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What is This?

Development of a High-Throughput Assay to Measure Histidine Decarboxylase Activity

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Histamine is a well-known mediator of allergic, inflammatory, and neurological responses. More recent studies suggest a role for histamine and its receptors in a wide range of biological processes, including T-cell maturation and bone remodeling. Histamine serum levels are regulated mainly by the activity of the histamine-synthesizing enzyme histidine decarboxylase (HDC). Despite the importance of this enzyme in many physiological processes, very few potent HDC inhibitors have been identified. HDC assays suitable for high-throughput screening have not been reported. The authors describe the development of a fluorescence polarization assay to measure HDC enzymatic activity. They used a fluorescein-histamine probe that binds with high affinity to an antihistamine antibody for detection. Importantly, they show that probe binding is fully competed by histamine, but no competition by the HDC substrate histidine was observed. The automated assay was performed in a total volume of 60 µL, had an assay window of 80 to 100 mP, and had a Z´ factor of 0.6 to 0.7. This assay provides new tools to study HDC activity and pharmacological modulation of histamine levels. (*Journal of Biomolecular Screening* 2006:816-821)

Key words: histidine decarboxylase, histamine, screening, fluorescence polarization

INTRODUCTION

HISTAMINE IS A POTENT BIOAMINE with multiple activities in various pathological and physiological conditions.[1] In addition to its well-characterized effects in the acute inflammatory and allergic responses, histamine regulates several aspects of antigen-specific immune responses.[2] Recent findings, such as the discovery of a novel histamine receptor (H4) on immunocompetent cells and the demonstration of a role for H1 and H2 receptors on T helper cell polarization, have generated much interest in the immune-regulatory mechanisms triggered by histamine.[2,3]

Histidine decarboxylase (HDC) is the rate-limiting enzyme in the biosynthesis of histamine.[4] Mammalian HDC is a member of a large family of pyridoxal 5-phosphate (PLP)–dependent enzymes.[5] HDC is expressed in most tissues, but the highest levels are found in the skin, the gastrointestinal (GI) tract, and the airways. HDC is a 74-kD enzyme that is converted to a shorter 53-kD form.[6] Both forms are active in vitro, but they are not found in the same subcellular compartments, with the 74-kD form being found predominantly in the endoplasmic reticulum.[7]

The recent generation of HDC-deficient mice provided a good system to study the role of endogenous histamine in a broad range of normal and disease processes.[4] The HDC^{–/–} mice have a reduced number of mast cells and reduced granular content such as mast cell proteases.[8] These mice show reduced airway hyperresponsiveness,[9] reduced vascular permeability,[10] reduced skin inflammation,[11] and increased bone density.[12] Thus, potent inhibitors of HDC activity might prove useful in allergic, inflammatory, immunological, bone, and cardiovascular disorders. Finally, histamine has also been shown to be a positive regulator of proliferation in some types of cancers such as melanoma.[13]

The biological role of histamine has been extensively studied with pharmacological approaches using histamine receptor–specific agonists or antagonists. Despite the important role of HDC in allergic and inflammatory responses, very few small-molecule inhibitors of this enzyme are known. Most of these inhibitors were discovered by rational design strategies and are histidine analogues. The best-characterized HDC inhibitor is the irreversible inhibitor α-fluoromethyl histidine.[14] The ability to identify novel classes of HDC inhibitors is limited by the lack of assays that are suitable for HTS. The most commonly used assay to measure HDC activity is based on the o-phthalaldehyde (OPT) method.[15] This assay is not selective for

histamine over histidine and involves a chromatographic separation of the enzyme product from the substrate. Another more sensitive HDC assay uses the conversion of [14C]-labeled histidine to [14C]-labeled histamine.[16] Thin-layer chromatography is then used to resolve substrate and product. Histamine enzyme-linked immunosorbent assay (ELISA) kits could potentially be adapted to measure HDC activity. However, these assays require an acetylation step (acetylated histamine) to reach any useful selectivity and sensitivity. Moreover, these procedures require many washing steps, rendering them less amenable for high-throughput screening (HTS). Recently, Claret et al.[17] reported on the development of a time-resolved fluorescence assay (TR-FRET) to measure histamine release from cells. The TR-FRET assay is based on the competition between histamine and cross-linked allophycocyanine (XL665)-conjugated histamine for binding to an antihistamine antibody labeled with Europium cryptate. This assay was reported to have a sensitivity threshold of 1.3 nM and a Z factor of 0.9. It is not clear to what extent the antibody used in this method discriminates between the substrate histidine and product histamine, and thus this approach may not be amenable to the assay of HDC activity.

