Method for Group Determination of Total *N*-Nitroso Compounds and Nitrite in Fresh Gastric Juice by Chemical Denitrosation and Thermal Energy Analysis

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A method for the determination of total *N*-nitroso compounds (NOC) and nitrite in fresh human gastric juice is described, which is based on earlier methods. A freshly obtained gastric juice sample is injected directly into refluxing ethyl acetate containing glacial acetic acid, HCl and HBr to determine the total concentration (A) of nitrite, total NOC and thermo- and acid-labile thermal energy analyser (TEA)-responsive compounds (TAC). Another fresh sample of the same juice (with or without the addition of sulfamic acid) is injected directly into refluxing ethyl acetate containing glacial acetic acid and HCl for determining the TAC level (B) and the total level (C) of nitrite and TAC. The NO released from nitrite, TAC and NOC is detected by TEA acting as a chemiluminescence detector. The differences between A and C and between C and B represent the concentrations of total NOC and nitrite, respectively. The method is rapid, reproducible (relative standard deviation 1–6%) and sensitive (detection limit 1.0 pmol). The behaviour of nitrite and NOC in the analytical system was studied and relevant techniques for the direct analysis of fresh gastric juice samples and for stabilization of fresh samples have been developed.

Keywords: N-*Nitroso compound determination; gastric juice; chemical denitrosation; thermal energy analysis*

N-Nitroso compounds (NOC) formed in the stomach have been postulated to be major aetiological agents for gastric carcinogenesis in man.[1,2] By using the *N*-nitrosoproline (NPRO) test it has been demonstrated that some high-risk populations for gastric cancer are exposed to higher levels of endogenous NOC.[3–8] However, there is controversy about the relationship between intragastric nitrosation and other intragastric factors, such as pH, bacterial count and nitrite concentration. It is well known that gastric hypochlorhydria, such as that associated with chronic atrophic gastritis, facilitates intragastric bacterial growth, also of nitrate-reducing bacteria, which convert nitrate into nitrite and increase nitrite concentration in gastric juice dramatically.[9] Walters *et al.*[10] were the first to devise a method to assay total NOC as a group in biological materials. Studies involving use of this method confirmed a positive relationship between nitrite and total NOC in gastric juice and intragastric pH and bacterial counts.[11–14] However, the results obtained by using an alternative method for the assay of total NOC[15] or using the NPRO test[16] suggested that total NOC in gastric juice or NPRO excreted in urine were unrelated or negatively related to intragastric pH and bacterial counts.[17–21] The methods of Walters and co-workers.[10,22] and Bavin *et al.*[15] have been examined and criticized,[23,24] and it would seem that the conflict was mainly due to a lack of reliability of both methods for the determination of total NOC in gastric juice.

As a result of their studies of the previous methods, Pignatelli *et al.*[23] developed a more reliable procedure, which is more selective than that of Bavin *et al.*[15] and more sensitive than those of Walters *et al.*[10] and Bavin *et al.*[15] The results obtained by use of their modified method did confirm that the total NOC concentration in gastric juice was related directly to intragastric pH. However, the response of the system to nitrite and NOC was not found to be stable enough in our laboratory and was especially unsuitable for the determination of total NOC in fresh gastric juice without the addition of sulfamic acid. Nitrosatable compounds, especially those leading to the formation of labile NOC, such as amides (including proteins and peptides), guanidines and ureas, are abundant in the human diet.[2,25] Animal and epidemiological studies suggest that the NOC that are most relevant to human gastric carcinogenesis are those labile compounds[2] that decompose very rapidly under acidic and alkaline conditions.[26–28] If true, then it would be essential to develop a method that could be used to analyse fresh gastric juice directly, without changing any conditions or adding any chemicals that might lead to decomposition of endogenously formed NOC or lead to artefactual nitrosation. Two thermal energy analysers (TEAs) are required when using the method of Pignatelli *et al.*[23] and so far there are no reports from other laboratories of the application of this method to the analysis of gastric juice. Here, we report a further improved method, which is simpler and more reliable and can be used to analyse fresh gastric juice directly.

Experimental

Reagents and Standards

The reagents used were of analytical-reagent or high-performance liquid chromatography grade and were purchased from BDH (now Merck) (Poole, Dorset, UK): glacial acetic acid, ethyl acetate, 45% m/v HBr in glacial acetic acid, acetone, 35.4% m/m HCl, sulfamic acid, sodium sulfate (anhydrous, granular), NaOH pellets, sodium nitrate and sodium nitrite.

