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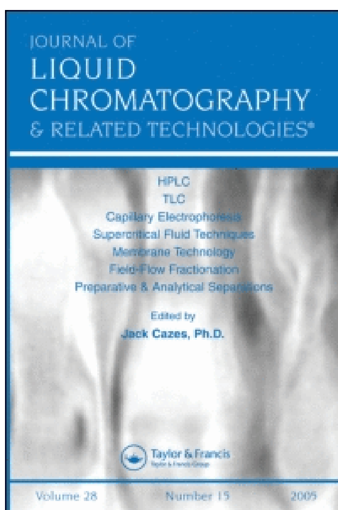

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SIMPLEX OPTIMIZATION FOR THE SIMULTANEOUS HPLC ASSAY OF THE ACTIVITIES OF PURINE-NUCLEOSIDE PHOSPHORYLASE AND HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE

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ABSTRACT

The modified sequential simplex procedure is shown to be effective for maximizing a complex enzyme assay. The optimum levels for two factors, pH and substrate concentration, for a coupled enzyme assay of hypoxanthine-guanine phosphoribosyl-transferase (HGPRTase) and purine nucleoside phosphorylase (PNPase) were found by searching a factor space made up of these variables. The performance index or response to be optimized was a function of the product of the two single activities. The maximum activity for this function was found at a pH of 7.9 and a concentration of inosine in the reaction mix of 0.84 mM.

INTRODUCTION

The optimization of a complex biochemical system requires the adjustment of a set of controlling variables to maximize or minimize a response. In the case of an enzyme assay, that

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response is the amount of substrate converted per unit time. Therefore, the response which is of interest for maximization is the activity of the enzyme. For a situation where no interaction between the variables exists, the traditional experimental approach of changing one variable at a time while holding all the others constant is acceptable. However, in cases where interaction among the variables exist, this univariate approach will frequently miss the optimum. The activity of an enzyme frequently shows interaction behavior. For example, the kinetics of HGRPTase for its substrate hypoxanthine are highly dependent upon pH. The enzyme can require more or less substrate given a certain pH of the reaction medium. Therefore, the Michaelis-Menten curve is sigmodial, indicative of allosteric or cooperative behavior (1).

Optimization techniques which can be used to deal with variable interaction include the so-called factorial designs (2,3). In these designs the experiment is modeled in such a way that the variables are changed simultaneously according to a prescribed recipe. A set of comparisons is then chosen which will permit estimates of the interactions between the variables.

While these designs are useful, they are subject to a number of disadvantages. For example, an excessive number of experiments is required to find all interactions. Factorial designs are best suited for application on nonordered, discrete possibilities. These designs frequently fail with variables that are continuous and ordered. Therefore, the correctness of a factorial model will be highly dependent upon the levels chosen for the initial experiments.

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Simplex search techniques are well-known methods for finding the optimum areas of response on an experimental surface (4-11). There are a number of variations of the simplex algorithm, such as sequential, modified, and super modified. These variations occcur in the steps the simplex takes to find an optimum. However, all these methods are similar in structure and experimental approach.

A simplex is by definition the simplest geometric figure which describes a factor space. The factor is made up of experimental variables. The dimensionality of a simplex for a given factor space is the number of experimental variables plus one. Thus, the geometry of a two-variable simplex is a triangle, a three variable simplex a tetrahedron, and so on. The simplex method acts as an optimization procedure through its evolutionary nature and uses a number of rules to dictate which experiments should be performed in the search for the optimum. Thus, each experiment both dictates the conditions for the next and uses the acquired data to define the optimum.

