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Simultaneous analysis of diphenylmethoxyacetic acid, a metabolite of diphenhydramine, and its deuterium-labeled stable isotope analog in ovine plasma and urine

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Abstract

Diphenylmethoxyacetic acid (DPMA) is a major metabolite of diphenhydramine in monkeys, dogs, and humans. The metabolic fate of diphenhydramine (DPHM) in sheep is not yet well understood; however, preliminary studies have demonstrated the presence of DPMA in the plasma and urine of sheep following an intravenous bolus of DPHM. Our current studies employ the simultaneous intravenous co-administration of DPHM and the stable isotope analog of DPHM to investigate the pharmacokinetics of DPHM in sheep. In these studies, in order to investigate the pharmacokinetics of the DPMA metabolite, measurement of both unlabeled and stable-isotope labeled DPMA is required. Thus, a stable isotope analog of DPMA ([$^2H_{10}$]DPMA) was synthesized, characterized, and purified for use as an analytical standard. The quantitative method for the gas chromatography–electron-impact mass spectrometry (GC–EI-MS) analysis of DPMA and [$^2H_{10}$]DPMA used a single step liquid–liquid extraction procedure using toluene for sample cleanup. The samples were derivatized with N-methyl-N-(*tert.*-butyldimethylsilyl) trifluoroacetamide. A 1.0-μl aliquot of the prepared sample was injected into the GC–MS system and quantitated using selected-ion monitoring (SIM). One ion was monitored for each compound, namely, m/z 165 for the internal standard diphenylacetic acid, m/z 183 for DPMA, and m/z 177 for [$^2H_{10}$]DPMA. The ion chromatograms were free from chromatographic peaks co-eluting with the compound of interest. The calibration curve was linear from 2.5 ng/ml (limit of quantitation) to 250.0 ng/ml in both urine and plasma. The intra-day and inter-day variabilities of this assay method were within acceptable limits (below 20% at the limit of quantitation and below 10% at all other concentrations). This method was used to measure the concentration of DPMA and

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[$^2H_{10}$]DPMA in plasma and urine samples from a ewe in which equimolar amounts of DPHM and [$^2H_{10}$]DPHM were administered by an intravenous bolus dose *via* the femoral vein. DPMA appeared to persist longer in the plasma and the urine as compared to DPHM. This method is robust and reliable for the quantitation of DPMA and [$^2H_{10}$]DPMA in biological samples obtained from sheep (e.g. plasma and urine).

1. Introduction

Diphenhydramine [2-(diphenylmethoxy)-N,N-dimethylethylamine] is a H_1 receptor antagonist that is used clinically for its antihistaminic, antitussive, and sedative properties [1–5]. The metabolism of diphenhydramine (DPHM) has been studied in several species including humans. The metabolites of DPHM that have been identified to date in dogs, humans, rhesus monkeys, and rats include N-demethyl DPHM, N,N-didemethyl DPHM, DPHM N-oxide, diphenylmethoxyacetic acid (DPMA), glycine and glutamate conjugates of DPMA, and a N-glucuronide of DPHM [6–10]. In several species (i.e. humans, dogs, and rhesus monkeys), DPMA and its various conjugates are the most abundant metabolites excreted in the urine [6–10]. DPMA (Fig. 1) has also been detected in both ovine plasma and urine following an intravenous bolus administration of DPHM (unpublished results). In order to gain a better insight into the metabolism and disposition of DPHM in sheep, further study regarding the quantitative importance of this metabolite is necessary.

The work conducted in our laboratory, examining the pharmacokinetics and metabolism of DPHM in pregnant and non-pregnant sheep, incorporates the simultaneous administration of both DPHM and stable isotope-labeled DPHM, namely [$^2H_{10}$]DPHM (in which the two aromatic rings are deuterium labeled). We have recently developed a method for the simultaneous quantitation of both DPHM and [$^2H_{10}$]DPHM in biological fluids from pregnant and non-pregnant ewes [11]. We now routinely examine the pharmacokinetics of DPHM and [$^2H_{10}$]DPHM following simultaneous and independent routes of administration (i.e. via different routes). The ability to examine the pharmacokinetics of the resulting metabolites, DPMA and [$^2H_{10}$]DPMA, following such an experiment would provide additional useful information regarding the in-vivo metabolism of DPHM in pregnant and non-pregnant sheep, and in late gestational fetal lambs. To date, there is no direct method of analysis for DPMA. Although there is an indirect method (i.e. colorimetric) for the quantitation of this acid metabolite, this method would

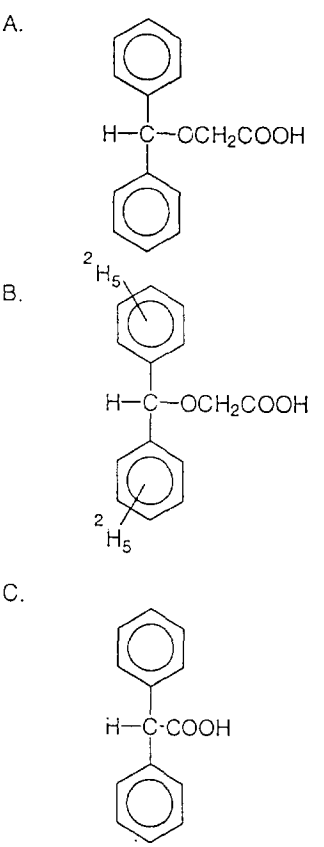

Fig. 1. The chemical structures of: (A) diphenylmethoxyacetic acid (DPMA), (B) stable isotopically (deuterium) labeled diphenylmethoxyacetic acid ([$^2H_{10}$]DPMA), and (C) the internal standard diphenylacetic acid (DPAA).

not be capable of simultaneously quantitating DPMA and [$^2H_{10}$]DPMA in a biological sample [8]. Mass spectrometry was chosen as the method of detection because this technique could provide the required selectivity between the unlabeled and stable isotope labeled DPMA, and sensitivity for quantitation. Prior to the development of this GC–MS analytical method, an analytical standard of stable isotope-labeled DPMA, with the label corresponding to the site of the label on the intact drug, had to be synthesized. The purpose of this paper is to describe the synthesis of the stable isotope [$^2H_{10}$]DPMA and the development of an analytical method by which both DPMA and [$^2H_{10}$]DPMA could be simultaneously measured in plasma and urine samples obtained from sheep dosed with a 50:50 mixture of DPHM and [$^2H_{10}$]DPHM.

