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Glycosylation study of the major genetic variants of human α_1-acid glycoprotein and of their pharmacokinetics in the rat

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Abstract

Human α_1-acid glycoprotein (AAG) is a mixture of at least two genetic variants, the A variant and the F1 and/or S variant or variants, which are encoded by two different genes. AAG is also an extensively glycosylated protein which possesses five N-linked glycans exhibiting substantial heterogeneity in their structures. The first objective of this study was to investigate the glycosylation of the two major gene products of AAG, i.e. the A variant and a mixture of the F1 and S variants (F1*S). To this end, we combined a chromatographic method for the fractionation of the AAG variants with a lectin-binding assay to characterise the glycosylation of purified glycoproteins. Secondly, because the oligosaccharides can influence the disposition of AAG, a kinetic study of the AAG variants was carried out in the rat. After intravenous administration of whole human AAG, the separation and quantification of the AAG variants in plasma was performed by application of specific methods by isoelectric focusing and immunonephelometry. The binding studies carried out on a panel of lectins showed significant differences in the lectin-binding characteristics of the separated F1*S and A variants, accounting for differences in the degree of branching of their glycan chains and substitution with sialic acid and fucose. The plasma concentration-time profiles of the F1*S and A variants were biphasic, and only small differences were observed between the variants for their initial and terminal half-lives, clearance and distribution volume. This indicates that the structural differences between the two AAG gene products do not affect their pharmacokinetics in the rat. Specific drug transport roles have been previously demonstrated for the F1*S and A variants, calling for further investigations into their effects on the disposition of drugs they bind in plasma. The present study shows that such investigations are possible without being complicated by kinetic differences between these variants.
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1. Introduction

Many drugs are bound, principally or in part, by the major plasma protein α_1-acid glycoprotein (AAG), and AAG is the principal carrier of basic drugs in plasma [1]. The extent of this binding can modify the distribution of drugs into tissues and their pharmacokinetic patterns [2]. AAG exists as a mixture of two or three genetic variants, the A variant and the F1 and/or S variant or variants, in the plasma of most individuals [3,4]. This polymorphism is the result of the existence of two different genes coding for the protein [5], of which the *AAG-A* gene encodes the F1 and S variants, and the *AAG-B*/*B′* gene encodes the A variant [6]. The two genes are structurally similar but differ by many base substitutions. Consequently, the A variant differs from the F1 and S variants by about 20 residues in the amino acid sequence, among a total of 181 residues [5,7]. F1 and S which are encoded by two alleles of *AAG-A*, are reported to differ by only one (1) amino acid residue [8]. We and others have shown that this polymorphism is important to consider when carrying out drug binding studies with AAG, as some drugs apparently bind similarly to each of the variants, while others bind predominantly, even exclusively to either the A variant or the F1 and S variants [9–12]. The

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functional heterogeneity between the AAG variants calls for further investigation into their effects on drug tissue transfer. Knowledge of the pharmacokinetics of these variants is, however, a prerequisite for such studies. In this respect, AAG is a heavily glycosylated protein, possessing five *N*-linked glycans which exhibit substantial heterogeneity in their structures [7,13]. The carbohydrate part is of importance for the in vivo circulatory life time of AAG [14,15]; it may also influence the AAG distribution into cells and tissues through its binding to receptors [16–18]. There is little known on the glycosylation of AAG in connection with its genetic expression. Nevertheless, it is possible that the amino acid substitutions between the variants encoded by *AAG-A* and *AAG-B*/*B′* may influence their glycosylation. Differences in glycosylation between these variants may produce kinetic differences which, in turn, may have implications for the disposition of the drugs they bind in plasma.

Therefore, the two main objectives of this work were to gain insight into the glycosylation of the AAG variants and study their pharmacokinetics in rats. A commercial human AAG was used as the protein source for these purposes. The combination of various methods used previously to fractionate and identify the AAG variants, and to characterise the glycosylation of purified glycoproteins, offered the potential to undertake these studies.

2. Experimental

2.1. Animals and reagents

Male Sprague-Dawley rats (OFA strain 230–250 g; 8 weeks) were obtained from Charles Rivers (L'Arbresle, France).

The following materials were used : purified human AAG (from Cohn fraction VI; lots 13H9336 and 125H9329), streptavidin-alkaline phosphatase conjugate and goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO); rabbit anti-(human AAG) immunoglobulins (Dako, Trappes, France); rabbit anti-(whole human plasma) antiserum (Behring, Marburg, Germany); *Clostridium perfringens* neuraminidase (Roche Diagnostics, Meylan, France); biotin-labelled lectins (Vector, Burlingame, CA); chelating (iminodiacetic acid linked) Sepharose Fast Flow Gel and Immobiline polyacrylamide gels (0.5 mm thick) in the pH range 4.5–5.4 (Amersham Biosciences, Orsay, France); nitrocellulose membrane (0.45 μm) (Sartorius, Göttingen, Germany); multiwell plastic plates (Maxisorp F16) (Nunc, Roskilde, Denmark). All other chemicals were of reagent grade and were purchased from Sigma.

2.2. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoelectrophoresis

These were carried out according to standard procedures, using 12% polyacrylamide gels and 1.5% agarose gels, respectively. Gels were stained with Coomassie Blue R-250 after electrophoresis.

2.3. Separation of the AAG variants by chromatography on immobilized copper(II) ions

The method used was the one we previously developed [19]. Briefly, chromatography was performed at room temperature (20–23 °C) using a column of iminodiacetic acid-Sepharose gel loaded with copper(II) ions (2.5 cm i.d., bed volume 80 ml) and equilibrated in 20 mM sodium phosphate/0.5 M NaCl, pH 7.0 (equilibration buffer E). AAG (40 mg in 3 ml buffer E) was loaded onto the column, and was then eluted at a constant flow rate of 40 ml/h. Fractions of 3.5 ml were collected. Their optical densities were measured at 280 nm. After elution of the non-bound component using buffer E, a second buffer (pH 7.0) consisting of 20 mM imidazole in buffer E, was applied to elute the bound component. The appropriate peak fractions were collected, concentrated on YM10 membrane filters (Amicon, Danvers, MA), then dialyzed against deionized water and finally lyophilized. The column was reconditioned between runs by removing chelated copper with EDTA, re-loading it with a fresh solution of copper(II) ions [19], and repeating the equilibration procedure.

2.4. Lectin binding studies and evaluation of the binding data

The glycosylation of AAG and its separated variants was characterised through lectin binding studies, using a multiwell plate technique [20]. Incubation times, temperatures and washing schedules were the same as those described in ref. [20]. Briefly, the wells in a microtiter plate were coated with different concentrations of each protein prepared in Tris-buffered saline (25 mM Tris-HCl/100 mM NaCl, pH 7.5; TBS). After removing non-bound protein, uncoated sites on the plate were blocked using 0.1% (v/v) Tween-20 in TBS (Tween-TBS). A biotinylated lectin was then added (0.2 or 0.3 μg per well) and allowed to interact with the carbohydrate on the adsorbed protein. The lectin was prepared in 25 mM Tris-HCl/1 mM $CaCl_2$/1 mM $MgCl_2$/1 mM $MnCl_2$/0.1% (v/v) Tween-20, pH 7.5. The non-bound lectin was removed and the amount of bound lectin was then measured by adding a streptavidin-alkaline phosphatase conjugate prepared in Tween-TBS (0.15 μg per well). The bound enzyme was used to develop a colour reaction by adding *p*-nitrophenyl phosphate (100 μg per well) prepared in a 0.1 M 2-amino-2-methyl-1-propanol buffer, pH 10.3. The enzyme reaction was stopped with 1 M NaOH (100 μl per well). Plates were read at 405 nm on a Titertek Multiscan MC/340 (McLean, VA).

