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Determination of Captopril in Human Blood by an Enzyme-linked Immunosorbent Assay

AHMED M. T. JEHANLI, TOWFIQ ARAFAT* AND MUNZIR AL-SHAMI†

*Thrombosis Research Institute, Emmanuel Kaye Building, Manresa Road, London, SW3 6LR, *School of Pharmacy, Jordan University for Women, Amman, Jordan and †School of Pharmacy, Jordan University, Amman, Jordan*

Abstract

An immunoassay for the quantitation of the angiotensin-converting enzyme inhibitor, captopril in human plasma is described.

Antisera very specific for captopril were produced by immunization with captopril conjugated to bovine serum albumin or porcine thyroglobulin via the drug's thiol group. The antibodies were used to develop an enzyme-linked immunosorbent assay (ELISA) with a detection limit of 0·3 ng mL^{-1} and intra- and inter-assay coefficients of variation of 7 and 12%, respectively. Apart from stabilizing captopril by the addition of *N*-ethyl maleimide, the assay was used to detect the drug in human plasma without further extraction or purification.

Our immunoassay provides a very sensitive and rapid (four hours) alternative for the study of captopril pharmacokinetics.

Captopril, (1-(3-mercapto-2-D-methyl-1-oxopropyl)-l-proline-(S,S), is an orally active ACE inhibitor, used for the treatment of hypertension and congestive heart failure. The quantitation of captopril in biological fluids has been carried out by radiochemical methods, gas chromatography, gas chromatography-mass spectroscopy and high-performance liquid chromatography. Captopril lacks a functional group that is directly detectable by UV-visible spectroscopy and sensitive chromatographic methods require chemical derivatization and preliminary extraction for determination in biological fluids. Radioimmunoassay techniques for captopril have been reported (Duncan et al 1983; Tu et al 1984) and the values obtained for captopril correlated well with those found by the standard gas chromatography/mass spectrophotometry techniques. However, radioimmunoassay techniques require expensive equipment, the radiolabelled drug has a short shelf-life, and radioactive work presents potential health hazards requiring licensing and special disposal of radioactive waste.

In this study, we describe the development of a sensitive, simple and rapid competitive enzyme linked immunosorbent assay (ELISA) for the determination of captopril.

Materials and Methods

Materials

All chemicals and the following reagents were obtained from Sigma Co., Poole, Dorset: porcine thyroglobulin, bovine serum albumin (BSA, A7888), rabbit serum albumin (RSA), γ-maleimidobutyric acid *N*-hydroxysuccinimide ester (GMBS), *N*-ethylmaleimide, Freund's complete adjuvant, Freund's incomplete adjuvant, alkaline phosphatase labelled goat anti-rabbit IgG antibody, proline, proline-glycine, alanine-proline and *p*-nitrophenyl phosphate. Captopril was a kind gift from the Jordan Pharmaceuticals and Medicines Co., Amman, Jordan. Sulphosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulpho-SMCC) was obtained from Peirce and Warinner Co., Chester, UK. Microtitre wells F8 modules for the immunoassay were purchased from Gibco-Nunc, Scotland.

Methods

Preparation of protein-captopril conjugates for immunization. Captopril was covalently linked, via its thiol group, to the primary amino groups of bovine serum albumin (BSA) and porcine thyroglobulin (Tg) using the heterobifunctional cross-linker sulpho-SMCC as described previously (Gudgeon et al 1991) (Fig. 1). Briefly, BSA (15 mg) was dissolved in 1 mL 0·1 M sodium phosphate buffer, pH 7·0. One hundred-fold molar excess of sulpho-SMCC (10 mg) dissolved in 1 mL phosphate buffer was added to the protein solution and the activation of the amino groups was allowed to proceed for 1 h at 30°C. The mixture was centrifuged at 10 000 *g* for 5 min at room temperature (21°C) and the excess cross-linker was removed by gel chromatography of the supernatant on a column (2 × 40 cm) of Sephadex-G50 fine, equilibrated in 0·1 M sodium phosphate buffer, pH 6·0. Eluted fractions (2 mL) were monitored for protein by measuring the absorbance at 280 nm. Fractions containing the protein were pooled and 100-fold molar excess (with respect to the protein) of captopril (4·2 mg) was added. The mixture was incubated with stirring for 24 h at room temperature to allow the coupling of captopril to the protein. Finally, free captopril was separated from the coupled drug by dialysing the protein conjugate against 0·15 M sodium chloride (2 × 2 L) for 48 h at 4°C. The protein concentration was measured (Markwell et al 1987) and the conjugate, BSA-captopril, was diluted to 1 mg mL^{-1}, divided into small portions and stored at −20°C. Captopril coupling to thyroglobulin was carried out similarly but using a 500-fold molar excess (with respect to the protein) of the heterobifunctional crosslinker and captopril. The conjugation ratio of captopril : protein was estimated using the method of

