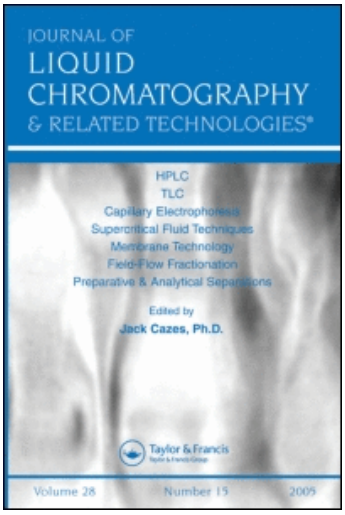

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Simultaneous Determination of Nine Water and Fat Soluble Vitamins After SPE Separation and RP-HPLC Analysis in Pharmaceutical Preparations and Biological Fluids

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SIMULTANEOUS DETERMINATION OF NINE WATER AND FAT SOLUBLE VITAMINS AFTER SPE SEPARATION AND RP-HPLC ANALYSIS IN PHARMACEUTICAL PREPARATIONS AND BIOLOGICAL FLUIDS

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ABSTRACT

An automated reversed phase high performance liquid-chromatography (RP-HPLC) method is described, for the simultaneous analysis of water soluble [ascorbic acid (C), nicotinic acid, nicotinamide, folic acid, cyanocobalamine (B_{12}), and riboflavin (B_2)] and fat soluble (retinol, α-tocopherol, α-tocopherol acetate) vitamins. The compounds are separated after solid-phase extraction (SPE) on C_{18} cartridges, where water soluble vitamins pass unretained, while fat soluble vitamins are retained on the sorbent.

After isolation of the two fractions, water soluble vitamins are separated on a Lichrosorb RP-18 250x4.0 mm, 5 μm analytical column, using a gradient elution system consisting of CH_3OH-0.05 M CH_3COONH_4 (5:95 v/v) changing to (30:70 v/v) over a period of 20 min at a flow rate 1 mL/min. Fat soluble vitamins

are separated on a Spherisorb RP-18 220x4.6 mm, 5 μm analytical column with an isocratic mobile phase consisting of CH_3OH-CH_3CN (30:70 v/v) at a flow rate of 1.5 mL/min.

A UV-vis detector operated at 270 nm and 290 nm for water soluble and fat soluble vitamins, respectively, is used for detection and quantitation of the analytes.

Xanthine is used as internal standard at a concentration of 4.2 ng/L for water soluble vitamins, while anthraquinone is used as internal standard at a concentration of 3.5 ng/mL for fat soluble vitamins.

Linearity is observed up to 10 ng/mL for ascorbic acid, folic acid, and riboflavin, up to 15 ng/mL for nicotinic acid, nicotinamide, and cyanocobalamine, while up to 20 ng/mL for all fat soluble vitamins. Limits of detection ranged from 2.5 - 5.0 ng for water soluble vitamins and 2.0 - 5.0 ng for fat soluble vitamins. Method's validation is achieved in terms of day-to-day and within-day reproducibility studies. Long term stability study is conducted during routine operation of the system over a period of ten consecutive days.

The developed method is applied to the analysis of pharmaceutical preparations: (tablets, injection solutions) and biological fluids: (blood serum, urine). SPE technique is used for the isolation of the vitamins from the matrix of human biological fluids: blood serum (40 μL) and urine (100 μL). High extraction recoveries are achieved using Merck Lichrolut RP-18 cartridges.

INTRODUCTION

Vitamins are vital to human life. The human metabolism is not able to synthesise these compounds, which have a catalytic function in anabolic and catabolic pathways. Therefore, a supplementation of these substances, most often only in μg or mg amounts per day, through food chain is necessary. Due to their function, vitamins are involved in developmental and fast reproducing processes like blood formation, maintenance of epithelial tissue, ossification of bone, eye functions, as well as, in the metabolic pathways of the central nervous system.

Malnutrition and metabolic diseases can lead to a vitamin deficiency, which shows very significant clinical symptoms, while excessive vitamin intake, particularly of fat-soluble vitamins, can result in different diseases.

Vitamins can be classified in two main groups: water-soluble and fat soluble vitamins. In the nomenclature of vitamins often abbreviations are used as common names, e.g., vitamin A, B, C, and so on. These abbreviations stand for the main active substance, but often compromise the biochemical effect of the vitamin itself plus its active metabolites.

The natural source of vitamins for human beings has always been food and drinks. Because artificial vitamin formulations, often complemented with minerals, can and will not replace the natural sources, our need for vitamins can be covered in the conventional way. Customer demands and marketing strategies have forced many food and drink producers to provide information on the vitamin content of their products in the product description.

Vitamins have been determined by a wide variety of techniques from which the most widely applied are the chromatographic ones: paper, thin-layer, column, gas-liquid, and high performance liquid chromatography (HPLC). The latter technique allows a rapid, simple, and selective determination of vitamins.

The analytical advantages of HPLC in comparison to the other analytical techniques are solvent economy, higher efficiency, mass sensitivity, easy coupling with other techniques, and finally small amounts of sample. This method requires relatively little clean-up and offers at once the possibility for the determination of several vitamins in the same run on the same column.[1,2]

Determination of vitamins can be done either by normal phase chromatography or by reversed phase chromatography which is the most common. Most of the analysts use very complex mobile phases like mixtures of three or four types of solvents[3-8] or phosphate buffers which are causing a lot of problems, if the analyst is not very cautious.[3,9-11] Many high performance liquid chromatographic (HPLC) methods for single or simultaneous multiple determinations of vitamins using either ultraviolet-visible absorbance (the most common techique for the detection of vitamins), electrochemical[12-16] or fluorimetric[13,17-21] detection have been published.

Most of the published methods involve the use of capillary columns,[22,23] gradient instead of isocratic elution,[8,24-26] two or three different detectors,[6,7,14,24,25,27,28] or changing the detection wavelength during the run.[26,29,30] Sometimes, diode-array detectors are used for the detection of

vitamins.[7,31] The complexity of these HPLC conditions, no doubt, effectively improves the selectivity and sensitivity of vitamin determinations, nevertheless, they are considered complicated and costly for routine analysis.

The first step in analysing vitamins is sample preparation. In most cases, they have to be extracted from the matrix, however, for the analysis of vitamins in additive raw material or soft drinks, a pre-treatment of the sample may not be necessary. It is important to verify that the chosen sample preparation method is suitable for the analysis of the vitamins of interest, because all vitamins are unstable during common sample preparation methods (boiling for deproteinisation, alkali- or acid-treatment).

For all vitamins it is recommended to use short-time and gentle extraction methods, which in some instances should be performed in a darkened place.

