

DEXAMETHASONE

Contents

I. Determinative Method

A. Introduction	1
B. Equipment	2
C. Reagents and Solutions	4
D. Standards	5
E. Extraction Procedure	6
F. Analytical Quantitation	10
G. Calculations	13
H. Hazard Analysis	14
I. Worksheet	15
J. Quality Assurance Plan	19

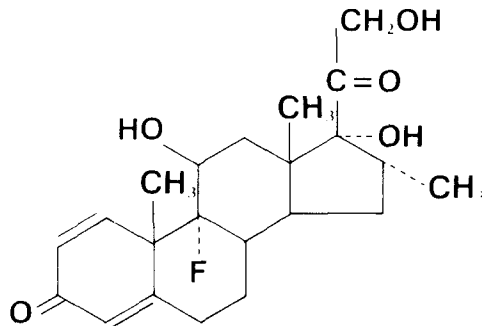
II. Confirmatory Method

A. Introduction	22
B. Equipment	23
C. Reagents and Solutions	24
D. Standards	24
E. Derivatization	25
F. Analytical Confirmation Procedure	27
G. [Reserved]	
H. Hazard Analysis	29
I. Worksheet	30
J. Quality Assurance Plan	33

I. DETERMINATIVE METHOD

A. INTRODUCTION

- 1. Theory and Structure** Liver and muscle tissue are extracted with a mixture of aqueous buffer and acetonitrile. Addition of hexane and dichloromethane to this extract produces a three-phase liquid system that provides a crude separation of compounds into three polarity classes. The layer containing dexamethasone is collected and subjected to coupled-column normal-phase HPLC, which performs further sample clean-up on-line with analytical determination. Time-programmable switching valves control the chromatographic events. The sample is injected onto the first column. For the time dexamethasone is known to elute, the flow from column 1 is diverted to a second column that collects dexamethasone along with any co-eluting matrix components. The contents of column 2 are then backflushed with a stronger eluent onto a third column for the final separation, which isolates dexamethasone. Meanwhile, column 1 is regenerated, i.e., retained polar materials are removed with a more polar eluent. [T. M. P. Chichila, P. O. Edlund, J. D. Henion, *Journal of Chromatography*, Biomedical Applications, 488 (1989)].



Dexamethasone
MW = 392.20

2. Applicability

This method will determine dexamethasone at concentrations above 10 ppb in bovine liver and muscle.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

If necessary, an equivalent may be substituted for all apparatus listed below.

- a. Homogenizer—Waring Blender—VWR Scientific.
 - b. 50 mL screw-cap polypropylene centrifuge tubes—Corning.
 - c. Pasteur disposable pipets, 10 mL.
 - d. Vortex mixer—Fisher Scientific Touch-Mixer.
 - e. Eppendorf pipets.
 - f. Disposable pipets.
 - g. pH meter—Fisher Scientific Model 815MP.
 - h. Roto-Rack—Fisher Scientific.
 - i. Constant temperature water bath (37° C)—American Optical Corp.
 - j. Centrifuges
 - i. Damon IEC Model K, equipped with 12-place angle rotor and capable of achieving a g force up to 4500.
 - ii. Fisher Micro-Centrifuge Model 59A.
 - k. Glass screw-cap test tubes, 25 mm i.d. × 150 mm.
 - l. Scintillation Vials, 20 mL—Wheaton.
 - m. Reacti-Therm Dry Block Heater with custom-drilled blocks—Pierce Chemical Co.
 - n. Volumetric flask, 10 mL.
 - o. Centrifugal filters—Microfilterfuge Tubes, 0.45 μm nylon—Rainin Instrument Co.
 - p. Graduated cylinders, 100 mL and 1 L.
 - q. Autosampler vials—1 mL size with conical bottom.
 - r. Ultrasonic bath—Metler Electronics Corp. Cavitator.
 - s. Disposable centrifuge tubes, 5 mL, screw-cap, with conical bottom—VWR Scientific.
-

I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

2. Instrumentation

-
- a. Perkin Elmer Model ISS-100 autosampler with 50 μ L sample loop.
 - b. Three Waters HPLC pumps. Model 500 and 600A.
 - c. Two Waters air-actuated automatic switching valves.
 - d. A Waters temperature control module to maintain columns 1 and 3 at 30° C.
 - e. A Hewlett-Packard Model 3390A integrator.
 - f. A Waters Model 680 gradient control module to program the pumps, switching valves, and integrator.
 - g. Columns:
 - i. Column 1: Spherisorb phenyl of lot #23/163, 4.6 mm i.d. \times 50 mm, 3 μ m, packed by Keystone Scientific (State College, PA).
 - ii. Column 2: Silica, 4 mm i.d. \times 12.5 mm, 5 μ m, packed by MacMod Analytical (Chadds Ford, PA).
 - iii. Column 3: Spherisorb nitrile, 4.6 mm i.d. \times 100 mm, 3 μ m, packed in-house. With slight adjustment of mobile phase strength, the commercially packed equivalent of this column (same lot not necessary) will provide the same operation.
-

