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Review

Chromatographic and electrophoretic analysis of biomedically important retinoids

Ronald Wyss

Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd, PRPK, 68/121A, CH-4002 Basel, Switzerland

Abstract

The determination of retinol (vitamin A) and its metabolites, as well as synthetic retinoids, in biological samples is a challenging task due to the sensitivity of these compounds to light, heat and oxygen, high protein binding, separation of geometric isomers and determination of low endogenous levels. Numerous procedures for sample preparation have been published for biological fluids and tissues, consisting of solvent extraction, solid-phase extraction (off-line) and HPLC with column switching (on-line solid-phase extraction). The last-mentioned technique has several advantages, including a high degree of automation, no evaporation of extraction solvents, protection from light and higher sensitivity. Due to the favourable UV characteristics of most retinoids, HPLC with UV detection is most often employed, and photodiode array detection is becoming more and more popular. Fluorescence and electrochemical detection have found only a limited field of application, but the use of LC–MS resulted in a few highly sensitive methods. Reconsideration of GC through the use of better deactivated columns and cold on-column injection and evaluation of new promising separation methods, such as supercritical fluid chromatography and capillary electrophoresis, have shown preliminary encouraging results, but appear to reach the required sensitivity only by coupling to MS. Therefore, HPLC with UV detection is still the method of choice for highly sensitive and selective retinoid determination, as well as for high sample throughput and robustness.

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List of abbreviations

AASP	Advanced automated sample processor
AC	Analytical column
AcOH	Acetic acid
AS	Autosampler
BHT	Butylated hydroxytoluene
CE	Capillary electrophoresis
DL	Detection limit
DLI	Direct liquid introduction
ED	Electrochemical detection
em	Emission
4-EPR	N-(4-Ethoxyphenyl)-retinamide
ex	Excitation
FAB	Fast atom bombardment
FID	Flame ionization detection
FL	Fluorescence
GC	Gas chromatography
HEPES	Hydroxyethylpiperazine ethanesulphonic acid
HPLC	High-performance liquid chromatography
4-HPR	N-(4-Hydroxyphenyl)-retinamide (fenretinide)
IUB	International Union of Biochemistry
IUPAC	International Union of Pure and Applied Chemistry
LC–MS	Liquid chromatography–mass spectrometry
MEKC	Micellar electrokinetic chromatography
4-MPR	N-(4-Methoxyphenyl)-retinamide
NARP	Non-aqueous reversed phase
NCI	Negative chemical ionization
PBS	Phosphate-buffered saline
PC	Precolumn
PDA	Photodiode array
PFB	Pentafluorobenzyl
QL	Quantification limit
RA	Retinoic acid
RAG	Retinoyl β-glucuronide
RAR	Retinoic acid receptor
RBP	Retinol binding protein
RE	Retinyl ester
ROG	Retinyl β-glucuronide
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SFC	Supercritical fluid chromatography
SIM	Selective ion monitoring
SPE	Solid-phase extraction
TBA	Tetrabutylammonium
TEA	Triethylamine
THF	Tetrahydrofuran
TPS	Tandem precolumn selector
UV	Ultraviolet

1. Introduction

For many years, retinol (vitamin A) and its metabolites have been well known for their importance in nutrition [1] and vision [2]. The term "retinoid" was introduced by Sporn et al.

[3] to extend this class of molecules and to show the relationship to the steroids. The fixation on structures closely related to retinol and the lack of knowledge of the nature of the receptors that mediate the biological activity of the retinoids led to the following definition by the IUPAC-IUB joint Commission on Biochemical Nomenclature in 1982 [4]: "Retinoids are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner; all retinoids may be formally derived from a monocyclic parent compound containing five carbon–carbon double bonds and a functional terminal group at the terminus of the acyclic portion." This very rigid definition, bearing in mind the structures of natural (Fig. 1) and synthetic retinoids (Figs. 1 and 2), was already obsolete by the time it was laid down, because arotinoids such as Ro 13-7410 (TTNPB, Fig. 3) had already been shown to be more active in typical retinoid assays. Therefore, a new definition was proposed by Sporn and Roberts in 1985 [5]: "A retinoid be defined as a substance that can elicit specific biologic responses by binding to and activating a specific receptor or set of receptors, with the program for the biologic response of the target cell residing in

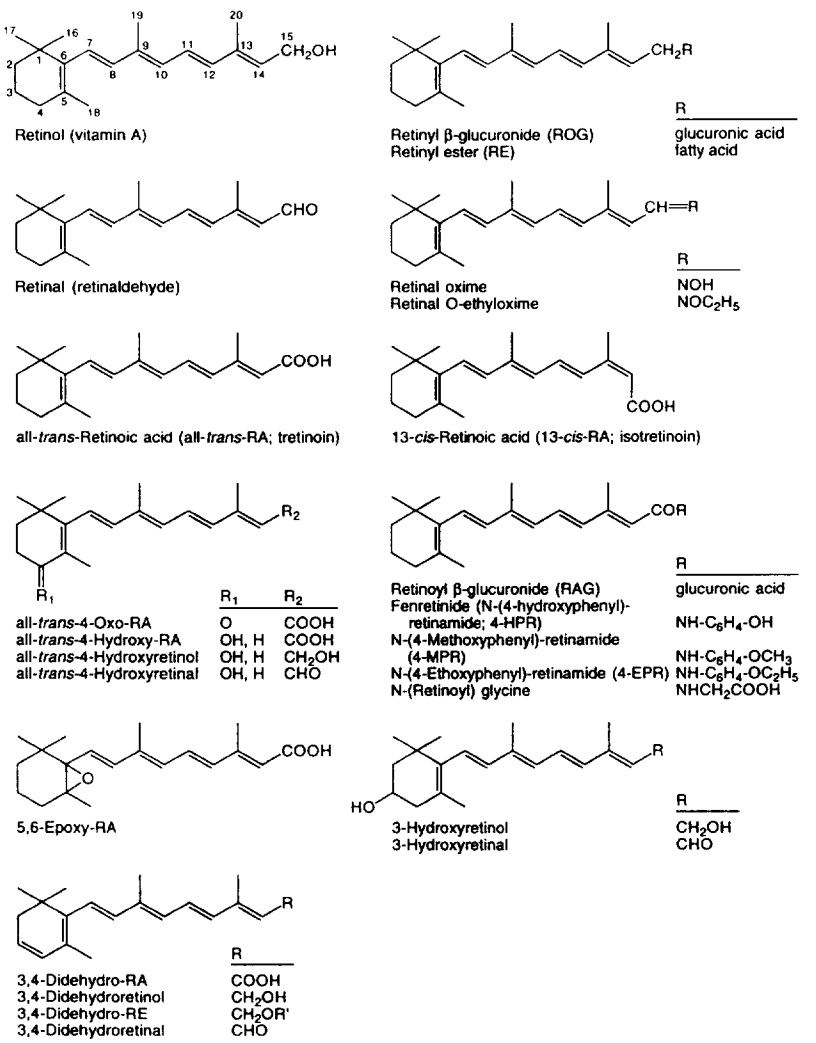

Fig. 1. Structures of natural and first-generation retinoids.

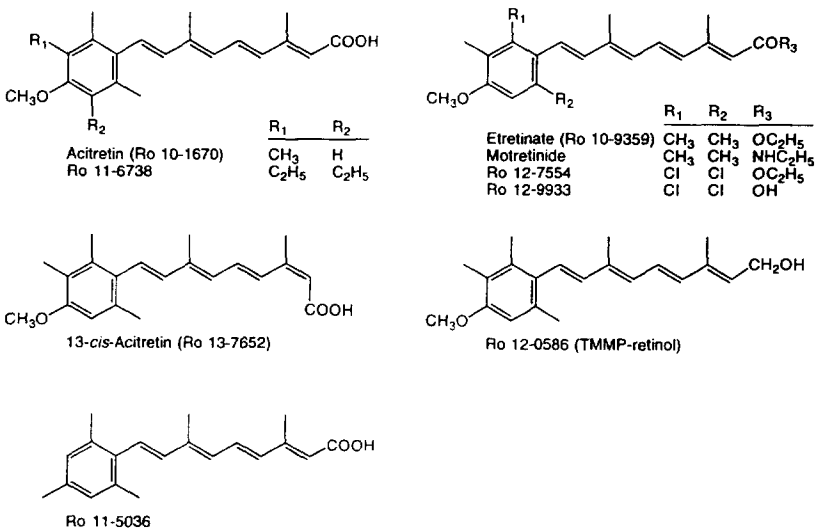

Fig. 2. Structures of second-generation retinoids.

the retinoid receptor rather than in the retinoid ligand itself."

However, the discovery of the retinoic acid receptors (RAR) [6,7] and the retinoid X receptors (RXR) [8], all members of the steroid/thyroid hormone-receptor superfamily, revolutionized retinoid research and revealed the retinoids as fundamental regulators of gene expression. 9-*cis*-Retinoic acid (9-*cis*-RA) was recently shown to act as a ligand for RXR [9–11]. A large number of publications clearly showed the important role of retinoids in cell differentiation and cell proliferation, morphogenesis, reproduction, vision, immunology, hematopoiesis, etc., and their effectiveness in the treatment of a variety of diseases in the fields of oncology [12] and dermatology [13]. However, retinoids are also toxic compounds manifesting typical problems ranging from mild hypervitaminosis to teratology. The state-of-the-art of retinoid research was recently reviewed in the 2nd edition of the monograph *The Retinoids* [14], and a large number of experimental methods may be found in two recently published volumes of *Methods in Enzymology* [15].

The great progress in retinoid research in the last 10–15 years has been brought about mainly by advances in techniques to separate these similar molecules, and would not have been possible without high-performance liquid chromatography (HPLC), which allowed differentiation of retinoid isomers, such as 11-*cis*- and all-*trans*-retinal or all-*trans*- and 9-*cis*-RA. Retinoids give rise to many analytical problems, including sensitivity to light, heat and oxygen, high and strong protein binding, insolubility in aqueous solutions, separation of geometrical isomers, and determination of low endogenous levels. However, in comparison to other lipids, the advantageous UV characteristics also enabled the development of highly sensitive and selective HPLC methods.

Substantial progress has been made in the separation of retinoids in biological samples, using chromatography and related techniques, since the author's last review in this journal [16]. The present review covers mainly the last five years, and also includes retinol, retinyl esters and retinal, as well as methods for multivitamin determinations. The term multivitamin determination is understood to mean the simultaneous determination of vitamin A, vitamin E (tocopherol) and/or carotenoids. However, emphasis is laid on the retinoids, and any special problems of the other vitamins are not discussed. The literature in this field has been reviewed since the appearance of the article of De Leenheer et al. [17]. Other reviews of retinoid

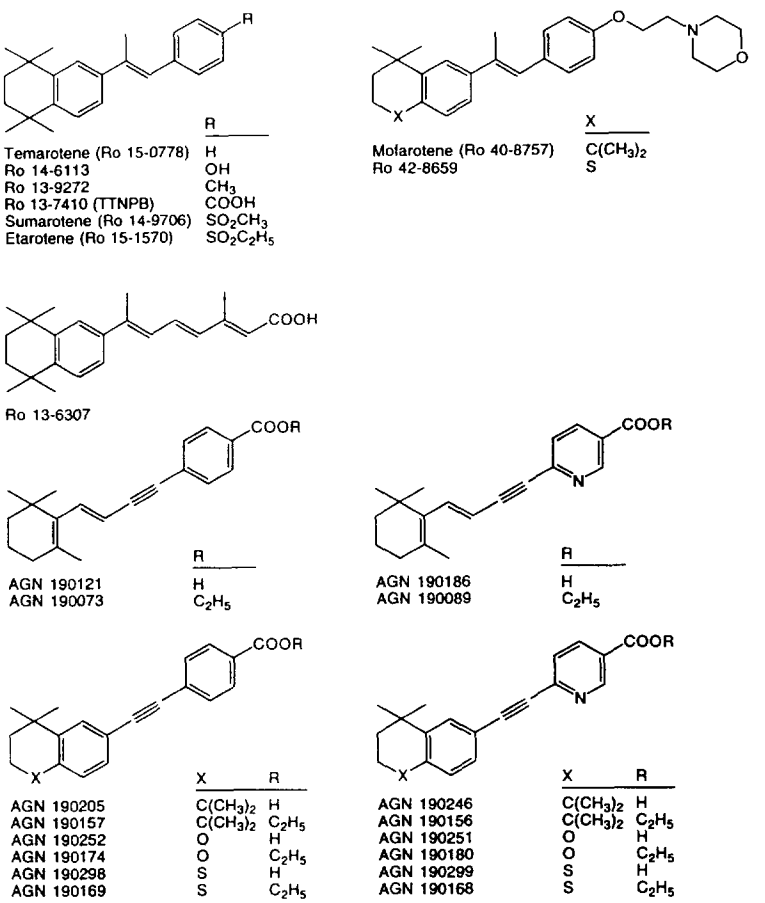

Fig. 3. Structures of third-generation retinoids.

analysis which appeared in the last few years include those of Bhat and Sundaresan [18], De Leenheer et al. [19] and Furr et al. [20,21].

In this article, validated assays for biological samples are emphasized, whereas applications or metabolic studies are only touched on. For a more comprehensive discussion of other aspects and the older literature, the reader is referred to the articles of Furr et al. [21] and Frolik and Olson [22].

2. Sample collection and storage

Retinoids are sensitive to light, oxygen and heat, all of which has to be considered in relation to sample collection and storage. Natural and first-generation retinoids (Fig. 1) are less stable than second-generation retinoids (Fig. 2). Third-generation retinoids (Fig. 3) give rise to less stability problems, but the precautions discussed below should still be taken. Photoisomerization of retinoids by irradiation was investigated for retinal [23–25], retinoic acids [26–29], as well as 13-*cis*-RA, acitretin and etretinate [30]. Photoisomerization in vivo, e.g. in skin, must also be considered, as was shown for etretinate, acitretin and 13-*cis*-acitretin [30], and, in vitro, for all-*trans*- and 13-*cis*-RA [31]. Therefore, reference compounds and biological samples should be protected from natural and fluorescent light; yellow light or at least subdued light should be used. Acitretin and 13-*cis*-acitretin were reported to be stable for 5 h under yellow light or in the

dark, but proved to be unstable under natural or artificial light [32]. Temarotene was stable under yellow and natural light for 6 h, but its metabolite Ro 14-6113 was only stable under yellow light and not under natural light during this time period [33].

Light conditions have also other consequences for sample collection; e.g., to investigate the composition of the retinoid isomers in the vision process, insects were dark-adapted for 2–24 h before enucleation of the eyes [34,35]. Isomerization of retinoids is a well-known in vivo process which may still be active under certain conditions after sample collection. Isomerization by thiol-containing compounds, such as the ubiquitous glutathione, has been reported for 13-*cis*- and all-*trans*-RA [36], as well as acitretin and 13-*cis*-acitretin [37]. This type of isomerization is influenced by the presence of acetonitrile which has been added to plasma samples before injection into an HPLC system with column switching [38].

Special precautions for storage of plasma or serum samples have been recommended [16], but apart from light protection, none appears to be essential. Craft et al. [39] found that a nitrogen purge of the vials before freezing did not affect the stability, when plasma samples containing retinol, tocopherols and carotenoids were stored for three months at −20°C. Retinol was found to be stable in the supernatant for 4 h at room temperature or 24 h at −20°C after precipitation of plasma proteins with 5% perchloric acid and ethyl acetate [40]. Retinol was also found to be stable in ethanol in the autosampler for 18 h at room temperature [39], but was unstable in water, PBS and culture medium at room temperature and at 37°C [40].

In whole blood, retinol proved to be stable for 24 h on ice [40] and in plasma for 4 h at room temperature or at 37°C [40]. Long-term stability of retinol in plasma was reported for up to eight years at −20°C [41]. Carotenoids were found to be unstable after 15 months at this temperature [39]. Therefore, and as a general precaution for long-term storage, plasma or serum used for multivitamin determinations should be stored at −70°C to ensure stability for five years [42] and probably for up to 15 years [41]. Retinol was reported to be stable in serum stored under nitrogen for 30 weeks at −70°C [43], and 13-*cis*-RA for 84 weeks at −90°C [44]. All-*trans*- and 13-*cis*-RA and their 4-oxo metabolites were stable in plasma of several species for 3 months at −20°C, but not after 9 months at this temperature. However, at −80°C, stability was still ensured after 9 months [45]. Tests on long-term storage for more than one year at −80°C are still in progress [45].

Etretinate was reported to be stable in plasma at −20°C for 8 weeks [46], and acitretin and 13-*cis*-acitretin for 90 days [47], at least. Large differences were found in the stability of the 16 acetylenic retinoids shown in Fig. 3 during short-term storage in acetonitrile, blood and urine [48]. Other third-generation retinoids showed good stability, as reported for temarotene for 60–70 days at −17°C [49], sumarotene for 3 months at −20°C [50] and Ro 13-7410 for 7.5 months at −20°C [51].

