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Determination of pyridoxal in human serum by matrix isopotential synchronous fluorescence spectrometry

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Abstract

The determination of pyridoxal in human serum was performed using a fluorimetric technique that is useful for the determination of compounds in samples with unknown background fluorescence, based on synchronous scans through a trajectory joining points of equal intensity of a fluorescence matrix three-dimensional spectrum. This technique, called matrix isopotential synchronous fluorescence can be improved by means of the application of derivatives. The determination of pyridoxal in human serum was performed with this technique without any prior separation steps. The validity, applicability and simplicity of the method were demonstrated. The measurements were performed in aqueous medium at pH 7.0 adjusted by adding 0.05 M phosphate buffer solution. A complete statisitical analysis of the experimental data was performed and the results showed that correlation coefficients are between 0.9901 and 0.9958 for all the calibration graphs, and in all cases the intercepts on the ordinate were negligible. The exprimental F (1.72) is smaller than theoretical value (3.89) as expected from an analysis of variance. The reproducibility of the method was tested and the results were a standard deviation of 0.0117 μg ml^{-1} and a relative error of 4.91%.

Keywords: Pyridoxal; Human serum; Matrix isopotential synchronous fluorescence spectrometry

1. Introduction

Fluorescence techniques have been developed in recent years to produce faster methods. An example is synchronous fluorescence spectrometry, and different types have been described such as variable-angle synchronous fluorescence spectrometry, introduced by Lloyd [1], and the non-linear variable-angle synchronous method developed by Kubic et al. [2]. These techniques offer several possibilities, e.g. (1) in a complex system, the maximum and minimum emission intensities can be explored by traversing the peaks and valleys; and (2) a curve trajectory can be followed through the emission and excitation matrix, allowing light scattering peaks to be avoided. Therefore, an overlapped system that cannot be resolved by linear scanning can be resolved by this technique. A special variation is constant-energy synchronous fluorescence, proposed by Inman and Winefordner [3] in which

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the energy between the measured excitation and emission radiation is kept constant. This technique reduces solvent interference by Raman scattering and has been applied to the spectral resolution of mixtures of polyaromatic hydrocarbon isomers and polyaromatic hydrocarbon alkyl homologues [4,5], extending to low-temperature conditions by reducing the sample temperature to that of liquid nitrogen of helium [6,7].

The combination of these techniques and derivative methods (suggested by John and Soutar [7]) improved the selectivity of the analysis. A derivative synchronous fluorescence method has been described for the resolution of compound mixtures with spectra of similar characteristics such as those provided by motor oil [8]. Constant-energy synchronous fluorescence was combined with a derivative fluorescence technique to determine four polynuclear hydrocarbons [9]. A study of the resolution of binary mixtures of nafcillin and methicillin using this technique has been reported [10].

In this work, we used a fluorescence technique [11–13] in which cuts are made in the total fluorescence spectrum through a trajectory joining points of equal intensity (isopotential trajectory). This trajectory is obtained by means of a program written in BASIC [14]. This technique is called matrix isopotential synchronous fluorescence.

Human serum is composed of a variety of components, but only a few of them make significant contribution to the overall fluorescence. Most compounds are present only in minor concentrations or have a low fluorescence efficiency. A three-dimensional plot is required for a complete description of the fluorescence.

The fluorescence of human serum is highly pH dependent, so it is necessary to keep the pH constant to make topograms comparable. At pH values between 6.5 and 4.0, precipitation of proteins does not allow reasonable measurements to be obtained. Wolfbeis and Leiner [15] described the assignment of peaks and shoulders for human serum in the ultraviolet and visible regions.

Sera which were used for the assignment were obtained from healthy persons because, as Wolfbeis and Leiner [15] recognized, sera relating to human diseases give rise to recognizable deviations in the topographic pattern. The procedures described in the literature are based on the addition of the analyte to healthy human sera. In order to develop this procedure, we added pyridoxal to healthy human sera.