The NOC standards used were: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Aldrich, Gillingham, Dorset, UK), *N*-methyl-*N*-nitrosourea (MNU), *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosodiethanolamine (NDELA) (Sigma, Poole, Dorset, UK), and NPRO, *N*-nitrosothiazolidine-4-carboxylic acid (NTCA), *N*-nitrososarcosine (NSAR) and *N*-nitroso-5-methyloxazolidine-4-carboxylic acid (MNOCA) [the last four compounds being kindly provided by Dr. R. C. Massey (MAFF Food Sciences Laboratory, Norwich, UK)]. The standard aqueous NOC solutions were freshly prepared.

[**Caution**: NOC are highly carcinogenic (except NPRO and possibly NTCA and MNOCA) and strictly controlled procedures should be adopted for all aspects of experimental work involving their use.]

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Instruments and Glassware

A TEA (Model 502, Thermo Electron, Waltham, MA, USA) was used as the NO chemiluminescence detector, connected to a Model 3392A integrator (Hewlett-Packard, Avondale, PA, USA).

The glassware and basic apparatus assembly used was as described previously,[22] except for the following modifications: (1) a trap containing NaOH pellets and a trap containing anhydrous sodium sulfate were positioned between the third trap containing NaOH solution (5 mol l^{-1}) and the first cold trap; (2) both cold traps were placed in a freezer (Electrolux, Helsinki, Finland) at −30 °C, the first containing ethyl acetate (40 ml) and the second containing acetone (40 ml); (3) a gas-stream filter (CTR, Thermo Electron) was used as previously described;[23] (4) the TEA flow meter was used instead of the glass pressure regulator and the connected trap; and (5) the needle valve was omitted. A schematic diagram of the apparatus assembly is shown in Fig. 1.

Operating Conditions

(1) Oxygen flow rate: 30 ml min^{-1}; (2) argon flow rate: 40 ml min^{-1}; (3) vacuum pressure: 2.0–4.0 mmHg; (4) temperature of working solutions: 31–33 °C, at scale 2 on the Electromantle heater (Electrothermal, Southend, Essex, UK) (when refluxing under reduced pressure); (5) cold trap temperature: −30 °C.

Preparation of Working Solutions

Under the above operating conditions, two working solutions were prepared as follows.

HBr solution.

Ethyl acetate (120 ml) was placed in the reaction flask; after the peak observed on the TEA decreased to a baseline, 10 ml of acetic acid containing 0.5 ml of 35.4% m/m HCl were injected into the flask. After the second peak on the TEA decreased to the baseline, 5 ml of 45% m/v HBr were injected leading to a large peak response. Water (0.2 ml) was then injected into the flask, and the reaction solution was ready when the small peak occurring from injected water decreased to the baseline. Injection of identical volumes (0.1 ml) of standard solutions of NOC and nitrite (2.5 μmol l^{-1}) should give rise to equal quantitative response peaks on the TEA.

HCl solution.

Ethyl acetate (120 ml) was placed in the reaction flask; after the peak observed on the TEA decreased to the baseline, 15 ml of acetic acid containing 0.5 ml of 35.4% m/m HCl were injected into the flask, and another peak was observed on the TEA until it returned to the baseline. A 0.2 ml aliquot of water was then injected into the flask as in the preparation of the HBr solution. Injection of nitrite standard solution should produce the same response peak on the TEA as with the HBr solution, but no response to injection of MNNG standard solution (0.1 ml; 2.5 μmol l^{-1}) should be observed.

Preparation of Samples

Fresh gastric juice samples were homogenized thoroughly, immediately after collection at endoscopy, with a magnetic stirrer (HI 200M, Hanna Instruments, Singapore), and the pH was measured (PHA 230, Whatman, Maidstone, Kent, UK). Samples of pH <6 were then stored at 2 °C for direct analysis. For the samples of pH >6, 1.0 ml of each sample was diluted (5- to 10-fold) with cold water (pH 7.4, 2 °C) for analysis in the HBr mode (see below) and another 1.0 ml was diluted (5- to 10-fold) with cold alkaline solution (NaOH, pH 11.0, 2 °C) for analysis in the HCl mode (see below) and kept at 2 °C. The dilution liquids were checked for their response on the TEA. A 0.1 ml aliquot of each of the prepared samples to be analysed was then immediately injected into the refluxing solutions.

Sulfamic acid was used for scavenging nitrite in gastric juice; 20 mg of sulfamic acid were added to 1.0 ml of homogenized fresh juice and mixed thoroughly for 1 min before injection of an aliquot of 0.1 ml into the reaction flask.

