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THE QUANTITATIVE DETERMINATION OF METABOLITES OF 6-MERCAPTOPURINE IN BIOLOGICAL MATERIALS

II. ADVANTAGES OF A VARIABLE-WAVELENGTH HPLC SPECTROPHOTOMETRIC DETECTOR FOR THE DETERMINATION OF 6-THIOPURINES

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SUMMARY

The advantages of a variable-wavelength spectrophotometric detector for use in high-performance liquid chromatography are demonstrated with the detection of 6-mercaptopurine metabolites in cell extracts. 6-Methylthiopurines, unsubstituted 6-thiopurines and 6-thioguanines are most sensitively detected at 291, 322 and 342 nm, respectively. Compared with detection at 254 nm, the sensitivity at these wavelengths is about one to two orders of magnitude greater. Furthermore, in the 291–355-nm range, common purines, which are normally contained in cell extracts as free bases, nucleosides and nucleotides exhibit only minute absorbances and so do not interfere in quantitative determinations of 6-thiopurine compounds.

INTRODUCTION

The separation of 6-mercaptopurine (6MP) metabolites on the base and (deoxy-)ribonucleoside level can easily be achieved by high-performance liquid cation-exchange chromatography[1]. However, the quantitative determination of these compounds, which are contained in neutralized, enzymatically digested perchloric acid (PCA)-extracts of biological materials, proved impossible when using UV detectors with a fixed wavelength of 254 or 280 nm, owing to the low molar absorbances ($\lambda_{max.}$) of the 6-thiopurines at 254 and 280 nm and to the minute 6-thiopurine concentrations in the digests. In addition, the common purine and pyrimidine derivatives which are contained in the cell extracts also exhibit high absorbances at these wavelengths.

In cultured cells grown with radioactively labelled 6MP, the 6MP metabolites can be detected and identified in the eluent by means of their label and elution volumes. Quantitative determinations can be effected according to the specific radioactivity of the 6MP batch[2].

If a radioactive label cannot be used, however, *e.g.*, in experiments with humans, qualitative and quantitative determinations of 6MP metabolites have to be carried out spectrophotometrically at wavelengths between 291 and 355 nm. In this region the 6-methylthiopurines, the unsubstituted 6-thiopurines, the 6-thioguanines and some oxidized 6-thiopurines, including 6-thiouric acid, exhibit their $\lambda_{max.}$ values[3–5]. The common purine and pyrimidine bases and ribonucleosides hardly show any absorbance above 290 nm[6,7].

In this paper, we report the determination of 6-thiopurine compounds in the 291–355-nm region with a variable-wavelength spectrophotometer designed for use in high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

The 6-thiopurine bases and (deoxy-)ribonucleosides were purchased from Papierwerke Waldhof-Aschaffenburg (Mannheim, G.F.R.), P.L-Biochemicals (Milwaukee, Wisc., U.S.A.), and Deutsche Wellcome (Grossburgwedel, G.F.R.). Common oxidized purines and their ribonucleosides were obtained from E. Merck (Darmstadt, G.F.R.). All reagents used were of the highest available purity.

About 30 mg of dry material of each purine or 6-thiopurine compound were dissolved in 1.0–2.5 l of the eluent. The amount of buffer thus depended on the

TABLE I

UV SPECTRAL DATA OF NORMALLY OCCURRING PURINES AND OF VARIOUS 6-THIOPURINES

Molar absorbances expressed as $\varepsilon a_M \cdot 10^{-3}$. For abbreviations of compounds, see Figs. 2 and 3.

Compound	$\lambda_{max.1}$ *(nm)*	$\varepsilon_{max.1}$* *($l \cdot mole^{-1} \cdot cm^{-1}$)*	$\lambda_{max.2}$ *(nm)*	$\varepsilon_{max.2}$ *($l \cdot mole^{-1} \cdot cm^{-1}$)*	*pH*	*Ref.*
Adenine	260.5	13.4	—	—	7.0	7
Guanine	276	8.15	246	10.7	7.0	7
Hypoxanthine	249.5	10.7	—	—	6.0	7
Xanthine	267	10.25	—	—	6.0	7
Uric acid	284	11.6	231	8.4	2.0	6
Inosine	248.5	12.3	—	—	6.0	7
Xanthosine	248	10.2	278	8.9	8.0	7
6MP	327	21.3	—	—	1.0	5
	311	19.7	—	—	12.0	5
	322	21.5	—	—	4.6	—**
6MPdR	322	26.1	—	—	4.6	—**
6MPR	322	27.3	—	—	4.6	—**
6MeMP	291	17.9	—	—	4.6	—**
6MeMPR	291	18.9	—	—	4.6	—**
2A6MP	342	25.6	255	7.2	4.6	—**
2A6MPR	342	26.7	258	9.7	4.6	—**
2A6MeMP	311	23.0	242	6.4	4.6	—**
6MNIMP	280	16.8	—	—	1.0	5
	280	18.2	—	—	4.6	—**
6TUA	355	28.65	263	9.4	1.0	3
	~347	~29	—	—	4.6	—**

* If two ε_{max} values are reported, $\varepsilon_{max.1}$ represents the higher value.

