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Molecular fractionation of gastric mucus component by high-performance liquid chromatography: application to pig gastric mucus *in vitro* and to human gastric mucus collected by aspiration during gastric endoscopy

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ABSTRACT

The glycoprotein molecular composition of antral and fundic adherent mucus has been studied by high-performance liquid chromatography on a silica gel column. Preliminary assays with pig gastric mucus allowed us to demonstrate the reproducibility of the method. The mucolytic activity of pepsin on this mucus demonstrates its ability to detect degradation of its glycoprotein components. This method was applied to control the state of pig antral mucosa that has previously been used in an *in vitro* antacid evaluation procedure, and also to study human fundic and antral mucus collected by aspiration from normal and diseased stomachs during upper gastrointestinal endoscopy. Different elution profiles were obtained with these samples, depending on the presence of non-degraded or degraded mucus or due to the lack of mucus on the mucosa.

INTRODUCTION

The soundness of gastric mucosa is due to a steady state between endogenous erosive factors and the mucus membrane defence. The aggressive factors include pepsin, material introduced into the stomach by gastroduodenal reflux, and physiological mechanical erosion by ingested foodstuff. The defensive factors include the cellular barrier, the endogenous bicarbonate and mucus secretions, and the mucosal blood flow. Leibur *et al.* [1] have suggested that qualitative changes in gastric mucus lead to defective functioning of the mucosal barrier in patients with recurrent ulcers and may be one of the causes of recurrences. Moreover, the susceptibility of rat stomach to chronic ulceration could be predicted by a pre-existent variability in a mucosal defence factor [2]. The protective quality of mucus is due to its content of high-molecular-mass (HMr) glycoprotein and to protein and lipid components

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that are implicated in its functional and rheological properties [3–6]. The thickness of the adherent mucus gel and its polymeric mucin content are decreased in peptic ulcer disease [7–9] and increased after prolonged administration of antiulcer agents and of mucus-layer strenghtening agents [10,11].

In humans, gastric secretion has been studied during functional exploration, and aggressive factors such as acid, pepsin and gastroduodenal reflux on mucus erosion have been demonstrated to play a role in duodenal ulcer disease [12,13]. Erosion of the mucus layer has been evaluated indirectly by the measurement of the sialic acid content of gastric juice, which reflects degradation of mucus glycoprotein [12], and by the establishment of the mucoprotective index using a pharmacological approach [14]. In humans, the adherent mucus layer has been analysed only for its rheological properties [15,16]. It therefore seemed necessary to develop a method capable of studying the quality of fresh human gastric mucus.

This paper describes a rapid method for the assessment of the molecular composition of gastric mucus. The technique was applied first to pig gastric mucus to validate it, then to human antral and fundic mucus collected *in vivo* during upper gastrointestinal endoscopy.

EXPERIMENTAL

Biological materials

Pig gastric mucus. Stomachs were obtained from pigs slaughtered at a local abbatoir (Cergy-Pontoise, France) and transported on ice in a 0.02% (w/v) sodium azide solution to the laboratory. They were opened along the greater curvature and washed, then antral and fundic mucus were gently scraped from nine mucosae. To avoid inter-sample variability, antral or fundic mucus samples from individual stomachs were pooled, then dispersed in 0.9% NaCl solution containing 0.02% (w/v) sodium azide (Prolabo, Paris, France) and 0.002% (w/v) pepstatin A (Sigma Chemie, France), and homogenized twice for 30 s in a Waring blender at low speed. To remove cellular debris, mucus was centrifuged at 3500 rpm (3200 *g*) for 30 min at 5°C. (Jouan K110N, France) and the supernatant was stored at −20°C. After thawing, the samples of mucus were diluted in the elution buffer before high-performance liquid chromatographic (HPLC) analysis.

