Disposition and metabolism of $[^{14}C]$ Sacubitril/Valsaltran (formerly LCZ696) an angiotensin receptor neprilysin inhibitor, in healthy subjects

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Abstract

1. Sacubitril/valsartan (LCZ696) is an angiotensin receptor neprilysin inhibitor (ARNI) providing simultaneous inhibition of neprilysin (neutral endopeptidase 24.11; NEP) and blockade of the angiotensin II type-1 (AT1) receptor.

2. Following oral administration, $[^{14}C]$LCZ696 delivers systemic exposure to valsartan and AHU377 (sacubitril), which is rapidly metabolized to LBQ657 (M1), the biologically active neprilysin inhibitor. Peak sacubitril plasma concentrations were reached within 0.5–1 h. The mean terminal half-lives of sacubitril, LBQ657 and valsartan were $\sim$1.3, $\sim$12 and $\sim$21 h, respectively.

3. Renal excretion was the dominant route of elimination of radioactivity in human. Urine accounted for 51.7–67.8% and feces for 36.9 to 48.3 % of the total radioactivity. The majority of the drug was excreted as the active metabolite LBQ657 in urine and feces, total accounting for $\sim$85.5% of the total dose.

4. Based upon in vitro studies, the potential for LCZ696 to inhibit or induce cytochrome P450 (CYP) enzymes and cause CYP-mediated drug interactions clinically was found to be low.

Keywords

Human ADME, LCZ696, sacubitril/valsartan, valsartan, sabubitril, and LBQ657

Introduction

LCZ696 (Figure 1) is an angiotensin receptor neprilysin inhibitor (ARNI) providing simultaneous inhibition of neprilysin (neutral endopeptidase 24.11; NEP) and blockade of the angiotensin II type-1 (AT1) receptor (Gu et al., 2010). Following oral administration, LCZ696 delivers systemic exposure to valsartan and sacubitril, which is rapidly metabolized to the biologically active neprilysin inhibitor LBQ657. The effects of neprilysin inhibition by LBQ657 are attributed to increased levels of biologically active natriuretic peptides (NPs) (Criscione et al., 1993; Rubattu et al., 2008; Volpe et al., 2014). The effects of AT1 receptor blockade by valsartan are related to inhibition of the deleterious effects of angiotensin II and its effectors on the cardiovascular system (Lee & Burnett, 2007; Rubattu et al., 2008). Since neprilysin also contributes to the catabolism of angiotensin II, neprilysin inhibitors could potentially increase circulating angiotensin II levels, thus, providing a therapeutic need for compounds that concurrently inhibit neprilysin and block the renin–angiotensin–aldosterone system (Vardeny et al., 2013).

In healthy subjects, LCZ696 was well tolerated following single oral doses 200–1200 mg and after multiple dosing 50–900 mg per day for two weeks (Gu et al., 2010). Based on pharmacokinetic analysis, LCZ696 delivers valsartan and sacubitril in human plasma (data not shown). The disposition and metabolism of valsartan in human are well characterized (Waldmeier et al., 1997), therefore, $[^{14}C]$LCZ696 with $^{14}$C radiolabels at both carbonyl carbons of the amino-4-oxo-butanoid acid moiety of sacubitril to evaluate the disposition and metabolism of sacubitril in human. Here we describe the first comprehensive assessment of metabolism and excretion of $[^{14}C]$LCZ696 in healthy volunteers after a single oral dose of LCZ696 200 mg. The primary objectives of the study were to determine the routes of excretion and mass balance of total radioactivity of LCZ696 in urine and feces, to determine the pharmacokinetics of LCZ696 and total radioactivity in blood/plasma, to identify and quantify the metabolites of sacubitril in plasma, urine and feces, and to elucidate key biotransformation pathways and clearance mechanisms of sacubitril.
Materials and methods

Radioactive drug substance

For [14C]LCZ696 (Figure 1) the 14C labels were located at both carbonyl carbons of the amino-4-oxo-butanoic acid moiety of sacubitril. The specific activity of [14C]sacubitril was 0.7718 μCi/mg (≈75 μCi/(200 mg × 411.5/847)) after adjusting for the differences in molecular weights of LCZ696 and sacubitril. The molecular weights of LCZ696, sacubitril and valsartan are 847, 411.5 and 435.5, respectively. The parent batch of radiolabeled drug substance (high specific activity) was prepared by the Novartis Isotope Laboratory and the analysis was performed by the Novartis Isotope Laboratory and Technical Research and Development (East Hanover, NJ).

Study design

Four male, nonsmoking Caucasian subjects, aged 23–30 years and in good health as determined by their past medical history, physical examination, vital signs, electrocardiogram and laboratory tests at screening, participated in this open-label study. The study was approved by the ethics committee and conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki (1964 and subsequent revisions), and all subjects gave written informed consent before participation.

Subjects were screened over a period of 21 days, prior to dosing continuing up until two days prior to dosing. Baseline assessments for safety and eligibility were conducted 16 h prior to the first dosing period. The planned duration spanned seven days, during which all subjects remained domiciled at the clinical site. Mass balance was considered complete if by the end of the seven day period >85% of the administered radioactive dose was recovered via feces and urine collection. An earlier release was also permitted, should the appropriate recovery (>85%) be achieved prior to the planned end of the study. This was left to the discretion of the study director.

