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QUANTITATION OF DEXAMETHASONE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific method for the quantitation of dexamethasone in plasma and urine is described. The specificity of the method is obtained using adsorption chromatography on a high-performance liquid chromatograph. The dexamethasone is detected with a variable-wavelength UV detector. An internal standard technique is used for quantitation of dexamethasone with a minimum sensitivity of 15 ng. Preliminary results of the application of the method to pharmacokinetic studies of dexamethasone in humans are reported.

INTRODUCTION

Several recent studies [1—3] have shown that glucocorticoids administered prenatally can significantly reduce the incidence of respiratory distress syndrome in premature infants. Dexamethasone [3] and betamethasone [1, 2] have been used for this indication. The disposition of dexamethasone in the maternal—placental—foetal system is under investigation in this laboratory.

Limitations in selectivity and sensitivity of thin-layer [4] and paper chromatographic [5] techniques prevent their application in the analysis of dexamethasone in plasma at clinically relevant concentrations. Analysis of dexamethasone using gas chromatography (GC) is complicated because steroids with a C-17 hydroxyacetone side chain undergo thermal degradation at the

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temperatures employed [6]. The assay of dexamethasone by GC would also seem to require the prior formation of a stable derivative.

Previous studies of the disposition kinetics of dexamethasone in humans have been effected by administering radioactively labelled drug and monitoring levels of radioactivity in various biological fluids [7—9]. The success of radiotracer methods in providing information on the disposition of the unchanged drug relies on the use of an additional technique which provides adequate separation of the unchanged drug from its metabolites. More recently, three radioimmunoassay procedures [10—12] have been reported for the determination of dexamethasone in plasma and urine and these have been used to monitor its plasma concentration [13, 14] and urinary excretion [13] after oral administration to humans. Since earlier studies had suggested that the major part of the dose of dexamethasone was metabolized in humans, the development of a suitable radioimmunoassay procedure for disposition studies in humans necessitates the preparation of a specific antibody having no significant cross-reactivity with coexisting metabolites. Since the metabolism of dexamethasone in man has not been elucidated, the possibility of these antibodies exhibiting significant cross-reactivity with unidentified metabolites cannot be discounted.

A radio-receptor assay has been reported for the determination of plasma glucocorticoid activity in man [15] and has been applied in a study of the glucocorticoid levels of maternal and cord serum after prenatal betamethasone therapy [16]. This technique provides an estimate of the amount of betamethasone in terms of cortisol equivalents and hence this analytical approach appears unsuitable for most pharmacokinetic studies.

The recent emergence of commercial high-performance liquid chromatographic (HPLC) equipment coupled with sensitive ultraviolet (UV) detectors has made available a new analytical approach for the assay of steroids such as dexamethasone. HPLC offers the advantages that (a) no derivatization steps are required prior to quantitation and (b) the dexamethasone is detected directly. This paper reports a method using HPLC and UV detection for the assay of dexamethasone in plasma and urine.

EXPERIMENTAL

Reagents and materials

Dexamethasone and prednisolone were gifts from Merck, Sharp & Dohme (Sydney, Australia) and Upjohn (Sydney, Australia). Dichloromethane (Merck, Darmstadt, G.F.R.) and 1-butanol (Ajax, Sydney, Australia) were analytical grade solvents. The heptane was spectroscopic grade (Ajax). All other reagents were analytical grade and were used without further purification as were the solvents.

All the glassware was cleaned with a chromic acid mixture and washed with distilled water. The glass evaporation tubes were then silylated with Siliclad (Clay Adams, Parsippany, N.J., U.S.A.), washed with distilled water and dried.