Determination of vitamins in pharmaceutical formulations (tablets, gelatine capsules, injections) is nearly always preceded by extraction with a solvent. Before the extraction can be carried out, these products have to be free from the tablet coating, capsule wall, or other shell vehicular. Sometimes the samples are saponificated.[3,10,32]

Separation of plasma-vitamins by HPLC is nearly always preceded by deproteination of the plasma with ACN, EtOH, MeOH and extraction with n-hexane or heptane.[7,9,14,17-18,23,27,33,34] Sometimes, sample preparation requires perchloric acid or trichloroacetic acid deproteinization of the plasma followed by centrifugation and filtration through a disposable filter.[9,11] To determine vitamin concentrations in tissues, homogenisation followed by the extraction with an organic solvent is the most widely used method.[5,18,19,31,35-38]

For the clean up procedure of biological samples, the technique of SPE extraction was applied in order to isolate the vitamins from the matrix. SPE is a rapid and solvent consuming technique, which leads to reduction in pollution and offers a wide sorbent selection.[25]

In the present paper, a simultaneous determination of water-soluble vitamins [ascorbic acid (C), nicotinic acid, nicotinamide, folic acid, cyanocobalamine (B_{12}), riboflavin (B_2)], and fat soluble vitamins (retinol, α-tocoferol, α-tocoferol acetate) is proposed. Solid-phase extraction is used as a pre-treatment technique of biological samples and also as an isolation method of the two fractions of vitamins (water soluble and fat soluble vitamins), in case the two fractions of vitamins exist in the same sample.

EXPERIMENTAL

Instrumentation

The chromatographic system which is used for the analysis of vitamins consists of the commercial components: a Spectra Physics 8800 HPLC ternary pump (California USA), a Spectra Chrom 100 UV/VIS detector, operated at 270 nm for the analysis of water-soluble vitamins and 290 nm for the analysis of fat-soluble vitamins and a sensitivity setting of 0.002 absorbance units full scale (AUFS), a Rheodyne 7125 (California, USA) Injection valve with a 10 μL loop, and a Spectra Physics SP 4290 integrator.

The analytical columns which are used are: a Lichrosorb RP-18, 250x4 mm, 5 μm for the determination of water-soluble vitamins (MZ Analysentechnik, Mainz, Germany) and a Spherisorb RP-18, 220x4.6 mm, 5 μm for the determination of fat-soluble vitamins (Brown Lee, a part of Perkin-Elmer/ABI, Deerfield, USA).

A glass vacuum-filtration apparatus obtained from Alltech Associates, was employed for the filtration of the buffer solutions, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) are employed for the pre-treatment of biological samples.

The SPE study is performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, a division of Varian (Harbor City, USA).

Lichrolut RP-18 SPE cartridges are supplied from Merck (Darmstadt, Germany).

All evaporations are performed with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

UV spectra for selecting the working wavelength of detection are taken using a Varian DMS 100S UV/VIS double-beam spectrophotometer. All computations are achieved using a VIP 312 Computer.

Materials and reagents

Vitamins were purchased from Merck and were used without further purification.

Xanthine, used as internal standard for water-soluble vitamins, was purchased from Sigma (St. Louis, MO. USA). Anthraquinone, used as internal standard for fat-soluble vitamins, was purchased from Serva (Heidelberg, Germany).

HPLC gradient grade methanol was obtained from Lab-Scan (Dublin, Ireland) and acetonitrile was obtained from Merck.

Ammonium acetate pro analysi was purchased from Merck. All other reagents used were of analytical grade. Bis de-ionised water was used throughout analyses.

Chromatographic Conditions

Water-soluble vitamins

A reversed phase C_{18} Lichrosorb RP-18 250x4 mm, 5 μm, is used for the separation of water-soluble vitamins at ambient temperature. A variety of mobile phases was tested in order to find out the optimum chromatographic system for the analysis of vitamins. The mobile phases, were in principle, binary mixtures of an aqueous solution of ammonium acetate with methanol in several ratios. The final mobile phase is chosen in terms of peak shape, column efficiency, and chromatographic analysis time, selectivity, and resolution. The mobile phase consisted of:

Time (min)	MeOH (%)	CH_3COONH_4 0.05 M (%)
0	5	95
6	15	85
13	30	70
20	30	70

Flow rate 1 mL/min.

Fat-soluble vitamins

A reversed phase C_{18} Spherisorb RP-18 220x4.6 mm, 5 μm, was used for the separation of the fat-soluble vitamins at ambient temperature. The mobile phase consists of CH_3OH- CH_3CN (30:70 v/v) at a flow rate of 1.5 mL/min.

The mobile phases were selected among others for leading to optimal resolution of compounds, as well as to convenience regarding total time of analysis.

Xanthine (XA) was chosen to be used as internal standard for water-soluble vitamins, after an assay of a wide variety of organic compounds, (Bamifylline, 3-methyl XA, 7-methyl XA, 1-methyl XA, Caffeine) taking into consideration the sufficient resolution, as well as the spectra criteria. For the same reasons, Anthraquinone was chosen to be used as internal standard for fat-soluble vitamins, after an assay of a wide variety of organic compounds (Codeine, Tolfenamic acid, Flufenamic acid, Mefenamic acid, Paracetamol, Hyrdoxy-anthraquinone, Anthranilic acid).

RESULTS AND DISCUSSION

Resolution Factors

The separation between water-soluble vitamins, as shown in chromatogram presented in Figure 1 and the separation between fat-soluble vitamins, as shown in chromatogram presented in Figure 2, are sufficient as signified, also, from resolution factors Rs which are:2.45 for Ascorbic acid-Nicotinic acid, 2.80 for Antraquinone-Retinol, 2.03 for Nicotinic acid-Xanthine, 15.50 for Retinol-αTocoferol, 4.76 for Xanthine-Nicotinamide, 2.75 for αTocoferol - α Tocoferol acetate 3.10 for Nicotinamide-Folic acid,17.82 for Folic acid-Cyanocobalamine and 7.28 for cyanocobalamine-Riboflavin.

Calibration Curves

Water-soluble vitamins

Calibration of the method was performed by injection of mixed standard of water-soluble vitamins covering the entire working range. Twelve concentrations were used in the range 0.5-20 ng/μL.

