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Capillary high-performance liquid chromatographic determination of lutein and zeaxanthin in aqueous humor from a single mouse eye

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Abstract

To protect the eye from ultraviolet phototoxicity caused by free radicals, ocular components such as the aqueous humor accumulate antioxidants, such as the carotenoids. Lutein and zeaxanthin are the only carotenoids known to be present in the aqueous humor. Due to the small sample volume, pooling of samples from an undesirable large number of animals is often required for sufficient sensitivity and statistically significant differences to be achieved. In this paper we present a rapid, sensitive and robust packed capillary high-performance liquid chromatographic visible detection method for the quantification of lutein and zeaxanthin in the aqueous humor of single mouse eyes.

Keywords: Lutein; Zeaxanthin

1. Introduction

Age-related macular degeneration and cataracts in particular, are the leading cause of visual loss worldwide. Taylor [1] stated as early as in the late 1980s that cataract afflicted more than 50 million persons worldwide. Recently, The World Health Organization (WHO) has singled out cataract as a priority disease in Vision 2020, a global initiative for the elimination of avoidable blindness [2].

Cataract, or opacification of the lens, is related to the precipitation of proteins or other constituents of the lens that occur upon aging, and ultimately leads to the loss of vision. Proteins in the lens are unusually long-lived, and can thus be subjected to extensive damage during their lifetime [3]. Laboratory and epidemiological data indicate that the physiological damage to lens constituents are due, in part, to short-wavelength light and subsequent formation of an environment rich in active forms of oxygen [4], commonly referred to as reactive oxygen species (ROS).

Opacification of the lens is non-reversible, and cataract can thus only be treated by surgical methods such as replacement of the opaque lens with a clear donor or synthetic lens. Estimates made by Taylor indicate that approximately half of the surgical cataract extractions performed can be avoided if the formation of cataract is delayed by 10 years [4].

There is evidence that cataract development can be delayed and possibly prevented by the presence or

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enhancement of antioxidants in the aqueous humor, the fluid that nourishes the lens. The aqueous humor is formed by the filtration of plasma constituents by the trabecular meshwork into the void between the lens and the cornea. Plasma levels of antioxidants, such as carotenoids, will thus affect the antioxidant status of the aqueous humor, and an elevated plasma level of antioxidants may thus reduce the risk of cataract development [5,6].

The six major known carotenoids present in human plasma are beta-carotene, lycopene, beta-kryptoxanthin, lutein, alpha-carotene and zeaxanthin, listed after decreasing plasma concentration levels. However, it is well established that the only carotenoids present in the human aqueous humor, and thus in the human lens, are lutein and zeaxanthin [7,8]. Structures of both compounds are shown in Fig. 1.

When present in the aqueous humor, lutein and zeaxanthin may effectively protect the lens from ultraviolet phototoxicity via quenching of the ROS [5,9]. This is supported by the observation that elevated long-term intake of lutein and zeaxanthin is associated with a reduced risk to develop lens opacities [10,11].

It is common practice for researchers to use the mouse eye as a model for human eyes [12]; however, there are some obvious limitations. The aqueous humor in mouse eyes is approximately 4–6 μl, and assessment of individual levels of lutein and zeaxanthin with the use of traditional analytical techniques as high-performance liquid chromatography (HPLC) with ultraviolet-visible (UV–VIS) or fluorescence (FLD) detection is extremely challenging due to the limited sample volume available for analysis. To our knowledge, mean values have up to now only been obtainable by pooling samples from multiple mice. Thus, individual differences have not been reported.

Our experience with the determination of carotenoids in human plasma (data not shown) indicates that the concentration levels of carotenoids can vary to a great extent between individuals and, as demonstrated in the present study, this is also the case for the concentration of the xanthophylls lutein and zeaxanthin in the aqueous humor of mice. Thus, there is a need for a robust and sensitive method for the determination of xanthophylls in aqueous humor in single mouse eyes.

With the possibility of measuring carotenoids in aqueous humor from single mouse eyes, investigators can then use eye research models that include control and exposed eye in the same animal, hence, reducing the difference between the treated and the control eye. Together, this will significantly reduce the number of animals included in research models, a factor of both ethical and economical value.