Drug extraction from biological samples

Plasma or urine (3 ml) and a methanolic solution of internal standard (1 μg prednisolone per 50 μl) were washed with *n*-heptane (7 ml) in a 15-ml glass centrifuge tube with a PTFE-lined screw cap, by shaking on a reciprocal shaker for 15 min. After centrifugation for 10 min at 1500 *g*, the organic phase was discarded. Sodium chloride (300 mg) was added to the aqueous phase which was then extracted with dichloromethane (10 ml). To minimise emulsion formation, this extraction was done with gentle shaking on a reciprocal shaker for 30 min. After centrifugation at 1500 *g* for 30 min, the aqueous phase was discarded. The organic phase was then transferred to another centrifuge tube leaving the residual emulsion on the walls of the first tube. The extract was then washed successively with aqueous solutions of sodium carbonate (3 ml, 0.05 *M*), hydrochloric acid (3 ml, 0.1 *M*) and water (3 ml) by vortexing for 1 min and centrifuging at 1500 *g* for 10 min. The dichloromethane extract was then transferred to an evaporation tube, a 15-ml glass tube with a 100-μl capillary at the base. The extract was concentrated in a water bath at 48°. When no liquid dichloromethane remained, the tube was stoppered and immersed in an ice—water bath. This allowed the dichloro-

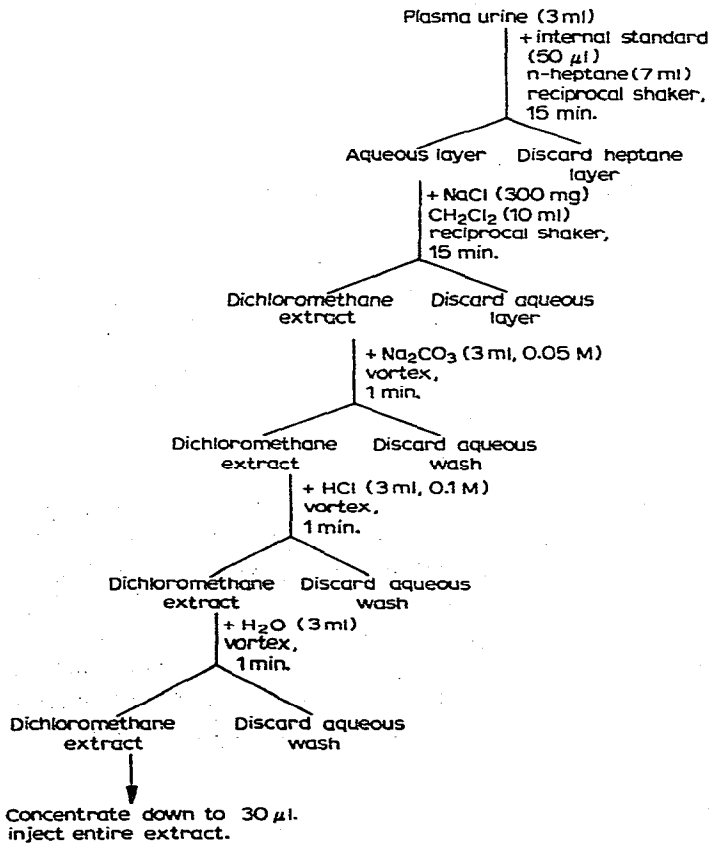

Fig. 1. Schematic diagram for the extraction of dexamethasone.

methane vapour to condense and wash down the internal walls of the evaporation tube. The evaporation and condensation procedures were repeated until approximately 30 μl of the dichloromethane extract remained. The entire extract was injected into the liquid chromatograph. The wall of the evaporation tube was again washed with 10 μl of the fresh dichloromethane. This was also injected into the liquid chromatograph. The use of a stop-flow injection system allows the dichloromethane rinse to be placed on top of the column with the original sample for each chromatographic run. Fig. 1 gives a schematic outline of the extraction procedure.

Chromatography

A Varian Aerograph Model 8500 high-performance liquid chromatograph equipped with a Varian Aerograph stop-flow, septum-less, high-pressure injection port and a Spectra-Physics Model 770 variable-wavelength UV detector operated at 240 nm was used. The column was 250 × 2 mm I.D. stainless-steel tubing packed with silica gel having an average particle diameter of 5 μm (Varian Aerograph Micropak Si-5). Analyses were performed using a mobile phase of dichloromethane containing 5% 1-butanol and 0.3% water at a flow-rate of 65 ml/h and an inlet pressure of 2000 p.s.i. Minor adjustments in flow-rate were made to maintain the retention times constant at 6.6 and 13.8 min for dexamethasone and prednisolone, respectively. The system was operated at an ambient temperature between 23° and 25°.

