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Sensitive fused-silica capillary gas chromatographic assay using electron-capture detection for indomethacin in ovine fetal fluids

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Abstract

A sensitive gas chromatographic (GC) method with electron-capture detection (ECD) has been developed to quantitate indomethacin (IND) in plasma, urine, amniotic, and tracheal fluids obtained from the pregnant sheep model. IND and the internal standard, α-methylindomethacin (α-Me-IND) are extracted by a simple liquid–liquid extraction procedure using ethyl acetate and derivatized with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) at 60°C for 50 min. The limit of quantitation (LOQ) is 1 ng/ml with a C.V. $< 10\%$ and signal-to-noise ratio > 10. Recoveries from all fluids were greater than 80%. Calibration curves were linear over the range of 1–32 ng/ml with a coefficient of determination (r^2) > 0.999. Inter- and intra-day coefficients of variation were $< 10\%$ at concentrations of 2–32 ng/ml, and $< 20\%$ at the LOQ. Applicability of the developed method is demonstrated for a pharmacokinetic study of IND samples collected following long-term infusion of IND in a chronically instrumented ovine fetus.

1. Introduction

IND, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid (Fig. 1), originally introduced in 1963 as a non-steroidal anti-inflammatory drug [1–3], is being increasingly used in pregnancy as a tocolytic and to treat polyhydramnios [4–7]. It is also a drug of choice for patent ductus arteriosus in the newborn [8]. Although its tocolytic potency is reported to be superior to the FDA approved drug, ritodrine hydrochloride, concern has been raised about its ability to cause untoward effects in the fetus, the most prominent being fetal and neonatal renal dysfunction [9]. IND crosses the placenta readily in the human and rabbit [10,11]; however, fetal clearance parameters (renal, total body, placental, and non-placental) have yet to be elucidated in any species.

Currently, we are investigating the disposition and effects of IND in chronically instrumented

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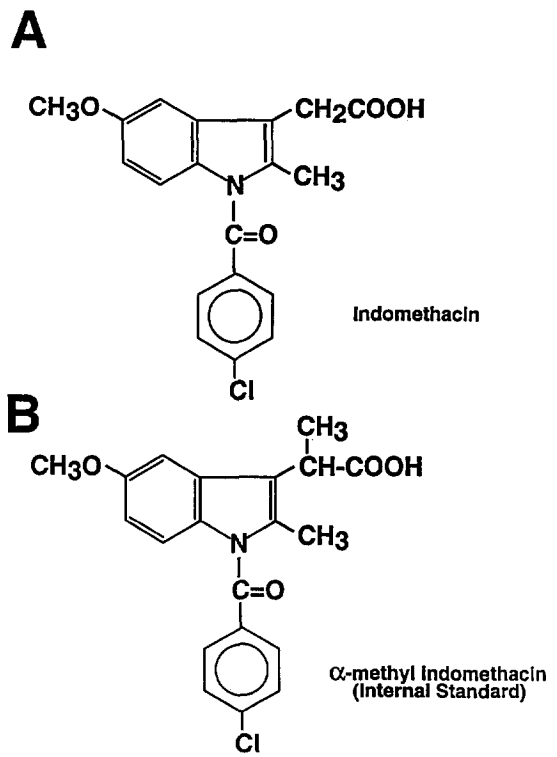

Fig. 1. Chemical structures of IND (A) and α-Me-IND (B).

fetal lambs in experiments involving a 3-day IND infusion intravenously to the fetus [12]. However, the study of fetal IND kinetics requires an analytical methodology with high sensitivity and specificity for sample volumes ≤ 1 ml. Several assay procedures have been reported for the measurement of IND in plasma, serum, and urine, namely, high-performance liquid chromatography (HPLC) [13–34], GC [35–44], gas chromatography–mass spectrometry (GC–MS) [45, 46], radioimmunoassay (RIA) [47], and spectrophotometry [48], but are unsuitable for fetal studies. The RIA method [47] is highly cross-reactive to glucuronide conjugates of IND and its metabolites. HPLC with UV detection lacks adequate sensitivity while fluorescence methods require post-column in-line hydrolysis necessitating auxiliary pump setups [16,22,26]. Current GC methods suffer from many disadvantages including cumbersome derivatization procedures, large sample volumes, poor extraction recoveries, high variability at lower concentrations, and multiple step extraction procedures. Of the available methods, GC with ECD would appear to provide the highest sensitivity. Several derivatizing reagents have been investigated for GC assays, including diazomethane [39,40,46], diazopropane [36], 1-ethyl-3-*p*-tolyltriazine [43], pentafluorobenzyl bromide [44], hexafluoroisopropanol and trifluoroacetic anhydride [45], bis-(trimethylsilyl) acetamide [46], ethyl iodide and extractive alkylation [42], pentafluoropropanol and pentafluoropropionic anhydride [37], and diazoethane [41]. The simplest of the derivatization reactions is with bis(trimethylsilyl) acetamide, a silylating agent producing trimethylsilyl ester of IND. Selection of internal standards for IND analysis by either HPLC or GC is yet another constraint. Several reports incorporate internal standards with no structural similarity to IND including phenylbutazone [27], phenacetin [15], itraconazole [13], penfluridol [40], while others use nonideal internal standards such as IND propyl ester [43] and IND methyl ester [36], and still others do not use any at all [38,41].

