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Pharmacokinetics of xylazine after 2, 4, and 6-hour durations of continuous rate infusions (CRI) in horses.

Running title: Pharmacokinetics of xylazine CRI in horses

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KEYWORDS

xylazine, pharmacokinetics, CRI, behavior, equine

Abbreviations

K_e = elimination rate constants

$t_{1/2e}$ = elimination half-life

V_c = volumes of central compartments

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Cl = clearance

AUC_0^∞ = area under the concentration-time curve

T_{max} = time to maximum concentration;

C_{max} = maximum plasma concentration

HR = heart rate

MAP = mean arterial pressure

Abstract

Intravenous (IV) bolus administration of xylazine (XYL) (0.5 mg/kg) immediately followed by a continuous rate infusion (CRI) of 1 mg/kg/h for 2, 4, and 6 h produced immediate sedation, which lasted throughout the duration of the CRI. Heart rate decreased and blood pressure increased significantly ($p > 0.05$) in all horses during the first 15 min of infusion, both returned to and then remained at baseline during the duration of the infusion. Compartmental models were used to investigate the pharmacokinetics of XYL administration. Plasma concentration-time curves following bolus and CRI were best described by a one-compartment model. No differences were found between pharmacokinetic estimates of the CRIs for the fractional elimination rate constant (K_e), half-life ($t_{1/2e}$), volume of distribution (V_d), and clearance (Cl). Median and range were 0.42 (0.15-0.97) /h, 1.68 (0.87-4.52) h, 5.85 (2.10-19.34) l/kg, and 28.7 (19.6-39.5) ml/min/kg, respectively. Significant differences were seen for area under the curve (AUC_0^∞) ($p < 0.0002$) and maximum concentration (C_{max}) ($p < 0.04$).

This indicates that with increasing duration of infusion, XYL may not accumulate in a clinically relevant way and hence no adjustments are required in a longer XYL CRI to maintain a constant level of sedation and a rapid recovery.

INTRODUCTION

The behavioral, sedative, analgesic, and cardiovascular effects of XYL were first described in the English literature in the 1960s (Clarke & Hall, 1969). Xylazine, 2-(2,6-dimethyl-phenylamino)-5,6 dihydro-4H-1,3-thiazine, serves as a prototype for a number of α_2 -adrenoceptor agonists and was the first in this group approved for use in horses (England & Clarke, 1996). The IV doses of XYL administered to the equine to obtain chemical restraint range from 0.5 to

1.1 mg/kg IV (Muir, 2009). The dose regimens used for a CRI range from 0.7 to 1 mg/kg/h after an IV bolus (Muller et al., 2012; Ringer et. al., 2012; Ringer et. al., 2013). Xylazine causes sedation primarily by stimulating the central nervous system presynaptic α_2 -adrenergic receptors. In addition, the drug has centrally mediated muscle relaxing properties and the intensity of these effects are dose-dependent (Kollias-Baker et. al., 1993). It produces an initial hypertensive response followed by a hypotensive phase (Hsu, Lu, & Hembrough, 1985). It also depresses cardiac minute work, mechanical index of myocardial oxygen demand, and coronary blood flow (Hennig, Court, & King, 1995). In the horse, following an IV administration, the maximum sedative effect occurs within 4 to 8 min.

The pharmacokinetic disposition in four domestic species has been described by 2-compartment elimination, small intraspecies differences, rapid absorption following IM administration, a rapid alpha distribution phase, and beta half-lives of 22 to 50 min (Garcia-Villar, Toutain, Alvinerie, & Ruckebusch, 1981). Significant differences were not seen following IV and intraosseous administrations (Santonastaso, Hardy, Cohen, & Fajt, 2014). In a more recent study, a 3-compartment disposition was described with beta and gamma half-lives of 2.79 and 26.0 h, respectively (Knych et al., 2017). Xylazine is rapidly metabolized and seven metabolites have been identified in urine of the horse, of which 4-hydroxy-XYL being the major metabolite (Spyridaki et al., 2004).

The purpose of this study was to define the pharmacokinetic properties of XYL when administered as a CRI over multiple duration times of 2, 4, and 6 h. We hypothesized that infusion times would have no impact on the elimination half-life.

