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DETERMINATION OF HISTAMINE, METHYLHISTAMINES AND HISTAMINE—*o*-PHTHALDIALDEHYDE COMPLEXES BY TWO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURES

APPLICATION TO BIOLOGICAL SAMPLES

J.C. ROBERT*, J. VATIER, B.K. NGUYEN PHUOC and S. BONFILS

Inserm U. 10, Unité de Recherche de Gastroentérologie, Hôpital Bichat, 170, Boulevard Ney, F-75877 Paris Cedex 18 (France)

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SUMMARY

Two high-performance liquid chromatographic procedures were proposed to measure histamine. The first, with UV detection and a strong acid cation exchanger (Partisil 10, SCX Whatman), made it possible to isolate histamine and some methylated derivatives. The second, with a C_{18} sorbent (μBondapak, Waters, 10 μm particle size) eluted with ion-pairing phases, made it possible to isolate the histamine—*o*-phthaldialdehyde complexes. This last procedure allied with a chromatographic purification step gave lower or identical amounts of histamine than those described in human urine (16 ± 7 μg per 24 h), canine whole blood (1.5 ± 1 ng/ml) and human gastric juice (2.3 ± 1.4 ng/ml). The two procedures gave the concentration of a histamine-like compound isolated from the antral mucosa.

INTRODUCTION

The technical difficulties in measuring histamine in biological samples have led scientists to use bioassay [1, 2], radioenzymatic assay [3] or fluorometric assay [4, 5]. For several years, the addition of chromatographic separations such as thin-layer chromatography [6], gas—liquid chromatography [7, 8], low-pressure liquid chromatography [9, 10], and high-performance liquid chromatography (HPLC) [11—21] has allowed the separation of histamine, its methylated derivatives and biogenic amines.

The isolation of a histamine-like compound from the antral mucosa [22—24] which possesses some biological differences from synthetic histamine made it necessary for our laboratory to have a specific analysis for this amine. We propose two HPLC procedures: the first allows us to analyse histamine and some methylated derivatives in UV light at 210 nm with a strongly acid

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cation-exchange column (Partisil 10, SCX Whatman); the second procedure analyses only histamine—*o*-phthaldialdehyde (OPT) complexes with ion-pairing reversed phases (on a μBondapak C_{18} column, particle size 10 μm). The addition of 1-octanesulfonic acid, sodium salt, was more effective than that of 1-heptanesulfonic acid, sodium salt.

In addition, we describe a rapid and sensitive assay of histamine in three biological fluids. It consists of a chromatographic purification step without the tedious two liquid—liquid extractions. The fluorescent complexes are then analysed with ion-pairing reversed-phase HPLC.

The antral histamine-like compound was measured by the two procedures; values for normal histamine excretion in human urine, human gastric juice concentration and canine whole blood concentration are presented and compared with those described in the literature.

EXPERIMENTAL

Chemical reagents

Sodium hydroxide, acetone, perchloric acid, sulfuric acid, acetic acid, hydrochloric acid, sodium acetate, sodium dihydrogen phosphate, potassium dihydrogen phosphate (Prolabo, Paris, France) were analytical grade; methanol RPE-ACS was obtained from Carlo Erba, Milan, Italy; the 1-octanesulfonic acid, sodium salt, its heptane and pentane analogues were products of Fisons, distributed by Interchim (Montluçon, France); OPT was a Fluka product, distributed by Interchim; histamine dihydrochloride and tris(hydroxymethyl)-aminomethane (Tris) were products of Merck (Darmstadt, G.F.R.). 3-Methylhistamine, 1-methylhistamine, N^{α}-methylhistamine were purchased from Calbiochem, distributed by Eurobio (Paris, France).

