Pharmacokinetics, Metabolism, and Routes of Excretion of Intravenous Irofulven in Patients with Advanced Solid Tumors

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ABSTRACT:
Irofulven is currently in Phase 2 clinical trials against a wide variety of solid tumors and has demonstrated activity in ovarian, prostate, gastrointestinal, and non–small cell lung cancer. The objectives of this study were to determine its pharmacokinetics and route of excretion and to characterize its metabolites in human plasma and urine samples after a 30-min i.v. infusion at a dose of 0.55 mg/kg in patients with advanced solid tumors. Three patients were administered i.v. 100 μCi of [14C]irofulven over a 30-min infusion on day 1 of cycle 1. Serial blood and plasma samples were drawn at 0 (before irofulven infusion) and up to 144 h after the start of infusion. Urine and fecal samples were collected for up to 144 h after the start of infusion. The mean urinary and fecal excretion of radioactivity up to 144 h were 71.2 and 2.9%, respectively, indicating renal excretion was the major route of elimination of [14C]irofulven. The Cmax, AUC0–t, and terminal half-life values for total radioactivity were 1130 ng-Eq/ml, 24,400 ng-Eq · h/ml, and 116.5 h, respectively, and the corresponding values for irofulven were 82.7 ng/ml, 65.5 ng · h/ml, and 0.3 h, respectively, suggesting that the total radioactivity in human plasma was a result of the metabolites. Twelve metabolites of irofulven were detected in human urine and plasma by electrospray ionization/tandem mass spectrometry. Among these metabolites, the cyclopropane ring-opened metabolite (M3) of irofulven was found, and seven others were proposed as glucuronide and glutathione conjugates.

Irofulven (6-hydroxymethylacylfulvene) is a semisynthetic acylfulvene derivative of illudin S. The sesquiterpenes, illudin S and illudin M, are toxic compounds obtained from the jack o’lantern mushroom Omphalotus illudens (McMorris and Anchet, 1965). These natural products possess antibacterial and antitumor properties but have an unfavorable toxicity profile when tested in animals (Kelner et al., 1987). Irofulven has a greater therapeutic index than the parent compound, illudin S (McMorris et al., 1996). Irofulven has strong concentration- and time-dependent cytotoxic effects in a broad variety of human cancer lines with natural and acquired resistance to classical and nonclassical alkylating agents (Poindessous et al., 2003). This derivative has been extensively investigated and is currently in Phase 2 clinical trials against a wide variety of solid tumors and has demonstrated activity in ovarian, prostate, gastrointestinal, and non–small cell lung cancer.

Irofulven is a derivative of acylfulvene; the stereoselective synthesis of this compound has been described by McMorris et al. (2004). The mechanism of action of irofulven is thought to involve enzymatic activation leading to an unstable intermediate that is subject to nucleophilic attack. Nucleophilic compounds such as proteins or DNA quench the reactive intermediate on the cyclopropane ring through opening and the concomitant loss of the tertiary hydroxyl in the C2 position.

Metabolism and excretion studies of irofulven (M) in rats after single i.v. administration of [14C]irofulven (M*) (see Fig. 1) have shown that the main excretion route was fecal, and two metabolites (M1 and M2) were identified in plasma and urine (Toda et al., 1998). Metabolism of irofulven by rat liver cytosol has produced four other metabolites identified by mass spectrometry and NMR studies (McMorris et al., 1999). In humans, the pharmacokinetics of irofulven has been characterized following i.v. administration over a wide range of doses from 1 to 17.69 mg/m² (Eckhardt et al., 2000; Alexandre et al., 2004).

The plasma concentrations of irofulven increased dose proportion-

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A. Paci and K. Rezai contributed equally to this work.

ABBREVIATIONS: irofulven, 6′-hydroxy-3′-hydroxymethyl-2′,4′,6′-trimethyl-spiro[cyclopropane-1,5′-5H-indenel]7′(6H)-one, 6-hydroxymethyla-
cylfulven; HPLC, high-performance liquid chromatography; GSH, glutathione; AUC, area under curve; CLp, total body clearance; Vdss, volume of distribution at steady state; MRT, mean residence time; AUMC, area under the first moment curve; ESI, electrospray ionization; MS/MS, tandem mass spectrometry.
ally during infusion and rapidly declined in a biexponential manner with a very short half-life of approximately 5 min and high body clearance up to 11 l/min. The mean volume of distribution was approximately 54 liters. The intersubject variability in pharmacokinetic parameters was moderate (Urien et al., 2003; Alexandre et al., 2004).

