system (Hewlett Packard, Palo Alto, CA, U.S.A.) comprising a microbore solvent delivery system, a sample loop injector of 250 μl capacity, autoinjector, autosampler and a computer for acquiring, integrating and storing data files. Separation was achieved on a Hypersil-ODS reversed-phase microbore column (200 mm × 2.1 mm, 5 μm particle size) (Hewlett Packard). A guard column (20 mm × 2.1 mm, Hypersil-ODS, 5 μm) (Hewlett Packard) and an on-line solvent filter assembly were also used in series with the column. The injector, column and detector were connected using 0.12 mm I.D. flexible stainless-steel tubing. The detector used was a HP 1046A programmable fluorescence detecor (Hewlett Packard).

The fluorescence intensity of labetalol was monitored at an excitational wavelength of 196 nm and an emission wavelenght of 412 nm with a 370-nm emission cut-off filter. The "Photo Multiplier Tube (PMT) Gain" (*i.e.* signal amplification) was set at 18 (maximum amplification) or 15 in a scale of 1–18. The optical system of the detector included a 2 mm wide excitation slit with two 4 mm wide emission slits.

Sample collection

Labetalol hydrochloride (Trandate®) was given as an intravenous bolus (100 mg) to a pregnant ewe, chronically instrumented as decribed by Rurak *et al.* [16], at 132 days gestation (term ≈ 145 days). Following drug injection, samples were collected from the maternal femoral vein, fetal tarsal vein, common umbilical vein and amniotic and fetal tracheal fluids at −5, 3, 10, 15, 20, 30, 45, 60 min and 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5, 6, 8, 10, 12 and 24 h. Blood samples were collected in heparinised tubes and following centrifugation, plasma was separated and stored in pyrex glass tubes with screw cap lined with polytetrafluoroethylene (PTFE) (Corning Glass Works, Corning, NY, U.S.A.). Amniotic and fetal tracheal fluids were collected and stored in pyrex glass tubes. Control or blank biological fluid samples were obtained from the sheep before the administration of drug and were used for calibration curves and extraction recovery studies. All samples were stored at −20°C until analysed.

Extraction of labetalol

Labetalol was extracted from plasma, amniotic and tracheal fluid as follows. To 0.25 ml of the biological fluid in a glass test tube, 0.25 ml of internal standard solution (40 or 300 ng/ml) and 0.5 ml of carbonate buffer (1 *M*), pH 9.5, were added and extracted with 6 ml of ethyl acetate by mixing for 20 min on a rotary shaker (Labquake Tube Shaker, Model 415-110, Lab Industries, Berkeley, CA, U.S.A.). The tubes were then refrigerated for 15 min to break any emulsion that might have formed during mixing. This was followed by centrifugation at 400 *g* for 6 min. The organic layer was transferred to a clean, dry screw-capped glass tube and mixed with 0.5 ml of 0.01 *M* phosphoric acid for 20 min, centrifuged, and 60 μl of the aqueous layer were injected into the HPLC system. To obtain

standard curves, blank samples were spiked with standard solutions of labetalol and extracted as decribed above. The efficiency of extraction of labetalol from each of the biological fluids was determined over the entire concentration range by comparing the peak-area ratio of the extracted samples with that of direct injection of standard solutions.

RESULTS AND DISCUSSION

Chromatography

Representative chromatograms (blank and spiked) of labetalol extracted from maternal plasma, amniotic fluid, fetal plasma and tracheal fluid are shown in Fig. 1. Satisfactory resolution of labetalol from the endogenous substances was obtained in all the biological fluids following the two-step extraction procedure (extractions with ethyl acetate followed by dilute phosphoric acid). The proportion of acetonitrile in the mobile phase had to be adjusted to 42% for analysing amniotic fluid samples, to resolve an endogenous interference eluting just before labetalol in the chromatogram, while 44% was optimal for the other biological fluid samples.

Calibration and extraction recovery

When maximum signal amplification (PMT = 18) was used, the detector response was near maximum with about 2 ng of labetalol (injected). Hence, to

