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COMPUTER-AIDED EVALUATION OF GASTRIC PROTEOGLYCANS BY HIGH-RESOLUTION GAS CHROMATOGRAPHY

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SUMMARY

A convenient procedure is described for the simultaneous separation and identification of monosaccharides and aminomonosaccharides constituting the proteoglycans of gastric mucosa. Computer assistance was used to obtain significant information from gas chromatographic data on proteoglycan modifications induced by anti-inflammatory drugs. Glass capillary chromatography was used for the separation and identification of saccharides and aminomonosaccharides of hydrolyzed mucus samples scraped from the stomachs of control and treated rats. The data, transmitted to a 48 K personal computer, were processed to obtain three-dimensional probability density functions or subjected to factor analysis to evaluate the suitability of the method to evidence modifications at the proteoglycan level induced by drugs, orally administered to rats. Indomethacin, zolimidine and acetylsalicylic acid were used as models for the applications.

INTRODUCTION

The gastric mucosa is coated by a layer of mucus, which plays an important role in protecting the underlying cells from the damaging effects of chemicals and enzymes. Many compounds that damage the stomach wall, including salicylates and other anti-inflammatory drugs, modify the mucus barrier, inducing back-diffusion of acid and subsequent development of mucosal erosion and haemorrhage.

The molecules on which the protective layer depends are glycoproteins, which are composed of proteins, monosaccharides (commonly xylose, fucose, mannose, galactose and glucose) and aminomonosaccharides (commonly N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid); The application of gas liquid chromatography (GLC) to the determination of the carbohydrate composition of glycoproteins and glycolipids has been extensively studied[1–6]. Our work deals with the automatic separation and computed-assisted evaluation of saccharides and aminosaccharides by high-resolution gas chromatography after methanolysis of the individual samples. The best analytical procedure and experimental parameters were chosen, according to our experience, and the results were transmitted to a 48 K personal computer for data processing.

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EXPERIMENTAL

Reagents and chemicals

Fucose, glucose, galactose, mannose, rhamnose, xylose, glucuronic acid, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, N-acetylneuraminic acid were obtained from Supelco (Bellefonte, PA, U.S.A.) as well as anhydrous methanolic hydrochloric acid (0.5 *M*), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylsilylimidazole (TMSIM) and anhydrous pyridine. Acetylsalicylic acid was purchased from Carlo Erba (Milan, Italy) and indomethacin from Merck Sharp and Dohme (Pavia, Italy). Zolimidine was a gift from Selvi (Milan, Italy).

Gas–liquid chromatography

All the GLC separations were accomplished on a 20 m × 0.3 mm I.D. glass capillary column, coated with SE-30 (film thickness 0.37 μm), in a Carlo Erba 2900 gas chromatograph, equipped with a flame-ionization detector and a Grob-type splitter injector. The column temperature was increased at 5°C/min from 150°C to 350°C. The injection splitter (splitting ratio 40:1) and detector were heated to 250°C and 370°C, respectively. The glass injection liner was filled with Chromosorb W HP (80–100 mesh). Helium was used as the carrier gas at a flow-rate of 2.0 ml/min.

Gas–liquid chromatography–mass spectrometry

An Hewlett-Packard Model 5985B GLC–mass spectrometry (MS) system was used, equipped with an HP 7906 Disk Drive. The GC injector was a splitter/splitless type, and the GC conditions were the same as described above. The mass spectra were registered at 70 eV (electron impact source) at 1000 resolution.

Data processing

The data processing was performed with a 48 K Apple II plus personal computer, connected to a Varian CDS-111 integration system via a RS-232 C interface and equipped with a dual floppy-disk drive and Watanabe WX4671 plotter. All the programs (RETIND, PROBDENS and FACTANAL) were written in BASIC Applesoft.

Sample collection

Mucus was scraped by means of a stainless-steel spatula from the stomach of Sprague–Dawley male rats (body weight 230 g), either 24 h after the oral administration of zolimidine (100 mg/kg), 4 h after an intraperitoneal injection of indomethacin (15 mg/kg) or after an oral administration of acetylsalicylic acid (200 mg/kg). It was immediately transferred into a 1-ml screw-capped vial and lyophilized. Its weight was recorded before (fresh mucus) and after lyophilization.