3. Sample preparation

3.1. General

Recommendations for the general handling of retinoids have been reported [21,52]. Care should be taken with reference compounds. For drug analysis, released batches are usually available. Other retinoids, especially unstable compounds, such as retinol, should be carefully checked for purity by HPLC and absorption spectroscopy before use, even when commercial sources state a high purity.

For the extraction from biological samples, protection of the retinoids from light, oxygen and heat has to be considered, as already mentioned for sample collection and storage. Although antioxidants were often used, it does not seem to be mandatory. No stability improvement was observed for retinol or other vitamins in plasma or serum, when butylated hydroxyanisole [53], butylated hydroxytoluene (BHT) [39,54,55] or ascorbic acid [39,42] were added before the extraction. On the contrary, interferences from

these antioxidants have been observed occasionally. Therefore, and because of the limited space, antioxidants are not listed in the tables or mentioned in the text. However, ascorbic acid was reported to improve the recovery during incubation of tissue samples with collagenase [56]. Owing to the special properties of the retinoids, use of an internal standard (I.S.) is highly recommended to compensate for variabilities in the protein binding or solubility during the extraction process. Typical internal standards are noted in Tables 1–5.

For most of the following extraction procedures, space does not permit the analytes to be specifically mentioned. However, not every extraction solvent is suitable for every retinoid, and the polarity of the analytes needs to be considered for each specific procedure.

3.2. Solvent extraction

3.2.1. Biological fluids

Only a few examples exist where no extraction was performed, and biological samples were injected onto the analytical column. These include injection of plasma samples for the determination of retinol bound to retinol binding protein (RBP) by size-exclusion chromatography [57], the injection of bile [58], cerebrospinal fluid [59] or lacrimal gland fluid and tears [60–64]. Protein precipitation of plasma or serum with a water-miscible organic solvent and injection of the supernatant into the HPLC system is a very fast and simple procedure. Disadvantages may be low sensitivity because of absence of a concentration step, or deterioration of the analytical column due to injected residual proteins. Furthermore, the solubility of the analytes in the supernatant may be critical, as well as peak broadening after injection of large volumes of solutions with high elution strength. The following solvents were used for deproteination of plasma, serum or bile, mainly for carboxylic acids: methanol [65–67], ethanol [68–71], acetonitrile [72–76], and methanol, acetonitrile plus perchloric acid [44]. Butanol–acetonitrile (1:1) was selected for blood, followed by evaporation of the supernatant and reconstitution in the mobile phase [48,77].

Usually the extraction is started with a protein precipitation step to liberate the analytes from the protein, followed by liquid–liquid extraction. The most popular procedure consists of ethanol for deproteination and one [39,54,63,78–85], two [86–90], three [56,91] or even four [92] consecutive hexane extractions. Addition of hydrochloric acid was used to extract acidic compounds [93], but the amount of acid must be low to avoid the hydrolysis of glucuronides [94,95]. Sometimes, an additional wash step with sodium hydroxide and hexane [96,97], or water addition [98–102] is incorporated. Petroleum ether was also used instead of hexane [103,104], as well as perchloric acid and ethyl acetate–THF (1:1) [105], or butanol–ethyl acetate (1:1), followed by addition of sodium sulphate [106]. After protein precipitation with ethanol, Barua et al. extracted RA, retinoyl β-glucuronide (RAG), etc., with ethyl acetate alone [94], or, after acidification with acetic acid, additionally with ethyl acetate and hexane [107–109]. The last procedure was also employed for retinol and other vitamins, but without acetic acid addition [55,110]. In another multivitamin extraction, 10 m*M* SDS was added to the plasma before the ethanol, and the final extraction was performed with heptane [111].

Methanol was another solvent used for the initial deproteination, followed by extraction with chloroform [43], dichloromethane [53] or petroleum ether–dichloromethane–2-propanol (80:19.3:0.7) [97]. The last mixture was identical to the mobile phase for normal-phase HPLC, and was directly injected. The same procedure was also possible with a butanol–methanol (95:5) extraction and injection into a reversed-phase HPLC system [112]. Acidic methanol was chosen as well, followed by hexane extraction [113,114]. Alternatively, saline was added prior to methanol, and the extraction was performed with chloroform [115] or hexane–butyl chloride–acetonitrile–acetic acid (90:15:5:0.01), followed by direct injection onto a silica column [116].

After deproteination with acetonitrile, the extraction was performed with hexane [97,117] or, in combination with the addition of water or

buffer, with hexane [118], hexane–2-propanol [119], diethyl ether [120] or *tert.*-butyl methyl ether [49]. The procedure of McClean et al. [121] consisted of deproteination of serum with butanol–acetonitrile (1:1), addition of saturated dipotassium hydrogenphosphate for phase separation and direct injection of the supernatant. It was adopted by others [122–126], including an initial acidification with acetic acid [127]. Another procedure for a multivitamin determination by direct injection of the supernatant consisted of deproteination with 2-propanol and extraction with dichloromethane [110].

Direct extraction of the biological fluid with an organic solvent is also possible. Hexane was used after acidification with formic acid [128]. Extraction with diethyl ether was performed after addition of ammonium acetate [129,130], phosphate buffer [131–136], and methyl acetate plus phosphate buffer [137–139]. Samples were also extracted with diethyl ether–ethyl acetate (1:1) after addition of phosphate buffer [32,47,140–142] or borate buffer [143], or with ethyl acetate after the addition of borate buffer [33] or methyl acetate and sodium sulphate [46,144]. Acidification with perchloric acid, followed by ethyl acetate extraction, was used for direct injection [40,59,145–147] or evaporation of the solvent [148]. Likewise, the extraction was performed either with ethyl acetate–ethanol (9:1) after addition of acetate buffer [149], or with ethyl acetate–THF (1:1), followed by addition of saturated ammonium acetate and direct injection [150]. Another extraction solvent was chloroform–methanol (2:1), used either alone [151] or followed by saline addition and hexane extraction [152]. Chloroform–methanol (2:1) was also employed after lyophilization of the serum sample [153–155].

Alkaline hydrolysis with potassium hydroxide and ethanol, followed by hexane extraction, may be suitable for total retinol determination of plasma samples containing different retinyl esters [156], but proved to be problematic for individual determination of RA, etretinate and acitretin [30,157–159] because of hydrolysis of these esters and isomerization.

Glucuronides of RA or acitretin and their metabolites were hydrolyzed in urine with β-glucuronidase, or with 0.5 *M* NaOH, followed by ether extraction at pH 6.0 [126]. In bile, incubation was performed with β-glucuronidase, followed by direct injection [160] or extraction with ethyl acetate [161,162] or diethyl ether [163]. A similar procedure was also followed for lacrimal gland fluid, using a final extraction with ethanol [64].

Human breast milk was extracted with *tert.*-butyl methyl ether–ethanol or, after saponification with potassium hydroxide–ethanol, with hexane [158]. Other retinoid determinations in food are not considered and have been reviewed recently [164].

3.2.2. Tissues

The homogenization of tissue samples prior to extraction is dependent on the type and amount of tissue. Cell cultures or very soft tissue often need no special homogenization, or ultrasonication may be sufficient [70,135]. Tough tissue is usually homogenized in water, buffer or directly in organic solvent, using either a mechanical mixer or a glass homogenizer (Potter type). Some older, more complicated procedures, such as lyophilization, grinding with sodium sulphate or alkaline hydrolysis are discussed below.

Tissue samples were extracted with methanol [165–167], ethanol [68–71,168,169], acetonitrile [75] or acetone [165]. Addition of a water-miscible, followed by a water-immiscible solvent, can also be used for liquid–liquid extraction, e.g., methanol and hexane [170], methanolic hydrochloric acid and hexane [171], methanol and chloroform [115] or ethanol and hexane [172–174]. The latter combination was also successful after initial acidification with hydrochloric acid [175,176] or tissue homogenization in phosphate buffer [177–179]. Other extraction conditions consisted of the addition of acetone, phosphate buffer and hexane–dichloromethane (9:1) [180], or homogenization in petroleum spirit and diethyl ether, followed by phosphate buffer addition [135]. The serum extraction procedure of McClean et al. [121], consisting of the addition of butanol–acetonitrile (1:1) followed by dipotassium hydrogenphosphate, was also applied to cell

cultures [9,11,181–183]. Likewise, the same method was employed after initial acidification with acetic acid [184].

Embryos were extracted with methanol–dichloromethane (2:1), including saline addition before the final phase separation [185]. Similarly, chloroform–methanol (2:1) was used with [186] or without [167,187] saline addition. Another extraction solvent was chloroform–methanol–formic acid [188]. Skin samples were extracted after disruption and acidification with hexane [100,189] or dichloromethane [31]. The first extraction procedure was also used with an additional hexane washing step at basic pH [190,191]. Skin samples were either directly homogenized in diethyl ether–ethyl acetate (1:1) [142,192], or after initial borate buffer addition [143]. The latter procedure was also used for hepatocytes [193]. Limb buds were extracted with ethyl acetate–methyl acetate (8:1) after addition of PBS, containing antioxidants, saturated sodium sulphate and ethanol [10,194–197].

A special problem is the analysis of retinal, retinol and retinyl esters (RE) in samples from visual pigments. The use of a denaturating solvent, such as methanol, would result in isomerization of 11-*cis*-retinal when liberated from the enzyme. Therefore, Groenendijk et al. [198] carried out the methanol extraction of a pigment suspension by adding a 1000-fold molar excess of 1 *M* hydroxylamine. In this way, retinal isomers were converted to the corresponding oximes without loss of the original configuration. After methanol, water and dichloromethane were added (final ratio 1:1:1) for the final extraction; dichloromethane extraction was repeated. This procedure was also used by others with small modifications [199–202]. Goldsmith et al. [203] started the extraction of lyophilized insect eyes with an initial hexane extraction to remove carotenoids and other lipids before the hydroxylamine was added. Others [34,35,204] supplemented the final extraction mixture, consisting of methanol, water and dichloromethane, with hexane. A similar procedure was used for the extraction of pineal glands after homogenization in buffer, although no retinal was detected in this organ [205]. Van Kuijk et al. [206] added O-ethylhydroxylamine to bovine retina homogenates, followed by methanol, containing BHT as antioxidant and cholesterol as a lipid carrier, before carrying out a threefold hexane extraction. The formation of the O-ethyl oximes avoided the co-elution of retinol and retinal oxime which occurred under their chromatographic conditions.

In another approach, Suzuki et al. [207] used 6 *M* formaldehyde in phosphate buffer, instead of hydroxylamine and methanol, before extraction with dichloromethane–hexane. In this way, also taken by others [201,208,209], reactive amino groups of proteins were blocked, and the formation of *syn* and *anti* conformers in the oxime method was circumvented. The different methods for the determination of retinoid isomers in the vision process were reviewed by Bridges [210].

Lyophilization of a tissue sample is time-consuming. The subsequent solvent extraction was performed with methanol [64,211,212], methanol plus hexane [91,213,214], chloroform–methanol (2:1) [153–155], diethyl ether [120] or hexane [215,216]. The last solvent was directly injected into a normal-phase HPLC system for retinyl ester determination. Another less common procedure consisted of grinding the tissue sample with sodium sulphate to remove water, and subsequent extraction with dichloromethane [63,217–219], ethyl acetate and dichloromethane [107], chloroform [166] or chloroform plus methanol [91].

Alkaline hydrolysis is a rather drastic procedure for the digestion of tissues, consisting of incubation of the samples at alkaline conditions (ethanolic potassium hydroxide) at 60–80°C, and subsequent extraction with an organic solvent. As all ester compounds are saponified under these conditions, this method allows, e.g., total retinol determination from a mixture of different retinyl esters [23,63,156,178,220]. However, the procedure is less suitable for the determination of retinoic acids, acitretin and especially etretinate, which is hydrolyzed to acitretin under these conditions, due to extensive isomerization. Nevertheless, this sample work-up was applied to several types of tissue samples, mainly skin, and

was used in combination with petroleum ether [221], hexane [30,63,156,157,159,178,222–225], diethyl ether [23] and hexane–toluene (1:1) [220] as extraction solvents.

Another method for digestion of tissue samples is incubation with specific enzymes. This can be performed under milder conditions at 37°C. Breast tissue was incubated in the presence of collagenase and hyaluronidase, and the retinoids were extracted with methanol [226], or with collagenase and porcine lipase, followed by ethanol addition and hexane extraction [56]. For lung, colon and skin tissue, collagenase alone was used before extraction [56]. Peng and Peng [227] performed digestion of mucosal cells by a protease, followed by addition of SDS and ethanol, and a final hexane extraction. Solid tissues were also incubated with collagenase, homogenized mechanically, incubated a second time with protease, followed by SDS–ethanol addition and hexane extraction [228]. Enzymatic digestion of tissue samples was reported to be superior to alkaline hydrolysis [56,228].

3.3. Solid-phase extraction

Only a few methods have been reported describing off-line solid-phase extraction (SPE) for retinoids. The reason may be lack of good recoveries, when untreated plasma samples are applied to reversed-phase stationary phases. This problem can now be easily overcome, and will be discussed in detail in the next section, dealing with on-line solid-phase extraction. McPhillips et al. [229] precipitated plasma proteins with acetonitrile and diluted the supernatant with acetic acid before SPE on C_{18} cartridges. Egger et al. [51] performed a two-step SPE prior to a highly sensitive determination of Ro 13-7410 by gas chromatography–mass spectrometry (GC–MS). Plasma was extracted with *tert.*-butyl methyl ether, using Extrelut columns, followed by a second clean-up on amino columns.

All other SPE procedures were used after a classical liquid–liquid extraction. For the determination of endogenous levels of all-*trans*- and 13-*cis*-RA in serum, aminopropyl columns were used after solvent extraction [95,230]. Alumina oxide columns allowed the separation of retinyl esters in plasma or tissue extracts from other lipids, such as triglycerides [156,231]. In a similar way, liver sample extracts, obtained after solvent extraction, were applied to silica columns to remove phospholipids, before retinol, 3,4-didehydroretinol and other lipids were determined by HPLC [177,232].

3.4. Column switching

HPLC with column switching is a technique which makes use of directing one or several mobile phases through two or more columns by means of switching valves. This can be performed as multidimensional chromatography, but it is used predominantly as on-line solid-phase extraction, coupling together SPE and HPLC in a fully automated way [233]. Column switching is nearly as old as HPLC, but became important only when Roth et al. [234] had shown that biological fluids (plasma or serum) can be injected directly onto a precolumn (also called pre-concentration column) without any sample pretreatment. Performed under optimal conditions, proteins are washed out to waste, while the analytes are concentrated on the precolumn. After valve switching, the analytes are transferred to the analytical column and separated as usual by reversed-phase HPLC. In routine analysis, the samples from, at least, one working day (40–50 or more) can be injected onto the same precolumn before it becomes clogged and has to be replaced.

Although injection of untreated biological fluid is not always possible, this technique has some important advantages, especially for retinoid analysis. These are: a high degree of automation, no evaporation of extraction solvents, no problems with redissolving of extracts before injection into the HPLC system, protection from light during analysis and higher sensitivity compared to traditional extraction methods. HPLC with column switching can be performed with simple equipment, consisting of one valve and one or two pumps only. A more sophisticated column-switching system from the author's laboratory is shown in Fig. 4. Samples

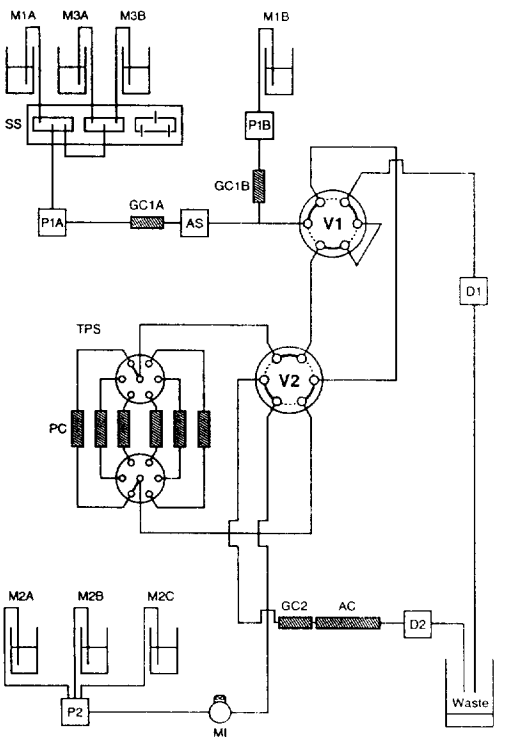

Fig. 4. Schematic representation of an HPLC column-switching system. P1A, P1B = HPLC pumps; SS = solvent selector; M1A, M1B = mobile phases (for injection); M3A, M3B = mobile phases (for purging); AS = autosampler; V1, V2 = switching valves; GC1A, GC1B, GC2 = guard columns; PC = precolumn; TPS = tandem precolumn selector; AC = analytical column; P2 = gradient pump; M2A, M2B, M2C = components of the gradient mobile phase; MI = manual injector (onto AC); D1, D2 = UV detectors.

are injected by an autosampler (AS in Fig. 4) and a mobile phase M1A onto one of the precolumns (PC). A second mobile phase (M1B) can be added, if required, by means of a T-piece, or other mobile phases (M3A and M3B) can be used for purging steps. Valve 1 allows forward- and backflush purging of the precolumn, and V2 is used for connecting the precolumn to the analytical column (AC), enabling backflush elution of the analytes from the precolumn and separation on the analytical column by the gradient system P2. The UV detector D2 is used for quantification, and an additional detector (D1, optional) may be used for monitoring of the precolumn out-flow. A tandem precolumn selector (TPS) enables automatic replacement of a precolumn when a defined pressure is reached.

