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Simultaneous Determination of Diphenhydramine, Its *N*-Oxide Metabolite and Their Deuterium-labeled Analogues in Ovine Plasma and Urine Using Liquid Chromatography/Electrospray Tandem Mass Spectrometry

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Our studies on drug disposition in chronically instrumented pregnant sheep involve simultaneous administration of the antihistamine diphenhydramine (DPHM), its deuterated analogue ([$^2H_{10}$]DPHM) and their metabolites to the mother or the fetus via various routes. Such studies require sensitive and selective mass spectrometric methods for quantitation of these labeled and unlabeled compounds in order to assess comparative maternal and fetal drug metabolism. The objective of this study was to develop and validate a liquid chromatographic/tandem mass spectrometric (LC/MS/MS) method for the simultaneous quantitation of DPHM, its *N*-oxide metabolite and their deuterium-labeled analogues in ovine plasma and urine. Samples spiked with the analytes and the internal standard, orphenadrine, were processed using liquid–liquid extraction. The extract was chromatographed on a propylamino LC column and MS/MS detection was performed in the positive ion electrospray mode using multiple reaction monitoring. The linear concentration ranges of the calibration curves for the *N*-oxides and the parent amines were 0.4–100.0 and 0.2–250.0 ng ml^{-1}, respectively. In validation tests, the assay exhibited acceptable variability ($\leqslant$15% at analyte concentrations below 2.0 ng ml^{-1} and $<$10% at all other concentrations) and bias ($<$15% at all concentrations), and the analytes were stable under a variety of sample handling conditions. Using this method, the labeled and unlabeled *N*-oxide metabolite was identified in fetal plasma after DPHM and [$^2H_{10}$]DPHM administration. This method will be used further to examine the comparative metabolism of diphenhydramine to its *N*-oxide metabolite in the mother and the fetus. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: diphenhydramine; diphenhydramine *N*-oxide; stable isotopes; liquid chromatography/electrospray tandem mass spectrometry; pregnant sheep

INTRODUCTION

Diphenhydramine or 2-(diphenylmethoxy)-*N*,*N*-dimethylamine (DPHM) is a potent histamine H1-receptor antagonist.[1] DPHM and other antihistamines are widely used during human pregnancy for the treatment of pregnancy-related urticaria, severe nausea and vomiting, insomnia, allergic rhinitis and common coughs and colds.[2–5] However, information on the pharmacokinetics and pharmacodynamics of DPHM during human pregnancy is lacking. Previous studies in our laboratory, using chronically instrumented pregnant sheep as a model of human pregnancy, demonstrated that DPHM readily crosses the placenta and is eliminated from the near-term fetus via both placental and non-placental pathways.[6] Our current objective is to systematically examine and compare the metabolic pathways responsible for DPHM clearance in adult and fetal sheep in order to elucidate the components of fetal and adult non-placental elimination. This will clarify the *in vivo* functional capacity of various drug-metabolizing enzyme systems in the fetal lamb during late gestation as compared to adult sheep.

The ability to simultaneously administer unlabeled and stable isotope-labeled drugs provides a number of interesting avenues for studying drug disposition during pregnancy. This approach essentially enables one to conduct two pharmacokinetic experiments (e.g. control and test experiments to study drug bioavailability or simultaneous study of maternal and fetal drug

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disposition) at the same time, thus offering advantages in terms of time and cost savings, number of biological samples to be collected and analyzed, number of animals required for the study and reduced inter-day biological variability. These advantages are especially important in studies with large chronically instrumented pregnant animals (e.g. sheep) during late gestation where there is a limited time window available for conducting experiments before the animals deliver ($\sim$1–2 weeks). Also, this stage of gestation is a very dynamic period in terms of fetal growth and a variety of other physiological and metabolic alterations in the maternal and fetal body such that the importance of eliminating inter-day variation in drug disposition becomes increasingly important. We have used simultaneous administration of unlabeled and deuterium-labeled DPHM ([$^2H_{10}$]DPHM) to study bi-directional maternal–fetal placental transport of the drug and to quantitate fetal hepatic first-pass diphenhydramine uptake.[7] We have also employed the simultaneous administration of a deuterium-labeled DPHM metabolite, diphenylmethoxyacetic acid ([$^2H_{10}$]DPMA), and unlabeled DPHM to simultaneously study parent drug and metabolite disposition in a single animal in one experimental protocol, thus reducing inter-day biological variability.[8–9]

DPHM-*N*-oxide (DPHMNOX) is a prominent metabolite of DPHM in many species (e.g. dog, rhesus monkey and man) and accounts for $\sim$5–10% of the administered dose.[10,11] In our preliminary studies in pregnant sheep, we have also observed the formation of DPHMNOX *in vivo* by both adult and fetal sheep. Flavin monooxygenases (FMOs) are a group of microsomal oxidative drug-metabolizing enzymes that are most commonly involved in the formation of tertiary amine *N*-oxide metabolites. In contrast to the cytochrome P450 and phase II conjugation enzyme systems, there is almost no information in the literature on the extent of development of these enzymes and the pharmacokinetics of *N*-oxides in the fetus of any species. We wish to examine DPHM as a model drug that is metabolized to its *N*-oxide in maternal and fetal sheep in order to elucidate the role of this group of enzymes in fetal drug metabolism and elimination. Our studies will involve quantitation of the *in vivo* importance of the DPHMNOX pathway in DPHM elimination and metabolism in the mother and the fetus by simultaneous administration of deuterium-labeled DPHM and unlabeled DPHMNOX or vice versa. This will be accompanied by a study of the enzymology of this metabolic pathway *in vitro* in maternal and fetal hepatic subcellular preparations. Previously, we developed an assay to simultaneously quantitate DPHM and [$^2H_{10}$]DPHM in ovine biological fluids using gas chromatography (GC) with mass-selective detection.[12] However, tertiary amine *N*-oxides are generally unstable at the high temperatures encountered in GC.[13] High-performance liquid chromatography (HPLC) coupled with electrospray tandem mass spectrometry (ESI-MS/MS) offers an obvious choice for the analysis of such compounds in biological matrices owing to its sensitivity, selectivity and chromatography and ionization at relatively lower temperatures. Hence we developed and validated an LC/MS/MS method for the simultaneous quantitation of DPHM, DPHMNOX and their deuterium-labeled analogues in plasma and urine samples obtained from chronically instrumented pregnant sheep.

EXPERIMENTAL

Instrumentation

The instrumentation consisted of a Hewlett-Packard (Avondale, PA, USA) Model 1090 II liquid chromatograph interfaced to a Fisons VG Quattro I triple-quadrupole tandem mass Spectrometer (Micromass, Cheshire, UK). The operation of both instruments and mass spectrometric data acquisition were controlled with a Windows-NT-based Pentium Pro 200 MHz personal computer using the mass spectrometric data handling software MassLynx® (Micromass). Chromatographic separations were carried out on a YMC propylamino (NH_2, 100 × 2.0 mm i.d., 5 μm) column (Wilmington, NC, USA) at ambient temperature. The HPLC autoinjector syringe and sample loop volumes were 25 and 250 μl, respectively.