I. DETERMINATIVE METHOD

C. REAGENTS

Reagent List

-
- a. B-Glucuronidase, Type H-2S—Sigma Chemical Corp. Activity: 133,000 units/mL.
 - b. Acetic acid, reagent grade—Fisher Scientific.
 - c. Acetonitrile, dichloromethane, ethyl acetate, hexane, methanol, 2-propanol, sodium acetate, water: HPLC grade—Fisher Scientific.
 - d. 0.04 M sodium acetate buffer: Weigh 5.44 g sodium acetate and dissolve in 1 L of HPLC-grade water.
-

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source	Dexamethasone analytical standard—Sigma Chemical Corporation, St. Louis, MO.												
2. Preparation of Standards	<p>a. Stock standard, 100 $\mu\text{g}/\text{mL}$: Dissolve 10.0 mg of dexamethasone stock standard in 100 mL methanol.</p> <p>b. Intermediate standard, 10 $\mu\text{g}/\text{mL}$ (or 10 $\text{ng}/\mu\text{L}$): Dilute 10 mL of the stock solution in 100 mL volumetric flask, using methanol.</p> <p>c. Fortification standard: Dilute the following standards in ethyl acetate:</p> <table border="1" data-bbox="477 716 1322 884"> <thead> <tr> <th data-bbox="477 716 725 772"><i>mL of Intermediate Standard</i></th> <th data-bbox="806 716 976 772"><i>Final Volume (mL)</i></th> <th data-bbox="1141 716 1322 772"><i>Solution Concentration</i></th> </tr> </thead> <tbody> <tr> <td data-bbox="594 800 608 821">1</td> <td data-bbox="875 800 904 821">10</td> <td data-bbox="1180 800 1278 821">1 $\text{ng}/\mu\text{L}$</td> </tr> <tr> <td data-bbox="594 827 608 848">2</td> <td data-bbox="875 827 904 848">10</td> <td data-bbox="1180 827 1278 848">2 $\text{ng}/\mu\text{L}$</td> </tr> <tr> <td data-bbox="594 854 608 875">3</td> <td data-bbox="875 854 904 875">10</td> <td data-bbox="1180 854 1278 875">4 $\text{ng}/\mu\text{L}$</td> </tr> </tbody> </table> <p>d. HPLC standard, 0.2 $\text{ng}/\mu\text{L}$: Dilute 0.2 mL of intermediate standard solution to 10 mL using HPLC mobile phase solution.</p>	<i>mL of Intermediate Standard</i>	<i>Final Volume (mL)</i>	<i>Solution Concentration</i>	1	10	1 $\text{ng}/\mu\text{L}$	2	10	2 $\text{ng}/\mu\text{L}$	3	10	4 $\text{ng}/\mu\text{L}$
<i>mL of Intermediate Standard</i>	<i>Final Volume (mL)</i>	<i>Solution Concentration</i>											
1	10	1 $\text{ng}/\mu\text{L}$											
2	10	2 $\text{ng}/\mu\text{L}$											
3	10	4 $\text{ng}/\mu\text{L}$											
3. Storage Conditions	All samples must be stored in the freezer at -10°C until ready to use.												
4. Shelf Life Stability	Not known at the present time. Peak signal of the same standard injected at different time has to agree within $\pm 10\%$. If not, prepare new standard.												

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

Sample Extraction

- a. Weigh 5.0 g homogenized beef liver or beef muscle into 50 mL polypropylene centrifuge tube.

NOTE: Tissue should be kept frozen (- 10° C) at all times until assayed.
- b. With 10 mL disposable pipet, add 10 mL of 0.04 M sodium acetate buffer.
- c. Vortex mix for 30 seconds.
- d. Using Eppendorf pipet, adjust pH to 4.2-4.7 with 75 µL glacial acetic acid, swirl, and check with pH meter.
- e. For liver or muscle fortification curve for linear regression analysis, add 100 µL of the following solutions of dexamethasone in ethyl acetate to achieve the desired tissue concentration of dexamethasone. For example, in liver:

<i>Tissue Concentration</i>	<i>Solution Concentration</i>
0 ppb	0 ng/µL
20 ppb	1 ng/µL
40 ppb	2 ng/µL
80 ppb	4 ng/µL

- NOTE: Separate curves are constructed using liver and muscle and only the same kind of tissue samples are analyzed by each curve.
- f. Add 100 µL of B-glucuronidase to each sample. Cap and mix by rotation (7 rotations/minute for 3 minutes on Roto-Rack).
 - g. Incubate for 8 hours at 37° C with gentle shaking in water bath.
 - h. After incubation, add 20 mL of acetonitrile to each tube and mix by rotation. For liver, 7 rotations/minute for 10 minutes. For muscle, 35 rotations/minute for 30 minutes and vortex-mix for 30 seconds.
 - i. Centrifuge samples: liver for 10 minutes at a g force of 3600, muscle for 30 minutes at a g force of 4500.

NOTE: $g \text{ force} = (1.118 \times 10^{-5})(\text{radius of rotation, cm})(\text{RPM})^2$

radius of rotation = distance from center of shaft to furthest inside tip of the tube.

- j. Decant supernatant into glass test tubes (25 mm i.d. x 150 mm).

NOTE: Liquid should pour off quite cleanly from plug, but if unavoidable, a small amount of decanted debris will not interfere.

