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A high-performance liquid chromatography–tandem mass spectrometric method for the determination of pharmacokinetics of ganaxolone in rat, monkey, dog and human plasma

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Abstract

A method for determining concentration levels of ganaxolone in rat, monkey, dog and human plasma was validated in the range of 5–1500 ng/ml using a 200-μl plasma sample volume. This validation report describes the linearity, specificity, sensitivity, reproducibility, accuracy, recovery and stability of the analytical method. The inter-day C.V. ranged from 0.5 to 9.2%, intra-day C.V. from 0.7 to 8.8% and intra-day accuracy (mean absolute percentage difference) ranged from 0.0 to 14.0% for rat, monkey, dog and human plasma. The method was used for the routine analysis of ganaxolone in rat, monkey, dog and human plasma and summary of the pharmacokinetic data are presented. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Therapeutically useful anticonvulsants, anxiolytics and sedative-hypnotics such as benzodiazepines (BZ) and barbiturates mediate their action by binding to distinct allosteric modulatory sites on the $GABA_A$ receptor-Cl^- channel complex. There is now a large body of evidence for an additional modulatory site on $GABA_A$ receptors that binds neuroactive steroids [1]. The prototypical ligand for this binding site is epianopregnanolone (3a-hydroxy-5a-pregnan-20-one) an endogenous metabolite of progesterone that demonstrates potent modulatory effects at the $GABA_A$ receptor.

Ganaxolone (CCD 1042) (**1**) is a 3β-methyl-substituted analog of the endogenous neuroactive steroid 3a-hydroxy-5a-pregnan-20-one (**2**). It is a high-affinity, stereoselective, positive allosteric modulator of the $GABA_A$ receptor complex in the brain through a unique recognition site and exhibits potent anticonvulsant activity in a broad range of animal seizure models [2].

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In radioligand-binding studies ganaxolone allosterically displaced [^{35}S]-*t*-butylbicyclophosphorothionate (TBPS) binding to the chloride channel and enhanced [^{3}H]-flunitrazepam binding to the benzodiazepine receptor in the presence of GABA. Electrophysiological studies demonstrate the ability of ganaxolone to potentiate the GABA-evoked currents in Xenopus oocytes expressing the human $GABA_A$-receptor subunits a_1, β_1 and γ_{2L} [2].

In order to correlate activity with plasma levels and to understand the pharmacokinetics of ganaxolone, it was necessary to develop a sensitive and specific method for the determination of ganaxolone in animal plasma with the limit of quantification (LOQ) of at least 5 ng/ml. Due to lack of a chromophore and extremely poor UV absorbance of ganaxolone, the development of an assay based on high-performance liquid chromatography (HPLC) with UV absorption detection was not feasible. An HPLC–UV method was developed using derivatization of the 3-hydroxy group to increase the sensitivity and lower the limit of detection (LOD) but this method had poor reproducibility and was limiting in many ways. A GC–MS method was successfully validated and used in assaying for ganaxolone in rat and dog plasma, but the run time was 39 min. and LOQ was only 15 ng/ml.

An assay procedure with liquid phase extraction and gas chromatography (GC) with electron capture detection (ECD) was developed for selectively assaying ganaxolone in plasma samples. Electron capture detectability and specificity for ganaxolone was achieved by derivatization with pentafluorobenzyl hydroxylamine (PFBHA) in pyridine at 65°C for 1 h to form stable oximes. The procedure was used to quantitate plasma levels of unchanged ganaxolone following oral and parental administration to mice, rats, dogs and humans. Plasma samples (1 ml) containing ganaxolone and an internal standard (I.S.) were extracted with hexane and derivatization was conducted. The extraction recovery over the standard curve range averaged around 98% for both ganaxolone and I.S. The GC analysis was carried out on 10 μl of the reconstituted extracts injected on to a DB17 capillary column with hydrogen as carrier gas. GC column and oven temperature programs were used over a run time of ~35 min. The retention times of ganaxolone and I.S. were 23.5 min. and 26.0 min. respectively. The assay was linear over the concentration range of 4–500 ng/ml with a minimum quantifiable limit was 4 ng/ml using 1 ml of plasma. Intra-day and inter-day coefficients of variation averaged around 4.2%. The absolute percentage differences found in the accuracy determination were less than 10.1%. The derivatization procedure [3] increased the sensitivity and lowered the limit of detection (LOD).

The above methods are limited in terms of long run times, having to deal with issues of stability of the derivatized product and the inability to detect metabolites. An alternative and more sensitive assay for ganaxolone and its metabolites in plasma was evaluated using HPLC with MS–MS detection method [4] with a run time of $<$5 min. ESI–LC–MS–MS is the method of choice for many classes of steroid compounds and is more sensitive a method [5]. Ganaxolone was found to be very sensitive in negative ion mode ESI [6,7] but only at extremely high pH conditions (pH$>$12) and was poorly ionized in positive ion mode ESI. Ganaxolone predominantly formed sodium ion adducts in positive ion mode ESI and attempts to fragment this sodium adduct ion were unsuccessful. This difficulty in fragmenting ganaxolone–sodium adduct ions is consistent with the behavior of many other sodium adduct ions. However, the high pH condition was not ideal for chromatographic separation.

