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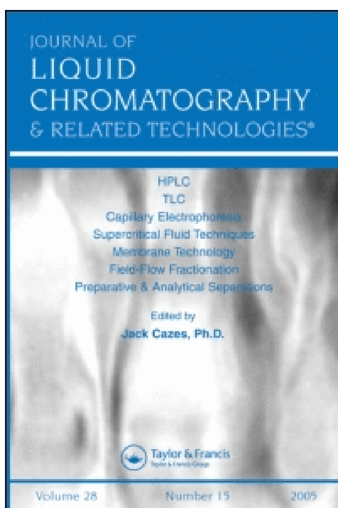

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RAPID DETERMINATION OF PRAZOSIN IN PERFUSION MEDIA BY HPLC WITH SOLID PHASE EXTRACTION

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ABSTRACT

A method is described for the quantitation of prazosin in tissue culture medium used for *in vitro* perfusion of human placental lobules. Prazosin was extracted using solid phase cartridges and the samples analysed by high performance liquid chromatography. The analysis utilised a C_{18} reversed-phase column maintained at 40°C with quantitation by fluorescence detection. The assay was linear to 100 ng/mL, intra-assay coefficients of variation measured at concentrations of 5 and 50 ng/mL were 5.8 and 6.2% respectively and inter-assay coefficients of variation were 4.9 and 2.7% for the same concentrations. The mean recovery of prazosin was 90.9 and 85.2% from solutions with concentrations of 5 and 50 ng/mL respectively. The minimum detectable limit was 0.1 ng/mL and the internal standard for this assay was propranolol. Analysis of a range of endogenous and

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exogenous compounds likely to be present in pregnancy plasma revealed only minor interference from lignocaine.

INTRODUCTION

Prazosin [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine], a selective post-synaptic alpha adrenergic antagonist, has been prescribed in pregnancy for the treatment of hypertension (1). This drug lowers peripheral resistance and decreases vasoconstrictor tone present in hypertensive patients.

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Prazosin has previously been quantitated by direct spectrofluorimetric analysis of samples (2-5) and by high performance liquid chromatographic (HPLC) assays using either ultraviolet or fluorimetric detection (6-17). The earlier direct fluorimetric measurement assays may be non-specific due to other fluorescent compounds in the matrix such as a reported metabolite of prazosin (13) whereas HPLC based techniques provide the specificity and sensitivity required to measure low concentrations of this drug. The majority of HPLC assays used solvent partitioning from alkaline plasma with ethyl acetate or another organic solvent followed by back extraction into dilute acid (6-8,10,12,13,15). One exception was dilution of plasma with acetonitrile to precipitate protein and analysis of an aliquot of supernatant (16). Solid phase extraction offers an alternative to the use and associated problems of disposal of large volumes of organic solvents as required by conventional liquid-liquid extraction procedures.

An assay for quantitation of prazosin in perfusion media has been developed as part of a study of metabolism and kinetics of maternofetal

transfer of antihypertensives in pregnancy. These studies utilise an *in vitro* perfused placental lobule model (18) with dosing of drug in either the maternal or fetal compartment. In this assay, prazosin and the internal standard (propranolol) are extracted directly from samples of tissue culture media from the perfusions using reversed-phase cartridges.

MATERIALS AND METHODS

Chemicals and Reagents

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Prazosin and propranolol (internal standard) were obtained from Sigma Chemical Company (St Louis, MO, USA). Sep-pak cartridges (C_{18}) were obtained from Millipore Corporation (Milford, MA, USA) and methanol was of liquid chromatography standard (FSE, Homebush, Australia). All other chemicals were of analytical reagent grade. The perfusion fluid, as described by Cannell *et al.* (18), consisted of tissue culture medium M199 (Sigma Chemical Company) augmented with glucose (2 g/L), heparin (25 IU/mL), gentamicin (100 mg/L, David Bull Labs., Melbourne, Australia) and dextran (approximate molecular weight = 40000, 7.5 g/L, Sigma Chemical Company). All assay validation studies were conducted using fresh tissue culture media as a sample matrix.