After the collection, the stomach wall was submitted to a histological examination in order to determine whether the gastric mucosa had been damaged by the scraping.

The three experiments were carried out separately, and each one was performed on ten treated and ten control rats.

Analytical procedure

Analysis samples. Each lyophilized mucus sample (about 5–10 mg) in the 1-ml

screw-capped vial with PTFE cup liner was treated with 0.5 ml of 0.5 *M* anhydrous methanolic HCl. The air was removed under a stream of nitrogen and the vial was left to stand at 50°C for 20 h in a heating block. After it had cooled at room temperature, 150 μl of anhydrous pyridine, followed by 100 μl of acetic anhydride were added. The vial was sealed and allowed to remain for 30 min at room temperature and then the solvents were removed under a stream of nitrogen at 80°C and the sample was taken to dryness in a vacuum desiccator at 40°C. As soon as it was dry, the residue was subjected to trimethylsilylation by adding 0.5 ml of BSTFA–TMSIM–pyridine (10:1:0.4). At this point, 25 μl of hydrocarbon reference mixture were added to the sample by a microsyringe. This reference solution was prepared by adding 25 μl of the liquid mixture containing in equal volumes C_{10}, C_{12}, C_{14} and C_{16} *n*-alkanes to 200 μl of a solution containing 500 mg of C_{18}, C_{20}, C_{22} and C_{24} *n*-alkanes in 3 ml of chloroform plus 2 ml of BSTFA. Aliquots of 2 μl can be injected after a few minutes.

Standards. An aliquot of a solution containing 0.2 mg of each sugar (fucose, glucose, galactose, xylose, glucuronic acid, rhamnose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, N-acetylneuraminic acid) in water was placed in a screw-capped Reacti-vial with PTFE cap liner and taken to dryness overnight under vacuum over potassium hydroxide pellets. Samples prepared in this way were subjected to the same treatment as described for analysis samples including methanolysis.

RESULTS AND DISCUSSION

Monosaccharides and aminomonosaccharides in the proteoglycans collected from the stomach of each rat were examined by glass capillary chromatography after methanolysis and derivatization. Basically, the method involves the conversion of the bound monosaccharides into free sugars, which can be analyzed by capillary GLC in the form of O-methyl N-acetyl O-trimethylsilyl derivatives. As is well known, the methanolysis reaction can yield four different glycosides of each sugar unit: the α- and β-anomers of both the methyl pyranosides and the methyl furanosides. The relative proportion of the different isomers is not altered in the reference mixture and in the samples, except for neuraminic acid, if the same experimental conditions are used.

The reference solution contained the known mixture of monosaccharides an aminomonosaccharides (see Experimental). A mixture of *n*-alkanes with even carbon numbers was added to each sample and regarded as a reference for the retention index calculations by non-linear interpolation[7]. The retention data of the reference solution were transmitted to a 48 K Apple II Plus personal computer via an RS-232 C interface; the calculated retention index values were stored on a floppy disk and used for analysis samples. The gas chromatograms of a standard mixture of an analytical sample are shown in Figs. 1 and 2, respectively. Only the most prominent and the most homogeneous peaks (from GLC–MS investigation) were used in the calculations. Such peaks are marked with an asterix in the figures. In addition, the ratios of the areas of the sugar peaks to the area of the C_{24} *n*-alkane were used as quantitative parameters. In this way, information about the absolute quantity of monosaccharides was lost but the data adequately account for modifications at the proteoglycan level, if the experimental conditions are exactly reproduced. Table I shows the reproducibility of identical samples of proteoglycans subjected to the same analytical procedure.