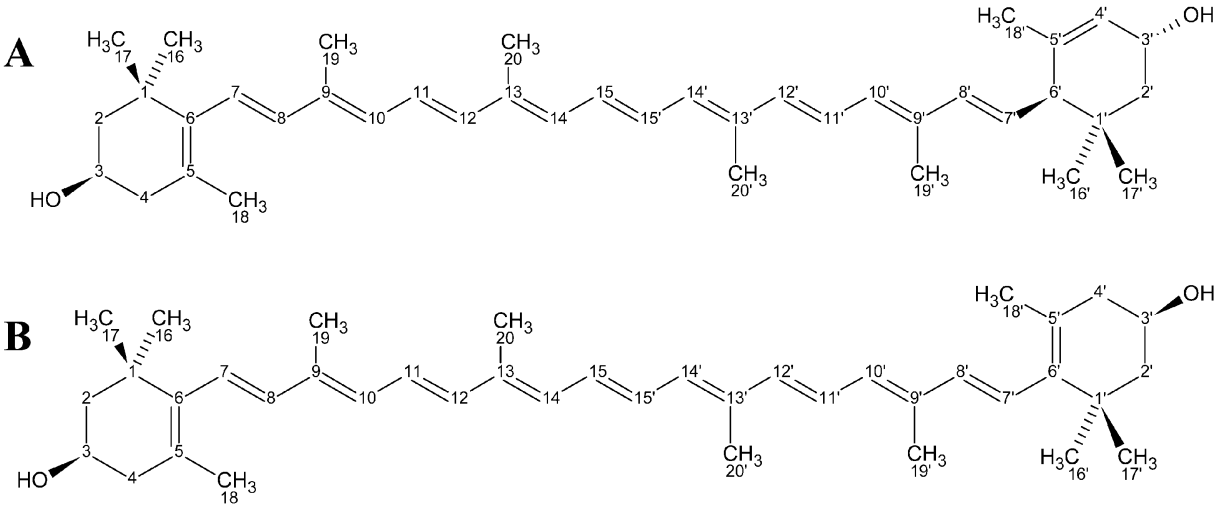

Fig. 1. Structure of lutein (A) and zeaxanthin (B).

In this paper we present a method for the determination of individual concentration levels of lutein and zeaxanthin in aqueous humor from single mouse eyes by the use of reversed-phase packed capillary HPLC.

Packed capillary HPLC offers several advantages, as compared to conventional HPLC. The main advantage associated with the use of packed capillary HPLC is the increased mass sensitivity obtained as a result of reduced dilution of the chromatographic band during separation. Thus, when only limited sample volumes are available, the increased mass sensitivity of capillary columns allows low concentrations of analytes to be determined and quantified [13].

2. Materials and methods

2.1. Chemical and materials

Butylated hydroxytoluene (BHT) astaxanthin, lutein, zeaxanthin, bovine serum albumin (BSA) and phosphate buffered saline (PBS) were purchased from Sigma Aldrich (Oslo, Norway). Benzene (p.a.), and HPLC-grade methanol were purchased from VWR (Oslo, Norway). Absolute ethanol and isopropanol was purchased from Arcus (Oslo, Norway). Bulk YMC C_{30}, 3-μm packing material, was obtained from YMC Europe (Schermbeck, Germany). Milli-Q-water was obtained from an in-house Millipore milli-Q-water station.

The YMC C_{30} column, 0.32×200 mm was kindly provided by Dr Pål Molander at the Research Group of Organic Analytical Chemistry, University of Oslo. Fused silica connecting tubing with polyetheretherketon (PEEK) protection layer was obtained from Agilent technologies (Palo Alto, CA, USA).

2.2. Sampling of aqueous humor from mice

Three healthy, adult mice were sacrificed by cervical dislocation. The eyeball was then removed, washed in phosphate-buffered saline (PBS), grasped by the optic nerve with a pair of forceps and placed in a micro funnel with the cornea facing the apex of the funnel. The eyeball was fixed from above with a pair of forceps and the cornea was punctured with a needle.

The funnel was then inserted into a micro vial and both were placed in an eppendorf tube, as shown in Fig. 2. This micro-apparatus was then centrifuged for 60 s at 2000 rpm to gravitate the aqueous humor into the micro vial leaving the remainder of the eye in the funnel that served as a stopper to prevent any other ocular constituents from entering the collection tube. An aliquot of 4–6 μl aqueous humor was possible to extract from each mouse eye. Samples were stored at −20 °C, and analyzed within 2 weeks.

