INDOMETHACIN: CAPILLARY GAS CHROMATOGRAPHIC - ELECTRON CAPTURE DETECTION ANALYSIS AND PHARMACOKINETIC STUDIES IN THE FETAL LAMB

by

RAJESH KRISHNA

B. Pharm., University of Kerala, Trivandrum, India, 1990
M. Pharm., Banaras Hindu University, Varanasi, India, 1992

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Faculty of Pharmaceutical Sciences
(Division of Clinical Pharmacy)

We accept this thesis as conforming to the required standard

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February 1995
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Department of Pharmaceutical Sciences

The University of British Columbia
Vancouver, Canada

Date 6 March 1995
ABSTRACT

Indomethacin is currently used in the treatment of patent ductus arteriosus, preterm labour, and for polyhydramnios. In order to investigate its clearance and disposition in the chronically instrumented ovine fetus, a sensitive assay was required.

The developed GC-ECD method involves the extraction of indomethacin and α-methyl indomethacin, the internal standard, from acidified plasma, urine, amniotic and tracheal fluids using a simple one-step liquid-liquid extraction procedure with ethyl acetate. The organic extract is evaporated and the residue derivatised with N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA). The derivatised samples were reconstituted with toluene and aliquots of 2 μL injected into the GC. Calibration curves were linear over the working range of 1-32 ng of indomethacin/mL with a coefficient of determination (r^2) > 0.99, following extraction of 0.1 mL of plasma, and 0.1-1.0 mL of urine, amniotic and tracheal fluids. Mean recoveries from plasma and urine were 96.99 ± 9.25 % and 94.74 ± 6.75 %, respectively. The limit of quantitation is 1 ng/mL (< 10% C.V., S/N > 10). Inter- and intra-day variabilities at each concentration point were < 11 % for concentrations between 2-32 ng/mL and < 20 % at the LOQ of 1 ng/mL.
The mean steady state fetal femoral arterial concentration of indomethacin was 181.67 ± 45.05 ng/mL in the high dose group (n=5), and 41.29 ± 3.14 ng/mL in the low dose group (n=2). The arterio-venous concentration difference for indomethacin at apparent steady state was minimal but statistically significant, suggesting a low placental permeability to indomethacin in sheep. The fetal total body clearance was estimated to be 44.55 ± 11.14 mL/min (n=7). The placental clearance of indomethacin, as determined by the Fick principle, was calculated to be 8.85 ± 0.81 mL/min/kg. The renal clearance was estimated to be 0.015 ± 0.009 mL/min (n=5). Indomethacin appeared in very low concentrations in amniotic fluid and fetal urine in high dose group fetuses. It was not observed to accumulate in tracheal fluid. The drug appeared to induce lactic acidosis and a reduction in fetal urinary output in some fetuses of the high dose group.

K. Wayne Riggs, Ph.D.

Research Supervisor
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<td>ACS</td>
<td>American Chemical Society</td>
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<tr>
<td>AMN</td>
<td>amniotic fluid</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
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<td>[A-V]_{indo(ss)}</td>
<td>aterio-venous indomethacin concentration difference at apparent steady state</td>
</tr>
<tr>
<td>BE</td>
<td>base excess</td>
</tr>
<tr>
<td>BSA</td>
<td>bis (trimethylsilyl) acetamide</td>
</tr>
<tr>
<td>BSTFA</td>
<td>N, O-Bis (trimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>C.V.</td>
<td>coefficient of variation</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CBF</td>
<td>cerebral blood flow</td>
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<td>C_{fa}</td>
<td>concentration of drug in fetal femoral arterial blood at steady state</td>
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<td>C_{fo}</td>
<td>fetal non-placental clearance</td>
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<tr>
<td>C_{lp}</td>
<td>placental clearance</td>
</tr>
<tr>
<td>C_{r}</td>
<td>renal clearance</td>
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<tr>
<td>C_{tb}</td>
<td>total body clearance</td>
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<td>C_{ss}</td>
<td>steady state plasma concentration</td>
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<tr>
<td>C_{um}</td>
<td>concentration of drug in umbilical venous blood at steady state</td>
</tr>
<tr>
<td>DA</td>
<td>ductus arteriosus</td>
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<tr>
<td>DBI</td>
<td>N-deschlorobenzoyl indomethacin</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
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<td>DMI</td>
<td>O-desmethyl indomethacin</td>
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<td>DPMA</td>
<td>diphenylmethoxyacetic acid</td>
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<td>DSC</td>
<td>differential scanning calorimetry</td>
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<td>EA</td>
<td>ethyl acetate</td>
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<td>ECD</td>
<td>electron capture detection</td>
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<td>electron-impact ionisation</td>
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<td>F-T</td>
<td>freeze-thaw</td>
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<td>F/M</td>
<td>fetal-to-maternal ratio</td>
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<td>FA</td>
<td>fetal femoral arterial</td>
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<td>[FA]_{indo(ss)}</td>
<td>fetal arterial plasma indomethacin concentration at apparent steady state</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FE_{Na+}</td>
<td>fractional excretion of sodium ion</td>
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<td>F_{ss}</td>
<td>fetal steady-state concentration</td>
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<tr>
<td>g</td>
<td>acceleration due to gravity</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GC</td>
<td>gas chromatography, gas chromatograph</td>
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<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
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<tr>
<td>h</td>
<td>hour</td>
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<td>H_{2}</td>
<td>hydrogen gas</td>
</tr>
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<td>HCO_{3}^{-}</td>
<td>bicarbonate ion</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HETP</td>
<td>height equivalent to a theoretical plate</td>
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<tr>
<td>HP</td>
<td>Hewlett-Packard</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>i.d.</td>
<td>internal diameter</td>
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<td>I.U.</td>
<td>international units</td>
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<td>i.v.</td>
<td>intravenous</td>
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<td>IPA</td>
<td>isopropyl alcohol</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
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<td>k_o</td>
<td>infusion rate</td>
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<tr>
<td>kPa</td>
<td>kilopascal</td>
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<tr>
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<td>liter</td>
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<td>LC/MS/MS</td>
<td>liquid chromatography/mass spectrometry/mass spectrometry</td>
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<td>LOQ</td>
<td>limit of quantitation</td>
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<td>M</td>
<td>maternal</td>
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<td>m/z</td>
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<td>MA</td>
<td>maternal arterial</td>
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<td>MAO</td>
<td>monoamine oxidase</td>
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<td>meq</td>
<td>milliequivalents</td>
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<td>milligram</td>
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<td>mL</td>
<td>milliliter</td>
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<td>mass spectrometry</td>
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<td>M_{ss}</td>
<td>maternal steady-state concentration</td>
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<tr>
<td>MSTFA</td>
<td>N-methyl-N-trimethylsilyl trifluoroacetamide</td>
</tr>
<tr>
<td>MTBSTFA</td>
<td>N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microliter</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>n</td>
<td>sample size</td>
</tr>
<tr>
<td>NCI</td>
<td>negative ion chemical ionisation</td>
</tr>
<tr>
<td>ND</td>
<td>not detectable</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectrometry</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>PCI</td>
<td>positive ion chemical ionisation</td>
</tr>
<tr>
<td>pCO₂</td>
<td>partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PDA</td>
<td>patent ductus arteriosus</td>
</tr>
<tr>
<td>PFB</td>
<td>pentafluorobenzyl</td>
</tr>
<tr>
<td>PFBBr</td>
<td>pentafluorobenzyl bromide</td>
</tr>
<tr>
<td>PFP</td>
<td>pentafluoropropanol</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>pH</td>
<td>negative logarithm (base 10) of the hydrogen ion concentration</td>
</tr>
<tr>
<td>pO₂</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
</tr>
<tr>
<td>Qum</td>
<td>umbilical blood flow</td>
</tr>
<tr>
<td>r</td>
<td>correlation coefficient</td>
</tr>
</tbody>
</table>
\( r^2 \)  
coefficient of determination

RBF  
renal blood flow

RIA  
radioimmunoassay

rpm  
revolutions per minute

R_t  
retention time

S/N  
signal-to-noise ratio

SD  
standard deviation

sec  
second

SEM  
standard error of the mean

T_{1/2}  
half life

TBDM-SIM  
N-t-butyldimethylsilylimidazole

TBDMCS  
t-butyldimethylchlorosilane

tBDMS  
tert-butyldimethylsilyl

TEA  
triethylamine

TMS  
trimethylsilyl

TR  
tracheal fluid

UHP  
ultra-high purity

UR  
fetal urine

UTV  
uterine vein

UV  
umbilical venous

\([UV]_{indo(ss)}\)  
umbilical venous plasma indomethacin concentration at apparent steady state

VP  
vasopressin

\[ y=mx + c \]  
linear regression statistics of a straight line
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DEDICATION

This thesis

is

dedicated

to my

parents

for their

love, support, and patience.
1. INTRODUCTION

1.1 Indomethacin

Indomethacin \( (1-[\text{p-chlorobenzoyl}]-5\text{-methoxy-2-methyl-indole-3-acetic acid}) \), introduced as a non-steroidal antiinflammatory drug (NSAID), is a potent prostaglandin synthetase inhibitor used in the treatment of gouty arthritis (O'Brien et al., 1968), ankylosing spondylitis (Kass, 1965), osteoarthritis (Bilka et al., 1964), rheumatoid arthritis (O'Brien et al., 1968), patent ductus arteriosus (Friedman et al., 1976), and preterm labour (Zuckerman et al., 1974).

![Chemical structure of indomethacin.](image.png)

Figure 1: Chemical structure of indomethacin.

1.1.1 Pharmacology Of Indomethacin

A. Mechanism Of Action
General Pharmacological Activity

Since its introduction in 1963, a number of mechanisms have been proposed to account for indomethacin's (and other Aspirin® like drugs) mode of action. These include uncoupling of oxidative phosphorylation, mucopolysaccharide biosynthesis inhibition, inhibition of platelet function, stabilisation of cellular and lysosomal membranes, and effects on leukocyte migration (Mörsdorf, 1964; Schönhofe, 1967; Whitehouse, 1968; Bryant et al., 1963). Structural-activity relationship studies at that time indicated the presence of a hypothetical antiinflammatory receptor site consisting of a cationic core and two noncoplanar hydrophobic regions (Shen, 1965; Shen, 1967). In the early 70s, the inhibition of prostaglandin (PG) synthetase by indomethacin and Aspirin® at therapeutically effective concentrations was established (Vane, 1971; Smith and Willis, 1971; Ferreira et al., 1971). Following this discovery, Ham et al., 1972, screened a series of substituted aryl acid compounds in a PG synthetase system. However, the elucidation of the exact mechanism of action was hindered largely due to the fact that PG synthesis systems in different tissues possess markedly dissimilar pharmacological and biochemical profiles, and that NSAIDs may inhibit the enzyme system at different sites in the biosynthetic pathway. At that time, it was also determined that the inactivation of PG synthetase was reversible and time-dependent (Smith and Lands, 1971; Raz et al., 1973). Indomethacin was observed to be a
competitive inhibitor of PG synthetase in sheep seminal vesicles with a $k_i$ of $6.5 \times 10^{-6}$ M (Ku and Wasvary, 1973). The mechanism of action is illustrated in Figure 2.

\[ \text{Figure 2: Inhibition of PG synthesis by indomethacin (Adapted from Shen and Winter, 1977)} \]

\textit{Patent Ductus Arteriosus}

Prostaglandin E$_2$ (PGE$_2$) is primarily responsible for maintaining the patency of the ductus arteriosus (DA) in the fetus (Friedman, 1978; Coceani and Olley, 1973; Coceani \emph{et al.}, 1975; Coceani \emph{et al.}, 1978a and 1978b; Clyman \emph{et al.}, 1980). Indomethacin treatment leads to constriction of the DA mediated via cyclooxygenase inhibition.
Premature Labour

Prostaglandins, PGE₁, PGE₂, and PGF₂α, all stimulate uterine contractions in pregnant subjects and are hence suitable candidates for the induction of labour (Embrey, 1970; Beazley and Gillespie, 1971; Roth-Brandel et al., 1970). Of these, prostaglandins, PGF₂α and PGE₂ are potent compounds used therapeutically for the induction of labour (Thiery and Amy, 1975). The two common formulations are PGE₂ gel for intracervical application and PGF₂α for slow i.v. infusion. If the gel cannot be employed or if the patient experiences primary dysfunctional labour (Andersson et al., 1983), then the i.v. PGF₂α formulation may be used. The exact mechanism by which these PGs stimulate uterine contractions is unclear.

It has been shown that during labour there is a rise in PG concentrations in blood (Karim, 1968) and amniotic fluid (Karim and Devlin, 1967; MacDonald and Casey, 1993). A subsequent paper reported that exogenously administered PG’s can induce labour (Karim et al., 1969). These reports suggested a physiological role of the PG’s in uterine motility. The critical step in the initiation of labour is the local production of PG’s, i.e., production of PGE₂ in the amnion, which then reaches the decidua. In the decidua, PGE₂ can either undergo conversion to PGF₂α or initiate the synthesis of further PGE₂ and PGF₂α (Nakala et al., 1986; McCoshen et al., 1990; Casey and McDonald, 1988; McDonald et al., 1991). Although the exact mechanism by which PGs stimulate uterine contractions is unclear, there is some
evidence to suggest that they affect transmembrane calcium flow (Huszar, 1989). It is speculated that PG’s may activate smooth muscle cell contractile protein and that the process involves an increase in calcium transport through the plasma membrane. PGF$_2\alpha$ stimulates Ca$^{++}$ influx into the smooth muscle cell. This influx of Ca$^{++}$ results in the activation of myosin light-chain kinase (MLCK) initiating phosphorylation. Shortening of smooth muscle cells is facilitated by actin-myosin interactions (Figure 3). Indomethacin inhibits PGF$_2\alpha$ production and thereby interferes with its modulatory action on transmembrane calcium flow. This results in an inhibition of uterine muscle activity. Therefore, by inhibiting uterine activity, PG synthetase inhibitors like indomethacin are indicated for tocolysis (Zuckerman et al., 1974; Wiquist et al., 1975; Niebyl et al., 1980).

**Figure 3:** Proposed role of PG’s in labour (Adapted from Miller, 1983).
B. **Clinical Uses**

**Anti-inflammatory, Analgesic, and Antipyretic Uses**

As previously mentioned, indomethacin is effective in rheumatic disorders (ankylosing spondylitis, osteoarthritis, rheumatoid arthritis) as well as for moderate pain such as dysmenorrhoea. The anti-inflammatory, analgesic, and antipyretic effects of indomethacin are the subject of several research papers: uses in Bartter's syndrome (Zancan et al., 1976; Haluska et al., 1977; Donker et al., 1977; Rado et al., 1978), in fever (Silberman et al., 1965; Kieley et al., 1969; Engerrall et al., 1986), in hypercalcaemia (Elliot and McKenzie, 1983; Dindogru et al., 1975; Blum, 1975), in osteoarthritis (Zachariae, 1966; Harth and Bondy, 1969; Woolf et al., 1978; Gordin et al., 1985), in pain (Holmlund and Slodin, 1978; Elder and Kapadia, 1979; Mattila et al., 1983; Ebbehoj et al., 1985; Jonsson et al., 1987), and in rheumatoid arthritis (Donnelly et al., 1967; Wright et al., 1969; Baber et al., 1979; Ekstrand et al., 1980).

**Patent Ductus Arteriosus**

The ductus arteriosus (DA) is a vascular shunt in the fetal circulation that diverts about 90% of the right ventricular output past the lungs into the descending aorta. PGE₂ is one of the most important determinants in the patency of the DA.
Closure of the ductus is imperative in the neonate as failure results in the development of a left-to-right shunt from the aorta to pulmonary artery. Other complications include hepatomegaly, hyperactive precordium, and congestive cardiac failure. The incidence of patent ductus arteriosus (PDA) is about 80% in neonates weighing less than 1000g and 10-15% in those weighing between 1500-2000g (Douider et al., 1988). PDA closure has been postulated to be age related (McCarthy et al., 1978).

Surgical ligation (Gay et al., 1973) and treatment with a prostaglandin synthetase inhibitor, such as indomethacin (Friedman et al., 1976), are two commonly used methods of correcting PDA. Increased oxygen tension and decreased prostaglandin concentrations are two common determinants of ductal closure at birth (Noerr, 1991). Intravenous indomethacin, in the sodium trihydrate form, is the drug of choice for neonatal PDA. However, renal dysfunction is claimed to be a prominent side effect of such a therapy. Decreased urine output and free water clearance, increased serum creatinine and blood urea nitrogen have all been reported during indomethacin use (Betkerur et al., 1981; John et al., 1984). A mean reduction in cerebral blood flow (CBF) by 24% has also been reported in a study involving pharmacological closure of the DA at an i.v dose of 0.2 mg/kg in human premature infants (Pryds et al., 1988). Changes in regional blood flow velocity have also been observed following indomethacin infusion in preterm infants with symptomatic PDA. These changes include a reduction in time-
averaged mean velocity, peak systolic velocity, and end-diastolic velocity in the anterior and middle cerebral artery (Austin et al., 1992). Reductions in CBF velocity to the extent of 50% in adult humans and 30% in newborn pigs have been observed (Wennmalm et al., 1981; Leffler et al., 1985). The apparent volume of distribution of indomethacin appears to be a useful marker of permanent PDA closure since it varies significantly pre- and post-PDA closure (Gal et al., 1991).

**Polyhydramnios**

Polyhydramnios is a complication arising from excess amniotic fluid and is determined by several methods including the maximum-vertical pocket technique (Chamberlain et al., 1984), amniotic fluid index (Phelan et al., 1987), and gestational age adjusted amniotic fluid index (Moore and Cayle, 1990). A 30% fetal mortality rate due to premature rupture of the membranes as a result of polyhydramnios has been reported (Cabrol et al., 1987). Conventionally, the management of polyhydramnios incorporates repeated transabdominal amniocentesis under tocolytic therapy and fluid withdrawal. Puncture complications include infection and membrane rupture. Prostaglandin synthetase inhibitors reduce amniotic fluid volume as well as inhibit premature labour (Cabrol et al., 1987). The reduction of amniotic fluid volume is thought to be due to a decrease in urinary flow (Cifuentes et al., 1979; Kirshon et al., 1988; Rosen et al., 1991; Kirshon et al., 1991; Walker et al., 1992a). One risk of such therapy,
however, is the possibility of oligohydramnios (Nordstrom et al., 1992; Hendricks et al., 1990; Krishon et al., 1991; Goldenberg et al., 1989).

Indomethacin is recommended in the treatment of symptomatic polyhydramnios (Krishon et al., 1990; Smith et al., 1990). The mechanism by which indomethacin's effects are thought to be mediated in polyhydramnios is through a reduction in fetal urinary output. The underlying mechanism by which indomethacin reduces amniotic fluid volume by a fall in urinary output, however, remains unclear. Suppression of vasodilatory renal PG's by indomethacin leading to a fall in renal blood flow (Matson et al., 1981; Kover, 1980) is one proposed mechanism for the reduction in fetal urinary output. However, recent results indicate that indomethacin may interact with arginine vasopressin (AVP) at the V2 receptor level and augment the action of AVP; an increase in fetal AVP concentration was observed when indomethacin reduced fetal urinary output at a dose of 0.0025 mg/kg/min five hour infusion (Walker et al., 1992a). When indomethacin (0.05 mg/kg, 5 h) was co-administered with the AVP-V2 receptor antagonist (d \([\text{CH}_2]_5\)-D-Phe-Ile, Arg8)-VP, the fetal oliguria that was observed with indomethacin alone was inhibited by the antagonist, suggesting that V2 receptor played a significant role (Walker et al., 1994). Indomethacin may increase circulating AVP levels and enhance the peripheral effects of AVP in the fetus. It is interesting to note that PGE2 inhibits cAMP production (Levenson et al., 1982), and this effect seems to be mediated via the V2 receptor of AVP (Levenson et al., 1982;
Somenburg et al., 1990). Considering the fact that PGE₂ is synthesised in the kidney in response to AVP and oxytocin leads one to speculate that there exists a negative feedback role for the PGs (Walker et al., 1993). Inhibition of PGE₂ synthesis by indomethacin will potentiate AVP action through release and peripheral action of AVP (Walker et al., 1993; Levenson et al., 1982).

Studies by Walker et al., 1992b, have shown that short-term infusion of indomethacin at a dose of 0.017 mg/kg/min for 5 hours leads to an increase in fetal urinary output, urinary osmolality, sodium and chloride. However, when the dose infused was reduced to 0.0025 mg/kg/min for 5 hours, there was a reduction in urinary output, suggesting fetal antidiuresis at low levels of indomethacin (Walker et al., 1992a).

**Preterm Labour**

One of obstetrics' major challenges lies in the therapy of preterm labour. The only tocolytic drug approved to date by the U.S. FDA is the β-sympathomimetic, ritodrine hydrochloride. A potential therapeutic alternative to ritodrine are the prostaglandin inhibitors as it is known that prostaglandins PGE₁, PGE₂, and PGF₂α stimulate uterine contractions and induce labour (Embrey, 1970; Roth-Brandel et al., 1970). In 1974, Zuckerman et al., demonstrated a marked inhibitory effect of indomethacin on uterine contractions in premature labour. In a randomised
comparative trial of indomethacin and ritodrine, Besinger et al (1991) reported that the two were equally effective in delaying preterm birth. In 1992, Carlan et al., compared two PG synthesis inhibitors, namely, indomethacin and sulindac for efficacy in refractory preterm labour. Sulindac was equally effective as indomethacin although the group that responded to indomethacin had a longer time to delivery, higher birth weight, and a lower number of days in the intensive care unit. The sulindac group, however, demonstrated a renal sparing effect speculated to be due to minimal placental transfer. Several fetal risks have been identified following indomethacin administration including premature ductal closure, renal dysfunction, oligohydramnios, and pulmonary hypertension (Arcilla et al., 1969; Manchester et al., 1976; Rudolph, 1977; Friedman et al., 1978; Cifuentes et al., 1979). Other complications include tricuspid regurgitation, right ventricular dysfunction, and pericardial effusion (Hallak et al., 1991). Maternal side effects included perspiration, hypertension, gastritis, dizziness, and nausea (Grella and Zanor, 1978).