The purpose of this study, then, was to develop a sensitive and selective analytical method capable of determining IND concentrations reproducibly from a variety of biological fluids (fetal arterial and umbilical venous plasma, urine, amniotic, and tracheal fluids) obtained following long-term fetal infusion studies in chronically instrumented pregnant sheep, and in other situations involving small sample volumes. Application of the assay is illustrated for IND measurement in fetal plasma, urine, and amniotic fluid following a fetal IND infusion.

2. Experimental

2.1. Materials

IND (lot 60H0448) was obtained from Sigma (St. Louis, MO, USA). α-Me-IND (Fig. 1) was a generous gift from Merck-Frosst (Kirkland, Canada). Sodium acetate (anhydrous, analytical reagent grade) obtained from BDH (Toronto,

Canada) and glacial acetic acid (American Chemical Society, ACS reagent grade), purchased from Allied Chemical (Pointe Claire, Canada), were used to prepare acetate buffers. N-Methyl-N-(*tert.*-dibutyldimethylsilyl)trifluoroacetamide (MTBSTFA, lot 930817150) was purchased from Pierce Chemical (Rockford, IL, USA). Ethyl acetate and toluene (distilled in glass) were purchased from Caledon Laboratories (Georgetown, Canada). Deionized, high-purity water (hereafter referred to in the text as water) was produced on-site by reverse osmosis using a Milli-Q water system (Millipore, Mississauga, Canada). Hydrogen, argon–methane (95:5), ultra-pure helium, and methane were obtained from Matheson Gas Products (Edmonton, Canada). Nitrogen NF was obtained from Praxair Canada (Mississauga, Canada).

2.2. Instrumentation

Gas chromatography

A Hewlett-Packard (HP) Model 5890 series II GC equipped with an HP Model 7673 autosampler, split–splitless capillary inlet system, Model HP 3365 chemstation, and a ^{63}Ni ECD (Hewlett-Packard, Avondale, PA, USA), was used for all analyses. A Pyrex glass inlet liner (78 mm × 4 mm I.D.) and Thermogreen LB-2 silicone rubber septa (Supelco, Bellefonte, PA, USA) were used. The optimized operating conditions for routine analyses were: column, Ultra-2 fused-silica capillary column cross-linked with 5% phenylmethylsilicone (25m × 0.31 mm, 0.52 μm film thickness); injection mode, splitless; injection port temperature, 200°C; initial column temperature, 210°C (1 min); oven programming rate, 40°C/min; final oven temperature, 300°C (6 min); detector temperature (ECD), 330°C; carrier gas, ultra high purity hydrogen at a column head pressure of 70 kPa (column flow-rate: 1 ml/min; linear velocity: 83.3 cm/s); purge delay time, 0.75 min; make-up gas, argon–methane, 95:5, at a flow-rate of 65 ml/min.

Gas chromatography–mass spectrometry

A HP Model 5890 series II GC (Hewlett-Packard, Avondale, PA, USA) with an HP 7673 automatic injector system, an HP Model 5989A MS, an HP 59827A vacuum gauge controller, an HP 98789A on-line computer, and an HP Ultra-2 fused-silica capillary column (25 m × 0.31 mm, 0.52 μm film thickness) was used to confirm derivative formation and identity. The carrier gas, helium, was operated at a column head pressure of 3 p.s.i. (ca. $2 \cdot 10^4$ Pa). In the chemical ionization mode, methane was used as the reagent gas. The electron impact-MS ionization energy was 200 eV, emission current 250 μA, and source pressure, 1.2 Torr (ca. 160 Pa).

