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DETERMINATION OF DIPHENHYDRAMINE IN BIOLOGICAL FLUIDS BY CAPILLARY GAS CHROMATOGRAPHY USING NITROGEN—PHOSPHORUS DETECTION

APPLICATION TO PLACENTAL TRANSFER STUDIES IN PREGNANT SHEEP

S.D. YOO and J.E. AXELSON*

Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, B.C. V6T 1W5 (Canada)

and

D.W. RURAK

Department of Obstetrics and Gynaecology, Faculty of Medicine, University of British Columbia, and Shaughnessy Research Center, Vancouver, B.C. V6T 1W5 (Canada)

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SUMMARY

A nitrogen—phosphorus detection—gas chromatographic method, which provides improved sensitivity and selectivity for diphenhydramine, is reported. A 25 m × 0.31 mm cross-linked, 5% phenylmethyl silicone-coated fused-silica capillary column (film thickness 0.52 μm) was used for all analyses. The splitless capillary injection mode was employed with a 2-μl sample being introduced by an automatic liquid sampler. Standard curves, using orphenadrine as an internal standard, were linear in the range 2–320 ng of diphenhydramine per 0.5 ml of sheep plasma. This represents an amount of diphenhydramine from ca. 40 pg to 6.4 ng at the detector. Chromatographic separation of diphenhydramine and orphenadrine was excellent, with no interference from endogenous plasma constituents. Applicability of the method was demonstrated by a placental transfer study in a chronically instrumented pregnant sheep following a 100 mg intravenous injection of diphenhydramine to the ewe.

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INTRODUCTION

Diphenhydramine hydrochloride, 2-(diphenylmethoxy)-N,N-dimethylethylamine (DPHM), is a potent and highly effective antihistaminic agent that posesses anticholinergic, antitussive, antiemetic and sedative properties [1, 2].

A number of analytical methods have been reported for the determination of DPHM in biological fluids including UV spectrometry [3], fluorescence dye techniques [4, 5], high-performance liquid chromatography (HPLC) [6], gas chromatography—mass spectrometry (GC—MS) [7, 8] and gas chromatography (GC) [9—16]. UV spectrometry and fluorescence dye methods lack specificity and sensitivity for trace level drug measurement. The HPLC method is convenient since no extraction procedure or derivatization step is required although sensitivity limitations require the use of relatively large volumes of plasma and injection of the entire sample volume into the chromatograph. GC methods employing conventional packed columns have been used for monitoring plasma concentrations in single-dose pharmacokinetic studies [9—12]. These methods require the use of plasma volumes larger than that normally collected during the study of placental transfer of drugs.

GC methods employing glass capillary columns and flame-ionization detection have recently been reported for the determination of several drugs, including DPHM [13, 14]. The combination of nitrogen—phosphorus-specific detection (NPD) with capillary GC has improved the detection limits for DPHM to facilitate DPHM pharmacokinetic studies in man [15, 16]. While these nitrogen-selective methods provide good sensitivity and selectivity, there are two significant limitations. Large plasma volumes (1.0—3.0 ml) and a very small final reconstitution volume (10 μl) for injection are required for the analysis.

In the present investigation of the placental transfer of DPHM in pregnant sheep, an assay with high sensitivity and selectivity is required for the small plasma volumes (0.5 ml) encountered during frequent serial blood sampling of the catheterized fetus and ewe. The aim of the present report is to describe a reproducible fused-silica capillary GC—NDP procedure with improved sensitivity and to demonstrate its applicability to the measurement of DPHM in sheep plasma.

EXPERIMENTAL

Materials

Diphenhydramine hydrochloride (Parke, Davis and Co., Montreal, Canada, Lot No. C429419) and orphenadrine hydrochloride (Pfaltz and Bauer, Stanford, CT, U.S.A., Lot No. D352) were used as reference standards. DPHM · HCl injectable 50 mg/ml (Benadryl® Injectable, 1-ml ampule) was obtained from the Hospital Pharmacy, Vancouver General Hospital (Vancouver, Canada). Triethylamine (TEA) was purchased from Pierce (Rockford, IL, U.S.A.). Toluene and dichloromethane, distilled in glass, were purchased from Caledon Labs. (Georgetown, Canada). A solution of 1 *M* sodium hydroxide was prepared from ACS reagent grade chemical (American Scientific Chemical, Seattle, WA, U.S.A.). Deionized, distilled water was used in the preparation of stock solutions throughout the analysis.

