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Journal of Chromatography A, 935 (2001) 13–43

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Qualitative and quantitative liquid chromatographic determination of natural retinoids in biological samples

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Abstract

Liquid chromatography continues to be the preferred method for determining retinoids in biological samples. The highly unstable nature of retinoids and the real possibility of artifacts or erroneous results have led to the development of rapid and highly automated protocols for retinoid extraction, separation and detection. Due to strong light absorbance in the ultraviolet region, UV detectors still predominate although mass spectrometric detection is gaining increased popularity. This paper reviews recent advances and provides major guidelines for using liquid chromatography to identify and quantify retinoids in biological samples.

Keywords: Reviews; Sample preparation; Retinoids; Vitamins

Contents

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)01043-3

1. Introduction

Many ancient sources, including the Greek philosopher Hippocrates, the explorer of Africa, David Livingstone, and many others, describe how night blindness and epithelial defects could be cured by administration of beef liver [1]. The dietary factor responsible for preventing these defects was identified between 1907 and 1913 by the important work of McCollum and Davis [2] and Osborne and Mendel [3,4]. The molecular structure of vitamin A or ''fat soluble factor A'', as it was initially named, was determined by Karrer et al. in 1931 [5]. Today, we know that a number of closely related compounds are naturally present in biological samples with all playing essential roles for almost every animal tissue and organ. As the field evolved, scientists started a search for synthetic chemical derivatives with the biological activity of retinol. Sporn et al. introduced the term retinoid in 1976 to cover all natural as well as synthetic structural analogs of retinol, including those with and without retinol bioactivity [6].

According to the definition of the International Union of Pure and Applied Chemistry (IUPAC), and the International Union of Biochemistry and Molecular Biology (IUB), retinoids are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner (http://www.chem.qmw.ac.uk/iupac/misc/ret.html). All retinoids may be formally derived from a monocyclic parent compound containing five carbon–carbon double bonds and a functional group at the terminus of the acyclic portion. The term vitamin A, on the other hand, should be used as the generic descriptor for those retinoids exhibiting qualitatively the biological activity of retinol [7].

Some new synthetic compounds, such as TTNPB, TTNN and Am-580 conform to the classical definition of vitamin A (i.e. having biological effects similar to retinol), but do not fit the IUPAC–IUB definition of a retinoid (i.e. structural relation to retinol). Sporn and Roberts have therefore suggested that a retinoid may be defined as a substance that can elicit specific biological responses by binding to and activate a specific receptor or a set of receptors [8]. We believe, however, that it is wise to keep the official IUPAC–IUB definition of retinoids and not include molecules that share the biological activity of retinol, such as TTNPB, TTNN and AM-580. Otherwise, we might also have to include the fatty acids docosahexaenoic acid [9] and phytanic acid [10] (and probably other unrelated molecules as well) that recently demonstrated their ability to bind and activate nuclear retinoid receptors.

The purpose of this paper is to review the literature of the last 5 years that deals with liquid chromatographic determination of natural retinoids in biological samples. Examination of this literature reveals that a number of different methods have been used for the extraction, separation, detection and

identification of retinoids over the years. Experts in the field have written several excellent reviews on the topic [11–13]. As most of these papers are published in literature that are not easily available to scientist outside or new to the retinoid field, we have tried to include descriptions of useful physical and chemical properties of retinoids with the most recent methodologies for their detection and quantification. This review will not however include a discussion of the few gas chromatographic, supercritical and electrophoretic separation techniques that have been published. In papers dealing with the simultaneous determination of several fat-soluble vitamins, only vitamin A will be referred to. In addition, papers that rely heavily on chromatographic procedures published before 1995 are generally not discussed.

2. Chemistry of retinoids

The skeleton of natural retinoids is made up of a non-aromatic six-carbon ring structure with a polyprenoid side chain which is, with a few exceptions, terminated with a carbon–oxygen functional group. Metabolic and catabolic alteration of any combination of these three parts of the skeleton will then give rise to several hundred analogs with potentially diverse biological effects. The structure of this skeleton (I) together with selected retinoids is given in Fig. 1.

2.1. Nomenclature and structure

Retinoids can be named by their chemical name (e.g. all-*trans*-retinoic acid) based on the numbering scheme depicted in Fig. 1 (I) or according to the IUPAC–IUB nomenclature depicted in Fig. 1 (II). According to IUPAC–IUB rules, for example all-*trans*-retinoic acid is named (all-*trans*-3,7-dimethyl-9-(2,6,6-trimethyl-cyclohex-1-en-yl)-nona-2, 2,6,8-tetraen-1-oic acid). Often retinoids also have a trivial name, e.g. tretinoin for all-*trans*-retinoic acid. When all four double bounds in the side chain are in the *trans* configuration, the molecule is said to be in all-*trans* configuration. If one or several double bonds are in the *cis* configuration only the *cis* bond is specified, e.g. 9,13-di-*cis*-retinoic acid. The terms Z and E are also occasionally used instead of *cis* and *trans*, e.g. all-E-retinoic acid instead of all-*trans*-retinoic acid.

The structures and chemical names of selected retinoids are shown in Fig. 1 while some trivial names can be found in Table 1.

2.2. Physical properties

Although they share the same molecular skeleton, retinoids vary considerably in their physical and chemical properties. Pure retinoids appear as yellow crystals or sometimes in the form of oil, as seen in long chained retinyl esters. The polarity and thereby the solubility of various retinoids range from very soluble to insoluble in polar solvents like water and vice versa in apolar solvent like hexane. For the ionisable retinoids, such as retinoic acid, solubility depends on the pH of the solvent. The point where the pH is such that there are equal amounts of the protonated and non-protonated form of an ionisable molecule is defined as the pK_a. Thus above pK_a retinoic acid is highly soluble in water. The pK_a of retinoic acid has been reported to be between 6 and 8 depending on the concentration and solvent composition. This is significantly higher than one would expect for a carboxylic acid and is due to intermolecular interactions [14,15]. Non-polar retinoids like retinyl esters which have more than forty carbon atoms, are poorly soluble in polar solvents like methanol and acetonitrile.

Retinoids are thermolabile, photosensitive and easily attacked by oxidants. This is mainly due to the electron-rich polyene chain. The polyene chain consists of several carbon–carbon double bonds in conjugation. In retinol, five double bounds are in conjugation, although the one in the ring is not in full resonance with the side chain due to steric hindrance. The λ_{max} of the retinoids dissolved in ethanol or methanol varies from below 300 to more than 400 nm with molar extinction coefficients (ε) in the range 20 000–100 000 (Table 1). Thus, very selective and sensitive detection with UV detectors can be achieved. The λ_{max} of at-retinol in ethanol is 325 nm, while in retro-retinoids all five bonds are in full resonance, which raises the λ_{max} approximately 25 nm, additionally a fine vibronic spectrum can be

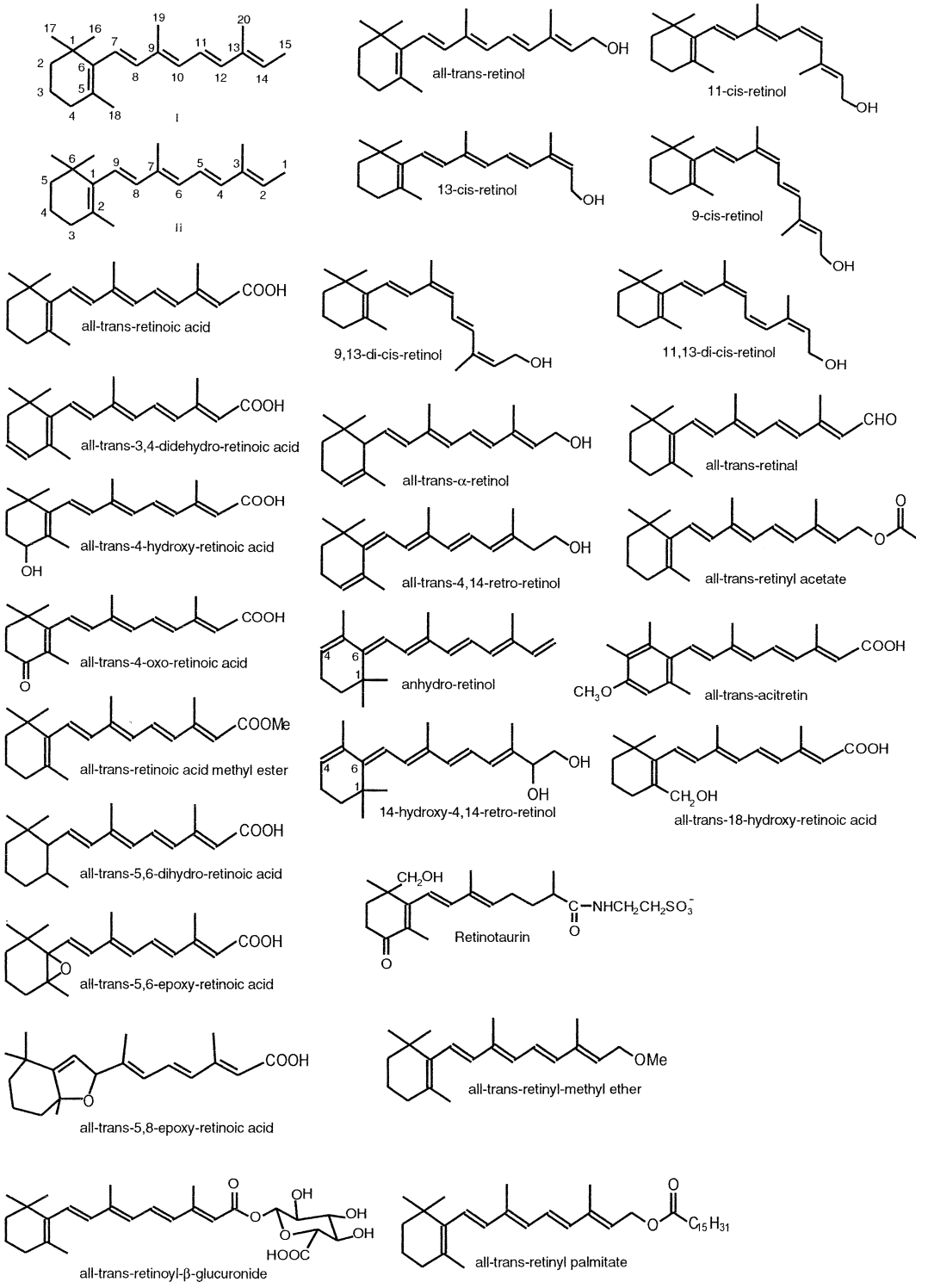

Fig. 1. Chemical structure of the carbon skeleton with classical numbering (I), numbering according to the IUPAC–UIB nomenclature (II) and chemical structure of selected retinoids.

Table 1
Roche substance number, molecular formula, molecular mass, light absorbance maximum (λ_{max}), molar extinction coefficient (ϵ) and ($E^{1\%}$) for selected retinoids

Retinoid	Ro No.	Molecular formula	M_r	λ_{max} (nm)	ε	$E^{1\%}_{1\ cm}$	Solv.
at-ROH (vitamin A)	01-4955	$C_{20}H_{30}O$	286.46	325	48305	1689	D
13-*cis*-ROH		$C_{20}H_{30}O$	286.46	328	51770	1810	D
11-*cis*-ROH		$C_{20}H_{30}O$	286.46	319	34890	1220	D
11,13-di-*cis*-ROH		$C_{20}H_{30}O$	286.46	311	29240	1024	D
9,13-di-*cis*-ROH		$C_{20}H_{30}O$	286.46	324	39500	1379	D
9-*cis*-ROH	4-4069	$C_{20}H_{30}O$	286.46	323	42300	1477	D
at-4-Oxo-ROH		$C_{20}H_{29}O_2$	301.44	347			A
at-3-OH-ROH		$C_{20}H_{30}O_2$	302.45	325			D
5,6-Epoxy-ROH		$C_{20}H_{30}O_2$	302.45	310	73140	2422	D
5,8-Epoxy-ROH		$C_{20}H_{30}O_2$	302.45	278	53390	1768	D
at-3,4-Didehydro-ROH (vitamin A_2)	4-3791	$C_{20}H_{28}O$	284.44	350	41320	1455	D
13-*cis*-3,4-didehydro-ROH	4-4264	$C_{20}H_{28}O$	284.44	352	39080	1376	D
9-*cis*-3,4-didehydro-ROH		$C_{20}H_{28}O$	284.44	348	32460	1143	D
9,13-Di-*cis* 3,4-didehydro-ROH		$C_{20}H_{28}O$	284.44	350	29950	1030	D
α-ROH		$C_{20}H_{30}O$	286.45	311	47190	1650	D
14-Hydroxy-4,14-retro-ROH		$C_{20}H_{30}O_2$	302.46	348	53960	1785	D?
at-Retinyl acetate		$C_{22}H_{32}O_2$	328.5	325	51180	1560	D
at-Retinyl palmitate	1-5852	$C_{36}H_{60}O_2$	524.9	325	49260	940	D
Anhydroretinol		$C_{20}H_{28}$	268.44	371	97820	3650	D
Anhydrovitamin A_2				370	79270	2980	D
Retinyl methyl ether		$C_{21}H_{32}O$	300.48	328	49800	1660	C
TMMP-ROH	12-0586	$C_{21}H_{32}O_2$	316.48	325	49800	1596	D
at-RA (tretinoin)	01-5488	$C_{20}H_{28}O_2$	300.42	350	45300	1510	D
13-*cis*-RA (isotretinoin)	13-7652	$C_{20}H_{28}O_2$	300.42	354	39750	1325	D
11-*cis*-RA		$C_{20}H_{28}O_2$	300.42	342			D
11,13-Di-*cis*-RA		$C_{20}H_{28}O_2$	300.42	346	25890	863	D
9,13-Di-*cis*-RA		$C_{20}H_{28}O_2$	300.42	346	34450	1150	D
9-*cis*-RA	04-4079	$C_{20}H_{28}O_2$	300.42	345	36900	1230	D
at-3,4-Didehydro-RA (vitamin A_2 acid)		$C_{20}H_{26}O_2$	298.43	370	41570	1395	D
13-*cis*-3,4-Didehydro-RA		$C_{20}H_{26}O_2$	298.43	372	38740	1300	D
9-*cis*-3,4-Didehydro-RA		$C_{20}H_{26}O_2$	298.43	369	36950	1240	D
9,13-Di-*cis*-3,4-didehydro-RA		$C_{20}H_{26}O_2$	298.43	366	32990	1107	D
α-RA		$C_{20}H_{28}O_2$	300.44	340	33000	1100	D
at-Methylretinoate	4-3781	$C_{21}H_{30}O_2$	314.46	354	44340	1415	D
13-*cis*-Methylretinoate		$C_{21}H_{30}O_2$	314.46	359	38310	1220	D
at-Retinoyl-β-glucuronide		$C_{26}H_{36}O_8$	476.56	360	50700	1065	B
13-*cis*-Retinoyl-β-glucuronide		$C_{26}H_{36}O_8$	476.56	369			B
9-*cis*-Retinoyl-β-glucuronide		$C_{26}H_{36}O_8$	476.56	353			B
5,6-Epoxy-RA		$C_{20}H_{28}O_3$	316.44	338	45280	1442	D
5,8-Epoxy-RA		$C_{20}H_{28}O_3$	316.44	298	39470	1257	D
3-OH-RA (Ro-14-7627/000)		$C_{20}H_{28}O_3$	316.44	355			A
at-4-Oxo-RA	12-4824	$C_{20}H_{26}O_3$	314.43	360	58220	1854	D
13-*cis*-4-Oxo-RA	22-6595	$C_{20}H_{26}O_3$	314.43	361	39000	1242	D
at-4-Oxo-retinoyl-β-glucuronide		$C_{26}H_{35}O_9$	491.55	364			B
13-*cis*-4-Oxo-retinoyl-β-glucuronide		$C_{26}H_{35}O_9$	491.55	367			B
9-*cis*-4-Oxo-retinoyl-β-glucuronide		$C_{26}H_{35}O_9$	491.55	356			B
Acitretin (TMMP-RA)	10-1670	$C_{21}H_{26}O_3$	326.44	361	41400	1270	D
13-*cis*-Acitretin	13-7652	$C_{21}H_{26}O_3$	326.44	361	40450	1241	D

