Determination of Dexamethasone in Bovine Liver by Chemiluminescence High-Performance Liquid Chromatography

Y. Iglesias, C. A. Fente,* B. Vázquez, C. Franco, and A. Cepeda

Laboratorio de Higiene e Inspección de Alimentos, Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Universidad de Santiago de Compostela, E-27002 Lugo, Spain

S. Mayo and P. G. Gigosos

Laboratorio de Salud Pública de Lugo, Consellería de Sanidade e Benestar Social, Xunta de Galicia, Lugo, Spain

A new method for the determination of dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) in bovine liver was developed. This new liquid−liquid extraction method comprises the addition of sodium hydroxide to the tissue sample followed by extraction with ethyl acetate. After centrifugation, the extract is evaporated to dryness and the residue dissolved in acetonitrile. The cleaning of the fat is performed with *n*-hexane, and the acetonitrile layer is evaporated. Analysis of the extracts is performed using high-performance liquid chromatography with chemiluminescence detection employing luminol as CL reagent. A series of recovery curves performed at spiking levels of 50, 30, 10, 5, and 2.5 ppb show that at least 80% of DEX can be recovered from liver and that the chemiluminescence detection yields satisfactory results with respect to sensitivity (LOD 0.2 ppb), reproducibility (CV% 10.7) and repeatability (CV% 6.2−8.9).

Keywords: *HPLC; chemiluminescence; dexamethasone; liver; luminol*

INTRODUCTION

Dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione, DEX; Figure 1) is a synthetic glucocorticoid with antiinflammatory and immunosuppressive actions (Ferguson and Hoening, 1995) that has found widespread application in veterinary medicine. Use of this synthetic corticosteroid is limited in the United States to non-food-producing animals (Code of Federal Regulation), and the EEC legislation limit for dexamethasone in bovine, porcine, and equine liver is 2.5 μg/kg (Comission Reglament 749/97). It has also been reported that this compound has been used illegally for increasing animal weight at slaughter (Shearan et al., 1991). The circumstantial evidence of illegal use of DEX in animal production makes it necessary to develop an analytical methodology for this residue in animal tissues.

Up to now methods have been developed for animal tissues, such as HPLC−UV (McLaugghlin and Henion, 1990; Shearan et al., 1991; Mallinson et al., 1995) and gas chromatography−mass spectrometry (Kayganich et al., 1990; Mallinson et al., 1995). Recently, a liquid chromatography−atmospheric pressure chemical ionization mass spectrometry method (Fiori et al., 1998) has been developed for feed additives.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is one of the most commonly used chemiluminescence (CL) reagents. In aqueous alkaline solution, luminol is oxidized to 3-aminophthalate, with the emission of light. The emission is blue, centered at about 425 nm, and arises from excited-state 3-aminophthalate (Baeyens et al., 1991). We know that reducing agents, such as glucose, ascorbic acid, uric acid, and corticosteroids, promote chemiluminescence by reaction with luminol in the presence of a catalyst in alkaline solution (Figure 2). Chemiluminescence (CL) has been employed as a efficacious method in the detection of corticoids (Toriba and Kubo, 1997).

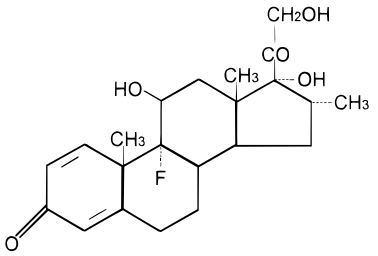

Figure 1. Structure of dexamethasone.

The purpose of this paper is to describe a method that permits rapid, sensitive, and reproducible determination of dexamethasone residues in liver by high-performance liquid chromatography with chemiluminescence detection employing luminol as a CL reagent.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Dexamethasone and luminol were purchased from Sigma (St. Louis, MO). Potassium hexacyanoferrate(II) and -(III), acetonitrile, and hexane (HPLC grade) and ethyl acetate (analytical grade) were supplied by Merck (Darmstadt, Germany). Sodium hydroxide pellets were from AnalaR (Poole, England).

Apparatus. All apparatus listed may be substitued with an equivalent if necessary: Tissue samples were homogenized with a Ultra-turrax, T25 homogenizer; ethyl acetate extracts were evaporated to dryness under nitrogen stream on a Liebisch heating block (Bielefeld, Germany).

* Corresponding author.

