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Improved analytical procedure for the measurement of captopril in human plasma by gas chromatography–mass spectrometry and its application to pharmacokinetic studies

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Abstract

An enhanced, sensitive GC–MS assay is presented for the highly specific angiotensin-converting enzyme (ACE) inhibitor, captopril. This method improves previously published assays by using solid NEM as stabilizer in the collection tubes, a rapid extraction technique with dichloromethane and back-extraction into base, a commercially available internal standard (thiosalicylic acid) and a capillary GC column. Captopril and the internal standard are measured as their bis-pentafluorobenzyl derivatives. The assay was linear from 10 to 5000 ng/ml with a mean recovery following solvent extraction at 50, 200 and 1000 ng/ml of 77%. At mean values of 45.9, 187 and 980 ng/ml inter-assay precision and accuracy were 4.0, 2.9 and 3.5% and 8.2, 6.5 and 3.1%, respectively. Analysis of captopril concentrations in plasma samples from 20 volunteers following oral administration of 100 mg of captopril provided the following pharmacokinetic data (mean±S.D.): C_{max}, 1470±467 ng/ml; $AUC_{0-\infty}$, 1736±481 ng/ml.h; T_{max}, 0.73 h; k_e, 0.468±0.122 h^{-1}; elimination half life, 1.58±0.41 h.

Keywords: Captopril

1. Introduction

Captopril ([(2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline], CTP, Fig. 1) is a highly specific competitive inhibitor of angiotensin I converting enzyme (ACE) which is used for the treatment of hypertension and heart failure. In humans, about half of the absorbed CTP is rapidly metabolized leading to low plasma levels of circulating parent drug. CTP is unstable in blood and plasma ex vivo due to oxidation and the rapid formation of disulfides [1,2]. Therefore a fixative must be added to each blood sample immediately following collection.

Published HPLC methods for CTP in plasma have utilized either UV detection following derivatization with *p*-bromophenacylbromide [1–3] or fluorescence

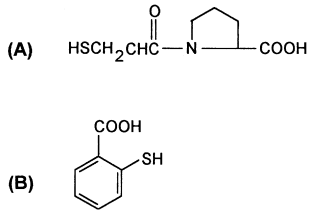

Fig. 1. Structures of (A) captopril and (B) thiosalicylic acid.

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detection after treatment with N-(1-pyrenyl)-maleimide [4,5]. These reagents served as both chemical stabilizers and chromophoric tags. As these reagents are not water soluble, an aliquot of the blood or plasma sample must be mixed volumetrically with a solution of the appropriate reagent in a water miscible organic solvent. This procedure is impractical when numerous samples must be collected over a short period of time, as typically occurs in clinical pharmacokinetic studies.

Most published GC assays use the more water soluble N-ethylmaleimide (NEM) as the stabilizing agent [6–10], which has the advantage of allowing non-volumetric addition of blood samples to tubes containing an excess of powdered or crystalline reagent. To enhance the volatility and GC elution properties of the CTP–NEM adduct, the carboxylic acid group is typically esterified using either methylation, pentafluorobenzylation or hexafluoroisopropylation [6–10] following extraction of the NEM adduct from plasma or blood. These derivatization procedures lead to the formation of structural isomers which are separated chromatographically into several peaks, rendering quantification difficult. To achieve more reliable quantification, Ito et al. [11] developed derivatization conditions which produced a bis-pentafluorobenzyl derivative of CTP by also displacing the NEM moiety, allowing the formation of a single chromatographic peak. That method, however, was unsuitable for high-throughput analysis due to the need for sample deproteinization and the requirement for the quantitative addition of NEM solution to collected blood. Furthermore, all of these GC assays required internal standards which were either custom synthesized or were not commercially available.

We required a rapid, sensitive method for assay of plasma captopril which eliminated the requirement for volumetric treatment of plasma samples at collection and used an internal standard which was easily obtainable, and which would therefore be suitable for the analysis of large numbers of plasma samples obtained in the conduct of bioequivalence or pharmacokinetic studies.

