

Absorption, metabolism and excretion of opicapone in human healthy volunteers

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Aims: The absorption, metabolism and excretion of opicapone (2,5-dichloro-3-(5-[3,4-dihydroxy-5-nitrophenyl]-1,2,4-oxadiazol-3-yl)-4,6-dimethylpyridine 1-oxide), a selective catechol-O-methyltransferase inhibitor, were investigated.

Methods: Plasma, urine and faeces were collected from healthy male subjects following a single oral dose of 100 mg [¹⁴C]-opicapone. The mass balance of [¹⁴C]-opicapone and metabolic profile were evaluated.

Results: The recovery of total administered radioactivity averaged >90% after 144 hours. Faeces were the major route of elimination, representing 70% of the administered dose; 5% and 20% were excreted in urine and expired air, respectively. The C_{max} of total radioactivity matched that of unchanged opicapone, whereas the total radioactivity remained quantifiable for a longer period, attributed to the contribution of opicapone metabolites, involving primarily 3-O-sulfate conjugation (58.6% of total circulating radioactivity) at the nitrocatechol ring. Other circulating metabolites, accounting for <10% of the radioactivity exposure, were formed by glucuronidation, methylation, N-oxide reduction and glutathione conjugation. Additionally, various other metabolites resulting from combinations with the opicapone N-oxide reduced form at the 2,5-dichloro-4,6-dimethylpyridine 1-oxide moiety, including nitro reduction and N-acetylation, reductive opening and cleavage of the 1,2,4-oxadiazole ring and the subsequent hydrolysis products were identified, but only in faeces, suggesting the involvement of gut bacteria.

Conclusion: [¹⁴C]-opicapone was fully excreted through multiple metabolic pathways. The main route of excretion was in faeces, where opicapone may be further metabolized via reductive metabolism involving the 1,2,4-oxadiazole ring-opening and subsequent hydrolysis.

KEYWORDS

COMT inhibitor, human, mass balance, metabolism, opicapone

1 | INTRODUCTION

Opicapone (also known as BIA 9-1067) is a reversible, peripherally selective high binding affinity and long-acting third-generation nitrocatechol catechol-O-methyltransferase (COMT) inhibitor^{1,2} that has recently been granted marketing authorization under the trade name Ongentys[®] by, among other regulatory agencies, the European Medicines Agency, the US Food and Drug Administration and the Japan Pharmaceutical and Medical Devices Agency as adjunctive therapy to the combination levodopa plus dopa decarboxylase inhibitor for the treatment of end-of-dose motor fluctuations in Parkinson's disease patients.³⁻¹¹

Opicapone was found not to induce cytotoxicity.¹² In humans, despite opicapone's short apparent terminal elimination half-life ($t_{1/2}$) of 0.8-3.2 hours, the half-life of erythrocyte COMT inhibition is in the range of 60-130 hours,^{13,14} far beyond the observable point of plasma opicapone clearance (6-10 hours), making it suitable for a once-daily regimen.^{13,14} Previous investigations have demonstrated that the pharmacokinetic profile of opicapone is dose-proportional up to 1200 mg and 3-O-sulfate-opicapone (BIA 9-1103) appeared to be the main metabolite of opicapone, with a relatively long $t_{1/2}$, ranging from 25.1 to 26.9 hours following a single dose and from 98.5 to 112.0 hours following repeated dosing.^{13,14} Along with BIA 9-1103 other opicapone metabolites were quantified in clinical studies, such as the N-oxide reduced form of opicapone (BIA 9-1079), the 3-O-glucuronide-opicapone (BIA 9-1106) and the 3-O-methyl-opicapone (BIA 9-1100). However, the definitive information concerning quantitative and comprehensive metabolic profiles of opicapone in human plasma and excreta is only provided by a clinical study with radiolabeled opicapone.¹⁵ This type of study is considered one of the most critical steps in drug development where the primary mechanism(s) of clearance, excretion, mass balance and metabolism are evaluated,¹⁵ contributing to the pharmacokinetics (PK) characterisation of a drug candidate. The further characterization of metabolites, such as their profile, potential for on-target pharmacological activity and drug-drug interactions (DDIs), are essential to better understand the safety of therapeutic agents. Previously, in an absorption, distribution, metabolism and excretion study with [¹⁴C]-opicapone in rats, we demonstrated that opicapone was rapidly absorbed after oral dosing and that the drug-derived radioactivity was excreted mainly into faeces.¹⁶ Furthermore, we found that opicapone underwent extensive metabolism via diverse pathways and various metabolites were detected in plasma and excreta. Although O-glucuronidation, -sulfation and -methylation of the nitrocatechol moiety were the principal metabolic pathways, a small amount of the N-acetyl derivative was detected as a result of reduction of the nitro group and subsequent conjugation. Other metabolic transformations include N-oxide reduction to the pyridine derivative and reductive cleavage of the 1,2,4-oxadiazole ring followed by further conjugation reactions. The pharmacological activity of some of those metabolites was also evaluated.¹⁶

In this study the absorption, metabolism and excretion of opicapone-related radioactivity were evaluated in healthy male subjects after oral administration of 100 mg of [¹⁴C]-opicapone. The total radioactivity and metabolic profile in urine and faeces were measured

What is already known about this subject

- Opicapone is a reversible, peripherally selective high binding affinity and long-acting third-generation nitrocatechol catechol-O-methyltransferase inhibitor that has recently been granted marketing authorization as adjunctive therapy to the combination levodopa plus decarboxylase inhibitor for the treatment of end-of-dose motor fluctuations in Parkinson's disease patients.

