Pharmaceutical Research, Vol. 23, No. 5, May 2006 (© 2006)
DOI: 10.1007/s11095-006-9777-8

Short Communication

Biantennary Glycans as Well as Genetic Variants of α_1-Acid Glycoprotein Control the Enantioselectivity and Binding Affinity of Oxybutynin

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Received July 12, 2005; accepted December 21, 2005

Purpose. The purpose of this study was to investigate the role of biantennary branching glycans of α_1-acid glycoprotein (AGP) and its genetic variants in the enantioselective binding of oxybutynin (OXY).
Method. Human native AGP was separated using imminodiacetate–copper (II) affinity chromatography into two fractions, the A variant and a mixture of the F1 and S variants (F1–S). These fractionated AGPs were further separated by concanavalin A affinity chromatography into two fractions, with and without biantenarry glycans. An on-line high-performance liquid chromatography (HPLC) system consisting of a high-performance frontal analysis column, an extraction column, and an analytical HPLC column was developed to determine the binding affinities of OXY enantiomers for respective AGP species.
Results. The total binding affinity as well as the enantiomeric selectivity of OXY in the F1–S mixed variant was significantly higher than that for the A variant, indicating that the chiral recognition ability of native AGP for the OXY enantiomers highly depends on the F1–S mixed variant. Furthermore, not only the genetic variants but also bianntenary glycans of AGP affect the binding affinity of OXY and are also responsible for the enantioselectivity.
Conclusions. Both genetic variants and glycan structures significantly contribute to the enantioselectivity and the binding affinity of OXY.

KEY WORDS: α_1-acid glycoprotein; biantennary branching glycan; genetic variants; high performance frontal analysis; oxybutynin; protein binding; sugar chain.

INTRODUCTION

The binding of plasma protein to drugs significantly influences their distribution in the body and their pharmacological activities (1,2). In addition, in the case of a racemic drug, the binding affinity potentially differs between the enantiomers, which may cause a difference in pharmacokinetic properties (3,4). Therefore, quantitative and enantioselective binding studies of individual proteins are important in the development of a racemic drug and its safe and rational use.

In this study, oxybutynin (OXY), which binds strongly to plasma proteins, especially α_1-acid glycoprotein (AGP) (5), was used as a chiral model. The analysis of strong protein binding using conventional methods such as ultrafiltration and equilibrium dialysis very often encounters problems such as difficulty in detecting low levels of unbound drugs, undesirable drug adsorption onto membranes, and the leakage of bound drugs, which cause a considerable overestimation of unbound drug concentrations. To overcome these problems, we developed a novel binding analysis, high-performance frontal analysis (HPFA), and showed that this method is useful for determining very low unbound drug concentrations (6–12). The principle and the features of HPFA were reviewed elsewhere (13).

AGP is an acute-phase reactant whose plasma concentration increases several fold during acute-phase reactions such as those during trauma, chronic pathological conditions, and tumor growth (14). The most well known properties of AGP are its ability to bind a variety of endogenous (e.g., steroids) and exogenous compounds (15), and its ability to modulate various immune reactions (16).

AGP has three major genetic variants and several minor ones (17,18), which can be identified by the isoelectrofocusing of desialylated forms. The three main AGP variants are designated F1, S, and A, according to their electrophoretic mobility (19). The polymorphism is generated by the presence of two different genes, *AGP A* and *B* (*B*/*B*′). Although the AGP phenotypes generally have only two or three of these major variants, all the minor variants account for less than 1% of the population. The F1 and S variants are encoded by the *AGP A* gene, and the A variant is encoded by the *AGP B* gene (20). These variants show different drug-binding properties as demonstrated by Herve *et al.* (21) and Nakagawa *et al.* (22). In addition, the glycoforms of AGP, which consist of complex diantennary, triantennary, and tetraantennary glycan chains, change during acute and chronic inflammation, pregnancy, estrogen treatment, cancer, liver disease, and autoimmune diseases, like rheumatoid arthritis and Graves disease (16). Therefore, it is important

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to investigate the difference in the drug-binding property among AGP glycoforms and variants.

Shiono *et al.* reported that sialic acid groups of AGP were responsible for the chiral recognition of propranolol enantiomers (23), suggesting that not only the peptide chains but also the carbohydrate chains play an important role in the stereoselective binding ability of AGP. Fraeyman *et al.* reported that the binding of oxplenolol to AGP is not affected by the branching glycan structure (24). However, these studies did not investigate the enantioselective drug-binding ability of branching glycan in AGP variants. Further study was therefore required to elucidate the effect of the carbohydrate moiety on the stereoselective binding abilities of AGP and its variant.

