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A gas chromatographic method for the indirect determination of hydroxylamine in pharmaceutical preparations: conversion into nitrous oxide

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Abstract

A simple, sensitive, and selective headspace gas-chromatographic method has been developed for measuring hydroxylamine (HA) in a variety of sample matrices including pharmaceutical formulations. This procedure relies on converting HA into nitrous oxide (N_2O), which is a single-step reaction that is carried out directly in a heated headspace vial. The gaseous products are then analyzed by headspace capillary gas chromatography. Several detection strategies were evaluated and electron capture provided the best sensitivity (4 parts-per-billion (ppb)) while the mass selective and thermal conductivity values were higher (14 ppb and 1.4 parts-per-million (ppm), respectively). The method's linear dynamic range spans two to four decades with a run-to-run precision that was better than 5% R.S.D. ($n = 7$). The reagent concentrations (oxidant, buffer) strongly impact the N_2O signal and the greatest response was obtained for solutions that contained equimolar amounts of reactants. HA was efficiently (98%) recovered from a sample matrix that contained only the active pharmaceutical ingredient (API) but the recovery was lower (83%) when excipients were present.
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Keywords: Hydroxylamine; Headspace gas chromatography; Pharmaceutical preparations; Matrix effects; Nitrous oxide

1. Introduction

A series of novel bicyclic hydroxamates [1] have recently been discovered that could be useful for treating a broad range of inflammatory disorders. Synthesis of these drugs involves the use of hydroxylamine (HA), which at modest levels can be toxic to humans, animals, and even plants [2]. These therapeutic agents are intended to be used in

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Table 1
Experimental HSGC parameters

	TCD	ECD	MSD
Gas chromatograph	HP 5890	PE Autosystem	HP 5890
Carrier flow (He, ml min^{-1})	3.0	3.0	1.2
Injection (heated, split)	15:1	15:1	40:1
Detector temperature (C)	150	350	150
Interface temperature (C)	N.A.	N.A.	250
Detector make-up gas (ml min^{-1})	He, 25	N_2, 15	N.A.
Headspace sampler	PE HS40	PE HS40	HP 7694
Injection	Timed, 3 s	Timed, 3 s	Fixed, 3.0 ml
Setpoints common to all experiments			
Oven ramp profile	28 °C (3 min) to 200 °C (1 min) at 45 °C min^{-1}		
Vial pressure (psi)	28		
Equilibration time (min)	30		
Equilibration temperature (C)	60		
Pressurization time (s)	45		

N.A., not applicable.

formulated drug products, therefore, a method is needed for measuring HA in a variety of matrices.

HA is thermally labile and not readily measured in its native form [3] although electrochemical detection (ED) has been successfully used in several cases [4–7]. More often HA is stabilized by conversion to an oxime [8], which can be readily analyzed using liquid or gas chromatography [8–13]. Although the oxime-based method can be quite sensitive (parts-per-billion, ppb) it is labor intensive and requires several steps to effect the conversion. Capillary electrophoresis and colorimetric methods have also been used to indirectly measure HA at the parts-per-million (ppm) level [14–16] but these methods might not be suitable for analyzing samples with a complex matrix or that contain non-volatile components, such as a pharmaceutical formulation.

In this report, we describe a simple method for converting HA into nitrous oxide (N_2O). The single-step reaction is carried out in a heated headspace vial and the product vapor transferred into a gas chromatograph for quantitative analysis. N_2O is sparingly soluble in most solvents and headspace sampling provides a simple means of collecting N_2O and separating it from a potentially complex matrix. The effects of reactant concentration and solvent composition on this conversion are presented. This methodology was used to measure HA in bulk active pharmaceutical ingredient (API) samples and a drug-product formulation.

2. Experimental

2.1. Apparatus

Data were collected on several instruments, which provided access to a variety of detection strategies. Studies using thermal-conductivity detection (TCD) were performed on a Hewlett Packard (HP) 5890 gas chromatograph (Wilmington, DE) that was interfaced to a Perkin Elmer (PE) HS40 headspace sampler (Norwalk, CT). The same HS40 sampler was also coupled to a PE Autosystem gas chromatograph (GC) equipped with an electron-capture detector (ECD). A J&W Scientific (Mountain View, CA) GS-Q megabore column (30 m × 0.53 mm ID, P/N 1153432) was used for all but the mass spectrometric studies. A capillary GS-Q column (30 m × 0.32 mm id, 5.0 μm film thickness, J&W Scientific, P/N 1133432) was installed in a HP 6890 GC that was interfaced to a HP 5973 quadrupole mass-selective detector (MSD) and HP 7694 headspace sampler. The MSD was operated in the single ion mode (SIM) for all quantitative work. The majority of experi-

mental data presented in this manuscript were collected using the PE HS40-HP5890 system. Table 1 presents the typical instrumental conditions used in this study. A N_2O gas standard (98% purity) was purchased from Matheson Gas Products (Montgomeryville, PA) and used for retention time matching. Peak areas were blank subtracted and all samples were prepared in triplicate unless otherwise noted. Kaleidagraph® (Synergy Software, Reading, PA) was used for data presentation and analysis.

