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Bromazepam – Ring Open or Ring Closed?

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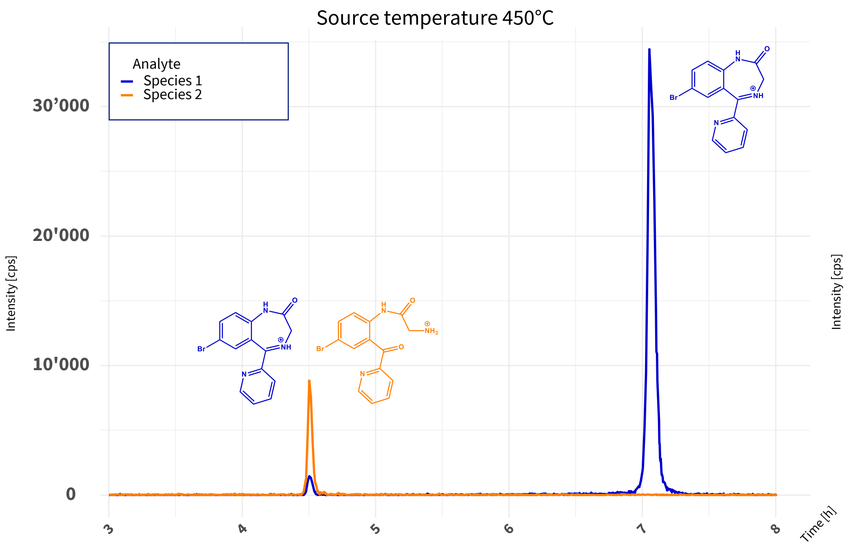
When comparing the fragmentation of different analytes, two peaks were observed for bromazepam. While this can easily be explained by the typical instability of benzodiazepines, leading to ring-opened and ring-closed species, the simultaneous occurrence of these two species at the first of the two peaks deserved our attention. In-source re-cyclization was hypothesized as a likely explanation. Correspondingly, an increase in the ion source temperature resulted in an increase of the signal of ring-closed bromazepam at the retention time of the ring-opened species.



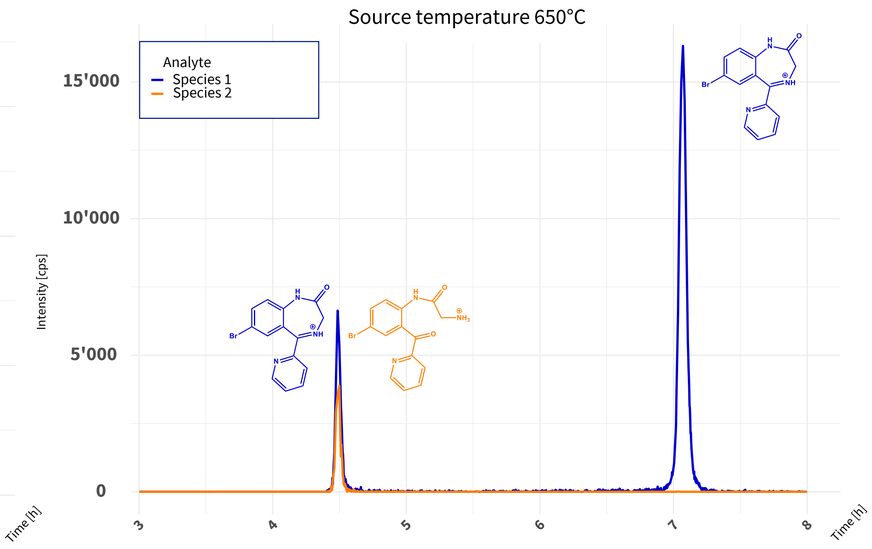


Fig.6: Extracted ion chromatograms of bromazepam and its ring-opened form, 20 hours after start of experiment (equilibrium was reached after approx. 13 h)

