquid chromatography

Normal-phase HPLC is older than reversed-phase HPLC. Therefore, normal-phase chromatography was first used for retinoid separations. Pure silica gel as stationary phase is especially suitable for apolar compounds, such as retinol and retinyl esters [3,5], and separation of geometrical isomers is better under these conditions. Another advantage could be the direct injection of organic extracts onto the analytical column, avoiding evaporation of the extraction solvent. For more polar compounds, such as retinoic acids and their metabolites, normal-phase HPLC was less often used, sometimes after esterification. However, for the simultaneous determination of polar metabolites, gradients are needed, and this

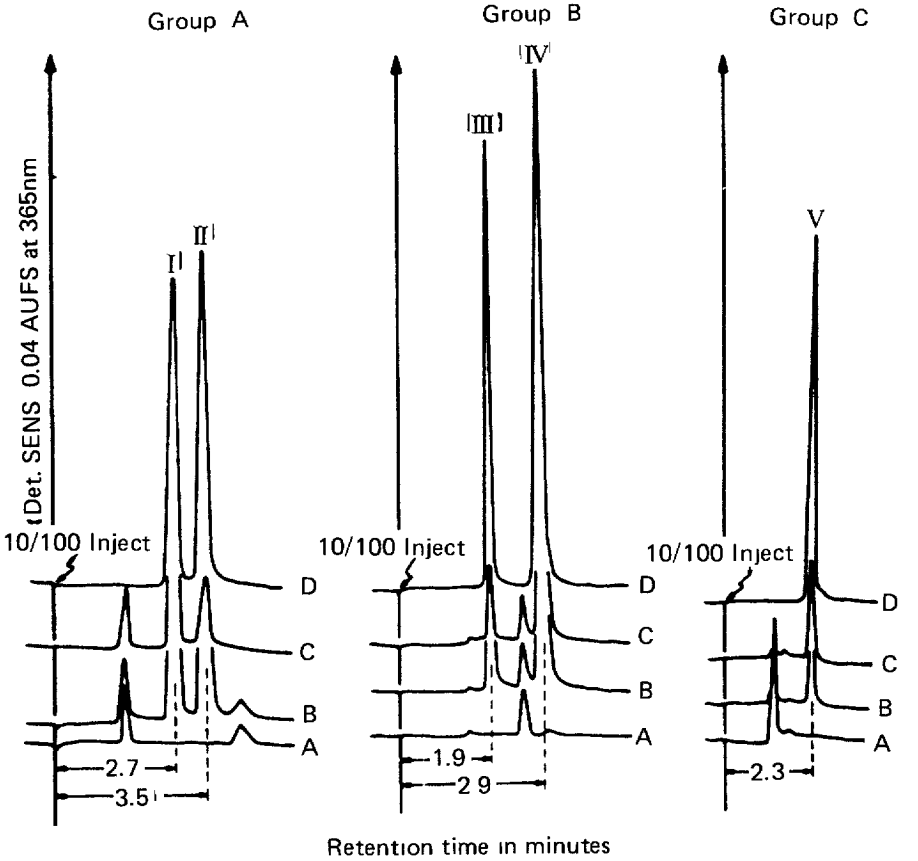


Fig 4 Normal-phase HPLC of (I) 13-*cis*-RA, (II) RA, (III) etretinate, (IV) acitretin, (V) ethylretinamide Column, 10-μm Partisil silica gel; mobile phase, dichloromethane–glacial acetic acid (99 5 0 5); flow-rate, 2 2 ml/min Traces: A = control blood; B = control blood extract containing recovered authentic standards, C = *in vivo* post-dosing specimen extracts, D = authentic standards (Reproduced with permission from ref 47.)

is easier with reversed-phase HPLC. Another disadvantage is poorly reproducible retention times.

Fig. 4 shows a normal-phase separation of several retinoid drugs. A list of drug assays is presented in Table 1, including chromatographic conditions. All these methods were validated to varying extents. Some of these methods also allow the simultaneous determination of retinol and, therefore, may not have been optimized for the determination of the analytes listed in Table 1. Several other assays were reported using normal-phase HPLC for metabolite purification and identification [38,41,43,53,67,68,72,74,75,87,88]. However, these methods are either too complicated for routine analysis, or have not been sufficiently validated or described.

