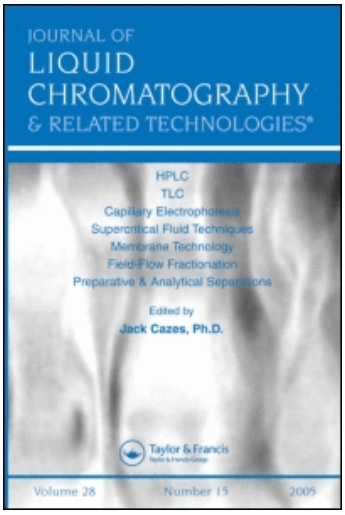

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR HISTAMINE IN PLASMA USING SOLID PHASE EXTRACTION AND FLUORESCAMINE DERIVATIZATION

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ABSTRACT

A reversed phase HPLC method for the determination of histamine in plasma was developed using solid phase extraction with fluorescamine derivatization. Betazole was used as an internal standard. Histamine and the internal standard were extracted from plasma using a cation exchange solid phase extraction cartridge, derivatized pre-column and the derivatized extracts were separated on a C_8 column. The solid phase extraction provided recoveries of 85-90%. The method was found to be linear through the range of 1-10 ng/ml and the histamine derivative was stable at 4°C for 18 hours. The method was found to be accurate to within 7% bias and precise to within 18% CV. Selectivity could not be rigorously assessed because of endogenous histamine, although comparison to RIA results suggested good selectivity. The limit

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of detection was calculated to be 13 pg on column, although the current method was limited by the small proportion of sample injected on column. The method is sufficient for measuring histamine levels expected during allergic response.

INTRODUCTION

The low baseline concentration of histamine in plasma requires highly sensitive analytical methodology for accurate measurement. Bioassay,[1,2] radioimmunoassay,[3,4] , enzyme isotope[5] and gas chromatography[6,7,8] have been utilized for quantitation of histamine. These methods have lacked specificity and/or sensitivity and most have been utilized for measuring histamine in tissue extracts or in urine. High performance liquid chromatography (HPLC) methods have been reported for determination of histamine in urine, animal tissues or body fluids.[9,10,11,12,13] Bettero et. al. reported a procedure for fluorescamine derivatization and detection of histamine in tears without prior sample preparation and suggested that this could be applied to plasma.[14] A previous study has shown, however, that many derivatization techniques lack specificity and that biological samples require a clean-up procedure prior to derivatization/HPLC analysis.[7] A method employing post column derivatization with o-phthaldehyde for analysis of histamine in plasma and brain microdialysates has been reported.[15,16] This work was based on previous work in which o-phthaldehyde was used as a precolumn derivatization reagent for analysis of histamine in plasma, urine and rat brain.[17,18] The current method utilizes ion-exchange sorbant extraction combined with fluorescamine derivatization and HPLC analysis for analysis of histamine in plasma. Advantages of this method over previous systems include obviation of ion pairing agents in the mobile phase, enhanced selectivity and automation capability of the solid phase extraction, obviation of multiple pumps and post column reactors and the use of a commercially available bonded phase column.

MATERIALS

Chemicals and Reagents

Imidazole, and histamine diphosphate (lot # 39208) were purchased from Sigma Chemical Company (Sigma Chemical Co. P.O. Box 14508 St. Louis MO 63178). Acetonitrile (ACN, B&J HPLC grade), nitric acid and hydrochloric acid (HCl), were purchased from Baxter (Baxter 8855 McGraw Rd., Columbia MD 21045) sodium borate was purchased from Baker Chemical Company (Baker Reagent Grade lot # 39208) betazole reference standard was obtained from the United States Pharmacopeia (USP Ordering Department Twinbrook Parkway, Rockville Maryland 20852). CBA (Carboxylic Acid) cartridges were obtained from Analytachem International, Harbor City, CA.

Apparatus

A Gilson pump (model #382) and manometric module (model #802-B) (Thomson Instrument Co., 9001 Braddock Rd., Suite 300, Springfield Va. 221151) was used for mobile phase delivery. A Rheodyne injector (Rheodyne Inc. P.O. Box 996 Cotata CA 94928) was used with a 50 μl injection loop. The analytical column was a Phenomenex Ultramex C-8 column 250 mm x 4.6 mm, 5μm (lot # pp/7116D) with a gaurd column packed with Pelligaurd LC-8 packing (lot # 1106 Supelco, Supelco Park, Bellefonte PA 16823-0048). Fluorescence detection was accomplished with the McPherson FL-750 Spectrofluorescence detector equipped with a high sensitivity attachment and a 150W short arc lamp power supply (model #750-03) to power a mercury xenon source (McPherson 53 Main St. Action Mass 72). The detector was set at λex 366nm and λem 440nm cutoff, gain 45. Chromatographic signals were acquired and processed using an HP integrator (model# 3396A, Hewlett Packard Corp. P.O. Box 1000, Avondale PA 16823-0048). An AASP prep station (Varian Instrument Group, Walnut Creek Division, 2700 Mitchell Drive, Walnut Creek, CA. 94598) was used for processing solid phase extractions cartridges.