MATERIAL & METHODS

Animals

The study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania, trial registry number 806338. Six university owned adult Thoroughbred horses including 3 geldings and 3 mares (age: 6 to 13 years; weight: 445 to 560 kg) were studied. Horses were determined healthy and free of disease by physical examination, complete blood count and a serum biochemistry panel. Food and water were available ad libitum throughout the study. No medications were administered to any horse for at least four weeks prior to the study.

Experimental design

Horses were assigned to three groups using a randomized cross-over design and received CRI treatments of 2, 4, and 6 h in duration. The washout time between treatments was 7 days. One hour prior to experimentation, horses were brought to a confined, climate-controlled area and restrained using a halter and a head rope. Two 14-gauge catheters (Angiocath, Becton Dickinson, UT, USA) were placed aseptically in both external jugular veins. One catheter was used for drug administration, while the contralateral was used for blood sampling. After being weighed, each horse received a 0.5 mg/kg IV bolus of XYL (X-Ject, Henry Schein, USA) followed by a CRI of 1 mg/kg/h of XYL for 2, 4, or 6 h.

Blood sampling

Blood samples were collected in Na-fluoride/K-oxalate anticoagulant (Johnson and Johnson, NJ, USA) before XYL bolus and CRI (baseline) and every hour during the CRI. Blood samples were also collected at 1, 2, 3, 6, 9, 12, 15, and 30 min, and 1, 2, 4, 6, 8, 12, and 24 h post CRI administration. Blood samples were centrifuged at 3000 g for 10 min. Plasma was immediately transferred into storage cryovials and stored at -80°C until analysis.

Physiological responses and behavioral monitoring

Horses were restrained for the duration of the CRIs. Physiologic and behavioral observations recorded. They included lowering of the head, shaking a can filled with marbles 3 feet away from the horse as an auditory stimulus, and firm touch of the operator against shoulder and neck as a tactile stimulus.

Heart rate (HR) and mean arterial blood pressure (MAP) were assessed before and 5, 10, 15 min and then every 15 min during drug administration. For MAP measurements an inflatable cuff was placed at the base of the tail (corrected to the level of the heart as described by Tunsmeijer et al., 2015) and the blood pressure was measured non-invasively using an oscillometric blood pressure measurement device (Dinamap 8100, GE Medical, Chicago, IL).

Quantification of XYL plasma concentration

Xylazine and xylazine- d_6 was purchased from Sigma-Aldrich (Sigma-Aldrich, MO, USA). Xylazine- d_6 was used as internal standard in the quantification analysis. Methyl tert-butyl ether (MTBE), ammonium hydroxide (57.6%) and optima grade water were purchased from EMD Chemical, Inc. (Gibbstown, NJ, USA). Formic acid (98%) was obtained from Merck KGaA

(Darmstadt, Germany). Methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA).

Sample preparation

Xylazine was extracted from equine plasma sample by liquid-liquid extraction using MTBE at neutral condition. Equine plasma samples (1.0 ml) were transferred into a 16 x 125 mm test tube and 10 μ l of XYL-*d*₆ IS solution was added. Five ml of MTBE was added, the tube capped and mixed on a roto-rack for 10 min prior to centrifugation at 3000 rpm (1610 x g) for 10 min. The resulting organic layer (top) in each tube was transferred to a pre-labeled culture tube and the content evaporated to dryness at 50° C (Techni Dri-Block DB-3, Duxford, Cambridge, UK) under a steady stream of air. The dried extract was reconstituted in 100 μ l of 5 mM ammonium formate:acetonitrile, (5:95, v/v) mixture from which 10 μ l was injected for LC-MS/MS analysis. If the concentration of a sample exceeded that of the upper calibrator (5.0 ng/ml), it was diluted with the blank plasma and re-analyzed.

Liquid chromatography-mass spectrometry

The liquid-chromatographic system was a Shimadzu Nexera X2 consisting of two binary pumps, a solvent degasser, an autosampler with sample compartment temperature at 40 °C and a column oven thermostatic at 40 °C (Shimadzu USA Manufacturing Inc., Canby, OR, USA). The mass spectrometer was a Sciex Qtrap 6500 instrument equipped with an IonDrive™ Turbo V electrospray ionization source (ESI) (AB Sciex LLC, Framingham, MA, USA).

