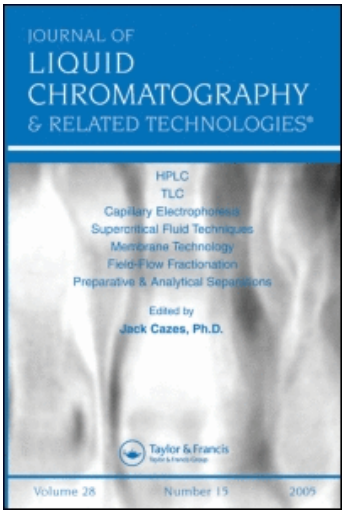

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CORTISOL, CORTISONE, AND THEIR 20-REDUCED METABOLITES IN PERFUSION MEDIA

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ABSTRACT

A reversed-phase liquid chromatographic assay to quantitate cortisol, cortisone and their respective 20α- and 20ß-dihydro reduced metabolites in tissue culture media from *in vitro* perfusions of the human placental lobule is described. The internal standard used in this assay was 6α-methyl-prednisolone. Steroids were extracted from the perfusion medium using Sep-Pak reversed-phase cartridges with the average recoveries of each

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steroid at 150 and 600 nmol/L ranging from 84.4 to 99.1% and 85.6 to 93.5% respectively. The separation was achieved by using two C_{18} columns linked in series at 40°C with a mobile phase of methanol/water (53/47 v/v) and a flow rate of 1.1 mL/min. The eluant was monitored by UV absorption at 242 nm. The assay was linear for each steroid to a concentration of 750 nmol/L with a lower detectable limit of 5 nmol/L. Intra-assay coefficients of variation were measured at 150 and 750 nmol/L with ranges of 4.0% (cortisone) to 5.5% (cortisol) and 2.8% (cortisol) to 4.0% (cortisone and 20α-dihydrocortisone) respectively. Inter-assay coefficients of variation were 6.0 (20α-dihydrocortisone) to 9.6% (cortisone) and 5.8 (20α-dihydrocortisol and cortisone) to 6.9% (20ß-dihydrocortisone) at these concentrations respectively. With this method prednisolone coelutes with cortisol however no other interferences, from endogenous steroids or drugs which may be used in pregnancy, were found.

INTRODUCTION

Human placental tissue contains significant concentrations of 11ß-hydroxysteroid dehydrogenase [1], an enzyme which catalyses the conversion of cortisol (11ß,17α,21-trihydroxypregn-4-ene-3,20-dione) to cortisone and may provide a protective function to limit the maternofetal transfer of maternal cortisol [2]. We have demonstrated that a related corticosteroid, prednisolone, is metabolised by the human placenta not only to prednisone, but also to 20α-dihydroprednisone, 20ß-dihydroprednisone and 20ß-dihydroprednisolone [3]. In that study a reversed-phase high performance liquid chromatography (HPLC) assay was used which quantitated prednisolone, prednisone and the related 20α- and 20ß- reduced metabolites [4].

A number of liquid chromatographic methods for the determination of corticosteroids in biological fluids have been reported using both reversed-phase and normal-phase modes of analysis. Reversed-phase

HPLC has been used to determine cortisol concentrations in serum [5–7] and plasma extracts [8,9] and normal–phase HPLC has been employed to assay prednisolone, prednisone and cortisol in human plasma with dexamethasone as the internal standard [10]. Blasco, Lopez–Bernal and Turnbull [11] described a reversed–phase HPLC method for the separation of cortisol and cortisone following incubation of cortisol with placental homogenates as a measure of the activity of 11ß–hydroxysteroid dehydrogenase. Other studies of placental corticosteroid metabolism have used Sephadex LH–20 columns or paper chromatography, which are relatively insensitive, for qualitative analysis [12,13] to identify metabolism at the 11 position in a series of corticosteroids incubated with placental minces and in *in vitro* tissue perfusions respectively. The only reported separation of cortisol, cortisone and their corresponding 20–reduced metabolites has been in human urine using a combination of reversed phase chromatography and gradient elution [14].

