Mass balance, metabolic disposition, and pharmacokinetics of a single oral dose of regorafenib in healthy human subjects

Authors
Michael Gerisch¹, Frank-Thorsten Hafner¹, Dieter Lang¹, Martin Radtke¹, Konstanze Diefenbach², Adriaan Cleton², John Lettieri³

Affiliations
¹Bayer AG, Wuppertal, Germany
²Bayer AG, Berlin, Germany
³Bayer Healthcare Pharmaceuticals, Whippany, NJ, USA

Corresponding author
Adriaan Cleton
Clinical PK ONC, Bayer AG, 13353 Berlin, Germany
Tel: +49 304 6819 2867
Email: Adriaan.Cleton@bayer.com
Appendix A: Isolation of Metabolites M-7 and M-8

Pooled urine (20 mL; 5 mL each from subject 001–004) from interval 0–12 h was applied onto solid-phase extraction (SPE) (Oasis HLB, 1 g, 20 mL, Waters, Eschborn, Germany). The cartridge was washed successively with water (10 mL), methanol/water mixtures (10 mL, 3:97, v/v; 10 mL, 1:9, v/v; 10 mL, 2:8, v/v, 10 mL, 3:7, v/v, 10 mL, 1:1, v/v, 10 mL, 9:1, v/v) and acetonitrile (10 mL) resulting in nine fractions. After radioactivity analysis, fractions 1–7 were discarded. Fractions 8 and 9 were pooled, concentrated to about 0.3 mL, and forwarded to preparative HPLC (Phenomenex Prodigy 3 ODS(3) column, 150 × 3 mm, 3 μm, ser. no. 522228-1) using phosphate buffer (2 g/L, pH 2.0) as mobile phase A and acetonitrile as mobile phase B. The chromatography was performed in a total of three runs (0.1 mL of the concentrated aqueous solution, each injected for chromatographic separation) using a gradient elution at a flow rate of 0.5 mL/min, starting with A:B (90:10), gradient to A:B (50:50, 0–10 min), gradient to A:B (45:55, 10–15 min), gradient to A:B (10:90, 15–16 min), and isocratic A:B (10:90, 16–20 min) at a flow rate of 0.5 mL/min and at a detection wavelength of 265 nm using DAD and a column temperature of 45°C. The first preparative run was divided into eight fractions. Runs 2 and 3 were divided into three fractions. Fraction 6 from the first run and fraction 3 from runs 2 and 3 were pooled and concentrated under vacuum to about 1 mL (M-7). Fraction 1 from runs 2 and 3 were pooled and concentrated under vacuum to about 1 mL (M-8). The pooled extracts were applied onto SPE for desalination (Oasis HLB, 0.1 g, 1 mL). The cartridges were washed with water (1 mL) and methanol (1 mL). Finally, about 10 μg of M-7 and M-8 was dissolved in DMSO-d6 and analyzed by NMR spectrometry.
Appendix B: HPLC Chromatogram after incubation of $[^{14}\text{C}]$regorafenib in human hepatocytes (2 µM, 2h)
Appendix C: Structural characterization of metabolites

Parent drug (regorafenib)

The high-resolution mass spectral data showed \([M+H]^+\) at \(m/z\) 483.084. The MS2 product ion spectrum showed major fragment ions at \(m/z\) 424.045 (loss of N-methylformamide \(\text{CHONHCH}_3\)) (Fig. S1), \(m/z\) 288.077, \(m/z\) 270.067, and \(m/z\) 201.986 with the urea amide bonds as sites of fragmentation.

M-1

M-1 was found in trace amounts in incubations with human hepatocytes. The high-resolution mass spectral data showed \([M+H]^+\) at \(m/z\) 515.073 (Fig. S2), which is 32 Da (+2O) higher than the parent drug. The MS2 product ion spectrum showed major fragment ions at \(m/z\) 497.063 (loss of H\(_2\)O), \(m/z\) 424.047 (loss of N-hydroxymethylformamide \(\text{CHONHCH}_2\text{OH}\)), \(m/z\) 302.057, and \(m/z\) 201.986 with the urea amide bonds as sites of fragmentation. Therefore, M-1 was interpreted as a pyridine N-oxide and N-methylhydroxylated product (combination of M-2 and M-3) of regorafenib.

