9. PŘÍLOHY

9.1. PŘÍLOHA I

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New approach for clinical monitoring of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 by Ultra High Performance Liquid Chromatography tandem Mass Spectrometry based on the Standard Reference Material 972

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Research Article

New approach for the clinical monitoring of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 by ultra high performance liquid chromatography with MS/MS based on the standard reference material 972

Biomarkers, 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2, are important indicators of the vitamin D general status and are monitored in several pathophysiological disorders, such as osteoporosis, diabetes, heart disease, etc. A novel ultra-HPLC with MS/MS methodology for the analysis of 25-hydroxyvitamin D derivatives coupled with a very simple and highly rapid sample preparation step was developed. Analytical parameters obtained showed linearity (R^2) above 0.999 for both vitamins with accuracies between 95.8 and 102%. The LODs were as low as 0.22 and 0.67 nmol/L for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2, respectively. Intra-assay precision (%RSD) was lower than 4.5%, and inter-assay precision (%RSD) was lower than 6.5%. The feasibility of the developed methodology to be applied in clinical routine analysis has been proved by its application in blood samples from nonagenarian patients, patients with familial hypercholesterolemia and patients suffering from age-related macular degeneration.

Keywords: Clinical research / Mass spectrometry / Standard reference material 972 / Vitamin D
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1 Introduction

Vitamin D is a hormone produced from sterols in the body by the photolytic reaction of UV light on the skin. However, there are some people who obtain this vitamin in the nutrients from their diets, such as those who live in the northern latitudes or spend most of their days indoors [1]. There are two different forms of vitamin D: ergocalciferol (D_2) and cholecalciferol (D_3). Ergocalciferol is derived from a plant and cholecaliferol is mainly endogenous [1, 2]. The liver, kidneys and other tissues activate this molecule into 25-hydroxyvitamin D (25-OH D) or calcidiol, the main biologically active hormonal form 1,25-dihydroxyvitamin D or calcitriol, which, finally, through the bloodstream reaches target tissues where it binds to its receptors [2].

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Abbreviations: ACN, acetonitrile; **FA**, formic acid; **MRM**, multiple reaction monitoring; **25-OH D**, 25-hydroxyvitamin D; **25-OH D_2**, 25-hydroxyvitamin D_2; **25-OH D_3**, 25-hydroxyvitamin D_3; **SRM®**, standard reference material®

The clinical investigation of serum vitamin D levels has undergone phenomenal growth over the past several years [3], and its measurement may be used diagnostically. Blood levels of 25-hydroxyvitamin D_3 (25-OH D_3) and 25-hydroxyvitamin D_2 (25-OH D_2) represent the best indicator of the general status of vitamin D, but the metabolite 1,25-dihydroxyvitamin D has no predicative information. A deficiency of vitamin D results in rickets or osteomalacia. There has been a considerable research interest in examining the association between low serum 25-OH D levels and other pathophysiological disorders, including heart disease, hypertension, diabetes, various cancers and autoimmune disorders [3–6].

In clinical studies, 25-OH D was measured in the serum [7–9], plasma [10, 11], breast milk [12], saliva [13] and cerebrospinal fluid [14]. Methods for the analysis of this compound in human serum by LC–MS have been presented by several studies [7–9, 15, 16].

Early clinical studies were based on available assays, and reference levels were often established based on findings using these methods. During LC–MS determination, reformulations of reagents and different antibody affinities for 25-OH D_2 versus 25-OH D_3 can occur to introduce some bias and require occasional recalibrations of immunochemical methods

Colour online: See the article online to view the Fig. 1 in colour.

[3]. Immunoassays usually cannot distinguish between 25-OH D_2 and 25-OH D_3, and are susceptible to matrix effects due to the lipophilic nature of the molecules [3, 17].

The first step of sample preparation of human serum for the determination of 25-OH D is protein precipitation, usually followed by SPE [7–9,18], liquid–liquid extraction [9,13,19–21] or turbo-flow [22,23]. In almost all presented studies, sample preparation is quite complicated and time consuming. SPE and liquid–liquid extraction require several extraction steps, which are not appropriate for routine analysis [24]. Other studies need automatisation, which is not available for all laboratories, such online SPE or Cohesive TX4 systems, which provide the determination of 40 samples per hour [3,25].

The analytical part of the determination of this compound is very often provided by MS detection in the positive mode. LC–MS methods report high sensitivity for 25-OH D, but due to lipophilic and few chargeable groups, there are ionisation problems [3]. Chromatographic conditions and columns varied, but very often ammonium acetate, formic acid (FA) or a combination of these was used to improve ionisation in the positive mode. The vast majority of presented studies use fragment monitoring in order to avoid matrix interferences. $[M+H]^+$ was formed from 25-OH D after positive ionisation (commonly ESI or atmospheric-pressure chemical ionisation). The precursor-monitored ions were 413 (fragments 395, 355) and 395 (fragment 377) for 25-OH D_2; 401 (159, 383, 365) and 383 (365, 159) for 25-OH D_3 [3].

In this study, the development, validation and clinical application of a new method for the fast determination of 25-OH D by ultra high performance liquid chromatography (UHPLC) coupled with fast MS/MS with simple and rapid sample preparation are presented. The new idea of this method is in the utilisation of standard reference material (SRM®) 972 as the calibration material containing serum matrix, which appears as a great advantage compared to other LC–MS methods. The method is very suitable for analysis of large sample sequences in clinical research and also in biochemical laboratories in routine practice.

2 Materials and methods

2.1 Chemicals

LC–MS water, acetonitrile and methanol were obtained from Sigma–Aldrich (Prague, Czech Republic). LC–MS FA was from Merck KgGa (Darmstadt, Germany). Zinc sulfate monohydrate as the precipitation reagent and standards of vitamin 25-OH D_2 and 25-OH D_3 were supplied by Sigma–Aldrich. Serum controls and calibrators with certificate of Standard Reference Material® (SRM®) 972 from the National Institute of Standards and Technology (NIST, Gaithersburg, USA) were obtained from Chromsystems (Munich, Germany).

2.2 Instrumentation

The analyses were performed using an UHPLC system Nexera (Shimadzu, Kyoto, Japan) composed of a degasser DGU-20A3, two pumps LC30-AD, autosampler SIL/30 AC, rack changer II (autosampler for microtiter plates), column oven CTO-20 AC, communication bus module CBM-20A and LCMS-8030 triple-quadrupole mass spectrometer detector (Shimadzu) coupled with nitrogen generator NM32LA by Peak Scientific (Frankfurt, Germany) and vacuum pump E2M28 by Edwards (Crawley, UK).

The sample preparation technique was developed using microtiter plates with filters AcroPrep 96 filter Plate 0.2 μm/350 μL Pall Corporation (Ann Arbor, USA), vacuum manifold Pall Corporation, vacuum pump VAC Space-50 Chromservis (Prague, Czech Republic), centrifuge 5810R and microcentrifuge MiniSpin plus, both from Eppendorf (Hamburg, Germany).

2.3 Preparation of standard and other solutions

Standard stock solutions of 25-OH D_3 and 25-OH D_2 were prepared by dissolving in methanol at a concentration of 242.50 and 249.79 μmol/L, respectively. Stock solutions were stored at −27°C and diluted to working solutions in methanol at concentrations of 0.1–10 μmol/L. Monohydrate zinc sulfate (4% w/v) as the precipitation reagent was prepared freshly in distilled water before the sample preparation.

Lyophilised serum controls and calibrators, based on human serum, were reconstituted in 2 and 1 mL of LC–MS water, respectively, homogenised for 15 min and stored at −27°C for up to three months.

2.4 Sample preparation

A total of 50 μL of 4% monohydrate zinc sulfate as the precipitation reagent and 400 μL of methanol were added to 200 μL of human serum in an Eppendorf tube. After 20 s of vortexing, the sample was incubated for 10 min at 4°C. The sample was then centrifuged (14 000× g, 5 min, 21°C) and 300 μL of supernatant was filtered using 0.2 μm microtiter plate filters and a vacuum manifold. The filtered solution was injected into the UHPLC system.

2.5 Chromatographic conditions

Analysis was performed by using an UHPLC Nexera set (Shimadzu) coupled with an LCMS-8030 triple-quadrupole mass spectrometer operating in positive ESI mode. Chromatographic separation was achieved on a Kinetex C_{18} analytical column (1.7 μm, 3 × 100 mm) connected to a security guard ultra cartridge C_{18}, both purchased from Phenomenex (Aschaffenburg, Germany). The column oven CTO-20 AC was used to set the temperature of the analytical column at 50°C. Acetonitrile and water, both with the addition of FA (c = 0.01 mol/L) were used as the mobile phase at a flow rate of 0.5 mL/min. The separation of metabolites 25-OH D_2 and 25-OH D_3 was realised in 6 min and under isocratic

Table 1. Optimised MRM parameters of 25-OH D_3 and 25-OH D_2

Precursor	Product	Dwell time (ms)	Pause time (ms)	Q1 (V)	CE (V)	Q3 (V)
25-OH D_3						
401.4	383.2	100	3	−16	−10	−30
401.4	107.1	100	3	−16	−25	−23
383.2	365.2	100	3	−20	−15	−27
383.2	105.0	100	3	−20	−50	−22
383.2	69.1	100	3	−20	−35	−16
25-OH D_2						
413.2	395.2	100	3	−16	−10	−29
413.2	83.1	100	3	−16	−25	−16
395.2	69.1	100	3	−30	−40	−28
395.2	119.0	100	3	−30	−25	−12
395.2	55.0	100	3	−30	−45	−25

conditions 72:28 v/v of acetonitrile/water. After analyte separation, the switching valve was activated and the pre-column and the analytical column were washed and equilibrated for 3 min into the waste by increasing the flow rate to 0.8 mL/min with 90:10 v/v of acetonitrile/water. The sample injection volume was 20 μL. The total analysis time was 9 min. The multiple-reaction monitoring (MRM) transitions used in MS detection are presented in Table 1 and spectra of both analytes are shown in Supporting Information Fig. S1. System operation, data acquisition and data processing were controlled using LabSolutions 5.41 SP1 software.

MS conditions were investigated using automated optimisation of software LabSolution as the first step of the analytical method development. Voltages of Q1, Q3, collision cell and five of the most intensive MRM transitions for identification and quantification of 25-OH D_3 and 25-OH D_2 were found. All data are presented in this section (Table 1). Other optimised conditions of ion source were as following: interface ESI positive polarity; interface temperature 350°C; desolvation line (DL) temperature 250°C; nebulising gas flow 3 mL/min; heat block temperature 400°C and drying gas flow 15 L/min.

3 Results and discussion

3.1 Optimisation of LC conditions

Several types of analytical columns, e.g. Acquity HSS T3 (1.8 μm, 2.1 × 50 mm), Ascentis Express amide (2.7 μm, 3 × 75 mm) and Kinetex C_{18} (1.7 μm, 3 × 100 mm), were tested for the separation of 25-OH D_2 and 25-OH D_3. The chromatographic conditions, such as column temperature of 25°C, flow rate of 0.5 mL/min, injection volume of 2 μL and gradient elution were elected for comparison of the columns. The gradient was set from time $t = 0$ min with the ratio of mobile phase 50:50 v/v of acetonitrile/water to time $t =$ 10 min with 90:10 v/v of acetonitrile/water. The fastest separation was provided by the Kinetex C_{18} column in 5.9 min, and separations of 6.5 and 8.5 min were achieved with Acquity HSS T3 and Ascentis Express amide columns, respectively. The analytical column Kinetex C_{18} was chosen based on the evaluation of the reproducibility and rapid separation of analytes in conjunction with the low solvent consumption during analysis. Furthermore, the gradient elution was substituted for isocratic elution with a mobile phase of 70:30 v/v of acetonitrile/water with the addition of FA and a flow rate of 0.3 mL/min. The final concentration of FA was $c = 0.01$ mol/L in both solvents. The method was successfully applied to the analysis of real samples, such as human serum, and the ratio of acetonitrile in water was increased to 72:28 v/v. The column temperature was increased to 50°C and the flow rate from 0.3 to 0.5 mL/min to shorten the analysis time. Any further changes of the above mentioned parameters caused a loss of the intensity of both analytes (see Section 3.3.4).

3.2 Optimisation of the sample preparation procedure

The development of the sample preparation procedure was based on the sample precipitation, centrifugation and filtration steps. Two steps were performed in one glass tube under centrifugation conditions (10 min, 3220× *g*). The glass tube was replaced with a disposable Eppendorf tube due to their use in a routine clinical laboratory, reduction of volumes, single use and faster centrifugation in a MiniSpin (5 min, 14 000× *g*).

Acetonitrile, *n*-hexane, ethanol, methanol and zinc sulfate monohydrate in various ratios were tested as precipitation reagents. Only the combination of methanol and zinc sulfate monohydrate emerged as suitable for the precipitation of serum. The volumes of the precipitation mixture components were optimised for the total available volume of the Eppendorf tube. The final volume of the mixture was 650 μL in a 1.5 mL Eppendorf tube. The incubation time was investigated as well, but times over 10 min did not affect the recovery. Microtiter plate filters with a pore size of 0.2 μm were used for the filtration step due to the traceability to the rack changer of the UHPLC system.

3.3 Method validation

The method validation was performed according to established guidelines [26, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf].

SRM® 972 was intended for the validation of new analytic methods and for use as an accuracy control in the critical evaluation of methods for determining the amount of substance concentration of vitamin D metabolites in human serum. This SRM® was also

Table 2. Linearity, LOD and LOQ, precision and accuracy of 25-OH D_3 and 25-OH D_2

Compound	25-OH D_3		25-OH D_2	
Regression equation	$y = 9038.92x + 35400.30$		$y = 3237.65x + 24896.50$	
Determination coefficient R^2	0.9998		0.9999	
LOD (nmol/L)/(ng/mL)[a]	0.22/0.09		0.67/0.29	
LOQ (nmol/L)/(ng/mL)[a]	0.73/0.28		2.24/0.92	
Intra-assay precision[b]	%RSD[d]	c (nmol/L)	%RSD[d]	c (nmol/L)
Level 1	3.1	82.9	4.5	15.9
Level 2	3.5	96.2	2.8	17.7
Level 3	2.2	34.6	2.9	51.5
Inter-assay precision[c]				
Level 1	4.4	82.3	4.4	32.6
Level 2	4.3	91.9	5.7	51.3
Level 3	6.5	121.6	6.5	106.6
Intra-assay accuracy	c (nmol/L)[g]	R (%)	c (nmol/L)[g]	R (%)
Level 1[e]	40.8	99.8	40.6	102
Level 2[f]	91.8	95.8	85.6	97.6

a) LOD and LOQ ($n = 5$).
b) Intra-assay precision ($n = 6$).
c) Inter-assay precision ($n = 3$).
d) %RSD of peaks area of 25-OH D_3/D_2 extracted from human serum samples.
e) Chromsystems serum control (LOT 0312) with range: 40.9 ± 8.2 nmol/L for 25-OH D_3 and 39.8 ± 8 nmol/L for 25-OH D_2.
f) Chromsystems serum control (LOT 0312) with range: 95.8 ± 19.2 nmol/L for 25-OH D_3 and 87.7 ± 17.6 nmol/L for 25-OH D_2.
g) Accuracy of Chromsystems serum control (each level extracted and analysed in triplet, $n = 3$).
R: recovery.

used as a quality assurance tool for assigning values to in-house control materials for these constituents [27, https://www-s.nist.gov/srmors/certificates/972.pdf]. The reference values are based on LC–MS/MS measurements and are certificated by NIST. By using the SRM, this method overcame problems with non-standardised materials and matrix effects because the calibration curves (Section 3.3.1.) were established with lyophilised calibrators containing the serum matrix. This is the biggest advantage of our method in comparison with other LC–MS methods.

3.3.1 Linearity

For both compounds, 3PLUS1 Chromsystem calibrators were used for 25-OH D_3/D_2 analysis. Level 1 of SRM® 972 was prepared from 'normal' human serum and was not altered. Level 2 was prepared by diluting level 1 with horse serum to achieve a lower 25-OH D concentration. Level 3 contains 'normal' human serum that has been fortified with 25-OH D_2. Level 4 contains 'normal' human serum [https://www-s.nist.gov/srmors/certificates/972.pdf]. The calibration curves were determined using four and three concentration levels for 25-OH D_3 and 25-OH D_2, respectively. Each point of measurement was from three individual sample extractions. The determination factor of both vitamins was higher than 0.999 (Table 2) with RSDs of 10.66 and 7.35% for 25-OH D_3 and 25-OH D_2, respectively. The linearity range of the calibration curves was in the range from the lower LOQ of 9.9 nmol/L to the upper LOQ of 174 nmol/L for 25-OH D_3 and from lower LOQ 37.5 nmol/L to upper LOQ 146 nmol/L for 25-OH D_2.

3.3.2 LOD and LOQ

The LOD was defined as the compound concentration that produced S/N = 3. The LOQ was evaluated as the concentration equal to ten times the S/N value in Table 2.

Recently, Adamec et al. and Higashi et al. published LC–APPI-MS/MS (APPI, atmospheric pressure photoionisation) and LC–ESI-MS/MS methods for the determination of vitamin D metabolites with an LOD of 1.5 ng/mL and an LOQ of 1–3 ng/mL, respectively [28, 29]. Our developed method showed multiple improvements to the sensitivity in comparison with the published methods.

3.3.3 Precision and accuracy

The precision was expressed as the RSD of the peak area and was demonstrated for three levels of non-spiked serum samples. Intra-assay (within-run) precision was evaluated using

six separately extracted samples from one source. Inter-assay (between-run) precision was evaluated over four days using three separately extracted samples from one source. Intra-day and inter-day precision did not exceed 6.5% (Table 2).

Accuracy control was measured by MassCheck® 25-OH D_3/D_2 (bi-level 1 + 2), and each level was extracted three times (Table 2). Analyte values of this control are traceable to certified substances and SRMs. The human-based serum controls were developed to monitor the accuracy and precision of the analytical procedures and can be used routinely. Serum control was provided with mean values and acceptable ranges that were determined from a representative sampling for this control lot. The recovery was in the range from 95.8 to 102% for both vitamins and did not exceed the limit of 100 ± 15%.

3.3.4 Ruggedness

The organic portion of the mobile phase (70–74% of acetonitrile in water), column temperature (48–52°C) and flow rate (0.3–0.7 mL/min) were tested for ruggedness. The concentration of added FA in water was tested in the range from 0.0086 to 0.0113 mol/L (Supporting Information Table S1).

The column temperature and the addition of FA did not influence the retention time more than ±6%. As supposed, the retention time was influenced by the flow rate and the percentage of ACN in the mobile phase. The retention time was changed by 160 and 73% for flow rates of 0.3 and 0.7 mL/min, respectively. The retention time changed from 116 to 88% for concentrations of ACN of 70 and 74%, respectively.

In all cases, the peak area was influenced considerably. However, the addition of FA to the mobile phase is crucial in chromatographic analysis in consequence of ionisation. Every change in the concentration of FA caused a decrease of the peak areas of the measured compounds in comparison with the optimal conditions of 0.01 mol/L. The column temperature changes had the same effect on the peak area. The increase of the flow rate and the percentage of ACN in the mobile phase led to a reduction of the peak area. On the contrary, a decrease of both parameters led to an increase of the peak area with unsuitable prolongation of the total analysis time.

3.3.5 Selectivity

Selectivity was defined as the ability of the method to measure and differentiate the analytes of interest from endogenous components in the sample. The selectivity was proved using six individual sources of human serum and serum controls. The RSD of the retention times was evaluated and did not exceed the limit of 2% for both vitamins. The relative intensity ratios of the MRM transitions 401.4→383.2, 383.2→365.2, and 413.2→83.1, 413.2→395.2 were evaluated for 25-OH D_3 and 25-OH D_2, respectively, and did not exceed the limit of 15%. The selectivity of the method is presented in Table 3.

Table 3. Selectivity of 25-OH D_3 and 25-OH D_2

	25-OH D_3		25-OH D_2	
	t_R (%RSD)[a]	MRM ratio (%RSD)[b]	t_R (%RSD)[c]	MRM ratio (%RSD)[d]
Serum sample	4.1 (0.2)	3.0 (5.8)	4.8 (0.2)	1.0 (5.5)
Serum control	4.0 (0.1)	3.1 (6.4)	4.7 (0.1)	0.9 (2.6)

a) %RSD of retention time of 25-OH vitamin D_3 extracted from human serum samples ($n = 6$).
b) Relative intensity ratio of MRM transitions of 25-OH D_3: 401.4 > 383.2/383.2 > 365.2 ($n = 6$).
c) %RSD of retention time of 25-OH vitamin D_2 extracted from serum controls ($n = 6$).
d) Relative intensity ratio of MRM transitions of 25-OH D_2: 413.2 > 83.1/413.2 > 395.2 ($n = 6$).
t_R: Retention time (min).

3.4 Application of the method

Because the monitoring of 25-OH D_3 and 25-OH D_2 is important, the newly developed UHPLC–MS/MS method was used for the determination of these compounds in three different patient groups from the University Hospital Hradec Králové. Group A, with 35 elderly patients, was divided into three subgroups A1 (patients over 90 years old, self-sufficient, without serious diseases, controlled at the department of the third Internal Gerontometabolic Clinic), A2 (patients of the same department as group A1 but approximately ten years younger, the control group) and A3 (patients over 90 years old, hospitalised in the hospice in Hradec Králové due to immobility (such as after a fracture of the femoral neck), mental health patients without dementia, controlled by the Cricht test). Group B included 26 patients with familial hypercholesterolemia treated by low-density lipoprotein apheresis, and group C included 18 patients with age-related macular degeneration treated by hemorheopheresis. The study protocol was approved by the Ethical Committee of the University Hospital at Hradec Králové. Informed consent was obtained from all participants. The research followed the tenets of the Helsinki Declaration.

