Determination of Prednisolone and Prednisone in Plasma, Whole Blood, Urine, and Bound-to-Plasma Proteins by High-Performance Liquid Chromatography

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Abstract

A high-performance liquid chromatographic technique for the simultaneous determination of prednisolone and prednisone in human plasma, whole blood, urine, and bound-to-plasma proteins, using betamethasone as internal standard, is presented. Liquid–liquid extraction is used for whole blood samples, and solid phase extraction is used for plasma, urine, and proteins bound to plasma. The accuracy, precision, specificity, linearity, and repeatability meet the requirements of current recommendations in bioanalytical method validation. The method is suitable for high altitude pharmacokinetic studies, in which the quantitation of drugs in those fluids is required. The results from healthy volunteers are presented.

Introduction

Prednisone and prednisolone are widely used synthetic corticosteroids in inflammatory conditions, neoplastic diseases, asthma, and as immunosuppressive agents. Prednisone is both a prodrug and a metabolite of the active drug prednisolone (1,2). A number of studies have been published in the literature describing their pharmacokinetics (1–4), and analytical methods to assay both compounds in plasma and urine have been reported (5–7).

High-altitude pharmacokinetic studies are a new and very important field in pharmacology because short- or long-term exposure to a high altitude generates a number of physiological changes that may alter the absorption and disposition of drugs in those circumstances. Acute mountain sickness has become a public health problem and has consequences for people who live and work at high altitudes and also in sport medicine and other groups of interest. Few studies have been published in the literature related to high-altitude pharmacokinetics (8–11). As physiological changes caused by high-altitude exposure may involve all the pharmacokinetic processes, the appropriate characterization of such changes needs to assay the drug in different biological fluids. Quantitation of drugs in plasma, whole blood, urine, and bound-to-plasma proteins (plasma proteins) allows the study of the influence of high altitudes on erythrocyte binding, excretion rate, and unbound drug fraction, all of which are relevant to the clinical effects of drugs. The high-performance liquid chromatography (HPLC) methods currently available are appropriate for measuring prednisone and prednisolone in plasma and urine, but an HPLC method that is simultaneously appropriate for the four different fluids previously mentioned has not been found in the literature. The purpose of this work has been to develop and validate an HPLC method that is easy to apply and serves to determine prednisone and prednisolone in humans in different biological fluids, as is required in pharmacokinetic studies.

Experimental

Apparatus

Liquid chromatograph

The HPLC system consisted of a Model L-6200 A, a Model L-7250 LaChrom autosampler, and a programmable L-4250 UV–vis absorbance detector (Merck, Darmstadt, Germany). Data recording was carried out by D7000-HSM software (Merck).

Column

The separation was performed by LichroCART 250-4 Lichrospher Si 60 (Merck) column (250- × 4.6-mm i.d., 5-µm particle size) and LichroCART 4-4 Si 60 (5-µm particle size) guard column (Merck).

Chemicals

Prednisolone (lot no. S-3908) was purchased from Münnich Pharma Medical (Santiago, Chile). Prednisone (lot no. 210802) and betamethasone (lot no. 601-9), used as internal standard, were donated by Laboratorio Chile (Santiago, Chile) and Laboratorio Bagó (Santiago, Chile), respectively. Acetonitrile, dichloromethane, methanol, and ethyl acetate were of HPLC grade (Merck). Sodium hydroxide and glacial acetic acid were of analytical-grade (Merck). Nitrogen was extra-pure grade 4.5 (Air Liquide, Santiago, Chile).

Analytical sample

Preparation of stock solution, internal standard, calibration standard, and quality control samples

Stock solutions of prednisolone and prednisone were prepared in mobile phase (20 µg/mL). Internal standard solutions of betamethasone were prepared in mobile phase (20 µg/mL). Ultrasonic was used for several minutes in order to accelerate the dissolution. The nominal plasma concentrations of the calibration standard were 100, 250, 500, 750, 1000, and 1500 ng/mL. The nominal concentrations of the calibration standard in urine, whole blood, and plasma proteins were 100, 250, 500, 750, 1000, 1250, and 1500 ng/mL). Three levels of quality control at 100, 500, and 1500 ng/mL were prepared.

