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Contributions to the determination of histamine rate by measuring out the histamine–orthophthalaldehyde complex in the absorption and fluorescence☆

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Abstract

Histamine is an important compound from a physiological point of view, but it is toxic since the absorption of low amounts of histamine can cause abdominal pains accompanied with vomiting. Why we have developed a new method for histamine analysis in order to improve Lerke and Bell method. From the absorption spectra, we showed that the stoichiometry of the complex histamine–orthophthalaldehyde (OPA) is 1:1. From the emission spectra, it was observed that the complex fluorescence is inhibited in acidic medium. In alkaline medium, an exaltation of fluorescence was observed, but the complex histamine–OPA was unstable. Nevertheless, kinetic study showed that good linear correlations between the fluorescence maxima of the formed complex and the histamine concentration could be obtained in this alkaline medium.

Keywords: Absorption; Spectrofluorimetry; Analysis; Fluorescence; Histamine; Orthophthalaldehyde; Complex; Calibration; Linear correlation; Medium solvent

1. Introduction

Many coastal countries live in most part to the ocean. The fishing products are regularly present in the meals since they satisfy the population food needs. Nevertheless, the expansion of this activity was accompanied by a great strictness of the consumer on the sanitary quality of these products. Hence, the halieutic resource producing countries must keep an eye open in the quality of their products. Within the context of '*population and environment*' theme, we have kept our interest in the analysis of histamine content in halieutic products [1–3]. Histamine is an amine obtained from the enzymatic decarboxylation of histidine, a natural amino acid [4]. Decarboxylases inducing

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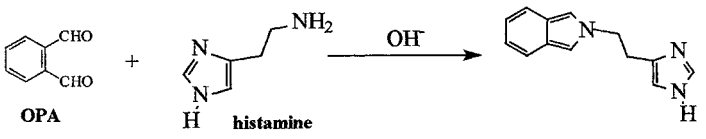
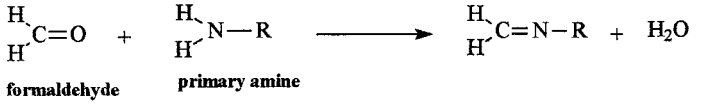
this transformation were present in animals' tissues and particularly in microorganisms. Bacteria such as *Morganella morganii*, *Klebsiella oxycota*, and *Klebsiella pneumonia* have been identified for their histamine-producing activity in tuno fish [5–7]. Histamine is also found in many foodstuffs. At low concentration level, it is essential to the organism for its important role in the nervous system where it acts as chemical mediator [5,8,9]. However, it is toxic at higher concentration, inducing severe symptoms [8,10]. The histamine production in fishes is also known to be increased in a range of temperature comprised between 24 and 30 °C during the post-mortem period [11]. Several families of fish have been itemized as the most important source of intoxication by histamine, namely the scombroids and scomberosocids (tunny, bonit, maquereau), the clupeids (sardine, herring) and the coryphenids (mahi–mahi) [5,8,9]. In consequence, the control of histamine content in food remains an important check of quality, since its tolerance level has been evaluated to be within 0.02–0.05% (m/m) [9,12].

