Development of a Sensitive Radioimmunoassay for Fab Fragments: Application to Fab Pharmacokinetics in Humans

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Anti-sheep Fab fragment antisera were produced in rabbits using sheep digoxin-specific Fab fragments (Digidot) as immunogen. These antisera were used for the development of a radioimmunoassay (RIA) of sheep Fab fragments in human plasma and urine using ^{125}I-labeled Fab fragments. Interference in the assays by digoxin, human proteins, and antibodies from different species was insignificant, but cross-reactivity between anti-sheep Fab antisera and goat IgG or Fab fragments was 22 to 67%. The limit of detection was 0.1 μg/mL and the assay was linear over a 0.6–28 μg/mL range of Fab fragments. Intra- and interassay coefficients of variation were less than 6.9 and 10.5%, respectively. Accuracy of plasma and urine assays at various Fab fragment levels ranged from 96 to 106%. RIA was applied to the pharmacokinetic study of sheep digoxin-specific Fab fragments in one patient acutely intoxicated by digitoxin and treated with Digidot. The Fab elimination half-life was 12.1 hr. Steady-state volume of distribution and total-body clearance were 10.8 L and 23.4 mL/min, respectively. Unchanged Fab fragments (50 kD) and degradation products (25 kD) isolated by gel filtration chromatography of a urine sample cross-reacted with the anti-Fab antiserum.

KEY WORDS: Fab fragment; radioimmunoassay; pharmacokinetics; urinary metabolites.

INTRODUCTION

Digoxin-specific Fab[3] fragments are effective in the treatment of acute poisoning by cardiac glycosides (1). However, despite the high cost of such biological products (2), dose and infusion time are not standardized and almost 50% of specific Fab fragments are cleared without neutralizing digoxin (3). Knowledge of the disposition of heterologous Fab fragments in humans could help to resolve these drawbacks. However, the pharmacokinetics of Fab fragments has not been extensively studied in humans because few suitable assays have been reported until now (3–6).

We report the development of a radioimmunoassay specific to sheep Fab fragments, based on the use of sheep Fab-specific antiserum raised in rabbits and ^{125}I-Fab tracer. Plasma disposition of Fab fragments and urinary excretion of Fab fragment metabolites were investigated in one patient treated with digoxin-specific Fab fragments.

MATERIALS AND METHODS

Chemicals

Digoxin-specific sheep Fab fragments (Digidot), molecular weight (MW) 50 kD, were from Boehringer Mannheim FRG. Carrier-free ^{125}I (sp act, 16.4 mCi/μg) and the donkey anti-rabbit magnetic separation reagent (Amerlex-M) were purchased from Amersham (Les Ulis, France). Freund's complete and incomplete adjuvant was obtained from Difco Laboratories (Detroit, MI), and Iodogen from Pierce-Touzart et Matignon (Vitry sur Seine, France). Goat and mouse immunoglobulins or Fab fragments were produced in our laboratory. The other proteins used for the cross-reactivity study (Table I) were supplied by Sigma (La Verpillère, France). Sephadex G25 PD 10 and XK 50/60 columns were obtained from Pharmacia (Saint Quentin en Yvelines, France), Ultrogel AcA44 from IBF (Villeneuve la Garenne, France), and molecular weight standards from Sigma. All other reagents of analytical grade were from Merck (Nogent sur Marne, France).

Preparation of the Reagents for the RIA

Anti-Fab antisera were raised in three male rabbits weighing approximately 3 kg (Charles de Bouscat, Charles River, Elbeuf, France). Each rabbit was immunized with 1 mL of emulsion containing 100 μg of digoxin-specific sheep Fab fragments in 9 g/L NaCl in Freund's complete adjuvant, by subcutaneous injections, in 10 sites on the back. Booster injections given at 3- to 4-week intervals were performed with Freund's incomplete adjuvant. Before and 10 days after the booster injections, rabbit blood was collected from the median artery of the ear into heparinized tubes and centrifuged at 2000*g* and 4°C for 15 min. Plasma was divided into aliquots and stored at −20°C.

