ORALLY ACTIVE INHIBITORS OF HUMAN LEUKOCYTE ELASTASE. III. IDENTIFICATION AND CHARACTERIZATION OF METABOLITES OF L-694,458 BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT:

The in vitro and in vivo metabolism of N-[1(R)-(1,3-benzodioxol-5-yl)butyl]-3,3-diethyl-2(S)-[4-{(4-methyl-1-piperazinyl)carbonyl}phenoxy]-4-oxo-1-azetidinecarboxamide (L-694,458) was studied in male Sprague-Dawley rats and rhesus monkeys. Analysis by LC-MS/MS and NMR revealed that the major metabolite generated in incubations with rat liver microsomes resulted from N-oxidation of the piperazine group, while the major metabolite generated in monkey liver microsomes was the catechol that resulted from O-dealkylation of the methylenedioxyphenyl group. Other metabolites observed in these incubations include the piperazine N-desmethyl, several monohydroxylated derivatives of the parent compound, and three products that resulted from cleavage of the \( β \)-lactam ring. Incubations of parent compound with rat hepatocytes in culture generated two major metabolites that resulted from cleavage of the piperazine ring with the loss of an ethylene group from one side of the ring; one of these metabolites retained the piperazine N-methyl group, while the other did not. The metabolite profiles in vivo were similar to those observed in vitro, but they were much more complex owing to secondary and, in some cases, tertiary biotransformations of many of the primary metabolites. Bile obtained from orally dosed rats contained more than 40 parent-related components, and many of these metabolites had arisen from piperazine ring cleavage.

HLE\(^1\) is a serine protease located in the azurophilic granules of the polymorphonuclear leukocytes (PMN). This enzyme has been implicated in the extracellular degradation of structural proteins such as elastin and collagen. Consequently, inhibition of HLE has been targeted as a potential therapy in diseases such as emphysema (1) and cystic fibrosis (2).

To date, numerous reports have been published concerning the synthesis and evaluation of nonpeptidyl, low molecular weight inhibitors of HLE (3–12). The potency of some of these potential inhibitors was screened in vitro, and then promising compounds were assayed for oral activity in hamsters and mice that had been instilled with HLE (8–11). Subsequently, pharmacokinetic studies were performed to determine whether leading drug candidates were bioavailable after oral administration to rats and monkeys. An example of one such orally active inhibitor of HLE is \( N \)-[1(R)-(1,3-benzodioxol-5-yl)butyl]-3,3-diethyl-2(S)-[4-{(4-methyl-1-piperazinyl)carbonyl}phenoxy]-4-oxo-1-azetidinecarboxamide (L-694,458). This report describes the in vitro and in vivo metabolism of this monocyclic \( β \)-lactam inhibitor in Sprague-Dawley rats and rhesus monkeys. These studies were performed in conjunction with a disposition study of this compound (13) in the two species that were used to determine its toxicological profile.

Materials and Methods

Chemicals. \( N \)-[1(R)-(1,3-benzodioxol-5-yl)butyl]-3,3-diethyl-2(S)-[4-{(4-methyl-1-piperazinyl)carbonyl}phenoxy]-4-oxo-1-azetidinecarboxamide (L-694,458, fig. 1A) was synthesized at the Merck Research Laboratories in Rahway, NJ (14). The radiolabeled [(butyl-3,4-[\( ^3 \)H]\( 2 \))] and pentadeuterated [(butyl-3,4-[\( ^3 \)H]\( 2 \)), piperazinyl-N-[\( ^2 \)D]) forms of L-694,458, and other reference standards (L-742,390 and L-740,447) were prepared at Merck Research Laboratories as well. Radiolabeled L-694,458 was 98% pure, as determined by HPLC analysis. Water was distilled in-house and purified by a Picosystem Ultra unit (Hydro, Garfield, NJ). Methanol and acetonitrile (optima grade), ammonium acetate (HPLC grade), and trifluoroacetic acid (reagent grade) were obtained from Fisher (Springfield, NJ). Argon and nitrogen collision gases were obtained from JWS Technologies (Piscataway, NJ).