Ring-closed bromazepam
Species 1
occurs at retention times (RT) 4.5 and 7 min

Ring-opened bromazepam
Species 2
occurs at retention time (RT) 4.5 only

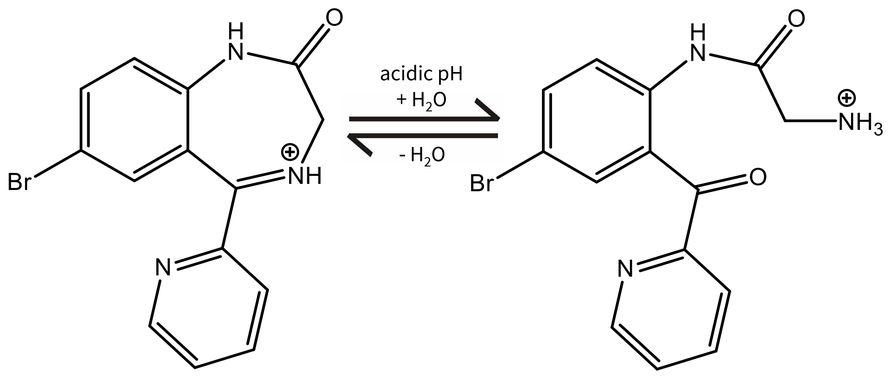


Fig.3: Reversible ring-cleavage reaction of bromazepam

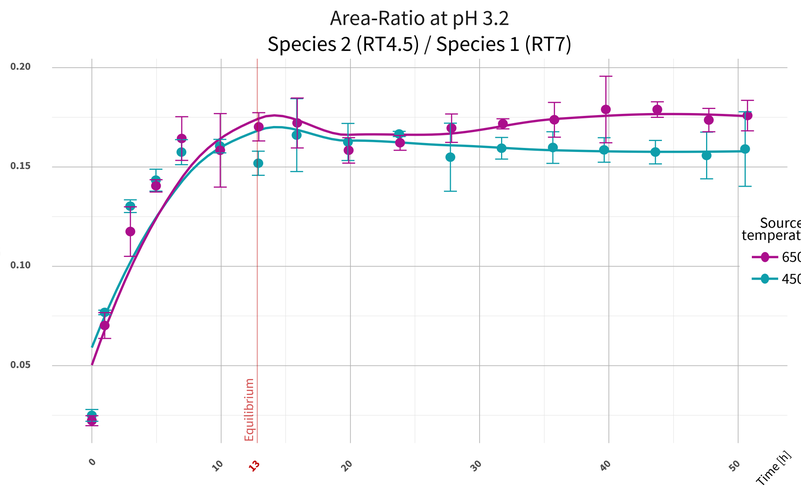


Fig.4: Development of the area of ring-closed divided by ring-opened form

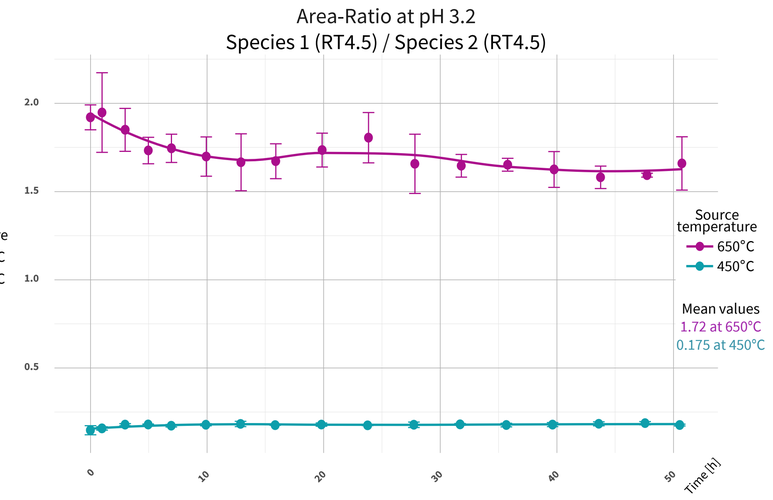


Fig.5: Development of the area of ring-closed divided by ring-open form

Methods

- Experiment conducted using HPLC-HRMS/MS approach
- Measurement employing Sciex ZenoTOF 7600 with reversed-phase chromatography
- Untargeted method in DDA mode
- Eluent A (10 mM ammonium formate:acetonitrile (99:1) + 0.1% formic acid) and eluent B (acetonitrile:ultrapure water (99:1) + 0.1% formic acid)
- Use of eluent A and eluent B with and without acid as solvents
- Analyte mix containing ten different benzodiazepines (bromazepam, pyrazolam, clonazepam, clonazolam, nitrazepam, diazepam, nordazepam, alprazolam, midazolam, triazolam) and two internal standards (lorazepam-d4 and trimipramine-d3)
- Sample measurement at ion source temperatures of 450 and 650 °C
- Repeated measurements in triplicates after incubation
- Higher density of measuring points at the outset
- Maximum incubation of 50 hours

