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Binding of Amitriptyline to α_1-Acid Glycoprotein and its Variants

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Abstract—Binding studies have been performed between amitriptyline and i) native α_1-acid glycoprotein (AAG); ii) its desialylated form; iii) its two variants, S-AAG and F-AAG; and iv) a mixture of S-AAG and F-AAG. Scatchard analysis revealed the presence of two classes of binding sites on AAG. For native AAG, the first class (of high affinity) has an association constant (K_{a1}) of 1.5×10^6 L mol^{-1} and a number of binding sites per mole of protein (n_1) of 0·25, while the second class (of low affinity) has a K_{a2} of 3.2×10^4 L mol^{-1} and a n_2 of 0·94. Similar data were found for desialylated AAG. S-AAG and F-AAG do not differ in their association constants measured with amitriptyline, but in their number of binding sites per mole of protein (n): S-AAG: $n_1 = 0.56$, $n_2 = 0.52$; F-AAG: $n_1 = 0.17$, $n_2 = 0.71$. These results confirm those of a previous study, in which a higher affinity of S-AAG towards various basic drugs in comparison with F-AAG has been found.

α-1 Acid glycoprotein (AAG) is a major binding protein for basic drugs in plasma (Piafsky & Borgå 1977; Paxton 1983). From the works of Schmid (1975), it is known that there are at least two variants of desialylated AAG, namely S-AAG and F-AAG, and three phenotypes can be determined according to the differences in density of the slow (S-AAG) or fast (F-AAG) migrating bands after isoelectric focusing of desialylated AAG. While the SS-pattern displays one major S and one minor F band, the FF-pattern shows one major F and one minor S band, and the FS-pattern one F and one S band of similar density. These three phenotypes are genetically determined (Johnson et al 1969).

Recently, binding studies revealed that isolated S-AAG displays a higher affinity for various basic drugs than F-AAG (Eap et al 1988). In the present study, binding experiments with a broad range of concentrations of amitriptyline have been performed to determine the association constant (K_a) and the number of binding sites per mole of protein (n) of amitriptyline with native AAG, desialylated AAG, S- and F-AAG isolated from desialylated AAG and a mixture of S-AAG and F-AAG.

Materials and Methods

Reagents

All chemicals were of analytical grade (E. Merck, Darmstadt, FRG). α_1-Acid glycoprotein (purity 99%) and neuraminidase (type V, protease impurities <0.001 units mg^{-1} solid) were from Sigma (St. Louis, USA). AAG concentrations were measured in triplicate by radial immunodiffusion using NOR-partigen plates (Behring, Marburg, FRG). Amitriptyline HCl was a gift from Lundbeck (Copenhagen, Denmark), and *N*-methylmaprotiline HCl from Ciba-Geigy (Basle, Switzerland). Immobilines were from LKB (Bromma, Sweden).

Desialylation of native AAG and purification of S- and F-AAG

These steps were performed according to Eap et al (1988). Briefly summarized, after extensive desialylation (more than 99% of bound sialic acid cleaved), AAG was submitted to isoelectric focusing in a 5·0 mm thick preparative immobilines gel (pH range 4·3–5·3), which was cast according to LKB application note 323. S- and F-AAG bands were then cut and electro-eluted into DEAE-Sephadex. After recovery from Sephadex, the two fractions were extensively dialysed and lyophilized.

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Dialysis

Buffer (66 mmol L^{-1} sodium phosphate, 50 mmol L^{-1} NaCl pH 7·4) was filtered through a 0·22 μm porosity Millipore filter and stored at 4°C. Dialysis was performed for 4 h in a bath set at 37°C. Native AAG, desialylated AAG, S- and F-AAG were diluted in buffer to a concentration of 22·5 μmol L^{-1}. The mixture of S-AAG and F-AAG ((S + F)-AAG) was made so that it contained 54% S-AAG and 46% F-AAG, which is the proportion found in desialylated AAG Sigma, as determined by laser densitometer. The dialysis membranes (mol. wt cut-off 10 000), dialysis cells (2×200 μL) and the drive unit (Dianorm) were from Diachema, (Munich, FRG).

