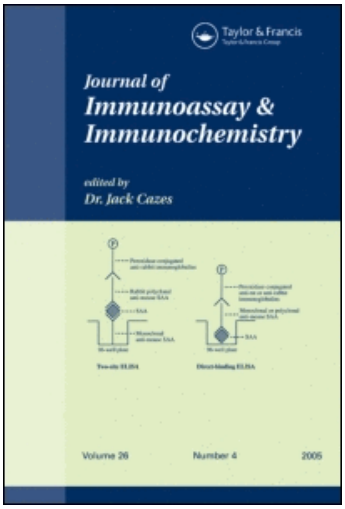

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Comparison of 5-Hydroxy-2, 3-Dihydrophthalazine-1, 4-Dione and Luminol as Co-Substrates for Detection of Horseradish Peroxidase in Enhanced Chemiluminescent Reactions

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COMPARISON OF 5-HYDROXY-2, 3-DIHYDROPHTHALAZINE-1, 4-DIONE AND LUMINOL AS CO-SUBSTRATES FOR DETECTION OF HORSERADISH PEROXIDASE IN ENHANCED CHEMILUMINESCENT REACTIONS

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ABSTRACT

The utility of 5-hydroxy-2, 3-dihydrophthalazine-1, 4-dione (HDP) as a co-substrate for the chemiluminescent detection of horseradish peroxidase was assessed. Several substituted aryl boronic acid derivatives (4-phenyl, 4-iodo) acted as potent enhancers of the peroxidase catalyzed reaction. Addition of chelating agents (EDTA) and surfactants (Tween-20 and [poly (vinylbenzyl)tributylphosphonium chloride-poly (vinylbenzyl) trioctylphosphonium chloride copolymer]) modulated background light emission and the intensity and duration of the signal from both HDP and luminol. However, HDP was found to be inferior to luminol in the peroxidase assay. Comparative studies revealed that at 500 amol of peroxidase the S/B was

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ten-fold higher using a commercial luminol-based signal reagent as compared with an HDP - EDTA - Tween-20 reagent (S/B t = 0 min 21.8 vs 1.7, S/B t =10 min 17.8 vs 2.0).

(KEY WORDS: Luminol, chemiluminescence, peroxidase, boronic acids)

INTRODUCTION

Recently, 5-hydroxy-2, 3-dihydrophthalazine-1, 4-dione (HDP) has been advocated as an alternative to luminol for the detection of horseradish peroxidase (HRP) labels using the enhanced chemiluminescent reaction. Various modifications of reaction conditions were described including the use of chelating agents and non-ionic surfactants (1, 2). We have undertaken a direct comparison of HDP and luminol in a 4-iodophenol-enhanced HRP reaction, and investigated HDP's performance in the presence of different substituted boronic acid enhancers.

MATERIALS AND METHODS

HDP was synthesized following a literature procedure (3). 1,1'-Biphenyl-4-yl boronic acid [CAS registry number 5122-94-1) and 4-iodophenol were purchased from Aldrich (Milwaukee, WI). Trans-4-(3-propionic acid) phenylboronic acid was obtained from Cookson Chemicals Ltd (Southampton, England). Luminol (Aldrich) was purified by recrystallization from sodium

hydroxide (4). HRP (Type VIA), dimethyl sulfoxide (DMSO), hydrogen peroxide (30 %, w/v), and EDTA were purchased from Sigma (St. Louis, MO) and Tween-20 from BioRad (Richmond, CA). Poly (vinylbenzyl)tributylphosphonium chloride-poly (vinylbenzyl) trioctylphosphonium chloride copolymer was obtained from Tropix Inc (Bedford, MA). Stock solutions (1 mg/mL) of HRP in Tris buffer (0.1 mol/L, pH 8.6) were prepared and stored at -20 °C. Amerlite Signal Reagent (ASR) was purchased from Amersham (Amersham, UK). Light emission was measured in a ML-3000 microplate luminometer (Dynatech Laboratories, Chantilly, VA) or in a Berthold Biolumat LB 9500C (EG & G Berthold, Nashua, NH). All reactions were performed at ambient temperature.

