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Hunt for NDSRIs: Unveiling Hidden Threats with the Novel ^{15}N-Enriched NAP Test

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ABSTRACT: Nitrosamine impurities, particularly nitrosamine drug substance-related impurities (NDSRIs), have emerged as a critical concern in pharmaceutical manufacturing due to their potential carcinogenicity. Regulatory agencies now require rigorous risk assessments and confirmatory testing to ensure product safety, considering all nitrogen atom alert groups. In this study, we present a novel strategy that combines the nitrosation assay procedure (NAP) with ^{15}N-enriched nitrosating reagents and ^{15}N NMR spectroscopy to detect and characterize nitrosamine formation. This method enables qualitative analysis of nitrosamines and provides valuable insight into the nitrosation reactivity of pharmaceutical compounds. The diagnostic chemical shift range for *N*−NO groups was validated, and the method was applied to a series of known nitrosamines and NDSRIs derived from active pharmaceutical ingredients and intermediates. The ^{15}N-enriched NAP test proved effective in identifying nitrosamines, even in complex matrices, and distinguishing between isomeric and degradation products. This integrated approach provides a robust and rapid tool for nitrosamine risk assessment and supports regulatory compliance by confirming or excluding nitrosamine formation under stress conditions.

KEYWORDS: *nitrosamine, ^{15}N NMR, NAP test, active pharmaceutical ingredient (API), nitrosamine drug substance related impurities (NDSRIs), sodium nitrite*

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1. INTRODUCTION

1.1. Nitrosamines, Introduction to the Chemistry and Related Problems. Nitrosamines, a subclass of *N*-nitroso compounds, are well-established genotoxic impurities with significant regulatory concern due to their carcinogenic potential. Their presence in pharmaceutical products, notably in sartan APIs such as valsartan, has prompted global regulatory agencies to implement stringent control strategies, including risk assessments and confirmatory testing.[1,2] Recently, focus has shifted to nitrosamine drug substance-related impurities (NDSRIs), which are structurally similar to APIs but are not known, and therefore have to be characterized.[3] The development of highly sensitive analytical methods simplifies the detection of these impurities.

Structurally, nitrosamines exhibit resonance between neutral and zwitterionic forms (Scheme 1), with restricted rotation around the N−N bond contributing to magnetic (and chemical) non-equivalence of the *N*-substituents in symmetric nitrosamines.[3]

Nitrosamines are typically formed via nitrosation reactions, which occur via the electrophilic attack of a nitrosonium ion (NO^+), generated in situ through nitrite salts (e.g., $NaNO_2$), in acidic aqueous media.[4,5] While secondary amines are the most common precursors, primary and tertiary amines can also yield nitrosamines through diazotization or nitrosative cleavage mechanisms.[6,7]

This study investigates the application of ^{15}N nuclear magnetic resonance (NMR) spectroscopy as a selective and sensitive technique for the detection and characterization of nitrosamines, supporting impurity profiling and risk mitigation in pharmaceutical development.

1.2. NAP Test. In the pharmaceutical industry, to achieve compliance with regulatory expectations, the risk of nitrosamine formation must be assessed, identifying structural alert groups, functional groups containing a nitrogen atom that may undergo nitrosation reactions. Alkylamine moieties are considered to be alert groups.

When the alert group is a secondary amine, the strategy usually applied is to synthesize the corresponding nitrosamine in the laboratory and then characterize the product with one or more appropriate analytical methods.

In ambiguous cases, where a secondary amine is not present or the nitrosamine formation is unlikely but non-negligible, literature research is conducted for known NDSRIs. This is followed by R&D work to search and test for any potentially present known NDSRIs, and more importantly, test API reactivity under nitrosation conditions to identify unknown NDSRIs.

Nitrosation conditions more commonly tested have been chosen following the NAP test according to the indications of the authorities. More specifically, EMA declares that, when no reactivity is observed, the risk is negligible:[8]

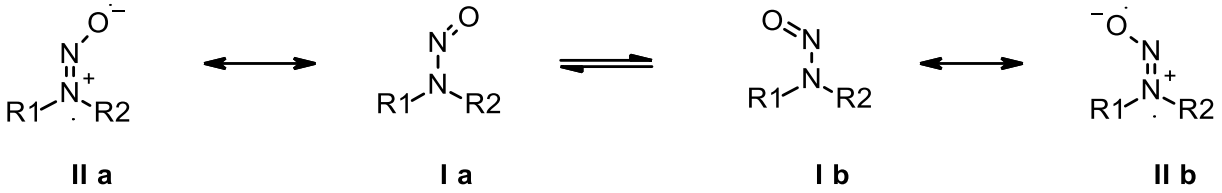
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Scheme 1. Resonance Structures of Nitrosamines



"If, despite extensive efforts, it becomes apparent that the relevant nitrosamine impurity cannot be synthesized, then this could be an indication that the nitrosamine either does not exist or that there is no risk of it being formed. In such cases, it may not be necessary to conduct confirmatory testing. This should be justified thoroughly on a case-by-case basis according to appropriate scientific principles."

Originally proposed by WHO in 1978, the NAP test, a general in vitro system, simulates worst-case nitrosation conditions under standardized protocols (Table 1), offering a practical and scientifically sound method to assess nitrosamine formation potential.[9]

Table 1. NAP Test Criteria Recommended by the WHO Expert Group in 1978

concentration of the drug	10 mmol/L
concentration of nitrite	40 mmol/L
reaction temperature	37 °C
pH	3−4
reaction times	1 and 4 h

Since the solubility and stability of chemical substances are highly variable and specific, different modifications of the original NAP test have been proposed recently to overcome these limitations.[10,11]

1.3. ^{15}N NMR Approach. Following the execution of the NAP test, one of the most challenging aspects of real-world applications is the selection of an appropriate analytical method for data acquisition and the critical interpretation of the results. This process is often far from straightforward. In many cases, highly sensitive techniques such as HPLC−MS are required to detect nitrosamines at trace levels. Nevertheless, even with advanced instrumentation, the results may remain inconclusive.

Other common analytical methods, including GC−MS and ^{1}H- and ^{13}C NMR, are generally sufficient for assessing the reactivity of secondary amines. However, their reliability diminishes when applied to complex structures such as NDSRIs, where ambiguous or nondefinitive outcomes are frequent. As such, determining the nitrosation potential of a given molecule remains a significant challenge.

This study was prompted by a regulatory requirement to perform a nitrosamine risk assessment on dexlansoprazole, an active pharmaceutical ingredient (API) manufactured by our company. Dexlansoprazole contains benzimidazole and pyridine rings, and although it lacks secondary dialkyl amines, all amine-containing moieties must be considered structural alert groups. Moreover, heterocyclic systems are known to undergo rearrangements or cleavage under stress conditions, potentially forming unexpected nitrosamines beyond the scope of standard NDSRIs.

The central question was whether the heterocyclic framework of dexlansoprazole could act as a precursor to nitrosamine formation. The NAP test was performed, and the reaction mixture was analyzed via HPLC−MS. No peaks corresponding to known nitrosamine fragmentation patterns were observed. However, the possibility of molecular rearrangements leading to nitrosamines with unpredictable molecular weights cannot be excluded.

Further analysis using ^{1}H- and ^{13}C NMR spectroscopy failed to yield conclusive structural information, as the complexity of the crude reaction mixture hindered interpretation. Literature reports indicate that dexlansoprazole is susceptible to degradation under acidic conditions, raising concerns about its behavior under nitrosating and oxidative environments.[12]

To address this uncertainty, an analytical technique capable of unequivocally confirming the presence or absence of nitrosamines in the reaction mixture was devised. The proposed solution was the application of ^{15}N NMR spectroscopy, which offers enhanced specificity for nitrogen-containing species and may provide definitive insights into the nitrosamine formation pathways.

Because of its low natural abundance (Table 2), low gyromagnetic ratio, low relative sensitivity, and often also

Table 2. Natural Abundance of ^{15}N Isotope Compared to Those of Other Common Nuclei Analysed with NMR

isotope	% natural abundance
^{1}H	99.99
^{13}C	1.07
^{15}N	**0.36**

because of long spin−lattice relaxation time (T_1, also called longitudinal relaxation time), long accumulation times are typically required for this type of ^{15}N NMR measurement. ^{15}N-enrichment can be used to overcome the problem of low sensitivity, and selective ^{15}N labeling is a very efficient assignment tool.[13]

^{15}N, being a nucleus with spin $I = 1/2$, in the presence of adjacent ^{15}N nuclei gives back a doublet signal. Hence, when an enriched ^{15}N atom is bonded to a naturally abundant ^{15}N, the latter will appear as a very weak (since only 0.3% of the nitrogen atoms are ^{15}N) doublet, coupled with the enriched one. At the same time, the enriched ^{5}N atom appears as a singlet (only a very small amount of the adjacent nitrogen is ^{15}N, and the very fast nuclear relaxation of ^{14}N averages the coupling with this isotope to zero).[14]

The ^{15}N chemical shifts in diamagnetic compounds extend over a range of 1500 ppm, significantly higher than those of ^{1}H and ^{13}C nuclei.[15] The ^{15}N NMR technique offers the possibility of investigating specific diagnostic ranges for different types of N-containing compounds and reduces the problem of signal overlap.

