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Breakthroughs and Views

^{1}H NMR analysis as a diagnostic probe for human saliva

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Abstract

The applications of high resolution ^{1}H NMR analysis as a diagnostic probe for human saliva are reviewed with special reference to diabetes mellitus, and a recently published report regarding the ability of this technique to detect advanced glycation endproducts (AGEs) in this biofluid [Biochem. Biophys. Res. Commun. 323 (2004) 377–381]. We also demonstrate that hypochlorous acid/hypochlorite ($HOCl/OCl^-$)-induced modifications to the ^{1}H NMR profiles of human salivary supernatants arise from the chlorination and, where appropriate, oxidation of amino acids and malodorous amines, together with the oxidation of carbohydrates and α-keto acid anions. The attack of $HOCl/OCl^-$ on carbohydrates yields formate (singlet, $\delta = 8.46$ ppm), the ^{1}H NMR signal of which was erroneously assigned to AGE species by the authors of [Biochem. Biophys. Res. Commun. 323 (2004) 377–381].

Keywords: NMR analysis; Human saliva; Metabolites; Hypochlorous acid/hypochlorite anion; Formate; Oxidation products

The paper published in your journal entitled 'Characterisation of advanced glycation endproducts in saliva from patients with diabetes mellitus' by Ming-Sung Yoon et al. [1] is of much concern to us since it contains many scientific flaws and misinterpretations, together with a complete misrepresentation of the authors' ^{1}H NMR results in their Discussion section and conclusions.

In their Introduction the authors state vaguely that 'Earlier attempts to use NMR spectroscopy [to the multicomponent analysis of biofluids] have partly been disappointing.' However, the technique has been successfully employed for the analysis of complex biofluid samples for greater than 20 years, and hence this statement is simply not true. They also pronounce that the metabonomics approach to this research area (involving principal component analysis or further multivariate statistical analysis methods), specifically to the comparison of biofluids from subjects with various clinical conditions with appropriate, corresponding controls [2,3], has advanced our ability to detect differences between such groups, which indeed it has, but to use this as an excuse for not making any ^{1}H NMR assignments whatsoever is extremely perturbing.

If the paper's authors had taken care to read our research report on the ^{1}H and ^{13}C NMR analysis of human saliva [4], which they reference, they would have been able to make unequivocal resonance assignments. Moreover, the chemical shift values of their highly putative advanced glycation end-product (AGE) resonances are only quoted to the nearest 0.1 ppm, and, even at an operating frequency of 400 MHz (400 Hz per ppm), this can preclude distinction between various components present in this biofluid. Furthermore, the coupling patterns (and coupling constants) of their signals are not even mentioned, let alone employed to facilitate any of their assignments.

The researchers claim to have found that saliva specimens from patients with diabetes mellitus had a substantially elevated intensity of ^{1}H NMR signals located at

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2.3, 7.2, and 8.4 ppm when expressed relative to those of healthy controls. From an examination of their spectra shown in Figs. 1 and 2, the resonance at 8.4 ppm is a singlet and undoubtedly assignable to formate ($\delta = 8.46$ ppm) [4], whereas that at 2.3 ppm (and probably also that at 7.3 ppm) appears to be a multiplet and/or composite signal representing two or more components. The 7.3 ppm signal is probably attributable (at least in part) to phenylalanine's H2,H6-aromatic ring protons (multiplet, $\delta = 7.32$ ppm) [3]. However, that at 2.3 ppm is potentially assignable to a range of components, including 4-hydroxyproline (γ-CHOH multiplet, $\delta =$ 2.23 ppm), acetone (-CH_3 groups' singlet, $\delta =$ 2.245 ppm), valine (β-CH multiplet, $\delta = 2.28$ ppm), γ-aminobutyrate (α-CH_2 triplet, $\delta = 2.29$ ppm), 3-D-hydroxybutyrate (-CH_2 doublet of doublets, $\delta =$ 2.34 ppm), proline (β-CH_2 multiplet, $\delta = 2.35$ ppm), iso-butyrate (α-CH septuplet, $\delta = 2.36$ ppm), or even glutamate (γ-CH_2 multiplet, $\delta = 2.37$ ppm). Indeed, the chemical shift values of at least some of these resonances are pH-dependent (albeit in a narrow but nevertheless significant δ value range), and hence without any salivary pH data (nor coupling patterns and their constants) supplied by the authors, such assignments are not facile. Of course, the application of two-dimensional NMR techniques (^{1}H–^{1}H or ^{1}H–^{13}C) would readily solve these assignment problems! [4].

