Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 2, pp. 197–206, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00
Pergamon Journals Ltd.

HPLC determination of glucocorticoid alcohols, their phosphates and hydrocortisone in aqueous solutions and biological fluids

H. DERENDORF,[1]* P. ROHDEWALD,[2] G. HOCHHAUS[2] and H. MÖLLMANN[3]

[1] *College of Pharmacy, University of Florida, Gainesville, FL 32610, USA*
[2] *Institut für Pharmazeutische Chemie der Westfälischen Wilhelms-Universität Münster, Hittorfstr. 58–62, D-4400 Münster, FRG*
[3] *Medizinische Universitätsklinik und Poliklinik "Bergmannsheil" der Ruhruniversität Bochum, D-4630 Bochum, FRG*

Abstract: High-performance liquid chromatographic (HPLC) assays are described for the determination of dexamethasone phosphate, dexamethasone and hydrocortisone and for the determination of triamcinolone, triamcinolone acetonide, triamcinolone acetonide phosphate and hydrocortisone in aqueous solutions and biological fluids. These assays allow quantification of the glucocorticoids in plasma down to a concentration of 100 ng ml^{-1} (dexamethasone phosphate 300 ng ml^{-1}, hydrocortisone 40 ng ml^{-1}). During the development of optimum extraction procedures the pK_a values of the esters were determined by extractions performed at different pH values. The stability *in vitro* of the phosphate esters in ampoules, plasma and blood was studied. The esters are stable in their formulated ampoules after long-term storage, whereas the half-life *in vitro* of dexamethasone phosphate in plasma at 37°C is 5 h and that of triamcinolone acetonide phosphate is 3.5 h. Determination by HPLC of endogenous hydrocortisone in samples from patients who received either dexamethasone phosphate or triamcinolone acetonide phosphate gave results identical with those obtained by radio-immunoassay (RIA).

Keywords: *Dexamethasone phosphate; triamcinolone acetonide phosphate; separation of glucocorticoid esters and alcohols; HPLC; ester hydrolysis in plasma; hydrocortisone levels in presence of exogenous glucocorticoids.*

Introduction

The esterification of glucocorticoid alcohols with phosphoric acid results in water-soluble derivatives which can be given intravenously. Dexamethasone is used as the disodium salt and triamcinolone acetonide as the dipotassium salt. Numerous methods have been developed for the determination of dexamethasone and triamcinolone acetonide as glucocorticoid alcohols [1–3]. However, thorough investigation of the pharmacokinetics of these drugs and their pro-drugs requires the determination of both ester and glucocorticoid alcohol levels in biological fluids [4]. Furthermore, the assay should

*To whom correspondence should be addressed.

enable the hydrocortisone content to be monitored in plasma; for triamcinolone acetonide phosphate the assay should enable triamcinolone to be determined as a possible product of hydrolysis. In addition to its application in pharmacokinetic studies, the assay might be used to investigate the stability of the phosphate esters *in vitro* and in ampoules following long-term storage. To achieve optimal conditions for the HPLC determination of glucocorticoid esters, free alcohols and hydrocortisone, various extraction procedures and chromatographic conditions were investigated.

Experimental

Materials

Dexamethasone and dexamethasone phosphate were gifts from E. Merck AG, Darmstadt, FRG; triamcinolone, triamcinolone acetonide and the phosphate ester of triamcinolone acetonide were gifts from von Heyden GmbH, Regensburg, FRG; 6α-methylprednisolone and hydrocortisone were gifts from Hoechst AG, Frankfurt, FRG; these glucocorticoids were used without further purification.

Solvents used for HPLC and extraction were double-distilled water, phosphoric acid (85%) (Merck, Darmstadt, FRG), acetonitrile chromasolv, ethyl acetate DAC, methanol p.a., ethanol p.a. (Riedel de Haen AG, Seelze, FRG), monosodium phosphate p.a., ammonium sulphate p.a. (Merck, Darmstadt, FRG).

