AN AUTOMATED UPLC/MS/MS APPROACH TO ENHANCE THE THROUGHPUT OF PHYSICO-CHEMICAL PROPERTY PROFILING IN DRUG DISCOVERY

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INTRODUCTION

The synthesis of large, focused chemical libraries allows pharmaceutical companies to rapidly screen large numbers of compounds against disease targets. Active compounds, or hits, resulting from these screens are traditionally ranked based on their activity, binding and/or specificity. Turning these hits into leads requires further analysis and optimization of the compounds based upon their physicochemical and ADME characteristics. The critical factor to consider in physicochemical profiling is throughput. The bottlenecks to throughput include MS method optimization for a large variety of compounds and data management for the large volume of data generated.

Currently, experiments including solubility, chemical and biological stability, water/octanol partitioning, PAMPA, Caco-2, and protein binding are used to generate physicochemical profiles of compounds in drug discovery. The measurement of physicochemical properties from these studies is easily enabled using chromatographic separation and quantitation using LC/MS/MS/UV. While the sample analyses may be efficient, the processing of the data and the interpretation of the results often requires tedious and time-consuming manual manipulation and calculation.

This paper describes an approach to solving these problems by the use of a novel software package that allows for the design of experiments, data acquisition, and the processing as well as report generation in a fully automated manner.

To demonstrate the usage of this software package we have developed an automated LC/MS/MS protocol for data generation. The data acquired from multiple assays were processed by a single processing method, all in an automated fashion. As a result, the physicochemical profiling process was significantly simplified and throughput increased.

Figure 1. ACQUITY® TQD system with PDA Detector.

METHODS

LC Conditions

Instrument: Waters® ACQUITY UPLC® System
Column: ACQUITY UPLC BEH C18 Column 2.1 x 50 mm, 1.7 µm
Column Temp: 40° C
Sample Temp: 20° C
Injection Volume: 5 uL
Mobile Phase: A. 0.1% Formic Acid in Water
B. 0.1% Formic Acid in Acetonitrile

Gradient:

Time	A%	B%	Curve	Flow
0.00	95%	5%	6	0.60 ml/min
1.00	5%	95%	6	0.60 ml/min
1.30	0%	100%	1	0.60 ml/min
2.50	95%	5%	11	0.60 ml/min

MS Conditions

Instrument: ACQUITY TQD Mass Spectrometer
Software: MassLynx™ 4.1 with ProfileLynx™
Tune Page Parameters:
ESI Capillary Voltage: 3.20 kV Polarity: Positive
Source Temp.: 150°C Inter-Scan Delay: 20 ms
Desolvation Temp.: 450°C Inter-Chan Delay: 5 ms
Desolvation Gas Flow: 900 L/Hr Dwell: 200ms
Cone Gas Flow: 50 L/Hr

Experimental

- A set of 27 commercially available compounds were randomly chosen to demonstrate the **ProfileLynx™ Application Manager, along with QuanOptimize™**.
- Each compound and a reference standard were analyzed by solubility, pH stability, LogP/LogD, protein binding and microsomal stability assays based on methods previously published.[1,2,3]
- For quantitative experiments, single point or multipoint calibration curves were used.
- To mimic the current practice in discovery labs, 96-well plate formats were used in this study.

Solubility Assay

- Solubility was determined at three pH's (pH 1.0, pH 7.4, and pH 9.4) to simulate conditions that could be observed in the stomach, blood, and colon.
- In order to simplify the filtration step, Sirocco™ protein precipitation plates were used to simultaneously filter all compounds in a 96-well plate mode, based on a modified solubility assay from Guo and Shen[1].

pH Stability Assay

- The library was run through a pH Stability assay at three different pH's (pH 1.0, pH 7.4, and pH 9.0) to simulate conditions that could be observed in the stomach, blood, and colon.
- Samples were incubated for a single 24 hour time point at 37° C and were quantitated against a single point calibration, based on an assay by Di et al[2].

Partitioning Coefficient

- Assay based on Wang et al[3].

Metabolic Stability

- The metabolic stability of the compound library was determined using pooled male rat liver microsomes.
- A single point incubation at 20 minutes was compared to a control to measure the metabolism of the samples , based on an assay from Di et al[4].