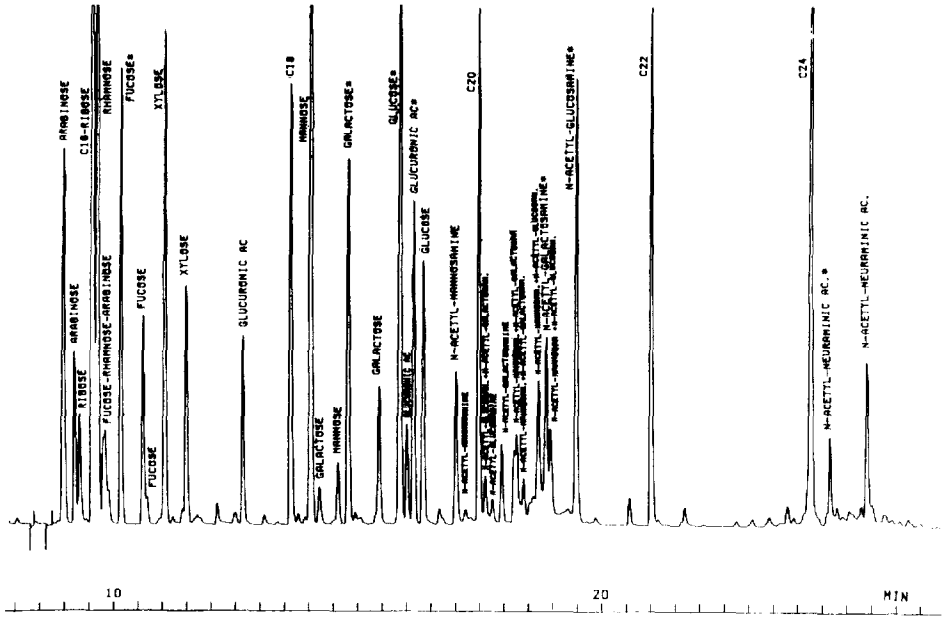

Fig. 1. GLC of the N-acetyl O-trimethylsilyl derivatives of O-methyl glycosides of standard sugars.

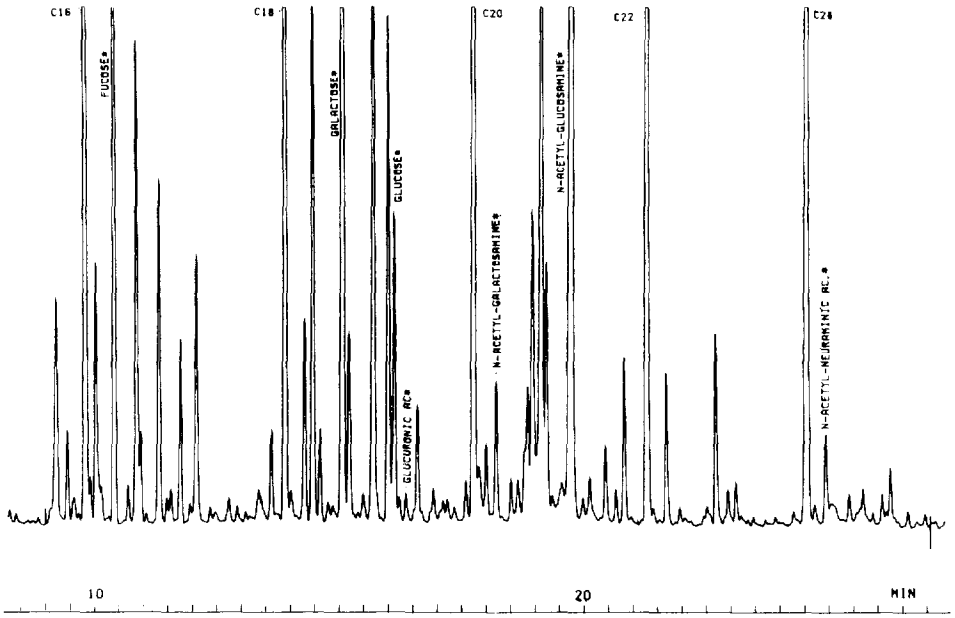

Fig. 2. GLC after N-acetylation and trimethylsilylation of the methanolysis residue from stomach proteoglycans.

