*Journal of Chromatography*, 338 (1985) 41—49
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2413

# COMPARATIVE EVALUATION OF THE RADIOENZYMATIC METHOD FOR THE DETERMINATION OF URINARY HISTAMINE WITH A MASS SPECTROMETRIC ASSAY

L. JACKSON ROBERTS, II*, KEN A. AULSEBROOK and JOHN A. OATES

*Departments of Pharmacology and Medicine, Vanderbilt University, Nashville, TN 37232 (U.S.A.)*

(First received June 5th, 1984; revised manuscript received October 17th, 1984)

## SUMMARY

Considerable inaccuracy and unreliability have recently been demonstrated to be associated with the widely used radioenzymatic methods for the determination of histamine in biological fluids. Urine appears to inhibit the methylation of histamine by histamine N-methyltransferase such that the radioenzymatic assay underestimates the concentration of histamine present in urine. Directly comparing the radioenzymatic assay with a recently developed reference method using mass spectrometry for the determination of urinary histamine, up to 34-fold differences in the levels of urinary histamine were found with the two methods.

## INTRODUCTION

The physiological and pathophysiological importance of histamine is well recognized. Because of its biological importance, a variety of methods has been developed to quantify histamine in biological fluids. Such methods include bioassay [1, 2], single and double isotope radioenzymatic assays [3—7], high-performance liquid chromatography [8, 9], and manual and automated fluorometry [10—13]. One of the most widely employed methods for the determination of histamine is the radioenzymatic assay. Although both the single and double isotope radioenzymatic methods were generally considered to be associated with an acceptable degree of accuracy, recent studies have suggested that substantial inaccuracy may at times be associated with these methods [14, 15].

Quantification of the urinary excretion of histamine is a valuable diagnostic indicator of the disorder mastocytosis, a disease we have recently found to be

0378-4347/85/$03.30 © 1985 Elsevier Science Publishers B.V.

much more common than previously recognized [16—18]. Because of the importance of using such determinations in the diagnosis of mastocytosis, the accuracy of the radioenzymatic method for the determination of histamine in human urine was examined and considerable inaccuracy was encountered which could not be readily rectified. Therefore efforts were directed towards the development of a more accurate and reliable method for quantification of urinary histamine. Because it is generally accepted that one of the most accurate methods for determination of biological compounds is stable isotope dilution assay with quantification by mass spectrometry (MS), we have recently adapted this methodology for the measurement of urinary histamine [19]. This gas chromatographic (GC) method employs negative-ion chemical-ionization MS which is associated with much greater sensitivity than previous methods reported for the determination of histamine using different methods of ionization [20—22]. The lower limits of detection of histamine with this method are in the range of 100—500 fg injected on-column [23]. The precision of the assay has a coefficient of variation of 2.5% and the accuracy of measuring histamine in urine is 97.6% [19].

Because the radioenzymatic method for quantification of urinary histamine has gained widespread use, it seemed to be a potentially valuable exposition to briefly outline some of the problems encountered with this particular method and to comparatively evaluate the radioenzymatic method for quantification of urinary histamine with the GC—MS method.

## MATERIALS AND METHODS

### *Materials*

Pentafluorobenzyl bromide and diisopropylethylamine were obtained from Pierce (Rockford, IL, U.S.A.). Histamine dihydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). [$\alpha,\alpha,\beta,\beta$-$^{2}H_4$]Histamine dihydrochloride was obtained from Merck Isotopes (Montreal, Canada). Poly I-110 GC column packing was purchased from Applied Science Labs. (State College, PA, U.S.A.). *S*-[Methyl-$^{3}$H]adenosyl-L-methionine (80 Ci/mmol) and *S*-[methyl-$^{14}$C]-adenosyl-L-methionine (56 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.).

### *Gas chromatograph and mass spectrometer conditions*

GC—MS analysis was performed using a Hewlett-Packard 5982A gas chromatograph—mass spectrometer modified to detect negative ions. Conditions: electron energy 25 eV, interface temperature 250°C, internal source temperature 225°C, direct inlet line, emission current 300 $\mu$A, methane as reagent and carrier flow gas, analyzer manifold pressure $1.6 \cdot 10^{-3}$ Pa, injection port temperature 250°C, conversion diode potential —3 kV. Analysis was performed using a 60-cm packed column of 3% Poly I-110 operated at 250°C.

