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Note

Highly sensitive high-performance liquid chromatographic technique for the simultaneous measurement of histamine, 1-methylhistamine and other biogenic amines

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Histamine has been measured by a variety of methods including bioassay [1], fluorimetry [2] and radioenzymatic assays using both single (SIREA) [3] and double-isotope (DIREA) [4] techniques. Although all these methods are comparably sensitive when applied to aqueous solutions of histamine, their selectivities appear to differ. Recent studies in our laboratory comparing all these techniques for the measurement of histamine in the same sputum samples demonstrated the DIREA to detect much lower levels of histamine than the other methods, suggesting it to be the most selective method [5]. We describe a new high-performance liquid chromatographic (HPLC) method for the estimation of histamine and other biological amines which offers the advantages of being relatively simple, rapid, highly selective and is able to measure other biological amines simultaneously.

EXPERIMENTAL

Chemical reagents

Methanol, tetrahydrofuran (THF) and ethanol were HPLC-grade (Rathburn Chemicals, Walkerburn, U.K.). [^{14}C]Histamine dihydrochloride, labelled in the ring C-2 position was supplied by Amersham International. The biogenic amines (5-hydroxytryptamine, octopamine, epinephrine, normetanephrine, norepinephrine, tyramine, dopamine and spermidine) were purchased from Sigma (Poole, U.K.). Histamine and 1-methylhistamine were prepared by Hoechst and supplied by Cambridge BioScience (Cambridge, U.K.). All other

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chemicals were supplied by BDH (Poole, U.K.) and were of the highest available purity. Beckman CP scintillation cocktail was supplied by Beckman.

HPLC procedure

An ACS HPLC Model 300 pump (Applied Chromatography Systems) regulated by an ACS Modular liquid chromatography LC 750 decilinear programmer and connected to a Rheodyne 7021 injection valve with a 20-μl loop (Applied Chromatography Systems) was used as the solvent delivery system. Chromatography was performed on a Spherisorb 3 ODS column (particle size 3 μm), 10 cm × 4.6 mm I.D. (HPLC Technology) with a mobile phase consisting of a mixture of 0.2 *M* sodium acetate in distilled water and tetrahydrofuran (75:25, v/v), adjusted to pH 5.1 with concentrated hydrochloric acid, pumped at a constant flow-rate of 1.0 ml min^{-1}. Detection of the *o*-phthalaldehyde (OPA) derivatised amines was achieved using a Perkin-Elmer LS-4 fluorescence spectrometer (Perkin-Elmer) fitted with a 3.0-μl flow cell. Fluorimetric intensity was monitored at an emission wavelength of 430 nm with the excitation wavelength set at 350 nm and chromatographic peaks were recorded and integrated with a Shimadzu Chromatopac C-RIB data processor (Shimadzu Corporation, Japan).

Preparation of o-phthalaldehyde reagent

OPA reagent was prepared according to the method of Skaaden and Greibrokk [6] except that 3-mercaptopropionic acid was substituted for 2-mercaptoethanol as suggested by Kucera and Umagat [7]. OPA (10 mg) and 50 μl 3-mercaptopropionic acid were dissolved in 1.0 ml of ethanol and then mixed with 9.0 ml 0.4 *M* boric acid solution adjusted to pH 10.8 with 2.0 *M* potassium hydroxide. The reagent was stored in a dark container and allowed to stand at room temperature for at least 24 h before use, to allow background fluorescence to minimise. The reagent was found to be stable for at least two weeks but was usually discarded if unused after seven days.

Standardisation

Standardisation of the assay was performed by the external standardisation method. Stock solutions of the appropriate amines were freshly prepared at concentrations of 1.0 mg ml^{-1} in 0.05 *M* hydrochloric acid and further diluted with distilled water as required. Derivatisation to fluorescent compounds was achieved by reacting 0.1-ml aliquots of the required dilution with 0.1 ml of OPA reagent. The derivatisation reaction was allowed to proceed for exactly 2 min before sample injection onto the column. Standard curves for histamine and 1-methylhistamine were constructed in the range 0.1—10 ng absolute.