The relationship between the amount of protein used for coating the wells, *Q*, and the absorbance of sample, *A*, was analysed with Triomphe software [21] using standard regression for a linear model corresponding to the following

equation:

$$A = S \times Q + A_0 \quad (1)$$

where S denotes the slope of the dilution curve and A_0 the y-intercept. The parameters S and A_0 were estimated with their standard errors.

Analysis of covariance [22] was used to both evaluate the intraday and interday variabilities of the S parameter for each combination of protein and lectin tested, and study the variation in the S parameter between the different proteins toward each lectin (statistical significance was set at the $P < 0.05$ level).

2.5. Lectin binding inhibition studies and determination of the inhibitory potencies (IC_{50}) of inhibitory sugars

The specificity of each lectin was checked by assaying AAG and its separated variants (used at a constant concentration) in the presence of increasing concentrations of an inhibitory sugar. Analysis of covariance [22] was used to evaluate the intraday and interday variabilities of the inhibition data.

The relationship between the binding percentage of the lectin and the concentration of inhibitory sugar was modeled with Eq. (2):

$$\text{binding}\,\% = \frac{A}{A_{\max}} = \frac{\text{IC}_{50}}{(\text{IC}_{50} + I_t)} \quad (2)$$

where A is the absorbance measured for each concentration of inhibitor, I_t, and $A_{\max}$ the maximum absorbance measured in the absence of inhibitor (i.e. $A = A_{\max}$ when $I_t = 0$). IC_{50} is the theoretical molar concentration of inhibitor producing a 50% decrease in $A_{\max}$. The parameter IC_{50} was estimated with its standard error by non-linear regression analysis using MicroPharm© software (Version 4.0; Institut National de la Recherche Scientifique et Médicale (INSERM), Paris, France) [23].

2.6. Plasma kinetics of AAG and its variants in the rat and pharmacokinetic determinations

Prior to treatment, the animals were maintained under standard conditions of temperature and lighting for 6 days with ad libitum access to food and water. The ethical rules of the French Ministry of Agriculture for experimentation with laboratory animals (law no. 87-848) were followed. Twenty seven rats were divided into nine groups of three rats, one group per sampling time. They all received a bolus dose of human AAG (50 mg/kg) as a 20 mg/ml solution in isotonic saline by tail vein injection, on the same day. They were treated after a hydric diet of 21 h, which was maintained 8 h after AAG administration. Blood was taken from anaesthetized rats by carotid bleeding at 5, 15 and 30 min and 1, 4, 8, 24, 30 and 48 h after AAG administration, then collected into glass tubes containing lithium heparinate, aprotinin and phenylmethyl sulfonyl fluoride, and finally centrifuged at 2000 × g for 10 min and at 5 °C. Harvested plasma samples were stored at −20 to −25 °C until analysis of the concentrations of AAG and variants.

The concentration (C)-time (t) data were fitted to a biexponential equation:

$$C = A \times \mathrm{e}^{-\alpha t} + B \times \mathrm{e}^{-\beta t} \quad (3)$$

using MicroPharm© [23]. The constants A and B (zero-time intercepts), α (distribution rate constant) and β (rate constant of the terminal elimination phase) were estimated with their standard errors.

The distribution half-life ($T_{1/2,\alpha}$), terminal half-life ($T_{1/2,\beta}$), total plasma clearance (CL), central distribution volume (V_c), steady state volume of distribution (V_{ss}) and the pseudo-equilibrium β phase volume of distribution (V_β) were calculated from the following equations:

$$T_{1/2,\alpha} = \frac{0.693}{\alpha} \quad (4)$$

$$T_{1/2,\beta} = \frac{0.693}{\beta} \quad (5)$$

$$\text{CL} = \frac{\text{dose}}{\text{AUC}_{0-\infty}} \quad (6)$$

$$V_c = \frac{\text{dose}}{A + B} \quad (7)$$

$$V_{ss} = \text{CL} \times \text{MRT} \quad (8)$$

$$V_\beta = \frac{\text{CL}}{\beta} \quad (9)$$

where $\text{AUC}_{0-\infty}$ is the area under concentration-time curve from zero to infinity and MRT the mean residence time.

2.7. Isoelectric focusing (IEF)

The genetic variants of AAG were determined by IEF in immobilized pH gradient (IPG) gels, as previously described [24]. The IEF-IPG gels (pH 4.5–5.4) were supplemented with 8 M urea, 2% (v/v) 2-mercaptoethanol and 8.7% glycerol. Samples applied to the gel were first desialylated with neuraminidase. Briefly, 20 μl neuraminidase solution (1 U/ml) in 5 mM sodium acetate/15.4 mM NaCl/0.9 mM $CaCl_2$, pH 5.5, were added to 50 μl AAG or variant samples (∼1.2 mg/ml in acetate buffer) or to 5 μl plasma samples, and incubated for 24 h at 37 °C. Samples (20 μl) were applied on the gel using filter paper tabs. The desialylated plasma samples were analysed after dilution to avoid an excess of antigen [24]. IEF was run with a LKB 2117 Multiphor II electrophoresis apparatus equipped with a 2297 Macrodrive 5 constant-power supply. The gels were run first at 5 W, 5 mA and 500 V for 1 h, then the papers were removed and the gels were run overnight at 5 W, 5 mA and 2000 V. The gels were either stained with Coomassie Blue R-250 or

probed, after blotting onto nitrocellulose membrane, with a primary (rabbit anti-human AAG) antibody, and with the use of a secondary (goat anti-rabbit) antibody labelled with alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium for detection. The gel and membrane were scanned with a LKB 2202 Ultroscan laser densitometer. Each track was determined in three positions. The relative scanned proportions of the variants in each plasma were multiplied by the total AAG level in corresponding plasma to obtain the relative concentrations of the variants.

2.8. AAG concentration of all samples

The specific assay of AAG was carried out by an immunonephelometric method, with a nephelometer analyser (Model 7571 ARRAY TM Protein System) and standards from Beckman Instruments (Fullerton, CA). The anti-AAG antibody was the same used for the immunoblotting procedure. The assay did not cross react with rat plasma. All plasma samples were assayed in duplicate. The assay sensitivity was 0.05 mg/ml (number of determinations, $n = 6$). The coefficients of variation at 0.05, 0.25 and 1 mg/ml were 11.8, 2.6 and 2.3%, respectively, within days ($n = 6$ per concentration), and were 11.8, 3.6 and 2.9% between days ($n = 4$ per concentration). The accuracy of the assay was −17.3% at 0.05 mg/ml, +10.2% at 0.25 mg/ml, and +2.7% at 1 mg/ml.

3. Results

3.1. Purity of AAG

The commercial AAG samples used showed only one protein band corresponding to the molecular mass of AAG (ca. 40 000) on SDS–PAGE (results not shown). Immunoelectrophoresis confirmed their purity. Each sample showed a single arc-like immunoprecipitate when a rabbit antiserum to whole human plasma was used (results not shown).

3.2. Separation of the AAG variants and identification

After affinity chromatography on immobilized copper(II) ions (Fig. 1A), the recovery of AAG measured from the comparison between the absorbance values in the starting material and the sum of each fraction was quantitative (>95%), with ∼75% eluted in the non-bound fraction and ∼25% in the bound fraction. IEF-IPG analysis, after desialylation of the proteins, showed that the non-bound fraction consisted of a mixture of the F1 and S variants, and the bound fraction of the A variant in a pure form (Fig. 1B). There was no apparent degradation of the variants which showed an isoelectric point (p*I*) identical to that of the starting material (Fig. 1B). The relative scanned proportions of variants F1 and S in the F1*S fraction were ∼60 and ∼40%. Quantification by IEF-IPG and immunonephelometry of the respective amount of the separated variants indicated that each was present after separation in the same ratio as in the starting material, i.e. the relative scanned proportions of F1, S and A in AAG-lot 13H9336 were 45, 28 and 27%, respectively, and 42, 31 and 27% in lot 125H9329.