Apparatus

For the HPLC assay the following instruments were used: a high-pressure pump (Model 100, Altex, Berkeley, USA) equipped with a 20-μl injection loop (Model 7105, Rheodyne, Cotati, USA), a 10-μm octadecylsilane column (Macherey & Nagel, Düren, FRG), a variable wavelength-detector (CE 2012, Cecil Instruments, Cambridge, UK) and an integrator (HP 3390A, Hewlett & Packard, Avondale, USA). For the extraction procedure a vortex mixer (Bender and Hobein, Zurich, CH) and a laboratory centrifuge (minifuge 2, Heräus Christ, Osterode, FRG) were used.

Extraction procedure

Plasma, urine or saliva (0.5–2 ml depending on the estimated drug concentrations) was mixed with 100 μl of internal standard (20 mg l^{-1} 6α-methylprednisolone in methanol) and 1 g of ammonium sulphate. The mixtures were extracted twice by shaking for 15 min with 3-ml portions of ethyl acetate. Samples were centrifuged at 5000 r.p.m. for 5 min.

Chromatographic conditions

In vitro *experiments*. For the HPLC separation of the glucocorticoid alcohols and their phosphate esters in stability tests the following conditions were used for dexamethasone and for triamcinolone acetonide.

Mobile phase: Acetonitrile–0.05 M phosphate buffer (pH 2) (30:70, v/v); flow rate 2 ml min^{-1}; wavelength 232 nm; temperature 40°C; injection volume 20 μl; internal standard, 6α-methylprednisolone.

Examples of separations in stability tests *in vitro* are given in Fig. 1.

Biological samples. Dexamethasone and dexamethasone phosphate were separated by HPLC following extraction at pH 1 from plasma constituents in samples from patients and volunteers (Fig. 2).

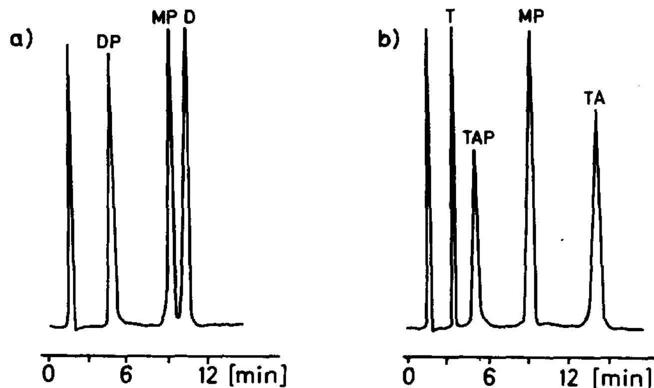


Figure 1
Separation of glucocorticoid alcohols and their phosphate esters in stability tests. (DP) dexamethasone phosphate, (D) dexamethasone, (MP) 6α-methylprednisolone, (T) triamcinolone, (TA) triamcinolone acetonide, (TAP) triamcinolone acetonide phosphate.

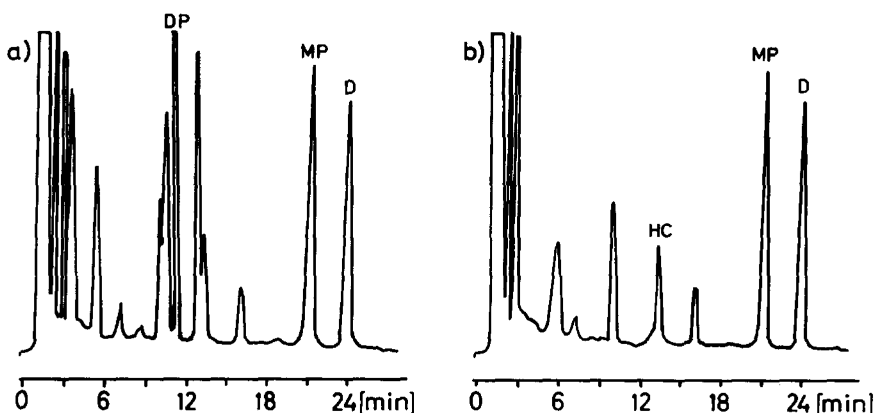


Figure 2
HPLC for the determination of dexamethasone phosphate (DP) in plasma (a) and for the separation of hydrocortisone (HC) and dexamethasone (D) in plasma (b). Internal standard, 6α-methylpredisolone (MP).