Analyses of 25-OH D_3 and 25-OH D_2 were done before and after treatment by low-density lipoprotein apheresis and hemorheopheresis. The significance of the differences before and after extracorporeal therapy was studied. The results were statistically evaluated using software NCSS 2004 (Number Cruncher Statistical Systems, Kaysville, USA). The significance of the differences between groups A1–A2, A2–A3 and A1–A3 was examined by the Mann–Whitney U test. In the case of groups B and C, the significance of the differences was studied before and after extracorporeal therapy by the Wilcoxon Signed-Rank Test. The decision about statistical significance was based on $p \leq 0.05$. The chromatogram of human serum analysis using the newly developed UHPLC–MS/MS method is shown in Fig. 1.

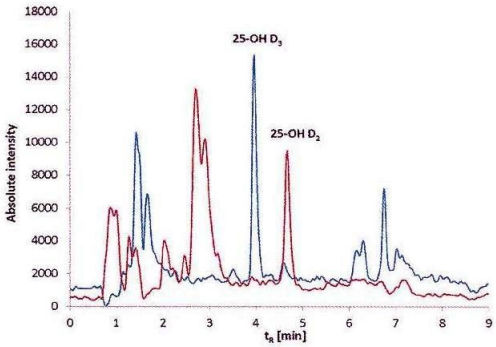

Figure 1. Chromatogram of patient's serum 25-OH D_3 (c = 19.9 nmol/L, t_R = 3.96 min) and 25-OH D_2 (c = 33.5 nmol/L, t_R = 4.67 min).

Table 4. Statistical evaluation of 25-OH D_3 levels of patients from group A, B, C [nmol/L]

Groups	n[f]	Mean	Median	SD	Statistical significance p
A_1[a]	11	12.57	10.94	11.41	1 vs. 2 = 0.353516 (n.s.)
A_2[b]	13	16.91	16.53	13.42	2 vs. 3 = 0.045159 (s.)
A_3[c]	11	10.53	1.73	22.85	1 vs. 3 = 0.146263 (n.s.)

		Before procedure		After procedure		
		Mean	SD	Mean	SD	
B[d]	26	53.34	29.33	46.56	29.57	0.002963 (s.)
C[e]	18	26.11	13.13	23.07	11.03	0.001715 (s.)

a) Patients over 90 years old, self-sufficient, without serious diseases, controlled at the department of the third Internal Gerontometabolic Clinic.
b) Patients of the same department as group 1 but about 10 years younger, the control group.
c) Patients over 90 years old, hospitalized in the hospice in Hradec Králové due to immobility (such as after a fracture of femoral neck), mental health patients without dementia, controlled by Cricht test.
d) Patients with familiar hypercholesterolemia analyzed before and after LDL apheresis.
e) Patients with age-related macular degeneration analyzed before and after hemorheopheresis.
f) Number of patients.

Analyses of 25-OH D_3 and 25-OH D_2 in the serum of elderly patients and patients suffering from familial hypercholesterolemia and age-related macular degeneration treated by extracorporeal elimination brought new pathophysiological knowledge. There was a significant difference of 25-OH D_3 levels between groups A2 and A3 (p = 0.045159), whereas the differences between groups A1–A2 and A1–A3 were not statistically significant ($p > 0.05$). Significant differences were found in the case of groups B (p = 0.002963) and C (p = 0.001715), and it follows that the extracorporeal elimination method can be considered a safe procedure in relation to the serum levels of vitamin D (Supporting Information Fig. S2). All data are summarised in Table 4. 25-OH D_2 was present only in 6.3% of patients and, therefore, was not statistically evaluated. The results of 25-OH D_3 and 25-OH D_2 were in the target range (75–150 nmol/L) in 12.7% of patients, mildly deficient (50–74 nmol/L) in 6.3% of patients, deficient (25–49 nmol/L) in 27.8% of patients and insufficient (<25 nmol/L) in 53.2% of patients [http://www.ceva-edu.cz/mod/data/view.php?d=13&rid=178].

4 Concluding remarks

In the present study, a new, simple and rapid UHPLC–MS/MS method for the selective and sensitive determination of 25-OH D_3 and 25-OH D_2 in human serum for clinical monitoring was developed and validated. The method was based on SRM® 972, which was intended for use as a calibration and a control material in the evaluation of the method. By using SRM® 972, this method overcame problems with non-standardised materials.

The very fast sample preparation consisted of simple precipitation and filtration steps using microtiter plates, which allowed handling 96 samples in one operation; therefore, it is suitable for the routine analysis of a high number of samples. The newly developed method was applied for the real analysis of 25-OH D_3 and 25-OH D_2 in human serum in various groups of patients and can be used in clinical research and also in routine practice.

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9.2. PŘÍLOHA II

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Recent trends in the analysis of vitamin D and its metabolites in milk – a Review

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Review

Recent trends in the analysis of vitamin D and its metabolites in milk – A review

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ABSTRACT

Vitamin D plays an important role in calcium metabolism and affects other metabolic pathways. Despite the intense interest in vitamin D, no comprehensive overview addressing the analysis of vitamin D in milk has been published.

Historically, immunoassay techniques have been mainly used for the routine quantification of vitamin D and its metabolites. However, the greater accuracy and precision of chromatography makes it one of the most important methods in the analysis of vitamin D. The determination of vitamin D and its metabolites by LC–MS is the gold standard for its assessment. LC–MS has unique advantages for vitamin D determination and quantification due to its high sensitivity and specificity.

In this review, the current status of vitamin D and its metabolites analysis in milk, human and bovine, including sample pre-treatment and chromatography analysis, are critically discussed and summarised.

Contents

1. Introduction

Vitamin D is a hormone precursor with a steroidal structure and can be present in either of two different forms, vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol), in plants and animals, respectively. Both vitamin D_3 and vitamin D_2 are inactive in the human body and must undergo a series of metabolic transformations before effects are manifested in the target tissues (Kanis, 1982). Vitamin D_2 and its provitamin form (ergosterol) occur mainly in plants, fungi, moulds and certain invertebrates (Combs, 2008). Vitamin D_3 is synthesised from its precursor (7-dehydrocholesterol) in the skin by the action of ultraviolet light (295–300 nm) (Higashi, Shimada, & Toyo'oka, 2010).

Vitamin D is transported in the plasma bound to the specific protein transcalciferin or to vitamin D-binding protein (DBP) (Combs, 2008). DBP transports both forms of vitamin D to the liver, where they are converted by hydroxylation to 25-OH-D. This metabolite is an inactive form of vitamin D and thus undergoes

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further identical conversion. 25-OH-D is the major circulating form of the vitamin and storage form of vitamin D in the human body. Its metabolic pathway is connected with 1α-hydroxylase in the kidney tubules, although this enzyme activity has been demonstrated in the placenta, skin, lymp nodes, pancreas, colon, lung, bone and other (DeLuca, 2004; Holick, 2004; Weisman et al., 1979). The 25-OH-D form is hydroxylated by the enzyme 1α-hydroxylase to its active form, 1,25-$(OH)_2$-D, is responsible for the biological activity of vitamin D (Christakos, Ajibade, Dhawan, Fechner, & Mady, 2010). 25-OH-D can also be hydroxylated at carbon 24 by the mitochondrial enzyme 24-hydroxylase that is present in the kidney tubules, intestine, cartilage, and placenta. The product of this hydroxylation is 24,25-dihydroxyvitamin D (24,25-$(OH)_2$-D), a relatively inactive metabolite compared to 1,25$(OH)_2$D. Calcitriol (1,25$(OH)_2$-D_3) plays a central role in the metabolism of calcium and phosphorus (Christakos et al., 2010). The complete metabolic pathway of vitamin D is presented in Fig. 1.

Breast milk contains about 3 times less vitamin D than the woman's circulating concentration of vitamin D and 100 times less vitamin D than the circulating concentration of 25-OH-D (Greer, Hollis, & Napoli, 1984). Colostrum, the milk produced in the first few days after birth, is richer in protein, vitamins A, B12, and K and immunoglobulins than is mature milk (Emmett & Rogers, 1997).

Breastfed infants are at risk for vitamin D deficiency due to the poor penetration of vitamin D and 25-OH-D into milk (Haggerty, 2011; Kovacs, 2008). Vitamin D deficiency in children can lead to the soft, thin, and brittle bones, a disease known as rickets. There are increasing reports worldwide of vitamin D deficiency and rickets amongst breastfed infants who lack adequate sunlight exposure and do not receive vitamin D supplementation (Dawodu & Tsang, 2012). The human breast milk contains all the required nutrition and the World Health Organisation (WHO) recommends full breastfeeding for the first 6 months of life. Breastfed children are healthy on average and are more resistant to infections and allergies than their peers who are not breastfed (Taylor, Wagner, & Hollis, 2008). Nevertheless, the concentration of liposoluble vitamins in breast milk is very low and depends on many factors, including the diet and age of the mother, the stage of lactation, and other parameters (Kasparova et al., 2012). Standard growth milks in general contain 7–10 μg of vitamin D in 100 g of powder milk (information achieved for Czech Republic market).

The vitamin D concentration of milk is expressed as antirachitic activity (ARA), based on the biological activity values of vitamin D and 25-OH-D in human breast milk, as established in 1982 (Reeve, Jorgensen, & Deluca, 1982; Specker, Tsang, & Hollis, 1985). In general, the mean ARA of human milk in healthy lactating women ranges from 10 to 80 IU/L (Dawodu & Tsang, 2012). The American Academy of Pediatrics (AAP) recommended using 200 IU of vitamin D for the first 2 months of life in 2003 and increased this recommendation to 400 IU (10 μg/day) for all infants in 2008 (Kulie, Groff, Redmer, Hounshell, & Schrager, 2009).

In 1990, Zheng et al. (1990) demonstrated differences in the concentrations of vitamin D and its metabolites in mothers who delivered pre-term infants (preterm milk) compared with mothers who delivered full-term infants (term milk). Preterm milk showed a higher concentration of 25-OH-D than full term milk, but no difference in 1,25-$(OH)_2$-D levels was observed.

Specker et al. (1985) studied the effect of race and diet on human milk concentrations of vitamin D and 25-OH-D and reported that increased skin pigmentation was related to a decrease in the content of vitamin D and 25-OH-D in human milk.

Other dietary source of vitamin D is bovine milk, which is very often enriched with vitamin D (Kaushik, Sachdeva, Arora, Kapila, & Wadhwa, 2014a; Trenerry, Plozza, Caridi, & Murphy, 2011). However, the levels are still quite low – in the range of 4–40 IU/L (0.1–1.0 μg/l) (Trenerry et al., 2011), which means 1–10% of AAP recommendation dose per day is in 1 L of the milk. Bovine milk also contains even lower levels of 25-hydroxyvitamin D_3 as well as lesser amounts of 24,25-(OH)-D_3 and 1,25-(OH)-D_3, which together, account for about 15% of vitamin D activity (Perales, Alegria,

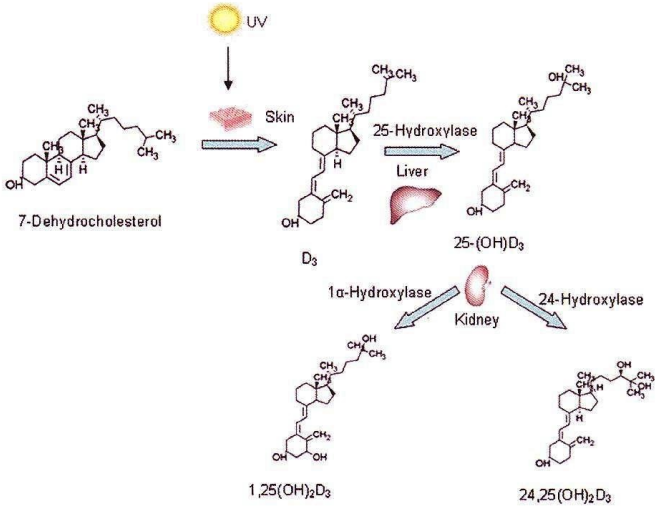


Fig. 1. Metabolism of vitamin D_3 (modified according to Higashi et al., 2010).

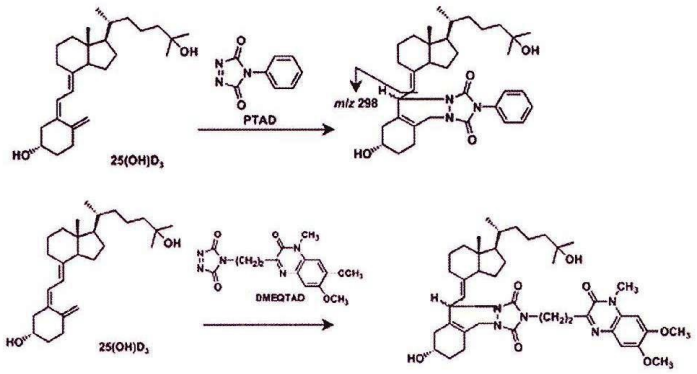


Fig. 2. Derivatisation of 25-OH-D_3 with PTAD and DMEQTAD (Higashi et al., 2010).

Barbera, & Farre, 2005). Moreover, several studies proved, that vitamin D is unstable in milk due to light, heat or oxidation (Cremin & Power, 1985; Kutsky, 1981). Contrary, some other studies proved, that vitamin D is stable for the mentioned conditions (Blanco, Fernandez, & Gutierrez, 2000; Kreutler, 1980). Kaushik, Sachdeva, and Arora (2014b) investigated milk stability in glass, plastic bottles and in polyethylene pouches. Vitamin D2 content decreased due to sorption in polyethylene pouches during storage; it decreased 5% and almost 10% after 3 days and 7 days, respectively.

Several reviews on vitamin D analysis have been published. Vitamin D determination in plasma or serum by LC–MS has been reported by Higashi (Higashi et al., 2010). The extraction of vitamin D and other fat-soluble vitamins from food, human fluids and pharmaceutical preparations was covered in a review by Musteata and Musteata (2011) and in an overview by Luque-Garcia and de Castro (2001). However, determination in milk was not addressed. Our review presents an overview of the determination of vitamin D and its metabolites in bovine and human breast milk and additionally discusses methods of sample preparation.

2. Methodology of determination of vitamin D and its metabolites

The quantification of vitamins and their metabolites in human fluids has been considered one of the most difficult goals in clinical chemistry (Luque-Garcia & de Castro, 2001). Optimisation of the sample preparation process plays an important role in enhancing sensitivity and reducing matrix interference in biological samples, such as bovine and human breast milk.

Techniques for the determination of vitamin D in milk can be categorised into immunological techniques (competitive protein binding assay – CPBA, enzyme immunoassay – EIA, radio immunoassay – RIA) and non-immunological techniques (High-Performance Liquid Chromatography – HPLC, Liquid Chromatography–Mass Spectrometry – LC–MS). Vitamin D and its metabolites have traditionally been measured by immunological techniques in low cost routine analyses. However, these methods have several problems, such as cross-reactivity due to polyreactive antibodies, the ability to analyse only one analyte at a time, the inability to achieve structural validation of the analyte, and inadequate sensitivity. Immunological techniques are not usually able to distinguish between 25-OH-D_2 and 25-OH-D_3 due to the cross-reactivity of antibodies, and it is not possible to achieve the same information that is provided by chromatographic determination.

Currently, the most popular vitamin D detection methods are based on liquid chromatography coupled with mass spectrometry or with ultraviolet detection. These methods are able to distinguish between 25-OH-D_2 and 25-OH-D_3, in contrast to common immunological techniques. Chromatographic methods provide advantages such as sensitivity, flexibility and specificity. The separation is usually performed on a reversed-phase analytical column packed with C18 particles. The best results are achieved by using isotope-labelled internal standards and MS detection (Musteata & Musteata, 2011).

2.1. Sample preparation

Vitamin D and its metabolites possess non-polar and non-ionic characteristics that enable the use of reversed phase purification and separation strategies. The chemical structure of these compounds is similar to that of steroid hormones (Musteata & Musteata, 2011) (Table 1). A sample pretreatment prior to the analytical determination of vitamins is normally required (Tadeo, Perez, Albero, Garcia-Valcarcel, & Sanchez-Brunete, 2012). The most commonly used extraction technique is saponification, liquid–liquid extraction (LLE) followed by preparative chromatographic clean-up to eliminate contaminants (Musteata & Musteata, 2011).

Internal (IS) and external standards are used in vitamin D determination, however internal standardisation is more desirable to compensate for any losses of vitamin D that may be incurred during the multistep sample preparation procedure (Perales et al., 2005). An internal standard is also needed to eliminate analytical errors due to losses of vitamin D during sample preparation and to compensate for signal variation if using MS detection. For quantification of vitamin D, an internal standard is even more essential due to reversible isomerization with the corresponding previtamin D (De Gara, Grusak, & Murgia, 2014).

Alternatively, one of the vitamins D_2 or D_3 can be used as an internal standard to quantify the other supplemental vitamin during HPLC determination. However, this is not the best approach when vitamin D_2 and vitamin D_3 occur in milk simultaneously (Nollet & Toldrá, 2013). For quantification by MS, isotopic labelled compounds are ideal internal standards, because of the complete resemble with the analyte (De Gara et al., 2014).

Table 1
Vitamin D and its metabolites with their molecular weights, pKa and log P values.

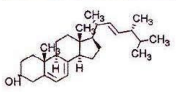
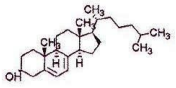
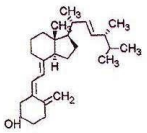
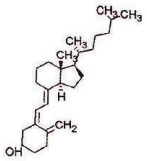
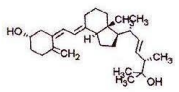
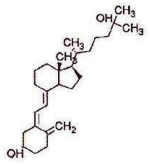
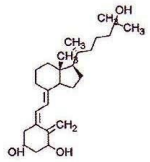
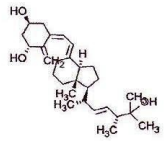
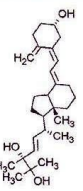
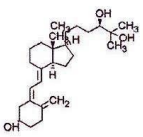
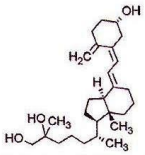
Abbreviation	Name	MW	pKa	log P	Structure	CAS number
Ergosterol	Vitamin D_2 precursor	396.6484	–	7.39		57-87-4
7-Dehydrocholesterol	Vitamin D_3 precursor	384.6377	–	8		434-16-2
Vitamin D_2	Ergocalciferol	396.6484	–	7.59		50-14-6
Vitamin D_3	Cholecalciferol	384.6377	–	7. 5		67-97-0
25-OH-D_2	25-Hydroxyvitamin D_2, Ercalcidiol	412.6478	19.0	6		21343-40-8
25-OH-D_3	25-Hydroxyvitamin D_3, Calcidiol	400.6371	18.53	6		19356-17-3
1,25-$(OH)_2D_3$	1,25-Dihydroxyvitamin D_3, Calcitriol	416.64	15.29	5.51		32222-06-3
1,25-$(OH)_2D_2$	1,25-Dihydroxyvitamin D_2, Ercalcitriol	428.6472	15.3	5.45		60133-18-8

Table 1 (*continued*)

Abbreviation	Name	MW	pKa	log P	Structure	CAS number
24,25-$(OH)_2D_2$	24,25-Dihydroxyvitamin D_2, 24,25-Dihydroxyergocalciferol	428.6472		6.319		71183-99-8
24,25-$(OH)_2D_3$	24,25-Dihydroxyvitamin D_3, 24,25-dihydroxycholecalciferol, Secalciferol	416.6365	15.3	5.47		55721-11-4
25,26-$(OH)_2D_3$	25,26-Dihydroxyvitamin D_3, 25,26-dihydroxycholecalciferol	416.6365	15.5	5.14		29261-12-9

2.1.1. Liquid–liquid extraction

LLE was one of the first tools for the extraction/purification of vitamin D and its metabolites in milk and remains a common approach (Luque-Garcia & de Castro, 2001). An overview of LLE methods is presented in the Table 2.

Saponification is commonly the first step of the LLE of vitamin D and its metabolites and is used to remove neutral lipids, especially triglycerides. Hot saponification consists of treating the sample with aqueous, methanolic or ethanolic KOH in a temperature range from 60 to 100 °C for 20–45 min. Overnight cold saponification consists of treating the sample with aqueous or ethanolic KOH at room temperature under slow constant stirring. To avoid the oxidation of vitamin D pyrogallol, ascorbic acid or butylated hydroxytoluene as antioxidants are used (Perales et al., 2005).

Thermal isomerization of vitamin D to previtamin D can occur during saponification, which usually complicates LC/MS analysis. The reversibility of the isomerization reaction is very slow, and therefore the percentage of the previtamin will remain virtually unchanged during the subsequent stages of the analytical procedure. This equilibrium allows the potential vitamin D to be calculated from measurements of the vitamin D peak alone, and the same principle applies to the hydroxylated metabolites of vitamin D (Nollet, 2013).

Blanco et al. (2000) and Gomis, Fernandez, and Alvarez (2000) used extraction with hexane and washing with two portions of methanol–water and butylated hydroxytoluene as an antioxidant to simultaneously determine vitamin D_2, D_3 ergosterol and 7-dehydrocholesterol. These extractions provided very good recoveries of 85–110%, low sample and solvent consumption and extraction of vitamin D_2, D_3 and its precursors in one procedure. LoA recovery of less than 72% was reported by methanol–dichloromethane, ethyl-acetate, and ethanol (see Table 2). Recoveries of 91–105% for vitamin D_2, D_3 and 25-OH-D_2, 25-OH-D_3 were obtained after double extraction with hexane–ethyl acetate and pyrogallol–ethanol in a study by Kamao et al. (2007). The analysis was time consuming due to the several steps required for sample preparation. A comparison of the methods is quite complicated, since some studies do not provide all the parameters, such as recovery values and LOD/LOQ. In several studies published after the year 2000, the authors employed similar methodologies as those used in previous studies in the 1980s.

