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Chemiluminescence and LC–MS/MS analyses for the study of nitric oxide release and distribution following oral administration of nitroaspirin (NCX 4016) in healthy volunteers

Marina Carini [a,*], Giancarlo Aldini [a], Marica Orioli [a], Angela Piccoli [a], Paola Tocchetti [b], Roberto Maffei Facino [a]

[a] *Istituto Chimico Farmaceutico Tossicologico, University of Milan, Viale Abruzzi 42, 20131 Milan, Italy*
[b] *NicOx Research Institute S.r.l., Via Ariosto 21, 20091 Bresso, Milan, Italy*

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Abstract

The metabolic fate of nitric oxide (NO) released from nitroaspirin, benzoic acid, 2-(acetyloxy)-3-[(nitrooxy)methyl]phenyl ester (NCX 4016), the lead compound of a new class of NO-releasing non-steroidal anti-inflammatory drugs (NO-NSAIDs) has been studied in eight healthy male Caucasian subjects following p.o. administration of 1600 mg (single dose), by monitoring at different times in plasma the bioactive storage forms of NO, *S*-nitrosothiols (RSNO) and its oxidation products (NO_x). Plasma levels of NO_x and RSNO and urinary levels of NO_x were determined by an ozone-based chemiluminescent assay using a sensitive Nitric Oxide Analyzer (LOQ: 10 pmol NO injected). In parallel plasma samples were analyzed by a newly developed LC–MS/MS method for analysis of NCX 4015, the metabolite bearing the nitrate ester function. Using MS/MS with multiple reaction monitoring (MRM) in negative ion mode for NCX 4015 and the internal standard (NCX 4015-^{13}C-D_2) it was possible to detect with sufficient accuracy and precision the metabolite in plasma with a quantification limit of 78.1 ng ml^{-1}. Concentration versus time profile of plasma NCX 4015 gave a C_{max} value of 161.94 ± 47.4 ng ml^{-1} and a t_{max} 4.5 ± 1 h. The results indicate that both NO_x and RSNO (these last for the first time determined in vivo in man following oral administration of a NO-donor drug) are effective plasma markers of NO release in vivo, the latter being an earlier indicator of NO distribution (t_{max} 2.0 ± 0.6 h versus 5.4 ± 1.2 h).

Keywords: Nitroaspirin; NCX 4016; Human studies; Nitric oxide; Plasma *S*-nitrosothiols; Chemiluminescence; Liquid chromatography–tandem mass spectrometry

1. Introduction

Nitroaspirin (NCX 4016), benzoic acid, 2-(acetyloxy)-3-[(nitrooxy)methyl]phenyl ester (Fig. 1) is a nitric oxide releasing derivative of aspirin currently under clinical development for cardiovascular diseases. Nitroaspirin consists of two active moieties and

* Corresponding author. Tel.: +39-02-50317532; fax: +39-02-50317565.
E-mail address: marina.carini@unimi.it (M. Carini).

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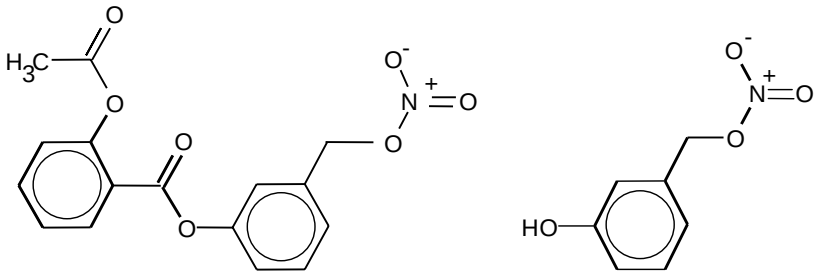

Fig. 1. Structure of nitroaspirin (NCX 4016) and of its NO-carrying metabolite (NCX 4015).

can thus exert anti-inflammatory and anti-thrombotic effects through multiple mechanisms, extending beyond the cyclooxygenases (COX) pathways [1]. Preclinical studies have shown that NCX 4016 is devoid of the gastrointestinal toxicity typical of non-steroidal anti-inflammatory drugs (NSAIDs) [2] while retaining, and in some cases extending the pharmacological properties of the parent drug, including the anti-inflammatory [3], analgesic [4], and antithrombotic activities [5].

The gastrointestinal safety and the inhibitory effects on COX-1 and platelet aggregation of the drug have been recently confirmed in man in a parallel-group, double blind, placebo-controlled pilot study in healthy volunteers following oral chronic treatment [6].

Since many of the NCX 4016 effects are NO-mediated, the knowledge of the metabolic fate of NO in vivo in man, i.e. of the rate of NO release, distribution and elimination, is of paramount importance in the definition of the pharmacokinetic and pharmacodynamic profiles of the drug.

