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AN ENZYMATIC KINETIC METHOD FOR THE DETERMINATION OF 2′-DEOXYCOFORMYCIN IN BIOLOGICAL FLUIDS

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Abstract—An analytical method for determination of 2′-deoxycoformycin (2′-DCF) concentrations in plasma and urine was developed based upon a modification of adenosine deaminase (ADA) inhibition assays described in the literature. The method involves the spectrophotometric monitoring of the rate of deamination of adenosine by the enzyme in the presence of various concentrations of the inhibitor 2′-DCF, and relating the deamination rate to the 2′-DCF concentration. In the course of developing the method, it was found that adenosine deaminase appears to lose activity after dilution with phosphate buffer (pH 7.2). Enzyme inactivation was found to occur mono-exponentially with time and, in order to accommodate for this inactivation, a method was developed for quantitating 2′-DCF which takes into consideration the relative activity of the enzyme in the incubation mixtures. The results obtained from the analysis of samples containing known concentrations of 2′-DCF were fitted to a three-dimensional standard surface by means of a nonlinear least-squares regression computer program. Quantitation of 2′-DCF in patient samples is accomplished by an ADA inhibition titration technique in which the spectrophotometrically determined absorbance change is related to the two independent variables, the concentration of 2′-DCF in the standards and the relative time of the analysis. As little as 1 ng/ml of 2′-DCF in plasma can be quantitated with the assay.

2′-Deoxycoformycin (2′-DCF, NSC 218321) [*R*-3-(2-deoxy-β-D-erythropentofuranosyl)-3,6,7,8- tetrahydroimidazo[4,5-*d*][1, 3]diazepin-8-ol; covidaribine; pentostatin] which is currently in Phase I–II clinical trials is a potent inhibitor of adenosine deaminase (EC 3.5.4.4.) (ADA). 2′-DCF, a fermentation product isolated from *Streptomyces antibioticus*, was characterized by Woo *et al.* [1]. The "tight-binding" inhibition of ADA by 2′-DCF has been attributed [2, 3] to the structural resemblance of the tetrahedral carbon at C-8 of 2′-DCF to the transition state intermediate postulated to exist during the deamination of adenosine by ADA [4].

The clinical use of ADA inhibitors stems from two factors. First, the report by Giblett *et al.* [5], correlating a deficiency of ADA in two children with severe combined immune deficiency (SCID), suggested the requirement of this enzyme in lymphocytes. Second, as a transition-state inhibitor of ADA [2, 3], 2′-DCF not only blocks the conversion of adenosine to inosine but also the metabolism of any drugs which are deaminated by the enzyme, most notably adenine arabinoside (ARA-A) [6–8], an antiviral and antitumor agent.

Since 2′-DCF is a "tight-binding" inhibitor of ADA [2, 3], an enzyme inhibition titration assay can be developed employing any method which monitors the enzymatic reaction. Assays which have been described previously in the literature may be divided into three general classes. The first class of procedures [9–13] measured the ADA activity by quantifying the amount of inosine formed by use of a chromatographic technique to separate the product, inosine, from the substrate, adenosine. These procedures have used both radiolabeled and unlabeled adenosine as the substrate for ADA. The second class of procedures [14, 15] quantitated the amount of ammonium ion liberated in the conversion of adenosine to inosine. A spectrophotometric procedure [16–18] was used in the third class of procedures to monitor the rate of the enzymatic conversion of adenosine to inosine.

The basic method of Chassin *et al.* [17] and McConnell *et al.* [18] was adopted for determining the plasma pharmacokinetics of 2′-DCF in patients. In performing the assay on replicate plasma samples containing 2′-DCF, the results were poorly reproducible when identical samples were analyzed at various times over a period of several hours. An investigation of the origin of the failure to reproduce the assay results indicated that the enzyme was being inactivated in the phosphate buffer solution during the time course of the analyses. For the assay of a large number of plasma samples during a given day, it was necessary to develop an analytical method which corrected for the ADA inactivation. A model is presented which satisfactorily explained the observed log-linear relationship between the enzyme activity and 2′-DCF concentration. The observed enzymatic activity, following incubation with 2′-DCF, was a function of both the 2′-DCF concentration and the time interval between the assay of the first sample and a subsequent sample.