2.3. Preparation of stock solutions

IND

Approximately 10 mg of IND was accurately weighed and transferred to a 50-ml volumetric flask and made up to volume with methanol. A 0.5-ml aliquot of solution was diluted with water to volume in a 50-ml volumetric flask. The latter solution (1 ml) was further diluted to volume with water in a 50-ml volumetric flask to provide a final working stock solution with a concentration of 0.04 μg/ml. Volumes of 25, 50, 200, 400, and 800 μl were used to prepare the calibration curve.

α-Me-IND (internal standard)

Approximately 5 mg of α-Me-IND was accurately weighed and made up to 1 ml with methanol. An aliquot of 100 μl was transferred to a 10-ml volumetric flask and made up to volume with methanol. A 100-μl aliquot of the latter solution was transferred to another 10-ml volumetric flask and made to volume with water to produce a working solution with a concentration of 500 ng/ml. A volume of 50 μl (25 ng) was used in the assay.

2.4. Preparation of reagent solutions

Acetate buffer pH 5.0 was prepared according to British Pharmacopoeia [49] standards by dissolving 13.6 g of sodium acetate and 6 ml of glacial acetic acid in sufficient water to produce 1000 ml. An aliquot of 2 ml (excess) was used in

the assay to produce an aqueous phase pH of ca. 5.0.

2.5. Sample preparation

Ovine fetal fluids (plasma, urine, amniotic and tracheal fluids), typically 0.1–1.0 ml, were pipetted into clean 15-ml borosilicate Kimax culture tubes with polytetrafluoroethylene (PTFE)-lined screw caps. To the biological fluid were added 50 μl of internal standard, α-Me-IND, and 2 ml of acetate buffer pH 5.0 and the mixture adjusted to a final volume of 3.0 ml with water (final pH of the aqueous phase ca. 5.00). The mixture was gently vortex-mixed and 5 ml of ethyl acetate was added. The aqueous phase was extracted for 20 min on a rotary shaker. The samples were then placed in a freezer at −5°C for 10 min to facilitate breakage of any emulsion formed in the extraction step. This was followed by centrifugation for 10 min at 3000 *g*. The upper organic layer was transferred to clean 15-ml tubes and evaporated to dryness in a water bath maintained at 37°C under a gentle stream of nitrogen gas. To the residue was added 100 μl of toluene containing 8 μl of MTBSTFA, and the samples vortex-mixed and placed in an oven at 60°C for 50 min. The samples were allowed to cool to room temperature after which 200 μl of toluene was further added, vortex-mixed, and transferred to automatic sampler injection vials. Aliquots of 2 μl were injected into the GC.

2.6. Preparation of calibration curves

Serial quantities of the working IND stock solution (1, 2, 8 or 10, 16, and 32 ng) were added to 0.1 ml of blank fetal sheep biological fluids (plasma, urine, amniotic and tracheal fluids). A 50-μl aliquot of the internal standard (α-Me-IND) was added, and the solution made up to a fixed volume of 1 ml with water. The samples were then extracted and derivatized as described previously. Determination of IND concentrations was made by plotting the peak-area ratios of the *tert.*-butyldimethylsilyl (tBDMS) derivatives of IND and α-Me-IND against the known amount of IND added to each sample.

2.7. Extraction recovery studies

Extraction recoveries of IND were determined at four concentration points, i.e., 2, 8, 16 and 32 ng/ml. Two groups of samples were used in this study, namely the test and the control group. Samples in both groups contained blank ovine fetal biological matrix (plasma or urine), and the internal standard, α-Me-IND (50 μl). Only samples from the test group were spiked with IND to produce final concentrations of 2, 8, 16 and 32 ng/ml. Both test and control group samples were subjected to the liquid–liquid extraction procedure described above after which aliquots of IND, prepared in methanol, were added to control samples to produce concentrations of 2, 8, 16 and 32 ng/ml. Control and test group samples were then dried under nitrogen, derivatized with MTBSTFA, reconstituted with toluene, and chromatographed as described earlier. Extraction recovery at each concentration point was calculated as the ratio of peak-area counts observed with test (extracted) to that in control (unextracted) and expressed in percentage.

