Quantitation of metabolites in plasma samples by UV-MS correction using a dual-cell-linear ion trap mass spectrometer

Tim Stratton[1], Yingying Huang[1], August Specht[1], Julie Horner[1]

[1]Thermo Fisher Scientific, San Jose, CA, USA

Overview

Purpose: Quantitation of the desmethyl metabolites of dextromethorphan in plasma using a UV-MS correlation established through standard curve generated *in vitro*.

Methods: Metabolites of dextromethorphan from an *in vitro* incubation were identified by MS^n fragmentation. The desmethyl metabolites in the incubation were quantified by LC-UV using dextromethorphan as the standard. The quantified *in vitro* incubate was then diluted to prepare a standard curve for LC-MS quantitation of plasma samples containing multiple levels of both desmethyl metabolites.

Results: Spiked plasma samples of dextrorphan and 3-methoxymorphinan were successfully quantified within 25% of the known levels by using a UV-MS correlated standard curve.

Introduction

The quantitative assessment of metabolites in samples from development *in vivo* studies has become an area of increasing study with the release of guidances from regulatory bodies (MIST and M3 R2) and their subsequent interpretation and implementation. The hurdles faced in metabolite identification are joined by obtaining quantitative levels on relevant metabolites for which absolute structure may not be known and for which reference standards are not available. The challenges of matrix suppression, analyte stability, and MS detector response factor make quantitation of metabolites based on a parent drug standard almost impossible. We have applied a UV correlation to assign concentrations to metabolites from *in vitro* generated samples and used the results to quantify plasma samples on the same instrument.

Methods

Sample Preparation

To create a metabolite standard for MS analysis, dextromethorphan (50 µM) was incubated in human liver microsomes (HLM, 1 mg/mL) for 1 hour. Blank microsomal matrix was also prepared similarly and used as the diluent for subsequent standard preparations. The concentration of dextrorphan and 3-methoxymorphinan was determined by UV quantitation (280 nm) against a standard curve of dextromethorphan prepared in quenched microsomal matrix. The microsomal incubate was diluted to provide a standard curve using quenched microsomal matrix. A series of 12 spiked plasma samples were created with known levels of dextrorphan and 3-methoxymorphinan and prepared by precipitation with ACN. In order to account for matrix effects, both matrixes were mixed 1:1 with blank matrix of the opposite type.

Liquid Chromatography

The initial analysis of the incubate mixture, both for metabolite identification and for UV-MS quantitation, was performed using an Open Accela™ autosampler, an Accela 1250 UHPLC pump, an Accela PDA and a Velos Pro™ linear ion trap (Thermo Fisher Scientific, San Jose CA). Separation was performed on a 100X2.1 Synergi Max-RP column (Phenomenex) using LC method A in Table 1.The analysis of the diluted MS standard curves and plasma samples was performed using the same analytical system with the separation performed using LC method B on a 100X2.1 Hypersil™ Gold aQ column (Thermo Fisher Scientific).

Table 1. LC Methods

Method A			Method B		
Time (min)	% A	% B	Time (min)	% A	% B
0.0	98	2	0.0	95	5
0.5	98	2	0.5	95	5
6.0	5	95	2.5	5	95
6.2	5	95	3.0	5	95
7.0	98	2	3.5	95	5
8.0	98	2	5.0	95	5

Mass Spectrometry

All quantitative and qualitative analyses were performed on an Velos Pro linear ion trap connected to an Accela LC system described above. The mass spectrometer was operated in positive ionization mode with HESI-II probe (Sheath Gas: 40, Aux Gas: 15, 450 °C)

Data Analysis

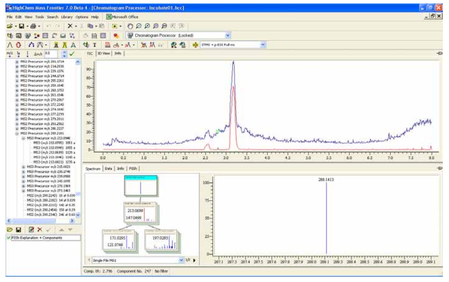
Metabolite detection and structure interpretation was performed using Mass Frontier™ 7.0 (HighChem, Bratislava Slovakia). Quantitation was performed using Xcalibur™ 2.1 (Thermo Fisher Scientific, San Jose CA)

Results

Detection of Dextromethorphan Metabolites from Human Liver Microsomes

The first step toward quantitation of metabolites based on UV-MS correlation is to identify the metabolites observed in the *in vitro* incubations and correlate their presence in the *in vivo* samples. For those present in the *in vivo* samples, the structure is identified based on the MS^n fragmentation pattern obtained. In this application the high concentration of the *in vitro* incubation made detection of the abundant metabolites relatively simple. However, in complex matrices or at low concentrations, detecting metabolites can be difficult. This can be aided by the application of processing software such as Mass Frontier. The fragment ion search (FISh) function in Mass Frontier software detects components based on common fragment ions between parent compound and its metabolites. This approach is shown in Figure 1.