4.4. Reversed-phase high-performance liquid chromatography

Reversed-phase HPLC is the method most often used for retinoids, because it is rapid and non-destructive, *i.e* it gives quantitative recoveries. Furthermore, sensitivity and resolution are good, and the method can cope with compounds within a wide polarity range. Gradient elution is quite common, and direct injection of biological fluids is also possible in combination with column-switching techniques. Fig. 5 shows the wide polarity range of first-generation retinoids that can be analysed using reversed-phase HPLC with gradient elution For very li-

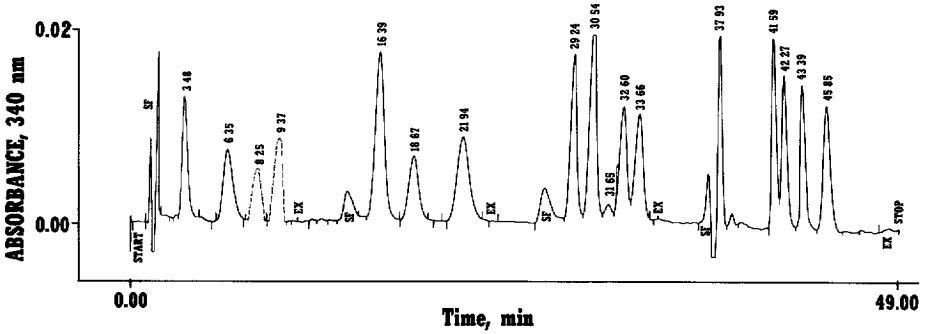


Fig 5 Reversed-phase HPLC of first-generation retinoid standards with a wide difference in polarity Two columns were arranged in tandem a 70 mm × 4 6 mm I D precolumn filled with Co-Pell ODS and a 250 mm × 4 6 mm I D Partisil ODS-2 column A multi-step gradient was used as mobile phase methanol–water (70 30), containing 0.01 *M* ammonium acetate for 12 min, methanol–water (80 20), containing 0 01 *M* ammonium acetate for 12 min, methanol–water (88 12) for 10 min, methanol–chloroform (83 17) for 15 min SF is the solvent front and EX is a signal to change to the next solvent. Broken lines refer to biologically generated standards Retention times correspond to the following compounds 3 48 min, 4-oxo-RA, 6 35 min, 5,6-epoxy-RA; 8 25 min, 13-*cis*-retinoyl β-glucuronide, 9 37 min, retinoyl β-glucuronide, 16 39 min, 13-demethyl-RA, 18 67 min, 13-*cis*-RA, 21.94 min, RA, 29 24 min, 4-HPR; 30 54 min, 13-*cis*-ethylretinamide; 31 65 min, 13-*cis*-retinol, 32 60 min, retinol, 33 66 min, retinal, 37 93 min, retinyl acetate, 41 59 min, retinyl linolenate, 42 27 min, retinyl linoleate, 43 39 min, retinyl palmitate and retinyl oleate, 45.85 min, retinyl stearate (Reproduced with permission from ref 40)

