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# Mitigation of On-Column Conversion of Ivabradine to Desmethylivabradine Metal Chelation With Citric Acid Modifier

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## OVERVIEW

### Purpose

Mitigation of on-line N-demethylation of ivabradine to its metabolite by incorporating citric acid in the mobile phase for liquid chromatography/multiple reaction monitoring (LC-MRM) detection.

### Method

In the present study, liquid-liquid extraction of ivabradine and N-desmethylivabradine using chlorobutane was optimized. Both analytes were chromatographed on a hydrophilic interaction liquid chromatography (HILIC) column and injected on a SCIEX API 5000 in ESI (+) mode.

### Results

The stock interference check of ivabradine demonstrated on-column N-demethylation to its metabolite, caused by the presence of metal in the chromatographic setting. The incorporation of citric acid in the mobile phase reduced on-column demethylation of ivabradine to N-desmethylivabradine.

## INTRODUCTION

Ivabradine is used for the symptomatic management of patients with chronic angina pectoris and is extensively metabolized by cytochrome P450 3A4 (CYP3A4) to its major metabolite N-desmethylivabradine (Figure 1). A sensitive and specific LC-MRM method was successfully developed and validated for ivabradine and N-desmethylivabradine for ranges of 0.100 to 100.0 ng/mL, and 0.100 to 10.0 ng/mL, respectively. As part of the validation, a cross-interference check between analytes demonstrated that acceptance criteria was met (i.e. <5%). However, during sample analysis, an on-column conversion of ivabradine to N-desmethylivabradine was noted. Given the ten-fold disparity in ULQ response for N-desmethylivabradine, the response contribution from ivabradine ranged between 1% and 30%.

In the current research, we report on the effectiveness of citric acid as a mobile phase modifier to mitigate the on-column conversion of ivabradine to its primary metabolite.

## METHODS

**Sample Extraction**

**Liquid-Liquid Extraction**

- Sample Volume 150 µL human plasma
- Add 50 µL ISWS, add 150 µL of 0.5M $NH_4HCO_3$ pH 10 vortex
- Add 5 mL chlorobutane and shake
- Transfer organic layer and evaporate to dryness at 50° C
- Reconstitute with 250 µL ACN:MeOH:$H_2O$ 45:5:50% (v:v:v)
- Transfer 150 µL of reconsituted sample into 350 µL of acetonitrile (ACN) and inject

**Chromatography**

- High-performance liquid chromatography (HPLC): Agilent Technologies 1100 Series
  Column: XBridge HILIC 50 x 2.1 mm, 3.5 µm (Waters)
- Mobile Phase
  - A (15%): 50 mM $CH_3COONH_4$ pH 6.0 + 25 µM Citric Acid (Final condition): B (85%): ACN
- Run time: 3.5 minutes

**Detection**

- SCIEX API 5000 ESI Positive mode
- Ion Spray Voltage: 3500
- Multiple reaction monitoring (MRM) transitions
  - Ivabradine: *m/z* 469.26 → 262.20
  - N-desmethylivabradine: *m/z* 455.25 → 262.20
  - Ivabradine-D6 (IS1): *m/z* 475.31→ 268.20
  - N-desmethylivabradine-D6 (IS2): *m/z* 461.29 → 268.20

**Concentration Range(s)**

Ivabradine: 0.10 ng/mL - 100.00 ng/mL

N-desmethylivabradine: 0.10 ng/mL - 10.00 ng/mL

**Preparation**

Ivabradine, N-desmethylivabradine, and stable-labelled isotopic internal standards were dissolved in MeOH.

The stock interference check was prepared in $H_2O$:ACN 15:85% v/v at a concentration of 18.0 ng/mL for ivabradine and 1.8 ng/mL for N-desmethylivabradine, representing post-extract concentrations (Figure 2).

