Physicochemistry, Pharmacokinetics, and Pharmacodynamics of *S*-Nitrosocaptopril Crystals, a New Nitric Oxide Donor†

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Abstract □ S-nitrosocaptopril (CapNO) has been proposed as a compound possessing capacities of both a nitric oxide (NO) donor and an inhibitor of angiotensin converting enzyme (ACE). In the present study, we characterized the physicochemical, pharmacokinetic, and pharmacological properties of the crystalline CapNO. The novel stable crystals are in a red flake form. Spectroscopic analyses of CapNO revealed its UV/visible λ_{max} and the corresponding extinction coefficients, and characteristic infrared frequencies for the N═O and S–N stretch. The NMR signals corresponding to the protons attached to the carbon (C–S) and the carbon itself were remarkably shifted downfield upon S-nitrosylation. Mass and HPLC analyses, solubility, and melting point of CapNO were determined. Simultaneous on-line analyses of pharmacodynamic and pharmacokinetic profiles of CapNO in catheterized awake rats of spontaneous hypertension (SHR) showed acute decreases in mean arterial pressure (MAP), concomitant with the corresponding increases in plasma levels of CapNO after po or iv administration. The pharmacokinetic parameters for CapNO, i.e., $t_{1/2}$, T_{max}, C_{max}, V_d, AUC, and oral bioavailability were analyzed to understand the dose-dependent potency and effective period of CapNO. The highest concentrations of oral CapNO distributed in tissues were found in kidney, liver, lung, and small intestine. CapNO was excreted predominantly via urine, and second via feces in the detectable forms of thiols and nitrogen oxide although a small portion of CapNO was found in bile. The results provide the evidence of in vivo cleavage of the S–N bond and biotransformation of CapNO.

Introduction

The endothelium-derived relaxing factor, known as EDRF,[1] is generally considered to be a labile NO-containing precursor. Evidence has accumulated implicating that reduced generation of NO has been involved in several diseases.[2,14] Thus, there is a great demand for exogenous, potent NO donors to treat NO-deficient diseases. However, it is difficult to reliably introduce NO into most biological systems for therapeutic purposes without premature decomposition. Due to a growing appreciation for NO-related responses that are not mediated directly by NO itself, there is an increasing interest in compounds which generate NO, mimic its biological function, and might have pharmacological effects in a controlled manner.

Water-soluble RSNOs, where R can be any one of a large range of chemical entities, might meet these major requirements as possible storage and transport forms for the otherwise highly reactive NO molecule.[3−5] Many efforts have been made to prepare a solid powder of RSNOs; however, most RSNOs are reported to be too unstable to isolate as pure solids. It has long been known that RSNOs decompose to yield NO and the corresponding disulfide RSSR;[7−9] the latter could then be reduced by either an intracellular spontaneous recovery mechanism[10] or a general protein disulfide reductase.[11] Therefore, an RSNO composed of an NO group and another pharmacological active component RSH would have considerable theoretical and practical significance. CapNO may be the best candidate due to its dual role as an ACE inhibitor and an NO carrier.[3,6,12,13]

We have recently developed a facile synthesis of CapNO in a good yield. The first synthesized red crystals, after being dried, could be stored in the dark at about 25 °C for at least two months and 4 °C for at least one year without a significant decay. The stability of the red crystals provides us with an opportunity to characterize the authentic physicochemical properties of the novel crystalline CapNO in the present report. Although numerous reports concerning the cardiovascular pharmacology of RSNOs have been published,[3−6,11−13] none of them has addressed the pharmacokinetic profiles and oral bioavailability of RSNOs. The exemplary CapNO, that nonenzymetically releases NO in a manner similar to most of RSNOs,[8,15] is certainly a very useful tool for exploring pilot information regarding in vivo biotransformation and functional significance of RSNOs. Therefore, we investigated the overall pharmacokinetical profiles and antihypertensive effects of CapNO simultaneously and evaluated the drug's tissue distribution and elimination.

Experimental Section

Spectral Analyses—The preparation of crystalline CapNO was accomplished via a S-nitrosylation reaction of captopril (Cap) as described in detail.[12] The resulting red crystals were dissolved in distilled water at a final concentration of 1 mM. Five different spectrometers (Perkin-Elmer Lambda 2S, Hewlett-Packard 8452 A, Beckman DU-7, Shimadzu UV 2101PC, Shimadza UV 160) were calibrated with 0.5 mM of $K_2Cr_2O_7$ (Aldrich, Milwaukee, WI) before experiments. These spectrometers were then used to determine the peak wavelengths and the corresponding extinction coefficients of CapNO. Infrared spectra were analyzed using Nicolet Impact 400 Fourier Transform IR spectrometer (Nicolet Instruments, Inc., WI). The polyethylene film (KBr) was used to calibrate the full scale and features of the spectrometer. The purified CapNO in an NMR tube was dissolved in $CDCl_3$ for ^1H NMR analysis with a Varian 300 MHz NMR system (Oxford Instruments, Osney Mead, Oxford), or dissolved in D_2O for ^{13}C NMR analysis with a Unity 500 MHz system, respectively. Chemical shifts were recorded as parts per million relative to tetramethylsilane (TMS) as the internal standard (for ^1H NMR) and to $CDCl_3$ as the external

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† Abbreviations: CapNO, *S*-nitrosocaptopril; Cap, Captopril; RSNOs, *S*-nitrosothiols; ACE, angiotensin converting enzyme; MAP, mean arterial pressure; NO, nitric oxide.
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standard (for ^{13}C NMR). Mass spectral analysis was carried out with VG 70-SE analyzer (VG Analytical, Manchester, England) equipped with an electron-impact ionization source and a data-processing system.