The liquid-chromatographic separation was performed on a reversed-phase ACE C18 column (7.5 cm x 2.1 mm ID, 5 μ m particle size, (Mac-Mod Analytical, Chadds Ford, PA, USA) connected with a guard column (1 cm x 2.1 mm ID, 5 μ m particle size; (Mac-Mod Analytical, Chadds Ford, PA, USA). Mobile phase A consisted of 5 mM NH₄FA/FA, while mobile phase B was acetonitrile. The mobile phase was delivered at 0.3 ml/min flow rate by a gradient elution program as follows: 0 min, 80/20 (A/B); 4.0 min, 10/90 (A/B); 5.0 min, 10/90 (A/B); 5.1 min, 80/20 (A/B); 6.0 min, 80/20 (A/B). Total analysis time was 6 min.

The mass spectrometer was operated in positive electrospray ionization mode. The ion source was operated at 500°C and ion spray voltage was set at 5500 V. Curtain gases 1 and 2 were 40 and 65 psi, respectively. De-clustering potential and entrance potential were 60 and 10 V, respectively for all the analytes. The analytes were monitored using multiple reaction monitoring mode. The compound dependent parameters such as collision energy and collision

cell exit potential were optimized. Data acquisition and analysis were accomplished with Analyst software (v. 1.6.3; AB Sciex Foster city, CA, USA).

Quantification analysis

The MRM transitions of XYL and xylazine- d_6 (IS) for quantification analysis were m/z 221 $\rightarrow$ m/z 90 (CE 32; CXP 6) and m/z 227 $\rightarrow$ m/z 90 (CE 32; CXP 6), respectively. Calibration curve was constructed by plotting the peak area ratio of XYL vs. xylazine- d_6 (IS) against the concentration of XYL spiked in blank plasma. Linear regression model with $1/x^2$ weighting factor was used in describing the regression relationship. The quantification dynamic range was 0.01 to 5.0 ng/ml, with correlation coefficients (r) for the quadratic regression > 0.99 . Lower limits of quantification (LLOQ) of the method was 0.01 ng/ml. The assay was validated in horse plasma following FDA's Bioanalytical methods validation Guidance for industry¹. Two sets of quality control samples at three different concentrations: 0.2 ng/mL (low), 1.0 ng/mL (medium) and 10 ng/mL (high) were included at the beginning and the end of each assay. The accuracy ranged from 91.7% to 106% for concentration from 0.1 ng/mL to 10 ng/mL in equine plasma. The averaged accuracy was greater than 98.03%; also, intra-day and inter-day precision were less than 3.86%, and 5.48% (CV%) respectively.

Pharmacokinetic analysis

Compartmental analysis was used to describe the disposition and elimination of XYL following CRI administrations. Plasma concentration versus-time curves from each horse were analysed using conventional nonlinear least-squares regression analysis (Simulation, Analysis and Modeling Software, WinSaam.org). One and 2-compartment models were fitted to plasma concentrations of XYL. The model required to best describe the XYL plasma concentrations was based on the reduction in the sums of squares, minimization of fractional standard deviation of each compartmental parameter, and the converging of the observed and predicted plasma concentrations curves. The plasma concentrations used for the 3 infusions were the infusion time plus 24 h post infusion, for a total of 26, 28, and 30 h.

The apparent volume of central compartment (V_c), was calculated by the amount of drug in the body at time t , divided by the plasma concentration at time t (Toutain & Bousquet-Melou, 2004). The fractional standard deviation (FSD) was used for weighting the data ($W(K)$) in the fitting process. This was in the form of $W(K)=1/(C*QO(K)**2)$, where $QO(K)$ was the k th observed datum and C was the FSD. Data from each horse were iterated multiple times; reducing

the estimated FSD at each iteration until the sums of residual squares and the FSD of each compartmental parameter were no longer minimized and the observed and predicted lines converged. The fitting process (iterations) ceased when the improvement in the sums of squares of the residuals of the last iteration was $< 1\%$ (Wastney et. al., 1999). The elimination half-life (h) was calculated as natural log of two divided by the relevant elimination rate constant (K_e). The area under the concentration curve to the last sample (AUC_0^{lst}) was calculated by the linear trapezoid method. The area to infinite (AUC_0^∞) was the AUC_0^{lst} plus the end area correction calculated as $Cp_{(t^*)}/k_e$, where $Cp_{(t^*)}$, was last plasma concentration and K_e the elimination rate constants for the 2, 4, and 6 h CRI's (Riviere, 1999).

