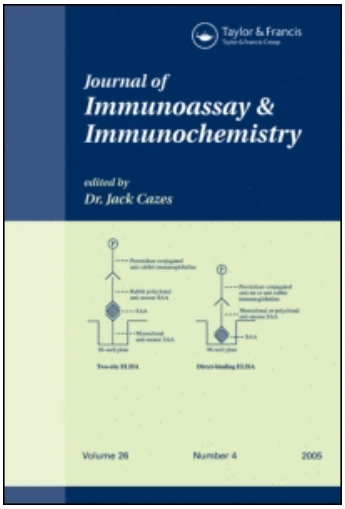

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Development and Validation of Enzyme-Linked Immunosorbent Assays for Quantification of Anti-Methotrexate IgG and Fab in Mouse and Rat Plasma

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ABSTRACT

This laboratory is investigating the use of anti-methotrexate IgG (AMI) and anti-methotrexate Fab fragments (AMF) within an inverse targeting strategy that is designed to enhance the pharmacokinetic selectivity of intraperitoneal (i.p.) chemotherapy. The goal of this study was to develop enzyme-linked immunosorbent assays (ELISAs) to determine concentrations of AMI and AMF in mouse and rat plasma. An antigen-specific ELISA was developed for AMI and AMF in mouse and rat plasma. The assay was validated with respect to precision and accuracy by evaluating the recovery of AMI and AMF from mouse and rat plasma samples. Preliminary pharmacokinetic studies of AMI and AMF

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were performed in Sprague-Dawley rats and Swiss Webster mice. The animals were instrumented with a jugular vein cannula and administered AMI or AMF, 15 mg kg^{-1} via the cannula. Plasma samples were taken at various time points and analyzed using the ELISA, and the observed concentration vs. time profiles were subjected to non-compartmental pharmacokinetic analyses. Standard curves for the ELISAs were found to be linear over concentration ranges of 0–250 and 0–350 ng mL^{-1} for AMI and AMF, respectively. Intra-assay and inter-assay recovery of AMI and AMF from plasma samples were found to be within 15% of theoretical values. Preliminary pharmacokinetic investigations of AMI allowed estimation of AMI clearance to be 0.017 mL kg^{-1} min^{-1} in the rat and 0.043 mL kg^{-1} min^{-1} in the mouse. AMF clearance was estimated to be 0.038 and 1.93 mL kg^{-1} min^{-1} in the mouse and rat, respectively. In conclusion, ELISAs have been developed and validated for quantitation of AMI and AMF in rat and mouse plasma. The assays will allow further investigations of AMI and AMF pharmacokinetics.

Key Words: Enzyme immunoassay; Methotrexate; Antibody; Pharmacokinetics; Targeting.

INTRODUCTION

The American Cancer Society estimates that, in 2003, approximately 25,000 women will be diagnosed with ovarian cancer in the United States, and that nearly 14,000 will die from this disease.[1] It is the leading cause of gynecologic cancer death in the United States.[2] In most cases, ovarian cancer is confined to the peritoneal cavity.[3] As such, it has been hypothesized that direct, intraperitoneal (i.p.) drug administration may allow increases in the therapeutic selectivity of chemotherapy, by bathing tumor cells with high drug concentrations, while minimizing systemic drug exposure.[4] However, i.p. chemotherapy has yielded only modest therapeutic benefit, largely due to the occurrence of systemic toxicities that result from the diffusion of drug out of the peritoneum and into the systemic circulation.[5–9]

This laboratory is investigating an inverse targeting strategy that is designed to optimize i.p. chemotherapy of ovarian cancer.[10,11] Our approach combines i.p. drug administration with intravenous administration of anti-drug antibodies. The presence of anti-drug antibodies in the systemic circulation is expected to lead to rapid binding of drug that diffuses out of the peritoneum (i.e., upon entry into blood), decreasing peak plasma unbound drug concentrations and reducing the rate and extent of drug distribution to systemic tissues. Thus, it is anticipated that anti-drug antibodies may be used to alter

systemic drug disposition, potentially increasing the pharmacokinetic and therapeutic selectivity of i.p. chemotherapy.

To test this hypothesis, polyclonal,[12] and more recently, monoclonal antibodies have been developed to bind to methotrexate, a commonly used chemotherapeutic. Preliminary studies have confirmed that anti-methotrexate IgG (AMI) and anti-methotrexate Fab fragments (AMF) produce regio-specific alterations in methotrexate disposition, increasing the pharmacokinetic selectivity of i.p. methotrexate chemotherapy, as desired.[11] For continued investigation and optimization of this targeting strategy, it will be necessary to characterize the pharmacokinetics of AMI and AMF in animal models.

