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Research Paper

The role of an alginate suspension on pepsin and bile acids – key aggressors in the gastric refluxate. Does this have implications for the treatment of gastro-oesophageal reflux disease?

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Abstract

Objectives During a reflux event the oesophagus is exposed to a heterogeneous mixture of gastric juice components. The role of non-acid components of the refluxate in causing damage to the oesophagus is now well established but no therapeutic option exists to address this.

Methods The role of Gaviscon Advance (GA), a raft-forming alginate suspension, in protecting the oesophagus from damage by pepsin and bile acids (aggressors) was investigated using a series of in-vitro models.

Key findings GA was able to dose-dependently inhibit pepsin activity over and above the neutralisation effect of the formulation. This was evident against both protein and collagen substrates using two distinct colorimetric assays. GA was able to retard the diffusion of pepsin and multiple bile acids using a Franz cell model. Using the raft-forming mode of action GA was able to remove both pepsin and multiple bile acids from a simulated reflux event. There was capacity in the GA raft to accommodate aggressors from multiple reflux events.

Conclusions GA can specifically remove both pepsin and bile acids from the refluxate, limit their diffusion and affect enzymatic activity of pepsin. There is a role for GA to reduce the damaging potential of the refluxate and thus protect the oesophagus.

Keywords alginate; bile acids; formulation; gastro-oesophageal reflux; oesophagus; pepsin

Introduction

The alginate-based formulations of Gaviscon (G) and Gaviscon Advance (GA) (Reckitt Benckiser (UK) Ltd) have been used successfully in the treatment of gastro-oesophageal reflux disease (GORD) for decades[1,2] and give a rapid onset of symptom relief.[3] The mode of action of GA is by forming a buoyant, CO_2-aerated alginate gel (raft) when the dose comes into contact with gastric contents.[4] The raft physically prevents reflux into the oesophagus and as such is often called a reflux suppressant.

The refluxate that enters the oesophagus during a reflux event comprises the gastric juice[5] and also the duodenal contents which are refluxed into the stomach.[5,6] This heterogeneous mixture includes gastric juice components such as acid (HCl), pepsins, mucus, bicarbonate, intrinsic factor, prostaglandins, hormones, and food and drink. Duodenal reflux components consist of bile acids and pancreatic enzymes (trypsin, chymotrypsin and pancreatin).

Pepsin is the major enzyme in gastric juice and the concentration in the stomach can reach 1 mg/ml.[7] There are several pepsin isoforms: 1, 3a, 3b, 3c and 5. Pepsin is frequently detected in oesophageal aspirates[5] and contrary to popular belief it, rather than acid, is the major damaging component of the gastric refluxate. In ex-vivo animal models it is well documented that acid alone (pH > 1.3) is unable to produce experimental damage to the oesophagus but that addition of pepsin to these solutions will result in significant damage equivalent to oesophagitis.[8–11] Currently there are no treatments for GORD that address the damaging potential of pepsin. Since pepsin remains active up to pH 5.5 and is not irreversibly

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denatured until pH 8.0, acid suppression is inadequate to combat proteolytic damage to the oesophagus.[12]

Bile acid reflux into the oesophagus is common.[5,6] Indeed it appears that the extent of bile reflux increases in reflux sufferers and also as the severity of reflux disease progresses.[13,14] Reflux of bile acids is believed to be a major aetiological factor associated with the development of complications of reflux, namely Barrett's oesophagus and oesophageal adenocarcinoma. There have been several studies to investigate the damaging potential of bile acids at different pH levels. The hypothesised mechanisms of damage by bile acids include alteration to membrane permeability,[10] changes in cell proliferation and differentiation,[15,16] initiation of oxygen-derived free radicals,[17,18] induction of DNA damage,[18–20] and up-regulation of oncogenes.[20,21]

The emergence of the 'weak-acid reflux' paradigm in the aetiology of reflux disease has highlighted the clinical importance of non-acid components of the refluxate. Technology such as 24-hour multichannel intraluminal impedance combined with pHmetry has shown that proton pump inhibitor (PPI) therapy does not stop gastro-oesophageal reflux and serves only to change the pH.[22–24] An optimal treatment for gastro-oesophageal reflux is one that prevents reflux *per se*, thus protecting the oesophagus from the multiple damaging components of the refluxate.

Here we investigate whether GA has a role in the protection of the oesophagus from gastro-oesophageal refluxate, and specifically the aggressors, pepsin and bile acids, using a series of in-vitro methods.

Materials and Methods

Materials

GA aniseed suspension (Reckitt Benckiser Healthcare (UK) Ltd) comprises 500 mg sodium alginate and 100 mg potassium bicarbonate per 5 ml dose (100 mg/ml alginate).

Chemicals were obtained from Sigma Aldrich (Poole, UK) or Fisher Thermo Scientific (Loughborough, UK) unless stated. Porcine pepsin A EC.3.4.23.1 was obtained from Sigma Aldrich (catalogue number P-7012: 2850 units/mg protein). Human gastric juice (pooled) was aspirated from patients undergoing gastroscopy and had a pepsin activity equivalent to 1 mg/ml porcine pepsin. Succinyl albumin was prepared by succinylation of bovine serum albumin (fraction V) with succinic anhydride at pH 7.5. Azo-dye-labelled Type I collagen, Azocoll (>100 mesh), was obtained from Calbiochem (San Diego, USA) (catalogue number 194933). Bile acids were obtained from Sigma Aldrich and were cholic acid (CA; 27010), taurocholic acid sodium salt hydrate (TCA; T4009), glycocholic acid (GCA; G2878) and deoxycholic acid (DCA; D2510).

Determination of pepsin activity

Pepsin activity was measured in the presence of GA using two colourimetric assays.

