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REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR DETERMINATION OF HISTAMINE AND ITS METABOLITES IN RAT URINE

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SUMMARY

A reversed-phase ion-pair high-performance liquid chromatographic procedure for quantitative determination of histamine and its metabolites in rat urine is described. This method allows simultaneous analysis of five major histamine metabolites. Good separations were obtained by using 1-pentanesulfonic acid as the ion-pair reagent. A gradient elution program was used; the total elution time was less than 22 min. Linear standard curves with high correlation coefficients were obtained. This procedure has the advantage of requiring little sample preparation time and handling, and therefore maximizes the recovery of metabolites.

INTRODUCTION

The principal pathways of histamine metabolism are well known. Histamine is primarily metabolized by two enzymes, diamine oxidase (DAO, histaminase) and histamine-N-methyltransferase (HMT), and their products are exreted in urine. The product of HMT, N^{τ}-methylhistamine (N^{τ}-MH), is mostly converted by monoamine oxidase (MAO) to N^{τ}-methylimidazoleacetic acid (N^{τ}-MIAA). The product of DAO, imidazoleacetic acid (IAA) can be conjugated to form imidazoleacetic acid riboside (IAA-riboside). At normal physiological levels, very little histamine is excreted unchanged. Trace amounts of N^{α}-methylhistamine (N^{α}-MH) have been found in human urine[1]. It is not known whether mammalian enzymes or bacteria are responsible for this product[2]. Histamine can also be converted to N-acetylhistamine (N-AH) by intestinal bacteria[3].

Many metabolism studies were done with parenteral[4–6] and oral[7–9] administration of histamine. In humans, large doses of histamine can be given orally without eliciting any adverse reactions[3,10]. The lack of toxicity is due to intestinal metabolism of histamine; HMT and DAO activities are quite high in the small intestinal mucosa of many species[11–13], and it is thought that the majority of an oral dose of histamine is metabolized as it traverses the intestinal mucosa or as it circulates through the liver[3]. A portion of orally administered histamine is metabolized by intestinal bac-

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teria to N-acetylhistamine[2,3]. Metabolism of orally ingested histamine is important because substantial amounts can be found in common foods, *i.e.*, fish, cheese and vegetables[14,15].

Many investigators have reported methods for analysis of one or two of the histamine metabolites in urine[16–22]. These methods give only a partial picture of the dynamics of histamine metabolism. Schwartzman and Halliwell[23] reported a thin-layer chromatographic method for quantitative analysis of histamine and three metabolites in urine of man and dog.

The importance of each metabolite may vary under different physiologic conditions[24], diets[14] and disease states[25,26]. Histamine is also a causative agent in some food poisoning cases[27–29]. In these food poisoning situations, evidence indicates that the toxicity of orally ingested histamine is potentiated by the presence of substances that inhibit the intestinal metabolism of histamine[30,31]. To obtain further evidence regarding the mechanism of oral histamine toxicity, the metabolism of large doses of histamine must be investigated by monitoring urinary metabolite excretion patterns. For that purpose, a high-performance liquid chromatographic (HPLC) procedure for simultaneous analysis of histamine and its metabolites (IAA, N^{τ}-MIAA, N-AH, N^{α}-MH and N^{τ}-MH) was developed.

EXPERIMENTAL

Apparatus

A Beckman Instrument (Berkeley, CA, U.S.A.) HPLC system was used for the analytical separations. This consisted of two Model 100A solvent metering systems, a Model 420 microprocessor, a Model 500 automatic sampler, and a Model 210 sample injection valve. The detector was a Micromeritics (Norcross, GA, U.S.A.) Model 788 dual variable wavelength detector. A Shimadzu (Kyoto, Japan) Model C-RIA data processor was used for recording and data analysis. Two HPLC columns were used. An Ultrasphere-ODS column (25 cm × 4.6 mm I.D.) with a particle size of 5 μm (Altex Scientific, Berkeley, CA, U.S.A.) was used for analytical separation, and an Ultrasphere-ODS column (25 cm × 10 mm I.D.) with a particle size of 5 μm (Altex Scientific) was used for purification of the aqueous part of mobile phase.

