Analysis of Propofol (2,6-Diisopropylphenol) and its Metabolites in One Injection using the DuoSpray™ Ion Source

AB SCIEX QTRAP® 4500 LC-MS/MS System and Gerstel, Inc. MultiPurpose Sampler

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Introduction

Propofol (2,6-diisopropylphenol) is a short acting anesthetic drug used most commonly for both the induction and maintenance of general anesthesia. There is an importance for the analysis of propofol in the forensic toxicology setting especially with increased reports of abuse, accidental overdose, suicide and criminal purpose.[1] Propofol rapidly metabolizes in the liver to hydrophilic and inactive compounds, which requires monitoring of both the parent drug and its metabolites. While GC/MS is traditionally used to analyze for the parent drug, it cannot be used to detect the thermally labile metabolites (e.g., glucuronides). Alternatively, an alternative LC-MS/MS approach can monitor both the parent drug and metabolites in a single experiment, providing both financial and time savings. A number of references have described developed LC-MS/MS methods for the analysis of propofol,[2-4] some methods include the analysis of the metabolites as well.[5-7]

This work focused on developing an LC-MS/MS method for highly selective and sensitive quantification of Phase II metabolites (propofol glucuronide, propofol sulphate), Phase I metabolites (4-hydroxypropofol, propofol quinone) and parent drug (propofol) in one injection. To accomplish this, we utilizied the triple quadrupole technology of the AB SCIEX QTRAP® 4500 LC/MS/MS system operated in Multiple Reaction Monitoring (MRM) mode. In addition to the quantitative aspects of these analyses, an interesting behavior of ion formation (radical anion) for one of the propofol metabolites was observed and will be described.

The final developed method utilized the GERSTEL Multi-Purpose Sampler (MPS) 2 XL autosampler equipped with a solid phase extraction (SPE) module to allow automated sample cleanup of human fluid samples and direct injection into the LC that is coupled to an AB SCIEX QTRAP® 4500 LC/MS/MS System for quantification of propofol and its metabolites.

Figure 1. GERSTEL MPS 2XL multi-purpose sampler configured for On-Line SPE LC-MS/MS with an AB SCIEX QTRAP® 4500 LC-MS/MS system for automated sample clean up and LC-MS/MS analysis.

Experimental

Materials.

Standard solutions of propofol (1 mg/mL in methanol; Product No. P-076,), d-17 propofol (100 µg/mL,Product No. P-077), and propofol β-D glucuronide (100 µg/mL, Product No. P-82) were purchased from Cerilliant Corporation (Round Rock, TX,). 4-Hydroxypropofol (1 mg, Cat. # H950770,) was procured from Toronto Research Chemicals (Toronto, ON).

Blank serum was spiked with drug mixture stock solutions making concentrations ranging from 5 to 2000 ng/mL to prepare the calibrators; similar solutions with drugs and metaboltes present at 10 and 1000 ng/mL were used as QCs.

Samples were obtained from a local hospital that had also been analyzed by GC/MS. These samples were processed in the same way as the calibrators and QCs and analyzed by the developed LC-MS/MS method.

