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Determination of pteroic acid by high-performance thin-layer chromatography

Contribution to the investigation of 7,8-dihydropteroate synthase

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ABSTRACT

A TLC method was developed that allows the evaluation of the 7,8-dihydropteroate synthase reaction via determination of the stable pteroic acid (PtA), obtained by chemical oxidation of the enzymatic product dihydropteroic acid (H_2PtA). Using amino-bonded HPTLC plates and solution of 52% acetonitrile in 100 m*M* Tris–HCl buffer (pH 8.6) as the mobile phase it is possible to separate all enzyme substrates and competitively formed drug analogues of PtA from the oxidized enzymatic product. The method allows the detection of PtA at levels down to 0.5 ng/μl.

INTRODUCTION

The reaction shown in Fig. 1 is catalysed by 7,8-dihydropteroate synthase (SYN) (EC 2.5.1.15) of the folate pathway. Our interest was in the isolation of this enzyme from different organisms and in the investigation of its inhibition. Several methods for following this enzymatic reaction have been published: a biological detector system [1] and chromatographic systems (TLC, HPLC), analysing radiolabelled [2], fluorescent [3] or UV-absorbing compounds [4,5]. Other workers have described chromatographic separation techniques for different pteroyl derivatives, which were detected by radiolabelling [6] or a biological overlay technique [7,8]. For the detection of pteroic acid only, these procedures are difficult in handling and quantification, and especially the expenditure of time is high. Hitherto we employed a time-consuming HPLC method [5] for low-level detection and a TLC method [3] measuring a

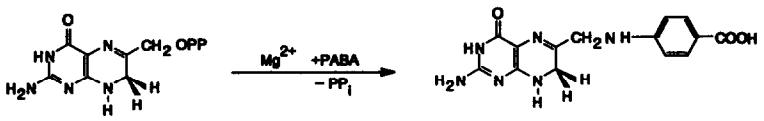

Fig. 1. Reaction catalysed by 7,8-dihydropteroate synthase. 7,8-Dihydropteridine alcohol pyrophosphate, enzymatically formed in a preceding reaction, is converted into 7,8-dihydropteroic acid by incorporation of *p*-aminobenzoic acid.

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fluorescent product that could not be quantified. The HPTLC method described here, however, combines fast enzyme detection with quantitative measurements and is adaptable to the study of the enzyme's inhibition by different drugs.

EXPERIMENTAL

Materials

The chemicals used were of analytical-reagent grade and were purchased from Merck (Darmstadt, Germany), as were amino-bonded HPTLC plates (20 × 10 cm). Pteroic acid (PtA) applied as a standard was obtained from Aldrich (Steinheim, Germany) and acetonitrile (ACN) from Baker (Gross-Gerau, Germany). 3,4-Dimethyl-5-sulphaisoxazole (SA) and its PtA analogue N^1-5-(3,4-dimethylisoxazolyl)-N^4-(6-pterinylmethyl)sulphanilamide (Pt-SA) were synthesized in our laboratory according to ref. 9. For chromatographic measurements, a Nanomat III, a horizontal development chamber (20 × 10 cm) and a TLC Scanner II working with CATS software 3.12, obtained from Camag (Muttenz, Switzerland) were used.

Methods

For system development and subsequent investigations 7,8-dihydropteroic acid (H_2PtA), pteroic acid (PtA), 7,8-dihydropteridine alcohol (H_2PtOH) and *p*-aminobenzoic acid (PABA) were used singly and in artificial mixtures. For the optimization of the mobile phase the method of Issaq and Seburn [10] was applied. To determine R_F values and for the determination of PtA, the diffuse reflectance signal at 280 nm was used.

As PtA is much more stable than the SYN product H_2PtA, the latter was oxidized to PtA according to ref. 4. However, we avoided trichloroacetic acid (coprecipitation of PtA and protein). Further, the excess of the oxidizing agent was destroyed by 1,4-dithiothreitol (DTT) (6 mmol/l). The volumes of the reagents were adapted to the amount of PtA. Samples of 1 μl were applied to the TLC plates (20 × 10 cm) and developed horizontally at room temperature in an unsaturated chamber.

