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SIMULTANEOUS DETERMINATION OF BETAMETHASONE, BETAMETHASONE ACETATE AND HYDROCORTISONE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, specific, reproducible and rapid high-performance liquid chromatographic method for the simultaneous quantitation of betamethasone, betamethasone 21-acetate and hydrocortisone in biological fluids is described. Hydrocortisone acetate is used as an internal standard and the samples are extracted with dichloromethane before chromatographing on a reversed-phase system. Detection at two ultraviolet wavelengths (254 nm and 240 nm) was used to assess the specificity of the system, and the sensitivity was found to be greater than 10 ng for all steroids. The speed with which this assay can be performed makes it particularly useful for pharmacokinetic studies, and plasma concentration—time profiles resulting from the administration of betamethasone phosphate and betamethasone acetate are presented.

INTRODUCTION

Betamethasone (I) is commonly administered antenatally in an attempt to reduce the incidence of respiratory distress syndrome in premature infants. In this indication betamethasone is usually given intravenously or intramuscularly as the disodium salt of its 21-phosphate ester, or intramuscularly using a formulation which includes both the phosphate ester described and the 21-acetate ester of betamethasone (II). The phosphate and acetate esters are expected to be hydrolysed in vivo to betamethasone, the presumed active compound. The disposition ⌐ I in the maternal—foetal system is being investigated in this laboratory, an͏ ͏nce pharmacokinetic studies of this nature involve the analysis of several h: ͏ired plasma, blood and urine samples they require a sensitive, specific, rep ͏lucible and rapid analytical method for the drug of interest. After admin͏ ͏ation of glucocorticoids it is also advantageous to be able to measure concentrations in biological fluids of endogenous hydrocortisone (III),

as well as the esters of betamethasone which are present in the commercially available dosage forms.

A high-performance liquid chromatographic (HPLC) technique which allows the simultaneous determination of I, II and III in blood and plasma, and I and II in urine has been developed. This method, which is reported here, is sensitive and precise, and allows very rapid analysis of samples. Betamethasone phosphate is water soluble and, using conventional solvent extraction, cannot be efficiently removed from biological samples with the other compounds of interest. Therefore its analysis is not described here.

EXPERIMENTAL

Reagents and materials

Betamethasone (I) and its 21-acetate ester (II) were gifts from Essex Laboratories (Sydney, Australia); hydrocortisone (III) and hydrocortisone 21-acetate (IV) were purchased from Sigma (St. Louis, MO, U.S.A.). Standard solutions of these compounds were made in distilled water (5 μg/ml) and stored at 4°C. The solutions of II and IV contained the disodium salt of EDTA at a concentration of 0.1% w/v, to stabilise the esters. Potassium fluoride (Ajax Unilab, Sydney, Australia) was kept as a 50% w/v solution in distilled water and sodium arsenate (Sigma) was stored as an aqueous solution (2 M). Dichloromethane (GR grade; Merck, Darmstadt, G.F.R.) was used without further purification.

Glass tubes were used for all steps in the analysis, and were cleaned in a 2% solution of Extran 300 (Merck). The glass extraction tubes (20 ml) were fitted with screw caps with PTFE liners.

A Varian Aerograph (Palo Alto, CA, U.S.A.) Model 8500 HPLC-pump equipped with a loop injection system (Valco, Houston, TX, U.S.A.) and a 250 mm × 4.6 mm I.D. RP-8 column (Brownlee, Santa Clara, CA, U.S.A.) with an average particle diameter of 10 μm was used. For all analyses a Waters Assoc. (Milford, MA, U.S.A.) Model 440 ultraviolet detector with a 254-nm filter was used and this was connected in series with a Spectra-Physics (Santa Clara, CA, U.S.A.) Model 770 variable-wavelength ultraviolet detector set at 240 nm when dual wavelength monitoring was required. A dual-pen recorder (Cole-Parmer, Chicago, IL, U.S.A.) with inputs set at 10 mV and a chart speed of 8 in./h was used. The mobile phase, HPLC grade methanol (Waters Assoc.)—filtered distilled water (60:40), was pumped through the column at a flow-rate of 1.25 ml/min, resulting in an inlet pressure of 700 p.s.i.g.

