

Sensitive, Robust Quantitative Analysis of Small Molecule Mixture in Plasma

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Small molecules still occupy a significant portion in most pharmaceutical organisation's product list and pipeline. With the ever increasing requirement to address diseases with novel efficacious compounds, targeted quantitation of small molecule drugs is one of the most critical parts of the drug discovery and development process. Targeted quantitation enables analysis of small molecules in biological matrices to investigate efficacy and toxicity for accurate determination of the potential marketability of a drug. New methods using LC-MS/MS for the quantitation of compound mixtures in biological matrices have become more robust, accurate, and reliable, instilling higher confidence in results and furthering development of targeted drug candidates.

Introduction

Inspired by chemistry, however, guided by pharmacology and the clinical sciences, small molecule (molecular weight < 1000 Da) drug research has contributed more to the progress of medicine during the past century than any other scientific factor [1,2]. However, in today's world, owing to the increasing complexity of the diseases and hence drug targets [14,15], pharmaceutical drug discovery and development has become increasingly complex in terms of discovering and optimising new chemical entities into novel drugs with desirable efficacy and low toxicity - while ensuring compliance with regulatory requirements [3-7]. Despite the cost of development of small molecule based drugs going up constantly [4,8], they offer some advantages owing to their structural diversity. From using natural products [16,17] to new chemical entities offering novel targeted functionality, the inherent variability in small molecule based drugs still hold an important position in the world of drug discovery and development [9-12, 18-21].

Addressing regulations to ensure competency in procedures and drug safety before release to market is a critical process [13]. Such regulatory control studies require high quality and reproducible safety data for each drug tested. Regulatory agencies such as the United States Food and Drug Administration (FDA) and European Medical Agency (EMA) have regulations

in place for laboratories within pharmaceutical organisations to follow in order to produce detailed analytical results supporting the development of new drugs. The typical regulatory controls include Good Manufacturing Practices (GMPs), Manufacturing and Importation Authorization (MIA) and Qualified Person (QP) declarations, among others.

Most small molecule drugs are chemically synthesised and so have a well-defined structure that can be thoroughly characterised. However, in order to test the efficacy and safety of a new drug, it must be tested and analysed in a model system. In addition to the metabolite identification (Met ID) studies, targeted quantitation is an essential piece of the drug development workflow to determine compound levels of known analytes in biological systems. Analysing drug concentration in biological tissues presents additional challenges with navigating clean extraction and obtaining an adequate yield to provide precise quantitation of compounds in a mixture. In order to resolve these challenges, robust and reliable bioanalytical methods are needed to help

Table 1: Chromatography gradients

Time (min)	Flow Rate (mL/min)	%A	%B
0	0.6	95	5
0.4	0.6	95	5
0.5	0.6	65	35
1.5	0.6	64	36
1.6	0.6	55	45
2.2	0.6	53	47
2.3	0.6	5	95
2.95	0.6	5	95
2.995	0.6	95	5
3.5	0.6	95	5

generate comprehensive pharmacokinetic (PK) data for drug safety analysis.

Previous targeted quantitation efforts have been performed using older generation mass spectrometry (MS) systems or ligand binding assays (LBA). While these assays are able to clearly quantify small molecules, they are unable to provide the high levels of sensitivity, selectivity, and specificity to address the complexity of the target analytes and matrices. Conversely, LBA assays are matrix and assay selective but may not have the flexibility to quantitate a wide variety of compounds or mixtures.