Calibration and reproducibility

Known quantities of dexamethasone (15—350 ng) were added to blank plasma and urine samples. The samples were then assayed for dexamethasone. Calibration curves were constructed by plotting the peak height ratios between dexamethasone and the internal standard versus the amount of dexamethasone added. To check the reproducibility of the analytical procedure, three different plasma calibration curves were constructed on three different days using human plasma samples from four different sources: a healthy male, a healthy female and two healthy pregnant women near term. A calibration curve using human urine was similarly prepared.

Drug disposition study

Dexamethasone phosphate (8 mg, Decadron®; Merck, Sharp & Dohme) corresponding to 6.66 mg of dexamethasone was administered intravenously over 5 sec to a healthy female volunteer (age 25 years, weight 60 kg). Blood (10 ml) was withdrawn via a cannula from the antecubital vein and placed in tubes containing 100 units of ammonium heparin and separation granules at 12, 19, 34, 65, 123, 153, 190, 247, 298, 357, 417 and 468 min. The plasma was separated immediately and stored frozen until analysis. All urine passed in the 24 h after dosing was collected as a bulk sample and stored frozen prior to analysis.

RESULTS AND DISCUSSION

The major problem encountered in the development of this method was

devising a combination of an extraction procedure and a chromatographic system that consistently resolved dexamethasone from endogenous compounds which absorbed significantly at the $\lambda_{max.}$ of dexamethasone (240 nm). The system reported here has overcome this problem reliably and has been applied in the analysis of over 300 biological samples.

The extraction of dexamethasone from biological fluids using dichloromethane, chloroform and ethyl acetate was investigated, but dichloromethane was found to be the most suitable solvent since (a) it extracted the least number of endogenous compounds, (b) the recovery of dexamethasone was approximately 75% and (c) this solvent was easily evaporated on a water bath. It was necessary, however, to wash the dichloromethane extract with solutions of sodium carbonate and hydrochloric acid to remove the endogenous acids and bases which were unresolved from either dexamethasone or prednisolone on all the chromatographic systems that were examined. The instability of the C-17 side chain in the presence of base [17] necessitated the use of a mild alkaline solution (Na_2CO_3) in this washing step.

The activity of the silica column is dependent on the water content of the mobile phase [18]. It was found that the incorporation of 0.3% water into the mobile phase stabilized the activity of the column and improved the peak shape of both dexamethasone and prednisolone.

A significant loss of activity occurred when the recycled mobile phase became polluted and the resolution of dexamethasone from the endogenous compound eluting before it, decreased significantly. The column was then re-

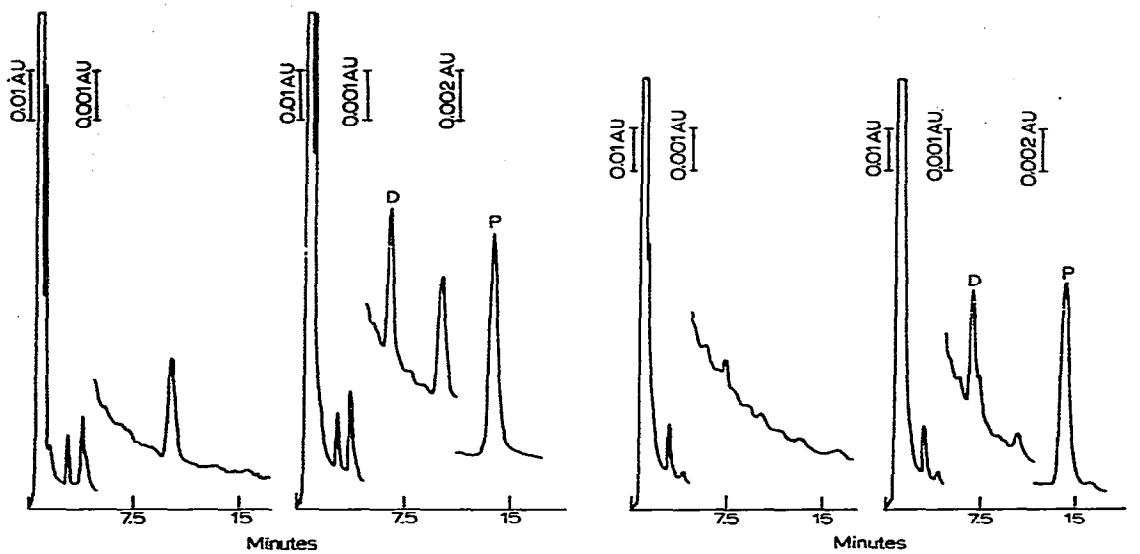

Fig. 2. Chromatograms of blank human plasma and human plasma to which were added 150 ng of dexamethasone (D) and 1 μg of prednisolone (P). Chromatographic conditions are given in the text.

