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Short communication

Simultaneous determination of captopril and hydrochlorothiazide in human plasma by reverse-phase HPLC from linear gradient elution

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Abstract

A simple, rapid and sensitive high-performance liquid chromatographic method for the simultaneous determination of captopril and hydrochlorothiazide in human plasma samples was developed. Captopril was derivatized with 2,4′-dibromoacetophenone (pBPB) to form a captopril-pBPB adduct. From acidified serum plasma samples, the hydrochlorothiazide and derivatized captopril was extracted with 5 ml ether, then with 5 ml dichloromethane. Effective chromatographic separation was achieved using a C_{18} column (DIAMONSIL 150 mm × 4 mm i.d., 5 μm) based on an acetonitrile–trifluoroacetic acid–water gradient elution at a flow rate of 1.2 ml/min.

The internal standard (IS), derivatized captopril and hydrochlorothiazide were detected at 263 nm and were eluted at 4.2, 6.8 and 16.9 min, respectively. No endogenous substances were found to interfere. The limit of quantification for hydrochlorothiazide and derivatized captopril in plasma were 3.3 and 7 ng/ml. The calibration curve for derivatized captopril showed linearity in the range 20–4000 ng/ml, with a regression coefficient corresponding to 0.9993 and the coefficient of the variation of the points of the calibration curve being lower than 10%. The calibration curve for hydrochlorothiazide showed linearity in the range 10–1200 ng/ml, with a regression coefficient corresponding to 0.9999 and the coefficient of the variation of the points of the calibration curve being lower than 10%. The method was suitably validated and successfully applied to the determination of captopril and hydrochlorothiazide in human plasma samples.
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1. Introduction

Captopril, 1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline, is an angiotensin-converting enzyme inhibitor that is used in the treatment of hypertension and congestive heart failure [1]. Hydrochlorothiazide (or 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide) is a diuretic agent within the class of benzothiadiazine drugs. Fig. 1 shows the structural formulae of captopril (I), hydrochlorothiazide (II) and sulphadimidine (internal standard, IS).

A new combination dosage form of captopril and hydrochlorothiazide is indicated for the treatment and management of hypertension. To our knowledge, there have been no reports for the simultaneous determination of both drugs in biological fluids. Therefore, it is necessary to establish a new analytical method for their simultaneous determination.

There are a number of reported methods for the determination of the two drugs. Captopril has been determined by several methods, including gas chromatography (GC) [2], gas chromatography–mass spectrometry (GC–MS) [3,4], high-performance liquid chromatography (HPLC) [5–11] and liquid chromatography–mass spectrometry (LC–MS) [12]. However, the GC method is limited by sensitivity and the GC–MS or LC–MS methods may not be widely accessible. Of these methods, HPLC is the most extensive and highly sensitive. HPLC methods have been described for captopril determination in biological fluids, but many of these methods require specific detectors, such as a fluorometer [13], and others require benzene for washing [14]. Ultraviolet-light detection at a sufficiently low detection wavelength is not possible for detection of captopril. Bahmaei et al. used pBPB as the derivatization reagent to determine the concentration of captopril. The reagent can avoid captopril conversion into a disulphide dimer and prevent the

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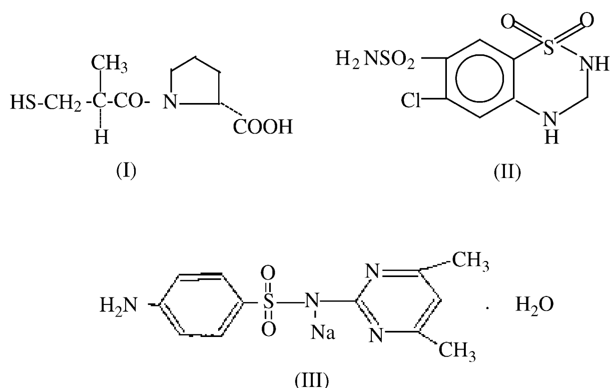

