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Note

Determination of ritodrine in blood and plasma by high-performance liquid chromatography with fluorescence detection

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Ritodrine is a β_2-sympathomimetic amine used to arrest preterm labour and prevent preterm delivery in pregnant women [1]. The pharmacokinetics of ritodrine in pregnant women require detailed characterisation in order to establish whether the present intravenous infusion regimen [1] is the most appropriate to rapidly achieve therapeutic plasma concentrations while minimising significant maternal side-effects such as tachycardia. Ritodrine is used widely in obstetrics; however, little information has been reported concerning its disposition in pregnant or non-pregnant women, presumably owing to the lack of sensitive and specific assay methodology. Reliable pharmacokinetic data in pregnant women in preterm labour have still to be reported.

Ritodrine concentrations in plasma have been determined by radioimmunoassay [2] and reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection [3, 4]. The radioimmunoassay [2] has excellent sensitivity; however, its use has been limited by the poor availability of ritodrine antiserum. In addition, the wide variability in maternal serum concentrations

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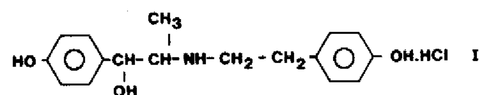

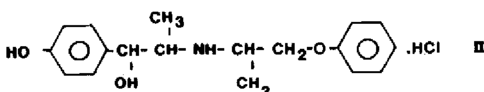

Fig. 1. Structures of ritodrine hydrochloride (I) and the internal standard isoxsuprine hydrochloride (II).

reported [5] using this technique has been attributed [4] to cross-reactivity of the antiserum with endogenous substances. The specificity and sensitivity of the HPLC methods using electrochemical detection were good [3, 4]. The application of these techniques is limited, however, to laboratories equipped with electrochemical detectors.

The determination of blood ritodrine concentrations, though of considerable importance, has not yet been reported. Appreciable blood cell uptake of ritodrine may be expected to occur as a result of association with β_2-adrenoceptors recently identified on cellular membranes [6].

The native fluorescence of ritodrine has been investigated and employed as a selective means of detection as has been utilised for other β-sympathomimetics such as salbutamol [7] and prenalterol [8]. In this study a sensitive and specific method has been developed to determine ritodrine concentrations in blood and plasma by reversed-phase HPLC with fluorescence detection. This method is sufficiently sensitive to enable detailed characterisation of ritodrine pharmacokinetics in pregnant and non-pregnant women.

EXPERIMENTAL

Reagent and materials

Ritodrine hydrochloride (>99.5% pure) and the internal standard isoxsuprine hydrochloride (>99.5% pure) (Fig. 1), generously donated by Ethnor Australia, were of analytical reference standard. Acetonitrile (Mallinckrodt, Sydney, Australia) was of HPLC grade. All other chemicals were of analytical grade and obtained from Ajax Chemicals (Sydney, Australia). Carbonate buffer, 1 *M*, pH 9.48, consisted of 26.5 g sodium carbonate and 21 g sodium bicarbonate in 500 ml of distilled water. Ethyl acetate, for extraction, was freshly distilled prior to use. Glass extraction tubes (15 ml) fitted with screw caps with PTFE liners and glass evaporation tubes (15 ml) were soaked in 2% Liquid Pyroneg® (Diversey, Sydney, N.S.W., Australia), washed with 1 *M* sodium hydroxide and 1 *M* hydrochloric acid, rinsed thoroughly with double-distilled water and dried prior to use.