M-2 (BAY 75-7495)

M-2 was found as a major metabolite in incubations with human hepatocytes. The high-resolution mass spectral data showed \([M+H]^+\) at \(m/z\) 499.079 (Fig. S3), which is 16 Da (+O) higher than the parent drug. The MS2 product ion spectrum showed major fragment ions at \(m/z\) 424.045 (loss of N-
methylformamide and oxygen $\text{CHONCH}_3 + \text{O}$, m/z 304.072, m/z 247.051, and m/z 201.986 with the urea amide bonds as sites of fragmentation. Therefore, M-2 is interpreted as a pyridine N-oxide product of regorafenib. Furthermore, all mass spectral data were identical to the synthetic reference material BAY 75-7495.

M-3 (BAY 81-8753)

Only small amounts of M-3 were found in human hepatocytes. The high-resolution mass spectral data showed $[\text{M+H}]^+$ at m/z 499.077 (Fig. S4), which is 16 Da (+O) higher than the parent drug. The MS2 product ion spectrum showed major fragment ions at m/z 424.045 (loss of N-hydroxymethylformamide $\text{CHONCH}_2\text{OH}$), m/z 286.061, and m/z 201.986 with the urea amide bonds as sites of fragmentation. Therefore, M-3 is interpreted as an N-methylhydroxylated product of regorafenib. Furthermore, all mass spectral data were identical to the synthetic reference material BAY 81-8753.

M-4 (BAY 75-1098)

Only small amounts of M-4 were found in human hepatocytes. The high-resolution mass spectral data showed $[\text{M+H}]^+$ at m/z 469.067 (Fig. S5), which is a loss of 14 Da (-CH$_2$), compared with the parent drug. The MS2 product ion spectrum showed major fragment ions at m/z 424.045 (loss of formamide $\text{CHONH}_2$), m/z 274.061, m/z 247.050, and m/z 201.986 with the urea amide bonds as sites of fragmentation. Therefore, M-4 was interpreted as an N-demethylated product of regorafenib. Furthermore, all mass spectral data were identical to the synthetic reference material BAY 75-1098.
M-5 (BAY 81-8752)

M-5 was found as a metabolite in incubations with human hepatocytes. The high-resolution mass spectral data showed [M+H]+ at m/z 485.061, which is 2 Da (-CH$_2$, +O) higher than the parent drug. The MS2 product ion spectrum (Fig. S6) showed fragment ions at m/z 424.045 (loss of formamide and oxygen CHONH$_2$ and O), m/z 290.056, m/z 247.050, and m/z 201.986 with the urea amide bonds as sites of fragmentation. Therefore, M-5 was interpreted as a pyridine N-oxide and N-demethylated product (combination of M-2 and M-4) of regorafenib. Furthermore, all mass regorafenib 16 spectral data were identical to the synthetic reference material BAY 81-8752.

M-6 (BAY 1005352)

Only small amounts of M-6 were found in human hepatocytes. The high-resolution mass spectral data showed [M+H]+ at m/z 470.051 (Fig. S7), which is a loss of 13 Da (-NH$_2$CH$_3$, +H$_2$O) compared with the parent drug. The MS2 product ion spectrum showed major fragment ions at m/z 424.045 (loss of CO$_2$), m/z 231.055, m/z 247.050, and m/z 201.986 with the urea amide bonds as sites of fragmentation. Therefore, M-8 was interpreted as an N-demethylated and hydrolyzed carboxylic acid derivative of regorafenib. Furthermore, all mass spectral data were identical to the synthetic reference material BAY 1005352.

M-7
M-7 was found as a metabolite in incubations with human hepatocytes. The high-resolution mass spectral data showed [M+H]+ at m/z 659.113 (Fig. S8), which is 176 Da (+C₆H₈O₆) higher than the parent drug. The MS2 product ion spectrum showed fragment ions at m/z 641.103 (loss of H₂O), m/z 483.082 (loss of -C₆H₈O₆, anhydro glucuronic acid, 176 Da), which is the pseudomolecular ion of parent drug, m/z 288.077, and m/z 270.067 with the urea amide bonds as sites of fragmentation. Therefore, M-7 was interpreted as a glucuronic acid conjugate of parent drug regorafenib. For further structure elucidation, M-7 was isolated and purified by HPLC from human urine (GCM 2082-01), and subjected to LC-MS and NMR analysis (Fig. S10). From these data it was proposed that M-7 is N-glucuronidated at the urea nitrogen adjacent to the trifluoromethyl-chloro phenyl moiety.