2.2. Reagents

A variety of solvents (diluents) were evaluated including methanol (MeOH), ethanol (EtOH), isopropanol (IPA), dimethylsulfoxide (DMSO), acetonitrile (AcN), and water (H_2O). All solutions were made from chromatographic-grade solvents that were purchased from VWR Scientific Products (New Brunswick, NJ). Hydroxylamine hydrochloride ($NH_2OH \cdot HCl$, 98%), ferric ammonium sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 97%), ferric chloride ($FeCl_3$, 98%), sodium acetate trihydrate ($CH_3CO_2Na \cdot 3H_2O$, 95%), and sodium hydroxide (NaOH, 94%) were obtained from Sigma Aldrich (St. Louis, MO) and used without further purification.

2.3. Sample preparation

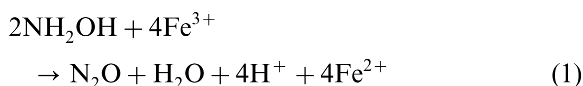
Separate stock solutions of the oxidant ($FeCl_3$, 250.3 mmol l^{-1}), buffer (sodium acetate, 265.3 mmol l^{-1}), and hydroxylamine hydrochloride (21.6 mmol l^{-1}) were prepared in MeOH. HA standards were prepared fresh daily whereas all others were made weekly. Calibration standards were prepared by diluting the HA stock solution with MeOH. A 10 ml aliquot of the HA standard was dispensed into a 20 ml headspace vial and then 100 µl of each reagent solution (oxidant, buffer) was added to the vessel. The vial was immediately sealed upon addition of the final reagent. A Whatman 2.7 µm disposable syringe filter (VWR, P/N 6888-2527) was used to filter the drug product, drug substance, and placebo solutions prior to analysis. All stock solutions were stored at 5 °C.

In these studies, the reagent concentrations were found to strongly influence the production of N_2O. Experiments were performed whereby the concentration of one reagent was systematically altered while holding the others constant. The reagent stock solution was serially diluted with methanol and then used as the sample solvent (diluent). As before, the HA standards were prepared in the HS vial except 250 µl of the HA stock solution was added to the diluent (10 ml). Data from these experiments are presented in Section 3.

3. Results and discussion

3.1. Conversion of hydroxylamine into nitrous oxide

The formation of N_2O via the oxidation of HA was first studied in detail by Rao and Somidevamma [17]:



$$2NH_2OH + 4Fe^{3+} \rightarrow N_2O + H_2O + 4H^+ + 4Fe^{2+} \quad (1)$$

Since then, this reaction has been used to measure HA in a variety of sample matrices including marine and fresh waters [18,19]. This approach can also be used to measure HA in pharmaceutical formulations and would be a simple way to separate the analyte from a potentially complex matrix. However, the key experimental parameters had to first be identified before this method could be used to analyze samples of drug substance or drug product.

3.2. Instrumental variables

The performance of this method was strongly influenced by the instrumental setpoints and sample-preparation procedure. Analyzer-specific variables such as injection time, vial-pressurization time, and sample volume (injection time) were optimized empirically. For these initial studies, a 75 ppm (2.3 mmol l^{-1}) HA standard was prepared

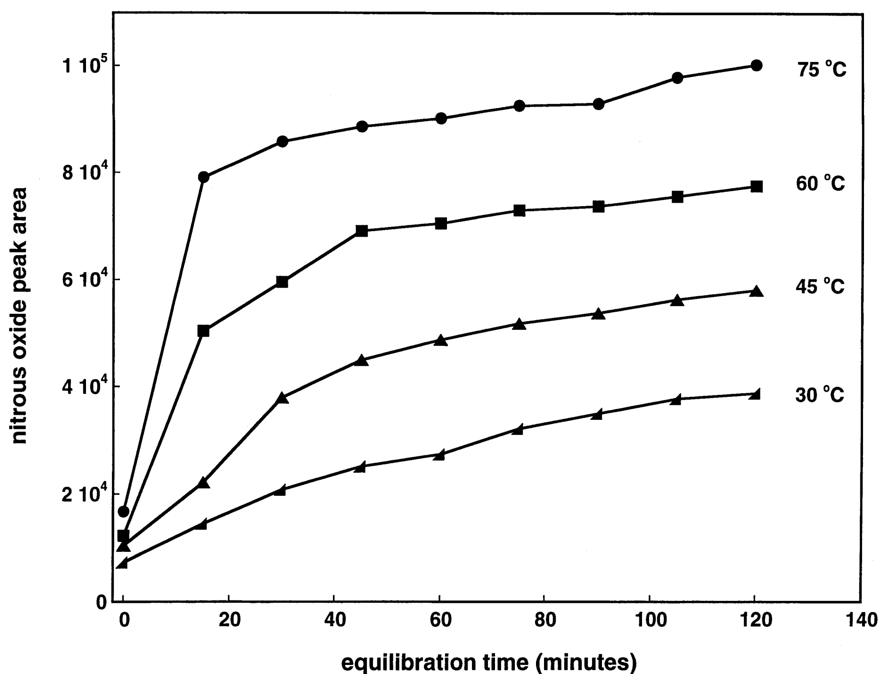


Fig. 1. N_2O peak area as a function of the sample equilibration temperature and equilibration time. The samples contained 75 ppm (2.3 mmol l^{-1}) HA and 3.0 mmol l^{-1} Fe^{3+} (as $FeCl_3$) in MeOH. The TCD signal was used to collect these data.

in a methanol solution that also contained Fe^{3+} (as ferric ammonium sulfate (FAS), 3.0 mmol l^{-1}). Several iron salts were evaluated as oxidants and these results will be discussed later.

