Determination of Tritiated Dexamethasone in Rat Liver and Muscle: Comparison of Two Sample Preparation Techniques, Combustion and Solubilization, Prior to Liquid Scintillation Counting

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Rats were injected intramuscularly with 20 μCi/kg [^{3}H]dexamethasone, which is a synthetic therapeutic glucocorticoid. After 2 h, the rats were sacrificed, and liver and muscle tissues were taken for assay. ^{3}H radioactivity measurements were performed in a liquid scintillation counter. The objective of this study was to compare two sample preparation techniques, namely, alkaline solubilization versus dry combustion, to release the incorporated radioactivity quantitatively from rat liver and muscle; sample combustion has a recovery of 99 ± 1.0%. In addition, the effect of bleaching the samples with hydrogen peroxide was evaluated and quench correction curves were established. The current data demonstrated that the efficiencies of both methods were similar in the measurement of [^{3}H]dexamethasone incorporated in rat liver and muscle tissues. For muscle, differences in disintegrations per minute (dpm) reached only 0.18%; for liver, the score was 0.78%. In conclusion, knowledge of the total radioactivity present in rat liver and muscle makes these tissues suitable as standard materials for the determination of [^{3}H]dexamethasone extraction recoveries. The high solubilization efficiency of alkaline solubilization strengthens the notion that this method can be used to release glucocorticoid residues from biological samples.

Keywords: *Dexamethasone; sample preparation; combustion; solubilization; liquid scintillation*

INTRODUCTION

Dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) is a synthetic glucocorticoid used therapeutically to treat primary ketosis and inflammatory diseases. When these glucocorticoids are applied to animals intended for food production, residues of these drugs can be introduced into the food chain. Nowadays, several of these glucocorticoids, for example, dexamethasone, triamcinolone, flumethasone, and prednisolone, are frequently misused as illegal growth promoters to improve zootechnical performances. Recently, for dexamethasone, the European Union established for horses, cattle, and pigs definitive maximum residue limits (MRLs) of 0.3 ppb in milk, 0.75 ppb in muscle and kidney, and 2.0 ppb in liver [Commission Regulation (EC) 1837/97, 1997].

A literature search revealed that all dexamethasone extraction recovery studies from animal tissues (McLaughlin and Henion, 1990) and fluids (Hochhaus et al., 1992) are based upon spiking control tissues with [^{3}H]dexamethasone. No studies were found using incurred tissue samples to determine dexamethasone recoveries. Therefore, laboratory animals (rats) were injected with [^{3}H]dexamethasone. The production of biological tissues wherein [^{3}H]dexamethasone was incorporated in vivo allows these samples to be used as standard material for dexamethasone recovery studies. To be able to calculate recovery percentages, determination of total radioactivity in homogenized tissue samples is necessary.

[^{3}H]Dexamethasone measurements were performed in a liquid scintillation counter. One of the most important steps in the scintillation process is the sample preparation procedure. Various methods such as acid (Bukowski et al., 1992) or alkaline digestion (Huskisson et al., 1982; Sweeney et al., 1996), wet combustion (Amato, 1983), and dry combustion (Kripalani et al., 1975; Saito et al., 1990) have been used to solubilize various types of biological materials. However, because acid digestion (Bukowski et al., 1992) and wet combustion (Kessler, 1989a) use reagents that exhibit a large amount of chemical quenching, counting efficiency is impaired. In addition, these reagents produce chemiluminescence (Bukowski et al., 1992; Kessler, 1989a), which interferes with radioactivity measurements. As such, to count tritiated products in a reproducible and reliable way, quenching and chemiluminescence should be minimized. Therefore, acid digestion and wet combustion were rejected. Dry combustion has a practical advantage (Saito et al., 1990) because it produces

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colorless and homogeneous samples with minimum chemiluminescence and quenching.

