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Determination of retinol and retinyl esters in human plasma by high-performance liquid chromatography with automated column switching and ultraviolet detection

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Abstract

A HPLC method with automated column switching and UV detection is described for the simultaneous determination of retinol and major retinyl esters (retinyl palmitate, retinyl stearate, retinyl oleate and retinyl linoleate) in human plasma. Plasma (0.2 ml) was deproteinized by adding ethanol (1.5 ml) containing the internal standard retinyl propionate. Following centrifugation the supernatant was directly injected onto the pre-column packed with LiChrospher 100 RP-18 using 1.2% ammonium acetate–acetic acid–ethanol (80:1:20, v/v) as mobile phase. The elution strength of the ethanol containing sample solution was reduced by on-line supply of 1% ammonium acetate–acetic acid–ethanol (100:2:4, v/v). The retained retinol and retinyl esters were then transferred to the analytical column (Superspher 100 RP-18, endcapped) in the backflush mode and chromatographed under isocratic conditions using acetonitrile–methanol–ethanol–2-propanol (1:1:1:1, v/v) as mobile phase. Compounds of interest were detected at 325 nm. The method was linear in the range 2.5–2000 ng/ml with a limit of quantification for retinol and retinyl esters of 2.5 ng/ml. Mean recoveries from plasma were 93.4–96.5% for retinol (range 100–1000 ng/ml) and 92.7–96.0% for retinyl palmitate (range 5–1000 ng/ml). Inter-assay precision was ≤5.1% and ≤6.3% for retinol and retinyl palmitate, respectively. The method was successfully applied to more than 2000 human plasma samples from clinical studies. Endogenous levels of retinol and retinyl esters determined in female volunteers were in good accordance with published data.

Keywords: Retinol; Retinyl esters

1. Introduction

Vitamin A, a fat-soluble vitamin, is an essential nutrient for animals and humans [1]. Vitamin A and its metabolites function in vision, cell differentiation, immune response and also embryonic development [2]. Measuring plasma retinol concentrations is a common method to assess vitamin A status. Since plasma retinol levels are, however, homeostatically controlled over a wide concentration range, the interpretation of retinol concentrations alone can be difficult. For the determination of vitamin A absorption from the gut, however, the absorption kinetics of retinyl esters in plasma can be used as good approximation.

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High-performance liquid chromatography (HPLC) with UV detection is the technique most commonly used for the determination of retinoids in biological samples [3–8]. However, the determination of retinoids at endogenous concentrations often requires more sensitive methods. The use of a microbore column switching system with UV detection substantially improved the detection limits for retinoic acid [9]. Molander et al. determined retinoic acids by packed-capillary liquid chromatography in combination with large volume on-column focusing [10]. Sample volumes up to 100 μl were injected resulting in a detection limit of 5 ng/ml. A similar system using temperature-programmed packed capillary liquid chromatography (LC) on a C_{30}-based stationary phase was recently developed for the determination of retinyl esters in arctic seal liver samples [11]. With this method a concentration limit of detection (LOD) of 2.7 ng/ml with respect to retinyl palmitate was obtained. Andreoli et al. investigated the suitability of UV, particle beam mass spectrometry (MS), and electrochemical detection for the HPLC analysis of fat-soluble vitamins including the vitamin A esters retinyl acetate and retinyl palmitate [12]. They reported detection limits in standard solutions of 80 pg vitamin A and 500 pg vitamin A palmitate using electrochemical detection in combination with standard-bore columns. Electrochemical detection was also applied to the quantification of endogenous retinoids in mouse embryos and mass limits of detection of 10 pg and 25 pg were reported for retinoic acids and retinol, respectively [13]. In an LC–MS system using atmospheric pressure chemical ionization mass spectrometry Van Breemen et al. were able to detect retinol and retinyl palmitate in human serum at levels down to 34 and 36 fmol/μl [14].