METHODS

Extraction and Derivatization Procedure

Spiked Plasma samples were prepared in concentrations of 10, 7.5, 5.0, 2.5, and 1 ng/ml by dilution of an aqueous 100 ng/ml stock solution with normal plasma. Controls were prepared in concentrations of 8, 4, and 1.5 ng/ml in plasma. Solid phase extraction was performed using the CBA cartridges. Each cartridge was conditioned with 1.0 ml of MeOH, followed by 2 ml of 0.01 M phosphate buffer pH = 7. Five hundred microliters of spiked plasma with 100 μl of a 1.0 mg/ml solution of the betazole internal standard added, were diluted with 2 ml of ice cold phosphate buffer and were passed through the cartridge at a rate of 0.2-0.3 ml/min. The cartridges were allowed to dry for 30 seconds and were subsequently rinsed with 1.0 ml of hexane. The samples were then eluted with 1.0 mL of 40/60 0.1 M HCl/MeOH. Samples were dried under nitrogen at 60°C and reconstituted in 100 μl of sodium borate buffer (0.2 M pH = 9). The reconstituted sample was combined with 50 μl of 20 μg/ml fluorescamine, in acetonitrile and vortexed. Samples were refrigerated at 4°C until injection.

Chromatographic Conditions

HPLC conditions for the fluorescamine derivative of histamine were optimized by a series of experiments which evaluated solvent strength, ionic strength and pH. The optimal HPLC conditions were found to be a mobile phase of 20:80 ACN: Imidazole buffer (0.50 M pH = 7). The injection volume was 50 μl and the flow rate was 1.0 ml/min.

RESULTS

Stability of the Fluorescamine Derivative

At room temperature the sample maintained at least 90 % or better of its initial concentration for the first hour, followed by a decline to 20 % of the initial signal within 2 hours. Refrigeration at 4°C was found to increase the stability defined as 90% retention of original concentration to 18 hours. Sodium metabisulfite (100 μl of 0.1M) and benzoic acid (50 μl of 0.1M) were added as preservatives without any significant effect.

Selectivity and Recovery

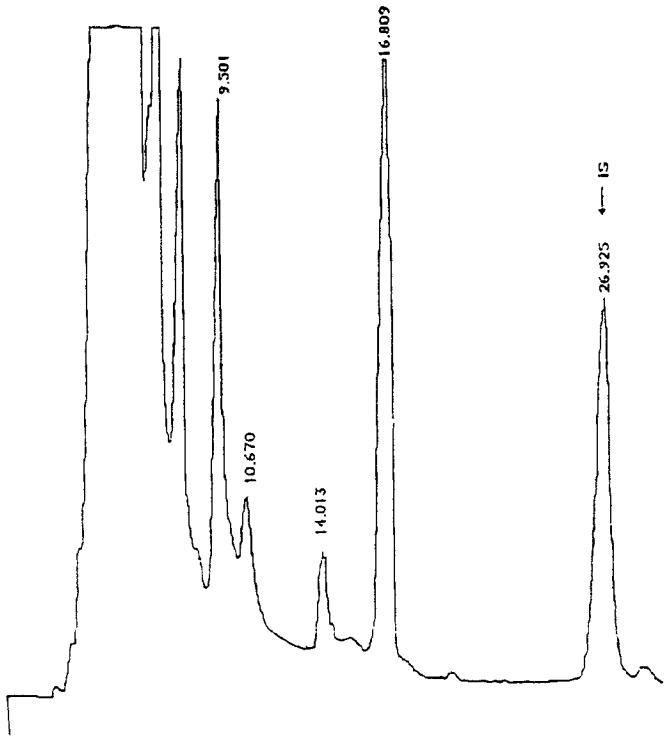
The absolute recovery of the extraction method was determined by comparing the mean area counts (n = 5) for unextracted samples in water to the mean area counts for samples extracted from plasma. Recoveries of 86.9, 89.5% and 87.2 were obtained for concentrations of 10, 7.5 and 5 ng/ml, respectively. Selectivity was evaluated by examining normal plasma extracted, derivatized and injected prior to each run. Low level peaks were observed in some of these samples. Analysis of these plasmas by RIA for histamine demonstrated reasonable correlation between the baseline peaks and histamine concentration. This suggested that these peaks were due to endogenous histamine. Chromatograms of normal plasma and plasma spiked with 4.0 μg/ml of histamine are shown in Figures 1 and 2, respectively.