Study medication

All the subjects received a single oral dose of 200 mg [14C]LCZ696 containing a mean radioactivity of 75 μCi. The radioactive dose was 2.78 MBq (75 μCi), was considered sufficient for analytical sensitivity to perform the required analyses in the collected samples. The whole body committed effective dose (CED) was estimated to be ≤0.89 mSv, which was lower than the recommended dose limits for the public of 1 mSv per year and was in compliance with the International Commission on Radiological Protection (ICRP) as a minimal risk.

In the determination of the CED certain assumptions were made based on the recommendations in the 2007 Recommendations of the International Commission on Radiological Protection ICRP103 (Valentin, 2007). We assumed the levels of uptake and retention by tissues will be the same in man as in the experimental animals. We took data from the whole body radiography study where rat organs were extrapolated to human organs on the basis of (1) the known human plasma levels of LBQ657 (primary metabolite of labeled moiety of LCZ696), (2) the observed ratio of 14C to LBQ657 in animals, (3) the ratio of 14C in individual organs to 14C in plasma in rats, (4) LBQ657 was major circulation component in plasma (>80% based on AUC) in vivo and (5) LBQ657 was the only major metabolite in the liver (rat, dog, monkey and human) slices incubations. The whole body CED (mSv) is then determined from the individual organ and tissue equivalent doses HT (human equivalent dose) and Wt (weighting factor) according to the equation CED = ΣWt × HT (man) as provided in ICRP 103. Only a standard list of mandatory 12 organs with defined weighting factors is taken into account, plus one additional organ, which exhibits a substantial exposure in excess of the highest dose in any of the 12 organs. Other organs were excluded.

Sample collection

Serial blood samples were collected to assess LCZ696 pharmacokinetics and total radioactivity over a seven-day period at pre-dose, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h following the end of the oral dose. Blood samples collected in BD (Franklin lakes, NJ) Vacutainer™ tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant, and plasma was prepared by centrifugation within 30 min of blood collection. Urine collection was collected and pooled over a period of 8 days prior to dosing to determine a baseline. Following pre-dose collections pools were collected between 0–4, 4–8, 8–12, 12–24, 24–48 and every 24 h post-dose for seven consecutive days. Fecal samples, including used toilet paper, were collected pre-dose and in 24 h intervals after administration of [14C]LCZ696 for up to seven days. All samples were stored.
at 20°C or –80°C. Measures were taken to protect samples from light exposure during collection, handling, storage and sample analysis.

**Determination of radioactivity and sample preparation for characterization of LCZ696 metabolites**

Measurement of total radioactivity in plasma, blood, feces and urine was performed using liquid scintillation counting (LSC). LSC for plasma, urine and other clear samples used Flo-Scint II (PerkinElmer, Inc., Waltham, MA) scintillation fluid. Whole blood and fecal samples were combusted before LSC (Packard Tri-Carb 306 sample oxidizer, Carbo-Sorb CO₂ absorbing fluid, and Permafluor E+ scintillation fluid). For extracts, extraction efficiency was calculated based on the total radioactivity in the original aliquot and the resulting extract.

**Plasma:** For metabolite profiling, equal volumes of plasma from each subject per time point were extracted with 2–3 volumes of acidified acetonitrile. Samples were vortex mixed, sonicated and centrifuged at 3500 rpm (~1000 g) for 5 min at 10°C. The supernatant was concentrated to dryness under nitrogen. The residue was reconstituted with 200 μL of water/acetonitrile with 0.1% formic acid (1/1, v/v), vortex mixed and centrifuged. An aliquot of 20 μL was used to determine the radioactivity by LSC. A 75 μL aliquot was analyzed by high performance liquid chromatography (HPLC) to obtain the metabolite profile.

**Urine:** Frozen urine samples were thawed at room temperature and centrifuged at 3000 rpm for 5 min. Duplicate (0.2 mL) aliquots of urine were placed into separate scintillation vials containing 10 mL of cocktail and measured by LSC. A pooled urine sample was prepared from each subject by combining approximately 2.5 (%v/v) aliquots from each subject at pooled intervals of 0–48 h to allow consistent comparisons of metabolite profiles. Aliquots for the urine analysis were taken at pooled intervals of 0–48 h to allow consistent comparisons of urine profiles. The aliquots were then centrifuged at 3500 rpm (~1000 g) for 10 min at 10°C. An aliquot of 20 μL of each from the original 200 μL was injected on to an HPLC system with offline radioactivity detection to generate the urine chromatographic profiles.

**Feces:** For metabolite profiles, aliquots of fecal samples from each subject were pooled to encompass ≥90% of the total fecal radioactivity excretion. This was expected to be representative of the original sample. Each pooled sample (~1–2 g) was then extracted with 10 mL of 1:1 acetonitrile:methanol, vortex mixed, followed by centrifugation at 3500 rpm and 10°C for 10 min. Two extracts were taken and combined prior to concentrating them to dryness on a Zymark Turbo-Vap LV (Hopkinton, MA). The residue was reconstituted in 5 mL of 1:1 water:acetonitrile, of which 50 μL aliquots were injected onto the HPLC system with off-line radioactivity detection to generate the feces chromatographic profiles.

