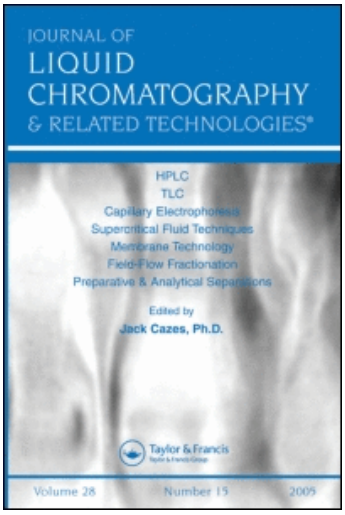

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Performance Liquid Chromatog-Raphy for the Separation of Histamine, Its Precursor, and Metabolites: Application to Biological Samples

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To cite this Article Hermann, K. , Frank, G. and Ring, J.(1995) 'High Performance Liquid Chromatog-Raphy for the Separation of Histamine, Its Precursor, and Metabolites: Application to Biological Samples', Journal of Liquid Chromatography & Related Technologies, 18: 1, 189 — 204
To link to this Article: DOI: 10.1080/10826079508009231
URL: http://dx.doi.org/10.1080/10826079508009231

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(1), 189-204 (1995)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF HISTAMINE, ITS PRECURSOR, AND METABOLITES: APPLICATION TO BIOLOGICAL SAMPLES

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ABSTRACT

Histamine was separated from its precursor L-histidine and its metabolites 1-methylhistamine and methylimidazole acetic acid on a TSK SP-5 PW cation exchange column and gradient elution. A baseline separation of histamine, 1-methylhistamine and methylimidazole acetic acid was also achieved under isocratic elution conditions on a reversed phase C_8 column with a mobile phase of 15 % methanol and 85 % of an aqueous solution of 0.05 M NaH_2PO_4 pH 3.1 which contained 0.5 mM EDTA-Na_4 and 0.005 M octan-1-sulfonic acid sodium salt as an ion paring reagent. The most sensitive UV detection of histamine and 1-methylhistamine with the highest detector signal was obtained at a wavelength of 210 nm. In the absence of the ion pairing reagent and the organic modifier methanol, histamine and 1-methylhistamine were not retained on a reversed phase C_{18}, C_8 or C_4 column. Octadecasilyl-silica cartridges were used to purify histamine from other constituents present in human urine and a commercially available heparin formulation. The analytical recovery of ^{3}H-labeled histamine after the purification on octadecasilyl-silica cartridges was 95.16 ± 0.92 % (mean ± SEM, n=22). The concentration of the

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histamine-like material in urine samples from healthy volunteers was 46.28 ± 15.42 µg/24h (mean ± SEM; n=7). In the heparin formulation the histamine concentrations were 81.93 ng/ml before and 279.24 ng/ml after the purification on octadecasilyl-silca cartridges. The histamine-immunoreactive material in urine samples could be characterized on a TSK SP-5 PW cation exchange column as 19.69 ● 7.86 % histamine and 46.74 ± 12.89 % 1-methylhistamine (mean ± SEM, n=7). Rechromatography of the 1-methylhistamine peak from the ion exchange column on the reversed phase C_8 column disclosed a substance of unknown nature with a different retention time than 1-methylhistamine. Histamine purified from heparin eluted from the ion exchange column as a single peak with the same retention time as the histamine standard.

INTRODUCTION

The biogenic amine histamine (H) is predominantly found in mast cells and basophil leukocytes. Besides its numerous biological effects on blood vessels, the heart, the bronchial tract and the stomach, H is an important mediator of immediate type hypersensitivity and a marker for allergic reactions (1-4). The determination of histamine in blood, serum, urine or cell cultures as a diagnostic parameter is therefore not only of scientific interest but also of significance for the routine allergy practice. Numerous analytical procedures such as radioenzymatic (5), photometric (6) or gas chromatographic-mass spectrometric assays (7) have been developed for the qualitative and quantitative measurement of H in plasma, urine and cerebrospinal fluid. These approaches are either tedious or require expensive equipment and specially trained personal. Likewise, radioimmunoassays (RIA) have been introduced to determine H in urine, whole blood and cell supernatants after in-vitro H-release (8-10). Although the radioimmunological determination possess high sensitivity and specificity, crossreactions with H-metabolites, the H-precursor or other components may occur. Therefore, other procedures such as HPLC are needed for the accurate evaluation of the radioimmunological data.

