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Simultaneous Analysis of Diphenhydramine and a Stable Isotope Analog ($^2H_{10}$)Diphenhydramine Using Capillary Gas Chromatography with Mass Selective Detection in Biological Fluids from Chronically Instrumented Pregnant Ewes†

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This report describes both the synthesis of a stable isotope analog of the H_1 receptor antagonist diphenhydramine (DPHM), and the simultaneous quantitation of DPHM and a deuterated stable isotope analog of DPHM, viz. ($^2H_{10}$)DPHM in biological fluids from the chronically instrumented pregnant ewe. ($^2H_{10}$)DPHM was synthesized and purified, and both its structure and purity were verified. Biological samples were prepared for analysis using liquid–liquid extraction prior to capillary gas chromatography/mass spectrometry. The method employed electron impact ionization with selective ion monitoring of ions with *m/z* 165 for DPHM and *m/z* 173 for ($^2H_{10}$)DPHM. The minimal quantifiable concentration of DPHM and ($^2H_{10}$)DPHM from a 1.0 ml sample was 2.0 ng ml^{-1} in fetal and maternal plasma, fetal tracheal fluid and amniotic fluid. The method was validated from 2.0 ng ml^{-1} to 200.0 ng ml^{-1} for both DPHM and ($^2H_{10}$)DPHM in plasma, fetal tracheal fluid and amniotic fluid. Differences in the disposition between DPHM and ($^2H_{10}$)DPHM were not apparent during a control experiment in which both labeled and unlabeled DPHM were administered to a chronically instrumented fetal lamb. This method provides the required sensitivity and selectivity for the simultaneous quantitation of unlabeled and labeled DPHM during pharmacokinetic experiments conducted in near-term pregnant sheep.

INTRODUCTION

Diphenhydramine (2-(diphenylmethoxy)-*N*,*N*-dimethylethylamine) (Fig. 1) is an H_1 receptor antagonist used clinically for its antihistaminic, antitussive and sedative properties.[1–5] Pharmacokinetic studies conducted in chronically instrumented pregnant sheep at 120–140 days gestation (term 145 days) have shown that diphenhydramine (DPHM) rapidly and readily crosses the ovine placenta, resulting in significant fetal exposure.[6] In addition, it has also been shown that the fetus clears DPHM *in utero* by both placental and non-placental routes.[7] To date, it is unclear whether fetal hepatic, renal, pulmonary and/or other routes of clearance are predominant components in the measured fetal non-placental clearance. This aspect of DPHM disposition in chronically instrumented pregnant sheep near-term requires further study.

Pharmacokinetic studies employing stable isotope labeled drugs, aside from circumstances where the label influences drug disposition, offer many advantages over the more conventional use of unlabeled drugs.[8,9] A pharmacokinetic study in which a labeled and an unlabeled drug are simultaneously and independently administered (e.g. labeled drug is administered to the fetus and unlabeled is administered to the ewe) is advantageous since it reduces to inter-day variability in the measured pharmacokinetic parameters. The advantages of this approach include an increase in the statistical power of the study, yielding an overall reduction in the number of study subjects, and also a diminished risk due to a reduction in the exposures to the drug. In addition, the overall time required to conduct experiments, and hence the number of samples to be collected for analysis, may also be substantially reduced.[9–11] The benefits of a reduced number of animals required in experiments conducted with near-term pregnant sheep are of considerable importance owing to the high cost associated with the animal preparation and the narrow time window for experimentation before the ewe delivers (i.e. 7–14 days). This experimental design also allows essentially two experiments, namely a test and a control experiment, to be conducted simultaneously during one experimental period. This is in comparison to an experimental design where only unlabeled drug is available; in

† Chronically instrumented pregnant sheep: Surgically prepared pregnant sheep (120–140 days gestation) in which experimental instruments (e.g. catheters for serial sampling of vascular and non-vascular compartments, catheters for monitoring blood, amniotic and tracheal pressures, and vascular flow probes) are maintained for extended periods of time (i.e. 10–20 days post surgery).

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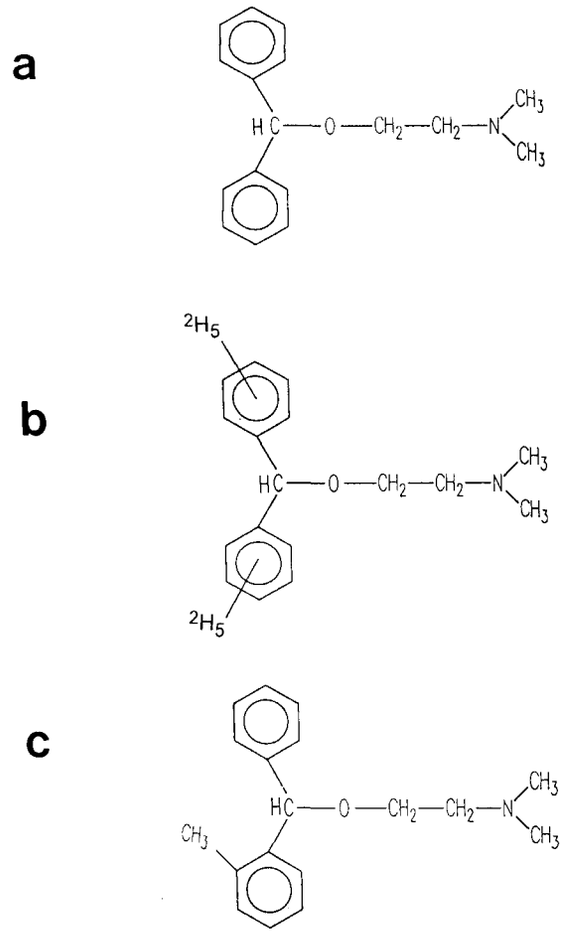


Figure 1. Chemical structures of (a) diphenhydramine, (b) ($^2H_{10}$) diphenhydramine and (c) orphenadrine, the internal standard.

this case, the control and test experiments are conducted on different days, separated by an appropriate wash-out period. Thus, an experimental design incorporating the simultaneous and independent administration of both unlabeled and stable isotope labeled drug substantially reduces the impact of time-dependent physiological and metabolic changes which may alter the measured pharmacokinetic parameters. These time-dependent changes, due to fetal maturation and altered fetal body composition, occur rapidly during the last gestational term during which our experiments are conducted.[12] Therefore, the concurrent and independent administration of a stable isotope labeled analog of DPHM and unlabeled DPHM was deemed necessary to conduct the best possible study of the pharmacokinetics and metabolism of DPHM in the ovine mother and fetus.

