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Application of 2-halopyridinium salts as ultraviolet derivatization reagents and solid-phase extraction for determination of captopril in human plasma by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method was developed for the determination of captopril in human plasma. 1-Benzyl-2-chloropyridinium bromide (BCPB) was used as a precolumn derivatizing reagent. The mercapto group of captopril was arylated by the reagent to generate a stable UV-sensitive product. The derivative was solid-phase extracted (SPE), separated on a C_{18} column using reversed-phase ion-paring chromatography and monitored by a spectrophotometric detector at 314 nm. The method enabled sensitive determination of captopril and its disulphides in human plasma in patients after oral administration. Disulphides of captopril with captopril itself and with endogenous thiol compounds are reduced with triphenylphosphine to form captopril, followed by derivatization with the same reagent. The quantification limit was 10 ng/ml. Calibration curves were prepared for human plasma samples spiked with captopril and captopril disulphide. The calibration curves were linear in the range of 10 to 500 ng/ml for captopril and 10 to 1000 ng/ml for captopril disulphide.

Keywords: Derivatization, LC; solid-phase extraction; Captopril; 1-Benzyl-2-chloropyridinium bromide

1. Introduction

Captopril, an orally active antihypertensive drug, has been widely used for the treatment of hypertensive disease and congestive heart failure since 1980 [1]. As an angiotensin converting enzyme inhibitor, captopril was designed to mimic a natural peptide and as such it is effective in relatively small doses. Analytical methods employed to determine this unstable and hardly detectable aliphatic mercapto compound are various and complex. Methods so far available for the quantification of captopril in plasma include radioimmunoassay [2], enzyme immunoassay [3], thin-layer chromatography [4], gas chromatography [5,6], gas chromatography–mass spectrometry [7,8], and, above all, column liquid chromatography with ultraviolet [9,10], fluorescence [11,12], and electrochemical detection [13].

In spite of existing methods, clinical studies are mostly not supported by captopril plasma measurements reflecting the problems of assay development. Generally, these methods are time consuming procedures involving several consecutive steps; double derivatization, double liquid–liquid extraction, and/or the use of sophisticated equipment which is not widely available in clinical or research laboratories.

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The objective of our study was the development of a procedure which allows the determination of captopril in human plasma using conventional HPLC-equipment and solid-phase extraction. Therefore, we elaborated a procedure with the use of one-step extraction on a disposable octadecyl column, prior to separation in the isocratic mode on a reversed-phase column with detection by ultraviolet absorption. In order to minimise the contributions of sample preparation and injection variations to the final results, the internal standard approach was used. The method described here is based on our previous experiences in determination of biologically important aminothiols [14] and preliminary application of 2-halopyridinium salts for determination of captopril [15].

2. Experimental

2.1. Reagents

Derivatization reagent (BCPB, I, Fig. 1), internal standard (BCMPB, c, Fig. 1) and captopril disulphide were synthesized in our laboratory. Captopril substance and captopril drug were supplied by Jelfa, (Jelenia Góra, Poland). Acetonitrile, acetone and methanol were of HPLC grade (J.T. Baker, Deventer, Netherlands). All other chemicals used were of analytical-reagent grade. Triple distilled water was used throughout the experiments. All liquids used for HPLC system were filtered through 0.2 μm membranes.

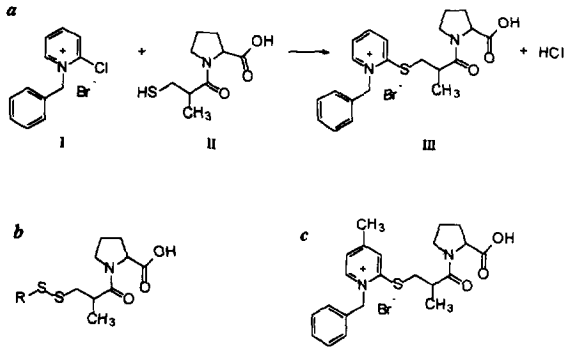

Fig. 1. Chemical derivatization reaction of captopril (a); general structure of captopril disulphides (b); RS=captopril, cysteine, glutathione, proteins and other endogenous thiols; chemical structure of the internal standard (c).