Creech Kraft et al. [235] utilized a tandem precolumn system, consisting of two precolumns which were used simultaneously. Whereas one was loaded with a sample, the second one, connected in series with the analytical column, was eluted by mobile phase 2. This configuration reduces the total run time for one sample, but does not allow forward- and backflush purging of the precolumns or automatic precolumn replacement. Eckhoff and Nau [236] used a different system of on-line SPE. Samples were applied to C_2 cartridges in a cassette pre-conditioned in the usual manner. After one or several washing steps, the cassette was loaded onto an automated sample processor (AASP, Varian), and each cartridge was eluted, on-line, onto the analytical column. This modification has several advantages (batchwise sample application, flexibility in washing steps) and disadvantages (possible variations in recoveries between cartridges, limited sample loading capacity, higher price).

Because of the high protein binding of the retinoids, recoveries were disappointingly low (40–50% for all-*trans*- and 13-*cis*-RA), when untreated plasma samples were injected for the first time into the HPLC column-switching system. However, measures have been developed to overcome these difficulties [237]. As a result, the injection conditions described in Sections 3.4.1 to 3.4.3 have been used. Details of the most important methods can be found in Table 1.

3.4.1. Dilution

Addition of acetonitrile to the plasma sample improves the transfer of the retinoid from the protein to the stationary phase through an aqueous phase. When the final acetonitrile content is lower than 20%, plasma proteins are not precipitated. For the determination of RA and metabolites, plasma (0.5 ml) was diluted with 9 m*M* sodium hydroxide–acetonitrile (8:2) (0.75 ml) and 0.5 ml were injected [238,239], or, alternatively, diluted with 2% ammonium acetate–acetonitrile (5:1) before applying to an AASP cartridge [236,240,241]. Water or buffer addition can be omitted and acetonitrile only added to

Table 1
HPLC methods with column switching

Analyte	Matrix (amount used)	Pretreatment	Precolumn	Mobile phase 1	Analytical column	Mobile phase 2	Detection wavelength (nm)	DL or QL	Ref.
13-*cis*-, all-*trans*-RA, 13-*cis*-, all-*trans*-4-oxo-RA, acitretin (I.S.)	Plasma (0.5 ml)	+ 0.75 ml 9 m*M* NaOH–acetonitrile (8:2) 0.5 ml injected	Corasil C_{18}	1% Ammonium acetate–acetonitrile (9:1)	Spherisorb ODS 1	Gradient acetonitrile–water–10% ammonium acetate–AcOH (600:400:4:30 to 850:146:4:10)	360	QL 2 ng/ml	[238,239]
(A) 13-*cis*-, all-*trans*-RA, 13-*cis*-, all-*trans*-4-oxo-RA, 13-*cis*-acitretin (I.S.). (B) + RAG, ROG	Plasma (0.35 ml)	+ 0.6 ml 2% Ammonium acetate, 0.6 ml 2% ammonium acetate–acetonitrile (2:1)	C_2 cartridge AASP	0.5% Ammonium acetate–acetonitrile (85:15)	Spherisorb ODS 2 60°C	Gradient methanol–0.06 *M* ammonium acetate (57.5:42.5 to 95:5)	340 + 356	QL 0.5 ng/ml	(A) [236] (B) [240,241]
(A) Acitretin, 13-*cis*-acitretin, Ro 11-6738 (I.S.) (B) 13-*cis*-, all-*trans*-RA, 13-*cis*, all-*trans*-4-oxo-RA	Plasma (1 ml)	+ 0.2 ml Acetonitrile 1 ml injected	Corasil C_{18}	1% Ammonium acetate–acetonitrile (9:1)	Spherisorb ODS 1	(A) Gradient acetonitrile–methanol–water–10% ammonium acetate–AcOH (400:400:200:4:3 to 850:0:120:4:3 to 990:0:10:0:1) (B) excl. methanol (600:400:4:30 to 960:40:4:10 to 990:10:0:1)	360	QL 0.3 ng/ml	[242]

Table 1 (*continued*)

Analyte	Matrix (amount used)	Pretreatment	Precolumn	Mobile phase 1	Analytical column	Mobile phase 2	Detection wavelength (nm)	DL or QL	Ref.
Sumarotene, Ro 18-6776 (*Z*-isomer), etarotene (I.S.)	Plasma (1 ml)	+ 0.2 ml Acetonitrile 0.5 or 1 ml injected	Corasil C_{18}	1% Ammonium acetate–acetonitrile (85:15)	Spherisorb ODS 1	Gradient acetonitrile–water (70:30 to 95:5 to 99:1)	303	QL 1–2 or 0.5 ng/ml (0.5 or 1 ml)	[50]
Acitretin, 13-*cis*-acitretin, Ro 11-6738 (I.S.)	Plasma (1.2 ml)	+ 5 μl I.S. 1.0 ml injected	Corasil C_{18}	1% Ammonium acetate–acetonitrile (100:2, M1A) and (6:4, M1B)	Spherisorb ODS 1	Gradient acetonitrile–water–10% ammonium acetate–AcOH (600:400:4:30 to 950:50:4:10 to 980:20:0:1)	360	QL 0.3 ng/ml	[38]
Etretinate, acitretin, 13-*cis*-acitretin, Ro 12-7554 (I.S.), 13-*cis*-RA (I.S.)	Plasma (0.5 ml)	1 ml Ethanol 0.5 ml injected	Corasil C_{18}	1% Ammonium acetate + AcOH–acetonitrile (8:2)	Spherisorb ODS 1	Gradient acetonitrile–water–10% ammonium acetate–AcOH (700:300:4:3 to 850:146:4:1)	360	QL 2 ng/ml	[243,239]
Mofarotene, Ro 42-8659 (I.S.)	Plasma (0.5 ml)	1 ml Ethanol 0.9 ml injected	Corasil C_{18}	1% Ammonium acetate + AcOH–acetonitrile (9:1)	Superspher 100 RP-18	Gradient acetonitrile–water–10% ammonium acetate–AcOH (700:300:8:15 to 980:20:5:10 to 990:10:0:5)	300	QL 10 ng/ml	[244]

(*Continued on p. 394*)

Table 1 (*continued*)

Analyte	Matrix (amount used)	Pretreatment	Precolumn	Mobile phase 1	Analytical column	Mobile phase 2	Detection wavelength (nm)	DL or QL	Ref.
(A) 13-*cis*-, all-*trans*-RA, 13-*cis*-, all-*trans*-4-oxo-RA, retinol, (B) 13-*cis*-, all-*trans*-RAG, (C) acitretin, 13-*cis*-acitretin, etretinate, (D) motretinide	(E) Plasma, amniotic fluid (0.1–0.2 ml) (F) embryo, placenta, yolk sac	(E) 1 vol. 2-propanol, liquid nitrogen (F) 1 vol. ethanol, 2 vol. 2-propanol, liquid nitrogen, sonication 0.1–0.2 ml injected	LiChrosorb RP-18	0.04 *M* Ammonium acetate (pH 5.8)	Spherisorb ODS 2	(1) Gradient methanol–0.04 *M* ammonium acetate (55:45 to 100:0) (2) dito (71:29 to 100:0)	354	QL 5 ng/(ml or g) (0.1 ml or g sample)	(A,1) [235,248] (B,1) [249,250] (C,2) [246,247] (D,2) [245]
13-*cis*-, all-*trans*-RA, 13-*cis*-, all-*trans*-4-oxo-RA, retinol, retinyl acetate, laurate, myristate, palmitate/oleate, stearate, linoleate, linolenate, arachidinate	(A) Plasma, extraembryonic fluid (0.1 ml) (B) embryo, yolk sac (100 mg) (C) placenta (50 mg) (D) liver	(A) 3 vol. 2-propanol, 0.24 ml + 1.2 ml acetate buffer injected (B) dito, incl. sonication (C) dito, +50 μl water (D) dito, after homogenization in 9 vol. water	C_2 cartridge AASP	Sodium acetate buffer (pH 4.75)	Spherisorb ODS 2 45°C	Gradient 0.01 *M* TBA hydrogensulphate, 0.02 *M* HEPES (pH 7.6)–methanol–2-propanol (17:20:3 to 1:20:19)	325	(A) QL 5 ng/ml (B) 5 ng/g (C) 10 ng/g (D) 50 ng/g	[254]
13-*cis*-, all-*trans*-RA, 13-*cis*-, all-*trans*-4-oxo-RA, acitretin (I.S.)	(A) Plasma (0.5 ml) (B) embryo	(A) 0.8 ml 2-propanol, 1.2 ml + 1 ml 0.5% AcOH injected; (B) 3 vol. 2-propanol, sonication, 1 vol. + 4 vol. 2% ammonium acetate injected	C_2 cartridge AASP	2% Ammonium acetate–acetonitrile (85:15)	Spherisorb ODS 2 35°C	Gradient 0.06 *M* ammonium acetate (pH 6.8) to methanol	360/340	?	[259]

Table 1 (*continued*)

Analyte	Matrix (amount used)	Pretreatment	Precolumn	Mobile phase 1	Analytical column	Mobile phase 2	Detection wavelength (nm)	DL or QL	Ref.
9-*cis*-, 9,13-di*cis*-, 13-*cis*-, all-*trans*-RA, 9-*cis*-, 13-*cis*-, all-*trans*-RAG	Plasma (0.125 ml)	3 vol. 2-propanol, 0.4 ml + 1.2 ml 2% ammonium acetate	C_2 cartridge AASP	0.5% Ammonium acetate–acetonitrile (85:15)	Spherisorb ODS 2 60°C	Gradient acetonitrile–water (30:70 to 99:1) cont. 0.2% trifluoroacetic acid	340 + 356	DL 0.25 ng	[256]
13-*cis*-, all-*trans*-RA, 13-*cis*-, all-*trans*-4-oxo-RA, acitretin (I.S.)	Plasma (0.4 ml)	1.5 ml Ethanol, 1.4 ml injected	LiChrospher 100 RP-18	1.25% Ammonium acetate + AcOH–ethanol (8:2, M1A), 1% ammonium acetate + 2% AcOH–ethanol (102:4, M1B)	Superspher 100 RP-18 endcapped 26°C	Gradient acetonitrile water–10% ammonium acetate–AcOH (600:300:60:10 to 950:20:5:20 to 990:5:0:5)	360	QL 0.3 ng/ml	[45]
Acitretin, 13-*cis*-acitretin, Ro 11-6738 (I.S.), etretinate, Ro 12-7554 (I.S.)	Plasma (0.4 ml)	1.5 ml Ethanol, 1.4 ml injected	LiChrospher 100 RP-18	1.25% Ammonium acetate + AcOH–ethanol (8:2, M1A), 1% ammonium acetate + 2% AcOH–ethanol (102:4, M1B)	Superspher 100 RP-18 26°C	Gradient acetonitrile–water–10% ammonium acetate AcOH (700:270:30:5 to 950:20:5:5 to 990:9:0:5)	360	QL 0.3 ng/ml	[260]
13-*cis*-, all-*trans*-RA, 13-*cis*-, all-*trans*-4-oxo-RA, acitretin (I.S.)	Skin, fat (1–70 mg)	Homogenization in 1 ml ethanol, 0.6 ml injected	Corasil C_{18}	Ethanol (M1A), 1% ammonium acetate + 2% AcOH–ethanol (102:4, M1B)	Superspher 100 RP-18 endcapped 26°C	Gradient acetonitrile–water–10% ammonium acetate–AcOH (600:300:60:10 to 950:20:5:20 to 990:5:0:5)	360	QL 4 ng/g (50 mg sample)	[267]
Acitretin, 13-*cis*-acitretin, Ro 11-6738 (I.S.), etretinate, Ro 12-7554 (I.S.)	Skin, fat (1–70 mg)	Homogenization in 1 ml ethanol, 0.6 ml injected	Corasil C_{18}	Ethanol (M1A), 1% ammonium acetate + 2% AcOH–ethanol (102:4, M1B)	Superspher 100 RP-18 26°C	Gradient acetonitrile–water–10% ammonium acetate–AcOH (700:270:30:5 to 950:20:5:5 to 990:9:0:5)	360	QL 8 ng/g (50 mg sample)	[268]

improve the sensitivity, as demonstrated by Wyss and Bucheli for 13-*cis*-RA, acitretin and their metabolites [242]. Acetonitrile (0.2 ml) was added to 1 ml of plasma, and 1 ml was injected after centrifugation. For sumarotene [50], 0.5 or 1 ml of diluted plasma were injected without any problem, whereas isomerization in the autosampler vials was sometimes observed for first- and second-generation retinoids. Therefore, 1 ml of plasma was directly injected after internal standard addition in a 5-μl volume only. Acetonitrile was then added, on-line, during injection onto the precolumn by means of pump P1B and the T-piece, as shown in Fig. 4 [38]. No isomerization occurred using this procedure.

3.4.2. Protein precipitation

Plasma proteins can be precipitated with a water-miscible organic solvent, and the supernatant directly injected. For highly lipophilic retinoids, Wyss et al. added 2 vol. of ethanol to 1 vol. of plasma [239,243,244]. The method of Creech Kraft et al. [235] was applied to several studies, using 1 vol. of 2-propanol and 1 vol. of plasma [245–251] or embryonic fluid [252]. Alternatively, ethanol was added instead of 2-propanol [253]. In a similar way, Eckhoff, Nau and coworkers deproteinated plasma with 3 vol. [254–256] or 5.5 vol. [257,258] of 2-propanol before diluting the supernatant with buffer and applying to the cartridge. To improve the sensitivity, 0.5 ml plasma was deproteinated with 0.8 ml of 2-propanol, and 1.2 ml of the supernatant were diluted with 1 ml of acetic acid before injection [259].

The approach of Wyss and Bucheli for highly sensitive methods for 13-*cis*-RA and metabolites [45] and acitretin and metabolites [260] consisted of deproteination of plasma (1 vol.) with ethanol (3.75 vol.) and injection of 1.4 ml of the supernatant. This large volume of ethanol would prevent pre-concentration of the analytes on the precolumn. Therefore, ammonium acetate was added, on-line (M1B in Fig. 4), to reduce the elution strength of the injection solution. This procedure had several advantages compared to dilution of the injection solution prior to injection. Furthermore, it proved to be very flexible and allowed injection volumes of 4 ml, when less sensitive UV detectors were available, or even up to 24 ml for in vitro metabolism studies [261].

3.4.3. Tissue homogenates

Tissue samples can be homogenized, centrifuged and the supernatant directly injected onto the precolumn. Creech Kraft et al. analysed embryos by adding 1 vol. of ethanol and 1–2 vol. of 2-propanol, storing in liquid nitrogen overnight, followed by sonication and injection of 0.1–0.2-ml volumes [235,248,250,252]. Alternatively, only 2-propanol was added, either 2 vol. [245–247] or 1–3 vol., but without freezing in liquid nitrogen [251,262–265]. Embryos were also sonicated in 2 vol. of ammonium acetate, followed by addition of an equal vol. of ethanol and subsequent injection of 0.2 ml of the supernatant [253]. After sonication of embryos in 3 vol. of 2-propanol, the supernatant was diluted with buffer (3–5 vol.) and analysed by the AASP system [241,254,255,259]. Placental tissue was first mixed with 1 vol. of water, liver was homogenized in 9 vol. of water, before further work-up as described for embryos [254].

