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Development of ultrasensitive chromatographic tools for high-throughput analysis of retinoids in biological samples

By

Thomas E. Gundersen

Department of Nutrition
Institute of Basic Medical Sciences
Faculty of Medicine
University of Oslo
Norway

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PREFACE AND ACKNOWLEDGEMENTS

This work was conducted at the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo supervised by Prof. Rune Blomhoff and initially partly at the Department of Chemistry, University of Oslo, supervised by Prof. Elsa Lundanes. The Project has been financially supported by the Norwegian Cancer Society and the University of Oslo, Institute of Basic Medical Sciences. This support is gratefully acknowledged.

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Oslo, December 2006

Thomas E. Gundersen

ABBREVIATIONS

Acitretin	9-(4-methoxy-2,3, 6-trimethyl-phenyl)- 3,7-dimethyl-nona-2,4,6,8-tetraenoic acid
ADH	Alcohol dehydrogenase
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric pressure chemical ionisation
ARAT	Acyl:CoA retinol acyltransferase
CAT	Chloramphenicol acetyltransferase
CE	Capillary electrophoresis
CEC	Capillary electro chromatography
CM	Chylomicron
CMR	Chylomicron remnants
CRABP	Cellular retinoic acid binding protein
CRAD	*Cis*-retinol-androgen-dehydrogenase
CRBP	Cellular retinol-binding protein
DAD	Diode array detection
DHA	Docosahexaenoic acid
dpc	Days post coitum
ED	Electrochemical detection
ESI	Electrospray ionisation
EtOH	Ethanol
FCS	Foetal calf serum
FLD	Fluorescence detection
GC	Gas chromatography
GFP	Green fluorescent protein
GSH	Glutathione
HPLC	High performance liquid chromatography
IMH	Isomerhydrolase
IPM	Interphotoreseptor matrix
IRBP	Interphotoreceptor retinoid-binding protein

IS	Internal standard
IUB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
JCBN	Joint Commission on Biochemical Nomenclature
LLE	Liquid-liquid extraction
LOD	Limit of detection
LRAT	Lecithin:retinol acyltransferase
MeOH	Methanol
mLOD	Mass limits of detection
MP	Monophase
MPE	Monophase extraction
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NEM	N-ethylmaleimide
NMR	Nuclear magnetic resonance
OPG	Osteoprotegenin
PCR	Polymerase chain reaction
PEEK	Poly ether ether ketone
PPAR	Peroxisome proliferator activated receptor
PrOH	Propanol
RA	Retinoic acid
RAL	Retinal
RALDH	Retinal dehydrogenase
RALR	Retinal reductase
RANKL	Receptor activator of NF-κB ligand
RAR	Retinoic acid receptor
RARE	Retinoic acid responsive element
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
RE	Retinyl ester
REH	Retinyl ester hydrolase

ROH	Retinol
RP	Reversed phase
RPE	Retinal pigment epithelium
RT	Reverse transcriptase
RXR	Retinoic X receptor
SDR	Short-chain dehydrogenase/reductase
SDS	Sodium dodecyl sulphate
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
TMMP-ROH	all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol
TR	Thyroid hormone receptor
TTR	Transthyretin
UGT	Uridine (5'-)diphosphate-glucuronosyltransferase
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
VAD	Vitamin A deficiency
VDR	Vitamin D receptor
WAT	White adipose tissue

LIST OF PAPERS INCLUDED

I. **Gundersen TE, Lundanes E, Blomhoff R.** Quantitative high-performance liquid chromatographic determination of retinoids in human serum using on-line solid-phase extraction and column switching. Determination of 9-*cis*-retinoic acid, 13-*cis*-retinoic acid, at-retinoic acid, 4-oxo-at-retinoicacid and 4-oxo-13-*cis*-retinoic acid. J Chromatographic B Biomedical Sciences and Applications. 1997 Mar 28;691(1):43-58.

II. **Gundersen TE, Blomhoff R.** On-line solid-phase extraction and isocratic separation of retinoic acid isomers in microbore column switching system. Methods in Enzymology. 1999;299:430-41.

III. **Sakhi AK, Gundersen TE, Ulven SM, Blomhoff R, Lundanes E.** Quantitative determination of endogenous retinoids in mouse embryos by high-performance liquid chromatography with on-line solid-phase extraction, column switching and electrochemical detection. Journal of Chromatography A. 1998 Dec 18;828(1-2):451-60.

IV. **Gundersen TE, Bastani NE and Blomhoff R.** High-throughput analysis of endogenous retinoids in human serum using triple stage LC-MS/MS. Manuscript 2006.

V. **Gundersen TE, Blomhoff R.** Qualitative and quantitative liquid chromatographic determination of natural retinoids in biological samples. Journal of Chromatography A. 2001 Nov 23;935(1-2):13-43.

VI. **Hoover F*, Gundersen TE*, Ulven SM, Michaille JJ, Blanchet S, Blomhoff R, Glover JC*.** Quantitative assessment of retinoid signalling pathways in the developing eye and retina of the chicken embryo. Journal of Comparative Neurology. 2001 Jul 30;436(3):324-35.

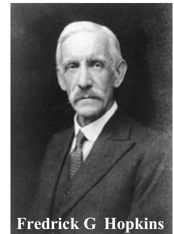
VII. **Ulven SM*, Gundersen TE*, Weedon MS, Landaas VO, Sakhi AK, Fromm SH, Geronimo BA, Moskaug JO, Blomhoff R.** Identification of endogenous retinoids, enzymes, binding proteins, and receptors during early postimplantation development in mouse: important role of retinal dehydrogenase type 2 in synthesis of at-retinoic acid. Developmental Biology. 2000 Apr 15;220(2):379-91.

VIII. **Ulven SM, Gundersen TE, Sakhi AK, Glover JC, Blomhoff R.** Quantitative axial profiles of retinoic acid in the embryonic mouse spinal cord: 9-*cis* retinoic acid only detected after at-retinoic acid levels are super-elevated experimentally. Developmental Dynamics. 2001 Nov; 222 (3):341-53.

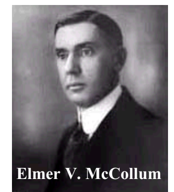
* These authors contributed equally

INTRODUCTION

History of vitamin A


Fredrick G Hopkins

In the year 1906 the British biochemist Frederick Hopkins postulated that foods contained essential "growth factors" in addition to proteins, carbohydrates, fats, minerals, and water [1]. Six years later, he published a paper with the title “Feeding experiments illustrating the importance of accessory factors in normal dietaries” [2]. Hopkins received the Nobel Prize in medicine in 1929. In 1911 the Polish chemist Casimir Funk discovered that the anti-beriberi (motor neuron paralysis, beriberi means “I cannot” in Singhalese) substance in unpolished rice was an amine (later identified as thiamine), thus Funk proposed that it should be named vitamine, for "vital amine" a growth factor present in food which was essential for life [3,4]. Later it became apparent that there was more than one growth factor, and the term vitamine soon came to be applied to the growth factors in general. McCollum and Davis, and Osborne and Mendel simultaneously discovered that some growth factors could be extracted into lipids and divided them into two classes 'fat-soluble A' and 'water-soluble B' [5,6]. Objecting to the chemical implications of the suffix '-ine', Drummond later suggested deletion of the final 'e', renamed McCollum's two groups vitamin A and vitamin B, and proposed that further future members of this series should be called vitamin C, vitamin D, etc [7].


Elmer V. McCollum

In 1934 Wald extracted and isolated from the retina of animals a substance he named retinene [8]. Morton later suggested that this compound was the aldehyde of vitamin A [9], and called it retinaldehyde which later became retinal (RAL) [10].

The correct structure of vitamin A was deduced in 1931 by Karrer who proposed the name axerophthol based on its action in preventing the eye disease xerophthalmia, an excessive dryness of the conjunctiva and cornea often leading to blindness [11]. Karrer received the Nobel Prize in chemistry in 1937. Since then, numerous closely related compounds belonging to the family of vitamin A have been described.

Paul Karrer

Nomenclature of retinoids

According to the Joint Commission on Biochemical Nomenclature (JCBN), including the International Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry and Molecular Biology (IUB), the term vitamin A should be used as the generic descriptor for retinoids exhibiting qualitatively the biological activity of retinol (ROH). Retinoids, on the other hand, are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner. All retinoids may be formally derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion [12-14]. The chemical structures of the most common retinoids are given in figure 1, whereas a more comprehensive listing may be found in Paper V, figure 1, page 16.

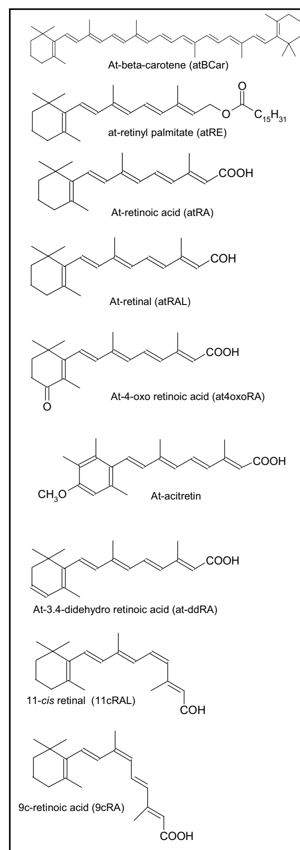


Figure 1. Structures of the most common retinoids.

The major forms of vitamin A are ROH, RAL, retinyl esters (REs) and retinoic acid (RA). RA contains an ionisable carboxylic acid moiety and is soluble in alkaline aqueous solution. RE, ROH and RAL are lipophilic compounds with limited solubility in aqueous solutions. Most of the retinoids have 4-6 double bonds in conjugation, accordingly they absorb light very well (ε= 40-60 000) in the 300-400 nm region. Ultraviolet (UV) detection is consequently often the detection principle of choice in retinoid analysis. Each double bond can exist in either *cis* or *trans* configuration. If all double bonds are in *trans* configuration, the term all-*trans* is used (e.g. all-*trans* retinol (atROH)). If the double bond between carbon 9 and 10 is in *cis* configuration the name 9-*cis* ROH (9cROH) is used. A more detailed description of the physiochemical properties can be found in Paper V, page 15-18.

Intestinal uptake of retinoids

Vitamin A is a true essential nutrient that has to be supplied through the diet for most animals. The natural source of vitamin A is plant carotenoids with pro-vitamin A activity, meaning that the molecule has at least one intact β-ionone ring in one end of the molecule, producing at least one molecule of vitamin A upon cleavage in the enterocytes of the intestine. Of the more than 600 carotenoids present in nature, between 50 and 60 display provitamin A activity, and β-carotene is the most important and abundant of these. Although an enzyme responsible for cleavage of β-carotene was characterised 40 years ago [15,16], until recently it has been controversial whether the cleavage follows a central or eccentric pathway. It is now established that both pathways in this key step of vitamin A production actually occur. The central cleavage of absorbed β-carotene [17], producing two molecules of RAL, is catalyzed by 15,15'-mono-oxygenase (βCMOOX) [18] also called 15,15'-di-oxygenase (EC 1.13.11.21), while the eccentric pathway producing β-ionene, β-apo-carotenals and subsequently RAL by β-oxidation, is catalyzed by β,β-carotene-9',10'-dioxygenase [19]. βCMOOX expression was recently demonstrated to be regulated by RA in enterocytes, indicating a possible feedback regulation of ROH production from β-carotene [20]. This enzyme also cleaves lycopene into its corresponding apolycopenals. RAL produced in intestinal enterocytes by either pathway will then be oxidized to some extent to RA by retinal dehydrogenase (RALDH) [21], but the majority will be reduced to ROH by retinal reductase (RALR), esterified to RE by lecithin:retinol acyltranferase (LRAT) or acyl:CoA retinol acyltransferase (ARAT), and incorporated into chylomicrons (CMs) [22].

