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The determination of hexoses in rat gastric mucus by high-performance liquid chromatography*

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Introduction

Gastric mucus, which protects underlying mucosa against digestive secretions and irritants, is a complex and still not well understood biological matrix [1]. In particular, the major role in protective activity seems to be played by glycoproteins (mucins), each consisting of a peptide backbone covered, for the most part, by short carbohydrate chains linked to the peptide region by *O*-glycosidic bonds [2]. The carbohydrate chains may contain up to five different monosaccharides, namely: galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acids.

As an alternative to the determination of the content of intact mucins, a content that may be altered by pathological events or by mucoactive drugs, many authors measure the monosaccharides liberated by methanolysis [3] or by hydrolysis [4]. The complexity of the gastric mucus *per se* and the potential contaminations due to the possible presence of other sugar containing molecules such as polysaccharides and glycoproteins originating from blood, cell debris and bile, tend to make this analytical approach difficult. In fact, determination of sugars after mucus hydrolysis can be a marker of mucin production provided that there is at least one monosaccharide peculiar to these mucins and that the analysis is carried out by means of a specific and selective method. Colourimetric assays, which are still widely used, give poor and doubtful results, whereas gas chromatographic analysis, which requires complex and time consuming pre-column sample handling, produces a multiplicity of peaks for each monosaccharide [5] and often generates spurious peaks [6].

Using an HPLC assay, based on neutral hexoses derivatisation with dansyl hydrazine, UV detection and reversed-phase gradient elution, it has been found that fucose may be

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determined without interference and that mannose is absent, or present at low level, in rat gastric mucus but abundant in rat plasma and bile. The selective and specific LC determination of these markers enabled the discrimination of polluted samples and the evaluation, in the rat gastric mucus, of stimulating effects of drugs endowed with cytoprotective activity.

Experimental

Rat gastric mucus obtained by scraping the gastric mucosa [7] was homogenised with Polytron (Kinematica GmbH, Kriens-Luzern, Switzerland) for 30 s; 0.5 ml of the suspension was added to 0.5 ml of 2 M aqueous trifluoroacetic acid and hydrolysed for 7 h at 100°C in sealed tubes. Then, each sample was added to 2 ml of acetonitrile and dried under a stream of nitrogen at 60°C. The dried samples were resuspended in 100 μl of water, and 50 μl of a 2.5% dansyl hydrazine (Pierce Chemical Co., Rockford IL, USA) solution in acetonitrile and 10 μl of 5% aqueous trichloroacetic acid added. Each tube was sealed, coated with aluminium sheet and immediately placed into a thermostatic bath at 60°C for 30 min. After cooling to room temperature, 140 μl of water was added to give a final volume of about 300 μl. Then, to remove excess reagents, the samples were washed twice with 2 ml of toluene and immediately analysed.

The same procedure also was used when aqueous standards or other biological samples such as blood or bile were processed.

HPLC analyses were carried out by means of a Varian 5000 instrument (Palo Alto, CA, USA) equipped with a Varian UV-100 variable wavelength detector operating at 254 nm, with a Rheodyne injector mounting a 20 μl loop and with a RP-8, 5 μ Hibar (E. Merck, Darmstadt, FRG) column (25 × 0.46 cm i.d.). The analyses were performed at room temperature and at flow rate of 0.8 ml min^{-1} with gradient elution, using mixtures of acetonitrile (A) and of 0.5% aqueous acetic acid (B). The following gradients were used: from 95 to 75% B in 35 min; isocratic at 75% B for 5 min; 75 to 20% B in 10 min; isocratic at 20% B for 5 min; 20 to 95% B in 15 min. The latter part of the gradient serves to elute strongly retained substances including unreacted reagent.

All monosaccharide standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). For the assay of the sugars, aqueous standards were used. Calibration plots were derived by the least squares regression technique using peak areas obtained for a series of sugar concentrations.