Only a few studies compared oxidation with digestion for ^{14}C (Niimi et al., 1979; Whipps et al., 1984). No studies were found comparing results from different sample preparation techniques for tritiated products. The aim of this study was to investigate the performances of alkaline digestion as compared to dry combustion on rat liver and muscle samples for the radioactivity measurement of tritiated dexamethasone. For alkaline sample solubilization, it is known that many factors such as quenching, chemiluminescence, bleaching, and the type of solubilizer influence the results by liquid scintillation counting (LSC). Therefore, it was felt to be necessary to evaluate such factors for alkaline digestion to achieve the same efficiency as via oxidation of the samples. This will enable us to perform further experiments in which alkaline digestion will be used to release glucocorticoids from biological samples.

MATERIALS AND METHODS

Apparatus. A Packard Tri-Carb liquid scintillation analyzer model 1600CA (Canberra Packard, Zellik, Belgium) equipped with a ^{133}Ba external standard was used to measure the amount of β-energy of tritiated products. The transformed spectral index of the external standard (tSIE value) (Kessler, 1989b), which was calculated by the counter, was used as quench parameter. Spiked and incurred samples were counted for 3 and 7 min, respectively. Combustion of the samples was carried out in an updated Packard 306 sample oxidizer (Canberra Packard); collecting recovery for ^3H is 99 ± 1.0% (Kessler, 1989a).

Drugs and Chemicals. [6,7-^{3}H(N)]Dexamethasone (specific activity = 47 Ci/mmol) was purchased from NEN Life Science Products (Brussels, Belgium) and unlabeled dexamethasone from Diosynth (Oss, The Netherlands). Ultima Gold (scintillation fluid), Solvable and Soluene-350 (solubilization fluids) and tritiated water, used as an internal standard (441 700 dpm/g), were purchased from Canberra Packard (Groningen, The Netherlands). Oxiluma (scintillation fluid) was from Lumac LSC (Mechelen, Belgium). All reagents were of analytical grade.

Dose Preparation and Administration. Unlabeled dexamethasone was dissolved in a mixture of ethanol/propylene glycol/physiological saline (2:1:7 v/v/v). [^{3}H]Dexamethasone was added to obtain a solution containing 12.5 μCi of [^{3}H]-dexamethasone and 0.25 mg of cold dexamethasone per milliliter (19.6 μCi/μmol). This solution was used to inject a male Wistar rat (IFFA Credo Belgium, Brussels, Belgium), maintained in a cage with food and water ad libitum, intramuscularly with 20 μCi of [^{3}H]dexamethasone/kg (0.167 μg of [^{3}H]dexamethasone/kg) and 0.4 mg of cold dexamethasone/kg.

Tissue Collection and Preparation. The rat was sacrificed 2 h after injection. Liver and muscle tissues were collected, homogenized, and kept at −10 °C for subsequent analysis. Both tissues were minced in a CH100 PK032/AP Kenwood mixer. Muscle was minced again once the tendons were removed as much as possible. Liver was further homogenized in a PT 10TS Polytron mixer.

Sample Preparation by Dry Combustion. Liver (40−230 mg) and muscle (75−400 mg) samples were mixed with 120−170 mg of microcrystalline cellulose (Merck, Darmstadt, Germany) and wrapped into 100−150 mg of tissue paper (Kimberly-Clark, Duffel, Belgium). Cellulose and tissue paper helped to obtain a homogeneous combustion. The sample was put in a combusto-cone (Canberra Packard, Groningen, The Netherlands) and lyophilized to obtain a homogeneous combustion. Lyophilization was performed with a Savant refrigerated condensation trap RT4104 (Ankersmit, Belgium) with an Edwards high-vacuum pump (Smagghe and Degheele, 1993). Samples were combusted (Smagghe and Degheele, 1993) in an oxygen atmosphere in an updated Packard 306 sample oxidizer. Carbon dioxide and tritiated water were formed, and the tritiated water was trapped in Oxiluma. The resulting samples were shielded from temperature and light influences in a Packard Tri-Carb 1600CA counter for at least 1 h before counting.