C. **Renal Effects Of Indomethacin**

Production of large quantities of hypotonic urine (amniotic fluid), excretion of sodium in large amounts, low renal blood flow (RBF), and low glomerular filtration rate (GFR) with greater fractional blood flow to mature inner nephrons are some of the characteristics of fetal renal function (Kleinman, 1978). As gestation
advances, there is a progressive increase in urinary flow rate, RBF, and GFR, and a reduction in fractional excretion of Na\(^+\) (FE\(_{\text{Na}^+}\)). Postnatal renal adaptation includes increases in GFR and RBF, redistribution of intrarenal blood flow from inside to the periphery of the cortex, reduction in FE\(_{\text{Na}^+}\), and a greater ability to concentrate urine. AVP levels are high at birth and during hypoxia and hemorrhage (Kleinman, 1978; Gleason et al., 1988; Robillard et al., 1982). Optimal urine concentrating ability depends upon the GFR, the AVP level, and the ability of the renal collecting tubule to respond to AVP. AVP enhances water resorption from the collecting tubule leading to anti-diuresis. The exact role by which renal prostaglandins modify AVP's effects is still unclear. In 1968, Grantham and Orloff proposed that AVP was controlled by a negative feedback loop wherein it stimulates intrarenal biosynthesis and production of PGs, and these PGs inhibit AVP's effects at the collecting tubule. While the exact mechanism(s) remain to be elucidated, several studies have demonstrated that the prostaglandin inhibitors enhance the anti-diuretic effect of AVP (Anderson et al., 1975; Berl et al., 1977; Fejes-Toth et al., 1977; Gullner et al., 1980; Lum et al., 1977).

When indomethacin was administered to close the PDA, the observed neonatal renal dysfunction was characterised by reduced urine output, decreased free water clearance, and increased blood urea nitrogen and serum creatinine. Early studies related these events to reduced renal PG production (Betkerur et al., 1981; Cifuentes et al., 1979; Levenson et al., 1982; Terragno et al., 1977).
Maternal indomethacin administration often leads to adverse renal effects in the human fetus and neonate (Mogilner et al., 1982; Veerema et al., 1983; Vanhaesebrouk et al., 1988; VanDer Heijden et al., 1988). A reduction in fetal urinary output has been demonstrated to be the underlying mechanism by which indomethacin acts in polyhydramniotic states (Hickok et al., 1989; Krishon et al., 1988; Krishon et al., 1991). These effects of indomethacin seem to be dose dependent as fetal urinary output increased when indomethacin was administered at a dose of 0.017 mg/kg/min, but decreased when the dose was reduced to 0.0025 mg/kg/min in the pregnant sheep model, suggesting fetal antidiuresis at lower plasma indomethacin concentrations (Walker et al., 1992a, 1992b). This incidence of fetal oliguria has been attributed to reduced RBF following inhibition of renal prostaglandins (Matson et al., 1981; Millard et al., 1979). Walker et al (1994) proposed, however, that this fetal oliguria is a result of an effect of AVP on the fetal kidney and not due to RBF. Co-administration of indomethacin and the AVP-V2 receptor antagonist \{(d[CH_2]_5-D-Phe-Ile_4,-Arg_8)-Vassopressin\} resulted in inhibition of oliguria due to indomethacin alone (Walker et al., 1993, 1994), suggesting that oliguria was due to activation of the AVP-V2 receptor and not to a reduction in RBF.
D. *Fetal Effects Of Indomethacin*

Indomethacin has been reported to exert marked fetal side effects following either fetal or maternal drug administration. The latter scenario is widely perceived as a consequence of easy and high degree of placental transfer. Indomethacin increased fetal pulmonary arterial pressure at a dose of 0.05 mg/kg i.v in pregnant ewes (Ohara *et al.*, 1991). The study reported that ductal constriction by indomethacin caused increases in the systolic pulmonary artery-aortic pressure difference as well as fetal heart rate. A marked fall in placental blood flow was noted after indomethacin was given in pregnant rabbits (Katz *et al.*, 1981). Indomethacin reduced CBF in the human fetus by about 40% (Vanbel *et al.*, 1990). A drastic decline in fetal urine output was noted when short term oral indomethacin was used for therapy of preterm labour (Krishon *et al.*, 1988). Indomethacin also stimulates sleep state independent fetal breathing which is abolished by PGE$_2$ infusion in fetal lambs (Jansen *et al.*, 1984). A similar study demonstrated that PG synthesis inhibitors stimulated breathing movements in fetal sheep (Kitterman *et al.*, 1979). Stevenson *et al.*, 1992 evaluated the effects of indomethacin on the ovine fetus. They administered indomethacin at a dose of 10 mg/kg i.v to the ewe and 12 mg/kg i.v to the fetus. Increases in fetal arterial PO$_2$, placental blood flow, and urine osmolality, and decreases in lung liquid flow and free water clearance were observed. Walker *et al.*, 1992b, administered indomethacin as a 0.35 mg/kg bolus followed by a 0.017 mg/kg infusion over 5 h in chronically catheterised ovine
fetuses and observed a marked increase in fetal urinary output (about 84%) and urinary osmolality as well as sodium and chloride concentrations. Fetal heart rate was also increased.

Though there are a number of studies illustrating fetal indomethacin effects there does not exist a comprehensive database on the drug's dose-response characteristics which would rationalise the continued use of the drug to a therapeutic advantage.

1.1.2 Pharmacokinetics Of Indomethacin

1.1.2.1. Absorption

Following oral administration, indomethacin is readily and almost completely absorbed from the gastrointestinal tract with peak plasma concentrations appearing within 120 minutes (Duggan et al., 1972; Alvan et al., 1975). Dissolution of indomethacin capsules was observed to be greater in a low acidity condition compared to a more acidic environment (Aoyagi et al., 1985). Bioavailability of indomethacin is markedly altered by various diets; absorption being faster with a high protein and a high lipid diet compared to a high carbohydrate diet (Wallusch et al., 1978). Bioequivalence studies have indicated that the bioavailability of many indomethacin formulations are far from being equal. A 30% variation in
bioavailability was observed from formulations marketed in Finland (Turakka and Airakinsen, 1974) and in India; two out of ten products studied were found to be bioinequivalent (Chaudhari et al., 1984). However, this bioavailability information should take into account the extensive enterohepatic recirculation indomethacin undergoes as approximately half an intravenous dose of indomethacin is subjected to biliary recycling (Kwan et al., 1976). Bile facilitates the dissolution of poorly soluble indomethacin, due to micellar solubilisation, and thereby increases its rate of absorption (Miyazaki et al., 1980; Miyazaki et al., 1981).

Significant species differences exist in the pharmacokinetics of intravenously (bolus) administered indomethacin. Plasma levels of $^{14}$C-indomethacin were markedly higher in dogs and rats than in guinea pigs and rhesus monkeys, and tissue levels of $^{14}$C were greater in the guinea pig than in the rat (Baer et al., 1974). Indomethacin can easily penetrate the skin of humans (Snyder, 1975; Eckard, 1982), rabbits (Naito and Tsai, 1981), rats (Shima et al., 1981; Wada et al., 1982), and guinea pigs (Inagi et al., 1981), from various formulations (gels, ointments, etc.) indicating the potential for transdermal delivery. Indomethacin is detected in the aqueous fluid, iris, and cornea following ocular absorption in the rabbit (Hanna and Sharp, 1972; Green et al., 1983). Circadian changes, as a determinant of drug administration time, have been observed with indomethacin; its chronopharmacokinetics being well studied in humans (Clench et al., 1981) and in rats (Guissou et al., 1987). The former study indicated that indomethacin
administered in the morning had the highest plasma concentration, and shortest time to peak in addition to greater elimination (Clench et al., 1981).

1.1.2.2. **Protein Binding**

Indomethacin, a weakly acidic organic compound, binds strongly with plasma proteins, the binding being in the order of 90% as determined by equilibrium dialysis (Hucker et al., 1966; Hvidberg et al., 1972). An association constant of $0.86 \times 10^3$ L/mol with a total number of binding sites of 15 has been determined for human serum albumin (Hvidberg et al., 1972). An ultracentrifugation method yielded a considerably higher value of about 99%, with an association constant of $3 \times 10^5$ L/mol and an indication of a single primary binding site and seven secondary binding sites with association constants in the order of $1.4 \times 10^4$ L/mol (Mason and McQueen, 1974). Binding of indomethacin was reduced by aspirin and cinmetacin, and increased by phenylbutazone (Mason and McQueen, 1974; Montero et al., 1986). In another study, using indomethacin as a model drug, Zona et al., 1986, suggested that the conformation of the protein in plasma played a key role in drug-protein interaction, and that binding was favoured when human serum albumin adopted a “loose” structure. Binding of indomethacin to human serum albumin, studied by the dynamic dialysis method, yielded an association constant of $1.4 \times 10^6$/M with 2.71 as the number of primary binding sites (Montero et al., 1986). Indomethacin was bound to proteins in the human cerebrospinal fluid in the order of
40%, as determined by equilibrium dialysis, with albumin being the protein of importance (Muller et al., 1991).

Protein binding studies using radiolabeled indomethacin in maternal and fetal plasma ultrafiltrates in sheep have revealed that 97.6% of the fetal and 98.5% of the maternal drug is bound to plasma proteins (Anderson et al., 1980c). In another study involving unlabeled indomethacin and an equilibrium dialysis procedure, the binding in maternal, fetal, and nonpregnant control was determined to be 85.6%, 43.8%, and 92.1%, respectively (Harris and Van Petten, 1981). Binding of indomethacin in the postnatal stage is speculated to be related to bilirubin levels while levels of albumin in the mother and fetus appear to be responsible for the maternal-to-fetal binding gradient (Nau et al., 1983).

1.1.2.3. Distribution

Indomethacin appears in synovial fluid, peak concentrations occurring about a hour later than in serum in patients with rheumatoid arthritis (Emori et al., 1973). Similar results have been obtained with intraarticularly administered indomethacin (Neander et al., 1992). Following intramuscular administration, the levels of indomethacin in synovial fluid and blood were identical after an hour (Dittrich et al., 1984). Indomethacin was not detected in spinal fluid in the same study, while minimal amounts were detected in another study (Hucker et al., 1966).
Indomethacin was detected in saliva following intravenous administration to dogs, the levels in parotid saliva and mandibular-sublingual saliva being 7.4 and 4.4% of the levels in plasma respectively (Watanabe et al., 1981). Binding of indomethacin to salivary proteins was greater in parotid saliva. Indomethacin appears in breast milk; the milk:plasma ratio being 0.37 (Lebedevs et al., 1991). Indomethacin readily and rapidly crosses the placental membrane in humans (Moise et al., 1990), rabbits (Parks et al., 1977; Harris and Van Petten, 1981), and rats (Klein et al., 1981). Transfer in sheep, however, appears to be more limited (Harris and Van Petten, 1981; Anderson et al., 1980c).

1.1.2.4. Metabolism and Fate

Metabolism of Indomethacin in The Adult

The metabolism of indomethacin in several species (monkey, guinea pig, dog, rats, and humans) has been the subject of numerous reports (Harman et al., 1964; Duggan et al., 1972; Hucker et al., 1966; Yesair et al., 1970; Baer, 1972; Vree et al., 1993). Two major metabolites of indomethacin are N-deschlorobenzoyl-indomethacin (DBI) and O-desmethyl-indomethacin (DMI) (Harman et al., 1964). Both are excreted in unconjugated as well as glucuronide conjugated forms. Therefore, the metabolic pathways in man appear to be O-desmethylaion and N-deacylation as well as conjugation with glucuronic acid. The
major pathway, however, appears to be desmethylation mediated via the hepatic microsomal enzyme system which is followed by a deacylation step that is extramicrosomal in nature (Duggan et al., 1972). These metabolites are found in high concentrations in plasma. Metabolites of indomethacin are devoid of prostaglandin synthesis inhibiting activity (Shen, 1965).

Significant species variation has been observed with the metabolism of indomethacin. The glucuronide of N-deschlorobenzoyl-indomethacin is primarily eliminated in the urine of monkeys and guinea pigs. The primary product of excretion in rabbit urine was indomethacin glucuronide. O-desmethyl-indomethacin was not detected. Neither free nor conjugated indomethacin was detected in rat urine (Harman et al., 1964). There was also minimal urinary excretion of indomethacin or its metabolites in the dog. In another study (Yesair et al., 1970), it was found that dogs excreted most of the drug given i.v. unchanged in the feces. In monkeys indomethacin was extensively metabolised to DBI and excreted in the urine, while in rats DMI was equally excreted in urine and feces.

In human subjects, less than 33% of the administered dose is excreted as indomethacin with 50% of that as the conjugate. Unconjugated desmethyl-indomethacin and desmethyl-desbenzoyl indomethacin contributed to total fecal excretion which was in the order of 21-42% of the dose (Duggan et al., 1972). Following the oral administration of indomethacin (100 mg) in a single human
subject (Vree et al., 1993), the percentage of the dose excreted can be individualised as follows: indomethacin (0.76%), DMI (0.58%), indomethacin acyl glucuronide (32.1%), DMI acyl glucuronide (7.3%), and DMI ether glucuronide (29.8%) (Figure 4).

Figure 4: Metabolism of indomethacin in the adult human (Adapted from Vree et al., 1993)
Hucker et al., 1966, reported that less than 5% of an oral dose of indomethacin was excreted unchanged in the urine. In other studies (Duggan et al., 1972; Kwan et al., 1976), the amount of indomethacin recovered in urine was in the order of 27%, half of which was in the form of glucuronide conjugate. Urinary free metabolites recovered were about 18% and 13% of the dose administered respectively, with the remainder as conjugates. Indomethacin, desmethyl-indomethacin, and deschlorobenzoyl-indomethacin are found in human plasma and urine in high quantities while desmethyl-N-deschlorobenzoyl-indomethacin appears primarily in the feces (Shen and Winter, 1977; Duggan et al., 1972). Indomethacin and its metabolites undergo enterohepatic circulation in humans, rats, dogs, and monkeys (Kwan et al., 1976; Yesair et al., 1970). The metabolic pathway for indomethacin in humans is summarised in Figure 4, while the routes of formation are presented in Table 1.

**Table 1: Metabolites of Indomethacin**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Product of Metabolism</th>
<th>Location of Metabolic Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-Demethylation</td>
<td>DMI</td>
<td>Microsomal oxidation involving cytochrome P450</td>
</tr>
<tr>
<td>Deacylation</td>
<td>DBI</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Glucuronidation</td>
<td>Glucuronides (acyl, ether)</td>
<td>Endoplasmic reticulum</td>
</tr>
</tbody>
</table>
Maternal/Fetal/Neonatal Indomethacin Metabolism

In rabbits, there is a significant increase in the production of DMI after 2 weeks of life (Evans et al., 1981). In the same study, indomethacin was found to be mainly metabolised to DBI in neonatal rabbit hepatocytes by non-microsomal deacylation. The major components of eliminated drug in adults were the glucuronides of indomethacin and its metabolites, however, less than 8% of the total metabolites were conjugated in hepatocytes from 25 day old rabbits indicating that glucuronidation was not the major metabolic pathway in the neonate (Evans et al., 1981). The rate of production of DBI and DMI in fetal rabbits is in the order of 0.23 and 0.13 nmoles/10^6/min, respectively. A very high maturational increase in DBI production was determined on day 12 of postnatal life with a value of 1.3 nmole/10^6/min (Evans et al., 1981). In newborn rats the metabolism of indomethacin is minimal and has been attributed to reduced affinity between enzyme and substrate as well as to the presence of competitive inhibitors. There is, however, a progressive increase in the formation of DMI from day 1 of postnatal life to 60 days of age (Clozel et al., 1986). Endogenous cytosolic factors appear to selectively inhibit microsomal oxidation of indomethacin in neonatal rabbit liver (Evans et al., 1981). This finding is consistent with the changes in K_m observed in the neonatal rat study (Clozel et al., 1986).
1.1.2.5. **Elimination Kinetics**

Pharmacokinetic reports on indomethacin have been extensive ever since its introduction in 1962. However, early studies depended heavily on spectrophotometric and spectrofluorimetric methods of analysis and reported elimination half-lives were in the range of 1.5-2.3 hours (Duggan et al., 1972; Hucker et al., 1966; Hvidberg et al., 1972; Traeger et al., 1972). Given the sensitivity limitations of these assay methods it seems highly probable that the true elimination phase was not accurately determined. Using a more sensitive mass fragmentographic method, however, the mean elimination half-life of indomethacin was found to be in the range of 6-7.6 hours following either oral, i.v., or rectal administration (Alvan et al., 1975).

While differences in assay methods and sensitivity account for some of the inter- and intra-individual variability in elimination kinetics of indomethacin, the majority of this is considered to be due to extensive enterohepatic recycling and the irregularity of biliary discharge (Helleberg, 1981).

Plasma concentration-time data obtained from single dose studies were fit to a two compartment open model (Alvan et al., 1975). Decay of indomethacin from plasma was characterised by a rapid initial phase (t$_{1/2}$ ~ 90 min) and a slower elimination phase (t$_{1/2}$ ~ 10 hours). The apparent volume of distribution of
indomethacin was in the order of 0.34 L/Kg - 1.57 L/Kg, and plasma clearance varied from 0.044 - 0.109 L/Kg (Alvan et al., 1975). Steady state concentrations in plasma ranged from 0.3-0.6 µg/mL. The long elimination phase was speculated to be due to enterohepatic recycling of the drug. There is no indication of dose-dependent elimination (Alvan et al., 1975; Duggan et al., 1972).

Using fluorimetric methods, the half-life of indomethacin in adults and newborns was found to be 2.2 h and 14.7 h respectively (Traeger et al., 1973). Using a gas chromatographic (GC) method, Friedman et al., 1978, determined the half-lives of indomethacin in two premature infants with patent ductus arteriosus to be 21 and 24 h respectively, with a volume of distribution of 4.4 L/Kg and plasma clearance of 0.145 L/Kg/h in one of the infants. While drug elimination in urine was not determined, it was suggested that the prolonged half-life could be due to renal immaturity. In another study involving nine preterm infants, the half-life following oral administration was found to be 11-20 hours (Bhat et al., 1979). These same authors report that protein binding of indomethacin was in the order of 95%. The kinetic parameters obtained for indomethacin in neonates from various studies are summarised in Table 2.
Table 2: Pharmacokinetics of Indomethacin in Neonates

<table>
<thead>
<tr>
<th>Postnatal Age (days)</th>
<th>Birth Weight (g)</th>
<th>$t_{1/2}$ (h)</th>
<th>$V_{area}$ (L/Kg)</th>
<th>CI (mL/Kg/h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-14</td>
<td>1301 ± 72</td>
<td>14.5 ± 3</td>
<td>0.80</td>
<td>38</td>
<td>Thalji et al., 1979</td>
</tr>
<tr>
<td>10.8 ± 6.0</td>
<td>1253 ± 389</td>
<td>20.7 ± 8</td>
<td>0.35 ± 0.21</td>
<td>13 ± 9.5</td>
<td>Thalji et al., 1980</td>
</tr>
<tr>
<td>9.5 ± 2.2</td>
<td>1300 ± 200</td>
<td>33.9 ± 11.7</td>
<td>0.35 ± 0.16</td>
<td>7.6 ± 3.0</td>
<td>Vert et al., 1980</td>
</tr>
<tr>
<td>8.8 ± 6.8</td>
<td>1213 ± 309</td>
<td>32</td>
<td>0.19-0.61</td>
<td>7.0</td>
<td>Brash et al., 1981</td>
</tr>
<tr>
<td>10.2 ± 2.2</td>
<td>1200 ± 400</td>
<td>21.6</td>
<td>0.3</td>
<td>9.2</td>
<td>Yeh et al., 1985</td>
</tr>
<tr>
<td>5.7 ± 4.2</td>
<td>1125 ± 399</td>
<td>-</td>
<td>0.280</td>
<td>2.63</td>
<td>Wiest et al., 1991</td>
</tr>
<tr>
<td>9.5 ± 1.3</td>
<td>1455 ± 119</td>
<td>16.2 ± 2.9</td>
<td>0.36 ± 0.01</td>
<td>17.1 ± 2.8</td>
<td>Bhat et al., 1980</td>
</tr>
</tbody>
</table>

The elimination half-life is markedly prolonged in neonates when compared to adults and is related inversely to gestational age (Vert et al., 1980; Evans et al., 1979). Data, however, is severely lacking for indomethacin in-utero pharmacokinetics in the fetus.

Following a single oral dose of indomethacin, the total clearance in the elderly (79.5 ± 1.3 y) was 0.8 mL/min/Kg compared to 1.4 mL/min/Kg in younger subjects (36.9 ± 3 y) with an elimination rate constant of 0.23 h⁻¹ and 0.32 h⁻¹, respectively (Oberbaur et al., 1993). The fall in clearance in the elderly has been attributed to a decline in renal function and a reduced renal excretion of indomethacin since about 27% of the dose is eliminated through the kidneys.
(Duggan et al., 1972; Kwan et al., 1976; Traeger et al., 1973; Oberbaur et al., 1993).

1.1.3. Placental Transfer

The maternal-placental-fetal unit is a complex pharmacokinetic system comprised of two independent circulations. The lipoprotein nature of the placenta is, however, very similar to other biological membranes in the body and plays an important role in controlling drug transfer. The unbound lipophilic drug in the unionised form can easily cross the placenta by passive diffusion while other mechanisms such as facilitated diffusion, active transport, and pinocytosis are more prevalent for placental passage of nutrients and other endogenous substances. The mechanisms of placental transfer and subsequent degree of fetal drug exposure have been the subjects of numerous reviews over the years (Moya and Thorndike, 1962; Green et al., 1979; Krauer et al., 1980; Levy, 1981; Szeto et al., 1982b, 1982d; Mihaly and Morgan, 1984; Reynolds and Knott, 1989; Szeto, 1993).

Several factors may potentially affect maternal-fetal drug exchange and fetal exposure during the progression of normal pregnancy including the following: (i) physico-chemical properties such as lipophilicity, molecular weight, and ionisation, (ii) placental permeability, stage of gestation, and type of placentation, (iii) orientation of maternal and fetal placental blood flows, (iv) placental metabolism of
drugs, (v) fetal circulation, (vi) pH, (vii) uterine and umbilical blood flows, and (viii) protein binding.

1.1.3.1. Pharmacokinetic Characterisation

Pharmacokinetic evaluation of maternal/fetal drug disposition generally involves consideration of the mother and fetus each as a single compartment. Such a system assumes quick equilibration of the drug. If the drug enters fetal tissues slowly with concurrent elimination from the maternal compartment, a deep compartment is presumed to exist. The pharmacokinetic data of meperidine was treated using the mother and fetus each as a single compartment (Szeto et al., 1978). Such a system assumes fetal and maternal drug concentrations to be at steady state. If a biexponential decay of the drug is observed in the fetus, then the fetus should be considered as a two compartmental model to account for fetal distribution kinetics (Szeto, 1982). The placenta (Krauer and Krauer, 1977) and the amniotic fluid (Brien and Clarke, 1988) have also been considered as distinct kinetic compartments.

1.1.3.2. The Chronically Instrumented Pregnant Sheep Model

Several experimental animal models, such as rats, guinea pigs, baboons, goats, pigs, sheep, and monkeys, have been used in placental transfer studies due to
ethical and technical constraints precluding such studies in humans. The commonly used animal models in fetal medicine have been reviewed (Nathanielsz, 1980). Small animal models such as rats and guinea pigs are often inadequate when serial sampling is required due to low blood volume. Of the available models, the anatomy and physiology of the monkey closely parallels the human situation and theoretically would seem to be the best to conduct studies on placental transfer. However, several complications such as the cost of the preparation, ease of handling, and size limitation of the fetus restrict the use of this model.