In this study we report the use of HPLC to separate H from its precursor L-histidine and its metabolites 1-methylhistamine (MH) and methylimidazole acetic acid (MIAA) using ion exchange and reversed phase paired ion chromatography. The influence of C_{18}, C_8 and C_4 reversed phase columns and penta-1- and octan-1-sulfonic acid as ion pairing reagents upon the resolution of H from MH and the effect of different wavelengths on the detector signal was investigated. Octadecasilyl-silica gel (ODS) cartridges were used to purify H in urine and heparin. HPLC in combination with a specific radioimmunoassay (RIA) for H was applied to measure and characterize H in partially purified human urine samples and a commercially available heparin formulation.

EXPERIMENTAL

High performance liquid chromatography (HPLC)

An ERC HPLC equipment model ABEO 88 (ERC, Alteglofsheim, FRG) was used. All separations were performed at room temperature. The mobile phases were passed through a RC 55 membrane filter (Schleicher and Schüll, Dassel, FRG) prior to use.

Ion exchange chromatography

Ion exchange chromatography was carried out on a cation exchange TSK SP-5 PW column (75 x 7.5 mm, TasoHass, Stuttgart, FRG) with gradient elution. The mobile phases were solution A: HPLC water and solution B: 0.5 M NaH_2PO_4 solution in HPLC water, adjusted to pH 5.0 with concentrated phosphoric acid. Initially, 10 % of solution B corresponding to 0.05 M was delivered for 9 min followed by an exponential increase in solution B to 60 % corresponding to 0.325 M for 16 min. At 25 min the column was flushed isocratically with 60 % of solution B. The column was equilibrated under the initial conditions for 20 min. The separations were carried out at a flow rate of 0.6 ml/min. The column was calibrated with H, MH, L-histidine and MIAA using UV detection at 210 nm and a sensitivity of 0.1 absorbance units full scale (AUFS).

Paired ion chromatography

Paired ion chromatography was conducted on a reversed phase C_{18}, C_8 or C_4 column (Nucleosil C_{18}, C_8, C_4, 300 Å , 7 µm, 250 x 4 mm, Macherey and Nagel, Düren, FRG). The column was developed isocratically at a flow rate of 1.0 ml/min. The mobile phase was 15 % methanol and 85 % of an aqueous solution of 0.05 M NaH_2PO_4 pH 3.1 which contained 0.5 mM EDTA-Na_4 and 0.005 M pentan-1-sulfonic acid or octan-1-sulfonic acid sodium salt. The column was calibrated with MIAA, L-histidin, H and MH using UV detection at 205, 210, 215, 225 and 254 nm and a sensitivity of 0.1 AUFS.

The standard substances were dissolved in 0.01 N HCl to yield a concentration of 1 mg/ml. For all separations the injection volume of each standard was 1-2 µl corresponding to 1-2 µg.

Radioimmunoassay (RIA)

RIA for H was carried out using a test kit delivered by IBL (Immuno Biological Laboratories, Hamburg, FRG). Briefly, 0.1 ml samples or standards were incubated for 30 min at room temperature with an acylation reagent to form acyl-H. Then 0.05 ml ^{125}I-labeled H and 0.05 ml anti-H-antiserum were added and the mixture was incubated over night in the refrigerator at 6-8°C. The next day 1.0 ml of second antibody solution was added. After incubation at room temperature for 15 min the precipitate was spun for 15 min at 2 000 x g and 20°C. The supernatant was aspirated and the pellet was counted in a gamma counter. The crossreactivity of the antibody with acyl-H was *100 % and with MH*

0.01 %. L-histidine, imidazole acetic acid, MIAA, serotonin and 5-hydroxyindole acetic acid did not crossreact with the antibody.

Collection of urine samples

Urine was collected in individual fractions over 24 h from 7 healthy volunteers, 4 females and 3 males, mean age 30 ± 6 years. Each time the individual had to urinate the urine was collected in a 1 L plastic flask. The time and the volume was recorded. From the total urine volume 40 ml were poured from the 1 L flask into a 50 ml plastic tube which contained 0.32 ml of 25 % HCl. The samples were thoroughly mixed and stored at -80°C.

Purification of histamine from urine and heparin

H was purified from urine using octadecasilyl-silica cartridges (ODS, Sep Pak C_{18}, Millipore Waters, Eschborn, FRG). The cartridges were first rinsed with 3 ml methanol followed by 3 ml 0.01 N HCl.