Although there are distinct advantages in using a stable isotope labeled analog of DPHM for pharmacokinetic and drug metabolism studies, the synthesis of such an analog and the simultaneous quantitation of labeled and unlabeled DPHM has not previously been reported. Numerous methods have been reported for the quantitation of unlabeled DPHM, including high-performance liquid chromatography (HPLC) with fluorescence detection,[13] gas chromatography (GC) with nitrogen/phosphorous specific detection,[14,15] and GC/MS[16–18] methods. Unfortunately, none of the reported methods to date have demonstrated the ability to simultaneously quantitate both labeled and unlabeled DPHM. The purpose of this report is to describe the synthesis of a deuterated stable isotope analog of DPHM (($^2H_{10}$)DPHM) (Fig. 1), and also to report a sensitive and selective analytical method for the simultaneous quantitation of both DPHM and ($^2H_{10}$)DPHM using GC with mass selective detection (MSD) in biological fluid samples obtained from chronically instrumented pregnant ewes. This method is intended for use during a course of experiments in the study of fetal and maternal pharmacokinetics and metabolism of DPHM in chronically instrumented pregnant ewes.

EXPERIMENTAL

Reagents and materials

Diphenhydramine hydrochloride and orphenadrine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Deuterated benzene was obtained from MSD Isotopes (Montreal, Canada). Anhydrous aluminum chloride, anhydrous sodium sulfate, anhydrous magnesium sulfate, carbon tetrachloride, diethyl ether, ethyl alcohol, hydrochloric acid, isopropyl alcohol, HPLC-grade methanol, petroleum ether, sodium hydroxide and *para*-toluene sulfonic acid were purchased from BDH (Toronto, Canada). Dueterium oxide was acquired from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Triethylamine (sequanal grade) was obtained from Pierce Chemical Co. (Rockville, Illinois, USA). Toluene and n-hexane distilled in glass were purchased from Caledon Laboratories (Georgetown, Canada). Deionized, high-purity water was produced on-site by reverse osmosis and subsequent filtration using a Milli-Q® water system (Millipore, Bedford, Massachusetts, USA).

Synthesis and purification of deuterated diphenhydramine

The stable isotope analog of DPHM (($^2H_{10}$)DPHM) was synthesized in three steps (Fig. 2). The initial step involved the synthesis of ($^2H_{10}$)benzophenone. This was carried out as described previously with the minor modification[19] that deuterium-labeled benzene was used rather than unlabeled benzene and ^2H_2O was used in transformation of the ($^2H_{10}$)dichloridiphenylmethane to ($^2H_{10}$)benzophenone. In addition, column flash chromatography (in a glass column of dimensions 6 cm × 75 cm packed with Silica gel 60; mesh 240–400; mobile phase 97% n-hexane:3% diethyl ether) was used for the purification of the product rather than fractional distillation. The second step in the synthesis involved the conversion of the ($^2H_{10}$)benzophenone to ($^2H_{10}$)benzhydrol as described previously for the undeuterated benzophenone.[20] The purified ($^2H_{10}$)benzhydrol was then used to synthesize the ($^2H_{10}$)DPHM.[21] Following synthesis, the ($^2H_{10}$)DPHM was extracted from the reaction mixture and converted

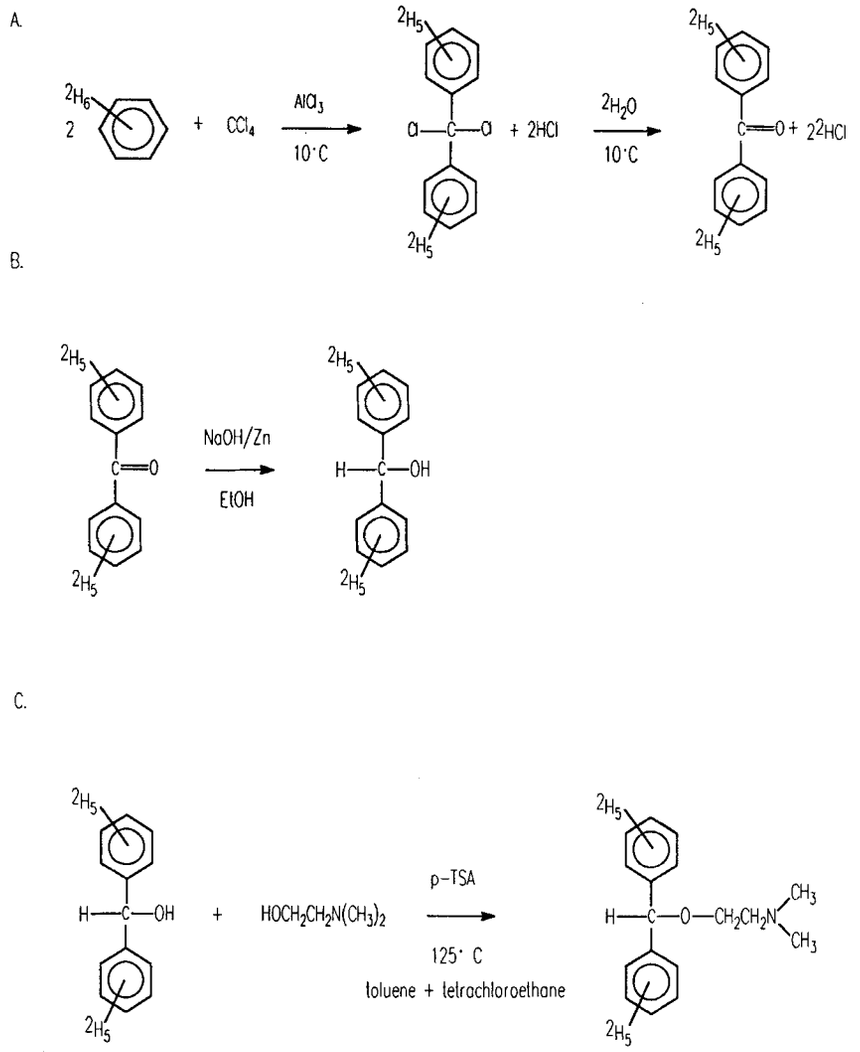


Figure 2. The synthesis of the labeled stable isotope analog of diphenhydramine (($^2H_{10}$)diphenhydramine). The reaction proceeded in three steps: (A) synthesis of ($^2H_{10}$)benzophenone; (B) conversion of ($^2H_{10}$)benzophenone to ($^2H_{10}$)benzhydrol; (C) synthesis of ($^2H_{10}$)diphenhydramine. EtOH = 95% ethanol; p-TSA = *para*-toluene sulfonic acid monohydrate.