Stop Point: Samples may be stored at 4° C for as long as 12 hours, perhaps longer. Allow them to return to room temperature before proceeding.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

-
- k. With 10 mL disposable pipets, add 8 mL of hexane and 2 mL of dichloromethane to the supernatant.
 - l. Roto-Rack (7 rotations/minute) for 3 minutes.
 - m. Centrifuge at a g force of 1200 for 4 minutes.
 - n. With 10 mL disposable pipets, transfer 15 mL of the middle layer to a scintillation vial, leaving 1-2 mL of the middle layer behind to avoid contamination by the other layers.
 - o. Evaporate samples to dryness under nitrogen at 60° C.

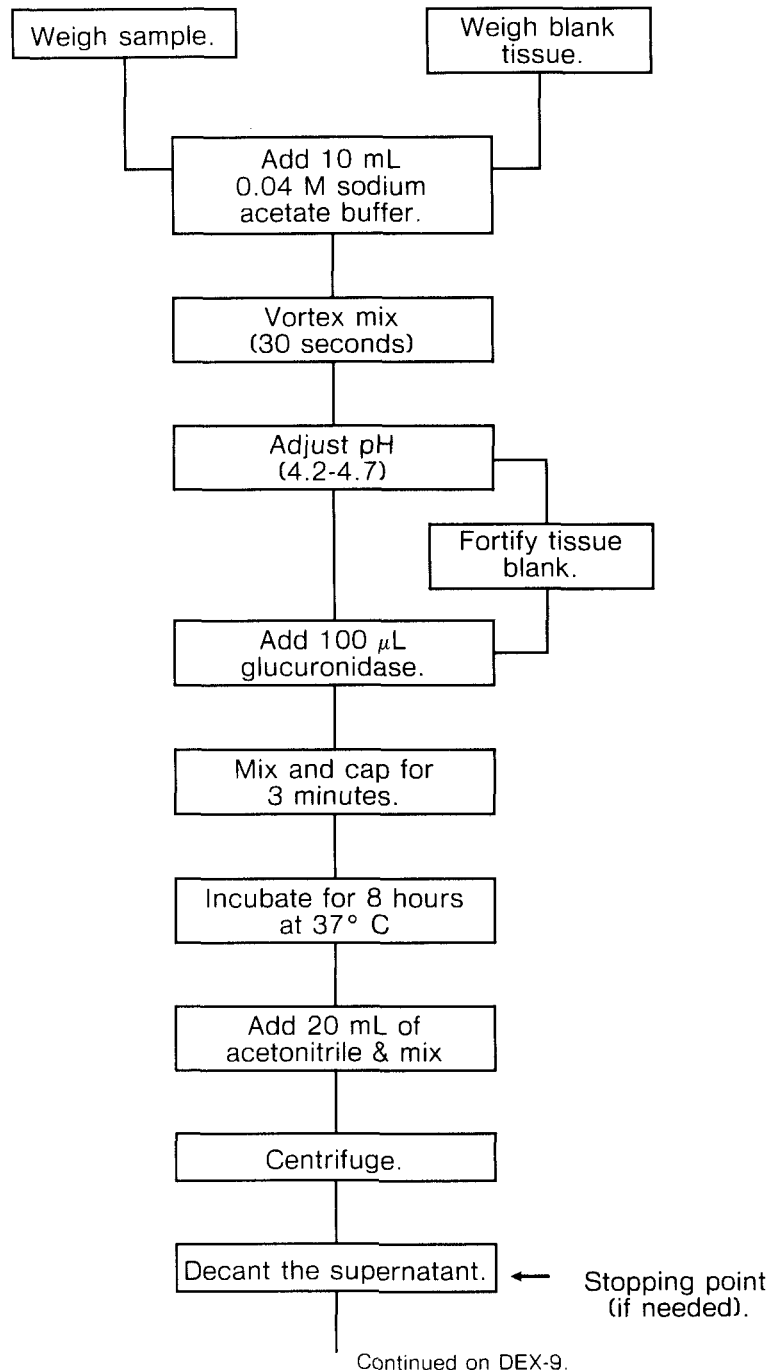
NOTE: All acetonitrile must be removed; this takes 45-60 minutes.

Stop Point: If time permits, proceed to section F. Otherwise, resulting residues can be stored in the freezer. Stability of dexamethasone and matrix components in these residues was not studied, but no degradation was observed after several days of freezer storage.

I. DETERMINATIVE METHOD

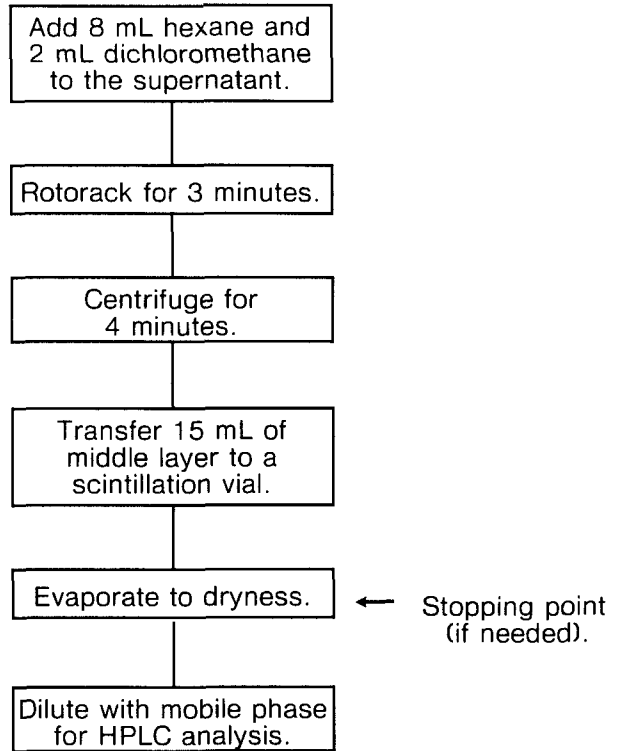
E. EXTRACTION PROCEDURE (Continued)