Evaporation at reduced pressures in a vacuum rotatory evaporator or under a nitrogen stream is mostly used for sample preconcentration. The temperature during sample preparation is maintained below 50 °C because of the danger of degradation of vitamin D at higher temperatures (Perales et al., 2005).

In the majority of studies, internal standards were added to the milk sample prior to extraction to monitor the recoveries of the extraction and chromatography procedures (Abernethy, 2012; Ballester, Cortes, Moya, & Campello, 1987; Greer, Ho, Dodson, & Tsang, 1981; Hollis, 1983; Jakobsen & Saxholt, 2009; Kamao et al., 2007; Weisman, Bawnik, Eisenberg, & Spirer, 1982).

Takeuchi et al. (1988, 1989) applied alkali-saponification followed by benzene extraction and the addition of an ethanolic pyrogallol solution as an antioxidant for the analysis of vitamin D and 25-OH-D_2. One of the two fractions was extracted with ethyl acetate to obtain 24,25-$(OH)_2$-D and 1,25-$(OH)_2$-D and then passed through a preparative HPLC column for further clean-up. The second fraction was evaporated and injected onto an analytical HPLC column for the determination of vitamin D_2, D_3 and 25-OH-D_2, 25-OH-D_3. Both recoveries were higher than 90%. However, the proposed procedures for sample preparation were not suitable for routine analysis, due to time demands of two different extraction procedures of vitamin D, 25-OH-D and dihydroxy metabolites.

Gong and Ho (1997) used simple LLE of a milk sample to measure the fat soluble vitamins and their different forms prior to HPLC analysis. A large volume of sample (50 ml) was required for the detection of vitamins in this method.

Table 2
LLE methods used for the determination of vitamin D and its metabolites.

Analyte	Matrix, sample volume	Sample preparation	Extraction solvent	Method	Recovery (%)	References
Vitamin D_3,D_2, ergosterol,7-dehydrocholesterol	Bovine milk, 5 ml	LLE	15 ml hexane	Microcolumn LC	87–110	Gomis et al. (2000)
Vitamin D_3	Whole milk, 50 ml	LLE/TLC	50 ml hexane	HPLC	88–99	Grace and Bernhard (1984)
Vitamin D_2, vitamin D_3	Nonenriched milk, enriched milk, 20–25 g	LLE	2 × 50 ml or 100 ml diethyl ether/2 × 50 ml or 100 ml hexane	HPLC		Barba et al., 2011
Vitamin D_3	Milk, 4 ml	LLE	10 ml isooctane	UPLC/MS	103.2	Abernethy, 2012
24,25-$(OH)_2D_3$,1,25-$(OH)_2D_3$, vitamin D_3, 25-OH-D_3	Bovine milk, 100 ml	LLE, prep. chromatogr. (Sephadex LH-20/Lipidex 5000)	600 ml methanol–chloroform (1:1)	HPLC/CBA		Reeve, Chesney et al. (1982) and Reeve, Jorgensen et al. (1982)
Vitamin D_3,25-OH-D_3, vitamin D_2, 25-OH-D_2	Human breast milk, 10 ml	LLE, HPLC	2 × 30 ml hexane–ethyl acetate (9:1)	LC–MS/MS	91–105	Kamao et al. (2007)
Vitamin D, 25-OH-D, 24,25-$(OH)_2$D,1,25-$(OH)_2$D, 25,26-$(OH)_3$D	Human breast milk, bovine milk, 5 ml	LLE, prep. HPLC (Lipidex 5000)	Methanol–methylene chloride (2:1)	HPLC/CPBA	53–72	Hollis et al. (1981)
Vitamin D_2, vitamin D_3, 25-OH-D_2, 25-OH-D_3,24,25-$(OH)_2$D, 1,25-$(OH)_2$D	Human breast milk, 100 ml	LLE, prep. chromatogr. (Sephadex LH-20/Lipidex 5000)	350 ml methanol–chloroform	HPLC/CPBA		Reeve, Chesney et al. (1982) and Reeve, Jorgensen et al. (1982)
VITAMIN D_2, vitamin D_3, 25-OH-D_2, 25-OH-D_3,24,25-$(OH)_2D_2$, 1,25-$(OH)_2D_2$,24,25-$(OH)_2D_3$, 1,25-$(OH)_2D_3$	Human breast milk, bovine milk, 25 ml (vit D + 25-OH-D), 5 ml (dihydroxy Vit D)	LLE, prep. HPLC, SPE	Vit D + 25-OH-D-benzene, dihydroxy Vit D-2 × 10 ml ethyl-acetate	HPLC/CPBA/RRA	>90	Takeuchi et al. (1988, 1989)
Vitamin D, 25-OH-D, 1,25-$(OH)_2$D 25-OH-D, 1,25-$(OH)_2$D	Human breast milk, bovine milk, 30 ml Human breast milk, 10 ml	LLE, prep. HPLC, SPE LLE, SPE	Methanol–dichloromethane (2:1) Chloroform–methanol	HPLC/CPBA HPLC/CPBA/RRA	54–72	Kunz et al. (1984) Zheng et al. (1990)
25-OH-D, 24,25-$(OH)_2$D,1,25-$(OH)_2$D	Human breast milk, 25–50 ml	LLE, prep. HPLC (Sephadex LH-20)	Chloroform–methanol (2:1)	HPLC/CPBRA		Weisman et al. (1982)
25-OH-D,1,25-$(OH)_2$D	Human breast milk, 2 ml	LLE, prep. HPLC (Sephadex LH-20/Lipidex 5000)	Ethanol	HPLC/CPBA	60–65	Greer et al. (1981)
Vitamin D_3,25-OH-D_3, vitamin D_2,25-OH-D_2	Human breast milk, 5 ml	LLE, prep. HPLC	Methanol–methylene chloride (2:1)	HPLC/CPBA	>90	Hollis, 1983
Vitamin D_3, D_3	Bovine milk, 50 ml	LLE	2 × 10 ml hexane	HPLC		Gong and Ho (1997)
Vitamin D_2, D_3	Bovine milk, 10 ml	LLE/SPE	40 ml ethylacetate	HPLC	50–70	Holick et al. (1992)
Vitamin D_2, D_3	Bovine milk, 25 ml	LLE	100 ml light petroleum	PB LC–MS		Careri et al. (1995)
Vitamin D, 25-OH-D	Human breast milk, bovine milk, 5 ml	LLE/SPE	Methanol–methylene chloride	HPLC/CPBA	>90	Parviainen et al. (1984)
Vitamin D_3, vitamin D_2, ergosterol, 7-dehydrocholesterol	Bovine milk	LLE/SPE	15 ml hexane	HPLC	85–105	Blanco et al. (2000)
Vitamin D_2	Bovine + buffalo milk, 3 ml	LLE	2 ml hexane	HPLC	99.6%	Kaushik et al. (2014c)

Reeve, Chesney, and Deluca (1982) and Reeve, Jorgensen et al. (1982) reported a method for the determination of 24,25-$(OH)_2$-D and 1,25-$(OH)_2$-D, but LODs were lower than the concentrations found in real samples. Vitamin D and its metabolites were isolated and/or washed with a MeOH/chloroform mixture and the recovery was confirmed by adding labelled standards. This procedure was unable to separate vitamin D_2 from D_3 and was complicated and time consuming.

Barba, Esteve, and Frigola (2011) compared hexane and diethyl ether as extraction solvents for the determination of vitamin D_2 and vitamin D_3. The greatest yield was obtained using a double hexane extraction. Using hexane instead of diethyl ether was advantageous because diethyl ether is more inflammable and unstable than hexane. Furthermore, in this study, the addition of ascorbic acid or butylated hydroxytoluene (BHT) as antioxidants was compared. The optimal conditions were found to comprise the addition of BHT and saponification with KOH in ethanol. This method can be used in advance for the determination of vitamin D and E in different liquids, and the same extraction procedure for both vitamins in all types of samples makes it suitable for routine assessment.

A recently published study by Abernethy (2012) presented a rapid method for the analysis of vitamin D_3 in milk using Diels–Alder derivatisation. The vitamin was extracted from the sample in one step into isooctane. Deuterium-labelled vitamin D_3 was used to correct for losses in extraction. The recovery of spiked vitamin D_3 averaged approximately 103.2%. This study is one of the best liquid liquid extraction with advantages of using approximately 35 ml of solvents and only 4 ml of sample. The only critical step for accurate analysis is the exact volume of internal standard and the slow reaction of derivatising reagent with sample residue.

Some complicated methods for the determination of vitamin D and its metabolites in milk have been designed by Kunz, Niesen, Vonlilienfeldtoal, and Burmeister (1984), Zheng et al. (1990), Weisman et al. (1982), Greer et al. (1981). Additionally, all mentioned studies reported low or no recoveries.

The other suitable extraction procedure with great recovery and low sample and solvent consumption was designed by Kaushik, Sachdeva, Arora, and Wadhwa (2014c). However, both these extraction processes were proposed only for one form of the vitamin D, either vitamin D_3 or D_2.

The most appropriate methods based on LLE demonstrated recoveries near 100% with less than a 15% coefficient of variation. The above mentioned extraction methods are suitable for the analysis of vitamin D and its metabolites in milk. Despite of this fact, the main problems, such as high consumption of solvents during the multistep extraction and the long times required for sample preparation still persist.

2.1.2. Solid-phase extraction

Previously developed methods for the determination of vitamin D have most frequently employed Sep-Pak silica and C-18 as the sorbents for the milk samples (Table 1). Sep-Pak silica was favoured by several authors for the extraction of vitamin D and its derivatives (Kunz et al., 1984; Parviainen, Koskinen, Ala-Houhala, & Visakorpi, 1984; Takeuchi et al., 1988, 1989; Zheng et al., 1990) and provided recoveries of approximately 90%, unlike the results of Kunz et al. (1984), where the recoveries of vitamin D, 25-OH-D, and 1,25-$(OH)_2$D were lower, ranging from 50% to 72%. This sorbent is successfully used at present with recoveries of 60–90% (Ballester et al., 1987; Jakobsen & Saxholt, 2009; Sakurai et al., 2005; Trenerry et al., 2011). An overview of the SPE methods is shown in the Table 3.

C-18 cartridges (Delgado-Zamarreno, Sanchezperez, Gomezperez, & Hernandezmendez, 1995; Holick, Shao, Liu, & Chen, 1992; Parviainen et al., 1984) provide similarly good results for the determination of vitamin D_2 and D_3 as does the Sep-Pak silica. C-18 cartridges are used because of the strong capacity of this material to extract vitamins. However, recoveries have varied for this type of sorbent (Holick et al., 1992). The most recent recoveries reported were in the range of 74–116%, as published by Blanco et al. (2000).

Earlier, elution of vitamins from the Sep-pak silica was mainly accomplished by hexane with the addition of isopropyl alcohol, as described in several studies (Ballester et al., 1987; Sakurai et al., 2005; Takeuchi et al., 1988, 1989). Parviainen et al. (1984) divided the elution of milk samples into two steps. Samples containing vitamin D were passed through Sep-Pak C18 cartridges and eluted with chloroform. Next, further purification with Sep-Pak silica cartridges and elution was performed with a mixture of hexane-ether. The recoveries were, on average, approximately 100%. Vitamin D metabolites (25-OH-D, 1,25-$(OH)_2$-D) passed only through SPE cartridges with elution by acetonitrile achieved recoveries of 94–120%.

Additionally, recently, several authors have used more than one step to purify samples of milk using SPE. To achieve a cleaner extract, LLE was used after a silica SPE clean-up step suitable for MS detection by Trenerry et al. (2011). In contrast, Kunz et al. (1984) used LLE prior to SPE and finally used preparative chromatography to separate vitamin D from substances with similar polarities. This complicated multistep purification achieved the best sensitivity, but the recoveries after extraction and chromatography were only 54–72%.

SPE is commonly performed off-line. Only in the study of Delgado-zamarreno et al. (1995) was SPE extraction applied on-line coupled to chromatographic determination. In this way, sample treatment was speed up, and together with the HPLC determination of vitamin D_3 in the milk sample, required only 25 min. In addition, the vitamin recovery was good, in the range of 88–105%. Other studies with immunological determination used very complicated sample preparation procedures, such as preparative HPLC or SPE orthogonal purification (Parviainen et al., 1984; Takeuchi et al., 1988, 1989; Zheng et al., 1990). In general, these types of methods reached high recoveries, except from the study of Holick et al. (1992).

Blanco et al. (2000) used C18 SPE cartridge for sample preparation and also reversed phase for the HPLC determination of vitamin D and its metabolites. In both procedures methanol and water were used for the wash step, elution and as the mobile phase (Jakobsen & Saxholt, 2009). More specifically, silica amino and cyano HPLC columns were used for purification. Orthogonal purification also provided clean extract. However, the methods are complicated, time-consuming and not suitable for routine analysis (see Table 3), due to time demands, higher solvent consumption and the necessity for increased human manipulation. Delgado-Zamarreno et al. (1995) developed an automated method, which provided good validation parameters, low sample consumption and it is suitable for routine milk analysis, but only for vitamin D_3, unlike the extraction method designed by Blanco et al. (2000).

2.1.3. Direct extraction

The direct extraction of vitamin D has been used in several studies (without previous saponification) with organic solvents. Hexane is frequently used as an organic solvent. To better disrupt the fat globule membranes that encapsulate the fat soluble vitamins, ultrasonication has been used together with the addition of ethanol to the milk sample (Blanco et al., 2000; Gomis et al., 2000). Vitamin D recoveries using direct extraction have achieved similar results as has saponification (89–107%). Recoveries reported by Blanco et al. (2000) and Gomis et al. (2000) were tested by addition of standards at three different concentration levels.

Table 3
SPE methods used for the determination of vitamin D and its metabolites.

Analyte	Matrix, sample volume	Sample preparation	Clean up cartridges	Conditioning	Elution	Method	Recovery (%)	Ref.
Vitamin D_3	Bovine milk, 10 ml	SPE	Silica		6 ml hexane/ethylacetate (80:20)	LC/MS[a], LC–MS/MS	61–86	Treneery et al. (2011)
Vitamin D_3,25-OH-D_3, vitamin D_2, 25-OH-D_2	Bovine milk,1 l	SPE	Silica			HPLC	93–101	Jakobsen and Saxholt (2009)
Vitamin D_3	Human breast milk, 4 ml	SPE	Sep-pak silica		0.4% IPA in hexane	HPLC		Sakurai et al. (2005)
vitamin D	human breast milk, 2 ml	SPE	Sep-pak silica	4 ml ethylacetate/hexane (7:93)	18 ml ethylacetate/hexane (7:93)	HPLC/CPBA	90	Ballester et al. (1987)
1,25-$(OH)_2$D, 24,25$(OH)_2$D	Human breast milk, bovine milk, 25 ml	SPE	Sep-pak silica	2.5% IPA in hexane	10 ml 2.5% IPA in hexane + 10 ml 10% IPA in hexane	UV/CPBA/ RRA	>90	Takeuchi et al. (1988, 1989)
Vitamin D, 25-OH-D, 1,25-$(OH)_2$D	Human breast milk, bovine milk, 30 ml	SPE	Sep-pak silica		Hexane/ethylacetate (95:5, 75:25, 20:80)	HPLC/CPBA	54–72	Kunz et al. (1984)
25-OH-D, 1,25-$(OH)_2$D	Human breast milk, 10 ml	SPE	Sep-pak silica			HPLC/ CPBA/RRA		Zheng et al. (1990)
Vitamin D_2, D_3	Bovine milk, 10 ml	SPE	C-18	Distilled H_2O, 70% MeOH in distilled H_2O, acetonitrile	MeOH	HPLC	50–70	Holick et al. (1992)
Vitamin D_3	Bovine milk, 1 g	PFSPE	Electrospun polymer nanofibres	100 µl EtOH, 100 µl H_2O	100 µl EtOH	HPLC	>90	Chen et al. (2011)
Vitamin D, 25-OH-D	Human breast milk/ bovine milk, 5 ml	SPE	(a)C-18 Sep-Pak (b)Sep-pak silica	5 ml MeOH, 10 ml H_2O	(a) 3 ml chloroform (b) 10 ml hexane–ether (67:33)	HPLC/CPBA	81–149	Parviainen et al. (1984)
Vitamin D_3, vitamin D_2, ergosterol,7-dehydrocholesterol	Bovine milk, 1 ml	SPE	Mega Bond Elut C 18	25 ml MeOH, 10 ml Milli-Q H_2O	6 ml MeOH	HPLC	74–116	Blanco et al. (2000)
Vitamin D_3	Bovine milk, 1 ml	On-line SPE	Sep-Pak Plus C-18	MeOH:H_2O (60:40)	MeOH	HPLC	88–105	Delgado-Zamarreno et al. (1995)

Table 4
An overview of the determination methodology of vitamin D and its metabolites.

Analyte	Matrix	Sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min)	LOD (ng/L)	References	Number of citations
Vitamin D_2, vitamin D_3, ergosterol,7-dehydrocholesterol	Bovine milk	LLE	Microcolumn LC	Hypersil C18 BDS (150 × 0.3 mm, 3 μm)	Gradient elution A:MeOH:water (99:1) B:MeOH:THF (70:30)	UV	15–17	200–46,000	Gomis et al. (2000)	37
Vitamin D_3	Whole milk	LLE/TLC	HPLC	Guard column (40 × 2 mm), RP C18 (250 × 4.6 mm,10 μm)	MeOH:water (93:7)/1 ml/min	UV	20		Grace and Bernhard (1984)	9
Vitamin D_3	Fresh bovine milk, commercial milk	SPE	LC/MS^n, LC–MS/MS	Guard column C18, Polaric C18-A (150 × 2.1 mm, 5 μm)	LC/MS^n –MeOH:water (92:8)/0.2 ml/min, LC–MS/MS – MeOH:water with 5 mM AmFormate (92:8)/0.2 ml/min	LC/MS^n –APCI (+)-Q-IT (SRM), LC–MS/MS–ESI (+) (MRM)	21–24	330 (LC/MS^n), 660 (LC–MS/MS)	Trenerry et al. (2011)	5
Vitamin D_2, vitamin D_3	Nonenriched milk, enriched milk	LLE	HPLC	Kromasil 100 C18 (150 × 4.6 mm, 5 μm)	ACN:MeOH (90:10)/1 ml/min	UV	30	8.2–15.7	Barba et al. (2011)	4
Vitamin D_3	Milk	LLE	UPLC/MS	Kinetex RP C18 (50 × 2.1 mm, 2.6 μm core shell)	Gradient elution A:water with 0.2% AmFormate B:MeOH/0.6-1 ml/min	ESI (+) (MRM)-Q-IT	4.5	50	Abernethy (2012)	1
24,25-$(OH)_2D_3$,1,25-$(OH)_2D_3$, vitamin D_3, 25-OH-D_3	Bovine milk	LLE, prep. chromatogr. (Sephadex LH-20/Lipidex 5000)	HPLC/CBA	Zorbax-SIL, Zorbax-ODS (250 × 4.6 mm)	Hex:$CHCl_3$ (9:1), IPA:Hex (3:97), water:MeOH (1:49)	UV/CPBA			Reeve, Chesney et al. (1982) and Reeve, Jorgensen et al. (1982)	41
Vitamin D_3,25-OH-D_3, vitamin D_2,25-OH-D_2	Semi-skimmed milk, whole milk, organic milk	SPE, prep. HPLC	HPLC	RP		DAD, UV	22		Jakobsen and Saxholt (2009)	9
Vitamin D_3,25-OH-D_3, vitamin D_2,25-OH-D_2	Human breast milk	LLE, HPLC	LC–MS/MS	Capcell Pak C18 UG120	Gradient elution ACN:water (30:70)/20 μl/min	QqQ, APCI (MRM)		20–40	Kamao et al. (2007)	26
Vitamin D_3	Human breast milk	SPE	HPLC	Hitachi Gel 3056 (250 × 4.6 mm, 5 μm)	Hex:IPA (99.6:0.4)	UV			Sakurai et al. (2005)	17
Vitamin D	Human breast milk	SPE	HPLC/CPBA	RP μBondapak C18 (300 × 3.9 mm)	MeOH:water (90:10)/1.3 ml/min	UV/CPBA	30		Ballester et al. (1987)	11
Vitamin D, 25-OH-D, 24, 25-(OH)2D,1,25-(OH)2D, 25, 26-$(OH)_2$D	Human breast milk, bovine milk	LLE, prep. HPLC (Lipidex 5000)	HPLC/CPBA	Guard column CO:PELL PAC (4 × 50 mm), μ-Porasil (300 × 4 mm)	Vit D-Hex:IPA (99.5:0.5)/2 ml/min, 25-OH-D-Hex:IPA (98:2)/2 ml/min, Dihydroxyvitamin D (concave gradient, initial c:Hex:IPA (97:3)/1 ml/min	UV/CPBA	30		Hollis et al. (1981)	167
vitamin D_2, vitamin D_3, 25-OH-D_2, 25-OH-D_3,24,25-$(OH)_2$D, 1,25-$(OH)_2$D	Human breast milk	LLE, prep. chromatogr. (Sephadex LH-20/Lipidex 5000)	HPLC/CPBA	Zorbax-SIL, Zorbax-ODS (250 × 4.6 mm)	Vit D-IPA:Hex (1:99), water:MeOH (5:95), 25-OH-D-IPA:Hex (3:97), 24,25-(OH)2D-IPA:Hex (3:47), 1,25-(OH)2D-IPA:Hex (1:9)	UV/CPBA			Reeve, Chesney et al. (1982) and Reeve, Jorgensen et al. (1982)	112
Vitamin D_2, vitamin D_3, 25-OH-D_2, 25-OH-D_3,24,25-$(OH)_2D_2$, 1,25-$(OH)_2D_2$,24,25-$(OH)_2D_3$, 1,25-$(OH)_2D_3$	Human breast milk, bovine milk	LLE, prep. HPLC, SPE	HPLC/CPBA/RRA	Nucleosil 5C18 (300 × 7.5 mm), Zorbax-SIL (250 × 4.6 mm)	Vit D-MeOH:ACN (1:1)/2 ml/min, 25-OH-D- IPA (2.5%, v/v) in Hex/1, 5 ml/min, 24,25-(OH)2D + 1,25-(OH)2D- IPA (10%, v/v) in Hex/1.2 ml/min	UV/CPBA/RRA	25		Takeuchi et al. (1988, 1989)	21, 26