2.2. Preparation of derivatization reagent and internal standard

2.2.1. Derivatization reagent, BCPB

Benzyl bromide (8.5 g, 0.05 mol) was added to stirred 2-chloropyridine (4.5 g, 0.04 mol) and the mixture was heated on an oil bath (60°C) overnight. After cooling, the resulting white crystals were filtered off and washed with acetone followed by drying in vacuo to yield 5.3 g [47%, m.p. 187–191°C; ^{1}H NMR, 60 MHz, D_2O, δ ppm: 6.08 (s, 2H, CH_2), 7.56 (s, 5H, ϕ), 7.93–9.22 (m, 4H, pyr. ring)]. For captopril derivatization prior to HPLC a 20 mg/ml aqueous solution of BCPB was used.

2.2.2. Internal standard, BCMPB–captopril derivative (c, Fig. 1)

A 30 mg (0.14 mmol) of captopril and 80 mg (0.27 mmol) of 1-benzyl-2-chloro-4-methylpyridinium bromide (BCMPB), synthesized in the same manner as BCPB and characterized by ^{1}H NMR, 60 MHz, D_2O, δ ppm: 2.74 (s, 3H, CH_3), 5.97 (s, 2H, CH_2), 7.52 (s, 5H, ϕ), 8.00 (t, 2H, pyr. ring), 8.90 (d, 1H, pyr. ring), in 5 ml of 0,5 *M* pH 8.2 Tris buffer were stirred for 1 h. After the addition of 1.6 ml 0.1 *M* solution of sodium sulphide in order to bind the excess of BCMPB, the mixture was allowed to stand for 10 min followed by extraction according to the following procedure: Condition the C_{18} (1 g) column with two 1-ml portions of methanol and rinse with 1 ml of water. Immediately, pass the reaction mixture through the column and wash with two 1-ml portions of water. After drying by suction of the air through the column for 10 min, wash with three 1-ml portions of acetonitrile (greenish ring of 1-benzyl-2-tiopyridone elutes). Again suck the air for 5 min and elute BCMPB–captopril with 5 ml of 80% methanol directly into a 10-ml volumetric flask. Dilute to the volume with water and standardise by HPLC. Working internal standard solutions were prepared by appropriate dilutions with water.

2.3. Apparatus

The isocratic HPLC system consisted of a Hewlett-Packard Series 1050 pump, a Rheodyne Model 7125 injection valve with a 20-μl injection loop, a Hewlett-Packard Series 1050 variable-wavelength detector, and a Hewlett-Packard Type 3395 A integrator. UV spectra were recorded on a Carl Zeiss Jena UV-Vis spectrophotometer. The Bakerbond SPE disposable extraction columns each filled up with 100 mg of sorbent and vacuum manifold SPE-12 G were supplied by J.T. Baker.

2.4. Extraction of derivatization product from plasma matrix

Octadecyl Bakerbond disposable columns were used for solid-phase extraction of *S*-pyridinium derivative of captopril. The columns were fitted into a 12-place vacuum manifold. Syringe barrels were connected to the Bakerbond columns to act as reservoirs for large volumes of samples and extraction solvents. Extraction experiments were first performed using standard captopril solutions after derivatization with BCPB in 10-ml volumetric flasks, and the procedure was checked with human plasma spiked with captopril and internal standard. The final concentration of captopril in the form of its BCPB derivative (III, Fig. 1) in 10-ml volumetric flask was 2 μg/ml. A 20-μl aliquot was analysed by HPLC and the rest was passed through the SPE column; the extract was reconstituted with mobile-phase to the same volume and again chromatographed in order to find out extraction recovery. The recovery ranged from 95–100%. The following SPE procedure was adopted: Condition the C_{18} (100 mg) column with two 200-μl portions of methanol and wash twice with 200 μl of water. Immediately, pass the sample, acidified to pH 2.5–3.0 (indicator paper) with 4 *M* phosphoric acid through the column followed by rinsing with 1 ml of water and drying by means of suction of the air for 10 min. Wash with three 100-μl portions of acetonitrile and dry again for 5 min. Elute the analyte with one 200-μl portion of a methanol–acetic acid mixture and two 200-μl portions of a methanol–water mixture, both 80:20 (v/v).

