Improved Metabolite Isolation from Samples of Biological Origin Using Liquid Chromatography with Sub-2-Micron Particle Columns

Application

Pharmaceuticals

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Abstract

In recent years, a sense of urgency has been placed on increasing the rate at which drugs are brought to market. [1] Therefore, every aspect of analysis should be achieved at an increased rate as well, without sacrificing the quality of the analysis. The recent adoption of small sub-2-micron particle columns has enabled faster and higher resolution liquid chromatography. When isolating metabolites from complex matrices such as plasma and urine, it is imperative that if the rate of sample isolation is to be increased, the overall purity or recovery of the collected metabolites must not be compromised. The advantages of small-particle columns in the application of isolating low-level metabolites are demonstrated.

Introduction

In drug metabolism and pharmacokinetics (DMPK), the goal of both in vitro and in vivo studies is to identify drug-related components. [2,3] When more structural detail is needed for specific metabolites than can be provided by LC-MS alone, isolation using LC becomes a critical step in providing samples for additional characterization by nuclear magnetic resonance (NMR) analysis. Metabolites are isolated from a variety of complex biological matrices such as plasma, urine, feces, liver, and bile. Driven by the promise of faster run times and higher resolution with smaller sub-2-micron particle columns, conventional HPLC methods have been reevaluated as a way of reducing the time requirements for metabolite isolation. [4, 5]

Experimental

The Rapid Resolution LC

A number of experiments were performed to determine if the use of 1.8-micron columns on the Rapid Resolution LC (RRLC) system could offer improved chromatography. The RRLC system used is shown in Figure 1.

- 1200 Series SL Binary pump equipped with micro vacuum degasser
- 1200 Series High Performance Autosampler
- 1200 Series Thermostatted Column Compartment
- 1200 Series High Speed Variable Wavelength Detector
- 1100 Series Analytical Fraction Collector

The columns used were ZORBAX SB-C18 in several column lengths and packing sizes. The inner diameter remained fixed at 4.6 mm in order to maintain constant loading capacity and flow rate. A flow rate of approximately 1 mL/minute is ideal to obtain fractions of a practical volume. Details on the columns used are listed below:

- 4.6 x 250 mm, 5 µm, PN 880975-906
- Rapid Resolution 4.6 x 150 mm, 3.5 µm, PN 863953-902
- Rapid Resolution HT 4.6 x 150 mm, 1.8 µm, PN 829975-902
- Rapid Resolution HT 4.6 x 100 mm, 1.8 µm, PN 828975-902

Pertinent method parameters include mobile phase A: 10 mM ammonium acetate pH 5.0 using acetic acid and mobile phase B: 75:25 ACN: MeOH. The variable wavelength detector (VWD) monitored λ 235 nm, with a peak width setting of > 0.005 min or a 0.12-second response time, and equipped with the standard flow cell (PN G1314-60086).

The gradient table for the original method (4.6 x 250 mm column) is as follows:

Time	%B
0	20
35	35
50	55
55	100
55.1 to 60	20

The time was reduced proportionally for the shorter Rapid Resolution (RR) and Rapid Resolution High Throughput (RRHT) columns.

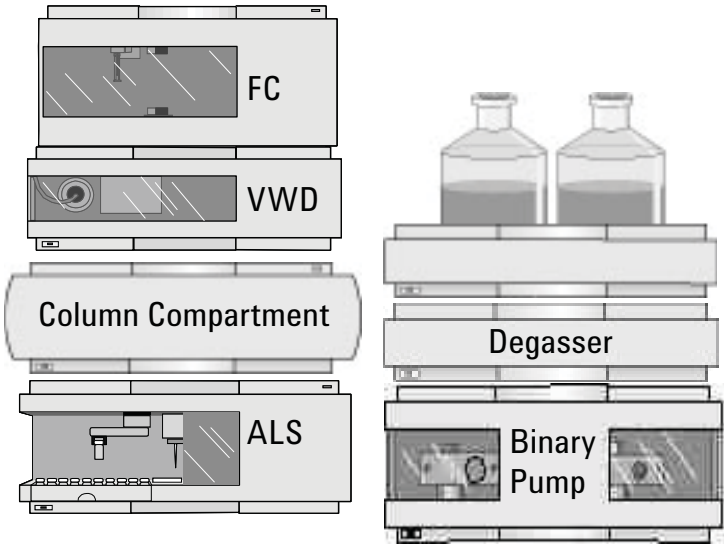

Figure 1. RRLC system for rapid simultaneous detection and isolation of drug metabolites.