Sheep, on the other hand, are docile, have large fetuses conducive for chronic preparation and similar physiology and biochemistry (Comline and Silver, 1974) compared to humans. Both sheep and primate models demonstrate similar fetal behavioural states (viz. fetal breathing movements, eye movements, etc) in the last trimester of gestation (de Vries et al., 1982; de Vries et al., 1985; Nijhuis et al., 1982). They have been used to study in-utero fetal physiology and placental transfer of a number of endogenous compounds (Szeto et al., 1978; Van Petten et al., 1978; Rurak et al., 1991). Sheep do, however, possess a different type of placentation, and care must be exercised when extrapolating data to the human situation, as is necessary with any animal model.
1.1.3.3. **Placental Transfer of Indomethacin**

Indomethacin has been reported to traverse the placental membrane in rabbits (Parks *et al.*, 1977; Harris and Van Petten, 1981), rats (Klein *et al.*, 1981), sheep (Harris and Van Petten, 1981; Anderson *et al.*, 1980c), and humans (Moise *et al.*, 1990), however, detailed information is lacking.

When indomethacin was given orally at a dose of 2 mg/kg in pregnant rabbits, in a study period of two hours, the drug was detected in maternal (M) and fetal (F) blood and amniotic fluid within 15 minutes; the F/M ratio at the end of the study being 0.568. Although indomethacin did appear in the amniotic fluid, its concentration relative to maternal and fetal blood levels was much lower. At the end of the study period detectable concentrations of free drug were seen in maternal urine, i.e., 19.2 μg/mL, and bile, i.e., 0.35 μg/mL (Parks *et al.*, 1977). In another study involving pregnant rabbits, indomethacin was administered subcutaneously in a dose of 10 mg/kg for a study period of six hours (Harris and Van Petten, 1981). In contrast to the former study where fetal plasma levels remained lower than maternal levels throughout the experiment, fetal plasma indomethacin concentrations exceeded maternal levels and remained elevated over a period of time before falling. At the end of the 1st hour, the F/M ratio was 0.795. Indomethacin was detected in the amniotic fluid 2 hours after dosing and its concentration at the end of six hours was comparable to that of maternal plasma. A major concern as to the reliability of
these data is the analytical methodology involved, namely, spectrophotometric and spectrofluorimetric methods of analysis.

In a study with pregnant rats, indomethacin was administered at a dose of 4 mg/kg by gastric intubation. At the end of the 3rd hour, maternal and fetal plasma indomethacin concentrations were 16.8 ± 1.6 µg/mL and 4.6 ± 0.3 µg/mL, respectively, with a F/M ratio of 0.274 (Klein et al., 1981).

In 1980c, Anderson et al. were the first group to attempt description of placental transfer in quantitative terms. Their study involved simultaneous administration of $^{14}$C-indomethacin as bolus in the fetus and $^{3}$H-indomethacin as an infusion in the ewe. Maternal and fetal blood were analysed for radioactivity in a scintillation counter and placental blood flows measured using radiolabeled microspheres. Placental clearance ($Cl_p$) was determined by the formula, $Cl_p = C_{tot} \times \frac{F_{ss}}{M_{ss}}$, where $C_{tot}$ is the total clearance, and $F_{ss}$ and $M_{ss}$ are fetal and maternal steady state concentrations. Fetal and placental tissue clearances were estimated to be 3.63 ± 0.58 ml/min/kg and 2.10 ± 0.32 ml/min/kg, respectively, with a F/M ratio of 0.28. Major criticisms of this method include: (a) the requirement that steady state conditions prevail, (b) it only provides single point estimation, (c) the model is limited to cases where fetal clearance is zero and simultaneous solving of the equations is impossible (Szeto, 1982b), (d) it is applicable only in instances where drug clearance by the placenta from mother and fetus are equal, (e) the issue of an
isotope effects not addressed, and (f) the method cannot differentiate intact drug from its metabolites.

When indomethacin was administered as an intravenous infusion at a dose of 10 mg/kg into pregnant ewes, maternal and fetal plasma levels at the end of infusion were $13.5 \pm 0.7 \, \mu g/mL$ and $0.6 \pm 0.1 \, \mu g/ml$ with a F/M ratio of 0.04 (Harris and Van Petten, 1981). Indomethacin concentrations in amniotic fluid were markedly higher than fetal and maternal plasma levels at the end of the study period. A major concern is again the non-specific analytical methodology involved.

Moise et al., 1990, administered 50 mg of indomethacin orally to pregnant human patients. Maternal and fetal indomethacin levels (cordocenteses) were $218 \pm 21 \, ng/ml$ and $219 \pm 13 \, ng/ml$, respectively, with a F/M ratio of 1.004. The fetal plasma to amniotic fluid concentration ratio was $10.0 \pm 1.2$.

1.1.4. Analytical Methods

Indomethacin has been analysed in biological fluids, for a variety of study requirements, by several methods including liquid chromatography (HPLC), gas chromatography (GC), mass fragmentography, radioimmunoassay (RIA), and spectrophotometric methods. The detection limits of most HPLC assays fall in the range of 10 - 100 ng/mL, GC assays in the range of 1-50 ng/mL, and RIA methods
in the range of 50 ng/mL. Spectrophotometric methods of analysis are sensitive in the μg range with poor selectivity.

Major disadvantages of available methods include:

1. Radioimmunoassay is highly cross reactive to glucuronide conjugates of indomethacin and its metabolites (Hare et al., 1977).

2. HPLC assays with fluorescence detection require post-column in-line hydrolysis necessitating auxiliary pump setups (Bernstein and Evans, 1982; Mawatari et al., 1989), and those with UV detection are not sensitive in the lower nanogram range.

3. GC methods are plagued by long retention times (Nishioka et al., 1990), multi-step derivatisation, lack of internal standards (Helleberg et al., 1976; Ferry et al., 1974), long derivatisation reaction times (Nishioka et al., 1990), use of hazardous and corrosive derivatising agents (Evans, 1980; Helleberg et al., 1976), and inadequate internal standards (Evans, 1980; Guissou et al., 1983). Other complications include large sample size (≥1 mL), inadequate recovery, multiple extractions, and high variability at lower concentrations.

Of the available methods, GC with electron-capture detection would appear to provide the best sensitivity and selectivity; only one GC method has applied
fused silica capillary column technology to indomethacin measurement to date (Nishioka et al., 1990).

Several derivatising reagents have been investigated for GC assays, viz., diazomethane (Guissou et al., 1983; Giachetti et al., 1983; Plazonnet and Van den Heuvel, 1977), diazopropane (Arbin, 1977), 1-ethyl-3-p-tolyltriazine (Nishioka et al., 1990), pentafluorobenzyl bromide (Sibeon et al., 1978), hexafluoroisopropanol and trifluoroacetic anhydride (Matsuki et al., 1983), bis (trimethylsilyl) acetamide (Plazonnet and Van den Heuvel, 1977), ethyl iodide and extractive alkylation (Jensen, 1978), pentafluoropropanol and pentafluoropropionic anhydride (Evans, 1980), and diazoethane (Helleberg, 1976). The simplest of the derivatisation reactions is with bis (trimethylsilyl) acetamide, a silylating agent producing trimethylsilyl esters of indomethacin.

Selection of internal standards for indomethacin, by either HPLC or GC, is yet another problem encountered in the literature. Several reports incorporate internal standards with no structural similarity to indomethacin including phenylbutazone (Mehta and Calvert, 1983), phenacetin (Avgerinos and Malamataris, 1989), testosterone (Kwong et al., 1982), itraconazole (Al-Angary et al., 1990), and penfluridol (Guissou et al., 1983). Others use inappropriate internal standards such as indomethacin propyl ester (Nishioka et al., 1990) and indomethacin methyl ester (Arbin, 1977), and still others do not use any at all.
(Helleberg, 1976; Ferry et al., 1974). Some suitable internal standards include α-methyl indomethacin (Stubbs et al., 1986; Bayne et al., 1981), and 5-fluoro indomethacin (Sibeon et al., 1978).

1.2. Rationale and Specific Aims

1.2.1. Rationale

Available analytical methods for indomethacin were impractical for use in the current study design with respect to sensitivity, selectivity, speed, sample volumes, and variability at lower concentrations. Therefore, there is a need to develop a sensitive, selective, and rapid analytical procedure.

Available data on placental transfer were limited and may be unreliable because of the analytical methodology used and inadequate experimental design. The chronically instrumented pregnant sheep model is a versatile animal model for studying placental drug transfer and maternal/fetal drug pharmacokinetics. The model closely simulates the human system with respect to fetal renal, cardiorespiratory, and behavioural states (de Vries et al., 1982; de Vries et al., 1985) and fetal biochemistry and physiology (Van Petten et al., 1978). The model also allows for serial sampling to an extent which is impractical in small animal models such as rats, rabbits, and guinea pigs.
Reports on the limited placental transfer of indomethacin in sheep preclude the use of the Szeto model (Szeto et al., 1982b) where measurable steady-state concentrations of drug must be obtained in both the ewe and fetus following individual maternal and fetal infusion. This coupled with the disadvantages of the Anderson method (Anderson et al., 1980c) of clearance estimation illustrate the need to determine placental clearance values by an alternate technique. The Fick diffusion model, employing direct fetal administration to provide plasma concentrations corresponding to those observed in studies of human tocolysis (Moise et al., 1990), appeared then, to be a more suitable and reliable method for our studies.

1.2.2. Specific Aims

1. To develop and optimise a sensitive and selective gas chromatographic method with electron-capture detection for indomethacin in sheep biological fluids using fused-silica capillary column technology.

2. To apply the developed method for the determination of plasma, urine, amniotic and tracheal fluid indomethacin concentrations following long term infusion of the drug in chronically instrumented sheep fetuses, and to calculate placental, non-placental, and renal clearances.
2. EXPERIMENTAL

2.1 Materials and Supplies

2.1.1 Chemicals

Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione, lot 110H0632), acemetacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid carboxymethyl ester, lot 38F0022), sulindac ([Z]-5-fluoro-2-methyl-1-[p-(methylsulfinyl) benzylidene] indene-3-acetic acid, lot 101H0643), indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole 3-acetic acid, lot 60H0448), carprofen (lot 28F0440), zomepirac sodium (5-[p-chlorobenzoyl]-1,4-dimethylpyrrole-2-acetic acid [sodium salt, lot 13H0228) and flufenamic acid (lot 61H3562) were obtained from Sigma Chemicals Co., St. Louis, MO, USA. 5-Fluoroindole-2-carboxylic acid (lot 10130DF) was purchased from Aldrich Chemicals Co., Milwaukee, WI, USA. 5-Fluoroindomethacin (Merck Research Laboratories, West Point, PA, USA) and α-methylindomethacin (Merck-Frosst Inc., Kirkland, Quebec) were generous gifts. Injectable ampicillin (Penbritin\textsuperscript{R}), injectable gentamicin sulphate (Garamycin\textsuperscript{R}), injectable atropine sulphate, halothane (Fluothane\textsuperscript{R}), and injectable heparin (Hepalean\textsuperscript{R}), were obtained from the Pharmacy Department of Women's Hospital, Vancouver, BC. Sodium chloride for injection USP was
obtained from Abbott Laboratories, Montreal, Quebec. The enzyme, β-glucuronidase (Bovine liver, type B-1), lot 117F7256, was purchased from Sigma Chemicals Co., St. Louis, MO, USA.

2.1.2 Reagents

Sodium acetate (anhydrous, analytical reagent grade) was obtained from BDH (Toronto, Ontario) and glacial acetic acid (American Chemical Society, ACS reagent) from Allied Chemical (Pointe Claire, Quebec). Triethylamine was purchased from Pierce Chemical Co., Rockford, Illinois, USA.

Pentafluorobenzylbromide (PFBBr, lot 930413082), N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA, lot 930817150), and N,O-Bis-(trimethylsilyl) acetamide (BSA), were purchased from Pierce Chemical Co. (Rockford, Illinois, USA). 2,2,3,3,3-Pentafluoropropanol (97%) and pentafluoropropionic anhydride (99%) were obtained from Aldrich Chemical Co., Milwaukee, WI, USA.

2.1.3 Solvents
The following (distilled in glass) were purchased from Caledon Laboratories Ltd., Georgetown, Ontario: ethyl acetate, ethyl ether, hexane, toluene, dichloromethane, and acetonitrile. Deionised, high-purity water (hereafter referred to as water) was produced on-site by reverse osmosis using a Milli-Q® water system (Millipore, Mississauga, Ontario).

2.1.4. Gases

Hydrogen, carrier gas for GC-ECD, and argon-methane (95:5), make-up gas for ECD, and ultra-pure helium, carrier gas for GC-MS, were obtained from Matheson Gas Products, Edmonton, Alberta. Nitrogen NF was obtained from Praxair Canada Inc., Mississauga, Ontario.

2.1.5. Supplies

Membrane filters (0.45) (Millipore, Mississauga, Ontario); disposable plastic pipet tips (National Scientific, San Rafael, CA, USA); needles and disposable plastic syringes (Luer-Lok®) for drug administration and sample collection (Beckton-Dickinson Canada, Mississauga, Ontario); borosilicate glass pasteur pipets (John Scientific, Toronto, Ontario); heparinised blood gas syringes (Marquest Medical Products Inc., Englewood, CO, USA); heparinised Vacutainer®
tubes (Vacutainer Systems, Rutherford, NJ, USA); Pyrex® 15 mL disposable culture tubes (Corning Glass Works, Corning, NY, USA); polytetrafluoroethylene (PTFE) lined screw caps (Canlab, Vancouver, British Columbia); polystyrene tubes (Evergreen Scientific International Inc., Los Angeles, CA, USA); polyvinyl tubing for catheterisation (Dow Corning, Midland, MI, USA).

2.2 Instrumentation

2.2.1 Gas Chromatography

A HP (Hewlett-Packard, Avondale, PA, USA) model 5890 series II gas chromatograph equipped with a HP model 7673 autosampler, split-splitless capillary inlet system, model HP 3365 chemstation, and a 63Ni electron-capture detector, was used for all analyses. A Pyrex® glass inlet liner (78 mm x 4 mm, i.d.) and Thermogreen® LB-2 silicone rubber septum (Supelco, Bellefonte, PA, USA) were used for all analyses.

The optimised operating conditions for routine analyses were: column, Ultra-2® fused silica capillary column cross-linked with 5% phenylmethylsilicone, 25m x 0.31 mm x 0.52μm film thickness, (Hewlett-Packard, Avondale, PA, USA); 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; column head pressure, 10 psi (i.e., 70 kPa, flow rate ~1 ml/min); purge delay time, 0.75 min; make-up gas, argon-methane, 95:5 (flow rate, 65 mL/min).

2.2.2. Gas Chromatography-Mass Spectrometry

A HP model 5890 series II gas chromatograph (Hewlett-Packard, Avondale, PA, USA) equipped with a HP 7673 automatic injector system, and an HP model 5989A mass spectrometer, HP 59827A vacuum gauge controller, and an HP 98789A on-line computer, with a HP Ultra-2® fused silica capillary column (25 m x 0.31 mm x 0.52μm film thickness) was used for mass spectrometric studies. The carrier gas, helium, was operated at a column head pressure of 3 psi. In the chemical ionisation mode, methane was used as the reagent gas. The electron-impact-MS ionisation energy was 200 eV, emission current 250 μA, and source pressure, 1.2 torr.

2.2.3. Columns
Ultra-2® fused silica capillary column cross-linked with 5% phenylmethylsilicone (25 m x 0.31 mm, i.d., x 0.52 μm film thickness) and Ultra-2® (25 m x 0.2 mm x 0.33 μm film thickness) narrow-bore columns were obtained from Hewlett-Packard, Avondale, PA, USA; a DB-1701 column (30 m x 0.25 mm, i.d., x 0.25 μm film thickness) was obtained from J & W Scientific, Folsom, CA, USA.

2.2.4. Liquid Chromatography-Mass Spectrometry/Mass Spectrometry

A Varian VG Biotech LC/MS/MS system (Fisons Instruments, VG Biotech, Altrincham, UK) equipped with an electrospray interface, VG Quattro triple quadrupole mass spectrometer (two high performance quadrupole mass analysers, and a hexapole collision cell between the two mass analysers), a Dynolite™ detector system, with a PC-compatible computer to run the VG MassLynx software system (Mass Spectrometry Data System, VG Biotech, Fisons Instruments, Pointé Claire, Québec, Canada).

2.2.5. Differential Scanning Calorimeter
A 910 Differential Scanning Calorimeter module interfaced to a Du Pont Series 99 Thermal Analyzer (Du Pont, Wilmington, DE, USA) was used for indomethacin melting point and water content determinations.

2.2.6. **Nuclear Magnetic Resonance Spectrometry**

Proton NMR spectra were obtained on a Bruker WH-200 spectrometer (200 MHz) in the Department of Chemistry, University of British Columbia, Vancouver, BC. D₆-dimethylsulfoxide was used as the dissolution solvent.

2.2.7. **General Equipment**

Vortex-type mixer (Vortex-Genie®), incubation oven (Isotemp® 500 series), Accumet® pH meter 915, Fisher Scientific Industries, Springfield, MA, USA; IEC Model HN-SII centrifuge, Damon/IEC Division, Needham Hts. MA, USA; rotating-type tube mixer (Labquake® Tube Shaker, model 415-110), Labindustries, Berkeley, CA, USA; infusion pump (Harward model 944), Harvard Apparatus, Millis, MA, USA.

2.2.8. **Physiological Monitoring**
Polygraph recorder to monitor fetal arterial pressure, venous pressure, amniotic fluid pressure, heart rate, and breathing rate (Sensormedics Inc., Anaheim, CA, USA); Apple IIe computer and computer data acquisition system consisting of Interactive Systems (Daisy Electronics, Newton Square, PA, USA), analog to digital converter and clock card (Mountain Software, Scott’s Valley, CA, USA); Instrument Laboratory System 1306 pH/blood gas analyser (Instrument Laboratory System, Lexington, MA, USA); Advanced Digimatic Osmometer 3D2 to measure urine osmolality, Needham Heights, MA, USA; American Optical hand-held refractometer to measure plasma protein concentration (American Optical); YSI Glucose/Lactate Analyser (Yellow Springs Instruments, Yellow Springs, CO, USA) to measure glucose and lactate concentrations; Nova 5 Electrolyte Analyser (Newton, MA, USA) to measure urine electrolyte concentrations.

2.3. **Preparation of Stock Solutions**

2.3.1. **Preparation of Drug Stock Solution**

2.3.1.1 **Indomethacin**

Approximately 10 mg of indomethacin was accurately weighed, transfered 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. One mL
of the latter solution was further diluted to volume with water in a 50 mL volumetric flask to give a final working stock solution with a concentration of 0.04 \( \mu \text{g/mL} \). Volumes of 25, 50, 200, 400, and 800 \( \mu \text{L} \) were used to prepare the calibration curve.

2.3.2 Preparation of Internal Standard Solutions

2.3.2.1. Zomepirac Sodium

Approximately 27.04 mg of zomepirac sodium (equivalent to \( \approx 25 \) mg of zomepirac free acid) was accurately weighed and transferred to a 250 mL volumetric flask and made up to volume with water. A 1 mL aliquot of this solution was transferred to a 100 mL volumetric flask and made up to volume with water to produce a working stock solution with a concentration of 1000 ng/mL. A 100 \( \mu \text{L} \) aliquot of this solution was used in the assay.

2.3.2.2. Sulindac

Approximately 10 mg of sulindac 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 producing a
concentration of 2 μg/mL. A volume of either 100 μL (200 ng) or 50 μL (100 ng) was used.

2.3.2.3 α-Methyl Indomethacin

Approximately 5 mg of alpha-methyl indomethacin 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.

2.3.2.4 Other Experimental Internal Standards

Working solutions of 2 μg/mL were separately prepared for: 5-fluoro indole-2-carboxylic acid, 5-fluoro DL tryptophan, 5-fluoro indole-3-acetic acid, flufenamic acid, carprofen, acemetacin, phenylbutazone, and 5-fluoro-indomethacin.

2.3.3 Preparation of Reagent Solutions
Acetate buffer pH 5.0 was prepared according to British Pharmacopoeia 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 ≈ 5.00.

Acetate buffer pH 2.45, B.P., was prepared by mixing 200 mL of 1M hydrochloric acid with 200 mL of 1 M sodium acetate and diluting to 1000 mL with water. The pH of the final solution was adjusted to 2.45 with 1 M hydrochloric acid.

Acetate buffer pH 3.5 was prepared by dissolving 25 g of ammonium acetate in 25 mL of water and adding 38 mL of 7 N hydrochloric acid.

Acetate buffer pH 4.6 was prepared by dissolving 5.4 g of sodium acetate in 50 mL of water, adjusting to pH 4.6 with glacial acetic acid and diluting to 100 mL with water.

Acetate buffer pH 6.0 was prepared by dissolving 100 g of ammonium acetate in 300 mL of water, adjusting to pH 6.0 with 4.1 mL of glacial acetic acid and diluting to 500 mL with water.
2.4. Sample Preparation

2.4.1. Analysis of Intact Indomethacin Levels

Ovine fetal fluids (plasma, urine, etc.), typically 0.1 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-methyl indomethacin, 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 ≈ 5.00). The mixture was gently vortex-mixed and 5 mL of ethyl acetate was added. The aqueous phase was extracted for 20 minutes on a rotary shaker. The samples were then placed in a freezer at -5 °C for 10 minutes to facilitate breakage of any emulsion formed in the extraction step. This was followed by centrifugation for 10 minutes 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. This was vortex-mixed and placed in an oven at 60 °C for 50 minutes. 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 gas chromatograph. Scheme-I summarises the sample preparation protocol.
Biological Sample
(fetal plasma, urine, amniotic, or tracheal fluid)
0.1-1.0 mL
+
Internal Standard
(α-methyl indomethacin, 50 μL, 25 ng)

2.0 mL pH 5.0 Acetate Buffer
+
Distilled Water, q.s. to 3.0 mL total aqueous phase
+
5.0 mL Organic Solvent (Ethyl Acetate)

Mix 20 minutes on rotary shaker

Freeze at -5 °C for 10 minutes

Centrifuge at 3000 g for 10 minutes

Aqueous
(Waste)

Organic

Evaporate to dryness under nitrogen gas at 37 °C

Derivatise residue with MTBSTFA (8 μL) and toluene (92 μL) at 60 °C for 50 minutes

Cool to room temperature and add toluene to obtain a final reconstitution volume of 300 μL

2.0 μL for injection

Scheme 1: Sample Preparation Procedure For Indomethacin
2.4.2. Analysis of Glucuronide Conjugates

The glucuronide conjugates in urine and amniotic fluid samples were analysed by enzyme hydrolysis using β-glucuronidase. The method for preparation of samples is similar to that described above. About 200 mg of β-glucuronidase was accurately weighed and made to volume in a 25 mL volumetric flask with pH 5.00 acetate buffer, resulting in a concentration of 5000-Units/mL. Prior to extraction with ethyl acetate, 0.5 mL of the enzyme solution was added to the buffered urine samples, and incubated at 37°C for 3 hours. Due to limited volume of urine samples, it was not possible to conduct a rigorous study to determine optimum hydrolytic conditions (incubation time and amount of enzyme) with a satisfactory “n” value. However, a study was carried out with these limitations along with input from Bayne et al., 1981, and Vree et al., 1993 (Vree et al., 1993, determined optimum conditions for indomethacin conjugates in urine to be 10,000 Units of enzyme activity and an incubation time of 2 hours, while the conditions reported by Bayne et al., were 1500 Units and 30 minutes, respectively). The concentration of glucuronide-conjugated indomethacin was calculated by subtracting the non-conjugated indomethacin concentration (as determined by the method described in section 2.4.1 above) from the samples measured following hydrolysis.
2.5. **Preparation of Calibration Curve**

2.5.1. **Calibration Curve with α-Methyl Indomethacin**

Serial quantities of the working indomethacin stock solution (1, 2, 8, 16, and 32 ng) were added to 0.1 mL of blank fetal sheep biological fluid (plasma, urine, amniotic, or tracheal fluid). A 50 μL aliquot of the internal standard (α-methyl indomethacin) was added, and the solution made up to a fixed volume of 1 mL. The samples were then extracted and derivatised as described previously. Determination of indomethacin concentration was made by plotting the peak area ratios of the tert-butyldimethylsilyl derivatives of indomethacin and α-methyl indomethacin against the known amount of indomethacin added to each sample.