The determination of pyridoxal and its derivatives by various methods has been reported [16–18]. All are based on a prior chomatographic treatment or derivatization reactions [19–21], owing to the multiple forms and similar structures of these compounds. The determination of pyridoxal, which is one of the active derivatives of vitamin B_6, in biological fluids is interesting. The main method was based on the natural fluorescence of vitamin B_6 and its derivatives. Human serum is composed of numerous organic substances, only some of which are fluorescent, but these are sufficient to provide a noticeable background fluorescence and to interfere with direct determination of pyridoxal in human serum. Yamata et al. [22,23] described a method for the determination of pyridoxal in body fluids but it required a tedious separation and prior reactions. Kraut and Inhoff [24] described a method to determine vitamin B_6 and its derivatives in body fluids, but a prior chromatography separation is necessary in order to characterize each compound.

More recently, Petidier et al. [25] described a method for the characterization of pyridoxal and its derivatives in human serum, but it required a prior derivatization reaction with beryllium in ammonia medium, and deproteinezed human sera. Linares et al. [26] described a method involving fluorimetry and flow injection analysis for determining pyridoxal and pyridoxal 5-phosphate in human serum based on their oxidation in the presence of cyanide. Deproteination of serum was necessary and the average error was as high as ± 10–15%.

The fluorescence spectra of pyridoxal and human serum showed overlapping so the conventional fluorimetric method does not permit the direct determination of this compound, even if first-derivative synchronous fluorescence spectrometry is applied.

In this work, the determination of pyridoxal in human serum was performed by matrix isopotential synchronous fluorescence. The results obtained show that this technique is a rapid and straightforward method for resolving a mixture without any prior separation procedures and for determining analytes in complex fluorescent matrices, e.g. biological fluids.

2. Experimental

2.1. Reagents

Pyridoxal was purchased from Merck. A stock standard solution of 500.0 $\mu g\ l^{-1}$ was prepared by dissolution in ultra-pure water obtained with a Milli-Q system (Millipore). This stock standard solution was used to prepare working standard solutions by suitable dilution.

Buffer solution of pH 7.0 was prepared by mixing an appropriate amount of sodium dihydrogenphosphate and sodium hydroxide.

Human serum was obtained from fasting and healthy people in the morning in a clinical analysis laboratory, where blood was treated in order to obtain serum. Human serum was frozen under at $-18°C$ until used.

2.2. Apparatus

Fluorescence measurements were performed on a Perkin-Elmer LS 50 spectrofluorimeter equipped with a xenon lamp, connected to an Ataio S 3000 ST 386 computer fitted with Perkin-Elmer F. L. Data Manager (FLDM) software and linked to an Epson FX-850 printer. A thermostat and a Crison Model 2001 pH meter with a glass–saturated calomel combination electrode were also used. Ftotal software [14] was used for the treatment of total luminescence spectra.

2.3. Procedures

A 0.25 ml volume of human serum, fresh or defrosted, containing between 0.52 and 5.0 μg of pyridoxal was transferred into a 10.0 ml volumetric flask, 1.0 ml of phosphate buffer solution of pH 7.0 was added and the mixture was diluted to volume with Milli-Q water.

For each sample, 61 emission spectra of 240 nm width were measured, varying the excitation wavelength in 4 nm steps. The total luminescence spectra were obtained using the Ftotal software [14]. Subsequently, suitable trajectory and matrix isopotential synchronous spectra were obtained for pyridoxal using the same program. The determination of pyridoxal is possible by measuring the first derivative of the spectra.

Calibration graphs was obtained by using pyridoxal-free human serum spiked with different amounts of pyridoxal. Standards were treated as indicated above.

3. Results and discussion

One of the major disadvantages of fluorimetric methods is that it is not easy to remove background fluorescence. This means that direct determinations of fluorescent compounds, e.g. in important matrices such as urine, serum or plasma, have to be approached by means of fluorescence derivatization or by prior extraction procedures, with the disadvantages that they involve.

If the matrix for which we wish to perform a spectrofluorimetric determination displays an almost invariable composition, even though its fluorescence intensity may vary, it is possible to maintain a constant background signal if a cut is made in the matrix total luminescence spectrum following one of the trajectories which join points of equal fluorescence intensity (iospotential trajectory) from initial excitation and emission wavelengths.