Alternatively, if vitamin A in the form of preformed RE is ingested (from an animal in which REs have been formed from carotenoids as described above), RE is converted to ROH by retinyl ester hydrolase (REH) [23] in the intestinal lumen, taken up by enterocytes, bound to cellular retinol-binding protein (CRBP) II, reesterified by LRAT or ARAT and incorporated into CMs. The CMs are then exocytosed into the lymphatic system carrying the absorbed provitamin A in the form of 60-70 % RE and 20-30 % unchanged β-carotene [24].

Figure 2. Major pathways of retinoid uptake and transport in the body.

After entering the general circulation, CMs are converted to chylomicron remnants (CMR) with most of the RE intact. CMR-RE are mainly cleared by the liver (~75 %) but uptake in extrahepatic tissue, through lipoprotein receptors, occurs to some extent [25]. Direct delivery of dietary lipoprotein-RE, and not through retinol binding protein (RBP) bound ROH from the liver, has recently been shown to be the preferred route for delivery of vitamin A to the developing embryo [26], as well as for secretion into milk [27,28]. The mechanisms involved in the absorption of dietary vitamin A have recently been reviewed by Harrison [29].

Liver storage and plasma transport

In the liver, parenchymal (hepatocytes) and stellate cells play a central role in vitamin A uptake and storage. CMR-RE entering the space between the endothelial cells and the hepatocytes, which is called the "Space of Disse", undergo lipolytic processing and a receptor-mediated endocytosis into the hepatocytes where RE is hydrolyzed by REH to ROH. Within a few hours, ROH is transferred from hepatic parenchymal cells to the stellate cells. It has been assumed that

RBP was important for facilitating the transport of ROH from parenchymal cells to the stellate cells, but disruption of the gene coding for RBP revealed that RE stores were no different from that of the wild type [30,31]. CRBP I null type mice [32] on the other hand do not synthesise or accumulate RE in the liver, suggesting that CRBP I, and not RBP, is the key mediator of ROH transport within the liver. In stellate cells, the ROH is esterified with long-chain fatty acids, predominantly palmitic, stearic and oleic, to form hepatic RE stored in the form of lipid droplets [33]. In mammals, the liver stores 50-80 % of the total body vitamin A, with the stellate cells holding 90-95 % of this. Although hepatic stellate cells are particularly important for vitamin A storage in many animals, interstitial cells and some other cells of organs such as the lungs, kidneys and intestines of higher vertebrates may accumulate REs in lipid droplets after ingestion of large amounts of vitamin A [34,35]. Such extrahepatic storage of REs may even be an important local supply of vitamin A for organs with a huge demand, e.g. RE storage in retinal pigment epithelial cells as a prerequisite for normal visual function.

The mechanisms for mobilization of vitamin A from liver stores are not well characterised. ROH complexed with RBP is secreted into the bloodstream. Whether the RBP originates from the stellate cells or from the parenchymal cells still remains unclear [31,36]. As ROH has limited solubility in aqueous blood, the ROH is transported in the blood stream bound to RBP [37]. Approximately 95 % of the ROH-RBP complex is again bound to transthyretin (TTR) in a 1:1 molar ratio, which increases the size of the whole complex considerably, and thereby reduces the excretion of ROH into the urine [38-40] and maintains a steady plasma concentration of 1-3 μM. In the target tissues, ROH is taken up on the cell surface, probably by a receptor mediated mechanism [41], although this has not been clarified as yet. ROH is then oxidized enzymatically to RAL by members of the short-chain dehydrogenase/reductase (SDR) family of microsomal enzymes including retinol dehydrogenase (RDH)1, RDH5, RDH11, *cis*-retinol-androgen dehydrogenase (CRAD)1, CRAD2, CRAD3 and retSDR1 [42]. The SDRs utilize atROH bound to CRBP-I as substrates. CRBP-I acts as a chaperone for retinol and retinal, and directs them to these metabolizing enzymes [43]. RAL is then oxidized further to RA by several types of RALDH [44,45].

RA also circulates in the bloodstream bound to albumin (4-14 nM) [46], which is ~0.5 % of circulating ROH levels [47]. It is, however, generally assumed, based on the relatively high levels of ROH in tissues and plasma, and the abundance in tissues of enzymes able to oxidize ROH to

RA, that *in situ* oxidation of ROH to RA is the principal route through which tissue needs for RA are fulfilled. Cytoplasmic RA may then diffuse into the nuclei or, as suggested by some studies, be translocated by CRABP I and CRABP II to the nucleus, where it can bind to retinoic acid receptors (RARs).

Catabolism of retinoids

In addition to the RA producing metabolic system, there is a catabolic system that serves to remove excess RA from cells and the body. The production of RA from RAL, the "on-switch", is a non-reversible reaction so RA cannot be recycled and stored for reuse. Instead, the RA molecule is inactivated by different enzymes, the "off-switch", and the rate of excretion from the body is increased. The modifications known to occur are; oxidation at the 4 position of the ring [48], oxidation at C-18 [49], 5,6-epoxydation [50] and glucoronidation [51] (figures 1 and 4). The cloning of the enzyme P450RAI-1, also named CYP26A1 [52], provided valuable insights into the catabolism of RA. P450RAI produces 4-OH-RA, 4oxoRA, 18-OH-RA and 5,6-epoxy-RA from RA [53]. Some of these retinoids activate the nuclear receptors [54], and it has been debated whether they have an important function or are merely waste products on their way out of the body. Results from experiments using P450RAI-1 knockout mice suggest that the latter suggestion is largely correct. Embryos from these mice have shown teratogenic effects, similar to those seen in the presence of excess RA. It is, however, possible that these effects could be caused by a deficiency in some of the metabolites produced by P450RAI-1, whose roles are presently unknown.

In a publication by Niederreither et al [55], P450RAI-deficient mice were crossed with a Aldh1a2 (RALDH) deficient strain. RALDH-deficient mice have very low RA concentrations and their embryos suffer from severe malformations. If the teratogenic effects observed in the P450RAI-1-deficient mice had been caused by lack of metabolites normally produced by P450RAI-1, absence of both P450RAI-1 and Aldh1a2 in the double transgenic mice should exacerbate the malformations. Alternatively, if this theory was incorrect, the malformations should be reduced in the double transgenic mice, due to reduced RA production compensating for the reduced RA catabolism. The findings of Niederreihter at al were that the teratogenic effects observed with P450RAI-1 knockout were almost fully alleviated by disruption of the Aldh1a2 gene in addition

to P450RAI. In this way, Niederreither et al elegantly demonstrated that 4oxoRA is primarily a waste product from P450RAI-1 type enzymes.

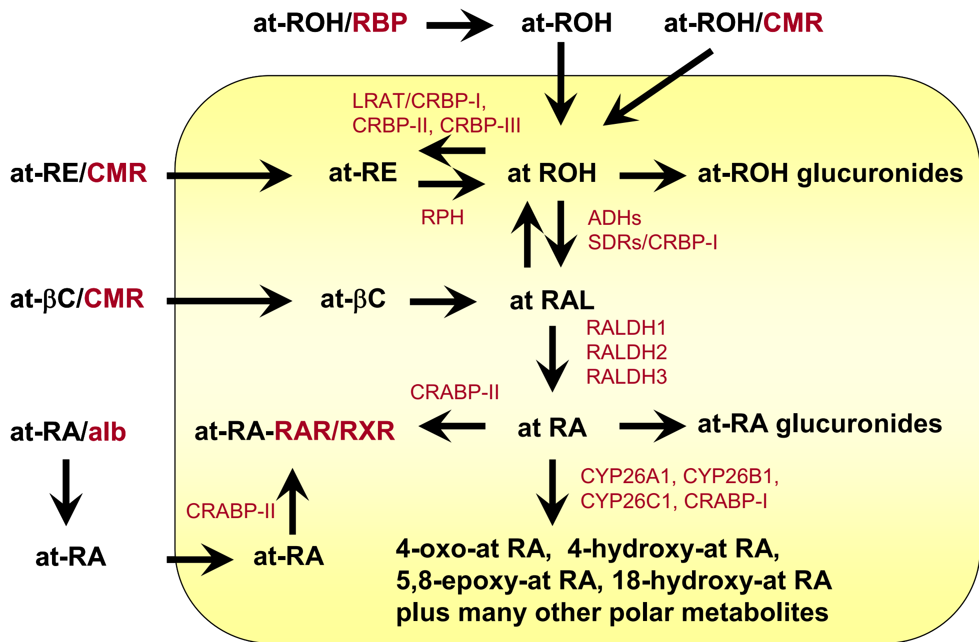


Figure 3. Schematic representation of major pathways for cellular retinoid metabolism and the main enzyme families involved.

Endogenous retinoids in various tissues

To my knowledge there is no publication listing the retinoid content of all, or at least most, of the organs in an animal. Retinoid concentrations in a few organs from mice, rats and humans can be found in the literature, typically from control animals used in experiments or as applications in methodological papers. A thorough screening of the retinoid levels in mice, rats, chickens, and salmon with standardised diets, age and sex would be of value to the field. In table 1, retinoid values taken from various sources in the literature, and supplemented with some data from our own lab, are shown.

Table 1. Mean concentrations and individual variations of retinoids in various tissues from rats, mice and humans.