2.5.2. **Calibration Curve with Zomepirac Sodium**

Serial quantities of the working indomethacin stock solution (1, 2, 8, 16, and 32 ng) were added to 0.1 mL of blank fetal sheep biological fluid (plasma, urine, amniotic or tracheal fluid). A 100 μL aliquot of the internal standard (zomepirac sodium) was added, and the solution made up to a fixed volume of 1 mL. The samples were then extracted and derivatised as described previously. Determination
of indomethacin concentrations was made by plotting the peak area ratios of the tert-butyldimethylsilyl derivatives of indomethacin and zomepirac against the known amount of indomethacin added to each sample.

2.6. Chemistry

2.6.1. Derivatisation Procedures For Indomethacin


A specified amount of indomethacin (1 µg/mL) was evaporated to dryness under oxygen-free nitrogen in a water bath maintained at 40 °C. The residue was dissolved in 90 µL of ethyl acetate and 10 µL of BSA added. The contents were derivatised for 15 min at 60 °C in a hot air oven, cooled to room temperature and diluted to 1 mL with ethyl acetate before injecting 1 µL into the GC.

[2] Reaction with N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA)
A methanolic solution of indomethacin (0.5 μg/mL) was evaporated to dryness under oxygen-free nitrogen gas in a water bath maintained at 40 °C. The residue was derivatised with 100 μL of toluene containing 8 μL of MTBSTFA at 60 °C for 30 minutes. The solution was cooled to room temperature and 1 or 2 μL injected into the GC.


A methanolic solution of indomethacin (0.5 μg/mL) was evaporated to dryness under oxygen-free nitrogen gas in a water bath maintained at 40 °C. To each tube about 25 mg of potassium carbonate (catalyst) and 0.5 mL of a stock solution of PFBBBr (10 μL of supplied solution is made up to 10 mL with acetone) were added. The reaction was carried out at 60 °C for 30 minutes. Excess derivatising agent was removed using a stream of nitrogen gas at room temperature. To each tube was then added 1 mL of distilled water and 1 mL of hexane. The tubes were mixed on a shaker for 10 minutes and centrifuged at low speed for a further 10 minutes. About 2 μL of the upper hexane layer was injected into the GC.

A methanolic solution of indomethacin was evaporated to dryness under oxygen-free nitrogen in a water bath maintained at 40 °C. To the residue was added 10 μL of a mixture of 2,2,3,3,3,-pentafluoro-1-propanol in pentafluoropropionic anhydride (1:4, v/v) and derivatised at 75 °C for 20 min. The solution was allowed to cool to room temperature, evaporated to dryness under nitrogen, and reconstituted in 10 μL of ethyl acetate and 1 or 2 μL injected into the GC.

2.7. Optimisation of Assay Parameters

2.7.1. Derivatisation of Indomethacin

The following derivatisation parameters were examined:

1. Selection of a suitable derivatising agent: reaction with BSA, MTBSTFA, PFBBr, or PFP (see section 2.6.1.).
2. Volume of derivatising agent-2 μL to 100 μL.
3. Temperature of derivatisation-27 to 90 °C.
4. Time course of derivatisaton-10 min-1 hour.
5. Selection of derivatisation solvent (toluene, acetonitrile, etc.)
6. Reconstitution volume (0.1-1.0 mL)
2.7.2. **Chromatography**

1. Selection of a suitable internal standard (sulindac, indole derivatives, zomepirac, carprofen, acemetacin, phenylbutazone, 5-fluorooindomethacin, α-methylindomethacin).

2. Injection mode (split vs splitless).

3. Injection port temperature (140-300 °C).

4. Oven temperature programming (various initial temperatures, ramp rates, and final temperatures and times were examined).

5. Column (nonpolar vs polar, wide bore vs narrow bore).

6. Column head pressure (5-15 psi).

7. Injector port liner configuration (packed vs unpacked inlet liner).

8. Purge activation time (10-120 seconds).

9. Detector temperature (300-360 °C).

2.7.3. **Extraction**

1. Solvent for extraction (ethyl acetate, hexane, hexane-ethyl acetate mixtures, toluene, toluene-triethylamine (0.0125M) mixtures, hexane-isopropylalcohol (10%) mixtures, diethyl ether, dichloromethane).
2. Volume of solvent (1-5 mL).

3. pH of extraction medium (pH 2-6.00, acetate buffers).

4. Time of extraction (5-60 minutes).

2.7.4. Validation

2.7.4.1. Extraction Recovery Studies

Extraction recoveries of indomethacin were determined at five concentration points, i.e., 1, 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, urine, amniotic or tracheal fluid), and the internal standard, α-methyl indomethacin. Only samples from the test group were spiked with IND to produce final concentrations of 1, 2, 8, 16, and 32 ng/ml. Both test and control group samples were subjected to the liquid-liquid extraction procedure (see section 2.4.1.) after which, aliquots of indomethacin, prepared in methanol, were added to the control samples to produce concentrations of 1, 2, 8, 16, and 32 ng/ml. Control and test group samples were dried under nitrogen, derivatised with MTBSTFA, reconstituted with toluene, and chromatographed as described earlier (section 2.4.1.). 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.

2.7.4.2. **Inter-day & Intra-day Variability**

Intra-day and inter-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 three separate days (values are reported as % C.V.).

2.7.4.3. **Linearity**

The linearity of the calibration curves was investigated over the concentration range of 1-500 ng/mL for plasma and urine samples.

2.7.4.4. **Stability Studies**

Studies were performed periodically to assess the stability of the samples during storage and analysis. Freezer storage stability was determined at one concentration point (8 ng/ml): a batch of biological matrices (plasma and urine)
were spiked with a known amount of indomethacin, stored at -20°C, removed periodically (over 14 months) and analysed. In order to assess the stability of indomethacin during freeze-thaw cycles, blank biological samples (plasma) were spiked with indomethacin (8 ng/ml), frozen at standard freezer temperatures (-20°C), and thawed at room temperature on the bench-top for four consecutive cycles, and analysed. A bench-top stability study (ambient conditions) was performed which involved spiking plasma and urine samples with a known amount of indomethacin, and allowing them to stand at room temperature for 0, 12, and 24 h. At the end of each study, the internal standard, α-methyl indomethacin, was added and the samples extracted and analysed as described above. The stability of indomethacin 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), spiking with a known amount of indomethacin and allowing the samples to remain on the bench-top for 0, 6, 12, and 24 h. The internal standard, α-methyl indomethacin, was then added and the 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 ten days. Stability studies were also performed on the indomethacin formulation used in the fetal infusion studies, at room temperature and at 4 °C.

2.8. Animal Experiments
2.8.1. Animal Handling & Surgical Preparation.

Ten time dated pregnant sheep of Dorset/Suffolk breeds were used. These pregnant ewes were kept in the animal unit at the Children's Variety Research Center in groups of two or more in different but adjacent pens in full view of one another. The animals were placed on a standard diet and water. Ethical approval for these studies was obtained from the UBC Animal Care Committee and the guidelines of the Canadian Council on Animal Care were followed.

The sheep were operated on between 124 and 125 days of gestation (term ≈ 145 days). Atropine (6 mg) was administered as an intravenous bolus to control salivation followed by (~15 min later) induction of anaesthesia with sodium pentothal (1 g). The ewes were intubated with an endotracheal tube and anaesthesia maintained using a mixture of halothane (1-2%), nitrous oxide (60%), and oxygen. The abdominal area was shaved and the surgical area swabbed with a 10% povidone-iodine topical solution following which the animal was draped with sterile sheets. Following a midline abdominal incision, access to the fetus was gained through an incision of the uterine wall free of placental cotyledons and major blood vessels. The fetal hind limbs were extracted and 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.

2.8.2. Recording and Monitoring Procedures

The analog signals were also processed by an on-line computerised data acquisition system, which stored one minute averages of the variables on floppy diskettes. Fetal arterial pressure, amniotic fluid pressure, heart rate, and fetal tracheal pressure were continuously monitored using a polygraph recorder (Sensormedics Inc.). Fetal arterial and venous pressures were adjusted to zero
reference by subtracting amniotic fluid pressure continuously using the computer. Initial studies involved determination of urinary flow rate twice daily using an intermittent measurement system. In later studies, fetal urine flow rate was measured continuously by the computer by means of a bladder catheter which drained by gravity into a sterile reservoir, the hydrostatic pressure of which was monitored by the computer. When the preset pressure was exceeded, a calibrated peristaltic pump was activated to pump urine from the reservoir into the amniotic cavity via an amniotic catheter. The volume pumped each minute was stored on diskette and provided a measurement of urine flow. Fetal arterial blood (0.7 mL) samples were collected for measuring pH, pCO₂, pO₂, HCO₃⁻, and base excess at 39.5 °C (Instrument Laboratory System 1306 pH/Blood Gas Analyser, Lexington, Mass., USA). Glucose and lactate concentrations in fetal blood samples were also determined using a YSI 2300 Glucose/Lactate Analyser. Hematocrit was estimated using a micro-capillary centrifuge. Blood O₂ saturation and hemoglobin concentrations were determined using an OSM-2 Hemoximeter. Plasma protein concentration was determined using an American Optical hand-held refractometer.

2.8.3. Determination of Organ Blood Flows

Fetal organ blood flows were determined by injecting 15±3 μm diameter microspheres containing either ¹⁵³Gd, ⁸⁵Sr, ⁵¹Cr, ⁴⁶Sc, or ⁹⁵Nb radioisotopes
incorporated in their structure and suspended in 10% dextran. A different isotope was used on each day of the 5-day protocol. Before injection, the microspheres were mixed and placed in an ultrasonic bath for 5 min to minimize aggregation of the spheres. Immediately after collecting the second (pm) sample of the day, 0.5 mL of the microsphere solution containing $1.2 \times 10^6$ microspheres was injected via the fetal tarsal venous catheter and the catheter flushed with 3 mL of heparinised saline. Lower and upper body reference blood samples via the fetal aortic and carotid catheters were obtained during three minutes, beginning about 30 sec prior to injecting microspheres, at a 2.2 mL/min withdrawal rate using a Harvard pump. Animals were euthanised after each experiment with pentobarbital sodium (100 mg/Kg) and the operated fetus removed and weighed. Fetal tissues and organs of interest were obtained at autopsy, weighed and carbonised in an oven at 300°C for 3-5 days. The gamma emission generated by the radioactive microspheres was measured from the fetal organs (kidneys, brain, adrenals, bone, liver, placenta, amnion, chorion, gut, atria, ventricles, spleen, fetal aortic and carotid reference samples) using a multi-channel gamma counter, isotope separation being achieved using computer software.

2.8.4. Experiment Protocol For Fetal Infusion Studies
At least 24 h before each experiment, the ewe was placed in an experimental pen in full view of companion ewes with access to standard diet and water. Each experiment was composed of five days with the first day being the control day when no drug was given. Indomethacin in a solution of 1.1% ethanol and 0.75% sodium bicarbonate in normal saline was infused into the fetal vein at a dose of 0.0025 (high dose) or 0.0006 (low dose) mg/Kg/min (2 mL/h) or vehicle alone (control) on days 2 through 4. The infusion was terminated on day 5 and the fetus also monitored during this 24 h recovery period. Samples (2 mL) of fetal arterial blood, umbilical venous blood, amniotic and tracheal fluids, and fetal urine were collected twice daily at six hour intervals and stored at -20 °C pending assay. Blood samples were collected in heparinised Vacutainer R collection tubes, centrifuged at 3000 g for 10 minutes at -10 °C and the plasma transferred into Pyrex R 15 mL culture glass tubes for storage and analysis. Withdrawn fetal blood was promptly replaced with an equal volume of heparinised drug-free maternal blood via the tarsal vein catheter.

2.9. **Data Analysis**

2.9.1. **Pharmacokinetic Calculations**

[1] Fetal total body clearance (Cl_{tb}) of indomethacin was estimated as:

\[ Cl_{tb} = k_\alpha/C_{ss} \]
where $k_o$ was the infusion rate and $C_{ss}$ the apparent fetal arterial steady-state indomethacin concentration.

[2] Placental clearance ($Cl_p$) was estimated as:

$$Cl_p = Q_{um} \times (C_{fa} - C_{um})/C_{fa}$$

where $Q_{um}$ is the umbilical blood flow rate, $C_{fa}$ the fetal femoral arterial indomethacin concentration, and $C_{um}$ the umbilical venous indomethacin concentration at apparent steady state.

[3] Fetal non-placental indomethacin clearance ($Cl_{fo}$) was then calculated as:

$$Cl_{fo} = Cl_{tb} - Cl_p$$

[4] Renal clearance ($Cl_r$) was estimated by plotting the average urinary excretion rate versus the drug concentration at the midpoint of the collection interval and fitting the least squares straight line forced through the origin. The slope of the line provided the renal clearance value in mL/min.

2.9.2. **Statistical Calculations**
Statistical evaluations were performed on analytical, physiological, and pharmacokinetic parameters using established statistical tests as outlined in each section. Data are expressed as the mean ± S.E.M. or 1 S.D as indicated. Coefficient of variation is reported in percentage. Linear regression statistics are presented for analytical data, and the paired $t$-test was used to determine whether fetal umbilical venous and femoral arterial indomethacin concentrations were significantly different. A significance level of $P<0.05$ was used.
3. RESULTS


3.1.1. Purity assessment of indomethacin

A proton NMR spectrum of indomethacin in deuterated dimethylsulfoxide was taken at 200 Mhz (Figure 5). The spectrum was compared to a spectrum obtained with an authentic (Merck standard) sample (O'Brien et al., 1984). The chemical shift values of the test indomethacin sample (Table 3) compare well with Merck standard indomethacin sample.

A differential scanning calorimetry (DSC) analysis of indomethacin is illustrated in Figure 6. Indomethacin was observed to melt at a temperature of 162°C. The melting point of indomethacin has been reported to be 153-154 °C (Shen et al., 1963). However, official monographs indicate the melting point to be 155 and 162 °C for two polymorphic forms of indomethacin (NF XIII), while the BP (1980) specifies a m.p. of 158-162°C. There are two crystalline forms of indomethacin, Form I which melts at 160-162°C, and Form II which melts at 153-155°C, the former being more stable than the latter (O'Brien et al., 1984).
Figure 5a: NMR spectrum of indomethacin in DMSO
Table 3: Chemical shift values of indomethacin and their respective group assignment

<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Indomethacin Sigma Chemical Co.</th>
<th>Indomethacin Merck Standard+</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 (s)</td>
<td>2.22 (s)</td>
<td>2-CH₃</td>
<td></td>
</tr>
<tr>
<td>3.62 (s)</td>
<td>3.67 (s)</td>
<td>3-CH₂CO₂H</td>
<td></td>
</tr>
<tr>
<td>3.78 (s)</td>
<td>3.74 (s)</td>
<td>OCH₃</td>
<td></td>
</tr>
<tr>
<td>6.7-6.9 (m)</td>
<td>6.55-7.10 (m)</td>
<td>H₄, H₆, H₇</td>
<td></td>
</tr>
<tr>
<td>7.6-7.8 (m)</td>
<td>7.45-7.80 (m)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 6: DSC scan of indomethacin: (a) open pan, and (b) closed pan methods
The LC/MS/MS spectra of indomethacin is illustrated in Figure 7. The salient features of the spectrum are: $m/z$ 139-141 due to the $p$-chlorobenzoyl group, and molecular ion at $m/z$ 358.

3.1.2. Optimisation of Derivatisation

Derivatising Agents

Indomethacin was derivatised with four different derivatising reagents to determine the most suitable agent with respect to reaction time, chemical safety of the agent, completeness of the reaction, chromatographic behaviour of the resulting derivative, as well as peak geometry and shape. The type of reactions selected were (I) silylation (with BSA, MTBSTFA), and (ii) fluorination (PFBBBr, PFP).

The chromatographic behaviour of the resulting derivatives following derivatisation with BSA, MTBSTFA, and PFBBBr is illustrated in Figures 8 and 9. Derivatisation with PFP was incomplete and was, therefore, not pursued further. The silylation reactions were much easier to perform. Peak response was best with MTBSTFA and PFBBBr. Derivatisation with PFBBBr afforded compounds with high volatility and short retention times (Figure 9). The response was slightly greater than that obtained using MTBSTFA, however, the procedure was comparatively more cumbersome and time consuming involving the use of a catalyst. Overall, the
Figure 7: LC/MS/MS spectrum of indomethacin
Figure 8: GC-ECD chromatograms of indomethacin derivatised by (a) BSA, and (b) MTBSTFA.
Figure 9: GC-ECD chromatogram of indomethacin derivatised by PFBBr.
reaction with MTBSTFA was quick, safe, complete and produced peaks of superior geometrical shape and integrity.

**Reaction Conditions**

The effect of temperature on the derivatisation reaction was examined, from room temperature (27°C) to 90°C and is shown in Figure 10. Maximum variability was noted with lower temperatures (27°C, 45°C). Area counts were maximal at 60°C and did not increase following any further increase in temperature, and thus this was chosen as the optimal temperature for derivatisation.

Different reaction times were also examined, from 10 min to 60 min, at a constant temperature of 60°C (Figure 11). Mean area counts increased as derivatisation time was prolonged from 10 min to an hour, with a plateau appearing at 50 minutes. Keeping sample preparation time in mind as well as noting the least variability at 50 minutes, the reaction time was set at 50 min.

Various concentrations of MTBSTFA were also examined, i.e., from 2% to 100% solution, the solvent being toluene (from 2 μL in 100 μL toluene to 100 μL MTBSTFA). The area counts did not differ significantly over the volume range, however, an effect on peak height was observed. The optimal volume was
Figure 10: Determination of optimum derivatising temperature for indomethacin with MTBSTFA (data are mean values ± 1 S.D., n=4).
Figure 11: Determination of optimum reaction time between indomethacin and MTBSTFA (data are mean values ± 1 S.D., n=4).
determined as 8 µL MTBSTFA in 100 µL toluene (8%), since this afforded the greatest peak response in terms of peak height.

3.1.3. Selection of Internal Standards

Ideally, the internal standard should possess close structural resemblance to the analyte, have similar chromatographic behaviour, and elute in a region free from endogenous interference. Several compounds were screened, viz., sulindac, 5-fluoro indole 2 carboxylic acid, 5-fluoro dl tryptophan, 5-fluoro indole 3 acetic acid, zomepirac, acemetacin, carprofen, 5-fluoroindomethacin, and α-methyl indomethacin (Figure 12).

The indole analogs (Figure 12) are chemicals of fairly low molecular weight and did not appear on the chromatogram when derivatised with MTBSTFA. When these same compounds were derivatised with PFBBr, retention times of the resulting derivatives were very short with interfering bridged peaks as shown in Figure 13a. The indomethacin analogs, namely, 5-Fluoroindomethacin and α-methyl indomethacin produced excellent peaks with MTBSTFA as shown in Figure 13b. Sulindac produced a satisfactory peak when derivatised with either MTBSTFA or PFBBr with good retention times as well as pilot calibration curves yielding an r>0.999. However, an unidentified peak of significant magnitude eluted with
Figure 12: Chemical structures of tested internal standards
Figure 13: (A) Derivatisation of 5-fluoro DL tryptophan with PFBBr for use as a potential internal standard for indomethacin.
Figure 13: (B) GC-ECD chromatograms of indomethacin analogs, 5-fluorooindomethacin, and α-methyl indomethacin, derivatised with MTBSTFA.
sulindac. Association of this peak with sulindac was confirmed by injecting indomethacin alone, indomethacin and sulindac, sulindac alone, and blank plasma (Figure 14). Oven temperature programming changes did not affect the presence of peak "X", nor did recrystallising sulindac help. Figure 15 illustrates the DSC scan of sulindac and its recrystallised form. It is speculated that sulindac may be oxidised to a sulphone in the injection port, and this might have eluted as peak 'X' on the chromatogram. Because of this additional peak we were unable to use this compound as an internal standard for this assay.

Zomepirac is closely related to indomethacin and provided a fairly good response with MTBSTFA (Figure 16). However, an extraneous peak in experimental fetal plasma samples co-eluted with it, precluding the use of this compound as the internal standard. This interference was not observed in fetal or maternal plasma samples stored beyond two years (blank samples), amniotic and tracheal fluids, or in fetal or maternal urine samples. Following analysis of samples collected from the present infusion studies, however, the interference was detected, leading to a re-examination of chromatographic conditions (oven temperature programming, column stationary phase [polar/nonpolar], column diameter [wide/narrow bore]), use of PFBBBr as a potential derivatising agent, as well as screening of additional internal standards.
Figure 14: Illustration of a decomposition peak "X" associated with sulindac.

Chromatograms indicate: (1) indomethacin and sulindac, (2) sulindac only, and (3) blank.
Figure 15: DSC scan of sulindac and recrystallised sulindac.
Figure 16: Chromatogram of indomethacin and zomepirac spiked in fetal plasma.
α-methyl indomethacin (Figure 17), a generous gift of Merck Frosst Inc, provided all the qualities required for an ideal internal standard, i.e., it was a very close structural analog of indomethacin, eluted close to the analyte in a region free from endogenous interference (Figure 13B), and produced a response factor of \( \approx 1.00 \) at a concentration equal to that of indomethacin. On this basis it was chosen as the internal standard for all further studies.