Chromatographic Analyses—Thin-layer chromatography (TLC) was performed on Analtech silica gel F_{254} plates (0.25 mm thick). A 5-μL of CapNO (1 mM in H_2O) was spotted on the origin of the plates, which were developed a distance of 6 cm in a solvent-developing system consisting of isopropyl alcohol−acetic acid−water (4:1:1, by volume). CapNO, its parent compound Cap, and the corresponding disulfide were analyzed using a reversed phase HPLC system (Shimadzu, Kyoto, Japan) consisting of a LC-10A liquid delivery module, a SPD-10A ultraviolet detector, a CTO-10A column oven, and a Shim-pack CLC-ODS column (150 mm × 6.0 mm i.d., 5 μm particle size). The system was controlled with an SCL-10A system controller under the following conditions: flow rate, 1.0 mL/min; column temperature, 25 °C; UV detection wavelength, 220 nm. A 5-μL aliquot of CapNO, Cap, or its corresponding disulfide was injected into the injection loop, respectively, and chromatographed using the C_{18} (octadecylsilane) column eluted isocratically with a mobile phase of 50% methanol and 50% phosphoric acid (0.1%). The area under each peak was calibrated with a Shimadzu data processor. The detection limits were 1 μg/ mL on the basis of peak:noise ratio at 3:1. All samples were diluted with 0.1 N HCl immediately before injection.

Simultaneous Measurement of CapNO Plasma Level and Effect Relationship in Awake SHR—Male SHR rats, weighing 365 ± 32 g, were anesthetized with sodium thiopentone (50 mg/kg, ip) and underwent cannulation of the left femoral artery (PE 50), which was used for periodic blood sampling and monitoring of the MAP with a transducer. After recovery from general anesthesia, the catheterized SHR rats were placed in individual restraining cages with free access to food and drinking water. For intravenous administration, the femoral vein was used for the drug injection. For oral administration, the rats were fasted for 4 h before CapNO was given by gavage. CapNO solutions (50 mg/mL) were prepared using saline (for iv) or water (for po) immediately before administration. Serial blood samples (0.2 mL) were collected each time with heparinized 1 mL syringes and replaced with 0.2 mL of normal saline at 0, 1, 5, 30, 60, 120, and 240 min after each iv injection, or 0, 10, 20, 30, 60, 120, 240, and 480 min after the oral dosing. Between samplings, the arterial cannula was connected to a pressure transducer for measurement of MAP. Blood samples were immediately centrifuged at 10000*g* for 5 min. The plasma was prepared and diluted with ice-cold Milli Q water in order to observe the appropriate area of signals. The diluted plasma was collected in airtight syringes and introduced via a HPLC pump (Shimadzu, Kyoto, Japan) into a photolysis−chemiluminescence system at a fixed flow rate of 1 mL/min, where the homolytic cleavage of NO from CapNO was completed. The CapNO concentration is directly proportional to detected signal area, which could be converted to the plasma level of CapNO by using the peak−concentration standardization curve of CapNO. The pharmacokinetic study was performed in each individual rat with the PharmK kinetic software.[16] Pharmacokinetic parameters for individual plasma concentration−time data were determined by a nonlinear least-squares regression program of the PharmK, using a one-compartment open model for single oral dosing, and a two-compartment model for single bolus iv dosing (Table 1). The goodness of fit was assessed from the distribution of residuals. The bioavailability was calculated using the values of area under the concentration−time curve (po versus iv).

Six calibration standard concentrations ranging from 2.5 to 500 ng/mL of CapNO were prepared by spiking blank plasma with CapNO working standards. To determine sensitivity and specificity of the assay prior to the initiation of the study, CapNO and its major contaminants, $NaNO_2$, the starting compound Cap, and the corresponding disulfide, were detected in Milli Q water or rat blank plasma. The diluted rat blank plasma itself gave no signal peaks, indicating no interfering materials present. The detection limit for CapNO was approximately 2.5 ng/mL, which was sensitive enough to measure the predicted plasma levels of CapNO for at least three $t_{1/2}$ periods after single oral dose (5 mg/kg). However, the detection limit for $NaNO_2$ was 2.5 μg/mL with the retention time remarkably shifted, and the system did not respond to the parent Cap and its disulfide, indicating the assay specificity for plasma CapNO. To assess accuracy, precision, and reproducibility, five sets of quality control samples were analyzed together with the diluted plasma and urine samples, or kidney homogenates. The mean predicted quality control concentrations were within 3.6% of the nominal values. The within-day and between-day coefficients of variation were determined for triplicate spiked samples of CapNO at 12.5, 25, and 250 ng/mL and resulted in values of 4.1−8.4%. The recovery of CapNO spiked to rat plasma, urine, bile, and various tissue homogenates was >89%. The relationship between peak areas and CapNO concentrations (2.5, 12.5, 25, 62.5,125, and 500 ng/mL) in rat plasma, urine, bile, and tissue homogenates was linear with correlation coefficients > 0.99.