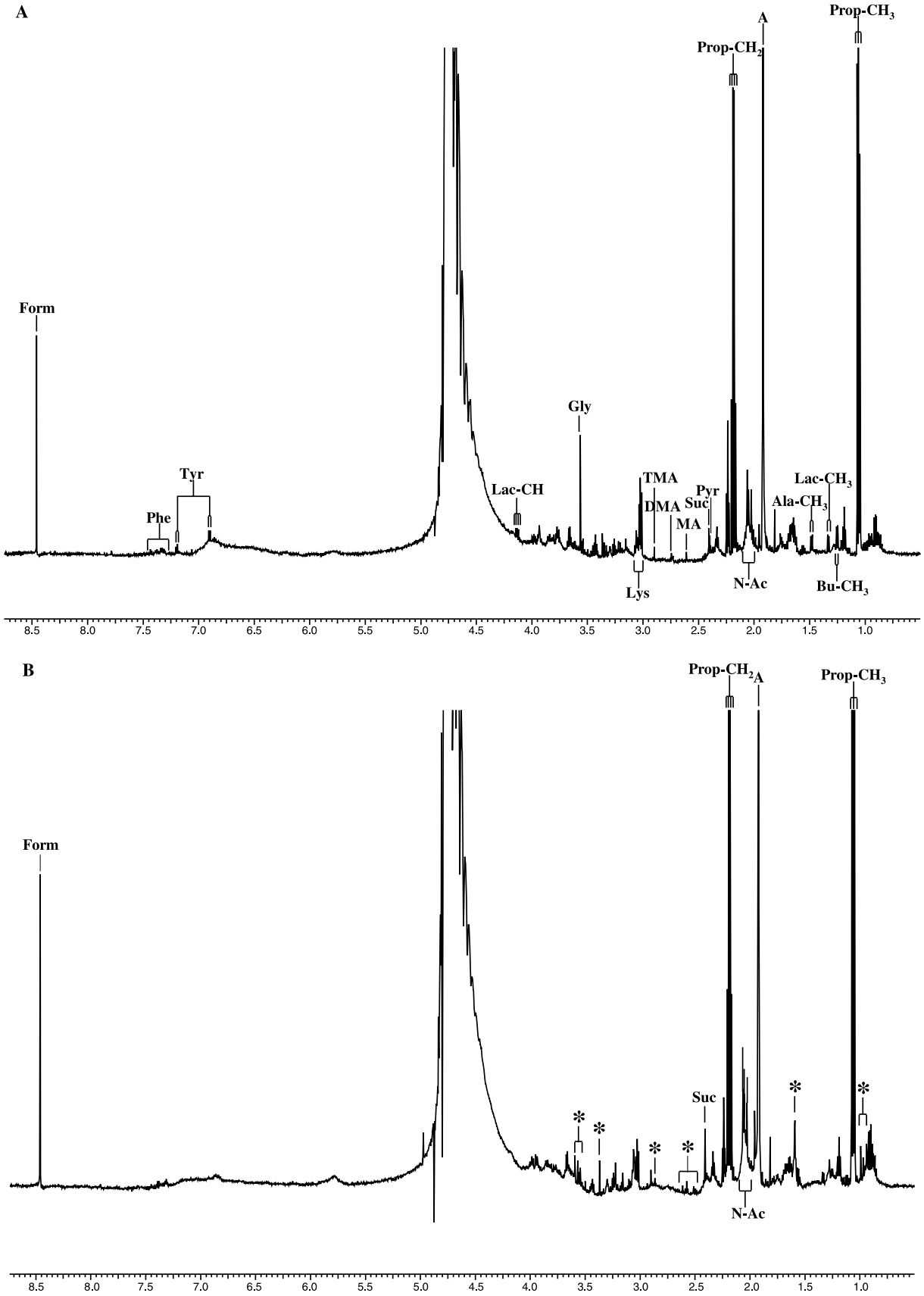
The Methods section states that 50 μl HOCl was added to saliva specimens and the mixtures were incubated at room temperature for a 2 h period. What were the concentration and pH value of this added reagent solution, [the hypochlorous acid/hypochlorite anion ($HOCl/OCl^-$) system has a pK_a value of 7.40], and did the addition of it alter the pH of saliva samples? The latter question is clearly important on consideration of the above comments regarding the pH-dependence of biomolecule chemical shifts.

