

Eliminating Hydrolysis for Pain Management Monitoring of

Benzodiazepines by LC/MSMS

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ABSTRACT

Laboratories are under constant pressure to reduce costs per sample and increase productivity. The hydrolysis step to convert drug metabolites back to parent and free metabolite compounds is the most time consuming and costly step in the process. Developing an LC/MS/MS assay that is able to detect both parent and metabolite(s) free and conjugated in urine would have several advantages. First, it would remove many of the associated costs and problems with hydrolysis. Second, it would give a better picture of the patient's metabolic process, which combined with some of the new personalized medicine techniques, may allow doctors to modify their treatment plan to achieve better outcomes.

However there are still many challenges, the first being that we may not know all of the potential metabolites that are present in urine, making it difficult to obtain analytical reference standards to properly set up the method. The metabolites are also quite polar making them hard to chromatograph using the same analytical conditions as the parent drugs. Finally, the interpretation of the results by both the pathologist as well as the patient's physician must be considered.

Initial attempts to develop a direct analysis of benzodiazepines showed very promising results with standards and QC samples. As validation proceeded, patient samples compared using the old hydrolysis procedure and the new direct analysis showed discrepancies for some benzodiazepine drugs, e.g. alprazolam. Repeated experiments also lead to the conclusion that there were additional metabolites that were not commercially available as reference standards.

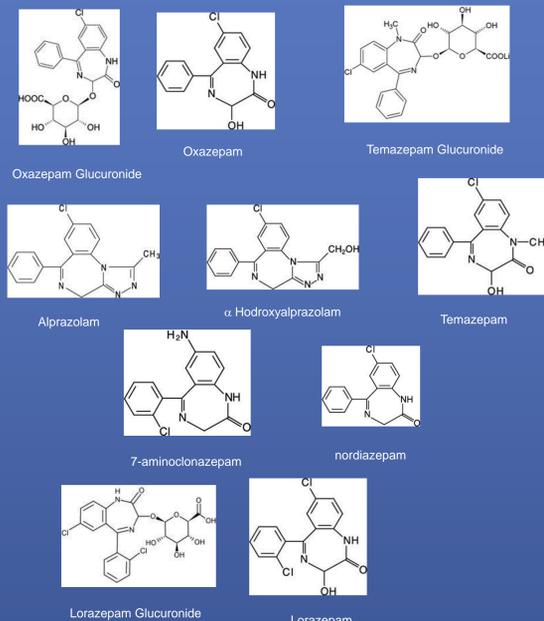
In this poster we will discuss our on-going efforts to eliminate hydrolysis for LC/MS/MS confirmations and the challenges and success we have had for benzodiazepines.

INTRODUCTION

- Benzodiazepines are sedative hypnotics used to treat depression. They are highly abused and include a wide variety of drugs such as diazepam and chlordiazepoxide. Many of the benzodiazepines metabolize to nordiazepam and oxazepam. These drugs and metabolites undergo phase II biotransformation and form glucuronide conjugates (Figure 1.)¹.
- Analysis of these molecules generally consists of enzyme hydrolysis of the glucuronides as well as other metabolites back to the unbound parent drugs. This adds significant time and cost to the analysis of the benzodiazepines and can add significant variability^{2,3}.
- Separation of the more polar glucuronide conjugates from each other and from the more hydrophobic parent molecules presents a nontrivial challenge in routine analysis⁴.
- The work reported herein uses a Kinetix C18 core shell HPLC column to resolve glucuronides and parent drugs from each other followed by MSMS analysis of each compound.
- The method is relatively fast with excellent separation of the analytes in question.

INTRODUCTION

• Figure 1. Molecules of interest to this study are shown below



MATERIALS AND METHODS

- The HPLC method consists of a gradient from 85% A (90:10 20mM Ammonium Formate in water:Methanol)/15% B (100% Methanol) to 100% B over 1.17 minutes at a flow rate of 0.5mL/min.
- Study conducted on TSQ Quantum Access Triple Stage Quadrupole mass spectrometer by Thermo Fisher Scientific operating in positive ESI mode.
- Two transitions were selected for each Benzodiazepine, Benzodiazepine conjugate and deuterated internal standard to be monitored following optimization via direct infusion of each compound on the Quantum Access.
- Urine samples (225µL) were diluted 3X with 0.1% formic acid in water:Methanol (75:25) and centrifuged.
- Prepared samples were injected (50µL) on to a Phenomenex Kinetex 2.6µ C18 100A 50 x 3.00mm analytical column with a Phenomenex SecurityGuard ULTRA C18 (3.0mm ID) guard cartridge in place. Analysis occurred with Agilent 1200 series HPLC pump and Quantum Access Max Triple Quadrupole Mass Spectrometer.
- Method was validated using previously analyzed positive patient urine samples from existing 4.5 minute LC-MS/MS method. Comparisons were made to demonstrate abundance of glucuronide-metabolites in non-hydrolysed patient samples.

