ORIGINAL ARTICLE

Metabolite V, an epoxide species is a minor circulating metabolite in humans following a single oral dose of deflazacort

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Abstract

Deflazacort (Emflaza) was approved in the United States in 2017 for the treatment of the Duchenne muscular dystrophy in patients aged 2 years and older. Several deflazacort metabolites were isolated and identified from rats, dogs, monkeys, and humans. Among them, 1ß,2ß-epoxy-3ß-hydroxy-21-desacetyl deflazacort, referred to as Metabolite V, was reported to be one of the major circulating metabolites in humans. However, its quantitative distribution in plasma was not fully characterized. The objective of this study was to determine deflazacort plasma pharmacokinetics, metabolite profiles and their quantitative exposures in humans following a single oral dose. Six healthy male subjects were each administered a single oral dose of 60 mg [^{14}C]-deflazacort. Plasma and urine were collected and deflazacort metabolites in plasma were quantified by high performance liquid chromatography radio-profiling followed by liquid chromatography-mass spectrometry characterization. Metabolite V was isolated from urine and its structure was further confirmed by nuclear magnetic resonance analysis. These analyses demonstrated that deflazacort was not detectable in plasma; of the eight circulating deflazacort metabolites identified or characterized, the pharmacologically active metabolite 21-desacetyl deflazacort and inactive metabolite 6ß-hydroxy-21-desacetyl deflazacort accounted for 25.0% and 32.9% of the 0-24 hours plasma total radioactivity, respectively, while Metabolite V, an epoxide species, was a minor circulating metabolite, representing only about 4.7% of the total plasma radioactivity.

KEYWORDS

1β,2β-epoxy-3β-hydroxy-21-desacetyl deflazacort, 21-desacetyl deflazacort, deflazacort metabolism, Metabolite V

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1 | INTRODUCTION

Duchenne muscular dystrophy (DMD) is a recessive X-linked form of muscular dystrophy which results in muscle degeneration, difficulty walking, breathing, and eventually, death.[1] The incidence is approximately one in 3500 live male births,[2,3] Deflazacort (DFZ), a glucocorticoid with anti-inflammatory and immunosuppressive effects, is used in treating a variety of diseases, including the inflammation associated with DMD. DFZ is currently approved by the US Food and Drug Administration (FDA) as a treatment for patients with DMD aged ≥2 years,[4] and is the only approved small molecule drug in the US for use in DMD patients.

DFZ is an inactive pro-drug which is rapidly converted by esterases to the active metabolite 21-desacetyl deflazacort (21-desDFZ) after oral administration. This metabolite is further metabolized to several other inactive metabolites mainly by CYP3A4.[4,5] Following a single 5 mg/kg oral dose in rats, dogs, and monkeys, oral bioavailability of 21-desDFZ was 92%, 37%, and 43%, respectively; and of the dosed radioactivity, 54.3%, 18.0%, and 49.9% excreted in urine and 37.2%, 82.2%, and 39.4% was recovered in feces in rats, dogs, and monkeys, respectively.[5] After a 50 mg oral dose of [^{14}C]-DFZ to human subjects, 68.4% of the dose was present in urine and 30.7% of the dose was found in feces.[5]

The characterization of DFZ metabolites was first reported by Martinelli and colleagues.[6] Five metabolites, designated as Metabolites I to V, on the basis of their decreasing normal phase thin layer chromatographic (TLC) retention factor values, were isolated from rat cytosolic incubation sample or from bile and urine samples following a single oral [^{14}C]-DFZ dose in rats, dogs, and humans. Based on ultraviolet (UV), infrared (IR), mass spectrometry (MS), and proton nuclear magnetic resonance (^{1}H-NMR) spectral analyses, Metabolites I to IV were identified as: 4,5-dihydro-21-desDFZ (I), 21-desDFZ (II), 6β-OH-21-desDFZ (III), and 3-OH-1,2,4,5-tetrahydro-5-en-21-desDFZ (IV). Due to impurity interference, the structure of Metabolite V was not determined but was proposed to be a mono-oxidation product, probably in the B-ring (Figure 4), of dihyrdro-21-desDFZ.[6]

Similarly, following a single 5 mg/kg [^{14}C]-DFZ oral dose in monkeys, six metabolites were isolated and identified from urine: 21-desDFZ (II), 6β-OH-21-desDFZ (III), 6α-OH-21-desDFZ (VI), 4,5-dihydro-6α-OH-21-desDFZ (VII), 20-hydroxy-21-desDFZ (VIII), and 11-keto-21-desDFZ (IX).[7] Quantitative TLC-autoradiography analysis indicated that 21-desDFZ (II) and 6β-OH-21-desDFZ (III) were the major metabolites in plasma and urine, respectively in rats, dogs, monkeys, and humans.