Fig. 3. Chromatograms of blank human urine, and blank human urine to which were added 150 ng of dexamethasone (D) and 1 μg of prednisolone (P). Chromatographic conditions are given in text.

equilibrated with fresh mobile phase and resolution was restored within a few hours.

A similar loss in resolution also occurred when the same grade of dichloromethane from another manufacturer was used to prepare the mobile phase. The identity of the impurity in the solvents that caused this difference in resolution is unknown.

The selectivity of the extraction procedure and chromatographic system is demonstrated by the chromatograms from human plasma and urine extracts in Figs. 2 and 3, respectively. The analysis of blank plasma and urine indicated that there was no significant interference from endogenous compounds. The possibility that metabolites of different polarity [7] from dexamethasone, if extracted, would co-chromatograph with the unchanged drug in this chromatography system is remote. Attempts to confirm the homogenity of the quantitated dexamethasone by mass spectral analysis was frustrated by the thermal instability of the C-17 hydroxyacetone side chain.

Reproducible quantitation of dexamethasone was achieved by using prednisolone as the internal standard. This compound's physical and chemical properties were similar to those of dexamethasone and resulted in a similar recovery for prednisolone (72%) and dexamethasone (75%), and hence contributed to the acceptable linearity of the calibration data (Fig. 4). The calibration plots derived during the application of this method were superimposable within the methodological variation whether the biological fluid being analyzed was plasma or urine. The method enabled the reliable quantitation of 15 ng of dexamethasone and thus provided a lower sensitivity limit of 5 ng/ml for a 3-ml plasma sample. This sensitivity has permitted studies of the pharmacokinetics of dexamethasone in healthy volunteers and patients receiving parenteral doses of 8 mg of dexamethasone phosphate. Fig. 5 presents a typical plasma concentration—time profile of dexamethasone after intravenous administration to a healthy female adult. The terminal phase half-life was 154 min and 2.4% of the dose was excreted unchanged in urine in 24 h. These plasma concentration—time data for dexamethasone were amenable

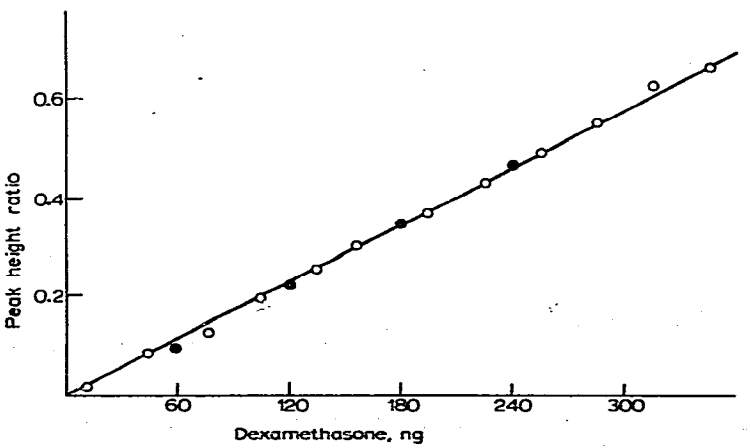

Fig. 4. Calibration curve for dexamethasone in (0) plasma and (•) urine.

