efpia
European Federation of Pharmaceutical Industries and Associations

18 February 2022

1 Hydrochlorothiazide HCTZ- Investigation into potential *N*-Nitrosation and the specific nature of Nitroso HCTZ

1.1 INTRODUCTION

Hydrochlorothiazide (HCTZ) is a thiazide-type diuretic medication approved in 1959 for the treatment of hypertension and edema (Herman LL, 2020; Reynolds, 1989; Rosendorff, 2011). It is the most commonly prescribed antihypertensive drug and used both as a single agent and in combination with other APIs such as beta blockers, ACE inhibitors, angiotensin II receptor blockers, calcium channel blocking agents, statins, and other diuretics. In 2019 it was reported to be the second most commonly prescribed drug (as part of a combination product) in the US with some 40 million prescriptions and close to 10 million patients

1.1.1 Preparation

Developed in the 1950s, hydrochlorothiazide was granted FDA approval on 12 February 1959.

Hydrochlorothiazide is synthesized by either the reaction of paraformaldehyde with 5-chloro-2, 4disulfamoylaniline in nonaqueous media, or the reaction of formaldehyde with 6-chloro-7-sulfamoyl2H-1, 2, 4-benzothiadiazine-1, 1-dioxide in aqueous alkaline solution (Deppeler, 1981).

As part of the Article 5(3) (CHMP, 25 June 2020) risk process a step 1 assessment concluded that there was a potential risk of formation of nitroso hydrochlorothiazide in the formulated drug product as a result of Nitrosation of HCTZ through interaction with traces of nitrite in excipients; concerns relating primarily to the low pK_a and anilinic nature of the secondary amine present within the molecule.

1.2 SCOPE & PURPOSE OF THIS POSITION PAPER

- *Scope:* This position paper summarizes EFPIA-member-companies' past- and current activities related to nitrosamine investigation surround the HCTZ containing products and the lessons learned (scientific-, regulatory-, compliance-wise).

- *Purpose:* The paper is constructed to provide an update to the NIOG (Nitrosamine Impurities Oversight Group)/QWP (Quality Working Party) and Safety working party and other interested parties, about the current status of scientific knowledge related to *N*-nitrosamine impurities in HCTZ and to allow the scientific understanding gained to be used to inform future discussions about) formation of *N*-nitrosamines in HCTZ-containing products, and their hazard classification and control.
- *This reflects the current state of the analysis, review is still on going, and therefore, the EFPIA-membercompanies reserve the right to further amend/revise going forward.*
- Contributing companies to the scientific experimentation and evaluation include: Novartis, AstraZeneca, Takeda, Teva, Pfizer, Merck Healthcare KGaA, Organon.

1.3 EXECUTIVE SUMMARY

Industry has conducted an extensive amount of root cause investigations into the potential formation of *N*-nitroso-HCTZ (NO-HCTZ), (figure 1) and the toxicology, specifically mutagenicity of this compound.

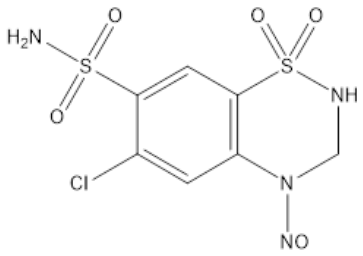


Figure 1: *N*-Nitroso Hydrochlorothiazide

- These investigations relate to formation, stability and specific fate of NO-HCTZ in vitro, in vivo and within the HCTZ containing products. They also look at the risk in terms of likely in vivo species formed and pre-clinical studies used to investigate in vivo relevance of the potential presence of NO-HCTZ within HCTZ based products

1.3.1 ANALYSIS

- The analysis of NO-HCTZ and the challenges associated with quantifying levels of NO-HCTZ within a matrix containing HCTZ, a highly reactive secondary anilinic amine, and measurement of an intrinsically unstable NO-HCTZ are also discussed.

- Detectable levels of NO-HCTZ have now been observed in several drug products. Investigations in levels and root causes is ongoing. Development and validation activities to support Step 2 testing for the remaining HCTZ containing drug products are being actively pursued.

1.3.2 PERMISSIBLE LIMIT

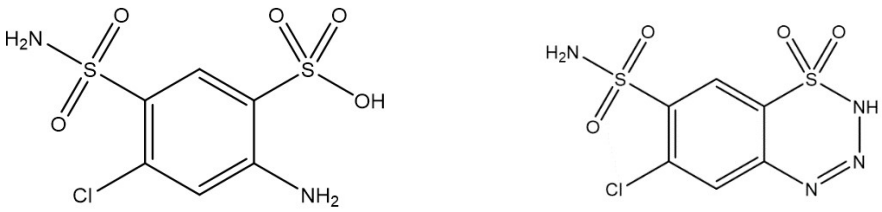
- Establishing a permitted limit is also challenging. There are no existing data for NO-HCTZ and only one closely related analogue, Nitrosomethylphenylamine (NMPA). NMPA is itself complex in terms of reported limit, there being discrepancies between limits defined by authorities, see section 1.5.

1.3.3 SAFETY AND MECHANISTIC INVESTIGATIONS

- These investigations conclude that HCTZ and its' associated impurity NO-HCTZ are complex and unusual and warrant specific consideration in determining suitable controls.
- HCTZ as an anilinic secondary aromatic amine, is highly susceptible to nitrosation; this, combined with the presence of nitrites in excipients, gives rise to a high theoretical risk of NO-HCTZ being generated in HCTZ related products. On this basis HCTZ containing products were reported as being 'at potential risk' to HAs (Health Authority) in March 2021 and step 2 investigation, consistent with Article 5(3) (CHMP, 25 June 2020) were instigated
- Samples of NO-HCTZ was synthesized and tested in a series of Ames tests. Described in details below these included testing with and without hamster and rat S9, with pre-incubation and plate incorporation and with and without presence of DMSO. Results showed a positive response with both rat and hamster S9. However, a positive response was also seen in some strains without activation. This is not expected for a potent *N*-nitrosamine where the mechanism if toxicity is via formation of a reactive diazonium species following metabolic activation. *N*-Nitrosamines are normally stable unreactive, non-mutagenic compounds without metabolic activation. This finding is consistent with an earlier report (Andrews, Lijinsky, & Snyder, 1984)
- *N*-Nitrosamines are metabolically activated carcinogens (Guengerich & Shimada, 1998), the ultimate species formed being an alkyl / or aryl diazonium ion. Consequently, the fact that a positive response was seen without activation indicates an unusual toxicity associated with this nitrosamine that needed further evaluation.
- In parallel stability assessments (25°C, 2 hours) were performed using a purified standard of the NO-HCTZ in the solvent system used in the pre-incubation stage of the Ames test. This was conducted on a timescale analogous to that of the pre-incubation step performed in an Ames test.

These showed the NO-HCTZ to be extremely unstable, semi-quantitatively only < 20% remained after 2 hours at 25°C. The level of the nitrosamine remaining after preincubation at 37°C was subsequently found to be less than 5% of the initial level.

