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Short Communication

Difference enzyme spectrophotometric determination of 6-mercaptopurine in urine

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Introduction

6-Mercaptopurine (6-MP) has been used since the early fifties in the treatment of acute lymphoid leukaemia of childhood [1]. From the very beginning there have been great efforts to develop suitable methods for its determination in body fluids. As a consequence of the low plasma levels (a few ng ml^{-1} to a few hundred ng ml^{-1}) these analyses require particularly sensitive and selective methods. The earliest published methods were based on colorimetry [2] and later on fluorimetry [3, 4], but more recently chromatographic procedures have become available. A GC–MS method requiring derivatization with pentafluorobenzyl bromide excels with its high selectivity and sensitivity [5]. In recent years several HPLC methods have been described which do not require derivatization. Although a method based on electrochemical detection has been described [6] the majority of papers report on the use of UV detectors. As a consequence of the low concentration of 6-MP in plasma either very high volumes have to be injected [7, 8] or lengthy extraction procedures have to be carried out [9, 10]. The use of fluorimetric detection after pre-column [11] or post-column [12] derivatization has also been described and to some extent this has resulted in improved sensitivity.

Much less attention has been paid to the determination of 6-MP in urine; this task is simpler because of the higher concentrations which are in the μg ml^{-1} range. This enabled UV spectroscopy to be used after ion-exchange chromatographic separation and the addition of mercuric chloride which shifts the spectrum of 6-MP; this serves as the basis of a fairly specific assay [13]. Another interesting method is an indirect volumetric procedure based on the titration of iodine formed in the iodide-azide reaction catalyzed by 6-MP [14, 15]. Of course HPLC can also be used for this purpose [8] and an isotope dilution assay has also been described [34].

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The aim of the present study was to develop as an alternative to the HPLC procedure a simple difference spectrophotometric assay for the determination of 6-MP in urine without preliminary extraction or separation. The method is based on the spectral shift caused by the enzyme-catalyzed oxidation of 6-MP to thiouric acid (6-TUA).

Experimental

Apparatus

Spectra were recorded and the assay was carried out using a Pye-Unicam SP-1800 UV-Vis spectrophotometer. Silica cells (10 mm) were used.

Materials

6-Mercaptopurine was the gift of Chinoin Pharmaceutical Works (Budapest, Hungary). Xanthine oxidase (EC 1.2.3.2; Grade IV) was purchased from Sigma (St. Louis, USA). The activity of the enzyme suspension according to the label claim was 0.1 unit mg^{-1} protein. All other reagents were of analytical reagent grade and were purchased from Reanal (Budapest, Hungary).

Reagents

EDTA solution (0.05 M). Phosphate buffer solution (0.1 M, pH 7.9). 26.8 g of disodium hydrogen phosphate heptahydrate was dissolved in 800 ml of water, the pH was adjusted to 7.9 with 0.1 M hydrochloric acid and the solution was diluted to 1000 ml with water.

Procedure

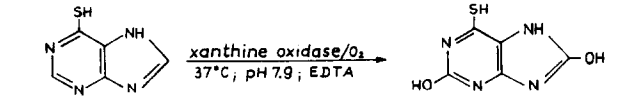
Phosphate buffer 10 ml solution, 0.4 ml of 0.05 M EDTA solution and 25 µl of the enzyme suspension were transferred to a 25-ml volumetric flask. 2.0 ml of the urine sample was added and the mixture diluted to volume with water (solution A). A similar solution was prepared but the enzyme was omitted (solution B).

Both solutions were allowed to stand at 37°C for 5 h. Solutions A and B were then cooled to room temperature and transferred to the sample and reference cells, respectively, and the difference spectrum was scanned. The 6-MP concentration was calculated from the absorbance difference at the maximum near 348 nm.

For the HPLC assay the procedure of Whalen *et al.* [10] was adopted.

Results and Discussion

The basis of the new method is the hydroxylation of 6-MP at positions 2 and 8 (reaction scheme) by atmospheric oxygen, catalyzed by the enzyme xanthine oxidase [16].



6 - mercaptopurine

6 - MP

6 - thiouric acid

6 - TUA

This reaction has previously been used for the determination of 6-MP. Tawa and Hirose [4, 17] oxidized 6-MP enzymatically followed by oxidation with chromic acid to form the highly fluorescent 6-sulphonate derivative.