Table 1—Pharmacokinetic Data for CapNO after Dosing to SHR

route	parameter	dose 5 mg/kg	dose 10 mg/kg	dose 50 mg/kg
iv	$t_{1/2(\alpha)}$ (min)	1.9 ± 0.5	1.9 ± 0.3	5.3 ± 0.4
	$t_{1/2(\beta)}$ (min)	24.6 ± 3.5	19.7 ± 5.9	26.2 ± 5.9
	K_{12} (min^{-1})	0.17 ± 0.03	0.12 ± 0.03	0.05 ± 0.01
	K_{21} (min^{-1})	0.24 ± 0.03	0.28 ± 0.05	0.31 ± 0.03
	K_{10} (min^{-1})	0.07 ± 0.02	0.06 ± 0.01	0.04 ± 0.01
	V_d (L/kg)	0.09 ± 0.01	0.11 ± 0.01	0.21 ± 0.02
	$CL_{(T)}$ (mL/min/kg)	16.0 ± 0.7	22.4 ± 3.2	25.0 ± 1.7
	AUC (μg/mL·min)	247 ± 13	408 ± 25	1122 ± 165
po	K (min^{-1} 10^{-3})	12.3 ± 2.1	13.3 ± 1.0	8.9 ± 0.5
	$K\alpha$ (min^{-1} 10^{-3})	54.9 ± 10.9	62.5 ± 12.1	67.7 ± 18.4
	$t_{1/2}$ (min)	64.4 ± 12.3	70.6 ± 10.0	78.8 ± 5.0
	$CL_{(T)}$ (mL/min/kg)	15.8 ± 0.7	16.4 ± 0.7	23.6 ± 1.8
	V_d/F (mL/kg)	52.7 ± 2.1	56.4 ± 2.2	106.5 ± 8.5
	T_{max} (min)	45.3 ± 2.6	41.8 ± 5.2	37.7 ± 4.4
	C_{max} (μg/ mL)	393 ± 81	606 ± 40	1199 ± 105
	AUC (μg/mL·min)	60 ± 5	89 ± 24	277 ± 29
	bioavailability (%)	24.3	21.8	24.7

For iv route, $t_{1/2(\alpha)}$ and $t_{1/2(\beta)}$ were the half-life of distribution and elimination, respectively. K_{12} was the rate constant for the movement of drug from central to tissue compartment; K_{21}, rate constant for the movement of drug from tissue to central compartment; K_{10}, elimination rate constant. For po route, K was elimination rate constant, and $K\alpha$, absorption rate constants. $t_{1/2}$ was elimination half-life. $CL_{(T)}$, systemic clearance; V_d, apparent volume of distribution; F, bioavailability; T_{max}, time to attain maximum plasma concentration; C_{max}, maximum plasma concentration. AUC, the total area under plasma concentration-time curve from 0 to 4 h.

Tissue Preparation for CapNO Distribution Studies—CD-1 mice, 25 ± 6 g, eight males and seven females were used. Mice were fasted overnight with water available ad libitum. Tissues were excised following ether anesthesia at 0.5, 1, and 5 h after oral administration of CapNO. The intestine was cut opened and rinsed with cold saline to remove the contents. Tissues were washed three times with cold saline, weighed, and minced with scissors. Homogenization of each tissue (0.2 g) was performed in five volumes by tissue weight of ice-cold 10 mM potassium phosphate buffer (pH 7.4) for 5 min. The homogenates were then centrifuged at 14000*g* for 15 min (0−4 °C). The supernatants were removed and diluted with 10-fold excess of Milli Q water. An aliquot of 100-μL diluted supernatants was assayed for CapNO by the photolysis−chemiluminescence method described above. CapNO concentrations in the various tissue samples were determined with standardization curves ranging from 2.5 to 500 ng/mL prepared in the corresponding tissue samples.

Urine, Feces, and Bile Collection—SD rats individually resided in stainless steel metabolism cages, where urine and feces were separated by a cone-shaped device. Pooled urine and feces were collected overnight prior to drug administration and then at 5, 10, 24, and 48 h after a single oral dose. Feces were homogenized in a mortar in two volumes by fecal weight of cold water. Both fecal homogenates and urine were centrifuged at 14000*g* at 0 °C for 15 min. The supernatants of urine and feces were diluted with 10^3−10^4 fold excess of Milli Q water, and the diluted supernatants were analyzed by Ellman test[17] for SH groups, and by Griess assay[18] for nitrite determination. Rats were anesthetized with sodium thiopentone (50 mg/kg, ip), and the peritoneal cavity was opened by an incision along the bloodless midabdominal line. The common bile duct was cannulated with tubing PE50 toward the liver. The abdominal wall was then closed by suturing to prevent hypothermia and dehydration. The open end of the tubing was kept in a sample tube for bile collection before and at 20, 30, 60, 120, 240, and 360 min after oral administration of CapNO (50 mg/

