Identification of Buprenorphine and Norbuprenorphine in Postmortem Urine and Liver by Orthogonal-Axis Time-of-Flight

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ABSTRACT

In this study, confirmation of Buprenorphine (BUP), a semisynthetic thebaine opioid derivative and its metabolite Norbuprenorphine (NBUP) were done on 5 postmortem urine and 3 postmortem liver case samples using orthogonal-axis TOF analysis. The unique design of the oa-TOF holds the energy values and the ion travel distance constant, allowing accurate measurement of flight time, thus producing an accurate ion mass value. In contrast to other scanning mass analyzers, all ions are simultaneously detected by the oa-TOF, providing high time resolution and further improving ion sensitivity for exact mass. Identification of BUP and NBUP within case samples was done ultilizing LC mobile phases 0.5% formic acid/H_2O and methanol while simultaneously infusing a leucine-enkephlin lockmass, 556.2771 m/z, by ESI. Samples were compared to precursor ions BUP, 468.3114 m/z, and NBUP, 414.2664 m/z, by overall retention time (RT) of the BUP/NBUP standards and part-per-million (ppm) error calculated by MassLynx®. Due to low therapeutic levels of BUP, high resolution and sensitivity of the oa-TOF detected postmortem case urine when set at a maximum error threshold of 8 ppm. BUP was identified in all 5 postmortem case urine samples, ranging from 2.1 to 7.5 ppm, with NBUP detected in 3 of the 5 samples from 0.7 to 8.9 ppm. BUP and NBUP were not identified within the 3 postmortem liver cases.

INTRODUCTION

- Identification of Buprenorphine (BUP) and its metabolite Norbuprenorphine (NBUP) was done on 5 postmortem urine and 3 postmortem liver case samples using oa-TOF analysis.
- BUP is a semisynthetic thebaine opioid derivative, closely related to morphine. In phase 2 metabolism, buprenorphine undergoes N-dealkylation in the liver, forming NBUP (Figure 2).
- Therapeutic BUP concentrations have typically been found in blood to be 0.3-5 ng/ml while fatal levels have been found from 1.1-29ng/ml.
- In contrast to GC/MS, the oa-TOF is capable of detecting much lower analyte concentrations, increasing the number of analytes identified within a single sample.

METHODS

Solid Phase Extraction

- Urine and liver samples were prepared for SPE by adding 1ml of each sample to a clean 15 ml centrifuge tube.
- 200µl of 20% Na-$_2CO_3$ was added to each tube and vortexed for 60 s.
- 10 ml of n-butyl chloride was added to each sample and rocked for 20 min.
- Centrifugation was performed at 3,000 rpm for 10 min.
- The organic layer was dried under nitrogen for 10 minutes at 45°C.
- Samples were reconstituted in 100µl mixture of 80% A (0.1% formic acid/H_2O) and 20% B (methanol)

Liquid Chromatography (Table 1)

- Chromatic separation was achieved by 5µl sample injection onto a Waters Acquity UPLC® HSS T3 (2.1 × 50 mm, 1.8 µm) column (Milford, MA, USA).
- A gradient elution was performed with mobile phase A and B. Initial flow conditions were set at 20% B and 80% A at 450 µl for 3 min.
- After 3min, B was increased to 75% for 6s and then increased to 100% for 1 min at a continuous flow of 450 µl/min.
- At 4 min, A and B mobile phases were returned to initial conditions for 6s, and the column was allowed to equilibrate for 1 min.
- The total run time was 5 min.

Mass Spectrometry (Table 2)

- Mass spectrometric data was collected using a Waters LCT XE™ oa-TOF Mass Spectrometer (Manchester, UK), equipped with a Z-spray™ electrospray source and a lockmass sprayer, operating in positive ion mode.
- The source temperature was set at 150 °C with a cone gas flow of 30 L/h, a desolvation gas temperature of 350 °C, and a nebulization gas flow of 800 L/h.
- The capillary voltage was set at 2.252 kV and the cone voltage to 60 V.
- All analyses were acquired using the lockspray to ensure accuracy and reproducibility; leucine-enkephalin was used as the lock mass (m/z 556.2771 or 554.2615) at a concentration of 0.5ng/mL and flow rate of 5 µL/min.
- Data were collected in centroid mode, the lockspray frequency was set at 5 s, and data were averaged over 10 scans.
- The mass spectrometric data were collected in full-scan mode from m/z 100–1000 from 0–5 min in positive ion mode.