An older procedure in the author's laboratory consisted of homogenization of skin and fat samples in 0.6 ml of ethanol, followed by addition of 0.3 ml water, and injection of a 0.5-ml aliquot of the supernatant. Although the injection conditions were identical with those for plasma samples [243], a decrease of peak heights after repeated injections was observed, especially for etretinate in fat samples. This appeared to be due to coating of the precolumn by lipids, which became insoluble in the injection solution after water addition [266]. This effect was no longer seen when the samples were homogenized in 1 ml of ethanol, and 0.6 ml of the supernatant were injected, using ethanol as mobile phase M1A and ammonium acetate as M1B (Fig. 4). In this way, a time-consuming pre-separation step for co-extracted lipids could be avoided. This improved method was applied routinely to the determination of 13-*cis*-RA and metabolites [267] and acitretin and metabolites [268] in skin and subcutaneous fat samples.

Overall, it can be said that protein precipi-

tation of biological fluids is more robust than direct injection of diluted samples. However, a large volume of the supernatant (or of tissue homogenate) has to be injected to obtain high sensitivity, and a suitable method for pre-concentration on the precolumn has to be found. This can be achieved by on-line addition of buffer through a T-piece.

4. Chromatographic techniques

4.1. General

The vast majority of published methods for the separation of retinoids uses HPLC, which is discussed in Section 4.2. GC was regarded for years to be a technique unsuitable for retinoids because of extensive isomerization, especially of first- and second-generation retinoids. However, in recent years, better deactivated columns and cold on-column injection have led to a reconsideration of its use. This will be discussed in Section 4.4. Supercritical fluid chromatography (SFC, Section 4.5) and the non-chromatographic capillary electrophoresis (CE, Section 5) are promising new techniques for which only limited experience has been gained. Liquid or thin-layer chromatography have been only rarely used in the last years and have been discussed elsewhere [21,22].

Only one publication [269], which has appeared during the last years, dealt with the separation of retinoid enantiomers by HPLC. This is not surprising, since only a few retinoids are chiral. All-*trans*-3-hydroxyretinal enantiomers were either separated directly on a Chira-Spher column with hexane–diethyl ether–2-propanol (69.3:30:0.7) as mobile phase, or were converted to diastereomers of all-*trans*-3-hydroxyretinal camphanates, and separated by normal-phase HPLC on LiChrosorb Si 60 with cyclohexane–benzene–ethyl acetate (47.5:47.5:5) [269].

The use of an internal standard for the determination of retinoids has already been recommended. It is not within the scope of this review to discuss validation of analytical methods in detail. However, for calibration, the procedures for the determination of unknown concentrations in the published methods were not always state-of-the-art. For instance, single-point calibration should be replaced by multi-point calibration, and the calibration standards should be prepared in the biological matrix of the unknown samples [270]. It may be possible that in special cases not all analytes are available as reference standards. Ross [156,231] used retinol and Furr [219] retinyl palmitate for calibration of different retinyl esters. Several isomers of 3-hydroxyretinol and 3-hydroxyretinal were determined, using all-*trans*-retinol and *syn* all-*trans*-retinal oxime as calibration standards [203]. On the other hand, not every matrix may be available in sufficient amounts for use as calibration samples. In this case, an appropriate alternative should be found and validated, for example 10% rabbit plasma in water instead of embryo [259]. An additional problem is the occurrence of endogenous levels, which requires a standard addition method [93,239] or substitution by an analyte-free matrix.

4.2. High-performance liquid chromatography

4.2.1. Normal-phase HPLC

Normal-phase HPLC is especially suitable for apolar compounds and is preferred for the separation of geometrical isomers of retinol and retinal. Organic extracts can be injected directly onto silica columns, and redissolving an evaporated extract in hexane for normal-phase chromatography is usually easier than in methanol–water, as used for reversed-phase HPLC. However, some disadvantages are also associated with normal-phase chromatography, including problems with gradients, varying retention times and need for more clean-up.

A series of normal-phase HPLC methods is presented in Table 2, showing typical examples of validated and, mostly, well described methods. However, a variety of other chromatographic conditions, often used in applications or metabolic studies, has also been published. Hexane–dioxane as mobile phase was used not only in the pioneering work of Groenendijk et al. [198] for

Table 2
Normal-phase HPLC methods

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
Retinol, α-tocopherol, α- + β-carotene, retinyl acetate (I.S.)	Serum (0.2 ml)	Methanol, dichloromethane, water	LiChrosorb Si 60 20°C	Hexane–dioxane (95:5)	320 (retinoids) 296 (tocopherol) 450 (carotenoids)	DL 0.2 μM (retinoids)	[53]
Retinol, α-tocopherol, lipids	Liver (1 g)	Methanol–chloroform (2:1), chloroform, sat. sodium chloride, SPE SiOH	Zorbax CN	0.1% 2-propanol in heptane	190–370 PDA		[232]
Retinyl palmitate, retinol, retinyl acetate (I.S.)	Plasma, lipoprotein fraction (0.1–0.5 ml)	Methanol, mobile phase	Supelcosil LC-SI	Hexane–butyl chloride–acetonitrile–AcOH (90:15:5:0.01)	330	QL 1 ng/ml	[116]
all-*trans*-Retinyl palmitate, stearate, oleate, palmitoleate, linoleate, 9-*cis*-, 11-*cis*-, 13-*cis*-retinyl palmitate, 11-*cis*-, 13-*cis*-retinyl stearate	Guinea pig tissues (0.5–100 mg)	Lyophilization, hexane	Spherisorb silica	Hexane–diisopropyl ether (98.5:1.5)	325 FL ex 332, em 472	DL 0.8 pmol (UV) DL 0.3 pmol (FL)	[215,216]

Table 2 (*continued*)

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
all-*trans*-, 9-*cis*-, 11-*cis*-, 13-*cis*-Retinal	Pigment membranes (0.1 ml suspension)	1 *M* Hydroxylamine pH 6.5, methanol, dichloromethane	MicroPak Si-5	Hexane–dioxane (100:6)	360	?	[198]
11-*cis*-, all-*trans*-Retinal and -3,4-didehydroretinal	Rod outer segment (0.1 ml suspension)	6 *M* Formaldehyde, dichloromethane, hexane	Zorbax SIL	Hexane–diethyl ether–ethanol (93.95:6:0.05)	360	?	[207]
all-*trans*-, 9-*cis*-, 11-*cis*-, 13-*cis*-, 9,11-di*cis*-, 9,13-di*cis*-Retinol, all-*trans*-, 7-*cis*-, 9-*cis*-, 11-*cis*-, 13-*cis*-retinal	Retina	Hanks' buffer pH 6.5, methanol, 1 *M* hydroxylamine pH 6.5, dichloromethane, water	LiChrosorb Si 60	Hexane–ethyl acetate–dioxane–octanol (854:112:20:14)	325 + 360	DL 2–3 pmol	[199,200]
(A) 13-*cis*-, all-*trans*-RA (B) retinol, etarotene (I.S.)	Plasma (0.5 ml)	Ethanol, water, hexane, 2 *M* HCl	Spherisorb S5W silica	Hexane–2-propanol–AcOH (200:0.7:0.135)	350	(A) DL 0.5 ng/ml (B) DL 10 ng/ml	[93]
Etretinate, acitretin, 13-*cis*-acitretin, retinyl propionate (I.S.)	Plasma (0.5 ml)	Ethanol, 2 *M* HCl, water, hexane	Chromspher Si	Dichloromethane–2-propanol–AcOH (250:0.55:0.4)	350	DL 3 ng/ml	[98]
Acitretin, 13-*cis*-acitretin, 13-demethyl-RA (I.S.)	(A) Plasma (B) blister fluid (0.5 ml) (C) blister roof	(A,B) Ethanol, 2 *M* HCl, water, hexane (C) preceding freeze-thawing and ultrasonication	Chromspher silica	Hexane–methylsalicylate AcOH (200:18:0.6)	360	(A) QL 3–4 ng/ml (B) DL 2–3 ng/ml (C) DL 50–70 ng/g	(A) [99] (A,B,C) [100]
Acitretin, 13-*cis*-acitretin, etretinate, 13-*cis*-RA (I.S.)	(A) Plasma (0.5 ml) (B) skin (ca. 75 mg) (C) fat (ca. 124 mg)	(A) Ethanol, 2 *M* HCl, water, hexane (B,C) preceding freeze-thawing and ultrasonication	Chromspher silica	Hexane–methylsalicylate–AcOH (200:18:0.3)	360	(A) DL 2 ng/ml (B) DL 10 ng/g (C) DL 7.5 ng/g	(A) [101] (A,B,C) [189]

the separation of isomers of retinal oximes and in a multivitamin determination [53] (Table 2), but also by others mainly for retinol and retinal analysis [75,95,173,185,195,202,203,209]. Hexane, with small amounts of THF and acetic acid, was introduced by Hänni et al. [271] for the separation of etretinate and acitretin, and was also chosen by others for the same compounds [46,138], as well as for Ro 12-7554 and Ro 12-9933 [128]. Other mobile phases comprised hexane and 2-propanol [23,220] or 2-propanol–acetic acid [93] (Table 2), hexane–octanol–trifluoroacetic acid [188] and hexane–ethyl acetate [177,200]. Hexane–ethyl acetate was also used in combination with acetic acid [30], ethanol [157,201] or both [224,225]. Gradient elution with the last mentioned mobile phase enabled the determination of acidic retinoids [224,225] or the separation of oximes of retinal, 3,4-didehydroretinal and 4-hydroxyretinal. Isocratic elution with hexane–ethyl acetate–dioxane–octanol allowed the complete separation of 13 isomers of retinol, retinal and retinal oximes by Landers and Olson [199,200]. A chromatogram of this method is shown in Fig. 5, and additional details may be found in Table 2 and in the literature [205,210].

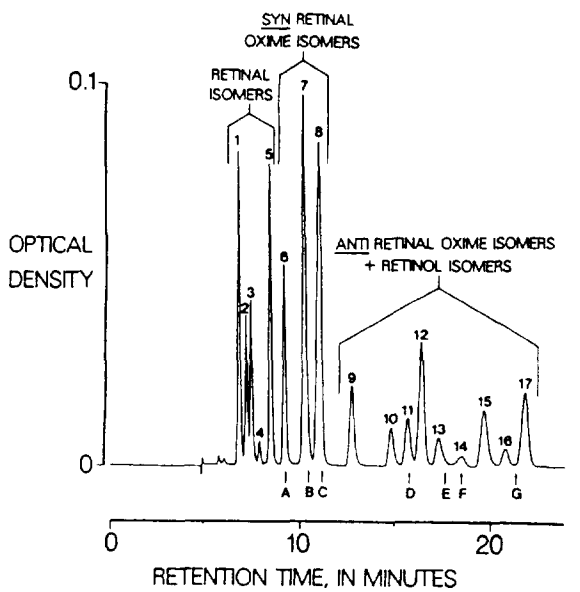

Fig. 5. Normal-phase HPLC of retinal, retinal oxime and retinol isomers. Peaks: 1 = 13-*cis*-retinal; 2 = 11-*cis*-retinal; 3 = 9-*cis*-retinal; 4 = 7-*cis*-retinal; 5 = all-*trans*-retinal; 6 = *syn* 11-*cis*-retinal oxime; 7 = *syn* all-*trans*-retinal oxime; 8 = *syn* 9-*cis*-retinal oxime + *syn* 13-*cis*-retinal oxime; 9 = *anti* 13-*cis*-retinal oxime; 10 = *anti* 11-*cis*-retinal oxime; 11 = 11-*cis*-retinol; 12 = 13-*cis*-retinol; 13 = *anti* 9-*cis*-retinal oxime; 14 = 9,11-di*cis*-retinol + 9,13-di*cis*-retinol; 15 = *anti* all-*trans*-retinal oxime; 16 = 9-*cis*-retinol; 17 = all-*trans*-retinol. Additional elution positions are marked for compounds not represented by peaks: A = *syn* 9,11-di*cis*-retinal oxime; B = *syn* 7-*cis*-retinal oxime; C = *syn* 9,13-di*cis*-retinal oxime; D = *anti* 9,13-di*cis*-retinal oxime; E = *anti* 9,11-di*cis*-retinal oxime; F = *anti* 7-*cis*-retinal oxime; G = 7-*cis*-retinol. Two columns, LiChrosorb Si 60 (250 × 4 mm I.D. each), 5 μm, in series; mobile phase 11.2% ethyl acetate, 2% dioxane and 1.4% octanol in hexane, flow-rate 1 ml/min; detection 325 nm. (Reproduced with permission from Ref. [199].)

Hexane–methyl benzoate–propionic acid [37,137,139] and hexane–methyl salicylate–acetic acid [99–101,189] (Table 2) were employed for the separation of acitretin, 13-*cis*-acitretin and etretinate. Furthermore, hexane was successfully used in combination with acetonitrile–acetic acid [171,194], acetone [190], *tert.*-butyl methyl ether [208] and diethyl ether–ethanol [204,207,272]. Using this last composition, Suzuki et al. [207] separated 11-*cis*- and all-*trans*-retinal and 3,4-didehydroretinal directly (without oxime formation), after treatment of the sample with an excess of formaldehyde to avoid the formation of a Schiff base between retinal and amino groups of the pigment protein. In this way, the formation of *syn* and *anti* conformers of retinal oximes is prevented, and the complexity of the chromatograms is reduced. Chromatograms of retinyl ester isomer separations, using hexane–diisopropyl ether as mobile phase and direct injection of hexane extracts, are shown in Figs. 6 and 7 [215,216] (Table 2 for further details). Napoli et al. employed hexane–acetic acid and dichloromethane [114] or 1,2-dichloroethane [114,190,191] for the separation of retinoic acids.

Heptane–*tert.*-butyl methyl ether was recommended, since it is less toxic than hexane–dioxane mixtures [273]. Older methods employed petroleum ether and acetonitrile–acetic acid or dichloromethane–2-propanol, as demonstrated by De Leenheer et al. for RA [96,97], and retinol [97], respectively. Other mobile phases consisted of dichloromethane with addition of acetic acid [131] or 2-propanol–acetic acid [98] (Table 2),

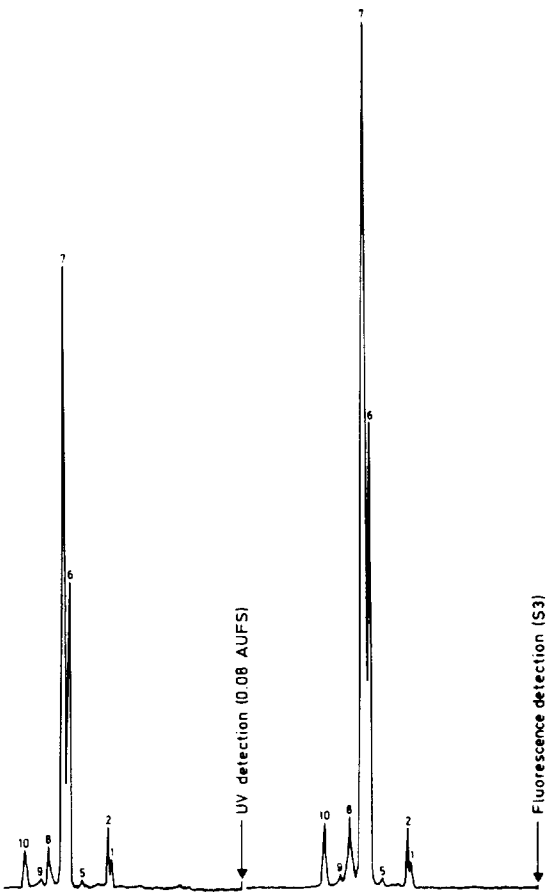

Fig. 6. Normal-phase HPLC of retinyl esters in a liver sample from a guinea pig. Peaks: 1 = 13-*cis*-retinyl stearate; 2 = 13-*cis*-retinyl palmitate; 3 = 11-*cis*-retinyl stearate; 4 = 11-*cis*-retinyl palmitate; 5 = 9-*cis*-retinyl palmitate; 6 = all-*trans*-retinyl stearate; 7 = all-*trans*-retinyl palmitate; 8 = all-*trans*-retinyl oleate; 9 = all-*trans*-retinyl palmitoleate; 10 = all-*trans*-retinyl linolate. Column, Spherisorb Silica (250 × 4.6 mm I.D.) and guard column (25 × 4.6 mm I.D.), both 3 μm; mobile phase hexane–diisopropyl ether (98.5:1.5), flow-rate 2 ml/min; UV detection 325 nm; fluorescence detection 332 nm (excitation), 472 nm (emission). (Reproduced with permission from Ref. [215].)