N-terminal assay

The N-terminal assay is a quantitative colorimetric assay of proteolytic activity and is a sensitive, accurate method that is able to detect a single peptide bond cleavage.[25] Peptide bond hydrolysis, and therefore generation of new N-terminal groups, is detected by trinitrophenylation of the amino group. The use of this method to show inhibition of pepsin by alginates has been previously described in detail by Strugala *et al*.[26]

Porcine pepsin was diluted in 0.01 M HCl, pH 2.2 to a concentration of 50 μg/ml (142.5 units) and a standard curve was produced ranging from 0 to 50 μg/ml pepsin (total volume 200 μl). Five hundred microlitres of 10 mg/ml succinyl albumin substrate in 0.01 M HCl, pH 2.2, was added to the pepsin solutions and incubated at 37°C for 30 min. The reaction was halted by addition of 500 μl 4% $NaHCO_3$. Colour was developed with 500 μl 10 mM trinitrobenzenesulfonic acid (TNBS) and incubated at 50°C for 10 min. After addition of 500 μl 10% sodium dodecyl sulfate and 250 μl 1 M HCl the absorbance was read at 340 nm (A340) against a reagent blank.

For test solutions, 100 μl of aqueous GA solution (1 : 10–1 : 100 dilution) was added to 500 μl succinyl albumin substrate followed by a prepared mixture of 100 μl pepsin standard. It was essential to follow this strict order of addition since GA had some neutralisation effect on the small volume of pepsin used, although this was negated by the larger volume of substrate. Even so, the pH of the reaction mixture was recorded and a pH-matched pepsin control was performed to allow accurate assessment of the effect of product on pepsin activity. The background effect of GA on the assay system was controlled by carrying out a reagent blank in which pepsin was only added after halting the reaction with 500 μl 4% $NaHCO_3$. The mean A340 of the reagent blank was then subtracted from the test values.

Azocoll assay

The Azocoll digestion assay is a quantitative colorimetric assay of pepsin activity.[27] Azocoll is a commercially available azo-dye-labelled collagen (Type 1 from skin). Azocoll is insoluble, but after digestion with a protease the azo dye is liberated into a soluble form proportionally to the extent of proteolysis, and the extent of release can be measured spectrophotometrically.

Porcine pepsin was diluted in 0.01 M HCl to a concentration of 100 μg/ml (285 units) and a standard curve produced between 0 and 100 μg/ml pepsin (total volume 400 μl). In the case of human gastric juice the sample was diluted with 0.01 M HCl to a concentration equivalent to 100 μg/ml porcine pepsin and used in the same way as with porcine pepsin. One thousand microlitres of 2.5 mg/ml Azocoll substrate in pH 2 glycine/HCl buffer was added and incubated at 37°C for 2 h with frequent agitation of the tubes. The tubes were centrifuged (4000 rpm 5 min) to sediment the insoluble collagen and absorbance at 540 nm (A540) of 200 μl of the supernatant was measured.

For test solutions, 200 μl of aqueous GA solution (1 : 5–1 : 50 dilution) was added to 1000 μl Azocoll substrate and mixed well, after which a prepared mixture of 200 μl pepsin standard was added. It was essential to follow this strict order of addition since GA had some neutralisation effect on the small volume of pepsin used that was negated by the larger volume of buffered substrate. The mean A540 of the GA

reagent blank (0 pepsin) was subtracted from the test values to account for background due to GA alone.

Positive and negative controls

Pepstatin A, a specific pepsin inhibitor, was used as a positive control in both assays. Five micrograms per milliliter of pepstatin A in 0.01 M HCl, pH 2.2, was added to pepsin standard, giving a final molar concentration of 1.7 μM (which was at least twofold molar excess to pepsin). The negative control was deionised water.

Calculations

Reactions were tested in duplicate and mean absorbance (A) calculated. Data was normalised such that 0 μg/ml of pepsin had an absorbance of 0.000. Percentage inhibition of pepsin activity was calculated at 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml and 50 μg/ml pepsin using the formula:

$$(A_{\text{pepsin std curve}} - A_{\text{test}})/A_{\text{pepsin std curve}} \times 100$$

Each dilution of GA was repeated on at least five separate occasions and mean (SD) percentage inhibition of pepsin activity quoted.

Determination of diffusion

The horizontal Franz-type diffusion cell is an established technique to evaluate diffusion and drug delivery. The characteristics of the Franz cell used in this study were: donor chamber, 1.5 ml; membrane, Whatman grade 4 filter paper; receptor chamber, 5 ml; aperture: 9 mm diameter; area for diffusion, 63.6 mm^2.

The sections of the Franz cell relate to the following in-vivo components of the gastro-oesophageal reflux model: (1) donor chamber: oesophageal lumen representing refluxate, (2) membrane: oesophageal squamous cell membrane, (3) receptor chamber: oesophageal cell cytoplasm.

The Franz cell was maintained at 37°C using a thermostatically controlled heating block with built-in magnetic stirrer plate. Detection of the compound of interest in the receptor chamber was by continual closed system UV spectrometry, with output to a chart recorder and response measured in millimetres or by sampling at selected time intervals from the sample port for further analysis.

Pepsin diffusion

The receptor chamber was filled with 0.01 M HCl and 500 μl of 3 mg/ml porcine pepsin in 0.01 M HCl was applied to the donor chamber. Appearance of pepsin in the receptor chamber was detected by absorbance at a wavelength of 280 nm (A280) over 30 min. The influence of GA on pepsin diffusion was assessed by application of formulation (0.05 ml, 0.1 ml or 0.2 ml, or 0.1 ml 1 : 5 or 1 : 10 dilutions) to the membrane prior to application of the pepsin dose.

A background effect of GA at A280 nm was observed but this stabilised after 2 h. Therefore the GA dose was left to equilibrate for 2 h prior to addition of the pepsin dose.