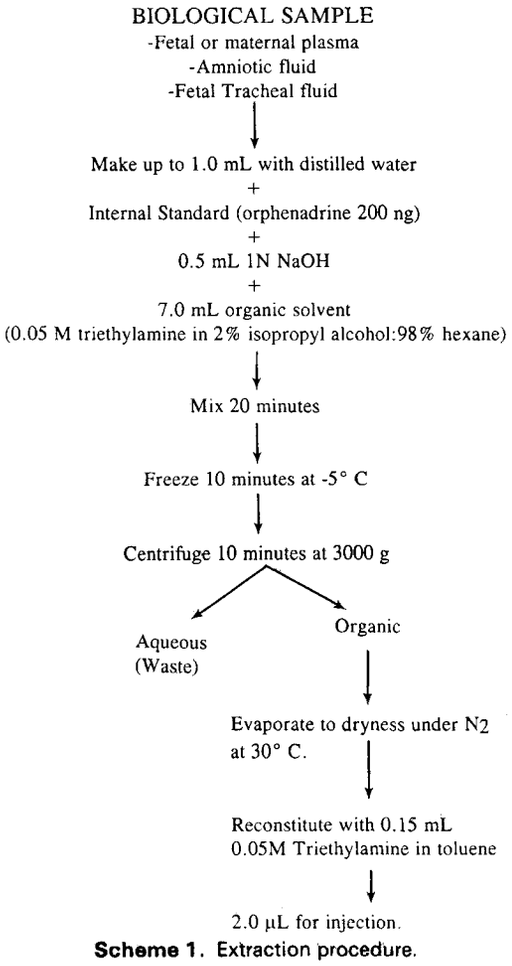
to the hydrochloride salt.[21] The ($^2H_{10}$)DPHM HCl was further purified by recrystallization in acetone. The yield of the recrystallized vacuum-dried ($^2H_{10}$)DPHM HCl was roughly 50% based on the weight of the starting material (($^2H_{10}$)benzhydrol). Identification of the ($^2H_{10}$)DPHM was confirmed using ^{1}H-nuclear magnetic resonance (NMR) (Bruker AC-200 (200 MHz), Department of Chemistry, University of British Columbia) (^{1}H-NMR: (D_2O)δ5.38 (s, 1H, CHO), 3.58 (t, 2H, OCH_2), 2.62 (t, 2H, CH_2), 2.28 (br s, 6H, $N(CH_3)_2$)). In addition, GC/LC/MS was also used in the identification of ($^2H_{10}$)DPHM (Hewlett Packard Model 5989 MS Engine, 5890 Series II gas chromatograph, 5989 electron impact/positive chemical ionization/negative chemical ionization (EI/PCI/NCI) mass spectrometer, and 1090 high-performance liquid chromatograph). Characteristic fragments of ($^2H_{10}$)DPHM following GC EI were *m/z* 58, 77, 159, 173, 177 and 193. LC/MS, which was conducted using a thermospray interface with the ion source in positive ion mode, identified the ion at *m/z* 266 corresponding to [M + H].

The purity of the DPHM HCl standard and the synthesized ($^2H_{10}$)DPHM HCl standard was assessed in the following fashion. Firstly, aqueous solutions of DPHM HCl or ($^2H_{10}$)DPHM HCl (100 μg ml^{-1}) were extracted with 2% isopropyl alcohol:98% hexane with 0.05 M triethylamine (TEA). The organic phase was dried and reconstituted with 0.05 M TEA in toluene. One aliquot of the reconstituted samples (i.e. DPHM and ($^2H_{10}$)DPHM) was subjected to GC with nitrogen/phosphorus specific detection (GC NPD, Hewlett Packard HP 5890 Series II), while the other aliquot of the reconstituted samples was assessed using GC/MS (scan mode) (Hewlett Packard 5971A mass selective detector, Hewlett Packard, Avondale, Pennsylvania, USA). Only one chromatographic peak, other than those present in the blank, was seen with GC NPD and in the total ion chromatogram (GC/MS) following the injection of the prepared DPHM HCl and ($^2H_{10}$)DPHM HCl standards. Standard aqueous solutions of DPHM HCl and ($^2H_{10}$)DPHM HCl (100 μg ml^{-1}) were subjected to LC/MS via direct introduction into a thermospray interface (HP 5989 GC/LC/MS Engine, Hewlett Packard, Avondale, Pennsylvania, USA) (carrier phase (50 ammonium acetate buffer 10 mM:50 acetonitrile, pH 7.0), thermospray capillary

temperature 120 °C, fragmenter off, ion source in the positive ion scanning mode). DPHM and ($^2H_{10}$)DPHM did not fragment under these conditions, and therefore only one ion was observed in each sample, which corresponded to the [M + H] ion, namely *m/z* 256 and *m/z* 266 of the DPHM HCl and ($^2H_{10}$)DPHM HCl standards, respectively. Thermal analysis was conducted using differential scanning calorimetry (Dupont Instruments Series 99 Thermal Analyzer). Data obtained showed only one sharp peak corresponding to a melting point of 167 °C. The absence of other peaks during the thermal analysis also suggest the lack of any polymorphic forms, and/or solvates of the ($^2H_{10}$)DPHM HCl standard.

Extraction procedure

Samples were prepared for analysis by a single-step liquid–liquid extraction procedure, as shown in Scheme 1. Aliquous of biological samples (0.1–1.0 ml) including maternal and fetal plasma, amniotic fluid and tracheal fluid were individually pipetted into clean test tubes. The samples were made up to volume (1.0 ml) with distilled water; internal standard (orphenadrine 200 ng) and 0.5 ml of 1 N NaOH were added to the test tube along with 7.0 ml of solvent (0.05 M triethylamine in 2% isopropyl alcohol in hexane). The samples were capped with Teflon-lined lids and mixed for 20 min on a Labquake® Tube Shaker, Model 415–110 (Lab Industries, Berkeley, California, USA), cooled for 10 min at −5 °C in a freezer in order to break the emulsion formed during mixing, and centrifuged for 10 min at 3000 × *g* on an IEC model HN-SII centrifuge (Damon/IEC Division, Needham Heights, Massachusetts, USA). The organic phase was then transferred to a clean test tube and dried in a water bath at 30 °C under a gentle stream of nitrogen gas. The dried samples were reconstituted with 150 µl of 0.05 M triethylamine in toluene. The reconstituted samples were then transferred to clean borosilicate microvial inserts from which a 2.0 µl aliquot was used for injection.



Scheme 1. Extraction procedure.