2. Experimental

2.1. Chemicals

Captopril (USP Reference Standard) was obtained from Activon Scientific (Sydney, Australia). Thiosalicylic acid (TSA, internal standard, Fig. 1), N-ethylmaleimide, pentafluorobenzylbromide (Sigma Chemical Company, St Louis, MO, USA), acetone (AR grade), methanol (HPLC grade), dichloromethane (nanograde, Mallinckrodt, Paris, KY, USA) and chloroform (AR grade, Rhone-Poulenc) were all used as received. All other chemicals were of AR or higher grade and were used without further purification.

2.2. GC–MS

A Hewlett–Packard model 5890 Series II Gas Chromatograph was used in conjunction with a model 7673 automatic injector operated in on-column mode and a model 5971 mass selective detector operated in selected ion monitoring (SIM) mode with an electron impact source. The injector had a septum purge of helium at a flow rate of approximately 14 ml/min and the mass spectrometer was autotuned using the maximum sensitivity option. The column used was a 25 m×0.2 mm I.D.×0.33 μm film thickness HP-5 capillary column preceded by a 0.5 m×0.53 mm I.D. phenylmethyl deactivated silica guard column (Hewlett–Packard). The operating conditions for the GC–MS were as follows: oven, initial temperature 80°C for 0.5 min, ramp 40°C/min to 260°C, ramp at 10°C/min from 260 to 290°C, hold at 290°C for 5 min; injector, initial temperature 160°C for 0.5 min, ramp 50°C/min to 280°C, hold at 280°C; GC–MS transfer line 310°C. Helium was used as the carrier gas at 0.8 ml/min. Data was acquired from the detector using MS Chemstation software (Hewlett–Packard). SIM groups monitored for the pentafluorobenzylated derivatives of each analyte were as follows: captopril, m/z 294, 396; thiosalicylic acid, m/z 333, 514. Injection volume was 1 μl for all analyses.

2.3. Standard solutions

Stock solutions (1 mg/ml) and appropriate dilutions of captopril and internal standard were prepared in phosphate buffer (0.2 *M*, pH 7.0) and stored in amber glass vials at 4°C. Because captopril can form disulfides in solution, NEM (0.5%) was added to the phosphate buffer to increase drug stability. Under these conditions, the solutions were found to be

stable for a minimum of three months. TSA was diluted in the same buffer solution for addition to the sample tubes.

2.4. Dosing and sample collection

A single 100 mg oral dose of CTP (Capoten®, Bristol–Myers Squibb, 2×50 mg tablets) was administered to 20 healthy young adult volunteers and blood samples were drawn at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6 and 8 h post-dose. Blood (10 ml) was collected by syringe and immediately transferred into lithium heparin tubes containing powdered NEM (50±10 mg) and gently mixed. Samples were centrifuged immediately at 2000 *g* and the plasma transferred to 5 ml tubes containing additional NEM (25±5 mg), mixed again and stored at −20°C. Drug free plasma used during assay validation was collected from healthy volunteers and treated in an identical manner.

2.5. Sample assay

Plasma samples (1.0 ml) which had been treated with NEM were dispensed into 15 ml glass tubes with Teflon-lined caps and were spiked with 1 μg internal standard (0.1 ml of the TSA aqueous solution), acidified with HCl (3 *M*, 0.1 ml) and extracted with dichloromethane (5 ml). The tubes were mixed by inversion for 5 min and the organic layer transferred to a clean tube. The analytes were then back extracted into $NaHCO_3$ (5%, 2 ml), the aqueous layer reacidified with HCl (3 *M*, 0.5 ml) and re-extracted with fresh dichloromethane (2 ml). This extract was evaporated to dryness under an air stream at ≤40°C and the residue derivatized by the addition of pentafluorobenzylbromide (2% in acetone, 0.5 ml) in the presence of base (0.5 *M* NaOH in methanol, 0.1 ml). The tubes were then capped and incubated at 60°C for 1 h. Excess derivatizing agents were evaporated under air and the residue reconstituted in chloroform (0.2 ml).