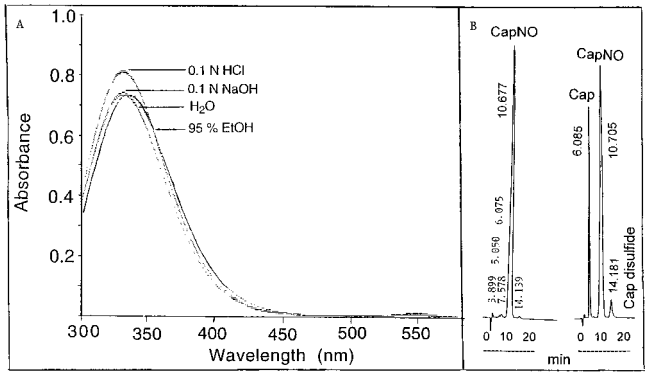

Figure 1—UV-visible spectroscopic and chromatographic characterization of CapNO. A. UV–visible absorption spectra revealed the presence of two maxima at 332 and 546 nm when CapNO was prepared in Milli Q water. HCl caused a hyperchromic shift, and ethanol resulted in a bathochromic shift, whereas NaOH did not have significant effects on the absorption maxima. B. Representative HPLC analysis using UV detection at 220 nm revealed that CapNO solution (50 mM) prepared with its flake crystals in 0.1 N HCl showed only one peak at t_R = 10.6 min without the corresponding monosulfide and disulfide (left tracing), whereas a mixture solution consisting of CapNO, its monosulfide, and its disulfide exhibited three peaks at different retention times (right tracing).

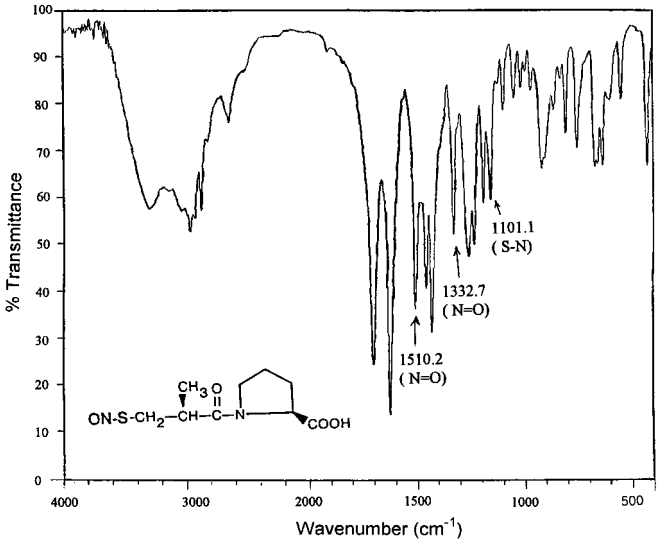

Figure 2—Infrared spectra observed in CapNO molecules showed the most important frequencies at 1332 and 1510 cm^{-1} corresponding to the functional group N═O; 1101 cm^{-1} was assigned to the S–N stretch.

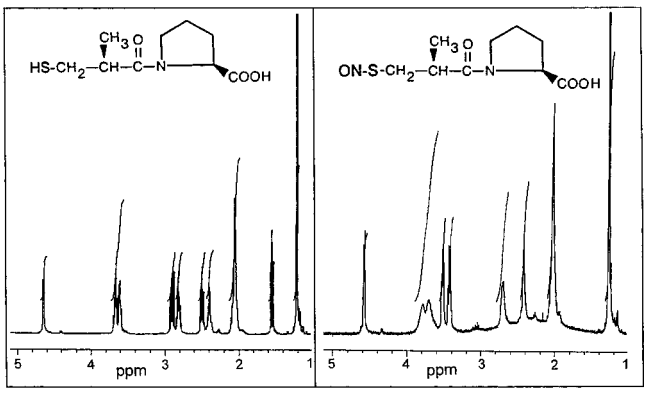

Figure 3—Representative ^1H NMR spectra of CapNO crystals (right) and its starting compound Cap (left). The substitution of SH group with SNO was confirmed by an electron-withdrawing effect of the SNO group, which shifted the signals of the methylenic protons (SCH_2) from δ 2.8–2.9 (in Cap) to δ 3.4–3.5 (in CapNO). In addition, the peak at about 1.5 ppm corresponding to the HS proton disappeared.

kg). CapNO levels in rat bile were determined by the photolysis–chemiluminescence method using the corresponding standardization curve.

Statistical Analyses—Unless otherwise noted, all results are presented as mean ± SE. In some figures, only one SE is shown either above or below the mean to improve clarity. Differences were analyzed by Student's *t* test, a $p < 0.05$ was accepted as significant.

