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Time-resolved fluoroimmunoassay for the determination of lisinopril and enalaprilat in human serum

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Abstract

A solid-phase immunoassay with detection based on time-resolved fluorescence (TR-FIA) has been developed for the determination of lisinopril and enalaprilat in human serum. The immunogen was prepared by coupling lisinopril to bovine serum albumin through a two-step reaction with difluorodinitrobenzene. An antiserum specific to both lisinopril and enalaprilat was used. The assay is based on the competitive immunoassay principle in which the drug competes with biotin-labeled drug for a limited quantity of primary antibody bound via sheep anti-rabbit globulin to the wells of microtitration strips. At the end of the first incubation, the unbound biotin-labeled drug is washed away. In the second step, europium-labeled streptavidin (specific to biotin) reacts with the biotin already bound to the solid-phase antibody. After a washing step, the addition of an enhancement solution dissociates the europium ions from the labeled streptavidin into solution. The fluorescence from each sample is inversely proportional to the concentration of the drug in the sample. The assay demonstrates good accuracy, reproducibility and specificity at serum concentrations down to 0.5 ng ml^{-1}. However, the useful concentration range of TR-FIA is much narrower than that obtained by double antibody radioimmunoassay (RIA).

Keywords: Lisinopril; Enalaprilat; Time-resolved fluoroimmunoassay; Radioimmunoassay

1. Introduction

Enalapril maleate, (-(*S*)-(1-[*N*-[1C)ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline, (*Z*)-2-butene dioate (1:1) salt) (Fig. 1), a relatively poor angiotensin-converting enzyme (ACE) inhibitor, gives rise to a very potent inhibitor, enalaprilat, via esterolysis. Unlike its ester, enalaprilat is poorly absorbed orally. Lisinopril is the lysine analog of enalaprilat and is at least as potent as enalaprilat. Both enalapril maleate and lisinopril inhibit angiotensin-converting enzyme after oral administration to animals and man [1–3].

A radioimmunoassay (RIA) has previously been established for the quantitation of lisinopril and enalaprilat in biological fluids [1,2]. The RIA was selected based on the need for high assay sensitivity, the absence of metabolism of lisinopril and enalaprilat in man which leaves little or no concern about the assay lacking specificity, and the ability of the assay to be readily automated. However, the use of radioisotopes as labels has several disadvantages, including a short shelf-life

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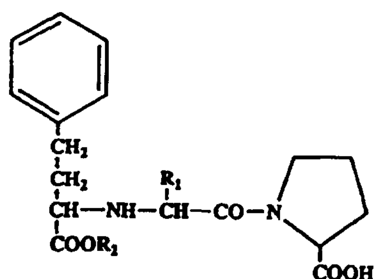

	R_1	R_2
Lisinopril	$(CH_2)_4NH_2$	H
Enalaprilat	CH_3	H
Enalapril	CH_3	C_2H_5

Fig. 1. Structures of lisinopril, enalaprilat and enalapril.

(^{125}I-label) and disposal concerns. An enzyme immunoassay (EIA) using a fluorogenic substrate was subsequently developed, which did not achieve the RIA's sensitivity. This lack of sensitivity was partially attributed to the background fluorescence from both the test samples and the plastic apparatus [4].

The time-resolved fluoroimmunoassay (TR-FIA) appears to have potentially overcome some of the disadvantages associated with the conventional fluoroimmunoassay (FIA) [5,6]. The highly fluorescent lanthanide (europium) complex, widely separated excitation and emission wavelengths (namely a large Stokes shift) and the long fluorescence life-time specifically enables the TR-FIA to match the sensitivity of the RIA [7].

In this report, we describe the development of a TR-FIA for lisinopril in which biotin was covalently bound to the drug. Europium-labeled streptavidin was added after the competitive binding of the drug and labeled drug to antibodies which were fixed by adsorption to the surface of polystyrene microtiter strips. The assay was sensitive to below 1 ng ml^{-1} (Fig. 2).

The radioimmunoassays for enalaprilat and lisinopril are virtually identical (unpublished data). Lisinopril was selected arbitrarily for the evaluation of the present TR-FIA procedure.