- This degradation was investigated and using both high resolution accurate mass spectrometry (HRAM-MS/MS) and NMR (Nuclear Magnetic Resonance) it was confirmed that at neutral pH NOHCTZ eliminates formaldehyde to generate two products, a primary amine and a thiatriazine (Figure 2) (the proposed mechanism is shown in Figure 13)



Amine **Thiatriazine**

Figure 2 – Principal NO-HCTZ degradation products

  - As an aromatic amine the amine sulphonic acid alerts as a potential mutagenic impurity (Class 3), however as this is only present because of degradation of the Nitrosamine of HCTZ the levels are not of concern being significantly below the TTC.
  - The thiatriazine does not give an alert. (DEREK Nexus / LeadScope Modeller) ○ Further studies were performed that demonstrated that formaldehyde was formed rapidly and stoichiometrically by the degradation of NO-HCTZ at neutral pH.
  - Another Ames test was performed running the NO-HCTZ side by side with formaldehyde itself (see figure 3). This showed a clear correlation indicating that the results of the Ames test can be attributed to the generation in situ (CYP independent) formation of formaldehyde from the NO-HCTZ, see Figure 14, 15, 16.
- It is concluded, based on the studies described that, if formed within HCTZ related products, NOHCTZ is intrinsically unstable at physiological pH and that therefore control of NO-HCTZ as a member of the cohort of concern is not warranted. Instead, this impurity should be understood as a source of formaldehyde, an aromatic amine and the thiatriazine, none of which belong to the cohort of concern and thus require no specific control as levels formed from traces of the Nitrosamine of HCTZ are well below the appropriate TTC for each species.

1.4 Safety investigations – Ames test outcomes

To assess the mutagenicity of *N*-nitroso hydrochlorothiazide an authentic reference material was synthesized by four separate organizations. In each case the materials were shown to be >96% pure and organic impurities were confirmed to be non *N*-Nitrosamine in nature (thus avoiding the risk of a false outcome originating from the presence of another related *N*-Nitrosamine species).

1.4.1 Evaluation of NO-HCTZ Ames test findings

HCTZ is licensed by many different organisations who have conducted the risk assessment required in accordance with Article 5(3) requirements – Step 1, resulting in the conduct of several Ames tests on NNitroso-HCTZ.

As consensus on the optimal Ames protocol for evaluation of an N-Nitrosamine is still under discussion, the testing protocol utilized varied between testing organizations.

The following protocols were applied to the assessment of N-Nitroso-HCTZ in the Ames.

Company A – low DMSO loading, pre- incubation, Hamster S9

Company B – DMSO, pre-inc, rat S9

Company C – DMSO, plate incorporation, TA100, TA1535 rat S9

Company D - DMF (Dimethyl formamide), pre-incubation, both rat and hamster S9 – the test was performed twice

Despite some differences in the results profile from these studies in all cases a positive outcome for NNitroso-HCTZ was concluded.

Ames testing on the putative degradation products of N-Nitroso HCTZ (formaldehyde and a thiatriazine compound) under Ames test conditions conducted by company D revealed considerable overlap between test result profiles for formaldehyde and N-Nitroso HCTZ.

Of note was the positive results observed in TA98, TA100 and e. coli strains in the absence of activation. Such a profile is inconsistent with the usual profile of a potent *N*-nitrosamine, figure 3 showing the associated mechanism for NDMA (Nitroso dimethylamine).

Table 1: Summary of Ames test results

Condition	Strain	NO-HCTZ Company A result	Maximum fold revertant increase	NO-HCTZ Company B result	Maximum fold revertant increase	NO-HCTZ Company C result	Maximum fold revertant increase	NO-HCTZ Company D results Experiment 1	Maximum fold revertant increase	NO-HCTZ Company D result Experiment 2 (repeat))	Maximum fold revertant increase	Thiatriazine Company D result	Maximum fold revertant increase	Formaldehyde Company D results	Maximum fold revertant increase
	1535			Neg		Neg		Neg		Neg		Pos	4.0	Neg	
Rat S9 (Aroclor-induced)	1537 98			Neg Neg				Neg Pos	 2.4	Neg Pos	 2.5	Neg Neg		Neg Neg	
	100			Pos	2.1	Pos	2.4	Pos	4.7	Pos	4.1	Neg		Pos	4.2
Hamster S9 (Aroclor-induced)	e. coli 1535	Pos	5.2	Neg		Neg		Pos Neg	2.4	Pos Neg	2.6	Neg Pos	4.4	Pos Neg	2.4
	1537	Neg						Neg		Neg		Neg		Neg	
	98	Pos	5.5					Pos	2.3	Pos	2.2	Neg		Pos	2.6
	100 e. coli	Pos Pos	4.8 5.5			Pos	3.0	Pos Pos	5.1 2.5	Pos Pos	5.1 2.2	Neg Neg		Pos Neg	4.2
None	1535 1537	Neg Neg		Neg Neg		Neg		Neg Neg		Neg Neg		Pos Neg	4.0	Neg Neg	
	98 100	Pos Pos	2.2 4.2	Neg Neg		Pos	2.7	Neg Pos	1.7 4.6	Pos Pos	2.0 2.9	Neg Neg		Pos Pos	2.5 7.4
	e. coli	Pos	2.8	Pos	2.1			Pos	3.0	Pos	3.7	Neg		Pos	3.8

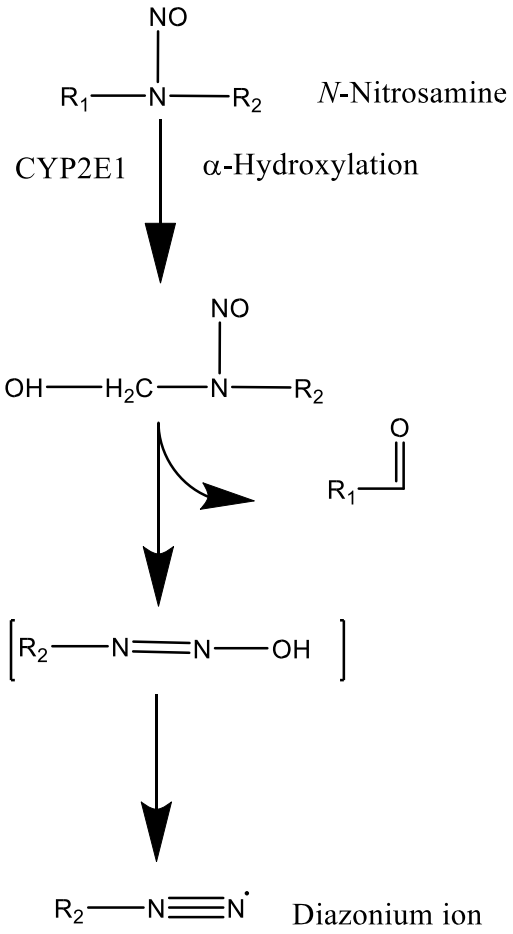


Figure 3: Metabolism of N-Nitrosamines (NDMA) in vitro / in vivo

Based on this outcome further experiments were conducted, these included the parallel running of an Ames study comparing the profile of NO-HCTZ, with that of formaldehyde and Impurity D (Thiatriazine) see table 1.

The results without activation show a truly clear correlation between NO-HCTZ and formaldehyde (Table 1). Stability studies have clearly shown the rapid decomposition of NO-HCTZ at physiological pH, NMR studies clearly showing a near stoichiometric correlation between loss of NO-HCTZ and levels of formaldehyde

(Figure 17). It is therefore postulated that the positive outcome of the Ames test of NO-HCTZ both and without activation are a result of the presence of formaldehyde. The *N*-nitrosamine of HCTZ therefore does not contribute to the toxicity other than through generation of formaldehyde.