Results

Physicochemical Properties of CapNO—The red flake crystals of CapNO were not hygroscopic, and the stability of the crystals was significantly improved after purification. The red flake crystals were freely soluble in ethanol, chloroform, acetone, and ether and soluble in H_2O, NaOH (0.1 N), and HCl (0.1 N). The melting point of CapNO was found in the range of 49–52 °C, which was determined in open capillary tubes with a melting point apparatus. After being heated over the melting point, the red crystals instantaneously decomposed to an off-white color, which was demonstrated to be captopril disulfide according to the disulfide's retention time of HPLC. The off-white material exhibited a beehive-like appearance when the temperature passed through 104 °C.

Spectral Analyses of CapNO—Scanning by the five UV–visible spectrometers at every ±0.5 nm around the two maxima revealed the peak wavelengths for CapNO at 332 and 546 nm with a molar extinction coefficient (mol^{-1} m^2) of 874.8 ± 12.1 and 15.5 ± 0.2, respectively. Solvent effects on the absorption maxima of CapNO were compared (Figure 1A). TLC spots were detected with UV light (254 nm) or visualized with iodine vapors. TLC showed Cap and CapNO with R_f values of 0.90 and 0.72, respectively (not shown). From the HPLC study (Figure 1B), in each case a single major peak was observed, indicating that the crystalline CapNO has a purity of >99% without detectable monosulfide and disulfide Cap residues. Retention time ranged from 10.6 min for CapNO to 6.1 min for the starting compound Cap to 14.1 min for the major metabolite Cap disulfide. The HPLC analysis revealed that the peak area generated was linear over a range of 1.64 μg/mL to 1.64 mg/mL ($r = 0.99$).

The presence of a S–NO group in CapNO can be readily determined from the strong IR band at 1332 and 1510 cm^{-1}, which corresponds to the stretching vibration of the N═O bond. An absorption band at 1101 cm^{-1} is characteristic of the vibration of the S–N bond. The other absorption bands were identical by IR (KBr) to the bands found in the starting compound Cap although the frequency associated with a particular group varies slightly owing to the influence of the molecular environment. These bands appeared at 1626 cm^{-1} for the amide C–N, 2962, 2930, 2883, and 1460 cm^{-1} for the stretching vibration of the C–H bond, and 3400, 1703, 1433, and 922 cm^{-1} corresponding to the COOH group (Figure 2).

The ^1H NMR spectrum of CapNO is shown in Figure 3. Upon completion of S-nitrosylation of Cap, the signals corresponding to the methylenic protons (SCH_2) of the side chain of Cap were shifted downfield from δ 2.8–2.9 to δ 3.4–3.5. This shift is expected because a thiol is transformed into a more electron-withdrawing nitrosothiol group. The disappearance of the triplet signals of the sulfur proton of Cap at δ 1.53 is indicative of the conversion of Cap to CapNO as well. Reported below are the ^1H NMR data for the CapNO: δ 1.25 (3H,d, CH_3), 2.01(3H, m, 3,4-CH_2CH in proline), 2.42 (1H, q, 4-CH in proline), 2.70 (1H, m, CH), 3.50 (2H, m, SCH_2 in side chain), 3.75 (2H, t, CH_2 in side chain), 4.58 (1H, t, 5-CH in proline). The replacement of SH with S–NO induced the ^{13}C NMR signals of

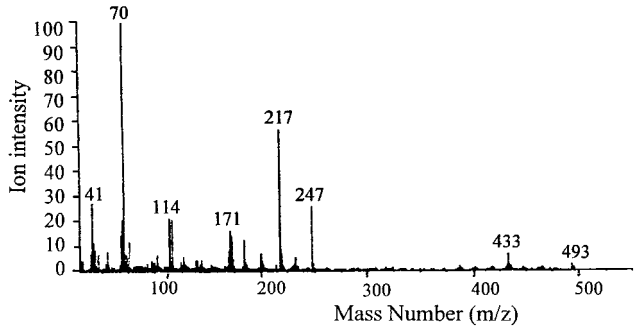

Figure 4—Mass analysis of CapNO crystals using fast atom bombardment mass spectrum analysis of CapNO. The analysis revealed *m/z* 247 and 493, which corresponds to CapNO and a dimer of Cap, respectively. The ion intensity is expressed in an arbitrary unit by taking the height of the base peak at *m/z* 70 as 100.

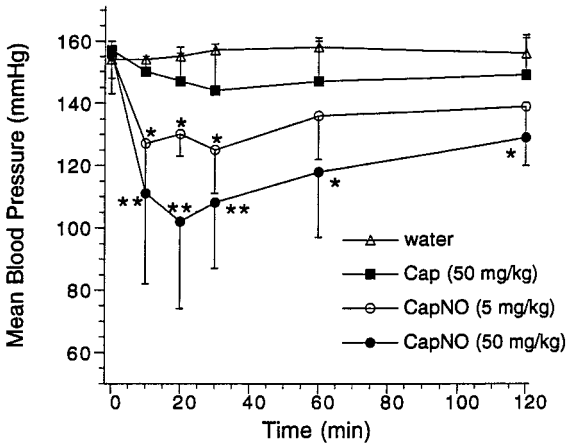

Figure 5—Time course of mean arterial pressure after oral administration of water, Cap, and CapNO to SHR rats. Points and vertical bars represent the mean ± SD (n = 4–6). *p < 0.05, **p < 0.01, compared with water group.

the methylenic carbon (S−CH_2) to shift from δ 42.437 to δ 37.801 (data not shown).

