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Note

High-performance liquid chromatographic technique for the rapid determination of histamine in both the plasma and cellular components of blood

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Histamine (HA) has important physiological roles in neurotransmission, allergy, and in the microcirculation.

The application of high-performance liquid chromatography (HPLC) with fluorimetric detection to the measurement of HA in biological samples is a recent development [1] Its main advantages over the more established radioenzymatic [2] and other fluorimetric methods [3] are those of cost efficiency and relative simplicity.

As many biological compounds co-chromatograph with HA [4,5] it is necessary to incorporate a clean-up stage prior to analysis The two most commonly employed are liquid chromatography using ion-exchange resins [6] and alcohol extraction [2]. However, these procedures can be time-consuming and are relatively inefficient [4]. The use of a Millipore Sep-Pak C_{18} cartridge overcomes these disadvantages by the rapid extraction of interfering compounds based on their polarity differences to HA. The cartridge also cleans up the sample for the subsequent HPLC analysis.

The HPLC technique using *o*-phthalaldehyde (OPA) to convert HA to a HA-OPA fluorophore combines the methods of Skotfitsch et al [7] and Ronnberg et al. [8]

The HA inactivating enzyme diamine oxidase (DAO) [9] has been used to assess the specificity of the assay [10] Incubation of plasma with DAO just

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prior to HA analysis causes any HA present to be degraded. Other interfering substances tend not to be affected in this manner; however, DAO is not entirely specific to HA and may also inactivate other biogenic amines [9].

The immunoglobulin E antigen (anti-IgE) is a useful tool in demonstrating the reliability of a HA assay [11]. Interaction of this antigen with IgE causes HA release by the Type 1 reaction from the basophils into the surrounding medium [12]. After a 'challenge' of whole blood with this antibody, the extracellular HA concentration increases and intracellular basophil HA concentration is reduced by the same proportion, leaving the whole blood HA concentration unaffected [13].

This method incorporates all of the above-mentioned techniques in order to ascertain the specificity, reproducibility and sensitivity of the assay.

EXPERIMENTAL

Reagents

Pentanesulphonic acid, OPA, DAO, piperazine-N,N′-bis-2-ethanesulphonic acid (PIPES)–albumin–glucose buffer, anti-IgE (developed in goat) and human serum albumin were purchased from Sigma (Poole, U.K.); glacial acetic acid, methanol, hydrochloric acid (all AR grade), acetonitrile (HPLC grade), sodium hydroxide, calcium chloride, sodium chloride and potassium chloride (all AR grade) were purchased from FSA (Loughborough, U K.); Sep-Pak C_{18} cartridges were purchased from Millipore U.K. (High Wycombe, U.K)

Preparation of blood extract

At least 1 ml of lithium-heparinised blood was required for analysis [13]. This could be stored for up to 3 h at 4°C with no significant HA decay or extracellular release from the basophils. Blood was separated into its plasma and cellular fractions by centrifugation at 2000 *g* for 10 min taking care not to disturb the leucocyte layer above the erythrocytes which contains the basophils. After separation, 250 μl of each fraction were analysed for HA content. All subsequent work was carried out using disposable sterile polypropylene test tubes. The cellular fraction was washed twice in PIPES–albumin–glucose buffer pH 7.4 [14] by adding 250 μl of buffer, gently mixing, centrifuging at 2000 *g* for 10 min and discarding the same volume of supernatant after each wash. Both the plasma and cellular aliquots were made up to 1 ml with PIPES and after gentle mixing deproteinised with 1 ml of 0.8 *M* perchloric acid. This was followed immediately by vortex-mixing for 1 min, incubation at room temperature for 30 min and centrifugation at 3000 *g* for 15 min. The supernatant containing the HA was then removed and 1 ml was diluted with an equal volume of water, so that the sample volume was 2 ml in 0.2 *M* perchloric acid. HA standard ranging between 2 and 200 nmol/l freshly made up in PIPES and a reagent blank were also taken through the assay.