Bile acid diffusion

The receptor chamber was filled with HCl of the same pH as the bile acid tested and 500 μl of 0.5 mM bile acid solution was applied to the donor chamber. Appearance of bile acid in the receptor chamber was detected every 5 min for 30 min. The influence of GA on bile acid diffusion was assessed by application of 0.1 ml formulation to the membrane prior to application of the bile acid dose. Bile acids were detected by colorimetric total bile acid assay kit (Diazyme, San Diego, USA) in which the enzyme 3α hydroxysteroid dehydrogenase converts bile acids to 3-keto steroids and NADH. The NADH is reacted with nitro-blue tetrazolium (NBT) and diaphorase to generate a formazan dye, which is detectable at 540 nm. The extent of the generation is proportional to the bile acid concentration and linear between 0 and 0.2 mM bile acid. The calibration curve was prepared with the specific bile acid and pH tested, in order to enable millimolar bile acid diffused to be calculated. GA did not interfere with bile acid detection using this methodology (results not shown).

As positive control, 0.1 ml of a 20 mg/ml suspension of cholestyramine resin, a known bile acid binder, was used in the place of GA.

Calculations

Each test condition was repeated on at least four separate occasions and the mean (SD)% diffusion of applied dose quoted. The area under the curve (AUC) was calculated as a measure of amount of diffusion over the 30 min time frame.

In-vitro modelling of a reflux event

An in-vitro model of Gaviscon product raft formation is well established.[4] The model consists of applying a 10 ml dose (maximum dose) of GA to a 250 ml glass beaker containing 150 ml of 0.1 M HCl prewarmed to 37°C. The GA formulation reacts with the acid to form an alginic acid gel and releases CO_2, which becomes entrapped in the alginate gel matrix, allowing it to become buoyant. After 30 min of maturation the raft can be harvested for further testing as required.

An in-vitro model to assess the ability of a GA raft to remove aggressors from the refluxate was devised. The raft was placed on top of a filter paper disc within a porcelain Buchner funnel inserted into a side-arm flask to which a mild vacuum was applied using a diaphragm vacuum pump. A simulated gastric refluxate (SGR), containing either 1 mg/ml pepsin or 1 mM bile acid in HCl, was applied to the raft. The resulting fluid was collected in the flask, syringe filtered (0.2 μm), and assessed for the presence of pepsin or bile acids using UV spectrometry (A280 nm for pepsin and A203 nm for bile acids). Background due to GA alone was obtained by application of HCl alone.

Calculations

Using a calibration curve, the amount of pepsin or bile acid in the SGR was determined and the amount removed by the raft was calculated and expressed as a percentage of aggressor removed.

Viscometry

Solution viscosity was measured using a Bohlin CVO50 controlled stress rheometer using cup and bob geometry at 25°C with an applied shear stress of 0.1–10 Pa.

Statistical analysis

Data were analysed using one-way ANOVA followed by Student's unpaired *t*-tests when appropriate. Data were considered statistically significant if $P < 0.05$. Data are expressed as means (SD) unless otherwise stated.

Results

Pepsin inhibition

Pepsin activity was measured using the N-terminal assay and the Azocoll assay. Figure 1 shows the activity of porcine pepsin and human gastric juice in these two assay systems and also the ability of pepstatin A to inhibit enzyme activity.

GA (1 : 5–1 : 100 aqueous dilutions) was tested for the ability to inhibit pepsin activity. GA was able to inhibit pepsin activity over and above the neutralisation effect of the formulation on reaction pH. Data from both pepsin activity methods, the N-terminal assay using a soluble protein substrate and the Azocoll assay using an insoluble collagen substrate, are shown in Figure 2.

Complete inhibition of pepsin activity on breakdown of collagen was seen at dilutions above a level equivalent to 15 mg/ml alginate. On further dilution there was a linear decrease in inhibition until no effect was observed at 3.3 mg/ml alginate.

Using the protein substrate, maximal inhibition was 78% (at 10 mg/ml) as it was not possible to evaluate more concentrated product. Similarly, there was a decrease in inhibitory ability with dilution but even at 1 mg/ml some inhibition of pepsin was seen (28%). This suggests a minor preferential substrate protection advantage for smaller molecular weight protein rather than large particle size collagen by GA. Alternatively the difference between the two methods may be a consequence of the pH of the substrate (pH 2.2 vs pH 2.0).

In all experiments a positive control was included. Pepstatin, a known inhibitor of pepsin, was included at a constant level of 2.5 μg/ml (1.7 μM) which was at least in twofold molar excess of pepsin. Mean (SD) pepsin inhibition by pepstatin was 97.4 (6.2)% in the N-terminal assay ($n = 126$) and 94.8 (4.2)% in the Azocoll assay ($n = 26$).

Dilutions of GA (1 : 5–1 : 20) were able to inhibit the pepsin activity in human gastric juice using the Azocoll assay (Figure 3). There was a dose-dependent decrease in inhibition of human gastric juice pepsin activity from complete at 20 mg/ml alginate to 85% at 5 mg/ml alginate. It is interesting that the slope of the dose–response curve was far shallower with human gastric juice than with porcine pepsin. This may