When this trajectory is applied to the total luminescence spectrum of a component in the presence of the fluorescente matrix, the same spectrum is obtained as that of the component in isolation, increased by a constant equivalent to the matrix intensity in that isopotential trajectory.

The intensity of the samples will be

$$I_{S(\lambda_{ex},\lambda_{em})} = I_{C(\lambda_{ex},\lambda_{em})} + I_{B(\lambda_{ex},\lambda_{em})}$$

where $I_{S(\lambda_{ex},\lambda_{em})}$, $I_{C(\lambda_{ex},\lambda_{em})}$ and $I_{B(\lambda_{ex},\lambda_{em})}$ are the fluorescence intensities of the sample, component and background, respectively.

By definition, if the trajectory is isopotential, then $I_{B(\lambda_{ex},\lambda_{em})}$ is constant (K) during the whole scan of wavelengths, that is,

$$I_{S(\lambda_{ex},\lambda_{em})} = I_{C(\lambda_{ex},\lambda_{em})} + K$$

Although the quantitative composition may vary in different determinations, the constant K can be ascertained as the value towards which at least one of the extremes of the spectrum of the component to be determined tends asymptotically and, in consequence, it is possible to obtain the fluorescence intensity corresponding only to the component to be determined:

$$\lim_{\lambda_{ex}\to\lambda_{exL}} I_{S(\lambda_{ex},\lambda_{em})} = \lim_{\lambda_{ex}\to\lambda_{exL}} I_{C(\lambda_{ex},\lambda_{em})} + K$$

or

$$\lim_{\lambda_{ex}\to\lambda_{exI}} I_{S(\lambda_{ex},\lambda_{em})} = \lim_{\lambda_{ex}\to\lambda_{exI}} I_{C(\lambda_{ex},\lambda_{em})} + K$$

where $\lambda_{ex\,L}$ and $\lambda_{ex\,I}$ are the last and initial excitation wavelength, respectively. If

$$\lim_{\lambda_{ex}\to\lambda_{exL}} I_{C(\lambda_{ex},\lambda_{em})} \to 0$$

or

$$\lim_{\lambda_{ex}\to\lambda_{exI}} I_{C(\lambda_{ex},\lambda_{em})} \to 0$$

then

$$K = \lim_{\lambda_{ex}\to\lambda_{exL}} I_{S(\lambda_{ex},\lambda_{em})}$$

or

$$K = \lim_{\lambda_{ex}\to\lambda_{exI}} I_{S(\lambda_{ex},\lambda_{em})}$$

If this is not possible because the spectrum of the component in isolation according to this trajectory does not adopt the value zero at the extremes, as in the previous case, the problem of not knowing the matrix constant value can be avoided by applying the first derivatives, that is,

$$\frac{dI_{S(\lambda_{ex},\lambda_{em})}}{d\lambda} = \frac{dI_{C(\lambda_{ex},\lambda_{em})}}{d\lambda} + \frac{dI_{B(\lambda_{ex},\lambda_{em})}}{d\lambda}$$

as the trajectory is isopotential, and then

$$\frac{dI_{B(\lambda_{ex},\lambda_{em})}}{d\lambda} = 0$$

and

$$\frac{dI_{S(\lambda_{ex},\lambda_{em})}}{d\lambda} = \frac{dI_{C(\lambda_{ex},\lambda_{em})}}{d\lambda}$$

It is always possible to find the matrix trajectory which passes through the maximum fluorescence excitation and emission wavelengths of the component to be determined, and so the same sensitivity is achieved as in a direct determination in the absence of background fluorescence.

The only condition which is necessary and sufficient for the application of this technique is that the fluorescence of the matrix and the analyte should be additive, as is the case with all techniques applied to samples containing more than one fluorescent compound. Even if this condition is not fulfilled, this technique could be used by comparing the fluorescence values with a calibration graph constructed with the analyte in isolation.

This technique was applied to the determination of pyridoxal in serum.

3.1. Factors affecting fluorescence intensity

Chemical variables were studied to obtain the optimum measurement conditions and maximum fluorescence signal.