Capillary GC and mass spectrometry

The samples were analyzed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a Hewlett Packard Model 7673 auto sampler, capillary split–splitless inlet system and a Hewlett Packard Model 5971A mass selective detector (Hewlett Packard, Avondale, Pennsylvania, USA). A 2.0 µl aliquot of prepared sample was injected through a Thermogreen LD-2® silicone rubber septa (Supelco, Bellefonte, Pennsylvania, USA) into a Pyrex glass inlet liner (78 mm × 4 mm i.d.) in the splitless mode. Chromatographic separation of DPHM, ($^2H_{10}$)DPHM and orphenadrine from endogenous materials and the demethylated metabolites of DPHM and ($^2H_{10}$)DPHM was achieved using a 30 m DB-1701 0.25 mm i.d. (0.25 µm film thickness) capillary column (J&W Scientific, Folsom, California, USA). Column head pressure was optimized at 10 psi. Gas chromatographic operating conditions were optimized as follows. The injection port temperature was held at 180 °C. The initial oven temperature was maintained at 140 °C for 1 min, then the oven temperature was ramped at 30 °C min^{-1} to 200 °C. The oven temperature was again ramped at 17.5 °C min^{-1} from 200 °C to 265 °C, where it was held for 5.0 min. The temperature program resulted in a total run time of 12.7 min. The transfer line temperature was held at 280 °C. The mass selective detector (MSD) was manually tuned with the tuning reagent perflourotributylamine (PFTBA) to ions at *m/z* 100, *m/z* 131 and *m/z* 219. The GC/MSD operating in the EI mode (voltage 70 eV) with selective ion monitoring (SIM) was used to quantitate DPHM and ($^2H_{10}$)DPHM by monitoring ions *m/z* 165 and 173, respectively. The dwell time was set at 50 ms for each ion being monitored to ensure adequate sampling of the chromatographic peak of interest. The electron multiplier voltage was programmed to +300 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.

Extraction recovery

Extraction recoveries of both DPHM and ($^2H_{10}$)DPHM were determined at low, moderate and high concentrations (2.0, 50.0 and 200.0 ng ml^{-1}, respectively) from a variety of biological matrices

(maternal plasma, amniotic fluid and tracheal fluid). Two groups of samples were used to assess extraction recovery, namely a test group and a control group. Both groups of samples contained blank biological matrix (plasma, amniotic fluid and fetal tracheal fluid) and internal standard. However, the samples from the test group were spiked with both DPHM and ($^2H_{10}$)DPHM to yield final concentrations of 2.0, 50.0 and 200.0 ng ml^{-1}, whereas the samples in the control group were not spiked with DPHM and ($^2H_{10}$)DPHM at this point of the experiment. Following liquid–liquid extraction of both the test and control group samples, aliquots of DPHM and ($^2H_{10}$)DPHM standards, made up in methanol, were added to the control group samples to yield drug concentrations of 2.0, 50.0 and 200.0 ng ml^{-1}. Control and test samples were then dried, reconstituted and chromatographed as described above. The concentrations of the test and control samples were determined from standard curves extracted from the corresponding biological matrices. 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. Since no differences in the extraction recoveries were evident at the three concentrations tested, the mean recovery of all the samples in that particular biological matrix (i.e. plasma, fetal tracheal fluid and amniotic fluid) was reported. The mean recovery of DPHM and ($^2H_{10}$)DPHM from plasma was 98 ± 2% and 105 ± 3%, from amniotic fluid was 100 ± 5% and 110 ± 6%, and from fetal tracheal fluid was 97 ± 5% and 104 ± 5%, respectively.

Calibration curve

An eight-point calibration curve was constructed from aqueous standard solutions of DPHM and ($^2H_{10}$)DPHM at concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 150.0 and 200.0 ng ml^{-1}. The aqueous standard solution of DPHM HCl was prepared in distilled deionized water to yield a final concentration of 200 ng ml^{-1} of DPHM free base. The aqueous standard solution of ($^2H_{10}$)DPHM HCl was prepared as outlined above, but was weight corrected to yield 208 ng ml^{-1} of ($^2H_{10}$)DPHM free base (molar equivalent to 200 ng ml^{-1} unlabeled DPHM). The blank plasma, fetal tracheal fluid or amniotic fluid was added to the calibration curve samples. The samples were then extracted and quantitated as described above. Weighted linear regression (weighting function = $1/y^2$) was performed between the drug response (DPHM or ($^2H_{10}$)DPHM peak area/internal standard [orphenadrine] peak area) and the spiked drug concentrations of DPHM and ($^2H_{10}$)DPHM.

Sample stability determinations

Maternal sheep plasma was spiked with DPHM and ($^2H_{10}$)DPHM at known concentrations of 5.0, 25.0 and 100.0 ng ml^{-1}, frozen at −20 °C, and stored frozen for a duration up to 3 months. Samples were periodically removed and the concentrations of DPHM and ($^2H_{10}$)DPHM determined using the GC MSD method described above.

Method validation

Intra-day variability was determined by quantitating six replicates at concentrations of 2.0, 20.0, 100.0 and 200.0 ng ml^{-1} using the GC MSD method reported above on one experimental day. Inter-day variability was determined by quantitating one sample in duplicate at concentrations of 2.0, 20.0, 100.0 and 200.0 ng ml^{-1} using GC MSD on six different experimental days.

The GC MSD method for the quantitation of DPHM and ($^2H_{10}$)DPHM was independently cross-validated by quantitating samples of DPHM and ($^2H_{10}$)DPHM individually by the GC MSD method developed, and by a published capillary GC analysis for the quantitation of DPHM using GC NPD.[15] This GC NPD method utilized a single-step liquid–liquid extraction with 0.05 M triethylamine in methylene chloride with splitless sample injection.[15] Chromatographic separation was achieved on a 25 m × 0.31 mm i.d. cross-linked 5% phenylmethyl silicone fused silica capillary column.[15] This method was validated over the concentration range of 2.0–320.0 ng ml^{-1} [15] In order to be able to quantitate both the DPHM and the ($^2H_{10}$)DPHM using GC NPD, calibration curves and samples were prepared individually, i.e. DPHM and ($^2H_{10}$)DPHM were not present together in the same sample (only for cross-validation samples). This was done because the GC NPD method could not differentiate between labeled and unlabeled DPHM if they were present in the same sample.

