METHODS IN MOLECULAR BIOLOGY™

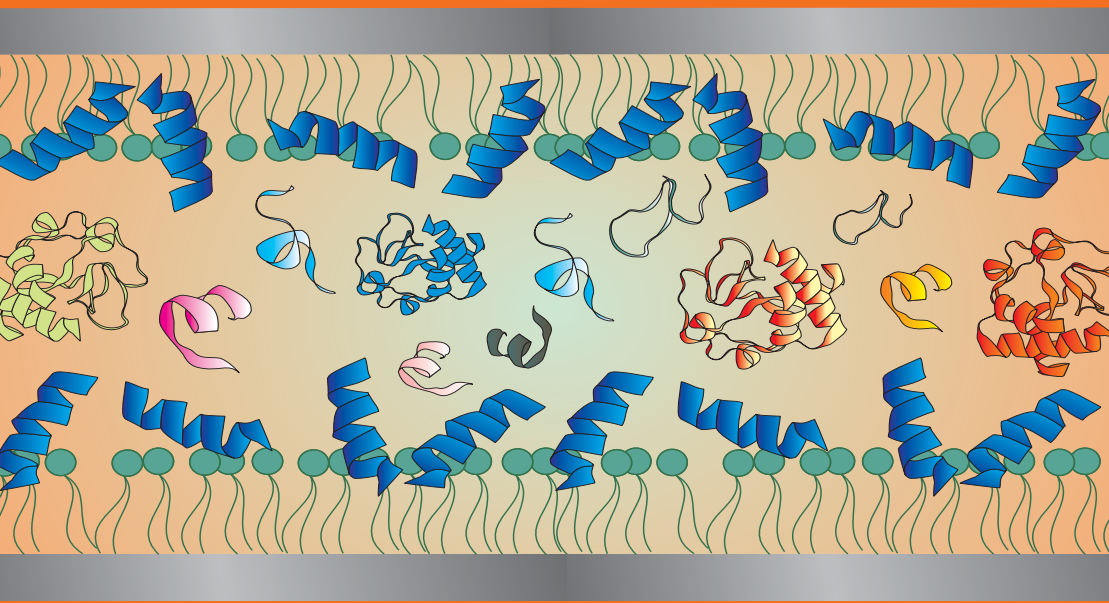
Volume 251

HPLC of Peptides and Proteins

Methods and Protocols

Edited by

Marie-Isabel Aguilar



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Sensitive Enzymatic Analysis of Histidine Decarboxylase Using HPLC

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1. Introduction

High-performance liquid chromatography (HPLC) can be used in enzymatic analysis in order to achieve a good purification of the reaction product. In this chapter, we will see how to use HPLC to measure histamine formation, a process catalyzed by the enzyme histidine decarboxylase (EC 4.1.1.22). Often the tissue under analysis is a poor source of this enzyme, expressed only by a few cell types such as histaminergic neurons, mast cells, and gastrointestinal enterochromaffin-like cells. For this reason, a method of high sensitivity is needed to determine histamine synthesis in tissue slices or histidine decarboxylase activity in low homogenate volumes. However, it may be difficult to eliminate the precursor histidine from histamine purifications because of their similar chemical properties. The method we describe here is based on an HPLC purification of histamine ***(1)*** that eliminates histidine much more selectively than previous methods based on cation-exchange gravity columns ***(2–5)***. A radiolabeled substrate is used to obtain to the maximum sensitivity and specificity of the assay.

Because decarboxylation of histidine spares the imidazole ring, a suitable radiolabelled precursor would be commercial [2,5-^{3}H]L-histidine. Unfortunately, imidazole rings show a partial tautomerism that favors tritium exchange. This causes a decomposition rate of this substrate of about 1–3% per month or even higher according to the manufacturers. This problem can be overcome if substrate specific activity is routinely monitored after storage. We show in **Subheading 3.1.** how to purify periodically by HPLC one aliquot of original commercial [^{3}H]-histidine and assess its specific activity. This is also

From: *Methods in Molecular Biology, vol. 251, HPLC of Peptides and Proteins: Methods and Protocols*
Edited by: M.-I. Aguilar © Humana Press Inc., Totowa, NJ

important because the sensitivity of histamine purification can be limited if the blanks contain excessive disintegration per minute (dpm) from tritium contamination.

