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Determination of ritodrine in biological fluids of the pregnant sheep by fused-silica capillary gas chromatography using electron-capture detection

M. R. WRIGHT

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

J. E. AXELSON*

Division of Biopharmaceutics and Pharmaceutics, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, B.C. V6T 1W5 (Canada) and Department of Obstetrics and Gynaecology, The Children's Variety Research Centre, Faculty of Medicine, The University of British Columbia, 950 West 28th Avenue, Vancouver, B.C. V5Z 4H4 (Canada)*

F. S. ABBOTT

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

K. W. RIGGS

Division of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, B.C. V6T 1W5 (Canada)

and

M. P. VAN DER WEYDE, S. M. TAYLOR, G. H. McMORLAND and D. W. RURAK

Department of Obstetrics and Gynaecology, The Children's Variety Research Centre, Faculty of Medicine, The University of British Columbia, 950 West 28th Avenue, Vancouver, B.C. V5Z 4H4 (Canada)

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ABSTRACT

A sensitive and selective gas chromatographic assay method employing splitless injection, fused-silica capillary columns and electron-capture detection is reported for the quantitation of the tocolytic drug, ritodrine, in a variety of biological fluids obtained from the pregnant ewe and fetus. This method has improved sensitivity and selectivity over previously published assay procedures. A 25 m × 0.31 mm I.D., cross-linked 5% phenylmethylsilicone, fused-silica capillary column was employed for all analyses. Linearity of response was observed over the range 2.5–75 ng of ritodrine base per 0.05–0.5 ml of biological fluid, representing ≈1–75 pg at the detector. The coefficient of variation was less than 10% over the range 2.5–75 ng of added ritodrine. The minimum quantifiable amount is ≈2.5 ng from a 0.5-ml biological fluid sample. Applicability of this method to biological fluids, obtained from ovine subjects, is demonstrated by the analysis of samples obtained during the course of ritodrine placental transfer studies.

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INTRODUCTION

Ritodrine hydrochloride (Yutopar® (*erythro-p*-hydroxy-α[1-[(*p*-hydroxyphenethyl)amino]ethyl]benzyl alcohol hydrochloride) is a potent β_2-adrenoreceptor agonist widely employed as a tocolytic agent. Little detailed study has been made of the pharmacokinetics of this drug in pregnancy or its placental transfer. Several assay procedures have been published previously but these are generally unsuitable for the study of the placental transfer of this drug due to limited sensitivity and/or selectivity [1–4]. The study of ritodrine maternal/fetal kinetics *in utero* and in the neonate requires an assay with high sensitivity and specificity for repetitive sampling where only small volumes (< 1 ml) are available (*e.g.* fetal and neonatal plasma).

Thomas *et al.* [1] reported the development of a radioimmunoassay for ritodrine. While this method displays reasonable sensitivity (~ 0.1 ng/ml from 100-μl serum samples), precision of measurement is poor (coefficient of variation, $> 10\%$ below 2.5 ng/ml) and the kinetic parameters obtained in studies using this assay have shown large inter-subject variability [2]. This variability has been ascribed to cross-reactivity of the anti-sera with endogenous substances [2].

Lin *et al.* [2] and Kuhnert *et al.* [3] developed high-performance liquid chromatographic (HPLC) methods which employ electrochemical detection of the oxidation of the phenolic hydroxyl groups of ritodrine. Although both methods show acceptable selectivity, the sample volume required in both instances (≥ 1 ml) is impractical for repetitive sampling where fluid volume is limited. Gross *et al.* [4] have recently reported an HPLC method using the native fluorescence of ritodrine. Although the selectivity of this method is excellent it requires large sample volumes for extraction (> 1 ml plasma) and a small reconstitution volume (100 μl) which does not allow for multiple injections of the same sample.

