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# Comparison of the *N*-glycoloylneuraminic and *N*-acetylneuraminic acid content of platelets and their precursors using high performance anion exchange chromatography

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*N*-Acetylneuraminic acid (Neu5Ac) and *N*-glycoloylneuraminic acid (Neu5Gc) are distributed widely in nature. Using a Carbopac PA-1 anion exchange column, we have determined the ratios of Neu5Ac and Neu5Gc in hydrolysates of platelets and their precursors: a rat promegakaryoblastic (RPM) cell line and a human megakaryoblastic leukemia cell line (MEG-01). The ratio of Neu5Gc:Neu5Ac in cultured RPM cells is 16:1, whereas in platelet rich plasma and cultured MEG-01 cells it is 1:38 and 1:28, respectively. The nature of these sialic acids from RPM cells was verified using thin layer chromatography and liquid secondary ion mass spectrometry. The relevance of increased Neu5Gc levels in early stages of development is discussed.

*Keywords*: *N*-glycoloylneuraminic acid, *N*-acetylneuraminic acid, promegakaryoblast, platelet, high performance anion exchange chromatography.

*Abbreviations*: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid; RPM, rat promegakaryoblast; MEG-01, human megakaryoblastic leukaemia cell line; PAD, pulsed amperometric detection; WGA, wheat germ agglutinin; FCS, foetal calf serum; PPEADP, phosphatidylethanolamine dipalmitoyl; LSIMS, liquid secondary ion mass spectrometry; HPAEC, high performance anion exchange chromatography; TBA, thiobarbituric acid.

The immortal rat bone marrow derived cell line (RPM) is an analogue of the promegakaryoblast, which is the first stage in the sequence of maturation of the bone marrow megakaryocyte [1]. Megakaryocytes are then thought to fragment in the pulmonary circulation to form platelets [2]. Sialic acids play an important role in sialoglyconjugates in platelets. They are involved in functional events such as aggregation and adhesion as well as platelet lifespan [3]. *N*-Acetylneuraminic acid (Neu5Ac) is the simplest and most ubiquitous member of the diverse group of sialic acids that occur in nature; the second most common sialic acid is *N*-glycoloylneuraminic acid (Neu5Gc). Human platelets contain sialic acid exclusively of the Neu5Ac type, whereas platelets from other vertebrate species are known to contain both Neu5Ac and Neu5Gc in varying amounts [4]. The reason for this diversity is unknown, although recent investigations have revealed an increase in Neu5Gc in human and chicken tumours [5]. Other authors have shown sialic acid variation during maturation and development [6].

In this study, we aim to investigate the relative concentrations of Neu5Ac and Neu5Gc in the early stages of rat platelet development, i.e., the promegakaryoblast, and compare this with the equivalent human megakaryocytic cell line (MEG-01) [7] and the more mature rat platelet. Separation of sialic acids was carried out using high performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD).

## Materials and methods

The immortal RPM cell line (from J. Martin, Wellcome) was grown in suspension in 100 mm cell culture dishes in