Boronate enhancers

The luminol-hydrogen peroxide and HDP-hydrogen peroxide reagents were prepared as follows: sodium luminol or HDP (12.5 mg) was dissolved in 50 mL of Tris buffer (0.1 mol/L, pH 8.6). Hydrogen peroxide (15.5 µL, 30 %, w/v) was mixed with 0.5 mL of the same buffer, then these two solutions were combined and protected from light. Trans-4-(3-propionic acid) phenylboronic acid (20 mmol/L) and 1,1'-biphenyl-4-yl boronic acid (20 mmol/L), stock solutions were prepared in DMSO and diluted in Tris buffer (0.1 mol/L, pH 8.6). The following reagents were added to a well of a white microwell plate (Dynatech): luminol-hydrogen peroxide reagent or HDP-hydrogen peroxide reagent

(100 μL), boronate enhancer (10 μL, 0 - 5 mmol/L), and either HRP Type VIA (10 μL, 20 μg/L) or Tris buffer (10 μL) as a blank. The reagents were mixed and the light emission was measured for 30 minutes.

Phenol enhancer

The luminol-hydrogen peroxide and HDP-hydrogen peroxide reagents were prepared as described above. 4-Iodophenol (20 mmol/L) stock solution was prepared in DMSO and diluted in Tris buffer (0.1 mol/L, pH 8.6). The luminol-hydrogen peroxide or HDP-hydrogen peroxide reagent (100 μL), 4-iodophenol (10 μL, 0 - 10 mmol/L), and either HRP Type VIA (10 μL, 20 μg/L) or Tris buffer (10 μL, as blank) were added to a microwell and the light emission measured for 30 minutes.

Comparison of HDP and luminol in enhanced chemiluminescent reactions

Effect of EDTA and Tween-20

A luminol (3 mmol/L) or HDP (3 mmol/L) and 4-iodophenol (4.8 mmol/L) reagent and a solution of sodium perborate tetrahydrate (6.5 mmol/L) in 0.1 mol/L Tris buffer pH 8.8 were mixed together in a 1:1 ratio. EDTA (5 mmol/L) and Tween-20 (10 % w/w) stock solutions were prepared in Tris buffer (0.1 mol/L, pH 8.8). The reagent (200 μL), together with either

EDTA (0 - 10 μL) or Tween-20 (0 - 20 μL), and either HRP Type VIA (5 μL, 0.1 μg/mL) or Tris buffer (5 μL, blank) were added to a microwell and the light emission measured for 30 minutes.

Effect of the phosphonium polymer

A luminol (3 mmol/L) or HDP (3 mmol/L) and 4-iodophenol (4.8 mmol/L) containing Tween-20 (1.0 % w/w) with or without poly (vinylbenzyl)tributylphosphonium chloride-poly (vinylbenzyl) trioctylphosphonium chloride copolymer (0.05 % w/w) reagent and a solution of sodium perborate tetrahydrate (6.5 mmol/L) containing EDTA (0.1 mmol/L) were mixed together in a 1:1 ratio. The reagent (200 μL), and HRP (10 μL, 0 - 2 μg/L) were added to a microwell and the light emission measured for 30 minutes.

HRP detection

Serial, freshly prepared dilutions of HRP in Tris buffer were analyzed using the following detection reagents: HDP - Tween-20 - EDTA, Amerlite Signal Reagent (ASR), and ASR - 1,1'-biphenyl-4-yl boronic acid. The HDP - Tween-20 - EDTA was prepared by mixing HDP (3 mmol/L) and 4-iodophenol (4.8 mmol/L) reagent containing Tween-20 (1.0 % w/w) and a solution of sodium perborate tetrahydrate (6.5 mmol/L) containing EDTA (0.1 mmol/L) together in a 1:1 ratio. ASR was prepared by dissolving one tablet A and B in a bottle of signal

reagent buffer, and ASR - 1,1'-biphenyl-4-yl boronic acid reagent prepared by mixing 1,1'-biphenyl-4-yl boronic acid (100 μL, 0.625 mmol/L) with ASR (2 mL).