Sample preparation

Derivatisation of sputum samples was carried out using the same procedure described above for aqueous samples. However, preliminary experiments revealed the presence of large amounts of interfering compounds which co-chromatographed with histamine and 1-methylhistamine and it was therefore necessary to subject the samples to a clean-up procedure. Following thawing of the frozen samples, homogenisation was carried out by sonication. Aliquots

(1.0 ml) were then de-proteinated by the addition of an equal volume of 0.4 *M* perchloric acid. Following the addition of 0.7 g of sodium chloride, the mixture was mixed by vortexing for 15 min and then centrifuged at 1500 *g* for 15 min. The supernatants obtained after centrifugation were diluted 1:10 with 0.5 *M* sodium acetate, pH 6.5, and then passed through an ion-exchange column of 1.0 ml Amberlite CG-50 prepared according to the method of Oates et al. [8] and contained in a 10-ml polypropylene Econo column (Bio-Rad). The column was then washed serially with 5.0 ml distilled water, 7.5 ml of 0.5 *M* sodium acetate, pH 6.5, and finally 5.0 ml of 5 m*M* hydrochloric acid. Recovery of the amines from the column was achieved by elution with 2.0 ml of 0.5 *M* hydrochloric acid and the eluate then blown to dryness. This method is similar to that employed by Robert et al. [9] but modified by the initial ten times sample dilution which in our hands improved the final recovery of histamine. The dried samples were re-suspended in 250 μl of 0.4 *M* borate buffer, pH 10.8, before reacting with OPA solution.

Correction for any losses and final recovery of amines after the processing procedure was performed by spiking two duplicate sputum samples with a known amount of [^{14}C]histamine and processing in parallel with samples of interest. Counting of the final eluate obtained from the spiked samples was performed using a Beckman LS-1800 scintillation counter (Beckman) allowing mathematical correction for any losses incurred in the processing procedure.

Separation of biogenic amines

Aqueous stock solutions containing 1 mg ml^{-1} of dopamine, norepinephrine, octopamine, 5-hydroxytryptamine, normetanephrine, spermidine, histidine and tyramine in addition to histamine and methyl-histamine were prepared. The solutions were diluted suitably, mixed together, derivatised with OPA as described above and analysed. Following the appearance of peaks due to histamine, a THF gradient was applied in order to assist elution.

Double-isotope radioenzymatic assay

Estimation of histamine by DIREA was performed on all samples by the method of Brown et al. [4].

RESULTS AND DISCUSSION

Preliminary experiments for separation of the OPA derivatives of histamine and 1-methylhistamine were conducted using varying proportions of sodium acetate, THF and methanol in the mobile phase. However, the best separation of these amines was obtained using a solvent system comprising sodium acetate and THF (75:25, v/v). In general, retention times were approximately 7.5 and 8.5 min for 1-methylhistamine and histamine, respectively and retention times varied by only a few sec from day to day. Standard curves show linearity for histamine and 1-methylhistamine in the range 0.1—10 ng ml^{-1}. The limits of detection for histamine and 1-methylhistamine with this system were found to be 5.0 pg 20 μl^{-1} and 10.0 pg 20 μl^{-1} of injected sample, respectively at a signal/noise ratio of 10 to 1. Quantitatively the assay was found to be highly reproducible with intra-assay coefficients of variation of 10% and 7% and inter-

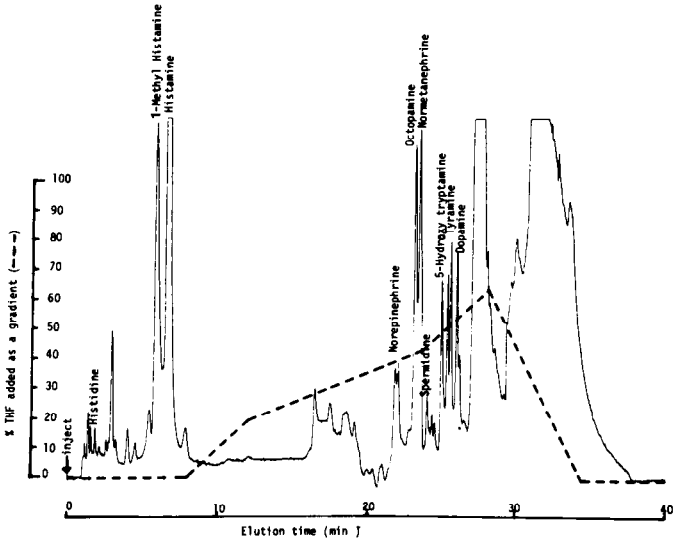

Fig. 1. HPLC chromatogram of a mixture of biogenic amines. A step-up gradient of THF was applied at 8 min to assist elution.

assay coefficients of variation of 9% and 10% for 1-methylhistamine and histamine, respectively.