3.1.4. Optimisation of GC conditions

In order to obtain maximal sensitivity, the splitless mode of injection (suitable for trace level analyses) was used throughout. Injection port temperature is another critical parameter since it influences greatly the thermal stability of the derivatised compound as well as the degree of drug transfer onto the head of the column. Hence, the effect of various temperatures (140-300 °C) on indomethacin's response were examined. This evaluation was conducted using a low (20 ng/mL) and a high (100 ng/mL) sample concentration of indomethacin. As illustrated in Figure 18, maximal absolute area counts were obtained at injection port temperatures in the range 180-200 °C. A drastic decline in area counts was observed when the temperature was increased from 200-300 °C. The profile was similar at both concentrations likely indicating thermal decomposition at higher
Figure 17: Chemical structures of indomethacin and α-methyl indomethacin.
Figure 18A: Effect of injection port temperature on the peak area counts of (A) 20 ng/mL, and (B) 100 ng/mL indomethacin concentrations (n=4).
Figure 18B
temperatures. While a temperature of 180 °C resulted in the highest response the chromatogram contained many peaks from endogenous compounds. The chromatograms, however, were much cleaner at 200 °C and therefore, this temperature was selected for use in the assay. Figure 19 illustrates the effect of various initial column temperatures on the retention time of indomethacin at a constant programming rate of 8 °C/min. An initial column temperature of 210 °C and a final temperature of 300 °C with a programming rate of 40 °C/min provided optimal resolution, peak geometry, run times, and response and were therefore, chosen for the final assay.

Various column types were also investigated, viz., an Ultra-2 wide bore non-polar column [stationary phase: 5% phenylmethylsilicone], Ultra-2 narrow bore column, and a DB-1701 polar column [stationary phase: methyl (7% phenyl, 7% cyanopropyl) silicone]. Use of the DB 1701 column was observed to be more appropriate with PFBBr derivatised samples, and did not appear to be useful for MTBSTFA derivatised samples as illustrated in Figure 21. Representative chromatograms obtained for the narrow bore Ultra-2 column are illustrated in Figure 20 and for the wide bore column in Figures 13B and 16. While both Ultra-2 non-polar columns provided rapid run times and excellent chromatography, the wide-bore column allows for a greater volume of sample to be injected (Figures 13B, 16) and was therefore, selected for use in the assay.
Figure 19: Effect of various initial column temperatures on peak retention times.
Figure 20: GC-ECD chromatograms of indomethacin and zomepirac using a narrow bore fused silica Ultra-2 column.
Figure 21: GC-ECD chromatogram of indomethacin and zomepirac using a narrow bore DB-1701 column.
The time delay following injection for inlet purging to occur is critical in determining the degree of sample transfer onto the column and, therefore, needs to be optimised. To examine this, purge delay times ranging from 10-120 seconds were examined (Figure 22). A plateau was reached at 40-120 seconds. Therefore, a purge delay time of 0.75 min was chosen.

Various detector temperatures were also examined, ranging from 300-360 °C (Figure 23). Detector temperature had no effect on overall indomethacin peak area counts over the range studied so 330 °C was selected, since at lower temperatures the ability to maintain an uncontaminated detector was in question. There was no significant effect of make-up gas (argon-methane; 95/5) flow rate on the detector response, therefore a flow rate of 65 mL/min was used.

Various column head pressures (5-15 psi) were also investigated (Figures 24 and 25). Maximal peak area counts and area ratios were noted at a column head pressure of 10 psi. Furthermore, as column head pressure was increased from 5 to 15 psi, there was a proportionate fall in retention time and peak width. Hence, a column head pressure of 10 psi was chosen since this afforded maximum peak area counts and intermediate peak width and peak retention time.
Figure 22: Effect of purge delay times on the peak area counts of indomethacin tBDMS derivative ($n=4$).
Figure 23: Effect of various detector temperatures on the peak area counts of indomethacin tBDMS derivative (n=4).
Figure 24A: Determination of optimum column head pressure: Effects on (A) peak indomethacin area counts and (B) peak area ratio.
Figure 24B
Figure 25: Determination of optimal column head pressure with reference to peak retention time (A), and peak width (B).
Figure 25B
3.1.5. **Optimisation of drug extraction**

The extraction efficiency of various organic solvents was examined by comparing the ratio of extracted samples/control samples following extraction from a model biological fluid matrix (tracheal fluid) using acetate buffer pH 5.00 (Figure 26 and Table 4). Of the solvents examined, only ethyl acetate and dichloromethane were able to extract 1 ng indomethacin effectively. Solvent mixtures were also examined, namely, (a) toluene-TEA (0.0125M), (b) hexane-IPA (10%), and (c) hexane-ethyl acetate (1:4, 2:3, 3:2, 4:1). As expected, the addition of triethylamine (TEA) to toluene reduced extraction efficiency drastically. This is contrast to the fact that addition of TEA to toluene markedly improves the recovery of basic compounds (Wright, 1992). While the addition of isopropyl alcohol (IPA, 10%) to hexane improved the overall extractability of indomethacin recoveries were still not satisfactory with this solvent system. The addition of hexane to ethyl acetate was observed to reduce the recovery of indomethacin (Table 4). For ethyl acetate (EA), extraction efficiency improved with increasing volume (Figure 27), but increasing solvent volume had no effect with dichloromethane. The recoveries following extraction with 2 mL EA, 4 mL EA, and 5 mL EA, were 53.66 ± 4.32%, 73.01 ± 2.76%, and 84.59 ± 5.63%, respectively (Table 4). Based on these observations, ethyl acetate, in a volume of 5 mL, was selected for use in the assay since maximal recovery and extraction efficiency were noted at these conditions.
Figure 26: Extraction efficiency of various organic solvents (n=4) using tracheal fluid as the model biological fluid at an indomethacin concentration of 12 ng/mL.
Helleberg (1976), and Ou and Frawley (1984) suggested that attainment and maintenance of an aqueous phase pH of 5.00 was essential for optimal recovery of indomethacin. To examine this, a series of acetate buffers were prepared in the pH range 2-6 (BP, 1988). As shown in Figure 28, attainment of pH 5.00 was essential for optimal extraction; a lower pH reduced extraction efficiency considerably. This pH agrees well with the pKa of 4.5-4.7 for indomethacin.

The effect of extraction times ranging from 5-60 min on indomethacin response were also examined (Figure 29). The highest variability was observed at 5 min and the lowest at 20 min. Maximal area counts were also observed at 20 min and hence an extraction time of 20 minutes was chosen.
Table 4: Extraction efficiency (%) of organic solvents used to extract indomethacin at different concentrations using tracheal fluid as the model biological fluid.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Volume [mL]</th>
<th>Indomethacin Concentration [ng/mL]</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>6</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>2</td>
<td>58.43</td>
<td>52.56</td>
<td>50.00</td>
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</tr>
<tr>
<td>Toluene</td>
<td>2</td>
<td>-</td>
<td>52.56</td>
<td>55.25</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>2</td>
<td>-</td>
<td>20.26</td>
<td>23.57</td>
<td></td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>4</td>
<td>75.90</td>
<td>70.38</td>
<td>72.75</td>
<td></td>
</tr>
<tr>
<td>Diethyl Ether</td>
<td>2</td>
<td>-</td>
<td>49.66</td>
<td>39.05</td>
<td></td>
</tr>
<tr>
<td>Toluene-TEA*</td>
<td>2</td>
<td>-</td>
<td>16.90</td>
<td>13.30</td>
<td></td>
</tr>
<tr>
<td>Hexane-IPA**</td>
<td>2</td>
<td>-</td>
<td>36.25</td>
<td>35.66</td>
<td></td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>2</td>
<td>80.72</td>
<td>55.79</td>
<td>61.11</td>
<td></td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>5</td>
<td>91.04</td>
<td>80.60</td>
<td>82.13</td>
<td></td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>4</td>
<td>-</td>
<td>55.94</td>
<td>61.58</td>
<td></td>
</tr>
</tbody>
</table>

* Triethylamine (0.0125 M)
** Isopropyl alcohol (10%)
Figure 27: Effect of solvent volume on the extraction efficiency of ethyl acetate.
Figure 28: Determination of optimum pH on the extraction of indomethacin

(n=4).
Figure 29: Determination of optimum extraction time for indomethacin from tracheal fluid matrix using ethyl acetate at pH 5.00 (data are mean ± 1 S.D., n=4).
3.1.6. **Gas chromatography-Mass spectrometry (GC-MS)**

GC-MS was performed on the tBDMS, TMS, and PFB derivatives of indomethacin in the EI, NCI, and the PCI modes (Figures 30-32) to confirm chemical structure. The GC-MS of indomethacin-TMS has been previously reported (Plazonnet and Vandenheuvel, 1977), but no data exits for indomethacin-tBDMS.

Following derivatisation of indomethacin with MTBSTFA and PFBBBr, both GC-ECD and GC-MS demonstrate the formation of a single species as evidenced by a single, symmetrical sharp peak in the respective GC-ECD and total ion chromatogram (GC-MS), Figures 8 and 9. Derivatisation with BSA produced a single peak, however, the peak geometry was poor compared to the tBDMS and PFBBBr derivatives (Figure 8).

The tBDMS derivative of indomethacin was subjected to detailed GC-MS analysis to obtain the EI, NCI, and PCI spectrums (Figure 30). Following EI, the most intense peak occurs at \(m/z\) 139 due to the p-chlorobenzoyl fragment. The characteristic \([M-57]^+\) peak at \(m/z\) 414 represents the loss of the \(C(CH_3)_3^+\) fragment. The EI scan also shows a molecular ion peak at \(m/z\) 471. The spectrum following chemical ionisation shows the molecular ion peak at \(m/z\) 471 (NCI, \(M^+\)), and \(m/z\) 472 (PCI, \(MH^+\)).
Figure 30A: GC-MS spectra of the indomethacin tBDMS derivative in the EI (A), NCI (B), and PCI (C) modes.
Figure 30B
Figure 30C
The GC-MS of indomethacin-TMS derivative (Figure 31) is similar to that of the tBDMS derivative. There is extensive fragmentation under EI conditions, and the base peak at \( m/z \) 139 is again due to the \( p \)-chlorobenzoyl fragment. The molecular ion was observed, with significant abundance, at \( m/z \) 429. The spectrum following chemical ionisation shows a molecular ion peak at \( m/z \) 429 (NCI, \( M^+ \)) and \( m/z \) 430 (PCI, \( MH^+ \)).

In EI-MS, indomethacin-PFB ester produces a base peak of \( m/z \) 139 corresponding to the \( p \)-chlorobenzoyl fragment. Pentafluorobenzyl esters undergo dissociative electron capture under NCI conditions as a part of the electron-molecule interaction. The characteristic \([M-181]^+\) is observed at \( m/z \) 356 which is the base peak in the NCI spectrum (Figure 32). The molecular ion peak under PCI conditions (\( MH^+ \), PCI) is observed at \( m/z \) 538. A mass spectrometric assay operated in the negative-ion chemical ionisation mode would appear to provide the best sensitivity for indomethacin derivatised by PFBBR.
3.2. Validation of the GC-ECD method

3.2.1. GC Operating Conditions

The optimised operating conditions for routine analyses were: column, Ultra-2 fused silica capillary column cross-linked with 5% phenylmethylsilicone (25 m x 0.31 mm x 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); purge delay time, 0.75 min; and detector make-up gas, argon-methane (95/5, 65 mL/min).
Figure 31A: GC-MS spectra of the indomethacin-TMS derivative.
Abundance Average of 15.207 to 15.437 min. from x3Sep0101001.d (KLnde-TNS)

Figure 31B
Figure 32A: GC-MS spectra of the indomethacin-PFB derivative.
Figure 32B
3.2.2. **Drug extraction from biological fluids**

The extraction and derivatisation procedure for indomethacin is summarised in Scheme 1. Figures 33-36 illustrate the representative chromatograms of extracts of biological fluids from the chronically instrumented ovine fetus. Both the tBDMS derivatives of indomethacin and α-methyl indomethacin elute in regions free from endogenous interference (Figures 33-35). The retention time of the internal standard α-methyl indomethacin (Figure 33, peak a) is ~4.8 min and that of indomethacin (Figure 33, peak b) is ~5.0 min. Figure 36 is a representative chromatogram of indomethacin and zomepirac (the retention time of zomepirac being ~3.5 min and that of indomethacin ~5.1 min) spiked in amniotic fluid obtained during initial testing of zomepirac as a possible internal standard.
Figure 33: Representative chromatograms of the tBDMS derivative of indomethacin and alpha-methyl indomethacin spiked in fetal plasma (the inset shows the magnified baseline). The superimposed blank indicates no interference from endogenous components.
Figure 34: Representative chromatograms of the tBDMS derivatives of indomethacin and α-methyl indomethacin spiked in fetal urine.
Figure 35: Representative chromatograms of the tBDMS derivative of indomethacin and α-methyl indomethacin spiked in amniotic fluid along with the superimposed control.
Figure 36: Representative chromatograms of indomethacin and zomepirac spiked in amniotic fluid.
3.2.3. **Recovery studies**

The extraction recoveries for plasma and urine of the fetal lamb, determined by the matrix-compensation method, are presented in Table 5a. Mean recoveries from plasma and urine were 96.99 ± 9.25% and 94.74 ± 6.75% respectively. This is greater than the solid-phase extraction procedure of Nishioka *et al.*, 1990 (about 87%), and the liquid-liquid extraction procedure, with ethylene dichloride, of Sibeon *et al.*, 1978 (about 85%), reported for plasma. However, recoveries comparable to the present study have also been reported: viz 95% from serum using dichloromethane (Jensen, 1978), and ~ 92% from plasma with ethyl acetate (Evans, 1980). In amniotic fluid, the recovery of indomethacin averaged 78.42 ± 2.57% (Table 5b).

3.2.4. **Stability studies**

Indomethacin was found to be stable for at least four freeze/thaw cycles, at least 24 h on bench-top, at least 24 h in acidified plasma and urine, and at least 1 week on the autosampler tray (Table 6). Indomethacin is also stable in plasma and urine samples stored in the freezer following sampling in the time periods examined to date (14 months). Indomethacin is also stable in the formulation prepared for the infusion studies (pH 7.8) for at least 96 h as illustrated in Figure 37.
Table 5a: Extraction recoveries (n=4), inter-day variability (n=4) and intra-day variability (n=3) for indomethacin in (A) fetal plasma, and (B) fetal urine, using α-methyl indomethacin as the internal standard.

(A) Fetal Plasma

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Inter-day variability % CV&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Intra-day variability % CV</th>
<th>Recovery % (S.D.&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7.54</td>
<td>4.10</td>
<td>101.77 (2.05)</td>
</tr>
<tr>
<td>8</td>
<td>1.49</td>
<td>3.66</td>
<td>104.82 (3.24)</td>
</tr>
<tr>
<td>16</td>
<td>5.44</td>
<td>1.94</td>
<td>97.52 (3.33)</td>
</tr>
<tr>
<td>32</td>
<td>4.18</td>
<td>1.03</td>
<td>83.85 (1.29)</td>
</tr>
<tr>
<td>slope</td>
<td>4.00</td>
<td>1.13</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Coefficient of Variation; 2 One Standard Deviation

(B) Fetal Urine

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Inter-day variability % CV&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Intra-day variability % CV</th>
<th>Recovery % (S.D.&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>9.05</td>
<td>11.14</td>
<td>100.71 (9.41)</td>
</tr>
<tr>
<td>8</td>
<td>7.75</td>
<td>1.09</td>
<td>100.35 (12.51)</td>
</tr>
<tr>
<td>16</td>
<td>7.01</td>
<td>0.37</td>
<td>87.83 (3.09)</td>
</tr>
<tr>
<td>32</td>
<td>3.92</td>
<td>3.56</td>
<td>90.07 (3.44)</td>
</tr>
<tr>
<td>slope</td>
<td>3.11</td>
<td>4.11</td>
<td>-</td>
</tr>
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</table>

1 Coefficient of Variation; 2 One Standard Deviation
Table 5b: Inter- and intra-day variability parameters for indomethacin in amniotic fluid of fetal sheep, with zomepirac as the internal standard.

**Intra-day variability**

<table>
<thead>
<tr>
<th>Conc. ng/mL</th>
<th>Calibration curve 1</th>
<th>Calibration curve 2</th>
<th>Calibration curve 3</th>
<th>Mean</th>
<th>% C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0172</td>
<td>0.0169</td>
<td>0.0165</td>
<td>0.01686</td>
<td>2.08</td>
</tr>
<tr>
<td>2</td>
<td>0.0315</td>
<td>0.0334</td>
<td>0.0330</td>
<td>0.03263</td>
<td>3.09</td>
</tr>
<tr>
<td>8</td>
<td>0.125</td>
<td>0.1228</td>
<td>0.1250</td>
<td>0.1242</td>
<td>1.02</td>
</tr>
<tr>
<td>16</td>
<td>0.2238</td>
<td>0.2139</td>
<td>0.2223</td>
<td>0.2200</td>
<td>2.42</td>
</tr>
<tr>
<td>32</td>
<td>0.4324</td>
<td>0.4168</td>
<td>0.4247</td>
<td>0.4247</td>
<td>1.84</td>
</tr>
<tr>
<td>r</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
<td>-</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0086</td>
<td>0.0099</td>
<td>0.0100</td>
<td>0.0095</td>
<td>8.22</td>
</tr>
<tr>
<td>Slope</td>
<td>0.0133</td>
<td>0.0127</td>
<td>0.01307</td>
<td>0.01304</td>
<td>2.04</td>
</tr>
</tbody>
</table>

**Inter-day variability**

<table>
<thead>
<tr>
<th>Conc. ng/mL</th>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 3</th>
<th>DAY 4</th>
<th>DAY 5</th>
<th>% C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0174</td>
<td>0.0184</td>
<td>0.0207</td>
<td>0.0164</td>
<td>0.0164</td>
<td>10.02</td>
</tr>
<tr>
<td>2</td>
<td>0.0321</td>
<td>0.0333</td>
<td>0.0324</td>
<td>0.0323</td>
<td>0.0297</td>
<td>4.18</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>0.1120</td>
<td>0.1157</td>
<td>0.1167</td>
<td>0.1057</td>
<td>4.43</td>
</tr>
<tr>
<td>16</td>
<td>0.1998</td>
<td>0.2019</td>
<td>0.2065</td>
<td>0.2081</td>
<td>0.1836</td>
<td>4.87</td>
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<tr>
<td>32</td>
<td>0.3778</td>
<td>0.4100</td>
<td>0.3751</td>
<td>0.3995</td>
<td>0.3280</td>
<td>8.36</td>
</tr>
<tr>
<td>r</td>
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<td>0.999</td>
<td>0.998</td>
<td>0.999</td>
<td>0.997</td>
<td>-</td>
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<tr>
<td>slope</td>
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<td>0.0125</td>
<td>0.0115</td>
<td>0.0123</td>
<td>0.0100</td>
<td>8.48</td>
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</table>
Table 5b (Continued): Extraction recoveries of indomethacin spiked in amniotic fluid using zomepirac as the internal standard.

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
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<td>2</td>
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<td>16</td>
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<td>32</td>
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<td>78.42</td>
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<tr>
<td>Standard Deviation</td>
<td>2.57</td>
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</table>
Table 6: Stability of indomethacin in acidified sample matrix, during freeze-thaw cycles (with α-methyl indomethacin as the I.S.), and on the GC autosampler tray (with zomepirac as the I.S.).

(A) Acidified biological sample (urine) matrix at pH 5.0 (n=2)

<table>
<thead>
<tr>
<th>Time</th>
<th>Area Ratio</th>
<th>Mean ( % C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.2187</td>
<td>0.2183 (6.13)</td>
</tr>
<tr>
<td>6 hours</td>
<td>0.2371</td>
<td></td>
</tr>
<tr>
<td>12 hours</td>
<td>0.2468</td>
<td></td>
</tr>
<tr>
<td>24 hours</td>
<td>0.2302</td>
<td></td>
</tr>
</tbody>
</table>

(B) Freeze-thaw (F-T) cycle stability of indomethacin in plasma (n=2)

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Control</th>
<th>1st F-T</th>
<th>2nd F-T</th>
<th>3rd F-T</th>
<th>4th F-T</th>
<th>Mean ( % C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 ng/mL</td>
<td>0.2307</td>
<td>0.2774</td>
<td>0.2491</td>
<td>0.2742</td>
<td>0.2618</td>
<td>0.2586 (7.43)</td>
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</tbody>
</table>

(C) GC autosampler tray stability of indomethacin (n=6)

<table>
<thead>
<tr>
<th>Conc. ng/mL</th>
<th>AREA</th>
<th>RATIO</th>
<th>Mean</th>
<th>% C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=0 h</td>
<td>t=24h</td>
<td>t=60h</td>
<td>t=6d</td>
<td>t=10d</td>
</tr>
<tr>
<td>1</td>
<td>0.0114</td>
<td>0.0146</td>
<td>0.0153</td>
<td>0.0163</td>
</tr>
<tr>
<td>2</td>
<td>0.0230</td>
<td>0.030</td>
<td>0.0289</td>
<td>0.0304</td>
</tr>
<tr>
<td>4</td>
<td>0.044</td>
<td>0.051</td>
<td>0.0541</td>
<td>0.0567</td>
</tr>
<tr>
<td>6</td>
<td>0.076</td>
<td>0.079</td>
<td>0.0781</td>
<td>0.0803</td>
</tr>
<tr>
<td>8</td>
<td>0.0902</td>
<td>0.1065</td>
<td>0.1013</td>
<td>0.1006</td>
</tr>
<tr>
<td>10</td>
<td>0.119</td>
<td>0.134</td>
<td>0.1248</td>
<td>0.1228</td>
</tr>
<tr>
<td>20</td>
<td>0.249</td>
<td>0.2694</td>
<td>0.2402</td>
<td>0.2250</td>
</tr>
<tr>
<td>30</td>
<td>0.313</td>
<td>0.3607</td>
<td>0.3389</td>
<td>0.3061</td>
</tr>
<tr>
<td>40</td>
<td>0.485</td>
<td>0.5529</td>
<td>0.4769</td>
<td>0.3833</td>
</tr>
<tr>
<td>r</td>
<td>0.999</td>
<td>0.996</td>
<td>0.999</td>
<td>0.997</td>
</tr>
<tr>
<td>slope</td>
<td>0.0122</td>
<td>0.0132</td>
<td>0.0115</td>
<td>0.0099</td>
</tr>
</tbody>
</table>
Figure 37: Stability study of indomethacin infusate formulation at room temperature, and at standard refrigeration conditions.
3.2.5. **Inter-day and intra-day variability**

Inter-day and intra-day variabilities (as % C.V.) for plasma were less than 8% (less than 4% for slope), and 5% (less than 1.5% for slope) at all concentration points studied, respectively (Table 5a). For urine (Table 5a), the values were below 10% and 11% for inter- and intra-day variability, respectively. In amniotic fluid, respective CV's for intra- and inter-day variability were < 4% and 11% (Table 5b). The variability parameters for the LOQ are presented in Table 7: In urine and plasma, respective CV's for inter- and intra-day variability parameters were below 10% and 20%, respectively, for both fluids.

