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# Determination of histamine in the whole blood of colon cancer patients

Maurizio Previati[a,*], Andrea Raspadori[b], Lucia Bertolaso[a], Alina Parmeggiani[a], Debora Bindini[a], Cristina Vitali[a], Irene Lanzoni[a], Elisa Corbacella[a], Massimo Saviano[b], Francesco Fagioli[c], Gabriella Blo[c], Silvano Capitani[a]

[a]*Dipartimento di Morfologia ed Embriologia, Università degli Studi di Ferrara, Via L. Borsari 46, 44100 Ferrara, Italy*
[b]*Dipartimento di Chirurgia, Università degli Studi di Modena, Via del Pozzo 71, 41100 Modena, Italy*
[c]*Dipartimento di Chimica, Università degli Studi di Ferrara, Via L. Borsari 46, 44100 Ferrara, Italy*

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**Abstract**

The aim of the present work is to investigate whether histamine assay could be useful in detecting the presence of primary cancer. The high-performance liquid chromatographic (HPLC)-based *o*-phthalaldialdehyde (OPA) histamine derivatization assay was investigated with respect to several variables, dramatization reagent concentration, organic solvent requirement, derivatization time and counter-ion effect on chromatographic separation.The OPA histamine assay, in the absence of added –SH groups, was found to detect histamine in whole blood samples with relative standard deviations $<14\%$ and recoveries not less than 90%. The assay showed high selectivity towards other aminic-containing compounds and a detection limit of 18 n*M* of histamine was evaluated. Calibration curves in the range 50–500 n*M* were obtained by using histamine standards in 0.1 *M* HCl with a regression coefficient value ($r^2$) of 0.9969. In order to assess the usefulness of this assay in primary tumor monitoring, two groups of individuals, 29 controls and 29 colon cancer patients were selected, and serum levels of histamine, carcinogen embrionary antigen (CEA), carcinogen antigen 19.9 (CA19.9), and tumor staging, were determined. A significant histamine reduction ($P=0.028$) between controls ($180.12\pm70.4$ n*M*) and patients ($134.5\pm90.3$ n*M*) was found, and a cut-off value of 157.5 n*M* was extrapolated as intercept point of sensitivity and specificity curves. Fifty percent of patients showed a histamine value below the cut-off, while 45.8 and 8.3% of patients were positive for CEA and CA19.9, respectively. No correlation was found between Tumor Node Metastasis staging and histamine amount, indicating that this marker is not related to the tumor mass. Our data suggest that histamine level, together with other classical tumor markers, could be a potentially interesting tumor marker in colon cancer monitoring.


*Keywords:* Colon cancer; Histamine determination; Tumor markers; Histamine; OPA

## 1. Introduction

Histamine is a decarboxylation product of histidine, which is released from activated granulocytes and mast cells. Histamine is considered one of the

*Corresponding author. Dipartimento di Morfologia ed Embriologia, Sezione di Anatomia Umana Normale, Università degli Studi di Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy. Tel.: +39-0532-291-193; fax: +39-0532-207-351.
*E-mail address:* prm@dns.unife.it (M. Previati).

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most important mediators of inflammation and also plays an important role as mediator of numerous physiological processes, such as neurotransmission, allergic reactions, microcirculation regulation.

In addition to these widely recognized functions, histamine is also released at tumor sites [1], where it has been demonstrated to cooperate in the regulation of some important parameters of host response to cancer. In fact, during the development of neoplasm, several components of immune response, as such as basophilic granulocytes cytotoxic macrophages and T and B lymphocytes, can migrate from the intravascular compartment toward the tumor site. At the tumor site, histamine liberation occurs and cooperates in regulating at least three important factors: (1) blood delivery and tumor vascularization [2–6], (2) feedback regulation of immune response [7–14] and (3) regulation of cancer cell growth [15–21].

A decreasing of histamine blood levels was observed as a consequence of the granulocytes' migration from the intravascular compartment to the tumor site. This event was regarded as a potential marker of tumor presence, and several papers evaluated the histamine as a usefulness predictor of tumor presence and recurrence in different neoplasms. These studies assayed histamine in whole blood by fluorometry after water–butanol partition and re-extraction into HCl. This step was followed by *o*-phthalaldialdehyde (OPA) derivatization in absence of added thiolic moieties, leading to a highly specific histamine assay [16].