The mobile phase comprised acetonitrile–water–phosphoric acid (85%) (28:72:0.15, v/v/v); flow rate 1 ml min^{-1}; wavelength 254 nm; temperature 25°C; injection volume 20 μl; internal standard, 6α-methylprednisolone. Under these conditions hydrocortisone could not be resolved from plasma constituents.

The HPLC separation of hydrocortisone and dexamethasone was possible following extraction at pH 6–7 under the same chromatographic conditions as given for the separation of dexamethasone and its phosphate (Fig. 2), because the plasma constituents which overlapped with hydrocortisone after acidic extraction were not present in neutral extracts. Dexamethasone phosphate was not extracted at pH 6–7. Thus samples that contained the phosphate ester had to be divided for the analysis and two separate extractions were needed for each sample.

Simultaneous separation of triamcinolone acetonide, triamcinolone, triamcinolone acetonide phosphate and hydrocortisone could not be achieved in one step. The plasma samples were divided into two parts: one part of the sample was extracted after acidification to pH 1 with phosphoric acid for the analysis of the phosphate ester (Fig. 3); the other part was extracted without acidification to determine triamcinolone, triamcinolone acetonide and hydrocortisone without interference from the plasma constituents which were present following extraction at pH 1. Triamcinolone acetonide phosphate was not extractable at neutral pH (Fig. 3). For both extracts the chromatographic conditions were identical: the mobile phase was acetonitrile–water–phosphoric acid (85%) (28:72:2, v/v/v); flow rate 1.5 ml min^{-1}; wavelength 254 nm; temperature 25°C; injection volume 20 μl; internal standard, 6α-methylprednisolone.

The organic phases were evaporated under nitrogen at 45°C and the drug residue was dissolved in 100 μl of ethanol–water (1:1, v/v).

Calibration curves were derived from pooled human plasma by adding appropriate amounts of a mixture of the glucocorticoids in methanol and 100 μl of internal standard solution. The concentration ranges of the calibration curves were 0.08–0.4 μg ml^{-1} for hydrocortisone, 1–20 μg ml^{-1} for dexamethasone phosphate, and 0.5–10 μg ml^{-1} for dexamethasone, triamcinolone, triamcinolone acetonide and triamcinolone acetonide phosphate. Correlation coefficients for the standard curves (peak area ratio against concentration) were between 0.999 and 0.995. The precision (from three different determinations) was 6.4–8.7% for hydrocortisone, triamcinolone acetonide, triamcinolone acetonide phosphate and dexamethasone and 13.8% for triamcinolone. The lower limit for the quantitative determination in 1 ml of plasma (relative standard deviation ± 30%) was 40 ng ml^{-1} for hydrocortisone, 300 ng ml^{-1} for dexamethasone phosphate and 100 ng ml^{-1} for triamcinolone, triamcinolone acetonide, triamcinolone acetonide phosphate and dexamethasone.

Results and Discussion

Extractibility in acidic medium

As previously observed for prednisolone and 6α-methylprednisolone [5], the extractibility of the glucocorticoid alcohols hydrocortisone, dexamethasone, triam-