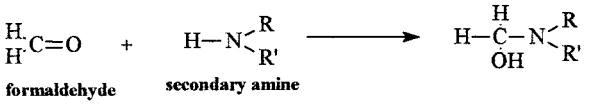
Since its discovery in 1916 by Gugenheim and Lofler, a variety of techniques and detectors have already been used for determining histamine in various matrices. The originally developed methods are based on enzymatic or radio enzymatic assays. The use of fluorescence for histamine residue analysis is due to Shore in 1959 [13]. The aim of this early fluorimetric assay was to investigate the possibility of quantifying low levels of histamine in biological matrices. The promising results of this first attempt has led to the development of a number of other fluorimetric methods utilizing all the same condensation reaction scheme (Eq. (1)), allowing the transformation of the non-fluorescent histamine into a fluorescent derivative. Although the described method is sensitive and presents some interest for analytical applications, its applicability to real biological fluid samples analysis is limited, as it requires extensive real sample preparation and laborious clean-up steps in order to avoid interferences from sample coextractives. Several extraction procedures were developed for histamine determination [12,14,15]. Most of them start with a deproteinization process. Afterwards, biological fluids or tissues samples are homogenized into about 10 volumes of an acid. The acidic solutions usually used are: perchloric, phosphoric, chlorhydric or trichloroacetic acids (TCA). The obtained suspension is allowed to settle, then centrifuged and filtered before being transferred into the extraction solvent [14]. Additional washing with a NaOH solution saturated with NaCl allows removal of the presumed histidine [15]. For clean-up steps, chromatographic columns equipped with appropriate ion exchanging resins with pH switching are generally used. The following resins are the most employed: IRC-50, Amberline CG-50, Dowex-1, Cellex-P, Phosphated cellulose, etc. Buffer pH solutions are prepared from phosphates, acetates, borates, etc. [12]. This real sample preparation procedure, although time consuming, has been used for a long time before it was questioned in recent studies. Indeed, carbonyled compounds and nitrogen oxides can alterate histamine according to the Eqs. (2a), (2b) and (3)), respectively [16,17].



(1)

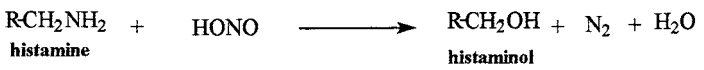


(2a)



(2b)

utilized for the analysis of histamine because of their great sensitivity and selectivity. Direct fluorimetry cannot be applied to histamine since it is naturally non-fluorescent. However, in the presence of some fluorogenic labelling reagents such as orthophthalaldehyde (OPA), a fluorescent complex can be generated. In this derivatization reaction condition, interference with others amines



(3)

In addition, the contamination of real samples by enzymes such as the diamine oxidase and the histamine methyltransferase can lead to an under estimation of histamine concentration [18]. In contrast, an over estimation is expected if these samples are contaminated by the decarboxylases, which provoke the release of histamine in the reaction medium [4]. Physical factors such as temperature and UV radiation have also significant effects on histamine [11,14,18]. Therefore, the histamine sample to be analysed must be kept in drastic conditions i.e., non-bacteria environment, acidic media and low temperature (about $-10\ ^{\circ}C$) are required.

Other spectroscopic methods have been utilized for determining histamine content in various matrices including fishes, drinks, drugs and many biological fluids [18–20]. Among these techniques, absorption and infrared spectroscopy have shown their usefulness.

Chromatographic methods, mainly HPLC and GC, were also used [21]. However, the two methods suffer from interferences of spectral band overlapping effect and poor resolution. Indeed, HPLC/UV–visible detector is more subject to interference in the shorter UV band where histamine does absorb as well as many amines. Histamine also presents very similar retention times, both in GC and HPLC with numerous amines. Therefore, fluorimetric methods have been proposed in the last years and have been widely can occur only if (i) the later compounds react with OPA, and (ii) both the analytical excitation and emission wavelengths band of the derivatized amines are similar to that of histamine–OPA complex. The only major inconvenient of the fluorimetric method is that the solution must be kept in the dark or protected against light with aluminium foil or stored in dark flasks [11,14,18].

The most widely used fluorimetric method of histamine–OPA complex analysis was described by Lerke and Bell [1–3]. In this method, histamine was extracted from real sample with TCA 10% (m/v). The acidic extract is then passed through a chromatographic column packed with a strong cation exchanger resin (e.g. Amberline CG-50) in which the histamine is retained by the mean of an acetate buffer pH 4.62. The elution is achieved by a 0.2 N chlorhydric acid solution. The condensation reaction takes place in alkaline condition, and an acidification process always precedes the fluorescence measurements.

The method of Lerke and Bell, as is the case for many other similar based methods, shows a succession of several chemical steps. Although the acidification phase preceding the fluorescence measurements was said to stabilize the obtained complex [15,22], the present investigation was necessary in order to improve the fiability of Lerke and Bell method.