Table 1. Continued

Retinoid	Ro No.	Molecular formula	M_r	λ_{max} (nm)	ε	$E^{1\%}_{1\ cm}$	Solv.
at-RAL	1-6015	$C_{20}H_{28}O$	284.44	383	42880	1510	D
13-*cis*-RAL		$C_{20}H_{28}O$	284.44	375	35500	1250	D
11-*cis*-RAL		$C_{20}H_{28}O$	284.44	380	24935	878	D
11,13-di-*cis*-RAL		$C_{20}H_{28}O$	284.44	373	19880	700	D
9,13-di-*cis*-RAL		$C_{20}H_{28}O$	284.44	368	32380	1140	D
9-*cis*-RAL		$C_{20}H_{28}O$	284.44	373	36100	1270	D
at-3,4-Didehydro-RAL	4-6273	$C_{20}H_{26}O$	282.4	401,314	41450	1470	D
9-*cis*-3,4-Didehydro-RAL		$C_{20}H_{26}O$	282.4	391,315			D
13-*cis*-3,4-Didehydro-RAL		$C_{20}H_{26}O$	282.4	395,314			D
11,13-Di-*cis*-3,4-didehydro-RAL		$C_{20}H_{26}O$	282.4	386,269			D
3-OH-RAL		$C_{20}H_{28}O_2$	300.43	379			A
4-Oxo-RAL		$C_{20}H_{27}O_2$	299.43	381			A
5,6-Epoxy-RAL		$C_{20}H_{28}O_2$	300.43	365	45330	1511	D
α-RAL		$C_{20}H_{28}O_2$	300.43	368	48800	1720	D
Etretinate	10-9359	$C_{23}H_{30}O_3$	354.49				D
Arotinoid (TTNPB)	13-7410	$C_{24}H_{28}O_2$	348.49				D
TTNN		$C_{25}H_{26}O_2$	358.48				D
AM 580		$C_{22}H_{25}NO_3$	351.45				D

Values are collected from Refs. [13,16,20,21,27].
ROH, retinol; RAL, retinal; RA, retinoic acid.
A: Acetonitrile–methanol–formic acid (65:15:0.12).
B: Methanol.
C: 2-propanol.
D: Ethanol.

seen. In retinal and retinoic acid, the polyene chain is extended by the carbonyl group and the λ_{max} is increased to 380 and 350 nm, respectively. Exchanging the π-electron donating carboxylic acid group of retinoic acid with the π-electron withdrawing hydroxyl group of retinol lowers the λ_{max}. In 3,4-didehydro retinoids, another double bond is introduced, adding 20 nm to the λ_{max}, while in 5,6- or 5,8-epoxides the polyene chain is broken and the λ_{max} is reduced substantially. The all-*trans* isomers will generally give the highest λ_{max}. *cis* Configuration in one or more of the double bonds will lower the λ_{max} a few nanometers, and an additional ''*cis* band'' will appear between 233 and 265 nm [16]. The intensity of this band will be higher if the *cis* configuration is located in the centre of the side chain. Thus, 9-*cis*-RA will have a higher ''*cis* band'' than 13-*cis*-RA. The highest intensity is seen for 9,13-di-*cis*-RA. The light absorbance properties of selected retinoids are shown in Table 1. Additional details on the physiological properties of retinoids will be given in subsequent chapters. The reader is also referred to several comprehensive books on the topic [17,18].

2.3. Standards

Only a few pure retinoid reference compounds are available commercially. These are at-RA, 13-*cis*-RA, 9-*cis*-RA, at-ROH, 13-*cis*-ROH, at-RAL, 13-*cis*-RAL, 9-*cis*-RAL, at-retinyl acetate, at-retinyl palmitate. (Sigma–Aldrich, BioMol). Other retinoids will have to be synthesized, isolated from natural sources or obtained from colleagues and industrial sources such as Hoffman-La Roche. Carotenoids or xanthophylls can be cleaved to their corresponding retinoid derivatives by oxidation with osmium tetroxide [19–21]. This is a simple way of generating retinoids if the pro-vitamin A compound is available. For example, oxidation of canthaxanthin generates 4-oxoretinal, which in turn can be reduced to 4-oxoretinol with sodium borohydride or oxidised to 4-oxoretinoic acid by sodium chlorite treatment [20]. Waldmann et al. have described a simple H_2O_2/

iron(III) porphinate mediated conversion of retinol (10 mg) to 4-hydroxyretinol (65 μg), 4-oxoretinol (70 μg), 5,8-epoxyretinol (55 μg), 5,6-epoxyretinol (65 μg), 3-dehydroretinol (35 μg), retroretinol (100 μg) and anhydroretinol (95 μg) [22]. Retinyl esters can be prepared by reacting retinol or 3,4-didehydroretinol with the proper fatty acyl chloride or fatty acid anhydride [23–26]. For more detailed information on the synthesis of retinoids the reader is referred to some excellent books and papers on the topic [13,17,18,27].

cis Isomers of various retinoids can be generated by irradiating all-*trans*-retinoids with UV light. *cis*/*trans* Configuration of the resulting isomers will subsequently have to be confirmed by NMR. Detailed information on this topic can be found in several excellent papers by Nöll and co-workers [28–31] and others [32,33]. Under identical conditions, these procedures can be used for generation of pure cis isomers of retinoids.

2.4. Internal standards

The use of an internal standard usually strengthens the method and adds better precision and accuracy to a quantitative assay. However, care should always be taken when an internal standard is chosen. A sub optimal internal standard may well result in poorer assay performance. Assay performance may, in fact turn out to be better without an internal standard included in the extraction and calibration step, however adding a volumetric internal standard prior to injection is always an aid to the chemist. The internal standard should, of course, differ as little as possible from the retinoid in question but still be well resolved during the chromatographic run, except for mass spectrometry, where the internal standard preferably should co-elute with the analyte. The internal standard should have the same functional group at the end of the side chain and not be present in the sample. The choice of internal standards in retinoid analysis is however often limited by the fact that few retinoids are available commercially. Retinyl acetate, being one of the few commercially available retinoids not present endogenously, is often used. As it is a methyl ester of retinol, and lacks the hydroxyl group with proton donor properties, this retinoid will behave quite differently from the polar retinoids during extraction. If the sample preparation step includes saponification, retinyl acetate cannot be used as it will be hydrolyzed and converted to retinol. Other internal standards with retinoid structure will either have to be synthesized, isolated from natural sources, or obtained from colleagues or industrial sources as described previously.

The most often used internal standard for retinoic acid and its analogs is the aromatic retinoid TMMP-retinoic acid (Acitretin) or one of its *cis* isomers. Similarly, TMMP-retinol is used for retinol or its analogs. For retinyl esters the use of odd-numbered retinyl esters (C-15, C-17, C-19) has been suggested [34]. Odd retinyl esters have however been found in animal tissue [23]. For mass spectrometric detection stable isotope labeled retinoids are the preferred internal standard. In electrospray ionisation mass spectrometry (ESI–MS) the problem with variable signal suppression caused by variations in the matrix, makes the use of isotope labeled internal standards almost unavoidable for accurate quantitative results to be obtained [35], however, only retinyl acetate is available (Cambridge Isotope Labs., Andover, MA, USA). Others will have to be synthesized. Difficulties with ion-suppression can be resolved by performing post-column injection of the internal standard. In this way, natural retinoids with a different mass present in the sample can be used as the separation has already taken place. The internal standard mix can contain several internal standards and be injected at multiple time points during the chromatographic run [36]. Internal standards recently used can be found in Table 2.

2.5. Calibration

Concentrations of retinoid standards should always be measured spectrophotometrically in addition to or instead of weighing. Common solvents for spectrophotometric measurement of retinoids are methanol, ethanol or hexane. One should be aware of the fact that retinoic acid has very different spectroscopic characteristics in different solvents. Retinoic acids will self-associate in some solvents, which can lead to extension of the chromophore and a higher λ_{max}. Differences may be observed between 96% ethanol and absolute alcohol [37]. Large variations can also be observed if the pH of the solvent is changed. The

Table 2
Recent reversed-phase and normal-phase liquid chromatographic methods for natural retinoids

Sample type (amount used)	Retinoid	Extraction	Column	Mobile phase	Detection	LOD/LOQ	IS	Ref.
Milk powder, flour (1.25 g)	ROH	LL: Saponification, diethyl ether, petroleum ether	Lichrosorb RP-18, 5 μm, 125×4.5 mm	Acetonitrile	UV: 292 nm DAD	LOD:0.02 mg/l LOQ: 0.06 mg/l		[41]
Animal feed (1 g)	Retinyl acetate	LL: acetone–chloroform (30:70)	Novapak C_{18}, 150×3.9 mm	Methanol	UV: 290 nm	LOD 10 ng/g		[126]
Serum, blood (50 μl)	ROH	CPE: Genapol X-080 NaCl (s) acetonitrile	C_{18} (Burdick and Jackson), 5 μm, 150×4.6 mm	Ethanol–acetonitrile–water (45:45:10)	UV: 325 nm	LOD: 1 ng/ml	Retinyl acetate	[64]
Serum (100 μl)	ROH	On-line SPE: BSA-80Ts 13 μm, 10×4.6 mm, 200 n*M* SDS–ethanol (70:30) with 300 with 200 m*M* EDTA and 0.3% H_3PO_4	TSKgel ODS-80Ts, 5 μm, 150×4.6 mm:	Ethanol–water (80:20)	FL: E_x 340 nm E_m 460 nm	LOD: 0.56 μg/dl		[91]
Serum (1 ml)	at-ROH, at-retinyl palmitate	LL: Saline, ethanol, hexane	YMC C-30, 100×2.0 mm	Gradient methanol–water–acetic acid (50:50:0.5) to methanol–MTBE–acetic acid (50:50:0.5)	APCI-MS	LOD: 34 fmol/μl LOD: 36 fmol/μl LOQ: 500 fmol/μl LOQ: 250 fmol/μl	Retinyl acetate	[59]
Plasma (0.4 ml)	13-*cis*-4-oxo-RA at-4-oxo-RA 13-*cis*-RA at-RA	On-line SPE after Ethanol precipitation, LiChrospher 100 RP-18, 5 μm, 4×4 mm, 1.25% ammonium acetate and acetic acid–ethanol (8:2), on-line dilution with 2% acetic acid–ethanol (102:4)	2×Supersher 100 RP-18e, 5 μm, 250×4 mm	Gradient acetonitrile–water–10% ammonium acetate–acetic acid (60:30:6:1) to (95:2:0.5:2) to (99:0.5:0:0.5)	UV 360 nm	LOQ: 0.3 ng/ml	at-acitretin	[65]
Rat Brain, placenta, liver (50 mg)	at-ROH, at-retinyl palmitate	LL: 2×Hexane, sonication	Nucleosil 120, 5 μm, 150×4.6 mm, 40°C	Methanol–water (96.5:3.5)	UV 325 nm		Vitamin K	[60]