The PE HS-40 does not employ a fixed volume gas-sampling loop; instead, the headspace vial and column are connected briefly (2–6 s) by a timed switching valve. The amount of sample vapor that is transferred into the GC is determined by the vial pressure and valve-switching time. An elevated HS-vial pressure (28 psi) was used in these studies to reduce sampling artifacts that might otherwise result from testing solvents with vastly different vapor pressures.

The sample's equilibration temperature and equilibration time directly impact the vapor-phase concentration of N_2O [20,21]; these data are presented in Fig. 1. The highest carousel temperature (75 °C) produced the largest N_2O signal in the shortest period of time. Conversely, when the sample was equilibrated at 30 °C the N_2O signal changed more gradually and never reached the same levels that were observed at higher temperatures. HA was converted into N_2O more quickly [22] as the temperature increased while there was a concomitant decrease in its condensed-phase solubility. Unless otherwise specified, samples were heated (equilibrated) at 60 °C for 30 min.

3.3. Sample reagents

The reagent (oxidant, buffer) concentrations can also impact the production of N_2O. The effect of [$FeCl_3$] on the N_2O peak area is presented in Fig. 2; the HA concentration (1.9 mmol l^{-1}) was held constant throughout this experiment. The N_2O signal rises sharply until the [Fe^{3+}] reaches 3.4 mmol l^{-1}, then HA becomes the limiting reagent and the signal hardly changes. These findings are supported by Eq. (1), which states that two equivalents of iron (i.e. two electrons) are needed to oxidize each mole of HA. Clearly, the greatest N_2O signal cannot be realized if there is a limited supply of iron. Similar results were obtained when

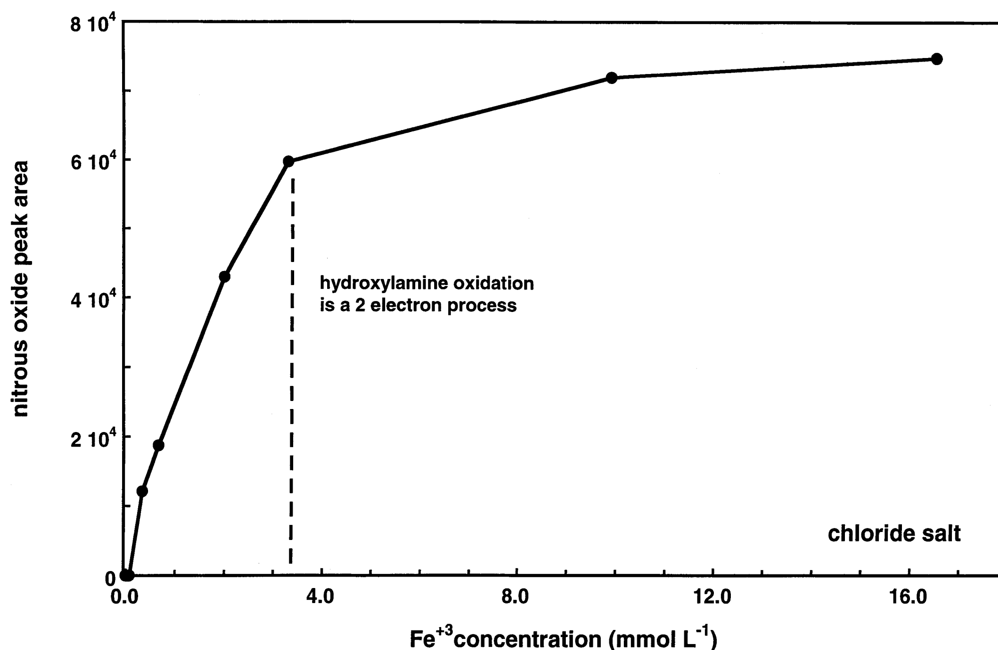


Fig. 2. Relationship between N_2O peak area and [Fe^{3+}] (as iron chloride) in the sample solution. The HA concentration was 1.9 mmol l^{-1}. A linear relationship was observed until HA became the limiting reagent (~ 3.8 mmol l^{-1}). Similar results were obtained using FAS as the source of iron (data not shown). Samples were heated at 60 °C for 30 min. Data collected through use of the TCD.

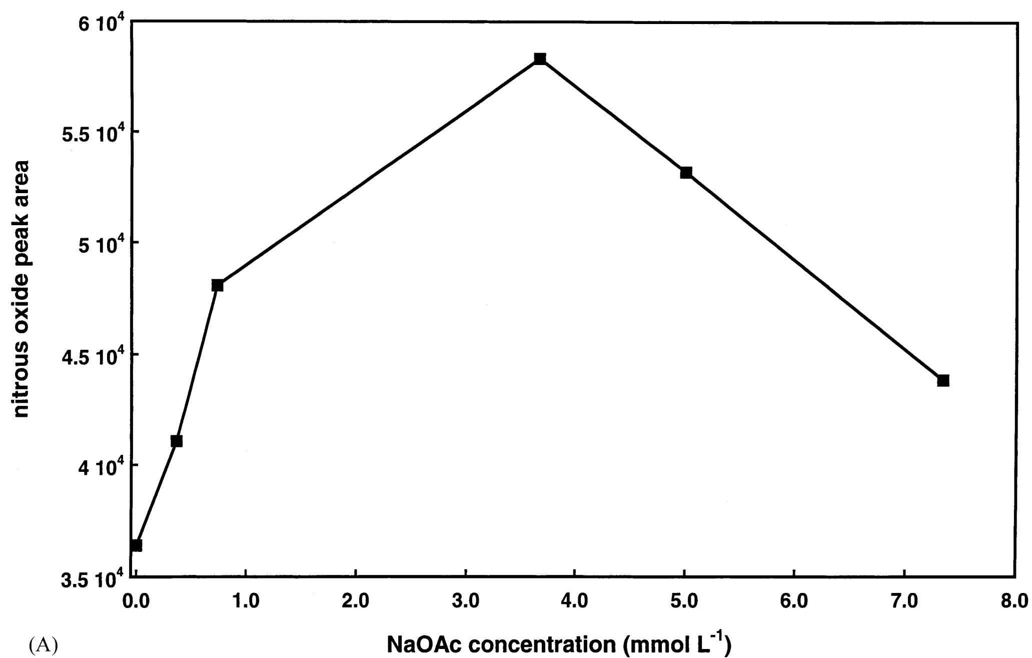
FAS was used as the oxidant instead of $FeCl_3$ (data not shown). Rao and Rao report that for reaction (1) to proceed quickly the molar ratio of iron must be at least twice that of HA [23].