Besides a sensitive detection technique sample preparation is a crucial step in retinoid analysis. Liquid–liquid extraction is widely used to extract retinoids from plasma or serum. Usually the plasma samples are diluted with water, deproteinized with ethanol, and extracted with *n*-hexane [3,4,6,8,14]. This methodology of extraction shows a high degree of reliability of results as determined by inter-assay precision and accuracy, as well as recovery experiments with spiked plasma [15]. However, liquid–liquid extraction always involves the danger of losses due to air and light exposure and adsorption to glassware. Got et al. observed hydrolysis of retinyl esters to retinol during the hexane layer evaporation step [5]. In addition, with increasing number of samples the time consuming liquid–liquid extraction procedure becomes the limiting factor. Therefore, solid-phase extraction with on-line HPLC analysis becomes more and more the technique of choice. Modern column switching systems with on-line solid-phase extraction offer a number of benefits including a high degree of automation (e.g., time consuming manual extraction steps are avoided), no evaporation of extraction solvents, high efficiency, and protection from light and oxygen [16]. The point last mentioned is especially important for photo-, thermo-, and oxidation-sensitive compounds such as retinoids. An additional advantage is the improved sensitivity. High enrichment factors allow the use of the less sensitive but robust UV detection.

On-line solid-phase extraction with column switching and UV detection was successfully applied to the quantitative analysis of retinoic acids and their 4-oxo metabolites in human serum [17] and in human and animal plasma [18]. Eckhoff et al. described the simultaneous determination of retinol and retinyl esters in human plasma using an automated device for solid-phase extraction followed by HPLC analysis [19]. Samples were prepared off-line with a set of 10 extraction cartridges. After preconcentration and clean-up up to 100 cartridges were loaded onto a liquid chromatography module where on-line elution and separation of analytes was performed [20,21]. Although parts of the method were already automated several manual sample preparation steps were still required. A further drawback is that retinyl palmitate and retinyl oleate coeluted under the chromatographic conditions described.

In the present paper we present a highly automated isocratic HPLC method with on-line solid-phase extraction and column switching for the quantitative determination of retinol and major retinyl esters in human plasma. Our aim was to develop a sensitive and robust method which is suitable for the routine analysis of large numbers of samples from clinical studies. Therefore we adapted a system that was previously developed by Wyss and Bucheli for the determination of retinoic acids and their metabolites in human and animal plasma [18]. The new pro-

cedure requires only minimal sample pre-treatment: following protein precipitation with ethanol and centrifugation the samples are directly injected. Further sample clean-up and separation of analytes is accomplished as part of the automated column switching process. The method enables the simultaneous determination of retinol and retinyl esters with a good resolution of retinyl palmitate and retinyl oleate. Although considerable different in polarity, good recoveries for both, retinol and retinyl esters, are obtained. Limits of quantification (LOQs) in plasma as low as 2.5 ng/ml are achieved for the analytes investigated.

2. Experimental

2.1. Materials, reagents and solvents

Ethanol (absolute analytical-reagent and HPLC grade), methanol, 2-propanol (both analytical-reagent grade), and ammonium acetate (analytical-reagent grade), were purchased from E. Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, UK). *N*-Ethyldiisopropylamine (analytical-reagent grade) was obtained from Fluka (Buchs, Switzerland). Water was distilled twice from an all-glass apparatus. Retinol and fatty acid anhydrides were obtained from Sigma (Buchs, Switzerland). Retinyl palmitate and retinyl propionate were provided by F. Hoffmann–La Roche (Basel, Switzerland) and were kept under argon at −20°C. Spiked plasma samples were prepared using fresh frozen plasma from a blood bank (Blutspendezentrum SRK, Basel, Switzerland).

2.2. Synthesis of retinyl acylates as reference compounds

Commercially unavailable retinyl esters (retinyl linoleate, retinyl oleate, and retinyl stearate) were synthesized from retinol and the anhydride of the respective fatty acid. The reaction was catalyzed with the hypernucleophilic acylation catalyst 4-dimethyl-amino-pyridine (DMAP) as described by Höfle et al. [22].