3. Flow Chart Summary



I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

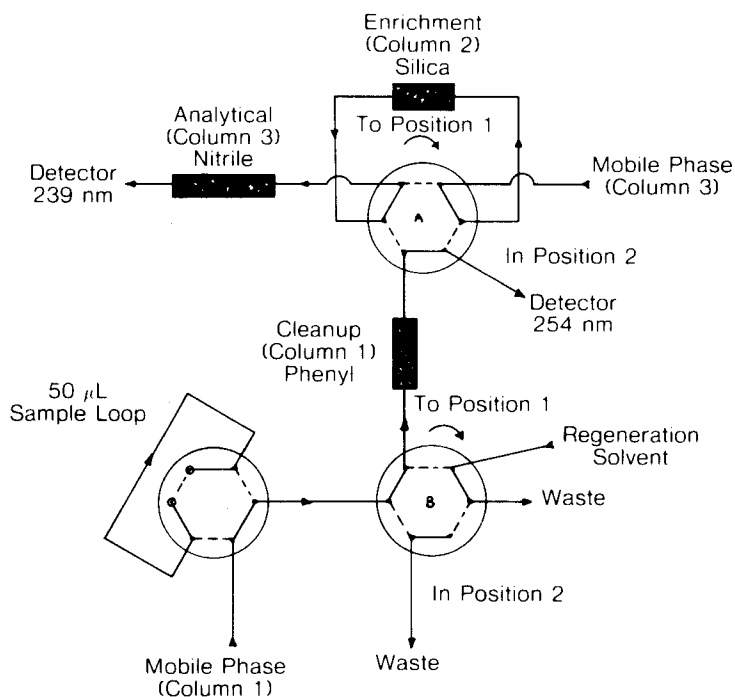


I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTIFICATION

1. Instrumental Settings and Conditions

A schematic of the system is shown in Figure 1.



a. Column-Switching Timetable.

<u>Time (Min)</u>	<u>Valve A</u>	<u>Valve B</u>	<u>Event</u>
0	Position 2	Position 2	Injection.
3.2	Position 1		Begin collection of dex on column 2.
4.1	Position 2		End collection; begin integrator to monitor column 3 separation.
4.2		Position 1	Begin regeneration of column 1.
11.2		Position 2	End regeneration and begin equilibration of column 1.
15.2	Position 2	Position 2	End equilibration; ready for next injection.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTIFICATION (Continued)

b. HPLC Operating Conditions.

i. Mobile phases

- (a) Column 1: 0.1% water, 0.1% acetic acid, 5.8% 2-propanol in hexane (% by volume).
- (b) Column 3: 0.1% water, 0.1% acetic acid, 12.8% 2-propanol in hexane (% by volume).
- (c) Regeneration solvent: 2% water, 49% methanol in dichloromethane (% by volume).

NOTE: Mobile phases were sparged with helium for several minutes before use. For example, with an Eppendorf pipet, 1 mL of acetic acid and 1 mL of water were added to the 100 mL graduated cylinder and brought to 60 mL with 2-propanol. This mixture was transferred to the 1-L graduated cylinder. The smaller cylinder was rinsed with hexane which is poured into the 1-L cylinder and the solution brought to 1 L with hexane. This method provided adequate batch to batch reproducibility, but volumetric flask may be preferred.

ii. Flow rate

1.5 mL/min for analysis on columns 1 and 3; 2.0 mL/min for regeneration of column 1.

iii. Attenuation

0.004 AUFS or as needed.

iv. Detection

Two absorbances detectors. If possible, monitor column 3 at 239 nm (lambda max for dexamethasone); 254 nm is acceptable for column 1.

v. Chart speed

1.0 cm/min

vi. Retention time

Approximately 3.5 minutes on column 1 and approximately 7.6 minutes on column 3. For any given day and batch of mobile phase, the reproducibility of these retention times should be within ± 0.5 minutes. After many days of sample injections, the retention time of dexamethasone on column 1 generally increases. To regain the original retention time, increase the percentage of 2-propanol in column 1 mobile phase.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTIFICATION (Continued)

2. HPLC Analysis

-
- a. For HPLC use, prepare 0.2 ng/ μ L dexamethasone solution by diluting an aliquot of the methanol stock solution with column 1 mobile phase.
 - b. Inject 10 ng of dexamethasone (50 μ L of above solution) to determine the retention time of dexamethasone on column 1.

