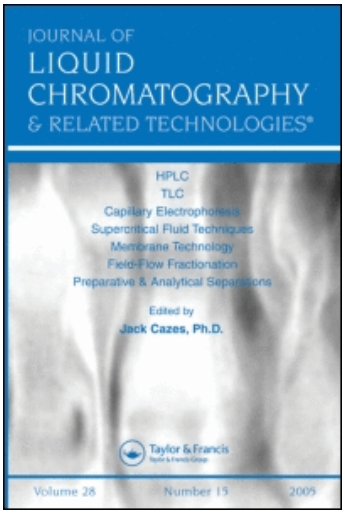

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SIMPLE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF CAPTOPRIL IN HUMAN PLASMA AND CEREBROSPINAL FLUID

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ABSTRACT

A simple HPLC assay has been developed for free captopril in plasma and CSF, and for its mixed disulphides in CSF. Captopril is derivatized with p-bromophenacylbromide (p-BPB), and the adduct formed is extracted and then assayed using a reversed-phase column and UV detection. Captopril mixed disulphides are reduced with tributylphosphine to the free form which in turn is trapped with p-BPB. No clean-up procedures are necessary for this method which shows satisfactory accuracy and precision. A sensitivity level of 10ng/mL has been reached for free and total captopril in CSF, and for free captopril in plasma.

INTRODUCTION

Captopril {[1-(D-3 mercapto-2-methyl-1-oxopropyl)-L -proline} (SQ 14,225) is the first orally active inhibitor of angiotensin-converting enzyme and is used for the treatment of hypertension [1] and congestive heart failure [2]. In plasma, this drug is readily converted into its disulphide dimer and forms disulphide conjugates with endogenous thiol compounds [3]. There is also good evidence from animal research that free captopril crosses the human blood-brain barrier [4].

Several reports indicate that brain angiotensin II participates in the regulation of blood pressure [5,6]. It has been proposed, from animal data, that part of the hypotensive effect of captopril may be due to ACE inhibition within CNS structure [4,7,8]. Furthermore, captopril represents a potential therapeutic advance for CNS disabling conditions, such as melancholia [9,10].

However, there is only one study involving a small number of patients which suggests that one acute oral dose of captopril is able to inhibit the CSF angiotensin-converting enzyme [11]. On the other hand, no direct measurement of the drug was done. To monitor captopril blood and urine levels, radiochemical [12], GC-MS [13-15] and HPLC [16-20] methods were reported. The lack of a sensitive chromatographic assay to determine free and total (free and bound) captopril in human CSF prompted us to develop a simple, fast and sensitive HPLC technique using p-bromophenacyl bromide (p-BPB) as a coupling reagent for the unstable mercapto group of free captopril. Quantitation of the latter in plasma is also described in this paper.

EXPERIMENTAL SECTION

Instruments and chromatographic conditions

A Hewlett-Packard HP 1090 L liquid chromatograph, equiped with a PV5 ternary solvent delivery system, was used. A HP 1040A photodiode-array UV detector was utilized to monitor the column effluent at 260nm. A confirmation wavelenght of 270nm was added in order to determine the purity index of the compounds analyzed. A reversed- phase C-18 column was used (lichrosorb RP-18 30 X 0.4cm) with a mean particle diameter of 5 μM. The peak areas were measured by a HP 3392 A integrator, in an attenuation range of -1 to +1. The mobile phase consisted of a water-acetonitrile - acetic acid mixture (180: 220: 2,5) at a flow rate of 1mL/min., at ambiant temperature.

Materials

Captopril powder in its free form was donated by Squibb Pharmaceuticals (Montreal, Quebec). As a thiol protectant agent, p-bromophenacyl bromide (p-BPB) was used and it was purchased from Aldrich Chemicals (Milwaukee, WIS). Tributylphospine (TBP) was obtained from the same supplier. Phenylacetic acid, used as an internal standard, was purchased from Sigma Chemicals (St-Louis, MO). Sodium chloride was bought from Fisher Scientific (Toronto, Canada). All reagents were reagent grade. HPLC grade acetonitrile was purchased from Caledon (Georgetown, Canada). Ethyl acetate and benzene were supplied by the same manufacturer. They were both of HPLC purity. Human plasma was given by the Canadian Red Cross and fresh CSF (120mL) was obtained by direct puncture of the brain ventricles of one patient, immediately after his death.