The metabolic profile and the routes of excretion of irofulven in humans are not completely known. Characterization of the pharmacokinetics, metabolism, and routes of excretion of irofulven in humans is of potential benefit in understanding the biotransformation of irofulven and may aid in further development of this drug. Therefore, the objectives of this study were to determine the pharmacokinetics, metabolism, and the route of excretion of irofulven after an i.v. dose of [14C]irofulven in patients with advanced solid tumors.

Materials and Methods

Chemicals. Commercially available chemicals and solvents used were high-performance liquid chromatography (HPLC) or analytical grade. Alltima, HPLC analytical columns were obtained from Alttech (Templemars, France). Instagel, Hionic-Flour, FLO SCINT III scintillation mixtures, and Solvène-350 were supplied by Perkin Elmer Life Science and Analytical Instruments Inc. (Villebon-sur-Yvette, France). Methylen chloride and pentane were purchased from Aldrich (St-Quentin Fallavier, France). Acetonitrile and isopropanol were obtained from VWR International Inc. (Fontenay-sous-Bois, France). Methanol was supplied by Carlo Erba (Val de Reuil, France). β-Glucuronidase was purchased from Sigma (St-Quentin Fallavier, France).

Reference Standards. [14C]Irofulven was supplied by Amersham (Cardiff, UK) and possessed a specific activity of 136.3 μCi/mg. The [14C]irofulven is labeled on the C-14 position (Fig. 1). Irofulven (HMAF, MGI-114) and its metabolites, the glutathione (GSH)-conjugate M1 and the cyclopropane ring-opened derivative M2, were provided by MGI PHARMA Inc. (Minneapolis, MN) as reference standards.

Study Design. Radioactive study. This was an open-label Phase 1, single-center, pharmacokinetic study of [14C]irofulven. Three patients were administered an i.v. infusion over 30 min with a mixture of 0.55 mg/kg dose of irofulven containing 100 μCi of [14C]irofulven on day 1 of cycle 1. Pharmacokinetic parameters were calculated from plasma irofulven concentration-time and plasma radioactivity concentration-time data using noncompartamental methods by WinNonlin version 4.1. The mean maximal plasma concentration of irofulven and total radioactivity (Cmax) and the time to reach Cmax (Tmax) were recorded from individual subject concentration-time curve. The terminal phase elimination half-life (t1/2) was determined as ln2/Kel. The area under the plasma concentration-time curve from time 0 to the last measurable concentration at time t (AUC0-t) was calculated using the linear trapezoidal method. For irofulven, AUC0-t was computed as AUC0-t plus the extrapolation from the last time point to infinity using C/Kel, where C is the last quantifiable concentration. For irofulven, the total body clearance (CLint) was calculated from the dose/AUC0-t. The volume of distribution at steady state (Vss) was calculated as CLint × MRT, where mean residence time (MRT) was calculated as [AUMC/AUC0-t] − τ2, where τ is the duration of infusion. The area under the first moment curve (AUMC) was determined using the linear trapezoidal rule and extrapolated to infinity as AUMC0-t + t × C/Kel + C/(Kel)2.

Nonradioactive study. To study the metabolic profile, nonradioactive plasma and urine samples were obtained because the mass spectrometry could only be performed with cold irofulven. Samples were obtained from three patients who were enrolled in another Phase 1 clinical and pharmacokinetic study of irofulven in combination with oxaliplatin in patients with advanced solid tumors.

Subjects. In the pharmacokinetic study (radioactive), three patients (two males, numbers 1 and 3/one female, number 2) were enrolled. In the metabolite percentages, the mean and standard deviation for each of the parameters are shown in Table 1. The cumulative excretion of total radioactivity in patients is shown in Fig. 2.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>% Cumulative Excretion (n = 3)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Urine (0–144 h)</td>
<td>71.2</td>
</tr>
<tr>
<td>Feces (0–96 h)</td>
<td>2.9</td>
</tr>
<tr>
<td>Urine + feces</td>
<td>74.1</td>
</tr>
</tbody>
</table>

Fig. 1. Structure of irofulven (M) and [14C]irofulven (carbon-15 is radiolabeled).

Fig. 2. Mean radioactivity concentrations in blood (○) and plasma (●), and mean irofulven in plasma concentration (□)-time profiles following 0.55 mg/kg dose containing 100 μCi [14C]irofulven administered over 30 min in patients with solid tumors.
olism study (nonradioactive), three patients (A, B, and C) were treated with 0.55 mg/kg cold irofulven.