Retinol (5 μmol), fatty acid anhydride (15 μmol), and DMAP (5 μmol) were dissolved in 3 ml pyridine in a pointed flask and allowed to react for 16 h. The reaction was carried out at room temperature in the dark under an argon atmosphere. Then the reaction mixture was transferred with 30 ml *n*-hexane–diethyl ether (1:1, v/v) into a separatory funnel containing 25 ml 1% acetic acid in water and 5 ml ethanol. After phase separation the lower aqueous layer was discarded. The organic phase was washed with water until neutral and evaporated to dryness by means of a rotary evaporator (35–40°C). The residue was reconstituted in *n*-hexane, applied on two alumina thin-layer chromatography (TLC) plates and developed with ethyl acetate–*n*-hexane (1:2, v/v). Retinyl acylate (R_F 0.85) was scrapped off, desorbed with diethyl ether and the extraction solution evaporated to dryness. Quantitative determination of synthesized compounds was performed by UV–Vis spectroscopy at 325 nm using a molar extinction coefficient in hexane of 52 275 M^{-1} cm^{-1} for all retinyl esters as published by Ross [23]. Purity of retinyl acylates was examined by reversed-phase (RP) HPLC using the chromatographic system described in this paper. The contribution of *cis* isomers to total retinyl ester was not investigated. As a consequence synthesized compounds were only used as qualitative and not as quantitative standards.

2.3. Standard solutions

2.3.1. Spike solution

A stock solution (100 μg/ml) of the internal standard was prepared in an amberized volumetric flask by dissolving 10 mg of retinyl propionate in 100 ml ethanol. A 1-ml volume of the stock solution was further diluted with ethanol to 1000 ml to give a spike solution of the internal standard (100 ng/ml). Stored in the dark at 4°C stock and spike solutions were stable for several months.

2.3.2. Calibration solutions and quality control samples

Two stock solutions of retinol and retinyl palmitate were prepared in amberized volumetric flasks by dissolving 10 mg of retinol and retinyl palmitate, respectively, in 10 ml of ethanol (1000 μg/ml). Appropriate amounts of each stock solution were combined and diluted with ethanol to give working solutions in the range of 0.25–200 μg/ml. Cali-

bration solutions were prepared by adding 0.1 ml of each working solution to 75 ml spike solution followed by the addition of 10 ml ethanol, corresponding to theoretical plasma concentrations of 2000, 1000, 500, 100, 50, 20, 5 and 2.5 ng/ml. The calibration standards were stored in the dark at 4°C and were stable for several months.

Quality control (QC) samples were prepared by spiking blank plasma with small volumes (≤1%, v/v) of adequately prepared standard working solutions. QCs were stored frozen in small portions at −80°C until analysis.

2.4. Sample preparation

As a precaution all operations were carried out under yellow light. A 1.5-ml volume of the internal standard spike solution was added to 0.2 ml of plasma for protein precipitation. After vortex mixing the sample was kept in a deep freezer at −20°C for 30 min. Following centrifugation at 1000 *g* and 10°C for 10 min, the supernatant was transferred to an autosampler vial. Samples were stored at 4°C in the autosampler until 1.2-ml aliquots were injected for analysis. For calibration, 1.2 ml of the calibration standards was directly injected into the chromatographic system.

2.5. Chromatographic system and conditions

2.5.1. Instrumentation

A schematic representation of the column switching system is given in Fig. 1. HPLC pump P1 (L-6000, Merck) delivered mobile phases M1A, M1B and M1C via a solvent selector, SS (Labsource, Reinach, Switzerland) to the pre-column, PC. Samples were cooled by a Peltier cooling system (PCU 1000, Labsource) and were injected by the autosampler, AS (Model AS-4000A, Merck) onto one of the pre-columns, PC. In order to inject large volumes, the autosampler was used with a 5-ml syringe, a 3-ml sample loop, and a slow needle-down-speed mode. The injected sample plug was diluted on-line with mobile phase M2 delivered by HPLC-pump P2 (L-6000, Merck) via T-piece (Valco Instruments, Houston, TX, USA; 1/16 in., bore 0.25 mm) (1 in.=2.54 cm). Gradient pump P3 (L-6200A, Merck) delivered mobile phases M3A and M3B for the separation of compounds of interest on the analytical

