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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HYDROCORTISONE AND METHYLPREDNISOLONE AND THEIR HEMISUCCINATE ESTERS IN HUMAN SERUM

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SUMMARY

A high-performance liquid chromatographic method is described for the simultaneous determination of methylprednisolone (MP) and methylprednisolone hemisuccinate (MPHS), or hydrocortisone (HC) and hydrocortisone hemisuccinate (HCHS) in human serum. Reversed-phase liquid chromatography was performed on a microparticulate C_{18} column (Spherisorb, 5 μm) using a mobile phase of 2% glacial acetic acid, 30—35% acetonitrile, 70—65% water with ultraviolet detection (254 nm). The method uses 17α-hydroxyprogesterone as the internal standard for the determination of methylprednisolone and its hemisuccinate ester, or 11-deoxy-17-hydroxycorticosterone as the internal standard for the determination of hydrocortisone and its hemisuccinate ester. The sensitivity is 0.03 μg/ml for HC, 0.07 μg/ml for MP, 0.04 μg/ml for MPHS, and 0.10 μg/ml for HCHS, with a detection limit of 0.02 μg/ml for all four steroids. Calibration curves are linear up to 3 μg/ml for MP or MPHS (as equivalent MP) and up to 4 μg/ml for HC and 7 μg/ml (as equivalent HC) for HCHS. The pooled relative standard deviation for replicate samples for each steroid is <7%. Plasma concentration—time curves are reported for MP and MPHS or HC and HCHS of two human subjects following intramuscular administration of 125 mg of methylprednisolone sodium succinate for injection, U.S.P., or 250 mg of hydrocortisone sodium succinate for injection, U.S.P.

INTRODUCTION

The sodium salts of methylprednisolone hemisuccinate (MPHS) and hydrocortisone hemisuccinate (HCHS) are water-soluble esters of the 21-hydroxycorticosteroids, methylprednisolone (MP) (11β,17α,21-trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione) and hydrocortisone (HC) (11α,17,21-trihydroxypregn-4-ene-3,20-dione). After parenteral administration of a solution of these esters, they are hydrolyzed to their respective parent corticosteroids. Several methods have been described to determine the parent 21-hydroxycortico-

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steroids, such as the Silber—Porter photometric method [1] or radioimmunoassay [2—4]. However, these methods would require the prior separation of the 21-hydroxycorticosteroid from its hemisuccinate ester and subsequent hydrolysis of the hemiester to its parent steroid. Thin-layer chromatography, which has also been used in the analysis of corticosteroids [5—8], was considered as a possible method for the simultaneous analysis of the hemiester and its parent steroid. However, this method lacked the desired reproducibility.

High-performance liquid chromatography (HPLC) offered the advantage of simultaneous analysis of hemiester and parent steroid [9]. HPLC has been used extensively in the analysis of corticosteroids using either normal phase [10 –12] or reversed-phase chromatography [13—15] in biological fluids. This paper reports a method using reversed-phase HPLC with ultraviolet detection for the simultaneous determination of either MP and MPHS, or HC and HCHS, in human serum or plasma.

EXPERIMENTAL

Reagents

Hydroxycortisone hemisuccinate and methylprednisolone hemisuccinate, U.S.P. reference standards, and methylprednisolone and hydrocortisone, N.F. reference standards, and pooled human serum were used to prepare spiked serum samples. 17-α-Hydroxyprogesterone (Upjohn, Kalamazoo, Mich., U.S.A.) and 11-desoxy-17-hydroxycorticosterone (Abbott, North Chicago, Ill., U.S.A.) were used as internal standards. Distilled-in-glass spectroscopic grade ethyl acetate, hexane, methyl alcohol, and acetonitrile (Burdick and Jackson, Muskegon, Mich., U.S.A.) were used without further purification. Glacial acetic acid (Mallinckrodt, St. Louis, Mo., U.S.A.) was used to prepare the 5% acetic acid solution and the mobile phases.

