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*Research Article*

# Gas Chromatographic–Mass Spectrometric (GC-MS) Determination of MDL 72,222 and Four Metabolites in Monkey Plasma

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MDL 72,222, a potent serotonin antagonist, is being developed for use as an antiemetic drug in cancer chemotherapy. An assay method has been developed for the determination of MDL 72,222 and four metabolites: *N*-desmethyl-MDL 72,222 (**1**), 3,5-dichlorobenzoic acid (**2**), glycine conjugate of **2** (**3**), and MDL 72,222-*N*-oxide (**4**). The method involves liquid–liquid extractions, derivatization with trifluoroacetic anhydride for metabolite **1**, methylation with diazomethane for metabolites **2** and **3**, reduction with titanous chloride for **4**, and detection of each analyte by GC-MS. In this method $d_3$-MDL 72,222, a 3-methyl-5-chlorobenzoate analogue of **1** (**5**), and 3,4-dichlorobenzoate analogues of **2–4** (**6–8**) are used as internal standards for the determination of MDL 72,222 and metabolites **1**, **2**, **3**, and **4**, respectively. The method is suitable for quantification of MDL 72,222 and the metabolites **1–4** over a concentration range of 1–150, 0.5–75, 1–150, 0.5–75, and 1–150 ng/ml, respectively. The intraday precision and accuracy values are within 10% RSD and 92–110%, respectively. The interday precision and accuracy values are within 14% RSD and 87.6–116%, respectively. The method is specific and sensitive for the analysis of MDL 72,222 and four metabolites in monkey plasma. The assay method has been utilized in analyzing pharmacokinetic study samples.

KEY WORDS: MDL 72,222; gas chromatographic–mass spectrometric method; MDL 72,222-*N*-oxide; *N*-desmethyl-MDL 72,222; 3,5-dichlorobenzoic acid; glycine conjugate of 3,5-dichlorobenzoic acid; metabolites.

## INTRODUCTION

MDL 72,222 (8-methyl-8-azabicyclo[3,2,1]octan-3-yl 3,5-dichlorobenzoate; Fig. 1) is a potent serotonin ($5\text{-}HT_3$) antagonist (1) currently under evaluation as an antiemetic drug in cancer chemotherapy. In ferrets, this compound inhibits cisplatin-induced vomiting (2). We report here a specific and sensitive GC-MS method for the quantification of MDL 72,222 and four metabolites, *N*-desmethyl-MDL 72,222 (**1**; Fig. 1), 3,5-dichlorobenzoic acid (**2**; Fig. 1), glycine conjugate of **2** (**3**; Fig. 1), and MDL 72,222-*N*-oxide (**4**; Fig. 1), in citrated monkey plasma.

## MATERIALS AND METHODS

### Materials

The reference standards of MDL 72,222 and compounds **1–4**, $d_3$-MDL 72,222, a 3-chloro-5-methylbenzoate analogue of compound **1** (**5**; Fig. 2), and 3,4-dichlorobenzoate analogues of compounds **2–4** (**6–8**; Fig. 2), were synthesized at the Merrell Dow Research Institute (Cincinnati, OH). Acetonitrile, hexane, and methanol were obtained from Burdick and Jackson Laboratories (Muskegon, MI) and were HPLC grade. Titanous chloride (20% solution) was obtained from Fisher Scientific (Fairlawn, NJ). Sodium carbonate (anhydrous, granular) was obtained from the J. T. Baker Chemical Co. (Phillipsburg, NJ). Hydrochloric acid (5 *N*) and sodium hydroxide (5 *N*) were obtained from Red Bird Services Inc. (Metamora, IN) and the Ricca Chemical Co. (Arlington, TX), respectively, and were diluted to 2.5 *N* with HPLC water. Sodium bicarbonate (GR grade), methylene chloride (glass distilled), diethyl ether (anhydrous, GR grade), sodium hydroxide (1 *N*), and sodium sulfate (anhydrous, GR grade) were obtained from EM Science (Cherry Hill, NJ). Pyridine and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) were obtained from the Aldrich Chemical Co. Inc. (Milwaukee, WI). Iso-octane (capillary GC/GC-MS grade) and trifluoroacetic (TFA) anhydride were obtained from Burdick and Jackson Laboratories and the Regis Chemical Co. (Morton Grove, IL), respectively. Hexamethyldisilazane was obtained from Alltech Associates Inc. (Deerfield, IL). Monkey plasma was obtained from an in-house colony.