Maximum plasma concentration (C_{max}), and time to reach the maximum plasma concentration (T_{max}), were derived directly from the plasma concentration time-curves not the fitted curves.

Clearance (Cl_c) was calculated as:

$$Cl = \text{Total Dose} / AUC_0^\infty$$

Statistical analysis

Plasma concentrations, HR and MAP were expressed as mean $\pm$ standard deviation (SD). A two-way analysis of variance and Tukey's post hoc test were used for comparing the measured variables HR and MAP between treatment groups and by period-of-time (repeated measurements). Pharmacokinetic parameter estimates of XYL were expressed as median and range. Normality of the parameter values were determined by the Shapiro-Wilk W Test. Values normally distributed were compared using 2-way analysis of variance and differences in means using LSMeans Student's t test. Values not normally distributed (V_d , C_{max} , post CRI) were analyzed using the nonparametric Wilcoxon/Kruskal-Wallis Tests (JMP Statistical Discovery 13.2.1 Cary, NC). Significance was indicated when $p < 0.05$.

RESULTS

Physiological responses and behavioral changes

Xylazine induced sedation, including lowering of the head, wide based stance, and lack of arousal was noted in all horses upon bolus administration of XYL, and lasted throughout the CRI.

Compared to baseline, HR decreased significantly (group 2h $p < 0.002$, group 4h $p < 0.01$, group 6h $p < 0.003$) at 5, 10, and 15 min, and returned to baseline at 30 min in all three groups (**Figure 1**). A significant increase in MAP occurred for 30 minutes (group 2h $p < 0.01$, group 4h $p < 0.01$, group 6h $p < 0.005$) after the XYL bolus (**Figure 1**).

Pharmacokinetic analysis

The plasma concentration time curves of XYL following a bolus injection and CRI administrations for 2, 4, and 6 h were best described by a 1-compartment model (**Figure 2a, 2b, 2c**). Despite the durations of three different CRIs, significant differences were only seen for the AUC_0^∞ ($p < 0.0002$) and C_{max} ($p < 0.04$). Table 1 shows the median and range of values for each CRI. No significant differences were seen for K_e , $T_{1/2}$, V_c , and Cl . The total doses for the three CRI for 2, 4, and 6 h were 1362.5 ± 175.9 , 2452.5 ± 316.6 , and 3542.5 ± 457.3 mg, respectively.

DISCUSSION

Horses in all groups showed signs of deep and stable sedation after bolus administration of XYL and this lasted for the entire duration of drug infusion. Head height or nose-to-ground distance are commonly used in horses to assess degree of sedation (Muller et al., 2012; Ringer et al., 2013). The degree of sedation (no response to tactile or acoustic stimuli) suggest that horses would have had a lower head position for the entire infusion periods. To prevent horses from developing severe head and upper airway edema by having their head in a low position for up to 6 hours or longer, horses were cross tied in their stalls to keep the head and nose at the level of the shoulder joint or higher.

After bolus administration of XYL an increase in blood pressure was noticed for about 30 min. Alpha-2-agonists like XYL induce peripheral vasoconstriction by activating peripheral α -receptors and this vasoconstriction results in an increase in blood pressure. The arterial blood pressure response to α_2 -agonists after bolus administration is usually biphasic. An immediate hypertensive response is followed by a long-lasting (>60 min) decrease in MAP to below baseline values (Link et al., 1996). This decline in arterial blood pressure can be explained by the stimulation of α_2 -adrenoreceptors located in the brain stem producing a long-lasting decline in sympathetic nervous activity and hence vasomotor tone (Scheinin et al., 1994). In the study horses, blood pressure decreased after the initial peak increase but never dropped below baseline values. We assume that during the XYL infusion a balance between central depression of