Apparatus

The chromatographic apparatus consisted of a high-pressure liquid chromatograph (Spectra-Physics Chromatronix, Model 3500, Santa Clara, Calif., U.S.A.), equipped with a 100 μl value injection loop, a fixed wavelength detector (Model 8200), and a strip chart recorder (Hewlett-Packard Model 7130A). The column was an ODS reversed stationary phase (Sperhisorb 5 μm ODS, 3 × 250 mm, Spectra-Physics). Peak areas and retention times were determined with an integrator (Autolab Minigrator, Spectra-Physics).

The apparatus used in the extraction procedure was a reciprocal shaker (Eberbach, Ann Arbor, Mich., U.S.A.), a refrigerated centrifuge (Sorvall RC-3, Norwalk, Conn., U.S.A.), and a drying block (Driblock DB-3, Techne, Cambridge, Great Britain).

Mobile phase

The mobile phase was prepared by mixing exact volumes of acetonitrile, filtered distilled water, and glacial acetic acid. The solution was stirred and degassed. For the determination of MP and MPHS, the ratio of acetonitrile—water—glacial acetic acid was 35:65:2. For the determination of HC and HCHS, the ratio of acetonitrile—water—glacial acetic acid was 30:70:2.

Chromatographic operation conditions

The ultraviolet detector was fixed at 254 nm. The sensitivity of the detector was 0.04 a.u.f.s. (absorbance units full scale) for MP and MPHS and 0.08 a.u.f.s. for HC and HCHS. The flow rate was held constant at 1 ml/min. The column pressure was 2000—2500 p.s.i. Chart speed was 0.5 cm/min.

Extraction procedure

One-half milliliter of spiked serum or human sample, 1 ml of 5% acetic acid solution containing 1 μg per ml of internal standard, and 10 ml of hexane were added to a 20 ml screw-cap test tube. The tubes were shaken for 15 min at 100 cycles/min and then centrifuged for 5 min at 1400 *g* at 10°.

The hexane (upper) layer was aspirated off. Six milliliters of ethyl acetate were then added to the remaining aqueous layer. The tubes were shaken at 100 cycles/min for 20 min and centrifuged at 1400-*g* at 10° for 5 min. Five milliliters of the ethyl acetate layer were transferred to a conical tube and evaporated to dryness with filtered air at 40°. The residue was redissolved in 500 μl of mobile phase and then injected on to the column.

Internal standard

17α-Hydroxyprogesterone was used as the internal standard in the procedure for MP and MPHS. 11-Desoxy-17-hydroxycorticosterone was used as the internal standard in the procedure for HC and HCHS. The internal standards were prepared by diluting a methanol solution of each of the above (100 μg/ml) with 5% acetic acid solution to a concentration of 1 μg/ml.

Preparation of spiked serum samples

Methylprednisolone—methylprednisolone hemisuccinate. Methanolic solutions of MP (500 μg/ml) and MPHS (400 μg/ml, equivalent MP) were diluted (8:100) with water and then further diluted with pooled human serum to concentrations from 0.10 to 2.95 μg/ml for MP and 0.08 to 3.20 μg/ml for MPHS. Each spiked sample contained both MP and MPHS. MPHS concentrations are reported as equivalent MP concentrations.

Hydrocortisone—hydrocortisone hemisuccinate. Methanolic solutions of HC (500 μg/ml) and HCHS (800 μg/ml, equivalent HC) were diluted (8:100) with water and then further diluted with pooled human serum to concentrations from 0.08 to 4.05 μg/ml for HC and 0.12 to 6.97 μg/ml for HCHS. Each spiked sample contained both HC and HCHS. HCHS concentrations are reported as equivalent HC concentrations.