In Vitro Incubations. Rat and monkey liver microsomes. Subcellular fractions from rat and monkey livers were prepared by differential centrifugation using established procedures (15). Liver microsomes from Sprague-Dawley rats and rhesus monkeys (2 mg protein/ml) were incubated with L-694,458 (<1 to 100 \( \mu \)M) at 37°C for up to 1 hr in a pH 7.4 phosphate buffer and in the presence of an NADPH-regenerating system (1 mM NADP, 10 units glucose-6-phosphate dehydrogenase, and 20 mM glucose-6-phosphate). The microsomal protein fraction was precipitated with acetonitrile and the mixture was centrifuged. The supernatant was removed and dried under nitrogen, and the dried extract was reconstituted in 1:1 acetonitrile:water prior to HPLC-UV (220 nm) or LC-MS analysis.

Primary rat hepatocyte culture. Hepatocytes were isolated from a male Sprague-Dawley rat and plated in a petri dish at 1.5 \( \times 10^6 \) cells/ml for 48 hr with 10 \( \mu \)M dexamethasone (16). Cell viability was determined using trypan blue exclusion and was found to be 93%. The medium was changed at 2, 24, and 48 hr. The substrate was dissolved in DMDSO and mixed with culture medium to achieve a final concentration of 50 \( \mu \)M. The final concentration of DMDSO did not exceed 1% (v/v). Three ml of medium containing the substrate

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1 Abbreviations used are: HLE, human leukocyte elastase; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; LC-MS, liquid chromatography-mass spectrometry; API, atmospheric pressure ionization; TIC, total ion current; EIC, extracted ion current; TIS, turbo-ionspray; CID, collisionally induced dissociation; SIF, source-induced fragmentation; CYP, cytochrome P450.

2 L-694,458 has been licensed to DuPont Merck and is also known as DMP 777.
Fig. 1. (A) Product ion mass spectrum of parent compound (L-694,458). (B) Source-induced fragmentation mass spectrum of parent compound. Proposed structures of designated fragment ions shown in fig. 2.
was added to the plated hepatocytes and incubated for 48 hr at 37°C. At the end of the incubation, the cells were scraped from the plate with a cell scraper. The medium and cell mixture was removed and the plate was washed with 3 ml of methanol. The methanol wash was combined with the medium and cell mixture and the entire volume was dried under nitrogen. Acetonitrile (5 ml) was used to resuspend the parent-related material and the supernatant was transferred to a clean tube. The remaining solid material was resuspended in 2 ml water and extracted with 5 ml ethyl acetate. The ethyl acetate and acetonitrile extracts were combined, dried under nitrogen, and resuspended in 1:1 acetonitrile:water for LC-MS analysis.

Rat intestinal homogenate. Intestines were obtained from six fed male Sprague-Dawley rats. Small intestines were isolated from below the duodenum to just above the cecum. Large intestines were isolated from below the cecum. The intestinal segments were sliced open longitudinally and emptied by rinsing the contents in ice cold sodium chloride solution (0.9%; pH 5.6). Homogenates were prepared by cutting up the intestines into 3- to 5-mm segments and dropping these segments into a hand-held glass homogenizer filled with 5 ml NaCl solution. After homogenization of the tissue, the soluble fraction with buffer was removed and kept on ice until ready for use.

An aliquot of substrate solution was added to 10 ml of either small or large intestinal homogenate for a final concentration of 50 μM. Incubations were carried out for 4 hr in polypropylene tubes placed in a shaking water bath (60 rpm, 37°C). At the end of the incubation, the incubates were frozen at −20°C. Extraction of 1 ml of thawed homogenate was performed twice using 5 ml ethyl acetate each time. The extract was dried under nitrogen and resuspended in 1:1 acetonitrile:water for LC-MS analysis.