Correspondence: A. Jehanli, Thrombosis Research Institute, Emmanuel Kaye Building, Manresa Road, London, SW3 6LR.

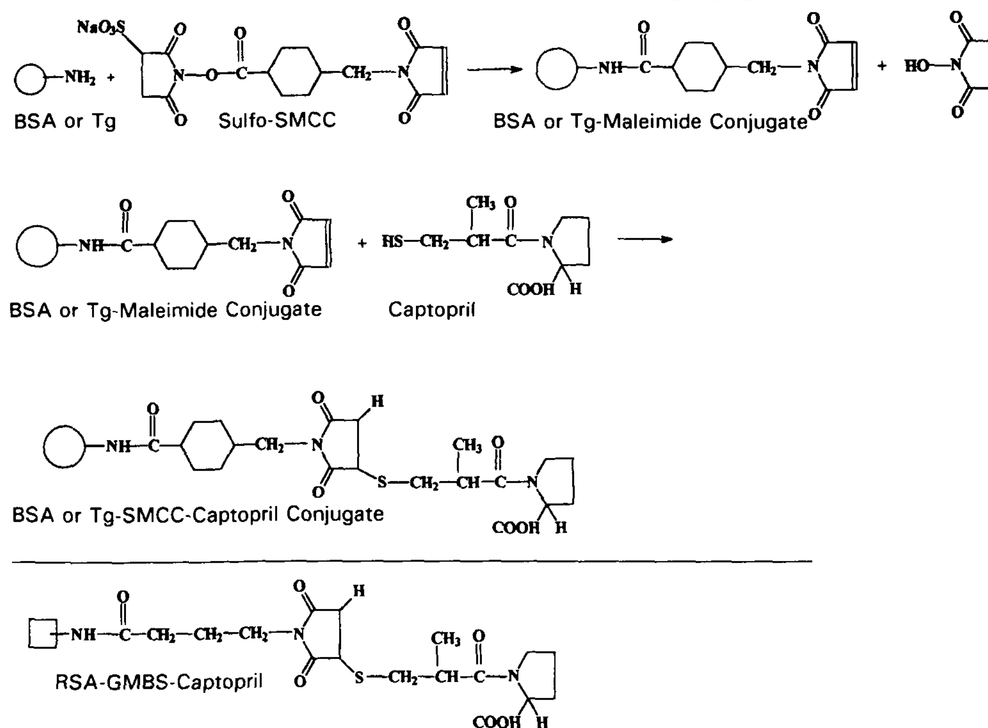

FIG. 1. Chemical coupling of captopril to BSA or Tg, and to RSA using the hetero-bifunctional crosslinkers sulpho-SMCC and GMBS.

Jones et al (1989). A ratio of 20–25 : 1 and 200–225 : 1 was obtained for captopril : BSA and captopril : thyroglobulin, respectively.

Immunisation protocol. New Zealand White rabbits (two per conjugate) were used. For each rabbit, 100 μg conjugate in 0·5 mL 0·15 M NaCl was vigorously mixed with an equal volume of Freund's complete adjuvant. The suspension was injected intramuscularly in four sites. The rabbits were boosted after 8 and 16 weeks with further injections as above but using Freund's incomplete adjuvant. Final bleeds were obtained 10 days after the last injection. Serum was obtained by centrifuging the clotted blood at 5000 *g* for 30 min at room temperature, then divided into small portions and stored at −70°C.

Preparation of RSA-captopril conjugate for the immunoassay. For use in microtitre plate immunoassays, captopril was coupled to rabbit serum albumin (RSA) using the general procedure described above with one modification; the hetero-bifunctional crosslinker γ-maleimidobutyricsuccinimide ester (GMBS) was used in place of sulpho-SMCC (Fig. 1). A different crosslinker was used to avoid interference in the ELISA that would arise if antibodies to the crosslinker were produced in the immunized rabbits. A coupling ratio of 15–20 : 1 (captopril : RSA) was obtained.