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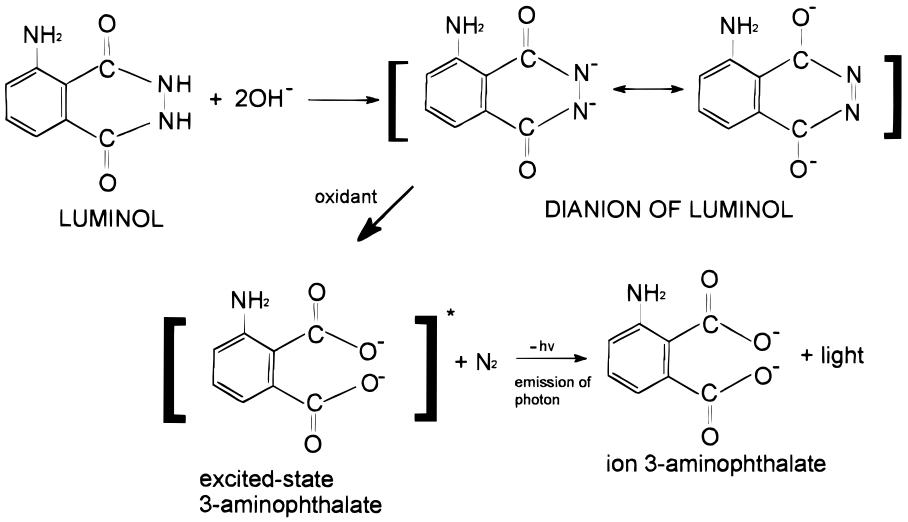

Figure 2. Mechanism for luminol chemiluminescence reaction.

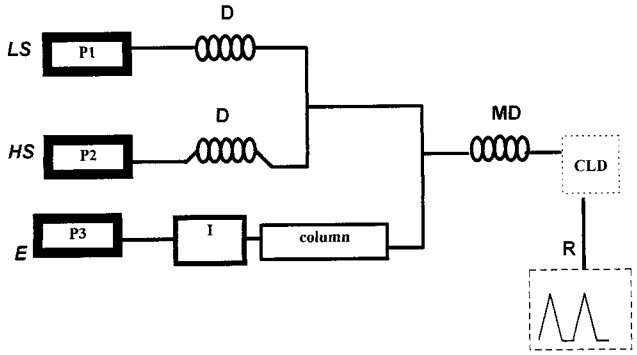

Figure 3. Diagram of chemiluminescence HPLC system. LS: luminol solution. HS: hexacyanoferrate(III) solution. E: eluent. P1, P2, P3: pumps. D: damper. I: injector. C: column. MD: mixer damper. CLD: chemiluminescence detector. R: recorder.

All static emission measurements were carried out at 25 ± 2 °C with a Perkin-Elmer LS 50B model luminescence spectrometer (Norwalk, CT) with CL accessory.

Figure 3 illustrates the flow diagram for a HPLC system, consisting of two reagent delivery pumps, each attached to a damper (P_1 and P_2), so as to achieve flow stabilization, and one mobile phase delivery pump [all pumps were JASCO model 980-PU (Tokyo, Japan)]. The injection device was a Rheodyne injector model 7725 (Cotati, CA), with a 100 μL sample loop, and the column was a Spherisorb ODS (2), 5 μm, 25 × 0.46 cm (Teknokroma, Barcelona, Spain). The column eluate was mixed at the mixing device with the chemiluminescence reagent solutions. The chemiluminescence detector was a Camspec (Cambridge, U.K.) with a flow cell volume of 120 μL and a wavelength range from 320 to 1000 nm.

Static Studies. The luminol solution, prepared in 0.4 M sodium hydroxide solution, contained 1.0 mM luminol and 250 mM hexacyanoferrate(II). On the other hand, hexacyanoferrate(III) solution was 0.2 mM hexacyanoferrate(III) in aqueous solution (Toriba et al., 1997) was the CL reagent. With the static system the percentages were calculated for each of the two CL reagents, so that the CL response was maximum. The reaction kinetics was measured, to estimate the time necessary for reaching the maximum intensity peak when the CL reagents react with dexamethasone.

Chromatographic Conditions. The mobile phase was acetonitrile−water (35 + 65, v/v), carefully degassed for 15 min with helium and filtered prior to use. The CL reagents are the same used in static studies. The flow rate of the mobile phase was 0.8 mL/min, and those of the luminol solution and the hexacyanoferrate(III) solution were 1 and 0.3 mL/min, respectively.