Human gastric mucus. Fundic and antral mucus was collected by aspiration during routine endoscopic investigation of sixteen subjects, six women and ten men (55.3 ± 16.0 years). Two samples each of antral and of fundic mucus were collected in each of these subjects: three antritis patients, three oesophagitis patients, three duodenal ulcer (DU) patients, one fundic atrophy patient, four normal subjects undergoing diagnostic endoscopy and two patients on non-steroidal anti-inflammatory drug (NSAID) treatment. A single upper gastrointestinal endoscopy was performed, during which the tip of a D-8 catheter (Olympus C PWIL, Scop, France) filled with a sterile saline solution (0.9% NaCl) was applied to the antral and fundic wall, and manual suction was then performed with a 10-ml plastic syringe to collect the mucus. Mucus collected *in vivo* must be analysed within 2 h after collection to avoid rearrangement of the mucin subunits. The collected mucus was stored at 4°C in 1.0 ml of 0.9% NaCl buffer with 0.002% (w/v) pepstatin A (Sigma) and 0.02% (w/v) sodium azide before being analysed. Immediately before sample injection, the mucus was solubilized in a Potter homogenizer, then centrifuged at 10 000 rpm (9700 *g*) for 10 min (Sigma-202MC, Bioblock, France), and 200 μl of the supernatant were directly subjected to HPLC analysis.

HPLC analysis

Components of crude mucus were fractionated on a silica gel column by HPLC. The chromatographic system (LKB Pharmacia, France) included an isocratic pump (Model 2150), a detector (Uvicord SII 2238) with a 206-nm filter, a recorder (Model 2210), a fraction collector (Helirac 2212) and a manual injector (Rheodyne). Mucus samples of 200 μl were chromatographed at a flow-rate of 0.8 ml/min on a 600 × 7.5 mm I.D. column of TSK G 4000 SW silica gel (Toyosoda,

Japan), equilibrated and eluted with a degassed and filtered 0.15 *M* Tris–HCl buffer (pH 6.5) (Aldrich Chemie, St. Quentin Fallavier, France). The gastric mucus components were eluted according to their relative molecular mass (M_r). Qualitative analysis of the chromatographic profile was based on the number of peaks detected at 206 nm and on the apparent M_r of mucus components determined using standard M_r markers (MW-GF 1000, Sigma Chemie Sarl, St. Quentin Fallavier, France) (Fig. 1).

Procedure validation

Reproducibility of the technique. To test stability of the silica gel, M_r markers were applied once a month during fifteen months. To test the resolving capacity of the silica gel, a pig fundic mucus sample (280 μg/ml of total carbohydrates) was subjected to five repeated HPLC analyses. The repeatability was expressed in terms of relative standard deviation (R.S.D.) values.

Qualitative analysis of eluted fractions. Pig antral and fundic mucus samples were subjected to HPLC analyses (n = 5) under the same elution conditions, and the mucus components were detected at 206 nm. The corresponding elution fractions (5 × 2 ml) were pooled, dialysed overnight against water, then concentrated 2.5 times and assayed for protein content (Bio-Rad protein assay) [17] with bovine albumin as standard (Sigma Chemie Sarl) and for total carbohydrate content by the Mantle and Allen method [18] with mucin type II as standard (Sigma, Coger, Paris, France).

Peptic degradation of gastric mucus. Pig mixed antral and fundic mucus (1 ml) was incubated with pig gastric pepsin solution (1 ml at 400 μg/ml) (Sigma) for 30 min at pH 2.2 and 37°C. The hydrolysis was stopped by the addition of 1 *M* NaOH to a pH of 9.0 [19]. A 1.0-ml sample was withdrawn from the supernatant and subjected to HPLC analysis.

Quality control. The pig antral mucosa used for studying the interaction between mucus and an aluminium-containing antacid [20] was cleared of adherent mucus by gently manual scraping, then stored at 4°C in a 0.9% NaCl 0.02% NaN_3 solution. The state of this mucosa was controlled monthly by HPLC analysis of the mucus excreted *post-mortem* on the mucosal surface.