2.6. Assay validation

Linearity of the assay was demonstrated on three separate occasions by processing plasma standards in triplicate at nine separate concentrations over the range 10–5000 ng/ml. Peak area ratios (CTP:TSA) were plotted against CTP concentration and analyzed using weighted (1/concentration) least-squares linear regression. Precision and accuracy were assessed in conjunction with the linearity studies on three separate occasions using six spiked plasma samples at each of three concentrations (nominally 50, 200 and 1000 ng/ml). Measured concentrations were determined by application of the appropriate standard curve obtained on that occasion. Precision was assessed in terms of the relative standard deviation of the measured concentrations in a replicate set, while accuracy was determined from the mean relative error in a replicate set (i.e. difference between measured and nominal concentrations of the spiked samples).

Recovery of CTP from plasma was assessed by comparison of the slopes of calibration curves for CTP:internal standard from extracted versus non-extracted samples. All samples were assayed in triplicate, and the internal standard was not extracted in both sample sets. The recovery of the internal standard was determined at the assay concentration in triplicate extracted versus non-extracted samples, with CTP serving as the unextracted reference standard. Specificity in relation to endogenous compounds was demonstrated by analysis of a series of randomly selected drug-free plasma samples ($n=10$). In addition, lignocaine, commonly used at the site of insertion of forearm venous cannulas, was also investigated for possible interference due to co-elution. The stability of CTP under conditions of storage and handling relevant to the conduct of clinical pharmacokinetic studies (16 h at room temperature and 99 days at −20°C) was also investigated.

3. Results and discussion

The mass spectra of the bis-pentafluorobenzyl derivatives of CTP and TSA are shown in Fig. 2. Ito et al. [11] observed an M^+ for derivatized captopril at m/z 577 with an extremely weak signal strength at a 50-fold amplification. No molecular ion was observed for the CTP derivative on our system. The observed signals at m/z 396, 294 and 250 were as observed by Ito et al. [11], with the signals at 396 and 294 resulting from the respective losses of one PFB moiety and of the side chain following cleavage