Table 1. Alkaline Solubilization of Rat Liver and Muscle Samples: Comparison of Solvable and Soluene-350

	Solvable	Soluene-350
counting efficiency (%)		
50−200 mg of muscle (n = 14)	47−41	47−35
25−100 mg of liver (n = 6)	45−35	ND[a]
chemiluminescence (%)		
50−200 mg of muscle (n = 14)	<2	10−15
25−100 mg of liver (n = 12)	<2	10−15
solubilization time (h)		
50−200 mg of muscle (n = 14)	2−3.5	1.5−4
25−100 mg of liver (n = 12)	0.5−2	1−4

[a] ND, not determined.

Sample Preparation by Alkaline Solubilization. Liver (25−100 mg) and muscle (25−250 mg) samples were incubated with 1 mL of Solvable at 60 °C for 4 h, with occasional swirling. This yielded green and brown solutions, respectively, which needed to be bleached to obtain acceptable counting efficiencies. After cooling to room temperature, 100 and 200 μL of 30% hydrogen peroxide (Merck) were added in two equal aliquots to muscle and liver samples, respectively. Due to addition of hydrogen peroxide, foaming occurred. Therefore, samples were swirled for 20 min after each addition of hydrogen peroxide to subdue foaming. For liver samples that were weighed >75 mg, 300 μL of hydrogen peroxide was added in three equal aliquots of 100 μL. After bleaching, the samples were incubated overnight at 60 °C to complete bleaching and to eliminate the excess of hydrogen peroxide. After cooling to room temperature, 10 mL of Ultima Gold was added. The samples were shielded from temperature and light influences in a Packard Tri-Carb 1600CA counter for at least 1 h before counting. The same procedure was used for Soluene-350.

Plotting a Quench Correction Curve. Blank rat liver (100−400 mg) and muscle (100−500 mg) samples were spiked with 50 μL of tritiated water with known activity (441 700 dpm/g) and subjected to the combustion method. For the solubilization method, samples (25−150 mg of liver, 40−300 mg of muscle) were subjected to the described method, cooled after overnight incubation, and then spiked. The samples were weighed and corrected for the weight of ^3H_2O added. The radioactivity of the samples was measured in a Packard Tri-Carb 1600CA counter and the counting efficiency calculated. The relationship between tSIE and counting efficiency percentages was plotted in a quench curve.

RESULTS AND DISCUSSION

Effect of Alkaline Solubilization. The performances of two alkaline solubilizers, Solvable and Soluene-350, were investigated for liver and muscle samples. Samples should be prepared such that the radiation is released quantitatively from the tissues and that the liquid scintillation counter is quantitating a homogeneous mixture of sample and scintillation solution. Tests on sample solubilization time, counting stability, counting efficiency, and chemiluminescence production were all in favor of Solvable (Table 1). Chemiluminescence and solubilization time for liver and muscle samples solubilized with Solvable were significantly different ($p \leq 0.05$) from those of samples solubilized with Soluene-350. The difference in sample solubilization time was especially marked for liver samples; Solvable solubilized liver samples twice as quickly as Soluene-350. The counting stability of biological samples solubilized with Soluene-350 measured by counts per minute (cpm) was low due to precipitation of the

solubilized material as a function of time when scintillation cocktail was added. Counting efficiency was higher when Solvable was used, particularly for high sample sizes. In addition to all of these advantages of Solvable, the most important reason Solvable was the solubilizer of choice was that for the described methodology, samples solubilized with Soluene-350 produced a high amount of chemiluminescence, which was in contrast to those solubilized with Solvable. For samples solubilized with Soluene-350, 24 h after cooling chemiluminescence was still present in large amounts (>10%), interfering with radioactivity measurements because LSC is based upon conversion of radioactivity into photons. Chemiluminescence caused by hydrogen peroxide was minimized by overnight incubation.