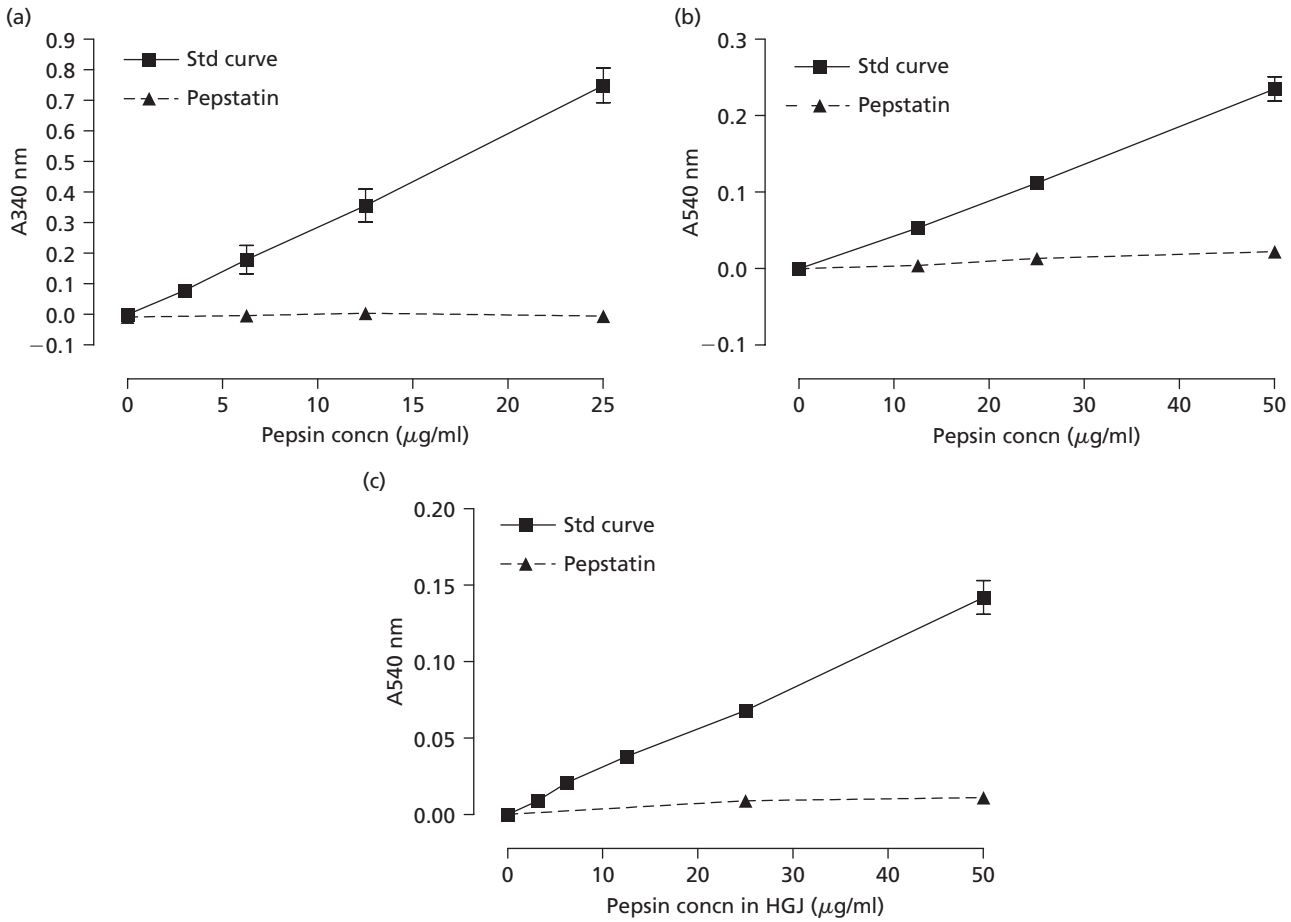

Figure 1 Enzyme activity of pepsin. (a) N-terminal assay with 0–25 μg/ml porcine pepsin (0–71 units), (b) Azocoll assay with 0–50 μg/ml porcine pepsin (0–142 units), (c) Azocoll assay with human gastric juice (HGJ) equivalent to 0–50 μg/ml porcine pepsin (0–142 units). Inhibition of enzyme activity by pepstatin A (2.5 μg/ml) is shown. Data are means (SD).

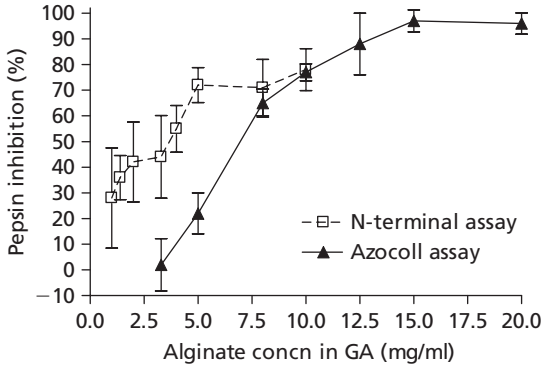

Figure 2 Inhibition of pepsin activity by Gaviscon Advance. Inhibition of pepsin activity by Gaviscon Advance (GA) is shown as a function of alginate concentration (active). Data are means (SD), n = 5–10.

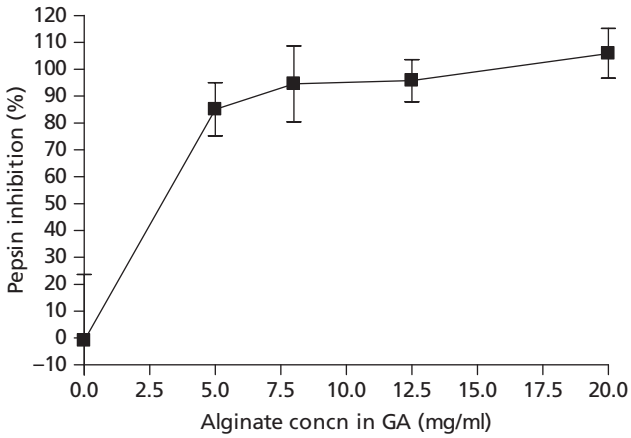

Figure 3 Inhibition of human gastric juice pepsin activity by Gaviscon Advance. Percentage inhibition of human gastric juice pepsin activity by Gaviscon Advance (GA) is shown as a function of alginate concentration (active). Data are means (SD), n = 10.

be related to the more complex mixture of pepsin isoenzymes found in gastric juice.