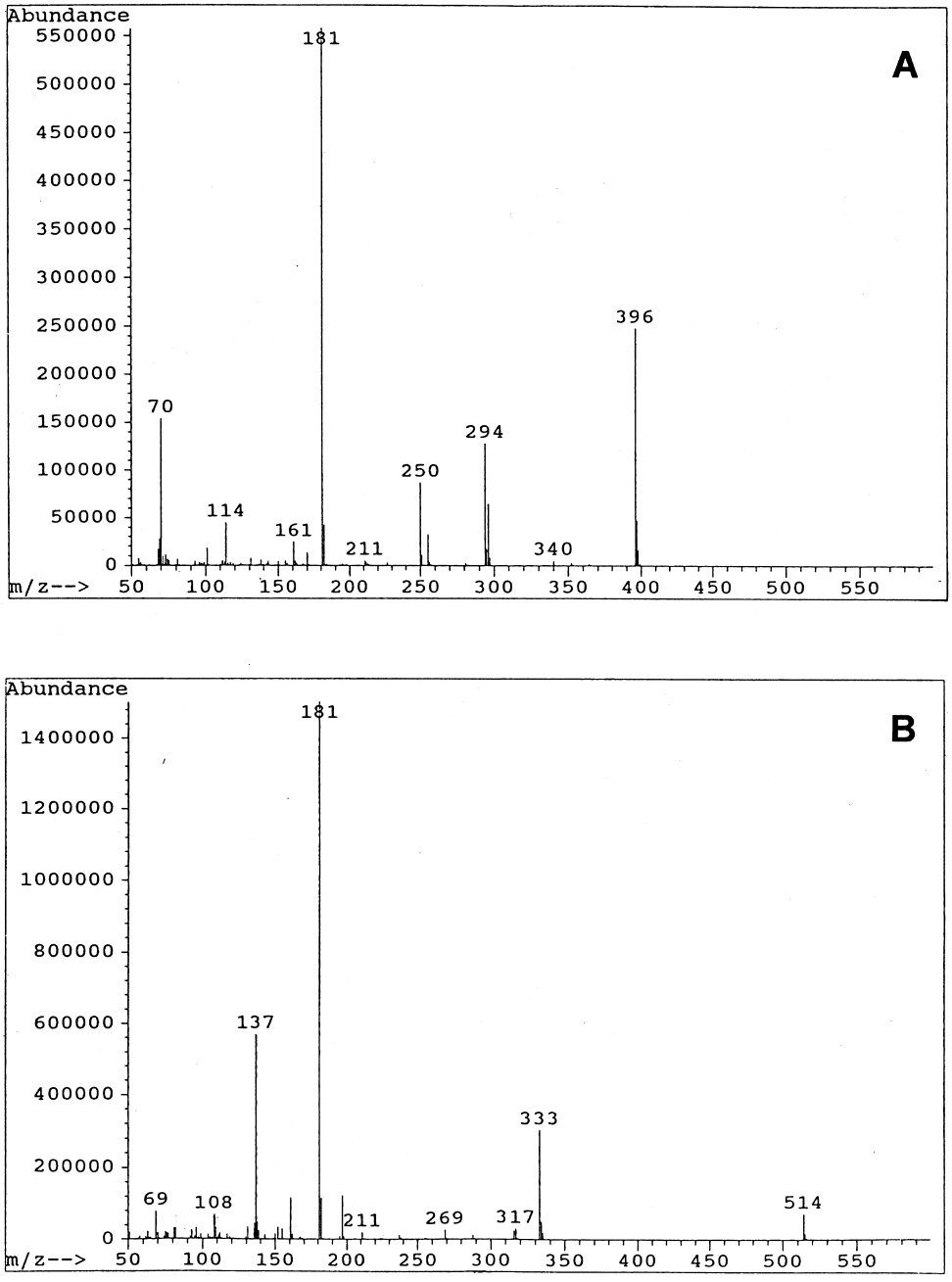

Fig. 2. Mass spectra of the bis-pentafluorobenzyl derivatives of (A) captopril and (B) thiosalicylic acid.

of the C–N bond. For bis-pentafluorobenzyl-TSA, an M^+ of 514 was observed as was a signal at 333 $[M-181]^+$ representing loss of a PFB moiety. The signal at m/z 181 in both mass spectra results from free pentafluorobenzyl groups. TSA proved to be a suitable internal standard, as it contained both functional groups present on captopril which were involved in the stabilization reaction with NEM and