This paper describes a simple and convenient solid-phase, antigen-specific enzyme-linked immunosorbent assay (ELISA) procedure that allows quantification of AMI and AMF in mouse and rat plasma samples. The assay was validated with respect to precision and accuracy, and preliminary studies in mice and rats were conducted to confirm the utility of the assay for pharmacokinetic investigations. The ELISA will be used in future studies to thoroughly characterize AMF and AMI pharmacokinetics; resultant data will be used to guide efforts to optimize i.p. methotrexate chemotherapy via the inverse targeting approach.

EXPERIMENTAL

Production and Purification of AMI and AMF

Briefly, hybridoma cells secreting a murine monoclonal IgG1 anti-methotrexate antibody (AMI) were derived from mice immunized with a methotrexate-keyhole limpet hemacyanin immunogen. These hybridoma cells were grown at 37°C within 1-L spinner flasks containing serum free media (Hybridoma-SFM, Invitrogen, Grand Island, NY). The cell culture supernatant was centrifuged, passed through a 0.22-μm filter, and AMI was purified via protein-G affinity chromatography (Pharmacia Biotech Hi-Trap Protein G column, Piscataway, NJ) using an automated, medium pressure chromatography system (Biologic, BioRad Laboratories, Hercules, CA). The loading buffer was 20 mM Na_2HPO_4 (pH 7.0, Sigma Chemical, St. Louis, MO), and bound protein was eluted from the column using a glycine buffer (pH 2.8, 0.1 M, BioRad Laboratories). The eluate was neutralized with Tris buffer (pH 9, 1M, BioRad Laboratories) to minimize AMI precipitation. AMI concentrations in buffer were determined by assessing absorbance at 280 nm, assuming that 1.35 AU = 1 mg mL^{-1} AMI.[13] AMF was prepared by papain digestion of AMI, and was then purified using hydroxyapatite chromatography (BioRad Laboratories). Endotoxins were removed by passing

the concentrated AMI and AMF solutions through a Detoxi endotoxin removing gel (Pierce, Rockford, IL), three times before use.

ELISA Procedure

Bovine serum albumin–methotrexate conjugates were prepared as described previously.[12] Nunc Maxisorp 96 well microplates (Nunc model #439454, Roskilde, Denmark) were incubated overnight at 4°C with 0.250 mL of a 10 $\mu g\,mL^{-1}$ bovine serum albumin–methotrexate solution in Na_2HPO_4 buffer (20 mM, no pH adjustment, Sigma Chemical). Following incubation, the microplate was washed three times with a phosphate–Tween buffer (PB–Tween) consisting of 20 mM Na_2HPO_4 (Sigma Chemical, no pH adjustment) and 0.05% Tween 20 (Sigma Chemical). Plates were then rinsed with distilled water two times, and incubated with samples and standards, 0.25 mL, for 2 hr at room temperature. The standards were made by diluting a stock solution of AMI or AMF to the appropriate concentration (0, 25, 50, 100, 175 and 250 $ng\,mL^{-1}$ for AMI and 0, 25, 50, 100, 250 and 350 $ng\,mL^{-1}$ for AMF) with phosphate buffered saline (PBS, pH 7.4), with the addition of rat or mouse plasma to a final concentration of 1% (v/v). Following incubation with the samples and standards, plates were washed with PB–Tween three times, rinsed with distilled water twice, and then incubated for 1 hr at room temperature with Fab specific goat anti-mouse-antibody–alkaline phosphatase conjugate (Sigma Chemical) diluted 1 : 500 in PB–Tween and 1% bovine serum albumin (Sigma Chemical). The microplate was washed and rinsed, as described above, and 0.2 mL *p*-nitro phenyl phosphate (Pierce), 4 $mg\,mL^{-1}$, diluted in diethanolamine buffer (pH 9.8, Sigma Chemical), was added to each well. The change in absorbance over the change in time (dA/dt) was determined using a microplate reader at 405 nm (SpectraMax 250, Molecular Devices, Sunnyvale, CA). Standard curves were generated from each assay plate by plotting the dA/dt vs. AMI or AMF concentration. The assay was validated with respect to precision and accuracy by determining the recovery of AMI or AMF from rat or mouse plasma samples.

Pharmacokinetic Studies

Female Sprague Dawley rats (Harlan, Indianapolis, IN), 0.23–0.25 kg, and male Swiss-Webster mice (Harlan), 20–25 g, were instrumented with jugular vein cannulas under ketamine/xylazine anesthesia. The animals were allowed to recover for 2 days following surgery and then were administered AMI or AMF, 15 $mg\,kg^{-1}$, in PBS, pH 7.4 through the cannula. Blood samples

(300 μL) were taken from the rats using the cannula at 1, 3, 6, 12, 24, 48, 96, and 168 hr and samples from the mice (25 μL) were taken from the saphenous vein at 30, 60, 120, 180, 360, 720, and 1440 min. The blood was placed in microcentrifuge tubes containing 0.5 μL heparin, 5000 U mL^{-1} (Elkins-Sinn Inc., Cherry Hill, NJ) and centrifuged at 13,000 rpm for 2 min. The plasma was isolated and stored at 4°C until analyzed with the ELISA. After analysis, a concentration vs. time profile was generated; and non-compartmental pharmacokinetic analysis was performed using WinNonlin, version 2.1.