Diffusion of pepsin

Thirty-eight per cent of the expected amount of dosed pepsin was detected in the receiver phase after 30 min. The amount of pepsin reaching the receiver phase was reduced in the presence of GA (Figure 4). Neat GA was able to retard pepsin diffusion by a mean (SD) of 53 (8.0)% (n = 16) after 30 minutes but with no influence of dose applied (0.05 ml – 57%, 0.1 ml – 49%, 0.2 ml – 54%). Area under the curve (AUC) analysis of diffusion over the entire 30 min time period showed the effect of GA to be statistically significant ($P < 0.001$; 759.5 vs 320.1). Diluted GA (1 : 5–1 : 10) was also able to retard pepsin diffusion compared to control with a mean (SD) of 82 (2.6)% (n = 8). AUC analysis of diffusion over the entire 30 min time period showed pepsin diffusion across dilute GA to be statistically significant compared to the control ($P < 0.001$; 759.5 vs 138.3) and surprisingly also compared to neat GA ($P < 0.01$; 320.1 vs 138.3). Dilutions of GA of 1 : 12 and above had no significant influence on pepsin diffusion (data not shown).

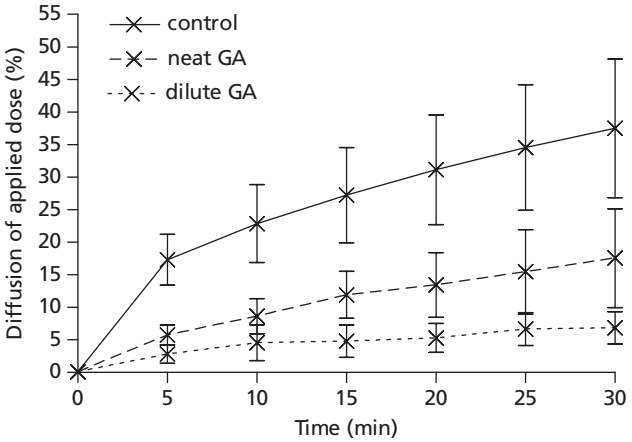

Figure 4 Appearance of diffused pepsin in the receiver chamber as a function of time in the presence and absence of Gaviscon Advance. Data are means (SD), n = 8–16. GA, Gaviscon Advance.

To investigate this interesting phenomenon of improved barrier function by dilute GA the viscosity of the donor phase in the presence of GA was assessed. Pepsin/HCl alone had a mean (SD) viscosity of 2.723 (0.030) mPas (n = 3), which increased to 4.373 (0.457) mPas in the presence of neat GA (n = 9) but significantly increased further still with dilute GA to 7.874 (1.259) mPas (n = 6) ($P < 0.001$ compared to neat).

It was hypothesised that GA was able to influence diffusion of pepsin by two mechanisms. Firstly, the formulation provided a physical barrier to diffusion across the artificial membrane, and the more viscous the better. Secondly, unstirred, slightly diluted formulations were capable of influencing the viscosity of the small volume of pepsin solution in the donor chamber and therefore slowed down access of the pepsin to the membrane. At 1 : 5–1 : 10 dilutions both factors were in play, whereas with the neat formulation only the physical barrier to diffusion was apparent. At 1 : 12 and below neither factor was in play, explaining the inability to retard diffusion.

Bile acid diffusion

GA was able to significantly ($P < 0.05$) retard the diffusion of the four bile acid conditions tested over 30 min (Figure 5).

AUC analysis of diffusion of TCA at pH 2 showed that GA significantly reduced diffusion ($P < 0.001$; 2074 vs 333.3) and also when TCA was dissolved in pH 5 HCl ($P = 0.002$; 1124 vs 742.3). Two other bile acids were evaluated at pH 5 and AUC analysis shows that GA significantly reduced diffusion of GCA ($P < 0.001$; 496.6 vs 120.5) and CA ($P < 0.001$; 826.7 vs 478.4).

The positive control cholestyramine resin (20 mg/ml), a known bile acid binder, was able to statistically significantly ($P < 0.05$) retard diffusion of all bile acid conditions tested (data not shown).

Removal of pepsin and bile acids from the refluxate

By application of 5 ml simulated gastric refluxate (SGR) containing 1 mM bile acid or 1 mg/ml pepsin the GA raft was