RESULTS AND DISCUSSION

In the analysis of MP and MPHS, the parent steroid and its hemiester were resolved from endogenous hydrocortisone, the internal standard and other endogenous components. The retention times were about 3.5, 5, and 7 min for MP, MPHS and internal standard (17α-hydroxyprogesterone). The retention time for HC was about 2.5 min. Chromatograms of extracted serum spiked with MP and MPHS and a blank serum extract containing internal standard only are given in Fig. 1.

In the analysis of HC and HCHS, the parent steroid and its hemiester were resolved from endogenous components and the internal standard (11-desoxy-17-hydroxycorticosterone). The retention times were about 3.5, 5.2, and 6.5 min for HC, HCHS and internal standard. Chromatograms of extracted serum spiked with HC and HCHS and a blank serum extract containing internal standard only are given in Fig. 2. Since all substances identified as true metabolites of hydrocortisone retain the 11β-hydroxyl function, either as such or as a keto group [16], 11-desoxy-17-hydroxycorticosterone should not be a metabolite of hydrocortisone.

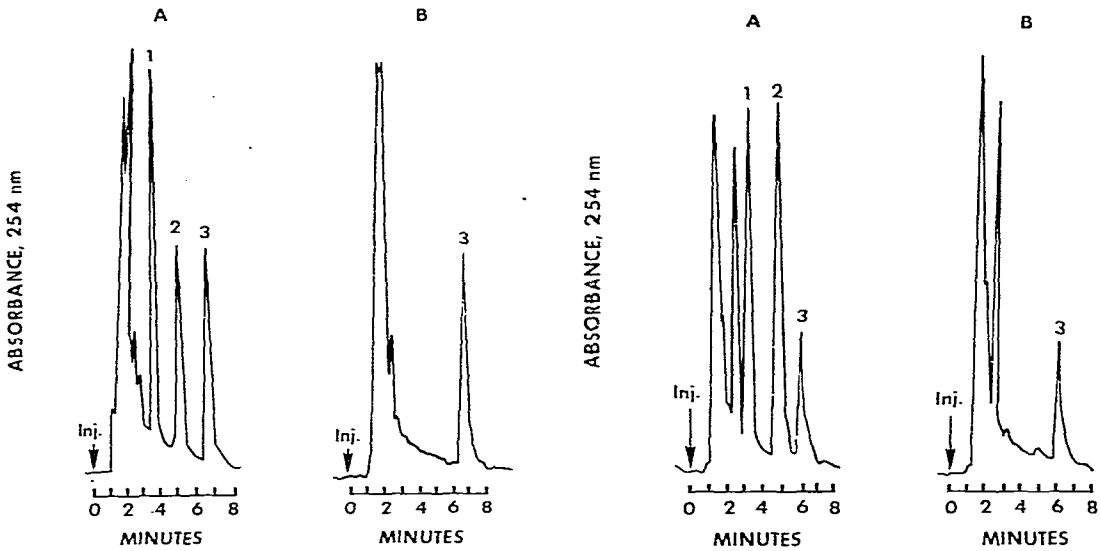

Fig. 1. (A) Chromatogram of extracted human serum spiked with methylprednisolone 2.11 μg/ml (1), methylprednisolone hemisuccinate 1.59 μg/ml (2), and internal standard (3). (B) Extracted "blank" serum containing only internal standard (3); 0.04 a.u.f.s.

Fig. 2. (A) Chromatogram of extracted human serum spiked with hydrocortisone 3.04 μg/ml (1), hydrocortisone hemisuccinate 4.65 μg/ml (2), and internal standard (3). (B) Extracted "blank" serum containing only internal standard (3); 0.08 a.u.f.s.

The peak heights of an extracted serum sample, adjusted for actual volumes, were compared to peak heights of an unextracted aqueous solution of each steroid at known concentrations to determine the percent recovery in the extraction procedure. In the analysis of MP and MPHS, the average percentage recovery was 80% for MP, 94% for MPHS, and 66% for 17α-hydroxyprogesterone. In the analysis of HC and HCHS, the average percentage recovery was 80% for HC, 85% for HCHS and 83% for 11-desoxy-17-hydroxycorticosterone.

