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ANALYTICAL® CHEMISTRY

Reprinted from
Volume 73, Number 20, Pages 4972–4976

Anal. Chem. **2001**, *73*, 4972–4976

Technical Notes

Determination of Cabergoline by Electrospray Ionization Tandem Mass Spectrometry: Picogram Detection via Column Focusing Sample Introduction

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An electrospray ionization tandem mass spectrometric method was developed for low-picogram detection of an ergot alkaloid, cabergoline, in coyote plasma extracts. Cabergoline is under investigation as an abortifacient in canid species. Central to the successful development of this method was the ability to introduce relatively large sample volumes into the mass spectrometer. This was achieved by focusing the analyte on a conventional high-performance liquid chromatography guard column prior to elution into the spectrometer. Volumes up to at least 900 μL could be injected onto the guard column using a 100% aqueous mobile phase. Cabergoline retained on the column was eluted as a discreet band into the mass spectrometer by the rapid addition of methanol (30%) to the mobile phase. As compared to flow injection sample introduction, the ability to inject larger sample volumes led to a greatly lowered detection limit. Using this technique and a modification of a previously reported extraction procedure, cabergoline could be determined in coyote plasma at concentrations as low as 9 pg of cabergoline/mL of plasma.

Cabergoline, a prolactin-inhibiting ergot derivative, is used for the treatment of hyperprolactinemia and Parkinson's disease in humans.[1] Because of prolactin's unique role in maintaining pregnancy in felines, canines, and rodents, cabergoline is also being investigated as a reproductive control drug in such species.[2–7] Scientists at the National Wildlife Research Center, a U.S. Department of Agriculture research facility, are investigating cabergoline as a contraceptive tool to reduce coyote (*Canis latrans*) predation on livestock and game species. Contraception is considered a viable component of integrated predation management because coyotes without pups are less likely to kill larger prey species such as lambs, calves, and fawns.[8,9]

To examine uptake and retention of cabergoline administered to coyotes, a method for its determination in plasma was required. Studies have indicated that doses as small as 5 μg/kg were effective in terminating pregnancies in dogs and cats.[6,10] Thus, we anticipated that a method capable of detecting cabergoline in plasma at concentrations in the low parts-per-trillion would be required.

Methods for the determination of cabergoline in human plasma have previously been reported.[1,11] The more recent method employed high-performance liquid chromatography (HPLC)/tandem mass spectrometry (MS–MS) using a triple quadrapole analyzer to greatly improve sensitivity versus the former. Many studies have demonstrated the applicability of tandem mass spectrometry paired with flow injection,[12] reversed-phase HPLC,[13–15] and fast gradient HPLC.[16] Nonchromatographic introduction of the sample is often applicable to clinical analyses because of the increased selectivity of tandem techniques. Furthermore, flow injection decreases instrumental run time and increases sample throughput. Accordingly, we investigated a method employing flow injection sample introduction for the determination of cabergoline in plasma extracts by MS–MS.

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10.1021/ac0103367 Not subject to U.S. Copyright. Publ. 2001 Am. Chem. Soc.
Published on Web 08/24/2001

We modified the extraction method of Allievi and Dostert[1] for the determination of cabergoline in coyote plasma. After evaluating flow injection sample introduction of plasma extracts, we developed a technique for focusing cabergoline on a guard column and eluting it into an ion trap mass spectrometer for MS–MS analysis. This column focusing technique allowed for injection volumes of at least 900 μL, which in turn resulted in a detection limit for cabergoline in coyote plasma that rivals that reported for human plasma using a triple quadrapole instrument.[1]

EXPERIMENTAL SECTION

Solutions. Methanol (HPLC grade, EM Scientific, Gibbstown, NJ) and acetic acid (Fisher Scientific, Fair Lawn, NJ) were used to prepare the mobile phases. The aqueous portion of the mobile phase consisted of 1% acetic acid in water, while the organic portion was 100% methanol. Concentrated cabergoline (Pharmacia & Upjohn Inc., Peapack, NJ) standards were prepared in methanol. Intermediate and working standards were prepared from the concentrated standard with 1% acetic acid in water. Ethyl acetate (HPLC grade, Fisher Scientific), methylene chloride (HPLC grade, Fisher Scientific), and a borate buffer solution (pH 10, Fisher Scientific) were used in the extraction of plasma samples.