Table 2. Continued

Sample type (amount used)	Retinoid	Extraction	Column	Mobile phase	Detection	LOD/LOQ	IS	Ref.
Plasma (0.35 ml)	13-*cis*-4-oxo-RA 13-*cis*-RA at-RA	On-column focusing after acetonitrile precipitation	Sperisorb ODS2 C-18, 3 μm, 10×4.6 mm, 30°C	Gradient 40 m*M* ammonium acetate–buffer pH 5.75–methanol (50:50) to methanol	UV 340 nm	LOQ: 0.6, 0.3, 0.3 pmol		[45]
Calf liver (0.15 g)	Retinyl palmitate	SFE: CO_2/Hydromatrix, 41 min at 2 ml/min and 80°C	Beckman ODS C-18, 3 μm, 80×4.6 mm	Gradient acetonitrile–methanol–THF–ammonium acetate–BHT (see Ref. [7] I paper)	UV 325 nm DAD			[127]
Rat liver (1 g)	ROH 15 different retinyl esters	LL: homogenized in buffer extracted with *n*-hexane–dichloromethane (5:1) Fractions dried and dissolved in acetone applied to gold target for LDI	Suplex pKb-100, 5 μm, 250×4.6 mm	Gradient acetonitrile–methanol–dichloromethane–*n*-hexane (88:4:4:4) to (70:10:10:10)	UV 325 nm LDI–TOF–MS	Qualitative assay		[23]
Plasma (0.5 ml)	13-*cis*-4-oxo-RA, at-4-oxo-RA, 13-*cis*-RA, 9-*cis*-RA at-RA, at-ROH	LL: Ethanol, saturated ammonium sulphate, water+*n*-hexane–dichloromethane-2-propanol (80:19:1)	Nucleosil 100, 5 μm, 200×4 mm	Gradient *n*-hexane–2-propanol–acetic acid (400:1:0.27) to (400:6:0.27)	UV 350 nm	LOD: 0.5 ng/ml RA, 10 ng/ml ROH	Arotinoid ethylsulfone	[128]
Urine (5 ml)	Retinoyl-β-glucuronides of 13-*cis*-4-oxo-RA at-4-oxo-RA 13-*cis*-RA at-RA, 9-*cis*-RA	SPE: Bond-Elut C-18, 500 mg	Zorbax ODS C-18, 5 μm, 250×4.6 mm, 45°C	Gradient 1 *M* ammonium acetate–water–methanol-(4:496:600) to methanol–ethyl acetate (400:100)	UV 363 nm	LOD: 0.01 μg/ml	Acitretin-β-glucuronide	[46]
Plasma (ml)	13-*cis*-4-oxo-RA, at-4-oxo-RA, 13-*cis*-RA, 9-*cis*-RA at-RA, at-ROH	LL: diethyl ether–ethyl acetate (50:50) pH=7	Nova-Pack C-18, 4 μm, 250×4.6 mm	Gradient 2% acetic acid to methanol–acetonitrile (65:35)+5% THF	UV 350 nm	LOQ 2 ng/ml	at-acitretin, 13-*cis*-acitretin	[47]
	at-RA, at-RAL, at-ROH, at-Retinyl acetate		YMC C-30, 5 μm, 250×4.6 mm	Gradient methanol–water (50:50:0.5) to methanol–MTBE (50:50) with 0.5% acetic acid (positive) or 5 m*M* ammonium acetate (negative)	UV 360 nm AP-ESI-MS	LOD 23 pg, 1.0 ng, 0.5 ng, 10 ng		[95]

Table 2. Continued

Sample type (amount used)	Retinoid	Extraction	Column	Mobile phase	Detection	LOD/LOQ	IS	Ref.
Plasma (0.4 ml)	13-*cis*-3-OH-RA, at-3-OH-RA, 13-*cis*-3-OH-4-oxo-RA, at-3-OH-4-oxo-RA	On-line SPE after Ethanol precipitation, LiChrospher 100 RP-18, 5 μm, 4×4 mm, 0.02% ammonium acetate and acetic acid–ethanol (100:3:4) also used for on-line dilution.	2×Purospher 100 RP-18e, 5 μm, 250×4 mm	Gradient acetonitrile–water–10% ammonium acetate–acetic acid (540:450:2:30) to (600:350:2:30) to (950:40:2:30)	UV 360 nm	LOQ 1 ng/ml	Ro 12-7310/000	
Plasma (0.2 ml)	at-4-oxo-RA,5,6-epoxy-RA, 13-*cis*-RA, 9-*cis*-RA at-RA, at-ROH	Acetonitrile precipitation	Spherisorb ODS-2, 5 μm, 250×4.6 mm	Isocratic acetonitrile–water–methanol–*n*-butyl alcohol (56:37:4:3) with 100 m*M* ammonium acetate and 70 m*M* acetic acid pH=6.3	UV	LOD 5–50 ng/ml		[68]
Plasma	13-*cis*-RA, 9-*cis*-RA at-RA	SPE Accubond Methyl-C1, 100 mg. Conversion to pentafluorobenzyl ester	Nova-Pak C-18, 225×3.9 mm	Gradient 0.1 *M* ammonium acetate–acetonitrile pH=5.0 with acetic acid (80:20) to (80:10)	DAD PB–MS	LOD 25 pg		[77]
Serum (0.01–0.1) Liver (100–200 mg)	at-4-oxoretinoyl-β-glucuronide, at-retinoyl-β-glucuronide, at-4-oxoRA, at-5,6-epoxy RA, 13-*cis* RA, 9-*cis* RA, at-RA, at-ROH, at-RAL, retinyl esters	LL 2-propanol-dichloromethane (2:1)+acetic acid or ethyl acetate, hexane	Microsorb-MV, 3 μm, 100×4.6 mm	Gradient methanol–water (3:1) 10 m*M* ammonium acetate to methanol–dichloromethane (4:1)	DAD		Retinyl acetate	[101]
Plasma (0.2 ml)	at-ROH, retinyl esters	On-line SPE after ethanol precipitation (1.5 ml). LiChrospher 100 RP-18, 5 μm, 4×4 mm, 1.2% ammonium acetate–acetic acid–ethanol (80:1:20) on-line dilution with (100:2:4)	Superspher 100 RP-18e, 12.5×4 mm+250×4 mm	Isocratic acetonitrile–methanol–ethanol–2-propanol (1:1:1:1)	UV 325 nm	LOQ 2.5 ng/ml	Retinyl propionate	[48]

Table 2. Continued

Sample type (amount used)	Retinoid	Extraction	Column	Mobile phase	Detection	LOD/LOQ	IS	Ref.
Xenopus embryos	4-oxo-ROH, 4-oxo-RAL, 4-oxo-RA	Acetonitrile: 1-butanol+BHT, saturate K_2HPO_4	Vydac 201TP54, C_{18}, 250×4.6 mm	Buffer–acetonitrile (60:40) to acetonitrile (100)	UV DAD	LOD 0.5 ng		[119]
Mouse embryos	13-*cis*-RA, 9-*cis*-RA, at-RA, at-ROH,	On-line SPE after acetonitrile precipitation, Bondapak C_{18}, 37–53 μm, 10×2.1 mm acetonitrile–methanol–2% ammonium acetate–glacial acetic acid (79:2:16:3)–water (1:3)	Suplex pKb-100, 5 μm, 250×4.6 mm	Isocratic acetonitrile–methanol–2% ammonium acetate–glacial acetic acid (79:2:16:3)	ECD+0.7 V	LOD 10 pg RA LOD 25 pg ROH	13-*cis*-acitretin	[80]
Serum (0.5)	13-*cis*-4-oxo-RA, at-4-oxo-RA, 13-*cis*-RA, 9-*cis*-RA at-RA, at-ROH	On-line SPE after acetonitrile precipitation, Bondapak C_{18}, 37–53 μm, 10×2.1 mm, acetonitrile–*n*-butanol–methanol–2% ammonium acetate–glacial acetic acid, (69:2:10:16:3) on-line dilution with water	Suplex pKb-100, 5 μm, 250×2.1 mm	Isocratic acetonitrile–*n*-butanol–methanol–2% ammonium acetate–glacial acetic acid, (69:2:10:1 6:3)	UV 350 nm DAD	LOD 30–60 pg	At-acitretin	[81]
Serum (0.5)	13-*cis*-4-oxo-RA, at-4-oxo-RA, 13-*cis*-RA, 9-*cis*-RA at-RA, at-ROH	On-line SPE after acetonitrile precipitation, Bondapak C_{18}, 37–53 μm, 10×2.1 mm 0.05% TFA-acetonitrile (85:15)	Suplex pKb-100, 5 μm, 250×4.6 mm	Isocratic acetonitrile–*n*-butanol–methanol–2% ammonium acetate–glacial acetic acid, (69:2:10:16:3)	UV 350 nm DAD	LOD 1 pg	At-acitretin	[50]
Liver	Retinyl esters	On-column focusing	YMC C-30, 40 cm×320 μm	Isocratic acetonitrile–dichloromethane (70:30) temperature program	UV 327 nm	LOD 27 pg	Retinyl nonadecanoate	[72]

RP, reversed-phase; at, all-*trans*; NP, normal-phase; IS, internal standard; LL, liquid–liquid; LOD, limit of detection; LOQ, limit of quantification; CPE, cloud point extraction; THF, tetra hydro furan; TFA, trifluoro acetic acid; BHT, butylated hydroxytoluene; MTBE, methyl *tert*.-butyl ether; PB-MS, particle beam mass spectrometry; DAD, diode array detection; FLD, fluorescence detection; ED, electrochemical detection; SPE, solid-phase extraction; AP, atmospheric pressure; CI, chemical ionisation; LDI, laser desorption ionisation; TOF, time-of-flight; ESI, electrospray ionisation; RA, retinoic acid; RAL, retinal; ROH, retinol.

λ_{max} of RA in a basic water solution is 340 nm while it is 380 nm in an acidic water solution [15]. In alkaline or acidic methanol solutions the values are 340 and 355 nm, respectively [17]. For this reason the spectrum should always be scanned and the absorbance at the peak of the spectrum should be used for quantification. Commercial preparations are easily degraded and often it is necessary to purify them by one or several chromatographic steps before they can be used.

When preparing calibration standards for quantitative determination of retinoids from biological samples one should consider the way the retinoid is carried physiologically. In the cells, retinoic acid may be bound to the cellular retinoic acid binding proteins (CRABP) CRABPI or CRABPII while retinol is carried by CRBPI or CRBPII [38]. In the blood stream, retinol is carried by retinol binding protein (RBP) while retinoic acid is carried by albumin [39]. The more lipophilic retinoids, like the retinyl esters, are incorporated into chylomicrons and into lipoproteins. In various tissues, especially in the liver, highly concentrated areas of lipids with high amounts of retinyl esters form what are called lipid droplets. The optimal way to make up a calibration standard is to enrich a blank sample having identical matrix composition with known amount of pure retinoids. As retinoids are present in all cells throughout the body, this is rarely possible. Treating plasma with UV radiation, charcoal or by dialysation can provide a retinoid free matrix, but is seldom used. A better way is to enrich a 1% fatty acid free albumin solution in phosphate-buffered saline (PBS), as most retinoids will bind to albumin. This will ensure that the concentration of retinoids stays the same by keeping them in solution bound to albumin. If a carrier is not used for the retinoids, they tend to stick to the container wall making them hard to recover and susceptible to degradation. This is especially pronounced for retinoids containing functional groups or when the concentration is low. Calibration curves intended for quantifying a specific retinoid should also preferably be made with the same retinoid. Alternatively, other retinoids coupled with the use of response factors can be used. Since the UV response vary considerably among retinoids, even for *cis–trans* isomers, and literature ε values are only given for the λ_{max}, response factors will usually have to be obtained by the chemist.

3. Sample collection, handling and storage

3.1. General considerations

The electron rich polyene chain in retinoids makes them extremely sensitive to: light below 500 nm; oxygen; trace metals; strong acids; and excessive heat. If retinoids are exposed to daylight, extensive isomerisation will occur within a short time [40,41]. All sample treatment should therefore preferably be performed in amber containers under red or yellow light. Containers for storage of samples should be sized so that the sample nearly fills the container, leaving little air at the top of the sample. Purging the sample with argon gas and addition of an antioxidant like butylated hydroxytoluene (BHT) can be beneficial for long-term storage, but care should be taken since BHT can interfere with some chromatographic systems. Sensitivity towards photo-induced isomerisation appears to be less pronounced when the retinoids are bound to its transport protein in plasma or tissue. The light filtering properties of plasma and blood will also provide some protection. After homogenisation, retinoids will be exposed to biological activity in the form of enzymes and other catalysing factors such as glutathion and other thiols [42–44]. Efficient means to minimize this type of potential artifact generation is the addition of denaturing solvent or snap freezing in liquid nitrogen. Samples should be kept cold on ice during sample treatment and preferably, a cooled HPLC injector should be used during the analysis of long series. Samples to be analysed the same day can be kept at 4°C otherwise, they should be stored at −20°C or lower temperatures.

3.2. Stability of retinoids

Retinol has been found to be stable for 5 days stored in the dark at ambient temperature after extraction from powdered milk with light petroleum [41]. Teerlink et al. found that RA and its 4-oxo metabolites were stabile for 60 h when stored cooled in the dark after extraction from human plasma with acetonitrile [45]. Retinoyl-β-glucuronide concentration was shown to be highly unstable in rat urine when stored at room temperature. Large variations were also found at −20° and −70°C if the storage time exceeded 3 days [46]. Disdier et al. [47] found

an alcoholic solution of retinoic acid to isomerise or degrade to less than 50% of the initial concentration in 1 h under natural light. After 24 h, less than 10% was left. When the same alcoholic solution was stored under yellow light, no isomerisation or degradation was observed [47]. Wyss and Bucheli investigated the stability of retinoids in plasma, and found all-*trans* retinoic acid, 13-*cis*-retinoic acid and their 4-oxo metabolites to be stable for only 3 months when stored at −20°C. This was extended to 9 months when stored at −80°C [12]. Hartman et al. found no significant degeneration of retinol and retinyl esters in human plasma when stored at −80°C for 6 months [48].

4. Sample preparation

4.1. Direct injection

Plasma, serum, urine, tear fluid, cerebrospinal fluid, bile fluid are all samples in liquid form. In theory, after removal of particulate matter by filtration or centrifugation samples already in the fluid form can be injected directly into the liquid chromatographic system without further pre-treatment. HPLC columns used for this purpose are commonly called restricted access media (RAM) and are constructed such that the hydrophobic material is shielded by a hydrophilic layer, surface, or network. Upon injection of bio-fluids large molecules such as proteins will not be retained, while small molecules can penetrate the outer layer and be retained by the underlying RP material. Examples of RAM columns are shielded hydrophobic phase (SHP), internal surface reversed-phase (ISRP), semi-permeable surface (SPS) and alkyl-diol silica (ADS) [49].

Limitations to these columns are low chromatographic efficiency and a limited loading capacity. Furthermore, highly protein bound compounds such as retinoids (99.9%), are not efficiently extracted [50]. This can partially be overcome by addition of displacers (such as TCA), dilution of the sample, adjusting the pH, or addition of organic modifiers to loosen the retinoid protein interaction. The use of RAM based analytical columns has found little or no use for retinoid determination in bio-fluids.