In this study, two iron (III) salts were used as oxidants ($FeCl_3$ and FAS) and their effectiveness was compared. The two salts were used to make HA standards that had the same [Fe^{3+}] and the resulting N_2O data were indistinguishable (six replicates each). As compared with FAS, ferric chloride was more soluble in the solvents that were tested so it was used for all remaining studies. A nitrous-oxide signal was not observed for HA standards prepared without iron.

Under basic conditions, iodine has been used to convert HA into N_2O and could be employed for analyzing acid-sensitive compounds [16,24,25]. Vanadium and cuprous salts reportedly catalyze the oxidation of HA although they have not been tested in our laboratory [23,26]. All of our experiments were conducted at temperatures well above ambient so the reaction proceeded relatively quickly, however, a catalyst might be useful for analyzing thermally-labile compounds.

The buffer (or base) concentration is another variable that can affect the N_2O signal; furthermore, the solution chemistry can even influence which products are formed [18,23,26]. HA standards were prepared for analysis that varied only in their sodium acetate concentration (0.0–7.0 mmol l^{-1} OAc^-). As noted in Fig. 3A, the N_2O peak area increased with [OAc^-] until it reached 3.6 mmol l^{-1} then the N_2O response quickly declined. A 6-fold reduction in the N_2O signal was observed at 38 mmol l^{-1} OAc^- as compared with its highest value (data not shown). Butler and Gordon [18] report that a strong correlation exists between solution pH, reaction efficiency, and the recovery of N_2O from marine waters; their best results were obtained when the solution pH was below 6.7 [18]. Likewise, in these studies the greatest N_2O signals were observed when the solution pH was less than 6.1.

The N_2O signal was also monitored as a function of the sodium hydroxide concentration



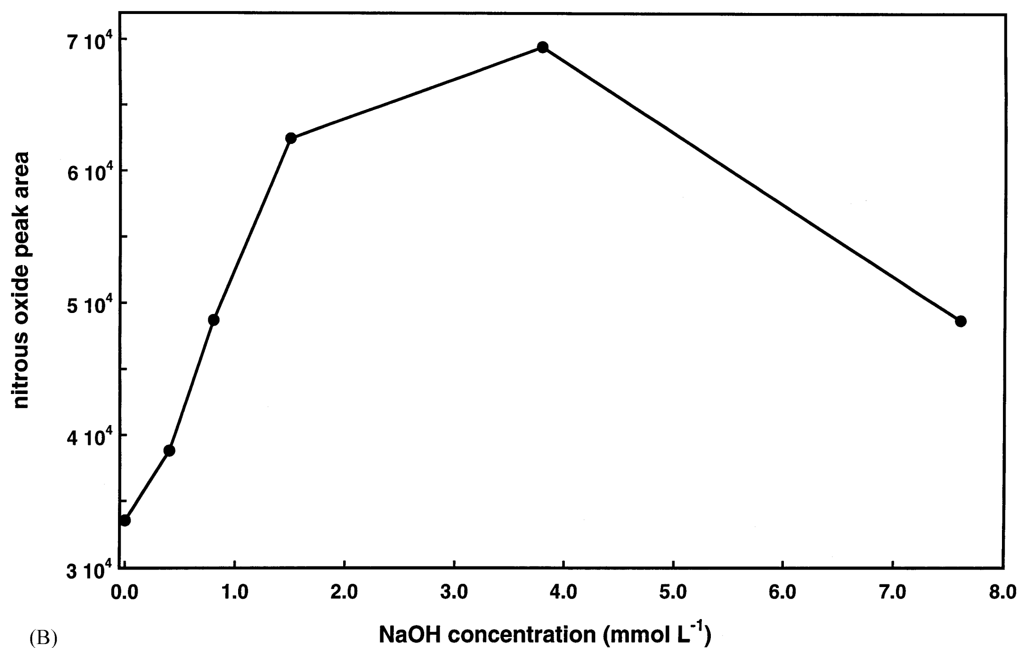
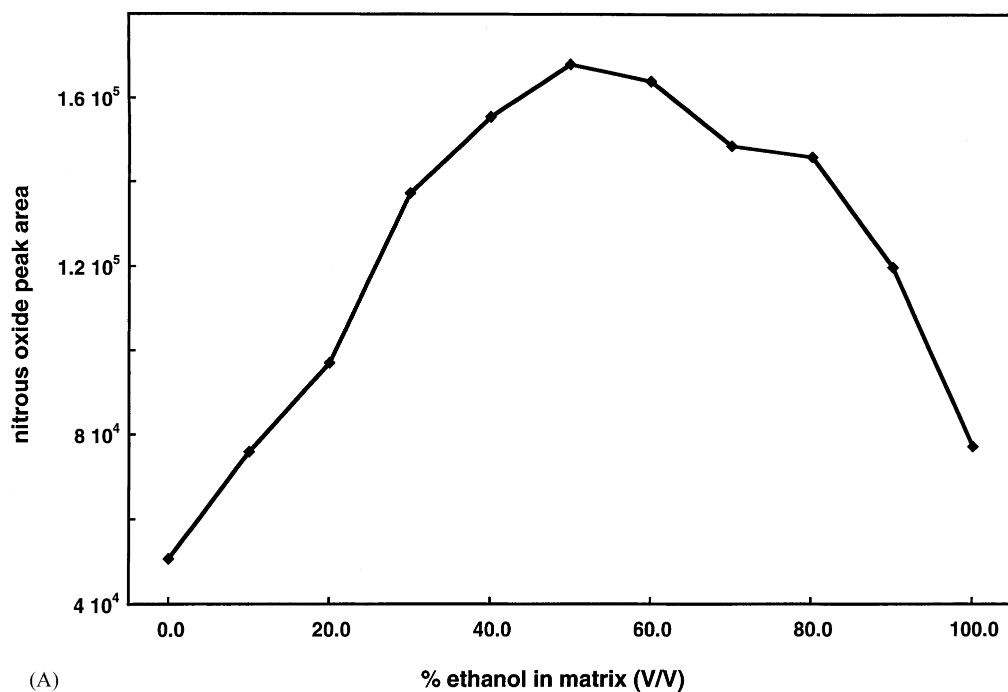