Bleaching by Hydrogen Peroxide. After solubilization with Solvable, liver and muscle samples gave green and brown solutions, respectively, thus drastically reducing counting efficiency. Particularly, tritium measurements are affected by quenching because the β-particle energy is so low that relatively few photons are produced even without quenching. In this work, the maximum counting efficiency for tritium, even under ideal circumstances of no quenching agent besides scintillation cocktail, was not more than ~55%. To reduce color quenching, bleaching with hydrogen peroxide solution was necessary. Bleaching of muscle and liver samples with hydrogen peroxide was satisfactory. All samples had a white-yellow color after overnight incubation. Hydrogen peroxide should be added in small portions because the reaction with the colored samples can be vigorous; almost all samples foamed when hydrogen peroxide was added. Addition of hydrogen peroxide has the disadvantage that it causes chemiluminescence. Overnight incubation enabled the elimination of the excess of hydrogen peroxide added. This minimized chemiluminescence. No significant long-term chemiluminescence was detected. In tests with shorter incubation periods, long-term chemiluminescence was still detected, resulting in bad radioactivity measurements. Overnight incubation also optimized bleaching.

Quench Correction Curves. For comparison of both sample preparation methods, quench correction curves were required. Hereto, blank samples were spiked with tritiated water with known activity. The measured cpm values together with the corresponding dpm values (added) allowed us to calculate the counting efficiency. This is shown in Tables 2 and 3 for liver samples solubilized with Solvable and for combusted samples, respectively. Counting efficiency for combusted samples (35−37%) was less than expected but varied less than for solubilized samples (33−45%). This clearly indicated that for tritiated products, combustion is susceptible to quenching, although to a lesser degree than solubilization. By measuring a series of quenched standards, all with the same activity added but with differing levels of quenching, a relationship between the tSIE and the counting efficiency could be established. This relationship provided an efficiency correlation curve for these standards as a function of the calculated tSIE value of each standard. Table 2 shows the equation of the efficiency correlation curve for liver samples solubilized with Solvable and spiked with tritiated water. This enabled the conversion of cpm into dpm for incurred tissues with unknown radioactive content. Specific quench curves were constructed for each sample

Table 2. Determination of the Counting Efficiency for Liver Samples Spiked with 50 μL of Tritiated Water[a]

liver wt (mg)	Solvable (mL)	dpm added	cpm[b] counted	counting efficiency[c] (%)	tSIE[c]
0	0	21776	11715 ± 125	53.80 ± 0.57	695
0	0.1	22041	11607 ± 124	52.66 ± 0.56	663
0	0.2	21820	11232 ± 122	51.48 ± 0.56	643
0	0.5	21776	10948 ± 121	50.28 ± 0.55	603
0	1	21511	10546 ± 119	49.02 ± 0.55	578
0	1.5	21334	10025 ± 116	46.99 ± 0.54	542
25	1	21731	9827 ± 114	45.22 ± 0.53	491
57	1	21820	8753 ± 108	40.11 ± 0.50	428
65.3	1	21599	8220 ± 105	38.06 ± 0.48	399
72.1	1	21776	8125 ± 104	37.31 ± 0.48	385
81.4	1	21599	7780 ± 102	36.02 ± 0.47	370
95	1	21776	7558 ± 100	34.71 ± 0.46	353
112.3	1	21687	7388 ± 99	34.06 ± 0.46	343
146	1	21731	7179 ± 98	33.03 ± 0.45	332

[a] Solvable was used to solubilize the samples. Corrections for the weight of ^3H_2O added were carried out. [b] Values are means of 3 min of counting ± 2 SD. [c] The equation of the quench curve was $y = -0.00007342x^2 + 0.1321x - 2.7424$, $r^2 = 0.9986$, with x = tSIE value and y = counting efficiency.