The so-called modified simplex is a method which allows the size of the simplex to change during the search (9,10). Therefore, the search can move more quickly to an area of higher response, and once there, quickly "home in" on the optimum. The speed and simplicity of this technique make the simplex method highly attractive for optimizing a complex procedure such as a coupled enzyme assay. Therefore, we report on the optimization of the simultaneous measurement of activities of purine nucleoside phosphorylase (PNPase) and hypoxanthine-guanine phosphoribolsy-transferase (HGPRTase) using the simplex search technique. In this

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assay, PNPase converts the nucleoside, inosine, to the base, hypoxanthine. The hypoxanthine is then used as a substrate for the HGPRTase. This reaction converts the hypoxanthine to the nucleotide, inosine-5-monophosphate (IMP). This coupled assay requires that the product from the PNPase reaction, hypoxanthine, be present in sufficient quantities to saturate the second enzyme HGPRTase. The pH optima and behavior of these enzymes are vastly different and complicate the substrate requirements of both reactions. Therefore, these factors which will predominate in the assay make up the factor space in which the combined enzyme activities were maximized.

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EXPERIMENTAL

Chromatographic Conditions

The liquid chromatographic system consisted of an M6000 pump, a model 440 absorbance detector, and U6K injector (Waters Associates, Milford, MA), and a Hewlett-Packard 3380A Integrator (Avondale, PA). Separations were performed on a 4 mm x 25 cm Partisil 5-ODS analytical column preceded by a guard column containing Co-Pell-ODS (Whatman, Incorporated, Clifton, NJ). Separations were performed isocratically at ambient temperature. The mobile phase consisted of 0.02 M KH_2PO_4 (pH 4.70), in which there was 5% methanol.

Reagents

Inosine-5'-monophosphoric acid (IMP), hypoxanthine (Hyp), inosine (Ino), guanine (Gua), tris(hydroxymethyl)amine methane

(Trizma base), magnesium sulfate ($MgSO_4$), and 5-phosphoribosyl pyrophosphate (PRPP) were obtained from Sigma Chemical Co. (St. Louis, MO). All the water for preparation of the eluents and standard solutions was doubly distilled, deionized and filtered through 0.45 micromembrane filters (Millipore Corp., Meford. MA). Distilled in glass methanol was obtained from Burdick and Jackson (Muskegon, MI). Potassium dihydrogen and disodium hydrogen phosphate were HPLC grade and purchased from Fischer Scientific Co. (Waltham, MA)

Enzyme Assays

PNPase and HGPRTase activities were measured by HPLC. The method is based upon the individual optimizations of these enzymes and is reported in detail elsewhere (12,13). The assay was performed on erthrocyte lysates. Red blood cells from 15 donors were pooled, washed in 0.9% w/v saline, packed by centrifugation (20 minutes at 1154 RCF) and resuspended in saline to a hematocrit of 5%. The suspension of cells served as the stock of enzymes for all of the optimizations and was used throughout the simplex search.

For each experiment, 50 microliters of the cell suspension were pipetted into glass test tubes. The cells were lysed by addition of 100 microliters of cold water, followed by freezing in liquid nitrogen. Lysates were preincubated at 37°C for 5 minutes. At time zero, the reactions are initiated by addition of the PNPase substrate, inosine, in 0.05 M phosphate solution. The concentration and pH of the inosine solution changed as the simplex search dictated. The reaction was incubated for exactly 5 minutes in a

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thermostated bath. After 5 minutes, the second reaction, that of HGPRTase, was initiated by addition of 200 microliters of a solution which contained 10 mM PRPP and 20 mM $MGSO_4$. The pH of this solution was the same as that of the inosine solution for a given measurement. The second reaction proceeded in the temperature-controlled bath for 10 minutes. Reactions were terminated by addition of 25 microliters of 3 N HCl. After reaction termination, the mixtures were neutralized and analyzed by HPLC.

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RESULTS AND DISCUSSION

The first requirement for the optimization is the development of a response function or performance index. Generally, in an enzyme assay, the activity of an enzyme is maximized. In this case, the function must represent the combined activities of two enzymes; PNPase and HGPRTase. The response functions which were examined were the sum of the activities, the product of the activities, and a quadradic built from the activities. It was found that the additon of the activities simply maximized PNPase at the expense of HGPRTase. Both the quadradic and the product relationship provided a reasonable response function. The product was a simpler relationship and was chosen for the response function.

Response Function = Product of PNPase Acitivity and HGPRTase Activity.