3.2.6. **Calibration Curves**

The calibration curve parameters of indomethacin from plasma, urine, and amniotic fluid of fetal sheep are presented in Tables 8-10 and Figures 38-40. Working calibration curves of 1-32 ng/mL were linear with coefficients of determination ($r^2$) ≥ 0.99 [linear regression statistics were: Y=0.05042X + 0.00543 for plasma, Y=0.03574X + 0.05947 for urine and Y=0.02637X + 0.03694 for amniotic fluid]. The coefficient of variation at each concentration point as well as for the slope, were below 11% for all three fluids. The limit of quantitation in both fluids was 1 ng/mL with a signal-to-noise ratio of >10.
Table 7: Variability parameters for the limit of quantitation (LOQ) of 1 ng/mL

(A) Inter-day variability (n=10)

<table>
<thead>
<tr>
<th>Biological Fluid*</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Mean</th>
<th>% C.V.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>0.03260</td>
<td>0.03415</td>
<td>0.03338</td>
<td>0.0337</td>
<td>2.32%</td>
</tr>
<tr>
<td></td>
<td>(10.435%)</td>
<td>(7.58%)</td>
<td>(7.99%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.05732</td>
<td>0.08356</td>
<td>0.07175</td>
<td>0.07087</td>
<td>18.54%</td>
</tr>
<tr>
<td></td>
<td>(13.269%)</td>
<td>(15.51%)</td>
<td>(11.744%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* blank fluid samples are from the fetal lamb.
NB: Numbers in paranthesis indicate the coefficient of variation in %.
# Coefficient of variation for days 1, 2, and 3.

(B) Intra-day variability (n=3)

<table>
<thead>
<tr>
<th>Biological Fluid*</th>
<th>Set 1</th>
<th>Set 2</th>
<th>Set 3</th>
<th>Mean</th>
<th>% C.V.#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>0.03486</td>
<td>0.03160</td>
<td>0.03048</td>
<td>0.03231</td>
<td>7.04%</td>
</tr>
<tr>
<td></td>
<td>(15.47%)</td>
<td>(1.574%)</td>
<td>(6.137%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.05928</td>
<td>0.05991</td>
<td>0.05739</td>
<td>0.05886</td>
<td>2.23%</td>
</tr>
<tr>
<td></td>
<td>(5.081%)</td>
<td>(14.41%)</td>
<td>(14.291%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* blank fluid samples are from the fetal lamb.
NB: Numbers in paranthesis indicate the coefficient of variation in %.
# Coefficient of variation for sets 1, 2, and 3.
Table 8: Calibration curve data for indomethacin in fetal plasma (n=4).

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Area Ratio ± S.D.(^1)</th>
<th>% C.V.(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0579 ± 0.0048</td>
<td>8.41</td>
</tr>
<tr>
<td>2</td>
<td>0.1043 ± 0.0088</td>
<td>8.45</td>
</tr>
<tr>
<td>10</td>
<td>0.5087 ± 0.0342</td>
<td>6.74</td>
</tr>
<tr>
<td>16</td>
<td>0.7831 ± 0.0682</td>
<td>8.71</td>
</tr>
<tr>
<td>32</td>
<td>1.6247 ± 0.0864</td>
<td>5.32</td>
</tr>
</tbody>
</table>

\([r^2=0.999, \text{int.}=0.00543, \text{slope}=0.05042]\]

1: values are mean ± one standard deviation
2: coefficient of variation
Table 9: Calibration curve data for indomethacin in fetal urine (n=4).

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Area Ratio ± S.D(^1).</th>
<th>% C.V(^2).</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0672 ± 0.0004</td>
<td>0.69</td>
</tr>
<tr>
<td>2</td>
<td>0.0974 ± 0.0029</td>
<td>3.01</td>
</tr>
<tr>
<td>8</td>
<td>0.3882 ± 0.0347</td>
<td>8.95</td>
</tr>
<tr>
<td>16</td>
<td>0.6877 ± 0.0252</td>
<td>3.67</td>
</tr>
<tr>
<td>32</td>
<td>1.1683 ± 0.0446</td>
<td>3.82</td>
</tr>
</tbody>
</table>

\([r^2=0.990, \text{int.}=0.05947, \text{slope}=0.03574]\)

1: values are mean ± one standard deviation
2: coefficient of variation
Table 10: Calibration curve data for indomethacin in amniotic fluid (n=8).

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Area Ratio ± S.D. (^1)</th>
<th>% C.V. (^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05429 ± 0.00338</td>
<td>6.24</td>
</tr>
<tr>
<td>2</td>
<td>0.08985 ± 0.00945</td>
<td>10.52</td>
</tr>
<tr>
<td>8</td>
<td>0.24974 ± 0.00456</td>
<td>1.82</td>
</tr>
<tr>
<td>16</td>
<td>0.47369 ± 0.01367</td>
<td>2.88</td>
</tr>
<tr>
<td>32</td>
<td>0.87350 ± 0.01983</td>
<td>2.27</td>
</tr>
</tbody>
</table>

1 values are mean ± one standard deviation
2 Coefficient of variation
[r = 0.9996, r\(^2\) = 0.999, int. = 0.03694, slope = 0.02637]
Figure 38: Representative calibration curve for indomethacin in fetal plasma.
Figure 39: Representative calibration curve of indomethacin in fetal urine.
Figure 40: Representative calibration curve of indomethacin in amniotic fluid.
3.3. Fetal Infusion Studies

Table 11A lists the ewes on which experiments were performed, samples collected, and the dose of indomethacin administered. In two animals (E 1137 and E 1115), the dose of indomethacin infused was one-third of that normally administered (i.e., low dose ewes). In all of the animals studied, hemodynamic and metabolic parameters were within normal limits at the start of experimentation (Figures 41 A-F). Indomethacin was observed to induce lactic acidosis in the high dose group of fetuses (E 1221, E 2160), as evidenced by markedly high blood lactate levels (Figure 41D), and a reduction in pH (Figure 41E) and base excess (Figure 41F), while blood glucose levels remained constant (Figure 41C). This was not seen in low dose group (E 1137, E 1115).

3.3.1. Analysis of Plasma Samples

The concentration of indomethacin was determined in FA and UV plasma samples (Table 12) obtained following administration of indomethacin in seven chronically instrumented ovine fetuses and the mean profiles are illustrated in Figures 42 and 43. Table 11B summarises the attainment of steady state concentrations, i.e., time achieved, samples used, the average concentrations and the variability. The indomethacin concentrations in the fetus, in the high dose group, are similar to those observed during human tocolysis (Moise et al., 1990) with mean values ranging
Table 11A: Animal experiment sampling and dosing protocol.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Samples Collected</th>
<th>Dose of Indomethacin (mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 796</td>
<td>UV, FA, MA, UTV, AMN, TR, UR</td>
<td>0.0075</td>
</tr>
<tr>
<td>E 984Y</td>
<td>UV, FA, UR, AMN, TR</td>
<td>0.0075</td>
</tr>
<tr>
<td>E 2160</td>
<td>UV, FA, UR, AMN, TR</td>
<td>0.0075</td>
</tr>
<tr>
<td>E 1221</td>
<td>UV, FA, UR, AMN, TR</td>
<td>0.0075</td>
</tr>
<tr>
<td>E 1211</td>
<td>UV, FA, UR, AMN, TR</td>
<td>0.0075</td>
</tr>
<tr>
<td>E 1137</td>
<td>UV, FA, UR, AMN, TR</td>
<td>0.0019</td>
</tr>
<tr>
<td>E 1115</td>
<td>UV, FA, UR, AMN, TR</td>
<td>0.0019</td>
</tr>
<tr>
<td>E 562V</td>
<td>UV, FA, UR, AMN, TR</td>
<td>Vehicle only (Control)</td>
</tr>
<tr>
<td>E 2182</td>
<td>UV, FA, UR, AMN, TR</td>
<td>Vehicle only (Control)</td>
</tr>
<tr>
<td>E 2221</td>
<td>UV, FA, UR, AMN, TR</td>
<td>Vehicle only (Control)</td>
</tr>
</tbody>
</table>

Abbreviation: UV, umbilical venous; FA, fetal femoral arterial; UR, fetal urine; MA, maternal arterial; AMN, amniotic fluid; TR, tracheal fluid; UTV, uterine vein.

Table 11B: Determination of apparent steady state plasma concentration.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Sample</th>
<th>Duration of SS(^1) attained (h)</th>
<th>Mean(^2) (ng/mL)</th>
<th>S.D.(^3)</th>
<th>% C.V(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 796</td>
<td>FA(^5)</td>
<td>23-69</td>
<td>195.07</td>
<td>16.83</td>
<td>8.63</td>
</tr>
<tr>
<td>E 984Y</td>
<td>FA</td>
<td>5-69</td>
<td>113.12</td>
<td>8.40</td>
<td>7.43</td>
</tr>
<tr>
<td>E 2160</td>
<td>FA</td>
<td>5-69</td>
<td>222.25</td>
<td>13.05</td>
<td>5.87</td>
</tr>
<tr>
<td>E 1221</td>
<td>FA</td>
<td>23-69</td>
<td>161.69</td>
<td>16.58</td>
<td>10.25</td>
</tr>
<tr>
<td>E 1211</td>
<td>FA</td>
<td>23-69</td>
<td>216.21</td>
<td>45.02</td>
<td>20.82</td>
</tr>
<tr>
<td>E 1137(^6)</td>
<td>FA</td>
<td>5-69</td>
<td>39.07</td>
<td>5.69</td>
<td>14.58</td>
</tr>
<tr>
<td>E 1115(^7)</td>
<td>FA</td>
<td>5-69</td>
<td>43.51</td>
<td>7.93</td>
<td>18.22</td>
</tr>
</tbody>
</table>

1: steady state  
2: mean steady state plasma indomethacin concentration  
3: standard deviation  
4: coefficient of variation  
5: fetal femoral arterial plasma sample  
6, 7: fetuses of the low dose group
Figure 41A: Effect of indomethacin on fetal arterial blood gas parameters.
Figure 41B: Effect of indomethacin on fetal arterial glucose and lactate concentrations.
Figure 41C: Effect of indomethacin on fetal arterial pH and base excess.
Table 12: Mean concentration-time data for indomethacin in fetal femoral arterial (FA) plasma, umbilical venous (UV) plasma, amniotic fluid, and fetal urine samples in high dose ewes (n=5)

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>FA [ng/mL]</th>
<th>UV [ng/mL]</th>
<th>FETAL URINE [ng/mL]</th>
<th>AMNIOTIC FLUID [ng/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.E.M.</td>
<td>Mean</td>
<td>S.E.M.</td>
</tr>
<tr>
<td>1</td>
<td>110.96</td>
<td>22.51</td>
<td>117.67</td>
<td>20.94</td>
</tr>
<tr>
<td>5</td>
<td>183.61</td>
<td>27.12</td>
<td>199.74</td>
<td>38.71</td>
</tr>
<tr>
<td>23</td>
<td>168.05</td>
<td>18.49</td>
<td>136.78</td>
<td>21.90</td>
</tr>
<tr>
<td>29</td>
<td>175.19</td>
<td>19.95</td>
<td>134.01</td>
<td>14.91</td>
</tr>
<tr>
<td>46</td>
<td>193.03</td>
<td>21.15</td>
<td>149.34</td>
<td>35.94</td>
</tr>
<tr>
<td>52</td>
<td>201.18</td>
<td>28.39</td>
<td>208.91</td>
<td>30.03</td>
</tr>
<tr>
<td>69</td>
<td>168.60</td>
<td>20.71</td>
<td>136.72</td>
<td>13.62</td>
</tr>
<tr>
<td>70*</td>
<td>86.12</td>
<td>14.66</td>
<td>89.37</td>
<td>14.46</td>
</tr>
<tr>
<td>76*</td>
<td>22.29</td>
<td>6.44</td>
<td>21.48</td>
<td>5.56</td>
</tr>
<tr>
<td>94*</td>
<td>12.79</td>
<td>-</td>
<td>7.95</td>
<td>-</td>
</tr>
</tbody>
</table>

* post-infusion samples
Figure 42: Mean concentration-time profiles of indomethacin in plasma, urine, and amniotic fluid for high dose ewes (n=5).
Mean (S.E.M.) Concentration-Time Profile

--- FA
--- UV

Figure 43: Mean concentration-time profiles of indomethacin in FA and UV plasma for low dose ewes (n=2).
from ~111-208 ng/mL during the infusion period. MA and UTV samples from one ewe (E796) were analysed but no drug was detected in them, i.e., the concentration was below the detection limit of the assay. The arterio-venous concentration difference for indomethacin at apparent steady state in the high dose ewes averaged 11.72 ± 1.53 ng/mL (mean ± 1 S.D.) (Table 13), and while small, was significantly different (α=0.05, t-test, Table 14). These results suggest a low placental permeability to indomethacin in sheep.

Fetal total body clearance was estimated to be 44.55 ± 11.14 mL/min (n=7, Table 15). It was only possible to calculate placental and non-placental clearances in two animals because of incomplete umbilical blood flow data. The placental clearance in E 796 was determined to be about 32.00 mL/min, and the non-placental clearance to be 6.44 mL/min (Table 16). In a low dose ewe (E 1115), the values were 26.47 mL/min and 22.16 mL/min, respectively. These values are much lower than those reported for either diphenhydramine or metoclopramide (Rurak et al., 1991). Ritodrine, on the other hand, had a fetal placental clearance of about 5 mL/min/kg and a markedly higher fetal non-placental clearance of 63 mL/min/kg (Rurak et al., 1991).
Table 13: Arterio-venous concentration difference for indomethacin at apparent steady state.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>$[FA]_{indo \ (SS)}$ ng/mL$^1$</th>
<th>$[UV]_{indo \ (SS)}$ ng/mL$^2$</th>
<th>$[A-V]_{indo \ (SS)}$ ng/mL$^3$</th>
<th>Extraction Ratio$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>796</td>
<td>195.07</td>
<td>181.71</td>
<td>13.37</td>
<td>0.068</td>
</tr>
<tr>
<td>984Y</td>
<td>113.12</td>
<td>101.67</td>
<td>11.45</td>
<td>0.101</td>
</tr>
<tr>
<td>2160</td>
<td>222.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1221</td>
<td>161.69</td>
<td>151.35</td>
<td>10.34</td>
<td>0.064</td>
</tr>
<tr>
<td>1211</td>
<td>216.21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1137*</td>
<td>39.07</td>
<td>36.67</td>
<td>2.39</td>
<td>0.061</td>
</tr>
<tr>
<td>1115*</td>
<td>43.51</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Low dose group.

1: Mean apparent steady-state fetal arterial (FA) indomethacin concentration.
2: Mean apparent steady-state umbilical venous (UV) indomethacin concentration.
3: Mean arterio-venous (A-V) indomethacin concentration difference.
4: Extraction ratio = $C_{FA}-C_{UV}/C_{FA}$, where $C$ is the concentration of indomethacin at apparent steady-state.
Table 14: Arterio-venous concentration difference: statistical evaluation

To determine whether fetal umbilical venous and femoral arterial indomethacin concentration differences are statistically significant.

**Two-tailed paired sample t-test**

<table>
<thead>
<tr>
<th>Ewe</th>
<th>FA Indo. steady state conc. [ng/mL]</th>
<th>UV Indo. steady state conc. [ng/mL]</th>
<th>[A-V] Indo. at steady state [ng/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>796</td>
<td>195.078</td>
<td>181.71</td>
<td>13.368</td>
</tr>
<tr>
<td>984</td>
<td>113.123</td>
<td>101.673</td>
<td>11.450</td>
</tr>
<tr>
<td>1221</td>
<td>161.694</td>
<td>151.356</td>
<td>10.338</td>
</tr>
</tbody>
</table>

\[ n=3, v=n-1=2 \]
\[ \bar{d}=11.718 \text{ ng/mL} \]
\[ S_d = 0.8849 \]
\[ t = \frac{\bar{d}}{S_d} = 13.241 \]
\[ t_{0.05(2),2} = 4.303 \]

Reject \( H_0 \) (i.e., \( \mu_d=0 \)), at \( \alpha=0.05 \)
Table 15: Determination of fetal total body clearance.

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Infusion Rate mg/min</th>
<th>[$FA_{ind0}(SS)$] ng/mL*</th>
<th>$CL_{tb}$ mL/min**</th>
</tr>
</thead>
<tbody>
<tr>
<td>796</td>
<td>0.0075</td>
<td>195.07</td>
<td>38.45</td>
</tr>
<tr>
<td>984Y</td>
<td>0.0075</td>
<td>113.12</td>
<td>66.29</td>
</tr>
<tr>
<td>2160</td>
<td>0.0075</td>
<td>222.25</td>
<td>33.75</td>
</tr>
<tr>
<td>1221</td>
<td>0.0075</td>
<td>161.69</td>
<td>46.38</td>
</tr>
<tr>
<td>1211</td>
<td>0.0075</td>
<td>216.21</td>
<td>34.69</td>
</tr>
<tr>
<td>1137</td>
<td>0.0019</td>
<td>39.07</td>
<td>48.63</td>
</tr>
<tr>
<td>1115</td>
<td>0.0019</td>
<td>43.51</td>
<td>43.67</td>
</tr>
</tbody>
</table>

* Mean steady-state fetal arterial (FA) concentration of indomethacin

** Total body clearance.

Mean total body clearance: $44.55 \pm 11.14$ mL/min
**Table 16: Calculation of placental and non-placental clearances for indomethacin**

<table>
<thead>
<tr>
<th>Ewe</th>
<th>Fetal Weight [kg]</th>
<th>([F_{a_{ind}}]^{ss}) [ng/mL]</th>
<th>([A-V]) [ng/mL]</th>
<th>(Q_{um}^*) [mL/min]</th>
<th>(Cl_{tb}) [mL/min]</th>
<th>(Cl_p) [mL/min]</th>
<th>(Cl_fo) [mL/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>796(^1)</td>
<td>3.393</td>
<td>195.078</td>
<td>13.368</td>
<td>466.99</td>
<td>38.446</td>
<td>32.001</td>
<td>6.445</td>
</tr>
<tr>
<td>1137(^2)</td>
<td>3.194</td>
<td>39.073</td>
<td>2.398</td>
<td>431.25</td>
<td>48.626</td>
<td>26.467</td>
<td>22.159</td>
</tr>
</tbody>
</table>

* flow values are mean values, calculated over the respective steady state periods.
1: High dose ewe
2: Low dose ewe
3.3.2. Analysis of Urine Samples

The results from the analysis of indomethacin in urine samples are summarised in Table 17 and Figure 42. As Table 17 indicates, the levels of intact indomethacin in fetal urine are, in general, very low. The urine samples were also subjected to enzyme (β-glucuronidase) hydrolysis to determine if there were conjugates present. While there is considerable variability, the results indicate that the fetal lamb does have some ability to conjugate indomethacin. Figure 44 illustrates the urine flow rate (mL/min) as a function of time during indomethacin infusion. In three of the fetuses (E 2160, E 984Y, and E 796), there was a marked reduction in urine flow rate within 23 hours (~50% reduction) of initiating the indomethacin infusion. However, in the other two fetuses, urine flow was observed to increase.

The estimated fetal renal clearance of indomethacin, in the high dose group, is about $0.015 \pm 0.009$ mL/min (Table 18) (Figure 45), and is not significantly different from zero. This finding is consistent with observations for other weak organic acids in the fetal lamb, viz., diphenylmethoxyacetic acid, a metabolite of diphenhydramine (Tonn et al., 1994), and valproic acid (Gordon et al., 1994). Cumulative amounts of drug excreted could not be calculated since urine samples
were only collected over a one-half to one hour period at each blood sampling interval.

3.3.3. **Drug Disposition in Amniotic and Tracheal Fluid Compartments**

The concentration of indomethacin in amniotic fluid, in the high dose experiments, was significantly lower than the plasma concentrations and slightly higher than fetal urine levels as illustrated in Figure 42. Amniotic fluid indomethacin concentrations were below detection limits in E 1137 and E 1115, the two low dose ewes. These findings are in sharp contrast to basic drugs such as metoclopramide, labetalol, and ritodrine which have been shown to accumulate considerably in amniotic fluid (Rurak *et al.*, 1991). Conjugates were not measured in amniotic fluid due to limitations in sample volume.

Indomethacin levels in fetal tracheal fluid samples were below the detection limit of the assay. Again, this is in sharp contrast to basic drugs, such as metoclopramide, ritodrine, and labetalol, wherein the levels in tracheal fluid far exceeded those in fetal plasma, the extent of accumulation being the greatest for metoclopramide (Rurak *et al.*, 1991).
Table 17: Concentration of indomethacin in fetal urine.

<table>
<thead>
<tr>
<th>Ewe</th>
<th>E 796</th>
<th>E 984</th>
<th>E 2160</th>
<th>E 1221</th>
<th>E 1211</th>
<th>E 1137#</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unhyd* ng/mL</td>
<td>Hyd** ng/mL</td>
<td>Unhyd ng/mL</td>
<td>Hyd ng/mL</td>
<td>Unhyd ng/mL</td>
<td>Hyd ng/mL</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
<td>1.729</td>
<td>5.748</td>
<td>7.351</td>
<td>3.992</td>
<td>0.932</td>
</tr>
<tr>
<td>23</td>
<td>ND</td>
<td>4.127</td>
<td>5.351</td>
<td>7.877</td>
<td>11.03</td>
<td>26.69</td>
</tr>
<tr>
<td>29</td>
<td>ND</td>
<td>5.226</td>
<td>2.781</td>
<td>2.826</td>
<td>19.51</td>
<td>41.57</td>
</tr>
<tr>
<td>46</td>
<td>0.609</td>
<td>6.098</td>
<td>1.847</td>
<td>7.036</td>
<td>8.805</td>
<td>25.20</td>
</tr>
<tr>
<td>52</td>
<td>1.628</td>
<td>7.714</td>
<td>2.125</td>
<td>6.327</td>
<td>10.81</td>
<td>10.16</td>
</tr>
<tr>
<td>70</td>
<td>4.330</td>
<td>5.280</td>
<td>7.258</td>
<td>2.444</td>
<td>18.56</td>
<td>-</td>
</tr>
<tr>
<td>76</td>
<td>3.246</td>
<td>8.257</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>94</td>
<td>-</td>
<td>1.853</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Unhydrolysed, i.e., intact indomethacin levels

** Hydrolysed, i.e., sample treated with the enzyme β-glucuronidase.

ND: level of indomethacin was below detection limit of the assay.