2.8. Stability studies

Studies were performed periodically (over 14 months) to assess the stability of the samples during storage and analysis. Freezer storage stability was determined at a concentration of 8 ng/ml by spiking plasma and urine samples with this known amount of IND. The samples were then stored at −20°C, removed periodically and analysed. In order to assess the stability of IND during freeze–thaw cycles, blank biological samples (plasma) were spiked with IND (8 ng/ml), frozen at standard freezer temperatures (−20°C), and thawed at room temperature on the bench-top for four consecutive cycles, and subsequently analysed. A bench-top stability study, at ambient conditions, was also performed by spiking biological test fluids with a known amount of IND (8 ng/ml). The samples were allowed to stand at room temperature for 0, 12 and 24 h. At the end of each study, the internal standard was added and they were processed as previously described. The stability of IND in

acidified biological matrix (pH 5.00) was investigated by adding an excess of pH 5.00 acetate buffer to the biological fluid (plasma/urine) and spiking the acidified matrix with a known amount of IND. The samples were allowed to remain on the bench-top for 0, 6, 12 and 24 h, following which the internal standard was added and samples processed as before. Finally, the stability of processed samples on the GC autosampler tray was investigated by repeatedly injecting the sample vials at 24-h intervals for one week.

2.9. Inter-day and intra-day variability

Inter-day and intra-day variability were determined at five concentration points, namely, 1, 2, 8, 16 and 32 ng/ml. For evaluating intra-day variability, three calibration curves were prepared on the same day, and for inter-day variability, calibration curves were prepared on each of three separate days.

2.10. Animal experiments

A pregnant (Dorset/Suffolk) ewe (125 days, term ca. 145 days) was surgically prepared for experimentation as described previously [51]. Briefly, the ewe was anesthetized with halothane/nitrous oxide in oxygen following induction with sodium pentothal intubation of the ewe. Access to the fetus was gained via a midline abdominal incision and then through an incision of the uterine wall free of placental cotyledons and major blood vessels. Polyvinyl catheters were placed in the inferior vena cava and descending aorta through the femoral and tarsal vessels, respectively. The fetal bladder was cannulated through a suprapubic incision, followed by non-occlusive catheterisation of the common umbilical vein at the umbilicus. Two catheters were placed in the amniotic cavity and anchored to the abdominal skin of the fetus. A carotid artery was also catheterised, via a separate uterine incision, and a non-occlusive catheter was inserted into the trachea, via an incision below the larynx. A catheter was also placed in the amniotic cavity, and anchored to the skin on the neck. The hysterotomy and laparotomy incisions were then closed. The maternal inferior vena cava and descending aorta were cannulated via maternal femoral vessels. All catheters were passed subcutaneously to a small incision in the maternal abdominal wall on the left flank where they exited. They were stored in a denim pouch attached to the flank. Postoperatively, ampicillin (500 mg) was administered as an intramuscular prophylactic antibiotic for three days to the ewe, and ampicillin (500 mg) intra-amniotically to the fetus at the time of surgery and daily into the amniotic fluid for the duration of the preparation. Each catheter was flushed daily with heparinised (12 units/ml) 0.9% sodium chloride for injection. The experiment was conducted over a period of five days with the first day being the control day when no drug was given. IND, in a solution of 1.1% ethanol and 0.75% sodium bicarbonate in normal saline (pH 7.8), was infused into the fetal lateral tarsal vein at a dose of 0.0025 mg kg^{-1} min^{-1} (2 ml/h) on days 2 through 4. The infusion was terminated on day 5 and the fetus further monitored over a 24-h recovery period. Samples (ca. 2 ml each) of fetal arterial (FA) and umbilical venous (UV) blood, amniotic and tracheal fluids, and urine were sampled twice daily at 6-h intervals and stored at −20°C until assay. Blood samples were collected in heparinised Vacutainer collection tubes, centrifuged at 3000 *g* for 10 min at −10°C and the plasma transferred into borosilicate glass tubes with PTFE-lined screw caps until analysis.

3. Results and discussion

3.1. Extraction, derivative formation, and chromatography

The method presented here has several advantages over existing assay procedures for IND in terms of sensitivity, selectivity, reproducibility, and robustness. The previously reported capillary column GC method reported by Nishioka et al. [43] has the major disadvantages of long run times (ca. 20 min), lengthy derivatization reaction times (ca. 6 h), and inappropriate internal standard (IND propyl ester). The propyl and methyl

esters of IND are sensitive to hydrolysis and are, therefore, not ideal internal standards. Fig. 1 illustrates the chemical structures of IND and the internal standard, α-Me-IND. The latter compound closely resembles the analyte providing very similar chromatographic characteristics and detector response, with a response factor of ca. 1.0 at a concentration equal to that of IND. Both compounds elute in regions free from endogenous interference as illustrated in Fig. 2 which depicts a chromatogram obtained from pregnant sheep plasma. Retention times (total) of the tBDMS derivatives of α-Me-IND (peak a) and IND (peak b) are 4.8 and 5.0 min, respectively. Very similar patterns were observed in fetal urine, amniotic and tracheal fluids.