Activity = Units/ml packed cells.

One Unit = One micromole of substrate converted per minute.

Once the response function has been chosen, the optimization

using the modified simplex algorithm can proceed. The technique requires that experiments be performed based upon a set of rules. The application of these moves forms the simplex. The moves are made as follows:

1. The initial starting coordinates of pH and inosine concentration are chosen.
2. A move is made after each experiment is performed and activity determined. By move we mean the definition of new factor levels to be tried in the next activity determination.
3. A simplex moves by removing the point in the current simplex of lowest response and replacing it by its mirror image across the plain of the figure.
4. If the new vertex lies outside any boundary established at the beginning of the experiment, the response function will be assigned an extremely low value, regardless of the actual response at that point. This assignment will force the simplex back into an area which is experimentally desireable. For example, HGPRTase has an optimum pH at approximately 9.2 and PNPase has little activity there. Thus, if the search over the combined activity surface were to extend into that region, the response function would be assigned the worst value possible. This technique will make the simplex circle the optimum, provided that the simplex is not so small as to approximate the indeterminate error.

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Table 1

Initial Coordinates and Boundaries

The simplex search was started at pH 8.5 and .33 mM Ino. The search was restricted to the bounds listed.

Factor	Lower	Upper	Start
pH	7.0	9.0	8.50
mM INO	0.1	3.0	0.30

Table 1 lists the initial coordinates and boundaries established for this search.

The values are chosen by consideration of the behavior of the individual enzymes. The pH optimum of HGPRTase is 9.2, while that of PNPase is 7.5. Additionally, the overall behavior is different for these enzymes. PNPase has very little activity above pH 8.5; therefore, this pH was used as an upper bound in the search. Likewise, HGPRTase has very little activity below pH 7.0. This value was used as the lower boundary. The upper and lower boundary for the concentration of inosine were chosen to fall in the range of the two Michaelis constants (Km) of the substrates. After a five-minute incubation of the lysate with inosine, sufficient hypoxanthine is produced to react with HGPRTase.

The movement and progress of the simplex on the combined enzyme surface is graphed in Figure 1. The data for this search is compiled in Table 2. The initial simplex is midsize. The size is increased through expansions as the simplex moves to higher numbers, from simplex 1 to simplex 4. At simplex 4, the figure starts to contract. This contraction continues until the change

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TABLE 2

Movement and Progress of Simplex
on Combined Enzyme Surface

Data from a simplex search on the coupled enzyme response surface. The units of factor 1 are mM inosine. The units of factors 2 are pH. The units of response are activity units, Unit/ml of RBC.

Vertex	Factor 1	Factor 2	Response
1	0.300	8.50	0.68
2	0.397	8.52	1.32
3	0.321	8.52	0.22
4	0.370	8.42	1.87
5	0.393	8.34	2.64
6	0.483	8.37	3.25
7	0.584	8.30	4.29
8	0.581	8.12	5.26
9	0.670	7.92	5.99
10	0.860	7.88	6.48
11	1.100	7.66	5.18
12	0.953	7.51	4.19
13	0.676	8.10	5.83
14	0.868	8.06	6.24
15	1.056	7.85	6.05
16	0.961	7.91	6.38
17	0.957	7.73	5.93
18	0.890	7.98	6.44
19	0.794	7.95	6.44
20	0.835	7.94	6.49