Approximately 0.01–0.5% of the substrate histidine is converted into histamine *(1,4)*. Such a low degree of conversion may cause difficulties in the purification of the product histamine in the presence of a large excess of substrate. To facilitate purification of histamine, we eliminate most of the histidine after incubation by binding to an anion-exchange resin added to deproteinized samples (*see* **Subheading 3.3.**). As the carboxylate of the histidine binds to the resin, unfortunately there is also a low degree of nonspecific binding of histamine to the resin. We overcome this problem by estimating histamine recovery in every sample through the addition of nonradiolabeled histamine as an internal standard. The use of unlabeled histamine as an internal standard ensures a very high reproducibility and also facilitates the HPLC fraction collection.

For HPLC purification of histamine, we have chosen an inexpensive reversed phase C_{18} column and a mobile phase with an ion-pair reagent, but it should also be possible to use ion-exchange HPLC. Our mobile phase has a high content of salt and methanol to facilitate quick elution of histidine in samples. Histamine retention in the column is caused by the high ion-pair concentration in the mobile phase which is run isocratically at low pH. The low dpm obtained in blank samples (*see* Table 2) makes further development of histamine purifications using gradient elution unnecessary. The HPLC is automated by a sample injector and a fraction collector. Collection is started by ultraviolet (UV) detection of the histamine internal standard. HPLC separation is straightforward provided that routine checks of tritium in the system and adequate clean-ups are performed, especially if the same HPLC system has been used previously for [^{3}H]-histidine purification. Finally, dpm are counted in the histamine fraction by scintillation.

Our method can also be applied with minor modifications to study histamine synthesis and release in synaptosomes and slices. Because the tissue slices maintain relative synaptic integrity, histidine decarboxylase activity in homogenates may not correlate with histamine synthesis quantified in slices *(6)*. Slices must take up ^{3}H-histidine into histaminergic terminals before being decarboxylated. Also, newly formed histamine in slices could be subject to regulatory processes *(7)*.

2. Materials

2.1. [^{3}H]-Histidine Purification

1. Ring-labeled [2,5-^{3}H]L-histidine stocks (1 mCi, 50 Ci/mmol) obtained from Amersham and stored at 4°C. Stocks can be kept for several years if they are purified 1–3 mo before use.

2. Nonradiolabeled histidine (Sigma).
3. Mobile phase for HPLC gradient: prepare fresh solutions A and B in Milli-Q water and vacuum filter them through nylon membranes of 0.2 µm pore size. Solution A: 25 m*M* NaH_2PO_4 pH 3, 0.1 m*M* octanesulfonic acid (Sigma), and 2% methanol (HPLC grade). Solution B: 0.1 m*M* octanesulfonic acid and 2% methanol.
4. HPLC system: Kontron 325 pump, Rheodyne injector model 7125 and Kontron 432 UV detector.
5. Reverse-phase C_{18} column, 25 × 0.46 cm (Tracer Extrasil ODS-2, of 5 µm particle size; Teknokroma, Spain), equipped with a 2 × 20 mm guard column (Upchurch; Teknokroma).
6. HPLC cleaning solutions: 0.1 *M* nitric acid and methanol/Milli-Q water (70/30%) (*see* **Note 1**.)

2.2. Enzymatic Assays and Slice Preparations

1. 10 m*M* Potassium phosphate buffer, pH 7.4 (stored as 1 *M* stock at 4°C for several months).
2. 0.2 m*M* Pyridoxal phosphate (stored aliquoted at –20°C).
3. 10% (v/w) Trichloroacetic acid (stored as 50% stock solution at 4°C for months).
4. 10 m*M* Histamine (make 1 mL aliquots and freeze).
5. Modified Krebs–Ringer bicarbonate medium (KRM) made of 120 m*M* NaCl, 0.8 m*M* KCl, 0.67 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 2.6 m*M* $CaCl_2$, 27.5 m*M* $NaHCO_3$, 10 m*M* glucose, dissolved in Milli-Q water (*see* Note **2** for precautions on preparation). Bubble O_2/CO_2 (95:5) through solution before use and periodically during use.
6. Depolarizing medium: 2 NaCl m*M*, 119 m*M* KCl, 0.67 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 2.6 m*M* $CaCl_2$, 27.5 m*M* $NaHCO_3$ and 10 m*M* glucose, dissolved in Milli-Q water (*see* **Note 2**). Bubble O_2/CO_2 (95:5) through solution before and during use. (Depolarizing medium is a KRM rich in KCl and poor in NaCl to maintain isomolarity.)
7. McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Surrey, England).
8. Sonic-dismembrator DYNATECH model 300 (ARTEK Systems Co., Formingdale, NY).

