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Development and implementation of an electrochemiluminescence immunoassay for the determination of an angiogenic polypeptide in dog and rat plasma

A.S. Yuan *, M.L. Morris, K.C. Yin, J.Y.-K. Hsieh, B.K. Matuszewski

Merck Research Laboratories, WP75A-203, West Point, PA 19486, USA

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Abstract

A quantitative method based on electrochemiluminescence immunoassay for the determination of the angiogenic agent aFGF-S117 has been developed and validated. Two polyclonal antibodies specific to aFGF-S117 and a wild-type aFGF antibody were selected for the analysis. The assay was based on the non-competitive sandwich immunoassay principle in which the drug is trapped with a biotinylated antibody that is immobilized on a streptavidin magnetic particle. The drug is then sandwiched with a ruthenium chelated second antibody. The assay demonstrates good accuracy and reproducibility at plasma concentration of 0.5 ng/ml.

Keywords: aFGF; Immunoassay; Electrochemiluminescence

1. Introduction

Fibroblast growth factor-1 (FGF-1; also known as acidic FGF) is a potent angiogenic polypeptide that belongs to a family of structurally related proteins characterized by a high affinity for glycosaminoglycans [1,2].

A recombinant form of acidic FGF that differs from the human form by two amino acid molecules (aFGF-S117) has been synthesized in genetically engineered yeast. As compared to wild-type acidic FGF protein, aFGF-S117 protein has a longer half life in vitro both in the absence and the presence of heparin; this property may allow for a more stable formulation and potentially, a more convenient dosing regimen and better efficacy relative to wild-type acidic FGF [3,4].

A sensitive method for the quantitation of aFGF-S117 in biological fluids was required to support the preclinical studies necessary to develop this drug candidate [5]. An electrochemiluminescence immunoassay was developed for this new compound and the details of the methodology are presented in this paper.

* Corresponding author. Tel.: +1-215-652-7659; fax: +1-215-652-2410.
E-mail address: al_yuan@merck.com (A.S. Yuan).

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2. Experimental

2.1. Reagents

Goat anti-aFGF IgG, recombinant human aFGF and bFGF were obtained from R&D Systems (Minneapolis, MN); rabbit anti-aFGF IgG was from RDI (Flanders, NJ); bovine serum albumin (BSA) was obtained from Calbiochem (San Diego, CA); potassium phosphate, ethylenediaminotetraacetic acid, gelatin and goat IgG were from Sigma (St. Louis, MO); *N*-hydroxysuccimidebiotin (NHS) was from Pierce (IL, USA); streptavidin dynabeads, TAG–NHS Ester, Igen assay and washing buffers were obtained from Igen (Gaithersburg, MA); aFGF-S117 was obtained from Merck Research Laboratories (West Point, PA). The following buffer solutions were used: 50 mmol/l potassium phosphate buffer (pH 7.5) containing 5% BSA and 0.05% sodium azide (buffer A); 50 mmol/l potassium phosphate buffer (pH 7.5) containing 1% BSA and 0.1% Triton X-100 (buffer B); 10 mmol/l potassium phosphate buffer (pH 7.5) containing 0.1% BSA, 0.1% Triton X-100 and 0.05% sodium azide (buffer C); 10 mmol/l potassium phosphate buffer (pH 7.5) containing 0.1% gelatin and 0.05% sodium azide (buffer D).

2.2. Preparation of biotinylated anti-aFGF IgG

Anti-aFGF IgG was conjugated with NHS. An affinity purified anti-aFGF goat IgG (0.5 mg) was dissolved in 0.5 ml of 0.1 mol/l sodium bicarbonate solution (pH 8.7) and to this solution 20 μl of 2 mg NHS/ml in dimethyl sulfoxide was added. The solution was stirred 4 h at room temperature. Dialysis was carried out at 0–4 °C overnight against 2 × 2 l of 10 mmol/l potassium phosphate (pH 7.5) containing 0.05% sodium azide. Anti-aFGF IgG–biotin conjugate was stored at −20 °C in buffer D.

