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Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 1011–1016

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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An ELISA for quantification of murine IgG in rat plasma: application to the pharmacokinetic characterization of AP-3, a murine anti-glycoprotein IIIa monoclonal antibody, in the rat

Ryan J. Hansen, Joseph P. Balthasar *

Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84108, USA

Received 2 February 1999; received in revised form 19 July 1999; accepted 26 July 1999

Abstract

An enzyme-linked immunosorbent assay (ELISA) has been developed to determine concentrations of murine IgG in rat plasma. Specifically, the assay was developed to measure a murine anti-glycoprotein IIIa antibody (AP-3) in rat plasma to facilitate future investigations of AP-3 pharmacokinetics and pharmacodynamics in the rat. The working range of the assay is 15–100 ng ml^{-1}, corresponding to a limit of quantification of 1.5 μg ml^{-1} in rat plasma. The assay was validated with respect to accuracy, precision, and cross-reactivity with both pooled rat and mouse IgG. Intra-assay recoveries of AP-3 in rat plasma ranged from 93 to 103% with CV% values ranging from 5.2 to 8.5%. Inter-assay recoveries of the plasma AP-3 samples ranged from 107 to 119% with CV% values ranging from 17.7 to 25.1%. The assay has no appreciable cross reactivity with pooled rat IgG and full cross reactivity with pooled mouse IgG, making this an ideal assay to determine plasma pharmacokinetics of mouse antibodies in the rat. The assay was used to determine the pharmacokinetics of AP-3 in a Sprague–Dawley rat.

Keywords: Murine; IgG; ELISA; Validation; Pharmacokinetics; Rat plasma; GPIIIa; AP-3

1. Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune condition characterized by an increase in platelet destruction by the mononuclear phagocyte system due to the interaction of anti-platelet autoantibodies with platelet membrane proteins [1,2]. In addition to the use of immunosuppressants or splenectomy, administration of intravenous high-dose pooled human immune gamma globulin (HD-IgG) has been used to treat patients with ITP [3]. Although several mechanisms have been proposed to explain the therapeutic activity of HD-IgG in ITP, a complete understanding of the mechanism(s) of this treatment has not yet been achieved [4–6].

* Corresponding author. Fax: +1-801-585-3614.
E-mail address: jbalthasar@pharm.utah.edu (J.P. Balthasar)

0731-7085/99/$ - see front matter
PII: S0731-7085(99)00227-7

The platelet membrane glycoprotein IIb/IIIa complex is a common target of autoantibodies in patients afflicted with ITP [2,7]. AP-3, a mouse monoclonal IgG_1 antibody directed against human glycoprotein IIIa (GPIIIa), was first produced and characterized in 1984. This antibody was shown to bind human platelet GPIIIa with an affinity of 1.4×10^9 M^{-1}, without inhibiting induced platelet aggregation by such agents as adenosine diphosphate, thrombin, collagen, or arachidonic acid [8].

A rat model of ITP is being developed in this laboratory to better understand the interaction of anti-platelet antibodies with platelet antigens, to investigate relationships between anti-platelet antibody concentrations and the kinetics of platelet destruction, and to test proposed hypotheses about the mechanism of HD-IgG in ITP treatment. This model attempts to induce immune thrombocytopenia through the administration of AP-3 to Sprague–Dawley rats. Preliminary results have demonstrated that AP-3 administration leads to thrombocytopenia with a corresponding increase in tail-vein bleeding time (unpublished results). Quantitative pharmacokinetic and pharmacodynamic models will be created to facilitate testing of proposed hypotheses.

Quantitative modeling of the time course and effects of AP-3 requires the ability to accurately and precisely measure AP-3 levels in rat plasma. Radio-labelled IgG has been commonly used to determine mouse IgG concentrations in rat plasma for pharmacokinetic analyses [9,10]. However, use of an ELISA to measure plasma concentrations of IgG in the rat circumvents the problems encountered using radio-labelled mouse IgG to study pharmacokinetics, such as difficulty separating metabolized antibody from intact antibody, and the safety issues involved with using radioactive substances.

Several researchers have reported using ELISAs to measure mouse IgG concentrations hybridoma supernatants, ascites [11], mouse serum [12], and human serum [13]; some groups have even reported use of an ELISA to determine mouse monoclonal antibody concentrations in rat plasma [14,15]. However, to the authors' knowledge, this work represents the first report of an appropriately validated ELISA used to quantify a murine monoclonal antibody in rat plasma. The assay presented in this paper was validated with respect to intra-assay and inter-assay precision and accuracy, and was tested for cross-reactivity with pooled mouse and rat IgG.