The mucus of each control and treated rat (5–10 mg) was subjected to lyophilization, methanolysis and derivatization and analyzed by GLC. The results are reported in Tables II, III and IV. The GLC data corresponding to each animal can conveniently be represented as points on a two-dimensional diagram by a computer program, by plotting the sugar content (expressed as the sum of each monosaccharide to C_{24} *n*-

TABLE I

REPRODUCIBILITY OF THE ANALYTICAL METHOD

Sugar	*Sugar content (Ratio of the area of sugar peak to C_{24} n-alkane)*		
	Mean (n = 5)	*S.D.*	*R.S.D.(%)*
Fucose	0.637	0.019	3.63
Galactose	1.266	0.032	2.51
Glucose	0.257	0.011	4.48
Glucuronic acid	0.039	0.001	1.36
N-Acetylgalactosamine	1.086	0.097	9.03
N-Acetylglucosamine	1.326	0.072	5.43
N-Acetylneuraminic acid	0.146	0.012	8.09

TABLE II

EFFECT OF ZOLIMIDINE ON THE SUGAR CONTENT (AREA RATIO OF SUGAR PEAK/C_{24} *n*-ALKANE) OF STOMACH PROTEOGLYCANS OF ORALLY TREATED RATS.

Mean ± S.E.

Sugar	*Sugar content*	
	Control (n = 9)	*Zolimidine (n = 12)*
Fucose	0.432 ± 0.066	0.640 ± 0.143
Galactose	0.837 ± 0.127	1.247 ± 0.252
Glucose	0.162 ± 0.042	0.260 ± 0.086
Glucuronic acid	0.025 ± 0.006	0.039 ± 0.009
N-Acetylgalactosamine	0.080 ± 0.010	0.103 ± 0.030
N-Acetylglucosamine	1.027 ± 0.108	1.609 ± 0.306
N-Acetylneuraminic acid	0.027 ± 0.020	0.049 ± 0.020

TABLE III

EFFECT OF INDOMETHACIN ON SUGAR CONTENT (AREA RATIO OF SUGAR PEAK/C_{24} *n*-ALKANE) OF STOMACH PROTEOGLYCANS IN ORALLY TREATED RATS:

Means ± S.E.

Sugar	*Sugar content*	
	Control (n = 8)	*Indomethacin (n = 9)*
Fucose	0.805 ± 0.203	0.549 ± 0.073
Galactose	1.446 ± 0.365	1.025 ± 0.137
Glucose	0.327 ± 0.127	0.216 ± 0.059
Glucuronic acid	0.029 ± 0.008	0.025 ± 0.007
N-Acetylgalactosamine	0.117 ± 0.040	0.089 ± 0.022
N-Acetylglucosamine	1.974 ± 0.458	1.480 ± 0.234
N-Acetylneuraminic acid	0.087 ± 0.033	0.061 ± 0.023

TABLE IV

EFFECT OF ACETYLSALICYLIC ACID ON SUGAR CONTENT (AREA RATIO OF SUGAR PEAK/ C_{24} *n*-ALKANE) OF STOMACH PROTEOGLYCANS IN ORALLY TREATED RATS

Mean ± S.E.

Sugar	*Sugar content*	
	Control (n = 12)	*Acetylsalicylic acid (n = 13)*
Fucose	0.326 ± 0.039	0.274 ± 0.034
Galactose	0.655 ± 0.078	0.596 ± 0.077
Glucose	0.140 ± 0.026	0.260 ± 0.043
Glucuronic acid	0.020 ± 0.005	0.019 ± 0.007
N-Acetylgalactosamine	0.563 ± 0.103	0.551 ± 0.144
N-Acetylglucosamine	0.713 ± 0.081	0.629 ± 0.087
N-Acetylneuraminic acid	0.091 ± 0.021	0.088 ± 0.020

alkane) against the aminosugar content (expressed as the sum of peak area ratios of each aminomonosaccharide to C_{24} *n*-alkane. Fig. 3a, c, and e show the graphs obtained from the experiments carried out with zolimidine, indomethacin and acetylsalicylic acid, respectively. As expected, the points are distributed around a straight line. The ellipsoids are the regions of equal density of probability, centered at the mean values (C for control and D for treated), and the axes of the ellipses correspond to the standard errors. In a three-dimensional space are also represented the probability functions[8] of the mean values for each group of data for visualization of the pharmacological effect (Fig. 3b, d and f). It is evident that, as expected, zolimidine increases and indomethacin decreases significantly of the mucus barrier on the stomach of rats. In contrast, no significant modification in mucus quantity was observed in acetylsalicylic acid-treated animals.