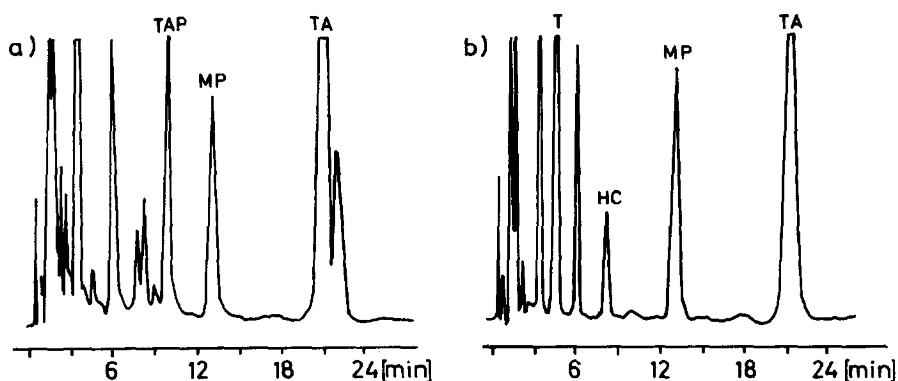


Figure 3
HPLC for the determination of triamcinolone acetonide phosphate (TAP) in plasma (a) and for the separation of triamcinolone (T), hydrocortisone (HC) and triamcinolone acetonide (TA) in plasma (b). Internal standard, 6α-methylprednisolone (MP).

cinolone and triamcinolone acetonide from buffer solutions (0.05 M, pH 1–7, concentration 10 μg ml^{-1} for each substance) into ethyl acetate was not dependent on pH and the partition coefficients of these alcohols were constant (Fig. 4). With an increase in pH, the amount of the phosphate esters extracted diminished greatly so that the partition coefficients became smaller (Fig. 4). No free glucocorticoid alcohol could be detected at pH 1–4 in the analysis of the aqueous phase of extracts containing the phosphate esters of dexamethasone and triamcinolone acetonide; thus the phosphate esters were stable under both the extraction and the chromatographic conditions.

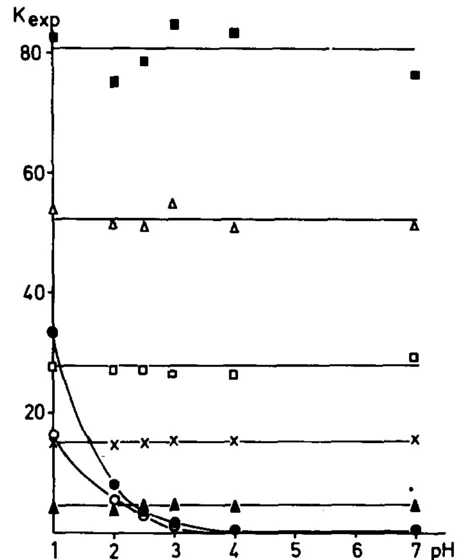


Figure 4
Partition coefficients of the phosphate esters and glucocorticoid alcohols as function of pH. ■ Triamcinolone acetonide, ● triamcinolone acetonide phosphate, ▲ triamcinolone, × hydrocortisone, △ dexamethasone, ○ dexamethasone phosphate, □ 6α-methylprednisolone.

Determination of the pK_a by the extraction method

From the pH-dependence of the experimentally determined partition coefficients for the two phosphates (Fig. 4) it is possible to determine their pK_a values. The dissociation constant K of an acid HA is defined as:

$$K = \frac{[H^+]\,[A^-]}{[HA]}. \tag{1}$$

Only the undissociated species is able to partition into the organic phase. The ratio of undissociated drug in organic and aqueous phase can be defined as the intrinsic partition coefficient, k_{intr} [6]:

$$k_{intr} = \frac{[HA]_{org}}{[HA]_{aq}}. \tag{2}$$

When the partition coefficient is determined experimentally, the drug concentration in the aqueous phase represents both ionized and non-ionized form. This value is defined as the experimental partition coefficient, k_{exp}:

$$k_{exp} = \frac{[HA]_{org}}{[HA]_{aq} + [A^-]_{aq}} . \quad (3)$$

Substitution of (1) and (2) into (3) gives:

$$\frac{1}{k_{exp}} = \frac{1}{k_{intr}} + \frac{[HA]_{aq} K}{[H^+] [HA]_{org}} \quad (4)$$

or

$$\frac{1}{k_{exp}} = \frac{1}{k_{intr}} + \frac{K}{k_{intr}} \cdot \frac{1}{[H^+]} . \quad (5)$$

When the reciprocals of the experimental partition coefficients are plotted against the reciprocals of the hydrogen ion concentration, a straight line can be obtained. It is possible to determine k_{intr} from the intercept and the dissociation constant K from the slope if it is assumed that no side-reactions occur in the aqueous and organic phases.