However, later studies pointed out the existence of contaminants in the solvents used for extraction as well as of interferences from the blood matrix, which is constituted by serum and cell bodies [22,23]. In addition, different methods also showed different and non-quantitative recovery of the autacoid after liquid partitioning.

In the present work, since selectivity and recovery are very important parameters in the analytical determination of a marker that is expected to decrease instead of increase during neoplasm, we describe the set-up of the high-performance liquid chromatography (HPLC)-based OPA histamine assay for histamine determination in blood samples, testing derivatization, separation performances, sensitivity, accuracy, repeatability and detection limits.

Then, in order to investigate the usefulness of whole blood histamine content as a tumor marker, we analyzed whole blood samples of both control and solid tumor cancer patients, and evaluated positivity for histamine decrease versus other classical colon cancer markers as carcinogen embrionary antigen (CEA) and carcinogen antigen 19.9 (CA19.9).

## 2. Experimental

### 2.1. Chemicals

Inorganic reagents (RPE) and organic solvents (HPLC grade) were purchased by J.T. Baker (Milan, Italy). Histamine, spermine, spermidine, 1,8-diaminooctane, DL-3,4-dihydroxyphenylalanine (DOPA), all the amino acids and OPA (99% purity) were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Analytical procedures

#### 2.2.1. Subject selection

All subjects were asked for their informed consent before the beginning of the study. Patients treated by $H_1$ and $H_2$ antagonists, and antidepressant steroids, as well as patients referred to surgery after preoperative therapy or treated by radiochemiotherapy within 6 months, were excluded from this study.

Clinical status allowed the division of patients into two groups:

Group 1: Absence of any neoplasm; 29 volunteers or patients hospitalized for non-neoplastic conditions. No CEA or CA19.9 determination was performed in this group.

Group 2: Presence of unresected primary cancer without known metastasis; 29 individuals affected by colon cancer were subjected to clinical inspection, instrumental analysis and tumor marker detection.

#### 2.2.2. Blood collection and preparation

Blood samples were collected before any surgical or anesthesiological procedure, since administration of drugs and anesthetics can lead to histamine liberation [24]. Blood samples were taken from 07.00 to 09.00 h to minimize circadian histaminemia variations. The first 10 ml of blood was not used because needle penetration can liberate histamine.

The following 5 ml was collected in polystyrene tubes with EDTA as anticoagulant and conserved on ice for no more than 1 h before the next treatment. The use of heparin as anticoagulant was avoided since it has been shown that it may be occasionally contaminated by histamine [25]. Subsequently, 5 ml of whole blood was mixed with 2 ml of 2 *M* $HClO_4$, kept on ice for 10 min and centrifuged at 3000 *g* at 4 °C for 10 min. The separated supernatant was again centrifuged, separated and extracted by liquid–liquid partition according to already reported procedures [26]. The final 0.1 *M* HCl extract was derivatized or alternatively, awaiting derivatization, stored at −20 °C.

*2.2.3. Derivatization procedure*

Derivatization was performed according to [26], with slight modifications. Briefly, 200 μl of the acid blood extract or an appropriate volume of standard histamine, taken up to 200 μl with 0.1 *M* HCl, was brought to 25 °C in a water bath. A 20-μl volume of 1 *M* NaOH was added, and incubation started with the addition 25 μl of a freshly made 10 mg/ml methanolic solution of OPA, corresponding to a final concentration of 5 m*M* OPA. After 4 min of incubation, 30 μl of 3 *M* HCl was added and incubation continued for at least 10 min. All the derivatization volume was loaded into a 100 μl loop and injected into the HPLC apparatus.