Results and Discussion

Various methods have been used to accomplish the selective detection of neutral sugars present in glycoproteins by means of HPLC with either pre-column derivatisation with dansyl hydrazine [8, 9] and dabsyl hydrazine [10] or by post-column reaction [11]. However, very few quantitative applications to crude biological samples have been described.

With the main aim of developing a routine method for the analysis of large numbers of raw biological samples pre-column derivatisation of sugars with dansyl hydrazine followed by RP–HPLC with UV detection was selected. This method enabled the determination of the neutral hexoses obtained by hydrolysis of complex biological matrices such as gastric mucus, serum and bile without any particular pretreatment. Figure 1 shows a typical chromatogram obtained by processing a standard mixture of all

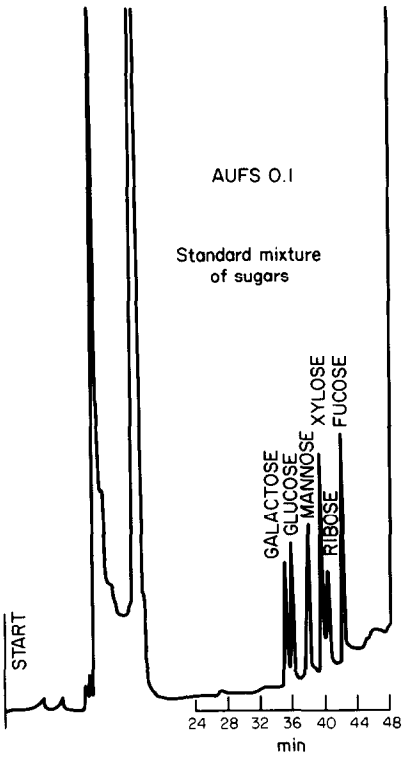

Figure 1
Chromatogram of a standard mixture of hexoses (0.5 ml of a solution containing 0.05 μM of each sugar was processed).

the hexoses expected to be present in biological samples. As can be seen each sugar is eluted as a single and well resolved peak.

Qualitative comparison between the chromatograms of rat gastric mucus (Fig. 2c) and those of the potential contaminants such as serum (Fig. 2a) and bile (Fig. 2b) shows that glucose, galactose and mannose are always present, whereas fucose is found only in the gastric mucus. These findings led to the decision to use fucose measurements as the main marker of gastric mucus glycoproteins output. Appropriate calibration curves for the hexoses were established for each experiment using aqueous standards (six concentrations in the range 5–80 μg of sugar per ml). Typical examples of calibration lines obtained by least squares regression analysis are: fucose $y = -11.60 + 6.07x$ ($r = 0.9954$); galactose $y = 2.84 + 2.95x$ ($r = 0.9969$), where the xs are μg ml^{-1} of the hexose and the ys are hexose peak areas (arbitrary units). The detection limits are about 0.1 μg ml^{-1} of sugar using aqueous standards and about 1 μg ml^{-1} using gastric mucus. Moreover, processing different volumes of gastric mucus from a homogeneous pool resulted in very similar levels of hexoses per ml of mucus, showing that the derivatisation yield is independent of the volume of gastric mucus processed.

As an illustrative example, Fig. 2d shows a representative chromatogram of a gastric mucus sample obtained from a rat treated with the new antiulcer drug esaprazole [12]. Comparison with Fig. 2c reveals a remarkable increase of the concentration of all the hexoses identified. In addition, Table 1 confirms the trend quantitatively. The total output of fucose is consistent with a significant stimulation by the drug of glycoprotein production. In this table the total output per rat of galactose also is reported, because

