A REVERSE DOUBLE-ISOTOPE ENZYMATIC HISTAMINE ASSAY:

ADVANTAGES OVER SINGLE-ISOTOPE METHODS

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SUMMARY

High concentrations of codeine necessary to demonstrate hista-
mine release from leukocytes interfere with histamine methylation,
producing a concentration-dependent flattening of the histamine
standard curves obtained from single-isotope (^{3}H) assays. For this
reason, the use of uniformly labeled ^{14}C-histamine was investigated
as an internal standard. This isotope serves as an internal standard
and accurately predicts the recovery of histamine in the presence of
up to 70% enzyme inhibition. The use of this reverse double-isotope
procedure allows the assay of histamine without direct external
recovery measurements in the presence of various drug concentrations.

The radioenzymatic assay for histamine was introduced by Snyder et al.
(1) as a sensitive and specific method. The assay is based on the enzymatic
methylation of histamine using histamine methyltransferase and [^{14}CH$_3$] S-
adenosyl-L-methionine (SAM) as the methyl donor. Labeled (^{3}H) histamine is
added to each tube as an internal standard and the labeled methylhistamine
is isolated and counted. This double-isotope assay has been modified by
Taylor and Snyder (2) and by Beaven et al. (3) to improve sensitivity.
Nevertheless, difficulties with reproducibility have been reported (4). The
single-isotope method using ^{3}H-SAM instead of the double isotope method has
been reported to be more reliable and sensitive (4,5). This assay has been
used to measure brain histamine (6), as well as histamine release from human
basophils (5).

We have adapted the single-isotope assay for the investigation of drug-
induced histamine release from isolated basophils. The results reported
here demonstrate the difficulties encountered with this method when assays
are carried out in the presence of high concentrations of potential releas-
ing agents. Because of variable histamine methylation, the recovery in each
tube must be determined using external histamine standards. Alternatively,
all samples from release experiments must be adjusted to contain identical
drug concentrations prior to assay for histamine. Both methods are laborious
and introduce additional sources of error. Therefore, we have investigated
the use of uniformly labeled ^{14}C-histamine as an internal standard within the
^{3}H-single-label assay and report success with this 'reverse' double-isotope
method.

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MATERIALS AND METHODS

Histamine assays using the single-isotope method were carried out in heparinized normal human plasma, 0.05 M sodium phosphate buffer (pH 7.9), or a tris-buffered solution of albumin, calcium and magnesium (Tris ACM). The latter is useful in histamine release studies and was prepared according to the method of Lichtenstein (7). The appropriate solution was added to 400 μl polyethylene tubes (KEW Sci.) containing 0-2.0 ng histamine base in a final volume of 10 μl. Codeine phosphate was also included to make final concentrations between 0 and 0.01 M. All solutions and tubes were kept at 4° C prior to incubation and at room temperature thereafter. The enzyme-isotope mixture was prepared by transferring an aliquot of ^{3}H-[CH$_3$] S-adenosyl-L-methoinine (8.8 Ci/mmol, New England Nuclear) to a glass culture tube and evaporating to dryness at 4° C under a stream of nitrogen. This isotope is routinely stored at 4° C. A sufficient amount was evaporated for 0.5 μCi (1 μl) per assay tube. Histamine methyltransferase was prepared by the method of Snyder (8) and contained 6.7 units activity/ml and 4.8 mg/ml protein. The enzyme was transferred to the isotope residue with mixing in an amount equivalent to 2 μl/tube. Each assay tube received 2 μl of this enzyme-isotope solution, mixing and incubation at 37° C for 1 hr. The reaction was terminated by the addition of 10 μl 0.4 N HClO$_4$ containing 1 mg/ml methylhistamine dihydrochloride (Calbiochem). Each tube received 10 μl 10 N NaOH, 200 μl CHCl$_3$, and vigorous mixing on a microvortex for 30 seconds. The layers were separated by a brief, low speed centrifugation and the aqueous fractions were removed by aspiration. The organic fractions were washed with 50 μl of 3 N NaOH and the centrifugation and aspiration steps were repeated. One hundred μl aliquots were transferred from each tube to polyethylene mini-vials (Rochester Sci.) and evaporated at room temperature under a nitrogen stream. Each mini-vial received 5 ml of toluene scintillation solution (2 g POP + 0.1 g POPOP/liter toluene), and the samples were counted in a Beckman LS-200 liquid scintillation counter. Unless other specified, results represent the mean of three samples and are expressed in cpm.