Retinoid	Species		
	Mouse	Rat	Human
Retinyl esters			
-Serum/plasma (nM)	352±142 [a]	69.1±8.5 [a]	78.4±22.3 [a]
-Liver (µmol/g)	4.19±0.53 [a]	0.22±0.02 [a]	0.29±0.16 [a]
-Kidney (µmol/g)	0.80±0.17 [a]	37.4±13.5 [a]	
-Brain (nmol/g)	0.52±0.30 [a]		
-Testis (µg/g)	0.57±0.2 [c]	0.81±0.13 [a]	
-White adipose tissue (WAT) (µg/g)	0.35±0.1 [c]	352±142 [c]	
-Lung (µmol/g)	0.6±0.27 [c]		
-Eye (nmol/g)	14.0±6.0 [c]		
-Ovary (nmol/g)	24.0±7.0 [c]		
-Epidimis (nmol/g)	0.5±0.20 [c]		
Retinol			
-Serum/plasma (µM)	0.59±0.04 [a]	1.81±0.24 [a]	3.42±0.38 [a]
-Liver (µg/g)	11.0±1.3 [a]	1.62±0.16 [a]	1.05±0.38 [a]
-Kidney (µg/g)	0.41±0.04 [a]	12.5±1.3 [a]	
-Brain (µg/g)	0.65±0.44 [a]		
-Testis (µg/g)	0.14±0.04 [c]	0.81±0.13 [a]	
-WAT (µg/g)	0.267±82 [c]		
-Thymus (µg/g)	0.16±0.042 [c]		
-Lung (µg/g)	8.63±5.1 [c]		0.13±0.27 [d]
-Heart (µg/g)	0.22±0.09 [c]		
-Spleen (µg/g)	0.92±0.28 [c]		
-Muscle (µg/g)	0.041±0.036 [c]		
-Eye (µg/g)	1.23±0.012 [c]		
-Bone (µg/g)	0.049±0.01 [c]		
-Uterus (µg/g)	0.18±0.04 [c]		
-Ovary (µg/g)	1.90±0.45 [c]		

-Epidimis (µg/g)	0.208±0.1 [c]		
13-*cis*-4-oxo-RA			
-Serum/plasma (ng/mL)			2.44±1.77
13-*cis*-retinoic acid			
-Serum/plasma (ng/mL)	0.32±0.14 [a]	0.39±0.17 [a]	1.80±0.97 [a]
-Liver (ng/g)	1.22±0.17 [a]		1.41±0.36 [a]
-Kidney (ng/g)	0.66±0.15 [a]		
-Brain (ng/g)	1.26±0.39 [b]		
-Testis (ng/g)	0.87±0.27 [b]	0.44±0.12 [a]	
-WAT (ng/g)	0.22±0.09 [c]		
at-retinoic acid			
-Serum/plasma (ng/mL)	1.08±0.33 [a]	0.66±0.05 [a]	1.39±0.30 [a]
-Liver (ng/g)	5.55±1.78 [a]	5.08±0.72 [a]	15.8±8.3 [a]
-Kidney (ng/g)	2.37±0.95 [a]	4.06±0.84 [a]	
-Brain (ng/g)	1.60±0.27 [a]		
-Testis (ng/g)	2.8±0.42 [b]	3.85±0.37 [a]	
-WAT (ng/g)	0.70±0.17 [c]		
-Spleen (ng/g)	0.22±0.08 [c]		

a: Schmidt et al [56] , b: kane et al [57], c: Gundersen et al, own data (not published) mean concentration and individual variations from three different animals, , d: Redlich et al [58]

Mechanisms of action of vitamin A

Gene transcription

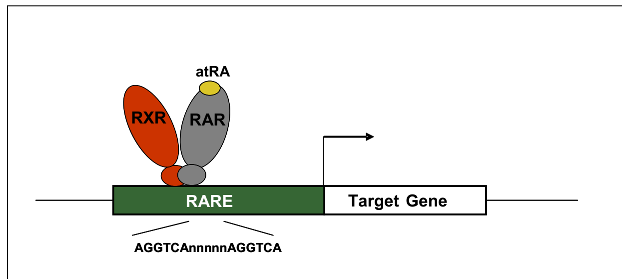


Figure 4. Classical activation of RARE by RA with subsequent alteration of gene transcription

RA functions by binding to nuclear receptor proteins. The nuclear retinoid receptors can be divided into two families, the retinoic acid receptors (RARs) (α,β,γ) and the retinoid X receptors (RXRs) (α,β,γ), both belonging to the steroid hormone superfamily of nuclear receptors. These receptors are ligand-dependent transcription factors that regulate the expression of numerous genes by binding to sequences of DNA called RA responsive elements (RAREs) close to the target genes, thereby altering their transcription [59] (fig 3). RAREs consist of direct repeats of the consensus half-site sequence "(a/g)g(g/t)tca" separated most commonly by five or two nucleotides (DR5 and DR2, respectively), whereas RXRs are typically separated by one nucleotide spacing (DR1) [60].

The receptors mainly mediate their effect via heterodimers (RAR-RXR), but homodimers (RXR-RXR) have also been described. RXRs also form heterodimers with other members of the nuclear receptor family like peroxisome prolifeator activated receptor (PPAR), vitamin D receptor (VDR) and thyroid hormone receptor (TR) [61]. It is assumed that RARs need to be activated by a ligand, while a RXR can also function as a silent partner, stabilizing the heterodimers. Members of the RAR bind, and are experimentally activated to various degrees, by a number of retinoids, including atRA, 9cRA, atddRA, at4oxoRA, at4oxoROH and at4oxoRAL. atRA is regarded as the physiological ligand in mice and humans, while both atRA and at-ddRA are important in chickens. Members of the RXR family have been experimentally activated by 9cRA, 9-*cis*-3,4-didehydro RA (9cddRA) and 9c4oxoRA [62-64]. It has, however, been difficult to demonstrate the existence of 9cRA *in vivo* [65-67].

In addition to the binding of ligands, RARs recruit a multitude of positive and negative regulatory proteins, referred to as co-activators or co-repressors, to carry out various transcriptional functions. These co-factors serve as bridges between the nuclear receptors and the basal transcription machinery [68,69]. The net effect may either be gene repression, the release of gene repression, or gene activation. For a detailed description of this topic, see a comprehensive review by Perissi and Rosenfeld [70]. Important knowledge about the roles of the various RARs and RXRs has been obtained from studies in mice, where one or several of the receptor genes have been deleted from their genomes. Interestingly, many, but not all, symptoms of vitamin A deficiency (VAD) can be recapitulated in such mice [71]. When mutations only affect one receptor, the mice survive and abnormalities are limited, suggesting a functional redundancy between various receptors and isoforms. In double knock-outs, lacking either two RAR subtypes or both RAR and RXRα, the abnormalities are more severe and the animals die [72]. Altogether, more than 500 genes have been suggested to be regulatory targets of RA.

In some cases the regulation of these genes is direct, driven by a liganded RAR-RXR heterodimer bound to a DNA response element. In many cases, however, the gene regulation appears to be indirect, reflecting the actions of intermediate transcription factors, non-classical associations of receptors with other proteins, or even more distant mechanisms. Twenty-seven genes are unquestionably direct targets of the classical RAR-RXR-RARE pathway in permissive cellular contexts and approximately 100 other genes appear to be good candidates [73].

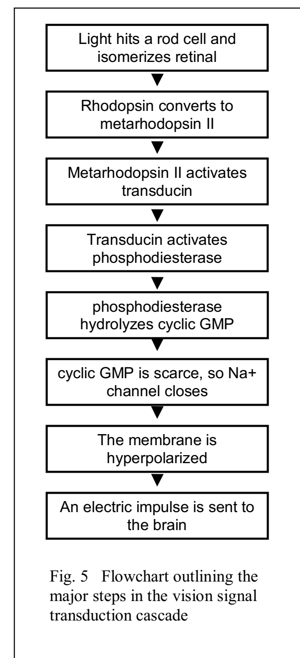


Fig. 5 Flowchart outlining the major steps in the vision signal transduction cascade

Visual chromophore

Vitamin A plays a pivotal role in vertebrate vision [reviewed in [74]] and its function in the visual cycle is executed via a non-genomic mechanism. The retinoids involved are atREs, 11cREs, atRAL, 11cRAL, atROH and 11cROH. The ability of the polyene chain of the retinoids to change confirmation from *cis* to *trans* and visa versa over the different double bonds is crucial

in this respect. The outer rods and cones of the retina, located at the hind wall of the eye, contain an apoprotein called opsin. This apoprotein is connected by a Schiff base linkage to 11cRAL. The complex in rods is called rhodopsin, while other opsins are expressed in cones. The rods function is to generate black and white images in dim light, whilst the cones, constituting only 5 % of the retina photoreceptors, generate colour images in daylight.

The initial step in the generation of a visual image in the brain, is the isomerization of 11cRAL to atRAL, as incoming light reflected from the visual object hits RAL and causes a *cis trans* isomerization. The *trans*-RAL is a longer molecule than 11cRAL and fits differently in the binding pocket of opsin. The pocket is no longer optimal for the RAL molecule and it is expelled from the protein, yielding free opsin plus free atRAL. As the protein changes its conformation, it initiates a cascade of biochemical reactions (figure 5) that result in the closing of Na^+ channels in the cell membrane, creating a polarization across the plasma membrane. An electrical impulse is passed along the optic nerve, to the brain, where the visual information is interpreted [75].

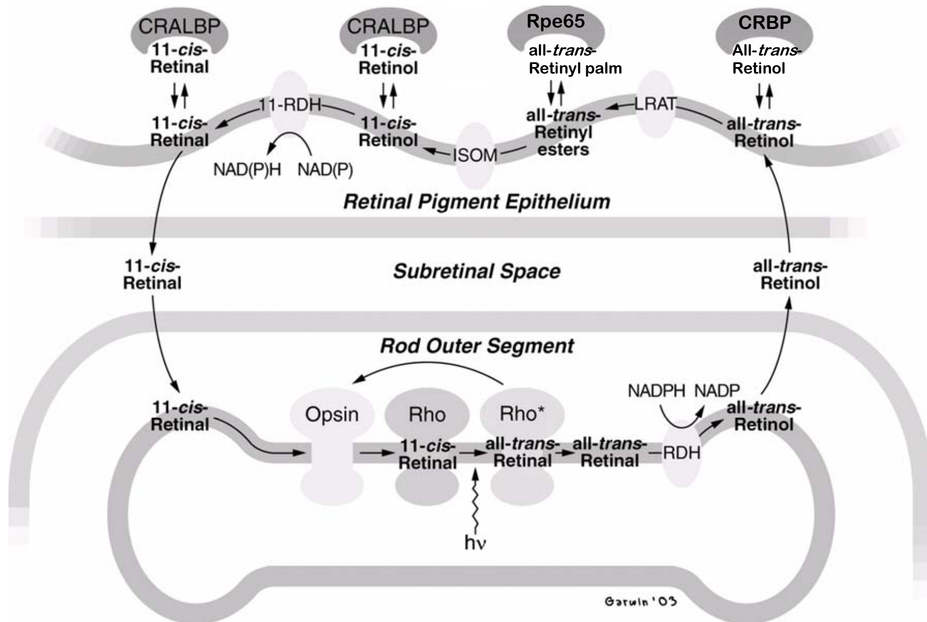


Figure 6. Schematic presentation of our current understanding of the rod visual cycle. Reproduced and updated from [76].

The 11cRAL is then recycled back to atRAL, so that it can be reused in the same process over again. This process, referred to as the visual cycle (figure 6), is a complex process involving the photoreceptors, several forms of vitamin A, isomerases, hydrolases, reductases and vitamin A binding proteins. The regeneration starts with reduction of atRAL to atROH in the outer rod segments. The ROH is then transported to the extracellular space surrounding the photoreceptors (interphotoreseptor matrix (IPM), subretinal space) where it is taken up by interphotoreceptor retinoid-binding protein (IRBP) [77]. The ROH is then translocated to the retinal pigment epithelium (RPE), transverses the membrane and it is escorted to LRAT by CRBP. After esterification to atRE by LRAT, 11*c*ROH is formed by an isomerhydrolase (IMH) [78]. The conversion of atRE to 11cROH was recently found to be dependent on a RE binding protein named Rpe65 [79]. Rpe65 is thought to deliver the highly lipophilic RE to IMH. The Rpe65 knockout accumulates RE in the RPE and 11cROH is absent [80]. Recently, it was demonstrated that RPE65 is in fact the IMH in the retinoid visual cycle [81,82]. 11cROH is then oxidized to 11cRAL by the alcohol dehydrogenases (ADHs) RDH4/5 or 11-*cis* dehydrogense (RDH11). Finally the generated 11cRAL re-enters the rods to re-associate with opsin, forming rhodopsin, and the circle is closed [76,83].