Among the other metabolites, 4,5-dihydro-21-desDFZ (I) was isolated from rat cytosolic incubation samples; 3-hydroxy-1,2,4,5-tetrahydro-5-en-21-desDFZ (IV) was found in rat and human urine in a small amount (1.5%-2.5% of dose), while Metabolites VI to IX isolated from monkey urine together accounted for about 10% of dose.[5]

Metabolite V, reported to be accounting for 13%-20% of the administered dose in urine and for 25% of the plasma radioactivity following a single oral dose in humans,[6] was later isolated from human urine and its structure was proposed to be

1β,2β-epoxy-3β-hydroxy-21-desacetyl deflazacort (1,2-epoxy-3-OH-21-desDFZ) using IR, MS, and NMR analyses.[8]

Though several DFZ metabolites have been isolated and characterized from rat, dog, monkey, or human urine samples, their relative distribution was estimated using TLC-autoradiography analysis. Circulating DFZ metabolites including Metabolite V, have not been fully and systematically addressed in humans so far. In a previous study, the amount of deflazacort metabolites in humans were quantified using TLC-autoradiography.[6] Compared with the modern chromatographic separation techniques such as high performance liquid chromatography (HPLC), by which, complex analytes can be well separated from each other and then accurately quantified using sensitive radioactivity detection equipment such as microplate scintillation and luminescence counter technology, the use of TLC autoradiography, the only available tool at the time of the above studies, was not sensitive enough to detect minor metabolites. It also had the fundamental limitation that it overestimates the metabolite distribution due to limited resolution power of TLC as a tool for complex analytes separation. There could be potentially prominent metabolite(s) of DFZ that have not yet been identified or accurately quantified. In particular, Metabolite V, an epoxide metabolite, was reported as one of the major plasma metabolites following 50 mg single oral dose in three healthy volunteers,[6] which triggered an FDA postmarketing requirement (PMR) study to characterize and quantify DFZ circulating metabolites. Consequently, a clinical study was conducted to characterize and quantify DFZ plasma metabolites, in particular Metabolite V, following a single 60 mg oral dose of ^{14}C-DFZ in healthy male subjects.

The results presented here demonstrate that deflazacort was not detectable in plasma; the pharmacologically active metabolite 21-desacetyl deflazacort and inactive metabolite 6ß-hydroxy-21-desacetyl deflazacort were the major circulating metabolites; however, in contrast to a previous report, Metabolite V, an epoxide species, was a minor circulating metabolite following a single 60 mg oral dose of ^{14}C-DFZ in healthy male subjects.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Deflazacort (>99% purity) was manufactured by Sterling S.p.A. (Perugia, Italy), and [^{14}C]-DFZ (>129 μCi/mg, ≥99% radiochemical and chemical purity) was synthesized by ALMAC Sciences; 6α-OH-21-desDFZ and 6β-OH-21-desDFZ were supplied by Toronto Research Chemicals; Polyethylene glycol 400 and polysorbate 80 were obtained from Spectrum Chemical Mfg. Corp.; Formic acid, hydrogen peroxide, and ammonium hydroxide were purchased from JT Baker; Ascorbic acid was purchased from Sigma-Aldrich; Potassium phosphate buffer was purchased from Teknova; Acetonitrile and methanol were obtained from EM Science; SOLVABLE^{TM} was purchased from PerkinElmer; Liquid scintillation cocktail was obtained from the R. J. Harvey Instrument Corporation (Tappan); ACS aqueous counting scintillant was purchased from Amersham Biosciences.

2.2 | Methods

2.2.1 | Study design and sample collection

This was an open-label, nonrandomized, single-dose, 1-period, absorption, and metabolism study. The study was carried out in accordance with the Declaration of Helsinki and the International Council for Harmonisation Good Clinical Practices (ICH GCP). The institutional review board (IRB) reviewed and approved the clinical study protocol, clinical study protocol amendments, subject information sheets, written informed consent forms (ICFs), and other relevant documentation. Prior to participation in the study, each subject was apprised of the nature and purpose of the study, and ICF was obtained.

Six healthy, adult, male subjects, 21 to 52 (median 36.5) years of age, 64.5-101 (median 80.7) kg of body weight and a body mass index of 23.1-28.7 (median 24.9) kg/m^2 were enrolled. On Day 1, subjects reported to the clinic. On Day 1, after a 10-hour overnight fast, each subject received a single 60 mg (100 µCi) oral dose of ^{14}C-DFZ and remained confined in the clinical research unit until at least Day 3 and up to a maximum stay of 6 days postdose (Day 7).