The pharmacokinetic profile of the drug and its major metabolites following single or repeated oral doses in the rat is known: no unchanged drug was detectable in plasma, but only salicylic acid (SA) [2,7]. More recently [8,9] we have demonstrated that in rats orally treated with 100 mg kg^{-1} of NCX 4016, the SA plasma concentration versus time profile parallels the increase of the bioactive storage forms of NO (blood nitrosylhemoglobin and plasma *S*-nitrosothiols) and that of its oxidation products nitrites/nitrates (NO_x), recognized as direct and indirect markers of NO delivery, respectively. These results have provided for the first time clear cut analytical evidence, at least in the rat, that NCX 4016 is able to release slowly, but progressively the vasodilating moiety NO after oral administration. To our knowledge, no data about NO release and distribution from NCX 4016 in man are available. The aim of the present work was to evaluate the pharmacokinetics of NO and/or its derivatives following NCX 4016 oral administration to human volunteers. This was achieved by monitoring *S*-nitrosothiols (RSNO) and nitrites/nitrates (NO_x) plasma levels and the NO_x urine excretion using a chemiluminescent assay. Their kinetics of formation were compared with the metabolic profiling of the drug in plasma by a reverse-phase LC–MS/MS assay. To our knowledge, the results of this study provide the first unequivocal identification and quantitative determination of NO delivery after oral administration of NCX 4016 in man.

2. Materials and methods

2.1. Chemicals

HPLC grade and analytical grade organic solvents were purchased from Sigma-Aldrich (Milan, Italy). HPLC grade water was prepared with a Milli-Q water purification system. Benzoic acid, 2-(acetyloxy)-3-[(nitrooxy)methyl]phenyl ester (NCX 4016) and benzenemethanol,3-hydroxy-α-nitrate (NCX 4015; Fig. 1) were kindly provided by NicOx S.A. (Sophia Antipolis, France); *N*-ethylmaleimide (NEM), *ortho*-phosphoric acid (85%), potassium iodide, sodium nitrate, vanadium(III) chloride, sulfanilamide (*p*-amino-benzenesulfonamide), copper(II) sulfate and ethylene-diaminetetraacetic acid were purchased from

Fluka Chimica (Milan, Italy); *S*-nitrosoglutathione (GSNO) from Cayman Chemical (Cabru s.a.s., Milan, Italy). The internal standard NCX 4015-^{13}C-D_2 was from MDS Pharma Service (Quebec, Canada).

2.2. Apparatus

Chemiluminescence analyses of plasma/urine NO_x and *S*-nitrosothiols were performed with a Sievers Instruments Model 280 Nitric Oxide Analyzer (NOA^{TM}; Sievers, Boulder, CO, USA; Sensor Medics, Milan, Italy). HPLC–MS/MS analyses of NCX 4015 were carried out on a Perkin-Elmer HTS Pal system (a micropump Series 200, an autosampler Series 200) connected to an API 3000 triple quadrupole mass spectrometer equipped with an ionspray source (Perkin-Elmer, Ontario, Canada). LC separations were done on a Prism RP column (50 mm × 2.1 mm i.d.; particle size 5 μm) in isocratic conditions using methanol–ammonium acetate (5 mM in water) (60:40, v/v) as mobile phase delivered at a flow rate 0.2 ml min^{-1}. The following mass spectrometer settings were used: turbo ionspray interface maintained at 350 °C with nitrogen nebulization; nitrogen at a pressure of 10 psi; detection (negative ion mode) of the analytes using the multiple reaction monitoring (MRM) scan mode was performed using the collision activated dissociation gas (CAD) at 6 psi; curtain gas (CUR) at 10 psi; turbo ionspray voltage at −4000 V and channel electron multiplier (CEM) 2800 V. MacQuan® 1.6 (PESCIEX) software was used to process the quantitative data.

2.3. In vivo studies: experimental protocol

Eight healthy male volunteers, mean age 27 years (range 22–39 years), mean BMI 23 (range 19–27) participated in the study. Subjects were healthy based on a pre-study medical examination, including ECG, vital signs, routine clinical laboratory tests on blood and urine, and screening for drug of abuse, hepatitis B and HIV. All volunteers gave their written informed consent before the study start. The clinical study protocol and the written informed consent were approved by the Ethics Committee of Canton Ticino, Switzerland and the study was conducted according to the Declaration of Helsinki (and subsequent revisions).

This was a Phase I, open, single and multiple doses, pharmacokinetic, metabolism and excretion study. In the first part of the study each subject received a single oral dose of NCX 4016 (1600 mg, sachet) in the morning after a standard breakfast. In the second part of the study, each volunteer received NCX 4016 800 mg b.i.d. after breakfast (at approximately 8:00 a.m.) and dinner (at approximately 8:00 p.m.) for 7 consecutive days, starting from the evening administration. Subjects were asked to follow a nitrate-controlled diet from 3 days prior to each study period. All procedures and results reported in the present paper are related to the first part of the study (single oral dose).