2. Materials and methods

2.1. Reagents

The following reagents were purchased from the indicated vendors: *N*-hydroxysuccinimide-LC-biotin and streptavidin from Pierce Co. (Rockford, IL, USA); rabbit IgG and sheep anti-rabbit gamma-globulin from Arnel Products Co. (New York, NY, USA); Sephadex G-25, Sepharose 6B, the europium–chelate of *N'*-(*p*-isothiocyanatobenzyl)diethylene-triamine-*N,N,N,N*-tetraacetic acid (Eu^{3+}–DTTA) (labeling compound) and the enhancement solution from Wallac (Turku, Finland). *N*-hydroxysuccinimide affinity filters were from Affinity Technology (NJ, USA); bovine serum albumin (BSA) was obtained from Calbiochem (San Diego, CA. USA), and the europium standard (100 μM in 0.1 M acetic acid), diethylenetriamine–pentaacetic acid (DTPA), ethanolamine, triethylamine, difluorodinitrobenzene, *N*-hydroxysuccinimide and 1,3-dicyclohexylcarbodiimide were from Sigma (St. Louis, MO, USA). The following compounds were obtained within the Merck Research Laboratories: enalapril (MK-0421), enalaprilat (MK-0422) and lisinopril (MK-0521). The remaining agents were widely available and not dependent on the source.

2.2. Buffers

The assay buffer was 50 mM phosphate (pH 7.5) containing 0.5 M EDTA and 1 g of BSA per liter. The streptavidin–europium dilution buffer consisted of 50 mM Tris–HCl solution (pH 7.8), containing 1.5 mmol of sodium chloride, 5 g of BSA, 0.5 g of bovine globulin, 0.1 ml of Tween 40, 8 mmol of sodium azide and 20 μmol of DTPA, all per liter. The enhancement solution consisted of 0.1 M phthalate–HCl buffer (pH 3.2) containing 15 mM 2-naphthoyltrifluoroacetone, 5 mM tri-*N*-octylphosphine oxide and 0.1% Triton X-100. The washing solution was 10 mM phos-