Collection of biological samples

The 21-acetate and 21-phosphate esters of betamethasone are unstable in biological fluids and steps must be taken to ensure that no hydrolysis to I occurs in vitro. Samples for plasma, blood and urine analysis were collected as follows.

Plasma. Blood samples (10 ml) were drawn by venipuncture or through a Dwellcath cannula (Tuta Lab., Lane Cove, Australia) using disposable plastic syringes (Pharmaplast, AHS/Australia, Balgowlah, Australia) and placed in

chilled plastic tubes (10 ml; Disposable Products, Sydney, Australia) containing 100 units of ammonium heparin and separation granules. These tubes also contained 100 μl of sodium arsenate solution which inhibited the hydrolysis of the phosphate ester. Each tube was placed in an ice bath, and centrifuged (1000 g, 5 min) within 5 min. The resulting plasma was immediately transferred to plain plastic tubes (Disposable Products) which contained 1% v/v of the potassium fluoride solution to inhibit hydrolysis of II.

Blood. Samples for whole blood analysis were collected exactly as for plasma samples, but instead of centrifuging, a measured quantity of blood was transferred immediately to the plain plastic tubes which contained 1% v/v of potassium fluoride solution. The blood was diluted with an equal volume of distilled water before freezing.

Urine. Urine was collected into large glass bottles containing sodium arsenate and potassium fluoride (6 ml of each solution per 12-h urine collection) and aliquots were transferred to plain plastic tubes for storage.

All samples were frozen at $-22°C$ prior to analysis.

Extraction procedure

Samples (1 ml) of plasma and diluted blood were extracted directly, but urine was first diluted with an equal quantity of Sorenson's phosphate buffer pH 7.4 to standardise the pH of the samples. The internal standard, IV, (150 ng/30 μl) was added, then all samples were extracted with 5 ml dichloromethane by vortexing for 1 min. After centrifugation for 7 min at 1000 g, the upper aqueous layer was aspirated and the organic phase transferred to a 7-ml glass evaporation tube which had a 50-μl capillary base. A boiling chip (BDH Chemicals, Port Fairy, Australia) was added and the tube placed in a 45°C water bath. Immediately after dryness had been achieved, the tube was stoppered and placed in an ice bath to allow the dichloromethane vapour to condense and wash down the internal walls of the evaporation tube. Just before injection into the chromatograph the condensed dichloromethane was evaporated in the water bath, and the residue reconstituted with 100 μl of the mobile phase by vortex mixing for 20 sec. The whole extract was injected onto the HPLC column.

Calibration and reproducibility

Known quantities of I, II and III ranging from 10 ng to 300 ng were added to blank plasma, blood and urine samples. The samples were extracted and chromatographed as outlined above. Calibration curves were constructed by calculating the ratio of the peak height of each compound (I, II and III) to that of the internal standard (IV), and plotting the ratio against the amount of compound added to the sample. The calibration curves for III in plasma and blood were adjusted by subtracting the ordinate intercept, equivalent to the endogenous hydrocortisone in the sample, from all the peak height ratios calculated for III.

The reproducibility of the method was assessed for plasma and urine by spiking eight blank samples with 50 ng of I and II, and for blood by spiking six blank samples with 100 ng of each compound. Plasma and blood were analysed for endogenous hydrocortisone, I and II; urine was analysed for I and II only.

RESULTS

Stabilisation of the esters

Betamethasone phosphate, II and IV are all subject to both enzymic and chemical hydrolysis to their corresponding alcohols. The hydrolysis of the phosphate ester in vitro must be prevented so that the levels of I measured are not spuriously high. The conversion in biological fluids of betamethasone phosphate to I is rapid, but was prevented by the collection of samples into vessels which contained 1% v/v of a 2 M sodium arsenate solution [1]. The procedure did not cause haemolysis of red blood cells and did not alter the distribution of I between the blood cells and plasma.