Our goal was to develop a homogeneous HDC assay suitable for HTS. We report herein the development and validation of a robust fluorescence polarization (FP) assay for HDC and its use in HTS for inhibitors for this enzyme.

METHODS

Reagents

FITC-Histamine (Thiourea, N-[3´,6´dihydroxy-3-oxospiro] isobenzofuran-1 (3H),9´-[9H]xanthene-5(or 6-yl)-N´-[2-(1H-imidazol-4-yl)ethyl]-2,4-dimethyl-, disodium salt) was obtained from Invitrogen/Molecular Probes (Eugene, OR). Histamine monoclonal antibody 22.12 was obtained from Argene (Varilhes, France). L-Histidine, histamine, potassium phosphate (1M mono- and dibasic solutions), polyethylene glycol 400 molecular weight, ethylene glycol tetraacetic acid (EGTA), dithiothreitol, pyridoxal-5-phosphate, and sodium chloride were from Sigma Chemical Co. (St. Louis, MO). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from Pierce Chemical Co. (Rockford, IL). DMSO was from Baker Chemical Co. (Phillipsburg, PA). Black opaque polystyrene 384-well plates were obtained from Corning-Costar (Cambridge, MA). The known HDC inhibitors, histidine-methyl ester, and His-Phe were from Sigma Chemical Co., and α-fluoromethyl histidine was obtained from the Boehringer Ingelheim Pharmaceuticals compound library.

Buffers

HDC buffer is composed of 200 mM potassium phosphate (pH 6.8), 2% PEG-400, 0.2 mM EGTA, and 0.03% CHAPS. FP buffer is 16.6 mM Tris-HCl (pH 7.5) and 50 mM NaCl.

HPLC determination of histamine content

HPLC separations were performed on an Agilent 1090M equipped with a diode array detector. A Delta-Pak HPI C4 300 Å, 2.0 × 150-mm column (Waters, Milford, MA) was used. The mobile phase consisted of 20 mL PIC® B-8 Low UV Reagent (Waters) in 1 L 10-mM triethylamine phosphate, pH 3.0. All separations were carried out at room temperature (22 °C) with a flow rate of 0.2 mL/min and were monitored at a wavelength of 215 nm.

Histamine concentrations were calculated by linear regression from standard curves for histamine concentrations (in μM) versus the area response. The standard curves were generated using duplicate injections of histamine in control buffer at concentrations of 0 to 200 or 600 μM. Six equally spaced concentrations were used. Correlation coefficients were greater than 0.999.

Baculoviral expression and purification of GST-HDC

A gene fragment containing the first 1431 bases (amino acid residues 1-477) of human HDC (accession number: NM_002112) was amplified by PCR using total RNA from the human mast cell line HMC-1.17. A thrombin cleavage site was incorporated at the 5´ end adjacent to the first base, and this construct was subcloned into pDEST20 using Gateway Cloning Technology (Invitrogen). This GST-HDC construct was made into a bacmid and transfected into Sf9 cells for viral amplification according to standard protocols. The bacmid DNA was verified by sequencing. HDC protein was produced in Sf9 insect cells using a 25-L working volume–stirred tank bioreactor with a standard biomass yield of ~300 g. Then, 25 mg of leupeptin was added 24 h into the 48-h infection. The HDC was purified using a glutathione sepharose affinity column (Amersham, Uppsala, Sweden) with a standard yield of 33 mL of HDC at 1.8 mg/mL.

Histidine decarboxylase assay

In the standard assay, HDC, diluted to 90 nM in HDC buffer plus 0.9 mM DTT and 99 μM PLP, was added to a black opaque 384-well plate in 20 μL. Test compound in HDC buffer plus 6% DMSO or buffer alone was added to the plate in 10 μL. Then, 6 nM FITC-histamine and 3.6 mM histidine were combined in FP buffer and transferred to the plate in 10 μL. Finally, 90 nM antihistamine antibody was added in 20 μL of FP buffer. Thus, the final concentrations in the assay were as follows: HDC 30 nM, FITC-histamine 6 nM, histidine 600 μM, antihistamine antibody 30 nM, and DMSO 1%. The plate was incubated at 37 °C for 90 min. The fluorescence polarization signal was read on an LJL Analyst (Molecular Devices, Sunnyvale, CA) with excitation at 485 nm, emission at 530 nm, a fluorescein dichroic mirror at 505 nm, and G factor set to 1. A 96-well plate version of the assay, used in assay development, was performed as described above with twice the volumes indicated for 384-well plates.