For the reverse double-isotope method, the protocol is identical to the one above except that U-^{14}C histamine (290 mCi/mmol, Amersham-Searle) is evaporated along with the SAM solution in sufficient quantity for 1 nCi (0.02 μl) per assay tube.

Following the removal of the final aqueous phases, aliquots of the CHCl$_3$ fractions do not have to be taken, since the results are expressed as the ^{3}H/^{14}C ratio. For the purposes of accurately determining the individual isotopes, however, aliquots were taken in these experiments. Samples were counted with appropriate window settings for ^{3}H and ^{14}C determinations and the activity in dpm of both isotopes was determined. Results are expressed as the mean ratio ^{3}H/^{14}C for each group of samples. Linear regression analyses were performed on each standard curve.

RESULTS

Fig. 1 shows a standard curve obtained from a single-isotope histamine assay in phosphate buffer. The curve demonstrates excellent linearity from 0.02-2.0 ng and easily allows the detection of 0.1 ng in a volume of 10 μl.

Fig. 2 shows standard curves for the single-isotope histamine assay performed in 3 buffer systems and the effect of increasing codeine concentrations on these curves. The drug-free curves in phosphate buffer and Tris ACM are similar and in contrast to the flatter plasma curve. Thus, histamine can be measured in all 3 systems although the sensitivity is less when

FIG. 1
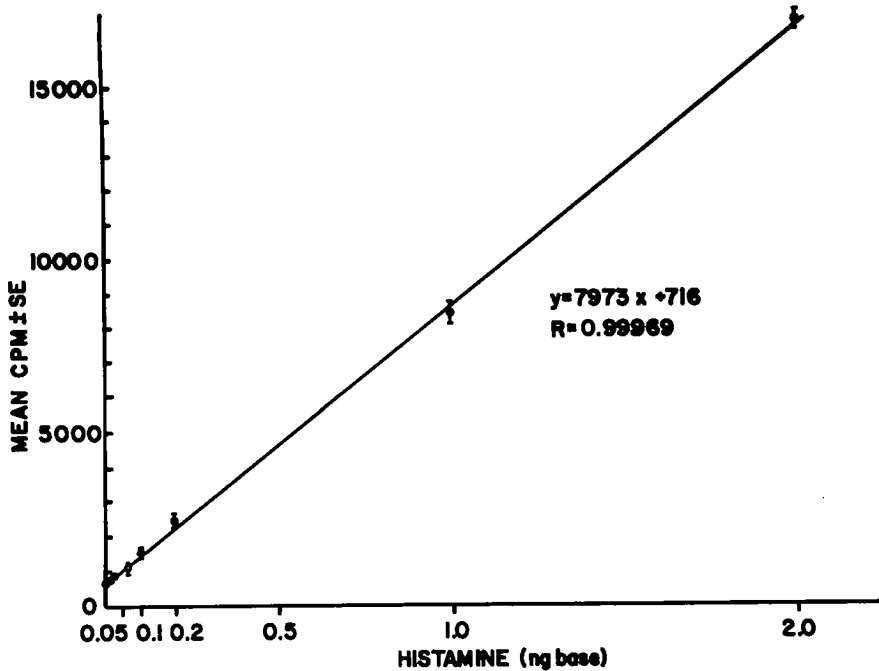
Single-isotope histamine standard curve. Samples containing
0.01-2.0 ng histamine in ten μl of 0.05 M sodium phosphate
buffer, pH 7.9, were assayed as described in Methods. The
mean ± S.E. of triplicate determinations are shown.

the assay is done directly in plasma. The slopes of all 3 curves, however,
are depressed in a concentration-dependent manner by codeine, although the
assay performed in Tris ACM is unaffected by 10^{-4} M codeine. At 10^{-2} M
codeine, no histamine methylation occurred in any buffer and thus no
histamine was detected.