To evaluate NCX 4016 safety and tolerability, hematological and urinary laboratory tests, ECGs and vital signs carried out for each subject at entry, were repeated at the end of the study and compared for significant changes. Finally, the occurrence, severity, duration and time of onset of adverse events as well as their relationship with the study medication were closely monitored throughout the study.

2.4. Blood and urine collection

Blood samples were taken pre-dose, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48 and 72 h post-dosing using Li-heparin Vacutainer^{TM} tubes and centrifuged at 2000 × *g* for 10 min at 4 °C. The plasma samples were then transferred to 2 ml cryovials and stored at −80 °C until the analysis of NO_x (chemiluminescence) and drug and metabolites (HPLC).

Blood samples used for measurement of RSNO levels were collected into tubes containing EDTA (final concentration 2 mM) and NEM (final concentration 5 mM). All the samples were then centrifuged and stored as above described.

Urine samples were collected before treatment (pre-dose) and at the following time intervals: 0–6, 6–12, 12–24, 24–48 and 48–72 h.

2.5. NO_x and RSNO determination

Plasma NO_x and RSNO were determined in pre-dose, 0.5, 1, 2, 3, 4, 6, 12 and 24 h samples. NO_x levels were determined in plasma aliquots deproteinized with cold ethanol (1:2, v/v) and centrifuged at

$2000 \times g$ (5 min) or on urine samples diluted with distilled water (1:100) according to the method described by Braman and Hendrix [10]. In both cases 10 μl aliquots were injected. The NO_x content in plasma and urine was determined on calibration curves prepared by adding known amounts of sodium nitrate to blank samples in the concentration range 0.5–100 μM and expressed as μM (plasma) and μmol mmol^{-1} creatinine (urine).

RSNO were determined using 400 μl plasma aliquots by the method of Marley et al. [11] in the presence of 100 μl sulfanilamide (0.5% solution in 0.1 M HCl) to eliminate interference by nitrites. The RSNO content in plasma was determined on calibration curves prepared by adding known amounts of GSNO (concentration range 50–5000 nM) to blank samples, calculated as pmol ml^{-1} of NO and expressed as nM RSNO. The storage stability of RSNO in plasma was checked by replicate analyses ($n = 3$) of native plasma fortified with known amounts (50, 500 and 2500 nM) of GSNO and stored at −80 °C for 8 weeks. The recoveries were from 94.7 to 102.5% (data not shown). Data collection and analysis was performed using the NOAnalysis™ software (Sievers, Boulder, CO, USA).

2.6. Analysis of NCX 4015

Stock solutions of NCX 4015 and of the internal standard NCX 4015-^{13}C-D_2 (IS, 1 mg ml^{-1}) were prepared separately in CH_3OH–CH_3CN–H_2O (4:20:76, v/v/v) and stored at −20 °C. Standard plasma samples were prepared by spiking 50 μl of drug-free plasma with known amounts of NCX 4015 (78.1, 156.3, 312.5, 625, 1250, 2500, 5000 and 10,000 ng ml^{-1}) and 100 μl of IS (40 μg ml^{-1}). Samples were added with 1 ml formic acid (1% solution in water), thoroughly vortexed, extracted with diethylether (3 vol.) by shaking and centrifuged at $3000 \times g$ (room temperature). The organic phase was evaporated to dryness under N_2 and the residue reconstituted with 50 μl of CH_3CN–H_2O (80:20, v/v). Ten microliters aliquots were injected at 5 °C and the MRM experiments were performed by transmitting the $[M-H]^-$ ions of NCX 4015 at *m*/*z* 167.8 and of the IS at *m*/*z* 171.1 through the first quadrupole (Q1) and into the collision cell (Q2). The product ions for NCX 4015 (*m*/*z* 121.1) and for the IS (*m*/*z* 93.1) were transmitted in turn through the third quadrupole (Q3). During sample analysis, a pre-dose sample from each subject was extracted with internal standard to detect potential interference from contaminants or endogenous compounds at the mass transition and the retention time of NCX 4015. Peak-area ratios obtained from MRM of NCX 4015 and IS were used for quantification. The calibration lines were constructed by weighted ($1/x$) least-square linear regression analysis of the peak area ratios. The precision and accuracy of the method (expressed as above reported) were evaluated intra- and inter-day by determining NCX 4015 in three quality control (QC) samples prepared at nominal concentrations of 312.5, 937.5 and 5000 ng ml^{-1} in quadruplicate. Three different calibration curves of eight plasma concentrations of NCX 4015 were prepared to determine the QC samples. Percentage recoveries, determined at the two extremes of the calibration ranges ($n = 6$ each) were satisfactory, ranging from 85 to 95% for the compound (data not shown). The storage stability of NCX 4015 in plasma was determined by replicate analysis ($n = 3$) of native plasma containing known amounts of the analyte (312.5, 937.5 and 5000 ng ml^{-1}) stored at −20 °C for 8 weeks (recoveries were from 95.7 to 98.5%; data not shown).