### Instrumentation

All analyses were performed with a Hewlett–Packard (HP) Model 5988A mass spectrometer and 5890 gas chromatograph (Palo Alto, CA) equipped with a 7673A autosam-

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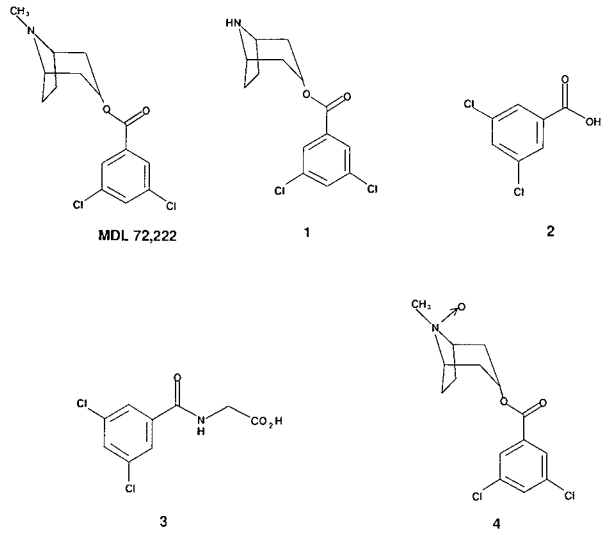


Fig. 1. Chemical structures of MDL 72,222, *N*-desmethyl-MDL 72,222 (1), 3,5-dichlorobenzoic acid (2), glycine conjugate of 2 (3), and MDL 72,222-*N*-oxide (4).

pler. The MS and GC parameters and data integration were controlled by HP Chemstation software. The chromatographic column was a fused-silica capillary column, 15 m × 0.25-mm I.D., DB-1 bonded phase at 0.25-μm film thickness (J & W Scientific, Folsum, CA). Helium was used as the carrier gas.

### GC-MS Conditions

Samples were introduced to the capillary through a splitless injector that was set at 250°C. Following injection of a 1-μl sample, the purge valve was closed for 1 min. Initially, the oven temperature was held at 80°C for 1.5 min, then brought up to 300°C at 30°C/min and held at 300°C for 0.17 min. The effluent from the chromatographic column was routed directly to the ion source set at 110°C. The interface between the GC oven and the mass spectrometer was set at 300°C. Analyses were performed in the negative chemical ionization mode with methane (1.05-Torr source pressure) as the reagent gas. The ions that were monitored were *m/z* 313 for MDL 72,222 and the reduced products of compounds 4 and 8, *m/z* 316 for $d_3$-MDL 72,222, *m/z* 375 and 395 for the TFA derivatives of compounds 1 and 5, *m/z* 204 for the methyl ester derivatives of compounds 2 and 6, and *m/z* 261 for the methyl ester derivatives of compounds 3 and 7. These ions are the molecular ions and base peaks of these analytes.

### Silanization

All of the glassware used for this work was gas phase silanized with hexamethyldisilazane (16 hr at 135°C in an evacuated oven).

### Preparation of Standards

Stock solutions of MDL 72,222 and compounds 1–4 (1 mg/ml) were prepared in acetonitrile. Compound 1 (hydrochloride salt) was dissolved in a minimum amount of methanol (200 μl/10 mg sample) and was then diluted to volume with acetonitrile. These solutions were stable for 1 month in a refrigerator at 3°C. Mixed working standard solutions containing the five analytes in acetonitrile were prepared on each day of analysis. Plasma standards were prepared by spiking 1 ml of citrated control monkey plasma with 20 μl of the appropriate working standard solution to yield concentration ranges of 0.5–10 and 10–75 ng/ml (for compounds 1 and 3) as well as 1–20 and 20–150 ng/ml (for MDL 72,222 and compounds 2 and 4). These standards were analyzed using the procedure described below.

