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Orthogonal method development using hydrophilic interaction chromatography and reversed-phase high-performance liquid chromatography for the determination of pharmaceuticals and impurities

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Abstract

A hydrophilic interaction chromatography (HILIC) method has been developed and validated as a secondary or orthogonal method complementary to a reversed-phase HPLC (RP-HPLC) method for quantitation of a polar active pharmaceutical ingredient and its three degradation products. The HILIC method uses a diol column and a mobile phase consisting of acetonitrile/water and ammonium chloride. The compounds of interest show significant differences in retention behaviors with the two very different chromatographic systems, which are desired in developing orthogonal methods. The HILIC method is validated and has met all validation acceptance criteria for the support of drug development activities.
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Keywords: Hydrophilic interaction chromatography; HILIC; Reversed-phase HPLC; Pharmaceutical analysis; Orthogonality; Specificity; Validation

1. Introduction

For HPLC methods used in pharmaceutical analysis, the specificity, also referred to as selectivity or stability indicating ability, is a critical attribute that should be thoroughly investigated and demonstrated in method development and validation. In simple terms, the specificity is the method's ability to separate and detect known, unknown, and potential impurities. The specificity is especially important for methods intended for early-phase drug development [1] when the chemical and physical properties of the active pharmaceutical ingredient (API) are not fully understood and the synthetic processes are not fully in control. Therefore the assurance of safety in clinical trials of an API relies heavily on the ability of analytical methods for the detection and quantitation of unknown impurities that may pose safety concerns.

Various approaches have been adopted to ensure method specificity. A feasible and reliable approach to check specificity is to develop a secondary method to separate peaks of interest using a different separation mechanism. Ideally, the secondary method should be orthogonal to the primary method.

To develop orthogonal methods, separation techniques other than HPLC, such as gas chromatography (GC), supercritical fluid chromatography (SFC) or capillary electrophoresis (CE) may be used [2–5]. Hydrophilic interaction chromatography (HILIC) is another technique that offers a different retention mechanism and is, therefore, a good candidate for orthogonal HPLC method development. In HILIC, a hydrophilic column is eluted with a hydrophobic mobile phase and retention increases with increased polarity of solutes [6]. HILIC has been used for the analysis of polar compounds such as proteins [6], peptides [7,8], amino acids [6], oligonucleotides [6], carbohydrates [9], histones [10] and natural product extracts [11,12]. It has also been

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used to determine polar pharmaceutical compounds that are difficult to retain and separate by reversed-phase HPLC (RP-HPLC) [13–16].

This paper describes orthogonal method development for a polar API currently under development by the Johnson & Johnson Pharmaceutical Research and Development, L.L.C. Specificity of the methods was demonstrated by analyzing forced degradation samples. Factors affecting method development for the HILIC method on a diol column are discussed. The HILIC method has been validated based on the ICH (International Conference on Harmonization) guidelines [17–20]. To the best of our knowledge, this is the first reported HILIC method that has been validated for assay and purity analysis of an active pharmaceutical ingredient.

2. Experimental

2.1. Instrumentation

An Agilent (Wilmington, Delaware, USA) 1100 HPLC system equipped with a photodiode array detector was used. Data acquisition and processing were conducted using the Waters (Milford, MA, USA) Empower software.

2.2. Chemicals

HPLC-grade acetonitrile was from EMD Chemicals (Gibbstown, NJ, USA). Phosphoric acid (85%) and ammonium chloride were from EM Science (Gibbstown, NJ, USA) or Aldrich (Milwaukee, WI, USA). 0.1 M HCl and 0.1 M NaOH solutions were from Mallinckrodt (Phillipsburg, NJ, USA). This study also involves a proprietary Johnson & Johnson Pharmaceutical Research and Development compound.

2.3. Preparation of solutions

The drug substance solution was prepared at a concentration of 7.5 mg/mL in 0.1 M HCl and 0.1 M NaOH, respectively. After 24 h, the samples were diluted with water or acetonitrile to contain 0.75 mg/mL API (Compound 2) for RP-HPLC or HILIC (unless otherwise specified). A mixture of stressed samples in 0.1 M HCl and 0.1 M NaOH was prepared as the specificity solution to contain all possible impurities.

2.4. Chromatographic conditions

A solution of 0.09% phosphoric acid was prepared by mixing 1mL phosphoric acid (85%) with 1000 mL water. The HILIC mobile phase was prepared by dissolving a salt in water followed by addition of acetonitrile. The injection volume was 10 μL unless otherwise specified. A Waters XTerra MS C_{18} column, 100 mm × 4.6 mm, 3.5 μm particles, and a YMC-pack Diol-120-NP column, 250 mm × 4.6 mm, 5 μm particles, were used for RP-HPLC and HILIC, respectively.