Fig. 1 illustrates a chromatogram of a mixture of biogenic amines in aqueous solution. Of all the amines tested, 1-methylhistamine, histamine and the chemically closely-related L-histidine elute much earlier under isocratic conditions within approximately 10 min. The excellent separation of these compounds from the other biogenic amines is illustrated by the necessity for application of a step-up gradient of THF in order to elute the latter within a reasonable time.

Estimation of histamine and 1-methylhistamine in sputum

Histamine and 1-methylhistamine were estimated in sputum samples from patients with asthma and chronic bronchitis. On each occasion when sputum samples were analysed, a check on the retention times for these two compounds was made by spiking duplicate samples with known amounts of both substances.

Recoveries for both histamine and 1-methylhistamine after sample processing were found to be 86 ± 5% (mean ± S.D.).

We consistently detected large quantities of histamine in the sputum of asthmatic and chronic bronchitic patients. The amounts ranged from 17.2 to 184.1 ng ml^{-1}. There was good correlation of the values obtained with those obtained using the DIREA (r = 0.93) (Table I). However, there was no detectable 1-methylhistamine present in any of the samples. This is shown in Fig. 2 where spiking of the sputum sample with 1-methylhistamine results in the detection of a new peak whereas no peak was seen in this region for the unspiked sample.

The method we have developed provides a relatively simple means for the simultaneous measurement of histamine and one of it's most important metab-

TABLE I

CORRELATION BETWEEN MEASUREMENT OF HISTAMINE BY DIREA AND HPLC

Coefficient of correlation (r) = 0.93, $p < 0.001$.

Patients	Sample	Method of determination of histamine (ng g^{-1})	
		DIREA	HPLC
Asthma	1	37.58	36.70
	2	36.78	36.10
	3	20.29	18.00
	4	37.30	35.58
Pneumonia	5	143.34	121.80
	6	46.59	65.80
	7	36.17	48.12
	8	50.75	72.97
	9	8.10	11.10
Acute exacerbations of chronic bronchitis	10	23.58	18.40
	11	131.68	184.10
	12	37.87	28.10
	13	31.44	17.20

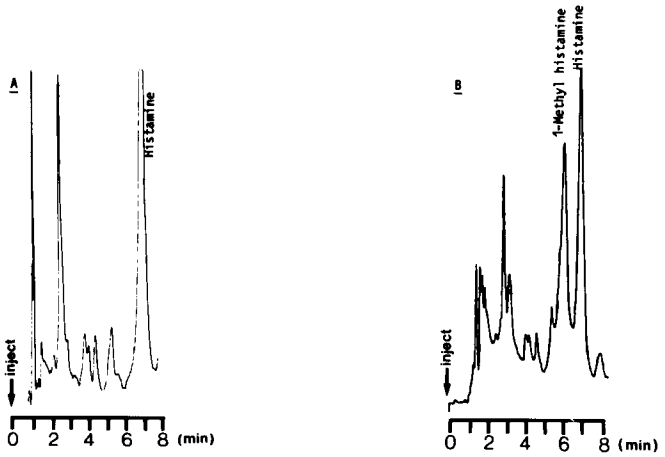

Fig. 2. (A) HPLC chromatogram of a sputum sample obtained from a patient with chronic bronchitis; (B) the absence of 1-methylhistamine in the sample was demonstrated by the appearance of a new peak following the addition of 1-methylhistamine to the sample.

olites [10]. It is reproducible and measurements of histamine correlate well with those obtained by the DIREA, with which it compares well in terms of sensitivity. It is also highly selective, as demonstrated by its ability to discriminate between closely related amines. Narrow peak width allowed good separation in the region of interest and short column retention times enabled the rapid analysis of a large number of samples.

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