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Monitoring the effect of triamterene and hydrochlorothiazide on dihydrofolate reductase activity using a new spectrophotometric method*

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Abstract: A new spectrophotometric method is developed and applied for the study of the inhibitory effect of triamterene, hydrochlorothiazide and their combinations on the *in vitro* activity of dihydrofolate reductase enzyme. The method is based on incubating the drug (0.1–1.0 μM) or a buffer control with a solution containing reduced nicotinamide adenine dinucleotide phosphate (0.5 mM), magnesium chloride (1.29 mM), and folic acid as a substrate (0.01–0.1 mM) with the dihydrofolate reductase (0.25 unit). The resulting tetrahydrofolic acid is determined by first hydrolysing it by a methanol–hydrochloric acid mixture to produce *p*-aminobenzoyl glutamic acid, then adding *p*-dimethylaminocinnamic aldehyde reagent to form a stable pink coloured product. The colour is found to develop within 5 min and is stable over 12 h, with a maximum absorption at 545 nm. A linear calibration curve is formed by using standard solutions of tetrahydrofolic acid. The presence of the studied drugs did not interfere with the determination. Lineweaver–Burk plots of the reaction kinetics, in the presence of triamterene and/or hydrochlorothiazide showed a competitive inhibition of the dihydrofolate reductase in the presence of triamterene with or without hydrochlorothiazide. A 100% inhibition is obtained by 1 μM solution of triamterene at a folic acid concentration of 0.01 mM. No measurable effect of hydrochlorothiazide at the studied concentration range is demonstrated.

Keywords: *Tetrahydrofolate analysis; triamterene; hydrochlorothiazide; dihydrofolate reductase;* p-*dimethylaminocinnamic aldehyde; competitive inhibition of dihydrofolate reductase.*

Introduction

The enzyme dihydrofolate reductase (DHFR) catalyses the reduction of the 5,6-double bond in dihydrofolate (FH_2) to form tetrahydrofolate (FH_4). The latter serves as an electron donor in the synthesis of thymidylate and in the conversion of phenylalanine to tyrosine [1]. Compounds which have structural similarity to folic acid, act as folic acid antagonists inhibiting DHFR [2]. The DHFR activity has been routinely measured by non-specific spectrophotometric methods based on the decrease in absorbance that

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occurs at 340 nm when nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and FH_2 are converted to $NADP^+$ and FH_4, respectively [3–5]. More sensitive and selective methods have been suggested using radioisotope assays for DHFR [6] or radiochemical binding assays [7, 8] but they have the disadvantages inherent in radioisotope handling and disposal requirements.

The objective of this paper is to develop a simple spectrophotometric method capable of studying the effect of drugs on the *in vitro* activity of DHFR-enzyme. Triamterene (TMT), hydrochlorothiazide (HCT) and their combinations, widely used as diuretics and in the treatment of various types of oedema, [9] were the drugs chosen for this study.

Experimental

Materials

Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) tetrasodium salt, type III 98% purity; folic acid (pteroylglutamic acid), 98% purity; tetrahydrofolic acid (5,6,7,8-tetrahydropteroyl-L-glutamic and), 70% purity; DHFR from chicken liver, supplied as solution (25 units ml^{-1}) in 50% glycerol containing 1 M $(NH_4)_2SO_4$ and 0.1 M potassium phosphate, pH 6.4; triamterene, 98% purity and crystalline HCT were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). The reagent, *p*-dimethylaminocinnamic aldehyde, of 98% purity was purchased from Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin, USA). Magnesium chloride ($MgCl_2 \cdot 6H_2O$) 100.9% purity was purchased from J. T. Baker Chemical Co. All other chemicals used were analytically pure.

Reagents

Phosphate buffer, 0.02 M, pH 6 was used as a solvent in all cases, except where otherwise specified.

Folic acid solution. A 0.2 mM stock solution was prepared by dissolving 25.0 mg of folic acid in 250 ml of the phosphate buffer. The stock solution was then diluted by the same buffer to prepare the working solutions in the range 0.02–0.2 mM.

Magnesium chloride solution. A 12.9 mM solution was prepared by dissolving 262.3 mg in 100 ml of the phosphate buffer.

NADPH solution. A 5 mM solution was prepared by dissolving 9.5 mg in 2 ml of the phosphate buffer.