**Analysis of LCZ696 (sacubitril, LBQ657 and valsartan) concentrations in plasma**

The sample preparation was performed using liquid–liquid extraction with incorporation of a Quadra 96 Tomtec system for solvent transfers. An aliquot of spiking solution in methanol/water (50/50, v/v) was added to human plasma (treated with EDTA) to make the standard and QC pools. A 100 μL aliquot of each plasma sample (standard, QC and study sample) was added to a 96-well plate. A 50 μL aliquot of internal standard solution (475/495/500 ng/mL [14C]-sacubitril/[13C4]-LBQ657/D9-valsartan in methanol/water, 50/50, v/v) was added to each well, except for human plasma blanks, and the plate was vortex mixed briefly. To each well, 50 μL of 0.5 N HCL was added followed by mixing. A 500 μL aliquot of MTBE was added followed by vortex mixing for approximately 5 min. The plate was centrifuged at 3500 rpm (approximately 2000 g) for 5 min at approximately 10°C. A 250 μL aliquot of the organic layer was transferred to a separate 96-well plate. The organic layer was evaporated to dryness under nitrogen at 40°C. The extract was reconstituted with 200 μL of methanol:water:formic acid (10:90:0.1, v/v/v). A 5–15 μL aliquot was injected onto the LC–MS/MS system.

Valsartan was monitored by the 436.3 → 235.0 m/z transition, while internal standard D9-valsartan was monitored by the m/z 445.3 to m/z 235.0 transition. Sacubitril was monitored by the 412.2 m/z to 266.1 m/z transition, while internal standard [13C4]-sacubitril was monitored by the m/z 416.2 to m/z 266.1 transition. In addition, the active metabolite LBQ657 was monitored by the 384.1 m/z to 266.1 m/z transition, while internal standards [13C4]-LBQ657 was monitored by the m/z 388.1 to m/z 266.1 transition. Instrument responses for the three analytes were calculated by dividing the intensity (peak area) of their mass spectrometric responses by the intensity of the mass spectrometric response of the respective internal standard. Calibration curves for the analytes were constructed by plotting the instrument responses of the calibration standards verses their nominal concentrations (μg/mL). The calibration curves were fit to the instrument response × concentration using a quadratic regression with 1/x² weighing. The lower limit of quantification (LLOQ) for each analyte was 1 ng/mL, using 50 μL of human plasma. Concentrations below the LLOQ were reported as 0 ng/mL.

**Metabolite profiling analysis by HPLC**

[14C]LCZ696 (sacubitril component) and its metabolites in the plasma and excreta were analyzed by HPLC with off-line radioactivity detection. A Waters Acquity UPLC system (Milford, MA) was used. The chromatographic separations were performed on a MAC-MOD ACE-5 AQ column 150 × 4.6 mm, 5 μm (Chadls Ford, PA) maintained at a temperature of 30°C. Sacubitril and its metabolites were resolved with gradient elution consisting of solvent A (5 mM ammonium formate with 0.1% formic acid, pH ~3) and B (acetonitrile with 0.1% formic acid), at a flow rate of 0.5 mL/min. The gradient elution program was as follows (all steps were linear): 0–5 min, 10% solvent B, 5–35 min, 10–80% solvent B, 35–40 min, hold at 10% solvent B, 40–45 min, 80–10% solvent B, 45–55 min, 10% solvent B, for re-equilibration. Chromatograms were evaluated using the Laura 4 radiochromatography software version 4.0275 (LabLogic, Sheffield, England).
After the injection of plasma, urinary or fecal extracts, column eluant from the analysis of the extracts was split (ratio 4:1) to deliver 400 μL/min-1 for collection into LumaPlates at a rate of 0.14 min/well or to waste and ~100 μL min-1 to a mass spectrometer. LCZ696 (sacubitril component) and its metabolites were analyzed by electrospray in positive ionization mode. Metabolites in excreta extracts were characterized by full scan MS, MS/MS and selected reaction monitoring (SRM) analyses. The LumaPlates were sealed after drying and counted for 15 min per well on a Packard TopCount NXT microplate scintillation counter (Downer’s Grove, IL).

Metabolite identification by liquid chromatography/mass spectrometry
Metabolites were identified by liquid chromatography–mass spectrometry (LC–MS and LC MS/MS) on an LTQ-Orbitrap hybrid mass spectrometer (with high resolution and accurate mass capabilities) interfaced with a Waters Acquity separation module which included an autosampler and solvent delivery system. Qualitative analyses (metabolite structure identification) were carried out using electrospray ionization (ESI) in the positive ion mode. The heated capillary temperature was 275 °C, the normalized collision energy was 35%, the resolution was 30 000 with an isolation window of 3 Da, the sheath gas flow rate was 60 arbitrary units and auxiliary gas flow rate was 30 arbitrary units. The ion spray voltage, the capillary voltage and tube lens offset were adjusted to achieve maximum sensitivity using the parent ion. The HPLC separation method was identical to that used for metabolite profiling. The flow rate was 1.0 mL/min with a 1:4 split to the mass spectrometer ion source and 96-well LumaPlates. The MS/MS spectra were obtained by high-energy collision-induced dissociation (HCD) of [M+H]+.

