Increased Sample Throughput for Ketoconazole Analysis by Automated 96-Well Sample Preparation and Multiplexed Liquid Chromatography

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An existing ketoconazole method involved manual protein precipitation followed by LC-FL analysis, with a run time of approximately 15 minutes. Sample preparation was converted to the 96-well format and automated using the TomTec Quadra96. In addition, a multiplexed LC system was configured to cut the LC run times in half. The LC configuration enabled alternate sample injections to travel down one of two LC columns. The injections were staggered such that the eluting peaks could be diverted to a single FL detector. This approach significantly reduced the analysis time of the method while simultaneously demonstrating the feasibility of injecting one batch of samples across more than one LC column. Additional data utilizing a fast flow monolithic silica rod column is also presented.

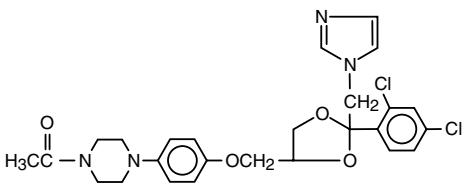
Ketoconazole (***F1***) is an important antifungal agent used in the treatment of infections. It is most often taken in oral form, but can also be an ingredient in externally applied products such as topical creams, hair shampoos and foot sprays (1). Ketoconazole has also been co-administered with protease inhibitors to AIDS patients who can develop fungal infections due to lowered immune function (2).

Recently, there has been additional interest in ketoconazole because this compound is a potent inhibitor of CYP3A (3-4), an enzyme which metabolizes a wide range of chemical compounds. The inhibition of CYP3A4 by ketoconazole is time-dependent and slowly reversible. In addition, the degree of CYP3A inhibition by ketoconazole may vary with different individuals. Therefore, it is necessary to measure the concentration of ketoconazole in plasma so proper dosing regimens may be established for co-administrated drugs that are also metabolized by CYP3A. Ideally, analytical methods for measuring plasma ketoconazole would be both rapid and reliable.

The original ketoconazole method developed in our lab involved a simple protein precipitation followed by LC with fluorescence (FL) analysis. A chromatographic run time of about 15 minutes was necessary because plasma from some individuals exhibit endogenous interference peaks at shorter retention times. This report demonstrates that sample preparation time can be decreased using the TomTec Quadra96, and that sample throughput can be increased using multiplexed LC. The multiplexing configuration is discussed in detail, and the performance statistics (precision and accuracy data) from the resulting validated methods are shown. In addition, a nonvalidated fast-flow LC method is presented as part of an investigation to further improve sample throughput.

F1

Ketoconazole structure.



Ketoconazole

Experimental

Matrix: 250 µL heparinized human plasma

LC Column: Waters Symmetry C18 (150 x 3.9 mm) with Javelin BDS C18 precolumn (20 x 3 mm) or Merck Chromolith Performance RP-18e column (100 x 4.6 mm)

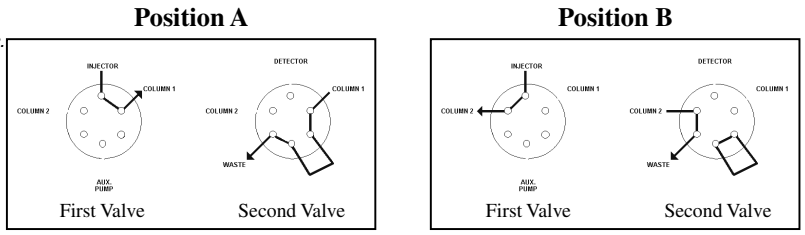
Mobile Phase: 40% ACN : 60% 50 mM Phosphate Buffer, pH 7

Detector: Hitachi L-7480 fluorescence detector. λ_{ex} = 260, λ_{em} = 375

Validated Range: 0.2 to 50 µg/mL

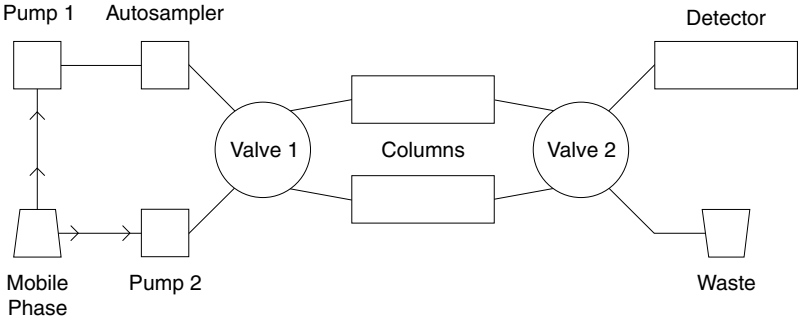
F2

Switching valve schematic.