Drug administration and sample collection

An intravenous bolus dose of DPHM HCl and ($^2H_{10}$)DPHM HCl equivalent to 4.0 mg of free base was administered simultaneously via the fetal tarsal vein to a chronically instrumented fetal lamb which was surgically prepared as described by Rurak *et al.*[22] Serial samples were drawn from the fetal femoral and carotid arteries (2.0 ml), and fetal tracheal fluid (2.5 ml) and amniotic fluid (5.0 ml) at −5, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210, 240, 300, 360, 480 and 600 min. Blood samples were collected in heparinized tubes. Following centrifugation, plasma was separated and stored in Pyrex glass tubes with screw caps lined with polytetrafluoroethylene (PTFE) (Corning Glass Works, Corning, New York, USA). Amniotic and fetal tracheal fluids were collected, stored in Pyrex glass tubes, and capped as described above. Control or blank biological fluid samples were obtained from the sheep before the administration of drug and were used for calibration curves and extraction recovery studies. All samples were stored at −20 °C until analysis.

RESULTS AND DISCUSSION

The introduction of an experimental design in which both stable isotope labeled DPHM and unlabeled

DPHM were to be administered simultaneously to chronically instrumented pregnant sheep presented an analytical challenge. That is, the method previously used in our laboratory for the analysis of DPHM employed capillary GC with nitrogen/phosphorus specific detection.[15] Unfortunately, nitrogen/phosphorus specific detection cannot distinguish between stable isotope labeled and unlabeled DPHM. Consequently, GC with MSD was selected to developed a new analytical method. This mode of analysis could provide both the necessary selectivity between labeled and unlabeled DPHM, and the sensitivity required for trace level analysis of DPHM and ($^2H_{10}$)DPHM in biological samples from experiments conducted in pregnant sheep.

Mass spectra of DPHM and ($^2H_{10}$)DPHM standards following EI resulted in extensive fragmentation (Fig. 3a, b) as reported previously in the literature for DPHM.[16] Preliminary attempts were made to quantitate both DPHM and ($^2H_{10}$)DPHM using GC with methane PCI in order to avoid extensive fragmentation. Unfortunately, methane PCI also resulted in substantial fragmentation of DPHM and ($^2H_{10}$)DPHM. In addition, methane PCI also resulted in a reduction in sensitivity. Since methane PCI did not offer any substantial advantages over EI in the quantitation of DPHM and ($^2H_{10}$)DPHM, EI was used as the mode of ionization. The extensive fragmentation of DPHM and ($^2H_{10}$)DPHM results in a base ion of *m/z* 58 with no apparent molecular ions (Fig. 3a, b). A previously reported GC/MS method uses the base ion (*m/z* 58) in selective ion monitoring (SIM) to quantitate DPHM.[17] However, in the case where the deuterium label does not reside on the part of the molecule which corresponds to this *m/z* fragment, SIM of this fragment would not provide the necessary differentiation between labeled and unlabeled DPHM (Fig. 3a, b). In the currently reported method, two ions, specifically *m/z* 165 (DPHM and orphenadrine) and *m/z* 173 (($^2H_{10}$)DPHM) were used for SIM (Fig. 3a, b). These ions were employed since they provide the necessary differentiation between the labeled and unlabeled DPHM, and give the required sensitivity for trace level analysis.

The chromatographic parameters and temperature program used resulted in retention times for DPHM and ($^2H_{10}$)DPHM of 7.67 and 7.64 min, respectively. The internal standard (orphenadrine) for both DPHM and ($^2H_{10}$)DPHM elutes at 8.05 min (Fig. 4). The use of a temperature program instead of an isothermal run for separation and quantitation of these compounds results in a total analysis time of 12.7 min. A slight rise in the baseline was noted in the chromatograms; this is likely due to some column bleed resulting from the temperature programming. A potential complication of chromatographic interference may occur when using low *m/z* fragments during quantitation by GC/MS with SIM. This could result when chromatographic peaks containing the same *m/z* ions co-elute with DPHM, ($^2H_{10}$)DPHM, or the internal standard, and thus interfere with the analysis (e.g. incomplete resolution of chromatographic peaks). The most likely source of these interfering peaks is from biological endogenous components which may co-extract with DPHM and ($^2H_{10}$)DPHM. This did not appear to be a problem with the use of *m/z* ions 165 and 173 with the biological matrices utilized in these experiments (Fig. 4).

The extraction recovery of DPHM and ($^2H_{10}$)DPHM following liquid–liquid extraction using 0.05 M triethylamine (TEA) in 98% hexane:2% isopropyl alcohol from ovine plasma, fetal tracheal fluid and amniotic fluid was nearly complete. No apparent concentration-dependent change in the extraction recovery was noted since the extraction recovery at the three different concentrations tested (i.e. 2.0, 50.0 and 200.0 ng ml^{-1}) was the same. A common problem encountered during

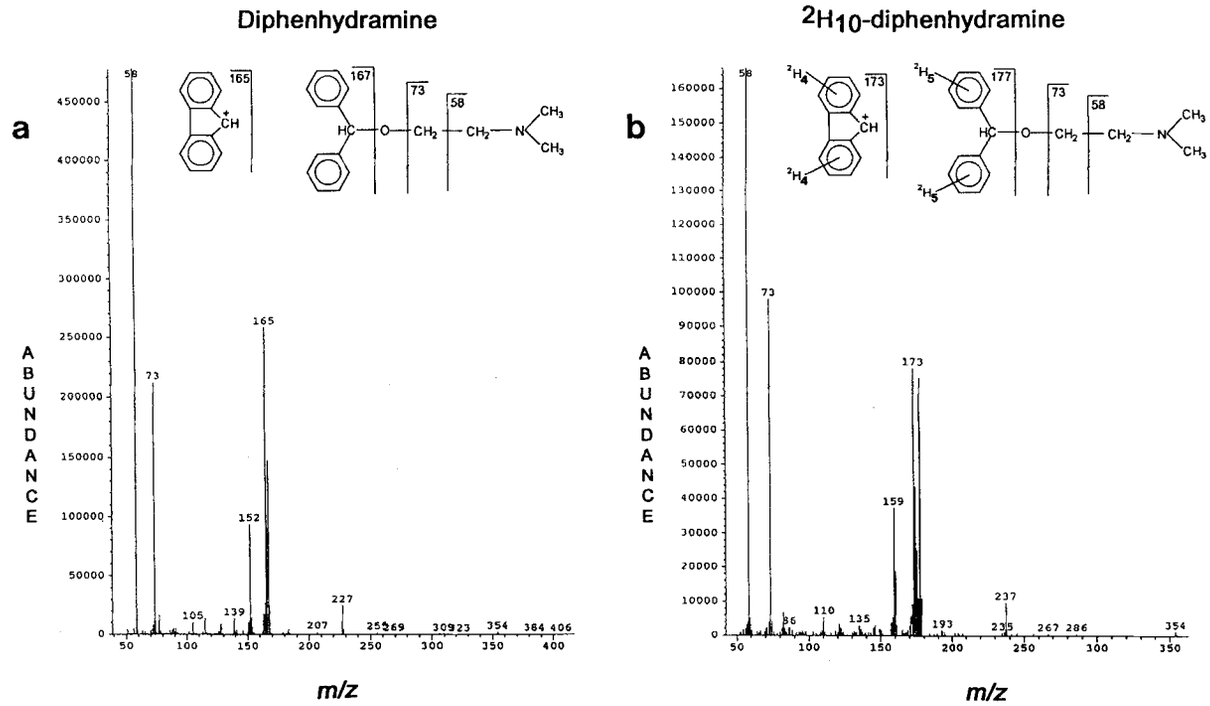


Figure 3. Mass spectrum and fragment assignments of (a) diphenhydramine and (b) ($^2H_{10}$)diphenhydramine.