# low dose group

In Ewe 1115 (low dose group), no intact or conjugated drug was detected in any sample.
Figure 44: Effect of indomethacin on the fetal urinary flow rate.
Figure 45: Representative plot for the determination of renal clearance.
Table 18: Determination of renal clearance of indomethacin in high dose group fetuses

<table>
<thead>
<tr>
<th>Ewe No.</th>
<th>Renal Clearance (CL_r) mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 796</td>
<td>0.00169</td>
</tr>
<tr>
<td>E 984Y</td>
<td>0.00544</td>
</tr>
<tr>
<td>E 2160</td>
<td>0.00817</td>
</tr>
<tr>
<td>E 1221</td>
<td>0.05200</td>
</tr>
<tr>
<td>E 1211</td>
<td>0.01200</td>
</tr>
</tbody>
</table>
4. DISCUSSION

4.1. Development of a capillary GC-ECD method

Purity assessment of indomethacin

According to O'Brien et al., 1984, indomethacin is defined as the crystalline, non-solvated free acid entity of the compound (Form I; see section 3.1.1.). The indomethacin sample we procured from Sigma Chemical Co., St. Louis, MO, was subjected to a variety of physical tests to authenticate its purity for our experimental studies. NMR studies indicated that the chemical shift values obtained with our sample compared well with those obtained with Merck reference standard (Table 3) (O'Brien et al., 1984). DSC studies indicated that the melting point of our indomethacin sample was at 162°C (Figure 6). The Form I (crystalline) indomethacin has a melting point of 162°C (NF XIII). Since no other peak was observed in the DSC scan, other than that which melted at 162°C, we can attribute the melting temperature as observed to Form I. This is in full agreement with that observed with the Merck standard; the recorded temperature being 162°C (O'Brien et al., 1984). The mass spectrum obtained in an LC/MS/MS system was characterised by a molecular ion at \( m/z \) 358 (the molecular weight of indomethacin is 357.81) and a characteristic base peak at \( m/z \) 139 corresponding to the \( p-\)
chlorobenzoyl group (Figure 7). These findings confirmed that indomethacin, as obtained from Sigma, is comparable in its physical assessment to the Merck standard. Indomethacin was therefore used in our experimental studies without further purification.

Assay development

Several packed column GC assay procedures have been described for the determination of indomethacin in human plasma/serum and urine (Guissou et al., 1983; Sibeon et al., 1978; Matsuki et al., 1983; Plazonnet and Vandenheuvel, 1977; Jensen, 1978; Arbin, 1977; Evans, 1980; Ferry et al., 1974; Helleberg, 1976). These packed column methods suffer from serious limitations of poor resolution and chromatography, since they are prone to interference from endogenous peaks due to fewer theoretical plates compared to capillary columns. There is also one report on the use of fused silica capillary column technology for indomethacin (Nishioka et al., 1990) determination in plasma. However, this method has a number of disadvantages including: high sample volume (1 mL), lengthy derivatisation step with 1-ethyl-3-p-tolyltriazine (i.e., 6 hours), lengthy retention times (~20 min), a moderate recovery of 87% using a solid-phase extraction procedure, and a detection limit of 2 ng/mL.
For our detailed pharmacokinetic studies with indomethacin there is the potential for interference from components in biological matrices (e.g., tracheal and amniotic fluids, plasma, and urine) as well as limitations on fetal fluid sampling volume, in particular blood (=250-500 μL of plasma). The existing analytical methods were, therefore, not suitable and it was necessary to develop an analytical method with high sensitivity, specificity, simplicity, reproducibility and robustness.

Selection of derivatising agent

Although the conjugated p-chlorobenzoyl group in indomethacin affords the inherent electron-capture property in the molecule, derivatisation is necessary to increase volatility and thereby improve chromatography. This subsequently results in enhanced sensitivity. A number of derivatising agents have been used for indomethacin including the explosive diazoalkanes (Arbin, 1977; Giachetti et al., 1983; Guissou et al., 1983; Helleberg, 1976; Plazonnet and Vandenheuvel, 1977), and poisonous fluorine containing reagents (Matsuki et al., 1983; Evans, 1980; Sibeon et al., 1978). There have been only two instances where the derivatising agent is reportedly non-toxic, i.e., bis (trimethylsilyl) acetamide (Plazonnet and Vandenheuvel, 1977), and 1-ethyl-3-p-tolyltriazine (Nishioka et al., 1990). However, bis (trimethylsilyl) acetamide (BSA) derivatisation produces trimethylsilyl esters that are unstable to hydrolysis, and thus require protection from
moisture, and the use of freshly derivatised samples for GC runs. The use of ethyl tolyltriazine is also not desirable since the derivatisation step is very lengthy, i.e., in the order of 6 hours. Since BSA as a silylating agent provides the advantages of safety and simplicity of the derivatisation step, further newer silylating agents were explored.

Silylation of organic compounds improves volatility greatly by blocking protic sites thereby reducing dipole-dipole interactions, as illustrated below:

\[
\begin{align*}
\text{H-Y} & \quad + \quad \text{Si} \quad \text{X} \\
& \quad \rightarrow \quad \text{silyl donor} \quad \text{reactant} \\
& \quad \rightarrow \quad \text{product} \quad \text{byproduct}
\end{align*}
\]

There is a nucleophilic reaction between the electronegative heteroatom and the silicon atom of the silyl donor resulting in a bimolecular transition state. The derivatisation proceeds to completion if the basicity of the leaving group (X) is greater than that of Y. Silylation with MTBSTFA confers several advantages over other silylating agents (BSA, MSTFA, BSTFA, TBDM-SIM, and TBDMCS) such as:

(a) increased reactivity, especially its ability to silylate carboxyls,

(b) short derivatisation reaction times, and

(c) superior hydrolytic stability (while TMS derivatives need to be freshly prepared and injected into the GC, tBDMS derivatives are stable to hydrolysis as was
observed in this study where the processed samples were stable on the GC autosampler tray for at least one week)

Based on our studies, MTBSTFA derivatisation was rapid and produced tBDMS derivatives with superior hydrolytic stability in agreement with previous studies by Mawhinney and Madson, 1982, for other compounds with reactive carboxyl groups.

Derivatising with PFBBBr produced comparable sensitivity to MTBSTFA. Because sample preparation was more cumbersome and time consuming with PFBBBr we chose to use MTBSTFA for our assay. This is the first instance wherein a silylating agent like MTBSTFA has been used for indomethacin with high sensitivity electron-capture detection.

The electron-capture properties of tBDMS-indomethacin derivatives can be attributed to the conjugated p-chlorobenzoyl group, more specifically the carbonyl group in resonance with an imide structure, i.e., the two aromatic ring systems enhancing charge delocalisation (Plazonnet and Vandenheuvel, 1977; Matin and Rowland, 1972). The carbonyl carbon acts as the first site of electron acceptance while the final electron-capture response is dependent upon resonance stabilisation as illustrated in Scheme 2.
Although the GC-MS spectra of indomethacin derivatised by BSA has been reported (Plazonnet and Vandenheuvel, 1977), there are no detailed reports in the literature of mass spectra of tBDMS derivatised indomethacin. The tBDMS derivative of indomethacin was subjected to GC-MS analysis to obtain EI, NCI, and PCI spectrums (Figure 30). Following EI, the most intense peak occurs at $m/z$ 139 due to the $p$-chlorobenzoyl fragment (Figure 30 A). The characteristic $[M-57]^+$ peak at $m/z$ 414 represents the loss of the $C(CH_3)_3^+$ fragment. The EI scan also shows a molecular ion peak at $m/z$ 471. The spectrum following chemical
ionisation shows the molecular ion peak at $m/z$ 471 (NCI, M$^+$) and $m/z$ 472 (PCI, MH$^+$) (Figure 30, B and C).

During optimisation of the extraction conditions for this assay, a number of solvents were examined (viz., ethyl acetate, toluene, hexane, diethyl ether, toluene-0.0125M TEA, hexane-10% IPA, hexane-ethyl acetate, and methylene chloride), with ethyl acetate providing the highest extraction efficiency (Table 4). In order to improve the selectivity of extraction, various mixtures of hexane-ethyl acetate were examined. However, the addition of hexane markedly reduced the extraction efficiency of ethyl acetate and provided only a marginal improvement in selectivity. Of all of the solvents tested ethyl acetate alone was found to provide the best recovery of indomethacin from sheep biological fluids and the cleanest chromatograms.

A number of solvents have been used in existing assay procedures for indomethacin, the most common being dichloroethane (Sibeon et al., 1978; Bayne et al., 1981; Helleberg, 1976), ethyl acetate (Matsuki et al., 1983; Evans, 1980), hexane-ethyl acetate (4:1), (Plazonnet and Vandenheuvel, 1977), dichloromethane (Jensen, 1978), and hexane-amyl alcohol 10% (Ferry et al., 1974). Nishioka et al., 1990, are the only group who have attempted a solid-phase extraction procedure for indomethacin, however, this method does not offer any advantages over the
conventional liquid-liquid extraction, either in extraction recovery (87%), or in terms of cleanliness of the chromatogram.

The extractability of indomethacin is highly pH-dependent. Our results indicated that attainment and maintenance of pH 5.00 was required for optimal recovery. These results agree well with similar observations of Helleberg (1976), and Ou and Frawley (1984). Recovery is drastically reduced when pH is less than 4 or higher than 6. The pKa of indomethacin is 4.5-4.7 (O’Brien et al., 1984), and this likely explains the optimal recovery around pH 5.00. Varying times of extraction have been reported in the literature for indomethacin including 5 min (Giachetti et al., 1983), 15 min (Ferry et al., 1974; Helleberg, 1976), and as high as 40 min (Jensen, 1978). In the present study, however, extraction time was optimised as 20 minutes.

Mean recoveries from plasma and urine in the present study were 96.99 ± 9.25% and 94.74 ± 6.75%, respectively. These values are comparable with those reported in the literature: 95% from serum using dichloromethane (Jensen, 1978), 92% from plasma using ethyl acetate (Evans, 1980), 95% from serum with dichloroethane (Helleberg, 1976), and 95% from plasma with a 50:50 mixture of petroleum ether-dichloromethane (Johnson and Ray, 1992). The values are greater than the solid-phase extraction procedure of Nishioka et al., 1990, i.e., about 87% in
plasma and the liquid-liquid extraction procedure with dichloromethane of Sibeon et al., 1978, i.e., about 85% from plasma.

The column used in this assay is a fused silica wide bore capillary column cross-linked with 5% phenylmethylsilicone (25 m x 0.32 mm x 0.52 μm film thickness). Chromatographic behaviour of indomethacin and the internal standard, in terms of resolution, retention times, sensitivity, and peak geometry, was optimal with this relatively non-polar column stationary phase ($R_t^{\text{indo}} = \sim 4.8$ min). When a narrow bore column with the same stationary phase was used (25 m x 0.2 mm x 0.33 μm film thickness), retention times ($R_t$) were longer and sensitivity was lower. The decrease in sensitivity is likely in part due to the lower sample capacity of the narrow column as only a 1 μL volume can be injected compared to 2 μL for the wide bore column. With a narrow bore column of the same phase, there is both a reduction in internal diameter was well as in stationary phase thickness. Equation 1 illustrates the relationship between phase ratio, $\beta$, internal radius, $r$, of a wall coated open tubular column, and stationary phase film thickness, $d_f$.

$$\beta = \frac{r}{2d_f} \quad (1)$$

From this equation, it can be observed that a decrease in $d_f$ (if $r$ is constant) will result in a reduction in partition ratio and a decrease in component retention and resolution with reduction in run times (Hyver and Sandra, 1989). However, with the narrow-bore column, the internal diameter is also smaller, with run times being
dramatically longer. When a polar DB-1701 column was used (narrow-bore, methyl [7% phenyl, 7% cyanopropyl] silicone) of dimensions, 30 m x 0.25 mm x 0.25 μm, however, the elution behaviour of the analytes were reversed. Zomepirac, being the more polar compound was retained longer in the column than was indomethacin.

The splitless mode of injection was used for all analyses since this method of sample introduction is generally very suitable for trace level analysis. This is because 100% of the injected sample is introduced into the column (Hyver and Sandra, 1989).

The time delay following inlet purging to occur is critical for the following reasons and was therefore optimised. The purge delay time is dependent upon the solvent and solute characteristics, capacity of the vaporising chamber, sample volume, speed of injection, and the velocity of the carrier gas. Short purge delay times will prevent complete transfer of solute into the column while long delay times will prevent venting of residual waste vapour resulting in their transfer onto the column and solvent tailing. A purge delay time of 0.75 min was chosen from the tested range of 10-120 seconds as this provided optimal peak response (Figure 22).

The overall peak characteristics (peak shape, geometry, etc.) of the analyte are dependent upon the injection port temperature. Stability of the derivatised
compound is also important as it may decompose at higher temperatures resulting in a loss of sensitivity. A very low injection port temperature may result in peak broadening as a result of slower sample vapourisation with the time for transfer into the column being longer. In this study, it was determined that maximal peak area counts of indomethacin were observed over the 180-200°C range and that any further increase in temperature resulted in a drastic decline in absolute indomethacin area counts. Usually the injection port temperature is higher than the column temperature. However, in the present study an injection port temperature of 200°C and an initial column temperature of 210°C were chosen as these provided optimal response.

The column temperature has a direct effect on run time and peak geometry. It also has a marked effect on peak separation. The log of partition ratio (k) is inversely related to the temperature. The lower the column temperature, the larger the partition ratio, thereby increasing the time the analyte spends in the stationary phase. This increased retention of the analyte in the liquid phase confers greater selectivity. Various column temperatures were examined and an initial column temperature of 210°C was selected with quick ramping at 40°C/min to 300°C, the final column temperature. Maximal absolute indomethacin area counts were observed at this final column temperature. These column temperatures also resulted
in short analysis times (~9 min) and are within the maximum 400 °C temperature rating recommended for these columns.

The carrier gases commonly used in GC are hydrogen, helium, and nitrogen. Nitrogen is a denser gas compared to either hydrogen or helium. Of the three gases, the major advantage of using hydrogen is that the value for the theoretical plate height increases very slowly when one increases the average linear gas velocity through the column. This results in a faster analysis with little loss in resolution. In the current study, hydrogen was used as the carrier gas for these reasons. A variety of carrier gases have, however, been used for the GC analysis of indomethacin including nitrogen (Sibeon et al., 1978; Arbin, 1977; Evans, 1980; Ferry et al., 1974; Helleberg, 1976); argon-methane, 90:10 (Plazonnet and Vandenheuvel, 1977; Jensen, 1978; Guissou et al., 1983); and helium (Nishioka et al., 1990). Except for the method of Nishioka et al., 1990, these assays involve the application of packed column technology.

Make-up gases are frequently used in many capillary GC methods since the low column flow rates (1-5 mL/min) employed do not sufficiently maintain cell volume or effectively sweep the electron capture detector cell (ECD). The ECD is a concentration sensitive detector, the sensitivity being inversely related to flow. Flow through the cell then, must be sufficient to maintain ECD cell volume in order
to accommodate the high surface area of the $^{63}$Ni source as well as to efficiently sweep the cell so that solute mixing does not occur. Use of the make-up gas also assures an equilibrium concentrations of thermal electrons in the ECD. Argon-methane (95:5) was chosen as a make-up gas at a flow rate of 65 mL/min, since this provided the greatest sensitivity, the lowest S/N ratio, and minimal band spreading.

The length of the capillary column entering the detector cell appeared to influence absolute indomethacin area counts markedly. In spite of the recommended depth of 72 mm into the ECD cell (Hewlett-Packard support line), a depth of about 40 mm provided maximal area counts and was therefore chosen for the assay. This could be due to effective sweeping of the make-up gas at the detector base.

In summary, the optimised operating conditions for routine analyses were: column, Ultra-2 fused silica capillary column cross-linked with 5% phenylmethylsilicone (25 m x 0.31 mm x 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 gas at a column head pressure of 70 kPa (column flow rate ~1 mL/min); purge delay time, 0.75 min; make-up gas, argon-methane, 95:5, at a flow rate of 65 mL/min.
Internal Standards

Several compounds were screened as potential internal standards in this assay (Figure 12 illustrates some of these). A variety of internal standards have been reported in the literature including compounds that are not structurally related to indomethacin, such as phenylbutasone (Mehta and Calvert, 1983), phenacetin (Avgerinos and Malamataris, 1989), testosterone (Kwong et al., 1982), itraconazole (Al-Angaary et al., 1990), penfluridol (Guissou et al., 1983), esters of indomethacin, such as indomethacin propyl ester (Nishioka et al., 1990), and indomethacin methyl ester (Arbin, 1977), and close structural analogs of indomethacin, such as \( \alpha \)-methyl indomethacin (Stubbs et al., 1986; Bayne et al., 1981), and 5-fluoroindomethacin (Sibeon et al., 1978).

Ideally the internal standard should:

1. Be a close structural analog of indomethacin, so that it exhibits similar extraction and chromatographic behaviour.

2. Possess an active carboxylic acid site for derivatisation like indomethacin.

3. Elute in a region free from endogenous interference and close to the analyte of interest.
4. Produce a peak response equivalent to the analyte’s, resulting in area ratio of unity at equivalent concentrations.

5. Not exhibit any chemical interactions with indomethacin.

6. Possess good chemical stability following derivatisation.

With these requirements in mind several compounds were evaluated as potential candidates. Sulindac, a close structural analog of indomethacin, was one of the first compounds to be tested (Figure 12). Chemically, it had an ideal halogen (i.e., fluorine) function on the 5th position of the heterocyclic ring and an acetic acid function on the 3rd. It also produced a satisfactory peak when derivatised with either MTBSTFA or PFBBr, and calibration curves when derivatised with the former, were linear with \( r > 0.999 \). However, it was speculated that sulindac was oxidised to a sulphone in the injection port, due to the appearance of a coeluting peak in the chromatogram (Figure 14). Use of lower injection port temperatures did not affect peak “X” as it continued to elute. Recrystallising sulindac from chloroform also did not help, although one polymorph was selectively isolated as depicted in the DSC scan in Figure 15. The fact that there is no reported GC method for sulindac in the literature would also appear to indicate that this compound is not stable under these conditions. Based upon these observations this compound was not considered further.
Zomepirac, a structurally similar compound to indomethacin, provided a fairly good chromatographic response when derivatised with MTBSTFA and appeared to satisfy most of the requirements mentioned above. Figure 16 illustrates a representative chromatogram of indomethacin and zomepirac spiked in fetal plasma. When spiked in stored plasma, amniotic, tracheal fluids, and urine (fetal and maternal) as well as freshly collected amniotic and tracheal fluids and fetal urine, both indomethacin \( (R_t = 5.9 \text{ min}) \) and zomepirac \( (R_t = 3.9 \text{ min}) \) eluted in regions free from any significant interference. However, the analysis of experimental samples and use of freshly collected blank fetal plasma samples resulted in a co-eluting endogenous peak that interfered with that of zomepirac. It was, therefore, not possible to use zomepirac as an internal standard for the analysis of experimental samples. Zomepirac was used, however, as the internal standard during initial validation studies with amniotic fluid (Tables 5b and 6c). Figure 36 provides a representative chromatogram of indomethacin and zomepirac spiked in amniotic fluid and illustrates that no interference was present for either compound.

A further re-examination of chromatographic conditions, columns, derivatising agents, and screening of other internal standards (carprofen, acemetacin, 5-fluorooindomethacin) was then conducted. However, none of these proved to be satisfactory. \( \alpha \)-methyl indomethacin, on the other hand, met all the requirements of an ideal internal standard outlined above. It is a very close
structural analog of indomethacin (Figure 17), elutes in a region free from any endogenous interference (Figure 33) and close to the analyte, and yields an area ratio of about unity at equivalent concentrations. α-methyl indomethacin was chosen, therefore, as the internal standard for the final assay procedure.

Using α-methyl indomethacin, calibration curves were linear over the working range of 1-32 ng of indomethacin \( \text{mL}^{-1} \) with a coefficient of determination \( r^2 > 0.99 \), following extraction of 0.1 mL of plasma, and 0.1-1.0 mL of urine, amniotic and tracheal fluids. Linearity was also observed over a concentration range of 1-500 ng/mL \( r^2 > 0.99 \). Inter- and intra-day variabilities at each concentration point were < 11 % for concentrations between 2-32 ng/mL and < 20 % at the LOQ of 1 ng/mL. The limit of quantitation is 1 ng/mL \(< 10\% \text{ C.V., S/N} > 10 \). The developed method is therefore reproducible and robust for accurate determination of indomethacin concentrations in biological fluids.

*Derivatisation Conditions*

Derivatisation of indomethacin and α-methyl indomethacin with 100 μL of toluene containing 8 μL of MTBSTFA (8% v/v) at 60°C for 50 minutes was found to be rapid, constant, and reproducible with maximal indomethacin area counts. No significant differences in peak response were observed between the temperatures of
60-90°C (Figure 10) and between reaction times of 50-60 minutes (Figure 11). A reaction time of 50 minutes and temperature of 60 °C was chosen to ensure complete derivatisation. This is the first reported use of MTBSTFA as a derivatising agent for indomethacin. Plazonnet and Vandenheuvel, 1977, used BSA to silylate indomethacin producing the trimethylsilyl ester. They derivatised indomethacin with 10 μL of BSA and 90 μL of ethyl acetate (10%), and reconstituted the final samples with 3% BSA in ethyl acetate since the resulting TMS esters were prone to hydrolysis. However, this problem is not evident for MTBSTFA since this molecule contains sterically crowded alkyl substituents on the silicon atom that confers hydrolytic stability. In this assay, 100 μL of a 8% v/v solution of MTBSTFA (8 μL) in toluene (92 μL) produced optimal reaction conditions.

A solvent is usually used in the reaction medium for derivatisation and for reconstitution, i.e., dilution prior to injection into GC. Some of the recommended solvents in silylation reactions include pyridine, dimethylformamide (DMF), dimethyl sulphoxide, tetrahydrofuran, and acetonitrile (Blau and Halket, 1993). However, toluene appeared to be a better medium for MTBSTFA derivatisation and for sample dilution than any of these solvents (DMF, Acetonitrile, or ethyl acetate), and on this basis was selected for the assay.
It has been suggested that addition of t-butyldimethylchlorosilane (TBDMCS, 1% v/v) as a catalyst improves the silylation power of MTBSTFA (Mawhinney and Madson, 1982; Ballard et al., 1990; Lewis and Yudkoff, 1987). However, addition of this agent did not have any effect on indomethacin area counts.

MTBSTFA has been popularly used over the recent years to derivatise various chemicals and drugs for GC determinations. The carboxylic acid derivatisations reported in the literature include: carboxylic and hydroxyl groups in tartaric acid and γ-resorcylic acid (Kim et al., 1989), and valproic acid and its metabolites (Abbott et al., 1986).