The isocratic HPLC assay described here was developed to separate cortisol, cortisone and their related 20α– and 20ß–reduced metabolites in order to investigate further the metabolism of cortisol in the *in vitro* perfused human placental lobule.

MATERIALS AND METHODS

Chemicals and Reagents

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

(Rheodyne Inc., California, USA) fitted with a 100 μL sample loop and a Kortec K95 variable wavelength UV detector set at 242 nm. Two Brownlee Spheri-5 RP-18 columns (250 x 4.6 mm), linked in series and a RP-18 newguard precolumn (15 x 3.2 mm, Brownlee Labs., San Jose, CA, USA) were used for the separation. Column temperature was set at 40°C with a TC1900 Temperature Controller (ICI Instruments, Sydney, Australia).

The mobile phase was 53% (v/v) methanol (HPLC grade, Mallinckrodt, Paris, KY, USA) in reagent grade water, filtered under vacuum through a 0.45 μm nylon membrane (Alltech Associates, Sydney, Australia). The mobile phase flow rate for the analyses was 1.1 mL/min. C_{18} Sep-Pak cartridges were purchased from Millipore (Brisbane, Australia). Cortisol, cortisone, their respective 20-reduced metabolites and 6α-methylprednisolone were obtained from Sigma Chemical Company (St. Louis, MO., USA). All validation studies used perfusate from a placenta not perfused with cortisol or prednisolone as substrate. The perfusion fluid was tissue culture medium M199 (Sigma) with glucose (2 g/L), heparin (25 IU/mL), gentamicin (100 mg/L, David Bull Labs., Melbourne, Australia) and dextran (7.5 g/L, Sigma).

Standard Separations

Stock solutions (2 mg/mL) of each steroid were prepared in methanol. A working mixture was prepared by combining aliquots (100 μL) of each stock solution and diluting to 10 mL with methanol to give a final concentration of 20 μg/mL for each steroid. A 10 μL aliquot of this solution was then analysed by HPLC.

Sample preparation

A solid-phase technique was used to extract the steroids from the perfusion media [16]. The C_{18} reversed-phase cartridges were washed with methanol (10 mL), followed by water (10 mL). The perfusion samples (3 mL) were loaded on the cartridges, the cartridge washed with water (3 x 10 mL) and the steroids eluted with methanol (5 mL). The solvent was evaporated (35°C) with nitrogen, the residue reconstituted in mobile phase (100 μL) and an aliquot (50 μL) injected for analysis. Glassware used during the extraction procedure was silanised.

Linearity

Increasing volumes of the working mixture, together with a constant amount (500 ng) of the internal standard, 6α-methylprednisolone, were added to perfusate (3 mL) to prepare concentrations of each steroid in the range of 150 to 750 nmol/L. The samples were prepared in duplicate for extraction. Mean peak height ratios (steroid : internal standard) were then determined and plotted as a function of increasing steroid concentration.

Recovery

The recovery of each steroid was determined at 150 and 600 nmol/L. Samples of perfusate (3 mL) at each concentration were prepared in duplicate and extracted using reversed phase cartridges. A constant amount (500 ng) of internal standard, 6α-methylprednisolone, was added to each

methanol eluate prior to evaporation under nitrogen. The recoveries were calculated by comparison of the peak height ratios (steroid : internal standard) of the samples with those of non-extracted standards of the same concentrations.

Specificity

A range of endogenous and exogenous steroids and drugs which may be used therapeutically in pregnancy were investigated for interference with quantitation of the steroids being analysed in this assay.

Precision studies

Precision studies were conducted at two concentrations, 150 and 750 nmol/L. Samples of perfusate (3 mL) were spiked with internal standard (500 ng) and with aliquots of the working solution, and were extracted and analysed as described above. Intra-day coefficients of variation were calculated from a set of five separate extractions repeated at each concentration and the inter-day coefficients of variation from corresponding sets of analyses repeated over five days.