Dihydrofolate reductase solution. 1 ml of the enzyme solution was diluted to 10 ml with phosphate buffer.

Tetrahydrofolic acid standard solution. A 10 mM stock solution was prepared by dissolving 28.57 mg in 5 ml of 0.5 M HCl in methanol. The working solutions 1 μM–1 mM were obtained by appropriate dilutions with 0.5 M HCl in methanol.

Triamterene solution. A stock 0.1 mM solution, prepared by dissolving 6.33 mg in 250 ml of phosphate buffer, was used to prepare working solutions in the range 0.4–4 μM using the same buffer.

Hydrochlorothiazide solution. Stock 0.05 mM solution was prepared by dissolving 7.44 mg in 500 ml of the phosphate buffer. Working solutions in the range 0.4–4 μM were then prepared by appropriate dilutions with phosphate buffer.

Triamterene–hydrochlorothiazide solution. Working solutions in the range 0.4–4 μM were prepared by appropriate dilutions of stock solutions of triamterene and hydrochlorothiazide with phosphate buffer.

p-*Dimethylaminocinnamic aldehyde solution.* A 0.1% (w/v) solution in methanol was used.

Hydrochloric acid 0.5 M, solution in methanol was used.

Apparatus

A Beckman DU-40 single-beam recording spectrophotometer with 10.0 mm cells was used for scanning and absorbance measurements.

Procedure

Sample. Aliquots, 0.2 ml each, of the 12.9 mM magnesium chloride and 5 mM NADPH were transferred to 10-ml test tubes. A 1.0 ml of folic acid solution (0.02, 0.06, 0.1 or 0.2 mM) and 0.5 ml of HCT, TMT or their combination (0.4, 2.0 or 4.0 μM) were added. The mixed solutions were incubated for 5 min at 37 ± 0.5°C. A 0.1 ml DHFR solution was added and incubated for an additional 5 min. An accurately measured 0.1 ml of the reaction mixture was transferred to 0.4 ml of methanolic HCl 0.5 M solution, followed by 2.0 ml solution of *p*-dimethylaminocinnamic aldehyde. The absorbance of the solution was measured after 5 min at 545 nm against a blank solution, which had been similarly treated.

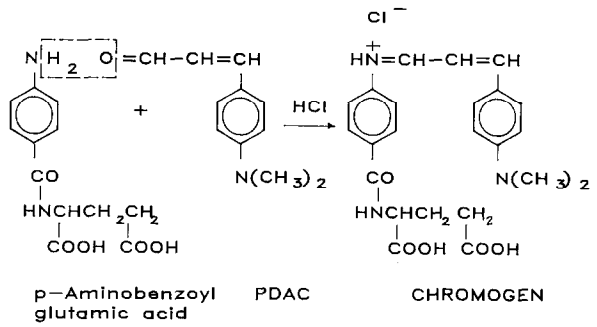
Blank. The blank solution was prepared by following the same procedure for the sample solution, but replacing the DHFR solution by 0.1 ml phosphate buffer.

Control. The sample procedure was followed using 0.5 ml phosphate buffer in place of the inhibitor solution (HCT, TMT or their combination).

Standard curve. A standard curve was prepared using standard solutions of tetrahydrofolic acid over a concentration range of 1 μM–1 mM. The previous procedure was used for the chromogen development. Standard solutions were spiked with the inhibitors and other reagents used.

Results and Discussion

This study is based on the reduction of folic acid to tetrahydrofolic acid when reacted with NADPH in the presence of DHFR enzyme [10]. The determination of the resulting tetrahydrofolic acid is a direct monitor to the DHFR activity. Tetrahydrofolic acid is cleaved non-enzymatically in acid medium, with the liberation of *p*-aminobenzoylglutamic acid [11]. This arylamine derivative was used to form a highly coloured Schiff's base upon reacting it with *p*-dimethylaminocinnamic aldehyde as illustrated in the following scheme:



The resulting chromogen has an absorption maximum at 545 nm, which was well removed from any possible interference from the reactants or from the drugs under investigation.

