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Simultaneous determination of glucocorticoid alcohols, their succinates and hydrocortisone in plasma

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Abstract: A reversed-phase high-performance liquid chromatographic (HPLC) assay is described for the simultaneous determination of methylprednisolone, methylprednisolone-21-hemisuccinate and endogenous hydrocortisone in biological fluids. This assay is also applicable to the determination of prednisolone-21-hemisuccinate in the presence of prednisolone and hydrocortisone in biological fluids. Prednisolone and hydrocortisone are determined by normal-phase HPLC. The stability of hemisuccinate esters in ampoules, in saline and in plasma has been studied. Whereas the esters were stable in saline at 37°C, they were considerably hydrolysed in plasma at the same temperature. Comparison of hydrocortisone levels obtained by HPLC and radioimmunoassay (RIA) showed the large influence of the cross-reactivity of methylprednisolone and hydrocortisone in the presence of high serum concentrations of methylprednisolone.

Keywords: ***Hydrocortisone; exogenous glucocorticoids.***

Introduction

Esterification of glucocorticoids is often carried out with phosphoric acid but succinic acid can also be used to form water-soluble esters of glucocorticoids. The two most commonly used glucocorticoid hemisuccinates are the sodium salts of methylprednisolone-21-hemisuccinate and prednisolone-21-hemisuccinate. Various methods have been reported for the simultaneous determination of alcohols and esters [1] as well as for the simultaneous determination of exogenous and endogenous glucocorticoid alcohols [2]. A single assay using simple extraction methods for hydrocortisone, the water-soluble glucocorticoid ester and for the glucocorticoid alcohol would be useful in pharmacokinetic studies.

Extraction procedures and chromatographic conditions have been studied in the development of an HPLC assay for hydrocortisone, methylprednisolone or prednisolone and their hemisuccinates in biological fluids.

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Experimental

Materials

Prednisolone, methylprednisolone and their hemisuccinates as well as hydrocortisone were gifts from Hoechst AG (Frankfurt, FRG), triamcinolone acetonide from von Heyden GmbH (Regensburg, FRG), fluocortolone from Schering AG (Berlin, FRG); these were used without further purification.

Solvents for HPLC and extraction were: double-distilled water; glacial acetic acid p.a. (E. Merck, Darmstadt, FRG); acetonitrile chromasolv, absolute ethanol p.a. and ethyl acetate DAC (Riedel de Haen AG, Seelze, FRG); *n*-hexane for HPLC, Baker Chemikalien (Groß Gerau, FRG); methylene chloride for HPLC, Roth K.G. (Karlsruhe, FRG).

Apparatus

HPLC equipment. This comprised a high-pressure pump (model 100, Altex, Berkeley, USA), a 20-µl injection loop (7105 Rheodyne, Cotali, USA), a variable wavelength detector (CE 2012, Cecil Instruments, Cambridge, UK), and an integrator (3390 A, HP, Avondale, USA). Two types of columns were used: 10-µm octadecylsilane, 300 × 6 mm o.d.; 5-µm silica, 200 × 6 mm o.d.; (Macherey & Nagel, Düren, FRG).

For the extraction procedure a vortex mixer (Bender and Hobein, Zürich, CH), and a centrifuge (Minifuge 2, Heräus Christ, Osterode, FRG) were used.

Extraction procedure

For the extraction of methylprednisolone-21-hemisuccinate, methylprednisolone and hydrocortisone, 0.5–2-ml of plasma, urine or saliva, depending on the estimated drug concentrations, or 2–10 ml of bronchoalveolar lavage fluid was mixed with 0.10 ml of internal standard (20 mg l^{-1} triamcinolone acetonide in methanol) and 1 g of ammonium sulphate. For the neutral extraction of prednisolone, prednisolone-21-hemisuccinate and hydrocortisone, a solution of paracetamol (10 mg l^{-1}) in methanol was added as internal standard. The paracetamol standard showed no evidence of degradation during storage under the chromatographic conditions of this assay. For the separate extraction of prednisolone-21-hemisuccinate, the samples were acidified to pH 1 with sulphuric acid; 1 g of ammonium sulphate and 0.10 ml of a methanolic solution of fluocortolone (10 mg l^{-1}) as internal standard were added. Samples (0.5–2-ml) were extracted twice with 3 ml of ethyl acetate; 10-ml samples were extracted with 5 ml of ethyl acetate by shaking for 15 min. Samples were centrifuged at 5000 r.p.m. for 5 min. The organic phases were evaporated to dryness under nitrogen at 45°C and the residue was dissolved in 100 µl of ethanol–water (1:1, v/v).