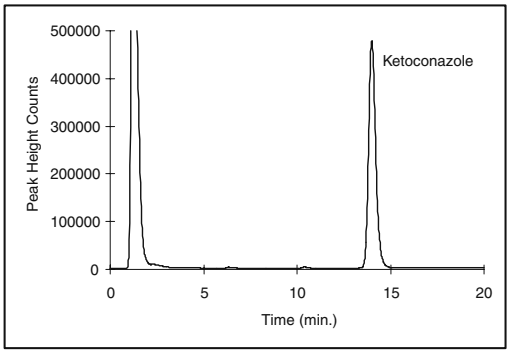
F3

Multiplexed-LC configuration.



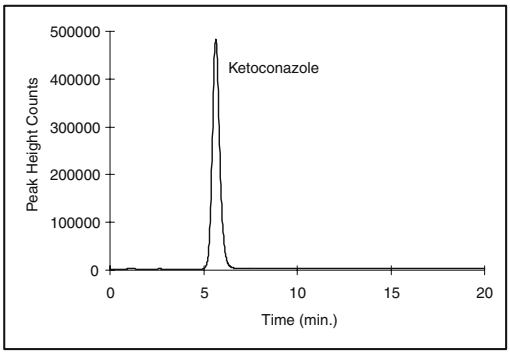
F4

Typical chromatogram from "Manual-sequential" assay configuration.



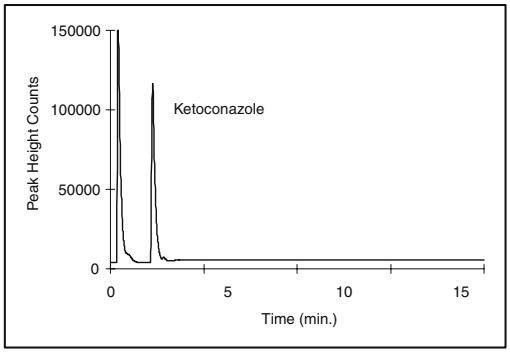
F5

Typical chromatogram from "TomTec-multiplexed" assay configuration.



F6

Typical chromatogram from "TomTec-chromolith" assay configuration.



Manual Sample Prep: Protein precipitation by combining 250 µL heparinized human plasma and 500 µL acetonitrile. Vortex mix, then centrifuge for 10 minutes. Transfer 500 µL of the supernatant to an autosampler vial, dilute with an equal volume of water, vortex mix, then inject 100 µL.

96-Well Sample Prep: Aliquot 250 µL of each sample into a 1.2 mL 96-well plate. The TomTec Quadra96 adds 500 µL acetonitrile. Cap plate, vortex, and centrifuge for 5 minutes. The TomTec then transfers 250 µL of the supernatant to a fresh plate, dilutes with an equal volume of water, and mixes. Centrifuge plate for 5 minutes, apply foil seal, then inject 100 µL.

Multiplexing Configuration: An autosampler alternately injects onto one of two columns via a switching valve. Another synchronized switching valve selects one column to the detector and one column to waste (see valve schematic in ***F2***). ***F3*** shows the overall setup. The injections are staggered such that the eluting peaks can be diverted to a single FL detector. The mobile phase going through the two columns comes from the same reservoir. Two pumps, one of them connected to the autosampler, flow through the first switching valve. The outlets from the columns go through the second valve. After the first injection (both valves in position A), the stream from the autosampler goes to column 1 and a sample is injected onto this column. Meanwhile, the auxiliary pump (pump 2) delivers mobile phase to column 2. The effluent from columns 1 and 2 go through valve 2 (also in position A). Immediately after the first injection, the eluent from column 1 goes to waste and the effluent from column 2 goes to the detector. When the time approaches for the analyte to elute from the first column, both valves are switched simultaneously to position B so the analyte from column 1 can be detected and a new sample can be injected, this time onto column 2. In position B the effluent from column

T1

"Manual-sequential" assay performance.

Nominal Concentration (ng/mL)	40000	20000	600
Average Concentration (ng/mL)	40279	20356	623
Standard Deviation	763	561	36.0
Precision (%)	1.9%	2.8%	5.8%
Accuracy (%)	100.7%	101.8%	103.9%
N	18	18	18

T2

"TomTec-multiplexed" assay performance.

Nominal Concentration (ng/mL)	40000	20000	600
Average Concentration (ng/mL)	40424	20134	598
Standard Deviation	961	334	17.5
Precision (%)	2.4%	1.7%	2.9%
Accuracy (%)	101.1%	100.7%	99.6%
N	18	18	18

T3

"TomTec-chromolith" assay performance.

Nominal Concentration (ng/mL)	40000	20000	600
Average Concentration (ng/mL)	39319	18997	627
Standard Deviation	1151	341	27.5
Precision (%)	2.9%	1.8%	4.4%
Accuracy (%)	98.3%	95.0%	104.6%
N	18	18	18

T4

Total analysis time for 96-sample batch.