Reagents

Diphenhydramine hydrochloride and orphenadrine hydrochloride were obtained from Sigma Chemical Co. (St Louis, MO, USA). Deuterium-labeled DPHM ([$^2H_{10}$]DPHM) was synthesized and purified in our laboratory as reported previously.[12] DPHM *N*-oxide hydrochloride was generously provided by Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI, USA). Deuterium-labeled DPHM-*N*-oxide hydrochloride was synthesized and purified as described below. Chloroperoxybenzoic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Acetonitrile, methanol, acetone, diethyl ether, ethyl acetate and hexane were purchased from Caledon Laboratories (Georgetown, ON, Canada) and were of HPLC or GC grade. Ammonium acetate, sodium carbonate and glacial acetic acid were obtained from BDH Chemicals Toronto, ON, Canada) and were of analytical reagent grade. Triethylamine (Sequanal grade) was purchased from Pierce Chemical Co. (Rockford, IL, USA). High-purity deionized water was produced in our laboratory by reverse osmosis and subsequent filtration using a Milli-Q water system (Millipore, Bedford, MA, USA).

Synthesis and purification of deuterium-labeled DPHM-*N*-oxide hydrochloride

The [$^2H_{10}$]DPHMNOX metabolite was synthesized from [$^2H_{10}$]DPHM by its oxidation with 3-chloroperoxybenzoic acid using slight modification of a method described for the synthesis of *S*- and *N*-oxides of phenothiazine antipsychotics.[14] For this purpose, [$^2H_{10}$]DPHM hydrochloride was converted to its free base by alkalinization of an aqueous solution of the hydrochloride salt with sodium hydroxide. The free DPHM base was extracted with diethyl ether and the

solvent was evaporated under vacuum. The DPHM base (10 mmol) was then dissolved in 30 ml of dry dichloromethane. To this solution, 3-chloroperoxybenzoic acid (12 mmol) was added and the mixture was stirred in an ice-bath for 1 h. At the end of the reaction, unreacted 3-chloroperoxybenzoic acid was consumed by addition of an excess of triethylamine (12 mmol). The crude product was purified by flash column chromatography over silica gel using benzene–methanol (85:15) as the eluent. The fraction containing [$^2H_{10}$]DPHMNOX was collected and the solvent was removed under vacuum. The residue was then washed repeatedly with hexane and cold methanol (−20 °C). The washed residue was dissolved in dry acetone, cooled to −20 °C and [$^2H_{10}$]DPHMNOX hydrochloride was precipitated by the addition of propan-2-ol saturated with hydrogen chloride gas. The precipitate was recrystallized from acetone to give a white crystalline powder. The final product gave a single spot on thin-layer chromatographic plate and only one peak on a number of HPLC columns under a variety of elution conditions, indicating acceptable purity for the synthesized metabolite. Also, the HPLC retention time and daughter ion mass spectra of the purified metabolite were similar to those of the Parke-Davis DPHMNOX standard except for an expected 10 a.m.u difference in some fragment masses (Fig. 1; see below).

Stock standard solutions

An aqueous stock standard solution of analytes containing 2.5 μg ml^{-1} of DPHM, 2.6 μg ml^{-1} of [$^2H_{10}$]DPHM, 1.0 μg ml^{-1} of DPHMNOX and 1.04 μg ml^{-1} of [$^2H_{10}$]DPHMNOX was prepared by dissolving appropriate amounts of the analytes (based on free base) in deionized water. Two additional solutions were prepared as 10- and 25-fold dilutions of the above stock standard solution. An internal standard (I.S.) solution containing 250.0 ng ml^{-1} orphenadrine was prepared by dissolving an accurately weighed amount of orphenadrine hydrochloride in deionized water.

Sample extraction

The analytes of interest were extracted from the biological fluid samples using a single-step liquid–liquid extraction procedure. Sheep plasma or urine samples (up to 1.0 ml) or the spiked standards were pipetted into clean borosilicate glass tubes with polytetrafluoroethylene (PTFE)-lined caps. The sample volume was adjusted to 1.0 ml with deionized water. A 100 μl aliquot of the I.S. solution (containing 25.0 ng of orphenadrine) was added to each sample and the samples were alkalinized (pH 11.5) by adding 0.5 ml of saturated sodium carbonate solution. Ethyl acetate (6 ml) containing 0.05 M (0.72%, v/v) triethylamine (TEA) was then added to each sample and the tubes were capped. The samples were vortex mixed for 10 s, mixed with a slow rotary motion on a Labquake tube shaker (Model 415-110, Laboratory Industries, Berkeley, CA, USA) for 20 min, cooled to −20 °C for 10 min (to break any emulsion formed during mixing) and then

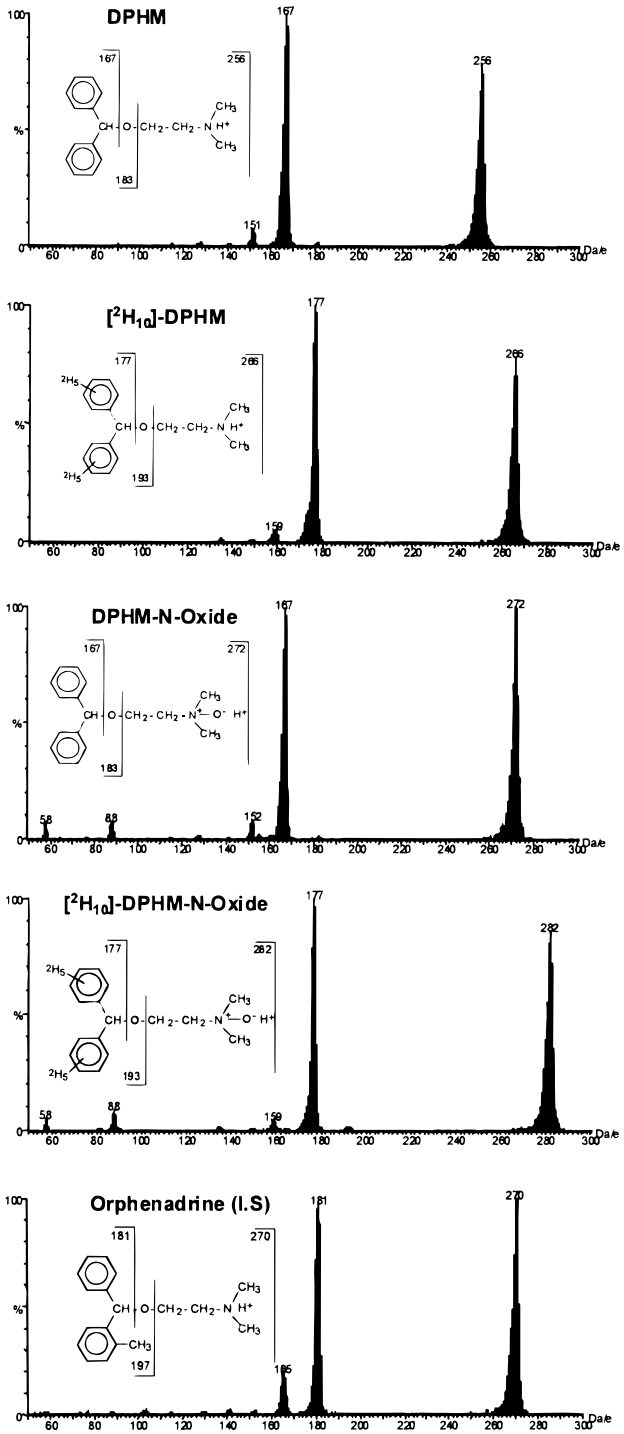

Figure 1. Positive ion electrospray fragment ion mass spectra of DPHM, [$^2H_{10}$]DPHM, DPHM-*N*-oxide, [$^2H_{10}$]DPHM-*N*-oxide and the internal standard, orphenadrine.