The purpose of this study was to develop a sensitive and selective analytical method capable of reproducibly determining ritodrine concentrations from a wide variety of biological fluids (*e.g.* plasma, tracheal, amniotic and allantoic fluids) obtained from pregnant sheep during the course of placental transfer studies with sample volumes limited to ≤ 0.5 ml.

EXPERIMENTAL

Reagents and materials

Ritodrine hydrochloride ($> 99.5\%$ pure) and ritodrine free base ($> 95\%$ pure) were generously provided by Duphar (Weesp, The Netherlands). The internal standard, 5-hydroxypropafenone, was a gift from Knoll Pharmaceuticals Canada (Mississauga, Canada). The identity of all compounds was confirmed using 300-Mhz ^{1}H nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS) and differential scanning calorimetry (DSC). The solvents and fine chemicals — ethyl acetate (Omnisolv®), methanol (Omnisolv), potassi-

um carbonate, sodium bicarbonate, disodium hydrogen orthophosphate, and sodium dihydrogen orthophosphate (all analytical grade) — were obtained from BDH (Toronto, Canada). Heptafluorobutyric anhydride (HFBA) and triethylamine (TEA) (sequanal grade) were obtained from Pierce (Rockville, IL, U.S.A.). Toluene (distilled-in-glass) was obtained from Caledon Labs. (Georgetown, Canada). Deionized, high-purity water was produced on-site by reverse osmosis using a Milli-Q water system (Millipore, Mississauga, Canada).

Stock solutions

Ritodrine hydrochloride (100 ng/ml, equivalent to base) and 5-hydroxypropafenone (200 ng/ml, equivalent to base) were prepared by dissolving these compounds in deionized water. Both solutions were stored at 4°C for up to one month without evidence of degradation.

Instrumentation and chromatographic conditions

A Hewlett Packard (Avondale, PA, U.S.A.) Model 5890 gas chromatograph equipped with a Model 7673 autosampler, split–splitless capillary inlet system, Model 5895 GC workstation and an electron-capture detector was used for all analyses.

A Hewlett Packard 25 m × 0.31 mm I.D. cross-linked fused-silica capillary column (5% phenylmethylsilicone, film thickness 0.52 μm, Hewlett Packard) was used for all analyses. The splitless injection mode, using a pyrex glass inlet liner (78 mm × 4 mm I.D.) and Thermogreen® LB-2 silicone rubber septa (Supelco, Bellefonte, PA, U.S.A.) was employed to introduce a 1-μl sample volume onto the analytical column. The operating conditions were: injection port temperature, 210°C; initial column temperature, 145°C (held for 3 min); temperature programming rates, 15°C/min (to 190°C and then held for 1 min), 5°C/min (to 220°C) and 50°C/min (to 280°C and then held for 1 min); electron-capture detector temperature, 350°C; carrier gas flow-rate (hydrogen), 2 ml/min; make-up gas flow-rate (argon–methane, 95:5), 60 ml/min.

Extraction and derivative formation

To a 10-ml disposable culture tube with a polytetrafluoroethylene (PTFE)-lined screw cap were added 0.05–0.5 ml of biological fluid (either maternal or fetal plasma, amniotic fluid or fetal tracheal fluid), 0.5 ml of the internal standard (200 ng/ml), an aliquot (0.95–0.5 ml) of deionized water and 0.5 ml of 1 *M* carbonate buffer (pH 9.5) (final pH of the aqueous phase was $\approx$9.5). The mixture was gently vortex-mixed and 6 ml ethyl acetate were added. The aqueous phase was extracted for 20 min on a rotary shaker (Labquake tube shaker, Model 415-110, Lab Industries, Berkeley, CA, U.S.A.). The samples were then placed in a freezer at −20°C for 15 min to facilitate breakage of any emulsion formed in the extraction step. This cooling was followed by two centrifugations of 5 min duration at 2300 *g*. The organic layer was transferred to clean 15-ml disposable tubes and evap-

orated to dryness in a water bath (30°C) under a gentle stream of nitrogen. The residue was reconstituted in 0.4 ml of 0.0125 *M* TEA in toluene. A 20-μl volume of HFBA was added to each tube, the tube vortex-mixed and then placed in an oven at 55°C for 60 min. The samples were allowed to cool to room temperature and the excess derivatizing reagent was neutralized with 2 ml (0.067 *M*) phosphate buffer (pH 6) (vortex-mixing for 30 s). Following centrifugation (2300 *g*) for 1 min the organic layer was transferred to automatic sampler injection vials (Hewlett Packard) and diluted to 1 ml with toluene. Aliquots of 1 μl were injected into the GC apparatus.