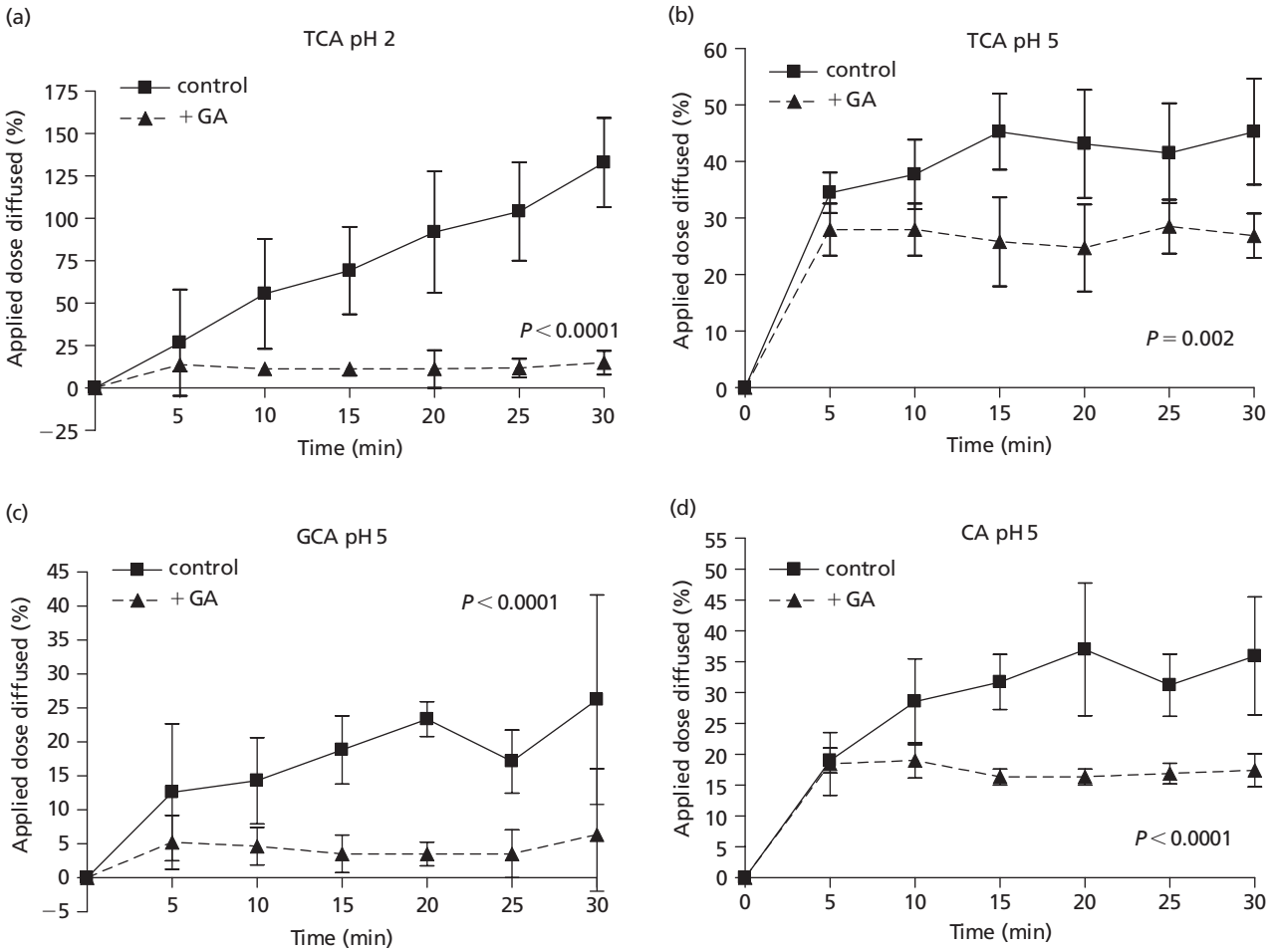

Figure 5 Appearance of diffused bile acids in the receiver chamber as a function of time in the presence and absence of Gaviscon Advance. The bile acids studied were taurocholic acid (TCA) at pH 2 and at pH 5, glycocholic acid (GCA) at pH 5 and cholic acid (CA) at pH 5. GA, Gaviscon Advance. Data are means (SD), $n = 6$.

capable of removing virtually all of the aggressors (Table 1). A superior ability was observed with conjugated bile acids (TCA and GCA) and pepsin but there was more variable data seen with the unconjugated bile acids (CA and DCA).

The amount of aggressor removed by the raft was a consequence of the volume of the SGR. As the volume of SGR increased, the percentage of pepsin removed decreased, with 40% of the pepsin removed from a 50 ml reflux event.

Using cholestyramine resin (20 mg/ml) as a positive control for bile acid binding, experimental data showed complete removal of all bile acids from a 5 ml SGR.

In the absence of the raft (filter paper only), 100% of pepsin in the SGR was retrieved. Control experiments showed that the presence of the raft had no influence on volume of SGR retrieved.

Multiple reflux events

In the pathological situation, GA is given to GORD sufferers who may experience multiple reflux events within the lifetime of the therapeutic raft (up to 4 h). Ten 5 ml SGRs containing either pepsin or bile acids were applied and the amount of aggressors retained in the raft were calculated.

Essentially all of the pepsin in the first reflux event was removed by the raft and after each subsequent event a small decrease in the capacity of the raft was seen. However, even after 10 reflux events approximately half of the pepsin was removed (Table 2).

A similar pattern was seen with bile acids, in which all of the conjugated bile acids (TCA and GCA) were removed from the first reflux event, with a small decrease in capacity observed with each subsequent reflux episode. Approximately half of the bile acid was removed from the tenth reflux event (Table 2). In comparison, cholestyramine resin had a more prolonged full capacity to remove bile acids (Figure 6).

There was no difference between the profiles of any bile acid or pepsin, suggesting a broad role for GA in preventing aggressors in the refluxate reaching the oesophagus (Figure 6).

Discussion

GORD is commonly treated by addressing the acidity of the gastric contents. However, the role of non-HCl acid components, and in particular pepsin and bile acids, in causing damage to the oesophagus is now well established.

Table 1 Mean (SD) percentage of aggressor in a 5 ml simulated gastric refluxate removed by a Gaviscon Advance raft

Condition	Mean percentage removed by raft	SD (%)
TCA pH 2	100	1
TCA pH 5	90	5
DCA pH 6	100	10
GCA pH 5	92	4
CA pH 5	76	52
Pepsin pH 2	100	6

CA, cholic acid; DCA, deoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid.