centrifuged at 3000*g* for 10 min using an IEC Model NH-SII centrifuge (Damon/IEC Division, Needham Heights, MA, USA). The top organic ethyl acetate layer was separated, transferred into a clean set of tubes and evaporated to dryness under a gentle stream of nitrogen at 25 °C using a Zymark Turbo Vap LV® evaporator (Zymark Corporation, Hopkinton, MA, USA). The residue was reconstituted in 200 μl of acetonitrile–water (9:1) and the tubes were vortex mixed for 30 s. Samples were transferred to the HPLC autosampler vials with

0.35 ml glass inserts and a 10 μl volume was injected into the HPLC system.

High-performance liquid chromatography

The samples were chromatographed on a Hewlett-Packard Model 1090 II LC instrument using a 100 × 2.0 mm i.d., 5 μm YMC amino column, employing normal-phase chromatography. Precolumn filters, with replaceable 2 μm frits, were installed in the LC instrument between the sample loop and the column. Gradient elution was used to achieve a quick run time and also optimal retention of the compounds on the HPLC column. The chromatographic run began with acetonitrile–2 mM ammonium acetate buffer (95:5) containing 1% glacial acetic acid (pH 3.0). The proportion of aqueous buffer was increased to 25% in a 6 min linear gradient, held there for 0.5 min, brought back to the initial 95:5 proportion at 7.0 min and held there for 3 min before the next injection. The mobile phase flow-rate was 0.4 ml min^{-1} with a 50:50 split to the mass spectrometer and waste. This HPLC procedure resulted in a total run time of 10 min for all five compounds of interest.

Electrospray tandem mass spectrometry

The eluate from HPLC column was split, and 50% (0.2 ml min^{-1}) was introduced into the Fisons VG Quattro I triple-quadrupole tandem mass spectrometer for detection of the analytes. Nitrogen was used as the nebulizing and bath gas. The compounds were ionized in the positive ion electrospray mode and detected using multiple reaction monitoring (MRM). The ion transitions monitored were m/z 256 → 167 (DPHM), m/z 266 → 177 ([$^2H_{10}$]DPHM), m/z 272 → 167 (DPHMNOX), m/z 282 → 177 ([$^2H_{10}$]DPHMNOX) and m/z 270 → 181 (orphenadrine). These transitions were selected based on the predominant fragmentation pathways of various compounds in their daughter ion spectra (Fig. 1). The dwell time for each transition was set at 0.2 s with an interchannel delay of 20 ms to provide optimal sampling of each peak of interest (12–15 scans per peak). Collisionally induced dissociation (CID) was achieved with argon at a pressure of 3×10^{-4} mbar in the collision cell. For maximal sensitivity, the collision energy, ion source temperature and cone voltage of the mass spectrometer were optimized at 70 eV, 110 °C and 30 V, respectively.

Calibration curves and the regression model

Calibration standards for both amines and *N*-oxides were prepared at the concentrations given in Table 1 by adding appropriate amounts of the stock standard solutions to 1 ml of blank ovine plasma or urine. The I.S. (25.0 ng of orphenadrine) was then added to each sample and the samples were extracted and analyzed using LC/MS/MS as described above. Weighted linear regression (weighting factor = $1/y^2$) was performed between the ratio of peak area of each analyte to that of the I.S. *vs.* the corresponding spiked concentration in order to reduce bias at the lower concentrations. Linearity of calibration curves was demonstrated by calculating the regression bias. This was accomplished by analyzing six sets of calibration curve samples and back-calculating the concentration of each standard from the obtained slope, intercept and the peak area ratios. The bias (%) was calculated as

$$\text{bias (\%)} = \frac{\text{back-calculated concentration} - \text{nominal concentration}}{\text{nominal concentration}} \times 100$$

A bias of $< \pm 15\%$ at each concentration was considered evidence of linearity of the calibration curves.

Extraction recovery

Absolute recoveries of all analytes in plasma and urine were determined at four different concentrations representing the entire range of the calibration curves (Fig. 2). Two sets of samples, the control group and the recovery group, were prepared and processed as outlined in Fig. 2. The concentrations of the analytes in the control and recovery group samples were measured against the extracted duplicate standard curves prepared in the corresponding biological matrix. The absolute recovery was calculated as the ratio of measured concentration of recovery samples to that of the corresponding control samples at each different analyte concentration.

Analyte stability in biological fluid samples

A number of tests were carried out according to the scheme shown in Fig. 3 in order to establish the stability of the analytes under the routine sample handling conditions in the laboratory. In addition, the stability of processed samples was evaluated by repeatedly injecting the samples on the autosampler tray three times during a 48 h period after extraction. The area counts of peaks and their ratios to those of the I.S. were evaluated.

In all stability tests, the analytes were considered 'stable ' if the measured concentration after the treatment was within ±10% of the nominal value.

Table 1. Concentrations of calibration curve standards for DPHM, [$^2H_{10}$]DPHM, DPHM-*N*-oxide and [$^2H_{10}$]DPHM-*N*-oxide

DPHM or [$^2H_{10}$] DPHM (ng ml^{-1})	0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 125.0, 250.0
DPHM-*N*-oxide or [$^2H_{10}$]DPHM-*N*-oxide (ng ml^{-1})	0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 50.0, 100.0

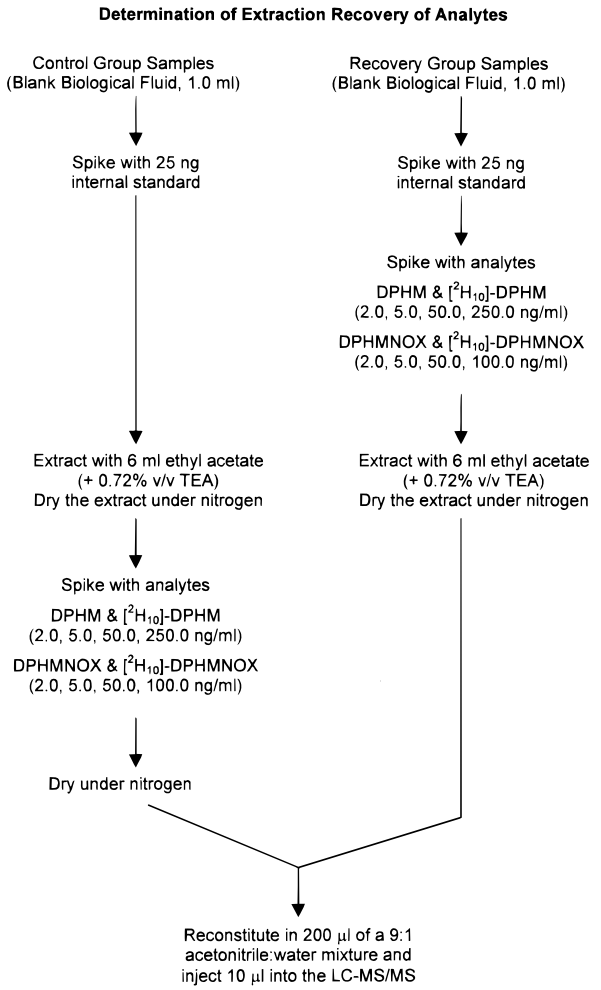

Figure 2. Sample processing scheme for the determination of extraction recovery of analytes in ovine plasma and urine.