HPLC apparatus

The liquid chromatographic system consisted of a Model 848 pump module (Dupont, Orsay, France) equipped with a Rheodyne 7021 injection valve with a 100-μl loop (Touzart et Matignon, Vitry sur Seine, France), an absorbance monitor Isco Model 1840 (Roucaire, Velizy, France) with an 8-μl flow-cell unit, a JY3 spectrofluorometer (Jobin Yvon, Longjumeau, France) with a 20-μl flow-cell unit, and an Ifelec IF 3802 recorder (Jobin Yvon). The system was operated at room temperature.

Histamine analysis with UV detection

HPLC procedure. Chromatography was performed on a Partisil 10 SCX Whatman column, 25 × 0.4 cm I.D., particle size 10 μm, with a mobile phase of 0.4 *M* potassium dihydrogen phosphate aqueous solution (pH 4.5). The histamine compounds were detected at 210 nm. The flow-rate was 2 ml/min (61 bar).

Sample preparation. Antral histamine was isolated from the mucosa as described earlier [22]. After the last step of purification on a carboxymethyl-cellulose column (CM-52; Whatman, Orléans, France) with an ammonium formate gradient of 0.1 to 0.3 *M*, the antral histamine solution was divided into batches and kept at −30°C. Each batch contained an equivalent part of

10 g of fresh antral tissue per ml. From 10 to 50 μl could be injected into the loop.

Histamine analysis with fluorometric detection

HPLC procedure. Chromatography was performed on a μBondapak C_{18} column, particle size 10 μm, 30 × 0.4 cm I.D. (Waters, Paris, France) with a mobile phase of methanol, 0.02 *M* sodium acetate in distilled water, acetic acid (55:43:2, v/v) and 0.15 × 10^{-3} *M* of 1-octanesulfonic acid, sodium salt, as the ion-pairing agent. The flow-rate was 1.5 ml/min (136 bar). Fluorometric intensity was monitored at the emission wavelength of 450 nm with the excitation wavelength set at 360 nm.

Sample preparation. Antral histamine solution (10 μl) was diluted 30-fold with 0.25 *N* hydrochloric acid to obtain an approximately 10^{-7} *M* histamine concentration; the fluorometric assay was developed according to the general procedure (see below).

A 1-ml volume of human urine was adjusted to pH 7.5 with either 0.5 *M* Tris or 10% acetic acid and was diluted to 2 ml with distilled water. A 2-ml volume of canine whole blood or 1 ml of human gastric juice were adjusted to the same pH. Before purification clear gastric juice was obtained by filtration on XAD-2 adsorbent; Servachrom XAD-2, 100—125 μm particle size (distributed by Tebu, Versailles, France), was prepared as previously described [25] and 1 ml was poured into a 10-ml Econo Column (Biorad, Touzart & Matignon). The gastric juice was filtered through the XAD-2 column and 1 ml of 0.050 *M* Tris buffer (pH 7.5) was also added to separate all histamine from the adsorbent.

Sample purification of biological fluids. The histamine was extracted according to the chromatographic procedure described by Oates et al. [26] with some changes in technical conditions; Amberlite CG-50 type I (Prolabo) was prepared as described [26] and purification was carried out as follows. A 0.4-ml volume of Amberlite was poured into a 10-ml Econo Column, rinsed with 2.5 ml of 0.5 *M* sodium phosphate, pH 7.5, and 2.5 ml of distilled water. The sample was gently deposited on the resin, then cleaned with 2.5 ml of distilled water, 3 × 2.5 ml of 0.5 *M* sodium acetate pH 6.5, 2 × 2.5 ml of distilled water and 0.5 ml of 0.5 *N* hydrochloric acid. Histamine was eluted with 2 × 1 ml of 0.5 *N* hydrochloric acid.

Two other extraction procedures were studied on a urine sample. The liquid—liquid extraction [26] was added to the above-mentioned chromatographic purification. Histamine was also extracted as described by Huff et al. [27]; they eluted histamine from Amberlite CG-50 with 0.5 *N* sodium hydroxide instead of hydrochloric acid.

