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Short communication

Analysis of thiopurines using aqueous normal phase chromatography

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ABSTRACT

The chromatography of several thiopurines is investigated using aqueous normal phase (ANP) conditions in conjunction with a silica hydride-based column. Both isocratic and gradient elution modes are tested. Detection of higher concentration samples is done by UV to demonstrate feasibility in this format while lower concentration samples utilize mass spectrometry (MS). Repeatability of successive runs is also tested with particular attention to gradient methods where the equilibration time of the stationary phase can be evaluated.

1. Introduction

Thiopurines are an important class of biologically relevant compounds. In terms of active agents used for the treatment of disease, they have proved useful as immunosuppressants and cytotoxic drugs for inflammatory bowel disease [1–5] and acute lymphoblastic leukemia [6]. 6-Mercaptopurine (6-MP) and azathioprine (Aza) are two thiopurine drugs currently used in clinical practice for treatment of these disorders. The biochemical properties Aza (the 1-methyl-4 nitro-5 imidazole sulphur derivative of 6-MP) are actually attributable to its cleavage to form 6-MP and the resulting 6-MP metabolism [7]. Extensive transformation of these compounds occurs in the intestine and liver through several well-established pathways. Both 6-MP and Aza remain for only a short time in plasma following oral administration. Therefore measurement of their plasma levels is seldom used for clinical purposes. More relevant are the metabolites such as 6-thioguanine nucleotides (6-TGNs), 6-methylmercaptopurine (6-MMP) nucleotides and 6-methylmercaptopurine nucleotides (6-MMPNs) that are either the active cytotoxic or are inactive species used to measure hepatotoxicity [8].

Because of their polar nature, thiopurines and their metabolites are difficult to retain and analyze by traditional reversed-phase methods. Therefore, to use a typical C18 column, most of the compounds must be derivatized in order to diminish the hydrophilic properties.

Aqueous normal phase chromatography (ANP) is a new methodology that has been recently developed for the retention and separation of hydrophilic compounds [9–11]. The most versatile approach for successfully utilizing ANP involves the use of silica hydride-based stationary phases. These separation materials have been shown to be applicable to a broad range of hydrophilic species including amino acids, carbohydrates, small organic acids [12], amines, nucleotides [13,14], drugs [15] and peptides [16,17]. The term ANP is defined as the ability of a stationary phase to retain both hydrophobic and hydrophilic compounds with polar retention occurring when the mobile phase composition has a high percentage of organic solvent and reversed-phase properties being manifested with high aqueous content mobile phases. Similar behavior is found in mixed-mode stationary phases but the term ANP is used to emphasize the unique properties of the silica hydride material. Thus normal phase behavior of silica hydride materials is similar to hydrophilic interaction liquid chromatography but these stationary phases also possess reversed-phase properties that are either not found or only minimally present HILIC. In the case of polar compound retention and selectivity, the silica hydride phases have the advantage of rapid equilibration after gradient runs or changes in mobile phase composition, reproducibility on an intraday, interday and intercolumn basis, ruggedness and ease of use with mobile phases that are compatible with detection by mass spectrometry. In addition, it is relatively easy to keep the stationary phase free from contaminants by using a 50:50 mixture of water/methanol or water/isopropanol as the strong solvent for ANP. Thus biological samples which often result in rapid column deterioration are more amenable to analysis by ANP on silica hydride-based stationary phases.

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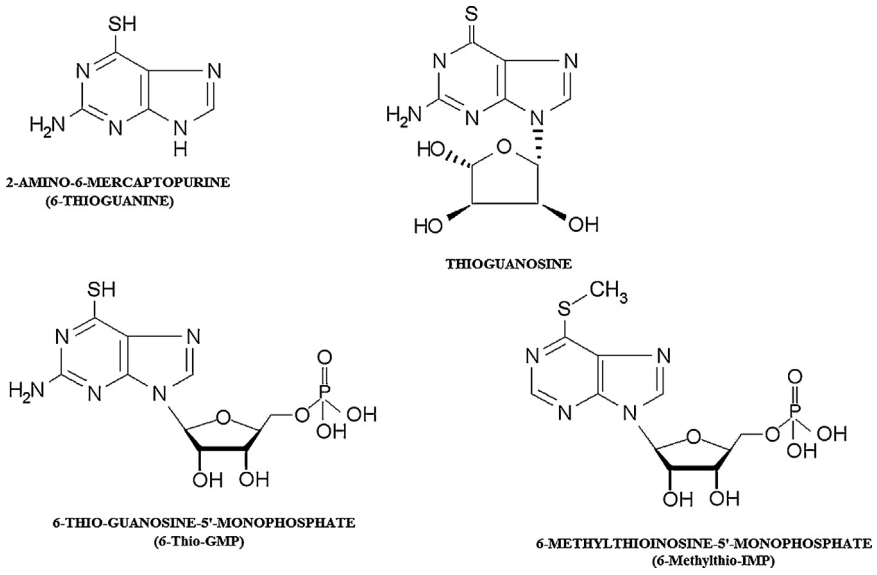

Fig. 1. Structures of several thiopurines investigated in this study.