NOTE: This is done at the start of each day of HPLC analysis and is checked approximately every two hours thereafter during an analysis.

- c. Based on peak observed in step b, program collection window, allowing approximately 0.15 minutes on each side of peak base.

NOTE: The collection window was typically 0.9 minutes long.

- d. Inject 10 ng dexamethasone and, using column-switching program, determine retention time on column 3.

NOTE: One detector monitors column 1 and the other monitors column 3. The appropriate detector must be connected to the recorder before each run. Column 1 is monitored for checking retention time of dexamethasone standard; column 3 is always monitored when samples are injected using switching program.

- e. Prior to injection, dissolve liver and muscle residues in 500 μ L of column 1 mobile phase respectively.
 - f. Cap vials and sonicate for 2 minutes.
 - g. Filter centrifugally, and transfer filtrate to auto sampler vials. Cap the autosampler vials if preparing many samples for an automated run.
 - h. Make duplicate injections of control and fortified tissues and record peak height to construct calibration curve. Before control tissue is injected, a blank (mobile phase) injection is made, though no ghosting was ever observed.
 - i. Make duplicate injections of each sample to be analyzed and record peak height.
 - j. For GC-MS confirmation, collect dexamethasone in 5 mL centrifuge tubes from approximately 0.4 minutes before to 0.4 minutes after its column 3 retention time. Immediately after collection, store tubes in freezer and perform derivatization no more than 24 hours later.
-

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

-
- a. Using data from the control (0 ppb) and fortified tissue samples, construct a least squares linear regression standard curve by plotting fortified tissue concentration against peak height (average from duplicate injection) for the resulting equation, $y = mx + b$.

x = concentration (ppb) of dexamethasone in tissue

y = dexamethasone peak height (average value from duplicate injections)

m = slope

b = y-intercept

- b. Using the established regression line, determine the concentration of dexamethasone in each sample (x) from the average value peak height from duplicate injections (y).

NOTE: For liver, a standard curve was constructed using tissue concentrations of 0, 20, 40, and 80 ppb. The correlation coefficient was typically 0.995.

For muscle, a standard curve constructed using fortified tissue at concentrations of 0, 3, 5, and 10 ppb gave poor quantitative results. Quantification above 10 ppb may be possible but was not evaluated by this method.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination and Confirmation of Dexamethasone in Bovine Tissues		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat, fume hood.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	Methanol Dichloromethane Acetonitrile Hexane Ethyl acetate 2-propanol	Organic solvents are flammable, irritating, and can cause toxic injuries.	Work in fume hood, away from any combustion devices.
	Glacial acetic acid	Combustible, irritating, and can cause toxic injuries.	Work in fume hood, away from any combustion devices.
	B-Glucuronidase		Avoid contact and inhalation.
4. Disposal Procedures	Organic solvents	See above.	Place chlorinated and nonchlorinated solvents in separate labeled waste containers until disposal by in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Dexamethasone Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

This section is only applicable for beef liver matrix. Further study has to be done for the evaluation of muscle.

1. Performance Standard

Analyte	Analytical Range (ppb)	Acceptable Recovery %	Repeatability CV, %
Dexamethasone	20	80-130	≤ 10
	40	80-130	≤ 10
	80	90-110	≤ 10

NOTE: Data for acceptable recovery in muscle tissue is unavailable at the present time.

2. Critical Control Points and Specifications

Record	Acceptable Control
a. Tissue condition	Should be kept frozen until assayed.
b. Sample weight	5.0 ± 0.1 g
c. pH adjusted (using glacial acetic acid)	4.2-4.7
d. B-glucuronidase: volume added	100 µL ± 10 µL
e. Incubation temperature	37° C ± 5° C
f. Evaporation temperature	60° C ± 5° C
g. Evaporation condition	Sample must be evaporated to dryness; all acetonitrile must be removed.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—Duplicate standard curve at each of the following concentration levels on 3 consecutive days:

Solution Concentration	Tissue Concentration
0 ng/µL	0 ppb
1 ng/µL	20 ppb
2 ng/µL	40 ppb
4 ng/µL	80 ppb

- ii. Phase II: Self-fortified samples—Duplicate analyses on fortified samples at 0, 20, 40, and 80 ppb on 3 different days, using beef liver.

NOTE: Phase I and Phase II may be performed concurrently.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iii. Phase III: Check samples for analyst accreditation.

(a) 6 samples submitted by the supervisor with the following specifications:

(i) Matrix—beef liver.

(ii) Fortification level in tissue must be 20-80 ppb.

(b) Report data to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

**4. Intralaboratory
Check Samples**

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst fortified at 20-80 ppb.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on differences between replicates.

(c) All percent recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

I. DETERMINATIVE METHOD**J. QUALITY ASSURANCE PLAN (Continued)****5. Sample Acceptability and Stability**

-
- a. Matrix: Beef liver.
 - b. Sample receipt size: Minimum of 25 g.
 - c. Condition upon receipt: Frozen.
 - d. Sample storage:
 - i. Time: To be determined.
 - ii. Condition: -20° C.
-

6. Sample Set

-
- a. Screening method.

For screening purposes, a sample set can include a blank tissue, a blank tissue fortified at the middle range of the standard curve, and up to 8-12 samples per set.