Fig. 1. The structural formulae of captopril (I), hydrochlorothiazide (II) and sulphadimidine (III).

formation of conjugated disulphide and improve the sensitivity of captopril. On the other hand, various methods have been reported for the assay of hydrochlorothiazide, individually or in combination with other drugs, in pharmaceutical formulations and biological fluids. These methods are based on voltammetry [15], capillary-zone electrophoresis [16], spectrophotometry [17], HPLC [11,18–21] and liquid chromatography–mass spectrometry (LC–MS) [22,23]. In addition, a HPLC method has been reported for the simultaneous determination of captopril and hydrochlorothiazide by derivatization [11]. However, a derivatized temperature of 105 °C is too high for the captopril to be easily oxidized. Moreover, the sensitivity is not high enough for the determination of plasma samples.

As a crucial part of the drug development process, a rapid, sensitive and selective assay is required to measure drug concentrations in human plasma samples from clinical pharmacokinetic studies. We developed an accurate and sensitive validated HPLC assay with ultraviolet detection for the simultaneous determination of captopril and hydrochlorothiazide in human plasma samples based on the work by Manoochehr et al. [14].

2. Experimental

2.1. Apparatus

Chromatographic analyses were performed on an Agilent 1100 LC system (Agilent, USA) that was equipped with a G1310A bin pump, a G1322A vacuum degasser, a G1316A thermostated column compartment, a VWD variable wavelength UV/VIS detector and an HP 1100 series manual injector 20 μl fixed loop. The detector was set at 263 nm and peak areas were integrated automatically using the Hewlett-Packard Chem. Station software program. Other apparatus used included a radiometer NEL pH 890 digital pH meter that was equipped with a combined glass–calamol electrode and ultrasound generator.

2.2. Chemicals used

Compound captopril tablets (containing 10.0 mg captopril and 6.0 mg hydrochlorothiazide/tablet; lot no. 030321), captopril and hydrochlorothiazide were kindly supplied by Xi'an Lv-Gu Pharmacy Ltd. Co. (Shanxi, China). Methanol (HPLC grade), 2,4′-dibromoacetophenone (pBPB) and acetonitrile (HPLC grade) were purchased from Merck (New Jersey, USA). Trifluoroacetic acid, ethylenediaminetetraacetic acid disodium salt (EDTA·2Na), hydrochloric acid, ascorbic acid, sodium hydroxide and dichloromethane were obtained from Sinopharm Medicine Chemical Reagent Ltd. Co. (Shanghai, China). Sulphadimidine (internal standard, IS) was kindly supplied by the Department of Eye and ENT Hospital of Fudan University (Shanghai, China). Ether was purchased from Chang-Shu Yang-Yuan Chemical Industry Ltd. Co. (Jiangshu, China). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Procedure for high-performance liquid chromatography

2.3.1. Chromatographic conditions

Solutions and mobile phases were freshly prepared before use. The analytical column was a C_{18} column (DIAMONSIL 150 mm × 4 mm i.d., 5 μm). Elution was obtained by using the following gradient steps of solvents A [0.2% trifluoroacetic acid (pH 1.8)] and B (acetonitrile): 87:13 (A:B) for 4 min, then 60:40 for 8 min at a flow rate of 1.2 ml/min. After the run was complete, the column re-equilibration time was 4 min. All analyses were carried out under isothermal conditions at 30 °C.

2.3.2. Standards

Stock solutions of captopril and hydrochlorothiazide were made daily by dissolving the appropriate amount of pure drug in methanol to yield a final drug concentration of 400 and 200 μg/ml, respectively. This solution was further diluted with methanol to give a series of standards with concentrations of 1, 2, 7.5, 16, 40, 120 and 200 μg/ml (20, 40, 150, 320, 800, 2400 and 4000 ng/ml in blood, respectively) for captopril and 0.5, 1, 2, 5, 10, 20 and 60 μg/ml (10, 20, 40, 100, 200, 400 and 1200 ng/ml in blood), respectively, for hydrochlorothiazide. A stock solution of sulphadimidine (200 μg/ml) for the internal standard (IS) was prepared in methanol and diluted to a concentration of 40 μg/ml with methanol.