2. Experimental

2.1. Production and purification of AP-3

Hybridoma cells producing the desired mouse anti-human GPIIIa antibody (AP-3) were obtained from the American Type Culture Collection (ATCC # HB-242, Manassas, VA). Balb/C mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were prepared for ascites production by an intraperitoneal injection of pristane (Sigma Chemical, St. Louis, MO), 0.5 ml/mouse, approximately one week prior to injection of antibody producing cells. Hybridoma cells, 5×10^5 cells/mouse, were then injected intraperitoneally into the mice. Mice were sacrificed 10–20 days after injection of the cells, and ascitic fluid was obtained.

The ascitic fluid obtained from the mice was centrifuged and the straw-colored supernatant was isolated. Anti-GPIIIa IgG was purified from the supernatant by Protein G chromatography (Pharmacia Biotech Hi-Trap protein G column, Uppsala, Sweden) on a Bio-Rad medium pressure chromatography system. The loading buffer for the protein G chromatography was a 20 mM Na_2HPO_4 (Sigma Chemical) buffer, pH 7.0; the elution buffer was a 0.1 M citric acid (Sigma Chemical) buffer, pH 2.7. Sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) was utilized to ascertain the purity of the IgG pool, and a Micro-Bicinchoninic Acid (BCA) assay kit was used to determine protein concentration (Pierce Kit, # 23235, Rockford, IL).

2.2. ELISA procedure

Assays were performed in Nunc Maxisorp 96 well microplates (Nunc model # 442404, Roskilde, Denmark). Fc specific goat anti-mouse antibody (Pierce M-3534, 1:500 in 0.02 M

Na_2HPO_4, no pH adjustment), 0.2 ml, was placed in each of the wells to be tested and incubated at 4°C overnight. The plate was then washed three times with a tween containing phosphate buffer (PB-Tween) consisting of 0.05% Tween 20 (Sigma Chemical) and 0.02 M Na_2HPO_4 (Sigma Chemical, no pH adjustment). After the wash, the plate was incubated with samples and standards (0.1 ml) for 2 h at room temperature. The plate was washed three times with PB-Tween, and then incubated with 0.2 ml Fab specific goat anti-mouse-antibody–Alkaline Phosphatase conjugate (Pierce A1682, 1:500 in 0.02 M Na_2HPO_4, no pH adjustment) for 1 h at room temperature.

Finally, after washing again with PB-Tween three times, 0.2 ml of p-nitro phenyl phosphate (Pierce, 5 mg ml^{-1} in distilled water) was added to each of the wells and the change in absorbance with time was monitored via a microplate reader at 405 nm (Spectra Max 250, Molecular Devices, Sunnyvale, CA). A standard curve was generated by plotting concentration of AP-3 versus the change in absorbance with time (dA/dt). Standards were made by diluting a stock AP-3 solution to the appropriate concentration (0, 15, 25, 50, 75, and 100 ng ml^{-1}) with phosphate buffered saline (PBS, pH 7.4), with addition of rat plasma (Hilltop Laboratories, Scottdale, PA) to a final concentration of 1% (v/v). Standard curves included a blank standard; consequently no additional correction for non-specific binding was required to evaluate sample concentrations from the standard curve. The assay was validated for precision and accuracy by evaluating the recovery of AP-3 from rat plasma samples. The concentrations of AP-3 in rat plasma samples were initially 1.5, 2.5, 5.0 and 10.0 μg ml^{-1}; samples were diluted by a factor of 100 in PBS immediately before analysis.

2.3. Cross-reactivity of the ELISA with mouse and rat IgG

Mouse and rat IgG were purified from pooled mouse and rat plasma (Hilltop Laboratories). Purification was performed using protein G chromatography as described for AP-3. SDS-PAGE and BCA analysis were performed as described previously. Serial dilutions were made of the mouse or rat IgG, and assay response was determined using the procedures described above. Assay response to pooled mouse IgG was tested over a concentration range of 16.2–108 ng ml^{-1}. Assay response to pooled rat IgG was tested over a concentration range of 15–400 μg ml^{-1}.

2.4. Specific activity of AP-3 preparation

AP-3, 5 μg ml^{-1}, was incubated with 3×10^9/ml outdated human platelets (American Red Cross, Salt Lake City, UT) in PBS, overnight while shaking. Samples were then centrifuged at $13\,000 \times g$ for 6 min to pellet out the platelets and the platelet-bound AP-3. After appropriate dilution in PBS, samples were analyzed for free AP-3 concentration using the ELISA. Samples of pooled mouse IgG were incubated with the platelets as a negative control, and samples of AP-3 and pooled mouse IgG incubated without platelets were used as a reference concentration for 100% recovery. The specific activity was defined as the percent of the IgG bound to the platelets.