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MATERIALS AND METHODS

Materials. Adenosine and ADA (Type III) were obtained from the Sigma Chemical Co. (St. Louis, MO). 2′-DCF was produced by the Warner Lambert Co. and provided by Dr. Robert E. Engle, Pharmaceutical Resources Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Stock solutions of adenosine (11.2 mM) and 2′-DCF (10.52 μg/ml) were prepared in phosphate buffer (pH 7.2, 50 mM). An adenosine deaminase working solution (0.0005 units/μl) in phosphate buffer (pH 7.2, 50 mM) was prepared daily and stored in the refrigerator.

2′-Deoxycoformycin assay. 2′-DCF concentrations were determined by spectrophotometrically monitoring the inhibition of adenosine deaminase by following the conversion of adenosine to inosine at 265 nm. Reference standards were prepared by adding 2′-DCF to blank plasma to give a concentration range of 10 to 100 ng/ml (37.3 to 373 nM). Patient plasma samples were diluted, when necessary, with phosphate buffer (pH 7.2, 50 mM) to provide a concentration within the range of the standards. Samples for incubation with ADA were prepared by mixing the plasma sample (10 μl) with phosphate buffer (1.0 ml). The samples were then heated in a boiling water bath for 5 min in order to inactivate any endogenous adenosine deaminase. The samples were centrifuged at 1000 *g* for 2 min and then refrigerated for a minimum of 2 hr before the start of the analysis.

An incubation solution was prepared by adding ADA (37.5 mU) to a sample, selected at random from the set being analyzed. The mixture was incubated at 25° in a Dubnoff metabolic shaking incubator for 5 min. The adenosine solution (8 μl) was then added to the incubated sample with shaking to give a final concentration of 82 μM. After exactly 0.5 min during which the reaction mixture was mixed and the sample inserted into a Beckman model 35 spectrophotometer, the conversion of adenosine to inosine was monitored at 265 nm for 1.5 min.

RESULTS

Tight-binding inhibitor kinetics. Pseudo-zero-order conditions for the assay were obtained by measuring the initial reaction rates for only a short period of time. The substrate concentration is observed to decrease linearly with time with a slope equal to $-v$. Under these conditions the rate of the reaction is given by:

$$\text{RATE} = v = k_2' [E]_o'' \tag{1}$$

where $[E]_o''$ is the enzyme concentration remaining after incubation with the inhibitor and k_2 is the pseudo-first-order rate constant during the short incubation period when the adenosine concentration $[S]_o$ changes minimally.

$$k_2' = \frac{k_2 [S]_o}{K_m + [S]_o} \tag{2}$$

During the assay, the same amount of enzyme is added to each sample. After incubation with 2′-DCF, the concentration of enzyme available to react with adenosine decreases as the concentration of 2′-DCF increases, since 2′-DCF is a "tight-binding" inhibitor of ADA. Using the enzyme inhibition titration assay, $[E]_o''$ decreases with increasing inhibitor concentration which results in a proportional decrease in the observed v. Therefore, a family of curves for the absorbance change as a function of time are obtained for differing concentrations of inhibitor, with the rates inversely proportional to the inhibitor concentration.

A plot of the rate of adenosine deamination by adenosine deaminase as a function of 2′-DCF concentration was found to be nonlinear. Upon plotting the data semi-logarithmically (Fig. 1), a log-linear dependency of the deamination rate on 2′-DCF concentration was observed.

A linear relationship [18] has been reported between the degree of ADA inhibition and 2′-DCF concentration. However, a logarithmic relationship [17] has also been reported.

The apparent log-linear dependency of the deamination rate on 2′-DCF concentration can be explained in terms of the two steps of the assay procedure. These steps are: (1) incubation of ADA with 2′-DCF, and (2) determination of the uninhibited ADA.

In the first step, an aliquot of enzyme is added to a sample containing inhibitor. The reaction of enzyme with inhibitor is represented by Equation 3:

$$I + E \xrightarrow{k_i} EI \tag{3}$$

where I is the inhibitor, E is the enzyme, EI is the inactive enzyme–inhibitor complex, and k_i is the rate constant for the reaction. There is only a forward rate constant, since 2′-DCF is considered to be an irreversible inhibitor of ADA [3, 15, 19]. The rate equation for the reaction of 2′-DCF with ADA can be derived from the above scheme and is given in Equation 4:

$$\frac{d[E]''}{dt} = -k_i[I]\,[E]'' \tag{4}$$

where the brackets denote the concentration of I and the activity of E. Equation 4 is written in terms of the disappearance of enzyme, because the observed v for the conversion of adenosine to inosine by ADA determined in the second part of the analysis procedure is dependent upon the uninhibited enzyme concentration remaining after incubation with 2′-DCF.

