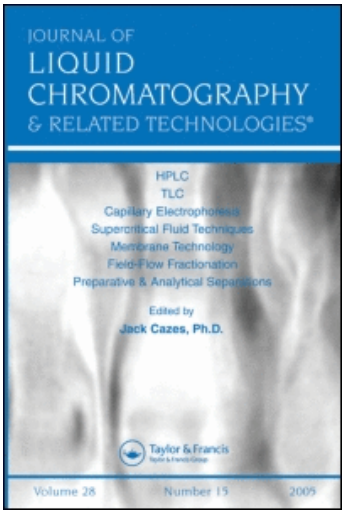

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New Approaches for Analysis of Metabolism Compounds in Hydrophilic Interaction Chromatography

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Abstract: Hydrophilic interaction chromatography (HILIC) was developed by Andrew Alpert for separation of highly polar compounds. HILIC is characterized by a high organic mobile phase concentration and the hydrophilic stationary phase. Thus, the retention times of highly polar compounds are increased with the increase of the hydrophilicity of the solutes.

Recently, it became necessary to determine highly polar compounds, such as pharmaceutical metabolism products. Therefore, HILIC has been promoted by the demand to analyze polar compounds in a complex matrix. Another reason for the increase in popularity is the widespread use of mass spectrometry (MS) coupled to liquid chromatography (LC). It is easy to promote ionization of several compounds, because there is low concentration of aqueous mobile phase.

This review attempts to summarize the ongoing discussion of the separation mechanism and gives an overview of the stationary phases used and the applications addressed with this separation mode in LC.

Keywords: Hydrophilic interaction chromatography, Metabolism compounds

INTRODUCTION

High performance liquid chromatography (HPLC) is powerful tool for determining highly polar compounds in several matrices, instead of gas

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chromatography (GC). Particularly, reversed phase liquid chromatography (RPLC) on an octadecyl silica column is an indispensable technique, i.e., one of the major advantages of the RPLC technique, of which aqueous and organic solvent are the most frequently employed.

HPLC is the most widely used analytical technique in the pharmaceutical industry because of its versatility and ability to retain and resolve a number of different types of compounds. However, the retention of polar analytes often requires a highly aqueous mobile phase to achieve retention, which can cause a number of issues such as dewetting of the stationary phase and decreased sensitivity in electrospray ionization mass spectrometry (ESI-MS).

Although it is a powerful separation mode, a major limitation of RPLC is the lack of adequate retention of very polar molecules. In this case, normal phase liquid chromatography (NPLC), which includes silica or alumina as the stationary phase, is used. It is generally used to separate highly polar compounds with no water in the mobile phase, e.g., hexane, chloroform, or benzene, under isocratic elution conditions. NPLC is a useful separation technique, as it provides different selectivity than RPLC. However, it is difficult to dissolve hydrophilic compounds such as peptides and nucleosides in non-aqueous mobile phase. Thus, the application of NPLC to biological samples is limited.

On the other hand, ion exchange or ion pair reagents have been used to separate and retain polar compounds. However, each of these techniques has certain drawbacks to ionization. In addition, ion pair reagents are difficult to use with mass spectrometry (MS) because they will cause ion suppression. The precision and accuracy of quantitative methods can be affected by these suppression effects.

In 1990, Alpert described a new separation method, i.e., hydrophilic interaction chromatography (HILIC), for the separation of polar compounds. In HILIC, retention increases with increasing polarity of the stationary phase and solutes and with decreasing polarity of predominantly organic solvent systems used for elution; this is in contrast to the trends observed with RPLC. Published methods of HILIC applications are given in Table 1.[1–9]

This review summarizes the ongoing discussion on the separation mechanism and applications of metabolism compounds.