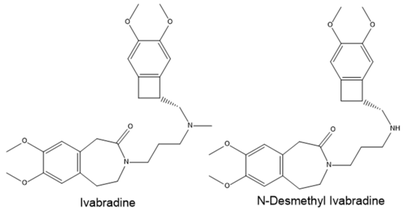


**Figure 1.** Molecular Structures of Ivabradine and N-desmethylivabradine

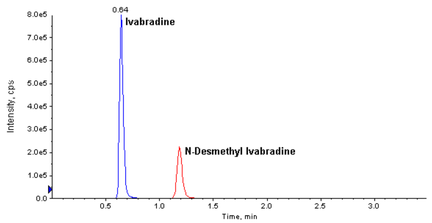


**Figure 2.** ULOQ Ivabradine 100.0 ng/mL and N-desmethylivabradine 10.0 ng/mL

## RESULTS

Ivabradine and N-desmethylivabradine were successfully validated; however, during samples analysis, interference from ivabradine to its metabolite was observed. During the interference check, the presence of N-desmethylivabradine was observed between 0.1% and 3% (**Figure 3**) when analyzing ivabradine only at equal concentration. The primary objective was to therefore determine the root cause for N-demethylation of the parent compound to its metabolite. Several investigations eliminated the potential for conversion during sample preparation, including evaporation. The chromatography was next considered for possible on-column conversion with several XBridge HILIC columns evaluated. Results indicated ivabradine on-column conversion ranging from 0% to 3%, representing 0% to 30% (**Table 1**) at the ULQ concentration of N-desmethylivabradine. Multiple organic solvents, buffers and acids were investigated to condition and/or replenish the column without success.

**XBridge HILIC Columns**

| Column Identification | % On-column conversion |
|---|---|
| 1 | 18 % |
| 2 | 30 % |
| 3 | 25 % |
| 4 | 1.4 % |

**Table 1.** Percent On-column Conversion for N-desmethylivabradine (ULQ) from Ivabradine ULQ only

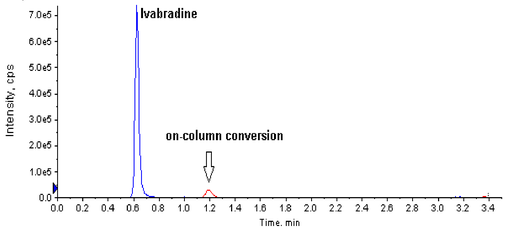


**Figure 3.** On-column conversion of ivabradine to N-desmethylivabradine from the stock interference check of ivabradine only .

The initial chromatographic conditions for ivabradine and N-desmethyl ivabradine were performed on an XBridge HILIC column with 25 mM ammonium bicarbonate: ACN 15:85% v/v for optimal sensitivity and separation from an isobaric metabolite. New columns showed acceptable conversion; however after 12-hours of injection, N-demethylation gradually occurred *ca* 5% (ULQ) when injecting ivabradine ULQ only (3 lots tested) (**Figure 4**). Eventually the conversion yielded above 20% conversion during the stock interference check.

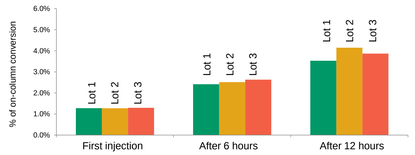


**Figure 4.** On-line Percent Conversion Injection without Citric Acid in the Mobile Phase

We hypothesized that N-demethylation was catalyzed by the presence of trace metals present on column frits, housing and packing. The hypothesis was evaluated via introduction of a metal chelator to the mobile phase, with ethylenediaminetetraacetic acid (EDTA) demonstrating promising results, albeit significant variable analyte response was observed (data not shown). The mobile phase was modified for 50 mM ammonium acetate pH 6.0 with 25 µM citric acid. The column was conditioned with the new mobile phase and ivabradine stock interference check was injected over a 12-hour period. The on-column conversion of ivabradine to its metabolite remained stable. In contrast, citric acid (25 µM) reduced on-column conversion (0% to 3% at the metabolite ULQ) (**Figure 5**) while conserving sensitivity and linearity (**Table 2**).

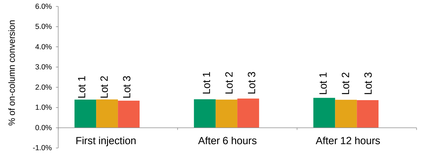


**Figure 5.** On-line Percent Conversion Injection With Citric Acid in the Mobile Phase

| Calibrant | Concentration (ng/mL) | % Bias |
|---|---|---|
| STD 1 | 0.100 | 1.8 |
| STD 2 | 0.200 | -2.3 |
| STD 3 | 0.400 | -0.2 |
| STD 4 | 0.600 | -1.0 |
| STD 5 | 0.800 | -2.6 |
| STD 6 | 1.500 | -3.0 |
| STD 7 | 2.500 | -1.2 |
| STD 8 | 4.000 | 6.3 |
| STD 9 | 7.000 | -3.0 |
| STD 10 | 8.500 | -2.5 |
| STD 11 | 10.000 | 7.7 |

**Table 2.** Calibration Curve for N-desmethylivabradine in Human Plasma (with Citric Acid in the Mobile Phase)

## CONCLUSIONS

Interference check is an important assessment during method validation. Although on-column conversion from ivabradine to N-desmethylivabradine is minor at equal concentration, this may impact a bioanalytical assay with disparate ranges. Different columns showed different N-demethylation conversions, which was controlled with the employment of citric acid as a metal chelator. The method was successfully validated under the new chromatographic conditions for ivabradine and N-desmethylivabradine in human plasma.