Table 2 Mean (SD) percentage bile acid or pepsin from 10 × 5 ml simulated gastric reflux events removed by a Gaviscon Advance raft

	TCA pH 2	TCA pH 5	GCA pH 5	Pepsin pH 2
Reflux 1	95 (3)	91 (1)	93 (3)	87 (35)
Reflux 2	90 (3)	87 (1)	89 (2)	83 (26)
Reflux 3	81 (2)	78 (2)	80 (1)	83 (23)
Reflux 4	74 (3)	70 (2)	74 (1)	76 (23)
Reflux 5	69 (3)	64 (3)	69 (2)	65 (29)
Reflux 6	66 (3)	61 (3)	66 (1)	71 (25)
Reflux 7	61 (3)	56 (3)	63 (2)	58 (31)
Reflux 8	58 (3)	53 (3)	61 (2)	52 (36)
Reflux 9	55 (3)	50 (3)	58 (2)	43 (36)
Reflux 10	51 (3)	46 (3)	56 (2)	44 (35)

GCA, glycocholic acid; TCA, taurocholic acid.

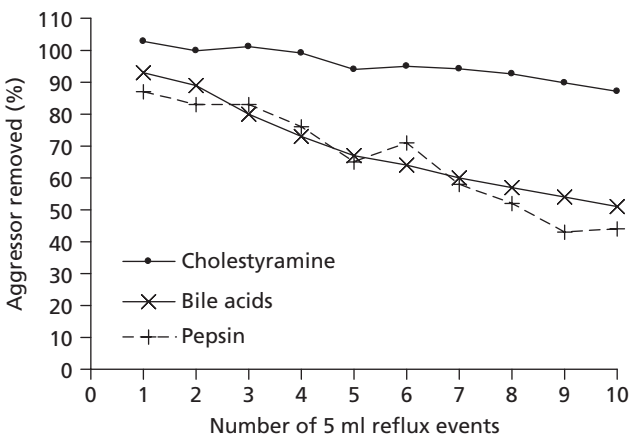

Figure 6 Reduction of bile acid and pepsin from simulated gastric reflux events by Gaviscon Advance. The percentage of bile acid and pepsin in successive 5 ml reflux events removed by a Gaviscon Advance raft are shown, alongside a comparison to the positive control, cholestyramine. Data are means, $n = 6$.

GA acts in a non-systemic way to suppress reflux in general but here we have investigated whether there was a more specific role against pepsin and bile acids.

We clearly demonstrated that GA can inhibit pepsin activity *in vitro* using two distinct colorimetric assays. These two methods differ in the substrates used and both have clinical relevance. The N-terminal assay utilises a protein substrate, succinyl albumin. Albumin is a common component of the cell and plasma but also protein in general makes up a high proportion of the cell membrane (e.g. ion channels, receptors). The Azocoll assay utilises a collagen substrate, which is of particular relevance to the oesophagitis model. Collagen is the main component of the basement membrane of the gastrointestinal mucosa and is exposed in the case of ulceration. Digestion of collagen by pepsin therefore leads to severe oesophageal damage.

The current study indicated that dilute solutions of GA were able to inhibit pepsin activity completely in the Azocoll assay and by about three-quarters in the N-terminal assay. The highest dilution test (1 : 5) is equivalent to a 10 ml dose in 50 ml gastric volume, The lowest dilution (1 : 100) is equivalent to a 10 ml dose in 1 litre of gastric volume, which is below realistic therapeutic levels. There was a strong dose-dependency exhibited. This finding was not related to the changes in pH that the formulation caused and was specifically related to preventing enzyme activity and/or substrate protection. Alginate, the active ingredient in GA, has previously been shown to inhibit pepsin activity,[26] as has carbopol,[28] a non-active component. It appears that the superior pepsin inhibition by the GA product may be a consequence of having dual pepsin inhibiting roles and perhaps through a synergistic interaction.

Interestingly, GA was shown to inhibit pepsin activity in a source of human gastric juice. In fact the ability of the formulation to inhibit gastric juice activity was superior to that against porcine pepsin when using the Azocoll assay. Human gastric juice is a complex mixture of several different pepsin isoenzymes (pepsin 1, 3a, 3b, 3c and 5) all of which have distinct substrate and pH preferences and, in particular, pepsin 1 has a preference for collagen.[29] This in-vivo complexity may suggest that GA could have a greater effect on the pepsin 1 component than on pepsin 3b.

GA, in the form of a small layer of formulation, exhibited a considerable ability to retard the diffusion of pepsin and bile acids. Using the Franz cell model, GA could reduce the amount of pepsin from the refluxate reaching the 'oesophageal cellular compartment' by approximately half. The product was also efficient in reducing diffusion of bile acids, including taurocholic acid, glycocholic acid and cholic acid.

Using the buoyant raft mode of action of GA, experimental data was able to show that pepsin and bile acids could be removed from a reflux event. It is not known what the volume of a reflux event is, but it is likely to be relatively small and of approximately 5 ml.[30,31] It was shown that all of the pepsin and bile acids in a 5 ml reflux event could be removed by the GA raft, thus preventing the aggressors from reaching the oesophagus. Even after repeated reflux events, a single raft was capable of removing a large proportion of the aggressors in the reflux event.

Since the amount of damage done to the oesophagus by pepsin is dose-dependent[10,11] and because there may be a threshold level for damage by bile acids,[20] any reduction in the amount of aggressor reaching the oesophageal mucosa will have a marked effect on patient symptomatology and disease pathology. Importantly, GA does not have any influence on normal physiology. Both pepsin and acid are

vital components of the digestive process, being essential for the initial digestion of food and also bacteriocidal function. The mode of action of GA is such that it only affects pathological exposure of pepsin and bile acids (and gastric acid) to the oesophagus, giving a distinct mode of action compared to the other therapeutic options for GORD, which alter the gastric conditions (e.g. PPIs and H_2-receptor antagonists).

Conclusion

In conclusion, the experimental data presented here indicate that GA could specifically remove both pepsin and bile acids from the refluxate and limit the diffusion of these damaging agents. This gives GA a role to play in reducing the causticity of the refluxate and protecting the oesophagus from damage. In addition, GA had a strong ability to affect the enzymatic activity of pepsin, thus giving a second mode of action to combat damage to the oesophagus in GORD.