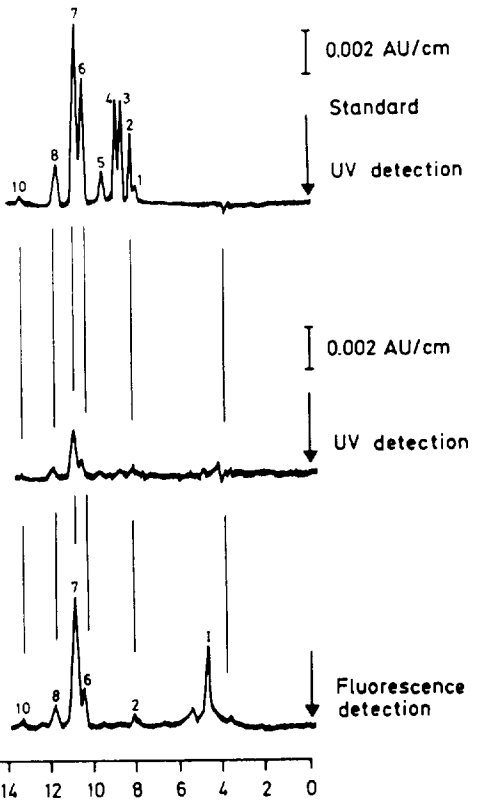

Fig. 7. Normal-phase HPLC of retinyl esters in the sensory cells (organ of Corti) of the inner ear from a guinea pig. Peak identification and conditions (except detector scales) as in Fig. 6. (Reproduced with permission from Ref. [215].)

and 1,1,2-trichlorotrifluoroethane–*tert.*-butyl methyl ether [24]. Seki et al. used step gradients of benzene–*tert.*-butyl-methyl ether–ethanol for the separation of isomers of retinol, retinal oximes and their 3-hydroxy metabolites [34,35], whereas Ito et al. [269] separated diastereoisomers of all-*trans*-3-hydroxyretinal camphanates with benzene–cyclohexane–ethyl acetate. However, chlorinated solvents or benzene in the mobile phase should, whenever possible, be replaced for ecological and safety reasons.

4.2.2. Reversed-phase HPLC

Reversed-phase chromatography is the most popular separation mode in HPLC, and is especially suitable for the analysis of biological samples. Compared to normal-phase chromatography, no problems arise from the presence of water or high amounts of lipids. Furthermore, retinoids with a wide range of polarity can easily be analysed in a single run, often with the help of gradient elution. HPLC column-switching methods are discussed separately (Sections 3.4 and 4.2.3), but the final separation consists of reversed-phase HPLC. Therefore, these methods should also be considered here, including the

Table 3
Reversed-phase HPLC methods for natural and first-generation retinoids

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
Retinyl laurate, myristate, palmitoleate, linoleate, pentadecanoate, palmitate, oleate, heptadecanoate, stearate	Rat tissues	Lyophilization, methanol, hexane	Ultrasphere ODS	Methanol–water (98:2)	325 Radioactive	DL 40 pmol	[213]
Retinol, retinal, retinyl palmitate, stearate, acetate (I.S.)	Retina, eye cups	Homogenization in buffer (pH 7), 0.1 *M* O-ethylhydroxylamine, methanol, cholesterol, hexane	Econosphere C_{18}	Gradient 0.5% ammonium acetate in methanol–2-propanol (100:0 to 50:50)	325	DL 1 ng (retinal)	[206]
Retinyl octanoate, decanoate, laurate, γ-linolenate, myristate, palmitoleate, linolenate, palmitate, oleate, elaidate, stearate, pentadecanoate (I.S.)	(A) Chylomicrons, plasma, (B) liver, milk	Ethanol, hexane, water, SPE alumina oxide	Supelcosil LC-8	Gradient acetonitrile–water (88:12 to 98:2)	340	75 pmol	(A,B) [156] (A) [231]
Retinyl myristate, pentadecanoate, palmitoleate, palmitate, heptadecanoate, stearate, oleate, linoleate, 6,9,12-octadecatrienoate, eicosanoate, 11,14-eicosadienoate, 8,11,14-eicosatrienoate, arachidonate, 13,16-docosadienoate, laurate (I.S.), retinol	(A) Liver (B) plasma	Chloroform–methanol (2:1), 0.1% sodium chloride	Ultrasphere ODS	(A) 1–3.5% water in methanol (B) 12% water in methanol	325	?	[186]
Retinol, retinyl linolenate, arachidonate/laurate, linoleate, myristate/palmitoleate, pentadecanoate, oleate, palmitate, heptadecanoate, stearate, eicosanoate (I.S.)	Liver (0.5 g)	Grinding with anhydrous sodium sulphate, dichloromethane	Resolve C_{18}	Acetonitrile–dichloroethane (80:20) containing 0.1% cyclohexene or gradient acetonitrile–water (85:15) to acetonitrile–dichloroethane (80:20) cont. 0.1% cyclohexene	325	DL 4 pmol (retinyl palmitate)	[219]
Retinyl palmitate, *dl*-tocol (I.S.)	Liver (ca. 50 mg), liver-based foods	Water, ethanol, hexane	Nucleosil C_{18}	Methanol	295 PDA	?	[174]

Table 3 (*continued*)

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
13-*cis*-, all-*trans*-RA, -retinol, -retinal, -retinyl acetate	(A) Plasma (0.1 ml) (B) liver, lung, mamma	(A) 0.9% NaCl, methanol, chloroform (B) homogenization in AcOH, ascorbic acid, EDTA, methanol, chloroform	Nucleosil C_{18}	Acetonitrile–0.1 *M* ammonium acetate (80:20)	350	(A) DL 10 ng/ml	[115]
13-*cis*-, all-*trans*-RA, retinol	Serum (0.2 ml)	0.1 *M* Ammonium acetate–acetonitrile (3:1), hexane	Chemcosorb 5-ODS-H 50°C	Acetonitrile–methanol–0.1 *M* ammonium acetate (46.7:23.3:30)	340	QL 0.5 ng/ml	[118]
(A) Retinol, 3,4-didehydroretinol, TMMP-retinol (I.S.), (B) all-*trans*-, 13-*cis*-RA, acitretin (I.S.)	Skin (20–50 mg)	(A) Ethanol, 14 *M* KOH, water, hexane (B) dito + 5 *M* HCl, water, hexane	Nucleosil C_{18}	(A) Acetonitrile–water (82:12) (B) acetonitrile–water–AcOH (82:18:0.05)	326 + 360	DL 10–20 ng/g	[157]
(A) Retinol, 13-*cis*-RA, 13-*cis*-4-oxo-RA (B) +all-*trans*-RA, all-*trans*-4-oxo-RA	Plasma (0.5 ml)	5% Perchloric acid, ethyl acetate	(A) Ultrasphere ODS + μ-Bondapak (B) Ultrasphere + Resolve C_{18}	Acetonitrile–1% ammonium acetate (95:5)	340 + 365	(A) ? (B) DL 0.03 μM	(A) [40] (B) [147]
13-*cis*-, all-*trans*-RA, acitretin (I.S.)	Serum (1.5 ml)	Methanol, NaCl, chloroform–methanol (2:1), SPE aminopropyl	Pecosphere 3 × 3CR ODS	Gradient methanol–water (65:35) cont. 1% ammonium acetate to 100% methanol	340	DL 0.1 ng	[95]
all-*trans*-, 13-*cis*-RA, acitretin (I.S.)	Serum (3.5 ml)	Ethanol, 2 *M* NaOH, hexane, 2 *M* HCl, hexane, SPE aminopropyl	Spherisorb ODS 2	10% THF in methanol–water–17 *M* AcOH (84.85:15:0.15)	350	DL 2.7 n*M*	[230]
13-*cis*-RA, 9-methylanthracene (I.S.)	Serum (0.5 ml)	Methanol, acetonitrile, 0.1 *M* perchloric acid	Zorbax ODS	Acetonitrile–0.5% AcOH (85:15) cont. 0.05% sodium hexanesulphonate	365	DL 12 ng/ml	[44]

(*Continued on p. 404*)

Table 3 (*continued*)

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
all-*trans*-RA, all-*trans*-retinyl acetate (I.S.)	Plasma (0.5 ml)	Acetonitrile	LiChrosorb RP-18	Acetonitrile–methanol–water–10% ammonium acetate (60:16:20:4)	340	QL 10 ng/ml	[76]
13-*cis*-, all-*trans*-RA, 13-*cis*-4-oxo-RA, 13-*cis*-acitretin (I.S.)	Plasma (0.5–2 ml)	Phosphate buffer (pH 7), diethyl ether–ethyl acetate (1:1)	Nucleosil C_{18}	Methanol–1% AcOH (85:15)	(A) 350 (B) 360	DL ca. 2 ng/ml	(A) [32] (B) [140]
(A) all-*trans*-ROG, all-*trans*-RAG, all-*trans*-RA (B) +retinol (C) +3,4-didehydro-RA, -retinol, -RE, retinyl oleate, palmitate, stearate, palmitoleate	(A) Serum (2 ml) (C) Cultured keratinocytes	Ethanol, ethyl acetate, water, 10% AcOH, ethyl acetate, hexane	(A) Resolve C_{18} (C) Nova Pak C_{18}	Methanol–water (68:32) cont. 0.01 *M* ammonium acetate or gradient as above to 100% methanol–dichloromethane (4:1)	(A) 325 or 335 (C) 340 PDA radioactive	?	(A) [108,109] (B) [108,109] (C) [291]

Table 4
Reversed-phase HPLC methods for second- and third-generation retinoids

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
(A) Acitretin, 13-*cis*-acitretin, etretinate, all-*trans*-RA (I.S.) (B) excl. etretinate	(A) Plasma (0.5–2 ml) (B) skin (20–300 mg)	(A) Phosphate buffer (pH 7), diethyl ether–ethyl acetate (1:1) (B) homogenization, diethyl ether–ethyl acetate (1:1)	Nucleosil C_{18}	Methanol–1% AcOH (85:15)	(1) 350 (2) 360	(A) DL ca. 2 ng/ml (B) QL 10 ng/g (200 mg sample) DL 5 ng/g	(A1) [32,47] (A2) [140] (B) [192]
Etretinate/acitretin, all-*trans*-RA (I.S.)	Skin (20–50 mg)	Ethanol, 14 *M* KOH, incubation, water, hexane, 5 *M* HCl, hexane	Nucleosil C_{18}	Acetonitrile–water–AcOH (82:18:0.05)	360	DL 10–20 ng/g	[157,30]
Acitretin, 13-*cis*-acitretin, etarotene (I.S.)	(A) Plasma (0.3–1 ml) (B) skin, blister skin, blister fluid	(A) Phosphate buffer (pH 7), diethyl ether–ethyl acetate (1:1) (B) homogenization, diethyl ether–ethyl acetate (1:1)	Nucleosil C_{18}	Methanol–acetonitrile–1.5% AcOH (595:255:150)	350	(A) QL 1 ng/ml (B) QL 5 ng/g (200 mg sample)	(A) [141] (B) [142]
Temarotene, Ro 14-6113, Ro 13-9272 (I.S.)	Plasma (1 ml)	Acetonitrile, *tert*-butyl methyl ether	Sepralyte C_{18}	Methanol–acetonitrile–water (90:6:4)	280	QL 20 ng/ml	[49]
Temarotene, Ro 14-6113, Ro 13-9272 (I.S.)	Plasma (0.2 ml)	Water, acetonitrile, hexane–2-propanol (9:1)	2 × RP-8, column switching	Acetonitrile–water (77:23) cont. 0.01 *M* Tris-HCl (pH 4.1)	280	QL 100 ng/ml	[119]
Temarotene, Ro 14-6113, etretinate (I.S.)	(A) Plasma (0.2–1 ml) (B) +skin (200–300 mg)	Borate buffer (pH 11), diethyl ether–ethyl acetate (1:1), (B) dito, incl. homogenization	Nucleosil C_{18}	Acetonitrile–water (90:10)	300	(A) QL 2 ng/ml (B) QL 10 ng/g	(A) [33] (B) [143]

Table 5
Reversed-phase HPLC methods for multivitamin determinations

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
Retinol, α-tocopherol, retinyl palmitate, tocol (I.S.)	Plasma (0.2 ml)	Ethanol, hexane	Superspher RP-18	Gradient acetonitrile–water (9:1) to ethyl acetate–2-propanol (9:1) (60%)	305	DL 0.1 μg/ml (retinol)	[82]
Retinol, retinyl palmitate, α- + β-carotene, retinyl acetate (I.S.)	Serum (0.5 ml)	Ethyl acetate–THF (1:1)	IBM C_{18}	Acetonitrile dichloromethane–methanol–THF (61:19:17:3)	325 (retinoids) 440 (carotenoids)	?	[150]
Retinol, retinyl palmitate, β-carotene	Buccal mucosa cells	Methanol, sonication, water, hexane	Ultrasphere ODS	Gradient acetonitrile–THF–methanol–1% ammonium acetate (71:15:6:8 to 67:25:6:2)	340 + 436	DL 0.35 ng (retinol) DL 1.43 ng (retinyl palmitate)	[170]
Retinol, retinyl acetate (I.S.), α- + γ-tocopherol, lycopene, α- + β-carotene	Serum (1 ml)	Ethanol (A) petroleum ether or (B) hexane	Biophase ODS	Acetonitrile chloroform–2-propanol–water (78:16:3.5:2.5)	292 (retinoids, tocopherols) 460 (carotenoids)	?	(A) [104] (B) [88]
Retinol, α-tocopherol, canthaxanthin, zeaxanthin, β-cryptoxanthin, lycopene, α- + β-carotene	Serum (0.2 ml)	Water, ethanol, hexane	(A) Nova Pak C_{18} (B) Ultrasphere ODS	Acetonitrile–dichloromethane–methanol (7:2:1)	325 (retinol) 292 (tocopherol) 436 (carotenoids) 450 (β-carotene)	(A) ? (B) DL 16 n*M* (retinol)	(A) [80] (B) [54]
Retinol, α- + γ-tocopherol, lycopene, α- + β-carotene	Plasma (0.25 ml)	Water, ethanol, hexane	Ultrasphere ODS 28°C	Acetonitrile–THF–methanol–1% ammonium acetate (684:220:68:28)	450/472 (carotenoids) FL ex 330, em 470 (retinol)	DL 20 ng/ml (retinol)	[81]
Retinol, α-tocopherol, lutein, cryptoxanthin, lycopene, α- + β-carotene	Lung, skin, colon, breast (15–50 mg), plasma (0.2 ml)	Collagenase (+ lipase for breast), incubation, ethanol, hexane	Ultracarb ODS	Acetonitrile–THF–methanol–1% ammonium acetate (65:25:6:4)	325 (retinol) 292 (tocopherol) 450 (carotenoids)	DL 16 ng/g (50 mg tissue) DL 42 n*M* (retinol in plasma)	[56]
Retinol, α-tocopherol, lutein, lycopene, α- + β-carotene, retinyl acetate (I.S.)	Plasma (0.2 ml)	Ethanol, hexane	Nucleosil C_{18}	Acetonitrile–THF–methanol–1% ammonium acetate (68:22:7:3)	325 (retinol) 290 (tocopherol) 450/470 (carotenoids)	DL 10 ng/ml (retinol)	[83]

Table 5 (*continued*)

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
Retinol, retinyl acetate, palmitate, α-tocopherol, α-tocopheryl acetate, apo-carotenoids (5), α-, β- + γ-carotene	(A) Plasma (0.1 ml) (B) liver, lung, mamma	(A) 0.9% NaCl, methanol, chloroform (B) homogenization in AcOH, ascorbic acid, EDTA, methanol, chloroform	Hypersil ODS	Acetonitrile dichloromethane–methanol water (70:10:15:5)	350 (retinoids) 292 (tocopherols) 445 (carotenoids)	DL 10 ng/ml (retinoids and carotenoids)	[115]
Retinol, retinyl hexanoate (I.S.), α- + γ-tocopherol, lutein, zeaxanthin, α- + β-cryptoxanthin, lycopene, α- + β-carotene	Serum (0.1 ml)	(A) Ethanol, ethyl acetate, hexane (B) or 2-propanol, dichloromethane	Resolve C_{18}	(A) Acetonitrile–dichloromethane–methanol–octanol (90:15:10:0.1), (B) acetonitrile dichloroethane–methanol (85:10:5) cont. 0.05% ammonium acetate	300 (retinoids, tocopherols) 450 (carotenoids), PDA	DL 0.21 pmol (retinol)	(A) [55,110] (B) [110]
Retinol, retinyl linoleate, oleate, palmitate, stearate, butyrate (I.S.), α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α- + β-carotene	Serum (0.2 ml)	Ethanol, hexane	Ultramex C_{18} 29°C	Acetonitrile ethanol (1:1) cont. 0.01% diethylamine	325 (retinoids) 300 (tocopherols) 450 (carotenoids)	DL 17 n*M* (retinol) DL 28 n*M* (retinyl palmitate)	[85]
Retinol, α-tocopherol, tocopherol acetate (I.S.), cryptoxanthin, lycopene, α- + β-carotene	Plasma (0.25 ml)	0.01 *M* SDS, ethanol, heptane	Spherisorb ODS 2	Acetonitrile–methanol–chloroform (47:47:6)	325 (retinoids) 292 (tocopherols) 450 (carotenoids)	?	[111]
Retinol, retinyl palmitate, α- + γ-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α- + β-carotene, *cis*-β-carotene	(A) Buccal mucosa cells (B) plasma	(A) Protease, digestion, SDS–ethanol–BHT, hexane (B) plasma without digestion	Nova Pak C_{18}	Gradient acetonitrile–THF–methanol–1% ammonium acetate–BHT (85:5:5:5:0.05 to 55:35:5:5:0.05)	PDA, 325 (retinoids) 300 (tocopherols) 452 (carotenoids)	?	[227]