noradrenergic neuronal activity and direct peripheral vasoconstriction by the α_2 -agonist was reached. Blood pressure measurements were discontinued at the end of the drug infusion. Therefore, no blood pressure data are available beyond the drug infusion period. One might speculate that arterial blood pressures might have decreased immediately after discontinuing the infusion of XYL with drug plasma concentrations decreasing and consequently direct stimulation of vascular α -receptors diminishing, while central depression continuing for some time. Simultaneously with the rise in blood pressure, HR decreased upon XYL bolus administration and then returned to baseline as soon as arterial blood pressures returned to baseline values. The decrease in HR after XYL administration is a well described baroreceptor-mediated response to the increase in arterial blood pressures that was observed initially after drug administration (Kirchheim, 1976). The baroreflex mechanism is a fast response to changes in blood pressure, which would explain why the HR returned to baseline after normalization of the blood pressure. For the blood pressure measurements, a non-invasive, oscillometric approach was used. Although invasive measurement techniques are considered being a gold standard for arterial blood pressure measurements and usually more reliable compared to non-invasive measurement techniques, multiple studies have shown acceptable reliability of oscillometric devices in sedated and anesthetized horses (Tunsmeyer, Hopster, Feige, & Kastner, 2015; Yamaoka et al., 2017). Further, it has been shown that non-invasive devices allow good estimation and differentiation of marked blood pressure trends (Heliczzer, Lorello, Casoni, & Navas de Solis, 2016).

Studies describing XYL plasma concentrations in the horse after CRIs of various durations have not been described. Following a IV bolus administration, the disposition of XYL has been described by 2-compartmental models (Garcia-Villar, Toutain, Alvinerie & Ruckerbusch, 1981; Santonastaso, Hardy, Cohn, Fajt, 2014) and recently by a 3-compartment model (Knych et al., 2017). This third compartment was defined based on the increased sensitivity of the analytical method employed. This third compartment, with a gamma half-life of 26 h, suggests that XYL was distributed to a deeper third compartment. The production and recovery of central nervous effects (i.e. sedation, central muscle relaxation, and analgesia), are related to the drug concentration in the central compartment; deeper compartments become relevant only when they contribute to maintaining the concentration high enough in the central compartment and the target organ to sustain the effect or prolong recovery.

In this study, the plasma concentrations during and following the 2, 4, and 6 h CRI periods were best described by a 1-compartment model and the addition of a second compartment to the model was attempted. Its addition worsened the indices of goodness-of-fit and was not verified based on the criteria used to define the compartmental rate-constants k_{12} and k_{21} .

The results of this study were contrary to what would be expected, longer duration of CRI should result in a longer period of recovery (Shafer & Varvel, 1991) and pharmacokinetic parameters influenced by the duration of infusion (Sano et al., 2006). Other aspects of this study were puzzling, inconsistency in values with increasing durations of CRI's and considerable variation in our data. The increase in AUC_0^∞ and C_{max} were significant but not proportional to dose, the 6 h CRI durations were not the highest concentrations. The predicted and observed data for each curve showed less than ideal conversion (Figures 2a, 2b, 2c). The end-of-infusion plasma concentrations determined from the plasma samples whether 2, 4, or 6 h in duration were within the same range of values. The median and ranges of the CRI's estimated pharmacokinetic and secondary parameters overlapped (Table 1). The authors have no definitive conclusions for these discrepancies, but a possible explanation. In our study few plasma samples ($n=3$) were hemolyzed during collection and when analyzed showed very high concentrations of xylazine. They were re-analyzed with the same results. These samples were not used in the study.

We suspect the discrepancies seen in this study may be related to the uptake of xylazine by the RBC. A drug related to xylazine, the alpha-2-agonist of adrenergic receptor romifidine, showed partitioning into RBC following a single IV bolus, with the RBC concentrations higher than plasma (Romagnoli et al., 2017).

The drug ketamine following a single IV bolus also showed uptake by RBC. The uptake of ketamine by RBC was reported in the dog following an intravenous administration (Roncada et al., 2006). An intravenous study in the horse reported similar concentrations of ketamine in RBC and plasma, but more rapid elimination by the RBC (Roncada et al., 2007). A later study reported higher concentrations in RBC which paralleled the plasma concentrations (Sori et al., 2013). It has been suggested that the role RBCs in pharmacokinetics is unclear and may be a storage for drugs (Hinderling, 1997). This possible uptake by RBC would explain the many puzzling aspects of this study. We were quantifying the plasma concentrations but could not account for a possible second parallel compartment.