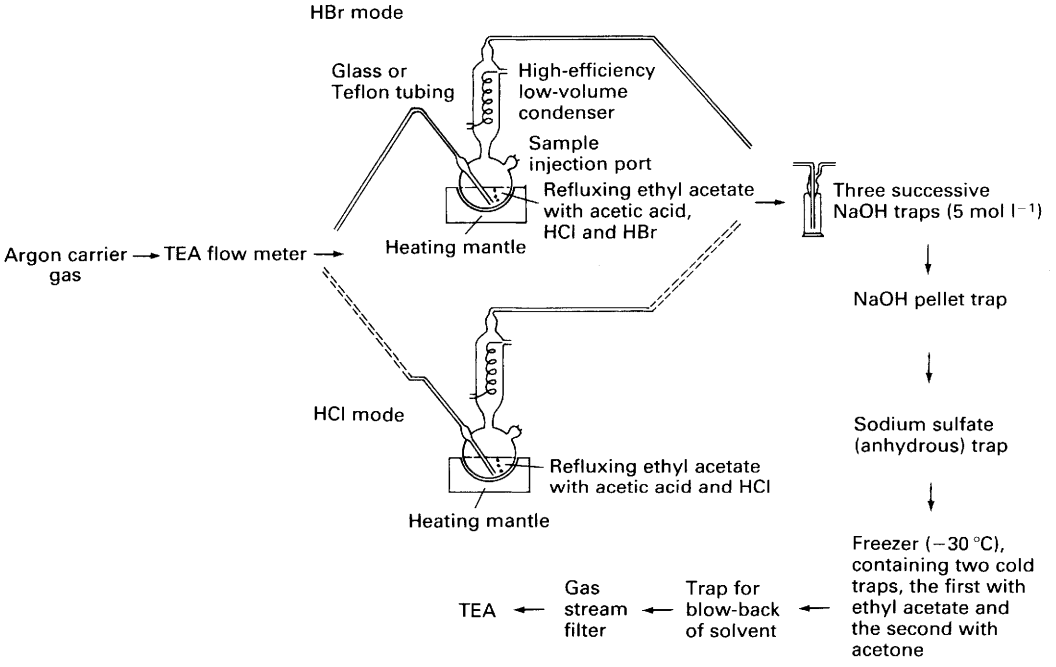

Fig. 1 Assembly for determination of nitrite and total NOC in fresh gastric juice using a TEA as a chemiluminescence detector for NO

Analytical Procedures

The HBr solution was used in the HBr mode to determine (a) the total concentration of NOC + nitrite + thermo- and acid-labile TEA-responsive compounds (TAC) in fresh gastric juice, and (b) the total concentration of NOC + TAC in fresh gastric juice with added sulfamic acid. The HCl solution was used in the HCl mode to determine (c) total concentration of nitrite + TAC in fresh gastric juice, and (d) the TAC concentration in fresh gastric juice with added sulfamic acid. The differences in the results between the two modes ($a - c$; $b - d$) and that of $c - d$ represent the concentrations of total NOC, NOC detected in the same samples with added sulfamic acid (SNOC) and nitrite, respectively (Table 1). The calculation method was the same as that reported previously by Pignatelli *et al.*[23] Regular use of the standard nitrite solution is recommended for both the HBr and HCl modes as a control to ensure accurate results in routine analyses. The TEA response to each sample injection should be kept within the range of that from the injection of standard nitrite solution (2.5 μmol l^{-1}; 0.1 ml), by adjusting the injection volume of samples between 10 μl and 0.2 ml to permit correct and accurate determination.

Because of the instability of NOC in gastric juice, fresh samples should be analysed in the HBr mode as soon as possible after collection, usually within 2–10 min. The switch between the HBr and HCl modes was effected by (1) disconnecting the argon gas inlet tube with the reaction flask slowly; and (2) changing the glass inlet tubes, condensers and reaction flasks containing the different solutions. The switch between modes usually took about 10 min.

The traps need be changed only at the end of each day, although the ethyl acetate in the first cold trap should be checked after about 1.5 h running to avoid overflow. Under normal working conditions, the temperature of the upper part of the flask should be below 30 °C, otherwise it indicates that there are leaks or blockages in the tubing assembly.

Conditions to Minimize Artefactual Nitrosation and Decomposition of NOC

All samples were kept at 2 °C and analysed within 30 min of collection. Aliquots of the samples of pH ≥ 6 were diluted with cold water (for analysis in the HBr mode, pH 7.4, 2 °C) or cold alkaline solution (for analysis in the HCl mode, pH 11.0, 2 °C), and kept at 2 °C.