(*continued on next page*)

Table 4 (*continued*)

Analyte	Matrix	Sample preparation	Method	Column	Mobile phase/flow rate	Detector	Analysis time (min)	LOD (ng/L)	References	Number of citations
Vitamin D, 25-OH-D, 1, 25-$(OH)_2$D	Human breast milk, bovine milk	LLE, prep. HPLC, SPE	HPLC/CPBA	2× μ-Porasil (300 × 4 mm)	Vit D-Hex:IPA (99:1)/2 ml/min, 25-OH-D-Hex:IPA (97:3)/2 ml/min, 1, 25-(OH)2D-Hex:IPA (88:12)/1.5 ml/min	UV/CPBA		8(vit D), 20 (25-OH-D), 0.6 (1.25-(OH)2D)	Kunz et al. (1984)	27
25-OH-D, 1, 25-$(OH)_2$D	Human breast milk	LLE, SPE	HPLC/CPBA/RRA						Zheng et al. (1990)	
25-OH-D, 24, 25-$(OH)_2$D,1, 25-$(OH)_2$D	Human breast milk	LLE, prep. HPLC (Sephadex LH-20)	HPLC/CPBRA	μ-Porasil, RP μBondapak C18	25-OH-D, 1,25-(OH)2D-Hex:IPA (93:7) 1 ml/min, 24,25-(OH)2D-water: MeOH (20:80)	UV/CPBA			Weisman et al. (1982)	38
25-OH-D,1, 25-$(OH)_2$D	Human breast milk	LLE, preparative HPLC (Sephadex LH-20/Lipidex 5000)	HPLC/CPBA	μ-Porasil (300 × 3.9 mm)	1,25-(OH)2D- IPA (10%, v/v) in Hex	UV/CPBA			Greer et al. (1981)	27
Vitamin D_3,25-OH-D_3, vitamin D_2,25-OH-D_2	Human breast milk	LLE, preparative HPLC	HPLC/CPBA	NP:Zorbax-SIL (250 × 4 mm), RP:2× Ultrasphere ODS (250 × 4 mm)	NP-Hex:IPA (99:1)/2 ml/min, RP-water:MeOH (2:98)/1.25 ml/min	UV/CPBA	32		Hollis (1983)	59
Vitamin D_2, D_3	bovine milk	LLE	HPLC	C18 (250 × 4.6 mm, 5 μm)	EtOH:water (95:5)	UV	>20		Gong and Ho (1997)	12
Vitamin D_2, D_3	bovine milk	LLE/SPE	HPLC	Vydac C18 (250 × 4.6 mm, 5 μm)	ACN:MeOH (75:25)/1.1 ml/min	UV			Holick et al. (1992)	112
Vitamin D_3	Bovine milk	PFSPE	HPLC	Zorbax SB-C18 (250 × 4.6 mm, 5 μm)	100% MeOH/1 ml/min	PDA	20	10000	Chen et al. (2011)	1
Vitamin D_2, D_3	Bovine milk	LLE	PB LC–MS	LiChrosorb RP-8 (250 × 4.6 mm, 10 μm)	Water:MeOH (3:97)/0.6 ml/min	UV/CI (+/−), EI(+/−) (SIM)		30,000–35,000	Careri et al. (1995)	17
Vitamin D, 25-OH-D	Human breast milk, bovine milk	LLE/SPE	HPLC/CPBA	Nucleosil 50-7silic acid (250 × 4.5 mm),	Vit D-IPA:water:MeOH (4:96)/2 ml/min, 25-OH-D-IPA:Hex (4:96)/2 ml/min	CPBA		20–30	Parviainen et al. (1984)	
vitamin D_3, vitamin D_2, ergosterol,7-dehydrocholesterol	Bovine milk	LLE/SPE	HPLC	Extrasil ODS2 (150 × 2.1 mm, 3 μm)	Gradient elution A:MeOH:H2O (99:1) B:MeOH:THF (70:30)/0.15 ml/min to 5.5 min and 0.2 ml/min after to 6.3 min	UV	<33	820 * 10^3–60 10^3	Blanco et al. (2000)	25
Vitamin D_3	Bovine milk	On-line SPE	HPLC	Precolumn RP18 (15 × 3.2 mm, 7 μm), OD-224 RP18 (220 × 4.6 mm, 5 μm)	Water:MeOH (1:99) with 2.5 mM acetic acid-sodium acetate/1 ml/min	UV	25	680.7 * 10^3	Delgado-zamarreno et al. (1995)	27
Vitamin D_2, D_3	Bovine milk	LLE/SPE	HPLC	Vydac C18 (250 × 4.6 mm, 5 μm)	ACN:MeOH (97:3)/1.0 ml/min	UV	30	1.6	Hagar et al. (1994)	8
Vitamin D_2	Bovine + buffalo milk	LLE	HPLC	Guard column C18 (5 um), Phenomenex C18 (250 × 4.5 mm, 5 μm)	ACN:MeOH:chloroform (88:8:4)/1 ml/min	PDA	15	1 pg/100 μl	Kaushik et al. (2014c)	0

PFSPE – Packed-fiber solid phase extraction, CPBA – Competitive protein binding assay, RRA – radioreceptor assay, PB – particle beam.

* Derivatisation with PTAD.

** Derivatisation with DMEQ-TAD.

However vitamin D can be bound as an ester and the true recovery is difficult to be tested by standard addition.

2.2. Analytical methods

Many studies describing the analysis of vitamin D and its metabolites in human fluids have been published, but several problems remain to be discussed. For example, the concentration of vitamin D in milk is extremely low (usually in the μg/L to ng/L concentration range), and therefore a selective separation method and sensitive detection are required. Published methods are summarised in the Table 4. Currently, the most important methods used for vitamin D characterisation are LC–MS, LC–UV and immunological techniques. LC is considered to be the primary method for the separation of vitamin D. Various types of detection methods have been used with LC, including MS–MS, MS and UV. LC–MS and LC–MS–MS are the methods of choice in vitamin D analysis since this methodology is sensitive, accurate and provides high specificity.

2.2.1. Chromatographic techniques

Chromatographic methods are the most commonly used analytical techniques for characterising vitamin D and its metabolites in milk. These methods can separately and simultaneously quantify derivatives of vitamin D. Both normal phase and reverse phase chromatography have been used for the assessment of vitamin D and its metabolites in milk, with normal phase HPLC used more commonly before the 2000.

2.2.1.1. Normal phase HPLC. Isocratic elution was carried out in almost all studies. Hexane or a mixture of hexane with a small percentage of a more polar solvent, such as isopropyl alcohol or methyl dichloride, is the most utilised mobile phase for normal phase HPLC (Greer et al., 1981; Hollis, 1983; Hollis, Roos, Draper, & Lambert, 1981; Kunz et al., 1984; Reeve, Chesney et al., 1982; Reeve, Jorgensen et al., 1982; Sakurai et al., 2005; Takeuchi et al., 1988, 1989; Weisman et al., 1982).

The measurement of vitamin D and its metabolites in milk was conducted in several steps in this case, including the initial separation of vitamin D from the metabolites using preparative chromatography, further purification on normal or reverse phase HPLC and quantification by immunoassays or HPLC coupled with UV detection (Greer et al., 1981; Hollis, 1983; Hollis et al., 1981; Kunz et al., 1984; Reeve, Chesney et al., 1982; Reeve, Jorgensen et al., 1982; Takeuchi et al., 1988, 1989; Weisman et al., 1982). Normal phase HPLC was used mainly to isolate the specific fraction of vitamin D before the final quantification step by CPBA (competitive protein binding assay). In the work of Hollis (1983), vitamin D_2 and vitamin D_3 were found to co-elute in normal phase HPLC using hexane:isopropanol (99:1) as the mobile phase. These vitamins were finally applied to reverse phase HPLC (mobile phase – methanol:water), and vitamin D_2 was separated from vitamin D_3. Furthermore, resolved peaks were observed for 25-OH-D_2 and 25-OH-D_3 D using a single normal phase HPLC separation with hexane:isopropanol (97:3). The HPLC fractions were quantified using a rat serum CPBA. This method does not allow the quantification of vitamin D and its metabolites by UV detection and requires additional procedural steps, making this method more time consuming.

Other studies have reported the quantification of 25-OH-D_3 using UV HPLC without CPBA (Reeve, Chesney et al., 1982; Reeve, Jorgensen et al., 1982; Weisman et al., 1982) and detected concentrations that correlated with the levels of 25-OH-D as quantified by CPBA (Weisman et al., 1982).

Normal phase sorbent methods facilitate the washing of triglycerides and other non-polar material from the column (Perales et al., 2005). However, the methods for the determination of vitamin D in milk were complicated and require more than 2 steps for purification, determination and subsequent quantification. Additionally, these proposed procedures were time and solvent consuming, not sufficiently sensitive and efficient.

2.2.1.2. Reverse phase HPLC. Currently, RP-HPLC is the most widely used chromatographic technique. This technique utilises a non-polar stationary phase, such as an octadecyl column, and a polar mobile phase. In almost all studies, a C18 column was used. The particle size of the analytical columns used ranged from 2.6 μm to 10 μm. In some cases, a pre-column (Delgado-Zamarreno et al., 1995) or guard column (Grace & Bernhard, 1984; Trenerry et al., 2011) was used to protect the analytical column. However, using a narrow-bore column (Blanco et al., 2000; Gomis et al., 2000) decreased the solvent consumption in comparison with common columns and allows the injection of small amounts of sample.

Barba et al. (2011) compared three columns for the quantification of vitamins D_2 and D_3. C18 (150 × 4.6 mm, 5 μm), C18 (250 × 4.6 mm, 5 μm) and C8 (250 × 4.6 mm, 5 μm) columns were assayed. C8 did not obtain good peak resolution, and the vitamin D_2 and vitamin D_3 peaks overlapped. The best peak resolution and the fastest analysis were achieved with a C18 column (150 × 4.6 mm, 5 μm). Further details of the chromatographic conditions and columns can been observed in Table 4.

The mobile phase for RP stationary phases commonly consists of a mixture of water with methanol, ethanol or acetonitrile. Moreover, non-aqueous mobile phases, such as acetonitrile–methanol (Barba et al., 2011; Hagar, Madsen, Wales, & Bradford, 1994; Holick et al., 1992; Takeuchi et al., 1988, 1989) or 100% methanol, have been used (Chen et al., 2011). Acetic acid–sodium acetate was another component of the mobile phase (Delgado-Zamarreno et al., 1995). Separation has been carried out mainly using gradient elution of the mobile phase.

A study published by Gong and Ho (1997) can be used as an example for the determination of vitamin D in milk. In this study, the authors developed an isocratic separation method for ten different forms of fat-soluble vitamins in milk with two mobile phases on an analytical C18 column (250 × 4.6 mm, 5 μm). A mixture of ethanol and methanol was unable to distinguish vitamins D_2 and D_3. Better separation was achieved with a mobile phase containing ethanol and water; however, the analysis time was over 20 min. Two solvents, methanol–water (solvent A) and methanol–tetrahydrofuran (solvent B), were successfully used as the mobile phase in gradient elution to assess fat-soluble vitamins and provitamins (Blanco et al., 2000; Gomis et al., 2000). Tetrahydrofuran was added to methanol and was found to decrease the retention time in this study.

Kaushik et al. (2014c) optimised the mobile phase for the estimation of vitamin D_2. Three different mobile phases were investigated – acetonitrile:methanol:ethyl acetate (88:8:4), acetonitrile:methanol:chloroform (88:8:4) and acetonitrile:methanol (80:20). The best resolution was obtained by acetonitrile:methanol:chloroform (88:8:4), with a flow rate of 1 ml/min. This method offers low detection and quantification limits and good reproducibility for vitamin D_2 (see Table 4).

2.2.1.3. LC–UV. Ultraviolet detection is one of the most commonly exploited methods for the quantification of vitamin D. A UV detector is used since all vitamin D active compounds possess a broad UV absorption spectrum within the range of 250–265 nm (Perales et al., 2005). The wavelengths of the detector for vitamins D_2 and D_3 were set at 264 nm (Blanco et al., 2000; Gomis et al., 2000), 250 nm (Gong & Ho, 1997), 265 nm (Barba et al., 2011; Grace & Bernhard, 1984; Hagar et al., 1994; Jakobsen & Saxholt, 2009) or 254 nm (Holick et al., 1992).

DAD and UV detection were used by Jakobsen and Saxholt (2009), and the detection limits for vitamin D and 25-hydroxy vitamin D using the DAD detector were approximately two-fold less than those for the UV detector. The method of Barba et al. (2011) with UV detection proved to be sufficiently sensitive with an LOD 0.82–1.57 ng/100 ml for the detection of vitamin D_2 and D_3.

A UV detector with a narrow-bore column used by Blanco et al. (2000) achieved detection limits ranging from 0.80 to 4.1 ng/injection (5 μL) for vitamin D_2 and D_3 and their provitamins. This method appears to be inadequate for the determination of the vitamins levels in unprocessed milk, although for several types of fortified milk this method is suitable. Capillary LC separation according to Gomis et al. (2000) with UV detection provided sensitive determination of vitamin D_2 and D_3 and their provitamins in milk with a LOD 0.02–46 ng/ml for detection. More sensitive LC–UV method were used by Barba et al. (2011) for vitamin D_2 and D_3. The limits of detection were 0.82–1.57 ng/100 ml. The LC–UV system provides a solution for the problems associated with the separation of vitamin D_2 and D_3, allowing the determination of both vitamins and thus permits their individual quantification.

PDA detector was used for the detection and quantification of vitamin D_2. Different λ_{max} (228, 254, 265 nm) were assayed for vitamin D_2 analysis in milk. The maximum peak area for vitamin D_2 during HPLC analysis was determined at 254 nm. This method offers very low detection limit, only 1 pg/100 μl, for vitamin D_2 in milk (Kaushik et al., 2014c).

LC–UV and LC–DAD are convenient for the determination of vitamin D, however, LC–MS provides better results in terms of sensitivity and specificity.

2.2.1.4. LC–MS. Over the last few years, there has been an increased interest in using liquid chromatography coupled to mass spectrometry (LC–MS) to determine the levels of vitamin D and other metabolites (Trenerry et al., 2011). This method is increasingly used as a routine analytical technique in clinical laboratories and provides an attractive complementary method to traditional methodologies for routine applications (van den Ouweland & Kema, 2012). One major advantage that LC–MS offers over immunoassays is the ability to accurately quantify multiple analytes in a single assay (El-Khoury, Reineks, & Wang, 2011).

Both ESI (electrospray ionisation) and APCI (atmospheric-pressure chemical ionisation) techniques have been used in LC–MS for the determination of vitamin D and its metabolites (Abernethy, 2012; Kamao et al., 2007; Trenerry et al., 2011). ESI positive-ion mode (+) was used in most cases. Only Careri, Lugari, Mangia, Manini, and Spagnoli (1995) used electron impact and chemical ionisation in both the positive-ion and negative-ion modes. Triple quadrupole (QqQ) and ion trap (IT) are the most commonly used analysers.

The method reported by Kamao et al. (2007) includes two extraction methods and a sensitive LC–APCI–MS/MS (QqQ) detection step using stable isotope-labelled internal standards for the determination of vitamins D_2, D_3, 25-OH-D_2, and 25-OH-D_3 in human breast milk. This method allows the determination of the principal fat-soluble vitamins in breast milk, which contains more interfering compounds compared to plasma or serum samples. All analytes were detected in MS/MS-multiple reaction monitoring (MRM) mode with unit resolution at both Q1 and Q3. Vitamin D and 25-OH-D were measured after DMEQ-TAD (4-[2-(3,4-Dihydro-6,7-diMethoxy-4-Methyl-3-oxo-2-quinoxalinyl)ethyl]) derivatisation to improve the ionisation efficiency (see Fig. 2). DMEQ-TAD derivatisation enhanced the measurement sensitivity of D and 25-OH-D by approximately 40 times over the intact form. Additionally, excellent recoveries for the vitamins of approximately 91–105% were achieved. However, this method is time consuming because prior to LC–MS, the samples are concentrated and purified by normal phase HPLC, and the retention times (RT) of vitamin D and 25-OH-D after derivatisation are 36 min and approximately 22 min, respectively. Additionally, greater amounts of solvents were used during these procedures.

Trenerry et al. (2011) compared the methods of liquid chromatography – linear ion trap mass spectrometry (LC–MS^n) and liquid chromatography – tandem mass spectrometry (LC–MS/MS) to measure the levels of vitamin D_3 in several types of bovine milk. The LC–MS^n instrument was operated in the positive ion APCI because it gave a 20-fold increase in sensitivity over ESI. The tandem mass spectrometer was operated in the positive ion ESI mode, which provided better sensitivity than did APCI. The levels of vitamin D_3 were nearly identical in the two methods and were comparable to the levels stated on the label. A slightly better limit of quantification (LOQ) for vitamin D_3 was achieved by LC–MS^n, at 0.01 μg/100 ml and 0.02 μg/100 ml, than by LC–MS/MS. The recovery of vitamin D_3 was in the range of 61–86%. The disadvantages of these methods were the time of analysis, with the RT of vitamin D_3 being about 18 min, and their low recovery.

A faster analytical method with a comparable LOQ for vitamin D_3 in bovine milk was developed by Abernethy (2012). Ammonium formate was added to water in the mobile phase to enhance the ionisation in MS. This method for the determination of vitamin D_3 utilises the Diels–Alder derivatisation reaction with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (see Fig. 2). Detection was carried out by (+)ESI–MS/MS in MRM using positive ESI, and the RT for vitamin D_3 was about 3.5 min. This method boasted low solvent consumption and low waste generation of <45 ml (including both extraction and chromatography). This method is rapid and cost effective, with high recoveries of spiked vitamin D_3 at an average of 103.2%.

In recent years, LC–MS/MS has become the most widely used chromatographic method for the assay of vitamin D and its metabolites. Currently, it is considered as the "gold standard" method (Garg, Munar, Frazee, & Scott, 2012). On the other hand, the equipment is expensive and requires specialised staff.

3. Conclusions

The development of analytical methods for the accurate determination of vitamin D and its metabolites is an important area in analytical chemistry because of the importance of these vitamins in the human body. In this review, the methods used for sample pretreatment and the analysis of vitamin D and its metabolites in human and bovine milk were critically discussed.

Sample preparation is carried out mainly by LLE since SPE is more complicated and does not provide any huge advantages compared to LLE. This is demonstrated using the most frequent milk sample preparation, i.e., saponification with KOH followed by extraction with hexane, which satisfactory validation parameters. LC–MS has unique advantages for vitamin D determination and quantification due to its high sensitivity and specificity. To improve the LC–MS detection, isotope-labelled internal standards can be used. Optimisation plays an important part in enhancing the sensitivity and reducing the matrix effects. Complicated methods such as multistep sample preparations including HPLC purification, LLE or SPE will be replaced by automatic or online sample preparation with the same outcomes. Future trends in sample preparation are focused mainly on the automation of the extraction step, where human manipulation can be minimised to avoid operator errors and biological hazards. In addition, reductions in the used volume of the sample and solvent will lead to cost reductions and decrease the waste burden for the environment. Immunoassays could still be used for the routine quantification of vitamin D and its metabolites because they are relatively inexpensive compared with chromatographic methods. However, the greater accuracy and

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9.3. PŘÍLOHA III

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Blood levels of antioxidant agents during age-related macular degeneration treatment by rheohaemapheresis

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Blood levels of antioxidants during age-related macular degeneration treatment by rheohaemapheresis

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Aims. Rheohaemapheresis treatment influences rheological markers and most likely improves metabolism in affected retinal areas, resulting not only in absorption of soft drusen but also reduction or complete disappearance of drusenoid retinal pigment epithelium detachments. However, the character of the treatment process has raised suspicion that there is a decrease not only in cholesterol but also in antioxidants, such as vitamin E and vitamin A.
Methods. Twenty-three patients with the progressive dry form of age-related macular degeneration were each treated with 8 procedures of rheohaemapheresis. We measured levels of vitamin E (α-tocopherol), the vitamin E/cholesterol ratio in serum and lipoproteins (VLDL, LDL, HDL). Vitamin E in erythrocyte membrane and serum vitamin A (retinol) were also measured. These parameters were determined before and after rheohaemapheresis. Erythrocyte superoxide dismutase, erythrocyte glutathione peroxidase and serum malondialdehyde were analysed as markers of antioxidant activity and lipid peroxidation, respectively.
Results. In serum, the VLDL and LDL fraction ratios of vitamin E/cholesterol increased significantly. Additionally, the HDL fraction ratio showed an increase but this was not statistically significant. The patients showed no clinical signs of vitamin E deficiency, and their serum concentrations of vitamin E did not differ from normal values. The results show that rheohaemapheresis in addition to causing a significant reduction in atherogenic LDL cholesterol, may have favourable additive anti-atherogenic effects due to a relative increase in the content of vitamin E in the lipoprotein fractions.