All these characteristics make the NMR instrument a valued ally of the chemists and a promising candidate for the nitrosamine "hunt".

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A useful but little explored application of ^{15}N NMR spectroscopy is the detection of *N*-nitroso compounds whose typical chemical shift range of *N*−NO nitrogen is 120−190 ppm (referred to as CH_3NO_2). Moreover, using ^{15}N-enriched reagents (for example, $Na^{15}NO_2$), it is possible not only to identify if the nitrosamine has formed, but also to determine the nitrogen atom involved in the reaction, but this is not a focus of the current work.

The easy approach of the NAP test was therefore combined with the added value of the ^{15}N NMR technique in this work, leading to the ^{15}N-enriched NAP test as an additional tool for nitrosamine risk assessment. When analyzing the outcome of a nitrosation experiment, the attribution of observed signals can be less than trivial if using only mass analysis, especially in the presence of other nucleophilic residues like pyridine, imidazole, or tetrazole ring nitrogens, which can potentially react.

Sometimes the products obtained from nitrosation reactions can be identified unequivocally, characterizing them with HPLC−MS, ^{1}H-spectroscopy, and ^{13}C NMR, especially if they are simple molecules carrying a secondary amine. When the substrate is a complex molecule with different nitrogen-containing functional groups, the resulting products of the nitrosation reaction can be difficult to identify or characterize through common analytical methods. Among these are some NDSRIs that give ambiguous results through HPLC−MS and ^{1}H NMR.

In these cases, ^{15}N NMR studies were very helpful. As previously mentioned, each nitrogen-containing group has a characteristic chemical shift range in the ^{15}N spectra, allowing for easy functional group identification. For example, the signal of the nitrogen atom of an NO group of a nitrosamine (*N*−NO) is found in the chemical shift range from 120 to 190 ppm (Table 3).

Table 3. ^{15}N NMR Tabulated Chemical Shift (Referred to CH_3NO_2)

^{15}N NMR-tabulated chemical shifts		
secondary amines		−380/−310 ppm
nitrosamines	R_2N−NO	−170/−100 ppm
	R_2N−NO	120/190 ppm

The NAP test and modified NAP test performed in D_2O using $Na^{15}NO_2$ as the source of nitroso group allow for the direct analysis of the reaction mixture, therefore rapidly determining whether the nitroso group has reacted with the molecule and consequently if a nitrosamine has been formed. Indeed, carrying out the reaction in the presence of $Na^{15}NO_2$, the nitroso group that will attach to the target molecule will certainly be the one coming from the ^{15}N-enriched nitrosating agent. With isotopic enrichment in ^{15}N close to 100%, the only signal that will be clearly seen in the ^{15}N NMR spectrum is that of the nitrogen belonging to the nitroso group. It will therefore be important to understand whether one or more nitrosamines of the target molecule or of its derivatives have been formed. Moreover, it is possible to unequivocally assign the $Na^{15}NO_2$ signal (229 ppm) and *t*-BuO^{15}NO signal (298 ppm).

2. RESULTS AND DISCUSSION

The approach that was followed was first the generalization of the NMR range for *N*−NO detection, second to verify its validity for known nitrosamines of interest for the pharmaceutical industry, and the last to test the usefulness of the method with a selection of NDSRIs, to define a model (Figure 1).

Initially, a series of known nitrosamines were reviewed by searching the literature for reports of ^{15}N NMR spectra and verifying whether the *N*−NO chemical shift aligned with the expected range described in the literature.[13,16] The next step involved ^{15}N NMR characterization of previously synthesized nitrosamines derived from APIs containing secondary amines. This was followed by the synthesis of each of these nitrosamines using a ^{15}N-enriched nitrosating agent. This data collection allowed for the creation of a library of nitrosamines with corresponding *N*−NO chemical shifts, confirming the specific chemical shift region where the nitroso group of the nitrosamine signals is located, and ensuring that other nitrogen signals in the molecule do not interfere with the *N*-nitroso (*N*−NO) signal. Once this approach was validated as suitable for identifying nitrosamines in organic molecules, particularly APIs, it was applied to the study of NDSRIs potentially derived from more complex APIs containing one or more amino groups.

This review allowed us to establish a diagnostic range for the signal of the nitrosamine NO group, which falls in the range 120−190 ppm, according to tabulated values.[13,16]

2.1. Known Nitrosamines. The preliminary choice of nitrosamines of interest for the pharmaceutical industry was based on information on known nitrosamines (Figure 2) regulated by authorities.[7] The corresponding ^{15}N NMR data are reported in literature;[17,18] therefore, the tabulated chemical shifts related to the NO nitrogen bonded to the amine were

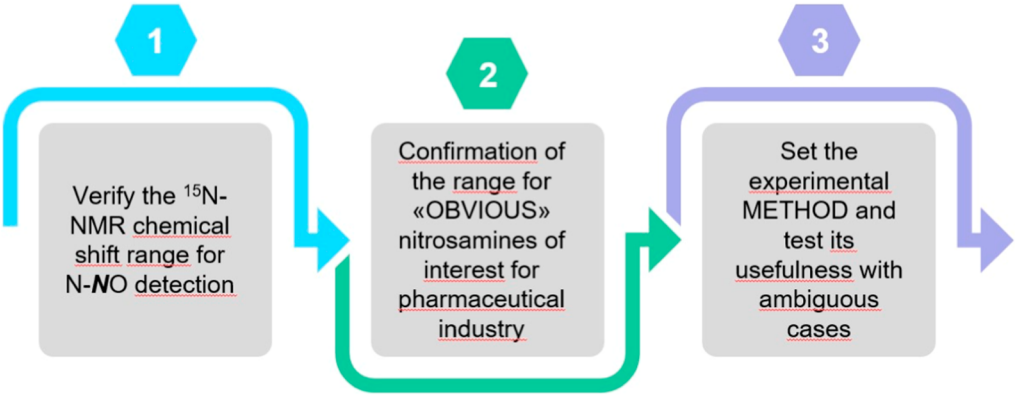


Figure 1. Three-step strategy to evaluate the applicability of the ^{15}N NMR as an analytical tool for interpreting the NAP test.

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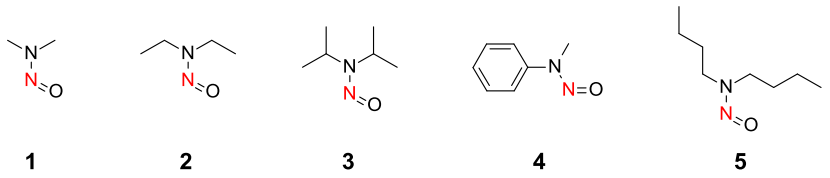


Figure 2. Chemical structures of five known nitrosamines chosen among those regulated by authorities.

used (Table 4). These values of chemical shifts were found to fit the ^{15}N NMR spectrum range that was recognized as diagnostic for the NO group of nitrosamines.

Table 4. Tabulated Chemical Shifts of Five Known Nitrosamines[a]

	N-nitrosamine	ppm (*N*–NO)
1	*N*-nitrosodimethylamine (NDMA)	149.7
2	*N*-nitrosodiethylamine (NDEA)	160.6
3	*N*-nitrosodiisopropylamine (NDIPA)	162.3
4	*N*-methyl-*N*-nitrosoaniline (NMPA)	153.6
5	*N*-nitrosodibutylamine (NDBA)	156.5

[a]The others of the known regulated nitrosamines are not reported due to the absence of experimental spectra references in the literature. We considered their synthesis in the laboratory unnecessary, also due to the known mutagenic and carcinogenic properties of these nitrosamines.

2.2. Secondary Amines from APIs—Likely Nitrosamines. The next step involved retrieving data from specific compounds of pharmaceutical interest containing a secondary amine that can form the corresponding nitrosamine under nitrosating conditions. By performing the NAP test or one of the modified condition NAP tests, we were able to synthesize a selection of molecules for the study. The use of sodium nitrite enriched with 95% ^{15}N ($Na^{15}NO_2$) in the generation of the nitrosating species enables a fast and easy identification of the diagnostic signal of the nitrogen atom bonded to the secondary amine moiety in the ^{15}N NMR spectrum.