For each fraction, dialysis was carried out in triplicate at each concentration. For the working solutions of amitriptyline, the stock solution (1 mg base amitriptyline mL^{-1} 0·1 mol L^{-1} HCl) was diluted in phosphate buffer pH 7·4 at a final concentration ranging from 200 ng mL^{-1} (0·72 μmol L^{-1}) to 200 000 ng mL^{-1} (720 μmol L^{-1}). Preliminary experiments have shown that 4 h of dialysis were sufficient to obtain equilibrium, and no significant changes in the volumes of the two compartments were observed after dialysis. Losses of amitriptyline due to adsorption to the cells or dialysis membranes were around 5–10% of the initial amount added.

Calculation

At the end of equilibrium dialysis, the concentration of amitriptyline was measured in each compartment by gas chromatography on a Hewlett-Packard 5880 (Eap et al 1988) to determine the free (F) and bound (B) drug concentration.

To calculate the association constant (K_a) and the number

of binding sites per mole of protein (n), the data were subjected to non-linear regression analysis, using the LIGAND program (Munson & Rodbard 1980). This program generates final parameters with their standard errors, standard errors which should only be considered as a rough guide of the parameters' stability (McPherson 1985). To obtain the initial parameters for LIGAND, another program, EBDA (McPherson 1983) was used in conjunction with LIGAND on an Amstrad PC 1512. These two programs were from Elsevier Biosoft, (Cambridge, UK). For statistical comparisons between the data, the F-test was applied, as recommended by Munson & Rodbard (1980). For all data, the two-site model was used as it was shown by the F-test to be the most adequate.

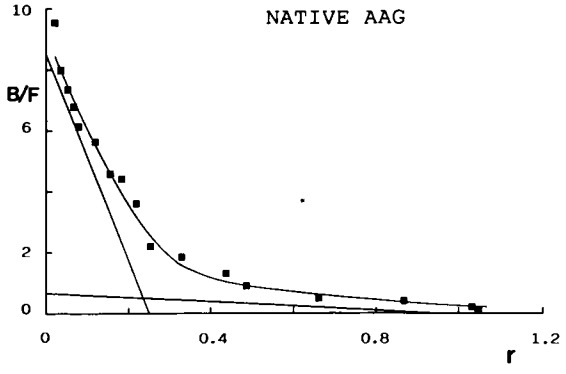
Results

All problems relating to the desialylation and isolation processes for S- and F-AAG have been discussed previously (Eap et al 1988).

The results of the binding experiments of amitriptyline to AAG are summarized in Table 1, with the association constants (K_a) and number of binding sites per mole of protein (n) of native, desialylated, S-, F- and (S+F)-AAG. A graphic presentation of the data in the form of Scatchard plots, as given by the program for the five fractions, reveals the existence of two classes of binding sites (Fig. 1). Native AAG has a K_{a1} of $1{\cdot}5\times10^6\pm4\times10^5$ L mol^{-1} and a n_1 of $0{\cdot}25\pm0{\cdot}04$ (Table 1). Although n_2 is more than three times higher than n_1, the second class of binding sites contributes only a little to the overall binding capacity of AAG due to the very low K_{a2} in comparison with K_{a1}. Furthermore, it has to be emphasized that the existence of the second class is only revealed at very high, unphysiological concentrations of amitriptyline. The association constants (K_{a1}) of desialylated

Table 1. Association constants (K_a) and number of binding sites per mole of protein (n) of amitriptyline with native α_1-acid glycoprotein (AAG), desialylated AAG, S-AAG, F-AAG, and (S+F)-AAG. Values are given with their standard errors as generated by LIGAND.