**Inclusion criteria.** The inclusion criteria were histologically or cytologically confirmed malignant solid tumor, disease refractory to anticancer treatment or for which no standard treatment exists, and Eastern Cooperative Oncology Group Performance Status ≥ 2 (in bed <50% of time).

**Exclusion criteria.** The exclusion criteria were patients who have had radiation therapy to more than 30% of their bone marrow before entry into study; and previous chemotherapy with nitrosourea or high-dose carboplatin (>6 AUC), previous mitomycin C cumulative dose ≥25 mg/m², previous bone marrow transplantation, or intensive chemotherapy with stem cell support.

**Study Drug (Preparation, Administration).** A mixture of \[^{14}C\]irofulven and cold irofulven was dissolved with 150 ml of dehydrated ethyl alcohol and 2.85 ml of 5% dextrose in water and then administrated over 30 min as an i.v. infusion into a 100-ml minibag of 5% dextrose injection on day 1 of cycle 1. A total of 100 μCi of \[^{14}C\]irofulven (0.45 mg of irofulven), combined with cold irofulven to achieve a dose of 0.55 mg/kg, was administered.

**Sample Collection for Radioactive Study.** Blood samples were drawn at 0 (immediately before initiation of the irofulven infusion), 5, 10, 20, 30, 35, 40, 50, 60, 70, and 90 min, and 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, and 144 h after the start of infusion of \[^{14}C\]irofulven. Samples of blood (11 ml) were drawn in EDTA tubes from a peripheral venous access in the arm opposite drug administration. The blood sample was centrifuged for 5 min at 10,000 rpm at 4°C. The plasma was removed and divided into three equal volumes in transfer tubes and immediately stored at −70 to −80°C until analysis.

**TABLE 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total Radioactivity</th>
<th>Irofulven</th>
</tr>
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<tbody>
<tr>
<td>(C_{\text{max}}) (ng/ml)</td>
<td>1130 ± 174</td>
<td>82.7 ± 46.0</td>
</tr>
<tr>
<td>(T_{\text{max}}) (h)</td>
<td>0.72 ± 0.12</td>
<td>0.29 ± 0.10</td>
</tr>
<tr>
<td>(\text{AUC}_{\text{0-24}}) (ng·h/ml)</td>
<td>15,000 ± 634</td>
<td>30.6 ± 25.8</td>
</tr>
<tr>
<td>(\text{AUC}_{\text{0-24}}) (ng·h/ml)</td>
<td>24,400 ± 1780</td>
<td>65.5 ± 2.4</td>
</tr>
<tr>
<td>(t_{1/2}) (h) terminal half-life</td>
<td>116.5 ± 27.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>(V_{\text{diss}}) (l/kg)</td>
<td>11.8 ± 2.1</td>
<td>8.48 ± 0.14</td>
</tr>
<tr>
<td>(\text{AUC}_{\text{0-24}}) ratio(^a)</td>
<td>0.00209 ± 0.00180</td>
<td></td>
</tr>
<tr>
<td>(^{14}C)-AUC(_{\text{0-24}}) blood/plasma ratio</td>
<td>1.58 ± 0.737</td>
<td>0.820 ± 0.205</td>
</tr>
<tr>
<td>(^{14}C)-(C_{\text{max}}) blood/plasma ratio</td>
<td>0.820 ± 0.205</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Geometric mean.

\(^b\) Ratio of irofulven to radioactivity.

**FIG. 3.** Electrospray mass spectra of metabolite M₁ (A), metabolite M₂ (B), and irofulven standards (C).
to 72 h after the start of infusion. Tear samples were collected up to 48 h postdose using absorbent paper at 0 (baseline before the start of infusion), 28 (just before the end of infusion), and 30 min, and 6, 24, and 48 h after the end of infusion. Expired air was collected from each patient in scintillation vials at 0 (just before the start of infusion), 25 to 28 (just before the end of the infusion), and 30 min, and 6 and 24 h after the end of the infusion. The patient was allowed to blow gently into the pink solution of scintillation vials containing benzethonium hydroxide (approximately 1 M in methanol) with absolute ethanol and 1% phenolphthalein solution through a tubing set equipped with one-way valve to prevent aspiration of the solution (the pink color disappeared when approximately 2 mM CO2 was captured). All the samples were labeled and immediately frozen at −70 to 80°C until analysis.