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Dulbecco's modified Eagles medium containing penicillin ($100\ \mathrm{U\,ml^{-1}}$) and streptomycin ($50\ \mu\mathrm{g\,ml^{-1}}$) and 10% foetal calf serum (FCS) in a humidified cabinet containing 5% $CO_2$. The human MEG-01 cell line (from J. Martin, Wellcome) was seeded in tissue culture flasks in RPM1 medium supplemented with 10% FCS and penicillin G ($100\ \mathrm{U\,ml^{-1}}$) and streptomycin ($50\ \mu\mathrm{g\,ml^{-1}}$) and incubated as above. For experimentation, cells were spun down at 150 × **g** and washed three times in phosphate buffered saline (20 mM $NaH_2PO_4$, 150 mM NaCl, pH 7.4). Rat blood was collected by cardiac puncture into a 10 ml plastic syringe containing 1 ml 3.8% trisodium citrate, and platelet rich plasma (PRP) was prepared by centrifugation [8]. Protein was determined using the Lowry microprotein assay [9]. To liberate sialic acids, cells/platelets (10 mg protein $\mathrm{ml^{-1}}$) were resuspended in 0.05 M $H_2SO_4$ and heated at 100 °C for 1 h [10]. After cooling and centrifugation, the neutralized supernatant was removed and lyophilized ready for analysis. Separation and purification of sialic acids was carried out using a Carbopac PA1 anion exchange column of pellicular resin with pulsed amperometric detection (PAD). The response of the system was calibrated using known concentrations of individual standards of Neu5Ac and Neu5Gc, respectively (obtained from Sigma Chemical Company, Poole, Dorset, UK). The response was linear over the range 0–1 µg sialic acid. Chromatography was carried out under two sets of conditions. 1, A neutral solvent system [11] was employed with PAD following post-column addition of NaOH. Carbopac PA-1 columns (4 × 250 mm) were eluted at 30 °C in the isocratic mode for 5 min with 5 mM sodium acetate followed by a 30 min linear gradient to 50% 5 mM sodium acetate, 50% 5 mM acetic acid, continued by washing with 100% 5 mM acetic acid for 10 min and 100% 5 mM sodium acetate for 15 min. The flow was $1\ \mathrm{ml\,min^{-1}}$ and post-column addition with 300 mM NaOH was $\approx 0.4\ \mathrm{ml\,min^{-1}}$. Typical back pressure was between 1000 and $1200\ \mathrm{lb\,in^{-2}}$. PAD (Gold) settings were $E_1 = 0.05$, $E_2 = 0.6$, $E_3 = -0.6$, $T_1 = 5$, $T_2 = 2$, $T_3 = 1$; response time = 1 s, range = 1000 nA. 2, An isocratic solvent system was used under basic conditions. The Carbopac PA-1 column was eluted at 30 °C in isocratic mode for 20 min using 150 mM sodium acetate and 100 mM NaOH. The column was washed with 500 mM sodium acetate and 100 mM NaOH for 5 min before resuming initial isocratic condition. PAD settings and flow rate were as previously described.

In preparative runs following PAD detection, the sample was passed through a cationic micro membrane suppressor to remove cation contamination from the eluent. Recovered fractions were lyophilized and analysed by a modified thiobarbituric acid (TBA) assay [12].

Standard samples of Neu5Ac and Neu5Gc, taken through the chromatography and cation removal steps, and peak fractions 1 and 2 eluted from the Carbopac PA-1 column (Fig. 1) were converted to phosphatidylethanolamine dipalmitoyl (PPEADP) derivatives by the method previously described for neutral sugars [13]. The PPEADP conjugates were separated on high performance Silica Gel 60 plates (Merck) using the solvent system chloroform–methanol–water, 130:50:9, by vol. The sample bands were located under long-wavelength ultraviolet light following spraying with primulin reagent (0.0001% primulin in a mixture of acetone and water, 4:1 by vol). Bands were cut from the plate and submitted to liquid secondary ion mass spectrometry (LSIMS) as described previously [14].