Results

Effect of HCl Concentration in Working Solutions on Response of the System to Nitrite and NOC

The HCl content of the working solutions significantly affected the capacity of the solutions to transform nitrite and NOC into detectable NO. When 120 ml of ethyl acetate were injected with 20 ml of acetic acid containing 0.1% v/v HCl (35.4% m/m), the response to nitrite on the TEA was poor, as the standard nitrite solution (2.45 μmol l^{-1}) was measured as 1.50 μmol l^{-1}, and after 15 min running the response to the same nitrite standard became even worse with a much smaller and broader peak, as measured as 0.70 μmol l^{-1} (Fig. 2), owing to loss of HCl under low pressure. Injection of 0.1 ml of acetic acid containing 4% v/v HCl improved the response markedly. When the accumulated volume of 4% v/v HCl in acetic acid reached 1.0 ml, the maximum response was achieved with a sharp peak. Additional HCl did not improve the response. Acetic acid (15 ml) containing 35.4% m/m HCl (0.4 ml) used in 120 ml of ethyl acetate maintained the proper capacity for about 40 min. By increasing the HCl content to 0.5 ml, a stable, sensitive reactive system could be maintained for about 1.5 h.

When the HBr solution contained 5 ml of 45% m/v HBr in acetic acid and 0.4 ml of 35.4% m/m HCl in 15 ml of acetic acid, the system responded to standard MNNG, NPRO, NSAR, NTCA, MNOCA, NDMA, NDEA and NDELA with sharp peaks; however, the response to MNU showed a lower, broad peak, which was much improved and acceptable when the HBr solution contained 0.5 ml of 35.4% m/m HCl. A 135 ml portion of the HBr solution containing 5 ml of 45% m/v HBr and 10 ml of acetic acid with 0.5 ml of 35.4% m/m HCl could maintain an efficient denitrosating capacity of the system for at least 3 h. Although an increase in temperature of the solutions (to over 35 °C) increased the response of the system, this decreased rapidly, leading to a shortening of the effective reaction time, owing to rapid loss of acidity at higher temperatures.

Response of the System to Nitrite, NOC and Nitrate, and Analysis of Fresh Gastric Juice

At the beginning of each session the response of the system was poor, but as the saturation of NaOH pellets and sodium sulfate with ethyl acetate vapour occurred, it progressed rapidly to a stable and sensitive condition. In general, it took about 30 min for the system to provide an appropriate response to nitrite and NOC, as shown for NPRO in Fig. 3. A typical example of the analysis of standard MNNG and fresh gastric juice sample for nitrite, total NOC and TAC is shown in Fig. 4. Total NOC, TAC and nitrite in the sample were well separated in the HBr and HCl modes, which has been confirmed by the results from analyses of more than 100 fresh samples (unpublished data). No response to nitrate was observed at a concentration of 1000 μmol l^{-1} in samples, but when nitrate concentrations were >1200 μmol l^{-1} a low, non-

Table 1 Analysis of fresh gastric juice with or without added sulfamic acid in the HBr and HCl modes

	Sulfamic acid	HBr mode	HCl mode	Yield*
a	—	√	—	NOC† + TAC + nitrite
b	√	√	—	NOC + TAC
c	—	—	√	TAC + nitrite
d	√	—	√	TAC

* a − c = total NOC, b − d = SNOC; c − d = nitrite.

† NOC, *N*-nitroso compounds; SNOC, NOC detected from samples with added sulfamic acid; TAC, thermo- and acid-labile TEA-responsive compounds.

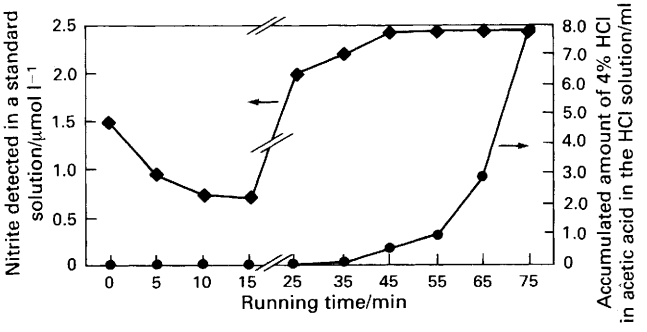

Fig. 2 Effect of HCl content in refluxing ethyl acetate–acetic acid on response of the HCl mode system to nitrite. Experimental protocol, 120 ml of ethyl acetate in the reaction flask, added with 15 ml of 0.1% v/v HCl in acetic acid as the HCl solution at the beginning. Various amounts of 4% HCl in acetic acid (ml) were injected at intervals. Aliquots of 0.1 ml of standard nitrite solution (2.45 μmol l^{-1}) were injected at intervals of 5–10 min

