Response to the Letter to the Editor of Heliyon Re: determination of dimethylamine and nitrite in pharmaceuticals by ion chromatography to assess the likelihood of nitrosamine formation Heliyon. 2021; 77: e06179

Dear Editor,

We would like to thank the letter authors for reading and reviewing our paper. This letter responds to the individual points made in their letter. First, we stand by our published method for measuring nitrite in drug products and the reported values. Our statement at the end of Section 3.4 is a good summary of how we feel about our methods for nitrite and amines: “Therefore, in our opinion these methods are best used for developing processes that limit the amount of DMA or other amine and nitrite during the synthesis of the API and formulation of the product.” Importantly we are stating these methods can be used to develop processes. We are not a pharmaceutical company and do not have some of the resources necessary to ensure we have the appropriate sample preparation and an optimized chromatography method for a given drug product or related sample, and that will be reflected in some of our responses that follow. In fact, the authors note our sample limitation in their discussion: “The undiluted APIs were sourced from a life science company and may therefore not be fully equivalent to pharmaceutical grade API in terms of quality, purity, and impurity profile.”

With respect to the Discussion, from its start to the paragraph that suggests possible methodology flaws, we have no issues and trust the authors’ expertise.

The letter suggested that there were possibly some methodology flaws that could have led to artificially high nitrite values. Here we address each of those possible methodology flaws.

a. Sample preparation:

Point i: A significant number of syringe filters contain levels of nitrite and therefore could have led to overestimation of the nitrite levels reported during analysis.

Our response: Though we cannot completely discount this possibility, we use PES syringe filters. We have tested these, and they do not contain detectable levels of nitrite. We used these filters for all the seven samples. Sample #1 had no nitrite. If nitrite originated from the filter, we would expect nitrite in sample #1.

Point ii: The manuscript highlights that the samples were sonicated until they were dissolved. Not all products would have been fully dissolved, even after extended sonication as excipients such as magnesium stearate (used in Metformin products) are insoluble in water. It is likely that for this reason the centrifugation and filtration step were included.

Our response: The drug substances were fully dissolved, but as suggested, drug products are not. For this reason, we centrifuged and filtered each sample. We believe nitrite should be dissolved in water solution after sonication. If we were not capturing all the nitrite it would result in lower reported values rather than values that are too high as suggested.

Point iii: The sonication time is also not specified which causes serious concerns regarding the viability of the procedure. The formation of both nitrite and nitrate upon sonication of aerated water is well-known. Analysis within the consortium highlighted that extended sonication for certain excipients including magnesium stearate generates significant levels of artefactual nitrite during sample preparation.

Our response: We did not specify the sonication time because the times vary for drug substances and drug products. For drug substances, the times were short while for some drug products 30 min was required. We were not aware that nitrite could be generated by sonicating magnesium stearate. We will note that we detected nitrite (27 ug/g) in sample #2, a drug substance, which does not contain magnesium stearate and has a short sonication period.

b. Methodology

Point i: It is claimed that the methods are validated however the tests discussed do not provide sufficient data to demonstrate that the methods are fit for purpose, for example:

• Specificity, the most important validation parameter has not been demonstrated for the analysis of nitrite in the different sample matrices. Data has been collected using both a UV and conductivity detector, representative chromatograms should be provided to demonstrate the method is specific for the analyte. Additionally, there was access to IC-MS technology, which could have been used to demonstrate specificity and peak purity. Specificity might have been demonstrated for common inorganic anions; however, many organic compounds absorb UV light at 210 nm and specificity for common organic impurities for each product has not been demonstrated. Furthermore, the chromatographic method does not seem to be robust as the baseline does not reach the starting height once a run is completed, see Figures 6 and 7 of the original publication.

Our response: For specificity we used retention time of the reference standard and two detection techniques. This approach is also consistent with identification tests used in United States Pharmacopeia monographs. We also understand the selectivity of the anion-exchange column we used for this application. In other words, we know where common anions and small organic acids elute on the column. The IC-MS experiment would certainly help, and we could have used the MS had we felt it necessary as we did for the amine portion of the publication.
If we had collected the data for a few more minutes you would see that the baseline returns to the starting height (background). After 30 min the system is preparing the next injection, by time the next injection occurs the baseline has returned to the starting background. If we had not designed a method to return to the starting background, we would have observed poor retention time reproducibility.