### *Radioenzyme assays of urinary histamine*

The single isotope radioenzymatic assay was used essentially as described by Beaven et al. [3] except that in some experiments *S*-[$^{3}$H]adenosylmethionine was used rather than *S*-[$^{14}$C]adenosylmethionine. Results reported are the mean of duplicate or triplicate determinations of a single assay.

*Mass spectrometric assay of urinary histamine*

The stable isotope dilution GC—MS assay was used as recently described [19]. Briefly, to 1 ml of urine is initially added 40 ng of $[^2H_4]$histamine followed by addition of 150 $\mu$l of 1 *M* sodium hydroxide and extraction into 2 ml of butanol. Then 2 ml of heptane are added, mixed, centrifuged and the butanol—heptane layer is separated from the aqueous layer and extracted with 100 $\mu$l of 1 *M* hydrochloric acid. The aqueous hydrochloric acid phase is evaporated under a stream of nitrogen and the residue dissolved in 30 $\mu$l acetonitrile, 10 $\mu$l diisopropylethylamine, and 15 $\mu$l of a 25% solution of pentafluorobenzyl bromide in acetonitrile. After 30 min at room temperature excess reagents are evaporated under a stream of nitrogen and the residue is dissolved in 150 $\mu$l of 10% sodium carbonate followed by extraction with 250 $\mu$l methylene chloride. The upper aqueous layer is aspirated, discarded and residual sodium carbonate removed by washing the upper part of the vial with distilled water. The methylene chloride is evaporated under a stream of nitrogen and the residue dissolved in ethyl acetate for injection and analysis by GC—MS. Quantification is accomplished by selected-ion monitoring of the ratio of intensity of the *m/z* 430 and *m/z* 434 ion peaks for $[^2H_0]$- and $[^2H_4]$-$(CH_2C_6F_5)_3$-histamine, respectively.

## RESULTS

Because of our interest in determining the urinary excretion of histamine as a diagnostic indicator of the disease mastocytosis, the accuracy of the single isotope radioenzymatic assay for urinary histamine was initially examined by measuring the concentration of histamine present in urine collected from five patients suspected of having mastocytosis before and after the addition of 60 ng/ml histamine. These results are listed in Table I. In each instance, the radioenzymatic assay underestimated the amount of histamine that was added to these urines. The degree of underestimation was quite variable between different urine samples but in each the magnitude of underestimation was substantial. Thus, it appeared that urine collected from these patients interfered greatly with the radioenzymatic determination of histamine.

TABLE I

URINARY HISTAMINE MEASURED BY RADIOENZYMATIC ASSAY (ng/ml)

Concentrations of histamine were measured by single isotope radioenzymatic assay in urine obtained from five patients suspected of having mastocytosis. Histamine was measured before and after the addition of 60 ng/ml histamine and the percentage of the added histamine detected by the assay calculated.

| Patient | No addition of histamine | Addition of 60 ng/ml histamine | Percentage of added histamine measured |
|---|---|---|---|
| 1 | 2 | 12 | 17 |
| 2 | 20 | 41 | 35 |
| 3 | 7 | 40 | 55 |
| 4 | 8 | 24 | 27 |
| 5 | 3 | 18 | 25 |

Whether urine collected from five normal individuals also interfered with the radioenzymatic assay, as was found with the urine obtained from the mastocytosis patients, was then examined. In this experiment, the urine was initially diluted 1:20 prior to the assay with 0.1 *M* phosphate buffer, pH 7.4, to which was added 20 ng/ml histamine. Compared to the standard curve of histamine assayed in 0.1 *M* phosphate buffer, pH 7.4, the assay again underestimated the 20 ng/ml histamine present in each of the 1:20 diluted urine samples by as much as 35—70% (Fig. 1). The other important information that was obtained from this experiment was that diluting the urine by as much as 1:20 prior to analysis did not successfully remove the interference of urine with the assay.