Standard Solutions. Stock standard solutions (0.1 mg/mL) in the mobile phase were prepared monthly and stored at −4 °C in the dark. Working standard solutions (1 μg/mL, 0.1 μg/mL, etc.) were prepared daily in the mobile phase too.

Sample Preparation. Liver samples were stored in a freezer (temperature −15 °C) until the liver was to be analyzed. LC chromatograms of samples stored for longer than 3 weeks may contain more matrix components.

A 5 g sample of liver samples was homogenized with 10 mL of 10^{-3} M NaOH in the ultraturrax, and the homogenate was placed in a 50 mL extraction tube. The samples used in the recovery studies were fortified by the addition of a known concentration of dexamethasone in 500 μL of mobile phase (50, 30, 10, 5, and 2.5 ppb). A 10 mL portion of ethyl acetate was then added to the tube, which was stirred by rotation for 5 min (7 rotations/min) and centrifuged at 2000 rpm for 10 min. The lower aqueous layer was frozen and the upper organic layer poured into a 25 mL extraction tube. The aqueous layer was thawed and re-extracted with an additional 10 mL of ethyl acetate, as described above. The combined extracts (20 mL) were evaporated to dryness at 50 °C under nitrogen and the residue dissolved in 10 mL of acetonitrile and stirred with a vortex mixer. Thereafter, the fat from liver is washed off by addition of hexane (5 mL), in three consecutive times, removing the hexane organic layer in each of them. The acetonitrile layer was evaporated to dryness at 50 °C under nitrogen and the residue dissolved in 200 μL of HPLC mobile phase.

Sample extracts (100 μL) were then injected into the column.

RESULTS AND DISCUSSION

Liver Extraction. The initial step of this extraction procedure disrupts the binding of albumin and α-globulin to dexamethasone (Mallinson et al., 1995). The use of a sodium hydroxide solution in the initial extraction step prevented the introduction of partially soluble matrix components into subsequent extraction steps. The effectiveness of this partition was demonstrated by a comparison of ethyl acetate supernatants of liver samples extracted with different sodium hydroxide solutions. The concentrations of NaOH that were surveyed were 10^{-2}, 2×10^{-3}, 10^{-3}, and 6.6×10^{-4} M.

During the extraction of dexamethaxone in bovine liver, polar and ionic components partition into the bottom aqueous layer (10 mL), and dexamethasone along with other components of the tissue partition into the upper ethyl acetate layer (20 mL), while nonpolar components partition into the hexane layer (15 mL). Other authors such as Shearan et al. (1991) or Mallinson et al. (1995) use an SPE columns method for the

Table 1. Extraction Recovery of Dexamethasone from Liver

concentrated liver (ppb)	*n*	% recovery (mean ± SD)	CV%
50	9	83.2 ± 8.4	10.1
30	9	82.1 ± 9.9	12.1
10	9	84.6 ± 6.7	7.9
5	9	83.7 ± 6.1	7.3
2.5	9	81.6 ± 7.2	8.8

final cleanup of the extracts. For CL-HPLC analysis no further cleanup was necessary. Average liver recoveries with the method proposed in this work over spiking levels ranged from 81.6 to 84.6% (see Table 1), more than the percentage obtained for the other authors for DEX in this tissue (McLaughin and Henion, 1990; Shearan et al., 1991; Mallinson et al., 1995).

Static Measurements. Corticosteroids, such as dexamethasone, yield chemiluminescence with luminol in the presence of a catalyst in alkaline solution (Toriba and Kubo, 1997). Under static conditions, using the LS 50B spectrometer, with a CL accessory, the percentages for each of the luminescent reagents were optimized so that the obtained CL response was maximum. Similar to what Toriba and Kubo (1997) have found, in the presence of hexacyanoferrate(III) as catalyst for the chemiluminescence reaction, the CL intensity is very high, but the background intensity is large too. With the addition of hexacyanoferrate(II) onto the luminol solution, in the presence of hexacyanoferrate(III), chemiluminescence intensity decreases, but the background intensity could be controlled. Therefore, only the proportion of luminol reagent was varied, keeping constant the proportion of catalyst in the final mixture. From the obtained results, it can be deduced that an increase in the percentage of luminol reagent implies an increase of emission intensity, until the proportion of luminol is twice that used by Toriba and Kubo (1997). With larger percentages it is observed that, although the intensity is still maximum, the background noise also increases notably. The best conditions, as referred to the flow to be used in the chromatographic system, were those shown in the Experimental Procedures.