Mass analysis indicated an apparent molecular ion of *m/z* 217 and *m/z* 247, both of which provided valuable diagnostic evidence for the proposed structure of CapNO. First, the base peak of molecular ion at *m/z* 30 (NO) and the largest *m/z* 217 (Cap) strongly suggested the homolysis of the S−NO bond in the CapNO after electron-impact ionization. Second, analysis of the molecular weight of CapNO by fast atom bombardment mass spectrometry (Figure 4) yielded values of *m/z* 247 and 493 $[M + H]^+$, which correspond to CapNO and a dimer of Captopril, respectively. Third, high-resolution mass spectrum analysis of CapNO using glycerol as the reference indicated that the ion at *m/z* 247 (calculated 247.07525) represented a $C_9H_{15}O_4N_2S$ formula for the tested compound.

Pharmacodynamics and Pharmacokinetics of CapNO—After collection of blank blood samples, CapNO was administered (po or iv) to the conscious SHR (n = 6, each group). CapNO (iv) immediately resulted in hypotensive responses in awake SHR. MAP fell to the nadir within 2 min. At doses of 5 and 50 mg/kg, CapNO decreased MAP from 160 ± 7 to 102 ± 8 mmHg (p < 0.01), and 154 ± 5 to 82 ± 9 mmHg (p < 0.001), respectively. The hypotensive effects lasted for more than 4 h. The plasma concentrations of CapNO at 1 min after injections reached the peak of 35.9 ± 1.3 μg/mL (5 mg/kg), 64.4 ± 1.4 μg/mL (10 mg/kg), 219.6 ± 18.8 μg/mL (50 mg/kg); thereafter, the drug concentrations declined with time (Figure 6). Oral administration of CapNO to catheterized awake SHR produced reduction in MAP from 153 ± 10 to 126 ± 18 mmHg (p < 0.05, 5 mg/kg), from 151 ± 5 to 130 ± 5 mmHg (p < 0.05,

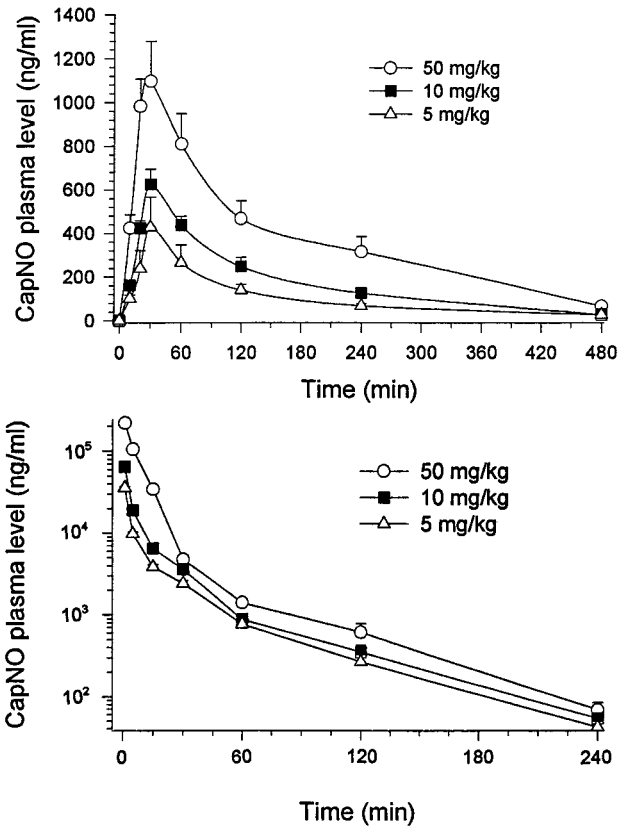

Figure 6—Plasma concentration–time course of CapNO after oral (upper) or intravenous (lower) administration to awake SHR rats (n = 6).

10 mg/kg). As shown in Figure 5, CapNO 50 mg/kg (po) produced a marked and sustained hypotension. In contrast, both vehicle (H_2O) and Cap (50 mg/kg) did not produce significant hypotensive effects in SHR rats (Figure 5).

The plasma concentration−time curves for CapNO after po and iv administration are shown in Figure 6. Concomitant with this decrease in blood pressure, CapNO level in plasma reached 425 ± 62 ng/mL at 10 min and 1092 ± 183 ng/mL at 30 min after oral administration. The time course of decreases in blood pressure and increases in CapNO plasma levels paced almost simultaneously. Peak effect and peak plasma level were noted synchronously by 30 min intervals (Figures 5 and 6). The relationship between single oral CapNO doses (5, 10, and 50 mg/kg) and the area under the plasma concentration−time curves was linear. The pharmacokinetic parameters of CapNO after po and iv dosing are listed in Table 1. The bioavailability of oral CapNO, as determined by the ratio of oral AUC to intravenous AUC, ranged from 22 to 25% (Table 1). The results suggest that CapNO is subject to either incomplete absorption or hepatic first-pass metabolism when administered orally.