Materials and reagents

Histamine dihydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.); N^{τ}-methylhistamine dihydrochloride, N^{α}-methylhistamine dihydrochloride, and N^{π}-methylhistamine dichydrochloride[32] were from Calbiochem (LaJolla, CA, U.S.A.), N-acetylhistamine was from Pfaltz and Bauer (Stamford, CT, U.S.A.); [^{14}C]histamine dihydrochloride (56 mCi/mmole) was from Amersham (Amersham, U.K.); 1-pentanesulfonic acid, sodium salt was from Eastman-Kodak (Rochester, NY, U.S.A.).

Mobile phase. Aqueous solvent: a 1-l solution which contained 30 g of 1-pentanesulfonic acid, 10 ml of 85% (w/w) phosphoric acid and 6.9 g of sodium dihydrogen phosphate was prepared. The pH of this solution was adjuted to 2.8 with sodium hydroxide (1 *M*). This solution was purified by passing through an Ultrasphere-ODS column (25 cm × 10 mm I.D.) at a flow-rate of 1 ml/min. The purified solution was diluted ten times with deionized distilled water before use. Organic

solvent: acetonitrile from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) was used.

Urine sample preparation

Female Sprague-Dawley rats (200 g) were dosed orally with 0.5 mmole/kg of [^{14}C]histamine dihydrochloride (10 μCi/mmole), and urine samples were collected in bottles which contained 1 ml of 2 *M* hydrochloric acid. Urine samples were obtained every 4 h over a 12-h period. They were stored frozen at -15°C until analysis.

To 0.25 ml of urine, 1.70 ml of the aqueous part of the mobile phase and 50 μl of N^{π}-methylhistamine (10 μmole/ml) were added. N^{π}-methylhistamine was used as an internal standard. This mixture was filtered and 10 μl aliquots were used for HPLC analysis. A standard curve for each metabolite was prepared from various concentrations (50–250 nmole/ml) of the metabolites in water.

In order to analyze conjugated IAA-riboside, an acid hydrolysis procedure to convert the conjugate to free acid was performed. To 0.5 ml of urine, 0.5 ml of 2 *M* hydrochloric acid was added. This mixture was heated in a sealed tube for 5 h at 150°C[4,7]. At the end of this hydrolysis period, 0.5 ml of the hydrolyzed sample was taken, and 1.45 ml of the aqueous part of the mobile phase and 50 μl of N^{π}-methylhistamine (10 μmole/ml) were added. This mixture was filtered and 10 μl aliquots were used for HPLC analysis.

Chromatographic separation

A gradient elution program was used. Solvent A was prepared with 3% acetonitrile and 97% aqueous solvent (described above). Solvent B was prepared with 8% acetonitrile and 92% aqueous solvent. After sample injection, the elution was first done isocratically with solvent A from 0 to 7 min. A linear gradient was then performed to go to 100% solvent B over a period of 10 min. An isocratic elution of solvent B was continued until complete elution was obtained. The flow-rate was kept constant at 1 ml/min. Metabolites were detected at 212 nm.

RESULTS AND DISCUSSION

A chromatogram of the mixture of histamine and its metabolites is shown in Fig. 1. The total elution time was less than 22 min. The pH of the mobile phase was 2.8, and at this pH, ion-pairing can occur between pentanesulfonic acid and the positively charged groups on the solutes. The acidic metabolites (IAA and N^{τ}-MIAA) were eluted first at 6.04 min and 7.79 min, respectively. They were followed by N-AH (14.19 min), histamine (17.69 min), N^{π}-MH (18.49 min), N^{α}-MH (20.29 min), and N^{τ}-MH (21.49 min). The compounds were well separated with this elution program. N^{π}-methylhistamine was used as an internal standard in this procedure because this compound has not been found in biological samples[33].