Method validation

Method validation was performed by evaluating the intra- and inter-assay variance and bias (inaccuracy) in the quantitation of quality control samples (QCs). The QC samples were prepared by spiking blank plasma or urine with analytes at concentrations representing the limit of quantitation and low, medium and high ranges of the standard curve (see Tables 4 and 5).

Intra-assay variance and bias were estimated by analyzing six QCs at each concentration using a duplicate standard curve in one batch. For inter-assay variance and bias, six batches of samples, each consisting of six QCs at each concentration and a duplicate standard curve were analyzed on six separate days.

The assay method was also independently cross-validated for the quantitation of DPHM and $[^2H_{10}]$DPHM with our earlier GC/MS assay.[12] This was accomplished by comparing the results obtained from the two methods for the analysis of plasma samples spiked with three different concentrations (5.0, 50.0 and 250.0 ng ml^{-1}) of DPHM and $[^2H_{10}]$DPHM. The cross-validation of the quantitation of *N*-oxide metabolites could not be performed because, to our knowledge, no methods exist for the measurement of these compounds in biological fluids.

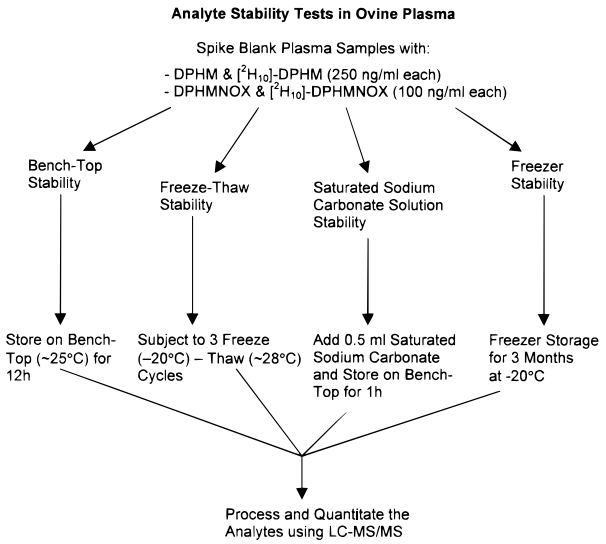

Figure 3. Scheme for analyte stability studies in ovine plasma.

Application of the assay to a study of DPHM, $[^2H_{10}]$DPHM, DPHMNOX and $[^2H_{10}]$DPHMNOX disposition in the ovine maternal–fetal unit

A pregnant sheep (125 days gestation, term 145 days) was surgically prepared under halothane anesthesia by placing fluid sampling polyvinyl catheters and other monitoring devices (e.g. ultrasonic blood flow probe) in maternal and fetal blood vessels as described earlier.[15] After a recovery period (4 days), an equimolar dose of DPHM (2.5 mg) and $[^2H_{10}]$DPHM (2.6 mg) was simultaneously administered as a bolus via the fetal lateral tarsal vein. Serial fetal (~2 ml each) and maternal (~4 ml each) femoral arterial blood samples were collected at 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, 120, 140, 160, 180, 210, 240, 300 and 360 min after drug administration. Plasma was separated by centrifugation and the plasma samples were stored in borosilicate glass tubes at −20 °C until analysis. Blank plasma samples were also collected just before drug administration (−5 min) for use in calibration curve sample preparation. Maternal and fetal plasma samples were then analyzed for DPHM, $[^2H_{10}]$DPHM, DPHMNOX and $[^2H_{10}]$DPHMNOX concentrations using the LC/MS/MS method described above.

RESULTS AND DISCUSSION

As discussed in the Introduction, the ability to simultaneously administer unlabeled and stable-isotope labeled drugs and metabolites has significantly improved our ability to study maternal–fetal drug disposition in a scientifically unbiased, efficient and cost-effective way.[7–9] Our current focus is to elucidate *in utero* fetal development and functional capacity of various drug-metabolizing enzyme systems as compared to the adult. We plan to achieve this by studying *in vivo* maternal–fetal drug pharmacokinetics and metabolite formation

using a combination of stable isotope-labeled compounds and mass spectrometry. The rationale for studying DPHM-*N*-oxide lies within the fact that *N*-oxides are most commonly formed via the microsomal flavin-monooxygenase system and there is almost no information in the literature on the ontogenetic development of this group of enzymes in any species. These studies require a rapid, sensitive and selective assay method capable of determining low concentrations (in the range of ng ml^{-1}) of the parent drug, its metabolites and their stable isotope-labeled analogues in a number of biological fluids (e.g. plasma and urine).

High-performance liquid chromatography of diphenhydramine and the *N*-oxide metabolite

The simultaneous determination of the tertiary amine diphenhydramine (DPHM and [$^2H_{10}$]DPHM) and its *N*-oxide metabolite (DPHMNOX and [$^2H_{10}$]DPHMNOX) in a single run using LC/MS/MS presented an interesting analytical challenge. The parent amines and their *N*-oxides metabolites have widely differing polarities. In general, the tertiary amine *N*-oxides are some of the most polar drug metabolites whereas the parent amines have a predominantly lipophilic character.[13] This presented us with some difficulties with respect to the choice of a single stationary phase for the optimal chromatographic retention of both the parent drug and the metabolites. The *N*-oxide metabolites exhibited little retention on many conventional reversed-phase columns such as C_{18}, C_8, C_2 and phenyl- and cyano-bonded types. The extreme reversed-phase conditions (>99% aqueous content in the mobile phase) necessary for only a minimal retention of the *N*-oxides led to severe adsorption of the parent amines to the column and subsequent slow elution. This resulted in extremely long run times, severe peak tailing for the parent amines and also 'carry-over' problems. Since reversed-phase chromatography utilizes interactions between the lipophilic moieties of the analytes with the non-polar stationary phase for analyte retention on the HPLC column, we concluded that the *N*-oxide metabolites do not exhibit sufficient lipophilic character for optimal interaction with these non-polar stationary phases.

Some investigators have used ion pairing as an approach to improve the retention of tertiary amine *N*-oxides on reversed-phase columns in HPLC methods with UV detection.[16] This was not possible with our method, however, because ESI-MS precludes the use of non-volatile additives such as ion-pairing agents. This led us to consider the use of normal-phase chromatography in order to utilize the polar component of the analyte molecules for their interaction with and retention on a relatively polar column. For normal-phase chromatography, the propylamino (NH_2) phase provides a useful alternative to silica. In contrast to silica, the NH_2 phase is compatible with the aqueous components of the mobile phase. This stationary phase essentially allows the use of the same solvents as in traditional reversed-phase chromatography (in different proportions compared to the reversed-phase mode with water being the stronger solvent) without any restriction to purely organic mobile phases (e.g. hexane, dichloromethane) as is the case with silica. This is important because a complete lack of an aqueous or buffer component in the mobile phase could lead to inadequate ionization of the analytes during the electrospray ionization process in the mass spectrometer.