In the retinas of chickens and ground squirrels, cones represent ~60 % of the photoreceptors. The less researched cone visual process has recently been studied in greater detail by Mata and colleagues [84-86] and differs from the rod cycle in two main respects. atROH is not transported to the RPE, but to the Müller cells were the atROH conversion to 11cROH occurs. 11cROH is then transported to the cones, presumably by IRBP, and oxidized to 11cRAL by a novel cone-specific dehydrogenase that regenerates the chromophore up to 2000 times faster than in the rods [85].

Function of vitamin A

Vitamin A plays an important role in a vast number of physiological processes, including reproduction, bone growth, immune function, vision, embryonic development, cell growth and differentiation [22]. RAL is the functional form of vitamin A in the visual process, while the multitude of remaining functions are so far believed to mediated through RA. Good health depends on maintaining vitamin A levels within a normal range, as either too little or too much of this vitamin leads to serious disease. Because the liver stores rather large amounts of vitamin A,

deficiency states typically take several months to develop. Nevertheless, VAD (hypovitaminosis A) is a public health problem in 118 countries, especially in Africa and South-East Asia. Globally, between 100 and 140 million children are assumed to be vitamin A deficient [87] and VAD contributes to the death of over 1 million children every year [88].

Cases of excessive amounts of vitamin A are more rare, but have been known to occur as a result of intake of foods particularly rich in vitamin A, like liver from arctic animals, use of pharmaceutical preparations, or excessive intake of vitamin A supplements [89,90]. Vitamin A toxicity (hypervitaminosis A) in adults manifests itself as one or several of the following symptoms; red blood cell damage, abdominal cramps, blurred vision, irritability, bone pain, loss of appetite, diarrhoea, nosebleeds, growth retardation, hair loss, skin rashes, dry skin, enlargement of the liver, nausea, vomiting, headache and increased cerebrospinal pressure. In a recent metaanalysis [90] of case reports on toxicity claimed to be induced by excessive intake of vitamin A (ROH or RE), 248 cases published in the scientific literature were identified. The authors reported that water-miscible, emulsified, and solid forms of ROH supplements are more toxic than oil-based preparations and vitamin A in foods [91]. Use of preparations containing various forms of RA (Accutane/Roaccutane, Acitretine/Soriatane) during pregnancy often result in miscarriage or severe embryonic malformations [92-96]. More than two million prescriptions of these drugs are given every year and during recent years Roaccutane (13cRA) has been associated with depression, anxiety and suicide ideation as well as impaired night vision [97-103].

Vision

Vitamin A, in the form of RAL, is a necessary structural component of rhodopsin, the light sensitive pigment within the rods of the retina. Related opsin proteins, with covalently bound RAL, are found in the three types of cone cells, which are responsible for colour vision. If inadequate quantities of vitamin A are present, vision is impaired. Moderate deficiency leads to deficits in vision under conditions of low light ("night blindness"), while severe deficiency can result in excessive dryness and opacity of the cornea (xerophthalmia) and subsequently blindness. An estimated 250 000 to 500 000 vitamin A-deficient children become blind every year, half of them dying within 12 months of losing their sight (WHO 2003) [87].

Immunesystem

The immune system is highly dependent on a sufficient supply of vitamin A to function properly. In several infectious diseases studied, VAD has been shown to increase the frequency and severity of disease. Additionally, infections will increase the risk of VAD yet further. Population-based studies have shown that vitamin A supplements decrease the risk of death from infectious diseases [104-106]. Vitamin A is essential to maintain intact epithelial cells in the surface linings of the eyes and the respiratory, urinary, and intestinal tracts. Lack of vitamin A leads to dysfunction - the skin becomes keratinized and scaly, mucus secretion is suppressed and bacteria can more easily penetrate the epithelia and enter the body. VAD decreases T-lymphocyte mediated antibody responses in mice [107], rats [108] and humans [109]. VAD weakens T-helper cell type 2 (Th2) immune responses, while enhancing T-helper cell type 1 (Th1) responses, by decreasing and increasing the number of the two cell types respectively [110].

Bone growth

Normal functioning of the osteoblasts and osteoclasts of bones, is dependent upon vitamin A. Deficiency can result in bone malformations. On the other hand, in recent years too much vitamin A has been linked to bone loss and an increased risk of hip fracture [111-113]. In vitro, vitamin A increases osteoclast proliferation and activity, and stimulates bone resorption [113,114]. Several nuclear RARs are expressed in osteoblasts, but it still is uncertain whether the bone-resorptive effect of atRA is mediated via any of these receptors [115-117]. Recently, expression of two of the regulatory components in the paracrine signalling necessary for osteoclast differentiation, (i.e. osteoprotegenin (OPG) and receptor activator of NF-κB ligand (RANKL)), were shown to be affected by atRA through a RAR-mediated mechanism [118].

Reproduction

Normal levels of vitamin A are required for several aspects of reproduction. Vitamin A is required for spermatogenic epithelial (Sertoli) cells. VAD results in failure of spermatogenesis that can be restored by vitamin A replacement within 10–11 weeks and beyond [119]. Severely vitamin A deficient rats ovulate, but the oocytes have been shown to be degenerated, with failure of blastogenesis [120]. Cornification of the vaginal epithelium also occurs with subsequent

failure of implantation [121]. Vitamin A also seems to play an important role in placenta and yolk sac development and maintenance, although its role in these contexts is still rather unclear.

Preimplantation embryo

The developing embryo crucially requires a well-balanced supply of vitamin A for the proper development of the whole range of its organ systems. Both excess and deficiency of RA during embryonic development, results in congenital malformations. RA is produced when ROH is oxidized by RDH enzymes to RAL and then irreversibly to RA by RALDH enzymes [44,45]. These enzyme types have been demonstrated to play an essential role in retinoid signalling and show discrete domains of expression during embryonic development [122-124].

As the sperm approach an egg, it must first migrate through a layer of follicle cells and then bind to, and cross, the egg coat (zona pellucida). Finally, the sperm must bind to and fuse with the plasma membrane of the oocyte and donate several components, including its chromatin, to the oocyte. This fertilized oocyte (zygote), which is a single cell embryo, subsequently undergoes a series of divisions, progressing through 2-cell, 4-cell, 8-cell and 16 cell stages, and eventually forming a blastocyst. The attachment of the mature blastocyst to the uterus wall ends the period known as the preimplantation period, which lasts 3-4 days post coitum (dpc) in the mouse. Prior to implantation, the mammalian embryo is probably not supplied with many nutrients by the mother. Available knowledge about retinoids and retinoid signalling during the preimplantation period is scarce. However, sperm from several species, including rats and humans, have been demonstrated to contain both RE, atRA and 9cRA [125,126]. To my knowledge neither human nor mouse oocytes have ever been successfully examined for retinoid content. Mohan et el examined bovine oocytes and pre-attached blastocysts, fertilized *in vitro*, for expression of mRNAs for RBP, RARα, and RARγ and the RARα and RARγ using RT-PCR [127], as well as the expression and spatial distribution of RXRs, ADH-I and RALDH2 using whole mount *in situ* hybridization, and immunohistochemistry [128]. Expression of mRNA for RBP, RARα, RARγ, and of the RARα and RARγ2 as well as transcripts for RXRα, RXRβ and RALDH2 were detected in all stages, from the oocyte to the hatched blastocyst. Strong immunostaining was seen for both RARα and RARγ2 proteins. It was later shown that the embryos responded when RA was added and were able to produce RA from added ROH [129]. In mouse embyros, only RARα,

RARβ and RARγ mRNA have been demonstrated [130]. Exposure of mouse embryos to 10 μmol/l of atRA at oocyte stage or the blastocyst stage were found to cause different degrees of retardation of development and embryonic death [131]. Taken together, there seems to be sufficient data to support the fact that the preimplantation embryo has the ability to synthesise and make use of RA, but so far no one has been able to clearly demonstrate the presence of RA in the preimplantation embryo and it remains unclear whether RA signalling occurs or not. The data presented in this thesis support the absence of RA signalling during the preimplantation stage.

Postimplantation embryo

During gastrulation (6-7 dpc), axes are established within the mouse embryo. Head, tail and limbs become defined, and by ten days after fertilization all the organs have begun to develop in mice. During this period, there is an increased demand for RA, and ROH is therefore supplied to the embryo from the maternal bloodstream through the placenta. RBP plays some role in the delivery of ROH [132], but alternative mechanisms are also likely to occur as homozygote RBP knockout mice are viable and fertile [30]. Recently it was demonstrated that dietary RE bound to lipoproteins is the primary source for the foetus to support embryogenesis, and that RBP bound ROH functions more as a backup system in case of dietary vitamin A insufficiency [26]. Although RBP seems dispensable, RALDH2, the enzyme responsible for oxidation of RAL to RA before E9 (in mouse) seems a requisite as offspring of homozygotic mice deficient in RALDH2 (RALDH -/-) die *in utero* before E10.5 [133].

In the remaining nine days before birth in the mouse, organogenesis continues and the embryo increases in size. Chemical analysis of retinoid content in individual organs at early stages of development has proven difficult due to the very limited amounts of tissue. In whole mouse embryos, however, the major retinoids present seem to be atROH and atRA, at 6.5-9.5 dpc [134] and 9.5-14.5 dpc [65]. At later stages viscera, spinal cord, somites, frontonasal mass + branchial arches, forebrain, midbrain, hindbrain, tail bud [65] and limb buds [135] have all been found to contain high concentrations of atROH and some atRA. Data on embryonic concentrations of REs are very limited. The embryo is known to store REs after implantation of the embryo, and vitamin A transport via the placenta has been established. Eckhoff et al found 19.1 ng/g RE at 11.5 dpc in the mouse [136], while the limbs of the porcine embryo were shown to contain 2-4 nmol/g at gestation days 22-30 [137]. As the pigment of the eye is developed, atRE and 11cRE

become detectable in all species, but prior to implantation of the mouse embryo, there seems to be no available data on RE distribution.

Analysis of retinoids

For a number of years following the report on the structures of ROH and RAL, spectrophotometric, fluorometric and calorimetric methods were the only methods available for measuring vitamin A. Of these, the Carr-Price method was, until recently, the official procedure of the Association of Official Analytical Chemists (AOAC) for ROH analysis [138] but it is seldom used nowadays. ROH and its esters produce a blue colour upon reaction with antimony trichloride and the intensity of the colour can be used to estimate the ROH concentration in a sample. Limitations cited for this method include rapid fading of the colour, inability to differentiate between ROH derivatives, and interference from carotenoids [139]. The use of UV absorbance, also known as the Bessey-Lowry method [140], was an accepted procedure for more than 50 years [141]. The absorbance of the extract is determined, the ROH is irradiated with UV light, and the absorbance is determined again. The difference in absorbance is the concentration of ROH in the sample. Furthermore, the intense native fluorescence of ROH bound to RBP may be utilized in direct fluorometric methods. Serum or plasma analysis based on this technique appeared in the literature as late as 1997 [142]. As no sample preparation is needed at all, these techniques are quite interesting, even today, and especially for screening large series of samples [143,144]. Direct fluorometry on plasma/serum correlates quite well (R=0.7-0.85) with chromatography-based methods, but intensity of the fluorescence is strongly affected by the degree of hemolysis and turbidity caused by lipaemia in the sample [142,145] .