^{14}C-DFZ was formulated as a solution of 3 mg/mL in polyethylene glycol 400/polysorbate 80/purified water. Blood (for whole blood and plasma) samples was collected at predose and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 16, 20, 24, 36, 48, 72, 96, 120, and 144 hours postdose; urine samples were collected at predose and every 24 hours up to 120 hours postdose. Starting on Day 3, subjects could be discharged from the clinic, if ≥3 collected blood samples contained acceptably low radioactivity levels (eg, plasma total radioactivity <3% of the C$_{max}$) and ≥2 consecutive urine samples contained radioactivity levels <1% of the administered dose. If these criteria were not met by Day 3, subjects continued to be confined until the discharge criteria were met or up to a maximum stay of 6 days postdose (Day 7).

2.2.2 | Sample preparation for radioactivity determination

Duplicate aliquots of blood, plasma, and urine samples were taken for total radioactivity (TRA) measurement by liquid scintillation counting (LSC). Plasma samples at 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 hours from six subjects, 66 samples in total, were selected for radio-profiling individually based on the overall plasma radioactivity—time profile. Urine samples (0-24 hours) from all six subjects were selected for Metabolite V isolation.

2.2.3 | Plasma sample preparation for metabolite profiling

A portion (2.2 mL to 4.0 mL, depending on sample availability) of each completely thawed plasma sample was diluted to 10 mL with 10 mmol/L potassium phosphate buffer (pH 7.4). The mixture was centrifuged at 10 000g at 4°C for 10 minutes. The supernatant was collected for solid phase extraction (SPE) cleanup. The SPE cartridge (Waters Oasis® HLB 6cc) was conditioned by 6 mL methanol followed by 12 mL water. The supernatant was loaded into the cartridge, followed by washing with 10 mL of 1.8% w/v ascorbic acid in water. The loading and washing solutions were collected and combined. Deflazacort and metabolites were eluted with 10 mL of 0.09% w/v ascorbic acid in acetonitrile:water (98:2, v/v), followed by additional 5 mL of 0.09% w/v ascorbic acid in acetonitrile:water (98:2, v/v). The eluates were combined and evaporated to dryness under a nitrogen stream. The residues were reconstituted with an appropriate volume of 0.1% formic acid in water: methanol (1:1, v/v) for radio-profiling.

2.2.4 | Determination of plasma metabolite profiles

The metabolite profiles were determined by HPLC radio-chromatography using a Waters 2695 HPLC system. An Ace C18 Excel column (3 µm, 150 × 4.6 mm; MAC-MOD Analytical, Inc.) maintained at 35°C, and two solvent systems of 0.4% formic acid in water, pH 3.2, adjusted with ammonium hydroxide (A) and 100% acetonitrile (B) were used. The flow rate was 0.7 mL/min and the linear gradients were: 0% B for 1 minute; 0% to 5% B in 2 minutes; 5% to 30% B in 52 minutes, 30% to 100% B in 5 minutes, hold 100% B for 2 minutes; 100% to 0% B in 1 minute and hold 0% B for 15 minutes. HPLC fractions were collected by time (15 sec/fraction) to Deepwell LumaPlate-96 plates (PerkinElmer Life Sciences). The plates were subsequently dried by a SpeedVac concentrator (Savant Instruments Inc.) for up to 8 hours. The radioactivity in each fraction was determined by Packard TopCount NXT Microplate Scintillation and Luminescence Counter technology (PerkinElmer Life Sciences). LC radio-chromatograms were reconstructed using Laura™ software (LabLogic). Radioactivity peaks were integrated to determine the percent distribution of individual radioactivity peaks or regions in each sample.

2.2.5 | Metabolite identification

LC-MS analysis

Plasma extracts were analyzed by liquid chromatography-mass spectrometry (LC-MS) using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was equipped with an electrospray ionization source operated in positive ion mode with a capillary temperature of 275°C and ion spray voltage of 3.5 kV. The sheath gas, auxiliary gas, and sweep gas pressures were 45, 20, and 10 units, respectively. The resolving power was set at 70 000 for full scan and 17 500 for product ion scan.

