ELSEVIER

Journal of Chromatography B, 674 (1995) 237–246

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

Simultaneous determination of prednisolone, prednisolone acetate and hydrocortisone in human serum by high-performance liquid chromatography

S.A. Döppenschmitt[a,*], B. Scheidel[a], F. Harrison[b], J.P. Surmann[c]

[a]ACC, Analytical Clinical Concepts GmbH, Industriestr. 14–16, 64319 Pfungstadt, Germany
[b]Harrison Clinical Research GmbH, Albrechtstr. 43, 80636 München, Germany
[c]Humboldt-Universität zu Berlin, Math. Nat. I Fak., Institut für Pharmazie, Goethestr. 54, 13086 Berlin, Germany

First received 5 April 1995; revised manuscript received 13 July 1995; accepted 20 July 1995

Abstract

A method for the simultaneous determination of prednisolone, prednisolone acetate and hydrocortisone has been established to monitor the serum levels of these three compounds in healthy volunteers following intramuscular administration of prednisolone acetate. Serum samples of 0.75 ml were extracted with ethyl acetate after addition of the internal standard, dexamethasone. The compounds were separated using a LiChrosorb Si 60 column and detected by UV absorbance. Specificity, linearity, as well as the repeatability, intermediate-precision and accuracy of the method were established. The lower limit of quantification was 2.0 ng/ml for prednisolone (C.V. = 14.7%, $n = 6$) and 5.0 ng/ml for prednisolone acetate (C.V. = 13.9%, $n = 6$) and hydrocortisone (C.V. = 11.7%, $n = 6$). Data on the recovery of the compounds and the internal standard are provided. The results of quality control samples determined during routine analysis ($n = 114$) are presented. Serum levels of the compounds after intramuscular administration of 25 mg of prednisolone acetate are discussed.

Keywords: Prednisolone; Prednisolone acetate; Hydrocortisone
COMPOUND INDEX:

1. Introduction

Prednisolone is a widely used synthetic glucocorticoid with reduced mineralocorticoid side effects compared to the physiological steroid hydrocortisone. The esterification of glucocorticoids, e.g. as prednisolone acetate, leads to prodrugs with modified pharmacokinetic properties [1,2]. One of the general side-effects of the exogenous supply of glucocorticoids is the suppression of the blood levels of endogenous corticoids such as hydrocortisone.

Several HPLC methods for the determination of prednisolone and hydrocortisone have been published [e.g. 3–10]. The lower limits of quantification for prednisolone of these methods ranged from 25.0 to 10.0 ng/ml for UV absorbance detection; one method, using a more

* Corresponding author.

0378-4347/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(95)00317-7

sophisticated derivatization and fluorescence detection was able to detect 3.0 ng/ml [5,7]. None of these methods took prednisolone acetate into consideration.

The aim of this study was to develop an efficient HPLC method for monitoring serum levels of prednisolone acetate, prednisolone and the endogenous steroid hydrocortisone following intramuscular administration of prednisolone acetate. For this purpose it was necessary to improve the limit of quantification for prednisolone in comparison to values cited in the literature. The validation of the method and the quality assurance during routine analysis was carried out according to published papers [11–18].

2. Experimental

2.1. Reagents

Prednisolone and prednisolone acetate were purchased from Sigma (Deisenhofen, Germany), hydrocortisone from E. Merck (Darmstadt, Germany) and dexamethasone, used as internal standard, from Caesar and Lorentz (Hilden, Germany). Methanol, *n*-hexane, dichloromethane were of HPLC grade (Fisons Scientific Equipment, Loughborough, UK). Ethyl acetate and acetic acid 96% were of analytical grade ACS (E. Merck). Nitrogen was of quality 5.0 (Messer-Griesheim, Frankfurt am Main, Germany).

2.2. Instrumentation

The chromatographic system from Jasco (Gross-Umstadt, Germany) consisted of a Pu-980 pump, an 851-AS autosampler and a programmable UV-975 UV–Vis absorbance detector. The separation was performed by a LiChrosorb Si 60, 5 μm, 10 × 4 mm I.D. (Knauer, Berlin, Germany) precolumn and a LiChrosorb Si 60, 5 μm, 250 × 4 mm I.D. (Knauer) analytical column. Data recording was carried out by Chromcard Software (Fisons Instruments, Rodano, Italy).