2.3. Preparation of the ruthenium tag

Ruthenium–anti-aFGF IgG tag was prepared by conjugation of a *N*-hydroxy succinimide ester of a ruthenium(II) tris-bibyridine chelate (TAG–NHS Ester) with affinity purified anti-aFGF rabbit IgG. TAG–NHS Ester (75 μg) was dissolved in 50 μl of dimethyl sulfoxide and, to this solution 0.8 mg of anti-aFGF IgG in 0.8 ml of 10 mmol/l phosphate buffer (pH 7.9) was added. The mixture was incubated for 90 min with mixing, at room temperature in the dark. The reaction was stopped by the addition of 20 μl of 2 M glycine and the mixture was incubated at room temperature for 10 min in the dark. Purification of the tag was carried out by using a prepacked Sephadex G-25 column (Pharmacia). The fractions containing the conjugation product were tested for immunoreactivity and stored at −20 °C.

2.4. ECL immunoassay

The stock standard was prepared in distilled water at a concentration of 1 mg/ml. Working standards were stored as 10 μg/ml solution in assay buffer B. The standard curve (Fig. 1), diluted from this stock, covered the range of 0.5–50 ng/ml. The biotinylated anti-aFGF IgG was titrated with minimum amount of streptavidin coated magnetic beads to provide a linear binding capacity for the standard range. Ruthenium–anti-aFGF IgG tag was titrated to a best percent binding with minimum non-specific binding within 1 h of incubation period. To minimize the sample matrix effect, multiple washes with buffer C were used after the first incubation with samples. Reagents were added to 12 × 75 mm glass culture tubes using an automatic pipetting station (Multiprobe II, Packard, USA) at room temperature as follows: 15 μl of preincubated biotinylated anti-aFGF and streptavidin coated beads in 200 μl of buffer B were vortexed and trapped magnetically for 5 min decanting for 2 min. Standard or plasma samples (25 μl) followed by 50 μl of buffer B were added to the tubes. After 60-min incubation, particles were trapped magnetically and washed with 500 μl of buffer C. Following the decant, 25 μl of ruthenium–anti-aFGF tag and 25 μl of buffer B were added to the tube. After a 60-min incubation, 300 μl of buffer B was added to the assay mixture followed by ECL detection on ORIGEN Analyzer (Igen, USA). All samples were assayed in duplicate. Using a five parameter logistic curve fit, a

calibration curve of net control binding versus concentration with optimal weighting was constructed. The concentration of aFGF-S117 in test samples was calculated by interpolation from the calibration curve.

3. Results

3.1. Assay precision and working range

The intra-assay precision was determined from four sets of standards at concentration of 0.5–50 ng/ml. All samples were assayed in one batch. Four sets of blank (drug-free) dog and rat plasma containing aFGF-S117 at nominal concentrations of 1, 5 and 20 ng/ml were prepared as quality controls and assayed as unknowns on two successive occasions. Results are shown in Table 1.

The inter-assay precision was determined from the coefficients of variation at each concentration during the analysis of four sets of standards (Table 1). The data showed acceptable values for precision in rat and dog plasma samples.

A representative calibration curve is shown in Fig. 1. The assay binding was considered to be satisfactory over the range of 0.5–50 ng/ml. Although the precision values outside this range were acceptable, the percent of binding was regarded as too low or too high to provide reliable data. The average non-specific binding was less then 5% of the high standard binding. The assay precision at the lower quantifiable limit (0.5 ng/ml) was 9.9% ($n = 4$) of intra-assay CV. Test plasma containing aFGF-S117 at the concentration in

Table 1
Intra- and inter-assay accuracy and precision for the determination of aFGF-S117 in dog and rat plasma by sandwich ligand electrochemiluminescence immunoassay

Concentration (ng/ml)	Replicate determinations	Mean found	Accuracy %[(Found)/(Actual)]100	Precision %CV
Dog plasma				
Intra-assay				
0.5	4	0.48	95.3	9.3
1	4	0.99	98.6	7.4
2	4	2.16	108.2	7.2
5	4	4.89	97.9	11.0
10	4	9.82	98.2	8.8
20	4	19.60	98.0	2.7
50	4	51.34	102.7	6.4
Inter-assay				
1	4	0.94	94.3	12.1
5	4	5.19	103.8	8.1
20	4	20.82	104.1	5.9
Rat plasma				
Intra-assay				
0.5	4	0.46	91.8	9.9
1	4	0.97	96.9	6.2
2	4	2.02	100.8	5.2
5	4	5.24	104.7	4.6
10	4	10.36	103.6	5.3
20	4	19.90	99.5	7.4
50	4	48.42	96.8	13.6
Inter-assay				
1	4	0.92	91.9	8.1
5	4	5.10	101.9	2.0
20	4	20.02	100.1	6.0

