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CLEARANCE TIMES AND BEHAVIORAL EFFECTS OF CLENBUTEROL IN THE HORSE

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ABSTRACT

The urinary excretion pattern of clenbuterol (1.6 μg/kg i.v.) was studied in 4 horses. Basic liquid extraction of clenbuterol was performed at pH 9.5 which yielded a 70% receovery of drug from equine urine. Drug levels were determined using a gas chromatograph equipped with a 2% OV-101 column and nitrogen phosphorus detector. Lower limit of detection by this method was 5 ng/ml. Post-dose samples were obtained at 0.5, 1, 2, and 6 hours and every 12 hours thereafter until drug could no longer be detected. A plot of peak height versus drug concentration yielded significant linearity over a one log unit range of concentrations. Peak urinary levels were observed within 6 hours following drug administration and declined linearly over the next 24 to 36 hours.

The effects of clenbuterol (0.8 μg/kg i.v.) administered alone and 30 minutes prior to fentanyl (10 μg/kg i.v.) were studied on locomotor behavior in 4 horses. Data were analyzed according to the method of Combie et al (J. Equine Med. Surg. 3:377-385 (1979)). Preliminary results indicate that clenbuterol may prolong fentanyl-induced locomotor stimulation. However, clenbuterol alone does not alter this parameter.

SUMMARY

The urinary excretion pattern of clenbuterol (1.6 μg/kg i.v.) was studied in 4 horses. Basic liquid-liquid extraction of clenbuterol was performed and drug levels were analyzed by both gas and thin-layer chromatography. Samples were obtained ½ hour through 36 hours after dosing. Peak urinary concentration of clenbuterol was observed 4 hours post-dose and concentrations declined thereafter with a half-life of 6½ hours

The effect on locomotor behavior of clenbuterol (0.8 μg/kg i.v.) administered 30 minutes prior to fentanyl (10 μg/kg i.v.) or saline was studied in 4 horses. A Latin-square cross over design was followed. Clenbuterol failed to alter either the magnitude or duration of fentanyl-induced locomotor stimulation. Clenbuterol alone did not elicit a locomotor response.

INTRODUCTION

Clenbuterol belongs to the class of therapeutic agents known as B2 adrenoreceptor agonists. Actions of B_2 agonists include bronchodilation, the desired therapeutic effect of clenbuterol, as well as peripheral vasodilation, which results in perspiration and pooling of blood. B_2 agonists may also show some B_1 activity such as increased heart rate[2]. Clenbuterol is useful in relieving respiratory disorders such as obstructive lung disease[1] and may have prophylactic value in the treatment of exercise-induced pulmonary hemorrhage. Certain B_2 agonists such as salbutamol have central effects such as antidepressant activity. This action is thought to reflect incresed serotonin metabolism (turnover) in the brain. Both salbutamol and clenbuterol increase central serotonin metabolism[5] Tremulousness is a frequently reported side effect of administration of B_2 agonists[1,4] and is a result of somatomotor excitation. The chemical structure of clenbuterol is shown in Figure I. Since clenbuterol is structurally related to the central locomotor stimulants amphetamine and ephedrine, it may augment locomotor behavior. This is undesirable for a drug which may be used in racing horses.

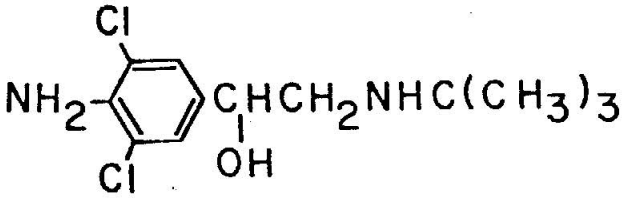


Fig. I: Chemical structure of clenbuterol

The narcotic analgesics produce locomotor stimulation in certain species including the rat and horse. The proposed mechanism for this action is via the release of brain monoamines[3]. Fentanyl produces a clear locomotor stimulation which is dose-dependent and reproducible[7]. Stimulants such as methamphetamine potentiate the response to fentanyl[8]. Potentially, by further increasing brain monoamine, clenbuterol could produce a greater locomotor response than that seen with narcotics alone.