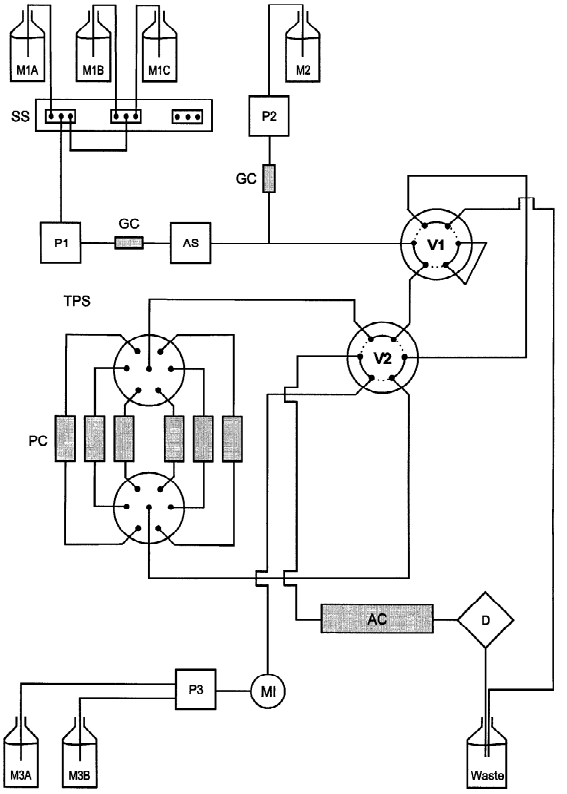

Fig. 1. Schematic representation of the column switching system showing the configuration during sample injection (abbreviations: AC: analytical column; AS: autosampler; D: detector; GC: guard column; MI: manual injector; PC: pre-column; P1/2/3: pump 1/2/3; SS: solvent selector; TPS: tandem pre-column selector; V1/2: valve 1/2).

column, AC. A manual injector, MI (Model 7125, Rheodyne, Cotati, CA, USA) with a 200-μl loop was used for direct injection onto the analytical column (e.g., for recovery experiments). A column oven (LC-Pelcooler, Labsource) allowed the analytical separation to be performed at a constant temperature of 25°C. Detection of the eluted compounds was carried out at 325 nm with a UV detector (SPD-10A, Shimadzu, Japan). The two electrically-driven switching valves, V1 and V2 (high-speed valve 7000E, Labsource) and the solvent selector were controlled by P3. Pre-columns were automatically replaced using a tandem pre-column selector, TPS (EA6 port valve 7066/CPR, Labsource) after 150 injections or when the pressure limit was exceeded. A HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany), in connection with a laboratory-de-

veloped LIMS (Unichrom and Kinlims [24]) was used for data acquisition and processing.

2.5.2. Columns and mobile phases

LiChroCART HPLC cartridges (all 4×4 mm I.D.) packed with LiChrospher 100 RP-18 (5 μm) were used as guard columns, GC, and as pre-columns, PC. The analytical column, AC, consisted of two LiChroCART HPLC cartridges (125×4 mm I.D. and 250×4 mm I.D.) connected by a manu-CART coupling unit (all Merck). The cartridges were packed with Superspher RP-18 (endcapped). Mobile phase compositions are summarized in Table 1. Mobile phase M1A was prepared by mixing 100 ml of 10% ammonium acetate with 700 ml of water, 200 ml of ethanol (analytical-reagent grade) and 10 ml of acetic acid. Mobile phases M1B and M1C consisted of ethanol–water (8:2, v/v) and ethanol, respectively. Mobile phase M2 was prepared by mixing 100 ml of 10% ammonium acetate with 900 ml of water, 40 ml ethanol and 20 ml of acetic acid. Mobile phase M3A consisted of acetonitrile–methanol–ethanol–2-propanol (1:1:1:1, v/v) plus 100 μl/l *N*-ethyldiisopropylamine. During pre-concentration of retinol and retinyl esters onto the pre-column, M3B consisting of 1% ammonium acetate in water was added on-line for pre-conditioning of the analytical column.

2.5.3. Procedure

The total sequence of automated sample analysis required 35 min. Details of the parameters used are shown in Table 2. The autosampler started the gradient program of P3. The latter started the HPLC ChemStation and controlled the flow of P1. In addition, the timer signals of P3 were also used for: (a) switching the valves, (b) the solvent selector, and (c) to start or to stop P2.

2.6. Calibration and quantification

Within each analytical series eight calibration standards, covering the expected concentration range, and four quality control samples were processed together with the unknown samples. The calibration curve ($y=a+bx$) of retinol was obtained by weighted linear least-squares regression (weighting factor $1/x^2$) of the measured peak-area ratios (y) retinol/retinyl propionate (internal standard) versus the concentrations of retinol (x). The calibration curve of retinyl palmitate was established accordingly.