2.7. Pharmacokinetic analyses

NCX 4015, NO_x and RSNO plasma concentrations versus time data were analyzed using the KINETICA™, version 4.1 (Innaphase, USA) software, by a standard non-compartmental model. Maximum measured plasma concentrations (C_{max}) and the corresponding times (t_{max}) were taken directly from the raw data. The area under the plasma concentration-time curve up to the last measurable time-point (AUC_t) was calculated using the trapezoidal rule. The AUC_t was extrapolated to infinity (AUC) by adding the portion C_{last}/λ_z where C_{last} was the predicted concentration at the last measurable sample given by regression analysis and λ_z was the elimination rate constant of the terminal linear phase of the plasma concentration-time curve. The terminal elimination half-life ($t_{1/2}$) was calculated as $\ln 2/\lambda_z$. Pharmacokinetic parameters and safety and tolerability data were summarized by descriptive statistics.

3. Results

3.1. NO_x and RSNO levels

The chemiluminescent assay of NO_x in plasma (Fig. 2a; individual data and mean ± S.D. of eight subjects) indicates a time-dependent increase over basal levels (46.71 ± 20.04 μM) up to a C_{max} of 101.95 ± 42.49 μM (a two-fold increase), reached at a t_{max} of 5.4 ± 1.2 h. The presence of endogenous levels and the small quantity of sampling times in the terminal elimination phase of the concentration versus time profile did not allowed to determine a consistent half-life. NO_x levels were also determined in urine samples from all the subjects at different time intervals. As reported in Fig. 2b, where the values are shown as individual data and as a mean ± S.D. of eight subjects, most of the NO_x excretion takes place in the first 12 h post-dose, while at the following time intervals (12–24 h), urinary NO_x levels returned to the pre-dose values (116.6 ± 145.0 μmol $mmol^{-1}$ creatinine).

The kinetic profile of plasma RSNO, reported in Fig. 2c, results from the analysis on six of the eight subjects: subjects 5 and 8 were excluded since the majority of plasma samples were contaminated by hemolized erythrocytes (the presence of hemoglobin traces hampers *S*-nitrosothiols determination). *S*-nitrosothiols seem to be an early marker of NO distribution, since a 1.7-fold increase over basal levels (133.12 ± 16.04 nM) was observed already at 0.5 h (230.98 ± 55.2 nM) and peak concentration (C_{max} 352.26 ± 66.50 nM, 2.6-fold increase compared to basal levels) was obtained at a t_{max} of 2.0 ± 0.6 h, a back shift of 3.4 h in respect to NO_x.

The main pharmacokinetic parameters obtained for NO_x, RSNO (and NCX 4015) are summarized in Table 1.

3.2. Plasma levels of NCX 4016 metabolite: NCX 4015

Preliminary experiments carried out with plasma samples from the same subjects (data not shown) indicated that no unchanged drug was detectable at all sampling times, but only NCX 4015 (low levels) and SA (NicOx S.A., data on file). In the present study our attention was focussed only on the evaluation of plasma levels of NCX 4015, the metabolite that, bearing the nitrate ester function, can be considered the carrier for NO. We tried to apply the RP-HPLC method (UV-DAD detection) previously described by us for the study of the in vitro metabolism of the drug in rat liver subcellular fractions [12], but the method, based on UV-DAD detection, although able to efficiently separate all the NCX 4016 metabolites, was unsuited for analysis of NCX 4015 in plasma (due to the low extinction coefficient of the analyte; LOD in plasma = 200 ng ml^{-1}). For this reason we have developed a new HPLC–MS/MS method with negative ion detection to increase sensitivity.

NCX 4015 was determined by LC–MS/MS analysis in a very short time (R.T. = 0.94 min). No co-eluting interfering peaks were ever observed for the metabolite (and the internal standard) throughout the entire study. Fig. 3 shows representative MRM chromatograms for blank plasma (a), plasma spiked with NCX 4015 (312.5 ng ml^{-1}) (b), and the response for a sample from subject 2 (5 h post-dosing; (c));

Table 1
Pharmacokinetic (PK) parameters of NCX 4016

PK parameter	Plasma markers		
	NO_x	RSNO	NCX 4015
C_{max}	101.95 ± 42.49 μM (42%)	352.26 ± 66.50 nM (19%)	161.94 ± 47.44 ng ml^{-1} (29%)
t_{max} (h)	5.4 ± 1.2 (18%)	2.0 ± 0.6 (32%)	4.5 ± 0.8 (17%)
AUC	NE	NE	784.85 ± 498.13 ng ml^{-1} h (63%)
AUC_t	1765.46 ± 651.66 μM h (37%)	4418.26 ± 1188.64 nM h (27%)	550.86 ± 305.54 ng ml^{-1} h (55%)
$t_{1/2}$ (h)	NA	NA	3.1 ± 0.4[a] (13%)

NA: not applicable due to endogenous levels; NE: not evaluated; C_{max}: peak plasma concentration; t_{max}: time to reach C_{max}; AUC: area under the plasma levels – time curve extrapolated to infinity; AUC_t: area under the plasma levels – time curve to the last detectable plasma concentration at time *t*; $t_{1/2}$: elimination half-life.