**Stability studies on indomethacin**

Indomethacin is a pale yellow crystalline powder. It may exist in amorphous or crystalline forms [Form I (mp 160-162 °C) and Form II (mp 153-155°C), with Form I being more stable]. Form II converts to the more stable Form I on storage. Indomethacin is sparingly soluble in water and early pharmaceutical formulations incorporated a small amount of mild alkali (sodium carbonate or bicarbonate) to improve solubility. However, in the presence of strong alkali such as sodium
hydroxide, indomethacin (I) decomposes to 2-methyl-5-methoxy-indole-3-acetic acid (II) and p-chlorobenzoic acid (III), as shown below in Scheme-3.

\[
\text{Scheme-3: Degradation of indomethacin in alkali}
\]

The following Table illustrates some of the stability data on indomethacin in aqueous solution:

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Degradation Half-life (T(_{1/2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.00</td>
<td>RT*</td>
<td>1 min</td>
</tr>
<tr>
<td>10.00</td>
<td>RT</td>
<td>1-1.5 h</td>
</tr>
<tr>
<td>9.40</td>
<td>50</td>
<td>0.38 h</td>
</tr>
<tr>
<td>8.14</td>
<td>50</td>
<td>3.13 h</td>
</tr>
<tr>
<td>8.00</td>
<td>RT</td>
<td>195 h</td>
</tr>
</tbody>
</table>

* room temperature
In our study, indomethacin was administered in a vehicle of 1.1% ethanol and 0.75% sodium bicarbonate in normal saline. The pH of this infusate is about 7.8-7.9. Stability studies on indomethacin in this vehicle indicated that the drug was stable for at least 96 hours at both room temperature and standard refrigerator temperature (4°C) (Figure 37). This observation is consistent with that of Shen and Winter, 1977, who determined the $T_{1/2}$ of indomethacin in an aqueous solution, at pH 8.00, to be about 195 h at room temperature.

Several pharmaceutical formulations have been designed for indomethacin administration in animal experimentation from the combined standpoints of stability and solubility. It is strongly recommended that these solutions be freshly prepared before each study. These indomethacin formulations usually contain sodium carbonate, mannitol, and Emulphor-620, with the pH of the resulting solution being between 8.4-9.1 (Shen and Winter, 1977).

Indomethacin powder solubilised in a vehicle of simple syrup with 10% alcohol (pH 5.2) for pediatric administration appeared to be stable for at least 224 days at 24°C (Das Gupta et al., 1978). Curry et al., 1982, reported that a solution of indomethacin in 0.1 M sodium carbonate (pH 10.7) lost 75% of its content in 80 minutes: Adjustment of the pH to 7.4 resulted in stability for at least 24 h. These authors also found that a solution of indomethacin in polyethylene glycol, PEG 400,
was stable. However, Curry et al., 1982, also indicated that inclusion of sodium carbonate in a vehicle can be potentially dangerous, and recommended that while preparing a solution containing $\text{Na}_2\text{CO}_3$, the sodium carbonate must be added very slowly to the indomethacin slurry to avoid rapid degradation in a high alkalinity environment. It appears that vehicles containing PEG or alcohol would be highly desirable from the standpoint of stability, but not quite so from the pharmacological perspective.

The maximal stability of indomethacin in aqueous solution at 25°C is at pH 4.9, and solutions at this pH are predicted to be stable for at least 2.0 years (Kahns et al., 1989). A 3% aqueous indomethacin solution was prepared in 30% each of ethyl carbamate and ethylurea and the mixture boiled for 30 secs (pH 6.95) resulting in stability for 25 months at 20°C (Pawelczyk and Knitter, 1978).

Indomethacin was observed to be most stable in surfactant systems solubilised in Tween 80 or Brij 99. The $T_{1/2}$ in these solutions being more than 6 months (Ghanem et al., 1979). The following Table summarises some of these vehicles:
Table 20: Stability of indomethacin in surfactant solubilised systems (Adapted from Ghanem et al., 1979)

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Condition</th>
<th>T&lt;sub&gt;50&lt;/sub&gt; (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 80</td>
<td>RT*</td>
<td>9.8</td>
</tr>
<tr>
<td>Tween 80</td>
<td>5°C</td>
<td>12.6</td>
</tr>
<tr>
<td>Brij 99</td>
<td>RT</td>
<td>10.7</td>
</tr>
<tr>
<td>Brij 99</td>
<td>5°C</td>
<td>15.5</td>
</tr>
<tr>
<td>Tween 80, 40% syrup, sodium benzoate</td>
<td>RT</td>
<td>6.8</td>
</tr>
<tr>
<td>Tween 80, 60% syrup, sodium benzoate</td>
<td>RT</td>
<td>12.4</td>
</tr>
<tr>
<td>Tween 80, 20% glycerol, 40% propylene glycol</td>
<td>RT</td>
<td>11.9</td>
</tr>
<tr>
<td>Tween 80, 10% ethanol, 10% glycerol</td>
<td>RT</td>
<td>11.9</td>
</tr>
<tr>
<td>Brij 99, chlorocresol</td>
<td>5°C</td>
<td>17.7</td>
</tr>
<tr>
<td>Brij 99, sodium thiosulphate</td>
<td>5°C</td>
<td>17.7</td>
</tr>
</tbody>
</table>

* room temperature

It appears then, that the following parameters are important to ensure drug stability: pH of the infusate (it is preferable to keep the pH from exceeding 8.0 units); mode and order of ingredient mixing (as determined by Curry et al., 1982); and use of surfactants (these will aid against degradation). I also strongly recommended that freshly prepared solutions be used. Use of the sodium trihydrate salt of indomethacin, which is presently being employed in the treatment of patent ductus arteriosus, however, may be preferable as it would be completely soluble in aqueous solutions.

The stability of indomethacin in biological fluids and extracted samples was also assessed. The drug was found to be stable for at least four freeze-thaw cycles,
at least 24 h on the bench-top, at least 24 h on the acidified sample matrix, and at least one week on the autosampler tray (Table 6). Indomethacin is also stable in plasma and urine samples stored in the freezer in the time periods studied to date (over 14 months).

Data on the stability of indomethacin during analytical development in existing assays have been sparse. Al-Angary et al., 1990, found that indomethacin was stable for at least 1 month in serum at -20°C. Indomethacin was found to be stable for at least 30 minutes in various extraction solvents including ethyl acetate, hexane 2 propanol, chloroform, benzene-cyclohexane, and hexane-diethyl ether, all acidified to pH 2.0 with HCl (Catrula and Cusido, 1992).

4.2. Disposition of indomethacin in sheep

Following assay development and validation the method was applied to the study of indomethacin disposition in the chronically instrumented pregnant sheep model. The indomethacin concentrations in the fetus, obtained in the high dose group, were similar to those observed during human tocolysis, i.e., ~ 200 ng/mL (Moise et al., 1990). Steady state levels in fetal plasma were achieved over the 5-69 hour period following indomethacin infusion, while in some fetuses this was delayed and was observed over the 23-69 hour period. Since samples were only
collected twice daily, six hours apart, it was not possible to determine the exact time and/or duration of the steady state achieved. The mean steady state fetal femoral arterial concentration of indomethacin was 181.67 ± 45.05 ng/mL in the high dose ewes (n=5), and 41.29 ± 3.14 ng/mL in the low dose ewes (n=2). Fetal total body clearance was estimated to be 44.55 ± 11.14 mL/min (n=7). Indomethacin was detected in very low levels in urine (Section 4.2.3.). The urine samples were also subjected to enzyme hydrolysis with β-glucuronidase, and the results indicate the presence of glucuronide metabolites of indomethacin, suggesting that the fetal lamb does have some ability to conjugate indomethacin (Table 17). This aspect of fetal drug metabolism (i.e., conjugation), however, requires further investigation.

4.2.1 Placental transfer of indomethacin in sheep

Indomethacin is a polar and fairly medium molecular weight (MW 357.8) organic acid that crosses the placental membrane easily in humans (Moise et al., 1990) and rabbits (Parks et al., 1977; Harris and VanPetten, 1981). In the present study with the pregnant sheep model, however, there is a limited placental permeability to indomethacin. The sheep placenta has been shown to be relatively impermeable to polar compounds (Boyd et al., 1976). There is a low but statistically significant arterio-venous indomethacin concentration difference at apparent steady state indicating placental uptake of the drug (Figures 42, and 43;
Tables 13 and 14). Indomethacin levels were below detection limits in maternal arterial and uterine vein plasma samples collected in one ewe suggesting little or quantitatively insignificant placental transfer of indomethacin. In other studies, the fetal-to-maternal (F/M) drug ratio has been reported to be 0.28 (Anderson et al., 1980c) and 0.04 (Harris and Vanpetten, 1981) in sheep, indicating limited placental transfer of indomethacin in this species. The epitheliocorial placenta in sheep has a placental pore radius of 4-5 Å, while the hemochorial placenta in rabbits and humans behaves as if it has a placental pore radius of ~300 Å. This difference could explain, in part, the limited placental permeability in sheep. In general, the sheep placenta has been reported to be relatively impermeable to polar compounds (Boyd et al., 1976). The partition coefficient of the nonionised form of indomethacin and that of the ionised form of the drug, between 1-dodecanol and water at 32°C, has been determined to be 2400 and 1.1, respectively (De Vos et al., 1994). Ionised indomethacin is also reported to form ion-pairs with such ions as Na\(^+\), K\(^+\), and NH\(_4\)\(^+\) (De Vos et al., 1994). It is likely that indomethacin partitions into a lipophilic phase predominantly in the un-ionised form. Indomethacin could form ion-pairs with cations in the physiological milieu, and thus its implication in absorption through biological membranes may be underestimated (De Vos et al., 1994).

A relatively high degree of plasma protein binding has been reported for indomethacin in maternal (98.5%) and fetal (97.6%) sheep (Anderson et al., 1980c).
This is markedly higher than that observed in pregnant rabbits, where the maternal binding of indomethacin (85.6%) is significantly higher than fetal binding (43.8%) (Harris and Vanpetten, 1981). However, these differences cannot be attributed solely to species differences because: (a) the methods of protein binding used are different; ultracentrifugation (Anderson et al., 1980c), and equilibrium dialysis (Harris and Vanpetten, 1981), and (b) use of radiolabeled indomethacin (\(^{14}\text{C}\)) by Anderson et al., 1980c, and unlabeled indomethacin by Harris and Vanpetten, 1981.

4.2.2. Placental clearance

To date, three major methods have been reported in the literature for determining placental clearance: the Fick principle (Meschia et al., 1967), the Szeto (Szeto et al., 1982b) and the Anderson methods (Anderson et al., 1980c). Placental clearance is defined as the ratio of flux in the concentration difference between the maternal artery and fetal umbilical artery (Meschia et al., 1967), while fetal clearance may be defined as the number of mL of plasma from which a drug is completely removed per unit of time (Anderson et al., 1980c).

The Fick principle involves sampling of maternal arterial and uterine venous blood or fetal femoral arterial and umbilical venous blood to determine drug concentrations in these samples following a respective maternal or fetal steady state infusion. It also requires quantitative data on uterine or umbilical blood flow,
respectively. Placental clearance is then calculated as a product of the blood flow rate and the ratio of arterio-venous concentration difference to the arterial drug concentration at apparent steady state. The Anderson method involves the simultaneous administration of a fetal intravenous bolus dose of labeled drug and maternal i.v. infusion of unlabeled drug. The total fetal body clearance ($C_{tot} = C_p + C_f$) can be determined from the fetal bolus. Placental clearance, then, is determined as the product of the total body clearance ($C_{tot}$) and the ratio of steady state fetal to maternal concentrations of the drug ($F/M$). However, a major limitation is the inability of the method to differentiate between the intact drug and its metabolites. Other limitations include the difficulty of procuring a stable isotope or radiolabeled form of the drug and possibility of an "isotope effect" occurring, i.e., selectivity of the placenta between the isotope and its natural element. The Szeto method involves the representation of the maternal-fetal unit as a two compartment pharmacokinetic system that requires paired drug infusions to steady state to both the mother and the fetus. A potential limitation again, is the availability of a stable isotope labeled drug and an analytical method that will selectively quantitate the unlabeled compound and the stable isotope. If the stable isotope labeled drug is not available, then the experiments need to be performed on different days with a suitable washout period between them. This may result in considerable variability due to time-dependent changes in both maternal and fetal physiology. The fetus, for example, grows at the rate of about 5% per day (Kong et al., 1975). Apart from these limitations, the requirement that detectable drug concentrations should be seen
in the maternal circulation across the placenta during fetal infusion studies, and vice versa, must be met. In the current fetal indomethacin infusion studies, drug concentrations were below detection limits in MA and UTV samples precluding therefore, the application of the Szeto method to determine placental clearance.

The Fick principle is also not without problems. It requires more extensive surgical catheterisation of the umbilical venous or uterine blood vessel apart from either MA or FA vessels. It also requires determination of either the umbilical or uterine blood flow. Another problem lies in the complexities of the fetal circulation as there may be a difference between observed and actual concentrations leaving the sites of exchange, e.g., uterine venous blood may be a mix of myometrical and placental venous blood.

In spite of these potential problems the Fick principle may be used to determine placental clearance. However, it must be borne in mind that the method allows for the estimation of either the fetal to maternal placental clearance or vice versa, at any given time. Examples of its use include the estimation of placental clearance of glucose analogs (Stacey et al., 1978), sodium and chloride (Armentrout et al., 1977), and ritodrine (Wright, 1992) in the fetal lamb.

In this study, there is a low but statistically significant difference between the indomethacin concentrations in FA and UV plasma at apparent steady state (mean
difference: \(11.718 \pm 0.8849\ \text{ng/mL; mean} \pm \text{s.e.m., } n=3\), indicating placental uptake of indomethacin (Tables 13 and 14). Placental clearance values were determined only for two ewes, one at the high dose, and the other at the low dose. This was due to surgical failures of cannulation, catheter failures during experimentation (especially UV sampling), and the unavailability of umbilical blood flow data. The placental clearance of indomethacin as determined by the Fick method was calculated to be \(9.43\ \text{mL/min/kg in E 796 and 8.28 mL/min/kg in E 1137}\). These values are only somewhat higher than the value of \(2.10 \pm 0.32\ \text{mL/min/kg reported by Anderson et al., 1980c}\). The mean placental clearance value for indomethacin in this study, i.e., \(8.85\ \text{mL/min/kg},\) is similar to that determined for ritodrine, i.e., \(9.2 \pm 2.7\ \text{mL/min/kg (Wright, 1992)},\) but markedly lower than the values reported for other drugs in the fetal lamb, namely, metaclopramide - \(103 \pm 13\ \text{mL/min/kg (Riggs et al., 1989)},\) diphenhydramine - \(124 \pm 22\ \text{mL/min/kg (Rurak et al., 1991)},\) and methadone - \(168 \pm 29\ \text{mL/min/kg (Szeto et al., 1982a)}.\) The observed placental clearance of indomethacin in E 796 (high dose ewe) accounts for more than 80% of the total fetal body clearance while in the low dose ewe (E 1137) it accounts for about 55%. 
4.2.3. Elimination of indomethacin by the fetal kidney

It was evident from the present study that the pathway for elimination of indomethacin, a weak acid, by the fetal kidney are not matured. Very low levels of indomethacin were observed in fetal urine samples collected from the high-dose ewes, and indomethacin concentrations were below detection limits in urine samples from the low dose ewes. Similar observations have been reported for other weakly acidic agents such as valproic acid (Gordon et al., 1994), diphenylmethoxy acetic acid or DPMA (Tonn et al., 1994), and p-aminohippuric acid (Elbourne et al., 1990) in the ovine fetus. It has been speculated that the pathways for the excretion of organic acids by the fetal kidney mature after birth in contrast to that for organic bases which appear to mature much earlier (Elbourne et al., 1990). The estimated fetal renal indomethacin clearance value in this study (Table 18) was very low (0.015 ± 0.009 mL/min) and not significantly different from zero. This is again, consistent with findings for valproic acid (Gordon et al., 1994) and diphenylmethoxyacetic acid, an acid metabolite of diphenhydramine (Tonn et al., 1994), in the fetal lamb.
4.3. **Drug disposition in amniotic and tracheal fluids**

4.3.1. **Amniotic fluid**

Indomethacin concentrations in amniotic fluid were significantly lower than FA plasma levels in the present study. Amniotic fluid indomethacin concentrations were below detection limits in E 1137 and E 1115, the two low dose ewes. These findings are also consistent with those observed for valproic acid and DPMA (Gordon *et al.*, 1994; Tonn *et al.*, 1994). However, they are in sharp contrast to observations for basic drugs (metoclopramide, diphenhydramine, labetalol, and ritodrine) which have been shown to accumulate considerably in amniotic fluid (Rurak *et al.*, 1991). Renal excretion of drug and subsequent appearance in amniotic fluid has been observed for meperidine (Szeto *et al.*, 1978), lidocaine (Morishima *et al.*, 1979), and ethanol (Clarke *et al.*, 1987) in the fetal lamb. It has been suggested that basic drugs accumulate in amniotic fluid due to the mechanism of "ion-trapping" due to the lower pH of amniotic fluid as compared to fetal plasma (Szeto *et al.*, 1978). The sources of drug appearance in the amniotic fluid are two-fold, i.e., fetal renal drug elimination (time delay in the appearance of drug in the amniotic fluid following dosage is characteristic for this type), and diffusion across chorio-allantoic membranes. The latter route was suggested to be a predominant pathway for the appearance of meperidine in amniotic fluid (Szeto *et al.*, 1978). Renal elimination, on the other hand, has been suggested to be important for
compounds such as mannitol (Basso et al., 1977). Metabolites have also been detected in amniotic fluid with the glucuronide conjugates of ritodrine serving as an example (Wright et al., 1991). The presence of glucuronide conjugates in amniotic fluid for indomethacin in the present study, however, could not be confirmed due to limited sample volume.

4.3.2. Tracheal fluid

Indomethacin levels in fetal tracheal fluid samples were below assay detection limits. While above detectable limits, the concentrations of valproic acid (Gordon et al., 1994) and DPMA (Tonn et al., 1994), two other acidic compounds, in tracheal fluid have been reported to be very low. This is in sharp contrast to basic drugs, such as metaclopramide, ritodrine, labetalol, and dyphenhydramine, wherein the drug levels in tracheal fluid were equivalent or even higher than those in fetal plasma, the extent of accumulation being greatest for metoclopramide (Riggs, 1989; Wright et al., 1991; Yoo, 1989; Yeleswaram, 1992).

Basic drugs are known to accumulate in the adult lung. Amphiphilic amines are preferentially taken up by the lung which appears to be a major organ of clearance for these agents. It has been suggested that molecules containing the amino group and a lipophilic alkyl function are necessary for lung specificity and uptake (Fowler et al., 1976) and that predictions can be made on the basis of drug...
basicity and lipid solubility. Okumura et al., 1978, have implied the existence of active transport system or a specialised binding system as a mechanism for accumulation of basic drugs in the lung. There is also evidence to suggest that mitochondrial monoamine oxidase (MAO) is a binding site for tertiary basic drugs in the lung (Yoshida et al., 1989) and may hence act as a reservoir. Carrier-mediated transport processes for the uptake of organic anions (Schanker, 1978) and amino acids (Lin and Schanker, 1981) have been reported in the rat lung. However, there is no reported accumulation of weakly acidic drugs in the tracheal fluid, suggesting that the lung plays a minor role in the elimination of these compounds.

4.4. **Effect of indomethacin on physiological factors**

Indomethacin appears not to influence arterial pO2 and arterial pCO2 in both high and low dose ewes, as they remain constant throughout the study period in relation to control (Figures 41 A, B). Indomethacin also did not appear to affect fetal arterial blood lactate levels in low dose ewes. However, in three high dose ewes lactate levels were markedly higher than respective controls (Figure 41 D). It was interesting to note that the drug did not influence arterial glucose levels in either of the two dose groups (Figure 41 C). Significant decreases in arterial pH as well as base excess were observed when indomethacin was given in higher doses, whereas the values remained more or less constant in low dose ewes compared to the respective controls (Figures 41 E, F). It can be observed that indomethacin
caused lactic acidosis in some high dose ewes (E 1221, E 984, and E 2160) and that the effect might be dose related as the same was not observed in low dose ewes. Further, a severe metabolic acidosis was observed when indomethacin was administered as a 6 hour intravenous infusion at a dose of 17 μg/min in hypoxic ovine fetuses (Hooper et al., 1992). The severe lactic acidosis observed in E 1221, E 2160, and E 984 (high dose group fetuses) were characterised by markedly reduced fetal pH. All the three fetuses died following cessation of drug infusion. That the deaths of these fetuses was attributable solely to drug effect is questionable, as death due to infectious etiology cannot be ruled out. Further, high dose group fetuses that survived the experimental protocol demonstrate that the drug does not cause significant lactic acidemia. Also, no significant reduction in arterial pH, pO₂, pCO₂, or base excess was observed. Further investigation is underway to elucidate the relationship between indomethacin and lactic acidemia.

In three of the high dose ewes (E 2160, E 984Y, and E 796), there was a significant reduction in fetal urine flow rate, in the order of 50%, within 23 hours of initiating indomethacin infusion (Figure 44). A similar reduction in urinary flow rate, in the order of 55%, was observed within 2 hours of a 5 h indomethacin infusion at an identical dose (Walker et al., 1992a). This effect on urinary flow rate appears to be dose related. When indomethacin was infused at a rate of 0.017 mg/kg/min for 5 h, fetal urinary output significantly increased by an order of ~85%
within 3 hours (Walker et al., 1992b). Further studies are being carried out to elucidate the mechanism of oliguria and its physiological implications.
5. SUMMARY AND CONCLUSIONS

The following points summarise the salient features of the developed assay method:

1. Simple one step liquid-liquid extraction procedure.
2. High efficiency fused silica capillary column technology.
3. Rapid run times (9.25 min)
4. Simple and rapid derivatisation with the relatively safer MTBSTFA.
5. Small sample volumes (0.1-1.0 mL).
6. Sensitivity of 1 ng/mL (Limit of quantitation, < 10% C.V., S/N >10).
7. Excellent chromatography with both the analyte, indomethacin, and the internal standard, α-methyl indomethacin, eluting in regions free from endogenous interference.

Overall the developed method offers several advantages over existing analytical techniques for indomethacin in biological fluids, the most important being: improved sensitivity, simpler sample preparation, improved selectivity, and a smaller biological fluid sample requirement.
Application of the method to indomethacin measurement in plasma, urine, tracheal and amniotic fluid samples was demonstrated by investigating its disposition in the ovine fetus. The following observations were made:

1. The fetal plasma concentrations of indomethacin in the high dose animals were similar to those seen during human tocolysis (Moise et al., 1990).
2. The low levels of indomethacin in amniotic fluid and fetal urine are consistent with our observations for other organic acids (e.g., valproic acid, diphenylmethoxyacetic acid) in the fetal lamb.
3. Unlike our observations with basic amine compounds (e.g., metoclopramide, diphenhydramine), indomethacin was not observed to accumulate in tracheal fluid.
4. The mean steady-state fetal femoral arterial concentration of indomethacin was $181.67 \pm 45.05$ ng/mL in the high dose group ($n=5$) and $41.29 \pm 3.14$ ng/mL in the low dose group ($n=2$).
5. The arterio-venous concentration difference for indomethacin, at apparent steady state, was minimal but statistically significant suggesting a low placental permeability to indomethacin in sheep. This finding is consistent with observations in other studies (Anderson et al., 1980; Harris and Vanpetten, 1981).
6. The fetal total body clearance of indomethacin was estimated to be 44.55 ± 11.14 mL/min (n=7).

7. The placental clearance of indomethacin as determined by the Fick principle was calculated to be 8.85 ± 0.81 mL/min/kg.
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