In Vivo Samples. Male Sprague-Dawley rats were used in all experiments as described in the preceding paper (13). Rats weighing 300—400 g (3 to 5 per dose) were dosed with [3 H]-labeled L-694,458 orally at 10 mg/kg and iv at 5 mg/kg. Intravenous dosing was via the tail vein or via a previously implanted catheter in the femoral vein for biliary excretion studies, and oral dosing was by gavage.

HPLC-UV and HPLC-Radiometric Analysis. Chromatographic separation of microsomal metabolites was performed isocratically at a flow rate of 1 ml/min using 40% acetonitrile in water with 10 mM ammonium acetate and 0.1% TFA on a DuPont Zorbax SB-CN column (4.6 × 250 mm). In experiments in which mass spectrometry was not used, the UV absorbance of the eluate was monitored at 220 nm and the radioactivity was monitored on-line with a radiometric detector.

Chromatographic separation of biliary metabolites was achieved on a DuPont Zorbax Phenyl column (4.6 × 250 mm) using a linear gradient from 30% acetonitrile with 0.2% TFA (solvent A) and 70% 2 mM ammonium acetate in water with 0.07% TFA (solvent B) to 70% A in 24 min, at a flow rate of 1 ml/min. In this case the entire effluent was transported into the mass spectrometer via the Sciex turbo-ionspray (TIS) interface, which uses the heated nebulizer probe to heat the ionized effluent after it exits the ionspray source. The nebulizing air pressure was 60 psi, with an auxiliary air flow of 6 ml/min through the heated probe, which was operated at 600°C. The nitrogen curtain gas flow was 1.2 ml/min to minimize condensation of droplets at the orifice.

Chromatographic separation with on-line MS analysis of biliary metabolites was achieved on a DuPont Zorbax Phenyl column (4.6 × 250 mm) using a linear gradient from 30% acetonitrile with 0.1% TFA (solvent A) and 70% 3 mM ammonium acetate in water with 0.07% TFA (solvent B) to 70% A in 24 min, at a flow rate of 1 ml/min.

NMR Analysis. NMR spectra were acquired in CD3OD at 25°C on a Varian Unity 400 MHz spectrometer. Chemical shifts are on a ppm scale relative to the residual solvent CD3H signal set at 3.30 ppm.

Metabolite Designations. The designation of the individual metabolites has been coded to specify the structure and the source of the biotransformation product. The initial letter R or M refers to the animal source: rat or monkey. The second letter, H, P, or B refers to the tissue source or incubation medium: microsomes, hepatocytes, plasma, or bile, respectively. The number given to a metabolite corresponds to a specific chemical entity regardless of the source in which it was identified. For example, metabolite 1 is invariably the catechol, whether it is found in rat hepatocytes (RH1) or in monkey liver microsomes (MM1).

Results

Mass Spectrometric Analysis. The [M+H]+ ion of parent compound appeared at m/z 565. The product ion mass spectrum and structure of L-694,458 are shown in fig. 1A. The SIF mass spectrum shown in fig. 1B was obtained under TIS conditions, and it is similar to the conventional CID product ion mass spectrum shown in fig. 1A. The SIF spectra can be extremely useful because they often obviate the need to isolate fractions or repeatedly inject a complex mixture of metabolites under conventional CID conditions to obtain structural information on the individual metabolites.

Proposed structures for eleven principal product ions of L-694,458 are shown in fig. 2, and this information has been summarized in table 1. Fragment ion a appears in the conventional CID product ion mass spectrum as well as in the SIF mass spectrum. Figs. 1A and 2 show that fragment ion a includes the β-lactam ring and the phenoxacyclopiperazine substituent; therefore, if the difference between the [M+H]+ ion of a metabolite and parent compound does not correspond to the same difference in fragment ion a, this suggests that some biotransformation has occurred on the 4-butyl-methylenedioxyphenyl portion of the molecule (fragment ion i).