Configuration	Sample Prep	LC Run	Total time
Manual – Sequential	1.5 hr	26.0 hr	27.5 hr
TomTec – Multiplexed	0.5 hr	13.0 hr	13.5 hr
TomTec – Chromolith	0.5 hr	6.0 hr	6.5 hr

1 goes to the detector and the autosampler loads column 2. When the time approaches for the analyte to elute from the second column, the valves are switched back to position A so the analyte from column 2 goes through the detector. This sequence is then repeated with each new sample. Since the peak of interest elutes near the end of the chromatogram, the first half of each run is always diverted to waste.

Results and Discussion

The original ketoconazole method involved manual protein precipitation and dilution before LC-FL analysis. The manual sample preparation scheme was easy with few steps. An internal standard was not necessary. Even so, converting to the 96-well format and automating with the TomTec Quadra96 cut the sample preparation time to one-third of the original. The plasma had to be individually pipetted into the 96 sample wells, just as in the manual discrete-tube method, but everything else was done automatically. So, automated sample preparation provides a distinct advantage in that the whole batch is prepared essentially at once, and thus all samples are treated the same.

Our initial method could be called "manual-sequential": manual sample preparation followed by sequential injections onto the LC column. With this approach, one Waters C18 symmetry column was used with one Javelin BDS precolumn. The flow rate was 1ml/min. Performance statistics for spiked plasma samples are shown (***T1***), along with a sample chromatogram (***F4***). The nominal concentration represents the amount of ketoconazole added to the plasma. The average concentration was obtained from linear regression of the observed peak areas using 1/concentration weighting, then averaging the back-calculated concentrations at the each level. The accuracy was calculated as the observed average divided by the nominal concentration, with the resulting value being expressed as a percentage. The precision was obtained by dividing the standard deviation of the measured concentrations by the measured average, with the resulting value being expressed as a percentage. The values for the "manual-sequential" method were all well within the typical Good Laboratory Practice acceptance criteria of ±15%.

Multiplexing, or processing two or more samples simultaneously on the same LC system, is a common means of increasing sample throughput. Our lab typically multiplexes LC/MS/MS assays, employing a dual column set-up that isolates one set of samples onto a single LC column. Historically, we have avoided splitting a single batch of samples across two LC columns due to concerns that the chromatographic performance on both columns might not match throughout the course of a large batch. Since multiplexing is a generic strategy and modern LC columns are quite reproducible, it was reasonable to extend the multiplexing concept to a conventional LC assay while simultaneously testing the feasibility of injecting a single set of samples across two LC columns.

To this end, a "TomTec-multiplexed" method was developed. This involved automating the original sample preparation using the 96-well format on a TomTec Quadra96. Two Waters C18 Symmetry columns were used with two Javelin BDS precolumns, as shown in ***F3***. The flow rate was 1 ml/min. A typical chromatogram (***F5***) and statistical results (***T2***) are also shown for this approach. The "TomTec-multiplexed" data indicate the method performed well. Even at the lowest concentration tested, the precision and accuracy were well within acceptable limits and comparable to values obtained with the "manual-sequential" approach.

Based on the assay's performance statistics, the automated multiplexed method was found to be robust, even though two separate analytical columns were used for the same batch. By multiplexing, two samples could be analyzed in the same time required for one sample

by sequential injection, thus throughput was doubled. As shown in ***T4***, a 96-sample batch analyzed using the "TomTec-multiplexed" method could be finished in 13.5 hours, compared with 27.5 hours for the "manual-sequential" technique.

A fast flow monolithic silica rod column was also evaluated to see if throughput could be increased further. A Chromolith Performance RP-18e column was used for this purpose. This column utilizes a novel highly porous silica that is able to tolerate increased flow rates without compromising the resolution between components (5). It is especially well-suited for separations that have few peaks in the mixture. Therefore, the Chromolith column seemed fitting for our application, since only ketoconazole and a few interfering plasma peaks are present. The flow rate used was 4 mL/minute. Again, the TomTec robotic sample preparation was used, but the multiplexed chromatography setup was omitted. Only one column and one pump were connected. With this setup, referred to as "TomTec-Chromolith," the retention time for ketoconazole was about three minutes (***F6***). This decreased the analysis time of a batch to 6.5 hours, compared to 13.5 hours for the TomTec-multiplexed method (***T4***). Even though the run time was significantly reduced, the accuracy results were still well within acceptable limits (***T3***). Thus, the Chromolith LC column appears to have definite speed advantages, cutting analysis times by at least four-fold compared to the original method.

Conclusions

Several conclusions can be drawn from this study. First, the use of the TomTec Quadra96 and multiplexed LC allows significant time savings without a loss of quality. Furthermore, although typically associated with LC/MS/MS assays, multiplexed LC is useful with other detectors as well. In addition, the data clearly demonstrate the ability to inject a single batch onto two LC columns without compromising the assay. Finally, the results also indicate that the Chromolith LC column appears to provide significant speed advantages without sacrificing chromatographic performance.

References

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