In order to investigate whether the original chromatographic data contain significant information about possible changes of sugar and aminosugar composition induced by the drugs investigated, we also considered other mathematical methods. Interesting results were obtained by application of factor analysis[9] to each experiment based on the original GLC data, represented by the peak area ratios (sugar/C_{24}), to form a *n* samples × *n* sugars data matrix. Factor and discriminant analyses, which are multivariate statistical methods, are able to examine all the variables in the data matrix simultaneously and to compact the data by removing redundancy and noise from the measurements. These statistical analyses provide a set of variables fewer in number that in the original set, while still preserving the integrity and information content of the original. Each factor is a linear combination of sugar data and carries different amounts of explanation of variance. From factor plots of factor 1 *vs.* factor 2 and factor 2 *vs.* factor 3 it is evident that factor 2 and factor 3 clearly separate control from treated cases in the acetylsalicylic acid experiment (Fig. 4a, b) whereas no separations are observed for zolimidine (Fig. 4c,d) and indomethacin (Fig. 4e,f). Factor 1, which accounts for 99.67% (indomethacin), 99.43% (zolimidine) or 98.97% (acetylsalicylic acid) of the total variance, can be associated with the total proteoglycan quantity. Factor 2 and factor 3, which account for 0.18% and 0.09% (indomethacin), 0.40% and 0.15% (zolimidine), 0.52% and 0.32 (acetylsalicylic acid), respectively, of the total variance, may