### Preparation of Internal Standards

Stock solutions of $d_3$-MDL 72,222 and compounds 5–8 (1 mg/ml) were prepared in acetonitrile. Compound 5 (hydrochloride salt) was dissolved in a minimum amount of methanol (400 μl/10 mg sample) and was then diluted to volume with acetonitrile. These solutions were also stable at 3°C. Mixed working internal standard solutions containing the five internal standards (5 μg/ml for the high-concentration standard curve and 0.5 μg/ml for the low-concentration standard curve) were prepared in acetonitrile on each day of analysis.

### Assay Method

Frozen samples were thawed at room temperature, then mixed, and aliquots (10–1000 μl) were transferred to 16 × 100-mm silanized disposable screw-capped culture tubes. Citrated control monkey plasma was added to any sample of less than 1 ml to make the volume equal to 1 ml.

To standards and samples a 20-μl aliquot of the appropriate dilute internal standard solution (5 μg/ml for standards and samples expected to fall on the high-concentration standard curve and 0.5 μg/ml for standards and samples on the low-concentration standard curve) was added, and the standards and samples were mixed by vortexing. The pH was adjusted to 10 with 19 μl of 1 *N* NaOH, followed by vortexing. All were extracted three times with 3 ml of hexane for 5 min by rotation (Rugged Rotator Model RD250, Glas-Col Apparatus Co., Terre Haute, IN). The hexane layer was separated from the plasma by centrifugation at 3500 rpm.

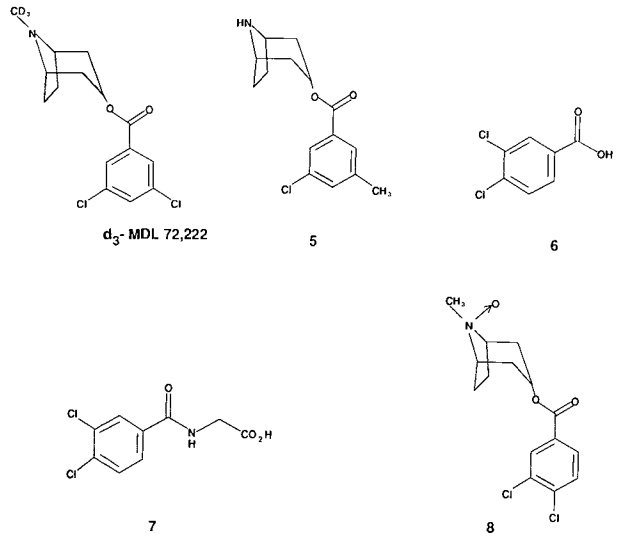


Fig. 2. Chemical structures of $d_3$-MDL 72,222 and compounds 5–8.

The hexane layer from the third extraction was discarded. After the third extraction, residual hexane was removed from the aqueous phase by evaporation under a stream of dry nitrogen at 30°C. The pH of the plasma layer was adjusted to 7 with 12 μl of 2.5 *N* HCl and samples were stored overnight at −10°C for the subsequent analysis of metabolites **2**, **3**, and **4**. The combined hexane layer from the first and second extraction was evaporated under dry nitrogen. The residue was derivatized in 10 min at room temperature with 250 μl of a mixture containing trifluoroacetic anhydride/methylene chloride (1:4, v/v) and 0.1% (v/v) pyridine as a catalyst. The derivatizing reagent was evaporated under nitrogen and the residues were redissolved in 1 ml of 0.2 *M* carbonate/bicarbonate buffer (pH 10). The aqueous solution was extracted twice with 3 ml of hexane. The combined extracts were evaporated to dryness and the residues were reconstituted in 300 μl of iso-octane containing 1% pyridine (solution A). Usually, solution A was injected onto the GC-MS overnight for the determination of MDL 72,222 and metabolite **1**.