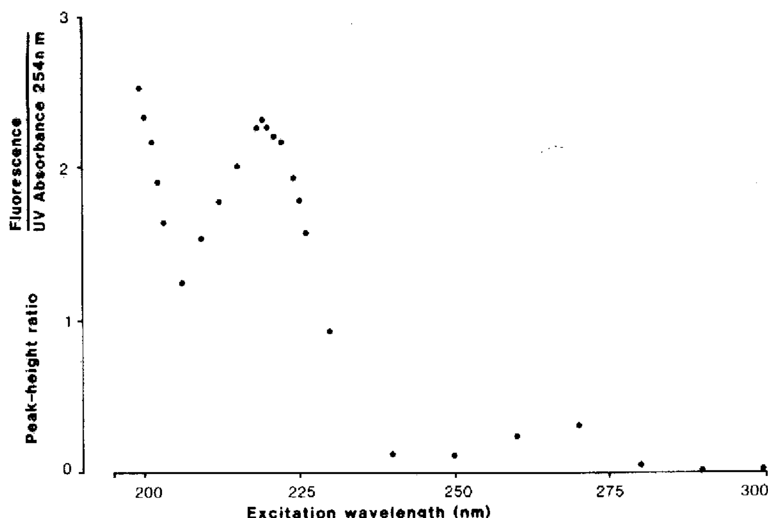

Fig. 2. Variation in the ratio of the fluorescence detector response to that of the UV absorbance at 254 nm as a function of excitation wavelength with repeated injections of 200 ng ritodrine hydrochloride.

Chromatographic equipment

An M45 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) was used to pump mobile phase through a Rheodyne 7125 loop injector (Cotati, CA, U.S.A.) fitted with a 100-μl loop, and onto either a μBondapak phenyl column (300×3.9 mm I.D., particle size 10 μm, Waters Assoc.) for the plasma assay or a Spheri-5 RP-18 column (250×4.6 mm I.D., particle size 5 μm, Brownlee Labs., Santa Clara, CA, U.S.A.) for the blood assay. An FS970 spectrofluorimeter (Schoeffel, Westwood, NJ, U.S.A.) at a time constant of 2, and a Sekonic SS250F chart recorder (Technico Electronics, Sydney, Australia), with an input voltage of 10 mV and a chart speed of 15 cm/h, completed the chromatographic equipment used.

Optimal excitation and emission wavelengths

The optimal excitation wavelength to quantify ritodrine was determined by connecting the FS970 fluorescence detector in series with a Waters 440 ultraviolet (UV) absorbance detector at 254 nm. An aliquot of ritodrine hydrochloride, 200 ng in distilled water, was injected while the excitation wavelength was increased in increments from from 199 to 300 nm. No emission filter was used. The ratio of the peak height of ritodrine fluorescence, emitted at each excitation wavelength, to the peak height of the UV absorption at 254 nm was calculated and graphed as a function of the excitation wavelength (Fig. 2). The maximal responses occurred at very low wavelengths. An excitation wavelength of 200 nm was selected and used for all analyses.

The total fluorescence emitted by ritodrine was collected in all analyses. The use of emission filters at the excitation wavelength 200 nm diminished the detector response (the 320-nm filter decreased the response by 51%, 340-nm filter by

60% and 370-nm filter by 97%) and increased the detector baseline noise, thereby compromising sensitivity.

Mobile phase

The mobile phase used for quantification of ritodrine in plasma, using the phenyl column, consisted of 0.05% orthophosphoric acid in water–acetonitrile (83:17, v/v). The flow-rate was constant at 2 ml/min resulting in an inlet pressure of 60 bar. The low mobile phase pH, 2.5, did not adversely affect column life (up to two years). The mobile phase used to assay ritodrine in blood, using the RP-18 column required a higher proportion of acetonitrile to ensure resolution from endogenous compounds and a rapid analysis time. The optimal mobile phase was 0.05% orthophosphoric acid in water–acetonitrile (37:63, v/v). The flow-rate was again 2 ml/min resulting in an inlet pressure of 150 bar. The mobile phase was always passed through a 0.5-μm filter (Millipore, Bedford, MA, U.S.A.) and thoroughly degassed prior to use. All analyses were performed at ambient temperature.