During optimization of the extraction conditions, various solvents were investigated (viz., ethyl acetate, toluene, toluene–triethylamine, hexane, hexane–isopropanol, diethyl ether, hexane–ethyl acetate, and dichloromethane). Recoveries were maximal with ethyl acetate at a volume of 5 ml. We also examined the optimum pH conditions for IND extraction over the range of 2–6 pH units. Like Helleberg [41] and Ou and Frawley [28], we found the extractability of IND to be pH-sensitive and maximal at pH 5.00. Extraction times ranging from 5 to 60 min were examined with 20 min providing optimal area ratio values. The extraction recoveries ($n = 4$) were determined by the matrix-compensation method. On this basis, recoveries in plasma averaged $101.77 \pm 2.05\%$, $104.82 \pm 3.24\%$, $97.52 \pm 3.33\%$, and $83.85 \pm 1.29\%$, respectively, at IND concentrations 2, 8, 16, and 32 ng/ml. In urine, the respective values were $100.71 \pm 9.41\%$, $100.35 \pm 12.51\%$, $87.83 \pm 3.09\%$, and $90.07 \pm 3.44\%$. This is greater than the solid-phase extraction procedure of Nishioka et al. [43], i.e., about 87% in plasma, and the liquid–liquid extraction procedure of Sibeon et al. [44], i.e., about 85% recovery from plasma using ethylene dichloride. Recoveries comparable to ours have also been reported: 95% from serum using dichloromethane [42] and about 92% from plasma using ethyl acetate [37].

The active carboxylic acid site on the IND molecule was derivatized with bis(trimethylsilyl)

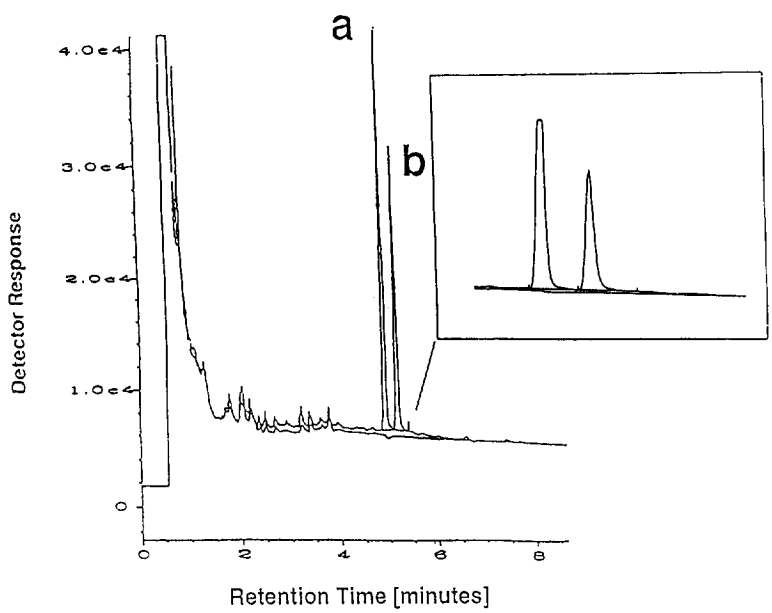

Fig. 2. Representative chromatograms of α-Me-IND (peak a, 25 ng/ml) and IND (peak b, 16 ng/ml) spiked in fetal plasma with the superimposed blank. The inset shows the magnified baseline.