Lower limit of detection

This value was determined for each steroid by determining the concentration at which the ratio of sample response to background was 2.0.

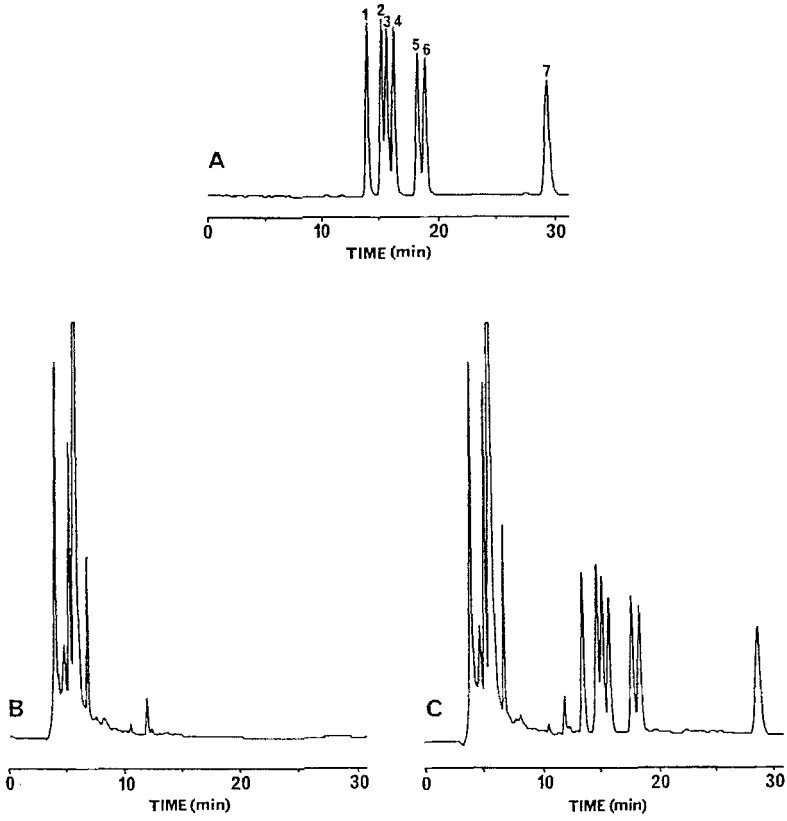

FIGURE 1. HPLC separation of (A) standard steroid mixture with internal standard, (B) extracted blank perfusate and (C) perfusate containing standard steroids and internal standard. Peaks: 1 = 20α–dihydrocortisone, 2 = 20ß–dihydrocortisone, 3 = 20α–dihydrocortisol, 4 = cortisone, 5 = 20ß–dihydrocortisol, 6 = cortisol and 7 = 6α–methylprednisolone (internal standard).

RESULTS

Typical examples of the separation of standards, extracts of blank perfusate and perfusate containing the standard steroids are shown in Figure 1. Although not all the steroids were base–line resolved, accurate

TABLE 1

Recoveries of Steroids from Perfusion Medium

Steroid	Recovery (%)		
	150 nmol/L	600 nmol/L	Mean
Cortisol	86.6	93.5	90.1
Cortisone	99.1	85.6	92.4
20α-Dihydrocortisol	84.4	93.4	88.9
20ß-Dihydrocortisol	95.1	90.7	92.9
20α-Dihydrocortisone	90.6	92.2	91.4
20ß-Dihydrocortisone	94.2	90.3	92.3

quantitation was obtained. The assay was linear for all the steroids in the range 0 – 750 nmol/L with a typical calibration graph for cortisol giving a regression of $y = 1.79 \times 10^{-2} + 1.30 \times 10^{-3}x$, $r = 0.981$, where y is the peak-height ratio, x the concentration of steroid (nmol/L) and r is the correlation coefficient. Mean recoveries of each steroid, using the solid-phase extraction procedure at concentrations of 150 and 600 nmol/L, ranged from 88.9% for 20α-dihydrocortisone to 92.9% for 20ß-dihydrocortisone (Table 1).