- b. Quantitation method.

To be used for any samples found positive from the screening method above. Each set must contain:

- i. A blank tissue.
- ii. A fortified standard curve including:
 - (a) 20 ppb.
 - (b) 40 ppb.
 - (c) 80 ppb.

NOTE: Separate curve is needed for different tissue.

- iii. All samples found positive from the screening method.
-

7. Sensitivity

- a. Lowest detectable level (LDL): 10 ppb.
 - b. Lowest reliable quantitation (LRQ): 20 ppb.
 - c. Minimum proficiency level (MPL): not determined.
-

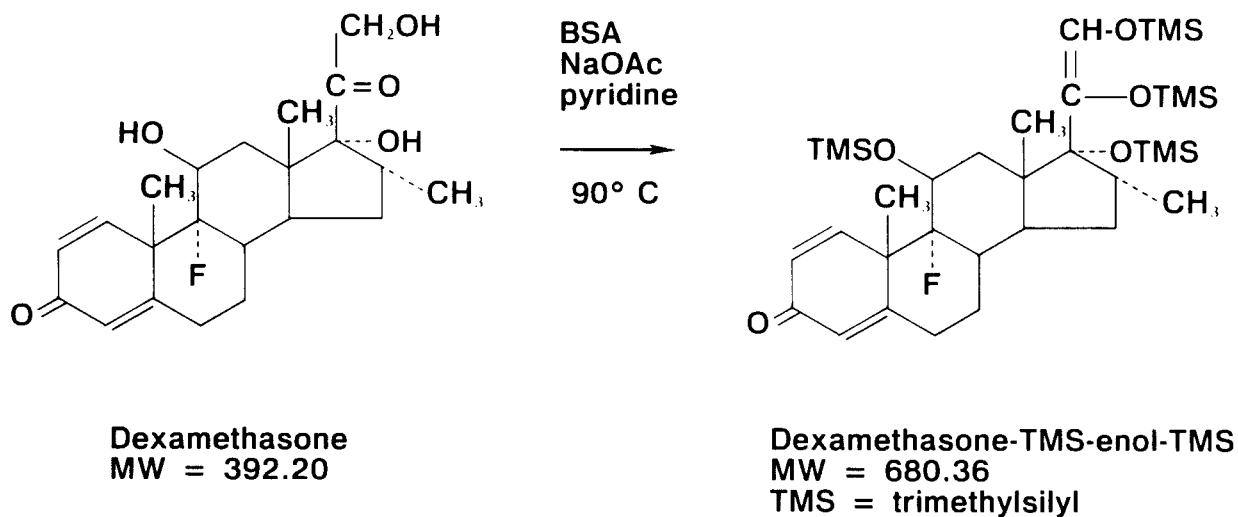
II. CONFIRMATORY METHOD

A. INTRODUCTION

Theory and Structure

The presence of dexamethasone in extracts of bovine liver and muscle obtained by the determinative method can be confirmed by using the HPLC-prepared extract converted to its TMS-enol-TMS derivative with BSA in the presence of sodium acetate and pyridine.

The derivative of dexamethasone is separated by capillary gas chromatography and four ions characteristic of it are monitored by electron ionization mass spectrometry in the selected ion monitoring (SIM) mode. Confirmation is based on retention time and presence of the four selected ions in the correct relative abundances.



II. CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus

An equivalent can be substituted if necessary for all apparatus listed below:

- a. Disposable glass centrifuge tubes, 5 mL, screw cap, with conical bottom—VWR Scientific.
- b. Parafilm—American Can Co.
- c. Syringes for volumetric additions, 10 μ L and 100 μ L—Hamilton Co.
- d. Reacti-Therm Dry Block Heater—Pierce Chemical Co.

2. Instrumentation

-
- a. Gas chromatograph—Carlo Erba Model 5160 Mega Series high resolution GC.
 - b. Mass spectrometer—Hewlett-Packard 5970 Series Mass Selective Detector with direct capillary interface.
 - c. Injector—Scientific Glass Engineering Model OC1-3 On-Column with On-Column Control Module (SGE, Austin, TX).

NOTE: Grob-type heated split-splitless injector may also be suitable but was not utilized in this work.

- d. Syringe for GC injection—5 μ L on column syringe.
 - e. Oxy-Purge nonindicating oxygen trap—Alltech Associates. Helium gas passes through trap before reaching GC column.
 - f. Capillary GC column—J&W Scientific DB-1, 0.25 mm \times 15 m, 0.1 μ m film thickness (Folsom, CA).
 - g. Data station—Hewlett-Packard 59970C Chem Station.
-

II. CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

All reagents listed below can be purchased from an alternate source if available (with equivalent specifications).

- a. N, O-bis-(trimethylsilyl)-acetamide (BSA) in mL ampules—Pierce Chemical Co.
 - b. Acetone—ACS certified Fisher Scientific.
 - c. Pyridine—ACS certified Fisher Scientific.
 - d. Cyclohexane, hydrous sodium acetate ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$).
 - e. Methanol—Fisher Scientific, HPLC grade.
 - f. Solution of hydrous sodium acetate in methanol, 1 $\mu\text{g}/\mu\text{L}$.
-

D. STANDARDS

See Determinative Method, section D.

