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Journal of Pharmacology & Clinical Toxicology

Research Article

Interactions of Linezolid with Two Major Serum Proteins, Human Serum Albumin and Alpha-1 Acid Glycoprotein

Kazuaki Taguchi[1], Keishi Yamasaki[1,2], Akiko Shiga[1], Masaki Otagiri[1,2,3]* and Hakaru Seo[1,2#]*

[1]*Faculty of Pharmaceutical Sciences, Sojo University, Japan*
[2]*DDS Research Institute, Sojo University, Japan*
[3]*Department of Biopharmaceutics, Kumamoto University, Japan*

***Corresponding authors**
Hakaru Seo, Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 862-0082, Japan; Tel: +81-96-326-4076; Fax: +81-96-326-5048; E-mail: seo@ph.sojo-u.ac.jp

Masaki Otagiri, Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 862-0082, Japan; Tel: +81-96-326-3887; Fax: +81-96-326-3887; E-mail: otagirim@ph.sojo-u.ac.jp
#Both the authors contribute equally

Submitted: 20 October 2014
Accepted: 14 January 2015
Published: 17 January 2015
ISSN: 2333-7079

Keywords
- Linezolid
- Albumin
- Alpha1-acid glycoprotein
- Protein binding

Abstract

Linezolid (LZD) is an oxazolidinone antibiotic agent that acts against gram-positive bacteria. It was recently reported that LZD can induce severe hematologic toxicity, which is thought to result in high LZD concentrations in plasma. Although many factors can affect the disposition of drugs, the binding of drug to plasma proteins, such as albumin and alpha1-acid glycoprotein (AGP), is one of the most important factors due to the competitive displacement of drugs from proteins. However, little is known about the interactions of LZD with these proteins. The aim of this study was to elucidate the binding characteristics of LZD to human serum albumin (HSA) and AGP, including binding parameters and the binding site. Based on the results of fluorescence studies and ultrafiltration experiments, it appears that LZD binds to both HSA and AGP, but the affinity of LZD for both proteins was low. Competitive protein binding analyses using an ultrafilitration method clearly indicated that LZD binds to the digitoxin binding site on HSA and the basic and/or hydrophobic drug binding site on AGP. These detailed analyses of LZD-HSA or LZD-AGP interactions provide valuable information in terms of understanding the pharmacokinetics properties of LZD in clinical settings.

ABBREVIATIONS

LZD: Linezolid; AGP: Alpha1-Acid Glycoprotein; HSA: Human Serum Albumin; WF: Warfarin Potassium; Auo: Auramine O; DNSS: Dansylsarcosine; BR: Bilirubin; IDP: Iodipamide; PBZ: Phenylbutazone; DIG: Digitoxin; NR: Nile Red; ANS: 8-Anilino-1-Naphthalenesulfonic Acid

INTRODUCTION

Linezolid (LZD; Figure 1), a member of the oxazolidinone family, exhibits a broad spectrum of activity against gram-positive bacteria, including methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci, and has very favorable rates of penetration into tissues [1]. It is available in both intravenous and oral formulations and can be switched from intravenous to oral formulations (or oral to intravenous formulations) without dose modification due to its 100% absolute bioavailability of oral administration. Furthermore, LZD, unlike other anti-methicillin-resistant Staphylococcus aureus drugs, can be safely administered without the need to adjust the dosage, based on therapeutic drug monitoring, even in patients with impaired renal function, because the pharmacokinetic profiles of LZD in patients with and without renal insufficiency were found to be quite similar [2]. However, recent clinical studies have shown that LZD can induce severe hematologic toxicity, particularly thrombocytopenia, in patients with renal insufficiency [3], which is thought to be associated with high LZD concentrations in the plasma [4]. Therefore, it is frequently recommended that therapeutic drug monitoring of LZD would be

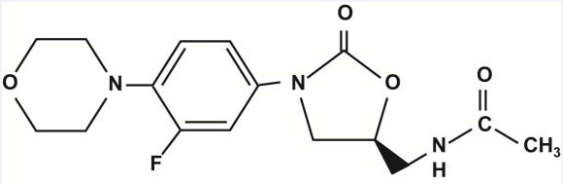

Figure 1 Chemical structure of LZD ((-)-N-[[(S)-3-(3-fluoro-4-morpholinophenyl)-2-oxo-5- oxazolidinyl]methyl]acetamide).

especially helpful for dosage adjustment. In fact, Pea *et al*., based on results from a retrospective study reported that adequate maintenance of the LZD trough concentration can be helpful in avoiding the hematological toxicity induced by LZD [5]. Thus, it is important to regulate the pharmacokinetics, especially the plasma concentration, of LZD.