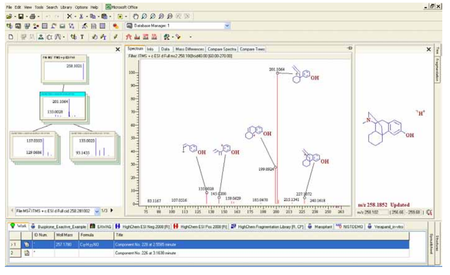
FIGURE 2. Fragment Ion Search (FISh) Results on *In Vitro* Incubation Sample. The Raw Trace is Shown in Blue, the FISh Extracted Trace of Metabolites in Red, and a Minor Oxidative Metabolite Found is Highlighted in Green.



Identification of Dextromethorphan Metabolites in Microsomal Incubations

The combination of HCD and CID at multiple MS^n stages provides extensive fragmentation data for structure elucidation. Mass Frontier aids the fragment interpretation process (Figure 2). The MS^n spectral trees of the metabolites were interpreted by comparison with the spectral tree of Dextromethorphan. FISh explanations for prominent fragment ions were used to assign putative structures.

FIGURE 2. Mass Frontier Annotation of the Fragmentation of Dextrorphan HCD MS^n Data.



Establishing a UV-MS Correlation Calibration Curve – Quantfying Metabolites

After metabolites of interest have been identified, their levels in the *in vitro* incubate must be established. This can be performed by UV based quantitation with a standard curve of Dextromethorphan. For this, the assumption was made that the molar absorptivity of the metabolites and the parent are essentially the same. Quantifying the metabolites based on their mass spectrometer signal against a dextromethorphan standard curve would be limited by the potential significant difference in ionization efficiency between metabolites and their parent structures. UV for this purpose is assumed to be a more "universal detection".

FIGURE 3. 20 µM UV Calibration Standard of Dextromethorphan.

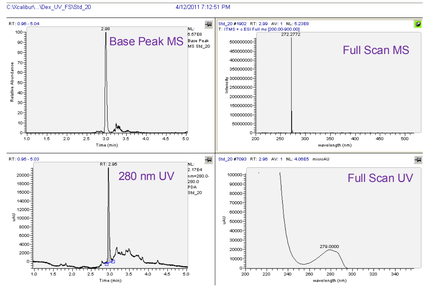
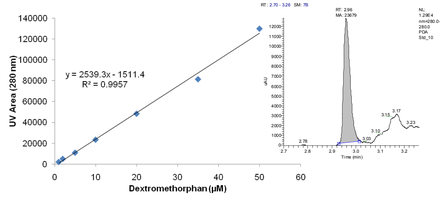


FIGURE 4. UV Calibration Curve of Dextromethorphan and Example Peak Integration of a 10 µM Standard Injection.



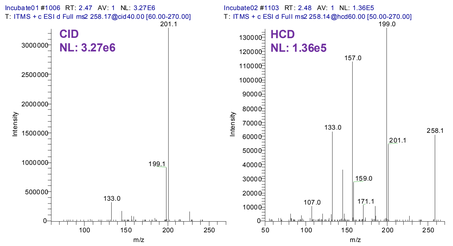
UV Quantitation of Dextrorphan and 3-Methoxymorphinan

Dextrorphan and 3-methoxymorphinan were quantified by triplicate injections and determined to be 1,852 ng/mL for dextrorphan and 1,484 ng/mL for 3-methoxymorphinan.

Mass Spectrometer Quantitation – Flexibility in Selecting Transitions

Accurate quantitation of metabolites in a complex mixture was achieved via an SRM approach. The access to both HCD and CID fragmentation mechanism on the Velos Pro provided flexibility and more options to improve the results. Figure 5 shows a comparison between the CID optimized fragmentation and the HCD optimized fragmentation of dextrorphan. The signal of the most abundant ion in the CID spectrum is more than twice as the most abundant ion from the HCD spectrum. As a result, CID was chosen for quantitation of dextrorphan. A similar analysis for 3-methoxymorphinan showed that a unique fragment from HCD was optimal. Therefore, 3-methoxymorphinan was quantified using HCD. Since both HCD and CID fragmentation spectra were acquired in the same run, the choice of which fragment ion, i.e., transition to use was made during data review and did not require any reinjections.