Identification of In Vitro Metabolites. Incubations with liver microsomes. HPLC-UV profiles obtained after the incubation of L-694,458 with liver microsomes from male Sprague-Dawley rats and male rhesus monkeys suggested that several metabolites had been generated (13). Subsequent on-line LC-MS analysis of these samples was used to characterize the metabolites. Fig. 3 shows EIC chromatograms obtained from LC-MS analysis of the monkey liver microsomal extract. Subsequent CID experiments were performed on each of the suspected metabolites to produce MS/MS spectra to aid in their structural characterization. Table 1 summarizes the [M+H]+ ion observed for each of these metabolites and the fragment ions observed in each of their product ion mass spectra.

Fig. 3 shows that a peak was observed in the extracted ion current
FIG. 2. Proposed structures of fragment ions generated by collisionally induced dissociation of parent compound.

<table>
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<tr>
<th>Structures</th>
<th>Metabolite Designation</th>
<th>[M+H]^+</th>
<th>Fragment ions m/z (label)</th>
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<tr>
<td>Parent drug</td>
<td></td>
<td>565</td>
<td>389 (a), 346 (b), 303 (c), 221 (d), 289 (e), 203 (f), 169 (g), 345 (h), 177 (i), 135 (j), 121 (k)</td>
</tr>
<tr>
<td>1</td>
<td>553</td>
<td></td>
<td>389 (a), 221 (d), 289 (e), 203 (f), 169 (g), 195 (h, j - 12), 121 (i - 12), 121 (k)</td>
</tr>
<tr>
<td>2</td>
<td>581</td>
<td>139 (a), 221 (d), 289 (e), 203 (f), 169 (g), 149 (h, j + 14), 121 (k)</td>
<td></td>
</tr>
<tr>
<td>3, 4</td>
<td>581</td>
<td></td>
<td>(isomers) [Both metabolites exhibited very weak spectra]</td>
</tr>
</tbody>
</table>

TABLE 1—Continued

<table>
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<th>Structures</th>
<th>Metabolite Designation</th>
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<th>Fragment ions m/z (label)</th>
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<td></td>
<td></td>
<td>319 (a), 135 (j), 115, 77</td>
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<tr>
<td>8</td>
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<td></td>
<td>319 (a), 147, 135 (j), 100, 72</td>
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<td>221 (k, 101), 99, 93, 86, 65, 58, 45</td>
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<tr>
<td>10</td>
<td></td>
<td>539</td>
<td>363 (a + 25), 332, 277 (c + 25), 246, 195 (d + 25), 164, 26, 203 (f), 169 (g), 246</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>525</td>
<td>349 (a + 40), 332, 263 (c + 40), 246, 181 (d + 40), 164, 264, 203 (f), 169 (g), 246</td>
</tr>
<tr>
<td>12, 13</td>
<td></td>
<td>553</td>
<td>375 (a + 14), 289 (e), 203 (f), 169 (g), 246, 179 (h, j + 2), 137 (f + 2), 121 (k)</td>
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</table>
chromatogram of m/z 553, which is 12 Da less than parent compound. This metabolite was determined to be the catechol that results from O-dealkylation of the methylenedioxyphenyl group. Based on HPLC analysis with UV and radiometric detection, the catechol (MM1) was estimated to be the major product when L-694,458 was incubated with monkey liver microsomes (13). The assigned structure of MM1 and the other microsomal metabolites discussed below are summarized in table 1.

Several peaks were observed in the ion current chromatogram of m/z 581 (fig. 3), which represents oxidized metabolites of L-694,458. For example, MM2 has been assigned as hydroxylated on the 4-butyl substituent of the methylenedioxyphenyl group based on the unique fragment ions observed at m/z 149 and 193. Metabolites MM3 and MM4 have also been assigned as hydroxylated derivatives on the basis of the product ion at m/z 405, which corresponds to an increase of 16 Da in fragment ion a (table 1). The small amounts of MM3 and MM4 generated in the incubation with monkey liver microsomes generated very weak product ion mass spectra, which precluded their further structural characterization.

The product ion mass spectrum of MM5 (m/z 551) was similar to that of parent compound, except for fragment ions a, c, and d, which are 14 Da less (table 1). Consequently, MM5 (L-742,390) has been assigned as the piperazine N-desmethyl metabolite of L-694,458.