PRINCIPLE OF HYDROPHILIC INTERACTION CHROMATOGRAPHY

A major limitation of reversed phase liquid chromatography (RPLC) is the lack of adequate separation of extremely high polar molecules. In this case, normal phase liquid chromatography (NPLC), which is performed with silica or alumina as the stationary phase, is used to separate polar compounds with non-aqueous mobile phases such as hexane, toluene, or chloroform. However, it was difficult to dissolve high polar compounds in non-aqueous solutions and mobile phases. At this point, extremely polar compounds, such as peptides,

Table 1. Theses about metabolism compounds using hydrophilic interaction chromatography

Analyte	Sample matrix	Instrument	Reference
Folic acid	Plasma	Tandem mass spectrometry	[1]
Tetrahydrofolate 5′-methyletrahydrofolate 5′-formyltetrahydrofolate			
Neomycin	Plasma	Tandem mass spectrometry	[2]
Isoniazid	Plasma	Tandem mass spectrometry	[3]
Cetirizine			
Cocaine	Blood	Mass spectrometry	[4]
Norcocaine	Urine		
Benzoylecgonine	Hair		
Methylecgonine	Brain		
Anhydroecgonine methylester	Bile		
Marijuana			
Fluoxetine			
Buprenorphine			
Delta9-tetrahydrocannabinolic acid			
Amikacin	Serum	Tandem mass spectrometry	[5]
Gentamicin			
Kanamycin			
Neomycin			
Paromomycin			
Tobramycin			
Acetylcholine	Brain	Tandem mass spectrometry	[6]
Choline			
Omeprazole	Plasma	Tandem mass spectrometry	[7]
5-OH omeprazole			
Zebularine	Plasma	Radioactivity detection	[8]
Uridine			
Uracil			
Dihydrouracil			
N-glycans	Immunoglobulin	Mass spectrometry	[9]
N-glycopeptides			

nucleosides, and saccharides were not amenable to NPLC. In 1990, the name hydrophilic interaction chromatography (HILIC) was first published by Andrew Alpert to distinguish this technique from normal phase chromatography.[10] HILIC is characterized by the presence of a high initial concentration of organic modifier to favor hydrophilic interaction between the solute and the hydrophilic stationary phase. Briefly, HILIC, based on silica columns, is normal phase chromatography, and it utilizes conventional reversed phase (RP) mobile phases. HILIC is run with polar stationary phases such as silica,[11–13] amino,[14,15] diol,[16] and polyhydroxyethyl aspartamide.[17] The HILIC mechanism involves partitioning between the adsorbed polar component of the mobile phase and the remaining hydrophobic component of the mobile phase. In this mechanism, the polar analyte partitions into and out of the adsorbed water layer on the negatively charged silica surface. In addition, depending upon the pH of the mobile phase, a positively charged analyte, which is a basic compound, can undergo cation exchange with the negatively charged silanol groups.

For example, allantoin, which is a metabolism compound of uric acid, is a very highly polar compound. It is difficult to retain by RPLC. The results of this study are shown Figure 1. HILIC very readily retains these polar compounds. On the other hand, retention times becomes long as mobile phases contain a high concentration of organic solvent (Figure 2).[18] Therefore, from this relationship, it might be assumed that the retention mechanism in HILIC is similar to that of more traditional NPLC methods.

Thus, the retention times of highly polar compounds are increased as their hydrophilicities are increased. HILIC is easy to use with MS because a hydrophilic stationary phase is used in combination with an organic mobile phase, and elution is usually performed by increasing the concentration of aqueous phase.

BIOLOGICAL AND PHARMACEUTICAL METABOLIC ANALYSIS

Recently, analytical methods for biological samples have been developed. Especially, there were focuses on metabolism pathways. Metabolic profiling is an important selective criterion for interpreting the efficacy outcome, for explaining the toxicity of lead compounds, and for explaining the toxicity of the specific drugs during either drug discovery or drug development. These metabolic compounds have highly polar characteristics. Therefore, HILIC is useful for determining polar metabolic compounds.