Moreover, in their Introduction, the authors state that treatment of blood plasma with $HOCl/OCl^-$ is a 'generally acceptable method to generate advanced glycation endproducts from plasma of healthy subjects'; in view of the rich redox and chlorination chemistry of this reagent on reaction with biomolecules, it certainly is not! The molecular mechanisms involved in the development of AGEs are already complex enough, without complicating them further via the introduction of $HOCl/OCl^-$-induced oxidised and chlorinated protein amino acids, which have little or no relevance to the subject.

How can it be that the addition of $HOCl/OCl^-$ to human saliva generates exactly the same ^{1}H NMR resonances assigned (erroneously) to AGEs [specifically formate (singlet, $\delta = 8.46$ ppm), probably phenylalanine (multiplet, $\delta = 7.32$ ppm), and possibly acetone (singlet, $\delta = 2.245$ ppm)]? During the last 10 years, we have acquired a considerably large quantity of ^{1}H NMR data on a very wide range of saliva specimens (>2000, a conservative estimate!), and, with the exception of only one or two, formate has been detectable in every sample examined! We can understand that $HOCl/OCl^-$ will certainly degrade AGEs to products, which are likely to be ^{1}H NMR-detectable (indeed, our spectrophotometric studies have recently shown that brown-coloured melanoidin glycation products, components at least partially responsible for extrinsic tooth discolouration, are readily degraded and decolourised by this oxidant), but to hypothesise that AGEs are actually generated in saliva on treatment with this agent, and that ^{1}H NMR signals assignable to common salivary metabolites such as formate and phenylalanine are ascribable to such AGEs is beyond all sense of scientific reasoning! Indeed, it is of no surprise that the addition of 5.00 mmol dm^{-3} glucose, pentosidine, and carboxymethyl-lysine to saliva samples by the authors did not give rise to increases in the intensities of these resonances! Moreover, the only other sound conclusion arising from this work is that an ^{1}H NMR spectrum of an aqueous solution of $HOCl/OCl^-$ did not contain resonances which they have wrongly assigned to AGEs. This, again, is of course not unexpected! The authors appear to lack any form of understanding and/or consideration of even elementary NMR phenomena, let alone those required for the analysis of multicomponent biofluid specimens and the interpretation of high field ^{1}H NMR spectra derived therefrom.

Despite performing oral irrigation with water (100 ml) prior to sample collection, it appears that the researchers involved did not take any acceptable precautions regarding potential analytical interferences arising from the introduction of exogenous agents into the oral environment and their rate of clearance therefrom. In order to avoid such interferences, the patients recruited should have been directed to completely refrain from all oral activities (eating, drinking, smoking, tooth-brushing, oral rinsing, etc.) for a period of at least 4 h prior