Fig. 3. N_2O peak area as a function of the salt used to adjust pH. The 63 ppm (1.9 mmol l^{-1}) HA standard was prepared in MeOH/water and heated at 60 °C for 30 min. Ferric chloride (4.0 mmol l^{-1}) was used as the oxidant but similar results also were obtained with ferrous ammonium sulfate. The TCD signal was recorded for this study. (A) sodium acetate, (B) sodium hydroxide.



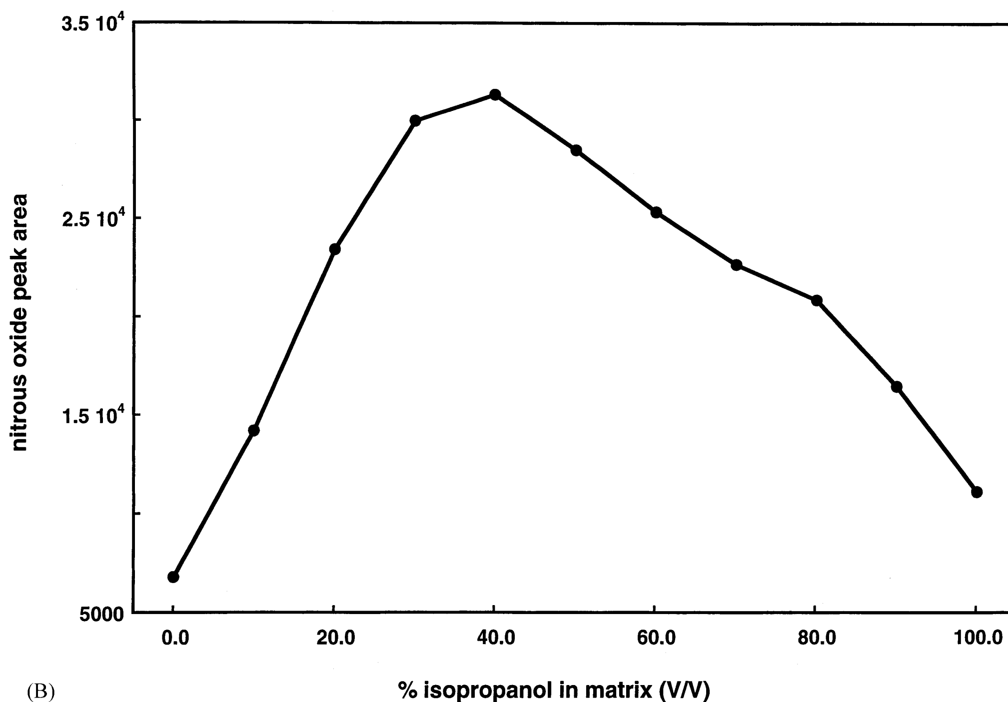


Fig. 4. Detailed study showing the effect of sample matrix on N_2O signal level (TCD). The HA, $FeCl_3$, and NaOAc concentrations were the same for all samples (2.3, 4.5 and 4.4 mmol l^{-1}, respectively). Solutions were heated at 60 °C for 30 min. (A) Ethanol/water, (B) isopropanol/water.

and these data are presented in Fig. 3B. The acetate and hydroxide solutions performed similarly at lower concentrations, however, at 38 mmol l^{-1} $[OH^-]$ the N_2O signal was reduced by 60-fold (data not shown). N_2O can be converted into nitrite and nitrogen under alkaline conditions, which might explain why the greatest hydroxide (and acetate) concentration yielded the lowest N_2O signal. [18,23]. Overall, the best N_2O response was obtained when the reaction mixture contained equimolar amounts of iron and base (or buffer).

