Snake $F(ab')_2$ Antivenom from Hyperimmunized Horse: Pharmacokinetics Following Intravenous and Intramuscular Administrations in Rabbits

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Purpose. The pharmacokinetics of a currently available horse $F(ab')_2$ antivenoms to *Vipera aspis, V. ammodytes,* and *V. berus* (Ipser Europe) and a new more purified and pasteurized preparation (SAV) was investigated in the rabbit.
Methods. An immunoradiometric assay using an affinity-purified goat IgG horse $F(ab')_2$ specific and the same IgG labelled with iodine 125 as a tracer was developed. The limit of quantification in plasma was 0.032 μg/ml. Specificity study showed that mouse $F(ab')_2$ and Fab did not cross-react.
Results. Pharmacokinetic analysis showed that the plasma $F(ab')_2$ concentration followed a biexponential decline after intravenous bolus administration with distribution and elimination half-lives of 2.66 ± 0.18 hrs and 49.69 ± 4.13 hrs, respectively. The total volume of distribution (Vdss or Vdβ) was between 209 and 265 $ml.kg^{-1}$ and was similar to the volume of the extracellular fluid in the rabbit (300 $ml.kg^{-1}$). Total body clearance ranged from 3.33 to 3.96 $ml. h^{-1} \cdot kg^{-1}$. After intramuscular administration which was only investigated with SAV, Tmax was 48 hrs and the absolute bioavailability was 42%.
Conclusions. No difference in pharmacokinetics was observed between the two antivenom preparations following the intravenous administration. In contrast, a reduced rate and extent of absorption was shown following intramuscular administration.

KEY WORDS: horse $F(ab')_2$; antivenoms; immunoradiometric assay; rabbit pharmacokinetics; absolute bioavailability.

INTRODUCTION

Specific antibodies are currently used to reverse toxicity from drugs (1) and toxins. Hyper-immunization of horses with venom antigens stimulates the production of IgG (IgG: immunoglobin G) antibodies to various venom proteins and peptides. These antibodies have been identified as a subclass of horse IgG and are called IgG_T (2). *In vivo* studies with purified IgG_T have demonstrated their efficacy in neutralizing the effects of *Bothrops jararaca* venom (3). The preparation of $F(ab')_2$ ($F(ab')_2$: bivalent fragment antigen binding) from IgG_T was undertaken to minimize the antigenicity of the whole molecule. Moreover, more purified and pasteurized horse $F(ab')_2$ preparations are now being developed to replace the currently available antivenom antibodies. The improvement in clinical efficacy and safety with the new generation of $F(ab')_2$ antivenoms must be assessed in different clinical trials. Therefore, we undertook to investigate the pharmacokinetics of $F(ab')_2$ in the animal as a first step.

In the present study, an immunoradiometric assay (IRMA) was developed and validated to quantitate the plasma concentrations of horse $F(ab')_2$. The IRMA technique is based on the use of affinity-purified goat IgG horse $F(ab')_2$ specific for plate coating and the same IgG labelled by iodine 125 as a tracer. Finally, plasma disposition of $F(ab')_2$ fragments was investigated in rabbits using a currently available antivenom to *Vipera aspis, V. ammodytes, and V. berus* (Ipser Europe) and the new corresponding preparation (SAV). The aim of the pharmacokinetic study was to (i) describe $F(ab')_2$ pharmacokinetic parameters; (ii) assess the pharmacokinetic equivalence between the two generations of antibodies; (iii) estimate the bioavailability after intravenous (i.v.) and intramuscular (i.m.) administration.