What this study adds

- The absorption, metabolism and excretion of opicapone were elucidated and revealed that it had substantial systemic availability after oral administration. The recovery of >90% radioactivity was obtained after 144 hours mainly through the faeces. The major circulating metabolite of opicapone was the pharmacologically inactive 3-O-sulfate-opicapone.

using the conventional liquid scintillation counting method; in plasma, due to the low amount of radioactivity, the metabolic profile was evaluated by accelerator mass spectrometry (AMS), an extremely sensitive method for the detection and determination of isotopic ratios.¹⁷

2 | MATERIALS AND METHODS

2.1 | Reagents

The unlabelled opicapone was manufactured by Patheon (formerly DPx Fine Chemicals, Linz, Austria GmbH & Co KG; Batch no 129059-2-2). The labelled drug substance, [¹⁴C]-BIA 9-1067 (opicapone), was manufactured by Pharmaron Inc., Cardiff, UK. Quotient Clinical (Nottingham, UK) manufactured the [¹⁴C]-BIA 9-1067 drug in capsule for use in this clinical trial. [¹⁴C]-opicapone (Figure 1), specific radioactivity 2.09 GBq/mmol (5.03 MBq/mg), was supplied by GE Healthcare (Little Chalfont, UK) and by Quotient Bioresearch, (Nottingham, UK) at a radiochemical purity of 99.1%. Opicapone and its metabolites were synthesized in the Laboratory of Chemistry, BIAL (Coronado [S. Mamede, S. Romao]), Portugal (additional information on the compound structure is given in the Supporting Information).

2.2 | Clinical study conduct

The clinical study (EudraCT number 2016-004454-15) was conducted by Quotient Clinical, Nottingham, UK in accordance with the ethical principles outlined in the Declaration of Helsinki, the ICH Good

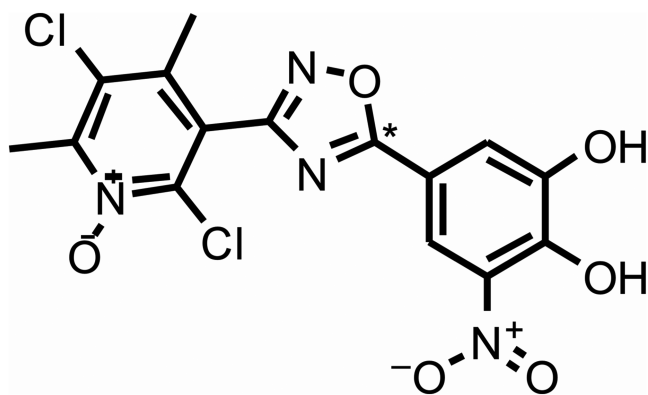


FIGURE 1 Structure of [^{14}C]-opicapone. * represents the ^{14}C labelling position

Clinical Practice (GCP) guideline (ICH Topic E6, GCP), the study protocol, local legislation regarding the rights and welfare of human participants in biomedical research, and applicable regulatory requirements. All participants provided written informed consent. The protocol and the associated documents were reviewed and approved by an independent ethics committee and the Clinical Trial Authorisation application was approved by the UK Medicines and Healthcare Products Regulatory Agency (MHRA). Quantification of opicapone and its metabolites was conducted in accordance with the principles of Good Laboratory Practice at SYNLAB Analytics and Services, Switzerland AG. The mass balance and the metabolite profiling and identification were performed at Quotient Bioresearch, Pharmaron, UK and Pharmaron, United States (former Xceleron), respectively.

2.3 | Clinical study design

This was an open-label, single-dose, single-period study designed to assess the mass balance recovery, metabolite profile and metabolite identification of [^{14}C]-opicapone in healthy male subjects (aged 30–65 years). The seven enrolled subjects were dosed with a 100 mg [^{14}C]-opicapone capsule containing not more than 3.3 MBq (89.2 μCi) carbon-14 on the morning of day 1 following an overnight fast of approximately 8 hours. Blood, urine, faeces and expired air were collected at predefined time points for mass balance and PK analysis. After 22 days of residence in the clinical unit all subjects met the discharged criteria (>90% mass balance recovery, <1% of the administered dose eliminated in excreta for two consecutive 24-hour periods) and no additional residency period was required. All subjects attended four further return visits for PK sampling on day 28, day 42 (± 3 days), day 56 (± 3 days) and day 77 (± 3 days).

2.4 | Sample collection and storage

Venous blood samples were collected at predose and then at regular intervals from 0.5 to 648 hours post-oral [^{14}C]-opicapone administration. Whole blood was collected at predose and up to 96 hours post-

dosing; Urine, faeces and expired air were collected at predose and then at regular intervals until 504 hours post-oral [^{14}C]-opicapone dosing (additional information on collection timepoints is given in the Supporting Information).

2.5 | Measurement of total radioactivity

The residual radioactivity in the dosing bottles was quantified and the recovery of total radioactivity was less than 0.0333%. Total radioactivity in urine and plasma was measured after pooling and mixed with phosphate-buffered saline.

Total radioactivity in the faecal samples was determined by the combustion method using an automatic sample oxidiser (Model 307, Perkin Elmer, Waltham, MA, USA). The combustion products were absorbed into CarboSorb E and mixed with the scintillator cocktail PermaFluor E⁺ for measurement of radioactivity. The same procedure was applied to whole blood. Total radioactivity in expired air was measured in air-trapping solution obtained from individual expired air. Measurements of radioactivity in urine, faeces, whole blood and plasma were performed in duplicate (single measurement for expired air) using liquid scintillation counting (2300TR, 2900TR or 3100TR Tri-Carb, Scintillation Counter, Perkin Elmer) (additional information on total radioactivity measurements is given in the Supporting Information).