CapNO Tissue Distribution and Excretion—Tissue levels of CapNO were shown in Table 2. The kidney, liver, lungs, and small intestine were found to contain the highest levels of CapNO, while brain and fat tissues contained almost no CapNO. The urinary levels of CapNO were not detectable 5 h after dosing with 50 (data not shown) and 500 mg/kg of CapNO by using either the photolysis−chemiluminescence or Saville assay[4] to quantify the *S*-nitroso content of CapNO. This result reflected the in vivo biotransformation of CapNO caused by the cleavage of the S−NO bond. However, the major CapNO metabolites such as NO_2^- and thiols, were detected in the pooled urine and feces by the Griess and Ellman assays (Table 3). The data indicate that CapNO was subjected to extensive

Table 2—Tissue Distribution of CapNO (50 mg/kg) after Oral Administration to Mice

time (h)	*n*	kidney	liver	lungs	small intestine	heart	spleen	skeletal muscle	brain	fat
0.5	5	389 ± 165	366 ± 24	317 ± 65	619 ± 76	189 ± 49	112 ± 69	27 ± 9	26 ± 18	ND[a]
1	5	211 ± 109	279 ± 45	192 ± 73	238 ± 109	178 ± 42	80 ± 30	19 ± 9	25 ± 15	ND[a]
5	5	43 ± 23	34 ± 29	49 ± 34	10 ± 23	23 ± 20	35 ± 23	8 ± 2	5 ± 6	ND[a]

[a] Not detected. Results are the mean ± SD (ng/g) for five experiments.

Table 3—Cumulative Excretion (% of dose) of Thiols and NO_2^- in Pooled Rat Urine and Feces after Oral Dosing of CapNO (500 mg/kg, $n = 5$)

time after dosing (h)	SH compounds[a]		NO_2^- [b]	
	urine	feces	urine	feces
0–5	3.54 ± 0.43		15.42 ± 2.93	
0–10	6.80 ± 0.92		25.37 ± 4.72	
0–24	7.52 ± 1.08	3.04 ± 0.74	40.17 ± 5.62	18.25 ± 2.13
0–48	7.91 ± 1.03	3.18 ± 0.91	40.60 ± 5.78	19.42 ± 3.16

[a] Determined by Ellman assay. [b] Determined by Griess assay.

Table 4—Excretion Rate and Recovery of CapNO (50 mg/kg) in Rat Bile after Oral Dosing

time after dosing (min)	excretion rate (ng/mL/h)	recovery of dose ($\times 10^{-5}$)
0–20	912 ± 81	1.38 ± 0.12
20–30	1461 ± 546	0.54 ± 0.21
30–60	511 ± 73	0.93 ± 0.13
60–120	262 ± 10	1.78 ± 0.06
120–240	75 ± 10	2.95 ± 0.19
240–360	26 ± 7	0.45 ± 0.05
0–360		8.03[a]

[a] Total recovery of the dose in the 0–360 min excreta.

metabolism. CapNO excretion via the rat bile within 6 h after a single dose was shown in Table 4.

Discussion

This is the first report systematically characterizing physicochemistry of the novel crystalline CapNO by spectral UV−visible, IR, MS, and NMR. The two absorption maxima at 332 nm (UV, assigned to B band) and 546 nm (visible) are indicative of S-nitrosylation. The λ_{max} of CapNO solution at 546 nm indicates that the solution absorbs a green light, and thus the solution color looks like pink, which is complementary to the color of green. The formation of CapNO could be conveniently monitored by the visible pink and quantified at 332 and 546 nm. Neither such maxima nor the pink color could be observed when the S-nitrosylation reactions were carried out using equimolar concentrations of $NaNO_2$ and disulfides (either Cap disulfide or glutathione disulfide dimer) under the same conditions of reaction. This indicates a specific replacement of SH with SNO.

Infrared and NMR spectra of crystalline CapNO are excellent probes for the nitrosation of thiols in structural determinations. Chemical shifts of CapNO are significantly different from those of the parent Cap. The greater electronegativity of the *S*-nitroso group has a deshielding effect, which is readily identified by downfield shifts of resonance of the α-protons and α-carbon atom attached to the SNO group. The characteristic infrared frequencies assigned to the stretching vibration of the N=O bond are indicative of the presence of S-nitrosylation (Figure 2).