With advancements in both liquid chromatography (LC) and triple quadrupole MS (MS/MS), LC-MS/MS is often a preferred technology that is extensively used for

Table 2: Mass spectrometer parameters

Parameter	Setting
Run Time	3.5 min
Ion Source	HESI
Spray Voltage	3500 V
Sheath Gas	40 Arb
Auxiliary Gas	15 Arb
Sweep Gas	0 Arb
Ion Transfer Tube Temperature	350 °C
Vaporizer Temperature	325 °C
Experiment Type	SRM
Cycle Time	0.3 s
Chromatography Peak Width	6 s
Collision Gas Pressure	1.5 mTorr
Q1 Resolution	0.7 FWHM
Q3 Resolution	0.7 FWHM

targeted quantitation. LC-MS/MS offers the unique ability to systematically configure an assay specific to any molecule or mixture, as well as the ability to perform multiplexed analysis of many compounds in a single assay. LC-MS/MS also has the added benefit of using internal standards to correct for analytical variability, resulting in improved precision and accuracy of results.

Analysis of Drug Candidates in Plasma Using LC-MS/MS

In this assay, targeted quantitation was performed on a mixture of known compounds using LC-MS/MS with a Thermo Scientific TSQ Altis triple quadrupole mass spectrometer. By adding 8 small molecule compounds in pooled rat plasma to simulate the analysis of a potential drug candidate in the plasma of an animal model, LC-MS/MS can be verified for a valid and improved assay for small molecule drug candidate testing.

Sample Preparation

Rat plasma was used in combination with 8 small molecule compounds. Acetonitrile (ACN) was added to plasma in the ratio of 3:1 (v/v) to precipitate out abundant serum proteins. Although the protein precipitation clean-up process offers less efficiency in terms of yield, removal of unwanted analytes, etc. compared to some other sample preparation processes (such as solid phase extraction, etc.), this is a highly preferred option owing to its reduced cost/sample offering and increased ease-of-

use. The resulting supernatant taken after processing was added to an equivalent volume of water to make the final crashed plasma stock solution. Stock solutions of each standard compound were then made at a concentration of 1 mg/mL and diluted in a pooled mix of all compounds with the crashed plasma stock to generate concentration ranges of 1 pg/mL to 25,000 pg/mL and 10 pg/mL to 100,000 pg/mL. Isotopically labelled internal standards for each compound were added to each calibration level to produce a final internal standard concentration of 0.5 ng/mL.

Liquid Chromatography

Chromatographic separation was performed using a Thermo Scientific Vanquish Horizon HPLC system. The column used was a Thermo Scientific Hypersil GOLD aQ C18 Polar Endcapped LC column (100 × 2.1 mm, 1.9 µm particle size). Mobile phases

A and B consisted of 10 mM ammonium formate in Fisher Chemical Optima grade water (pH ~3.5), and 0.1% formic acid in Fisher Chemical Optima grade acetonitrile, respectively. The column temperature was 50°C, with a total run time of 3.5 minutes. Chromatography gradients for analysis are summarised in Table 1.

Mass Spectrometry

Mass spectrometry analysis was performed on a Thermo Scientific TSQ Altis triple quadrupole mass spectrometer equipped with the Thermo Scientific OptaMax NG source housing. TSQ Altis offers several advantages over any other triple quadrupole MS with the ability to perform sensitive assays in – segmented quadrupoles, which offer better ion transmission efficiency (reduced fringe field effects), ion beam guide with neutral blocker, preventing neutrals from contaminating the quadrupoles and ensuring increased robust performance, a novel detector with increased lifetime while delivering the capability to perform robust, sensitive, fast quantitation assays.

In this study, Table 2 shows the mass spectrometer parameters and settings. Table 3 gives the selection reaction monitoring (SRM) properties used in the experimental setup.

Data analysis was performed using Thermo Scientific TraceFinder software.