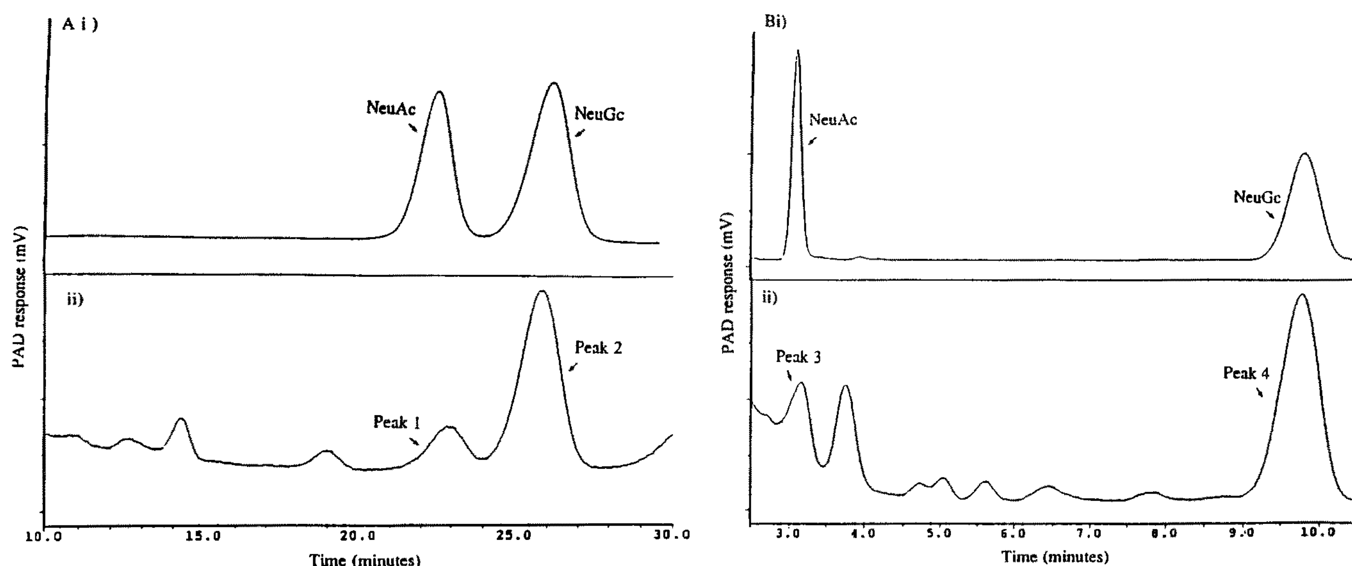


**Figure 1.** Elution profile of standard sialic acids, Neu5Ac and Neu5Gc (500 ng each in 10 µl) separated using a neutral system (Ai) and an isocratic system (Bi) on a Carbopac PA1 column with PAD. Elution profile of RPM hydrolysate separated using a neutral (Aii) or isocratic (Bii) system as above. Peaks 1 and 3 compare with Neu5Ac standard and peaks 2 and 4 compare with the Neu5Gc standard, respectively.

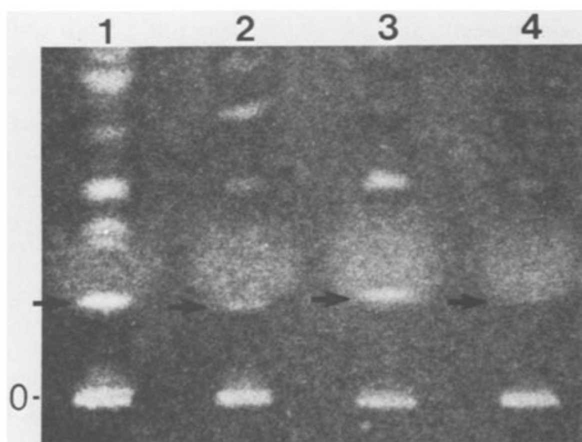


**Figure 2.** Thin layer chromatogram of PPEADP derivatives of fractions isolated by HPLC on Carbopac PA1 column from RPM hydrolysate peaks 1 and 2, see Fig. 1 (lanes 1 and 2, respectively) and from standard samples of Neu5Ac (lane 3) and Neu5Gc (lane 4). 0 indicates the origin and arrows indicate the bands taken for TLC-LSIMS analysis. The band in lane 4 was weak and its UV absorption was partially suppressed by co-eluting salt in this region, although this did not affect the LSIMS detection.