The alginate-containing anti-reflux product GA acts to suppress reflux in general and combats not only acid, but also pepsin and bile acids that are responsible for much of the damage to the oesophageal mucosa during GORD.

Declarations

Conflict of interest

IGJ and LMJ are employees of Reckitt Benckiser Healthcare (UK) Ltd.

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References

1. Chatfield S. A comparison of the efficacy of the alginate preparation, Gaviscon Advance, with placebo in the treatment of gastro-oesophageal reflux disease. *Curr Med Res Opin* 1999; 15: 152–159.
2. Dettmar PW *et al*. The suppression of gastro-oesophageal reflux by alginates. *Int J Clin Pract* 2007; 61: 1654–1662.
3. Dettmar PW *et al*. Rapid onset of effect of sodium alginate on gastro-oesophageal reflux compared with ranitidine and omeprazole, and relationship between symptoms and reflux episodes. *Int J Clin Pract* 2006; 60: 275–283.
4. Hampson FC *et al*. Alginate rafts and their characterisation. *Int J Pharm* 2005; 294: 137–147.
5. Gotley DC *et al*. Composition of gastro-oesophageal refluxate. *Gut* 1991; 32: 1093–1099.
6. Koek GH *et al*. Mechansims underlying duodeno-gastric reflux in man. *Neurogastoenterol Motil* 2005; 17: 191–199.
7. Ten Kate RW *et al*. Effect of high dose omeprazole on gastric pepsin secretion and serum pepsinogen levels in man. *Eur J Clin Pharmacol* 1988; 35: 173–176.
8. Goldberg HI *et al*. Role of acid and pepsin in acute experimental esophagitis. *Gastroenterology* 1969; 56: 223–230.
9. Salo JA *et al*. Morphological alterations in experimental esophagitis. Light microscopic and scanning and transmission electron microscopic study. *Dig Dis Sci* 1983; 28: 440–448.
10. Johnson LF, Harmon JW. Experimental esophagitis in a rabbit model. Clinical relevance. *J Clin Gastroenterol* 1986; 8: 26–44.
11. Tobey NA *et al*. The role of pepsin in acid injury to esophageal epithelium. *Am J Gastroenterol* 2001; 96: 3062–3070.
12. Johnston N *et al*. Activity/stability of human pepsin: implications for reflux attributed laryngeal disease. *Laryngoscope* 2007; 117: 1036–1039.
13. Kauer WK *et al*. Composition and concentration of bile acid reflux into the esophagus of patients with gastroesophageal reflux disease. *Surgery* 1997; 122: 874–881.
14. Nehra D *et al*. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. *Gut* 1999; 44: 598–602.
15. Roman S *et al*. Downregulation of p63 upon exposure to bile salts and acid in normal and cancer esophageal cells in culture. *Am J Physiol (GI Liver Physiol)* 2007; 293: G45–G53.
16. Zhang T *et al*. A rat surgical model of esophageal metaplasia and adenocarcinoma-induced by mixed reflux of gastric acid and duodenal contents. *Dig Dis Sci* 2007; 52: 3202–3208.
17. Boni L *et al*. Free radical production in the oesophago-gastro-duodenal mucosa in response to acid and bile. *Dis Esophagus* 2006; 19: 99–104.
18. Dvorak K *et al*. Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus. *Gut* 2007; 56: 763–771.
19. Fein M *et al*. Carcinogenesis in reflux disease – in search for bile-specific effects. *Microsurgery* 2007; 27: 647–650.
20. Jenkins GJ *et al*. The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kB activation in oesophageal cells, with a mechanism of action involving ROS. *Mutagenesis* 2008; 23: 399–405.
21. Tselepis C *et al*. Upregulation of the oncogene c-myc in Barrett's adenocarcinoma: induction of c-myc by acidified bile acid in vitro. *Gut* 2003; 52: 174–180.
22. Vela MF *et al*. Simultaneous intraesophageal impedance and pH measurement of acid and nonacid gastroesophageal reflux: effect of omeprazole. *Gastroenterology* 2001; 120: 1599–1606.
23. Tamhankar AP *et al*. Omeprazole does not reduce gastro-esophageal reflux: new insights using multichannel intraluminal impedance technology. *J Gastrointest Surg* 2004; 8: 888–896.
24. Hemmink GJM *et al*. Esopageal pH-impedance monitoring in patients with therapy-resistant reflux symptoms: 'on' or 'off' proton pump inhibitor? *Am J Gastroenterol* 2008; 103: 2446–2453.
25. Hutton DA *et al*. Separation of pepsin in human gastric juice: analysis of proteolytic and mucolytic activity. *Biochem Soc Trans* 1986; 14: 735.
26. Strugala V *et al*. Inhibition of pepsin activity by alginates in vitro and the effect of epimerization. *Int J Pharm* 2005; 304: 40–50.
27. Will PC *et al*. Quantification of pepsin A activity in canine and rat gastric juice with the chromogenic substrate Azocoll. *Clin Chem* 1984; 30: 707–711.
28. Foster SNE *et al*. Interaction of polyacrylates with porcine pepsin and the gastric mucus barrier: a mechanism for mucosal protection. *Clinical Science* 1994; 87: 719–726.
29. Allen A *et al*. Pepsins. In: Wallace JL, ed. *Endogenous Mediators of Gastrointestinal Disease*. CRC Press, 1990: 53–69.
30. Rhee PL *et al*. Measuring esophageal distension by high-frequency intraluminal ultrasound probe. *Am J Physiol (GI Liver Physiol)* 2002; 283: G886–G892.
31. Sifrim D. Relevance of volume and proximal extent on gastro-oesophageal reflux disease. *Gut* 2005; 54: 175–178.