HPLC Instrumentation

The HPLC system consisted of a Kortec model K35D pump (ICI Instruments, Sydney, Australia), a Rheodyne model 7125 injector

(Rheodyne, Cotati, CA, USA) fitted with a 100 µL sample loop and a Hitachi F1000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan). Samples were analysed on an Alltima C_{18} column (5 µm particle size, 250 mm x 4.6 mm ID, Alltech Associates Inc., Deerfield, IL, USA). The column was maintained at 40°C in a model TC1900 column oven obtained from ICI Instruments (Sydney, Australia).

HPLC Analysis

The mobile phase used was methanol (55%, v/v) in 0.05 M phosphate buffer (pH 5.8, final pH = 5.1) and was filtered through a 0.45 µm nylon membrane filter (Alltech). The flow rate was 1 mL/min and the fluorescence detector was set at an excitation wavelength of 280 nm and an emission wavelength of 395 nm.

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Sample Extraction

Reversed-phase cartridges were primed by washing with methanol (10 mL) followed by water (10 mL). Internal standard (1 µg) was added to samples (1 mL) which were diluted with water (2 mL) before being applied to reverse-phase cartridges for extraction. The loaded cartridges were washed with water (10 mL) and excess water removed by air aspiration. Prazosin and internal standard were eluted from the cartridges with methanol (5 mL), collected in a silanised glass tube and solvent removed by a gentle stream of nitrogen at 40°C. The residue was reconstituted in mobile phase (50 µL), centrifuged for 5 minutes (700 g) and a 30 µL aliquot analysed by HPLC.

Standard Solutions

Stock solutions of prazosin and propranolol (1 mg/mL) were prepared in methanol. Standard prazosin solutions were diluted in perfusate to provide solutions for other validation studies.

Linearity

A solution of prazosin in perfusate was serially diluted to provide samples with concentrations ranging from 2.5 to 100 ng/mL. Duplicate aliquots (1 mL) were spiked with a constant amount (1 μg) of internal standard and were extracted and analysed as described above. Peak height ratios (prazosin to internal standard) were calculated and plotted as a function of increasing prazosin concentration.

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Recovery

The recovery of prazosin was determined at concentrations of 5 and 50 ng/mL. Duplicate samples (1 mL) at each concentration were spiked with internal standard (1 μg), extracted using solid phase cartridges and analysed as described earlier. Peak height ratios (prazosin to internal standard) were determined and compared with those obtained by analysis of a corresponding set of nonextracted standards. The recovery of the internal standard, propranolol, was determined using this procedure at a concentration of 1 μg/mL.

TABLE 1

Drugs Tested for Interference in the Assay for Prazosin

Alcuronium	Hydrocortisone	Phenytoin
Aminophylline	Hyoscine	Prednisolone
Atenolol	Hyoscine-N-Butylbromide	Prednisone
Atropine	Labetolol	Promethazine
Betamethasone	Lignocaine	Propylthiouracil
Bupivicaine	Methimazole	Pyridoxine
Cortisone	Metoclopramide	Ranitidine
Dexamethasone	Noradrenaline	Salbutamol
Diazepam	Phenobarbitone	Verapamil
Diltiazem	L-Phenylephrine	

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Specificity

A series of drugs and endogenous compounds (Table 1) were tested for potential interference with the analysis of either prazosin or the internal standard under the conditions of the assay. Stock solutions of each compound to be analysed were prepared in ethanol, the solvent evaporated and the residue dissolved in mobile phase. Retention times were then determined by HPLC analysis. Perfusate samples from six hour recirculating placental perfusion experiments, which did not use prazosin as substrate, were extracted and analysed to determine if any compounds from the tissue culture medium, or endogenous compounds released from the placental tissue during perfusion, would interfere in the assay.

Precision Studies

Intra-assay and inter-assay coefficients of variation were determined for samples with prazosin concentrations of 5 and 50 ng/mL. Five perfusate samples (1 mL) were extracted and analysed for intra-assay precision. This procedure was repeated over 5 days to determine inter-assay precision.