1.4.2 Thiatriazine

A further study was performed in parallel on the major degradation product, the Thiatriazine – see figure 2. This was tested and the profile compared to that of NO-HCTZ and formaldehyde, see Table 1. The results show a weak positive in one strain, TA 1535, with and without S9 activation. Importantly these do not correlate with the profile of a *N*-Nitrosamine or with that of formaldehyde. Ames result show the Thiatriazine to be weakly positive, within one strain TA1535. The structure of the Thiatriazine, confirmed to be that shown in Figure 2, cannot follow the metabolic pathway shown in Figure 3 i.e. that of a NNitrosamine, specifically it cannot form a diazonium ion. This result, combined with the inability of the molecule to generate a reactive diazonium ion metabolically, rules this out as a cohort of concern *N*nitrosamine.

1.5 Analysis of *N*-Nitrosamines - NO-HCTZ

Trace analysis of *N*-Nitrosamines in pharmaceutical products in has proven to be extremely challenging this being due to a series of factors:

- a requirement for high femtogram to low nanogram per milliliter level limits of quantitation (LOQ) and limits of detection (LOD)
- the critical levels of specificity and analytical rigor required to minimize the risk of false positives and false negatives during both confirmatory and QC laboratory testing at these ultra-trace levels
- the requirement for advanced analytical instrumentation, to deliver robust validated methods for the targeted acceptable intakes
- challenging method development, validation and technical transfer driven by the nature of the *N*-Nitrosamines targeted from the risk assessment process

Hydrochlorothiazide has a particularly reactive anilinic amine functionality with a low pK_a and as such it is very reactive and thus is prone to generating analytical artifacts, both formation and decomposition during sample preparation, during the analytical workflow (Myers et al., 2013) (Fritzsche et al., 2022) its low pK_a and the relative instability of NO-HCTZ to photolysis and under a range of solution conditions (Gold & Mirvish, 1977; Sieh & Perlman, 1984).

As a result extensive work hase been performed across EFPIA members and other parties to resolve and understand the analytical challenges specific to NO-HCTZ.

1.5.1 Current Status

1.5.1.1 LOD and LOQ

Measurement of levels as low as high femtogram to low nanogram per milliliter level remain challenging even with the most advanced LC-MS and GC-MS (encompasses MS/MS, high resolution MS and MS triple quadrupole) technologies. Increasing the sample loading is an option, however, this must only be considered whilst addressing sample preparation and resultant matrix effects in the broadest terms. It is of note that achieving these levels robustly involves a level of sample preparation and analytical complexity not routinely encountered in a QC (release) laboratory environment. This is further complicated by the need to consider the impact on the analysis of the drug substance as well as the effect each drug product formulation matrix upon the analysis. The inherent reactivity of the HCTZ API and the instability of the NO-HCTZ analyte further add to this complex analytical challenge. Conservative LOD and LOQ values have been targeted using a class specific threshold of theoretical concern (TTC) of 18 ng/d.

1.5.1.2 Sample preparation

Method development strategies have observed that sample preparation is linked to a more robust final analytical method, and that preparation choices are broadly linked to each manufacturers drug product formulation. Minimizing sample loading i.e. leveraging the most sensitive MS systems available, has also been proved to improve analytical performance, potentially by limiting the amount of matrix the LC-MS/MS or the LC-HRAMS system is exposed to. In development strategies to date more involved sample preparation approaches such as liquid-liquid extraction, or dispersive liquid extraction, have not been pursued due to an inability to control degradation

and NO-HCTZ formation in these more complex approaches. Simpler dissolution of the major API components with limited ultrasonication and then centrifugation has been the general approach to API and drug product analysis so far. These approaches have been constrained by the observed risk of generating artefactual false negatives or false positives.

1.5.1.3 Method Development and validation considerations

The instability of NO-HCTZ under specific solution condition and at ambient temperatures must be borne in mind during development. Adequate control of formation of and degradation of NOHCTZ must be developed into the method and demonstrated. Measures such as exposing samples preparations to ambient temperatures for the minimum amount of time and ensuring preparations are kept refrigerated, also helps manage the risk of artefactual formation or degradation of NO-HCTZ, but this complicates analytical development. The limited commercial availability of stable labeled standards further constrains the approaches that can be taken and increases the potential for uncertainty at the ultra-trace levels targeted. A QbD approach to investigation of factors potentially impacting robustness has been utilized to manage the complexity. In addition, thorough structured suppression profiling of the various matrices, and their individual components, has been required to gain understanding of the challenges observed during NO-HCTZ analytical method development. Appropriate validation considering the uncertainty inherent in ultra-trace analysis at fg/mL to ng/mL levels (European Reference laboratories for residues of pesticides, 2020; Horwitz, 1982) is applied for N-Nitrosamine analysis.

1.5.1.4 Sources of false positives

A potential source of false positives is the reaction of nitrite, present in drug product excipients, with the secondary aniline amine present in HCTZ API. The use of liquid-liquid extraction in dichloromethane in certain scenarios can cause or accelerate nitrosation [1]. It is particularly challenging to quench/scavenge the formation of NO-HCTZ due to its high reactivity and low pKa.

1.5.1.5 Preliminary Outcomes

Detectable levels of NO-HCTZ have now been observed in several drug products. Investigations in levels and root causes is ongoing. Development and validation activities to support Step 2 testing for the remaining HCTZ containing drug products are being actively pursued.

1.6 Further Studies

Further studies are being conducted to support the proposed hypothesis that NO-HCTZ is extremely labile and that reported Ames positives correlate with the generation of Formaldehyde. These include a repeat of the Ames test in the presence of formaldehyde dehydrogenase and a study using NO-HCTZ to measure in vivo exposure in rats.