Stock solutions

DPHM · HCl (160 ng/ml, equivalent to base) and the internal standard, orphenadrine hydrochloride (1.0 μg/ml, equivalent to base) were prepared by dissolving these compounds in distilled water. The solutions were stored at 4°C for up to two months.

Instrumentation and chromatographic conditions

A Model 5830A Hewlett-Packard (Hewlett-Packard, Avondale, PA, U.S.A.) gas—liquid chromatograph equipped with a nitrogen—phosphorus-selective detector and a Model 18835B HP capillary inlet system were used for all analyses. A Model 7671A HP automatic sampler was used for sample injection into the gas chromatograph.

A 25 m × 0.31 mm I.D. cross-linked fused-silica capillary column (5% phenylmethyl silicone, film thickness 0.52 μm, Hewlett-Packard) was used in the analysis. The splitless injection mode employing a fused-silica insert (78 mm × 2 mm I.D.) was used, with a 2-μl sample being injected. Thermogreen® LB-2 silicone rubber septa (Supelco, Bellefonte, PA, U.S.A.) were used to provide low bleed at high inlet temperatures. The operating conditions for routine analysis were: injection port temperature, 250°C; initial oven temperature, 190°C; programming rate, 2°C/min at 1 min and 5°C/min at 8.5 min; final oven temperature, 240°C; nitrogen—phosphorus-selective detector temperature, 300°C; carrier gas (helium) flow-rate, 1.0 ml/min; septum purge flow-rate, 0.6 ml/min; make-up gas flow-rate, 2 ml/min; hydrogen/air flow-rate ratio, 3:50.

Extraction

To 0.3—0.5 ml of plasma were added 0.2 ml of internal standard (1.0 μg/ml) and 0.5 ml of 1 *M* sodium hydroxide in a 15-ml disposable culture tube with a screw cap lined with polytetrafluoroethylene (PTFE). The aqueous phase was adjusted to a total volume of 2.5 ml (pH ca. 13) with distilled water and mixed on a vortex mixer. To this mixture, 6.0 ml of 0.05 *M* TEA in dichloromethane were added and the aqueous phase was extracted by shaking the mixture for 20 min on a rotary shaker (Labquake Tube Shaker, Model 415-110; Lab Industries, Berkeley, CA, U.S.A.). The samples were placed in a freezer (−20°C) for 5 min and then centrifuged at 2300 *g* for 10 min. After centrifugation, the aqueous layer was aspirated and discarded. The remaining organic layer was transferred to a clean 15-ml culture tube and dried under a gentle stream of nitrogen in a 30°C water bath. The residue was reconstituted to a volume of 100 μl with toluene. The sample was mixed on a vortex mixer for 10 s. Aliquots of 2 μl were used for GC—NPD analysis.

Preparation of the calibration curve

A 0.5-ml sample of blank sheep plasma was spiked with varying amounts of DPHM (2, 4, 8, 16, 32, 64, 128, 192, 256 and 320 ng) using the prepared stock solution (160 ng/ml) and diluted stock solution (20 ng/ml), and then 0.2 ml of the orphenadrine solution (1.0 μg/ml) and 0.5 ml of 0.5 *M* sodium hydroxide were added. The aqueous phase was adjusted to a total volume of 2.5 ml (pH ca. 13) with distilled water and the samples were extracted as described above.

Quantitative estimation of DPHM in plasma was accomplished by plotting the area ratios of DPHM and orphenadrine against the range of indicated DPHM concentrations. For a DPHM recovery study, DPHM was dissolved in methanol and the solution further diluted to provide concentrations ranging from 2 to 320 ng/ml. The amount of DPHM extracted from plasma by methylene chloride was calculated using calibration curves obtained from direct injection of the prepared methanolic solutions.

Gas chromatography—mass spectrometry

Capillary GC electron-impact mass spectrometry (EI-MS) and chemical ionization mass spectrometry (CI-MS) were carried out using a Model 5987A HP gas chromatograph—mass spectrometer equipped with a 25 m × 0.32 mm I.D. (0.25 μm film thickness) OV-1701 fused-silica column (Quadrex). Methane was used as the ionizing gas and the splitless injection mode was employed in the study. The following conditions were used for GC—MS of DPHM: initial oven temperature, 50°C; time 1, 0 min; rate, 30°C/min; final oven temperture, 260°C; time 2, 10 min; injection port temperature, 240°C; carrier gas (helium) flow-rate, 1.0 ml/min; electron multiplier voltage, 2200 V; emission current, 300 μA.