Fab fragments were radiolabeled using the Fraker and Speck method (7): 420 μCi of Na^{125}I was reacted with 100 μg Fab in a tube coated with 10 μL iodogen (1 mg/mL). Free iodine was separated from iodinated Fab through a Sephadex G25 by elution with 0.01 *M* phosphate-buffered saline (PBS) with 0.15 *M* NaCl, pH 7.4. Aliquots of iodinated Fab were frozen at −20°C, and further dilutions were made in PBS, pH 7.4, with 0.1% Tween 20. Purity of the iodinated Fab preparation was ensured by counting the trichloracetic acid-precipitable ^{125}I fraction (gamma-scintillation counter Minaxi 5000, Packard Instruments, Rungis, France).

RIA Procedure

Nine standard Fab fragment solutions (0.625 to 25 μg/mL) were prepared daily from a stock solution at 4 mg/mL in 9 g/L NaCl and diluted in normal human plasma or urine. All measurements were performed in duplicate, using an auto-

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[3] Abbreviations used: RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; Fab, fragment antigen-binding; Ig, immunoglobulin; B_0, bound fraction of plasma or urine sample without Fab; SD, standard deviation; CV, coefficient of variation; sp act, specific activity; *T*, total radioactivity; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Table I. Cross-Reactivity of the Rabbit Antisera

Compound	Cross-reactivity (%)[a]
Polyclonal sheep digoxin-specific Fab fragments	100
Sheep γ-globulins	194
Polyclonal goat colchicine-specific IgG	35–81[b]
Polyclonal goat colchicine-specific Fab fragments	22–67[b]
Rabbit γ-globulins	<1.6
Monoclonal mouse digitoxin-specific IgG	<0.02
Monoclonal mouse digitoxin-specific Fab fragments	<0.01
Mouse γ-globulins	<0.01
Human γ-globulins	<0.01
Human albumin	<0.01
Human transferrin	<0.01
Human α_1-acid glycoprotein	<0.01

[a] The cross-reactivity is expressed as the molar ratio of the sheep digoxin-specific Fab fragment to the cross-reacting substance at 50% displacement of the antibody-bound tracer.
[b] According to the rabbit antiserum tested.

mate for diluting and dispensing reagents and samples (Probe 1000, Packard, Les Ulis, France). The reaction mixture for RIA procedure contained 0.1 mL of ^{125}I-Fab (5.2 ng/tube, 20,000–30,000 cpm), 0.05 mL of standard or sample, and 0.05 mL of a dilution of antibody that binds 50 to 60% of iodinated Fab fragments. The volume of the reaction mixture was made up to 0.3 mL with PBS pH 7.4. Determinations of the total radioactivity (T), the nonspecific binding of the tracer (NS), and the zero standard (B_0) were included in each series. After incubation for 60 min at 37°C with stirring (equilibrium was reached in 45 min), separation of bound (B) and free fractions was achieved by the addition of 0.5 mL of anti-rabbit IgG linked to magnetizable particles and application of a magnetic bar. Radioactivity of the pellet (B) was counted for 60 sec. Fab concentrations of unknown samples were calculated relative to the standard curve ($\%B/B_0$ versus log Fab concentrations) fitted by weighted least-squares regression.

Characteristics of the Rabbit Anti-Fab Antisera

The affinity constant of the rabbit anti-sheep Fab antiserum was calculated according to the method of Müller (8). Specificity of the antisera was assessed by calculating cross-reactivity with human proteins that can interfere in the human plasma assays and with immunoglobulins of different species (goat, rabbit, mouse). Because Fab fragments are used for the treatment of digoxin-intoxicated patients, the influence of digoxin (7.8 to 250 μg/mL) on the measurement of a constant Fab fragment concentration (9 μg/mL) was studied by RIA. The means of the Fab concentrations without digoxin (n = 12) and with digoxin (n = 12) were compared using Student's t test.

Validation of the RIA in Human Plasma and Urine

Validation criteria for the RIA were determined with Fab standards in human plasma or urine. Linearity was assessed by dilution parallelism, using three standards of high Fab concentrations (28, 22, and 18 μg/mL) in blank plasma or urine at dilutions from 1:2 to 1:32, measured in triplicate. The correlation between the theoretical concentrations and the experimental concentrations was analyzed by linear regression. The accuracy was evaluated by calculating the percentage recovery of standards at three Fab concentrations. Intraassay and interassay precision was determined by repeatedly assaying aliquots of Fab samples. The limit of detection was calculated as previously described (9), by measuring the zero standard with 20 replicates.

