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Comprehensive Evaluation of BTZ-043: From hAME Study to Metabolite Exposures - Handling the Challenge of Unstable Hydride Meisenheimer Complex Metabolites

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Introduction

Tuberculosis (TB) is a severe infectious disease caused by Mycobacterium tuberculosis, primarily affecting the lungs and spreading through airborne droplets. Symptoms include cough, fever, night sweats, and weight loss. Treatment involves a long course of antibiotics, but drug-resistant strains are challenging. BTZ-043, a promising new drug in development belongs to the class of benzothiazinone and works by inhibiting the synthesis of the bacterial cell wall, leading to the death of the bacteria. BTZ-043 has shown potent activity against both drug-sensitive and drug-resistant TB strains, making it a crucial candidate in the fight against multidrug-resistant TB, and could shorten TB treatment duration and improve outcomes. (Ref 1).

During initial DMPK *in vivo* studies, pronounced instabilities of several main metabolites required considerable efforts to develop accurate bioanalytical methods. This challenge proved even more demanding during the preparation and conduct of the human mass balance and metabolite study.

Ex-Vivo Instability of Meisenheimer Complexes & Related Analytes

The major circulating BTZ-043 metabolite M2 was sensitive to even traces of ambient oxygen and back-oxidized to parent (Fig. 1), rendering quantification of **both** components inaccurate. Addition of ascorbic acid largely prevented this interconversion at 0°C (Fig. 2). However, lability of M2 authentic compound made any accurate LC-MS/MS calibration impossible. Reliable quantification was only amenable by measuring M0 in a) 'M2-stabilized' samples (yielding $M0_{actual}$) and in b) M2 fully back-oxidized samples (yielding $M0_{total}$): M2 = $M0_{total}$ – $M0_{actual}$. The quantitative transformation of M2 to M0 was enforced by adding oxyhemoglobin (traces of hemolysate) and formic acid.

Instability of the Meisenheimer complex M3 was noticed even in ascorbate plasma (Fig. 1 & 2) and back-oxidation of M3 to M4 could not be prevented by any pretreat. Only $M4_{total}$ (sum of M3 + M4) could be reliably quantified. Lability of other minor metabolites were minimized in addition by shock freezing of samples.

Cluster Approach

In a pragmatic attempt to address the major labilities, the main drug-related components were grouped in two clusters of associated components: (**Cl-1**: M0, M1 and M2; **Cl-2**: M3, M4 and M10; Fig. 6), assuming that the sum of concentration within each cluster to be constant independent from instabilities.

Design of the Human Mass Balance & AME Study

The in-life part of the human mass balance and AME study was performed at ICON (Groningen, Netherlands): Four healthy male subjects were administered a co-crystallized blend of 500 mg BTZ-043 and 100 µg [^{14}C]BTZ-043 as liquid suspension. Prospective human dosimetry suggested a human whole-body dose of 0.16 mSv. Blood, urine, and feces samples were collected for 7 days until the discharge criteria were met.

Sample Collection & Processing

Blood and excreta were immediately cooled down, split into aliquots and treated in different ways to guarantee the stability of jointly analyzed metabolites as best possible: **Plasma** samples were processed individually; native & ascorbate-stabilized aliquots were shock-frozen. **Urine** samples were pooled and stabilized 'online' by diluting aliquots into 9 vol. of native or ascorbate-stabilized plasma in micturition layers (for details see Fig. 3). Entire **feces** samples were shock-frozen, and homogenate pools prepared per subject and day w/o stabilizing agents, since the critically labile metabolites had hardly been observed in rat feces during the ADME study.

Sample Analysis

Total radioactivity measurements were performed at ICON using liquid scintillation counting in a validated assay for native plasma, urine and feces aliquots.

Cluster-1 (BTZ-043, M2, and M1): LC-MS/MS methods for quantification of $M0_{actual}$ (BTZ-043), $M0_{total}$ (enabling calculation of M2 concentrations), and metabolite M1 were developed and validated in appropriately pretreated plasma and urine aliquots.