Samples Preparation

Procedure to obtain drug bound to plasma proteins

A 1-mL sample of plasma was filtered with an YM 50 Centricon filter (Amicon, Bedford, MA) for 25 min at 2500 *g*; the filtered (plasma–water) was discarded. The biological matrix obtained was named plasma proteins.

Extraction procedure for plasma, urine, and plasma proteins

A 100-µL sample of internal standard solution (20 µg/mL) was added to 500 µL of plasma, 100 µL of urine or 500 µL of plasma proteins prior to solid-phase extraction (SPE). The extraction system consisted of a Lichrolut RP-18 solid-phase column (Merck). Methanol, ethyl acetate, and acetonitrile were used for SPE. Sodium hydroxide (0.2N) was used to wash the eluate after SPE. The SPE column was activated with 1 mL methanol and washed with 1 mL of distilled water. The sample was passed slowly through the SPE column and then washed with 1 mL of distilled water for plasma and plasma proteins and 2 mL for urine. The sample was eluted with 3 mL of acetonitrile for plasma and plasma proteins, and with ethyl acetate (9 mL) for urine. In the case of urine, the eluate was washed with 3 mL sodium hydroxide (0.2N). The samples were evaporated to dryness under nitrogen stream at 37°C. The residue was reconstituted with 300 µL of mobile phase and vortex mixed for 30 s. The solution was filtered through a 0.22 µm GVWP Millipore filter (Bedford, MA).

Extraction procedure for whole blood

A 1.5-mL sample of distilled water was added to 500 µL of whole blood and mixed by shaking. A 100-µL sample of internal standard solution (20 µg/mL) was added to the sample prior to the liquid–liquid extraction. Sodium hydroxide and ethyl acetate were used for liquid–liquid extraction. Ethyl acetate (9 mL) was added and shaken for 15 min. The sample was centrifuged for 10 min at 1800 *g*; the organic phase was removed and washed by shaking with 3 mL of 0.2N NaOH. The organic phase was separated and evaporated to dryness under nitrogen stream at 37°C. The residue was reconstituted with 300 µL of mobile phase and vortex mixed for 30 s. The solution was filtered through a 0.22-µm GVWP Millipore filter.

Chromatographic conditions

Mobile phase

The mobile phase was a mixture of methanol–glacial acetic acid–dichloromethane (1.5:8.0:90.5, v/v/v). The flow rate was 1.8 mL/min and the room temperature fluctuated from 15°C to 20°C. The eluate was monitored by UV absorbance at 254 nm. The injection volume was 100 µL.

Defining assay characteristics

Specificity

To demonstrate the specificity of the method, blanks of plasma, urine, and whole blood were analyzed. As recommended (12), plasma, urine, and whole blood samples from at least six different sources were used during validation. Each blank sample was tested for interfering substances, mainly endogenous matrix components.

Linearity

For the evaluation of linearity, a linear regression model in a standard curve with seven concentrations between 100 ng/mL and 1500 ng/mL and three quality control samples (100 ng/mL, 750 ng/mL, and 1500 ng/mL) was used. In accordance with the Food and Drug Administration's recommendations for evaluation of linearity, a plot of concentration versus signal and the mean relative error (RE) of the interpolated concentration of the quality control standards were taken into consideration.

$$RE = \frac{\text{interpolated concentration of standard} - \text{nominal concentration}}{\text{nominal concentration}} \times 100 \qquad \text{Eq. 1}$$

The following criteria were taken into account to assess linearity: the values for the medium- and high-quality control samples should be within 15% of the actual value, only low quality control samples could be within 20%, four to six quality control samples fulfilled the same criteria and a correlation coefficient ≥ 0.95 was considered adequate (12).

Precision and accuracy

Accuracy and precision for the assay were determinated by calculating the interday variation at three concentrations (100, 750, and 1500 ng/mL) in six replicates for plasma and urine, in five replicates for whole blood, and in three replicates for plasma proteins.