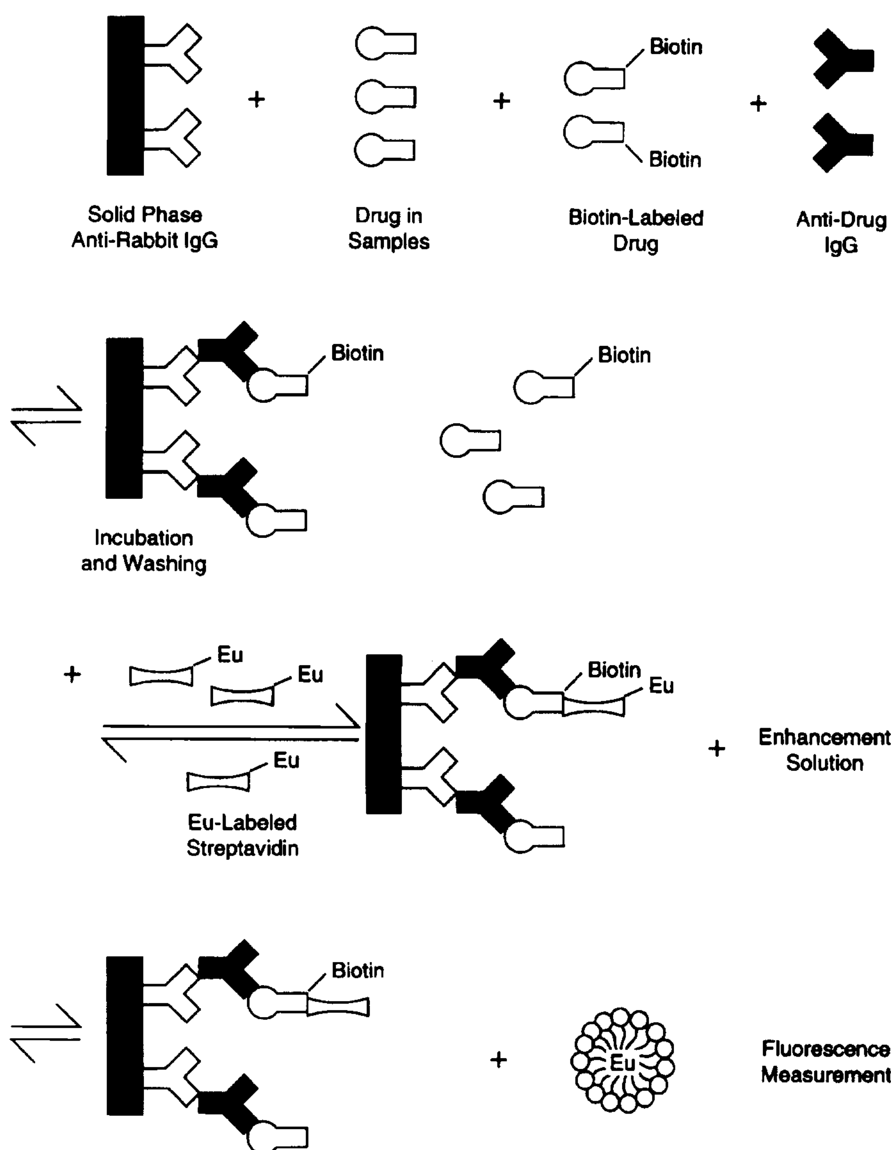

Fig. 2. Schematic presentation of the indirect TR-FIA.

phate (pH 7.5), containing 0.15 M sodium chloride and 0.5 ml l^{-1} of Tween 20.

2.3. *Biotinylation of lisinopril*

N-hydroxysuccinimide-LC-biotin (8.6 mg; 15 μmol) was added directly to lisinopril (3 mg; 6.8 μmol) in 0.5 ml of 50 mM bicarbonate (pH 8.5). The reaction was allowed to proceed on ice for 2 h and the unreacted NHS-LC-biotin was removed by HPLC using an Altex Ultrasphere Octyl column (4.6 mm × 250 mm; 3 μm; Beckman, Fullerton, CA). A linear gradient from 20 to 80% methanol–phosphate buffer (pH 7.0; 10 mM) in 20 min was used and the product was eluted after a retention time of 9 min (Fig. 3). The biotinylated lisinopril was vacuum-dried, reconstituted in assay buffer and stored at −20°C until use.

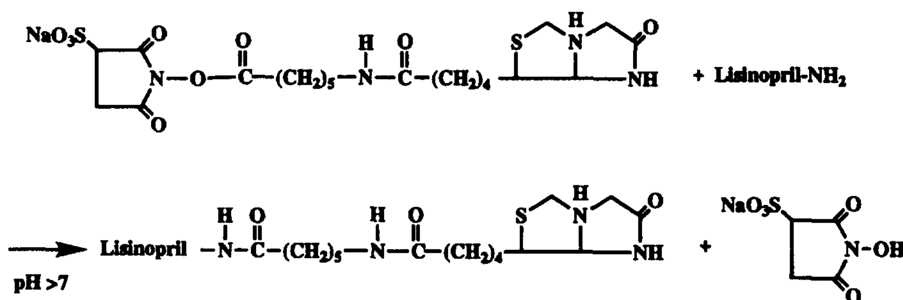

Fig. 3. The reaction of *N*-hydroxysuccinimide-LC-biotin with lisinopril.

2.4. Labeling of streptavidin with europium

Streptavidin was reconstituted in distilled water and buffer exchanged using a G-25 column (1 cm × 10 cm) with a labeling buffer consisting of 50 mM sodium carbonate (pH 8.5), containing 0.9% (w/v) sodium chloride. The labeling of streptavidin was performed by mixing 0.2 mg of the labeling compound with 1 mg of streptavidin in 0.5 ml of the labeling buffer. The mixture was allowed to stand overnight at room temperature. The purification of the europium-labeled streptavidin was accomplished by gel filtration using a Sepharose 6B column (1.5 cm × 30 cm) eluted with 50 mM Tris–HCl buffer (pH 7.8) containing 0.9% (w/v) sodium chloride and 0.05% (w/v) sodium azide. One-milliliter fractions were monitored by their UV absorbance at 280 nm and the europium concentration was determined after appropriate dilution. The incorporation of europium into streptavidin was estimated at 1.8 (mol/mol). The binding characteristics and the background fluorescence counts of the labeled streptavidin were determined experimentally (Fig. 4).