Table 3. Determination of the Counting Efficiency for Tissue Samples Spiked with 50 μL of Tritiated Water[a]

tissue paper	cellulose	tissue wt (mg)	dpm added	cpm[b] counted	counting efficiency[c] (%)	tSIE[c]
			19622	7535 ± 100	38.40 ± 0.51	436
+		0	19494	7459 ± 100	38.27 ± 0.51	437
+	+	0	19708	7420 ± 99	37.65 ± 0.50	428
+	+	199.5[d]	19537	7123 ± 97	36.46 ± 0.50	415
+	+	335[d]	19324	6951 ± 96	35.97 ± 0.50	408
+	+	420.9[d]	19025	6764 ± 95	35.55 ± 0.50	404
+	+	519.2[d]	19366	6873 ± 96	35.49 ± 0.49	402
+	+	411.6[e]	18385	6599 ± 94	35.89 ± 0.51	407
+	+	107.9[e]	18215	6370 ± 92	34.98 ± 0.51	398
+	+	317.2[e]	18727	6847 ± 96	36.57 ± 0.51	409

[a] Samples were combusted in a sample oxidizer. Corrections for the weight of ^3H_2O added were carried out. [b] Values are means of 3 min of counting ± 2 SD. [c] The equation of the quench curve was $y = 0.0833x + 2.0042$, $r^2 = 0.9748$, with x = tSIE value and y = counting efficiency. [d] Muscle tissue. [e] Liver tissue.

type for both sample preparation methods. The use of a standard quench curve for all kinds of measurements is not advisable because every sample preparation technique has its own specific quenching agents. Even the choice of another scintillation cocktail requires a new quench curve. In the current assays, cpm conversions based upon quench curves plotted using sealed standards gave incorrect results. This indicated, in agreement with Rundt (1991), that different quenching agents have different quenching properties. Only in a few cases, where virtually no variation in quenching is encountered, is a quench curve unnecessary. In that case, within the same laboratory, results in cpm can be compared. In practice, it is difficult to prepare samples with identical counting efficiencies, especially when one is dealing with biological material such as in this study. Therefore, to be able to evaluate counting data, cpm values always need to be converted into dpm values. This can be achieved using specific quench curves, as in this study, or by the internal standardization method (Kessler, 1989c).

Muscle and Liver Tissue of Rats Injected with [^{3}H]Dexamethasone. The radioactivity measurements in rat muscle after solubilization or combustion are shown in Figures 1 and 2. Similar graphs were constructed for liver tissues. The results were plotted as a relationship between the sample weight and the calculated dpm. Cpm values were converted to dpm

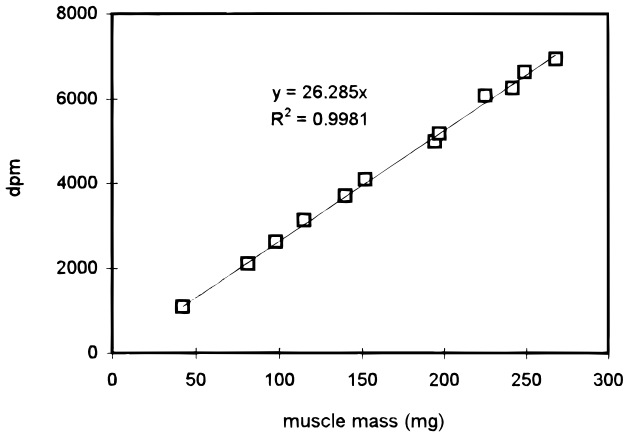

Figure 1. Radioactivity found in muscle samples of a male rat 2 h after a single intramuscular injection of [^{3}H]dexamethasone. Dpm values were plotted as a function of muscle weight. The samples were solubilized with Solvable, and dpm values were calculated by using a specific quench curve (n = 12).