RESULTS

Figure 2 shows chromatography of Benzodiazepines and metabolites at a level of 250 ng/mL. These data were acquired on a fully multiplexed system such that the data window is less than 2 min.

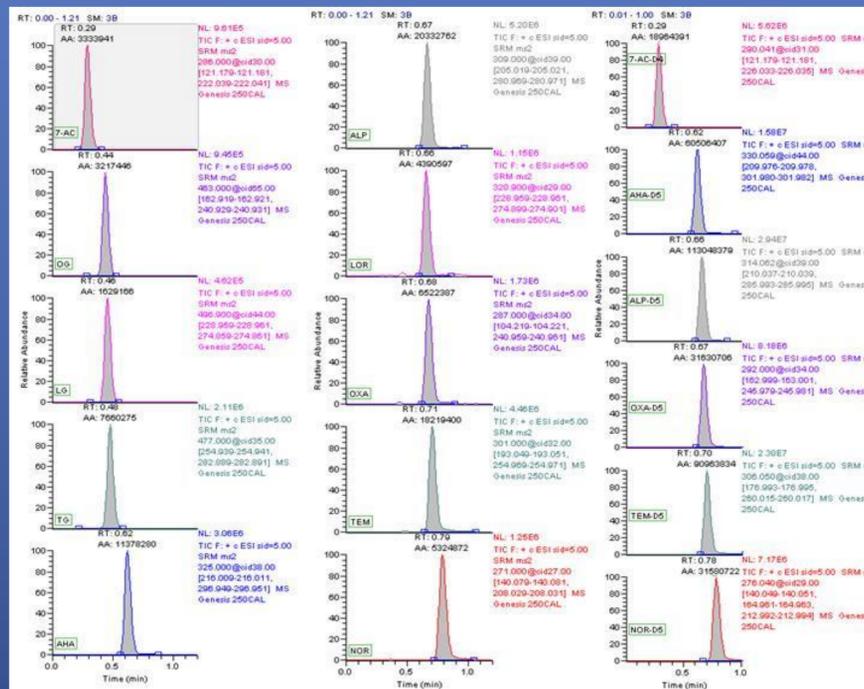


Figure 3. Representative calibration curves for Oxazepam Glucuronide and Temazepam

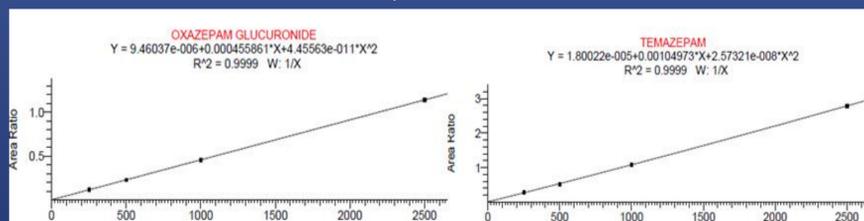


Table 1. Representative Patient Data (ng/mL)

Sample #	Oxazepam	Oxazepam Glucuronide	Temazepam	Temazepam Glucuronide	Lorazepam	Lorazepam Glucuronide
53					20	1304
511	0	190	0	137	11	1990
772					8	714
578	0	158	73	4014	31	521
232	27	4988	82	617		
74	3	709	37	1654		
974	201	5463	539	5122		
767					11	447
571	0	140	0	15	63	0
653	20	649	16	877		
870	16	3217	87	4218		
499	6	1026	10	1852		
2					15	1167
261	10	1975	19	2198		

RESULTS

- Using the Thermo TLX-4 systems, the following parameters were achieved:
LOD = 50 ng/mL
LOQ = 75 ng/mL
ULOL = 5,000 ng/mL
Carryover Limit = 10,000 ng/mL
- Linearity was >0.99 in all cases for quadratic responses over the concentration ranges of interest.
- Matrix effects were generally not significant with the exception of Temazepam glucuronide where sample presented in Normal Human Urine was 35% greater in response than when presented in mobile phase.
- Patient samples exhibited higher levels of glucuronide conjugates than parent drugs as expected (see Table 1).

DISCUSSION

- Analysis of Benzodiazepines has traditionally required enzyme hydrolysis which is documented to have varying levels of efficiency as the compounds change^{2,3}. This method avoids this contribution to variability and yet is fast and selective.
- Glucuronide levels ranged from 0 to over 7,000 ng/mL*.
- In every case where glucuronide is reported, the parent drug is always less than 10% of that level and is often less than LOQ which begs the question of why analyze for the parent drug?
- A more difficult issue is ensuring that all (significant) metabolites are accounted for in the analytical method. For example, α-hydroxyalprazolam glucuronide was not commercially available and thus not accounted for in this method.
- As expected, Temazepam and Oxazepam glucuronides are present together

CONCLUSION

- Analysis of Benzodiazepines and their corresponding glucuronide conjugates is possible in a single method.
- Separation of these compounds can be fast and selective
- Separation is effective using HPLC conditions
- The time and cost of enzyme hydrolysis not to mention the added variability resulting from enzyme hydrolysis can be avoided.

REFERENCES

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* Sample diluted 2x for additional analysis