High-throughput screening

The assay was automated on a Zymark Allegro robotics system (Caliper-Zymark, Hopkinton, MA), using a Multidrop to add enzyme, the Sciclone to add substrate/probe and test compound, and a Multidrop to add the antibody. The plates were incubated at 37 °C in a humidified environment, and fluorescence polarization was read on an LJL Analyst integrated into the Allegro system using the settings described above. Compounds were screened at a concentration of 5 μg/mL. The percentage of control (POC) values were calculated relative to an assay blank containing complete reaction minus HDC and a 100% control containing HDC buffer with 1% DMSO in place of compound.

RESULTS AND DISCUSSION

An important parameter contributing to the performance of an FP assay is the affinity of the fluorescent probe for its receptor or target molecule.[18] As a general rule, the Kd for probe binding to its receptor is inversely proportional to the fraction bound. Thus, high-affinity binding allows for an optimal fluorescent-ligand/receptor stoichiometry and a robust FP signal. We screened multiple antihistamine antibodies to find one that had a suitable affinity for our histaminyl-fluorescein probe (data not shown). Only 1 of these antibodies, D22.12, had an affinity that was high enough for the development of an FP assay. Interestingly, the D22.12 antibody was generated by immunizing mice with 2-histaminyl-1,4-benzoquinone coupled to albumin,[19] whereas all the other antibodies we tested were generated by immunization with histamine or acetylated histamine coupled to albumin. Thus, the high-binding affinity of D22.12 for histaminyl-fluorescein could result from a structural homology between the immunogen used to obtain D22.12 (histaminyl benzoquinone) and the histaminyl-fluorescein probe.

We first examined the affinity of the probe for the D22.12 antibody and the specificity of the interaction, both of which are crucial to constructing a robust assay in a competitive mode. **Figure 1A** shows a binding curve of the probe to an increasing amount of antibody, as determined by measurement of anisotropy. The probe concentration was held at 6 nM, and the antibody concentration varied over approximately 3 logs. A dissociation constant of 3.9 nM was determined upon fitting the data.

The specificity of the antibody for histamine over histidine was then tested (**Fig. 1B**). Probe and antibody concentrations were held constant at 6 and 50 nM, respectively, whereas the concentrations of histamine and histidine were varied as shown. Histidine was unable to compete with FITC-histamine for binding to the antibody over the 5 log range examined. Histamine, however, freely competed with the probe for antibody binding, yielding an IC_{50} of 135 μM.

The K_m of HDC (53-kD form) for its substrate histidine is 275 μM.[20] Thus, an important requirement for the development of an HDC assay is the selectivity for histamine over histidine.
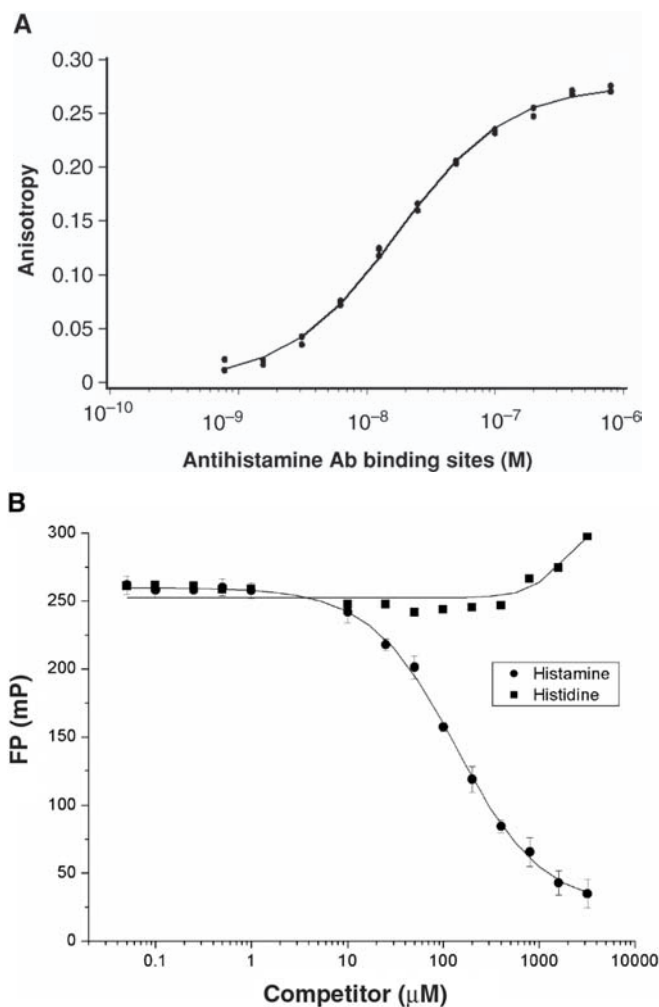