Sample Collection for Metabolite Profiling and Identification. Urine and plasma samples were collected from three patients (A, B, and C) enrolled in another Phase 1 clinical and pharmacokinetic study of irofulven in combination with oxalaplatin.

Urine samples. For patients A and B, a predose urine sample was collected before the infusion of cold irofulven, and urine samples were collected 45 min after the start of infusion. An aliquot (1.5 ml) of each urine sample was extracted with 5 ml of the extraction mixture (methylene chloride/pentane, 50:50 v/v). After centrifugation for 10 min at 4000 rpm, the supernatant was extracted with 5 ml of the extraction mixture (methylene chloride/pentane, 50:50 v/v). After centrifugation for 10 min at 4000 rpm, the supernatant was evaporated. Enzymatic hydrolysis of the same urine samples of patients A and B was performed to detect glucuronide metabolites. Five hundred units of β-glucuronidase was added to 1.5 ml of each urine sample. The mixture was incubated at 37°C for 2 h. The reaction was stopped by the addition of the extraction mixture.

Plasma samples. Blood samples were drawn on the first dosing day at 0 min (before infusion of irofulven), 10 min, before the end of infusion (30 min), and 5, 10, 15, and 30 min postinfusion into a 7-ml lavender-top (EDTA-K3) evacuated tube, inverted to mix, and placed in an ice bath. The tubes were centrifuged within 30 min of collection at 4000 rpm for 10 min at 4°C. The plasma (approximately 2–2.5 ml) from each tube was immediately stored centrifuged within 30 min of collection at 4000 rpm for 10 min at 4°C. The plasma (approximately 2–2.5 ml) from each tube was immediately stored centrifuged within 30 min of collection at 4000 rpm for 10 min at 4°C. The plasma (approximately 2–2.5 ml) from each tube was immediately stored "vertical at (−70°C) until analysis.

For patients A and C, two extracts of control plasma (i.e., collected before the perfusion of irofulven) and two extracts of plasma (i.e., collected 60 min after the start of infusion of irofulven) were obtained according to the same procedure.

Determination of Radioactivity. Radioactivity in samples was measured by liquid scintillation counting. Aliquots of each plasma, whole blood, urine, feces, saliva, expired air, and tear sample (in duplicate) described above were placed into vials and mixed with a scintillation mixture and counted in a liquid scintillation counter for 5 min (Packard Tri-Carb 1900TR; Perkin Elmer Life Science and Analytical Instruments Inc.). Aliquots of feces and whole blood were dissolved with a mixture of Soluène-350 (1 ml) and isopropanol (0.5 ml) and decolorized with 30% hydrogen peroxide solution.

The radioactivity in the dose was expressed as 100%, and the radioactivity in the urine and feces at each sampling time was defined as the percentage of dose excreted in the matrices at that sampling time.

HPLC with Radioactivity Detection. Plasma and urine samples obtained during this study were assayed for irofulven by a validated HPLC method with a β-counter detection. The analytical HPLC system (Waters, St-Quentin en Yvelines, France) consisted of a 717 plus autosampler, a 515 pump and controlled by Millennium software for data handling, and a radioactive detector (β-RAM; IN/US system). Chromatography was performed on a reversed-phase column C18, 5 μm Alltima, 250 × 4.6 mm i.d. (Alltech) with a mobile phase containing a mixture of methanol, acetonitrile, and water (15:25:60, v/v/v) at 1 ml/min flow rate. Standard curve was prepared by isotopic dilution and was linear over 2.5 to 2500 ng/ml concentrations of irofulven.

HPLC with UV Detection. The HPLC system (Waters) consisted of two 510 pumps, a 710 autosampler, and a 486 UV detector, controlled by Millennium software. The standards and the extracted urine and plasma samples were analyzed on a reversed-phase column C18, 5 μm Alltima, 250 × 4.6 mm i.d. (Alltech) and detected at 260 nm UV wavelength. Isocratic elution (methanol/acetonitrile/water, 10:20:70, v/v/v) was carried out at 1 ml/min flow rate. The extracts were dissolved in 100 μl of solvent (acetonitrile/water, 50:50, v/v) before analysis of an aliquot by HPLC.