3.3. Lectin binding studies

When, in preliminary experiments, the separated F1*S and A variants and (unfractionated) AAG were assayed for their adsorption onto the multiwell plates using a two-antibody technique for quantification (the antibodies were the same used for the immunoblotting procedure), only small and non significant differences in the amount of adsorbed protein were observed between these samples ($P > 0.05$). Therefore, any lectin-binding difference found between them must be ascribed to different amounts of the carbohydrate groupings under investigation.

Some dilution curves for the separated F1*S and A variants and corresponding AAG (lot 13H9336) measured by the lectin-binding assay are presented in Fig. 2. Table 1 shows the parameters of the curves, the slope indicating the reactivity of each protein for the tested lectin. A good linear relationship was always observed between the amount of glycoprotein and the colour intensity ($r \geq 0.98$; Table 1). The intraday variability of the *S* parameter was between 2.3 and 5.1%, and the interday variability between 6.4 and 27.4% (Table 2). It is important to point out that the percentages presented take into account the variability of the binding data for all tested concentrations of protein.

The A variant had a higher reactivity than the F1*S variants for both *Aleuria aurantia* (AAL) (Fig. 2A) and *Ricinus communis* (RCA-I). This was also true for Concanavalin A (Con A) (Table 1). The difference in Con A binding between the variants was confirmed in other experiments after treating them with neuraminidase. Although the reactivity of each variant for Con A was increased after desialylation, that of the A variant was still higher (Table 1). The results of the binding study of *Maackia amurensis* (MAL) (Fig. 2B) showed, however, that higher amounts of this lectin were bound by the F1*S variants than the A variant (Table 1). Data for AAL (Fig. 2A), RCA-I, Con A and MAL (Fig. 2B) binding to commercial AAG were consistent with the preference of these lectins for binding to either of the variants, with respect to their proportions in the unfractionated protein (i.e. 27% A and 73% F1*S). Finally, both variants had similar reactivities for *Sambucus nigra* (SNA) (Fig. 2C) and these results were supported by the binding data of this lectin to unfractionated AAG (Table 1; Fig. 2C). It is important to emphasize that we came up with similar binding results using other samples of commercial AAG (lot 125H9329) and corresponding F1*S and A variants in assays with AAL, Con A, MAL and SNA.

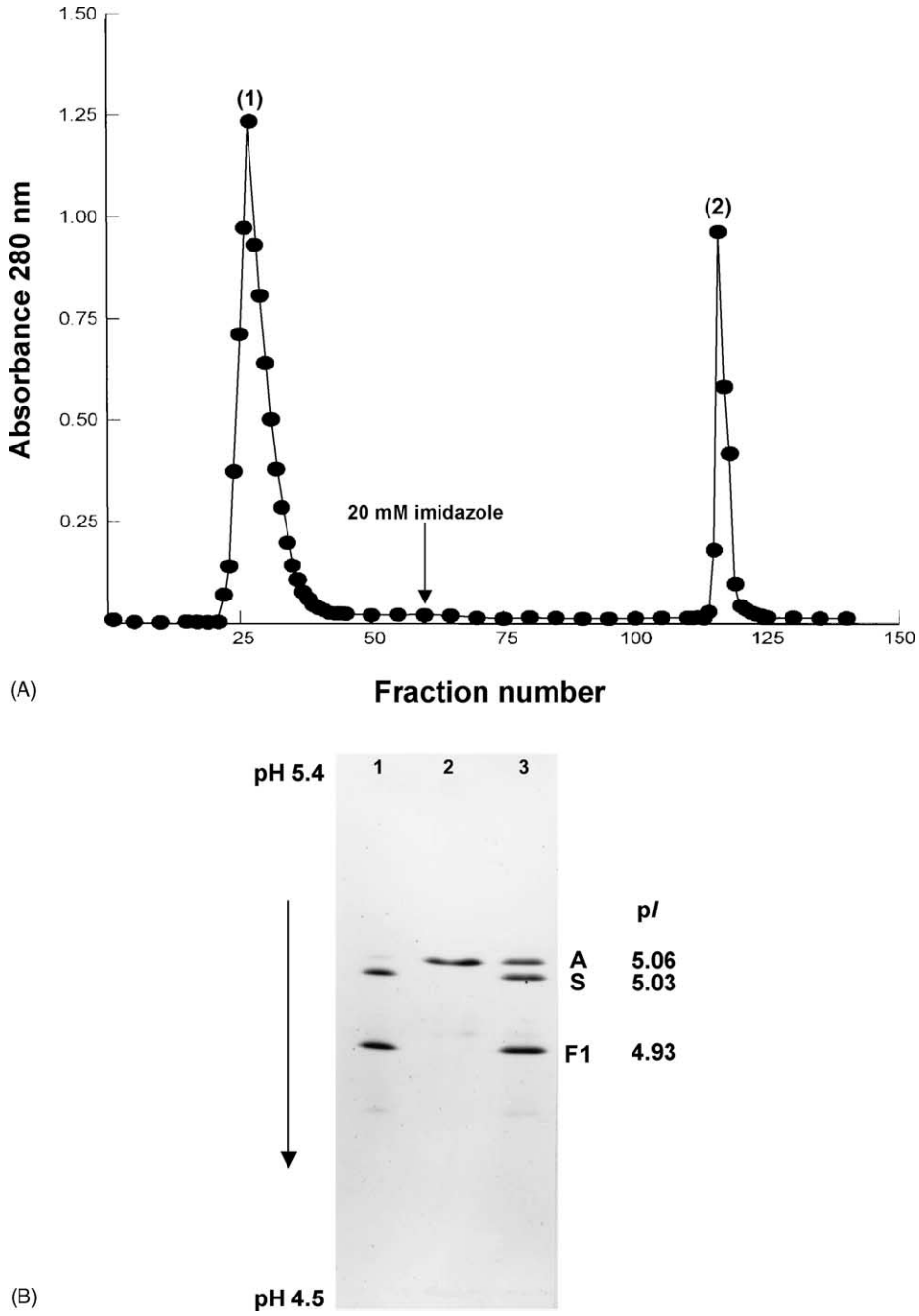

Fig. 1. Chromatographic separation of the AAG variants (A) and identification of the separated variants by isoelectric focusing (B). In (A), elution profile (absorbance 280 nm) of commercial AAG (~40 mg) applied to a copper-iminodiacetate column (i.d. 2.5 cm; bed volume, 80 ml). Following sample application, the column, equilibrated with 20 mM sodium phosphate/0.5 M NaCl, pH 7.0, was developed with the same buffer. After elution of the non-bound fraction (peak 1), imidazole (20 mM) was introduced in the buffer (indicated by an arrow) to elute the bound fraction (peak 2). The flow rate of the buffers was 40 ml/h. Fractions of 3.5 ml were collected. In (B), isoelectric focusing analysis of the AAG fractions separated by affinity chromatography on immobilised copper(II) ions as indicated above. Following desialylation, aliquots from whole AAG and the fractions separated were run on an IPG gel (pH 4.5–5.4). After completion of the run, the gel was stained with Coomassie Blue R-250. Tracks 1 and 2, the variants from peak 1 (i.e. variants F1 and S, 20 μg) and peak 2 (i.e. variant A, 20 μg). Track 3, commercial AAG (20 μg) used for the separation. The relative proportion of each AAG variant was determined by laser densitometry.