2.3.3. Preparation of plasma samples

The plasma samples were stored at −20 °C and allowed to thaw at room temperature before processing. To 1 ml of plasma in a glass-stoppered 15-ml centrifuge tube (tube I), the following were added: 40 μl of 0.1 M ascorbic acid; 0.1 M ethylenediaminetetraacetic acid disodium salt. To this mixture, 40 μl of internal standard solution (40 μg/ml of sulphadimidine), 40 μl of 0.1 M sodium hydroxide and 40 μl 2,4′-dibromoacetophenone (pBPB) (1 mg/ml) in methanol were added. The tube was vortex-mixed for 30 s and allowed to stand at room temperature for 30 min. A total of 0.4 ml of 1 M hydrochloric acid and 5 ml ether were added. After vortexing for 2 min, and after 15 min centrifugation at 2700 × *g*, the organic phase was transferred into a new borosilicate glass tube (tube II) and evaporated until completely dry under a nitrogen stream. In the aqueous phase (tube I), 5 ml dichloromethane was added for the second extrac-

tion. After vortexing for 2 min, and after 15 min centrifugation at 2700 × g, the organic phase was transferred into tube II and evaporated until completely dry under a nitrogen stream. The residue was dissolved in 50 μl acetonitrile and 20 μl of solution was injected into the liquid chromatograph.

2.3.4. Calibration and linearity

To determine calibration curves, plasma spiked with captopril within the concentration range 20–4000 ng/ml and hydrochlorothiazide within the concentration range 10–1200 ng/ml were prepared. The calibration curve was obtained by plotting the peak area ratio of captopril-pBPB and IS to hydrochlorothiazide and IS.

2.3.5. Extraction recovery

Extraction recoveries were calculated in plasma samples ($n = 5$) that had been spiked with internal standard at a concentration of 20, 320 and 4000 ng/ml for captopril, and 10, 100 and 400 ng/ml for hydrochlorothiazide. The experimentally determined concentrations of captopril and hydrochlorothiazide in spiked plasma samples were compared with theoretical values.

2.3.6. Precision and accuracy

The intra- and inter-day precision and accuracy of the developed method were evaluated in plasma samples spiked with captopril, hydrochlorothiazide and internal standard. For intra- and inter-day precisions and accuracies, the samples ($n = 5$) that had been spiked at a concentration of 20, 320 and 4000 ng/ml for captopril, and 10, 100 and 400 ng/ml for hydrochlorothiazide were assayed. The inter-day precision and accuracy were evaluated on five continuous days in a week. Standard deviations (S.D.s) were calculated using standard methods.

2.3.7. Application to plasma samples

The assay was applied to an open-label, single-dose (50 mg captopril, 30 mg hydrochlothiazide) pharmacokinetic study in two young healthy volunteers. After drug administration, blood samples for analytical determinations were collected in heparinized tubes containing 40 μl of 0.1 M ascorbic acid and 0.1 M ethylenediaminetetraacetic acid disodium salt. Within 30 min after blood collection, blood plasma was separated by centrifugation at 2700 × g for 10 min. To 1 ml plasma samples, the following were added: 40 μl of 0.1 M sodium hydroxide; 40 μl 2,4′-dibromoacetophenone (pBPB) (1 mg/ml). To the mixture, 40 μl of internal standard solution (40 μg/ml of sulphadimidine) was added. The tubes were vortex-mixed for 30 s and allowed to stand at room temperature for 30 min, then stored at −20 °C until analysis.