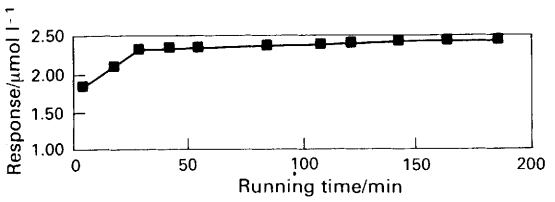

Fig. 3 Response of the new replaced system to NPRO as a function of time. A 10 ml volume of 5% v/v HCl in acetic acid and 5 ml of 45% m/v HBr in acetic acid were injected into 120 ml of refluxing ethyl acetate as a working solution in the reaction flask. An aliquot of 0.1 ml of standard NPRO solution (2.45 μmol l^{-1}) was injected at intervals

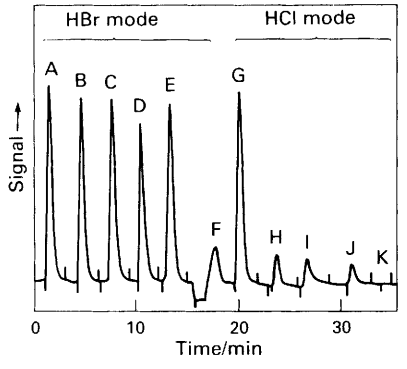

Fig. 4 Thermal energy analyser (TEA) response during analysis of fresh gastric juice and standard MNNG in the HBr mode and HCl mode. HBr mode: A, sodium nitrite (standard), 4.9 μmol l^{-1} (40 μl); B, fresh gastric juice M (0.1 ml), 1.93 μmol l^{-1}; C, fresh gastric juice M, duplicate (0.1 ml), 1.93 μmol l^{-1}; D, fresh gastric juice M + 2% m/v sulfamic acid for 1 min (0.1 ml), 1.76 μmol l^{-1}; E, MNNG 1.92 μmol l^{-1} (0.1 ml); and F, switch from the HBr mode to the HCl mode. HCl mode: G, sodium nitrite (standard), 4.9 μmol l^{-1} (40 μl); H, fresh gastric juice M (0.1 ml), 0.32 μmol l^{-1}; I, fresh gastric juice M, duplicate (0.1 ml), 0.32 μmol l^{-1}; J, fresh gastric juice M + 2% m/v sulfamic acid for 1 min (0.1 ml), 0.23 μmol l^{-1}; and K, MNNG 1.92 μmol l^{-1} (0.1 ml). Total NOC (μmol l^{-1}) = (B + C)/2 − (H + I)/2 = 1.61; SNOC (μmol l^{-1}) = D − J = 1.53; nitrite (μmol l^{-1}) = (H + I)/2 − J = 0.09; and TAC (μmol l^{-1}) = J = 0.23

Table 2 Decomposition of MNNG, MNU and other NOC in the HCl mode*

NOC/ μmol l^{-1}	Content (ml) of 35.4% m/m HCl in the HCl solution				
	0.60	0.50	0.40	0.30	0.20
N-Nitrosoguanidine and N-nitrosourea—					
MNNG:					
4.2	—†	—	—	—	—
10.8	↑‡	—	—	—	—
27.2	↑	↑	—	—	—
54.4	↑ ↑	↑	—	—	—
108.7	↑ ↑	↑ ↑	↑	—	—
217.4	↑ ↑ ↑	↑ ↑	↑	↑	—
MNU:					
145.0	—	—	—	—	—
N-Nitrosamino acids—					
NPRO:					
113.8	—	—	—	—	—
NSAR:					
128.0	—	—	—	—	—
NTCA:					
2.5	↑	—	—	—	—
4.9	↑	↑	—	—	—
MNOCA:					
2.5	↑	—	—	—	—
4.1	↑	↑	—	—	—
Volatile N-nitrosamines—					
NDMA:					
148.4	—	—	—	—	—
NDEA:					
107.6	—	—	—	—	—
NDELA:					
6.7	↑	—	—	—	—
13.4	↑	↑	—	—	—

* Mixed solution (135 ml) of ethyl acetate (120 ml) and acetic acid (15 ml) containing different amounts of 35.4% m/m HCl; temperature of the refluxing solution: 32 °C. The injection volume of the standard solutions was 0.1 ml.

† —: No response observed.

‡ ↑ Representing the baseline rising as a measure of the extent of decomposition.

peak response was obtained and resulted in the baseline rising slightly in both HBr and HCl modes. Therefore, nitrate interference is negligible as both modes yield the same response, and a nitrate concentration of >1000 μmol l^{-1} is rarely encountered in gastric juice samples.

