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Detailed insights into the retention mechanism of caffeine metabolites on the amide stationary phase in hydrophilic interaction chromatography

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ABSTRACT

The amide phase was investigated using a wide range of acetonitrile content in the mobile phase in both the HILIC and RPLC modes. Using caffeine metabolites as the model compounds, the retention, thermodynamic and kinetic data was obtained under various mobile phase conditions and supported the previous postulation that there might be a transition of the predominant retention mechanism in relation to the acetonitrile content in HILIC. On the amide phase, hydrophilic partitioning seemed to be the predominant retention mechanism below 85% acetonitrile; and a different retention mechanism (presumably surface adsorption) made more and more significant contributions to the overall retention when the acetonitrile content reached above 85%. This study also provided more direct evidences to explain the effect of salt concentration on the retention of non-charged solutes in HILIC. In addition, the retention, thermodynamic and kinetic data suggest that the amide phase behaved very differently from the conventional C18 phase in the RPLC mode.

1. Introduction

Hydrophilic interaction chromatography (HILIC) has gradually matured into a valid and valuable chromatographic technique for the analysis of polar compounds over the past two decades [1–3]. Parallel to tremendous success in application [4–6], significant progress has also been made in understanding fundamental aspects of HILIC in terms of separation mechanism and kinetic performance [7–9]. The hydrophilic partitioning model as originally proposed by Alpert postulates that the retention in HILIC is based on partitioning of the polar solutes between the mobile phase and a water-rich layer immobilized on the polar surface of the stationary phase [10]. It is generally accepted that the actual retention mechanism of HILIC is more complicated than the simple hydrophilic partitioning model [8]. Other types of molecular interactions, such as adsorption and electrostatic interactions also play significant roles in HILIC separation. It has been demonstrated that the electrostatic interactions between charged solutes and stationary phases are a major factor in determining the retention and selectivity of charged compounds [11,12], but other retention mechanisms (e.g., surface adsorption) may also be involved in the retention of non-charged compounds [13,14]. There are evidences that the predominant retention mechanism for HILIC separation may depend on the content of organic solvents in the mobile phase. Karatapanis et al. investigated the transition of retention mechanism as the organic content in the mobile phase varied from 50 to 90% (v/v) using a group of selected water soluble vitamins (WSVs) [15]. Based on the retention data, Karatapanis et al. argued that the predominant retention mechanism might have shifted from partitioning to surface adsorption after the acetonitrile content reached 75–85% for neutral WSVs on the silica, amino and diol columns; and this transition also occurred to the ionizable WSVs that had the same charges as the stationary phase when the acetonitrile content was above 85%. However, only retention data was reported and no thermodynamic and kinetic studies were performed.

Amide phase is widely used in HILIC in addition to amino, diol and silica phases. TSKgel Amide-80 column contains an amide functional group attached to the silica surface through a linker group [4]. A number of studies have investigated the retention mechanism of the amide phase in HILIC [7,16–18]. Such mechanistic studies typically take the approach of examining the effect of organic solvent content in a wide range (e.g., 60–95% acetonitrile), but at a fixed column temperature and flow rate. The thermodynamic and kinetic studies were conducted by varying column temperature and flow rate, but only using one fixed mobile phase composition (i.e., specific acetonitrile percentage and salt concentration). Considering possible retention mechanism shift with the content of organic solvents, this conventional approach of conducting thermodynamic or