3.4. Lectin inhibition studies

Examples of the inhibition of lectin binding to the separated variants and to whole AAG by addition of an inhibitory sugar are illustrated in Fig. 3. The intraday and interday variabilities of the inhibition assays are presented in Table 2. We point out once again that these values take into account the variability of the inhibition data for all tested

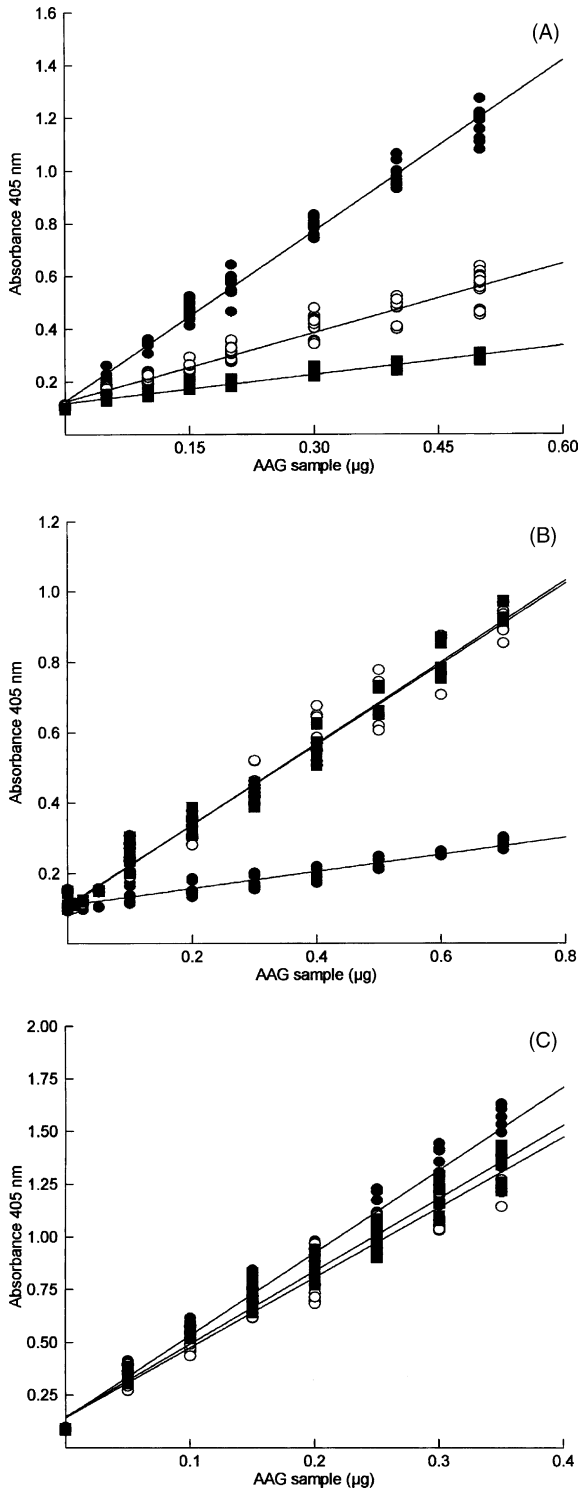

Fig. 2. Dilution curves for the F1*S variants (■), the A variant (●) and (unfractionated) commercial AAG (○) measured by the lectin binding assay using the biotinylated lectins AAL (A), MAL (B) and SNA (C). The data of three quadruplate binding experiments are shown. The solid lines are computer-estimated values. The parameters of the dilution curves are presented in Table 1, and the results for the variability of the binding assays in Table 2. All other details are described in the experimental section and the legend to Table 1.

concentrations of inhibitory sugar. Table 3 shows the IC_{50} values of the (free) sugars used as inhibitors estimated from the inhibition curves.

Higher concentrations of the sugars were needed to inhibit the binding of AAL, Con A (Fig. 3) and RCA-I to the A variant relative to the F1*S variants (Table 3). These results were consistent with the larger amounts of these lectins bound to the A variant (Table 1). When lactose was used to inhibit SNA binding, we found similar inhibitory potencies for F1*S and A (Table 3). This concurred with the variant binding reactivities for SNA (Table 1). However, lactose did not completely inhibit the binding of MAL in each AAG variant assayed (i.e. using 200 mM lactose, the binding was reduced by 45% or less), and no IC_{50} values could be calculated. This should, perhaps, not be surprising since lactose has a low potency to inhibit MAL agglutination of red blood cells [25], and to displace bound oligosaccharides from MAL columns [26]. Data for the binding inhibition of the same series of lectins to unfractionated AAG (Table 3; Fig. 3) agreed with those for the separated variants.

3.5. Plasma kinetics of the AAG variants in normal adult rats

Fig. 4 shows the results for the AAG patterns obtained over a 48 h period in rats treated with human AAG (lot 13H9336), after IEF-IPG analysis of the plasma which was followed by immunoblotting (tracks 2-10). Each rat plasma showed three main AAG bands, which were identified as the F1, S and A variants by running control human plasma (track 11 in Fig. 4). No other AAG bands than those produced by the addition of fresh AAG to control rat plasma (track 1 in Fig. 4) were detected. Thus, it is unlikely that metabolic products of AAG altered the estimation of the pharmacokinetic parameters in the current study.

The plasma concentration-time profiles of AAG and its variants in treated rats are shown in Fig. 5. The estimates of the pharmacokinetic parameters are presented in Table 4. As the analyses of the concentration time data obtained for the F1 variant and the S variant yielded very similar results, only those obtained for both variants (i.e. F1*S) are presented.

The peak concentration of human AAG in this study (1.04 ± 0.07 mg/ml; $n = 3$) was at the upper limit of the endogenous AAG concentration observed in rats (from 0.10 to 0.80 mg/ml) [27]. The average maximum plasma concentration was 0.77 ± 0.08 mg/ml for the F1*S variants and 0.27 ± 0.02 mg/ml for the A variant. The concentration-time data obtained for AAG and its variants were correctly described by a bi-exponential equation (Fig. 5), the adequacy of this model being supported by small standard errors affecting the constants A, B, α and β (Table 4). The standard errors for the other parameters were not estimated, as will be discussed below. After an initial phase of distribution of 1.5 h, the terminal half-life of AAG was 16.9 h, the slow decline of this protein in blood being the consequence of its low clearance (4.7 ml/h/kg). AAG also showed

Table 1
Parameters of the dilution curves for the separated F1*S and A variants and (unfractionated) AAG measured by the lectin-binding assay using the series of tested lectins

Lectins	A variant			F1*S variants			Commercial AAG			Variation in the S parameter		
	S	A_0	r	S	A_0	r	S	A_0	r	A vs. F1*S	A vs. AAG	F1*S vs. AAG
AAL	2.15 ± 0.02	0.13 ± 0.01	0.99	0.36 ± 0.01	0.12 ± 0.01	0.98	0.87 ± 0.02	0.12 ± 0.01	0.98	**	**	**
Con A	2.22 ± 0.03	0.17 ± 0.01	0.99	0.78 ± 0.02	0.16 ± 0.01	0.98	1.33 ± 0.02	0.16 ± 0.01	0.99	**	**	**
Con A[a]	6.38 ± 0.09	0.16 ± 0.01	0.99	2.69 ± 0.08	0.17 ± 0.01	0.98	4.44 ± 0.12	0.15 ± 0.01	0.98	**	**	**
MAL	0.24 ± 0.01	0.11 ± 0.01	0.98	1.15 ± 0.02	0.11 ± 0.01	0.99	1.14 ± 0.02	0.11 ± 0.01	0.98	**	**	NS
RCA-I	1.37 ± 0.02	0.09 ± 0.01	0.99	0.25 ± 0.01	0.09 ± 0.01	0.98	0.50 ± 0.01	0.08 ± 0.01	0.98	**	**	**
SNA	3.91 ± 0.07	0.14 ± 0.01	0.99	3.45 ± 0.06	0.15 ± 0.01	0.99	3.33 ± 0.08	0.14 ± 0.02	0.98	*	*	NS