TABLE 2

REVERSED-PHASE HPLC METHODS FOR FIRST-GENERATION RETINOIDS

Analyte	Sample (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref
RA, 13-*cis*-RA	Plasma (0 5 ml)	Lyophilization, methanol	Partisil ODS-2	Acetonitrile–1% ammonium acetate (65 35)	350	DL 50 ng	20
13-*cis*-RA	Plasma (1 ml)	Methanol, 0 2 *M* acetate buffer, hexane–dichloromethane–2-propanol (80.19 1)	LiChrosorb RP-18	Acetonitrile-1% ammonium acetate (80 20)	350	DL 25 ng/ml	37
RA	Plasma (0 2 ml)	Lyophilization, methanol	Partisil ODS	Acetonitrile–1% ammonium acetate (55 45)	365 Radioactive	DL 10 ng/ml ?	31
RA, 13-*cis*-RA, 2-HER	Serum, urine, tissue	Homogenization, butanol–methanol (95 5)	Spherisorb ODS or Partisil ODS-2	Acetonitrile–1% ammonium acetate (5·3 or 2 1)	340 Radioactive	QL 2 ng DL 50–100 ng/g	15 64
13-*cis*-RA 13-*cis*-4-oxo-RA	Blood (1 ml)	1 *M* Phosphate buffer (pH 6), diethyl ether	Partisil ODS	Gradient methanol–water (58 42 to 86 14) containing 0 77 g/l ammonium acetate and acetic acid (pH 6 65)	365	QL 10 ng/ml QL 50 ng/ml	22
RA, 13-*cis*-RA	Serum (0 1 ml)	Acetonitrile	Spherisorb ODS	Acetonitrile 1% ammonium acetate (75 25)	340	QL 100 ng/ml	35
RA, 13-*cis*-RA	Serum (1 ml)	Butanol acetonitrile (1 1), dipotassium hydrogenphosphate	Partisil ODS-2	Acetonitrile–water–acetic acid (79 5 20 0 5)	360	DL 20 ng/ml	9
RA, 13-*cis*-RA	Plasma (0 5 ml), urine (1 ml)	5% Perchloric acid, ethyl acetate	Bio-Sil ODS-10	Acetonitrile–1% ammonium acetate (75 25)	340	DL 20 ng/ml	11,54
13-*cis*-RA, 4-oxo-RA	Plasma (1 ml)	Methanol	C_{18} Radial-PAK	Gradient acetonitrile–1% ammonium acetate (40 60 to 80·20)	340	DL 15 ng/ml	17

RA, 13-*cis*-RA	Blood, serum (1 ml)	1 *M* Phosphate buffer (pH 6), diethyl ether	Partisil ODS-2	Gradient acetonitrile–water (8 2 to 9 1) containing 0 5% acetic acid	360	?	49
RA, 13-*cis*-RA, 4-oxo-RA, 5,6-epoxy-RA, retinoyl β-glucuronide, 13-*cis*-retinoyl β-glucuronide, 13-demethyl-RA, 4-HPR, 13-*cis*-ethylretinamide	Plasma, serum (0.1 ml) Tissue (0.1–1 g)	Lyophilization, methanol, hexane and others	Partisil ODS-2	Gradient methanol–water (70.30) containing 0 01 *M* ammonium acetate to methanol–chloroform (83 17)	340 Radioactive	QL 5 ng DL 10 pg	8 40
RA, 13-*cis*-RA	Serum (0 1 ml), liver, fat, skin (2–20 mg)	Acetonitrile–tetrahydrofuran–acetic acid (780 80 5) containing ammonium acetate and (for tissue) sodium sulphate	Brown-Lee RP-18	Acetonitrile–tetrahydrofuran–water–acetic acid (780 80 160 5) containing 1.6 g/l ammonium acetate	340	DL 0 5 pmol	36
13-*cis*-RA, 13-*cis*-4-oxo-RA	Blood (0 5 ml)	Butanol–acetonitrile (1 1), dipotassium hydrogenphosphate	Zorbax ODS	Gradient acetonitrile–water- acetic acid (1000·1000·10 to 1900·100·0 8) containing 0 77 g/l ammonium acetate	365	QL 10 ng/ml	12
RA	Skin	Chloroform–methanol (1.1)	Spherisorb ODS	Gradient methanol–0 01 *M* ammonium acetate (70 30 to 80 20)	340 Radioactive	DL 10 pmol	85
RA, 13-*cis*-RA, 13-*cis*-4-oxo-RA	Plasma (0 5–3 ml)	Phosphate buffer (pH 7), diethyl ether–ethyl acetate (1·1)	Nucleosil C_{18}	Methanol–1% acetic acid (85 15)	360	DL 2 ng/ml	52
RA, 13-*cis*-RA, 4-oxo-RA, 13-*cis*-4-oxo-RA	Plasma (0.5 ml)	9 m*M* Sodium hydroxide–acetonitrile (8 2), solid-phase C_{18} on-line	Spherisorb ODS 1	Gradient acetonitrile - water–acetic acid (60 40 3 to 85 15 1) containing 0 04% ammonium acetate	360	QL 2 ng/ml	21,57

(Continued on p 498)

Table 2 (*continued*)

