A NEW WAY OF CHEMICAL BLOOD-TYPING OF HUMAN SINGLE INDIVIDUALS

Identification of a blood group Le^b-active glycosphingolipid in plasma by mass spectrometry and NMR spectroscopy

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Received 28 November 1980

1. Introduction

The presence of blood group Lewis (Le) antigens of glycosphingolipid nature in human plasma was made probable through the work of Marcus et al. [1]. Recently, several Le active glycosphingolipids were isolated and partially characterized from 8 l pooled human plasma obtained from 40 donors [2,3]. The sparse occurrence on cell surfaces or in body fluids of these substances is a major problem in chemical studies of the individual expression of blood group glycolipids. We have developed specific and sensitive techniques based on mass spectrometry [4,5] and NMR spectroscopy [6–8] for the identification of blood group antigens. Mass spectrometry may give conclusive information on separate saccharide sequences also in mixtures [5]. NMR spectroscopy may establish the anomerity along the saccharide chain and also discriminate between type 1 (Gaβ1 → 3 GlcNAc) and type 2 (Galβ1 → 4 GlcNAc) saccharide chains [6–8]. These methodological developments were essential for the specific detection of an Le^b hexaglycosylceramide in plasma of a human donor.

2. Materials and methods

One transfusion unit (450 ml) of fresh human blood (O, Rh-, Le(a–b+), secretor) in acid–citrate–dextrose solution was received from the blood bank at Sahlgren's hospital in Göteborg. Plasma was immediately removed, carefully avoiding the buffy coat after centrifugation and recentrifugation. The cell-free plasma was stored at −20°C before lipid extraction. The cells were washed 5 times with 1 vol. saline. Cell membranes were prepared by toluene flotation [9] and lipid extraction took place within 24 h after blood donation.

Methanol (250 ml) was added to ~250 ml plasma or toluene-flotated red blood cell membranes of 1 transfusion unit and the mixture was heated to +70°C under constant stirring for 30 min. The extract was filtered and the filtrate was transferred back to the extraction bottle. The procedure was repeated twice with 250 ml chloroform:methanol, 2:1 (v/v) and one with 250 ml methanol. The combined extracts were evaporated to dryness with the addition of small volumes of toluene. The dry lipid extract was subjected to mild alkaline hydrolysis, dialysis, DEAE-cellulose and silicic acid chromatography [10]. Alkali-stable non-glycolipids were removed by silicic acid chromatography of acetylated lipids [11].

An MS 902 mass spectrometer (AEI Ltd, Manchester) was used, equipped with a direct inlet system and a separate probe heater. The mass spectrometer was connected on-line to a computer system (Datamass One, Kratos-Instem Ltd, England). A Bruker 270 MHz NMR instrument was used in the pulsed Fourier transform mode. Spectra were taken up in 8 K memory with a band-width of 3000 Hz and an acquisition time of 1.37 s. Chemical shift values are given in ppm relative to tetramethylsilane and the residual solvent peak was used as an internal standard.

Part of this work was presented at the 11th FEBS Meeting in Copenhagen, August 1977, and at the XV Congress of the International Society of Blood Transfusion in Paris, July 1978

3. Results and discussion

Fig.1 shows the thin-layer chromatographic pattern of the total non-acid glycosphingolipids of cells and plasma, respectively. One-fourth of the plasma fraction (2 mg) was permethylated [12] and subjected to mass spectrometry. By a successive recording of spectra during heating of the sample in the ion source the presence of major glycolipids with 1–4 sugars was established (cf. fig.1). These have been characterized chemically before [13]. Late in the evaporation a spectrum was recorded (fig.2) which is very similar to the spectrum of an Le^b-like hexaglycosylceramide in [14]. The only terminal sugar found