Steroids have been commonly analyzed using liquid–liquid extraction and APCI–LC–MS–MS [8,9]. Ganaxolone was found to be quite fragile in APCI interface due to the fact that APCI probes were typically operated at high temperatures (above 400°C). Subsequently, ganaxolone loses one molecule of water easily regardless of the conditions (such as lower probe and source temperature and lower cone voltage) and thus the conditions were carefully optimized to gain maximum sensitivity for the protonated molecular ion. This could be due to the fact that dehydration of ganaxolone would involve the formation of a stable tertiary carbocation, which makes it easier for ganaxolone to lose a molecular of water as compared to any secondary alcohol. We were therefore left with a choice of developing a method based on the dehydrated molecular ion. In one respect, this increased the specificity of the method, since it enhanced selectivity in the MS–MS detection.

Influence of eluent composition and ionization

efficiency has been extensively studied [10]. In the process of optimizing conditions for dehydrated ganaxolone, it was found that small amount of formic acid (0.05%, other acids such as acetic acid showed the same effect) helped the sensitivity. Additional amounts of acid (adding up to 0.5% formic acid in the mobile phase was tried) did not increase sensitivity. This effect could be explained easily, as the presence of small amount of acid could facilitate the dehydration process.

The objective of the studies was to develop a rugged, specific method with sufficient sensitivity that can be applied to analyze large amount of pre-clinical and clinical samples. A fully validated [11] bioanalytical method including inter and intra-day precision and accuracy was used to determine the pharmacokinetics of ganaxolone in rats, monkeys, dogs and human volunteers after oral administration.

2. Experimental

2.1. Materials

Ganaxolone (CCD 1042) was received from Diosynth (Oss, The Netherlands) and internal standard (I.S., P3830) 3β-hydroxy-5α-pregnan-20-one was purchased from Steraloids (Wilton, NH, USA) (Fig. 1). HPLC-grade solvents methanol (MeOH), hexane, formic acid and K_2-EDTA Vacutainer® tubes were obtained from VWR Scientific (Bridgeport, NJ, USA or Ville Mont-Royal, Quebec, Canada). Deionized water Type I, Elgastat UHQ-PS, was supplied by Elga (Northbrook, IL, USA). Nitrogen and refrigerated liquid nitrogen were obtained from Keen Compressed Gas (Wilmington, DE, USA). Blank Sprague–Dawley rat, Cynomolgus monkey and Beagle dog plasma containing EDTA as the anticoagulant were purchased from Cocalico Biologicals (Reamstown, PA, USA). Human plasma was Biologicals Specialties (Landsdale, PA, USA).

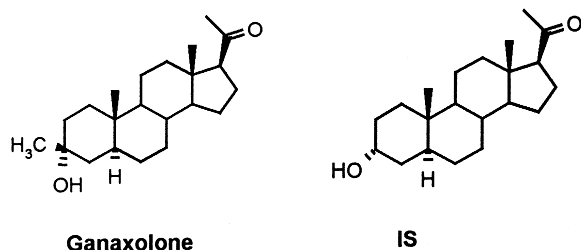

Fig. 1. Structures of ganaxolone and I.S.

2.2. Instrumentation

A Micromass Quattro II (Micromass, Beverly, MA, USA) triple quadruple mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface, a HP 1100 binary pump solvent-delivery system (Hewlett-Packard Company) along with a HP 1100 Vacuum Gegasser and HP 1100 Autoinjector was used for the HPLC–MS–MS analysis. The data were processed using MassLynx version 3.0 (Micromass) software.

2.3. Chromatographic conditions

The analysis was performed on a Genesis C_{18}, 4-μ, 30×3.0 mm analytical column (Jones Chromatography, Lakewood, CO, USA) and a mobile phase consisting of methanol–water–formic acid (80:20:0.05) at a flow-rate of 750 μl/min. The total run time was 3 min. Under these conditions the retention time of ganaxolone and the I.S. were 1.8 min and 1.1 min, respectively and well separated from the solvent front.

2.4. HPLC–MS–MS conditions

A Micromass Quattro II (Micromass, Inc.) mass spectrometer was interfaced via an APCI probe in the positive mode with the HPLC system. The source was maintained at a temperature of 150°C and APCI probe at 550°C with drying gas (nitrogen) at a flow-rate of 450 l/h and ionization was effected with a corona voltage of 3.85 kV. The sample-cone energy was set at 17 V, with skimmer at 1.5 V, skimmer lens at 5 V, RF lens at 0.2 V and APCI sheath gas pressure at 15 l/h. The vacuum running pressure was maintained at $\sim 1.5 \times 10^{-5}$ mBar. The mass spectrometer was programmed to monitor the transitions (MRM) m/z 315→m/z 297 (for ganaxolone) and m/z 301→m/z 283 (for I.S.), with collision induced fragmentation (collision gas argon at 9.0×10^{-4} mBar). The dwell time was 300 ms and the collision energy was set at 12 eV for ganaxolone and I.S. The electron multiplier setting was 650 V.

