Application Note

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Ultrafast Plasma Protein Binding Analysis

Using the Agilent RapidFire high-throughput mass spectrometry system and accurate mass Q-TOF

Authors

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Abstract

There has been increased demand for higher throughput *in vitro* ADME analyses to enable earlier optimization of drug candidates. One analysis that is key to assessing both the pharmacokinetics and pharmacodynamics of a drug candidate is plasma protein binding (PPB). A single, ultrafast method for analysis using the Agilent RapidFire High-Throughput Mass Spectrometry system and an Agilent 6550 iFunnel Q-TOF Mass Spectrometer was developed. More than 40 compounds were subjected to rapid equilibrium dialysis, then analyzed using a 9 second/sample SPE/TOF/MS method as well as by LC/MS/MS. Excellent correlation was determined between the two analytical methodologies with an R^2 value of 0.977. However, the SPE/TOF/MS method provided 20 times greater throughput compared to LC/MS/MS with a capacity of 400 samples/hour.

Introduction

During plasma protein binding, the fraction of drug that is bound to plasma proteins is no longer available to interact with the biological target or site of action. The remaining unbound and bioavailable fraction, therefore, is an important factor in pharmacokinetic properties including distribution and clearance. LC/MS/MS has become the method of choice for determining PPB due to its selectivity and sensitivity. However, disadvantages include the need to develop individual MRM methods for every compound and analysis times of several minutes per sample. In this study, these time-consuming disadvantages were overcome by eliminating the need for individualized compound method development and dramatically increasing the speed of analysis. The ability to develop a single, ultrafast method of analysis using the RapidFire High-Throughput Mass Spectrometry system and a 6550 Q-TOF Mass Spectrometer was investigated.

Experimental

Analytical systems

An Agilent 1260 Infinity Binary LC system was coupled with an Agilent 6460 Triple Quadrupole Mass Spectrometer and used to analyze each sample. Samples were analyzed at a rate of 3 minutes per sample using the conditions shown in Table 1. Individual compound multiple reaction monitoring (MRM) methods were optimized offl ine by infusion using a syringe pump from KD Scientific, Holliston, MA.

Identical samples were also analyzed by a SPE/TOF/MS system, consisting of a RapidFire 360 High-throughput Mass Spectrometry system, and a 6550 iFunnel Q-TOF Mass Spectrometer. Samples were analyzed at a rate of 9 seconds or less per sample using the conditions shown in Table 2.

Table 1. LC-triple quadrupole instrument conditions.

LC Conditions	
Analytical Column	Agilent Poroshell 120 EC-C18, 2.1 mm × 50 mm, 2.7 µm (p/n 699775-902)
Column Temperature	25 °C
Injection Volume	5 µL
Mobile Phase	A) 0.1 % formic acid in water B) 0.1 % formic acid in acetonitrile
Gradient	10 % B 0.0 minutes 10 % B 0.2 minutes 95 % B 0.8 minutes 95 % B 2 minutes
Run Time	2.0 minutes
Flow Rate	0.6 mL/min
Post Time	1 minute
Triple Quadrupole Conditions	
Acquisition Mode	Agilent JetStream, positive ionization, MRM
Sheath Gas Temperature	350 °C
Sheath Gas Flow Rate	11 L/min
Drying Gas Temperature	300 °C
Drying Gas Flow Rate	10 L/min
Nebulizer	35 psi
Nozzle Voltage	500 V
Capillary Voltage	3,500 V

Table 2. RapidFire-Q-TOF instrument conditions.