Metabolite V isolation and identification

Urine samples (0-24 hours) from all six subjects were selected for Metabolite V purification. The urine sample from each subject (ca. 1 L) was partitioned twice by liquid-liquid extraction with ethyl

acetate (1.5×, v/v). The ethyl acetate fractions from all six subjects were combined, followed by concentration under rotary evaporator. The concentrated ethyl acetate fraction was dried under nitrogen, and re-dissolved with ca. 1.5 mL of methanol:water (1:1, v/v). The reconstituted sample was subject to HPLC purification. The HPLC conditions were the same as used for plasma metabolite profiling except for the mobile phase and gradient changes: mobile phase A was 10 mmol/L ammonium acetate in water, pH 5.6 adjusted with acetic acid and mobile phase B was 100% acetonitrile. The flow rate was 0.7 mL/min and the linear gradients were: 0% to 25% B in 3 minutes; 25% to 31% B in 17 minutes, 31% to 100% B in 3 minutes, hold 100% B for 5 minutes; 100% to 0% B in 1 minute and hold 0% B for 15 minutes. HPLC fractions were collected by time (10 sec/fraction) into 15 mL centrifuge tubes. Fractions from 12.0 to 13.8 minutes containing Metabolite V were combined, dried under the nitrogen stream, and reconstituted in ca. 400 μL methanol:water (1:1, v/v). The reconstituted sample was subject to a second step HPLC purification. The HPLC conditions were the same as used for plasma metabolite profiling except for the gradient changes: the flow rate was 0.7 mL/min and the linear gradients were: 0% to 25% B in 3 minutes; 25% to 30% B in 17 minutes, 30% to 100% B in 3 minutes, hold 100% B for 3 minutes; 100% to 0% B in 1 minute and hold 0% B for 15 minutes. HPLC fractions were collected by time (10 sec/fraction) into 15 mL centrifuge tubes. Fractions from 13.0 to 13.8 minutes containing Metabolite V were combined, dried under the nitrogen stream, and the residue was reconstituted in ca. 500 μL chloroform-d3 for NMR analysis. All NMR experiments were collected on a Bruker 500 MHz spectrometer (Bruker BioSpin) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C equipped with a 5 mm QNP probe. 1D ^{1}H- and ^{13}C-NMR and 2D heteronuclear single quantum coherence spectrometry (HSQC) data were collected at room temperature.
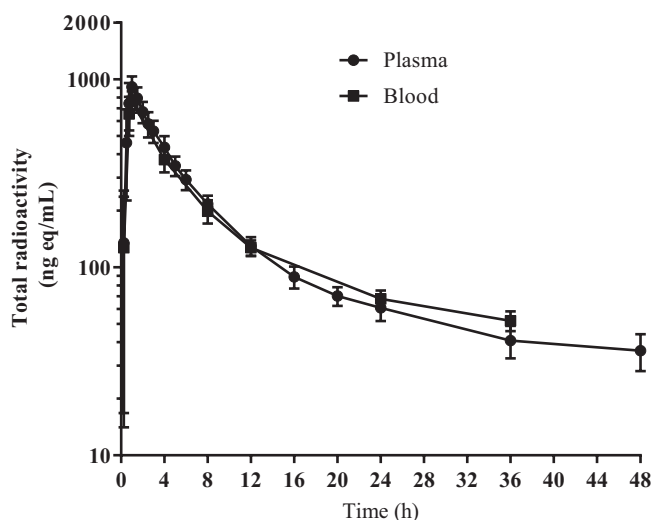
FIGURE 1 Mean ± SD plasma and blood concentration of total radioactivity vs time profile after 60 mg oral administration of [^{14}C]-deflazacort to six healthy male human subjects

2.2.6 | Pharmacokinetic analysis

Plasma pharmacokinetic (PK) parameters were calculated using noncompartmental method within Phoenix WinNonlin (version 6.3, Pharsight Corporation). The PK parameters were obtained using the plasma concentrations (expressed as ng-Eq./mL) of deflazacort and its metabolites or TRA. The area under the plasma concentration-time curves (AUC_{0-t}) were computed using the linear trapezoidal method. T_{max} and C_{max} were observed values.

3 | RESULTS

3.1 | Compliance and adverse events

All six male healthy subjects who received a 60 mg or 100 μCi oral dose of [^{14}C]-DFZ completed the study. Deflazacort was well tolerated and no adverse events were reported in this study.