2.5. Stock solutions

2.5.1. Primary stock solutions SS-A and SS-B for standards

The primary stock solution SS-A (500 μg/ml) was prepared by carefully transferring 25.0 mg (accurately weighed on Denver Analytical Balance, Model M220-D, Denver Instrument Company, Arvada, CO, USA) of ganaxolone to a 50-ml volumetric flask and diluting to volume with methanol. The primary stock solution SS-B (20 μg/ml) was prepared by diluting 1 ml of stock solution A to 25 ml with MeOH. The solutions were stored at ≤−20°C.

2.5.2. Working stock solutions for standards (SS1–SS7)

The working stock solutions SS1–SS7 (5–1500 ng/ml) were prepared by diluting the stock solutions with methanol as shown in Table 1. The solutions were stored at ≤−20°C.

2.5.3. Internal standard stock solutions (I.S.-A and I.S.-B)

The primary stock solution I.S.-A (400 μg/ml) was prepared by carefully dissolving 10.0 mg of I.S. in 25 ml methanol with the aid of a volumetric flask. The working stock solution I.S.-B (2 μg/ml) was prepared by diluting 0.25 ml of stock solution I.S.-A to 50 ml with MeOH. The solutions were stored at ≤−20°C.

2.5.4. Primary stock solutions QC-A, QC-B and QC-C for quality control samples

The primary stock solution QC-A (1000 μg/ml) was prepared by carefully transferring 10.0 mg of ganaxolone to a 10-ml volumetric flask and diluting to volume with methanol. The primary stock solution QC-B (20 μg/ml) was prepared by diluting 1 ml of stock solution QC-A to 50 ml with MeOH. The primary stock solution QC-C (250 ng/ml) was prepared by diluting 0.125 ml of stock solution QC-B to 10 ml with MeOH. The solutions were stored at ≤−20°C.

2.6. Rat pharmacokinetics

Sprague–Dawley rats (Charles River Canada, Inc., Québec, Canada) catheterized in the femoral vein were dosed 10 mg/kg of ganaxolone as a βCD complex suspension (2.5 mg/ml) by oral gavage. Serial blood samples (approximately 0.5 ml) were collected at pre-dose, 30 min, 1, 2, 4, 6, 8, 12, 24 and 30 h post-dose. Following each blood sampling (except after the last sampling time point), each animal was administered via the cannula a volume of blood from the donor rats (consistent with the strain

Table 1
Preparation of working stock solutions for calibration standards and quality control plasma samples

From stock solution conc.	Take (ml)	QS w/MeOH (ml)	Working stock solution conc. (ng/ml)	QC plasma conc. (ng/ml)[a]	Stds. # / QCP #
20 μg/ml	0.75	10	1500	1500	SS7
20 μg/ml	0.75	10	–	1500	QCP4
20 μg/ml	0.5	10	1000	1000	SS6
20 μg/ml	0.25	10	500	500	SS5
20 μg/ml	0.125	10	250	250	SS4
20 μg/ml	0.125	10	–	250	QCP3
1000 ng/ml	0.5	10	50	50	SS3
1000 ng/ml	0.1	10	10	10	SS2
250 ng/ml	0.4	10	–	10	QCP2
500 ng/ml	0.1	10	5	5	SS1
250 ng/ml	0.2	10	–	5	QCP1

[a] Stock solution evaporated to dryness and 10 ml of pooled blank plasma added.

of rats), equal to the volume of blood withdrawn. The blood sample was collected in powdered K_2-EDTA Vacutainer® tubes and centrifuged at 1500 *g* for 10 min between 0 and 4°C. The plasma was then transferred to a 2-ml cyrostorage tube and stored at ≤−20°C until analyzed.

2.7. Monkey pharmacokinetics

Cynomolgus monkeys (Covance., Alice, TX, USA) were dosed ganaxolone by oral gavage as a βCD complex suspension (25 mg/ml) at a dose of 10 mg/kg. The animals were fasted overnight until 30 min prior to dosing. Serial blood samples (approximately 2.0 ml) were collected after oral dosing at pre-dose, 30 min and 1, 2, 4, 6, 8, 12 and 24 h post-dose. The blood sample was collected in powdered K_2-EDTA Vacutainer® tubes and centrifuged at 1500 *g* for 10 min between 0 and 4°C. The plasma was then transferred to a 2.0-ml cyrostorage tube and stored at ≤−20°C until analyzed.