In this paper, we investigated spectrophotometrically and spectrofluorimetrically the properties

of the histamine–OPA complex in different media including water, water/HCl, water/NaCl, water/NaOH, and water/NaOH/HCl. We evaluated the analytical usefulness of each media for the fluorescence determination of histamine residues in biological samples. Therefore, in this study, we showed that fluorescence measurements of the complex OPA–histamine conducted in alkaline medium were also possible after a kinetic study.

2. Experimental

2.1. Reagents

Histamine dihydrochloride (98%, m/m), OPA (99%, m/m) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and used as received. Sodium hydroxide (97%, m/m), commercials chlorhydric acid (36%, m/m) and TCA, (99%, m/m) were obtained from Labosi (Oulchy-le-château, France). Distilled water was used for preparing aqueous stock solutions of histamine and OPA.

2.2. Apparatus

All fluorescence measurements were performed at room temperature on a Kontron SFM-25 (Zurich, Switzerland) spectrofluorimeter interfaced with an IBM microcomputer, Model Aptiva. Uncorrected fluorescence spectra were acquired and processed using a K-Wind 25 data control and acquisition program. A standard Hellma (Mulheim, Germany) 1-cm pathlength quartz fluorescence cuvette was used.

All absorption measurements were performed on a Jasco spectrophotometer Model 7800 UV/Vis controlled by a Philips microcomputer, Model Monitor 80. Corrected absorption spectra were acquired and printed using a Jasco PTL-3965 printer.

2.3. Procedures

2.3.1. Solutions preparation

Stock solutions of histamine (1×10^{-3} M) and OPA (1×10^{-3} M) were freshly prepared by dissolving the compounds in water. Serial dilutions were performed to obtain working standard solutions. All solutions were protected against light with aluminium foil and stored in a refrigerator. Stock solutions of HCl (0.5 M) and NaOH (0.1 M) were prepared with distilled water and used for serial dilutions. The histamine–OPA complex was prepared by transferring 25–250 μl aliquots of the OPA working standard solution into the quartz cuvette and adjusting to 2.5 ml volume, with the required histamine solution volume, NaOH and/or HCl solutions when needed and distilled water. The solutions were then shaken before analytical measurements.

2.3.2. Analytical measurements

An aliquot of the OPA working standard solution was placed in the quartz cuvette in which known and increasing amounts of histamine were added.

For the fluorimetric method, the fluorescence intensity was monitored at the fixed analytical excitation (λ_{ex}) and emission (λ_{em}) wavelengths of the complex. For the absorptiometric method, the absorbance signal was measured at the fixed analytical absorption maximum (λ_{ab}) wavelength of the complex. For both methods, linear calibration curves were obtained at these λ_{ex}, λ_{em} and λ_{ab} values by measuring the spectra height signal.

All fluorescence measurements were corrected for the solvent signal with the appropriate blank. Fluorescence measurements were carried out in triplicate while the absorption intensities were measured on at least two histamine concentrations. In all cases, the results were expressed as mean value. MICROCAL ORIGIN, version 5.00, application software was used for the statistical treatment of the data.

3. Results and discussion

In this study, two methods were used to investigate the physicochemical properties of the OPA–histamine complex: the absorptiometric and the fluorimetric methods. Our preliminaries experiments showed that the first method is not suitable for quantitative studies, principally for two reasons: because of the high sensitivity re-