The percentage of each steroid partitioning into the hexane layer was also determined. The hexane layer was transferred to a conical test tube, evaporated to dryness, reconstituted with 0.5 ml of mobile phase and injected onto the column. The peak heights were compared to that of an unextracted aqueous solution. In the analysis of MP and MPHS, 16% of the 17α-hydroxy-

progesterone and <1% of MP and MPHS were found in the hexane layer. In the analysis of HC and HCHS, 6% of 11-desoxy-17-hydroxycorticosterone and <1% of HC and HCHS were found in the hexane layer.

Linearity of response and sensitivity

Pooled serum spiked with MP and MPHS from 0.10 to 2.95 μg/ml and 0.8 to 3.20 μg/ml, respectively, were extracted and injected onto the HPLC column. Peak height ratio (peak height of MP or MPHS divided by peak height of internal standard) was used as the response. Least-squares linear regression analysis was used to determine the slope, y-intercept, and correlation coefficient. Using peak height ratio, $Y = 0.007 + 0.752X$ ($r = 0.996$). Using peak area ratio, $Y = 0.02 + 0.592X$ ($r = 0.996$).

Pooled serum spiked with HC and HCHS from 0.08 to 4.05 μg/ml and 0.12 to 6.97 μg/ml, respectively, was extracted and injected onto the HPLC column. Peak heights of HC were corrected for blank serum response representing endogenous HC. Peak heights of HCHS were also corrected for blank serum response representing an endogenous serum component. The corrected peak height ratio was used as the response. Least-squares linear regression analysis was used to determine the slope, y-intercept and correlation coefficient. Using peak height ratio, $Y = 0.004 + 0.831X$ ($r = 0.998$). Using peak area ratio, $Y = 0.04 + 0.587X$ ($r = 0.998$). The concentration of endogenous HC for the pooled serum was calculated to be 0.10 μg/ml.

In the analyses of either MP and MPHS or HC and HCHS, the relationship between serum concentration and peak height ratio was linear up to at least 3 μg/ml of MP and MPHS, 4 μg/ml for HC and 7 μg/ml for HCHS. Peak area ratios were also linear in these ranges. Analysis of 13 standard curves over a period of three weeks indicated that all correlation coefficients of peak height ratio and serum concentration for MP, MPHS, HC and HCHS were 0.99 or greater. The day-to-day coefficient of variation in the slope of the calibration curves was 4.1% for MP, 4.2% for MPHS, 3.2% for HC and 3.5% for HCHS.

The sensitivity of this method was determined to be 0.07 μg/ml for MP, 0.04 μg/ml for MPHS, 0.03 μg/ml for HC and 0.10 μg/ml for HCHS ($p = 0.05$). Sensitivitity is defined as the concentration calculated by linear regression to give a y-response greater than zero 95% of the time. The detection limit was empirically estimated to be 0.02 μg/ml for both steroids and their hemiesters.

Since the serum standards were spiked with both MP and MPHS or HC and HCHS, the effect of the parent steroid response on the hemiester response and the effect of hemiester on the parent steroid response was studied. Serum was spiked with different concentrations of parent steroid only and with hemiester only. These results were compared to standards containing both parent steroid and hemiester. The responses of parent steroid with hemiester were the same as those without hemiester. The responses of hemiester with parent steroid were the same as those without parent steroid. Spiked serum containing only MPHS or HCHS showed no peak at the MP retention time or only endogenous levels of HC.

These results showed that the responses of parent steroid and hemiester were

independent of one another and that MPHS and HCHS were not hydrolyzed to MP and HC during the assay procedure.