The various amounts of each protein used for coating the wells ranged between 0.05 and 0.5 μg (AAL), 0.1 and 0.7 μg (MAL), 0.05 and 0.35 μg (SNA), 0.05 and 0.5 μg (Con A), or between 0.1 and 0.7 μg (RCA-I). The amounts of each desialylated protein ranged between 0.03 and 0.15 μg (Con A). The amount of Con A, MAL, SNA, or RCA-I added to each well was 0.2 μg, and that of AAL 0.3 μg. The colour reaction was developed for 25 min (AAL), 120 min (MAL), 10 min (SNA), 25 min (Con A), or for 60 min (RCA-I). The slope, S, and y-intercept, A_0, of each dilution curve were calculated from the results of three quadruplate experiments ($79 \leq n \leq 93$), and are given with their standard errors. S is expressed in absorbance units per μg of protein, and A_0 in absorbance units. The A_0 value calculated for each curve was very similar to the absorbance value measured without protein but with all other reagents (results not shown). r is the coefficient of correlation between the amount of protein and the absorbance.

[a] The results correspond to those of the desialylated proteins.

The variation in the S parameter between the different proteins was studied by analysis of covariance with the type of protein as the covariable: NS, non significant differences; $^{*}P < 0.01$; $^{**}P < 0.001$.

Table 2
Intraday and interday variabilities of the binding and inhibition assays using the series of tested lectins

Lectins	Variability of the binding assays						Variability of the inhibition assays					
	A variant		F1*S variants		Commercial AAG		A variant		F1*S variants		Commercial AAG	
	Intraday (%)	Interday (%)	Intraday (%)	Interday (%)	Intraday (%)	Interday (%)	Intraday (%)	Interday (%)	Intraday (%)	Interday (%)	Intraday (%)	Interday (%)
AAL	4.3	7.7	2.3	9.8	3.5	19.5	6.3	12.4	7.4	34.4	5.0	36.1
Con A	5.1	6.4	4.0	12.7	4.2	7.9	14.2	33.7	9.1	24.0	9.6	30.4
Con A[a]	4.4	10.0	3.8	15.8	3.4	21.1	13.9	–	23.7	–	11.1	–
MAL	2.3	25.6	3.1	23.8	4.7	27.4	3.7	6.5	2.5	11.7	2.8	9.4
RCA-I	3.3	14.2	3.7	19.8	3.0	23.2	8.5	22.8	14.4	32.6	12.6	34.6
SNA	4.0	12.1	4.2	17.2	3.2	12.4	7.2	28.0	13.1	28.8	9.9	23.9

The variability values were estimated by analysis of covariance of the binding/or inhibition data for all tested concentrations of protein/or inhibitory sugar.

[a] The results correspond to those of the desialylated proteins. For other details, see the legends to Tables 1 and 3.

Table 3
Inhibitory potencies (IC_{50}) of the free sugars used as inhibitors of lectin binding to the separated variants and (unfractionated) AAG

Inhibitory sugars	Lectins	A variant		F1*S variants		Commercial AAG	
		IC_{50} (mM)	r	IC_{50} (mM)	r	IC_{50} (mM)	r
Fucose	AAL	0.643 ± 0.050	0.99	0.233 ± 0.026	0.99	0.519 ± 0.062	0.98
α-D-Methylmannoside	Con A	0.116 ± 0.006	0.99	0.046 ± 0.002	0.99	0.070 ± 0.004	0.99
α-D-Methylmannoside	Con A[a]	0.074 ± 0.003[a]	0.99	0.039 ± 0.003[a]	0.99	0.047 ± 0.005[a]	0.99
Lactose	MAL	[b]	–	[b]	–	[b]	–
Lactose	RCA-I	0.148 ± 0.011	0.99	0.023 ± 0.003	0.98	0.038 ± 0.002	0.99
Lactose	SNA	5.96 ± 0.67	0.99	6.22 ± 0.84	0.98	4.89 ± 0.33	0.99

The results are presented with respect to each combination of lectin and protein assayed. The amount of protein used for coating the wells was 0.5 μg (AAL), 0.7 μg (MAL), 0.25 μg (SNA), 0.5 μg (Con A), or 0.7 μg (RCA-I). The amount of desialylated protein was 0.15 μg (Con A). The concentrations of fucose and α-D-methylmannoside ranged from 0.05 to 5 mM; those of lactose ranged from 2 to 200 mM with MAL and SNA, and from 0.1 to 10 mM with RCA-I. All other details are as in the legend to Table 1. For the evaluation of the inhibition data, the absorbance values were corrected for background absorbance. The inhibitors' IC_{50} values were calculated from the data of one to two triplicate inhibition experiments ($19 \leq n \leq 38$), and are given with their standard errors. r is the coefficient of correlation between the theoretical and observed values.

[a] The results correspond to those of the desialylated proteins.

[b] No IC_{50} values could be calculated because the inhibition percentage was less than 50%.

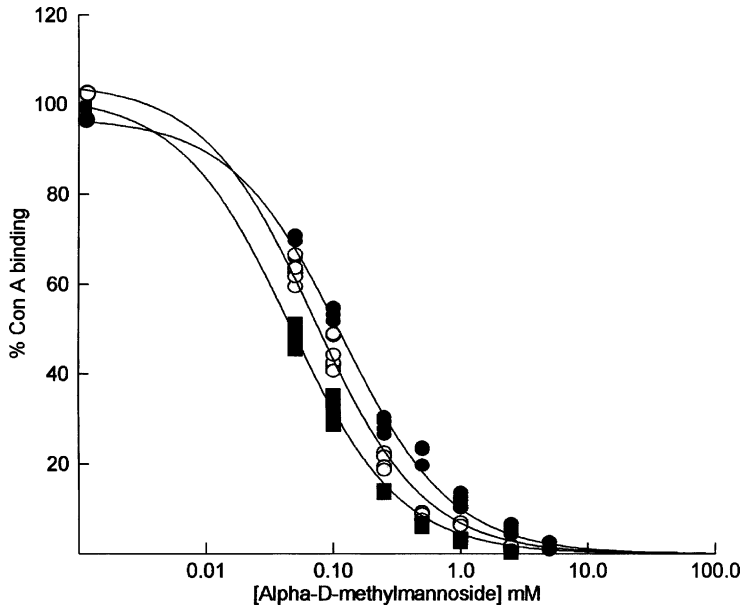

Fig. 3. Inhibition of Con A binding to the F1*S variants (■), the A variant (●) and (unfractionated) commercial AAG (○) by addition of an inhibitory sugar. The data of two triplicate inhibition experiments are shown. The solid lines are computer-estimated values. The inhibitor IC_{50} values calculated from the inhibition data are shown in Table 3. The results for the variability of the inhibition assays are presented in Table 2. All other details are described in the experimental section and the legend to Table 3.

a low steady-state volume of distribution (∼100 ml/kg) similar to the pseudo-equilibrium β phase volume of distribution. Small differences were seen between AAG and the F1*S variants or the A variant with respect to their kinetic parameters (Table 4), with the exception of AUC whose decrease is explained by the smaller dose of each variant relative to AAG (i.e. 36.5 mg/kg for F1*S and 13.5 mg/kg for A versus 50 mg/kg for AAG). These results indicate that the two major genetic variants of human AAG, F1*S and A, exhibit similar plasma kinetics in the rat.

