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Thin-layer chromatography and fibre-optic fluorimetric quantitation of thiamine, riboflavin and niacin

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ABSTRACT

Thiamine (vitamin B_1), riboflavin (vitamin B_2) and niacin (nicotinic acid) were separated by thin-layer chromatography and fluorimetrically determined by using a commercially available fibre-optic-based instrument. Under fluorimetric monitoring riboflavin shows native fluorescence, but nicotinic acid and thiamine had to be pre-chromatographically converted to fluorescent derivatives. A new fluorescent tracer, fluoresceinamine, isomer II, was used to label the nicotinic acid. Thiamine was converted to fluorescent thiochrome by oxidizing with potassium ferricyanide solution in aqueous sodium hydroxide. The analytes were separated on HPTLC silica gel plates using methanol–water (70:30, v/v) as mobile phase. In these conditions the R_F values for the thiamine, riboflavin and niacin derivatives were, respectively, 0.73, 0.86 and 0.91. The developed plate was scanned by a bifurcated fibre-optic that both transmits emission radiation to the plate and collects the emission signal to the fluorimeter. Calibration curves for the determination of thiamine 300–750 ng, riboflavin 48–320 ng and niacin 10–100 ng were established.

INTRODUCTION

Thiamine, riboflavin and niacin frequently occur together in foods. High-performance liquid chromatography (HPLC) can separate and determine these vitamins [1,2]. Thin-layer chromatography can be used to determine simultaneously thiamine, riboflavin and pyridoxine [3].

High-performance thin-layer chromatography (HPTLC) permits comparable separations to HPLC [4]. The high resolution obtained by HPTLC might remain unexploited without reliable instrumentation for *in situ* quantitation of planar separations [5]. Although measurements are possible by using mechanical densitometers, recent advances in fibre-optics have made it possible to couple these devices to commercial fluorescence spectrometers that permit spectroscopic measurements remote from the detector head. Consequently, absorption [6] or fluorescence [7] measurements are now possible in places where it is difficult or impossible to bring the sample to the spectrometer, such as a TLC plate.

Thiamine, riboflavin and niacin absorb in the UV range, but not strongly enough to be detected with sufficient sensitivity. Measurement of fluorescence is generally more sensitive than measurement of absorption. However, whereas riboflavin exhibits a high fluorescence quantum yield, thiamine and niacin do not fluoresce, so these compounds must be converted into fluorescent derivatives, either before or after chromatographic separation.

In pre-column derivatization of thiamine to fluorescent thiochrome [1,8–14] sample extracts are injected at high pH and in the presence of an excess of potassium ferricyanide. Both high pH and ferricyanide damage liquid chromatography columns. They must be removed before chromatographic analysis.

Moreover, when post-column derivatization [15–18] is used, the presence of an excess of oxidizing reagent in the mobile phase gives rise to fluorescent impurities that generate noise and

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reduce the sensitivity of the detection method. These phenomena complement the existing disadvantages of post-column derivatization: they lengthen retention times, widen peaks and require a second pump system.

Derivatization of niacin with N,N′-dicyclohexyl-O-(7-methoxycoumarin - 4 - yl) -methylisourea for the vitamin determination by HPLC with fluorescent detection has been reported [19].

The simultaneous determination of water-soluble vitamins by HPLC has been demonstrated using photometric detection [2], but if sensitive fluorescence detection is required two detectors must be used [1,9,11,15–18]. The recoveries and reproducibility are good but the approaches are rather complex. Moreover, recently simultaneous determination of thiamine, riboflavin and pyridoxine by TLC with fluorimetric detection has been described [3].

In this work thiamine, riboflavin and niacin were separated by TLC before fluorescence analysis. Thiamine was pre-chromatographically converted to fluorescent thiochrome. Riboflavin revealed natural fluorescence, and fluoresceinamine, isomer II, was used to label niacin before chromatography [20].

EXPERIMENTAL

Materials

Thiamine hydrochloride, riboflavin, nicotinic acid, fluoresceinamine isomer II and N,N-dicyclohexylcarbodiimide (DCC), were obtained from Aldrich (Milwaukee, WI, USA) or Sigma (St. Louis, MO, USA). Potassium ferricyanide, sodium hydroxide and the solvents were purchased from Merck (Darmstadt, Germany). All samples were prepared in distilled–deionized water or in ethanol and were sonicated for at least 10 min.