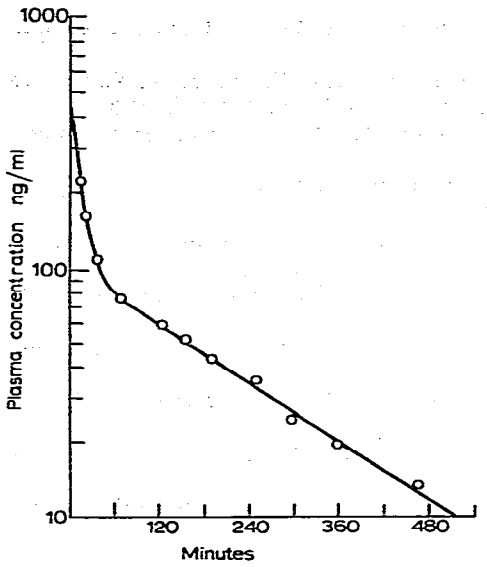

Fig. 5. Plasma concentration—time profile of an 8-mg intravenous dose of dexamethasone phosphate (equivalent to 6.66 mg of dexamethasone) to a healthy female volunteer. o Experimental data; —, computerized non-linear regression fitting to a biexponential equation.

to pharmacokinetic analysis and this and urinary excretion studies will be reported elsewhere.

The results obtained with this HPLC technique suggest that, in humans, there may be unidentified metabolite(s) which are cross-reactive with the antibodies used in the radioimmunoassay of dexamethasone. The most pronounced discrepancy is in the estimates of the fraction of the dose excreted in urine. Duggan et al. [14] report the urinary excretion of 10.6% of the intravenous dose unchanged after 48 h while English et al. [13] found 31% of an oral dose unchanged after 24 h. It also appears that Duggan et al. [14] may be correct in their suspicion that the shoulder appearing in their plasma concentration—time curve after intravenous dosage may be caused by the appearance of metabolite(s) to which the antibody is cross-reactive.

This HPLC method is simple, direct, sensitive and accurate for the quantitation of dexamethasone concentrations in biological fluids and has been found to be adequate for clinical applications.

REFERENCES

1 G.C. Liggins and R.N. Howie, Pediatrics, 50 (1972) 515.
2 R.N. Howie and G.C. Liggins, in C.A. Villee, D.B. Villee and J. Zuckerman (Editors), Respiratory Distress Syndrome, Academic Press, New York, 1973, p. 369.
3 E. Caspi, P. Schreyer, Z. Weinraub, R. Reif, I. Levi and G. Mundel, Brit. J. Obstet. Gynaecol., 83 (1976) 187.
4 *United States Pharmacopeia XIX*, Mack Publ. Co., Easton, Pa., 1975, p. 122.
5 C.A. Johnson and S. Fowler, J. Pharm. Pharmacol., 16 (1964) Suppl. 177.
6 W.J.A. VandenHeuvel, in M.S. Kroman and S.R. Bender (Editors), Theory and Applications of Gas Chromatography in Industry and Medicine, Green and Stratton, New York, 1968, p. 120.

7 N. Haque, K. Thrasher, E.E. Werk, H.C. Knowles and L.J. Sholiton, J. Clin. Endocrinol., 34 (1972) 44.
8 Y. Araki, O. Yokota, T. Kato, M. Kashima and T. Miyazaki, in G. Pincus, T. Nakao and J.F. Tait (Editors), Steroid Dynamics, Academic Press, New York, 1966, p. 463.
9 S.M. Brooks, E.E. Werk, S.J. Ackerman, J. Sullivan and K. Thrasher, New Eng. J. Med., 286 (1972) 1125.
10 A.W. Meikle, L.G. Lagerquist and F.H. Tyler, Steroids, 22 (1973) 193.
11 M. Hichens and A.F. Hogans, Clin. Chem., 20 (1974) 266.
12 M.C. Dumasia, D.I. Chapman, M.S. Moss and C. O'Connor, J. Biochem., 133 (1973) 401.
13 J. English, J. Chakraborty, V. Marks and A. Parke, Eur. J. Clin. Pharmacol., 9 (1975) 239.
14 D.E. Duggan, K.C. Yeh, N. Matalia, C.A. Ditzler and F.G. McMahon, Clin. Pharmacol. Ther., 18 (1975) 205.
15 P.L. Ballard, J.P. Carter, B.S. Graham and J.D. Baxter, J. Clin. Endocrinol. Metab., 41 (1975) 290.
16 P.L. Ballard, P. Granberg and R.A. Ballard, J. Clin. Invest., 56 (1975) 1548.
17 S.K. Wamba, S.W. Amin and N. Rafael, J. Pharm. Sci., 57 (1968) 1231.
18 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York, 1973, p. 269.