Precision was expressed as the coefficient of variation (CV) of the interpolated concentrations. A CV ≤ 15% for medium and high controls and ≤ 20% for low range is acceptable (12).

Accuracy was expressed as the RE of the interpolated concentration of samples. A RE ≤ 15% for medium and high controls, and ≤ 20% for low range is acceptable (12).

Limit of quantitation

The limit of quantitation (LOQ) was determined by repeated analysis of spiked plasma, urine, whole blood, and plasma protein samples (n = 6). Precision and accuracy were calculated. Following international recommendations a CV ≤ 20% is acceptable at the LOQ (12).

Recovery

The assay recovery for each steroid was determined at three concentrations (n = 3). A minimum of 50% of recovery was used as criteria. The absolute recovery (AR) was calculated using peak areas of extracted biological samples (n = 3 at each concentration) and directly injecting solutions of the same concentrations. AR

was calculated using the same matrix as a reference, which was purified before the addition of the standard solutions.

$$A.R. = \frac{\text{Peak area}_{\text{extracted analyte}}}{\text{Peak area}_{\text{unextracted analyte}}} \times 100 \qquad \text{Eq. 2}$$

Stability of the extracted samples

After the extraction procedure, the spiked samples with known concentrations (100, 750, and 1500 ng/mL) were stored for three months at –20°C and analyzed using the same HPLC method. Quality control samples were compared with a calibration curve prepared freshly, after 1, 2, and 3 weeks, and 1, 2, and 3 months.

Results

Specificity

Figure 1 shows the chromatograms of a blank individual volunteer whole blood sample (A), a blank whole blood sample spiked with prednisone, prednisolone, and betamethasone (B), and a whole blood sample of a volunteer after an oral administration of 80 mg of prednisolone (C). The chromatograms demonstrated that the compounds of interest could be detected separately and without interference with endogenous compounds. Chromatograms of plasma, urine, and plasma proteins showed very similar behavior, with fewer signals from the biological matrix than whole blood. Total time of analysis was 20 min for all the samples.

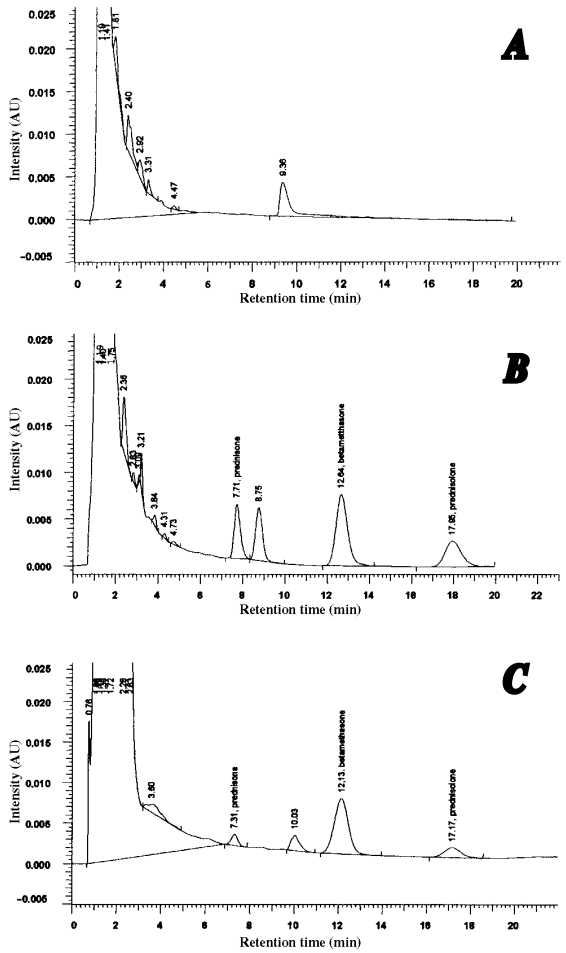

Figure 1. Blank individual volunteer sample of whole blood (A). Whole blood spiked with prednisone, prednisolone, and betamethasone (B). A whole blood sample of a volunteer after an oral administration of 80 mg of prednisolone (Prelone) (C).