** Calculated by us.

solubility of each substance, which, for the 6-thiopurine compounds, is extremely low.

The UV spectra of the compounds were measured in a 1-cm cuvette both manually (5-nm steps, except for 0.5-nm steps in the maximum and minimum regions of each spectrum) and with a self-recording Zeiss Type PMQII spectrophotometer (for more detailed information, see ref. 1). A Varian LCS 1000 high-performance liquid chromatograph was used, equipped with a Zeiss Type PM2DLC variable-wavelength spectrophotometric HPLC detector with 8-μl cuvettes. The 1-m stainless-steel column (0.18 cm I.D.) was filled with strongly acidic cation-exchange resin, Type M71, particle diameter 10–12 μm, obtained from Beckman (Munich, G.F.R.), according to the slurry method of Scott and Lee[8]. The column was eluted with 0.4 *M* (with respect to the NH_4^+ concentration) ammonium formate solution, pH 4.6. At a constant pressure of 2,800 psi (200 bar), the flow-rate was adjusted at 8.0 ml·h^{-1} (flow velocity 5.2 cm·min^{-1}). The column oven temperature was 50°. The samples were injected on to the column at intermittant flow with a 10- or 50-μl Hamilton syringe with a 7-cm needle.

RESULTS AND DISCUSSION

According to the spectral data (Table I) that were obtained from our spectral analyses (see Fig. 1) and from reports on 6-thiopurines and common purines[3–7], four groups of compounds were distinguished: (1) the common purines and their oxidized

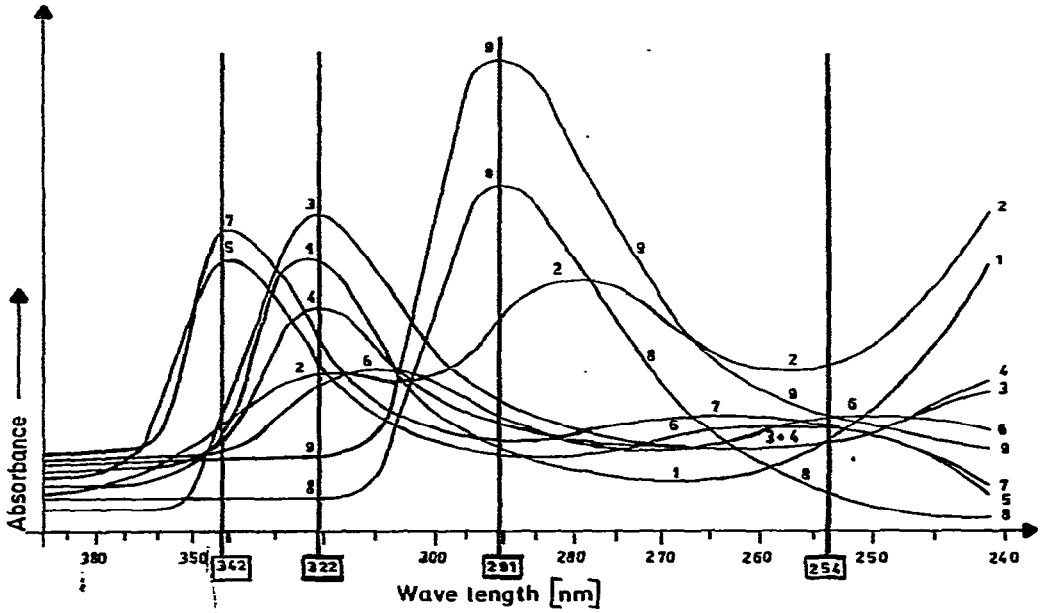

Fig. 1. UV spectra of 6-thiopurine compounds. 1 = 6-Mercaptopurine; 2 = 6-(1-methyl-4-nitro-imidazole)mercaptopurine (Azathioprine–Wellcome); 3 = 6-mercaptopurine ribonucleoside; 4 = 6-mercaptopurine 2′-deoxyribonucleoside; 5 = 6-thioguanine; 6 = 6-methylthioguanine; 7 = 6-thioguanine ribonucleoside; 8 = 6-methylmercaptopurine; 9 = 6-methylmercaptopurine ribonucleoside. The vertical lines indicate the $\lambda_{max.}$ values of 6-thiopurine compounds and of the normally used 254-nm detection.