MATERIALS

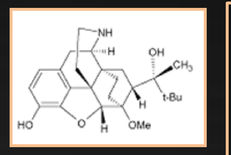
Instrumentation

- LCMS analysis performed on a Waters LCT XE™ oa-TOF Mass Spectrometer (Manchester, UK); equipped with a Z-spray™ electrospray source and a lockmass sprayer, interfaced with a Waters ACQUITY UPLC System™ autosampler and LC pump (Milford, MA, USA).
- LCMS data analysis performed using Waters MassLynx® and Waters ChromaLynx® Software (Waters Corporation, Milford, MA, USA).

Reagents

- Buprenorphine (BUP) and Norbuprenorphine (NBUP) standards were obtained from Cerilliant (Austin, TX, USA).
- Reagent grade methanol, 88% formic acid, n-butyl chloride, isopropanol, sodium carbonate, and sodium hydroxide were from Sigma Chemicals (St. Louis, MO, USA). All solvents were of HPLC grade

Figure 1. Waters LCT XE™oa-TOF Mass Spectrometer



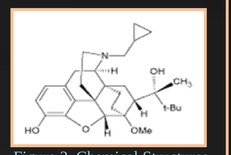


Figure 2. Chemical Structures BUP (top) and NBUP (bottom)

Table 1. TOF LC System and Gradient Method

UPLC	Waters ACQUITY UPLC™ System
Column	Waters Acquity UPLC™ HSS T3 (2.1X 50mm, 1.8um)
Injection Volume	5 µl
Mobile Phase A	0.5% Formic Acid/H_2O
Mobile Phase B	100% Methanol
Flow Rate	450µl/min
0-3 min	80% A 20% B
3-3.10 min	25% A 75% B
3.10-4.20 min	100% B
4.20-4.30 min	80% A 20% B
4.30-5min	80% A 20% B
Total Run Time	5 min

Table 2. TOF MS system and MS Method

Mass Spectrometer	Waters LCT XE™oa-TOF
Ionization	ESI Z-Spray™
Lock Mass	Leucine-enkephalin (m/z 556.2771)
Scan Range	100-1000 m/z
Scan Frequency	10 scans/5s
Desolv/Cone Gas	Nitrogen
Capillary Voltage	2.252 kV
Sample Cone	60 V
MS Inlet Temp	150°C
Desolv. Temp.	350°C

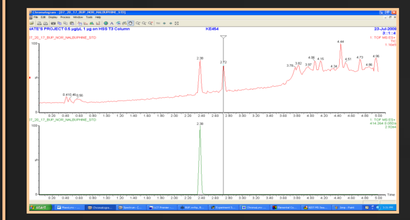


Figure 3. NBUP standard. TIC (top) of BUP and NBUP standard run. EIC (414.2664 m/z, corresponding to NBUP – bottom) with RT 2.38.

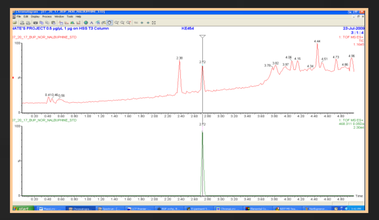


Figure 4. BUP standard. TIC (top) of BUP and NBUP standard run. EIC (468.3114 m/z, corresponding to BUP – bottom) with RT 2.72

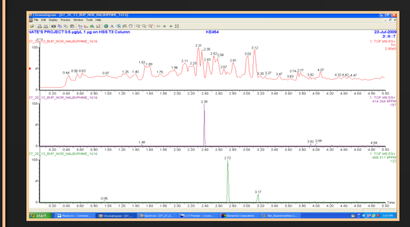


Figure 5. 09-1616 urine TIC (top). EIC (414.1988 m/z, corresponding to NBUP – middle). Identification error of NBUP was 3.6 ppm. EIC (468.3138 m/z, Corresponding to BUP – bottom). Identification error of 5.1 ppm.

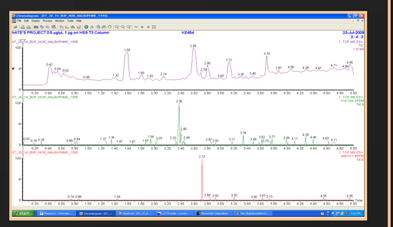


Figure 6. 08-1990 liver TIC (top). EIC (corresponding to NBUP – middle). RT peak at 2.39 is shown, however co-eluted with other analytes. An exact mass for this peak was N/A due to the high background noise. EIC (468.2336 m/z, corresponding to BUP – bottom). Upon further examination of elemental composition (not shown), a corresponding molecular formula of $C_{28}H_{42}N_3O_3$ was observed. At a ppm error identification threshold of 15, BUP could not be identified within this liver sample.