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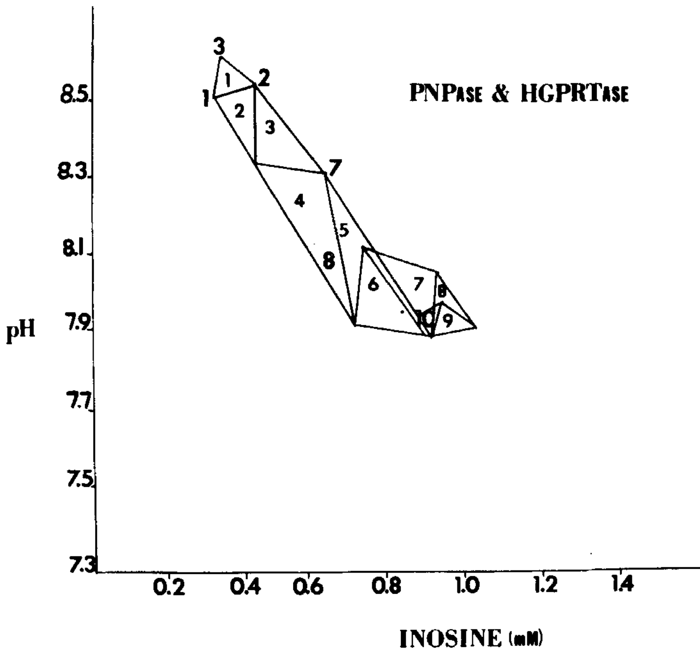

Figure 1: Simplex search on the surface of the coupled enzyme assay. The search was started at the coordinates listed in Table 1 and continued until the values converged.

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in response with change in simplex size becomes too small to measure. Thus, the change in the response function is at the level of experimental error. The last few simplex figures show little change, thus the centroid of simplex 10 is the optimum. The centroid, which is the midpoint of the figure, is a convenient estimate of the best point in the best simplex. The coordinates of this point will be used in future assays. The coordinates are 0.84 mM inosine at pH 7.9. This optimum was achieved in 20 experimental trials; however, after 9 moves the response function took values greater than 99% of the optimum. These data illustrate

the application of the simplex algorithm. In regions far from optimum, the expansion and contractions of the figure are fairly large. However, as the optimum is approached, the simplex makes smaller and smaller moves. Thus, as expected, the size of the simplex contracts around the optimum. Table 3 shows the contribution of the individual enzymes to the response function. These data illustrate that the optimum found is a combined optimum and not unduly weighted toward either enzyme.

One of the advantages of the technique is that it is functional in the presence of indeterminate error. Therefore, replication of the data is not required. The search can be started in virtually any place in factor space and still converge upon the optimum. Experimental or determinate errors do not grossly interfere with this search until they approach the step size of the simplex. In this coupled assay, the factors are constrained due to the kinetic requirements of the individual enzymes. The individual behavior of PNPase and HGPRTase was examined independently by using the same simplex search. Figures 2 and 3 show the progress of the respective simplex for the activities of PNPase and HGPRTase uncoupled. The simplex for the HGPRTase lead the search in the direction of highest pH. This search for <u>highest</u> pH is important since, alone, HGPRTase achieves maximum activity far above physiological pH. The search of the PNPase response surface also demonstrates the correctness of the direction. This enzyme has a pH optimum much closer to physiological pH, and thus the simplex was directed through experiments to areas of lower pH.

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TABLE 3

Contribution of Individual
Enzymes to Response Factor (Activity in Units/ml)

The individual activities are listed of both HGPRTase and PNPase which were obtained at each vertex of the simplex and used for the calculation of the total response.

Vertex	PNPase	HGPRTase	Total
1	1.20	0.57	0.69
2	2.44	0.542	1.33
3	0.44	0.507	0.22
4	3.13	0.60	1.88
5	4.14	0.64	2.65
6	5.35	0.62	3.26
7	6.89	0.62	1.29
8	7.75	0.68	5.28
9	8.57	0.70	6.00
10	9.88	0.66	6.47
11	8.84	0.59	5.18
12	7.05	0.66	4.19
13	8.77	0.66	5.03
14	9.63	0.65	6.36
15	9.63	0.65	6.00
16	9.94	0.64	6.24
17	9.45	0.63	5.83
18	9.92	0.65	6.35
19	9.68	0.67	6.44
20	9.85	0.66	6.49
21	9.93	0.65	6.49