Methanolic HCl solution was necessary to induce cleavage of tetrahydrofolic acid and to stop the enzymatic reaction [12]. The acid concentration greatly affected the intensity of the measured absorbance. High concentrations of methanolic hydrochloric acid solution, though increasing the absorption intensity rendered the chromogen unstable. The 0.4 ml of 0.5 M HCl in methanol was recommended for colour development and to ensure stability for at least 12 h.

The use of 2.0 ml of 0.1% (w/v) of PDAC solution was considered satisfactory for the tetrahydrofolic acid concentration levels encountered in the present study. The time required for maximum formation of the derivative was 5 min and the absorbance was stable for at least 12 h.

The Beer–Lambert plot was found to be linear over a concentration range of 0.2–20 μM of FH_4: $A_{545} = a + bC$,

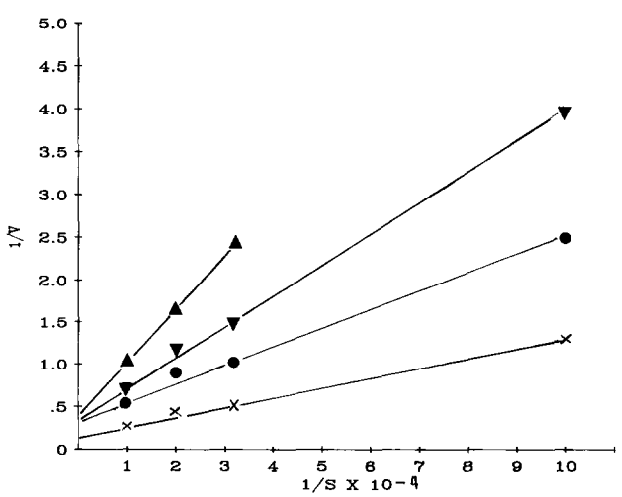


Figure 1
Lineweaver–Burk plot of folic acid–TMT interaction. TMT concentration: ×, 0.0 μM; ●, 0.1 μM; ▼, 0.5 μM; ▲, 1.0 μM.

where C is the concentration of FH_4 in μM, $a = 0.001$, and $b = 0.21$; the regression coefficient, $r = 0.9995$ for $n = 6$. The molar absorptivity was found to be 2.1×10^5. The above regression equation for calibration series of standard FH_4 solutions did not change when spiked with TMT or HCT. Therefore the developed method was used to monitor the effect of TMT, HCT and their combination on the activity of DHFR enzyme without any measurable interference.

The Lineweaver–Burk graphical method [12] was used to study the kinetics of the enzymatic reaction in the presence and absence of the studied drugs. The data obtained by measuring the velocity of the reaction with variable amounts of substrate, folic acid, were plotted as the conventional Lineweaver–Burk double reciprocal plot in absence and presence of TMT (Fig. 1), HCT (Fig. 2) and their mixture (Fig. 3). The velocity of

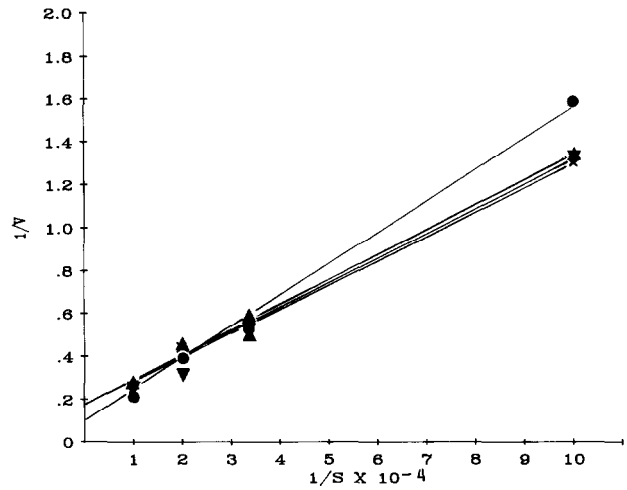


Figure 2
Lineweaver–Burk plot of folic acid–HCT interaction. HCT concentration: ×, 0.0 μM; ●, 0.1 μM; ▼, 0.5 μM; ▲, 1.0 μM.