M-8

M-8 was found as a metabolite in incubations with human hepatocytes. The high-resolution mass spectral data showed [M+H]+ at m/z 675.109 (Fig. S9), which is 192 Da (+C₆H₈O₆, +O) higher than the parent drug. The MS2 product ion spectrum showed fragment ions at m/z 657.099 (loss of H₂O), m/z 499.077 (loss of -C₆H₈O₆, anhydro glucuronic acid, 176 Da), which is the pseudomolecular ion of the hydroxylated parent drug, and m/z 304.072 with the urea amide bonds as sites of fragmentation. The fragment ion m/z 304.072 is characteristic for M-2; therefore, M-8 was interpreted as a glucuronic acid conjugate of M-2. For further structure elucidation, M-8 was isolated and purified by HPLC from human urine (GCM 2082-02) and subjected to LC-MS and NMR-analysis. From these data it was proposed that M-8 is N-glucuronidated at the urea nitrogen adjacent to the trifluoromethyl-chloro phenyl moiety.
Fig. S1 Product ion spectrum of regorafenib (BAY 73-4506)

**Drug**

BAY 73-4506  
\[m/z \, ^{12}C^+ = 483\]  
\[m/z \, ^{14}C^+ = 485\]

**MS² (483)**

- \(C_{19}H_{11}O_2N_3F\)  
- \(C_{19}H_{11}O_2N_3ClF_4\)  
- \(C_{19}H_{11}O_2N_3Cl\)

**MS² (485)**

- \(C_{20}H_{14}O_3N_4ClF_4\)  
- \(C_{20}H_{14}O_3N_4F\)  
- \(C_{20}H_{14}O_3N_4Cl\)
Fig. S2 Product ion spectrum of M-1

**M-1**
- m/z $^{12}$C$^+$ = 515
- m/z $^{14}$C$^+$ = 517

**MS$^2$ (515)**

**MS$^2$ (517)**
Fig. S3 Product ion spectrum of M-2

**M-2**

\[ m/z \left[^{12}\text{C}\right]^+ = 499 \]
\[ m/z \left[^{14}\text{C}\right]^+ = 501 \]

**MS\(^2\) (499)**

**MS\(^2\) (501)**
Fig. S4 Product ion spectrum of M-3

**M-3**

m/z $[^{12}\text{C}]^+ = 499$

m/z $[^{14}\text{C}]^+ = 501$

**MS2 (499)**

**MS2 (501)**
**Fig. S5** Product ion spectrum of M-4

**Top**

- **RT:** 18.33
- **AV:** 1
- **NL:** 1.06E5
- **F:** FTMS + p ESI d Full ms2 471.07 [50.00-480.00]

**Middle**

- **MS2 (469)**

**Bottom**

- **MS2 (471)**

---

**M-4**

- **m/z** $^{12}\text{C}^+$ = 469
- **m/z** $^{14}\text{C}^+$ = 471

---

**Details:**

- Compound structures and masses are annotated, including chemical formulas and isotope distributions.

---

**Notes:**

- Detailed analysis of product ions, peak intensities, and mass spectrometric data.
Fig. S6 Product ion spectrum of M-5

M-5
m/z $[^{12}\text{C}]^+ = 485$
m/z $[^{14}\text{C}]^+ = 487$

**MS² (485)**

**MS² (487)**
Fig. S7 Product ion spectrum of M-6

### MS2 (470)

- **M-6**
  - m/z $[^{12}\text{C}]^+$ = 470
  - m/z $[^{14}\text{C}]^+$ = 472

### MS2 (472)

- **M-6**
  - m/z $[^{12}\text{C}]^+$ = 470
  - m/z $[^{14}\text{C}]^+$ = 472

---

**RT:** 13.20
**AV:** 1
**NL:** 1.21E6

**F:** FTMS + p ESI Full ms [100.00-1000.00]

---

**MS2 (470)**

- **M-6**
  - m/z $[^{12}\text{C}]^+$ = 470
  - m/z $[^{14}\text{C}]^+$ = 472

### MS2 (472)

- **M-6**
  - m/z $[^{12}\text{C}]^+$ = 470
  - m/z $[^{14}\text{C}]^+$ = 472
Fig. S8 Product ion spectrum of M-7