2. Experimental

2.1. Reagents and materials

Deuterated benzene (d^6-benzene, 99.5% purity) was obtained from MSD Isotopes (Montreal, Canada). Bromoacetic acid, anhydrous aluminum chloride, anhydrous sodium sulfate, carbon tetrachloride, diethyl ether, hydrochloric acid, HPLC-grade methanol, ethyl alcohol, isopropyl alcohol, and sodium hydroxide were purchased from BDH (Toronto, Canada). Diphenylacetic acid (DPAA) and deuterium oxide were acquired from Aldrich Chemical Co. (Milwaukee, WI, USA). N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and pentafluorobenzyl bromide (PFBBr) were obtained from Pierce Chemical Co. (Rockville, IL, USA). Toluene distilled in glass was purchased from Caledon Laboratories (Georgetown, Canada). Ultra-high purity helium was obtained from Matheson Gas (Edmonton, Canada). Deionized, high purity water was produced on-site by reverse osmosis and subsequent filtration using a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Synthesis, identification and purification of diphenylmethoxyacetic acid and deuterated diphenylmethoxyacetic acid

The unlabeled diphenylmethoxyacetic acid (DPMA) was synthesized from benzhydrol and bromoacetic acid as described previously, with only a minor modification (Fig. 2) [12]. That is, rather than the removal of toluene by steam distillation following the reaction, the toluene and aqueous layers were separated using a separatory funnel. The stable isotope analog of diphenylmethoxyacetic acid ([$^2H_{10}$]DPMA) was synthesized as shown in Fig. 2. The initial step in the synthesis of [$^2H_{10}$]DPMA involved the synthesis of deuterium labeled benzhydrol ([$^2H_{10}$]benzhydrol) as described previously [11]. The [$^2H_{10}$]benzhydrol and bromoacetic acid were used to synthesize [$^2H_{10}$]DPMA as shown in Fig. 2. DPMA and [$^2H_{10}$]DPMA were purified using column flash chromatography [in a glass column of dimensions 75 × 6 cm I.D. packed with silica gel 60; mesh 240–400; mobile

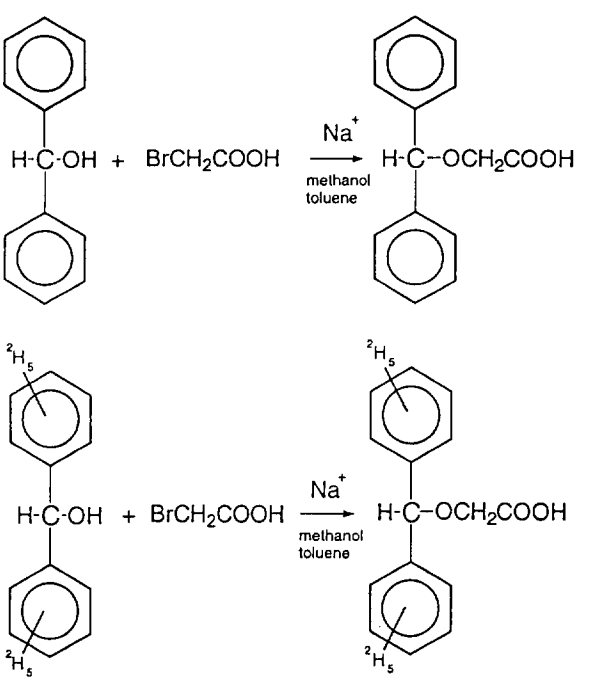

Fig. 2. The synthesis of unlabeled DPMA and stable isotopically labeled DPMA ([$^2H_{10}$]DPMA).

phase diethyl ether–hexane–isopropyl alcohol (50:48:2, v/v)] followed by re-crystallization with hexane and acetone. The yield of vacuum-dried DPMA and [$^2H_{10}$]DPMA was approximately 25% based on the weight of the starting material, benzhydrol, and [$^2H_{10}$]benzhydrol, respectively.

The identification of DPMA and [$^2H_{10}$]DPMA was confirmed using ^1H nuclear magnetic resonance (NMR) (Bruker AC-200, 200 MHz; Department of Chemistry, University of British Columbia): DPMA ^1H NMR: ($CDCL_3$); δ 4.20 (S, 2H, OCH_2); 5.50 (S, 1H, CH); 7.30 (M, 10H, ArH) and [$^2H_{10}$]DPMA ^1H NMR: ($CDCL_3$); δ 4.20 (S, 2H, OCH_2), 5.50 (S, 1H, CH). In addition, electron ionization GC–MS and negative chemical ionization GC–MS were used in the identification of DPMA and [$^2H_{10}$]DPMA. Pure DPMA and [$^2H_{10}$]DPMA were dissolved in toluene, derivatized with MTBSTFA, and injected onto the GC–MS (electron-impact mode) which was used in the scanning mode. Characteristic fragments following GC–EI-MS for DPMA were *m/z* 165, 167, 183, and 299, and for [$^2H_{10}$]DPMA were 173, 177, 193, and 309, respectively. Pure samples of both DPMA and [$^2H_{10}$]DPMA were dissolved together in distilled water. These samples were extracted in duplicate using 400 μl of 1.0 *M* HCl and 5.0 ml of toluene. The organic layer was transferred to a clean test tube, and the organic layer removed with a gentle stream of nitrogen at 40°C. The residue was reconstituted with acetone–PFBBr (100:1). The mixture was incubated at 60°C for 60 min. The acetone was evaporated and the residue reconstituted with a mixture of 200 μl of toluene and 500 μl of distilled water. The test tube containing the sample was mixed for one minute on a vortex-mixer and centrifuged at 3000 *g* for 2 min. The top organic layer was removed, and a 1.0-μl aliquot of that layer was injected into the GC–MS in the negative chemical ionization (NCI) mode with methane as the reagent gas. The total-ion scan showed only one fragment for DPMA (*m/z* 241) and [$^2H_{10}$]DPMA (*m/z* 251) corresponding to the loss of the pentafluorobenzyl group from the molecular ion (i.e. [M − 181]).

The purity of DPMA and [$^2H_{10}$]DPMA was assessed in the following fashion. DPMA and [$^2H_{10}$]DPMA were dissolved in toluene, an aliquot was removed and derivatized with MTBSTFA, and this aliquot was injected directly into the GC–MS (electron impact mode). The total-ion chromatogram showed only the peaks corresponding to DPMA and [$^2H_{10}$]DPMA. No chromatographic peaks other than those present in the blank were detected. In addition, DPMA and [$^2H_{10}$]DPMA were dissolved in distilled water. These samples were extracted as described above. The reconstituted residue was derivatized with either MTBSTFA (GC–EI-MS) or PFBBr (GC–NCI-MS). The total-ion chromatograms showed only the chromatographic peaks corresponding to the compound in question, and no other peaks than those found in the blank. Thermal analysis was conducted using a differential scanning calorimeter (Dupont Instruments Series 99 thermal analyzer). Data obtained showed only one sharp peak corresponding to a melting point of 77°C and 78°C for DPMA and [$^2H_{10}$]DPMA, respectively. The lack of other peaks during thermal analysis also suggests the lack of any polymorphic forms, and/or solvates and hydrates of DPMA and [$^2H_{10}$]DPMA analytical standards.