2.6 | Quantitation of concentrations in plasma samples by liquid chromatography-tandem mass spectrometry

Concentrations of opicapone and its metabolites, BIA 9-1103, BIA 9-1079, BIA 9-1106 and BIA 9-4588, in plasma were measured using a validated liquid chromatography-tandem mass spectrometry. Briefly, plasma samples (100 mL) were mixed with 200 mL of acetonitrile and internal standards. The samples were vortex and centrifuged, and the supernatant was diluted with water to be injected into a liquid chromatography-tandem mass spectrometry (LC-MS/MS) TSQ Quantum (Thermo Fisher Scientific, Waltham, MA, USA). The limit of quantification for all metabolites was 10 ng/mL. Calibration curves and a set of quality control (QC) samples were analyzed with each batch of study samples. The analytical method was demonstrated to be precise (<15%, coefficient of variation) and accurate ($\pm 15\%$) over the analytical range.

2.7 | Pharmacokinetic and statistical analyses

The PK of total radioactivity in plasma and blood, opicapone and its metabolites in plasma were analyzed using WinNonlin (version 6.3; Certara USA, Inc., USA) for concentration versus time for each subject. Pharmacokinetic parameters were calculated by noncompartmental analysis (additional information on pharmacokinetic parameters is given in the Supporting Information).

2.8 | Metabolite profiling and identification

A single area under the curve (AUC) pool was prepared for each subject (using the method of Hamilton et al¹⁸) to include as a minimum the last time point containing quantifiable radioactivity. In addition, a plasma pool across subjects at the total radioactivity t_{\max} time point was prepared. The plasma (5 g) was precipitated with acetonitrile and the supernatant collected. After a second wash the mixed supernatant was evaporated and resuspended with dimethyl sulfoxide:acetonitrile:water (20:20:60 v/v) to be analysed by LC-AMS and LC-MS and off-line radiodetection. For urine, the samples were pooled for each subject to account for at least 80% of the total recovered radioactivity and concentrated prior analysis. For faeces, the samples were pooled for each subject to account for greater than 90% of the excreted radioactivity. Additionally, pooled samples across all subjects at the 48-72 hours and 96-120 hours time points were prepared to evaluate the change in profile with time. The faeces pools were weighed, extracted with acetonitrile, shaken, centrifuged and the combined supernatants were dried and reconstituted in acetonitrile:water (20:80 v/v).

The resulting samples from urine, faeces and the t_{\max} cross-subject plasma pool were analyzed using accurate mass LC-MS and off-line radiodetection (additional information on LC and the radiodetection methods is given in the Supporting Information).

The metabolic profile in the plasma AUC prepared for each subject was performed separately by Xceleron Inc. (Germantown, MD, USA) using AMS. For the AMS analysis, the total carbon content of the plasma pool ($n = 2, 30 \mu\text{L}$) and each plasma extract ($n = 2, 30 \mu\text{L}$) was determined using a PerkinElmer 2400 Series 2 C,H,N analyzer (additional information on the AMS method is given in the Supporting Information).

2.9 | Structural analysis

Metabolite identification were obtained by LC-MS/MS Q-Exactive (Thermo Fisher Scientific, Waltham, Massachusetts, EUA) using accurate mass, positive and negative ion full scan, and product ion analyses was carried out on the supplied samples to screen for the presence of 'typical' or 'predicted' metabolites that corresponded to regions of radioactivity. Typically the MS methods contained two full-scan experiments (positive/negative switching) performed at 70 000 resolution over the range 100-800 amu. A full scan mass range of 100-1000 amu was used on one occasion to ensure high m/z species were not being excluded. Additional Tandem mass spectrometry experiments on pre-determined accurate m/z values were also included at 17 500 resolution. Product ions were obtained using higher-energy collisional dissociation at (typically) 20, 40 and 60 units (additional information on structural analysis is given in the Supporting Information).

2.10 | Extraction and column recovery

The recovery of radioactivity following extraction and reconstitution was determined for all samples by comparing the radioactivity in the

initial sample pool to that recovered in the supernatant and in the reconstituted extract (additional information on recovery is given in the Supporting Information).

2.11 | Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY, and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20).^{19,20}

3 | RESULTS

3.1 | Mass balance

Following a single oral dose of 100 mg [¹⁴C]-opicapone, a mean of approximately 96% (range 91.2-105.1%) of the total radioactivity administered was recovered by the end of the sampling period (504 hours). The cumulative excretion of ¹⁴C in urine, faeces and expired air with time is shown in Figure 2. An average of approximately 5% (range 3.65-7.17%) of the total radioactivity was recovered from the urine, approximately 70% (range 50.46-82.21%) was recovered from the faeces and approximately 20% (range 7.58-38.32%) was recovered in expired air samples by the end of the sampling period. On average, more than 90% of total radioactivity was recovered within 144 hours (6 days) of dosing. Within the first 24 hours post-dose on average approximately 3% and 7% of the total radioactivity was recovered in the urine and faeces, respectively. Based on urine and expired air data alone, minimum absolute bioavailability was approximately 25%, but with inclusion of faecal data (post 48 hours only) this value increased to approximately 70%.