2.5. Pharmacokinetic studies

A female Sprague–Dawley rat (Harlan Sprague–Dawley), 0.2 kg, was instrumented with an abdominal aorta cannula. AP-3, 1 ml of 0.5 mg ml^{-1}, was administered to the rat through the cannula. Blood samples (300 μl) were taken at 1, 3, 6, 12, 26.5, 49.5, 80.5, 104.5, and 169 h, and placed into 50 μl of an acid-citrate–dextrose anticoagulant (25 g l^{-1} trisodium citrate dihydrate, 20 g l^{-1} dextrose, 13.7 g l^{-1} anhydrous citric acid). Plasma was isolated, and stored at 4°C until analyzed. AP-3 concentrations were determined with the ELISA. The concentration versus time profile was fit to a standard two compartment mammillary pharmacokinetic model using 'The Scientist' software (MicroMath, Salt Lake City, UT), with the data weighted by a factor of y^{-1}.

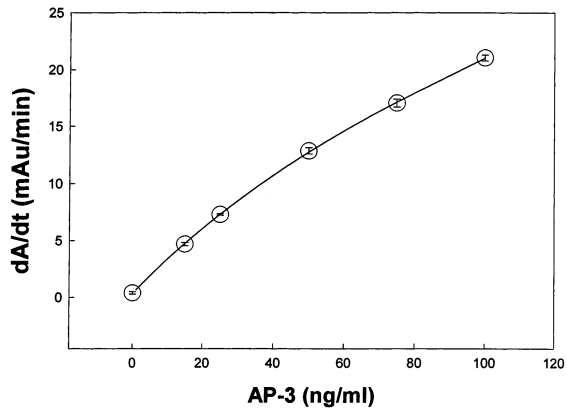

Fig. 1. Representative standard curve for AP-3 over the range 0–100 ng ml^{-1}. Curve is fitted with a cubic equation, $r^2 = 0.9999$. Error bars represent the standard deviation about the mean of three replicates.

3. Results and discussion

3.1. Assay validation

A typical standard curve for AP-3 is shown in Fig. 1. The best fit line shown in this figure was obtained by fitting the data to a cubic polynomial ($r^2 = 0.9999$). Intra-assay and inter-assay recovery of AP-3 from rat plasma samples is shown in Table 1. The intra-assay recovery ranged from 93 to 103%, and the coeffficient of variation (CV) ranged from 5.2 to 8.5%. The inter-assay recovery ranged from 107 to 119% and the CV% ranged from 17.7 to 25.1%. The working range of the assay was 15–100 ng ml^{-1}, resulting in a limit of quantification for AP-3 in rat plasma of 1.5 μg ml^{-1}.

Few studies have reported using an ELISA to measure murine IgG_1 concentrations in rat plasma. To the authors' knowledge, this is the first report of intra-assay and inter-assay variability for an ELISA measuring mouse IgG in rat plasma. Due to a lack of appropriate data, it is difficult to directly compare the precision and accuracy of this assay to other assays previously developed. Fleming et al. report CV% values of less than 7.6% for intra-assay and less than 15.6% for inter-assay variabilities for their ELISA determining mouse IgG levels in hybridoma supernatants and ascites [11]. The intra-assay variability of this ELISA is similar to the Fleming assay, and the inter-assay variability is slightly larger. The larger inter-assay variability may be due to the more complex plasma matrix compared to the supernatant and ascites matrices in the Fleming study. The limit of quantification and the working range of the assay is comparable with other assays for mouse IgG reported in the literature [11,13,16].

3.2. Cross reactivity with mouse and rat IgG

The response of the assay to pooled mouse and rat IgG is shown in Fig. 2, with plots of actual versus assayed concentration. The slope of the pooled mouse IgG plot is 1.04 and is not significantly different from 1 ($P = 0.65$). The assay demonstrated complete cross reactivity with

Table 1
Variability of ELISA with respect to recovery of AP-3 from plasma samples

Actual concentration (ng ml^{-1})	Recovered concentration (ng ml^{-1})	%R	CV%
Intra-assay variability ($n = 5$)			
15	13.9	93	8.5
25	23.6	95	5.2
50	51.0	102	5.2
100	103.1	103	6.4
Inter-assay variability ($n = 4$)			
15	17.9	119	17.8
25	27.1	108	25.1
50	54.5	109	18.5
100	107.4	107	17.7