On the second day of analysis, samples were thawed, a 110-μl aliquot of 2.5 *N* HCl was added to adjust the pH to 1, and 40 μl of titanous chloride was added. The mixture was stored in the dark for 40 min and extracted twice with 3 ml of a mixture of hexane/diethyl ether (7:3, v/v). Samples were centrifuged at 3500 rpm and the combined extracts were evaporated to dryness under nitrogen. The residues were methylated in tightly capped vials for 10 min at room temperature with 100 μl of 0.33 *M* ethereal diazomethane which was made from MNNG according to an established procedure (Technical Information Bulletin AL-132, Aldrich Chemical Co. Inc.). The vials were uncapped and the ether and diazomethane were allowed to evaporate at room temperature. It should be noted that MNNG is a potent mutagen and that diazomethane also is rather toxic. All work should be performed in a fume hood.

To the remaining aqueous phase, 217 μl of 2.5 *N* NaOH was added to adjust the pH to approximately 9.5, samples were vortexed, 500 μl of 0.2 *M* carbonate/bicarbonate buffer was added to adjust the pH to 10, and samples were again vortexed. The aqueous solution was extracted twice with hexane (3 ml each). The extracts were transferred to a culture tube containing 150 mg of anhydrous sodium sulfate, rotated for 5 min, and centrifuged for 5 min at 3500 rpm. The dry extracts were transferred to a vial and evaporated to dryness. The corresponding residues following reduction and methylation of the same sample were combined into 150 μl of reconstituting solvent (iso-octane containing 1% pyridine) and transferred to a conical autosampler vial for analysis (solution B).

Solutions A and B were injected separately for the determination of MDL 72,222 and metabolite **1** as well as metabolites **2**, **3**, and **4**, respectively. In each analytical run, the standards were assayed in duplicate. At the end of each run, the regression of the peak area ratios of analytes/internal standards versus the corresponding analyte concentrations in the standards were calculated by least-squares linear regression analysis. Data from standards in the lower analysis ranges of either 0.5–10 or 1–20 ng/ml, depending on the analyte, were weighted by various schemes such as the reciprocal of the concentration or the reciprocal of the concentration squared, to determine the best fit. The concentrations of analytes in unknown samples were determined by interpolation from the appropriate regression lines.

### Validation Procedure

Individual plasma samples from 10 monkeys were analyzed for possible chromatographic interference with each analyte or internal standard peak. Possible interference from endogenous plasma was also evaluated by analyzing predose samples from a single-dose study.

The extraction recovery of each analyte was determined by comparing the slope of a standard curve (peak area versus concentration) extracted from monkey plasma to the slope of a nonextracted standard curve.

The limit of detection was determined as follows: two 1.0-ml aliquots of citrated control plasma from each of 10 normal monkeys were transferred into 20 separate culture tubes. To one tube of each pair, 20 μl of acetonitrile containing 0.5 ng of MDL 72,222, 0.25 ng of **1**, 0.5 ng of **2**, 0.25 ng of **3**, and 0.5 ng of **4** was added and samples were mixed (spiked sample). The other tube was spiked with 20 μl of acetonitrile (blank). All of the samples were then analyzed by the described assay procedure. The measured peak area at the retention time of each compound for each sample was recorded and the difference in response for spiked versus blank sample pairs was evaluated with Student's *t* test for paired samples.

The stability of the compounds at −10°C was evaluated by preparing a large set of 1-ml stability samples spiked at concentrations of 5 and 50 ng/ml. At various times, groups of three samples were analyzed to determine the stability.

The intraday accuracy and precision of the method were determined as follows: monkey plasma containing 1, 50, and 100 ng of MDL 72,222 and compounds **2** and **4** as well as 0.5, 25, and 50 ng/ml of compounds **1** and **3** were prepared and analyzed in five replicates. The observed mean, percentage theoretical recovery, standard deviation (SD), and percentage relative standard deviation (%RSD) were calculated.

Interday precision and accuracy were assessed by analyzing five replicates of monkey plasma samples spiked at three different concentrations (1.0, 50, and 100 ng/ml for MDL 72,222 and compounds **2** and **4** as well as 0.5, 25, and 50 ng/ml for compounds **1** and **3**) on 3 different days. The observed mean, percentage theoretical recovery, SD, and %RSD were calculated.

## RESULTS AND DISCUSSION

### Method Development

Development of the proposed method of analysis of MDL 72,222 and metabolites **1–4** was aided by the range of acidities, basicities, and polarities found in these analytes. The basic MDL 72,222 and its *N*-demethylated metabolite, compound **1**, were extracted from plasma at pH 10 with two hexane extractions, while the acidic metabolites, compounds **2** and **3**, and the more polar *N*-oxide metabolite, compound **4**, remained in the aqueous phase.