1.7 EXPERIMENTAL INVESTIGATIONS

1.7.1 SOLUTION STABILITY

The stability of NO-HCTZ at pH 7 was investigated, see Figure 4

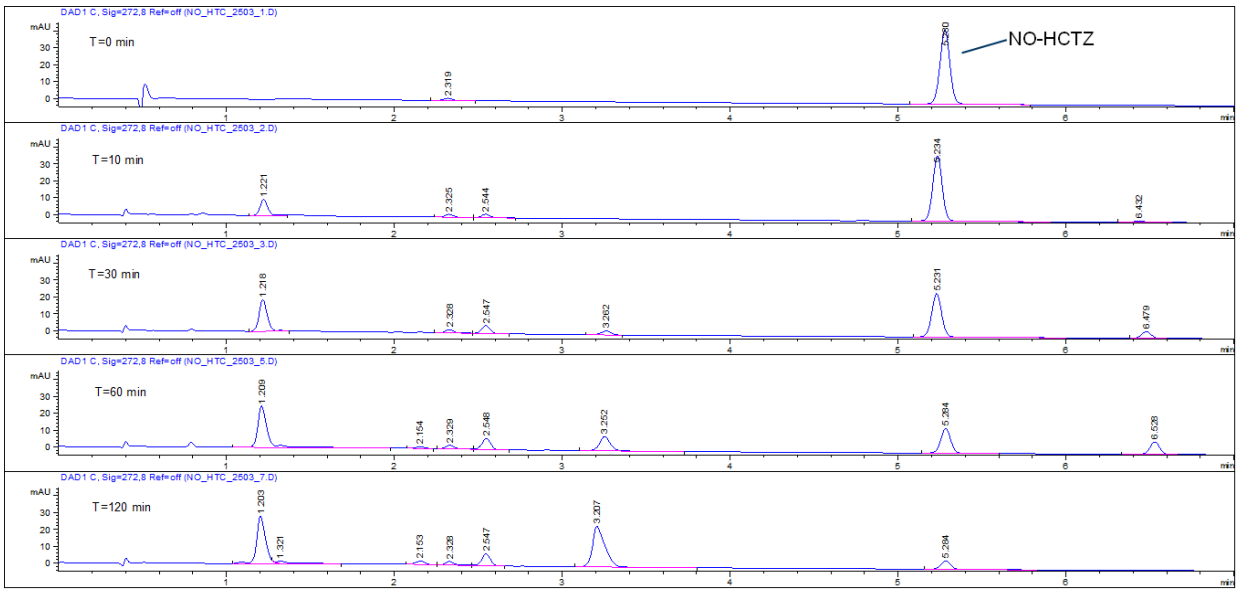
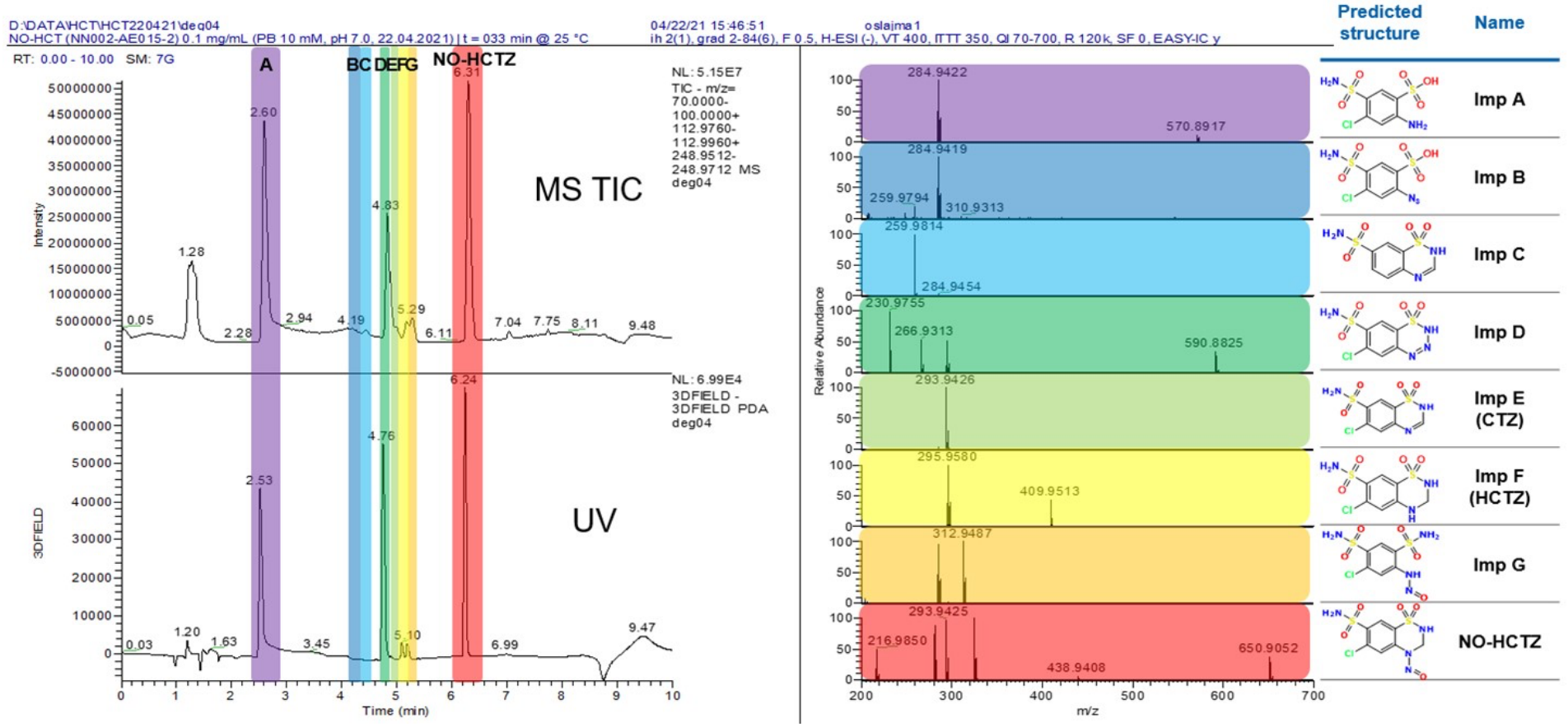


Figure 4: Degradation of NO-HCTZ in phosphate buffer pH 7, 22° C

Further investigation, including mass based identification of species present, see figure 5, showed a series of products, the most significant based on semi-quantitative analysis, were defined as Impurity A and Impurity D.

Figure 6 defines tentative structures for the products concerned. In relation to impurity A – based simply on mass, two possible structures were identified, these were investigated further through a series of NMR studies

Figure 5: Degradation of NO-HCTZ after 0.5 h in phosphate buffer pH 7.0 @ 22 °C (HPLC-UV-HRMS)



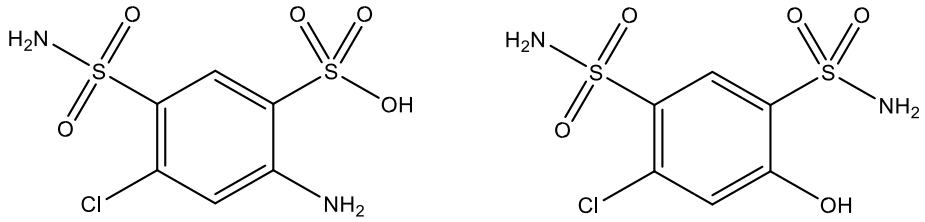
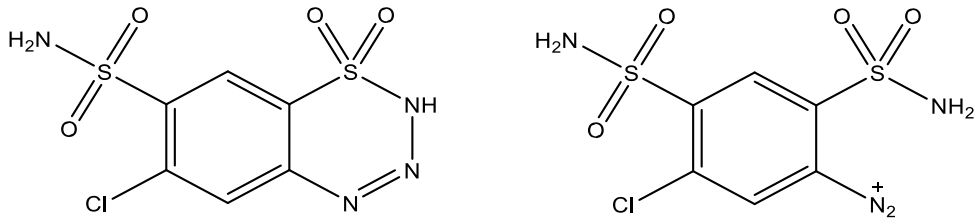


Figure 6: Two possible structures of NO-HCTZ-impurity A according to MS/MS data

Similarly two possible structures were identified for Impurity D – figure 7



Thiatriazine **Diazonium**

Figure 7: Two possible structures of NO-HCTZ-impurity D according to MS/MS data

1.8 Experimental – Clarification of Degradation product structures

In order to determine the specific nature of the degradants of Nitroso HCTZ are series of spectroscopic studies were conducted

1.8.1 Impurity A NMR studies

NMR Studies were conducted on Impurity A in order to differentiate between the two possible structure. An initial experiment was conducted whereby the ^{13}C NMR spectrum of Impurity A was compared to that of the synthetic intermediate (4-amino-6-chlorobenzene-1,3-disulfonamide). Alpha amino carbon atoms (marked with arrow in Fig. 8) have very simillar ^{13}C chemical shifts proving the position of the amino functional group (figure 9).