Precision studies at concentrations of 150 and 750 nmol/L showed the assay to be reproducible for all steroids under study with intra-assay coefficients of variation ranging from 4.0 (cortisone) to 5.5% (cortisol) and from 2.8 (cortisol) to 4.0 (cortisone and 20α-dihydrocortisone) for the two concentrations respectively. The corresponding inter-assay coefficients of variation varied from 6.0 (20α-dihydrocortisone) to 9.6% (cortisone) and 5.8 (cortisol and 20α-dihydrocortisol) to 6.9% (20ß-dihydrocortisone)

TABLE 2

Coefficients of Variation for Steroids at Two Concentrations

Steroid	Coefficient of variation (%)			
	Intra-assay		Inter-assay	
	150 nmol/L	750 nmol/L	150 nmol/L	750 nmol/L
Cortisol	5.5	2.8	8.5	6.0
Cortisone	4.0	4.0	9.6	5.8
20α-Dihydrocortisol	5.2	2.9	8.5	5.8
20ß-Dihydrocortisol	5.0	3.9	8.8	6.7
20α-Dihydrocortisone	4.3	4.0	6.0	6.6
20ß-Dihydrocortisone	5.2	3.0	9.4	6.9

TABLE 3

Compounds Tested for Interference in this Assay

Steroids	Other drugs
Prednisone	Bupivicaine
Prednisolone	Carbamazepine
Progesterone	Clonazepam
17α-Hydroxyprogesterone	Diazepam
Estradiol	Hyoscine
Estriol	Methimazole
Betamethasone	Paracetamol
Dexamethasone	Phenobarbitone
Testosterone	Ritodrine
Dehydroepiandrosterone	Salbutamol
Cholesterol	

(Table 2). The lower limit of detection observed was 5 nmol/L for each of the steroids. No interference was found from any of the steroids or drugs listed in Table 3 except for prednisolone which coeluted with cortisol.

DISCUSSION

We have developed a specific and reproducible HPLC assay to separate cortisol, cortisone and the 20–reduced metabolites of these compounds in samples from *in vitro* perfusions of the human placenta. Other assays have been described for the resolution of metabolites of prednisolone [4] in tissue culture medium from perfusions in placental tissue and for the metabolism of corticosteroids in adrenal cell cultures [15]. There was no interference from endogenous compounds originating from the perfused tissue, as measured by analysis of 6 hour perfusate samples from perfusions in which cortisol or prednisolone had not been used as substrates. Potential interferences from this source were minimised by removing any blood remaining in the tissue before the tissue culture medium was recirculated through the placental lobule. The only exogenous compound which interfered was prednisolone which co–eluted with cortisol. The partition coefficients of the metabolites of cortisol are similar and two C_{18} reversed phase columns linked in series were required to effect the separation. A column temperature of 40°C was used to lower the back pressure and reduce the retention times.

Although baseline separation of two sets of steroids (20ß–dihydrocortisone, 20α–dihydrocortisol and cortisone; 20ß–dihydrocortisol and cortisol) was not achieved, the resolution was sufficient for quantitation

of these components. Use of other modifiers and solvents in the mobile phase did not improve the resolution.

A high recovery of each of the steroids was achieved using reversed phase cartridges for extraction of the perfusion matrix. At a concentration of 150 nmol/L, the recoveries were in the range 84.4 to 99.1% whereas at 600 nmol/L the corresponding recoveries ranged from 85.6 to 93.5%. These recoveries are comparable with those of McBride *et al.* [9] who extracted 99.3 to 108% of cortisol from plasma, Dawson *et al.* [16] who obtained up to 81% of cortisol, cortisone and corticosterone from plasma and Cannell *et al.* [17] who obtained a 98% recovery of cortisol and cortisone from plasma using the same reversed-phase extraction technique. This isocratic assay is simpler to perform than the gradient elution HPLC method of Schoneshofer *et al.* [14] and will be applied to the separation of metabolites following *in vitro* perfusions of placental tissue with cortisol as substrate.

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