Urine samples were thawed at 4°C and centrifuged for 10 min at 1 000 x g and 4°C. The pellets were discarded and the clear supernatants of the urine samples were further processed. The distinct urine samples collected over 24 h from the 7 volunteers were pooled to obtain a 24 h urine pool of the 7 volunteers. From the 7 pooled urine samples, 2 ml of the clear supernatants were applied to the primed cartridges and the flow through of the cartridges were collected (fraction # 1). The cartridges were washed 3 times with 1 ml 0.01 N HCl and the eluates of the 3 washing steps were collected in single fractions (fraction # 2 to 4). The samples (fractions # 1 to 4) were dried in a vacuum centrifuge. The dried residues, fractions 1 and 2 were dissolved in 1 ml HPLC water each. Fractions 1 and 2 were pooled and spun at 20°C, 10 000 x g and 2 min.

H was also purified from a heparin formulation using the ODS cartridges. The heparin sample (1.0 ml) was applied to the primed cartridge and 1 ml of the flow through was collected. The cartridge was then washed once with 1 ml 0.01 N HCl and the eluting fraction was collected. The two eluting fractions were combined and dried in a vacuum centrifuge. The dried residues were dissolved in 1.0 ml HPLC water and spun at 20°C, 10 000 x g for 2 min. The heparin formulation contained 25 000 I.E. heparin/5ml.

H was measured radioimmunologically in the reconstituted urine or heparin samples.

Recovery of ^{3}H-Histamine in urine samples

The analytical recovery of the ODS cartridges for H in urine samples was determined by the addition of ^{3}H-labeled H (^{3}H-H) to the urine samples before purification. Fractions 1 to 4 were collected and aliquots of 0.1 ml of the radioactive ^{3}H-H were mixed with 5.0 ml scintillation fluid and counted for 1 min in a liquid scintillation counter.

HPLC characterization of H-like material

For the characterization of H-like material, 1 ml urine or 1 ml of heparin which were purified on ODS cartridges were injected and separated on the cation exchange TSK SP-5 PW column. Fractions of 0.6 ml were collected. Aliquots of 0.1 ml of each fraction were withdrawn for the radioimmunological measurement of H. Rechromatography was performed on a reversed phase C_8 column using paired ion chromatography as mentioned above.

Chemicals

All chemicals used were of analytical grade. L-Histidine, H and MIAA were purchased from Sigma Chemie, Deisenhofen, FRG. MH was delivered by Calbiochem, Frankfurt, FRG. All other chemicals including HPLC solvents were delivered by Merck AG, Darmstadt, FRG.

RESULTS

HPLC separation of histamine on an ion exchange column

On the cation exchange TSK SP-5 PW column and gradient elution, a baseline separation was achieved for H, its metabolites MH and MIAA and its precursor L-histidine (Fig. 1A). The retention time for H was 32.54 ± 0.04 min, for MH 30.57 ± 0.02 min, for L-histidine 8.11 ± 0.12 min and for MIAA 4.05 ± 0.01 min (mean ± SEM; n=3).

HPLC separation of histamine on different reversed phase columns

Using an aqueous mobile phase of 0.05 M NaH_2PO_4 which contained 0.5 mM EDTA-Na_4 (in the absence of an organic modifier and the ion pairing reagent pentan-1-sulfonic acid or octan-1-sulfonic acid), H and MH were not retained on a reversed phase C_{18}, C_8 or C_4 column and eluted in the void volume of the columns. The addition of pentan-1-sulfonic acid had no influence of the retention times for H and MH on C_{18} column. H and MH still eluted in the void volume of the column with retention times of 1.78 and 1.96 min, respectively. Although, the retention times of H and MH increased on a C_8 or C_4 column, H could not be separated from MH (Tab. 1). Replacement of the pentan-1-sulfonic acid by the octan-1-sulfonic acid significantly increased the retention time of H (62.79 min) and MH (82.48 min) on the C_8 column (Tab. 1). Substitution of the C_8 column with a C_4 column induced a notable reduction of the retention times of H and MH from 62.70 to 18.16 min and 82.48 to 21.76 min (Tab. 1).