Cluster-2 ($M4_{total}$ and M10): $M4_{total}$ (sum of M3 and M4) was determined within the same LC-MS/MS method as used for $M0_{total}$. Metabolite M10 was quantified by a separate LC-MS/MS method in native plasma and urine.

Met-ID: Native plasma, urine and feces aliquots were pooled and extracted by LLE. Drug-related components were profiled by UPLC-HR-MS^E and quantified either by LC-MS (Cluster compounds, in plasma and urine), or by offline SSC (all other) plasma or urine. Only the non cluster related metabolites were reliably quantified by that approach (Tab 1).

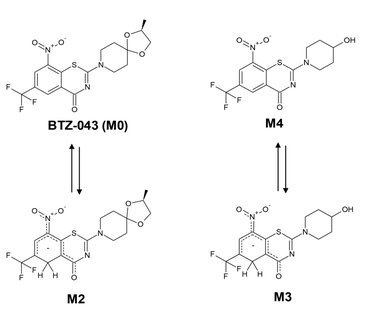


Figure 1: BTZ-043 (M0) and its Meisenheimer complex metabolite M2, as well as its homolog M4 (formed by ketal hydrolysis) and the derived Meisenheimer complex metabolite M3.

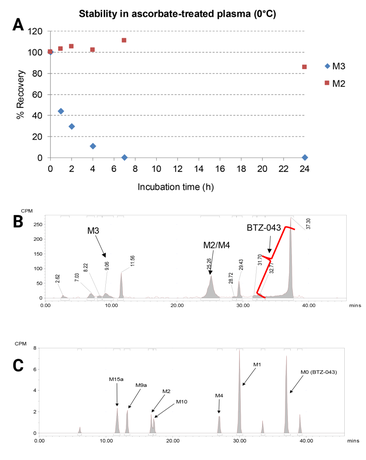


Figure 2: Stability of metabolites M2 and M3 observed in **A:** ascorbic acid supplemented plasma and **B:** during metabolite profiling of native plasma (direct injection) showing on-column instability of M2 and M3 **C:** Chromatogram following LLE

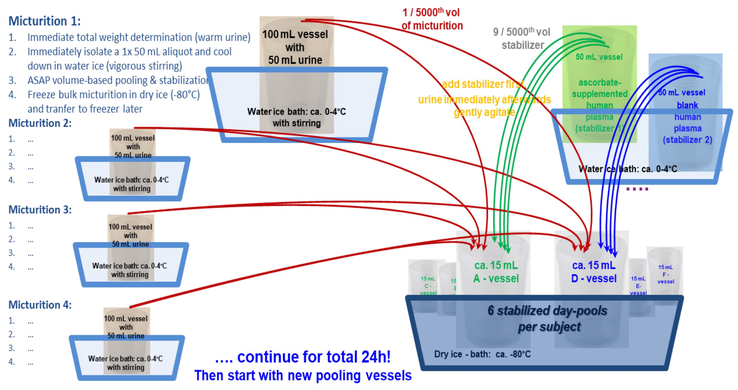


Figure 3: Sample collection scheme for urine 'online' pooling w/wo native plasma and ascorbic acid as stabilizing agent (ordinary sample pooling just prior to bioanalysis was considered dubious, since thawing/pooling requires substantial 'unfrozen' time triggering labilities even in stabilized urine matrix on ice).