Analyte	Sample (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref
RA, 13-*cis*-RA, 4-oxo-RA, 13-*cis*-4-oxo-RA	Plasma, amniotic fluid (0 1–0 2 ml), embryo	2-Propanol or ethanol–2-propanol (1 2) and liquid nitrogen, solid-phase C_{18} *on-line*	Spherisorb ODS 2	Gradient methanol–40 m*M* ammonium acetate (1 1) to methanol	354	QL 5 ng/ml or g	29
RA, 13-*cis*-RA, 2-HER	Tissue (0 5 g), serum (0.4 ml)	Homogenization, methanol	Spherisorb ODS	Acetonitrile–1% ammonium acetate (48·52)	365	DL 100 ng/ml	34
4-HPR, 13-*cis*-4-HPR, 4-MPR, 4-EPR, RA, 13-*cis*-RA, 5,6-epoxy-RA	Serum, tissue	Lyophilization, chloroform–methanol (2·1)	Partisil ODS-2	Gradient methanol-water (70 30) to methanol	350	DL 5–10 ng	70
RA, 2-HER	Plasma (0 25–0 5 ml)	Water, acetonitrile, acetic acid, solid-phase C_{18} off-line	Spherisorb ODS 1	Methanol–water (85 15) containing 30 m*M* ammonium acetate	340	QL 2 ng/ml	16
4-HPR	Plasma (0 2 ml), tissue	Lyophilization, methanol	Partisil ODS-2	Acetonitrile–water (4·1)	365 Radioactive	QL 2 5 pmol	60
4-HPR, 4-MPR	Plasma (0.5 ml)	Ethanol, 5% perchloric acid, ethyl acetate	Ultrasphere ODS	Acetonitrile–1% ammonium acetate (88 12)	365 340	DL 10 ng/ml	55
N-Acetylretinylamine, retinal acetylhydrazone	Tissue	Lyophilization or grinding with anhydrous sodium sulphate, diethyl ether	Spherisorb ODS	Acetonitrile–water (70 30 to 90·10)	325	QL 1 nmol/g	71

pophilic substances, *e.g.* retinyl esters, the use of non-aqueous reversed-phase (NARP) systems is an elegant solution [89].

As is shown in Table 2 for first-generation retinoids, C_{18} bonded phases are used with acetonitrile or methanol as organic modifiers in the mobile phase, usually in combination with ammonium acetate to improve the peak shape of carboxylic acid compounds. The appropriate stationary phase material should be used with either acetonitrile or methanol in the mobile phase, as investigated by Curley *et al.* [90]. These authors found that methanol should be used in combination with fully end-capped material, and acetonitrile with non-end-capped material, for optimal isomer separation