The interference of urine with the radioenzymatic determination of histamine over a range of known histamine concentrations in urine was then evaluated. Urine from a normal individual was initially diluted 1:20 with 0.1 *M* phosphate buffer, pH 7.4, to which was then added histamine to give concentrations of 20, 50, and 100 ng/ml prior to analysis. The analysis of these urine samples was then compared to the analysis of samples of 20, 50, and 100 ng/ml histamine in 0.1 *M* phosphate buffer, pH 7.4, alone (Fig. 2). The curve relating [$^3$H]methylhistamine recovered to histamine concentrations in the urine samples was linear over this concentration range but the slope was considerably

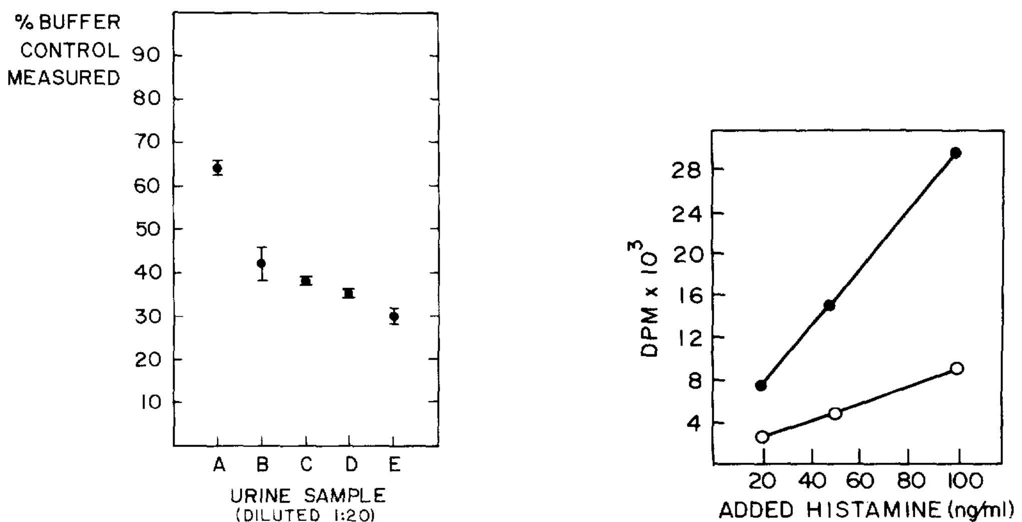


Fig. 1. Urine was obtained from five normal volunteers and diluted 1:20 with 0.1 *M* phosphate buffer, pH 7.4, to which was subsequently added 20 ng/ml histamine. The histamine concentration was then determined in the urine samples by single isotope radioenzymatic assay by comparison to a standard curve of histamine concentrations prepared in 0.1 *M* phosphate buffer, pH 7.4. Plotted is the percentage of radiolabeled methylhistamine recovered in the analyses of the urine samples compared to that recovered in the standard curve analysis of 20 ng/ml histamine in phosphate buffer.

Fig. 2. Comparison of the analysis by single isotope radioenzymatic assay of 20, 50, and 100 ng/ml of histamine in 0.1 *M* phosphate buffer, pH 7.4, and in urine. The urine was initially diluted 1:20 with 0.1 *M* phosphate buffer prior to addition of 20, 50, and 100 ng/ml histamine before analysis. Plotted are the curves relating recovered [$^3$H]methylhistamine dpm to histamine concentration present in phosphate buffer (●) and urine (○). Each data point represents the mean of duplicate or triplicate determinations.

more flat than the curve obtained from the analysis of histamine in phosphate buffer alone. These data again illustrated the inhibitory influence of urine with the radioenzymatic determination of histamine and again confirmed the failure of diluting the urine by as much as 1:20 before analysis to successfully remove this interference of urine with the assay.

Because diluting urine by as much as 1:20 prior to analysis did not successfully eliminate the interference of urine with the assay, a few additional simple maneuvers were examined in an attempt to eliminate this interference including briefly boiling the urine before analysis, using increased enzyme concentrations, and altered incubation times and temperature. However, none of these procedures successfully removed the interference of the urine with the radioenzymatic assay. It was also confirmed that the apparent interference could not be attributed to differences in the extraction recoveries of radiolabeled methylhistamine from buffer and urine (data not shown).