Absolute recovery study

Ritodrine free base was dissolved in distilled water and methanol, respectively, to provide solutions of 100 ng/ml. Various volumes representing 75, 50, 25, 10, 5 and 2.5 ng of ritodrine free base were transferred into two sets of glass tubes, respectively. To the set of ritodrine in water tubes was added blank maternal sheep plasma followed by extraction as outlined previously. The internal standard, 5-hydroxypropafenone, dissolved in methanol, was then added (500 ng) to both sets of samples. The two sets of samples were then subjected to the same derivatization reaction. A standard curve, relating the peak-area ratio of the ritodrine derivative to the internal standard derivative *versus* the amount of added ritodrine base, from the samples dissolved in methanol was then produced. The area ratios from the extracted samples were then determined and the amount of ritodrine calculated from the standard curve.

Preparation of the calibration curves

Serial amounts of the prepared ritodrine hydrochloride stock solution (75, 50, 25, 10, 5 and 2.5 ng) were added in a fixed volume (1 ml) to either 0.1-, 0.25- or 0.5-ml samples of blank pregnant sheep biofluid (either maternal or fetal arterial plasma, amniotic fluid or fetal tracheal fluid). A 0.5-ml aliquot of internal standard solution (5-hydroxypropafenone, 200 ng/ml) was added and the samples were then extracted and derivatized as previously described. All samples were prepared in duplicate and a standard curve, in the appropriate biofluid was prepared daily. Determination of ritodrine concentrations was made by plotting the peak-area ratios of the heptafluorobutyryl derivatives of ritodrine and 5-hydroxypropafenone against the known amount of ritodrine added to each sample.

Gas chromatography–mass spectrometry

Capillary GC electron-impact (EI), positive and negative chemical ionization (PCI, NCI) were carried out using a Hewlett Packard Model 5987A quadrupole GC–MS system. Chromatographic conditions were as described previously except that helium was used as the carrier gas. In the chemical ionization mode, methane was used as the reagent gas. The MS operating conditions were: electron ionization energy, 70, 110 and 130 eV, respectively, for the EI, PCI and NCI modes; emission current, 0.3 mA; ion source temperature, 240°C.

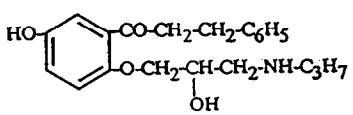
Sheep experiments

A study of the placental transfer and maternal and fetal effects of ritodrine, following maternal intravenous bolus administration (50 mg ritodrine hydrochloride), was carried out in a pregnant ewe with vascular cathethers implanted in the mother and fetus [5]. Samples of fetal arterial (1.5 ml), maternal arterial (3.0 ml), umbilical venous (1.5 ml) and uterine venous (3.0 ml) blood as well as amniotic (3.0 ml) and fetal tracheal (3.0 ml) fluids were drawn at −15, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 480, 600, 720 and 1440 min relative to the dose being given. All blood samples were immediately centrifuged and the plasma was transferred to PTFE-lined screw-cap disposable culture tubes and stored at −20°C until analysis. All other biofluids (amniotic and fetal tracheal fluids) were immediately transferred to PTFE-lined screw-cap disposable culture tubes and stored at −20°C until analysis.