Because the incubation period is very short and the amount of inhibitor is in excess, the concentration of inhibitor remains relatively unchanged throughout the incubation period. Therefore, at any time, the concentration of I can be considered equal to its initial concentration ($[I]_o$). Substituting for I in Equation 4 gives the rate expression shown in Equation 5.

$$\frac{d[E]''}{dt} = k_i\,[I]_o\,[E]'' \tag{5}$$

Equation 5 can be integrated to give Equation 6, which describes the activity of enzyme remaining

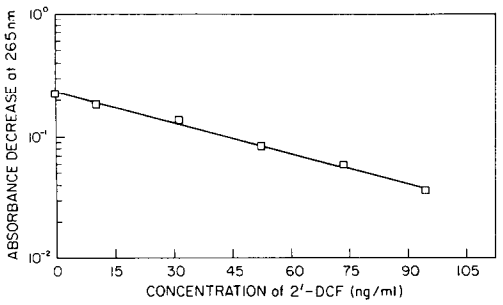

Fig. 1. Typical semi-logarithmic standard curve for 2′-DCF concentration versus absorbance decrease at 265 nm.

after incubation with inhibitor, ($[E]''_o$).

$$[E]''_o = [E]'_o e^{-k_i[I]_o t_i} \quad (6)$$

In Equation 6, $[E]'_o$ is the activity of enzyme available for reaction with the inhibitor and t_i is the length of the incubation period.

In step 2 of the assay, the residual uninhibited enzyme from step 1 is reacted with the substrate, adenosine. Equation 1 describes the enzymatic kinetic behavior under pseudo-zero-order conditions. Substitution of Equation 6 into Equation 1 gives Equation 7, which describes the dependency of the reaction rate for the conversion of adenosine to inosine upon the

$$\text{RATE} = k'_2 [E]'_o e^{-k_i[I]_o t_i} \quad (7)$$

inhibitor concentration. Under the conditions of the assay, the incubation time is held constant and, therefore, the observed reaction rate appears to be inversely proportional to the inhibitor concentration only. A logarithmic transformation of Equation 7 results in Equation 8 which predicts a log-linear relationship between the reaction rate for the conversion of adenosine to inosine and the inhibitor concentration.

$$\ln(\text{RATE}) = \ln(k'_2 [E]'_o) - (k_i t_i)[I]_o \quad (8)$$

When the time of incubation of ADA with 2′-DCF was held constant, the observed standard curve (Fig. 1) appears to be consistent with Equations 7 and 8. One method of verifying Equations 7 and 8 is to maintain the inhibitor concentration constant and vary the incubation time. A log-linear relationship

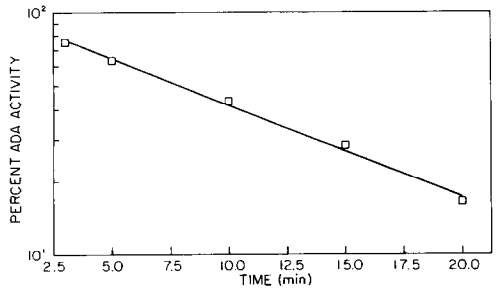

Fig. 2. Effect of incubation time on the percent activity of ADA in the presence of 196.2 nM 2′-DCF at 25°.

is predicted when the logarithm of the reaction rate for the conversion of adenosine to inosine is plotted as a function of the incubation time. Figure 2 shows that a log-linear relationship was obtained when the 2′-DCF concentration was held constant and only the incubation times were varied. A log-linear relationship has also been shown for ADA activity in human erythrocytes and mouse blood as a function of incubation time with 2′-DCF [3, 15, 20].

Kinetics of adenosine deaminase inactivation. An examination of results for the analysis of 2′-DCF over a 6-hr time interval indicated that there was a problem with reproducibility in the assay. Potential sources of this lack of reproducibility include random error, degradation of the drug, and inactivation of the enzyme.