Emission measurements were made with a Perkin-Elmer LS-50 luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK) equipped with a plate-reader accessory. A 1-m glass fibre-optic bifurcated bundle (Oriel, Stratford, CT, USA) with a light transmission range of 390–1500 nm was used to transfer the excitation and emission energies between the plate and the spectrometer. Information was sent via the RS232C interface of the fluorescence instrument to an external computer. Instrumental parameters were controlled by Fluorescence Data Manager (FLDM) software (Perkin-Elmer). Three-dimensional and contour plots were made using SURFER software (Golden Software, Golden, CO, USA). Graphical print-out was from a NEC Silentwriter 2 laser printer (NEC, Tokyo, Japan).

Thin-layer chromatography

Samples were applied by the spray-on technique using a Linomat IV microprocessor-controlled device (Camag, Muttenz, Switzerland). Sample aliquots of 15 μl were applied to the plates in narrow 5-mm-long bands separated by 10 mm. The compounds were chromatographed on HPTLC Silicagel 60 plates without fluorescent indicator (Merck, No. 5631). The plates were developed in a Camag horizontal developing chamber for some 15 min over a distance of about 50 mm from the origin using methanol–water (70:30, v/v) as mobile phase. *In situ* measurements of thiamine, riboflavin and niacin were made at $\lambda_{ex} = 390$ nm, $\lambda_{em} = 440$ nm; $\lambda_{ex} = 464$ nm, $\lambda_{em} = 520$ nm, and $\lambda_{ex} = 501$ nm, $\lambda_{em} = 523$ nm, respectively. Blanks without analyte were measured in the same way as the analyte. Quantitative data were obtained using fluorescence intensity at the centre of the spots (peak heights). The calibration curves were constructed by plotting peak heights against concentration of standards.

Derivatization procedure

To samples containing thiamine, riboflavin and niacin were added 3 ml of 0.03 *M* potassium ferricyanide solution of 3.75 *M* aqueous sodium hydroxide; pH was adjusted to 7. To the neutralized solution were added fluoresceinamine and DCC in a molar ratio twice that of nicotinic acid. The reaction was carried out at room temperature for 24 h, and the precipitated cyclohexylurea was filtered through 0.2-μm nylon filter. The final solution was made up to 50 ml with ethanol before being subjected to thin-layer chromatography.

RESULTS AND DISCUSSION

Only riboflavin exhibits natural fluorescence. Nicotinic acid and thiamine were pre-chromatographically converted to fluorescent derivatives. A post-chromatographic derivatization of thiamine to fluorescent thiochrome on silica gel plate can be readily done, but for niacin this is difficult. Conversion of thiamine to the fluorescent derivative, thiochrome, is extensively used to detect this analyte by HPLC [1,8–18], and potassium ferricyanide in sodium hydroxide is used as the reagent. The same reagent concentration was found to be optimal to derivatize thiamine on the silica gel plate.

Fluorescent amines can be used to label the carboxylic groups. Because fluorescein derivatives offer high quantum efficiency, fluoresceinamine (isomer II) was selected to label the nicotinic acid. However, the carboxylic acid group had to be activated before coupling with the amines [21–23]. Carbodiimide derivatives such as N,N-dicyclohexylcarbodiimide (DCC) can be used for this.

$$RN=C=NR + R'\text{-}COOH \rightarrow RNH\text{–}\underset{\displaystyle OCOR'}{\underset{|}{C}}=NR$$

$$RNH\text{–}\underset{\displaystyle OCOR'}{\underset{|}{C}}=NR + R''NH_2 \rightarrow R'CONHR'' + RHNCONHR \text{ (cyclohexylurea)}$$

where R is the substituent of carbodiimide, R′ is the structure containing the carboxylic group and R″ is the structure that contains the amino group. The insoluble cyclohexylurea reaction product was filtered through a nylon filter. The reaction yield, as determined by preparative TLC, extrusion of the derivative band and subsequent fluorescent readings, appeared to be about 60–80%. An increase in the reaction temperature might improve this procedure, but this could lead to sample degradation.

The fluorescence spectra of thiamine, riboflavin and niacin and their derivatives were obtained. The excitation and emission maxima of riboflavin, thiochrome and niacin derivatized in ethanolic solutions and adsorbed on silica gel are shown in Table I. Only thiochrome modified their maxima with adsorption on silica gel.

