Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 9, pp. 761–767, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

A selective LC/RIA for dexamethasone and its prodrug dexamethasone-21-isonicotinate in biological fluids

GÜNTHER HOCHHAUS,*† HARTMUT DERENDORF,† HELMUT MÖLLMANN‡ and JÜRGEN BARTH‡

†*College of Pharmacy, Box J-494, University of Florida, Gainesville, FL 32610, USA*
‡*Medizinische Universitätsklinik und Poliklinik "Bergmannsheil" der Ruhruniversität Bochum, D-4630 Bochum, Germany*

Abstract: A combined LC/RIA procedure is described for the selective determination of dexamethasone (DEX) and its prodrug dexamethasone-21-isonicotinate (DIN) in plasma. The low affinity of the employed dexamethasone antiserum for DIN (cross-reactivity <0.5%) allowed the direct determination of DEX in plasma extracts. For the determination of DIN, both substances of interest were separated by LC, the DIN containing fraction was collected, hydrolysed and the generated DEX was consequently assayed by radioimmunoassay. The assay detection limits were 0.1 ng ml^{-1} for DEX and 0.75 ng ml^{-1} for DIN. For both substances, inter- and intra-day variabilities (RSDs) were 6 and 12%, respectively.

Keywords: *Dexamethasone; dexamethasone-21-isonicotinate; liquid chromatography; radioimmunoassay.*

Introduction

Dexamethasone-21-isonicotinate is a glucocorticoid prodrug that induces a receptor-mediated effect after cleavage of its 21-ester function [1]. It is frequently used in patients with asthma or after acute pulmonary intoxication. While its clinical efficacy is well documented [2–5], pharmacokinetic studies have been limited to investigations in live-stock [6, 7].

A number of analytical procedures, including LC and LC coupled with mass spectroscopy and radioimmunological assays (RIA), have been developed to measure dexamethasone in biological fluids [8–14]. Among these, RIA are most frequently used for measuring dexamethasone [10–13] in the sub-ng ml^{-1} range. However, RIAs are generally not able to differentiate between active drug and prodrugs and are therefore of limited value in pharmacokinetic studies. A sensitive LC/RIA procedure is presented here that allows the selective determination of dexamethasone and dexamethasone-21-ester in biological samples.

Experimental

Materials

Dexamethasone (DEX) and dexamethasone-21-isonicotinate (DIN) were provided by Thomae KG, (Biberach, Germany). 6,7 (N)-[^{3}H]-dexamethasone (43 Ci $mmol^{-1}$) was obtained from New England Nuclear Research (Boston, MA, USA). Two dexamethasone antisera were purchased (antiserum 1: IgG-Incorp., Nashville, TN, USA; antiserum 2: Paesel & Lorei, Frankfurt, Germany). All other chemicals (HPLC or analytical grade) were purchased from Fisher (Springfield, NJ, USA) or Sigma Chemicals (St. Louis, MO, USA). Blank plasma was obtained from a local blood bank.

Instrumentation

The LC system consisted of a Constametric IIIG pump (LDC/Milton Roy, St Petersburg, FL, USA), a Perkin–Elmer model ISS-100 auto-injector (Perkin–Elmer, Norwalk, CT, USA), a C18 reversed phase column (Spherisorb ODSII, Aldrich, 15 μm, 15 cm length, 4.6 mm dia.), a Spectromonitor D variable

*Author to whom correspondence should be addressed.

UV-detector (LDC Milton Roy), and a programmable Gilson model 203 fraction collector (Gilson, Middleton, WI, USA). Organic extracts were concentrated on a N-Evap evaporator (Organomation, South Berlin, MA, USA). A Beckman LS 5000 TD (Beckman Instr., Fullerton, CA, USA) was used for radioactivity measurements.