2.3. Elimination of Excess Substrate

1. Amberlite IRA-900 strong anion-exchange resin (Supelco) was purchased from Teknokroma (Spain) and stored at room temperature. It should be prepared as described in **Subheading 3.3.1.** before use.
2. Microspin filter Ultrafree-MC tubes with low-binding durapore membrane 0.45 µm pore size, obtained from Millipore (Spain).
3. Eppendorf tube mixer 5432 (Netheler + Hinz GmbH, West Germany).

2.4. HPLC Purification of Histamine

1. Mobile phase: Make an aqueous solution of 0.3 *M* NaH_2PO_4 and 10 m*M* octanesulfonic acid in Milli-Q water, adjust it to pH 3.0 and store it (for months) at room

temperature. On the day of use, mix up 79% of aqueous solution with 21% methanol and filter it through nylon filter membranes 0.2 µm pore size. Methanol may evaporate if mixed with aqueous solutions longer than a week before use, which would change the retention time of histamine.
2. HPLC cleaning solution: methanol/Milli-Q water (70/30%). Add 10 m*M* octanesulfonic acid to the water to facilitate posterior equilibration of column with the mobile phase.
3. 0.1 *M* Nitric acid for the HPLC autosampler (*see* **Note 5**).
4. OptiPhase "HiSafe"-3 liquid scintillation cocktail (Wallac, EG&G Company, Turku, Finland).
5. HPLC MERCK-HITACHI: L-6200A Pump, L-4000 UV Detector, L-5200 Fraction Collector, and L-7200 Autosampler (Merck KgaA, Darmstadt, Germany).
6. Hercule 2000 Chromatography Interface with Borwin Chromatography Software (JMBS Developments, France).
7. Reversed-phase C_{18} column 25 × 0.46 cm (Tracer Extrasil ODS-2 of 5 µm particle size; Teknokroma) equipped with a 2 × 20 mm guard column (Upchurch, Teknokroma, Spain).

3. Methods

3.1. Purification of [2,5-^{3}H]L-Histidine

1. In order to avoid excessive radiolysis and check its specific activity, aliquots of the commercial [^{3}H]-histidine standard should be purified 1–3 mo before use.
2. The HPLC system is set to perform a linear gradient from 1 to 6 m*M* of sodium phosphate buffer in 12.5 min (solution A from 4% to 24%) at a flow rate of 1 mL/ min. A low percent of methanol (2%) and octanesulfonic acid (0.1 m*M*) are constant throughout the gradient because they are present in both A and B solutions. Under these conditions, histidine elutes at 9–10 min (*see* **Fig. 1A).**
3. Set UV wavelength at 225 nm and sensitivity at maximum.
4. Stabilize the column in initial conditions for 15 min before every injection.
5. Perform several injections of 0.2–20 nmol of nonradiolabeled histidine as external standard. Then inject about 50 µCi (2 nmol) of [^{3}H]-histidine. The whole histidine fraction should be collected manually into a polypropylene tube while watching histidine peak signal in the UV detector display.
6. Count 5 µL of the collected fraction by scintillation counting and calculate total dpm recovered.
7. To determine the amount of [^{3}H]-histidine, calibrate the [^{3}H]-histidine peak area against a linear regression of nonradiolabeled histidine areas.
8. To calculate specific activity, divide total dpm in histidine fraction by the amount of histidine detected. Specific activity should be similar to that reported by the vendor, of about 50 Ci/mmol. For cleaning of the HPLC, *see* **Note 1**.

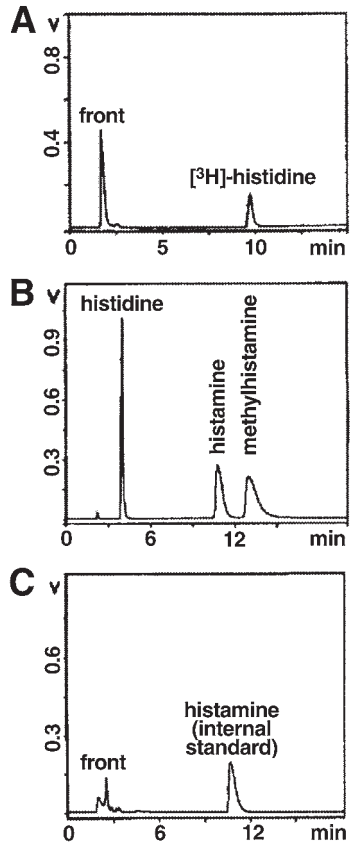

Fig. 1. Chromatograms of **(A)** [^{3}H]-histidine purification, **(B)** standard mixture, and **(C)** sample.