**MS2 (659)**

**MS2 HCD (659)**
**Fig. S9** Product ion spectrum of M-8

**M-8**

m/z $[^{12}\text{C}]^+ = 675$

**MS\textsuperscript{2} (675)**

**MS\textsuperscript{2} HCD (675)**
Fig. S10 NMR analysis of M-7

Parent compound: DAV 73-4506
Metabolite: M-7
Sample: GCM 2082-01
Origin: Human urine
Chemical structure: DMBO

Chemical structure of GCM 2082-01, M-7

NMR, results:

$^1$H NMR (DMSO-δ6, 600.13 MHz): δ=8.80 (d, J=4.9 Hz, 1H), 8.53 (d, J=5.5 Hz, 1H), 8.11 (brs, 1H), 7.83 (d, 2H), 7.58 (d, J=8.9 Hz, 1H), 7.50 (q, 1H), 7.40 (d, J=2.4 Hz, 1H), 7.27 (dd, J=10.8, 2.7 Hz, 1H), 7.17 (dd, J=5.5, 2.3 Hz, 1H), 7.03 (d, J=8.7, 2.5 Hz, 1H), 2.78 (d, J=4.9 Hz, 3H)

$^1$H NMR (DMSO-δ6, 600.13 MHz): δ=8.80 (d, J=4.9 Hz, 1H), 8.53 (d, J=5.5 Hz, 1H), 8.11 (brs, 1H), 7.83 (d, 2H), 7.58 (d, J=8.9 Hz, 1H), 7.50 (q, 1H), 7.40 (d, J=2.4 Hz, 1H), 7.27 (dd, J=10.8, 2.7 Hz, 1H), 7.17 (dd, J=5.5, 2.3 Hz, 1H), 7.03 (d, J=8.7, 2.5 Hz, 1H), 2.78 (d, J=4.9 Hz, 3H)

$^1$H NMR (DMSO-δ6, 600.13 MHz): δ=8.80 (d, J=4.9 Hz, 1H), 8.53 (d, J=5.5 Hz, 1H), 8.11 (brs, 1H), 7.83 (d, 2H), 7.58 (d, J=8.9 Hz, 1H), 7.50 (q, 1H), 7.40 (d, J=2.4 Hz, 1H), 7.27 (dd, J=10.8, 2.7 Hz, 1H), 7.17 (dd, J=5.5, 2.3 Hz, 1H), 7.03 (d, J=8.7, 2.5 Hz, 1H), 2.78 (d, J=4.9 Hz, 3H)

$^1$H NMR (DMSO-δ6, 600.13 MHz): δ=8.80 (d, J=4.9 Hz, 1H), 8.53 (d, J=5.5 Hz, 1H), 8.11 (brs, 1H), 7.83 (d, 2H), 7.58 (d, J=8.9 Hz, 1H), 7.50 (q, 1H), 7.40 (d, J=2.4 Hz, 1H), 7.27 (dd, J=10.8, 2.7 Hz, 1H), 7.17 (dd, J=5.5, 2.3 Hz, 1H), 7.03 (d, J=8.7, 2.5 Hz, 1H), 2.78 (d, J=4.9 Hz, 3H)

$^1$H NMR (DMSO-δ6, 600.13 MHz): δ=8.80 (d, J=4.9 Hz, 1H), 8.53 (d, J=5.5 Hz, 1H), 8.11 (brs, 1H), 7.83 (d, 2H), 7.58 (d, J=8.9 Hz, 1H), 7.50 (q, 1H), 7.40 (d, J=2.4 Hz, 1H), 7.27 (dd, J=10.8, 2.7 Hz, 1H), 7.17 (dd, J=5.5, 2.3 Hz, 1H), 7.03 (d, J=8.7, 2.5 Hz, 1H), 2.78 (d, J=4.9 Hz, 3H)

$^1$H NMR (DMSO-δ6, 600.13 MHz): δ=8.80 (d, J=4.9 Hz, 1H), 8.53 (d, J=5.5 Hz, 1H), 8.11 (brs, 1H), 7.83 (d, 2H), 7.58 (d, J=8.9 Hz, 1H), 7.50 (q, 1H), 7.40 (d, J=2.4 Hz, 1H), 7.27 (dd, J=10.8, 2.7 Hz, 1H), 7.17 (dd, J=5.5, 2.3 Hz, 1H), 7.03 (d, J=8.7, 2.5 Hz, 1H), 2.78 (d, J=4.9 Hz, 3H)