2.5. Chromatographic conditions

Chromatographic separations were carried out under isocratic mode on a microbore ODS Hypersil (5 μm, 100×2.1 mm I.D.) reversed-phase column fitted with a Hypersil (5 μm, 20×2.1 mm I.D.) precolumn at 50°C and mobile-phase flow-rate of 0.2 ml/min. For routine determination of captopril in human blood plasma, a mobile-phase consisting of 25% of acetone and 75% (v/v) of 0.2 *M* pH 2.5 citrate buffer containing 10 mmol/l sodium octanesulphonate and 15 mmol/l sodium chloride was used. The detector wavelength was 314 nm.

2.6. Standard solutions of the analyte

Stock solution (1 mg/ml) was prepared by dissolving 50 mg of captopril and captopril disulphide in a 50-ml volumetric flask in water containing 5 ml of 0.1 *M* hydrochloric acid. Working standard solutions were obtained by appropriate dilutions of the stock solution with water.

2.7. Assay procedure

Freshly drawn blood was centrifuged without delay in order to obtain plasma, followed by deep freezing if analysis was not done immediately. A schematic diagram of the assay procedure for captopril, protein-conjugated captopril and total captopril in human plasma is shown in Fig. 2.

2.7.1. Captopril

To 1 ml of plasma add 100 μl of 0.2 *M* disodium EDTA solution, 100 μl of 0.2 *M* ascorbic acid solution and 200 μl of internal standard solution (1 μg/ml). Deproteinize the sample with 400 μl of 3 *M* perchloric acid solution and centrifuge for 15 min (4000 *g*) followed by rinsing the precipitated proteins with three 500-μl portions of water. Neutralize the combined supernatant and washings with 1 *M* solution of sodium hydroxide (indicator paper), add 3 ml of pH 8.2 1 *M* Tris buffer, and 100 μl of 20 mg/ml BCPB solution. After 15 min render the mixture acidic by adding 4 *M* phosphoric acid solution (pH 2.5–3.0, indicator paper) and centrifuge again. Extract the supernatant and washing according

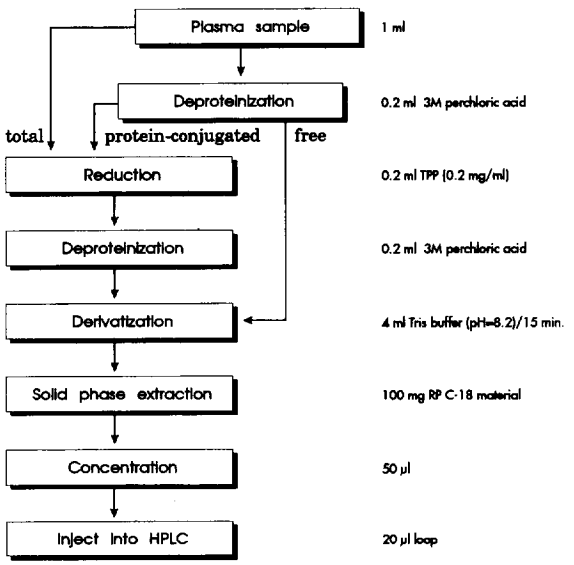

Fig. 2. Schematic diagram of the assay procedure for captopril in human blood plasma.

to the procedure described in Section 2.4. Evaporate the extract to dryness at 60°C, reconstitute with 50 μl of the mobile-phase and inject 20 μl onto the liquid chromatograph.