Fig. 1. Six hundred megahertz ^{1}H NMR spectra of a human salivary supernatant sample (A) before, and (B) after treatment with $HOCl/OCl^-$ (3.10 mM) at pH 7.00. Spectra were acquired on a Bruker AMX-600 spectrometer operating at a frequency of 600.13 MHz and a probe temperature of 298 K. Samples were prepared by adding deuterium oxide (0.07 ml) and a 5.00 mM solution of sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TSP) in deuterium oxide (0.03 ml, chemical shift reference, $\delta = 0.00$ ppm) to 0.60 ml of control or $HOCl/OCl^-$-treated supernatant. *Abbreviations:* A, acetate-CH_3; Ala-CH_3, alanine-CH_3; Bu-CH_3, β-hydroxybutyrate-CH_3; DMA, dimethylamine-CH_3; Form, formate-CH; Gly, glycine-CH; Lac-CH_3 and -CH, lactate-CH_3 and -CH group protons, respectively; Lys, lysine-ε-CH_2; MA, methylamine-CH_3; N-Ac, spectral region for acetamido methyl groups of *N*-acetyl sugars; Phe, phenylalanine aromatic ring protons; Prop-CH_3 and -CH_2, propionate-CH_3 and -CH_2 group protons, respectively; Pyr, pyruvate-CH_3; Suc, succinate-CH_2; and TMA, trimethylamine-CH_3; Tyr, tyrosine aromatic ring protons. The asterisk denotes a series of new (unassigned) resonances generated from the addition of $HOCl/OCl^-$.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to sample collection, although we do, of course, understand that this is not easily achievable in patients with type-I diabetes! We routinely request subjects to collect all saliva immediately after waking, and also to refrain from all oral activities in the short period between awakening and expectoration (usually <5 min). This method would avoid any complications arising from the carbohydrate demand of insulin- or oral hypoglycaemic agent-dependent diabetics.

There is no mention in the Methods section that the collected saliva samples were centrifuged prior to analysis. Without this preliminary preparation step, solution-phase spectra acquired will be of a poor quality in view of the (relatively) large amount of particulate matter present in this biofluid when intact, and this would appear to account for the sub-standard spectra exhibited in Figs. 1 and 2 [especially Figs. 1B and 2B] at an operating frequency of 500 MHz. We have acquired ^{1}H NMR spectra on human saliva samples at lower operating frequencies which are much more informative, i.e., they contain a multitude of clear, sharp resonances.

Prior to storage and analysis, the samples collected were not pre-treated with fluoride in order to prevent microbial metabolism and proliferation, and hence modifications in the concentrations and therefore intensities of resonances arising from selected salivary microbial catabolites normally detectable in this biofluid are expected to occur during sample preparation and storage periods (this phenomenon is well known to those working in the area of biomedical NMR spectroscopy).

Our laboratory has recently monitored the oxidative attack of HOCl/OCl$^-$ on biomolecules present in intact human salivary supernatants using this multicomponent analytical technique at an operating frequency of 600 MHz (Fig. 1). Addition of a 3.10 mM concentration of HOCl/OCl$^-$ to this biofluid at pH 7.00 ($n = 6$) gave rise to clear and substantial reductions in the intensities of many free amino acids detectable (e.g., alanine, glycine, lysine, phenylalanine, and tyrosine) in view of their ready conversion to mono- and dichloroamine species by the oxohalogen oxidant and, where appropriate, oxidation and aromatic ring chlorination. Additionally, α-keto acid anions were oxidatively decarboxylated (i.e., pyruvate to acetate and CO_2, 2-oxoglutarate to succinate and CO_2). Moreover, the concentration of ^{1}H NMR-detectable formate was elevated to values much higher than that of untreated control samples, an observation presumably ascribable to its generation as a terminal end-product from the oxidative attack of the added reagent on salivary carbohydrates (e.g., glucose, glycosaminoglycans, and the molecularly mobile carbohydrate side-chains of glycoproteins), as indeed it is from the attack of hydroxyl ($^{\bullet}$OH) radical on these targets [5] (further modifications to the spectra acquired were also notable, e.g., consumption of malodorous amines such as methyl- and dimethylamine, which are presumably converted to their corresponding mono- and/or dichloroamine derivatives, and the oxidation of lactate and 3-D-hydroxybutyrate, the former to pyruvate and subsequently acetate and CO_2, the latter presumably to acetoacetate).