end-products, which exhibit maximum absorbance ($\lambda_{max.}$) in the range 240–280 nm; (2) the 6-methylthiopurines with $\lambda_{max.}$ at 291 nm; (3) the unsubstituted 6-thiopurines with $\lambda_{max.}$ at 322 nm; and (4) the 6-thioguanines and some 6-thioxopurines with $\lambda_{max.}$ in the range 342–355 nm. Additionally, the 6-thioguanines exhibit a second $\lambda_{max.}$ between 240 and 260 nm. All values given here were measured in 0.4 *M* ammonium formate solution at pH 4.6.

Using these wavelengths for the quantitative determination of the various 6-thiopurines, each compound can be detected with high sensitivity. For this purpose, however, a variable-wavelength spectrophotometric HPLC detector must be available.

Fig. 2 gives an example of the increase in sensitivity obtained when using the spectrophotometric HPLC detector. Five common purines and three 6-thiopurines

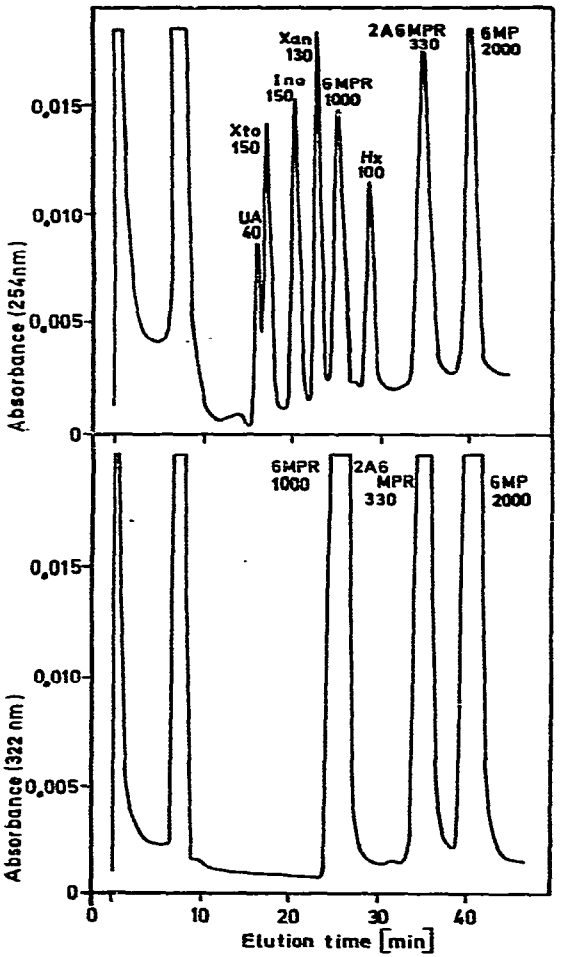

Fig. 2. Elution profiles of a separation of some common purines and of three 6-thiopurines as detected at 254 and 322 nm. UA = Uric acid; Xto = xanthosine; Ino = inosine; Xan = xanthine; 6MPR = 6-mercaptopurine ribonucleoside; 2A6MPR = 6-thioguanine ribonucleoside; 6MP = 6-mercaptopurine. The numbers on the peaks indicate the amount of each compound (in picomoles).

are separated from each other. Detection is performed at 254 and 322 nm. Amounts less than 300 pmole of each common purine are not detected at 322 nm. Relatively large amounts of the 6-thiopurines had to be injected on to the column in order to obtain peak heights comparable to those of the common purines at 254 nm. These amounts, however, cause huge peak heights and full-scale deflections of the recorder pen at 322 nm.

At 322 and 342 nm, about 30 pmole of each unsubstituted 6-thiopurine and of each 6-thioguanine are detected quantitatively. The sensitivity of detection is greater by a factor of 70 for the 6-thiopurines and 10 for the 6-thioguanines compared with detection at 254 nm.