2.7.2. Protein-conjugated captopril

Resuspend the precipitated protein pellet (from 1 ml of plasma, as described in Section 2.7.1) with 2 ml of 0.1 *M* perchloric acid solution, add 200 μl of internal standard solution, 100 μl of triphenylphosphine (TPP) solution in acetonitrile (40 mg/ml) and heat on a water bath at 50°C for 40 min with occasional shaking. After cooling, deproteinize the sample and proceed further as described in Section 2.7.1.

2.7.3. Total captopril

To 1 ml of plasma sample add 100 μl of 0.2 *M* disodium EDTA solution, 100 μl of 0.2 *M* ascorbic acid solution, 200 μl of internal standard solution, 2 ml of 0.1 *M* perchloric acid solution and 100 μl of acetonitrile TPP solution followed by incubating at 50°C for 40 min. Deproteinize the sample after cooling, and process it further as described in Section 2.7.1.

2.8. Standard curves

Standard curves were prepared by spiking of drug-free human plasma (1 ml) with 200 μl of a 1 μg/ml internal standard solution and various amounts of captopril or captopril disulphide ranging from 10 to 500 ng and 10 to 1000 ng, respectively, and assaying in the manner described in Section 2.7. The blank specimens were prepared by spiking control plasma with only the internal standard solution. The peak-area ratio of captopril derivative to that of internal standard was plotted against the plasma concentration of captopril.

2.9. Patient study

Captopril was administered as an oral dose of 12.5, 25.0 or 50.0 mg, three times a day, to 20 patients ranging in age from 45 to 76 years, as part of treatment for hypertensive disease and/or congestive heart failure. The treatment was continued for at least seven days. Blood samples were drawn at 1.5 h after the final dose. Free, protein-conjugated and total captopril levels were measured.

2.10. Effect of time on the derivatization reaction

To determine the adequate reaction time for the captopril–BCPB reaction, 200 μl of a 1 mg/ml standard captopril solution (0.92 μmol) was placed in a 10-ml volumetric flask. A 70-μl volume of a 20 mg/ml solution of BCPB (4.96 μmol) and 2 ml of 0.1 *M* pH 8.2 phosphoric buffer were added. The mixture was vortex-mixed, diluted to the volume with water and mixed again. A 1-cm cell was filled with the reaction mixture, placed in a spectrophotometer and the absorbance was measured for 30 min at 314 nm.

2.11. Stability of the derivative

In order to test the stability of captopril–BCPB adduct, four 1-ml plasma samples were spiked with 200 ng of captopril and 200 ng of internal standard. All the four samples were processed according to the assay procedure (Section 2.7) until derivatization with BCPB. One sample was then solid-phase extracted and chromatographed without delay. The

other three samples were kept refrigerated at 5°C and processed further in the same manner after 1, 4 or 7 days, respectively.

3. Results and discussion

3.1. Derivatization

Because of the hydrophilic nature of captopril, the compound can not be separated on hydrophobic reversed-phase columns. More importantly, captopril does not have suitable UV absorption properties for its measurement in biological samples using UV detection. Both of these problems can be solved by derivatization.

2-Halo-1-methylpyridinium salts have been used as derivatization reagents for the analysis of thiols by electrophoresis [16], spectrophotometry [17] and HPLC [14]. Accordingly, we have synthetised several 2-halopyridinium salts, thiol reacting compounds, with various substituents in the pyridine ring in order to choose the most suitable one for the derivatization of captopril. After several trials 1-benzyl-2-chloropyridinium bromide (BCPB) was chosen and has been used in routine assay to react with captopril (I, Fig. 1) prior to extraction and introduction of the sample into the HPLC system. Captopril reacts with BCPB under slightly alkaline conditions, in a manner which appears identical to that of cysteine and metabolically related compounds [14], to generate a UV-sensitive derivative. The chemical reaction is illustrated in Fig. 1a. Due to the specificity of the reaction, no interference from other non-thiol plasma matrix compounds is expected. The derivatization results in a largely enhanced absorption in the relatively clean ultraviolet region enabling sensitive detection. The advantage of BCPB is that it gives a captopril derivative with sufficient lipophilicity to be separated under ion-pair reversed-phase HPLC conditions.