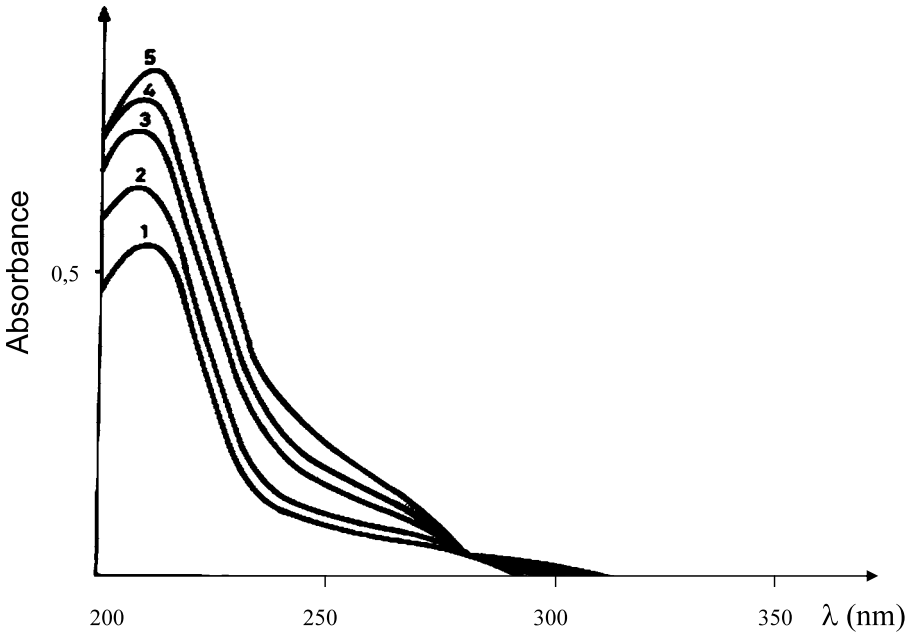


Fig. 1. Absorption spectra of the complex histamine–OPA in water. Concentrations used: OPA 1×10^{-4} M and histamine (1) 0; (2) 3.33×10^{-5}; (3) 6.61×10^{-5}; (4) 11.44×10^{-5}; (5) 14.59×10^{-5} M.

quired for histamine residue measurements, and, due to band overlapping effect, since the two compounds (histamine and OPA) and their reaction product (the histamine–OPA complex) do absorb at the same UV–visible region band. Hence, the absorptiometric method is more useful for characterizing the complex formation.

3.1. Absorptiometric study

We studied the effect of two media (Water and NaOH) on the absorption spectral properties of the individual compound and also, for the formed complex.

The results obtained in water exhibited for OPA the presence of two bands: the most intense appeared at 209 nm with a shoulder near 223 nm, while the second small peak was located at 260 nm (Fig. 1). When the absorption spectrum of histamine was recorded in the same conditions, a single band appeared with the maximum located at 210 nm. The calculated molar extinction coefficient values for all the compounds at the different peaks were larger than 1000 M^{-1} cm^{-1}. Therefore, these observed absorption bands might be attributed to $\pi \rightarrow \pi^*$ transitions. The spectra of the complex histamine–OPA at different histamine concentrations were obtained by adding increasing amounts of histamine to a known OPA solution.

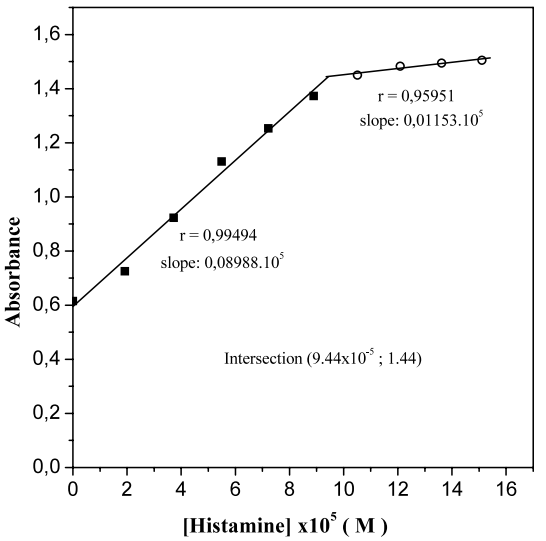


Fig. 2. Calibration graph of histamine. Medium: OPA (10^{-4} M) in water/NaOH (0.41 M) $\lambda_{max} = 209$ nm.

The effect of increasing histamine concentration was to enhance progressively the absorption peak at 210 nm, with the appearance of an isobestic point located at 280 nm. There are no shifts of the maximum wavelengths, but the existence of the isobestic point proved the formation of a complex between histamine and OPA (Fig. 1).

When aqueous NaOH solution is used instead of water, the addition of increasing histamine amounts to the OPA aqueous solutions resulted in a small blue-shift (208 nm) of the absorption peak and a strong enhancement of the absorption intensity, with the appearance of a shoulder at 240 nm.

