Dear JPTM Editors,

The manuscript was updated based on comments from the reviewers. Excellent suggestions were provided by the various reviewers. We hope the revised manuscript will be considered suitable for publication in the Annual Special Issue on Safety Pharmacology.

Yours sincerely,

The authors

Response to reviewer’s comments JPM-S-16-00072

The authors would like to thank the reviewers for the very valuable comments and suggestions. The manuscript was edited to include this important feedback.

Reviewer #1: This is an interesting manuscript on qualitative analysis of cardiovascular data. It also cannot be overlooked that it also represents a set of responses on contractility in the conscious rat. as the authors mention there is considerable interest in this topic at present.

There is a clear relationship between the index of contractility and heart rate. Conventional techniques would generally simply average each parameter over chosen time intervals. This contrasts with QT interval measurement in large animals and man where there is an attempt made to correct QT for heart rate changes. These rate corrections again are fraught with difficulty and many fail to adequately correct. Visualization of the clouds can make up for this deficit to some extent and some appreciation of the slope or shape of the relationship between the two parameters can be gained. However, the density of the data can pose an issue - sparse data a long way from the cloud can be over interpreted whilst the density within areas of the cloud is difficult to see. In SAS or JMP the density can be represented as elliptical or nonparametric contours to aid in interpretation but the use of the marginal distributions seems an easier means of adding that component. The separation of the data in to time blocks adds another layer to the potential value in interpretation. The heart rate data under control conditions and in the full 0-24 hours in
particular illustrates another issue with averaging. The marginal distribution shows more than one peak. Under those circumstances averaging would create a numerical value between the two peaks where in actual fact no physiological data exists. This could pose a problem for the correct interpretation of the effects and confound any potential interpretation.

Authors: Thanks for the valid perspective on contractility data analysis. We fully concur with the summary provided by the reviewer.

There are a few ways I believe the manuscript should be improved. In particular since the title suggests that marginal distribution is a main thrust of the paper it is odd that it only appears in the second-half of the discussion and its treatment in the results section is meager. The whole manuscript would be improved if there was some particular insight in to the action of these drugs only available when the marginal distributions. To that end it looks as if there may be some differences in the slope of the contractility versus HR relationships for some of the compounds. Having relatively short distributions along the x-axis means liner regression may not be the most useful technique. Is there something which can be done using the marginal distributions. Perhaps superimposing distributions for two drugs at doses having similar mean values in the 2-4 hour window but different marginal distributions? Whilst a reasonable amount of data is necessary to create reasonable marginal distributions the fact that this analysis uses beat-2-beat data and the heart rates often exceed 300bpm there would still be a large number of points allowing the marginal distribution to be scanned in time with a moving 30-minute average. Whilst this would be difficult to convey in a 2D paper presentation would such a scan as a movie of GIF file offer any further insight. At present the 2-4 hour window appears to be centered on the PD Tmax is there any information on the PK Tmax for these compounds?

Authors: The reviewer provided excellent comments on the application and limitations of marginal distribution. The discussion was modified to introduce to the concept of marginal distribution curves earlier. The selected window was based on the anticipated PD Tmax of the drugs. Similarly, PK Tmax was expected to occur during the selected window but no formal PK profile was generated in this study. The discussion was modified to present potential bioavailability limitations, especially with itraconazole which did not induce the expected effects on contractility.

Minor comments: there is a "cannot" where I believe it should read "can" in the discussion sentence "But the timing and duration of pharmacological effects........". Since the text mentions contractility index and dP/dt max can the authors clarify exactly what the contractility index is?
Authors: Thanks for the valuable comments. The manuscript was corrected or clarified to address the points that were raised. Contractility index was defined.

Reviewer #2: Cardiovascular (CV) testing in rodents is critical in drug discovery and lead candidate selection, especially as the focus on safety testing moves away from a QT prolongation centric view. The rodent offers a cost-effective model to answer a number of questions regarding CV safety. This manuscript presents important and necessary data on several positive control compounds. The methods and analysis are sound, in particular, the marginal distribution curves are a nice visual representation of multiple parameters.

Authors: We would like to thank the reviewer for the positive comments. The manuscript was edited to address the points that were raised.

Comments/suggestion:
1. The first paragraph of the discussion should be re-written or re-structured, perhaps broken into a few separate paragraphs. It appears to this reviewer to be a "run-on" paragraph, basically lumping discussion of all of the compounds together.

Authors: The first paragraph of the discussion was broken into a few separate paragraphs as suggested by the reviewer.

2. The predictability of a model is important and a false negative is troubling. It would be helpful if the authors could provide more discussion as to why intraconazole did not give the expected results. It is noted that two citations are provided in the discussion, but any additional speculation or insight would assist the reader in evaluating this model for preclinical safety testing.