RESULTS

Validation of the HPLC assay

Stability of the silica gel column. There was no change in the elution volume of any of the M_r

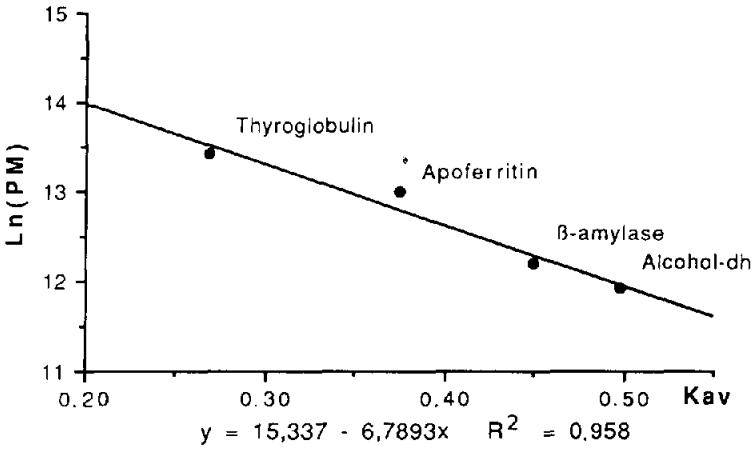

Fig. 1. Calibration curve. M_r markers (1 mg/ml) were eluted at a flow-rate of 0.8 ml/min from a TSK G 4000 SW silica-gel column equilibrated with 0.15 *M* Tris–HCl (pH 6.5) and detected at 206 nm. The V_0 (11.09 ml) was determined with the elution of dextran blue or haemocyanin (and V_t = 31.6 ml).

TABLE I

STABILITY OF SILICA GEL COLUMN

After fifteen chromatographic elutions (once a month) of molecular mass markers, there was no significant variation in the elution volume of any of the M_r markers. Column: TSK G 4000 SW (600 mm × 7.5 mm I.D.).

Marker	Molecular mass	Number of assays	Elution volume (ml)		R.S.D. (%)
			Mean ± S.D.	Minimum–maximum	
Dextran blue	2 000 000	15	11.09 ± 0.28	10.50–11.40	2.5
Thyroglobulin	669 000	14	16.61 ± 0.40	15.56–17.00	2.4
Apoferritin	443 000	14	18.78 ± 0.46	17.75–19.20	2.4
β-Amylase	200 000	15	20.32 ± 0.37	19.50–20.80	1.8
Alcohol dehydrogenase	150 000	15	21.30 ± 0.44	20.75–22.00	2.1

markers over the fifteen analyses (Fig. 1 and Table I). The R.S.D. of the elution volumes did not exceed 2.5%. These results show the stability of the silica gel column. The results obtained with pig fundic mucus show that the silica gel column used was capable of separating the components of crude mucus. There was no significant difference in the number of peaks detected at 206 nm and in the elution volume of each peak detected in successive fractionations (Table II).

Analysis of the elution fractions of pig crude antral and fundic mucus. After separation of the mucus components, four major components were detected at 206 nm (Fig. 2): a HMr (> 1 300 000) component eluted in the leading peak (antral mucus) or in the first two peaks (fundic mucus); an intermediate M_r (IMr) (500 000–600 000) component eluted in peak II; a low M_r (LMr) (150 000) component eluted in peak III and very low M_r (VLMr) (< 80 000) components eluted in peak IV. Qualitative analysis of these elution fractions showed that the total carbohydrates were mainly eluted in peak I and that proteins were mainly eluted in peak III.

These results indicate that (i) the silica gel column could separate the HMr and LMr components in crude mucus, (ii) peak I and peak II detected at 206 nm correspond to HMr mucin and

TABLE II

REPEATABILITY OF FIVE SUCCESSIVE SEPARATIONS OF PIG FUNDIC MUCUS COMPONENTS

After five successive separations of crude pig fundic components, there was no significant difference in the number of peaks detected at 206 nm or in the peak elution volumes.