Xylazine is rapidly metabolized in the horse and 7 phenolic metabolites have been identified in urine, the major being 4-hydroxy XYL. Following an IV bolus administration, XYL was detected in urine for only 3 h (Spyridaki et al., 2004). In a more recent study, following a bolus injection, XYL was detected in urine for only 4 h post injection, but the main metabolite 4-hydroxy XYL was detected for 96 h. The mean urinary concentration of 4-hydroxy xylazine at 4 h compared to XYL was 1538 ng/ml vs 4.9 ng/ml, which represents a ~300-fold difference. The plasma concentrations of XYL was ~100 fold higher than 4-hydroxy XYL (Knysch et al., 2017). The lack of differences in plasma concentrations following different CRI may also be explained by rapid metabolism to 4-hydroxy XYL and elimination of the metabolite.

In summary, this study shows that during a XYL 2-, 4- and 6-h CRI no difference in depth of sedation was noticed. In this study, the plasma concentrations during the 2, 4, and 6 h CRI's and post infusion periods were best described by a 1-compartment model. No differences were seen in the estimated pharmacokinetic parameters. This indicates that with increasing duration of infusion, XYL does not seem to accumulate in plasma or tissues and hence no adjustments are required in a longer xylazine CRI to maintain a constant level of sedation and a rapid recovery.

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CONFLICTS OF INTEREST

Authors have no conflicts of interest

AUTHORS CONTRIBUTIONS

All authors have read and approved the final manuscript. KH senior and corresponding author; LRS Pharmacokinetic analysis; XL quantification of plasma concentration; CHI study period coordination and assistance; RCB model review; BD study design and planning.

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Table 1: Pharmacokinetic parameters for xylazine (median and range) following intravenous administration of 0.5 mg/kg xylazine followed by a constant rate infusion (CRI) of 1 mg/kg/h for 2, 4, and 6 h (n = 6 horses).

Parameter	2 hours	4 hours	6 hours	Mean $\pm$ SD
K_e (/h)	0.53 (0.20-0.97)	0.36 (0.15-0.79)	0.39 (0.23-0.52)	0.45 ± 0.22
$T_{1/2}$ (h)	1.34 (0.71-3.52)	2.01 (0.87-4.52)	1.80 (1.34-2.98)	1.95 ± 1.04
Post (ng/ml)	398 (298-729)	411 (335-530)	462 (393-526)	440.4 ± 99.3
C_{max} (ng/ml)	398 (358-729) ^a	572 (348-864) ^b	503 (393-718) ^a	NA
T_{max} (h)	2	2.5 (1-4.25)	6.1 (2-6.17)	NA
V_c (l/kg)	3.65 (2.14-11.57)	8.43 (2.10-19.33)	5.96 (4.45-12.66)	7.07 ± 4.69
AUC_0^∞ (ng•h/ml)	1402.6 (1054.0-1850.7) ^f	3147.3 (2199.2-4878.9) ^g	4102.6 (2952.6-5526.3) ^g	NA
Cl (ml/min/kg)	29.7 (22.5-39.5)	23.9 (15.1-33.5)	26.6 (19.6-36.7)	40.56 ± 12.07
Cl (l/h/kg)	1.78 (1.35-2.37)	1.43 (0.92-2.05)	1.595 (1.18-2.20)	2.43 ± 0.72
Dose (mg)	1375 (1075-1675)	2200 (1720-2600)	3300 (2580-3900)	NA

K_e = fractional elimination rate constant; $T_{1/2}$ = elimination rate half-life; Post = plasma concentration at 2, 4, 6 h; C_{max} = maximum concentration; T_{max} = time to maximum concentration; V_c = volume of central compartment; AUC_0^∞ = area under the concentration curve Cl = clearance from central compartment; Dose = total dose

C_{max} - $p > 0.04$ a, b, a; AUC_0^∞ - $p > 0.0002$ f, g, g

Values with the same letter were not significantly different.

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