3.2. Incubation of Tissue

3.2.1. Histidine Decarboxylase Enzymatic Assay in Homogenates

1. Obtain a fresh brain from a rat, dissect the region of interest, and homogenize it manually using a glass-Teflon homogenizer in 10 vol ice-cold 10 m*M* potassium phosphate buffer (pH 7.4).
2. Determine protein content of homogenates by the method of Lowry, using bovine serum albumin as a standard. Keep samples at 0–4°C.
3. Prepare deproteinizing mixture containing one volume of 10% trichloroacetic acid with 2 vol 10 m*M* histamine to be used as internal standard. Total volume should be at least 15 µL × total number of samples.

4. Working on ice, mix aliquots of approx 0.2 mg protein (20–40 µL of homogenate) with 10 µM pyridoxal 5′-phosphate (5 µL 0.2 m*M* stock) and homogenization phosphate buffer (pH 7.4) to make a final incubation mix of 100 µL.
5. Add to blank samples 15 µL of previously prepared deproteinizing mixture.
6. Add prepurified [^{3}H]-histidine to all samples (to make 0.25 µ*M*, or 1.25 µCi per sample).
7. Incubate at 37°C for 1 h in a shaking water bath. Do not incubate blank samples, just keep them on ice.
8. Stop incubation by placing all samples on ice and immediately add 15 µL of deproteinizing mixture. Each sample should contain 100 nmol histamine and 0.5% trichloroacetic acid.
9. Vortex thoroughly and centrifuge at 12,000*g* at 4°C for 10 min.
10. Recover supernatants and treat them as described in **Subheading 3.3.**

3.2.2. Histamine Synthesis in Slices of Rat Brain Cortex

1. Obtain a fresh brain from a rat and place it into ice-cold modified Krebs Ringer bicarbonate medium (KRM) for 5 min.
2. Working in a cold environment (4°C), remove meninxs, dissect both cortical lobes and remove white matter.
3. Place cortices over the tissue chopper platform and chop at 0.3 mm.
4. Turn platform 90° and chop again to obtain miniprisms.
5. Place miniprisms in a glass bottle containing 20 mL of ice-cold KRM.
6. Wash miniprisms three times with ice-cold KRM to remove debris of damaged cells and released proteases.
7. Finally, settle miniprisms at the bottom of the bottle and remove excess KRM.
8. Prepare a deproteinization mixture containing 2.5 vol 10% trichloroacetic acid mixed with 1 vol of 10 m*M* histamine to be used as internal standard. Final volume of mixture should be at least 35 µL × number of samples.
9. Distribute 100 µL aliquots of the slice suspension (2–3 mg protein, but *see* **Note 3**) into polypropylene 2 mL tubes.
10. Bubble O_2/CO_2 (95 : 5) through solutions, cap and preincubate for 25 min at 37°C in a shaking water bath.
11. Add to blank samples 35 µL of deproteinization mixture and place them on ice.
12. Then add prepurified [^{3}H]-histidine to all samples (6.25 µCi, to make a final concentration of 0.5 µ*M*) and vortex gently.
13. Five minutes later, make final volumes equal to 250 µL by adding either KRM or depolarizing medium.
14. Maintain incubation/depolarization for 30 min unless otherwise desired. The slices should be treated with O_2/CO_2 periodically during the procedure.
15. Stop incubations by placing all samples on ice and immediately add 35 µL of deproteinization mixture. Each sample should contain finally 100 nmol histamine and 1% trichloroacetic acid.
16. Sonicate samples for 10–20 s at 4°C.

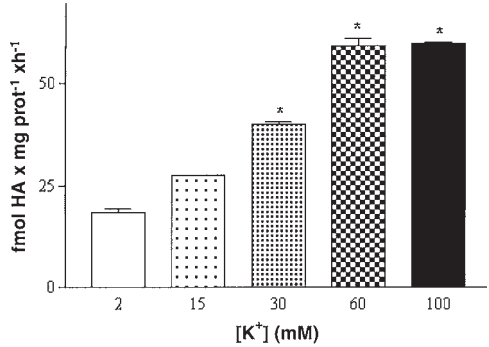

Fig. 2. K^+ - induced depolarization stimulates histamine synthesis in rat brain miniprisms. * $p < 0.01$ as compared to $K^+ = 2$ m*M*. Each column represents the mean ± SEM of 4 to 12 experiments.