RESULTS AND DISCUSSION

Assay Validation

Figure 1 shows a representative standard curve for AMI in rat plasma. A linear relationship exists between dA/dt and concentration between 0 and

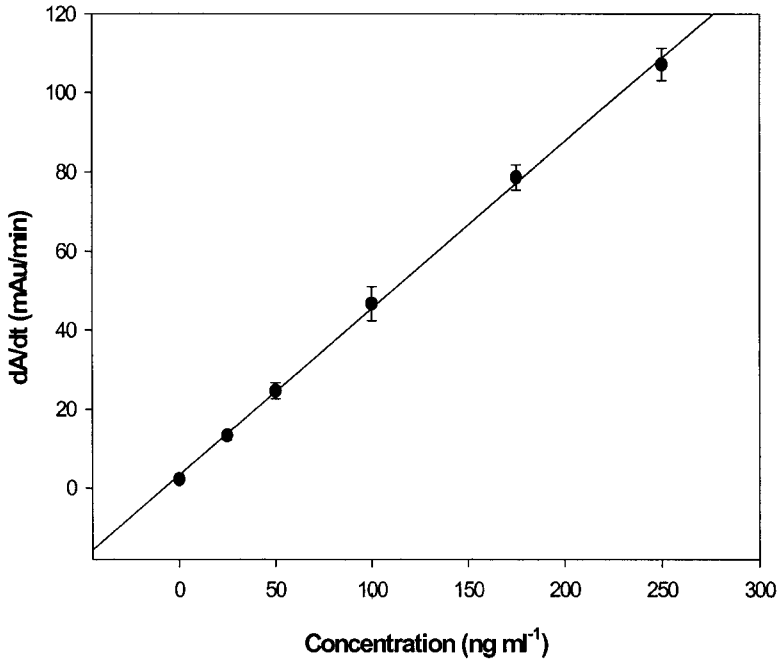

Figure 1. Representative standard curve for AMI in rat plasma over the range of 0–250 ng mL^{-1}. Analysis of linear regression shows an $r^2 = 0.999$. Error bars represent the standard deviation of the mean of the three replicates.

250 ng mL^{-1}. The curve is fitted with a linear equation, $r^2 = 0.999$. A similar relationship is observed for AMI in mouse plasma over the same concentration range (results not shown). For AMF in rat and mouse plasma, a linear relationship is noted between 0 and 350 ng mL^{-1} (results not shown). Each assay was validated with respect to accuracy and precision by quantifying concentrations of AMI or AMF from rat or mouse plasma samples. Intra-assay and inter-assay recovery of AMI from rat plasma samples is shown in Table 1. The intra-assay recovery was between 111% and 114%, and the coefficient of variance (CV%) ranged from 3.62% to 8.09%. The inter-assay recovery was between 99% and 102%, and the CV% ranged from 3.03% to 9.67%. Tables 1 and 2 list validation data for AMI in mouse plasma and AMF in rat and mouse plasma.

Pharmacokinetics of AMI and AMF in the Mouse and Rat

The concentration vs. time profile for AMI in the mouse and rat following an i.v. bolus dose of 15 mg kg^{-1} is shown in Fig. 2. Table 3 lists the clearance,

Table 1. Variability of ELISA with respect to recovery of AMI from mouse and rat plasma samples.

Actual concentration (ng mL^{-1})	Recovered concentration (ng mL^{-1})	Recovery (%)	CV (%)
AMI in rat plasma			
Intra-assay variability ($n = 4$)			
250	277.7	111	5.92
150	169.8	112	3.62
25	28.5	114	8.09
Inter-assay variability ($n = 3$)			
200	198.9	99	3.03
50	50.8	102	7.01
30	30.3	101	9.67
AMI in mouse plasma			
Intra-assay variability ($n = 3$)			
250	248.5	99	4.32
150	149.2	99	5.49
25	23.8	95	4.78
Inter-assay variability ($n = 3$)			
250	240.5	96	6.64
150	157.2	105	8.89
25	28.3	113	7.02

Note: %CV, percent coefficient of variation.

Table 2. Variability of ELISA with respect to recovery of AMF from mouse and rat plasma samples.