Because of these problems with the radioenzymatic determintion of urinary histamine which could not be easily rectified, efforts were directed towards the development of a reliable method for the quantification of urinary histamine by stable isotope dilution assay using GC—MS [19]. To further evaluate and elucidate the problems associated with the radioenzymatic assay of urinary histamine, levels of urinary histamine measured by the two assays were compared in a series of urines obtained from patients with mastocytosis. Urines were selected for analysis by GC—MS in which the levels of urinary histamine measured by the radioenzymatic assay ranged from normal to markedly increased. This was done to assess whether the ability of the radioenzymatic assay to measure increased concentrations of histamine in some urines could be attributed to less interference of these particular urines with the assay. The results of these determinations of urinary histamine by the two methods are shown in Table II. In urine obtained from two patients (2 and 4), the levels

TABLE II

HISTAMINE LEVEL MEASURED

Comparison of the levels of urinary excretion of histamine (μg per 24 h) determined by both single isotope radioenzymatic assay and stable isotope dilution assay with GC negative-ion chemical-ionization MS in 24-h urine collections obtained from ten patients with mastocytosis.

| Patient | Radioenzymatic assay | GC—MS assay |
|---|---|---|
| 1 | 20 | 272 |
| 2 | 11 | 11 |
| 3 | 13 | 67 |
| 4 | 15 | 8 |
| 5 | 49 | 335 |
| 6 | 44 | 65 |
| 7 | 83 | 231 |
| 8 | 563 | 867 |
| 9 | 526 | 1123 |
| 10 | 519 | 1882 |

of urinary histamine quantified by the two assays were in close agreement. However, in urine from all of the other patients, the GC—MS assay consistently measured levels of histamine that were higher than the levels quantified by the radioenzymatic assay. The magnitude of the differences measured by the two methods was quite remarkable. The overall mean level measured by the GC—MS assay in these urines was 4.6-fold greater than that obtained with the radioenzymatic assay with a range of 1.5-fold (patients 6 and 8) to as high as 13.6-fold (patient 1). In addition, it is apparent that the interference of the urine matrix with the radioenzymatic determination of histamine was not limited to the urines in which the radioenzymatic assay measured low levels of histamine but also occurred in urines in which the radioenzymatic assay measured very high levels.

Perhaps an even more straightforward demonstration of the disparity between these two methods for the analysis of urinary histamine is illustrated in Fig. 3. In this figure is shown the urinary excretion of histamine as measured by the two assays in a patient with mastocytosis in whom severe episodes of flushing were provoked by the ingestion of aspirin. Although we have found that to ameliorate the symptoms of mastocytosis requires treatment with high doses of aspirin to inhibit the release of prostaglandin $D_2$ from mast cells [16, 17], analogous to the asthmatic population, a small subset of patients with mastocytosis exists in whom attacks of flushing are initially triggered by the ingestion of small doses of aspirin or other non-steroidal antiinflammatory drugs. In this patient repeated doses of 10 mg of aspirin were administered over

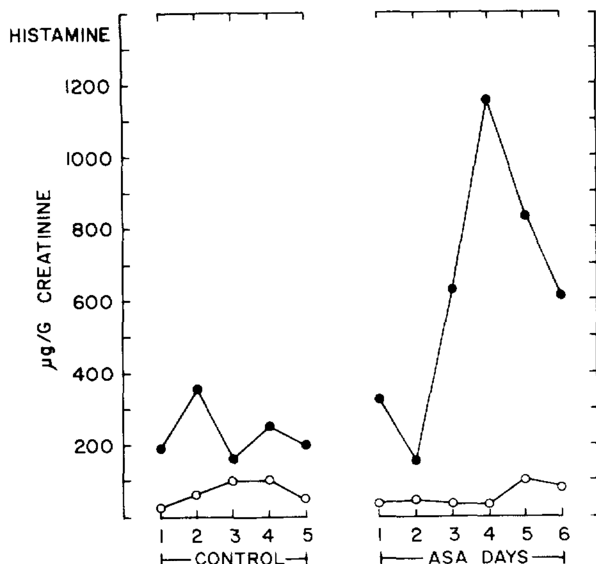


Fig. 3. Levels of urinary excretion of histamine (μg/g creatinine) determined by both single isotope radioenzymatic assay (○) and stable isotope dilution assay with GC negative-ion chemical-ionization MS (●) in a patient with mastocytosis and aspirin hypersensitivity. On the left are levels determined during control periods in the absence of aspirin ingestion and episodes of flushing. On the right are levels of urinary excretion of histamine determined when repeated doses of 10 mg of aspirin (ASA) were administered at varying intervals over six days during which time repeated episodes of severe flushing occurred.