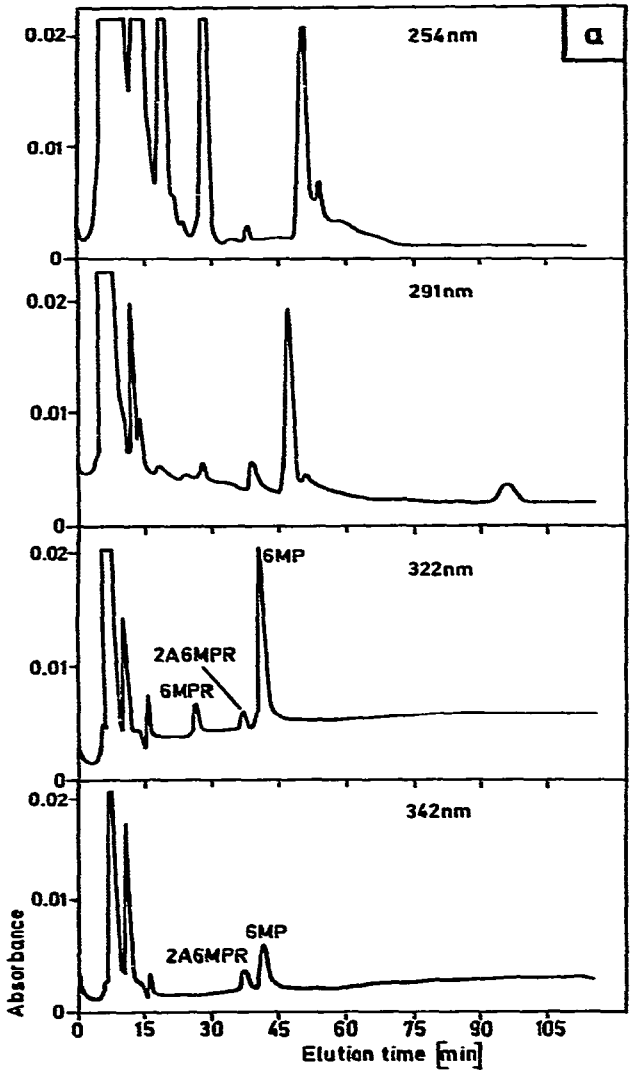

Fig. 3 (a).

(Continued on p. 66)

Fig. 3 shows a series of chromatograms of a PCA-extract of cells that were incubated with 6MP: (a) extract with no enzymatic digestion, (b) after phosphatase digestion and (c) after phosphatase plus purine nucleoside phosphorylase digestion. The common purine and pyrimidine free bases and ribonucleosides and some related compounds that exhibit $\lambda_{max.}$ in the 240–280-nm range are eluted at the beginning of the chromatograms. Detection at 254 nm hardly gives any resolution between the different compounds and 6-mercaptopurine metabolites are not detected. On switching, however, to 291, 322 and 342 nm, the chromatograms become increasingly simple. Common purines are no longer detected, but 6-methylthiopurines, unsubstituted 6-thiopurines and 6-thioguanines are recorded as sharp peaks, which can serve

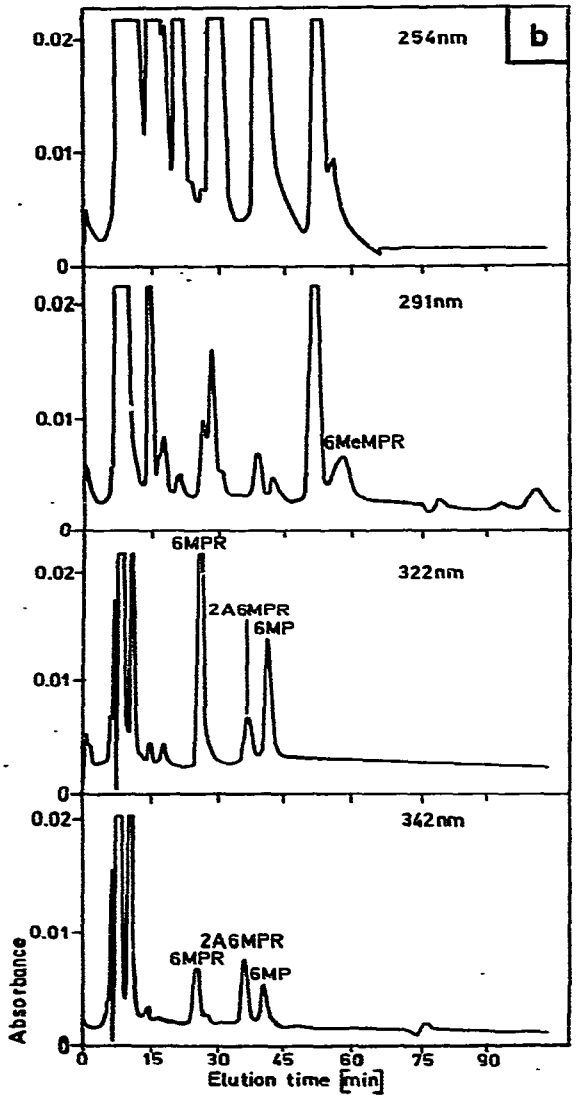

Fig. 3 (b).

for quantitative evaluation. More detailed information will be given in Part III[2].