HPLC with Mass Spectrometry. The 1100 series HPLC system (Agilent Technologies, Massy, France) consisted of a binary pump, an autosampler fitted with a microbore column C18, 5 μm Uptisphere, 150 × 1 mm i.d. (Interchim, Montluçon, France). The flow rate of 50 μl/min was achieved with an elution gradient composed of solvent A (acetonitrile/water with 0.1% formic acid, 20:80, v/v/v) and of solvent B (acetonitrile/water with 0.1% formic acid, 80:20, v/v/v). After an initial 5-min step with 100% of solvent A, elution gradient was running from 0 to 70% solvent B up to 18 min and then back again to initial conditions up to 30 min. The standards and extracted samples were diluted in solvent A and then analyzed with the triple quadrupole mass spectrometer Quattro LCZ (Micromass-Waters, Manchester, UK) equipped with the Z-spray electrospray source. The HPLC/electrospray ionization tandem mass spectrometry (ESI-MS/MS) chromatograms were obtained by scanning in positive-ion mode over the m/z 150 to 600 range and processed with Masslynx NT software. ESI-MS/MS was performed with collision-induced dissociation of selected parent ions. The capillary and cone voltages were set at 3500 and 30 V, respectively. The collision energy was set at 10 eV, using argon as collision gas.

Results

Mass Balance. Mass balance after i.v. infusion of 0.55 mg/kg dose of irofulven containing 100 μCi of [14C]irofulven to human subjects was achieved with a 74.1% mean recovery of the administered dose in urine and feces. As shown in Table 1, the administrated radioactivity was eliminated predominantly in the urine. The mean urinary excretion...
tion amounted to 71.2% with the majority (88%) of radioactivity excreted in the first 24 h. The radioactivity in the urine was excreted up to 144 h after the start of infusion. The mean fecal recovery was 2.9%. Radioactivity was also measured in the expired air, tears, and saliva samples (data not shown). Urinary excretion was a major route of elimination of [14C]irofulven from the body.

**Pharmacokinetics.** Mean radioactivity and irofulven concentration time profiles are shown in Fig. 2. Plasma concentrations of irofulven increased during the 30-min infusion period and rapidly declined in a biexponential manner following discontinuation of infusion. A summary of plasma pharmacokinetic parameters is presented in Table 2. The mean $C_{\text{max}}$ was 1130 and 82.7 ng/ml for total radioactivity and irofulven, respectively. The mean total radioactivity AUC$_{0-\infty}$ was 24,400 ng·h/ml, and mean irofulven AUC$_{0-\infty}$ was 65.5 ng·h/ml. In plasma, irofulven was a minor circulating moiety representing approximately 0.27% of total radioactivity AUC. The plasma radioactivity disappeared with an initial distribution phase followed by a slow elimination phase and was measurable up to 144 h. The mean terminal phase elimination half-life was 116.5 and 0.3 h for total radioactivity and irofulven, respectively. The mean total body clearance and the apparent volume of distribution for irofulven were 8.48 l/h/kg and 3.12 l/kg, respectively. The blood/plasma distribution ratio of radioactivity was 1.6. Plasma irofulven pharmacokinetic profile and parameters are consistent with previous Phase 1 studies (Eckhardt et al., 2000; Urien et al., 2003).

**Analyses of Standards by HPLC/UV and HPLC/ESI-MS/MS.** Irofulven and metabolite standards $M_1$ (GSH conjugate) and $M_2$ (the cyclopropane ring-opened product) were analyzed by HPLC/UV at 260 nm and by HPLC/ESI-MS/MS. The HPLC/UV retention times ($t_R$) for irofulven, $M_1$, and $M_2$ were 15.7, 10.6, and 9.2 min, respectively.