varying time intervals for six days. This provoked recurrent episodes of severe flushing which, as determined by the GC—MS assay, were accompanied by a marked elevation in the urinary excretion of histamine compared to control days in the absence of aspirin and flushing reaching a maximum level of 1158 $\mu$g/g creatinine on day 4. However, using the radioenzymatic assay, no appreciable increase in the urinary excretion of histamine during these episodes of severe flushing provoked by aspirin could be detected. Although the interference of urine with the radioenzymatic assay during control quiescent days was of similar magnitude to that found in urine from the patients listed in Table II, the interference in urine collected from this patient during recurrent episodes of severe flushing was remarkably greater. On day 4 of aspirin administration when the urinary excretion of histamine determined by the GC—MS assay was 1158 $\mu$g/g creatinine, the radioenzymatic assay detected only 34 $\mu$g/g creatinine. This was a 34-fold difference in the levels of histamine measured in this urine by the two assay methods or expressed differently, the radioenzymatic assay only measured 2.9% of the urinary histamine actually present as determined by the GC—MS assay.

The data obtained in this patient indicate that greater amounts of substances which can interfere with the radioenzymatic determination of histamine were excreted at times when the patient was experiencing severe attacks of flushing and hypotension compared to quiescent days. Whether this same phenomenon can be generalized to other patients with mastocytosis remains to be determined.

## DISCUSSION

The results of these experiments demonstrate that urine frequently interferes with the radioenzymatic determination of histamine. The magnitude of interference in most instances was quite substantial, although variable between urine samples analyzed. The interference appears to result from inhibitory substances in urine which prevent complete methylation of the histamine present by the histamine N-methyltransferase in the assay. Thus, the radioenzymatic assay frequently underestimated the concentration of histamine that was actually present in urine. At present the nature of these inhibitory urinary substances is not known. However, we could not satisfactorily eliminate the interference of urine with the assay by a few simple procedures, including a brief boiling of the urine prior to analysis, diluting the urine by as much as 1:20 prior to analysis, increasing the amount of enzyme in the assay, prolonging the incubation time, and altering the incubation temperature.

A recent report suggests that extraction of urinary histamine, in addition to analyzing diamine oxidase treated and untreated urine, does improve the accuracy of the double isotope radioenzymatic assay to an extent, but some interference and unreliability still remains [15]. Whether additional chromatographic purification following extraction of urinary histamine prior to analysis would satisfactorily remove interfering urinary substances remains to be determined but deserves investigation. Although we chose to circumvent these problems by developing the GC—MS method of analysis, these studies should help investigations into procedures which can potentially improve the radio-

enzymatic assay as mass spectrometer instrumentation may not be universally available to all laboratories interested in the quantification of urinary histamine.

It also seems important to mention that although the single isotope radioenzymatic assay was used in these studies to investigate the inhibitory influences of urine with the determination of histamine, it would not seem that simply using a double isotope radioenzymatic assay would successfully overcome the problems of inaccuracy with the method. Although the double isotope radioenzymatic assays incorporate a control for incomplete methylation of histamine in the assay, the accuracy of this control becomes substantially reduced with increasing degrees of interference of urine with the assay. With increasing interference, the slope of the curve relating histamine concentrations to recovered methylhistamine radioactivity becomes very flat. This phenomenon is illustrated in Fig. 2. An even flatter curve than depicted in Fig. 2 would be obtained with urines in which there was an even greater degree of interference such as the urine on day 4 of aspirin administration in Fig. 3. In other words, in the presence of substantial interference, a large increase in histamine concentration only produces a very small absolute increment in radiolabeled methylhistamine recovered. Because the accuracy of scintillation counting is proportional to the number of disintegrations per minute detected, the assay would be insensitive to small but meaningful differences in the concentration of histamine present in such urines and such differences could not be accurately and reliably measured. The correction factor that is incorporated into the calculations in the double isotope assay to control for incomplete methylation of histamine would, in this situation, only multiply the error associated with the scintillation counting. For analogous reasons, running a standard curve of histamine concentration in each individual urine sample analyzed, in addition to being very time-consuming, would also not overcome the inaccuracy of the radioenzymatic determination of histamine in urines which profoundly interfere with the assay.

