*Journal of Chromatography*, 529 (1990) 431–436
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5314

# Note

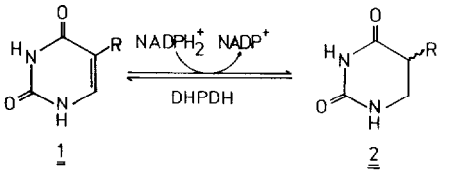
# New high-performance liquid chromatographic method for the determination of low activities of dihydropyrimidine dehydrogenase

CLAUS MICHAEL KLEIN and HERMANN JOSEF HAAS*

*Fachrichtung Medizinische Biochemie der Universitat des Saarlandes, D-6650 Homburg/Saar (F R G.)*

(First received October 6th, 1989; revised manuscript received March 5th, 1990)

Dihydropyrimidine dehydrogenase (EC 1.3.1.2, DHPDH), localized in the cytoplasm of numerous cells [1–10], catalyses the first step in the degradation of uracil and thymine to the corresponding 5,6-dihydro derivatives [11] (Fig. 1). Recent reports have described some children suffering from lack of DHPDH [12–16], three of whom exhibited neurological symptoms (absences, epilepsis, mental retardation). They showed, compared with healthy children, lower activities of leucocyte DHPDH (ca. 1%) and higher renal excretion of uracil and thymine. By gas chromatography, Bakkeren et al. [12] found large amounts of uracil and thymine in the urine of a six-year-old girl with undefined convulsive fits. Three members of the girl's family showed a complete absence of




1 2

Fig. 1. DHPDH-catalysed hydrogenation of uracil (**1**, R=H) and thymine (**1**, R=$CH_3$) to the corresponding 5,6-dihydro derivatives (**2**).

0378-4347/90/$03.50 

fibroblast DHPDH; two of the homozygotes and one heterozygote were epileptics [15].

DHPDH activities in leucocytes and fibroblasts have been determined photometrically by measuring the decrease of $NADPH_2^+$ [3,14,17] or by radiochemical detection of the 5,6-dihydro derivatives of $^{14}C$-labelled uracil and thymine following high-performance liquid chromatographic (HPLC) separation. The first method is not sensitive enough to determine pathologically reduced activities and the second one is relatively complicated.

The HPLC method described here can measure very low DHPDH activities without using radiochemicals. The method was established for DHPDH from rat liver.

## EXPERIMENTAL

### *Chemicals*

5-Bromouracil was purchased from Sigma (Deisenhofen, F.R.G.), disodium hydrogenphosphate dihydrate, potassium dihydrogenphosphate, sodium chloride and trichloroacetic acid from Merck (Darmstadt, F.R.G.), $NADPH_2^+$ from Boehringer (Mannheim, F.R.G.) and trioctylamine from Merck-Schuchardt (Darmstadt-Hohenbrunn, F.R.G.).

### *Preparation of DHPDH*

The enzyme was prepared from rat liver homogenate according to Fritzson [3] and kept in 1–4 ml of water at −20°C (stock solution). The protein concentration was determined by the Biuret method. To obtain lower protein concentrations the stock solution was diluted with aqueous sodium chloride (0.9%).

### *Chromatographic conditions*

The HPLC system consisted of two M 510 pumps, an U6K injector, a gradient control system (Waters-Millipore, Milford, MA, U.S.A.), a 2152 UV detector (LKB, Munich, F.R.G.) and a C-R3A integrator (Shimadzu, Düsseldorf, F.R.G.). The stationary phase (Hypersil ODS 2, particle size 5 μm, Grom, Herrenberg, F.R.G.) was kept between 28 and 30°C. The mobile phase (1.0 ml/min) was 0.02 *M* potassium dihydrogenphosphate (pH 5.6)–methanol (94:6, v/v).

### *Quantitation*

A 0.20-ml aliquot of the stock solution and 0.25 ml of $NADPH_2^+$ (2.5 mg/ml) were mixed with 2.30 ml of Sörensen buffer (pH 7.4). The reaction was started by adding 0.05 ml of 5-bromouracil (0.9 mg/ml). For measuring the activities of diluted enzyme solutions the volumes of the reaction mixture were reduced to 1.0 ml. At intervals, 50 μl (100 μl) of the mixture containing the stock solution (diluted enzyme solution) were mixed with 150 μl (100 μl) of

TABLE I

DETERMINATION OF THE SPECIFIC ACTIVITIES OF DHPDH BY HPLC

| Dilution | $n$ | Specific activity | |
|---|---|---|---|
| | | nmol/h/g of liver | nmol/h/mg of protein |
| Undiluted | 4 | 482.6–666.9 | 66.50–91.90 |
| 1:100 | 3 | 3.6–5.8 | 0.50–0.80 |
| 1:200 | 3 | 1.7–2.5 | 0.23–0.35 |
| 1:500 | 3 | 0.6–1.0 | 0.08–0.14 |
| 1:1000 | 3 | 0.3–0.6 | 0.04–0.08 |