To evaluate the analytical interest of this method, we have established the calibration graphs under alkaline conditions for the complex formed (Fig. 2). Plots of the absorbance of the complex versus the histamine concentrations give two different calibration curves, which were both linear with correlation coefficient values of 0.995 and 0.959, respectively. The first line characterizes the formation of the complex histamine–OPA, which was achieved at the breaking point. The intersection point indicates clearly the end of the complexation reaction, and consequently, suggests that the stoichiometry of the complex is 1:1. The second line represents the addition of excess amount of histamine on the complex formed.

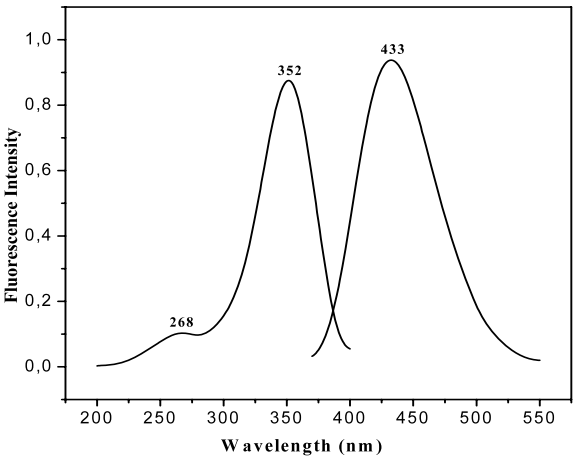


Fig. 3. Excitation and emission spectra of the histamine–OPA complex in the studied media (water, NaCl, NaOH, HCl).

Unfortunately, histamine, OPA and their resulting complex absorb in the same UV–visible region. Hence, it would be difficult to conduct a reliable quantitative analytical study using the absorption method. Fluorimetry is therefore proposed.

3.2. Fluorimetric study

Histamine and OPA were found to be naturally non-fluorescent in all solvents or media under study, whereas an intense fluorescence band appeared upon mixing the two compounds. Consequently, the fluorescence excitation and emission spectra were obtained after adding into the OPA solution placed in the quartz cuvette, a known amount of histamine. Preliminaries results showed that the excitation and emission spectra of the formed histamine–OPA complex have similar shape in all the media under study, i.e., water, water/NaOH, water/HCl, water/NaCl and water/NaCl/NaOH. The excitation spectra present two peaks, located at 270 nm for the weakest, and 350 nm for the strongest, while the emission spectra gave a single peak located at 430 nm. No wavelengths shift of these band occurred when going from one medium to another (Fig. 3).

3.2.1. Effect of aqueous medium

Histamine and OPA react slowly in water to form a fluorescing complex (Fig. 4). For instance, the complex formation is achieved within approximately 10-min reaction time.

Calibration graphs representing the plots of fluorescence intensity of the complex versus increase amounts of histamine in the presence of 1×10^{-4} M solution of OPA give two straight lines (Fig. 5). The first line is related to the progressive formation of the complex, which was achieved at the crossing point of the two lines. The second, indicating too the signal growth, may be attributed to another process characterizing the formation of a second type of fluorescent complex.

3.2.2. Effect of alkaline medium (NaOH)

To evaluate the fluorophore formation kinetics in alkaline medium (NaOH 10^{-2} M), we investigated the evolution of fluorescence intensity with