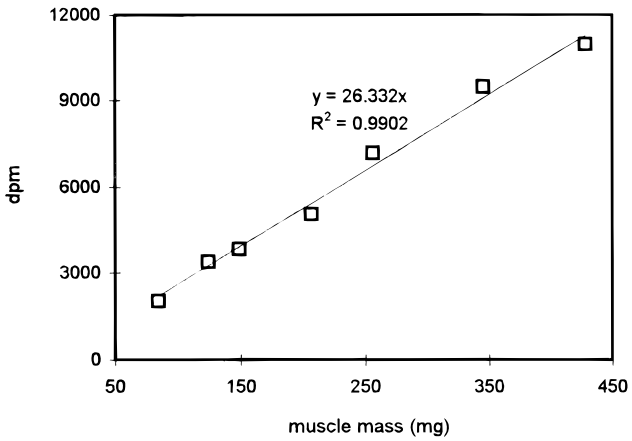

Figure 2. Radioactivity found in muscle samples of a male rat 2 h after a single intramuscular injection of [^{3}H]dexamethasone. Dpm values were plotted as a function of muscle weight. The samples were combusted, and dpm values were calculated by using a specific quench curve (n = 7).

values with the use of a specific quench curve plotted for each sample type. We found a linear relationship between sample weight and dpm value for liver and muscle samples subjected to the two sample preparation techniques. This indicated that the samples were homogeneous and that LSC is directly proportional to ^{3}H.

The equations of the linear regression found for liver were for

alkaline solubilization:

$$y = 173.02x \quad (r^2 = 0.9958)\,(n = 6) \quad (1)$$

and

dry combustion:

$$y = 174.38x \quad (r^2 = 0.9814)\,(n = 6) \quad (2)$$

Similarly, we established a linear regression curve in muscle for

alkaline solubilization:

$$y = 26.285x \quad (r^2 = 0.9981)\,(n = 12) \quad (3)$$

and

dry combustion:

$$y = 26.332x \quad (r^2 = 0.9902)\,(n = 7) \quad (4)$$

with x = sample weight (mg) and y = dpm.

For liver as well as for muscle, the linear regression curves were plotted through the origin. Equations 1 and 2 and eqs 3 and 4 have similar slopes, indicating that both methods are able to release all of the incorporated radioactivity from the selected biological samples, because sample oxidation has a recovery of 99 ± 1.0% (Kessler, 1989a). The linear regression curves allowed us to predict the total radioactivity present in a certain amount of sample. The dpm values obtained with eqs 1 and 2 for 1 g of rat liver differed by only 0.78%; those obtained with eqs 3 and 4 for 1 g of muscle differed by 0.18%. It should be remarked that this difference was independent of sample size because linear regression curves were plotted through the origin. A Student t test was carried out to compare both sample preparation methods. For liver samples as well as for muscle samples dpm values were not significantly different ($p \leq 0.05$).

When counted by LSC, both techniques gave similar results, indicating that for tritiated dexamethasone alkaline sample solubilization is suitable to solubilize tritiated dexamethasone quantitatively from rat liver and muscle samples. Likewise, it should be feasible to achieve the solubilization of dexamethasone from tissues other than muscle and liver by the described methodology.

Further experiments will be focused on extraction recovery studies for [^{3}H]dexamethasone incorporated in rat liver and muscle. The availability of standard material is for this purpose essential. The high efficiency of solubilization through alkaline solubilization strongly suggests that this method can be used to release glucocorticoid residues from biological samples. Extraction of [^{3}H]dexamethasone after alkaline solubilization will be studied. HPLC measurements will give an idea of the formation of metabolites in both tissues and of possible formation due to alkaline solubilization. In this study, it was already shown that no tritiated water was formed because lyophilization, necessary for dry combustion, did not cause differences in results between the two preparation techniques.

The investigation of alkaline solubilization is a first step in the development of analytical detection methods for dexamethasone in liver and muscle and will contribute to an improved food control, thereby enhancing consumer confidence in the proper surveillance of foodstuffs from cattle, horses, and pigs.

ABBREVIATIONS USED

MRL, maximum residue limit; LSC, liquid scintillation counting; tSIE, transformed spectral index of the external standard.

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