Clenbuterol has not been approved for use in the United States. However, it is being used on the tracks in the U.S. and has been detected in routine screening by the Kentucky Equine Drug Testing Program. Since little is known about the pharmacokinetics of clenbuterol in the horse and considering its abuse potential, we examined the detectability of clenbuterol in equine urine and characterized its urinary excretion pattern. In addition, the behavioral effects of clenbutrol and its interaction with narcotics were studied using he locomotion assay described by Tobin and co-workers[7,8].

MATERIALS AND METHODS

Clenbuterol is detectable in equine urine by both gas chromatography using a 3% OV-101 column and a nitrogen-

phosphorus detector, and thin-layer chromatography. Figure II shows typical gas chromatograms of clenbuterol in urine. Note the strong peaks of clenbuterol at a retention time of 1.1 minutes. Extraction for thin-layer chromatography was performed according to the method used by the Kentucky Equine Drug Testing Program. This method is outlined in Figure III. For gas chromatography, a basic extraction method optimized for pH was developed. Spiked urine samples (50 ng/ml) were made basic by the addition of saturated aqueous solutions of sodium tetraborate in which the pH was varied from 9 to 11. The optimal pH for recovery was 10.5. The full extraction procedure is outlined in Figure IV. Using this extraction procedure, a standard curve was constructed. In spiked urine, clenbuterol can be detected in concentrations as low as 10 ng/ml. A plot of concentration versus peak area gives a staight line through the range of concentrations found in dosed horse urine, as seen in Figure V.

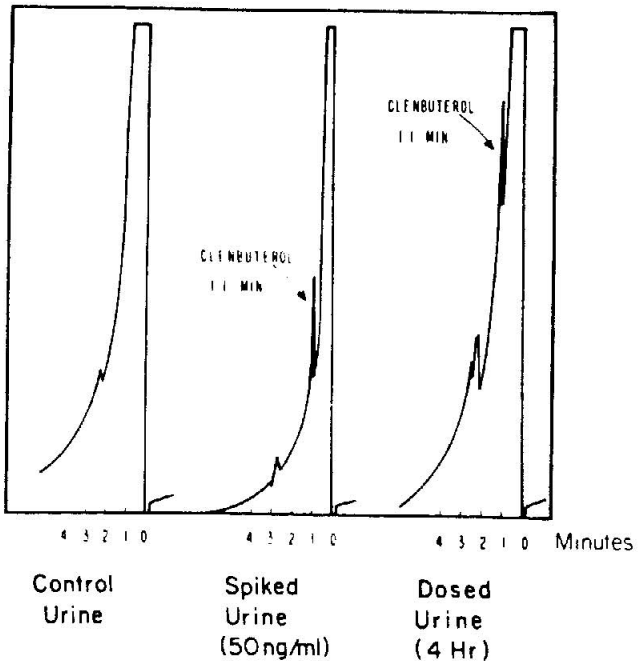


Fig. II: Gas chromatograms of clenbuterol in horse urine. Typical Chromatograms of extracted samples of (left) control (blank) urine; (center) urine spiked with clenbuterol (50 ng/ml); and (right) urine obtained from a horse dosed with 1.6 µg/kg clenbuterol (urinary concentration approx. 50 ng/ml). All chromatograms were generated by a Perkin-Elmer 900 with a 6-foot OV-101 column and a nitrogen-phosphorus detector. Retention times (min.) are indicated on the abscissa. Clenbuterol retention time 1.1 min. Temperatures: Injector = 25 C; Column = 220 C; Manifold = 350 C; Detector = 590 C.

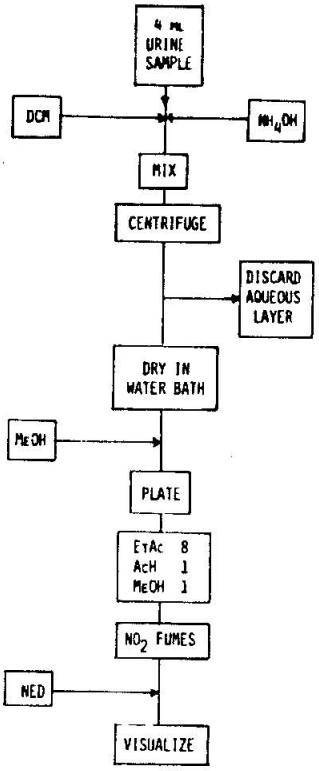


Fig. III: Method: extraction of clenbuterol from equine urine for thin-layer chromatography. DCM = dichloromethane; NED = naphthlethylenediamine; MeOH = methanol; EtAc = ethyl acetate; AcH = acetic acid.