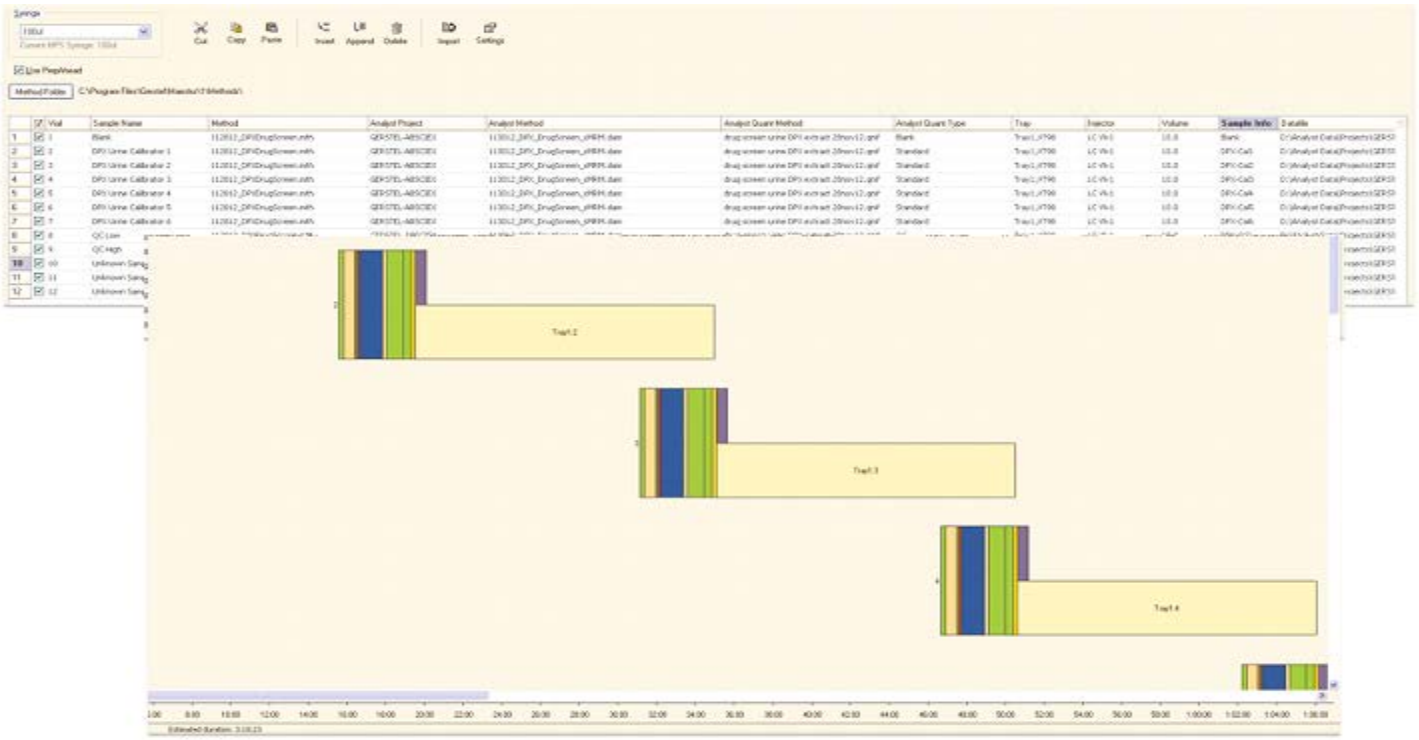

Figure 2. Maestro Sequence Scheduler with Prep Ahead coupled to Analyst® Software MS acquisition software.

Instrumentation

All automated SPE and injections were performed using a dual-head MPS 2XL multi-purpose sampler with the GERSTEL SPE Option as shown in Figure 3. Automated sample preparation utilized Phenomenex Strata-X strong cation exchange cartridges modified to be accommodated by the SPE module. All analyses were performed using an Agilent 1260 HPLC pump with a 2.1 x 50 mm Inertsil Ph-3, 2-µm, phenyl column (GL Sciences, Tokyo, Japan) and an AB SCIEX QTRAP® 4500 LC-MS/MS System.

Sample pretreatment

The automated SPE sample cleanup was programmed using Maestro software coupled to Analyst® 1.6.1 Software. Enabling the Prep Ahead functionality in Maestro allowed high-throughput "just in time" sample preparation for analysis (Figure 3). The automated SPE-LC-MS/MS serum cleanup and analysis method included the following steps:

Figure 3. GERSTEL Multi-Purpose Sampler (MPS) 2XL - equipped with an SPE module

Automated SPE Prep Sequence:

- Addition of 500 µL 0.05% NH_4OH to elution vial
- 1st Conditioning: MeOH
- 2nd Conditioning: H_2O
- Added 500 µL of the sample to the SPE cartridge
- Wash: 95:5 H_20/MeOH (2% NH_4OH)
- 1st Elution: 500 µL AcN/MeOH (75:25)
- 2nd Elution: 500 µL AcN/MeOH (75:25 2%FA)
- Mixed the sample in the elution vial for 10 seconds

LC Conditions:

LC column: 2.1 x 50 mm Inertsil Ph-3, 2-µm, phenyl column, GL Sciences, Tokyo, Japan.