Behaviour of Various NOC in the Working Solutions

The NOC standards MNNG, MNU, NDMA, NDEA, NDELA, NPRO, NTCA, NSAR and MNOCA were systematically examined in the HBr and HCl modes (injection volume 0.1 ml) (Table 2). No decomposition of MNU at a standard solution concentration of 145.0 μmol l^{-1} was observed even when the HCl solution contained 0.60 ml of 35.4% m/m HCl. The compound MNNG was much more susceptible to the HCl solution. Although no response was encountered with the standard MNNG solution at concentrations of up to 10.8 μmol l^{-1} in the HCl solution containing 0.5 ml of 35.4% m/m HCl, a higher concentration of MNNG (27.2 μmol l^{-1}) gave rise to a low, non-peak response, resulting in an elevated baseline. The responses of the HCl mode to MNOCA and NTCA were similar to that with MNNG, but at a concentration of 2.5 μmol l^{-1} no visible response occurred when the HCl solution contained 0.5 ml of 35.4% m/m HCl. Similarly, no responses to the system were observed from NPRO, NSAR, NDMA and NDEA at concentrations of <100 μmol l^{-1} and from NDELA at a concentration of 6.7 μmol l^{-1} in the HCl solution containing 0.5 ml of 35.4% m/m HCl.

All NOC tested (except MNU) responded in the HBr mode with sharp peaks similar to nitrite, but in contrast, in the HCl mode there was only either a clear baseline or a non-peak, elevated baseline response when labile NOC concentrations were very high. The results reveal that decomposition of NOC in mixed HCl, acetic acid and ethyl acetate solutions is closely related to the character of the NOC, together with the concentrations of HCl and NOC.

Reproducibility, Recovery and Limit of Detection

Samples (1.00 ml) of gastric juice were spiked with standard NPRO at 1.40 μmol l^{-1} and detected as 1.383 ± 0.066 μmol l^{-1} (n = 10), with a relative standard deviation (RSD) of 4.8%. From the analysis of fresh gastric juice samples, the RSDs for duplicate TEA measurements were 1–6%. When 990 or 980 μl samples (concentration of total NOC in the sample, 0.55 μmol l^{-1}), obtained by using an Eppendorf (Hamburg, Germany) Varipette, were spiked with 10 or 20 μl of standard NPRO solution (7.00 μmol l^{-1}), the mean recovery was 98.7 ± 4.9% (n = 10). The limit of detection (signal-to-noise ratio 3:1) was 0.01 μmol l^{-1} at an injection volume of 0.1 ml, *i.e.*, 1.0 pmol.

Stability of NOC and Nitrite in Fresh Gastric Juice

The stability of total NOC and nitrite in fresh gastric juice has been studied. Total NOC concentrations in both low pH (<2.0) and high pH (>7.0) samples decreased rapidly at 4 °C

(Fig. 5). No increase in total NOC, nitrite or TAC levels in fresh samples at pH <5 was observed at 20 and 4 °C. However, increases of nitrite concentrations in fresh samples of gastric juice at pH 6.0–8.5 were observed at 4 °C (Fig. 5). Adjusting the pH of the high pH samples to >10 with cold alkaline solution (pH 11.0, 2 °C) and storing at 2 °C could effectively keep nitrite stable in these samples. Owing to the instability of nitrite and NOC in fresh gastric juice, such samples must be analysed as soon as possible after collection. In our study almost all samples were analysed within 30 min, and only a few samples were stored at −20 °C and analysed within 2 h.

Scavenging Efficiency of Sulfamic Acid on Nitrite

The efficiency of sulfamic acid in scavenging nitrite was tested. The efficiency was affected by sulfamic acid concentration, nitrite concentration and reaction time at room temperature (Fig. 6). Direct addition of solid sulfamic acid to samples for the removal of nitrite was very effective. When 20 mg of sulfamic acid were added to 1.0 ml (2% m/v) of standard nitrite solution (613.0 μmol l^{-1}) the nitrite was completely decomposed within 10 s (Fig. 6). Nitrite at concentrations of 337.5– 650.3 μmol l^{-1} in fresh gastric juice samples (pH 7.57 and 7.75) could be completely decomposed by sulfamic acid (2% m/v) within 40 s. Therefore, 1 min with vigorous shaking was selected as the appropriate scavenging time of sulfamic acid for sample preparation.