Sheep experiment

A preliminary experiment on the placental transfer of DPHM was carried out in a pregnant ewe chronically implanted with maternal and fetal vascular catheters [17]. A 100-mg intravenous dose of DPHM · HCl was administered over a 30-s period via a maternal femoral venous catheter and the catheter flushed with 3.0 ml of heparinized saline. Blood samples for DPHM determination were simultaneously withdrawn from the maternal and fetal femoral arterial catheters at 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300 and 360 min. The blood samples were transferred to heparinized Vacutainer® collection tubes (Becton-Dickinson, Mississauga, Canada) and immediately centrifuged. Following centrifugation, the plasma was transferred to a 15-ml PTFE-lined screw-capped disposable culture tube, and stored at −20°C until extracted and analyzed.

RESULTS

Representative GC chromatograms obtained from blank plasma and a plasma sample from a DPHM-treated pregnant sheep are shown in Fig. 1. Extraneous peaks from endogenous plasma constituents were negligible and did not interfere with the analysis (Fig. 1A). The peaks with retention times (t_R) of 7.85 and 9.31 min were identified as DPHM and orphenadrine, respectively.

The data for a representative calibration curve used in the quantitation of DPHM in maternal and fetal sheep plasma are presented in Table I. Linearity was observed over the concentration range studied (2—320 ng/ml) with the line of best fit being described by $y = 0.0050x + 0.0004$ with a correlation coefficient (r) of 0.9996. An average DPHM recovery of 103.19% (coefficient of variation, 5.14%) was obtained over the concentration range studied.

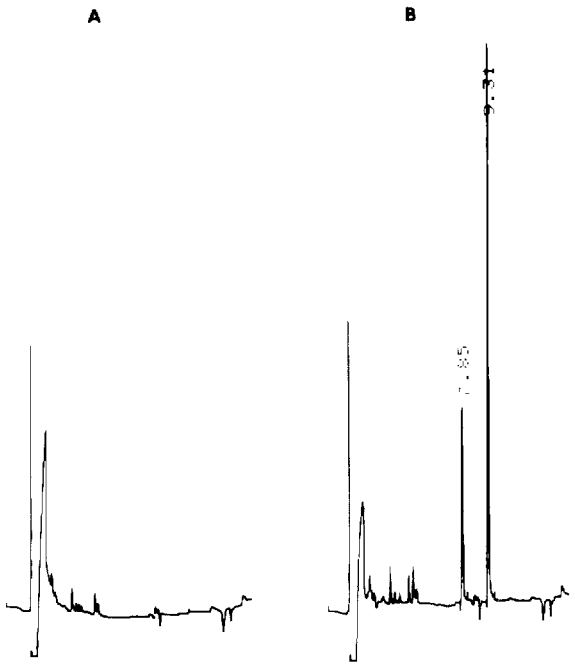

Fig. 1. Representative capillary gas chromatograms obtained from blank sheep plasma (A) and a plasma sample from a pregnant sheep (B) after administration of 100 mg of diphenhydramine hydrochloride intravenously. The plasma sample from the drug-treated sheep contained diphenhydramine (t_R = 7.85 min) and spiked internal standard, orphenadrine, (t_R = 9.31 min). Attenuation 2^5, nitrogen-phosphorus detector voltage 14 V.

TABLE I

CALIBRATION CURVE DATA FOR SHEEP PLASMA

Linear regression line for diphenhydramine: $y = 0.0050x + 0.0004$, $r = 0.9996$.

Amount of DPHM spiked (ng)	Diphenhydramine/orphenadrine area ratio* (mean ± 1S.D.)	Coefficient of variation (%)
2	0.010 ± 0.001	10.00
4	0.018 ± 0.001	5.56
8	0.037 ± 0.003	8.11
16	0.080 ± 0.006	7.50
32	0.152 ± 0.010	6.58
64	0.324 ± 0.025	7.72
128	0.662 ± 0.038	5.74
192	0.956 ± 0.024	2.51
256	1.238 ± 0.047	3.80
320	1.609 ± 0.024	1.49

*Four samples, mean of two injections.