Five compounds were taken into account (Figure 3): bupropion (**6**), nortriptyline (**7**), protriptyline (**8**), vortioxetine (**9**), and methyl 2-[[(2′-cyano[1,1′-biphenyl]-4-yl)-methyl]amino]-3-nitrobenzoate (MBN) (**10**), an intermediate in the synthesis of candesartan API.

From the number of peaks in the nitrosamine region in the ^{15}N NMR spectrum, it is possible to identify how many nitrosamines have formed.

In some cases, nitrosamine exists as a mixture of two detectable isomers, cis and trans. In fact, the nitrosamine of bupropion (**11**) precipitates as a white solid that is a mixture of two isomers. The ^{15}N NMR spectrum of the freshly dissolved compound shows two distinct signals in the nitrosamine's diagnostic region, one at 155.9 ppm and the other at 174.6 ppm (Table 4). Additionally, the ^{1}H NMR spectrum shows the presence of two isomers. The two isomers of the nitrosamine of bupropion (**11**), when in solution for about 15 min, equilibrate to only one form. This behavior is observed in all the NMR analyses; in particular, with ^{15}N NMR the only isomer detected is the one that has the signal at 174.6 ppm. This behavior suggests that the precipitate obtained from the reaction mixture is a combination of both the kinetic and thermodynamic products. When dissolved in the deuterated solvent for NMR analysis, the kinetic product then evolves into the more stable thermodynamic nitrosamine.

Another notable case of isomerism is observed with organic molecules belonging to the class of tricyclic antidepressant (TCAs).[19] These compounds share a common structural feature: a seven-membered ring flanked by two aromatic rings and a short side chain terminating in a dimethylamine moiety. The lack of planarity in the central seven-membered ring system results in conformational flexibility and interconversion. Three primary types of internal conformational mobility are possible for TCA drugs: ring inversion of the tricyclic moiety, flexing of the CH_2–X bridge within the central seven-membered ring, and flexibility of the alkyl side chain.[19] The possibility of observing different NMR signals for different conformers depends on whether the chemical exchange is slow or fast for this spectroscopic technique. Bridge flexing is a highly localized motion of the CH_2–X moiety in the central seven-membered ring, expected to occur on the nanosecond time scale. In contrast, ring inversion induces a global change

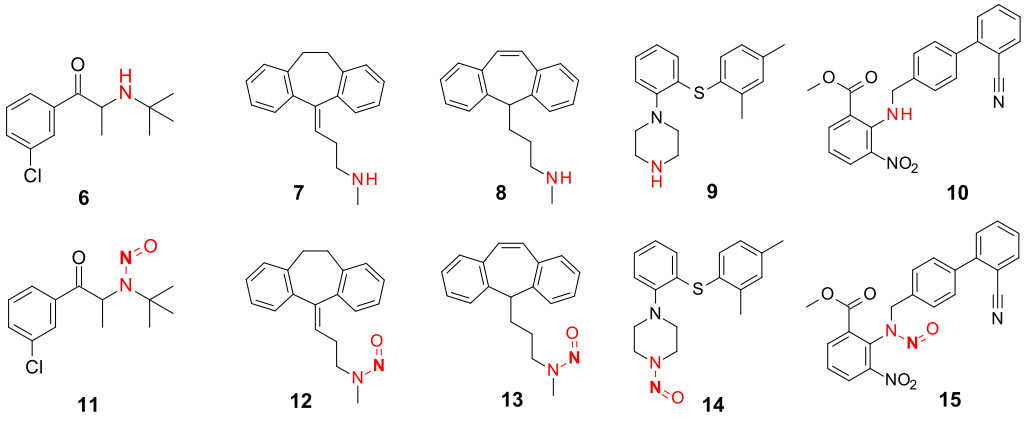


Figure 3. Secondary amines and related *N*-nitroso compounds obtained from the nitrosation reaction.

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in the TCA framework and is anticipated to occur on a much slower, millisecond time scale. This bridge flexing leads to a broadening of the two methylene group ^{1}H NMR signals, when acquired at higher temperatures (40−50 °C), which are chemically distinct. However, when the spectrum is acquired at room temperature, these signals do not split. Due to this behavior, the ^{1}H NMR spectrum exhibits a series of complex signals. This is the case for nortriptyline (**7**) and its corresponding nitrosamine *N*-nitrosonortriptyline (**12**). The ^{1}H NMR spectrum of nortriptyline shows broad signals corresponding to the bridging *ethylene* group. Moreover, the corresponding nitrosamine exists as two isomers, making the ^{1}H NMR spectra difficult to interpret. In conclusion, it is possible to assert that *N*-nitrosonortriptyline exists as a mixture of two isomers, cis and trans, due to the partial double bond character of the *N*−NO bond. In both isomers, the bridging ethylene group exhibits broad signals due to bridge flexing.

Another case of isomerism is found with the nitrosamine of protriptyline (**13**). The ^{15}N NMR spectrum shows four ^{15}NO signals at 155.1, 155.3, 155.4, and 155.6 ppm (Table 5), attributable to the four isomers (isomer ratio 1:0.26:0.25:0.10). In fact, the molecule of protriptyline (**8**) exists as a mixture of two conformational isomers, and each of the corresponding nitrosamines exists as a mixture of two isomers, cis and trans, resulting in a total of four molecules. Another significant example comes from the elucidation of the structure of the nitrosamine in MBN (**15**). The product isolated from the reaction of nitrosation of MBN is a yellow solid, which exhibits a particular type of isomerism. ^{1}H-, ^{13}C-, and ^{15}N NMR analyses show splitting of the signals, derived from the presence of nitrosamine in its two isomeric forms *Z*/*cis* and *E*/*trans*, also confirmed by an HPLC analysis that shows two peaks with different retention times. While the presence of two signals in the ^{15}N NMR nitrosamines diagnostic region, at 167.7 ppm and at 171.4 ppm (Table 5), is justified by the two isomers, this interpretation alone does not explain the complexity of the ^{1}H NMR observed. In fact, due to the partial double bond character of the N−N bond and the steric hindrance of the neighboring moiety, a couple of rotamers are observed. This endows chirality to the molecule, which results in an A-B pattern from the coupling of the geminal hydrogens of the methylene adjacent to the nitrogen.

The synthesis of these ^{15}N-enriched nitrosamines and the subsequent analysis with ^{15}N NMR technique allowed the verification and the confirmation of the chemical shift range of the *N*−NO nitrogen atom (Table 5). This range falls entirely within that reported in the literature.

Table 5. ^{15}N Chemical Shifts of Nitroso Nitrogen Atom of the Five Nitrosamines Obtained from Secondary Amines

	nitrosamine	ppm (*N*−NO)
11	*N*-nitrosobupropion	155.9
		174.6
12	*N*-nitrosonortriptyline	155.9
		156.1
13	*N*-nitrosoprotriptyline	155.1
		155.3
		155.4
		155.6
14	*N*-nitrosovortioxetine	153.2
15	*N*-nitrosoMBN	167.7
		171.4

2.3. Test the Usefulness of the Method with Ambiguous Cases. Sometimes, performing a reaction under nitrosating conditions may lead to a different, unexpected compound. It is well-known that nitrous acid (generated in nitrosation conditions from sodium nitrite in an acidic environment) can act as both an oxidizing and nitrosating agent, so oxidation can also occur during the NAP test. An additional ^{15}N NMR analysis can help with the correct interpretation and explanation of the outcome of the exposure to nitrous acid.

Two cases were investigated, namely, nifedipine (**16**) and tetrahydropterin (**18**) (Figure 4), where it was hypothesized that the secondary amine in the molecule could form the corresponding nitrosamine under nitrosation conditions. In both cases, the NAP test was conducted with $NaNO_2$ and $Na^{15}NO_2$. Both ^{15}N NMR and HPLC−MS analyses were performed, as the combination of data obtained with the two techniques helps to determine the presence or absence of a nitrosamine, particularly when the interpretation of ^{1}H NMR is not easy.