A plot of ^{3}H-dpm alone from the reverse double-isotope method re-
produces results obtained with the single isotope assay. The effect of
codeine is likewise reproduced. Fig. 3A reveals the progressive decline in
^{3}H-dpm for all values of histamine as the codeine concentration is increased.
The curves remain linear, however. The ability of ^{14}C-histamine to serve
as an internal standard under these circumstances is demonstrated in Fig. 3B.
When the ^{3}H-dpm in each tube is corrected for the ^{14}C-methylhistamine re-
covered in that tube, essentially no change in the histamine standard
curves can be seen throughout the 4 concentrations of codeine used. This
implies that the loss of ^{14}C activity parallels that of ^{3}H activity which
is evident in Table I.

Codeine produces concentration-dependent losses in the methylation of
histamine, as reflected by the slopes of the ^{3}H-dpm curves. A 70% reduction
in slope occurs with 3×10^{-3} M codeine. A parallel loss of the methylation

of ^{14}C-histamine is also produced by codeine, with a 71.5% reduction at
the same concentration. The ability of the ^{14}C-dpm to accurately predict
the histamine methylation is evidenced by the relatively stable slopes of
[3]H/^{14}C ratio curves. Although concentrations of codeine as high as 10^{-3} M
inhibit histamine methylation up to 50%, the slopes of the ratio curves
vary only from 75-88 and in a non-dose-dependent manner. The slope obtained
with 3×10^{-3} M was inhibited by 28%.

FIG. 2
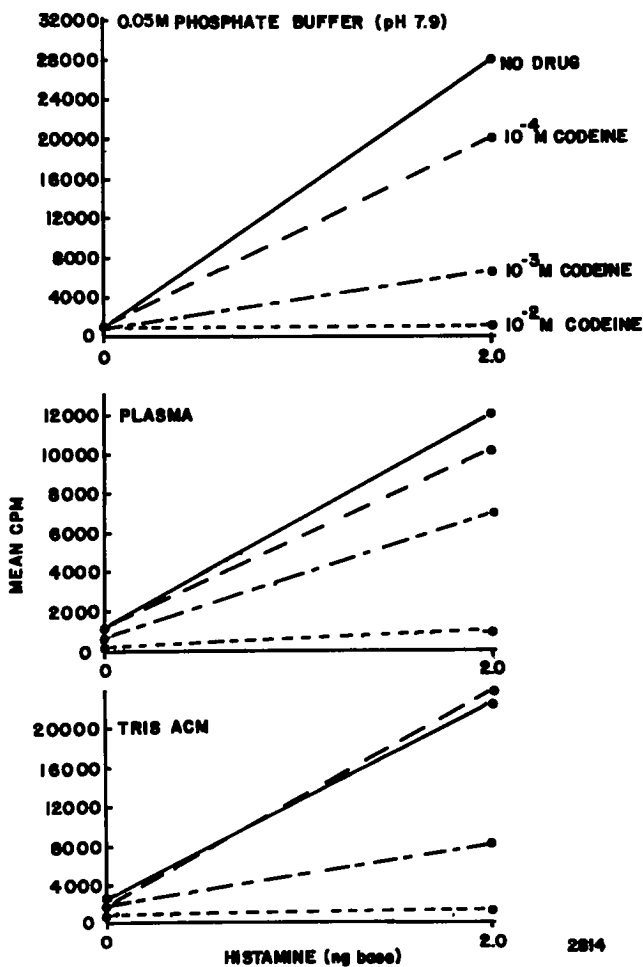
Effect of codeine on single-isotope histamine standard
curves in phosphate buffer, plasma and Tris ACM. Assays
were performed in the presence of 3 codeine concentra-
tions and each point represents the mean of duplicate
determinations. A concentration-related inhibition is
evident in all buffer systems.