MATERIALS AND METHODS

Chemicals

Horse $F(ab')_2$ antivenoms to *Vipera aspis, V. ammodytes, and V. berus* (Ipser Europe and SAV) were supplied by Pasteur-Mérieux Sérums et Vaccins, (Lyon, France). Briefly, Ipser Europe is a commercial product obtained from hyperimmunized horse plasma by a precipitation procedure and pepsin hydrolysis: a first step of ammonium sulfate precipitation yields a globulin concentrate which is submitted to pepsin hydrolysis in order to obtain $F(ab')_2$ from the immunoglobulins. The second step of ammonium sulfate precipitation is associated with a thermocoagulation and an aluminium gel adsorption step in order to remove impurities from $F(ab')_2$. SAV was obtained from hyperimmunized horse plasma by a two chromatography step procedure and pepsin hydrolysis. First, horse IgG_T were isolated on a anion-exchange column and submitted to hydrolysis in order to obtain $F(ab')_2$. A second-anion exchange column achieved the purification of $F(ab')_2$. Finally, a liquid pasteurization step was added as an additional vial safeguard. The purity of two $F(ab')_2$ preparations was 88% for Ipser Europe and 97% for SAV in exclusion HPLC analysis. Contaminants were mainly low molecular weight components (<20 kDa) for 9% (Ipser Europe) and 2% (SAV). The remaining percentages were polymeric structures (>200 kDa). Affinity-purified goat IgG horse $F(ab')_2$ specific (2.4 mg/ml), chromatography-pure horse $F(ab')_2$ (12.1 mg/ml, 100% of purity in exclusion HPLC analysis) were from Jackson ImmunoResearch Laboratories (Interchim, Montluçon, France). Mouse $F(ab')_2$ and Fab (Fab: Fragment antigen binding) were prepared by Wahyono *et al.* (4). Carrier-free ^{125}I (specific activity, 15.2 mCi/μg) was purchased from Amersham (Les Ulis, France), and Iodogen from Pierce-Touzart et Matignon (Vitry sur Seine, France). Sephadex G25 PD 10 was obtained from Pharmacia, (Saint Quentin en Yvelines, France). Bovine serum albumin (BSA) was from Sigma, (Saint Quentin Fallavier, France) and all other chemical reagents were purchased from Merck, (Darmstadt, Germany).

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Preparation of the Reagents for IRMA

Affinity-purified goat IgG horse F(ab')$_2$ specific was radiolabeled using the Fraker and Speck method (5): 0.5 mCi of Na^{125}I was reacted with 100 μg IgG in a tube coated with 10 μg iodogen. Free iodine was separated from iodinated IgG by chromatography through a Sephadex G25 and elution with 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl (phosphate buffered saline, PBS). Aliquots of iodinated IgG were frozen at −20°C and further dilutions were made in PBS with 0.2% Tween 20. Purity of the iodinated IgG preparations was ensured by counting the trichloracetic acid precipitable ^{125}I fraction (gamma-scintillation counter Minaxi gamma 5000, Packard Instruments, Rungis, France). Iodination of affinity-purified goat IgG horse F(ab')$_2$ specific by the iodogen method provided a yield of 90% and a specific activity of 2.1 μCi/μg.

Standards were prepared by dilution of a stock solution of chromatography-purified horse F(ab')$_2$ (12.1 mg/ml) from Jackson ImmunoResearch Laboratories in PBS-Tween and rabbit plasma were spicked with the same stock solution.

IRMA Procedure

Microtitration plates (Falcon microtest III U50, Polylabo, Strasbourg, France) were coated with 200 μl of 100 mM sodium carbonate pH 9.6 containing 5 μg/ml of affinity-purified goat IgG horse F(ab')$_2$ specific by overnight incubation at 4°C. The plates were washed three times with PBS containing 0.2% Tween-20 (PBS-Tween). Overcoating was achieved by a 30 min incubation at room temperature with 100 μl/well PBS containing 3% BSA (PBS-BSA) to reduce non-specific serum protein binding. Plates were then washed three times. Sensitized plates were stored at 4°C for several weeks until required. 100 μl of standard samples or plasma samples diluted in PBS-Tween were added to the sensitized plates and incubated for 2 hrs at room temperature. The plates were washed and incubated 1 hr at room temperature as before with 100 μl of appropriate optimally diluted conjugate (affinity-purified goat IgG horse F(ab')$_2$ specific conjugated to sodium iodine, 35 000 cpm). Plates were washed 3 times and dried. Each well was cut out and placed in a 5 ml plastic tube. All tubes were capped and placed in a gamma counter. The radioactivity in each tube was counted and results expressed as % binding, i.e.: (B-NS)/(T-NS) versus log F(ab')$_2$ concentrations where B = bound, T = total radioactivity and NS = the nonspecific binding of the tracer.

Validation of the IRMA

The linearity was assessed by measuring in triplicate the IRMA response of two plasma samples from rabbit donors containing different horse F(ab')$_2$ concentrations (1.0 μg/ml; 0.8 μg/ml) at dilutions ranging from ½ to 1/32. The correlation between theoretical concentrations and experimental measurements was analyzed by linear regression using the GRAPHPAD program (ISI, California). Parallelism of standard curves was checked between the two tested Ipser Europe and SAV F(ab')$_2$ and the reference F(ab')$_2$ from Jackson ImmunoResearch Laboratories.