<table>
<thead>
<tr>
<th>Irofulven and Metabolites</th>
<th>Mass (M)</th>
<th>Mass ESI-MS/MS Mass Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irofulven ($M_1$)</td>
<td>246</td>
<td>247, 229 (–H$_2$O), 211 (–2 H$_2$O)</td>
</tr>
<tr>
<td>M-GSH conjugate ($M_1'$)</td>
<td>535</td>
<td>536, 518 (–H$_2$O), 308, 229</td>
</tr>
<tr>
<td>M$_2$-GSH conjugate ($M_2'$)</td>
<td>537</td>
<td>538, 520 (–H$_2$O), 308</td>
</tr>
<tr>
<td>M$_2$-GSH conjugate ($M_2''$)</td>
<td>553</td>
<td>554, 536 (–H$_2$O), 518 (–2 H$_2$O)</td>
</tr>
<tr>
<td>M-glucuronide conjugate ($M_1$)</td>
<td>422</td>
<td>423, 405 (–H$_2$O)</td>
</tr>
<tr>
<td>Cyclopropane ring-opened metabolite ($M_2$)</td>
<td>248</td>
<td>249, 231 (–H$_2$O), 213 (–2 H$_2$O)</td>
</tr>
<tr>
<td>M$_2$-glucuronide conjugate ($M_2'$)</td>
<td>432</td>
<td>435, 407 (–H$_2$O), 249, 231</td>
</tr>
<tr>
<td>Hydroxylated metabolite ($M_3$)</td>
<td>262</td>
<td>263, 245 (–H$_2$O), 227 (–2 H$_2$O)</td>
</tr>
<tr>
<td>Hydroxylated metabolite ($M_3''$)</td>
<td>262</td>
<td>263, 245 (–H$_2$O), 227 (–2 H$_2$O)</td>
</tr>
<tr>
<td>M$_2$-glucuronide conjugate ($M_2''$)</td>
<td>438</td>
<td>435, 421 (–H$_2$O), 403 (–2 H$_2$O), 263</td>
</tr>
<tr>
<td>Reduced metabolite ($M_4$)</td>
<td>230</td>
<td>231, 213 (–H$_2$O), 203</td>
</tr>
<tr>
<td>Reduced and hydroxylated metabolite ($M_4$)</td>
<td>246</td>
<td>247, 229 (–H$_2$O), 219, 211 (–2 H$_2$O)</td>
</tr>
<tr>
<td>M$_2$-glucuronide conjugate ($M_2'$)</td>
<td>422</td>
<td>423, 405 (–H$_2$O)</td>
</tr>
</tbody>
</table>

Electrospray mass spectra of standards (Fig. 3) provided the following molecular masses of metabolites ($M_1 = 535.2$ and $M_2 = 248.2$) and irofulven ($M = 246.1$). The product ion mass spectrum (Fig. 3A) of irofulven-GSH conjugate $M_1$ (m/z 536) showed a characteristic fragment ion m/z 308 corresponding to the [GSH + H]$^+$ ion of GSH and another fragment ion (m/z 229), indicating the linkage between irofulven and GSH. The mass spectrum (Fig. 3B) of $M_2$ standard showed a molecular ion at m/z 249 and an intense m/z 231 fragment ion (249 – 18), corresponding to the water loss likely from the allylic hydroxyl at carbon 14 (Fig. 1) of metabolite $M_2$, and another ion (m/z 213) indicating an additional water loss from the hydroxyl on the opened cyclopropane. The mass spectrum (Fig. 3C) of
irofulven showed the molecular ion at m/z 247 and that two hydroxyls were present on the molecule by two successive losses of water (m/z 229 and 211). The HPLC/ESI-MS/MS analyses of the standards mixture were obtained by reconstructed ion mass chromatograms (m/z 536, 249, and 247) and indicated that M1 and M2 compounds eluted with a close polarity at acidic pH (tR/M3 15.4 min; tR/M2 15.5 min) and that the retention time of irofulven was 16.6 min.

Characterization of Metabolites in Urine Samples. The HPLC/UV analysis (Fig. 4A) of patient A urine extract, collected at 45 min after the start of infusion, showed the presence of several chromatographic peaks corresponding to irofulven (tR = 15.3 min), M1 (tR = 9.8 min), and M2 (tR = 9.3 min) and to other unknown products that were different from endogenous peaks detected in control urine (Fig. 4B).

The HPLC/ESI-MS/MS analyses (Fig. 5) of patient A urine (collected after and before the perfusion) confirmed these observations and detected several significant products. Seven metabolites of irofulven were characterized in the urine of patient A by their retention times, their molecular masses, and their fragment ions indicated by their ESI-MS/MS mass spectra corresponding to the selective ions m/z 536 (M1), 538 (M1'), 249 (M2), 263 (M3, M4), 231 (M6), and 247 (M3) (Fig. 5; Table 3).