Metabolite **1**, being structurally very similar to MDL 72,222, eluted as a tailing peak which was poorly resolved from MDL 72,222 by the chromatographic conditions de-

scribed above. Acylation resulted in the formation of a less polar trifluoroacetyl derivative which was easily separated from MDL 72,222 and had good peak shape. However, trifluoroacetic acid produced from this derivatization protonates MDL 72,222 molecules, making them insoluble in the reconstituting solvent. Therefore, MDL 72,222 was deprotonated with dilute aqueous base prior to the extractions with hexane.

After isolating MDL 72,222 and metabolite 1 from the plasma with two hexane extractions, the aqueous phase was extracted a third time with hexane for MDL 72,222, and the extract was discarded. These three extractions resulted in nearly quantitative extraction of MDL 72,222, which ensured that little or no residual MDL 72,222 was carried into the next portion of procedure where the *N*-oxide of MDL 72,222 was reduced to MDL 72,222 with titanous chloride. Any carryover of MDL 72,222 would inflate the assay value for the *N*-oxide. After reduction, the acidic metabolites, compounds 2 and 3, were extracted, derivatized with diazomethane, and combined with the MDL 72,222 formed from the *N*-oxide for GC-MS analysis.

*N*-Oxides such as metabolite 4 are very polar, difficult to extract from an aqueous phase, and often thermolabile (3). It was found that metabolite 4 could not be extracted from plasma with hexane. Thus, hexane was used to extract MDL 72,222, metabolite 1, and their internal standards in the presence of metabolite 4. Because metabolite 4 could decompose to MDL 72,222 in the heated injection port of a gas chromatograph or on the GC column, reduction of metabolite 4 was chosen to make this compound amendable to isolation and quantification.

In a method described by Beckett *et al.* (4) for the determination of the *N*-oxide metabolite of nicotine, this metabolite was reduced to nicotine with titanous chloride at an acidic pH. The reduced metabolite was subsequently isolated by liquid–liquid extraction from basified samples. Thus, a very polar and possibly nonextractable metabolite was isolated as a less polar entity in the reduced form. Similarly, metabolite 4 was reduced to MDL 72,222 with titanous chloride and was quantitated in a separate analysis. Alternatively, zinc powder could have been used as the reducing agent. The reaction with zinc powder, however, would have been heterogeneous and would probably require a longer reaction time (5); thus, titanous chloride was used for this reduction.

Because titanous chloride is light sensitive, samples were stored in the dark during reduction. A time-course study of the reduction reaction showed that 30 min was probably adequate for complete reduction of metabolite 4, but an additional 10 min was allowed to ensure complete reduction.

## Method Validation

Typical chromatograms of extracted plasma sample are shown in Fig. 3. The retention times of MDL 72,222 and compounds 1–4 were 6.89, 7.31 (detected as TFA derivative), 3.61 (detected as methyl ester), 6.01 (detected as methyl ester), and 6.89 (detected as MDL 72,222) min, respectively. The retention times of their corresponding internal standards were 6.89, 7.21 (detected as TFA derivative of compound 5), 3.78 (detected as methyl ester of compound 6),