Random error, by definition, implies that the results are randomly distributed about the mean for identical samples. However, when replicate samples containing 2′-DCF were quantitated with a time difference greater than 30 min, the observed reaction rate for the conversion of adenosine to inosine became consistently slower. Therefore, the lack of reproducibility cannot be attributable to random error.

When a series of standard curves obtained at various analysis times was plotted, each individual curve appeared to be log-linear. An Analysis of Variance test [21] showed that the slopes were identical, but that the intercepts were statistically different. The slope of the standard curve (Eq. 8) is equal to $k_i t_i$, which is a function of both the incubation time and the rate constant for the formation of the enzyme–inhibitor complex. Since the slopes and the incubation times for each sample are identical, the enzyme–inhibitor rate constant (k_i) appears to remain unchanged during the time-course of the analysis. The control samples containing no 2′-DCF also showed a decreasing rate with the time of analysis, so that the lack of reproducibility of the assay results was not attributed to degradation of 2′-DCF in the solutions, but instead of inactivation of ADA.

Schrader *et al.* [22] observed that dilution of ADA isolated from human erythrocytes resulted in inactivation of the enzyme which could be prevented by the addition of bovine serum albumin. Hall *et al.* [23] observed a steady decrease in enzyme activity over 96 hr for ADA extracted from sheep lymphocytes. The stability of the enzyme in dilute solutions has been reported to be proportional to protein concentration [24]. The increased instability observed for the enzyme in the working solution is apparently due to the dilution of protein contained in the commerical preparation.

It was concluded that inactivation of the enzyme during the time-course of the analyses occurs, since a continual decrease in the conversion rate of adenosine to inosine as a function of time was observed in both the samples containing 2′-DCF and control samples, with no inhibitor present. A plot of the enzyme activity in the absence of inhibitor as a function of time is log-linear and therefore the inactivation of ADA in the phosphate buffer is first-order. Equation 9, in which $[E]_o$ is the enzyme activity when the first sample is analyzed and k_d is the first-order rate constant for the inactivation of enzyme, is used

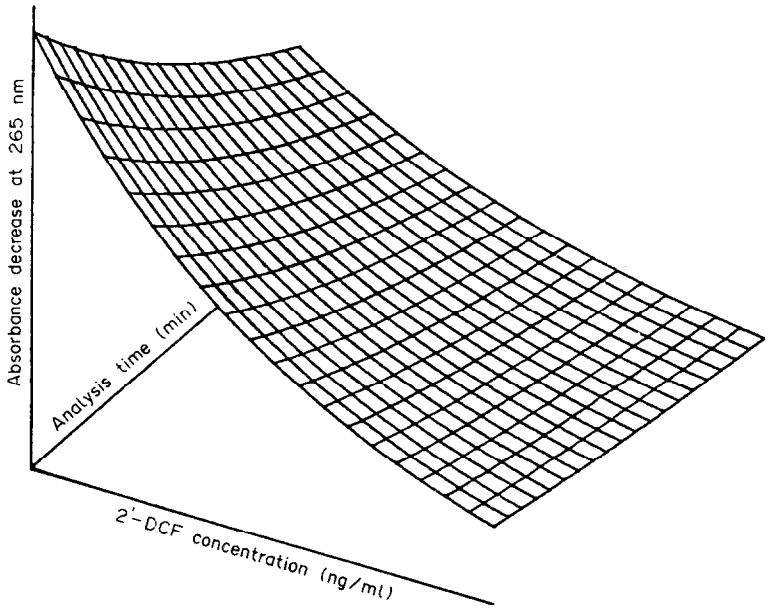

Fig. 3. Three-dimensional standard surface for 2'-DCF concentration, for one set of patient samples, as a function of both the enzymatic rate and analysis time, obtained by using Equation 10.

to predict the enzyme activity ($[E]'_o$) at any time (t_d)

$$[E]'_o = [E]_o e - k_d t_d \quad (9)$$

Combining Equations 7 and 9 results in Equation 10 which describes the enzyme activity as a function of both the relative time of analysis (t_d) and the inhibitor concentration ($[I]_o$). Solving for the coefficient and rate

$$\text{RATE} = k'_2 [E]_o e\, k_d t_d + k_i [I]_o t_i) \quad (10)$$

constants in Equation 10 was achieved with the use of a nonlinear regression program. Employing the nonlinear least-squares computer program NONLIN [25], the observed data was fitted to the model described by Equation 10. Because there are two independent variables (t_d and $[I]_o$), the standard curve represented by a graph of Equation 10 is a three-dimensional standard surface (Fig. 3).