Alternatively, bio-fluids can also be applied directly to a regular solid-phase extraction (SPE) column, after liberation of the substance as described above.

This approach was used by Wyss et al. and later by others. Acetonitrile (<20%) was added to plasma either before injection [50–52] or on-line through a T-piece prior to the SPE column in a column switching system [53]. Adachi et al. diluted bovine serum with ethanol containing 0.2 *M* SDS to keep proteins in solution in the presence of ethanol. This mixture was then injected on a BSA-ODS on-line extraction column (Tosoh, Tokyo, Japan). Proteins were washed to waste but retinol was retained and transferred to the separation column by means of column switching [54].

4.2. Homogenisation

Samples not already in the fluid form need to be homogenised before further sample pre-treatment can be done. Homogenisation is often done in an equal volume of aqueous buffer such as saline or PBS or it can be performed directly in an organic solvent. For soft tissues like cells from culture or embryonic tissue, sonication with a probe or in an ultrasound bath often is sufficient. For other tissues a more rigorous rupture of the tissue is obtained with manual or motorised glass or teflon homogenisers of the potter type. Alternatively, an ultra-turrax can be used. A higher volume of buffer or solvent is also often required. For minute samples such as embryonic tissue, a motorized pellet grinder (Kontes, http://www.kontes.com) with exchangeable tips is convenient. Care should always be taken to ensure that the equipment is clean and blanks should always be generated, with the utilization of all equipment used for preparation of the real sample.

4.3. Protein precipitation

Precipitation of protein is performed by lowering their solubility in the aqueous medium by the addition of a water miscible organic solvent or by lowering the pH of the solution. Addition of water immiscible solvents like hexane, chloroform, ethyl acetate, diethyl ether or methyl *tert*.-butyl ether (MTBE) will not lead to precipitation of proteins. Depending on the solvent, 2–4 volumes of water miscible organic solvents will have to be added to achieve a near quantitative removal of proteins.

Dilution of plasma or serum before precipitation will therefore increase the total volume substantially. Quantitative removal of proteins is especially important when no further sample clean up is performed prior to injection. Residual proteins will lead to rapid pressure build up and deterioration of the column performance. On the other hand, dilution should be kept to a minimum to maximise the sensitivity of the assay. Common solvents for protein precipitation, listed in their effectiveness in precipitating proteins are acetonitrile, acetone, methanol, ethanol and 2-propanol. The order is inversed relative to their polarity [55]. In Table 3 protein precipitation efficiency at different volumes of some common solvents and acids are given. Acetone should be avoided for determination of retinal due to the risk of aldol condensation reaction forming a C-23 ketone [13]. Precipitation with acids should be avoided because of the risk to hydrolyse the glucuronides of retinols and retinoic acids. Acids may also dehydrate retinols to anhydroretinols.

4.4. Liquid–liquid extraction

The classical way of extracting retinoids is by addition of a water immiscible solvent after protein precipitation, vigorous shaking for 5–10 min, centrifugation and removal of the organic phase. This procedure is repeated one to three times, the organic layers are pooled and the solvent is removed either by vacuum or with heating and careful purging with an inert gas. The residue is then dissolved in the mobile phase or in another solvent such as methanol. Common solvents for extraction are hexane, acetone, petroleum ether, chloroform, dichloromethane, ethyl acetate, diethyl ether or mixtures of these. Standard procedures are the Bligh Dyer extraction [56] or modifications of this and the Folch extraction [57]. Liquid–liquid extraction is still frequently used today, despite drawbacks which include: the use of toxic solvents, time-consuming procedures, problematical automation, multiple transfers and need for removal of solvent. Kitagawa and Hosotani compared the use of hexane, ethyl acetate and chloroform for extraction of retinol and retinyl palmitate from rat liver and serum [58]. They found that hexane was the most efficient in the extraction of retinol while ethyl acetate gave seven times higher recovery of retinyl palmitate, compared to hexane. They also found that repeating the extraction three times was adequate to achieve quantitative recovery. Different solvents for dissolving the residue after N_2 evaporation at 40°C were examined. Large differences in recovery depending on the solvent used, for both tissue and serum, were found. Others have found that hexane extracted retinol with recoveries in the range 94–101% from human serum [59–61]and retinyl esters with 100% recovery [62]. Disdier et al. used a mixture of diethyl ether–ethyl acetate (1:1) [47] while others used butanol–ethyl acetate (1:1) [63]. Petroleum ether was used after saponification for retinol in powdered milk [41].

A special extraction technique called cloud point extraction (CPE) was used for extraction of retinol from human serum. This technique involves the addition of a detergent, in this case Genapol X-080, to the sample. When heated over a certain temperature a phase separation will occur and the retinoid are extracted into the detergent. After removal of the water phase and dilution of the remainder, excessive detergent and hydrophobic proteins are precipitated with acetonitrile. Recovery of retinol was reported to

Table 3
The relative efficacy of some precipitants given as percentage of plasma proteins precipitated

Precipitant	pH of supernatant	0.6 vol	1 vol	2 vol	3 vol
Acetonitrile	8.5–9.5	45.8	97.2	99.7	99.8
Acetone	9.0–10	33.6	96.2	99.4	99.2
Methanol	8.5–9.5	32.2	73.4	98.7	98.9
Ethanol	9.0–10	41.7	91.4	98.3	99.1
10% TCA	1.4–2.0	99.6	99.5	99.8	99.8
6% $HCLO_4$	<1.5	98.9	99.1	99.1	99.1
5% HPO_4	1.6–2.7	98.1	98.3	98.4	98.2

The data are adapted from Blanchard [55].

be 86%. CPE is an environmentally friendly, almost solvent-free, alternative to traditional solvent extraction [64].

4.5. Mono-phase liquid extraction

Although liquid–liquid extraction with water immiscible solvent works well for the non-polar retinoids, its ability to extract polar and acidic retinoids is more questionable. When two phases are generated, there will always be a partitioning of the retinoid between the phases. The solubility of neutral retinoids in water (pH 7.4) is reported to be as high as 0.1 μ*M* [37]. Extraction of retinoic acid requires low pH to avoid extensive partitioning into the water phase. An alternative to liquid–liquid extraction is mono-phase extraction. A water miscible solvent or a mix of several solvents is added in 1–10 volumes to the biofluid or the homogenised tissue. This will precipitate proteins, liberate the retinoids and if the composition and volume is right, it will give quantitative extraction in to a single phase [65]. The combined precipitation and extraction solutions are normally supplemented with antioxidants and internal standards. After centrifugation, injection can be performed directly from above the protein pellet. Benefits are simplicity, speed, no transfers and high recoveries. The use of internal standard is less important and excellent precision is obtained due to few steps. Retinoids present in high concentrations such as retinol [66] in plasma and retinyl esters in liver can readily be determined by this extraction technique in combination with HPLC. Mono-phase extraction has also been used for RA in bovine or human serum [67,68].

For retinoids present at low concentrations, drawbacks of mono-phase extraction are low selectivity and dilution instead of concentration of the sample. Removal of the solvent either by N_2 evaporation or by vacuum cannot be achieved without strong heating when the extract contains substantial amounts of water. However, by addition of a saturated salt solution, the polarity and ion strength of the mono phase are increased severely. If an aprotic solvent such as acetonitrile or acetone is used, phase partition will occur rapidly. The retinoids will partition into the organic phase and both clean-up and concentration is achieved. In a frequently used procedure by McClean et al., 0.4 volumes of butanol–acetonitrile (1:1) is added to 1 volume of serum. After vortex mixing 0.3 volumes of saturated dipotassium monohydrogen phosphate is added. After mixing and centrifugation, the supernatant is injected directly into the HPLC [69]. This procedure was used by others for perfusate, bile, and hepatic tissue [70] and mouse plasma and liver [71].

4.6. On-column focusing

Volumes injected on normal bore HPLC columns (4.6 mm I.D.) usually are in the range 5–100 μl. Exceeding this volume often leads to distorted peaks or even split peaks. However, larger volumes can be applied if the elution strength of the injection solution and the mobile phase is lowered by dilution with a buffer. Large volumes can then be applied and the retinoids will be focused at the entrance of the column. Elution is then achieved by increasing the elution strength of the mobile phase or in some instances by temperature programming [72].

Teerlink et al. focused 2000 μl of the diluted supernatant resulting from acetonitrile precipitation of plasma on a 4.6 mm column. The method allowed quantification of endogenous levels of at-RA, 13-*cis*-RA and 13-*cis*-4-oxo-RA in human plasma and cell cultures [45]. Molander et al. used a 320 μm I.D. capillary column packed with 5 μm Suplex pkb-100 material for the separation of at-retinol, at-RA and 13-*cis*-RA. Operation at elevated temperature provided reduced column backpressure, and allowed higher volumetric flow-rates to be used during sample introduction. Injection volumes up to 100 μl were successfully applied and a mass limit of detection (mLOD) of 0.5 ng of the retinoic acids, corresponding to a concentration limit of detection (cLOD) of 5 ng/ml, was found using on-column UV detection (320 μm light path) at 360 nm. The authors estimated that a mLOD of 12 pg would be possible if a Z-cell with 8 mm light path was used [73]. A similar approach was used for determination of retinyl esters in arctic seal liver samples. On-column focusing and large volume injection on a C-30 capillary column under isocratic non-aqueous conditions gave a mLOD of 27 pg. Elution of retinyl esters were aided by temperature programming and the detection was performed with a ''U-shaped'' flow

cell. Focusing of large injection volumes can also be achieved by installing a large particle size SPE column in the loop of the injector. The auto sampler is then used as a SPE robot, programmed to perform automated SPE. Elution is then achieved by the mobile phase when the injection valve is turned.

4.7. Solid-phase extraction

An additional technique for concentration and further clean up after protein precipitation is SPE. This technique has gained much popularity in the last few years, but surprisingly not as much in retinoid analysis. The supernatant is applied, either directly, or after dilution and pH adjustment, to the pre-conditioned SPE cartridge. The solid-phase is most often an alkyl modified silica material. C_1, C_2, NH_2, strong anion-exchange (SAX) and mixed mode materials have been used. The cartridge is then washed with several volumes of acetonitrile–water or methanol–water eluted by gravity, vacuum or by positive pressure. Elution of the extracted substances is then achieved by passing through 250–500 μl of a solvent with strong eluting power like methanol or chloroform. The eluate can be injected directly or evaporated with N_2 and dissolved in a smaller volume. SPE can be performed either manually or by automated systems like Prospect (Spark, The Netherlands) and AASP (Varian, USA). Retinoyl-β-glucuronide was extracted from urine by applying urine directly to a SPE cartridge after dilution and acidification with acetic acid [46]. In numerous publications dealing with the toxicology of retinoids [74], Nau and co-workers have used 2-propanol monophase extraction with subsequent on-line SPE on AASP C_2 columns to determine a wide range of retinoids from most types of tissues and biofluids [75,76], while others have used C_1 [77] or Bakerbond C_{18} [78]. Horst et al. used the SPE step to remove retinol (ROH) and retinyl ester (RE) prior to determination of 9,13-di-*cis*-RA in bovine plasma [79].

4.8. Column switching

Multidimensional HPLC systems, where several columns and two or more pumps are connected by manually, pneumatically or electrically driven switching valves is often referred to as column switching systems. Flow from the pumps is directed through different columns by changing the position of the valves. Column-switching systems can be used for the automation of a wide range of sample preparation steps, derivatisation and separation techniques that are used in HPLC analysis of retinoids. The instrumentation can be rather complex but once it is established, a very flexible system that can be used for most applications will be available. Column-switching systems are especially suited for retinoid analysis because the sample is protected from the exposure to light and air throughout the entire analytical procedure. The classical way of using this technique in retinoid analysis is on-line solid-phase extraction. The difference between semi automated SPE equipment like Prospect and AASP is that SPE column-switching system is a high-pressure system that uses short HPLC columns. High sensitivity can be obtained by concentrating large injection volumes onto narrow bore columns [65,80,81].

A schematic presentation of a typical on-line solid-phase extraction system is shown in Fig. 2. The injected sample is concentrated on the concentrating column by a mobile phase with weak eluting strength. When the injection volume is large and of a strong eluting strength, the sample plug is diluted on line through a T-piece. Kraft was the first to describe the use of column-switching systems for retinoid analysis [82]. This technique has been adapted and further developed by others over the years. Wyss and co-workers described their use of the column switching technique in a series of excellent publications, where they analysed both natural and synthetic retinoids [51–53,65,83–89]. Recently, Wyss et al. described a method for determination of 13-*cis*-3-hydroxy RA, all-*trans*-3-hydroxy RA and their 4-oxo metabolites in human and animal plasma [83]. Polar retinoids were concentrated after ethanol (1.5 ml) precipitation of plasma (0.4 ml) on an 4×4 mm I.D. LiChrospher 100, 5 μm C_{18} column, with a mobile phase containing only 4% organic modifier. With this high water content, polar retinoids were successfully concentrated with high recoveries (97–107%). A slightly different method was used for determination of endogenous levels of 13-*cis*-RA, all-*trans*-RA, and their 4-oxo metabolites in human and non-