The product ion mass spectrum of MM6 (m/z 581) exhibited diagnostic fragment ions that were suggestive of piperazine ring oxidation; however, the MS/MS spectrum did not provide sufficient information to distinguish between hydroxylation and N-oxidation. Subsequent NMR analysis of the parent compound (fig. 4A) and the metabolite (fig. 4B) demonstrated that attack on the amino nitrogen of the piperazine ring was indicated from the downfield displacement of 1.06 ppm and 0.60 ppm for the attached N-methyl (peak 11) and methylenes (peak 10), respectively (figs. 4A and 4B). Since the remaining spectral features closely resembled those of the parent drug, the findings strongly supported the N-oxide, as suggested by mass spectrometric analysis. Based on HPLC analysis (13), the N-oxide (L-752,688) was estimated to be the major metabolite produced when L-694,458 was incubated with rat liver microsomes.

Additional in vitro metabolites were identified in incubations of L-694,458 with rat liver microsomes. Fig. 5 displays EIC chromatograms of four metabolites that have arisen from alternate biotransformation pathways than those described above. The peak at m/z 237 (RM7) was determined to be the monosubstituted urea derivative that has been expelled as a result of β-lactam ring cleavage (see table 1). This structural assignment was confirmed by comparing the chromo-
metabolite with a reference standard (L-740,447). This metabolite was also identified as a major degradation product (D1) of [2H]-labeled L-694,458 after a 24-hr incubation at 37°C in rat plasma (13).

The product ion mass spectrum of RM8 (m/z 319) provided only minimal structural information, which was insufficient to assign its structure (table 1). This metabolite was also observed as a degradation product (D2) of [3H]-labeled L-694,458 after a 24-hr incubation at 37°C in rat plasma (13). Consequently, the structure of RM8 was determined by NMR and details of the identification of this metabolite are reported elsewhere (13). Like RM7, this metabolite also resulted from β-lactam cleavage, but unlike RM7, RM8 retained part of the β-lactam ring (see table 1).

Based on the identification of two metabolites that resulted from cleavage of the β-lactam ring (RM7 and RM8), the 4-methylpiperazinyl-carbonyl-phenol by-product was also expected to be generated in the incubation with rat liver microsomes. The early-eluting peak at m/z 221 in fig. 5 represents this metabolite (RM9; see fig. 6A and table 1). Although authentic standard was not available for comparison, an experiment was performed in which pentadeuterated (butyl-3,4-[2H]2, piperazinyl-N-[3H]3)] L-694,458 was incubated with rat liver microsomes. The corresponding deuterium-labeled analog of RM9, with [M+H]+ of 224 Da, was expected to retain the deuterium-labeled piperazinyl N-methyl group ([3H]3); the product ion mass spectrum of this analog was consistent with the assignment of RM9 (fig. 6B).

The small peak at m/z 539 in fig. 5 was originally assumed to be an impurity unrelated to parent compound; however, based on incubations of L-694,458 with rat hepatocytes in culture, this component was determined to be a true biotransformation product of parent compound (RM10). Details concerning the identification of RM10 are included in the following section.

*Incubations with rat hepatocytes in culture.* Selected EIC chromatograms obtained from a 48-hr incubation of L-694,458 in cultured rat hepatocytes are shown in Fig. 7. A component with [M+H]+ of 539 Da (RH10) that co-eluted with the parent compound was observed in this incubation, and this was believed to be the metabolite that had been observed previously in the incubation of L-694,458 with rat liver microsomes (RM10). Fig. 7 also shows that the N-desmethyl metabolite (RH5) was accompanied by a co-eluting metabolite (RH11) with [M+H]+ of 525 Da. These two components were unusual in that they were 26 and 40 Da lower in molecular weight than parent compound, which was not suggestive of any standard route of biotransformation. Subsequent CID analyses of RH10 yielded the product ion mass spectrum shown in fig. 8. Intact fragment ions i and j indicate that the 4-butyl-methylenedioxyphenyl portion of the molecule was not altered, and intact fragment ions e and g suggest that the β-lactam ring was not cleaved (see fig. 2). Assignment of the fragment ions at m/z 164, 195, 277, 332, and 363 suggest that an ethylene group is missing from the piperazine ring, corresponding to a net loss of 26 Da from the molecule (table 1). The structural assignment of RH11 was established in a similar manner using the fragment ions listed in table 1.