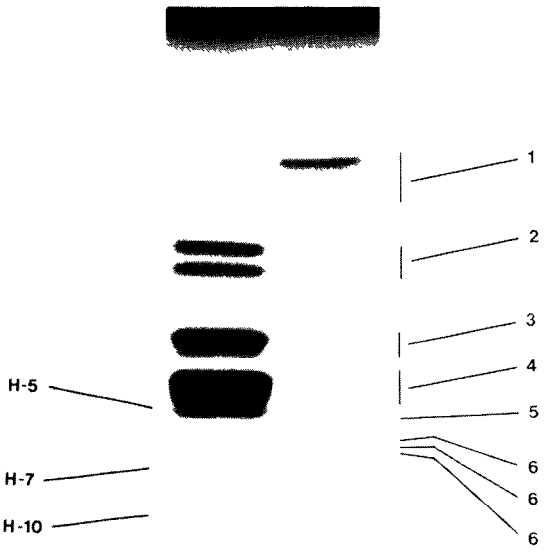

Fig.1. Thin-layer chromatogram of the total non-acid glycosphingolipid fractions obtained from red blood cell membranes, lane 1, and plasma, lane 2, of a blood group O, Le(a–b+) human donor. The amounts spotted on the thin-layer plate correspond to the same amount of whole blood (1 ml). The figures to the right indicate the number of sugars found in the carbohydrate chain. The 3 bands for 6 sugars probably represent glycolipids with different ceramide species but with the same oligosaccharide. The major component in the membrane fraction, lane 1, probably globotetraosylceramide (4 sugars). The band just below this is an H-active pentaglycosylceramide (H-5). H-active glycosphingolipids with 7 (H-7) and 10 (H-10) sugars are also indicated. For anlytical thin-layer chromatography HPTLC-Fertigplatten with Kieselgel 60 were used (Merck). The solvent was chloroform:methanol:water, 60:35:8 (by vol.), and detection reagent was anisaldehyde [10].

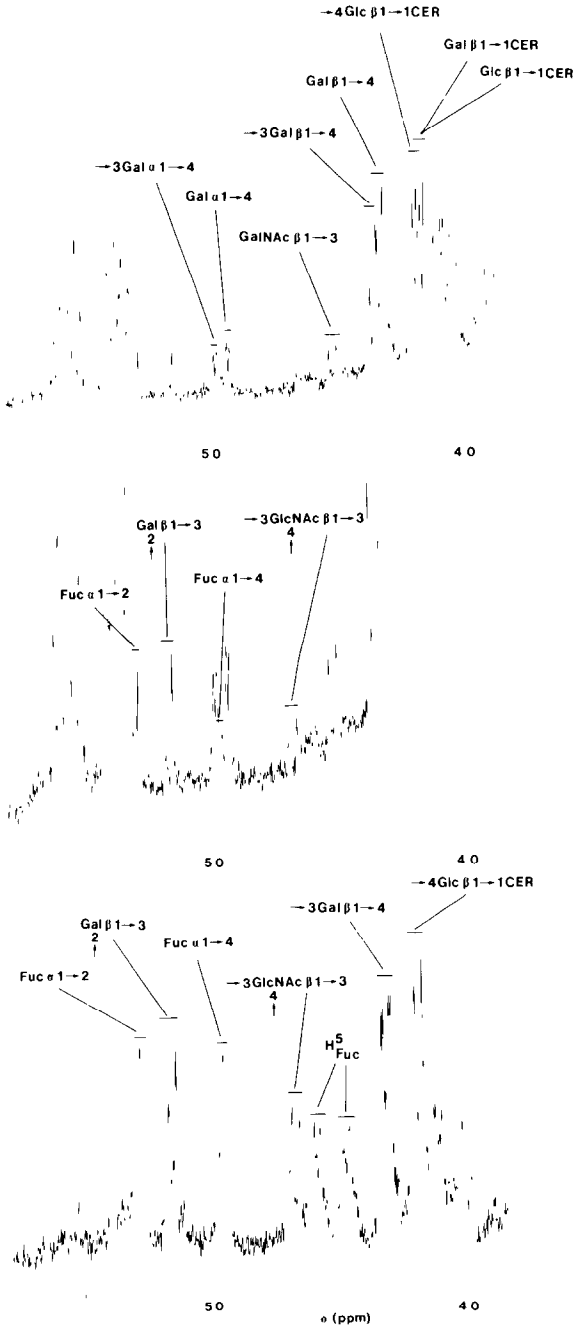

Fig.3. Top: Partial NMR spectrum of a permethylated-reduced glycosphingolipid mixture from plasma. The spectrum was recorded in ^2H–chloroform solution (1.4 mg in 0.5 ml). 5600 pulses were recorded at 40°C. Middle: The same as top spectrum but reproduced with higher amplification. Bottom: Spectrum of permethylated-reduced Le^b active hexaglycosylceramide, Fucα1 → 2 Galβ1 → 3 GlcNAc(4 ← 1αFuc)β1 → 3 Galβ1 → 4 Glcβ1 → 1 ceramide [8]. 1.2 mg in 0.5 ml ^2H–chloroform. 1750 pulses at 40°C.