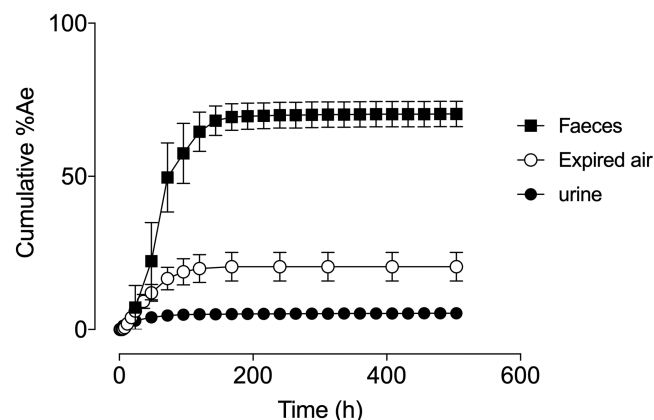


FIGURE 2 Cumulative recoveries (mean \pm SD) of ¹⁴C (urine, faeces and expired air) after a single oral dose of 100 mg [¹⁴C]-opicapone capsule containing not more than 3.3 MBq (89.2 μCi). Each point represents the mean \pm SD of seven subjects

3.2 | Pharmacokinetic profiles of total [^{14}C]-opicapone and metabolites

The plasma and blood concentrations versus time profiles of total ^{14}C together with opicapone and its major metabolite, BIA 9-1103, plasma concentrations are shown in Figure 3. Pharmacokinetic parameters of total ^{14}C contents in plasma and of opicapone and its metabolites BIA 9-1103 and BIA 9-1106 are summarized in Table 1. Maximum plasma concentrations occurred between 1 and 4 hours post-dose and remained quantifiable for between 72 and 648 hours (only in two out of seven subjects) post-dose. Whole-blood concentrations of total radioactivity were quantifiable by 1 hour in most subjects and

remained quantifiable for the duration of the sampling period (96 hours) for the majority of subjects. Geometric mean whole blood to plasma concentration ratios were 0.652 at 1 hour and 0.713 at 96 hours, with no apparent trend with respect to time.

Opicapone maximum plasma concentrations occurred between 0.75 and 4 hours post-dose and remained quantifiable for between approximately 4 and 12 hours post-dose. The geometric mean elimination half-life was 1 hour (range 0.476-2.19 hours). BIA 9-1103 maximum plasma concentrations occurred between approximately 6 and 8 hours post-dose and then declined in a multiphasic manner. Plasma BIA 9-1103 levels remained quantifiable for between approximately 120 and 408 hours post-dose. The geometric mean terminal half-life

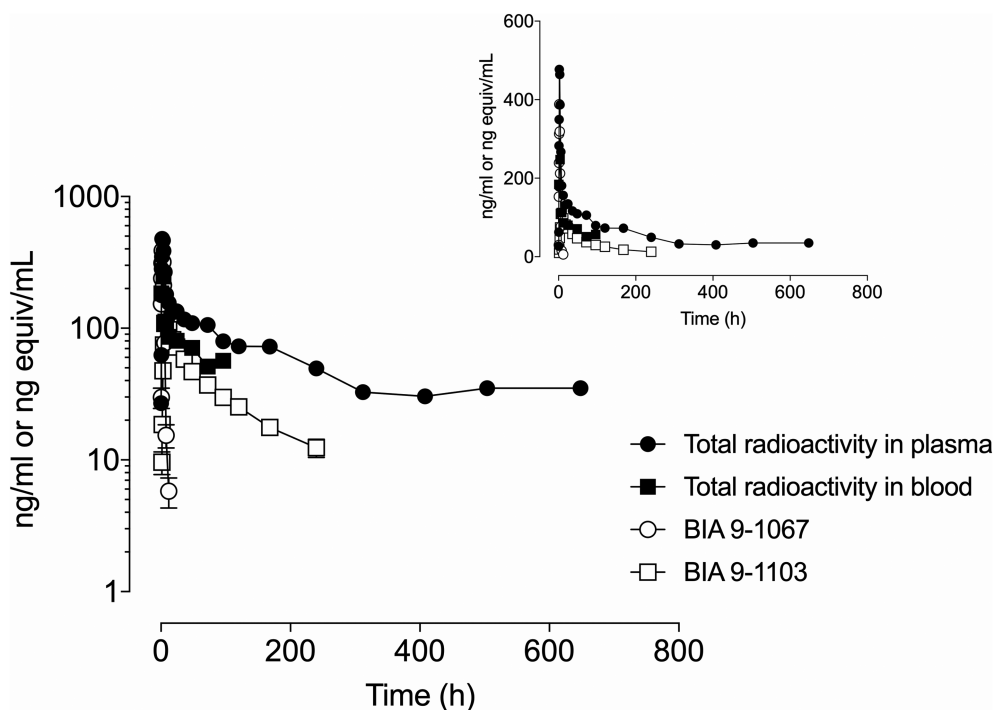


FIGURE 3 Mean concentrations of radioactivity in blood and plasma, and mean concentrations of opicapone (BIA 9-1067) and its major metabolite (BIA 9-1103) in plasma following single oral dose of 100 mg [^{14}C]-opicapone capsule containing not more than 3.3 MBq (89.2 μCi). Each point represents the mean \pm SD of seven subjects

TABLE 1 Summary of pharmacokinetic parameter estimates of opicapone (BIA 9-1067), BIA 9-1103 and BIA 9-1106 in plasma and total radioactivity in plasma after oral administration of a single 100 mg dose of [^{14}C]-opicapone

	Plasma $^{14}\text{C}^{\text{a}}$	BIA 9-1067	BIA 9-1103	BIA 9-1106
Tlag (h) ^b	0.500 (0.250-1.50)	0.250 (0.250-0.500)	1.05 (1.00-2.00)	0.75 (0.500-2.00)
t _{max} (h) ^b	2.00 (1-4)	2 (0.75-4.00)	6 (6.00-8.00)	2 (1.50-6.00)
C _{max} (ng/ml)	718 (34.8) ^a	600 (39.1)	115(48.7)	33.6(34.2)
AUC _t (ng.h/ml)	23 800 (101.8) ^a	1830 (52.6)	8120 (75.7)	75.0 (67.1)
AUC _{inf} (ng.h/ml)	NC	1890 (62.7) ^c	16 200 (31.9) ^d	NC
t _{1/2} (h)	NC	1.00 (59.5) ^c	138 (3.9) ^d	NC

Note: Geometric mean (% coefficient of variation).