The detection reagent (200 μL), and HRP (10 μL, 0 - 2 μg /L) were added to a microwell and the light emission measured for 30 minutes.

RESULTS

Substituted boronic acid and phenol enhancers

The substituted boronic acids, 1,1'-biphenyl-4-yl boronic acid and trans-4-(3-propionic acid) phenylboronic acid, enhanced the HRP catalyzed oxidation of HDP and luminol. The intensity of light emission from luminol in the presence of HRP (5 fmol) and the substituted boronic acid was much higher, and the background in the absence of HRP was lower compared to HDP (Figure 1). The signal/background ratio for the 1,1'-biphenyl-4-yl boronic acid and trans-4-(3-propionic acid) phenylboronic acid enhanced luminol reaction was 55-fold and 87-fold higher than with HDP.

The maximum intensity of light emission from luminol was higher than obtained from HDP in the 4-iodophenol enhanced reactions, and the background for luminol was 9-fold lower than with HDP at the optimum 4-iodophenol concentration (0.38 mmol/L final concentration) (Figure 2). Similar results were

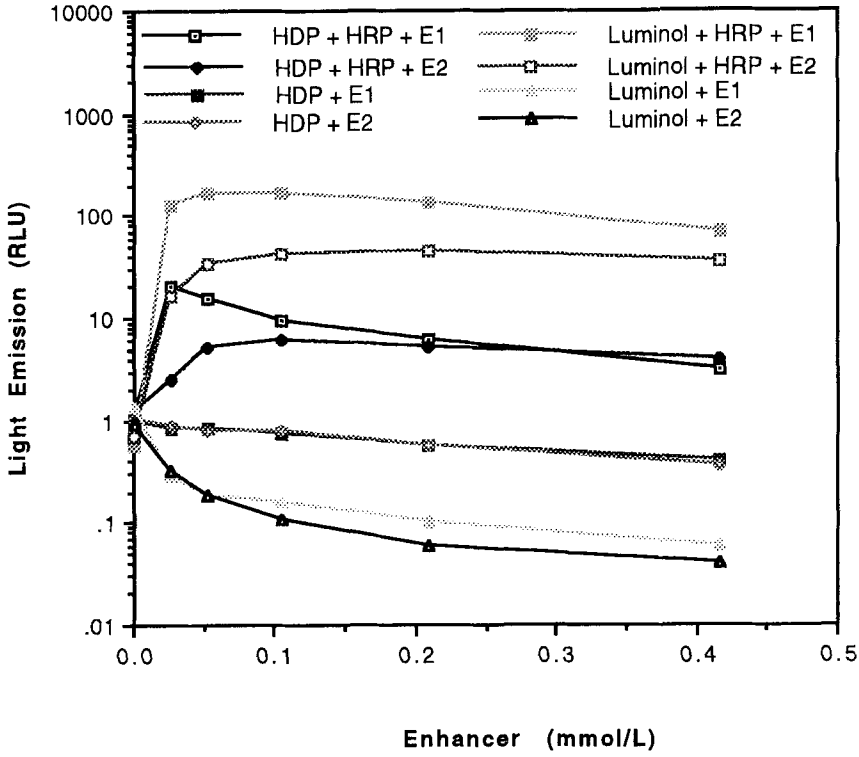

FIGURE 1. Effect of 1,1'-biphenyl-4-yl boronic acid and trans-4-(3-propionic acid) phenylboronic acid enhancers on HRP-catalyzed (5 fmol) HDP and luminol reactions.
(E1, 1,1'-biphenyl-4-yl boronic acid; E2, trans-4-(3-propionic acid) phenylboronic acid; RLU, relative light units)

obtained with samples of HDP synthesized by two different laboratories.