Fluorometric procedure. Samples of 1 ml were made alkaline (pH 12.1) by the addition of 0.2 ml of 2 *N* sodium hydroxide and the derivatisation was performed by mixing 0.1 ml of 0.1% OPT dissolved in methanol.

The fluorometric reaction was developed for 40 min at 0°C in darkness as described by Håkanson et al. [28] and then stopped by acidification (pH 2.2) with 0.2 ml of 1 *N* sulfuric acid. Fluorescence was stable for 2 h.

Standardisation and quantitation

All the histamine standard solutions were prepared daily from stock solutions. With UV absorption, 50 μl of standard solutions (2×10^{-5} to 16×10^{-5} *M*) were injected into the loop; with fluorometric detection, 15 μl of the histamine—OPT standard solutions (2.5×10^{-8} to 80×10^{-8} *M*) were injected. One blank was always run with the histamine range and a second one was prepared during the purification by substituting distilled water for the biological fluids.

There was a linear relationship between the fluorometric intensity, UV absorbance (peak height) and the concentration of histamines; the reproducibility of the two methods was tested by ten repeated injections of the same histamine solutions (see Analytical results). The recoveries of histamine (%) and the relative standard deviations (R.S.D. %) of ten assays on pooled samples were checked by adding known amounts of histamine: 100 pmol/ml of urine or gastric juice, 50 pmol/ml of whole blood, 100 pmol/ml or 300 nmol/ml of antral histamine solution (see Biological results). A 75-μl volume of biological histamine—OPT complexes injected corresponded to 25 μl of human urine or human gastric juice, to 50 μl of canine whole blood, and to 3.3 mg of fresh tissue. The results were expressed as histamine base.

Nature and collection of the samples

Pig antral mucosae were obtained from a slaughterhouse and stored at —30°C before histamine extraction. Biological fluids were obtained from normal humans or animals; human urine was collected in plastic containers with sodium metabisulfite [15], human gastric juice and canine whole blood as described by Lorenz et al. [5, 29].

ANALYTICAL RESULTS

HPLC procedure with UV absorption of histamine

Histamine (H), N^{α}-methylhistamine (N^{α}-MH), 1-methylhistamine (1-MH) and 3-methylhistamine (3-MH) were well separated at 210 nm in 0.4 *M* potassium dihydrogen phosphate aqueous solution (see Fig. 1).

Table I gives the capacity ratio (k') values of histamines expressed in terms of dihydrogen phosphate concentration; pH had no effect on the values in the pH range 2.5—5.5. When the buffer concentration was lower than 0.3 *M*, methylated histamines began to be retained on the column. With a 0.1 absorbance unit full-scale (a.u.f.s.), the limits of detection were: 0.2 nmol for H, N^{α}-MH and 3-MH, and 1 nmol for 1-MH, per 50 μl of injected sample at a signal-to-noise ratio of 3:1.

The reproducibility of the assays was tested by ten repeated injections of H, N^{α}-MH, 3-MH (10 nmol of each) and 1-MH (50 nmol); the means of the calculated peak heights showed a relative standard deviation of 1%, 1.3%, 1.3% and 1.5%, respectively.

HPLC procedure for the histamine—OPT complexes

In order to measure histamine in biological fluids with high sensitivity, it was necessary to form fluorescent compounds. Table II shows the values for

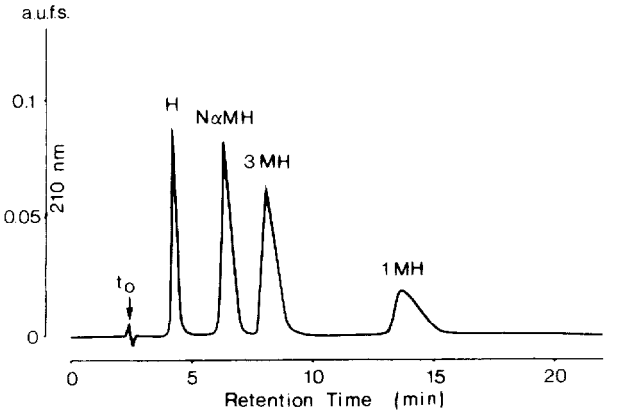

Fig. 1. Chromatogram of standards on Partisil 10, SCX Whatman: 8 nmol of each; H = histamine, N^{α}-MH = N^{α}-methylhistamine, 3MH = 3-methylhistamine, 1MH = 1-methylhistamine. Injection volume = 50 μl. The mobile phase consisted of 0.4 *M* potassium dihydrogen phosphate. Flow-rate: 2 ml/min; t_0 corresponded to $k' = 0$.