Standards and sample processing

Stock solutions (1 mg ml^{-1}) of DEX or DIN were prepared in ethanol. Further dilutions were made in blank plasma to yield standards covering concentration ranges relevant for the pharmacokinetic studies (high concentration range for DEX: 0.5–250 ng ml^{-1}; low concentration range for DEX: 0.05–25 ng ml^{-1}; concentration range for DIN: 0.25–75 ng ml^{-1}). DIN standards were spiked with phenylmethylsulphonyl fluoride (PMSF, 2 mM), an esterase inhibitor, and stored in aliquots at −80°C.

For the assay validation, additional blood samples were obtained from healthy volunteers after administration of DIN. These samples were stored at −80°C. Immediately before extraction, the plasma samples were thawed and PMSF added (2 mM).

Extraction

Plasma (500 μl) (standards of samples) was extracted with 1.5 ml of ethyl acetate; the mixture was agitated for 10 min, followed by a 10-min centrifugation at 2000 rpm. After removal of the supernatant, the extraction was repeated and the combined organic phases were evaporated under a stream of nitrogen. The resulting residues were reconstituted in 500 μl of 50% ethanol, vortexed for 1 min, followed by a 5-min centrifugation (Microfuge Model 235 A, Fisher, full speed). Aliquots of the supernatant (250 μl) were used either for the LC separation of DIN and DEX or for the radioimmunological determination of DEX (sample preparation 1). For DEX samples in the high concentration range (see standards and sample processing), it was necessary to dilute the supernatant 10 fold with 50% ethanol before it could be assayed.

The extraction efficiency for DIN and DEX was determined by LC with the system described below. Peak areas of the reconstituted extract were compared with those of controls.

LC

As mobile phase a mixture of water–acetonitrile (1:1, v/v) was used with a flow rate of 1 ml min^{-1} and UV detection at 254 nm. Retention times for DEX and DIN were verified before every experiment using UV detection. Aliquots (200 μl) of the extracted and reconstituted samples (see extraction) were injected onto the column. A 5–8 min window was collected as the DIN fraction.

Cleavage of the ester

The DIN containing LC fraction (3 ml) and 300 μl of 0.01 N NaOH were incubated for 30 min at 40°C. Acetonitrile was removed under a stream of nitrogen (20 min at 40°C), followed by extraction with ethyl acetate (as described above). The evaporated organic phase was stored overnight at −20°C and used in the RIA procedure after reconstitution in 600 μl of ethanol–water (sample preparation 2).

RIA procedure

All determinations were performed in duplicate. Phosphate buffer (450 μl, pH 7.4, 100 mM) was mixed with 100 μl of the antiserum (diluted with phosphate buffer as recommended by the manufacturer), 50 μl of sample preparation 1 or 2 and 100 μl of radioactivity (^{3}H-dexamethasone in PBS, 2000 CPM). The mixture was incubated on ice for 24 h. Bound and unbound radioactivity was separated by charcoal adsorption; 100 μl ice-cold dextran-coated charcoal (0.1% dextran 70, 1% Norit in PBS) was added to the incubation mixture and the suspension was incubated for 5 min at 0–4°C, followed by a 3-min centrifugation at 10,000*g* (Fisher Microfuge). Aliquots of the supernatant (600 μl) were mixed with 4 ml of Scintiverse II, and the radioactivity (CPM) was measured by liquid scintillation counting.

Using the non-linear curve fitting procedure MINSQ (Micromath, Salt Lake City, Utah), displacement curves were fitted to the logistic function:

$$B = T - TC^N(C^N + IC_{50}{}^N) + NS \quad (1)$$

with B, bound tracer in the presence of competitor (CPM); T, total specific binding (CPM in the absence of competitor minus nonspecific binding); C, competitor concentration; N, Hill slope factor; NS, non-specific binding (CPM). By knowing the estimates of T, N, NS and IC_{50} (ng assay $tube^{-1}$), the

concentrations of the unknown (Cx) was consequently derived from:

$$Cx = IC_{50}{}^{N}\left(\frac{100 - \%B}{\%B}\right)^{1/N} DF \qquad (2)$$

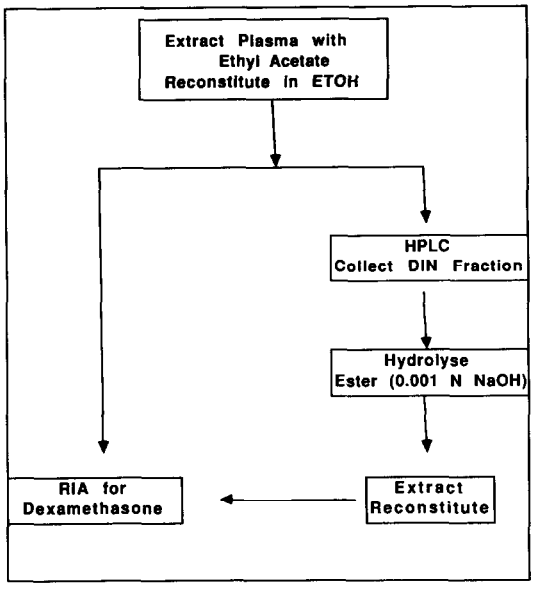
with $\%B$, specific binding in the presence of competitor (expressed as per cent of total specific binding T); and DF, the dilution factor, necessary for the conversion into plasma concentrations.

For the assay characterization, uniform sets of calibrations curves were derived at different days. Raw data were used to determine the relevant binding curve parameters [N, T, IC_{50}, NS, equation (1)]. The same data (CPM) were consequently treated as unknowns and the concentrations were calculated using the above parameters and equation (2). The comparison of theoretical (spiked) and measured values allowed the assessment of sensitivity, working range and reproducibility. The working range of a given assay version was defined as the concentration range for which estimates showed less than 10% inter-day variability. The limit of quantification was defined as the concentration for which the inter-day variability was less than 25%.

Finally, clinical samples covering defined concentration ranges were used to assess intra- and inter-day variability. Here, double determinations were performed on the same (intra-day) or two different days (inter-day). The per cent deviation from the mean and its SD was calculated for all sample pairs and used as indicator for intra- or inter-day variability.

Results and Discussion

The developed LC/RIA procedure for dexamethasone (DEX) and dexamethasone-21-isonicotinate (DIN) involved extraction of samples with ethyl acetate (Scheme 1). Because of the low affinity of the employed antiserum for DIN (see below), one portion of the reconstituted extract could be assayed directly for DEX. For the determination of DIN, both substances were first separated by LC. The DIN containing LC fraction was collected, the ester was cleaved in base, the generated DEX extracted and assayed by RIA. The development and validations steps resulting in the above outlined design are described below.



Scheme 1
In the final assay, plasma samples are extracted with ethyl acetate. One portion of the reconstituted extract is assayed directly for DEX by RIA, while the second is injected onto an LC. The collected DIN fraction is cleaved at basic pH, extracted and the reconstiuted extract is assayed for DEX.

Extraction

The extraction efficiency for DEX and DIN, determined at concentrations suitable for LC analysis (2 μg ml^{-1} for DEX, 6 μg for DIN) was 96.5% for DEX (n = 5) and 97.8% for DIN (n = 5). However, additional data (see below) suggested the possibility of decreased recoveries at lower concentrations.

Stability of DIN in plasma or solvents

To rule out a possible ester cleavage during the assay procedure, the stability of DIN (6 μM) was tested in plasma at room temperature. No degradation of DIN could be detected over a period of 2 h (data not shown). Hence, degradation was of no concern during the thawing and extraction process; especially as PMSF was added as an esterase inhibitor on a routine basis. In contrast, DIN was unstable in a methanol–water mixture (a possible reconstituting solvent) with a significant degradation of about 30% DIN over a period of 72 h. The rate of degradation was significantly reduced to less than 0.5% per 24 h in ethanol–water (1:1, v/v). This mixture was consequently used for reconstituting the ethyl acetate extract.

