Altasciences CLINICAL RESEARCH
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# A Novel Strategy for the *In-Process* Stabilization of N-Oxide Metabolites in Hemolyzed Plasma Determined by LC-MS/MS

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## OVERVIEW

### PURPOSE

The primary objective of the study was to determine optimal extraction procedures which negate N-oxide conversion to parent drug during sample processing. Conversion of the N-oxide metabolite was evaluated at concentrations equimolar to that of the parent compound, and the conversion percent determined in both plasma and hemolyzed human plasma (5%).

### METHOD

Dasatinib/dasatanib N-oxide, pramoxine/pramoxine N-oxide and bupivacaine/bupivacaine N-oxide were fortified in plasma and hemolyzed plasma (5%) and extracted by protein precipitation using MeOH and ACN in a matrix:solvent ratio of 1:3. Acidified (0.1% formic acid) MeOH and ACN were also evaluated as precipitating solvents for dasatinib N-oxide and pramoxine N-oxide, the latter was also examined by liquid-liquid extraction (LLE) using chlorobutane, chlorobutane/MTBE and MTBE/hexane.

### RESULTS

We have demonstrated that N-oxide conversion to the parent drug is not only dependent upon matrix composition (i.e. plasma vs. hemolyzed plasma), but that judicious selection of the extraction approach can effectively limit conversion. Of the diagnostic compounds evaluated herein, it was concluded that protein precipitation using ACN represented the most efficient extraction technique to circumvent N-oxide decomposition to the parent drug.

## INTRODUCTION

Regulatory agencies require that hemolyzed matrix be evaluated during the course of validation to gauge the impact on drug/metabolite degradation, recovery and matrix effect in the presence of blood components. It is recognized that N-oxide metabolites can contribute to the parent drug response by in-source fragmentation and/or degradation during sample processing. To circumvent the impact of in-source fragmentation, the N-oxide must be chromatographically resolved from the parent drug, and its stability monitored during sample processing. In the current investigation, the conversion of pramoxine N-oxide, bupivacaine N-oxide and dasatinib N-oxide to their parent counterparts was noted in hemolyzed plasma, but absent in plasma. Therefore, sample preparation strategies were investigated to minimize N-oxide conversion in hemolyzed plasma.

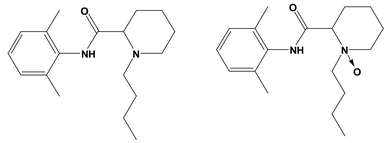


**Figure 1. Structures of Bupivacaine and Bupivacaine N-Oxide.**

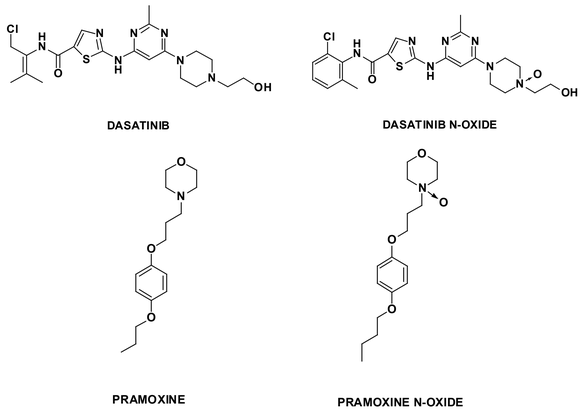


**Figure 2. Structures of Dasatinib, Dasatinib N-Oxide, Pramoxine and Pramoxine N-Oxide.**

## METHODS

### SAMPLE PROCESSING

Analyte and N-oxide metabolites were prepared in both plasma and non-hemolyzed human plasma in a 1:1 concentration ratio. Samples were extracted by either protein precipitation or liquid-liquid extraction.

**Protein Precipitation of Dasatinib and Bupivacaine**

- 100 µL of plasma was aliquoted and precipitated with 300 µL of ACN or MeOH with or without 0.1 % $HCO_2H$.
- Samples were vortexed, centrifuged and further diluted for LC-MS/MS analysis.

**Liquid-Liquid Extraction of Pramoxine**

- 200 µL of plasma was fortified with 50 µL of ISWS in EtOH and buffered with 100 µL of $Na_2CO_3$ (0.5 M, pH 10.5).
- Samples were extracted with 3 mL of chlorobutane, chlorobutane:MTBE (4:1) or MTBE:hexane (4:1).