[a] $n = 4$; CV% in parentheses.

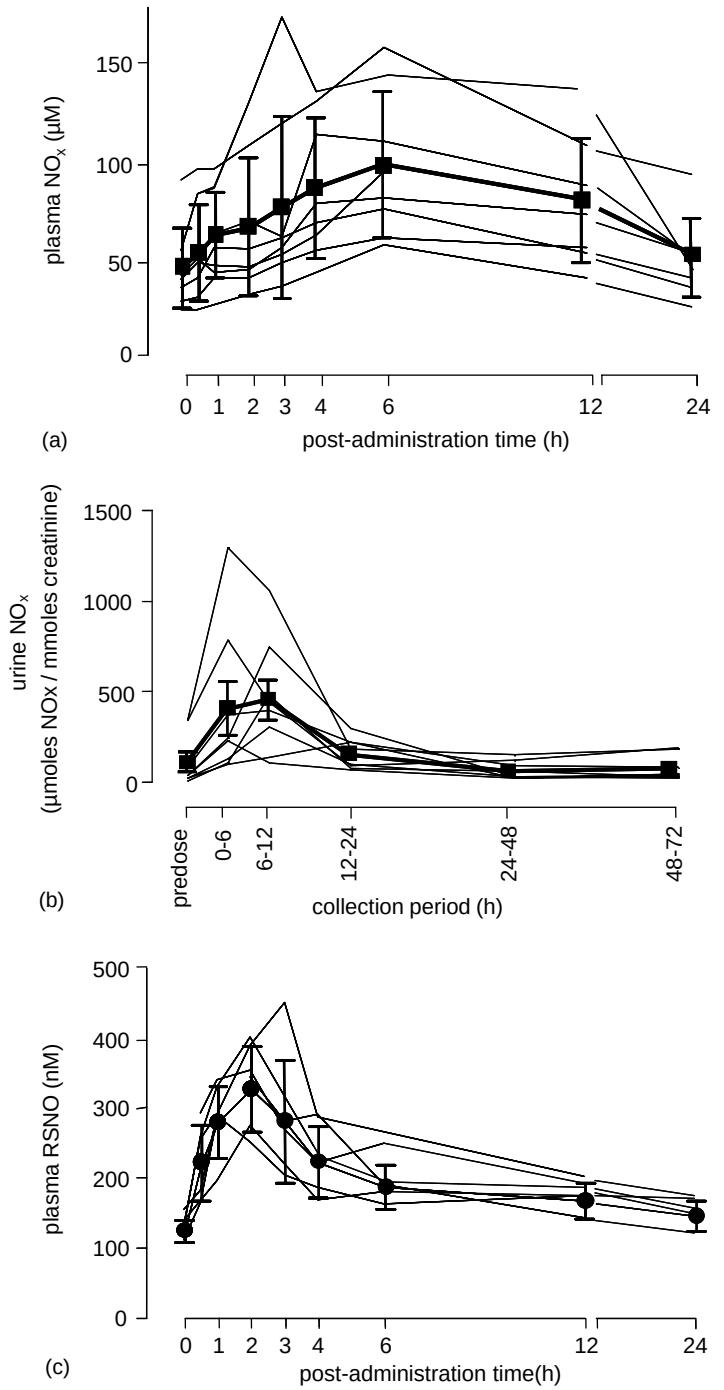

Fig. 2. Time-course of plasma (a) and urine (b) NO_x formation and of plasma RSNO (c) in human volunteers following single oral dose (1600 mg) of NCX 4016. Values are reported as individual data (thin lines) and as mean ± S.D. (bold line) of eight subjects (a and b) and as mean ± S.D. (bold line) of six subjects (c: subjects 5 and 8 excluded). Mean pre-dose values: 46.71 ± 20.04 μM (a); 116 ± 145 μmol mmol^{-1} creatinine (b); 133 ± 16 nM (c).