Protein Binding

- Protein Binding of the compound library was determined by equilibration dialysis with both rat plasma and human plasma.
- A RED Device (rapid equilibration device), purchased from Pierce (Rockford, IL, USA) was utilized to carry out the dialysis.

RESULTS

MS/MS Method

- The 27 compound library was processed by QuanOptimize to determine the optimum MS/MS conditions and MRM transitions for each of the compounds. The ACQUITY TQD was run in ESCi mode so that ESI+, ESI-, APCI+, and APCI- were evaluated for the best mode of ionization for each compound. Table 1 lists the results obtained from the optimization runs.
- All compounds in the library were found to exhibit acceptable MS response using electrospray positive ionization. After completion of the MS/MS optimization, QuanOptimize was utilized to create the MS/MS MRM methods for each of the compounds and these methods were subsequently used in the various screening assay analyses.

Compound	Ion	Transition	Mode	CV	CE
Alprenolol	1st	250.18 > 116.14	ESP+	53	16
Amitriptyline	1st	278.19 > 91.05	ESP+	53	30
Atenolol	1st	267.17 > 56.11	ESP+	53	30
Benzimidazole	1st	119.02 > 92.05	ESP+	53	23
Betaxolol	1st	308.23 > 55.22	ESP+	60	30
Caffeine	1st	194.99 > 116.98	ESP+	5	2
Colchicine	1st	400.20 > 358.27	ESP+	53	23
Diltiazem	1st	415.19 > 178.06	ESP+	13	23
Diphenylamine	1st	170.52 > 109.03	ESP+	21	9
Doxepin	1st	280.17 > 107.03	ESP+	53	23
Lidocaine	1st	235.18 > 86.12	ESP+	13	16
Loperamide	1st	478.24 > 267.16	ESP+	37	23
Metoprolol	1st	268.19 > 56.10	ESP+	5	30
Nephazoline	1st	211.12 > 141.11	ESP+	21	30
Nortriptyline	1st	264.18 > 91.05	ESP+	45	16
Oxprenolol	1st	266.19 > 72.12	ESP+	13	23
Oxybutynin	1st	358.25 > 72.15	ESP+	60	37
Pindolol	1st	249.16 > 116.08	ESP+	13	16
Procainamide	1st	236.17 > 163.12	ESP+	37	16
Propranolol	1st	260.17 > 56.08	ESP+	13	30
Sotalol	1st	273.13 > 133.15	ESP+	21	30
Sulfadimethoxine	1st	311.07 > 92.07	ESP+	45	37
Timolol	1st	317.17 > 74.13	ESP+	45	23
Tolazamide	1st	312.14 > 91.08	ESP+	45	30
Tolbutamide	1st	271.07 > 91.12	ESP+	60	37
Verapamil	1st	455.31 > 165.11	ESP+	53	30
Zolpidem	1st	308.18 > 235.16	ESP+	21	37

Table 1. QuanOptimize Results for 27 Compound Library

Solubility Assay

- Because the solubility assay of the compounds is carried out at 250µM and the samples are diluted 1:100 before UPLC analysis, the concentrations determined by UPLC/MS/MS must be multiplied by a factor of 100 to get the final solubility. If this final measured concentration is 250µM, then the compound has a solubility greater than or equal to 250µM at the pH of the buffer.
- The ProfileLynx results indicate a wide range of solubility for the various compounds in the 27 compound library.
- The ProfileLynx browser, in Figure 1,shows the results for diltiazem, including a chromatogram and calibration curve. The solubilities of diltiazem at the three different pH's are very similar which agrees with the literature.
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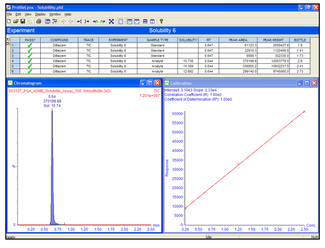

Figure 1. ProfileLynx Browser results for diltiazem

pH Stability Assay

- When using a single point calibration, ProfileLynx calculates the % Stability as the ratio of the amount of compound (or peak area) in the pH buffer divided by the amount of compound in the standard.
- Figure 2 shows an example of the manner in which ProfileLynx processes and displays pH stability data and results.
- The results for metaprolol, shown here, indicate that although quite stable, some degradation has taken place, particularly at pH 7.4 and pH 9.4. This is in agreement with literature.