RapidFire Conditions	
Buffer A	Water with 0.09 % formic acid, 0.01 % trifluoroacetic acid
Buffer B	Acetonitrile with 0.09 % formic acid, 0.01 % trifluoroacetic acid
Injection Volume	10 µL
SPE Cartridge	Agilent RapidFire cartridge C (reversed-phase C18 chemistry, p/n G9205A)
RF State 1	Sip sensor
RF State 2	3,000 ms
RF State 3	3,000 ms
RF State 4	500 ms
MS Conditions	
Ion Mode	Dual AJS ESI, positive ion polarity
Drying Gas Temperature	200 °C
Drying Gas Flow Rate	18 L/min
Nebulizer	30 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow Rate	12 L/min
Nozzle Voltage	300 V
Capillary Voltage	5,000 V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
OCT1 RF Vpp	750 V
RF Voltages	High pressure funnel: 180 V Low pressure funnel: 80 V
Acquistion Parameters	2 GHz dynamic range MS mode 125 to 1.000 *m/z* acquistion 5 spectra/s

Chemicals and reagents

Rapid Equilibrium Dialysis (RED) device plates and inserts were purchased from Thermo Scientific (Rockford, IL). Pooled human plasma was purchased from Bioreclamation Inc. (Westbury, NY). Phosphate buffered saline (PBS), test compounds, and solvents were purchased from Sigma-Aldrich (St. Louis, MO).

Sample preparation

Human plasma (100 µL) was spiked with individual test compounds at a concentration of 5 µM and inserted into the sample chamber of the RED device. PBS (300 µL) was inserted into the buffer chamber of the RED device. Triplicate assays were conducted for each test compound. The test plates were sealed and incubated for 5 hours at 37 °C on an orbital shaker. After incubation, 50 µL from each sample chamber was transferred to a well of a 96-well plate containing 50 µL of PBS. The corresponding 50 µL from each buffer chamber was transferred to a well of the 96-well plate containing 50 µL of human plasma. Cold acetonitrile (200 µL) containing 0.1% formic acid and internal standard (1 µM bucetin) was added to each sample to precipitate proteins. The samples were centrifuged, and the resulting supernatants were diluted 1:1 with water and transferred to a 96-well analysis plate.

Data analysis

Agilent MassHunter Quantitative Analysis software (B.05.00) was used to integrate samples acquired from LC/MS/MS analysis. Samples acquired from SPE/TOF/MS were extracted by exact mass using a 10 ppm window and integrated using RapidFire Integrator Software. All integrated values were normalized by their corresponding internal standard values. Percentage bound was calculated for each test compound using the following equation:

% bound = 100 – [(average buffer/ average plasma) × 100]

Results and discussion

More than 40 different test compounds with diverse chemical properties including a xLogP range of –0.4 to 7.1 and molecular weight range of 160 to 733 g/mol were subjected to rapid equilibrium dialysis. Identical samples were analyzed by SPE/TOF/MS and by LC/MS/MS. A small, chemically diverse, subset of the test compounds was used to develop a single generic SPE/TOF/MS method. Sample-to-sample rates for this method were 9 seconds or less, providing a throughput of 400 samples an hour. This rate is 20 times greater than the throughput of the LC/MS/MS method. In addition, the LC/MS/MS method with gradient chromatography required MRM method development before analysis for every compound. This bottleneck in method development was eliminated by the use of mass accuracy provided from the high resolution of the 6550 Q-TOF.

The analytical results for the SPE/TOF/MS method were found to be comparable to traditional LC/MS/MS despite the differences in methodology. The percentage bound determined for each compound by both analyses is shown in Table 3. There was excellent correlation between the two methods with a R^2 value of 0.977 (Figure 1). There was also good agreement for both methods with previously reported values obtained using RED devices in the literature.[1-3]

Table 3. Human plasma protein binding values.

Compound	LC-Triple Quadrupole, % Bound	RapidFire-TOF, % Bound	Difference	RED Lit.
Amitriptyline	98.0	97.1	0.90	98.9
Amodiaquine	99.0	94.9	4.15	
Amoxapine	92.3	93.6	-1.26	
Buspirone	89.5	86.5	2.97	
Carbutamide	91.4	91.2	0.19	
Chloropromazine	99.9	99.8	0.12	
Cinnarizine	99.6	99.3	0.25	
Desipramine	90.7	91.9	-1.23	
Dextromethorphan	69.7	70.4	-0.74	
Diclofenac	99.7	99.7	-0.03	
Diltiazem	87.9	82.6	5.29	
Diphenhydramine	84.8	84.0	0.79	
Erythromycin	82.8	80.4	2.41	79.1
Fluconazole	20.7	17.6	3.05	21.2
Fluphenazine	99.4	98.8	0.62	95.7
Imipramine	94.6	93.6	0.96	93.4
Ketoconazole	99.4	99.3	0.03	
Lansoprazole	99.0	98.9	0.13	
Levofloxacin	46.1	50.5	-4.31	60.3
Metoprolol	24.3	16.7	7.60	3.5
Nadolol	23.0	17.7	5.29	25.3
Nicardipine	99.9	99.8	0.05	
Nicotine	54.4	42.5	11.94	