Pharmacokinetic analyses and statistics
All the subjects were included in the pharmacokinetic data analysis. Descriptive statistics was provided for the pharmacokinetic data and mean concentrations versus time plots were prepared for all the relevant data. Concentrations were given in mass per volume units. Missing values or those below the limit of quantification were indicated in the data listings and treated as zero in data presentations and calculations. Conventional pharmacokinetic parameters were determined from individual plasma concentration versus time data using non-compartmental method(s) with WinNonlin Pro (Version 5.2); these include maximum concentration (Cmax), time to maximum concentration (Tmax), elimination half-life (T1/2), area-under-the-curve from time zero to infinity (AUCinf), volume of distribution (Vd) and clearance (CL). No formal statistical analysis was done due to small subject size.

In vitro inhibition and induction of cytochrome P450 (CYP) enzymes
The ability of sacubitril and its metabolite, LBQ657, to inhibit CYP enzyme activity was assessed using pooled human liver microsomes (n = 50 donors, mixed gender; Xenotech, LLC, Lenexa, KS). To determine individual CYP activities, several probe substrate reactions were used that are known to be CYP enzyme-selective. The reactions used and corresponding probe substrate concentrations included phenacetin O-deethylation (5 μM, CYP1A2), paclitaxel 6x-hydroxylation (10 μM, CYP2C8), diclofenac 4’-hydroxylation (5 μM, CYP2C9), S-mephenytoin 4’-hydroxylation (15 μM, CYP2C19), bufuralol 1’-hydroxylation (5 μM, CYP2D6), clorozazone 6-hydroxylation (10 μM, CYP2E1), midazolam 1’-hydroxylation (5 μM, CYP3A4/5) and 6β-hydroxytestosterone formation (25 μM, CYP3A4/5). The probe concentrations used were less than or approximately equal to their reported Km values. Increasing concentrations of sacubitril or LBQ657 (up to 100 μM) were incubated at 37 °C individually with human liver microsomes (0.2 or 0.5 mg microsomal protein/mL) and one probe substrate in 100 mM potassium phosphate buffer, pH 7.4, 1 mM NADPH, 5 mM ΜgCl2, 1 mM EDTA and 0.2% DMSO (final concentrations). After incubation, the reactions were quenched by the addition of acetonitrile. Probe substrate turnover was determined by LC/MS/MS analyses of metabolite formation (AB Sciex API4000 mass spectrometer; Applied Biosystems, Foster City, CA). Reference standards for probe metabolites were obtained from commercial sources as follows: acetaminophen and 6β-hydroxytestosterone (Sigma-Aldrich, St. Louis, MO); 1’-hydroxybufuralol, 6-hydroxychlorozazone, 4’-hydroxy-S-mephenytoin and 1’-hydroxymidazolam (Ultrafine Chemicals, Manchester, UK); and 6-hydroxyplacetaxel and 4’-hydroxydiclofenac (BD Biosciences, San Jose, CA). Chromatographic separation was achieved using an Agilent Technologies (Piscataway, NJ) Zorbax SB-C18 (50 × 2.1 mm, 4 μm) column. The column temperature was ambient. The mobile phase consisted of two solvents: A = 5 mM ammonium acetate containing 0.1% acetic acid (pH ~4.5) and B = 90/10 (acetonitrile/methanol (1/1, v/v))/water containing 0.1% acetic acid. Probe metabolites derived from phenacitin, midazolam, bufuralol and paclitaxel were analyzed using ESI in positive ion mode, whereas the metabolites of the remaining probes (diclofenac, chlorozazone and S-mephenytoin) were analyzed in negative ion mode. The gradient elution program (%B) for the positive mode ion analytes was 0 → 1 min (5%), 1 → 2 min (5%), 2 → 3 min (5–95%), 3 → 4.5 min (95%), 4.5 → 4.8 min (95–5%) and for negative mode ion analytes was 0 → 1 min (10%), 1 → 3 min (10–95%), 3 → 4.5 min (95%), 4.5 → 4.8 min (95–10%). Sacubitril was found to be relatively metabolically stable under the incubation assay conditions with ≥60% remaining at the end of the incubation periods. LBQ657 was stable with little or no loss of parent compound in these studies (data not shown).