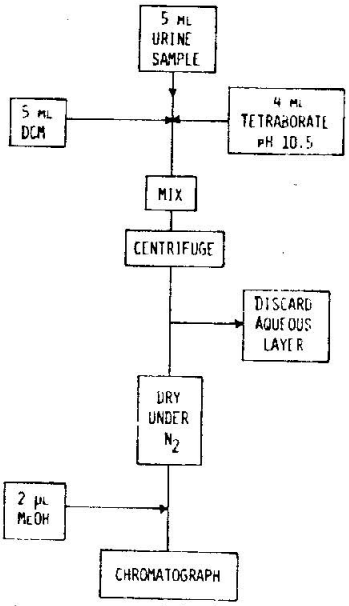


Fig. IV: Method: extraction of clenbuterol from equine urine for gas chromatography. DCM = dichloromethane; MeOH = methanol.

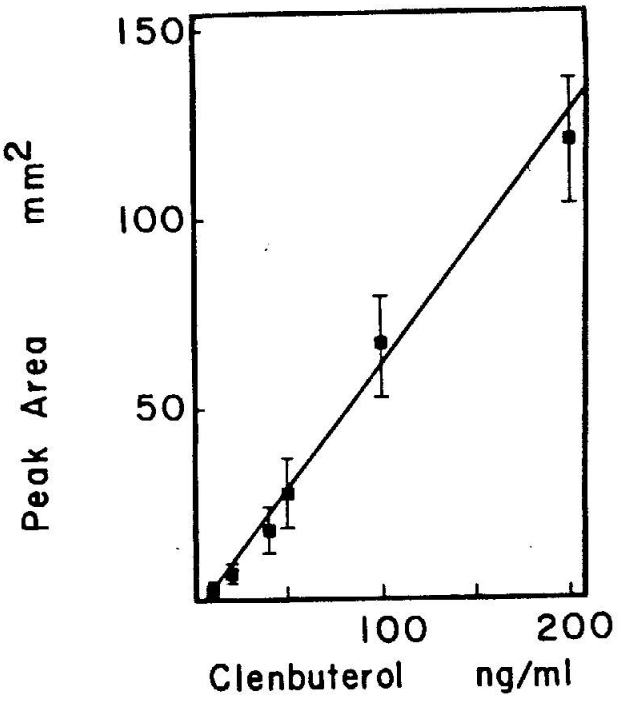


Fig. V: Clenbuterol standard. Mean peak area of chromatogram versus clenbuterol concentration in spiked urine. Each point represents the mean of six trials (±S.E.M.). Correlation coefficient, r = +0.9796, significant at p = 0.01.

For the kinetics portion of the study, four mature Thoroughbred and Standardbred mares were taken in from pasture and put in box stalls eighteen hours before dosing. They were allowed hay and water *ad libitum*. Each mare was dosed with 1.6 μg/kg clenbuterol into the left jugular vein. Samples were obtained by urinary catheter and the bladder was drained after each sample. Control urines were drawn just prior to dosing. Samples were taken at 1/2 hour, 1, 2, 3, 4, 6, 8, 12, 24 and 36 hours post-dosing. All samples were frozen until analysis.

For the behavioral portion of the study, 4 mature Thoroughbred and Standardbred geldings were brought in from pasture eighteen hours before the study. They were placed in stalls which minimized interactions between horses and between horse and observer. The geldings were allowed hay and water *ad libitum*. Clenbuterol was given at a dose of 0.8 μg/kg and fentanyl at a dose of 10 μg/kg as the citrate. The control was normal saline. All injections were made into the jugular vein.

The experiment followed a Latin-square crossover design. Initially, each horse was dosed with either clenbuterol or saline. Each gelding eventually received each of the four combinations at approximately one week intervals.

Observations began immediately after the second injection and lasted 76 minutes. Steps taken per two minute period were recorded. A "footstep" was counted each time the left foreleg was lifted and placed down in a different location. Acute effects of clenbuterol were observed approximately 30 minutes after dosing. Therefore, the clenbuterol or saline treatment was administered 30 minutes prior to fentanyl or saline to maximize the potential for observing an interaction between treatments. Behavioral data were analyzed using a threeway analysis of variance in which variances between treatments, horses, and weeks were segregated.