Around 1950 various chromatographic techniques were developed for vitamin A analysis of biological samples. Chromatography is not a measuring technique in itself, but a separation technique used in front of a measuring device, the detector. The use of chromatography in retinoid analysis will be described more thoroughly in the next chapter. The topic has been reviewed by experts on several occasions [146-149]. Paper V in this thesis is a recent comprehensive review on the topic, and covers the period from 1995-2001 [150]. Gas chromatography (GC) techniques are rarely used for vitamin A analysis as there are several problematic issues, including dehydration and isomerisation related to the inherently high temperatures used, and the high chemical activity of the silica-based stationary phases in the GC

columns. Stable isotope tracer techniques, however, based on GC-mass spectrometry (MS) is an important tool for studying the bioavailability and bio-efficacy of dietary carotenoids [151]. A few capillary electrophoresis (CE) [152-154] and electro chromatography (CEC) [155] based methods have also been reported, but have found little use.

Reporter assays, with RAREs (figure 4) upstream of genes expressing proteins (reporter proteins), that can be visualized, developed or detected in some way when RA or other RARE activating substances are present have been described [156]. The most common reporters are chloramphenicol acetyltransferase (CAT), LacZ (beta-galactosidase), green fluorescent protein (GFP) and luciferase. For example, when LacZ-producing cells are treated with the substrate X-gal (5-bromo-4-chlor-3-indolyl-β-d-galactopyranoside) they will display a dark blue staining in areas expressing the reporter gene, as LacZ catalyses the conversion of the colourless X-gal to the deep blue 5,5'-dibromo-4,4'-dichloroindigo in the presence of O_2. If a RARE is used as the promoter, staining indicates that RA or another compound with ability to bind and activate RAR-RXR is present.

Reporter constructs can be expressed in different cell types and used as an indirect method for quantification of retinoids. Pieces of tissue or whole embryos are placed on a layer of cells to detect active substances released from the tissue. These reporter systems are very sensitive and have given valuable insights on the spatial and temporal distribution of retinoids, but provide limited information about the identity and concentration of the retinoid. The assays are limited by the facts that the retinoids need to be released from the tissue, that the reporter cells themselves can metabolize and isomerise the secreted retinoids, and that retinoids other than the ones activating the RARE will not be detected at all.

By incorporating the reporter gene into the genome of transgenic animals, indications of ligand-induced activation can be obtained *in vivo*. However, it should be emphasized that ligand-independent regulatory mechanisms for nuclear receptor activation, e.g., by phosphorylation [157], have been demonstrated and may also be detected by most types of reporter models. Furthermore, *in vivo* patterns of activation of reporter genes based on short response elements should be interpreted with caution since DNA and chromosome context, and integration site are clearly involved in control of gene expression.

Liquid chromatographic analysis of retinoids

Chromatography (meaning "writing in colours" in Greek), was first described by the Russian botanist Michael Tswett in 1906 [158]. Tswett's work is relevant in the context of this thesis as he applied the technique for the separation of pigments in leaves, the carotenoids, xanthophylls, and chlorophyll, some of which have provitamin A activity. Chromatography has matured immensely as a technique during the last hundred years, with high performance liquid chromatography (HPLC) initiated in the mid 60s by Horvàth and co-workers [159,160]. The new separations techniques were rapidly adopted by biochemists for the separation of various constituents of biological samples like steroids and amino acids, as well as vitamin A compounds.

Most biological samples are complex; they contain more than 20 000 different molecules, many in mM concentrations, while the concentration of RA usually is in the low nM area. Several geometrical isomers of retinoids exist, and interconversion between these are induced by several factors including daylight and thiols [161,162]. Additionally retinoids are heat and air sensitive. Consequently, successful quantification of retinoids requires extensive clean up and concentration, with simultaneous protection from heat, light and air. Furthermore, when analyzing biological samples, geometrical isomers sometimes need to be separated in the presence of 50-1000 fold concentrations of other retinoids. Therefore accurate quantitative measurement of endogenous concentrations of RA in bio-samples is still regarded as an especially challenging task.

The typical procedure for extraction of lipids, including retinoids, from aqueous samples was and still is: precipitation of proteins by addition of alcohol, salt or acid; repeated extraction with a water-immiscible solvent, such as hexane or ethyl acetate; pooling the supernatants; evaporation of the solvent; and reconstitution in a smaller volume compatible with the chromatographic step. This manual, laborious, solvent-consuming and lengthy approach often involves use of heat, and exposure to light and air - factors that are generally incompatible with the labile nature of retinoids, and consequently there is the risk of erroneous results and artifacts. Light exposure can be avoided by working under red or yellow light, although these are not ideal working conditions.

Solid phase extraction (SPE), a less solvent-consuming, but still manual, technique, has been developed but has not been used extensively for retinoid analysis.

SPE coupled in-line via an automated diversion valve was originally described in 1981 by Roth et al for extraction of drugs in bio-fluids [163]. This technique was later adapted by Kraft et al [164] and researchers at Hoffman-La Roche in Basel for the analysis of RA and its main metabolite, 4oxoRA [165]. This paper was the first to describe the separation of low levels of RA and 4oxoRA *cis* trans isomers in the same run. However a binary gradient, producing a severe baseline drift at the end of the run, was needed and the cycle time of the method was 45 minutes. As the on-line SPE technique may provide full protection from light, heat and air during the entire analytical procedure, as well as the possibility of fully automation, we aimed at utilizing this technique for retinoid analysis. This technique is challenging and requires advanced technical expertise due to the high degree of complexity. It also has a very high initial cost. However, if these hurdles can be overcome, a very powerful and flexible analytical tool is available.

AIM OF THE PROJECT

The aim of this project was to establish high-throughput chromatographic analytical tools for the measurement of endogenous retinoid concentrations in biological samples. As active forms of retinoids accumulate in picomolar or low nanomolar concentrations in tissues, it was of particular importance to develop highly sensitive methods that would allow detection of very small quantities of retinoids in minute tissue samples. In particular, to elucidate the role of retinoids in embryonic development, it was important to be able to measure retinoids in early stage embryos and small quantities of tissue samples from embryos. Application of the developed methods to blood samples in clinical studies will provide insight on the role of retinoic acids in various diseases. Additionally, it was equally important that the developed methods protected and conserved the extremely labile retinoids during the sample collection, preparation and analysis. Thus, the major objectives can be summarised as follows:

1. To develop and optimize high-throughput, robust, reliable and very sensitive methods for the identification and quantification of retinoids in biological samples.

2. To use the developed methods to study the distribution of endogenous retinoids in the early stages of vertebrate development, blood samples, and other types of biological samples.

SUMMARY OF RESULTS

Paper I: Gundersen TE, Lundanes E, et al. Quantitative high-performance liquid chromatographic determination of retinoids in human serum using on-line solid-phase extraction and column switching. Determination of 9-*cis*-retinoic acid, 13-*cis*-retinoic acid, at-retinoic acid, 4-oxo-at-retinoic acid and 4-oxo-13-*cis*-retinoic acid. J Chromatographic B Biomedical Sciences and Applications. 1997 Mar 28;691(1):43-58.

In this work, we established a sophisticated, fully automated, two-dimensional, chromatographic system with integrated simultaneous sample extraction, concentration and clean up for the quantitative determination of trace amounts of endogenous retinoids in plasma or serum. Isocratic separation of RA isomers and two 4oxoRA isomers as well as atROH and 13cROH was achieved for the first time in the same run, eliminating the baseline disturbances experienced with gradient elution. Cycle time was 28 min. Limit of detection for atRA was 23 pmol injected. Minimal loss and artifact generation due to light induced isomerization, air and heat exposure and adsorption to glassware during pre-treatment was achieved. The establishment of this initial method was an important basis for further development of the chromatographic methods in the Blomhoff lab, and in subsequent years was used for the analysis of several thousand samples.

Paper II: Gundersen TE, Blomhoff R. On-line solid-phase extraction and isocratic separation of retinoic acid isomers in microbore column switching system. Methods in Enzymology. 1999;299:430-41.

Extensive experience with the method described in Paper I eventually resulted in the development of the next generation of methodology used for retinoid analysis in our lab. Quantitative recovery and increased robustness was achieved by precipitation of proteins prior to injection, and on-line dilution in front of the SPE column by a third HPLC pump. The inner diameter of both the SPE and the separating column was reduced and comparable separation to that in Paper I was achieved in less then half the time by optimization of the eluent. Altogether, the changes allowed for the quantification of as little as 100 fmol atRA from human serum. As well as contributing to higher sensitivity, the reduced cycle time allowed handling of more samples. A slightly modified version of this method was later used for analysis of RAs and ROHs, as well as 3,4-didehydro retinoids, in chicken embryonic ocular tissues and in mouse

embryos. In additional papers, not included in this thesis, the candidate, in collaboration with others, explored further possible benefits of reducing the inner diameter to capillary dimensions (320 µm) in combination with on-line SPE and column switching [166,167].

Paper III: Sakhi AK, Gundersen TE, et al. Quantitative determination of endogenous retinoids in mouse embryos by high-performance liquid chromatography with on-line solid-phase extraction, column switching and electrochemical detection. Journal of Chromatography A. 1998 Dec 18;828(1-2):451-60.

To increase the method sensitivity further we investigated the potentially very sensitive detection technique, electrochemical detection (ED), in combination with the automated column switching system. As ED reacts with severe baseline shifts to even small changes in eluent composition, gradient elution is not possible and, for the same reason, column switching has rarely been attempted. We succeeded in combining the two, resulting in a method with a limit of detection for atRA of 33 fmol injected. Isocratic separation of RA isomers was a prerequisite for the successful combination. The method was applied to RA analysis in mouse embryos and allowed quantification of atRA in a single mouse embryo 9.5 dpc.

Paper IV: Gundersen TE, Bastani NE, et al. High-throughput analysis of endogenous retinoids in human serum using triple stage LC-MS/MS. Manuscript 2006.

In this paper, tandem mass spectrometry (MS/MS) with atmospheric pressure chemical ionisation (APCI) and isotope dilution was applied to further improve the identification and quantification of retinoids in complex biological samples. MS/MS introduces specificity and sensitivity beyond that possible with UV detection. As an additional dimension is used during detection, the possibility of contaminated chromatographic peaks is reduced considerably. Sample preparation is performed by fast and gentle precipitation with ethanol (EtOH) and direct injection of the resulting supernatant. The developed method is slightly more sensitive than the ED system described in Paper III, even without the column switching system. By applying modern 1.8 µM particle rapid resolution columns, high temperature, high flow, and rapid gradients, we achieved the required separation within 6 minutes. This method thus, facilitates robust analysis of 150 samples per day, including sample preparation.

Paper V: Gundersen TE and Blomhoff R. Qualitative and quantitative liquid chromatographic determination of natural retinoids in biological samples. Journal of Chromatography A. 2001 Nov 23;935(1-2):13-43.

During the development of the methods described in Papers I-III, considerable experience and knowledge about the chemistry, handling and analysis of retinoids were gained. Paper V is a detailed tutorial in retinoid analysis and a comprehensive review of the literature published between 1995-2001.

Paper VI: Hoover F, Gundersen TE, et al. Quantitative assessment of retinoid signalling pathways in the developing eye and retina of the chicken embryo. Journal of Comparative Neurology. 2001 Jul 30;436(3):324-35.