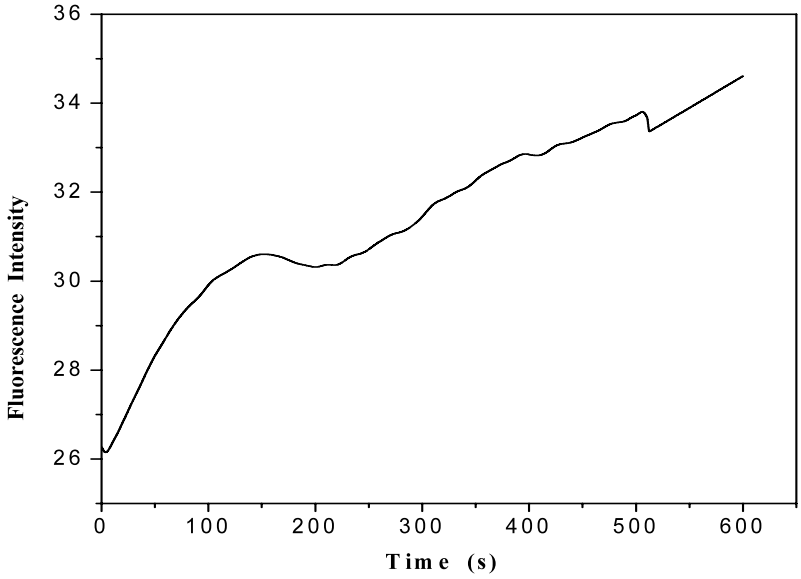


Fig. 4. Kinetic of the histamine–OPA complex formation in water.

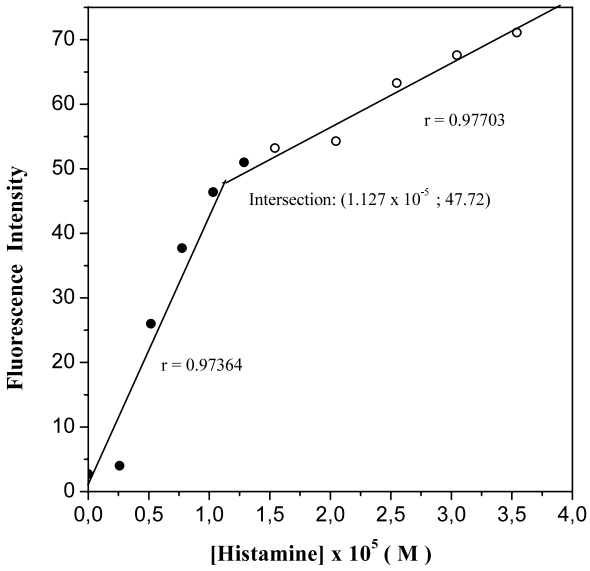


Fig. 5. Calibration graph of histamine. Medium: OPA (1×10^{-4} M) in water: $\lambda_{ex} = 350$ nm, $\lambda_{em} = 430$ nm.

reaction time. For all the examined histamine concentration, a two-step mechanism was found, i.e., the fluorescence intensity initially increased with increasing reaction time, and then reached a maximum value and, finally decreased continuously. The stage of signal increase corresponded to the complex formation, the maximum obtained indicated the end of the reaction, and the decrease of signal was probably characterized by the complex unstability.

To evaluate the quantitative aspect of our study, we established a calibration graph of the fluorescence intensity of the complex versus histamine concentration for fixed amounts of OPA, under optimum reaction time condition. The curve was similar to those previously observed in other media, i.e., two straight lines were obtained. The first line corresponded to the histamine quantitation, of which the linear dynamic range (LDR) within 0–160 fluorescence units was about 50–100 times larger relative to that obtained in water. This feature showed that determining histamine in the alkaline medium is more sensitive than the aqueous one. However, the extraction process of histamine from real samples is always accomplished with chlorhydric acid solution [3,11]. It would therefore be interesting to study the physicochemical properties of the later medium on the fluorescence of the formed complex.

3.2.3. *Effect of acidic medium (HCl)*

Chemical reaction between histamine and OPA do not occur in acid medium. Therefore, the