3.4. Sample matrix

Matrix-effects are well known in headspace analysis [20,27–30] and many factors can influence the vapor-phase composition, especially the sample solvent. HA standards (75 ppm, 2.3 mmol l^{-1}) were prepared in five solvent systems and the N_2O peak areas normalized to ethanol with the following results; EtOH (1.0), IPA (0.75), AcN (0.76), DMSO (0.64), MeOH (0.59), water (0.48). Diluents were prepared by mixing together equal volumes of organic solvent and distilled water. The reagent concentrations (ferric chloride = 4.5 mmol l^{-1}, sodium acetate = 4.4 mmol l^{-1}) were identical in all cases. The average N_2O peak areas were within a factor of two for all solvents except methanol/water, which yielded a slightly lower result.

The matrix-effect was studied in greater detail by tracking the N_2O signal while systematically varying the EtOH–water ratio (v/v). Three standards were prepared for each mixture (HA = 2.3 mmol l^{-1}, $FeCl_3$ = 4.5 mmol l^{-1}, NaOAc = 4.4 mmol l^{-1}) and the averaged data are presented in Fig. 4A. The N_2O signal increased with the [EtOH] until equal amounts of ethanol and water (v/v) were present, then it tracked the decline in $[H_2O]$. As seen in Fig. 4B, similar results were obtained when ethanol was replaced by isopropanol. An ethanol-based solvent (1:1 ratio, v/v) was used throughout the rest of this study because the API was most stable and soluble in this diluent.

Inorganic salts are often used in HSGC as a way to improve sensitivity by reducing the analyte's solubility in the condensed phase [20]. The salting-out effect was tested by preparing two groups of samples that contained identical reagents except sodium sulfate (~ 3 g) was added only to the first set. The N_2O peak areas were identical for all samples (data not shown) thus sodium sulfate was not used in any other experiments.

3.5. Linearity, precision, and detection limits

The linear dynamic range of this method is dependent on the GC detector and solution chemistry. The performance characteristics of common detectors are well defined and these specifications are readily available [31], however, to realize the greatest N_2O signal the reagent levels must be matched to that of HA (e.g. Fig. 3A and B). A typical calibration curve qualitatively resembles Fig. 2 as it becomes non-linear once the iron has been stoichiometrically consumed. In these studies, the detection limit (DL) was calculated as:

$$\mathrm{DL} = \frac{3(\text{standard error of the } y - \text{axis intercept})}{\text{calibration line slope}} \tag{2}$$

A 1.5 ppm DL for HA was obtained through use of the TCD with a response that was linear across three to four decades ($y = 824 + 513x$, $R = 0.9994$). The instrumental figures-of-merit for the HSGC method are summarized in Table 2.

The ECD's calibration range was more restricted (two to three orders) because this detector is easily saturated (flat-top peaks) by high N_2O levels, however, it yielded a 1.4 ppb limit of detection ($y = 1655 + 2.36 \times 10^8 x$, $R = 0.9995$). HA standards were prepared in an EtOH/water solution as before but the oxidant and buffer concentrations were reduced to 0.21 and 0.34 mmol l^{-1}, respectively. Reactant concentrations much greater than HA can inhibit the production of N_2O so their amounts were decreased in order to achieve the best signal.

The MSD provided a DL of 14 ppb for HA, which was slightly higher than that for the ECD. In these studies the MSD was set to monitor the most abundant ion for N_2O (i.e. m/z 44). The