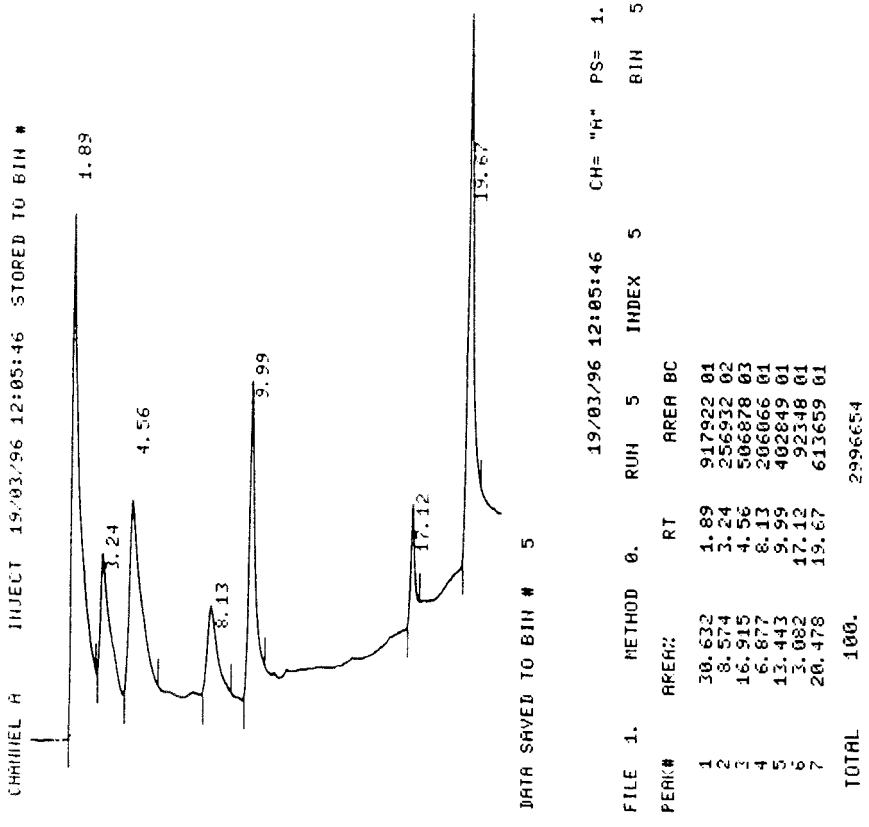

Figure 1. High performance liquid chromatogram of water soluble vitamins. Ascorbic acid (4.12ppm) 1.89min, Nicotinic acid (4.16ppm) 3.24min, Xanthine (4.20ppm) 4.56min, Nicotinamide (4.16ppm) 8.13min, Folic acid (3.12ppm) 9.99min, Cyanocobalamine (4.88ppm) 17.12min, Riboflavin (4.02ppm) 19.67min.

These working solutions were prepared and frozen at -18°C. The working solutions were stable about five to six days. The sensitivity setting of the UV/VIS detector was adjusted to give almost full scale deflection for the highest standard concentration. Each sample was injected five times.

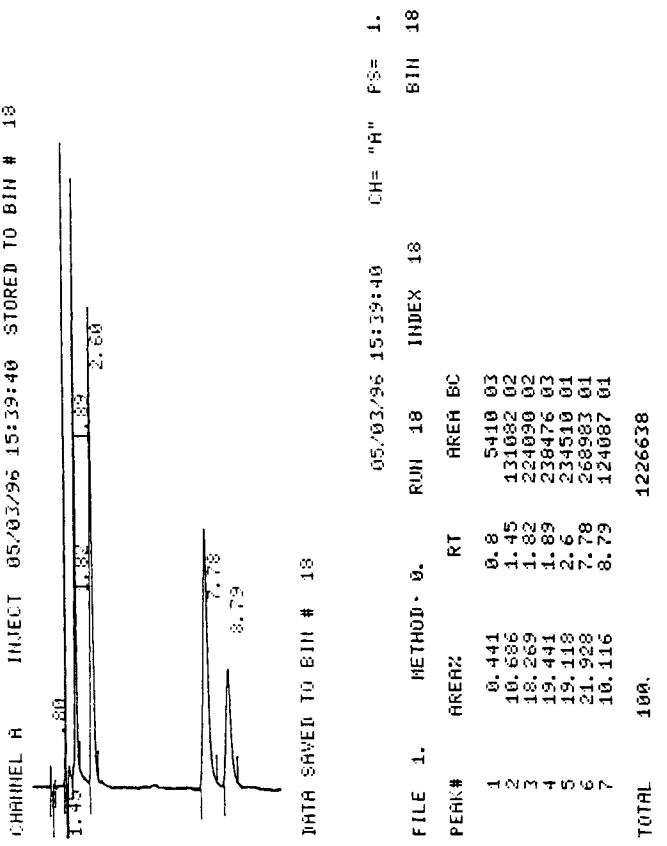

Figure 2. High performance liquid chromatogram of fat soluble vitamins. Anthraquinone(3.50 ppm) 1.89 min, Retinol (2.00 ppm) 2.60 min, α-Tocoferol (5.00 ppm) 7.78 min, α-Tocoferol acetate(4.50 ppm) 8.79min.

Linear correlation between absolute injected amount or concentration and peak area ratio was obtained for all water-soluble vitamins, using xanthine as internal standard, at a concentration of 4.20 ng/μL. The correlation coefficients of the calibration curves had a range of 0.99722-0.99931.

Fat-soluble vitamins

The same procedure described for the calibration of the method of water-soluble vitamins, was followed for fat-soluble vitamins as well. Stock standard and working solutions were prepared and frozen at -18°C. All solutions were

stable at that temperature, for at least one month. Linear correlation, between absolute injected amount or concentration and peak area ratio was obtained for all fat-soluble vitamins, using Antraquinone as internal standard, at a concentration of 3.50 ng/μL.

The correlation coefficients of the calibration curves had a range of 0.99926-0.99948.

Linear range

Linearity was observed up to 10.0 ng/μL for ascorbic acid, folic acid and riboflavin and up to 15.0 ng/μL for nicotinic acid, nicotinamide, cyanocobalamine.

In the case of fat-soluble vitamins linearity was observed up to 20.0 ng/μL.

Limits of detection

The detection limits were assessed in the presence of the internal standards. Those were considered to be the quantities which are producing a signal of peak height three times the size of background noise.

The detection limits are: 2.5ng for Ascorbic acid, 5.0 ng for Nicotinic acid, 5.0 ng for Nicotinamide 3.0 ng for Folic acid 5.0 ng for Cyanocobalamine, 2.5 ng for Riboflavin, 2.0 ng for Retinol, 5.0 ng for α-Tocoferol and 5.0 ng for α-Tocoferol acetate.

Precision and Accuracy

Method validation regarding reproducibility was achieved by replicate injections of standard solutions at low and high concentration levels where peak areas were measured in comparison to the peak area of the internal standard.

Statistical evaluation revealed relative standard deviations at different values for eight injections. Long term stability study was conducted during routine operation of the system over a period of ten consecutive days. Results are illustrated in Table 1.

Table 1

Precision and Accuracy for the Analysis of Vitamins

	Within-Day Precision and Accuracy for the Analysis of Vitamins (n=8)					Day-to-Day Precision and Accuracy for the Analysis of Vitamins (n=7)			
Analyte	**Added (ng)**	**Found (ng)**	**SD**	**RSD (%)**	**Recovery (%)**	**Found (ng)**	**SD**	**RSD (%)**	**Recovery (%)**
Water-Soluble Vitamins									
Ascorbic acid	41.2	42.2	0.6	1.4	102.4	41.0	0.2	0.5	99.5
	51.5	51.4	1.4	2.7	99.8	51.1	1.1	2.2	99.2
	61.8	60.1	1.0	1.7	97.2	61.2	1.6	2.6	99.0
Nicotinic acid	41.6	40.1	1.6	4.0	96.4	42.3	1.4	3.3	101.7
	52.0	51.3	1.7	3.3	98.6	50.9	2.8	5.5	97.9
	62.4	60.3	2.1	3.5	96.6	60.6	1.6	2.6	97.1
Nicotinamide	41.6	40.1	0.7	1.7	96.4	40.3	1.8	4.5	96.9
	52.0	51.2	2.8	5.5	98.5	51.9	3.1	6.0	99.8
	59.8	61.1	1.0	1.6	102.2	63.1	3.4	5.4	101.1
Folic acid	42.7	41.1	0.7	1.7	96.2	40.0	2.0	5.0	96.2
	48.8	50.0	1.4	2.8	102.5	50.6	1.0	2.0	97.3
	60.3	61.0	1.1	1.8	101.2	60.8	3.1	5.1	101.7
Cyanoco-balamine	40.2	39.9	0.9	2.2	99.2	42.8	1.2	2.8	100.2
	53.6	51.5	3.6	7.0	96.1	47.3	1.9	4.0	96.9
	61.0	60.4	2.9	4.8	99.0	60.8	2.6	4.3	100.8
Riboflavin	40.2	38.6	0.5	1.3	96.0	41.2	2.3	5.6	102.5
	53.6	53.6	0.8	1.5	100.0	53.2	3.6	6.8	99.2
	61.0	60.6	0.4	0.7	99.3	60.2	1.1	1.8	98.7
Fat-Soluble Vitamins									
Retinol	30.0	28.9	1.5	5.2	96.3	29.1	0.5	1.7	97.0
	40.0	38.2	1.0	2.6	95.5	39.6	1.1	2.8	99.0
	50.0	50.0	1.3	2.6	100.0	49.9	0.5	1.0	99.8
α-Tocoferol	34.2	32.2	1.0	3.1	94.2	33.1	0.6	1.8	96.8
	45.6	44.2	1.0	2.3	96.9	44.9	0.9	2.0	98.5
	57.0	55.6	0.5	0.9	97.5	55.2	0.8	1.4	96.8
α-Tocoferol acetate	31.2	29.4	0.4	1.4	94.2	30.1	1.1	3.6	96.5
	39.0	37.7	1.0	2.6	96.7	38.7	1.3	3.4	99.2
	54.6	54.0	1.9	3.5	98.9	54.2	0.5	0.9	99.3

