MS/MS system. The metabolites were separated on a Phenomenex Aqua analytical column (250 x 4.6 mm, 5 µm particle size), protected by a guard column (SecurityGuard C18 ODS; 4 x 3.0 mm; Phenomenex). Solvent A consisted of 50 mM HCOOH (pH 2.6) and solvent B consisted of methanol. The eluent from 5.8 to 8.0 min was introduced into the mass spectrometer. A Quattro II tandem mass spectrometer (Micromass) was used in the negative Electrospray ionization (ESI) mode and nitrogen was used as the nebulizing gas. Multiple-reaction monitoring was used to detect the metabolites by the specific m/z transition of precursor ion to fragment. Analysis of 5FU levels in plasma was also performed using a reversed-phase HPLC-UV method, as described before (3).

Results

The detection of 5FU and the internal standard 1,3-$^{15}N_2$-5FU was performed using multiple-reaction monitoring with an *m/z* 129→42 and *m/z* 131→43, respectively. The optimal settings of the mass spectrometer for the detection of 5FU and 1,3-$^{15}N_2$-5FU were a cone voltage of 35V and a collision energy of 15 eV. The intra-assay variation and inter-assay variation of plasma with added 5FU (1 µM, 10 µM, 100 µM was less then 6%. Recoveries of the added 5FU (1 µM, 10 µM, 100 µM) were > 97% and the detection limit of 5FU was 0.05 µM. Figure 1 shows that the analysis of the 5FU levels in plasma samples from patients with the HPLC tandem mass spectrometry method and a reversed phase HPLC-UV method yielded comparable results ($r^2 = 0.98$).

Discussion

5FU has a relatively narrow therapeutic index and a strong correlation has been described between exposure to 5FU and both hematological and gastrointestinal toxicity. Despite the many different treatment schedules that exist for 5FU, comparable AUC thresholds have been observed for the onset of severe toxicity. In the case of a deficiency of DPD, profound alterations in the metabolism of 5FU can be expected with an increased likelihood of developing severe toxicity (1, 2). Controlling the AUC of 5FU might therefore be an attractive approach.

In this study we demonstrate that with HPLC-tandem mass spectrometry, 5FU could be measured within 16 min with a detection limit of 0.05 µM, which is at least 6 times more sensitive than the HPLC-UV method (3). The use of stable-isotope-labeled 5FU enabled the correction of the signal for quenching by coeluting compounds, resulting in high recoveries of >97%. The reproducibility of our method is demonstrated by the low intra- and inter-assay variation (< 6%). Thus, the highly specific and sensitive HPLC tandem mass spectrometry method allows the rapid and unambigious analysis of 5FU levels in plasma and could, therefore, be used for therapeutic drug monitoring.

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Determination of thymidine phosphorylase activity by a non-radiochemical assay using reversed-phase high-performance liquid chromatography

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Thymidine phosphorylase (TP) catalyses the first step in the degradation of the pyrimidine deoxynucleosides thymidine and deoxyuridine. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disease which is caused by a thymidine phosphorylase deficiency (1). Clinically, MNGIE is characterised by ptosis, progressive external ophthalmoplegia, severe gastrointestinal dysmotility, cachexia, peripheral neuropathy and skeletal myopathy (2). In patients with MNGIE, no or a severely reduced TP activity was detected in leukocytes. A serious drawback of the applied spectrophotometric assay is the fact that the non-specific absorbance of interfering substances of crude tissue extracts hampers the accurate determination of the TP