Table 5 (*continued*)

Analyte	Matrix (amount used)	Extraction	Column	Mobile phase	Detection wavelength (nm)	DL or QL	Ref.
Retinol, retinyl palmitate, acetate (I.S.), lutein/ zeaxanthin, cryptoxanthin, lycopene, α- + β-carotene, *cis*-β-carotene, γ-carotene (I.S.)	Serum (0.1 ml)	Chloroform methanol (2:1), 0.85% NaCl, hexane	Pecosphere-3 C_{18}	Gradient acetonitrile–THF–1% ammonium acetate (50:29.6:20.4 to 50:44:6)	340 (retinoids) 450 (carotenoids), PDA	DL 3 ng/ml (retinoids)	[152]
Retinol, retinyl palmitate, α-, γ- + δ-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α- + β-carotene	Serum (0.2 ml)	Ethanol, hexane	Bakerbond C_{18} 29°C	Gradient acetonitrile–0.05 *M* ammonium acetate in methanol–ethyl acetate (98:2:0 to 68:25:7) cont. 0.05% TEA	325 (retinoids) 450 (carotenoids) FL ex 295, em 335 (tocopherols)	?	[89]
Retinol, α-tocopherol, β-carotene, tocol (I.S.)	Serum (0.25 ml)	Ethanol, hexane	Vydac polymeric C_{18}	Gradient methanol–water–butanol (75:15:10 to 88:2:10) cont. 0.02 or 0.05 *M* ammonium acetate	325 (retinol) 295 (tocopherol) 450 (carotene) ED + 900 mV	DL UV/ED 6/4.1 ng/ml (retinol), 96/ 0.65 ng/ml (tocopherol), 29/2.1 ng/ml (carotene)	[86] (ED) [87]
Retinol, retinyl acetate (I.S.), α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α- + β-carotene, echinenone (I.S.)	Plasma (0.5 ml)	Ethanol, hexane	Spherisorb ODS 1	Gradient methanol–acetonitrile–water (72:18:10 to 80:20:0) cont. 0.1 *M* ammonium acetate	325 (retinol) 292 (tocopherol) 450 (carotenoids)	DL 0.35 μM (retinol)	[90]

chromatograms presented in Section 4.2.3. Typical methods for natural and first-generation retinoids are presented in Table 3. Second- and third-generation retinoids are compiled in Table 4. A series of newer assays for multivitamin determinations is listed in Table 5. However, the conditions for these methods may have been optimized for tocopherol (vitamin E) or carotenoids and are, therefore, not discussed in detail.

A wide variety of eluents for reversed-phase chromatography has been used (in combination with C_{18} columns, when not otherwise mentioned), and the preference for a mobile phase composition may also depend on the personal experience of the analyst. Acetonitrile- and methanol-based systems are both popular. The first solvent has the advantage of producing less back pressure and is, therefore, more suitable for coupling of columns. Isocratic acetonitrile–water mixtures were used for neutral compounds [33,143,177,178,222,274]. Addition of an acid, usually acetic acid, results in ion suppression of carboxylic acids, but does not disturb the simultaneous determination of retinol or other neutral compounds. McClean et al. [121] separated all-*trans*- and 13-*cis*-RA with acetonitrile–water–acetic acid (79.5:20:0.5), which was also adopted by others [43,127,157]. The same conditions were also applied to acitretin and metabolites [30,123,132,157,159], acetylenic acid or ethyl ester retinoids (Fig. 3) [48,77] and fenretinide and 4-MPR [73–75]. For the last two analytes, 4-EPR is a suitable internal standard [74,75]. Acetonitrile and 1% or 0.1 *M* ammonium acetate buffer were often used, improving the peak form and separation of RA [43,58,59,65,72,145,181,275–278] (Table 3), acitretin and etretinate [129,130,279,280] and Ro 13-7410 [67]. This mobile phase was also chosen for the separation of fenretinide and 4-MPR [105,146] and, in combination with a C_8 column, for retinol and retinyl esters [120].

Acetonitrile as the major component, in combination with methanol and acetic acid or ammonium acetate, was often employed for the separation of RA, its isomers and metabolites [10,29,68,188,194,195,197] (Table 3), as well as all-*trans*-3,4-didehydro-RA and all-*trans*-3,4-didehydroretinol [196]. Acetonitrile in combination with other organic solvents is suitable for very lipophilic compounds. Examples of mobile phases still containing some water or buffer are presented in Tables 3 and 5. Further mobile phases include acetonitrile–methanol–2-propanol–1.2% acetic acid for the separation of RA isomers [27,197] and THF as additive to acetonitrile–ammonium acetate [175,176,281]. Nelis et al. [117] were the first to try non-aqueous reversed-phase (NARP) chromatography for the determination of retinol. The mobile phase consisted of acetonitrile–dichloromethane–methanol (70:15:15) and it was especially suitable for very lipophilic compounds to prevent precipitation in the mobile phase. Acetonitrile–dichloromethane with [97,282] or without methanol [63,218] was also used in later applications (see Table 5 for further examples).

Gradients of acetonitrile–water were used in combination with C_8 columns [156,231,283] and of acetonitrile–acetic acid in combination with C_{18} columns [134,135]. However, gradient elution with acetonitrile–ammonium acetate (in combination with C_{18} columns) was more popular, as demonstrated by Buggé et al. [122] and others for RA and metabolites [9,11,126,165,167, 183, 284], 3,4-didehydro-RA [9,11], retinol and 4-hydroxyretinol [212], and acitretin and etretinate [122,125,144,161,162]. Several gradient methods consisting of acetonitrile and additional solvents can be found in Tables 3 and 5.

Methanol has also been widely used as the main component of reversed-phase mobile phases, even without modifiers (100% methanol), as demonstrated mainly for retinyl esters, retinol and tocopherols [39,79,112,172,174, 177, 178]. De Ruyter and De Leenheer [285] added silver salts (58.9 m*M* $AgNO_3$) to the methanol for the separation of retinyl esters and retinol. Complexation of the double bonds of retinyl esters, consisting of unsaturated fatty acids, can be used for altering the capacity factors, whereas retinyl esters with saturated fatty acids were not influenced. The addition of silver salts to a methanol–perchloric acid mobile phase was investigated by Baillet et al. [28] for a rapid separation (24 min) of RA isomers after photo-

isomerization. Methanol–water was suitable for retinol or retinyl ester separations [156,179] (Table 3), whereas methanol–acetic acid was chosen for carboxylic acids or its methyl esters [31,149,160] (Tables 3 and 4). However, methanol–ammonium acetate was usually preferred for RA [26,229] and especially for the simultaneous determination of its 4-oxo, 4-hydroxy and 5,6-epoxy metabolites or RAG [64,286,287] (Table 3). However, it was also used for etretinate/acitretin [169] and retinol [60,62,63]. Methanol, together with acetonitrile and buffer, acid or water, was applied to temarotene [49,165], Ro 13-6307 [69], N-(retinoyl) glycine and retinoic acid [71], retinoic acid, its metabolites and retinol [31,70], and acitretin and 13-*cis*-acitretin [141,142]. In addition, methanol was chosen in combination with THF [230], chloroform [92] and butanol [25], mainly for the separation of retinol and more lipophilic compounds. Retinol and tocopherols were also chromatographed with 2-propanol–water [288,289].

Fenretinide and 4-MPR were analysed by gradients of methanol–water (70:30) to 100% methanol [153–155,187,226]. Methanol–ammonium acetate gradients were applied to etretinate/acitretin [136] and RA [95,107,133, 163,168,185,290] and their metabolites. Gradients starting with methanol–ammonium acetate or water and ending with a portion of a more lipophilic solvent, such as chloroform [91,166,214], dichloromethane [108,109,291] or 2-propanol [206], were also reported. MacCrehan and Schönberger [86,87] used a gradient mobile phase consisting of methanol, butanol and ammonium acetate. Chromatograms of this separation, obtained with absorbance and electrochemical detection (ED), are shown in Fig. 8. ED will be discussed in detail in Section 4.3.4.

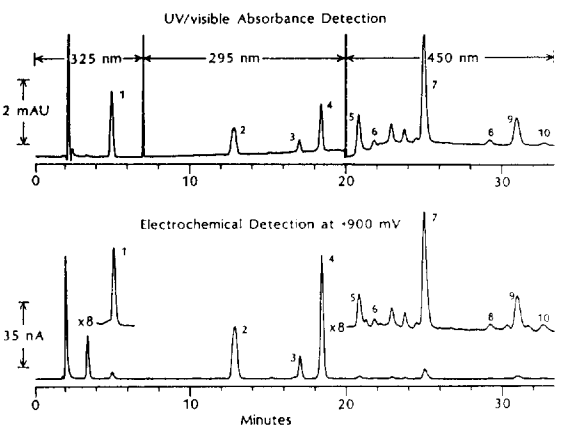

Fig. 8. Reversed-phase HPLC, using absorbance and electrochemical detection, for a multivitamin determination in serum. Peaks: 1 = all-*trans*-retinol; 2 = tocol (I.S.); 3 = γ-tocopherol; 4 = α-tocopherol; 5 = lutein; 6 = zeaxanthin; 7 = cryptoxanthin; 8 = α-carotene; 9 = all-*trans*-β-carotene; 10 = cis-β-carotene. Column, Vydac polymeric C18 (201TP) (250 × 4.6 mm I.D.), 5 μm; gradient mobile phase methanol–water–butanol (75:15:10 to 88:2:10) containing 0.02 *M* ammonium acetate (pH 3.5), flow-rate 1.5 ml/min; programmed absorbance detection 325 nm, 295 nm, 450 nm; amperometric detection at +900 mV. (Reproduced with permission from Ref. [86].)

The numerous examples mentioned above show that reversed-phase chromatography is also well suited for isomer separation of retinoids. Curley et al. [292] demonstrated that methanol should be used in combination with a fully end-capped reversed-phase material, whereas acetonitrile is preferred for non-end-capped stationary phases.

4.2.3. HPLC with column switching

HPLC with automated column switching is a new technique which has been used for retinoids, mainly for the purpose of on-line solid-phase extraction, as discussed in Section 3.4. However, it can be also used in other ways. Rissler et al. [119] extracted temarotene, its metabolite Ro 14-6113 and the internal standard Ro 13-9272 with hexane–2-propanol (9:1) after initial deproteination with acetonitrile. The analytes were then chromatographed with acetonitrile–water (77:23), containing 0.01 *M* Tris-HCl (pH 4.1), on two C_8 columns (125 × 4.6 mm I.D. each), coupled in series. After elution from the first column, the analytes were separated on the second column. In the meantime, late-running peaks were eluted in the backflush mode from the first column, allowing a considerable reduction of the overall run time.

The chromatographic conditions of the column-switching methods discussed in Section 3.4 were similar to other reversed-phase separations. Examples can be found in Table 1. In general,

gradient elution was employed, which allowed difficult separations of metabolites, as demonstrated by Eckhoff et al. [240] for the simultaneous determination of all-*trans*- and 13-*cis*-RA, their 4-oxo metabolites, ROG and RAG. Fig. 9 shows chromatograms of a monkey plasma sample and reference standards, obtained by direct injection of diluted plasma into the AASP system [236,240].

A fully automated HPLC separation of acitretin and 13-*cis*-acitretin in human plasma samples using column switching is shown in Fig. 10. To achieve the remarkable quantification limit of 0.3 ng/ml, 1 ml of plasma was injected onto the precolumn without any off-line pretreatment

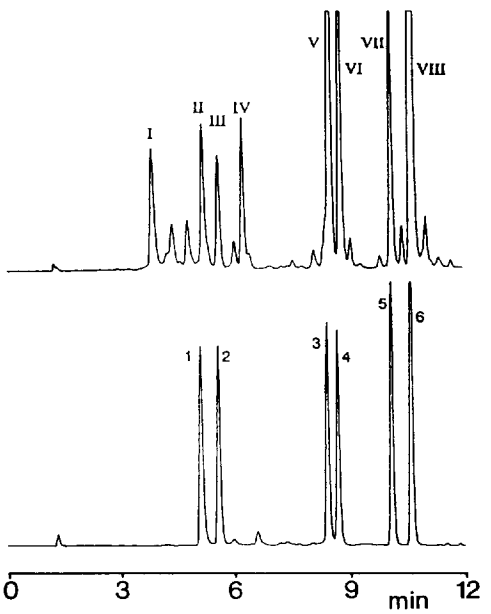

Fig. 9. Reversed-phase HPLC with column switching of a monkey plasma sample, obtained 2 h after administration of 150 000 I.U./kg of vitamin A (upper chromatogram). The fractions of peaks I–VIII were collected for identification by thermospray LC–MS. The lower chromatogram shows a reference standard with all-*trans*-4-oxo-RA (1), 13-*cis*-4-oxo-RA (2), RAG (3), ROG (4), 13-*cis*-RA (5) and all-*trans*-RA (6). Coelution was observed between II and 1, III and 2, V and 3, VI and 4, VII and 5, VIII and 6. I was identified as all-*trans*-4-oxo-RAG by LC–MS, whereas the structure of IV could not be elucidated. The plasma sample (0.35 ml) was diluted with 2% ammonium acetate (0.6 ml) and 2% ammonium acetate–acetonitrile (2:1) (0.6 ml) and applied to an AASP C_2 cartridge. After washing twice with 1.5 ml of 2% ammonium acetate–acetonitrile (85:15), the cartridge was loaded onto the AASP and eluted on-line. Analytical column, Spherisorb ODS 2 (120 × 4 mm I.D.), 3 μm, kept at 60°C; gradient mobile phase methanol–60 m*M* ammonium acetate (pH 7.5) (57.5:42.5 to 95:5), flow-rate 0.7 ml/min; detection 340 nm. (Reproduced with permission from Ref. [240].)

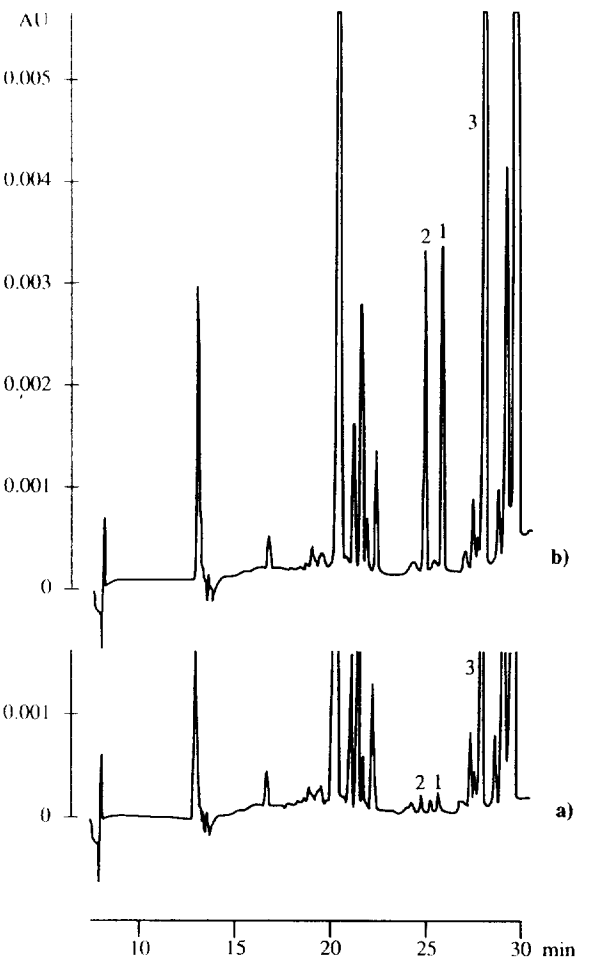

Fig. 10. Reversed-phase HPLC with column switching of human plasma samples, spiked with (a) 0.3 ng/ml and (b) 5 ng/ml of acitretin (1) and 13-*cis*-acitretin (2). Peak 3 is the internal standard Ro 11-6738. After addition of 5 μl I.S., plasma (1.0 ml) was directly injected into the HPLC column-switching system shown in Fig. 4. PC = Corasil C18 (14 × 4.6 mm I.D.), 37–50 μm; AC = three Spherisorb ODS 1 (125 × 4 mm I.D.), 5 μm, in series; GC2 = Spherisorb ODS 1 (30 × 4 mm I.D.), 5 μm; M1A = 1% ammonium acetate–acetonitrile (100:2), flow-rate 1.4 ml/min; M1B = 1% ammonium acetate–acetonitrile (6:4), flow-rate 0.7 ml/min; M2 = gradient acetonitrile–water–10% ammonium acetate–acetic acid (600:400:4:30 to 950:50:4:10 to 980:20:0:1), flow-rate 1 ml/min; D2 = 360 nm. (Reproduced with permission from Ref. [38].)