Abbreviations: NC, not calculated.

^aNanograms equivalent for total radioactivity.

^bMedian (minimum – maximum).

^cFive out of seven subjects included in calculations.

^dthree out of seven subjects included in calculations.

was 138 hours (range 133-143 h). As shown in Table 1, low levels of BIA 9-1106 were quantified and BIA 9-1079 was below the limit of quantification (BLQ, <10 ng/mL) in almost all time points.

3.3 | Metabolite profiling and identification in plasma, urine and faeces

Plasma AUC sample radiochromatograms were generated using AMS radiodetection. A representative radiochromatogram for plasma is shown in Figure 4. Structural assignment and MS data are summarized in Table 2. The proposed metabolic pathway is shown in Figure 5.

3.4 | Plasma

As presented in Figure 4 and Table 2, although in total 13 radioactive regions of interest were observed in the plasma, no *m/z* and structure

were attributed to regions P1 and P5. The largest region of interest (P6), at a retention time of 18.6 minutes, was identified as BIA 9-1103, the 3-*O*-sulfate of opicapone, with a minor peak corresponding to the BIA 9-1103 isomer. BIA 9-1103 resulted on average in 58.6% of the total radioactivity in the profiles. The second largest region of interest, at a retention time 22.6 minutes, corresponded to opicapone, which on average resulted in 12.1% of the radioactivity in the profiles.

Other less abundant circulating metabolites that accounted for more than 2% of the average total circulating radioactivity were detected in circulation. Those included two metabolites formed by 4-*O*- and 3-*O*-methylation of opicapone, BIA 9-1104 (P10) and BIA 9-1100 (P11), which represented an average of 6.59% and 2.96% of total circulating radioactivity, respectively. Two co-eluted metabolites (region P2) with a characteristic $^{35}\text{Cl}:$ ^{37}Cl ratio consistent with a singly chlorinated molecule, were tentatively assigned as a dicycysteine conjugate and cysteine-cysteine-glycine conjugate of either BIA 9-1104 or BIA 9-1100. These metabolites collectively

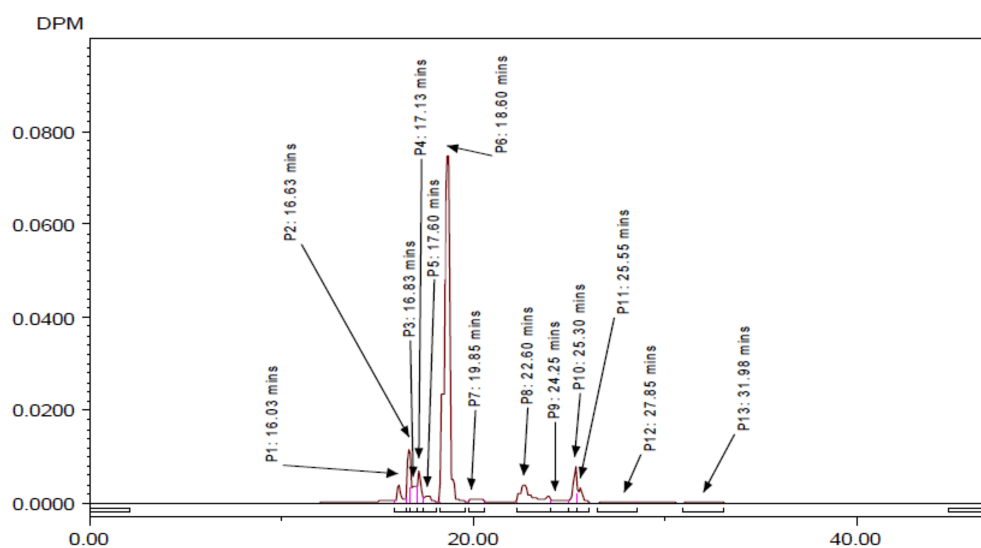


FIGURE 4 Representative UV chromatogram (250 nm) generated from the injection of diluted plasma spiked with synthetic standards of opicapone (P8), BIA 9-1100 (P11), -1079 (P12), -1101 (P13), -1103 (P6), -1106 (P3), -4588, -1104 (P10), -5048, -5049 (P4) and -4584 (P7), overlaid against a ^{14}C radiochromatogram based on the AMS detection of fractions and fraction pools collected from direct injection of 0- to 504-hour AUC pool plasma after administration of a single 100-mg (89.2 μCi) oral dose of ^{14}C -opicapone to healthy subjects (A), zoom of Figure 4A in the time interval between 15 to 32 min (B)

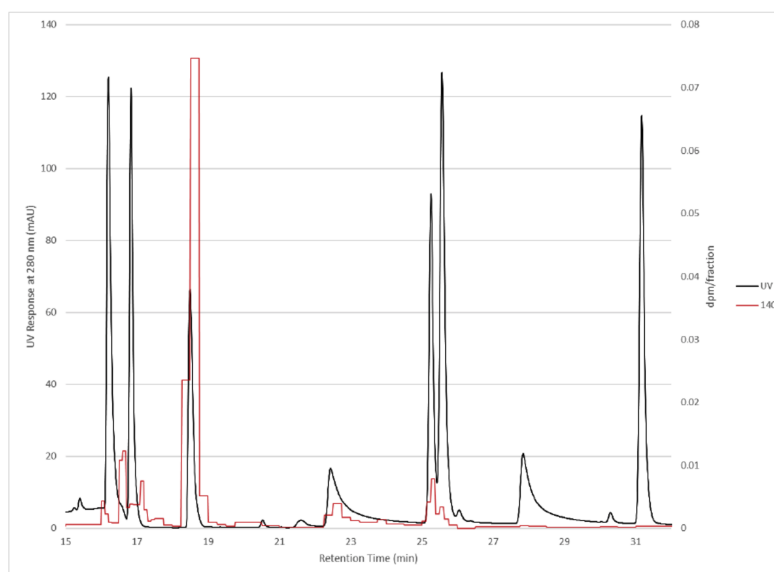


TABLE 2 Mass spectral analysis of opicapone metabolites detected in human plasma and faeces after a single 100 mg oral dose of [¹⁴C]-opicapone to healthy humans