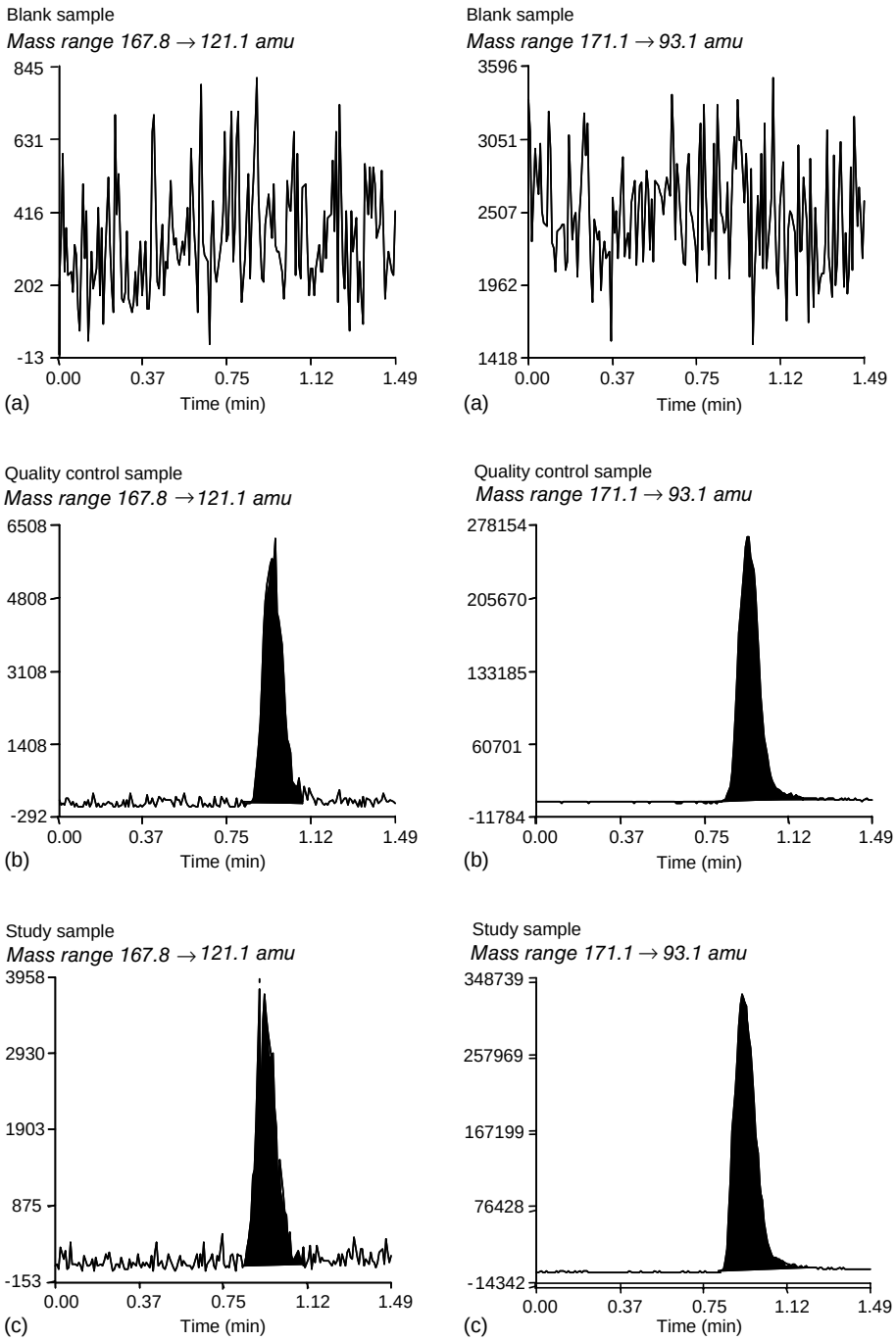

Fig. 3. LC–MS/MS analysis of NCX 4015 in plasma: MRM chromatograms of blank plasma (a); QC sample spiked with NCX 4015 (312.5 ng ml^{-1}) and IS (40 μg ml^{-1}) (b), study sample from subject 2 at the 5th h post-dose (c). Left panels: mass transition $[M - H]^-$ NCX 4015 *m*/*z* 167.8 → 121.1 Right panels: mass transition $[M^{13}C\text{-}D_2 - H]^-$ IS *m*/*z* 171.1 → 93.1.

Table 2
Intra- and inter-day precision and accuracy for determination of NCX 4015 in plasma

	Nominal concentration (ng ml^{-1})	Mean measured concentration (ng ml^{-1})	CV (%)	Accuracy (%)
Intra-day[a]				
Day 1	312.5	318.15 ± 11.24	3.53	101.81
Day 2		302.45 ± 19.95	6.60	96.78
Day 3		296.60 ± 15.60	5.26	94.91
Day 1	937.5	948.35 ± 41.93	4.42	101.15
Day 2		894.57 ± 36.46	4.08	95.42
Day 3		909.43 ± 42.53	4.68	97.00
Day 1	5000	5227.80 ± 245.37	4.69	104.56
Day 2		5079.45 ± 263.68	5.19	101.60
Day 3		5119.50 ± 254.53	4.97	102.39
Inter-day[b]	312.5	303.25 ± 17.24	5.68	97.04
	937.5	917.45 ± 43.55	4.75	97.86
	5000	5125.14 ± 233.98	4.57	102.50

[a] Four replicates at each concentration level ($n = 4$).
[b] Three runs, four replicates at each concentration level over a period of 3 days ($n = 12$).

there was very little background noise, and a stable baseline was maintained throughout.