2.3. Preparation of standard stock solutions

The aqueous standard solutions of DPMA and [$^2H_{10}$]DPMA were prepared with distilled deionized water to yield a final concentration of 500 ng/ml and 520 ng/ml (mass corrected for the mass of the stable isotope label), respectively. The internal standard DPAA was dissolved in methanol. An aliquot of the methanolic solution was diluted with distilled deionized water to give a final concentration of 2.0 μg/ml.

2.4. Extraction procedure

Samples were prepared for analysis using a single-step liquid–liquid extraction as shown in Fig. 3. Aliquots of biological samples (0.10–1.00

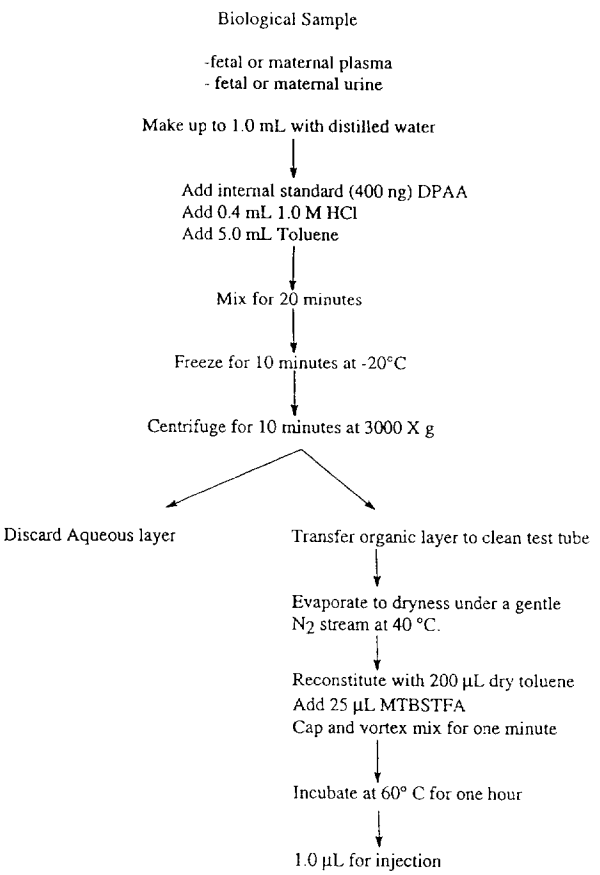

Fig. 3. The extraction procedure of DPMA and [$^2H_{10}$]DPMA from a biological matrix.

ml of plasma and 0.05–1.00 ml of urine) were pipetted into clean test tubes. The samples were made up to a volume of 1.0 ml with distilled water. A 200-μl aliquot of internal standard (diphenylacetic acid: DPAA 2.0 μg/ml), 400 μl of 1.0 *M* HCl, and 5.0 ml of toluene was added to the biological sample. The test tubes were capped with polytetrafluoroethylene (PTFE)-lined lids (Corning Glass Works, Corning, NY, USA) and mixed for 20 min on a Labquake Tube Shaker, Model 415-110 (Lab Industries, Berkeley, CA, USA), cooled for 10 min at −20°C in a freezer (to break any emulsion formed during mixing), and centrifuged at 3000 *g* on an IEC Model NH-SII centrifuge (Damon/IEC Division, Needham Heights, MA, USA). The organic layer was transferred to clean test tubes and evaporated to dryness in a water bath at 40°C under a gentle stream of nitrogen gas. The dried samples were reconstituted with 200 μl of dry toluene (toluene stored on anhydrous sodium sulfate) and 25 μl of the derivatizing reagent MTBSTFA were added. The tubes were capped, mixed for one minute on a vortex-mixer, and incubated at 60°C for one hour. After the samples cooled, the derivatized mixture was transferred to clean borosilicate microvial inserts (placed in standard borosilicate autosampler vials) from which a 1.0-μl aliquot was used for injection.

2.5. Capillary gas chromatography and mass spectrometry

The samples were analyzed using a Hewlett-Packard 5890 Series II gas chromatograph (GC) equipped with a Hewlett-Packard Model 7673 autosampler, a capillary split–splitless inlet system, and a Hewlett-Packard Model 5971A mass selective detector (MSD) (Hewlett-Packard, Avondale, PA, USA). A 1.0-μl aliquot of prepared sample was injected through a Thermogreen LD-2 silicone rubber septum (Supelco, Bellefonte, PA, USA) into a Pyrex glass inlet liner (78 × 4 mm I.D.) with the capillary inlet operated in the splitless injection mode. Chromatographic separation of the analytes ([$^2H_{10}$]DPMA, DPMA, and DPAA) from endogenous materials was achieved using a HP Ultra-2 25 m × 0.2 mm I.D. (0.33 μ film thickness) capillary column (Hewlett-Packard, Palo Alto, CA, USA). Column head pressure was optimized at 100 kPa (0.6 ml/min at the initial temperature). The gas chromatographic system operating conditions were optimized as follows: The injection port temperature was held at 280° C. The initial oven temperature was maintained at 125°C for 1 min, the oven temperature was ramped at 12.5°C/min to 280°C where it was held for 4.0 min. The temperature program resulted in a total run time of 17.4 min. The transfer line temperature was held at 285°C. The mass selective detector (MSD) was manually tuned with the tuning reagent perfluorotributylamine (PFTBA) to ions *m/z* 100, 131, and 219. The GC–MSD operating in the electron-impact

ionization mode (voltage 70 eV) with selective-ion monitoring (EI-SIM) was used to quantitate DPAA, DPMA, and [$^2H_{10}$]DPMA by monitoring ions m/z 165, 183 and 177, respectively. The dwell time was set at 125 ms for each ion being monitored to ensure adequate sampling of the chromatographic peak of interest. The electron multiplier voltage was programmed to +200 V relative to the tune value during the elution of the compounds of interest. The voltage was programmed to reset to −1000 V relative to the tune value at all other times to maximize the life span of the electron multiplier.

2.6. Extraction recovery

Extraction recoveries of DPMA and [$^2H_{10}$]DPMA were both determined at low, moderate, and high concentrations (5.0, 50.0, and 500.0 ng/ml, respectively) from plasma and urine. Two groups of samples were used to assess extraction recovery (i.e. test and control groups). Both groups of samples contained blank biological matrix (plasma, or urine), and internal standard (DPAA). However, the samples from the test group were spiked with both DPMA and [$^2H_{10}$]DPMA to yield final concentrations of 5.0, 50.0, and 500.0 ng/ml, whereas the samples in the control group were not spiked with DPMA and [$^2H_{10}$]DPMA at this point of the experiment. Following liquid–liquid extraction of both the test and control group samples, aliquots of DPMA and [$^2H_{10}$]DPMA standards, made up in methanol, were added to the control group samples to yield drug concentrations of 5.0, 50.0, and 500.0 ng/ml. Control and test samples were dried, reconstituted, derivatized, and chromatographed as described above. The concentrations of the test and control samples were determined from standard curves extracted from the corresponding biological matrices (i.e. plasma and urine). The extraction recovery was calculated as the ratio of the measured concentration of the test samples over the measured concentration of the control samples at the low, medium, and high concentrations.