In this study, an on-line high-performance liquid chromatography (HPLC) system involving the HPFA method was applied to investigate the role of the branching glycan structure and the genetic polymorphism of AGP in the enantioselective binding of OXY.

MATERIALS AND METHODS

Materials

Enantiomers of OXY were obtained from Sepracort (Newton, MA, USA) and human AGP (Cat. No. G-9885) was purchased from Sigma (St. Louis, MO, USA). Iminodiacetate (IDA)–copper (II) and concanavalin (Con) A immobilized on sepharose gel were obtained from Pharmacia Biotech (Uppsala, Sweden). Develosil 100 Diol 5 was purchased from Nomura Chemicals (Seto, Japan). YMC-Pack ODS-AM was purchased from YMC (Kyoto, Japan).

Sample Preparation

Native AGP was separated into the A variant and the F1–S mixed variant by IDA–Cu (II) affinity chromatography according to Herve (25). One milliliter of the native AGP solution (70 mg/mL) was applied to a 2.6 × 60 cm IDA–Cu (II)-packed column. Sodium phosphate buffer containing 0.5 M NaCl (pH 7.0) was then permitted to flow at 1.0 mL/min and the F1–S variant was eluted after 250 to 360 min. After complete elution of the F1–S variant, 20 mM imidazole supplemented with the elution buffer was pumped at 1.0 mL/min and the A variant was eluted after 750 to 820 min. Both fractions were then dialyzed using Spectra/Por 7 membranes against excess volumes of Milli-Q water at 4°C, and lyophilized. Two milliliters of the F1–S or A solution (16 mg/mL) was then applied to a Con A–Sepharose-packed column (2.6 × 12 cm, 4°C), and eluted with 50 mM Tris/HCl buffer containing 1.0 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. The untreated AGP (UR-AGP) was eluted after about two column volumes of the starting buffer. After complete elution of UR-AGP, 0.15 M methyl α−D (+)-glucose was added to the starting buffer to elute the retained AGP (R-AGP). The fractions were dialyzed using Spectra/Por 7 dialysis membranes (molecular weight cut off 10,000) (Spectrum, Houston, TX) against Milli-Q water at 4°C, and lyophilized. The isoelecric focusing in immobilized pH gradient analysis (26) showed these fractions were pure. We conformed the purity (91–102%) using UV absorbance and gravimetry.

OXY and fractionated AGP mixed solutions were prepared in sodium phosphate buffer (pH 7.4, ionic strength of 0.17) and these mixed solutions were kept at 37°C before analysis.

Table I. HPFA Conditions

Subsystem		Condition
HPFA	Column	Develosil 100 Diol 5 (5 cm × 4.6 mm i.d.)
	Mobile phase	Sodium phosphate buffer (pH 7.4, I = 0.17)
	Flow rate	1.0 mL/min
Extraction	Column	Develosil ODS 10 (1 cm × 4.6 mm i.d.)
Analytical	Column	YMC-Pack ODS-AM (15 cm × 4.6 mm i.d.)
	Mobile phase	Sodium phosphate buffer (pH 4.6, I = 0.04)/MeOH= 4:6 (pH 3.0) (v/v)
	Flow rate	1.0 mL/min
	Detection	UV 220 nm

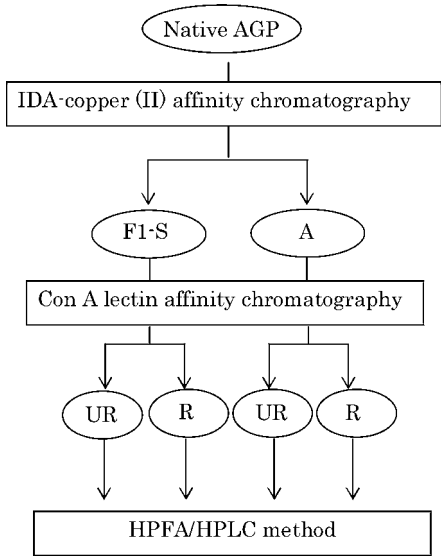

Fig. 1. Flow chart for the preparation of AGP.

On-Line HPFA–HPLC System

The instruments used were as follows: HPLC pumps, LC 9A (Shimadzu, Kyoto, Japan) and A-30-S (Eldex Laboratories, San Carlos, CA, USA); UV detector, SPD-6A (Shimadzu); injector, Rheodyne Type 8125; integrated data analyzer, Chromatopac C-R3A and C-R6A (Shimadzu); and column oven, CS-300C (Chromato-Science, Osaka, Japan).