In our experiments, both the parent amines and their *N*-oxide metabolites exhibited excellent retention on a relatively short (10 cm) NH_2 column [Fig. 4(B)]. However, owing to the greatly different polarities of the parent drug and the metabolites, and in order to achieve a balance between adequate retention of all analytes and a fast analysis time (~10–15 min), gradient elution was found to be necessary. In general, low mobile phase flow-rates (~50–100 µl min^{-1}) provide maximal sensitivity in LC/MS/MS assays. However, it is difficult to use gradient elution at these low flow-rates because of the relatively large void volume between the HPLC pump and the column (~1 ml in the HP 1090

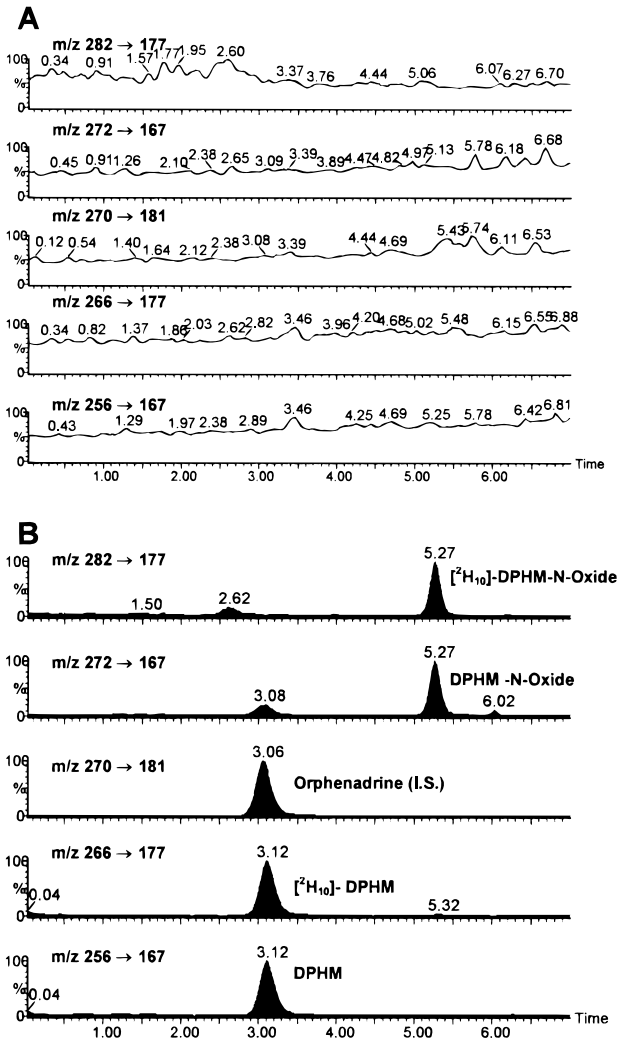

Figure 4. LC/MS/MS MRM ion chromatograms of (A) an extracted blank sheep plasma sample and (B) an extracted plasma calibration standard containing 0.4 ng ml^{-1} each of [$^2H_{10}$]DPHM-*N*-oxide and DPHM-*N*-oxide and 1 ng ml^{-1} each of [$^2H_{10}$]DPHM and DPHM. The *y*-axis scales in (A) have been magnified to show clearly the baselines at different MRM ion transitions. The HPLC and MS/MS conditions and specifications are described in the text.

Series II chromatograph). Hence we decided to use a higher mobile phase flow-rate (0.4 ml min^{-1}) with a 50:50 split to the mass spectrometer and waste. This provided sufficient sensitivity for all analytes for our purposes and a fast run time (10 min) for all five compounds of interest [Fig. 4(B)]. Using normal-phase chromatography and the described gradient timetable, the relatively lipophilic amines orphenadrine, DPHM and [$^2H_{10}$]DPHM eluted earlier (at 3.06, 3.12 and 3.12 min, respectively) than the more polar *N*-oxides (at 5.27 min). The variations in the retention times of different analytes within a single run were <5%. Although the two groups of compounds (tertiary amines and *N*-oxides) were well resolved from each other, it was neither possible nor necessary to resolve different compounds chromatographically within the amine or the *N*-oxide group [Fig. 4(B); also see below].

Tertiary amine drugs may exhibit severe tailing on many reversed-phase columns. However the use of an NH_2 column, in addition to providing optimal retention for all the compounds, also resulted in excellent symmetrical peak shapes [Fig. 4(B)]. The use of acidic ammonium acetate buffer of pH 3.0 (2 mM ammonium acetate and 1% acetic acid) instead of pure water led to an improvement in peak shape for all the compounds and significantly reduced peak tailing for the parent amines.

Tandem mass spectrometric detection of the analytes

Figure 1 shows daughter ion mass spectra of all the analytes and the internal standard, orphenadrine, in the positive ion electrospray mode. All analytes appear to fragment predominantly at the ether linkage on the aliphatic side chain of the molecules, thus forming analogous fragment ions. Some additional fragmentation also takes place and corresponding *m/z* assignments are depicted in Fig. 1. Our initial attempts to quantitate these compounds involved the use of single-ion recording (SIR) by monitoring the $[M + H]^+$ ion for each analyte in the first quadrupole analyzer of the mass spectrometer (Q1). However, for some ions, this resulted in significant cross-over of signal from one ion to the other (e.g. *m/z* 266 and 270 for [$^2H_{10}$]DPHM and orphenadrine, respectively), especially at higher analyte concentrations. Mass spectrometric resolution of these interferences was important because it was not possible to resolve many of these compounds from one another chromatographically owing to very subtle differences in their chemical structure (Figs 1 and 4). The use of a multiple reaction monitoring mode (MRM) helped to eliminate these interferences by utilizing the enhanced selectivity of MS/MS detection as compared with the single mass spectrometer configuration in SIR. Also, the MRM mode provided much cleaner baselines than SIR, leading to an increase in signal-to-noise ratio. The molecular ion $[M + H]^+$ and the daughter ion formed by fragmentation at the ether linkage are the major ions in the mass spectra for all the analytes (Fig. 1) and would be expected to provide maximal sensitivity for the analysis of these compounds. Based on this, the mass transitions of *m/z* 256 → 167 (DPHM), *m/z* 266 → 177 ([$^2H_{10}$]DPHM), *m/z* 272 → 167 (DPHMNOX), *m/z* 282 → 177 ([$^2H_{10}$]DPHMNOX) and *m/z* 270 → 181 (orphenadrine) were selected (Figs 1 and 4).