The Sample

Urine analyses are shown in Figures 2 and 3. Sample preparation of urine samples consisted of concentrating the urine 10-fold to dryness by centrifugation under vacuum at ambient temperature and reconstituting in the starting mobile phase. The sample was then centrifuged once more to remove particulates prior to injection.

This approach can be generalized for plasma, liver homogenate, and fecal homogenate. Sample preparation would consist of extracting, in triplicate, with three volumes (1:3 sample: acetonitrile), vortex mixing, and centrifugation. The supernatants would be decanted, combined, and reduced to dryness. Extracts would then be reconstituted in starting mobile phase conditions, centrifuged, and injected. Most often, bile is injected directly with no sample work up.

The results from urine analyses are shown in Figures 2 and 3. The drug metabolites of interest are identified here as M5, M13, M15, and M17 in the chromatograms.

Results and Discussion

In our laboratory, due to the complexity of samples being analyzed, chromatographic run time can range from 60 minutes up to 3 hours using 4.6 mm x 250 mm, 5-micron columns. The goal here was to take an existing method and reduce the run time while maintaining resolution and throughput.

Column diameter is partially determined by the amount of material being loaded per injection. Generally metabolites of interest are present in nanogram to microgram per mL of matrix, and it is

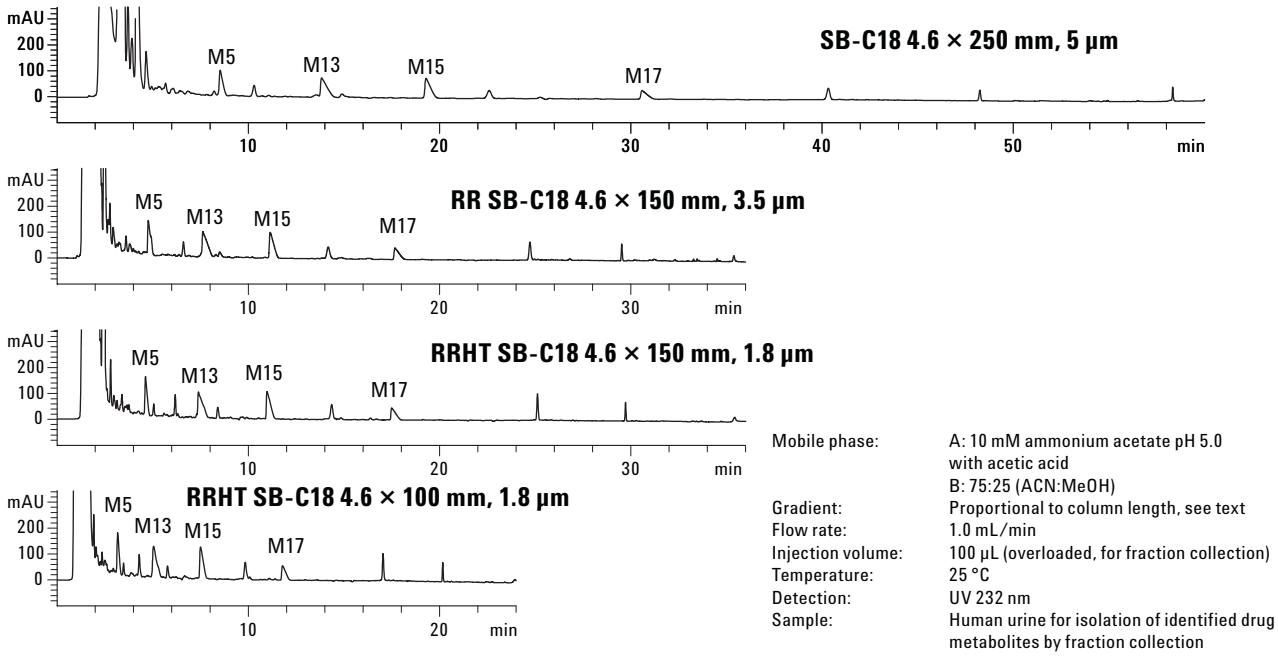

Figure 2. Scalability of SB-C18 columns increases throughput of DMPK metabolite isolation method.