Key words: rheohaemapheresis, age related macular degeneration, vitamin E, vitamin E/cholesterol ratio, antioxidants

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INTRODUCTION

Age-related macular degeneration (AMD) is a highly complex disease, with demographic, environmental and genetic risk factors[1]. It affects the central region of the retina and choroid, which can result in loss of central vision[2]. AMD is a bilateral disorder; choroidal neovascular membranes develop in more than one fourth (26%) of fellow eyes that are initially free of exudative AMD over a 5-year period[3]. AMD is also the leading cause of vision loss in the developed world, such as in Europe, the USA and Australia, among people older than 55/ 65 years old, accounting for up to 50% of all cases. Additionally, its prevalence is likely to increase as a consequence of increasing longevity[2-6]. The prevalence in adults is approximately 3% (ref.[2]). It is estimated that approximately 30% of adults older than the age of 75 years old have some signs of AMD and that approximately 10% of these patients demonstrate advanced or late stages of the disease[7]. A comparison of statistical data from the Czech Republic with those from other countries provided difficulties because in the Czech Republic, the limit for legal blindness is 1/60, while in other countries, it is 6/60 (ref.[5]).

Clinically, AMD appears in two forms - a non-exudative dry form and an exudative wet form - which in an individual patient can also represent stages of the disease. The non-exudative form involves a variety of presentations, including hard drusen, soft drusen, and geographic (areolar) atrophy of the retinal pigment epithelium (RPE). This non-exudative form of AMD accounts for 80-90% of AMD cases[5,8].

Greater intake of fish, nuts, and dark green, leafy vegetables has been associated with lower risk for AMD. An increase of intake of vegetable fat, mono- and polyunsaturated fatty acids, and linoleic acid was associated with increased risk for AMD (ref.[7]). The Age-Related Eye Disease Study (AREDS), a multi-centre, randomised, controlled clinical trial, demonstrated that oral supplementation with a combination of vitamin C, vitamin E, β-carotene, zinc oxide and cupric oxide in patients with intermediate or advanced AMD in one eye resulted in a 25% relative risk reduction of developing advanced AMD

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in the other eye[5,8]. A comparison with placebo demonstrated a statistically significant odds reduction for the development of advanced AMD with antioxidants plus zinc[9]. The US Veterans Administration's LAST (Lutein Antioxidant Supplementation Trial) study found that lutein supplementation, alone or in combination, significantly augmented macular pigment, improved near visual acuity and contrast sensitivity and demonstrated a lack of disease prógression over the one-year study period. Gale et al. found that the serum concentration of zeaxanthin was significantly lower in individuals with AMD, compared to those without the disease. Serum concentrations of lutein and of lutein and zeaxanthin combined were also lower but not significantly[10-12].

Bláha et al. observed, after rheohaemapheresis (RH), not only absorption of soft drusen but also a reduction in or complete disappearance of drusenoid retinal pigment epithelium detachments (RPEDs). RH influenced rheological markers and most likely improved metabolism in the affected retinal areas, leading to the positive results[8]. RH results in a decrease in high-weight molecules, such fibrinogen, immunoglobulins (mainly IgM), LDL cholesterol and other compounds. For this reason, the viscosity of the blood decreases, and the proportions of cytokines and adhesion molecules also change positively. Moreover, the deformability of erythrocytes decreases. Clinical studies on implementing RH in AMD have been guided by evidence-based medicine[13].

However, the character of the treatment process has raised the suspicion that RH also causes a decrease in antioxidants, such as vitamin E and vitamin A.

METHOD

Patient group

From June 2008 to July 2011, twenty-three patients with the non-neovascular form of AMD were randomised. Patients were treated with 8 procedures of RH (9 men, 14 women, mean age 67 years old, range 41-85 years).

Patients admitted to the study had to have received a diagnosis of AMD in both eyes, including dry AMD in one or both eyes confirmed by fluorescein angiography and fundus photography. The exclusion criteria were retinal or choroidal disorders other than AMD, optic nerve disorders, glaucoma, conditions limiting examination of the fundus, and acute bleeding in the study eye. The general exclusion criteria for RH treatment were the usual exclusion criteria for extracorporeal circulation or therapeutic haemapheresis and the absence of peripheral veins suitable for establishing an extracorporeal circuit. Regarding randomisation, patients with the late-stage, high-risk, preangiogenic form of AMD with soft drusen, confluent soft drusen and DPEDs were assigned to RH therapy.

Rheohaemapheresis

The cascade method of plasma filtration was used as our modification of rheotherapy (named rheohaemapheresis by Borberg et al., 2006) (ref.[14-17]) (Fig. 1). After

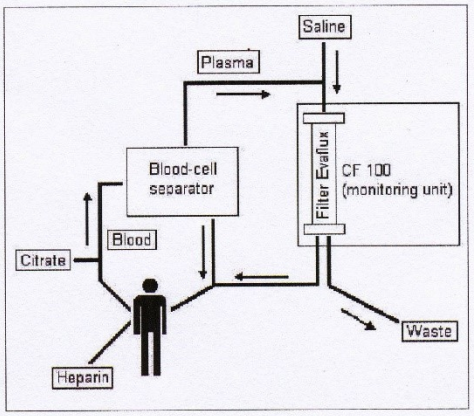

Fig. 1. Scheme of rheohaemapheresis with our modification.

plasma separation (blood-cell separator, Cobe-Spectra, Denver, CO, USA), the separated plasma was pumped through the rheofilter (Evaflux 4A, Kuraray, Japan) to remove high molecular weight rheologic factors. The time schedule of the study was the same as that of the largest published MIRA-1 study[18], i.e., 2 procedures weekly and then a 14-day pause, with the procedure repeated four times. Altogether, 8 procedures were performed within 10 weeks, and 1 to 2 procedures were recently added after one year of follow-up, if needed (if suspicion or symptoms of disease progression were discovered). We treated 1.5 volumes of plasma in one session. Critical values were considered to be decrease in fibrinogen levels to less than 0.7 g/L. In these cases, the volume of filtered plasma was decreased (from 1.5 x body volume to 1 x body volume). The patients were required to have peripheral veins allowing for vascular access to establish the extracorporeal circuit. All of the filters and tubing systems for extracorporeal circulation were for a single use only.

Analytical determination of retinol and α-tocopherol in serum, lipoprotein fractions and erythrocyte membranes

The blood samples were collected before and after the extracorporeal elimination process. The study protocol was approved by the Ethics Committee of University Hospital in Hradec Králové. Informed consent was obtained from all of the participants. The research followed the tenets of the Declaration of Helsinki.

The blood samples were first centrifuged (2000 × g, 10 min, 4 °C), and the serum was separated and analysed immediately or was frozen at -20 °C until analysis.

Lipoprotein fractions were prepared from fresh serum by a gradient ultracentrifugation technique, using saline solution with a determined density with 0.1% EDTA added to avoid oxidation of lipoproteins during ultracentrifugation.

Serum lipoproteins were separated into very low-density lipoprotein (VLDL d < 1.006), low-density lipoprotein (LDL d < 1.063) and high-density lipoprotein (HDL d >

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Table 1. Levels of cholesterol and TAG in serum and lipoprotein fractions before and after RH treatment.

Analyte	N	Before Average (±SD) mmol/L	After Average (±SD) mmol/L	Decreased by (%)	Statistical significance
Cholesterol-serum	66	4.25 (±1.35)	1.78 (±0.49)	58.12	0.001
Cholesterol-VLDL	66	0.81 (±0.34)	0.30 (±0.22)	62.96	0.001
Cholesterol-LDL	64	2.28 (±0.71)	0.81 (±0.29)	64.47	0.001
Cholesterol-HDL	66	1.02 (±0.21)	0.64 (±0.15)	37.26	0.001
TAG-serum	65	1.48 (±0.64)	0.74 (±0.44)	50.00	0.001
TAG-VLDL	65	0.93 (±0.49)	0.48 (±0.37)	48.39	0.001
TAG-LDL	64	0.35 (±0.11)	0.17 (±0.08)	51.43	0.001
TAG-HDL	65	0.13 (±0.08)	0.09 (±0.04)	30.77	0.001

Table 2. Levels of vitamin E in serum and lipoprotein fractions before and after RH treatment.

Analyte	N	Before Average (±SD) µmol/L	After Average (±SD) µmol/L	Decreased by (%)	Statistical significance
Vit E-serum	68	22.23 (±6.75)	11.00 (±3.25)	50.52	0.001
Vit E-VLDL	66	6.31 (±3.14)	2.56 (±1.28)	59.43	0.001
Vit E-LDL	66	9.45 (±3.45)	3.83 (±1.34)	59.47	0.001
Vit E-HDL	67	6.45 (±1.81)	3.88 (±1.20)	39.85	0.001

1.063) fractions, using an OPTIMA MAX-XP ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) (ref.[19]).

The method used in this study for the analysis of vitamin A (retinol) and vitamin E (α-tocopherol) in serum and lipoprotein fractions was modified from the method previously published by Urbanek et al.[20].

The level of α-tocopherol in erythrocyte membranes was analysed by the modified HPLC method previously published by Solichova et al.[21].

Analytical determination of cholesterol and triacylglycerols

Serum cholesterol and triacylglycerols (TAG) were determined on a MODULAR analyser (Hoffmann-La Roche, Basel, Switzerland) using commercially available kits, according the manufacturers' instructions. The same methods were used for the evaluation of cholesterol and TAG contents in the lipoprotein fractions.

Analytical determination of enzymes

Erythrocyte glutathione peroxidase (GPx) was determined spectrophotometrically using a commercial kit (Ransel, Randox, United Kingdom), according the manufacturer's instruction, with the elimination of absorbance at a 340 nm wavelength (Cobas Mira, Roche, Switzerland).

Serum malondialdehyde was measured as a red complex with thiobarbituric acid at 485, 532 and 560 nm using a Beckman DU 640 spectrophotometer (Beckman, Palo Alto, USA).

Erythrocyte superoxide dismutase was determined spectrophotometrically with a commercial kit (Ransod, Randox, United Kingdom) according the manufacturer's instructions, as the elimination of absorbance at a 505 nm wavelength (Secomam, Ales, France).

Statistical analysis

NCSS 2007 software (Kaysville, USA) was used for statistical evaluation of the changes in each measured parameter before and after extracorporeal elimination therapy. Evaluation was performed with the nonparametric Mann-Whitney U test and Wilcoxon's signed-tank test. Statistical significance was based on $P \leq 0.05$.

RESULTS

Cholesterol and triacylglycerols

Decreases in cholesterol levels are presented in Table 1. Cholesterol and TAG were measured in serum and different lipoprotein fractions, including VLDL, LDL and HDL fractions. The results are presented in Fig. 2.

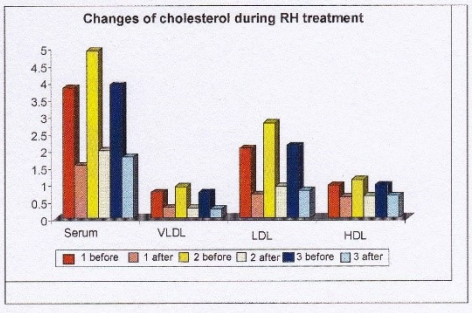

Fig. 2. Changes of cholesterol levels in serum and lipoproteins during RH treatment.

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Table 3. Vitamin E/cholesterol ratios in serum and lipoprotein fractions before and after RH treatment.

Vitamin E/ cholesterol ratio	N	Before Average (±SD) 10^{-3}	After Average (±SD) 10^{-3}	Increased by (%)	Statistical significance
Serum	65	5.42 (±1.23)	6.28 (±1.34)	15.87	0.001
VLDL	64	7.88 (±2.29)	9.80 (±5.09)	24.37	0.000309
LDL	57	4.16 (±0.99)	5.11 (±3.64)	22.84	0.000006
HDL	65	6.36 (±1.21)	6.48 (±4.12)	1.89	0.043457

Table 4. Levels of MDA, SOD and GPx before and after RH treatment.

Analyte	N	Before Average (±SD)	After Average (±SD)	Statistical significance
Malondialdehyde (μmol/L)	50	0.48 (±0.41)	0.56 (±0.22)	0.002358
Superoxide dismutase (U/g Hb)	50	1595.78 (±729.02)	1505.74 (±676.66)	0.593154
Glutathione peroxidase (U/g Hb)	50	35.79 (±11.29)	34.46 (±10.20)	0.557875

Vitamin E

Levels of vitamin E before and after the procedure and its decreases in serum and lipoprotein fractions are presented in Table 2.

However, a decrease in the vitamin E/cholesterol ratio was not observed. In serum and in the VLDL and LDL fractions, the vitamin E/cholesterol ratio increased significantly in range, from 1.9% to 24.4%. In the HDL fraction, the ratio showed weak but statistically significant changes. The results are presented in Table 3.

The results of our study confirmed an increase (not statistically significant) in vitamin E levels in the membranes of 2% after rheohaemapheresis (Fig. 3).

Vitamin A

The average levels of vitamin A in serum before and after treatment were 1.56±0.38 μmol/L and 1.22 ± 0.27 μmol/L, respectively. The results constitute a statistically significant decrease (P = 0.001). Nevertheless, the measured values after treatment were at physiological levels.

Other metabolites

During the rheohaemapheresis treatment, there were reductions observed in the levels of erythrocyte superoxide dismutase and erythrocyte glutathione peroxidase, but these changes were not statistically significant. The serum level of malondialdehyde after rheohaemapheresis increased significantly by 17%, as presented in Table 4 and also in Fig. 4.

DISCUSSION

Until now, there have been no effective pharmacological therapies for the dry form of AMD that have been documented by multicentre, randomised, double-blind studies[8,22].

Cholesterol and triacylglycerols

An exactly defined spectrum of high molecular weight, rheologically relevant plasma proteins (i.e., LDL-cholesterol, lipoprotein) is removed by rheohaemapheresis. As described above, this process can lead to sustained microcirculatory recovery and can significantly alter the natural course of this chronic disease. This process should improve the metabolic exchange between the RPE and

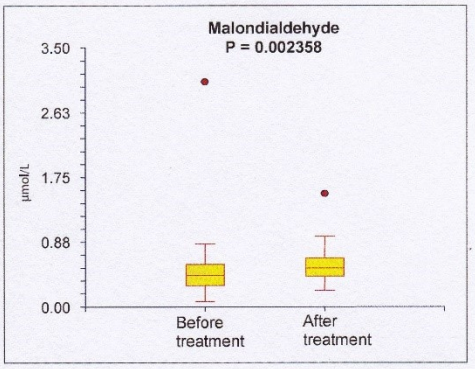

Fig. 3. Levels of MDA in serum before and after RH treatment.

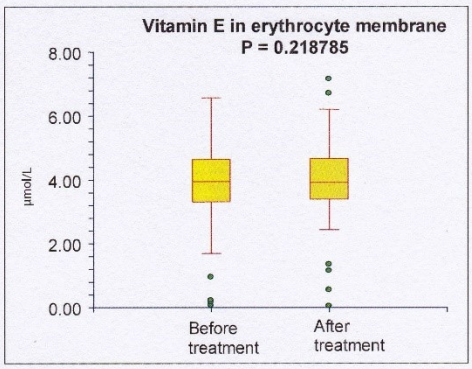

Fig. 4. Levels of vitamin E in erythrocyte membranes before and after RH treatment.

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the choriocapillaris and the nutrition of RPED cells and of the neuroepithelium, reducing ischaemia and the production of vascular endothelial growth factor (VEGF). VEGF and pigment epithelium-derived factor (PEDF) are regulated by tissue oxygenation. Expression of VEGF is induced by hypoxia, thus promoting neovascularisation, while PEDF is induced by increase of oxygen, thus inhibiting neovascularisation[23]. However, optimal quantification of the surface structures of erythrocytes (e.g., stability and elongation), granulocytes (e.g., migration and penetration of the vessel wall), lymphocytes e.g., role of regulatory T cells), monocytes (e.g., migration and penetration of the vessel wall) and platelets (e.g., adhesion to endothelial cells) has not yet been achieved[13,24].

In this work, high molecular weight molecules, such cholesterol and TAG, decreased by 31-65%.

Vitamin E

Antioxidant defence systems, such as enzymes or vitamins, protect the host directly and indirectly against the damaging influence of oxidants[25]. Tocopherols react with free radicals, notably peroxyl radicals, and with singlet molecular oxygen, which forms the basis of their function as antioxidants[26].

The term vitamin E covers 8 different forms (α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol) (ref.[27]), and in general, it is the major chain-breaking antioxidant of cellular membranes. α-tocopherol is the most active scavenger of free radicals and the most predominant tocopherol in the human retina and plasma[26].

There have been some studies reporting an association between high total vitamin E intake and the risk of late AMD or indistinct soft or reticular drusen and between higher intake of dietary α-carotene and the risk of neovascular AMD (ref.[28]).

There has been much evidence supporting the concept that vitamin E protects against retinal oxidative damage, including that vitamin E deficiency results in retinal degeneration and that darkening, which results in increased vulnerability to retinal light damage, is associated with reduced ascorbate and vitamin E levels in the rat retina[4].

AREDS demonstrated that supplementation with high-dose antioxidant supplements (vitamin C, vitamin E, and β-carotene) and zinc for an average of 6.3 years could reduce the risk of progression to advanced AMD in persons with intermediate AMD or advanced AMD in 1 eye. Nevertheless, AREDS could not determine whether the zinc and antioxidant combination could delay progression of early-stage AMD or could prevent the onset of AMD in persons at usual risk[29]. However, there has been accumulating evidence that taking vitamin E or β-carotene supplements will not prevent or delay the onset of AMD, and some researchers have failed to demonstrate that vitamin E and selenium protect against photochemical damage to the retina[4,26,29].

The optimal combination of antioxidants has yet to be formulated, as has whether antioxidant therapy will be part of a multifaceted approach to the treatment of AMD (ref.[30]). The results of 3 trials, enrolling primarily persons at usual risk, found little evidence that supplementation with vitamin E for 4 to 6 years or with β-carotene for 10 years could materially reduce the risk of AMD; with a treatment duration of 10 years, the current findings extended these earlier findings by showing that very long-term supplementation with vitamin E alone was unlikely to have an important effect on AMD occurrence[29].

Vitamin E does not appear to have a specific carrier protein in the plasma. It is rapidly transferred from chylomicra to plasma lipoproteins, to which it binds non-specifically, whereupon it is taken up by the liver and is incorporated into nascent VLDL (with selectivity in favour of α-tocopherol over the γ-vitamer), which is secreted by the liver. Although the majority of triglyceride-rich VLDL remnants are returned to the liver, others are converted by lipoprotein lipase into LDL. It appears that, during this process, vitamin E also transfers spontaneously to apolipoprotein B-containing lipoproteins, including VLDL, LDL and HDL. Therefore, plasma tocopherols are distributed among these three lipoprotein classes, with the more abundant LDL and HDL classes comprising the major carriers of vitamin E (ref.[31]). Fasting blood levels of vitamin E, after multivariate adjustment, showed a weak negative association with AMD (ref.[31]). Lipid-standardised plasma α-tocopherol had significant inverse relationships with early and late AMD, representing a risk reduction in AMD of 82% for those in the highest quintile versus the lowest quintile, in a study by Delcourt et al.[32].

However, Christen et al. reported that, in a large-scale, randomised trial of male US physicians, alternate-day use of 400 IU of vitamin E and/or daily use of 500 mg of vitamin C for 8 years had no appreciable beneficial or harmful effects on the risk of incident diagnosis of AMD (ref.[33]).

Nevertheless, among the components of LDL investigated in this study, an increased vitamin E-to-cholesterol ratio was the strongest predictor of increased resistance of the lipoprotein to metal ion-dependent oxidation[34], which was why we examined the proportions of vitamin E and cholesterol in serum and in different lipoprotein fractions, and the results showed no decreases in the lipoprotein fractions.

In general, the highest accumulation of vitamin E is in the adipose tissue[35]. In most non-adipose cells, vitamin E is localised almost exclusively in the membranes. Kinetic studies have indicated that such tissues have two pools of the vitamin: a labile, rapidly overturning pool; and a fixed, slowly overturning pool[31].

Very important is the effect on the levels of vitamin E in membranes. As a model of the circumstances in membranes, erythrocyte membranes were selected. Erythrocytes are the major cellular component of the blood, and erythrocyte membrane fluidity can be affected by human diseases associated with oxidative stress. Free radicals are commonly formed in erythrocytes due to oxidation of haemoglobin. Erythrocyte membrane fluidity can thus be viewed as an indirect marker of oxidative stress[36]. For this reason, we measured vitamin E levels in erythrocyte membranes and confirmed no decrease.

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Vitamin A

Vitamin A exists in the following three oxidation states: an alcohol (retinol), an aldehyde (retinal), and an acid (retinoic acid) (ref.[4]). Retinol is essential for vision since it must be available in the retina as a precursor of 11-*cis*-retinal for the regeneration of rhodopsin.

In healthy individuals, plasma retinol is maintained within a narrow range (0.53 - 2.1 μmol/L in adults; typically approximately half of that in new-born infants) despite widely varying intakes of vitamin/provitamin A (ref.[31,37]).

Vitamin A is also involved in the repair of cells that have been oxidatively damaged. Notably, in the retina, vitamin E is believed to protect vitamin A from oxidative degeneration[4].

Nutritional treatment of retinal disease has proved at least partially successful in common retinitis pigmentosa (vitamin A), Bassen-Kornzweig disease (vitamins A, E, and K), and Sorsby fundus dystrophy (vitamin A) (ref.[10]). Nevertheless, regarding plasma retinol and AMD, the POLA study failed to detect a significant association[4].