	K_a L mol^{-1}	n
Native AAG	$K_{a1}: 1{\cdot}5\times10^6\pm4\times10^5$	$n_1: 0{\cdot}25\pm0{\cdot}04$
	$K_{a2}: 3{\cdot}2\times10^4\pm6\times10^3$	$n_2: 0{\cdot}94\pm0{\cdot}04$
Desialylated AAG	$K_{a1}: 1{\cdot}2\times10^6\pm5\times10^5$	$n_1: 0{\cdot}25\pm0{\cdot}07$
	$K_{a2}: 2{\cdot}9\times10^4\pm1\times10^4$	$n_2: 1{\cdot}15\pm0{\cdot}09$
S-AAG	$K_{a1}: 1{\cdot}3\times10^6\pm3\times10^5$	$n_1: 0{\cdot}56\pm0{\cdot}06$
	$K_{a2}: 3{\cdot}4\times10^4\pm1{\cdot}3\times10^4$	$n_2: 0{\cdot}52\pm0{\cdot}05$
F-AAG	$K_{a1}: 1{\cdot}2\times10^6\pm8\times10^5$	$n_1: 0{\cdot}17\pm0{\cdot}09$
	$K_{a2}: 5{\cdot}8\times10^4\pm2{\cdot}5\times10^4$	$n_2: 0{\cdot}71\pm0{\cdot}07$
(S+F)-AAG	$K_{a1}: 1{\cdot}3\times10^6\pm4\times10^5$	$n_1: 0{\cdot}25\pm0{\cdot}04$
	$K_{a2}: 5{\cdot}3\times10^4\pm1{\cdot}3\times10^4$	$n_2: 0{\cdot}63\pm0{\cdot}04$



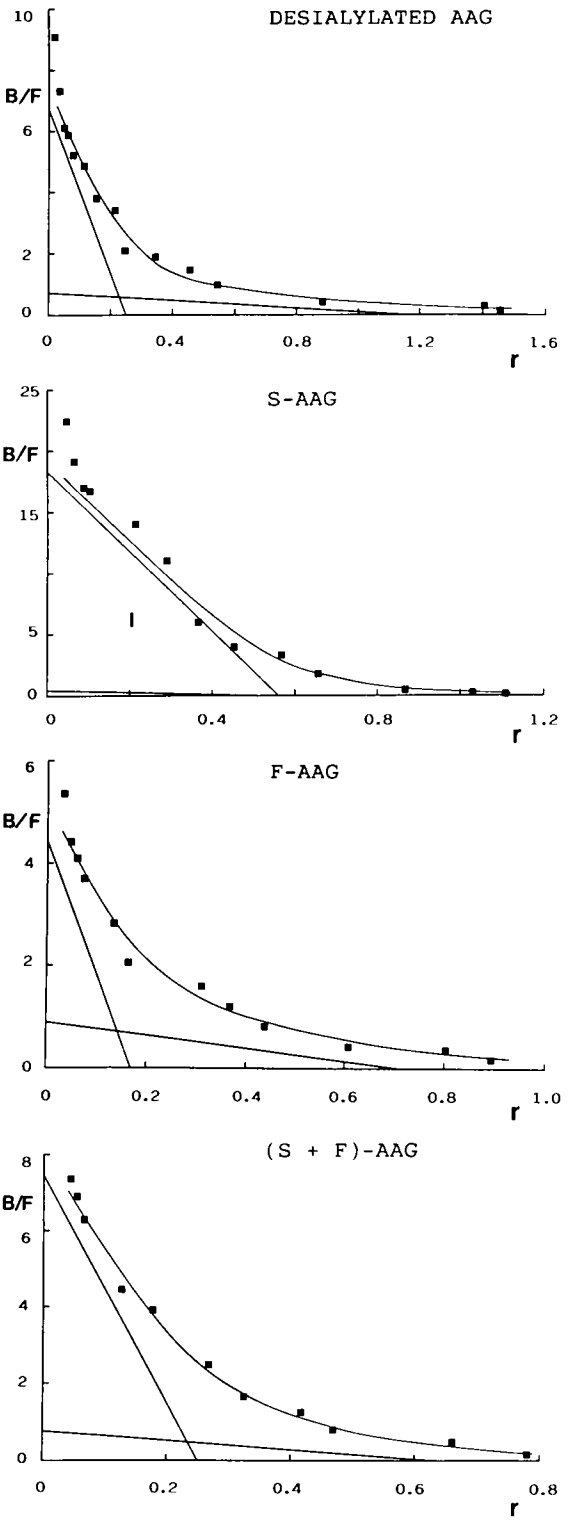


FIG. 1 Binding of amitriptyline (0·72 μmol L^{-1}—720 μmol L^{-1}) to native AAG, desialylated AAG, S-AAG, F-AAG, and (S+F)-AAG (22·5 μmol L^{-1}). The curves were analysed by nonlinear regression with a two-class binding-site model, using the LIGAND program.

AAG, S-, F-, and (S + F)-AAG are lower than those of native AAG, but the differences are not statistically significant. For example, $F_{(2,24)} = 0.11$, n.s., when the data were analysed from native AAG and desialylated AAG with K_{a1} kept constant and equal for these two fractions.