acetamide (BSA), MTBSTFA, pentafluorobenzylbromide (PFBBr), and pentafluoropropanol (PFP) to select the most appropriate derivatizing agent. The area counts of derivatized IND with PFBBr were not significantly different from those with MTBSTFA, and both reactions produced peaks of excellent geometry and sharpness. However, derivatization with PFBBr was both labour-intensive and time-consuming, while derivatization with PFP was incomplete and was not pursued further. Since MTBSTFA derivatization (producing *tert.*-butyldimethylsilyl [tBDMS] ester of IND) provided the best chromatography along with simplicity and safety of the reaction, it was chosen for our assay. There have been no reports in the literature of the mass spectra of IND derivatized by MTBSTFA. During assay development the spectra of IND, derivatized with MTBSTFA, were characterised using electron-impact ionization (EI), positive-ion chemical ionization (PCI), and negative-ion chemical ionization (NCI) GC–MS (Fig. 3) to confirm derivative formation and identity. The mass spectrum of IND-tBDMS derivative following EI resulted in extensive fragmentation with a base peak of m/z 139 due to the *p*-chlorobenzoyl fragment (Fig. 3a). The characteristic $[M-57]^+$ peak at m/z 414 is due to the loss of the $C(CH_3)_3$ fragment. The EI scan also shows the molecular ion of the tBDMS derivative at m/z 471. The spectrum following PCI and NCI, yielded an intense molecular ion at m/z 472 ($[M+H]^+$) and m/z 471 (M^+), respectively, confirming the formation of the IND-tBDMS derivative (Figs. 3b and 3c). Derivatization conditions were optimal with a time of 50 min and a reaction temperature of 60°C as maximal absolute IND area counts were observed under these conditions.

3.2. Calibration curves, validation, and sample stability

Working calibration curves over the range of 1–32 ng/ml, in plasma, were linear with coefficient of determination (r^2) in the order of 0.999, and linear regression statistics of $y = 0.05042x + 0.00543$. The coefficients of variation (C.V.) at each concentration point were below 10% (range: 5.32–8.71%; LOQ: 8.41%). Similarly, C.V. for the slopes of the calibration curves were $< 10\%$. Calibration curves for IND spiked in fetal urine provided similar results with an r^2 of 0.990 and regression statistics of $y = 0.03574x + 0.05947$. As observed with plasma, C.V. at each concentration point were below 10% (range: 0.69–8.95%; LOQ: 0.69%). The LOQ in both biological fluids was 1 ng/ml requiring 0.1 ml of sample with a signal-to-noise ratio of > 10. Inter-day and intra-day variabilities for IND concentrations ranging from 1 to 32 ng/ml are shown in Table 1. Very similar validation results were obtained for IND in amniotic fluid. Assay accuracy at three different concentration points (low, medium, and high) in plasma and urine was determined as [(amount measured/amount added) × 100] − 100 (bias, %). In fetal plasma, accuracies for IND at 1, 16, and 32 ng were +4.65, −0.43 and +0.21, respectively. In fetal urine, the respective values were +0.33, +0.21 and +0.54. IND was found to be stable in

Table 1
Inter-day ($n = 4$) and intra-day variability ($n = 3$) for IND in fetal plasma and fetal urine

Concentration (ng/ml)	Coefficient of variation (%)	
	Inter-day	Intra-day
Fetal plasma		
1	18.54[a]	2.23
2	7.54	4.10
8	1.49	3.66
16	5.44	1.94
32	4.18	1.03
Slope	4.00	1.13
Fetal urine		
1	2.32[a]	7.04
2	9.05	11.14
8	7.75	1.09
16	7.01	0.37
32	3.92	3.56
Slope	3.11	4.11

[a] $n = 10$.