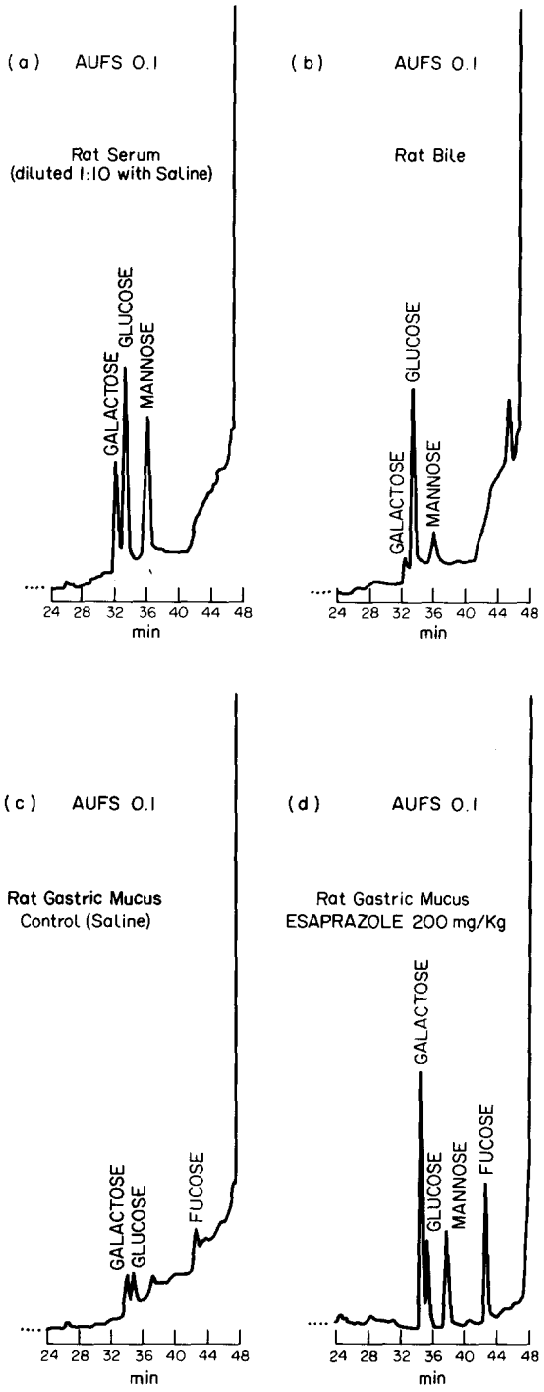

Figure 2
Chromatograms of processed biological samples: (a) rat serum; (b) rat bile; (c) rat gastric mucus; (d) gastric mucus from rat treated with esaprazole.

Table 1
Fucose and galactose total output per rat in controls and in animals treated with 200 mg kg^{-1} per os of esaprazole

Rat	Fucose output (μg)*		Galactose output (μg)*		Galactose/fucose*	
	Supernatant†	Pellet†	Supernatant†	Pellet†	Supernatant†	Pellet†
Controls ($N = 5$)	11.39 ± 3.8	30.4 ± 9.3	24.65 ± 16.8	71.98 ± 16.4	2.05 ± 0.73	2.56 ± 0.52
Treated with esaprazole ($N = 5$)	58.4 ± 22.6	81.7 ± 27.6	146.3 ± 58.6	156.5 ± 71.6	2.53 ± 0.31	2.00 ± 0.38

*Data ± standard deviation.

†Mucus samples were centrifuged to separate the soluble mucus fraction (supernatant) and the insoluble mucus that sedimented as a gelatinous pellet [7]. The pellet is resuspended in the same volume of the supernatant. Both samples are treated as described in the Experimental section.

literature data [13] indicate that galactose:fucose ratio typically ranging around a value 2 for glycoproteins. The present work gave evidence of a similar ratio for both control and treated rats, as shown in Table 1. However this only applies if contamination from bile and/or blood is absent (data of contaminated samples have been discarded).

Conclusions

Quantitative analysis of fucose in rat gastric mucus, after acidic hydrolysis, by this selective and sensitive HPLC method gives a specific index of neutral gastric mucins production. This index may be suitable for the screening of new drugs endowed with potential cytoprotective effects.

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