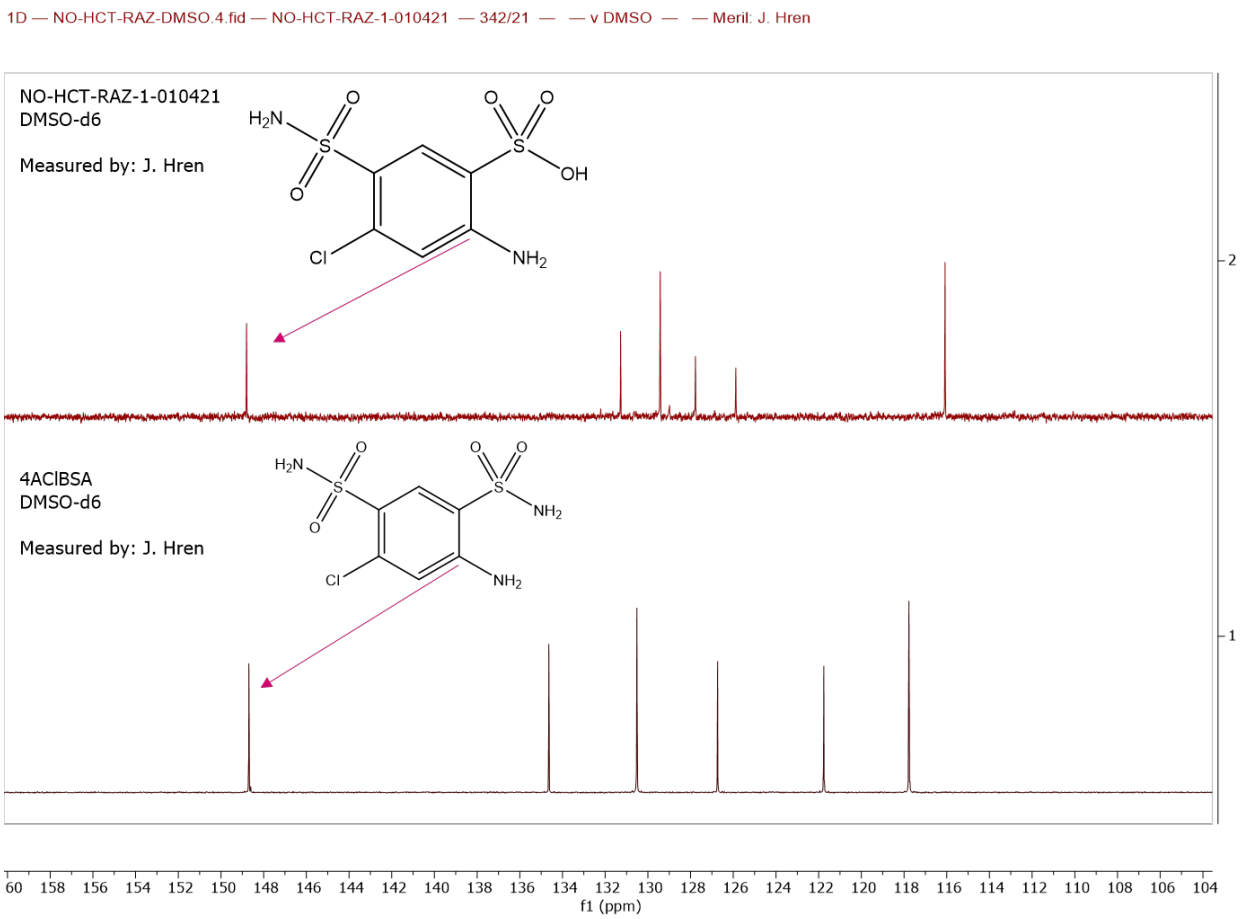


Figure 8: Comparison of ^{13}C NMR spectrum of known compound (4AClBSA) and NO-HCTZ-impA

This was then confirmed by carrying out a 2D ^{1}H-^{15}N HMBC experiments for both NO-HCTZ-imp A and 4amino-6-chlorobenzene-1,3-disulfonamide respectively, see figures 9 and 10.