Enzyme-linked immunosorbent assay (ELISA). Wells of microtitre strips were coated by incubation overnight at 4°C with RSA-captopril (0·1 mL, 50 ng mL^{-1}) diluted in 0·05 M sodium carbonate/bicarbonate buffer, pH 9·5. Wells were washed three times with 50 mM Tris/HCl buffer pH 7·4, containing 0·05% Tween-20, 0·15 M NaCl and 20 mM sodium azide using Wellwash 2 Instrument (Denly Co.,Billinghurst, UK) and free sites blocked with the same buffer by incubation at ambient temperature for 30 min. For anti-captopril antibody titration, serial dilutions of the antisera in assay buffer (wash buffer containing 0·1% casein) were added to the wells in triplicate (200 μL/well) and the strips incubated at room temperature for 2 h on an orbital shaker. The wells were washed as before and alkaline phosphatase-labelled goat anti-rabbit IgG diluted in assay buffer was added (1 : 1000 dilution, 100 μL/well). The strips were incubated for 1 h at room temperature on the shaker. After 3 washes as before, bound antibody was revealed by the addition of 100 μL/well of 1 mg mL^{-1} *p*-nitrophenyl phosphate in substrate buffer (10% diethanolamine/HCl buffer, pH 9·8 containing 1 mM $MgCl_2$ and 20 mM NaN_3). Colour development was terminated after 30 min by the addition of 3 M NaOH (50 μL/well) and absorbance read at 405 nm using a Denly plate reader.

Construction of standard captopril solutions. A 1-mg mL^{-1} stock solution of captopril was prepared using 0·1 M sodium phosphate buffer, pH 7·0 containing 25 mM *N*-ethylmaleimide. The solution was divided into portions and stored at − 20°C. Serial dilutions of the standard captopril were made in assay buffer containing 25 mM *N*-ethylmaleimide, or in human plasma pooled from 10 normal subjects not taking any medication, and treated with 0·125 M *N*-ethylmaleimide (final concentration 25 mM). A standard curve was constructed every time an assay was performed using standards that were frozen and thawed only once.

Determination of intra- and inter- assay coefficients of variation (CV). Intra-assay CVs were determined by assaying 8-replicates of human plasma samples containing various concentrations of *N*-ethylmaleimide-treated captopril. Inter-assay CVs were determined by measuring captopril concentration in the same plasma samples on 6 consecutive weeks.

Competitive ELISA. In inhibition experiments, microtitre wells were coated with RSA-captopril as described above.

Serially diluted (in assay buffer or plasma) standard captopril, known amounts of inhibitors to assess antibody specificity, or test samples were added to the wells followed by 100 μL antiserum appropriately diluted in assay buffer. The rest of the assay was carried out as described above. Maximum absorbance values (100% binding) were determined using assay buffer or captopril-free pooled plasma in place of inhibitor and control wells included buffer in place of anti-captopril antiserum. Concentration of captopril in test samples was determined from a plot of absorbance versus log concentration of standard solutions.

Captopril stability in plasma. Captopril was added to pooled normal human plasma at 2000 ng mL^{-1} and then stored for up to 24 h at room temperature, 4°C or −20°C in the presence or absence of *N*-ethylmaleimide (25 mM final concentration). The amount of captopril remaining in the plasma was determined by ELISA.

Sample preparation and handling. Freshly drawn blood samples (1 mL) were mixed with 0·2 mL freshly prepared *N*-ethylmaleimide solution (0·125 M in 0·1 M sodium phosphate buffer, pH 7·0) to give a final concentration of 25 mM. The blood was centrifuged at 10 000 *g* for 10 min and the plasma aspirated and stored at −20°C. At the time of assay, the plasma was thawed in a water bath at 37°C, centrifuged as before, diluted with one volume of assay buffer and added to the microtitre wells (100 μL/well).