The hydrolysis of II following the collection of samples was found to be considerable. When whole blood containing II (100 ng/ml) was left at room temperature for 30 min, 22% of II converted to I, and 15% conversion occurred if the sample was stored on ice for the same time. The addition of 1% v/v of sodium arsenate (2 M) did not fully prevent the hydrolysis, however the incorporation of 1% v/v of a 50% w/v potassium fluoride solution into the blood did inhibit the degradation [2]. Unfortunately, this was not suitable for blood samples which were drawn for plasma analysis since the potassium fluoride caused haemolysis of the red blood cells.

The collection methods described were found to be quite satisfactory. When blood was collected into chilled heparinised tubes containing sodium arsenate, placed on ice, centrifuged within 5 min, and the plasma immediately aspirated into tubes containing potassium fluoride, no detectable degradation of II occurred, even if the plasma remained at room temperature for 1 h. Similarly the collection methods for whole blood and urine were found to prevent any detectable hydrolysis of II. The addition of the potassium fluoride to plasma not only avoided the problem of haemolysis, but prevented any possible effects of the salt on the distribution of I, II and III between plasma and red blood cells.

Chemical hydrolysis of II and IV in stock aqueous solutions was prevented by adding 0.1% disodium salt of EDTA and storing them at 4°C. The chemical hydrolysis is base catalysed and therefore basification during the extraction procedure may result in degradation of the esters. No hydrolysis of the esters occurred during the analysis of spiked samples using the analytical method reported here.

Calibration and reproducibility

Chromatograms resulting from the analysis of blank human plasma, blood and urine are shown in Fig. 1, along with a chromatogram of blank plasma to which 50 ng of I, 100 ng of II and III and 150 ng of IV had been added. The retention times of III, I, IV and II were 5.3, 6.5, 7.2 and 9.0 min respectively. The small peak which chromatographs near the internal standard has been of the same magnitude in all samples studied and does not compromise the analysis since it is swamped by the internal standard peak.

The calibration plots obtained were linear over the concentration range from 10 ng to 300 ng for I and II and from 10 ng to 200 ng for III. The signal-to-noise ratio obtained when quantitating 10 ng was 30—40:1 which indicated
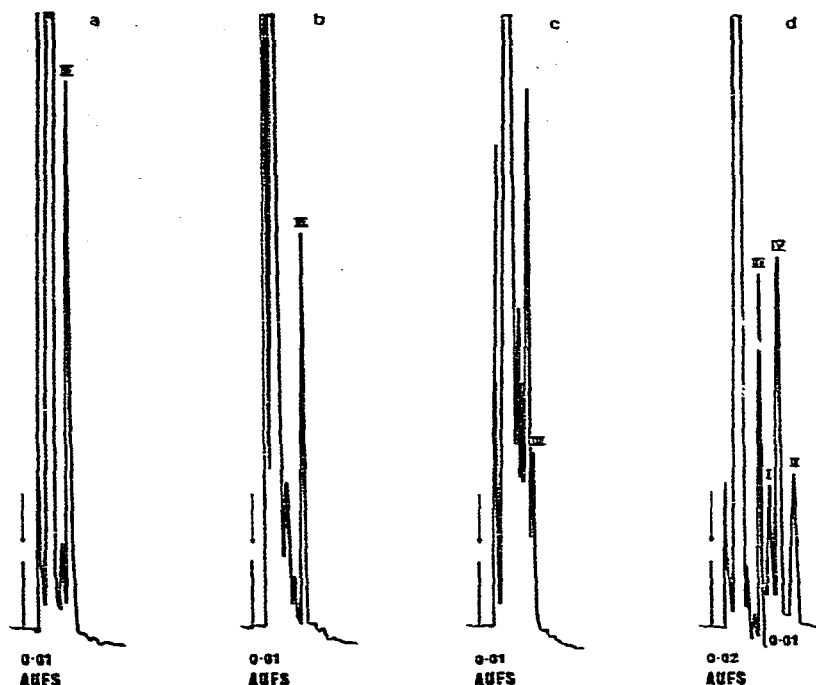
Fig. 1. Chromatograms of extracts of blank human (a) plasma, (b) blood, (c) urine and (d) plasma spiked with 50 ng betamethasone (I), 100 ng betamethasone acetate (II) and hydrocortisone (III) and 150 ng hydrocortisone 21-acetate (IV) as internal standard. The retention times of III, I, IV and II are 5.3, 6.5, 7.2 and 9.0 min respectively. The arrows mark the points of injection.