Clinical Application of the Fab Fragment RIA

A 54-year-old woman who had ingested 10 to 20 mg of digitoxin was successfully treated with 800 mg of antidigoxin Fab fragments (cross-reactivity of 33% with digitoxin) infused over a period of 1 hr. Blood samples were collected on dry heparin before Fab infusion and 0.5, 1, 2, 4, 6, 10, 14, 20, 26, 35, 43, 60, and 72 hr after the end of the infusion. Urine was collected during the first 48 hr. Plasma and urine samples were frozen at −20°C until assayed.

Pharmacokinetic Analysis

Pharmacokinetic parameters were determined by a model-independent method using classical equations (10).

Qualitative Analysis of Urinary Fab Metabolites

First, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of a urine sample corresponding to the 0- to 5-hr period after Fab infusion was carried out using the PhastSystem (Pharmacia, Saint-Quentin en Yvelines, France), with a 20% gel, under nonreducing and reducing (5% 2-mercaptoethanol) conditions. Second, 800 μL of the same urine sample was fractionated on an Ultrogel AcA44 column at 10°C, previously calibrated using dextran blue (2000 kD) and molecular weight standards (6.5, 14, 29, 50, and 66 kD). Elution was performed at a flow rate of 0.3 mL/min, with a 50 mM Tris–HCl buffer at pH 7.5. Elution was monitored by UV detection (single path monitor UV-1, 280-nm filter, Pharmacia) and 10-mL fractions were collected using a Gilford collector (Villiers Le Bel, France), concentrated sixfold on PEG 6000, and extensively dialyzed at 4°C against PBS at pH 7.4. Each fraction collected was assayed for immunoreactivity to anti-sheep Fab fragment antiserum by RIA.

RESULTS

Development of the RIA

Iodination of digoxin-specific sheep Fab fragments by the Iodogen method provided a yield of 75% and a specific activity of 97 μCi/nmol.

For the assays, one of the antisera was selected for its convenient specificity, high titer (1:9100), and high-affinity constant ($4.1 \cdot 10^9\ M^{-1}$). Figure 1 presents the linear range of the standard curve as a function of the tracer amount and the Fab-binding percentage of the tracer to the antiserum (B_0/T). A high specific activity (97 μCi/nmol) and low B_0/T (34%) resulted in a high sensitivity and a short linear range of the standard curve (0.05 to 5 μg/mL). In contrast, lower specific activity (0.7 μCi/nmol) and higher B_0/T (60%) re-

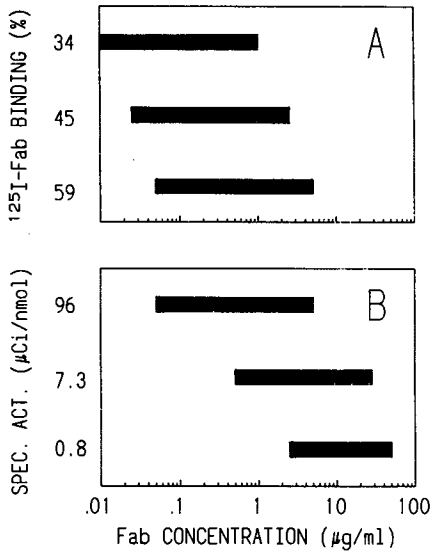

Fig. 1. Influence of the percentage of binding to the tracer and tracer specific activity on the linear range of the standard curve. (A) Constant specific activity (97 μCi/nmol). (B) Constant percentage of binding to the tracer (60%).

sulted in a weaker sensitivity but over a wider linear range of the standard curve (2.5–50 μg/mL). With a specific activity of 7 μCi/nmol and a B_0/T of 60%, we obtained sufficient sensitivity and a suitable Fab concentration range for the standard curve (Fig. 2). The nonspecific binding percentage was lower than 6%.

RIA Validation

Human proteins did not interact with anti-Fab antiserum (Table I). Study of species specificity showed that mice IgG did not cross-react (<0.02%), while goat IgG and Fab fragments reacted at 22 and 67%, respectively, with anti-sheep Fab antibodies. A 1.6% cross-reactivity was observed with rabbit γ-globulin. Fab measurement was not affected by high digoxin concentrations: 9.4 ± 0.3 μg/mL (mean ± SD, without digoxin) vs 9.2 ± 0.4 μg/mL (with digoxin).