2.7. Calibration curve

A seven-point calibration curve was constructed from aqueous standard solutions of DPMA and [$^2H_{10}$]DPMA at concentrations of 2.5, 5.0, 10.0, 25.0, 50.0, 125.0, and 250.0 ng/ml with 400 ng/ml of DPAA as the internal standard. Aliquots of blank plasma or urine were added to the calibration curve samples. The samples were extracted and quantitated as described above. Weighted linear regression (weighting function = $1/y^2$) was performed between the drug response [DPMA or [$^2H_{10}$]DPMA peak area/internal standard (DPAA) peak area] and the spiked drug concentrations of DPMA and [$^2H_{10}$]DPMA.

2.8. Sample stability determination

Numerous studies were carried out in order to determine the stability of the samples during storage and analysis. The freezer stability of these samples was determined by spiking blank plasma with DPMA and [$^2H_{10}$]DPMA at a known concentration of 100.0 ng/ml. These samples were frozen at −20°C and removed at specific intervals and analyzed. The freeze–thaw stability of DPMA and [$^2H_{10}$]DPMA in plasma samples was assessed as follows. Blank plasma samples were spiked with 100.0 ng/ml of DPMA and [$^2H_{10}$]DPMA. These samples were frozen at −20°C and thawed at 22°C on the bench-top. This cycle was continued for a total of three cycles. On the final cycle, the samples were frozen at −20°C and stored until analysis. The bench-top stability of DPMA and [$^2H_{10}$]DPMA in a plasma matrix was determined in the following fashion. Blank plasma was spiked with DPMA and [$^2H_{10}$]DPMA to yield a concentration of 100 ng/ml. The samples were left on the bench-top at 22°C for various periods of time (i.e. 0, 1, 2, 4, 6, 12, and 24 h). The samples were immediately frozen at −20°C and stored frozen until the time of analysis. Because DPMA and [$^2H_{10}$]DPMA appeared to be sensitive to acid, the stability of the samples in an acidified matrix (i.e. acidified to the same degree as

during the extraction procedure) was examined. Briefly, spiked samples in a distilled water, plasma, and urine matrix were acidified with 400 μl of 1.0 *M* HCl. These samples were vortex-mixed for one minute and left on the bench-top for the following periods of time: 0, 0.5, 1.0, 2.0, 4.0, 6.0, and 21.0 h. Following the desired incubation time, the internal standard was added and the samples extracted as described above. The degradation half-life of DPMA, and [$^2H_{10}$]DPMA was determined in the biological matrices examined. A stability study of the extracted and derivatized samples stored on the autosampler tray of the GC–MSD was conducted. Prepared samples at the concentrations of 2.5 and 250.0 ng/ml were extracted and analyzed. These samples were repeatedly injected at 24 h intervals for a total of 96 h (i.e. 0, 24, 48, 72, and 96 h).

2.9. Intra-day and inter-day variability

Intra-day variability was determined by quantitating four replicates at concentrations of 2.5, 25.0, 125.0, and 250.0 ng/ml using the GC–MSD method reported above on the same day. Inter-day variability was determined by quantitating one sample in duplicate at concentrations of 2.5, 25.0, 125.0, and 250.0 ng/ml using GC–MSD on four different days.

2.10. Drug administration and sample collection

Using methods previously described, a non-pregnant Dorset and Suffolk cross-breed ewe was prepared for experimentation using aseptic surgical techniques under 1–2% halothane (Fluothane, Ayrest Laboratories, Montreal, Canada)/nitrous oxide (60%) anesthesia [13]. Silicone rubber (Silastic, Dow Corning, Midland, MI, USA) catheters were surgically implanted in the femoral artery and femoral vein of a non-pregnant ewe. Several days post-surgery, on the morning of the experiment, a Foley bladder catheter (16 fr, Bard Ltd., Sunderland, UK) was inserted for cumulative urine collection. A simultaneous dose bolus of 57.2 mg of DPHM-HCl and 59.5 mg [$^2H_{10}$]DPHM-HCl was simultaneously administered via the femoral vein. Serial blood samples were collected from the femoral artery at −5, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270, 300, 360, 480, 720, and 1440 min. These samples were transferred to clean heparinized tubes (Vacutainer, Vacutainer Systems, Rutherford, NJ, USA) and centrifuged for 20 min at 3000 *g*. The plasma layer was transferred to clean borosilicate test tubes with PTFE-lined screw caps. Cumulative urine collections were made by draining the urine collected in sterile polyvinyl urine collection bags at −5, 30, 60, 90, 120, 150, 180, 240, 360, 480, 720 and 1440 min. The volume and pH of the urine were measured. A 10-ml aliquot of urine was transferred to clean borosilicate test tubes with screw caps lined with PTFE. All samples were stored frozen at −20°C until analysis. The concentration of DPHM and [$^2H_{10}$]DPHM in plasma and urine was determined by a previously published method [11]. Following the measurement of intact DPHM and [$^2H_{10}$]DPHM, the concentrations of DPMA and [$^2H_{10}$]DPMA were measured using the method described above.

3. Results

3.1. GC–MS

The mass spectra of the *tert.*-butyldimethylsilyl (TBDMS) derivatives of DPMA and [$^2H_{10}$]DPMA following electron-impact ionization show extensive fragmentation resulting in numerous small fragment ions with no molecular ion present at *m/z* 356 for DPMA and at *m/z* 366 for [$^2H_{10}$]DPMA, respectively (Fig. 4). The prominent ions for the TBDMS derivatives of DPMA and [$^2H_{10}$]DPMA are *m/z* 167 and 183, and *m/z* 177 and 193, respectively. Fragment ions of low intensity corresponding to [M − 57] or the loss of the *tert.*-butyl group from the derivatized DPMA and [$^2H_{10}$]DPMA (i.e. *m/z* 299 and 309) were also observed (Fig. 4). The