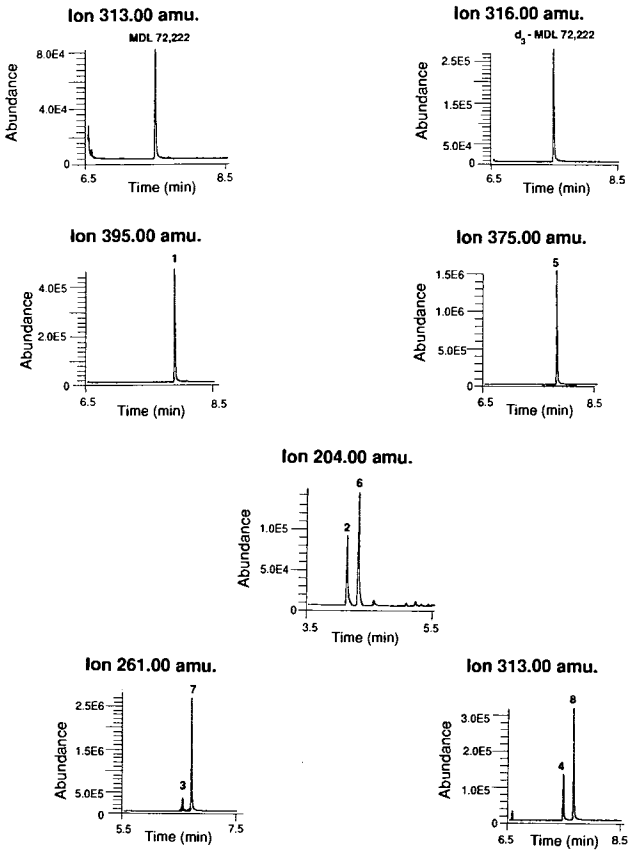


Fig. 3. Typical selected-ion chromatograms of monkey plasma spiked with 20 ng/ml each of MDL 72,222, and compounds 2 and 4, 10 ng/ml of compounds 1 and 3, and 10 ng/ml each of $d_3$-MDL 72,222 and internal standards 5–8.

6.16 (detected as methyl ester of compound 7), and 7.08 (detected as reduced product of compound 8) min. Analysis of blank control plasma from 10 normal monkeys indicates that there are no endogenous plasma components that interfere with peaks of these compounds or their internal standards. Predose monkey plasma samples also showed no interferences.

Because the detector response was not linear in the range of 1.0 to 150 ng/ml of MDL 72,222, metabolite 2, or metabolite 3 or in the range of 0.5 to 75 ng/ml of metabolites 1 or 4, two standard curves in the concentration range of either 0.5–10 and 10–75 ng/ml or 1.0–20 and 20–150 ng/ml were used to quantify MDL 72,222 and its four metabolites in monkey plasma. Analysis of plasma standards, in duplicate, showed excellent linearity. Linear regression analysis gave a correlation coefficient close to unity for each standard curve on each day of analysis. The slopes of the two standard curves for each compound were reproducible from day to day with an RSD of $<10\%$ ($N = 8$).

The mean detector responses for the plasma samples spiked with 0.5 ng/ml of MDL 72,222, 0.25 ng/ml of 1, 0.5 ng/ml of 2, 0.25 ng/ml of 3, and 0.5 ng/ml of 4 were significantly greater than those for the corresponding blank samples ($P < 0.001$). Therefore, these concentrations were chosen as the detection limits of the method. These levels of sensitivity for MDL 72,222 and compound 1 could not have been achieved without silanized glassware.

Table I. Intraday Precision and Accuracy

| Compound | Concentration (ng/ml) Nominal | Found | % recovery ± % RSD |
|---|---|---|---|
| MDL 72,222 | 1.00 | 1.01 ± 0.01[a] | 101 ± 0.1 |
| | 50.0 | 51.3 ± 1.3 | 103 ± 2.5 |
| | 100.0 | 100.5 ± 1.9 | 101 ± 1.9 |
| 1 | 0.50 | 0.44 ± 0.04 | 92.0 ± 9.3 |
| | 25.0 | 23.5 ± 0.1 | 94.0 ± 0.5 |
| | 50.0 | 46.7 ± 1.5 | 93.0 ± 3.3 |
| 2 | 1.00 | 1.10 ± 0.06 | 110 ± 5.5 |
| | 50.0 | 50.8 ± 2.6 | 102 ± 5.2 |
| | 100.0 | 93.5 ± 3.0 | 93.5 ± 3.2 |
| 3 | 0.50 | 0.50 ± 0.03 | 100 ± 5.9 |
| | 25.0 | 23.2 ± 0.23 | 92.8 ± 1.0 |
| | 50.0 | 46.8 ± 1.4 | 93.6 ± 2.9 |
| 4 | 1.00 | 1.02 ± 0.09 | 102 ± 8.6 |
| | 50.0 | 47.5 ± 0.7 | 95.0 ± 1.5 |
| | 100.0 | 93.8 ± 3.0 | 93.8 ± 3.2 |

[a] Mean ± SD; $N = 5$.