Linearity was observed between 0.6 and 28 μg/mL Fab fragments for both urine and plasma specimens: linear regression analysis gave $y = 1.008x + 0.012$ with $r = 0.999$ (plasma) and $y = 0.980x + 0.028$ μg/mL with $r = 0.999$ (urine). The limit of detection was 0.1 μg/mL. Recovery ranged from 96.0 to 105.8% (Table II). Intra- and interassay coefficients of variation (CV) ranged from 2.4 to 6.9 and 4.7 to 10.5%, respectively (Table III).

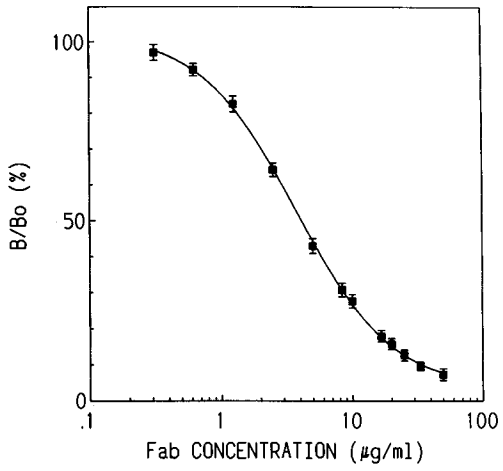

Fig. 2. Standard curve of a sheep Fab fragment RIA using plasma Fab fragment standards (mean ± SD; $n = 5$), at 60% binding to the tracer and 7.3 μCi/nmol specific activity.

Pharmacokinetic Study of the Fab Fragments

On admission of the patient to the hospital and prior to Fab fragment infusion, plasma digitoxin was at a toxic level of 287 nmol/L. Figure 3 shows the plasma concentration–time curve of sheep Fab fragments measured by RIA. Fab fragment concentration was maximal (252 μg/mL) at the end of the infusion (1 hr) and then decreased biexponentially, with an elimination half-life of 12.1 hr. Fab was detectable in the plasma up to 44 hr (1.1 μg/mL). Mean residence time was 7.7 hr. Steady-state distribution volume and total-body clearance were 10.8 L and 23.4 mL/min, respectively.

Qualitative Analysis of Fab Fragments in Urine

SDS-PAGE of urine of the Fab-treated patient revealed two bands of 50 and 25 kD under nonreducing conditions and a single band of 25 kD in the presence of 2-mercaptoethanol. After gel filtration separation, one peak (50 kD) was detected at 280 nm. Among the concentrated fractions assayed by RIA, two fractions reacted with anti-Fab antiserum. They corresponded to molecular weights of 50 and 25 kD according to gel filtration chromatography. The 25 kD fraction represented 8% of the urinary protein amount in terms of Fab equivalents determined by RIA.

DISCUSSION

Few data on Fab pharmacokinetics are available in humans because of the lack of specific and sensitive analytical methods for Fab measurement. Indirect quantitation based on digoxin binding capacity has been used by Schaumann *et al.* (3). On the other hand, three immunological methods have been described: immunonephelometry (4), radial immunodiffusion (11), and ELISA (5,6). However, radial immunodiffusion using anti-human Fab fragment antiserum cannot be used to assay Fab in humans, and ELISA developed by Timsina *et al.* cannot be performed for plasma containing digoxin.

Table II. Recovery of Sheep Fab Fragments by RIA in Human Plasma and Urine

Expected conc. (μg/mL)	Measured conc. (μg/mL), mean ± SD ($n = 3$)		Recovery (%)	
	Plasma	Urine	Plasma	Urine
2.0	1.9 ± 0.1	2.1 ± 0.1	96.0	105.0
8.5	9.0 ± 0.3	8.3 ± 0.2	105.8	98.2
19.0	19.7 ± 1.5	18.5 ± 1.2	103.7	97.5

Table III. Analytical Precision for RIA of Sheep Fab Fragments in Human Plasma and Urine

	Assay precision in plasma		Assay precision in urine	
	Mean ± SD (μg/mL)	CV (%)	Mean ± SD (μg/mL)	CV (%)
Intraassay (n = 12)	2.6 ± 0.1	4.8	1.9 ± 0.1	6.9
	10.1 ± 0.4	4.3	8.2 ± 0.2	2.4
	19.8 ± 0.6	3.0	15.8 ± 0.6	3.8
Interassay (n = 8)	3.0 ± 0.3	9.4	1.7 ± 0.1	8.1
	9.7 ± 0.8	7.9	8.5 ± 0.4	4.7
	20.3 ± 2.1	10.5	16.1 ± 1.2	7.5