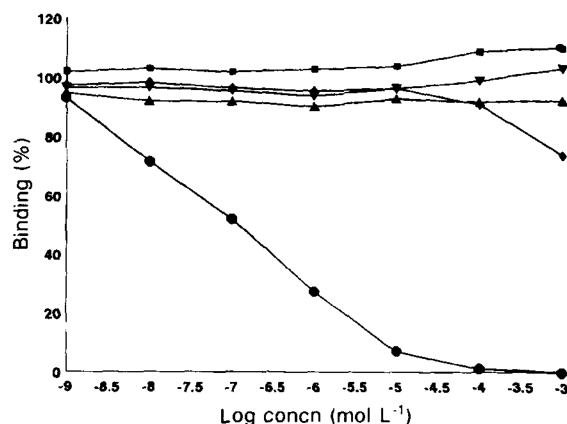

FIG. 2. Inhibition of anti-captopril antibodies (TA20) binding to RSA-captopril coated plate wells with captopril (●), proline (■), proline-glycine (▲), alanine-proline (◆) and phenylalanine-proline (▼).

Results

Production and specificity of anti-captopril antibodies

Both conjugates, Tg- and BSA-captopril proved to be highly immunogenic, with antibodies to captopril detectable after the first boost. All four antisera were similar in terms of affinity (data not shown) and therefore, only the results obtained with one, TA20, will be described here. In all competitive assays, antiserum TA20 was used at a final dilution of 1 : 20 000.

The specificity of the antibodies was tested using a number of structurally related ligands in competitive ELISA as shown in Fig. 2. The antibodies were highly specific to captopril. The closely related dipeptide alanine-proline showed a very small crossreactivity (IC50 > 10^{-3} M).

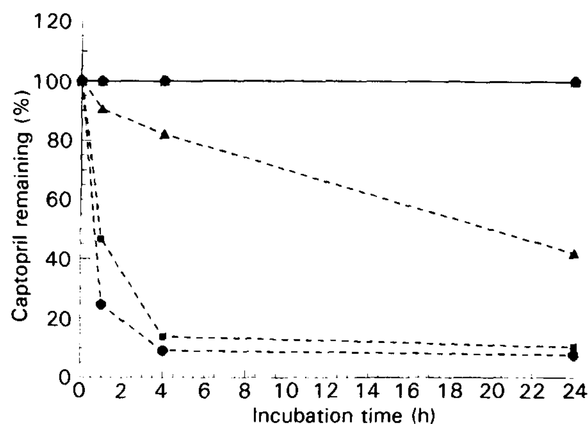

FIG. 3. Stability of captopril in human plasma. The percentage of captopril remaining in human plasma was determined by competitive ELISA in samples incubated at room temperature 21°C (●), 4°C (■) and −20°C (▲) in the presence (——) and absence (----) of 25 nM *N*-ethylmaleimide.

Stability of captopril in human plasma

In plasma, captopril is oxidized rapidly to the dimeric form and also binds to proteins via a disulphide bond formation (Kubo & Cody 1985). We therefore examined the stability of the drug in plasma in the absence and presence of *N*-ethylmaleimide. This compound inhibits captopril oxidation and protein binding by attaching a maleimido-group to the thiol group (Funke et al 1980). Fig. 3 shows that in the absence of *N*-ethylmaleimide, captopril immunoreactive signal disappeared very rapidly in human plasma stored at room temperature and 4°C (half-life < 2 h) and at a slower rate in plasma stored at −20°C (half-life approximately 20 h). The addition of *N*-ethylmaleimide protected captopril and the immunoreactivity was unchanged for at least 24 h, even in samples stored at room temperature. Hence, for assaying biological samples, captopril standards were constructed in *N*-ethylmaleimide-treated pooled plasma. This gave a detection limit of 0·3 ng mL^{-1}, corresponding to 90% binding (Fig. 2).

Recovery of captopril in plasma and intra- and inter-assay coefficients of variation

Human blood was obtained from 18 healthy laboratory workers, treated with *N*-ethylmaleimide as described in the methods section and the plasma assayed by the competitive ELISA against a captopril standard curve constructed in assay buffer.

The mean concentration ± standard deviation was 0·2 ± 0·2 ng mL^{-1} (range 0–0·9 ng mL^{-1}). This result indicated that there was no interfering material in human plasma.

Various concentrations of captopril in pooled human plasma were assayed against a standard curve constructed in buffer. The same samples were also assayed for intra- and inter-assay CV as described in the methods section. Table 1 shows that > 86% of the theoretically added captopril was detectable. The intra-assay CV was < 7% across the whole range of the calibration curve. The inter-assay CV was approximately 12% for concentrations below 500 ng mL^{-1} and between 15 and 20% for captopril concentrations higher than 500 ng mL^{-1}.