Pure authentic DPHM samples, DPHM-spiked control sheep plasma and plasma samples collected from DPHM-treated sheep have been examined using GC—MS techniques. The total-ion current mass chromatograms for DPHM

obtained from GC—MS studies of these samples all showed a single peak for DPHM in the positive ionization mode, providing evidence for the purity of the compound. Confirmation of the identity of DPHM was obtained using both EI and CI mass spectra from authentic drug and drug-spiked plasma. When CI mass spectra were obtained and evaluated throughout the DPHM peak elution from dosed sheep plasma, the results also confirmed the identity of DPHM and peak homogeneity. Prominent ion fragments for DPHM were observed at *m/e* 167, 256, 209 and 254 in the CI mode and at *m/e* 58, 73, 167, 165, 45, 168, 44 and 42 in the EI mode. The fragmentation patterns of DPHM were shown to be similar with those reported earlier [18].

Semilogarithmic plots of DPHM concentration in maternal and fetal sheep plasma versus time following administration of the 100 mg intravenous dose to the ewe are shown in Fig. 2. Initial estimates of the pharmacokinetic parameters for DPHM after an intravenous dose were obtained by computer analysis using the program AUTOAN [19]. The data were later fitted using the program NONLIN [20] and these initial estimates. The decline in the maternal and fetal plasma DPHM concentration with time was observed to follow a biexponential decay described by the following equation: $C_p = Ae^{-\alpha t}$

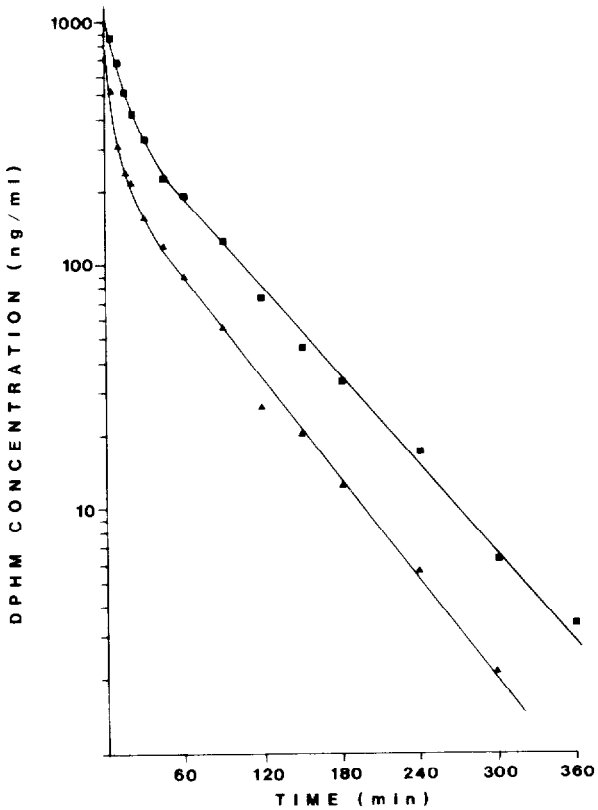

Fig. 2. A semilogarithmic plot of the diphenhydramine concentration versus time profiles obtained in maternal (■) and fetal (▲) plasma following a 100 mg intravenous dose to a pregnant ewe. The terminal elimination half-life was calculated to be 50 and 46 min in the ewe and fetus, respectively.

+ $Be^{-\beta t}$, where C_p is the plasma concentration at any time, t. A and B are the intercepts of the α and β phases, respectively. The parameters α and β represent the distribution and terminal elimination rate constants, respectively. The maternal half-life of the α phase ($t_{\frac{1}{2},\alpha}$) was calculated to be 8 min, indicating rapid distribution of DPHM following administration. A terminal elimination half-life ($t_{\frac{1}{2},\beta}$) of 50 min was calculated for DPHM in maternal plasma. The placental transfer of DPHM to the fetus was rapid, with peak fetal plasma concentrations being attained in less than 5 min following drug administration. The fetal DPHM decay curve followed a pattern similar to that observed in maternal plasma. Distribution and terminal elimination half-lives of 7 and 46 min were calculated for DPHM in the fetus.

DISCUSSION

Open tubular capillary column GC has been extensively used as an analytical tool in the analysis of complex matrices, such as environmental samples, fossil fuels, food and cosmetics, and biological materials.