TABLE I

k' VALUES OF THE HISTAMINES IN TERMS OF POTASSIUM DIHYDROGEN PHOSPHATE CONCENTRATION

— = Methylated histamines were retained on the column.

	Potassium dihydrogen phosphate concentration (mol/l)								
	0.5	0.4	0.3	0.25	0.2	0.15	0.125	0.1	0.075
H	0.55	0.68	0.83	1.04	1.20	1.80	2.36	3.75	5.2
N^{α}-MH	1.32	1.55	1.96	2.96	4.21	5.96	—	—	—
3-MH	1.96	2.24	3.35	5.98	—	—	—	—	—
1-MH	3.60	4.43	6.17	—	—	—	—	—	—

the capacity ratio (k') of the complexes with the addition of ion-pairing agents.

The use of 0.15×10^{-3} *M* 1-octanesulfonic acid, sodium salt, with methanol—0.020 *M* sodium acetate—acetic acid (55:43:2, v/v) gave a k' value of 1, a peak without shoulder and chromatographic separation within 4 min. The apparent pH was 3.8 and in the range 3—4.5; pH had no effect on k' values or on fluorescence intensity.

The limit of detection was 0.1 pmol per 15 μl of injected sample at a signal-to-noise ratio of 3:1. The reproducibility of the assay was tested by ten repeated injections of 6 pmol of histamine—OPT complexes; the mean of the calculated peak heights showed a relative standard deviation of 2.2%.

TABLE II

EFFECT OF THE ION-PAIRING AGENT ON k' VALUES OF HISTAMINE—OPT COMPLEXES WITH TWO MOBILE PHASES OF METHANOL—0.020 *M* SODIUM ACETATE—ACETIC ACID

1-Pentanesulfonic acid, sodium salt, had no effect on k' values of histamine—OPT complexes ($k' = 0$).

Mobile phase ratio	1-Heptane sulfonic acid ($\times 10^{-3}$ mol/l)	k'	1-Octane-sulfonic acid ($\times 10^{-3}$ mol/l)	k'
50:48:2 (v/v)	0	0	0	0
	0.025	0.5	0.050	0.9*
	0.050	0.65	0.100	1.4, 1.6**
	0.075	0.75*	0.150	1.7, 1.9**
	0.100	0.85*		
	0.150	0.91*		
55:43:2 (v/v)	0	0	0	0
	0.025	0.2	0.050	0.5
	0.050	0.35	0.100	0.8
	0.075	0.45	0.150	1.0
	0.100	0.5	0.200	1.15
	0.150	0.55		
	0.200	0.6		

*A shoulder appeared.
**Two peaks appeared in the ratio 6:1.

BIOLOGICAL RESULTS

Chromatographic purification of the biological samples and their histamine content

Antrum. The concentration of antral histamine was determined as follows: by the total fluorometric assay [22], mean ± S.D. ($n = 5$) 30 ± 1.5 μg per 10 g; by the HPLC fluorometric assay, mean ± S.D. ($n = 5$) 29.5 ± 0.9 μg per 10 g; by the HPLC UV assay, mean ± S.D. ($n = 5$) 30 ± 0.6 μg per 10 g; the means of the recovery (%) ($n = 5$) and the relative standard deviation (RSD %) were 98 ± 4%, 99 ± 2.5% and 101 ± 1.5%, respectively.