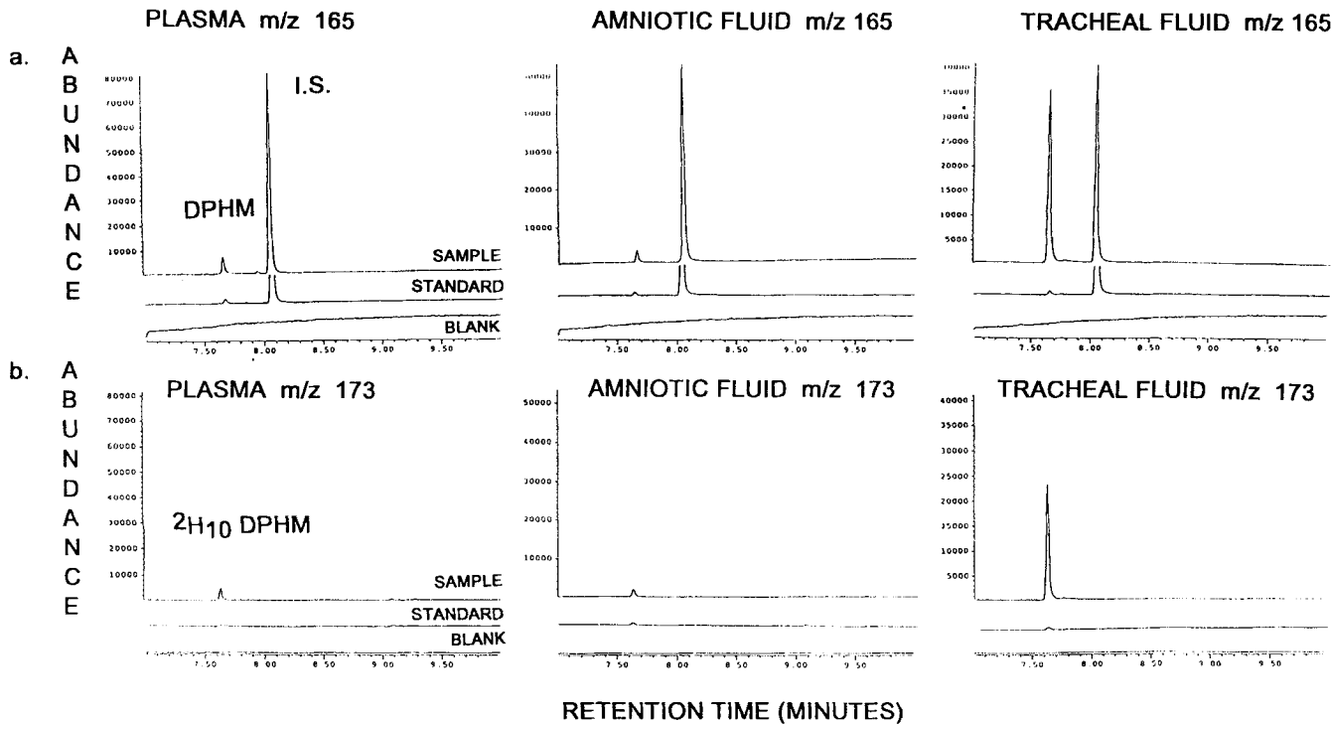


Figure 4. Sample ion chromatograms of (a) *m/z* 165 (diphenhydramine and orphenadrine (internal standard)) and (b) *m/z* 173 (($^2H_{10}$)diphenhydramine) in ovine plasma, ovine fetal tracheal fluid and ovine amniotic fluid. Sample chromatograms include a biological sample containing both DPHM and ($^2H_{10}$)DPHM spiked with internal standard, a blank biological sample spiked with 2.0 ng ml^{-1} of diphenhydramine and ($^2H_{10}$)diphenhydramine and the internal standard, and a blank biological sample. Retention times: DPHM 7.67 min; ($^2H_{10}$)DPHM 7.64 min; orphenadrine 8.05 min.

liquid–liquid extraction of tertiary amine drug entities is non-specific binding to glassware.[23,24] Numerous methods have been employed to prevent this phenomenon, including the use of silanized glassware; however, silanization is not always effective in preventing the absorptive loss of tertiary amine drugs.[24] The use of TEA in the extraction solvent has been suggested to prevent tertiary amines from binding to non-specific binding sites on glassware and other surfaces.[25] The use of 0.05 M TEA was found to significantly aid in the extraction recovery of DPHM and ($^2H_{10}$)DPHM. When only 98% hexane:2% isopropyl alcohol was used, i.e. TEA was omitted from the extraction solvent, the extraction recovery of DPHM and ($^2H_{10}$)DPHM was reduced by approximately 75%. Further, the use of silanized glassware did not result in a significant increase in the extraction recovery of DPHM and ($^2H_{10}$)DPHM. Since TEA was shown to significantly increase the extraction recovery of DPHM and ($^2H_{10}$)DPHM, it was used as a component of the extraction solvent (0.05 M) and the final reconstitution solvent (0.05 M) throughout the remainder of the experiments.

The calibration curve for DPHM and ($^2H_{10}$)DPHM showed good linearity over the range from 2.0 ng ml^{-1} to 200.0 ng ml^{-1} in all of the biological matrices examined. A sample calibration curve from plasma is shown in Fig. 5. The coefficients of variation were not more than 10% for each point of the calibration curve in plasma, fetal tracheal fluid and amniotic fluid. The regression coefficients in plasma, fetal tracheal fluid and amniotic fluid were, in most instances, greater than 0.999. The regression equation for DPHM was $Y = 0.0049X + (-0.0016)$, and for ($^2H_{10}$)DPHM was $Y = 0.0033X + (-0.0013)$. The slope of the DPHM calibration curve was greater than the slope for the ($^2H_{10}$)DPHM calibration curve, reflecting the difference in the relative abundance of the ions at *m/z* 165 and 173, respectively (Fig. 3a, b).