The retention times for H and MH could be significantly reduced from 62.70 to 11.14 and 82.48 to 12.60 min on a reversed phase C_8 column using a mobile phase which was composed of a mixture of 15 % methanol as an organic modifier and 85 % of an aqueous

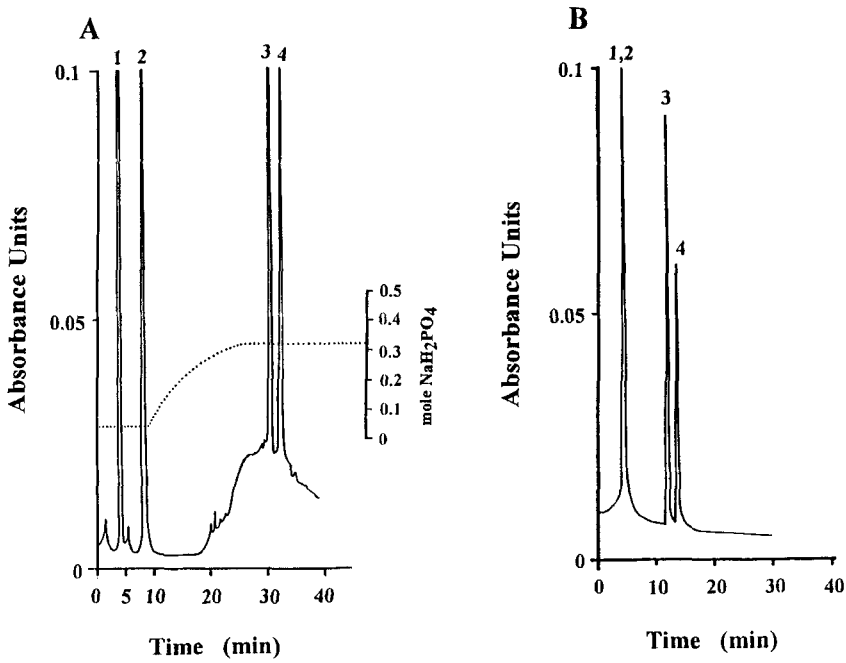

Figure 1

HPLC separation of H, L-histidine, MH and MIAA on an ion exchange TSK SP-5 PW column (A) and a reversed phase C_8 column (B) and UV detection at a wavelength at 210 nm. A: 1=MIAA, 2=L-histidine, 3=MH, 4=H. B: 1,2=MIAA and L-histidine, 3=H and 4=MH. For more detailed information see text.

solution of 0.05 M NaH_2PO_4 which contained 0.5 mM EDTA-Na_4 and 0.005 M octan-1-sulfonic acid (Tab. 1). With the use of a reversed phase C_8 column in combination with this mobile phase H could be separated from its precursor L-histidine and its metabolites MH and MIAA. However, MIAA and L-histidine could not be separated from each other (fig. 1B). The retention time for H was 11.14 ± 0.08 min, for MH 12.60 ± 0.09 min, for L-histidine 4.10 ± 0.02 min and for MIAA 4.34 ± 0.03 min (mean ± SEM; n=3). For this HPLC setup the detection limit for H was 20 ng at a detector sensitivity of 0.01 AUFS.

The influence of different wavelengths on the detector signal for H and MH for this HPLC setup were investigated. The highest detector signals for H and MH were obtained at a wavelength setting of 210 or 215 nm. The detector signals were significantly lower at 205 or 225 nm. At a wavelength of 254 nm no detector signal was obtained for H or MH (Fig. 2).

Table 1

Influence of pentan-1-sulfonic acid and octan-1-sulfonic acid on the retention times of H and MH using C_{18}, C_8 or C_4 columns in the absence of the organic modifier methanol. For more detailed information see text.

	Retention Times (min)			
Ion Pairing reagent	Histamine	1-Methylhistamine	Column	Experiments
Pentan-1-sulfonic acid	1.78	1.96	C18	n = 1
	3.50 +/- 0.01	3.95 +/- 0.04	C8	n = 3
	2.83 +/- 0.01	2.94 +/- 0.01	C4	n = 3
Octan-1-sulfonic acid	62.7	82.48	C8	n = 1
	18.16 +/- 0.45	21.76 +/- 0.19	C4	n = 3