RESULTS AND DISCUSSION

To date, there has been no GC method reported for the quantitation of ritodrine from biological fluids. The assay method presented here has advantages in sensitivity, selectivity and reproducibility over previously published techniques [1–4]. Fig. 1 shows the structures of ritodrine and the internal standard, 5-hydroxypropafenone. Fig. 2 illustrates representative chromatograms obtained from ovine biological fluids. No interference occurs from endogenous substances with either the drug (peak 1; retention time ~11.1 min) or internal standard (peak 2; retention time ~17.6 min).

During optimization of the extraction conditions for this assay, a number of solvents were examined (*viz.*, toluene, benzene, hexane, dichloromethane, ethyl acetate, methyl ethyl ketone and 1-butanol), with ethyl acetate providing the highest relative extraction ratio of all the tested solvents. In an effort to obtain



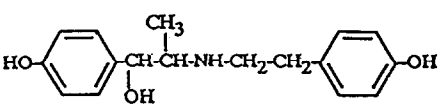


Fig. 1. Structures of 5-hydroxypropafenone (top) and ritodrine (bottom).

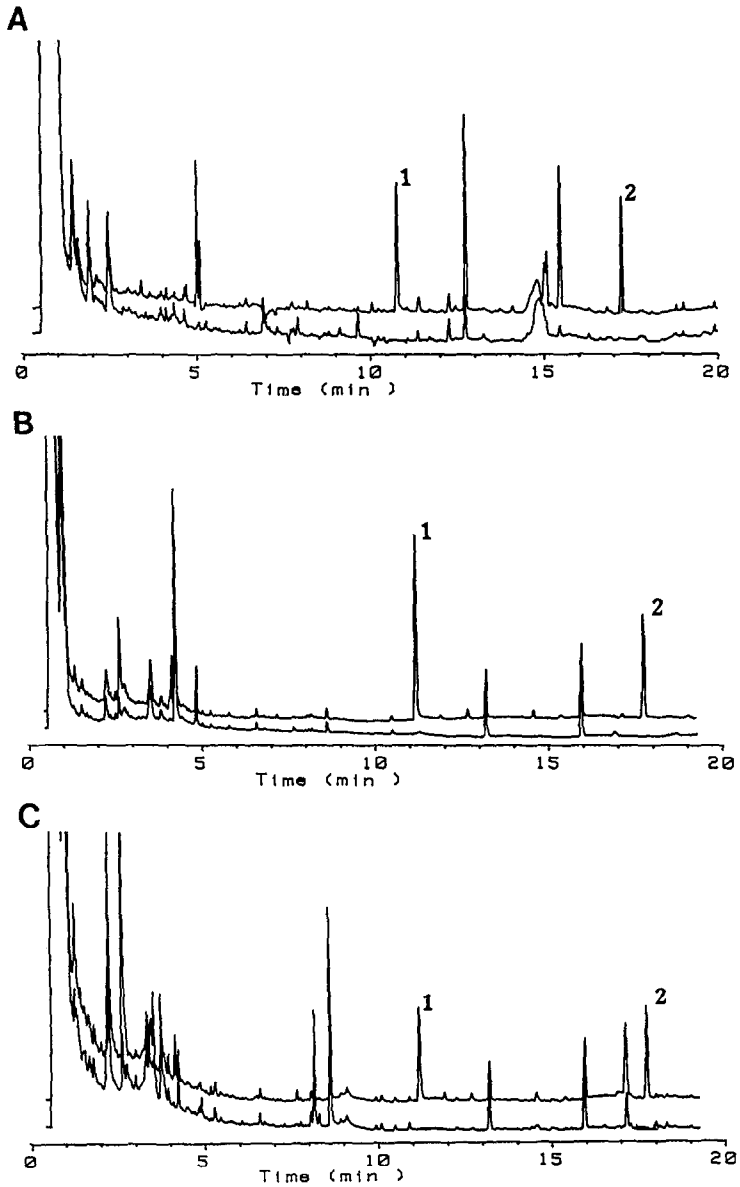


Fig. 2. Representative chromatograms from various biological fluids obtained from pregnant sheep (blank, bottom trace; following drug administration, top trace). (A) Maternal plasma; (B) fetal tracheal fluid; (C) amniotic fluid. The peaks labelled 1 and 2 are the HFB derivatives of ritodrine and 5-hydroxypropafenone, respectively.