The NAP test on nifedipine (**16**) actually resulted in the formation of dehydronifedipine (**17**). The oxidation product

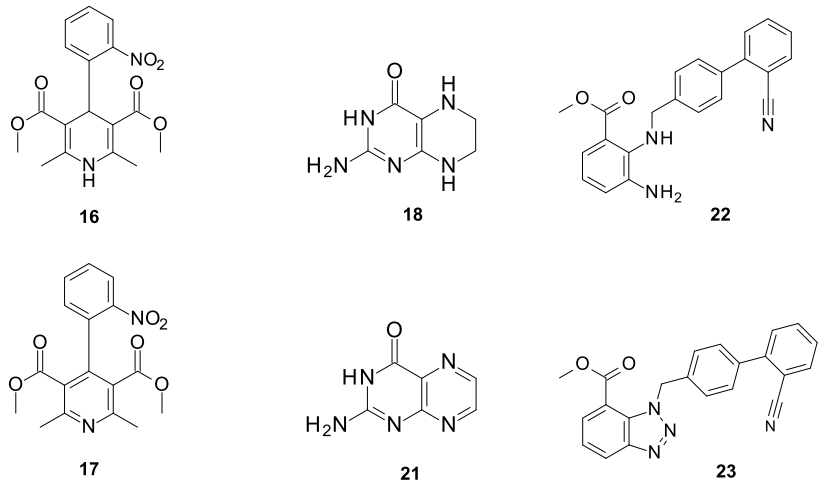
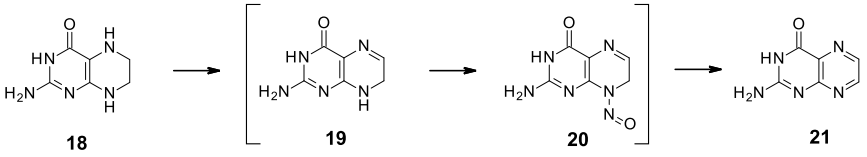


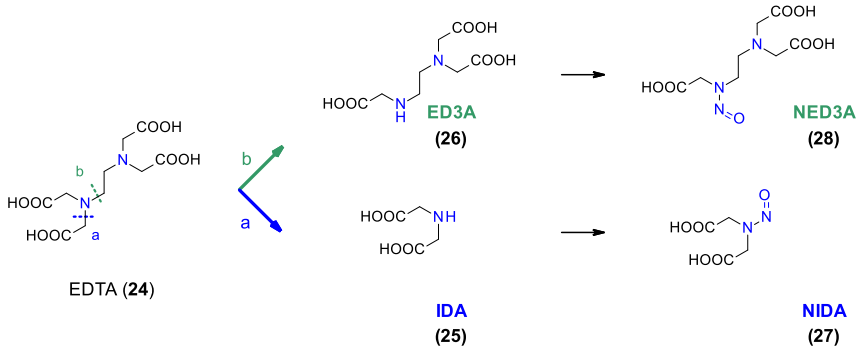
Figure 4. Ambiguous secondary amines and related products when posed in nitrosation conditions.

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Scheme 2. Hypothesis of the Conversion Pathway of Tetrahydropterin to Pterin



Scheme 3. Degradation Pathway of EDTA in the Two Corresponding Secondary Amines Which Can Generate the Corresponding Nitrosamines



was clearly identified by ^{1}H NMR, but a further confirmation was derived from the ^{15}N NMR analysis. The ^{15}N NMR spectrum of the compound treated with ^{15}N-enriched sodium nitrite did not show any signal in the nitrosamine diagnostic region. This result indicates that the NAP test on nifedipine (**16**) did not produce the corresponding *N*-nitrosamine, not even in traces, but only the oxidation product.

Regarding tetrahydropterin (**18**), the NAP test result was more complex. The ^{1}H NMR spectrum of the reaction mixture showed the presence of at least two compounds: one was the oxidation product, pterin (**21**) (Scheme 2). The other was attributable to the product of partial oxidation, dihydropterin (**19**) (Scheme 2), protonated on the nitrogen atom. The ^{15}N NMR spectrum showed a signal at 177 ppm, in the characteristic chemical shift region. However, this signal was only visible after the accumulation of a large number of scans, suggesting that it is an impurity, formed in low concentration. An additional clue in the nitrosamine investigation by means of the ^{15}N-enriched NAP test comes from HPLC−MS analyses. If a nitrosamine was formed with the NAP test, by analyzing the reaction mixture obtained with $NaNO_2$ or with $Na^{15}NO_2$, the HPLC−MS chromatograms would show the same peak at the same retention time (RT), but with two different *m*/*z* values. In fact, the chromatogram from the NAP test with $Na^{15}NO_2$ would show a peak with an *m*/*z* value +1 compared to the one with nonenriched $NaNO_2$. This allows the exclusion of every uncertainty in a nitroso-derivative attribution of a peak.

The interpretation of HPLC−MS chromatograms of the product from the NAP tests on tetrahydropterin (**18**) with $NaNO_2$ and $Na^{15}NO_2$ was carried out with this in mind; the two experiments showed minor peaks with *m*/*z* attributable to *N*-nitrosodihydropterin (**20a**) (*m*/*z* = 168 $[M + H]^+$) and ^{15}*N*-nitrosodihydropterin (**20b**) (*m*/*z* = 169 $[M + H]^+$). From these results, it was possible to conclude that tetrahydropterin (**18**), under nitrosation conditions, reacts to give the oxidized compound pterin (**21**). It is also possible to hypothesize the partial oxidation of the starting material to dihydropterin (**19**) as the intermediate (Scheme 2). However, *N*-nitrosodihydropterin (**20**) is also observed, but only as a byproduct or, more probably, as a fast-converting intermediate in the oxidation pathway.[20]

Another case of interest regards a compound containing both a primary and a secondary amine: methyl 3-amino-2-[[(2′-cyanobiphenyl-4-yl)methyl]amino]benzoate (MBA) (**22**) (Figure 4) (an intermediate in the synthesis of candesartan).

The ^{15}N-NAP test was performed on MBA (**22**) (Figure 4), and the reaction mixture was analyzed by ^{15}N NMR and HPLC−MS, showing the formation of a cyclization product. It is known that primary amines react with nitrous acid, forming the diazonium salt. In this case, the formation of the diazonium salt and the consequent cyclization with the secondary amine preceded the nitrosation of the secondary amine. Therefore, reacting MBA (**22**) with sodium nitrite under acidic conditions does not lead to the nitrosamine, but to the formation of the benzotriazole derivative, methyl 3-[[4-(2-cyanophenyl)-phenyl]methyl]benzotriazole-4-carboxylate (**23**) (Figure 4).[21] The ^{15}N NMR spectrum did not show any signal in the diagnostic region of nitrosamines, but showed only one signal at 5 ppm, a value compatible with the chemical shift of the N-2 nitrogen atom of benzotriazole (**23**).

2.4. Tertiary Amines—EDTA. Tertiary amines can pose a risk for nitrosamine formation due to potential C−N bond cleavage, forming secondary amines that act as nitrosamine precursors.[22] Several examples of pharmaceutical products with this behavior are reported.[23,24]

Ethylendiaminotetraacetic acid (EDTA) (**24**) serves very well as a model for the behavior of tertiary amines in the ^{15}N-enriched NAP test. Given its broad range of applications,[25] investigating whether EDTA (**24**) could act as a source of nitrosamines could be worthwhile. It is important to note that nitrous acid, the active nitrosating species in this context, also functions as an oxidizing agent, potentially influencing the degradation pathways of nitrogen-containing compounds.

The biological degradation of EDTA (**24**) in aqueous media, together with the identification of degradation intermediates, is described in the scientific literature.[26]

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EDTA (**24**) can undergo an oxidative C−N bond split, forming two secondary amines, iminodiacetic acid (IDA) (**25**) and ethylendiaminotriacetic acid (ED3A) (**26**), which can be the precursors of the corresponding nitrosamines (Scheme 3). The potential nitrosamines identified are *N*-nitrosoimminodiacetic acid (NIDA) (**27**) and *N*-nitrosoethylendiaminotriacetic acid (NED3A) (**28**).[27]

Starting from the amines IDA (**25**) and ED3A (**26**), the two corresponding nitrosamines NIDA (**27**) and NED3A (**28**), previously synthesized, were preliminarily characterized with HPLC−MS and ^{15}N NMR (Table 6).

Table 6. ^{15}N-NMR Chemical Shifts of NIDA and NED3A

	^{15}N NMR (ppm)
NIDA (**27**)	−141 ppm (***N***−NO) 162 ppm (*N*−**NO**)
NED3A (**28**)	−135 ppm (***N***−NO) 157 ppm (*N*−**NO**)

To investigate the behavior of EDTA (**24**) under nitrosation conditions, a ^{15}N-NAP test was performed. The reaction mixture was analyzed using ^{1}H NMR and HPLC−MS. The analytical results revealed predominantly unreacted EDTA (**24**), accompanied by only trace levels of unidentified impurities. Crucially, no HPLC−MS signals corresponding to nitrosamine standards NIDA (**27**) or NED3A (**28**) were detected.

This observation raised a critical question: can the presence of nitrosamines be definitively excluded, even at very low concentrations?

To address this, ^{15}N NMR spectroscopy was employed, comparing the spectrum of the reaction mixture with those of the NIDA (**27**) and NED3A (**28**) reference standards. The analysis showed no ^{15}N NMR signals attributable to either nitrosamine. The only detectable signal originated from residual sodium nitrite, confirming the absence of nitrosated degradation products (Figure 5).