It is necessary to know the time at which the CL reaction reaches a maximum intensity, since that must be the time between the moment of mixing the reagents with the dexamethasone exiting the column and the moment they arrive at the detector. If this is not taken into account, it is most probable that the reaction takes place before or after arrival at the measurement cell. In Figure 4 the CL emission of the studied reaction is plotted versus time. One can observe that the maximum response is obtained 10 s after the mixing of the reagents and the emission ends at 50 s.

Considering the diameter of the stainless steel tubing used and the total flow of eluent after mixing, a tubing distance of 6.9 m was obtained, which corresponds to a time for reaching the measurement cell of 10 s.

Chromatography. The chemiluminescence detection system with luminol in the presence of a catalyst (hexacyanoferrate(III)) in alkaline solution is proposed to be applicable to the determination of DEX in bovine liver without derivatization.

Figure 5 shows that a blank chromatogram of liver extract does not contain any responses that would interfere with the quantitation of DEX. Figure 6 shows the chromatogram of liver extract from an animal treated with DEX prior to sacrifice. The amount of DEX

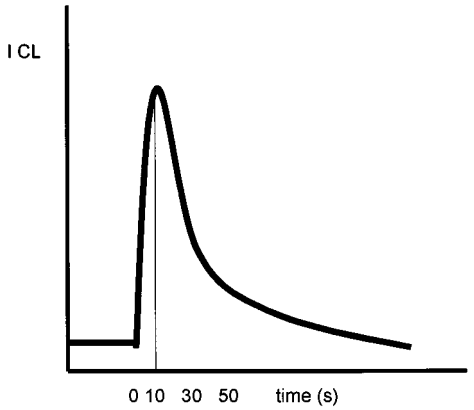

Figure 4. Chemiluminescence reaction emission.

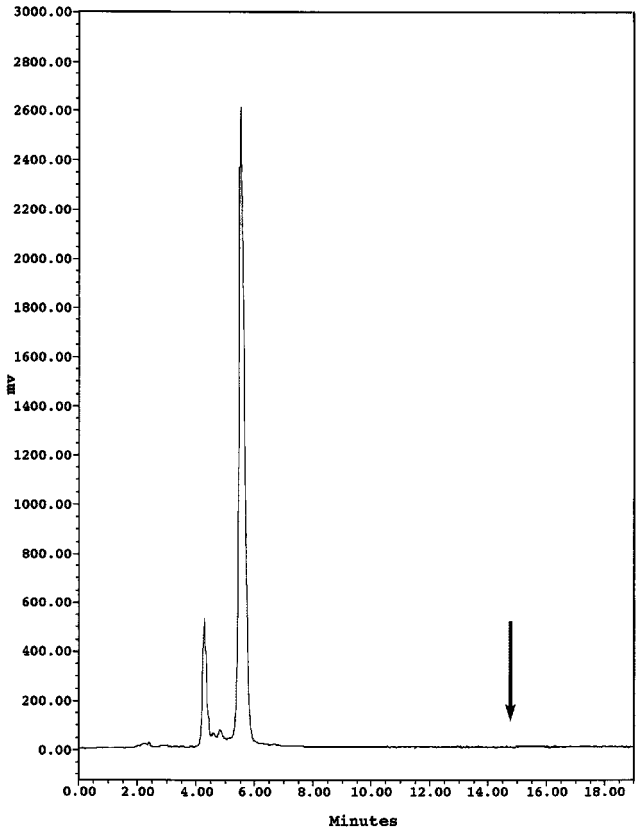

Figure 5. Blank chromatogram of liver extract.

(retention time 14.6 min) in that sample was 5 ppb, which is above the LMR established by the European laws (2.5 ppb) (Comission Reglament 749/97).

Analytical Figures of Merit. The instrumental calibration was made by injecting 150, 100, 30, 10, and 1 ng of DEX into the HPLC system. Five repetitions of each level injection were made for 5 days. Two analysts, using different solutions each day, generated the data. The chromatographic method was demonstrated to be linear from 150 to 1 ng of DEX injected. A linear curve described by the equation $Y = mX + b$, where Y represents the response value of the analyte in the sample (peak area) and X represents the number of nanograms of DEX on the column, was generated for each day. Repeatability (CV% 3.9−6.9) was calculated with the data for each day, and reproducibility (CV% 7.4) with the total data from 5 days (Table 2).