Instrument. Mobile phase was delivered with a HP1100 binary pump, from which the mixing column was removed (Agilent Technologies, Palo Alto, CA). A HP1100 autosampler (Agilent Technologies) equipped with a 1.0-mL injection loop was used to inject sample and standard solutions into the mass spectrometer. A guard column (4-mm i.d., Aquasil, Keystone Scientific, Bellefonte, PA) was placed between the autosampler and mass spectrometer. Solutions were analyzed with an ion trap mass spectrometer equipped with an electrospray ionization source (LCQ, ThermoQuest, San Jose, CA).

Extraction Procedure. Coyote plasma samples (2.0 mL) were placed in 25-mL culture tubes, and 2.0 mL of buffer solution (pH 10) was added. Ethyl acetate (8.0 mL) was added to the mixture, and the solutions were thoroughly mixed. Following centrifugation, the ethyl acetate was removed and the extraction repeated. The combined ethyl acetate extracts were dried under nitrogen at 60 °C in 25-mL culture tubes. Methylene chloride (350 μL) was added to tubes containing the dried extracts and mixed vigorously. The methylene chloride extracts were transferred to individual 2-mL microcentrifuge tubes, and 1.00 mL of 1% acetic acid was added to each tube. The microcentrifuge tubes were shaken thoroughly with a vortex mixer and centrifuged. The aqueous portion (top layer) was transferred to individual autosampler vials for injection into the instrument.

Column Focusing Sample Introduction. Sample extracts and standard solutions (5.0–900 μL) were injected into the aqueous mobile phase (1% acetic acid in water) delivered at a flow rate of 1.0 mL/min. These mobile-phase conditions were maintained for 3 min during which time the sample loop was swept with three loop volumes and the analyte was sorbed onto the packing material of the guard column (Table 1). The autosampler bypass valve was then actuated so that mobile-phase flow bypassed the sample loop. At the same time, a fast gradient program was employed to increase the organic (methanol) composition of the mobile phase from 0 to 30% over the course of 0.01 min. The new mobile-phase conditions were maintained for 0.5 min to perform the elution of the analyte. Cabergoline was eluted from the guard column at 3.8 min of the "chromatographic" run. The methanol composition of the mobile phase was rapidly increased from 30 to 90% at 3.5 min (Table 1). At a run time of 4.0 min, the autosampler bypass valve was switched back to the main pass position, allowing the mobile phase to sweep the sample loop. At 4.5 min, the mobile-phase methanol composition was rapidly decreased to zero percent. The entire run was 7.0 min in duration.

Table 1. Mobile-Phase Parameters Used for Sample Introduction of Cabergoline Solutions

run time, min	% MeOH	comments
0.00	0	sample injection
3.00	0	sample loop bypass
3.01	30	begin analyte elution
3.50	30	
3.51	90	begin column wash
4.00	90	sample loop mainpass
4.50	90	
4.51	0	begin equilibration for next injection
7.00	0	end run

Analyte Detection. The mass spectrometer was operated in the positive ion mode. The electrospray voltage was 3.5 kV, the orifice (heated capillary) temperature was 245 °C, and the capillary voltage was 8.0 V. Nitrogen sheath and auxiliary flows were 85 and 10% of maximum, respectively. The automated gain control parameters included 1 microscan and 750 ms maximum injection time. Tandem (MS–MS) experiments were conducted on the parent mass of 452 m/z (3.0 m/z isolation width) with a relative collision energy of 25%. The products of the tandem experiment were scanned from 125 to 385 m/z. The chromatographic response resulted from an extracted ion trace of the 381 m/z product.