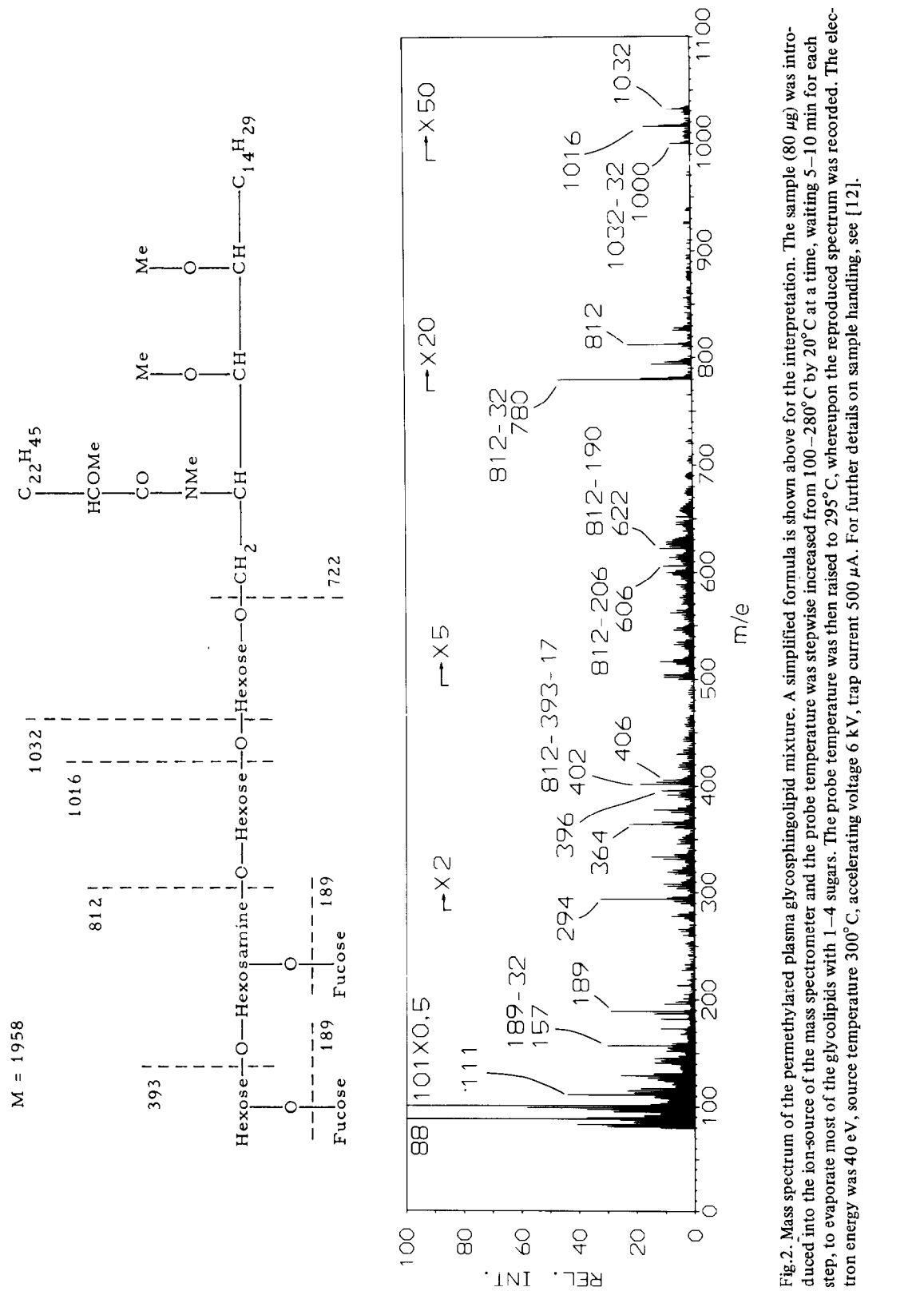

Fig.2. Mass spectrum of the permethylated plasma glycosphingolipid mixture. A simplified formula is shown above for the interpretation. The sample (80 μg) was introduced into the ion-source of the mass spectrometer and the probe temperature was stepwise increased from 100–280°C by 20°C at a time, waiting 5–10 min for each step, to evaporate most of the glycolipids with 1–4 sugars. The probe temperature was then raised to 295°C, whereupon the reproduced spectrum was recorded. The electron energy was 40 eV, source temperature 300°C, accelerating voltage 6 kV, trap current 500 μA. For further details on sample handling, see [12].