17. Then remove a 60-µL aliquot of each sample and determine protein content by the Lowry method, using bovine serum albumin as a standard (*see* **Note 4**).
18. Centrifuge samples at 12,000*g* for 10 min at 4°C.
19. Recover supernatants and treat them as described in **Subheading 3.3.**

The stimulation of histamine synthesis by depolarization is shown in **Fig. 2**.

3.3. Elimination of Excess Substrate

3.3.1. Preparation of Amberlite Anion-Exchange Resin

The strong anion exchange Amberlite resin (IRA 900, mesh 16–50) bears a quaternary amine that efficiently binds carboxylic groups such as that of histidine. However, the functional group of the resin is originally supplied equilibrated with chloride as a counterion, which cannot be efficiently displaced by carboxylate ions. In contrast, carboxylates displace hydroxyl groups, so the resin is regenerated to replace chloride by OH^- as the counterion as follows:

1. Pass 20 vol of 1 *M* NaOH through a large resin column (50 mL) and rinse subsequently with 20 vol Milli-Q water.
2. Recover the regenerated resin from the column and store it in Milli-Q water at room temperature.
3. Calibrate the efficiency of histidine removal by the resin by mixing 100 µL of resin with nonradiolabeled histidine and histamine (*see* **Table 1**). The resulting resin binding capacity (estimated about 0.5–1 meq/g) remains stable for six months of storage simply in Milli-Q water at room temperature.
4. Immediately prior to use, gently rinse the resin 10× in fresh Milli-Q water.

Table 1
Histidine Removal by the Amberlite Anion-Exchange Resin[a]

Resin volume (μL)	Histidine area (% recovery)	Histamine area (% recovery)
0	1920 (100%)	1727 (100%)
20	1459 (76%)	1737 (100%)
40	1559 (81%)	1712 (99%)
60	581 (30%)	1324 (76%)
80	392 (20%)	1278 (74%)
100	305 (16%)	1256 (72%)
120	255 (13%)	1246 (72%)
140	227 (11%)	1200 (70%)

[a]100 nmol of Histidine and histamine were mixed with the indicated resin volume, vortexed for 10 min and injected into the HPLC. The results are means of 2–3 injections per group. SEM did not exceed 1.5% of the mean.

3.3.2. Elimination of [^{3}H]-Histidine

1. Pipet 100 μL of resin bed into the top half of Ultrafree microspin tubes (use a cut yellow pipet tip as for miniprisms; *see* **Note 3**). This is equivalent to 15 mg dry-weight resin.
2. Add the deproteinized supernatants obtained in **Subheading 3.2.** and vortex for 10 min in the multitube shaker at room temperature. During this step, the resin binds most of the [^{3}H]-histidine through its carboxylate group, thereby clearing most excess precursor from the samples. The anion-exchange resin also binds the trichloroacetate ion, raising the pH of the sample from 2.0 to 8.0.
3. Centrifuge the tubes at low speed (4000*g*) for 5 min.
4. Recover the filtrate in the bottom half of the tube, to be injected into the HPLC system.
5. The efficiency of [^{3}H]-histidine removal by the resin can be controlled for each sample by counting a 10-μL aliquot prior to injecting the remainder into the HPLC. It should be noted that resin dilution by addition of deproteinized supernatant affects efficiency of removal. Smaller supernatant volumes (the case of histidine decarboxylase assays) work better than bigger volumes (as is the case for brain miniprisms).

3.4. Histamine Purification by HPLC

1. To purify [^{3}H]-histamine formed, set up HPLC with the mobile phase for histamine at 1 mL/min and let stabilize column for 30 min. Avoid using a column previously used for [^{3}H]-histidine purification.
2. Set up detector wavelength at 225 nm (imidazole ring absorbance).

Table 2
Typical dpm Obtained in Histamine Fractions Under Different Conditions[a]

	Nonlabeled histamine	Incubation blank	Sample	
Rat brain homogenate	12 ± 0.6 (6)	25 ± 0.9 (7)	633 ± 4 (7)	
Cortical miniprisms	123 ± 3 (13)	359 ± 2 (49)	**K+ = 2 m*M***	**K+ = 60 m*M***
			1347 ± 10 (30)	2710 ± 21 (30)

[a]The results are means ± SEM (N). More dpm are used in cortical miniprism experiments than in homogenate incubations, which explains the higher blank values. Nonlabeled histamine represents dpm carried out from a HPLC injection to the next.