2.5. Isolation of antibodies

The principal rabbit antiserum was that used in the previously reported RIA procedure [1,2]. The immunogen was prepared from lisinopril by coupling to bovine serum albumin (BSA) via a two-step reaction with difluorodinitrobenzene. The antiserum is specific for lisinopril and enalaprilat but not for enalapril (a greater than 0.1% cross-reactivity). The antiserum was used at 1:19000 dilution in the RIA, but prior to use in the TR-FIA, the antiserum required further purification by affinity chromatography to increase the antibody-capturing capacity on the solid-phase surface. Accordingly, an *N*-hydroxysuccinimide affinity-filter cartridge (Affinity Technology, New

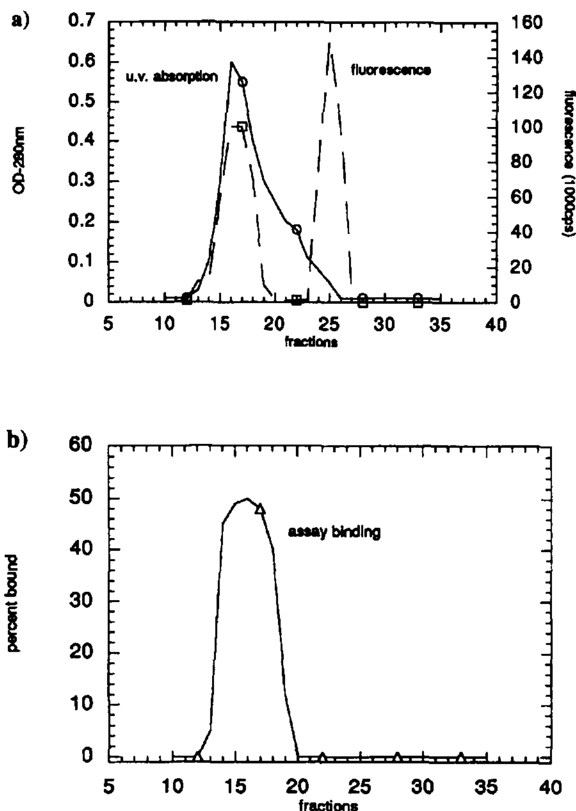

Fig. 4. Europium-labeled streptavidin elution profile. (a) UV absorption at 280 nm (circles) and fluorescence in counts per second (squares); (b) binding of europium-labeled streptavidin in the TR-FIA (per cent bound).

Table 1
Mean readback values of lisinopril concentrations (ng ml^{-1}) obtained from five successive analyses by TR-FIA

Lisinopril concentration (ng/ml^{-1})	Mean (B/B_0)	Readback value (ng/ml^{-1})						
		Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Mean	RSD (%)
0.4	0.91	0.4	0.40	0.40	0.39	0.39	0.40	1.8
1	0.75	1	1.01	1.00	1.05	1.10	1.03	4.3
2	0.62	2	1.88	2.00	1.99	2.00	1.97	2.6
4	0.46	4	4.01	4.00	4.01	4.18	4.04	1.9
10	0.28	10	10.00	10.00	9.74	9.99	9.94	1.2

Brunswick, NJ) was washed sequentially with cold distilled water and with 50 mM phosphate buffer (pH 7.0). Covalent coupling to the cartridge was achieved by recirculating a lisinopril solution (1 mg ml^{-1} in phosphate buffer). The excess lisinopril was eluted with phosphate buffer and the remaining active sites on the cartridge were blocked by treatment with 0.1 M aqueous ethanolamine at pH 7.0. Rabbit anti-lisinopril antiserum (1:10 in phosphate buffer) was then recirculated through the cartridge. After washing with phosphate buffer, antibodies were eluted with 0.1 M glycine–HCl (pH 4.0) into phosphate buffer (pH 7.5; 0.1 M).

2.6. *Coating of polystyrene microtiter strips*

Sheep anti-rabbit IgG (Arnel, New York, NY) was immobilized by adsorption to the well walls of polystyrene microtiter strips (Dynatech, Chantilly, VA). The wells were coated overnight with 0.3 ml of a 1 mg l^{-1} anti-rabbit–IgG solution in 0.1 M sodium carbonate buffer (pH 9.6). After

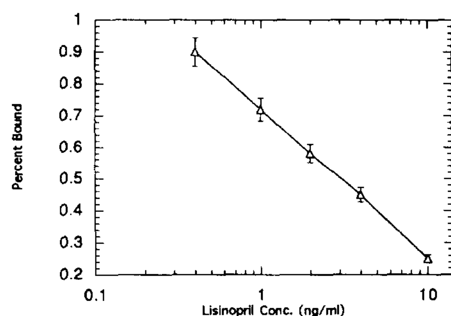

Fig. 5. A representative lisinopril TR-FIA standard curve,in the range 0.4–10 ng ml^{-1}.

coating, the wells were washed first with distilled water and subsequently with the washing solution. Saturation of the well surfaces was achieved by adding 0.3 ml of saturation buffer (phosphate (50 mM)–sodium chloride (0.9%)–BSA (5 g l^{-1}) (pH 7.4)). Finally, the wells were vacuum-dried and the strips were stored in plastic bags at 4°C for up to 3 months.