Effect of EDTA in enhanced chemiluminescent reactions

EDTA had an effect on light emission in the luminol and HDP reactions: it lowered the background and it stabilized the light

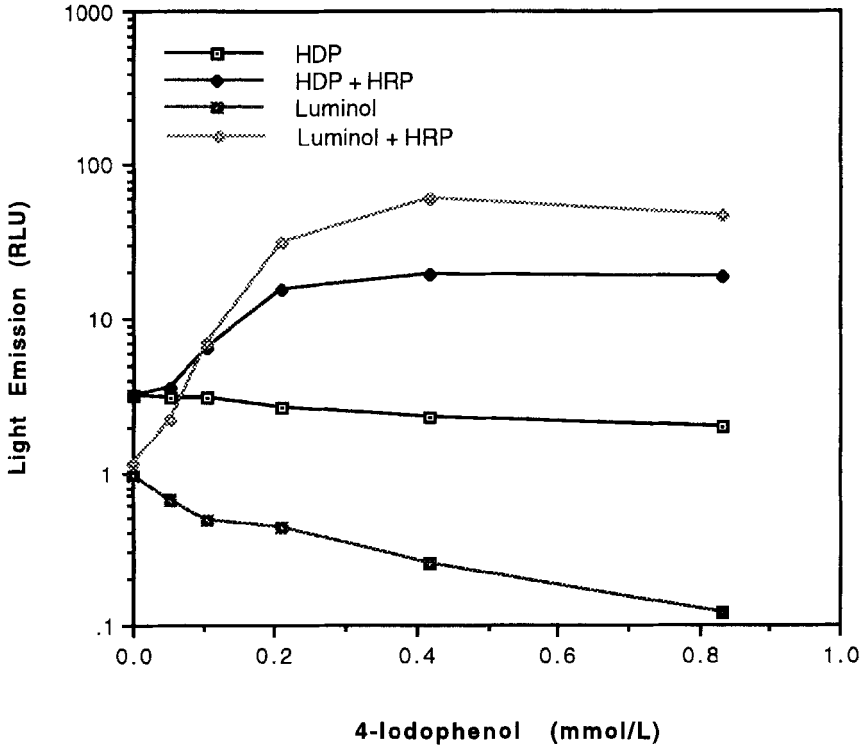

FIGURE 2. Effect of 4-iodophenol on HRP-catalyzed (5 fmol) HDP and luminol reactions.

emission (Figure 3). In the presence of EDTA (0.1 mmol/L) the background decreased 53-fold for HDP, but only 6-fold for luminol. The effect of EDTA on the light emission kinetics was most pronounced in the luminol reaction (Figure 4). Without EDTA the light from the 4-iodophenol enhanced reaction decayed very rapidly. Light emission decreased to 16% of its initial intensity within 25 min without EDTA. In the presence of EDTA, it only decreased to 72% of the initial signal.