*2.2.4. Chromatographic analysis*

The HPLC apparatus was a Varian 2510 isocratic pump (Varian, Turin, Italy), a Rheodyne (Rheodyne, Rohnert Park, CA, USA) sampling injection valve equipped with a 100 μl loop, a 250 mm×4.6 mm, 5 μm $C_{18}$ Ultrasphere column (Beckman, Milan, Italy) and a Varian 9070 fluorometric detector, set at 360 and 450 nm excitation and emission wavelengths, respectively. The detector output signal was integrated with the Flo-One software (Packard, Milan, Italy). The mobile phase consisted of 50 m*M* acetate buffer, pH 4.0–methanol–acetonitrile (44:20:36) containing 8 m*M* sodium decanesulfonate (DSF), and was delivered at 1 ml/min. Standard solutions of histamine were prepared in 0.1 *M* HCl and in whole blood matrix for external calibration and standard addition procedures respectively.

*2.2.5. Stability of standards and samples*

Extracted and standard histamine showed no variability after daily cycles of freezing and thawing or storage at 4 °C over a period of 14 days, and unextracted histamine was stable for the time employed in this procedure when kept at 4 °C, both in blood and in acid environments. This was specifically tested keeping the collected whole blood and $HClO_4$ precipitated samples on ice up to 5 h and then evaluating the residual histamine amount (data not shown).

*2.2.6. Statistical analysis*

Blood histamine levels, expressed as n*M*, were determined for the considered groups, and the values were compared by two-tailed Student's *t*-test. Sensitivity and specificity related to arbitrary cut-off values were established in the attempt to distinguish between the presence and the absence of primary cancer (groups 1 and 2). Correlation between histamine values and tumor stadiation was evaluated utilizing the Spearmann's coefficient of rank correlation [27].

*2.2.7. Histological evaluation*

The pathological classification of tumors pertaining to group 2 was made accordingly to the Tumor Node Metastasis (TNM) classification [28].

## 3. Results and discussion

The aim of our research was to investigate whether blood histamine level can be effectively used to monitor the presence and the progression of tumor disease. In the attempt to work out the most precise and reliable HPLC-based OPA histamine assay, each analytical step required for whole blood analysis, was carefully optimized.

Several variants related to the derivatization procedure were investigated in order to optimize the assay. The literature reported that the compound obtained by addition of one OPA molecule to one histamine molecule is unstable and minimally fluorescent. Furthermore, this compound could be subjected to a base-activated degradation, or converted, after the addition of a second OPA molecule

followed by acidification, into a very stable and highly fluorescent dihydrophenanthroline [29,30].

Existence of degradative routes for dihydrophenanthroline precursors suggested the need to optimize the reaction times in the presence of high pH. Consequently, both histamine standard and extracts from blood samples were incubated in presence of NaOH for different times before inducing the dihydrophenanthroline formation by addition of HCl. Two different temperatures were investigated. At 25 °C we found that the higher recovery of fluorescent adduct was obtained when acidification occurred after 4 min of incubation in presence of high pH, while longer incubation times resulted in a consistent reduction of fluorescent adduct recovery (Fig. 1). At 37 °C, both formation and degradative rates increased, and a higher recovery was reached in a shorter time. In order to have a reasonable time span to stop the reaction in presence of numerous samples, a standard condition of 25 °C and 4 min of incubation time was chosen.

The time needed to obtain the maximum increase of fluorescence after acidification and the stability of fluorescent adduct was explored. We found that 1 min acidification was required to develop the fluorescence maximum and that, at room temperature, the adduct was stable for 15 h, while it had a half life of 4 days at 4 °C (data not shown); consequently, a minimum time of 20 min after acidification before HPLC injection was arbitrary chosen.

In order to rule out the possibility of incomplete OPA solubility, we investigated the influence of methanol on the histamine assay in the low concentration range. Fig. 2 shows the formation of fluorescent histamine adduct in presence of constant OPA and different methanol concentrations. The reaction occurred at all the concentrations tested, requiring only 6.5% to reach a maximum. Over 6.5% methanol, the histamine recovery slightly decreased, so the optimal organic percentage was then set at 6.5% and used in all the following determinations.