Since retinyl palmitate was the only retinyl ester available in reference quality, other retinyl acylate concentrations were calculated using the palmitate calibration curve without correction for molecular mass. This simplification is justified because these retinyl esters show similar chemical behavior and the same molar extinction coefficient of 52 275 M^{-1} cm^{-1} at the detection wavelength [23]. Therefore a similar recovery can be expected. However, in cases the retinyl palmitate calibration curve is used to quantify other retinyl esters, the use of molar concentrations is suggested.

3. Results and discussion

3.1. Sample preparation and analytical system

The optimal utility of the column switching technique is in the direct application of undiluted plasma

Table 1
Mobile phases

	M1A	M1B	M1C	M2	M3A	M3B
Water	700 ml	200 ml		900 ml		
Ammonium acetate (10%)	100 ml			100 ml		
Ammonium acetate (1%)						1000 ml
Acetic acid	10 ml			20 ml		
Methanol					250 ml	
Ethanol	200 ml	800 ml	1000 ml	40 ml	250 ml	
Acetonitrile					250 ml	
2-Propanol					250 ml	
N-Ethyldiisopropylamine					100 μl	

Table 2
Column switching parameters and gradient program[a]

Step	Time (min)	P1 gradient			P1 flow (ml/min)	P2 flow (ml/min)	P3 gradient		P3 flow (ml/min)	P3 timer				Comment
		M1A (%)	M1B (%)	M1C (%)			M3A (%)	M3B (%)		V1	V2	SS	Others	
A	0				0.05	2.6	80	20	0.05	10	20			Injection of sample onto PC using M1A+
	0.1	100			1.8				0.7			30, 40		M1B for pre-concentration of the analytes
	2.5				1.8	0							62	P2 off
	2.6				4.5									PC is purged using M1A only
B	4.5				4.5					11				PC is purged in the backflush mode
C	5				2.0		100	0	0.7		21			Transfer of the retained components from PC to AC in the backflush mode Capillaries between P1 and V1 are purged with M1B
	5.1		100		1.0							31		
	10				0.5				1.0					
	18				0.5						20			PC is purged with M1B (backflush)
	18.1				2.0								72	TPS count/switch pre-column
	19			100								41		PC is purged with M1C (backflush)
	22										10			PC is purged with M1C (regular flow)
	23		100									40		PC is purged with M1B
D	24	100										30		Re-equilibration of PC with M1A
	27					2.6							52	P2 on
	28.9					0							62	P2 off
	30				2.0		100	0	1.0					

[a] The first digit of the timer signal represents the address and the second one the activation.

onto the pre-column. However, due to high lipoprotein binding of retinyl esters the direct injection of plasma results in low recoveries. This phenomenon is not only observed for retinol and retinyl esters but also for other retinoids [18,25,26].

In the present method the following approach was chosen to obtain the sensitivity needed to determine retinyl esters at endogenous concentrations: in an off-line step an excessive volume of ethanol (1.5 ml) was added to 0.2 ml of plasma for protein precipitation. Following centrifugation 1.2 ml of the supernatant was injected. Additional experiments with a reduced amount of ethanol for protein precipitation resulted in much lower recoveries. A low mass transfer of the lipophilic analytes to the stationary phase seems to be the main explanation for this observation. However, using a larger volume of ethanol would prevent pre-concentration of analytes onto the pre-column. Therefore, a 1% ammonium acetate solution (M2) was added on-line to reduce the elution strength of the ethanol containing sample. The small proportion of ethanol in mobile phase M2 was used to prevent bacterial growth in the solution.

The column switching system described in this paper offers a number of benefits compared to simple HPLC systems. Valve V1 allowed forward and backflush purging of the pre-column. In this way, precipitated proteins and other solid particles were backflushed to the waste, instead of being transferred to the analytical column. Pre-concentrated analytes were also eluted from the pre-column in the backflush mode in order to avoid any band-broadening during column transfer. Using a solvent selector (SS) the pre-column and all capillaries between the autosampler and the switching valves could be purged with solvents of different polarity to prevent memory effects from adsorbed material. The tandem pre-column selector (TPS) together with a pressure monitor automatically replaced a clogged pre-column, enabling routine overnight injections [27,28].