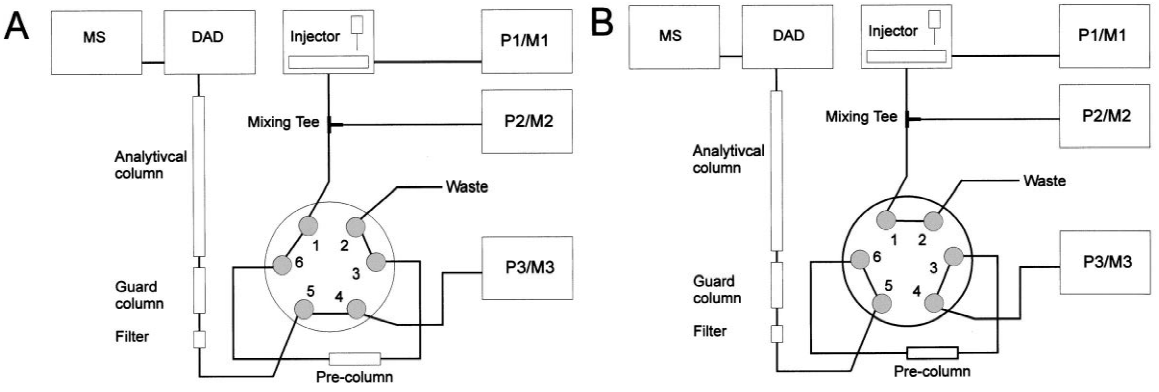

Fig. 2. Schematic representation of a column switching system for on-line dilution and on-line solid-phase extraction. (A) Sample is injected in mobile phase 1 (M1) delivered by pump 1 (P1), diluted on-line by P2 with M2 through the mixing tee to reduce the elution strength. Retinoids are extracted as the sample passes through the pre-column, while polar constituents are washed to waste. (B) The position of the six-port switching valve is changed and extracted retinoids are eluted from the pre-column by M3 delivered by P3. Retinoids are separated on the analytical column and detected by diode array detection (DAD) and mass spectrometric detection (MS).

human plasma [50,65]. Gundersen et al. used narrow bore columns for both concentration and separation. Sensitivity was increased three times compared to normal bore columns, and a mass limit of detection (mLOD) of 60 pg was found [81]. In another publication Sakhi et al. implemented coulometric electrochemical detection in combination with on-line SPE. An mLOD of 10 pg was obtained for retinoic acids and the method was later used to measure endogenous concentrations of retinoids in the developing mouse embryo [90]. Adachi and coworkers examined the use of detergents to keep proteins in solution under RP conditions. They found that after addition of 200 m*M* sodium dodecyl sulfate (SDS)–ethanol (7:3 v/v) retinol could be recovered quantitatively on a 10×4.6 mm I.D. BSA-80Ts column before transfer by column switching to the separating column. The addition of SDS was found to have no effect on separation over time [54,91]. A system for on-line alkaline hydrolysis of milk before concentration and separation is described by Delagado-Zemarreno et al.. Ethanolic sodium hydroxide is mixed with diluted milk on-line through a T-piece and hydrolysis allowed to occur in a reaction chamber. Exiting the reaction chamber the alkaline solution is neutralised with 2.5 *M* acetic acid to be compatible with the silica based C_{18} SPE column used for concentration. Elution is then achieved by methanol [92].

Solid-phase extraction of polar and non-polar retinoids under the same set of conditions has proven difficult because, in the highly aqueous mobile phase needed to retaine the polar retinoids, non-polar retinoids like retinyl esters seem to aggregate and form oily droplets and pass unretained through the SPE sorbent. Hartman et al. recently described the determination of retinol and several retinyl esters in human plasma with an on-line solid-phase extraction system [48]. Plasma (0.2 ml) is diluted with ethanol (1.5 ml) and 1.2 ml was injected and diluted on-line by 1% ammonium acetate–acetic acid–ethanol (100:2:4) to reduce the elution strength of the sample plug concentration with high recoveries is successfully achieved on a 4×4 mm LiChrospher 100 RP-18 column, before transfer to analytical column took place by column switching.

5. Chromatographic separation

Chromatography has always played a pivot role in retinoid research and high-performance liquid chromatography (HPLC) has become the predominant method for separation and quantification of retinoids in biological samples. Altogether, there is a vast number of possible natural retinoids spanning a wide range of polarity. To develop a method that could separate all in the same run would be almost an

impossible task. For this reason, chromatographic separation conditions are often tailored for each application.

HPLC is often divided into normal-phase, also called straight-phase, chromatography and reversed-phase (RP) chromatography. In normal-phase HPLC the stationary phase is a polar phase such as bare silica or silica modified with polar groups such as CN or NH_2 or short alkyl groups. The eluent is a non polar liquid such as hexane with small amounts of more polar solvents such as 2-propanol or chloroform. RP-HPLC is characterized by the partitioning of the analyte between a hydrophobic stationary phase and a polar eluent, but can also be operated in non-aqueous mode (NARP). Typical stationary phases are ODS (C_{18}) or C_8, but in recent years, C_{30} stationary phases also have been used. RP chromatography is often preferred because of its superior stability and robustness over normal-phase chromatography. Straight-phase chromatography is very sensitive to small changes in the mobile phase composition and needs long equilibrium time if a gradient elution is used. Normal-phase chromatography can, however, be of great use when the sample matrix is very high in lipid content, animal or plant oils for example, or when trace amounts of a retinoid need to be quantified in the presence of a high concentration of retinyl esters. Oil dissolved in hexane can be directly injected without further purification. If necessary, fractions can be collected, easily evaporated and injected on a RP system. *cis*/*trans* Isomers of retinal and retinol are often very difficult to separate in RP systems but are easily separated in normal-phase systems [30].

5.1. Reversed phase

Numerous reversed-phase (RP) chromatographic procedures have been described for the separation of retinoids. The majority of these are based on the use of C_{18} column eluted with methanol–water or acetonitrile–water in various proportions. Modern RP columns are very similar and few selectivity differences are found. However, there are a few special columns on the market offering very different selectivity. The need for selectivity varies considerably depending on the application, the complexity of the matrix, the concentration level of the analyte and the detection technique used. If only retinol is to be determined, virtually any RP column can be used in combination with UV detection or the more selective fluorescence detection (FLD). Similarly, different retinyl esters are easily separated on most RP columns and both UV and FLD are applicable. Separation of retinyl oleate and palmitate are sometimes reported to be a problem, however this can easily be achieved with acetonitrile–chloroform (80:20) in less than 10 min on a C_{18} column [93]. Care should be taken however, to make sure that there is no interference from carotenoids in general and especially phytofluene if fluorescence is used. Got et al. used a C_8 column and methanol–water (94:6) for the separation of retinol, retinyl acetate and the four major retinyl esters in human liver biopsies [62]. A 320 μm capillary column packed with C_{30} material was used to separate retinyl esters in arctic seal liver. Elution was achieved with acetonitrile–dichloromethane (70:30) and a temperature gradient (Fig. 3). Several additional peaks were separated compared to a normal bore column and a mLOD of 27 pg for retinyl palmitate was found [72]. A C_{30} capillary column was also used by Roed et al. in NARP electrochromatography of retinyl esters [94]. Van Bremen and Huang used the YMC C_{30} RP column for separation of retinoic acid, retinol, retinal, and retinyl acetate with gradient elution using mobile phases containing water, methanol, and methyl *tert*.-butyl ether (Fig. 4) [95]. The same column was compared with a C_{18} column for separation of RA isomers and 5,6-epoxy-RA before on-line nuclear magnetic resonance (NMR) [96]. The use of C_{30} columns was recently reviewed [97]. A gradient HPLC method was described for the separation of retinol and 15 related retinyl esters within 28 min. Analysis of rat liver extract showed the presence of retinyl myristate, pentadecanoate, palmitoleate, palmitate, heptadecanoate, linoleate, oleate, stearate, and 3,4-didehydroretinyl palmitate [23]. A recent paper by Hartmann et al. describes a column-switching system for on-line SPE of RE from human plasma. Isocratic separation of retinyl esters including oleat and palmitate was achieved with acetonitrile–methanol–ethanol–2-propanol (1:1:1:1) on a Superspher 100 RP-18 column [48]. The described method is one of a very few published methods that allows quantification of normal levels

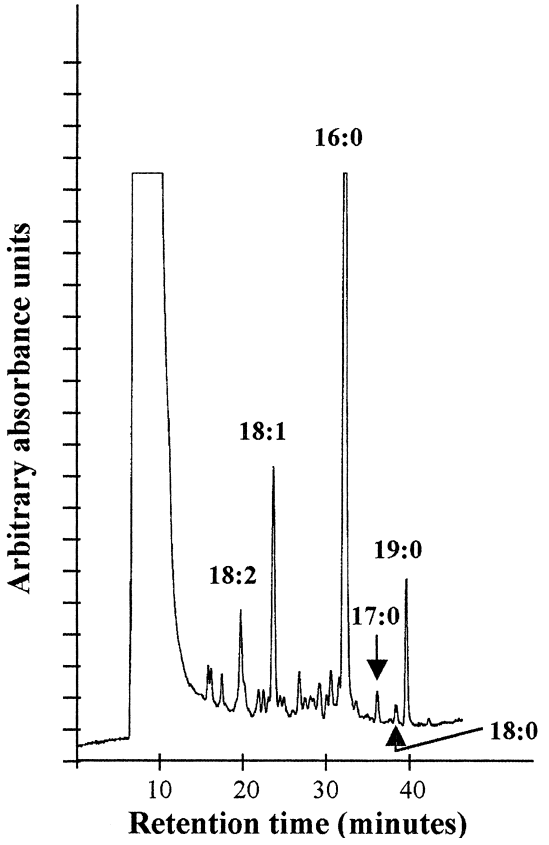

Fig. 3. Temperature programmed separation of retinyl esters in a polar seal liver sample. The injection volume was 10 μl. The column was of dimensions 40 cm×0.32 mm I.D. packed with 5 μm C_{30} particles. The mobile phase consisted of acetonitrile–dichloromethane (70:30, v/v). Temperature program: 10°C for 10 min, then 1°C/min to 30°C, then 2.5°C/min to 70°C. Peak identification; retinyl palmitate (16:0), retinyl heptadecanoate (17:0), retinyl stearate (18:0), retinyl oleate (18:1), retinyl linoleate (18:2) and retinyl nonadecanoate (19:0). (Reproduced with permission from Ref. [72]).

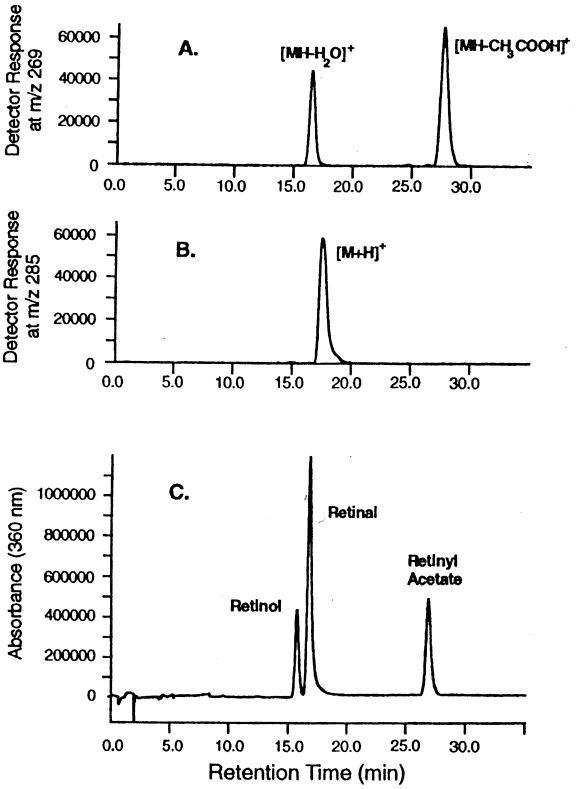

Fig. 4. Positive ion electrospray LC–MS analysis of a mixture of approximately 300 pmol each (after splitting the HPLC effluent 1/50) of all-*trans*-retinol, all-*trans*-retinal, and all-*trans*-retinyl acetate. HPLC separations were carried out on a C_{30} RP column with gradient elution using mobile phases containing water, methanol, and methyl *tert*.-butyl ether. Ammonium acetate (5 m*M*) was added to the mobile phase to facilitate ion pair formation during RP-HPLC of retinoic acid, and acetic acid (0.5%, v/v) was added to the mobile phase to enhance protonation during LC–MS analysis of nonacidic retinoids (A) Computer reconstructed mass chromatogram of the fragment ion of *m*/*z* 269 corresponding to the base peaks of the retinol and retinyl acetate electrospray mass spectra. (B) Computer reconstructed mass chromatogram of the protonated molecule, $[M+H]^+$, of retinal. (C) Absorbance chromatogram at 360 nm obtained on-line during LC–MS showing peaks for retinol, retinal and retinyl acetate. (Reproduced with permission from Ref. [95]).

of retinyl esters in human plasma in the post absorptive phase (Fig. 5). RP separation of the geometrical isomers of retinol is not easily performed, but has been achieved on a 250×4.6 mm I.D. Vydac 201TP54 C_{18} column (5 μm, 300 Å) eluted with methanol–*n*-butanol–water (65:10:25) containing 10 n*M* ammonium acetate, pH 3.2 [98]. Separation of the geometrical isomers of RA is usually performed with methanol–water or acetonitrile–water containing ammonium acetate and acetic acid on C_{18} columns.