2.8. Dog pharmacokinetics

Beagle dogs (Marshall Research Labs., North Rose, NY, USA) were dosed ganaxolone as a βCD complex suspension (2.5 mg/ml) by oral gavage at a dose of 10 mg/kg. The animals were fasted overnight and fed approximately 2 h after dosing. Serial blood samples (approximately 2–3 ml) were collected via venipuncture (jugular or cephalic) after oral dosing at pre-dose, 0.5, 1, 2, 4, 6, 8, 12, 24, 30, 48, 56 and 72 h post-dose. The blood sample was collected in powdered K_2-EDTA Vacutainer® tubes and centrifuged at 1500 *g* for 10 min between 0 and 4°C. The plasma was then transferred to a 2.0-ml cryogenic tube and stored at −70°C until analyzed.

2.9. Human pharmacokinetics

Human healthy volunteers were administered orally a 500 mg dose of ganaxolone. Sixteen blood samples for determination of the plasma concentration of ganaxolone were collected at pre-dose, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 16, 24, 36 and 48 h post-dose into EDTA Vacutainer tubes by venipuncture. Plasma was harvested from blood within 2 h by centrifugation at 1500 *g* for 10 min between 0 and 4°C. The plasma was then transferred to labeled, screw-top opaque 5 ml polypropylene tubes and stored at ≤−20°C until analyzed.

2.10. Sample preparation and extraction procedure

2.10.1. Preparation of quality control (QCP1–QCP4) plasma samples

The blank plasma was tested for interference prior to preparing standards or quality control samples. The plasma was stored frozen ≤−20°C until use. Known quantities of working stock solutions for QC samples (QC-B or QC-C) were transferred into 10-ml volumetric flasks and evaporate to dryness under a nitrogen steam (Turbo Vap LV evaporator, Zymark). They were reconstituted with 10.0 ml of blank rat, monkey, dog or human plasma and vortexed (Multi-tube vortexer, Model 12-810, VWR) gently for 30 min before sonicating for 10 min in a scintillation vial. The QC plasma samples (QCP1–QCP4) were stored in disposable screw top glass tubes as 200 μl aliquots at −20°C until required for analysis.

2.10.2. Preparation of plasma calibration standards (SP1–SP7)

Calibration standards were prepared in plasma at least 1 day before a study extraction. If this was not possible, they were quickly frozen in a dry ice–methanol solution and placed in a ≤−20°C freezer for at least 30 min. Plasma standards SP1–SP7 (in duplicate) were prepared by adding 200 μl aliquots of the working stock solutions (SS1–SS7) into separate 13×100 mm disposable screw-cap glass test tubes (VWR Scientific) with PTFE-lined caps (Fisher Scientific, Pittsburgh, PA) and evaporating off the solvent in a ≤40°C water bath under a stream of nitrogen. Blank EDTA plasma (200 μl) from one species was added and the tubes vortexed for 15 s, capped and stored in ≤−20°C freezer overnight. They were thawed 1 h prior to the run and spiked with 100 μl of I.S.-B working solution and vortexed for 15 s. Different sets of standards were prepared and run on three consecutive days for the assay validation.

2.10.3. Preparation of plasma blanks

Additionally, each standard curve included three blank samples. The plasma blank was prepared by transferring 200 μl of blank plasma into a disposable screw-cap glass test tube, spiked with 200 μl of methanol and vortexed for 15 s. The I.S. blank was prepared by transferring blank plasma (200 μl) and 100 μl of I.S.-B working solution to the tube and vortexed for 15 s. Lastly, the drug blank was prepared by spiking the tube with 200-μl aliquot of the working stock solution (SS7, 1500 ng/ml), evaporating it to dryness and then adding 200 μl of blank plasma, spiked with 200 μl of methanol and vortexed for 15 s.

2.10.4. Preparation of study samples

The study samples and a set of individually frozen aliquots of QC samples (QCP1–QCP4, Table 1) were thawed at room temperature for 1 h. Then 200 μl of study samples were transferred into disposable screw-cap glass test tubes. Where sample size was insufficient they were appropriately diluted with blank plasma. Lastly, 100 μl of I.S.-B working solution was spiked into each tube and vortexed for 15 s.

2.10.5. Plasma extraction procedure

To each of the plasma calibration standards, plasma blanks, study samples and QC samples was added hexane (5 ml) and the tubes shaken (Eberback Reciprocal Shaker, Two speed, VWR Scientific) for 15 min at high speed and then centrifuged (Marathon Bench top Refrigerated Centrifuge, Model 12KBR, Fisher Scientific) for 10 min at 1500 *g*. The tubes were then placed into a dry ice–methanol bath for 3 min and the bottom water layer frozen. The supernatant was transferred into properly labeled 13×100 glass culture tubes (VWR Scientific) and evaporated to dryness under a nitrogen stream at ≤40°C. The residue was reconstituted with 50 μl of mobile phase, vortexed for 1 min and then transferred to autosampler vials (autosampler glass vials with 200-μl conical glass inserts and snap caps, Pesce Lab Sales, Kennett Square, PA, USA) and arranged on the autosampler tray for LC–MS–MS analysis. Injections of 10/50 μl were made on the HPLC for analysis.