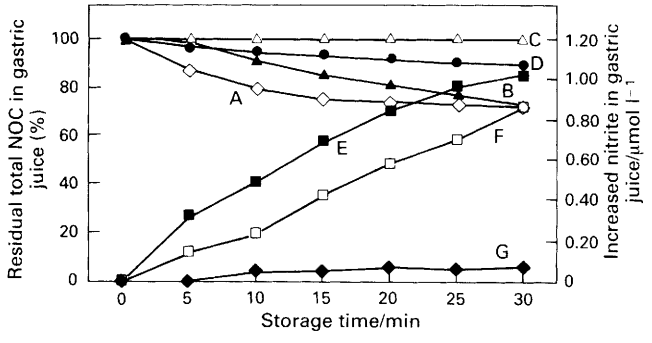

Fig. 5 Stability of total NOC and nitrite in fresh gastric juice samples at 4 °C. NOC (pH of samples): A, 1.69; B, 1.77; C, 3.42; and D, 7.34. Nitrite (pH of samples): E, 7.24; F, 7.76; and G, 10.57. Sample G was prepared from sample E (pH 7.24) by adjusting the pH with aqueous alkaline solution (pH 11.0, 2 °C)

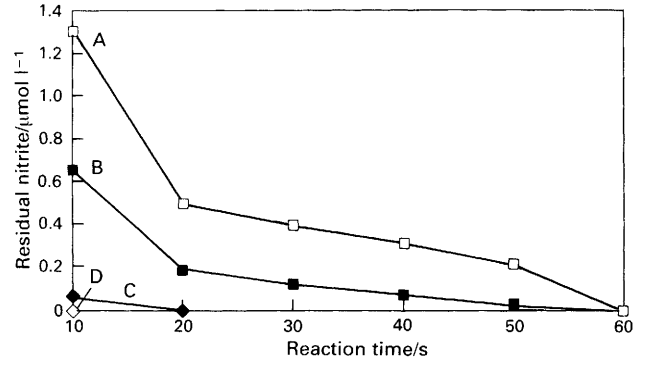

Fig. 6 Efficiency of sulfamic acid (SA) in destroying nitrite as a function of time. A, 2% m/v SA 0.5 ml + nitrite 6130 μmol l^{-1} 0.5 ml; B, 4% m/v SA 0.5 ml + nitrite 6130 μmol l^{-1} 0.5 ml; C, 2% m/v SA 0.5 ml + nitrite 613 μmol l^{-1} 0.5 ml; and D, 20 mg SA + nitrite 613 μmol l^{-1} 1.0 ml. pH of the solutions: 4% m/v SA solution = pH 1.25; 2% m/v SA solution = pH 1.50; nitrite solution = pH 7.70; 4% m/v SA solution + nitrite solution (1 + 1) = pH 1.65; and 2% m/v SA solution + nitrite solution (1 + 1) = pH 1.72. Reaction temperature, 22 °C

Discussion

In 1970, Eisenbrand and Preussmann[29] reported that HBr in acetic acid was highly effective in cleaving the N–NO bond by using a spectrophotometric Griess-type reagent to determine the denitrosation products. This procedure was modified by Downes *et al.*,[30] who used a chemiluminescence detector to measure the released NO. Walters and co-workers[10,22] further developed this method for the first time for the analysis of gastric juice samples. By using this method a positive relationship between total extractable NOC and intragastric pH, bacterial flora and nitrite in gastric juice was reported by several workers.[11–14] However, the addition of sulfamic acid, extraction of NOC from samples into ethyl acetate, possible decomposition of NOC in refluxing ethyl acetate, response interference from HBr solution and storage of samples were all suggested as potential interfering factors, in particular false-negative interference in this method.[15,24] In order to minimize the loss of NOC in gastric juice Bavin *et al.*[15] attempted to avoid acidic conditions for decomposing nitrite by using hydrazine sulfate and by raising the pH to 4.0 and directly analysed gastric juice samples, without extraction, soon after collection. Much higher gastric juice NOC concentrations with no relationship between total NOC and intragastric pH, bacterial flora and nitrite levels were reported by workers using this method.[15,17–19] As hydrazine sulfate is much less effective in decomposing nitrite, and *S*-nitroso compounds and other TAC were not differentiated from NOC in their procedure, false-positive interference could not be avoided, which was a factor in the significantly higher total NOC levels reported.[23,24,31,32] The method of gastric juice sample collection was also questioned.[32] A further improved method based on that of Walters *et al.*[22] was reported by Pignatelli *et al.*,[23] in which addition of 0.1% v/v HCl in acetic acid enhanced the sensitivity. These workers also observed a positive relationship between total NOC in gastric juice and intragastric pH.