3. Results and discussion

The aim of this work was to develop a new, simpler, more accurate, reproducible and sensitive HPLC method for the simultaneous determination of captopril and hydrochlorothiazide in human plasma. A satisfactory separation of each drug from biological endogenous components in human plasma was obtained. For separation of the examined drugs, various reverse-phase columns, isothermal and gradient mobile phase systems were attempted. The optimum wavelength for detection was 263 nm, at which much better detection responses were achieved. The mobile phases used were acetonitrile–trifluoroacetic acid–water gradient elution. Elution was obtained by using the following gradient steps of solvents A [0.2% trifluoroacetic acid (pH 1.8)] and B (acetonitrile): 87:13 (A:B) for 4 min, then 60:40 for 8 min at a flow rate of 1.2 ml/min. The composition of mobile phase was found to be essential for improving the sharpness of the captopril-pBPB and hydrochlorothiazide peaks.

The method was validated with regards to linearity, limit of detection and quantification, recovery, precision, accuracy and specificity.

Fig. 2 shows chromatograms of the extracted drug-free plasma, a calibration standard containing 320 ng/ml of captopril-pBPB and 400 ng/ml of hydrochlorothiazide, and a plasma sample obtained from a volunteer 1 h after a single oral dose of five compound captopril tablets. The retention times for the internal standard and investigated drugs were found to be 4.2 min (internal standard), 6.8 min (hydrochlorothiazide) and 16.9 min (captopril-pBPB), respectively. No endogenous plasma component eluted at the retention time of captopril-pBPB and hydrochlorothiazide.

Peak area ratio (Y) of captopril-pBPB of calibration standards versus internal standard were proportional to the concentration (x) of captopril-pBPB in plasma over the range tested (20–4000 ng/ml). The peak area ratio (Y) of hydrochlorothiazide calibration standards versus internal standard was proportional to the concentration (x) of hydrochlorothiazide in plasma over the range tested (10–1200 ng/ml). Blank human blood samples spiked with the corresponding compounds to give concentrations of 20, 40, 150, 320, 800, 2400 and 4000 ng/ml for captopril, and 10, 20, 40, 100, 200, 400 and 1200 ng/ml for hydrochlorothiazide were analysed. For all analytes, excellent linearity was obtained in the specified concentration range. The correlation coefficients for the calibration regression line were 0.999 or more. The equations of the calibration lines were as follows: $y = -0.00711 + 0.001663x$ for captopril and $y = -0.00578 + 0.00926x$ for hydrochlorothiazide.

The limit of detection (LOD) and the limit of quantification (LOQ) of captopril-pBPB and hydrochlothiazide were calculated for the peak area using the following equations [19]:

$$\mathrm{LOD} = \frac{3N}{B}, \qquad \mathrm{LOQ} = \frac{10N}{B}$$

where N, the noise estimate, is the S.D. of the peak areas (three injections) in the baseline signal of drug-free blood samples and B is the slope of the corresponding calibration curve. The limit of quantification and the limit of detection of captopril-pBPB and hydrochlorothiazide were found to be 20, 10, 7 and 3.3 ng/ml, respectively.

The extraction recovery was calculated by the ratio of the areas obtained from the spiked samples and the areas obtained from the standard solutions. These are illustrated in Table 1.

Intra-assay precision and accuracy of the method is illustrated in Table 2. Inter-assay precision and accuracy of the method is illustrated in Table 3.