3. Inject 100 nmol of each nonradiolabeled histidine and histamine.
4. Measure histamine peak area, as it will be used later to calculate recovery. Histamine retention time should be 10–11 min (*see* **Fig. 1B**). If it is not, adjust composition of mobile phase: Higher methanol content will decrease retention, whereas higher octanesulfonic acid concentration will increase it.
5. Check dpm in the postcolumn eluate before starting sample injection (typically less than 30 dpm/mL). In preliminary assays, it is advisable to monitor dpm baseline by recovering fractions at different times after injection of samples containing [^{3}H]-histamine. For routine determinations, it is sufficient to control for residual dpm in the system by injecting one nonradiolabeled standard of histamine every 10 samples.
6. Typical chromatograms are shown in **Fig. 1C**. Automated fraction collection is started upon detection of the histamine internal standard (8 min after injection, program will activate collection if slope exceeds 10 mV/min), and is completed automatically when the baseline is reached.
7. At the end of the assay, mix all fractions with Optiphase scintillation cocktail and count.
8. For each sample, compare internal standard histamine peak area with external standard histamine to obtain recovery. Use recovery to estimate dpm of histamine synthesized and subtract dpm in blank samples. Use specific activity of substrate to find out the amount of histamine synthesized. Use protein content and incubation time to quantify histidine decarboxylase activity in homogenates or histamine synthesis in slices.

The sensitivity of the assay will depend directly on the dpm in obtained for the blank samples. **Table 2** shows typical blanks obtained in homogenates and slices of rat brain cortex. Given that the specific activity of substrate is about 80 dpm/fmol, these blanks permit us to quantify as low as 2–5 fmol of histamine formed.

4. Notes

1. To clean up the HPLC after purification of [^{3}H]-histidine, remove the column and flow a few milliliters of 0.1 *M* nitric acid (pH = 1.0) through the HPLC system. This step will remove residual tritium in injection loops and the spectrophotometer. Next, wash with filtered Milli-Q water, and then with methanol (100%), to remove nitric acid. Check the system for residual dpm by scintillation counting. Next, reinstall the column and clean it with methanol/Milli-Q water (70/30%) at a flow rate of 0.8 mL/min until residual tritium is under 1000 dpm/mL. Avoid exposure of the column to nitric acid which would result in loss of the C_{18} material. Do not use the same column for [^{3}H]-histidine and [^{3}H]-histamine purifications.
2. It is possible store a solution of NaCl, KCl, $MgSO_4$,$7H_2O$, and KH_2PO_4 at 4°C for up to 2 mo. When required, add the rest of the solid reagents of the modified KRM. Do not add $CaCl_2$ and $NaHCO_3$ at the same time because Ca_2CO_3 will be produced and precipitate in the solution. The modified KRM has an approx pH 7.4 after gassing with O_2/CO_2.
3. Miniprisms in suspension can be pipeted into a standard yellow pipet tip cut at 1 cm from the tip. Although excessive variability should not be expected, it is still advisable to control for pipeting accuracy by determining the protein content in each tube after incubation. This will also be useful in order to express final results of histamine synthesis as per mg of protein.
4. The Lowry procedure requires an alkaline medium. When samples are acidic, add NaOH as required for the Lowry procedure and check alkaline pH with a paper pH indicator before adding other Lowry reagents.
5. To clean up between consecutive sample injections, the system washes the injection loop, needle, and syringe five times with 0.1 *M* nitric acid at pH 1.0. These washing steps do not introduce nitric acid into the column. Residual dpm carried from sample to sample can be controlled by placing a nonradiolabeled histamine standard to be injected in between (*see* **Table 2**). After the sample injection it is recommended to replace nitric acid solution in the injector by Milli-Q water in order to avoid corrosion and rusting of metals in the automatic injector. Finally, clean the column with methanol/water with 10 m*M* octanesulfonic acid (70/30%) at a flow rate of 0.8 mL/min for 2–3 h. This should remove residual [^{3}H]-dpm from the HPLC system, but this can be checked by counting dpm in the eluate (residual dpm are typically 20–30 dpm).

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