The bulk of described methods use gradient elution and only a very few papers describe the isocratic separation of RA isomers. An isocratic separation is difficult to achieve but can be of great advantage in some instances. Electrochemical detection is potentially very sensitive, but requires isocratic conditions, if not an expensive array system

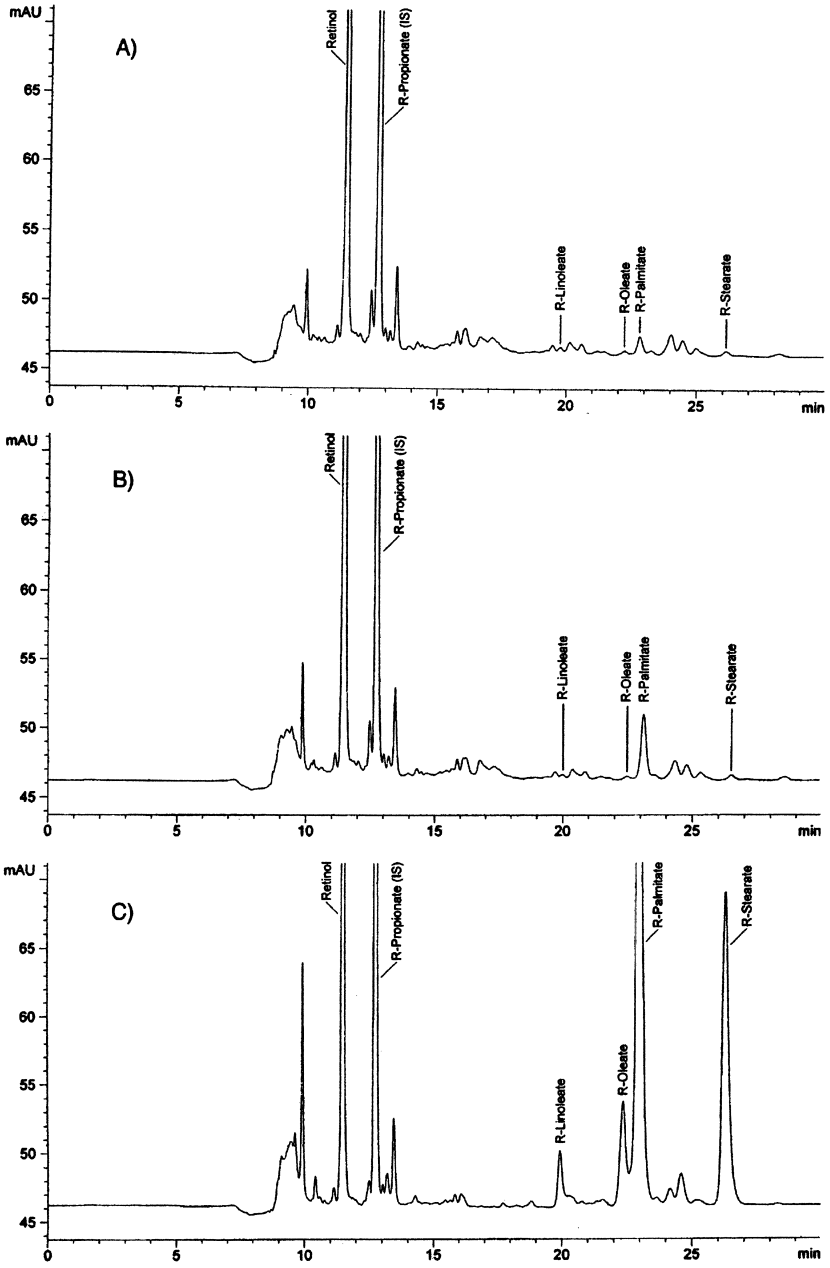

Fig. 5. Chromatograms of human plasma samples: (A) blank plasma, (B) blank plasma spiked with additional 200 ng/ml retinol and 80 ng/ml retinyl palmitate, (C) plasma from a volunteer 5 h after a oral dose of 4000 IU retinyl acetate administered together with a standard liver meal containing 100 000 IU vitamin A. Plasma (0.2 ml) was deproteinized by adding ethanol (1.5 ml) containing the internal standard retinyl propionate. Following centrifugation the supernatant was directly injected onto the pre-column packed with LiChrospher 100 RP-18 using 1.2% ammonium acetate–acetic acid–ethanol (80:1:20, v/v) as mobile phase. The elution strength of the ethanol containing sample solution was reduced by on-line supply of 1% ammonium acetate–acetic acid–ethanol (100:2:4, v/v). The retained retinol and retinyl esters were then transferred to the analytical column (Superspher 100 RP-18, endcapped) in the backflush mode and chromatographed under isocratic conditions using acetonitrile–methanol–ethanol–2-propanol (1:1:1:1, v/v) as mobile phase. Compounds of interest were detected at 325 nm. (Reproduced with permission from Ref. [48]).

is used (ESA Inc.). An isocratic separation is also very favorable in capillary chromatography. A capillary column with inner diameter of 180 μm were employed for the separation of geometrical isomers of RA using C_{18} stationary-phase and a mobile phase containing acetonitrile–water–methanol (65:32.5:2.5, v/v/v) with 1% tetrabutylammonium perchlorate and 0.174 *M* acetate buffered at pH 5 [67]. The Suplex pKb-100 column (Supelco) is superior for isocratic separation of RA isomers, and has been used by several for this purpose with excellent results [33,50,73,79–81,90]. Fig. 6 illustrates the isocratic separation of photo-isomerised RA and 4-oxo-RA on a Suplex pKb-100 column. Dimitrova et al. used Spherisorb ODS-2 (5 μm) column (250×4.6 mm I.D.) with acetonitrile–water–methanol–*n*-butanol (56:37:4:3, v/v) containing 100 m*M* ammonium acetate and 70 m*M* acetic acid as the elution system [68]. Details on these methods and other isocratic methods [99,100] is given in Table 2.

Several methods have applied gradient separation of retinoids. Barua and Olson described an RP-HPLC procedure that utilizes gradient elution to simultaneously analyse very polar retinoids and retinol, non polar retinoids present in human serum and liver and rat serum and tissues [101]. However, as is often the case, attempts to cover the whole range of retinoids including geometrical isomers of several classes of retinoids are seldom successful and separation of *cis*/*trans* isomers is poor (RA) or not shown (Fig. 7). Wyss et al. described a separation of several geometrical isomers of 3-hydroxy-RA and 4-oxo-RA together with the isomers of RA on two 250 mm columns connected in series, non-polar retinoids such as retinyl esters were not included (Figs. 8 and 9) [83].

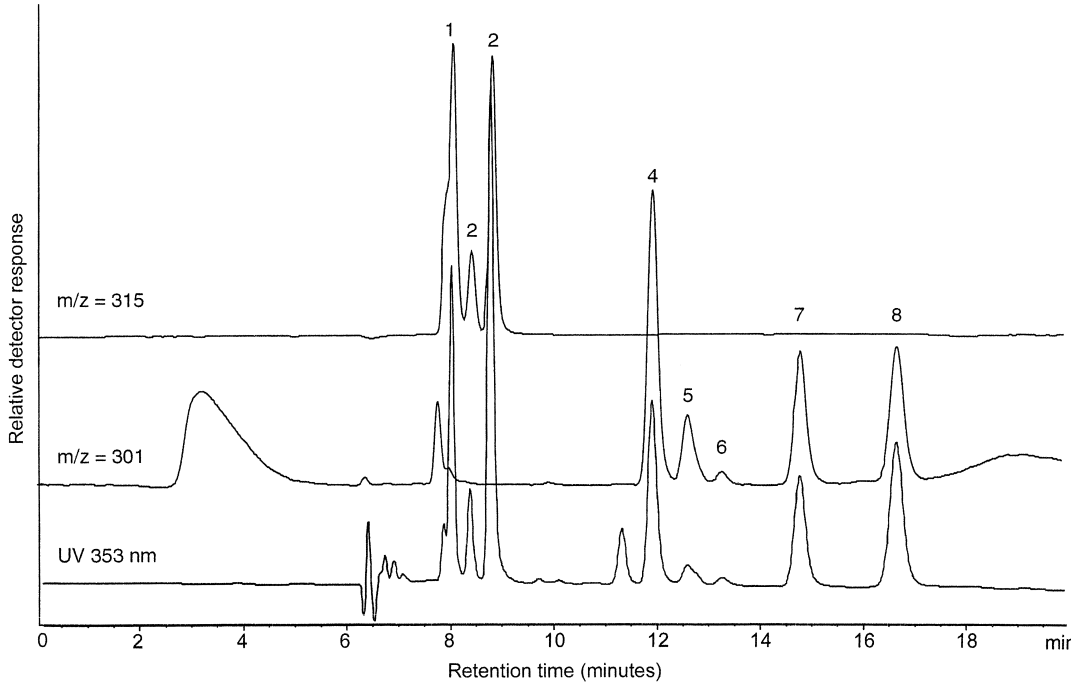

Fig. 6. Chromatogram showing isocratic separation of photo isomerised retinoic acid and 4-oxoretinoic acid on a 250×2.1 mm I.D. Suplex pKb-100 column. The mobile phase consisted of acetonitrile–methanol–2% ammonium acetate–glacial acetic acid (79:2:16:3, v/v), 0.4 ml/min, 40°C. The lower trace shows detection at 353 nm, while the middle and upper trace is obtained with a mass-selective detector and an electrospray interface. The mass-selective detector is operated in the selected ion monitoring (SIM) mode and at *m*/*z* 301 only protonated retinoic acid is detected (middle trace) while at *m*/*z* 315, only radical ions of the 4-oxo-retinoic acids are detected. Elution order of retinoids: 1=13-*cis*-4-oxo-RA, 2=9-*cis*-4-oxo-RA, 3=at-4-oxo-RA, 4=13-*cis*-RA, 5=11-*cis*-RA and 11,13-di-*cis*-RA, 6=9,13-di-*cis*-RA, 7=9-*cis*-RA, 8=at-RA.

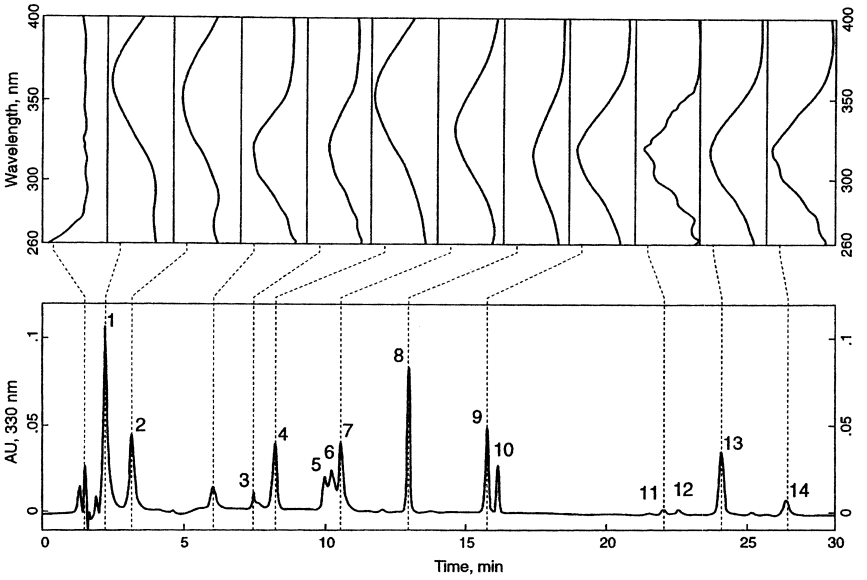

Fig. 7. Spectrum index plot of a standard mixture of retinoids obtained by reversed-phase gradient HPLC. Peak identification: 1, all-*trans*-4-oxo-retinoyl-β-glucuronide; 2, all-*trans*-4-oxo-retinoic acid; 3, all-*trans*-5,6-epoxyretinoic acid; 4, all-*trans*-retinoyl-β-glucuronide; 5, 13-*cis* retinoic acid; 6, 9-*cis* retinoic acid; 7, all-*trans*-retinoic acid; 8, all-*trans*-retinol; 9, all-*trans*-retinyl acetate; 10, methyl retinoate; 11, retinyl ester; 12, retinyl linolenate; 13, retinyl palmitate; and 14, retinyl stearate. A 3-μm Microsorb-MV column (100×4.6 mm I.D., Rainin, Woburn, MA, USA) was used, which was preceded by a guard column of C_{18} material (Upchurch Scientific, Omaha, NE, USA). The solvent system consisted of methanol–water (3:1, v/v) containing 10 m*M* ammonium acetate (solvent A) and methanol–dichloromethane (4:1, v/v) (solvent B). A linear gradient from solvent A (100%) to solvent B (100%) was applied over a period of 15–20 min, followed by isocratic elution with solvent B (100%) for an additional 15–20 min. The flow-rate was 0.8 ml/min. At the end of the run, the gradient was reversed to initial conditions by applying a linear gradient of 5 min. The column was then allowed to equilibrate for 10 min with solvent A before the next injection. Time scale is in minutes. (Reproduced with permission from Ref. [101]).

5.2. Normal phase

Nöll and Kalinowski studied the use of straight-phase chromatography in several papers dealing with the separation of *cis* and *trans* isomers of retinol, retinal and retinyl esters. Directions are given for the production of retinol and retinal isomers by photo-isomerisation. Elution orders of the different isomers with different mobile phases was investigated and the identity of isomers were identified by UV spectroscopy and NMR. The results were compared with previously published data and the authors found earlier data to be inconsistent and even contradictory [28,30]. Isocratic separation of retinol isomers were found to be best achieved on a Zorbax SIL Si-60 (250×4.6 mm I.D., DuPont) eluted with *n*-heptane–MTBE (98:2) while a LiChrosper Si-60 (250×4.0 mm I.D., Merck) eluted with *n*-heptane–MTBE (97:3) was found to be superior for separation of retinal isomers (Fig. 10). Partial separation of retinyl palmitate isomers with *n*-heptane–MTBE (99:1) was also achieved on the DuPont column. Brinkmann et al. applied an analytical column of 100×2 mm I.D. with a 3 μm stationary phase and a mobile phase consisting of 1-octanol in *n*-hexane as modifier, for the separation of seven geometrical isomers of retinol [102]. Lefebvre et al. separated all-*trans* and 13-*cis* retinoic acids and their 4-oxo metabolites by adsorption liquid chromatography on a 250×4 mm I.D. LiChrosper Si-60 (5 μm) eluted with hexane–dichloromethane–dioxane (78:18:4) containing 1% acetic acid [78]. A reversed-phase column eluted with a normal-phase mobile phase (16% diethyl ether in hexane) was used for separation of all-*trans*, 13-*cis* and 9-*cis* RAL isomers, all-*trans*-ROH, and 5,8-peroxyretinal and 5,6-epoxyretinal [103].