The binding of drugs to plasma proteins, such as albumin and alpha1-acid glycoprotein (AGP) is a major determinant in the disposition of a drug. However, the interaction of LZD with these proteins has not been well evaluated. In this study, the binding characteristics of LZD to human serum albumin (HSA) and AGP was investigated using a fluorescence method, a fluorescence probe displacement method and competition experiments, and the binding site, binding constant (*K*) and the number of binding sites (*n*) were also determined.

MATERIALS AND METHODS

Reagents

HSA (fatty acid free) and human AGP were purchased from Sigma-Aldrich (Missouri, USA). LZD (LKT Laboratories Inc., St. Paul, MN), warfarin potassium (WF) (Eisai Co., Tokyo, Japan), Auramine O (AuO) (Nakalai Tesque, Inc. Kyoto, Japan), dansylsarcosine (DNSS), bilirubin (BR) and iodipamide (IDP) (Sigma, St. Louis, MO, USA) were obtained as pure substances from the manufacturers. Phenylbutazone (PBZ), digitoxin (DIG), Nile Red (NR) and 8-Anilino-1-naphthalenesulfonic Acid (ANS) were purchased from Tokyo chemical industry CO., LTD (Tokyo, Japan). All other chemicals were of the highest grade commercially available. LZD solution was prepared using phosphate buffer (67 mM, pH 7.4).

Fluorescence Quenching Experiments

Fluorescence quenching experiments were carried out using a HITACHI F-2500 fluorescence spectrometer (Tokyo, Japan) in a 1 cm quartz cell using an excitation wavelength of 285 nm. Emission spectra in the absence and presence of LZD were recorded at 300–550 nm. Fluorescence titrations were performed by maintaining the HSA or AGP concentration (2.5 μM in 67 mM phosphate buffer, pH 7.4) constant and stoichiometrically

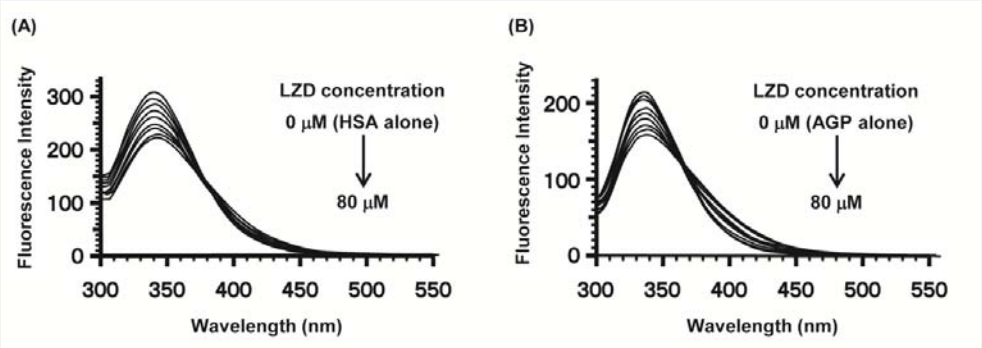

Figure 2 Fluorescence emission spectra of (A) HSA-LZD and (B) AGP-LZD in 67 mM phosphate buffer pH 7.4.
Free HSA or AGP alone (2.5 μM) and free HSA or AGP (2.5 μM) with different concentrations of LZD (5, 10, 20, 30, 40, 50, 60, 70 and 80 μM). Excitation wavelength = 285 nm, Emission wavelength = 300-550 nm.