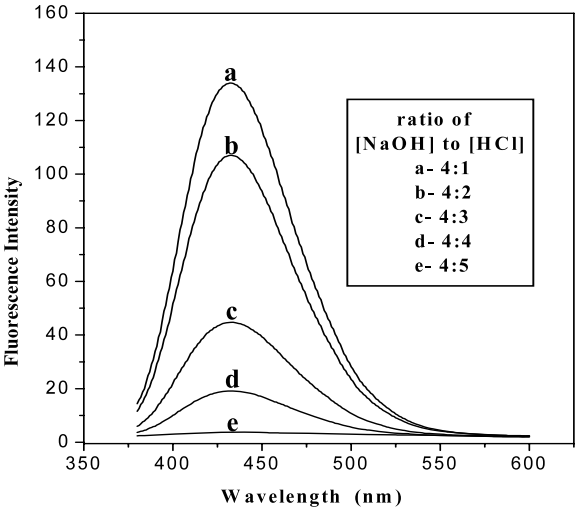


Fig. 6. Effect of progressive addition of HCl (2×10^{-4} M) concentration on the fluorescence intensity of the complex histamine–OPA (10^{-4} M/10^{-4} M) initially formed in aqueous alkaline NaOH (8×10^{-4} M) medium, $\lambda_{exc} = 350$ nm; $\lambda_{em} = 430$ nm.

complexation reaction was, in the first time, conducted in alkaline medium and afterwards, an excess of HCl solution was added in the reaction medium, as previously described in the literature [15,21]. Hence, fluorescence measurements have to be done in saline and acidic medium. The final results showed that fluorescence signal is quenched by HCl solution (Fig. 6).

3.2.4. Effect of saline medium (NaCl)

Since there is no significant fluorescence signal in acidic media, it would be useful to examine the influence of fluorescence intensity in saline medium. The excitation and emission spectra of the complex histamine–OPA in saline medium have similar shape with those recorded in water and alkaline media. No shifts of the maximum analytical wavelengths occurred on changing the media. In contrast, the optimum reaction time, t_{kin}^{opt} (defined as the reaction time corresponding to the maximum fluorescence intensity) and fluorescence intensity values exhibited important variations. Relative to NaOH medium, a significant decrease of fluorescence signal was observed in saline medium with concomitant increase in the reaction time of the complex formation, which was achieved in about 2 h. In consequence, saline medium appears to be the less convenient medium for analytical purposes.

Nevertheless, we have checked the effect of plotting fluorescence signal of the complex recorded after 10 min reaction, versus histamine concentration. A straight line is then obtained with correlation coefficient larger than 0.99. The LDR is too narrow, since it was scaled between 5 and 35 fluorescence units. Because of the low rate constant of the complex formation in this media, quantitative study of histamine using this curve were not feasible.

3.2.5. Effect of saline alkaline medium (NaCl + NaOH)

The study of fluorescence properties of the histamine–OPA complex in this medium was dictated by Lerke and Bell extraction and analysis methods of histamine [1–3,11,23]. A progressive addition of NaOH on the chlorhydric acid extract allows entire neutralisation of the acid, and in function of the amount of the excess NaOH added, we could determine the LDR of the calibration curves. For instance, the following mixture in which the concentration of NaOH is varied was studied: (NaCl 0.2 M + NaOH) or (NaCl 0.1 M + NaOH).

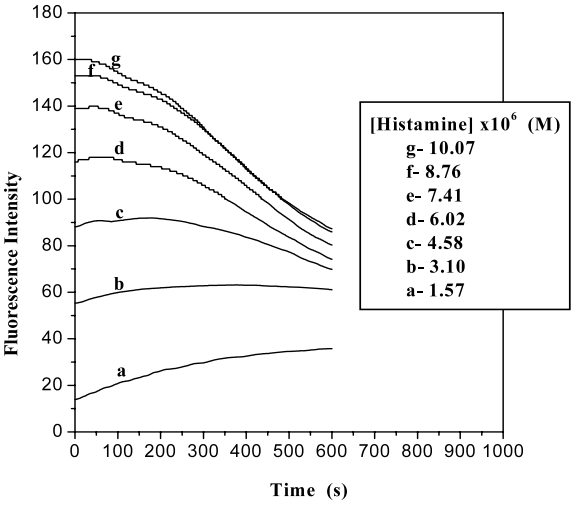


Fig. 7. Kinetic of the complex histamine–OPA formation in saline and alkaline medium (NaCl + NaOH) as a function of histamine concentration.