Sample Pre-Treatment for Water Soluble Vitamins Analyses in Pharmaceutical Preparations

Pharmaceutical preparations: tablets and injection solutions, were treated as follows, in order to examine the applicability of the HPLC method of water-soluble vitamins determination:

Tablets

According to the label, a tablet contains 50 mg nicotinamide and 15 mg riboflavin.

After finely powdering of a tablet in a porcelain mortar, a portion of 0.1014 g was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with water. The concentration of this solution was namely 98.1 ng/μL for nicotinamide and 29.4 ng/μL for riboflavin.

From this stock solution three working solutions were prepared by the proper dilution. Working solutions contained the internal standard xanthine, at a concentration of 4.2 ng/μL.

Aliquots of 10 μL were injected onto the HPLC analytical column.

High performance liquid chromatogram of water-soluble vitamins in tablet with xanthine as internal standard, is shown in Figure 3.

Injection solution 1

An ampoule of injection solution (volume 0.5 mL) contains 2 mg riboflavin and 100 mg nicotinamide, as stated on the label. Volume of 0.5 mL of this injection solution was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with water. The concentration of this solution was namely 20 ng/μl for riboflavin and 100 ng/μl for nicotinamide.

From this stock solution three working solutions were prepared by the proper dilution in order to obtain concentrations: 1, 2 and 3 ng/μl. Working solutions contained the internal standard xanthine at a concentration of 4.2 ng/μl.

Aliquots of 10 μL were injected onto the HPLC analytical column. High performance liquid chromatogram of water-soluble vitamins in injection solution 1, with xanthine as internal standard, is shown in Figure 4.

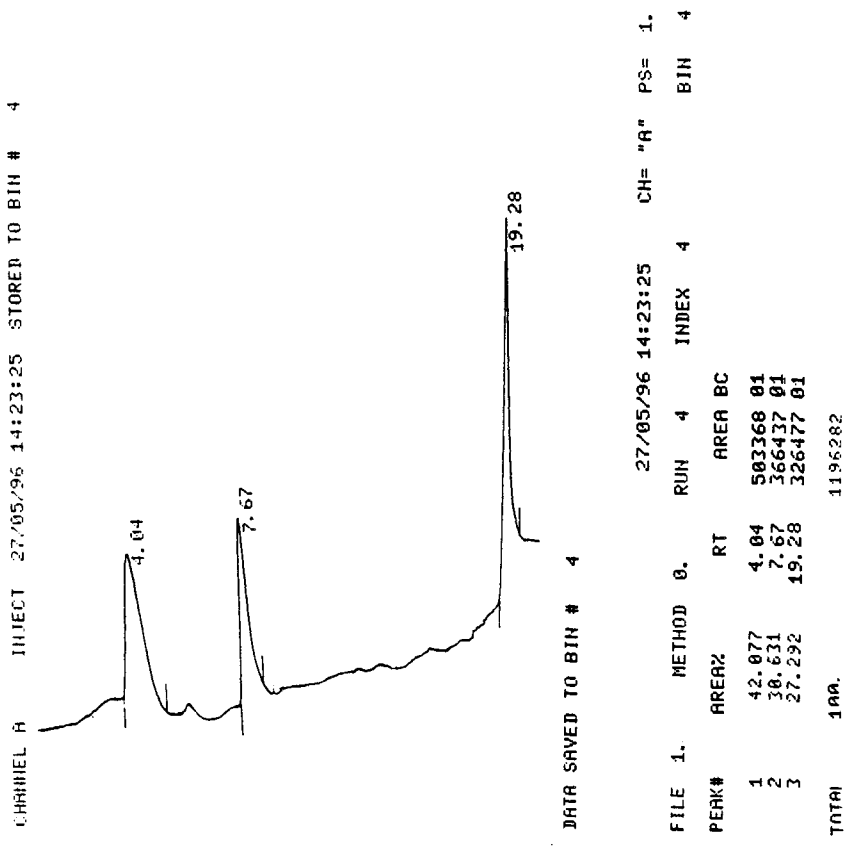

Figure 3. High performance liquid chromatogram of water soluble vitamins in tablet. Xanthine (4.2 ppm) 4.04 min., nicotinamide (7.85 ppm) 7.67 min. and riboflavin (2.35 ppm) 19.28 min.

Injection solution 2

According to the label, an ampoule of injection solution (volume 10 mL) contains 500 mg ascorbic acid and 50 mg nicotinamide. An aliquot of 2 mL was quantitatively transferred into an 100 mL volumetric flask and diluted to volume with water. The concentration of this solution was namely 100 ng/μL for ascorbic acid and 10 ng/μL for nicotinamide. From this stock solution three working solutions were prepared by the proper dilution, in order to obtain concentrations: 1, 2 and 3 ng/μL.