RESULTS AND DISCUSSION

Previous HPLC experiments using amino-bonded phases indicated a good separation of PtA. As the instability (hydrolysis) of this column material does not disturb TLC, amino-bonded HPTLC plates were selected. The two mobile phases fulfilling the requirements of the optimization method were Tris–HCl buffer + $CaCl_2$, which allowed the separation of PABA from the rest of the reaction mixture, and an aqueous solution of ACN (>75% ACN), which separated PABA and H_2PtOH from stationary H_2PtA and PtA. These experiments resulted in the selection of 100 m*M* Tris–HCl (pH 8.6) containing 120 m*M* $CaCl_2$ as mobile phase A and 65% ACN in 100 m*M* Tris–HCl (pH 8.6) as mobile phase B. Mixtures of 0, 25, 50, 75 and 100% A in B were prepared as mobile phases. Fig. 2 shows the R_F values for four chromatographic runs. The mixture with 75% A was not tested because of the expected similarity to the runs with 100% and 50% A. A solvent composition in the range 10–30% mobile phase A gave a good separation of all components. Table I shows the calculated compositions of the liquid phases. However, the actual concentration of $CaCl_2$ was lower than the calculated value. Some time after mixing, opacity was observed in liquid

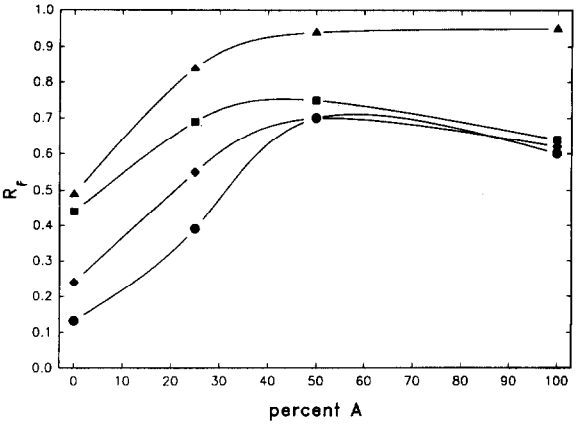

Fig. 2. Plot of R_F values *vs.* mobile phase composition. Amino-bonded HPTLC plates. ● = PtA; ◆ = H_2PtA; ■ = H_2PtOH; ▲ = PABA. Mobile phase A: 100 m*M* Tris–HCl (pH 8.6)–120 m*M* $CaCl_2$. Mobile phase B: 65% ACN in 100 m*M* Tris–HCl (pH 8.6).

TABLE I

COMPOSITIONS OF MOBILE PHASES

Solvent	A (%)	B (%)	Calculated mobile phase composition		
			ACN (%)	$CaCl_2$ (m*M*)	Tris–HCl (m*M*)
1	100	0	0	120	100
2	50	50	32.5	60	100
3	25	75	48.75	30	100
4	0	100	65	0	100

phases containing higher concentrations of ACN, indicating a decreased solubility of $CaCl_2$. Experiments with increasing $CaCl_2$ concentrations in the mobile phase using $CaCl_2$-impregnated and normal HPTLC plates showed that the addition of $CaCl_2$ influenced the R_F values only up to 5 m*M*. Higher concentrations were detrimental for the separation effect and for the peak symmetries on the impregnated plates. Hence $CaCl_2$ was not necessary for a good separation with the ranges of solvent composition used. In experiments with ACN-containing phases, solvent demixing resulted in a secondary solvent front [11]. The separation of the solutes was observed to occur below the secondary front. Therefore, in subsequent experiments the R_F values were calculated on the basis of this front and were labelled as R_x values.

Fig. 3 shows the R_x values at different Tris–HCl buffer concentrations in 52% ACN at pH 8.6. Lower buffer concentrations reduced the distance between the peaks. Concentrations above 40 m*M* were sufficient for baseline separation. To establish the pH dependence of the separation, a set of nineteen mobile phases were tested. Each phase was composed of 52% ACN and 100 m*M* Tris–HCl or phosphate buffer, with different pH values. Fig. 4 shows a plot of R_x *vs.* pH. The R_x values show differences in the influence of the buffer species. Phosphate buffers above pH 6.5 did not separate all compounds satisfactorily. Nevertheless, a separation over the total range of pH tested is possible. Fig. 5 shows an example using 100 m*M* Tris–HCl (pH 8.6) and 52% ACN.

The inhibition studies present an additional

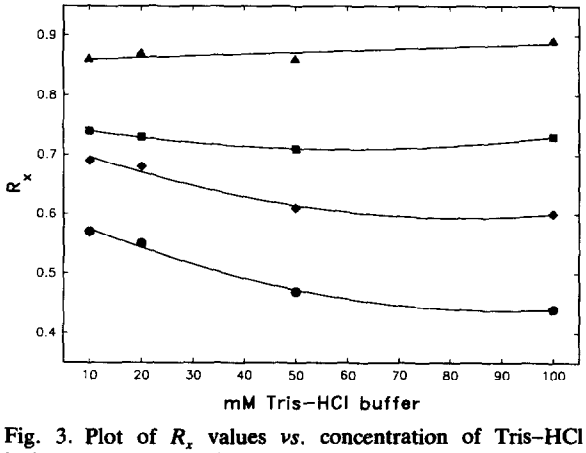

Fig. 3. Plot of R_x values *vs.* concentration of Tris–HCl buffer. Amino-bonded HPTLC plates. Symbols as in Fig. 2.

