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## Short Communication

# Liquid chromatographic analysis of prednisolone, prednisone and their 20-reduced metabolites in perfusion media

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ABSTRACT

A reversed-phase liquid chromatographic assay was developed to quantitate prednisolone, prednisone and the 20α-dihydro and 20β-dihydro reduced metabolites of both parent compounds in tissue culture media from *in vitro* perfusions of the human placental lobule. Steroids were extracted from perfusate, using reversed-phase cartridges, with average recoveries of 95.2% or greater. The internal standard for the analyses was 6α-methylprednisolone. In this assay cortisol coelutes with prednisolone, however, no other significant interferences were found. Assay of each steroid was linear in the range 0–1 μg/ml. Intra-assay coefficients of variation were measured at 10 and 750 ng/ml with ranges of 3.4% (20α-dihydroprednisone) to 8.8% (20β-dihydroprednisolone) and 4.1% (20β-dihydroprednisone) to 8.8% (prednisone). The corresponding inter-assay coefficients of variation were 3.3% (20α-dihydroprednisone) to 9.1% (20β-dihydroprednisolone) and 1.9% (prednisolone) to 3.5% (prednisone). The analyses utilized two $C_{18}$ columns which were linked together and maintained at 40°C.

## INTRODUCTION

When rat and rabbit kidneys are perfused with prednisolone (11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione), a synthetic steroid with enhanced glucocorticoid activity, both 20β-dihydroprednisone and 20β-dihydroprednisolone are formed [1,2]. In perfused human placenta [3] or placental minces [4] on the other

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hand the only reported metabolite is prednisone (17α,21-dihydroxypregna-1,4-diene-3,11,20-trione). While this may reflect intrinsic differences in metabolism, perfusate or medium was analysed in these latter studies by open-column chromatographic techniques which may not have had the power to resolve complex mixtures of metabolites with similar physical properties.

High-performance liquid chromatographic assays (HPLC) for prednisolone have been developed utilizing either the reversed-phase or normal-phase modes. Reversed-phase HPLC has been used to assay prednisolone in pharmaceutical preparations [5,6], while a number of reports have documented the use of normal-phase HPLC [2,7–13] to assay prednisolone in plasma or other biological fluids. Normal-phase HPLC separation of prednisolone, prednisone and their 20β-reduced metabolites has been reported [2] employing 6β-hydroxycortisol as the internal standard. The 20-reduced metabolites of the related corticosteroids, cortisol and cortisone, have been assayed in human urine using gradient elution analysis in a reversed-phase mode [14].

To investigate the question of more complex metabolism of prednisolone by the isolated, perfused human placental lobule, a sensitive and specific assay was developed for prednisolone, prednisone and related 20-reduced metabolites in perfusion media. This method is based on solid-phase extraction of the corticosteroids from perfusate followed by reversed-phase HPLC analysis.

## EXPERIMENTAL

### *Materials*

The HPLC equipment consisted of a Kortec Model K35D pump (ICI Instruments, Sydney, Australia), a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) fitted with a 100-μl sample loop, and a Kortec K95 variable-wavelength UV detector set at 242 nm. Separation was achieved using two Brownlee Spheri-5 RP-18 columns (250 mm × 4.6 mm I.D.) linked in series and a RP-18 Newguard precolumn (15 mm × 3.2 mm I.D., Brownlee Labs., San Jose, CA, U.S.A.). Column temperature was maintained at 40°C with a TC1900 temperature controller (ICI Instruments, Sydney, Australia).

The mobile phase was 57.5% (v/v) methanol (HPLC grade) in water, filtered under vacuum through a 0.45-μm nylon membrane (Alltech Assoc., Sydney, Australia). The flow-rate was 1.2 ml/min. Sep-Pak cartridges were purchased from Millipore (Brisbane, Australia). Prednisolone, prednisone and 6α-methylprednisolone were obtained from Sigma (St. Louis, MO, U.S.A.); 20α-dihydroprednisolone, 20β-dihydroprednisone and 20β-dihydroprednisolone were a gift from Schering (Kenilworth, NJ, U.S.A.), and 20α-dihydroprednisone from the MRC Steroid Reference Collection (London, U.K.).

All validation studies used perfusate stored from a previous, non-prednisolone perfusion. The perfusion fluid was tissue culture medium TC199 (Difco, Detroit, MI, U.S.A.) augmented with glucose (2 g/l), heparin (25 I.U./ml), gentamycin

(100 mg/l, David Bull Labs., Melbourne, Australia) and dextran (approximate molecular mass 40 500, 7.5 g/l, Sigma).

