Rapid Quan/Qual metabolic stability analysis with online oxidative metabolism synthesis

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Overview

Purpose: Demonstrate the utility of online oxidative synthesis as a tool for stability screening.

Methods: Study compounds were subjected to variable potentials in a synthesis cell in-line with an HPLC system. Potentials from -1100 to +1100 mV were applied and any products were subsequently separated by HPLC for analysis by HRAM MS. Potential vs stability analysis was performed by automatic processing of the results using a relative quan/qual software.

Results: A comparison of the results of the online synthesis system and a standard microsomal stability assay showed good correlation for compounds where oxidative enzymatic processes were the major determinate of stability.

Introduction

Screening of compounds for stability is an important part of nearly all pharmaceutical discovery programs. While this is most often done using various *in vitro* systems (hepatocytes, liver microsomes, or other tissue/subcellular fractions) the major products of enzymatic metabolism are typically oxidative. The use of an electrochemical system to generate oxidative conditions for compound reaction could theoretically mimic the products of these Phase I enzymatic processes. In this example we demonstrate the application of one such system in an online fashion. The electrochemical synthesis cell is placed in line with an HPLC-HRAM MS system such that oxidative products generated are subsequently separated by HPLC before analysis.

Methods

Sample Preparation

Stock solutions (1 µM) of five test compounds were prepared in 2:98 ACN:25 mM ammonium acetate to approximate initial chromatographic conditions.

Liquid Chromatography

The system as shown in Figure 1 consisted of an Thermo Scientific Open Accela™ autosampler and an Accela 1250 UHPLC™ pump to deliver the compounds to a synthesis cell (5021A / 5125) controlled by a Coulochem III (Thermo Fisher Scientific, San Jose CA). The products of the synthesis cell were separated on a 50X2 5µM C18. Mobile phase A consisted of 25 mM ammonium acetate buffer. Mobile phase B consisted of 80:20 ACN:25 mM ammonium acetate buffer.

Table 1. LC Method

Time (min)	% A	% B	Flow (µL/min)
0.0	98	2	1000
0.5	98	2	1000
4.5	0	100	1000
5.0	0	100	1000
5.5	98	2	1000
6.0	98	2	1000

Mass Spectrometry

Qualitative analyses was performed on Thermo Scientific Q Exactive benchtop Orbitrap mass spectrometer connected to the LC system described above. The mass spectrometer was operated in positive ionization mode with a HESI-II probe (Sheath Gas: 40, Aux Gas: 15, 450 °C). Acquisition included full scan (*m/z* 150-650, 70,000 resolution) followed by an all ions fragmentation (AIF) scan (*m/z* 50-650, 70,000 resolution). These scan pairs were used to perform a structure based neutral loss triggering of precursor ion selected MS^2 scans. Briefly, this involved analyzing every full scan AIF scan pair on-the-fly for any match to a provided neutral loss list created from the top six most intense observed fragment ions for each test compound. Any match found (± 5ppm) triggered a precursor ion selected MS^2 scan on the observed full scan peak (40% NCE, 17,500 resolution).

Data Analysis

Oxidative products were detected and relative quantitative formation was determined by analysis of the potential / formation relation by a beta version of Thermo Fisher Scientific MetQuest 1.2. Structure interpretation was performed using Mass Frontier™ 7.0 (HighChem, Bratislava Slovakia).

Results

Electrochemical Synthesis as a Surrogate for Metabolism

The use of electrochemical synthesis as a means to mimic oxidative metabolism has been used previously to generate potentially reactive metabolites(1). These studies demonstrated the ability of electrochemical systems to simulate enzymatic metabolism and for the synthesis of material for structure identification. In addition to such applications, we studied the application of online electrochemical synthesis as a tool for early quan/qual applications by using a synthesis cell in-line with an HPLC-HRAM MS system. While typical metabolic stability assays utilizing subcellular fractions use a time course of exposure to determine the enzymatic stability of a compound we utilized a voltage range (-100 to 1100 mV, 100 mV increments) to rapidly measure redox stability of the test compounds. For this ampherometry experiment the synthesis cell was activated and the fixed potential applied with a range of 1 mA and a filter interval of 100 2µS, the current was allowed to stabilize for ten seconds prior to the injection of the standard.

FIGURE 1. Schematic of the HPLC-Synthesis-MS System and Image of the Synthesis Cell Used.

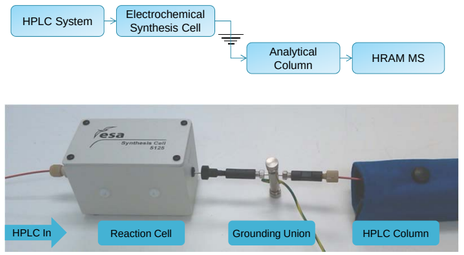
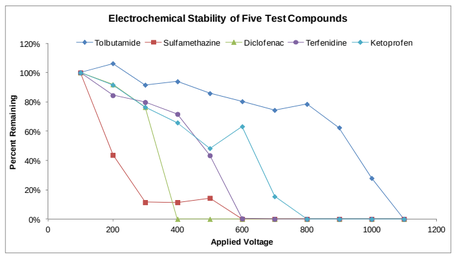


FIGURE 2. Stability Plot for Four Test Components.

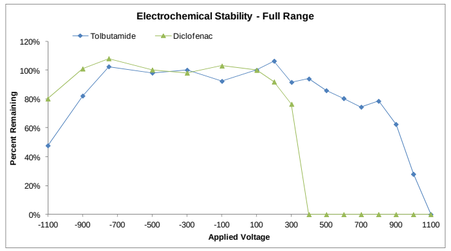


Potential Gradient Stability Profiles

The relative stability of compounds was determined graphically by comparing the detected area of the parent compound to the applied potential. A plot of the relative stability for the test compounds can be seen in Figure 2. Several compounds (sulfamethazine and diclofenac) displayed a strong potential / stability relationship. Tolbutamide was the most stable compound studied requiring potentials above +900 mV to achieve 50% depletion.