Several glass capillary GC—NPD methods [13, 14] have been reported for the determination of DPHM in street drug samples. Although glass capillary columns are considerably more inert than those of metal, they still possess significant column surface activity leading to absorptive interactions with the substances being chromatographed. The recent introduction of inert fused-silica capillary columns minimizes these problems [21], providing reproducible and highly sensitive analyses of a variety of drugs and their metabolites in tissues and biological fluids. As a consequence, these methods are being more frequently used for pharmacokinetic studies in man and animals, e.g. during placental transfer and neonatal kinetic studies, where small plasma volumes (0.5 ml or less) are frequently encountered.

Lutz et al. [16] have reported a GC method for DPHM measurement in human serum using a bonded-phase fused-silica capillary column. Meatherall and Guay [15] have also reported a GC—NPD method for the determination of DPHM employing direct sample injection onto a fused-silica capillary column. These methods provide good sensitivity and selectivity, however, they require the use of large plasma volumes (1.0–3.0 ml) and a very small final reconstitution volume (10 μl). The direct or on-column sample injection technique used by Meatherall and Guay [15] has been considered to be superior to split or splitless injection techniques in terms of precision and accuracy of quantitative analysis [22—24]. This injection mode is still, however, not readily amenable to automation owing to technical reasons thereby limiting its use for routine drug analysis and pharmacokinetic studies.

In the present study we report a GC—NPD method with improved sensitivity for a single-dose DPHM pharmacokinetic study in pregnant sheep. Splitless injection was employed to facilitate the automation of sample analysis. This injection mode has been found to be useful for trace analysis of dilute samples without pre-concentration and for samples with components eluting near the solvent peak [25—27]. While packing the injection port with glass wool can frequently improve volatilization and therefore apparent detector response, this technique was not used to avoid possible adsorption and injection port

discrimination of sample components. A purge activation time of 1 min was chosen since longer purge activation time did not provide increased peak area counts suggesting no further transfer of sample onto the column. As isothermal column operation caused peak broadening, multi-step temperature programming was used to improve peak shape and the resolution of the drug and internal standard.

Orphenadrine hydrochloride was chosen as an internal standard because of its structural similarity to DPHM. Resolution of DPHM and orphenadrine is excellent, as illustrated in Fig. 1B and no interference from endogenous sheep plasma components was observed (Fig. 1A). The developed assay method has been found to show good linearity over the concentration range studied (Table I). Within-run precision (repeatability) of a representative calibration curve showed good reproducibility with coefficients of variation ranging from 1.49 to 10.00% (Table I).

Adsorption of DPHM from the organic solvent, by glass surfaces, was observed to be a major source of drug loss during assay development. Various solvents including heptane, hexane, toluene, benzene and dichloromethane, in order of increasing polarity, were examined for their relative extraction efficiency. Dichloromethane was found to be the most efficient solvent for single-step plasma extraction of DPHM, providing low-level drug quantitation without any interference from plasma constituents. The addition of TEA in dichloromethane (0.01 *M*) minimized drug adsorption losses during extraction and evaporation processes.

The formation of an emulsion between plasma and dichloromethane during extraction occurred, but was effectively broken by placing samples in a freezer (−20°C) for 5 min prior to centrifugation. Since chlorinated solvents cause a reversible nitrogen–phosphorus detector sensitivity loss, toluene was used instead of dichloromethane for the reconstitution of the plasma extracts for sample injection.

A significant carry-over of DPHM and orphenadrine from the automatic sampler syringe needle was observed when toluene was used to wash the syringe. Among various solvents examined, acetone was found to eliminate this carry-over problem when the automatic sampler performed four wash cycles. The use of automatic liquid sample introduction has made it possible to perform routine analysis for a DPHM pharmacokinetic study.

The applicability of the method to the analysis of sheep plasma samples (0.3–0.5 ml) is illustrated in Fig. 2. A biexponential time course for the elimination of DPHM from maternal and fetal plasma was observed in the preliminary sheep experiment. The half-life of DPHM was 50 min in pregnant ewe and 46 min in the fetus (gestational age 129 days). These values are considerably lower than the half-life obtained in man (4.1–9.3 h) [12, 28, 29] but similar to other species, such as rabbits (1.6 h) [30], rhesus monkey (1 h) [31], and dog (1 h) [31]. The sensitivity limit of the present method (ca. 40 pg at the detector) has been found to be suitable for a preliminary pharmacokinetic assessment of plasma DPHM concentration versus time profiles in chronically catheterized maternal and fetal sheep.

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