Flow Injection Sample Introduction. Cabergoline solutions were also analyzed using flow injection sample introduction. Solutions were repeatedly injected into the aqueous mobile phase flowing at 1.0 mL/min. No guard column was present between the autosampler and mass spectrometer, and the mobile-phase composition did not include a gradient. The mass spectrometer and data acquisition parameters were identical to those employed for column focusing sample introduction.

Sample Introduction Comparison. A 2.20 ng/mL cabergoline solution was introduced into the mass spectrometer using both column focusing (previously described) and flow injection sample introduction (described above). Injection volumes of 25, 100, and 400 μL were employed and the resulting peak responses compared visually.

Mobile-Phase Strength. To study the effect of changing mobile-phase strength on column focusing sample introduction, 50 μL of a 2.20 ng/mL cabergoline solution was repeatedly injected into the instrument (n = 5). Three different elution conditions were evaluated: 50, 35, and 30% methanol in the mobile phase at the time of cabergoline elution. No other parameters were changed (Table 1). Data obtained from elution with 30% methanol were used to assess instrument repeatability.

Response Linearity. Detector response for column focusing sample introduction was assessed using both constant and variable sample volumes. In the constant-volume approach, 900 μL of five cabergoline solutions ranging in concentration from 12.8 to 2120 pg/mL were injected into the instrument in triplicate (Table 2). Conversely, a single cabergoline solution (2120 pg/mL) was

Table 2. Masses of Cabergoline Injected into the Instrument for Assessment of Response Linearity

solution concn, pg/mL	vol injected, μL	cabergoline concn, pg
2120	900	1910
1180	900	1060
108.0	900	97.2
61.5	900	55.4
12.8	900	11.5
2120	900	1910
2120	500	1060
2120	50.0	106.0
2120	25.0	53.0
2120	5.0	10.6

repeatedly injected into the instrument to assess response linearity when variable-injection volumes were employed. The solution was injected in triplicate at each of five different volumes ranging from 5.0 to 900 μL (Table 2). Analysis of variance (ANOVA) was used to assess the effect of injection method (constant versus variable volume) on peak height response. Injection method was a fixed effect and mass injected was included in the model as a covariate. A one-factor ANOVA was also conducted with data obtained from variable-volume injection to assess the impact of injection volume on peak height response factors.

Bias and Repeatability. Seven replicate coyote plasma samples (2.0 mL) were fortified with 100.0 μL of a 2210 pg/mL cabergoline solution and subjected to the extraction procedures previously described. The resultant samples had concentrations of 110 pg/mL. Additionally, three control plasma samples (no cabergoline) were similarly extracted. Extracts (400 μL) were analyzed by column focusing sample introduction tandem mass spectrometry.

Method Limits of Detection and Quantitation. Five replicate coyote plasma samples (2.0 mL) were fortified at a concentration of 15.9 pg/mL using a 2120 pg/mL cabergoline solution in 1% acetic acid. The samples were extracted, and 400 μL of the extract was injected into the instrument. Method limit of detection (MLOD) was determined from the chromatographic response of cabergoline, and the peak-to-peak chromatographic noise measured from each chromatogram. The MLOD was defined as the cabergoline concentration that would be required to produce cabergoline response equal to 2 times the chromatographic noise.

The method limit of quantitation (MLOQ) was calculated from the responses obtained from analysis of seven replicate cabergoline-fortified plasma samples used to assess method repeatability. The MLOQ was defined as the cabergoline concentration required to produce a response equal to 10 times the chromatographic noise. The MLOD and MLOQ were determined for each replicate and the mean values were calculated.

RESULTS AND DISCUSSION

The chromatograms produced by column focusing and flow injection sample introduction demonstrate the utility of column focusing as sample introduction technique (Figure 1). Superior peak shape, improved signal-to-noise ratio, and sample volume flexibility were observed with the column focusing technique. The relationship between mobile-phase flow rate and injection volume is critical in flow injection applications where chromatographic-like peaks are desired. This is why very small injection volumes (1−10 μL) are typically employed.[12] We found flow injection sample introduction ill-suited for our application because the desired level of detection was not possible. Improved detection could not be attained by increasing the injection volume (Figure 1). By comparison, the HPLC method of Allievi and Dostert[1] yielded very low parts-per-trillion detection from 150-μL injections.