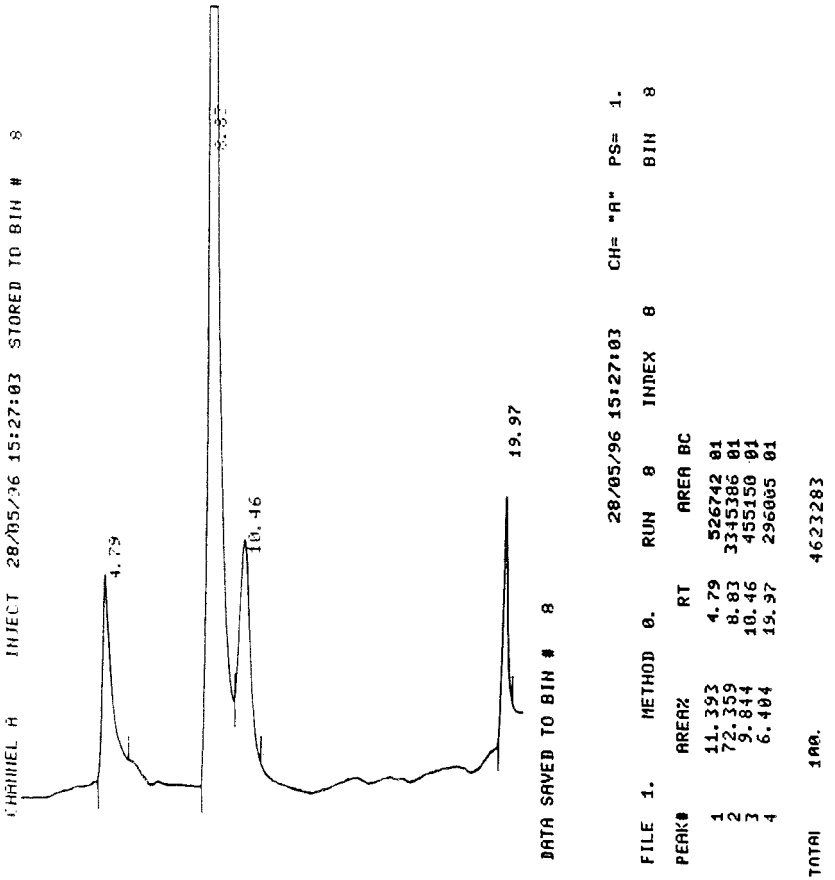

Figure 4. High performance liquid chromatograms of water soluble vitamins in injection solution 1. Xanthine(4.2 ppm) 4.79 min., Nicotinamide(80 ppm) 8.83 min., Riboflavin(1.6 ppm) 19.97 min., Unknown peak 10.46 min.

Working solutions contained the internal standard xanthine at a concentration of 4.2 ng/μL. Aliquots of 10 μL were injected onto the HPLC analytical column. High performance liquid chromatogram of water-soluble vitamins in injection solution 2, with Xanthine as internal standard, is shown in Figure 5. The experimental results from the analysis of water-soluble vitamins in pharmaceutical preparations are given in Table 2.

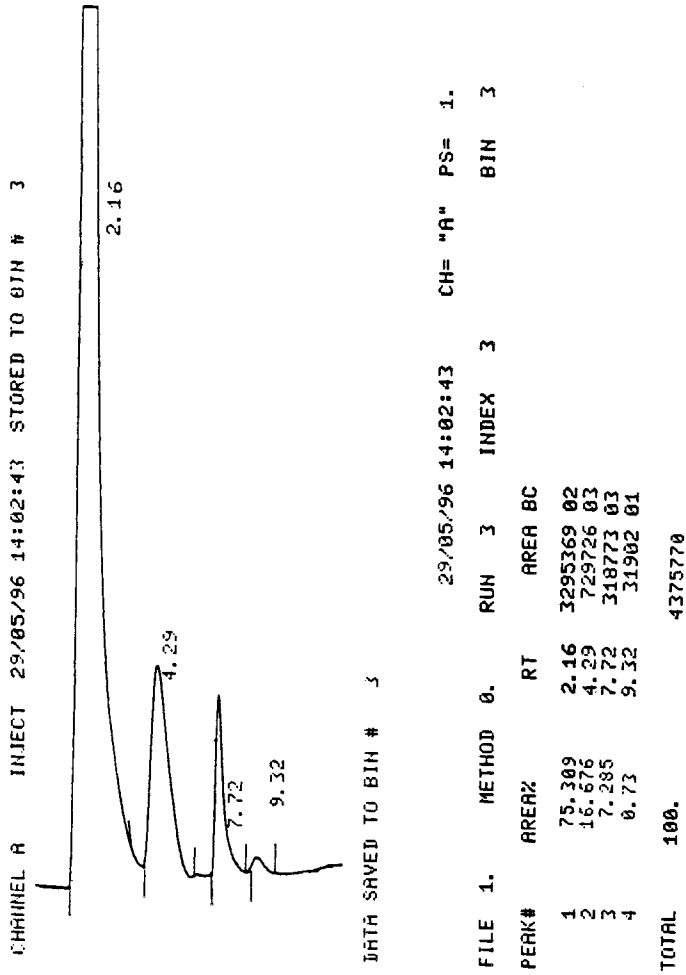

Figure 5. High performance liquid chromatogram of water soluble vitamins in injection solution 2. Ascorbic acid (45 ppm) 2.16 min., Xanthine (4.2 ppm) 4.29 min., Nicotinamide (4.5 ppm) 7.72 min., Unknown peak 9.32 min.

Solid-Phase Extraction

Solid-phase extraction cartridges (Lichrolut RP-18), fitted to the vacuum manifold, were conditioned by flushing with 3 mL methanol and 3 mL of water, prior to the addition of the sample. Then the sample (500 μL) was

Table 2

Experimental Results for the Analysis of Vitamins in Pharmaceutical Preparations by RP-HPLC with Xanthine as Internal Standard[a]

Sample	Analysed Quantity (ng)	Found (ng)±SD	RSD (%)	Labelled Amount (mg)	Found (mg)±SD	RSD (%)
Tablet						
Riboflavin	14.7	13.9±0.6	4.3	15.0	14.7±0.4	2.7
	23.5	23.1±0.9	3.9			
	35.3	35.4±1.0	2.8			
Nicotinamide	24.5	24.9±0.6	2.4	50.0	50.5±1.7	3.3
	49.0	47.7±1.0	2.1			
	78.5	77.9±2.7	3.5			
Injection Solution 1						
Riboflavin	10.0	9.5±0.5	5.3	2.0	1.9±1.1	4.7
	20.0	18.7±1.1	5.9			
	30.0	30.6±1.7	5.6			
Nicotinamide	10.0	10.1±0.4	4.0	100.0	98.0±2.6	2.7
	20.0	19.2±0.7	3.6			
	30.0	29.1±1.3	4.1			
Injection Solution 2						
Ascorbin acid	10.0	9.4±0.4	4.2	500.0	481.3±10.4	2.2
	20.0	19.7±0.6	3.0			
	45.0	44.6±1.9	4.3			
Nicotinamide	10.0	9.6±0.4	4.2	50.0	49.4±1.2	2.4
	20.0	20.2±0.7	3.5			
	30.0	30.1±1.2	4.0			

[a] (n=7)