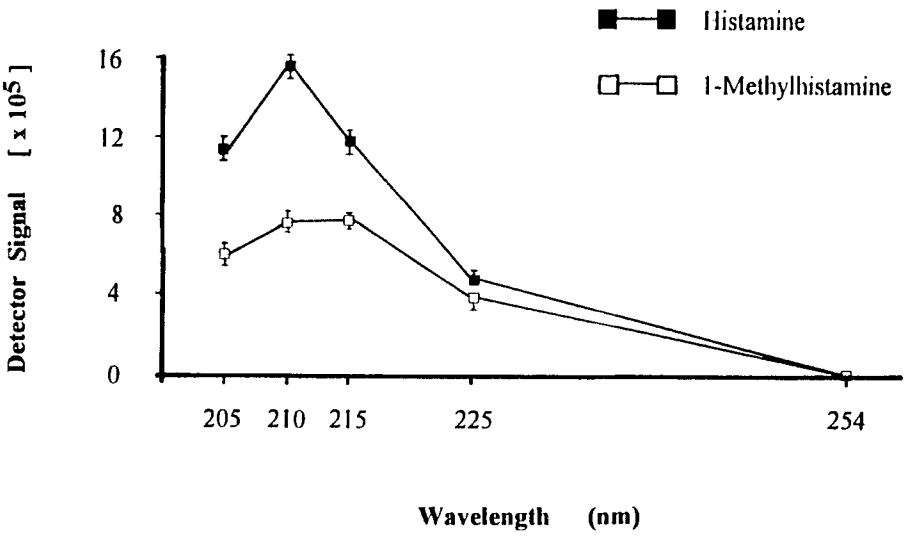

Figure 2

Relationship between the wavelength and the detector signals on the reversed phase C8 column. The highest detector signal for H and MH was noted at a wavelength of 210 nm.

Recovery of ^{3}H-H

The overall analytical recovery of ^{3}H-H in urine samples on ODS cartridges was 95.16 ± 0.92 % (n=22). In the fraction which was collected at the same time the sample was applied to the cartridge 63.10 ± 1.23 % of the radiolabeled H was recovered (fraction # 1). In the 1st wash 27.32 ± 1.26, the 2nd wash 3.79 ± 0.49 and the 3rd wash 0.99 ± 0.13 % could be recovered (Fig. 3 top panel). HPLC separation of ^{3}H-H in a pool of the application and the 1st wash fractions showed a single peak of intact ^{3}H-H indicating that H has not been degraded in the acidified urine (Fig. 3 bottom panel).

Histamine in human urine samples

H-immunoreactivity was present in human urine samples purified on ODS cartridges. The concentration of H-like material excreted in 24 h was 29.57 ± 10.16 µg (mean ± SEM; n=7).

HPLC characterization of H-like material on the cation exchange column showed the presence of H, a compound with the same retention time as MH and a variety of substances which crossreacted with the anti-H-antibody (Fig. 4).

With regard to the capability of the HPLC system separating H from MH, the urine

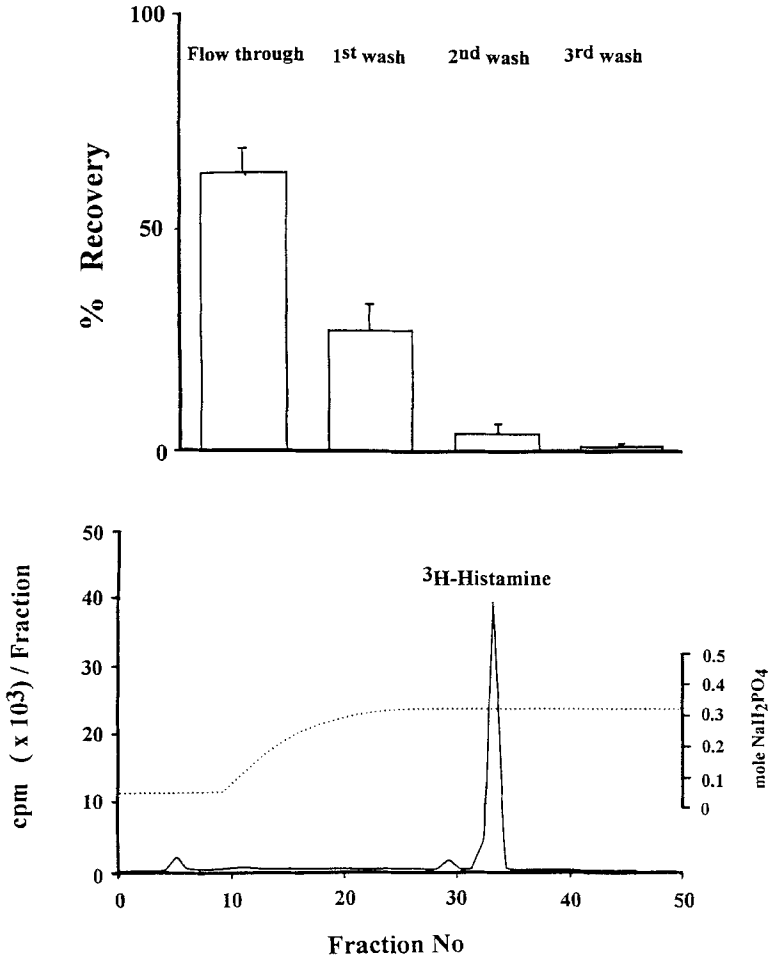

Figure 3

Top panel: Analytical recovery of ^{3}H-H in human urine samples on ODS cartridges. The overall recovery of ^{3}H-H on ODS cartridges was 95.16 ± 0.92 (n=22). Bottom panel: HPLC characterization of ^{3}H-H which was added to human urine samples prior to ODS purification on a TSK SP-5 PW cation exchange column. The chromatogram showed a single peak of intact H indicating that H was not degraded in the acidified urine samples. For more detailed information see text.