Mobile Phase A: 95% water + 5% acetonitrile + 1 mM ammonium acetate

Mobile Phase B: 95% acetonitrile + 5 % water + 1 mM ammonium acetate

Time (min)	Flow (mL/min)	%B
0.00	0.4	10
1.00	0.4	10
5.00	0.4	100
6.00	0.4	100
6.10	0.4	10
7.50	0.4	10

Injection volume: 50 µL
Column temperature: 40 °C

MS Conditions

AB SCIEX QTRAP® 4500 LC/MS/MS system

Operation: DuoSpray™ Ion Source Negative mode
Temperature: 450 °C
Ion Source Gas 1: 40
Nebulizer Current: -3
Curtain Gas: 25
CAD: High

The effluent from the Inertsil Ph-3, 2-µm, 2.1 x 50 mm column was sent to Port 4 of a Valco injection/diverter valve via 1/16" OD, 0.005" red PEEK tubing. This valve is a standard integrated part of the QTRAP® 4000 system. Port 3 was connected to the TurboIonSpray® probe, and Port 5 was connected to the Heated Nebulizer Probe via red PEEK tubing (as short as possible). The MRM experiment consisted of 2 periods. The first period involves the use of TurboIonSpray®; electrospray ionization (ESI) probe, and the second period was dedicated to Heated Nebulizer. During the method acquisition, the valve was switched to allow effluent from the LC column to flow through the TurboIonSpray® probe of the DuoSpray™ ionization source from 0 to 3.5 minutes of the LC-MS/MS method. The valve was then programmed to switch at 3.5 minutes to allow the LC effluent to run through the heated nebulizer probe of the DuoSpray™ ionization source for the rest of the LC gradient.

MRM Transitions and Ion Source Conditions

Compound	Q1	Q3	Ionization
Propofol Sulfate 1	257	*80*	*TurboIonSpray® Probe*
Propofol Sulfate 2	*257*	*227*	*TurboIonSpray® Probe*
Propofol Glucuronide 1	*353*	*113*	*TurboIonSpray® Probe*
Propofol Glucuronide 2	*353*	*177*	*TurboIonSpray® Probe*
Propofol 1	*177*	*145*	*Heated Nebulizer*
Propofol 2	*177*	*161*	*Heated Nebulizer*
4-Hydroxypropofol 1	*192*	*134*	*Heated Nebulizer*
4-Hydroxypropofol 2	*192*	*177*	*Heated Nebulizer*
2,6-Diisopropyl-phenyl isopropyl ether 1	*219*	*177*	*Heated Nebulizer*
2,6-Diisopropyl-phenyl isopropyl ether 2	*219*	*191*	*Heated Nebulizer*
3,3',5,5'-Tetraiso-propyldiphenol 1	*352*	*307*	*Heated Nebulizer*
3,3',5,5'-Tetraiso-propyldiphenol 2	*352*	*337*	*Heated Nebulizer*
d-17 Propofol 1	*194*	*154*	*Heated Nebulizer*
d-17 Propofol 2	*194*	*174*	*Heated Nebulizer*

Results and Discussion

In the development of the LC-MS/MS method, the mass spectrometric conditions of the parent propofol drug were first investigated. The aim of these experiments was to identify which ionization mode – Electrospray Ionization (ESI) or Atmospheric Pressure Chemical Ionization (APCI) - gives the best sensitivity for the compound. After direct infusion of a propofol standard with electrospray analysis in both positive and negative mode, the Q1 scans (Figure 4) showed little activity in positive mode for protonated propofol at *m/z* 179 or in negative mode for the de-protonated propofol at *m/z* 177.

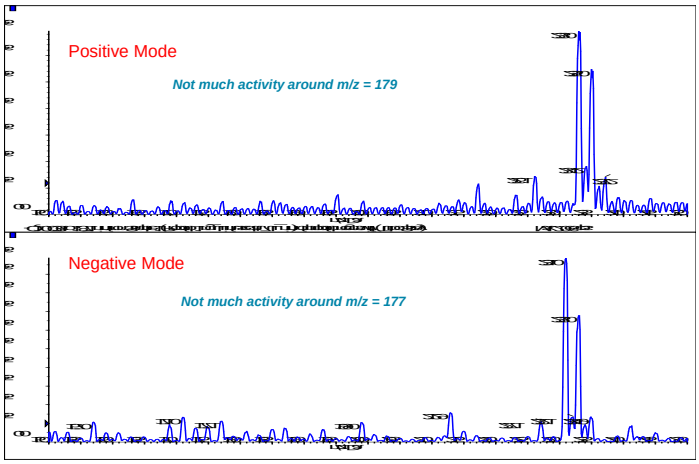

Figure 4. Positive (top) and negative (bottom) ion mode *TurbolonSpray*® Q1 spectra of propofol: 1 ng/µL solution infused

In APCI mode using the Heated Nebulizer, the expected ions were present in the Q1 scans for both positive and negative ion mode, with the negative ion mode producing the higher and cleaner signal (Figure 5).

