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Synthesis, Characterization, Kinetic Parameters, and Diagnostic Application of a Sensitive Colorimetric Substrate for β-Galactosidase (2-Chloro-4-Nitrophenyl-β-D-Galactopyranoside)

DENG R. HWANG[1] AND M. E. SCOTT

Diagnostic Division, Miles Inc., 511 Benedict Avenue, Tarrytown, New York 10591

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The synthesis and characterization of 2-chloro-4-nitrophenyl β-D-galactopyranoside, an improved chromogenic substrate for β-galactosidase, is described. The important kinetic parameters (K_m, V_{max} and K_p) for this substrate were compared with those of other substrates. The diagnostic utility of this substrate in a digoxin liposome immunoassay is discussed. The new substrate offers at least four times the sensitivity enhancement as that with *ortho*-nitrophenyl β-D-galactopyranoside in the assays for β-galactosidase. This substrate should find use in enzyme immunoassays where β-galactosidase is used as a label.

INTRODUCTION

β-Galactosidase fulfills a multiple physiological function in mammals. It plays an important role in carbohydrate metabolism since it brings about the hydrolysis of lactose. Furthermore, β-galactosidase is a key enzyme in the breakdown of glycolipids, mucopolysaccharides, and glycoproteins. In recent years, β-galactosidase has achieved importance in the field of immunodiagnosis. Thus, for example, this enzyme is employed to an increasing extent as a marker enzyme for enzyme immunoassay (*1*).

There are several known substrates for β-galactosidase including: 2-methoxy-4-(2-nitrovinyl)-phenyl-β-D-galactopyranoside (*2*), and phenosulphonphthaleinyl-β-D-galactopyranoside (*3*), and chlorophenolred-β-D-galactopyranoside (*4*), which were reported as chromogenic substrates for the assay of β-galactosidase; resorufin-β-D-galactopyranoside (*5*), 4-methyl-umbellifery-β-D-galactopyranoside (*6*), 2-naphtyl-β-D-galactopyranoside (*7*), and 6-hydroxy-fluoran-β-D-galactopyranoside (*8*), which were reported as fluorogenic substrates for β-galactosidase; ε-N-1 (1-deoxylactulosyl)-L-lysine, which was described as a substrate for determining the activity of β-galactosidase in the intestinal tract of mice (*9*); and two water-soluble high-molecular-weight substrates for β-galactosidase, each containing β-D-[^{3}H]galactopyranosyl moieties linked, through an aliphatic bridge, to either

[1] To whom correspondence should be addressed.