- The signal-to-noise ratio has only been calculated in standard solutions and does not provide a true representation in each sample matrix.

Our response: We do not have access to blank sample matrices, as a pharmaceutical company would and that is why we calculated S/N using a standard solution. In our experience when the chromatogram does not have several unknown peaks eluting near the peak of interest, the standard provides a good estimate of the detection limit. The sensitivity we are reporting is typical for determining nitrite by absorbance detection with the volume injected.

- The precision data reported is based on 3 standard injections (of a high-level standard) over 3 days. This does not provide any representation of the repeatability or precision of the method in the presence of the sample matrix.

Our response: We have reported precision as noted. We have confidence in the repeatability of our chromatography method for samples and that can be observed in Tables 3 and 4. Those tables show the results of sample and spiked sample analysis, respectively. Note that each data point is the average of six injections over three days. The highest RSD was 2.9% for the analysis of sample #6.

Point ii: There has been no mention around preparation blanks so cross-contamination of nitrite cannot be ruled out.

Our response: True blanks would use a drug product sample with the API as well as the individual excipients that make up the drug product. We do not have these. That said, we can consider sample #1 as a process blank. Though it contains sample, it went through the whole process and was found to contain no nitrite. We also know that our DI water contains no nitrite as well as the PES syringe filters.

Point iii: Due to the low sample concentration used any variability in the method impacts the reported results more significantly in comparison to using larger sample concentrations.

Our response: We do not believe this is pertinent to our method. Tables 3 and 4 show we have little variability in our measurements (<3% for all samples). We use a calibrated analytical balance to weigh the sample so are confident in the amount of material we are starting with.

Point iii: Analysis was performed on single tablets only; this does not provide any representative data due to tablet variability. Nevertheless, the levels of nitrite reported seem artificially high. We only had access to one tablet of metformin. It is possible that assaying multiple drug products would reveal overall lower nitrite concentrations. This does not change one of the central tenets of our publication that our method can be used to determine nitrite in these samples. Ironically, while developing this method one of our concerns was losing nitrite during sample preparation as it can be easily oxidized to nitrate.

Point iv: A quadratic fit for the trend line was used for the analysis of DMA. Was this also used for the analysis of nitrite? If so, this could cause inaccuracies at the extremes of the calibration curve. While the DMA calibration was fit with a quadratic function, the nitrite calibration was fit with a linear function. We agree that we did not specifically state that in Section 3.2 and the reader might assume we also used a quadratic function for nitrite.

Other points

The authors claim that their method is more sensitive than spectrophotometry and reference a publication from Narayana et al. (2009) with a LOD of 930 μg/L. Commercially available test kits for nitrite determination in aqueous solution reach LOQs of down to 30 μg/L (see e.g. https://www.sigmaaldrich.com/DE/de/product/mm/114547) which is about 30-fold lower than reported by the authors of this publication. Therefore, their statement is not valid.

Our response: The publication cited used a colorimetric test to determine nitrite in pharmaceuticals. There may be tests that have more sensitivity for pharmaceutical samples though these tests are subject to both negative and positive interferences. Regardless, our technique for measuring nitrite has more sensitivity than the commercially available test kit.

In the Introduction it is stated “For example, the nitrite level of 95.6 ppm in sample 7, Ranitidine (drug product), does not compare with any of the levels found in the nitrites in excipients database.”

While a higher level than has previously been recorded invites scrutiny, it does not in itself disqualify the measurement. Given that it was a measurement of a single sample it also does not mean it is the expected value for that product. Again, the intention of this publication was to show that nitrite and DMA (and other amines) can be measured in drug substances and drug products as surveillance methods for the possibility of nitrosamine formation.

Thanks again for your interest in our manuscript,

**Declarations**

**Author contribution statement**

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**Declaration of interests statement**

The authors declare no conflict of interest.

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