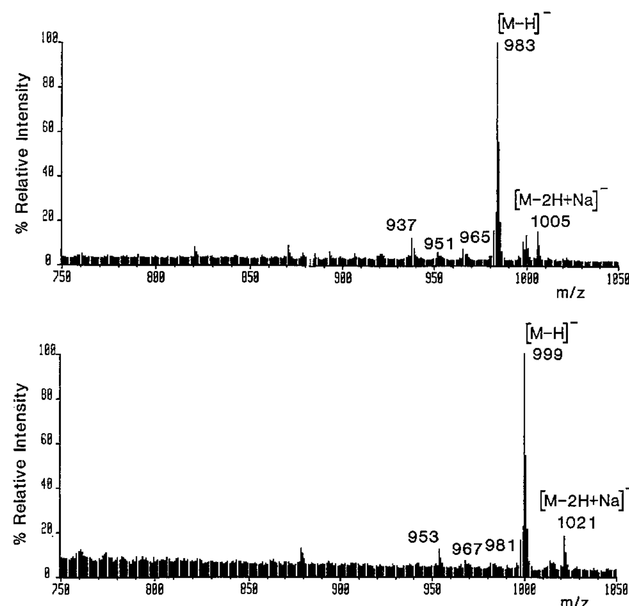


**Figure 3.** Negative ion spectra obtained by direct TLC-LSIMS of PPEADP derivatives prepared of RPM hydrolysate peak fractions 1 (upper) and 2 (lower).

## Results

Figure 1Ai shows the chromatographic separation of Neu5Ac and Neu5Gc standards with retention times of 23 and 26.5 min, respectively, using the neutral solvent system. Analysis of RPM hydrolysate (Fig. 1Aii) indicates two peaks (1 and 2) which elute at positions very close to those of the above standards. Occasionally after repeated use of the column the separation of Neu5Ac and Neu5Gc was less good. Re-equilibration of the column overnight restored the separation achieved in Fig. 1Ai. Separation using the isocratic basic solvent system gives retention times of 3.1 and 9.8 min for Neu5Ac and Neu5Gc standards (Fig. 1Bi). Peaks 3 and 4 from the RPM hydrolysate elute at similar retention times as standards (Fig. 1Bii).

Peaks 1 and 2 were collected for analysis following passages through the micro membrane suppressor and proved positive for sialic acid with the modified TBA assay.

The TLC of the PPEADP derivatives of Neu5Ac and Neu5Gc and peak fractions 1 and 2 are shown in Fig. 2. The relative mobility of Neu5Ac-PPEADP was slightly faster than that of Neu5Gc-PPEADP with the peak 1 derivative matching the mobility of Neu5Ac and the peak 2 derivative matching Neu5Gc. Other bands present in Fig. 2 arose from the presence of reagent-related components (top 3 bands) and from what appeared to be sialic acid degradation products (next 3 bands). The fainter blurr in front of the arrowed bands was due primarily to the presence of reagent-related salts which partially suppressed the UV absorbance of the Neu5Ac and Neu5Gc bands but did not affect MS detection. The LSIMS of Neu5Ac-PPEADP and Neu5Gc-PPEADP gave intense $[M-H]^-$ ions at *m/z* 983 and *m/z* 999, respectively, together with sodium attachment ions at *m/z* 1005 and *m/z* 1021, respectively. Both spectra showed typical carboxylic acid-related fragmentation in this case by fragment ions for $[M-18]^-$, $[M-32]^-$ and $[M-46]^-$. The mass spectra obtained from peaks 1 and 2 of the RPM hydrolysate are shown in Fig. 3 and were identical with the spectra from respective standards Neu5Ac- and Neu5Gc-PPEADP.

From calibration with the respective standards using HPAEC, the RPM hydrolysate was shown to contain $0.45 \pm 0.02$ µg Neu5Ac per mg protein and $7.25 \pm 0.5$ µg Neu5Gc per mg protein. Analytical PAD detection proved very reliable and sensitive, detecting sialic acid levels as low as 100 pmol. Recovery of sialic acids from preparative runs was tested with standards and was generally between 70 and 95% with the major loss occurring post-PAD detection in the micro membrane cation suppressor. Although the recovery of Neu5Gc tended to be lower than that of Neu5Ac (see Fig. 2) the TLC-LSIMS of the former was satisfactory.