3.2 | Excretion of dosed radioactivity in urine

Following a single 60 mg oral dose of [^{14}C]-DFZ, the mean ± SD recovery of the administered dose was 53.1 ± 6.32% and 57.6 ± 5.17% in 0-24 hours and 0-144 hours urine, respectively, which was slightly lower than the reported value in healthy human subjects following a single 50 mg oral dose, where 64.7% and 68.4% of the dose was excreted in 0-24 hours and 0-144 hours urine.[5]

TABLE 1 Pharmacokinetic parameters for total radioactivity in plasma following 60 mg oral administration of [^{14}C]-deflazacort to healthy human subjects

| Parameters | ^{14}C-deflazacort-derived total radioactivity | | |
	Mean	SD	n
AUC_{0-t} (ng eq × h/mL)	6389[a]	17.7[b]	6
AUC_{0-inf} (ng eq × h/mL)	7067[a]	9.9[b]	5
C_{max} (ng eq/mL)	924[a]	12.6[b]	6
T_{max} (h)	1.09 (0.78, 1.52)[c]	NA	5
$T_{1/2}$ (h)	22.2	6.97	6
T_{last} (h)	52.03	22.3	6

Abbreviations: AUC_{0-t}, area under the plasma concentration-time curve from 0 hour to last measurable concentration time point; AUC_{0-inf}, area under the plasma concentration-time curve from 0 hour to infinity; C_{max}, maximum concentration; eq, equivalent; NA, not applicable; $T_{1/2}$, the terminal half-life; T_{max}, time to maximum concentration; T_{last}, last measurable concentration time point.

[a]Geometric mean.

[b]Geometric CV%.

[c]T_{max} is median (minimum, maximum).

3.3 | Blood and plasma TRA and blood-to-plasma concentration ratios

Following a single 60 mg oral dose of [^{14}C]-DFZ, [^{14}C]-DFZ-derived TRA was measurable up to 36 hours post dose in both blood and plasma in all subjects and was nondetectable in the majority of subjects at 72 hours and beyond. [^{14}C]-DFZ-derived TRA was associated more with plasma or was equally dispersed between the plasma and blood cells with a blood-to-plasma concentration ratio ranging from 0.84 to 1.30. The mean ± SD blood and plasma TRA vs time profiles are shown in Figure 1 and the plasma PK parameters are summarized in Table 1.

3.3.1 | Identification of metabolites

Similarities in HPLC retention times, high accuracy mass data and the mass spectrometric fragmentation patterns to available reference standards facilitated the identification of metabolites 6α-OH-21-desDFZ (VI) and 6β-OH-21-desDFZ (III). Structures of other metabolites were proposed based on their high accuracy mass data and mass spectrometric fragmentation patterns relative to DFZ or the known metabolites.

Two plasma metabolites eluting at ca. 53 and 57 minutes (Figure 2) had a molecular weight identical to that of Metabolite V with fragmentation patterns similar to each other. Therefore, it was not possible to determine which one was Metabolite V by LC-MS alone considering that the reference standard of Metabolite V is not commercially available. To that end, the corresponding metabolite eluting at ca. 53 minutes in 0-24 hours urine was isolated and purified for NMR analysis. One- and two-dimensional NMR data of the isolated metabolite (Table 2) showed ^{1}H- and ^{13}C-chemical shift values similar to those of the reported values for Metabolite V; therefore, the plasma metabolite eluting at ca. 53 minutes was confirmed to be Metabolite V, that is, 1β,2β-epoxy-3β-hydroxy-21-desacetyl deflazacort.

3.3.2 | Metabolite profiles in plasma

Following a single 60 mg oral dose of [^{14}C]-DFZ, DFZ was not detectable in any plasma samples. In total, eight prominent plasma metabolites were identified or characterized and five of them, namely,
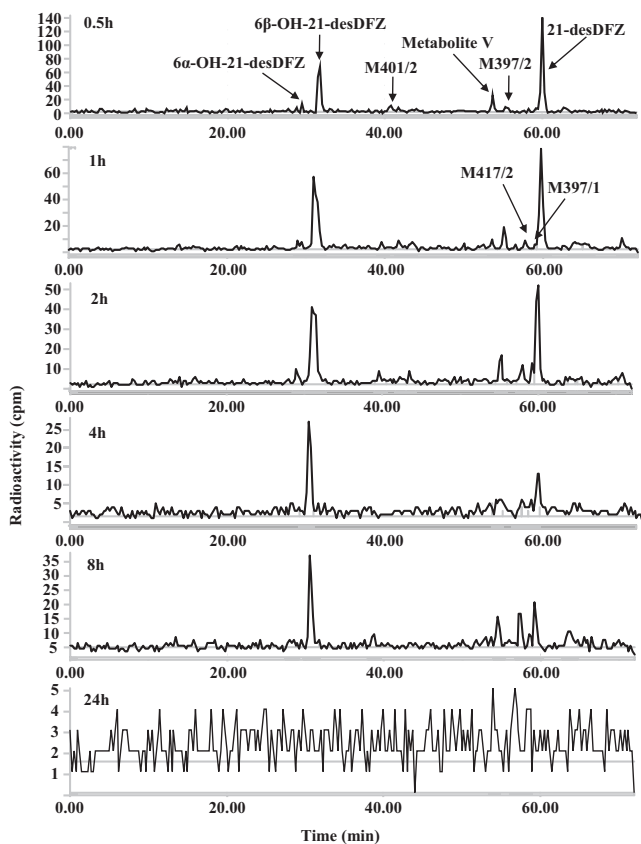
FIGURE 2 Representative plasma metabolite profile after 60 mg oral administration of [^{14}C]-deflazacort