This investigation reports on the retention of several thiopurines under ANP conditions using both UV and mass spectrometric (MS) detection. The basic format presented in this report could be further developed into specific analytical methods for various thiopurines in a variety of biological matrices.

2. Experimental

2.1. Materials

The silica hydride stationary phase used in this study was the Cogent Diamond Hydride (DH) material (dP 4.2 μm) in 75 mm × 4.6 mm for UV detection and 150 mm × 2.1 mm for MS detection columns (MicroSolv Technology, Eatontown, NJ, USA). The phase contains a small amount of an organic moiety (~2% carbon as reported by the manufacturer) on a silica hydride surface. The analytes evaluated in this study were purchased from either Jena Bioscience (Jena, Germany) or Sigma–Aldrich (St. Louis, MO, USA). Mobile phase solvents used were HPLC grade for UV detection, MS grade for mass spectrometric detection, and additives were obtained in the highest purity available.

2.2. Instrumentation

For UV-based analyses, a Hewlett–Packard (Palo Alto, CA, USA) 1050 HPLC system comprised of an autosampler, degasser, gradient pump, and variable wavelength UV detector set at 284 nm was used. The system was interfaced with Agilent Chemstation (Santa Clara, CA, USA) software. For MS-based analysis, the HPLC was an Agilent (Little Falls, DE, USA) 1200SL Series LC system, including degasser, binary pump, temperature-controlled autosampler and temperature-controlled column compartment. The mass spectrometer system was an Agilent Model 6220 MSD TOF with a dual sprayer electrospray source (ESI).

2.3. Methods

Stock solutions of the analytes were made in deionized (DI) water in the range of 0.2–0.7 mg/mL and used for experiments with UV detection. MS detection sample solutions were made by diluting the stock 1:100 in 50:50 acetonitrile/water or 2:1 methanol/water containing the mobile phase additive (acetic acid or formic acid) used in the analysis. The flow rate was 0.4 mL/min. The column temperature was 20 °C. The exact mass capabilities of the TOF system were used after calibration for identifying the correct analyte peak.

3. Results and discussion

Based on previous analyses of various polar compounds, the Diamond Hydride phase should be applicable to the analysis of thiopurines. Their structural features, some examples are shown in Fig. 1, such as primary amine groups, hydroxyls and phosphate moieties in some cases are typical of other compounds that have displayed good retention and peak shape in the ANP mode on silica hydride-based columns. For example, amine groups such as those found in amino acids and basic drugs and phosphates such as nucleotides have been analyzed with the Diamond Hydride stationary phase utilized in this study.

As a starting point, the chromatographic behavior of two thiopurines on the Diamond Hydride (DH) column was evaluated in the aqueous normal phase (ANP) mode using UV detection. Based on previous results with other strongly hydrophilic compounds, thiopurines should be well-retained under similar experimental conditions. Fig. 2 shows the chromatogram of 6-thioguanine (2-amino-6-mercaptopurine) and thioguanosine (TG) obtained on the DH column under ANP isocratic elution conditions. In this case adequate retention ($k \sim 1.0$ and 2.5 respectively) and good peak symmetry are found when an acetonitrile/water (95:5) mobile phase containing 0.1% formic acid is used. Both compounds have a primary amine group but stronger retention is observed for thioguanosine in comparison to 6-thioguanine due to the three hydroxyl groups on TG. The figure is an overlay of three consecutive injections of the mixture demonstrating the repeatability of the analysis in the ANP mode on the DH column. Other thiopurines tested also resulted in good retention on the DH stationary phase when using isocratic mobile phases containing at least 90% acetonitrile and 0.1% formic acid.

Gradient elution can be used to enhance selectivity and/or improve efficiency for various thiopurines that are difficult to separate or have undesirably long retention under isocratic conditions. Fig. 3 shows a gradient separation of two phosphate containing