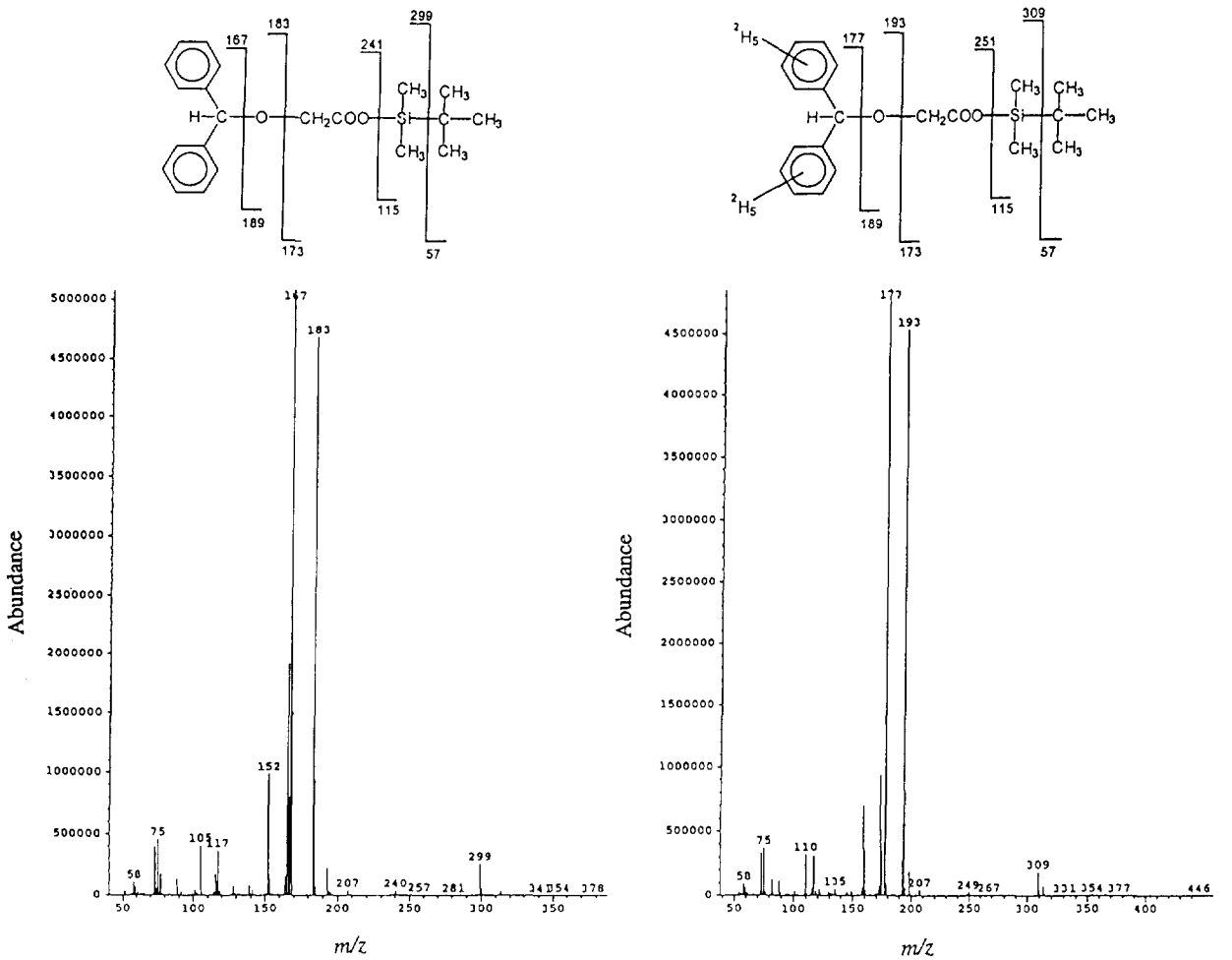

Fig. 4. The mass spectra of DPMA and [$^2H_{10}$]DPMA and the mass fragment assignments using gas chromatography–mass spectrometry with electron-impact (70 eV) ionization.

fragmentation of the *tert.*-butyldimethylsilyl derivative of the internal standard, DPAA, resulted in a base fragment ion [M − 57] of *m/z* 269 and a smaller fragment ion at *m/z* 165. For quantitation in SIM, fragment ions of *m/z* 165, 183, and 177 were monitored for DPAA, DPMA, and [$^2H_{10}$]DPMA, respectively. The ion chromatograms of plasma and of urine spiked with 250 ng/ml each of DPMA, [$^2H_{10}$]DPMA, and 400 ng/ml of DPAA and the corresponding blank matrices can be seen in Fig. 5. It should be noted that the *y*-axis for the blank biological samples is amplified to better show the ion chromatograms of the extracted blank matrices.

3.2. Extraction efficiency

The extraction efficiencies at various concentrations of DPMA and [$^2H_{10}$]DPMA from plasma were 78 ± 5 and 86 ± 11% at 5.0 ng/ml, 78 ± 1 and 75 ± 2% at 50.0 ng/ml, and 77 ± 2 and 74 ± 1% at 500.0 ng/ml, respectively. In urine, the extraction efficiencies for DPMA and [$^2H_{10}$]DPMA were 95 ± 6 and 99 ± 11% at 5.0