2.3. Preparation of hydrocortisone-free human serum

For preparation of hydrocortisone-free human serum 250 ml of pooled human serum were shaken for 40 h with pharmaceutical-grade active carbon (Caesar and Lorentz) for the absorption of hydrocortisone. Subsequently the serum was separated by filtration using a glass-sintered filter (porosity 10–16 μm).

2.4. Preparation of calibration standards and quality control samples

To 750 μl of hydrocortisone-free serum 75 μl of a methanolic solution of prednisolone, prednisolone acetate and hydrocortisone (resulting in the serum concentrations given in Table 2) and 100 μl of a methanolic solution of the internal standard (150 ng/100 μl) were added.

2.5. Preparation of serum samples of volunteers

To 750 μl of the individual serum sample 75 μl of methanol and 100 μl of a methanolic solution of the internal standard (150 ng/100 μl) were added.

2.6. Extraction procedure

After addition of 2 ml ethyl acetate the samples prepared as described above were shaken for 10 min and afterwards centrifuged (2500 *g*) for 10 min. A 1.9-ml aliquot of the organic layer was transferred into a glass tube and evaporated under a stream of nitrogen at 45°C. The residue was reconstituted in 100 μl of ethyl acetate. A 17-μl aliquot of this sample was injected into the HPLC system.

2.7. Chromatographic conditions

The mobile phase was *n*-hexane–dichloromethane–methanol–acetic acid (266:120:26:0.8, v/v). For the reproducibility of normal-phase HPLC it is important to obtain a constant content of water in the mobile phase. Therefore each batch of mobile phase was prepared by

mixing one aliquot of mobile phase saturated with water with two aliquots of water-free mobile phase, directly before degassing by sonication for 20 min. The flow-rate was 2 ml/min and the temperature was ambient. The eluate was monitored by UV absorbance at 242 nm (0.005 AUFS).

2.8. Data evaluation and calculations

All calculations were done on an IBM-type personal computer using Excel 4.0 software (Microsoft Corporation, USA).

2.9. Defining assay characteristics

Specificity

To demonstrate the specificity of the method, blank serum was analysed. As recommended [12] serum samples of six different individuals and in addition three pooled serum samples ($n = 5$) were examined during validation. For each individual volunteer a serum sample taken prior to the application of the drug was analysed [12].

Standard curve and linearity

The standard curve was determined on each day of a three-day validation ($n = 3$ at each concentration). The variance over the range of the standard curve was examined using Cochran's test [19], in order to choose the correct algorithm for linear regression (weighted or unweighted). For calculation of the standard curves peak-area ratios (substance: internal standard) were used. For the evaluation of linearity, a plot of concentration versus signal and the relative error (R.E.) of the interpolated concentration of the standards were taken into consideration.

$$\textit{Rel. error (R.E.)} = \frac{\textit{interpolated conc. of standard} - \textit{nominal conc.}}{\textit{nominal conc.}} \cdot 100\%$$

Precision and accuracy

The repeatability (intra-assay precision), the intermediate (inter-day) precision and the accuracy were calculated from data obtained during the three-day validation. Three concentrations were chosen from the high, medium and low range of the standard curve. Serum samples spiked at these three concentrations were analysed each day of three-day validation ($n = 6$ at each concentration). Precision was expressed as the coefficient of variation (C.V.) of the interpolated concentrations. All standard deviations of results obtained from standard curves were calculated considering the rule of error propagation [21]. Accuracy was expressed as the mean relative error (R.E.) of the interpolated concentration of the samples. According to international conference reports, a precision (C.V.) $\leq$15% and an accuracy (R.E.) $\leq \pm$15% are acceptable [11,12].

Limit of quantification (LOQ)

The limit of quantification was determined by repeated analysis of spiked serum samples ($n = 6$). Precision and accuracy of the determination at the limit of quantification were calculated as described above ("precision and accuracy"). Following international recommendations a precision (C.V.) $\leq$20% and an accuracy (R.E.) $\leq \pm$20% are acceptable *at the limit of quantification* [11,12].