Linearity and Detectability

A plot of concentration vs. response produced a straight line fitting the form $y = mx + b$, where the slope of the line (m) is the analytical sensitivity. All data were subjected to weighted linear regression with weighting factor of 1/concentration. The mean slope of three weighted calibration curves was 0.1838. A plot of mean peak height ratios (n = 3) versus spiked concentration produced a correlation coefficient of 0.989 and a log-log fit of this data produced a slope of 0.9246. This data indicates acceptable conformance to the linear model. Linearity was tested over the calibration range of 1.0 - 10 ng/ml which corresponds to the concentration range observed in patients exhibiting an allergic response. The limit of detection for the method was determined to be 13 pg on column and was calculated based on 3 times the standard deviation of the noise. The limit of quantitation based on k = 10 is 0.15 ng/ml in a plasma sample or 50 pg on column. Because of the variability at the low end of concentration, however (CV = 11.97 %, error = 17.09% at 1.5 ng/ml), the limit of quantitation was established at 1.0 ng/ml.

Accuracy and Precision

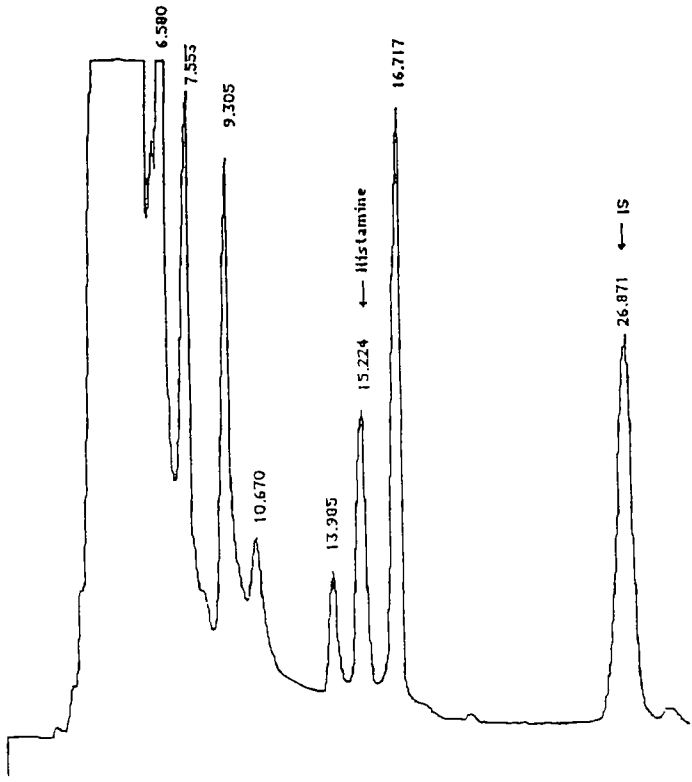
Accuracy was assessed by calculation of the percent bias of mean control results (n = 5) from their respective spiked concentrations. Controls were prepared in plasma



1. Chromatogram of normal plasma subjected to the procedure described.

Table 1. Accuracy and Precision

Concentration (ng/ml)	Concentration (ng/ml)	Percent CV		Percent Bias
		Within Run (n=9)	Between Run (n=5)	
8	8.3	16.6	4.0	4.0
4	4.3	17.7	4.3	7.0
1.5	1.6	12.0	16.4	5.6



2. Chromatogram of plasma spiked with 4.0 ng/ml of histamine and subjected to the procedure described.

samples that were shown to have no detectable levels of histamine and assayed independent of the standard curve on a between run basis. The method demonstrated acceptable accuracy with bias measured at all concentrations less than 20% (Table 1). Precision was assessed on both a within run (n = 9) and between run basis (n = 5). All coeffieients of variation were less than 18% showing acceptable precision. Recovery plots of predicted versus measured concentrations demonstrated no significant nonzero intercept, showing that the procedure of spiking normal plasma with undetectable levels of histamine was acceptable.

DISCUSSION

The described method has been shown to be accurate and precise to within acceptable limits for quantitation of histamine through the range of 1.0 - 10.0

ng/ml in plasma. This range is sufficient for determination of histamine levels expected from allergic responses but not sufficient for measurement of baseline levels of histamine. The method is not limited by detectability of the histamine derivative but by precision of the overall process below 1.0 ng/ml. If technical problems can be overcome, this approach may provide for analysis of histamine in plasma at the subnomogram per milliliter concentration levels observed for endogenous baseline histamine.

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