*Standard separations*

Stock solutions (2 mg/ml) of each steroid were prepared in methanol. Aliquots (100 $\mu$l) of each stock solution were combined and diluted to 10 ml in methanol to give a working solution with a final concentration of 20 $\mu$g/ml. An aliquot (20 $\mu$l) of this working solution was analysed.

*Extraction*

Sep-Pak cartridges were primed with methanol (10 ml) followed by water (10 ml). Samples (2 ml) of blank perfusate or perfusate spiked with the mixture of standard steroids were loaded onto the cartridge (flow-rate *ca.* 5 ml/min), washed with water (3 × 10 ml) and the steroids eluted with methanol (5 ml). The methanol was removed by a gentle stream of nitrogen at 35°C, the residue reconstituted in mobile phase (100 $\mu$l), and a 50-$\mu$l aliquot analysed. Glassware used during the extraction procedure was silanised.

*Linearity*

Perfusate (2 ml) was spiked with a constant amount (400 ng) of 6$\alpha$-methylprednisolone (internal standard) and varying volumes of the standard solution to give final concentrations in the range 0–1000 ng/ml. These samples were prepared in duplicate and extracted and analysed as described above. Peak-height ratios (prednisolone or metabolite to internal standard) were calculated and plotted as a function of increasing steroid concentration.

*Recovery*

The recovery of each steroid was determined at concentrations of 50 and 500 ng/ml. Perfusate samples (2 ml) were spiked with appropriate volumes of the working solution and extracted. A constant concentration (500 ng/ml) of internal standard was added to each methanol eluent prior to evaporation and analysis. Peak-height ratios were compared with those obtained upon analysis of a corresponding set of non-extracted standards.

*Specificity*

The retention times of a range of endogenous and exogenous steroids and drugs that may be present in the circulation of pregnant women were measured to check for co-elution with the steroid standards in this study.

*Precision studies*

Intra- and inter-day coefficients of variation (C.V.) for the assay were determined over five days at two steroid concentrations. Aliquots (2 ml) of perfusate were spiked with the standard steroids to give concentrations of 100 and 750

ng/ml together with 250 ng/ml 6α-methylprednisolone as internal standard. These samples were then extracted and analysed as described above. Five samples for each concentration were analysed on day 3 to determine intra-day precision while the mean from this day together with the mean results from triplicate analyses on days 1, 2, 4 and 5 were used to determine the inter-day precision.