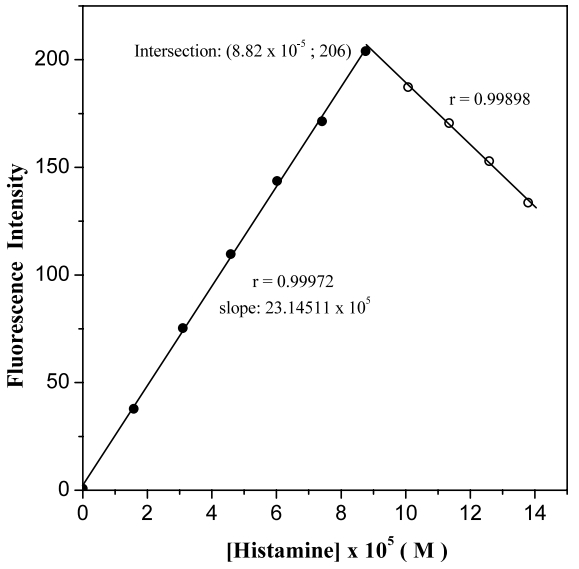


Fig. 8. Calibration graph of histamine. Medium: OPA (10^{-4} M) in water/NaCl (0.1 M)/NaOH (6×10^{-4} M); $\lambda_{ex} = 350$ nm, $\lambda_{em} = 430$ nm.

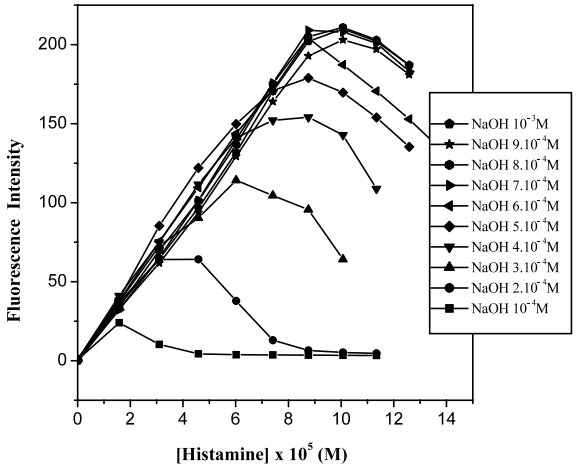


Fig. 9. Effect of NaOH concentration on the calibration graphs of histamine in the presence of OPA (10^{-4} M) in saline and alkaline medium, NaCl (0.1 M)+NaOH (variable).

Before any study, we first investigate the kinetic of complex formation in the mixture (NaCl+ NaOH) at different histamine concentrations (Fig. 7). The obtained results show that the optimum reaction time t_{kin}^{opt} of the complex formation is large for small amounts of histamine. But as the concentration of histamine increases, the reaction time diminishes, while the fluorescence intensity growth proportionally to the histamine concentration.

Calibration graphs established under optimal reaction times t_{kin}^{opt}, and representing the evolution of fluorescence signal of the complex versus histamine concentration (Fig. 8) were all linear, with correlation coefficient larger than 0.99. Another feature is that the LDR of the lines varies with both NaOH and OPA concentrations. At fixed OPA concentration, the LDR increases with increasing the amounts of NaOH and levelled off at NaOH concentration of about 7×10^{-4} M, reaching a plateau value (Fig. 9). In order to ensure complex formation, the optimal analytical medium selected for further studies was: OPA concentrations within $(1 \times 10^{-6}$–$1 \times 10^{-4})$ M, NaCl 0.1 M and NaOH 6×10^{-4} M. These final optimal conditions provided histamine residues concentrations determination in the range of 10 ng/ml and 100 μg/ml.

4. Conclusion

We have demonstrated in this work that fluorogenic labelling of histamine in several media yields strongly fluorescent products. For instance while neutral media i.e., water or NaCl give the less favourable reaction condition (large values of t_{kin}^{opt} and weak fluorescence signal), very alkaline solution (NaOH or NaCl+NaOH) provide better results. Representative calibration curves demonstrate the linear response of the method over the concentration range under study. In most instances, the accuracy was satisfactory. The use of this technique for real biological samples analysis is currently making progress and future developments of this method are expected.

Acknowledgements

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