FIG. 1. (**A**) Binding of FITC-histamine to the antihistamine antibody. The assay was run in duplicate in a 96-well plate as described in the Methods section, with FITC-histamine at 6 nM. The data were fit in SAS, and a Kd of 3.9 nM was determined. (**B**) Displacement of FITC-histamine binding to the antihistamine antibody by histamine (●) or histidine (■). Triplicate determinations in a 96-well plate were performed as described in the Methods section, with 6 nM FITC-histamine, 50 nM antihistamine antibody, and the indicated concentration of competitor ligand.

Our FP assay shows over a 100-fold selectivity for histamine over histidine. However, as shown in **Figure 1B**, the histidine concentration should be kept below 2 mM due to a nonspecific increase in the FP signal. This limitation should not be an issue in most applications measuring HDC activity given that the K_m of the enzyme for histidine is much lower than the maximal amount of histidine tolerated by the assay. Thus, the tight and specific binding of FITC-histamine to the histamine monoclonal antibody and the ability to compete for that binding with the product of the enzymatic reaction suggest that it is possible to develop a robust competitive FP assay for HDC.
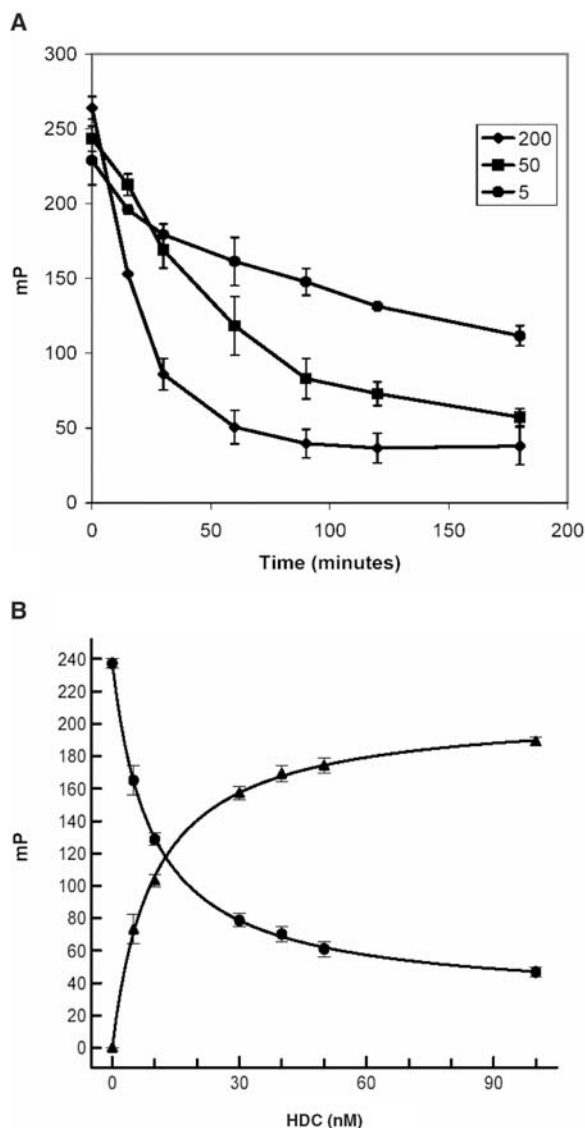
FIG. 2. (**A**) Time course of the histidine decarboxylase (HDC) assay at various enzyme concentrations. Triplicate reactions in a 384-well plate were initiated by the addition of the indicated concentration of HDC, and fluorescence polarization was determined at time points from 0 to 180 min of incubation. Probe, substrate, and antibody concentrations were as described in the standard assay. (**B**) HDC titration at 90 min. Assays (384-well) were performed in quadruplicate using the indicated concentration of HDC for 90 min at 37 °C on the Allegro system. Data represent the total mP signal (●) and the assay window (▲) corresponding to each HDC concentration tested. The assay window is defined as the difference in mP between the blank (no enzyme) and the reaction wells.