For the synthesis of the captopril p-BPB adduct, melting points were recorded on a a Gallenkamp apparatus, and NMR and mass spectra were taken on a Varian VXR-300 and a MS-50 TATC Kratos instruments, respectively.

Preparation of the standard captopril - pBPB adduct for the assay

The captopril-pBPB adduct was prepared in the Medicinal Chemistry Laboratory of the Faculty of Pharmacy, University of Montreal, Montreal, Quebec, Canada, according to a modification of the method described by Y. Kawahara et Al [16].

Adduct synthesis

Captopril (0.43g) and p-BPB (0.66 g) were dissolved together in 40mL of methanol. Triethylamine (0.40g) was then added and the mixture was refluxed for 1 hour. Vaccuum-evaporation of the solvent gave an oil which was dissolved in 100mL of water. The solution thus obtained was alcalinized to pH 10 by a dropwise addition of 2M NaOH. Then it was washed four times with 25mL ethyl acetate. The organic layer was discarded whereas the aqueous one was brouhgt back to pH 1 by addition of 1M HCl. This was finally extracted twice by ETOAc (20mL), dried over $MgSo_4$ and evaporated in vacuo. 500 mg (60%) of captopril-p-BPB adduct was obtained as a clear oil.

Thin layer chromatography performed on a silica gel $6F_{254}$ gave a R_f of 0.45, using a benzene-acetic acid 3:1 mixture as a solvent. Mass spectroscopy and nuclear magnetic resonnance analysis were used to characterize the product obtained.

Assay procedure

Free captopril in plasma and CSF

Freshly drawn blood (1mL) was mixed with 50 μL of a solution of EDTA (0.1M) and ascorbic acid (0.1M). The mixture was immediately centrifuged at 13,000 g for 2 minutes.

An aliquot of 0.5mL of the plasma obtained or CSF was added to a 16 X 125 mm screw-cap glass tube containing 50 μL of a 1mg/mL p-BPB solution in acetonitrile. The tube was vortexed for 15 sec and then left at room temperature for 30 minutes. Following this, 100 μL of 1N HCl and 50 μL of a 0.02% (w/v) methanolic solution of phenylacetic acid (internal standard) were added. Then, 5mL of a 1:1 mixture of ethyl acetate-benzene were poured in the tube as an extracting solvent. After a 3 minutes vortex-mixing the tube was shaken gently for 15 minutes. The aqueous phase was then saturated with sodium chloride and the tube was centrifugated in order to separate the phases. The organic layer was removed and evaporated to dryness under a stream of nitrogen, in a heating bath at 50° C. The dry residue was reconstituted in 250 μL of acetonitrile and a 20 μL-volume of this solution was injected into the HPLC. Typical chromatograms for plasma and CSF samples are shown in figure 1a. After elution of the captopril adduct the flow rate was increased to 3mL/min. for 3 min. to get rid of the excess p-BPB.

Total captopril in CSF

0.5mL of CSF and 1mL of a 0.05 M phosphate buffer, pH 7, were added to a 16 X 125mm screw-cap glass tube, which