Collection of biological samples

Blood samples were obtained at various times, via an indwelling cannula, from pregnant women receiving ritodrine hydrochloride by intravenous infusion to arrest preterm labour and from healthy non-pregnant volunteers administered a 10-mg intravenous infusion of ritodrine hydrochloride over 1 h. Drug-free blood and plasma for the preparation of calibration curves were obtained from healthy women by venepuncture. All blood samples were transferred to glass tubes containing 143 I.U. lithium heparin (Venoject, Terumo Medical Corp., Elkton, MD, U.S.A.). A 1-ml aliquot of blood was accurately transferred to a plain plastic tube, mixed with an equal volume of distilled water and stored at $-22°C$ pending analysis. The remaining blood was centrifuged for 10 min at 1750 *g* to separate plasma which was stored in plain plastic tubes at $-22°C$ prior to analysis. No degradation of ritodrine was observed under these storage conditions for at least three months in plasma and two months in blood (experiments continuing).

Extraction from plasma or blood

To a 1-ml aliquot of plasma or a 0.9-ml aliquot of diluted blood in a glass extraction tube were added 80 ng (50 μl) of the internal standard, isoxsuprine hydrochloride, 1 ml or 0.9 ml of 1 *M* carbonate buffer (pH 9.48) and 5 ml of ethyl acetate. The mixture was shaken for 1 min using a vortex mixer and subsequently centrifuged for 7 min at 1750 *g*. The organic phase was transferred to a glass evaporation tube and most of the ethyl acetate evaporated under a stream of nitrogen in a water-bath at 57°C. The final 0.5 ml of ethyl acetate was evaporated under a stream of nitrogen at room temperature. The residue was redissolved in 100 μl of acetonitrile by agitation on a vortex mixer for 15 s and injected onto the HPLC column.

Calibration and reproducibility

Known quantities of ritodrine hydrochloride ranging from 5 to 150 ng (equivalent to 4.5–133.5 ng of ritodrine base) and 80 ng of isoxsuprine hydrochloride

were added to drug-free plasma or blood and processed according to the analytical procedure described above. Calibration curves were constructed by plotting the ratio of the peak height of ritodrine to the peak height of isoxsuprine against the amount of ritodrine added. A calibration curve was constructed on each day that patient samples were assayed.

The within-day reproducibilities of the plasma and blood assays were determined by analysing replicate samples ($n=6$ or 7) at each of the concentrations of the calibration curve on the same day. The between-day reproducibility was assessed from the peak-height ratios for each standard, obtained from ten different calibration curves, generated over a three-month period.

RESULTS AND DISCUSSION

Ritodrine fluoresces strongly when excited at low wavelengths (Fig. 2). This property has enabled the development of a sensitive HPLC assay with fluorescence detection for ritodrine in both blood and plasma. The limit of sensitivity of the fluorescence technique was determined by injecting aqueous ritodrine standards directly onto the HPLC column. A signal-to-noise ratio of 3:1 was produced by 0.75 ng of ritodrine hydrochloride (equivalent to 0.67 ng ritodrine base). The limits of detection reported for the determination of ritodrine by radioimmunoassay [2] and HPLC with electrochemical detection [3, 4] were 0.1, 0.2 or 0.3 ng, respectively. The present HPLC assay with fluorescence detection, though less sensitive under the conditions reported here, has more than adequate sensitivity for the determination of ritodrine concentrations observed in biological fluids during routine administration.

Initial experiments were performed to establish a reliable and sensitive assay for ritodrine in plasma. A reversed-phase phenyl column was chosen because the polar packing material retains polar, basic compounds, like ritodrine, well. Numerous mobile phases, containing acetonitrile or methanol as the organic modifier, and phosphoric acid or phosphate buffers over a range of pH values (2.5–7.0) and ionic strengths (0.005–0.1 M) were investigated to establish the optimal conditions to quantify ritodrine reliably (capacity factor >2), ensure separation from endogenous substances and maintain a reasonably short assay time for both ritodrine and the internal standard.