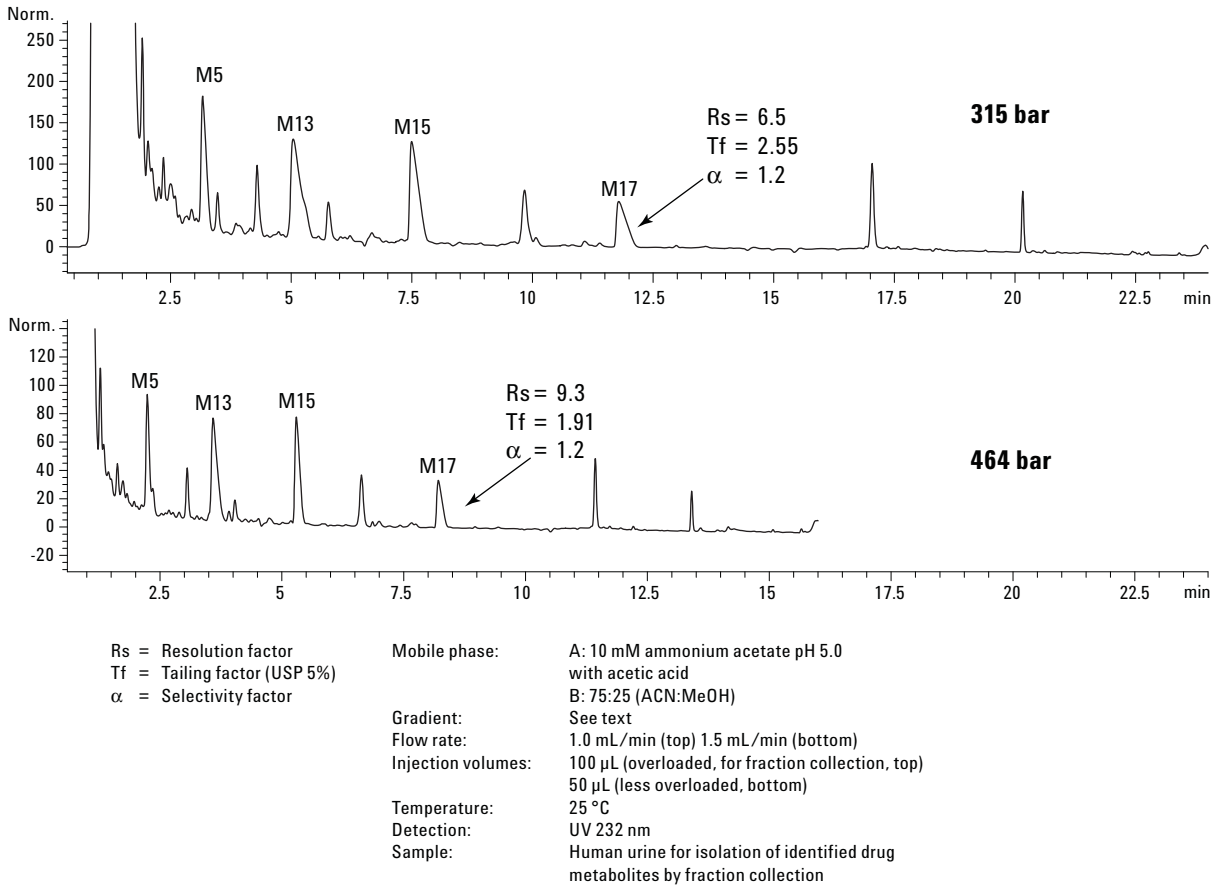

Figure 3. Effect of increasing flow rate and decreasing injection volume on RRHT analysis.

the endogenous material in the sample that makes up the vast majority of the mass. In order to obtain the maximum amount of metabolite free from endogenous material, columns are often overloaded. The amount of endogenous material loaded onto the column results in overload and loss of the chromatographic integrity. One must then resort to multiple small injections to reduce the coelution of endogenous material from the metabolites of interest. Multiple injections of these hour-long runs become quite time consuming when isolating very small amounts of material.