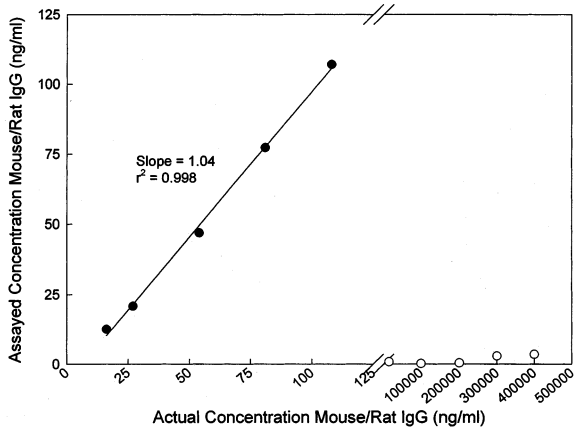

Fig. 2. Cross reactivity of ELISA with pooled mouse (●) and rat IgG (○). Concentrations of pooled mouse IgG ranged from 16.2–108 ng ml^{-1}, and concentrations of pooled rat IgG ranged from 15–400 μg ml^{-1}.

pooled mouse IgG, suggesting that this assay can be used to measure pooled mouse IgG concentrations in rat plasma.

A concentration of 0.4 mg ml^{-1} pooled rat IgG was needed for the assay to give a response of 3.5 ng ml^{-1}, a concentration well below the working range of the standard curve. The cross reactivity of the assay with rat IgG was therefore estimated to be less than $1:10^5$. Given this level of cross-reactivity and considering the normal range of IgG concentrations in undiluted Sprague–Dawley plasma (15.5 ± 3.6 mg ml^{-1} [17]), the presence of rat IgG in the assay matrix is expected to yield an assay response approximately 1 order of magnitude below that corresponding to the lowest standard in the standard curve, 15 ng ml^{-1}. In the present assay, standards were prepared in 1% rat plasma and samples were diluted minimally by a factor of 100 (i.e. to a final concentration of 1% rat plasma); however, the assay could easily be adapted to achieve a lower limit of quantification of AP-3 by reducing the extent of plasma dilution, if greater sensitivity is deemed necessary for applications other than our proposed pharmacokinetic and pharmacodynamic studies.

3.3. Specific activity of AP-3

Table 2 shows the results of the specific activity experiment. As shown in the table, 96% of the AP-3 preparation bound to the human platelets, and none of the pooled mouse IgG bound to the platelets. By SDS-PAGE, the AP-3 preparation was shown to be exclusively IgG (data not shown). These results, taken together, have shown that only ~4% of the preparation was non-AP-3 mouse IgG.

3.4. Pharmacokinetics of AP-3 in the rat

The concentration versus time profile for AP-3 in the rat is shown in Fig. 3, with a best fit line corresponding to a two-compartment mammillary model. Parameters generated using this model are shown in Table 3. The pharmacokinetic parameters for AP-3 are on the same order of magnitude as those reported by other researchers who have studied monoclonal mouse IgG_1 pharmacokinetics in the rat [9,14]. Fig. 3 demonstrates that the assay presented in this paper is well suited to determine the pharmacokinetics of AP-3 in the rat.

Table 2
Specific activity of AP-3

Sample	% Recovery[a]	SD	CV%	*n*	% Specific activity[b]
AP-3	4	1	20	2	96
Mouse	102	5	5	2	0

[a] Free AP-3/Mouse IgG recovered, compared to a control sample without platelets.
[b] Percent of AP-3/IgG bound to the platelets, representing the purity of the AP-3 samples.

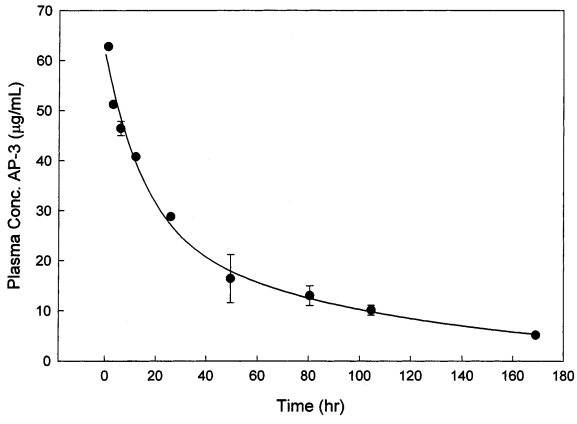

Fig. 3. Pharmacokinetics of AP-3 in a 0.2 kg Sprague–Dawley rat following a 0.5 mg dose of AP-3. Error bars represent standard error of means, using two dilutions of AP-3 per time point, and 3 replicates per dilution.

Table 3
Pharmacokinetic parameters for AP-3 in a 0.2 kg rat after administration of 0.5 mg AP-3 by IV bolus

Parameter[a]	Value
V1	8.2 ± 0.4 ml
V2	5.5 ± 1.6 ml
CL	0.15 ± 0.01 ml h^{-1}
CLD	0.18 ± 0.06 ml h^{-1}
Terminal $t_{1/2}$	71 ± 4 h

[a] V1 and V2 refer to central and peripheral compartment volumes, respectively. CL and CLD refer to systemic and distributional clearances.

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