The results clearly demonstrate that a variable-wavelength spectrophotometric detector is essential for the qualitative and quantitative determination of minor compounds that differ in their spectral characteristics from the major constituents of a given sample.

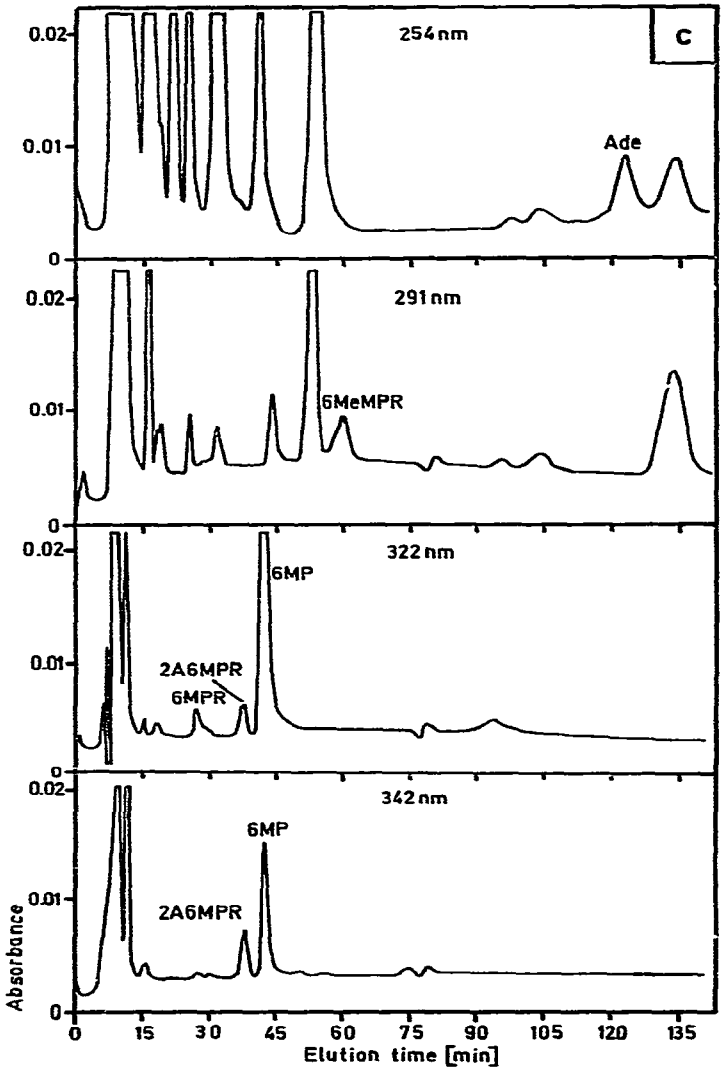

Fig. 3. Elution profiles of the separation of a PCA-extract of L5178Y mouse lymphoma cells grown in 6MP-containing medium as detected at the wavelengths indicated. (a) PCA-extract without enzymatic digestion; (b) PCA-extract digested with phosphatase, which liberates the ribonucleosides; (c) PCA-extract digested with phosphatase + purine nucleoside phosphorylase, which liberates the free bases except 6MeMP. 6MPR = 6-Mercaptopurine ribonucleoside; 2A6MPR = 6-thioguanine ribonucleoside; 6MP = 6-mercaptopurine; 6MeMPR = 6-methylthioguanine ribonucleoside; Ade = adenine. For details, see ref. 2.

HPLC, with its high-efficiency columns, proved superior to other chromatographic methods for the resolution of multi-component samples and, by use of the spectrophotometric detector, this advantage can even be enhanced.

A further application of the variable-wavelength spectrophotometric HPLC detector has recently been reported[9]. With intermittant stop–flow of the eluent, the absorbance of a substance that just passes the flow cell of the detector can be checked at several wavelengths. The identity and purity of the substance peak can then be established from its characteristic absorbance ratios which, throughout the peak, should be constant and of given values for a specified compound.

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