Table II. Distribution of UR-, R-AGP, and AGP Variants

	F1–S variants (%)	A variant (%)	Total (%)
UR-AGP	21.6	22.6	44.2
R-AGP	26.2	29.6	55.8
Total	47.8	52.2	100

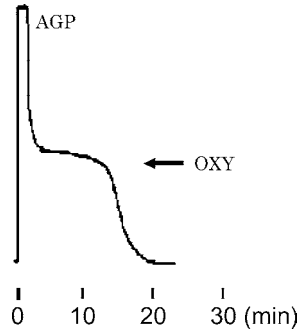

Fig. 2. HPFA profile of 80 μM OXY and 30 μM AGP mixed solution.

Table I lists the HPLC conditions. Sodium phosphate buffer (pH 7.4) was used as the mobile phase for HPFA without the addition of any organic modifiers so as not to disturb the drug–protein binding equilibrium. A hydrophilic HPFA column (diol–silica column) allowed the elution of hydrophobic OXY enantiomers with a mild mobile phase.

Determination of Unbound OXY Concentrations by HPFA–HPLC

The principle of HPFA was described in our previous articles (5,6,13). An HPFA column, an extraction column, and an analytical column were connected via a four-port and a six-port switching valve. A series of sample solutions of 2–10 μM OXY in 30 μM fractionated AGP solutions (1.5 mL) were directly injected onto the HPFA column to determine the unbound OXY concentrations. The HPFA column and the analytical column were kept at 37°C in a column oven.

Validation Studies

Calibration lines were prepared as follows: Ten-microliter portions of a series of standard solutions at 2, 5, 10, 20, 30, and 50 μM for (*R*)- or (*S*)-OXY in methanol were used to prepare calibration lines. Each 10-μL portion of the (*R*)- or (*S*)-OXY standard solution in methanol was directly injected into the extraction column. A calibration line was then prepared by plotting the peak area *vs*. the quantity of injected drug. The unbound OXY concentrations were calculated by dividing the amount of drug (picomoles) by the heart-cut volume. The resulting calibration curves showed good linearity ($r^2 > 0.994$).

The percent recovery of OXY from the extraction column was determined using standard samples of 2, 20, and 50 μM for each enantiomer. The peak-area ratios of three extracted samples were then compared with the unextracted samples to determine the percent recovery. The accuracy was evaluated by back-calculation and expressed as the percent deviation between the amount found and the amount added for each enantiomer of OXY for the three concentrations examined.

RESULTS AND DISCUSSION

AGP was separated into F1–S and A fractions by iminodiacetate (IDA)–copper (II) affinity chromatography according to Herve *et al.* (27). Con A lectin affinity chromatography can separate AGP having biantennary glycan chains. The lectin Con A has the ability to recognize and bind complex biantennary oligosaccharide chains. AGP containing one or more of these chains reacts readily with the α-mannosyl binding lectin, whereas AGP, containing only tri- and tetraantennary glycans, is unreactive (28). The AGP unretained on the Con A column (UR-AGP) is completely devoid of biantennary chains, whereas that retained (R-AGP) contains biantennary forms (29). In this study, native AGP was fractionated into four fractions by the two-step method (Fig. 1). Table II shows the distribution of these fractions. The proportions of the F1–S and A variants were almost the same between UR-AGP and R-AGP, and the proportions of UR-AGP and R-AGP were almost the same between the F1–S and A variants.

For HPFA, it is essential to obtain a plateau region in the chromatogram, for which HPLC conditions including the sample injection volume should be properly optimized. If the injection volume is insufficient or if the elution time of the analyte is too short or long, a clear plateau region cannot be obtained. Figure 2 shows a representative HPFA profile. A clear plateau zone due to unbound OXY was observed in the chromatogram, indicating that the experimental conditions were suitable for the frontal analysis.

A series of 30 μM fractionated AGP sample solutions containing 2, 4, 6, 8, and 10 μM (*R*)- or (*S*)-OXY were subjected to the on-line HPFA–HPLC analysis to determine the unbound OXY concentrations. Each sample was analyzed in triplicate. The coefficients of variation of the analytical values were less than 3.29%. The average recovery and accuracy for OXY were 98 and 98.5% over the three concentrations.

The binding affinity was calculated from the following equation: $nK = (C_t - C_u)/P \times C_u$, where nK, C_t, P, and C_u represent total binding affinity, total drug concentration, total protein concentration and unbound drug concentration, respectively.