Table 3. Analysis Results for Urine and Liver Samples

	NBUP	BUP				
Monoisotopic Mass	414.2664	468.3114				
Elemental Formula	$C_{25}H_{35}NO_4$	$C_{29}H_{41}NO_4$				
Urine Sample	NBUP Observed Exact Mass	BUP Observed Exact Mass	NBUP RT	BUP RT	NBUP ppm Error	BUP ppm Error
Standard	414.2664	468.3114	2.38	2.72		
09-1616	414.1988	468.3138	2.39	2.72	3.6	5.1
08-1990	414.2607	468.3102	2.38	2.72	8.9	2.6
08-2167	N/A	468.3104	N/A	2.73	N/A	2.1
08-3663	N/A	468.3149	N/A	2.72	N/A	7.5
08-3814	414.2647	468.3136	2.39	2.73	0.7	4.7
Average	414.2414	468.3126	2.39	2.72	4.4	4.4
Liver Sample						
08-1990	N/A	468.2336 $C_{28}H_{42}N_3O_3$*	N/A	2.72	N/A	0.0*
08-3663	N/A	N/A	N/A	N/A	N/A	N/A
08-3814	N/A	N/A	N/A	N/A	N/A	N/A

Table 3 corresponding to analysis results of 5 urine and 3 liver cases containing BUP. Experimental determination of BUP and NBUP monoisotopic mass and molecular formulas are shown (top). Urine and liver samples were analyzed for BUP and NBUP analytes by respective RT and observed exact mass compared with BUP and NBUP standards (middle). Identification at a 15 ppm error threshold was used for identification confirmation for each urine and liver sample (right). * At a perfect ppm error, elemental composition confirmed that BUP was in fact not present within liver sample 08-1990.

RESULTS

Buprennorphine/Norbuprenorphine Standards

- Results from the BUP and NPUP standards were obtained using MassLynx® elemental calculator, elemental composition and ChromaLynx® sample analysis.
- The exact masses for BUP and NBUP, at 468.3114 and 414.2644, were extracted from the total ion chromatogram (TIC). (Figures 3 and 4)
- Retention times (RT) for NBUP and BUP were observed at 2.38 and 2.72 min respectively. (Table 3)
- RT and Elemental composition of BUP, $C_{29}H_{42}NO_4$, and NBUP, $C_{25}H_{36}NO_4$, were confirmed using ChromaLynx®
- Extracted ion chromatograms (EIC) plotted for exact masses of BUP and NBUP.

Urine Case Sample

- Case 09-1616 urine was analyzed for BUP and NBUP at a maximum error threshold of 8ppm.
- NBUP and BUP RT times produced peaks at 2.39 and 2.72 min. respectively (Figure 5).
- Exact masses of NBUP and BUP were recorded at 414.1988 and 468.3138.
- Elemental composition using MassLynx® identified BUP, with a molecular formula of $C_{29}H_{42}NO_4$, and NBUP with a molecular formula $C_{25}H_{36}NO_4$
- ppm errors associated with NBUP and BUP identification were 3.6 ppm and 5.1 ppm. (Table 3)

Liver Case Sample

- Case 08-1990 liver was analyzed for BUP and NBUP at a maximum error threshold of 15 ppm
- Case 08-1990 liver presented RT peaks of 2.72 and 2.38 respectively. (Figure 6).
- Elemental composition of the 2.38 RT peak did not yield NBUP. Analysis of the 2.72 RT peak yielded an exact mass of 468.2336. Elemental composition of this exact mass produced a molecular formula of $C_{28}H_{42}N_3O_3$ (Table 3)

CONCLUSION

- Buprenorphine was identified in in all 5 postmortem urine cases. Case 09-1616 urine, when compared to the BUP and NBUP exact mass standards, presented exact mass RTs at 2.72 and 2.39 respectively. The ppm error based on the exact masses m/z of BUP (468.3114) and NBUP (414.2664) were 5.1 and 3.6 respectively.
- Buprenorphine nor its metabolite norbuprenorphine were not identified in any of the postmortem liver case samples. Liver in case 08-1990, when exact masses of BUP and NBUP were extracted from the TIC, presented RT exact mass peaks of 2.72 and 2.38. Upon further analysis using elemental composition, the 2.38 RT peak did not yield a m/z match for NBUP. Elemental composition of 2.72 RT peak produced an exact mass of 468.2336 and molecular formula $C_{28}H_{42}N_3O_3$. A close match to BUP, however not a definite identification.

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