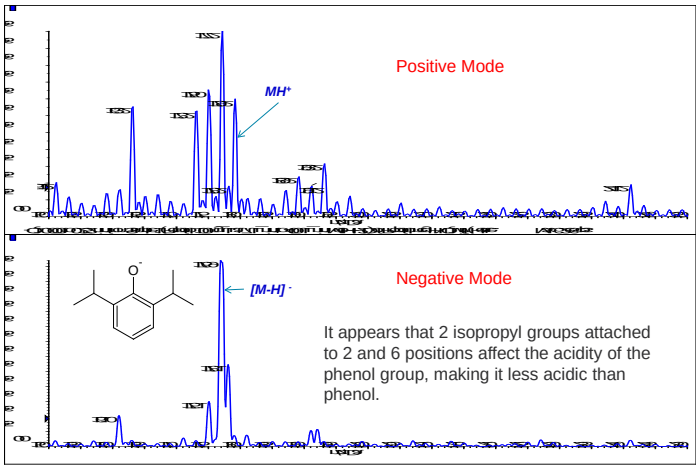

Figure 5. Positive (top) and negative ion mode Heated Nebulizer Q1 spectra of Propofol: 100 ng/µL x 7 µL/min Propofol added to 0.4 mL/min LC flow

Product ion experiments were performed to investigate the fragmentation of deprotonated propofol, to aid in the elucidation of metabolites (Figure 6). For example, protonated propofol (Figure 6, Top spectrum) fragments to lose the isopropyl side chains. If the hydroxylated metabolite of propofol forms similar fragments that are 16 Da greater than isopropyl group loss, then it can be postulated that metabolism occurred on the side chain. Such experiments were performed both in positive and negative modes to give different permutations to help derive the structures and also determine the MRM transitions to use in the final method.

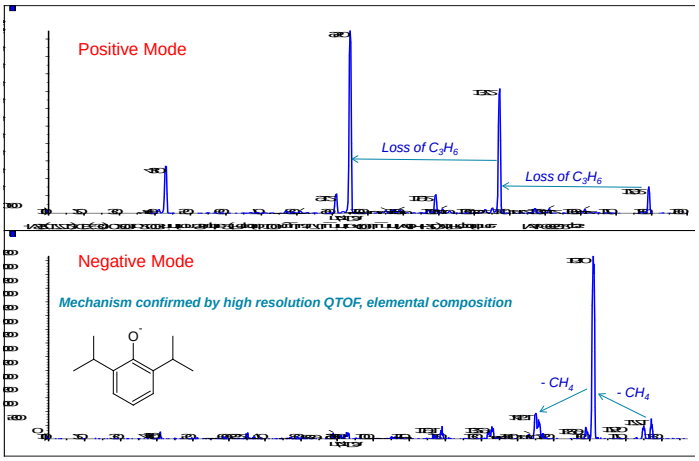

Figure 6. Heated Nebulizer Collision Induced Dissociation (CID) tandem mass spectrum of Propofol: positive (top) and negative (bottom) ion mode; 100 ng/µL x 7 µL added to 0.4 mL/min of LC flow

The mechanisms taking place in the negative ion mode MS/MS experiments of propofol were confirmed by the high resolution accurate mass of the fragment ions obtained by the use of the AB SCIEX TripleTOF® 5600+ LC-MS/MS system in TOF-MS/MS mode. Figure 7 shows the formula of the fragment ion at *m/z* 161.097 with the molecular formula being calculated as $C_{11}H_{13}O$ which is consistent with the loss of CH_4.