Intra- and inter-assay variations were determined at horse F(ab')$_2$ concentrations of 0.05 μg/ml, 0.1 μg/ml, and 0.2 μg/ml. The intra-assay variation was calculated from 8 measurements performed simultaneously with the same microtitration plate and the same standard samples and reagent solutions. The inter-assay variation was estimated from triplicate determinations performed over a three-week period using different standard samples and reagent solutions. The detection limit of the assay was determined by measuring in triplicate the IRMA response of 10 plasma (F(ab')$_2$-free) samples from different rabbits. The quantification limit was defined as the concentration of F(ab')$_2$ which gave an IRMA response in plasma equal to the mean of background values plus K standard deviation (for n = 10, K = 6.58, α = 5% and β = 1%). The specificity of the assay was assessed by examining cross-reactivity of the IRMA with mouse F(ab')$_2$ and Fab diluted in PBS-Tween.

Pharmacokinetics in Rabbits

Healthy adult male New Zealand white rabbits weighing 2.5–3 kg were divided into three groups of five animals. SAV was injected i.v. over 1 min. at the dose of 10.12 mg/kg via the marginal ear vein or i.m. in the back leg at the same dose. Ipser Europe was injected i.v. over 1 min. at the dose of 10.08 mg/kg. Samples of whole blood (4 ml) were collected in heparinized tubes just before and 0.017, 0.25, 1.5, 6, 24, 48, 96, 144, and 240 hrs after the end of injection. Plasma samples were stored at −20°C until analysis. Plasma concentrations of F(ab')$_2$ were determined in triplicate by the IRMA previously described.

Pharmacokinetic Analysis

Pharmacokinetic parameters were obtained using the SIPHAR program (SIMED, Créteil, France). Plasma concentration-time data were fitted using an extended least-squares algorithm. The primary pharmacokinetic parameter distribution volume (Vdβ), distribution volume at steady-state (Vd$_{ss}$) and total body clearance (Clt) were determined after i.v. administration using classical equations (6).

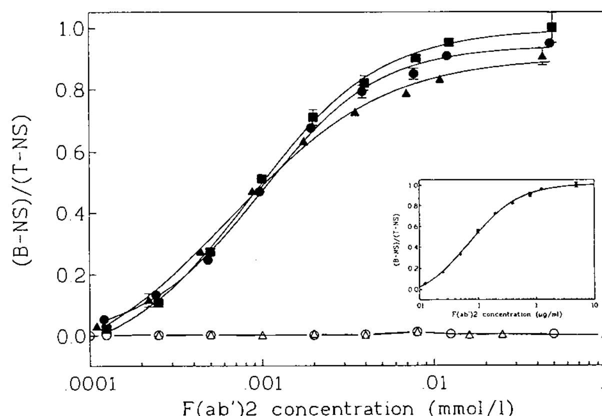

Fig. 1. Standard curves (signal vs molar concentrations) of the immunoradiometric assay horse F(ab')$_2$ in PBS-Tween were obtained to stock solution from Jackson ImmunoResearch Laboratories (■) or SAV (●) and Ipser Europe (▲). The other curves indicate the cross-reactivity of mouse F(ab')$_2$ (○) and Fab (△) in the assay. Insert shows the standard curve used for the pharmacokinetic study with F(ab')$_2$ concentrations expressed in μg/ml.

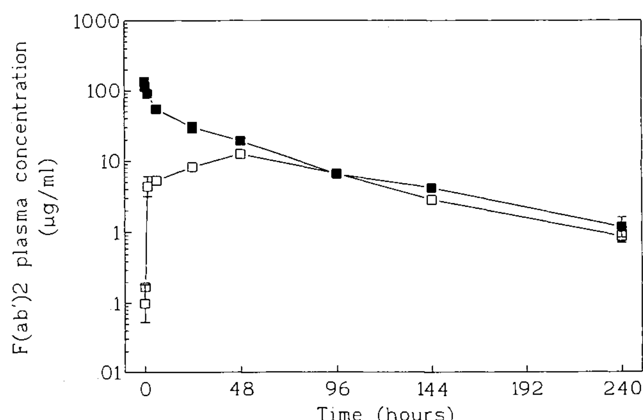

Fig. 2. Mean plasma concentration-time profile of SAV after intravenous (■) and intramuscular administration (□) of 10.12 mg/kg in the rabbit (mean ± SD, n = 5).