LC-assay

DIN and DEX were separated by reversed phase LC using a mobile phase (acetonitrile–

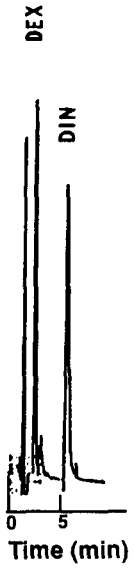


Figure 1
LC chromatogram showing the separation of DEX and DIN.

water; 50:50, v/v) which allowed simple pH adjustment in the post-LC ester cleavage (see Scheme 1). The system gave well resolved peaks and eluted DEX and DIN after 2.5 and 5.5 min, respectively (Fig. 1). To allow automatic over-night processing, a time window of 5–8 min post-injection was selected for the collection of DIN to account for possible fluctuations in the retention times.

Hydrolysis of DIN

The characteristics of the selected antiserum made it necessary to cleave DIN before the radioimmunological procedure (Scheme 1). At acidic pH, the ester cleavage was rather slow and the generated DEX degraded further (data not shown). At basic pH (final concentration: 0.001 N NaOH), DIN was hydrolysed efficiently at 40°C (Fig. 2), while the generated DEX was stable (Fig. 2). A 30 min hydrolysis at 40°C was selected for the final assay procedure.

Antibody binding of DEX and DIN

Two commercially available antisera were tested. While antiserum 1 was reported to have low cross-reactivity to endogenous steroids including hydrocortisone (<0.04%, information sheet of the supplier), tracer binding to antiserum 2 was reduced by ethyl acetate extracts of blank plasma. This allowed a determination of DEX only in diluted samples.

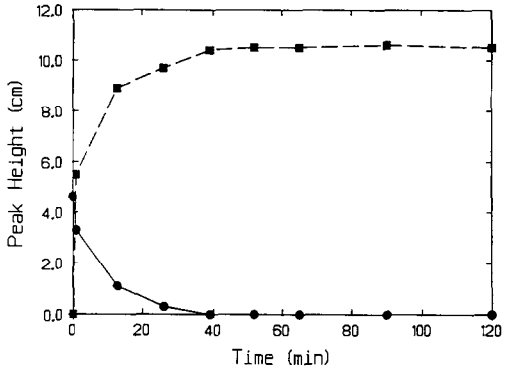


Figure 2
Time course of hydrolysis (5 μg ml^{-1}). DIN was incubated with 0.0001 N NaOH at 40°C and the incubation mixture was assayed by HPLC at the indicated time intervals for DEX (■) and DIN (●).

In addition, plasma extracts were fractionated by LC and 1-min fractions (extracted and reconstituted in PBS) were assayed for immunoreactivity. The major of LC fractions reduced tracer binding to antiserum 2, but not antiserum 1. Hence, antiserum 2 showed cross-reactivities to a number of plasma components. Antiserum 1 was selected for the final assay development.

A typical displacement curve for DEX (no extraction) and antiserum 1 is shown in Fig. 3. Analysis of a set of displacement curves revealed Hill slope factors of close to 1 and consistently high correlation coefficients ($r^2 > 0.995$). Hence, this antiserum displayed the behaviour of a homogeneous population of high affinity binding site and the adequate mathematical model was consequently used as basis for the analysis of the raw data. As

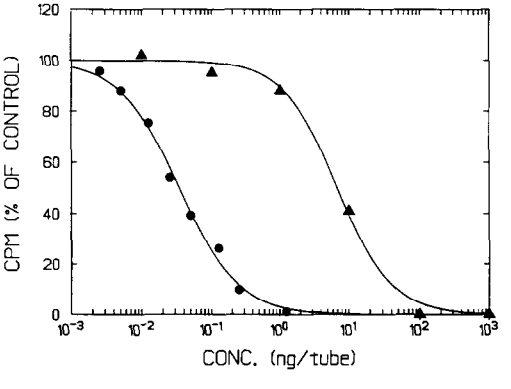


Figure 3
Competitive binding behaviour of DIN (▲) and DEX (●) for antiserum 1. Details of the experimental conditions are described in the Experimental Section. Note that DIN was not hydrolysed.