Metabolite number/code	RTmin	Matrix	ID	Ionization	m/z	Elemental composition	Characteristic fragment ions (m/z)
F3/F4:BIA 9-3752	8.5/9.0	F	Carboxylic acid likely formed by hydrolysis of the oxadiazole moiety of M4	[M + H] ⁺	212	C ₉ H ₁₀ O ₅ N	194, 170, 168, 152, 142, 126
F5: M41	10.8	F	N-oxide reduction, nitro group reduction and reductive ring opening of the oxadiazole moiety	[M + H] ⁺	369	C ₁₅ H ₁₅ O ₃ N ₄ Cl ₂	18, 201, 183, 152, 142
F9: M4	13.1	F	N-acetylated M41	[M + H] ⁺	411	C ₁₇ H ₁₇ O ₄ N ₄ Cl ₂	375, 218, 201, 194, 183, 152, 142
BIA 9-3679	9.5	F	Likely formed by hydrolysis of the oxadiazole moiety of M4	[M + H] ⁺	218	C ₈ H ₁₀ N ₃ Cl ₂	210, 183, 165, 138
F11: MWT 424	14.4/ 14.7	F	O-methylated M4	[M + H] ⁺	425	C ₁₈ H ₁₉ O ₄ N ₄ Cl ₂ C ₁₈ H ₁₉ O ₄ N ₄ Cl ₂	399, 218, 208, 201, 183, 166, 156, 138, 121 389, 218, 208, 201, 183, 166, 152, 142, 124
P2: MWT 630 and MWT 687	17.0 17.0	P	Dicysteine conjugate and cysteine-cysteine-glycine conjugate of O-methylation	[M + H] ⁺	631 688	C ₂₂ H ₂₄ O ₁₀ N ₆ Cl ₂ C ₂₄ H ₂₇ O ₁₁ N ₇ Cl ₂	544, 510, 494, 478, 448, 444, 404, 392 544, 510, 494, 478, 478, 448, 404, 392
P3/U15: MWT 568 and BIA 9-1106	17.3 17.3	P P/U	Cysteine/glycine conjugate and 3-O-glucuronide	[M-H] ⁻ [M + H] ⁺	569 587	C ₂₁ H ₂₂ O ₉ N ₆ ClS C ₂₁ H ₁₇ O ₁₂ N ₄ Cl ₂	466, 425, 408, 196, 145, 122, 99 375, 350, 230, 213 ^a
P4: BIA 9-5049	17.6	P	Cysteine conjugate of 4-O-methylated	[M + H] ⁺	512	C ₁₉ H ₁₉ O ₈ N ₅ ClS	495, 466, 425, 408, 213, 196, 165, 154 ^a
P6:1103 and 1103 isomer	19.0 19.01	P	3-O-sulfate 4-O-sulfate	[M-H] ⁻	491	C ₁₅ H ₉ O ₉ N ₄ Cl ₂ S	394, 350, 230, 213
P7: BIA 9-4584 and isomer of BIA 9-1106	20.4 20.5	P	Sulfate conjugate of methylated Glucuronide	[M-H] ⁻	505	C ₁₆ H ₁₁ O ₉ N ₄ Cl ₂ S	488, 425, 408, 393, 357, 213 ^a
P8/F18-Opicapone	22.8	P/F	Opicapone	[M-H] ⁻	411	C ₁₅ H ₉ O ₆ N ₄ Cl ₂	351, 230, 213, 200, 195, 153, 137 ^a
P9 MWT 490	24.5	P		[M-H] ⁻	489	C ₁₆ H ₁₁ O ₈ N ₄ Cl ₂ S	394, 348, 330, 320, 293, 256, 214
P10: BIA 9-1104	25.9	P	4-O-methylation	[M-H] ⁻	425	C ₁₆ H ₁₁ O ₆ N ₄ Cl ₂	357, 321, 304, 213 ^a
P11: BIA 9-1100	25.9	P	3-O-methylation	[M-H] ⁻	425	C ₁₆ H ₁₁ O ₆ N ₄ Cl ₂	367, 230, 213 ^a
P12: BIA 9-1079	28.3	P		[M-H] ⁻	395	C ₁₅ H ₉ O ₅ N ₄ Cl ₂	352, 214, 195, 165 ^a
P13: BIA 9-1101 isomer and BIA 9-1101	31.7	P	O-methylation of BIA 9-1079	[M-H] ⁻ [M-H] ⁻	409 409	C ₁₆ H ₁₁ O ₅ N ₄ Cl ₂ C ₁₆ H ₁₁ O ₅ N ₄ Cl ₂	394, 351, 320 ^a 394, 348, 330, 320, 293, 256, 214

Abbreviations: P, plasma; U, urine; F, faeces.