Sample stability upon storage is of utmost importance in quantitative analysis. Sample degradation may lead to spurious results which can subsequently lead to incorrect estimates of pharmacokinetic parameters. The frozen samples containing DPHM and ($^2H_{10}$)DPHM were shown to be stable for up to 3 months. The concentrations of DPHM and ($^2H_{10}$)DPHM determined by GC MSD described above were constant; i.e. the coefficient of variation for the samples was below 12% at 5.0

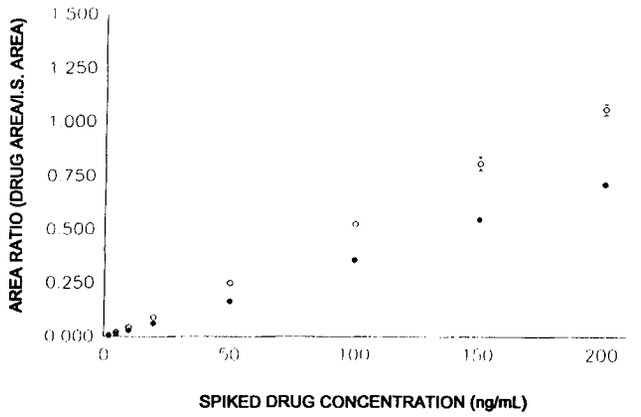


Figure 5. A representative standard calibration curve for diphenhydramine (○) and ($^2H_{10}$)diphenhydramine (●) in plasma.

ng ml^{-1} and below 10% for the 50.0 and 100.0 ng ml^{-1} samples. This would appear to suggest that the biological samples were stable when frozen at −20 °C for at least a duration of 3 months.

The validation of this method involved estimating intra-day and inter-day variability. In addition, this method was cross-validated with a previously published method for the quantitation of DPHM.[15] The estimates of intra-day variability for DPHM and ($^2H_{10}$)DPHM were below 17% at 2.0 ng ml^{-1}, and below 8% at all other concentrations investigated in all three of the biological matrices investigated (Table 1A). The measured inter-day variability for DPHM and ($^2H_{10}$)DPHM was below 15% at 2.0 ng ml^{-1} and below 10% for all other points (Table 1B). The published GC method used in the cross-validation studies employed NPD.[15] The response for both DPHM and ($^2H_{10}$)DPHM was essentially equivalent using this GC NPD method. The concentrations of DPHM and ($^2H_{10}$)DPHM were determined independently, since the nitrogen/

Table 1A. Intra-day variability of DPHM and ($^2H_{10}$)DPHM area ratios in plasma, amniotic fluid and fetal tracheal fluid

DPHM	Plasma	Amniotic	Tracheal
2.0 ng ml^{-1}	0.0088 ± 0.0008 (9.1%)	0.0078 ± 0.0013 (16.5%)	0.0076 ± 0.0008 (10.4%)
20.0 ng ml^{-1}	0.0902 ± 0.0026 (2.9%)	0.0853 ± 0.0063 (7.4%)	0.0870 ± 0.0053 (6.2%)
100.0 ng ml^{-1}	0.5235 ± 0.0135 (2.6%)	0.4914 ± 0.016 (3.3%)	0.5205 ± 0.0177 (3.4%)
200.0 ng ml^{-1}	1.0511 ± 0.0258 (2.4%)	1.0389 ± 0.0509 (4.9%)	1.0213 ± 0.0066 (0.7%)
($^2H_{10}$)DPHM			
2·0 ng ml^{-1}	0.0058 ± 0.0008 (13.8%)	0.0048 ± 0.0005 (10.1%)	0.0046 ± 0.0008 (16.4%)
20.0 ng ml^{-1}	0.0623 ± 0.0019 (3.1%)	0.0524 ± 0.0029 (5.4%)	0.0577 ± 0.0047 (8.1%)
100.0 ng ml^{-1}	0.3571 ± 0.0120 (3.4%)	0.3293 ± 0.0035 (1.1%)	0.3486 ± 0.0110 (3.2%)
200.0 ng ml^{-1}	0.7070 ± 0.0195 (2.8%)	0.6832 ± 0.0319 (4.7%)	0.6600 ± 0.0075 (1.1%)

Mean area ratios ± SD.
Numbers in parenthese are coefficients of variation.

Table 1B. Interday variability of DPHM and ($^2H_{10}$)DPHM area ratios in plasma, amniotic fluid and fetal tracheal fluid

DPHM	Plasma	Amniotic	Tracheal
2.0 ng ml^{-1}	0.0093 ± 0.0007 (7.5%)	0.0067 ± 0.0004 (6.0%)	0.0090 ± 0.0013 (14.4%)
20.0 ng ml^{-1}	0.0991 ± 0.0078 (7.9%)	0.0769 ± 0.0049 (6.4%)	0.0990 ± 0.0073 (7.4%)
100.0 ng ml^{-1}	0.5837 ± 0.0327 (5.8%)	0.4426 ± 0.0125 (3.4%)	0.5552 ± 0.0451 (8.1%)
200.0 ng ml^{-1}	1.1411 ± 0.0876 (7.7%)	0.9051 ± 0.0169 (1.9%)	1.1249 ± 0.1178 (9.5%)
($^2H_{10}$)DPHM			
2.0 ng ml^{-1}	0.0062 ± 0.0005 (8.1%)	0.0043 ± 0.0003 (7.2%)	0.0057 ± 0.0007 (12.3%)
20.0 ng ml^{-1}	0.0635 ± 0.0047 (7.4%)	0.0509 ± 0.0046 (9.0%)	0.0644 ± 0.0037 (5.7%)
100.0 ng ml^{-1}	0.3555 ± 0.0267 (7.5%)	0.2878 ± 0.0130 (4.5%)	0.3011 ± 0.0257 (7.1%)
200.0 ng ml^{-1}	0.7155 ± 0.0489 (6.8%)	0.5897 ± 0.0162 (2.7%)	0.7286 ± 0.0566 (7.8%)

Mean area ratios ± SD.
Numbers in parentheses are coefficients of variation.

phosphorus specific detector cannot differentiate between labeled and unlabeled DPHM. When the concentrations of DPHM and ($^2H_{10}$)DPHM were independently measured by GC NPD and plotted against the concentrations of DPHM and ($^2H_{10}$)DPHM independently measured by the proposed GC MSD method, the correlation was excellent ($r = 1.000$ DPHM and $r = 0.999$ ($^2H_{10}$)DPHM) (Fig. 6), suggesting that the two methods were highly comparable. The results of these validation experiments would appear to suggest that the method developed is robust, and measurements of DPHM and ($^2H_{10}$)DPHM concentrations made with this GC MSD method in the biological matrices examined can be made with a high degree of confidence.