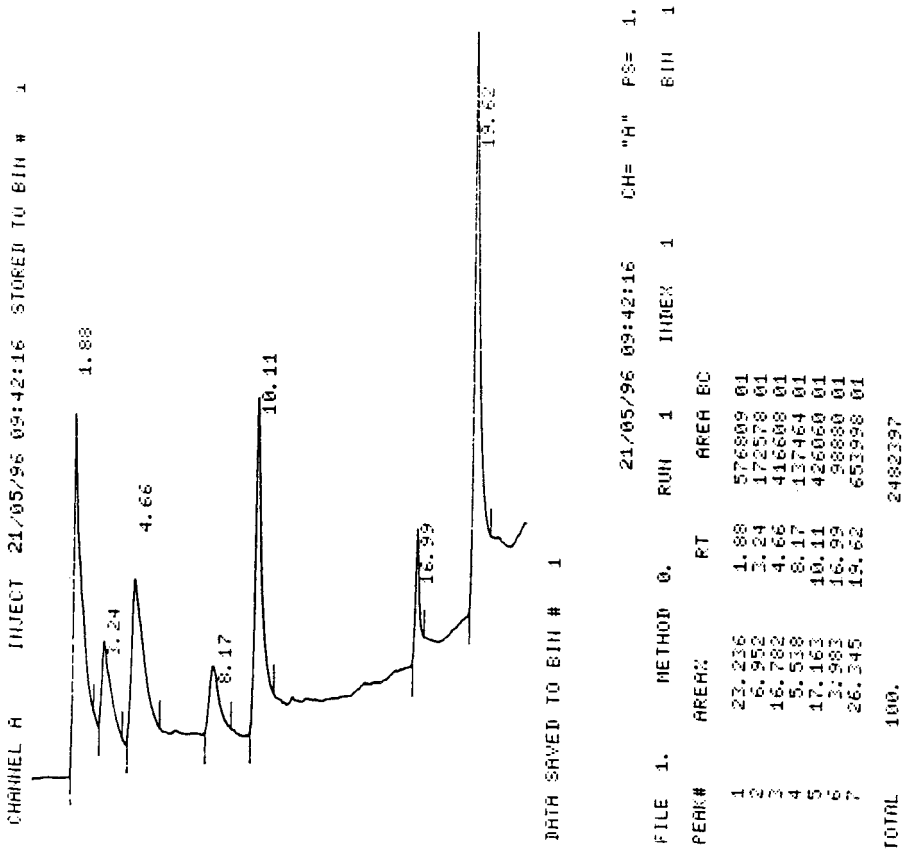

Figure 6. High performance liquid chromatogram of water soluble vitamins. Ascorbic acid (4 ppm) 1.88 min., Nicotinic acid (4 ppm) 3.24 min., Xanthine(4.2 ppm) 4.66 min., Nicotinamide (4 ppm) 8.17min., Folic acid (5 ppm) 10.11min., Cyanocobalamine min(5.5 ppm) 16.99 min., Riboflavin(5.05 ppm) 19.62 min.

applied to the cartridge. The fat-soluble vitamins were retained on the sorbent, while the water-soluble passed unretained. The water-soluble vitamins were collected into clean conical vials. Then fat-soluble vitamins were eluted by applying 3 mL of methanol and were collected into conical vials, as well. The solvent of the two selected fractions of vitamins was subsequently evaporated to dryness, at 45°C, under stream of nitrogen.

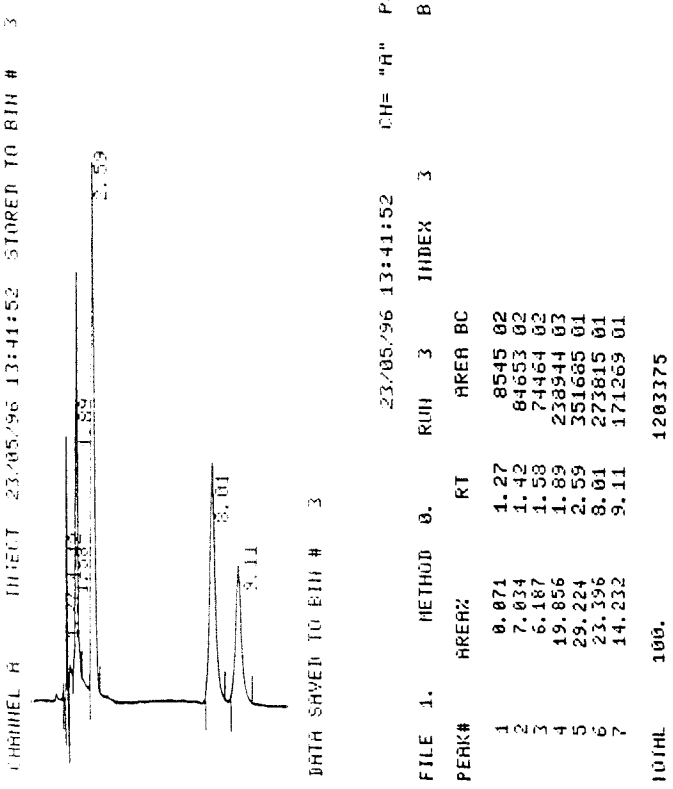

Figure 7. High performance liquid chromatogram of fat soluble vitamins. Anthraquinone(3.5 ppm) 1.89 min., Retinol (2.5 ppm) 2.59 min., α-Tocoferol (5 ppm) 8.01 min., α-Tocoferol acetate (6.5 ppm) 9.11 min.

The dry residue of water-soluble vitamins fraction was redissolved in 500 μL of methanolic solution of xathine (internal standard, 4.2 ng/μL), while the dry residue of fat-soluble vitamins fraction was redissolved to 500 μL of methanolic solution of Anthraquinone (internal standard, 3.5 ng/μL). Aliquots of 10 μL of each solution were injected onto the HPLC column.

Table 3

Recovery of Vitamins from Standard Solutions After SPE on C_{18} Cartridges Using Internal Standard

Analyte	Added (ng)	Found (ng)	SD	RSD (%)	Recovery (%)
Water-Soluble Vitamins					
Ascorbic acid	30.0	28.6	0.9	3.1	95.3
	50.0	49.1	1.7	3.5	98.2
Nicotinic acid	31.8	29.9	0.9	3.0	94.0
	53.0	52.0	1.9	3.6	98.1
Nicotinamide	29.4	28.0	1.3	4.6	95.2
	49.0	48.3	0.8	1.6	98.0
Folic acid	30.0	28.3	0.9	3.2	94.3
	50.0	48.3	1.0	2.1	96.8
Cyanocobalamine	30.0	28.3	0.6	2.2	94.3
	45.0	48.4	0.7	1.7	107.6
Riboflavin	30.3	26.7	1.7	6.4	88.1
	50.5	45.9	1.1	2.4	90.9
Fat-Soluble Vitamins					
Retinol	21.6	21.6	0.6	2.8	100.0
	32.4	31.8	1.3	4.1	98.2
α-Tocoferol	23.0	21.9	1.1	5.0	95.2
	34.5	31.4	1.2	4.1	91.0
α-Tocoferol acetate	25.3	23.2	0.8	3.5	91.7
	34.5	33.6	2.1	6.2	97.4