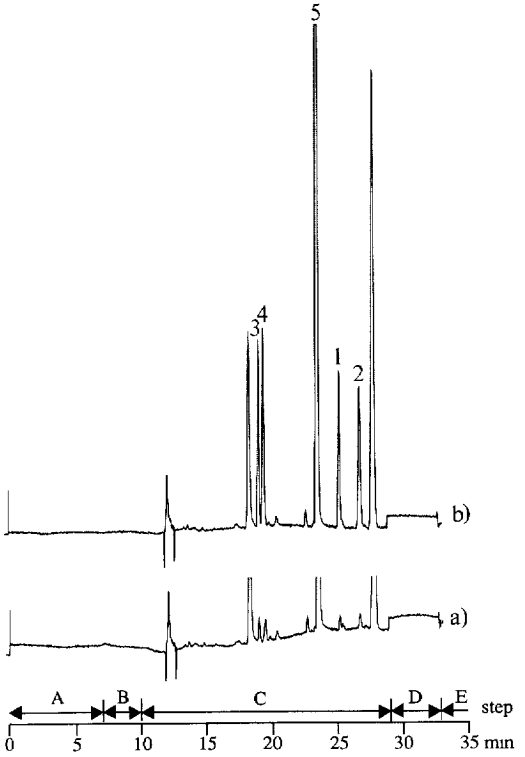


Fig. 6 First-generation retinoids in human plasma samples, analysed by reversed-phase HPLC with direct plasma injection and automated column switching (a) Blank plasma containing endogenous levels of 1 9–2 8 ng/ml of compounds 1–4, (b) blank plasma sample spiked with 20 ng/ml 1–4 and 150 ng/ml I.S Peaks 1 = 13-*cis*-RA, 2 = RA, 3 = 13-*cis*-4-oxo-RA, 4 = 4-oxo-RA, 5 = I S (acitretin) Apparatus and conditions as described in Fig 2 M1 = 1% ammonium acetate–acetonitrile (9 1), M2 = gradient acetonitrile–water–acetic acid (60·40 3 to 85 15 1) containing 0 04% ammonium acetate Samples of 0 5 ml of plasma were mixed with 0 75 ml of 9 m*M* sodium hydroxide–acetonitrile (8 2) containing the I S , and 0 5-ml aliquots were injected Step A proteins and polar components are washed out from PC, step B PC is purged in the backflush mode, step C after valve switching, the retained components are transferred from PC to AC1/AC2 in the backflush mode by M2, step D purge of the capillaries by M2, step E re-equilibration of PC and AC1/AC2 with M1 and M2, respectively (Reproduced with permission from ref. 57.)

TABLE 3

REVERSED-PHASE HPLC METHODS FOR SECOND-GENERATION RETINOIDS

Analyte	Sample (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref
Acitretin, etretinate	Plasma (1 ml)	0.1 *M* Phosphate buffer (pH 6), diethyl ether	LiChrosorb RP-18	Acetonitrile–water (8.2) containing 1% acetic acid	350	DL 10 ng/ml	13
Acitretin, etretinate	Plasma (1 ml)	1 *M* Phosphate buffer (pH 6), diethyl ether–ethyl acetate (75 25)	Spherisorb ODS	Acetonitrile–water (76 24) containing 1% ammonium acetate	350	DL 25 ng/ml	51
Etretinate	Serum (1 ml)	Butanol–acetonitrile (1 1), dipotassium hydrogenphosphate	Partisil ODS-2	Acetonitrile–water–acetic acid (79 5 20 0 5)	360	DL 30 ng/ml	9
Acitretin, etretinate	Blood, serum (1 ml)	1 *M* Phosphate buffer (pH 6), diethyl ether	Partisil ODS-2	Gradient acetonitrile–water (8 2 to 9 1) containing 0 5% acetic acid	360	?	49
Acitretin, 13-*cis*-acitretin, etretinate	Blood (0.5 ml)	Butanol–acetonitrile (1 1), dipotassium hydrogenphosphate	Zorbax ODS	Gradient acetonitrile–water–acetic acid (1000.1000 10 to 1900 100 0 8) containing 0 77 g/l ammonium acetate	365	QL 10 ng/ml	12

Acitretin, etretinate	Serum (0 1 ml), liver, fat, skin (2–20 mg)	Acetonitrile–tetrahydrofuran–acetic acid (780 80 5) containing ammonium acetate and (for tissue) sodium sulphate	Brown-Lee RP-18	Acetonitrile–tetrahydrofuran–water–acetic acid (780 80 160 5) containing 1 6 g/l ammonium acetate	340	DL 0 5 pmol	36
Acitretin, 13-*cis*-acitretin, etretinate	Plasma (1 ml)	1% Ammonium acetate (pH 6), diethyl ether	Nucleosil C_{18}	Acetonitrile–0.1 *M* ammonium acetate (8 2)	365	DL 1 ng/ml, 2 ng/ml	10
Acitretin, 13-*cis*-acitretin	Plasma, blood, urine (0 5–3 ml)	Phosphate buffer (pH 7), diethyl ether–ethyl acetate (1 1)	Nucleosil C_{18}	Methanol–1% acetic acid (85 15)	350 360	QL 2 ng/ml	24 52
Acitretin, 13-*cis*-acitretin, etretinate	Plasma (0.5 ml)	Ethanol, solid-phase C_{18} on-line	Spherisorb ODS 1	Gradient acetonitrile–water–acetic acid (700.300·3 to 850 150 1) containing 0 04% ammonium acetate	360	QL 2 ng/ml	28,57
Acitretin, etretinate	Blood (0 1 ml)	Butanol–acetonitrile (1 1), dipotassium hydrogenphosphate	Supelcosil LC-18	Acetonitrile–water (79 21) containing 0 8 g/l ammonium acetate and 10 ml/l acetic acid	360	QL 160 ng/ml, 80 ng/ml	18
Acitretin, 13-*cis*-acitretin, etretinate	Blood (1 ml), bile (0 5 ml)	1 *M* Phosphate buffer (pH 6 or 7), diethyl ether or ethyl acetate	Zorbax ODS	Gradient acetonitrile water–acetic acid (1000·1000 10 to 1900·100 0 8) containing 0 77 g/l ammonium acetate	365	QL 5 ng/ml, 10 ng/ml	50