Standard curves for NCX 4015 constructed on different working days showed good linearity over the entire calibration range (78–10,000 ng ml^{-1}), with coefficients of correlation (r^2) greater than 0.996. The equation of the calibration line was $y = 0.000109 (\pm 0.000042)x + 0.001867(\pm 0.000549)$. Based on a signal-to noise (S/N) level of 9–10, the limit of quantification (LOQ) was found to be 78.1 ng ml^{-1}: at this concentration the intra- and inter-day CVs were determined to be less than 12.9% and the accuracy was 105.9%. The intra- and inter-assay precision and accuracy of the method were determined on QC samples by analyzing four replicates at three concentration levels, and the relative data are reported in Table 2. The intra-day precision (CV%) was less than 6.7% and accuracy ranged from 94.9 to 104.5% of nominal concentrations; the inter-day CV values were less than 5.7% and the accuracy was in the range 97.0–102.5%.

The method was then applied to the analysis of plasma samples from eight human volunteers. Fig. 4

Table 3
Plasma levels (ng ml^{-1}) of NCX 4015 in 8 human volunteers

Sampling time (h)	Subjects							
	01	02	03	04	05	06	07	08
0	BLQ	BLQ	BLQ	BLQ	BLQ	NA	BLQ	BLQ
0.5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
1	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	96.5	NA
2	BLQ	BLQ	BLQ	102.0	BLQ	110.2	160.6	110.4
3	BLQ	BLQ	123.6	159.4	120.9	NA	204.1	121.3
4	BLQ	161.2	NA	182.6	NA	117.1	189.0	130.5
5	99.4	117.7	137.3	205.3	233.7	124.0	125.4	114.3
6	BLQ	108.3	99.0	147.3	142.6	93.0	111.5	BLQ
8	BLQ	BLQ	BLQ	103.6	98.0	BLQ	BLQ	BLQ
10	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
12	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

BLQ: below LOQ; NA: sample not available; in samples relative to 16, 24, 36, 48 and 72 h post-dosing, NCX 4015 levels were below LOQ in all the subjects.

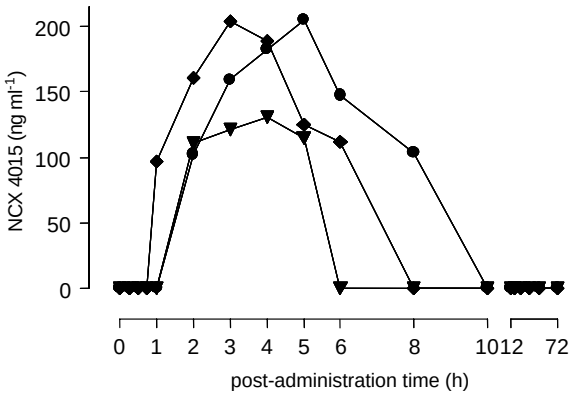

Fig. 4. Concentration vs. time profile of NCX 4015 formation: pharmacokinetic profiles from three representative subjects: 4 (●), 7 (◆) and 8 (▼).

reports the kinetic profiles of NCX 4015 in three representative subjects (4, 7 and 8) and Table 3 the plasma levels relative to all the subjects, indicating high inter-individual variability (at the first hour post-administration, NCX 4015 was quantified in subject 7 only and at the second hour in four of the eight subjects). The C_{max} value (161.94 ± 47.44 ng ml^{-1}) was reached at a t_{max} of 4.5 ± 0.8 h; no metabolite was detectable in all the subjects at the 10th h post-dosing and the elimination half-life ($t_{1/2}$) was 3.1 ± 0.4 h.

3.3. Safety and tolerability

NCX 4016 administered at a single oral dose of 1600 mg was well tolerated. No adverse events were experienced and no subject withdrew from the study. The measurements of vital signs, ECG and laboratory tests did not reveal any clinically relevant abnormalities.