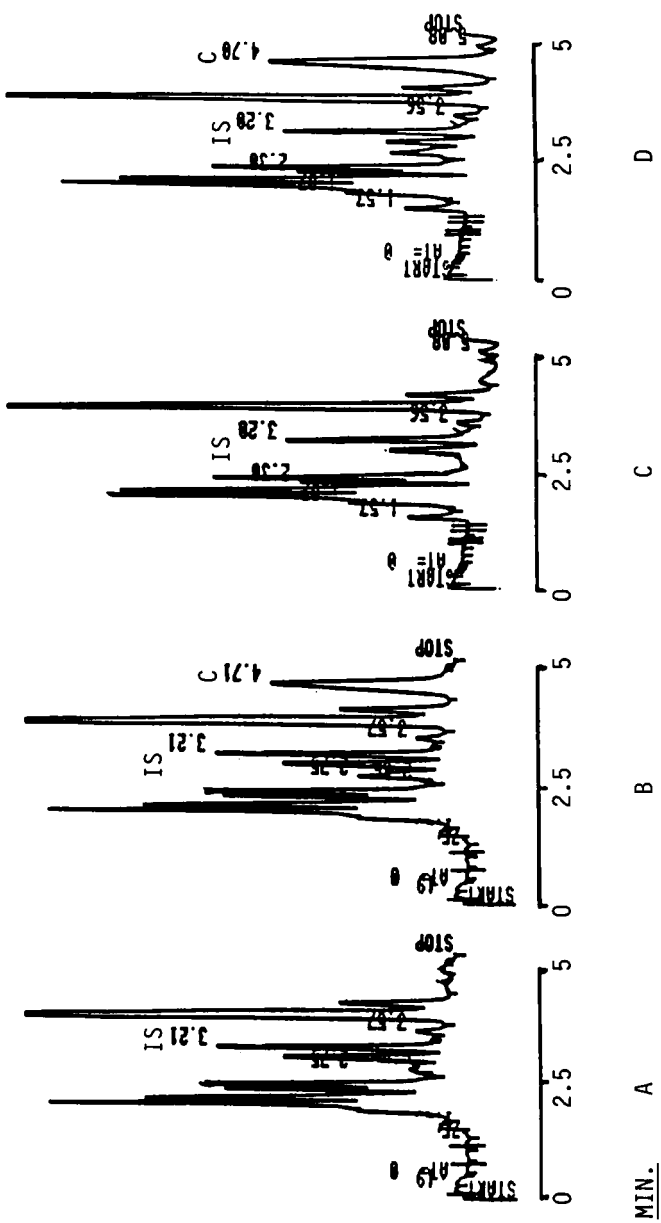

Figure 1a - Typical chromatograms for free captopril, obtained with blank (A) and spiked (B) plasma samples, and blank (C) and spiked (D) CSF samples. C = Captopril-pBPB adduct; IS = Internal standard.

contained 100 μL of a 2% (w/v) solution of tributylphosphine (TBP) in acetonitrile. The mixture was incubated at 50° C for 45 minutes. After incubation, the mixture was cooled to room temperature with cold water and then 100 μL of a 10mg/mL solution of p-BPB in acetonitrile were added. The sample was vortexed for 15 sec., left at room temperature for 30 minutes, extracted and assayed as for free captopril. Fig. 1b shows typical chromatograms obtained from captopril dimer. The flow rate was increased to 3mL/min. from 5 to 8 min. to get rid of the excess p-BPB and TBP.

RESULTS AND DISCUSSION

Derivatization of captopril

As a thiol protective agent, pBPB was used with success by Kawahara et al [16]. We found that no phosphate buffer was needed for free captopril in either plasma or CSF, because these biological fluids, even after the addition of the EDTA-ascorbic acid mixture, have a pH in the 6-8 range, which is optimal for the coupling reaction. A pH 7 buffer was used only with total captopril in CSF, in order to dilute the sample. Several maleimide derivatives were tried, but they did not work as well as pBPB, in terms of reaction time, recovery and elution of the adduct.