improved selectivity of extraction (*i.e.* to minimize co-extraction of endogenous compounds) various mixtures of ethyl acetate–toluene–isopropyl alcohol were examined. None of these mixtures offered significant improvements in selectivity

over ethyl acetate and all showed lower relative extraction of ritodrine. Ritodrine adsorption onto glass surfaces does not appear to be a major source of drug loss during extraction since addition of TEA to ethyl acetate in concentrations ranging from 0.001 to 0.5 *M* did not significantly improve ritodrine recovery. As suggested by Lin *et al.* [2], the extractability of ritodrine is highly pH-sensitive and the attainment and maintenance of pH 9.5 is crucial to optimal extraction and reproducibility (the relative recovery at pH 9.2 is ≈33% and at pH 10 is ≈60%). Previously published assay methods recommended extraction for periods of less than 20 min [2–4]. In the present study, however, extraction for 5, 10, 15, 20 and 30 min of uniform concentrations of drug and internal standard followed by derivatization showed that optimal recovery occurred after 20 min. The absolute recovery of ritodrine by the present assay method is 62 ± 5% which is similar to that obtained by Gross *et al.* [4] (68%) but slightly lower than that reported by Lin *et al.* [2] and Kuhnert *et al.* [3] (see Fig. 3). The absolute recovery of ritodrine may be increased to 81 ± 5.5% by re-extraction of the aqueous phase with 5 ml of ethyl acetate for 20 min followed by two centrifugations at 2300 *g* for 5 min. The organic layers were then pooled, evaporated and derivatized, as previously described. Improving the recovery in this manner, however, resulted in a ≈20% increase in the coefficient of variation at each sample concentration measured.

Underivatized ritodrine does not possess significant electron-capture properties, therefore, derivatization is necessary to maximize detector sensitivity. The molecular structures of both the β-agonists and β-antagonists when derivatized often result in compounds detectable in very low amounts [6]. In the development of the present assay both trifluoracetic anhydride (TFAA) and HFBA were examined for the preparation of a suitable derivative. It has been reported for the congener of ritodrine, isoxuprine, that the TFAA derivative and HFBA derivative provide similar sensitivity and also that TFAA reacts less readily with co-extracted endogenous substances [7]. HFBA was selected based on the observa-

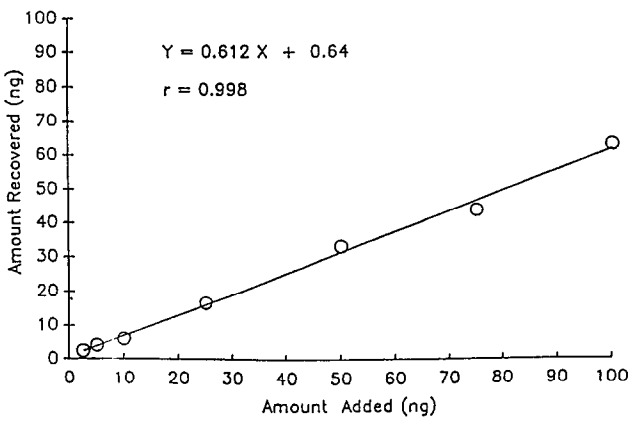


Fig. 3. Absolute recovery of ritodrine from maternal sheep plasma (mean ± S.D., $n = 3$).

tions that it increased detector response 10–100 fold over the TFAA derivative with no appreciable differences in reactivity with co-extracted solutes. Reaction conditions for the derivatization reaction and removal of excess derivatizing reagent were optimized following the procedures established by Walle and Ehrsson [8] and Ehrsson *et al.* [9].