Precision and accuracy

The reproducibility of this HPLC method for MP and MPHS and for HC and HCHS was determined. The mean calculated concentration ± S.D. was calculated at each concentration of MP and MPHS as well as HC and HCHS. The results of these calculations are given in Tables I and II. The relative standard deviation was also calculated. Above sensitivity limits, the pooled relative standard deviation is 6.8% for MP, 5.8% for MPHS, 2.9% for HC and 6.1% for HCHS.

The accuracy of this method was determined by spiking serum with MP and

TABLE I

REPRODUCIBILITY IN THE DETERMINATION OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE HEMISUCCINATE IN HUMAN SERUM

Methylprednisolone (μg/ml)		Methylprednisolone hemisuccinate (μg/ml)	
Theoretical	Calculated*	Theoretical	Calculated*
2.95	2.92 ± 0.18**	3.20	3.12 ± 0.07††
2.11	2.15 ± 0.12**	2.23	2.30 ± 0.19**
0.84	0.89 ± 0.04**	1.59	1.58 ± 0.06**
0.42	0.40 ± 0.03**	0.64	0.66 ± 0.05**
0.21	0.20 ± 0.02†	0.32	0.32 ± 0.02†
0.10	0.10 ± 0.03†	0.16	0.15 ± 0.01†
		0.08	0.08 ± 0†

*Mean ± S.D.
**$n = 5$.
†$n = 4$.
††$n = 3$.

TABLE II

REPRODUCIBILITY IN THE DETERMINATION OF HYDROCORTISONE AND HYDROCORTISONE HEMISUCCINATE IN HUMAN SERUM

Hydrocortisone (μg/ml)		Hydrocortisone hemisuccinate (μg/ml)	
Theoretical	Calculated*	Theoretical	Calculated*
4.05	4.02 ± 0.16**	6.97	6.99 ± 0.31**
3.04	3.08 ± 0.16†	4.65	4.66 ± 0.14†
2.43	2.46 ± 0.04†	3.72	3.69 ± 0.06†
1.22	1.22 ± 0.02**	1.86	1.86 ± 0.05**
0.61	0.59 ± 0.01**	0.93	0.91 ± 0.02**
0.30	0.30 ± 0.01**	0.47	0.47 ± 0.01**
0.15	0.16 ± 0.01†	0.24	0.24 ± 0.04†
0.08	0.06 ± 0.02†	0.12	0.12 ± 0.04†

*Mean ± S.D.
**$n = 5$.
†$n = 4$.

MPHS or HC and HCHS at random concentrations within the spiked standard limits. These samples were treated as unknowns and randomly assayed by HPLC. These results are given in Tables III and IV. The average % difference was 3.3% for MP, 4.8% for MPHS, 3.6% for HC and 3.9% for HCHS. It should be noted that spiked plasma samples gave the same response as spiked serum samples.

TABLE III

ACCURACY IN THE DETERMINATION OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE HEMISUCCINATE IN HUMAN SERUM

Methylprednisolone			Methylprednisolone hemisuccinate		
Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)	Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)
3.17	3.10	2.2	0.80	0.83	3.8
0.79	0.84	6.3	0.20	0.20	0
0.40	0.42	5.0	0.10	0.11	10
1.06	1.09	2.8	2.41	2.50	3.7
0.53	0.50	5.7	1.20	1.23	2.5
0.13	0.13	0	0.30	0.33	10
3.17	3.24	2.2	2.41	2.57	6.6
1.58	1.55	1.9	1.20	1.18	1.7

TABLE IV

ACCURACY IN THE DETERMINATION OF HYDROCORTISONE AND HYDROCORTISONE HEMISUCCINATE IN HUMAN SERUM

Hydrocortisone			Hydrocortisone hemisuccinate		
Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)	Theoretical concentration (μg/ml)	Calculated concentration (μg/ml)	Difference (%)
1.00	1.07	7	7.22	7.30	1
0.50	0.55	10	3.61	3.57	1
0.25	0.26	4	1.80	1.71	5
3.0	2.98	1	0.80	0.82	3
0.75	0.74	1	0.20	0.20	0
3.0	3.06	2	4.82	4.70	2
0.75	0.72	4	1.20	1.33	11
1.50	1.50	0	0.40	0.37	8

Human study

Plasma concentration—time curves for MP and MPHS for a single subject following intramuscular administration of 125 mg of Methylprednisolone Sodium Succinate for Injection, U.S.P. (A-methaPred, Abbott, Lot No. 79-822-AR) were determined by the above procedure and are shown in Fig. 3.