Table 1
Characteristics of calibration curves*

	DEX† spiked	DEX plasma extracted	DEX plasma extracted	DIN plasma extracted
Concentration range in plasma (ng ml^{-1})	n.a.	0.5–250	0.05–25	0.15–75
IC_{50} (ng tube^{-1})	0.032 (±0.001)	0.041 (±0.003)	0.049 (±0.003)	0.054 (±0.011)
T(CPM)	752 (±85)	709 (±35)	658 (±73)	688 (±73)
NS(CPM)	53.6 (±12.7)	64 (±11)	47 (±10)	62 (±16)
N	1.18 (±0.05)	1.2 (±0.11)	1.1 (±0.03)	1.12 (±0.12)
n	5	5	4	9

*Displacement curve parameters were obtained by non-linear curve-fitting (see Experimental). *n*, number of independent raplicates; *N*, Hill slope factor; *NS*, non-specific binding; *T*, total specific binding; IC_{50}, concentration of competitor, necessary to displace 50% of tracer binding.
†Plasma extracts were spiked with DEX.

Table 2
Determination of DEX in the presence of DIN

	DEX Measured (ng ml^{-1}) in the presence of		
Spiked (ng ml^{-1})	DIN (0 ng ml^{-1})	DIN (10 ng ml^{-1})	DIN (100 ng ml^{-1})
0	0	0.2	1.1
2.5	2.48	2.59	4.0
5	4.87	4.79	7.4
10	10.0	10.7	12.7

Plasma samples containing either 0, 10 or 100 ng ml^{-1} DIN were spiked with different concentration of DEX and were assayed for DEX as described in Materials and Methods. The theoretical (spiked) and measured DEX concentration is given.

indicated in Table 1, calibration curves were highly reproducible under these conditions with an average IC_{50} value of 0.032 ng tube^{-1} and a limit of detection of 10 pg assay tube^{-1}. Hence, the characteristics of antiserum 1 were comparable to, or better than, other characterized antisera [8–13].

In contrast, DIN showed a 200 times lower affinity to antiserum 1 (Fig. 3, IC_{50} value: 6.8 ng assay tube^{-1}). This suggested that DEX can be determined in the presence of moderate concentrations of DIN. For validation, plasma samples containing different concentrations of DEX were spiked with either 0, 10 or 100 ng of DIN. Samples were extracted and assayed by RIA. Table 2 indicates that DIN at a concentration of 10 ng ml^{-1} did not affect the determination of DEX, while the presence of 100 ng ml^{-1} resulted in a significant over-estimation, especially at low DEX concentrations. However, such high concentrations of DIN were not observed in clinical samples (data not shown).

Assay validation

DEX calibration curves obtained under the final assay conditions (including the extraction step) are shown in Fig. 4. IC_{50} values were 30–40% higher than those observed for spiked plasma extracts (Table 1). This might indicate a reduced extraction rate at low DEX concentrations, although a decrease in antiserum affinity during storage represents another possible explanation. However, the binding curve parameters of a given group of displacement curves (IC_{50}, *N*, total and non-specific bind-

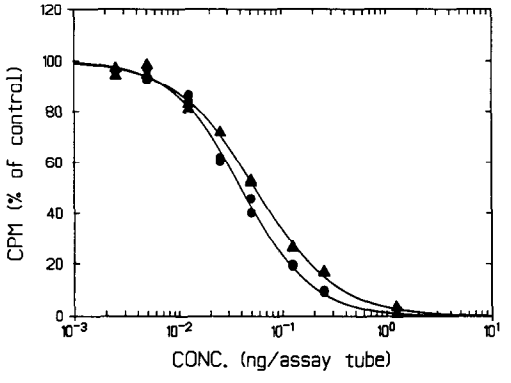


Figure 4
Calibration curves for DIN (▲) and DEX (●) obtained under established assay conditions (see Experimental). The bound radioactivity (CPM, per cent of controls) is plotted against DEX or DIN (ng assay tube^{-1}).