Recovery

For prednisolone, prednisolone acetate and hydrocortisone recovery was determined at two concentrations. The recovery of the internal standard was determined at the concentration used in this method (150 ng/ml). Therefore the absolute recovery (A.R.) was calculated using peak areas of extracted serum samples ($n = 6$ at each concentration) and directly injected solutions of the same concentrations:

$$\textit{Absolute recovery (A.R.)} = \frac{\textit{Peak area}_{\textit{extracted analyte}}}{\textit{Peak area}_{\textit{solution of analyte}}} \cdot 100\%$$

2.10. Volunteer samples

During routine analysis, each analytical run consisted of serum samples of volunteers, samples for the calculation of the standard curves

($n = 1$ at each concentration) and quality control samples in the high, medium and low range of the standard curve ($n = 2$ at each concentration).

To test the integrity of an individual run a limit of not more than ± 20% relative error for two of the six quality control samples ("not both at the same concentration") has been applied [12].

For further analysis of the quality control samples of each analytical run the mean of the two results was calculated at each concentration (high, medium, low) and plotted against the "day of analysis" ("mean-plot") [22]. In addition, the range of the two results obtained in each run was calculated and plotted against the "day of analysis" ("range-plot") [22].

3. Results

3.1. Specificity

Chromatograms of a blank individual volunteer sample, a pooled serum spiked with the compounds and a serum sample from a volunteer after the administration of prednisolone acetate are shown in Fig. 1. Chromatograms demonstrate that the compounds of interest could be detected separately from endogenous compounds.

3.2. Standard curve and linearity

During the three-day validation, it was shown that the variance over the range of the standard curve was not homogeneous (heteroscedastic data). So the standard curves had to be calculated using *weighted* linear regression algorithms [20]. Table 1 shows the characteristics of the standard curves during three-day validation. Table 2 gives the interpolated concentrations of the calibration standards and the relative errors (day one of the three-day validation) to demonstrate linearity.

3.3. Precision and accuracy

The values obtained during three-day validation for repeatability, intermediate precision and accuracy are summarised in Table 3. Inter-

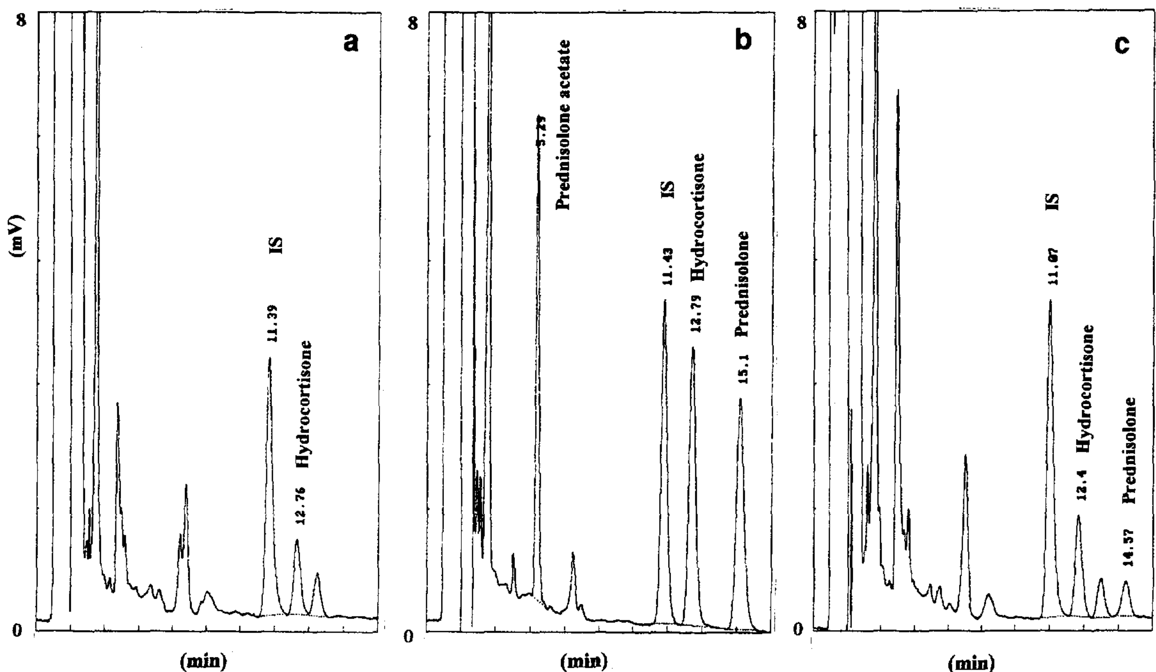

Fig. 1. Chromatograms of (a) a blank individual volunteer sample, (b) spiked standard (200 ng/ml of each compound), (c) individual volunteer sample (3 h after intramuscular administration of prednisolone acetate).