In conclusion, under the tested nitrosation conditions, EDTA (**24**) does not undergo oxidative degradation to form the secondary amines required for NIDA (**27**) or NED3A (**28**) synthesis. As NED3A (**28**) can be an impurity of EDTA, the corresponding nitrosamine can be present in EDTA, but it is not directly derived from EDTA under nitrosating conditions.

2.5. Determination of ^{15}N NMR Detection Limit. It is often not easy, when analyzing a reaction mixture with HPLC−MS, to determine with certainty whether a nitrosamine has actually been formed. The ^{15}N NMR technique allows for the easy determination of whether a given molecule contains the *N*−NO moiety. It is relatively simple to characterize a purified nitrosamine; a concentrated solution is required when molecules are not enriched with ^{15}N, but a much lower concentration is sufficient if the NO group is enriched in ^{15}N.

The aim of this work is not to detect nitrosamines in an API (because nitrosamines in the API will not be ^{15}N-enriched), but it is important to have an idea of the detection limit of the technique. This depends, among other factors, on the setting parameters, particularly the number of scans and the relaxation delay.

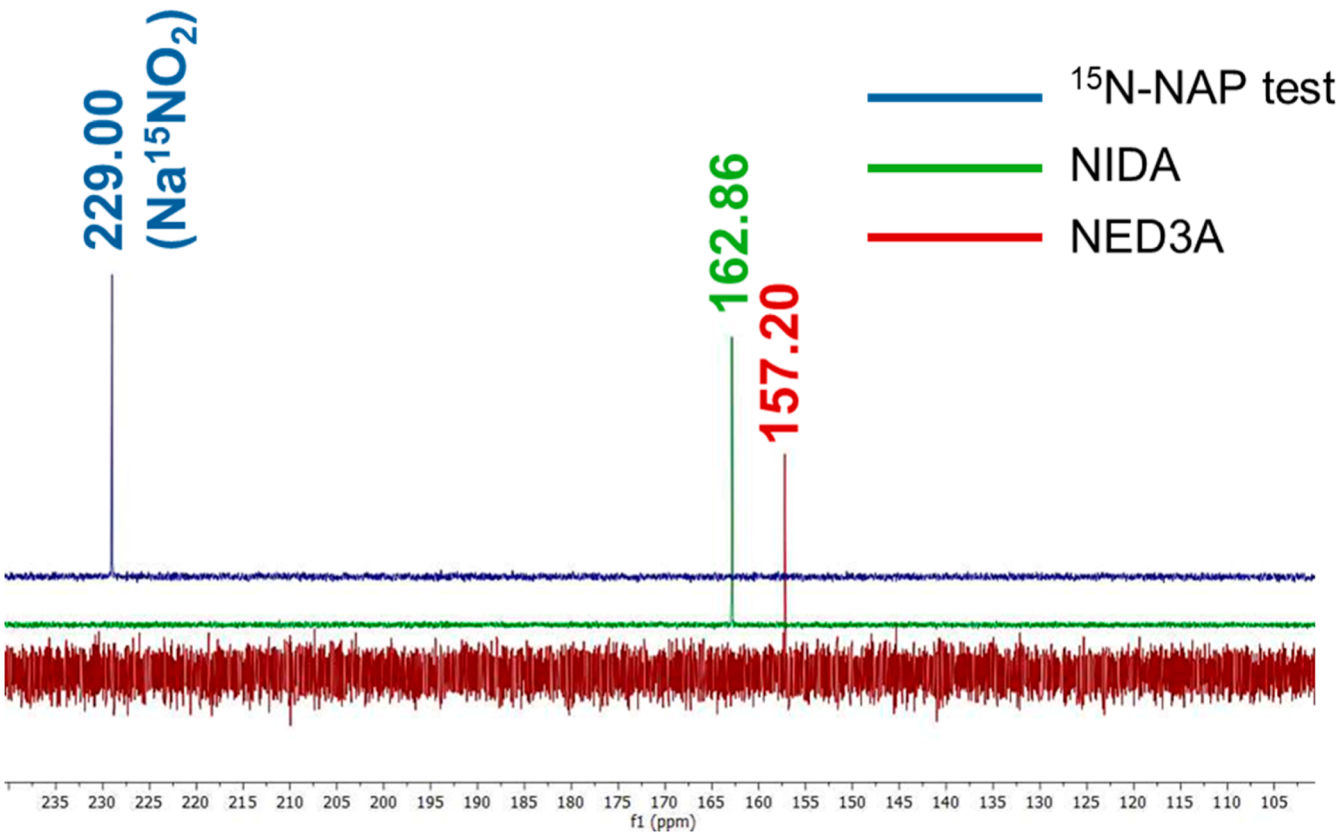


Figure 5. ^{15}N NMR spectra of NED3A (red) and NIDA (green) superimposed with the ^{15}N NMR spectra of the reaction mixture of the EDTA NAP test (blue).

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In order to evaluate the detection limit of the technique, an NIDA sample (^{15}NIDA) (**27a**) was synthesized using the ^{15}N-enriched NAP test conditions. The assay of the compound was determined through ^{1}H NMR, using 1,4-dimethoxybenzene as internal standard, on a sample in which ^{15}NIDA (**27a**) was 0.16 mol/L (a typical concentration used for standard NMR experiments) in DMSO-d_6. The assay of the compound resulted in 98%. Being the commercially available sodium nitrite ^{15}N-enriched for 98%, it is necessary to correct this value when calculating the molar concentration of the sample of ^{15}NIDA (**27a**), i.e., the concentration of NIDA ^{15}N-enriched.

The solution (0.16 M) was subsequently diluted to progressively lower concentrations to observe at which point the NO-nitrosamine signal could no longer be detected. When concentrations were reached, such that the peak of interest was no longer detectable and the number of scans gradually increased.

Table 5 reports the number of scans required to detect the ^{15}NIDA *N*−NO peak (162 ppm) in DMSO-d_6 at different concentrations. A spectral width from −200 to +300 ppm and a relaxation delay of 2 s were kept constant in all spectra (Table 7).

Table 7. NMR Conditions Required to Detect the ^{15}NIDA *N*−NO Peak at Different Concentrations

[^{15}NIDA] (mM)	[^{15}NIDA] (ppm)	acquisition time (min)	number of scans
160	26,000	3	64
50	8150	3	64
10	1630	3	64
5	815	7	128
1	163	60	860
0.5	**81.5**	**3752**	**68,544**

As shown in Table 7, 64 accumulations are sufficient for concentrations of 10 mM and above. For more diluted samples, it is necessary to increase the number of scans, up to 68,544 when [^{15}NIDA] was 0.5 mmol/L (81.5 ppm). Therefore, with the acquisition parameters used, the minimum detectable concentration of enriched NIDA is 81 ppm. The experimental conditions (such as the number of scans and relaxation delay) could be further optimized, but the detectable lower limit will not be significantly reduced unless very long acquisition times are employed.

2.6. Heterocyclic Nitrogen Compounds: Prazoles and Sartans. Two classes of pharmaceutical compounds that often raise concerns regarding nitrosamine contamination are prazoles and sartans. Prazoles contain a benzimidazole moiety, while sartans contain a tetrazole moiety. Both the benzimidazole and tetrazole rings contain nitrogen atoms; however, these are not secondary amines but rather part of the aromatic structure.

Regulatory authorities frequently require testing of sartan and prazole compounds to assess the potential risk of nitrosamine formation, which may result from nitrous acid used in the industrial process for the degradation of azides. Initially, the NAP test using $NaNO_2$ in acidic aqueous media and a modified NAP test with t-butylnitrite in organic solvent were performed on dexlansoprazole (**29**) and candesartan (**30**) APIs (Figure 6). The analysis of the reaction mixtures yielded complex results. The ^{1}H NMR spectra revealed a mixture of byproducts, including unreacted starting materials. HPLC−MS did not detect any peaks corresponding to the most predictable nitrosamine. However, it is well established that under acidic or oxidative conditions, molecules can fragment into various pieces that may potentially form a nitrosamine, complicating result interpretation and making it challenging to conclusively demonstrate the presence or absence of a nitrosamine-related substance.

To address this, the use of ^{15}N NMR was explored. NAP tests on dexlansoprazole (**29**) and candesartan (**30**) were conducted using $Na^{15}NO_2$ and *t*-Bu^{15}NO, both enriched with ^{15}N.

The NAP test, using $NaNO_2$ and $Na^{15}NO_2$ in acidic aqueous media on dexlansoprazole (**29**), resulted in a mixture of byproducts due to the molecule's instability in acidic conditions.[28] The reaction mixture was analyzed by ^{15}N NMR to check for the formation of nitrosamines; however, the spectrum, acquired with 80,960 scans, did not show any signals in the typical nitrosamine region. Further confirmation of the absence of nitrosamines came from HPLC−MS analysis, which did not detect any peaks corresponding to the predictable nitrosamine. Furthermore, no peaks with an m/z value of +1 were observed when comparing the HPLC−MS chromatogram with that of the reaction with nonenriched $NaNO_2$.