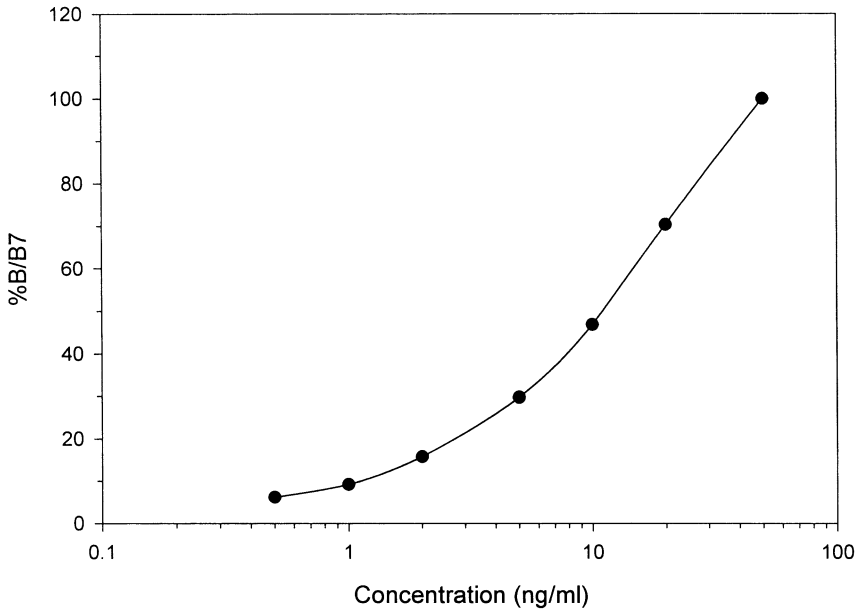

Fig. 1. A representative calibration curve for the determination of aFGF-S117 in dog and rat plasma.

excess of 50 ng/ml were further diluted prior to analysis.

3.2. Limit of quantitation

The intra and inter-assay precision and accuracy at 0.5 ng/ml of aFGF-S117 for dog and rat plasma were satisfactory. Accordingly, the lower quantifiable limit was at 0.5 ng/ml.

3.3. Recovery and parallelism

The assay recovery was determined by the analysis of aFGF-S117 spiked plasma samples for both dogs and rats. The results in Table 2 show analytical data that are not significantly different ($< 10\%$) from the expected values, confirming, the assay's accuracy was not compromised by the matrix.

Parallelism experiments were conducted on post-dose plasma samples from both rats and dogs that had received aFGF-S117 intravenously. Samples were assayed following sequential dilution in drug-free plasma matrix. The measured concentrations of aFGF-S117 were multiplied by the appropriate dilution factor, yielding the results shown in Table 2, showing no significant trend to higher or lower concentration with dilution.

3.4. Selectivity

No interference from endogenous plasma components was observed from control dog and rat plasma. The goat anti-FGF-1 antibody recognized epitope aa 137–155 mapping at the carboxy terminus. No reactivity with other members of the FGF family was observed.

3.5. Stability

The stability of the analyte was demonstrated over a period of 8 days at $-70\ ^{\circ}C$ in dog plasma. Similar stability of the analyte was observed in rats plasma (data on file). The representative data covering 8 days storage period in dog plasma are presented in Table 3.

4. Discussion

Wild-type aFGF and aFGF-S117 were used as immunogens. An attempt was made to produce

Table 2
Recovery and parallelism determination of aFGF-S117 in dog and rat plasma by electrochemiluminescence sandwich immunoassay

Subject #	Time	Endogenous (ng/ml)	Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)	[(Found)/(Expected)]100
Dog plasma						
Recovery						
001	0′	< 0.50	20.00	20.00	20.61	103.1
001	0′	< 0.50	10.00	10.00	10.11	101.1
001	0′	< 0.50	5.00	5.00	5.00	99.9
001	0′	< 0.50	2.50	2.50	2.43	97.2
Subject #	Time	No dilution (ng/ml)	Dilution (1:2) (ng/ml)	Dilution (1:4) (ng/ml)	Dilution (1:8) (ng/ml)	
Parallelism						
001	1′	15.82	15.07	14.85	15.63	
002	2′	12.76	13.50	13.89	12.00	
Subject #	Time	Endogenous (ng/ml)	Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)	[(Found)/(Expected)] × 100
Rat plasma						
Recovery						
001	0′	< 0.50	20.00	20.00	20.37	101.9
001	0′	< 0.50	10.00	10.00	10.19	101.9
001	0′	< 0.50	5.00	5.00	5.35	107.0
001	0′	< 0.50	2.50	2.50	2.63	105.2
Subject #	Time	No dilution (ng/ml)	Dilution (1:2) (ng/ml)	Dilution (1:4) (ng/ml)	Dilution (1:8) (ng/ml)	
Parallelism						
001	1′	18.90	18.89	19.17	18.11	
002	1′	16.15	17.75	18.00	18.16	