Results and Discussion

Relative abundance of each compound was determined using LC-MS/MS, providing robust and specific quantitation of the compounds isolated from the plasma matrix. Each of the eight compounds was analysed for proper detection and quantitation compared with isotopically labelled internal

Table 3: SRM properties and compounds used in the experiment

Compound Name	Start Time (min)	End Time (min)	Polarity	Precursor m/z	Product m/z	Collision Energy (V)	RF Lens
Desomorphine	0.760	1.060	Positive	272.062	215.054	26	69
Desmethyldoxepin	1.230	1.530	Positive	266.062	107.000	23	56
Flecainide	1.310	1.610	Positive	415.050	398.054	24	84
Midazolam	1.410	1.710	Positive	326.012	291.054	28	87
Imipramine	1.660	1.960	Positive	281.462	86.054	17	48
Amitriptyline	1.800	2.100	Positive	278.075	233.111	18	53
Fluoxetine	1.890	2.190	Positive	310.362	43.889	11	39
Diazepam	2.230	2.530	Positive	285.012	193.071	33	78

Table 4: Limits of quantitation for each drug candidate in plasma, and %CV values for the internal standards

Compound	LOQ (pg/mL)	IS %CV
Desomorphine	5	3.5
Desmethyldoxepin	2.5	3.5
Flecainide	1	3.5
Midazolam	2.5	4.4
Imipramine	2.5	4.4
Amitriptyline	2.5	4.4
Fluoxetine	5	5.1
Diazepam	2.5	3.4

standards. Lower limits of quantitation (LLOQ) were obtained for each drug candidate, as shown in Table 4. In all cases, the LLOQ values were significantly lower than those observed from previous MS assay systems, suggesting excellent sensitivity across all assays. Lower coefficients of variation percentage (%CV) values for the internal standards, with four quality control points measured at increasing concentrations, also indicate improved reliability and reproducibility for tandem MS/MS. Figure 1 demonstrates high selectivity using this LC-MS/MS method.

Linearity and Reproducibility

Extended linear dynamic range is an important characteristic to studying the activity and toxicity of new compounds and mixtures. Depending on the mixture and what compounds it contains, the dynamic range can change up to, or in excess of, four orders of magnitude. This increased dynamic range might exceed the ion detector saturation limit, making the impact of ion detection significant. However, high

saturation points allow for a larger dynamic range without losing low-end sensitivity. This provides the opportunity for complete pharmacokinetic (PK) profile analysis, which is essential to targeted quantitation.

In this assay, four orders of magnitude were needed in order to comprehensively cover all targets analysed, creating specific, precise, and accurate data points with no observed matrix effect. This also enables reproducibility, eliminating the need for repeated sample analysis or dilution series' due to results being obtained outside the range. Table 5 demonstrates linearity to at least 15,000 pg/mL and high levels of reproducibility across the four quality control points taken at increasing concentrations. The method shows precision with a <8 %CV for the initial quality control point at 30 pg/mL and <3 %CV for the final quality control point at 15,000 pg/mL.

Robust Quantitation Assays for Pharmaceutical Compounds

LC-MS/MS meets the increased sensitivity requirements for the analysis of small

molecule pharmaceutical compounds, particularly in biological matrices where compound concentrations can be extremely low. The assay successfully achieved targeted acquisition of data, precisely detecting and measuring compound concentrations with higher sensitivity and throughput than alternative methods. The limits of quantitation for the drug candidates in plasma varied from 1-5 pg/mL, depending on the drug candidate and its components. LC-MS/MS is able to leverage increased sensitivity capabilities to achieve lower detection limits for limited sample volumes typically observed in matrix testing, due to drugs that display low tissue dispersion or have poor bioavailability.

Operating LC-MS/MS in SRM mode involves a targeted approach to identify molecules of interest and their corresponding fragments, by continuously monitoring a set of transitions for each analyte. In this manner, SRM mode generates a greater level of specificity and sensitivity of quantitation.

Gain in Selectivity

Combining LC and MS/MS, especially triple quadrupole MS with high resolution SRM, enables the analysis of complex mixtures with increased selectivity, where a particular molecule or group of molecules can be isolated from a complicated matrix and accurately determined to be the correct component being measured. Since compounds are separated by their mass-to-charge ratio (m/z), only those peaks appear on the chromatogram, providing enhanced selectivity of the targeted compounds. Figure 1 reflects this unmatched selectivity, showing only the clean targeted peaks of interest with no background noise and no additional unknown peaks, allowing lower detection levels as well.