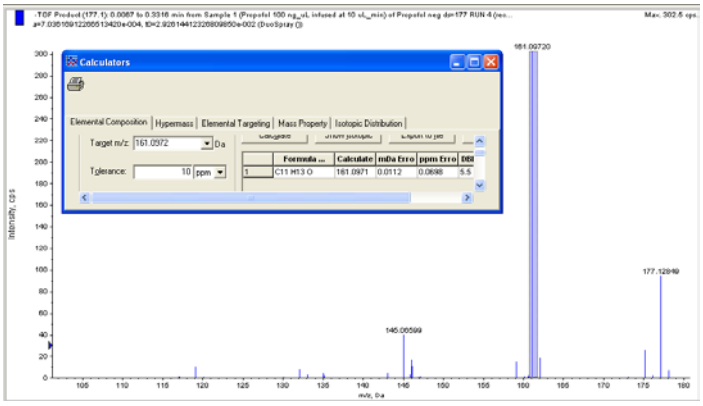

Figure 7. Heated Nebulizer; Negative ion mode CID tandem TripleTOF® mass spectrum of Propofol. Propofol fragment ion at *m/z* 161.09720 is due to loss of CH_4, as opposed to loss of O which would produce 328.7 ppm error.

Figure 8 shows the fragment ion at *m/z* 145.065 as having a formula of $C_{10}H_9O$ consistent with a further loss of a CH_4.

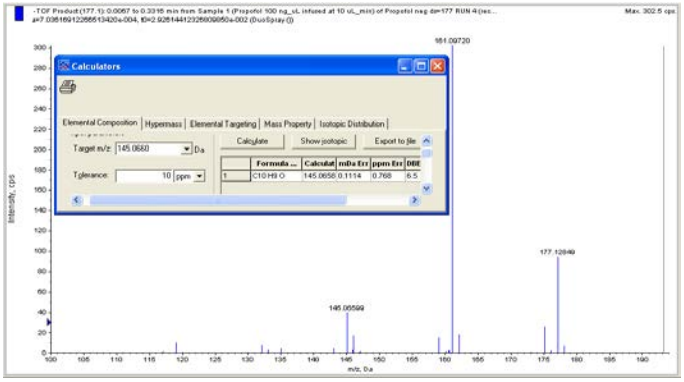

Figure 8. Heated Nebulizer; Negative ion mode CID tandem TripleTOF® mass spectrum of Propofol. Propofol fragment ion at *m/z* 145.06599 is due to loss of 2 x CH_4

Investigation into the mass spectrometric conditions of 4-hydroxypropofol was also performed A negative mode infusion in Q1 produced an intense ion at *m/z* 192. From the product ion experiment performed on this precursor ion (Figure 9) it was proposed that the 4-hydroxypropofol undergoes re-arrangement or in-source fragmentation to lose 2 hydrogen atoms, forming a stable 2,6-diisopropyl benzoquinone radical anion.

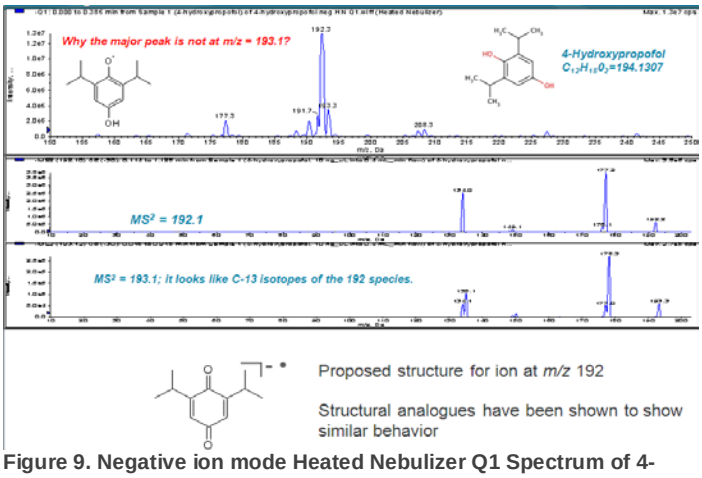

Figure 9. Negative ion mode Heated Nebulizer Q1 Spectrum of 4-Hydroxypropofol (top) and Negative ion mode Heated Nebulizer Collision Dissociation tandem mass spectrum of ions at *m/z* 192 (middle) and *m/z* 193 (bottom).

Phase II metabolites are easily detected in the negative ion mode using a nebulizer-assisted electrospray whereas the less polar parent molecule and 4-hydroxy propofol are better ionized using the negative ion mode APCI. Therefore, for the final method a source capable of providing both ionization methods in one analytical run was used - DuoSpray™ Ion Source (Figure 10).

Figure 10. DuoSpray Ion Source. Use of a source capable of providing both electrospray and heated nebulizer ionizations in one analytical run.