The authors state in their Methods section that 'Provided that the linewidths are comparable the resonance intensity can be used to determine the concentration of AGE. Calibration curves with AGE showed a relationship between concentration and resonance intensity. Therefore, the resonance intensity could be used for quantitation of the substance.' However, despite mention in the second paragraph of the Results and discussion section that 'The resonance intensities at 2.3, 7.2 and 8.4 ppm were compared between the two groups using Fisher's exact test,' no further mention of resonance intensities and derived concentrations of components detectable is made in the publication. Indeed, this test is only applicable to the detection of non-random associations between two categorical variables, i.e., as in contingency table (enumeration) data. As noted above, the quantification of formate ($\delta = 8.46$ ppm) by ^{1}H NMR spectroscopy may serve as a 'marker' of oxidative damage exerted by myeloperoxidase-generated HOCl/OCl$^-$ and/or $^{\bullet}$OH radical, but the separation of type-I diabetic patients with periodontal diseases into groups according to their severity, followed by the use of the presence/absence of this biomolecule's ^{1}H NMR signal as a diagnostic index, represents an extremely poor level of scientific reasoning, not least because it is ^{1}H NMR-detectable by us (and others) in virtually all saliva specimens examined! [was the value of 3 as a critical approximal plaque index (API) value selected prior to the collection of data, or decided subsequently?]. Hence, Fig. 3 in their publication, which shows the frequency of observation of the $\delta = 8.4$ ppm formate singlet resonance in the above two groups, fails to make any sense.

It is also of much importance to note that the addition of charged and/or polar reagents to biofluids can give rise to concentration-dependent increases (often substantial) in the intensities of many ^{1}H NMR resonances in view of the potential capacity of the added agent to displace biomolecules of like charge, polarity or H-bonding capacity from protein or alternative macromolecule-binding sites; signals of such macromolecule-bound components are not generally observable in spectra acquired in view of their short T_2 values (HOCl/OCl$^-$-mediated oxidative damage to such binding-sites is also likely to result in the release of these biomolecules). Indeed, addition of high levels of ammonium chloride to human plasma gives rise to marked increases in the intensities of lactate resonances [6] and hence treatment of saliva samples with sufficient concentrations of HOCl/OCl$^-$ (both before or after its

reaction with salivary biomolecules) could also give rise to this observation.

Also notable is the possible detection of acetone by the researchers which has a readily observable singlet resonance at $\delta = 2.245$ ppm and may correspond to their 2.3 ppm signal. If correct, this observation is of some importance in diabetes, but hardly represents a new marker of any diagnostic capacity since it is well known that this agent, a sign of poor glycaemic control which arises from the utilisation of lipids as a source of fuel, is readily detectable in human breath (organoleptically or otherwise). Presumably, salivary acetone levels will become elevated in cases of hyperglycaemia, but, as with blood plasma, its concentration will, of course, be dependent on the level of control exerted by the patient via the administration of insulin injections or oral hypoglycaemic agents, and dietary carbohydrate intake.

In conclusion, the study [1] clearly does not provide a new marker of oxidative protein modification: formate, its ^{1}H NMR signal unassigned by the researchers involved, is present as a normal catabolite in just about all human saliva samples (and alternative biofluids that both we and other research groups have subjected to ^{1}H NMR analysis, including blood plasma, urine, and inflammatory knee-joint synovial fluid). Although formate is also a common product from the oxidative attack of HOCl/OCl^- (and/or $^{\bullet}OH$ radical) on carbohydrates in general (salivary levels of which vary substantially between subjects and are, of course, markedly influenced by diurnal variation, e.g., how soon were the samples collected after the patients involved had eaten, to mention but one variable?) Formate is biologically ubiquitous, and the diet also serves as a potentially rich source of this agent: we have noted it as an ^{1}H NMR analyte in many foods. Furthermore, the results acquired certainly do not reveal the occurrence of oxidatively modified proteins in saliva from humans with diabetes mellitus, not least because any AGEs putatively generated in human saliva in vivo are expected to be of extremely low concentration, and hence their analysis by ^{1}H NMR spectroscopy is precluded by the technique's sensitivity at an operating frequency of 500 MHz; moreover, macromolecules of low-molecular-mobility and hence short T_2 values do, of course, have broad resonances which also severely restrict their detection (the conclusion by the authors that such adducts are detectable and offer valuable diagnostic indices is completely erroneous and of no relevance to their ^{1}H NMR data collected!). Consequently, these data also do not generate a new hypothesis regarding how periodontitis may be linked to cardiovascular diseases (in view of our comments, the authors can hardly extend the 'significance' of their results to the latter research area).

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