2.11. Precision, accuracy, specificity, sensitivity, recovery and stability

2.11.1. Specificity

Specificity was determined in blank plasma from six different lot numbers of each species that was extracted and analyzed on LC–MS–MS.

2.11.2. Sensitivity

Using 200 μl of plasma sample, the limit of quantification (LOQ), limit of detection (LOD) and signal-to-noise ratio were determined for ganaxolone.

2.11.3. Precision and accuracy

QC samples from at four concentration levels (5, 10, 250 and 1500 ng/ml) (QCP1–QCP4) were prepared for the determination of inter-day ($n=2$) and intra-day ($n=6$) reproducibility. The samples were extracted and analyzed on the same day during each of three consecutive runs and the peak-area ratios were compared to an independent standard curve for inter-day and intra-day precision and accuracy (during intra-day run).

2.11.4. Recovery

The extraction recovery of ganaxolone and I.S. was calculated by comparing the peak areas of extracted ganaxolone and I.S. plasma standard to the corresponding peak areas of unextracted standards of identical concentration in the same matrix. Recovery was performed on the second lowest standard (5 ng/ml) and the highest standard (1500 ng/ml), each in duplicate.

2.11.5. Stability

Bench-top stability of ganaxolone rat plasma standards was established at room temperature, over 96 h. Two sets of reconstituted standards were injected on the same day they were prepared. After injection, these samples were left on the bench for 4 days before being injected a second time.

Freeze/thaw stability for ganaxolone was done on the QC samples that had gone through at least three freeze–thaw cycles and analyzed in two runs.

2.12. Calibration

The peak area ratios of ganaxolone to I.S. were correlated with the standard concentration over the range of 5–1500 ng/ml in rat, monkey, dog and human plasma. After analysis, a standard curve for the compound was constructed by quadratic regression analysis with $1/X^2$; of weighting of peak area ratio (Y-axis) and compound concentration (X-axis) using MassLynx v3.0 software (Micromass).

2.13. Pharmacokinetic data analyses

Descriptive pharmacokinetic parameters [C_{max}, T_{max}, λ, $T_{1/2}$, $AUC_{0-T)}$ and $AUC_{(0-\infty)}$] were determined by standard model independent methods [12] based on the plasma concentration–time data. Results are presented as mean and standard deviation (SD), except $T_{1/2}$ which is expressed as harmonic mean and pseudo standard deviation based on jackknife variance [13]. All statistical and pharmacokinetic analyses were performed using Microsoft Excel Version 7.0 (Redmond, WA, USA).

3. Results and discussion

3.1. MS optimization

Even though ESI–LC–MS–MS is the more sensitive method of choice for many classes of steroid compounds, ganaxolone was found to sensitive only in the negative-ion mode at extremely high pH conditions (pH>12) which was not ideal for chromatographic separation and was poorly ionized in positive ion mode. Ganaxolone predominantly formed sodium ion adducts in positive-ion mode ESI and attempts to fragment this sodium adduct ion were unsuccessful like in the case of other sodium adduct ions. Ganaxolone lost one molecule of water easily that resulted in the formation of a stable tertiary carbocation and increased the specificity of the method. Formic acid (0.05%) helped the sensitivity, as it facilitated the dehydration process.

3.2. Linearity and calibration standard range

The peak area ratios of ganaxolone to I.S. were correlated with the standard concentration over the range of 5–1500 ng/ml in rat, monkey, dog and human plasma. The coefficient of determination (R^2) for the regression was greater than 0.9983 for rat, monkey, dog and human plasma (Table 2). Linearity of the calibration curves was demonstrated by calculating the mean absolute percentage difference and C.V. and both being $\leq\pm15\%$ considered as evidence of linearity. Excellent linearity was observed for each of the runs for rat, monkey, dog and human plasma, with mean absolute percentage difference ranging from 1.4 to 5.5% and C.V. from 1.5 to 7.8 (Table 3).

3.3. Specificity

LC–MS–MS analysis of blank plasma from five different lot numbers showed no endogenous peaks that interfered with the quantification of ganaxolone and its respective internal standard. Representative chromatograms of extracted blank dog plasma, with internal standard and with drug and internal standard are shown in Fig. 2. At the retention time of the I.S., a response is seen in the chromatographic trace for ganaxolone that arises from an impurity in the commercial supply of the I.S. It was separated from the ganaxolone and did not interfere with the quantification of ganaxolone.