The dielectric constant can modify the fluorescence characteristics of the compound. In this way, the effect of the ethanol content in the medium was investigated by preparing samples of pyridoxal and varying the ethanol concentration between 0 and 80% (v/v). The relative fluorescence intensity decreases when the ethanol content in the medium increases. Therefore, we chose to use an aqueous medium.

The influence of pH on the fluorescence intensity was studied by adding different amounts of $HClO_4$ or NaOH solutions. The fluorescence intensity of pyridoxal was maximum and constant between pH 5.5 and 9.5 and pH 7.0 was adopted. A pH of 7.0 was adjusted by adding phosphate buffer solution. The fluorescence intensity of pyridoxal was not affected by the concentration of buffer. A 0.05 M buffer concentration was selected to obtain an adequate buffering capacity.

Another factor that affects the fluorescence intensity is temperature: the fluorescence intensity decreases when the temperature increases from 3 to 50°C, with a temperature coefficient of about 1.05% $°C^{-1}$. This effect can be explained by the higher internal conversion when temperature increases, facilitating non-radioactive deactivation of the excited singlet state [27]. Therefore, the use of a thermostat is recommended, and a measurement temperature of 20°C was chosen as around room temperature.

Under these conditions, the influence of vitamin concentration on the fluorescence intensity was studied. A linear relationship between flluorescence intensity and pyridoxal concentration in the range 0.05–0.50 $\mu g\ ml^{-1}$ was obtained.

3.2. Determination of pyridoxal in human serum

Matrix isopotential synchronous fluorescence spectrometry was applied to the determination of pyridoxal in human serum, a fluorescent matrix, under the optimum chemical conditions described above.

As can be observed in Fig. 1, human serum and pyridoxal showed overlapped spectra, so a prior separation should be necessary to characterize pyridoxal in serum.

The fluorescence spectra of 20 samples of pyridoxal-free serum from healthy people of both

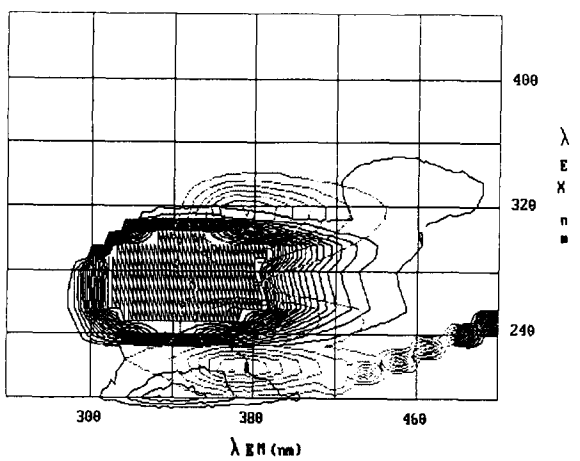


Fig. 1. Total luminescence spectra of diluted 40.0 fold of human serum (solid lines) and pyridoxal (dashed lines).