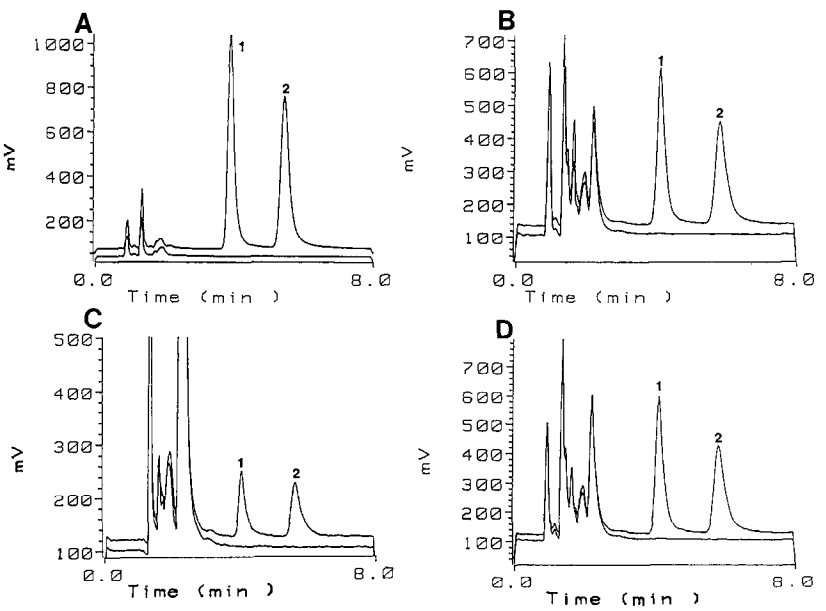

Fig. 1. Superimposed chromatograms of blank and spiked biological fluids. (A) Pregnant sheep plasma; (B) amniotic fluid; (C) fetal plasma; (D) fetal tracheal fluid. Peaks: 1 = labetalol; 2 = internal standard.

accommodate the wider range of concentrations seen in plasma, two calibration curves using two different PMT settings (18 and 15) were employed and both of them were found to be linear. In typical calibrations, the coefficients of correlation were 0.9985 and 0.9996 for the lower (0.5–20 ng) and higher (20–120 ng) concentration ranges in plasma, respectively. Similar correlations were also obtained with the other biological fluids. The mean intra-day coefficient of variation (C.V.) over the concentration range in plasma was found to be 2.95 ± 2.76%, while in the case of amniotic and fetal tracheal fluids the values were 3.85 ± 3.2 and 4.12 ± 2.7%, respectively. The ratio of the volume injected (60 μl) to the volume of the final extract (0.6 ml) was 0.1, which enabled multiple injections, unlike the case with some of the reported methods [12,13], where intra-sample variability could not be studied. We found this source of potential variability to be a negligible factor (C.V. consistently less than 0.5%) in our system. The mean extraction recovery of labetalol from plasma over the entire range studied (*i.e.* 0.5–120 ng) was 76.01 ± 2.8%. Good correlation was seen between the amount of labetalol added and the amount extracted ($r = 0.9998$) indicating that the extraction recovery was not concentration-dependent. Mean extraction recoveries from amniotic and tracheal fluids over the calibration range (0.5–20 ng) were found to be 70.56 ± 3.76 and 75.12 ± 8.8%, respectively.

Sensitivity

The use of a microbore column could significantly enhance mass sensitivity [17] and minimum detection limit [18] and thus is ideal for limited sample size application. Hence, in this assay, a microbore column (2.1 mm I.D.) and 0.12 mm I.D. connections (between injector, column and detector) were employed. Also, the potential peak diffusion that could occur at the detector was overcome with the use of a low-volume flow cell (5 μl capacity). The use of an emission cut-off (370 nm) filter resulted in significant reduction in baseline noise. Further optimisation of the signal-to-noise ratio was achieved with the use of a 2 mm wide excitation slit and 4 mm wide emission slits instead of the standard 1 mm and 2 mm wide slits, respectively. We defined the minimum quantifiable detection limit as the least amount that would produce a signal-to-noise ratio of at least 4, with a C.V. of not more than 10%. Accordingly, the minimum quantifiable limit in this assay was ≈ 30 pg of labetalol injected (absolute sensitivity). In terms of amount added (apparent sensitivity), this was equivalent to about 0.4 ng and in concentration terms was about 1.6 ng/ml (using a 250-μl sample volume). Fig. 2 shows a chromatogram obtained from plasma spiked with 0.5 ng of labetalol. The sensitivity/detection limit of the previously reported labetalol assays (in terms of plasma concentration) ranged from 1 to 20 ng/ml and the minimum quantifiable limit (calculated from the data provided in the reports) ranged from 0.45 to 3.0 ng of labetalol injected. Hence, the assay described here is at least one order of magnitude more sensitive than the existing labetalol assays.