The method described in Paper II was modified to include at-3,4-didehydro ROH (atddROH), 9cddRA and at-3,4-didehydro RA (atddRA). We studied the developing eye and retina of chickens using the HPLC method to quantify biologically active retinoids, immunohistochemistry to localize the retinoic acid synthetic enzyme RALDH2, and nucleic acid assays to quantify and localize retinoid receptor gene transcripts. Our results demonstrated spatial distinctions in retinoid synthesis and signalling that might be related to laminar differentiation in the developing retina. RAs, and their precursors ROHs, are the predominant retinoids in the developing chicken eye. The retinoid 9cRA was undetectable at all stages. AtddROH was detected only in the pigment epithelium and/or the choroid and sclera. RALDH2 immunoreactivity was intense in the choroid, low or absent in the pigment epithelium, and moderate in the neuroepithelium, where it was highest in the outer layers. Transcripts of all five chicken retinoid receptor genes were present in the neural retina and eye throughout development.

Paper VII: Ulven SM, Gundersen TE, et al. Identification of endogenous retinoids, enzymes, binding proteins, and receptors during early postimplantation development in mouse: important role of retinal dehydrogenase type 2 in synthesis of at-retinoic acid. Developmental Biology. 2000 Apr 15;220(2):379-91.

In this study we used the method presented in Paper III, and spectrometric detection as well as RT-PCR, to identify endogenous retinoids, enzymes, binding proteins, and receptors involved in

retinoid metabolism and signalling, in mouse embryos from 9.5 dpc down to 6.5 dpc (the egg cylinder stage), the early postimplantation period in mouse. At 9.5, 8.5 and 7.5 dpc atRA, atRAL and at ROL were detected along with the retinal generating RDH5, ADH4 and the RA generating RALDH2 as well as the retinoid nuclear receptors RAR and RXR. At the 6.5 dpc stage, all of these, except atRA and RALDH, were detected. Therefore, our results suggest that RALDH2 is a key regulator in initiating RA synthesis sometime between the mid-primitive streak stage and the late allantoic bud stage in mouse embryos.

Paper VIII: Ulven SM, Gundersen TE, et al. Quantitative axial profiles of retinoic acid in the embryonic mouse spinal cord: 9-*cis* retinoic acid only detected after at-retinoic acid levels are super-elevated experimentally. Developmental Dynamics. 2001 Nov; 222 (3):341-53.

In this work we used the method described in Paper III, as well as MS detection and DAD, in an attempt to identify definitively the endogenous retinoids present in different regions of the spinal cord at the stages when regional differences in RAR and RXR signalling have been reported [168]. We found a bimodal distribution of atRA, the ligand for RARs, and related this to the expression of several retinoid-synthesizing enzymes. However, we did not detect 9cRA, the putative RXR ligand, in any region of the spinal cord unless pregnant mice were gavage-fed massive doses of atRA. This study provided the first quantitative profiles of endogenous retinoids along the axis of the developing spinal cord. By RT-PCR we also detected transcripts coding for several enzymes involved in retinoid metabolism, of which CRAD1, a short chain dehydrogenase, and RALDH, were found to be distributed in a bimodal pattern. Immunohistochemistry, with antibodies against RALDH, revealed the presence of this protein along the entire spinal cord.

GENERAL DISCUSSION

Artifacts during sample handling and extraction

It has been a central theme of this work to develop methods that preserve the geometrical configuration of the retinoids, by protecting against degrading factors and by using as mild conditions as possible. We have developed methods that avoid isomerisations of RAs in plasma, serum and embryos of different species. In the visual cycle, 11cRAL is transformed to the all-*trans* form when absorbing the energy from one photon with wavelength below 500 nm. From this fact it is evident that light-induced isomerisation of retinoids is a matter of concern. In Papers I-III the problems with light-induced isomerisation are minimized by preparing samples in amber glassware, using specially designated rooms with red light, and ensuring full light protection after protein precipitation by using on-line sample handling and analysis in two-dimensional HPLC systems.

The yellow colour of blood plasma and the red colour of tissue and whole blood provide some protection from light. Addition of denaturating solvents will release and expose the retinoids in a colourless solution and from this point onwards, extreme care should be taken to avoid exposure to light. Concentrated solutions of pure RA isomers have been shown to be reduced to less than 50 % of initial concentrations upon exposure to daylight [169]. At very low concentrations, the degradation rate is even more rapid. Exposure to normal room light for 10 minutes gave mild isomerisation of a 50 nM solution of atRA [57]. The light source of the microscope used during dissection is normally strong white light. In Paper VI, figure 1, the importance of using suitable light conditions during micro dissection of embryonic tissue is illustrated. A 200 pg/ml solution of atRA was rapidly degraded in white light, but when a band filter eliminating wavelengths below 500 nm was used, the degradation was negligible. Filtering of microscope light was used by Kane et al [57], but is not mentioned in the majority of publications. In addition to their instability in light, retinoids are also thermo labile, degrade in the presence of strong acids, and oxidise relatively easily upon exposure to oxygen.

When preparing embryos for analysis in the work described in this thesis, embryos were snap frozen in liquid N_2, homogenised in small Eppendorf vials using a motorised homogeniser with disposable tips, and the proteins quickly denaturated with acetonitrile. The highly controlled

production of the active metabolite RA *in vivo* is likely to rely on the separation in space of substrates, like ROH and RAL from their corresponding oxidizing enzymes. Homogenisation of tissues prior to analysis has the potential to bring these together and alter the distribution of retinoids. Conditions in which the enzymes are inactive, like low pH and low temperature, should thus be preferably introduced shortly after tissue homogenisation.

Liver microsomes have been shown to contain a component that isomerizes pure RA isomers to a mixture of isomers [170]. The isomerization is non-saturable, non-stereo specific, and blocked by thiol-blocking reagents like N-ethylmaleimide (NEM), and is therefore likely to be thiol dependent [162]. Similar effects have also been demonstrated with glutathione (GSH) [161] [171] and albumin, as well as in mixtures of sodium dodecyl sulphate (SDS), and 1-dodecanthiol alone [162]. Lanvers at al [172] demonstrated that cell culture medium with 10 % heat deactivated foetal calf serum (FCS) alone, isomerizes single RA isomers into a mixture of isomers and that, additionally, atRA was much more efficiently taken up into the HL-60 cells than other isomers, thereby driving the chemical thiol-dependent isomerization reaction further. Schmidt [172] detected a peak co-migrating with 9cRA in human plasma that was caused by isomerisation from other RAs; the degree of isomerisation was ~7 % and the plasma matrix needed to be present for isomerisation to occur [56]. Kane et al demonstrated that contamination by acids, in addition to light exposure, will also cause isomerisation and samples displaying more than 10-15 % isomerisation should be discarded [57].

Internal standards (ISs) can be used to reveal and compensate for isomerisation caused by all these reasons. In Paper I we used a non-retinoid as IS as a retinoid IS was not available. This IS functioned as a volumetric IS. In Paper II we used all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (TMMP-ROH) as IS. This IS could not reveal isomerisation of RAs, as the isomers of retinols are not fully resolved in this system. Analysis of pure standards and plasma/serum indicated, however, that isomerisation had not occurred. In Paper III we used 13c-acitretin as IS. This aromatic retinoid behaves very similarly to the RAs, and isomerisation during analysis can be revealed. This IS (or another isomer) has also been used by others [56,173].

In Paper IV we applied a stable isotope of atRA as IS. This is the ideal IS for RA analysis, and any isomerisation during analysis will be revealed. This IS can be considered to behave identically to atRA, also with respect to possible enzymatic isomerisation. The use of MS/MS and a mass slightly different from atRA provides excellent control of isomerisation in every sample analysed. In Paper IV, figure 3, the plasma chromatograms clearly show that the added IS is not isomerised. Others have attempted to apply a stable isotope of RA as IS, but without success [174]. Kane at al also used MS/MS and a synthetic analogue of RA, 4,4-dimethyl-RA [57]. Experiments demonstrated that this IS behaved similarly to atRA when isomerisation was initiated by light or acid exposure.

Extraction of retinoids

Simple, fast and mild, monophase extraction (MPE), in combination with on-line concentration and clean up, is the basis for all methods and applications in this thesis. There are several benefits from this approach, but also drawbacks. The retinoids are a family of substances with polarity ranging from very apolar, and hence lipophilic, REs, to the water-soluble RAs and sugar or amino acid conjugated retinoids. Extracting retinoids from both ends of the polarity scale, and with simultaneous high recovery, is truly challenging. The methods described in Papers I, II and III are based on automated on-line SPE on short HPLC columns, in combination with column switching. In the original publications by Roth et al [163] addressing on-line SPE, it was stated that most drugs could be extracted with high recovery from unmodified bio-fluids. However, as most retinoids, except the sugar and amino acid conjugates, are incorporated into transport proteins (~99 % protein bound), release from the protein must be accomplished before extraction can take place efficiently [175]. In Paper I this was achieved by addition of 20 % acetonitrile. Addition of < 20 % organic modifier leaves the proteins in an altered conformation, loosening the protein-retinoid binding and increasing recovery on the SPE column, but still retaining the retinoid in solution by its interaction with the protein. Wyss et al [176] experienced problems with isomerisation caused by prolonged exposure to acetonitrile as the prepared samples queued up in the automated sample injector [176]. We circumvented this problem in Paper I by applying robotized addition and mixing of the acetonitrile and IS shortly before every injection.

In combination with this sample preparation we examined several 5 µm/100 Å particle commercial guard columns with 2 µm steel frits for use as the SPE column. None of them performed satisfactorily and the problems encountered were mainly low recoveries, low sample capacity, memory effects and rapid pressure build-up allowing only a few injections. Better results were achieved with a 20 mm x 4.6 mm poly ether ether ketone (PEEK) column with 2 µm titanium frits packed with a 30-50 µm C18 material with 300 Å pores. With this SPE column >200 injections of 1000 µL could be injected on the same column with 79-86 % recovery for all retinoids before extensive pressure build-up enforced column change. This was 4-6 times the number of injections reported by Wyss et al on similar systems [165,177]. In addition, use of titanium frits and a titanium needle in the injector reduced the memory effects obtained with their steel counterparts to 20 %, and therefore a complicated back-flush system to clean the SPE column, and used during development (not shown), was no longer necessary.

The mobile phase (M1) bringing the injected sample through the SPE column had a physiological pH to avoid precipitation of proteins and clogging of the SPE column, as well as facilitating phase transfer of the lipophilic retinoid through the aqueous M1 to the SPE phase. It was however, observed that recoveries were higher when an acidic pH was used and that trifluoroacetic acid (TFA) performed better than acetic acid. Equally important as trapping retinoids efficiently, is achieving transfer of the extracted retinoids to the separating column in a small volume, resulting in narrow peaks and improved sensitivity. Furthermore, it has to be considered that the chromatographic front, caused by elution of nonretained solvents, polar compounds and pressure changes during injections, is often more pronounced in column switching and can preclude the quantification of early eluting compounds. The uncapped C18 material used in the SPE column is rich in residual silanol groups, assumed to be important for high recovery of substances with hydrogen donor/acceptor functionalities. These R-Si-OH groups are ionisable and the pKa is 3-4. When acidic acid or formic acid is used in M1, residual silanol groups on the SPE column will probably be of both forms, resulting in a broader front as well as broader chromatographic peaks. Adjusting the separating mobile phase (M2), by exchanging some of the acetonitrile for methanol (MeOH) and increasing the concentration of ammonium acetate and using TFA in M1, resulted in narrower chromatographic peaks and a less distorted elution front. By monitoring the effluent from the pre-column during concentration and clean up

with a second UV detector it was found that a duration of ten minutes was optimal to obtain a clean extract with high recoveries of all retinoids.