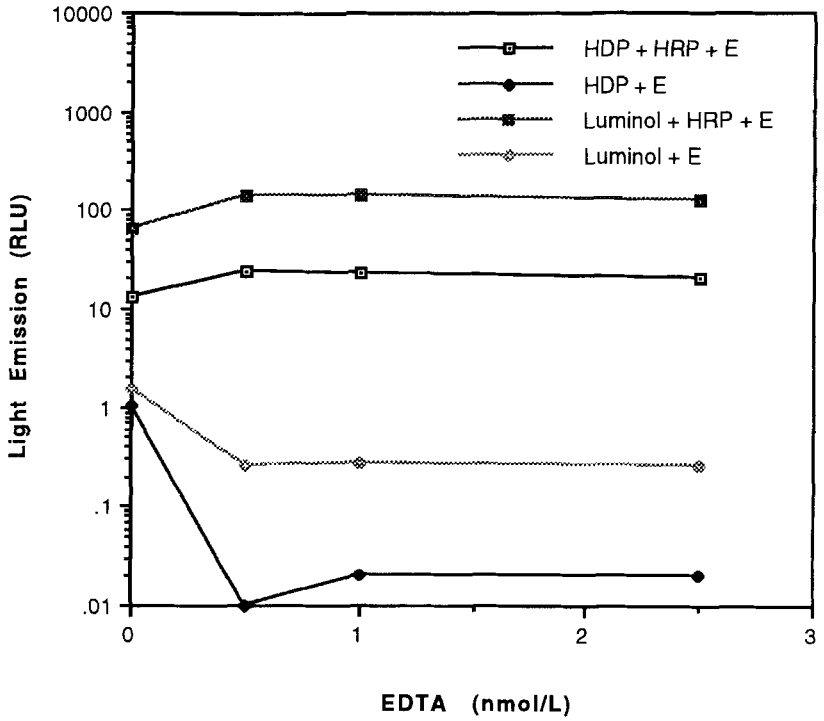

FIGURE 3. Effect of EDTA on 4-iodophenol-enhanced HRP-catalyzed (12.5 fmol) HDP and luminol reactions.
(E, 4-iodophenol (2.4 mmol/L); light emission measured 20 min after initiation of the reaction)

Effect of Tween-20 and the phosphonium co-polymer

Initially, Tween-20 inhibited light emission from the 4-iodophenol-enhanced HDP and luminol reactions, but after 5 min the light emission was increased and stabilized compared to reaction in the absence of Tween-20 (Figure 5). There was no significant effect on light emission and signal/background with

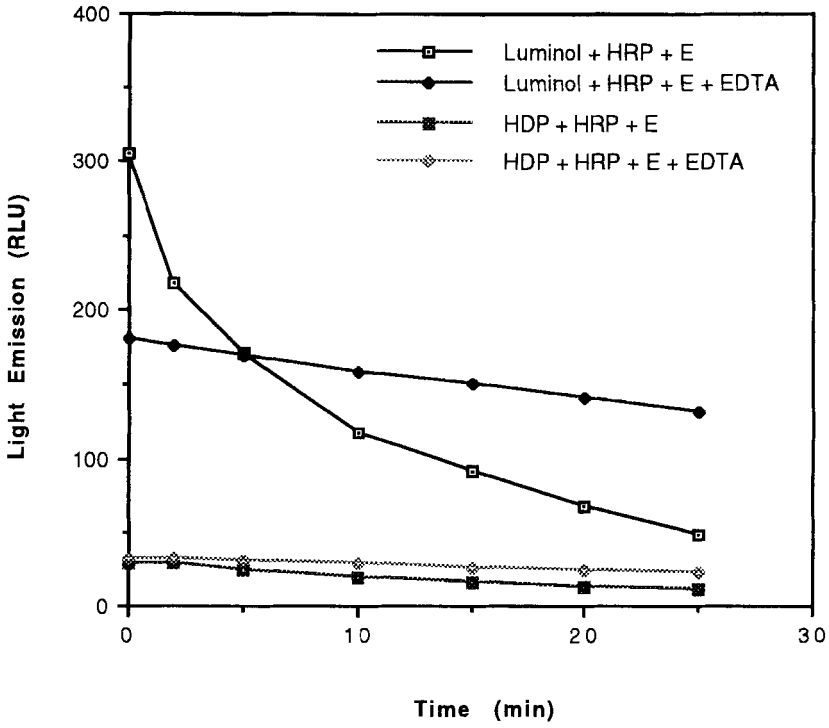

FIGURE 4. Kinetics of 4-iodophenol-enhanced HRP-catalyzed (12.5 fmol) HDP and luminol reactions in the presence of EDTA. [E, 4-iodophenol (2.4 mmol/L); EDTA (0.1 mmol/L)]

either HDP or luminol reactions (500 amole HRP) in the presence of the poly (vinylbenzyl)tributylphosphonium chloride-poly (vinylbenzyl) trioctylphosphonium chloride copolymer.