Although the product ion mass spectra were helpful in the assignment of RH10 and RH11, a derivatization approach was adopted to confirm that the piperazine ring had been cleaved. Derivatization of the rat hepatocyte extract with acetic anhydride in pyridine produced three principal acetylated derivatives that eluted with longer retention times than their underivatized precursors. For example, fig. 9A shows the product ion mass spectrum of acetylated RH10 with [M+H]+ of 581 Da, and fig. 9B shows the corresponding SIF mass spectrum. The conventional CID mass spectrum did not provide sufficiently intense product ions to permit a definitive structural assignment of the derivative. However, the SIF mass spectrum of the derivative contains prominent fragment ions at m/z 405 and m/z 319, which support the hypothesis that the piperazine ring had been cleaved and the resulting terminal secondary amine was acetylated. Similar SIF spectra were obtained for the acetyl derivative of RH11 ([M+H]+ at 567 Da) and the acetyl derivative of RH5 ([M+H]+ at 593 Da). The observation of acetylated RH5 served as a positive control for the derivatization reaction because this metabolite has a derivatizable secondary amine in the piperazine ring.

The EIC chromatogram of m/z 553 in fig. 7 contains two peaks that had not been observed in the microsomal incubations (RH12 and RH13). Subsequent analysis of RH12 under CID conditions indicated that fragment ion a had decreased by 14 Da, fragment ions i and j had increased by 2 Da, while the net decrease in [M+H]+ was 12 Da compared with parent compound (table 1). This fragmentation pattern
FIG. 6. Product ion mass spectra of metabolite 9 generated in incubations of L-694,458 (A) and [3H]-labeled L-694,458 (B) with rat liver microsomes.
suggests that the piperazine ring was \( N \)-demethylated and a methyl group has been added to one hydroxyl group of the catechol; analysis of RH13 under CID conditions revealed a similar pattern of fragmentation. Therefore, RH12 and RH13 have been assigned as mono-methyl ether regioisomers of this \( O \)-demethylenated and \( N \)-demethylated metabolite (table 1).

Incubations with rat intestinal homogenates. A 4-hr incubation of L-694,458 in homogenates of small and large intestine produced LC-MS profiles consisting mainly of the primary metabolites generated in liver microsomes of rats and monkeys (data not shown). These included the catechol, \( N \)-desmethyl, and \( N \)-oxide metabolites of parent compound. Minute amounts of the lactam-ring opened products (metabolites 7, 8, and 9) were observed in the ethyl acetate extract of the incubation of L-694,458 with homogenate of small intestine, but they were not detectable in the extract from homogenate of large intestine.

Identification of In Vivo Metabolites. The \( N \)-desmethyl and piperazine \( N \)-oxide metabolites were identified in plasma, lung, and liver obtained from rats dosed orally with \([3H]\)-labeled L-694,458.
The catechol resulting from O-dealkylation of the methylenedioxyphenyl group was observed in rat liver and lung, but was not detected in rat plasma. Two hydroxylated metabolites were also found in the plasma, lung, and liver samples. Plasma also contained the two metabolites that had resulted from β-lactam ring opening (RP7 and RP8); however, it is not clear if these components were true biotransformation products because they were also observed as degradation products (D1 and D2) in the in vitro incubations at 37°C with plasma.
Bile collected from rats at 2–4 hr after an oral dose of [³H]-labeled L-694,458 presented a complex metabolite profile that required further study by LC-MS (fig. 10). Figs. 11A and 11B display the EIC chromatograms corresponding to at least 40 parent-related components that were found in the acetonitrile extract of rat bile. Fig. 11A shows the presence of parent drug, RB1, RB12, RB13, RB5, RB10, RB11, RB6, RB2, RB3, as well as RB14 (hydroxylated on the 4-butyl-methyleneoxyphenyl portion) and RB15 (N-demethylated and O-dealkylated). The structures of these metabolites are included in table 1 and fig. 12. Fig. 11B depicts 26 other assigned metabolites and/or degradation products of L-694,458 in rat bile. The assigned structures of several in vivo metabolites are summarized in fig. 12 as well.