Table 2
Regression analysis

Species	LOD (ng/ml)	LOQ (ng/ml)	Mean quadratic equation (weighted $1/X^2$)	R^2
Rat	0.5	5	$Y=6.10\times10^{-8}X^2+3.56\times10^{-3}X-5.55\times10^{-4}$	0.99836
Monkey	0.5	5	$Y=7.99\times10^{-8}X^2+3.02\times10^{-3}X-3.77\times10^{-4}$	0.99921
Dog	0.5	5	$Y=6.95\times10^{-8}X^2+2.64\times10^{-3}X-9.98\times10^{-4}$	0.99911
Human	0.5	5	$Y=2.11\times10^{-7}X^2+2.66\times10^{-3}X+1.23\times10^{-3}$	0.99887

Table 3
Linearity of calibration curves based on mean absolute % difference and % C.V. of back-calculated values of calibration standards

Spiked (ng/ml) (SP1–SP7)	Rat		Monkey		Dog		Human	
	Abs mean % diff.	% C.V.	Abs mean % diff.	% C.V.	Abs mean % diff.	% C.V.	Abs mean % diff.	% C.V.
5	2.7	3.3	5.3	6.6	4.3	6.1	2.3	3.2
10	5.3	5.2	5.5	7.8	3.0	4.1	3.2	4.3
50	2.6	3.3	3.0	3.4	2.0	3.4	3.7	2.7
250	1.6	1.5	3.6	4.2	5.0	6.3	3.3	3.3
500	4.0	2.7	3.3	3.6	3.1	2.6	2.7	2.7
1000	1.7	2.3	1.4	1.7	1.8	1.8	2.0	2.5
1500	2.1	2.8	1.8	2.2	1.4	1.6	1.9	2.2

3.4. Sensitivity

The limit of quantification (LOQ) for ganaxolone using 200 μl of dog plasma sample was 5 ng/ml, with a signal-to-noise ratio of approximately 80:1. The limit of detection for this study was determined to be <0.5 ng/ml, based on a ratio of 6:1. Concentrations that were calculated to be below 5 ng/ml for ganaxolone calibration standard curve range 5–1500 ng/ml were reported as below quantifiable limit (bql) (Table 2).

3.5. Reproducibility

3.5.1. Inter-day precision and accuracy determination

Analysis of the peak area ratios from QC samples ($n=2$) for ganaxolone during each of three consecu-

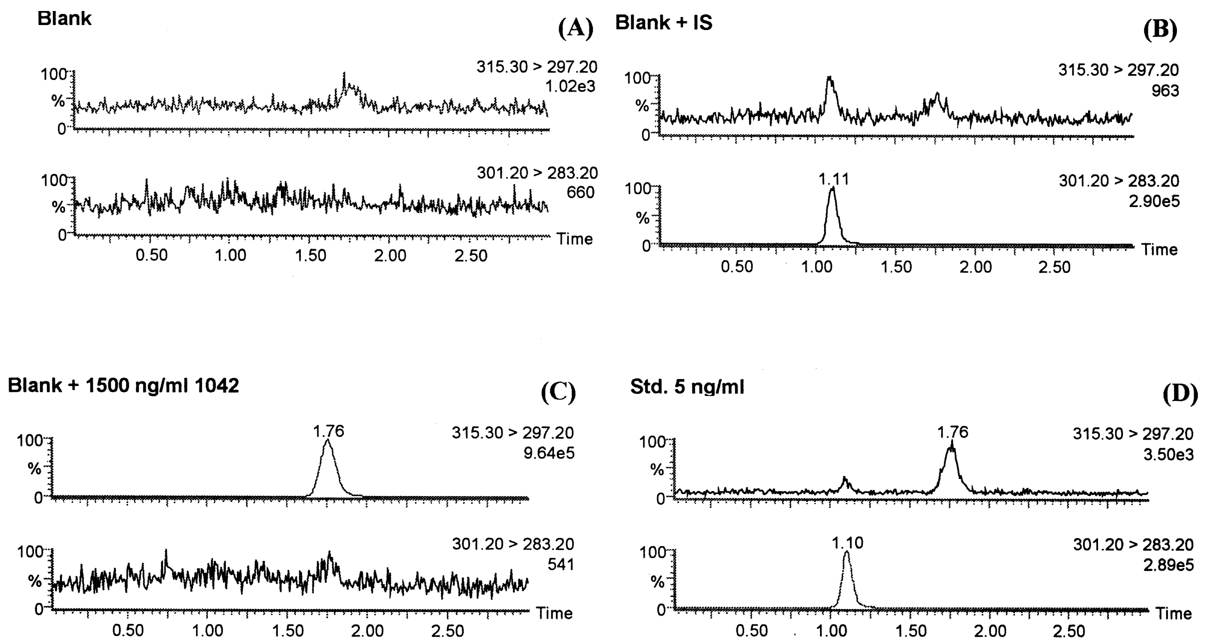

Fig. 2. Representative LC–MS–MS chromatograms from extracted blank human plasma (A), extracted blank human plasma with I.S. (B), extracted human plasma with I.S. (C) and extracted human plasma with I.S. and ganaxolone (5 ng/ml) (C). Internal standard and ganaxolone were monitored from m/z 301.20→283.20 and 315.30→297.20, respectively.