Analysis of the chromatogram did not indicate the presence of methylated histamines.

Urine. The histamine urinary excretion of three normal subjects was studied during non-consecutive days (number of samples: $n = 15$). The results were: mean ± S.D. = 16 ± 7 μg per 24 h, range 7—47 μg per 24 h.

The recovery and the relative standard deviation were tested on pooled urine ($n = 5$): mean ± S.D. = 14.5 ± 0.8 ng/ml; recovery ± RSD = 79.6 ± 5.4%; the limit of detection was 0.5 ng/ml of urine.

No histamine was seen in the blanks. Fig. 2 shows a chromatogram of urinary histamine—OPT. There was no interference with other amine compounds such as spermidine ($k' = 4$); an unidentified peak appeared before that of histamine—OPT.

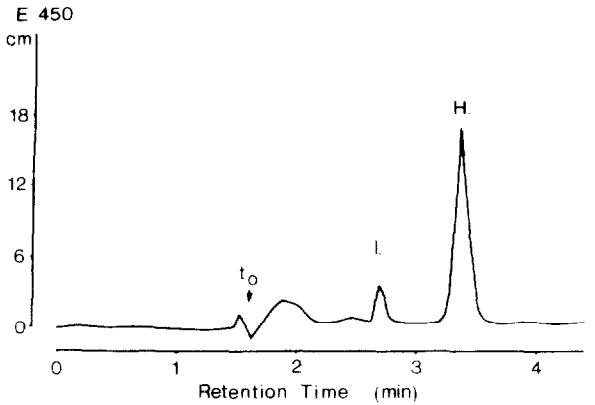

Fig. 2. Chromatogram of a urinary sample on μBondapak C_{18} (Waters), 10 μm particle size: I = unidentified peak; H = histamine—OPT complexes. Injection volume = 75 μl. The mobile phase consisted of methanol—0.020 *M* sodium acetate—acetic acid (55:43:2, v/v) and 0.15 × 10^{-3} *M* 1-octanesulfonic acid, sodium salt. Flow-rate: 1.5 ml/min; t_0 corresponded to $k' = 0$; emission wavelength 450 nm, excitation wavelength 360 nm; 100 fluorescence units corresponded to 30 cm.

Canine whole blood. The histaminemia of three dogs was measured six times. The mean ± S.D. was 1.5 ± 1 ng/ml, range 0.4—3 ng/ml.

The recovery (%) and the RSD (%) were tested on pooled canine whole blood (n = 10): mean ± S.D. = 0.9 ± 0.08 ng/ml; recovery ± RSD = 64 ± 8%; the limit of detection was 0.3 ng/ml of canine whole blood.

Human gastric juice. Amounts of 200 nmol of H, N^{α}-MH, 3-MH and 1 μmol of 1-MH dissolved in 0.1 ml of water were deposited on XAD-2 adsorbent which was then cleaned with 1.9 ml of 0.050 *M* Tris, pH 7.5. The HPLC UV assay indicated good recoveries: 99%, 98%, 101% and 98%, with RSD of 2%, 3%, 3.5%, and 4%, respectively.

The histamine concentrations of eight gastric juices were measured; the mean ± S.D. was 2.3 ± 1.4 ng/ml, range 0.9—4.2 ng/ml.

The recovery and the RSD were tested on pooled gastric juice (n = 10): mean ± S.D. = 2.1 ± 0.2 ng/ml; recovery ± RSD = 83 ± 9%; the limit of detection was 0.6 ng/ml of gastric juice.

DISCUSSION

HPLC procedure with UV detection

Gaetani and Laurei [13] were the first scientists to use a high-performance carboxylic resin to evaluate histamine compounds at 208 nm (they prepared their stationary phase themselves). At present, the use of the SCX Whatman column is known [20]. Both resins gave comparable results in the elution order of histamine and methyl derivatives. Even if UV detection was less sensitive than fluorometric detection, it had the great advantage of measuring histamine and its derivatives in tissues without the usual derivatisation with OPT [10, 30] or dansyl chloride [9].