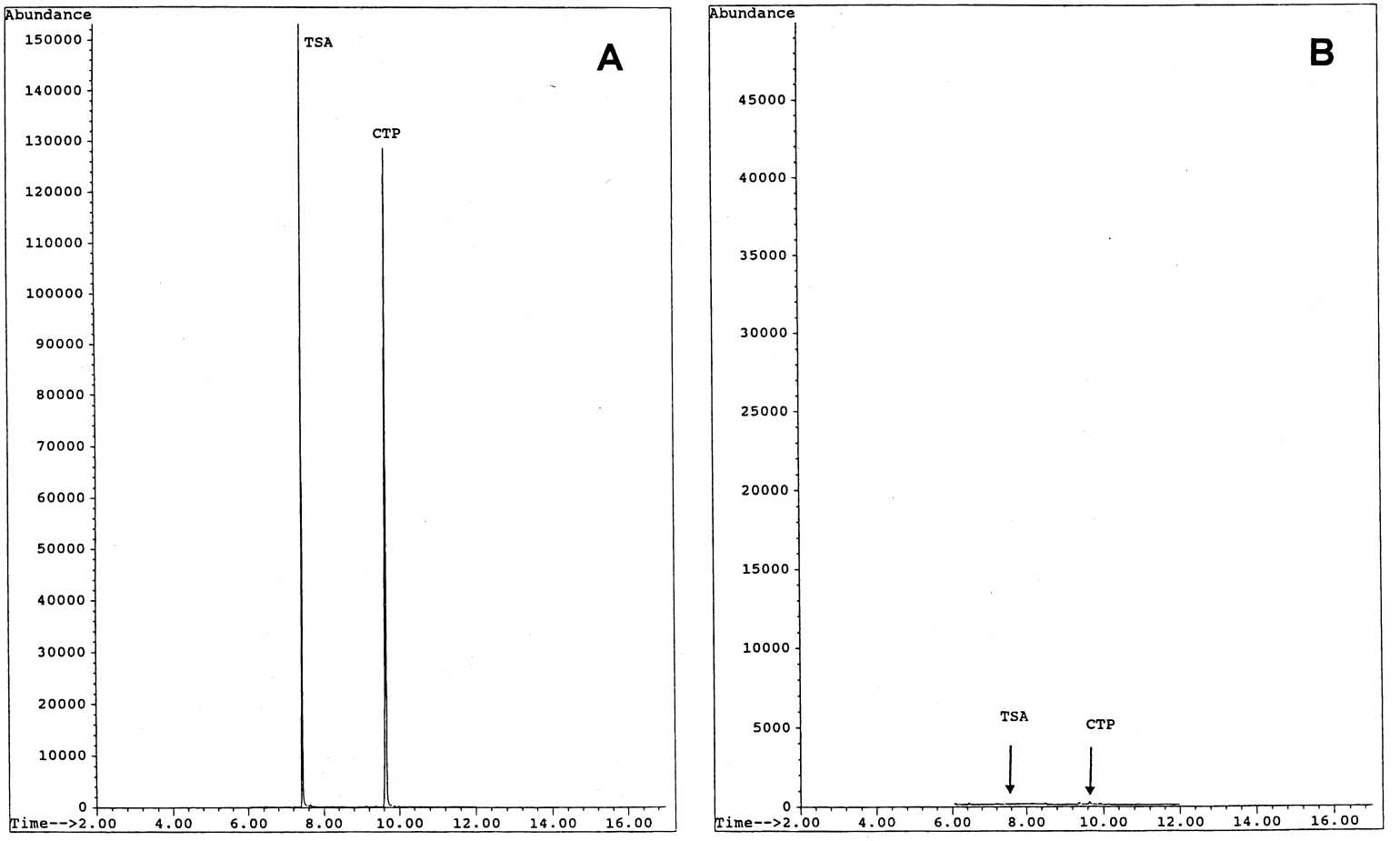

Fig. 3. Total ion chromatograms following analysis of (A) plasma sample spiked with captopril and thiosalicylic acid and (B) drug free plasma.

the derivatization reaction with PFBB, and eluted close to but with complete resolution from captopril.

The use of selected ion monitoring resulted in high signal to noise ratios for both compounds as evident in the total ion chromatogram of a plasma extract shown in Fig. 3A. Using the described chromatographic conditions, the retention times observed during assay development were: CTP, 7.1–7.4 min; TSA, 9.2–9.6 min, while the intra-batch coefficient of variation for these retention times during the three occasions of linearity evaluation was <1% for each analyte. The assay was linear from 10 to 5000 ng/ml with a typical calibration curve over this range producing a regression of $y=0.001493x-0.000750$; $r^2=0.9993$ (y=peak area ratio, x=concentration of analyte). This range, although not extending down to the 0.5 ng/ml level obtained by Ito et al. was much broader than that quoted by these workers and was more suited to the concentrations observed following analysis of plasma samples from pharmacokinetic studies.

Mean overall extraction recoveries for CTP and internal standard were 77 and 84%, respectively. These recoveries were considered acceptable given the requirement for three separate extraction steps in the sample workup. No interference from coeluting endogenous compounds was observed from analysis of ten drug-free plasma samples (Fig. 3B).

Intra-batch precision and accuracy were evaluated from assays of spiked samples ($n=6$) at three concentrations, and are shown in Table 1. In all instances both precision and accuracy were within 6.5%.

Inter-batch precision and accuracy were assessed from assays of six samples analyzed on three separate occasions and are shown in Table 2. In all instances both precision and accuracy were less than 9%.

The limit of quantitation (LOQ), defined as the lowest concentration at which both accuracy and precision is within 20%, was deemed to be 10 ng/ml during the use of this assay for pharmacokinetic studies. The data obtained for triplicate 10 ng/ml standards measured on three separate occasions (precision, 4.9%, accuracy, 5.7%) provided results which easily satisfied these criteria, indicating that a somewhat lower LOQ may have been achievable if required. CTP and TSA (as their derivatives) were found to be stable in vials at three concentrations on the autoinjector carousel for at least 16 h (Table 3), with peak area ratios differing by <1% from the initial observations, but with no detectable change in absolute peak areas for either CTP or TSA. Analysis of spiked plasma samples which had been stored at −20°C for 99 days (Table 4) indicated differences of <8% over this period which is within experimental variability. Stability for these periods were essential to encompass the times between collection of samples from pharmacokinetic studies to their final assay.