A number of β_2-sympathomimetic amines were investigated as potential internal standards. Salbutamol and terbutaline (retention times 2.2 and 2.3 min, respectively) were not resolved from endogenous peaks from plasma extracts and fenoterol (retention time 3.2 min) was not stable under the basic extraction conditions. Isoxsuprine (retention time 15.1 min) was chosen as the internal standard owing to its reliable extraction, strong fluorescence and good peak shape. The assay time (16 min) is reasonably short using the mobile phase finally selected and the capacity factors for ritodrine (retention time 3.7 min) and isoxsuprine were 2.1 and 11.7, respectively.

No chromatographic interference from endogenous compounds was observed in plasma samples collected from pregnant women or non-pregnant volunteers prior to (Fig. 3A) or after commencement of the ritodrine infusion (Fig. 3B, C).

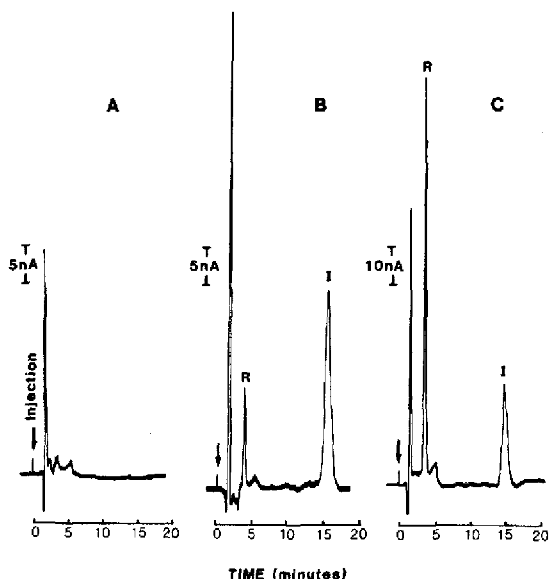

Fig. 3. Chromatograms of plasma extracts from (A) non-pregnant woman prior to ritodrine infusion, (B) pregnant woman 3.5 h after cessation of an infusion of 0.112 mg/min ritodrine hydrochloride (concentration, 8.4 ng/ml) and (C) pregnant woman during an infusion of 0.364 mg/min ritodrine hydrochloride (concentration, 114 ng/ml). Peaks: R = ritodrine; I = isoxsuprine, internal standard.

To test for potential interference with ritodrine or the internal standard by drugs commonly administered during preterm labour aqueous solutions of the most frequently used agents were injected into the HPLC system. Betamethasone, bupivacaine, caffeine, chloral hydrate, dexamethasone, diazepam, lignocaine, metoclopramide, morphine, nitrazepam, paracetamol, pethidine and salbutamol did not either fluoresce or elute at retention times which would interfere under the assay conditions used.

The HPLC conditions selected for the plasma assay were not appropriate for the analysis of ritodrine in blood because the simple, rapid extraction technique also extracted endogenous compounds which could not be resolved from ritodrine using the phenyl column. Other HPLC columns were therefore investigated and the Spheri-5 RP-18 column was found to separate ritodrine from endogenous interfering substances resulting from the extraction of blank blood. Both ritodrine and isoxsuprine were well retained while still exhibiting good peak shape. To ensure a reasonable analysis time the acetonitrile content of the mobile phase was increased. Using the optimal mobile phase, the retention times of ritodrine and isoxsuprine were 9.3 and 16.3 min, respectively, and the capacity factors for ritodrine and isoxsuprine were 10.6 and 19.4 respectively. Typical chromatograms of extracted blood samples collected prior to and during ritodrine administration are shown in Fig. 4.