Sep-Pak C_{18} cartridge treatment

The cartridge was prepared by passage of 4 ml acetonitrile and washed with 4 ml deionised water. It was equilibrated to the same pH as the samples with 6 ml of 0.2 *M* perchloric acid. The samples and standards were then slowly passed through the cartridge, the first 0.5 ml of the eluate being discarded and the rest collected for HA analysis. Storage of the samples was then possible at −20°C for up to two months. The acid environment prevented rapid HA degradation.

Fluorophore derivative formation

The OPA was freshly prepared each day by dissolving 200 mg of the solid in 10 ml of methanol. It was stored at 0°C. A 1-ml sample was made alkaline by the addition of 0 4 ml of 1 *M* sodium hydroxide. This was then reacted with 0.3 ml OPA solution, rapidly vortex-mixed and incubated at 0°C in the dark for 30 min to allow the HA fluorophore to develop. The fluorophore was made stable by adding 0.8 ml of 3 *M* hydrochloric acid to the reaction mixture. Due to instability of the fluorophore it was essential that the samples were analysed immediately after the fluorophore derivative formation was complete.

HPLC procedure

The HPLC system consisted of a Pye-Unicam LC3-XP pump and 4811 integrator. The detector was a Gilson 121 flow fluorimeter set at peak wavelengths for excitation 356 nm and emission 430 nm. The samples were injected using a Magnus M7110 pneumatic autosampler connected to a 0.5-ml sample loop. The column was an Altex C_8, 5 μm particle size, reversed-phase column (250 mm×4.6 mm I.D.). The buffer was acetate-based, pH 4.6, containing 0.6% glacial acetic acid, 20% acetonitrile and 0.1% pentanesulphonic acid as an ion-pair reagent. The flow-rate was 1.2 ml/min. With the blood cell preparation it was found necessary to flush the column between samples with 0.5 ml acetonitrile.

DAO and anti-IgE treatment

A preparation of stock freeze-dried DAO from porcine kidney was stored at 0°C for up to three months. Solutions of this were freshly made up when required to a volume activity of 5 U/l with PIPES buffer [9]. In triplicate, 50 μl of this DAO solution were added to 0.5 ml of human plasma and gently agitated for 1 h in a constant-temperature shaking incubator set at 37°C. The samples were then analysed for HA and compared against a control containing PIPES added to the plasma with no DAO

Stock anti-human IgE (minimum titer 1:8) was diluted to 1 ml with PIPES and stored at 4°C for up to one month. Duplicate 0.5-ml samples of freshly taken heparinised human whole blood were added to 50 μl of this anti-IgE solution and incubated as above, then separated into their plasma and cellular

components. Each component was analysed for HA and compared against a control containing no anti-IgE.

RESULTS AND DISCUSSION

The elution profile for the HA-OPA fluorophore revealed two peaks of retention times between 11.0 and 11.5 min and 14.0 and 14.5 min, respectively. The characteristics of the earlier peak proved the more suitable for analysis purposes and thus was used for all subsequent concentration calculations (Fig. 1).

The biogenic amines spermine, spermidine, histidine and putrescine did not interfere with the assay.