RESULTS

By gas chromatography, clenbuterol was detected in urine from 1 to 12 hours after dosing. Figure VI shows the urinary excretion pattern of clenbuterol. Peak drug concentrations occur in 4 hours, then the concentrations decline logarithmically with a 6 1/2 hour half-life. Clenbuterol was not detected in the 24 and 36 hour samples by gas chromatography. However, it was readily visualized on the thin-layer plates. Neither method detected any drug in the 1/2 hour urine sample.

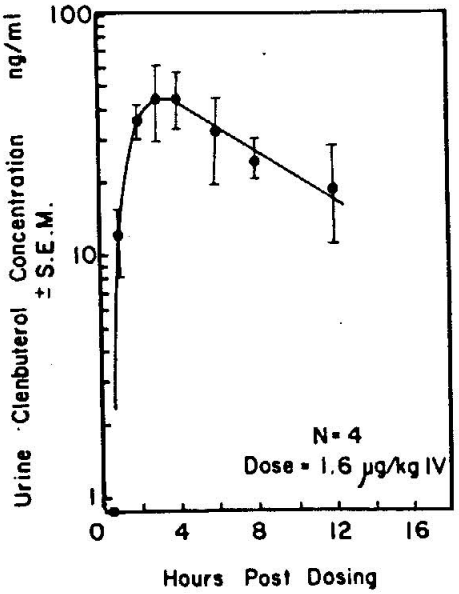


Fig. VI: Concentrations of clenbuterol in urine. Mean concentrations of urinary clenbuterol (±S.E.M.) following a single intravenous dose of 1.6 μg/kg.

Figure VII is a plot of mean footsteps taken per two minutes over time for the four geldings. Clenbuterol failed to alter the rate of rise or magnitude of the fentanyl locomotor response. Fentanyl locomotor stimulations dissipated within 40 minutes, which is consistent with the duration of effect reported previously by Tobin *et al.*[7].

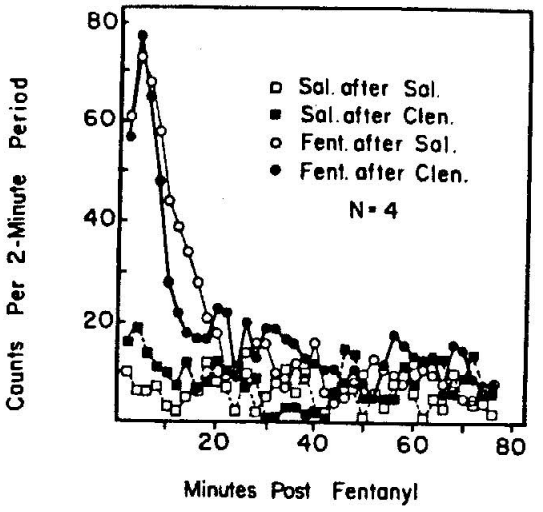
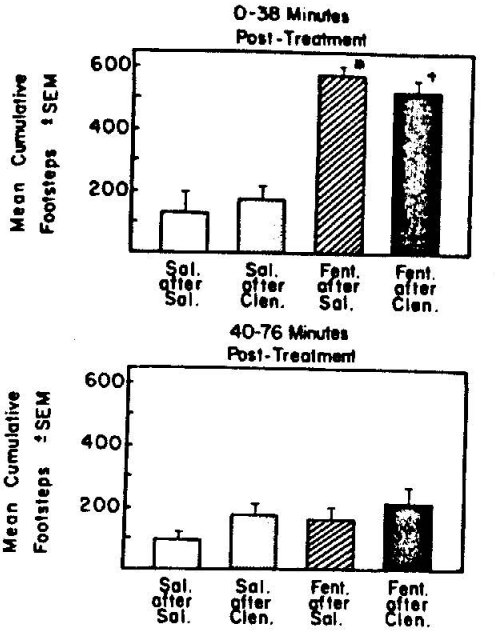


Fig. VII: Time course of the effects of clenbuterol on fentanyl-stimulated locomotor activity. Mean footsteps taken per 2-minute period versus time post-fentanyl or post-saline control.