TABLE I

REPRODUCIBILITY DATA

	Coefficient of variation (%)		
	Plasma (n=8)	Blood (n=6)	Urine (n=8)
Hydrocortisone	2.4*	6.6*	—
Betamethasone	2.1**	4.2***	1.8**
Betamethasone acetate	1.6**	3.2***	2.8**

*Endogenous hydrocortisone assayed.
**Concentration of steroids 50 ng/ml.
***Concentration of steroids 100 ng/ml.

that quantitation to substantially lower levels of these compounds is possible on this system. The analytical recovery of the compounds was measured by comparing the chromatographic peak heights from the analysis of eight biological samples which were spiked with 100 ng of each compound, and the peak heights resulting from a direct injection of aqueous standards. The recovery of all compounds from plasma, blood and urine was 80—85%, when approximately 85—90% of the dichloromethane phase was available for evaporation.

Table I gives the results of the reproducibility study. The precision of this

method was very good as is illustrated by coefficients of variation which were less than 3% for all compounds in plasma and urine, and less than 7% for the blood samples.

Confirmation of the homogeneity of the hydrocortisone peak was particularly important since the absence of interfering compounds could not be shown directly using a blank sample. It was also necessary to ensure that no metabolites of I chromatographed simultaneously with any of the compounds of interest. To check the specificity of the method, the ratio of the absorbance of each peak of interest at 240 nm to that at 254 nm was calculated, and compared to the ratio observed when authentic standards were injected directly into the chromatograph. The ratio was computed for fifteen plasma samples which were collected over an 8-h period after the administration of an 8-mg dose of betamethasone phosphate. The ratio for each peak of interest was always within 3% of the ratio obtained for the authentic standard. A further check for specificity was carried out by examining the retention characteristics of several drugs commonly administered to women who are receiving glucocorticoids antenatally. Table II lists the drugs which were examined, none of which interfered with the analysis of any of the compounds of interest.

TABLE II

COMPOUNDS SHOWN NOT TO INTERFERE WITH THIS ASSAY

Salbutamol	Bupivacaine
Ritodrine	Lignocaine
Hyoscine	Phenobarbitone
Diazepam	Paracetamol
Pethidine	Salicylic acid
Promazine	Betamethasone phosphate

DISCUSSION

The analytical method reported here is a rapid, precise, selective and sensitive way of simultaneously determining I, II and III in biological fluids. This method not only allows the disposition of betamethasone to be examined after various dosage regimes, but facilitates an examination of the time course of levels of the acetate ester which is administered and the effect these steroids have on endogenous hydrocortisone. Fig. 2 shows the plasma concentration—time profiles of I and III after the administration of (a) 10.6 mg of betamethasone phosphate intravenously and (b) 6 mg betamethasone (3 mg as betamethasone phosphate and 3 mg as betamethasone acetate) intramuscularly to pregnant patients. Following dose (b) no II was detected throughout the sampling period, however this may not be the case when different doses, formulations and muscle sites for injection are studied. Chromatograms resulting from the analysis of plasma samples collected just prior to dosing and 60 min after the dose was given are shown in Fig. 3.