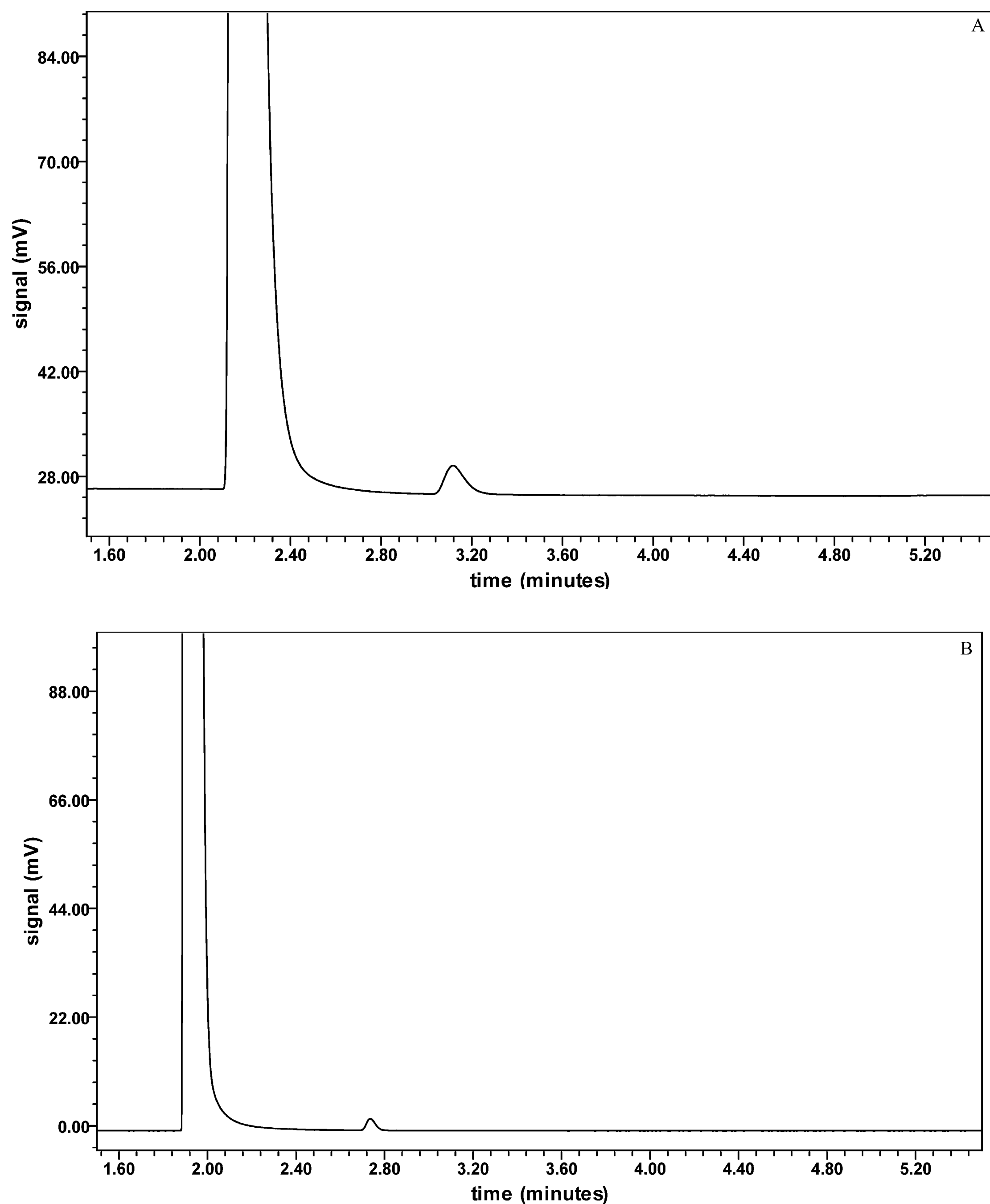


Fig. 5. Gas chromatographic analysis of a drug product formulation using three different detection modes. The samples were heated for 30 min at 60 °C. The peak identities are: $N_2/O_2 = 1.90$ min, $CO_2 = 2.75$ min, $N_2O = 3.05$ min (A) ECD, (B) TCD. An N_2O peak is not seen here because the HA concentration is too low. (C) MSD.

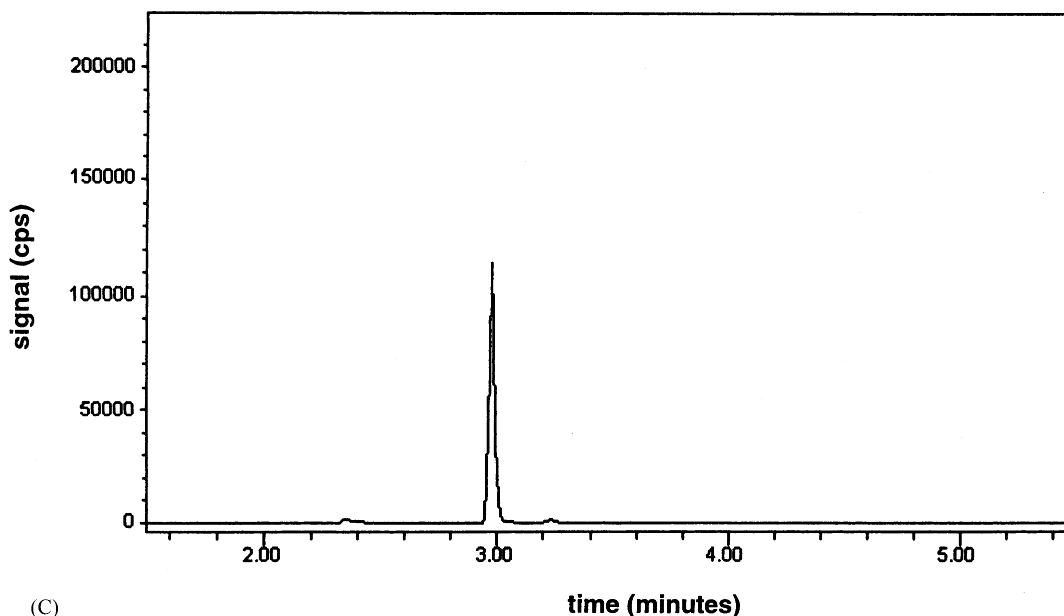


Fig. 5 (*Continued*)

acetate and iron (III) concentrations in the standard solutions were 1.6 and 2.1 mmol l^{-1}, respectively. The MSD's calibration curve was linear across three decades and flat-topped peaks were observed at higher analyte concentrations ($y = 360 + 2.39 \times 10^5 x$, $R = 0.9997$).

The nitrogen-phosphorus detector (NPD) was also tested, however, it showed no response towards N_2O even when 100 μl of a pure-gas standard was injected onto the column. The NPD normally responds only to compounds that contain organic nitrogen or phosphorus.

The run-to-run precision of this method was 4.9% R.S.D. ($n = 7$, MSD) with a day-to-day reproducibility of 6.3% R.S.D. ($n = 14$, MSD). Although not employed in this study, internal standardization might be used to further improve precision. The DLs could be lowered through use of a longer injection time (or larger injection loop), lower split-vent ratio, or higher equilibration temperature. The TCD and ECD are non-destructive detectors so collectively their dynamic range could be extended through tandem operation.