(except for the addition of 5 μl of I.S. solution). Acetonitrile, which is necessary for a good recovery, was added, on-line, with a second pump (P1B in the column-switching system shown in Fig. 4) [38]. An example of acetonitrile addition (1 vol. to 5 vol. of plasma) prior to injection, avoiding protein precipitation, is shown in Fig. 11 for the third-generation retinoid sumarotene and its Z-isomer (Ro 18-6778) [50]. An aliquot of 0.5 ml was injected. Pump 1B (Fig. 4) was not used in this method.

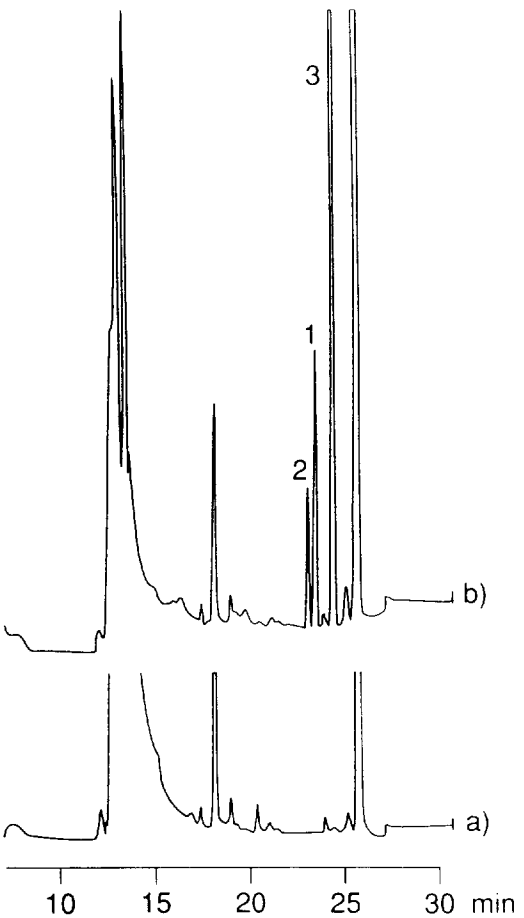

Fig. 11. Reversed-phase HPLC with column switching of human plasma samples. (a) Blank plasma sample; (b) blank plasma sample spiked with 20 ng/ml of sumarotene (1) and its Z-isomer (2), and 100 ng/ml of etarotene (3, I.S.). After addition of 0.2 ml of acetonitrile, containing the internal standard, to 1 ml of plasma, 0.5 ml were injected into an HPLC column-switching system similar to that shown in Fig. 4, but without P1B. PC = Corasil C18 (14 × 4.6 mm I.D.), 37–50 μm; AC = three Spherisorb ODS 1 (125 × 4 mm I.D.), 5 μm, in series; GC2 = Spherisorb ODS 1 (30 × 4 mm I.D.), 5 μm; M1A = 1% ammonium acetate–acetonitrile (85:15), flow-rate 1.5 ml/min; M2 = gradient acetonitrile–water (70:30 to 95:5 to 99:1), flow-rate 1 ml/min; D2 = 303 nm. (Reproduced with permission from Ref. [50].)

HPLC methods with automated column switching for retinoids have not only the advantages already mentioned in Section 3.4, but also have better precision and accuracy compared to conventional HPLC methods [102]. Furthermore, higher sensitivity can be achieved, because no analyte is lost by transfer of aliquots after injection onto the precolumn.

4.3. Detection in HPLC

4.3.1. Ultraviolet detection

The UV characteristics of most retinoids are quite favourable, especially for first- and second-generation retinoids. Absorption maxima of 325 and 350 nm, and extinction coefficients of 52 770 and 45 300 (in ethanol) for all-*trans*-retinol and all-*trans*-RA, respectively [21], enable selective and sensitive determination by HPLC–UV. Detection limits of less than 0.05 ng on column can now be achieved with the most sensitive UV detectors, which result in quantification limits of 0.3 ng/ml, using only 0.4 ml of plasma, for RA [45] or acitretin [260]. Quantification limits of less than 1 ng/ml are needed for the determination of endogenous levels of all-*trans*- and 13-*cis*-RA, which are in the range of 1.5 ng/ml with a high natural variability. During the last years, a few methods have been developed which have this required sensitivity, although not all are suitable for routine determinations (Tables 1–3).

Availability of more powerful computers in combination with photodiode array (PDA) detectors with higher resolution and sensitivity, resulted in an increasing number of applications in retinoid analysis. The sensitivity of the newest PDA detectors is now in the range of the most sensitive UV detectors. Even when not all possibilities of peak purity assessment and peak identification can be fully exploited at the highest sensitivity in biological samples, PDA detectors will become more and more important in method development, routine analysis and metabolite

identification. This was not only shown for purity determination of 13-*cis*-RA [293] and substance identification [24,31,185,212,291], but also for routine analysis [202]. PDA detectors were well suited for multivitamin determinations, where several wavelengths need to be monitored simultaneously [110,227,228,232]. Further improvements in sensitivity, resolution and data handling of PDA detectors which can be expected in the near future will prove to be extremely important for further progress in retinoid analysis.

4.3.2. Fluorescence detection

Retinol and retinyl esters are highly fluorescent in nonpolar organic solvents and, therefore, suitable for normal-phase HPLC with fluorescence (FL) detection, as demonstrated for retinol in liver [220]. FL detection was also used in series with UV detection [203], whereas Biesalski and Weiser [215,216] calculated detector quotients (FL/UV) for the identification of co-chromatographing isomers of retinyl esters. Chromatograms with both types of detection are shown in Figs. 6 and 7. FL detection also proved to be applicable to reversed-phase HPLC with mobile phases of low water [179] or buffer [81] content, and was also used in series with UV detection in an NARP method for multivitamin determination [89].

Furr and Olson [57] determined retinol (bound to plasma RBP) by direct injection of as little as 20 μl of plasma or serum using size-exclusion HPLC. A Toyo Soda TSK G3000SW column was eluted with 0.2 *M* NaCl and 0.01 *M* phosphate buffer (pH 6.8), followed by FL detection (excitation 334 nm, emission 425 nm). However, as the retinol–RBP complex eluted as two peaks, the peak-height ratios of which varied with the injection volume, this microassay may be problematic for routine determinations.

4.3.3. Chemiluminescence detection

Bryan and Capomacchia [294] investigated oxalate ester chemiluminescence detection of selected retinoids. After normal-phase HPLC separation, using hexane–THF–acetic acid (75:25:0.01) as mobile phase, the reagents 0.07 *M* bis(2,4,6-trichlorophenyl) oxalate in THF and 1 mg/ml of imidazole in THF–50% hydrogen peroxide (75:25) were added post-column. The conditions were optimized by stop-flow chemiluminescence. The detection limits obtained by chemiluminescence detection were about 10–15 times higher for retinol, retinyl palmitate and retinyl acetate, and about 1000–2000 times higher for etretinate, acitretin and all-*trans*-RA, compared to UV detection. Therefore, chemiluminescence is by far the least sensitive detection method considered in this review.

4.3.4. Electrochemical detection

MacCrehan and Schönberger [25] investigated the oxidation of retinol at a glassy-carbon electrode in methanol–water, using coulometry. The oxidation proved to be a multi-electron process that formed several products [25]. Electrochemical detection (ED) and simultaneous wavelength-programmed absorbance detection were successfully applied to a reversed-phase HPLC method for the determination of retinol, α-tocopherol and β-carotene in serum [86,87] (Table 5). Chromatograms with both absorbance detection and ED are shown in Fig. 8. Detection limits proved to be better for amperometric detection (at a potential of +0.9 V) with 4.1 ng/ml (0.103 ng absolute), 0.65 ng/ml (0.016 ng) and 2.1 ng/ml (0.053 ng) for retinol, α-tocopherol and β-carotene, respectively, compared to 6.0 ng/ml (0.15 ng), 96 ng/ml (2.4 ng) and 29 ng/ml (0.73 ng) using absorbance detection. The selectivity of absorbance detection was superior for retinol and β-carotene, whereas amperometric detection was more selective for α-tocopherol.

Wring et al. [78] studied the influence of buffer pH, ionic strength and the solvent on the voltammetric behaviour of retinol at a glassy-carbon electrode in more detail. The optimal conditions consisted of a supporting electrolyte, containing methanol–0.075 *M* acetate buffer (pH 5.0), which was used as mobile phase in combination with a Spherisorb ODS column. The potential of the amperometric detector was set at +1.1 V. The glassy-carbon working electrode and the Ag/AgCl reference electrode were in a wall-jet cell and not in a thin-layer cell, as used by

MacCrehan and Schönberger [25,86]. Whereas the thin-layer cell showed adsorption on the glassy carbon surface, probably by oxidation products, resulting in a decrease in peak heights, this did not occur with the wall-jet configuration [78]. The optimized conditions were applied to the determination of all-*trans*-retinol (other isomers or vitamins were not considered) in human serum. The HPLC–ED method proved to be about four times more sensitive than HPLC–UV. The detection limit for ED was 0.035 ng (absolute), compared to 0.13 ng with UV detection [78].

Bryan et al. [295] investigated normal-phase HPLC with amperometric detection. 13-*cis*- and all-*trans*-RA, acitretin and retinyl palmitate were chromatographed using hexane–THF–acetic acid (95:5:0.01) and similar mixtures as mobile phases; 0.04 *M tert.*-butylammonium tetrafluoroborate in dichloromethane was added as electrolyte post-column. The potential was set at +1.2 V. Detection limits for the investigated compounds were reported to be similar to UV detection (0.5–2 ng on column) [295]. However, these references are much higher than the state-of-the-art for UV detection (see above and Section 4.3.1).

4.3.5. Mass spectrometric detection

The coupling of a mass spectrometer to an HPLC column has become quite popular during the last years. However, because many retinoids have a carboxylic acid group which gives no stable molecular ion, derivatization is normally required to obtain high sensitivity. Because of the relatively poor sensitivity of LC–MS with thermospray, the most popular interface of the late eighties, a less common approach was chosen by Huselton and coworkers [296,297]. They developed a normal-phase microbore LC–MS method for the determination of all-*trans*- and 13-*cis*-RA in plasma, using a direct liquid introduction (DLI) interface. The same group [298] also reported the determination of acitretin and 13-*cis*-acitretin in human plasma, using the same methodology. In this method, the clean-up step, using reversed-phase HPLC and acetonitrile–water–acetic acid (75:24:1) as mobile phase, was performed immediately after the hexane extraction. The collected analyte fraction was evaporated and derivatized with pentafluorobenzyl bromide in the presence of 18-crown-6-ether and crystals of potassium acetate. The final normal-phase LC–MS analysis was similar to that described for retinoic acids, using hexane–toluene (65:35) as mobile phase. In negative chemical ionization (NCI) MS, the ion at m/z 325 ($[M\text{-}PFB]^-$) was used for quantification in selective ion monitoring (SIM) with trideuterated acitretin as internal standard. The limit of quantification was 1 ng/ml.

Although these sophisticated methods with modified instruments are difficult to transfer, Ranalder et al. [299] succeeded in establishing a modification of the retinoic acid method. Their method was validated for all-*trans*- and 13-*cis*-RA and their 4-oxo metabolites, using 13-*cis*- and all-*trans*-$^{13}C_9$-RA and -$^{13}C_6$-4-oxo-RA as internal standards, respectively. After a work-up procedure similar to that in the original method [296,297], the fractions of retinoic acids and the 4-oxo metabolites from the reversed-phase column were collected and analysed separately with the LC–MS system. This consisted of a capillary (250 × 0.32 mm I.D.) packed with a diol phase and two mobile phases of either hexane–toluene (95:5), at 4 μl/min, for the RA esters or hexane–THF (97.5:2.5), at 6 μl/min, for the 4-oxo-RA esters. Ammonia was used as reagent gas for NCI in an HP 5988 mass spectrometer. The column outlet was directed into the MS through a 50-μm fused-silica capillary, ending with a frit of porous waterglass (a restrictor used in SFC). Attempts to determine all four analytes in a single run, using acetonitrile as mobile phase, produced no negative ions and resulted in clogging of the SFC-frit. The final method had a limit of quantification of 0.3 ng/ml and a limit of detection of 1 pg injected. Selected ion chromatograms are shown in Figs. 12 and 13.

Besides the DLI LC–MS methods for quantification described above, several groups used LC–MS for identification of retinoids. DLI was employed for the identification of etretinate and 13-*cis*-etretinate [261], acitretin and metabolites [149], 9-*cis*-RA [9,11] and retinoyl β-glucuronide

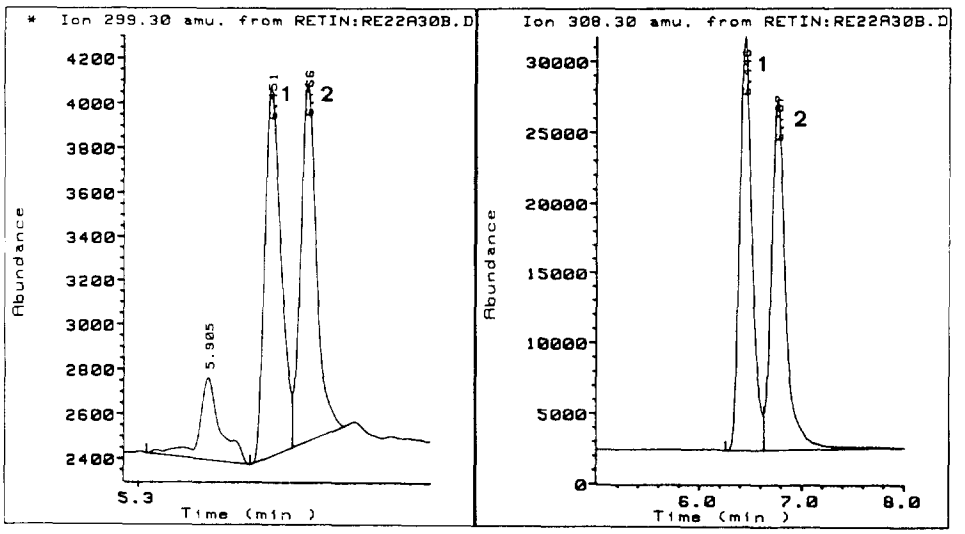

Fig. 12. Selected-ion chromatograms of the pentafluorobenzyl derivatives of endogenous 13-*cis*-RA (1) and all-*trans*-RA (2) in human plasma, obtained by normal-phase microbore LC–MS with direct liquid injection (DLI) and negative chemical ionization (NCI). At the left, analytes monitoring *m/z* 299.3. Found concentrations, 1.17 ng/ml for 13-*cis*-RA (1), 1.24 ng/ml for all-*trans*-RA (2). At the right, I.S. monitoring *m/z* 308.3, 20 ng/ml each of ^{13}C-labelled 13-*cis*- and all-*trans*-RA. Column, diol (250 × 0.32 mm I.D.) and guard column (1 × 0.25 mm I.D.), both 5 μm; mobile phase hexane–toluene (95:5), flow-rate 4 μl/min; DLI into an HP 5988 mass spectrometer; NCI, using ammonia as reagent gas. (Reproduced with permission from Ref. [299].)