The main result of this work is that S-AAG has the highest n_1 (0.56 ± 0.06) of all five fractions. Actually, the number of binding sites, n_1, of F-AAG is more than three times lower than that of S-AAG. As expected, dialysis with a mixture of S- and F-AAG gives values for n which are intermediate between S- and F-AAG. Nevertheless, when analysing the data from S-AAG together with those of native AAG, with the same fixed value for n_1-chosen so as to minimize the root mean square error (Munson & Rodbard 1980), we found that $F_{(2,22)} = 2.56$, $P = 0.10$. This means that the higher binding capacity of S-AAG may be due to two reasons: 1) the same n_1 for the two fractions, but a higher K_{a1} for S-AAG; 2) the same K_{a1} for the two fractions, but a higher n_1 for S-AAG.

The examination of the graphs and standard errors for the n values shows that the second hypothesis appears to be the more plausible.

Discussion

The results presented in this study demonstrate the higher binding capacity of S-AAG than F-AAG for amitriptyline. These findings are in agreement with those of a clinical (Tinguely et al 1985) and in-vitro investigation with pure commercially available AAG (Eap et al 1988), in which differences in the binding behaviour of S- and F-AAG towards basic drugs have been found.

AAG is a glycoprotein of 181 amino acids, whose sequence and 21 amino acid substitutions have been determined by Schmid et al (1973). The amino acids in position 21–31 may represent the binding region for progesterone on AAG (Kute & Westphal 1976). Kirley et al (1982) demonstrated the significant overlapping of the steroid-binding and basic-drug-binding domains. On the other hand, the amino acid substitution in position 20 (Arg vs Gln) is responsible for the formation of S- and F-AAG on starch gel electrophoresis at pH 5·1. (Nimberg et al 1971). So, Tinguely et al (1985) formulated the hypothesis that substitution of amino acid 20 might have an influence on the binding of basic antidepressants to AAG. Recently, Yuasa et al (1986) phenotyped a population and described the existence of two bands in the S- and two bands in the F- region. The existence of at least four variants of AAG could explain that in native AAG the high affinity binding site represents about one quarter of the total number of binding sites and in S-AAG about one half. Indeed, one hypothesis based on the present work is that only one of the two bands in the S- region could be responsible for the high affinity class. The small n_1 for F-AAG could be due either to a small sub-band in the F- region or to a contamination from the S- region, e.g. by the presence of polymers of AAG during the isolation process (Halsall & Kirley 1981; Halsall et al 1982). It has to be pointed out that, in the present binding studies, the variants recovered from the S- and F- region were used as a whole, due to the difficulty encountered in isolating the subbands. Some authors also found very low n_1 for the binding of some drugs to AAG. Haughey et al (1985) calculated a n_1 of 0·29 for the binding of disopyramide to AAG from Sigma, and Abramson (1982) found a n_1 of 0·38 for the binding of methadone to AAG from Sigma.

In the present study, excepting native AAG, the four other fractions are extensively desialylated (more than 99% sialic acid removed). Although the values of the high association constants (K_1) are lower for the four desialylated fractions than for the native AAG, the differences are not statistically significant ($F_{(2,24)} = 0.11$, n.s.) between native and desialylated AAG. In contrast, Primozic & McNamara (1985) calculated a lower association constant of desialylated AAG versus native AAG for propranolol, but Robert et al (1983) and Van der Sluijs & Meijer (1985) did not find any difference in the binding of nicergoline and some mono and quaternary ammonium compounds, respectively, to native AAG compared to desialylated AAG. It seems therefore that the role of the sialic acid residues in the binding of drugs to AAG is minor.

This is not the first report on two classes of binding sites for AAG; other authors like Brinkschulte & Breyer-Pfaff (1980) for amitriptyline, perazine and nortriptyline, El-Gamel et al (1982) for dipyramidole, Abramson (1982) for methadone, Schley & Müller-Oerlinghausen (1983) for imipramine, perazine and other psychotropic drugs, and Van der Sluijs & Meijer (1985) for *N*-methylimipramine, also found two classes.