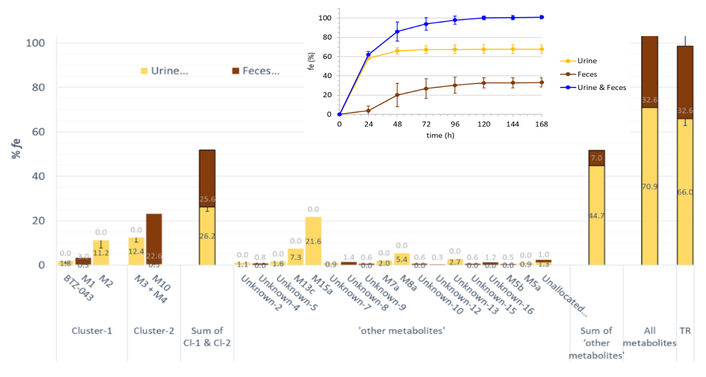
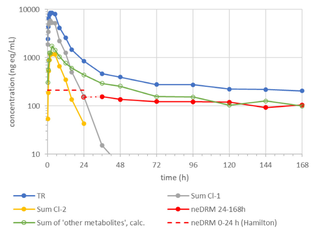


Figure 4: Mean cumulative excretion of TR (a), BTZ-043 and metabolites (b) into urine, (0-48 h) and feces (0-120 h; means ± SD of subjects 1-4)



TR: total radioactivity
Cl-1: Sum of Cluster-1 metabolites
Cl-2: Sum of Cluster-2 metabolites
neDRM: non-extractable drug-related material
Sum of other metabolites = TR – ∑ (Cl 1 + Cl 2 + neDRM)

Analytical Method Selected	Component		Mean ± SD AUC$_{0\text{-}24h}$ (h×ng eq/mL)	TR AUC$_{0\text{-}24h}$ (%)	Mean ± SD AUC$_{0\text{-}24h}$ (h×ng eq/mL)	TR AUC$_{0\text{-}24h}$ (%)
Black numbers UPLC-MS/MS (validated / qualified)	Cluster-1	BTZ-043 (M0)	18200 ± 2950	24.1	**4020 ± 830**	**4.77**
		M1	20700 ± 2770	27.3	2580 ± 413	3.06
		M2	733	1.11	40400 ± 6290	48.0
	Sum of Cl-1 components		*39600 ± 4050*	*52.5*	***47000 ± 6360***	***55.8***
Grey numbers Radio-profiling (*TopCount™*)	Cluster-2	$M4_{total}$	5310 ± 1370	6.88	10300 ± 3990	12.2
		M10	3520 ± 1600	4.57	439 ± 74.0	0.521
	Sum of Cl-2 components		*8830 ± 2110*	*11.5*	***10700 ± 3990***	***12.8***
	Sum of Cl-1 and Cl-2 (,main' components)		*48400 ± 4560*	*64.0*	***57700 ± 7510***	***68.6***
Radio-profiling (TopCount™)	M9a				6000 ± *1200*	7.13
	M15a				6270 ± *2090*	7.45
	Unknown-1				460	0.545
	Unknown-3				835	0.992
	Unknown-6				245	0.291
	Unknown-11				230	0.273
	Unknown-14				202	0.240
	Unknown-17				2880 ± *783*	3.42 [c]
	Unknown-18				230	0.273
	Unknown-19				460	0.546
	Unknown-20				3970 ± *1320*	4.71 [c]
	Unknown-21				1090	1.29
	Unallocated area				0	0.00
	Sum of ,other' metabolites (soluble)				22900 ± *2860*	27.2
LSC	Covalently protein-bound components				5090	6.05
	Sum of ,other' components				*28000*	*33.2*
Sum of structurally elucidated metabolites					**70000 ± *7880***	**83.1**
Total components detected					**85700**	**101.8**
LSC	Total radioactivity [b]				**84200**	**100.0**

Figure 5 and Table 1: Plasma exposure to BTZ-043 and its metabolites and comparison to total radioactivity (0-168 h & 0-24 h).

Results

Ex-vivo instabilities of several drug related components in plasma and urine could not be addressed by one single sample pre-treatment method due to their different redox traits. A number of stabilizing approaches were employed to sample aliquots (swift cooling and shock-freezing, ascorbate supplement and/or dilution into plasma (unique approach), 'online' layer pooling, back-oxidation) to accurately determine concentrations of the key components (M0, M1, M2, $M4_{total}$, and M10) by LC-MS/MS methods validated in line with current guidelines (Fig 1-3). Minor components were quantified by radio-profiling of untreated samples.