Linearity

Table I gives the slopes, intercept, and correlation coefficients for the four biological matrices. Correlation coefficients for plasma, urine, and plasma proteins were 0.99 and 0.97 for whole blood.

Precision and accuracy

Table II shows the values obtained during a 6-day validation for repeatability and accuracy for plasma. The CV for each biological fluid ranged from: 2.1% to 3.5% for prednisone and 3.5% to 4.6% for prednisolone in plasma; from 1.1% to 5.7% for prednisone and 3.8% to 8.9% for prednisolone in urine; from 4.3% to 14.6% for prednisone and 4.7% to 13.5% for prednisolone in whole blood; and from 4.3% to 6.7% for prednisone and 2.8% to 6.9% for prednisolone in plasma proteins.

LOQ

The LOQ determined by repeated analysis was 100 ng/mL for prednisolone for each biological fluid. For prednisolone the precision at the LOQ was determined as CV = 8.3% and for prednisone 5.3%; the mean RE was 13.0% for prednisolone and 17.0% for prednisone in plasma. The CV was 6.0% for prednisolone and 4.3% for prednisone; the mean RE was 17.7% for prednisolone and 18.0% for prednisone in urine. For whole blood, the CV was 11.7% for prednisolone and 10.8% for prednisone; the mean RE

Table I. Linear Correlation Parameters

Drug		Slope	Intercept	Correlation coefficient
Plasma				
Prednisone	Curve 1	3.699×10^{-4}	–0.020	0.9897
	Curve 2	4.077×10^{-4}	0.014	0.9901
Prednisolone	Curve 1	3.337×10^{-4}	–0.021	0.9927
	Curve 2	4.276×10^{-4}	–0.002	0.9949
Urine				
Prednisone	Curve 1	5.363×10^{-4}	–0.019	0.9938
	Curve 2	4.992×10^{-4}	0.063	0.9947
Prednisolone	Curve 1	4.901×10^{-4}	0.006	0.9932
	Curve 2	5.715×10^{-4}	0.072	0.9926
Whole blood				
Prednisone	Curve 1	5.678×10^{-4}	–0.017	0.9841
	Curve 2	4.998×10^{-4}	–0.035	0.9725
Prednisolone	Curve 1	4.467×10^{-4}	–0.047	0.9768
	Curve 2	4.001×10^{-4}	–0.009	0.9816
Plasma proteins				
Prednisone	Curve 1	4.125×10^{-4}	–0.063	0.9957
	Curve 2	4.147×10^{-4}	–0.024	0.9909
Prednisolone	Curve 1	3.941×10^{-4}	–0.061	0.9902
	Curve 2	4.035×10^{-4}	–0.098	0.9894

was 19.4% for prednisolone and 18.2% for prednisone. The CV was 6.3% for prednisolone and 6.0% for prednisone, the mean RE was 16.6% for prednisolone and 15.2% for prednisone in plasma protein.

Recovery

The absolute recoveries of prednisolone, prednisone, and betamethasone are listed in Table III. The CV ranged from 0.9% to 4.3% for plasma, 3.0% to 6.9% for urine, 2.3% to 4.5% for plasma proteins, and 5.6% to 11.3% for whole blood.

Stability

The samples were stable for at least 3 months.

Table II. Results of Interday Precision and Accuracy for Prednisone and Prednisolone in Plasma