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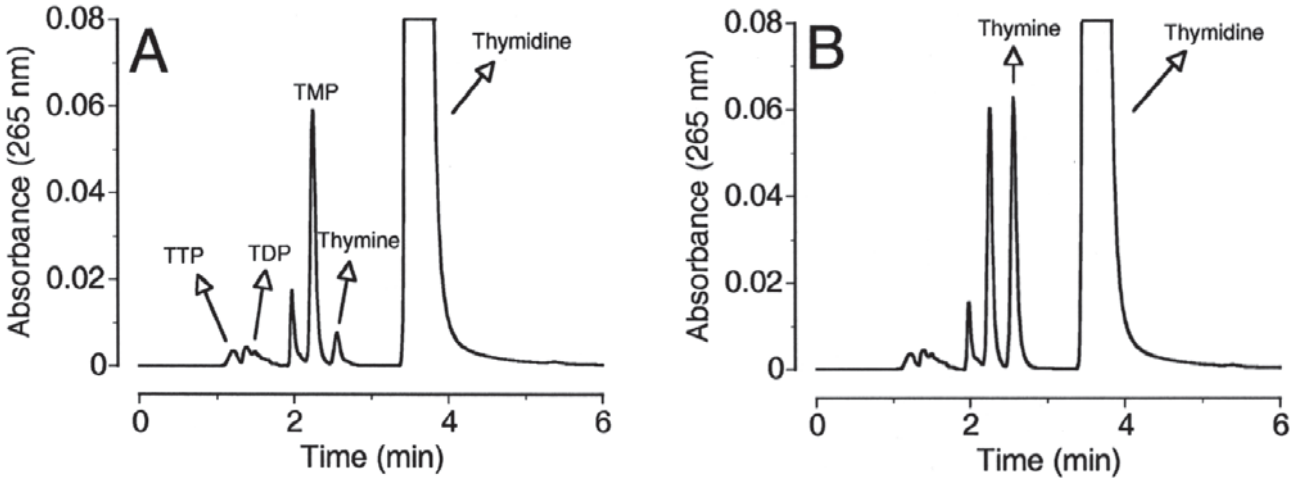

Figure 1. HPLC-elution profile of a reaction mixture. Panel A shows the elution profile of a reaction mixture prior to the start of the incubation. Panel B shows the elution profile of the same reaction mixture obtained after incubation at 37 ºC for 1 h.

activity. Therefore, we have developed a sensitive and fast assay of the TP activity which is based on the separation of thymine and thymidine using reversed-phase HPLC (3).

Materials and Methods

EDTA-anticoagulated blood (4 ml) was centrifuged at 450 x g at room temperature for 10 min and the buffy coat, containing the leukocytes, was collected and treated with ice-cold NH_4Cl to lyse the contaminating erythrocytes. The TP assay was performed in a reaction mixture containing an aliquot of cell sample, 35 mM potassium phosphate (pH 7.4), 1 mM dithiothreitol and 2 mM thymidine in a total volume of 0.5 ml. The reaction was started by the addition of the sample. After 15, 30, 45 and 60 min, 100 µl was removed from the reaction mixture and the reaction catalysed by TP was terminated by the addition of 11 µl of ice-cold 8 M $HClO_4$ and kept on ice for 10 min. After centrifugation, the resulting supernatants were saved for analysis by reversed-phase HPLC, as described before (2). The supernatant (75 µl) was injected into the HPLC system and separation of thymine from thymidine was performed isocratically [0.2% (w/v) acetic acid and 7% (v/v) acetonitrile] at a flow rate of 1 ml/min by HPLC on a reversed-phase column (Alltima C18 rocket, 250 x 4.6 mm, 3 µm particle size, Alltech Associates Inc., Deerfield IL) and a guard column (Alltima C18 5 µm particle size) with online UV detection at 265 nm.

Results

Figure 1 shows that a complete baseline separation was obtained within 6 min for thymine and the other compounds present in the assay mixture. The retention times of thymine and thymidine were 2.5 min and 3.6 min, respectively and the amount of thymine produced by TP from leukocytes was readily detectable (fig. 1B). The detection limit of thymine and thymidine in the HPLC system, defined as three times the value of the baseline noise, was approximately 0.8 and 0.7 pmol, respectively. The assay was linear with reaction times, up to at least 4 h, and protein concentrations up to at least 65 µg/ml. A normal Gaussian distribution was observed for the TP activity in leukocytes from controls. The TP activity did not differ with increasing age. The mean TP activity in this population was 316 ± 85 nmol/mg/h (n = 103) and was identical in man and women.

Discussion

MNGIE is an autosomal recessive disease which is caused by loss of function mutations in the gene encoding TP (1, 2). Since these patients present with no or a severely reduced activity of TP in leukocytes, the availability of an accurate assay to measure the TP activity is, therefore, of paramount importance. To date, the most frequently used assay of the TP activity is that in which the amount of thymine is measured spectrophotometrically at alkaline pH (1, 2). Unfortunately, the presence of interfering substances in the crude tissue extracts has been shown to hamper the accurate determination of the TP activity. In addition, the spectrometric assay was not linear over time which prevented the analysis of the steady-state kinetics of mutant TP enzymes (2). In this study, we developed an accurate assay procedure for TP, in which a complete separation of thymine from thymidine and other interfering metabolites was achieved in 6 min, using reversed-phase HPLC. Furthermore, the assay proved to be highly sensitive with a minimum amount of thymine that could be detected of 0.8 pmol.

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