peptide specific antibodies for the determination of intact molecule in a sandwich immunoassay. Using a pair of synthesized peptides at C-terminus: KAILFLPLPVSSD and N-terminus: YL-RILPDGTVDG conjugated with BSA as immunogens, antiserum raised in rabbit and mice for poly- and monoclonal antibodies were relatively inactive. In the case of monoclonal antibody,

Table 3
Stability determination of aFGF-S117 in dog plasma by electrochemiluminescence sandwich immunoassay

Time (h)	Day 1		Day 8		Ratio (Day 8/Day 1)		
	Dog #1	Dog #2	Dog #1	Dog #2	Dog #1	Dog #2	Average
0.083	452.92	573.84	463.71	556.46	1.02	0.97	1.00
0.25	187.12	479.88	235.84	455.18	1.26	0.95	1.10
0.5	92.44	332.17	87.96	331.87	0.95	1.00	0.98
4	4.63	6.59	4.91	5.05	1.06	0.77	0.91
6	1.03	1.70	1.05	1.45	1.02	0.85	0.94

After IV dosing with 100 μg/kg of aFGF-S117, concentrations are in ng/ml.

several active clones were identified but lost in the propagation process. The antibodies used in this assay were selected from the commercial source listed in Section 2.

Parallelism experiments were conducted on immunoassay in an attempt to detect unknown drug related substances which might interfere with the antigen antibody binding. Such substances, generally metabolites or degrades, frequently show cross-reactivity with respect to antigen, that are not constant but which varies with dilution. Plots of parallelism were constructed to the normalized found concentration versus dilution factor. There was no significant trend to increase or decrease concentrations in plasma, and hence no indication of interfering substances.

In our laboratory, we routinely use a more definitive analytical method to verify the selectivity of the immunoassay. Although the criteria for satisfactory cross-validation of bioanalytical methods are somewhat subjective, we found a verification with another method as an important step to assure the integrity of analytical results. In the case of a molecule such as aFGF-S117 with high molecular weight (MW ~ 16 000) and high affinity binding to heparin, the aFGF-S117 was exceeding the boundary to develop a sensitive non-immunoassay method such as Ion Spray MS, MALDI/TOF or HPLC. The extraction, chromatography and formation of multiply charged species were the major issues. The protein is relatively unstable at physiological condition and subject to severe denaturation under temperature or pH changes.

A semiquantitative SDS/PAGE assay was established with I^{125}-labeled aFGF-S117 to detect the intact drug. The mean plasma concentration of aFGF-S117 in dogs following an intravenous dose of 100 μg/kg were compared in both methods (Fig. 2). At higher concentrations (> 50 ng/ml) correlation between the two methods was good, but at the lower concentration correlation was unsatisfactory. This is probably due to the lack of sensitivity of the SDS/PAGE assay.

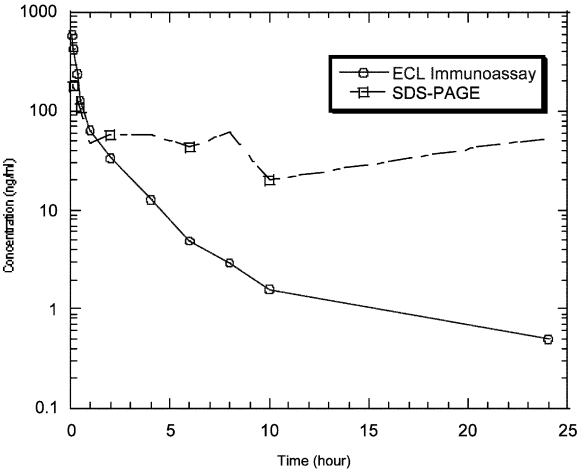

Fig. 2. Mean plasma concentration of aFGF-S117 in dogs following an intravenous dose of 100 μg/kg.

In summary, the aFGF-S117 electrochemiluminescence immunoassay showed satisfactory accuracy and precision along with good selectivity and demonstrated its usefulness for the quantitation of this drug in preclinical studies.

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