Because captopril oxidises very rapidly in plasma, a mixture of 0.1 M EDTA and ascorbic acid was added to freshly drawn blood. Even with this protection, the separation of the plasma fraction, available for coupling with p-BPB, should not last more than 20 minutes. Any additional delay will produce a loss of captopril greater than 5%, and rapidly increasing with

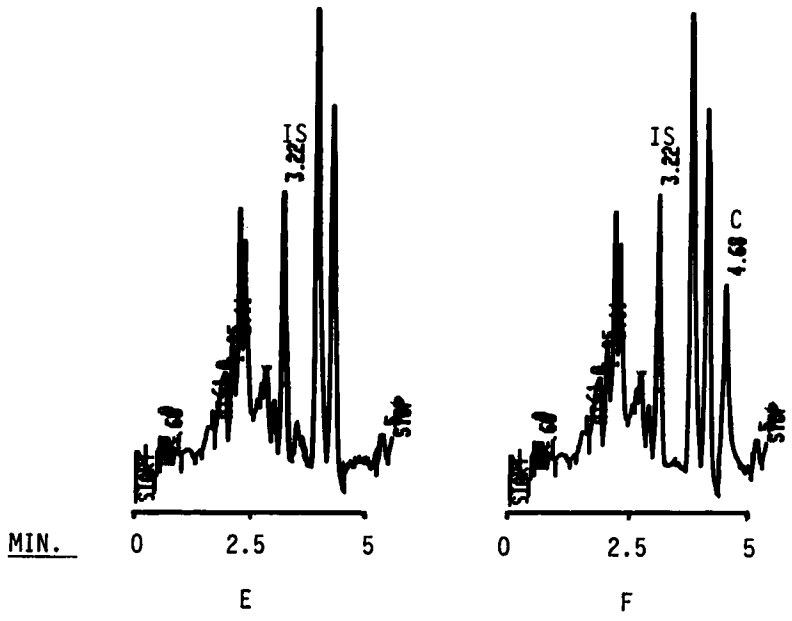

Fig. 1b: Typical chromatograms for total captopril, obtained with blank (E) and spiked (F) CSF samples. C and IS = as for fig. 1a.

time. The addition of the coupling agent directly into whole blood gave unsatisfactory results.

Reduction of captopril dimer

TBP has been successfully used by several investigators to reduce the disulphides bonds of captopril dimer and mixed disulphides [16,19,20]. Incubation times ranging from 30 to 60 minutes are described in the literature [16,19,20]. We found that 45 minutes was sufficient for the complete reduction of 10 to 10,000 ng/mL of captopril dimer in CSF.

However, a greater amount of p-BPB was necessary to achieve a complete coupling reaction with the free captopril released, because many endogenous disulphides seemed to be also reduced and reacted competitively with p-BPB.

Extraction step

Both solvents, ethyl acetate and benzene, used separately proved to be poor extracting solvents for the captopril-pBPB adduct, but used together in 1:1 ratio were found to be very efficient. To evaluate the recovery of the captopril-pBPB adduct and phenylacetic acid, drug free plasma and CSF were spiked with 50 and 1000 ng/mL of the adduct, and 20 μg/ml of the internal standard. Extraction recovery was calculated by dividing the peak areas after solvent extraction by the peak areas obtained with an acetonitrile solution containing the nominal values of the two subtances. The mean recovery was 99.8 $\pm$ 6.2% for the captopril-pBPB adduct (N=10) and 104.1 $\pm$ 3.5% for phenylacetic acid (N=10).

Excess p-BPB and TBP were not removed from the samples during the assay procedure, in order to avoid time-consuming clean-up steps described in other methods [13-18]. The two reagents, and the adducts theoretically formed with endogenous plasma and CSF thiols, did not interfere with the extraction and the elution of the compounds of interest.

Detector Wavelength

A fixed wavelength of 260nm was chosen from the UV absorbance scans of captopril-p-BPB and phenylacetic acid. In order to confirm the purity of the peaks obtained on the

chromatogram, a confirmation wavelength of 270nm (ref= 550nm) was used. Both wavelengths were used with a bandwidth of 20nm, according to the UV absorbance scans obtained with the mobile phase used as a reference. The mean purity index of the adduct was 0.99 ± 0.01 (SD) (N=5) confirming the MS and NMR data obtained with the pure standard. Both the scans "on the fly" and the purity indexes were identical for the pure standard and the adduct formed in plasma and CSF.