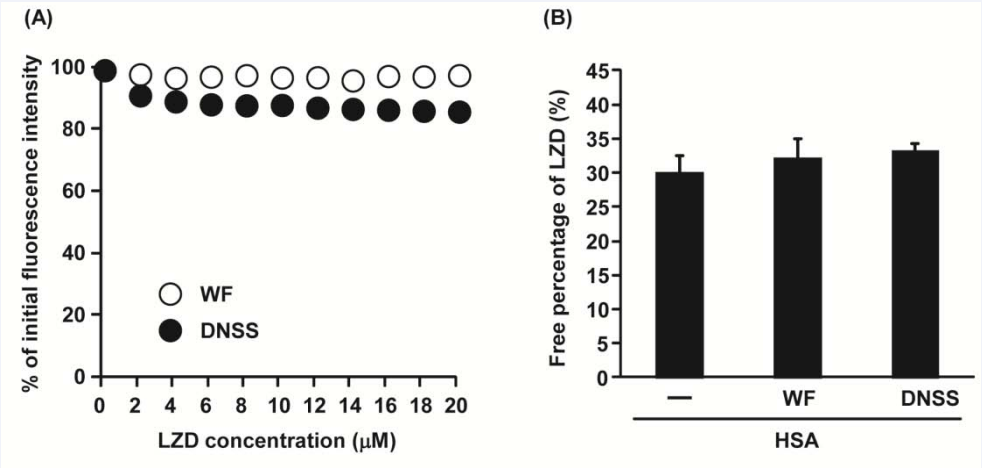

Figure 3 (A) Effect of LZD on the fluorescence intensity of WF (open circle) and DNSS (closed circle) bound to HSA. The following concentrations were used: HSA, 2 μM; WF and DNS, 2 μM.
(B) Competitive HSA binding of LZD and each site marker of HSA (WF and DNS) at pH 7.4 and 25**°C.** The binding of LZD to HSA in the presence of 60 μM site markers is shown. Values are expressed as the mean ± S.D. (*n* = 6).

varying the LZD concentration (0–80 μM). The fluorescence intensity derived from LZD is negligible.

Determination of the Bound Percentage of LZD to Human Plasma, HSA and AGP

The samples were prepared in Eppendorf tubes, where pooled human plasma (Interstate Blood Bank Inc., Lot#: UT075998), 600 μM HSA or 45 μM AGP were incubated with 100 μM of LZD. The fractions of free LZD and LZD bound to HSA or AGP were separated by ultrafiltration using Amicon Ultra Centrifugal Filters (Millipore, MW CO 10 kDa) and centrifugation for 10 min at 3,000 g. The free LZD concentrations were then measured by high performance liquid chromatography as described in a previous report [6].

Determination of LZD Binding Parameters

The samples were prepared in Eppendorf tubes, where 60 μM HSA or AGP were incubated with 6, 12, 18, 24, 30, 45, 60 and 120 μM of LZD. The fractions of free LZD and LZD bound to HSA or AGP were separated by ultrafiltration using Amicon Ultra Centrifugal Filters (Millipore, MW CO 10 kDa) and centrifugation for 10 min at 3,000 g. The free LZD concentrations were then measured by high performance liquid chromatography as described in a previous report [6]. All binding parameters were analyzed according to the Scatchard equation shown below:

$$r/[D_f] = nK\text{-}rK$$

Where r is the moles of drug bound per mole of protein, $[D_f]$ is the concentration of free drug, n is binding site multiplicity per class of binding sites, and K is the equilibrium binding constant.

Fluorescent Probe Displacement Experiments

WF and DNSS were used as fluorescent probes for site I and site II on HSA, respectively [7]. Both probes were dissolved in

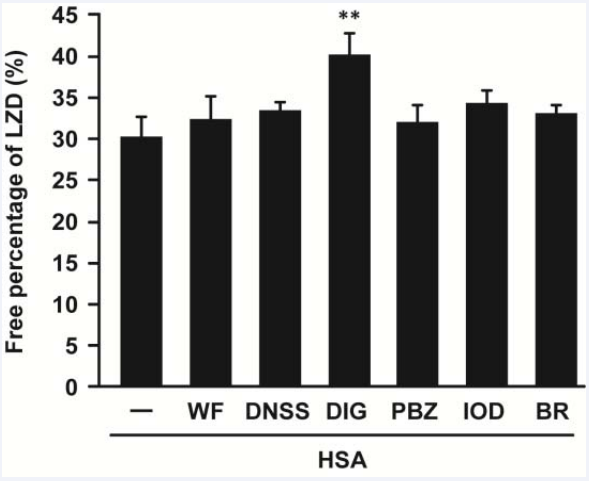

Figure 4 Effect of site markers for HSA (WF, DNSS, DIG, PBZ, IOD and BR) on free percentage of LZD at pH 7.4 and 25°C. The binding of LZD to HSA in the presence of 60 μM site markers are shown. Values are expressed as the mean ± S.D. (n = 3-6). **p<0.01 vs. HSA only.