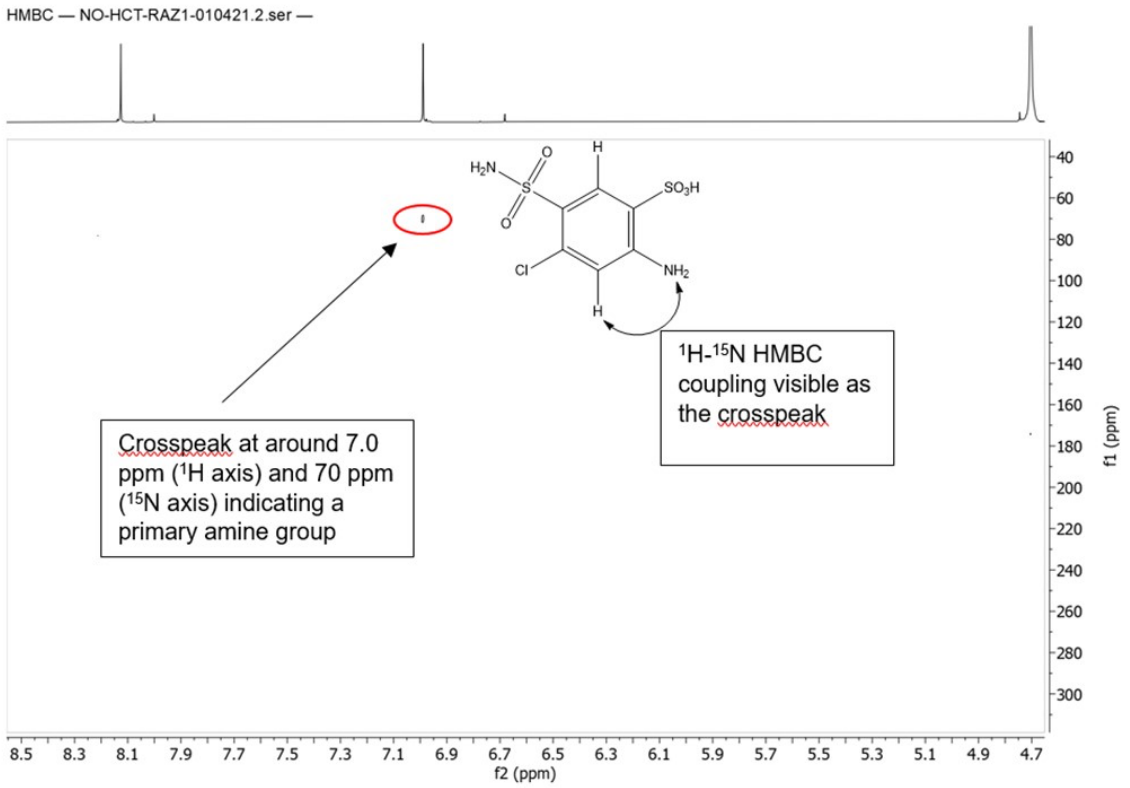


Figure 9: NO-HCTZ-imp A 2D ^{1}H-^{15}N HMBC spectrum

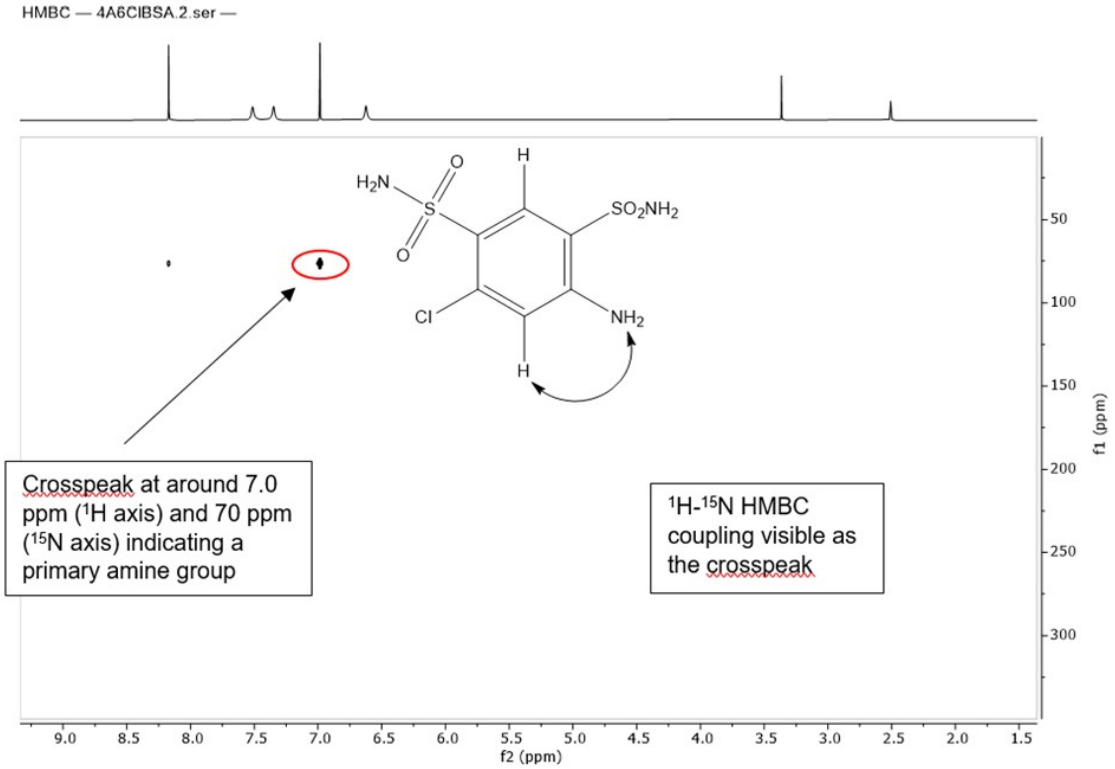


Figure 10: 4-amino-6-chlorobenzene-1,3-disulfonamide 2D ^{1}H-^{15}N HMBC spectrum for comparison

- comparison of ^{13}C NMR data of NO-HCTZ-imp A and 4-amino-6-chlorobenzene-1,3-disulfonamide show high chemical shift match between both carbon atoms bonded to amine group

- ^{1}H-^{15}N HMBC experiment data show long range ^{1}H-^{15}N correlations at similar chemical shifts in both dimensions pointing to primary amine functional group (^{15}N chemical shift at ~75 ppm). The results of these NMR experiments support the proposal that Impurity A is the amino compound as opposed to the isobaric phenol.

1.8.2 Impurity D

In an analogous manner an NMR experiment was conducted to determine the specific structure of Impurity D; as highlighted two possible structures were identified – see figure 6.

NMR experiments confirmed this to be the thiathiazine – the absence of signal of the compound at around 115 ppm in the ^{13}C NMR spectrum proving the compound is not a diazonium ion. Acquired spectra point to the thiatriazine structure presented in the spectrum (figure 11).

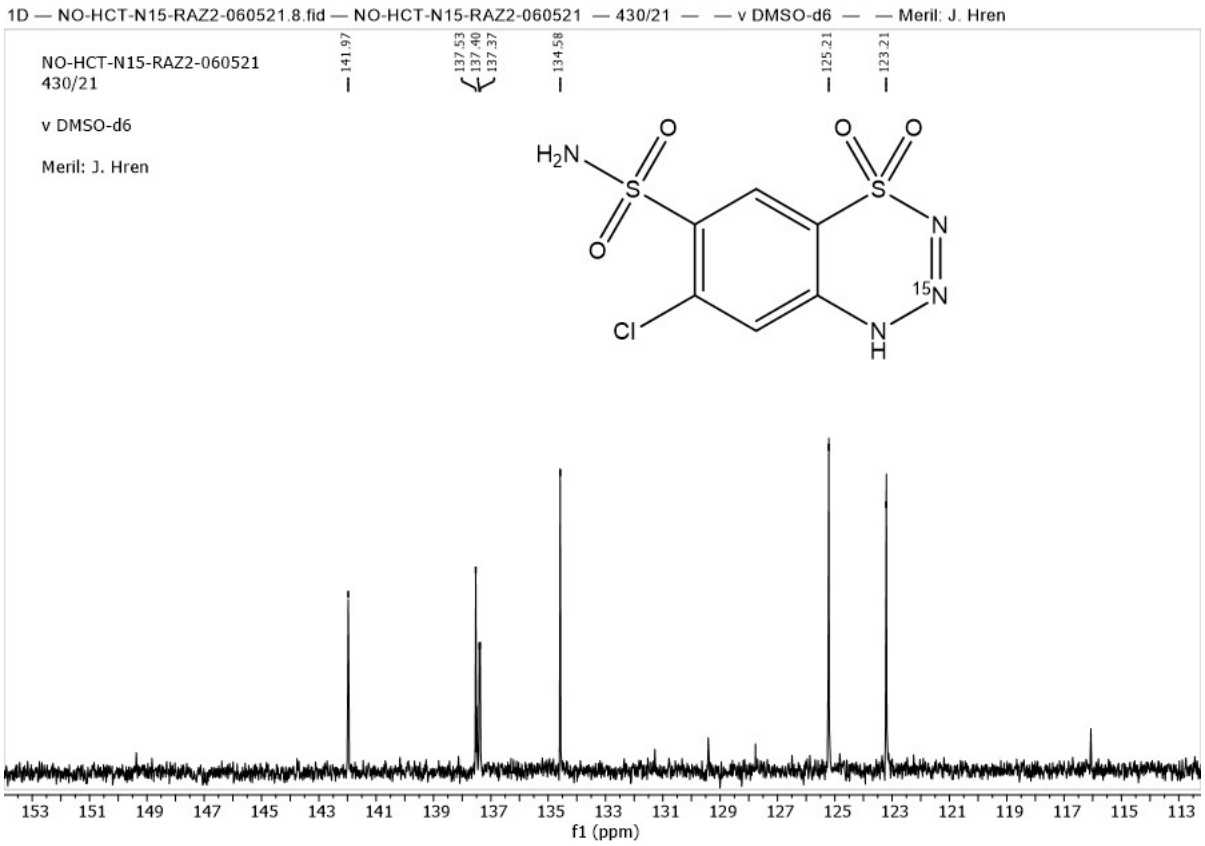


Figure 11 - ^{13}C NMR spectrum of NO-HCTZ-imp D isolated from decomposed ^{15}NO-HCTZ

1.8.3 Mechanism of degradation

Having identified the principal species formed by degradation of NO-HCTZ, it is possible to postulate a mechanism – figure 12