The mean purity indexes for phenylacetic acid, both in an acetonitrile solution and extracted from the biological matrices, were also identical, i.e. 0.98 ± 0.01 (N=5).

Linearity of the response

Free captopril

Drug-free plasma and CSF samples were spiked with increasing amounts of captopril, to give a concentration range of 10 - 750 ng/mL. A fixed amount of the internal standard was also added (see assay procedure). A standard curve was constructed by performing a linear regression analysis of peak area ratios (captopril-pBPB/internal std) versus captopril concentrations. Excellent linearity was found, as shown in table 1.

Total captopril

Drug-free CSF samples were spiked with increasing amounts of captopril dimer, used as a standard for captopril mixed disulphides. A concentration range of 10 - 1000 ng/mL was used. Again, the peak areas ratio method was applied. An equation similar to the ones obtained with free captopril was found, as shown in Table 1.

TABLE 1

Equations of the Standard Curves constructed from the Peak Areas Ratios (capr.-pBPB/int.std) against the Concentration of Captopril in ng/mL.

	Slope (± SD)	Intercept (± SD)	N^1	N^2	R^2
Free Form in plasma	y = 307.82 (±15.39)X	- 75.45 (±15.09)	3	6	0.99
Free Form in CSF	y = 311.78 (±15.59)X	- 15.95 (±3.20)	3	6	0.99
Total Form in CSF	y = 340.21 (±17.01)X	- 55.27 (±11.10)	3	7	0.99

N^1 : Number of standard curves constructed.
N^2 : Number of concentrations used for each calibration curve.

Precision and accuracy

Three different concentrations of free captopril namely 50, 300 and 500 ng/mL of plasma of CSF, were used to determine within and between - day precision, and accuracy. The same concentrations were used for captopril dimer in CSF. Results are shown in Table 2.

Sensitivity

Sensitivity, defined by a signal-to-noise ratio greater than 5, was 10 ng/mL of plasma or CSF for free captopril, and 10ng/mL of CSF for total drug. The detection limit for both forms was 5 ng/mL, considering a signal-to-noise ratio of 2.

TABLE 2

Accuracy, Within day (WD) and Between day (BD) Precision of the Determination of Captopril added to Human Plasma and CSF.

Sample	Nominal Value (ng/mL)	Mean determined values(ng/mL) n=6	Accuracy: mean as % of nominal	WD precision: coefficient of variation n=6	BD precision: coefficient of variation n=3
Free Captopril in plasma	50	54.5	109.0	9.0	6.6
	300	278.46	92.8	7.2	8.1
	500	536.1	107.2	7.0	6.8
Free Captopril in CSf	50	53.7	107.4	7.2	10.0
	300	279.1	93.0	3.5	9.3
	500	533.6	106.7	5.3	8.6
Total Captopril in CSF	50	52.9	105.7	6.1	9.0
	300	279.1	93.0	7.6	7.1
	500	530.1	106.0	6.1	8.4

The sensitivity of other reported methods ranges from 5 to 33 ng/mL [13-20]. Only Kawahara et al [16] obtained a sensitivity similar to ours, using UV detection. On the other hand, no data on CSF were presented. We tried to develop a method which would be as simple as possible, avoiding lengthly sample clean-up and washing procedures.

Also, the use of small plasma and CSF volumes (0.5mL) allows the application of our method to pharmacokinetic studies in children and small animals.

The determination of total captopril in CSF showed that the linear equation obtained is very close to the one calculated from free captopril data. An identical sensitivity was also reached. These results confirm that captopril dimer is fully reducted in its free form and subsequently behaves as the latter. Some authors describe different sensitivity levels for free and total captopril, while for example, 100ng of the dimer should generate exactly 100ng of the free form if a 100% reduction takes place with TBP [19]. Chromatographic interferences and/or incomplete reduction of the disulphide bonds could explain the reported differences.

Finally, we have developped a simple and reliable assay for the determination of free captopril in plasma and CSF, and total Captopril in CSF. This technique could then be used to clarify the neurobiological action of this drug in human subjects.

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