Figure 5 shows the graphs for dexamethasone phosphate and triamcinolone acetonide phosphate. Both are straight lines with a correlation coefficient of 0.999. Intrinsic partition coefficients determined from the intercept are 2.76 for triamcinolone acetonide phosphate and 1.39 for dexamethasone phosphate; the higher lipophilicity of the triamcinolone acetonide ester is expected from the longer retention time on the reversed-phase HPLC column. The pK_a values determined from the slopes for the two compounds are 1.70 for triamcinolone acetonide phosphate and 1.89 for dexamethasone phosphate. This method only determines the lower of the two pK_a values since the equilibrium between the mono- and di-anionic form has no significant influence on the extractibility. The pK_a values were also determined by potentiometric titration [7] to be $pK_{a_1} = 6.15$ and $pK_{a_2} = 1.5$ for triamcinolone acetonide phosphate and $pK_{a_1} = 6.38$ and $pK_{a_2} = 1.9$ for dexamethasone phosphate.

Stability of the phosphate esters in ampoules

Ampoules with storage times of 1–3 years were analysed for content of free glucocorticoid alcohols by injecting the solution from the ampoules directly into the

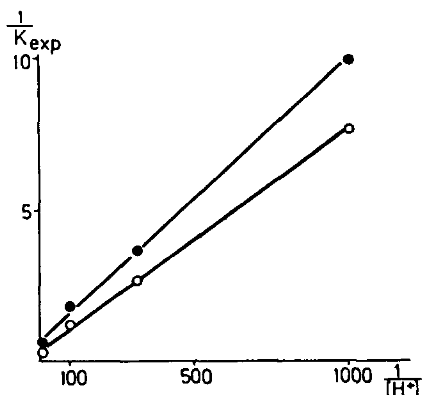


Figure 5
Determination of pK_a values for triamcinolone acetonide phosphate (○) and dexamethasone phosphate (●). For explanation see text.

chromatograph. Contents of glucocorticoid alcohols in samples of dexamethasone phosphate (Fortecortin-Mono-Amp., Merck, Darmstadt, FRG) and in ampoules of triamcinolone acetonide phosphate (Volon A solubile, von Heyden, Regensburg, FRG) were less than 1%. Thus these phosphate esters are much more stable than the succinate esters of prednisolone and 6α-methylprednisolone, which were hydrolysed to a greater extent [5] in the solid state than the phosphates in aqueous solution examined in the present work.

Stability in vitro *of the phosphate esters in blood and plasma*

To examine the influence of storage conditions of blood samples under the conditions of clinical blood sampling, including centrifugation and storage of the plasma, the stability *in vitro* of the phosphate esters were investigated in blood at 37°C and in plasma at 37° and 20°C. Blood from two healthy volunteers was withdrawn into citrated vials before breakfast to minimize dietary effects. The pooled blood was divided into two parts: one part was spiked with the esters dissolved in water to give concentrations of 10 $\mu g\ ml^{-1}$; the other part was centrifuged and the plasma was spiked with the glucocorticoid esters to give the same concentrations.

At hourly intervals plasma and blood samples were removed from the incubator, extracted as described and analysed by HPLC for the ester and the free glucocorticoid alcohol simultaneously. Results are given in Figs 6 and 7, the resulting rate constants for hydrolysis *in vitro* are given together with the respective values of the succinate esters of prednisolone and 6α-methylprednisolone [5] in Table 1.