Conclusion

An ultrafast SPE/TOF/MS method was developed to analyze plasma protein binding assay samples at a rate of 9 seconds per sample with analytical results comparable to those determined by LC/MS/MS. In addition to a 400 sample/hour throughput, this method provided increased efficiency by eliminating the need for offline MRM method development. This ultrafast, high-resolution approach may be useful for the analysis of other *in vitro* ADME assays.

References

1. van Liempd, S. *et al.* Development and Validation of a Higher-Throughput Equilibrium Dialysis Assay for Plasma Protein Binding. *Journal of the Association for Laboratory Automation* **2011**, *16(1)*, 56–67.
2. Waters, N. J. *et al.* Validation of a Rapid Equilibrium Dialysis Approach for the Measurement of Plasma Protein Binding. *Journal of Pharmaceutical Sciences* **2008**, *97(10)*, 4586–95.
3. Shanler, M. S. *et al.* Validation of an Automated High Throughput Plasma Protein Binding Assay. *BD Biosciences application note #474*, **2009**.

Compound	LC-Triple Quadrupole, % Bound	RapidFire-TOF, % Bound	Difference	RED Lit.
Nifedipine	97.6	91.6	5.91	
Nimodipine	98.8	98.4	0.37	
Nizatidine	33.4	18.0	15.39	
Norfloxacin	55.4	47.0	8.36	
Phenacetin	37.2	44.9	-7.70	
Promazine	97.7	97.9	-0.24	
Promethazine	99.1	98.6	0.47	98.1
Propafenone	95.9	94.0	1.91	
Propanolol	90.3	87.2	3.09	80.2-92.6
Pyrilamine	72.3	69.8	2.51	
Quinidine	88.5	85.7	2.85	
Tacrine	74.2	63.1	11.12	63.3
Tamoxifen	100.0	99.5	0.46	
Terfenadine	95.7	99.9	-4.20	99.5
Testosterone	95.2	97.1	-1.92	
Thioridazine	100.0	99.8	0.19	99.9
Ticlopidine	99.9	99.3	0.54	
Tolbutamide	96.3	97.7	-1.37	95.6
Triprolidine	91.3	93.1	-1.75	
Verapamil	96.3	94.2	2.08	90.6-94.8
Warfarin	99.4	99.5	-0.08	99.5

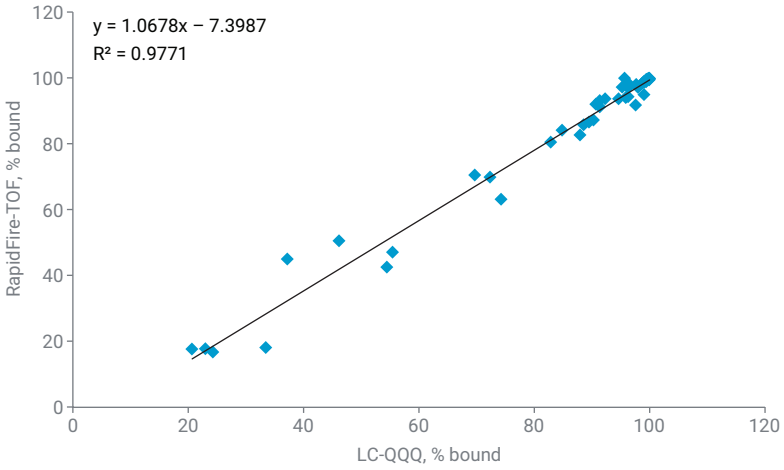

Figure 1. Comparison of human PPB values from LC-triple quadrupole versus RapidFire-TOF.

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