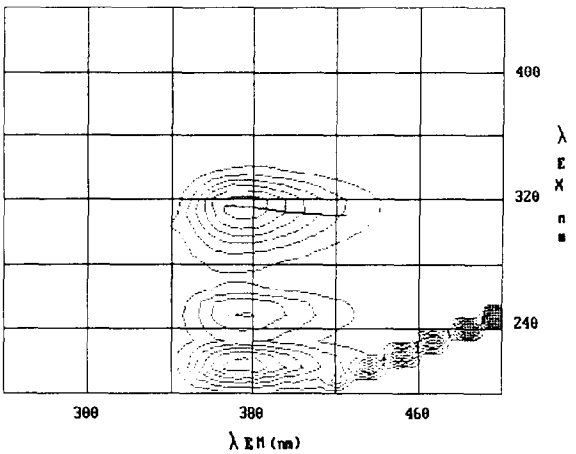


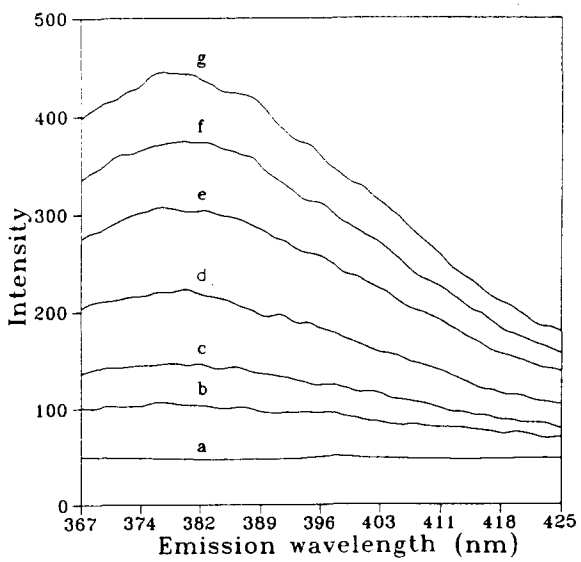
Fig. 2. Total luminescence spectrum of pyridoxal (dashed lines) and isopotential trajectory of human serum selected (solid lines).

sexes aged between 20 and 35 years and on different diets, were obtained in the form of a contour map in order to make a comparison. Different samples of serum showed the same type of fluorescence, with hardly any variation observed in either the form of the spectrum or the location of the fluorescence maxima, although a slight variation in their intensity was observed. The spectrum corresponding to the arithmetic mean of the 20 total luminiscence spectra of the different pyridoxal-free human sera was obtained by means of the Ftotal program.

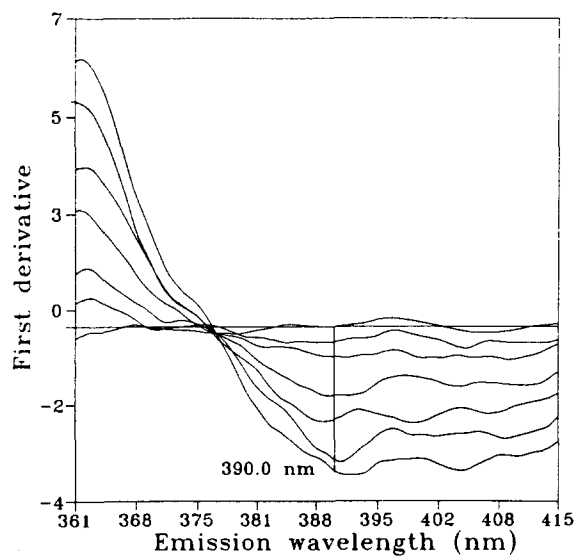
In order to determine pyridoxal, we calculated the isopotential trajectory in the human serum spectrum, which passed through the excitation and emission maxima of pyridoxal (315.0 nm excitation and 378.0 nm emission maxima) (Fig. 2).

Total luminiscence spectra of pyridoxal in different human sera were obtained in order to construct three calibration graphs. Calibration graphs were obtained by varying the pyridoxal concentration between 0.052 and 0.500 $\mu g\ ml^{-1}$ and the matrix isopotential synchronous fluorescence technique was applied to all the samples. The fluorescence intensity could not be measured because the extreme values in the spectrum are not constant (Fig. 3(A)), so the first derivative of the spectra according to the Savitzky–Golay algorithm [28,29] was calculated.

In the same way, we obtained the total luminescence spectra of pyridoxal at the same concentrations as applied in the above trajectory method in order to obtain their matrix isopotential synchronous fluorescence spectra, and we also obtained their first-derivative spectra, as for the serum samples.



(a)



(b)

Fig. 3. (A) Set of matrix isopotential synchronous spectra of human serum plus increasing concentrations of pyridoxal: (a) 0, (B) 0.052, (C) 0.104, (D) 0.206, (E) 0.306, (F) 0.404 and (G) 0.500 $\mu g\ ml^{-1}$. (B) First-derivative spectra.

In order to establish the appropriate number of points to obtain the first derivative by the Savitzky–Golay algorithm [28,29], spectra of serum and pyridoxal solutions were derivatized with 5, 9, 15, 21 and 25 points. Derivatized spectra with a suitable signal-to-noise ratio were obtained with 21 points.

The first-derivative signals of all the calibration graphs are displayed in Fig. 3(B), which shows that this signal is independent of the human serum (background signal). The measurement wavelength chosen was 390.0 nm, because the repeatability is maximal.