Test No.	Elution volume (ml)					
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
1	10.8	12.40	17.60	21.00	23.40	25.20
2	10.8	12.40	17.40	21.00	23.40	25.20
3	10.8	12.80	17.40	21.00	23.80	25.40
4	10.4	12.40	17.00	20.60	23.40	25.00
5	10.4	12.60	17.00	20.60	24.40	–
Mean ± S.D.	10.64 ± 0.22	12.52 ± 0.18	17.28 ± 0.27	20.84 ± 0.22	23.68 ± 0.44	25.20 ± 0.16
R.S.D. (%)	2.1	1.4	1.6	1.1	1.9	0.6

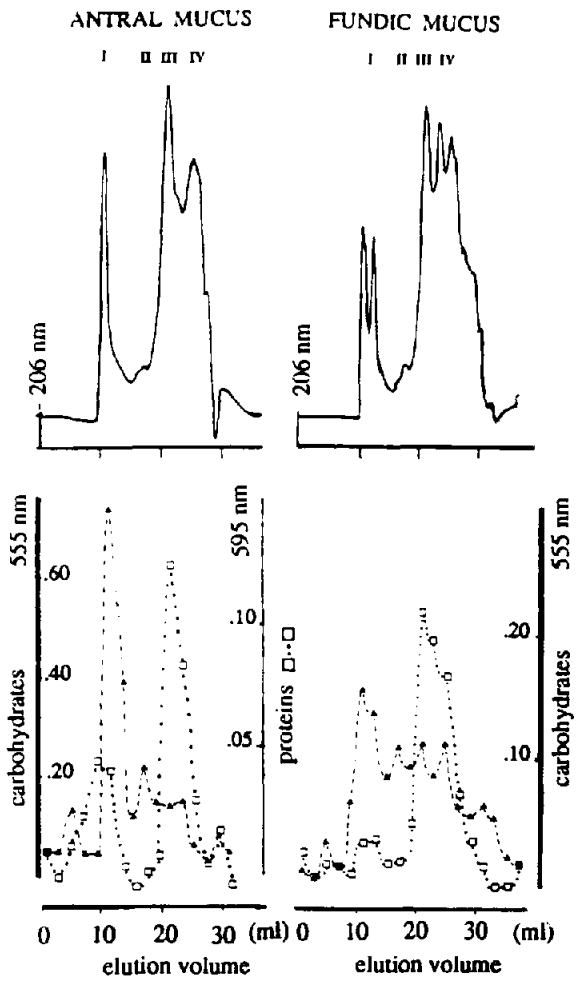

Fig. 2. Fraction elution analysis of pig crude antral and fundic mucus. Samples of the eluted fractions of components were concentrated, dialysed, then analysed for protein content at 595 nm (Bio-Rad assay) and for total carbohydrate content at 555 nm by the Mantle and Allen method [18]. The elution profiles of the proteins (□) and of the total carbohydrates (▲) are shown, together with that detected at 206 nm [peak I = HMr (>1 300 000); peak II = IMr (500 000–600 000); peak III = LMr (150 000); peak IV = VLMr (<80 000)].

mucin subunits, respectively [21], (iii) peak III detected at 206 nm corresponds to proteins whose apparent M_r could correspond to that of "link" glycopeptide [22].

Peptic degradation of gastric mucus. Before peptic digestion of pig gastric mucus, at least three peaks were detected at 206 nm (HMr, IMr and VLMr components) (Fig. 3). After peptic digestion at pH 2.2 for 30 min, peak I decreased and peak II disappeared, whereas the LMr and VLMr components detected in peaks III and IV increased. The peptic degradation of mucus components is detected at 206 nm. Clearly, pepsin has mucolytic activity on the crude pig gastric mucus.