FIG. 3
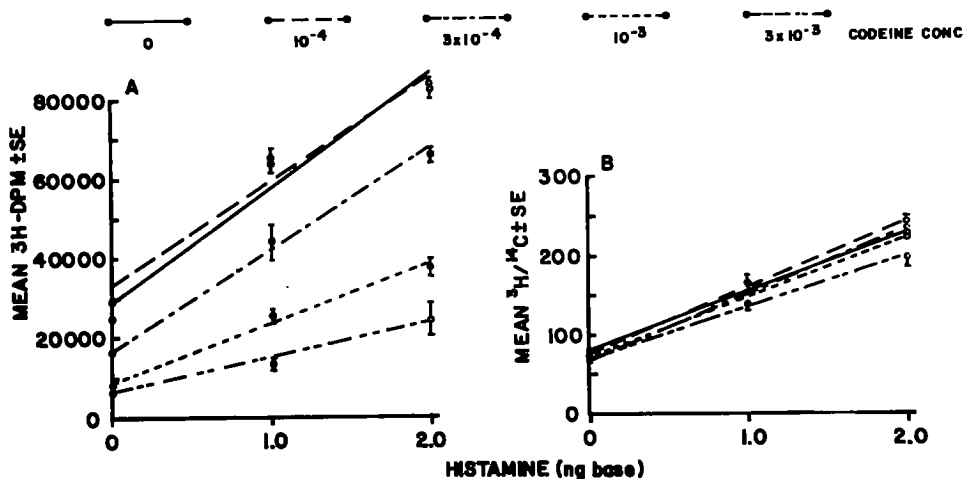
Effect of codeine on reverse double-isotope histamine standard
curves in phosphate buffer. The effect of codeine on ^{3}H-dpm
alone (panel A) is similar to that found by using the single-
isotope assay (Fig. 2). ^{14}C-histamine included in the assay
functions as an internal standard such that the ratio ^{3}H/^{14}C
remains unaffected by codeine (B).

TABLE I

Slopes and Mean ^{14}C-DPM for Standard Curves in Fig. 3

Codeine Conc. (M)	^{3}H-curve Slope	% Inhibition	^{14}C-DPM Mean ± S.E.	% Inhibition	^{3}H/^{14}C Slope	% Inhibition
0	28,677	-	374 ± 7.5	-	82	-
10^{-4}	26,862	6.3	367 ± 11.9	1.9	81	1.2
3×10^{-4}	25,083	12.5	279 ± 8.9	25.5	88	-7.3
10^{-3}	14,920	48.0	158 ± 7.0	57.8	75	8.5
3×10^{-3}	8,618	70.0	107 ± 8.7	71.5	59	28.0

The data shown are from Fig. 3. The slopes of the ^{3}H-standard
curves reveal a codeine-induced inhibition of the methylation
and thus detection of the total histamine present. Mean ^{14}C-
dpm values reflect similar effects on the detection of ^{14}C-
histamine. Minimal changes in the slope of the ^{3}H/^{14}C standard
curves result.

DISCUSSION

The use of β-^{3}H-histamine as an internal standard has been suggested by Beaven (9) as a modification of the original double-isotope histamine assay which uses ^{14}C-SAM. Studies in our laboratory with this modification confirm that the β-labeled isotope is more stable and more reproducibly methylated than the commercially available [3]H-(G)-histamine. Under conditions of enzyme inhibition, however, ^{3}H-histamine was found unsuitable as an internal standard, since ^{14}C/^{3}H standard curves were flattened in a dose-related manner using this method (unpublished observations). This was unexpected since the ratio should not have changed if ^{3}H-histamine served as an accurate index of histamine recovery. Furthermore, the sensitivity of this method is limited by the specific activity of the ^{14}C-SAM, which is much lower than the ^{3}H-SAM. These difficulties led to the use of the single-isotope method.

