Pituitary-dependent hyperadrenocorticism in dogs has historically been treated most commonly with mitotane, an adrenocorticolytic drug with preferential action on the zona fasciculata and zona reticularis. Much interest has been stimulated in recent years regarding the use of trilostane for treating canine hyperadrenocorticism in dogs. Trilostane is a competitive inhibitor of 3β-hydroxysteroid dehydrogenase, an essential enzyme in the production pathways of cortisol, corticosterone, aldosterone, and androstenedione, and perhaps other enzymes as well. In England, trilostane has been the approved drug of choice for treating hyperadrenocorticism in dogs for the past few years, and it has recently been approved by the FDA for use in the United States.

One of the challenges with successful use of trilostane for the treatment of canine hyperadrenocorticism is monitoring its effectiveness. History and clinical signs are invaluable in assessing a patient's response to treatment (eg, resolution of polyuria, polydipsia, polyphagia, and dermatologic signs). However, an adjuvant biochemical test marker that ensures patient response to treatment and avoidance of hypocortisolism would be advantageous.

Adrenocorticotropic hormone stimulation testing is the standard for monitoring treatment of hyperadrenocorticism; it provides information regard-

### Objective
To determine whether trilostane or ketotrilostane is more potent in dogs and determine the trilostane and ketotrilostane concentrations that inhibit adrenal gland cortisol, corticosterone, and aldosterone secretion by 50%.

### Sample
24 adrenal glands from 18 mixed-breed dogs.

### Procedures
Adrenal gland tissues were sliced, placed in tissue culture, and stimulated with 100 pg of ACTH/mL alone or with 5 concentrations of trilostane or ketotrilostane. Trials were performed independently 4 times. In each trial, 6 samples (1 for each time point) were collected for each of the 5 concentrations of trilostane and ketotrilostane tested as well as a single negative control samples. At the end of 0, 1, 2, 3, 5, and 7 hours, tubes were harvested and media and tissue slices were assayed for cortisol, corticosterone, aldosterone, and potassium concentrations. Data were analyzed via pharmacodynamic modeling. One adrenal slice exposed to each concentration of trilostane or ketotrilostane was submitted for histologic examination to assess tissue viability.

### Results
Ketotrilostane was 4.9 and 2.4 times as potent in inhibiting cortisol and corticosterone secretion, respectively, as its parent compound trilostane. For trilostane and ketotrilostane, the concentrations that inhibited secretion of cortisol or corticosterone secretion by 50% were 480 and 98.4 ng/mL, respectively, and 95.0 and 39.6 ng/mL, respectively.

### Conclusions and Clinical Relevance
Ketotrilostane was more potent than trilostane with respect to inhibition of cortisol and corticosterone secretion. The data should be useful in developing future studies to evaluate in vivo serum concentrations of trilostane and ketotrilostane for efficacy in the treatment of hyperadrenocorticism. (Am J Vet Res 2011;72:661–665)
ing adrenal secretory reserve. However, exogenous ACTH may override the competitive enzyme inhibition that trilostane provides, precluding its ability to indicate effective control.\(^3\) Therefore, identifying a therapeutic serum concentration of trilostane may provide valuable information that could be used in monitoring the efficacy of trilostane in patients with pituitary-dependent hyperadrenocorticism. However, a paucity of published information exists on the pharmacodynamics of trilostane in dogs. Furthermore, the most active metabolite of trilostane, ketotrilostane, is a more potent inhibitor of the target enzyme, compared with trilostane, in some species.\(^3,^4\) The relative potency of ketotrilostane versus trilostane and thus the importance of monitoring serum ketotrilostane concentrations in dogs are unknown.

The authors have developed an ex vivo model for assessment of adrenal gland function on the basis of tissue slicing that might provide guidance regarding target concentrations for monitoring purposes. The system offers an advantage over cell culture techniques. Adrenal slices can be maintained short-term and stimulated to secrete hormones, and trilostane administration inhibits cortisol production without any appreciable impairment in adrenal gland viability.\(^5\)

The objectives of the study reported here were to determine the EC\(_{50}\) of trilostane and ketotrilostane for adrenal gland secretion of cortisol, corticosterone, and aldosterone and to determine the relative inhibitory potency of trilostane and ketotrilostane for each hormone by use of the ex vivo adrenal gland tissue slice system.