Table 1
Results of the three-day validation for prednisolone, prednisolone acetate and hydrocortisone: slope ± S.D., intercept ± S.D. and coefficient of correlation of the standard curves ($n = 3$ at each concentration, for concentrations see Table 2)

Day	Slope (mean ± S.D.) $(ng/ml)^{-1}$	Intercept (mean ± S.D.)	Correlation coefficient
Prednisolone			
1	0.004941 ± 0.000097	0.002980 ± 0.000436	0.9929
2	0.004776 ± 0.000118	0.004785 ± 0.000561	0.9986
3	0.004666 ± 0.000101	0.005685 ± 0.000515	0.9926
Prednisolone acetate			
1	0.004477 ± 0.000103	−0.004894 ± 0.001325	0.9922
2	0.004536 ± 0.000112	−0.006845 ± 0.001437	0.9911
3	0.004157 ± 0.000069	−0.002169 ± 0.000884	0.9959
Hydrocortisone			
1	0.005536 ± 0.000143	0.007446 ± 0.001837	0.9902
2	0.005481 ± 0.000099	−0.000933 ± 0.001278	0.9951
3	0.005214 ± 0.000129	−0.000178 ± 0.001654	0.9910

Table 2
Concentrations of the standard curves and interpolated concentrations ($Conc_{int.}$) as well as relative error (R.E.) of the interpolated concentrations for prednisolone, prednisolone acetate and hydrocortisone (day one of the three-day validation)

Concentration (ng/ml)	Prednisolone		Prednisolone acetate		Hydrocortisone	
	$Conc_{int.}$ (ng/ml)	R.E. (%)	$Conc_{int.}$ (ng/ml)	R.E. (%)	$Conc_{int.}$ (ng/ml)	R.E. (%)
200.0	213.7	6.8	197.4	−1.3	193.6	−3.2
	197.1	−1.4	193.6	−3.2	194.7	−2.6
	192.5	−3.7	186.3	−6.8	196.5	−1.8
100.0	100.8	0.8	95.0	−5.0	99.4	−0.6
	96.4	−3.6	105.3	5.3	103.3	3.3
	101.1	1.1	100.0	0.0	100.9	0.9
50.0	47.1	−5.7	56.7	13.4	45.4	−9.2
	50.3	0.6	45.3	−9.5	47.5	−5.0
	46.4	−7.2	49.8	−0.3	47.8	−4.5
20.0	21.8	8.8	19.9	−0.7	20.5	2.5
	22.5	12.5	21.7	8.7	22.5	12.4
	21.5	7.3	18.7	−6.7	23.3	16.4
10.0	10.3	3.3	10.4	3.7	10.3	2.9
	10.7	7.5	9.6	−4.0	10.4	4.2
	10.2	2.2	11.3	13.4	8.8	−12.5
5.0	4.5	−9.7	5.2	3.6	4.8	−3.6
	4.3	−14.2	4.8	−4.2	5.5	9.1
	4.2	−16.3	4.7	−6.4	4.6	−8.7
2.0	2.1	6.5				
	1.8	−7.7				
	2.0	−0.4				

Table 3
Results of the three-day validation for prednisolone, prednisolone acetate and hydrocortisone: mean, standard deviation (S.D.) and coefficients of variation (C.V.) for documentation of repeatability and intermediate precision as well as the relative error (R.E.) for documentation of accuracy