The characterization of the developed method for liver analysis involved the repeated generation, every day,

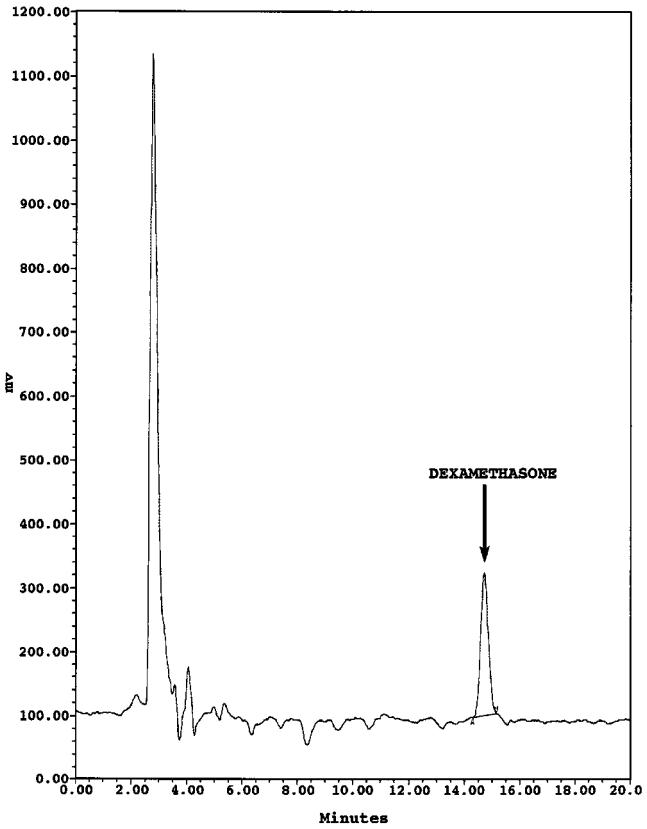

Figure 6. Chromatogram of liver extract from an animal treated with DEX prior to sacrifice.

Table 2. Statistics of the Chromatographic Method for Dexamethasone

day	calibration graph	calibration coefficient	repeatability (CV%)	reproducibility (CV%)
1	$Y = 1251197.6X + 62840.1$	0.996	4.7	
2	$Y = 1215632.9X + 101554.0$	0.999	4.5	
3	$Y = 1300997.9X + 7293.18$	0.998	6.9	
4	$Y = 1257204.1X + 96258.4$	0.997	4.4	
5	$Y = 1245326.2X + 129600.1$	0.993	3.9	
				7.4

Table 3. Statistics of the Method for Dexamethasone in Liver

day	calibration plot	calibration coefficient	repeatability (CV%)	reproducibility (CV%)
1	$Y = 8284.71X + 2316.2$	0.986	8.9	
2	$Y = 9645.7X + 122.13$	0.995	6.2	
3	$Y = 7833.7X + 2514.1$	0.993	8.3	
				10.7

for 3 days, of calibration curves for liver spiked with 50, 30, 10, 5, and 2.5 ppb of DEX. Two analysts, using different solutions each day, generated the data. Table 3 shows the data of calibration curves, repeatability, and reproducibility for the 3 days. The chromatographic method was demonstrated to be linear at the levels assayed. Similar linear curves as described in the preceding paragraph were generated for each day. Repeatability made with the date of each day (CV% 6.2−8.9) and reproducibility made with the date of 3 days (CV% 10.7) seemed to be acceptable.

According to the Official Journal of the European Community (1989) (Annex to council Directive 85/591/EEC, Section 1.27), the limit of detection (LOD) for this analytical method is equal to the mean of the measured content of representative blank samples ($n = 20$) plus 3 times the standard deviation of the mean and is established as 0.2 ppb of DEX in liver. This limit of detection is based on a calculated projection. The lowest spiking level that was detected and quantitated was 1.5 ppb, which is below the LMR (limit maximun of residue) of Reglament (CE) No. 749/97 and also below the LOQ obtained for other authors (Mallinson et al. 1995).

CONCLUSIONS

It has been demonstrated that in a liver sample this procedure can recover 84% of the DEX present in this tissue. The chemiluminescence detection system with luminol is proposed to be applicable to the determination of DEX in bovine liver without derivatization with a quantification limit of 1.5 ppb. This tecnique will be useful only as a screening method for detection of residues in veterinary inspection. For unequivocal identification of the residue it is necessary to use HPLC−MS or GC−MS

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