## METHODS

### CHROMATOGRAPHY

- Agilent Technologies Series 1100 binary pump and autosampler
- Reversed-phase chromatography

### DETECTION

- SCIEX API 5000 using Turbo IonSpray
- MRM acquisition (+ ESI):
  - Pramoxine: *m/z* 294.2 → 100.1
  - Dasatinib: *m/z* 488.2 → 427.2
  - Bupivacaine: *m/z* 289.2 → 140.1

## RESULTS

Bupivacaine N-oxide demonstrated 100% conversion to parent in hemolyzed plasma with MeOH as precipitating solvent, whereas < 5% conversion was observed with ACN; < 1% conversion was noted in plasma using either MeOH or ACN. Dasatinib N-oxide and pramoxine N-oxide conversion was < 0.5% in plasma with and without acidified MeOH or ACN. In hemolyzed plasma, conversion was < 3.8% in ACN or acidified ACN and up to 11.7% in MeOH, whilst acidified MeOH reduced conversion by ~ 5% **(Figures 3 and 4)**.

The LLE of pramoxine N-oxide in hemolyzed plasma revealed 78% conversion when using MTBE:hexane (4:1), while chlorobutane or a mixture of chlorobutane:MTBE reduced this conversion to 25%. In the case of plasma, < 2% conversion was observed regardless of extraction solvent. **(Figure 5)**.

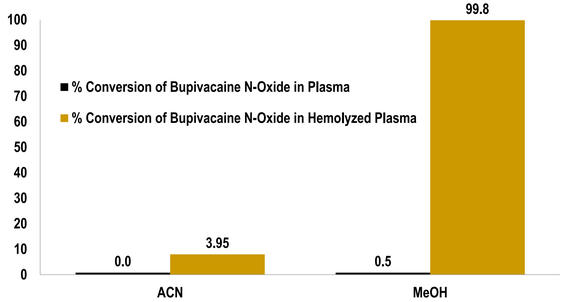


**Figure 3. Bupivacaine N-Oxide conversion in plasma and hemolyzed plasma when using protein precipitation.**

## RESULTS

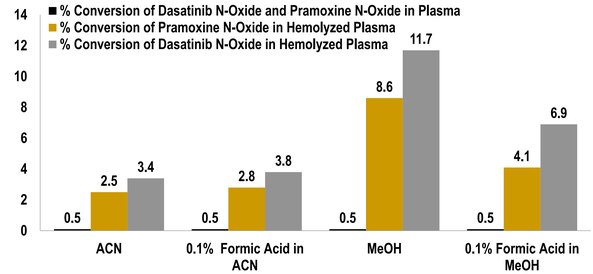


**Figure 4. Percentage of Pramoxine N-Oxide and Dasatinib N-Oxide conversion using protein precipitation extraction in plasma and hemolyzed plasma.**

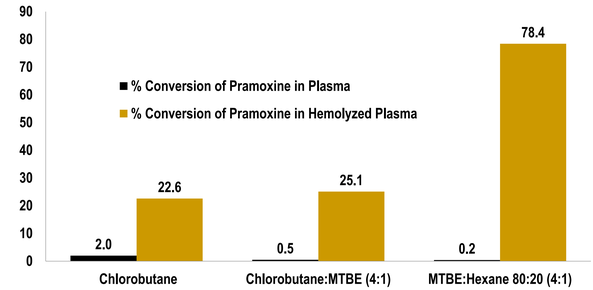


**Figure 5. Percentage of Pramoxine N-Oxide conversion by LLE in plasma and hemolyzed plasma.**

## Conclusion

Labile metabolites are an important consideration during method development, and their thorough characterization pre-empts potential over-estimation of parent drug concentrations in study samples. We have demonstrated in this research that N-oxide conversion to the parent drug is not only dependent upon matrix composition (i.e. plasma vs. hemolyzed plasma.), but that judicious selection of the extraction approach can effectively limit conversion. Of the diagnostic compounds evaluated herein, it was concluded that protein precipitation using ACN represented the most efficient extraction technique to circumvent N-oxide decomposition to the parent drug.