^aProduct ion spectra were generated for the authentic reference standard that matched the elemental formula and retention time with the radio and MS peak.

characteristic $^{35}\text{Cl}:$ ^{37}Cl ratio consistent with a di-chlorinated molecule was tentatively assigned as an *N*-acetylated M41, which represented on average 9.31% of the administered radioactivity. The metabolite BIA 9-3752 (F3), represented on average 9.29% of the administered radioactivity and was assigned to a carboxylic acid likely formed by hydrolysis of the oxadiazole moiety of M4. The metabolite BIA 9-3679 with a protonated molecule $[\text{M} + \text{H}]^+$ at m/z 218 was detected by MS at a retention time of 9.5 minutes. This component does not produce a radiochromatogram due to the loss of the [^{14}C]-label during cleavage to BIA 9-3752. The radioactive region F11 is composed of two peaks closely eluting with the full-scan spectrum showing a protonated molecule $[\text{M} + \text{H}]^+$ at m/z 425, with a characteristic $^{35}\text{Cl}:$ ^{37}Cl ratio consistent with a dichlorinated molecule. F11 was tentatively assigned as *O*-methylated M4, two isomers which collectively represent on average 5.40% of the administered radioactivity.

3.6 | Extraction and column recovery

The overall recovery of radioactivity from the t_{max} plasma was 91.3% and from AUC plasma extracts was 59.3%. The mean recovery from the urine samples following concentration was 94.7% and from faeces was 42.7%. A column recovery experiment performed for human urine was determined to be 97.2%. LC-AMS column recovery was between 80% and 120% of the total radioactivity injected onto the system (additional information on recovery results is given in the Supporting Information).

4 | DISCUSSION

The mass balance, excretion and metabolism of opicapone were determined in healthy subjects after oral administration of a single dose of 100 mg (containing 3.3 MBq (89.2 μCi) [^{14}C]-opicapone) of opicapone. On average, 96% of the radioactive dose of [^{14}C]-opicapone was recovered, mainly via faeces (approximately 70%). Low levels of total radioactivity related with opicapone were excreted in urine (approximately 5.3%) and approximately 20% of total radioactivity was detected in expired air. The excretion of the majority of the dose via faeces is consistent with that observed in preclinical species (rat and nonhuman primate [NHP]) in which the fraction of the dose recovered in faeces was more than 70% of the administered dose.²¹ [^{14}C]-Opicapone showed limited distribution to the whole blood, circulating preferentially in the plasma, where the C_{max} for total radioactivity was similar to the C_{max} of the unchanged parent compound. The much greater AUC_t of total radioactivity in comparison with opicapone suggested a strong contribution of opicapone metabolites to the total circulating radioactivity, which is corroborated by the long half-life of 3-*O*-sulfate-opicapone (BIA 9-1103), the only component that together with opicapone was observed at mean levels greater than 10% of total circulating radioactivity exposure, representing an average of 58.6% and 12.1%, respectively. Likewise, BIA 9-1103 is a

major metabolite in rats together with other conversions at the nitrocatechol moiety such as methyl-conjugations and the reduction of nitro-function.²¹ BIA 9-1103 peaked approximately 6 hours post-dose in plasma, declined with a long terminal elimination phase (geometric mean $t_{1/2}$ 138 hours), binds to plasma proteins by more than 98% and is pharmacologically inactive on COMT.¹⁶ Furthermore, clinically relevant drug interactions with BIA 9-1103 as well as by opicapone are considered unlikely when therapeutic exposure and plasma protein binding are taken into account.

While the parent compound and BIA 9-1103 represent the majority of the total drug-related material in plasma, 11 other less abundant circulating metabolites were identified. The latter accounted for more than 2% of total circulating radioactivity and included an *N*-oxide reduced form of opicapone (BIA 9-1079), an *O*-glucuronide conjugated (BIA 9-1106) and two *O*-methyl conjugates of opicapone (BIA 9-1104 and BIA 9-1100), both at the nitro catechol ring (Figure 5). From those metabolites only BIA 9-1079 and BIA 9-1104 were active against COMT with IC_{50} values of 128 nM and 429 nM, respectively, under experimental conditions where opicapone IC_{50} was 224 nM.¹⁶ Other circulating metabolites tentatively assigned to cysteine/glycine conjugates of methylated opicapone (P3), the co-eluted dicycysteine and cysteine-cysteine/glycine conjugates of methylated opicapone (P2), and the cysteine conjugate of 4-*O*-methyl-opicapone, BIA 9-5049 (confirmed against synthetic standards) suggested that the methyl conjugates of opicapone are further conjugated following the mercapturate pathway.²² Thus, the methyl conjugates of opicapone may undergo a transient conjugation with glutathione, not detected in circulation, that may arise via nucleophilic attack with a displacement of a single chlorine atom by glutathione (GSH) at the C-2 position of the 2,5-dichloro-4,6-dimethylpyridine 1-oxide moiety of the opicapone molecule. This is consistent with the detected cysteine/glycine and cysteine conjugates of methyl-opicapone with a characteristic mass spectrum signal $^{35}\text{Cl}:$ ^{37}Cl ratio of a singly chlorinated molecule. The displacement of the halogen atoms by GSH from the halogenates nitrobenzene derivatives has been previously described.^{22,23} Additionally, the assignment of cysteine/glycine derivatives further suggests the well-known conversion of glutathione *S*-conjugate to a cysteinylglycine *S*-conjugate by γ -glutamyltransferase and then to a cysteine *S*-conjugate (BIA 9-5049) by various peptidases.²² Although not confirmed with a synthetic standard, the co-eluted dicycysteine and cysteine-cysteine/glycine conjugates of methylated opicapone (P2) suggested a second conjugation with cysteine at the 4-methyl of the 4, 6-dimethylpyridine 1-oxide moiety. This mercapturate route observed in human plasma was also identified in rat¹⁶ and in other preclinical species, such as NHP and mini-pig (data on file). Methyl derivatives obtained from the *N*-oxide reduced form of opicapone (BIA 9-1079) were also identified in circulation, as well as the sulphate derivative of methyl conjugates of opicapone and methyl conjugates of BIA 9-1079. The methyl conjugation of opicapone by human COMT was described previously by Palma et al²⁴ as an important step in the mechanism of inhibition, demonstrating that the inhibition potency (K_i) is primarily dependent on the catalytic rate constant of the opicapone *O*-methylation by COMT.²⁴ Comparison of the