The stability of MDL 72,222 and its four metabolites in frozen samples (−10°C) was assessed by analyzing spiked plasma samples (5.0 and 50 ng/ml) in five replicates after various times of storage. MDL 72,222 and its four metabolites were found to be stable for at least 89 days in monkey plasma at −10°C. These compounds were also stable in the reconstituted extracts for at least 2 days. In addition, repeated freezing and thawing of the plasma samples had no apparent adverse effects on stability.

The extraction recovery was found to be 91% for MDL 72,222, 73% for metabolite **1**, 101% for metabolite **2**, 57% for metabolite **3**, and 93.6% for metabolite **4** reduced to and extracted as MDL 72,222.

The intraday precisions and accuracies of the assay method are presented in Table I. The intraday precision values (%RSD) were less than 10% and accuracy values were in the range of 92.0–110%. The interday precision values (%RSD) were less than 14% and accuracy values were in the range of 87.6–116% (Table II). Study results indicated that dilution of the samples did not significantly affect the precision and accuracy of the method.

Table II. Interday Precision and Accuracy

| Compound | Concentration (ng/ml) Nominal | Found | % recovery ± % RSD |
|---|---|---|---|
| MDL 72,222 | 1.00 | 0.88 ± 0.12[a] | 87.6 ± 13.4 |
| | 50.0 | 51.0 ± 1.1 | 102 ± 2.2 |
| | 100.0 | 100.5 ± 1.6 | 100 ± 1.5 |
| 1 | 0.50 | 0.52 ± 0.05 | 104 ± 10.3 |
| | 25.0 | 24.4 ± 1.1 | 97.6 ± 4.5 |
| | 50.0 | 47.7 ± 3.1 | 95.4 ± 6.4 |
| 2 | 1.00 | 1.16 ± 0.05 | 116 ± 4.5 |
| | 50.0 | 49.3 ± 1.7 | 98.5 ± 3.5 |
| | 100.0 | 90.0 ± 4.9 | 90.0 ± 5.4 |
| 3 | 0.50 | 0.50 ± 0.01 | 100 ± 1.0 |
| | 25.0 | 23.9 ± 0.8 | 95.6 ± 3.5 |
| | 50.0 | 46.6 ± 2.6 | 93.2 ± 5.6 |
| 4 | 1.00 | 0.99 ± 0.09 | 99.3 ± 9.4 |
| | 50.0 | 48.3 ± 0.7 | 96.6 ± 1.5 |
| | 100.0 | 94.3 ± 1.6 | 94.3 ± 1.8 |

[a] Mean ± SD; $N = 3$.

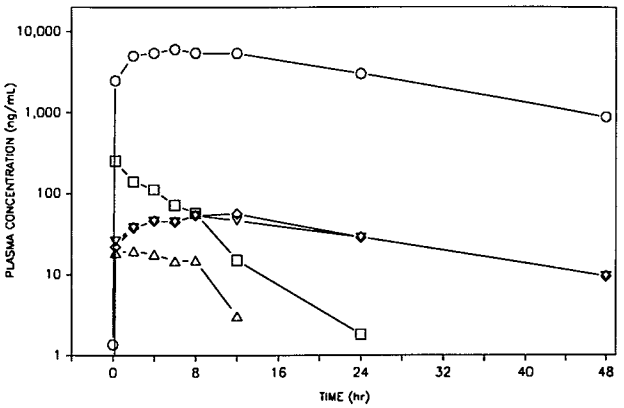


Fig. 4. Plasma concentration–time profiles of MDL 72,222 (□) and metabolites **1** (▽), **2** (○), **3** (◇), and **4** (△) in a monkey given a 5-mg/kg intravenous dose of MDL 72,222.

The assay method has been used to analyze pharmacokinetic study samples. An example of the plasma concentration–time profiles for one monkey is shown in Fig. 4.

In conclusion, a method based on GC-MS has been developed for analyzing MDL 72,222 and its four metabolites in 1 ml of citrated monkey plasma. This method has been shown to be specific, sensitive, precise, and accurate. This method can be easily modified to monitor MDL 72,222 and/or its metabolites in human plasma.

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