Single oral doses of CapNO are capable of reducing blood pressure in the awake SHR and causing the quick onset of hypotension (approximately 32% reduction in MAP), whereas Cap possesses no such quick activity. It is likely that the variation in the extent of hypotension between the two compounds arises from the NO moiety since only the crystalline CapNO produces immediate vasodilation in vitro[12] and an acute antihypertensive effect in vivo. Very importantly, the concomitant measurement of CapNO plasma concentration and efficacy in the awake SHR has been incorporated to establish the relationships between pharmacokinetics and pharmacodynamics for CapNO in the present studies. The potency and duration of action of CapNO were clearly dose-dependent and were paralleled to the plasma levels of CapNO (Figures 5 and 6). The mean time courses for decreases in blood pressure and increases in CapNO plasma concentration were parallel and concurrent (Figures 5 and 6). Also the oral plasma T_{max} of CapNO (Figure 6, Table 1) was close to the peak time of efficacy (Figure 5). The pharmacokinetic analyses for single doses showed that CapNO displayed a linear pharmacokinetic relationship between doses and dose-normalized AUC values (data not shown). The oral bioavailability of CapNO in the SHR was assessed 22−25% on the basis of the ratio of oral AUC to intravenous AUC. Consistent with these results was the comparison of antihypertensive effects produced by oral and intravenous administration of CapNO (both 5 mg/ kg). The oral effect was about 30% as potent as the intravenous effect calculated by dividing the area under the oral effect-time tracings by the intravenous ones. The differences in bioavailability determined by plasma data and pharmacological effects may be attributed to the additive effects caused by CapNO metabolites, which might not be detectable in plasma by the photolysis−chemiluminescence.

Distribution of CapNO in awake animals reached a peak at 30 min in tissues, where blood flow is relatively rapid. CapNO was eliminated 5 h after oral administration. The results indicate a rapid equilibrium of CapNO between the central and peripheral compartments. The negligible amounts of CapNO found in brain and fat tissues reflects the compound's poor penetration into brain and fat tissues owing most likely to the hydrophilic properties of CapNO. After oral administration, CapNO is excreted as its metabolites, i.e., reduced thiol and NO_2, predominantly in the urine and to a lesser extent in the feces (Table 3). The cumulative urinary recovery of CapNO within 48 h accounted for about 40% of the dose in the form of nitrogen dioxide, and to a minor portion in the form of thiols (Table 3). The relatively less elimination of thiols might be explained by the formation of disulfide dimers after dosing. Failure to detect the vulnerable S−NO bond of CapNO in the pooled urine and feces indicates the extensive metabolism due to the cleavage of S−NO bond when CapNO was eliminated out of the body. It is unlikely that the bile is a major excretion route for CapNO. We only recovered 8×10^{-5} of total CapNO from rat bile (Table 4) within 6 h although the CapNO bile excretion rate was fairly high within 1 h after oral dosing. The hepatic metabolism of RSNOs is worthy to be further investigated.

The mechanism by which CapNO reduced blood pressure in SHR may have involved the ability of CapNO to deliver NO to the blood vessels within a short period, and inhibit ACE[6,19] in vivo for a longer period. A more likely explanation of the antihypertensive action of CapNO can be divided into two phases: an early phase and a late phase. The early

phase involves a direct action of CapNO as an exogenous NO donor to restore the impaired intrinsic NO-mediated vasodilation and directly counteract the vasoconstrictive influences of angiotensin II and other vasoconstrictors that ACE inhibitors may not directly antagonize. The early phase represents a potent hypotensive effect of exogenous NO donors. The time course of this phase is described in Figure 6. The late phase displays a moderate antihypertensive effect produced by a number of bioactive metabolites of CapNO after homolytic cleavage of the S−N bond in CapNO. The metabolites may include Cap disulfides, Cap-cysteine, and Cap-glutathione disulfides.[20] Indirect evidence from the early studies of Cap suggests that these disulfides may play a role in the maintenance of the antihypertensive action of Cap.[21] Compounds containing S−NO group usually have a biotransnitrosylation effect, which involves conjugation with endogenous thiol-containing amino acids to form mixed S-nitrosothiol-thiols.[22] Hence, these potential effects produced by S-nitrosothiol-cysteine, -glutathione, and -serum albumin cannot be excluded from antihypertensive action of CapNO.

It has been known that the photolysis−chemiluminescence method is superior to others in accuracy, reproducibility, and convenience for determining NO and RSNOs.[4] Photolysis homolytically releases NO, and cold traps remove all nonvolatile parent compounds or derivatives. Therefore, the plasma, urine, and other biological samples do not need to be pre-prepared before measurement. This ensures our obtaining more accurate data without facing the problems such as sample processing and thermal and chemical decomposition of the target compound. The NO group exchange between a biochemical active thiol and a RSNO has been reported.[22−24] Because S-nitrosothiol-thiol exchange may produce a detectable signal in the photolysis−chemiluminescence, we cannot be absolutely certain of the nature of the derivative, especially when samples were obtained hours after CapNO was administered. Although it cannot unequivocally distinguish among different RSNOs, this methodology is valuable for studies of CapNO pharmacokinetics when coupled with pharmacodynamic studies.

Unlike glyceryl trinitrate, CapNO is not subject to tolerance,[25] and has no cross-tolerance with glyceryl trinitrate in vivo in dogs.[26] Our preliminary data proved that chronic oral administration of CapNO showed a potent effect in the SHR and SS/Jr hypertensive models (unpublished observation). CapNO is among the most advantageous of the known NO donor drugs from the therapeutic and toxicological point of view. The studies described herein is valuable in predicting effects of the related RSNOs in animals and planning clinical studies of CapNO.

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