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The use of methyl-β-cyclodextrin to solubilize cholesterol prior to coating onto a C18 stationary phase

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ABSTRACT

The use of methyl-β-cyclodextrin (MBCD) as a mobile phase additive in reversed-phase liquid chromatography is explored, with the primary goal of using MBCD to solubilize cholesterol in reversed-phase mobile phases for cholesterol-coating of C18 stationary phases. MBCD is shown to increase the solubility of cholesterol in typical reversed-phase mobile phases, especially when the stoichiometric ratio of MBCD to cholesterol exceeds 2:1. Additional equivalents of MBCD further increase solubility, or allow for weaker solvents to be used. The use of weaker solvents allows for larger coating levels of cholesterol onto a C18 stationary phase than are possible without the use of MBCD. Stationary phases coated with cholesterol using MBCD as a co-additive have different selectivity than uncoated phases, especially with regards to phenyl and shape selectivity. Further, the use of MBCD as a mobile phase additive for the elution of cholesterol is examined. It is seen via van't Hoff analysis that the reduction in retention of cholesterol when MBCD is added to the mobile phase is enthalpically driven.

1. Introduction

Alkyl stationary phases, such as C8 and C18, are commonly used in reversed-phase liquid chromatography. In order to introduce specific selectivity to a stationary phase, non-polar phases containing various functional groups have been developed. These include phenyl phases for enhanced *pi* interactions with solutes [1,2], embedded polar group (EPG) phases [3–8], and fluorinated phases [9,10], among others. While these phases are generally non-polar in nature and exhibit reversed-phase characteristics, the presence of a specific functional group alters their selectivity when compared to traditional alkyl phases such as C8 or C18.

Several reversed-phase stationary phases have been developed based on cholesterol [11–20]. In one methodology, cholesterol can be added to a stationary phase as a bonded ligand [11–17]. Bonded cholesterol phases have been investigated by several groups, using a variety of different synthetic methods. In general, these phases exhibit reversed-phase behavior but with different selectivity than alkyl phases. Most notably, bonded cholesterol phases have been shown to have enhanced selectivity for shape isomers [11,14]. This selectivity is thought to arise from alignment of cholesterol groups in a liquid-crystal fashion [14–16]. Bonded cholesterol phases are commercially available, and are a viable alternative when their specific selectivity is required.

A second methodology for preparing cholesterol-based stationary phases is to dynamically coat cholesterol onto an alkyl phase such as C18 [18–20]. This approach was first introduced by Cole [18], and further developed by Ogden and Coym [19,20]. Cholesterol is a significant component of the lipid bilayer membrane in cells; a similar lipophilic environment is present in an alkyl stationary phase. In this process, a solution containing cholesterol in a reversed-phase mobile phase, typically methanolic phases with 85–100% methanol, is pumped through a C18 column. Cholesterol intercalates to saturation into the stationary phase, after which it begins to elute and is detected. The "breakthrough curve" can then be used along with knowledge of the flow rate and cholesterol concentration in the mobile phase to calculate the mass of cholesterol coated onto the column. These dynamic coatings have been shown to be stable when relatively weak mobile phases (less than 70% methanol) are used. Coated phases show different selectivity than the base alkyl phase [19], and are somewhat similar in selectivity to bonded cholesterol phases. Using a neat methanol wash solvent, the cholesterol coating can be removed and the selectivity of the original C18 phase restored [19].

Several variables influence the amount of cholesterol that added to the stationary phase during the coating process. These include temperature, cholesterol concentration in the mobile phase, and composition of the mobile phase (fraction of methanol) [19]. As would be expected, larger amounts of cholesterol can be coated at low temperature, high cholesterol concentration, and low methanol content. Each of these has its drawbacks. At low temperature, the viscosity of the mobile phase increases, so relatively higher pressures are necessary for coating. However, this is generally not

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a problem with typical analytical columns and pumps, unless very long columns or very small stationary phase particles are used. The other two variables, which are related, present a challenge for loading higher amounts of cholesterol onto a column. Cholesterol is not particularly soluble in reversed-phase mobile phases, and its solubility decreases as the mobile phase is weakened. For example, mobile phases with 85–90% methanol can be used to coat large amounts of cholesterol, but the coating process is very time consuming because only a small amount of cholesterol will dissolve in the mobile phase [19,21]. As a result, our "typical" coating process uses a mobile phase of 95% methanol, but the amount of cholesterol loaded is not as large as it could be. An ideal scenario would be to use a weaker mobile phase (85–90% methanol) to drive the cholesterol onto the stationary phase, but have larger amounts of cholesterol dissolved in the mobile phase to speed up the process. Thus, a method to solubilize methanol in mobile phases containing less than 90% methanol is required.

The low solubility of cholesterol in water/methanol solutions can be increased by using a solubilizing agent as a co-additive in the mobile phase. Methyl-β-cyclodextrin (MBCD) is determined to be an appropriate co-additive based on its use in biological work in transporting active hydrophobic compounds, including cholesterol, in primarily polar environments [22–26]. Cholesterol forms an inclusion complex with various β-cyclodextrins, including MBCD, in polar solvents, allowing the cholesterol to be solubilized. Cholesterol can be removed from cell membranes by incubating them with a β-cyclodextrin, alternatively, cholesterol can be added to membranes in which it is depleted by adding a cyclodextrin-solubilized cholesterol to the culture media. The idea that the use of MBCD as a co-additive to cholesterol in HPLC will work is based on the alkyl ligand chains of the stationary phase being similar to the lipids in the cell membrane.