The same observation was also made by other authors [12,21] A good separation of two pairs of geometrical isomers with a rather high difference in polarity is shown in Fig. 6. Interestingly, the sequence of the 4-oxo metabolites, but not of the RAs, is reversed by replacing Spherisorb ODS 1 by Hypersil ODS [21]. A separation of 9-*cis*-, 11-*cis*-, 13-*cis*- and all-*trans*-RA was reported by Bhat and Lacroix [91], using a Zorbax NH_2 column and acetonitrile–dichloromethane (9:1) with 10 m*M* acetic acid. This is, therefore, an example of a bonded phase being used under straight-phase conditions. It should also be kept in mind that some of the methods presented in Table 2 include the separation of retinol and other retinoids not covered in this review, which has some influence on the chromatographic conditions.

Apart form these validated methods for routine determination, many other papers have appeared, reporting metabolic work or non-routine assays. These include determination of RAs in plasma or serum [39,42,43,63] and different retinoyl β-glucuronides in serum [39] or bile [19,31–33,66] Retinotaurine has also been investigated in bile [33,67], as well as 13-*cis*-RA and 13-*cis*-4-oxo-RA in lacrimal gland fluid or tears [30] and a series of polar metabolites of 13-*cis*-RA in blood [48]. A number of publications have dealt with the analysis of tissue samples of RA and its metabolites [6,7,61,72–75,78,80,81,92] or 4-hydroxyphenylretinamide and metabolites [59,82]. RAs and their metabolites have also been determined in skin [69], different cell cultures [88,93,94] or microsomes and other liver homogenates [76,77,95–98].

A list of validated drug assays of second-generation retinoids for use in several biological fluids is presented in Table 3. For the accurate and sensitive determination of etretinate and its two isomeric carboxylic acid metabolites, gradient elution is normally needed. Fig. 7 shows an example of such a separation using the same chromatographic conditions as for 13-*cis*-RA [12]. Etretinate and acitretin were also determined in skin samples However, because of alkaline hydrolysis before extraction, etretinate was converted into acitretin and could not be distinguished from its metabolite [65]. The only HPLC method reported so far for a third-generation retinoid was developed for temarotene and its hydroxy metabolite [25]. A chromatogram is shown in Fig. 8

4.5. Detection in high-performance liquid chromatography

Owing to the high absorption maxima (>300 nm) and high extinction coefficients ($>10^4$) of the retinoids, UV detection is widely used in connection with HPLC. Detection limits of 2–5 ng (on column) are normally easily achieved. However, because of different definitions for limit of detection (sometimes defined by a signal-to-noise ratio of *ca* 3) and limit of quantification (most often defined as the lowest concentration that can routinely be determined with acceptable precision and accuracy), it is difficult to compare the sensitivities of published methods. It appears that a quantification limit of 2 ng/ml represents the state-of-the-art, provided that not more than 1 ml of biological fluid is used. However,