To separate the vitamins on silica gel two mobile phases were analysed; methanol–water (70:30, v/v) and aqueous ammonia–chloroform–ethanol–acetone (2:2:2:1, v/v). Because similar separations were achieved with both these solvents, we choose the binary mixture methanol–water (70:30, v/v). With this mobile phase the R_F values for thiamine derivative, riboflavin, niacin derivative and fluoresceinamine were 0.73, 0.86, 0.91 and 1 respectively. The three-dimensional chromatogram and contour maps of the plate containing the separated vitamins, scanned at three different pairs of wavelengths are shown in Figs. 1 and 2. The fluorescence selectivity over that of HPTLC is well demonstrated because only the fluorescent vitamin gives a peak at each pair of wavelengths. The advantage of choosing

TABLE I

EXCITATION AND EMISSION MAXIMA OF THE INVESTIGATED VITAMINS IN ETHANOLIC SOLUTIONS AND ADSORBED ON SILICA GEL

	Ethanol		Silica gel	
	λ_{ex} (nm)	λ_{em} (nm)	λ_{ex} (nm)	λ_{em} (nm)
Thiamine derivative, $7.11 \cdot 10^{-6}$ *M* slit$_{ex}$ = 5 nm, slit$_{em}$ = 5 nm	369	415	390	440
Riboflavin, $4.25 \cdot 10^{-5}$ *M* slit$_{ex}$ = 2.5 nm, slit$_{em}$ = 2.5 nm	464	520	464	520
Nicotinic acid derivative, $4.8 \cdot 10^{-4}$ *M* slit$_{ex}$ = 2.5 nm, slit$_{em}$ = 5 nm	501	523	501	523

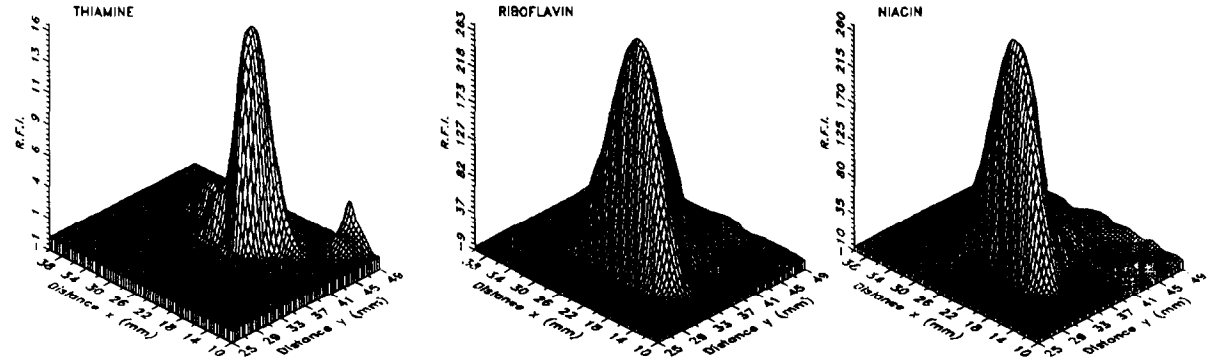

Fig. 1. Three-dimensional chromatogram with blank subtracted scanned at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 440$ nm (thiamine, 200 ng spotted); $\lambda_{ex} = 464$ nm and $\lambda_{em} = 520$ nm (riboflavin, 96 ng spotted); $\lambda_{ex} = 501$ nm and $\lambda_{em} = 523$ nm (niacin, 169 ng spotted). R.F.I = Relative fluorescence intensity.