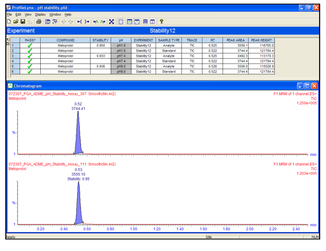

Figure 2. ProfileLynx Browser results for metaprolol

Partitioning Coefficient Assay

- Figure 3 shows an example of the manner in which ProfileLynx processes and displays LogP/LogD data and results.

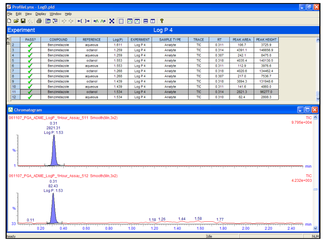

Figure 3. ProfileLynx Browser results for benzimidazole

- The results for benzimidazole shown here indicate that benzimidazole has partitioned mostly into the octanol phase resulting in a LogD7.4 = 1.53. The results for duplicate injections of all compounds were very reproducible.
- Since conditions were not chosen to ensure that all of the compounds would be in the unionized form, only a small number agree with the literature values of LogP obtained from the DrugBank[5] website.
- There is some disagreement in literature values of LogP from different sources for many of the compounds. For this reason, the majority of the values reported here should be considered LogD values determined at either pH 7.4 (buffer) or pH ~5.5-6 (water).

Metabolic Stability Assay

- When metabolic stability is run with a single point Sample List, the stability of the compound is calculated by a ratio of the peak area of the analyte (T_{20}) to the peak area of the standard (T_0).
- The ProfileLynx browser results for doxepin, shown in figure 9, indicate that doxepin is almost completely metabolized by the rat liver microsomes under these conditions with a stability of 0.003 (only 0.3% remaining).

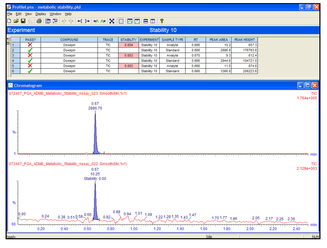

Figure 9. ProfileLynx Browser results for doxepin

Protein Binding Assay

- The example in figure 12 demonstrates how protein binding results are displayed for tolazamide.

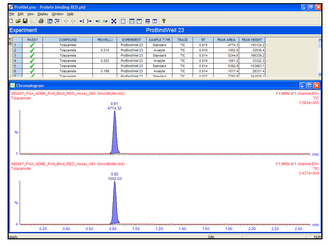

Figure 12. ProfileLynx Browser results for tolazamide

- The result column labeled PB(WELL) contains the fraction of compound that is free (not bound). The % of the compound bound by protein can be calculated by (1-PB(WELL)) *100%.
- The results for the entire set of compounds indicate a somewhat lower protein binding for samples in human plasma than for the same compounds in rat plasma.
- Overall, the protein binding results are very reproducible from injection to injection.

CONCLUSION

- **Optimal MS/MS conditions for each compound were easily determined using QuanOptimize.**
- **The ProfileLynx Application Manager was successful in the processing and management of data from a wide range of ADME assays.**
- **Processing the data was relatively easy and the interactive nature of the browser made small changes in integration for low level compounds a simple procedure.**
- **Assay results were comparable with literature values, and were reproducible.**
- **The use of the ACQUITY TQD system allowed for the fast analyses of samples to be achieved, with minimal cleanup, while increasing sensitivity and selectivity.**
- **By implementing QuanOptimize and ProfileLynx into existing LC/MS/MS workflows, the chemists reduced the amount of time it takes to perform these tests, in some cases from over 50 hours to just 20 hours.**

References

1. Y. Guo and H. Shen, from *Methods in Pharmacology and Toxicology, Optimization in Drug Discovery: In Vitro Methods*, ed. Z. Yan and G.W. Caldwell. Totowa, NJ: Humana Press Inc., pp 1-17.
2. L. Di et al., J. Biom. Sc., 11 (2006) 40-47.
3. X. Wang et al., Poster TPF204, ASMS, 2000.
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