In the present study the bathochromic shift which occurs as a result of the enzymatic hydroxylation reaction has been made use of. Figure 1 shows the spectrum of 6-MP (curve *a*), its enzymatic oxidation product, 6-TUA (curve *b*) and the difference spectrum (curve *c*). The strong bathochromic shift (34 nm) accompanied by a slight hyperchromic shift is a suitable basis for the difference spectrophotometric assay where the unoxidized form of the sample is placed into the reference cell and the enzymatically oxidized form of the same concentration into the sample cell; thus it is possible to eliminate interference originating from the spectra of any accompanying materials which do not change their spectra during the enzymatic treatment. Difference spectrophotometry is widely used in pharmaceutical analysis [18] and has been long employed by one of the authors (S.G.); methods include those based on acid–base equilibria [19–22], rearrangement and elimination reactions [23–27], reduction [28–30] and other reactions [31].

Curve *c* in Fig. 1 shows the difference spectrum at the positive maximum (λ_{max} = 348 nm; $\Delta\epsilon$ = 22.600) at which quantitative measurements can be carried out. In the

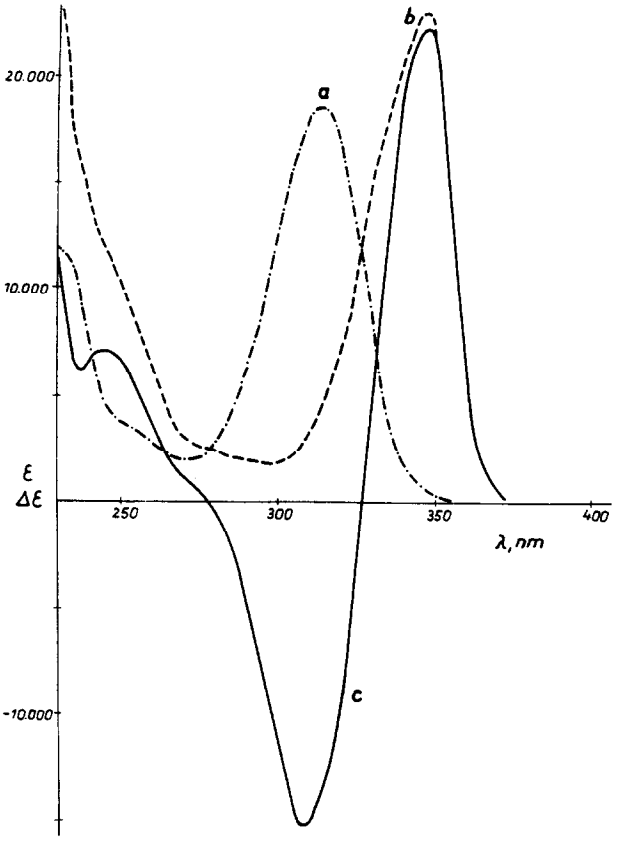


Figure 1
Spectra of 6-mercaptopurine. a. Spectrum without enzymatic oxidation; b. spectrum after enzymatic oxidation (6-TUA); c. difference spectrum (a/b). For details see text.

course of optimisation of the reaction, pH 7.9 was found to be the optimum value for the enzymatic reaction in accordance with the literature [16, 17]. This pH is suitable also for the difference spectrophotometric assay. The reason for this is that as a weak dibasic acid [32] 6-MP exists at pH 7.9 predominantly in the mono-anionic form (λ_{max} = 314 nm) and the spectrum is almost unchanged in the pH 7–9 range. The absorption maximum (324 nm) of the unionised form, which exists below pH 6, would be unfavourable for the difference spectrophotometric assay because it would decrease the bathochromic shift and therefore the sensitivity of the measurement. The di-anionic form is present at too high pH values (above 12) and does not show any advantage (λ_{max} = 310 nm). The spectrum of 6-TUA is also constant in the pH 6–9 range thus ensuring favourable conditions for the difference spectrophotometric assay.

Of the recommendations in the literature [16] the choice of temperature (37°C) and the addition of EDTA to the reaction mixture have been accepted; the latter influences favourably the reaction rate although the mechanism is unknown.