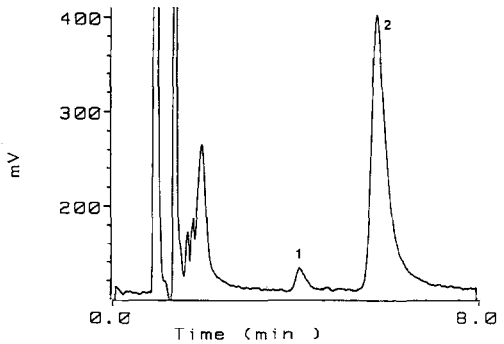

Fig. 2. Chromatogram obtained from blank pregnant sheep plasma spiked with 0.5 ng of labetalol (the lowest calibration point) and internal standard. Approximately 40 pg of labetalol were actually injected. Peaks: 1 = labetalol; 2 = internal standard.

Application to pregnant sheep pharmacokinetics

The disposition of labetalol in a chronically instrumented pregnant ewe following a 100-mg intravenous bolus is shown in Fig. 3. All the determinations were made in duplicate with C.V.s less than 10%. Labetalol concentrations in the

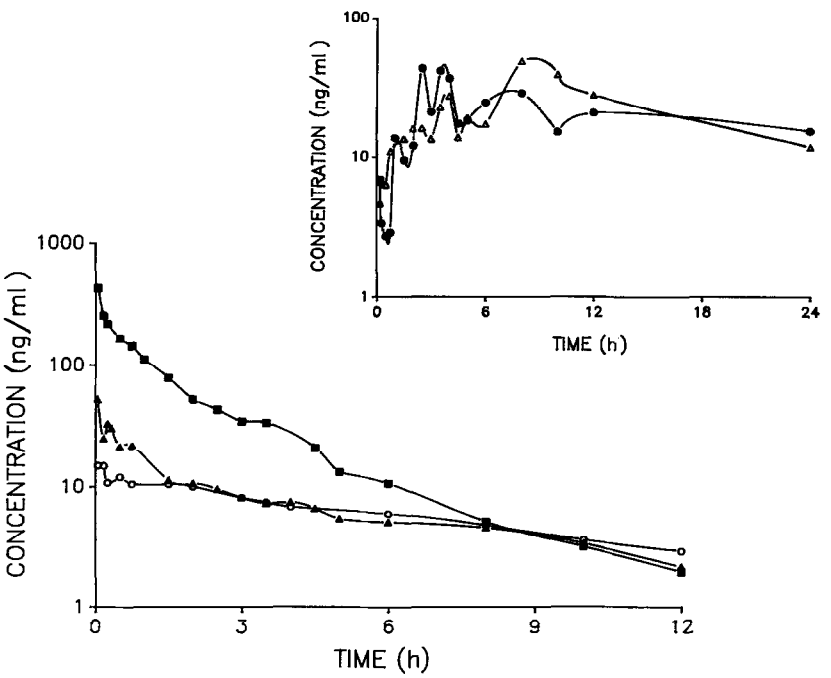

Fig. 3. Concentration–time curves for labetalol in the various biological fluids obtained from the chronically instrumented pregnant sheep following a 100-mg bolus to the ewe. The concentration–time profiles in the fluid compartments are shown in the inset. (■) Maternal femoral vein; (○) fetal tarsal vein; (▲) umbilical vein; (△) fetal tracheal fluid; (●) amniotic fluid.

maternal plasma were best fitted by a tri-exponential equation, while the disposition in the fetal plasma was found to be mono-exponential [19]. The elimination half-life of labetalol was found to be 2.0 and 3.1 h in the maternal and fetal plasma, respectively. The maternal total body clearance was about 18 ml/min · kg and the apparent volume of distribution was 0.9 l/kg. Labetalol could still be detected in amniotic and tracheal fluids after 24 h following drug administration, and the rate of elimination from these fluid compartments was much lower than that seen in maternal or fetal plasma.

CONCLUSION

A highly sensitive microbore HPLC assay with low-dispersion fluorescence detection has been developed for the rapid quantitative determination of labetalol in various biological fluids of the chronically instrumented pregnant sheep. Significant improvement over existing methods for the determination of labetalol in biological fluids has been made in terms of sample volume used (only 250 μl required), reduced variability (mean C.V. less than 5%) in quantitation, wider application of the method and higher sensitivity of detection of labetalol. Sensitivity of the assay has been optimised by (a) minimising the dead volume in the system through the use of microbore column and connections, (b) reducing the baseline noise with an appropriate cut-off filter, (c) enhancing the signal intensity by using wider excitation and emission slits and (d) using a low-dispersion fluorescence cell. In terms of amount injected, the lower limit for quantitation was 30 pg of labetalol. This assay has been applied to the study of placental transfer and maternal—fetal disposition of labetalol.

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