TABLE 2 Characteristic proton and carbon NMR data of 1β,2β-epoxy-3β-hydroxy-21-desacetyl deflazacort (Metabolite V)

Structure	H/C Number	Reported[a] (ppm)	Measured (ppm)
	H-1	3.45	3.36
	H-2	3.32	3.23
	H-3	4.46	4.37
	H-4	5.23	5.15
	H-21	4.31, 4.56	4.24, 4.45
	C-1	57.8	57.5
	C-2	55.5	55.5
	C-3	63.4	63.2
	C-4	114.7	115.0
	C-21	67.5	67.5

[a]Huber EW, Barbuch RJ. Spectral analysis and structural identification of a major deflazacort metabolite in man. *Xenobiotica* 1995; **25**:175-183.

TABLE 3 Pharmacokinetic parameters for plasma metabolites following 60 mg oral administration of [^{14}C]-deflazacort to healthy male human subjects

| Metabolite	Met. code in the literature[a]	Biotransformation	Mass difference (ppm)	C_{max} (ng eq/mL)		T_{max} (h)		AUC[b] (h × ng eq/mL)		%TRA by AUC[b]	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total radioactivity	NA	NA	NA	896	118	1.04	0.25	4700	537	100	0.00
Deflazacort	NA	Parent	−0.6792	Not detectable							
21-desDFZ	Met. II	Deacetylation	−0.0959	357	70.3	0.96	0.10	1170	179	25.0	2.56
M397/1	Met. IX[c]	Dehydrogenation of 21-desDFZ	−0.0766	22.3	13.3	3.83	2.04	89.9	57.7	1.87	1.21
M397/2	Met. IX[c]	Dehydrogenation of 21-desDFZ	0.2314	51.7	7.16	1.83	1.17	496	139	10.8	3.40
M401/2	Met. I[c]	Reduction of 21-desDFZ	0.6602	32.9	6.96	1.67	1.25	125	84.4	2.64	1.74
6α-OH-21-desDFZ	Met.VI	Mono-oxidation of 21-desDFZ	0.5157	40.6	6.58	1.46	0.51	168	40.2	3.59	0.81
6β-OH-21-desDFZ	Met. III	Mono-oxidation of 21-desDFZ	0.2745	289	38.5	1.13	0.31	1540	275	32.9	5.10
1,2-Epoxy-3-OH-21-desDFZ	Met. V	Reduction and mono-oxidation of 21-desDFZ	0.7248	62.9	17.0	1.54	2.19	229	156	4.74	2.80
M417/2	Met. VII[c]	Reduction and mono-oxidation of 21-desDFZ	0.5541	32.9	4.95	3.50	1.52	386	118	8.13	2.18

Abbreviations: AUC_{0-24}, area under the plasma concentration-time curve from hour 0 to 24 hours post dose; AUC_{0-t}, area under the plasma concentration-time curve from hour 0 to the last time point with measurable concentrations; C_{max}, maximum concentration; 21-desDFZ, 21-desacetyl deflazacort; 1,2-epoxy-3-OH-21-desDFZ, 1,2-epoxy-3β-hydroxy-21-desacetyl deflazacort; 6α-OH-21-desDFZ, 6α-hydroxy-21-desacetyl deflazacort; 6β-OH-21-desDFZ, 6β-hydroxy-21-desacetyl deflazacort; eq, equivalent; Met, metabolite; NA, not available or not applicable; T_{max}, time to maximum concentration; TRA, total radioactivity.

[a]Assandri A, Buniva G, Martinelli E, Perazzi A, Zerilli L. Pharmacokinetics and metabolism of deflazacort in the rat, dog, monkey and human. *Adv Exp Med Biol* 1984; **171**:9-23.

[b]AUC_{0-24} for plasma TRA or AUC_{0-t} for plasma metabolite.