**Materials and Methods**

Grossly normal adrenal glands were obtained within 10 minutes after healthy dogs used for practice surgeries performed by veterinary students were euthanized. Procedures were performed in accordance with published guidelines and as approved by the Auburn University Institutional Animal Care and Use Committee. The adrenal glands were stored for approximately 12 hours in medium (containing 500 kallikrein inhibitor units of aprotinin/mL and 100 pg of ACTH/mL)\(^3\) at 4°C. On the day of each trial, the glands were placed vertically in an aluminum cylinder and sliced at a 200- to 250- \(\mu\)m thickness with a tissue slicer.\(^1\) Adrenal slices were placed on ice in dishes containing the storage medium. Subsequently, slices were weighed and placed singly on type A titanium mesh roller inserts.\(^2\) The roller inserts were placed into scintillation vials containing Waymouth medium\(^4\) with 500 kallikrein inhibitor units of aprotinin/mL and 100 pg of ACTH/mL\(^3\) at 21°C. After a 30-minute incubation period, the vials were placed in a dynamic organ culture incubator and incubated down, allowing sectioning across the greatest diameter of the tissue slice. Tissues were sectioned with a microtome at 3 \(\mu\)m, stained with H&E, and examined histologically by 2 of the authors (EMW and ALM). Tissue slices can be maintained short-term and stimulated to secrete hormones, and trilostane administration inhibits cortisol production without any appreciable impairment in adrenal gland viability.\(^5\) Histologic examination. Adrenal slices were placed in neutral-buffered 10% formalin, sectioned transversely, and embedded in paraffin with the cut surface facedown, allowing sectioning across the greatest diameter of the tissue slice. Tissues were sectioned with a microtome at 3 \(\mu\)m, stained with H&E, and examined histologically by 2 of the authors (EMW and ALM). Tissues were evaluated and assigned a score on the basis of the following features: cell membrane (3+ = very crisp

700, 1,400, 4,200, and 7,000 ng/mL, and for ketotrilostane were 40, 200, 400, 1,200, and 2,000 ng/mL. Incubation continued after the addition of the drug for an additional 4 hours (\(T = 0\) through \(T = 4\) ). Samples were harvested at \(T = –3, –2, –1, 0, 2, \) and 4. Thus, for each compound in each trial, samples were collected at each of 6 time points for each of the 3 concentrations of trilostane and ketotrilostane tested as well as a single negative control. Two medium samples were collected at \(T = –2, –1, 0, 2, \) and 4 (ie, a total of 50 vials each for trilostane and ketotrilostane and 10 vials for the negative control, for a total of 110 medium samples/trial). Tissue slices were collected at \(T = –3, 0, \) and 4. Trials were performed independently 4 times. All slices treated with a single drug and concentration came from a single adrenal gland. All medium samples and tissue slices collected were divided for hormonal assays (cortisol, corticosterone, and aldosterone; medium samples were not collected at \(T = –3\) ); cytosolic potassium concentrations, and histologic examination.

Medium samples from both tubes per concentration were assayed for cortisol\(^1\) and aldosterone\(^2\) by use of validated assays.\(^6,^7\) The corticosterone assay\(^8\) was performed per manufacturer’s instructions. Four samples were randomly chosen and diluted 1:2, 1:4, and 1:8 in the diluent supplied with the kit and assayed. Mean percentage recoveries for the 1:2, 1:4, and 1:8 dilutions were 83.4%, 98.1%, and 83.1%, respectively. The slopes of the lines obtained from the dilutional studies were similar (\(t\) test; \(P > 0.05\) ) to those from the respective standard curves. Per the manufacturer, intra-assay CVs for samples of low, medium, and low concentration were 4.4%, 7.1%, and 10.3%, respectively, whereas the interassay CVs were 6.5%, 7.2%, and 7.1%, respectively. Cross-reactivities for cortisol and aldosterone were 0.05% and 0.03%, respectively (per the manufacturer). Sensitivity of the assay was 25 ng/mL. All samples were assayed in duplicate.