Minimum Detectable Limit

This value was defined as the concentration at which the ratio of sample response to that of the background was 2.0.

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Human Placental Perfusion

A peripheral lobule of a term human placenta was perfused in dual recirculating mode as described previously (18). Prazosin was added to the maternal circulation (50 ng/mL final concentration) and samples (1.2 mL) were drawn from both maternal and fetal circulations at regular intervals during the perfusion period (5 hours). These samples were extracted and analysed for prazosin concentration.

RESULTS

HPLC analyses of tissue culture medium from 6 hour placental perfusions are illustrated in Figure 1, with chromatograms from analyses of

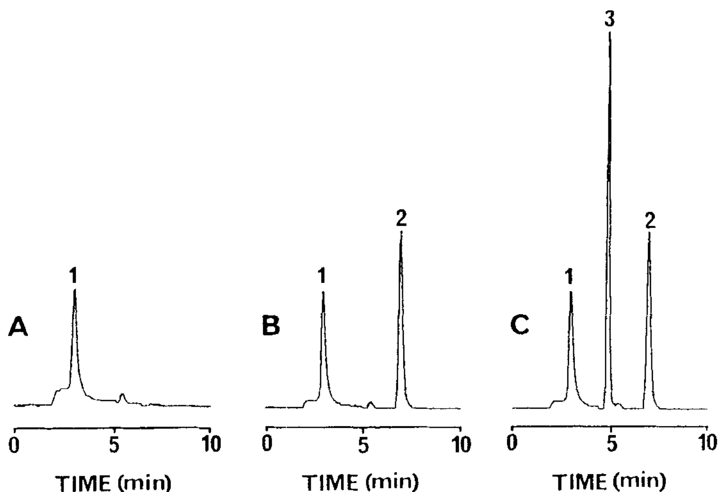

FIGURE 1. HPLC analysis of prazosin in tissue culture medium from 6 hour placental perfusions with (A) blank medium, (B) medium with internal standard and (C) medium with prazosin and internal standard.

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blank medium, medium containing internal standard and medium with prazosin and internal standard. Prazosin and the internal standard, propranolol, eluted at 4.9 and 7.1 minutes respectively and were baseline separated under the conditions of the assay.

The mean recoveries of prazosin using solid phase extraction, at concentrations of 5 and 50 ng/mL, were 90.9 and 85.9% respectively, while the recovery of the internal standard, propranolol, was 70.5% at a concentration of 1 µg/mL. The response was linear in the range 2.5 to 100 ng/mL with a typical calibration curve giving an equation of $y = 0.0178x + 0.0573$; $r = 0.995$, where y = peak height ratio of prazosin to internal standard, x = concentration (ng/mL) of prazosin and r = correlation coefficient..

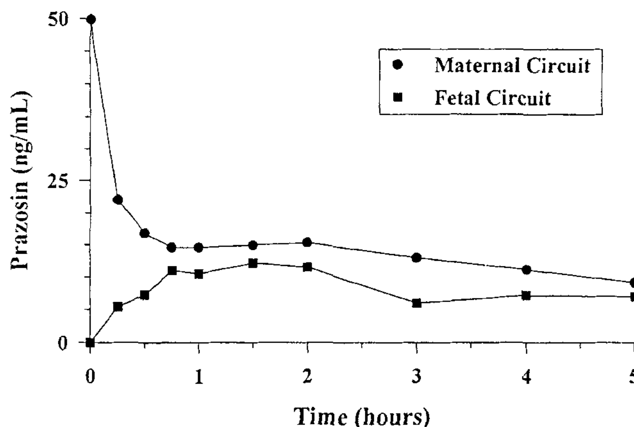

FIGURE 2. Prazosin transfer from the maternal to the fetal compartment following a 5 hour placental perfusion with dosing of prazosin in the maternal compartment.

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The assay was reproducible with intra-assay and inter-assay coefficients of variation respectively of 5.8 and 4.9% for samples with a prazosin concentration of 5 ng/mL and 6.2 and 2.7% for samples with a prazosin concentration of 50 ng/mL. The compounds listed in Table 1 were analysed under the conditions of the assay for interference due to coelution with prazosin or the internal standard. No compounds were found which interfered in the assay except for lignocaine which coeluted with prazosin, however the interference was not significant due to the low fluorescence response for lignocaine at therapeutic concentrations, at the wavelengths used in this assay.