Bile components were determined to be related to parent drug based on their SIE mass spectra obtained from a single injection of an ethyl acetate extract of the sample. For example, fig. 11B illustrates the extracted ion current chromatogram of six isomeric components of rat
bile with [M+H]^+ of 567 Da, which is 2 Da greater than parent compound. These metabolites were distinguished on the basis of their retention time and the diagnostic fragment ion \( a \) in their SIF mass spectra (data not shown):

a) **RB16**, **RB17**, and **RB18** were assigned as three hydroxylated derivatives of the \( N \)-desmethyl metabolite based on a decrease of 14 Da in fragment ion \( a \) (m/z 375), which implies that each of these metabolites has been oxidized at a distinct location on the 4-butyl-methylenedioxyphenyl portion of the molecule; 
b) **RB19** exhibited an increase of 2 Da in fragment ion \( a \) (m/z 391), which suggests that the methylpiperazine has been \( N \)-demethylated in conjunction with oxidation on either the piperazine ring, one of the two ethyl substituents on the \( \beta \)-lactam ring, or the phenyl ring; 
c) **RB20** and **RB21** have been assigned as the methoxy regioisomers of the catechol derivative of parent, based on unaltered fragment ion \( a \) at m/z 389.

Fig. 11B also illustrates that many second and third generation metabolites have arisen from primary metabolites that underwent cleavage of the piperazine ring. For example, the six metabolites with [M+H]^+ of 567 Da discussed above were accompanied by six corresponding metabolites with [M+H]^+ of 541 Da (**RB22** through **RB27**), which must have resulted from piperazine ring cleavage. The EIC chromatograms in fig. 11B at m/z 597 and 571 represent dihydroxylated derivatives of parent and a few of their piperazine ring fission products, respectively. The m/z 555 Da channel represents the piperazine cleavage products of **RB14**, **RB2**, and **RB3**, while the m/z 527 Da channel depicts those of **RB1**, **RB12**, and **RB13**.

**Discussion**

The present study has demonstrated the identification and characterization of the in vitro metabolites of L-694,458 in male Sprague-Dawley rats and male rhesus monkeys. This characterization was invaluable in determining the structures of the in vivo metabolites observed in a sample as complex as bile obtained from orally dosed rats. The results have shown that the major in vitro metabolites are formed via four primary metabolic pathways: 
a) \( O \)-dealkylation of the methylenedioxyphenyl group, which results in the catechol derivative (metabolite 1); 
b) \( N \)-demethylation of the methylpiperazine group (metabolite 5); 
c) \( N \)-oxidation of the methylpiperazine nitrogen (metabolite 6), which was confirmed by NMR analysis; and 
d) cleavage of the \( \beta \)-lactam ring, which results in three by-products: the 4-butyl-
methylenedioxyphenyl-substituted urea derivative (metabolite 7), the vinyl-substituted urea derivative, which retains part of the \( \beta \)-lactam ring (metabolite 8), and the \( C-4 \) leaving group, 4-methylpiperezinyl-carbonyl-\( p \)-phenol (metabolite 9). Based on HPLC analysis with UV and radiometric detection (13), the catechol was the major metabolite generated in monkey liver microsomes, while the \( N \)-oxide was the major metabolite produced in rat liver microsomes. The incubation of L-694,458 with rat intestinal homogenates generated metabolites 5, 6, while only the former was capable of producing minute amounts of metabolites 7, 8, and 9.

