The Quantitative Determination of Metabolites of 6-Mercaptopurine in Biological Materials

# IV. An Improved Separation Method for Twentytwo Compounds Related to Purine and 6-Thiopurine Metabolism Using High-Pressure Liquid Cation-Exchange Chromatography

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6-Mercaptopurine Metabolites, Separation of 6-Thiopurine Bases and Ribonucleosides, Separation of Common Purine Bases and Ribonucleosides, High-Pressure Liquid Cation-Exchange Chromatography

An improved method is described for the separation of 22 compounds normally related to purine and 6-thiopurine metabolism in biological materials using high-pressure liquid cation-exchange chromatography on strongly acidic exchange resin. The column (0.18×100 cm) is eluted with 0.4 M ammonium formate, pH 4.6, at a linear flow velocity of 5.2 cm·min$^{-1}$ at 50 °C. The elution volumes of sulphate anions, allopurinol, 6-thioxanthine, adenine, adenosine, and guanosine are demonstrated additionally to further 16 purine and 6-thiopurine compounds.

## Introduction

Many attempts have been made to determine 6-mercaptopurine (6MP) and its metabolites in biological materials, particularly in blood samples of patients as a measure of therapeutic efficacy or as a guide to more effective therapy. For this purpose the separation from plasma constituents of 6MP and of some of its metabolites was carried out either by paper [1], thin-layer [2], or liquid [3] and gas [4] column chromatography. The detection of 6MP and its metabolites was done by fluorimetry [5], colorimetry [6], UV-detection [3], radioactivity measurements [1], and mass spectrometry [7].

Recently, we reported a separation method by high-pressure liquid cation-exchange chromatography for 6-thiopurine bases and (deoxy-) ribonucleosides and for some common oxidized purines [8] and presented the advantages of a variable-wavelength HPLC detector for the identification and quantitative determination of these compounds on the picomole level [9]. These methods have frequently been applicated in our laboratory to routine determinations of 6-thiopurines in extracts from L5178Y murine lymphoma cells grown with radioactively labeled [8-$^{14}$C] or [6-$^{35}$S]6MP [10, 11].

Additionally to the 17 purine and 6-thiopurine compounds [8] we have now confirmed the location of the sulphate fraction within the void volume and the elution volumes of adenine, 2-hydroxy-6-mercaptopurine (6-thioxanthine), allopurinol, adenosine, and guanosine have been determined among the compounds which are retained on the exchange resin. The improved separation method for 20 compounds related to 6MP metabolism is reported in this paper.

## Materials and Methods

6-Mercaptopurine (6MP), 6-mercaptopurine ribonucleoside (6MPR), 6-methylmercaptopurine (6MeMP), 6-methylmercaptopurine ribonucleoside (6MeMPR), 6-thioguanine (2A6MP), 6-thioguanine ribonucleoside (2A6MPR), and 6-methylthioguanine (2A6MeMP) were purchased from Papierwerke Waldhof-Aschaffenburg, Mannheim (West Germany), 6-mercaptopurine deoxyribonucleoside (6MPdR) from P·L-Biochemicals, Milwaukee, Wi (USA), 6-(1-methyl-4-nitro-imidazolyl-) mercaptopurine (6MNIMP) from Deutsche Wellcome, Burgwedel (West Germany), 2-hydroxy-6-mercaptopurine (2OH6MP) from EGA-Chemie, Weinheim (West Germany), 6-thiouric acid (6TUA) and allopurinol (Alp) from Calbiochem, San Diego, Ca (USA). Uric acid (UA), xanthine (Xan), hypoxanthine (Hx), xanthine ribonucleoside (Xao), inosine (Ino), adenine (Ade), guanine (Gua), adenine ribonucleoside (rAdo), guanine ribonucleoside (rGuo), barium chloride, sulphuric acid, and further reagents were from E. Merck, Darmstadt (West Germany). All reagents used were of the highest available purity.

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Strongly acidic cation-exchange resin, type M71, particle diameter 10 – 12 $\mu$m, was purchased from Beckman, München (West Germany).

Separations were performed in a Varian LCS 1000 liquid chromatograph equipped with a 254 nm UV-flow cell-detector and a Varian Model 20 strip chart recorder. A 0.18 × 100 cm stainless steel tube was filled with strongly acidic cation-exchange resin according to the method of Scott and Lee [12]. The column was eluted with 0.4 M (with respect to the $NH_4^+$-concentration) ammonium formate, pH 4.6, at a flow rate of 8.0 ml·h$^{-1}$ (linear flow velocity 5.2 cm·min$^{-1}$) at 50 °C column oven temperature. The sensitivity of the recorder was set at 0.02 absorbance units full scale (AUFS).

Reference samples of purine and 6-thiopurine compounds which have been chromatographed for the determination of elution volumes have been prepared as described earlier [8]. Sulphuric acid and barium chloride were used as 1 mM solutions.

## Results and Discussion

Fig. 1 shows the elution pattern of the separation of twenty compounds [purine and 6-thiopurine bases and (deoxy-)ribonucleosides and allopurinol] related to the purine and 6-thiopurine metabolism in organic materials. Additionally the elution volumes of adenine- and guanine ribonucleoside are indicated.