However, the addition of sulfamic acid, the need for two sets of TEA instruments and the short effective duration are definite shortcomings of the method.[23] In particular, the variability of NO release from nitrite by acetic acid with added 0.1% v/v HCl observed in these studies, together with broad peaks, were a disadvantage of this procedure. It was found that the poor transformation capacity of nitrite into NO was mainly as a result of the acidity of the refluxing HCl solution being inadequate at the concentration used. The results of the present study have shown that this capacity was significantly affected by the HCl content in the solution as well as by the running time of the system. The size and shape of the response peak under given conditions was determined by this capacity; a high capacity produced a very tall, sharp peak, while reduced capacity resulted in peak height reduction and broadening. Injection of the samples containing sulfamic acid could also raise this capacity when the HCl content of the working solutions was too low, but this would inevitably lead to interference. A higher concentration of HCl was also needed for accurate determination of MNU in the HBr mode. Therefore, the inadequacy of the HCl concentration and the addition of sulfamic acid to gastric juice samples limit the use of the method of Pignatelli *et al.*[23] in the direct analysis of fresh samples. In order to obtain a sensitive and stable capacity of transformation, permitting a longer analysis run time, more HCl was needed in the working solutions. The minimal effective concentration of HCl in the working solutions was also determined by the differing behaviour of NOC in the solutions.

Many non-volatile NOC (*e.g.*, *N*-nitrosamides and some *N*-nitrosamino acids) are not stable in strongly acidic and alkaline solutions.[26–28] Therefore, it is important to ensure that all NOC produce NO maximally in the HBr mode, but remain stable in the HCl mode for accurate analysis. The degradation of various NOC in strong acid is very different

owing to their different characters. The results in the present study show that MNNG, MNOCA and NTCA are sensitive to higher concentrations of HCl in the HCl solution, and MNU is even strongly resistant to the HBr solution. Therefore, it is very important for the method to locate a suitable range of HCl contents in the working solutions to determine NOC in gastric juice samples correctly, *i.e.*, with labile NOC not being decomposed in the HCl mode to any marked extent, but with all NOC being detected in the HBr mode. Under the conditions in the present study when the HCl solution (135 ml) contained 15 ml of acetic acid and 0.5 ml of 35.4% m/m HCl, then MNNG, MNOCA and NTCA at concentrations of 2.5–10.8 μmol l^{-1} were still stable enough in the HCl mode, and MNU would give a proper peak response in the HBr mode, for correct analysis at a temperature of 31–33 °C. This capacity of transformation was stable for at least 1.5–3 h. The TAC concentrations detected in the gastric juice samples by use of this method were found (unpublished data) to be either similar to or lower than those reported by Pignatelli *et al.*,[23] who used 0.1% v/v HCl in acetic acid. This confirmed that no significantly increased decomposition resulted from the stronger acidity used in the HCl mode in the present method.

We observed that NOC in some fresh gastric juice samples decomposed rapidly after collection when kept at 4 °C (Fig. 5). This is in accord with the properties of most *N*-nitrosamides and many other more complex NOC.[26–28] Further, the addition of sulfamic acid to the samples resulted in marked acceleration of decomposition (unpublished data). Therefore, to avoid false-negative interference it is essential to analyse fresh samples directly, as soon as possible after collection and without the addition of any chemicals. The use of our method enabled us to analyse fresh gastric juice samples directly within 2 min after collection, thereby reducing the false-negative interference from the loss of NOC to a minimum. Gastric juice samples with pH ≥ 6 usually contained high concentrations of nitrite and potential nitrate-reducing bacterial flora. It was found that the nitrite concentration in some high pH samples could increase even at 4 °C during storage, possibly due to the activity of bacteria with a very high potential for nitrate reduction even at a low temperature. In order to avoid such interference we used a combination of low temperature storage (2 °C), cold neutral (for analysis in the HBr mode) and alkaline (for analysis in the HCl mode) aqueous solutions (2 °C, pH 7.4 and 11.0) to dilute aliquots of samples with pH values of 6–8.5[33,34] and analysed them in the HBr mode within 5 min and in the HCl mode within 30 min after collection, which effectively kept samples stable for obtaining accurate results.

Sulfamic acid had often been used as a stabilizer of nitrosation in biological samples, such as gastric juice. We observed that sulfamic acid could decompose unknown labile NOC in fresh gastric juice samples significantly, especially in those with a high pH (unpublished data). Therefore, the addition of sulfamic acid to gastric juice samples would result in false-negative interference and certainly should be avoided in the preparation of such samples for determination of total NOC. As sulfamic acid decomposed nitrite very rapidly and effectively, 1 min was selected as the standard reaction time for preparing samples for the determination of SNOC and TAC.

As the sensitivity was also very significantly enhanced in the present method, we were able to reduce the volume of most samples injected to 0.1 ml. This further avoided interference resulting from the injection of a large volume of water as well as maintaining a more stable system and prolonging the running time. The requirement of only one chemiluminescence detector makes our method more easily applicable in many more laboratories.

Applying this method to the analysis of fresh gastric juice samples and to the study of the stability of fresh gastric juice samples under different storage conditions is underway in this laboratory. We anticipate that the application of this method to the analysis of fresh gastric juice will provide more new data on the mechanism of endogenous *N*-nitrosation in man.

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