The incubation of L-694,458 with rat hepatocytes in culture produced many of the same metabolites identified in the microsomal incubations. A notable exception was the observation of metabolites 12 and 13, the two methoxy derivatives of the catechol (see table 1), which were not observed in incubations of L-694,458 with liver microsomes. This is because catechol-\( O \)-methyl transferase is a cytosolic enzyme (17), which should be present in the cultured hepatocyte system but not in the hepatic microsomes.

The hepatocyte culture system also produced substantial amounts of some unusual metabolites that do not possess an intact piperezine ring; instead, these biotransformation products appeared to contain an amido-ethyamine group. Acetylation was selected as a means of verifying the proposed ring fission product, which was expected to undergo \( N \)-acetylation readily, in contrast to the intact parent compound. Subsequent LC-MS/MS analysis of the acetylated derivatives in the hepatocyte extract indeed supported the assignment of metabolites 10 and 11 (see table 1).

While cleavage of the piperezine ring seemed to be an unusual metabolic pathway, there have been several precedents in the literature for this type of in vivo biotransformation. Studies have been made of the metabolism of several drugs containing a piperezine ring, including some phenothiazines (18–20), antihistamines (20, 21), an inotropic agent (22), and ketonozol (23–25). In addition, investigation of the in vitro metabolism of the hypotensive agent clondinone, which contains a five-membered imidazolidine ring, has indicated that ring scission may proceed either in an epoxide-diol pathway or after two successive hydroxylations at adjacent carbons in the ring (26).

It is interesting that oxidative cleavage of the methylpiperezine ring occurred with and without retention of the piperezine N-methyl group; this phenomenon was noted previously by Breyer et al. as well (19). This observation suggests that further studies of the enzymes that catalyze methylpiperezine N-demethylation and piperezine ring scission are warranted.

Several metabolites of L-694,458 observed in the plasma or tissues obtained from orally dosed rats have been characterized herein, and most of the primary metabolites identified in the in vivo samples had been observed in vitro. For example, the \( \beta \)-lactam ring-opened derivatives of L-694,458 (metabolites 7 and 8) were observed in the plasma of orally dosed rats, as well as in the in vitro incubations of L-694,458 with plasma from untreated rats (13). These results suggest that although these in vivo metabolites could have been generated in the liver, they also could have arisen from ex vivo degradation of circulating parent compound owing to the action of a type B esterase (13). Alternatively, the mechanism of formation of these metabolites in plasma may be pH-dependent, as evidenced by the incubation of several analogs of L-694,458 in basic media which produced the corresponding analogs of metabolites 7, 8, and 9 (27). These metabolites were also observed after incubation of related inhibitors with HLE in vitro (28). It should be noted that while analogs of metabolites 7, 8, and 9 have been generated as a consequence of the normal mechanism of HLE inhibition (27–30), it has not been established whether this same mechanism of inhibition has occurred in animals that have been dosed with L-694,458.

Rat bile yielded the most interesting and complex metabolite profile, which was comprised of at least 40 parent-related components. Many of these species were primary metabolites that had been observed in vitro, while several others were secondary metabolites that had not been seen before. The large number of metabolites circulating in bile is attributed to oxidation and subsequent cleavage of the piperezine ring of these primary and secondary metabolites. Detailed mass spectral analysis of the bile sample was useful in demonstrating that the large broadened peak in the radioprofile (fig. 10) corresponded to more than 20 parent-related components that eluted between 12 and 15 min (see figs. 11A and 11B).

In summary, incubation of L-694,458 with liver microsomes and cultured hepatocytes was used successfully to generate and characterize numerous primary and secondary metabolites by LC-MS/MS. The results from the in vitro studies were subsequently applied to the deconvolution of a very complex in vivo metabolite profile observed in bile obtained from orally dosed rats. Notably, a very unusual metabolic pathway resulting in numerous biliary metabolites was identified as cleavage of the piperezine ring, presumably owing to oxidation of adjacent methylenes in the ring. This pathway was interesting in that it occurred with and without molecular retention of the piperezine N-methyl group.

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