Compound	Conc. added (ng/mL)	Day	Conc. calculated (ng/mL)	Mean (ng/mL)	SD	RE (%)	CV (%) (n = 6)
Prednisone	100	1	123.3			23.3	
		2	117.8			17.8	
		3	111.6	117.0	4.1	11.6	3.5
		4	113.7			13.7	
		5	119.1			19.1	
		6	116.3			16.3	
Prednisone	750	1	768.3			2.4	
		2	763.2			1.8	
		3	742.6	757.0	15.8	–1.0	2.1
		4	756.5			0.9	
		5	776.4			3.52	
		6	734.8			–2.0	
Prednisone	1500	1	1578.1			5.2	
		2	1589.7			5.9	
		3	1562.7	1543.0	51.3	4.2	3.3
		4	1571.9			4.7	
		5	1483.1			1.1	
		6	1472.6			–1.8	
Prednisolone	100	1	109.2			9.2	
		2	117.5			17.5	
		3	113.7	113.0	4.8	13.7	4.2
		4	116.4			16.4	
		5	105.3			5.3	
		6	115.9			15.9	
Prednisolone	750	1	732.1			–2.4	
		2	764.9			2.0	
		3	781.3	756.4	34.9	4.2	4.6
		4	698.7			–6.8	
		5	768.6			2.5	
		6	792.7			5.6	
Prednisolone	1500	1	1463.4			–2.4	
		2	1593.7			6.2	
		3	1572.6	1559.3	54.0	4.8	3.5
		4	1531.5			2.1	
		5	1611.1			7.4	
		6	1583.4			5.6	

Volunteer samples

Figure 2 shows the concentration time profiles for plasma and whole blood, and Figure 3 shows the amount of prednisolone and prednisone excreted in urine, both from a healthy volunteer after the administration of 80 mg of prednisolone (Prelone, Asta Medica, Brasil)

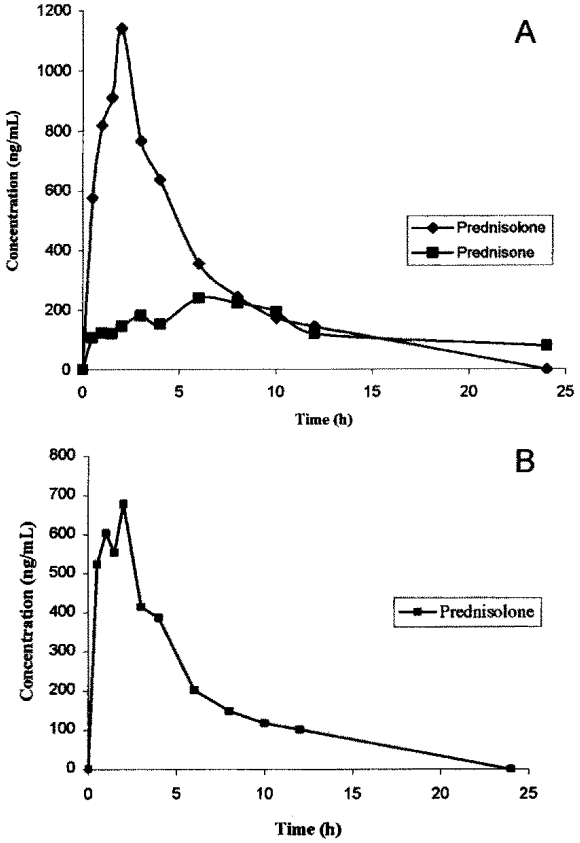

Figure 2. Concentration time profile of prednisolone and prednisone in plasma after the administration of 80 mg of prednisolone (Prelone) oral tablets (A). Concentration time profile of prednisolone in whole blood after the administration of 80 mg of prednisolone (Prelone) oral tablets (B).

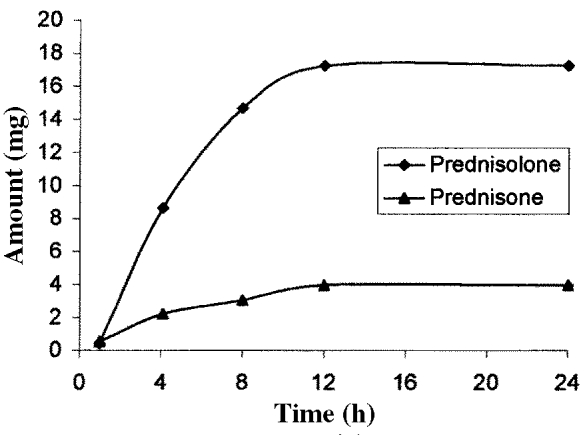

Figure 3. Amount of prednisone and prednisolone excreted in urine after the administration of 80 mg of prednisolone (Prelone) oral tablets.