Compound	Conc. added (ng/ml)	Day	Repeatability ($n = 6$ at each day)		Accuracy (R.E., %)	Intermediate precision ($n = 18$) (C.V., %)
			$Conc._{interpolated}$ (mean ± S.D.) (ng/ml)	C.V. (%)		
Prednisolone	200.0	1	197.4 ± 5.3	2.7	−1.3	
		2	198.4 ± 4.7	2.4	−0.8	2.5
		3	207.3 ± 4.2	2.0	3.7	
	50.0	1	45.8 ± 3.3	7.2	−8.3	
		2	47.5 ± 1.7	3.6	−5.0	6.7
		3	48.7 ± 4.4	9.0	−2.6	
	10.0	1	11.2 ± 1.10	9.9	11.6	
		2	9.9 ± 0.98	9.9	−0.8	9.6
		3	10.3 ± 1.10	10.7	3.4	
Prednisolone acetate	200.0	1	184.3 ± 4.1	2.2	−7.9	
		2	180.4 ± 7.4	4.1	−9.8	2.7
		3	196.5 ± 4.2	2.1	−1.7	
	50.0	1	49.0 ± 2.2	4.5	−2.0	
		2	50.5 ± 2.4	4.8	1.1	4.8
		3	51.3 ± 2.2	4.2	2.7	
	10.0	1	10.6 ± 1.06	10.0	6.3	
		2	10.8 ± 0.60	5.5	8.3	8.1
		3	10.6 ± 0.85	8.0	5.6	
Hydrocortisone	200.0	1	191.5 ± 1.7	0.9	−4.3	
		2	190.4 ± 6.0	3.2	−4.8	2.3
		3	203.8 ± 4.5	2.2	1.9	
	50.0	1	44.4 ± 1.5	3.3	−11.2	
		2	47.1 ± 0.7	1.5	−5.7	2.5
		3	48.7 ± 1.2	2.5	−2.6	
	10.0	1	9.6 ± 0.84	8.7	−3.9	
		2	10.4 ± 0.82	7.9	3.7	8.7
		3	9.9 ± 0.98	9.9	−1.1	

mediate precision ranged from 2.5 to 9.6% for prednisolone, 2.7 to 8.1% for prednisolone acetate and 2.3 to 8.7% for hydrocortisone (concentration 200–10 ng/ml, $n = 18$ for each). The accuracy was determined as −8.3 to 11.6% for prednisolone, −9.8 to 8.3% for prednisolone acetate and as −11.2 to 3.7% for hydrocortisone, respectively (concentration 200–10 ng/ml, $n = 6$ for each compound).

3.4. Limit of quantification

The limits of quantification determined by repeated analysis were 2.0 ng/ml for prednisolone and 5.0 ng/ml for prednisolone acetate and hydrocortisone. For prednisolone the precision at the lower limit of quantification was determined as C.V. = 14.7% ($n = 6$), for prednisolone acetate it was 13.9% ($n = 6$) and for hydrocortisone 11.7% ($n = 6$), respectively. The mean relative error (R.E.) was −5.3% for prednisolone, −6.8% for prednisolone acetate and −3.3% for hydrocortisone.

3.5. Recovery

The absolute recoveries of prednisolone, prednisolone acetate, hydrocortisone and dexametha-

Table 4
Absolute recoveries of prednisolone, prednisolone acetate, hydrocortisone and dexamethasone (I.S.) ($n = 6$ for each concentration)

Compound	Concentration added (ng/ml)	Absolute recovery (mean ± S.D.) (%)	C.V. (%)
Prednisolone	100.0	89.1 ± 4.3	4.8
	50.0	86.1 ± 0.7	0.8
Prednisolone	200.0	72.6 ± 2.1	2.9
acetate	50.0	75.4 ± 2.4	3.2
Hydrocortisone	100.0	86.6 ± 2.2	2.6
	10.0	88.0 ± 5.4	6.2
Dexamethasone	150.0	94.5 ± 2.4	2.6

sone are listed in Table 4. The C.V. ranged from 0.8 to 6.2%.

3.6. Volunteer samples

About 800 serum samples of volunteers, quality control samples and calibration standards in nineteen analytical runs have been analysed using this method. The results of the quality control samples for prednisolone analysed with each analytical run are shown in Fig. 2. The mean relative error was 4.6% (ranging from −4.9 to 12.6%), −0.5% (−12.5 to 14.0%) and −0.5% (−18.3 to 27.7%) for the high-, medium- and low-concentration range for prednisolone as well as 3.9% (−6.8 to 9.8%), 1.1% (−6.6 to 10.0%) and 4.5% (−16.3 to 24.5%) for hydrocortisone. For prednisolone 81% of the quality control samples ($n = 114$) were in the range of ± 10% of the nominal value, only three were outside ± 20%. For hydrocortisone 89% were in the range of ± 10% ($n = 114$) and only two samples deviated by more than 20% from the nominal value.