* Saline, Fentanyl vs. Saline, Saline ($p < 0.01$)

† Clenbuterol, Fentanyl vs. Clenbuterol, Saline ($p < 0.01$)

Dosages: Clenbuterol 0.8 µg/kg IV; Fentanyl 10 µg/kg IV

Fig. VIII: Effect of clenbuterol on fentanyl-stimulated locomotor activity. Cummulative footsteps for each treatment condition during the early (0-38 min.) and late (40-76 min.) epochs.

		CUMULATED COUNTS (FOOTSTEPS) $\bar{X} \pm$ S.E.M.	
PRE-TREATMENT	TREATMENT	0-38 MINUTES POST-TREATMENT	40-76 MINUTES POST-TREATMENT
SALINE	SALINE	124 ± 73	87 ± 27
CLENBUTEROL	SALINE	159 ± 69	157 ± 74
SALINE	FENTANYL	565 ± 48*	159 ± 66
CLENBUTEROL	FENTANYL	512 ± 65†	207 ± 111

* SALINE, FENTANYL VS. SALINE, SALINE, $P \ll 0.01$

† CLENBUTEROL, FENTANYL VS. CLENBUTEROL, SALINE, $P \ll 0.01$

N = 4 HORSES

DOSAGES: CLENBUTEROL (0.8 µg/kg) I.V., FENTANYL (10 µg/kg) I.V.

Fig. IX: Effect of clenbuterol on fentanyl-induced locomotor stimulation. Values indicate mean (±S.E.M.) cummulative footsteps taken by four horses during the early (0-38 min.) and late (40-76 min.) post-treatment epochs.

Since in previous experiments the period 30 minutes following fentanyl administration was the most sensitive to potentiation by other drugs[4,5], the observation time was divided into two epochs: 0-38 minutes and 40-76 minutes. Mean cummulative footsteps taken by the four horses in both the early (0-38 minutes) and late (40-76 minutes) epochs are shown in Figure VIII and IX. According to the analysis of variance, there was no significant variance between the footsteps taken by the saline and clenbuterol pretreated horses which were subsequently treated with saline for either the early or the late epoch. In the saline pretreated animals, fentanyl produced an appoximately five-fold increase in cummulative footsteps when compared to saline control ($p<.01$). A fentanyl response of similar magnitude was observed in horses pretreated with clenbuterol ($p<.01$), showing that clenbuterol did not alter the horses' response to fentanyl. The above treatment effects were observed only during the early post-treatment epoch. Although significant between horses variance was observed, no between weeks (period) variance was found.

No obvious signs of behavioral sedation or excitation were observed. Some animals perspired and exhibited brief periods of rapid shallow breathing following clenbuterol administration.

DISCUSSION

Clenbuterol is detectable in equine 36 hours after a single intravenous dose of 1.6 μg/kg. Because small quantities of clenbuterol are administered, very low concentrations of clenbuterol in physiological fluids result. This limits the detectability of clenbuterol. Clenbuterol may then persist in equine urine beyond 36 hours. Pilot studies show ^{14}C labelled clenbuterol is found in rat urine 72 hours after dosage.[5] No studies of clenbuterol's duration of action in the horse are in the literature. Shapland and co-workers reported clenbuterol produced a significant decrease in pulmonary pressure which showed no tendency to return to baseline after a three-hour testing period.[6] Conceivably, clenbuterol may still exert a pharmacologic action when urinary drug levels are below the chemist's limit of detection.

Despite anticipated central nervous system stimulation, clenbuterol produced no change in locomotor behavior, nor did it potentiate the narcotic-induced locomotor stimulation. In this respect, clenbuterol differs from other sympathomimetic bronchodilators (e.g. ephedrine) which augment locomotion, as well as potentiate narcotic-induced locomotor behavior. No observer noted clear signs of central stimulation or depression in any of the clenbuterol-treated horses. The rapid, shallow breathing and perspiration observed after clenbuterol are consistent with pharmacologic actions of B_2 agonists which include a decrease in airway resistance and peripheral vasodilation. Thus, clenbuterol produces bonchodilation[6] without enhancing locomotor activity.

ACKNOWLEDGEMENT

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DISCUSSION

MACDONALD: I'd like to know how long you can confirm a clenbuterol in the urine in a mass spec?