3.6. Analysis of drug substance and drug product

The method currently used in our laboratory to analyze HA in bulk API samples relies on ED following a liquid-chromatographic separation [6]. Unfortunately, the electrochemical method is not very robust and suffers from poor precision ($>$ 15% R.S.D.), baseline drift, and low sensitivity (4 ppm HA in API). One advantage of the ED technique is that the analyte can be directly measured.

The HSGC method was first tested on a solution that contained only the API (22 mg ml^{-1} in $EtOH/H_2O$) but the drug substance and Fe^{3+} formed a complex that produced a deep reddish-brown solution and precipitate. Furthermore, the N_2O response was the same for each sample even though they were spiked with different amounts of HA (standard addition). The molar concentrations of iron and buffer were further increased but this lowered the pH (< 2), which quickly degraded the molecule and released HA.

The reactivity problems mentioned above were overcome through the use of an aqueous slurry to extract, and pre-concentrate, the HA from the

bulk API (2.350 g drug, 40 ml H_2O). The filtrate was diluted with an equal portion of ethanol and then the reactants were added to the solution. Unlike before, the sample did not turn reddish brown or form a precipitate. Standard addition was used for analyte quantitation and the samples were spiked with HA at five levels ($n = 2$). The ECD provided good linearity ($y = 24\,858 + 815x$, $R = 0.9995$) across the calibration range of 3–250 ppb. A different detector cell was used for these spiking experiments so the slope of this calibration line does not match that previously reported. The API sample was found to contain 0.83 mg kg^{-1} HA, which is consistent with our electrochemical and in-process data. Analyte recovery from the matrix was found to be 98% and was measured by comparing the peak area of a 71 ppb HA standard against that of an API solution spiked at the same level. Several API samples were heated for a progressively longer period of time (in 30-min increments up to 3 h) and the N_2O signal did not increase suggesting that the molecule was stable under these conditions.

A similar standard-addition experiment was conducted using the TCD instead of the ECD. As before, an API extract was used to prepare the samples for analysis. The calibration curve exhibited good linearity ($R = 0.996$) across a higher concentration range (2–218 ppm). Recovery of HA from the sample matrix was 96%, measured by comparing the peak areas of a 36 ppm HA standard and API solution spiked at the same level. N_2O could not be detected in the native extract because the HA concentration was below the TCD's DL.

Spiking experiments were also performed on solutions that contained a drug-product placebo. The excipient blend (3.0 g) was added to 100 ml of diluent and the solution was placed on a wrist-action shaker for 20 min. After filtration, HA was spiked into these solutions (4–375 ppb) and the ECD response tracked N_2O with good linearity ($R = 0.998$) and precision (4.8% R.S.D., $n = 3$). HA recovery from the placebo solution was determined to be 83% at the 75 ppb level ($n = 3$), which is less than that obtained from a solution containing only the API. This lower recovery probably results from an interaction between the API and one of the sugars used in the formulation [32]. HA was not found in the solution that contained the placebo mixture.

The HSGC method was used to measure the concentration of HA in a drug product (DP) tablet formulation. Each 250 mg tablet contained 100 mg of the API and 150 mg excipients; these are the same components that were previously tested. As before, an aqueous slurry was used to extract and preconcentrate HA (2.50 g formulation, 10 ml water) from the powdered mixture. The extract was then analyzed using the three detection methods (TCD, ECD, MSD); example chromatograms are presented in Fig. 5A–C. The chromatographic patterns for these DP samples strongly resemble those of the standard HA solutions. The HA concentration in the DP samples was determined using external calibration and the results compared favorably to those of the bulk API (ECD = 0.73 mg kg^{-1}, MSD = 0.78 mg kg^{-1}, TCD = none detected). A previously documented interaction between HA and one of the excipients likely accounts for the lower-than-expected values [32].

The HA level was measured in another bulk drug sample that was structurally similar to the one previously tested. As before, an aqueous slurry was used to extract HA from the sample and aliquots were spiked with different amounts of HA. In this case, the iron and acetate levels were reduced even further (0.62 and 0.67 mmol l^{-1}, respectively) because this particular molecule binds strongly (and irreversibly) with transition metals [33]. Furthermore, from other studies we know that this molecule is susceptible to degradation by Fe(III). The calibration curve exhibited good linearity ($y = 1204 + 520.4x$, $R = 0.9994$) across the calibration range (10–300 ppb, 10 levels) and 0.092 mg kg^{-1} HA was found in the bulk API sample. The drug substance was in limited supply so we were able to prepare only a single HA spike per level. This API was also analyzed by the electrochemical method but the HA concentration was well below its DL [6].

4. Conclusions

A headspace gas chromatographic method has been developed to indirectly measure HA by first converting it into N_2O. Several detectors were evaluated and the ECD was found to be the most sensitive for N_2O (1.5 ppb). The linear dynamic range was two to four orders of magnitude for all detection strategies and the run-to-run precision was better than 5% R.S.D. The matrix composition (Fe^{3+}, base, buffer, solvent) strongly impacts the N_2O signal and the greatest response was obtained when equimolar amounts of iron-to-base (or buffer) were used to prepare the solutions. HA was efficiently recovered (98%) from a sample matrix that contained only the drug substance, however, a lower recovery was achieved (83%) when excipients were present.

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