Table 4
Inter-day precision and accuracy for the quantification of ganaxolone in plasma (n=2)

Spiked (ng/ml) (QCP1–QCP4)	Rat				Monkey				Dog				Human			
	Found		Abs mean % diff.	% C.V.	Found		Abs mean % diff.	% C.V.	Found		Abs mean % diff.	% C.V.	Found		Abs mean % diff.	% C.V.
	Mean	SD			Mean	SD			Mean	SD			Mean	SD		
5	5.0	0.3	4.0	5.8	5.6	0.4	14.0	7.2	5.2	0.3	6.0	4.8	4.9	0.2	6.7	4.1
10	10.1	0.9	7.0	9.2	10.6	0.6	7.5	5.6	9.9	0.4	4.0	4.4	9.9	0.9	5.7	8.6
250	248.9	2.1	4.4	0.8	262.0	7.6	4.6	2.9	256.7	10.6	2.6	4.1	266.5	5.6	6.9	2.1
1500	1483.1	27.0	4.6	1.8	1528.8	8.1	1.2	0.5	1431.2	116.4	6.4	8.1	1545.2	33.3	3.5	2.2

tive runs when compared to an independent standard curve gave C.V. that ranged from 0.8 to 9.2%, 0.5 to 7.2%, 4.1 to 8.1% and 2.1 to 8.6% for rat, monkey, dog and human plasma, respectively (Table 4). The inter-day accuracy was determined as the mean absolute percentage difference between the spiked and found concentrations for ganaxolone that ranged from 4.4 to 7.0%, 1.2 to 14.0%, 2.6 to 6.4% and 3.5 to 6.9% for rat, monkey, dog and human plasma, respectively. Assay reproducibility was observed (values ≤±15%) over the range of 5–1500 ng/ml.

3.5.2. Intra-day precision and accuracy determination

The intra-day precision for ganaxolone was evaluated by analysis of plasma QC samples (n=6) along with an independent standard curve for quantification. The results summarized in Table 5 show C.V.s that ranged from 1.2 to 4.1%, 1.7 to 8.8, 1.4 to 5.1% and 0.7 to 6.0% for rat, monkey, dog and human plasma, respectively. The accuracy of the assays for ganaxolone was determined during the intra-day precision determination (Table 5). The mean absolute percentage difference between the spiked and found concentrations for ganaxolone ranged from 1.2 to 5.0%, 0.7 to 14.0%, 1.8 to 9.2 and 0.0 to 7.9% for rat, monkey, dog and human plasma, respectively. Assay reproducibility was observed (values ≤±15%) over the range of 5–1500 ng/ml.

3.6. Recovery

The mean recovery (Table 6) for ganaxolone ranged from 70.5 to 117.7% while that for I.S. ranged from 87.0 to 102.8% in rat, monkey, dog and human plasma. No concentration-dependent recovery was evident.

3.7. Stability

3.7.1. Bench top stability

There was no significant difference (≤±15%) between the responses of standards at time zero and after 96 h in terms of mean absolute percentage difference (1.0–7.9), indicating stability of ganaxolone in mobile phase at room temperature over 96 h.

Table 5
Intra-day precision and accuracy for the quantification of ganaxolone in plasma (n=5)

Spiked (ng/ml) (QCP1–QCP4)	Rat				Monkey				Dog				Human			
	Found		Abs mean % diff.	% C.V.	Found		Abs mean % diff.	% C.V.	Found		Abs mean % diff.	% C.V.	Found		Abs mean % diff.	% C.V.
	Mean	SD			Mean	SD			Mean	SD			Mean	SD		
5	4.9	0.2	2.0	4.1	5.7	0.5	14.0	8.8	5.4	0.1	8.0	1.9	5.0	0.3	0.0	6.0
10	9.5	0.2	5.0	2.1	10.8	0.7	8.0	6.5	9.8	0.5	2.0	5.1	9.9	0.3	1.0	3.0
250	246.7	8.7	1.3	3.5	269.7	5.8	7.9	2.2	254.6	6.0	1.8	2.4	269.7	4.1	7.9	1.5
1500	1482.1	17.2	1.2	1.2	1489.8	25.0	0.7	1.7	1362.6	19.5	9.2	1.4	1568.3	10.7	4.6	0.7

Table 6
Mean absolute recovery of ganaxolone and is in plasma ($n=2$)

Spiked (ng/ml)	Ganaxolone				I.S.			
	Rat	Monkey	Dog	Human	Rat	Monkey	Dog	Human
5	91.8	70.5	101.2	117.7	90.5	89.3	102.8	101.9
1500	88.8	80.4	88.0	107.0	92.0	87.0	86.3	97.6

3.7.2. Freeze–thaw stability in plasma

The mean absolute percentage difference between the spiked and found concentrations for ganaxolone in QC samples that had gone through at least three freeze–thaw cycles ranged from 0.7 to 7.7% for rat, monkey, dog and human plasma indicating freeze–thaw stability.