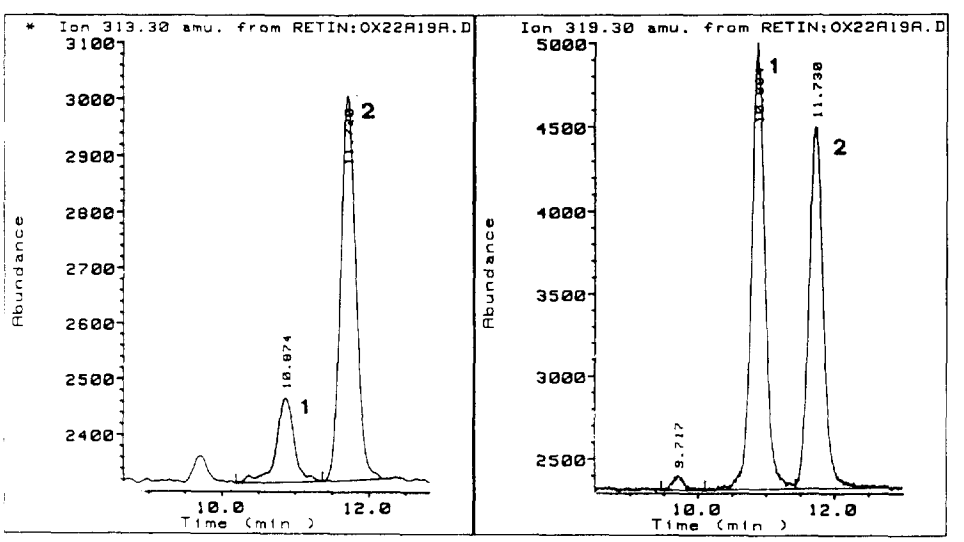

Fig. 13. Selected-ion chromatograms of the pentafluorobenzyl derivatives of endogenous all-*trans*-4-oxo-RA (1) and 13-*cis*-4-oxo-RA (2) in human plasma, obtained by normal-phase microbore LC–MS with direct liquid injection (DLI) and negative chemical ionization (NCI). At the left, analytes monitoring *m/z* 313.3. Found concentrations, 0.79 ng/ml for all-*trans*-4-oxo-RA (1), 6.36 ng/ml for 13-*cis*-4-oxo-RA (2). At the right, I.S. monitoring *m/z* 319.3, 20 ng/ml each of ^{13}C-labelled 13-*cis*- and all-*trans*-4-oxo-RA. Column, diol (250 × 0.32 mm I.D.) and guard column (1 × 0.25 mm I.D.), both 5 μm; mobile phase hexane–THF (97.5:2.5), flow-rate 6 μl/min; DLI into an HP 5988 mass spectrometer; NCI, using ammonia as reagent gas. (Reproduced with permission from Ref. [299].)

[297]. Thermospray was chosen for RA and metabolites, especially RAG [240,249], and acitretin and metabolites [160]. RA and metabolites were also identified by fast atom bombardment (FAB) LC–MS [287]. In addition, MS was also often used off-line, e.g. in a method, using direct exposure probe mass spectrometry for retinal and RA [300]. No paper has been published so far using atmospheric pressure ionization LC–MS–MS, which has become a very popular and powerful tool in drug analysis in the last few years.

4.4. Gas chromatography

For many years, GC proved to be problematic for the determination of retinoids, due to decomposition in hot injectors or at active sites on column surfaces [19–21]. However, derivatization followed by GC coupled to MS was used with some success for the determination of endogenous levels of all-*trans*- and 13-*cis*-RA in plasma [103,113,301], although none of these methods could finally give good separation and quantification of RA isomers. As discussed in the last review [16], and with even more experimental details by De Leenheer and Lambert [302], all these methods used packed columns. On the other hand, Egger et al. [51] chose two-dimensional capillary GC with zone-cutting, after derivatization with pentafluorobenzyl bromide, for the highly sensitive determination of the arotinoid Ro 13-7410 (QL 50 pg/ml). Capillaries were also used for the identification of RA as its methyl ester by GC–MS with SIM [10,31,151,194,196,236].

Progress in GC by using better deactivated columns and cold on-column injection made it possible to analyse thermolabile compounds without the need for derivatization. Smidt et al. [303] investigated this technique for simultaneous determination of retinol and α-tocopherol in liver and wheat flour. A fused-silica capillary (15 m $\times$ 250 μm I.D.), coated with a 0.25 μm film of methylsilicone, cold on-column injection, ultrapure hydrogen as carrier gas and flame ionization detection (FID), enabled this analysis to be carried out without derivatization. GC–MS was used for peak identification and was recommended instead of the FID for sensitive routine determinations of biological samples.

Furr et al. [304,305] applied this new approach for chromatographing underivatized retinol in deactivated capillaries by cold on-column injection. Using the same methylsilicone capillary, a series of retinoids and apo-retinoids was investigated, and the Kováts indices were determined. A thin-film methylsilicone column (DB-1, 5 m $\times$ 250 μm I.D., 0.1 μm film) and a cyanopropylphenylmethylsilicone column (DB-225, 30 m $\times$ 250 μm I.D., 0.15 μm film) were also employed, as well as GC–MS for peak identification. Attempts to chromatograph long-chain esters of retinol (retinyl tetradecanoate, hexadecanoate and octadecanoate), as well as apo-carotenoids and carotenoids, at high temperatures gave broad peaks, presumably caused not by degradation of the compounds, but by their condensation at the detector and/or by thermal isomerization during chromatography [304,305]. The GC conditions described above were also used by Clifford et al. [306] for the assessment of the vitamin A status in man, by measuring the dilution of standard doses of isotopically labelled vitamin A (retinol-d_4), using GC–MS and stable isotope dilution MS.

As retinol was found to undergo varying degrees of dehydration on exposed silica sites and, primarily, in MS ion sources during these analyses, the method was improved by Handelman et al. [307]. After precipitation of plasma proteins and extraction with hexane, followed by partitioning with acetonitrile, retinol was isolated by reversed-phase HPLC and converted to its *tert.*-butyldimethylsilyl derivative, which was analysed by GC–MS with SIM. A DB-1 fused-silica capillary (15 m $\times$ 250 μm I.D., 0.1 μm film) was used with splitless injection at 285°C and helium as carrier gas. The new method was reported to be more rapid, having fewer interferences and better reliability, sensitivity (DL for retinol-d_4 1.2 ng) and precision [307].

Meyer et al. [308] investigated capillary GC with cold on-column injection for acitretin and 13-*cis*-acitretin. Although importance was attached to derivatization with diazomethane and

careful selection of the capillary, a simultaneous determination of acitretin, 13-*cis*-acitretin and etretinate proved to be very difficult, and was only possible by using two capillaries in series, namely 25 m of biscyanopropylpolydimethylsiloxane (90% cyanopropyl; 250 μm I.D., 0.25 μm film) and 5 m of polydimethylsiloxane (HP Ultra-1; 320 μm I.D., 0.33 μm film). However, these conditions were rejected, and the authors planned to try only the second stationary phase (12.5 m × 250 μm I.D., 0.25 μm film) in combination with MS detection in the future. This was necessary because the FID could not distinguish the coeluting peaks of 13-*cis*-acitretin methyl ester and etretinate. However, taking into consideration the extremely laborious clean-up for plasma samples, consisting of liquid–liquid extraction [98], derivatization and HPLC purification, this planned GC–MS method appears to be more suitable for use as a reference method than for routine determinations.

In conclusion, it appears that the laborious sample clean-up, often including an HPLC step [301,306–308], is a disadvantage of GC and GC–MS, which cannot be compensated by the advances in capillary GC for retinoids using cold on-column injection.

4.5. Supercritical fluid chromatography

SFC has some advantages over GC for the separation of retinoids, because it is better suited to the analysis of thermolabile compounds. However, to our knowledge only two investigations have been published.

Walther et al. [309] compared the analysis of thermolabile compounds by capillary GC and wall coated open tubular column SFC. Whereas the pentafluorobenzyl and trimethylsilyl esters of all-*trans*- and 13-*cis*-RA showed decomposition in GC, when using a fused-silica column (10 m × 320 μm I.D.) with PS-086 (15% phenylmethyl polysiloxane), a temperature of 180–300°C at 10°C/min or 150–300°C at 10°C/min and hydrogen (50 cm/s) as carrier gas, a good separation was obtained by SFC. The conditions consisted of a fused-silica capillary (10 m × 50 μm I.D.) coated with SB methyl 100 (100% methyl polysiloxane, used at 100°C), carbon dioxide as mobile phase (0.2 g/ml to 0.75 g/ml at 0.01 g/min) and FID at 350°C.

Xie et al. [310] applied an on-line multidimensional open tubular column SFC system to the separation of all-*trans*- and 13-*cis*-RA in rat serum. A solvent vent injection technique allowed the injection of 5 μl ethanolic serum extract by making five sequential injections of 1 μl onto a deactivated, uncoated precolumn (2 m × 100 μm I.D.). Retinoic acids were separated from other serum components by a heartcut on the first column (3 m × 50 μm I.D.), deactivated with poly(cyanopropyl, hydro)siloxane and then coated (0.15 μm film) with an oligo(ethylene oxide)-substituted polysiloxane (glyme) phase. Afterwards, the isomers were separated on the second column (8 m × 50 μm I.D.), coated (0.15 μm film) with a liquid crystalline polysiloxane stationary phase. Detection was performed by FID. For flow-switching, an offset-cross interface with a small dead volume was placed between the first and the second column. Although several problems experienced with an earlier version of the two-dimensional SFC system were solved, further investigations with regard to resolution and shortening of the runtime will be needed for routine determinations of retinoids in biological samples. However, the favourable compatibility of carbon dioxide makes SFC especially suitable for multidimensional systems or hyphenated techniques such as SFC–MS.

5. Capillary electrophoresis

Chadwick and Hsieh [311] were the first to demonstrate the separation of all-*trans*- and 13-*cis*-RA by CE in buffer solution. A fused-silica, polyimide-coated capillary (60 cm × 75 μm I.D.) was used in combination with 10 m*M* sodium tetraborate decahydrate and 50 m*M* boric acid in acetonitrile–water (50:50) as running buffer. The applied voltage was +30 kV at 38 μA, and the UV detection wavelength 345 nm.

Bempong et al. [312] investigated the effect of the modifier type (acetonitrile, α-cyclodextrin and SDS) and concentration, the buffer type and

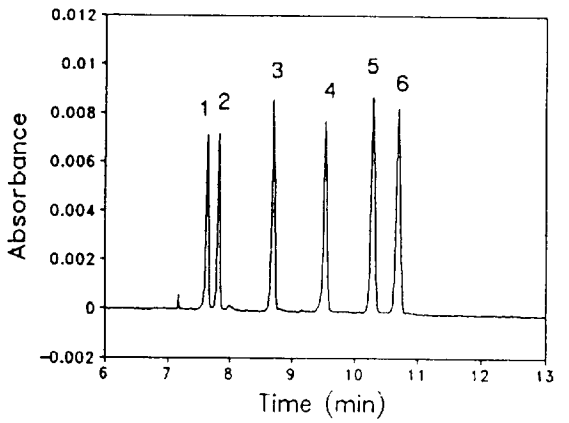

Fig. 14. MEKC separation of a retinoic acid standard mixture. Peaks: 1 = 13-*cis*-4-oxo-RA; 2 = all-*trans*-4-oxo-RA; 3 = 13-*cis*-acitretin (I.S.); 4 = 13-*cis*-RA; 5 = 9-*cis*-RA; 6 = all-*trans*-RA. Uncoated capillary (47 cm × 75 μm I.D.); buffer 20 m*M* Tris-borate (pH 8.5), 25 m*M* SDS and 20% acetonitrile; applied voltage 13 kV; detection 340 nm. (Reproduced with permission from Ref. [148].)

concentration, and the capillary length on the resolution of all-*trans*- and 13-*cis*-RA in CE and micellar electrokinetic chromatography (MEKC). The optimized conditions were applied to the separation of all-*trans*-RA (after exposure to light for 36 h) and its photodegradation products. The MEKC conditions consisted of a fused-silica capillary (122 cm × 50 μm I.D.), a 30 m*M* borate buffer (pH 8.5) containing 10 m*M* SDS, a constant voltage of 30 kV, a temperature of 30°C, and on-column UV detection at 345 nm.

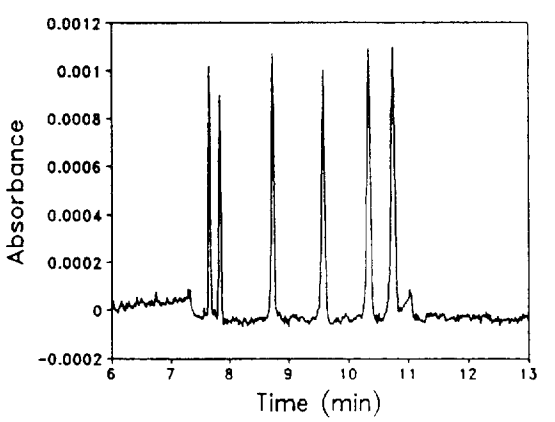

Fig. 15. Electropherogram of a rat plasma sample spiked with 15 μg/ml of a retinoic acid standard mixture. Conditions as in Fig. 14. (Reproduced with permission from Ref. [148].)

An excellent separation of all-*trans*-, 9-*cis*- and 13-*cis*-RA, all-*trans*- and 13-*cis*-4-oxo-RA and 13-*cis*-acitretin as internal standard using MEKC was reported by Chan et al. [148]. A 50% acetonitrile solution and spiked rat plasma (15 μg/ml), extracted as described by Peng et al. [40], were analysed, using an uncoated capillary (47 cm × 75 μm I.D.) and a buffer consisting of 20 m*M* Tris-borate (pH 8.5), 25 m*M* SDS and 20% acetonitrile. Electropherograms are shown in Figs. 14 and 15.

Ma et al. [313] took advantage of the low sample volume needed for CE by developing a microassay for the determination of serum retinol. A volume of 8–10 nl of serum was directly injected without any sample pretreatment, and the retinol–RBP complex (holo-RBP) was measured by laser-induced fluorescence detection. A fused-silica capillary (60 cm × 50 μm I.D.) was used for the separation, and the polymer coating was burned off 20 cm from the cathodic end to form a detection window. The buffer consisted of 50 m*M* sodium phosphate and 10 m*M* sodium chloride (pH 7.8), and the applied voltage was 24 kV. A helium–cadmium laser was used for excitation (325 nm), and the fluorescence was monitored at 465 nm. The linear range was 0.1–0.6 μg/ml, and the detection limit was 10 ng/ml serum. Because the method is much faster than conventional HPLC assays, and only minimal sample volume is needed, it may be ideally suited for finger-prick analysis of retinol.

6. Conclusions

The determination of retinoids in biological samples is a challenging task due to the lability of these compounds, and the difficult separations of geometric isomers or metabolites. The vast majority of analytical methods employs HPLC with UV detection. The numerous published extraction procedures, consisting mainly of liquid–liquid extraction, and different mobile-phase compositions could allow the conclusion that "anything goes". However, this is certainly not

the case, and each procedure has to be checked carefully regarding solubility and recovery of each analyte.

HPLC with column switching (on-line solid-phase extraction) has proven to have several important advantages over conventional methods, including a high degree of automation, no evaporation of extraction solvents, protection from light and higher sensitivity. The only sample pretreatment required for retinoids consists of either dilution of the biological fluid, or deproteination with an organic solvent, followed by injection of the supernatant. The latter is also suitable for tissue homogenates.

Owing to the favourable UV characteristics of most retinoids, HPLC with UV detection is the method of choice for retinoid determinations. Improvements in the sensitivity of UV detectors have lowered detection limits to less than 0.05 ng on column, resulting in plasma methods with limits of quantification of 0.3 ng/ml, using only 0.4 ml of plasma. The newest UV detectors also allow wavelength programming or multiwavelength detection, which is especially useful in multivitamin determinations. However, the most progress in this field has been realized by PDA detectors, which can deliver a huge amount of spectral information. Although PDA detectors have been rarely used till now, further developments of computer hard- and software and PDA detector resolution and sensitivity will allow this detection technique, coupled to HPLC, to become the method of choice for most retinoid investigations. However, this prediction is dependent on PDA detector sensitivity keeping pace with that of normal UV detectors.

Whereas fluorescence or electrochemical detection have only found a limited field of application, the use of HPLC–MS resulted in a few highly sensitive and interesting methods. However, the tricky instrumentation, using a direct liquid introduction interface connected to normal-phase HPLC and a modified MS apparatus, and the extensive sample pretreatment, resulted in a sample throughput which cannot compete in routine analysis. For future applications of LC–MS, atmospheric pressure ionization seems the most promising. However, except for some third-generation retinoids, this technique will also require derivatization for high sensitivity.

In contrast to other fields of lipid analysis, GC has proven to be problematic for retinoids for many years due to decomposition and isomerization. Even now, when better deactivated columns and cold on-column injection allow the analysis of labile retinoids, extensive sample clean-up, derivatization and coupling to MS will be needed for highly sensitive methods. Other new separation techniques, such as SFC, CE or MEKC, have shown preliminary encouraging results, but appear to reach the required sensitivity only by coupling to MS. This would require derivatization of most retinoids, as for all MS coupling techniques, to achieve high sensitivity.

Therefore, HPLC with UV detection, although used for years, is still the method of choice for highly sensitive and selective retinoid determination, as well as for high sample throughput and robustness. Improvements in sample preparation, as demonstrated by column-switching techniques, efficiency of columns, gradient elution and higher sensitivity of UV or PDA detectors will contribute to further progress in the separation of complex mixtures and the sensitive determination of retinoids in biological samples.

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