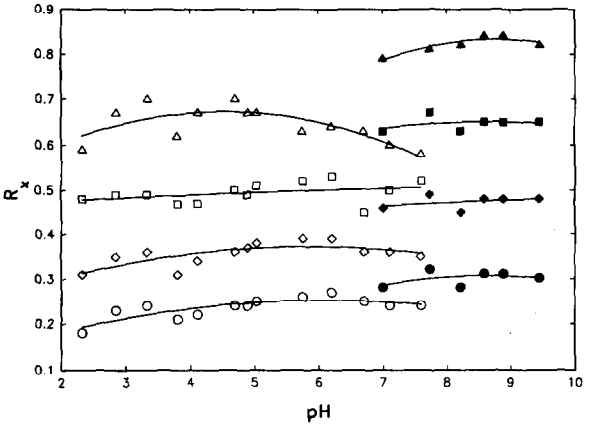

Fig. 4. Plot of R_x values *vs.* pH. Mobile phase: 52% ACN in 100 m*M* buffers of Tris–HCl (closed symbols) and phosphate (open symbols). Amino-bonded HPTLC plates. ●, ○ = PtA; ◆, ◇ = H_2PtA; ■, □ = H_2PtOH; ▲, △ = PABA.

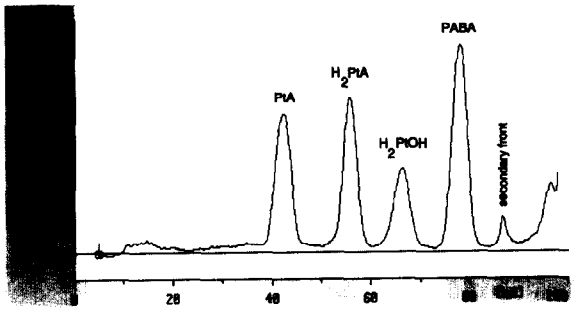

Fig. 5. Separation of PtA, H_2PtA, H_2PtOH and PABA on an amino-bonded HPTLC plate using 52% ACN in 100 m*M* Tris–HCl (pH 8.6) as mobile phase; 280 nm.

problem. It has been shown that the inhibition of *Escherichia coli* SYN by sulphonamides leads to the production of PtA analogues (Pt-SA) containing sulphonamides instead of PABA [9]. Therefore, two more solutes (Sa, Pt-SA) had to be separated. An analogue with 3,4-dimethyl-5-sulphaisoxazole (SA) was synthesized and analysed together with all the other substrates and the sulphonamide using the liquid phase described above. PtA was perfectly separated from all other solutes (Fig. 6).

Finally, it was difficult to examine samples quantitatively in the presence of proteins. After direct application of aqueous solutions, adsorption of PtA on the protein was observed, resulting in a disturbed chromatographic separation. Alkalinization of the sample by addition of 10 *M* NaOH was ineffective. Whereas the absorption of protein could be overcome, strong adsorption to magnesium hydroxide was ob-

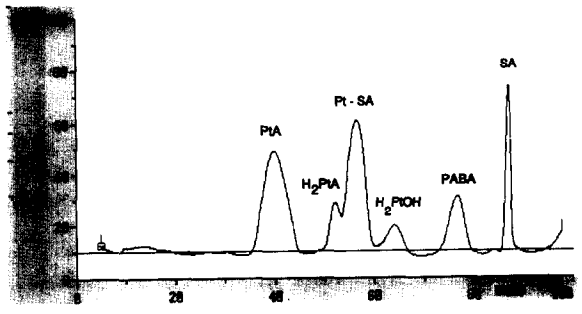

Fig. 6. Separation of PtA, H_2PtA, H_2PtOH, PABA, SA and Pt-SA on amino-bonded HPTLC plates using 52% ACN in 100 m*M* Tris–HCl (pH 8.6) as mobile phase; 280 nm.