The methods developed to determine ritodrine concentrations in plasma and blood use simple isocratic mobile phases. With judicious column selection it was not necessary to incorporate ion-pairing reagents in the mobile phase as required

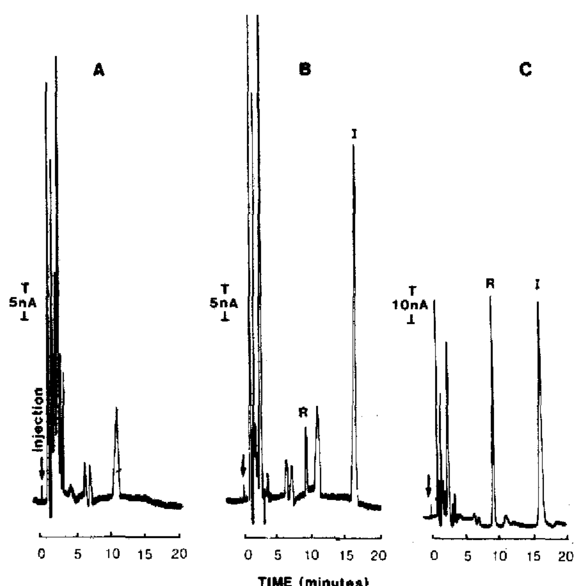

Fig. 4. Chromatograms of blood extracts from (A) pregnant woman prior to ritodrine infusion, (B) non-pregnant woman 2 h after cessation of an infusion of 0.171 mg/min ritodrine hydrochloride (concentration, 18.6 ng/ml) and (C) pregnant woman during an infusion of 0.112 mg/min ritodrine hydrochloride (concentration, 82.5 ng/ml). Peaks: R = ritodrine, I = isoxsuprine, internal standard.

by the previously described ritodrine HPLC assays [3, 4]. This results in considerable savings in column life and time since extensive washing to remove ion-pairing reagent is not necessary.

The extraction technique is simple and reliable. The recovery of ritodrine from ethyl acetate was superior to that from a number of other extracting solvents including diethyl ether and dichloromethane. The analytical recovery of ritodrine was determined by comparing the peak heights of extracted plasma and blood standards with the peak heights of aqueous standards of corresponding concentrations injected directly. The recovery of ritodrine from plasma was 68% ($n=6$, ritodrine hydrochloride concentration 90 ng/ml) and the recovery from blood was 58%. The lower recovery from blood is unrelated to a pH difference prior to extraction. After the addition of an equal volume of 1 *M* carbonate buffer the pH of plasma and blood are identical (pH 9.47, $n=5$ matched plasma and blood pairs).

The limit of detection of the ritodrine assay in both plasma and whole blood is 1 ng/ml. The assay is therefore suitable for the determination of both clinically relevant and pharmacokinetically significant blood and plasma concentrations.

The calibration curves for plasma (typically $r^2=0.996$, $y=0.026x+0.007$) and blood (typically $r^2=0.999$, $y=0.025x-0.003$) exhibited good linearity between peak-height ratio and ritodrine concentration from 4.5 to 133.5 ng/ml and the intercepts were not significantly different from zero. The within-day reproducibility of both the plasma [$n=6$ or 7, coefficient of variation (C.V.) = 4.9–8.8%] and the blood assays ($n=6$, C.V. = 3.6–7.5%) was good over the range of concen-

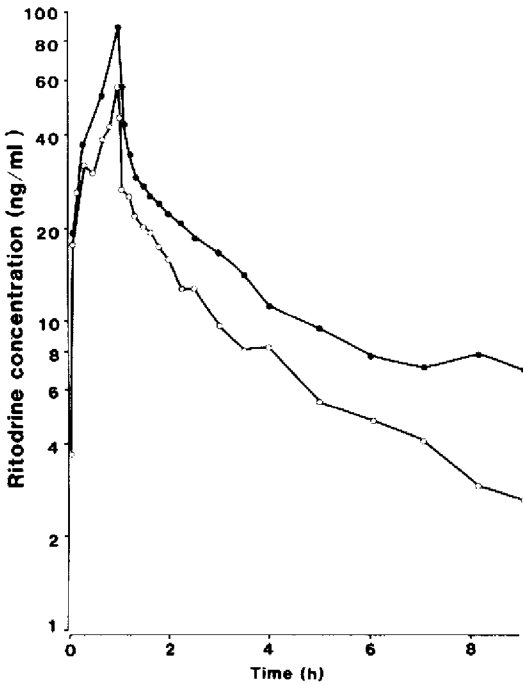

Fig. 5. Plasma (○) and blood (●) concentration-time profiles observed in a non-pregnant woman during and after an infusion of 10 mg of ritodrine hydrochloride over 1 h.