While high throughput is not a factor in our labs, increased throughput is. The results obtained by using the Rapid Resolution columns in combination with the Agilent 1200 Series Rapid Resolution LC are shown here. Often metabolism studies will involve the use of a ^{14}C-radioisotope in the compound as an effective way of identifying drug-related material in the presence of what might be an overwhelming background of endogenous compounds. These studies, accompanied with the use of sub-2-micron particle columns, will be the subject of a future paper. The samples analyzed here were from a first-time-in-human study where no ^{14}C-radioisotope was incorporated into the drug. For isolations purposes, only the UV response was monitored and reported.

In Figure 2, a comparison of an analyzed urine sample from this study is shown, where very-low-level metabolites need to be isolated. The original method specified a ZORBAX SB-C18 4.6 mm x 250 mm, 5-µm column and had a run time of 60 minutes. This same sample was separated on a 4.6 mm x 100 mm, 1.8-µm column and on 4.6 mm x 150 mm, 1.8-µm and 3.5-µm columns for comparison. The gradient profile was changed proportionally to column length.

As expected, with a decrease in particle size and column length, both the resolution and UV response increased. A direct comparison of each metabolite's UV response and peak width has been reported for each column in Table 1. For each of the metabolites, a marked improvement with peak shape was seen with an increase in theoretical plates. Note that column performance could likely be further improved by going to even smaller 2.1-µm id columns.

Figure 2 also demonstrates the method's ruggedness and the scalability on SB-C18 columns. The different column configurations, including particles sizes and lengths, had minimal effect on selectivity. This was seen by the same elution pattern of the four metabolites between the four separate analyses. The peaks of interest were tailing because the column was purposefully overloaded to maximize the amount of metabolite purified per run.

When an RRHT 4.6 x 100 mm is substituted for the conventional 4.6 x 250 mm, 5-µm column, peak height increased an average of 46% and peak width narrowed an average of 44% using the same amount of sample injected. This allows for minimal peak overlap when fractionating, negating the need to combine fractions. Additionally, analysis time was cut by over half. This benefit translates to increased laboratory productivity.

Further increased performance was easily achieved by increasing the flow rate from 1 to 1.5 mL/min and reducing injection volume from 100 µL to 50 µL. Figure 3 overlays the scaled down RRHT method in Figure 2 with a slightly optimized method. Analysis time was about 3.8 times quicker, and peak shape improved. System back pressure (maximum) was 464 bar for the 1.5 mL/min analysis and 315 bar for the 1 mL/min analysis, both well within the operating range (600 bar) of the Agilent 1200 RRLC. ZORBAX 1.8-µm particles have a specially engineered particle size distribution to provide lower pressures than other sub-2-micron particles.

Table 1. Peak Heights and Widths Using Various Column Configurations

	M5	M13	M15	M17
4.6 x 250 mm, 5 µm 60 minutes	105 mAU 30 seconds	75 mAU 48 seconds	75 mAU 48 seconds	25 mAU 60 seconds
4.6 x 150 mm, 3.5 µm 36 minutes	145 mAU 22 seconds	100 mAU 34 seconds	100 mAU 33 seconds	40 mAU 48 seconds
4.6 x 150 mm, 1.8 µm 36 minutes	165 mAU 18 seconds	105 mAU 34 seconds	105 mAU 30 seconds	45 mAU 36 seconds
4.6 x 100 mm, 1.8 µm 24 minutes	180 mAU 17 seconds	135 mAU 33 seconds	130 mAU 24 seconds	55 mAU 30 seconds

Conclusions

The RRHT technology has demonstrated improvements in laboratory efficiency. Substituting conventional columns with an RRHT column does increase throughput without compromising resolution. The fulfillment of higher resolution is the greatest gain from using small-particle (1.8- or 3.5-um) columns. Improvements in peak height and width are also gained when substituting a conventional-sized column with an RRHT column. Additionally, analysis time is shortened proportional to column length. These benefits will translate to increased laboratory productivity. The method is easily scalable between different SB-C18 column configurations, including different particle sizes, and works with multiple complex matrices, such as urine or plasma, with minimal sample preparation.

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