[c]Possible metabolite code in the literature.
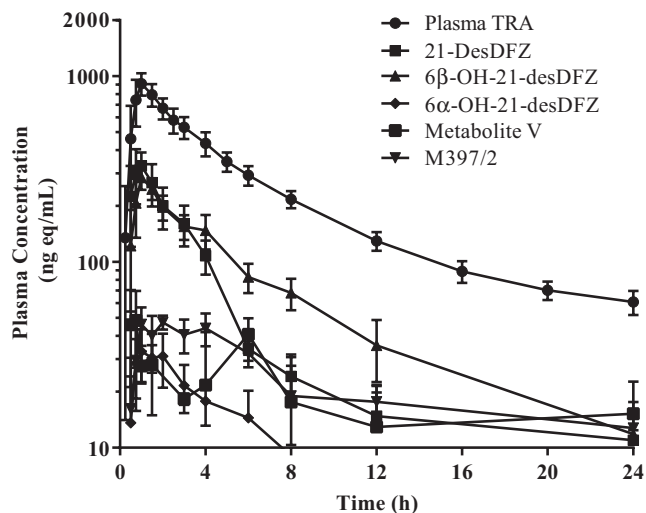
FIGURE 3 Mean ± SD plasma concentration vs time profile of prominent DFZ metabolites after 60 mg oral administration of [^{14}C]-deflazacort to six healthy male human subjects

FIGURE 4 Proposed metabolic pathways for deflazacort following a single oral administration in healthy male human subjects

6α-OH-21-desDFZ (VI), M397/1, M397/2, M401/2, and M417/2 were reported as minor human metabolites for the first time. Their corresponding metabolite codes in previous reports are shown in Table 3. All of these metabolites are oxidation or reduction products of 21-desDFZ (II), and together, they accounted for 89.6% of TRA in 0-24 hours plasma (Table 3). The representative metabolite profiles of DFZ in plasma from one subject are shown in Figure 2. The mean ± SD metabolite concentration vs time profiles are shown in Figure 3. The distribution of circulating metabolites and the PK parameters are summarized in Table 3. 6β-OH-21-desDFZ (III) was the most abundant circulating metabolite, accounting for 32.9% AUC_{0-24} of TRA, followed by 21-desDFZ (II), accounting for 25.0% of TRA. M397/2 was a less abundant metabolite, accounting for 10.8% of TRA. Other minor metabolites included M417/2, 1,2-epoxy-3-OH-21-desDFZ (V), 6α-OH-21-desDFZ (VI), M401/2 and M397/1, each accounting for 8.13%, 4.74%, 3.59%, 2.64%, and 1.87% AUC_{0-24} of TRA, respectively. Proposed metabolic pathways for the formation of the DFZ metabolites are shown in Figure 4.

4 | DISCUSSION

In the current study, following a single 60 mg [^{14}C]-DFZ oral dose in six healthy human subjects, 0.5 to 24 hours individual plasma samples from each subject were used for HPLC radio-profiling and eight prominent circulating metabolites accounting for 89.6% of TRA in 0-24 hours plasma were identified or characterized. Quantitative distribution analysis showed that 6β-OH-21-desDFZ (III) was the most abundant circulating metabolite, accounting for 32.9% plasma AUC of TRA, followed by 21-desDFZ (II), accounting for 25.0% of TRA, which are similar to previous results.[6] The major metabolites, 21-desDFZ and 6β-OH-21-desDFZ have been characterized for their in vitro cytochrome P450- and transporter-mediated drug interaction potential and were also monitored in toxicology and clinical
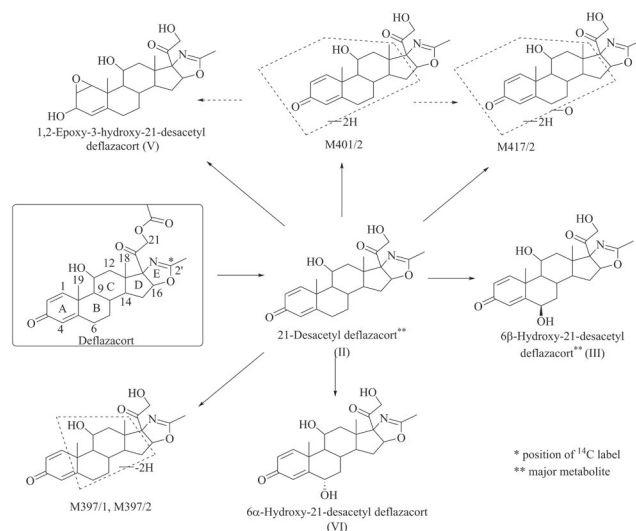
studies as well. The results will be published elsewhere. Of the minor human plasma metabolites, 6α-OH-21-desDFZ (VI) was previously detected in monkey only[5]; M397/1, M397/2, M401/2, and M417/2 are assumed to be the same metabolites as previously reported in animal species (Table 3), however, without reference standards, their identity cannot be confirmed by LC-MS alone, and the current MS data generated via electrospray ionization (ESI) are incomparable to previous MS data which were all generated with electron impact ionization, the popular MS ionization technique prior to wide use of ESI-based modern LC-MS instruments.