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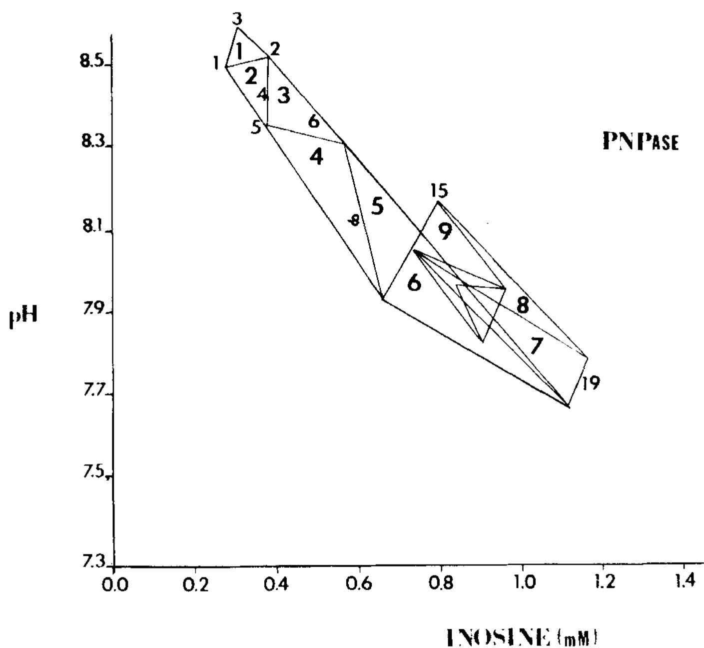

Figure 2: Simplex search conducted on the surface of the enzyme PNPase alone.

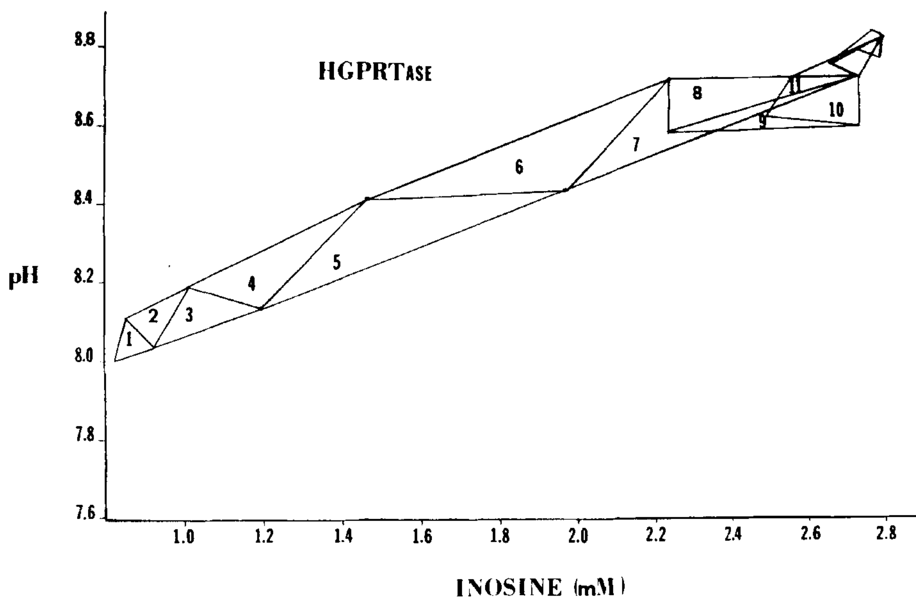

Figure 3: Simplex search conducted on the surface of HGPRTase alone.

The direction of the searches for the individual enzymes also shows that the simplex technique is sensitive to Michaelis-Menten behavior. That is, each search oriented itself in the direction of highest substrate concentration, but the behavior became less steep after a certain point. Thus, once the enzyme has been saturated with substrate and the activity is no longer changing radically with concentration, the slope of the search in the direction of concentration becomes less steep.

In conclusion, the simplex method of optimization is a powerful and rapid means of attaining experimental conditions. In the present application, the combined activities of PNPase and HGPRTase were optimized. This optimization was accomplished in the presence of strong interaction among the variables. The ability to search an area and determine optima in the case of interactions, and the small number of experiments required, are important characteristics of this technique.

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