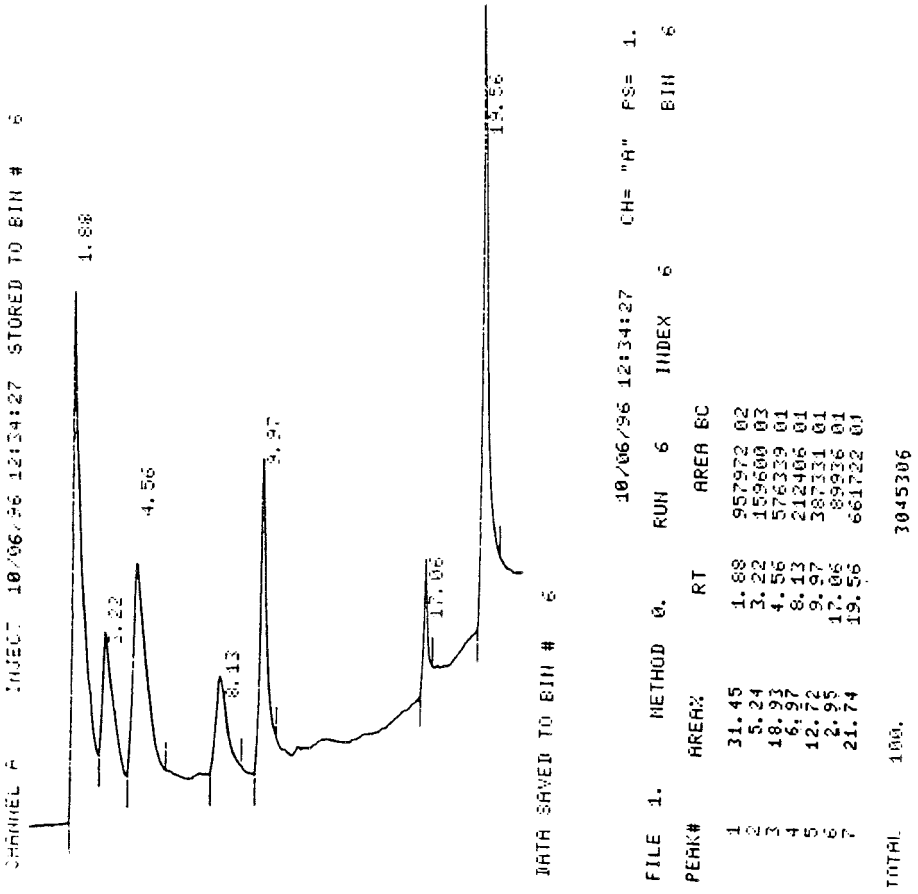

Figure 8. High performance liquid chromatogram of analysis of water soluble vitamins in spiked human blood samples. Ascorbic acid(4 ppm) 1.88 min., Nicotinic acid(3.19 ppm) 3.22 min., Xanthine(4.20 ppm) 4.56 min., Nicotinamide(4.40 ppm) 8.13 min., Folic acid(4.28 ppm) 9.97 min., Cyanocobalamine(4.50 ppm) 17.06 min. and Riboflavin(5.30 ppm) 19.56 min.

The correlation coefficients of the calibration curves had a range of 0.99917-0.99986 for water soluble vitamins and a range of 0.99904-0.99939 for fat-soluble vitamins. High performance liquid chromatograms of water-soluble and fat-soluble vitamins, after pre-treatment with SPE C_{18} cartridges are shown in Figures 6 and 7 respectively. The reproducibility and accuracy of solid phase extraction of vitamins were investigated. Results of recovery are shown in Table 3.

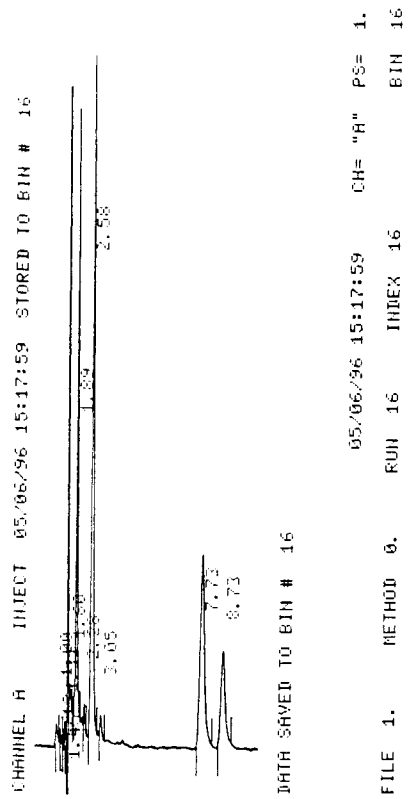

Figure 9. High performance liquid chromatogram of analysis of fat soluble vitamins in spiked human blood samples. Anthraquinone(3.50 ppm) 1.89 min., Retinol(3.41 ppm) 2.58 min., α-Tocoferol(5.10 ppm) 7.73 min. and α-Tocoferol acetate(4.90 ppm) 8.73 min.

Sample Preparation of Biological Fluids

Human blood plasma

Aliquots of 40 μL human blood plasma were treated with 80 μL of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was spiked with 100 μL of water soluble vitamins solutions, at concentration levels of 1, 3, 5, 7, 10 ng/μL.

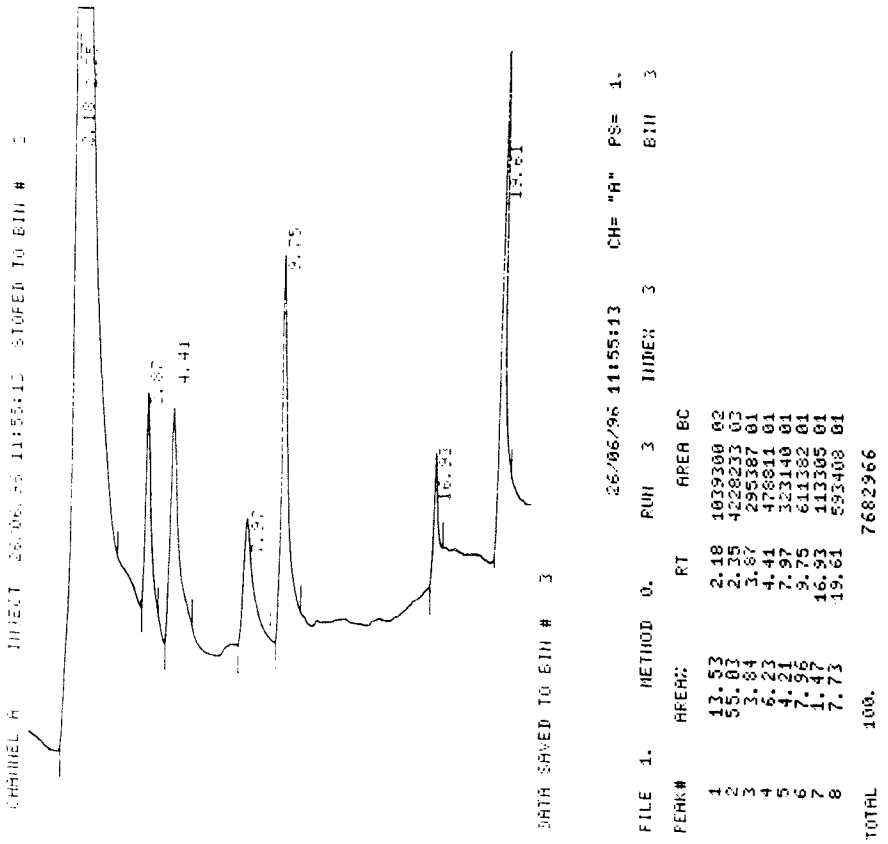

Figure 10. High performance liquid chromatogram of analysis of water soluble vitamins in urine samples. Nicotinic acid(6.24 ppm) 3.87 min., Xanthine(4.20 ppm) 4.47 min., Nicotinamide(6.60 ppm) 9.97 min., Folic acid(6.42 ppm) 9.75 min., Cyanocobalamine(6.0 ppm) 16.93 min. and Riboflavine(5.30 ppm) 19.61 min.