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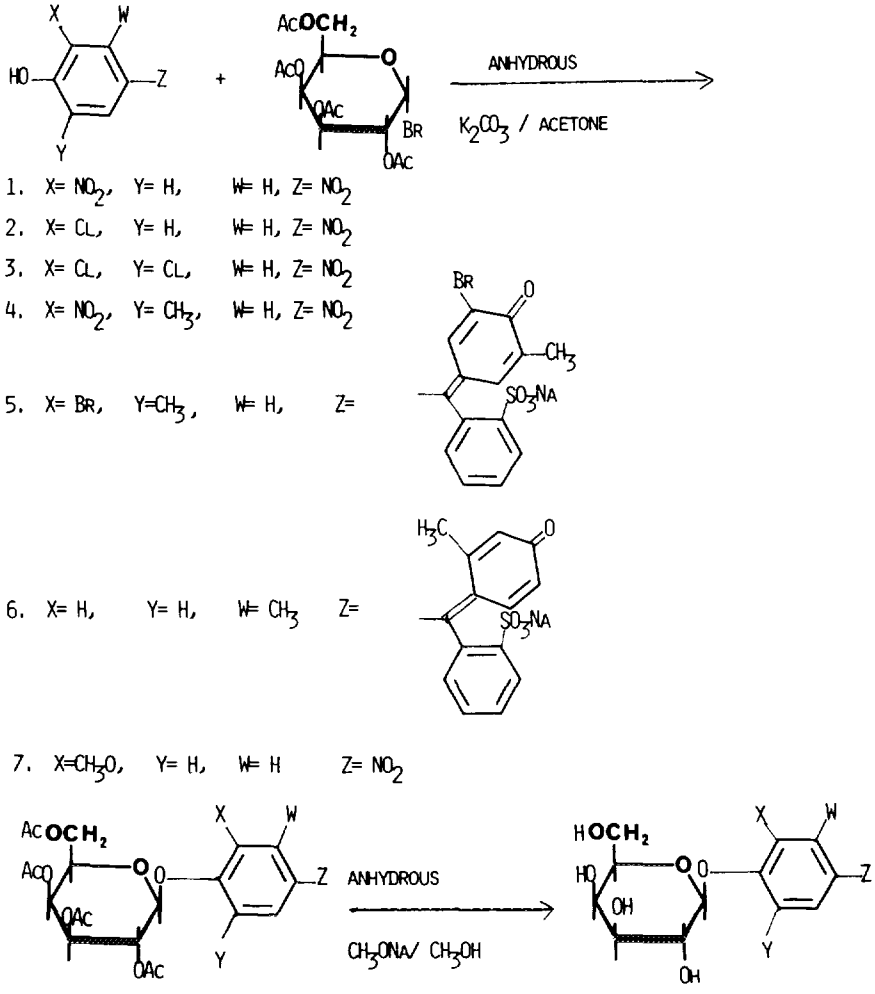
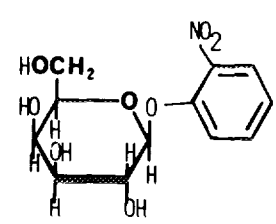


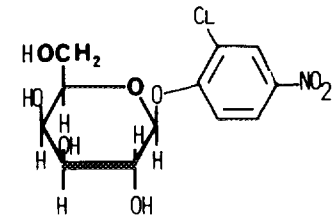
SCHEME 1. Preparation of β-galactosidase substrates.

poly-L-lysine or polymeric dialdehyde, which was reported by R. Madhan *et al.* (*10*).

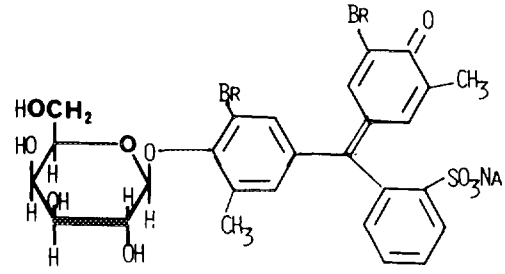
The most common substrate for beta-galactosidase currently in use is *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) which is preferred for its relatively fast enzyme turnover rate, good stability in aqueous buffers, and the relative ease by which it can be synthesized or commercially obtained. ONPG has a significant disadvantage, though, in that it has a relatively small molar absorptivity upon enzyme-catalyzed hydrolysis. Assays for digoxin and other clinically important low-concentration analytes which utilize β-galactosidase as an enzyme immunoassay label required a more sensitive substrate to achieve the required detection sensitivity.



ORTHO-NITROPHENYL-BETA-D-GALACTOPYRANOSIDE (ONPG)



2-CHLORO-4-NITROPHENYL-BETA-D-GALACTOPYRANOSIDE (CINPG)



BROMOCRESOL PURPLE-BETA-D-GALACTOPYRANOSIDE (BCPG)

SCHEME 2. β-Galactosidase substrates.

Therefore, it was desirous to develop a chromogenic substrate for β-galactosidase for use in the clinical diagnostic assay of digoxin and other clinically important low-concentration analytes which may be present in blood and other body fluids.

The desirable substrate should be kinetically equivalent to known substrates such as ONPG and stable in aqueous buffers, but exhibit a substantially increased molar absorptivity upon β-galactosidase-catalyzed hydrolysis than that with ONPG.