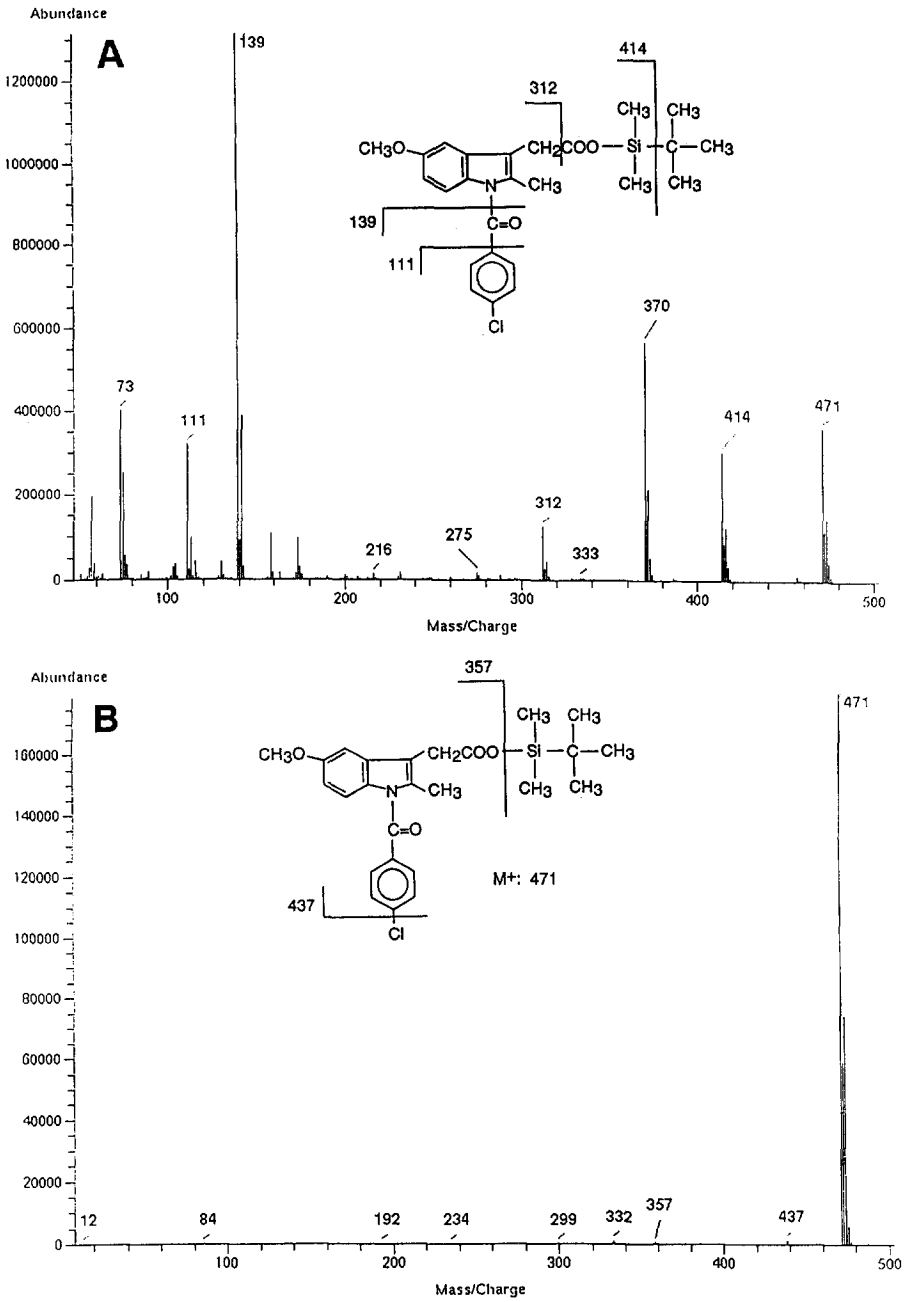

Fig. 3.

acidified biological sample matrix (pH 5.00) for at least 24 h, on the bench-top for at least 24 h, GC autosampler tray for at least one week, and for at least four freeze–thaw cycles.

3.3. Determination of IND in biological samples

Application of the method to IND measurement in plasma, urine, and amniotic fluid samples