The modified NAP test with t-Bu^{15}NO in organic solvent resulted in the formation of a precipitate. The ^{1}H NMR spectrum of the precipitate clearly indicated the presence of only one product distinct from the starting material. The deshielding of the chemical shifts of the aromatic signals in the pyridine moiety suggested that some reaction had occurred at this position. Additionally, the CH_2 group in the α-position to the sulfur atom has changed from a doublet of doublets to a singlet, indicating the possible loss of the stereogenic center, likely due to the oxidation of the sulfoxide to a sulfone. However, both the ^{1}H- and ^{13}C NMR spectra were insufficient to definitively determine the structure of the compound obtained. Furthermore, HPLC−MS did not reveal any peaks

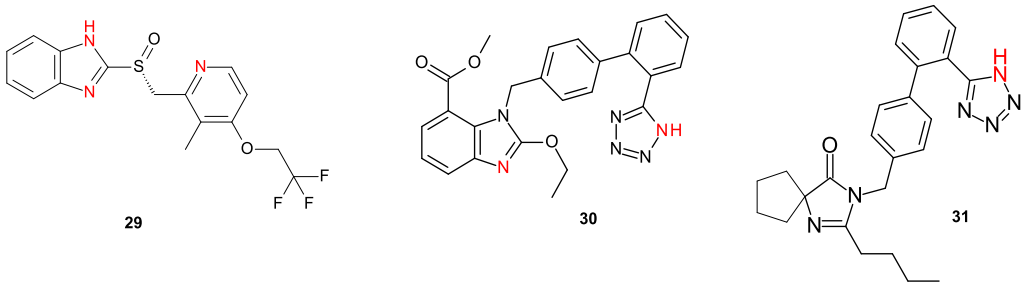


Figure 6. Chemical structures of dexlansoprazole (**29**), candesartan (**30**), and irbesartan (**31**).

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corresponding to the expected nitrosamines, but we cannot completely exclude the presence of a nitrosamine. ^{15}N NMR spectra were acquired for the precipitate and mother liquors, and no signals were observed in the diagnostic region for nitrosamines.

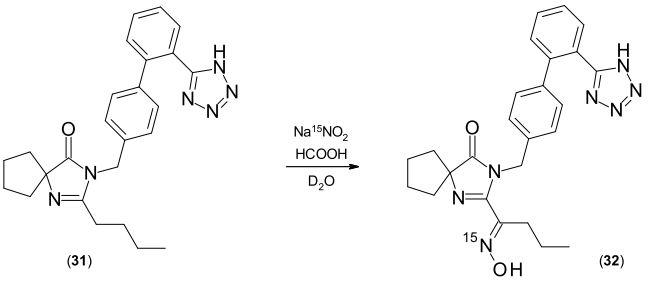
Following the previous discussion, this result excludes the presence of any nitrosamine when dexlansoprazole (**29**) reacts with nitrous acid. The same methodology was also applied to candesartan (**30**).

The NAP test with $Na^{15}NO_2$, in acidic aqueous media, resulted in unreacted candesartan (**30**), identified via ^{1}H NMR and HPLC−MS, and some unidentified byproducts. However, the ^{15}N NMR spectrum did not show any signal in the nitrosamine diagnostic region. To confirm the result, the modified NAP test with *t*-BuO^{15}NO was performed too. The ^{1}H NMR of the reaction mixture after 4 h showed unreacted candesartan and the same unidentified byproducts. Once again, the ^{15}N NMR spectrum confirmed the absence of any nitrosamines.

Another noteworthy case study is irbesartan (**31**), a member of the sartans class, whose behavior under nitrosation conditions has been extensively investigated and documented in the scientific literature.[29] A NAP test on this API was performed by using ^{15}N-enriched sodium nitrite as the nitrosating agent. The reaction mixture was analyzed by HPLC−MS, and the resulting chromatogram displayed a peak at 17 min with an *m*/*z* value of 459 $[M + H]^+$, corresponding to irbesartan (**31**) bearing an NO group. The same reaction mixture was also analyzed via ^{15}N NMR spectroscopy. The spectrum did not show any signal in the diagnostic region for nitrosamines. However, a signal was observed at −16 ppm, which may be compatible with a nitrogen atom in the oxime functional group. Worthy of note, the addition of an oxime group or a nitroso group would lead to a product having the same molecular weight. In order to confirm the presence of an additional nitrogen atom on the observed product, the reaction was repeated using nonenriched sodium nitrite. The HPLC−MS analysis exhibited the same chromatogram, with a peak at 17 min showing a mass of 458 $[M + H]^+$, corresponding to irbesartan (**31**) bearing an NO group. This mass differs by one unit compared with the reaction mixture obtained with $Na^{15}NO_2$. As discussed previously, this demonstrates the presence of an additional nitrogen atom on the product derived from sodium nitrite. Based on the data acquired, irbesartan seems to have reacted with sodium nitrite, generating a derivative bearing an oxime group. This is indeed the same conclusion reached by the studies reported in the literature.

This result provides further confirmation of the versatility of our method, leading to the exclusion of the formation of a nitrosamine on the tetrazole ring of irbesartan but confirming that the nitrosation reaction on this API leads to the formation of the corresponding oxime (**32**) (Scheme 4).

Scheme 4. Reaction of Irbesartan with Sodium Nitrite Leading to the Corresponding Oxime



What we report here is a fast NMR methodology to test the reactivity of an alert alkylamine moiety under nitrosation conditions. In the present work, an alert group for nitrosamine is considered any nitrogen that can potentially be nitrosated. By reacting a compound with a ^{15}N nitrosating agent, under NAP test or in its modified conditions, the formation of every predictable nitrosamine is easily confirmed. On the other hand, it is a rapid test for derisking because if there are no ^{15}*N*-nitroso signals, then the molecule can be considered not prone to nitrosation.

A method was hypothesized and tested, demonstrating that the ^{15}N NMR technique is a fast and reliable analysis, which allows counting all the ^{15}*N*-nitroso compounds in a short time (Figure 7). Moreover, NMR spectroscopy can help in the investigation because it also detects unpredictable nitrosamines and gives a hint in the search with other techniques such as MS spectroscopy for regio- and stereoisomers or degradation products. Furthermore, by repeating the NAP test both with natural and enriched sodium nitrite, it is possible to identify any nitroso-derivative by confrontation of the mass fragmentation searching for the one-unit difference in correspondence to the same retention time peak.

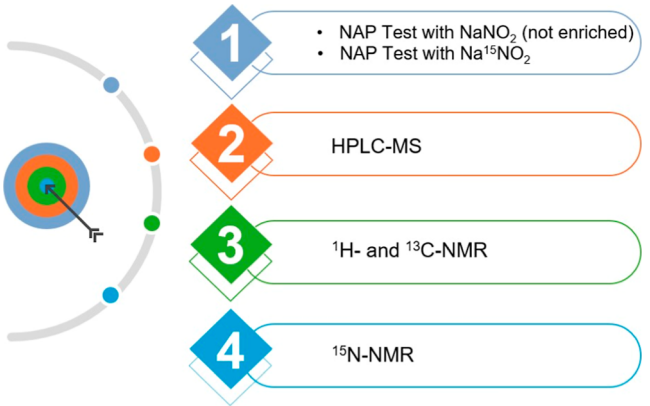


Figure 7. Model proposed to easily evaluate the reactivity of a compound to nitrosation conditions, using the ^{15}N-NAP test.

In conclusion, the ^{15}N-NAP test presented here combines the simple and fast approach of the NAP test, allowing to verify the reactivity of potential nitrosation substrates, with the added value of the powerful and selective ^{15}N NMR technique, normally hindered only by the extremely low natural abundance of ^{15}N. We believe that this approach can be a valid ally for scientists in debunking the misleading information published and the existence of unlikely impurities.

3. CONCLUSIONS

This work demonstrates the power of ^{15}N NMR analysis in the study of nitrosamine impurities, allowing one to determine how many nitrosamine-containing molecules are present in the reaction mixture. By consequence, if no peaks are present in the nitrosamine diagnostic region (120−190 ppm) of the ^{15}N NMR spectrum, then no nitrosamines are formed, and no confirmatory testing is required by authorities.