Then the sample was centrifuged at 3500 rpm for 15 min. and the supernatant was evaporated, at 45°C, under nitrogen stream, to remove organic solvents. Subsequently the sample was slowly applied to the solid-phase cartridge.

High performance liquid chromatograms of water-soluble and fat-soluble vitamins, extracted from human blood serum, are shown in Figures 8 and 9 respectively.

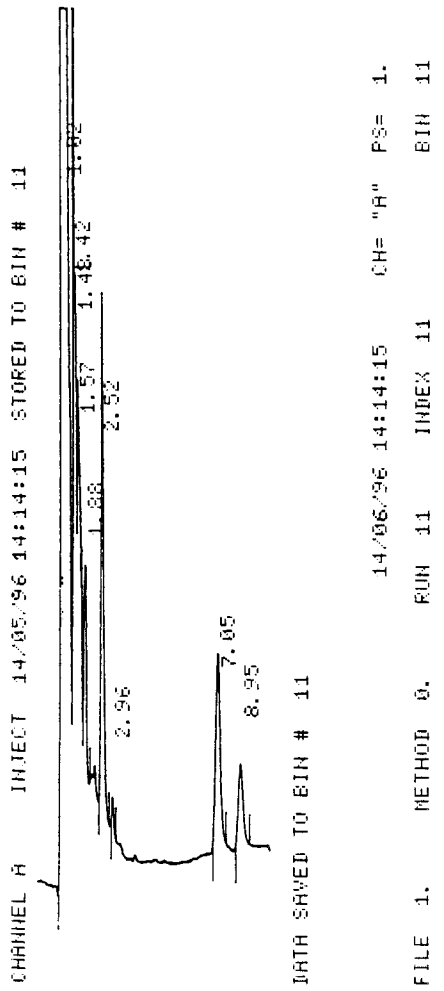

Figure 11. High performance liquid chromatogram of analysis of fat soluble vitamins in urine samples. Retinol(3.0 ppm) 2.52 min., α-Tocoferol(4.5 ppm) 7.75 min. and α-Tocoferol acetate(4.0 ppm) 8.95 min.

Urine

The same sample preparation method was followed for urine samples, after a small modification regarding sample volume. Thus in 100 μl of urine sample, 200 μl of acetonitrile were added and the procedure was followed as

described above. The serum and urine samples were pooled samples. No interference from endogenous compounds from sample matrix was observed in case of blood serum, in contrast to sample matrix of urine, in which some interferences observed. An interference in the fraction of water-soluble vitamin (retention time 2.48 min.) made the determination of vitamin C impossible. Also an interference in the fraction of fat-soluble vitamins (retention time 1.88 min) made the use of internal standard (Anthraquinone) impossible.

High performance liquid chromatograms of water-soluble and fat-soluble vitamins, extracted from human urine, are shown in Figures 10 and 11 respectively.

The correlation coefficients of the calibration curves for the analysis of vitamins in blood serum ranged between 0.99822 and 0.99982 for water soluble vitamins and between 0.99802 and 0.99968 for fat soluble vitamins. The correlation coefficients of the calibration curves for the analysis of vitamins in urine ranged between 0.99757 and 0.99943 for water soluble vitamins and between 0.99630 and 0.99954 for fat soluble vitamins. The precision and accuracy studies of SPE of vitamins from biological samples were conducted by spiking blood serum and urine samples, with known concentrations of the compounds and then by comparing obtained results, with those as calculated from regression equations. Results of recovery studies for serum and for urine samples are given in Table 4. Each value represents the mean of six measurements carried out.

CONCLUSIONS

Six water soluble vitamins (ascorbic acid, nicotinic acid, nicotinamide, folic acid, cyanocobalamine and riboflavin) and three fat soluble vitamins (retinol, α-tocopherol and α-tocopherol acetate) were separated into two fractions by means of solid-phase extraction and subsequently analysed by HPLC.

The developed method was further applied to multi-vitamin pharmaceutical preparations analysis and biological fluids analysis as well.

The binary eluent system used for water soluble vitamins and the isocratic eluent system used for fat soluble vitamins provide good separation, high selectivity and resolution within a minimum analysis time of 20 min for the fraction of water soluble vitamins and 9 min for the fraction of fat soluble vitamins.

Table 4a

Recovery of Vitamins from Human Blood Serum After SPE on C_{18} Cartridges[a]

Analyte	Added (ng)	Found (ng)	SD	RSD (%)	Recovery (%)
Water-Soluble Vitamins					
Ascorbic acid	10.0	8.3	0.6	7.2	83.1
	30.0	27.2	1.3	4.8	90.7
Nicotinic acid	10.4	8.8	5.2	5.8	84.6
	31.2	27.8	0.5	1.8	89.1
Nicotinamide	11.0	9.3	0.5	5.4	84.5
	33.0	31.8	0.4	1.2	96.4
Folic acid	10.7	10.7	0.7	6.5	100.0
	32.1	29.5	1.0	3.7	91.9
Cyanocobalamine	15.0	13.6	0.9	6.6	90.7
	30.0	29.2	0.9	3.1	97.3
Riboflavin	10.6	9.9	0.5	5.0	93.4
	31.8	29.6	1.0	3.4	93.1
Fat-Soluble Vitamins					
Retinol	11.2	10.2	0.3	2.9	91.1
	34.1	32.6	1.2	3.7	95.6
α-Tocoferol	12.6	11.1	0.6	5.4	88.1
	37.8	35.6	1.5	4.2	94.2
α-Tocoferol acetate	14.7	13.4	0.6	4.5	91.2
	34.1	33.6	1.1	3.3	98.5

[a] (n = 6)

Table 4b

Recovery of Vitamins from Human Urine Samples After Using Internal Standard[a]

Analyte	Added (ng)	Found (ng)	SD	RSD (%)	Recovery (%)
Water-Soluble Vitamins					
Nicotinic acid	10.4	9.4	0.5	5.3	90.4
	31.2	30.7	1.4	4.6	98.4
Nicotinamide	11.0	11.0	0.3	2.7	100.0
	33.0	32.4	1.6	4.9	98.2
Folic acid	10.7	10.2	0.2	2.0	95.3
	32.1	30.9	1.1	3.6	96.3
Cyanocobalamine	15.0	14.3	0.9	6.3	95.3
	30.0	29.1	1.7	5.8	97.0
Riboflavin	10.6	9.2	0.6	6.5	86.8
	31.8	28.6	1.9	6.5	89.3
Fat-Soluble Vitamins					
Retinol	11.2	10.3	0.6	5.8	92.0
	34.1	32.2	1.3	4.0	94.4
α-Tocoferol	12.6	11.4	0.5	4.4	90.5
	37.8	35.7	1.0	2.8	94.4
α-Tocoferol acetate	14.7	13.9	1.1	7.9	94.6
	34.1	34.1	1.8	5.3	100.0

[a] (n = 6)

Limits of detection are within 2-5 ng range for 10 μL injected sample volume. Day-to-day reproducibility was tested over ten consecutive days and repeatability (within day assay n=8) proved to be sufficient (RSD<7%).

Small volumes of biological fluids are required (40 μL of blood serum and 100 μL of urine).

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