3. Results and discussion

3.1. Specificity of HILIC versus RP-HPLC

For assay and purity methods in pharmaceutical analyses, method specificity is demonstrated by analyzing forced degradation samples. The forced degradation conditions included elevated temperature and humidity, light, oxidation, acidic and basic conditions. For the proprietary API, the major degradation products were generated in the acidic and basic media. Two major degradation peaks were identified in the 0.1 M NaOH-stressed sample as Compounds 1 and 3. Two major degradation peaks were identified in the 0.1 M HCl-stressed sample as Compounds 1 and 4. The structures of these degradation products were identified. The functional groups of Compounds 1, 2, 3 and 4 are listed in Table 1. A specificity solution was prepared for method development that contained all four compounds.

For RP-HPLC a solution of 0.09% phosphoric acid and acetonitrile were used as mobile phases A and B, respectively. A linear gradient was programmed to increase mobile phase B from 2 to 25% in 20 min. All four peaks were eluted in 16 min. As in a typical reversed-phase separation, the most polar compound (Compound 1) eluted first while the least polar compound (Compound 4) eluted last (Fig. 1a).

The HILIC method was isocratic with a mobile phase of 10 mM NH_4Cl in acetonitrile/water (95:5, v/v). The run time was 20 min. The elution order of the four compounds was much different from that in RP-HPLC (Fig. 1b), indicating good orthogonality in a practical sense.

3.2. HILIC method development and optimization

3.2.1. Effect of acetonitrile content on retention

Three types of mobile phases are commonly used in the HILIC mode, including acetonitrile/water, acetonitrile/salt solution, and acetonitrile/buffer [6–12]. The mobile phase should be chosen based on chemical properties of the analytes. For example, for neutral molecules the acetonitrile/water mobile phase is preferred but any of the three types can be used. For organic salts of strong bases, the acetonitrile/salt solution mobile phase can be used. The acetonitrile/buffer type of mobile phase is the best for weak acids or weak bases, as well as for samples containing unknown components.

The acetonitrile/salt solution mobile phase was chosen for this study. The acetonitrile concentration was varied from 70

Table 1
Functional groups of the studied compounds

Compound identification[a]	Functional groups
Compound 1	Aromatic, $-NH_2$, –OH
Compound 2 (API)	Aromatic, $-NH_2$, $-O-CONH_2$
Compound 3	Aromatic, $-NH-CONH_2$, –OH
Compound 4	Aromatic, –O–CONH–

[a] The molecular weights for these compounds are in the range of 160–200.

Fig. 1. Chromatograms of the specificity solution on: (a) RP-HPLC and (b) HILIC. RP-HPLC conditions: column 100 mm × 4.6 mm 5-μm Xterra MS C18; gradient elution with (A) 0.09% phosphoric acid and (B) acetonitrile (B, 2–25% in 20 min); column temperature 35 °C; flow rate 1 mL/min; UV detection 215 nm. HILIC conditions: column 250 mm × 4.6 mm 5-μm YMC-pack Diol-120 NP; mobile phase 10 mM NH_4Cl in acetonitrile/water (95:5, v/v); column temperature 30 °C; flow rate 1.5 ml/min; UV detection 215 nm.

to 95%. The plots of $\log k'$ versus acetonitrile concentration are presented in Fig. 2. For the less polar Compounds 3 and 4, the retention change versus acetonitrile concentration was small to modest. For Compounds 1 and 2, which have the ionizable amino functional group, the retention change was dramatic with increasing acetonitrile concentration. Interestingly, when acetonitrile is above 80%, the elution order of Compounds 1 and 2 was reversed. This reversal of elution order is beneficial in this case. Since Compound 2 is the major component, the quantitation is more reproducible and accurate for Compound 1 if it elutes before Compound 2.

3.2.2. *Effect of salt concentration*

When charged molecules (e.g., Compounds 1 and 2) are analyzed with HILIC, salt (or buffer) is an essential component in the mobile phase. Figs. 3 and 4 demonstrate the relationship between salt concentration and $\log k'$ for Compounds 1–4. In Fig. 3, the plots were obtained with mobile

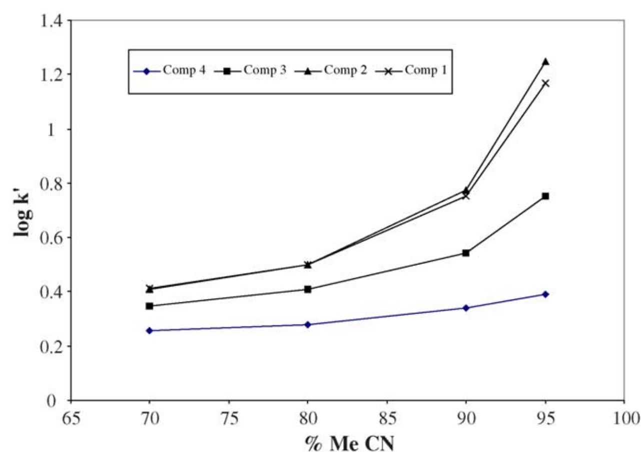

Fig. 2. Effect of acetonitrile concentration on $\log k'$. Other HILIC conditions: same as Fig. 1.