2.7. *Preparation of standards*

For calibration, lisinopril was first dissolved in distilled water at a concentration of 1 mg ml^{-1}. Working standards were stored as 10 μg ml^{-1} solutions in assay buffer (phosphate buffer with 0.1% BSA) at −70°C. Dilution of the working standard in buffer yielded standard concentrations of 0.4, 1, 2, 4 and 10 ng ml^{-1}. Quality control samples were prepared at concentrations of 0, 1, 2 and 4 ng ml^{-1}.

2.8. *Time-resolved fluoroimmunoassay*

Immediately prior to use, the strips were washed twice with the wash solution. Standards, controls and serum samples (10 μl) were pipetted in duplicate into the wells, 0.1 ml each of the diluted antibody solution and the working biotinylated lisinopril solution were added, and the strips were shaken in an automatic shaker for 5 min, then incubated for 3 h at room temperature. After the first incubation, the strips were washed three times with the wash solution and once with distilled water. A 0.2 ml volume of the diluted streptavidin–europium tracer was then added. After 45 min of incubation at room temperature on

the automatic shaker, the strips were washed five times with washing solution. Finally, 0.2 ml of enhancement solution was added to each well. After 20 min of slow shaking, the fluorescence of the 2-naphthoyltrifluoroacetone chelate was measured using an 1230 ARCUS fluorimeter (Wallac, Turku, Finland). The excitation wavelength was set at 340 nm, and the fluorescence was read at 613 nm. The absorbance of a blank (no drug added) was subtracted from each measurement and the assay results were calculated from the mean of the duplicates. A spline curve fitting data-reduction method (Wallac Inc.) was used for the construction of the calibration curve and the concentrations of unknown samples were determined by interpolation.

2.9. Radioimmunoassay

The RIA was conducted according to the procedure previously described [1,2]. Briefly, standards, controls and samples (15 μl) in triplicate were pipetted into assay tubes along with 0.1 ml of primary antiserum (1:19000 dilution), 0.1 ml of ^{125}I-labeled tracer with precipitation antiserum (sheep anti-rabbit globulin) and 0.58 ml of assay buffer. The assay was performed on an APS1 automatic pipetting station (Micromedic, Horsham, PA). The tubes were centrifuged after an overnight incubation at room temperature. The radioactivity was measured on a Micromedic 10/600 gamma-counter after decanting the supernatants. A polynomial curve fitting method [1] was used for the construction of calibration curves.

3. Results

3.1. Assay optimization

The capacity of the immobilized anti-rabbit IgG antibody on the microtiter wells is critical for the performance of the TR-FIA. The excess "capture" capacity is required to ensure the complete and reproducible binding of the primary antibody during the first incubation and thus minimize the variability of the solid phase. Various conditions for coating were tested, the optimal procedure being described in the text. The capacity of the solid phase was determined from the Scatchard plot analysis using competitive displacement of unlabeled vs. labeled rabbit IgG. The average antibody capacity of the wells is estimated at 40 ng (0.8 pmol) ml^{-1}.

The affinity-purified rabbit anti-lisinopril antibody was titered at 1:5000 dilution in assay buffer and added at 0.2 pmol per well. The biotin-labeled lisinopril was best used at 1:25000 dilution (1 pg per 0.1 ml). The europium-labeled streptavidin was used in two-fold excess to maximize the binding during the second assay incubation.

Table 2
Assay accuracy and intra-assay precision for the determination of lisinopril in serum by TR-FIA

Actual concentration (ng/ml^{-1})	Found concentration (ng/ml^{-1})	Recovery (%)	Mean Accuracy (%)	RSD (%)
1.0	1.08	108	108.3	4.8
2.0	1.92	96	95.8	2.4
4.0	4.10	102	102.3	4.5

3.2. Assay performance

3.2.1. Standard curve

The mean readback values from standard curves obtained on five different days are shown in Table 1. The inter-day standard curve reproducibility was assessed from the readback values of the five calibration curves. Relative standard deviations of the standards were satisfactory over the range 0.4 ng ml^{-1} (mean $B/Bo = 91\%$) to 10 ng ml^{-1} (mean $B/Bo = 27.6\%$) using 10 μl of serum. A representative standard curve is shown in Fig. 5. The range of the assay may be extended to higher concentrations by the dilution of test serum with normal human serum prior to analysis.