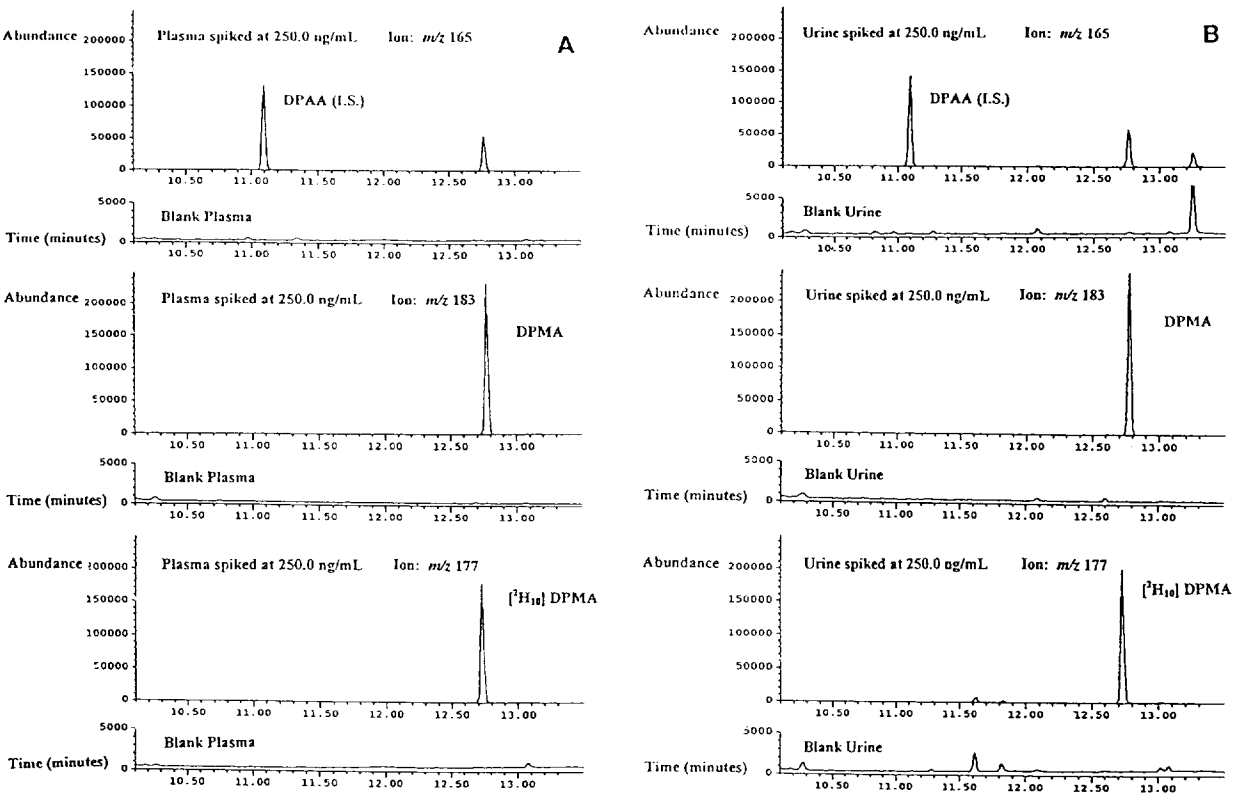

Fig. 5. (A) Ion chromatograms of m/z 165 (internal standard), 183 (unlabeled DPMA), and 177 (deuterium labeled DPMA) of blank plasma, and plasma spiked with 250.0 ng/ml of DPMA, 250 ng/ml of [$^2H_{10}$]DPMA, and 400 ng/ml of the internal standard DPAA. (B) Ion chromatograms of m/z 165 (internal standard), 183 (unlabeled DPMA), and 177 (deuterium labeled DPMA) of blank urine, and urine spiked with 250.0 ng/ml of DPMA, 250 ng/ml of [$^2H_{10}$]DPMA, and 400 ng/ml of the internal standard DPAA. Note that the y-axis scaling is increased in the blank to show more clearly the base-line of the ion chromatogram.

ng/ml, 73 ± 9 and 74 ± 14% at 50.0 ng/ml, and 79 ± 2 and 74 ± 2% at 500.0 ng/ml, respectively.

3.3. Calibration curve

The calibration curve for DPMA and [$^2H_{10}$]DPMA was linear over the concentration range examined (i.e. 2.5–250.0 ng/ml). Weighted linear regression was carried out using the weighting function of $1/y^2$. The resulting equations describe the linear regression for DPMA: $y = 0.0088x + 0.0009$; $r^2 = 1.000$, and [$^2H_{10}$]DPMA: $y = 0.0095x - 0.0013$); $r^2 = 1.000$. These regression constants resulted in a −9% bias at 2.5 ng/ml and + 1% at 250.0 ng/ml for DPMA and a + 4% bias at 2.5 ng/ml and a + 1% bias at 250.0 ng/ml for [$^2H_{10}$]DPMA. The minimum quantifiable concentration of this analytical method was 2.5 ng/ml (i.e. 11 pg at the detector). This minimum quantifiable concentration corresponds to a signal-to-noise ratio of 15 for DPMA and 20 for [$^2H_{10}$]DPMA.

3.4. Sample stability

Plasma samples containing DPMA and [$^2H_{10}$]DPMA appeared to be stable when stored frozen for up to a period of 3 months. Following three freeze–thaw cycles, the concentrations of DPMA and [$^2H_{10}$]DPMA did not differ significantly from the control values (Mann–Whitney U test; $P > 0.05$), suggesting that three freeze–

thaw cycles did not alter sample stability. The slope of the linear regression of the measured concentration of DPMA and [$^2H_{10}$]DPMA vs. time was not significantly different from zero (Students t-test; $P > 0.05$) following bench-top storage for up to and including 24 h, suggesting that bench-top storage did not adversely affect the stability of DPMA and [$^2H_{10}$]DPMA in a plasma matrix. DPMA was shown to be labile when stored in an acidified sample matrix for a prolonged period of time. The calculated degradation half-life was 16.5 h in water, 23.7 h in blank plasma, and 33.6 h in blank urine matrices. The area ratios of DPMA and [$^2H_{10}$]DPMA (i.e. DPMA/DPAA or [$^2H_{10}$]DPMA/DPAA) did not change for up to 96 h on the autosampler tray at room temperature (22°C).

3.5. Intra-day and inter-day variability

The results of the intra-day and inter-day variability studies for this analytical method are shown in Tables 1 and 2, respectively. The estimates of intra-day variability for DPMA and [$^2H_{10}$]DPMA were below 16% at the minimal quantifiable concentration of 2.5 ng/ml, and below 5% at all other concentrations investigated in plasma and urine (Table 1). The measured inter-day variability for DPMA and [$^2H_{10}$]DPMA was below 10% at the minimal quantifiable concentration of 2.5 ng/ml and below 8% for all other points (Table 2).

3.6. Disposition of DPMA and [$^2H_{10}$]DPMA

The disposition of both DPHM and [$^2H_{10}$]DPHM, and DPMA and [$^2H_{10}$]DPMA in plasma following a simultaneous intravenous bolus of DPHM and stable isotope-labeled DPHM is shown in Fig. 6. Whereas DPHM and [$^2H_{10}$]DPHM appear to undergo rapid removal from the plasma, DPMA and [$^2H_{10}$]DPMA show a much slower elimination from the plasma compartment. The cumulative DPHM and [$^2H_{10}$]DPHM amounts in the urine appear to

Table 1
Intra-day variability of assay method in plasma and urine

Spiked concentration	Plasma		Urine	
	DPMA	[$^2H_{10}$]DPMA	DPMA	[$^2H_{10}$]DPMA
2.5 ng/ml				
Mean	2.35	2.24	2.19	2.31
S.D.	0.23	0.06	0.35	0.13
C.V.	9.6	2.9	15.9	5.8
10.0 ng/ml				
Mean	8.5	8.3	8.8	9.3
S.D.	0.1	0.2	0.3	0.3
C.V.	1.0	1.9	3.3	3.6
50.0 ng/ml				
Mean	44.1	42.4	47.3	48.0
S.D.	0.4	0.7	1.0	1.2
C.V.	1.0	1.6	2.1	2.5
250.0 ng/ml				
Mean	235.4	219.7	251.5	249.1
S.D.	2.2	3.5	11.7	11.7
C.V.	0.9	1.6	4.7	4.7

Mean measured concentrations with standard deviation (S.D.) ($n = 4$).

Table 2
Inter-day variability of assay method in plasma and urine

Spiked concentration	Plasma		Urine	
	DPMA	[$^2H_{10}$]DPMA	DPMA	[$^2H_{10}$]DPMA
2.5 ng/ml				
Mean	2.30	2.39	2.25	2.51
S.D.	0.05	0.19	0.08	0.18
C.V.	2.2	8.1	3.6	7.2
10.0 ng/ml				
Mean	9.2	8.7	8.9	9.2
S.D.	0.6	0.3	0.3	0.1
C.V.	6.4	3.9	3.6	1.2
50.0 ng/ml				
Mean	46.5	45.5	47.3	47.8
S.D.	2.5	2.9	0.2	0.5
C.V.	5.4	6.3	0.3	1.1
250.0 ng/ml				
Mean	250.8	247.8	253.0	253.1
S.D.	11.2	19.1	2.3	4.2
C.V.	4.4	7.7	0.9	1.6

Mean measured concentrations with standard deviation (S.D.) ($n = 4$).