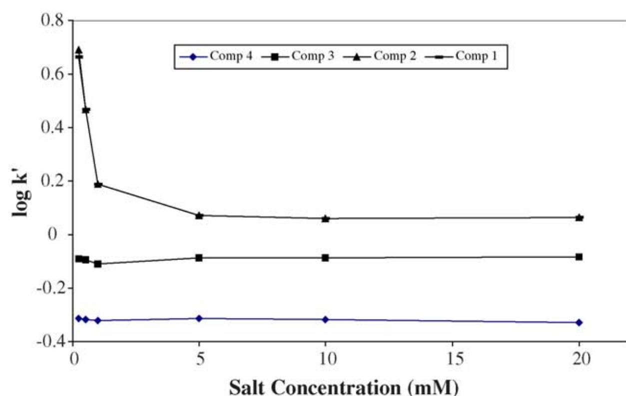

Fig. 3. Effect of salt concentration on log k'. Mobile phase: 80% acetonitrile with various NH_4Cl concentrations. Other HILIC conditions: same as Fig. 1.

phases containing 80% acetonitrile at different salt concentrations. For these compounds the log k' did not change when the salt concentrations were in the range of 5–20 mM. However, when the salt concentrations were <5.0 mM, Compounds 1 and 2, which co-eluted, showed much longer retention times. Poor peak shape was also observed at these conditions. The plots in Fig. 4 were obtained with mobile phase containing 95% acetonitrile. In the salt concentration range of 1–10 mM, the retention of Compounds 1, 2 and 3 increased slightly with increasing salt concentration. Below 1 mM salt concentration, poor peak shape was observed for Compounds 1 and 2. Based on the separation and peak shape, a mobile phase containing 95% acetonitrile and 10 mM NH_4Cl was selected for the final method.

The above-observed salt effects are consistent with the HILIC retention mechanism. It has been proposed that there may be a layer of stagnant water coated on the surface of the polar stationary phase (in this study the diol bonded phase) to facilitate the partition interaction for solutes [6,8]. In the absence of a salt or buffer, the charged solutes have a much greater tendency to reside in the water layer. Therefore, the solutes will have long retention time or are permanently retained on the column. As the salt concentration increases,

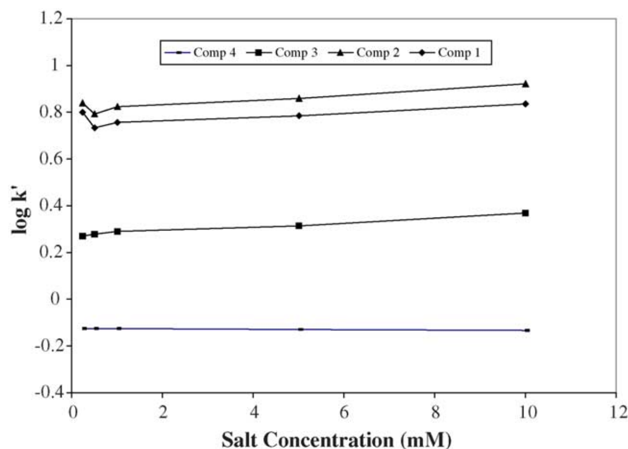

Fig. 4. Effect of salt concentration on log k'. Mobile phase: 95% acetonitrile with various NH_4Cl concentrations. Other HILIC conditions: same as Fig. 1.

the presence of excess amount of counter ions will promote the formation of ion-pairs for the charged solutes. The formed ion-pairs will have better solubility in the mobile phase, which results in shorter retention times.

3.3. Method validation

The validation was conducted according to the ICH guidelines [17–20]. The validated parameters include specificity, accuracy, limit of quantitation, linearity and precision. The optimized conditions of the HILIC method are described in Fig. 1.

The method specificity has been demonstrated by separating all known and potential degradation products. The specificity was further confirmed by using the RP-HPLC method. Accuracy of the method was determined by analyzing drug substance samples at multiple concentration levels (80.0–120.0%). The recovery was between 99.2 and 100.6% ($N = 9$). Linearity of the method was determined by preparing and analyzing a series of six standard solutions in the range of 0.05–120.0% of the nominal concentration. Regression analysis of the peak area versus concentration data yielded an $R^2 > 0.9999$ for the API. The system repeatability was assessed by multiple injections of the 80, 100, 120% standard solutions. For six injections of each solution, the relative standard deviation (RSD) was in the range of 0.4–0.8%. The limit of quantitation was estimated to be 0.05% for the impurities at a signal to noise ratio of 10.

3.4. Conclusion

A pharmaceutical compound and three of its related impurities have been separated using both RP-HPLC and HILIC conditions. It has been demonstrated that HILIC provides different selectivity than RP-HPLC and is a useful tool for orthogonal method development. Some of the parameters involved in the HILIC method development include the acetonitrile and salt concentration in the mobile phase. The validated method is suitable for the assay and purity analysis of the drug substance.

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