Cytosolic potassium concentrations were measured to assess adrenal gland slice viability.\(^8\) Homogenates of 1 slice/drug concentration were collected at all time points and assayed for cytosolic potassium concentration (ie, a total of 30 slices each for trilostane and ketotrilostane and 6 slices for the negative control, for a total of 66 slices/trial). To prepare the homogenates, adrenal gland slices were homogenized in 1.0 mL of distilled water at approximately 20,000 cycles/min for 30 to 45 seconds until adequately homogenized. Homogenates were stored at –80°C until assays could be performed. Potassium assays were performed within 12 weeks of collection via a commercial electrolyte analyzer\(^9\) after thawing and vortexing samples.

One adrenal gland slice exposed to each concentration of trilostane or ketotrilostane was submitted for histologic examination. Adrenal slices were placed in neutral-buffered 10% formalin, sectioned transversely, and embedded in paraffin with the cut surface facedown, allowing sectioning across the greatest diameter of the tissue slice. Tissues were sectioned with a microtome at 3 \(\mu\)m, stained with H&E, and examined histologically by 2 of the authors (EMW and ALM). Tissues were evaluated and assigned a score on the basis of the following features: cell membrane (3+ = very crisp...
With respect to cortisol, percentage inhibition attributable to the 4 higher concentrations of trilostane was significantly (P = 0.001) greater, compared with results for the negative control (Table 1). All 5 concentrations of ketotrilostane significantly (P < 0.001) inhibited cortisol secretion, compared with results for the negative control.

Pharmacodynamic modeling of trilostane and ketotrilostane effects on cortisol secretion identified an E<sub>max</sub> of percentage inhibition of 80.4% (90% CI, 69.2% to 91.3%) and 68.6% (90% CI, 11.3% to 125.9%), respectively, and an EC<sub>50</sub> of 480 ng/mL (90% CI, 142.3 to 817.7 ng/mL) and 98.4 ng/mL (90% CI, 38.9 to 157.9 ng/mL), respectively. Thus, ketotrilostane was 4.9 times as potent in inhibiting cortisol secretion as the parent compound trilostane. Additionally, pharmacodynamic modeling of trilostane and ketotrilostane effects on corticosterone secretion resulted in an E<sub>max</sub> of percentage inhibition of 81.4% (90% CI, 76.9% to 83.9%) and 78.3% (90% CI, 72.8% to 84.2%) and an EC<sub>50</sub> of 95.0 ng/mL (90% CI, 30.3 to 159.6 ng/mL) and 39.6 ng/mL (90% CI, 22.3 to 56.9 ng/mL), respectively. Thus, ketotrilostane was 2.4 times as potent in inhibiting corticosterone secretion as trilostane.

Cytosolic potassium concentrations decreased over the first hour of incubation and then remained stable throughout the remainder of the study (data not shown). Histologic scores for adrenal glands were totaled. Possible scores ranged from 3 (poorest) to 9 (best).

Statistical analysis—Results are reported as mean ± SD values. For all time points at which 2 medium samples were collected (ie, T = –2, –1, 0, 2, and 4), the CV was calculated. The mean sample pair CV was calculated for each of the hormones.

Percentage inhibition was calculated as the percentage difference between the T = 0 (maximal stimulation) and T = 4 (maximal inhibition) time points divided by the weight of the slice to correct for differences in slice size. To detect differences in percentage inhibition among drug concentrations, data were analyzed by use of a 1-way ANOVA with a commercial statistical program. If a significant difference was detected, post hoc comparisons were made by use of the Student-Newman-Keuls method (all-pairwise) and multiple comparison test.

The relationship between drug or metabolite concentrations and effect was assumed to be direct. The response-effect was subjected to pharmacodynamic modeling that determined the E<sub>max</sub> and EC<sub>50</sub> after removal of a baseline effect parameter. The best model was chosen on the basis of the goodness of fit of the estimated statistical model by use of Akaike information criterion.

For comparison of total morphological score over time and among treatments, a 1-way ANOVA was used. For all statistical analyses, values of P ≤ 0.05 were considered significant.

Results

For cortisol, corticosterone, and aldosterone, the mean sample pair CVs were 20%, 20%, and 35%, respectively. The CV for aldosterone data was judged to be unacceptable, and the data were not further analyzed.