Discussion

The method for the simultaneous determination of prednisolone and prednisone in plasma, whole blood, urine, and plasma proteins in healthy volunteers following oral administration of prednisolone is shown to be efficient.

Plasma protein binding to some drugs has been reported to be altered in subjects exposed to high altitudes (8). Data on the binding of prednisolone and prednisone to plasma proteins are important to better understand the pharmacokinetics of these drugs. Prednisolone and prednisone have a 90% rate of binding to protein (13). For this reason, it was necessary to develop a method to measure the amount of drug bound to plasma proteins. A Centricon filter (Amicon) was used for concentrated plasma proteins.

SPE was used for plasma, urine, and plasma protein purification, and the results were not appropriated in the case of whole blood samples. Because of the composition of such samples, the cartridges did not allow flow or elution. Liquid–liquid extraction was appropriate in this case.

In order to obtain an adequate separation of prednisone, prednisolone, and betamethasone and other endogenous matrix components, it was very important to maintain the room temperature between 15°C to 20°C. The retention times were 8, 11, and 14 min for prednisone, betamethasone, and prednisolone, respectively.

Precision and accuracy for prednisolone were comparable with other HPLC methods previously described in the literature. The LOQ for prednisolone (100 ng/mL) was higher than in other described methods (5–7). The precision and accuracy at the LOQ, the CV and mean RE were lower than 20%, as recommended in the literature (12)

This method is suitable for pharmacokinetic study purposes and, specifically, in high-altitude pharmacokinetic experiments, in which it is important to assay the drug in whole blood and other biological fluids. Figures 2 and 3 show the concentration-time curves (plasma and whole blood and urinary excretion, respectively) after a single oral dose of 80 mg of prednisolone (Prelone) is given to a healthy volunteer. The maximum and minimal concentrations of prednisolone were reached at 2 and 24 h, respectively. However, the prednisone concentration found in this study was 20% of the concentration of prednisolone. Therefore, prednisone concentration in whole blood was close to the LOQ or undetectable. This prednisolone–prednisone relationship concentration has also been reported by others (4).

Table III. Absolute Recoveries of Betamethasone (IS), Prednisone, and Prednisolone for Each Biological Fluid

Biological fluid	Compound	Concentration added (ng/mL)	Absolute recovery (mean ± SD) (%)	CV (%)
Plasma	betamethasone	100	86.8 ± 2.1	1.2
		750	91.7 ± 1.4	1.5
		1500	90.2 ± 0.8	0.9
	prednisone	100	83.4 ± 3.6	4.3
		750	86.7 ± 2.5	2.9
		1500	84.6 ± 3.7	4.3
	prednisolone	100	85.4 ± 3.3	3.9
		750	81.7 ± 2.1	2.6
		1500	84.2 ±.2.6	3.1
Urine	betamethasone	100	76.3 ± 3.1	4.1
		750	72.9 ± 2.6	3.6
		1500	75.7 ± 2.3	3.0
	prednisone	100	71.6 ± 4.2	5.8
		750	69.8 ± 3.8	5.4
		1500	73.6 ± 4.7	6.3
	prednisolone	100	73.5 ± 5.1	6.9
		750	76.1 ± 3.4	4.5
		1500	79.4 ±.3.9	4.9
Plasma proteins	betamethasone	100	81.3 ± 2.7	3.3
		750	82.4 ± 1.9	2.3
		1500	83.2 ± 2.4	2.9
	prednisone	100	82.7 ± 3.6	4.4
		750	83.6 ± 2.9	3.5
		1500	85.4 ± 3.1	3.6
	prednisolone	100	78.1 ± 2.9	3.7
		750	80.6 ± 3.6	4.5
		1500	84.1 ±.2.7	3.2
Whole blood	betamethasone	100	58.2 ± 3.8	6.5
		750	55.7 ± 3.1	5.6
		1500	57.6 ± 4.2	7.3
	prednisone	100	57.9 ± 5.1	8.8
		750	59.7 ± 3.9	6.5
		1500	60.1 ± 4.3	7.2
	prednisolone	100	56.2 ± 6.4	11.3
		750	61.3 ± 3.7	6.0
		1500	63.9 ± 4.9	7.7

Acknowledgments

This work was supported by Grant 1011072 Fondecyt, Chile. The authors wish to thank Asta Medica, Brazil, for the donated Prelone.