No analytical run had to be omitted considering the requirements mentioned above.

4. Discussion

An efficient method for the simultaneous quantitative determination of prednisolone, prednisolone acetate and hydrocortisone in serum of healthy volunteers ($n = 18$) following the intramuscular administration of prednisolone acetate is described.

Liquid–liquid extraction without any washing-steps was used for sample preparation. Various reversed-phase and normal-phase chromatographic systems are described in the literature [3–10]. The main problem of the chromatography is the separation of the chemically very similar compounds prednisolone and hydrocortisone, which differ by only *one double-bound*. In our experiments using various mobile and *reversed*-stationary phases no complete baseline-separation for prednisolone and hydrocortisone could be obtained. So especially at concentrations near the limit of quantification, the chromatographic resolution of prednisolone and hydrocortisone was not sufficient for the precise and accurate quantification of *low* concentrations of prednisolone in the presence of *high* concentrations of hydrocortisone and vice versa. The separation of the compounds of interest from serum components was therefore done by a *normal*-phase HPLC.

The standard curves obtained during three-day validation demonstrate linearity over the ranges of the standard curves. McLean et al. [23] used a "variance stabilising technique" for the calculation of standard curves to improve precision and accuracy in the low-concentration range of the standard curve. This method was applied to the data obtained during three-day validation, but gave no improvement in precision and accuracy compared to the used weighted linear regression method.