The mass spectra of peaks (m/z 536, tR = 14.7 min) and (m/z 249, tR = 14.9 min) were identical to those of M1 and M2 standards, respectively (Fig. 3, A and B). Based on these data, the metabolite (M4 = 535) was characterized as the GSH conjugate of irofulven (246 + 307 – 18 = 535). The metabolite (M2 = 248) corresponded to the known (cyclopropane ring-opened) derivative of irofulven. A polar compound M5 (tR = 10 min) has a molecular mass of 553. Its mass spectrum showed the fragment ions m/z 536 ([M5 + H – H3O]+) and m/z 518 (536 – 18), indicating the presence of two hydroxyls. The fragment ion m/z 308 ([GSH + H]+) suggested that the structure could be a GSH conjugate resulting from the addition on the C-8,9 double bond of irofulven as shown by Dick et al. (2004). The mass spectrum of another compound (tR = 14.4 min) showed the molecular ion m/z 538 (M1' = 537) and the fragment ion m/z 308 corresponding to a GSH conjugate of reduced irofulven M5 (248 + 307 – 18 = 537) as described in vitro by the same authors (Dick et al., 2004).

The M1 and M4 metabolites have an identical molecular mass of 262 (m/z 263) and the same fragment ions (m/z 247 and 227). They had respective retention times of 14.8 and 16.3 min, close to that of irofulven (16.1 min). The mass increase of 16 compared with the parent drug, irofulven, shows that these metabolites could be hydroxylated derivatives of irofulven. Hydroxylation could occur either at C-13 for metabolite M1 or at C-15 for metabolite M4. These identifications are supported by McMorris et al. (1999) with structural NMR analysis of hydroxymethylacylfulvene metabolites in rat.

The mass spectrum of metabolite M6 (tR = 18.8 min) showed a protonated molecular ion ([M6 + H]+) at m/z 231 and only one fragment ion (m/z 213) corresponding to water loss [M6 + H – H2O]+. The combined HPLC/ESI-MS/MS analyses of this metabolite (M6 = M – 16) suggested the presence of only one hydroxyl for M6. This hydrophobic compound compared with irofulven most likely could correspond to 6-methylyacylfulvene as characterized by McMorris et al. (1999). These authors described that the formation of M6 results from displacement of the primary allylic hydroxyl of irofulven by hydride from NADPH. The M6 metabolite has the same molecular mass of 246 (m/z 247) and the identical mass spectrum as irofulven, but it has a shorter retention time (15.2 min). The M6 metabolite is an isomer of irofulven, and its structure could be a hydroxylated derivative of the M6 compound with the hydroxyl at either C-13 or C-15. According to NMR analysis achieved by McMorris (1999), the expected position could be at C-15.

Characterization of Glucuronide Conjugates Metabolites in Urine. The HPLC/UV analyses of the urine sample, incubated with β-glucuronidase for patient B (Fig. 6, A and B), showed a significant increase in metabolite M2 (tR = 9.6 min) and metabolite M3 (tR =
19.1 min). Furthermore, in the case of the urine sample of patient B, peaks corresponding to irofulven \((t_R = 15.0 \text{ min})\) and to its hydroxy-
lated metabolite, \(M_1\) \((t_R = 5.6 \text{ min})\), seemed to be higher. Thus, the
HPLC/ESI-MS/MS analyses of the urine sample from patient B
allowed the detection of four glucuronide conjugates by their respec-
tive mass spectra (Fig. 7). \(M_2\)-glucuronide conjugate \((M'_{2})\) has a
molecular mass of 424 \((m/z 425 = 249 + 176)\); \(M_3\)-glucuronide \((M'_{3})\)
has a molecular mass of 422 \((m/z 423 = 247 + 176)\); \(M_4\)-glucuronide \((M'_{4})\)
has a molecular mass of 422 \((m/z 423 = 247 + 176)\); and
\(M_5\)-glucuronide \((M'_{5})\) has a molecular mass of 438 \((m/z 439 = 263 + 176)\). Moreover, these HPLC/ESI-MS/MS analyses (Fig. 5, A and B)
were used for peak area comparison for each compound. They showed
a large increase \((\times 50)\) in \(M_2\) metabolite \((m/z 249)\) and a relative
slightly increase \((\times 5)\) of irofulven \((m/z 247)\) and its metabolites \(M_4\)
\((m/z 263)\) and \(M_5\) \((m/z 247)\) in urine of patient B treated with
\(\beta\)-glucuronidase. Furthermore, the ions \([M + H]^+\) and \([M + H -
H_2O]^+\) confirmed the \(O\)-glucuronide linkage.

**Characterization of Metabolites in Plasma Samples.** The HPLC/
ESI-MS/MS analyses of plasma samples from two patients (A and C)
detected irofulven \((m/z 247; M = 246)\), its metabolite \((m/z 249; M_2 =
248)\), and its GSH conjugate \((M_1 = 535)\). Mass spectra of these
metabolites in the collected plasma of patient C (Fig. 8) were identical
to those obtained for standards (Fig. 3).