MATERIALS AND EXPERIMENTAL

Materials and Methods

Commercially available reagent-grade chemicals were used whenever possible. The lecithin, sphingomyelin, tocopherol, cholesterol, diacetylphosphate, dipalmitoyl phosphatidyl-ethanolamine (DPPE), acetobromo-α-D-galactose, phosphocho-

line, β-galactosidase, and *ortho*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma and Biosynth AG. The digoxin–DPPE conjugate and antibody were produced in-house. All phenolic compounds were purchased from Pfaltz & Bauer and Aldrich. All other substituted phenyl-β-D-galactopyranosides were prepared by following a synthetic procedure outlined under synthesis. Guinea pig complement was obtained from Cappel. Anhydrous methanol was prepared according to the established procedure (*11*). Anhydrous sodium methoxide and potassium carbonate were purchased from Aldrich. Methanol, chloroform, and acetone were HPLC-grade and were obtained from J. T. Baker. The EM Siliga Gel 60 F-254 TLC plate was used. Elemental analysis was performed by Galbrath Laboratories Inc. FT NMR spectra were obtained with the 7-T spectrometer at the Rockefeller University.

Synthesis

Preparation of 2-chloro-4-nitrophenyl-2,3,4,6-tetra-o-acetyl-β-D-galactopyranoside (TA-ClNPG). In a 200-ml round-bottom three-necked flask were dissolved 0.127 mol of 2-chloro-4-nitrophenol in 490 ml of dry acetone and added 0.4 mol of anhydrous potassium carbonate. To this suspension 0.122 mol of acetobromo-α-D-galactose was added with stirring. The mixture was heated for 36 h at a temperature of 55°C, kept under a blanket of nitrogen, and protected from light.

The reaction was monitored by TLC in hexane/chloroform/methanol (5/3/1 by volume). The desired product had an R_F value of 0.42 with 0.31 for the phenol and 0.52 for the acetobromo derivative. The spots were detected by spraying the TLC plate with methanol/sulfuric acid (9/1 by volume) and heating in the oven at 100°C for 5 min. After the reaction was judged complete by TLC, the mixture was cooled, filtered, and evaporated by rotary evaporation. The residue was dissolved in 600 ml of chloroform and washed three times with cold 1 N sodium hydroxide (200 ml) and then twice with saturated sodium chloride solution (200 ml).

The chloroform layer was dried over magnesium sulfate (2 g), filtered, and again evaporated by rotary evaporation. The crude product (60 g) was crystalized from 800 ml hot methanol with a typical yield of 76%. The pure product has a melting point of 147–149°C. Specific rotation α_D^{26} was determined to be −30°C (c = 1%, chloroform). NMR ($CDCl_3$): 2.0–2.4 (m, 12H), 4.1–4.3 (m, 3H), 5.1–5.7 (m, 4H), 7.35 (m, 1H), 8.15 (m, 1H), 8.35 (s, 1H).

Anal. Calcd: C 47.67, H 4.41, N 2.78, Cl 7.05, O 38.10. Found: C 47.6, H 4.45, N 2.68, Cl 6.91, O 38.02.

Preparation of 2-chloro-4-nitrophenyl-beta-D-galactopyranoside (ClNPG). In a two-necked 500-ml round-bottom flask, 0.063 mol (31.72 g) of TA-ClNPG was dissolved in 400 ml of a one to one mixture of dry chloroform and anhydrous methanol. To this solution 0.015 mol of sodium methoxide was added. The reaction mixture was stirred at room temperature for 36 h under nitrogen and protected from light. The reaction progress was monitored by TLC in chloroform/methanol (6/1 by volume) and the product detected as before, with a R_f of 0.18. After the

reaction was completed, the mixture was neutralized with 0.5 ml acetic acid and the solvent was evaporated. The residue was dissolved in 700 ml of hot methanol, filtered, and crystallized. A yield of 17.97 g (85%) was obtained. The melting point is 213–215°C. Specific rotation $[\alpha]_D^{26}$ was determined to be −50° (c = 0.9%, methanol). NMR (CD_3OD): 3.6–4.0 (m, 6H), 4.6–5.0 (m, 4H), 5.15 (d, J = 7.6 Hz, 1H), 7.5 (d, 1H), 8.2 (d, 1H), 8.35 (d, 1H).