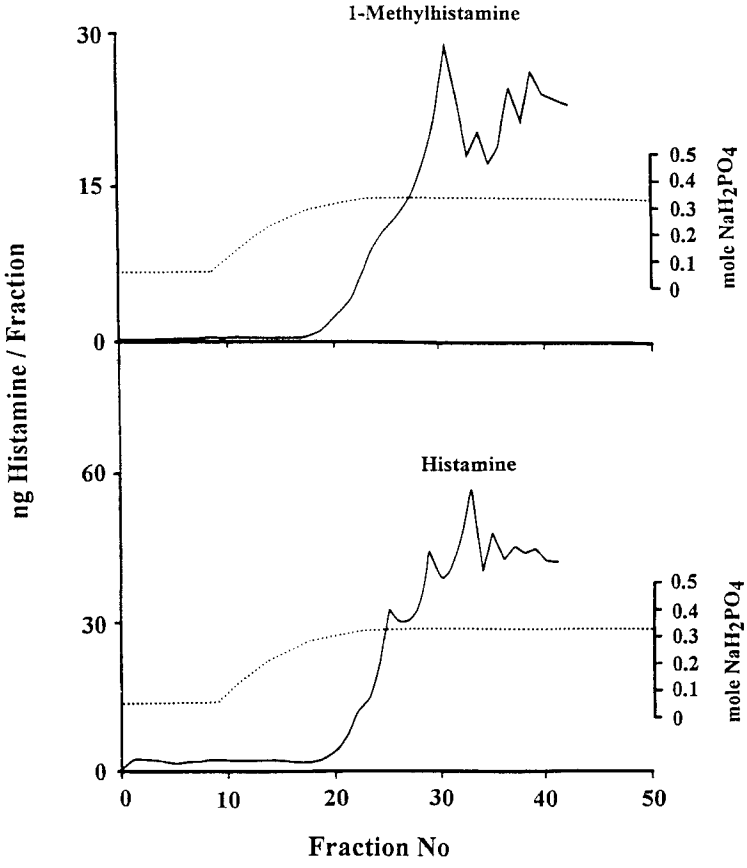

Figure 4

HPLC characterization of H-immunoreactive material in human urine samples on a TSK SP-5 PW cation exchange column. Two substances with the same retention times as H and MH were found in the HPLC fractions. For further information see text.

samples could be divided into 2 distinct types: one with mainly H (Fig. 4 bottom panel) and the other with MH (Fig. 4 top panel) as the prominently excreted product. Only H and no MH was identified in one subject (#4) while MH and no H was detected in another subject (#5). In one subject (subject #2) H was identified with minute amounts of MH. The H metabolite MH was mostly found in 4 of the 7 subjects (#1, 2, 6 and 7). The ratio of H and MH to the total amount of H-like material present in 7 urine samples from healthy volunteers was 19.69 ± 7.86 % for H and 46.74 ± 12.89 % for MH (Tab. 2).

Table 2

Concentration of H-immunorective material in partially purified human urine before and after HPLC separation on a TSK SP-5 PW cation exchange column. Numbers in parenthesis gives the percentage of H and MH in the HPLC fractions in relation to the H-immunorective material before HPLC separation. For further information see text.

Subject	before HPLC	after HPLC	
	H-like	MH	H
# 1	83.69	69.25 (82.75)	1.76 (2.10)
# 2	45.55	35.71 (78.39)	4.30 (9.43)
# 3	10.33	0.26 (2.56)	3.31 (32.05)
# 4	24.18	0	13.93 (57.59)
# 5	9.98	4.57 (45.83)	0
# 6	20.75	9.86 (47.55)	5.88 (28.33)
# 7	12.49	8.76 (70.13)	1.04 (8.35)
	29.57 ± 10.16	18.35 ± 9.65 (46.74 ± 12.89)	4.32 ± 1.77 (19.69 ± 7.86)