During the LC method development, a number of columns were investigated; the Inertsil Phenyl column from GL Sciences was found to provide the best separation of the compounds. The use of either acetonitrile or methanol as mobile phase constituents was also investigated, and the use of methanol was demonstrated to retain polar Phase II metabolites much better than acetonitrile.

Sample preparation consisted of an automated preparation in which the Phenomenex Strata-X Strong Anion Exchange Solid Phase Extraction cartridges were modified to be accommodated by the Gerstel Multi-Purpose Sampler (MPS) 2XL. The automated sample clean-up procedure included firstly adding 0.05% NH_4OH to the elution vial to aid in propofol mass spectrometric detection.

The LC-MS/MS conditions on the AB SCIEX QTRAP® 4500 LC-MS/MS system involved the use of the DuoSpray™ Ion Source. A built-in diverter valve allowed switching between directing LC eluent to either the TurboIonSpray® probe or the Heated Nebulizer Probe. For the first 3.5 minutes of the method the LC eluent was directed to the TurboIonSpray® probe after which the valve was switched to allow the LC effluent to run through the Heated Nebulizer probe of the DuoSpray™ ionization source for the rest of the LC gradient. The MS method consisted of 2 periods; the first monitored the elution of the propofol glucuronide and propofol sulfate in electrospray ionization mode up until 3.5 minutes. The second period, from 3.5 to 7.5 minutes, monitored the parent and Phase I metabolites in Heated Nebulizer ionization mode.