Anal. Calcd: C 42.93, H 4.21, N 4.17, Cl 10.56, O 38.13. Found: C 42.65, H 4.06, N 3.92, Cl 10.82, O 38.17.

Synthesis of Digoxin–DPPE Conjugates

The synthetic procedure for preparing the digoxin–DPPE conjugates is described elsewhere (*11*).

The Digoxin Liposome Immunoassay Protocol

The hapten liposome immunoassay protocol adapted for use on the Technicon RA-1000 clinical chemistry analyzer is typical of such protocols and is summarized below;

Sample	Reagent A		Reagent B
30 μl	350 μl pH 7.5		50 μl (1 : 700)
	Substrate	160 μl	Liposome 59
	Complement	80 μ l	
	PC	40 μl	
	Buffer	22 μl	
	Antibody	48 μl (1 : 2500)	
	4 min, 37°C		5 min, 37°C

where complement refers to serum protein, PC refers to phosphocholine, a stabilizer, and the hapten-modified liposome 59 component is prepared by the following film-deposition process. The membrane components consist of lecithin, sphingomyelin, tocopherol, diacetylphosphate, cholesterol, and the digoxin–DPPE conjugate. The membrane componets are dissolved in chloroform and evaporated on the inner surface of a 1-liter reaction vessel. The firm is then hydrated with an aqueous buffer solution containing β-galactosidase, which is the entrapped enzyme marker, in the same reaction vessel. Liposomes spontaneously form in high yield. Separation of reagent liposomes from unentrapped enzyme is accomplished by ultracentrifugation. The yield of liposome is sufficient for approximate 100,000 tests. The characterization of the liposome reagent requires special techniques which have been established in-house. The assay protocol involves the addition of sample and reagent A into the reaction cuvette, incubation for 4 min, addition of reagent B, and a second incubation of 5 min. The actual analyte concentrations are obtainable from a standard curve.

The Molar Absorptivity of the Phenolic Compounds

The molar absorptivity of the phenolic compounds was measured and calculated according to the formula $\varepsilon_{max} = A/c \times b$, where A is the absorbance, b is the

TABLE 1

Molar Absorptivity of Selected Phenolic Compounds

Phenol	Molar absorptivity (ε_{max})
Bromocresol purple	50.7 (585 nm, pH 8.5)[a]
4-Nitrophenol	17.0 (405 nm, pH 7.5)
2-Chloro-4-nitrophenol (ClNP)	16.9 (405 nm, pH 7.5)
2-Methoxy-4-nitrophenol	16.5 (430 nm, pH 9.5)[b]
2,6-Dichloro-4-nitrophenol	15.9 (405 nm, pH 7.5)
2-Chloro-4,6-dinitrophenol	11.5 (405 nm, pH 7.5)
2,4-Dinitro-6-methylphenol	11.0 (405 nm, pH 7.5)
2,4-Dinitrophenol (DNP)	10.7 (405 nm, pH 7.5)
Metacresol purple	8.4 (575 nm, pH 8.5)[a]
2-Chloro-6-nitrophenol	4.6 (405 nm, pH 7.5)
Ortho-nitrophenol (ONP)	3.0 (405 nm, pH 7.5)

[a] Fifty-micromolar Barbitol buffer.
[b] One-molar DEA–citric acid buffer.

path length through the sample, and c is the concentration of solute. The buffer for the phenolic compounds was 50 mM Tris–HCl + 150 mM NaCl, pH 7.5. The concentration of phenolic compounds was 0.0537 mM.

Kinetic Parameters of the Enzymatic Reaction

The detailed discussion of the enzymatic reaction can be found in a book edited by Hans Ultrich Bergmeyer (*13*). Various kinetic parameters of the enzymatic reaction were measured including K_m, V_{max}, and K_p.