Table 3
Accuracy and precision

DEX (ng ml^{-1})			DIN (ng ml^{-1})*	
Theoretical	Found (RSD)† Low concentration range‡	High concentration range§	Theoretical	Found (RSD)†
0	0 (0)		0	0
0.05	0 (±41%)			
0.1	0.13 (±24%)		0.15	0.25 (±118%)
0.25	0.25 (±6.8%)		0.3	0.25 (±31%)
0.5	0.65 (±11.6%)	0.61 (±47%)	0.75	0.77 (±21%)
1	1.1 (±6.3%)	1.1 (±21%)	1.5	1.53 (±11%)
2.5	2.6 (±9.6%)	2.5 (±17%)	3	3.0 (±9%)
5	4.8 (±7.7%)	5.25 (±7%)	7.5	7.4 (±9%)
10		9.9 (±2%)	15	15.0 (±10%)
25	15.6 (±20.3%)	26.2 (±9%)	75	67 (±48%)
50		47.4 (±6%)		

*The final extract, equivalent to 200 µl plasma, was reconstituted in 600 µl.
†Calibration curve parameters (see Table 1) were used to transform the binding data of the calibration curves (CPM) into DEX concentrations. The comparison of theoretical and calculated values gave some information on accuracy, limit of detection and reproducibility. See Table 1 for number of experiments.
‡500 µl spiked plasma was extracted and reconstituted in the same volume.
§Plasma extracts were diluted 10 fold.

Table 4
Inter- and intra-day variability

	High concentration range	Low concentration range
Intra-day:*		
Concentration range (ng ml^{-1})	1–50	0.1–3
Number of duplicates	190	200
Mean difference from mean	5% ± 3.6%	4.4% ± 3.6%
Inter-day:†		
Concentration range (ng ml^{-1})	1–50	
Number of double determinations	110	
Mean difference from mean	12% ± 11%	

*Duplicates of clinical samples were assayed during 1 day; resulting plasma concentrations were determined and the difference from the mean (expressed in per cent) was calculated.
†Inter-day variability was determined essentially as described above for intra-day variability.

ing), which were generated with the same antiserum stock, were highly reproducible (Table 1).

Using the assay conditions for low DEX concentrations (0.05–25 ng ml^{-1}, see Experimental), the limit of quantification was 0.1 ng ml^{-1} (Table 3, RSD < 25%). The working range was between 0.25 and 5 ng ml^{-1} (Table 3, RSD < 10%). Corresponding results were obtained for higher concentrated samples (Table 3; 0.5–250 ng ml^{-1}). Here, the limit of quantification was 1 ng ml^{-1} (RSD < 25%); the working range was between 5 and 50 ng ml^{-1} (RSD < 10%).

The intra-day variability (RSD) for two subgroups of clinical samples (high and low DEX concentration ranges) was 5 and 4.4%, respectively (Table 4). The average inter-day variability was 12%, a value often found for immunoassays. Hence, the assay is suitable for the determination of DEX in clinical samples.

The method was subsequently applied to the measurement of DEX after oral administration of DIN (18.75 mg). The resulting plasma-concentration–time profile for DEX is shown for one representative healthy volunteer in Fig. 5.