In the past, several methods have been used for the detection of I in biological fluids. These included radio-immunoassay (RIA) techniques which were preceded by column chromatography to reduce cross-reactivity problems [3]; and a combination [4] of a radioreceptor assay for total glucocorticoid activity
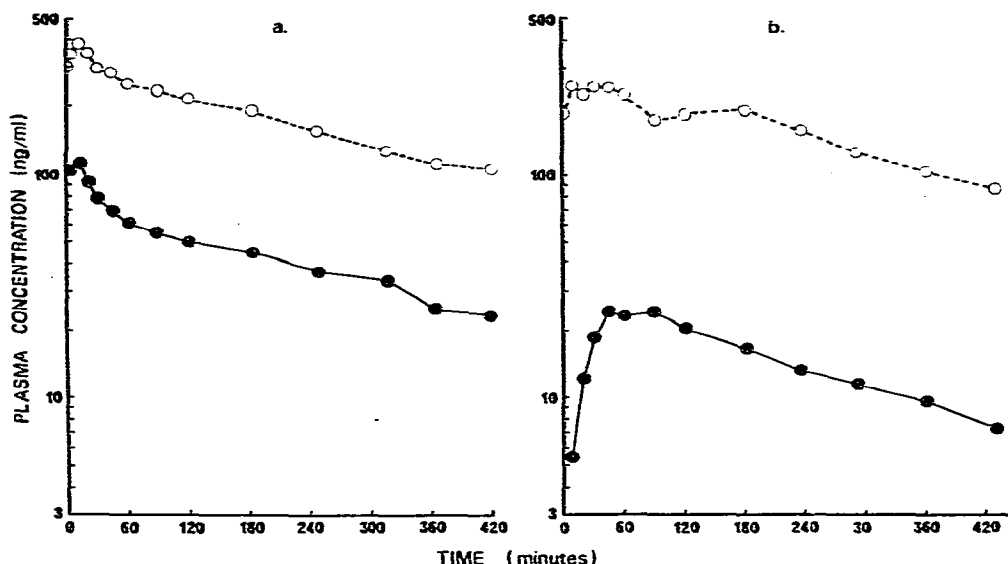
Fig. 2. Plasma concentration—time profiles of betamethasone (●——●) and hydrocortisone (o — — o) after the administration of (a) 10.6 mg betamethasone phosphate intravenously and (b) 3 mg betamethasone as the phosphate ester and 3 mg betamethasone as the acetate ester, intramuscularly, to pregnant patients.

[5] and the corticosteroid-binding globulin isotope assay of Murphy [6]. Similarly, III has been measured in plasma using competitive protein binding [6] and RIA [7] methods. However, these techniques cannot be used for simultaneous determination of steroids, and the time involved in the execution of these assays precludes their use in pharmacokinetic studies with large numbers of samples.

Recently, several HPLC assays for hydrocortisone and some synthetic steroids other than betamethasone in biological fluids have been reported [8—18]. The majority of these methods use normal-phase HPLC systems which are suitable for the analysis of the steroid alcohols, but the acetate esters are less polar and are not adequately retained on the column under these conditions. Additionally, these methods are relatively slow, involving several extraction steps, and usually include a basic wash with 0.1 N NaOH which is not suitable in this application since the chemical hydrolysis of II and IV is base catalysed.

Although the present method has been shown to be highly specific for the compounds of interest, it should be noted that one source of interference was encountered. When blood was collected in Monoject (Sherwood Medical Industries, Deland, FL, U.S.A.) or Terumo (Terumo Australia, Melbourne, Australia) disposable syringes, a large interfering peak eluted at a retention time of 6.5 min. Interference from this source has been reported before [19] and should be kept in mind since it is easy to erroneously label the peak as an endogenous biological substance. In this case the problem was solved by using Pharmaplast syringes for all dosing and sampling. An endogenous compound does elute after the steroids of interest but does not interfere with the subsequent injection provided that each sample is allowed to chromatograph for 12 min.
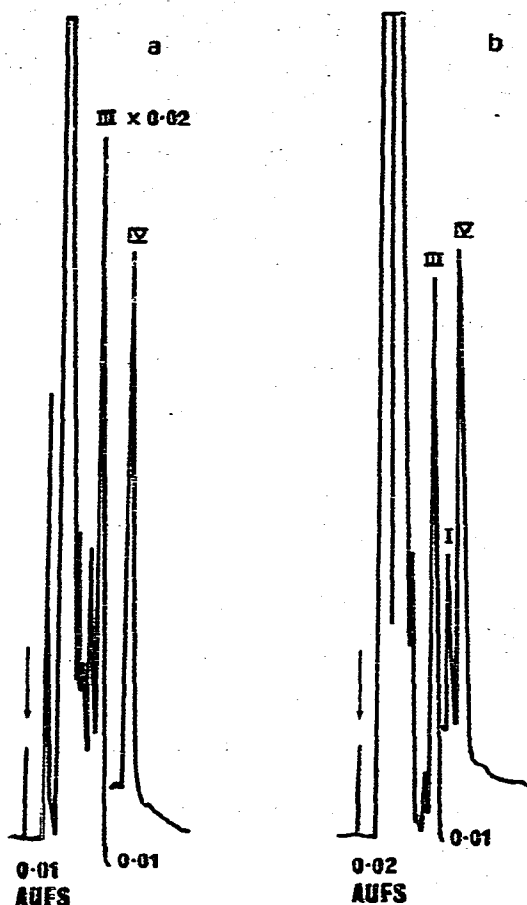
Fig. 3. Chromatograms of extracts of plasma from a pregnant patient (a) just before dosing and (b) 60 min after dosing with 10.6 mg betamethasone phosphate intravenously. Peaks: I, betamethasone; III, hydrocortisone and IV, internal standard (hydrocortisone 21-acetate). The arrows mark the points of injection.