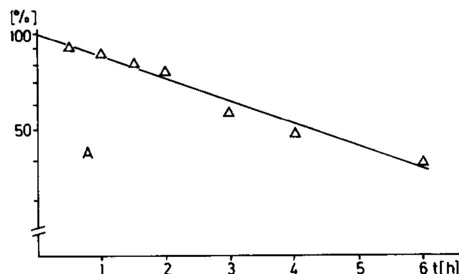
The half-lives *in vitro* at 37°C of the esters in blood and plasma are identical for the glucocorticoid phosphates (Figs 6 and 7). At 37°C the degradation in plasma is much more pronounced than at 20°C, showing a strong temperature dependency of hydrolysis of the phosphate esters. Because the phosphate esters were not hydrolysed at room temperature in neutral solution even after long-term storage, it is likely that plasma phosphatases are responsible for the hydrolytic cleavage of the phosphate esters in plasma and blood. However, from these experiments it could be concluded that use of a procedure of blood sampling with immediate centrifugation and freezing of the samples will result in values no more than 10% less than the actual concentration present at the time of sampling.

When the much faster hydrolysis *in vivo* of the phosphate esters and the inter-individual variation of the plasma half-lives *in vivo* for these esters are considered, the influence of the blood sampling conditions on the determination of the plasma half-lives *in vivo* can be neglected.

HPLC assay of phosphate esters, their glucocorticoid alcohols and hydrocortisone in biological fluids

The HPLC assays were developed for drug monitoring following high-dose therapy with water-soluble glucocorticoids.

In these clinical trials, dexamethasone phosphate was given in doses of 2 $mg\ kg^{-1}$ and triamcinolone acetonide phosphate in doses from 1 to 10 $mg\ kg^{-1}$. The resulting drug levels were high compared with those in studies with the usual small oral doses. Therefore sufficient data could be collected with the HPLC assays for pharmacokinetic analysis although the sensitivity of these assays was clearly lower than that of RIA methods [8]. According to the sensitivity of the assay the sample size varied between 0.5 ml for high drug concentrations and 10 ml for very low concentrations. The advantage of



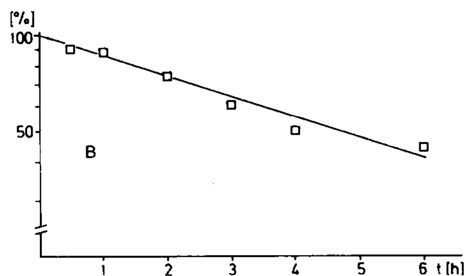
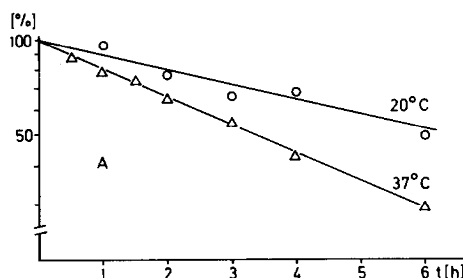


Figure 6
Hydrolysis in fresh human blood at 37°C of triamcinolone acetonide phosphate (A) and dexamethasone phosphate (B).



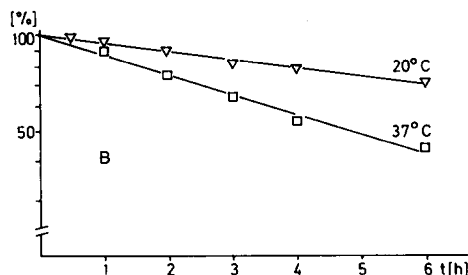


Figure 7
Hydrolysis in fresh human plasma at 20° and 37°C of triamcinolone acetonide phosphate (A) and dexamethasone phosphate (B).

Table 1
In vitro hydrolysis in plasma of water-soluble esters of glucocorticoid alcohols

Substance	Half-life (h) 20°C	37°C
Triamcinolone acetonide phosphate	7.4	3.5
Dexamethasone phosphate	11.7	5.0
Prednisolone hemisuccinate [5]	69.0	11.3
6α-Methylprednisolone hemisuccinate [5]	138.0	22.6

HPLC was the clear separation between the phosphate ester and the glucocorticoid alcohol formed *in vivo*. The necessity to divide the blood samples for the separate analysis of the phosphate ester and of the glucocorticoid alcohol was not a disadvantage since the phosphate esters were hydrolysed *in vivo* so rapidly that only the samples collected within the first 30 min after injection had to be analysed for both ester and alcohol. The samples collected later required only one extraction for the determination of the glucocorticoid alcohols.