Rearrangement of Equation 10, yielding Equation 11, permits the concentration ($[I]_o$) of 2'-DCF to be determined in an unknown sample.

$$[I]_o = \frac{\ln \left[\dfrac{\text{RATE}}{k'_2[E]_o}\right] + k_d t_d}{k_i t_i} \quad (11)$$

Both the enzyme activity and the time that a sample is incubated relative to the start of analysis of a set of samples is recorded. The 2'-DCF concentration is then solved as a function of both the observed enzymatic rate and the time of analysis.

The regression coefficients of the three-dimensional standard surface (Fig. 3) were used in Equation 11 to determine the 2'-DCF concentrations for the plasma concentration-time profile, shown in Fig. 4, of a patient who received 2'-DCF.

The mean values for the regression parameters (standard deviation), determined from the analyses of 2'-DCF concentrations in patient samples over a 6-month time interval, for k_i, k_d, and $k'_2[E]_o$ are 1.54×10^6 (1.66×10^5) $M^{-1}\,sec^{-1}$; 0.00407 (0.00184) min^{-1}; and 0.1781 (0.0663) absorbance units/min respectively. The value for k_i using calf intestinal ADA appears to agree with the values reported by Agarwal *et al.* [3] and Rogler-Brown *et al.* [15] for human erythrocytic ADA. The apparent k_i is expected to be a function of both the enzyme source and the incubation temperature. From the value of k_d, it can be determined that in the phosphate buffer there was an average 10% loss of the enzyme activity every 25 min. The rate of enzyme loss is expected to

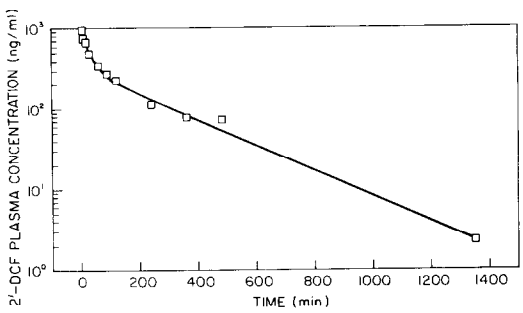

Fig. 4. Typical plasma concentration–time profile of 2'-DCF following the intravenous bolus administration of 10 mg/m² of 2'-DCF to a patient.

vary with the storage conditions (e.g. ice bath or room temperature) of the enzyme, during the time-course of the analysis.

DISCUSSION

The assay for the determination of 2′-DCF in biological fluids is dependent upon the inhibition of ADA activity and, therefore, may not be specific for 2′-DCF, if other ADA inhibitors are present. At present, no information is known regarding the metabolism of 2′-DCF in man. No metabolites of 2′-DCF were detected by Borondy *et al.* [6] in rats. The monophosphate of 2′-DCF has been observed in L1210 cells [26].

Cha [27, 28] proposed a model, identical with that described by Equation 5, for the interaction of enzyme with a hypothetical "tight-binding" inhibitor, when $[I]_0$ is much larger than $[E]_0$. The rates for association and dissociation between E and I are very slow compared to ordinary enzyme–substrate reactions. Therefore, the assumption of steady-state kinetics may not be applicable for "tight-binding" enzyme inhibitors, unless the inhibitor is allowed to interact with the enzyme for a much longer time period than was used in the present assay. The association of the "tight-binding" inhibitor coformycin with ADA also showed a log-linear relationship [2, 28] of the enzyme activity as a function of incubation time.

During the development of an assay for 2′-DCF, it was observed that the analytical results were not only dependent upon the drug concentration, but also upon the time interval from the start of the analyses of a set of samples. It appears that the enzyme inactivation occurs, after dilution to the working concentration. Standard curves for 2′-DCF which have been reported in the literature [17, 18] show the percent inhibition of enzyme as a function of 2′-DCF concentration. Assay results need to be corrected for the inactivation of the enzyme occurring during the analysis time period. The use of Equations 10 and 11 in the determinations of 2′-DCF in biological fluids permits a reduction in the total number of samples that are assayed, since fewer control samples containing no 2′-DCF need be analyzed.

A copy of the NONLIN subroutines used to fit the three-dimensional standard surface and to calculate the concentration of 2′-DCF in patient samples is available upon request from the authors.

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