Both the present data and those of Brinkschulte & Breyer-Pfaff (1980) are in agreement concerning the existence of two classes of binding sites, but their Ka_1 and Ka_2 were lower by a factor of three for amitriptyline. A possible explanation could be the different source of AAG, which was supplied from Sigma for the present study and from Behring for Brinkschulte & Breyer-Pfaff's (1980) work. Indeed, Haughey et al (1985) found large differences in the association constants of disopyramide to various sources of commercially available AAG. Different isolation methods of AAG, and different sources (urine, plasma, serum) could contribute to these variations. Moreover, this problem could explain the difference in number of classes of binding sites (1 or 2) appearing in the relevant publications. One has to consider this problem, when comparing data obtained for drug binding to different sources of AAG.

The question arises about the possible clinical consequences of this finding, especially in disease states, during which AAG concentrations increase dramatically (Kushner & Mackiewicz 1987), leading to alterations in the binding of some drugs (Tillement et al 1984; Svensson et al 1986). While in disease states, there are fluctuations in the proportion of the carbohydrate moiety of AAG, (Serbource-Goguel Seta et al 1986), it has been shown by Schmid et al (1968) and by Yoshizaki et al (1969) that, in spite of the considerable increase and variation in the concentration of AAG in blood following surgical trauma, irradiation, and under conditions of severe stress, the relative proportion of S- and F- forms of AAG does not change. It would be interesting to know whether an increase of AAG in a disease state would lead to different variations in drug binding between S-AAG and F-AAG patients.

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References

Abramson, F. P. (1982) Methadone plasma protein binding: Alterations in cancer and displacement from alpha-1 acid glycoprotein. Clin. Pharmacol. Ther. 32: 652–658

Brinkschulte, M., Breyer-Pfaff, U. (1980) The Contribution of Alpha-1 Acid Glycoprotein, Lipoproteins, and Albumin to the Plasma Binding of Perazine, Amitriptyline, and Nortriptyline in Healthy Man. Naunyn Schmiedeberg's Arch. Pharmacol. 314: 61–66

Eap, C. B., Cuendet, C., Baumann, P. (1988) Selectivity in the binding of psychotropic drugs to the variants of alpha-1 acid glycoprotein. Ibid. 337: 220–224

El-Gamel, S., Wollert, U., Müller, W. E. (1982) Optical studies on the specific interaction of dipyridamole with Alpha-1 acid glycoprotein (orosomucoid). J. Pharm. Pharmacol. 34: 152–157

Halsall, H. B., Kirley, T. L. (1981) Observations on the thermostability of orosomucoid. Biochem. Biophys. Res. Commun. 102: 761–765

Halsall, H. B., Kirley, T. L., Friedman, M. L. (1982) The preparation of orosomucoid from nephrotic urine. Prep. Biochem. 12: 111–120

Haughey, D. B., Steinberg, I., Lee, M. H. (1985) Protein binding of disopyramide-displacement by mono-N-dealkyldisopyramide and variation with source of Alpha-1 acid glycoprotein. J. Pharm. Pharmacol. 37: 285–288

Johnson, A. M., Schmid, K., Alper, Ch.A., Bissett, L. (1969) Inheritance of Human Alpha-1 Acid Glycoprotein (Orosomucoid) Variants. J. Clin. Invest. 48: 2293–2299

Kirley, T. L., Sprague, E. D., Halsall, H. B. (1982) The binding of spin-labelled propranolol and spin-labelled progesterone by orosomucoid. Biophys. Chemistry 15: 209–216

Kushner, I., Mackiewicz, A. (1987) Acute phase proteins as disease markers. Disease Markers 5: 1–11

Kute, T., Westphal, U. (1976) Steroid-Protein Interactions. XXXIV. Chemical Modification of Alpha-1 Acid Glycoprotein for Characterization of the Progesterone Binding Site. Biochim. & Biophys. Acta 420: 195–213

McPherson, G. A. (1983) A practical computer-based approach to the analysis of radioligand binding experiments. Computer Programs in Biomedicine 17: 107–114

McPherson, G. A. (1985) Kinetic, Ebda, Ligand, Lowry. A Collection of Radioligand Binding Analysis Programs. (Manual). McPherson (Ed.). Elsevier Science Publishers BV, Amsterdam, NL. Elsevier-Biosoft, Cambridge, U.K. (Distr.)