3.2.2. Precision and accuracy

Within-run precision was evaluated by assaying human serum controls containing added lisinopril at three different concentrations. The relative standard deviations for 12 samples at each concentration are presented in Table 2. The assay

Table 3
Inter-day precision and accuracy of lisinopril in serum by TR-FIA

Actual concentration (ng ml^{-1})	Found concentration (ng/ml^{-1})					Mean found/actual (%)	RSD (%)
	Day 1	Day 2	Day 3	Day 4	Day 5		
1.0	1.04	0.99	1.03	1.00	1.03	101.8	2.1
2.0	1.89	2.05	1.94	1.95	1.95	99.6	4.2
4.0	4.04	4.13	1.28	4.19	4.19	103.4	2.6

accuracy (mean percentage recovery) ranged from 95.8% at 2 ng ml^{-1} to 108.3% at 10 ng ml^{-1}. The intra-day assay relative standard deviations at 1 ng ml^{-1}, 2 ng ml^{-1} and 4 ng ml^{-1} were 4.8%, 2.4% and 4.5%, respectively. The inter-day relative standard deviations ($n = 5$) at the same analyte concentrations were 2.1%, 4.2% and 2.6%, respectively (Table 3).

3.2.3. *Proportionality*

Two serum samples obtained from a subject receiving lisinopril were serially diluted with normal human serum and assayed. The measured serum concentrations were multiplied by the appropriate dilution factor. The results indicate acceptable proportionality of the measured concentration with dilution (Fig. 6).

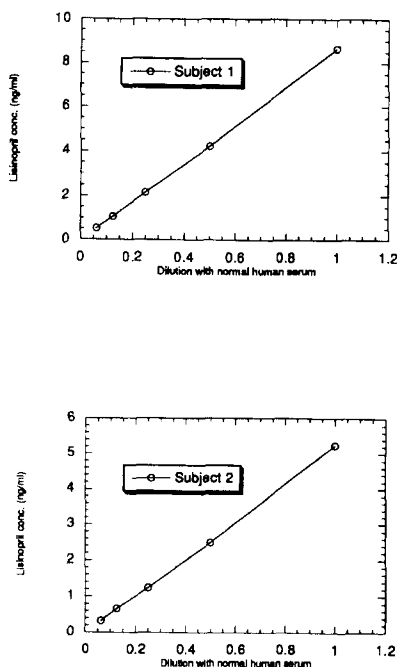

Fig. 6. Proportionality of measured lisinopril concentration (ng ml^{-1}) as a function of sample dilution with normal human serum.

3.2.4. *Spiked sample recovery*

Human serum samples were spiked with different known quantities of lisinopril and analyzed. The recovery ratio was defined as the found concentration divided by the actual spike. The ratios were 1.0, 0.9 and 0.9 for the first sample and 0.9, 1.0 and 0.9 for the second sample at spike concentrations of 0.5 ng ml^{-1}, 1.0 ng ml^{-1} and 2.0 ng ml^{-1}, respectively (Table 4a).

Table 4
(a) Recovery of lisinopril "spiked" into pre-dose human serum

Sample	Spike measured (ng ml^{-1})	Spike actual (ng ml^{-1})	Found/actual
1	0.50	0.5	1.00
	0.90	1	0.90
	1.80	2	0.90
2	0.47	0.5	0.94
	0.95	1	0.95
	1.82	2	0.93

(b) Accuracy of TR-FIA assay for lisinopril in patient's plasma using the method of standard deviation

Sample	Unspiked (ng/ml^{-1})	After spike (ng ml^{-1})	Difference (ng ml^{-1})	Recovery of spike (%)
1	0.00	1.01	1.01	101
2	2.93	4.09	1.16	116
3	4.81	5.75	0.94	94
4	6.78	7.72	0.94	94
5	11.50	12.40	0.85	85
6	16.60	17.52	0.90	90

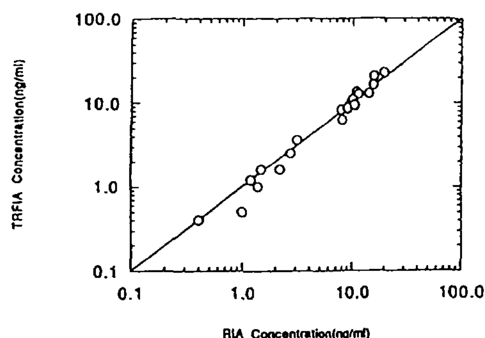

Fig. 7. Lisinopril in patient serum analyzed by the RIA (X axis) and the TR-FIA (Y axis). The linear concentration of Y is $0.57 + 1.1X$ and the correlation coefficient (R) is 0.98.