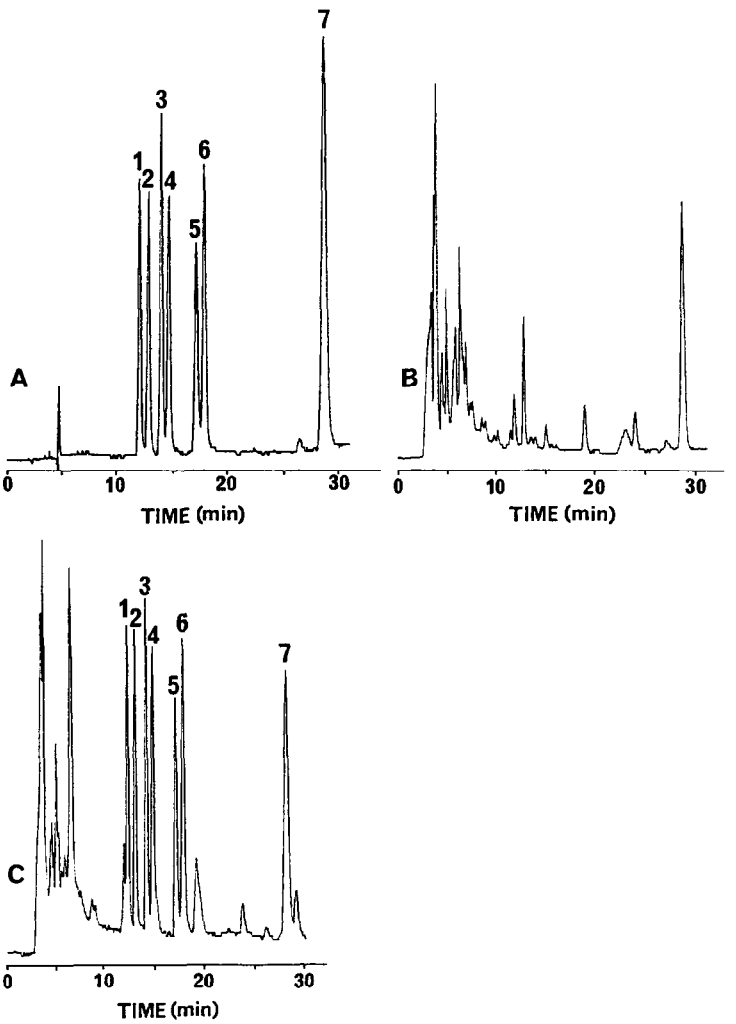


Fig. 1. HPLC separation of (A) standard steroid mixture with internal standard, (B) blank perfusate containing internal standard only and (C) perfusate containing standard steroids and internal standard. Peaks: 1 = 20α-dihydroprednisone; 2 = 20β-dihydroprednisone; 3 = prednisone; 4 = 20α-dihydroprednisolone; 5 = 20β-dihydroprednisolone; 6 = prednisolone; 7 = 6α-methylprednisolone (internal standard).

TABLE I

RECOVERIES OF STEROIDS FROM PERFUSION MEDIUM

| Steroid | Recovery (%) | | |
|---|---|---|---|
| | 50 ng/ml | 500 ng/ml | Mean |
| Prednisolone | 102.4 | 95.3 | 98.8 |
| Prednisone | 100.4 | 90.1 | 95.2 |
| 20α-Dihydroprednisone | 101.2 | 98.4 | 99.8 |
| 20β-Dihydroprednisone | 98.6 | 98.3 | 98.5 |
| 20α-Dihydroprednisolone | 104.1 | 101.0 | 102.6 |
| 20β-Dihydroprednisolone | 99.9 | 100.0 | 100.0 |

RESULTS

The chromatograms obtained following analysis of the working steroid solution and extracts of blank perfusate and perfusate containing the standard steroids are shown in Fig. 1. Virtual baseline separation was achieved for all steroids analysed. The assay was linear for all steroids tested in the range 0–1000 ng/ml with a typical calibration graph giving a regression of $y = 0.0398 + 0.003x$, $r = 0.9996$ where $y$ = peak-height ratio, $x$ = concentration of steroid (ng/ml) and $r$ = correlation coefficient. Mean recoveries of each steroid determined using the solid-phase extraction procedure at concentrations of 50 and 500 ng/ml ranged from 95.2% for prednisone to 102.6% for 20α-dihydroprednisolone (Table I), with little or no sample loss being observed in all cases.

Precision studies at concentrations of 100 and 750 ng/ml showed the assay to be reproducible for all steroids with coefficients of variation less than 10% (Table II). No interference due to co-elution was observed when any of the endogenous

TABLE II

COEFFICIENTS OF VARIATION FOR STEROIDS AT TWO CONCENTRATIONS

| Steroid | Coefficient of variation (%) | | | |
|---|---|---|---|---|
| | Intra-assay | | Inter-assay | |
| | 100 ng/ml | 750 ng/ml | 100 ng/ml | 750 ng/ml |
| Prednisolone | 6.3 | 4.8 | 4.4 | 1.9 |
| Prednisone | 4.7 | 8.8 | 6.5 | 3.5 |
| 20α-Dihydroprednisone | 3.4 | 4.4 | 3.3 | 1.3 |
| 20β-Dihydroprednisone | 5.4 | 4.1 | 7.3 | 1.4 |
| 20α-Dihydroprednisolone | 6.1 | 5.0 | 4.4 | 1.6 |
| 20β-Dihydroprednisolone | 8.8 | 6.4 | 9.1 | 3.0 |

TABLE III

COMPOUNDS TESTED FOR INTERFERENCE IN THE ASSAY

| Steroids | Other drugs |
|---|---|
| Estradiol | Pethidine |
| Estriol | Phenobarb |
| Progesterone | Lignocaine |
| 17β-Hydroxyprogesterone | Labetalol |
| Deoxycorticosterone | Promazine |
| Cholesterol | Diazepam |
| Betamethasone | Salicyclic acid |
| Dexamethasone | Salbutamol |
| Dehydroepiandrosterone | Hyoscine |
| Cortisol | Ritodrine |

or exogenous steroids or drugs listed in Table III were analysed, except for cortisol which co-eluted with prednisolone.