HRP detection

Table 1 compares the S/B for detection of 500 amole HRP using HDP and a commercial luminol-based signal reagent. Standard

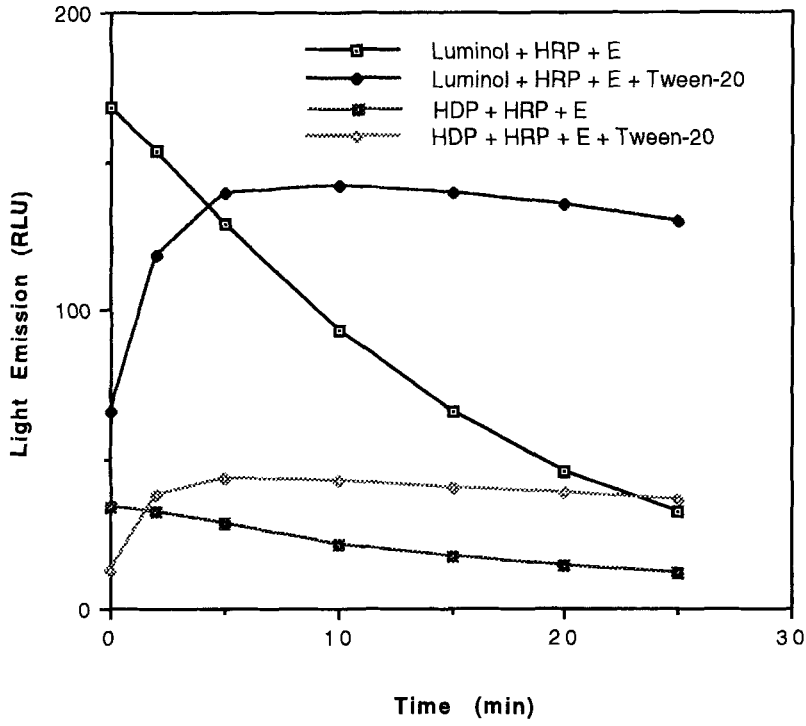

FIGURE 5. Kinetics of 4-iodophenol-enhanced HRP-catalyzed (12.5 fmol) HDP and luminol reactions in the presence of Tween-20.
[E, 4-iodophenol (2.4 mmol/L); Tween-20 (1.0%)]

curves for HRP using HDP, the commercial Amerlite Signal Reagent (ASR), and a synergistically enhanced signal reagent (ASR - 1,1'-biphenyl-4-yl boronic acid) are shown in Figure 6. Light levels were very low with HDP and the S/B was <2 for amounts of HRP <500 amole as compared with S/B = 21.8 for ASR and S/B = 105.8 for ASR - 1,1'-biphenyl-4-yl boronic acid.

TABLE 1

Signal to blank ratio for detection of 500 amole of HRP using HDP or luminol - based reagents

	S/B		
Luminometer	Berthold LB 9500C	Dynatech ML-3000	
Time (min)	0	0	10
HDP + Tween-20 + EDTA	1.5	1.7	2.0
Amerlite Signal Reagent	4.3	21.8	17.8
Amerlite Signal Reagent + 1,1'-biphenyl-4-yl boronic acid	134.2	105.8	88.1

DISCUSSION

Various substituted phthalazinediones have been tested as reagents for chemiluminescent detection of HRP in both enhanced and unenhanced reactions (5), and luminol has emerged as the cosubstrate of choice. Two recent reports