Application to pig and human gastric mucus

Estimation of the stability of pig antral mucosa. The HPLC elution profile of mucus obtained from pig antral mucosa kept at 4°C in 0.02% sodium azide for three months was not significantly different from that of fresh mucus. This shows

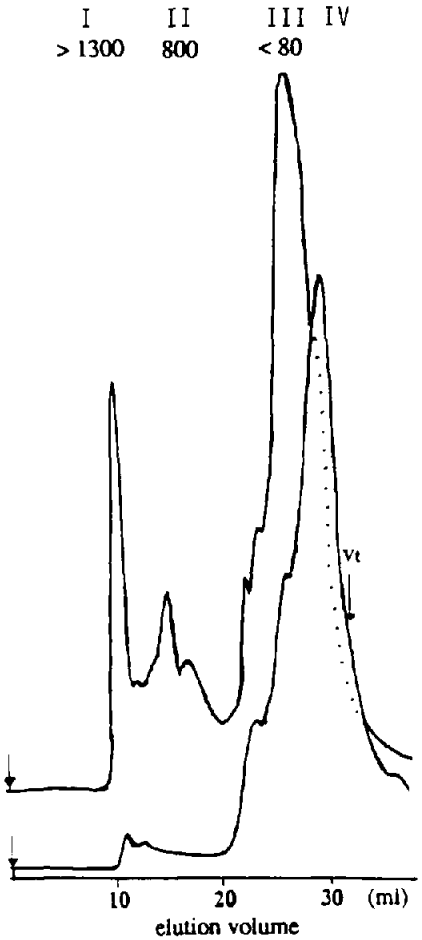

Fig. 3. Mucolytic activity of pepsin. Pig crude gastric mucus was incubated with pepsin (400 μg/ml) for 30 min at pH 2.2 and subjected to HPLC analysis. After proteolytic digestion, peak I (HMr mucin) and peak II (IMr mucin) were markedly decreased, whereas peaks III and IV (LMr and VLMr components) were increased. The upper elution profile is that of a control sample, and the lower one was obtained after mucolysis.

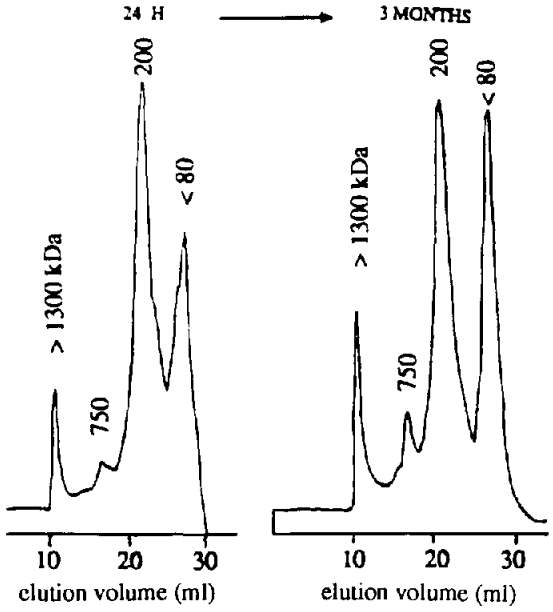

Fig. 4. Conservation of pig antral mucosa. Pig antral mucosa free of adherent mucus was stored at 4°C in 0.9% NaCl buffer with 0.02% NaN_3. The antral mucosa could excrete mucus onto the mucosal surface *post-mortem*. As judged by HPLC analysis of the excreted mucus, the mucosa remained stable for three months.

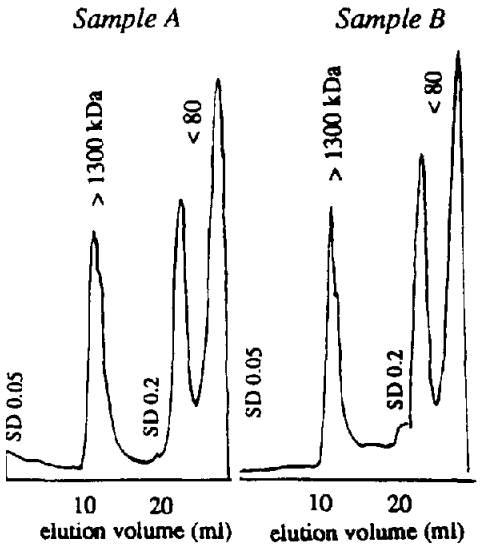

Fig. 5. Evidence for the homogeneity of the entire antral mucosal surface. Mucus samples A and B were collected from two different sites (separated by 3.0 cm) on the antral mucosa of a single patient. No difference was found between the mucus components from the two antral sites. Mucus appeared homogeneous throughout the antral mucosa. The same result was found for mucus covering the fundic mucosa.

that stored mucosa could excrete mucus onto the mucosal surface *post-mortem* and indicates that there were still intact mucus cells in the mucosa. The mucosal surface remained stable for at least three months (Fig. 4).