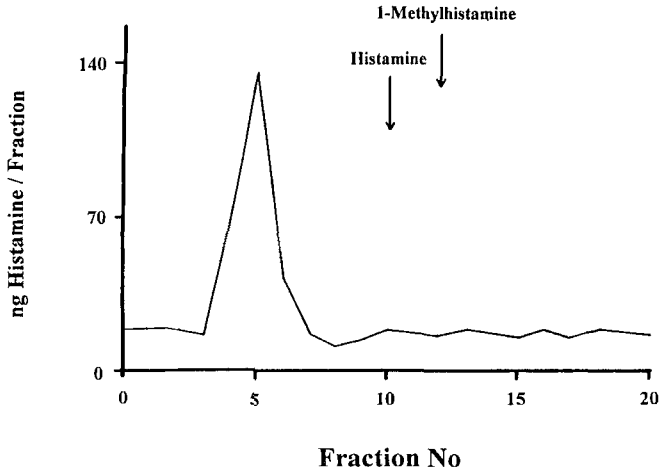

Figure 5

Rechromatograpy of the MH peak from the separation on the TSK SP-5 PW cation exchange column on a reversed phase C_8 column. The compound eluted with a retention time of 5 min which was different from the retention time of MH. Arrows indicate the retention times of H and MH. For further information see text.

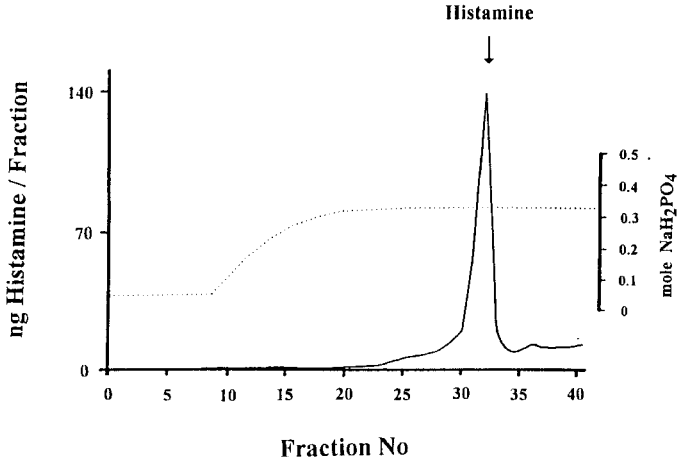

Figure 6
HPLC characterization of H extracted from a commercial heparin preparation using ODS cartridges on a TSK SP-5 PW cation exchange column. A compound with the same retention time as H could be identified in the HPLC fractions. For further information see text.

The substance which eluted from the cation exchange TSK SP-5 PW column with the same retention time as MH was submitted to a rechromatography on the reversed phase C_8 column with octan-1-sulfonic acid as anion pairing reagent. This compound showed a retention time different from MH (Fig. 5).

Histamine in heparin

In a commercially available heparin formulation 81.93 ng/ml H-immunoreactive material was detected. After ODS purification the concentration of the H-immunoreactive material was 279.24 ng/ml. HPLC characterization of the H-immunoreactive material on an ion exchange TSP SP-5 PW column demonstrated the presence of a substance with the same retention time as H (Fig. 6).

DISCUSSION

An ion exchange and reversed phase HPLC procedure for the separation of H from MH was developed and a combination of HPLC and RIA was utilized to characterize H and

MH in human urine samples and a heparin formulation. With an ion exchange TSK SP-5 PW column and gradient elution using a phosphate buffer pH 5.0 as a mobile phase H was separated from its precursor L-histidine and its metabolites MH and MIAA. In addition, H was separated from MH and MIAA on reversed phase C_8 column with isocratic elution in the presence of methanol as an organic modifier and octan-1-sulfonic acid sodium salt as an ion pairing reagent.

The effects of different stationary phases and the composition of the mobile phases upon the retention times of H and MH were studied. The results indicated a close interaction between the polarity of the mobile phases and the polarity of the column matrix which strongly effected the elution behavior of H and MH. The less polar the stationary phase was the less polar the ion pairing reagent had to be in order to retain H and MH. H and MH are very polar substances. They did not interact with the apolar matrix of a reversed phase C_{18}, C_8 or C_4 column in the absence of methanol and an ion paring reagent and eluted without retention in the void volume of the columns. In addition, H and MH were not retained on reversed phase C_{18} column even when the ion pairing reagent pentan-1-sulfonic acid was added to the mobile phase. Switching from a reversed phase C_{18} column to a C_8 or a C_4 column by decreasing the apolar column matrix induced an increase in the retention times for H and MH. However, no separation between H and MH was obtained. The use of octan-1-sulfonic acid which differs from the pentan-1-sulfonic acid only by 3 additional C-atoms had a marked influence on the retention of H and MH on the reversed phase C_8 column. H and MH were retained on the column for 63 and 82 min, respectively. Replacing the C_8 column with a C_4 column by increasing the polarity of the column matrix significantly reduced the retention times of H and MH to 18 and 22 min. The best chromatographic conditions with respect to an optimal retention time for H and MH and the ability for separating H from MH were obtained on a reversed phase C_8 column and a mobile phase which was composed of 15 % methanol in the presence of octan-1-sulfonic acid as an ion pairing reagent.