Various sulfonates with differing alkyl chain-lengths were investigated; the best resolution was obtained with pentanesulfonic acid. In general, increasing the size of the counterion will produce an increase in retention time. In this case, the compounds to be separated have only slight molecular differences, therefore an ion-pair reagent with small molecular size was selected in order to obtain a good separation. Retention of the solutes on the column also varies with the amount of organic solvent in the

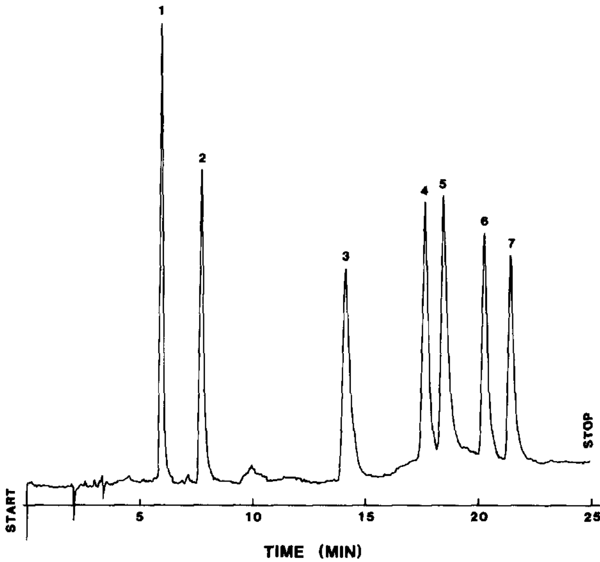

Fig. 1. Chromatogram of a standard histamine metabolite solution on Ultrasphere-ODS column (Altex): 2 nmoles of each; 1 = imidazoleacetic acid, 2 = N^{τ}-methylimidazoleacetic acid, 3 = N-acetylhistamine, 4 = histamine, 5 = N^{π}-methylhistamine, 6 = N^{α}-methylhistamine, 7 = N^{τ}-methylhistamine.

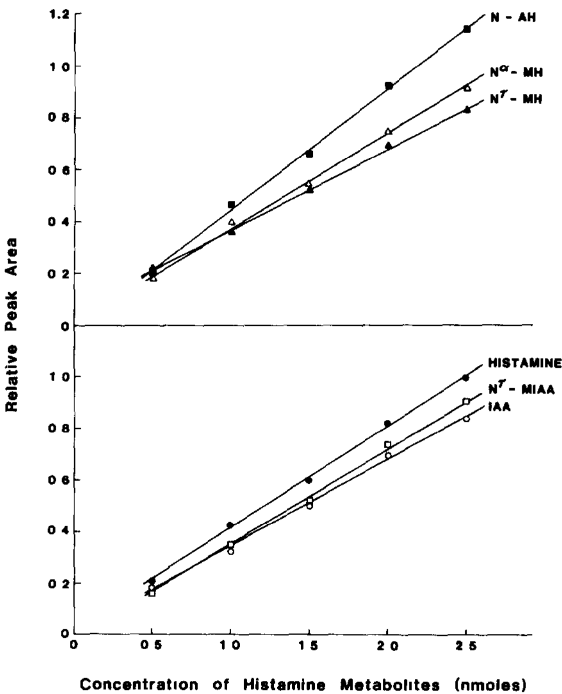

Fig. 2. Standard curve of histamine metabolites produced by using relative peak areas (compared to internal standard).

TABLE I

CORRELATION COEFFICIENTS FOR HISTAMINE AND ITS METABOLITE STANDARDS

	*Correlation coefficients**	
	1	*2*
Imidazoleacetic acid	0.999	0.998
N^{τ}-Methylimidazoleacetic acid	0.998	0.995
N-Acetylhistamine	0.998	0.998
Histamine	0.999	0.998
N^{α}-Methylhistamine	0.999	0.998
N^{τ}-Methylhistamine	0.999	0.998

* 1: Correlation coefficients obtained by using absolute peak areas for calculation; 2: correlation coefficients obtained by using relative peak areas for calculation.

mobile phase. An increase in retention time can lead to broadening of peaks. In this separation, a gradient of 3% acetonitrile to 8% acetonitrile was used to obtain good resolution and little peak broadening.