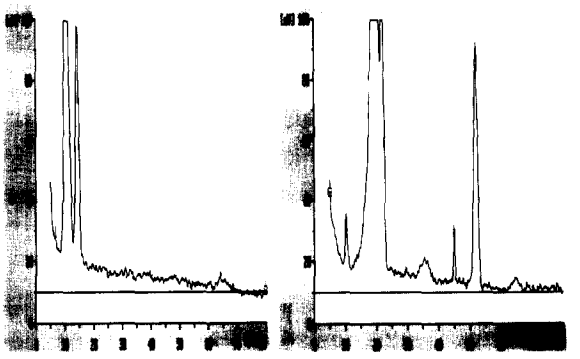

Fig. 7. Separation of PtA in the presence of protein. Untreated TLC plate on the left and spotted plate (1 μl of 1 *M* NaOH) on the right; 280 nm.

served that was not reversible by the addition of EDTA (*note:* magnesium ions are necessary for the enzyme reaction). However, this problem could be overcome as follows: before the sample was applied, the application point was directly alkalinized with 1 μl of 1 *M* NaOH ("spotting"). This procedure had two effects: first, the adsorption of PtA on the protein was terminated (up to 5 mg/ml of protein were tested), and second, the PtA peak was focused (Fig. 7). The shape of the calibration graph depends on the kind of sample application. The "spotting" of the TLC plate described above led to a non-linear dependence. However, a linear relation-

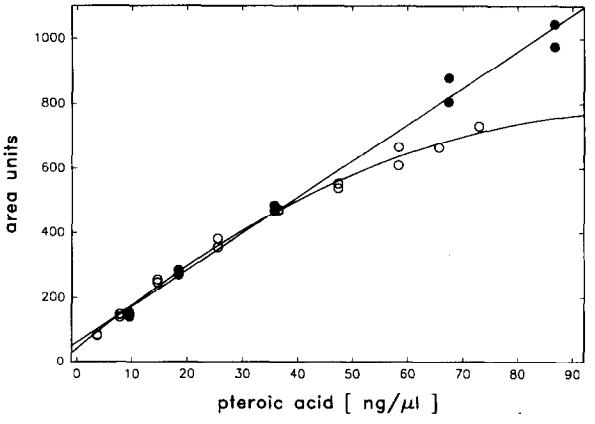

Fig. 8. Calibration graphs for the determination of pteroic acid. Plot of area units *vs.* concentration of PtA (ng/μl). ● = Aqueous solution of PtA: $y = 97.79 + 9.08x$, $r = 0.985$, S.D. = 41.44, $n = 18$. ○ = Spotted samples of PtA: $y = 42.77 + 14.21x - 0.069x^2$, $r = 0.997$, S.D. = 17.87, $n = 16$.

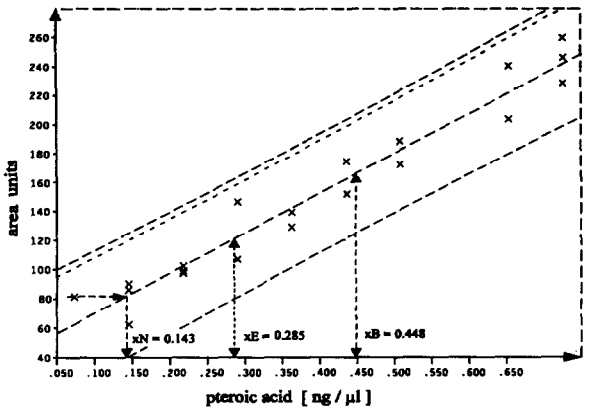

Fig. 9. Statistical calculation of the limit of determination [12]. xN = Limit of detection; xE = limit of identification; xB = limit of determination.

ship was obtained using solutions with pH < 9 (if another sample preparation is preferred, *e.g.*, solid-phase extraction) and untreated plates (Fig. 8). Sample treatment affects the shape of the PtA spot obtained. The untreated samples showed larger oval areas, whereas the "spotted" samples were separated into higher loaded bands instead of spots. For both preparations the determination limit of PtA was <0.5 ng/µl as determined by a serial dilution tests (Fig. 9). Samples containing 700 ng/µl were evaluated, but a suitable upper limit was 70 ng/µl. A linear relationship (area units/pteroic acid) is obtained in the lower range up to 7 ng/µl for both preparations.

CONCLUSIONS

The proposed HPTLC method is practical for the rapid detection of the SYN enzyme. The sensitivity is in the lower micromolar (ng/µl) range. The accessible pH range is large, and the system is tolerant to changes in the acetonitrile content in the mobile phase. It is possible to separate PtA from all other substrates of the SYN enzyme reaction and at least from one PtA analogue and its contaminating sulphonamide. The disadvantage of the method is the low dynamic range. To detect higher concentrations of PtA or enzyme concentrations, appropriate dilution of the sample is necessary. Higher sensitivity for biological systems [1,6,7] could be achieved by a concentration step using solid-phase extraction. Further, on-line automation should be feasible.

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