The detection of H and MH was highly influenced by the wavelength setting of the UV detector. A wavelength below 210 nm or above 225 nm resulted in a decrease in the detector signal. The most sensitive detection with the highest detector signal on the UV detector for H and MH was obtained at a wavelength of 210 nm.

The HPLC procedures were applied to characterize H in human urine samples and a heparin formulation. HPLC separation of native urine or heparin was hampered by the fact that these samples are composed of a variety of different substances which interfered with the radioimmunological identification of H in the HPLC fractions. Therefore, an extraction step to isolate and purify H from other constituents present in these samples was necessary. For this purpose, H was purified on ODS cartridges which have been used

previously for the purification of angiotensin peptides from brain tissue (11). Whereas angiotensin peptides were retained by the ODS cartridges, H was not. H passed through the cartridges leaving the yellow colored material in urine in the cartridges. With this purification step 63 % of ^{3}H-H was recovered in the flow through and another 27 % in the 1st washing of the cartridges. The overall recovery of ^{3}H-H which was added to the urine samples was 95 %. HPLC characterization of the recovered ^{3}H-H showed a single peak in the chromatogram indicating that H was not degraded in the acidified urine samples or during the sample handling. Moreover, the addition of HCl to the urine samples was essential to prevent degradation of H. In contrast, native urine samples with pH values between 5 to 6 displayed considerable degradation of H (data not shown).

H was identified radioimmunologically in the purified urine samples. The H-immunoreactive material could be characterized on the ion exchange column with subsequent radioimmunological detection as H and a substance with the same retention time as MH. However, the identity of MH could not be confirmed upon rechromatography of the MH peak on the reversed phase C_8 column. The nature of this substance is still unknown. Since the ion exchange column allowed a separation of H from MH the ratio of H to MH in human urine was calculated. H constituted about 20 % and MH about 47 % of the total H-immunoreactive material found in the urine samples without HPLC separation. The rest obviously accounted for material of unknown nature which crossreacted with the anti-H antiserum. This confirms findings of our previous study in urine of healthy volunteers where we also identified 50 % of the H-immunoreactive material as MH (12). It has been shown that H and MH were separated on a reversed phase C_{18} column with the addition of sodium dodecyl sulfate to the mobile phase using electrochemical detection (13). The authors could demonstrate a baseline separation and a highly sensitive detection for H and MH. Although, electrochemical detection is very sensitive it is also very susceptible to various interferences. We have tried to use electrochemical detection in our experiments but obtained variable and discouraging results and were unable to reproduce the results by Houdi et al (13).

H-immunoreactivity could be identified radioimmunologically in a commercially available heparin formulation. ODS cartridges were also used to purify H from other constituents. The partially purified H-immunoreactivity was clearly identified as H on the ion exchange TSK SP-5 PW column. Previously, Sjodin and Svensson reported the contamination of heparin with H. Administration of heparin in cats showed vasodepressor activity which could be blocked by a H_1-antagonist indicating the involvement of H (14). According to their study they estimated that 5 000 I.U. of heparin can be contaminated with as much as 100 ng H. Likewise, Adt et al showed that injection of H induced cardiopulmonary reactions and an increase in plasma H (15).

In summary: ion exchange and reversed phase HPLC in combination with a specific H RIA is fast, reproducible, specific and sensitive method for the determination of H in biological samples. This approach was successfully applied for the qualitative and quantitative determination of H in human urine and a commercially accessible heparin formulation.

Acknowledgments
The authors wish to thank Mrs. J. Grosch and P. Decker-Hermann for their excellent technical assistance. H-RIA-Kits were generously provided by Dr. B. Manz, IBL, Hamburg, FRG. The editorial help of Mrs. P. Decker-Hermann is gratefully acknowledged.

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Received: April 21, 1994
Accepted: September 20, 1994