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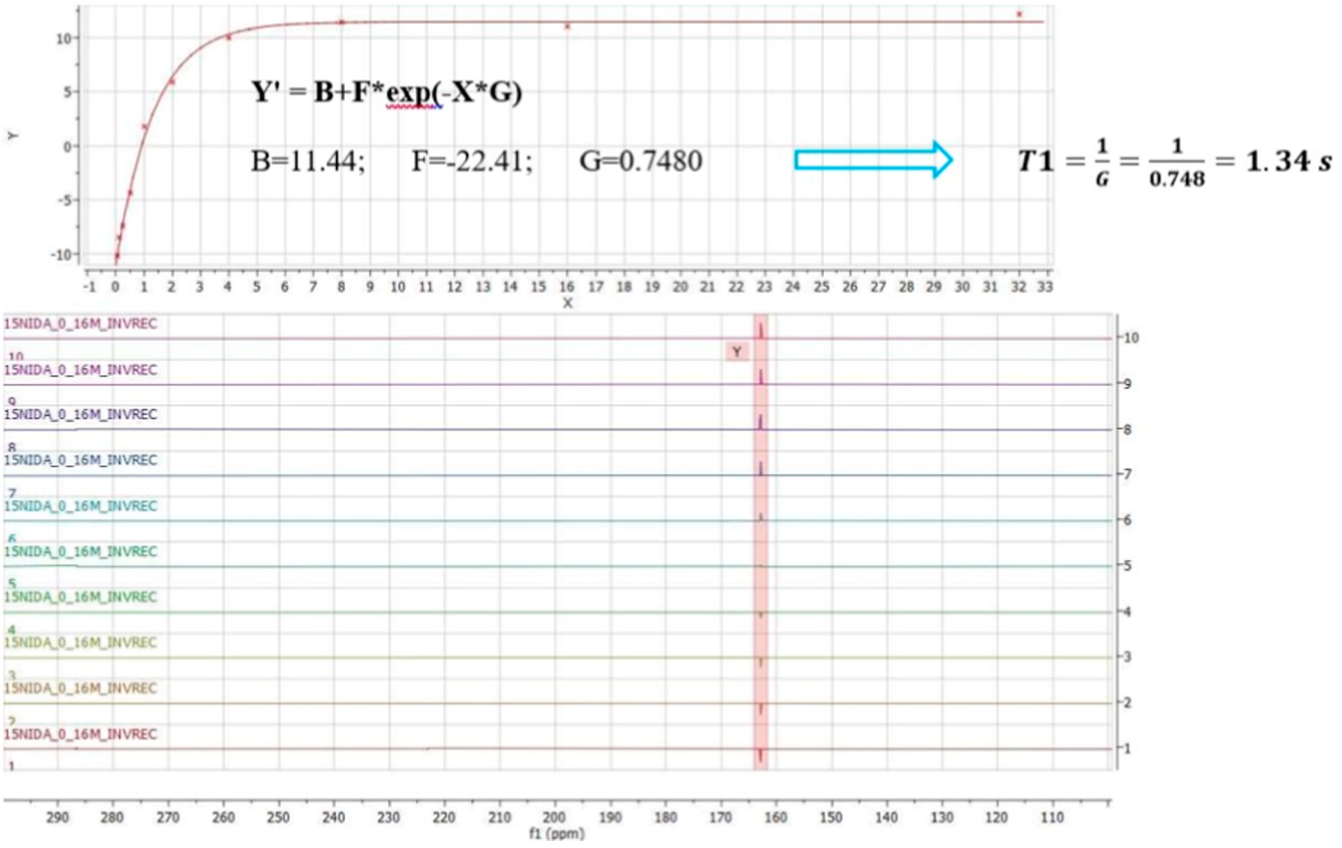


Figure 8. Integral graph obtained from the inversion recovery experiment, which gives the value G. The inversion of G $(1/G)$ is the longitudinal relaxation time (T_1).

4. FURTHER DEVELOPMENTS

The method described here has great potential for further work and development, especially in the optimization of acquisition parameters and FID processing procedures for specific purposes. Future investigations and explorations will be focused on, first of all, the possibility of a quantitative determination against an internal standard (as ^{15}N NMR can be used quantitatively with relative ease).

In quantitative ^{15}N NMR, there are two cases to consider:[30]

1. Determination of the correct number of various types of nitrogen atoms within the molecule;
2. Analysis of mixtures of nitrogen-containing compounds.

The combination of a low natural abundance and a small gyromagnetic ratio makes ^{15}N NMR spectra 2−4 orders of magnitude more difficult to obtain, in terms of experimental time, than ^{13}C spectra.[31] To carry out quantitative analyses, it is necessary to know the T_1 value of the specific ^{15}N nucleus. Another focus will be the exploration of the limit of detection, which is crucial in the analysis of potentially genotoxic compounds. A third potential field of investigation involves making use of the powerful ^{15}N NMR technique to identify the nitrosation site on the substrate. As the nitrogens on the substrate have a natural abundance of ^{15}N, this is not trivial.

4.1. T_1 Calculation with Inversion Recovery Experiment. To carry out quantitative analyses with ^{15}N NMR spectra, the delay between two scans must be longer than five times the longitudinal relaxation time (T_1). T_1 can be calculated through inversion recovery experiments, in which the waiting time between the first (180°) and the second (90°) pulse is changed.

The inversion recovery experiments acquired on the ^{15}N nucleus of ^{15}N-enriched NIDA (**27**) give, as the result of an exponential function, a relaxation time of 1.3 s. The obtained integral graph (Figure 8) has an adequate fitting and the intersection with the x axis occurs at a time just under 1 s, in agreement with the calculated T_1 value.

This result indicates that the relaxation of the ^{15}N nucleus of NIDA (**27**) is not slow, possibly due to its proximity to ^{14}N, a quadrupolar nucleus with I = 1 that generates an oscillating magnetic field by its rapid spin changes.

5. EXPERIMENTAL SECTION

5.1. General. Unless otherwise noted, all commercial solvents and reagents used for reactions were used without further purification. All solvents and reagents used in the HPLC analyses were of HPLC grade. All solvents and reagents employed in LC−MS analyses were of mass spectrometry grade.

^{1}H- and ^{15}N NMR spectra of the compounds were acquired on a Varian 500 MHz spectrometer at 25 °C. Chemical shifts are reported in ppm (δ units) relative to the internal standard of tetramethylsilane [$(CH_3)_4Si$, TMS] for ^{1}H and [CH_3NO_2] for ^{15}N. Multiplicities are as follows: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, and m = multiplet.

Sodium nitrite (CAS#: 7632-00-0), bupropion (CAS#: 34841-39-9), nortriptyline (CAS#: 894-71-3), protriptyline (CAS#: 438-60-8), vortioxetine (CAS#: 508233-74-7), MBN (CAS#: 139481-28-0), nifedipine (CAS#: 21829-25-4), tetrahydropterin (CAS#: 1008-35-1), MBA (CAS#: 136304-

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78-4), NED3A (CAS#: 862542-34-5), dexlansoprazole (CAS#: 138530-94-6), and candesartan (CAS#: 139481-59-7) are industrial production batches. Sodium nitrite ^{15}N enriched (CAS#: 68378-96-1), iminodiacetic acid (IDA) (CAS#: 142-73-4), *p*-toluenesulfonic acid (PTSA) (CAS#: 104-15-4), concentrated hydrochloric acid (37%, CAS#:7647-01-0), and dichloromethane (CAS#: 75-09-2) were sourced from commercial vendors.

5.2. Synthesis of ^{15}*N*-Nitrosobupropion (11). In a 10 mL round-bottom flask, 1.00 g of bupropion hydrochloride (3.63 mmol) was dissolved in 5 mL of D_2O, and hydrochloric acid (37%) was added dropwise until pH = 1. Subsequently, 0.51 g of ^{15}N-enriched sodium nitrite (7.26 mmol) was dissolved in 5 mL of water and added dropwise to the acidic aqueous solution of bupropion. The reaction solution was left at 25 °C under a nitrogen atmosphere for 4 h. After completion of the reaction, the reaction mixture was filtered, and the solid was analyzed with ^1H and ^{15}N NMR techniques and the HPLC−MS method.

5.3. Synthesis of ^{15}*N*-Nitrosonortriptyline (12). In a 25 mL round-bottom flask, 0.10 g of nortriptyline (0.33 mmol) was dissolved in 2 mL of D_2O, and hydrochloric acid (37%) was added dropwise until pH = 1. A white solid precipitates. Subsequently, 0.09 g of ^{15}N-enriched sodium nitrite (1.33 mmol) was dissolved in 3 mL of water and added dropwise to the acidic aqueous solution of nortriptyline. The reaction solution was left at 25 °C, under a nitrogen atmosphere, for 4 h. After completion of the reaction, the aqueous phase was extracted with dichloromethane, and the organic phase was analyzed with ^1H and ^{15}N NMR techniques.

5.4. Synthesis of ^{15}*N*-Nitrosoprotriptyline (13). In a 25 mL round-bottom flask, 0.50 g of protriptyline (1.66 mmol) was dissolved in 10 mL of DCM and PTSA was added. Subsequently, 0.46 g of ^{15}N-enriched sodium nitrite (6.64 mmol) was added to the reaction mixture. The reaction mixture was left at 25 °C under a nitrogen atmosphere for 4 h. After completion of the reaction, the PTSA was filtered, and the organic phase was analyzed with ^1H and ^{15}N NMR techniques.