To assess induction of CYP enzymes by sacubitril or valsartan, cryopreserved plateable primary human hepatocytes (In Vitro Technologies, Baltimore, MD) were plated onto collagen coated plates with no overlay (7 × 105 viable cells per mL). The cells were re-fed the day after plating with Complete Plating Media: InVitroGRO™ CP (plating) medium containing antibiotics (1 mL Torpedo Antibiotic mix per 45 mL media) and incubated for an additional ~24 h at 37 °C (5% CO2/95% air) prior to the induction treatment. The hepatocytes were treated with various concentrations of sacubitril (10, 50 or 100 μM), valsartan (1, 10, 25, 50 or 100 μM) or the positive controls, rifampin (rifampin, 5 μM) and indomethacin (5 μM) (IC50 of each). This was done in order to determine the appropriate concentration to be used in the microsome experiments.
10 μM), phenobarbital (PB, 1 mM), β-naphthoflavone (BNF, 10 μM) or omeprazole (OMP, 50 μM); or the vehicle control, (0.1% DMSO, final concentration), all of which were diluted in InVitroGRO™ HI (incubation) medium containing antibiotics as described above. Plates were treated identically for assessments of cell viability, mRNA isolation and measurement of CYP activity in situ. After the treatment period, the cells were assessed for cell viability using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (In vitro toxicology assay kit, Sigma-Aldrich). Relative quantification of human CYP mRNA was determined by real-time polymerase chain reaction (PCR) using the Comparative C_T Method (ABI Prism 7900 Sequence Detection System). CYP activity was measured in the cells after the treatment period. Briefly, after the induction treatment, the hepatocytes were washed once with InVitroGRO™ HI (incubation) media and pre-incubated in fresh media for 1 h at 37 °C. The media was aspirated and the cells were treated with CYP-selective probe substrates (200 μM phenacetin for CYP1A2, 400 μM bupropion for CYP2B6, 100 μM diclofenac for CYP2C9 and 100 μM midazolam for CYP3A) in the same media for 30 min. The reactions were quenched by the addition of an equal volume of 100% methanol to the wells and samples were transferred to wells of a 96-well plate. The cells in the wells were lysed and protein solubilized with 1 N sodium hydroxide at 70 °C for ~3h for protein concentration determination (Biorad protein assay, Biorad Laboratories, Hercules, CA). The media samples were evaporated in a 96-well evaporator and reconstituted in 200 μL of 20/800 (v/v) methanol/water containing internal standards. The sample plate was centrifuged and an aliquot (10 μL) was analyzed by LC–MS in the positive ion mode. Quantitative analysis of probe substrate metabolites was performed using LC/MS/MS essentially as described above for the CYP inhibition experiment using a AB Sciea QTrap 5500 mass spectrometer and chromatographic separation with a Phenomenex Synergi Polar-RP 80A column (50 × 2.0 mm, 4 μm, Torrance, CA). The column temperature was 30 °C. Solvents A and B were as described above. Probe metabolites derived from phenacetin, bupropion and midazolam, were analyzed using ESI in positive ion mode. The HPLC gradient elution conditions were either similar to those above or if all probe substrate metabolites were analyzed in the positive ion mode the following elution conditions were used: 0 → 1 min (5%), 1 → 2.5 min (5–95%), 2.5 → 4.5 min (95%), 4.5 → 4.8 min (95–5%), 4.8 → 6 min (5%).

The overall methods and materials were performed in accordance with typical Novartis procedures as similarly described in other published works (Dhuria et al., 2008; He et al., 2009; McKeage et al., 2012; Nodaa et al., 1996).

Results

Four male volunteers were enrolled with a mean age of 25.8 years (ranging from 23–30 years of age). No adverse events or clinically significant changes in vital signs, clinical chemistry, hematology or urinalysis were observed during the course of the study.

Dose administered

The administered dose (planned was 75 μCi or 2.78 MBq, actual was 79.0 μCi or 2.92 MBq) was derived from the weighted individual amounts (mg) of drug substance from the specific radioactivity (MBq/mg or μCi/mg) determined by the Novartis Isotope Laboratory (East Hanover, NJ).

Blood and plasma concentrations of radioactivity

Mean blood and plasma concentrations of radioactivity and key pharmacokinetic parameters are summarized in Table 1. The mean AUC_0–∞ of blood and plasma radioactivity was 44 529 ngEq/h/mL and 93 784 ngEq/h/mL, respectively. The mean C_{max} (at ~2 h post-dose) of radioactivity in blood and plasma was 5231 ngEq/mL and 10 858 ngEq/mL, respectively. The mean (range) of apparent terminal half-life of the total radioactivity was 10.1 (7.29–12) h in blood and 8.11 (7.16–9.24) h in plasma. Extraction recovery of radioactivity in plasma was 72–81%.

Plasma LCZ696 (valsartan and sacubitril) concentrations

Valsartan, sacubitril and its metabolite LBQ657 were the major drug-related circulating components in plasma, accounting for >95% of the radioactivity up to 24 h post-dose. Concentrations of valsartan, sacubitril and LBQ657 were quantified by LC–MS/MS and concentrations of

Table 1. Pharmacokinetic parameters of total radioactivity, valsartan, sacubitril and active metabolite LBQ657 in plasma following an oral administration of 200 mg [14C]LCZ696.

| Matrix pk parameter | Blood Radioactivity | Plasma b Radioactivity |
|---------------------|---------------------|------------------------|
|                     |                     |                        |
| T_{max} (h) a       | 2 (1.5–2)           | 2 (1–2)                |
| C_{max} (ng/mL or ngEq/mL) | 5231 ± 985       | 10 858 ± 1726          |
| AUC_{0–∞} (ng/mL or ngEq/mL) | 44 529 ± 9768     | 93 784 ± 16 437       |
| T_{1/2} (h)         | 10.1 ± 2.29        | 8.11 ± 1.04            |
| CL/F (L/h)         | NA                  | 4.22 ± 1.92            |
| Vz/F (L)           | NA                  | 101 ± 77.4             |

aMedian (range), NA: not applicable.

bRadioactivity was determined by scintillation counting and the concentrations of valsartan, sacubitril and M1(LBQ657) were quantified by LC–MS/MS.