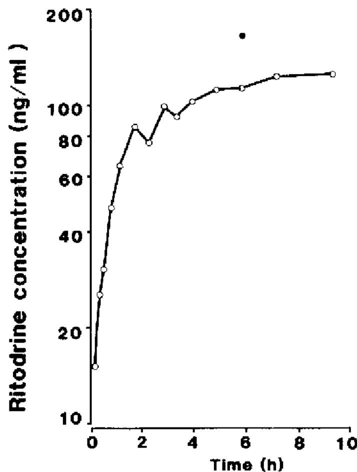

Fig. 6. Plasma concentration-time profile (○) observed in a 23-year-old pregnant woman (gestation 33 weeks), administered 230 mg ritodrine hydrochloride to arrest preterm labour. Infusion characteristics were: 0.182 mg/min for 10 min, 0.243 mg/min for 13 min, 0.364 mg/min for 15 min and 0.418 mg/min for 512 min. A single blood sample (●) was also assayed.

trations used in each calibration curve (4.5–133.5 ng/ml ritodrine). The between-day reproducibility was also good over a similar range of ritodrine concentrations for both the plasma ($n=10$, C.V.=4.7–10.2%) and the blood assays ($n=10$, C.V.=4–9.9%). The precision observed at low concentrations is comparable with

that of radioimmunoassay [2] and HPLC with electrochemical detection [3].

The methods described above have been used to measure the concentrations of ritodrine in over 150 blood samples and 600 plasma samples collected from pregnant and non-pregnant women participating in studies on ritodrine disposition. A typical plasma and blood concentration–time profile obtained in a non-pregnant woman administered at 10-mg infusion of ritodrine hydrochloride over 1 h is shown in Fig. 5. The plasma concentrations and a single blood concentration observed in a pregnant woman in preterm labour are shown in Fig. 6. It is interesting to observe that at all times blood concentrations of ritodrine are substantially higher than plasma concentrations indicating that ritodrine partitions in favour of blood cells.

In conclusion, a specific HPLC method has been developed to estimate ritodrine concentrations in both plasma and blood, with high sensitivity and reproducibility, utilising the native fluorescence of the ritodrine molecule. Unlike previous HPLC assays neither electrochemical detection nor an ion-pairing reagent in the mobile phase are required. The method is suitable for pharmacokinetic studies in pregnant and non-pregnant women. The distribution of ritodrine between blood cells and plasma and the pharmacokinetic significance of the observation that ritodrine blood concentrations are greater than plasma concentrations can be investigated using the methodology described here.

REFERENCES

1 T.P. Barden, J.B.Peter and I.R. Merkatz, Obstet. Gynecol., 56 (1980) 1-6.
2 K. Thomas, L. Vankrieken and M. Van Lierde, Gynecol. Obstet. Invest., 14 (1982) 151-155.
3 P. Kuhnert, P. Erhard, A. Dixon, B. Kuhnert and T. Gross, J. Liq. Chromatogr, 6 (1983) 2775-2783.
4 L.S. Lin, S.N. Caritis and L.K. Wong, J. Pharm. Sci., 73 (1984) 131-133.
5 R. Gandar, L.W. de Zoeten and J.B. van der Schoot, Eur. J. Clin. Pharmacol., 17 (1980) 117-122.
6 F. Bree, I. Gault, P. D'Athis and J.P. Tillement, Biochem. Pharmacol., 33 (1984) 4045-4050.
7 M.J. Hutchings, J.D. Paull and D.J. Morgan, J. Chromatogr, 277 (1983) 423-426.
8 C. Oddie, G. Jackman and A. Bobik, J. Chromatogr., 231 (1982) 473-477.