Cyclodextrins are characterized by their polar exterior and nonpolar interior cavity. The hydrophobic compounds essentially "jump" into this inner cavity, and the cyclodextrin acts as a ferry for the compound through the polar environment. The cavity of β-cyclodextrins, as compared with the other cyclodextrins, is the approximate size of cholesterol. However, native β-cyclodextrin is not very soluble in water-methanol solutions, so its derivative, methyl-β-cyclodextrin, is used.

Previous work with cyclodextrins as an additive to the mobile phase in HPLC has been done, as evident in Taghvaei and Stewart's work [27]. Cyclodextrins have the ability to complex and offer different selectivity. However, their research only involves β-cyclodextrin and not its methyl derivative. Spencer and Purdy used native and derivatized β-cyclodextrins as mobile phase additives for analysis of fat-soluble vitamins [28]. They found the methyl derivative provided the best selectivity. In addition, Leon et al. [29] used various β-cyclodextrins in the elution of various alkaloids. The use of cyclodextrin in the mobile phase allowed them to maintain chromatographic resolution while reducing analysis time and organic solvent consumption. Other, far more common uses of cyclodextrins in chromatography relate to their use as stationary phase chiral selectors [30–34]. A large variety of chiral stationary phases based on various cyclodextrins has been developed, characterized, and commercialized. This contrasts with our work, in which the primary use of MBCD is in the preparation of the coated stationary phase.

2. Experimental

2.1. Chemicals and reagents

Water was purified to a resistivity of 17 MΩ in-house using a Continental Water Systems Modulab purification system. Methanol and acetonitrile (Optima LC–MS grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). The two mobile phase additives of cholesterol (99%) and methyl-β-cyclodextrin (M_n average 1310) were obtained from Sigma Aldrich (St. Louis, MO, USA). Chromatographic test solutes including *o*-, *m*-, and *p*-terphenyl, naphthalene, anthracene, biphenyl, triphenylene, and 1,8- and 2,6-dimethylnaphthalene were also obtained from Sigma Aldrich. Benzene and biphenyl were obtained from Fisher Scientific. Thiourea (Sigma–Aldrich) was used as a void volume marker for calculation of chromatographic retention factors. Test solutions were made up in neat methanol.

2.2. Equipment and columns

Two chromatographic systems were used: one for preparing the cholesterol-coated stationary phases and another for chromatography. The system for coating the phases consisted of a Shimadzu (Kyoto, Japan) LC-20AD pump, a Torrey Pines Scientific (Carlsbad, CA, USA) model CO50 column oven, and a SSI Lab Alliance Model 500 UV–Vis detector (Sci-Con, Winter Park, FL, USA). Data collection for this system was via an SRI PeakSimple model 302 A/D converter, using PeakSimple v. 3.29 software (Grace Alltech, State College, PA, USA). The second HPLC, which was used for all other chromatography experiments was a Shimadzu Prominence system consisting of a model LC-20AT pump, DGU-20A degasser, CO-20A column oven, SPD-20A UV–Vis detector, and CBM-20A system controller. Data was collected and analyzed using Shimadzu LCSolution v. 1.24 software. All chromatography was performed using either a Kromasil C18 HPLC column, (Supelco, Bellafonte, PA, USA) or a Cosmosil Cholester HPLC column (Nacalai USA, San Diego, CA, USA). A 50 mm × 4.6 mm Kromasil column was used for van't Hoff analysis, while 100 mm × 4.6 mm columns were used for all other chromatography. All columns were packed with 5 μm C18 particles with 100 Å pores. Turbidity of solvent-cholesterol–MBCD solutions was measured using a LaMotte Model 2020 we/wi turbidity meter from Fisher Scientific.

2.3. Procedures

2.3.1. Solubility of cholesterol in MBCD-mobile phase solutions via turbidity

Solubility of cholesterol in mobile phases containing MBCD was assessed via measurement of the turbidity of the solution. Turbidity quantifies the clarity or haziness of a liquid. The more turbid a liquid, the less clear it is. Therefore, turbidity is not about color but rather the loss of transparency due to the effect of suspended particulate [35]. Turbidity is measured by illuminating the sample and measuring the reflected light off of the suspended particulates at a 90° angle to the light source. The more turbid a solution, the more light is scattered, and therefore a higher reading by the meter. The turbidity is given in Nominal Turbidity Units (NTUs), which is an arbitrary unit. Two methods were used for assessing solubility. In the first, solutions of a given mobile phase composition (water/methanol) and varying MBCD concentration (5, 10 or 15 mg/mL) were prepared. Then, cholesterol was added to 10.0 mL aliquots of these solutions. The solution was sonicated for 5 min, and the turbidity measured. Prior to saturation the turbidity of the solution was similar to that of a solution without cholesterol; once saturation of cholesterol was achieved the measured turbidity began to increase. (In effect, we are titrating the MBCD with cholesterol, with the endpoint indicated by an increase in turbidity). This procedure was repeated for mobile phases consisting of 85, 90, and 95% methanol or acetonitrile. In the second method, methanol-based solutions with fixed mole ratios of MBCD to cholesterol were prepared; these were then titrated with water until precipitation (indicated by a rapid increase in turbidity.) This process was used