Other metabolites

Other compounds, such as malondialdehyde (MDA), in the serum and the enzymes erythrocyte superoxide dismutase (SOD) and erythrocyte glutathione peroxidase (GPx) were investigated as markers of antioxidant activity and lipid peroxidation in this study. MDA is a common lipid peroxidation product that accumulates in many pathophysiological processes, including AMD (ref.[38]). Enzymes, superoxide dismutase and glutathione peroxidase demonstrated antioxidant activity[4].

SOD catalyses the quenching of the superoxide anion to produce hydrogen peroxide and oxygen. No association could be detected between systemic SOD activity and AMD in the POLA study[4]. In every way, high levels of SOD were also associated with an increased risk of nuclear cataracts[39].

GPx uses glutathione as an electron donor to reduce organic hydroperoxides. The POLA study analysed the relationship between antioxidant enzymes and age-related macular disease and found out that higher plasma levels of GPx were significantly associated with a nine-fold increase in the prevalence of late AMD, but they were unassociated with early AMD. Delcourt et al. demonstrated a strong association of high levels of plasma GPx with age-related eye diseases. More data are needed at the biochemical and epidemiologic levels for a better understanding of these findings[4,39].

MDA is an abundant oxidation-specific epitope that accumulates in a number of oxidative stress-related diseases. Chou et al. recently demonstrated that 15% of all IgM natural antibodies bound to MDA adducts in mouse plasma[40]. This observation illustrated the importance that the natural selection process of the innate immune system places on MDA. Recently, Weismann et al. identified complement factor H (CFH) as a major MDA-binding protein that could block both the uptake of MDA-modified proteins by macrophages and MDA-induced proinflammatory effects in vivo in mice[38].

As a summary of our study, we should mention that the levels of vitamin E in erythrocyte membranes did not decrease after rheohaemapheresis. These results suggest that tissue concentrations of vitamin E remained unchanged despite a reduction in the lipid carrier in the blood. In serum and lipoprotein fractions, vitamin E decreased. However, more informative data were obtained from the ratio of vitamin E to cholesterol in serum and in various lipoprotein fractions, in which the relative content of vitamin E even improved significantly. The monitored patients did not show any clinical signs of vitamin E deficiency, and their serum concentrations of vitamin E did not differ from normal values.

CONCLUSION

In conclusion, the level of serum vitamin A statistically decreased but not to less than physiological levels. Serum malondialdehyde significantly increased, and erythrocyte superoxide dismutase and erythrocyte glutathione peroxidase were not statistically significantly involved in rheohaemapheresis processes.

Rheohaemapheresis did not result in a negative antioxidant balance. On the contrary, the results show that, rheohaemapheresis might have had favourable additive anti-atherogenic effects due to the relative increase in content of vitamin E in the lipoprotein fractions, in addition to causing a significant reduction in atherogenic LDL.

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9.4. PŘÍLOHA IV

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The effect of LDL-apheresis and rheohaemapheresis treatment on vitamin E

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The Effect of LDL-Apheresis and Rheohaemapheresis Treatment on Vitamin E

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Summary Lipid apheresis (extracorporeal lipoprotein elimination) is administered to patients with familial hypercholesterolemia who fail to respond to standard therapy. The nature of the treatment process raises the suspicion that it decreases not only cholesterol but also antioxidants. A group of 12 patients (average age 47±17 y, 4 homozygous and 8 heterozygous individuals) with familial hypercholesterolemia treated by LDL-apheresis or rheohaemapheresis for 3–12 y was included in the study. In addition to cholesterol and triacylglycerol levels, vitamin E and vitamin A and also other markers of antioxidant activity were investigated. Nevertheless, the most important determined parameter was the vitamin E/cholesterol ratio in serum and lipoproteins. The results indicate that both extracorporeal elimination methods are effective and suitable ways to treat severe familial hypercholesterolemia, as the LDL fraction of cholesterol decreased by approximately 77% and 66% following LDL-apheresis and rheohaemapheresis, respectively. In addition, the serum vitamin E decreased by 54% and 57% and the decrease of the serum vitamin A was approximately 20%. However, the main marker of antioxidant capacity, vitamin E/cholesterol ratio, in the serum, VLDL and LDL significantly increased. The increase of vitamin E levels in the erythrocyte membranes of 2% following LDL-apheresis and a significant increase of 4% following rheohaemapheresis were confirmed. The presented results indicate that LDL-apheresis and rheohaemapheresis can be considered to be safe procedures according to the antioxidant capacity of the serum, VLDL and LDL lipoprotein fractions and the erythrocyte membrane.

Key Words vitamin E, LDL-apheresis, rheohaemapheresis, familial hypercholesterolemia

A complete change in lifestyle, a strict diet and intensive combined pharmacotherapy (HMG CoA reductase inhibitors are the most important components) are necessary for patients with familial hypercholesterolemia (FH) and provide excellent results in most patients. Unfortunately, these measures are not sufficient for all patients (*1–6*). Homozygous FH patients usually fail to show any response to pharmacotherapy. LDL-apheresis has become the standard treatment for individuals homozygous for FH (*7–9*). In homozygous FH, statins provide a range of responses, decreasing the plasma LDL anywhere from 0% to 48%, with an average of approximately 15% for receptor-negative individuals and approximately 26% for receptor-defective individuals (*5*). LDL-apheresis could be offered to treat symptomatic coronary heart disease in individuals who are heterozygous FH and whose LDL remains high (>160 mg/dL, 4.2 mmol/L) or decreases by <40% in response to maximal medical treatment (*1*).

Term LDL-apheresis was originally used in 1981 by Stoffel et al. (*10*) as an immunoadsorption elimination extracorporeal method (*10*). Nowadays, "LDL-apheresis" or "lipoprotein apheresis" is used for all types of extracorporeal eliminations of cholesterol. In our study we used a modification of the Stoffel and Borberg LDL-apheresis–immunoadsorption method and also double filtration plasmapheresis (the primary filter is replaced by a centrifugal separator), which additionally eliminates fibrinogen and has an influence on rheological properties. For our study we will use for the first mentioned method the term "LDL-apheresis" and for the second mentioned method the term "rheohaemapheresis."

LDL-apheresis is an invasive and relatively expensive but safe procedure (*11, 12*); it decreases cholesterol radically but it is also troubling to the patient's standard

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of living. Human LDL particles contain several types of antioxidants, which are important for the regulation of lipid oxidation (*13*). The treatment process is assumed to decrease not only cholesterol but also important antioxidants, such as vitamin E and vitamin A (*7, 13–16*). Additionally, other active compounds, such as enzymes, can be affected by certain methods of extracorporeal lipoprotein elimination. Superoxide dismutase (SOD) eliminates very reactive peroxide radicals and forms hydrogen peroxide with oxygen. Glutathione peroxidase (GP_x) ensures detoxification peroxides (*17, 18*). Malondialdehyde (MDA) is a biomarker that is formed by the lipid peroxidation of unsaturated fatty acids (*18*).

Antioxidant defence systems, such as enzymes or vitamins, protect the host directly and indirectly against the damaging influence of oxidants (*19–22*).

The antioxidant/radioprotective activity of α-tocopherol is thought to be associated with its ability to scavenge reactive oxygen species via its phenolic group (*23*). By controlling the production of free radicals, vitamin E affects free radical-mediated signal transduction events and ultimately modulates the expression of genes that are regulated by free radical signalling (*24*). Alternately, high concentrations of vitamin E can serve as a prooxidant (*25*).

Vitamin E is rapidly transferred from chylomicrons to plasma lipoproteins that it binds to non-specifically; it is then taken up by the liver and incorporated into nascent VLDL (with selectivity in favour of α-tocopherol over the γ-tocopherol), and then secreted by the liver. Vitamin E is also spontaneously transferred to apolipoprotein B-containing lipoproteins, including VLDL, LDL and HDL. Therefore, plasma tocopherols are distributed among these three lipoprotein types, with the more abundant LDL and HDL comprising the major carriers of vitamin E (*24, 26, 27*). For this reason, we investigated the levels of vitamin E in each lipoprotein fraction to more accurately assess pathophysiological processes. The aim of the present work was to evaluate the acute effect of extracorporeal elimination on the levels of vitamins E (α-tocopherol) and A (retinol) of hypercholesterolemic patients who received extracorporeal lipoprotein elimination treatments for a long term (3–12 y). The cholesterol, triacylglycerols (TAG), vitamin E, and vitamin E/cholesterol ratios in serum and the lipoprotein fractions were measured in groups of patients before and after extracorporeal lipoprotein elimination. Additionally, vitamin E was monitored in the erythrocyte membrane, which was considered to be a model of the cell membrane. Moreover, the erythrocyte levels of SOD, GP_x and serum level of MDA were analysed as markers of antioxidant activity and lipid peroxidation, respectively.

METHODS

Blood samples were collected before and after extracorporeal elimination was undertaken. The study protocol was approved by the Ethical Committee of University Hospital in Hradec Králové. Informed consent was obtained from all participants. The research followed the tenets of the Declaration of Helsinki.

The blood was drawn before being rinsed. The blood samples were first centrifuged (2,000 $\times g$, 10 min, 4°C) and then the serum was separated and analysed immediately or frozen at −20°C until analysis. Lipoprotein fractions were prepared from fresh serum using a gradient ultracentrifugation technique, with the addition of a sodium chloride solution of a determined density and 0.1% EDTA to avoid oxidation of the lipoproteins during ultracentrifugation. The serum lipoproteins were separated into very low-density lipoprotein (VLDL, $d<1.006$), low-density lipoprotein (LDL, $d<1.063$) and high-density lipoprotein (HDL, $d>1.063$) fractions using an OPTIMA MAX-XP ultracentrifuge (Beckman Coulter, Fullerton, CA) (*28*).

Patient group. A group of 12 patients received long-term treatments: 7 men and 5 women. The average age was 47±17 y (range, 21–63; median 52 y). The patients included 4 individuals who were homozygous for FH and 8 heterozygous (all heterozygotes were undergoing secondary prevention, with atherosclerotic complications, such as myocardial infarction, bypass, and arterial desobliteration). The MedPed criteria were applied to the patients (*29*) using cut-offs for total cholesterol and LDL-cholesterol levels above the 95 percentile within the Czech population (*30*), age, and family history. Furthermore, DNA-based evidence of a mutation in the low-density lipoprotein receptor (LDLR) gene was the criterion for homozygous FH. None of the patients had a mutation in the Apo-B gene. All patients were treated with high-dose statins, 1 patient in combination with fenofibrate, 2 patients in combination with bile-acid sequestrans, and 10 patients in combination with ezetimibe. The maximally tolerated doses of rosuvastatin or atorvastatin were chosen. Patients with increased levels of fibrinogen were treated by rheohaemapheresis in order to normalize this parameter.

The patients have been regularly treated with LDL-apheresis or rheohaemapheresis for 3–12 y (average, 7±3; range, 2–11; median, 7 y). Both procedures were carried out at an accredited facility, the Blood Cell Separator Centre, University Hospital in Hradec Králové, Czech Republic, using standardised methods and by experienced personnel.

LDL-apheresis technique. Plasma separation was performed using a Cobe-Spectra continuous centrifugal separator or Optia separator (Terumo BCT, Lakewood, CO). An automatic adsorption-desorption device (Adasorb, Medicap, Ulrichstein, Germany) was used to control the repeated fillings and washings of Lipopak 400® adsorbers (Pocard, Moscow, Russia). In two patients, Lipocollect adsorbers (Medicollect, Rimbach, Germany) were used for 32–54 mo. Initially, our target LDL-cholesterol level after the procedure was below 1 mmol/L; currently, a more effective rate of below 0.5 mmol/L has been applied. Normal values are 1.5–3.36 mmol/L.

Rheohaemapheresis technique. Plasma was collected using a Cobe-Spectra or Optia separator (Terumo BCT) following high-speed centrifugation. The plasma were then run through a "second stage" filter (Evaflux 4A or 5A, Kuraray, Tokyo, Japan) with ethylene-vinyl alcohol

hollow fibres with a hole size of 0.04 μm. Further, we ušed a CF-100 (Infomed, Geneva, Switzerland), which regulated inflow of plasma into the filter. The plasma flow was continuous, anticoagulation was carried out using heparin and ACD-A (Baxter, Munich, Germany), and the basic volume of processed plasma was 1.5 times the body volume, as calculated by the computer of the Cobe or Optia separator.

Analytical determination of retinol and α-tocopherol in serum, lipoprotein fractions and erythrocyte membrane. The method used in this study for the analysis of retinol and α-tocopherol in the serum and lipoprotein fractions was modified from the method previously published by Urbánek et al. and is briefly described below (*31*).

In the liquid-liquid extraction (LLE) procedure, 500 μL of serum was deproteinised using cold ethanol with 5% of methanol. Then, *n*-hexane was added to this mixture and extracted using a vortex apparatus. After centrifugation, an aliquot of the clean extract was separated and evaporated in a concentrator. The residue was dissolved in 400 μL of methanol and analysed using a Prominence HPLC system (Shimadzu, Kyoto, Japan). The separation of retinol and α-tocopherol was performed using the Chromolith Performance RP-18e monolithic column (Merck, Darmstadt, Germany). The detection of retinol and α-tocopherol was carried out at 325 and 295 nm, respectively, using a diode array detector.

The level of α-tocopherol in the erythrocyte membrane was analysed using a modified HPLC method previously published by Solichová et al. (*32*). A Chromolith Performance RP-18e monolithic column, 100×4.6 mm (Merck) with the same sample pretreatment that was applied prior to HPLC analysis was used for the chromatographic separation.

Analytical determination of cholesterol and triacylglycerols. The serum cholesterol and TAG were determined using a MODULAR analyser (Hoffmann-La Roche, Basel, Switzerland) with commercially available kits according to the manufacturer's instructions. The same methods were used to evaluate the cholesterol and TAG levels in the lipoprotein fractions.

Analytical determination of enzymes. The erythrocyte glutathione peroxidase was determined spectrophotometrically using a commercial kit (Ransel, Randox, Crumlin, United Kingdom) according to the manufacturer's instructions and the elimination of absorbance at a wavelength of 340 nm (Cobas Mira, Roche, Basel, Switzerland).

The serum malondialdehyde was measured as a red complex with thiobarbituric acid at 485, 532 and 560 nm using a Beckman DU 640 spectrophotometer (Beckman, Palo Alto, CA).

Superoxide dismutase was determined spectrophotometrically using a commercial kit (Ransod, Randox) according the manufacturer's instructions and the elimination of absorbance at a wavelength of 505 nm (Secomam, Ales, France).

Statistical analysis. The NCSS 2007 software (Kaysville, UT) was used to perform statistical evaluations of the changes in each measured parameter before and after extracorporeal elimination therapy. The evaluations were performed using the un-parametrical Mann-Whitney *U* and Wilcoxon Signed-Rank tests. Statistical significance was based on $p \leq 0.05$.

RESULTS

Cholesterol and triacylglycerols

The decreases in the cholesterol levels are presented in Table 1. These results indicate that both extracorporeal elimination methods are effective and suitable treatments, as the cholesterol decreased with both methods in the low-density lipoprotein (LDL) fraction by approximately 77% and 66% for LDL-apheresis and rheohaemapheresis, respectively. Cholesterol was measured for both methods in the various lipoprotein fractions. Levels in the very low-density lipoprotein (VLDL) fraction decreased by 68% and 66%. Levels in the high-density lipoprotein (HDL) fraction exhibited lower decreases relative to those of the other fractions, by 33% and 39% for apheresis and rheohaemapheresis, respectively.

The first three measurements and the last three measurements of the monitored period were compared, and no significant differences were observed for either extracorporeal elimination method. The means of the first and last three measurements of cholesterol levels were 5.86±1.32 mmol/L and 5.60±1.35 mmol/L, respectively.

Triacylglycerols (TAG) exhibited decreases in the serum and in the VLDL and LDL fractions in both methods. The initial levels decreased by approximately half (48–58%). Conversely, the level of TAG in HDL increased during LDL-apheresis by 16%, but this increase was not significant. Rheohaemapheresis decreased the level of TAG in the HDL fraction by 27%. All of the results for cholesterol and TAG are presented in Table 1.

Vitamin E

Both methods of extracorporeal lipoprotein elimination significantly decreased the levels of vitamin E in the serum and all lipoprotein fractions (Table 1). The decreases in the serum were by 54% and 57%, respectively. The levels in the various lipoprotein fractions exhibited decreases that ranged from 36–73%. However, decreases in the vitamin E/cholesterol ratio were not observed in the serum or in the VLDL and LDL fractions, which showed significant increases that ranged from 5–61%. There was only a significant decrease (1–6%) of the vitamin E/cholesterol ratio in the HDL fraction. These results are presented in Table 2.

The results (Table 1) confirmed a slight increase in the level of vitamin E in erythrocyte membranes of 2% during LDL-apheresis (not significant), as well as a significant increase of 4% during rheohaemapheresis.

Vitamin A and other metabolites

Both methods of extracorporeal lipoprotein elimination significantly decreased the levels of vitamin A in the serum (Table 1). The total decrease in this vitamin was approximately 20%.

The erythrocyte superoxide dismutase and erythrocyte glutathione peroxidase did not exhibit any significant changes. Similar results were observed for serum

Table 1. Levels of target analytes in serum, lipoprotein fractions and erythrocytes before and after extracorporeal elimination treatment.

Target analytes	Group	n*	Before procedure		After procedure		Statistical significance
			Average	SD	Average	SD	
Cholesterol-serum (mmol/L)	AP	155	5.34	1.27	1.73	0.89	$p<0.0001$
	RH	144	5.07	1.42	1.96	0.56	$p<0.0001$
	AP+RH	299	5.21	1.34	1.84	0.76	$p<0.0001$
Cholesterol-VLDL (mmol/L)	AP	160	1.03	0.50	0.33	0.27	$p<0.0001$
	RH	146	1.09	0.53	0.37	0.17	$p<0.0001$
	AP+RH	306	1.06	0.51	0.35	0.23	$p<0.0001$
Cholesterol-LDL (mmol/L)	AP	160	3.12	0.92	0.72	0.35	$p<0.0001$
	RH	147	2.86	0.98	0.97	0.44	$p<0.0001$
	AP+RH	307	2.99	0.96	0.84	0.42	$p<0.0001$
Cholesterol-HDL (mmol/L)	AP	160	1.01	0.33	0.68	0.22	$p<0.0001$
	RH	148	0.99	0.44	0.60	0.19	$p<0.0001$
	AP+RH	308	1.00	0.39	0.64	0.21	$p<0.0001$
TAG-serum (mmol/L)	AP	156	1.94	1.42	0.81	0.73	$p<0.0001$
	RH	145	2.28	1.64	1.17	0.69	$p<0.0001$
	AP+RH	301	2.20	1.54	0.98	0.73	$p<0.0001$
TAG-VLDL (mmol/L)	AP	160	1.35	1.22	0.57	0.61	$p<0.0001$
	RH	146	1.62	1.31	0.84	0.57	$p<0.0001$
	AP+RH	306	1.48	1.27	0.70	0.61	$p<0.0001$
TAG-LDL (mmol/L)	AP	160	0.38	0.15	0.18	0.19	$p<0.0001$
	RH	147	0.43	0.16	0.22	0.14	$p<0.0001$
	AP+RH	307	0.40	0.16	0.20	0.17	$p<0.0001$
TAG-HDL (mmol/L)	AP	160	0.12	0.07	0.14	0.19	0.940813
	RH	148	0.15	0.09	0.11	0.06	$p<0.0001$
	AP+RH	308	0.13	0.08	0.12	0.14	$p<0.0001$
Vitamin E-serum (μmol/L)	AP	159	27.00	7.02	11.70	3.36	$p<0.0001$
	RH	144	25.36	8.59	11.69	3.77	$p<0.0001$
	AP+RH	303	26.22	7.83	11.71	3.56	$p<0.0001$
Vitamin E-VLDL (μmol/L)	AP	159	8.88	5.98	3.28	2.49	$p<0.0001$
	RH	146	9.07	5.54	3.68	1.98	$p<0.0001$
	AP+RH	305	8.97	5.77	3.47	2.25	$p<0.0001$
Vitamin E-LDL (μmol/L)	AP	158	12.54	3.03	3.37	1.22	$p<0.0001$
	RH	144	10.97	4.38	3.90	1.67	$p<0.0001$
	AP+RH	302	11.79	3.82	3.62	1.47	$p<0.0001$
Vitamin E-HDL (μmol/L)	AP	160	6.80	2.41	4.34	1.71	$p<0.0001$
	RH	146	5.96	2.10	3.48	1.36	$p<0.0001$
	AP+RH	306	6.40	2.30	3.93	1.61	$p<0.0001$
Vitamin E-ery (μmol/L)	AP	154	3.54	1.47	3.62	1.45	0.128334
	RH	140	3.02	1.36	3.15	1.35	0.002414
	AP+RH	294	3.29	1.44	3.39	1.42	0.001683
Vitamin A-serum (μmol/L)	AP	158	1.57	0.50	1.23	0.43	$p<0.0001$
	RH	145	1.81	0.50	1.46	0.41	$p<0.0001$
	AP+RH	303	1.69	0.51	1.34	0.43	$p<0.0001$
MDA-serum (μmol/L)	AP	108	0.61	0.44	0.57	0.29	0.636777
	RH	40	0.69	0.41	0.73	0.24	0.256012
	AP+RH	151	0.62	0.43	0.61	0.29	0.252002
SOD-ery (U/gHb)	AP	78	1857.04	786.84	1878.26	734.43	0.423970
	RH	27	1958.67	692.09	1993.10	906.20	0.838187
	AP+RH	108	1874.80	756.30	1900.70	775.30	0.489416
GP_x-ery (U/gHb)	AP	73	40.36	11.77	41.05	12.34	0.161705
	RH	26	40.70	12.86	40.60	13.14	0.647540
	AP+RH	102	40.63	11.91	41.01	12.35	0.572619

AP: LDL-apheresis, RH: rheohaemapheresis, ery: erythrocytes.
*Number of pair measurements.