Munson, P. J., Rodbard, D. (1980) LIGAND: A Versatile Computerized Approach for Characterization of Ligand-Binding Systems. Analyt. Biochemistry 107: 220–239

Nimberg, R., Motoyama, T., Schmid, K. (1971) The Amino Acid Substitutions Found in the Genetic Variants of Alpha-1 Acid Glycoprotein. J. Biol. Chemistry 246: 5817–5821

Paxton, J. W. (1983) Alpha-1 Acid Glycoprotein and Binding of Basic Drugs. Meth. & Find. Exptl. Clin. Pharmacol. 5: 635–648

Piafsky, K. M., Borgå, O. (1977) Plasma protein binding of basic drugs. II. Importance of Alpha-1 acid glycoprotein for interindividual variation. Clin. Pharmacol. Ther. 22: 545–549

Primozic, S., McNamara, P. J. (1985) Effect of the Sialylation State of Alpha-1 Acid Glycoprotein on Propranolol Binding. J. Pharm. Sci 74: 473–475

Robert, L., Migne, J., Santonja, R., Zini, R., Schmid, K., Tillement, J. P. (1983) Plasma binding of an alpha-blocking agent, nicergoline—Affinity for serum albumin and native and modified α_1 acid glycoprotein. Int. J. Clin. Pharm. Ther. Toxicol. 21: 271–276

Schley, J., Müller-Oerlinghausen, B. (1983) The Binding of Chemically Different Psychotropic Drugs to Alpha-1 Acid Glycoprotein. Pharmacopsychiat. 16: 82–85

Schmid, K. (1975) Alpha-1 Acid Glycoprotein. In: F. W. Putnam (Ed.) The Plasma Proteins. Structure, Function, and Genetic Control. 2nd Ed. Vol. 1 (4). Academic Press, New York, San Francisco, London, pp 183–228

Schmid, K., Field, R. A., Yoshizaki, H. (1968) Constancy of Alpha-1 Acid Glycoprotein Variants and Caucasian Patients under Conditions of Severe Stress. J. Med. Genet. 5: 36–39

Schmid, K., Kaufmann, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M., Nanno, S. (1973) Structure of Alpha-1 Acid Glycoprotein. The Complete Amino Acid Sequence, Multiple Amino Acid Substitutions, and Homology with the Immunoglobulins. Biochemistry 12: 2711–2724

Serbource-Goguel Seta, N., Durand, G., Corbic, M., Agneray, J., and Feger, J. (1986) Alterations in Relative Proportions of Microheterogeneous Forms of Human Alpha-1 Acid Glycoprotein in Liver Disease. J. Hepatology 2: 245–252

Svensson, C. K., Woodruff, M. N., Baxter, J. G., Lalka, D. (1986) Free Drug Concentration Monitoring in Clinical Practice. Rationale and Current Status. Clin. Pharmacokin. 11: 450–469

Tillement, J.-P., Houin, G., Zini, R., Urien, S., Albengres, E. Barré, J., Lecomte, M., D'Athis, P., Sebille, B. (1984) The Binding of Drugs to Blood Plasma Macromolecules: Recent Advances and Therapeutic Significance. Adv. Drug Res. 13: 59–94

Tinguely, D., Baumann, P., Conti, M., Jonzier-Perey, M., Schöpf, J. (1985) Interindividual Differences in the Binding of Antidepressants to Plasma Proteins: The Role of the Variants of Alpha-1 Acid Glycoprotein. Eur. J. Clin. Pharmacol. 27: 661–666

Van der Sluijs, P., Meijer, D. K. F. (1985) Binding of Drugs with a Quaternary Ammonium Group to Alpha-1 Acid Glycoprotein and Asialo Alpha-1 Acid Glycoprotein. J. Pharmacol. Exp. Ther. 234: 703–707

Yoshizaki, H., Hunziker, K., Schmid, K. (1969) The Constancy of the Types of Alpha-1-Acid Glycoprotein Variants in Patients with Uterectomy and Irradiation. Clin. Chim. Acta 23: 147–151

Yuasa, I., Umetsu, K., Suenaga, K., Robinet-Lévy, M. (1986) Orosomucoid (ORM) typing by isoelectric focusing: evidence for two structural loci ORM1 and ORM2. Hum. Genet. 74: 160–161