In summary, these studies have elucidated substantial inaccuracy and unreliability with the radioenzymatic determination of histamine in urine. In laboratories where mass spectrometer instrumentation is available, the described stable isotope dilution GC—MS method enables quantification of urinary histamine that is not only accurate but also very efficient. However, for laboratories without access to mass spectrometer instrumentation, it seems likely that with further investigation, procedures can be found and incorporated which will improve the radioenzymatic methods for determination of urinary histamine to an acceptable degree. Until such procedures are determined, validated, and incorporated, however, one is left with substantial skepticism as to validity of levels of urinary histamine reported using radioenzymatic methods.

## ACKNOWLEDGEMENTS

This work was supported by Grants GM 15431 and 507-RR05424 from the National Institutes of Health. Dr. Roberts is a Burroughs Wellcome Scholar in Clinical Pharmacology. Dr. Oates is the Joe and Morris Werthan Professor of

Investigative Medicine. We would also like to acknowledge the generous contribution of Dr. David Beggs of Hewlett-Packard who supplied blueprints to allow modification of the HP-5982A mass spectrometer to detect negative ions.

REFERENCES

1 H. Dunner and B. Pernow, Scand. J. Clin. Lab. Invest., 8 (1956) 296.
2 R. Kappeler-Adler and B. Iggo, Biochim. Acta, 25 (1957) 394.
3 M.A. Beaven, S. Jacobsen and Z. Horakova, Clin. Chim. Acta, 37 (1972) 92.
4 R.E. Shaff and M.A. Beaven, Anal. Biochem., 94 (1979) 425.
5 C. Bruce, R. Weaterstone, A. Seaton and W.H. Taylor, Thorax, 31 (1976) 724.
6 R.L. Miller, C. McCord, M. Sanda, H.R. Bourne and K.L. Melmon, J. Pharmacol. Exp. Ther., 175 (1970) 228.
7 M.J. Brown, P.W. Ind, R. Causon and T.H. Lee, J. Allergy Clin. Immunol., 69 (1982) 20.
8 Y. Tsuruta, K. Kohashi and Y. Ohkura, J. Chromatogr., 224 (1981) 105.
9 G. Skofitsch, A. Saria, S.P. Holzer and F. Lembeck, J. Chromatogr., 226 (1982) 53.
10 P.A. Shore, A. Burkhalter and V.P. Cohn, J. Pharmacol. Exp. Ther., 127 (1959) 182.
11 J. Oates, E. Marsh and A. Sqoerdsma, Clin. Chim. Acta, 7 (1962) 488.
12 R.P. Siraganian, Anal. Biochem., 57 (1974) 393.
13 G. Meyers, M. Donlon and M. Kaliner, J. Allergy Clin. Immunol., 67 (1981) 305.
14 G.J. Gleich and W.M. Hull, J. Allergy Clin. Immunol., 66 (1980) 295.
15 K. Warren, J. Dyer, S. Merlin and M. Kaliner, J. Allergy Clin. Immunol., 71 (1983) 206.
16 L.J. Roberts, II, B.J. Sweetman, R.A. Lewis, K.F. Austen and J.A. Oates, N. Engl. J. Med., 303 (1980) 1400.
17 L.J. Roberts, II, J.P. Fields and J.A. Oates, Trans. Assoc. Amer. Physicians, XCV (1982) 36.
18 J. Turk, J.A. Oates and L.J. Roberts, II, J. Allergy Clin. Immunol., 71 (1983) 189.
19 L.J. Roberts, II and J.A. Oates, Anal. Biochem., 136 (1984) 258.
20 H. Mita, H. Yasueda and T. Shida, J. Chromatogr., 181 (1980) 153.
21 H. Mita, H. Yasueda and T. Shida, J. Chromatogr., 221 (1980) 1.
22 J.J. Keyzer, H. Breukelman, H. Elzinga, B.J. Koopman, B.J. Wolthers and A.P. Bruins, Biomed. Mass Spectrom., 10 (1983) 480.
23 L.J. Roberts, II, J. Chromatogr., 287 (1984) 155.