## DISCUSSION

The HPLC technique described here provides a simple and reproducible method of quantitating prednisolone, prednisone and their 20-reduced metabolites in perfusate samples. Excellent recoveries of all steroids were obtained using the solid-phase extraction procedure. Variation of the water content of the mobile phase, addition of the organic modifiers tetrahydrofuran, butanol or acetic acid and substitution of acetonitrile for methanol as the organic component did not improve selectivity of the system without loss of peak resolution. Maintenance of the column at 40°C was necessary to decrease retention times and reduce back-pressure in the system resulting from linking together the two analytical columns.

The peak eluting at 13.3 min in the analysis of blank perfusate from a prednisolone-free perfusion (Fig. 1B) co-elutes with 20β-dihydroprednisone. This unknown interference was shown to originate from the placental tissue and resulted in a response equivalent to less than 13 ng/ml 20β-dihydroprednisone for all perfusates analysed compared with concentrations of 20β-dihydroprednisone of 196 ng/ml in a typical prednisolone metabolism experiment. This interference was minimized by washing out both the maternal and fetal blood at set up before recirculating the perfusion media. The only other interference noted was the co-elution of cortisol with prednisolone which did not interfere due to the negligible levels of endogenous cortisol present in the perfusate samples.

The reversed-phase assay described above compares favourably with the report of Rocci and Jusko [2], who used normal-phase HPLC to analyse prednisolone, prednisone and their 20β-reduced metabolites in samples from kidney

perfusion studies. These workers obtained recoveries of up to 63.2% for these steroids using a solvent extraction precedure. The high recoveries (> 95%) recorded here parallel previous reports of steroid analyses using solid-phase extraction techniques. Cannell *et al.* [15] obtained greater than 83% recovery using Sep-Pak cartridges to extract a range of steroids from plasma, while Ramirez *et al.* [16] used these cartridges for the quantitative recovery of corticosteroids and the 20α-reduced series from culture media used in adrenal cell cultures. The linking of two analytical columns for separation of corticosteroids was also used by Althaus *et al.* [17] for the determination of radiolabelled triamcinolone acetonide, cortisol and their metabolites in biological samples.

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REFERENCES

1 M. L. Rocci, Jr., S. J. Szefler, M. Acara and W. J. Jusko, *Drug Metab. Dispos.*, 9 (1981) 177.
2 M. L. Rocci, Jr. and W. J. Jusko, *J. Chromatogr.*, 224 (1981) 221.
3 M. Levitz, V. Jansen and J. Dancis, *Am. J. Obstet. Gynecol.*, 132 (1978) 363.
4 A. T. Blanford and B. E. P. Murphy, *Am. J. Obstet. Gynecol.*, 127 (1977) 264.
5 V. Das Gupta, *J. Pharm. Sci.*, 68 (1979) 908.
6 P. A. Williams and E. R. Biehl, *J. Pharm. Sci.*, 70 (1981) 530.
7 J. C. K. Loo, A. G. Butterfield, J. Moffatt and N. Jordan, *J. Chromatogr.*, 143 (1977) 275.
8 J. Q. Rose and W. J. Jusko, *J. Chromatogr.*, 162 (1979) 273.
9 F. J. Frey, B. M. Frey and L. Z. Benet, *Clin. Chem.*, 25 (1979) 1944.
10 I. T. Asabeyoglu, J. G. Wagner and D. R. Kay, *Res. Commun. Chem. Pathol. Pharmacol.*, 28 (1980) 163.
11 N. R. Scott, J. Chakraborty and V. Marks, *Anal. Biochem.*, 108 (1980) 266.
12 L. Ost, O. Fal, O. Lantto and I. Bjorkhem, *Scand. J. Clin. Lab. Invest.*, 42 (1982) 181.
13 R. Hartley and J. T. Brocklebank, *J. Chromatogr.*, 232 (1982) 406.
14 M. Schoneshofer, B. Weber and S. Nigam, *Clin. Chem.*, 29 (1983) 385.
15 G. R. Cannell, J. P. Galligan, R. H. Mortimer and M. J. Thomas, *Clin. Chim. Acta*, 122 (1982) 419.
16 L. C. Ramirez, C. Millot and B. F. Maume, *J. Chromatogr.*, 229 (1982) 267.
17 Z. R. Althaus, J. M. Rowland and J. P. Freeman, *J. Chromatogr.*, 227 (1982) 11.