3.2. Selectivity

The determination of retinol and retinyl esters in a complex matrix like blood is a demanding task. A high selectivity is required to separate the analytes from endogenous compounds with similar retention times. In particular co-eluting carotenes (α-, β-, ζ-carotene, lycopene and phytofluene) may interfere with the determination of retinyl esters. With a stepwise empirical modification of the mobile phase a satisfactory selectivity for all analytes of interest could be achieved. Nevertheless, for some very complex biological samples it may be still difficult to obtain optimum conditions for all of the compounds of interest. The best resolution of retinyl esters and carotenes was reached with a quaternary mobile phase, consisting of acetonitrile, methanol, ethanol and 2-propanol, as described in the Experimental section. Using this mobile phase only ζ-carotene isomers showed some coelution with retinyl palmitate and retinyl oleate. ζ-carotene, however, is only present at trace levels in human plasma. Furthermore the absorbance of ζ-carotene at the detection wavelength of retinol and retinyl esters (325 nm) is less than 5% of its maximum absorbance. As a consequence the interference of this carotene on retinyl ester quantification may be neglected. Fig. 2A–C show representative chromatograms of human blank plasma, human blank plasma spiked with additional 200 ng/ml retinol and 80 ng/ml retinyl palmitate, and plasma from a volunteer 5 h after a oral dose of 4000 IU retinyl acetate administered together with a standard liver meal containing 100 000 IU vitamin A.

3.3. Linearity and limit of quantification

Standard solutions of retinol and retinyl palmitate were assayed at eight concentrations in the range of 2.5–2000 ng/ml. Calibration curves were calculated by a weighted least-squares regression procedure, using $1/x^2$ as weighting factor. Data of calibration sets assayed within a period of 3 months are presented in Table 3. Good linearity was observed for both analytes within the whole concentration range. The relative standard deviation (RSD) was $\leq$2.9% for retinol and $\leq$5.4% for retinyl palmitate showing good reproducibility. The LOQ, defined as a minimum signal-to-noise ratio of 10, was 2.5 ng/ml for both compounds, using 0.2 ml of plasma.

3.4. Recovery

Recoveries were determined using human plasma spiked with retinol and retinyl palmitate at different