4. Discussion

The release on NO and its metabolic fate has been studied measuring different markers (NO_x and RSNO) in different biological fluids obtained after oral administration of NCX 4016 to male healthy volunteers. NO_x levels, the intermediate (nitrites can be back converted to NO) and final (nitrates) oxidation products of NO metabolism, were measured both in plasma and urine. In plasma from subjects on nitrate-free diet, a 2.1-fold maximal increase was observed from basal levels, and most of NO_x were excreted in urine in the first 24 h, accounting for a 3–35% of dose. Plasma RSNO, the bioactive storage form of NO, displayed a time-dependent increase over basal values and an earlier t_{max} compared to NO_x (approximately 3 h back shift). This is consistent with the assumption that the first targets for NO, once liberated from the NO-donor, are the thiol-containing proteins, the well established carrier for NO distribution. It is worth noting that, to our knowledge, this is the first clear analytical evidence that plasma RSNO are increased after oral administration in man of NCX 4016, the lead compound of a new class of NO-donating compounds. Hence, both NO_x (universally considered indirect markers of nitric oxide disposition) and RSNO are effective indicators of NO release. It is important, however, to outline that the basal RSNO levels measured in healthy subjects were around 100 nM, which are in the range of those reported by Marley et al. [11,13] and Tsikas et al. [14], but greater than those recently found by Rassaf et al. [15] and Yang et al. [16]. This discrepancy may be due to contamination by nitrites, since it is well known that the chemiluminescent assay, adopted by us and routinarily used in several leading laboratories, is not specific for RSNO but determines concomitantly nitrites (nitrites interference is eliminated by sulfanilamide addition). However, in our experimental conditions, residual contamination by nitrites was unequivocally excluded since working even with higher sulfanilamide concentrations (up to 200 μl added to 400 μl of plasma), we have observed a slight decrease in the NO signal, but still within the standard error deviation. In any case, what is important to outline is that a two- to three-fold increase in RSNO over pre-dose values was observed in healthy volunteers following oral administration of the drug, an increase which was paralleled by an augmentation in NO_x values in both plasma and urine.

Another unequivocal marker of NO release, that also represents a delivery form of NO in the blood, is nitrosylhemoglobin HbFe(II)NO, and large experience was gained by our group in determining ex vivo this bioactive storage form after oral (acute and chronic) administration of NCX 4016 in the rat [8,17]. During the single dose phase of the present study, samples for HbFe(II)NO analysis were not

collected and the paramagnetic complex was measured (by electron spin resonance spectroscopy, ESR) only after the NCX 4016 multiple dose phase (second part of the experimental protocol, not reported in this study), but the blood HbFe(II)NO levels were below the limit of detection of the analytical method (approximately 0.5 μM) at all the sampling times. This indicates that nitrosylhemoglobin in man, determined by ESR, can not be employed as an effective marker of NO release from NCX 4016. Another important point to stress is that after multiple doses the levels of methemoglobin (Met-Hb), a typical oxidation reaction product of oxyhemoglobin by large excess of NO, did not change significantly in respect to the pre-dose values in all the subjects (Met-Hb not determined in the single dose phase of the study). The lack of methemoglobinemia indicates that NCX 4016 behaves as a slow NO-releasing drug. Details of all these findings will be reported elsewhere.

From an analytical point of view, a specific and sensitive LC–MS/MS method, suitably developed for this study, allowed the measurement in plasma of the NO-carrying metabolite NCX 4015, the spacer arising by hydrolysis of the ester bond linking aspirin to the NO-moiety. The pharmacokinetic characteristics of this metabolite (t_{max} 4.5 h and $t_{1/2}$ 3.1 h) are very similar to those of the main metabolite salicylic acid (t_{max} 4.5 h and $t_{1/2}$ 4.3 h). Although the C_{max} and the AUC are much higher for SA (18 μg ml^{-1} and 220 μg ml^{-1} h, respectively) (NicOx S.A. data on file), the presence of circulating NCX 4015 demonstrates that NO can be distributed throughout the body and further confirms its slow release.

It can be reasonably assumed that NCX 4016 undergoes substantial first pass effect to be rapidly metabolized to SA and NCX 4015, as previously demonstrated [12] in vitro with rat liver subcellular fractions. The highly polar SA is excreted from the hepatocyte into blood, while the more lipophilic NCX 4015 could be stored within the hepatocyte to undergo different metabolic pathways: denitration to give NO_2^-/NO; conjugation with glutathione to give a thioether adduct and nitrates [12] that can be excreted by the hepatocyte as such or undergo a stepwise conversion by liver xanthine oxidase to bioactive NO [18]; conjugation with glucuronic acid and excretion in urine, as indicated by preliminary data obtained by LC–MS/MS analysis of urine samples from the same subjects (this study). This might explain the low plasma levels of NCX 4015 versus SA. In conclusion, NO released from the NO-donating derivative of aspirin, is converted in part to its oxidation products nitrites and nitrates (NO_x) that are subsequently excreted in urine, and in part enters the physiological routes of NO disposition as RSNO: in this pathway the intermediate metabolite NCX 4015 seems to play a crucial role since it governs its formation and delivery, and further investigations will be carried out about the distribution, metabolism and excretion of this active metabolite.

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