The most commonly used method for calculation of K_m (Michaelis constant) is that of Lineweaver and Burk:

$$\frac{1}{v} = \frac{K_m}{V}\frac{1}{(S)}\frac{1}{V}.$$

Both important characteristics of an enzyme (K_m and V_{max}) are thus obtainable in a single operation.

K_p is the turnover number which is easily obtained if V_{max} (V_m) and enzyme concentration (E_c) are known. K_p is expressed as $K_p = V_m/E_c$.

Comparison of 2-Chloro-4-Nitrophenyl-β-D-galactopyranoside (ClNPG) and Ortho-Nitrophenyl-β-D-galactopyranoside (ONPG).

Free enzyme protocol. The enzymatic response of ClNPG was measured and compared with that of ONPG by the following free-enzyme protocol performed on a Technicon RA-1000 clinical chemistry analyzer: fifteen microliters of known concentration of beta-galactosidase in buffer (1 mg/ml) was added to 300 μl of substrate (2.5 mM in buffer.) The buffer used was 50 mM Tris–HCl, 150 mM NaCl, and 5 mM $MgCl_2$ (pH 7.5). The enzymatic reaction product was measured at 405

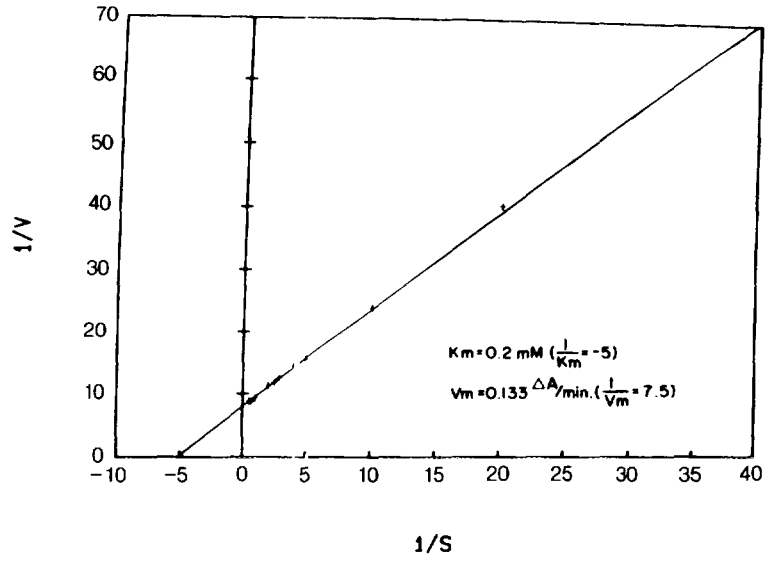


FIG. 1. Lineweaver–Burk plot (ClNPG).

nm after 30 sec and the ΔA 405 nm/min for three different concentrations of β-galactosidase was recorded for each substrate.

β-Galactosidase-encapsulated liposome protocol. The enzymatic rate of ClNPG was measured and compared with that of ONPG by the following β-galactosidase-encapsulated liposome protocol performed on a Technicon RA-1000 clinical chemistry analyzer: fifteen microliters (1/500) of liposome 35 was added to 300 ul of substrate (2.5 mM in buffer) and mixed for 30 s. The buffer used was same as above. The enzymatic rate of the mixture was then measured at 405 nm (−Triton). For chemical lysis, the identical mixture was prepared but 0.1% Triton X-100 was substituted for the buffer. The enzymatic rate of the mixture was

TABLE 2

Kinetic Parameters of Selected β-Galactosidase Substrates

Substrate	K_m (mM)	V_{max} (A/min)	K_p (min^{-1})
2-Chloro-4-nitro-phenyl-β-D-galacto-pyranoside (ClNPG)	0.20	0.13	7.40×10^4
Bromocresol purple-β-D-galacto-pyranoside (BCPG)[a]	8.0	0.11	3.10×10^2
Ortho-nitrophenyl-β-D-galacto-pyranoside (ONPG)	0.17	0.03	1.80×10^5
2,4-Dinitrophenyl-β-D-galacto-pyranoside (DNPG)	0.22	0.13	2.20×10^5

Note. The common buffer is 50 mM Tris + 150 mM NaCl + 5 mM $MgCl_2$; pH 7.5.
[a] Fifty-micromolar Barbitol + 150 mM NaCl + 5 mM $MgCl_2$; pH 8.5.