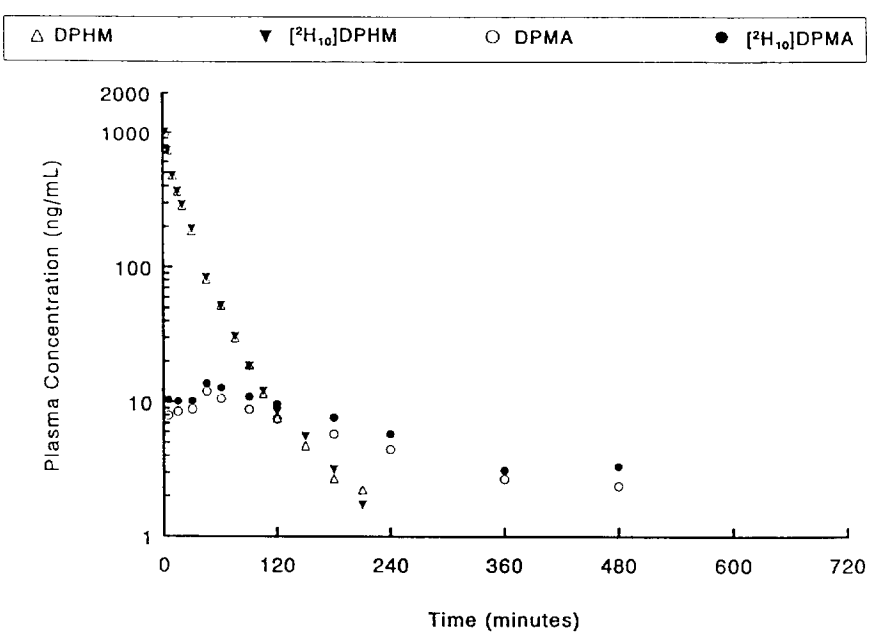

Fig. 6. Plasma concentration vs. time plot of labeled and unlabeled DPHM and labeled and unlabeled diphenylmethoxyacetic acid following a simultaneous bolus dose of 50 mg DPHM-HCl and 52 mg [$^2H_{10}$]DPHM-HCl.

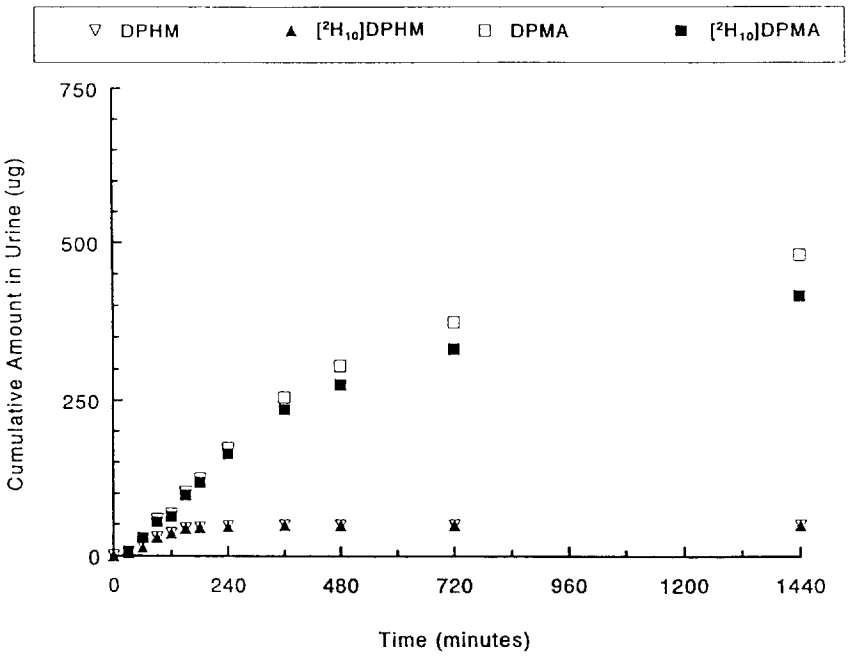

Fig. 7. The cumulative amount of labeled and unlabeled DPHM and labeled and unlabeled diphenylmethoxyacetic acid excreted in the urine following a simultaneous bolus dose of 50 mg DPHM-HCl and 52 mg [$^2H_{10}$]DPHM-HCl.

plateau 240 min following drug administration; however, the cumulative amounts of DPMA and [$^2H_{10}$]DPMA in the urine appear to continue to increase, even past the 24-h sampling time (Fig. 7).

4. Discussion

The simultaneous co-administration of labeled and unlabeled drug offers several advantages over traditional experimental designs in which only unlabeled drug is available. The ability to simultaneously conduct two experiments (i.e. a test and the corresponding control) on one occasion reduces the inter-day variability, the time required for experimentation, the number of incidences of drug exposure, and the number of samples requiring analysis [14–16]. The availability of [$^2H_{10}$]DPHM and an analytical method for the simultaneous quantitation of DPHM and [$^2H_{10}$]DPHM has enabled the application of stable isotope techniques to the study of the pharmacokinetics of this drug in pregnant, nonpregnant, and fetal sheep in our laboratory. A more thorough understanding of the in-vivo metabolism of DPHM in pregnant sheep can be obtained by the study of the resulting metabolites following the administration of the intact drug. The ability to measure DPMA and [$^2H_{10}$] DPMA is also of fundamental scientific importance in the process of interpreting data; in particular, data that suggest the presence of in-utero fetal metabolism of DPHM. Of the metabolites identified to date, unconjugated and various conjugates of DPMA have been shown to be prominent metabolites in dogs, rhesus monkeys, and humans [6–10]. Because this metabolic pathway also appears to be functional in sheep [i.e. unconjugated DPMA also appears to be present in the plasma and urine of adult sheep following the administration of unlabeled DPHM (unpublished results)], [$^2H_{10}$]DPMA was synthesized and purified. In addition, a GC–MS (SIM) analytical method was developed to simultan-

eously quantitate both [$^2H_{10}$]DPMA and DPMA to provide a tool to gain a better understanding of the in-vivo metabolism of DPHM in sheep.