The assay was applied to the analysis of prazosin transfer from the maternal to the fetal compartment of a human placental lobule perfused *in vitro* following dosing of prazosin in the maternal circuit. Samples were analysed at intervals during a five hour perfusion and the concentration of prazosin in both circuits is shown in Figure 2.

DISCUSSION

This assay utilises fluorescence detection with an excitation wavelength of 280 nm and emission intensity monitored at 395 nm. These values were chosen by experiment with samples from 6 hour perfusions, with and without prazosin as substrate, to minimise interference and maximise sensitivity for extracts from this tissue culture matrix. The combinations for other assays for prazosin extracted from plasma have generally used excitation and emission wavelengths in the ranges 243 to 258 and 370 to 390 nm respectively (6–18). The limit of detection for the assay was 0.1 ng/mL, which compares favourably with the 0.1–2 ng/mL limits described for other HPLC assays using fluorescence as the detection method (6–10).

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Prazosin and propranolol were extracted from the sample matrix using reversed-phase cartridges with reproducible and high mean recoveries for prazosin of 90.9% (5 ng/mL) and 85.9% (50 ng/mL) and 70.5% (1 μg/mL) for propranolol. This technique was rapid and large numbers were able to be processed using an extraction manifold. Prazosin has commonly been extracted with chloroform or ethyl acetate from plasma or serum made basic with potassium or sodium hydroxide. The organic phase is then extracted with dilute sulphuric acid and the drug extracted back into the organic phase and isolated following evaporation of the solvent. These time consuming liquid extraction techniques, with associated problems of disposal of organic solvents, have recovered prazosin with efficiencies in the range 44 to 99% (6–8, 10, 12, 13, 15). Acetonitrile was used in one report for simple protein precipitation of samples with essentially no drug loss, with an aliquot of the supernatant being analysed directly (16). Methanol was used in a similar manner in another report, however no quantitation of recovery was stated by these workers (14). Kelly *et al.* (19), in an assay for ondansetron

enantiomers from plasma using prazosin as the internal standard, employed solid phase extraction using cyanopropyl columns, while Jackman *et al.* (20) used C_{18} columns to extract the prazosin-related α-adrenoreceptor antagonist, doxazosin, from plasma with 75% efficiency.

Prazosin eluted in 6.2 minutes and the internal standard in 7.9 minutes (Figure 1) and the assay was linear in the range 2.5 to 100 ng/mL, thus covering the low concentrations which may be encountered in tissue perfusion experiments designed for kinetic or metabolic studies. Quantitation of prazosin was reproducible with intra-assay and inter-assay co-efficients of variation of 5.8 and 4.9% (5 ng/mL) and 6.2 and 2.7% (50 ng/mL) respectively. No endogenous compounds from either the sample matrix, compounds leached or released from the placental lobule, or other drugs interfered with the assay (Table 1). Although lignocaine coeluted with propranolol, no significant interference was recorded at therapeutic concentrations of this agent. The perfusion fluid collected at the beginning of the perfusion experiments from both the maternal and fetal circulations is discarded (19), thus minimising or eliminating any potential interferences from drugs present in the maternal or fetal blood.

The technique has been applied to a representative perfusion of prazosin in the *in vitro* perfused placental lobule with quantitation of drug levels in both the maternal and fetal circulations following dosing of prazosin in the maternal circulation (Figure 2). Prazosin equilibrated between the maternal and fetal compartments within 45 minutes illustrating that a proportional fraction of therapeutic doses of drug given to the mother would be rapidly transferred across the placenta to the fetal compartment.

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CONCLUSION

This assay is simple to perform, sensitive and reproducible and utilizes a rapid solid phase extraction technique, thereby eliminating the need for use and subsequent disposal of organic solvents. The assay is suitable for the study of prazosin disposition in perfusion experiments in placental lobules or other organs.

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