2.3. Preparation of standards and precipitation solution

Standards containing both compounds were prepared in absolute ethanol at a concentration of 70, 175 and 350 nmol/l and 70, 174 and 347 nmol/l for lutein and zeaxanthin, respectively.

A stock solution of the internal standard astaxanthin was prepared by dissolving 1 mg astaxanthin in benzene, to a final concentration of 0.25 mg/ml.

All solutions were prepared under red light, flushed with argon and stored in amber vials, and used for analysis within 1 month.

The precipitation solution was prepared in isopropanol containing the antioxidant BHT (12 mg/l) and the internal standard astaxanthin.

2.4. Sample preparation

An aliquot of 4 μl of the aqueous humor was sampled in an amber vial and 18 μl of the precipitation solution was added for protein precipitation. The sample was vortex mixed for 20 s and centrifuged at 3000 *g* and +4 °C in 15 min. An aliquot of 2 μl of the clear supernatant was injected into the chromatographic system. All samples were analyzed within 1 day of preparation.

2.5. Chromatographic system

The Agilent 1100 Series capillary liquid chromatography (LC) system was delivered by Agilent Technologies (Palo Alto, CA, USA). The capillary HPLC system consisted of an Agilent 1100 series; micro vacuum degasser, micro auto sampler, thermo-

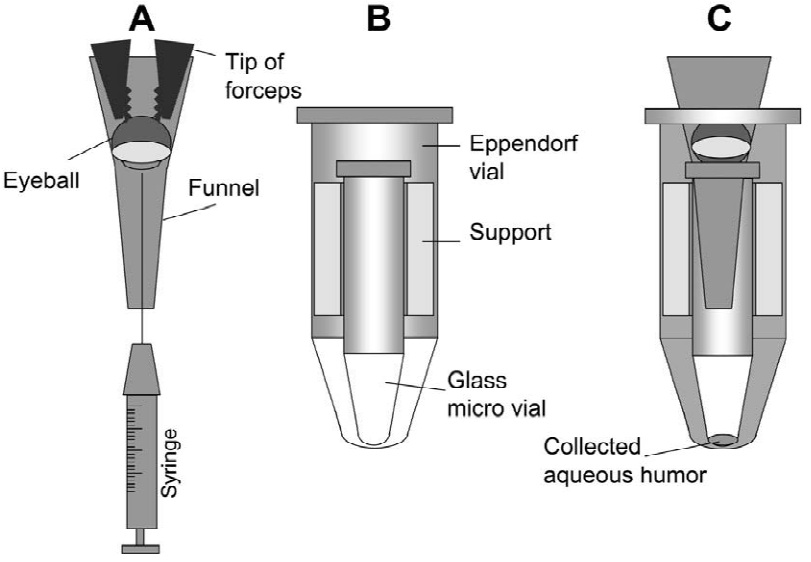

Fig. 2. Schematic representation of the micro-apparatus used for sampling of aqueous humor from mouse eyes. (A) Assembly used for fixing of the eyeball in the funnel and puncture with a syringe. (B) Empty eppendorf vial with inserted glass micro vial and support. (C) Funnel with punctured eye ball inserted into B. After centrifugation, aqueous humor from the eyeball is transferred to the bottom of the micro vial.

statted column compartment, capillary pump equipped with an electronic flow control (EFC), diode-array detector with 500-nl flow cell, PC hardware and dedicated software (Chemstation rev. 8.04) for instrument control and data analysis. All tubing was of fused-silica with a PEEK protection layer, with an internal diameter of 75 μm.

As analytical column, an YMC C30 column (0.32×200 mm) with 3-μm particles was used. The mobile phase consisted of water–methanol (2:98, v/v), and was delivered at a flow-rate of 10 μl/min. The injection volume was 2 μl, and visible detection was performed at 453 nm.