was fucose as shown by *m/e* 189 and 157. The sequence proposed in the formula is given by peaks at *m/e* 812 and 780 (terminal tetrasaccharide) and 1032, 1016 and 1000 (pentasaccharide). The rearrangement ion indicated at *m/e* 402 is probably composed of the hexosamine and fucose and is only formed from a type 1 chain.

The remaining part of the permethylated sample was reduced with $LiAlH_4$ and subjected to NMR analysis (fig.3). With a basis in reference spectra [6], anomeric signals originating from mono-, di-, tri- and tetraglycosylceramide are easily recognized (top spectrum). After amplification (middle spectrum) also specific signals of the less abundant hexaglycosylceramide are discernable. Two isomeric structures with the sequence proposed in fig.2 are known, one based on a type 1 (Le^b) and the other on a type 2 chain. NMR spectra of both have earlier been recorded and shown to have specific differences [8]. Weak but definite signals are indicated (middle spectrum) corresponding to the anomeric protons of the terminal tetrasaccharide of the reference Le^b glycolipid with a

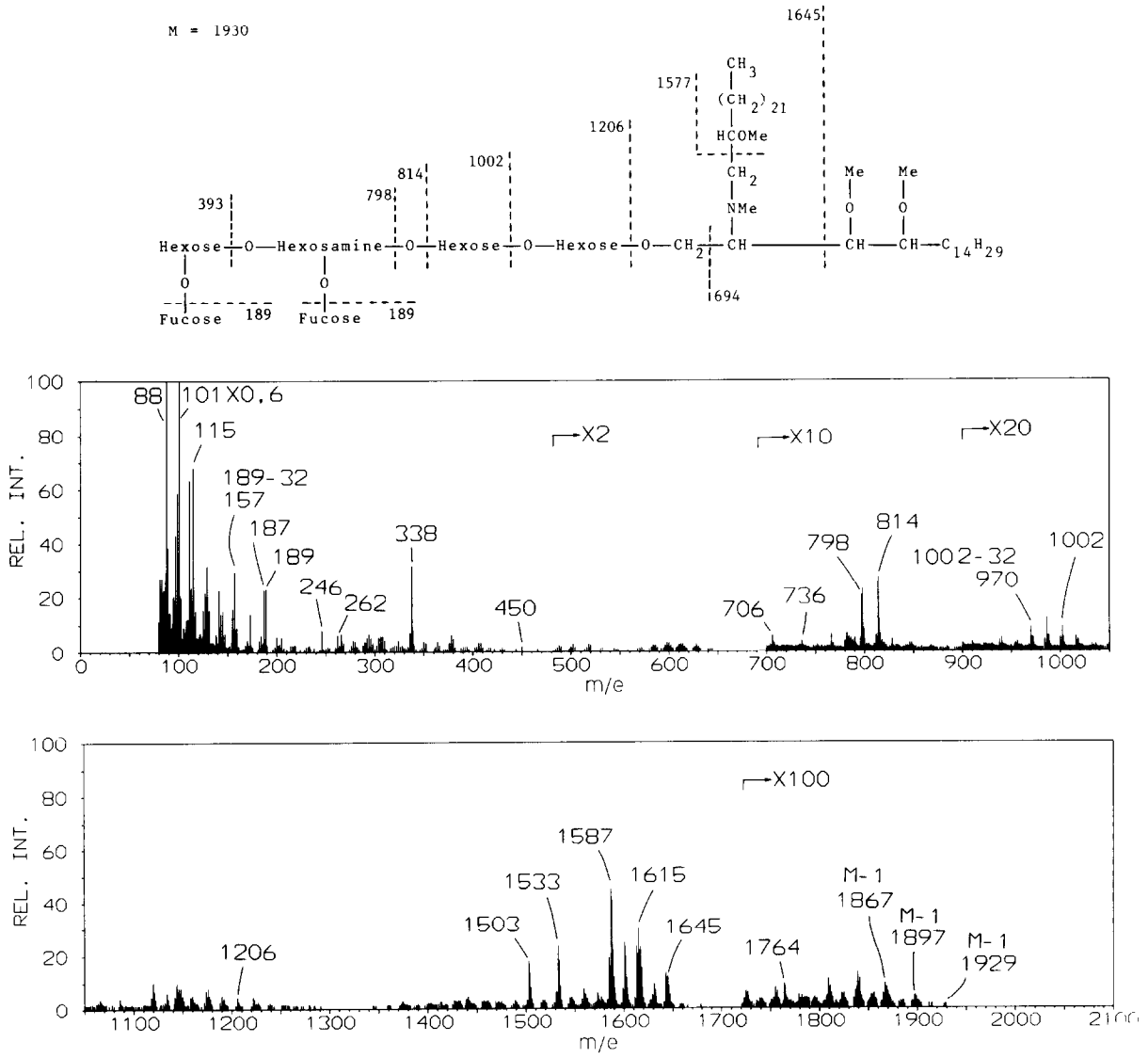

Fig.4. Mass spectrum of the permethylated-reduced plasma glycosphingolipid mixture. A simplified formula is shown for the interpretation. The spectrum was recorded at 275°C. For further details, see fig.2.

type 1 chain (bottom spectrum). The presence of the signal at 5.16 ppm and the absence of a Fucα1 → 3 signal at 5.78 ppm (type 2 chain) are highly diagnostic [8].

The reduced sample was finally subjected to mass spectrometry, using the same procedure as for the non-reduced mixture. During the evaporation a number of spectra were taken confirming the presence of mono-, di-, tri- and tetraglycosylceramides. At high temperature a spectrum was recorded (fig.4) which gave additional support for the presence of the hexaglycosylceramide proposed. Fragments represented by the series of peaks from *m/e* 1503 to 1645 contained the complete saccharide and a fatty acid, one series (1503, 1587, 1601 and 1615) with non-hydroxy fatty acids (16:0, 22:0, 23:0 and 24:0, respectively) and one series (1533, 1617, 1631 and 1645) with hydroxy fatty acids (16:0, 22:0, 23:0 and 24:0, respectively). Sequence information was given by *m/e* 157, 189, 798, 814, 970, 1002 and 1206.