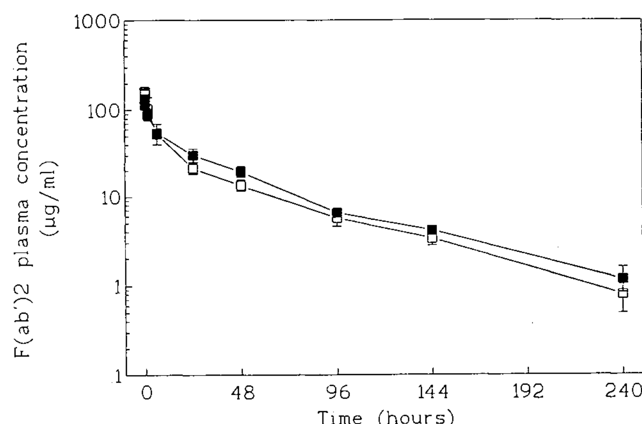

Fig. 3. Mean plasma concentration-time profile after intravenous administration of SAV (10.12 mg/kg) (■) and Ipser Europe (10.08 mg/kg) (□) in the rabbit (mean ± SD, n = 5).

The maximal plasma concentrations (C_{max}) at the time (T_{max}) after i.m. administration were the observed experimental values.

The absolute bioavailability (F) was determined as follows:

$$F = (AUC0-\infty_{IM} \cdot dose_{IV})/(AUC0-\infty_{IV} \cdot dose_{IM})$$

Pharmacokinetic parameters are expressed as mean ± SD (n = 5).

RESULTS

IRMA Procedure

Insert in Figure 1 shows a typical standard dose response curve expressed in µg/ml obtained for horse $F(ab')_2$ in PBS-Tween. Linearity was observed between 0.03 and 0.3 µg/ml horse $F(ab')_2$ (y = 1.01x − 0.01 with r = 0.99). The limits of detection and quantification in plasma were 0.013 µg/ml and 0.032 µg/ml, respectively. The intra-assay coefficient of variation ranged from 2.9 to 10.2% and from 6.9 to 11.1% for the inter-assays. SAV and Ipser Europe $F(ab')_2$ gave parallel response (Figure 1). Specificity showed that mouse $F(ab')_2$ and Fab did not cross-react (Figure 1).

Pharmacokinetic Study of Horse $F(ab')_2$ Fragments in Rabbits

Figure 2 shows the plasma horse $F(ab')_2$ disposition after i.v. bolus and i.m. administration of SAV. Figure 3 shows the plasma horse $F(ab')_2$ disposition after i.v. bolus of SAV and Ipser Europe. Horse $F(ab')_2$ declined biexponentially after i.v. administration. Intramuscular administration showed a resorption phase with Tmax at 48 hrs and then a monoexponential decline with a $t_{1/2}\beta$ similar to that in the i.v. group. The corresponding mean pharmacokinetic parameters (±SD) are presented in Table I. The absolute bioavailability was estimated at 42% for the intramuscular route.

DISCUSSION

The purpose of this study was to (i) describe horse $F(ab')_2$ pharmacokinetic parameters after i.v. and i.m. administration (ii) assess the pharmacokinetic equivalence between the two generations of antibodies and (iii) determine the absolute bioavailability after i.m. administration.

With these aims, a specific and sensitive IRMA was developed and validated in our study. All quality criteria complied with the standard norms recommended in pharmaceutical development (7) and finally the assay was specific of horse $F(ab')_2$.