Adherent human gastric mucus. Crude human gastric mucus was collected from normal and pathological stomachs as described above and analysed by HPLC. The amount of mucus collected via the catheter (50 μg) was sufficient for HPLC analysis. The inter-assay reproducibility was verified by chromatographic analysis of mucus collected from two different sites on the antral or the fundic mucosa (Fig. 5).

At least four different elution profiles were observed, depending on the number of mucin components detected:

(1) Mucus containing three mucin components that were strongly detected: HMr, IMr (600 000–500 000) and LMr components (300 000–200 000) (Fig. 6a).

(2) Mucus containing two mucin components: HMr and LMr (300 000) components (Fig. 6b). These first two types of elution profile were obtained with most of the endoscopically normal mucosa, with mucus from one patient with oesophagitis stage II and from one patient with duodenal ulcer.

(3) Mucus that appeared degraded, containing only HMr mucin weakly detected at 206 nm (Fig. 6c); this elution profile was seen with mucus obtained from pathological mucosa (antritis and fundic atrophy) and with one fundic mucus collected from an endoscopically normal mucosa or from DU patients, those with oesophagitis stage III and those with gastroesophageal reflux.

(4) A steady baseline elution profile without any detectable mucus components, because either no mucus was covering the mucosal surface or the aspiration was insufficient. (Fig. 6d). This type was observed with antral or fundic mucus from patients under NSAID treatment and with antral mucus collected from one patient with antritis and from one patient with normal gastric mucosa.

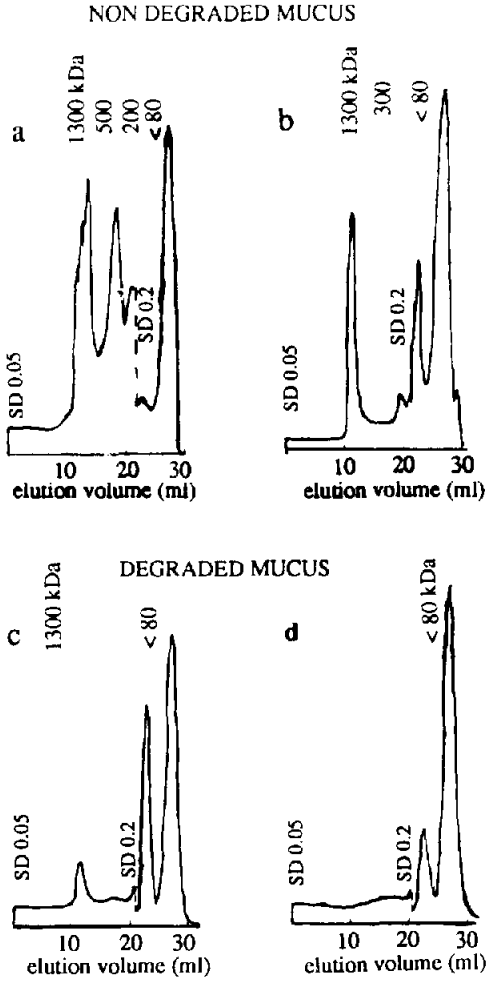

Fig. 6. HPLC analysis of human gastric mucus collected *in vivo* by aspiration through a D-8 catheter. In this preliminary assay, at least four HPLC profiles were seen, apparently independent of the pathological state of the patient: (a) mucus with a high level of HMr and LMr mucin; (b) mucus with a high level of HMr mucin and a low level of LMr mucin; (c) a degraded mucus with only a low level of HMr mucin; (d) lack of mucin.