Glutathione and its Oxidation Metabolism Compound

Biological thiol compounds are classified as high molecular weight protein thiols and low molecular weight free thiols. Endogenous low molecular

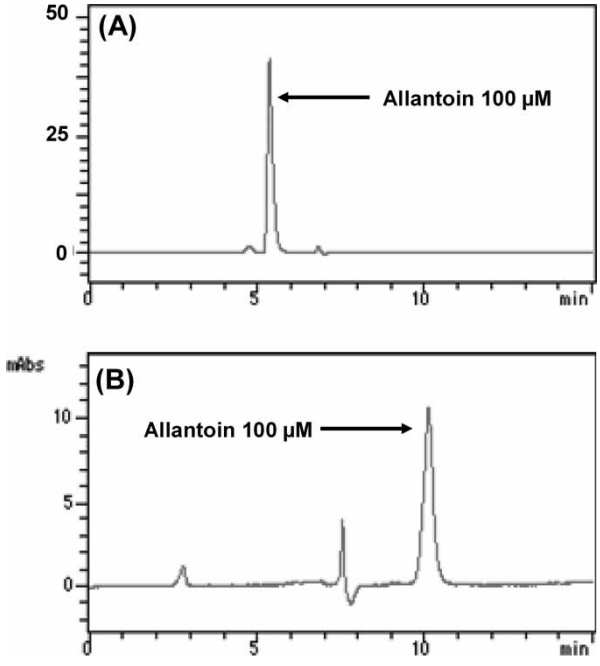

Figure 1. Chromatograms for allantoin (100 μM) by LC and HILIC methods with UV detection (A) Inertsil ODS-2 (250 × 4.6 mm, 5 μm; GL Sciences, Tokyo, Japan), (B) Atlantis™ HILIC Silica (250 × 4.6 mm, 5 μm; Waters, Tokyo, Japan). The UV detector was set at 220 nm. The mobile phase of (A) was water/acetonitrile (95:5, v/v). The mobile phase of (B) was water/acetonitrile (10:90, v/v). Flow rate of (A) and (B) were 0.5 mL/min.

weight thiol compounds, namely, glutathione (GSH) and its corresponding disulfide and oxidation metabolism product, glutathione disulfide (GSSG), are very important molecules that participate in a variety of physiological and pathological processes. However, it is difficult to determine reduced and oxidized thiol compounds simultaneously without derivatization because these compounds have very high polarities. However, the newly developed HILIC/MS method showed superior sensitivity for the determination of GSH and GSSG in human saliva samples.[19]

Morphine and its Metabolism

Morphine is a widely used opioid for treatment of severe pain. It is metabolized by liver UDP-glucuronosyl-transferases to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). The morphine glucuronides are hydrophilic compounds and are not retained in RP chromatography