Figure 11. Propofol Calibration Curve - Spiked Serum

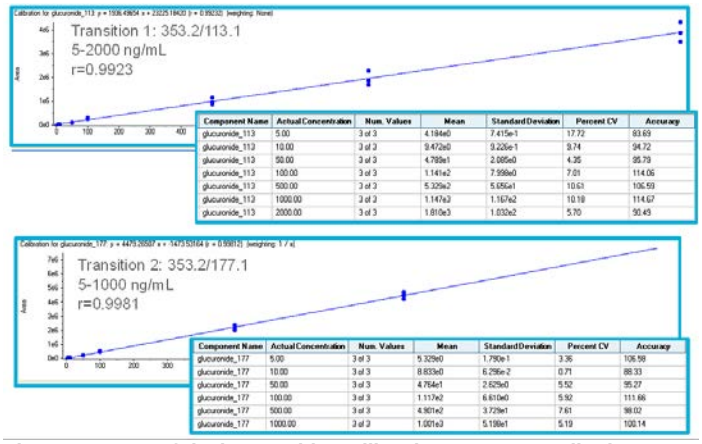

Figure 12. Propofol Glucuronide Calibration Curves - Spiked Serum

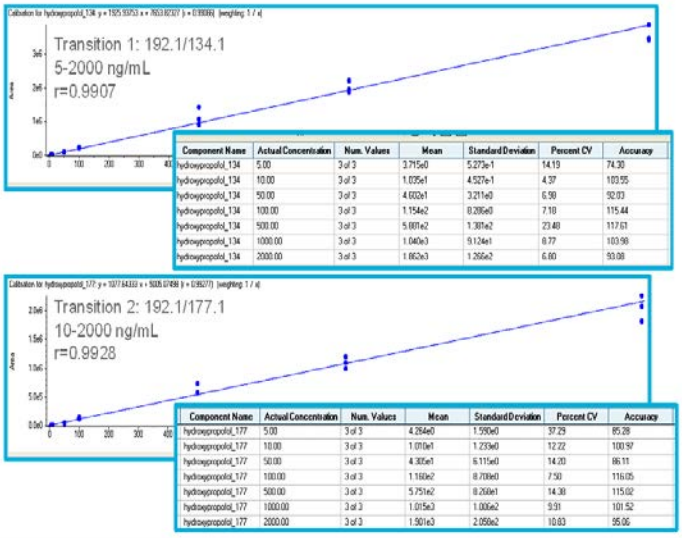

Figure 13. 4-Hydroxpropofol Calibration Curves - Spiked Serum

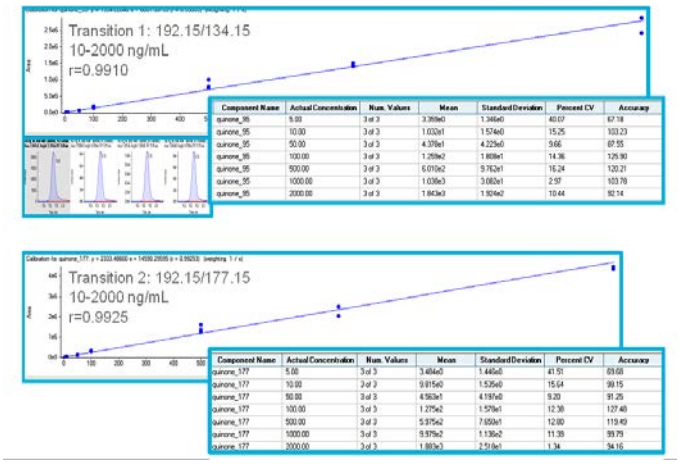

Figure 14. Propofol Quinone Calibration Curves - Spiked Serum

Figures 11 through 14 show the linearity for propofol and metabolites. Linearity was achieved between 5-2000 ng/mL for most analytes. Recoveries and %RSDs reported to be greater than 85% and less than 15%, respectively, in most cases.

A comparison of the LC-MS/MS method with a GC/MS method for propofol analysis showed good correlation (Figures 15 and 16). However, the LC-MS/MS method had the added benefit of being able to determine propofol glucuronide concentration from the same experiment. Figure 16 shows the excellent correlation between the LC-MS/MS and GC/MS experiments, with near equivalent amounts of propofol measured in each individual sample (on average within ~17%).

Sample	Propofol calculated concentration (ng/mL)		Propofol glucuronide calculated concentration (ng/mL)
	GC/MS	LC-MS/MS	LC-MS/MS
1	640	883.4	4062
2	800	933.8	8037
3	480	436.4	5348
4	230	362.2	7307
5	420	495.5	5098
6	200	189.7	5742
7	850	916.0	3068
8	220	293.0	3101
9	450	487.0	3315

Figure 15. LC-MS/MS Analysis Provides Propofol and Propofol Glucuronide Concentration Determinations from Serum Samples from the Same Injection

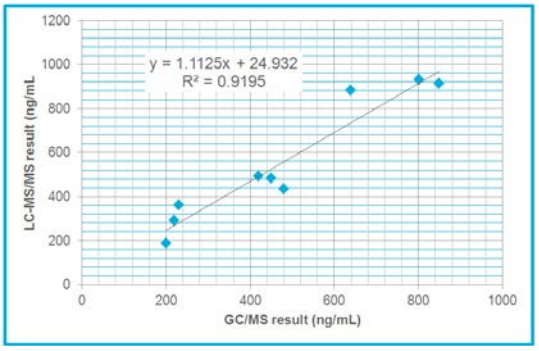

Figure 16. Analysis of Propofol: GC/MS cross validation. 9 real serum samples have been tested with this method to compare LC-MS/MS with a GC/MS method. Slope of 1.1 and correlation coefficient is 0.9195.

Matrix effects were evaluated at 1000 ng/mL using one lot of serum. % accuracy differences were 5.4 and 7.0 % for propofol and propofol glucuronide respectively and recoveries were determined to be greater than 85 % for all compounds.

Conclusions

- A forensic LC-MS/MS method has been developed for the analysis of Propofol and its metabolites.
- Propofol glucuronide and sulphate are ionized much better with TurboIonSpray® than with Heated Nebulizer
- Heater nebulizer provides better sensitivity for propofol, 4-hydroxypropofol and propofol dimer than the standard TurboIonSpray® source; propofol specifically by a factor of ~10 times.
- For the final method a source capable of providing both ionizations in one analytical run was utilized; the DuoSpray™ Ion Source.
- Automated On-Line SPE cleanup of samples was performed using Strata-X SPE cartridges, modified to enable automated extraction of serum samples on the Gerstel dual-head MPS 2XL multi-purpose sampler.
- The Gerstel dual-head MPS 2XL multi-purpose sampler serves as both the sample clean up module as well as the LC autosampler allowing direct injection of the cleaned up sample on to the LC column.
- Phenyl columns provide efficient separation of the polar Phase I and II metabolites and the parent drug.
- Linearity was achieved between 5-2000 ng/mL for most analytes. Recoveries and %RSDs reported to be greater than 85% and less than 15%, respectively, in most cases.
- A good correlation to GC/MS results was shown for the propofol concentrations but with the added benefit of being able to determine metabolite concentrations from the same LC-MS/MS injection.

Acknowledgements

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