COLLETT: We were unable to detect it with a mass spec in the urine samples from our horses. We had run mass spec on clenbuterol, we know what we are seeing is clenbuterol; however, on our urine samples we did not have enough remaining in our samples from the four horses to detect it.

MACDONALD: I am kind of confused, is the same procedure that you presented here today run into your routine laboratory?

COLLETT: The usual screening procedure in the laboratory is the TLC. If they call a positive, they do run a mass spec. I'm not exactly sure what the extraction procedure is for it.

MACDONALD: We had a case with you people, four clenbuterol samples were sent down and a report was sent back that you confirmed three and you didn't confirm the fourth one but your routine lab Director Jerry Blake stated he saw it and that's why I was wondering if there was a difference in your methodologies?

COLLETT: I am not at liberty to explain that, it's not exactly my area. Dr. Tobin could you help me?

TOBIN: I understand Dr. Blake wasn't able to confirm one of the samples as he stated.

WEBER: Did you see any locomotor stimulation with your 1.6 μg/kg dosage?

COLLETT: No.

SNOW: Did you say the peak levels occur four hours after intravenous injection?

COLLETT: That's correct.

SNOW: Do you have an explanation for that?

COLLETT: That was in urine.

SNOW: I'm sorry you said you couldn't make a detection in the plasma.

MAYLIN: Further up to Dr. Weber's question, you did your pharmacokinetic work with 1.6 μg/kg.

COLLETT: Yes.

MAYLIN: And you did your behavioral effects with half that dose.

COLLETT: Yes.

MAYLIN: Why did you select half a dose rather than the 1.6?

COLLETT: Actually, it's the other way around, 0.8 if you'll recall, is the clinically recommended dosage for clenbuterol in the horse and that's the dose that I understand is being used on the track at this time. We increased this kinetic study dose to 1.6 mainly because we wanted to make sure that we could detect it.

MAYLIN: Well is it not the effect of drugs, dose related in this particular case?

COLLETT: Yes.

MAYLIN: I'm wondering why you didn't use a higher dose rather than just one dose?

COLLETT: Because the 0.8 is the dose that is used on the track, the 1.6 is so we could better visualize the kinetics.

MAYLIN: Thank you.

SMITH: I'm curious about the origin of the clenbuterols being used. From what you said, or implied, it is not a licensed product in the United States or in Canada?

COLLETT: No, it is not.

SMITH: So, my question is an intriguing one, what is the source of this, is this from research samples, development samples, that are being provided to licensed investigators or sources overseas?

TOBIN: Sources to us?

SMITH: No, sources to the track. You said it's being used at the track illegally, illicitely, my question very simply is what do you think is the source of this?

COLLETT: It is probably imported but it's exact source I can't tell.

WEBER: I can maybe help you out a bit. In Canada, the drug is available for investigational use by obtaining permission from the Bureau of Veterinary Medicine and it is readily available to veterinarians, through that source.

SMITH: What speculation is the leak from there to the race track?

WEBER: I wouldn't want to comment on that.

WOODWARD: On your extraction procedure you say you adjust your pH with borate.

COLLETT: Yes, Sodium Tetra Borate.

WOODWARD: Borate is a very poor buffer at that pH, how do you know that you are actually extracting at that pH?

COLLETT: We don't but I do believe, okay we're using, about half the volume is the borate to begin with and secondly it is the easiest way for use to control our sampling and our extraction procedure.

WOODWARD: On your pharmacokinetic slide showing half-life, I noticed, what looks to me like some of your arrowbars are ten fold.

COLLETT: Can we go back to that slide please. On this slide the vertical scale is a logarithmic scale, so that would make it seem a little bit larger.

WOODWARD: It doesn't seem, it is, it's ten fold changes. How can you draw a curve like that?

COLLETT: By drawing the curve through the means of the data. That is a standard deviation, that is a linear regression analysis with a straight line.

WOODWARD: But it's not linear regression, it should be a logarithmic regression.

COLLETT: Logarithmic, excuse me.

WOODWARD: And one last question, did you measure stimulation by any other means besides foot steps?

COLLETT: No, we intend to be doing that later on in the year.

MAYLIN: Did you perform an error analysis under quantitative analysis due to systematic error analysis?

COLLETT: No.