A number of mass spectrometric parameters such as ion source temperature, argon pressure in the collision cell, collision energy and cone voltage were optimized to achieve maximal sensitivity. In general, lower ion source temperatures would be safer in terms of the stability of the *N*-oxide metabolites. In our experiments, lower temperatures (80–90 °C), however, resulted in a loss of sensitivity and deposition of material in the mass spectrometer, thus requiring frequent cleaning of the ion source. An increase in ion source temperature beyond 110 °C did not offer any significant increase in sensitivity and the *N*-oxide metabolites appeared to decompose at temperatures higher than 150 °C. Hence an ion source temperature of 110 °C was considered optimal for final analysis. Similarly, the argon pressure in the collision cell, the collision energy of the argon molecules and the cone voltage were optimized for maximal sensitivity at 3×10^{-4} mbar, 70 eV and 30 V, respectively.

Extraction method and recovery

A number of organic solvents and solvent mixtures such as dichloromethane, dichloromethane containing 2% propan-2-ol, hexane containing 2% propan-2-ol, toluene and ethyl acetate were evaluated for maximizing the extraction recovery of the analytes. Triethylamine (TEA; 0.05 M or 0.72%, v/v) was included in all the solvent systems in order to prevent non-specific binding of the analytes to glassware[12] (see also below). All solvent systems were very efficient in terms of extracting the parent amines. However, owing to the extreme polarity of the *N*-oxide metabolites, their recoveries were low (ranging from <20% for the hexane–2% propan-2-ol mixture to ~50% for the dichloromethane–2% propan-2-ol mixture). Extraction with ethyl acetate–0.05 M TEA (0.72%, v/v) provided maximal recoveries for the *N*-oxide metabolites and also resulted in clean extracts devoid of any chromatographic or mass spectrometric interference from the biological matrix [Fig. 4(A)]. Hence ethyl acetate–0.05 M TEA (0.72%, v/v) was chosen as the final extraction solvent. The recoveries for all the analytes were consistent (relative standard deviation (RSD) <10% for parent amines and <15% for the *N*-oxides) and independent of analyte concentration; hence an overall mean recovery was calculated. The mean recoveries for DPHM, [$^2H_{10}$]DPHM, DPHMNOX and [$^2H_{10}$]DPHMNOX from ovine plasma and urine are presented in Table 2.

Earlier we observed a dramatic improvement in DPHM and [$^2H_{10}$]DPHM recovery upon the addition of

Table 2. Extraction recoveries (%) of analytes from ovine plasma and urine

Sample	Analyte			
	DPHM	[$^2H_{10}$]DPHM	DPHM-*N*-oxide	[$^2H_{10}$]DPHM-*N*-oxide
Plasma	79.7 ± 6.6	76.6 ± 6.6	71.6 ± 9.4	69.0 ± 8.3
Urine	80.3 ± 5.8	82.3 ± 4.3	71.9 ± 6.5	72.2 ± 7.4

0.05 M TEA (0.72%, v/v) to hexane–2% propan-2-ol solvent, possibly due to a reduction in non-specific binding of the tertiary amines to active sites on glassware.[12] A similar phenomenon was observed with ethyl acetate extraction, the recovery of all analytes being reduced by ~20–30% when 0.05 M TEA (0.72%, v/v) was not included in the extraction solvent.

Some investigators have demonstrated decomposition of the *N*-oxide metabolites in plasma on alkalinization with sodium hydroxide.[13,17,18] These metabolites, however, seem to be stable with the use of saturated sodium carbonate solution.[17,18] Hence we decided to use saturated sodium carbonate solution for alkalinization of samples in this method. The diphenhydramine *N*-oxide metabolites were found to be stable in sodium carbonate (see below). Also, the use of a saturated sodium carbonate solution resulted in cleaner extracts as compared with sodium hydroxide, as evidenced by a reduction in baseline noise.

Analyte stability in biological fluid samples

A number of studies were conducted to evaluate the stability of the analytes in biological fluid samples under conditions simulated to match routine sample handling. The mean analyte concentrations measured in samples subjected to all the described stability tests were within ±10% of the nominal concentrations, with an acceptable RSD (<10%; see Ref. 19 for acceptability standards for bioanalytical assays). There was no significant change in the analyte to I.S. peak-area ratios of various compounds during repeated injections of the processed samples on the autosampler tray for up to 48 h after extraction. Based on these results, it appears that the analytes would be sufficiently stable in biological samples during actual freezer storage and analysis conditions.

Calibration curves and the regression model

The calibration curves for all the analytes showed good linearity in the concentration ranges tested (0.2–250.0 ng ml^{-1} for parent amines and 0.4–100.0 ng ml^{-1} for *N*-oxides). Weighted linear regression (weighting factor = $1/y^2$) was carried out on all the calibration curve data in order to reduce bias at the lower concentrations. This weighting function resulted in a relatively low regression bias for all analytes at both the lower and upper limits of the calibration curves (Table 3). The magnitude of this bias is within the limits established for bioanalytical assays by a number of regulatory agencies such as Health Protection Branch (Canada) and Food and Drug Administration (USA) (< ± 20% at the limit of quantification (LOQ) and < ± 15% at all other concentrations).[19] The peak area response for the *N*-oxides was non-linear at concentrations greater than 100.0 ng ml^{-1}. This could be due to fact that at higher concentrations, the *N*-oxides partly dimerize. Representative calibration curves for all analytes are shown in Fig. 5. The slopes of the [$^2H_{10}$]DPHM and [$^2H_{10}$]DPHMNOX standard curves were consistently higher than those for DPHM and DPHMNOX, respectively. This indicates that the fragmentation patterns of the compounds may have been quantitatively altered by the presence of deuterium labels.

The LOQs of 0.2 and 0.4 ng ml^{-1} were established for the parent amines and *N*-oxides, respectively, based on a signal-to-noise ratio of at least 20 and an acceptable variability and bias[19] at this concentration (see below). The LOQ of 0.2 ng ml^{-1} for DPHM and [$^2H_{10}$]DPHM achieved in this assay method is an

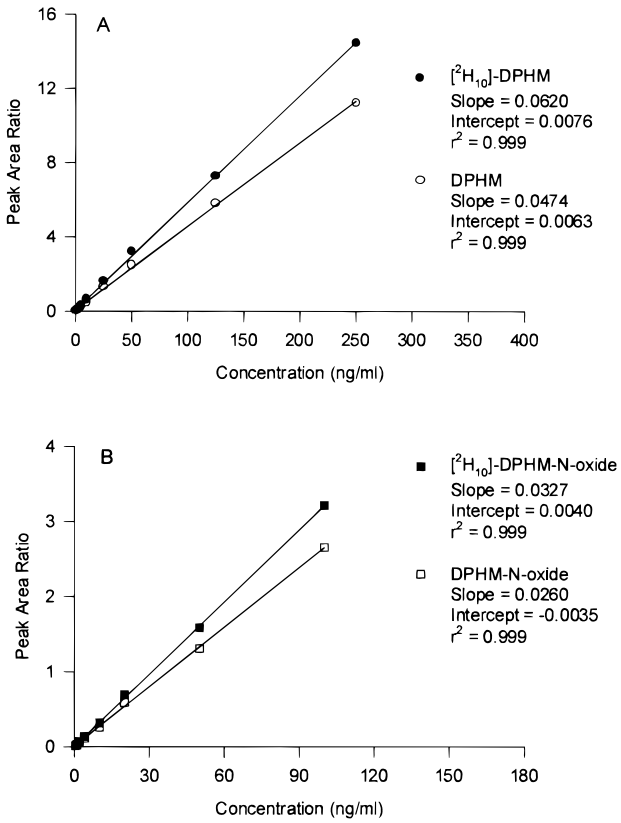

Figure 5. Representative calibration curves for (A) DPHM (○) and [$^2H_{10}$]DPHM (●), and (B) DPHM-*N*-oxide (□) and [$^2H_{10}$]DPHMN-*N*-oxide (■) in ovine plasma.