Precision and accuracy for the determination

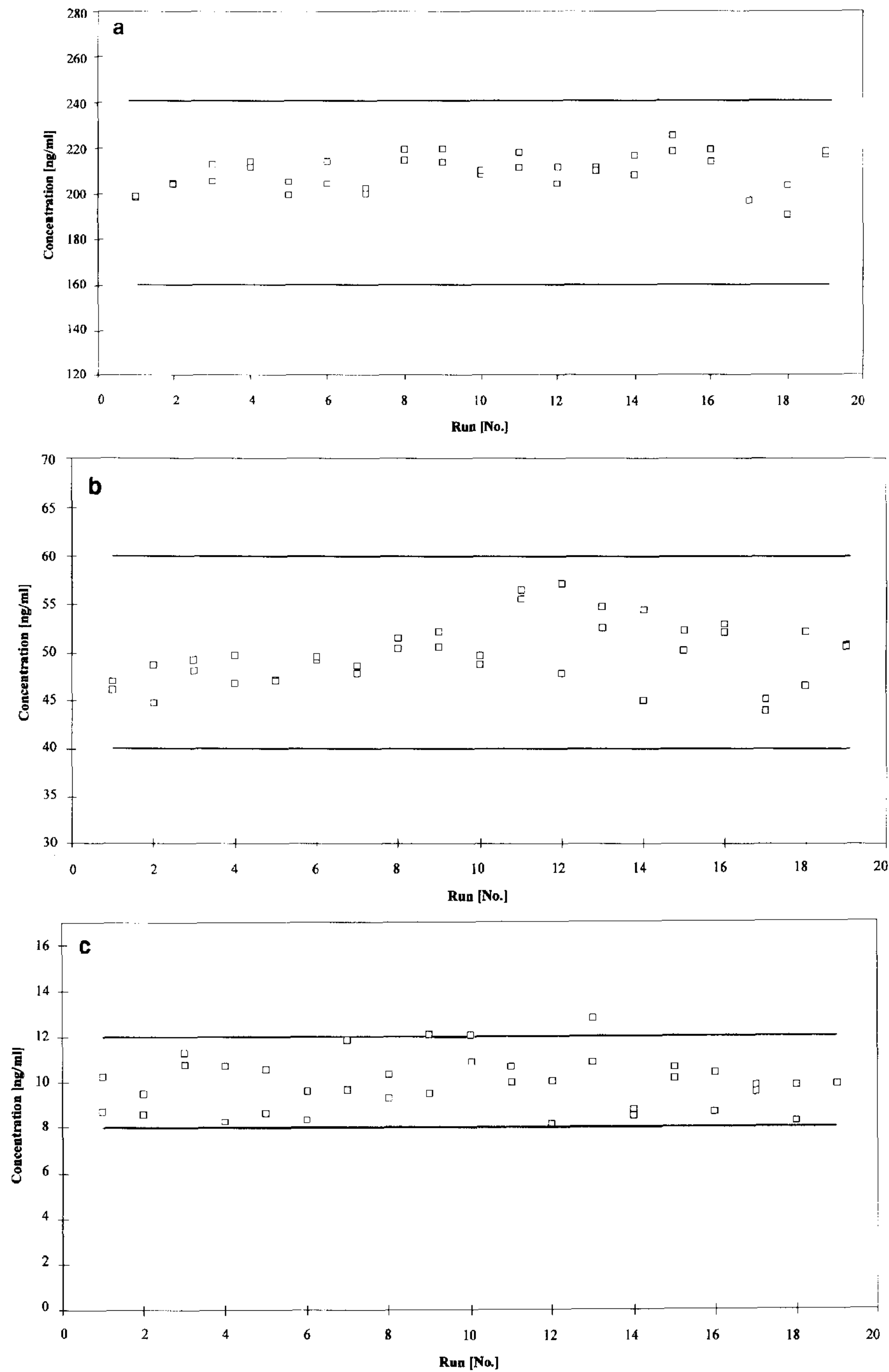

Fig. 2. Individual quality control samples (□) in the (a) high (200 ng/ml), (b) medium (50 ng/ml) and (c) low (10 ng/ml) range of the standard curve for prednisolone during the application to volunteer samples (the lines represent acceptance limits of nominal value ± 20%).

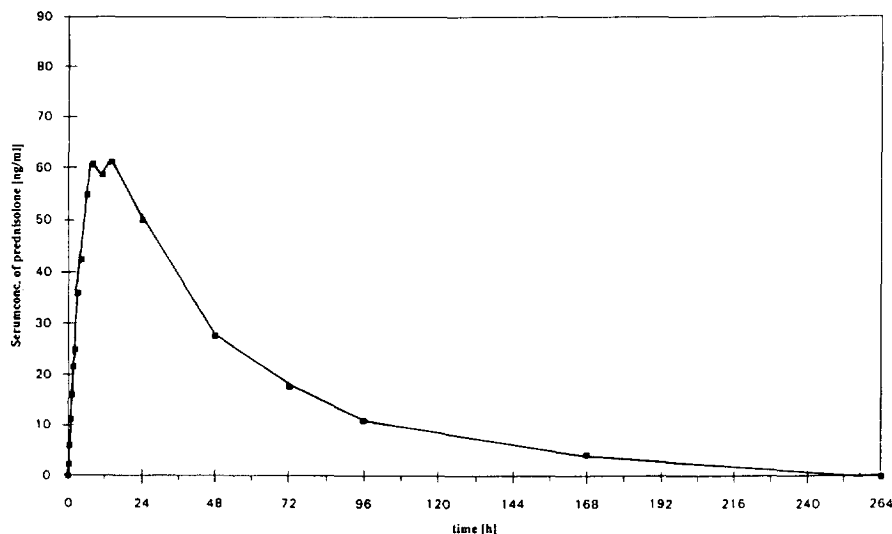

Fig. 3. Serum concentrations of prednisolone after intramuscular administration of 25 mg of prednisolone acetate to a healthy male volunteer.

of prednisolone were comparable or better than those of the methods already described in the literature [3–5].

The limit of quantification for prednisolone (2.0 ng/ml) could be improved 5- to 12.5-fold compared to the published HPLC methods [3–5], which report limits of quantification between 10.0 and 25.0 ng/ml. Precision and accuracy at the limits of quantification of the method presented meet the requirements of current recommendations in bioanalytical method validation [12]. Yamaguchi et al. [5] and Yoshitake et al. [7] published a limit of detection of 3.0 ng/ml, but they stated no values for precision and accuracy of the determination at this concentration.