References

1. M.E. Pickup. Clinical pharmacokinetics of prednisone and prednisolone. *Clin. Pharmacokin.* **4:** 111–28 (1979).
2. J.E. Gambertoglio, J.C. Amend, Jr., and L.Z. Benet. Pharmacokinetics and bioavailability of prednisone and prednisolone in healthy volunteers and patients: a review. *J. Pharmacokin. Biopharm.* **8:** 1–52 (1980).
3. S.J. Szefler, W.F. Ebling, J.W. Georgitis, and W.J. Jusko. Methylprednisolone versus prednisolone pharmacokinetics in relation to dose in adults. *Eur. J. Clin. Pharmacol.* **30:** 323–29 (1986).
4. A. Tanner, F. Bochner, J. Caffin, J.Halliday, and L. Powell. Dose-Dependent prednisolone kinetics.

Clin. Pharmacol. Ther. **25(5):** 571–78 (1979).
5. S.A. Döppenschmitt, B. Scheidel, F. Harrison, and J.P. Surmann. Simultaneous determination of prednisolone, prednisolone acetate and hydrocortisone in human serum by high-performance liquid chromatography. *J. Chromatogr. B.* **674:** 237–46 (1995).
6. W.J. Jusko, N.A. Pyszczynski, M.S. Bushway, R. D'Ambrosio, and S.M. Mis. Fifteen years of operation of a high-performance liquid chromatographic assay for prednisolone, cortisol and prednisone in plasma. *J. Chromatogr. B* **658:** 47–54 (1994).
7. V. Garg and W.J. Jusko. Simultaneous analysis of prednisone, prednisolone and their major hydroxylated metabolites in urine by high-performance liquid chromatography. *J. Chromatogr. B* **567:** 39–47 (1991).
8. W.A. Ritschel, C. Paulos, A. Arancibia, M. Pezzani, M.A. Argrawal, K.M. Wetzelsberger, and P.W. Lücker. Pharmacokinetics of meperidine in healthy volunteers after short-and long-term exposure to high altitude. *J. Clin. Pharmacol.* **36:** 610–16 (1996).
9. W.A. Ritschel, C. Paulos, A. Arancibia, M. Pezzani, M.A. Argrawal, K.M. Wetzelsberger, and P.W. Lücker. Urinary excretion of meperidine and normeperidine in man upon acute and chronic exposure to high altitude. *Meth. Find. Exp. Clin. Pharmacol.* **18(1):** 49–53 (1996).
10. W.A. Ritschel, C. Paulos, A. Arancibia, M. Pezzani, M.A. Argrawal, K.M. Wetzelsberger, and P.W. Lücker. Pharmacokinetics of acetazolamide in healthy volunteers after short- and long- term exposure to high altitude. *J. Clin. Pharmacol.* **38:** 533–39 (1998).
11. W.A. Ritschel, C. Paulos, A. Arancibia, M. Pezzani, M.A. Argrawal, K.M. Wetzelsberger, and P.W. Lücker. Urinary excretion of acetazolamide in healthy volunteers after short- and long-term exposure to high altitude. *Meth. Find. Exp. Clin. Pharmacol.* **20(2):** 133–37 (1998).
12. *Guidance for Industry, Bioanalytical Method Validation*. U.S. Department of Health and Drug Administration, Center for Drug Evaluation and Research (CDER), May 2001.
13. J.G. Hardman and L.E. Limbird. *Goodman and Gilman's, The Pharmacological Basis of Therapeutics*, 9th ed. McGraw-Hill Health Professions Division. New York, NY, 1996, pp. 697–701, 1472,1475–81,1475,1774.