Although all analyses were done on a mixture of glycosphingolipids, the structural identity of the hexaglycosylceramide with an Le^b determinant is very probably. This was in agreement with the Le^b activity of the total glycolipid fraction (unpublished). In addition, selected ion monitoring [5] of the reduced sample gave strong evidence for the presence of small amounts of a monofucosyl pentaglycosylceramide. This is probably an Le^a-like structure [3] and is seen as a faint band in fig.1.

The 3 bands in the 6-sugar region of the chromatogram (fig.1) may be explained to originate in the same hexasaccharide bound to a heterogeneous ceramide. Both hydroxy and non-hydroxy fatty acids were shown (fig.4) as well as dihydroxy- and trihydroxy-bases (shown by *m/e* 364 and 396 of fig.2, respectively). This ceramide composition differs from that of erythrocyte ABH fucolipids, which contain only non-hydroxy acids and dihydroxy base [12]. It is known that the weak Le activity of erythrocytes is taken up from plasma [1,3] but Le glycolipids of erythrocytes have not yet been reported.

The present preparation was done on 200 ml plasma equivalent to 1 transfusion unit but the actual analyses were performed on substance from ~50 ml. A number of blood group antigens reported to be present in human plasma [15] may now be chemically characterized from single donors. We have found that already at the stage of thin-layer chromatography in the analysis procedure (fig.1) there are interesting differences in the 5–8-sugar region for glycolipids of donors of separate blood groups (in preparation).

Acknowledgements

We thank Karin Nilson for expert technical assistance, Irmin Pascher for derivatizations, and Lena Sandberg, head of the Regional Blood Bank of Sahlgren's hospital in Göteborg, for valuable help. The work was supported by grants from the Swedish Medical Research Council (no. 3967), the Swedish Natural Science Research Council, Axel and Margaret Ax:son Johnson Foundation, Torsten and Ragnar Söderberg Foundation, Knut and Alice Wallenberg Foundation and the Tercentenary Fund of the Bank of Sweden.

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