CConcentration is expressed in ng equivalent/mL for radioactivity and ng/mL for valsartan, sacubitril and LBQ657.

dExposure is expressed in ng equivalent * h/mL for radioactivity and ng/h/mL for valsartan, sacubitril and LBQ657.
sacubitril and LBQ657 were also determined by radioactivity. In Figure 2, the cumulative recovery of radioactivity in urine and feces, expressed as % of dose, was plotted against the collection period (up to 7 days). Mean total recovery of radioactivity was 60.7% in urine and 41.8% in feces. As expected, concentration of sacubitril in plasma declined rapidly and concentration of the active metabolite LBQ657 increased concurrently. The concentration measurement by LC–MS/MS was nearly indistinguishable with that obtained by radioactivity.

The plasma clearance (CL/F) for valsartan and sacubitril was \(4.2 \pm 1.2\) and \(49.4 \pm 12.3\) L/h; mean \(C_{\text{max}}\) and mean AUC\(_{0-\infty}\) values for plasma valsartan, sacubitril and LBQ657 were 5360, 2115, 8623 ng/mL and 26913, 2561 and 71420 ng/h/mL, respectively. The terminal plasma half-lives of valsartan, sacubitril and LBQ657 were 20.8, 1.31 and 12 h (Table 1), respectively.

Excretion and mass balance in urine and feces

Overall, mass balance was considered complete in all the subjects. The cumulative excretion of radioactivity in urine and feces is shown in Figure 2. These plots indicate that excretion by both routes was complete by the last collection, consistent with the good mass balances values observed.

The mean contributions of the urinary and fecal routes to the overall elimination of total radioactivity were 60.7% and 41.8% of the administered dose, respectively. Mean extraction recovery for urine and feces averaged 77% and 90%, respectively.

Metabolism of LCZ696

Plasma profile

As expected, LBQ657 was the major component in plasma, accounting for 76.8% of total AUC. Sacubitril was only detectable at early time points (0.5 and 2 h) and accounted for 2.6% of total AUC. Two minor oxidative metabolites (M6 and M7) were also identified. Figure 3 shows the semi-log plots of sacubitril, LBQ657 and valsartan for both radioactivity and LC/MS concentrations. All three analytes give similar concentrations with both data sets. Although the LC/MS is more sensitive and providing more data points, a remarkable correlation between the two measurement techniques can be observed. Figure 4 shows representative radiochromatograms of metabolite profiles for subject 5101 at 0.5, 2 and 24 h. At 2 h, we see almost a complete conversion to LBQ657 was observed.

The pk data was generated with validated methods for valsartan, sacubitril and LBQ657. The LLOQ for the LCZ696 measurement was 1 ng/mL. Calibration standard responses were linear over the range of 1.00–5000 and 10–5000 ng/mL for LBQ657. A weighted \((l/c)\) under a quadratic calibration model was used. In plasma for valsartan, the interday assay accuracy, expressed as percent relative error for quality control (QC) concentrations, ranged from –11.2 to 0% in QC samples. Assay precision, expressed as the interday percent coefficients of variation (% CV) of the mean estimated concentrations of QC samples ranged from 2.5 to 11.4%. For sacubitril, the interday assay accuracy, ranged from –7.7 to 0.5% for QC samples. Interday assay precision, of the mean estimated concentrations of QC samples ranged from 2.8 to 10.9%. Similarly, for LBQ657 interday assay accuracy ranged from 0 to 14.8%. Interday assay precision ranged from 4.3 to 9.9%.

In all four subjects, the amount of the radioactivity excreted in urine ranged from 51.7 to 67.8% of the LCZ696 dose; the renal route was therefore identified as the primary route of excretion for parent and metabolites combined. The amount of
Figure 4. Representative metabolic profiles in human plasma following a single oral dose at 200 mg.
sacubitril recovered in the urine ranged from $\sim$0.82 to 2.82% of the dose, and renal clearance for sacubitril was judged moderate to high as a result.

In feces, the amount of radioactivity excreted ranged from 36.9 to 48.3% of dose. Sacubitril ranged from 0.1 to 0.96% with LBQ657 ranging from 31.6 to 41.5% for the fecal radioactivity across all four subjects. Excluding LBQ657, all other metabolites were below 1%. Figure 5 shows representative radiochromatograms of metabolite profiles for subject 5101 of the 0–24 h pooled urine and 0–96 h pooled feces samples. The LBQ657 is the predominant species in both the matrices.

Table 2 shows several proposed trace metabolites detected at low levels in urine and feces. These low level suspected metabolites seem to be the result of oxygenation, glucuronidation and combinations of both biotransformations. See Figure 6 for the biotransformation scheme. In general, the dominant ions in the product ion mass spectra of sacubitril and corresponding metabolites were associated with fragmentation about the same sacubitril moiety. These fragment ions were used to assign the ester hydrolysis, oxygenation and glucuronidation conjugation products. The characteristic ions and structures are shown in Table 2.