metabolic profiles of opicapone between humans and animals found that all metabolic pathways identified in circulation in human plasma were detected in preclinical species indicate no human-specific metabolites. In this study we demonstrate that the methyl conjugates of opicapone are further subject to multiple biotransformations, including the mercapturate and sulphate conjugation that in general should be more promptly excreted. However, with exception of BIA 9-1106, which was detected in urine (<2% of the dose), none of the circulating metabolites were observed in excreta, which maybe potentially related to instability or metabolism in excreta, slow elimination due to the high protein binding (>99%) or just not detected, further considering the low recovery of radioactivity in the faeces. However, the mechanisms underlying this finding have been unclear.

Overall, urine is a minor excretion route of opicapone-related compounds and only trace amounts of unchanged parent were detected. In contrast, in faeces 22.32% of the administered radioactivity corresponded to unchanged opicapone. As observed in rats,¹⁶ several metabolites were formed as a result of multiple (three or more) biotransformation processes that involved mainly reductive reactions. BIA 9-1079, the *N*-oxide reduced form of opicapone, was further reduced on the nitro moiety at the nitrocatechol ring (F5: M41) and subsequently *N*-acetylated into a tentatively assigned F9: M4, which undergoes either methylation to generate F11 or cleavage to generate BIA 9-3752 and BIA 9-3679 (Figure 5). The later metabolites, confirmed with synthetic standard, may be formed by reductive ring opening and hydrolysis of the oxadiazole moiety. The reductive cleavage of the N-O bond of the oxadiazole ring and the subsequent hydrolysis have been extensively investigated in isoxazole ring metabolism, suggesting that isoxazole ring-opening reactions could be catalysed under anaerobic conditions in many cases by liver enzymes.^{25,26} Furthermore the ring-opening reaction and in some cases ring-cleaving reactions were also described for 1,2,4-oxadiazole rings.^{27,28} There is evidence that the ring-opening reaction is enhanced by anaerobic conditions in human hepatocytes and in rat liver but enzymes including P450 might not contribute to this unique reaction,²⁸ however the mechanisms underlying ring-opening reactions are still unclear. The absence of any of those opicapone metabolites in circulation suggests that this reductive metabolism could be promoted at the intestinal levels and may involve the microbiome, supporting the moderate to high, dose independent, interindividual variability of the exposure parameters (C_{max} and AUC) observed after single and repeated dose of opicapone when administered to human healthy subjects (data on file). In recent years, a growing number of studies have confirmed the relevance of gut microbiome in the direct metabolism of drugs, mainly for the poor absorbed xenobiotics or indirectly following biliary excretion.²⁹⁻³¹ There is also evidence that drugs containing a nitro or azo group are chemical targets for metabolic modification, undergoing reductive reactions in anaerobic conditions by human gut bacteria.³⁰ Furthermore, the involvement of microbiome in the generation of $^{14}CO_2$, corresponding to ~20% of total radioactivity, cannot be excluded. Indeed, BIA 9-3752, an aromatic carboxylic acid metabolite observed in faeces but not in plasma, represents a likely

intermediate from which $^{14}CO_2$ could be generated. BIA 9-3752 could potentially be further metabolized by the microbiome to release CO_2 in the colon, which would then be absorbed and excreted in expired air. A recent publication on the metabolism of ozanimod, which shares the oxadiazole ring system with a ^{14}C label in the same position as in [^{14}C]-opicapone, showed that the loss of radioactivity and release of $^{14}CO_2$ does occur in human faecal homogenates after in vitro incubation for 96 hours under anaerobic conditions.³² In the presence of antibiotics, this loss was prevented, implicating the role of anaerobic bacterial metabolism leading to loss of $^{14}CO_2$. In addition, the corresponding product was formed only in incubations without antibiotics and not in control incubations or with antibiotics, showing that anaerobic bacterial metabolism leads to loss of CO_2 .³² Although the reductive metabolic pathway, including *N*-oxide reduction, nitro group reduction and reductive opening of the 1, 2, 4-oxadiazole moiety followed by a hydrolytic cleavage, was also identified in rats,¹⁶ the percentage of detected CO_2 in rats was significantly lower, which may suggest a difference in the extension of this metabolic reaction in both species.

5 | CONCLUSION

The total radioactivity is fully excreted after [^{14}C]-opicapone administrations involving multiple metabolic pathways such as sulfation, methylation, *N*-oxide reduction and GSH conjugation. The main opicapone circulating metabolites arise from 3-*O*-sulfate conjugation and main route of excretion is faeces, were opicapone may be further metabolized via reductive metabolism involving the ring-opening reduction and subsequent hydrolysis of the oxadiazole ring.

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COMPETING INTERESTS

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare all were employees of BIAL – Portela & C^a, S.A. in the previous 3 years.

CONTRIBUTORS

Participated in research design: A.I.L., F.R., N.S., P.S.-d-S. Conducted experiments: A.I.L., F.R., A.T.S., N.S. (Principal Investigator). Contributed new reagents or analytic tools: L.E.K. and R.P. Performed data analysis: A.I.L. Wrote or contributed to the writing of the manuscript: A.I.L., M.J.B. and P.S.-d-S.

CONSENT TO PARTICIPATE

Each patient provided written informed consent before participation. Each investigator received prospective approval of the study protocol, protocol amendments, informed consent documents and all other relevant documents from their individual institution's review board or ethics committee.

DATA AVAILABILITY STATEMENT

Due to the nature of this research, participants of this study did not agree for their data to be shared publicly, so supporting data is not available.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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