Controversial data for optimal histamine derivatization have been reported elsewhere regarding the OPA concentration, ranging from 74 μ*M* [26] to 18 m*M* [30]. In addition, there are disagreements on the appropriate histamine OPA ratio and on the existence of interfering effects of high OPA concentration [22,30]. Moreover, all these studies were carried out

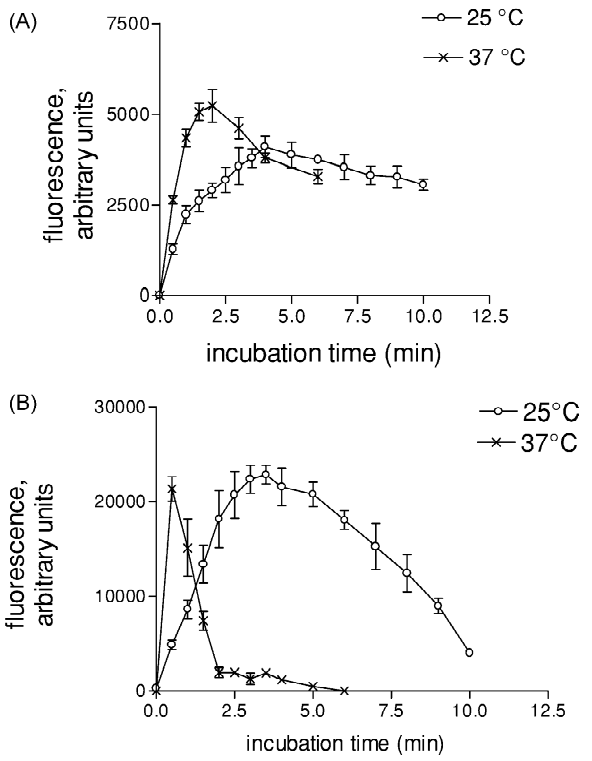


Fig. 1. Time and temperature dependence of histamine derivative production: (A) 71.4 n*M* histamine standard; (B) a whole blood sample. Data, in triplicate, are obtained from two separate experiments.

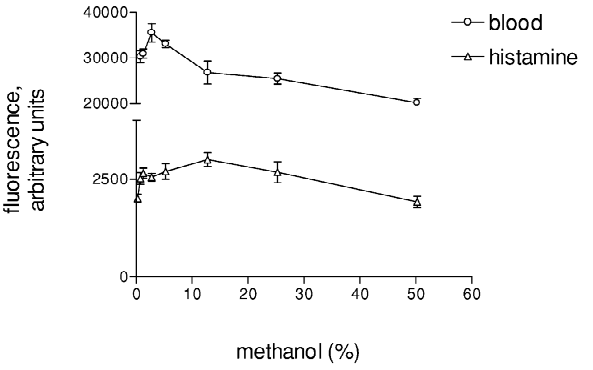


Fig. 2. The effect of the methanol concentration on the fluorescence of histamine adduct for a 45 n*M* histamine standard and a whole blood sample. Data, in triplicate, are obtained from two separate experiments.

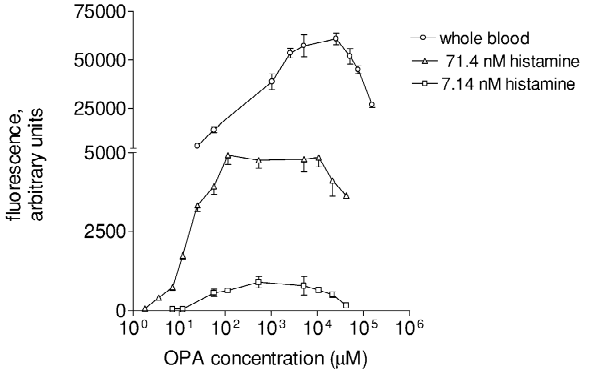


Fig. 3. OPA concentration dependence of histamine derivative fluorescence for two histamine standards: 71.4 n*M* (▽) and 7.14 n*M* (□), and for a whole blood sample (○). Data, in triplicate, are obtained from two separate experiments.

in presence of different histamine amounts, ranging from n*M* to m*M* concentrations.

In order to apply the assay at lowest blood histamine concentrations, the OPA concentration effects were investigated on two histamine standards (7.14 and 71.4 n*M*) and a blood sample. Fig. 3 shows, for the lower concentration standard, fluorescence increasing until 125 μ*M* OPA, and until 535 μ*M* OPA for the higher one, then slightly decreasing over 5.3 m*M* OPA for both standards. On the contrary, the blood sample required up to 27.6 m*M* OPA to reach the response maximum, while higher OPA concentrations reduced it. In order to get an optimal fluorescence signal, both for samples and standards, and to reduce the cost related to high OPA use, a concentration of 5 m*M* OPA was then chosen for the whole blood histamine assay.