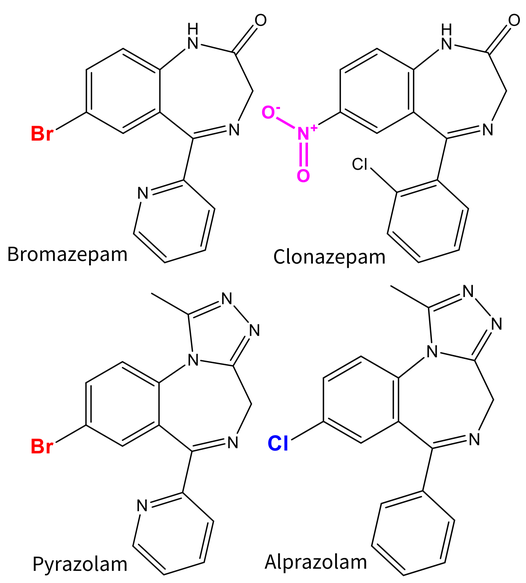


Fig.1: Exemplary analytes with structural differences

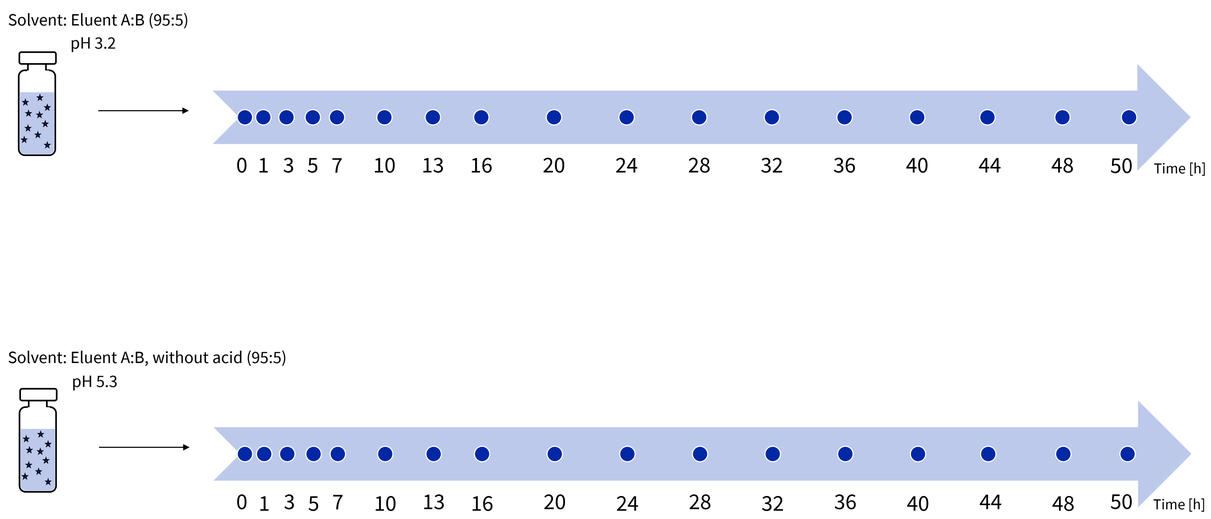


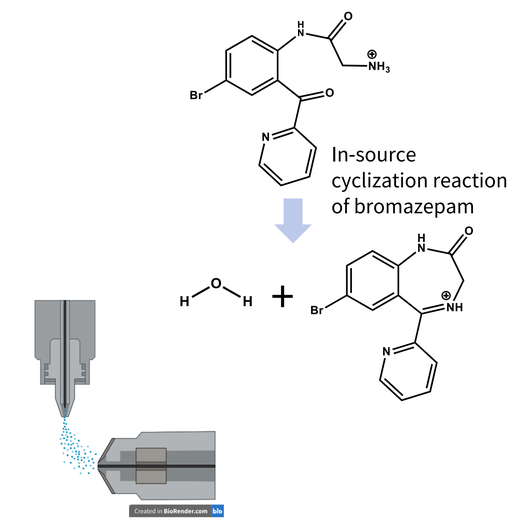
Fig.2: Schematic representation of experimental set-up
Ten analytes, 100 ng/mL each (two deuterated analytes, 50ng/mL each)

Results

- Two bromazepam species (ring-opened and ring-closed) at the same RT due to alleged in-source recyclization
- Pyrazolam (also with bromo substituent) showed no ring opening
- Other evaluated benzodiazepines showed expected ring opening, however without subsequent in-source recyclization
- Signal intensity for bromazepam reduced by 50% at higher temperature (650 °C)
- Increased source temperature led to tenfold increase of ratio species 1 / species 2 (RT4.5)
- Equilibrium for bromazepam and corresponding opened form reached after approx. 13 hours
- Few analytes showed ring opening at higher pH, bromazepam not included

Conclusion

- Second peak for species 1 at 4.5 min associated to in-source cyclization reaction of the ring-open form, enhanced at higher ion source temperatures
- Bromine-substituted benzodiazepines with pyridine ring showed unique behavior
- Need for further investigation of other compounds of this class, especially those with similar structural elements
- Potential serious implications for quantification of benzodiazepines in biological samples (especially in the case of targeted quantification in scheduled MRM mode)



Experiment repeated with source temperatures of 450 and 650 °C

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