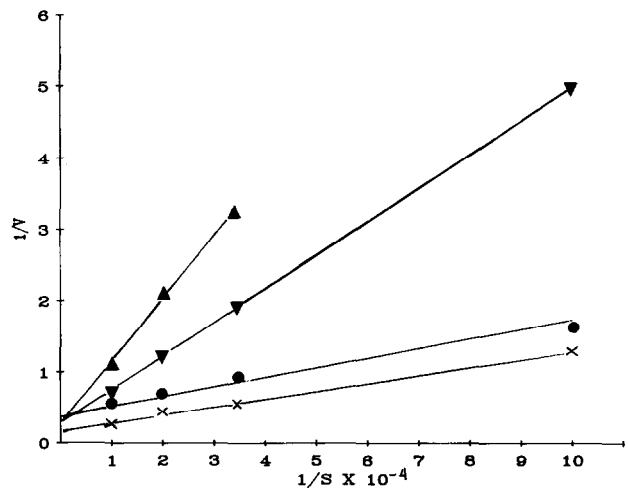


Figure 3
Lineweaver–Burk plot of folic acid–HCT–TMT interaction. HCT–TMT concentration: ×, 0.0 μM; ●, 0.1 μM; ▼, 0.5 μM; ▲, 1.0 μM.

the reaction V is represented by the number of micromoles of *p*-aminobezoylglutamic acid formed from the hydrolysis of FH_4 formed as measured by the developed method. These plots indicated that TMT alone or in combination with HCT has a competitive and reversible inhibitory effect for DHFR as shown by the intersect of the straight lines at a common intercept on the $1/V$ axis and by the reduced inhibition upon increasing the substrate concentration (Figs 4 and 5). There is no significant difference between the data obtained from TMT and from its combination with HCT. A 100% inhibition was obtained by 1 μM solution of TMT alone or in a mixture with HCT at a folic acid

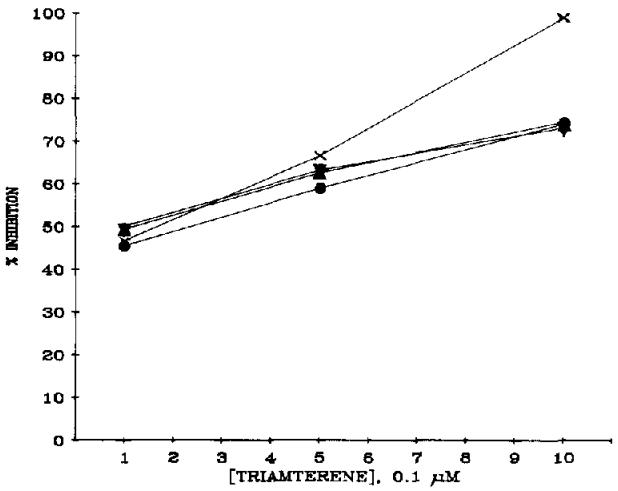


Figure 4
Effect of TMT concentration on the inhibition of DHFR activity. Folic acid concentration: ×, 0.0 μM; ●, 30 μM; ▼, 50 μM; ▲, 100 μM.

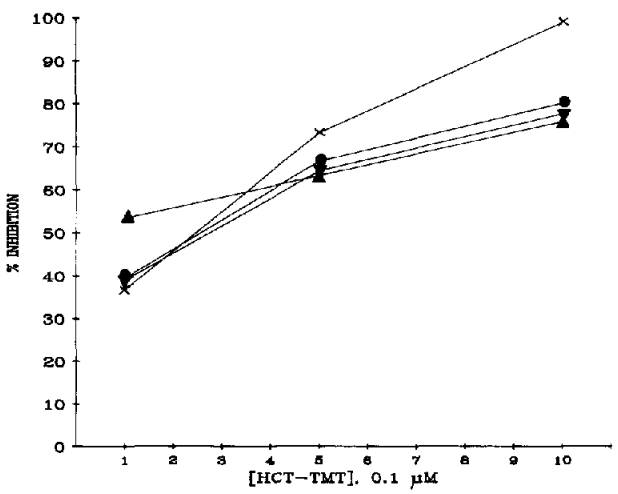


Figure 5
Effect of HCT–TMT concentration on the inhibition of DHFR activity. Folic acid concentration: ×, 0.0 μM; ●, 30 μM; ▼, 50 μM; ▲, 100 μM.

concentration of 0.01 mM. The superimposed plots in Fig. 2 for HCT and the control indicate non-inhibitory effect of HCT on DHFR activity.

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