The characterization of these metabolites \((M_1, M_2, M_3, M_4, M_5,
\text{and } M_6)\) observed in human urine and plasma samples and glucuro-
nide conjugates in human urine by HPLC/ESI-MS/MS suggests the
proposed in vivo metabolic pathways of irofulven in humans as shown
in Fig. 9.

**Discussion**

The pharmacokinetics, metabolism, and excretion of irofulven were
investigated in patients with advanced solid tumors after a 30-min i.v.
infusion of 0.55 mg/kg dose of irofulven containing \([^{14}C]\)irofulven.
The majority of the administrated radioactive dose was excreted in
urine \((71\%)\), suggesting that urinary excretion was the primary route
of elimination of irofulven-associated radioactivity in humans as
compared with excretion in rats, in which fecal excretion was pre-
dominant. Unchanged irofulven was not detected in the urine sample
possibly because of low renal elimination of irofulven and dilution of
sample as a result of collection over the larger interval. The pharma-
cokinetic profile of irofulven after the administration of a single dose
was characterized by a short mean half-life of 0.3 h and high total
body clearance, which is similar to the finding of the previous Phase
1 trial of irofulven (Eckhardt et al., 2000). As shown in Fig. 2, the
higher blood radioactivity concentrations for the last time points
resulted in higher AUC in blood with a blood/plasma ratio of 1.6 for
AUC but only 0.8 for \(C_{\text{max}}\). This blood/plasma distribution ratio of
radioactivity indicated a slight partitioning of irofulven-derived radio-
activity into the red blood cells. Plasma levels of total radioactivity
were higher than those for the unchanged irofulven and were mea-
surable up to 144 h after infusion, suggesting that the majority of the
circulating radioactivity was caused by the presence of metabolites.
Metabolite profiling using HPLC/UV and HPLC/MS in human plasma and urine was performed for three patients treated with irofulven in a separate Phase 1 clinical and pharmacokinetic study. In these samples, several peaks corresponding to the potential metabolites were detected. Several of these peaks were characterized as irofulven metabolites by HPLC/ESI-MS/MS in human urine and plasma samples. The structures of the observed metabolites in humans are proposed (Fig. 9) and seem to be in concert with the published results on metabolism of irofulven from in vitro and in vivo studies in rats (Toda et al., 1998; McMorris, 1999) and a study using NMR method by McMorris et al. (1999). These authors have shown that the stereochemistry of the tertiary hydroxyl does play a role in the enzyme-catalyzed activation step. The mechanism of action of its antineoplastic compound is thought to involve an activation step with a nucleophilic attack by thiols or NADPH, leading to a highly reactive intermediate. This latter reacts with nucleophilic compounds such as proteins or DNA on the cyclopropane ring with its opening and the concomitant loss of the tertiary hydroxyl in the C-2 position. The GSH conjugate (M1) of irofulven still retains the α,β-unsaturated ketone and cyclopropylmethylcarbinol moieties that can react with nucleophiles (McMorris et al., 2000, 2001). Therefore, M1 may still possess alkylating ability and hence antitumor activity (McMorris et al., 2004).

Twelve metabolites of irofulven were detected in human urine based on their retention times and their molecular masses. The known cyclopropane ring-opened metabolite (M2) and GSH conjugate metabolite (M1) of irofulven identified in rat plasma (Tanaka et al., 1994) have also been found in human urine and plasma. Four metabolites (M3, M4, M5, and M6) found in human urine in the present study were characterized by mass spectrometry, and their structures have been previously determined by NMR in the metabolism study of irofulven in rat liver cytosol (McMorris et al., 1999). The metabolites M3 and M4 are hydroxylated metabolites from irofulven formed via oxidation, whereas the metabolite M6 could be produced from irofulven via a presumed reduction. The M5 metabolite could be produced from M6 via oxidation on C-15 position (see Fig. 1). The glucuronide conjugates, detected in urine samples by their ESI-MS/MS mass spectra, probably resulted from association with irofulven and its primary metabolites (M1, M4, and M6). Thus, four new glucuronide conjugates (M', M''2, M''3, and
were found in the human urine. Finally, the formation of primary metabolites, GSH conjugates, and glucuronide conjugates in humans suggests the proposed biotransformation pathways in vivo of irofulven (Fig. 9). Further investigation on metabolism is ongoing to identify the enzymes involved in the formation of metabolites of irofulven.

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References


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