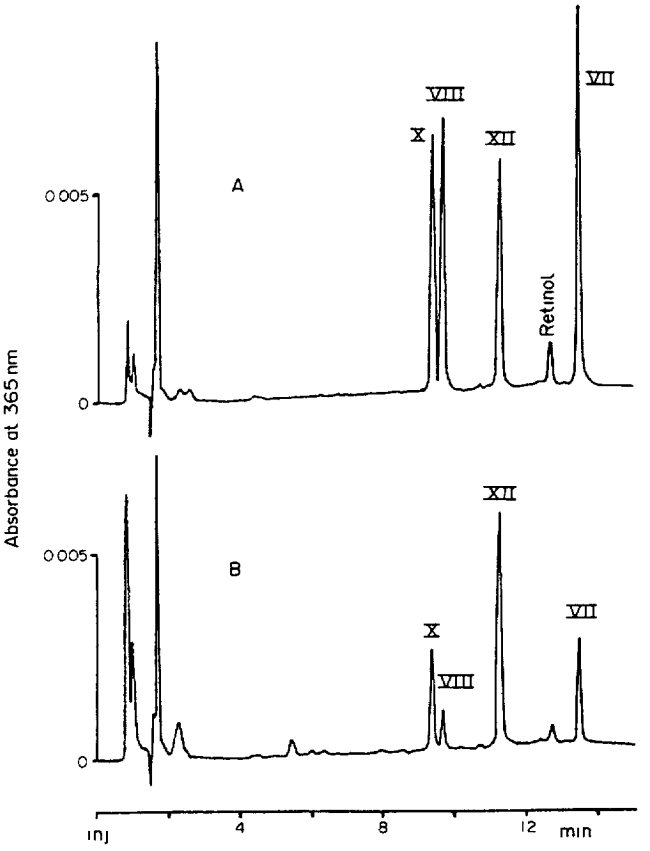


Fig 7 Reversed-phase HPLC of blood extracts of second-generation retinoids (A) Control blood spiked with 280 ng/ml VII, VIII and X, (B) blood from a psoriatic patient 8 h after a morning dose of 25 mg of VII during the eighth week of multiple-dose treatment Concentrations VII, 69 1 ng/ml, VIII, 41 1 ng/ml, X, 117 ng/ml Peaks: VII = etretinate; VIII = acitretin, X = 13-*cis*-acitretin, XII = I S (a synthetic second-generation retinoid, structure not shown) Column, Zorbax ODS, mobile phase, gradient acetonitrile–water–acetic acid (1000 1000·10 to 1900 100 0 8) in 10 min, containing 0 77 g/l ammonium acetate, flow-rate, I 5 ml/min (Reproduced with permission from ref 12)

it is possible to improve this limit using HPLC with column-switching and injection of high plasma volumes. In this way, a quantification limit of 0.3 ng/ml, using 1 ml of plasma, was achieved for acitretin and 13-*cis*-acitretin [58]. This is better than the most sensitive GC–MS method for first- or second-generation retinoids. The arotinoid structure is only about half as sensitive for UV detection at wavelengths of 280–300 nm, and, therefore, quantification limits are two to four times higher than those for first- and second-generation retinoids. Although fluorescence and electrochemical detection have been used for retinol [5], no methods using these techniques have been reported for RA or second- and third-generation retinoids. A great deal of metabolic work has been published, using

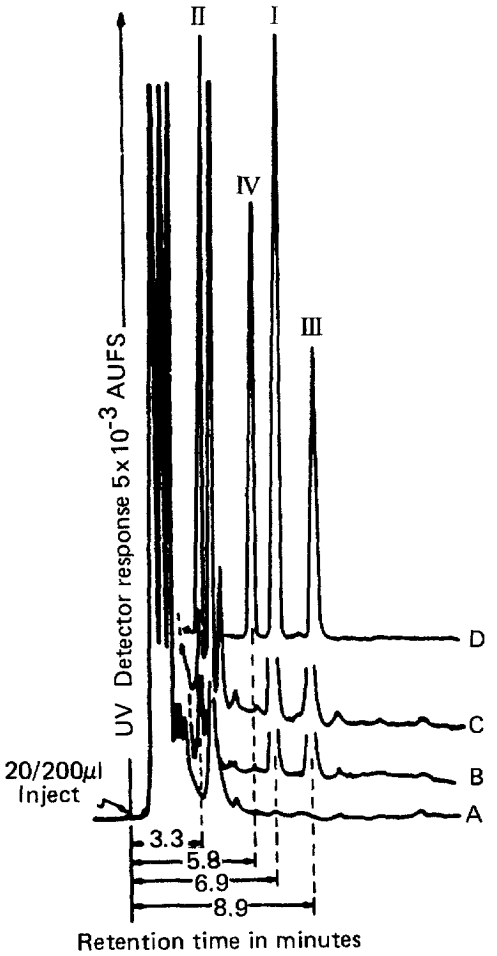


Fig. 8 Reversed-phase HPLC of third-generation retinoids (A) Control dog plasma, (B) dog plasma following oral dosing of I at 400 mg/kg on day 1, (C) authentic standards of I, II and III recovered from control dog plasma, (D) authentic standards of I–IV (equivalent to 500 ng/ml each, except III, which is equivalent to 200 ng/ml). Peaks I = temarotene; II = Ro 14-6113, III = Ro 13-9272 (I.S.); IV = *Z* isomer of temarotene Column, Sepralyte C_{18}, 5 µm, mobile phase, methanol–acetonitrile–water (90 6 4), flow-rate, 2 0 ml/min, detection, 280 nm (Reproduced with permission from ref 25)

either scintillation counting of collected HPLC fractions or a radioactivity detector on-line