Experiments were carried out to determine the reaction time necessary for completion of the derivatization. It was established that reaction occurs immediately and proceeds to a maximum in ca. 10 min. Based on this, for routine assay of plasma captopril a derivatization reaction time of 15 min is recommended before the reaction mixture can be solid-phase extracted and injected onto the HPLC system.

3.2. Reduction of captopril disulphides

Disulphides represent a fraction of captopril (Fig. 1b), covalently bound to thiol compounds such as captopril itself (captopril dimer), endogenous low-molecular-mass compounds such as cysteine, glutathione, and plasma proteins. Free captopril was generated from these disulphides by reductive cleavage with triphenylphosphine (TPP). Tributylphosphine (TBP) [9] gave the same results, but TPP was chosen for ease of handling. We have studied the effect of concentration of TPP and incubation time on the reduction of captopril disulphide in plasma at 50°C in the manner similar to that of Hayashi et al. [9] (kinetic data not shown). The yield of reduction was 95%. An attempt to use sodium borohydride for the reduction of the plasma captopril disulphides was not successful due to partial degradation of captopril to 3-mercapto-2-methylpropionic acid; after derivatization, a peak of its S-pyridinium derivative appeared on the HPLC chromatogram (data not shown).

Protein precipitated from plasma received from patients given captopril orally, treated with TPP followed by derivatization, extraction and HPLC separation showed peaks with a retention time corresponding to captopril derivative. Quantification was performed using an internal standard approach. We did not synthesize the human serum albumin-conjugated captopril, but based on our experiences with reduction of captopril disulphide we assumed (S–S linkage is weaker in the case of protein) that all captopril was released.

3.3. Chromatography

Under the experimental conditions used in this study, the captopril–BCPB was eluted after ca. 10 min. Typical chromatograms for human plasma can be seen in Fig. 3. The peak of captopril derivative was well separated from other peaks, including that of the internal standard and of plasma thiols, such as cysteine, which are known to react with 2-halopyridinium salts [14]. A relatively clean blank at the captopril derivative retention time can also be

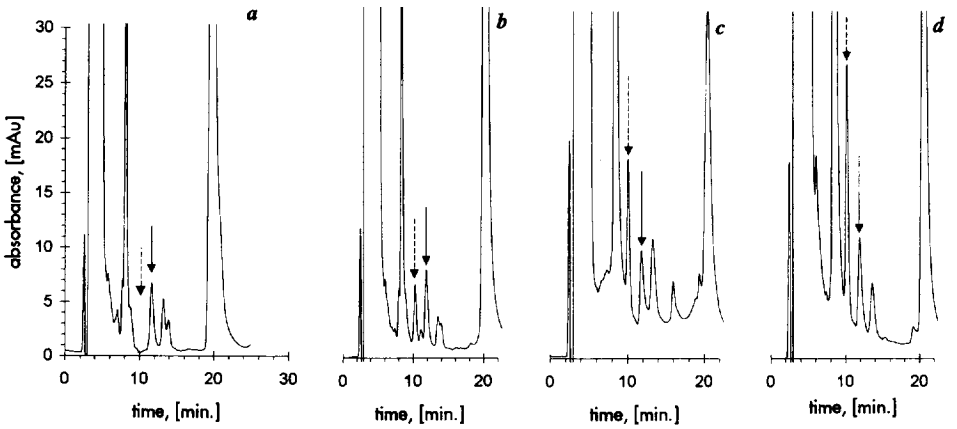

Fig. 3. Typical chromatograms of captopril (50 mg oral dose) in human plasma as BCPB derivative: a, biank; b, captopril (196 ng/ml); c, protein-conjugated captopril (427 ng/ml); d, total captopril (752 ng/ml). Peaks: broken arrow represents captopril and solid arrow represents the internal standard. Chromatographical conditions are described in Section 2.