Table 3. Regression bias (%) for the quantitation of analytes at the lower and upper limits of calibration curves

	Analyte			
	DPHM	[$^2H_{10}$]DPHM	DPHM-*N*-oxide	[$^2H_{10}$]DPHM-*N*-oxide
Lower limit	−11.6	−11.3	−7.1	+9.4
Upper limit	+1.5	−1.5	+3.0	−8.6

order of magnitude lower than that of our earlier GC/MS method (2.0 ng ml^{-1}),[12] indicating a significant improvement in sensitivity.

Method validation

The validation of the assay involved the estimation of intra- and inter-assay variability and bias in ovine plasma and urine. The results from plasma validation are presented in Tables 4 and 5. The variability and bias data in urine were similar and are not presented. The intra- and inter-assay variabilities (RSDs) for all analytes were $\leqslant 15\%$ at concentrations below 2.0 ng ml^{-1} and $<10\%$ at all other concentrations. The mean intra- and inter-assay bias (inaccuracies) ranged from -6% to $+12\%$ of the nominal concentration for the amines and from -10% to $+14\%$ for the *N*-oxides over the calibration curve range (Tables 4 and 5).

The cross-validation of the LC/MS/MS assay with our earlier GC/MS method yielded excellent agreement between the two methods; the concentrations of DPHM and [$^2H_{10}$]DPHM measured in spiked samples using the two methods were highly correlated (Pearson correlation coefficient $r = 1.000$ at all concentrations) and not significantly different (unpaired *t*-test, $p > 0.05$). However, as discussed above, the current method offers advantage of a lower LOQ (0.2 *vs.* ng ml^{-1}) compared with the earlier method. In addition, this method can also simultaneously quantitate the *N*-oxide metabolite of the drug with good sensitivity and selectivity.

Application of the assay to the study of DPHM, [$^2H_{10}$]DPHM, DPHMNOX and [$^2H_{10}$]DPHMNOX disposition in the ovine maternal–fetal unit

The developed assay was applied to a pharmacokinetic study in chronically catheterized pregnant sheep, designed to test the bioequivalency of DPHM and [$^2H_{10}$]DPHM in the fetal lamb in terms of the parent amine disposition, formation of DPHMNOX and [$^2H_{10}$]DPHMNOX from the two compounds and the clearance of DPHMNOX and [$^2H_{10}$]DPHMNOX. This bioequivalency is extremely important in order to obtain meaningful pharmacokinetic data by utilizing the stable isotope-labeled drug. Figure 6 shows the fetal femoral arterial plasma profiles of DPHM, [$^2H_{10}$]DPHM, DPHMNOX and [$^2H_{10}$]DPHMNOX after simultaneous equimolar fetal intravenous bolus administration of DPHM (2.5 mg) and [$^2H_{10}$]DPHM (2.6 mg) in a pregnant sheep. These data show that the plasma concentrations of DPHM and [$^2H_{10}$]DPHM decline rapidly after drug administration and are virtually superimposable. This indicates approximately equal rates of clearance of these two compounds from the fetal circulation. Also, the corresponding *N*-oxide metabolites (DPHMNOX and [$^2H_{10}$]DPHMNOX) were detectable in fetal plasma (Fig. 6). The concentrations of metabolites increased gradually and then declined rapidly in parallel with the parent drug. Similar to the parent drugs, the concentrations of the two metabolites were virtually identical, indicating a lack of any effect of the deuterium labels on this metabolic pathway. Hence these data demonstrate the

Table 4. Intra-assay variability and bias of the method in ovine plasma

Analyte	Parameter	QC-1	QC-2	QC-3	QC-4	QC-5
DPHM	Nominal concentration (ng ml^{-1})	0.20	1.0	5.0	50.0	250.0
	Measured concentration (ng ml^{-1})	0.20	1.04	5.0	55.3	239.9
	SD (ng ml^{-1})	0.02	0.05	0.32	1.8	6.8
	RSD (%)	9.7	5.1	6.4	3.3	2.8
	Bias (%)	−0.5	+3.8	+0.2	+10.6	−4.0
[$^2H_{10}$]DPHM	Nominal concentration (ng ml^{-1})	0.20	1.0	5.0	50.0	250.0
	Measured concentration (ng ml^{-1})	0.19	1.05	5.0	56.0	241.4
	SD (ng ml^{-1})	0.02	0.05	0.25	1.3	8.7
	RSD (%)	13.2	4.4	5.0	2.3	3.6
	Bias (%)	−5.4	+5.1	−0.8	+12.0	−3.4
DPHM-*N*-oxide	Nominal concentration (ng ml^{-1})		0.40	2.0	20.0	100.0
	Measured concentration (ng ml^{-1})		0.45	2.48	21.8	94.7
	SD (ng ml^{-1})		0.06	0.22	2.1	6.6
	RSD (%)		13.3	9.0	9.6	7.0
	Bias (%)		+12.8	+14.0	+9.2	−5.3
[$^2H_{10}$]DPHM-*N*-oxide	Nominal concentration (ng ml^{-1})		0.4	2.0	20.0	100.0
	Measured concentration (ng ml^{-1})		0.41	2.09	21.7	94.9
	SD (ng ml^{-1})		0.06	0.28	1.8	4.8
	RSD (%)		15.0	13.4	8.3	5.0
	Bias (%)		+3.0	+4.4	+8.6	−5.1

Table 5. Inter-assay variability and bias of the method in ovine plasma

Analyte	Parameter	QC-1	QC-2	QC-3	QC-4	QC-5
DPHM	Nominal concentration (ng ml^{-1})	0.20	1.0	5.0	50.0	250.0
	Measured concentration (ng ml^{-1})	0.21	1.06	5.5	54.5	237.7
	SD (ng ml^{-1})	0.01	0.04	0.3	0.9	4.3
	RSD (%)	5.6	3.5	5.9	1.7	1.8
	Bias (%)	+5.3	+6.3	+11.0	+9.0	−4.9
[$^2H_{10}$]DPHM	Nominal concentration (ng ml^{-1})	0.20	1.0	5.0	50.0	250.2
	Measured concentration (ng ml^{-1})	0.20	1.09	5.5	54.8	238.2
	SD (ng ml^{-1})	0.02	0.05	0.3	1.1	4.5
	RSD (%)	10.1	4.6	5.6	2.0	1.9
	Bias (%)	−1.1	+8.8	+10.1	+9.6	−4.7
DPHM-*N*-oxide	Nominal concentration (ng ml^{-1})		0.40	2.0	20.0	100.0
	Measured concentration (ng ml^{-1})		0.40	2.24	22.1	90.9
	SD (ng ml^{-1})		0.05	0.14	0.6	3.3
	RSD (%)		12.9	6.1	2.8	3.6
	Bias (%)		+0.6	+12.0	+10.4	−9.1
[$^2H_{10}$]DPHM-*N*-oxide	Nominal concentration (ng ml^{-1})		0.4	2.0	20.0	100.0
	Measured concentration (ng ml^{-1})		0.41	2.02	22.1	92.0
	SD (ng ml^{-1})		0.03	0.25	0.6	2.8
	RSD (%)		6.7	12.2	2.8	3.0
	Bias (%)		+2.6	+0.9	+10.4	−8.0