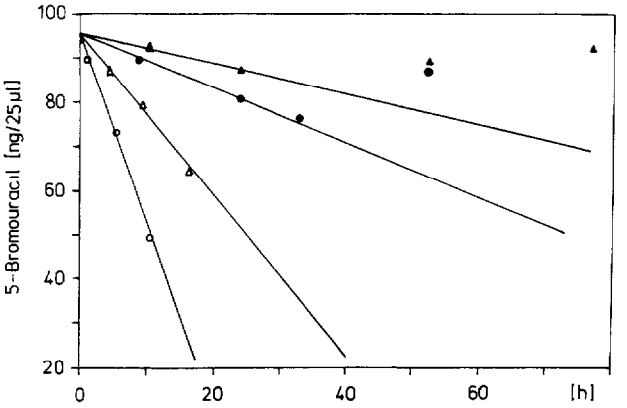



Fig. 2. Decrease of 5-bromouracil during incubation with $NADPH_2^+$ and DHPDH; (○) 1:100 diluted DHPDH solution; (△) 1:200 diluted DHPDH solution; (●) 1:500 diluted DHPDH solution; (▲) 1:1000 diluted DHPDH solution.

trichloroacetic acid (6%). After centrifugation at 8000 *g* for 2 min, the supernatant was shaken with 200 µl of a 0.5 *M* trioctylamine solution in freon and then centrifuged again for 2 min. Subsequently, 25 µl of the upper phase were injected. The wavelength for the determination of 5-bromouracil was 275 nm. The concentrations of 5-bromouracil in the samples were plotted against the incubation times. A regression line was drawn to calculate the change of 5-bromouracil concentration per hour and the specific activity (Table I; Fig. 2). The errors were minimized by multiple determinations ($n=3$ or 4).

## RESULTS

Fig. 3 shows three chromatograms illustrating the decrease of 5-bromouracil during incubation with $NADPH_2^+$ and DHPDH. In the range 5–200 ng the

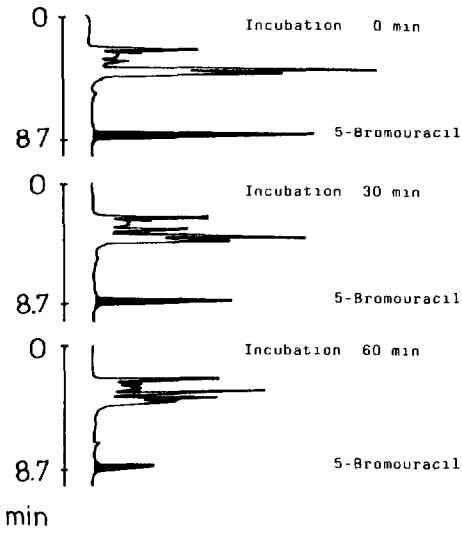



Fig. 3. HPLC profiles showing the decrease of 5-bromouracil during incubation with $NADPH_2^+$ and DHPDH. Conditions as in Experimental. Detection at 275 nm. Full scale deflection, 4 mV.

amounts of 5-bromouracil were linearly correlated to the corresponding integral areas.

Possible superpositions of the 5-bromouracil peak and contaminants or metabolites formed during the incubation at high enzyme activities were excluded by means of on-line spectra obtained with a photodiode-array detector. The activities for the undiluted enzyme were between 66.5 and 91.9 nmol 5-bromouracil per h per mg protein. Using diluted enzyme solutions (dilutions of 1:100, 1:200, 1:500 and 1:1000) in order to define the detection limit, the specific activities were so low that smaller incubation volumes, increased injection volumes as well as increased incubation times were necessary. Fig. 2 shows the results obtained.

For the calculation of the specific activities in the diluted enzyme solutions the protein content of the stock solution was used. This is acceptable because the total concentration of protein in the homogenates of leucocytes and fibroblasts is not much lower in the absence of DHPDH. The specific activity of the DHPDH decreases according to the degree of dilution. The dilution of 1:500 led to a specific activity of 0.08 nmol/h/mg protein, and the dilution of 1:1000 corresponded to an activity of 0.04 nmol/h/mg protein. The latter value is probably false, however, because 24 h later the concentration of 5-bromouracil was higher than before (Fig. 2). This error may be the result of a metabolite interfering with the 5-bromouracil peak.

## DISCUSSION

In order to establish a simple method for the determination of very low DHPDH activities, the DHPDH of rat liver homogenates was concentrated eight- to ten-fold [3] so as to minimize other $NADPH_2^+$-dependent processes. Enzyme solutions with lower activities were produced by simple dilution.