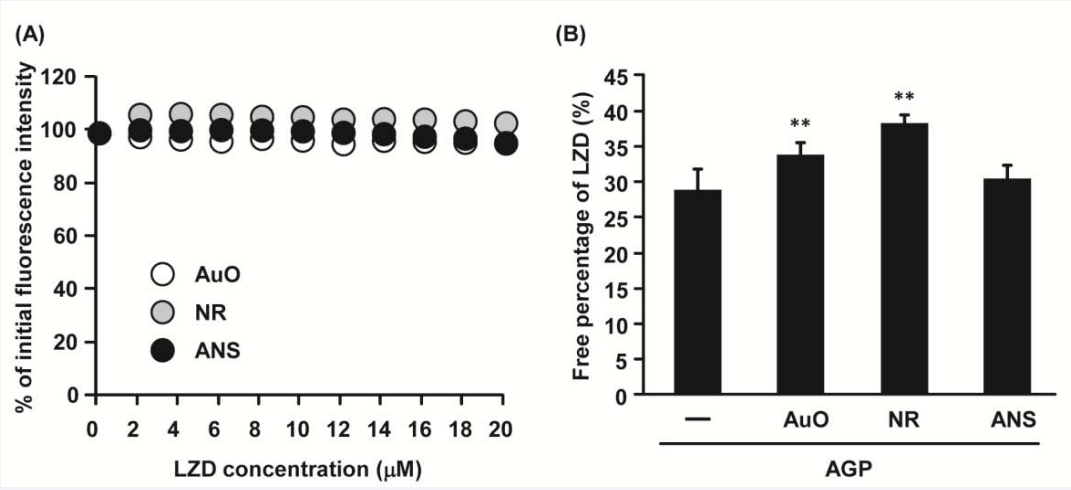

Figure 5 (A) Effect of LZD on the fluorescence intensity of AuO (open circles), NR (gray circles) and ANS (closed circles) bound to AGP. The following concentrations were used: AGP, 1.5 μM; AuO, 20 μM; NR, 1.5 μM; ANS, 10 μM.
(B) Effect of site marker for AGP (AuO, NR and ANS) on free percentage of LZD at pH 7.4 and 25°C. The binding of LZD to AGP in the presence of 60 μM site markers are shown. Values are expressed as the mean ± S.D. (n = 6). **p<0.01 vs. AGP only

100% ethanol. The displacement experiments for WF and DNSS by LZD were performed by recording the fluorescence of a solution containing 2 μM WF or DNSS and 2 μM HSA, while gradually increasing the concentration of LZD. The excitation wavelengths for WF and DNSS were 320 and 350 nm, respectively. The emission spectra for WF and DNSS were recorded in the range of 350–450 nm and 400–600 nm respectively.

For probe displacement experiments of AGP, an LZD solution was titrated into a 1 cm quartz cell containing a 2 μM AGP-probe complex solution. Three fluorophores were employed to purportedly probe the acidic (ANS), basic (AuO) and hydrophobic (NR) drug binding sites on AGP [8-10]. AGP and AuO were dissolved in 67 mM phosphate buffer (pH 7.4), and ANS and NR were dissolved in dimethyl sulfoxide. The excitation wavelengths specific for each probe were as follows: ANS (400 nm), Au O (428 nm) and Nile Red (550 nm). The emission spectrum for each probe was collected across the following wavelengths: ANS (420–600 nm), AuO (460–660 nm) and NR (590–750 nm).

Site Marker Competitive Experiments

Binding studies between LZD and HSA (or AGP) in the presence of site markers were measured using the ultrafiltration method. The 60 μM HSA or AGP solution, 60 μM LZD and 60 μM each site markers (HSA; WF, DNSS, PBZ, IDP, DIG and BR, AGP; ANS, NR and AuO) mixture were ultrafiltrered at 25°C and 3000 g for 10 min using Amicon Ultra Centrifugal Filters (Millipore, MW CO 10 kDa). Free LZD concentrations were measured by high performance liquid chromatography as described in a previous report [6]. The retention behavior of LZD was not affected by the presence of all site markers.

Statistical Analysis

Statistical analyses were performed by using one-way analysis of variance. A probability value of $p< 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Interaction of LZD with HSA and AGP

Drug-HSA or drug-AGP interactions were determined by fluorescence quenching of the protein upon drug binding [11,12]. Although the maximum fluorescence emission of protein arises from tryptophan, tyrosine and phenylalanine, phenylalanine has a very low quantum yield and the fluorescence of tyrosine is nearly completely quenched if it is ionized or located near an amino group, a carboxyl group or a tryptophan residue. Thus, the interactions of LZD with both proteins were first evaluated by measuring the intrinsic fluorescence intensity of tryptophan residues in HSA or AGP in the absence and presence of LZD. As a result, the maximum fluorescence of both HSA and AGP were quenched upon the binding of LZD in the presence of increasing concentrations of LZD (Figure 2). In addition, neither the shape nor the maximum wavelength of the HSA and AGP emission spectra were changed in the presence of LZD. These data indicate that LZD binds to both HSA and AGP, and micro environmental changes around tryptophan residues are induced in both HSA and AGP by the presence of LZD.