absence of any isotope effect in the disposition of [$^2H_{10}$]DPHM compared with DPHM, and also for [$^2H_{10}$]DPHMNOX compared with DPHMNOX. In addition to fetal plasma, the maternal femoral arterial plasma samples collected at the corresponding time points were also analyzed for the labeled and unlabeled parent drug and the metabolite. However, all compounds were present at much lower concentrations (near LOQ, data not shown) compared with the fetal plasma. The higher plasma concentrations of the *N*-oxide metabolites in fetal plasma compared with maternal plasma indicate the ability of the fetus to form this metabolite after DPHM and [$^2H_{10}$]DPHM administration. However, further experiments would be required to confirm these observations.

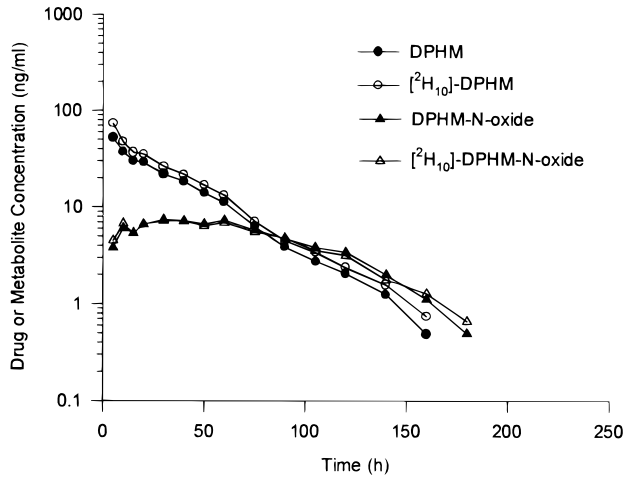

Figure 6. Femoral arterial plasma concentration *vs.* time profiles of DPHM (■), [$^2H_{10}$]DPHM (○), DPHM-*N*-oxide (▲) and [$^2H_{10}$]DPHM-*N*-oxide (△) in a fetal lamb after a simultaneous equimolar bolus dose of DPHM and [$^2H_{10}$]DPHM via the fetal lateral tarsal vein.

CONCLUSION

We have developed and validated an LC/MS/MS assay for the simultaneous quantitation of DPHM, [$^2H_{10}$]DPHM, DPHMNOX and [$^2H_{10}$]DPHMNOX in plasma and urine samples obtained from chronically instrumented pregnant sheep. The assay is rapid (fast sample processing, a 10 min run time), sensitive and selective (no interference from biological matrices, LOQs of 0.2 and 0.4 ng ml^{-1} for amines and *N*-oxides, respectively) and robust (acceptable variability and bias, sample stability). This method is currently being applied to detailed studies of the disposition kinetics of the diphenhydramine *N*-oxide metabolite within the ovine maternal–fetal unit.

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REFERENCES

1. J. C. Garrison, in *The Pharmacological Basis of Therapeutics*, edited by A. G. Gilman, T. W. Rall, A. S. Nies and P. Taylor, 2nd edn., Vol. I, Chapt. 23, p. 575. Pergamon Press, New York (1991).
2. J. O. Forfar and M. M. Nelson, *Clin. Pharmacol. Ther.* **14**, 632 (1973).
3. J. C. Brocklebank, W. A. Ray, C. F. Federspiel and W. Schaffner, *Am. J. Obstet. Gynecol.* **132**, 235 (1978).
4. W. Rayburn, J. Wible-Kant and P. Bledsoe, *J. Reprod. Med.* **27**, 569 (1982).
5. J. M. Piper, C. Baum and D. L. Kennedy, *Am. J. Obstet. Gynecol.* **157**, 148 (1987).
6. S. D. Yoo, J. E. Axelson, S. M. Taylor and D. W. Rurak, *J. Pharm. Sci.* **82**, 145 (1993).
7. S. Kumar, G. R. Tonn, E. Kwan, C. Hall, K. W. Riggs, J. E. Axelson and D. W. Rurak, *J. Pharmacol. Exp. Ther.* **282**, 617 (1987).
8. S. Kumar, D. W. Rurak, E. Kwan and J. E. Axelson, *Pharm. Res.* **12**, S-346 (1995).
9. S. Kumar, J. E. Axelson, K. W. Riggs and D. W. Rurak, *Pharm. Res.* **13**, S-425 (1996).
10. T. Chang, R. A. Okerholm and A. J. Glazko, *Res. Commun. Chem. Pathol. Pharmacol.* **9**, 391 (1974).
11. J. C. Drach, J. P. Howell, P. E. Borondy and A. J. Glazko, *Soc. Exp. Biol. Med. Proc.* **135**, 849 (1970).
12. G. R. Tonn, A. Mutlib, F. S. Abbott, D. W. Rurak and J. E. Axelson, *Biol. Mass Spectrom.* **22**, 633 (1993).
13. K. K. Midha, T. J. Jaworski, E. M. Hawes, G. McKay, J. W. Hubbard, M. Aravagiri and S. R. Marder, in *N-Oxidation of Drugs: Biochemistry, Pharmacology and Toxicology*, edited by P. Hlavica and L. A. Damani, Chapt. 3, p. 37. Chapman and Hall, New York (1991).
14. T. J. Jaworski, M. S. Sardessai, M. Aravagiri, G. Lin, Y. Y. Shi, E. M. Hawes, J. W. Hubbard, G. McKay and K. K. Midha, *J. Pharm. Sci.* **82**, 330 (1993).
15. D. W. Rurak, S. D. Yoo, E. Kwan, S. M. Taylor, K. W. Riggs and J. E. Axelson, *J. Pharmacol. Exp. Ther.* **247**, 271 (1988).
16. E. Koyoma, Y. Kikuchi, H. Echizen, K. Chiba and T. Ishizaki, *Ther. Drug Monit.* **15**, 224 (1993).
17. J. W. Hubbard, J. K. Cooper, E. M. Hawes, D. J. Jenden, P. R. A. May, M. Martin, G. McKay, T. Van Putten and K. K. Midha, *Ther. Drug Monit.* **7**, 222 (1985).
18. G. Lin, G. McKay, J. W. Hubbard and K. K. Midha, *J. Pharm. Sci.* **83**, 1412 (1994).
19. V. P. Shah, K. K. Midha, S. Dighe, I. J. McGilveray, J. P. Skelly, A. Yacobi, T. Layloff, C. T. Viswanathan, C. E. Cook, R. D. McDowall, K. A. Pittman and S. Spector, *Pharm. Res.* **9**, 588 (1992).