### 3.1. Selectivity

The selectivity of the OPA histamine assay was investigated toward several interferences: (1) amino acids, that are common constituents of plasma; (2) polyamines, that have been previously indicated as potential contaminants in this kind of assay [23]; (3) compounds, as such as DOPA, having structural similarities to histamine and that could efficiently react with OPA.

The selectivity, *S*, expressed as molar response ratio, has been calculated according to the following equation:

$$S = A_m(\text{histamine})/A_m\ (\text{interference}) \quad (1)$$

where $A_m$ is the molar peak area, calculated for each component on the chromatogram, as:

$$A_m = A/c \quad (2)$$

where *A* is the peak area of the component and *c* its concentration.

Selectivity data, reported in Table 1 for considered compounds, clearly indicate that, under optimal assay conditions, only a few compounds can be derivatized with an efficiency similar to histamine, while most of them show a molar response ratio at least two orders of magnitude greater than histamine.

In spite of this selectivity, when the derivatization approach was directly applied to the whole blood sample after $HClO_4$ precipitation, a high fluores-

Table 1
HPLC-based OPA histamine assay: selectivity (*S*) and capacity factor (*k′*) for several compounds investigated as assay interferences

| Compound | *S* | *k′*≤ | Compound | *S* | *k′*≤ | Compound | *S* | *k′*≤ |
|---|---|---|---|---|---|---|---|---|
| Alanine | 107.1 | 1.57 | Leucine | 219.1 | 1.57 | Tyrosine | 4444.9 | 1.57 |
| Arginine | 202.7 | 2.07 | Lysine | 34.7 | 1.57 | Valine | 3070.4 | 1 |
| Asparagine | 10 426.4 | 1.57 | Methionine | 266.8 | 1.57 | $CH_3$histamine | 230 727.27 | |
| Aspartic acid | 441.8 | 1 | Norleucine | 219.4 | 1.57 | Glutamine | 4478.82 | 1 |
| Citrulline | 124.3 | 1 | Norvaline | 159.4 | ≥3 | Spermine | 15 228 | 1 |
| Cysteine | 95 175 | 1 | Ornithine | 1919.3 | 1.57 | Spermidine | 846 000 | 1 |
| DOPA | 20.7 | 1 | Phenyalanine | 1471.5 | 1.57 | Putrescine | 27 192 | 1 |
| Glutamic acid | 159.7 | 1 | Proline | 10 186.6 | 1 | Diamine octane | 507 600 | 1 |
| Glycine | 481.2 | 1 | Serine | 1663.1 | 1 | | | |
| Histidine | 207.6 | 1 | Threonine | 5614.8 | 1 | Histamine | 1 | 2.07 |
| Isoleucine | 2179.7 | 1.57 | Tryptophan | 1761.8 | 2.07 | | | |

cence background was detected, to which histamine contributes only a minimal fraction.

In order to reduce this problem, first a liquid–liquid histamine extraction was applied before derivatization, and then the use of the DSF counter-ion in the carrier for chromatographic separation, was able to improve the histamine resolution from interferences. The retention data of 24 amino acids and some polyamines, reported as capacity factor $k'$ in Table 1, indicates that DSF-containing mobile phase greatly increases the retention of the dihydrophenanthroline histamine derivative towards interferences. Only arginine and tryptophan seem, in these conditions, to co-elute with the histamine peak, but fortunately they present high $S$ values; otherwise, lysine, histidine and DOPA, which show a molar response ratio closer to that of histamine, can be effectively resolved from it.

After setting-up, the whole analytical procedure was evaluated by performing repeatability, recovery and detection limit analysis.

### 3.2. Repeatability, accuracy and detection limit

As regard the evaluation of the assay repeatability, histamine determinations on blood samples, repeated five times, generally provided precision values $<14\%$, in terms of relative standard deviation (RSD), calculated according to:

$$\mathrm{RSD} = \frac{S}{\overline{m}} \tag{3}$$

where $S$ is the standard deviation and $\overline{m}$ the mean value calculated for repeated measures.