Recovery studies were performed. Mean recoveries ± relative standard deviations (RSD) were: hydrocortisone at 0.2 µg ml^{-1}, 80% ± 7.3% (n = 5); methylprednisolone at 2 µg ml^{-1}, 76% ± 18% (n = 10); methylprednisolone-21-hemisuccinate at 2 µg ml^{-1}, 91% ± 4.8% (n = 10); and triamcinolone acetonide at 2 µg ml^{-1}, 80% ± 8.2% (n = 10).

Calibration curves were established from the analysis of pooled human serum to which 0.10 ml of a mixture of the respective glucocorticoids in methanol and 0.10 ml of standard solution had been added.

The regression equations for peak area against concentration were calculated. For hydrocortisone at 0.080–0.40 µg/ml (n = 15), $y = 1.340x + 0.132$; Standard error (SE)

of gradient ± 0.258; SE of intercept ± 0.040. For methylprednisolone at 0.5–5.0 μg/ml (n = 15), $y = 0.587x + 0.103$; SE of gradient ± 0.017; SE of intercept ± 0.018. For methylprednisolone-21-hemisuccinate at 0.5–5.0 μg/ml (n = 15), $y = 0.264x + 0.002$; SE of gradient ± 0.042; SE of intercept ± 0.004. For prednisolone at 0.5–5.0 μg/ml (n = 15), $y = 0.317x + 0.193$; SE of gradient ± 0.033; SE of intercept ± 0.007. For prednisolone-21-hemisuccinate at 0.5–8.0 μg/ml (n = 20), $y = 0.345x - 0.0402$; SE of gradient ± 0.035; SE of intercept ± 0.07. Correlation coefficients were not less than 0.998. The RSD from three different curves were: 7% for prednisolone and hydrocortisone, 13% for prednisolone-21-hemisuccinate, 17% for methylprednisolone and 18% for methylprednisolone-21-hemisuccinate. The detection limits for determination (RSD ± 30%) were: 40 ng ml^{-1} for hydrocortisone (octadecylsilane), 100 ng ml^{-1} for methylprednisolone and 200 ng ml^{-1} for the hemisuccinate esters, prednisolone and hydrocortisone (silica).

Chromatographic conditions

In vitro *experiments*. For the separation of glucocorticoid alcohols and esters in stability tests, the octadecylsilane column was used at 40°C; the mobile phase was acetonitrile: phosphate buffer (pH 2, 0.05 M) (20:80, v/v); flow-rate 2 ml min^{-1}; detection wavelength 232 nm. Examples for separations in stability tests in serum are given in Fig. 1.

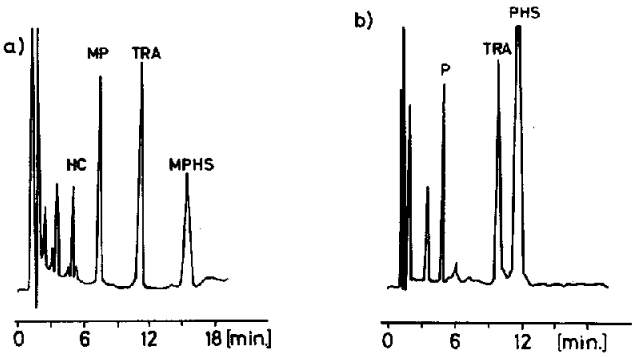

Figure 1
Separation of glucocorticoid alcohols and their 21-hemisuccinates from serum constituents in stability tests. HC = hydrocortisone, MP = methylprednisolone, TRA = triamcinolone acetonide, MPHS = methylprednisolone-21-hemisuccinate, P = prednisolone, PHS = prednisolone-21-hemisuccinate.