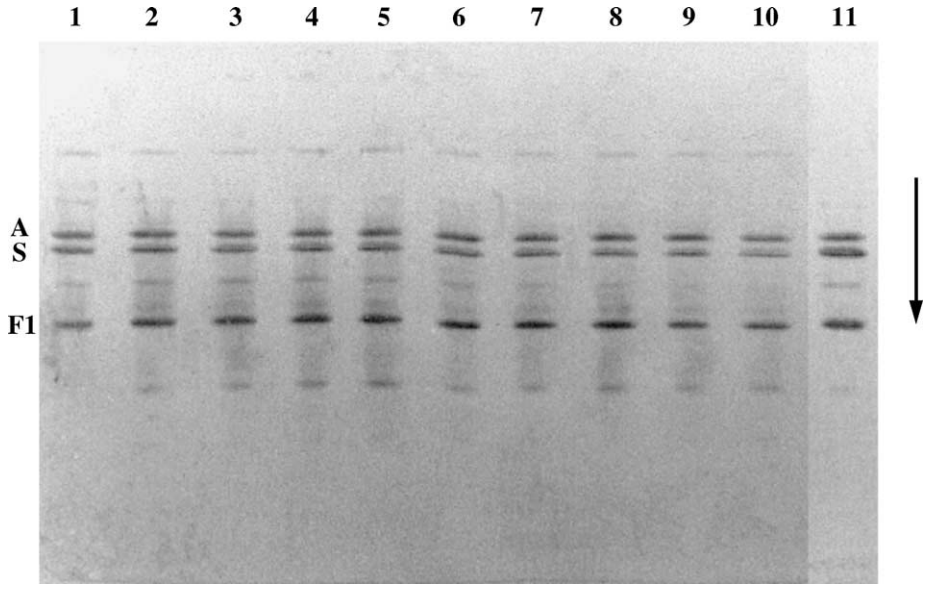

Fig. 4. Isoelectric focusing-immunoblot analysis of the AAG variants in plasma from rats treated with commercial human AAG. Following desialylation, aliquots from the plasma samples were run on an IPG gel (pH 4.5–5.4). After completion of the run, the gel was blotted on a nitrocellulose membrane which was revealed by immunostaining with two antibody steps (rabbit anti-human AAG antibody, and then goat anti-rabbit alkaline phosphatase-labelled antibody). Track 1: control (untreated) rat plasma added with fresh commercial AAG. Tracks 2–10: plasma from rats treated with AAG at 5, 15 and 30 min and 1, 4, 8, 24, 30 and 48 h after treatment. Track 11: standard human plasma containing AAG with the F1*S/A phenotype. The amount of AAG applied was 0.08 μg for all samples. The positions of the F1, S and A variants are indicated. The other faint bands appearing on the blot are probably due to incomplete desialylation of the variants. The relative proportions of F1, S and A in each plasma sample were determined by laser densitometry.

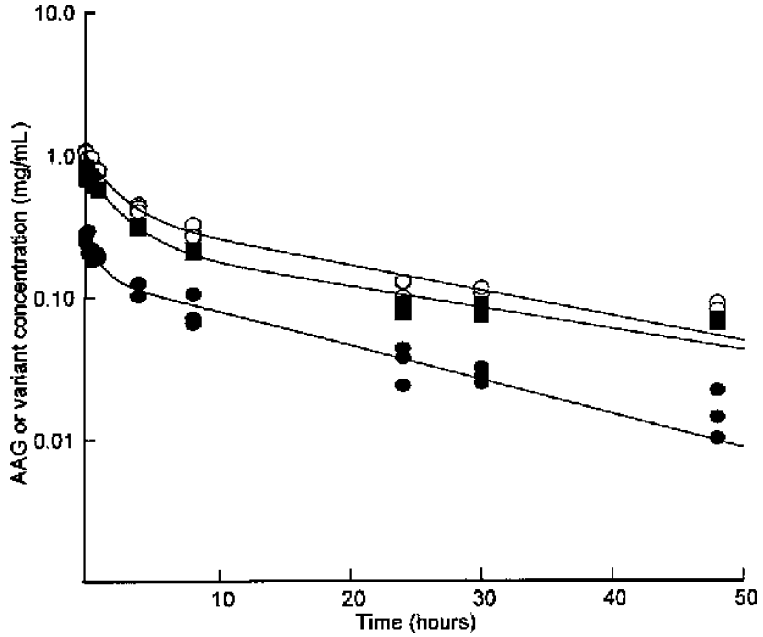

Fig. 5. Plasma concentration-time profiles of the F1*S variants (■), the A variant (●) and AAG (○) in normal adult rats, after intravenous administration of a bolus dose of commercial human AAG (50 mg/kg, corresponding to 36.5 mg/kg of F1*S and 13.5 mg/kg of A). The data obtained from the sacrificed rats at 5, 15 and 30 min and 1, 4, 8, 24, 30 and 48 h after AAG administration are shown (i.e. three rats per sampling time). The solid lines are computer-estimated values. The calculated values of the pharmacokinetic results for AAG and its variants are presented in Table 4.

Table 4
Pharmacokinetic parameters of AAG and its genetic variants, F1*S and A, after intravenous administration of a bolus dose (50 mg/kg) of commercial human AAG in adult rats ($n = 27$; three rats per sampling time)

Parameters	AAG	F1*S variants	A variant
A (mg/ml)[a]	0.675 ± 0.053	0.550 ± 0.053	0.137 ± 0.021
B (mg/ml)[b]	0.379 ± 0.058	0.237 ± 0.058	0.137 ± 0.020
α (h^{-1})[c]	0.477 ± 0.086	0.410 ± 0.088	0.917 ± 0.314
β (h^{-1})[d]	0.041 ± 0.008	0.035 ± 0.011	0.055 ± 0.013
$T_{1/2,\alpha}$ (h)[e]	1.5	1.7	0.8
$T_{1/2,\beta}$ (h)[f]	16.9	20.1	12.6
Clearance (CL) (ml/h/kg)[g]	4.7	4.5	5.1
V_c (ml/kg)[h]	47	46	49
V_{ss} (ml/kg)[i]	101	110	88
V_β (ml/kg)[j]	114	129	93
$AUC_{0-\infty}$ (mg × h/ml)[k]	10.7	8.2	2.6
MRT (h)[l]	21.5	24.7	17.2

The AAG dose administered consisted of 36.5 mg/kg of F1*S and 13.5 mg/kg of A. The parameters were calculated from concentration-time data fitted to a biexponential equation. The standard errors could be estimated only for the first four parameters.

[a] Zero-time intercept.
[b] Zero-time intercept.
[c] Distribution rate constant.
[d] Rate constant of the terminal elimination phase.
[e] Distribution half-life.
[f] Terminal half-life.
[g] Total plasma clearance.
[h] Central distribution volume.
[i] Steady state volume of distribution.
[j] Pseudo-equilibrium β phase volume of distribution.
[k] Area under concentration-time curve from zero to infinity.
[l] Mean residence time.

4. Discussion

Studies conducted on the pharmacological significance of the AAG polymorphism have shown that the variants determined by the two different genes coding for this protein bind many drugs with different affinities [9–12]. This binding affects the tissue transfer of drugs differently [28]. These findings pave the way for other research on the effects of the AAG variants on drug disposition. It would seem obligatory that any such research would take into account the pharmacokinetics of these variants. In such a case, we would also have to consider their glycosylation whenever the glycan moiety of AAG may be of importance for its biodisposition [14–18]. With these objectives in mind, we used commercial AAG as the protein source because the pharmacokinetic study of the variants called for large amounts of proteins, as would also further in vivo studies. It followed that the AAG variants used in the glycosylation studies were separated from commercial AAG.