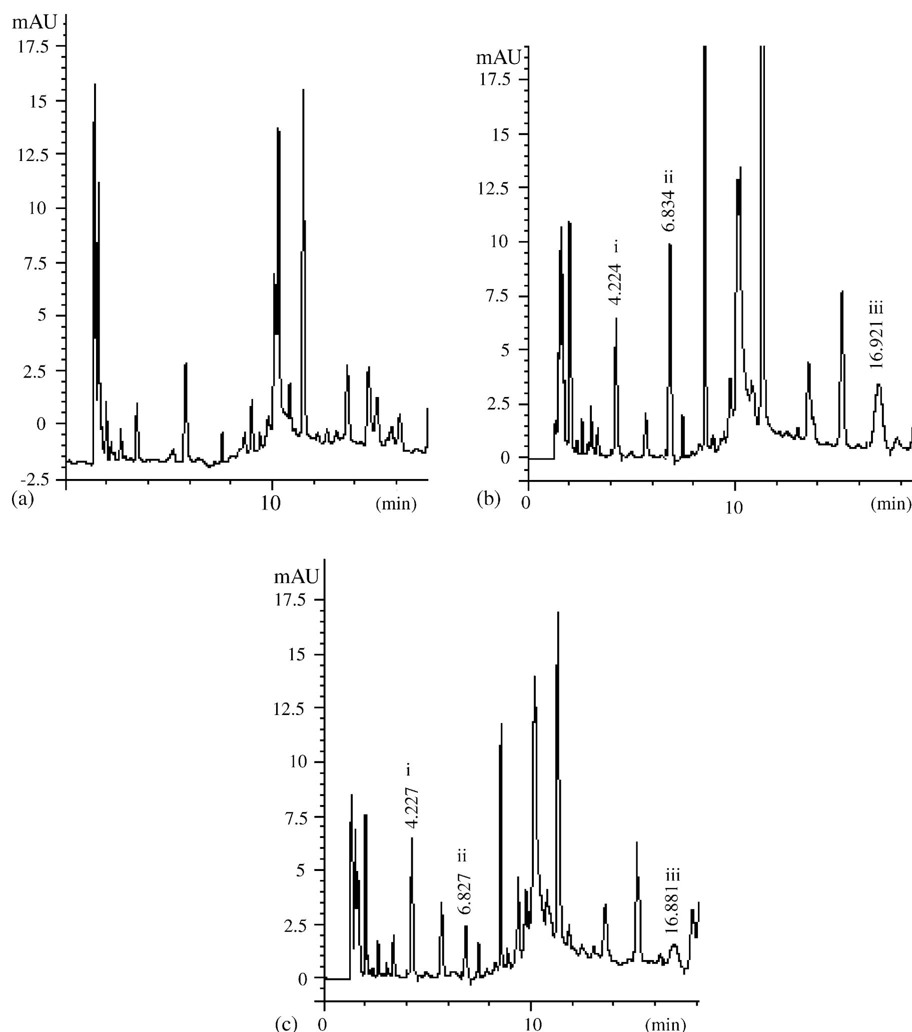

Fig. 2. (a) HPLC chromatogram of the extracted drug-free plasma. (b) HPLC chromatogram of a calibration standard containing 400 ng/ml of hydrochlorothiazide and 320 ng/ml of captopril-pBPB. (c) HPLC chromatogram of plasma sample obtained from a volunteer 0.75 h after a single oral dose of 50 mg captopril, 30 mg hydrochlorothiazide. (i) Internal standard (IS); (ii) hydrochlorothiazide; (iii) captopril-pBPB.

Table 1
The extraction recovery of captopril-pBPB (Cap-pBPB) and hydrochlorothiazide (HCTZ) in spiked human plasma

Captopril-pBPB		Hydrochlorothiazide	
Added (ng/ml)	Mean ± S.D.	Added (ng/ml)	Mean ± S.D.
20	74.2 ± 3.9	10	90.5 ± 8.8
320	82.5 ± 4.1	100	79.0 ± 10.8
4000	79.2 ± 4.7	400	89.3 ± 4.3

The samples were prepared in advance and stored at −20 °C. Slight variations in retention times were observed using the mobile phases that had been prepared on different days. The column-to-column reproducibility was evaluated by injecting the samples on two columns from different manufacturers containing the same brand of packing material. The elution order and the resolution of compounds were not affected and only slight variations in retention times were observed.