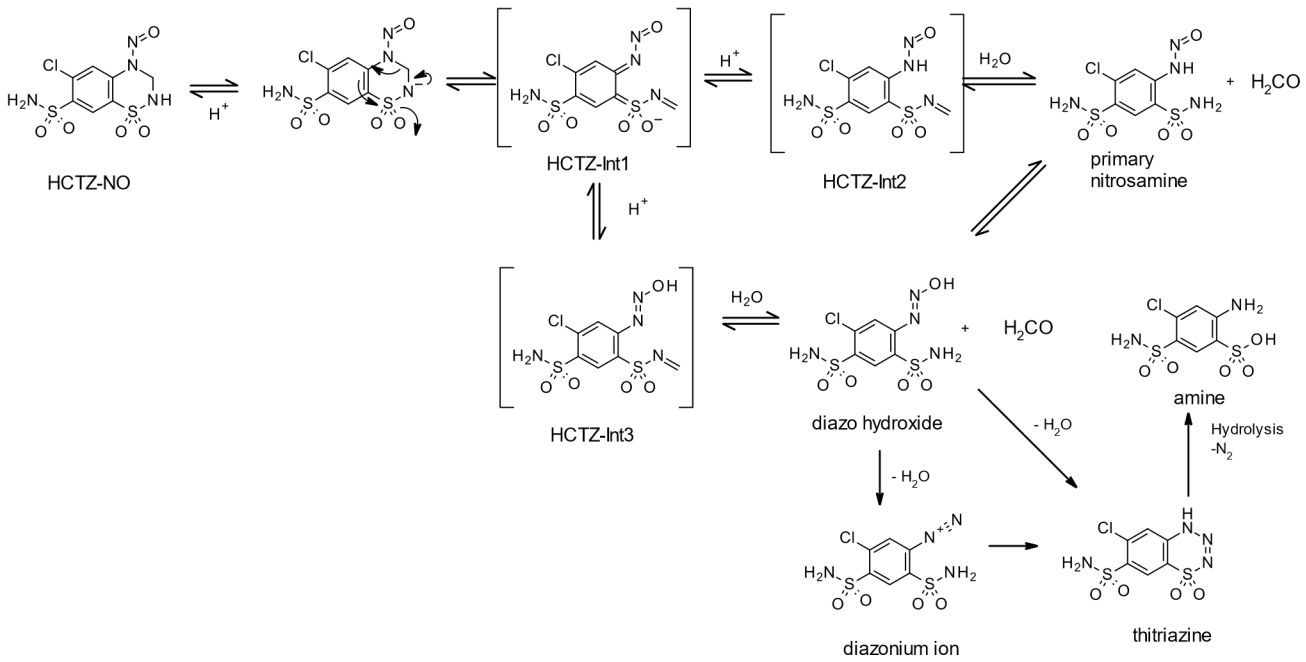


Figure 12 – Proposed Degradation pathway for NO-HCTZ

Such a mechanism is consistent with the weakly basic nature of NO-HCTZ, this being a sulphonamide and the facile formation of the associated anion and liberation of Formaldehyde

Critical to the understanding of the hazard associated with NO-HCTZ is understanding of the degradative profile and liberation of formaldehyde. Initial Chromagraphic studies of the degradation of NO-HCTZ showed a clear correlation between disappearance of NO-HCTZ as a function of time and the formation of formaldehyde, figure 13.

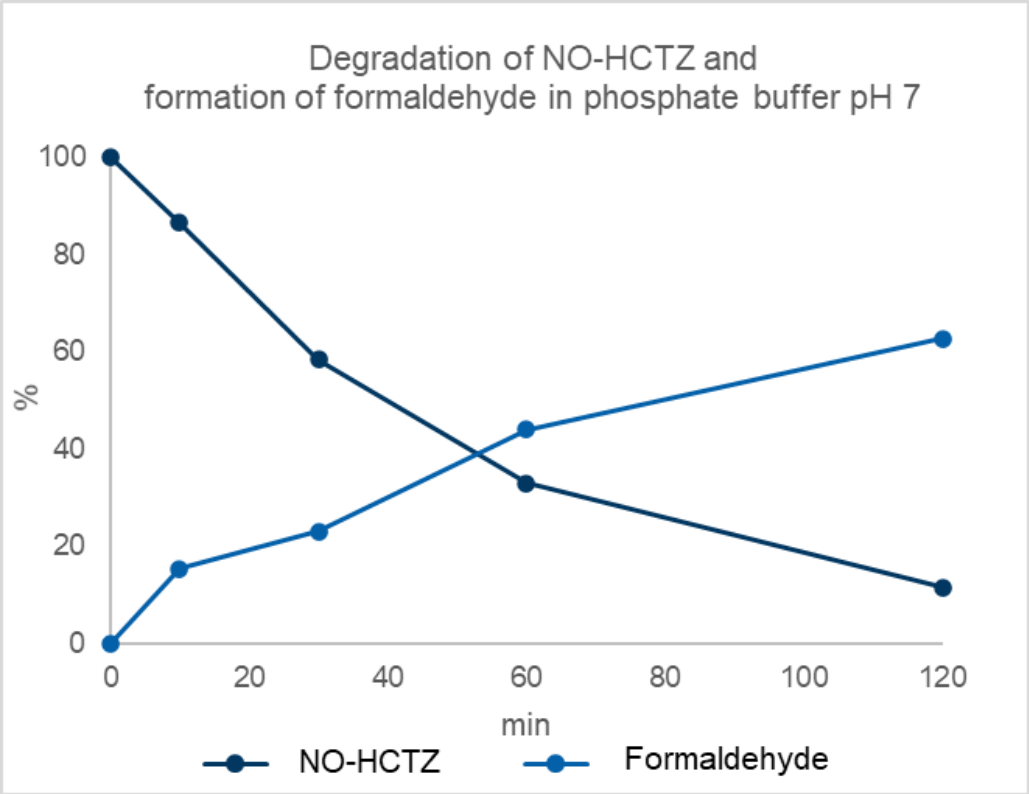


Figure 13: Degradation of NO-HCTZ and formaldehyde formation in phosphate buffer, pH 7

Also plotted is the relationship between formation of Formaldehyde and concentration of NO-HCTZ (figure 14).