AAG has five N-linked glycosylation sites (at asparagines 15, 38, 54, 75 and 85) which mostly are occupied by different complex oligosaccharide chains [7,13]. These chains may have two or more branches (di-, tri- and tetraantennary structures) and differ in the degree and the type of substitution with sialic acid and/or fucose. As a result AAG exists as an heterogenous population of glycoforms in normal human plasma. Methods employing lectins, such as binding assays in multiwell plates and affinity chromatography, have proven to be useful to study the microheterogeneities associated with the AAG glycans. The combination of one of these methods [20] with a chromatographic separation method of the AAG variants [19] constitutes the originality of our experimental approach to investigate into the glycosylation of AAG in relation to its genetic polymorphism. Moreover, the validity of the lectin-binding assay used was reinforced by a thorough statistical evaluation of the binding and inhibition results obtained by this method.

The major carbohydrate specificities of the five lectins used in our binding studies [26,29] are shown in Fig. 6. The lectin Con A has the ability to recognise and bind complex diantennary oligosaccharide chains. AAG containing one or more of these chains reacts readily with this α-mannosyl binding lectin, while AAG containing only tri- and tetraantennary glycans is unreactive [30]. It was seen that Con A had a binding preference to the A variant (Table 1), indicating a higher diantennary glycan content. This was confirmed in binding assays using the variants after their treatment with neuraminidase. Furthermore, the presence of peripheral sugars, such as sialic acid, on diantennary glycans can mask potential binding sites of Con A [29], the expected outcome of which would be a higher reactivity of the desialylated variants, as found in the present study (Table 1). The other lectins tested are useful for investigating the peripheral portions of oligosaccharide chains. The branches of the AAG glycan chains can be sialylated on some of the terminal galactoses either as α-2,3- or α-2,6-sialic acid [7,13]. The AAG variants were, therefore, assayed with SNA and MAL (Fig. 6). According to their lectin-binding characteristics (Table 1), both variants should have similar contents in α-2,6-linked sialic acid, while the content in α-2,3-linked sialic acid should be higher for F1*S than A. These results not only indicate differences in the type of glycan substitution with sialic acid, but also suggest differences in the overall degree of sialylation of the variants. This possibility was supported by the binding preference of RCA-I to the A variant (Table 1). This lectin is specific for terminal

Lectins	Monosaccharide specificity	Oligosaccharide specificity
Aleuria aurantia (AAL)	Fuc	Fuc(α1→ 3) Gal(β1→ 4)GlcNAcβ1→ R
Canavalia ensiformis (Con A)	Man	R→ GlcNAc(β1→ 2)**Man(α1→ 6)** **Man(β1→ 4)GlcNAc(β1→ 4)GlcNAc(β1→ N)Asn** R→ GlcNAc(β1→ 2)**Man(α1→ 3)**
Maackia amurensis (MAL)	NeuAc	NeuAc(α2→ 3)Galβ1→ R
Sambucus nigra (SNA)	NeuAc	NeuAc(α2→ 6)Galβ1→ R
Ricinus communis (RCA-I)	Gal	Gal(β1→ 4)GlcNAcβ1→ R

Fig. 6. Typical oligosaccharide structures of recognition sites for the series of tested lectins. All the structures shown have been found in AAG derived from pooled human plasma [7]. The oligosaccharide inner core common to all *N*-glycosylproteins is in bold characters. Abbreviations: Asn: asparagine; Fuc: fucose; Gal: galactose; GlcNAc: *N*-acetylglucosamine; Man: mannose; NeuAc: *N*-acetyl neuraminic acid (sialic acid); R: sugar.

β-galactosyl residues [26,29] (Fig. 6) and, since galactose is the sub-terminal sugar in fully formed *N*-linked glycans, RCA-I provides a means of identifying the asialo forms of glycoproteins. Finally, the data obtained for the binding of AAL, a fucose-specific lectin, indicated that A contained higher amounts of fucosylated glycans than F1*S (Table 1). Fucose occurs on AAG glycan chains in the α-1,3-linkage to the proximal *N*-acetylglucosamine [7] (Fig. 6). The presence of outer branch fucose in this linkage, if expressed on the same branch as sialic acid α-2,3-galactose, can potentially express the sialyl Lewis X (SLeX) antigen [16]. In spite of a relatively high fucose content, it is unlikely that the A variant expresses fucosylated structures of the type SLeX. The first reason being there is a low expression of SLeX on normal AAG [31], and secondly it was seen that A contained relatively low amounts of α-2,3-linked sialic acid (Table 1). It must be emphasised that the glycosylation presented represents an average of the various glycoforms making up each AAG variant. The specificity of the interaction with each lectin was confirmed in binding inhibition studies.

To our knowledge, this is the first study to bring evidence for the existence of significant differences in glycosylation between the two main AAG gene products. Previous investigations into the glycosylation of AAG in relation to its genetic polymorphism have been made, but the information collected was not conclusive. A study of the human AAG molecules occurring in the sera from transgenic mice expressing either the *AAG-A* and *AAG-B/B′* genes or only the *AAG-A* gene of human AAG, has shown that the glycosylation expressed by these molecules was strongly influenced by the host [32]. Besides, the observation that all three variants, F1, S and A, were present in the different chromatographic fractions of AAG that are reactive and unreactive to Con A-agarose had led to the proposal that the glycosylation expressed by these variants was similar [33]. However, the individual study of the A variant and the F1*S variants by Con A affinity chromatography has shown different reactivity ratios between these variants, suggesting differences in their glycosylation [19].

The glycosylation differences between the F1*S and A variants, as shown here, suggest that the glycosylation of AAG may be dependent on its genetic polymorphism. It is recognized that a polypeptide can exert control over its own glycosylation [34]. Moreover, even subtle changes in the amino acid sequence of glycoproteins can cause variations in the oligosaccharides attached to them [35]. The A variant differs from the F1 or S variant by 22 amino acid substitutions, some of which are located in the vicinity of glycosylation sites (positions 15, 75 and 85) [7]. Accordingly, it is possible that the substitutions in question may cause differences in glycosylation of the various gene products. We could not examine the glycosylation of the F1 and S variants individually, because they cannot be separated by chromatography on immobilized copper(II) ions when mixed [19]. Variants F1 and S differ by only one amino acid residue (position 20) in their sequence [8], but it cannot be excluded that this residue, because of its proximity to the asparagine 15, possibly influences the glycosylation at this site.

The possibility that the structural differences between the AAG variants may affect their pharmacokinetics was studied in the rat. To this end, we made use of a method by IEF-IPG enabling the separation and quantification of the variants directly starting from the plasma of AAG-treated rats: this is the first time that this method is applied to a kinetic study of AAG.

The pharmacokinetic parameters estimated for the F1*S variants differed only slightly from those estimated for the A variant (Table 4). It is not known whether or not these differences are significant because simple standard error calculations for these parameters were not possible due to the experimental protocol used. Different rats were used at each time of the kinetic study and, in this case, statistical evaluation of the data was not achievable by current computational techniques, and requested much more complex calculations (i.e. “population modeling” techniques). We considered that the similarity of kinetic results between the AAG variants did not call for such calculations.