Figure 4Aii shows separation of hydrolysates from rat platelet rich plasma (ii) and MEG-01 (iii) using the neutral solvent system. The peaks which elute in positions corresponding to Neu5Ac and Neu5Gc standards are arrowed accordingly. Figure 4B was obtained using the isocratic solvent system which again shows peaks directly comparable with the Neu5Ac standards (arrowed) and also approximating to the Neu5Gc position. From analytical PAD detection Neu5Ac and putative Neu5Gc were found in the ratio 28:1 in MEG-01 and in the ratio 38:1 in PRP.

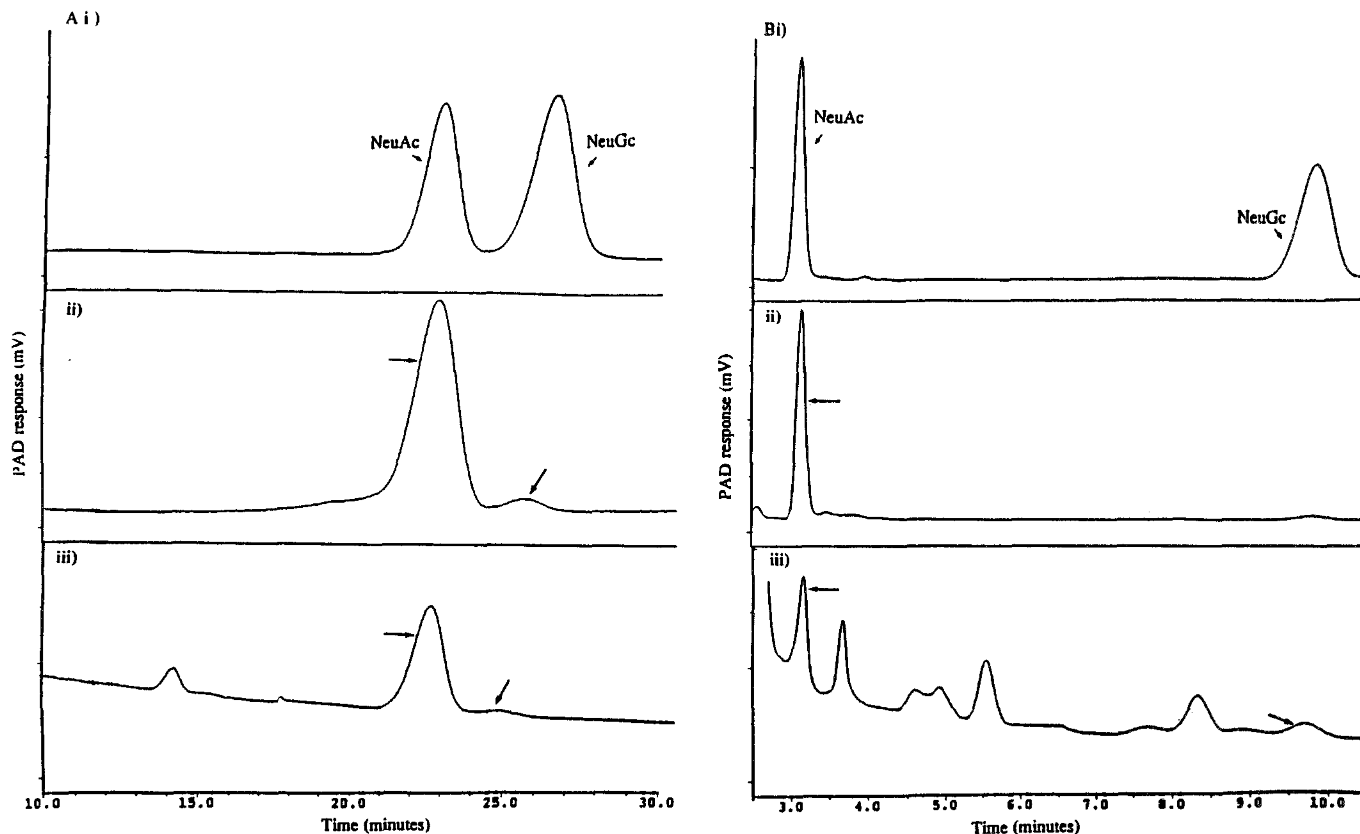


**Figure 4.** Elution profile of standard sialic acids, Neu5Ac and Neu5Gc separated using a neutral (Ai) and an isocratic (Bi) system on a Carbopac PA1 column with PAD. Elution profiles of PRP hydrolysate separated using a neutral (Aii) and an isocratic (Bii) system and MEG-01 hydrolysate separated using a neutral (Aiii) and an isocratic (Biii) system are shown. Peaks compared with sialic acid standards are arrowed.