A dedicated and well-trained lab team to instantly process samples from each subject was mandatory for the success of the study.

By introducing a Cluster approach, we confirmed that the $AUC_{0\text{-}24h}$ of Cluster 1 components (M0, M1 and M2) and of Cluster 2 metabolites (M3, M4 and M10), respectively, were similar (student´s t-test: Cluster 1: t = 1.94, p =0.099; Cluster 2: t = -0.406, p = 0.698387 statistically not different at p<0.05) when measured by LC-MS/MS as compared to (non-stabilized) metabolite radio-profiling.

Elimination: 33.2 ± 4.7 % of the radiolabeled dose was excreted into feces and 67.8 ± 4.7% into urine, indicating complete recovery of drug-related material within 168 h. BTZ-043 was almost completely metabolized, and excreted mainly in form of M2, M4, M10 and its conjugation products (Fig 4; ≥80% radioactivity structurally identified in each matrix).

Exposure: Cluster 1&2 components were in fact the main contributors to drug-related plasma exposure (68.6% AUC_{TR}), confirming the high effort invested in their method development. Other circulating metabolites were each <10% AUC_{TR}. Probably covalently protein bound material was in the range of 6% AUC_{TR} (Fig 5 & Tab 1).

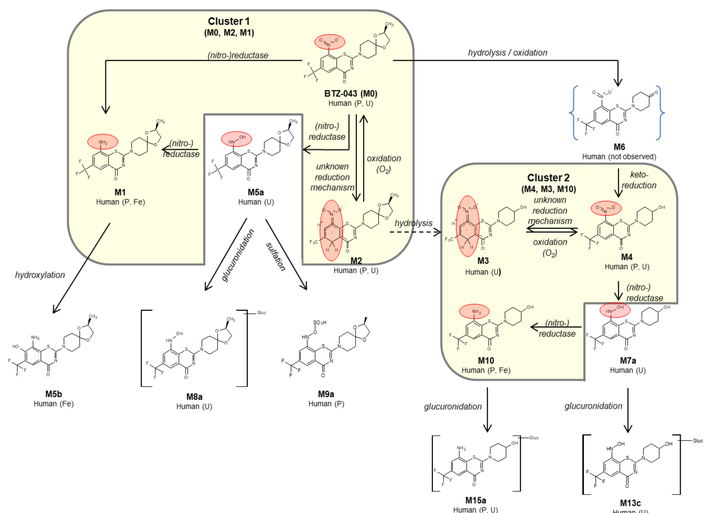


Figure 6: Proposed *in vivo* metabolic pathways of BTZ-043 in humans

Conclusions

- Aware of multiple metabolite instabilities from the rat ADME study, we developed several validated and stable LC-MS/MS assays crucial for the accurate exposure analysis of the main drug-related components. Only minor metabolites were reliably quantified with the standard hAME approach, i.e. radio-profiling. Given the instability of major metabolites during radiometric analysis, the exposures measured by LC-MS/MS are regarded as valid for the MIST assessment.
- Due to the complexity of the sample processing and the bioanalysis, the conduct of this hAME study was extremely challenging for all involved lab teams.
- Following fast absorption, BTZ-043 was rapidly cleared from the circulation and mainly eliminated by metabolism, yielding the Meisenheimer complex M2 as the major circulating component.
- Excretion of total radioactivity was quite fast and complete within 168 h, with 68% of the dose emerging in urine and 33% in feces.

References

1. Treu A et al. The clinical-stage drug BTZ-043 accumulates in tuberculosis lesions and efficiently acts against Mycobacterium tuberculosis. DOI: 10.21203/rs.3.rs-2615777/v1

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