For each analytical run individual standard curves and quality control samples were prepared as recommended for routine bioanalytical analysis [12,14,17]. Quality control samples were used to accept or reject every run. Acceptance criteria used were taken from published international conference reports on bioanalytical method validation [12,14].

Fig. 3 presents the serum concentrations of prednisolone of a representative volunteer. Maximum serum concentrations of prednisolone (56.5 ± 28.1 ng/ml, mean $\pm$ S.D.) were reached 10.7 ± 4.7 h after administration. Under the conditions of this study, prednisolone acetate was not observed in plasma samples of volunteers. Using this method it was possible to describe the pharmacokinetic properties of prednisolone after intramuscular administration of 25 mg prednisolone acetate in eighteen healthy volunteers and to estimate simultaneously the side-effect of the exogenous glucocorticoids on the serum levels of the physiological glucocorticoid hydrocortisone [24].

References

[1] H. Derendorf, H. Möllmann, A. Grüner, D. Haack and G. Gyselby, Clin. Pharmacol. Ther., 39 (1986) 313.

[2] H. Derendorf, P. Rohdewald, G. Hochhaus and H. Möllmann, J. Pharm. Biomed. Anal., 4 (1986) 197.

[3] W.J. Jusko, N.A. Pyszczynski, M.S. Bushway, R. D'Ambrosio and S.M. Mis, J. Chromatogr. B, 658 (1994) 47.

[4] H. Hirata, T. Kasama, Y. Sawai and R.R. Fike, J. Chromatogr. B, 658 (1994) 55.

[5] M. Yamaguchi, J. Ishida, T. Yoshitake and M. Nakamura, Anal. Chim. Acta, 242 (1991) 113.

[6] J.H. McBride, D.O. Rodgerson, S.S. Park and A.F. Reyes, Clin. Chem., 37 (1991) 643.

[7] T. Yoshitake, S. Hara, M. Yamaguchi and M. Nakamura, J. Chromatogr., 489 (1989) 364.

[8] N.R. Scott, J. Chakraborty and V. Marks, Anal. Biochem., 108 (1980) 266.
[9] J.Q. Rose and W.J. Jusko, J. Chromatogr., 162 (1979) 273.
[10] P. Rohdewald, J. Rehder, G.Drehsen, G. Hochhaus, H. Derendorf and H. Möllmann, J. Pharm. Biomed. Anal., 6 (1985) 565.
[11] H.T. Karnes, G. Shiu and V.P. Shah, Pharm. Res., 8 (1991) 421.
[12] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Jacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D., McDowall, K.A. Pittmann and S. Spector, J. Pharm. Sci., 81 (1992) 309.
[13] V.P. Shah, Clin. Res. Pract. Drug Reg. Affairs, 5 (1987) 51.
[14] A.C. Cartwright, Drug Inf. J., 25 (1991) 471.
[15] ICH Harmonised Tripartite Guideline, Text on validation of analytical procedures, 1994 ICH-Secretary, c/o-IFPMA, Geneva.
[16] CPMP Working Party On Quality Of Medical Products, Note For Guidance, Title: Analytical Validation, Brussels.
[17] A.G. Causey, H.M. Hills and L.J. Phillips, J. Pharm. Biomed. Anal., 8 (1990) 625.
[18] A.R. Buick, M.V. Doig, S.C. Jeal, G.S. Land, R.D. McDowall, J. Pharm. Biomed. Anal., 8 (1990) 629.
[19] H. Bubert and R. Klockenkämper, Fresenius Z. Anal. Chem., 316 (1983) 186.
[20] J.S. Garden, D.G. Mitchells and W.N. Mills, Anal. Chem., 52 (1990) 2310.
[21] L. Aarons, Analyst, 106 (1981) 1249.
[22] J.R. Lang and S. Bolton, J. Pharm. Biomed. Anal., 9 (1991) 435.
[23] A.M. McLean, D.A. Ruggirello, C. Banfield, M.A. Gonzalez and M. Bialer, J. Pharm. Sci., 79 (1990) 1005.
[24] S. de la Motte, B. Scheidel, B. Huber, S. Döppenschmitt, C. Rauch and F. Harrison, Naunyn-Schmiedeberg's Arch. Pharmacol., 351 (1995) R4.