Concentrations of cortisol and corticosterone progressively increased until T = 0, reflecting adequate recovery of the tissue slices from slicing (Figure 1). The decrease between T = 0 and T = 2 reflected the medium change; the concentrations at T = 4 indicated the effect of the drugs on hormone secretion, with the 5 concentrations of trilostane and ketotrilostane generally correlating with an increased degree of inhibition (Figures 1 and 2).

With respect to cortisol, percentage inhibition of the 4 higher concentrations of trilostane was significantly (P = 0.001) greater, compared with results for the negative control (Table 1). In addition, the 3 highest trilostane concentrations inhibited cortisol secretion significantly more than did the lowest concentration. With respect to ketotrilostane, the 4 higher concentrations caused significantly (P = 0.002) greater inhibition of cortisol secretion than did the negative control.
and 1.7 times as potent as trilostane, respectively. Interestingly, in the present study, ketotrilostane was 4.9 times as potent in inhibiting cortisol synthesis, as compared with its parent compound trilostane. Ketotrilostane in guinea pigs and rats, in comparison, is 2.4 and 1.7 times as potent as trilostane, respectively.

Thus, if serum drug concentration monitoring is proven to be useful with trilostane, it may be necessary to measure serum ketotrilostane concentrations in conjunction with or in place of trilostane.
much higher than obtained EC_{50} values, especially in the case of ketotriolostane. As stated, the concentrations were empirically selected on the basis of pharmacokinetic data obtained from the drug manufacturer. Given our data, a lower, narrower range of drug concentrations should be used for future studies. Lastly, in this study, the concentration of ACTH used was at the upper limit of the reference range; the concentration was chosen to simulate normal physiologic conditions in the ex vivo model and was proven in an initial study to adequately stimulate adrenal gland secretion. However, endogenous ACTH concentrations in patients treated with triolostane are substantially greater than pretreatment concentrations and greater than normal physiologic concentrations. One study revealed that endogenous ACTH concentrations were significantly different between pretreatment- and posttreatment time points, with median pretreatment values of 102 pg/mL and posttreatment values of 246 pg/mL. Increased concentrations of ACTH could cause increased adrenal gland stimulation that may theoretically override triolostane’s competitive inhibitory effects. Future studies could be performed with different ACTH concentrations to further evaluate the effect of concentration.

It was disappointing that the aldosterone data were not more informative. Given the high CV (35%), the variability between slices was deemed to be unacceptably high and the data were not further evaluated. A possible reason for the greater CV in the aldosterone data, compared with that calculated for cortisol and corticosterone, is that the zona glomerulosa layer, the outermost layer of the cortex, may have been more susceptible to damage during the harvesting or slicing portions of the study. Use of the ex vivo adrenal gland slice model to evaluate relative potencies of triolostane and ketotriolostane in inhibiting adrenocortical secretion of cortisol and corticosterone served as a useful technique in arriving at preliminary pharmacodynamic data. The data should be useful for future studies that evaluate in vivo serum concentrations of triolostane and ketotriolostane in further evaluating efficacy in the treatment of hyperadrenocorticism. Because of the greater potency of ketotriolostane, compared with triolostane, serum ketotriolostane concentrations must be measured to evaluate in vivo pharmacodynamics. In addition, because the EC_{50} and E_{max} may differ between hormones, the effect of triolostane and ketotriolostane should be evaluated for each hormone individually. The inhibition of cortisol secretion reflects efficacy, whereas the inhibition of aldosterone and possibly corticosterone secretion suggests toxicity and undesired adverse effects.

a. USB Corp, Cleveland, Ohio.
b. Cortrosyn, Amphastar Pharmaceuticals Inc, Rancho Cucamonga, Calif.
d. Sigma-Aldrich, Waymouth MB 752/1 Medium, St Louis, Mo.

g. Coat-a-Count aldosterone assay, Siemens Healthcare Diagnostics, Deerfield, Ill.
h. ImmuChem Double Antibody Corticosterone 125I Kit, MP Biomedicals LLC, Orangeburg, NY.
i. Medica EasyLyte, Bedford, Mass.
k. WinNonlin Professional, version 4.1, Pharsight Corp, Mountain View, Calif.

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