The HFBA derivative of 5-hydroxypropafenone shown in Fig. 4 has been previously confirmed by EI GC–MS [10]. Although details of the EI, PCI and fast atom bombardment (FAB) mass spectra of ritodrine following isolation via HPLC have been previously published [2,11], there have been no reports of the mass spectra of ritodrine derivatized by acylating reagents. In the development of this assay method both of the derivatives of ritodrine formed via acylation with TFAA and HFBA were characterized by EI, PCI and NCI GC–MS. Following derivatization with TFAA both GC–electron-capture detection (ECD) and GC–MS demonstrate the formation of a single species. Under NCI conditions this peak showed a molecular ion of m/z 671 a.m.u. which corresponds to a tetra-TFAA substituted derivative of ritodrine. Since the mass range of the quadrupole mass spectrometer employed was limited to 0–1000 a.m.u., it was not possible to observe a molecular or pseudo-molecular ion (m/z 1071) which would have been expected due to the formation of a similar tetra-substituted HFBA compound. Following derivatization with HFBA, analysis by GC–ECD and GC–MS demonstrated the formation of a single species as evidenced by a single, symmetrical and sharp peak in the GC–ECD pattern and also by a similar total ion chromatogram (GC–MS) demonstrating one symmetrical peak. The fragmentation patterns of HFBA derivatized ritodrine are shown in Fig. 4. Fragmentation in both EI and CI modes was extensive and complex due to multiple losses of all or part of the heptafluorobutyryl group (*viz.* F_7C_3COO, m/z = 213; F_7C_3CO, m/z = 197; F_7C_3, m/z = 169). Although no molecular ion could be observed, it is proposed that the derivative formed is tetra-substituted due to the similar size and spectrum of chemical reactivity of TFAA and HFBA as well as the characteristic fragments formed (m/z 542 and 548) under NCI conditions. The fragmentation patterns observed with the HFBA derivative were similar to those observed from the TFAA derivatization. Additionally, the EI fragmentation patterns observed in this study closely resemble those seen by Cova *et al.* [7] in their GC–MS analysis of TFAA-derivatized isoxuprine, a close congener of ritodrine.

In the present study a significant 'cold-trapping' effect was obtained by holding the initial column temperature at 145°C. Lower inlet temperatures resulted in significant band spreading of the solvent peak while significantly higher temperatures (*i.e.* >200°C) decreased the degree to which sample reconcentration was obtained. Since most commonly employed GC solvents expand 200–450 times during vaporization, the inlet liner used must have a volume large enough to contain the volatilized solvent [12]. The use of a 4 mm I.D. inlet liner improved both the sensitivity and reproducibility compared to that obtained with a more conventional 2 mm I.D. liner. In addition, the use of a 1-μl injection volume provided the best reproducibility.