The results shown with single-isotope assays in various buffer systems demonstrate the versatility of this method (Fig. 2). Histamine can be measured in solutions with wide ranges of pH and ionic strength, consistent with the studies of Brown et al. (10) and Kobayashi and Maudsley (4) on histamine methyltransferase (HMT) activity. However, there are definite differences in the degree of methylation in these systems, an important observation when assaying histamine in various physiological fluids using a standard curve constructed from one buffer. For example, the standard curve in phosphate buffer (Fig. 2) indicates that 2 ng of histamine yields 28,297 cpm. An identical amount of histamine assayed in plasma as an unknown would be measured as 0.89 ng using the phosphate curve since a 2 ng histamine standard yields only 12,174 cpm in plasma. Even within the same buffer system other factors can contribute to variable histamine methylation, such as the presence of various concentrations of drugs. This is the case for high concentrations of codeine. These observations point out the major limitation of the single isotope method: the need for external recovery measurements in each sample.

The results shown in Fig. 3 and Table I demonstrate that the use of ^{14}C-histamine as an internal standard allows accurate histamine measurements in the presence of up to 70% HMT inhibition by codeine. In a recent trial with compound 48/80, the double-isotope ^{3}H/^{14}C assay was accurate in the presence of 90% inhibition of methylation (unpublished data). As noted above ^{3}H-histamine did not serve as an internal standard under these circumstances as did ^{14}C-histamine. Thus, the use of this double-isotope assay eliminates the need for external recovery measurements in each sample.

Several aspects of the use of the double-isotope method merit further discussion. Both the ^{3}H-background and ^{3}H-sensitivity vary with the age and storage conditions of the ^{3}H-SAM. This has been observed by Beaven (9) who recommended storage in liquid nitrogen. In our laboratory, ^{3}H-SAM is stable over several months at 4°C as long as it is not frozen and thawed repeatedly. The use of ^{14}C-histamine in trace quantities would be ideal as an internal standard. Unfortunately, this is not possible despite the use of uniformly labeled isotope. Thus, the addition of as little as 900 dpm ^{14}C-histamine which results in 374 dpm recovered as product represents 0.16 ng histamine added to every sample. This has the effect of elevating the ^{3}H-background since 0.16 ng of histamine will produce several thousand cpm of ^{3}H-methyl-histamine (see Fig. 1). This reduces the sensitivity of the method as compared to the single isotope assay and makes it undesirable for the measurement of small quantities of histamine. The amount of ^{14}C-histamine used in these assays

may be varied somewhat but is limited by accurate ^{14}C-dpm determinations in the products. This problem is more evident under circumstances of depressed methylation. For example, 2 x 10^{-3} M codeine inhibited control methylation by 71.5%, yielding ^{14}C values of 107 dpm (64 cpm) (Table I). Although this method represents a substantial loss of sensitivity when compared to the existing enzymatic methods, it is unique in its ability to reproducibly measure histamine under conditions which make other methods unreliable or cumbersome. Thus the chief advantage of the reverse double isotope method is the enzymatic detection of moderate concentrations of histamine under circumstances of substantial enzyme inhibition.

The high concentrations of codeine used in these studies to inhibit HMT are not meant to suggest an in vivo effect on the enzyme but may be of relevance with regard to histamine release from mast cells. Concentrations of codeine in skin at the site of local injection may be higher than 10^{-4} M by our calculations. The use of codeine in these studies stems from our inability to demonstrate codeine-induced histamine release in isolated basophils. Our negative findings led us to the present systematic study of codeine on the assay itself. This, in turn, led to the development of the method reported here. The assay described here circumvents the previously necessary studies of drugs on the single-isotope assay itself. Because of the loss in sensitivity, this method is ideally suited for systems in which the histamine content can be controlled. Isolated basophils or mast cells are excellent examples. We are currently pursuing studies of codeine-induced histamine release using the reverse double-isotope method.

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