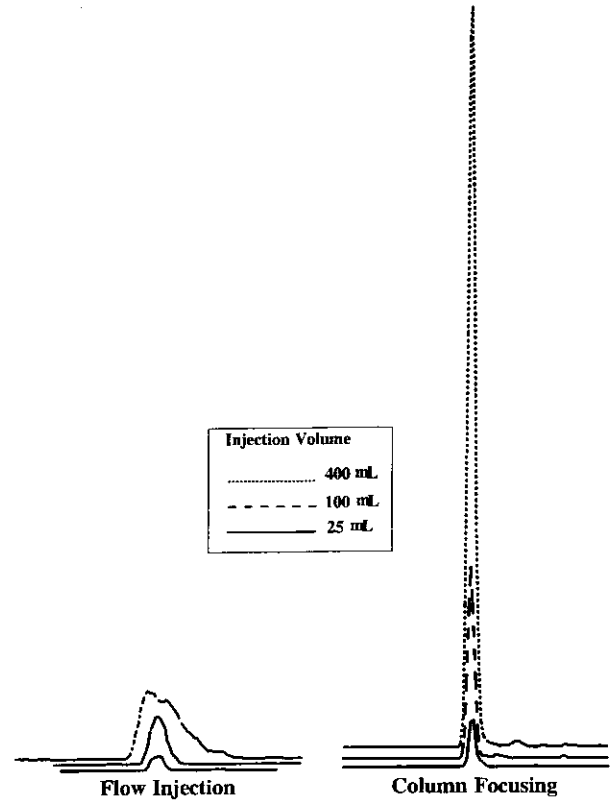

Figure 1. Flow injection sample introduction versus column focusing. Variable-volume injection of a 2.2 ng/mL cabergoline solution using identical mass spectrometric conditions, flow rate, and chromatographic scale. For flow injection introduction, a 100% aqueous mobile phase (1% acetic acid in water) was employed at a flow rate of 1.0 mL/min. Cabergoline was focused on the guard column with the identical 100% aqueous mobile phase and eluted with a 30:70 methanol/ water mobile phase during column focusing experiment.

Flow injection sample introduction on an ion trap instrument could not be expected to attain similar sensitivity because of two limiting factors: injection volume and mass analyzer. First, 150 μL is ~10 times greater than the typical injection volume employed in flow injection.[12] Both the inherent length of the sample plug and the effects of longitudinal diffusion conspire to produce poor chromatographic peak shape. Second, triple quadrapole instruments yield better sensitivity versus ion trap analyzers through use of selected reaction monitoring (SRM).[17] Conversely, SRM provides little added sensitivity over full-scan experiments with the ion trap because all ions are ejected from the trap according to m/z during its normal duty cycle. Instrument software takes advantage of the ion trap duty cycle by scanning all reaction products while allowing chromatographic traces to be produced from extracted ions. Loss in sensitivity is countered by the added qualitative data obtained during the scan event.

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The perception that tandem experiments often preclude chromatographic separation may be inaccurate.[18] Ion suppression and matrix effects may substantially impact analyte specificity and sensitivity, particularly with biological matrixes. However, our combination of sample extraction and column focusing reduced the likelihood of interference from ions or weakly basic matrix components. Our extraction procedure was a modification of Allievi and Dostert[1] that partitioned the free base into an organic solvent to prevent negative ions from accompanying the analyte in the final extract. Formation of the sodiated analogue of protonated cabergoline was similarly minimized.