The finding that Metabolite V was a minor circulating metabolite in this study is in contrast to a prior study which found it was a major metabolite in human plasma accounting for 25.2% of TRA in pooled (1, 2, 4, and 8 hours) plasma following a 50 mg oral dose in three healthy volunteers.[6] The higher plasma distribution of Metabolite V in the previous study probably resulted from 1) unrepresentative plasma sample pooling (1-8 hours uniform pool instead of 0-24 hours AUC pool) and 2) limitations of separation technique available at that time when TLC autoradiography analysis was the main tool for quantitative analysis. Due to the limited resolution capability of TLC, metabolites with similar physiochemical properties could be co-eluted leading to an overestimation of analyte of interests. In the current study, HPLC conditions were carefully optimized so that all [^{14}C]-DFZ metabolites were well separated using a long HPLC column with a long gradient, and the plasma concentrations of each metabolite were quantified using highly sensitive microplate scintillation and luminescence counter technology after HPLC fractionation. As a result, 89.6% of TRA in 0-24 hours plasma was identified or characterized and was attributed to these metabolites (Table 3).

Epoxides are oxidation products on aromatic or double bonds. The epoxides can be endogenous intermediates that are natural signaling molecules such as epoxyeicosatrienoic acids that are vasodilators and can influence both renal function and peripheral

vascular tone and are also involved in the long-term control of blood pressure[9]; epoxides can be toxic, such as aflatoxin B1 exo-8,9-epoxide that can react with guanine in DNA to cause DNA damage[10]; or like carbamazepine-10,11-epoxide, they can be pharmacologically active but toxic beyond certain level.[11] However, chemically and metabolically stable epoxides that are less reactive or nonreactive to epoxide hydrolase, resistant to nucleophilic attack or excreted as stable metabolites, have been reported.[12-15] This study and an earlier work from others[8] which found that 1,2-epoxy-3-OH-21-desDFZ (Metabolite V) is a stable metabolite as evidenced by successful isolation from human urine for structure elucidation; the absence of downstream metabolites, for example, epoxide hydrolysis products or glutathione adducts in the circulation may also suggest that 1,2-epoxy-3-OH-21-desDFZ be metabolically stable and less reactive toward nucleophiles. Attempts were made to synthesize authentic reference standard chemically or by using in vitro metabolic systems (bacteria, fungi, recombinant human CYPs or liver microsomes). However, due to the complex stereochemistry nature of 1,2-epoxy-3-OH-21-desDFZ, synthesis of authentic reference standard was not successful, which prevented us from further evaluating its biological activity, stability, and metabolism in vitro and in vivo. Nevertheless, 1,2-epoxy-3-OH-21-desDFZ is a minor metabolite following a single oral dose. There are extensive historical data regarding the exposure of deflazacort in DMD patients, as well as in patients with other inflammatory conditions. The side effects of DFZ in humans are well characterized and consistent with the glucocorticoid class, but deflazacort has demonstrated less weight gain compared with prednisone, the corticosteroid most commonly used in DMD patients in the US.[16,17]

The previous human radiolabeled study was conducted in 1979, where urinary metabolites were characterized using IR, MS, and NMR analyses after isolation and purification from urine samples,[6] while circulating metabolites were less characterized due to complex metabolite profiles and low plasma concentrations. As an FDA PMR study, the current study largely focused on the circulating metabolites of DFZ following a single oral dose in healthy human subjects, and as a result, approximately 90% of 0-24 hours plasma radioactivity was identified or characterized and was attributed to DFZ metabolites. Additional research such as in vitro and in vivo studies investigating biological and pharmacological activities of DFZ metabolites, DFZ metabolite formation pathways, and the metabolite profiles of DFZ in bile and/or feces will further benefit our understanding on the metabolism and disposition of deflazacort.

In summary, as an FDA postmarketing requirement, human circulating metabolites of deflazacort following a single oral dose were fully quantified and characterized for the first time. Among the eight circulating metabolites identified or characterized, 21-desDFZ and 6β-OH-21-desDFZ were the major metabolites. In particular, we demonstrate that Metabolite V, an epoxide previously reported as a major deflazacort metabolite, is actually a minor circulating metabolite in healthy human subjects following a single oral dose.

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