Table III lists the total binding affinities of the OXY enantiomers. Several interesting features were revealed. First, the affinities for the F1–S mixed variant were signifi-

Table III. Total Binding Affinities of OXY Enantiomers in AGP Variants

	F1–S		A	
Enantiomer	UR (M^{-1})	R (M^{-1})	UR (M^{-1})	R (M^{-1})
(*R*)-OXY	$(9.49 \pm 0.151) \times 10^6$	$(21.4 \pm 0.183) \times 10^6$	$(1.06 \pm 0.140) \times 10^6$	$(1.07 \pm 0.113) \times 10^6$
(*S*)-OXY	$(14.4 \pm 0.190) \times 10^6$	$(8.30 \pm 0.142) \times 10^6$	$(1.25 \pm 0.161) \times 10^6$	$(1.10 \pm 0.102) \times 10^6$
R/*S*	0.66	2.58	0.85	0.97

cantly higher than those for the A variant. Second, the affinities for the F1–S mixed variant were dependent on the chirality of the drug as well as the presence of biantennary chains, whereas those for the A variant were independent of these factors. The F1–S variant with biantennary chains showed selectivity for the R enantiomer, whereas that of F1–S-UR exhibited the opposite preference. These findings demonstrated for the first time that biantennary chains in AGP can influence the enantioselectivity for drug binding. It should be noted that the average binding affinities of (*R*)-OXY and (*S*)-OXY for the F1–S variants were similar between the UR and R fractions: Binding studies using racemic OXY would conclude that the biantennary glycan structure does not affect AGP binding, as in the case of oxprenolol (24).

OXY is clinically used as a racemate. (*R*)-OXY exhibits high antimuscarinic activity relative to the antispasmodic activity, whereas (*S*)-OXY exhibits relatively weak antimuscarinic activity (30). The total concentrations of (*R*)- and (*S*)-OXY in normal human serum were reported to be 4–13 and 1–3.5 ng/mL, respectively. Both enantiomers are highly bound to plasma proteins (99%). The free fraction of (*R*)-OXY is 1.6-fold higher than that of (*S*)-OXY (31). Changes in the serum levels and molecular forms of AGP can modulate pharmacological responses by changing free drug concentration. For example, the AGP concentration in normal human serum is 20–30 μM. In cirrhotic patients, total serum AGP concentration is decreased to 73.4% of the normal concentration, and the UR/R ratio increases to 11:8 from the normal value of 9:11, although the disease does not change the heterogeneity in genetic variants (24). Our results suggest that the unbound (*R*)-OXY concentration in cirrhotic patients should be significantly increased.

In IDA–Cu^{2+}affinity chromatography, AGP is retained by chelations of copper (II) ions to histidine residues (32). Both the F1–S and A variants have three histidine residues. Because AGP variants are divided into two fractions, F1–S and A, there must be some conformational difference between these variants. This may explain why the A variant and the F1–S mixture exhibit different binding properties (33). The differences in drug binding between the A variant and the F1–S mixed variant may also be related to the differences in their primary structure. The amino acid sequences deduced from the AGP A gene differ at 22 positions from those deduced from the AGP B gene (17). Genetic characterization of AGP variants has shown that F1 and S are encoded by two alleles of the same gene and should differ by only a few amino acids. However, the A variant is encoded by the other gene of AGP and should differ from F1 and S by more amino acids. The circular dichroism spectra for the wavelengths 200–260 nm showed no significant difference among these variants (data not shown), suggesting small and local conformational differences around the drug binding pocket on the AGP molecule. Furthermore, under pathological conditions such as in patients with malignant mesothelioma (34) and burn injuries (35), an increase in the AGP plasma concentration occurs, and the contribution of the F1 and S variants to the increase is greater than that of the A variant. Therefore, the alteration of plasma concentrations and distributions of genetic variants should affect the pharmacokinetic properties and the pharmacological activities of drugs. Additionally, the glycosylation pattern of AGP (36) and degree of microheterogeneity (37) is altered under such conditions. The former property may be related to the polypeptide moiety of AGP, whereas the latter seems to be glycosylation-dependent. Evidence exists that the F1–S and A variants have different ligand-binding properties (38) and that individual AGP glycoforms may have functional diversity during immunomodulation (39).

In conclusion, this study for the first time revealed biantennary glycans of F1–S variants affect the enantioselectivity of OXY. Further study is required to elucidate the enantioselective mechanism for drug–AGP binding.

ACKNOWLEDGMENTS

The authors wish to acknowledge the advice provided by Professor Terumichi Nakagawa (Setsunan University, Japan). The authors also thank Professor Irving W. Wainer (National Insitute on Aging, U.S.A.) for providing samples. This research was supported by the 21st Century Center of Excellence Program "Knowledge Information Infrastructure for Genome Science" from the Japan Society for the Promotion of Science.

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