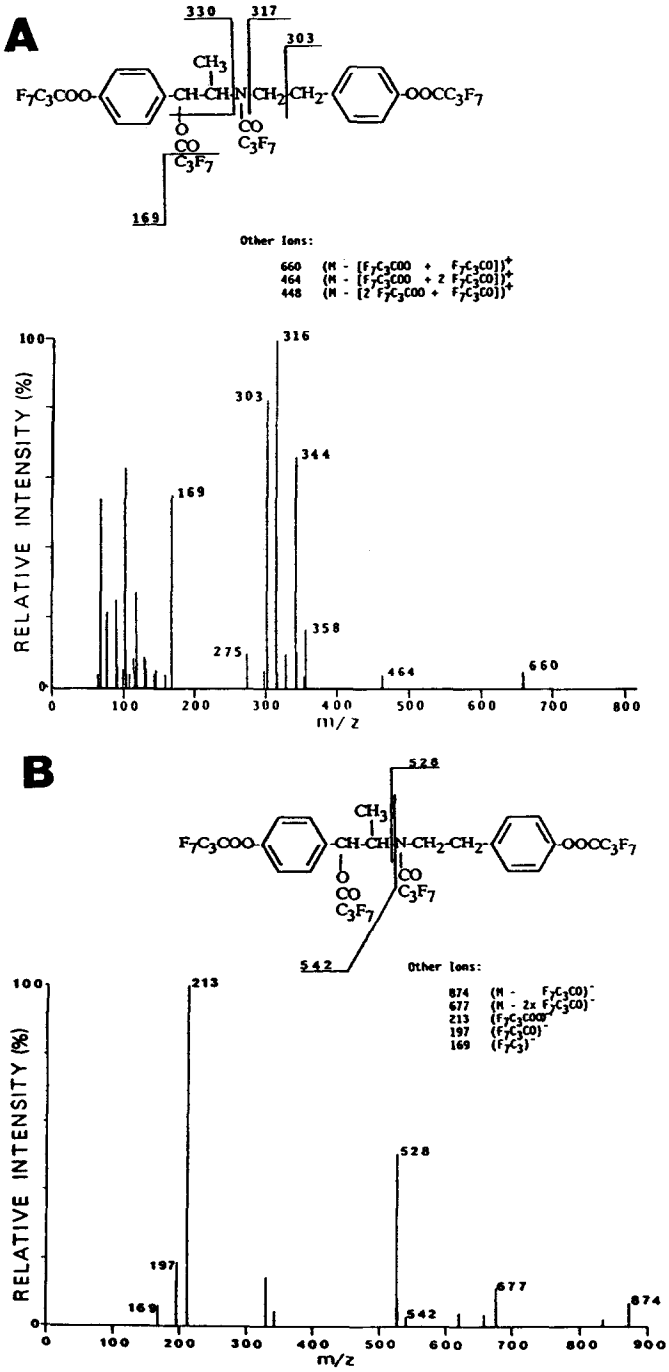


Fig. 4.

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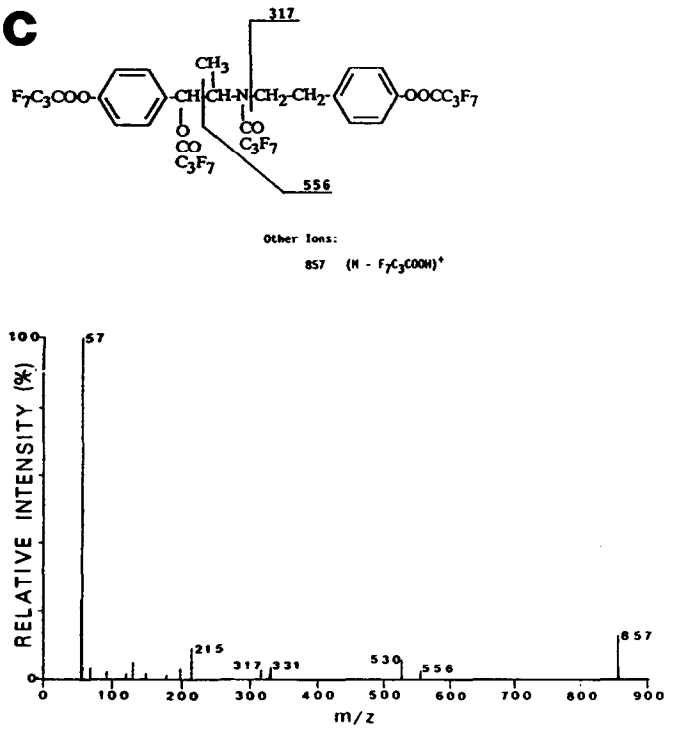

Fig. 4. GC–MS fragmentation patterns of the heptafluorobutyric anhydride derivatives of ritodrine. (A) Electron-impact ionization; (B) negative chemical ionization; (C) positive chemical ionization.

TABLE I

CALIBRATION CURVE FOR SHEEP PLASMA

n = 7–8. Linear regression statistics: $y = 0.0185\,x + 0.0534$; $r = 0.997$; $r^2 = 0.994$.