3.8. Pharmacokinetic study in mouse, rats, monkeys and dogs

The pharmacokinetics and absolute bioavailability of ganaxolone was determined in rats, monkeys, dogs and humans after oral dosing of ganaxolone (Table 7, Fig. 3).

Table 7
Oral pharmacokinetic parameters of ganaxolone in rats, monkeys, dogs and humans after a 10-mg/kg dose of Ganaxolone, mean±SD

Parameter	Rats	Monkeys	Dogs	Humans
Dose	10 mg/kg	10 mg/kg	10 mg/kg	500 mg
C_{max} (ng/ml)	37±36	28±14	1109±243	130±21
T_{max} (h)[a]	1.0 (1–2)	5.0 (2–6)	0.8 (0.5–1)	1.5 (1–2)
$AUC_{(0-inf)}$ (ng·h/ml)	126±124	232±92	12 875±5976	668±289
$T_{1/2}$ (h)[b]	3.1±1.8	4.9±1.0	25.0±5.7	6.1±7.3

[a] Expressed as median and range.
[b] Expressed as harmonic mean and pseudo SD based on jackknife variance.

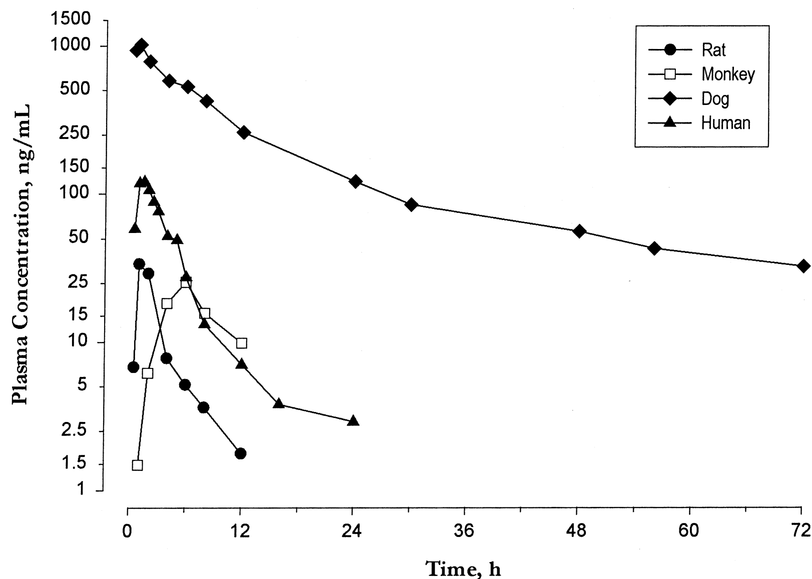

Fig. 3. Mean oral plasma concentration–time profiles of ganaxolone in rats, monkeys and dogs at 10 mg/kg and in humans after a 500-mg dose.

The plasma concentration–time curve in rats after oral dosing of 10 mg/kg showed a typical absorption profile. The plasma concentrations increased with time initially, reaching a maximum and then declined. The C_{max} values observed in male Sprague–Dawley rats were 37±36 with AUC values of 126±124 ng h/ml. The inter-animal variability of the plasma concentration–time profile gave rise to T_{max} values that ranged from 1 to 2 h and a mean terminal half-life of 3.1±1.8 h.

The plasma concentrations of ganaxolone in monkeys very extremely low with a C_{max} value of 28±14 ng/ml and AUC values of 232±92 ng h/ml. There was extensive inter-animal variability in terms of the plasma concentration–time profile giving rise to T_{max} values that ranged from 2 to 6 h and the terminal half-life was 4.9±1.0 h.

The plasma concentration–time curve in dogs after oral dosing of 10 mg/kg also showed a typical absorption profile. The plasma concentrations in dogs were much higher than in other species. They increased with time initially, reaching a maximum and then declined. The C_{max} values observed in male Beagle dogs were 1109±243 with AUC values of 12 875±5976 ng h/ml. T_{max} values ranged from 0.5 to 1 h with a mean terminal half-life of 25.0±5.7 h.

Following a single oral administration of 500 mg of ganaxolone to healthy female volunteers ganaxolone was detected in the majority of the plasma samples from each of the subjects and consistent with the absorption profile. Each subject had concentration–time profiles representing absorption and elimination. The mean C_{max} value was 130±21 ng/ml with a mean T_{max} value of 1.5 h and the AUC was 668±289 ng h/ml.

4. Conclusions

The liquid–liquid extraction method gave excellent recoveries of ganaxolone and I.S. and provided clean extracts. HPLC–MS–MS with APCI in the positive mode of detection appeared to be a very sensitive and selective method for the determination of ganaxolone and possibly its metabolites in rat, monkey, dog and human plasma.

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