II. CONFIRMATORY METHOD

E. DERIVATIZATION

1. Procedure

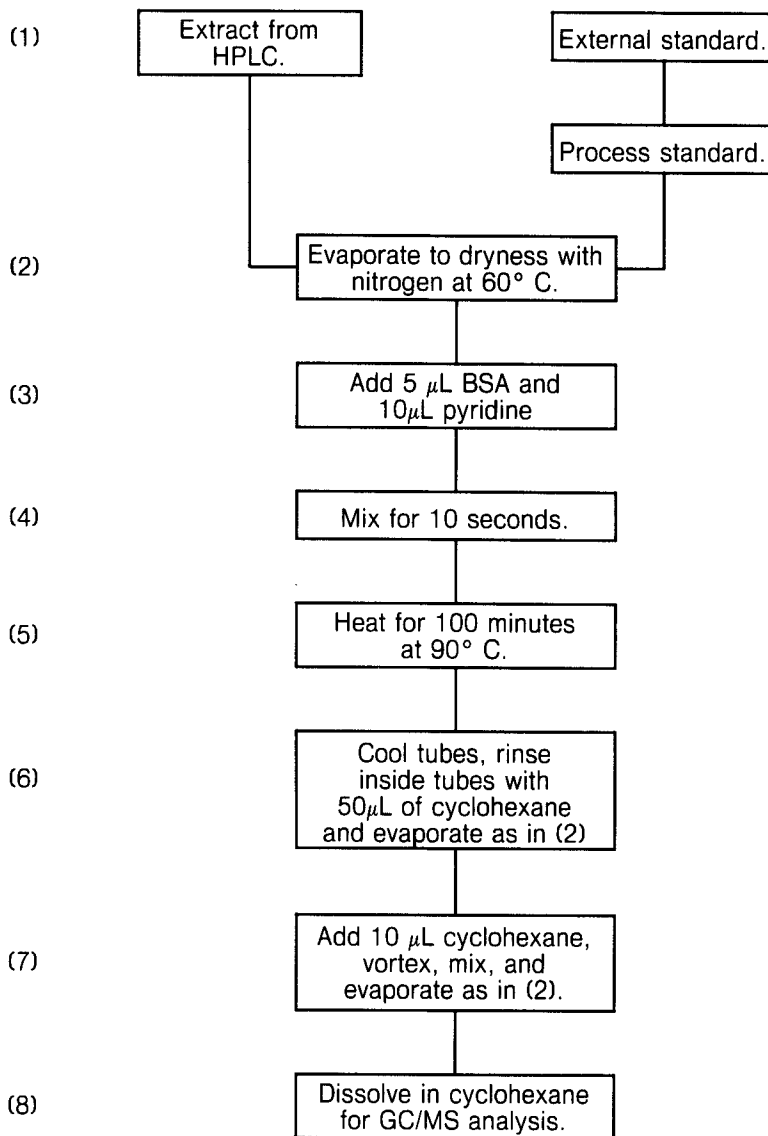
NOTE: Derivatization should be performed within 24 hours of HPLC sample collection in the Determinative Method.

- a. Evaporate the HPLC samples collected in conical centrifuge tubes to dryness under a gentle stream of nitrogen at 60° C.
 - b. For TMS-enol-TMS derivative of dexamethasone standard, place 100 ng (2.55×10^{-10} moles) of dexamethasone (100 μ L of methanol solution) in conical centrifuge tube and evaporate to dryness along with HPLC samples as in step a above.
 - c. Add 10 μ L sodium acetate/methanol solution to each tube, vortex-mix for 10 seconds, and evaporate to dryness as in step a above.
 - d. Add 50 μ L acetone to each tube, vortex-mix for 10 seconds, and heat in 90° C heater block for 100 minutes.
 - e. Add 5 μ L BSA and 10 μ L pyridine, cap vials, vortex-mix for 10 seconds, and heat in 90° C heater block for 100 minutes.
 - f. Allow tubes to cool to room temperature, then rinse condensation inside tubes with approximately 50 μ L of cyclohexane and evaporate to dryness as in step a above.
 - g. Add 10 μ L cyclohexane, vortex-mix for 10 seconds, and evaporate to dryness as in step a above.
 - h. Dissolve dexamethasone-TMS-enol-TMS standard in cyclohexane to obtain concentration desired for GC-MS injection. For instance, add 20 μ L cyclohexane to obtain a concentration of 8.67 ng/ μ L, assuming the derivatization reaction produces 173.49 ng of dex-TMS-enol-TMS from 100 ng of dexamethasone in 100% yield.
 - i. Dissolve sample derivatives in approximately 100 μ L cyclohexane for freezer storage.
 - j. Seal caps with Parafilm and store vials in freezer (-10° C) until time of analysis. Derivative is stable under these conditions for at least a week.
 - k. Prior to GC-MS analysis, evaporate cyclohexane from sample derivatives and redissolve in 3 μ L cyclohexane, all of which will be injected.
-

II. CONFIRMATORY METHOD

E. DERIVATIZATION (Continued)

2. Flow Chart Summary



II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions.

Injection: on-column with oven at 60° C.