TABLE 3

Enzymatic Rate of ClNPG and ONPG by β-Galactosidase[a]

β-Galactosidase concentration	405/min (ClNPG)	405/min (ONPG)	Ratio (ClNPG/ONPG)
0	0.0007	0.0003	—
1/2000	0.0427	0.0077	5.5
1/1000	0.0923	0.0159	5.8
1/200	0.4555	0.0795	5.7

[a] Protocol, under Materials and Experimental.

measured at 405 nm (+Triton). The ΔA405 nm/min for three different concentrations of liposome was recorded for each substrate.

RESULTS AND DISCUSSION

The synthetic scheme for β-galactosidase substrates is outlined in Scheme 1. The molar absorptivity (ε_{max}) of the phenolic precursor of several substrates was measured and calculated according to the protocol described under Materials and Experimental. The molar absorptivity of the phenolic precursor of ONPG was likewise measured and calculated for comparison purposes. The aim was to identify phenolic precursors which would provide enhanced molar absorptivity to that of *ortho*-nitrophenol (ONP). The results are set out in Table 1. These results show that some phenolic compounds display relatively high molar absorptivity upon chemical and enzymatic hydrolysis with the respective β-D-galactopyranoside, especially when compared to the known and preferred substrate (ONPG).

One objective of this work was to provide a new substrate for β-galactosidase which has a higher enzymatic rate than the existing preferred substrate. The calculated values of K_m, V_{max}, and K_p of certain new substrates, ClNPG and BCPG, are based on experimental data plotted in Figs. 1 and 2 respectively, and appear in Table 2 as do values for ONPG and DNPG. The molecular structures of β-galactosidase substrates are depicted in Scheme 2.

The advantage of the new substrate, ClNPG, over ONPG, the preferred known substrate, in terms of V_{max}, is evident from Table 2. While the K_m and K_p parameters of both substrates are similar, the V_{max} of ClNPG is at least four times that of ONPG. DNPG, while showing good kinetic parameters, is unstable in an aqueous environment. The introduction of a chloro substituent in the ortho position of the ClNPG has apparently exerted a stablization effect on the substrate because 4-nitrophenyl-β-D-galactopyranoside is also unstable in aqueous medium. It is worthwhile to note also that bromocresol purple, a hydrolysis product of BCPG, has the highest molar absorptivity among the phenolic compounds we have studied. Whereas the turnover number, K_p, of the BCPG is several hundredfold less than that of ClNPG. Thus, instability in aqueous buffers and low turnover number have excluded NDPG and BCPG respectively as useful agents in diagnostic applications.

TABLE 4

Chemical Lysis of β-galactosidase-Encapsulated Liposome by Triton X-100[a]

β-Galactosidase encapsulated liposome concentration (1/500 dilution)	405 nm/min (ClNPG)	405 nm/min (ONPG)	Ratio
0.1	0.0180	0.0030	6.0
0.5	0.0915	0.0138	6.6
1.0	0.1803	0.0279	6.5

[a] Protocol, under Materials and Experimental.

The results of enzymatic rate of ClNPG and ONPG by β-D-galactopyranoside and Triton X-100 induced chemical lysis of β-galactosidase-encapsulated liposomes are shown in Tables 3 and 4, respectively. These experiments were performed on a Technicon RA-1000 clinical chemistry analyzer, following the protocols outlined under Materials and Experimental.