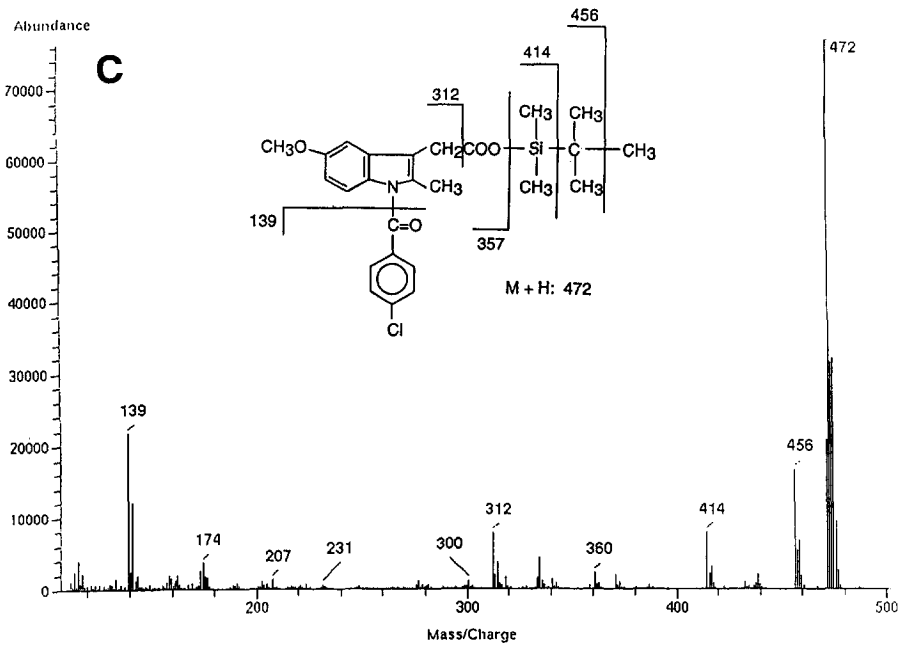

Fig. 3. GC–MS spectra of tBDMS derivatized IND: (A) EI mode, (B) NCI mode, and (C) PCI mode.

is shown in Fig. 4. The steady state fetal femoral arterial plasma IND concentrations averaged 216.2 ± 45.0 ng/ml (mean ± 1 S.D.) over the 23–69 h period following initiation of IND infusion. The fetal plasma concentrations of IND were similar to those seen during human tocolysis, i.e., ca. 200 ng/ml [11]. The fetal total body clearance (infusion rate/IND steady-state plasma concentration) was estimated to be 34.7 ml/min. Very low levels of IND were found in urine samples,

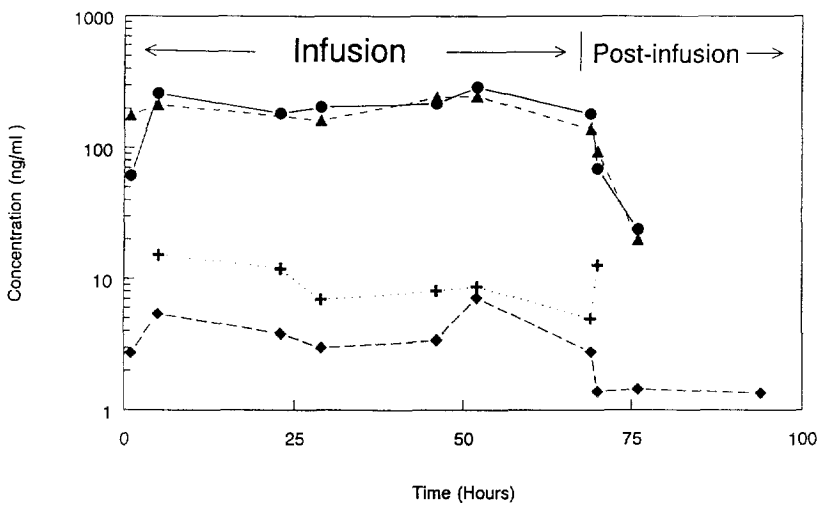

Fig. 4. Representative concentration–time profiles for IND in fetal femoral arterial (●) and umbilical venous (▲) plasma, urine (◆), and amniotic fluid (+) in a fetal lamb (Ewe 1211).

although the drug appeared rapidly in urine. The low levels of IND in amniotic fluid and fetal urine are consistent with our findings for other organic acids (e.g., valproic acid, diphenylmethoxyacetic acid) in the fetal lamb [50]. The renal clearance (slope of urinary excretion rate vs concentration of IND in plasma plot) was estimated to be 0.012 ml/min. This observation of minimal renal clearance is consistent with findings for other organic acidic compounds (e.g., valproic acid, diphenylmethoxyacetic acid, *p*-aminohippuric acid) in the fetal lamb [50,52]. Unlike our observations with basic amine compounds (e.g. metoclopramide, diphenhydramine), IND was not observed to accumulate in tracheal fluid [51]. A detailed pharmacokinetic analysis of IND in the chronically instrumented ovine fetus model will be presented elsewhere.

4. Conclusions

A reproducible, reliable, sensitive and selective GC-ECD method has been developed to quantitate IND in various biological fluids obtained from the pregnant sheep model. The method is superior to existing assay procedures for IND, as summarised below: (1) Simple one step liquid–liquid extraction procedure. (2) High-efficiency fused-silica capillary column technology. (3) Simple and rapid derivatization with MTBSTFA. (4) Small sample volumes (0.1–1.0 ml). (5) Rapid run times (9.25 min). (6) Sensitivity of 1 ng/ml (LOQ, $<10\%$ C.V., and $S/N > 10$). (7) Excellent chromatography with both the analyte, IND, and the internal standard, α-Me-IND, eluting in regions free from endogenous interference.

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