In order to evaluate the accuracy of the proposed assay, recovery analysis was performed adding histamine standard directly in blood samples in triplicate and at different concentrations.

Recoveries at different concentration values, calculated according to:

$$\mathrm{Recovery}\ (\%) = C_{\mathrm{exp}}/C_{\mathrm{add}} \times 10^2 \tag{4}$$

where $C_{\mathrm{exp}}$ and $C_{\mathrm{add}}$ are the concentrations of histamine standard, respectively, determined and added to blood sample, and are reported in Table 2. Data show that it is possible to obtain an almost complete recovery of histamine. This finding is substantially different from Ref. [31] which reported recoveries ranging from $47\pm8$ to $57\pm6\%$ after liquid partition in the presence of bis(2-ethyl-hexyl phosphoric acid). It noteworthy that our data demonstrate a requirement of 27.6 m$M$ OPA to reach the maximum fluorescence value in blood sample that have been subjected to the whole procedure. The higher OPA concentration could be required to overcome the interference due to the matrix effect, and the use of a more efficient derivatization protocol could reasonably account for the increase of recovery of histamine.

Table 2
HPLC-based OPA histamine assay recovery test

| Histamine (n$M$) | Recovery (%) |
|---|---|
| 100 | 100 |
| 200 | 95.6 |
| 400 | 89.4 |
| 500 | 101 |

Fig. 4 shows the calibration curves obtained both through external calibration method and standard addition method. The ratio between calibration curves slopes, closed to the unit, proved that, under the applied assay conditions, the matrix effect on the final signal is low. Then analyses are allowed to be performed with the external calibration procedure.

Linearity was satisfactory with a regression coefficient value ($r^2$) of 0.9969 in the considered concentration range. A concentration of 18 n$M$ was calculated as detection limit corresponding to a signal detection limit (DL) calculated according to:

$$\mathrm{DL} = 2N_{\mathrm{p-p}} \tag{5}$$

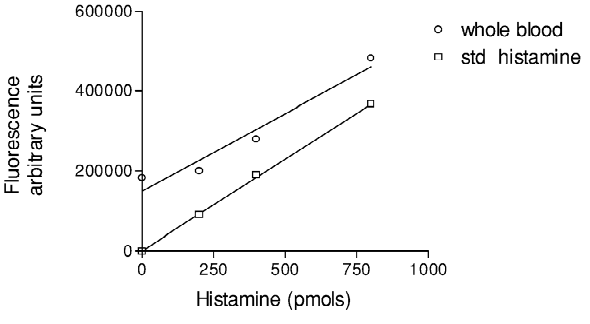


Fig. 4. Calibration curves for histamine determination in whole blood (○) and in 0.1 $M$ HCl (□). Linear regression equations are $y = 461.15x + 174.6$ (0.1 $M$ HCl) and $y = 390.8x + 149174.4$.

where $N_{p-p}$ is the peak-to-peak noise measured on the chromatogram baseline. A typical separation profile for histamine determination is shown in Fig. 5.

### 3.3. Analysis of cases

Histamine level was determined in the blood of 29 controls and 29 colorectal cancer patients. No significant differences were found when data where matched for sex and age, making it possible to pool the results. All the values fell in the range of the detectability of the assay, with the exception of two cases of group 2 that were below the detection limits. Histamine concentration in controls (group 1) was 180.12±70.4 n*M*. When histamine was determined in cancer patients with or without metastases (group 2), we found a significant reduction of mean histamine concentration to 134.5±90.3 n*M* ($P=0.028$).

In the attempt to distinguish between the presence and absence of primary cancer (groups 1 and 2) we extrapolated the number of true and false positive and negative in function of different arbitrary cut off values in groups 1 and 2 (Fig. 6). A cut-off of 157.5 n*M* of histamine, corresponding to a sensitivity of 65.9 and a specificity of 60.71 was extrapolated as point of intercept between sensitivity and specificity curves.

Among group 2, the 45.8% of cancer patients resulted positive for CEA while only 8.3% were positive for CA 19.9. On the contrary, the percentage of colon cancer patients, which showed a value lower than the indicated cut-off of 157.5 n*M* (see above), was 50%.