Similarly, plasma concentration—time curves for HC and HCHS for a single subject following intramuscular administration of 250 mg of Hydrocortisone Sodium Succinate for Injection, U.S.P. (A-hydroCort, Abbott, Lot No. 79-824-AR) were determined by the above procedure and are shown in Fig. 4.

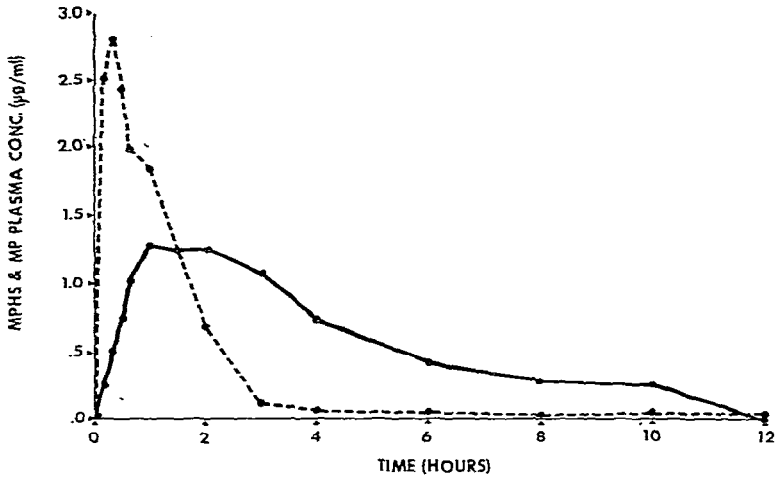

Fig. 3. Plasma concentration—time curve in human for methylprednisolone (——) and methylprednisolone hemisuccinate (- - - -) after a 125 mg intramuscular dose of Methylprednisolone Sodium Succinate for Injection, U.S.P.

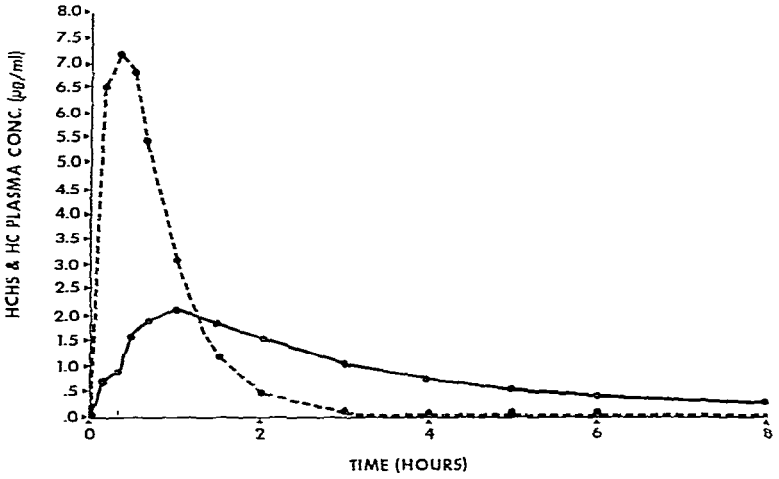

Fig. 4. Plasma concentration—time curve in human for hydrocortisone (——) and hydrocortisone hemissuccinate (- - -) after a 250 mg intramuscular dose of Hydrocortisone Sodium Succinate for Injection, U.S.P.

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