Ritodrine added[a] (ng)	Area ratio[b] (mean ± 1 S.D.)	Coefficient of variation (%)
2.50	0.0725 ± 0.0075	10.30
5.00	0.1292 ± 0.0095	7.32
10.00	0.2268 ± 0.0200	8.89
25.00	0.5714 ± 0.0380	6.66
50.00	1.0245 ± 0.0340	3.30
75.00	1.3961 ± 0.0384	2.75

[a] Calculated as free base.

[b] Area ratio, drug/internal standard ± 1 standard deviation.

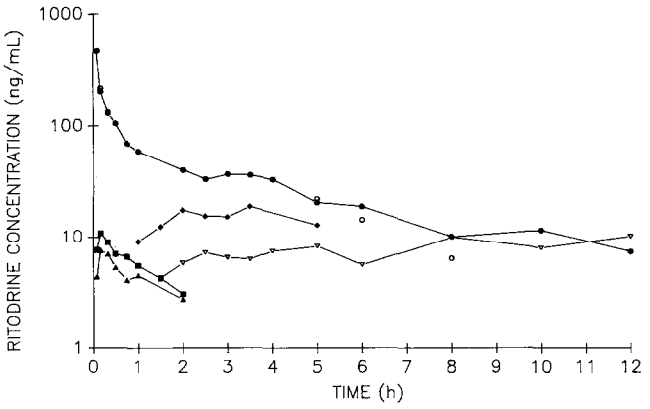

Fig. 5. Ritodrine concentration *versus* time profiles in the ewe and fetus following a 50-mg intravenous bolus dose to the mother. (●) Maternal arterial plasma; (○) maternal uterine venous plasma; (▲) fetal arterial plasma; (■) fetal umbilical venous plasma; (▽) amniotic fluid; (◆) tracheal fluid.

The data for a representative calibration curve used in the quantitation of ritodrine from maternal and fetal sheep plasma are presented in Table I. Linearity is observed over the concentration range studied (2.5–75 ng) with a coefficient of variation $< 10\%$ at all points.

Representative semilogarithmic plots of the ritodrine concentrations in maternal and fetal fluids are shown in Fig. 5. Maternal ritodrine elimination was observed to follow a triexponential decay described by the equation: $C_p(t) = P e^{-\pi t} + A e^{-\alpha t} + B e^{-\beta t}$ where $C_p(t)$ is the observed plasma concentration at time t. The parameters π and α represent distributional rate constants while β represents the terminal elimination rate constant. The parameters P, A and B are the intercepts of each phase. The apparent terminal elimination half-lives were 3.91 and 1.42 h from maternal and fetal arterial plasma, respectively. Fetal exposure, as calculated by the ratio of the area under the plasma concentration *versus* time curve for fetal arterial plasma to that of maternal arterial plasma, was very low ($\sim 3\%$) following maternal dosing. Peak fetal plasma concentrations were reached at 10 min indicating some delay in maternal–fetal transfer. There was also an apparent delay in the appearance of drug in the tracheal and amniotic fluids. The concentration of ritodrine noted in the tracheal fluid samples suggests drug accumulation in this fluid compartment. Such accumulation has been reported by Riggs *et al.* [13] for metoclopramide and diphenhydramine. Ritodrine appears to accumulate and persist in amniotic fluid at concentrations greater than those in fetal arterial plasma.

CONCLUSIONS

The assay method presented here is simple, reproducible, selective and sensitive enough to determine ritodrine during single-dose pharmacokinetic studies. Linearity of detector response was noted over the range 2.5–75 ng of added ritodrine base, which would represent approximately 1–100 pg mass at the detector. The minimum quantifiable concentration (coefficient of variation $< 10\%$) is 2.5 ng/ml from a 0.5-ml biological fluid sample. The proposed method has been used for the analysis of ritodrine from a variety of biological fluids obtained during experiments with chronically instrumented pregnant sheep.

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