HPLC procedure with fluorescence detection

Our goal was to obtain a rapid and specific separation of the histamine—OPT adducts. Furthermore, the use of a mobile phase in the low pH range permitted good stabilisation of the complexes [18, 19, 28].

Reversed phases containing hydrochloric acid, sodium chloride, or basic buffers are known [11, 12, 16, 18] but they were recommended neither by the manifacturers nor by Rabel [31]. Meil et al. [32] described a reversed-phase system where histamine—OPT was eluted near the solvent front, and they preferred to use a CN column [14]. Similarly, Davis et al. [15] analysed the OPT complexes on a phenyl column. The main disadvantage of these two procedures was the time necessary to analyse the purified histamine sample; analyses with an amino acid analyser [33] were also very time-consuming.

Under these conditions, the nature of the mobile phase was studied on the basis of the ratio buffer/methanol described in the literature [11, 12, 14, 18].

Phosphate was replaced by sodium acetate and acetic acid to obtain an apparent pH of 3.8; only the addition of 1-heptane- or 1-octanesulfonic acid, sodium salt, increased capacity ratios. The formation of two main products forced us to find a compromise between the resolution and the capacity ratio. We demonstrated that the addition of 0.15×10^{-3} *M* 1-octanesulfonic acid, sodium salt, to the mobile phase methanol—0.020 *M* sodium acetate—acetic acid (55:43:2, v/v), gave the best solution with $k' = 1$ and a "single peak".

The advantage of ion-pairing agent was demonstrated because histamine—OPT adducts could not be isolated from the SCX Whatman column with either phosphate buffers or water—methanol mobile phases.

Biological samples

Antrum. With UV absorption, the antral histamine solution showed a single histamine peak. 1-MH and 3-MH were not present although they do exist in the antral mucosa [34]. Both chromatographic analyses gave comparable antral histamine concentrations and demonstrated the close relationship between the fluorometric intensity and the UV absorption (peak heights).

Urine. Several methods might be used to purify the sample. If 2-mercapto-ethanol and OPT were added to crude urine, purification of the fluorescent complexes could be performed by the liquid—liquid extractions alone, but HPLC analyses were very time-consuming [14, 15] because the fluorogenic reagents reacted with primary amines. In order to develop a simple, simultaneous and shorter determination of histamine and 1-methylhistamine by HPLC, Tsuruta et al. [12] added a second purification step on a phosphocellulose resin.

When the fluorescence reaction was run without 2-mercaptoethanol, ammonia had to be eliminated from the urine with a cation-exchange resin; Oates et al. [26], Gilbert et al. [35] added the liquid—liquid extraction, Myers et al. [36] added a diamine oxidase reaction step and Endo [37] added a second chromatographic purification.

At the present time, HPLC analysis could replace the total fluorometric determination and the second purification step. Table III shows a comparative determination of histamine on pooled urine. Three purification methods were

TABLE III

COMPARATIVE VALUES OF HISTAMINE CONCENTRATION FOR POOLED URINE

Method	ng/ml ±	S.D.	Recovery (%)	R.S.D. (%)	n §
Huff's method [27] + total detection	47 ±	6.3	84	13.5	5
Huff's method [27] + HPLC analysis	*	*	*	*	5
CG-50** + L*** [26] + total detection	29 ±	2.7	54	9.5	5
CG-50** + L [26] + HPLC analysis	15 ±	1.2	55	7.8	5
CG-50** + total detection	27 ±	2.5	80	8.7	5
CG-50** + HPLC analysis	14.5 ±	0.8	79.6	6.2	5

*The HPLC chromatogram showed several peaks and a shoulder appeared in front of the histamine—OPT peak.
**The sample purification is described in the experimental part. CG-50 is a weakly acid cation exchanger.
***L = Liquid—liquid extractions.
§n = No. of samples.

allied to either a total fluorometric determination or an HPLC analysis. The total fluorometric determination always gave higher concentrations than those obtained with HPLC. Huff's [27] method gave the highest concentration with a shoulder in front of the histamine—OPT peak. We obtained the lowest concentration with a single chromatographic purification step allied with a selective and rapid HPLC analysis and the tedious liquid—liquid extractions could be eliminated.