3. Results and discussion

3.1. Chromatography

Separation of certain classes of constituent isomers, as lutein and zeaxanthin (Fig. 1), can represent a significant challenge. The use of C_{30} columns is more effective for the separation of constituent isomers than C_{18} materials [14]. Although separation of lutein and zeaxanthin will be possible with a C_{18} column, the C_{18} columns will not provide enough retention to fully resolve the constituent isomers lutein and zeaxanthin at the conditions described, due to the reduced thickness of the resin in C_{18} phases, as compared to C_{30} phases [15]. The use of a C_{30} material also provides increased sensitivity to the method, due to its superior loading capacity. Thus, a C_{30} column was selected for separation of lutein and zeaxanthin, and the compounds were fully resolved with the use of 2% water in methanol as mobile phase. Separation were performed at 40 °C, and completed within 8 min.

3.2. Choice of precipitation agent and internal standard

The main criteria for the selection of an internal standard is (1) that the compound is not present in the biological samples of interest; (2) that the compound has similar behavior as the analytes during sample work up, chromatography and detection; and (3) the internal standard will not interfere with the analytes during chromatography. Emphasis is put into finding a class- or structure-like compound that is not present in the samples of

Table 1
Limit of quantification and detection for lutein and zeaxanthin

	cLOD (nmol/l)	mLOD (pg)	cLOQ (nmol/l)	mLOQ (pg)
Lutein	13	15	38	43
Zeaxanthin	21	24	63	72

interest. Few carotenoids are available commercially, and astaxanthin was selected as an internal standard because this carotenoid is easily commercially available and not known to be present in the aqueous humor [16].

Furthermore, astaxanthin does not show any interference with the endogenous compounds of interest in aqueous humor and it is separated from lutein and zeaxanthin under the chromatographic conditions described above.

3.3. Method validation

Validation of the method for the determination of lutein and zeaxanthin in aqueous humor was performed using spiked solutions of 1% albumin in PBS. The albumin solutions were prepared in the same concentrations as the standard solutions. To determine the highest possible injection volume, a linear relationship between the peak width at half the peak height were established by the injection of increasing volumes of standard solutions in 1% albumin in PBS. At volumes above 2 μl, the relationship deviated from linearity.

The within-day parameters were established by injection of four sets of spiked samples and standards at three concentration levels within 1 day. The between-day parameters were established by injection of four sets of samples and standards at three concentration levels within 2 weeks, though not on the same day. The same analyst performed all analyses.

The mass limit of detection (mLOD) and mass limit of quantification (mLOQ) were determined by using a signal-to-noise ratio of 3:1 and 10:1, respectively, expecting 100% recoveries of all analytes. The mLOD and mLOQ are given in Table 1, along with the concentration limit of detection (cLOD) and the concentration limit of quantification (cLOQ).

The capillary HPLC method was linear in the investigated concentration range, 70–135 nmol/l, with coefficients of correlation for the method calibration curves, $R^2 \geq 0.998$ and 0.996 for lutein and zeaxanthin, respectively.

The within-assay and between-assay recoveries and precision data are shown in Table 2.

The method validation data demonstrate that the use of packed capillary HPLC provides a robust and sensitive determination of lutein and zeaxanthin in aqueous humor from individual mouse eyes. The method can be converted to other biological samples, as plasma or tissue homogenates. The small sample volume required for analysis allows determination of lutein and zeaxanthin when the sample volume is limited, i.e. plasma samples from whole blood drawn from the tail vein of small mice, or homogenates of adipose tissue from rat or mice. This possibility

Table 2
Within day and between day values for recoveries and precision for lutein and zeaxanthin

	Concentration level (nmol/l)	Within day recovery (%)	Between day recovery (%)	Within day precision (RSD, %)	Between day precision (RSD, %)
Lutein	70	96.6	91.2	13	16
	175	102.9	95.6	6	5
	350	98.5	92.7	2	3
Zeaxanthin	70	89.9	90.4	12	15
	174	95.3	95.2	5	6
	347	99.7	92.7	3	4

allows us to investigate the effects of interventions or treatments on animals, without sacrifice.

Capillary HPLC increases the sensitivity as compared to traditional HPLC techniques, and it is possible to determine low concentration levels in limited sample volumes, as for the xanthophylls in the aqueous humor from single mouse eyes. However, it is our experience that the use of capillary HPLC is extremely challenging in routine analysis of biological samples, due to the increased risk of clogging the capillary columns, caused by the accumulation of proteins and other sample constituents. For the analysis of multiple samples it is recommended to use columns with a larger internal diameter.