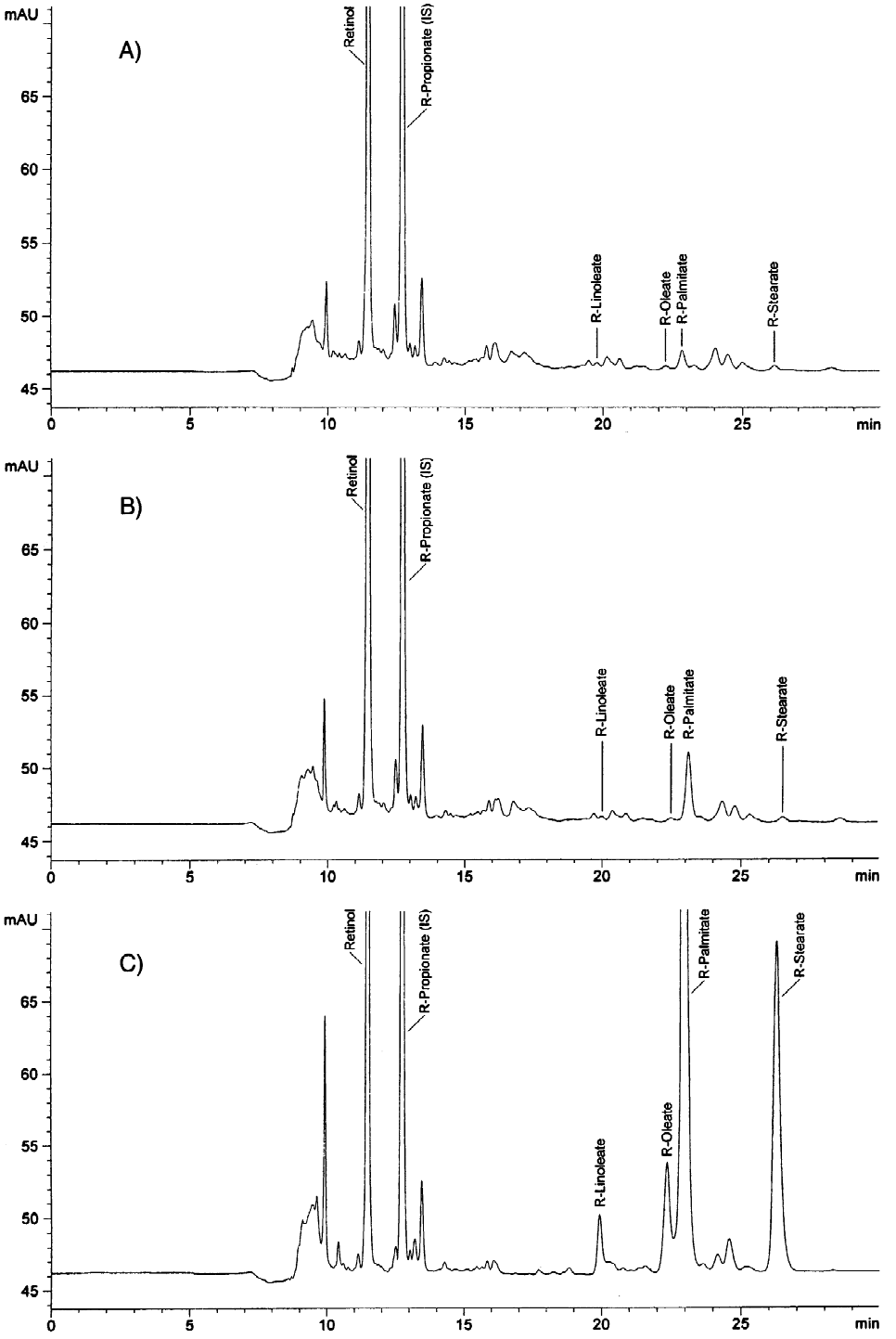

Fig. 2. Chromatograms of human plasma samples: (A) blank plasma, (B) blank plasma spiked with additional 200 ng/ml retinol and 80 ng/ml retinyl palmitate, (C) plasma from a volunteer 5 h after a oral dose of 4000 IU retinyl acetate administered together with a standard liver meal containing 100 000 IU vitamin A.

Table 3
Linearity (n=16)

Nominal concentration (ng/ml)	Retinol			Retinyl palmitate		
	Concentration found, mean±SD (ng/ml)	RSD (%)	Accuracy (%)	Concentration found, mean±SD (ng/ml)	RSD (%)	Accuracy (%)
2.5	2.50±0.04	1.4	99.9	2.41±0.07	2.8	96.5
5	4.99±0.15	2.9	99.8	5.30±0.29	5.4	105.9
20	20.3±0.2	0.9	101.4	21.0±0.8	3.9	105.1
50	49.8±0.4	0.8	99.6	49.6±1.2	2.5	99.1
100	99.4±0.8	0.8	99.4	98.9±2.2	2.2	98.9
500	510±4	0.7	102.0	506±8	1.6	101.2
1000	992±11	1.1	99.2	972±18	1.8	97.2
2000	1973±32	1.6	98.7	1922±48	2.5	96.1
R^2	0.9999±0.0002	0.02		0.9997±0.0004	0.04	

concentrations (QC samples). Due to high endogenous levels of the analytes in the plasma it was not possible to prepare spiked samples covering the whole calibration range.

Recovery from spiked plasma was assessed by comparison of peak areas of spiked plasma samples (QC) with the sum of peak areas obtained from non-spiked plasma (endogenous levels) and calibration standards containing the same amount of analyte as spiked to the QC samples. All plasma samples were processed as described in the Experimental section. The recoveries of retinol and retinyl palmitate from human plasma are presented in Table 4. Good recoveries were obtained for both compounds at all concentration levels examined, demonstrating the excellent operation of the column switching system.

Besides the determination of recoveries an additional test was conducted to check the performance of the solid-phase extraction step. Peak areas of calibration standards injected onto the pre-column were compared to those of solutions of the same amount of analyte directly injected onto the analytical column via a manual injector (MI). For manual injection analytes were dissolved in mobile phase M3A and 100 μl were injected. The experiment showed comparable peak areas for standards injected on the pre-column and manually injected solutions.