Actual concentration ($ng\,mL^{-1}$)	Recovered concentration ($ng\,mL^{-1}$)	Recovery (%)	CV (%)
AMI in rat plasma			
Intra-assay variability ($n = 3$)			
350	347.7	99	6.08
250	241.9	97	8.32
25	23.8	95	9.12
Inter-assay variability ($n = 3$)			
350	363.3	104	8.21
250	244.9	98	5.81
25	25.3	101	4.32
AMF in mouse plasma			
Intra-assay variability ($n = 3$)			
350	333	95	5.38
250	279	111	9.98
25	24.8	99	12.6
Inter-assay variability ($n = 3$)			
350	340	97	1.44
250	263	105	4.72
25	22.6	91	1.96

Note: %CV, percent coefficient of variation.

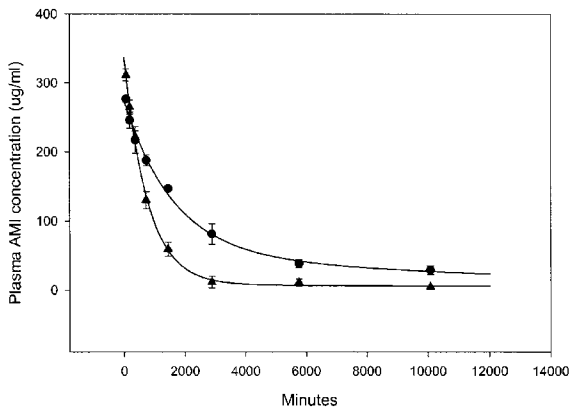

Figure 2. Plasma AMI pharmacokinetics following intravenous administration of $15\,mg\,kg^{-1}$ in one rat and one mouse. *Key*: (●), Rat; (▲), mouse. AMI concentrations were determined via ELISA. Error bars represent the standard deviation of the mean of three replicates per time point.

Table 3. Pharmacokinetic parameters for AMI and AMF in mice and rats following intravenous bolus administration of 15 mg kg^{-1}.

Species	CL (mL kg^{-1} min^{-1})	V_{ss} (mL)	$t_{1/2}$ (min)
AMI			
Rat	0.017	76.5	2950
Mouse	0.043	47.6	782
AMF			
Rat	1.93	117	82
Mouse	0.038	147	340

Note: CL, clearance; V_{ss}, volume of distribution at steady state; $t_{1/2}$, terminal half-life.

V_{ss}, and $t_{1/2}$ parameter values obtained from non-compartmental analyses of the pharmacokinetic data. The pharmacokinetic parameters generated for AMI are comparable with those reported in the literature for other mouse monoclonal antibodies.[14,15] Figure 3 shows the plasma AMF concentration vs. time profile following a 15 mg kg^{-1} i.v. bolus dose in the mouse and rat, with the pharmacokinetic parameters shown in Table 3.

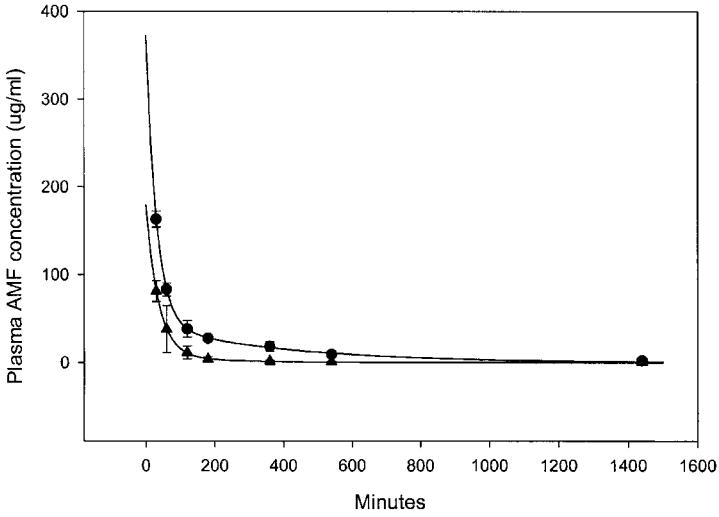

Figure 3. Plasma AMF pharmacokinetics following intravenous administration of 15 mg kg^{-1} in one rat and one mouse. *Key*: (●), Rat; (▲), mouse. AMF concentrations were determined via ELISA. Error bars represent the standard deviation of the mean of three replicates per time point.

CONCLUSION

This paper presents a sensitive assay for quantification of AMI and AMF concentrations in mouse and rat plasma samples. The assays have been shown to be well suited for determining the pharmacokinetics of AMI and AMF in the mouse and rat. These assays will allow further investigation of hypotheses regarding the disposition of AMI and AMF in these species, and may facilitate efforts to use these agents to optimize i.p. methotrexate chemotherapy.

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