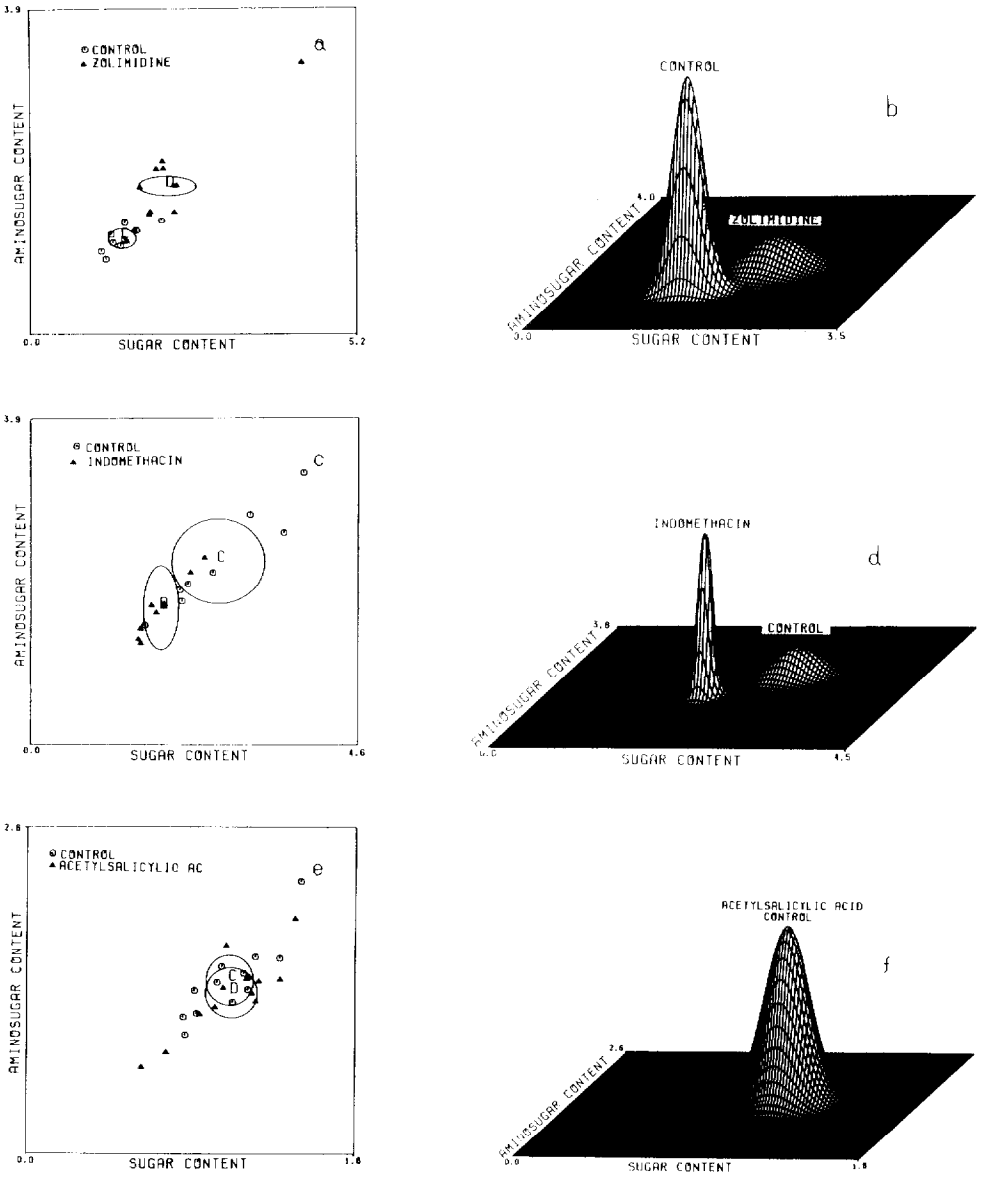

Fig. 3. a,c,e, Two-dimensional plot of sugar content (expressed as sum of peak area ratio of each saccharide to C_{24} *n*-alkane) against aminosugar content (expressed as the sum of peak area ratios of each aminosaccharide to C_{24} *n*-alkane) of the methanolysis residue from the stomach proteoglycans for control and treated rats. b,d,f, Probability density functions of the mean values for the cases considered.

be associated with a change in sugar composition for acetylsalicylic acid (eigenvalues with physical meaning) and with redundancy for the other cases.

In another simple mathematical approach, the per cent contribution of the peak ratio (sugar/C_{24}) for each sample was calculated to evaluate with the statistical *t*-test the significance of the differences between the mean values of control and treated cases.