The column focusing technique may also reduce electrospray interferences from other matrix components. While not as efficient as a HPLC separation, the digital elution process employed in this technique can discriminate among components varying in affinity to the stationary phase (capacity factor, k'). In other words, cabergoline is eluted from the guard column in a manner similar to solid-phase extraction (SPE).[19] Also similar to SPE, the analyte is eluted from the column in a discreet volume that can be substantially smaller than the injection volume. The benefit of this "concentration" process can be best observed when comparing flow injection and column focusing sample introduction (Figure 1).

Peak width, or more importantly, the number of data points defining the cabergoline peak, was another important aspect of this analysis that we attempted to optimize. Though the scanning rate of the mass analyzer was maximized, the number of data points defining the peak could be optimized chromatographically. The mobile-phase strength could be adjusted such that the cabergoline peak was defined by at least 10 data points. Peaks produced by elution with 50% methanol were quite narrow and consisted of only seven or eight data points (Figure 2). Elution with 35 and 30% methanol yielded wider peaks with more data points (8–9 and 10–11 points, respectively). Injection reproducibility was related to the number of data points that described the peak. The relative standard deviation (RSD) of peak height responses was lower when the 30% methanol mobile phase was employed (6.1%) versus the 35 and 50% elution conditions (16.2 and 13.0%, respectively). Some peak width variability was also observed among manufacturing lots of guard columns. Manipulation of the methanol composition allowed for optimization of peak width response when guard columns were changed.

Comparison of constant and variable injection demonstrated that cabergoline response was a function of the mass of cabergoline injected ($F_{1,25} = 3744$; $p < 0.001$), but independent of injection method ($F_{1,25} = 0.25$; $p = 0.62$). With peak height as the response and cabergoline mass (pg) the independent variable, similar detector response curves were obtained from both constant- and variable-volume injections (Figure 3). Injection volume did not impact cabergoline response factor ($F_{4,9} = 1.83$; $p = 0.21$), indicating that volumes ranging from 5.0 to 900.0 μL could be injected onto the guard column without loss of performance. This feature was useful for quantitative purposes, allowing calibration curves to be produced from injecting widely varying volumes of a single calibration standard.

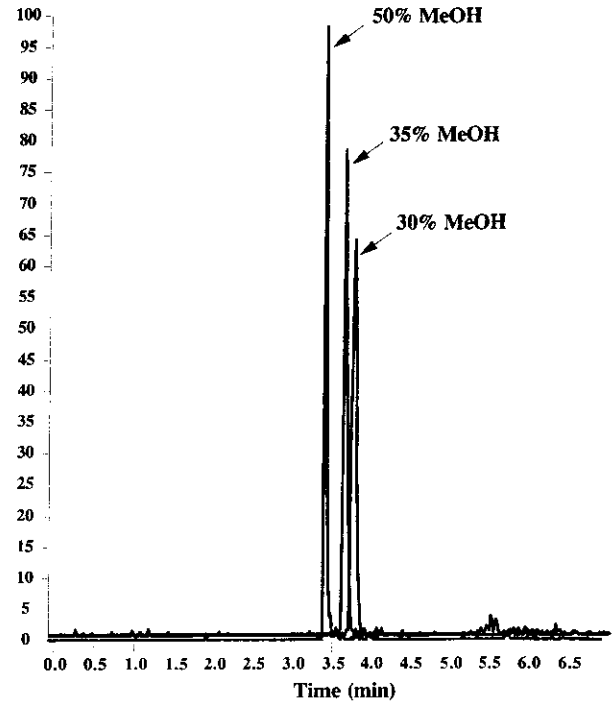

Figure 2. Cabergoline responses resulting from elution with 50, 35, and 30% methanol in the mobile phase (50-μL injections of a 2.2 ng/mL cabergoline solution; flow rate 1.0 mL/min).