Table 1. Intra- and inter-assay coefficients of variation (CV) of captopril ELISA.

Theoretical concn	Captopril concn (ng mL^{-1})			
	Intra-assay CV (n = 8)		Inter-assay CV (n = 6)	
	ELISA concn ± s.d.	CV	ELISA concn ± s.d.	CV
0·5	0·59 ± 0·018	3·0	0·43 ± 0·05	11·6
2	1·97 ± 0·124	6·3	2·12 ± 0·25	11·8
8	8·2 ± 0·508	6·2	8·1 ± 1·02	12·6
32	31·6 ± 1·327	4·2	31·9 ± 3·5	11·0
128	131·0 ± 4·847	3·7	151·1 ± 16·23	10·7
512	510 ± 22·44	4·4	522·4 ± 81·06	15·5
2048	1851 ± 105·507	5·7	1806·2 ± 369·1	20·4

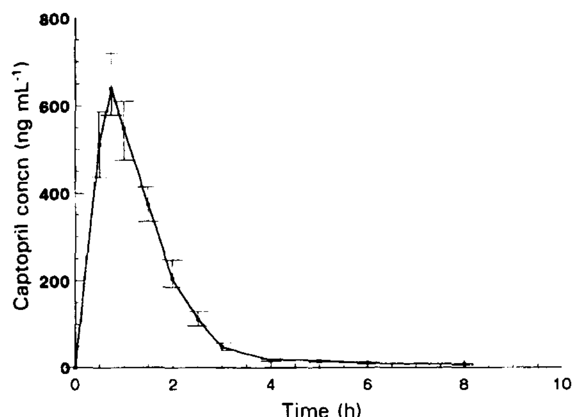

FIG. 4. Average ± s.e.m. concentration of plasma captopril in 18 volunteers receiving an oral dose of 50 mg captopril.

Assay of captopril levels in human plasma

Eighteen healthy fasting male volunteers received a therapeutic dose of captopril (Capoten, Squibb Co., 50 mg tablets). Blood samples were collected in heparinized glass tubes at 0, 0·5, 0·75, 1, 1·5, 2, 2·5, 3, 4, 5, 6, and 8 h and assayed (Fig. 4). The mean ± standard deviation and range of maximum plasma concentration (C_{max}) and time required to attain maximum plasma concentration (T_{max}) following the administration of captopril were 691 ± 311 ng mL^{-1}, 264–1540 ng mL^{-1} and 0·84 ± 0·28 h, respectively. These results are in accord with those obtained by HPLC and radiochemical methods (Shimada et al 1982; Kubo & Cody 1985; Duchin et al 1988; Shen et al 1992).

Discussion

In recent years, enzyme immunoassays have provided a convenient and alternative way of quantifying drugs and other small molecular weight substances and we have applied this technique to the assay of captopril. In our hands, captopril proved to be highly immunogenic once it was coupled to a large carrier protein BSA. The antibodies were specific to captopril and unlikely to react with other analytes in the plasma which are structurally related to captopril. The presence of a thiol group in captopril makes it unstable in biological fluids as it undergoes dimerization via S-S bridge formation and binding to plasma proteins that have free thiol groups. The stability study we performed showed that the addition of *N*-ethylmaleimide to plasma inhibited both reactions from happening and anti-captopril antibodies equally recognized the *N*-ethylmaleimide-captopril complex. The antibody also recognizes the dimeric captopril (data not shown) but not captopril bound to plasma proteins. In the captopril-protein conjugates used for immunization, the drug is linked via its sulphydyl group to the epsilon amine groups of lysine with a spacer (Fig. 1) while in plasma, the drug is directly coupled to protein thiol groups. In the former case, there is a considerable distance between the drug moiety recognized by the antibody and the carrier protein to allow the antibody-antigen binding to take place, while in the latter case, the drug molecule is too close to the bulk of the protein to allow antibody-antigen binding. This is confirmed by the gradual disappearance with incubation of the immunoreactive signal in plasma containing added captopril not treated with *N*-ethylmaleimide. Hence, the assay described here measures free captopril in plasma. It should be possible to use this assay to measure total captopril in plasma by first treating the sample with tri-*n*-butylphosphine to reduce all the di-sulphide bonds followed by treatment with *N*-ethyl maleimide (Tu et al 1984).

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