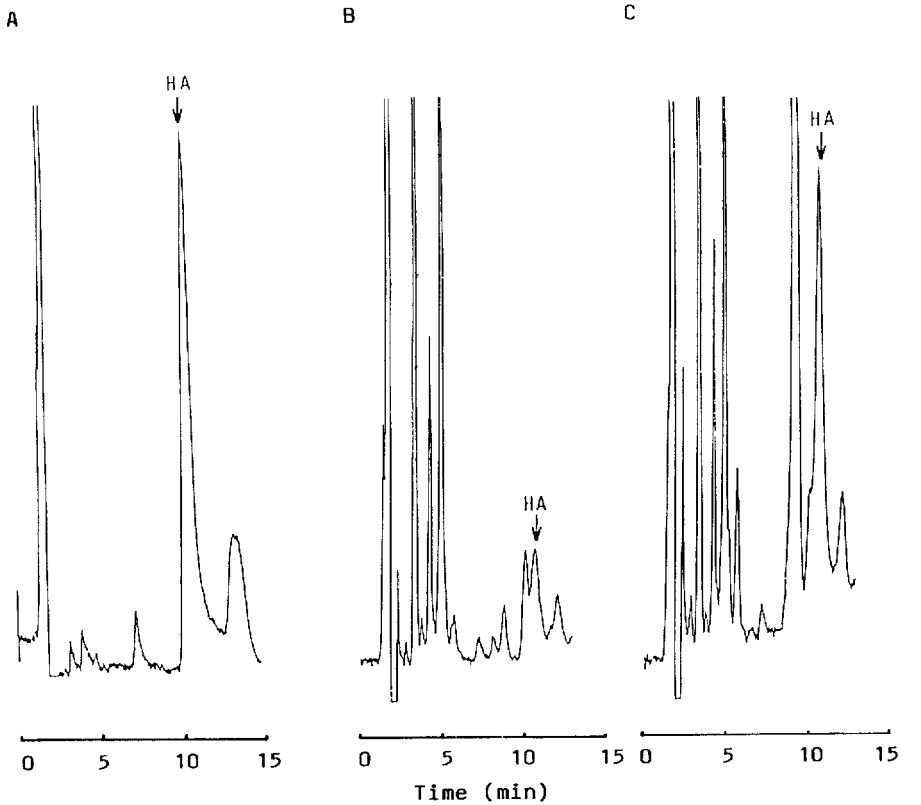

Fig 1 Chromatogram showing (A) histamine (HA) standard (100 nmol/l), (B) plasma histamine peak before challenge and (C) plasma histamine peak following whole blood challenge with anti-IgE

Standard curve

The lowest HA concentration for which a measurable peak could be obtained was 5 nmol/l. The standard curve was linear between 5 and 500 nmol/l However, for the convenience of peak measurement, concentrations greater than 100 nmol/l were diluted with 0.2 *M* perchloric acid just prior to derivative formation. Calibration curves obtained from the area under the peak gave the linear regression equation $y=1.35x-0.38$ and a coefficient of correlation of 0.94 To test for reproducibility in the HA standards, two concentrations were chosen having HA concentrations of 25 nmol/l ($n=8$) and 100 nmol/l ($n=8$). These gave (mean $\pm$S.D.) values from the standard curve of 27.0 ± 1.7 and $98\ 8\pm3.0$ nmol/l, respectively.

Serum and basophil HA

The (mean $\pm$S.D.) HA levels for the plasma and cellular fraction of a single sample blood which was separated, batched and taken through the assay ($n=12$) was 19.2 ± 2.6 and 52.4 ± 3.0 nmol/l, respectively.

A further series of batches from the same sample was spiked in triplicate with HA standards of 10, 20 and 50 nmol/l. The mean recovery of plasma and cellular HA from each spike was 107.7 and 95.8% (spike = 10 nmol/l), 108.7% and 104.7% (spike = 20 nmol/l) and 122.5 and 107.8% (spike = 50 nmol/l). This gave an overall mean HA recovery of 113 and 103% in the plasma and cellular fractions of blood, respectively.

TABLE I

HISTAMINE CONCENTRATIONS

1 = Plasma fraction of blood (no challenge), 2 = plasma fraction (anti-IgE challenge), 3 = cellular fraction of blood (no challenge), 4 = cellular fraction (anti-IgE challenge)

Subject No	Concentration (nmol/l)			
	1	2	3	4
1	31	183	675	531
2	21	66	276	210
3	29	60	398	354
4	24	77	127	68
5	13	83	270	182
6	19	116	423	312
7	59	243	410	219
8	35	153	509	402
9	42	81	191	167
10	24	116	366	271
Mean	29 7	117 8	364 5	271 2
S D	13 2	59 1	158 9	133 4

Table I shows the results of the plasma and cellular HA levels in ten subjects both with and without a whole blood anti-IgE challenge. It can be seen in all cases that HA release was initiated by the anti-IgE, thus there is a concentration rise in the plasma due to HA release from the basophils and a compensatory decrease in the cellular concentration.

In none of the subjects ($n=10$) was there any measurable peak produced at the retention time for HA following incubation of the plasma with DAO just prior to analysis.

This technique for HA analysis in human blood permits measurement to nanomole levels using equipment readily available in most biochemistry laboratories.

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