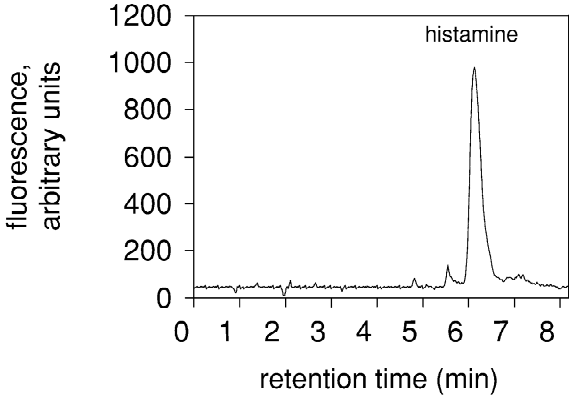


Fig. 5. HPLC separation for OPA histamine assay for a cancer patient blood sample (histamine concentration: 174.4 n*M*; other peak was not identified). For experimental conditions see Experimental.

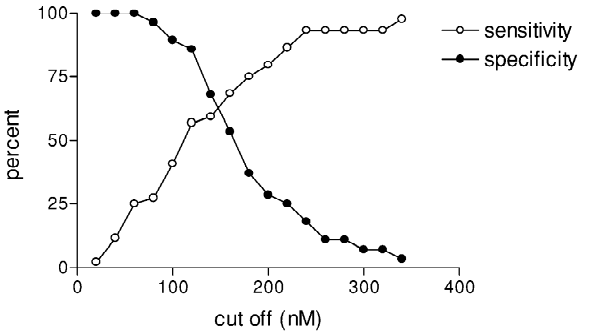


Fig. 6. Sensitivity and specificity of histamine OPA assay.

Correlation between histamine values and tumor stadiation was investigated. In group 2, histamine levels were grouped into ranks, and correlation to the tumor stadiation was evaluated utilizing the Spearmann's coefficient of rank correlation. No correlation was found (data not shown).

The histamine level, we found in control individuals, was lower than previously reported by Nielsen et al. [32] and Alvarez et al. [33] (275.3±151.5 n*M*), and was one third of that reported by Burtin et al. [34] (65.1±23.2 ng/ml, corresponding to 580±206.7 n*M*). This could suggest that analytical methods avoiding chromatographic separation steps, such as those used in Ref. [34], could be affected by interference of contaminants. This could lead to higher estimates of human blood histamine concentration in comparison to methods, like that described in the present paper or in Ref. [33], which employ chromatographic separation. In addition, Burtin et al. [34] did not discard the first fraction of the blood sample; it is reasonable to argue that they could have assayed the histamine liberated by mastocytes present in derma and periendothelium as well. Since in the neoplastic patients the anaphylactic reactivity is reduced with respect to controls [5], the different liberation of histamine in the zone of needle penetration could contribute both to the higher absolute values and the higher difference between controls and cases found in Ref. [34].

Analysis of controls and cases indicated that in colon cancer patients, the disease was associated with a significant reduction of histamine in the whole blood. The biological significance of whole blood histamine level reduction had been attributed to a granulocyte migration to the tumor site, where histamine is supposed to influence the tumor growth rate, either directly, by interaction with specific surface receptors, or indirectly, switching the balance of host immune response in favor of the disease [11].

Interestingly, no correlation of histamine level to tumor stadiation among untreated tumor patients was found. This could be explained by considering that the putative tumor marker is not directly produced by the tumor cell itself, not being so related to the tumor mass increase; on the contrary, it should rather depend on the granulocyte–tumor interaction. Accordingly, the individual variability in the tumor–host immune system interaction should be the source of the loss of correlation between histamine and tumor stadiation.

These observations give some indications on the potential usefulness of histamine assay in clinical tumor patient monitoring. It is well known that, unfortunately, several tumors produce relevant amounts of classical markers only when their extension is so high to make of reduced utility the surgical procedures. In this perspective, a marker not related to tumor mass could be of help in the monitoring of primary tumor presence and probably of recurrences [35,36]. Consistently, among our cases the 45.8% were positive or CEA and 50% for histamine reduction, supporting the hypothesis that histamine assay could be used in association with classical tumor markers, to improve early tumor detection.

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