3.5. Precision and accuracy

Inter-assay precision (defined as RSD of replicate samples) and accuracy (defined as deviation between found and added concentration) were evaluated for different plasma concentrations. At each concentration level a spiked plasma sample was prepared and analyzed on different days using a separate calibration set each day. The results summarized in Table 5 demonstrate good precision and accuracy for

Table 4
Recoveries of retinol and retinyl palmitate from human plasma

Analyte	Concentration added (ng/ml)	Recovery (%)	RSD (%)	n
Retinol	100	93.4	2.6	6
	1000	96.5	2.3	6
Retinyl palmitate	5	95.2	4.2	6
	20	92.7	3.1	6
	100	96.0	4.7	6
	1000	93.4	3.8	6

Table 5
Inter-assay precision and accuracy

Analyte	Concentration added (ng/ml)	Concentration found (ng/ml)	RSD (%)	Accuracy (%)	*n*
Retinol	0	691	5.1		6
	100	757	3.7	95.8	6
	1000	1587	3.1	93.9	6
Retinyl palmitate	0	26.8	5.2		6
	5	32.3	4.8	101.6	6
	20	45.2	3.3	96.6	6
	100	118	5.6	92.8	6
	1000	966	6.3	94.1	6

retinol and retinyl palmitate in human plasma over all concentration ranges analyzed.

3.6. Stability

The stability of retinol and retinyl esters was determined by analyzing plasma samples spiked either with 100 ng/ml retinol or 20 ng/ml retinyl palmitate. Subsequently, the samples were stored at −80°C and analyzed after 1, 2, 4 and 6 months. The results of the storage experiments are presented in Table 6. Neither for retinol nor for retinyl palmitate a considerable degeneration of more than 10% of the initial concentrations was detectable over the period of time.

3.7. Application and endogenous concentrations in human plasma

The method was successfully applied to clinical studies on vitamin A safety as well as to vitamin A status studies in volunteers. More than 2000 human plasma samples were analyzed, demonstrating the

Table 6
Stability of retinol and retinyl palmitate in plasma stored at −80°C

Storage time (months)	*n*	Retinol		Retinyl palmitate	
		Change in concentration after storage (%)	90% Confidence interval (%)	Change in concentration after storage (%)	90% Confidence interval (%)
1	6	−2.6	−0.3 to −5.1	−1.2	−4.3 to +3.2
2	6	−1.8	−8.3 to +5.2	+2.9	−4.7 to +10.3
4	6	+0.3	−2.3 to +3.1	−3.4	−5.8 to −0.8
6	6	−3.1	−5.9 to −0.7	−3.9	−7.2 to −0.4

Table 7
Endogenous concentration of retinol and retinyl palmitate in human plasma

Source	Subjects	*n*	Retinol (ng/ml)	Retinyl esters (ng/ml)
Eckhoff et al. [19]	Male volunteers	6	467±88.8	33.6±14.7[a]
Buss et al. [29]	Female volunteers	10	830±200	≈20.0[b]
Peiker et al. [30]	Female volunteers	6	477±71	10.5±7.1[a]
von Reinersdorff et al. [6]	Male volunteers	11	539±111	11.6±6.8[c]

[a] Retinyl palmitate/oleate.
[b] Retinyl palmitate concentrations taken from a graph.
[c] Total retinyl esters.

robustness of the method. Results of clinical studies will be reported elsewhere.

Endogenous concentrations of retinol and retinyl esters were determined in plasma from 35 untreated female volunteers. Mean plasma concentration of retinol was 714±134 ng/ml. Retinyl palmitate is the most abundant vitamin A ester in plasma, exhibiting a mean concentration of 24.2±9.1 ng/ml (46.2±17.4 n*M*). This accounts for approximately 60% of total retinyl esters endogenously found in plasma. Concentrations of retinyl stearate and retinyl oleate were considerably lower and achieved values of 6.61±3.07 ng/ml (12.0±5.6 n*M*) and 5.68±2.24 ng/ml (10.3±4.1 n*M*), respectively. Levels of retinyl linoleate were close to the LOD. The results were in good accordance with data published from other laboratories which are listed in Table 7.

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