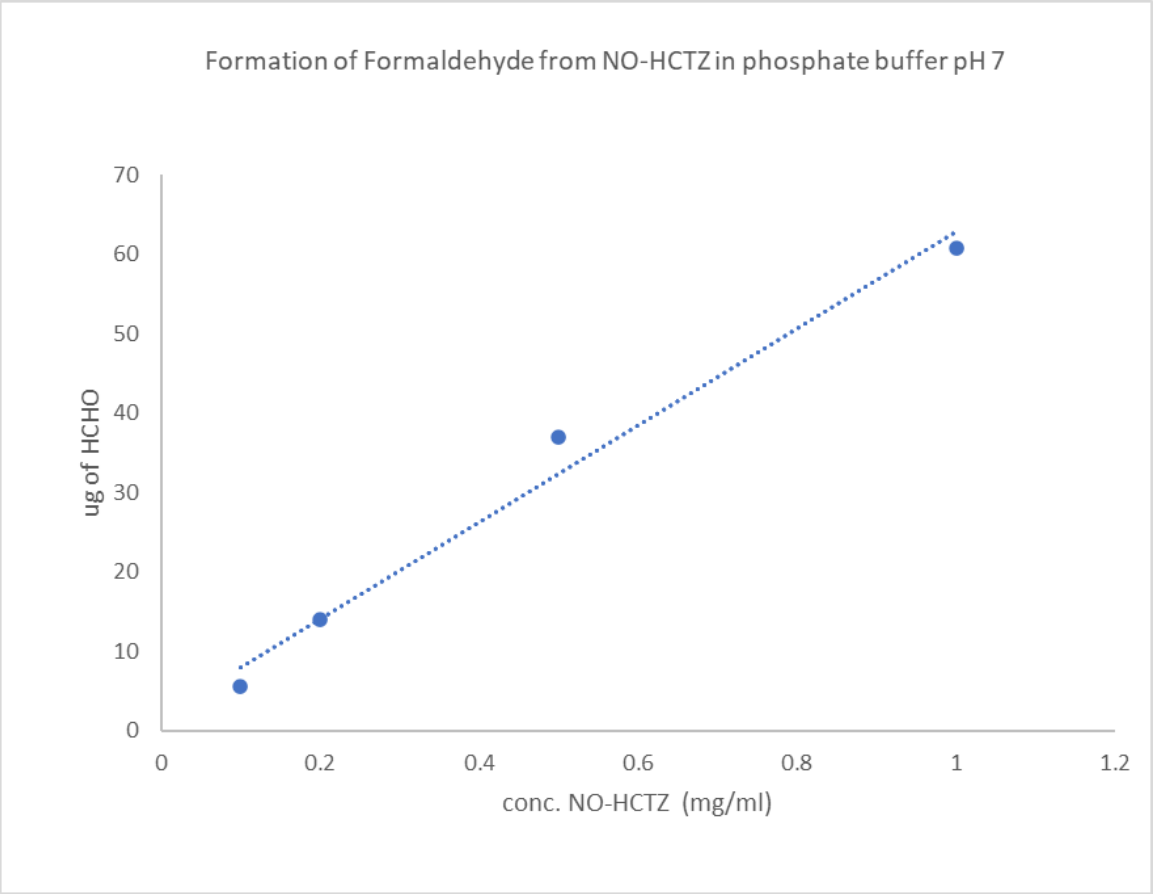


Figure 14- Linear relationship between formation of formaldehyde (HCHO) (µg) and concentration of NOHCTZ.

1.8.4 NMR Studies reaction (decomposition) monitoring studies

To evaluate this further a series of studies were performed using a sample of ^{13}C and ^{2}H labelled HCTZ-NO. To track decomposition the ^{2}H NMR integral versus time data was converted into fractional conversion versus time. Critically loss of NO-HCTZ and appearance of formaldehyde fitted to a first order rate law using Dynochem. A reasonable fit obtained $k=9.38\times10^{-4}$ s^{-1} (±13%) with a $t_{½}$ ~ 12 minutes. This is highly consistent with the earlier observed results that show rapid degradation at neutral pH (see figures 5 and 6) and implies that formaldehyde is formed from same manifold as HCTZ-NO degradation. Unfortunately only the later portion of the degradation of NO-HCTZ could be observed in this manner.

It was noted that while it was possible to profile the reaction using a Bruker InsightExpress system this proved challenging as the quality of the signal was impacted by the formation of bubbles of nitrogen within the narrow diameter flow tube. An observation that is consistent with the proposed route of formation of the Amine degradant.

A modified experimental approach using a conventional NMR tube and NO-HCTZ in a deuterated phosphate buffer system meant that loss of NO-HCTZ and formation of the main products could be monitored. Four significant species could be seen in the aromatic region including the thiatriazene believed to be the main product and Amine

Further experiments allowed the level of formaldehyde to be accurately quantified, indeed as shown in Figure 15, a good mass balance was achieved.

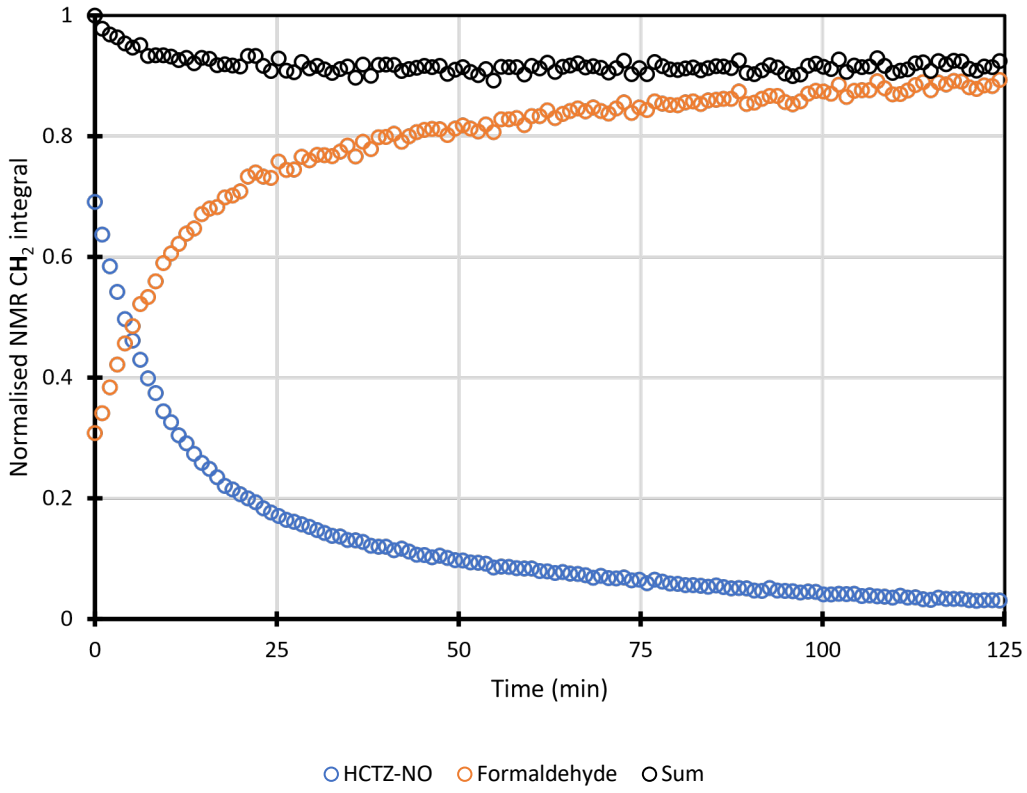
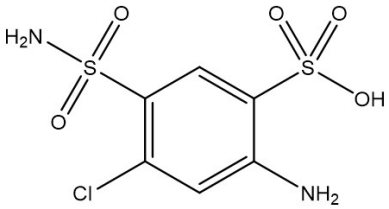


Figure 15: ^{1}H NMR based profile for the loss of NO-HCTZ in pH 7.2 phosphate buffer at 37 °C

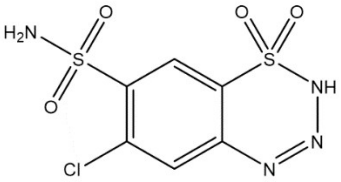
1.9 Conclusion of experimental investigations

From the investigations described the following key conclusions can be drawn

1. NO-HCTZ is inherently unstable with a half-life of ~12 minutes under physiological pH (at 22 °C)
2. Formaldehyde formation clearly linked to NO-HCTZ loss
3. Two significant products formed:
    a. Thiatriazene (impurity D)
    b. Amine (impurity A)



Amine



Thiatriazine

As well as the release of formaldehyde (limit 10 mg/day).

Neither of these are *N*-nitrosamines belonging to the cohort of concern and thus control of either to ng levels is inappropriate and instead they should be controlled based on the TTC, 1.5 µg/day. Given that the parent NO-HCTZ is present at ng level then there is no specific concern and there is no requirement for implementation of any additional controls.

1.10 References

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