**Inhibition and induction of CYP enzymes by LCZ696**

Sacubitril showed little or no in vitro inhibition of CYP enzyme activities ($\text{IC}_{50}$ values $>100 \mu\text{M}$) for CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4/5. Sacubitril inhibited CYP2C8 and CYP2C19 activities with $\text{IC}_{50}$ values of $\sim$15 and $\sim$20 $\mu\text{M}$, respectively. Of the CYP enzymes examined, LBQ657 only inhibited CYP2C9 with an $\text{IC}_{50}$ of $\sim$40 $\mu\text{M}$. As shown in Figures 7 and 8, treatment of human hepatocytes with sacubitril (10–100 $\mu\text{M}$) or valsartan (1–100 $\mu\text{M}$) did not result in induction of CYP1A2, CYP2B6, CYP2C9 or CYP3A4 mRNA or activity (induction levels were $<2$-fold) in the three donor hepatocytes (except one donor outlier for valsartan on CYP1A2 activity but no induction at mRNA level). Sacubitril was converted to its active metabolite, LBQ657 by $>50\%$ at 4 h incubation time point and by 100% at 24 h incubation time point (data not shown).
Table 2. Structures of LCZ696 metabolites.

| Peak ID Matrix                  | Measured mass [M + H]^+ (relative error) | Structure |
|--------------------------------|------------------------------------------|-----------|
| Sacubitril                      | 412.2118 (0.00 ppm)                       | ![Structure Diagram](image1) |
| LBQ657                          | 384.1807 (0.52 ppm)                       | ![Structure Diagram](image2) |
| Trace metabolite M6             | 400.1754 (−0.25 ppm)                      | ![Structure Diagram](image3) |
| Trace metabolite M7             | 400.1756 (0.25 ppm)                       | ![Structure Diagram](image4) |
| Trace metabolite M2             | 428.2065 (−0.700 ppm)                     | ![Structure Diagram](image5) |
| Trace metabolites M3            | 588.2439 (0.00 ppm)                       | ![Structure Diagram](image6) |

(continued)
| Peak ID Matrix   | Measured mass \([M + H]^+\) (relative error) | Structure |
|------------------|---------------------------------------------|-----------|
| Trace metabolite M8 | 400.1753/−0.500 ppm | ![Structure](image1) |
| Urine            | 400.1753/−0.500 ppm | ![Structure](image2) |
| Trace metabolite M4 | 560.2120/1.071 ppm | ![Structure](image3) |
| Urine            | 560.2120/1.071 ppm | ![Structure](image4) |
| Trace metabolite M5 | 560.2123/−0.535 ppm | ![Structure](image5) |
| Urine            | 560.2123/−0.535 ppm | ![Structure](image6) |
| Trace metabolite M9 | 604.2391/0.331 ppm | ![Structure](image7) |
| Urine            | 604.2391/0.331 ppm | ![Structure](image8) |
| Valsartan*       | 436.23401/0.633 ppm | ![Structure](image9) |

| Compound   | Diagnostic fragment ions$^b$ |
|------------|------------------------------|
| Sacubitril | 412, 394, 366, 348, 320, 266, 249, 221, 193 |
| M1(LBQ657) | 384, 366, 348, 320, 266, 249, 221, 193 |
| M2         | 428, 410, 382, 364, 294, 282, 265, 247, 209 |
| M3         | 588, 570, 412, 394, 348, 320, 266, 249, 221, 193 |
| M4         | 560, 542, 384, 366, 348, 266, 249, 221, 193 |
| M5         | 560, 542, 384, 366, 348, 320, 266, 249, 221, 193 |
| M6         | 400, 382, 364, 336, 282, 247, 237, 209 |
| M7         | 400, 382, 364, 336, 282, 237 |
| M8         | 400, 382, 364, 336, 282, 265, 247, 237, 209 |
| M9         | 604, 428, 410, 382, 364, 282, 265, 247, 237, 209 |

$^a$Valsartan was not a metabolite and was measured during analysis; it is included only for reference purposes.
$^b$The most abundant fragment ions are highlighted in bold.
Discussion

After the administration of a single oral 200 mg (79.0 µCi) dose of [14C]-radiolabelled LCZ696 200 mg to four healthy male subjects, recovery was complete and was safe and well tolerated. Renal excretion was the dominant route of elimination of LCZ696 in this study. Urine accounted for 51.7–67.8% and feces 36.9–48.3% of the total radioactivity. The majority of the drug was excreted in the urine as LBQ657 metabolite. Similarly, a significant portion of the radioactivity was also excreted in the feces in the LBQ657 form; LBQ657 accounted for ~85.5% of the dose.

The pk parameters in our hADME study (as captured in Table 1) correlated very well with single and ascending dose data (in-house data). Peak plasma concentrations for sacubitril were reached within 1–2 h indicating relatively fast absorption. The mean terminal half-life was short at 1.31 h and the mean plasma oral clearance (CL/F) was moderate at 49.4 L/h. The majority of radioactivity in plasma was LBQ657 (~76% of total AUC). Investigation of the biotransformation pathways...
Figure 7. Levels of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 mRNA (A) and activities (B) with respect to the vehicle control were measured after sacubitril or positive control treatment of human hepatocytes.
Figure 8. Levels of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 mRNA (A) and activities (B) with respect to the vehicle control were measured after valsartan or positive control treatment of human hepatocytes.
revealed that elimination was primarily in the form of the ester hydrolysis product of sacubitril, LBQ657. There was a minimal elimination in the form of M6 and M7 two trace oxygenated metabolites of LBQ657. Several other minor metabolites were detected in urine corresponding to mono-oxygenation, glucuronidation and combinations of both.