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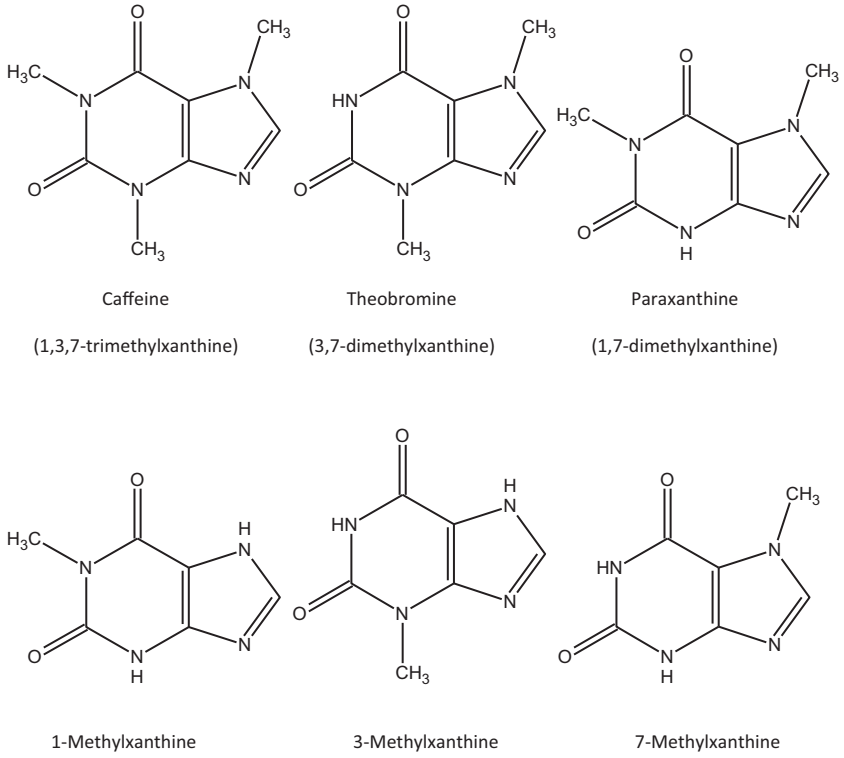

Fig. 1. Structures of caffeine and its metabolites used in the study.

kinetic studies in one mobile phase condition may not provide a full picture of the retention mechanism.

The stationary phases employed for HILIC separation are polar and typically operated in a mobile phase with high organic content (>60% by volume). Some polar phases (e.g., diol and polyethylene glycol phases) have been operated at low levels of organic solvent [19–21]. The separation is presumably based on reversed-phase (RP) mechanism. However, there have been no reports on thermodynamic and kinetic performance of the polar phases under the reversed-phase condition. Detailed studies on the polar stationary phase operated in a wider range of organic solvents (e.g., 0–95% acetonitrile) would provide much more insights into the behavior of the polar phases in different chromatographic modes.

This study focused on the amide phase and investigated its retention, thermodynamic and kinetic behaviors under various mobile phase conditions in order to gain a deep understanding of its retention mechanism. Caffeine metabolites including monomethyl and dimethylxanthines were selected as the model compounds in this study. These caffeine metabolites are formed through demethylation of caffeine and differ in the number of methyl groups resulting in varied polarity and also in the position of the methyl groups as shown in Fig. 1. They displayed moderate retention on the amide phase in both HILIC and RPLC modes. The behavior of geometric isomers was very helpful to reveal selectivity differences of the amide phase when operated in different modes. In addition, the effect of salt concentration was also studied at multiple levels of acetonitrile.

2. Materials and methods

2.1. Materials and reagents

TSKgel Amide-80 columns (3 μm particle size, 4.6 mm ID, 10 and 15 cm in length) were purchased from Tosoh BioScience (King of Prussia, PA, USA). Atlantis T3 column (3 μm particle size, 3.0 × 100 mm) was obtained from Waters (Milford, MA, USA). Water was obtained from an in-house Milli-Q water purification system (Millipore, Bedford, USA). HPLC grade acetonitrile (ACN) and toluene was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium acetate (ultra pure grade) was provided by Amresco (Solon, OH, USA). Stock ammonium acetate solutions (100 or 200 mM) was prepared by dissolving appropriate amounts of ammonium acetate in purified water. No pH adjustment was made.

Monomethylxanthines (1-, 3-, and 7-methylxanthine), dimethylxanthines (1,7-, and 3,7-methylxanthine) and caffeine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the model compounds (~0.5 mg/mL) were prepared by dissolving appropriate amounts of the model compounds in a mixture of water and acetonitrile (50/50, v/v). Test solutions were prepared by mixing various stock solutions and adding appropriate volumes of acetonitrile to reach the final concentration of approximately 0.1 mg/mL.

2.2. Equipment and methods

An Agilent 1260 HPLC system (Palo alto, CA, USA) equipped with an online vacuum degasser, a quaternary gradient pump, an autosampler, a thermostatted column compartment, a variable UV detector was used for the experiments. Chromatographic data (e.g., retention time and the number of theoretical plates) was processed by ChemStation for LC and LC/MS (Rev.C. 01. 06., Agilent Technologies). Detection was performed by UV set at 245 nm.

The mobile phase was prepared online by the quaternary gradient pump using acetonitrile, water and ammonium acetate stock solution at various proportions achieve desired acetonitrile content and ammonium acetate concentration. Toluene and a mixture of acetonitrile and water (50/50, v/v) were used as the marker for void time in HILIC and RP mode, respectively.