Table 2. Ratios of vitamin E and cholesterol in serum and lipoprotein fractions before and after extracorporeal elimination treatment.

Vitamin E/cholesterol (10^{-3}) in	Group	n*	Before procedure		After procedure		Statistical significance
			Average	SD	Average	SD	
Serum	AP	149	5.73	6.92	9.25	13.33	$p<0.0001$
	RH	142	5.11	1.29	6.22	2.74	$p<0.0001$
	AP+RH	291	5.43	5.03	7.77	9.03	$p<0.0001$
VLDL	AP	155	8.62	3.42	10.49	5.22	$p<0.0001$
	RH	144	8.42	2.58	10.41	3.75	$p<0.0001$
	AP+RH	299	8.50	3.09	10.45	4.57	$p<0.0001$
LDL	AP	155	4.29	1.45	5.02	1.55	$p<0.0001$
	RH	143	3.97	1.74	4.17	1.25	$p<0.0001$
	AP+RH	298	4.14	1.60	4.61	1.47	$p<0.0001$
HDL	AP	157	6.85	2.05	6.46	1.65	0.028095
	RH	146	6.11	1.49	6.05	3.12	0.007528
	AP+RH	303	6.49	1.84	6.26	2.47	$p<0.0001$

AP: LDL-apheresis, RH: rheohaemapheresis.
* Number of pair measurements.

malondialdehyde, which was monitored as a marker for the oxidative degradation or lipoperoxidation of cellular membranes (Table 1).

DISCUSSION

The lipid-soluble vitamin E (tocopherols) antioxidants, including α- and γ-tocopherol, are an important front line defense (*33*). We have confirmed that patients treated using extracorporeal methods undergo a significant decrease in their vitamin E levels in serum and lipoprotein fractions. However, this decrease was partly expected due to the disposition of the treatment methods. It is supposed that vitamin E has suppressive effects on atherosclerosis through antioxidant action (*14*). A reactive oxygen species attack can lead to a major depletion of antioxidants such as vitamin E (*33*). Vitamin E is essential, along with cholesterol, for the structural stability of membranes (*34, 35*). Indeed, a relation between the vitamin E content and resistance against oxidation of LDL has not been demonstrated, unless vitamin E was supplemented (*36*). This vitamin partially controls lipid peroxidation and oxidative stress-related disease including end-storage renal disease (*37*). Vitamin E-coated membranes are proved to increase vitamin E levels in plasma and therefore scavenge reactive oxygen species and reduce oxidative stress (*37*). Circulating tocopherol is well known to be distributed among all classes of lipoproteins, and between lipoproteins and red blood cell membranes (*38, 39*). For this reason, we observed the vitamin E/cholesterol ratios in the serum and in the various lipoprotein fractions. Antioxidant activity was not heavily affected, which was confirmed by examining the ratios of vitamin E/cholesterol, which significantly increased in the serum and the VLDL and LDL fractions and slightly decreased in the HDL fraction. Furthermore, we determined the levels of vitamin E in the erythrocyte membranes, as this vitamin is almost exclusively located in membranes (*24*). Wang and Quinn investigated rat plasma and reported that the levels reached a maximum after 48 h, and the liver was the main organ in which the vitamin had been accumulated. The levels in the kidney, heart, muscle and brain continued to increase throughout a 6-wk period (*40*).

In addition, erythrocytes are the major cellular component of blood, and erythrocyte membrane fluidity can be affected by diseases associated with oxidative stress (*41*). A study by Rajasekaran et al. proposed that the regulation of α-tocopherol levels in membranes is critically important to maintain the erythrocyte membrane structure and function (*42*). Recently, it has been hypothesized that α-tocopherol partitions into domains of membranes that are rich in polyunsaturated phospholipids, amplifying the concentration of the vitamin where it is most needed (*43*). The presented results indicated that vitamin E in erythrocytes did not change after LDL-apheresis and showed only a slight increase (although it was significant) after rheohaemapheresis. These results were also expected, as mentioned above.

Vitamin A is an important micronutrient that has an unusually wide range of vital biological functions (e.g., morphogenesis, vision, embryonic development, reproduction, immune function) in the mammalian system (*44*). In healthy individuals, plasma vitamin A is maintained within a narrow range (0.88–2.76 μmol/L in adults; typically approximately half that in newborn infants) despite widely varying intakes of vitamin/provitamin A. Vitamin A levels are significantly reduced, although not below the normal value, during extracorporeal lipoprotein elimination.

Ascorbate administration can be useful to lower the levels of hydrogen peroxide and proinflammatory mediators in patients undergoing apheresis (*45*). It was also initially hypothesized that ascorbic acid may decrease lipoprotein(a) concentrations; however, a study in 100 healthy people did not confirm this hypothesis (*46*). Though ascorbic acid proved strong scavenging H_2O_2

activity, in our study, we focused only on fat soluble vitamins.

Oxidation of LDL and the formation of lipid hydroperoxides are now widely accepted as a crucial step in atherogenesis. One important decomposition product of lipid hydroperoxides is MDA, which is derived from unsaturated fatty acids carrying more than two double bounds (*47*, *48*). However, according to observation of Schettler et al. (*48*) no difference between hyperlipidemic patients undergoing LDL-apheresis and coronary heart disease patients not undergoing extracorporeal treatment was confirmed. Nevertheless, an increased concentration in both patients groups has been claimed (*48*).

The antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GP_x) work together to remove reactive oxygen species from the cell in order to prevent oxidation of biological material. The enzyme SOD reduces the superoxide anion to hydrogen peroxide, which is further reduced to water by the actions of glutathione peroxidase. Exposure to fluoride induces increased lipid peroxidation measured as malondialdehyde by altering the activity of antioxidant enzymes: SOD, CAT, and GP_x (*49–51*). The major function of GP_x is to reduce soluble hydrogen peroxide and alkyl peroxides (*52*). GP_x uses glutathione as the hydrogen donor, and produces GSSG (glutathione disulfide) and water (*53*). Under normal physiological conditions the pathways of SOD and GP_x are complemented by the additional hepatocellular antioxidants, glutathione (GSH), vitamin E and ascorbate (*49*, *50*).

Protection against toxic effects of oxygen radicals is represented by antioxidant enzymes such SOD and GP_x. SOD seems to be the first line of defence against oxygen radicals generated by phagocytes. Schettler et al. (*48*) suppose that no critical concentration of oxygen radicals is achieved during LDL-apheresis process, since SOD activity is not affected. GP_x is a selenoprotein that reduces lipid and other organic hyperperoxides including peroxide. In agreement with our findings, GP_x was not affected by LDL apheresis. However, Schettler et al. (*48*) observed a decrease of selenium plasma concentration and explained it by partial removal of LDL, since LDL particles play an important role in selenium transportation in plasma.

According to our results, there were significant decreases in the serum vitamin E and lipoproteins. However, the vitamin E/cholesterol ratios significantly increased or slightly decreased in the HDL fraction. These ratios provide the most important information regarding oxidative processes. Additionally, it was confirmed that the levels of vitamin E remained constant in the membranes. The vitamin A levels were significantly reduced, but not below the baseline value. The other measured compounds, such as the serum MDA, erythrocyte SOD, and erythrocyte GP_x were not significantly affected by the LDL-apheresis or rheohaemapheresis processes.

The presented results indicate that, based on the serum oxidation activity of the lipoprotein fractions and the erythrocyte membrane, LDL-apheresis and rheohaemapheresis can be considered to be safe procedures for the treatment of familial hypercholesterolemia.

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9.5. PŘÍLOHA V

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Prognostic Significance of Serum and Urinary Neopterin Concentrations in Patients with Rectal Carcinoma Treated with Chemoradiation

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Prognostic Significance of Serum and Urinary Neopterin Concentrations in Patients with Rectal Carcinoma Treated with Chemoradiation

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Abstract. *Aim: To analyze the prognostic significance of serum and urinary neopterin concentrations in patients with rectal adenocarcinoma treated with (chemo)radiation. Patients and Methods: Urinary and serum neopterin and peripheral blood cell count were determined in 49 patients with rectal carcinoma before the start of (chemo)radiation. Results: Neopterin concentrations exhibited a significant inverse correlation with hemoglobin and positive correlation with leukocyte count, platelet count and platelet-to-lymphocyte ratio. Increased serum neopterin concentration was associated with significantly inferior relapse-free survival (RFS) and overall survival. However, a significant association was observed only in 28 patients treated in the neoadjuvant setting. Although increased urinary neopterin was also associated with inferior RFS and overall survival, this was not statistically significant. The neutrophil-to-lymphocyte ratio was also associated with poor prognosis. Conclusion: The data presented herein indicate a prognostic significance of serum neopterin concentrations in patients with rectal cancer treated with neoadjuvant chemoradiation.*

Rectal carcinoma is a common tumor associated with a relatively high mortality rate. In addition to surgery, external-beam radiation and systemic chemotherapy play an important role in the management of patients with rectal carcinoma. It has been demonstrated in prospective clinical trials that the concomitant combination of external-beam radiation and chemotherapy improves outcome (1, 2). However, this combined modality approach is associated with relatively high morbidity and serious, sometimes even fatal, adverse events. The identification of parameters that would predict prognosis or complications is therefore of potential importance in patient management. These prognostic and predictive factors may be determined clinically, as well as in the laboratory.

Laboratory biomarkers play an increasingly important role in medical oncology (3). While most attention so far has been focused on biomarkers associated with the activity of the tumor cells, there is mounting evidence that biomarkers that reflect the host response to neoplasia are also of paramount importance. These biomarkers can be assessed either in the tumor tissue (*e.g.* the presence of tumor-infiltrating lymphocytes) (4-6) or in body fluids (7, 8). Neopterin is a pteridine compound produced by macrophages activated by interferon-gamma. Urinary and serum neopterin concentrations have been established as a biomarker of systemic immune and inflammatory response across a range of different disorders (9-11). Notably, increased concentrations of neopterin have been reported in most malignant disorders, including colorectal carcinoma (11). Robust biomarkers of inflammatory response may be obtained by calculating the ratios of lymphocytes to other cellular components of peripheral blood (12). The neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR) and platelet-to-lymphocyte ratio (PLR) represent independent prognostic biomarkers across a spectrum of solid tumors, including colorectal carcinoma (13-17).

Although several studies have investigated the prognostic significance of neopterin concentration in patients with colorectal carcinoma, to the best of our knowledge no

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investigations have so far focused on the prognostic significance of neopterin in patients with rectal carcinoma treated with chemoradiation. The aim of the present study was to analyze the prognostic significance of serum and urinary neopterin concentrations in this clinical setting.

Patients and Methods

Forty-nine consecutive patients, 32 males and 17 females, aged (mean±standard deviation) 67±8 (range=54-85) years with histologically verified rectal carcinoma treated at the Department of Oncology, Palacký University Medical School and Teaching Hospital in Olomouc were included in the present study. Forty-seven patients had adenocarcinoma, while neuroendocrine carcinoma and squamous cell carcinoma were present in one case each. Eleven patients had stage IIA rectal carcinoma, four stage IIC, five stage IIIA, 17 stage IIIB and 12 had stage IIIC. All patients were treated with external-beam radiation administered as intensity-modulated radiation therapy or three-dimensional conformal radiotherapy as described before (18). All but five patients were treated with concomitant chemotherapy (infusional 5-fluorouracil or capecitabine). Twenty-eight patients were treated in the neoadjuvant and 21 patients in the adjuvant setting. Urinary neopterin, serum neopterin, serum creatinine and peripheral blood cell count were determined as described earlier (18, 19), and NLR, LMR and PLR were calculated. This was a part of an investigation studying biomarkers of toxicity in this population, and the results on changes of citrulline and neopterin concentrations during chemoradiation were reported separately elsewhere (18). The investigations were approved by our institutional ethical committee and the patients signed informed consent.

Correlations were analyzed using Spearman's rank correlation coefficient. Relapse-free survival (RFS) was defined as interval between the start of therapy and the diagnosis of relapse or death. Overall survival (OS) was defined as the interval between the diagnosis and death. Patients without a RFS or OS event were censored at the last visit. RFS and OS were evaluated with the Kaplan–Meier method, and the differences between subgroups of patients defined by clinical parameters or dichotomization of neopterin concentrations were studied by log-rank test. Cutoffs for dichotomization were selected based on prior reports (11, 13, 14, 18, 20) or upper limits of normal values. Multivariate analysis was performed using Cox regression. All dichotomized clinical and pathological variables were entered into a hierarchical forward and switching model and the results were expressed as hazard ratio (HR). The decision on statistical significance was based on $p=0.05$ level. The analyses were performed using NCSS software (Number Cruncher Statistical Systems, Kaysville, UT, USA).

Results

Apart from higher LMR and a trend for lower hemoglobin concentration in patients treated in the adjuvant setting, no difference was observed in serum neopterin, urinary neopterin, peripheral blood leukocyte counts and peripheral blood platelet counts and ratios between patient treated with preoperative (neoadjuvant) and postoperative (adjuvant) radiation (Table I). No significant difference in the investigated parameters was observed between patients with stage II and those with stage III tumors.

Serum and urinary neopterin concentrations exhibited a significant inverse correlation with hemoglobin and a positive correlation with age and platelet count (Table II). In addition, serum neopterin correlated significantly with the leukocyte count. A significant positive correlation was observed for urinary and serum neopterin/creatinine ratios with PLR, but not with NLR or LMR.

A trend for inferior RFS (median=17.4 months *vs.* not reached; $p=0.20$) and significantly inferior OS (29.7 months *vs.* not reached; $p=0.006$) was observed in patients treated in the neoadjuvant setting compared to patients treated with adjuvant chemoradiation. The staging of patients treated in the neoadjuvant and adjuvant setting was not comparable since the stage was determined based on clinical criteria in patients treated with neoadjuvant therapy, while pathological staging was used in the adjuvant setting.

In the entire cohort, a serum neopterin concentration of 3 μg/l (11.86 nmol/l) or more was associated with significantly inferior RFS (median 5.5 months *vs.* not reached; $p=0.049$; Figure 1) and OS (median 17.4 months *vs.* not reached; $p=0.009$; Figure 2). Serum neopterin 3 μg/l or more was a significant predictor of poor RFS (median 2.8 months *vs.* not reached; $p=0.001$; Figure 3) and OS (median 9.3 months *vs.* not reached; $p=0.0006$; Figure 4) in patients treated with neoadjuvant chemoradiation, but not among patients treated in the adjuvant setting (data not shown). In fact, none of the patients treated with neoadjuvant chemoradiation with neopterin concentrations 3 μg/l or more survived. A serum neopterin/creatinine value of 146 μmol/mol creatinine or more was associated with also significantly inferior OS (median=16.9 months *vs.* not reached; $p=0.016$), but the difference in RFS did not reach statistical significance (median 8 months *vs.* not reached; $p=0.093$). A serum neopterin/creatinine value of 146 μmol/mol creatinine or more was associated with significantly inferior RFS (median 3 months *vs.* not reached; $p=0.025$) and OS (median 9.3 months *vs.* not reached; $p=0.009$) in patients treated with neoadjuvant chemoradiation. Although increased (≥214 μmol/mol creatinine) urinary neopterin was also associated with inferior RFS (median 16.3 months *vs.* not reached) and OS (medians not reached), this was not statistically significant ($p=0.248$ and $p=0.337$, respectively). Age (<65 *vs.* ≥65 years), stage (stage II *vs.* III), grade (≤2 *vs.* 3), hemoglobin concentration (<120 g/l *vs.* ≥120 g/l), leukocyte (<10.0×10^9/l *vs.* ≥10.0×10^9/l) and platelet counts (<400×10^9/l *vs.* ≥ 400×10^9/l) were not associated with the outcome (data not shown). NLR of 3 or more was also associated with worse OS (median 29.7 months *vs.* not reached; $p=0.034$), but not RFS (medians not reached; $p=0.867$). LMR of 3 or more, and PLR of 150 or more were not predictive of RFS or OS (data not shown). When

Table I. *Values of biomarkers in patients treated in the neoadjuvant and adjuvant setting.*

Parameter	Setting		*p*-Value
	Neoadjuvant	Adjuvant	
Serum neopterin (μg/l)	3.2±4.2	2.3±1.2	0.518
Serum neopterin/creatinine ratio (μmol/mol creatinine)	170±237	120±59	0.739
Urinary neopterin (μmol/mol creatinine)	197±106	196±98	0.909
Age (years)	68±8	66±7	0.479
Hemoglobin (g/l)	129±21	119±14	0.055
Leukocyte count (10^9/l)	8.4±3.5	7.1±2.3	0.132
Platelet count (10^9/l)	278±91	277±89	0.906
NLR	4.0±2.8	3.1±1.4	0.193
LMR	2.5±0.8	3.2±0.9	**0.027**
PLR	188±85	183±73	0.897

NLR: Neutrophil-to-lymphocyte ratio; LMR: lymphocyte-to-monocyte ratio; PLR: platelet-to-lymphocyte ratio. Values shown represent the mean±standard deviation. Significant differences are highlighted by bold type.

Table II. *Spearman's rank correlation coefficients (corresponding p-value) between neopterin concentration and other biomarkers.*

	Serum neopterin (μg/l)	Serum neopterin/ creatinine ratio (μmol/mol creatinine)	Urinary neopterin (μmol/mol creatinine)
Age (years)	0.429	0.262	0.289
	(0.002)	(0.069)	**(0.046)**
	n=49	n=49	n=48
Hemoglobin (g/l)	−0.416	−0.497	−0.397
	(0.004)	**(0.0004)**	**(0.007)**
	n=47	n=47	n=46
Leukocyte count (10^9/l)	0.306	0.179	0.106
	(0.036)	(0.230)	(0.483)
	n=47	n=47	n=46
Platelet count (10^9/l)	0.379	0.413	0.406
	(0.009)	**(0.004)**	**(0.005)**
	n=47	n=47	n=46
NLR	0.151	0.182	0.085
	(0.311)	(0.220)	(0.576)
	n=47	n=47	n=46
LMR	−0.046	−0.131	−0.084
	(0.756)	(0.380)	(0.579)
	n=47	n=47	n=46
PLR	0.237	0.346	0.355
	(0.108)	**(0.017)**	**(0.015)**
	n=47	n=47	n=46

NLR: Neutrophil-to-lymphocyte ratio; LMR: lymphocyte-to-monocyte ratio; PLR: platelet-to-lymphocyte ratio. Significant correlations are highlighted by bold type.

evaluated separately in patients treated in neoadjuvant or adjuvant settings, age, stage, grade, hemoglobin concentration, leukocyte and platelet counts, NLR, LMR and PLR were not predictive of RFS nor OS (data not shown).

Because of significantly different RFS and OS in patients treated in the adjuvant and neoadjuvant setting, these cohorts of patients were evaluated separately. In multivariate analysis that included age, stage, grade, serum and urinary neopterin, hemoglobin concentration, leukocyte and platelet counts, NLR, LMR and PLR, a serum neopterin level of <3 μg/l was the only independent predictor of RFS (HR=0.14, 95% confidence interval=0.04-0.45; p=0.001) and OS (HR=0.12, 95% confidence interval=0.03-0.56; p=0.007) in patients treated in the neoadjuvant setting, but no independent predictor of OS was identified in patients treated with adjuvant chemoradiation.

Discussion

The data presented herein demonstrate a prognostic significance of neopterin concentrations in patients with rectal carcinoma treated with chemoradiation. In earlier studies, prognostic significance was demonstrated for patients with colorectal carcinoma presenting at different stages (21), as well as in patients with metastatic colorectal carcinoma (11). Urinary neopterin concentrations were studied in these previous reports (11, 21). Interestingly, prognostic significance was evident for serum, but not for urinary concentrations of neopterin in the present study. Moreover, serum neopterin predicted prognosis only in patients treated in the neoadjuvant setting. The absence of an association between neopterin concentrations and outcome in patients treated with adjuvant radiation could be explained by a confounding effect of inflammatory reaction induced by previous surgery. It is well known that surgical intervention results in a marked increase of neopterin concentrations (22, 23). On the other hand, in patients scheduled to be treated by neoadjuvant therapy, increased neopterin concentrations are predominantly the result of the host–tumor interaction that is associated with impaired immune response. Moreover, in a previous investigation in the present cohort of patients, we have reported an association between higher baseline serum neopterin/creatinine ratio and toxicity of therapy (18).