The minimal detectable amount, i.e. a signal produced by the analyte that is three times greater than the noise of a blank sample, of DPHM and ($^2H_{10}$)DPHM using the GC MSD parameters outlined above was 6.7 pg at the detector, corresponding to 0.5 ng ml^{-1} extracted from the biological matrices.[26] The minimal quantifiable amount, or the amount of analyte which results in a signal greater than ten times the noise recorded in the blank, was 27.6 pg at the detector, corresponding to 2.0 ng ml^{-1} of DPHM and ($^2H_{10}$)DPHM extracted from the biological matrices examined (plasma, fetal tracheal fluid and amniotic fluid).[26] Another stipulation placed upon the minimal quantifiable amount was that it had to fall within the acceptable limits of inter-day and intra-day variability (<20% relative standard deviation for the lowest concentration). The minimal quantifiable amount of the previously published GC NPD method was also 2.0 ng ml^{-1}; therefore, this method does not offer any advantage over the previously published methods in sensitivity.[15] Rather, the advantage of the current method over the previous published methods is the ability to simultaneously quantitate both labeled and unlabeled DPHM. In experiments where both the labeled and unlabeled drug are administered simultaneously and independently, and are therefore present in the same biological matrix, the published methods to date cannot differentiate between the amount of DPHM and the amount of ($^2H_{10}$)DPHM present in the sample. This would likely defeat the original purpose of the experiment. The advantage of the reported GC MSD method is that the amount of labeled and the amount of unlabeled DPHM in a biological sample could be measured independently and simultaneously during one chromatographic run.

The application of the reported GC MSD method involved the quantitation of DPHM and ($^2H_{10}$)DPHM concentrations in biological fluids obtained from the chronically instrumented pregnant ewe. An initial control experiment was conducted in order to rule out isotope effects in the disposition of ($^2H_{10}$)DPHM in the fetal lamb. These experiments involved a simultaneous bolus administration of labeled and unlabeled DPHM as a direct bolus to the fetus via the fetal lateral tarsal vein. The concentrations of DPHM and ($^2H_{10}$)DPHM in fetal plasma, fetal tracheal fluid and amniotic fluid were simultaneously measured, and are shown in Fig. 7a, b. The concentrations of DPHM and ($^2H_{10}$)DPHM in plasma, fetal tracheal fluid and amniotic fluid were similar, suggesting little or no apparent isotope effect influencing the disposition of the labeled and unlabeled DPHM in this ovine fetus. In order to unequivocally rule out isotope effects more control experiments must be conducted. Subsequent experiments will examine the extent of fetal hepatic first-pass

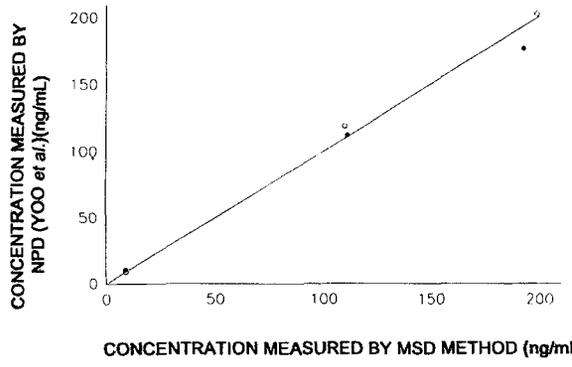


Figure 6. Correlation comparison between a published method for the analysis of diphenhydramine using GC NPD with GC MSD at 5.0, 100.0 and 200.0 ng ml^{-1}; (○) diphenhydramine, (●) ($^2H_{10}$)diphenhydramine.

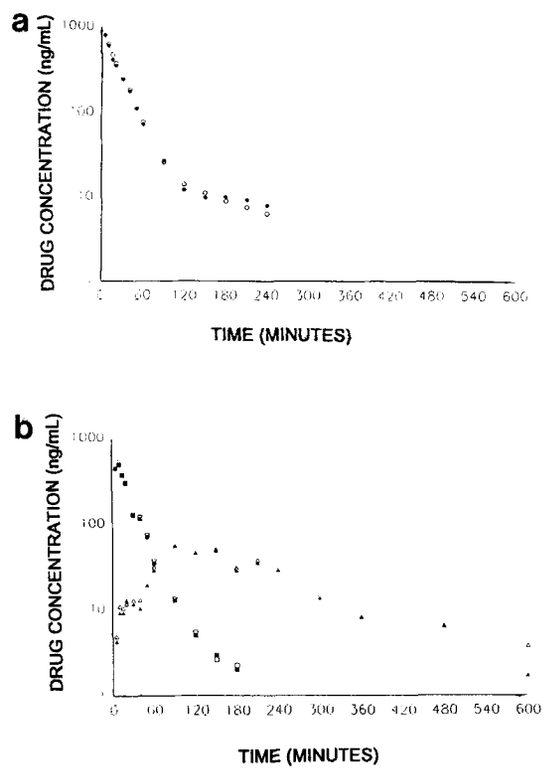


Figure 7. (a) A representative plot of the plasma diphenhydramine (○) and ($^2H_{10}$)diphenhydramine (●) following simultaneous bolus administration of 4.0 mg (equivalent to base) diphenhydramine and 4.0 mg of ($^2H_{10}$)diphenhydramine into the fetal lateral tarsal vein. (b) A representative plot of the amniotic concentrations of diphenhydramine (△), and ($^2H_{10}$)diphenydramine (▲), and tracheal fluid concentrations of diphenhydramine (□) and ($^2H_{10}$)diphenhydramine (■) following simultaneous bolus administration of 4.0 mg (equivalent to base) diphenhydramine and ($^2H_{10}$)diphenhydramine into the fetal lateral tarsal vein.

elimination *in utero* using the principle of simultaneous and independent administration of labeled and unlabeled DPHM to the fetal lamb.

In summary, we have reported the synthesis of a stable isotope labeled analog of DPHM, and a selective and sensitive method for the simultaneous quantitation of DPHM and this stable isotope labeled analog ($^2H_{10}$)DPHM using a GC MSD method. This method for the quantitation of DPHM and ($^2H_{10}$)DPHM will be used in a variety of pharmacokinetic and metabolic studies examining the fetal and maternal distribution and elimination of DPHM in the chronically instrumented pregnant sheep.

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