A time course of the enzymatic reaction at various concentrations of HDC was performed to establish optimal assay conditions (**Fig. 2A**). Histidine was used at a concentration of 600 μM, which is more than twice the Km for HDC.[20] At HDC

Table 1. IC$_{50}$ Values for Known Histidine Decarboxylase Inhibitors (μM)

Inhibitor	FP	HPLC	Reported
α-Fluoromethyl histidine	5	2	8[a]
Methyl ester histidine	9	5	2[b]
His-Phe	109	93	44[c]

IC$_{50}$ values (μM) were determined for all 3 inhibitors in both the fluorescence polarization (FP) and high-performance liquid chromatography (HPLC) assays. The corresponding IC$_{50}$ values obtained by other groups using a ^{14}C radiometric assay are also reported.

a. As reported by Duggan DE, Hooke KF, Maycock AL: Inhibition of histamine synthesis in vitro and in vivo by S-alpha-fluoromethylhistidine. *Biochem Pharmacol* 1984;33:4003-4009.

b. As reported by Kelly JL, Miller CA, White HL: Inhibition of histidine decarboxylase: derivatives of histidine. *J Med Chem* 1977;20:506-509.

c. As reported by Hammar L, Ragnarsson U: Peptide inhibition of mammalian histidine decarboxylase. *Agents Actions* 1979;9:314-318.

concentrations > 100 nM, the reaction is linear for only about 30 min. To balance the amplitude of the assay window (defined as the difference in mP between the blank without enzyme and reaction wells) with the enzyme requirements for a large-scale screen and linearity of the reaction, we chose to use 25 to 50 nM HDC and an incubation time of 90 min in the standard assay. This was further refined by performing a titration of HDC for a 90-min reaction period on the Allegro robotic system (**Fig. 2B**). From this experiment, we selected an enzyme concentration of 30 nM for further assays. Thus, the final standard assay conditions were set at 30 nM HDC, 30 nM antibody, 600 μM histidine, and 6 nM FITC-histamine in a total volume of 60 μL for 90 min at 37 °C. The PLP concentration was set at 33 μM to maintain saturation of the enzyme.

We sought to further validate our assay using 3 known HDC inhibitors. **Figure 3** shows the behavior of 2 of these inhibitors in the standard assay: methyl ester histidine and α-fluoromethyl histidine (α–FMH). In both cases, titration of the compound results in dose-dependent inhibition of the HDC activity. We determined an IC$_{50}$ of 5 μM for α–fluoromethyl histidine and 9 μM for methyl ester histidine. The IC$_{50}$ values for these 2 compounds and for the dipeptide inhibitor His-Phe are reported in **Table 1**. Importantly, the IC$_{50}$ values obtained in the FP assay are in good agreement with values we obtained using the high-performance liquid chromatography (HPLC) assay and with values reported by other groups using a radiometric method (**Table 1**).