Sacubitril in the mouse showed some similarities to human in terms of metabolism, but had clear differences with regard to excretion. Specifically, in the mouse sacubitril was rapidly converted to the active metabolite LBQ657 via ester hydrolysis and LBQ657 was the major circulating component in plasma, accounting for \( \sim 72.6\% \) of total AUC following oral dose. Unlike human, the major route of excretion of radioactivity was via fecal pathway (\( \sim 98\% \) of the dose). Urinary excretion of radioactivity (\( \sim 5–9\% \)) played a minor role by either dosing route. Recovery of radioactivity in excreta from mouse was complete within 168 h post-dose for p.o. administrations. LBQ657 was the major component in feces (\( \sim 96\% \) of the dose) and urine (\( \sim 5–8\% \) of the dose) by the p.o. dosing route. The major radiolabeled component in urine and feces was LBQ657. Several minor metabolites derived from hydroxylation, glucuronidation, sulfation, glycine and taurine conjugation were identified in feces and urine, each accounting for less than 1% of the dose.

In monkeys, following oral dosing of \( ^{14}\)LCZ696, the radioactivity was approximately equally excreted via feces (\( \sim 48\% \) of the dose) and urine (\( \sim 42\% \) of the dose). Total mean recovery of radioactivity in the excreta from monkeys was good (\( \sim 90\% \) of the dose) within 168 h post-dose. LCZ696 delivered valsartan and sacubitril in monkey plasma following oral dosing with sacubitril further converted to the active metabolite LBQ657 via ester hydrolysis. Both LBQ657 and sacubitril were the prominent circulating components in plasma, accounting for \( \sim 63\% \) and \( \sim 36\% \) of total AUC, respectively. The rate of conversion from sacubitril to LBQ657 was moderate in monkey following oral dosing of LCZ696. The major radiolabeled component was LBQ657 in urine and feces, accounting for \( \sim 29\% \) and \( \sim 41\% \) of the oral dose, respectively. Unchanged sacubitril was a minor component in urine and feces, accounting for \( \sim 1\% \) and 3% of the oral dose. The recovery of LBQ657 and sacubitril in urine might be underestimated because \( \sim 9–11\% \) of the i.v. or p.o. dose was recovered in cage wash and the samples were not analyzed. Similar to the mouse several minor metabolites derived from oxidation and conjugations were also identified in urine and feces, each accounting for less than 1% of the dose.

Considering the preclinical monkey ADME data, the AUC of the LBQ657 (LC/MS) ratio to total plasma radioactivity (radioactivity detection) correlates well to the human ratios at \( \sim 0.75 \). This is consistent with the observation that the LBQ657 was the major metabolite observed in human plasma as well as in monkey plasma. We see the plasma radioactivity was approximately two times higher than the blood. This supports the premise that the radioactivity distributes primarily to the plasma compartment rather than blood cells. In an independent plasma binding study, sacubitril plasma protein binding was greater in human (96.7 \( \pm \) 0.5%) than in monkey (91.3 \( \pm \) 0.8%) and appeared to be linear over the concentration range tested. Plasma protein binding can be characterized as being high in both species.

In rat, a dose of \( ^{14}\)C sacubitril showed the majority was recovered in the feces (74–91%) with a minor amount (9–24%) in the urine. The total radioactivity recovered in excreta within 168 h post-dose. The major circulating component in blood following both routes of administration was LBQ657, accounting for \( \geq 80\% \) of the total AUC \( \Delta \). Approximately 87% of the dose was excreted as LBQ657 in the feces and 7% was excreted in the urine. The primary biotransformation reaction, hydrolysis of the sacubitril ethyl ester group to form the dicarboxylic acid metabolite LBQ657, was again observed. Four additional minor oxidative metabolites of LBQ657 were also detected in feces (<1% of dose each). (see supplemental animal data for more information) A more detailed treatment of the nonclinical data is being prepared for an upcoming publication in this journal.

Sacubitril and LBQ657 showed very little potential to inhibit CYP enzyme activities. Of the CYP enzymes examined, only weak inhibition of CYP2C8 and CYP2C19 activities by sacubitril and of CYP2C9 by LBQ657 were found. Given the low unbound maximal plasma concentrations (<2 \( \mu \)M) of sacubitril and LBQ657, it is unlikely that sacubitril or LBQ657 would inhibit the metabolic clearance of medications metabolized by CYP enzymes. In a previous published study, valsartan was not found to be an inhibitor of CYP enzymes CYP1A2, CYP2A6, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 to any significant extent. It marginally inhibited CYP2C9 with a relatively high \( K_i \) value (135 \( \mu \)M) (Taavitsainen et al., 2000). Therefore, it is also unlikely to inhibit CYP enzymes \( \text{in vivo} \). In addition, both sacubitril (which also included LBQ657 in the incubation over time) and valsartan were also shown to have low CYP induction potential.

In summary, orally administered LCZ696 was well tolerated with LBQ657, the ester hydrolyzed product of sacubitril being the major single component in plasma. The LBQ657 metabolite is excreted \( \text{via} \) urine and feces, with renal excretion being the more dominant route of elimination. Traces of other extremely low level metabolites were detected at less than 1%. The potential for clinical drug-drug interactions (DDI) with respect to CYP inhibition or induction is deemed low.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.
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Supplementary material available online

Supplementary information