Samples from patients. For the determinations in biological fluids (clinical samples), methylprednisolone and its hemisuccinate and hydrocortisone were separated at 25°C on octadecylsilane columns; the mobile phase was acetonitrile: water: glacial acetic acid (70:30:2, v/v/v); flow-rate 1.5 ml min^{-1}; detection wavelength 254 nm (Fig. 2).

Prednisolone-21-hemisuccinate was determined in a separate run under the same conditions as those for methylprednisolone, methylprednisolone-21-hemisuccinate and hydrocortisone; prednisolone and hydrocortisone could not be separated with this system (Fig. 3). Prednisolone and hydrocortisone were separated on silica; the mobile phase was n-hexane: methylene chloride:ethanol:glacial acetic acid (195:90:15:06, v/v/v/v) at 25°C; flow-rate 1.5 ml min^{-1}; detection wavelength 254 nm (Fig. 3). Prednisolone-21-hemisuccinate could not be detected under these conditions.

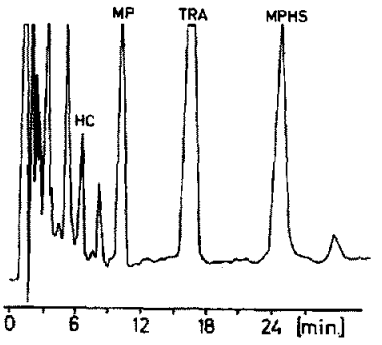

Figure 2
HPLC chromatogram for the separation of methylprednisolone (MP), its 21-hemisuccinate (MPHS), hydrocortisone (HC) and triamcinolone acetonide (TRA, internal standard) in serum, for pharmacokinetics studies.

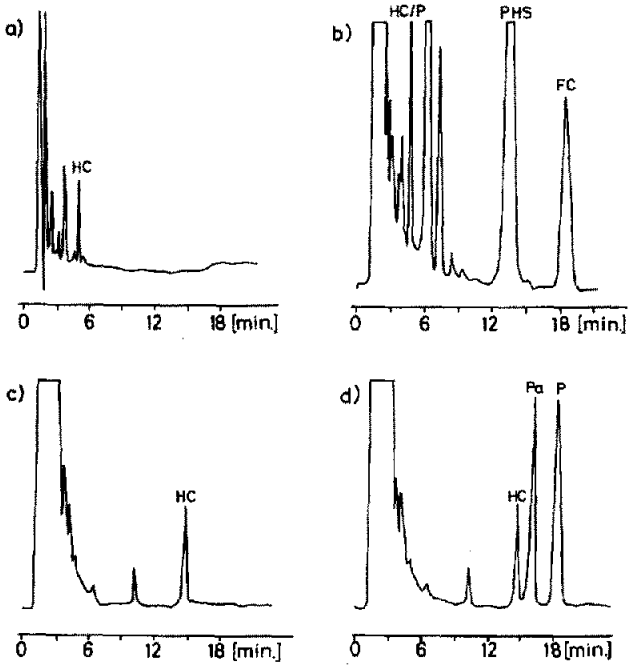

Figure 3
HPLC-chromatograms of: (a) blank serum containing hydrocortisone (HC); (b) sample containing hydrocortisone, prednisolone (P), prednisolone-21-hemisuccinate (PHS) and fluocortolone (FC, internal standard) using an octadecylsilane column. HPLC chromatograms of (c) blank serum containing hydrocortisone; (d) sample containing hydrocortisone, prednisolone, prednisolone-21-hemisuccinate and paracetamol (Pa, internal standard) using a silica column.

Results

Extractibility of the substances in acidic medium

The pH dependence of the extractibility of glucocorticoid alcohols and their hemisuccinates (10 μg ml^{-1}) from buffers with ethyl acetate (1:1 v/v) was investigated; pH 1–7 did not influence the extractibility of prednisolone and methylprednisolone, whereas the hemisuccinates could only be extracted efficiently at pH 1–5 (Fig. 4). The esters were stable in acidic solution during the extraction. Less than 0.1% of free alcohol was found in the solution that was extracted and this did not increase during extraction.