Accurate determinations of activity require a separation of 5-bromouracil from the other components in the incubation mixtures and a linear correlation between the amount injected and the integral area of the eluted 5-bromouracil. 5-Bromouracil is a better substrate than uracil because its affinity for DHPDH is 1.3 times higher [19] and because its HPLC signal can be exactly defined and analysed at 8.7 min (cf. Fig. 3). The optimal separation from the other components in the incubation mixtures was achieved by using an RP18 column and a mobile phase of 0.02 *M* potassium dihydrogenphosphate buffer (pH 5.6)–methanol (94:6, v/v).

Bakkeren et al. [12] and Berger et al. [13] showed by radiochemical detection that the leucocytes of healthy children exhibit DHPDH activities between 1.0 and 4.46 nmol uracil per h per mg protein. The specific activities of the leucocytes of DHPDH-lacking children were between 0.01 and 0.04 nmol uracil per h per mg protein. These low activities can also be measured by our HPLC method using 5-bromouracil as a substrate and by reducing the incubation volume and increasing the injection volume, as well as the time of incubation.

Furthermore we tried to determine the DHPDH activities photometrically by measuring the decrease of $NADPH_2^+$. This method was practicable for enzyme dilutions up to 1:100, but we were unable to observe changes of extinction for dilutions of 1:500 or 1:1000 during 16 h. The range of activity for which the method yielded reliable results was 0.6–1.1 nmol $NADPH_2^+$ per h per mg protein, which corresponds to a DHPDH activity in the lower part of the range for healthy children. This detection limit corresponds to the results of Piper et al. [20], who found a limit of 0.5–1.0 nmol/h/mg protein. The HPLC method affords reliable measurements of DHPDH activities as low as 0.08 nmol/h/mg protein, when a linear decrease of 5-bromouracil is detected within 24 h. Reliable determinations of 5-bromouracil are not possible later because 5-bromouracil begins to increase again.

## REFERENCES


1 T. Shiotani and G. Weber, J. Biol. Chem., 256 (1981) 219.
2 P. Fritzson, Acta Chem. Scand., 13 (1959) 2124.
3 P. Fritzson, J. Biol. Chem., 235 (1960) 719.
4 Y. Sanno, M. Holzer and R.T. Schimke, J. Biol. Chem., 245 (1970) 5668.
5 G. Weber, H. Kizaki, D. Tseng, T. Shiotani and E. Olah, Life Sci., 23 (1978) 729.
6 H.W Goedde, D.P. Agarwal and K. Eickhoff, Hoppe-Seyler's Z. Physiol. Chem., 351 (1970) 945.

7 H.W. Goedde, R. Hoffbauer and K.G. Blume, Biochem. Gen., 2 (1968) 93
8 J.C. Marsh and S. Perry, Arch. Biochem. Biophys., 104 (1964) 146.
9 L.L. Campbell, J. Bacteriol., 227 (1957) 693.
10 H. Kaspari, Microbiologica, 2 (1979) 231.
11 D. Keppler and A. Holstege, in H. Sies (Editor), Metabolic Compartmentation, Academic Press, London, 1982, p. 147.
12 J.A.Z.M. Bakkeren, R.A. De Abreu, R.C.A. Sengers, F.Z.M. Gabreels, J.M. Maas and W.O. Renier, Clin. Chim. Acta, 140 (1984) 247.
13 R. Berger, S.A. Stoker-de Vries, S.K. Wadman, M. Duran, F.A. Beemer, P.K. de Bree, Z.Z. Weits-Binnerts, T.Z. Penders and Z.K. van der Woude, Clin. Chim. Acta, 141 (1984) 227.
14 G. Berglund, J. Greter, S. Lindstedt, G. Steen, Z. Waldenstroem and U. Wass, Clin. Chem., 25 (1979) 1325.
15 J.P. Braakhekke, W.O. Renier, F.Z.M. Gabreels, R.A. De Abreu, J.A.Z.M. Bakkeren and R.C.A. Sengers, J. Neurol. Sci., 78 (1978) 71.
16 B. Wilcken, J. Hammond, R. Berger, G. Wise and C. James, J. Inher. Metab. Dis., 8 (1985) 115.
17 M.T. Dorsett, P.A. Morse and G.A. Gentry, Cancer Res., 29 (1969) 79.
18 J.X. Khym, Clin. Chem., 21 (1975) 1245.
19 T.E. Barman, Enzyme Handbook, Vol. I, Springer, Berlin, Heidelberg, New York, 1969, p. 150.
20 A.A. Piper, M.H.N. Tattersall and R.M. Fox, Biochim. Biophys. Acta, 663 (1980) 400.