3.4. Identification and quantification

Identification of lutein and zeaxanthin was based upon co-elution with pure authentic standards. Quantification of lutein and zeaxanthin was carried out by comparing the area between the analytes and the internal standard in samples. The area ratio was compared to the three-point calibration curve, which was generated on a daily basis.

3.5. Application

The presented method for the determination of lutein and zeaxanthin was successfully applied for the quantification of lutein and zeaxanthin in aqueous humor from three single mouse eyes; results are presented in Table 3.

A chromatogram of the determination of lutein and zeaxanthin in one single mouse eye is shown in Fig. 3.

The presented results show that the only detected carotenoids in the aqueous humor in mice are lutein and zeaxanthin. This is in agreement with the results presented by Yeum et al. [16], which demonstrated that the only identified carotenoids in the human lens are lutein and zeaxanthin. Yeum et al. [16] also determined the ratio between lutein and zeaxanthin in healthy, human lenses, and stated that this ratio is between 1.6 and 2.2. The results presented in this paper show that the ratio of lutein to zeaxanthin is higher in the aqueous humor of mice, ranging from

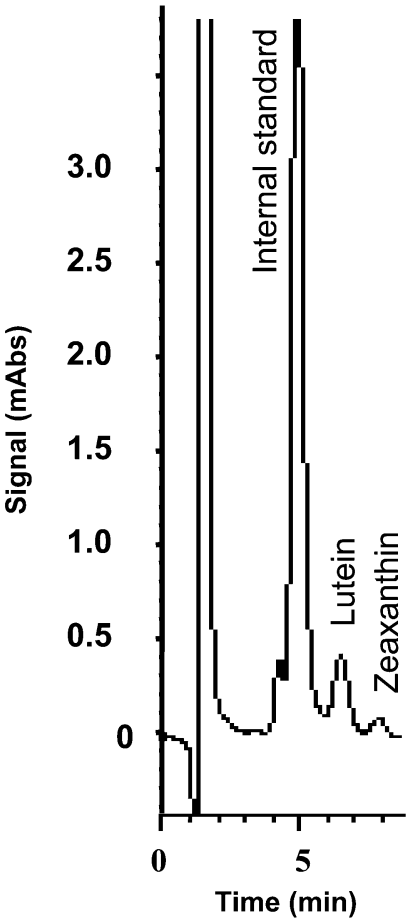

Fig. 3. Chromatographic determination of lutein and zeaxanthin in one single mouse eye. The column was an YMC C_{30}, 0.32×200 mm and the mobile phase consisted of water/methanol (2:98, v/v). The separation was performed at 40 °C. The injection volume was 2 μl and Vis-detection was performed at 453 nm.

Table 3
Lutein and zeaxanthin concentrations in single mouse eyes

	Lutein (μmol/l)	Zeaxanthin (μmol/l)	Ratio lutein/zeaxanthin
Mouse eye 1	0.088	0.029	3.03
Mouse eye 2	0.122	0.043	2.84
Mouse eye 3	0.130	0.031	4.19

2.8 to 4.2. It is also evident from the set of limited data presented here that both absolute carotenoid levels and the ratio varies to a great extent from animal to animal. It has not been possible to establish on an individual basis in mice due to the need to pool samples in traditional HPLC techniques.

Sampling and handling of minute biological samples containing degradable nutrients is an extremely challenging task. Emphasis should be placed on the sampling technique to prevent and avoid degradation of the light and oxygen sensitive nutrients during sampling and further handling. The novel centrifuge based micro sampling technique described in this paper allows rapid and reproducible extraction of a few microliters of aqueous humor from single mouse eyes, with following separation and sensitive detection. The method presented above allows the intriguing possibility of the simultaneous determination of a wide range of nutrients of low concentrations in samples of limited volumes.

4. Nomenclature

BSA	bovine serum albumin
WHO	World Health Organization
ROS	Reactive oxygen species
HPLC	high-performance liquid chromatography
UV–Vis	ultra-violet–visible
FLD	Fluorescence detection
PBS	phosphate buffered saline
PEEK	polyetheretherketone
BHT	butylated hydroxytoluene
LC	liquid chromatography
EFC	electronic flow controller
mLOD	mass limit of detection
mLOQ	mass limit of quantification
cLOD	concentration limit of detection
cLOQ	concentration limit of quantification

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