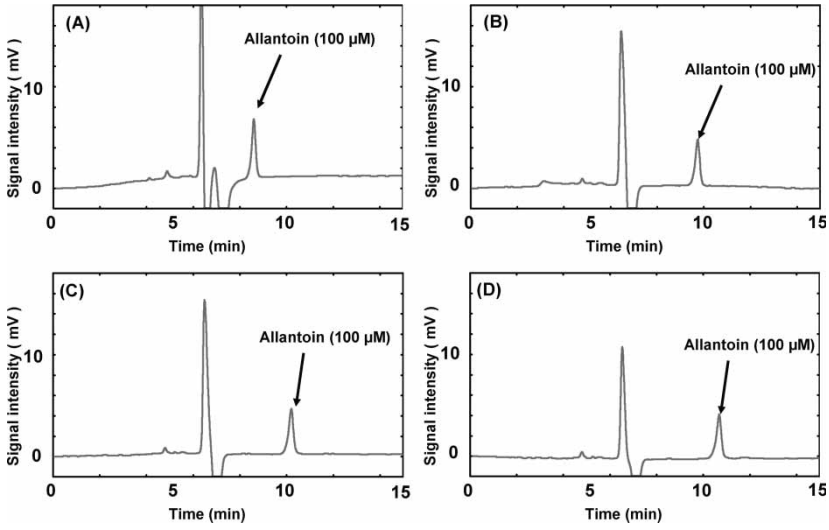

Figure 2. Chromatograms of allantoin (100 μM) by HILIC with UV detection. The UV detector was set at 220 nm. The mobile phase of (A) was acetonitrile/100 mM formic acid buffer (pH = 3.0) (90:10, v/v). The mobile phase of (B) was acetonitrile/100 mM formic acid buffer (pH = 4.5) (90:10, v/v). The mobile phase of (C) was acetonitrile/100 mM formic acid buffer (pH = 5.0) (90:10, v/v). The mobile phase of (D) was accetonitrile/100 mM formic acid buffer (pH = 5.5) (90:10, v/v). Flow rate of (A), (B), (C) and (D) were 0.5 mL/min.

without using ion pairing. Jörgen Bengtsson et al. developed a sensitive and reproducible method for the determination of morphine and its metabolites such as M3G and M6G.[20] The method was validated for perfusion fluid used in microdialysis, as well as for sheep and human plasmas. A C_{18} guard column was used to desalt the samples before analytical separation on an HILIC column and detection with tandem mass spectrometry (MS/MS). For human plasma, the lower limits of quantification (LLOQs) were 0.78, 1.49, and 0.53 ng/mL for morphine, M3G, and M6G, respectively. The methods described were used to analyze samples from animal and human studies. There were no interfering peaks in the chromatograms. Moreover, total plasma concentration versus a time profile from microdialysis sampling from a sheep fetus is presented in this publication.

Tobacco-Specific Nitrosamine Metabolite NNAL

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has been suspected as a potential lung carcinogen in

tobacco smoke. In vivo, NNK is rapidly metabolized to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is further metabolized via conjugation with glucuronide into NNAL-O-glucronide and NNAL-N-glucuronide.[21]

Nicotinic Acid and its Metabolites

Nicotinic acid, which belongs to the hydrophilic vitamin B complex, has a hypolipidemic agent possessing vasodilating and fibrinolytic properties. The separation of polar components is no easy task for RPLC. Yunsheng Hsieh et al. developed, for simultaneous determination of nicotinic acid and its metabolites in dog plasma by HILIC, system interfaces with atmospheric pressure ionization sources and MS/MS.[22] On the other hand, Christopherson et al. achieved separation of similar compounds between nicotinic acid and picolinic acid using HILIC.[23] The use of a silica column for the separation of nicotinic acid and its metabolites such as nicotinamide and nicotinuric acid to avoid mass spectrometric interferences was demonstrated.

Water Soluble Cellular Metabolites

A key unfulfilled need in metabolomics is the ability to efficiently quantify a large number of known cellular metabolites. An LC/MS/MS method is presented for reliable measurement of 141 metabolites, including components of central carbon, amino acid, and nucleotide metabolism.[24] The selected LC approach, HILIC with an amino column, effectively separated highly water soluble metabolites that failed being retained using standard RPLC. When applied to extracts of Escherichia coli grown in [^{12}C]-versus [^{13}C] glucose, the method enabled approximating 12Cand 13C-peaks for 79 different metabolites.

CONCLUSION

It is concluded that HILIC has been proven to be a useful analytical tool for the retention of small polar molecules. There are many publications about the metabolism of various compounds. Generally, the metabolites are highly polar compounds that are affected by metabolism in biological systems. These compounds are difficult to determine using RPLC. On the other hand, NPLC is not applied because the substances are not soluble in organic mobile phases. For these reasons, HILIC is the best way to separate these highly polar compounds. It is suggested to be used for the measurement of the metabolic products.

ABBREVIATIONS

HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
GC	Gas chromatography
RPLC	Reversed phase liquid chromatography
ESI-MS	Electrospray ionization mass spectrometry
NPLC	Normal phase liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

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