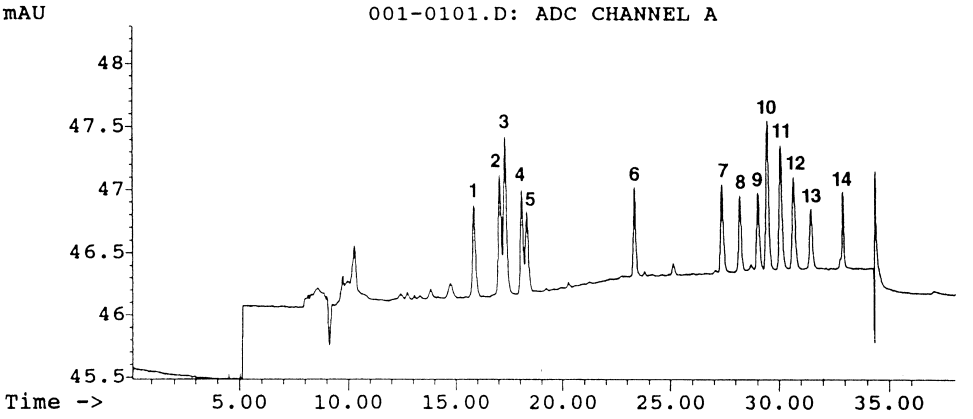

Fig. 8. Chromatogram of reference compounds. Peaks: 1=all-*trans*-4-hydroxy-RA, 2=all-*trans*-4-oxo-RA, 13-*cis*-4-hydroxy-RA, 4=13-*cis*-4-oxo-RA, 5=9-*cis*-4-oxo-RA, 6=all-*trans*-5,6-epoxy-RA, 7=all-*trans*-3,4-didehydro-RA, 8=all-*trans*-3,4-didehydroretinol, 9=13-*cis*-RA, 10=9,13-di-*cis*-RA, 11=9-*cis*-RA, 12=all-*trans*-RA, 13=all-*trans*-retinol, 14=all-*trans*-retinal. Retinoid standards were injected onto a precolumn packed with LiChrospher 100 RP-18 (5 μm). 1.25% ammonium acetate and acetic acid–ethanol (8:2, v/v) was used as mobile phase during injection and 1% ammonium acetate and 2% acetic acid–ethanol (102:4, v/v) was added, on-line, to decrease the elution strength of the injection solution. After backflush purging of the precolumn, the retained components were transferred to the analytical column in the backflush mode, separated by gradient elution and detected at 360 nm. Two coupled Superspher 100 RP-18 endcapped columns (both 250×4 mm I.D.) were used for the separation, together with a mobile phase consisting of acetonitrile–water–10% ammonium acetate–acetic acid: (A) 600:300:60:10 (v/v), (B) 950:20:5:20 (v/v), and (C) 990:5:0:5 (v/v). Time scale is in minutes. (Reproduced with permission from Ref. [65]).

6. Detection

6.1. Ultraviolet detection

Retinoids have multiple carbon–carbon bonds in conjugation and therefore absorb ultraviolet light in the range 300–400 nm. Molar extinction values are 30 000–60 000, permitting the detection of as little as 50 pg or less, with quantification limits around 300 pg/ml for retinoic acid [83]. The general trend in chromatography is miniaturisation, but normal bore column are still the standard column diameter used for retinoid analysis. Use of narrow columns has the potential of giving lower detection limits [104], especially in combination with column switching [81]. If the inner diameter of the column is reduced to less than 2 mm, a standard flow cell (8–12 μl) will often cause band broadening. A flow cell with a smaller volume (2–4 μl) will usually provide the expected increase in peak height. Use of ‘‘ Z-shaped’’ or ‘‘U-shaped’’ detection cells in capillary UV detectors, can when combined with on-column focusing of large injection volumes or in combination with column switching techniques, give a substantial increase in sensitivity [72,73]. The use of diode array detection has become more common as the sensitivity of this method now approaches that of the single wave detectors. However, tuned to this sensitivity the DAD looses all of its spectral capabilities but still is an excellent tool for both method development and use with retinoids present at higher concentrations. The UV properties of selected retinoids are given in Table 1. The majority of papers referred to in this review use UV detection either as the main detection technique or as a secondary detector.

6.2. Fluorescence detection

Retinol and its esters display a pale green fluorescence when excited with near-ultraviolet light [105]. The wavelength of excitation maxima is in the range 325–335 nm and emission maxima in the range 470–490 nm is only to some degree dependent on the solvent while the quantum yield is highly affected [17]. Generally, nonpolar hydrocarbon solvents hexane, xylene and dioxane provide strongest fluorescence while polar solvents such as acetonitrile and

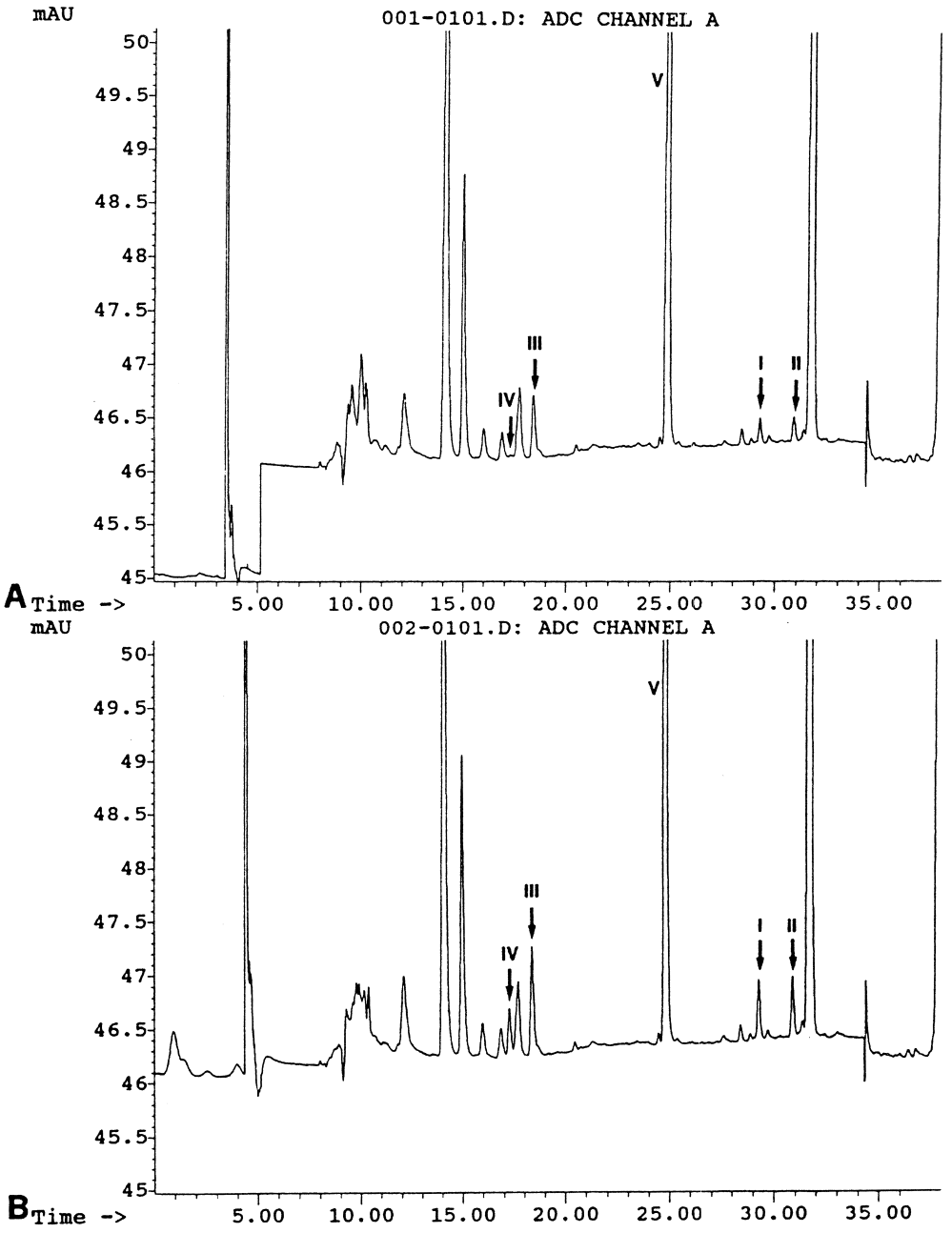

Fig. 9. Chromatograms of human plasma samples. (A) Volunteer plasma sample with endogenous levels of 1.36 ng/ml of 13-*cis*-RA (I), 1.20 ng/ml of all-*trans*-RA (II), 2.58 ng/ml of 13-*cis*-4-oxo-RA (III) and <0.3 ng/ml of all-*trans*-RA (IV). (B) Same plasma as in (A) but spiked with an additional 2 ng/ml of each of the four compounds. Peak V is the internal standard (Acitretin). Plasma (0.4 ml) was deproteinated by adding ethanol (1.5 ml) containing the internal standard acitretin. After centrifugation, 1.4 ml of the supernatant were directly injected onto the precolumn. Chromatographic conditions as in Fig. 8. Time scale is in minutes. (Reproduced with permission from Ref. [65]).

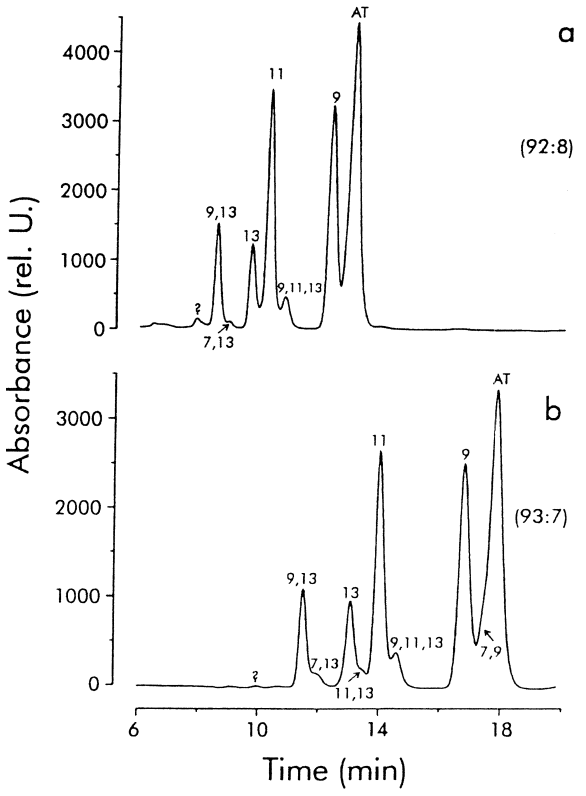

Fig. 10. Chromatogram of retinol isomers with the mobile phase *n*-heptane–MTBE in (a) (92:8, v/v), flow-rate 3 ml/min, 139 bar, 325 nm and in (b) (93:7, v/v), 3 ml/min, 136 bar. Elution order (retention time in min for (a); (b)): 9,13=9,13-di-*cis*-retinol (8.55; 11.50), 7,13=7,13-di-*cis*-retinol (8.96; 11.90), 13=13-*cis*-retinol (9.70; 13.06), 11,13-di-*cis*-retinol (-; 13.41), 11=11-*cis*-retinol (10.26; 14.00), 9,11,13=9,11,13-tri-*cis*-retinol (10.78; 14.60), 9=9-*cis*-retinol (12.31; 16.80), 7,9-di-*cis*-retinol (12.75; 17.33, hidden by AT) and AT=all-*trans*-retinol (13.07; 17.82). Column: DuPont Zorbax SIL (250×4.6 mm I.D.) packed with SI60, 5–6 μm particles. (Reproduced with permission from Ref. [30]).

methanol tend to quench the fluorescence [17]. When retinol is bound to RBP the uncorrected excitation wavelength is 334 nm and emission is 463 nm. The intensity of fluorescence is increased five to 15 times. Upon complexation with transthyretin (TTR) the intensity is increased with another 15% [106].

This increased sensitivity has been used in combination with laser-excited fluorescence for very sensitive detection of retinol in dried blood spots by HPLC [107] and capillary electrophoresis (CE) [108,109]. Others have used conventional fluorescence detectors in combination with HPLC for determination of retinol [61]. Interestingly Gatti and co-workers describe the use of fluorescence for the determination of retinoic acid. The authors reported that upon excitation at 350 nm, retinoic acids will emit at 520 nm, with detection limits in the range 1.5–3.1 ng for the different isomers of retinoic acid [99,100].

6.3. Electrochemical detection

HPLC in combination with electrochemical detection (ED) is widely accepted as a sensitive and selective technique for the determination of electroactive substances. ED is based on an electrochemical reaction of the analyte in the mobile phase and is therefore more sensitive to the composition of the mobile phase than spectroscopic detection techniques. The polyene chain of retinoids is regarded as an electroactive group and will be oxidised at a sufficiently high positive potential.

A few methods have been described where HPLC–ED is used for determination of retinoids. Hagen et al. described a method for separating and detecting retinoids by reversed-phase capillary liquid chromatography with amperometric ED [67]. The detection cell consisted of a carbon fiber barrel electrode held at +0.9 V versus an Ag/AgCl reference. Injection volumes of two μl produced detection limits of 2.73, 0.472, 0.428, and 0.267 fmol (or 410, 64.1, 60.9, and 38.2 pg/ml) for 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinaldehyde, and retinol, respectively. Sakhi et al. combined the advantage of on-line SPE with coulometric ED for detection of retinoic acids and retinol [80]. The retinoids were detected electrochemically at +750 mV using a coulometric electrochemical detector with a palladium reference electrode (ESA Inc.). The mass limits of detection were about 10 and 25 pg for the retinoic acids and all-*trans*-retinol, respectively. Others have used amperometric electrochemical detection [110] or coulometric ED (ESA Inc.) for determination of retinol in milk [104].