As an alternative to only partial denaturation of proteins prior to SPE, an excess amount of water-miscible organic solvents like MeOH, EtOH, propanol (PrOH) or acetonitrile can be added. Under these conditions the conformation of the protein is significantly altered, liberating the retinoid and leaving the protein in a non-soluble form. Two-three volumes are needed for near quantitative precipitation of proteins [178]. As the lipids are no longer retained in solution by protein binding, it is important that the lipids to be extracted are soluble in the new composition of the monophase (MP). When the sample is centrifuged, a protein pellet should form at the bottom of the vial, with a MP above containing all the retinoids.

The volume and composition of the MP can be designed to optimize recoveries for the various different retinoids. This type of extraction is called MPE or direct extraction. In MPE the sample is diluted, whereas in traditional liquid-liquid extraction (LLE) the sample is often concentrated by evaporation. Evaporation of aqueous solutions usually requires temperatures incompatible with the thermo labile nature of retinoids.

In Paper II we used MPE in combination with on-line SPE. The high organic content of the MP limits the volume that can be injected onto the SPE without causing broadening of the chromatographic peaks. By on-line dilution with water, prior to a 2.1 mm id SPE column with 5 μm PAT frits, 2 ml MP could be injected with a resultant 97-100 % recovery of the retinoids. The small elution volume from the narrow SPE column, in combination with the large volume injected resulted in a significant concentration of the sample. Problems previously encountered of isomerisation on storage in the injector when all plasma components were present were not observed, and this concurs with the finding that sulphydryl containing proteins, like albumin and GSH, might induce isomerisation of retinoids [162,171]. Although even more complex instrumentation was needed, the combination of MPE and on-line SPE increased recoveries, improved sensitivity and provided a more robust and flexible routine methodology. The combination of large volume injection and narrow columns increased the sensitivity considerably.

In Paper III MPE was used, but dilution with water after centrifugation was performed off-line, followed by injection of 1000 µl onto the SPE column. This was mainly due to limitations in available instrumentation. Recoveries were in the range of 86-103 % for ROH and RA isomers.

When developing the method described in Paper IV, we wanted to keep the simple MPE as it is very gentle and there is no risk of losing retinoids with high polarity into the water phase as occurs with two phase LLE. There is also no need to adjust the pH by addition of alkali or acids in MPE. The column switching based SPE system functions well, but it is complicated, and as the SPE column ages there is a risk that extraction recovery might be affected. It would therefore be advantageous if the SPE step could be avoided. There was also a real need for a fast method with a capability of handling large numbers of samples in a short time. By applying APCI-MS/MS in positive multiple reaction monitoring (MRM) mode we achieved a sensitivity and selectivity that allowed direct injection of 100 µL of EtOH-based MPE, allowing quantification of the important retinoids in plasma. To avoid peak distortion of early eluting retinoids, it was necessary to perform on-column focusing. This was achieved by using 75 % water in acetonitrile (0.1 % formic acid) as the initial conditions in the elution gradient. Recoveries were in the range of 87-105 % for ROH and RA isomers.

Separation of *cis trans* isomers of retinoids

We have developed rapid methods on specialty phases for separating geometrical isomers of retinoids. Most retinoids can exist in between 3 and 5 different geometrical cis trans isomers (see fig 1 in Paper V). Altogether several hundred different retinoids are possible. Separation of all retinoids, including the various isoforms, in the same run is not possible with HPLC, and rarely necessary, as only a few retinoids are normally present in the same type of sample. The most frequently used reversed phase (RP) columns, are the classical C8, C18 or C30 type columns. Separation of retinoid isomers often requires time-consuming gradient elution: However by using the chemical features of the various retinoid molecules, and by exploring different specialty stationary phases for chromatography, we are now able to manipulate their retention. Additionally, understanding these chemical interactions has enabled us to achieve the required separations in a shorter time. To achieve the lowest possible detection limits in trace analysis by UV detection or ED, it is advantageous to use isocratic separation. Isocratic separation of RA

geometrical isomers has been reported on a few types of column materials. The Suplex pKb-100 column (Supelco, Bellafonte, USA), used for most of this work, separates RA isomers remarkably well under isocratic conditions, and has been used by several groups for this purpose with excellent results [134,166,179-183]. The material has an amide functionality incorporated into the alkyl chain, close to the silica back-bone. This polar moiety probably forms strong secondary interactions with the polar end group of the retinioids. As a result, the retention of retinoids is stronger than that of other sample constituents, and high selectivity for retinoids has been achieved. Resolution between the geometrical isomers is probably due to this, combined with different degrees of hydrophobic interaction between the stationary phase alkyl chain and the polyene chain of the retinoid. *Cis* isomers have a shorter chain, and will therefore have less overlap in comparison to the all-*trans* isomer. A similar column, Supelcosil ABZ+Plus, was recently used by Kane at al [57]. In Paper IV another type of column was used. This method was designed for high-throughput analysis of polar retinoids in plasma.

High-throughput HPLC

In order to analyse a large number of samples in a short time, we have developed high-throughput methodology (Paper IV) for retinoid analysis. Also with MS/MS detection geometrical isomers have to be separated in time as the isomers fragmente in very similar fashion. The chromatographic separation time can be reduced by either increasing the flow rate and/or by shortening the length of the analytical column. There are two types of high-throughput HPLC columns, monolithic columns with very low backpressure at high flow rates, and short columns with smaller sized particles, typically 1-3 µm. Sub two micron columns will often generate very high backpressures at high flow rates. To handle this elevated pressure, ultra high pressure HPLC, or ultra performance liquid chromatography (UPLC) was introduced in 2004 [184]. We have used columns packed with 1.8 µm particles, in combination with high flow and high temperature. A high temperature is used to decrease the viscosity of the mobile phase, which consequently reduces the backpressure. Separation of the important geometrical isomers was achieved in 6 minutes.

When large number of samples are analysed, the second bottleneck is sample preparation. Previously we have used on-line sample preparation techniques in sequential mode. This means

that the total sample preparation time can be calculated as individual sample preparation time multiplied by the number of samples (e.g. 1000 samples x 10 min = 7 days). In Paper IV sample preparations were performed in parallel, in microtiterplates for up to 200 samples. This reduces the time spent on sample preparation significantly. Compared to classical approaches, like LLE or off-line SPE, this is a major improvement, facilitating the analysis of a large number of samples with little technical assistance necessary. The very short total analysis time is also advantageous for maintaining the integrity of the labile retinoids.

Detection of retinoids in chromatographic systems

In the methods and applications described herein, we have used UV detection in the form of single wave detectors and DAD. In Papers III and VII we have applied coulometric ED. On several occasions we have applied MS for qualitative purposes and in Paper VIII tandem MS was used for quantification as well.

UV and fluorescence detection (FLD)

We used UV as the primary detection technique in Papers I, II and VI. Most retinoids absorb UV light strongly in a region of the spectrum (300-400 nm) where few other natural compounds absorb light. Detection based on this technique is, in many cases, superior and is used in the majority of publications dealing with quantitative retinoid analysis [56,185,186]. The normal bore chromatographic system in Paper I generated a limit of detection (LOD) of around 2 ng/ml. When using the narrower columns in Paper II, the mass limits of detection (mLOD) for atRA was 60 pg on-column. In additional papers not included in this thesis, the candidate, in collaboration with others, explored further possible benefits of reducing the inner diameter to capillary dimensions (320 μm) in combination with on-line SPE and column switching [166,167]. The mLOD was 0.9 ng for retinyl palimitate [167] and 5 ng for atRA [166]. In comparison Wyss et al [173] reported mLOD of 50 pg for atRA while Schmidt [56] obtained a mLOD of 30 pg on-column for atRA.

Electrochemical detection (ED)

In Papers III, VII and VIII we used on-line SPE with coulometric ED for detection of RAs and ROH in embryonic tissue. ED is a potentially sensitive and selective technique, but it is rarely

applied for the determination of retinoids. The polyene chain of retinoids will be oxidized at a sufficiently high positive potential in amperiometric, as well as coulometric, EDs. A few methods using liquid chromatography (LC)-ED for determination of retinoids have, however, been reported. Hagen et al [187] describe a method for separating and detecting retinoids by reverse-phase capillary liquid chromatography with amperiometric ED. Detection limits of 410, 64.1, 60.9, and 38.2 pg/ml for 13cRA, atRA, RAL, and ROH, respectively, were reported. The mLOD were 10 pg and 25 pg for the RAs and atROH, respectively. ED has also been used for retinoid detection by others [188-194].

Mass spectrometric detection (MS)

We have used MS in Papers VII and VIII for qualitative purposes, as well as as a quantitative detection technique in Paper IV. The combination of LC and MS (LC-MS) provides a powerful analytical technique that combines the resolving power of HPLC, with the potentially high specificity and low LOD of MS. Historically only a handful of papers describe the use of LC-MS for the determination of retinoids in biological samples [195-198]. During the last decade, however, modern soft atmospheric pressure ionisation techniques like electrospray ionisation (ESI) and APCI have been explored for retinoid analysis. During recent years, hyphenated techniques like tandem MS (MS/MS), have also been available.

In Paper IV a method using positive ion APCI coupled to tandem MS detection (LC–MS/MS) was developed for the determination of at4oxoRA, 13*c*4oxoRA, 13*c*RA, atRA and atROH in human plasma using a stable isotope of atRAas the IS. The Applied Biosystem API 4000 Qtrap tandem mass spectrometer, equipped with a heated nebuliser (APCI) ionisation source, was operated in multiple reaction monitoring (MRM) mode, with the precursor-to-product ion transitions *m*/*z* 315.4→ 297 (4oxoRAs), 301.2 → 205 (RAs), 305.0 → 209 (IS) and 269.2 → 93 (ROH) used for quantification. The assay was fully validated and was found to have acceptable accuracy, precision, linearity, sensitivity and selectivity. The limit of quantification was in the range of 0.1-0.3 ng/ml andthe mLOD in the range 1-4 pg on-column. APCI was selected instead of ESI as ion suppression was less pronounced. Ion suppression will reduce the sensitivity of the assay as well as limit assay reproducibility and can be a serious problem in quantitative LC-MS analysis. Problems and solutions regarding ion supression/matrix effects in LC-MS have been discussed in several recent papers [199-202].

LC-APCI-MS/MS operated in MRM mode was used by Napoli and co-workers for RA analysis in adult mouse brain [57]. ROH and RE were removed by LLE during a rather extensive sample clean up before LC-MS/MS analysis. The assay allowed quantification of 6 pg atRA on-column (0.3 ng/ml). Wang et al [203] used a less selective, single-stage MS in positive single ion monitoring mode with APCI for the quantification of ROH and RA in rat prostate tissue. The sample preparation consisted of evaporation and reconstitution of an ethanolic supernatant resulting from protein precipitation. The method allowed quantification of 211 pg RA and 0.6 ng ROH on-column. Matrix effects were not studied. It is, however, rather supprising that the finding of massive amounts of 9cRA and 9cROH was not given more notice. A similar sample preparation was used by Rühl [204] in combination with MS/MS detection (triple quadrupole). The ethanolic supernatant was, in this case, concentrated by vacuum centrifugation and a LOD of 7 pg on-column was reported. In earlier publications Van Breemen and co-workers [205,206] used ESI-MS in positive mode for measuring ROH and RAL, and ESI in negative mode for RA, as well as positive APCI-MS for atROH and at-retinyl palmitate. ESI generated detection limits for RA, RAL and ROH of 23 pg, 1.0 ng and 0.5 ng, respectively, whereas APCI gave mLOD of 0.670 pmol atROH and 0.720 pmol at-retinyl palmitate on-column.