Two of these compounds, sulphate anions and 6TUA which are not retained on the exchange resin, are excluded from the porous resin beads possibly due to a strongly negative charge of their molecules. They elute within the void volume in front of the $^3H_2O$-fraction which indicates the elution volume of non-retained, but freely diffusible substances. Furthermore, sulphate anions and 6TUA within the void volume are clearly separated from each other. This has been proven using radioactively labeled [$^{35}$S]6TUA [8] and sulphate precipitation with dilute barium chloride solution. The accurate determination of the elution volume of sulphate anions seems noteworthy since this compound may generate at a certain extent from 6MP within living cells [1].

Allopurinol [4-hydroxypyrazole-(3,4-d)pyrimidine], an analogue of hypoxanthine in which the positions of N7 and C8 are reversed, in our cation-exchange system is not separated from hypoxanthine (Hx), but this has recently been reported by Brown and Bye [13] using HPLC on Aminex A-27 (Bio-Rad Labs.) anion-exchange resin with 1 M ammonium

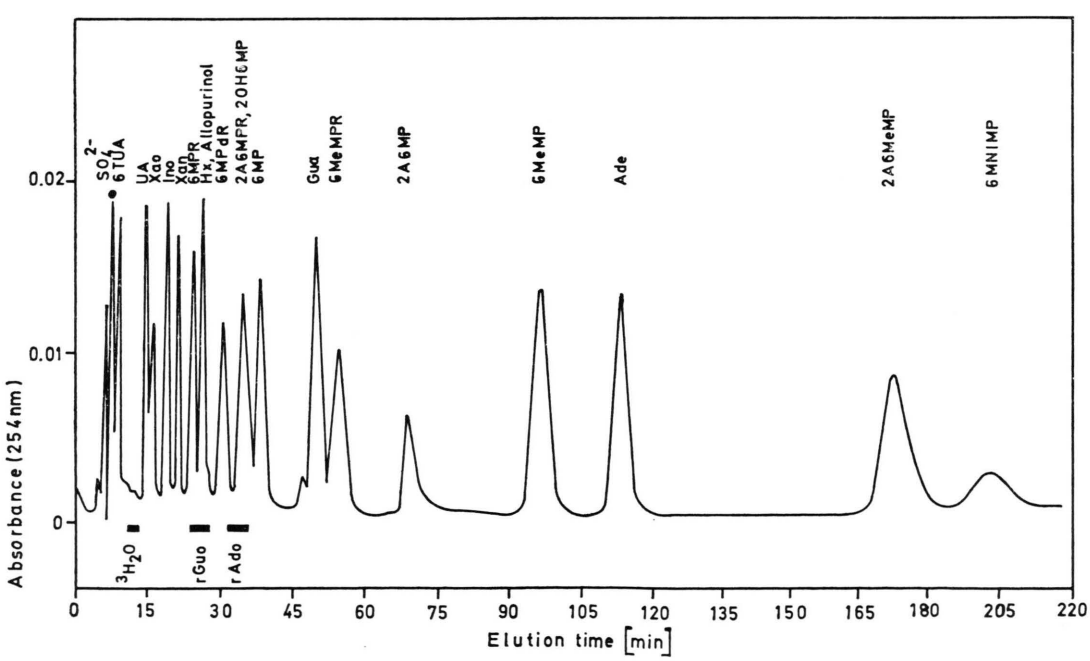


Fig. 1. Elution pattern of the separation of 20 compounds related to purine and 6-thiopurine metabolism. (The bars indicate the elution volumes of tritiated water, adenine and guanine ribonucleoside; ●, designates the pressure peak which represents initial changes in column pressure and flow; for abbreviations and separation conditions see Materials and Methods.)

acetate, pH 8.7, at 71 °C. Since Hx and allopurinol hardly possess any charge within their molecules at pH 4.6 they are retained by adsorption phenomena exclusively. This, however, gives no difference in elution volumes of Hx and allopurinol.

The purine ring of 6MP after desulphurization may be used for the synthesis of the common purines, adenine and guanine, and their (deoxy-) ribonucleotides. Since the determinations of purine and 6-thiopurine nucleotides in cell extracts are carried out on the (deoxy-)ribonucleoside (and base) level after the incubation of the extracts with alkaline phosphatase (and, in addition, purine nucleoside phosphorylase), the elution volumes of adenine- and guanine ribonucleoside and of adenine and guanine must be known. The desulphurized purine residue of [8-$^{14}$C]6MP which is used as the radioactively labeled precursor may appear as a common purine derivative.

The bases adenine and guanine do not interfere with 6-thiopurine compounds in the elution pattern, their ribonucleosides, however, are not separated from 6MPR and 2A6MPR. Therefore, accurate quantitative determinations of rAdo and rGuo should be carried out with 254 nm UV-detectors, whereas 6MPR and 2A6MPR are easily determined using a variable-wavelength UV-detector at 322 and 342 nm, respectively [9].

In contrast to previous suggestions [8] it seems realistic that 8-hydroxy-6-mercaptopurine, the oxidation product of 6MP by xanthine oxidase, is not eluted within the void volume but is retained on the exchange column, too, comparable to 2-hydroxy-6-mercaptopurine. Unfortunately, we still could not obtain a sample of pure reference material of 8-hydroxy-6-mercaptopurine. As to our knowledge, this compound has not yet been reported as an intracellular metabolite of 6MP.

6-Methylthioguanine and azathioprine which are most strongly retained on the exchange resin were not contained in the radioactively labeled cell extracts. Therefore, the doubling of the linear flow velocity after 75 min [8] was omitted and the column needed no regeneration interval after each chromatographic run.

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