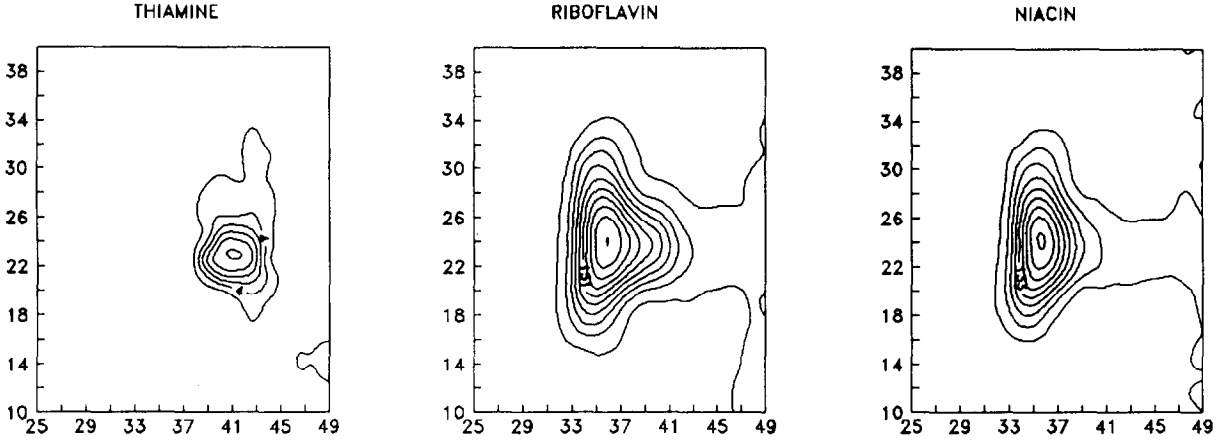

Fig. 2. Contour plots of the plate using the same parameters and concentrations as in Fig. 1.

the optimal detection wavelength for TLC by photodiode array spectrometry is described in ref. 6.

Quantitative analysis

Figs. 1 and 2 show that the peak widths of the different compound were very different. Because the sample substance was applied as a thin line at the origin, the concentration profiles of spots of separated substances exhibit a roughly Gaussian profile (cylinder character) [24]. The integral of the concentration density over the spot area was equal to the volume of the cylinder, which in turn is proportional to peak concentration value. In this way we can substitute integration by

TABLE II

STANDARD DEVIATION AND RELATIVE STANDARD DEVIATION

Compound	Concentration spotted (ng)	Mean peak height	S.D. ($n = 5$)	R.S.D. (%)
Thiamine	300	159	1.9	1.2
	450	245	5.9	2.4
	750	482	9.0	1.8
Riboflavin	48	35	4.6	13.1
	80	83	6.5	7.8
	160	210	11.0	5.2
Niacin	50	170	2.2	1.3
	70	212	1.8	0.8
	100	280	7.0	2.5

measuring the maximum fluorescence intensity (or peak height) at the centre of the spot.

The scanned relative fluorescence intensities (peak heights) as a function of spot concentration were found to be linear and ranged from 300 to 750 ng for thiamine, from 48 to 320 ng for riboflavin and from 10 to 100 ng for niacin. The respective regression coefficients were 0.9973 ($n = 5$), 0.9991 ($n = 5$) and 0.9992 ($n = 5$). Table II gives the mean peak heights, standard deviations (S.D.) and relative standard deviations (R.S.D.) for three different determinations at three concentration levels.

The repeatibility of the derivatization procedure was checked by applying three loadings (15 μl each) of six solutions each with the same vitamin concentrations (thiamine $1.32 \cdot 10^{-4}$ M, riboflavin $1.78 \cdot 10^{-5}$ M and niacin $4.76 \cdot 10^{-5}$ M) and then subjecting them to the derivatization procedure. Repeatability [expressed as relative standard deviation (S.D.) ($n = 6$)] was 5.1% for thiamine, 6.0% for riboflavin and 4.2% for niacin.

Although the yield of the derivatization reaction is not high (60–80%), repetitive between-batch experiments showed good standard deviations of the measurements, consequently the method appears to be a reliable approach for determining the assayed compound. A calibration graph should be made before each assay.

The detection limit (DL) is defined as that concentration which gives an analytical signal equal to three times the blank noise and is calculated using the expression $DL = 3s_{bk}/m$, where s_{bk} is the standard deviation of the blank signals ($s_{bk} = 1.8$) and m is the slope of the calibration curve. Values for thiamine, riboflavine and niacin were 0.73, 1.71, and 2.08, respectively. The values of the detection limits were 7.4 ng, 3.1 ng and 2.6 ng for thiamine, riboflavin and niacin, respectively.

CONCLUSIONS

This method has several advantages over those mentioned in the literature. It is not necessary to eliminate the oxidation reagent excess and this permits simultaneous determinations of vitamins with only one detector. The separation time is 15 min. The sensitivities and selectivities of fluorescence detection allow these vitamins to be quantified with low detection limits and also to discriminate between several compounds whose chromatographic resolution is poor by spectral resolution.

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