FIGURE 5. Fragmentation Spectra of Dextrorphan using CID and HCD.

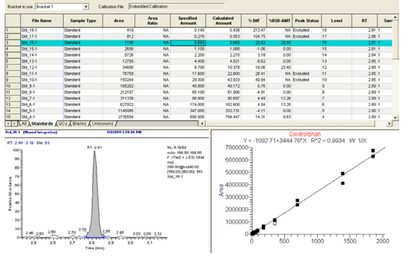


Mass Spectrometer Quantitation – Plasma Samples

The quantified *in vitro* microsomal incubation concentrate was used as the source of standard material for subsequent quantitation of dextrorphan and 3-methoxymorphinan in plasma. A standard curve of 18 points was prepared covering the range from 0.137 to 1852 ng/mL for dextrorphan and 0.110 to 1484 ng/mL for 3-methoxymorphinan by serial dilutions with a quenched microsmal blank matrix as the diluent. The lowest standards included for the quantitation was 0.550 ng/mL for dextrorphan and 0.881 ng/mL for 3-methoxymorphinan.

As the matrix of an LC-MS sample can significantly impact the signal intensity of the analytes, standards were mixed with a blank plasma extract (1:1) so that all samples would have the same matrix. A series of 12 spiked plasma samples were created with concentration ranges between 2 to 500 ng/mL (dextrorphan) and 2.5 to 800 ng/mL (3-methoxymorphinan). These samples were prepared for analysis by precipitation with acetonitrile. As with the standard curve, plasma supernatants were mixed with blank microsomal matrix (1:1) to provide the same matrix for analysis.

FIGURE 6. Quantitation of Dextrorphan by SRM (258 → 201, CID).



Quantitative Results for Dextrorphan and 3-Methoxymorphinan

An example of a calibration curve is shown in Figure 6 for dextrorphan. Points on the curve outside of ±20% were excluded and the simplest function that defined the points was applied for both dextrorphan and 3-methoxymorphinan. Spiked plasma samples were quantified against the standard curve prepared in the same matrix. Duplicate injections of the 12 samples were made and average results are reported in Table 2. With the exception of dextrorphan quantification in samples #9 and #12, all other measurements were within 20% of the actual value.

Table 2. Quantitation Results for Spiked Plasma Samples Quantified by MS Based on Standards Prepared by UV-MS Correlation.

	Average (ng/mL)		% of Actual	
Sample #	Dextrorphan	3-Methoxymorphinan	Dextrorphan	3-Methoxymorphinan
#1	500	2.79	99.9	112
#2	291	52.7	96.8	105
#3	244	357	97.4	102
#4	179	202	102	101
#5	167	4.24	111	84.8
#6	65.4	81.9	87.2	102
#7	65.0[1]	916[1]	130	115
#8	36.4	12.1	104	80.7
#9	41.2	527	165	105
#10	12.7	310	102	103
#11	6.93	166	92.4	110
#12	0.88	8.15	44.0	81.5
		Average % of Actual	103	100

[1] All values are n=2 except #7 were only one data point was available.

Conclusion

Quantification of the desmethyl metabolites of dextrorphan was successfully achieved on the Velos Pro through a UV-MS correlation method employing multiple fragmentation modes, as well as the creation of an LC-MS standard curve with dual plasma-microsomal matrix. This method provided acceptable accuracy for most samples with only 2 analyses outside of ±20% of expected values.

- A UV based quantification of metabolites based on parent is possible for compounds where metabolism does not significantly alter the chromaphore (and subsequently the molar absorptivity).
- Quantification of complex samples on an ion trap mass spectrometer is possible across a significant concentration range with acceptable performance.
- Multiple fragmentation methods (CID and HCD) available on the Velos Pro can be combined with its superior MS^n capability to provide comprehensive fragmentation information for unambiguous structural elucidation of the metabolites. In addition, the multiple fragmentation methods also enhance the instrument's quantification capability by providing multiple transition options to optimize specificity and sensitivity during post-acquisition data review without the need for re-injections.