Standard curves for each metabolite were prepared (Fig. 2). These compounds can be quantitated by using either absolute or relative (compared to internal standard) peak areas. The data for the standard curves were submitted to linear regression analysis; correlation coefficients are shown in Table I. A linear response was obtained for each compound for concentrations of 0.5–2.5 nmoles. The limit of detection was 50 pmoles for the first two peaks; due to a decrease in sharpness of the peaks with time, the limit of detection was 100 pmoles for the last peak.

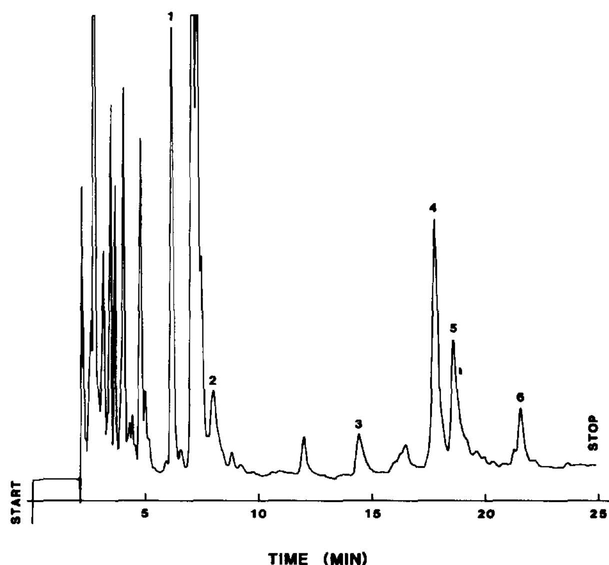

Fig. 3. Chromatogram of a urinary sample on Ultrasphere-ODS column (Altex): 1 = imidazoleacetic acid, 2 = N^{τ}-methylimidazoleacetic acid, 3 = N-acetylhistamine, 4 = histamine, 5 = N^{α}-methylhistamine, 6 = N^{τ}-methylhistamine.

A typical chromatogram of a urine sample is shown in Fig. 3. This sample was collected 4 h after oral dosing of the animal. Compounds excreted in urine are usually polar, and they were mostly eluted off the column in the first 5 min. IAA and N^{τ}-MIAA were separated very well from these interfering polar compounds. The identification of the peaks in urine samples as histamine metabolites was confirmed by measuring amounts of radioactivity in them. Histamine was radiolabelled at the 2 position of the imidazole ring, therefore, metabolism did not lead to loss of radioactivity.

Hydrolysis of urine samples at 150°C for 5 h was used to hydrolyze the IAA-riboside conjugate. However, this hydrolysis procedure also breaks the conjugated bond of N-acetylhistamine. Other metabolites were stable to this procedure, therefore, hydrolysis resulted in an increased amount of IAA and histamine.

This reversed-phase ion-pair HPLC procedure has the advantage of requiring little sample preparation time and handling, and therefore maximizes the recovery. For working with biological samples that require some clean-up steps, extraction procedures for both acidic and basic metabolites are available[23]. Various chemical derivatization procedures were investigated during the development of a suitable method. These procedures were not very useful due to the differences in the chemical properties of various metabolites. Many reported procedures for the analysis of histamine metabolites involve derivatization of the compounds[16–22], however, these methods are not good for simultaneous detection of both acidic and basic metabolites.

It is not the purpose of this paper to interpret the results of the urinary analysis of histamine metabolites. However, the availability of this HPLC method will allow investigators to study the metabolism of exogenous histamine in various physiologic and disease states.

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