The amounts of histamine we found in 24-h urine of normal persons (16 ± 7 μg per 24 h) were lower than those described with the fluorometric method by total detection [26, 34, 36, 37] and even with the help of HPLC [12], but were comparable to those obtained with Myers' method [36] and the enzymatic assay [39—41]. The results are shown in Table IV.

Canine whole blood. Purification and histamine analysis for canine whole blood were run as for human urine; the liquid—liquid extractions were eliminated. Our mean, 1.5 ng/ml, was close to the result obtained by Lorenz et al. [42], 2 ng/ml (see Table V).

Human gastric juice. The lack of fluidity of human gastric juices and, probably, the formation of histamine biliary salt complexes such as those formed in plasma with metals [43], proteins [44] and interactions with ascorbic acid [45], made it difficult to extract histamine.

XAD-2 adsorbent, which retains steroids [46] and biliary salts [25] at neutral pH, completely eluted histamine and its methylated derivatives; the XAD-2 filtrate was then treated like human urine and canine whole blood. Our mean, 2.3 ng/ml, was lower than that obtained by Lorenz et al. [42], 6 ng/ml, probably because the HPLC analysis was specific (see Table V).

CONCLUSION

Two HPLC methods were proposed to determine histamine. The first, with UV detection could be used particularly well for tissues; the second, allied with a simple purification step, could be used for three biological fluids.

TABLE IV

URINARY HISTAMINE LEVELS IN NORMAL SUBJECTS AS DETERMINED BY DIFFERENT ASSAYS

Authors [ref.]	Procedure*	Histamine level (μg per 24 h)
Oates et al. [26]	S → CG-50 → L → OPT → Total detection	45
Gilbert et al. [35]	S → CG-50 → L → OPT → Total detection	42
Huff et al. [27]	S → CG-50 → OPT → Total detection	45
Beall [38]	S → CM-50 → L → OPT → Total detection	46
Endo [37]	S → Dowex 50 → CM-cell. → OPT → Total detection	170—1100 (total hydrolysis)
Myers et al. [36]	S → Dowex 50 → OPT → DAO → Total detection	14
Mell et al. [14]	S → OPT + ME → L → HPLC	16—60 ng/ml
Tsuruta et al. [12]	S → L → P-cell. → OPT + ME → HPLC	
Beaven et al. [3]	S → Enzymatic method	16
Horakova et al. [39]	S → Enzymatic method	19
Rosenthal et al. [40]	S → Enzymatic method	17
Bruce et al. [41]	S → Enzymatic method	25
Present study	S → CG-50 → OPT → HPLC	16

*S = sample; L = liquid—liquid extractions; Dowex 50 = strongly acid cation exchanger; CG-50, P-cell., CM-cell. = weakly acid cation exchangers; ME = 2 mercaptoethanol; DAO = diamine oxidase.

TABLE V

HISTAMINE CONCENTRATION IN CANINE WHOLE BLOOD AND HUMAN GASTRIC JUICE

Biological fluid	Authors [ref.]	Procedure*	Histamine concentration (ng/ml)
Canine whole blood	Lorenz [42]	S → AG-50 → L ——→ OPT → Total detection	2
	Present study	S → CG-50 ——→ OPT → HPLC	1.5
Human gastric juice	Lorenz [42]	S → AG-50 → L ——→ OPT → Total detection	6
	Present study	S → XAD-2 → CG-50 → OPT → HPLC	2.3

*S = sample; L = liquid—liquid extractions; AG-50 = strongly acid cation exchanger; CG-50 = weakly acid cation exchanger; XAD-2 = polystyrene adsorbent.

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