The aim of this study was to develop a sensitive RIA specific to sheep Fab fragments. In contrast to nephelometry and ELISA using polyclonal *anti-Ig* antibodies, this RIA is performed with *anti-Fab* antiserum produced in the rabbit. The anti-sheep Fab antisera exhibited a significant cross-reactivity with goat IgG and Fab fragments but did not cross-react with human, rabbit, and mouse IgG and Fab fragments. This is presumably due to the phylogenetic relatedness between goat and sheep. Human proteins and digoxin did not interfere in the RIA of Fab in plasma and urine. The limit of detection of the RIA was 0.01 μg/mL with a 97 μCi/nmol specific activity of the tracer (data not shown). The affinity constant of the anti-sheep Fab antiserum was $4.1 \cdot 10^9$ L/mol. The RIA's sensitivity is superior to the sensitivity of immunonephelometry (4) and comparable to that of ELISA (6) and radial immunodiffusion (11). Nevertheless, in view of the wide range of Fab concentrations in biological samples of Fab-treated patients (up to 300 μg/mL) (4), the use of a tracer with a lower specific activity (7 μCi/nmol) was preferred. Thus, plasma and urine samples should not be diluted more than 1:30 and 1:70, respectively. Under these conditions, a wide range of linear standard curves (0.6 to 28 μg/mL) and a sufficient detection limit (0.1 μg/mL) were obtained. The RIA was found to be linear, accurate, and precise.

The RIA was applied to Fab pharmacokinetics in one patient. The value of Fab fragment elimination half-life of 12.1 hr is comparable to that observed by Urtizberea *et al.* (4) in two patients (12.6 and 10.7 hr). Schaumann *et al.* (3) reported elimination half-lives from 14.3 to 25.9 hr using an indirect method for Fab measurement that cannot be considered totally satisfactory. The steady-state distribution volume (10.8 L) is similar to values reported previously (3,4,12). The total-body clearance value (23.4 mL/min) is similar to that described by Schaumann *et al.* (3) (24.5 mL/min).

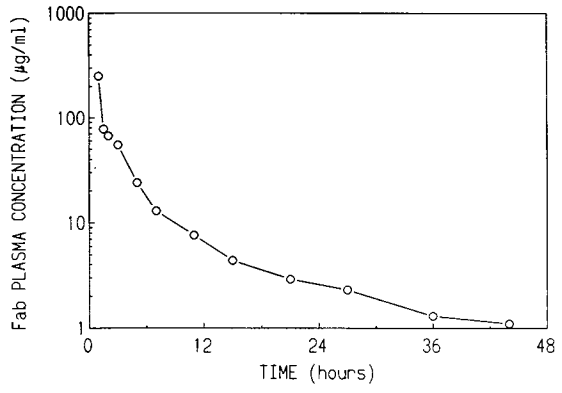

Fig. 3. Plasma concentration–time profile of digoxin-specific Fab fragments in a patient intoxicated by digitoxin. Fab fragments were administered (t = 0) over a 1-hr period.

After gel filtration separation of the urinary sample, proteins of 25 kD were found to cross-react with the anti-sheep Fab antiserum. These fragments could be light and heavy chains resulting from reduction of disulfide bridges or, less probably, Fv fragments (13). The failure of SDS-PAGE to detect 12.5-kD fragments, i.e., the molecular weight of reduced Fv, under reducing conditions suggests the presence of light and heavy chains. Further investigation would be required to characterize these compounds and to determine their immunoreactivity to digoxin.

In conclusion, this RIA provides a valuable assay for Fab fragments in human plasma and urine because of its high sensitivity and the absence of interference by digitalis or proteins. It should permit improvement of IgG and Fab fragment therapy. Because of the cross-reactivity of goat IgG and Fab fragments to the anti-sheep antiserum, this method can be extended to goat Fab measurement. This is of interest because of the use of goat colchicine-specific Fab fragments in the treatment of acute colchicine poisoning in humans (14,15). Further, the anti-sheep Fab antiserum can also serve as a tool for studying Fab metabolites in urine.

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