To confirm the results obtained by the fluorescence quenching experiments, the binding of LZD to HSA and AGP were further determined using an ultrafiltration method. The percentage bound to plasma was approximately 40%, a value that is close to the data obtained using ^{14}C-LZD described in the package insert [13]. In addition, LZD binds to HSA and AGP with the physiological concentration (HSA; 600 μM, AGP; 45μM), and the percentage of both HSA and AGP bound was approximately 42.2 ± 2.4% and 37.8 ± 2.2%, respectively. Taken together with fluorescence quenching and ultrafiltration experiment, it can be concluded that LZD interacts with both HSA and AGP.

Determination of Binding Parameters of LZD for HSA and AGP

The binding parameters of LZD for HSA and AGP were determined using an ultrafiltration method. The individual binding parameters, the *n* and *K* values, obtained by applying the Scatchard equation, are summarized in (Table 1). The data show that the binding affinities of LZD to both HSA and AGP are low. Moreover, there is only a single binding site for LZD in both HSA and AGP.

Identification of the Binding Site of LZD on HSA

It is well known that there are major specific drug binding sites, site I and site II, on HSA [14]. X-ray diffraction studies have shown that HSA is made up of three homologous domains, namely, I-III, each of which is comprised of two subdomains, referred to as A and B, and the these major specific drug binding sites on HSA are located in hydrophobic cavities in subdomains IIA and IIIA [15-17]. To identify the binding site of LZD to HSA, we first performed site marker displacement experiments using representative fluorescent probes, WF (a site I probe) and DNSS (a site II probe), based on the method of Sudlow *et al.* [18]. As shown in (Figure 3A), the fluorescent properties of both the WF-HSA and the DNSS-HSA complex were not changed by LZD. Because the binding affinity of WF (3.4×10^5 M^{-1}) [19] and DNSS (1.4×10^6 M^{-1}) [20] is 1~2 orders greater than that of LZD, it is possible that LZD did not displace the fluorescent probes. Thus, we then investigated whether these fluorescent probes competitively block the binding of LZD to HSA using an ultrafilitration method. The results indicated that WF and DNSS had no effect on the free percentage of LZD (Figure 3B). These data suggest that LZD does not bind to major specific drug binding sites (site I and II) but, rather, binds to other binding sites on HSA.

Site I is not simple binding region but is rather complex and is comprised of three subsites, namely, Ia, Ib and Ic [19]. In addition, not all substances, including drugs and endogenous substances, bind to sites I or II. Curry and collaborators demonstrated the presence of BR and 7 fatty acid binding sites through a crystallographic analysis [15,21]. In addition, a digitoxin binding

Table 1: Binding parameters of LZD to HSA and AGP as determined by ultrafiltration at pH 7.4 and 25°C.

	K	*n*
	$\times 10^4$ (M^{-1})	
HSA	4.3 ± 0.5	0.89 ± 0.15
AGP	4.0 ± 1.2	0.87 ± 0.14

K: binding constant; *n*: number of binding sites
Values are expressed as the mean ± S.D. ($n = 3$).

site has also been identified [22,23]. Thus, we finally investigated whether these substances competitively block the binding of LZD to HSA using an ultrafirtration method. PBZ (Ia~Ib) and IDP (Ia~Ic) are used as markers for subsite Ia, Ib and Ic [19]. As shown in Figure 4, the percentage of free LZD was significantly increased when DIG was used, whereas other markers (PBZ, IOD and BR) had no effect on these values. In addition, since there are 7 fatty acid-binding sites on HSA, we also carried out the competitively blocking of the binding of LZD to HSA in the presence myristic acid. As a result, the presence of a fatty acid (myristic acid) had no effect on the free percentage of LZD, even though the concentration of myristic acid was increased up to 7 times compared to the concentration of LZD (data not shown). These data suggest that LZD binds to the digitoxin binding site on HSA.