DISCUSSION

As previously indicated by Bagshaw *et al.* [2], a relatively non-invasive method should be available to study the quality of human gastric mucus so that epidemiological studies of gastric diseases can be undertaken. Generally, mucus quality has been evaluated by estimating the HMr glycoprotein content. Mucus glycoproteins can be partially or completely purified by ultracentrifugation in caesium chloride, the glycoproteins then being chromatographed on Sepharose CL2B or 4B [7,19]. The elution fractions are assayed for total carbohydrate content using the periodate acid–Schiff (PAS) reagent [18]. This tedious and time-consuming method, which is suitable for the study of purified glycoproteins, does not allow rapid analysis of crude human gastric mucus. The use of TSK G 4000SW silica gel to separate partially purified HMr glycoprotein from lower M_r molecules of sheep small intestine mucus was previously reported by Mukkur *et al.* [23]. The use of a catheter for human mucus collection *in vivo* was first described by Puchelle *et al.* [15].

Our data show that: (1) components of crude gastric mucus could be separated on TSK G 4000 SW silica gel and detected at 206 nm; (2) this technique could distinguish crude gastric mucus from mucus digested by pepsin; (3) HPLC analysis could be applied to adherent human gastric mucus collected *in vivo* by aspiration during upper gastrointestinal endoscopy; (4) the technique is reproducible and reliable. Variation in mucus elution profiles must be due to the quality of crude mucus rather than to the technique used.

The HPLC elution profile of pig fundic mucus differed from that of pig antral mucus by the presence of two HMr mucin components that eluted in leading peaks instead of only the single peak seen with antral mucus. PAS-reactive components were essentially eluted in the leading fractions, whereas most of the protein components were eluted in the later fractions.

The HPLC technique was applied to control the state of pig antral mucosa, especially to control the mucus quality. Indeed, an "artificial stomach" model has been used to assess the antacid characteristics of various antacid products. In addition, this model makes it possible to evaluate the interaction between aluminum-containing antacids and gastric mucosa by including a piece of pig antral mucosa in the "gastric reservoir" of that model [20]. This interaction could be explained by the fact that, in an acid medium, the electronegative charges of the sialic acid linked to mucus glycoproteins may bind cationic alumi-

num-containing compounds [24,25] and retard their discharge from the stomach. The importance of the mucus in this interaction was demonstrated by the use of gastric mucosa from which adherent mucus had been partially removed, as this resulted in a reduction of the duration of antacid activity [20]. For use of gastric mucosa in pharmacological assays, it therefore seems necessary to verify monthly the quality of the antral mucus coat on the mucosa, using the HPLC procedure described above.

The quality of human antral and fundic mucus from most of the mucosae studied was equivalent, but these results do not rule out the possibility that differences could exist between antral and fundic mucus from the same stomach. If that were the case, then such differences in molecular composition could be responsible for distinct rheological and functional properties of antral and fundic mucus. Four different elution patterns were obtained, indicating that at least four different molecular compositions could be exist in mucus from human mucosa: two types of non-degraded mucus containing HMr (1 300 000) mucin and LMr (200 000–300 000) components with or without IMr (500 000 600 000) mucin fractions and two types of degraded mucus without or with only a few HMr and IMr mucin fractions that maybe could be related to the aggressive nature of gastric secretions. When a steady baseline elution profile was obtained, it could be hypothesized that no mucus had covered the mucosal surface. These data could be explained either by mucous cell alteration, as in antritis, or by alteration of mucus synthesis, as in the response to a NSAID [2,26]. Also, these data plus the irregular and increased release of erosive secretions in ulcer disease and the limited number of subjects studied, could explain why the mucus elution profiles obtained in this preliminary assay appeared to be independent of the pathological state of the subjects and of the histological aspect of their mucosa. As previously reported by Leibur *et al.* [1] and Bagshaw *et al.* [2], variability in mucus synthesis and a pre-existent variability in some mucosal defence factor could also cause variability in the quality of mucus.

Further studies are necessary to determine whether the different elution profiles found in this work depend on pathological factors, cellular renewal or the quality of mucus synthesis.

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