As expected from previous reports (11), a significant correlation was observed between neopterin concentrations and hemoglobin and platelet count. The pathogenesis of anemia in patients with rectal carcinoma may be complex and may involve chronic blood loss, but the finding of a negative association between hemoglobin and neopterin concentrations supports the hypothesis that systemic immune activation is a major mechanism responsible for the anemia of chronic disease observed in rectal carcinoma. The positive

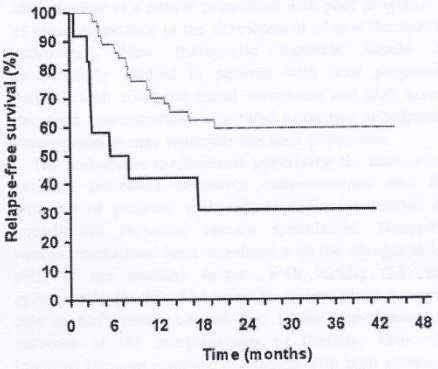

Figure 1. *Kaplan-Meier curve of relapse-free survival of patients with serum neopterin <3 μg/l (dashed line) compared to those with ≥3 μg/l (solid line; whole cohort): median not reached vs. 5.5 months; p=0.049 (n=49).*

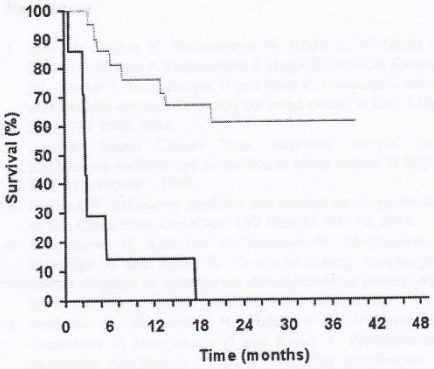

Figure 3. *Kaplan-Meier curve of relapse-free survival of patients with serum neopterin <3 μg/l (dashed line) compared to those with ≥3 μg/l (solid line) treated with neoadjuvant radiation: median not reached vs. 2.8 months; p=0.001 (n=28).*

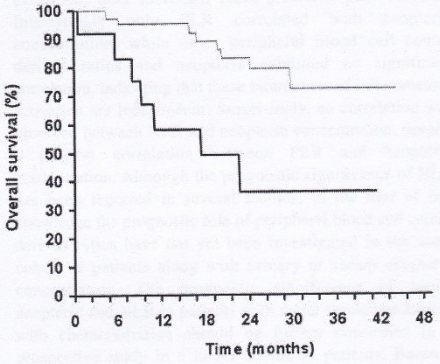

Figure 2. *Kaplan-Meier curve of overall survival of patients with serum neopterin <3 μg/l (dashed line) compared to those with ≥3 μg/l (solid line; whole cohort): median not reached vs. 17.4 months; p=0.009 (n=49).*

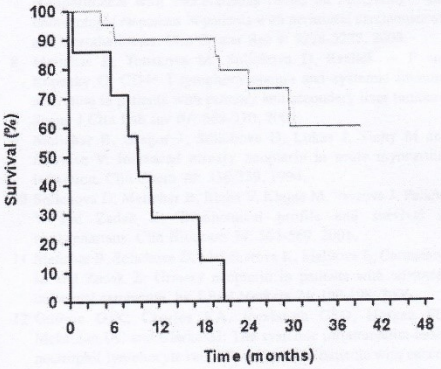

Figure 4. *Kaplan-Meier curve of overall survival of patients with serum neopterin <3 μg/l (dashed line) and ≥3 μg/l (solid line) treated with neoadjuvant radiation (median not reached vs. 9.3 months; p=0.0006; (n=28).*

association between platelet counts and neopterin may also be explained by the effect of systemic immune activation. Cytokines, *e.g.* interleukin-6, are potent inducers of thrombopoiesis.

The subgroups of patients treated in the neoadjuvant or adjuvant settings were not comparable, and the worse outcome of patients treated with neoadjuvant chemoradiation probably reflects more advanced disease that was understaged by imaging studies. The outcome of patients with increased serum neopterin treated in the neoadjuvant setting seems to be particularly poor. Obviously, these results need to be confirmed in a larger prospective cohort. The

identification of a patient population with poor prognosis is of great importance in the development of new therapeutic approaches. New therapeutic methods should be preferentially studied in patients with poor prognosis. Patients with advanced rectal carcinoma and high serum neopterin concentrations scheduled to receive neoadjuvant chemoradiation may represent one such population.

The pathogenic mechanisms underlying the association between increased neopterin concentrations and the outcome of patients with rectal carcinoma treated by neoadjuvant radiation remain speculative. Neopterin concentrations have been correlated with the changes in the cells of the immune system both locally (24) and systemically (8, 25). The immune system plays a crucial role in both tumor control and in the prevalence and outcome of the complications of therapy. Thus, the impaired immune response associated with high neopterin concentration could result in enhanced tumor growth, and a higher rate and severity of complications, resulting in decreased survival. Moreover, higher neopterin concentrations were shown to be associated with the presence of comorbid conditions in patients with cancer (26), which may also determine the patient outcome.

Among the peripheral blood cell count-derived ratios examined, only increased NLR predicted poor survival. Interestingly only PLR correlated with neopterin concentration, while other peripheral blood cell count-derived ratios and neopterin exhibited no significant correlation, indicating that these biomarkers of inflammatory activation are independent. Surprisingly, no correlation was observed between NLR and neopterin concentration, despite a positive correlation between PLR and neopterin concentration. Although the prognostic significance of NLR has been reported in several studies, to the best of our knowledge the prognostic role of peripheral blood cell count-derived ratios have not yet been investigated in the same cohort of patients along with urinary or serum neopterin concentration. The prognostic significance of serum neopterin and NLR in patients with rectal carcinoma treated with chemoradiation should be further confirmed in a prospective study in a larger cohort of patients. Because NLR and neopterin are independent biomarkers, it would be possible to combine these biomarkers in a more complex index reflecting immune and inflammatory activation.

In conclusion, the present data demonstrate a prognostic significance of serum neopterin concentration in patients with rectal cancer treated with chemoradiation, particularly for those treated in the neoadjuvant setting.

Acknowledgements

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9.6. PŘÍLOHA VI

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The effect of processing and storage on α-tocopherol and retinol levels in breast milk

Odesláno do časopisu Analytical Letters (IF2016 = 1,150)

The effect of processing and storage on α-tocopherol and retinol levels in breast milk

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Key words: pasteurisation; freezing storage; human milk; retinol; α-tocopherol

Abstract:

The effect of pasteurisation and storage on α-tocopherol and retinol levels in human breast milk from 25 healthy lactating mothers was studied. Both vitamins were determined in unprocessed human breast milk, in milk after pasteurisation and during the storage period.

Pasteurisation or freezing (-27 °C for 12 weeks) did not significantly decrease concentrations of α-tocopherol and retinol in comparison with their concentrations in fresh breast milk. Results obtained in our study demonstrated that pasteurisation and storage conditions in the Human Milk Bank of the University Hospital Hradec Králové did not have any damaging effect on the levels of target analytes in the human milk. It can be concluded, that infants feeded by stored human breast milk from the Milk Bank for 12 weeks will have the same content of of these important components as in fresh unprocessed breast milk.

1. Introduction

α-Tocopherol (major form of vitamin E) is one of the main antioxidants in the human milk. It is important for elimination of free radicals, prevents the propagation of lipid peroxidation, and protects tissues against chromosome damage and DNA oxidation (Francis et al. 2012; Martysiak-Zurowska et al. 2013). Retinol (vitamin A) belongs also to the antioxidant vitamins. Retinol is essential for vision, growth and differentiation of cells, immune system and embryogenesis (Goss et al. 1992). Vitamin A deficiency is also associated with increased morbidity and mortality in infants and children (Francis et al. 2012; Lindmark-Mansson et al. 2000).

Low-temperature long-time (LTLT) pasteurisation, known as holder pasteurisation is the most routinely used pasteurisation method in human milk banks (Updegrove 2005). However, this heat treatment can decrease levels of key biochemical components, such as immunoglobulins, lactoferrin or of some vitamins (da Costa, do Carmo et al. 2003; Mayayo et al. 2014; Romeu-Nadal et al. 2008; Vanzoerengrobben et al. 1987). Holder pasteurisation provides a compromise between microbiological safety and preservation of biological and nutritional components of the human milk (Abramovich et al. 2013). In this study the pasteurisation procedure elaborated in the Human Milk Bank of the University Hospital Hradec Králové was used (62,5°C for 20 minutes followed by rapid chilling) (Mericka et al. 2003, Spelina et at. 2007, Schlemmerova et al. 2009).

Changes in the components of the human milk (vitamins A, B, C and E, proteins and lipids) may also occur during storage. The losses of some micronutrients are mainly influenced by the temperature and the duration of storage (Giribaldi et al. 2013; Lacomba et al. 2012; Molinari et al. 2011). Human milk is usually cold stored by refrigeration at 4-6 °C for 48-72 h, or by freezing at temperatures around -20 °C for 15-90 days (Lacomba et al. 2012).

The aim of this work was to investigate the effects of holder pasteurisation and 12 weeks storage at -27 °C on the levels of α-tocopherol and retinol in human milk.

2. Materials and methods

2.1. Sample collection and pasteurisation

Human milk samples (volume 50 mL) originating from mothers in the range of 7th - 53rd day of lactation (25 healthy breastfeeding women, 26-39 years old) were collected into sterile glass bottles. Milk samples were transported to the Human Milk Bank of the University Hospital Hradec Králové and immediately divided into seven aliquots (4 mL). The first aliquot was used for the analysis before processing, the second one for analysis after pasteurisation. Another five aliquots were stored in a freezer at -27 °C for analyses after 1, 2, 4, 8 and 12 weeks of storage. All analyses were performed in triplicate, and all milk samples were thawed before processing in a warm water bath at 36 °C.

The study protocol was approved by the Ethical Committee of University Hospital Hradec Králové. Informed consent was obtained from all participating mothers.

Pasteurisation of the human milk was carried out using the holder pasteurisation (LTLT pasteurisation) technique, following the procedure of the Human Milk Banking Association of North America (HMBANA) (Updegrove, 2005). Samples of the human milk were placed in 250 mL glass bottles and submerged in the water bath that was preheated to 62.5 °C. A control bottle with a calibrated thermometer and saline water were placed into this water bath together with all the other bottles, to monitor the temperature of the milk during heat treatment. The heating process was allowed to continue for 20 min after reaching control bottle 5 values over 62.5 °C (Schlemmerova et al. 2009; Spelina et al. 2007). After this procedure, the donor milk was rapidly cooled in an ice bath. After pasteurisation, the milk was frozen and stored for a period not exceeding 3 months.

2.2.Instrumentation

Stirring of the samples before deproteinization and saponification was performed on Orbital test tube shaker LabDancer IKA (Staufen, Germany). The horizontal laboratory shaker LT1 Kavalier (Votice, Czech Republic) was used for extraction of vitamins. For heating during saponification Thermostat Stericell BMT (Brno, Czech Republic) was utilized. Centrifuge 5810-R Eppendorf

(Prague, Czech Republic) and the laboratory vacuum evaporator Concentrator 5301 Eppendorf (Hamburg, Germany) were used for separation and evaporation of organic layer.

HPLC analysis of the target vitamins was performed using a HPLC system Prominence Shimadzu (Kyoto, Japan) equipped with Rack changer/C autosampler for microtitrate plates, degasser DGU-20A5, pump LC20-AB, column oven CTO-20AC, diode array detector SPD-M20A and communication bus module CBM-20A.

2.3. α-Tocopherol and retinol analyses

α-Tocopherol and retinol were extracted from human milk according to the procedure previously developed by the authors (Kasparova et al. 2012). Briefly, 2 mL of cold ethanol and 1 mL of ascorbic acid were added to 0.5 mL of human milk in order to protect vitamins, followed by saponification with KOH (0.1M) for 30 min, at 80 °C in a thermostat. Then, 2 mL of hexane was added and the mixture was vigorously shaken for 5 min and centrifuged (10 min, 3 220 × g). The 1.5 mL of organic phase was poured into a new tube and evaporated, the residue was dissolved in 375 µL of methanol, and 20 µL was then injected into the HPLC system. Extraction of the vitamins from milk samples was done in low-light room and samples were protected by aluminium foil before light to prevent oxidation of the vitamins.

Concentrations of α-tocopherol and retinol were determined by RP-HPLC method Krcmova et al. (2010). Chromatographic separation of the vitamins was performed on the Chromolith Performance RP-18e, 100 mm × 4.6 mm monolithic column Merck (Darmstadt, Germany). Methanol was used as a mobile phase, and the flow rate of the mobile phase was 2.5 mL/min. The diode array detection of α-tocopherol and retinol was carried out at 295 nm and 325 nm, respectively. The overall time of analysis was 1.8 min.

The recovery (accuracy) of the method was tested on the three levels of spiked human milk samples. The recoveries of the retinol from milk samples were between 82-90 % and α-tocopherol 92-109 %. The precision was measured like repeatability on six samples of human milk. RSD (relative standard deviation) reached value 3 % and 5 % for retinol and α-tocopherol, respectively. Quantification was carried out using external standard (Sigma Aldrich, Prague, Czech Republic)

calibration curves included α-tocopherol in the range 0.5 – 50.00 μmol/L and retinol in the range 0.25 – 10.00 μmol/L. The linearity of the analytical method was confirmed by assessment the statistical significance of correlation coefficients (0.9998 for retinol and 0.9999 for α-tocopherol). Limit of detection and limit of quantification were 0.13 and 0.27 μmol/L for retinol, for α-tocopherol 0.09 and 0.19 μmol/L.

2.4. Statistical analyses

The NCSS 2007 software (Kaysville, USA) was used to perform statistical evaluations of the changes in α-tocopherol and retinol levels before and after pasteurisation and during 12 weeks of storage. Differences between the fresh milk samples, processed samples, and samples after different time of storage were evaluated by the one-way analysis of variance ANOVA. Nonparametric Wilcoxon Signed-Rank test was carried out to compare the median values of the analysed variables. The decision on statistical significance was based on $p < 0.05$.

3. Results and discussion

The mean values of vitamin concentrations with standard deviation detected prior to and after pasteurisation and during 12 weeks storage period at -27 °C are shown in Table 1. Graphically depicting of medians with standard deviation and range of variation of retinol and α-tocopherol values before and after pasteurization are presented on Fig.1 and Fig.2 and during freezing storage on Fig.3 and Fig.4. The human milk contains approximately 1.8 μmol/L of retinol and 13.8 μmol/L of α-tocopherol. The recommended vitamin A intake level for infants up to 6 months was set at 400 μg per day/ 1.4 μmol/L (Nancy et al. 2002). The recommended dose of vitamin E for infants is 3 mg per day/ 7 μmol/L (National Research Council) or 4 mg per day/ 9 μmol/L (Food and Nutrition Board) (Antonakou et al. 2011). These recommendation meets content of vitamin A and E in donor human milk from the Milk bank of the University Hospital Hradec Králové even after pasteurisation and 3 months after freezing storage. Holder pasteurisation and storage by freezing for 12 weeks did not decrease the levels (statistically not significant) of α-tocopherol and retinol in the banked human milk.

136 However, slightly (statistically not significant) lower levels of retinol in the pasteurised milk than in
137 the fresh milk samples taken before the heat treatment were noted.
138 The results of influence of pasteurisation and storage by freezing recorded in the present study
139 were very similar to the results published by Vanzoerengrobben et al. (1987) and by Molto-Puigmarti
140 et al. (2011). These authors claimed that holder pasteurisation had no effect on the levels of vitamins
141 studied in banked human milk.
142 In contrast of the results obtained in this study, a significant decrease in the concentration of α-
143 tocopherol about 17 % and 25 % in pasteurised human milk was shown by Romeu-Nadal (2008a) and
144 Delgado (2014) respectively. This degradation of α-tocopherol can be caused due to the oxygen and
145 light exposition during processing of the samples or to the different matrix composition. Under
146 different pasteurisation conditions (73 °C for 10 min.) Erb et al. (1981) reported a loss in levels of α-
147 tocopherol at about 34 % and showed that the higher temperature of pasteurisation can lead to greater
148 decreases in α-tocopherol levels in processed human milk.
149 The present results are in agreement with those reported by Jansson, Akesson et al. (1981),
150 Moffatt et al. (1987), Romeu-Nadal et al. (2008b) and Lacomba (2012), who demonstrated no
151 significant decrease of tocopherols in human milk during frozen storage. It seems that freezing can
152 assure higher stability of tocopherol levels than storage in refrigerators. Differences from 16 - 25 % in
153 the levels of α-tocopherol between the fresh human milk and human milk chilled at 4 °C (48 – 96 h)
154 were detected by Romeu-Nadal et al. (2008b).
155 The pasteurisation process followed by freezing and thawing used in the Human Milk Bank of
156 the University Hospital Hradec Králové for treatment of human milk did not affect the levels of
157 retinol, which is reported to be a very labile compound. These results are consistent with those of Goes
158 et al. (2002) and Vanzoerengrobben et al. (1987). However, the high reduction (34 %) of retinol levels
159 after pasteurisation was reported by Ribeiro et al. (2005). This could be affected by exposure to light
160 during freezing and re-bottling and by the defrosting in a microwave oven (Ribeiro et al. 2005; Sieber
161 et al. 1996).
162

4. Conclusions

The present study performed on breast milk samples collected from 25 mothers did not prove any negative effect of holder pasteurisation as well as of storage by freezing on the levels of α-tocopherol and retinol in human milk. According to these results, pasteurisation and storage in the freezer at -27 °C for 12 weeks, can be regarded suitable for maintaining the nutritional protective values of these substances for infant feeding.

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248

Table 1. Comparison of mean ± standard deviation of levels of α-tocopherol and retinol (μmol/L) in human milk as affected by pasteurisation and storage processes.

Analyte (μmol/L)	α-Tocopherol				Retinol			
	N[a]	Mean	SD[b]	Statistical significance	N[a]	Mean	SD[b]	Statistical significance
Fresh samples	25	14.91	10.88		25	1.95	1.31	
After pasteurisation	25	14.58	11.44	0.969039	25	1.88	1.14	0.084765
1 week after storage	19	13.63	7.08	0.136299	19	1.88	1.04	0.30422
2 weeks after storage	19	13.49	7.20	0.055816	19	1.90	1.08	0.951818
4 weeks after storage	19	13.97	7.41	0.793568	19	1.96	1.15	0.397202
8 weeks after storage	15	13.94	6.31	0.211016	15	1.82	0.99	0.255124
12 weeks after storage	15	13.17	6.38	0.292993	15	1.82	1.06	0.292603

[a] number of mother milk samples
[b] standard deviation

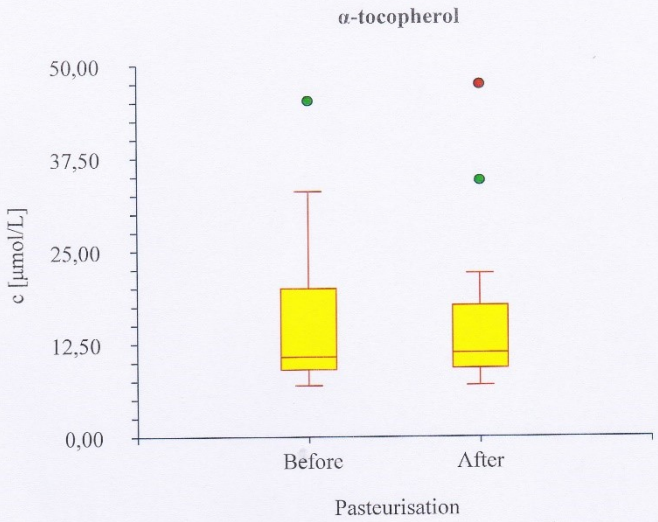

Fig.1. Levels of α-tocopherol in breast milk before and after pasteurisation.

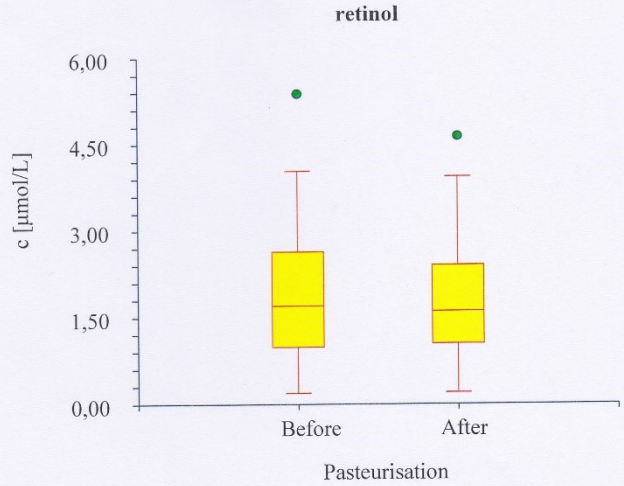

Fig.2. Levels of retinol in breast milk before and after pasteurisation.

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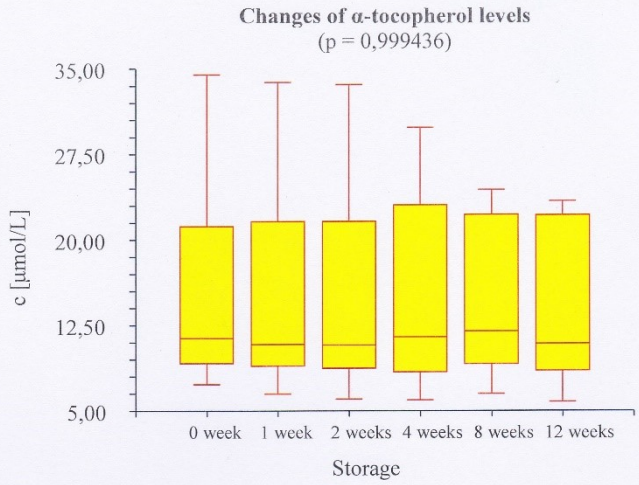

Fig.3. Changes of α-tocopherol levels in breast milk during freezing storage.

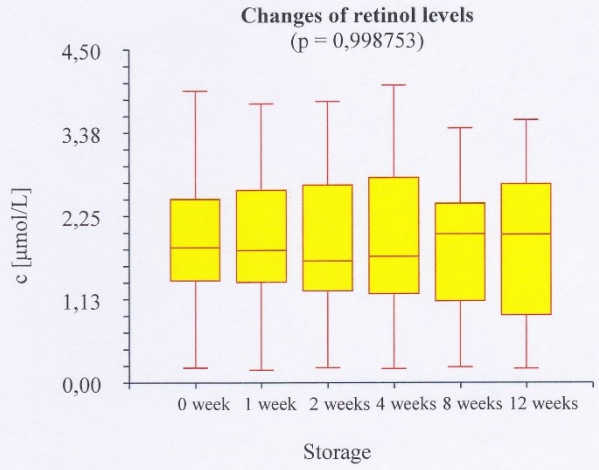

Fig.4. Changes of retinol levels retinol in breast milk during freezing storage

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