Linear regression equations ($y = a + mx$) for pyridoxal in human serum were obtained. The slopes, intercepts, correlation coefficients and standard deviations obtained for the determination of pyridoxal in human serum for the first-derivative matrix isopotential synchronous fluorescence spectra are summarized in Table 1. The significance of the intercept on the y-axis of the regression line obtained was evaluated by applying Student's t-test at the 95% confidence level. If the intercept on the y-axis for the line calculated by the least-squares method is negligible, it is necessary to repeat the fitting of the data to a function $y = m_0 x$ and therefore the new value of the slope (m_0) may be calculated. In all the calibration graphs the intercepts on the ordinate were negligible since the experimental t-value is smaller than the theoretical value, and for this reason new slopes were calculated.

When the results obtained for sera were compared with those obtained for aqueous solution, we obtained a recovery between 91.9 and 105.5%. This is why the slopes from the calibration graphs for pyridoxal in the presence of different human sera are lower than the slope obtained from calibration graph of pyridoxal in aqueous solution; This may occur because pyridoxal can be linked to proteins.

The accuracy of the method can be tested by using a statistical technique such as analysis of variance [30]. Variance is a statisitical measure of error or variation. The variance of a set of measurements $x_1, x_2, x_3, \ldots, x_n$ is defined as

Table 1
Results for the determination of pyridoxal in human serum by the first-derivative method

Calibration graph[a]	Slope	Intercept	Correlation coefficient	Standard deviation	
				Slope	Intercept
Pyridoxal	7.100	0.122	0.9913	0.47	0.14
Pa + serum 1	7.686	−0.216	0.9920	0.49	0.15
Pa + serum 2	6.591	0.095	0.9958	0.30	0.09
Pa + serum 3	6.706	−0.011	0.9901	0.48	0.15
Pa + serum 4	6.787	0.005	0.9917	0.44	0.13

[a] Pa = pyridoxal.

$$\frac{\Sigma(x+\bar{x})^2}{n-1}$$

where x is an individual value and $\bar{x}$ is the mean value given by $\Sigma x/n$. The divisor $(n-1)$ is termed the number of degrees of freedom since it represents the number of independent measurements available to calculate the variance (if the mean and $n-1$ of the values are given, the remaining nth value is automatically fixed). The square root of variance is termed the standard deviation, σ.

It may be shown that the expression for the variance is algebraically equivalent to

$$\frac{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}}{n-1}$$

which is simpler to calculate. This is the form of expression used to evaluate the different sources of variance in the analysis of variance. The numerator is usually referred to in analysis of variance tables as the sum of squares and the results when divided by the number of degrees of freedom are called mean squares.

The sequence of steps to be followed in determining the precision of a method which is subjected to collaborated testing is as follows:

(i) Reduce the results reported to give equal numbers of replicated results for each sample. This is necessary to produce a balanced set of data, otherwise the analysis of variance becomes extremely complex.

(ii) Eliminate any samples which give consistently high or low results and eliminate (or correct) any outlying data that are in error.

(iii) Examine the variances within samples or between replicates to see whether the reproducibility and repeatability are constant. The validity of the analysis of variance assumes that the residual error variance does not change from one sample to another or from one calibration graph to another.

(iv) Calculate the reproducibility and repeatability of the method and overall precision when one analyst carries out replicate determinations.

The variance ratio (experimental F) must be calculated and compared with a theoretical value of F, which must be smaller than the experimental F, for a suitable number of degrees of freedom at the 95% confidence level. Thus, the experimental value of F, was calculated (1.72) and found to be smaller then the theoretical value (3.29).

For four series of four standard samples containing 0.306 μg ml^{-1} of pyridoxal, the standard deviation and the relative error were calculated in order to ascertain the reproducibility of the proposed method and the results were 0.0117 μg ml^{-1} and 4.91% respectively, when first-derivative matrix isopotential synchronous fluorescence spectrometry was used.

4. Conclusions

Matrix isopotential synchronous fluorescence spectrometry is particularly useful for removing fluorescent matrix background effects and permits the determination of compounds in complex samples. This technique was used in combination with the first-derivative method in order to determine

pyridoxal in human serum without prior separation steps.

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