The *tert.*-butyldimethylsilyl (TBDMS) derivatives of DPMA and [$^2H_{10}$]DPMA underwent extensive fragmentation under electron-impact conditions at 70 eV, with the majority of fragments resulting from the breakage of the ether linkage of DPMA. No molecular ion was detected, and only small amounts of the characteristic fragments [M − 57] (i.e. the loss of the *tert.*-butyl group) were observed (Fig. 4). Extensive fragmentation of the trimethylsilyl derivative of DPMA was also noted by Chang et al. [8]. There were a number of fragment ions which retained the stable isotope label and showed good intensity for SIM quantitation of DPMA and [$^2H_{10}$]DPMA (i.e. *m/z* 167 and 183-DPMA, and *m/z* 177 and 193-[$^2H_{10}$]DPMA). Initially, fragment ions *m/z* 183 and 193 were chosen for the development of the analytical method. However, it was soon discovered that the fragmentation of DPMA also resulted in a small *m/z* 193 fragment ion of unknown origin (Fig. 4). This fragment ion from DPMA resulted in chromatographic interference in the ion chromatogram of *m/z* 193 used to quantitate [$^2H_{10}$]DPMA. Although fragment ions *m/z* 167 and 177 gave good sensitivity and retained the stable isotope label, co-extracted components from adult urine co-eluted with DPMA in the *m/z* 167 ion chromatogram, resulting in the inability to reliably quantitate DPMA in urine. As a result, the fragment ions *m/z* 183 and 177 were chosen to quantitate DPMA and [$^2H_{10}$]DPMA in plasma and urine matrices. Despite the fact that this choice of fragment ions was less optimal than fragment ions from the same fragment group (i.e. *m/z* 167 and 177, or 183 and 193), the assay method appeared to be free from complications resulting from this choice of ions (i.e. non-linearity etc.). In addition, intra-day variability studies conducted in plasma using ions *m/z* 167 and 177, 183 and 193, or 183 and 177 all gave similar results, suggesting that this choice of ions would be adequate for the quantitation of DPMA and [$^2H_{10}$]DPMA. Furthermore, the ion chromatograms (i.e. *m/z* 183 and 177) were free of chromatographic interference from the elution of endogenous components in ovine plasma and urine at the retention times of DPMA and [$^2H_{10}$]DPMA (Fig. 5). Despite the inability to use an ion-pair for the quantitation of labeled and unlabeled DPMA, the use of fragment ions *m/z* 183 and 177 for SIM appeared to be a suitable alternative.

The extraction recovery of DPMA and [$^2H_{10}$]DPMA from the plasma matrix appeared to be constant at different concentrations (i.e. 5.0, 50.0, and 500 ng/ml) of the analyte. The constant recovery in plasma appears to contrast the situation observed in urine. The extraction recovery in urine appeared to be dependent on the concentration of the analyte. A much greater extraction recovery was observed at the lowest concentration measured (i.e. 95% and 99% at 5.0 ng/ml of DPMA and [$^2H_{10}$]DPMA, respectively); however, at the higher concentrations the average recoveries observed for DPMA and [$^2H_{10}$]DPMA were 76% and 74% at 50.0 and 500.0 ng/ml, respectively. At the higher concentrations, the extraction recovery in urine and plasma were comparable. The reason for the apparent higher recovery in urine at the lower concentrations of DPMA and [$^2H_{10}$]DPMA is not clear.

Poor sample stability either during storage and/or sample work up, can result in erroneous quantitation, and thus, erroneous interpretation of pharmacokinetic data. The stability of DPMA and [$^2H_{10}$]DPMA was shown to be adequate in most circumstances; however, DPMA was found to be labile in the presence of acid. The extraction conditions employed in this assay required the acidification of the plasma and urine biological matrices with 1.0 *M* hydrochloric acid. Caution must be used during the extraction procedure to ensure that not too much hydrochloric acid is added, and that the extraction procedure be conducted rapidly. For our study, the addition of 400 μl of 1.0 *M* HCl was found to provide an adequate reduction in pH for plasma and urine matrices (i.e. below a pH of 2) without significant breakdown of DPMA and [$^2H_{10}$]DPMA. Excess acid (i.e. < than 1.0 ml of 1.0 *M* HCl) was found to reduce the recovery of

DPMA and [$^2H_{10}$]DPMA and substantially increase the sample to sample variability. Stability studies conducted under acidic conditions, similar to those encountered during extraction procedure, showed that DPMA was stable during the extraction procedure. The measured half-lives for the degradation of DPMA in sample matrices would translate into the degradation of 1.0%, 0.6%, and 0.4% of DPMA in water, plasma, and urine during the 30-min time interval required for the extraction procedure. Thus, despite the acid-labile nature of DPMA, the degradation of this compound during a rapid extraction (less than 30 min) would appear to only minimally contribute to a decrease in the recovery of DPMA and [$^2H_{10}$]DPMA using the extraction method outlined above.

The results of the method validation experiments (i.e. intra-day and inter-day variability studies) would appear to suggest that this method is robust and the concentrations of DPMA and [$^2H_{10}$]DPMA in ovine plasma and urine samples can be measured with a high degree of confidence. Following an intravenous bolus dose of DPHM and [$^2H_{10}$]DPHM, the plasma concentrations of the intact drug (DPHM and [$^2H_{10}$]DPHM) decline rapidly. As DPHM and [$^2H_{10}$]DPHM decline, the metabolites, DPMA and [$^2H_{10}$]DPMA, are formed, reach a plateau, and decline. The decline of DPMA appears to be much slower than the decline of DPHM (Fig. 6). A similar trend is also apparent in the urine. That is, the renal elimination of DPHM and [$^2H_{10}$]DPHM appears to be nearly complete by six hours, while after 24 h DPMA and [$^2H_{10}$]DPMA have not yet reached the plateau of the cumulative urinary excretion plot (Fig. 7). The prolonged persistence of the DPMA in plasma and urine following the administration of parent drug is similar to the results presented for the disposition of this metabolite in humans, rhesus monkeys, and dogs [6–10].

While the simultaneous co-administration of DPHM and [$^2H_{10}$]DPHM by the same route of administration is important to rule out possible isotope effects, the application of this methodology (i.e. measuring DPHM and [$^2H_{10}$]DPHM and the corresponding metabolites) becomes more powerful when applied to the simultaneous administration of DPHM and [$^2H_{10}$]DPHM via different routes of administration. In this case, the effects of different routes of administration on the disposition of intact drug and the corresponding metabolites can be examined during one experiment. The disposition profiles of DPHM, [$^2H_{10}$]DPHM, DPMA, and [$^2H_{10}$]DPMA may be of particular importance in tracing the source (fetal or maternal) of this acidic metabolite, which is found in the fetal circulation following simultaneous maternal (DPHM) and fetal ([$^2H_{10}$]DPHM) administration (unpublished results). The ability to now examine the pharmacokinetics and disposition of DPMA and [$^2H_{10}$]DPMA following administration of the parent drugs (i.e. [$^2H_{10}$]DPHM and DPHM) will aid in our understanding of the pharmacokinetics and in-vivo metabolism of DPHM and [$^2H_{10}$]DPHM in pregnant, non-pregnant, and fetal sheep.

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