Additional recovery experiments were performed on the actual clinical samples to verify the validity of the assay. Clinical samples with endogenous concentrations of 0–16.6 ng ml^{-1} were spiked with 1 ng ml^{-1} of lisinopril, and the recovery was assessed by analyzing the samples before and after the spike. The results presented in Table 4b show reasonable agreement between expected and observed observations.

3.2.5. Cross-validation of the FR-FIA and RIA methods

Twenty-four plasma samples obtained from patients dosed orally with lisinopril were assayed by both procedures (Fig. 7). The mean ratio (TR-FIA/RIA) was 1.00 with an RSD of 14.4%.

In a paired t-test, the observed value of t (-0.92 with 23 degrees of freedom) when compared with t-critical (2.07 for $p = 0.05$) showed no significant difference in assay results generated using the two different methods. Linear regression analysis showed an overall assay bias (mean TR-FIA − mean RIA) of 3.9%. The intercept was -0.568 with confidence intervals ranging from -1.54 to $+0.40$ indicating no fixed bias. The slope was 1.108 (1.006–1.211) showing the TR-FIA method to have a proportional bias of between 0.6 and 21%.

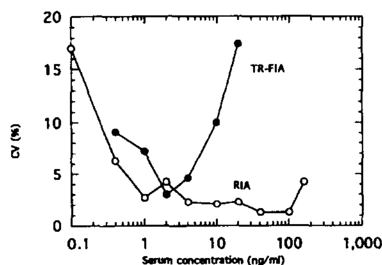

Fig. 8. Intra-assay precision profiles for the determination of lisinopril in human serum by the RIA and the TR-FIA.

4. Discussion

The performance characteristics of the TR-FIA meet the criteria for a sensitive and accurate test of lisinopril in serum. However, the TR-FIA has a short assay range (0.4–10 ng ml^{-1}) when compared to the established RIA (0.4–160 ng ml^{-1}) (Fig. 8). This is attributable partially to the limited solid-phase capacity but also to the quality of the europium–chelate streptavidin tracer.

The present assay is based on the principle of competitive immunoassay. Direct labeling of lisinopril with europium–chelate was first performed. The conjugation and separation of labeled lisinopril was found to be rather non-reproducible and the resulting tracer has high non-specific binding properties. It appears that the labeling compound (Eu^{3+}–DTTA) is more suited for conjugation with large proteins or peptides [8]. As an alternative, an indirect biotin–streptavidin labeling method was evaluated. Streptavidin rather than avidin was used because of its low non-specific binding properties. Streptavidin contains 13 available amino groups [9] and accordingly, the maximal incorporation of europium to streptavidin (13:1) can be achieved using the appropriate conditions [10]. It is conceivable that use of highly labeled streptavidin might enhance the assay sensitivity and the range of performance would be improved.

The biotin-labeled lisinopril and unlabeled lisinopril were tested in the RIA to verify their antigenicity with the antibody, and the biotin activity was evaluated in the TR-FIA. The quantitative recovery of the lisinopril from the biotin-la-

belled lisinopril indicated that the labeling procedure did not alter the avidity of lisinopril. The biotin-labeled lisinopril appears to be stable for more than 6 months at 4°C and 2 years at −20°C.

Several of the characteristics of the TR-FIA that have been published [10] were found also to apply to this assay. The indirect assay requires additional assay steps but the incubation time of those steps was short due to the assay design. The overall assay time required for the TR-FIA is comparable with that for the RIA. Much more manipulation is required by the TR-FIA than in the case with the RIA.

At low concentrations (0.4–10 ng ml^{-1}), the TR-FIA is comparable to the RIA in both sensitivity and precision. At higher concentrations, the TR-FIA is at a disadvantage because of its limited dynamic range. We recommend this TR-FIA assay for the determination of lisinopril and enalaprilat when the use of radioisotopes is prohibited.

Acknowledgments

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