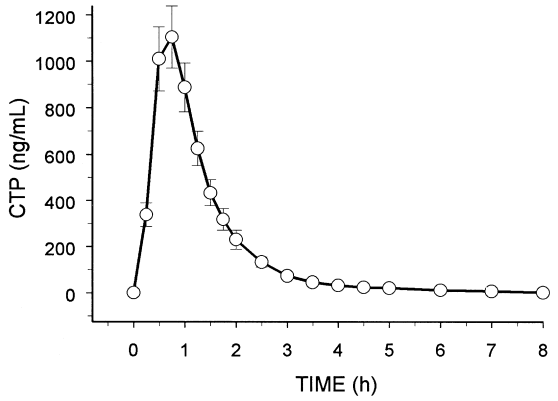

Fig. 4. Plasma concentration–time profile (mean±S.E.M.) for captopril following single oral 100 mg doses in twenty subjects.

Fig. 4 shows a plot of the mean CTP concentration at each sample collection time. Analysis of subject plasma samples following a 100 mg oral dose provided the following data ($n=20$, mean±S.D.): C_{max} 1470±467 ng/ml; $AUC_{0-\infty}$, 1736±481 ng/ml.h; T_{max}, 0.73 h; $t_{1/2}$, 1.58±0.41 h, k_e, 0.468±0.122 h^{-1}. These results compare favourably with previously published values for T_{max} (0.79±0.32 h, (50 mg dose), [3]) and $t_{1/2}$ (1.7 h, [12]).

4. Conclusion

We have described an enhanced, sensitive GC–MS assay for captopril, suitable for the analysis of large numbers of plasma samples obtained in the

Table 1
Intra-batch precision and accuracy for assay of CTP

Nominal CTP concentration (ng/ml)	Measured CTP concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
50.0	47.0	3.1	6.0
200	187	3.8	6.4
1000	973	4.3	3.7

[a] Assessed in terms of the relative standard deviation (R.S.D.) of the measured concentrations in a replicate set ($n=6$).
[b] Determined from the mean relative error in a replicate set ($n=6$).

Table 2
Inter-batch precision and accuracy for assay of CTP

Nominal CTP concentration (ng/ml)	Measured CTP concentration (ng/ml)	Precision (%)[a]	Accuracy (%)[b]
50.0	45.9	4.0	8.2
200	187	2.9	6.5
1000	980	3.5	3.1

[a] Assessed in terms of the relative standard deviation (R.S.D.) of the measured concentrations in a replicate set ($n=18$).
[b] Determined from the mean relative error in a replicate set ($n=18$).

Table 3
Stability of CTP for 16 h at room temperature on autoinjector carousel

Nominal CTP concentration (ng/ml)	Peak area ratio (CTP:TSA)		Difference (%)
	Initial ($t=0$)	$t=16.6$ h	
50.0	0.0688±0.0039	0.0682±0.0041	0.87
200	0.2806±0.0130	0.2784±0.0088	0.78
1000	1.4545±0.0401	1.4520±0.0770	0.17

Mean±S.D. of triplicate measurements.

Table 4
Stability of CTP following storage at −20°C for 99 days

Nominal CTP concentration (ng/ml)	Measured CTP concentration (ng/ml)		Difference (%)[a]
	Initial ($t=0$)	$t=99$ days	
50.0	51.7±2.2	50.7±0.9	1.9
200	200±5.5	215±2.3	7.5
1000	1051±29	1133±14	7.8

[a] Difference calculated from initial measured concentrations.
Mean±S.D. of triplicate measurements.

conduct of bioequivalence or pharmacokinetic studies. This method uses solid NEM as stabilizer in the collection tubes, therefore eliminating the requirement for volumetric treatment of plasma samples at collection, as well as a rapid extraction technique and a commercially available internal standard. The assay was used for the analysis of plasma samples from 20 volunteers following oral administration of 100 mg of captopril.

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