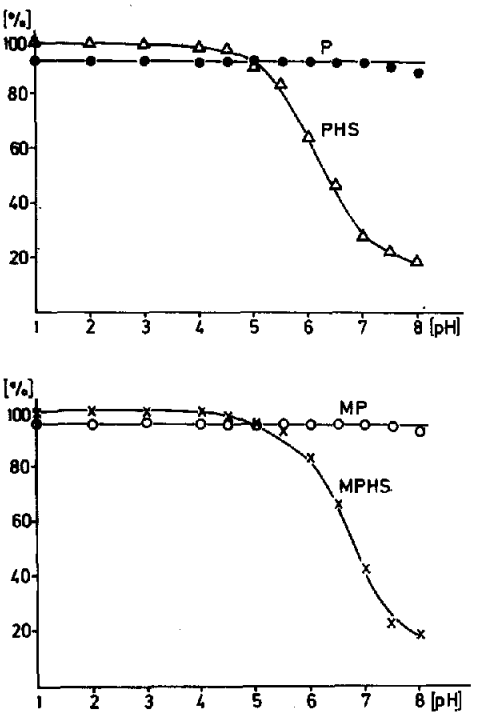

Figure 4
Extractibility of prednisolone (P) and methylprednisolone (MP) and their 21-hemisuccinates (PHS and MPHS) at different pH values.

Stability of the succinate esters in ampoules

The contents of ampoules from different lots of the sodium salts of methylprednisolone-21-hemisuccinate (Urbason Solubile, Hoechst AG, Frankfurt, FRG; Medrate Solubile, Upjohn GmbH, Heppenheim, FRG) and prednisolone-21-hemisuccinate (Solu-Decortin H, E. Merck, Darmstadt, FRG) were dissolved in water to give concentrations of 10 $\mu g\ ml^{-1}$ and analysed immediately by direct injection into the chromatograph. In addition to free methylprednisolone (Table 1) and the 21-hemisuccinate, another compound was present in the chromatogram (Fig. 5). With the HPLC assay of Anderson and Taphouse [3], this compound was identified as methylprednisolone-17-hemisuccinate by the retention time and the pH dependence of the chromatogram. The retention time observed (8.7 min) was identical to that reported [3]. Since the concentration of the 17-hemisuccinate is low (1%) compared with that of the 21-hemisuccinate, the 17-hemisuccinate could be neglected in pharmacokinetic studies. In samples from patients, small peaks for the 17-ester were detected after the injection of large amounts (10 $mg\ kg^{-1}$) of methylprednisolone-21-hemisuccinate.

Up to 7.4% of glucocorticoid alcohol was detected in the ampoules (Table 1) indicating hydrolysis of the succinates even in solid state; the ampoules contain the crystalline products only and the solutions for injection must be prepared immediately for use. Despite the relatively high concentration of the sparingly soluble alcohols, the solutions were not hazy, probably as the result of solubilization of the glucocorticoid alcohol by the large excess of the water-soluble ester.

Since the hemisuccinates are the pro-drugs, and are hydrolysed *in vivo* to glucocorticoid alcohols, the pharmacologically active form, formation of the glucocorticoid

Table 1
Free glucocorticoid alcohol in succinate esters in ampoules*

Ampoule	Lot number	Packing date	Glucocorticoid alcohol (%)
Methylprednisolone-21-hemisuccinate			
Medrate solubile 500	77 I 14/B 976D	Sept. 1977	6.7
Medrate solubile 1000	77 H 24/B 737D	Aug. 1977	7.4
Urbason solubile forte 250	230 U 305/U 427	Aug. 1978	5.4
Urbason solubile forte 1000	384 U 043/U 392	Jan. 1979	4.0
Urbason solubile forte 1000	173 C 050/U 077	Jun. 1979	2.3
Urbason solubile forte 250	296 W 356/W 029	Oct. 1981	2.0
Urbason solubile 20 mg	084 A 967 A 090	Mar. 1982	5.9
Urbason solubile 40 mg	293 A 632 A 112	Oct. 1982	0
Prednisolone-21-hemisuccinate			
Solu-Decortin H 250 mg	3179 A 2213458	Aug. 1979	5.7
Solu-Decortin H 250 mg	3287 U 0563868	Feb. 1980	5.6
Solu-Decortin H 1 g	2797 U 0783843	Mar. 1980	6.2
Solu-Decortin H 25 mg	8264 K 1817081	Jul. 1982	2.1

* Analyses were performed in June and July 1983.