In the study of the potential stability, only positive voltages were applied leading to positive current values. To further study the effect of applied potential on the stability of the test compounds, we studied two compounds over a full range of positive and negative potentials. Tolbutamide and diclofenac were injected with potentials from -1100 to +1100 mV to provide an overall range from positive to negative potentials. For both compounds, instability was observed at negative potentials however both compounds were significantly more stable than under positive potentials. Interestingly, while diclofenac displayed the greatest sensitivity to positive potentials, tolbutamide was more unstable under negative voltages.

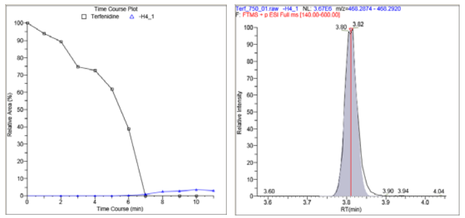
FIGURE 3. Full Positive / Negative Potential Range Stability.



Detection of Redox Synthetic Products

Electrochemical reaction of the test compound produced various redox products which were detected in the samples as the applied potential was increased. Detection of components was facilitated by the application of a relative quan/qual screening software, MetQuest™, which automatically searches for components in metabolic stability samples based on known Phase I and Phase II biotransformation products. In this application we used the Phase I biotransformations as our search criteria. The detected components were automatically integrated and areas were used to create a relative formation vs applied potential plot (Figure 4)

FIGURE 4. Automatic Detection and Integration of Terfenidine Products



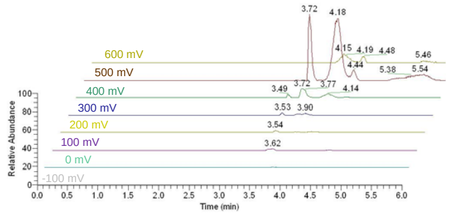
Appearance / Dissapearance Plot for Terfenidine and one Observed Product

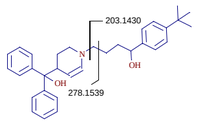
Automatically integrated peak for product at +750 mV

Synthetic Product Detection and Identification

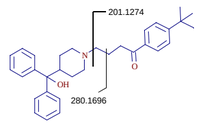
In addition to the automated detection of potential synthetic products described, a fragmentation-based approach was also used to detect more of these non-enzymatic products than a typical expected metabolite approach may. Briefly, the fragmentation observed for each drug was used to search through the fragmentation data for related products. This approach was complementary to the acquisition which used multiple known fragments for the parent drug to trigger precursor ion selected MS^2 scans based on the neutral loss values observed between every pair of full scan and all ion fragmentation scan collected throughout the entire run. This structure based acquisition and processing enabled the triggering of precursor selected MS^2 scans of minor synthetic products without the need for prior knowledge of what the electrochemical products might be. These precursor ion selected MS^2 scans were used to determine the structure of synthesized products. Figure 5 shows the voltage dependant formation of two terfenidine dehydrogenation products (t_R 3.7 and 4.2 min) across the potential range of -100 to +600 mV. In addition, the proposed structures for the two compounds are also shown.

FIGURE 5. Relative Formation and Structures of Dehydrogenation Terfenidine Products



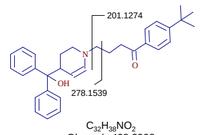


$C_{32}H_{38}NO_2$
Obs. m/z 470.3063
(+1.9 ppm)



$C_{32}H_{40}NO_2$
Obs. m/z 470.3057
(+0.6 ppm)

In addition to the two dehydrogenation products, a minor product was detected through the automated processing software. Despite the low level, the analytical approach used for acquisition triggered a precursor ion selected MS2 fragmentation scan which allowed for the assignment of a tentative structure. The product resulted from a combination of both dehydrogenations observed individually.

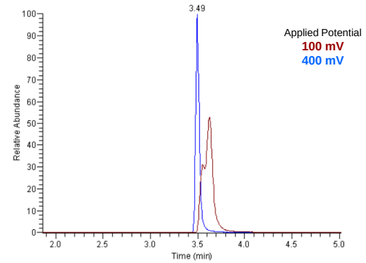


$C_{32}H_{38}NO_2$
Obs. m/z 468.2908
(+2.3 ppm)

Effect of Applied Voltage on Chromatographic Analyis

An unintended result of exposure to online electrochemical synthesis was noted for some of the test compounds. As voltage was varied from negative to positive through zero potential, the chromatographic peak shape for some compounds improved. As an example, the peak shape for terfenedine increased sharply as the voltage was increased from 100 mV to 400 mV. Although the absolute area of terfenedine had decreased by 28%, the intensity doubled due to a significant narrowing of the peak width.

Figure 6. Effect of Increasing Potential on Chromatography - Terfenedine.



Conclusion

We have demonstrated the use of an online electrochemical synthesis HRAM LC-MS/MS system to generate redox products from multiple compounds, separate the resulting products chromatographically, measure the relative stability/formation, and determine the structure of major products.

- Chromatographic separation of post synthesis cell products with HRAM MS analysis provided a rapid method for redox stability of compounds.
- Exposure to online electrochemical synthesis generated a number of identifiable products from the compounds studied, many of which were analogous to enzymatic products.
- Electrochemical synthesis provided a means to measure the relative redox stability potential of multiple compounds.
- Online electrochemical analysis effected the chromatographic behavior of some compounds in a voltage dependant manner.