The change in free drug concentration due to the displacement of drugs from the binding site may influence the pharmacological activity of a drug or the induction of side effects. In fact, Setoguchi *et al.* reported that pain relief in rheumatoid arthritis patients using a diclofenac suppository was increased by the simultaneous oral administration of nabumetone due to the competitive displacement of diclofenac from the binding site by the coadministered nabumetone, because both diclofenac and the active metabolite of nabumetone bind strongly to site II on HSA [24]. In addition, a similar phenomenon was observed in a study using rats on the combination therapy of flurbiprofen axetil and a lipid emulsion [25]. Thus, an increase in the free fraction of LZD may cause an enhancement in pharmacological activity (antimicrobial activity) or the induction of side effects (hematologic toxicity) when the competitive displacement of LZD from the binding site of HSA occurs as the result of the coadministration of drugs or endogenous substances. In this study, since the binding affinities of LZD to HSA are low, it is thought that LZD may be easily displaced from the binding site by competitive substances. However, since LZD binds to the digitoxin binding site on HSA (little is known regarding the binding of substances to the digitoxin binding site on HSA), LZD would not be insusceptible to competitive displacement on HSA by coadministered drugs or endogenous substances.

Binding interaction of LZD with AGP

To identify the binding site of LZD on AGP, site marker displacement experiments using representative fluorescent probes, ANS (an acidic drug binding site probe), AuO (a basic drug binding site probe) and NR (a hydrophobic drug binding site probe), were performed based on a previous report [26]. As a result, no fluorescent changes of any of the fluorescent probe-complexs were observed in the addition of LZD (Figure 5A). Because, the binding affinity of each fluorescent probe of AGP is much higher than that of LZD itself, it is possible that LZD is not capable of displacing the fluorescence probes. We next investigated whether these fluorescent probes competitively block the binding of LZD to AGP using an ultrafilitration method. As a result, AuO and NR caused a significantly increased free percentage of LZD, while ANS had no effect on the free percentage of LZD (Figure 5B). In a previous study, it was clarified the drug-binding sites on AGP, as determined by displacement experiments using fluorescent probes, indicated that the binding sites on AGP are not completely separated, but rather may be overlapped for basic, acidic and neutral drugs [27]. Thus, LZD was displaced by both AuO and NR. In addition, AGP is a mixture of at least two genetic variants: the A variant and the F1 and/or S variant. (F1*S variant). Recently, the X-ray crystallographic structures of the A and F1*S variants have been reported by our and other groups showed that the binding pocket of the F1*S variant consists of three lobes (I–III) whereas two lobes (I and II) are involved in the case of the A variant [28,29]. It is known that F1*S variant has wide, overlapping binding sites for basic, acidic and neutral drugs, while the A variant contains binding sites for basic and neutral drugs but not acidic drugs [27,30]. Based on these facts, LZD, which is a basic drug, would be located around the basic and hydrophobic drug binding sites on the binding pocket of both the F1*S and A variants.

Petain *et al.* reported that the concentration of AGP is significantly correlated with the clearance and/or volume of distribution of imatinib [31]. This indicates that the binding of drugs to AGP has the potential to play a crucial role in regulating its plasma concentration. In this study, the percentage of LZD that is bound to AGP (approximately 40%) was much lower than the binding of other drugs to AGP, such as imatinib (more than 96%) [32]. Therefore, it is unlikely that the binding of LZD to AGP affects on disposition or plasma concentration of LZD.

CONCLUSION

This study is the first report concerning the interaction of LZD with two major serum proteins, namely, HSA and AGP. Although LZD binds to both HSA and AGP, the percentage of bound molecules and the affinity of LZD for both proteins were low. Therefore, it is not necessary to take into consideration drug-drug interaction on proteins, even with the advent of the hematologic toxicity in patients associated with changes in LZD concentrations in plasma. However, the concentration of AGP may increase up to three- or four-fold under pathological conditions, including infections, because AGP is an acute phase reactant protein [33]. In addition, hypoalbuminemia can also occur under several pathological conditions, such as the nephrosis syndrome and chronic liver failure [34]. In these patients, the possibility of a temporary or a continuous change of the concentration of LZD in plasma cannot be excluded. Therefore, careful attention to changes in the LZD concentration in plasma is called for in these patients. Furthermore, it is also necessary to identify the preferential amino acid residues of HSA and AGP that play an important role for LZD binding. In the future, it should realize this point using site-directed mutagenesis such as chemical or genetic modified HSA and AGP.

ACKNOWLEDGEMENTS

This work was supported by a research grant from Sojo University.

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Cite this article

Taguchi K, Yamasaki K, Shiga A, Otagiri M, Seo H (2015) Interactions of Linezolid with Two Major Serum Proteins, Human Serum Albumin and Alpha-1 Acid Glycoprotein. J Pharmacol Clin Toxicol 3(2):1045.