5.5. Synthesis of ^{15}*N*-Nitrosovortioxetine (14). In a 10 mL round-bottom flask, 0.30 g of vortioxetine (free-base) (1.00 mmol) was dissolved in 10 mL of DCM and 0.20 g of PTSA (1.11 mmol) was added. Subsequently, 0.77 g of ^{15}N-enriched sodium nitrite (1.11 mmol) was added to the reaction mixture. The reaction mixture was left at 25 °C, under a nitrogen atmosphere, for 4 h. After completion of the reaction, the PTSA was filtered, and the organic phase was analyzed with ^1H and ^{15}N NMR techniques.

5.6. Synthesis of ^{15}*N*-nitrosoMBN (15). In a 10 mL round-bottom flask, 0.30 g of MBN (0.71 mmol) was dissolved in 3 mL of DCM and 0.13 g of PTSA (0.75 mmol) was added. Subsequently, 0.10 g of ^{15}N-enriched sodium nitrite (1.43 mmol) was added to the reaction mixture. The reaction mixture was left at 25 °C, under a nitrogen atmosphere, for 4 h. After completion of the reaction, the PTSA was filtered, and the organic phase was analyzed with ^1H and ^{15}N NMR techniques. (HPLC−MS of *N*-nitroso MBN was acquired for the non-^{15}N-enriched product.)

5.7. NAP Test Nifedipine (16) with $Na^{15}NO_2$. In a 25 mL round-bottom flask, 0.50 g of nifedipine (1.44 mmol) was dissolved in 7 mL of acetonitrile, then hydrochloric acid (37%) was added dropwise. 0.40 g of ^{15}N-enriched sodium nitrite (5.76 mmol) was dissolved in 1.5 mL of H_2O, and the obtained solution was added dropwise to the reaction solution. The reaction solution was left at 25 °C under a nitrogen atmosphere for 20 h. After the completion of the reaction, the aqueous phase was extracted with ethyl acetate (20 volumes) and the organic phase was analyzed with ^1H and ^{15}N NMR techniques.

5.8. NAP Test Tetrahydropterin (18) with $Na^{15}NO_2$. In a 10 mL round-bottom flask, 0.10 g of tetrahydropterin (0.60 mmol) was dissolved in 5 mL of D_2O, and then 3 drops of formic acid (pH = 1−2) and 0.17 g of ^{15}N-enriched sodium nitrite (2.40 mmol) were added. The reaction solution was left at 25 °C, under a nitrogen atmosphere, for 4 h. After 4 h, the reaction mixture presented a precipitate, which was filtered. Mother liquors and the solid phase were analyzed with ^1H and ^{15}N NMR techniques, and with the HPLC−MS method.

5.9. NAP Test MBA (22) with $Na^{15}NO_2$. In a 25 mL round-bottom flask, 0.50 g of MBA (1.40 mmol) was dissolved in 5 mL of DCM, and then 0.25 g of PTSA (1.47 mmol) was added. 0.40 g of ^{15}N-enriched sodium nitrite (5.60 mmol) was added to the reaction solution, obtaining a brownish suspension. The reaction mixture was left at 25 °C, under a nitrogen atmosphere, for 4 h. After 4 h, MBA was completely converted to the product. The reaction mixture was filtered, and the organic phase was evaporated and analyzed with ^1H and ^{15}N NMR techniques, and with the HPLC−MS method (HPLC−MS NAP test with $NaNO_2$).

5.10. NAP Test Dexlansoprazole (29) with $Na^{15}NO_2$. In a 25 mL round-bottom flask, 0.10 g of dexlansoprazole (0.27 mmol) was suspended in 10 mL of D_2O, then hydrochloric acid (37%) was added dropwise and dexlansoprazole dissolved. 0.08 g of ^{15}N-enriched sodium nitrite (1.08 mmol) was added, obtaining an orange solution. The reaction solution was left at 25 °C, under a nitrogen atmosphere, for 4 h. After 4 h, the reaction mixture was analyzed with ^1H and ^{15}N NMR techniques.

5.11. NAP Test Dexlansoprazole (29) with ^{15}N-Enriched *t*-Butyl Nitrite. In a 25 mL round-bottom flask, 0.40 g of dexlansoprazole (1.08 mmol) was suspended in 10 mL of DCM, and then 0.17 g of *t*-Bu$O^{15}NO$ (1.62 mmol) was added dropwise, obtaining a brownish solution. The reaction solution was left at 25 °C, under a nitrogen atmosphere, for 20 h. After 20 h, a precipitate was formed, which was filtered. The solid and mother liquors were analyzed with ^1H and ^{15}N NMR techniques.

5.12. NAP Test Candesartan (30) with $Na^{15}NO_2$. In a 25 mL round-bottom flask, 0.50 g of candesartan (1.15 mmol) was suspended in 15 mL of THF and 0.20 g of PTSA (1.19 mmol) was added, obtaining a solution. Subsequently, 0.32 g of ^{15}N-enriched sodium nitrite (4.6 mmol) was suspended in the reaction mixture. The reaction mixture was left at 25 °C, under a nitrogen atmosphere, for 4 h. After 4 h, a precipitate had formed and was filtered. The solid and mother liquors were analyzed with ^1H and ^{15}N NMR techniques.

5.13. NAP Test Candesartan (30) with ^{15}N-Enriched *t*-Butyl Nitrite. In a 10 mL round-bottom flask, 0.10 g of candesartan (0.23 mmol) was dissolved in 5 mL of THF, and 0.04 g of *t*-Bu$O^{15}NO$ (0.34 mmol) was then added dropwise. The reaction solution was left at 25 °C, under a nitrogen atmosphere, for 20 h. After 20 h, the reaction mixture was analyzed with ^1H and ^{15}N NMR techniques.

5.14. NAP Test Irbesartan (31) with $Na^{15}NO_2$. In a 25 mL round-bottom flask, 0.20 g of irbesartan (0.47 mmol) was dissolved in 4 mL of D_2O, and 0.13 g of $Na^{15}NO_2$ (1.87

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mmol), dissolved in 1 mL of D_2O, was then added dropwise. The reaction solution was left at 25 °C, under a nitrogen atmosphere, for 20 h. After 20 h, the reaction mixture was extracted with EtOAc (20 volumes). The aqueous and organic phases were analyzed with ^{15}N NMR and HPLC−MS techniques.

5.15. Synthesis of ^{15}N-Enriched *tert*-Butyl Nitrite (*t*-BuO^{15}NO). In a 10 mL round-bottom flask, 1.11 g of ^{15}N-enriched sodium nitrite (16.19 mmol) was loaded and dissolved in a mixture of water (1.70 mL) and 1.00 g of t-butanol (13.49 mmol).

The reaction solution was stirred under a N_2 atmosphere, and hydrochloric acid (37%) was dropped with a syringe pump over 15 min.

After 2 h, aqueous and organic phases were separated. The organic phase, which is the *t*-BuO^{15}NO isolated, was analyzed with the ^1H NMR technique, and it was stored at 4 °C.

5.16. Synthesis of ^{15}N-Nitrosoiminodiacetic Acid (^{15}NIDA) (26). In a 25 mL round-bottom flask, 0.20 g of iminodiacetic acid (1.50 mmol) was dissolved in 1 mL of H_2O and hydrochloric acid (37%) was added dropwise until pH = 1. Subsequently, 0.42 g of ^{15}N-enriched sodium nitrite (6 mmol) was dissolved in 1 mL of water and added dropwise to the acidic aqueous solution of iminodiacetic acid. The reaction solution was left at 25 °C, under a nitrogen atmosphere, for 4 h. After completion of the reaction, the aqueous phase was extracted with 3 × 10 volumes of ethyl acetate, and the organic phase was analyzed with ^1H and ^{15}N NMR techniques.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.oprd.5c00223.

^1H- and ^{15}N NMR spectra and HPLC−MS chromatograms of the obtained product. HPLC−MS analytical methods (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

EMA: European Medicine Agency; EDTA: ethylendiamminotriacetic acid; FDA: Food and Drug Administration; FID: free induction decay; ICH: International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

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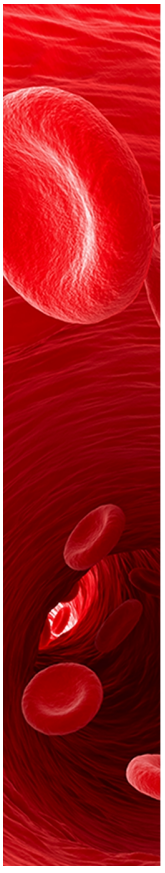
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