seen (Fig. 3a). The use of a microbore column after derivatization and solid-phase extraction provided conditions for a real trace analysis measurement. The quantification limit was 10 ng/ml.

3.4. Internal standard approach

In order to minimise the contributions of sample preparation and injection variations to the final results, the internal standard was used. The BCMPB–captopril derivative used here as an internal standard, possessed very similar chemical structure and chromatographic properties to that of BCPB–captopril derivative (Fig. 1) and eluted 2 min later then analyte. Although this approach did not include derivatization, it gave us satisfactory precision.

3.5. Calibration curves

The calibration graphs (peak-area ratio, y, vs. concentration, x) for captopril and captopril disulphide spiked human plasma are: $y=0.0029x - 0.0091$ ($r=0.999965$) and $y=0.0019x$ ($r=0.999976$), respectively. In both cases a good linearity over the working concentration intervals was observed. The calibration data are reported in Table 1.

3.6. Stability of the derivative in biological fluid

The captopril–BCPB derivative was found to be stable at room temperature for a reasonable time which allows for long unattended runs. The derivative was stable for at least 1 week when kept at 5°C

Table 1
Calibration data for captopril and captopril disulphide in human plasma

Dose ng/ml	Captopril ($n=4$)			Captopril disulphide ($n=4$)		
	Found	S.D.	R.S.D.	Found	S.D.	R.S.D.
10	12.9	0.9	9.3	7.3	0.9	8.7
50	51.3	1.0	2.0	52.4	3.1	6.2
100	101.5	3.2	3.2	98.7	3.1	3.1
200	197.9	5.3	2.7	200.9	3.2	1.6
500	500.5	16.4	3.3	495.3	17.0	3.4
1000				1000.8	35.5	3.6

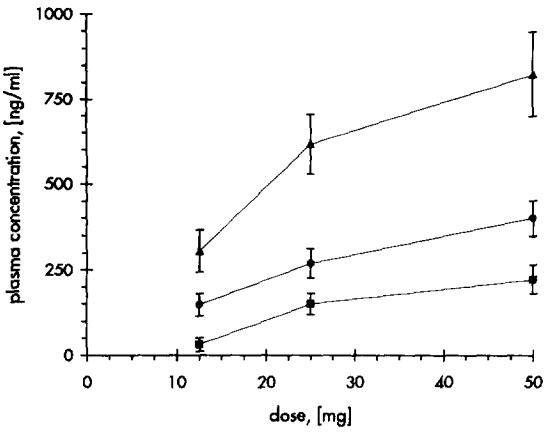

Fig. 4. Plasma captopril levels as a function of the oral dose administrated: 12.5 mg (n=6 patients); 25.0 mg (n=7 patients); 50.0 mg (n=6 patients); (■) captopril; (●) protein-conjugated captopril and (▲) total captopril. Vertical bars indicate standard deviation.

3.7. Patient study

The described analytical procedure has been applied to the measurement of blood plasma captopril levels in patients treated for hypertensive disease and/or congestive heart failure. Fig. 4 shows determined levels of captopril as a function of an oral dose. After oral dosing with 12.5, 25.0 and 50.0 mg captopril, twenty subjects attained a mean maximal concentration of captopril, protein-conjugated captopril and total captopril in blood plasma of 33, 150 and 250; 140, 280 and 625; 225, 400 and 825 ng/ml, respectively.

Acknowledgments

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