Oven temperature program: 60° C for 3 minutes, then programmed to 285° C at 40° C/min, held at 285° C until after dexamethasone-TMS-enol-TMS elutes.

Transfer line: 280° C.

Carrier gas: helium.

Flow rate (carrier gas): approximately 47 cm/second (measured at 285° C).

Retention time: 17.5 minutes (approximately).

b. MS conditions.

Set analyzer parameters with autotune program.

Adjust parameters necessary to meet decafluorotriphenylphosphine (DFTPP) specifications (15 ng in 1 μ L ethyl acetate injected).

Electron energy: 70 eV.

Electron multiplier voltage: 200-400 V above autotune voltage.

Selected ion monitoring parameters: From 12-20 minutes, monitor the following four ions: 305.2, 332.3, 345.2, and 680.5, using a 100 millisecond dwell time for each ion.

2. GC/MS Analysis

- a. Inject standard TMS-enol-TMS derivative in an amount comparable to amounts in suspect samples to be analyzed (i.e., within a factor of five). Make at least two injections; if time permits, more injections will provide better statistics for confirmation. For example, for confirmation in liver samples, 13 ng of dexamethasone-TMS-enol-TMS standard in 1.5 μ L cyclohexane was injected twice.

NOTE: In determining amount of standard to inject, note that yield of TMS-enol-TMS derivative may vary for reaction of standard versus reaction in sample in matrix.

It is probably better to arrange for all standard and sample injection volumes to be the same.

- b. Inject 3 μ L cyclohexane to check for ghosting of dexamethasone-TMS-enol-TMS derivative, as well as column and solvent background. No ghosting was ever observed.
- c. If control tissue samples are to be analyzed, inject them next, after the above solvent blank.
- d. Inject samples dissolved in 3 μ L cyclohexane.
- e. Using integrated peak heights, calculate selected ion ratios relative to the molecular ion for standards and samples. Calculate averages of standard ratios.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

-
- a. All four ions monitored must be present.
 - b. At least three of the four ratios must be within $\pm 20\%$ of corresponding average ratio determined by injections of dexamethasone-TMS-enol-TMS standard at a level comparable to the estimated concentration of the sample.
 - c. The retention time must be within ± 20 seconds of the average retention time of dexamethasone-TMS-enol-TMS standard.
-

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination and Confirmation of Dexamethasone in Bovine Tissues		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat, and fume hood.		
3. Procedure Steps	<u>Hazards</u>	<u>Recommended Safe Procedures</u>	
E. Derivatization			
Pyridine Acetone Methanol Cyclohexane	Organic solvents are flammable, irritating, and can cause toxic injuries.	Work in fume hood, away from any combustion devices.	
BSA *	Combustible, irritating, a possible inhalation hazard, a possible mutagen, and carcinogen suggestive.	Work in fume hood, away from any combustion devices; avoid inhalation and contact. Self-contained organic vapor respirator suggested.	
4. Disposal Procedures	Organic solvents	See above.	Place in labeled storage container until disposal by in-house specialist.
	BSA *	Reacts somewhat with water and reacts rapidly with alcohol.	Avoid mixing with alcohol.

*BSA = N,O-bis-(trimethylsilyl)-acetamide

II. CONFIRMATORY METHOD

I. WORKSHEET

The worksheet on the facing page, *Mass Spectrometry Data Report*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY DATA FORM

Analyte: _____ Tissue: _____

	R.T. (min)	Ion Abundance (m/z)				Ion Ratios			
	Base Peak								
Blank									
Standard									
Fortified Tissue									
Sample #									

Concentration:

Standard: _____

Fortified tissue: _____

Sample: _____

For each set of samples of a particular analyte submitted for confirmation, reference standards and blank and fortified tissue samples for the compound of interest must be included. This guideline applies to each analyte on a daily basis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

-
- a. No false positives at 0 ppb.
 - b. No false negatives at 20 ppb.
-

2. Critical Control Points and Specifications

<i>Procedure Step</i>	<i>Acceptable Control</i>
Derivatization of dexamethasone HPLC extract	Should be performed within 24 hours of HPLC sample collection
Volume BSA added	50 μ L \pm 1 μ L
Volume pyridine added	10 μ L \pm 1 μ L
Heating temperature	90° C \pm 10° C
Heating time	100 minutes \pm 10 minutes

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—By at least 3 replicate injections of standards, show that the instrument is functioning properly.
 - ii. Phase II: Minimum of 3 samples for each type of matrix, with at least 2 positives fortified at 20 ppb.

NOTE: Phase I and Phase II may be performed concurrently.
 - iii. Phase III: 3 check samples for analyst qualification meeting the same requirements as in 3.a.ii.
 - (a) Samples submitted by the supervisor.
 - (b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

- b. Acceptability criteria.

Refer to section F.4.
-

4. Intralaboratory Check Samples

Refer to Determinative Method, section J.4.

5. Sample Acceptability and Stability

Refer to Determinative Method, section J.5.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	Each set must contain a reagent blank, a blank tissue, a blank tissue fortified at 20 ppb, and all samples found positive from the quantification method.
7. Sensitivity	<ol style="list-style-type: none">a. Lowest detectable level (LDL): 20 ppb.b. Lowest reliable confirmation (LRC): 20 ppb.