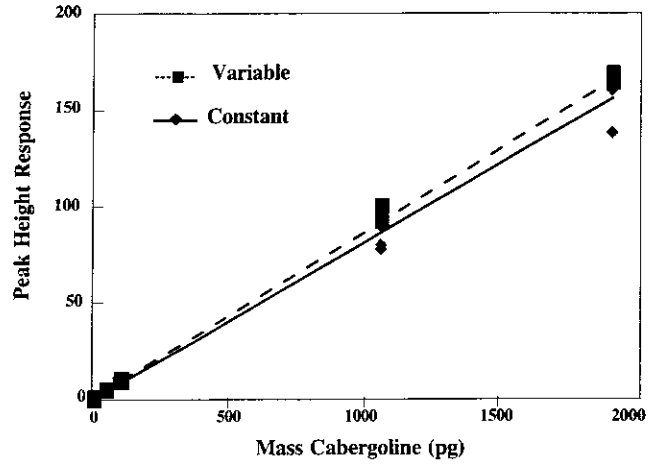

Figure 3. Cabergoline peak height response from two methods of sample introduction. Constant-volume injections were made by injecting 900 μL of cabergoline solutions of varying concentrations (Table 2). Variable-volume injections were made by injecting varying volumes of a 2120 pg/mL cabergoline solution (Table 2).

When plasma extracts were analyzed, it was typically not possible to recover the entire 1.00 mL of the aqueous solution from the final extraction step. Additionally, autosampler vials have a "void volume" (solution at the bottom of the vial inaccessible to the autosampler needle) limiting the sample volume available for automated analysis. Taken together, these factors limited maximum injection volume of extracts to 400 μL.

Owing to the digital elution process and selectivity of tandem mass spectrometry, extracts from the three control samples did not produce interfering peaks corresponding to cabergoline. Replicate analyses of seven plasma samples fortified at a cabergoline concentration of 110 pg/mL yielded a mean recovery of 52.4% with a RSD of 25.4%. The MLOD was determined to be 8.7

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pg cabergoline/mL plasma and the MLOQ was 59.7 pg/mL. Considering the low fortification level tested, we were pleased with 52% analyte recovery from cabergoline-fortified coyote plasma samples. Our recovery compares favorably with the 65% extraction efficiency reported by Allievi and Dostert[1] from similarly fortified human plasma samples. However, they were able to report much better overall accuracy (near 100%) and repeatability (10%) by employing a ^{14}C-labeled surrogate standard. Our poorer than expected repeatability (RSD = 25.4%) was greatly influenced by a replicate fortified sample that yielded only 28% recovery and effectively doubled the relative standard deviation.

Instrument variation did not contribute to the poor precision in analyte recovery from fortified samples. As reported, examination of replicate injections of 110 pg of cabergoline under the conditions employed for extract analysis (for the purposes of evaluating mobile-phase strength) demonstrated a RSD of 6.1% ($n = 5$). Analyte recovery lower than 65% may have resulted from our modifications to the published method.[1] Specifically, we used ethyl acetate as the extraction solvent because it was less likely to form emulsions (vs 2:3 methylene chloride/isooctane) during the liquid–liquid extraction step. Further, our modification included complete drying of the organic solvent, prior to partitioning the analyte back into the aqueous phase. While this additional step may have led to a slight reduction in cabergoline recovery, we found it helpful because our study required that the samples be extracted at a field animal facility and transported to the analytical laboratory.

CONCLUSIONS

The improved sample introduction technique described here can be achieved without specialized hardware. We employed a guard column typically used for routine analytical separations. Though we found some irreproducibility in peak height and width response among manufacturing lots of columns, this could be overcome by adjusting mobile-phase strength to produce peaks of required quality. This sample introduction technique offered improved selectivity and sensitivity versus flow injection and shorter run times as compared to conventional HPLC.

ACKNOWLEDGMENT

Mention of specific products does not constitute endorsement by the United States Department of Agriculture. We thank Pharmacia & Upjohn Inc. for providing several milligrams of cabergoline and Amy Seglund, Krista Wenning, J.B. Minter, Dena Jones, and Nicole Seleno for their assistance in the field and laboratory. The comments of Rick Mauldin, Randy Stahl, and two anonymous reviewers were greatly appreciated.

Received for review March 20, 2001. Accepted July 17, 2001.

AC0103367