In addition to the determination of both the ester and the glucocorticoid alcohol, the simultaneous determination of the endogenous hydrocortisone and the exogenous glucocorticoid alcohols is advantageous. RIA (Gammacoat, Travenol, München, FRG) could not be used for the determination of hydrocortisone in the presence of high concentrations of prednisolone or 6α-methylprednisolone because of cross-reactivity.

For dexamethasone and triamcinolone acetonide the correlation between the results by HPLC and RIA (Fig. 8) show that even in the presence of large quantities of the exogenous glucocorticoid, when the relatively high morning levels of hydrocortisone are initially unaffected, both methods give the same results. Thus no cross-reactivity of the hydrocortisone occurs with dexamethasone and triamcinolone acetonide and the RIA may be used for the hydrocortisone determination even after injection of high doses of these glucocorticoids.

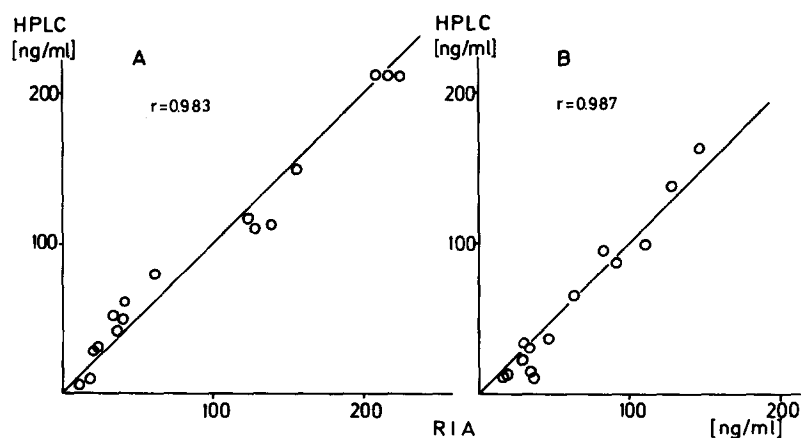


Figure 8
Correlation between HPLC and RIA determinations of hydrocortisone in the presence of dexamethasone (A) and triamcinolone acetonide (B).

Acknowledgements: The skilful assistance of Alwine Schulze-Elfringhoff, Jörg Rehder and Joe Ault is greatly appreciated.

References

[1] D. H. Silh, in *Analytical Profiles of Drug Substances* (K. Florey, Ed.), pp. 615–650. Academic Press, New York (1982).
[2] M. D. Smith, in *Steroid Analysis by HPLC* (M.P. Kautsky, Ed.), pp. 105–144. Marcel Dekker, New York (1981).
[3] V. Das Gupta, *J. Pharm. Sci.* **72**, 1453–1456 (1983).

[4] M. E. Tenneson and T. Cowen, in *Trace-Organic Sample Handling* (E. Reid, Ed.), *Methodological Surveys* (A): *Analysis*, Vol. 10, pp. 218–219. Ellis Horwood, Chichester (1981).
[5] P. Rohdewald, J. Rehder, G. Drehsen, G. Hochhaus, H. Derendorf and H. Möllmann, *J. Pharm. Biomed. Anal.* **3**, 565–573 (1985).
[6] E. R. Garrett, A. van Peer, H. Mahrous and W. Schuermann, *J. Pharm. Sci.* **71**, 387–395 (1982).
[7] A. Albert and E. P. Serjeant, in *The Determination of Ionization Constants*, pp. 28–42. Chapman & Hall, London (1971).
[8] D. Haack and P. Vecsei, *Arzneim-Forsch/Drug Res.* **32**, 832–834 (1982).

[*Received for review 27 September 1984; revised manuscript received 22 February 1985*]