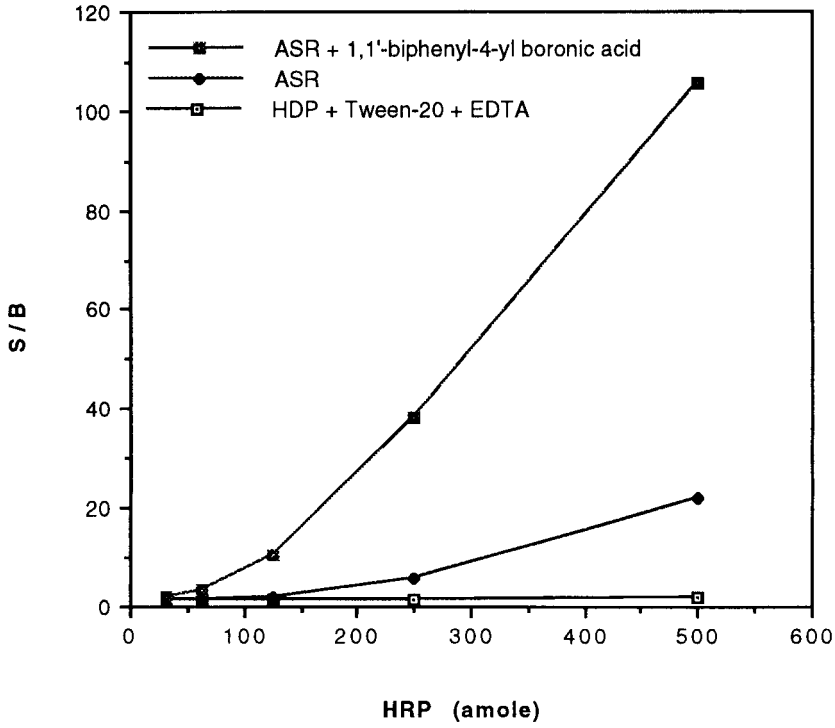

FIGURE 6. Comparison of standard curve for detection of HRP using HDP - Tween-20 - EDTA, ASR and ASR - 1,1'-biphenyl-4-yl boronic acid reagents.
(ASR, Amerlite Signal Reagent; light emission measured immediately after initiation of the reaction)

suggest that HDP may be a viable alternative to luminol as a reagent for HRP detection in a substituted phenol-enhanced chemiluminescent assay (1, 2).

HDP is a chemiluminescent co-substrate for HRP and we have shown that it can be used with the new boronic acid enhancers as well as substituted phenol enhancers (6). It produced less

light than sodium luminol in these enhanced reactions. Although the low background light emission was generated the presence of EDTA and Tween-20, the low signal intensity obtained under these conditions is a disadvantage for most applications. The detection limit for HRP was inferior to that using luminol-based reagents (Table 1 and Figure 6). Light emission measurements were performed immediately after initiation and at 10 minutes after the initiation of the HRP-catalyzed reactions. This ensured that peak light emissions could be compared for the rapid luminol-based assays (peak light emission < 2 minutes) and the slower HDP - EDTA - Tween-20 based assays (peak light emission > 8 minutes).

Early studies of the properties of HDP investigated different catalysts (hemin, hemoglobin) (7, 8), and showed that it ranked fourth in brightness behind luminol in the presence of hemoglobin (7). Chemiluminescence quantum yield measurements also confirm that HDP is inferior to luminol (CL quantum yield 1.1% vs 2.4 %) (9).

The effect of surfactants on CL luminol reactions has been the subject of several studies (10, 11). Recently, surfactants (eg, CTAB) and vinyl addition homo- and copolymers (eg, poly [N-cyclohexyl-N,N-dimethyl-N-(m-and p-vinylbenzyl) ammonium chloride] have been used in 4-hydroxyacetanilide-enhanced assays for HRP using luminol as the cosubstrate. The surfactants stabilized light emission (12). Chelating agents such as diethylenetriaminepentaacetic acid (DTPA) have been

included in buffers used in enhanced CL assay for HRP (12). One role of this additive is presumably chelation of trace metal impurities that would otherwise catalyze the oxidation of luminol and produce a background light emission.

The stated benefits of HDP, namely photochemical stability and ease of purification (1, 2), are outweighed by its relatively poor analytical performance. Other analogs of luminol such as 8-amino-5-chloro-7-phenyl-pyridopyridazine (14) and 3-substituted-7,8-dihydropyridazino[4,5-g]quinoxaline-2,6,9(1H)-trione (15) have been synthesized and these may have greater utility in chemiluminescent assays for HRP.

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