Pharmacokinetic analysis showed that the plasma $F(ab')_2$ concentration followed a biexponential decline after i.v. bolus administration with distribution and elimination half-lives of 2.66 ± 0.18 hrs and 49.69 ± 4.13 hrs, respectively. These values are in the range of those previously published in humans (8,9,10). Few data are available in animals, especially rabbits. The total volume of distribution (Vdss or Vdβ) was between 209 and 265 $ml.kg^{-1}$ and was

Table I. Kinetic Parameters of SAV and Ipser Europe Following Intravenous and Intramuscular Administration in Rabbits

	Dose $\mu g \cdot kg^{-1}$	Administration	$AUC_{0-\infty}$ $hr \cdot \mu g \cdot ml^{-1}$	C_{max} $\mu g \cdot ml^{-1}$	T_{max} hr	F %	$t_{1/2}\alpha$ hr	$t_{1/2}\beta$ hr	Vdβ $ml \cdot kg^{-1}$	Vd_{ss} $ml \cdot kg^{-1}$	Clt $ml \cdot kg^{-1} \cdot hr^{-1}$
SAV	10120	intravenous	3072	125.66	—	—	2.66	49.69	224	209	3.33
		route	± 341	± 4.64			± 0.18	± 4.13	± 28	± 24	± 0.38
	10120	intramuscular	1299	12.67	48	42	—	59.63	—	—	—
		route	± 64	± 1.10	± 0			± 2.88			
Ipser	10080	intravenous	2618	159.82	—	—	2.66	46.93	265	231	3.96
Europe		route	± 490	± 21.26			± 0.74	± 5.92	± 34	± 37	± 0.75

Table II. Pharmacokinetic Parameters of Antibodies and Fragments in the Rabbit

	Analytical methods	$t_{1/2}\alpha$ hr	$t_{1/2}\beta$ hr	Vd_{ss} $ml \cdot kg^{-1}$	Clt $ml \cdot kg^{-1} \cdot hr^{-1}$
Rabbit IgG[a]	Total ^{125}I-labelled IgG	1.06	43.3	—	—
Goat Fab[b]	Immunoassay	0.21	2.3	189.1	86.5
Sheep Fab[c]	TCA precipitable ^{125}I-Fab	0.52	12.27	278.4	23.4
	Immunoassay	0.68	6.97	151.4	38.7

[a] According to Ismail *et al.* (12).
[b] According to Timsina *et al.* (13).
[c] According to C. Vo Than (unpublished data).

similar to the volume of extracellular fluid in the rabbit (300 $ml.kg^{-1}$) (11). These data indicate that $F(ab')_2$ are able to diffuse across the capillary wall and reach body compartment equilibrium in about 15 hrs. This long delay is easily explained by the relatively high molecular weight of $F(ab')_2$ (100 kDa). Distribution comparisons are possible in rabbits between the whole IgG and its $F(ab')_2$ and Fab (12,13) (Table II). Whole IgG (150 kDa) has a very small volume of distribution (twice plasmatic volume), though the Fab and $F(ab')_2$, with molecular weights of 50 kDa and 100 kDa respectively, have similar Vdss corresponding to the physiological values of the water extracellular space in the rabbit. Due to their relatively high molecular weight, total body clearance of $F(ab')_2$ is quite moderate, $F(ab')_2$ are not subject to renal excretion because their molecular weight is higher than the cut-off limit of 50 kDa allowing glomerular filtration (1).

Though the new SAV preparation is more purified than Ipser Europe and is subjected to a pasteurization process, the pharmacokinetic studies showed no difference between the two antivenoms following intravenous administration. Removal of non IgG_T components does not modify the pharmacokinetics of the new antivenom preparation, but would reduce the risks of serious allergic side effects such as anaphylactic shock and serum sickness (14).

The last objective was to assess the bioavailability of i.m. compared to i.v. administration. Intramuscular injection is frequently used in clinical practice for antivenom therapy and the rate and extent of $F(ab')_2$ resorption into the systemic circulation remain questionable. Our data clearly indicate that about 42% of the $F(ab')_2$ dose is resorbed slowly as Tmax was observed 48 hrs after the administration. These data are consistent with the fact that most high molecular weight proteins are released and relatively slowly from the injection site following intramuscular or subcutaneous administration (15). A consequence of these bioavailability data could be that $F(ab')_2$ has less antivenom potency after i.m. than after i.v. administration. Because of the lower rate of entry into systemic circulation, venom toxins could be less efficiently neutralized following i.m. administration of $F(ab')_2$. This last hypothesis needs further experimental investigation.

In conclusion, the validation of a specific and sensitive immunoradiometric assay for horse $F(ab')_2$ has enabled a pharmacokinetic study of $F(ab')_2$ based antivenoms to be performed. The resulting data may be helpful for determining the optimal use of such antivenoms in the *Vipera aspis, V. ammodytes,* and *V. berus* envenimation therapy.

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