MS/MS, in the form of an iontrap mass spectrometer, has been used for the characterisation of 14-hydroxy-retro-retinol and 4-hydroxyretinol in the liver of neonatal rats at days 3 and 11 after birth, and the novel retinoid 9-*cis*-4-oxo-13,14-dihydro-RA in adult mouse liver and also in human liver [207]. Others have used normal phase LC-APCI-MS/MS (iontrap) for quantification of ROH and RA in liver from 8-week old rats and retina from embryonic mice on days 13-15 [174]. The lower limit of detection was 50-100 fmol on-column. The concentration of liver ROH in three different animals was 13.3, 22.4 and 16.6 nmol/g, and that of RA was 1.0, 1.9 and 2.2 nmol/g. atRA concentrations in mouse retina were found to be 20.8 (s.d. 2.0) pg/µg retina protein. Matrix effects were not discussed, but the authors found that use of a stable deuterated isotope of atRA as an IS caused larger variations than when no IS was used. When soft ionisation techniques are used, there is a real risk of overloading the ionisation capacity in the spray chamber and an IS can be very helpful. In Paper IV, we have successfully applied a novel ^{13}C (10,11,14,15-^{13}C-at RA)-labelled stable isotope of atRA. In spite of the very simple preparation, and three times dilution of the samples, the detection limits of the method described in Paper IV are comparable to, or even better than, other methods.

Unequivocal identification of retinoids

Identification of retinoids found in biological samples has, in work included in this thesis, been performed by matching chromatographic retention with pure standards, by recording of on-line UV spectra and comparing these with spectra from pure standards, and by MS and tandem MS. Unequivocal identification of a specific retinoid by chromatographic techniques is not an easy task. There is a multitude of possible retinoids, and the number of commercially available authentic retinoid standards is very limited. The analytical chemist can obtain these as gifts from other researchers or institutions, isolate them from natural sources, or synthesise them. The subsequent utility of this retinoid is therefore highly dependent on full initial characterisation of the molecular structure, something that is not always performed, or at least the data may not be available to researchers. Ideally, a certificate stating the identity and purity should accompany the retinoid, as for commercial standards. A thorough spectral characterisation should include high-resolution MS, nuclear magnetic resonance (NMR) and UV spectroscopy. If a pure authentic standard is available, matching the chromatographic retention time of an unknown in a specified system with that of a standard is an indication, although not proof, of its identity. Further identification can be done by collecting the fraction containing the substance and re-injecting it on a second chromatographic system, then again comparing the retention of the standard with the unknown. This is, of course, not possible when using destructive detectors like ED and MS.

Many retinoids absorb strongly in the 300-400 nm region of the UV spectrum, where few other substances do. However, as the concentrations of bioactive retinoids are extremely low, there is often substantial noise at the baseline. Thus, UV spectra of endogenous concentrations of retinoids collected on-line with DAD, if visible at all, often lack the quality needed for identification purposes. Single quadrupole MS seems to be less selective for retinoids compared with UV. This is especially true when ESI is used. APCI is, in general, a more selective ionisation technique and will generate cleaner chromatograms. MS/MS detection is very selective and provides increased specificity compared with UV detection of retinoids. Unequivocal identification of the geometrical form of a retinoid isolated from biomaterial can, however, only be obtained with NMR. Because the amount of substance needed for NMR is relatively high, and the endogenous amounts of retinoids are very low, this is rarely feasible. NMR can also be performed on-line with HPLC. HPLC-NMR is starting to become available in some labs, and has

been used for the structural characterisation of some retinoids [208-211]. Some, or several, of these approaches are often used during method development and validation of assays. For practical reasons, however, it is less common to apply full qualitative analysis to every sample, even when analysing different kind of tissue. There will always be a risk that new interferences appear in some samples or in different types of tissues and the amount of interfering substances might also change and lead to falsely low or elevated results.

Variations in the retention behaviour of similar chromatographic columns or even between batches of the same type of column have been reported. For example, Noll et al recently revealed extensive inconsistencies and errors in the elution order of retinal and retinol isomers in straight phase chromatographic systems [212].

Retinoids and embryological development in mice and chickens

In Papers VI, VII and VIII the methods developed in this thesis have been applied to study the role of retinoids in embryonic development mice and chickens.

In Paper VI the developing eye of the chicken was studied. In addition to the common retinoids ROH, RAL, RE and RAs, the chicken has a second set of retinoids, namely the didehydroretinoids (Vitamin A2). Vitamin A2 standards were obtained from F.Hoffmann-La Roche Ltd and Dr. Anders Vahlquist and the method described in Paper II was adapted to include these retinoids. Epithelium, choroid and sclera (stages: E8.5, E7.5, E6.5 and E4.5) were isolated from chick eyes at different stages of development and analysed for retinoids. The whole eye was also analysed at the earlier stage (E4.5). RAs and their precursor ROHs were found to be the predominant retinoids in the developing chick eye. AtRA and all-*trans*-3,4-didehydro-RA were present in the neuroepithelium in approximately equal amounts from early stages of neurogenesis until shortly before hatching. The RXR ligands 9cRA and 9c4oxoRA were not detected at any of these stages. RAs were not detected in the pigment epithelium at all. AtROH was present in the neuroepithelium and pigment epithelium, whereas all-*trans*-3,4-didehydro-ROH was detected only in the pigment epithelium and/or the choroid and sclera. These results demonstrated spatial distinctions in retinoid synthesis and signalling, that may be related to laminar differentiation in the developing retina.

In Paper VII developing mice were examined for retinoid content. Whole embryos from stages 9.5 dpc, 8.5 dpc, 7.5 dpc and 6.5 dpc were analysed for 16 different retinoids. Only atRA, atROH and atRAL were detected at the various stages. atRA and at-retinol were detected at 9.5, 8.5, 7.5 dpc, but not at 6.5 dpc. However, at this stage, and at stage 7.5 dpc, its precursor atRAL was present. The method described in Paper III was used for quantitative analysis. A similar system, with DAD, was used for retinoid identification. In addition, LC-MS was used to confirm the identity of RAL at the earlier stages. Prior to this publication, no chemical analysis data existed on stages earlier than 9.0 dpc. The novel finding of RAL at the early stages of development, together with the detection of RALDH2 (also Paper VI, RT-PCR) and atRA at stage 7.5 dpc, indicates that it is at this stage that RA signalling becomes active in the developing mouse and that RALDH2 is the key regulator.

In Paper VIII regions of the mouse spinal cord were studied. This publication was the first to describe the quantitative profile of specific endogenous retinoids along the axis of the developing mouse spinal cord. Following an intriguing publication by Solomin et al [213], where strong evidence for the presence of RXR activation was demonstrated in different regions of the mouse spinal cord at stage 10.5 dpc, we dissected these regions from mouse embryos to determine whether 9cRA occurred there, but could only detect atRA. Only after the mother was fed large toxic amounts of atRA, could small amounts of 9*c*RA be detected, together with several other retinoids including 13cRA and 13c4oxoRA. Although no comparable specific quantitative data obtained by chemical analysis are available, our findings are supported by data obtained by other techniques. Bioassays and reporter mice have demonstrated the presence of retinoids in the developing neuroaxis, including the spinal cord, with concentrations at lumbar and brachial levels relative to thoracic levels [213-218].

The physiological role of 9-*cis* retinoic acid is questionable

Following the original publication in January 1992 demonstrating that 9cRA acts as a ligand for the RXRs [219], more than a thousand investigations have been published establishing the potent effects of this compound in numerous biological processes. It is now generally accepted that 9cRA represents one of the signalling pathways that comprise the mechanisms of action of ROH. However, a major premise for this biological role remains unconfirmed and has been largely

neglected by the research community: that is, the unequivocal identification of 9cRA as an endogenous compound. Although only a few applications are presented in this thesis, the methodology presented herein has been applied to a multitude of sample types from many species of animals and we have never detected endogenous 9cRA in any tissue sample, unless high toxic amounts of retinoids have been administered.

By using the ultra-sensitive HPLC method described in Paper III we detected ample amounts of atRA, but no 9cRA, in any region of the mouse embryonic spinal cord. We could only demonstrate 9cRA in mouse embryos when massive, teratogenic doses of RA were fed to the pregnant dams. We also tried to reproduce the original identification of 9cRA in mouse kidney and liver [219], but were unable to detect 9cRA in normal mouse kidney or liver, nor in human plasma. Furthermore, polar bear liver, which is known to accumulate near toxic amounts of vitamin A, contains many different retinoids but not 9cRA (data not shown). We have also, in collaborative projects not referred to in this thesis, examined: plasma from rats, salmon, dogs, and monkeys, and tissue samples from rats, monkeys and dogs without detecting 9cRA. Thus, demonstration of 9cRA has to date only been tentatively demonstrated in humans following administrations of about 100 mg vitamin A [220] and in mice embryos after maternal ingestion of toxic, teratogenic doses of atRA [180].

Should we then conclude that 9cRA is only present during vitamin A intoxication and teratogenicity? Although this might be the case, it may be wise to defer such a conclusion at present. Firstly, 9cRA might only be present in very localized regions, or in very few cells, but at concentrations that are high enough to drive a biological response (about 1 nM). Secondly, detection of 9cRA could be missed because sufficient concentrations are only present transiently. As it is documented that RXR signalling does occur [221,222], ligands other than 9cRA might be responsible. In fact, phytanic acid, eicosanoids and docosahexaenoic acid (DHA), have been shown to activate RXRs [223-225]

CONCLUSIONS

We have developed a set of chromatographic methods for the quantititative and qualitatative identification of a number of retinoids in minute amounts of various biological samples. The methods have been specially designed to preserve the very labile retinoids. To minimize the risk of artifact formation of geometrical isomers, a simple protein precipitation and MPE in containers protected from light has been the basis for all the methods used. Further treatment, like clean up, concentration, separation and detection have been automated in multidimensional HPLC systems with various detectors. The methods used have high resolving powers, ultra high sensitivities and full protection from critical variables at all times. Paper IV represents state-of-the-art methodology, and includes increased sensitivity, real-time control of possible isomerisation, and high-throughput capabilities by implementing LC-MS/MS technology, stable retinoid isotopes, and sub two micron HPLC columns for fast separation of geometrical isomers.

The developed methods have been applied to several challenging projects, studying important biological functions during embryonic development of retinoids in mouse and chicken. As a result of the increased sensitivity and optimal control of possible artifact generation during analysis, several novel findings have been possible. These include: identifying atRAL and the onset of RA signalling at 7.5 dpc in the developing mouse embryo; demonstrating the lack of 9cRA in regions of the mouse spinal cord where RXR signalling is known to occur; obtaining the first quantitative profile of specific endogenous retinoids along the axis of the developing mouse spinal cord; defining the spatio-temoral distribution of endogenous retinoids in several regions of the developing eye of the chicken.

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