An interesting technique is the coupling of a mass spectrometer to an HPLC column; such a method was recently developed for the quantification of endogenous RA in human plasma [46]. After an extensive extraction and clean-up procedure, consisting of three liquid–liquid extractions with hexane, evaporation of the solvent, conversion into the pentafluorobenzyl ester and a second evaporation step, RAs were purified using reversed-phase HPLC. The evaporated fractions

were dissolved in the mobile phase, hexane–toluene (85:15), and separated on a diol column (1 mm I.D.). The flow (50μl/min) was directly introduced into the mass spectrometer. Quantification was performed at *m/z* 299 using NICI-MS. The limit of quantification was 0.5 ng/ml using 1 ml of plasma. In view of the very complicated sample work-up and some recovery problems, resulting in a slight underestimation of the amount of RA, this method still has scope for improvement. Using this HPLC–MS method, 0.9 ng/ml RA and 1.6 ng/ml 13-*cis*-RA have been found in human plasma [46] These endogenous levels are in agreement with those found by other groups (1–4 ng/ml) [38,39,42,43,57]. High levels of RA (*ca.* 4 ng/ml [38]) may be due to hydrolysis of retinoyl β-glucuronide by the acid added during the extraction [39]. Therefore, the question of true endogenous levels of RA and 13-*cis*-RA may still be open, and milder methods with a minimum of sample preparation are needed. This demand could be met by HPLC column-switching techniques with direct plasma injection [58].

5 CONCLUSIONS

Specific and sensitive determination of retinoids is a difficult task, owing to isomerization and oxidation. High and strong protein binding and insolubility in aqueous solutions hamper the extraction from biological samples. Nevertheless, many extraction procedures have been developed during recent years, using mainly lyophilization of tissue samples or liquid–liquid extraction of biological fluids. Direct injection of these fluids or tissue homogenates, using HPLC column-switching techniques, simplifies sample work-up and provides much more gentle conditions. This new on-line extraction technique employs reversed-phase HPLC with UV detection, currently the method of choice for specific and sensitive determination of retinoids, including separation of geometric isomers and polar metabolites. GC–MS is not appropriate for first- and second-generation retinoids because of isomerization. However, third-generation retinoids can be determined with this technique at the picogram per millilitre level because of the greater stability of the arotinoid structure, although very extensive sample clean-up and derivatization are necessary. A similar extensive sample pretreatment has been used for the first HPLC–MS method. Provided that routine application is possible, GC–MS, HPLC–MS or even supercritical fluid chromatography–mass spectrometry (SFC–MS) will be of importance in the future, especially as reference methods. However, direct injection of high plasma volumes into HPLC systems, using automated column switching, appears to be sufficiently sensitive, and may become the method of choice for routine work.

6 SUMMARY

This article reviews the determination of retinoic acids and their metabolites (first-generation retinoids), aromatic retinoids (second generation) and aroti-

noids (third generation) in biological samples. Because of the sensitivity of the retinoids to isomerization and oxidation, special care has to be taken from sample collection and storage, throughout extraction, till the final chromatographic separation. High and strong protein binding, and insolubility in aqueous solutions hamper the extraction from biological samples. Various extraction procedures are discussed, mainly involving liquid–liquid extraction of biological fluids or lyophilized tissue samples. The new technique involving direct injection of biological fluids or tissue homogenates, using high-performance liquid chromatography (HPLC) with automated column switching, provides full protection from light and simplifies sample work-up.

HPLC with ultraviolet detection is the method of choice for the determination of retinoids, because it is rapid, sensitive and allows separation of geometric isomers and metabolites within a wide polarity range. Gas chromatography–mass spectrometry is not appropriate for first- and second-generation retinoids because of isomerization, but allows very sensitive determination of third-generation retinoids, although very extensive sample clean-up and derivatization are necessary. However, direct injection of large volumes of biological fluids into HPLC systems, using on-line solid-phase extraction and automated column-switching, results in very sensitive methods even with simple ultraviolet detection and may become the method of choice for routine analyses.

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