## Discussion

Neu5Gc frequently occurs in oligosaccharides and glycoconjugates in many vertebrates and a high proportion has been found in platelet membranes from pig, horse, donkey and mule. Lamb platelets contain Neu5Ac:Neu5Gc in the ratio 1:1 [15]. It is also of relevance that in the foetal calf serum glycoprotein fetuin, Neu5Gc accounts for 7% of total sialic acid [16]. Certain species such as human and chicken lack Neu5Gc in normal tissues. Recently, investigations have revealed trace amounts of Neu5Gc in ganglioside rich fractions from human cancerous tissues (0.02–0.5% of total sialic acid) and avian lymphoma cell lines contained Neu5Gc ranging from 0.03–0.11% of total sialic acid, suggesting that the presence of Neu5Gc is associated with oncogenesis [5].

In this study we have used two different HPAEC techniques to separate Neu5Ac and Neu5Gc in cultured RPM cells. Their separation and quantitation by both a basic isocratic and neutral solvent system gave consistent ratios of Neu5Ac:Neu5Gc, although the neutral system gave greater separation of the two compounds. The sialic acids from RPM cells were characterized by TLC–LSIMS as being Neu5Ac and Neu5Gc, respectively. Neu5Gc was shown to comprise the majority of sialic acid released from cultured RPM cells which at $7.25 \pm 0.5$ µg per g protein represented 94% of the total.

The possibility that the Neu5Gc is derived from the FCS in the growth medium of these cells is unlikely. First, in other experiments, SDS-PAGE of RPM cells cultured and recovered as described here failed to show the presence of FCS proteins (data not shown). This is supported by the marked differences reported here in the ratio of Neu5Ac to Neu5Gc between RPM and MEG-01 cells, both of which were grown in the presence of FCS.

It is interesting that such a high content of Neu5Gc is found in the immature stage of rat platelet development, although RPM is a transformed cell line from Long Evans rat bone marrow, and an increase in tumorgenicity as noted in continuous cell lines may account for the high proportion of Neu5Gc. The mature platelet in the same rat species, however, contained mainly Neu5Ac. Correspondingly, the sialic acid content of the similar cell line to RPM from human promegakaryoblasts MEG-01 also consisted of a majority of Neu5Ac as shown by both chromatographic techniques. In this case a maximum of only 3% of the sialic acid was Neu5Gc. Unfortunately, insufficient Neu5Gc was recovered from platelet and MEG-01 cells to carry out

TLC-LSIMS. The chromatographic identification of Neu5Gc in these cells must therefore be viewed as provisional.

From studies with WGA, the guinea-pig megakaryocyte exhibited cell surface changes in sialoglycoproteins during maturation; WGA labelling increasing with maturation and ploidy [6]. The affinity of WGA to Neu5Gc is much lower than to Neu5Ac [17]. Therefore it is possible that the low levels of WGA binding in the early stages of development are associated not with the loss of sialic acid but with a higher ratio of Neu5Gc to Neu5Ac. The Neu5Gc could therefore be of developmental significance in rat species.

The expression of Neu5Gc and Neu5Ac levels is thought to be due to the relative amounts of CMPNeu5Gc and CMPNeu5Ac available to the sialyltransferases within the Golgi, which is in turn due to the activity of Neu5Ac hydroxylase [18]. An increase in activity of this enzyme would increase Neu5Gc levels.

Clearly the expression and role of Neu5Gc is of significance in biological processes. The techniques demonstrated will be important in qualitatively and quantitatively determining Neu5Ac and Neu5Gc in biological material.

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