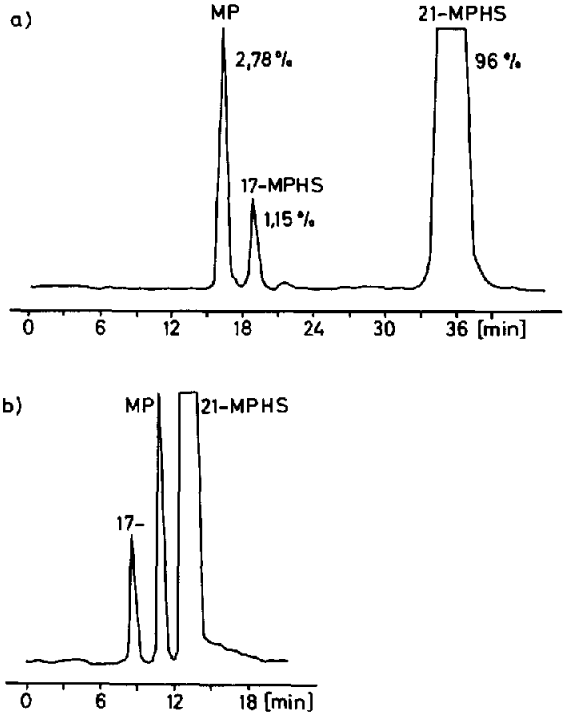

Figure 5
Identification of the methylprednisolone-17-hemisuccinate (17-MPHS) in a solution of methylprednisolone-21-hemisuccinate (21-MPHS) and methylprednisolone (MP). (a) Acetonitrile–water–glacial acetic acid (30:70:2, v/v/v). (b) Acetate buffer (0.05 M, pH 5.4)–acetonitrile (67:33, v/v) (3).

alcohols *in vitro* results in administration of a small initial dose of the active drug itself. Therefore the relatively small amounts of hydrolysed pro-drug should not be interpreted to represent deterioration of the formulation.

Stability of the succinate esters in saline and plasma

To check the influence of body temperature on hydrolysis, solutions of the hemisuccinates in saline were stored for 6 h at 37°C and analysed at intervals of 1 h. No significant formation of prednisolone or methylprednisolone could be detected. The stability in plasma was investigated *in vitro* to follow the influence of storage temperature of plasma samples in clinics. Plasma was spiked with the esters at 20°C or 37°C, at 10 μg ml^{-1}; at hourly intervals the concentrations of the ester as well as of the glucocorticoid alcohol were determined (Fig. 6). From both concentrations the rate constants for hydrolysis could be calculated. The half-lives *in vitro* of the esters based on these results are 11.3 h for methylprednisolone-21-hemisuccinate and 22.6 h for prednisolone-21-hemisuccinate at 37°C. At 20°C the corresponding values are 69 and 138 h. From these data only insignificant changes in ester concentrations would be expected if samples of biological fluids were cooled from 37°C to 0°C during 30–60 min before deep freezing. In addition the process of thawing the samples after storage at −20°C should not influence the results. The plasma half-life *in vitro* will be compared later with the plasma half-life *in vivo* to elucidate the effects of liver enzymes on ester hydrolysis *in vivo*.

The HPLC assay of the succinates and glucocorticoid alcohols in biological fluids

The assay was developed for the determination of exogenous and endogenous glucocorticoids following the administration of doses of 80–1200 mg of water-soluble glucocorticoid esters in clinical studies.