The kinetic parameters shown for AAG (Table 4) are comparable to those reported in previous studies [36,37] where the doses of (commercial) AAG administered to rats was up to ∼50 times larger than in the present study [37]: the similarity of results would, therefore, tend to indicate that the pharmacokinetics of AAG is not dose-dependent. More interestingly, Parivar et al. [38] also came up with similar kinetic results using Con A-reactive and Con A-unreactive AAG forms separated by lectin affinity chromatography of commercial AAG. Both forms were seen to have almost identical kinetic parameters in the rat, whereas the Con A-reactive form contained di-, tri- and tetraantennary glycans, and the Con A-unreactive form only tri- and tetraantennary glycans. The average values estimated for the terminal half-life, steady-state volume of distribution and clearance of these AAG forms (i.e. 13.5 h, 91 ml/kg, and 5.4 ml/h/kg), respectively) are similar to the values of the corresponding parameters estimated for AAG and its genetic variants in this study (Table 4). It would follow from the results of Parivar et al. and ours that different diantennary glycan contents between AAG molecular forms or between AAG variants do not produce differences in their plasma kinetics.

Finally, in spite of differences in the amino acid primary sequence [7] and also in the glycosylation, as shown here, the A and F1*S variants exhibited similar pharmacokinetics. It is not possible to say from the present results whether the glycosylation alone or the glycosylation and the peptide chain affect the kinetics of these variants. Investigating this question more in depth seems to be difficult since the removal of oligosaccharide chains from AAG, or even simply splitting the terminal sialyl residues from these chains, has dramatic effects on the AAG in vivo circulatory life-time

[14,36]. In any case, the present data indicate that studies on the effects of the two major genetic variants of AAG on drug disposition and action can be performed in the rat as an animal model, without the interpretation of the results being complicated by kinetic differences between these variants. It should be stressed, however, that our kinetic results were obtained in normal rats, and similar behaviours between the variants may not be expected when carrying out studies in rats under conditions likely to induce an acute phase response. The differences in glycosylation between F1*S and A, and more specifically those in diantennary glycan content, might then produce differences in their kinetics [38].

References

[1] J.M.H. Kremer, J. Wilting, L.H.M. Janssen, Pharmacol. Rev. 40 (1988) 1.
[2] F. Hervé, S. Urien, E. Albengres, J.-C. Duché, J.-P. Tillement, Clin. Pharmacokinet. 26 (1994) 44.
[3] C.B. Eap, P. Baumann, in: P. Baumann, C.B. Eap, W.E. Müller, J.-P. Tillement (Eds.), α1-Acid Glycoprotein. Genetics, Biochemistry, Physiological Functions and Pharmacology, Alan R. Liss Inc., New York, 1989, p. 111.
[4] I. Yuasa, S. Weidinger, K. Umetsu, K. Suenaga, G. Ishimoto, C.B. Eap, J.-C. Duché, P. Baumann, Vox Sang. 64 (1993) 47.
[5] L. Dente, M.G. Pizza, A. Metspalu, R. Cortese, EMBO J. 6 (1987) 2289.
[6] L. Tomei, C.B. Eap, P. Baumann, L. Dente, Hum. Genet. 84 (1989) 89.
[7] K. Schmid, in: P. Baumann, C.B. Eap, W.E. Müller, J.-P. Tillement (Eds.), α1-Acid Glycoprotein. Genetics, Biochemistry, Physiological Functions and Pharmacology, Alan R. Liss Inc., New York, 1989, p. 7.
[8] I. Yuasa, K. Umetsu, U. Vogt, H. Nakamura, E. Nanba, N. Tamaki, Y. Irizawa, Hum. Genet. 99 (1997) 393.
[9] F. Hervé, E. Gomas, J.-C. Duché, J.-P. Tillement, Br. J. Clin. Pharmacol. 36 (1993) 241.
[10] F. Hervé, J.-C. Duché, P. d'Athis, C. Marché, J. Barré, J.-P. Tillement, Pharmacogenetics 6 (1996) 403.
[11] F. Hervé, G. Caron, J.-C. Duché, P. Gaillard, N.A. Rahman, A. Tsantili-Kakoulidou, P.-A. Carrupt, P. d'Athis, J.-P. Tillement, B. Testa, Mol. Pharmacol. 54 (1998) 129.
[12] L.P. Cogswell, D.E. Raines, S. Parekh, O. Jonas, J.E. Maggio, G.R. Strichartz, J. Pharm. Sci. 90 (2001) 1407.
[13] H. Yoshima, A. Matsumoto, T. Mizuochi, T. Kawasaki, A. Kobata, J. Biol. Chem. 256 (1981) 8476.
[14] V. Gross, P.C. Heinrich, D. vom Berg, K. Steube, T. Andus, T.-A. Tran-Thi, K. Decker, W. Gerok, Eur. J. Biochem. 173 (1988) 653.
[15] V. Gross, K. Steube, T.-A. Tran-Thi, W. Gerok, P.C. Heinrich, Biochem. Soc. Trans. 17 (1989) 21.
[16] G. Walz, A. Aruffo, W. Kolanus, M. Bevilacqua, B. Seed, Science 250 (1990) 1132.
[17] M. Qin, S. Øie, J. Pharm. Pharmacol. 46 (1994) 896.
[18] D.N. Predescu, S.A. Predescu, T. McQuistan, G.E. Palade, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 6175.
[19] F. Hervé, E. Gomas, J.-C. Duché, J.-P. Tillement, J. Chromatogr. 615 (1993) 47.
[20] M.T. Goodarzi, G.A. Turner, in: J.M. Walker (Ed.), The Protein Protocols Handbook, Humana Press, Totowa, 1996, p. 619.
[21] M. Gex-Fabry, L.P. Balant, in: L.P. Balant, P.G. Welling (Eds.), Handbook of Experimental Pharmacology, vol. 110, Springer Verlag, New York, 1994, p. 507.
[22] G.W. Snedecor, W.G. Cochran, in: Statistical Methods, sixth ed., The Iowa State University Press, Ames, 1957, p. 419.
[23] S. Urien, Pharmacol. Res. 12 (1995) 1225.
[24] C.B. Eap, P. Baumann, Electrophoresis 9 (1988) 650.
[25] W.-C. Wang, R.D. Cummings, J. Biol. Chem. 263 (1988) 4576.
[26] T. Endo, J. Chromatogr. A 720 (1996) 251.
[27] F.M. Belpaire, M.G. Bogaert, P. Mugabo, M.T. Rosseel, Br. J. Clin. Pharmacol. 88 (1986) 697.
[28] P. Jolliet-Riant, M.F. Boukef, J.-C. Duché, N. Simon, J.-P. Tillement, Life Sci. 62 (1998) 219.
[29] T. Osawa, T. Tsuji, Ann. Rev. Biochem. 56 (1987) 21.
[30] M.F. Bierhuizen, M. De Wit, C.A. Govers, W. Ferwerda, C. Koeleman, O. Pos, W. Van Dijk, Eur. J. Biochem. 175 (1988) 387.
[31] T.W. De Graaf, M.E. Van der Stelt, M.G. Anbergen, W. Van Dijk, J. Exp. Med. 177 (1993) 657.
[32] W. Van Dijk, M.E. Van der Stelt, A. Salera, L. Dente, Eur. J. Cell Biol. 55 (1991) 143.
[33] W. Van Dijk, O. Pos, M.E. Van der Stelt, H.J. Moshage, S.H. Yap, L. Dente, P. Baumann, C.B. Eap, Biochem. J. 276 (1991) 343.
[34] T.W. Rademacher, R.B. Parekh, R.A. Dwek, Ann. Rev. Biochem. 57 (1988) 785.
[35] R.T. Schwarz, H.-D. Klenk, Virology 113 (1981) 584–593.
[36] P.P. van Rijk, C.J. van den Hamer, J. Lab. Clin. Med. 88 (1976) 142.
[37] D.E. Keyler, P.R. Pentel, D.B. Haughey, J. Pharm. Sci. 76 (1987) 101.
[38] K. Parivar, L. Tolentino, G. Taylor, S. Øie, J. Pharm. Pharmacol. 44 (1992) 447.