Mass-to-charge ratio separation also enables LC-MS/MS to include isotopically labelled internal standards, which might pass through LC but can then be sorted by their mass difference. The use of stable isotopically labelled internal standards can help regulate variability when studying quantitation.

Analysis on a triple quadrupole instrument operated in SRM mode acts by targeted high resolution fragment ion analysis that generates a large gain in selectivity with more data points at high SRM rates. The high resolving power increases compound discrimination by separating ions of interest from interferences, thus improving the selectivity of measurements and overall quantitation function.

Matrix effect is caused by other non-targeted components in a sample affecting

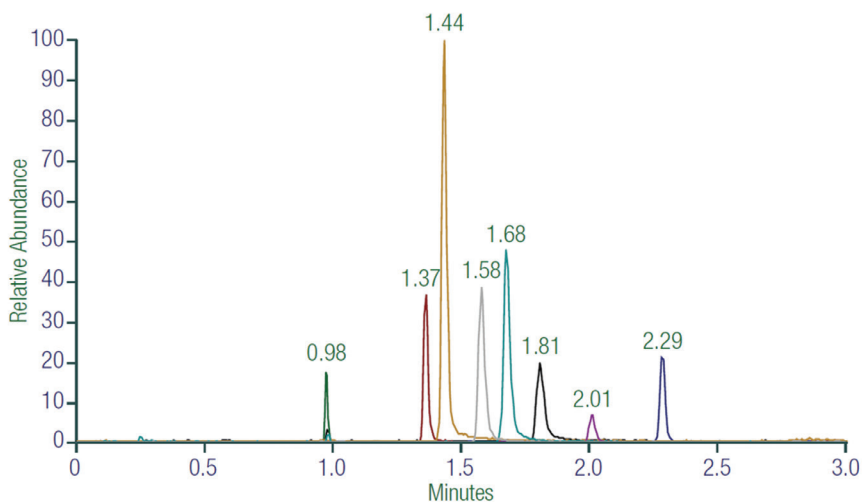


Figure 1: Chromatogram of QC 2, measured at 300 pg/mL

Table 5: Linearity and reproducibility data measured at four quality control points per calibration curve

Compound	QC 1 %CV 30 pg/mL	QC 2 %CV 300 pg/mL	QC 3 %CV 3000 pg/mL	QC 4 %CV 15,000 pg/mL	R ² Linear Fit
Desomorphine	7.93	4.72	3.47	1.15	0.9945
Desmethyldoxepin	5.28	1.55	0.67	1.01	0.9904
Flecainide	4.20	4.88	2.46	2.97	0.9924
Midazolam	2.96	1.52	1.71	2.77	0.9917
Imipramine	2.50	1.26	0.38	1.24	0.9913
Amitriptyline	7.04	3.16	0.68	0.83	0.9908
Fluoxetine	3.15	2.80	2.03	2.87	0.9901
Diazepam	5.77	3.15	0.53	2.69	0.9927

the analysis and quantifiable outcomes of an assay. Matrix effects and selectivity issues tend to be problematic when dealing with biological sample sources. In order to improve reproducibility and robustness of methods that are subjected to matrix effect, LC-MS/MS using a triple quadrupole instrument demonstrates significantly improved robustness of complex bioanalytical methods.

Conclusion

The development of a sensitive and reliable LC-MS/MS analysis method for targeted quantitation of a mixture of pharmaceutical-based compounds in biological matrices offers laboratories a novel platform for systematic, reproducible, and robust compound detection and quantitation. Applying this method to a known set of multiple drug standards suspended in rat plasma provides evidence that LC-MS/MS performed in this way can assist pharmaceutical labs with small molecule quantitation for preclinical efficacy studies.

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