In addition, a digoxin liposome immunoassay was performed by using protocol outlined in experimental section D to compare the enzymatic rate utilizing ClNPG and ONPG. The antibody titration curve, as shown in Fig. 3, clearly illustrates that the performance of ClNPG was superior to that of ONPG. The former offers a fourfold increase in assay sensitivity over the latter for a wide range of antibody.

It is apparent from the above that the use of ClNPG in both β-galactosidase

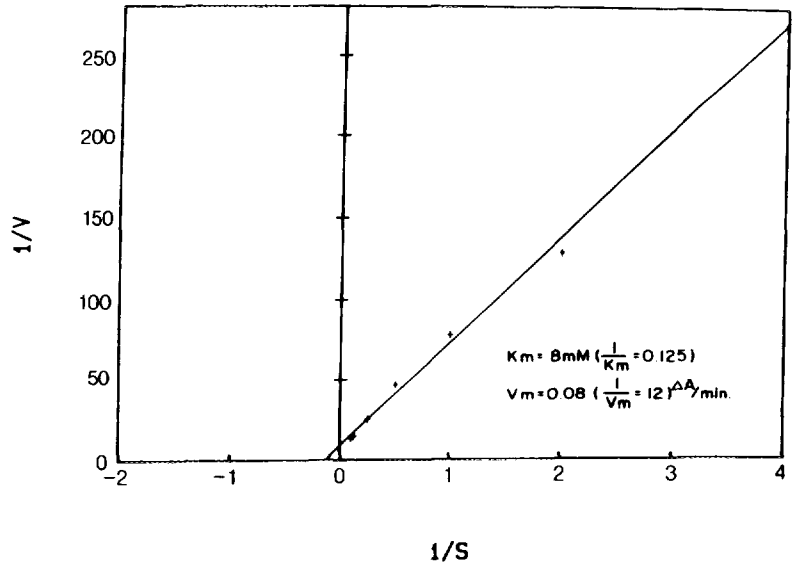


FIG. 2. Lineweaver–Burk plot (BCPG).

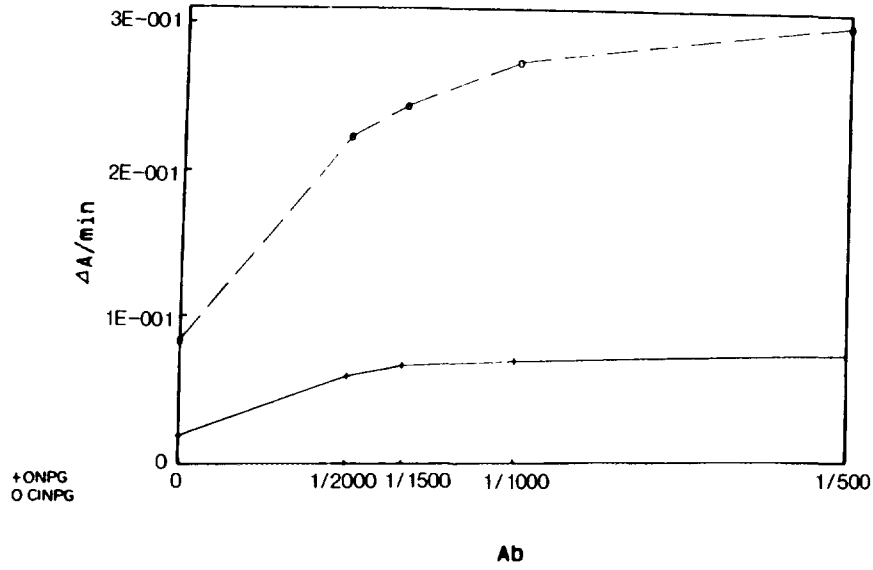


FIG. 3. Digoxin immunoassay–antibody titration.

and β-galactosidase encapsulated liposome assays provides a very significant improvement in detection sensitivity.

Similarly, this substrate should be useful for enzyme immunoassays which utilize β-galactosidase conjugates where increased detection sensitivity is desired.

ACKNOWLEDGMENTS

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