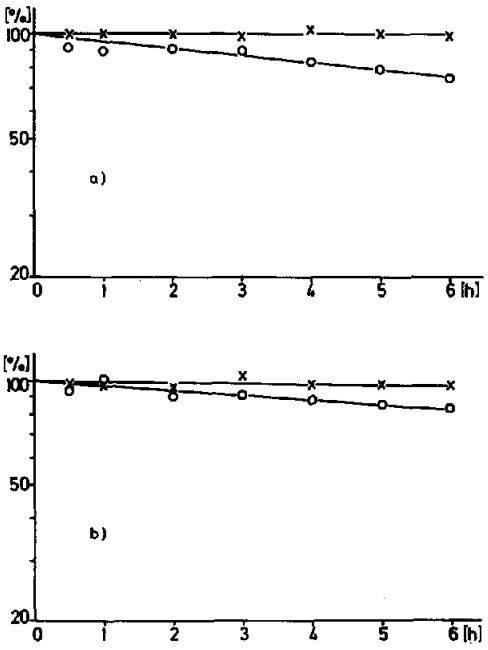

Figure 6
Hydrolysis in fresh human plasma at 20°C and 37°C for (a) methylprednisolone-21-hemisuccinate and (b) prednisolone-21-hemisuccinate. × = 20°C, ○ = 37°C.

Table 2
Hydrocortisone concentrations (μg/ml) in serum in the presence of methylprednisolone

Glucocorticoid	Method	Time after injection (h) 0	0.5	1	1.5	2	4	8	12	16	24	36	48	60	72	96
Patient 1																
Hydrocortisone*	RIA	0.41	2.41	2.41	1.75	1.35	1.08	0.39	0.19	0.08	0.02	0.05	0.08	0.14	0.30	0.20
	HPLC	0.35	0.23	0.31	0.27	0.22	0.14	0.07	0	0	0	0.06	0.15	0.15	0.32	0.27
Methylprednisolone*	HPLC	0	10.6	9.17	7.36	5.67	3.05	1.49	0.53	0.09	0	0	0	0	0	0
Patient 2																
Hydrocortisone*	RIA	0.2	>0.6	>0.6	>0.6	>0.6	>0.6	>0.6	0.33	0.01	0.02	0.02	0.03	0.02	0.05	0.14
	HPLC	0.16	0.20	0.25	0.24	0.23	0.23	0.13	0.05	0	0	0.07	0.01	0.07	0.06	0.16
Methylprednisolone*	HPLC	0	11.35	9.46	7.81	6.21	4.91	2.50	1.31	0.39	0	0	0	0	0	0

*Concentrations at different times after injection are given in μg/ml.

For hydrocortisone determinations, the calibration curve was corrected for the hydrocortisone present in the pooled serum by taking the hydrocortisone concentration from the calibration curve and subtracting the intercept on the *y*-axis.

Since the calibration curves cover only a small concentration range, smaller volumes (0.5 ml) were extracted from concentrated samples (high-dose therapy), whereas from dilute urine samples or from bronchoalveolar lavages 10 ml samples had to be extracted. Compared with those in the literature [1, 2], the sensitivity and reproducibility of the present assays is satisfactory only for the examples given, i.e. in studies on the pharmacokinetics of relatively high doses of glucocorticoid esters and alcohols and the simultaneous measurement of the resulting decrease of the hydrocortisone level.

Whereas the assay for methylprednisolone, its succinate and hydrocortisone is simple, acceptable separation of prednisolone and hydrocortisone could not be achieved on different octadecylsilane columns in the analysis of clinical samples.

The separation of prednisolone and hydrocortisone was possible on silica but with a sensitivity lower than that on octadecylsilane; however a separate determination was needed for prednisolone-21-hemisuccinate. Because two different HPLC assays were required for the analysis of prednisolone and hydrocortisone and for the succinates, the method is time-consuming and a relatively large volume of the biological fluid is needed.

In the analysis of hydrocortisone in serum samples from some patients after the injection of 10 mg kg^{-1} of methylprednisolone hemisuccinate, by both HPLC and RIA (Travenol GmbH, München Gammacoat), large differences were found in the hydrocortisone concentration [4] (Table 2). For RIA cross-reactivity of hydrocortisone with methylprednisolone caused these differences when more than 200 ng ml^{-1} of the exogenous glucocorticoid was present in the samples. Therefore, RIA could not be applied for the hydrocortisone determination on the day following the injection. One or more days later, however, the two methods gave nearly identical results for hydrocortisone (Table 2). Similarly, since cross-reactivity between prednisolone and hydrocortisone also occurs (87.5%), the use of HPLC is preferred to RIA for that assay of those substances.

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