The assay as described was then used to screen compounds from the Boehringer Ingelheim library at a final concentration of 5 μg/mL. 384-well plates were set up to contain 352 compound wells, 16 control wells (no compound), and 16 blank wells (no enzyme) per plate. Compounds, in neat DMSO, were diluted in buffer to give a final DMSO concentration of 1% in the assay. This concentration of DMSO was shown to have no effect on the enzymatic activity or stability (data not shown). The assay was fully automated on the Allegro robotic system,
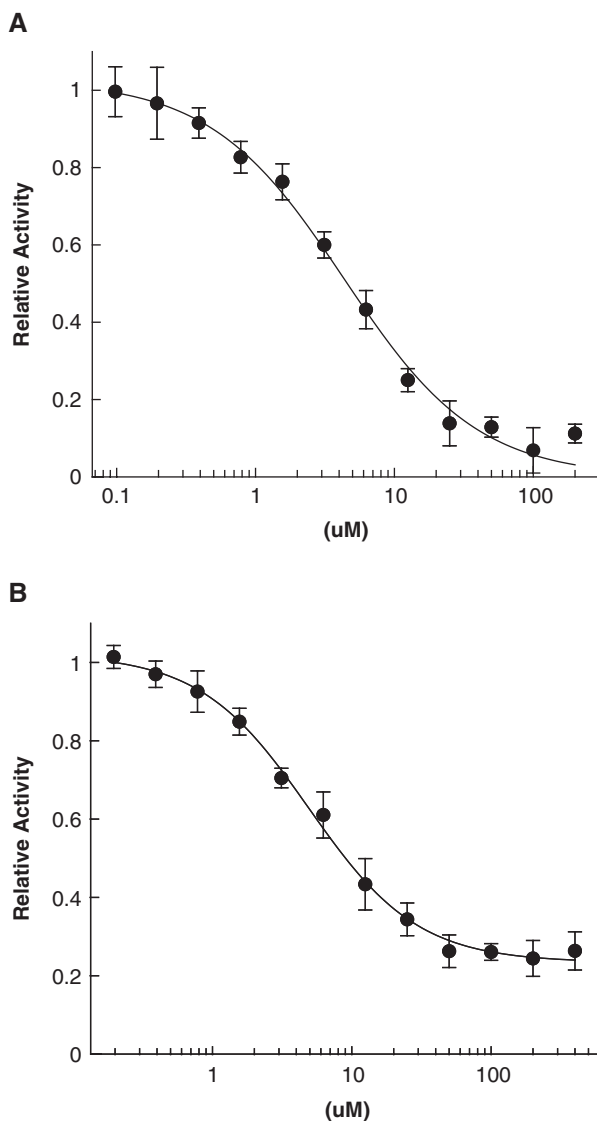
A

B

FIG. 3. Inhibition of histidine decarboxylase (HDC) by (**A**) methyl ester histidine and (**B**) α-fluoromethyl histidine. Reactions were run using the standard conditions as described in the Methods section and the indicated concentration of inhibitor. Data points are expressed as enzyme activity relative to control wells containing no compound (1% DMSO). IC_{50} values were obtained by fitting the data using XLFit4 (IDBS Software). Error bars show the mean ± SD of triplicate determinations.

FIG. 4. Scatterplot of blank/no enzyme (○) and positive control (●) values from a single day's screening run of 90 plates.

Decarboxylases form a large family of enzymes playing important physiological roles. For example, DOPA decarboxylase is responsible for the synthesis of the key neurotransmitters dopamine and serotonin via decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan, respectively.[21] Current methods used to measure transmitters such as serotonin and dopamine are analogous to the histamine detection techniques. Thus, the assay technique we describe here for HDC could be applied to related enzymes such as DOPA decarboxylase and allow for the development of new inhibitors with better pharmacological characteristics.

In conclusion, the novel HDC assay described herein was shown to be useful for the determination of HDC activity in a high-throughput setting. This simple, homogeneous assay has the potential to play a valuable role not only in basic research on this enzyme but also in the discovery of novel HDC inhibitors as potential therapeutics.

ACKNOWLEDGMENTS

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enabling throughputs of approximately 100 plates per day. **Figure 4** shows a scatterplot of blank and control wells for a single screening run of 90 plates, with an average Z´ of 0.7 and an assay window of approximately 100 mP. In excess of 600,000 compounds were screened in the assay with a confirmed hit rate of 0.05% using 60% of control (1% DMSO) as the hit criteria. Confirmed hits were subsequently assayed in 10-point dose response to assess potency.
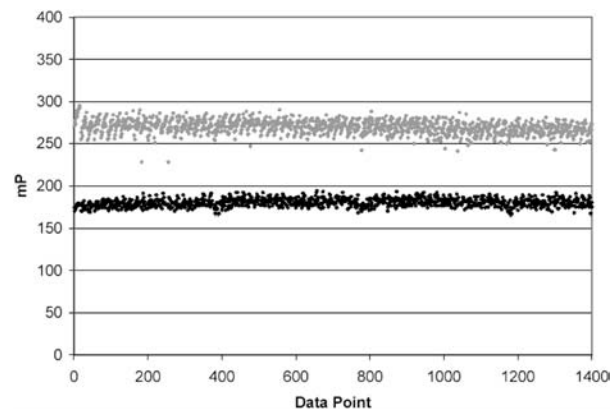
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