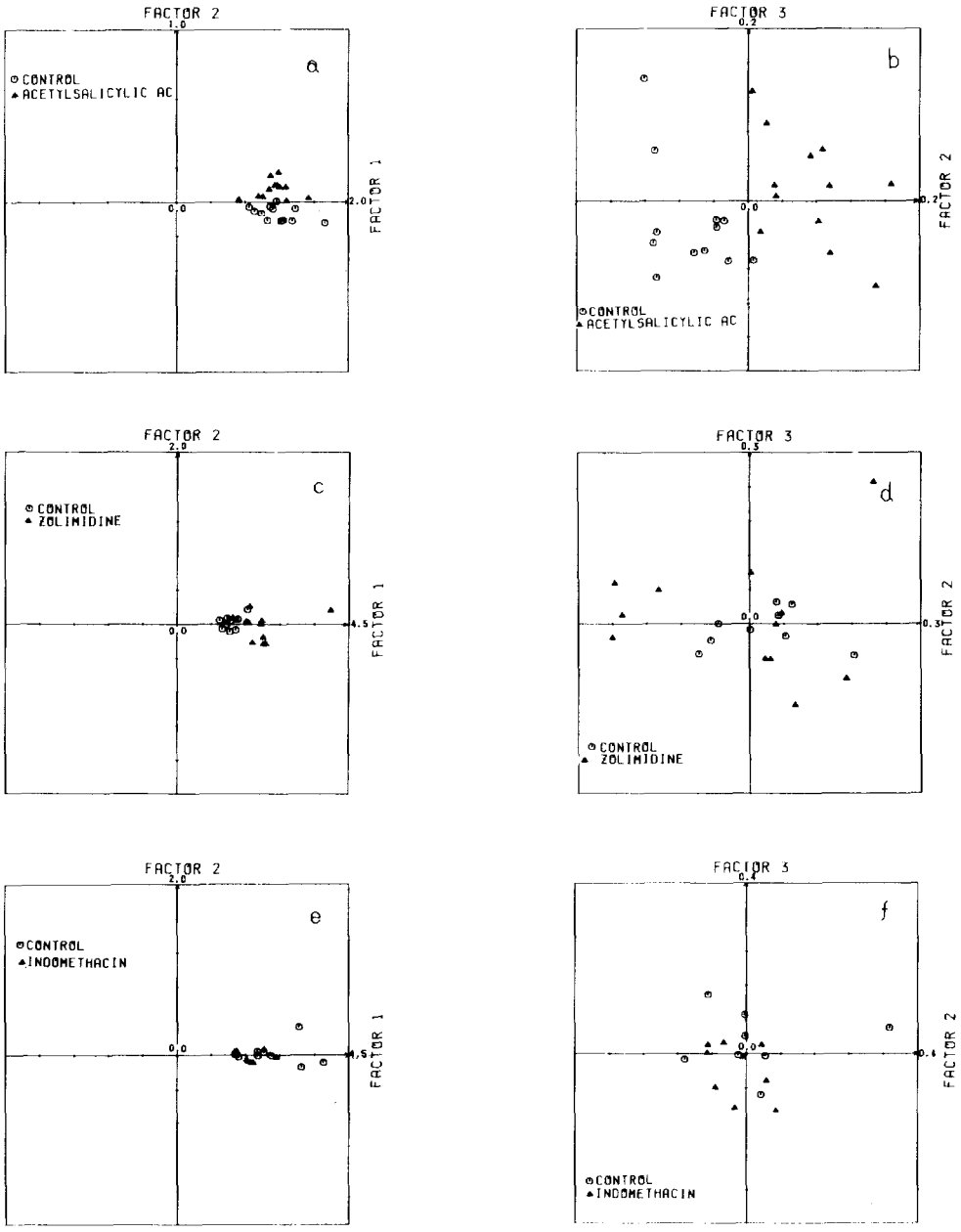

Fig. 4. Factor analysis of GLC data (peak area ratios of each saccharide to C_{24} *n*-alkane) of the methanolysis residue from stomach proteoglycans for control and treated rats. a and b, Experiment with acetylsalicylic acid; c and d., Experiment with zolimidine; e and f, Experiment with indomethacin.

In all these experiments only acetylsalicylic acid showed a significant effect on changing the composition of the sugars of proteoglycans. Fucose, glucose and N-acetylglucosamine showed significant changes in peak area ratio composition for acetylsalicylic acid-treated compared to control cases. The mean values are reported in Table V.

TABLE V

EFFECT OF ACETYLSALICYLIC ACID ON THE SUGAR COMPOSITION (PERCENTAGE OF AREA RATIO OF SUGAR PEAK / C_{24} *n*-ALKANE) OF STOMACH PROTEOGLYCANS IN ORALLY TREATED RATS

Sugar	*Sugar composition*	
	Control (n = 12)	*Acetylsalicylic acid (n = 13)*
Fucose	13.03 ± 0.45	11.44 ± 0.64
Galactose	26.20 ± 0.88	24.75 ± 0.95
Glucose	5.62 ± 0.91	10.78 ± 1.14
Glucuronic acid	0.79 ± 0.19	0.75 ± 0.24
N-Acetylgalactosamine	22.21 ± 1.35	22.77 ± 1.38
N-Acetylglucosamine	28.53 ± 1.24	26.01 ± 0.81
N-Acetylneuraminic acid	3.59 ± 0.55	3.57 ± 0.56

CONCLUSION

The analytical procedure described has been successfully employed in our laboratory for the routine analysis of glycoprotein samples in order to ascertain sugar modifications induced by drugs at the proteoglycan level. Ratios of peak areas were used as quantitative data for computer processing but, if needed, absolute amounts of sugars can be calculated and used.

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