6.4. Mass spectrometric detection

HPLC–mass spectrometry (MS) has gained much popularity the last years and is fast becoming the preferred tool for liquid chromatographers. Liquid

chromatography (LC)–MS is a powerful analytical technique that combines the resolving power of HPLC with the detection specificity of MS. Charged ions are generated and separated by their mass to charge ratio. Only a handful of papers describe the use of LC–MS for the determination of retinoids in biological samples [111–114]. More recently, modern atmospheric pressure ionisation techniques like electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) have been explored for retinoid analysis. ESI and APCI are based on the formation of ions in solution. When the mass spectrometer is set to detect positive ions, an organic acid, usually formic acid, is added to the mobile phase for protonation of the retinoids. All retinoids will generate positive ions under acidic condition. For retinoic acid, the MS can also be operated in negative mode. As the pK_a of RA can be as high as 8.0, highly basic conditions are required for complete ionisation. Silica columns will dissolve at this pH, therefore the solution is often to use an ammonium acetate based buffer. The pH can however be increased by adding a strong base like triethylamine post-column through a T-piece. Special columns that can tolerate high pH, such as the X-terra column (Waters), are now also available. Retinol and its esters will be dehydrated in the MS system under acidic conditions and the same fragment with m/z 269 $[M-17]^+$ will be observed. The 4-oxo-retinoids and the 4-hydroxyretinoids can in addition to $[M+1]^+$ or $[M-1]^-$, generate radical ions (M^{*+}). Van Bremen et al. applied HPLC–ESI-MS for the retinoids: RA, ROH, RAL, and retinyl acetate [95]. RA was detected in negative mode (Fig. 11) while the other retinoids were detected as protonated molecules in positive mode (Fig. 4). Limits of detection for RA, RAL, ROH, and retinyl acetate were 23 pg, 1.0 ng, 0.5 ng, and 10 ng, respectively [95]. Later, a method for the quantitative analysis of all-*trans*-retinol and all-*trans*-retinyl palmitate was developed using LC–APCI–MS [59]. The limit of detection of LC–APCI–MS for at-ROH and at-palmitate was determined to be approximately 34 and 36 fmol/μl, respectively. Tzimas et al. used a HPLC–APCI-MS method for identification of the retro-retinoid 14-hydroxy-4,14-retro-retinol as a major metabolite found in rodent tissue and human plasma after a high vitamin A intake [115]. Due to extensive fragmentation of all retinyl esters leading to a single fragment with m/z 269 in ESI and APCI,

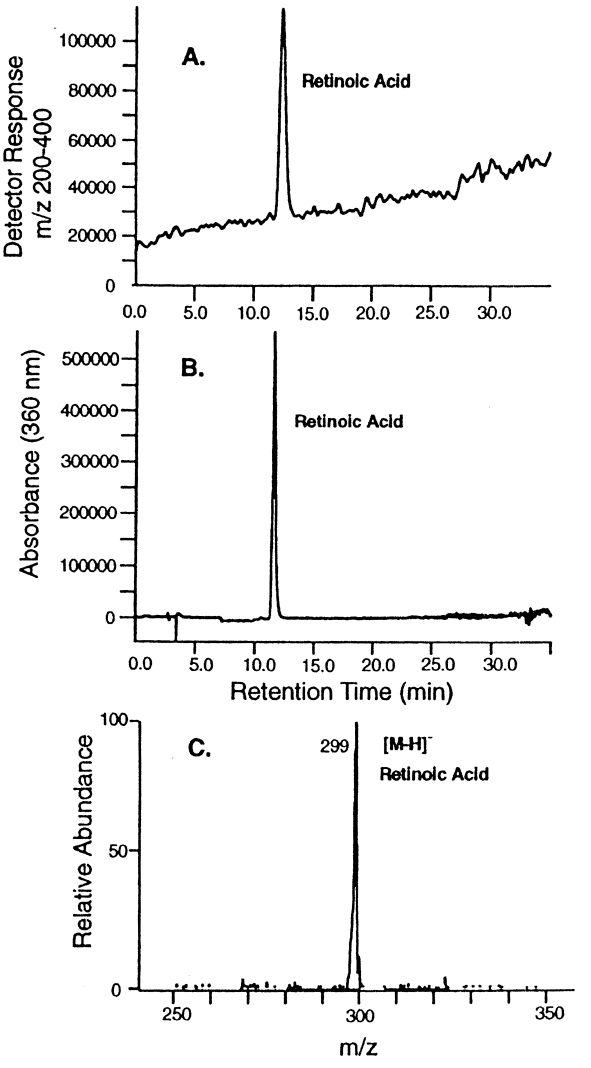

Fig. 11. Negative ion electrospray LC–MS of 340 pmol all-*trans*-retinoic acid. (A) Total ion chromatogram showing elution of retinoic acid at 13 min. (B) Absorbance chromatogram of all-*trans*-retinoic acid obtained on-line during LC–MS. (C) Negative ion electrospray mass spectrum of retinoic acid obtained at a retention time of 13 min. Chromatographic conditions as in Fig. 4. (Reproduced with permission from Ref. [95]).

no information on the molecular ion is obtained. By use of laser desorption ionisation (LDI) time-of-flight (TOF) MS or matrix-assisted laser desorption ionisation (MALDI) TOF-MS, retinyl esters formed radical molecular ions (M^{*+}) in addition to the fragment at m/z 269. LDI-MS and MALDI-MS were used to study ROH, RA, and RAL and their analogs 3-hydroxy-ROH, 3-hydroxy-RA, 3-hydroxy-RAL, 4-

oxo-ROH, 4-oxo-RA, 4-oxo-RAL, 3,4-didehydro ROH, 3,4-didehydro-RA, 3,4-didehydro RAL, acyclo-ROH, acyclo-RA, and acyclo-RA. Under the conditions of LDI-MS the compounds formed abundant radical molecular ions (M^{*+}), whereas in the MALDI mass spectra abundant protonated molecular ions $[M+H]^+$ were observed [20]. LDI-MS was found to be more appropriate than MALDI-MS for the described analytical task [23]. A particle beam (PB) interface was used by Andreoli et al. for determination of retinol in milk [104]. A narrow bore C_8 column was used and detection limits for retinol, retinyl palmitate and retinyl acetate were 2.5, 1.0 and 12.0 ng, respectively. An HPLC–PB-MS system was used for determination of at-RA and 13-*cis*-RA in human plasma after derivatisation to their pentafluorobenzyl esters. Selected ion monitoring (SIM) at m/z 299 carboxylate anion produced by negative chemical ionisation using methane reagent gas achieved minimum detection limits of 25 pg injected [77].

7. Identification

7.1. General considerations

Identification of the specific retinoid analysed by chromatographic techniques, is not an easy task. New retinoids appear frequently and the number of commercially available retinoids is very limited. This forces the chemist to obtain these as gift from other researchers or institutions or isolate them from natural sources or to synthesise them. It is of utmost importance that an unequivocal identification of the retinoid is performed before the retinoids is passed on to other scientists. This is not always performed and can lead to wrong assignment of the identity of retinoids found in biological samples. Ideally, a certificate stating the identity and purity should follow the retinoid as for commercial standards. A thorough spectral characterisation should include high-resolution MS, NMR and UV spectroscopy. If a pure authentic standard is available, matching of an unknown substances retention time in the chromatographic system with the standard is an indication of its identity. Further identification can be done by collection of the fraction containing the substance and re-inject it on a second chromatographic system, again comparing the retention with the standard. This is of course not possible with destructive detection methods like ED and MS. On-line collection of several types of spectral data is possible. Alteration of the molecule by chemical means, electrochemical or photo reactor is also possible before re-chromatography. Methylation of RA will in most cased lead to higher retention in RP systems. Methylation can be achieved by reaction with diazomethane [116]. Kits for the in situ generation of diazomethane can be bought (Sigma–Aldrich). Nöll and Kalinowski recently revealed extensive inconsistency and errors in the elution order of retinal and retinol isomers in straight-phase chromatographic systems [28]. Upon analysis of eggs from *Xenopus* with a single-wavelength UV detector, all-*trans*- and 9-*cis*-RA were claimed to be present [117,118]. By use of DAD, others later found that the substances in question had UV spectra distinct from RA [119].

7.2. Ultraviolet spectroscopy

As stated in Section 2.2, retinoids have multiple carbon–carbon double bonds in conjugation and show a characteristic adsorption in the UV region. The use of DAD allows on-line collection of the UV spectra of substances separated by chromatography. The UV spectra of unknowns can then be compared with the UV spectra of the standard. Identical UV spectra together with matching retention are a strong indication as to the identity of the retinoid. It is important that the spectra are taken up under identical conditions. DAD will also reveal contamination of other UV absorbing species in the chromatographic peak. Failure to separate other UV absorbing co-eluting substances will of course lead to overestimation of the concentration. This is detectable only if DAD is used in the spectral mode or at least with comparison of two detection wavelengths (ratio chromatogram). Often DAD is used only during method development while single-wavelength detectors are used in routine. Co-eluting substances will not be detected in this way, and erroneous results may be obtained. As retinoids have a λ_{max} varying with up to 100 nm, detection with a single wavelength can result in failure to detect some retinoids. For instance, detectors set to 325 nm for the quantification of retinol will most likely fail to detect small amounts of retinal (λ_{max}=380 nm). The response of

the retinoid and the detection wavelength vs. the λ_{max} of the retinoid should always be considered when comparing chromatograms visually.

7.3. Mass spectrometry

Mass spectrometry is an excellent tool for identification of retinoids. The only limitation of MS is its inability to distinguish between *cis* and *trans* isomers since their mass as well as their fragmentation pattern is identical. The molecular formula of a totally unknown substance can be found by high-resolution MS, provided that the substance is pure. Retinoids purified by HPLC can be introduced into an MS system directly, or by gas chromatography (GC) interfaced to MS. The use of GC requires derivatisation of hydroxyl and carboxylic acid moieties. Alternatively, the mass spectrometer can be connected on-line with the HPLC. Most MS instruments used in combination with HPLC are not high-resolution instruments. Data obtained with low-resolution MS provides the molecular mass and only minor structural information. High-resolution MS is, however, also available for HPLC. As a detector for HPLC, MS offers high selectivity and sensitivity as well as important qualitative data.

7.4. Nuclear magnetic resonance spectrometry

NMR has played a major role in identifying and elucidating the structures of retinoids. NMR is the only spectroscopic technique capable of distinguishing between different *cis* and *trans* isomers of retinoids. Unequivocal identification of the geometrical form of a retinoid isolated from biomaterial can only be done with NMR [28,30]. Because the amount of substance needed for NMR is rather high, and the endogenous amounts of retinoids is very low, this is rarely possible. NMR can also be performed on-line with HPLC. HPLC–NMR is starting to become a routine technique in some laboratories, and has been used for structure characterisation of retinoids [96,120–122].

For detailed NMR data on retinoids the reader is referred to a comprehensive book on the topic [18] and a recent review on LC–NMR in general [123].

8. Conclusions

Despite continuous development and improvement of analytical equipment and instrumentation, the chromatographic determination of retinoids in biosamples still is among the most challenging tasks in analytical chemistry. This is mainly due to the low concentrations combined with the complexity of the biosamples as well as instability towards several factors such as daylight, acids, high temperatures, trace metals and oxygen. Furthermore, recent works have demonstrated that a number of retinoids are present in biological samples. A generic method for their extraction and separation seems impossible to develop. Depending on the actual sample, its matrix composition, the concentration level of the retinoids present, the chemist will have to modify existing methods to obtain the selectivity, sensitivity and reproducibility required for his or her special application. This is probably the reason for the multitude of methods in use for quantitative and qualitative determination of retinoids in biological samples. Nevertheless, some general guidelines can be drawn and some trends seem to be evident from reading the recent literature. Simple protein precipitation or mono-phase extraction directly on a biofluid, after homogenisation of tissue or with acetonitrile, 2-propanol, ethanol or acetonitrile–1-butanol (1:1) are used more frequently for extraction of retinoids. A wide range of retinoids including the polar retinoids 3-OH-RA, 4-oxo-RA [83] and the glucuronides of RA [124] and the non polar retinyl esters [48] have been showed to be extracted efficiently. Increased concentration of the sample was obtained by addition of salt [69], on-column focusing [45,72,73], SPE [46,75,76] or on-line SPE with column switching [50,65,80,81,83]. Separation of non polar retinoids has been obtained on C_{18} [23,48,93] or C_{30} columns with non aqueous mobile phases [59,72] based on acetonitrile, methanol or ethanol modified with strong eluting solvents like chloroform, dichloromethane, 2-propanol, 1-butanol or MTBE. Separation of polar retinoids including geometrical isomers of retinoic acid was achieved on C_{18} columns either by gradient [65,83] or isocratic elution [50,73,80,81], with combinations of acetonitrile–water–ammonium acetate–acetic acid. Geometrical isomers of retinol and retinal were readily separated by normal-phase

chromatography [29,30,102], but RP can also be applied [98]. UV detection is the preferred detection technique for retinoids [65] although fluorescence has been used for retinol [107] and retinyl esters [61] while electrochemical detection has been shown to be a very sensitive technique in some instances [67,80]. As the price of bench top mass spectrometers is constantly reduced, it will probably find wide spread use in retinoid field. MS detection has been used often in combination with diode array for detection and identification of retinoids [20,23,59,77,90,95,115].

Liquid chromatography–tandem mass spectrometry (LC–MS–MS) is potentially very selective and sensitive and was recently applied for the determination of the retinoids Ro-6791 and Ro-6792 in plasma. On-line solid-phase extraction and use of 300 μm columns in combination with MS–MS gave a quantification limit of 1 pg/ml [125]. Despite its high price the potential of LC–MS–MS is so promising that an increasing number of methods will most likely be presented in the near future.

9. Nomenclature

ADS	alkyl-diol silica
AM-580	4-[(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]-benzoic acid
APCI	atmospheric pressure chemical ionisation
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
cLOD	concentration limit of detection
CE	capillary electrophoresis
CPE	cloud point extraction
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DAD	diode array detection
ED	electrochemical detection
ESI	electrospray ionisation
FLD	fluorescence detection
HPLC	high-performance liquid chromatography
ISRP	internal surface reversed-phase
LDI	laser desorption ionisation
MALDI	matrix-assisted laser desorption ionisation
mLOD	mass limit of detection
MS	mass spectrometry
MTBE	methyl *tert*.-butyl ether
NARP	non-aqueous reversed-phase
NMR	nuclear magnetic resonance
ODS	octadecylsilane
PB	particle beam
PBS	phosphate-buffered saline
RA	retinoic acid
RAL	retinal
RAM	restricted access media
RBP	retinol binding protein
RE	retinyl ester
ROH	retinol
RP	reversed-phase
SAX	strong anion-exchange
SDS	sodium dodecyl sulfate
SHP	shielded hydrophobic phase
SIM	selected ion monitoring
SPE	solid-phase extraction
SPS	semi-permeable surface
TCA	trichloroacetic acid
TMMP	4-methoxy-2,3,6-trimethylphenyl
TOF	time-of-flight
TTNN	6-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)naphthalene-2-carboxylic acid
TTNPB	4-[E-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid
TTR	transthyretin
UV	ultraviolet

Acknowledgements

We are grateful to George Alexander and Anders Dahlgren for critical reading of the manuscript. This work was supported by the Norwegian Cancer Society (DNK) and The Research Council of Norway (NFR).

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