The stability of captopril-pBPB and hydrochlorothiazide was determined by diluting samples in mobile phase and storing them at +4 °C. The samples were checked after three successive days

Table 2
Intra-assay precision for the determination of captopril-pBPB and hydrochlorothiazide

Captopril-pBPB			Hydrochlorothiazide		
Added (ng/ml)	Measured ± S.D.	R.S.D.	Added (ng/ml)	Measured ± S.D.	R.S.D.
20	19.0 ± 2.4	2.62	10	10.2 ± 1.2	7.6
320	306.8 ± 20.0	5.31	100	93.3 ± 5.2	5.2
4000	4489.0 ± 166.2	3.67	400	416.7 ± 27.8	6.6

Table 3
Inter-assay precision for the determination of captopril-pBPB and hydrochlorothiazide

Captopril-pBPB			Hydrochlorothiazide		
Added (ng/ml)	Measured ± S.D.	R.S.D.	Added (ng/ml)	Measured ± S.D.	R.S.D.
20	19.1 ± 1.8	9.3	10	11.1 ± 1.1	10.2
320	308.4 ± 32.1	10.4	100	90.7 ± 5.6	6.2
4000	4234.1 ± 389.5	9.2	400	418.4 ± 37.1	8.9

of storage and the data were compared with freshly prepared samples. In each case, the R.S.D. (relative standard deviation, is expressed in percent and is obtained by multiplying the standard deviation by 100 and dividing this product by the average) values of assays were found to be below 3%. This indicates that captopril-pBPB and hydrochlorothiazide are stable in the mobile phase for at least 3 days.

The stability of captopril-pBPB, captopril and hydrochlorothiazide in plasma was determined by periodic analysis of spiked samples at +4 °C. The results indicated that no degradation occurred. In addition, the selected clinical plasma samples, which were assayed repeatedly for three successive days, showed high stability of captopril-pBPB, captopril and hydrochlorothiazide at −20 °C. Captopril-pBPB, captopril and hydrochlorothiazide were also shown to be stable in plasma for at least 3 days. Furthermore, captopril-pBPB, captopril and hydrochlorothiazide were stable through at least two freeze–thaw cycles.

The proposed method was applied to the determination of captopril and hydrochlorothiazide in plasma samples. Plasma samples were periodically collected up to 9 h after oral administration of five compound captopril tablets to two healthy, young, male volunteers. Fig. 3 illustrates the mean plasma-concentration/time-profile of captopril-pBPB and hydrochlorothiazide, following an oral dose containing 50.0 mg captopril and 30 mg hydrochlorothiazide. The plasma level of captopril and hydrochlorothiazide reached a maximum at 0.75 and 2 h, respectively, after the administration. The human pharmacokinetic profiles that were obtained correspond to pharmacokinetic profiles that were previously reported in the literature [4,18].

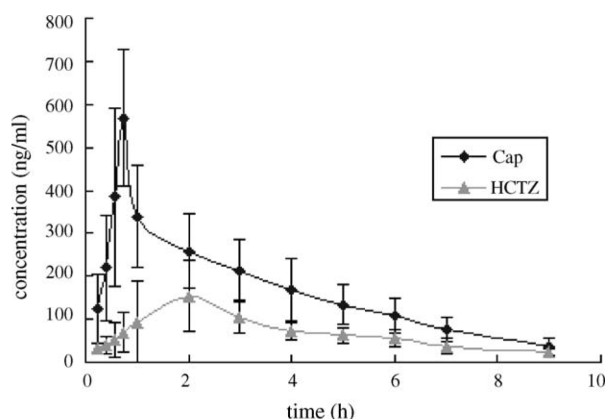

Fig. 3. Mean plasma concentrations of captopril (Cap) and hydrochlorothiazide (HCTZ) after single oral dose five compound captopril tablets. Each point represents the mean ± S.D. ($n = 2$).

4. Conclusion

The procedure was successfully applied to the simultaneous determination of the studied compounds (compound captopril tablets) in human plasma without any interference from additives and endogenous substances. It is a simple and accurate procedure requiring inexpensive reagents that could be used for rapid and reliable clinical and pharmacokinetic studies of compound captopril tablets.

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