Figure 4 shows a calibration curve for DIN generated under the final assay conditions. Table 1 summarizes the resultant binding parameters. The inter-day variability for most of the displacement curve parameters (N, IC_{50}, T, NS) (Table 1) was slightly higher than for DEX. The average IC_{50} value was 54 pg assay tube^{-1}, which corresponds to an IC_{50} value of 45 pg dexamethasone assay tube^{-1}. Hence, IC_{50} values for DIN were similar to those of DEX (Table 1), despite the complexity of the

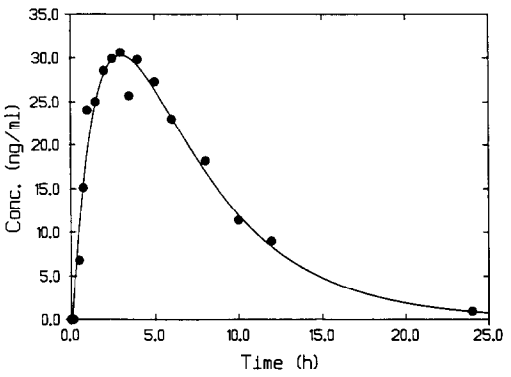


Figure 5
Plasma concentration–time curve for DEX after oral administration of DIN (15 mg). DIN could not be detected under the assay conditions.

DIN assay. The limit of quantification was 0.75 ng ml^{-1} plasma with a working range of 1.5–15 ng ml^{-1}. The average intra-day variation for double determinations (n = 50) was 6.4% (RSD 5.8%).

Application of this method for the determination of DIN after oral administration revealed DIN levels below the limit of quantification (0.75 ng ml^{-1}; data not shown), quite in contrary to resulting DEX levels (Fig. 5). It was not attempted to further increase the assay sensitivity for DIN, as the knowledge of DIN levels at such a low DIN/DEX ratio was not of pharmacokinetic interest.

Conclusions

The LC/RIA procedure allows the sensitive and specific determination of DEX and DIN in plasma at DEX and DIN concentrations as low as 100 and 750 pg ml^{-1}, respectively. The assay is reliable and robust and suitable for processing sample sizes of up to 150 per run. It will be used in this laboratory for the pharmacokinetic characterization of DIN after several modes of administration.

Acknowledgements — This study was supported by Thomae KG, Biberach, Germany. The technical help of Renate Hochhaus, Haneke Schreurs and Christian Barmeyer is acknowledged.

References

[1] G. Hochhaus and H. Möllmann, *J. Barth. Akt. Rheumatol.* **15**, 66–69 (1990).
[2] F. Laumen, *Beitr. Klin. Erforsch. Tuberk. Lungenkr.* **138**, 56–67 (1968).
[3] J. Turiaf, C. Guerot and M. Amsel, *Poumon Coeur* **26**, 769–790 (1970).
[4] S. Makino, T. Miyamoto and Y. Oshima, *Acta Allerrgol.* **27**, 364–371 (1972).
[5] G. Engelhardt, *Arzneimittelforschung/Drug Res.* **37**, 519–523 (1987).
[6] P.L. Toutain, R.A. Brandon, H. de Pomyers and J.D. Baggot, *Am. J. Vet. Res.* **45**, 1750–1756 (1984).
[7] P.L. Toutain, M. Alvinerje and Y. Rukebusch, *Am. J. Vet. Res.* **44**, 212–217 (1983).
[8] H. Derendorf, P. Rohdewald, G. Hochhaus and H. Möllmann, *J. Pharm. Biomed. Anal.* **4**, 197–206 (1986).
[9] S.E. Tsui and J.L. Ashley, *J. Chromat.* **145**, 213–220 (1978).
[10] F. Hoelsboer, D. Haack, A. Gerken and P. Vescei, *Biol. Psychiatr.* **19**, 281–291 (1984).
[11] E.S. Lo, G. Huttinot, M. Fein and T.B. Cooper, *J. Pharm. Sci.* **78**, 1040–1044 (1989).
[12] A.W. Meikle, L.G. Lagerquist and F.H. Tyler, *Steroids* **22**, 193–202 (1973).
[13] C. Lejeune-Lenain, D. Bosson and P. Linkowski, *Clin. Chim. Acta* **179**, 13–22 (1989).
[14] K. Kayganich, J.T. Watson, C. Kilts and J. Ritchie, *Biomed. Environ. Mass Spectrom.* **19**, 341–347 (1990).

[*Received for review 29 May 1991; revised manuscript received 26 June 1991*]