This method is particularly suitable for pharmacokinetic studies since it is both rapid and sensitive. The ability to measure I, II, and III in plasma and blood is essential for studies where the distribution of drugs between plasma and the red blood cells is to be investigated. The amount of drug which is excreted by the kidneys as I and II can also be measured using this method.

REFERENCES

1 L.E. Hare, K.C. Yeh, C.A. Ditzler, F.G. McMahon and D.E. Duggan, Clin. Pharmacol. Ther., 18 (1975) 330.
2 M. Rowland and S. Riegelman, J. Pharm. Sci., 56 (1967) 717.
3 A.B.M. Anderson, G. Gennser, J.Y. Jeremy, S. Ohrlander, L. Sayers and A.C. Turnbull, Obstet. Gynecol., 49 (1977) 471.
4 P.L. Ballard, P. Granberg and R.A. Ballard, J. Clin. Invest., 56 (1975) 1548.
5 P.L. Ballard, J.P. Carter, B.S. Graham and J.D. Baxter, J. Clin. Endocrinol. Metab., 41 (1975) 290.
6 B.E.P. Murphy, J. Clin. Endocrinol. Metab., 27 (1967) 973.

7 P. Vecsei, B. Penke, R. Katzy and L. Baek, Experientia, 28 (1972) 1104.
8 J.C. Touchstone and W. Wortmann, J. Chromatogr., 76 (1973) 244.
9 C. Hesse, K. Pietrzik and D. Hotzel, Z. Klin. Chem. Klin. Biochem., 12 (1974) 193.
10 F.K. Trefz, D.J. Byrd and W. Kochen, J. Chromatogr., 107 (1975) 181.
11 J.C.K. Loo, A.G. Butterfield, J. Moffatt and N. Jordan, J. Chromatogr., 143 (1977) 275.
12 J.C.K. Loo and N. Jordan, J. Chromatogr., 143 (1977) 314.
13 D.C. Garg, J.W. Ayres and J.G. Wagner, Res. Commun. Chem. Pathol. Pharmacol., 18 (1977) 137.
14 G. Schwedt, H.H. Bussemas and Ch. Lippmann, J. Chromatogr., 143 (1977) 259.
15 S.E. Tsuei, J.J. Ashley, R.G. Moore and W.G. McBride, J. Chromatogr., 145 (1978) 213.
16 J.Q. Rose and W.J. Jusko, J. Chromatogr., 162 (1979) 273.
17 G.E. Reardon, A.M. Caldarella and E. Canalis, Clin. Chem., 25 (1979) 122.
18 P.M. Kabra, L.L. Tsai and L.J. Marton, Clin. Chem., 25 (1979) 1293.
19 N.K. Athanikar, G.W. Peng, R.L. Nation, S.-M. Huang and W.L. Chiou, J. Chromatogr., 162 (1979) 367.