The rate of the enzymatic reaction depends largely on the enzyme used. The batch-to-batch variation in activity of enzyme from the same source is rather high and the activity depends also on the age of the enzyme preparation. The time sequence of the transformation shown in Fig. 2 is characteristic of an enzyme preparation of medium activity within its category (Grade IV). It can be seen that the reaction is practically complete within 4 h. If the activity of the enzyme had decreased below this level, the enzyme was not used; with some of the fresh preparations two- to three-fold activity was found. The stability of the reaction product (6-TUA) is practically unlimited under the conditions of the spectrophotometric measurement. Beer's law was found to be obeyed in the 0–2 difference absorbance range corresponding to about 0–1.5 mg/100 ml of 6-MP:

$$A_{348} = a + b.c$$

where c is the concentration of 6-MP in mg/100 ml. For an aqueous solution a = 0.003 and b = 1.41; for human plasma spiked with 6-MP at a concentration range of 0.15–15

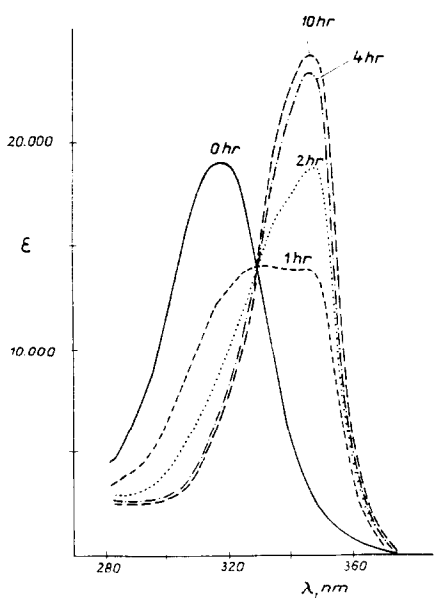


Figure 2
Time sequence of the enzymatic transformation of 6-mercaptopurine to 6-thiouric acid.

mg 6-MP/ml plasma $a = 0.005$ and $b = 1.26$; the values for human urine spiked with the above concentrations of 6-MP were 0.006 and 1.34 ($r > 0.999$ in all cases) indicating 90 and 95% apparent recoveries, respectively. The reason for the decrease in the slopes in the presence of plasma and urine is unknown.

The lowest measurable concentration of 6-MP in body fluids is about 500 ng ml^{-1} corresponding to a ΔA value of 0.06. As a consequence of the extremely low plasma concentrations of 6-MP [33] this sensitivity is by no means sufficient to monitor plasma levels after oral administration of the drug; it is, however, satisfactory for measuring 6-MP in urine. In accordance with the basic principles of difference spectrophotometry, 6-TUA, which is not only the product of enzymatic oxidation, but is also the main urinary metabolite of 6-MP [34] does not interfere with the assay and neither do other important metabolites such as 6-MP riboside and 6-MP riboside phosphate since these substances are not oxidized by xanthine oxidase.

Figure 3 shows the difference spectrum of the urine of a 9-year-old boy (M.T.) before (curve *a*) and 6 h after (curve *b*) the oral administration of 50 mg ml^{-1} of 6-MP. From curve *a* it is evident that no background absorption in the difference spectrum of urine not containing 6-MP has to be taken into account under the conditions of the enzymatic assay. The comparison of curve *b* with curve *c* of Fig. 1 indicates that the urine background of the metabolites does not cause any distortion of the difference spectrum; both maxima are at 348 nm and the wavelengths where the difference spectral curves intersect the wavelength axis are almost identical (327 and 328 nm, respectively). The situation is the same with plasma but, as previously mentioned, it is not possible to make use of this because the plasma levels of 6-MP are too low to enable the method to be used for this purpose.

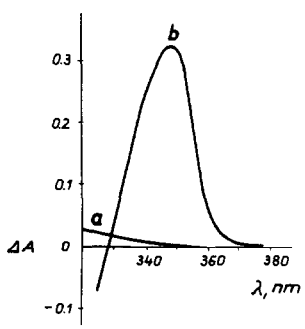


Figure 3
Difference spectra of urine samples. a. Sample collected before administration of 6-MP; b. sample collected 6 h after administration of 6-MP. For details see text.

The 6-MP concentration of the urine sample (M.T.) calculated from curve *b* of Fig. 3 was 2.04 mg ml^{-1}. The relative standard deviation (±2.7%; $N = 8$) is characteristic of the precision of the method.

A concentration of 1.95 mg/100 ml of 6-MP was found for the same urine sample using the HPLC method [10]. On the basis of the assay of several urine samples by both methods ($N = 15$) it can be stated that the agreement of the results is good ($r = 0.96$):

$$C_{Enz} = 1.03.\ C_{HPLC} + 0.05$$

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