Authors: This is a good point which deserves attention. The manuscript was edited to discuss the potential cause for the lack of effect observed with itraconazole.

Reviewer #3: Manuscript C01810 describes the use of marginal distribution methodology to data collected from telemetrized, conscious rats. The authors provide dose-dependent changes from a number of different, well characterized drugs. They evaluated drug effects on blood pressure, left-ventricular pressure, and the EKG and body temperature. The authors applied data cloud and developed marginal distribution curves to both heart rate
and contractility measures. The paper has some interesting data, interesting methods but I have some comments.

Authors: The comments from the reviewer are much appreciated and the manuscript was edited to address the points that were raised.

General Comments:

1. This manuscript describes the cardiovascular effects of a number of diverse compounds; however, my primary concern relates to no background information or true discussion about either cloud data or marginal distribution curves. I am certain the authors are well versed in analyzing data with these methods; however, many in Industry have only a peripheral, at best, understanding of these methods. The information described will be highly valuable to the industry regarding use of the these analysis processes but you will have to add some text to the Introduction about these methods, describe some details in the actual Methods section and follow up with how/why such data analysis should be conducted in the Discussion.

Authors: The manuscript was updated to add a paragraph in the discussion to introduce the concept of marginal distribution curve and its common applications. The last paragraph of the discuss also presents considerations related to this analysis process.

2. Does this methodology provide additional information to better inform with regard to the safety profiling of an NCE?

Authors: This is an excellent question. The authors believe that beat-to-beat cloud analysis with marginal distribution curve can be considered as a complement to LVP data analysis. Averaging which is typically used may dilute important characteristics of multivariate datasets including the relationship between parameters. Using beat-to-beat cloud analysis with marginal distribution curve enables the pharmacologist to evaluate these relationship.

3. Is this methodology applicable to dogs and NHP? Do you intend to conduct similar studies in these non-clinical safety pharmacology species?

Authors: Absolutely. The method can be applied to all LVP data. We presented similar dataset from dog and NHP at the 2016 SOT meeting.

Specific Comments:

1. Please paginate the manuscript
This was updated.

2. In the abstract - electrocardiography should be electrocardiogram

This was updated.

3. In the Introduction, paragraph 1 - "...over the last decade, providing safety pharmacologists with a ..."

This was updated.

4. I am confused with your statement: The rat remains the most frequently used model for regulatory drug safety assessments...?" Not in safety pharmacology, perhaps discovery pharmacology and regulatory toxicology as the primary non-rodent species. Please modify as appropriate for the safety pharmacology audience.

This was updated.

5. "...well established cv testing model.." - The rat is used only for hemodynamic assessments, not for ECG evaluation or characterizing safety profiles - in safety pharmacology.

The rat is mostly used for hemodynamic assessments but PR and QRS effects are also evaluated in rats in some cases.

6. Describe clouds and marginal distribution curves in the text. Even a definition of marginal distribution would help (note I read Wikipedia but that did not help!)

This was updated.

7. I have read that 'Marginal distributions tell us nothing about the relationship between two variables' and rather a Conditional distribution of a variable may be more appropriate as it actually describes the values of the variable among individuals who have a specific value of another variable. Is this correct?

The marginal distribution is a simple methodology to assess the relationship between two variables. Conditional distribution of a variable is a statistical analysis that statistically assesses the relationship between 2 (or more variables). Both methodology could be applied to safety pharmacology.

8. Why were animals 22 weeks old used? These are not standard age/weight?
Larger animals have a higher success rate for LVP surgery.

9. How were doses selected for testing?

Doses were selected based on data obtained in our facility with rats over the last 10 years along with discussion with colleagues, safety pharmacologists.

10. Note that in the manuscript text use Figure and not Fig.

Noted. It seems JPM accepts both Figure and Fig.

11. Would not 'observed time' in Figure 5 be better presented at hours rather than minutes?

Our standard display is to present the x-axis as minutes. If the reviewer has a strong preference for hours to be displayed, we can update.

12. In the discussion, paragraph 1 - "The absence of an inotropic effect..."

This was updated.

13. Good discussion about drug effects, stats designs etc but please add some information about marginal distribution. Since this is the first paper, that I am aware of using this method in this manner, some information about the relation of this parameter to standard parameters should be discussed.

The discussion was updated to included additional information on marginal distribution. Possible advantages when compared to analysis using data bins are now presented.

14. Please format all references according to Journal standard (you need to spell out the entire name of the journal and italicize) as follows:


15. In Figure 5 - the lines are very thick and obscure the symbols, please correct.

The line style was adjusted. Note that no symbol was used in this figure.
16. In Table 1 - just add some info about dose selection.

This was updated.

We would like to thank the reviewers, once again for the valuable feedback and suggestions on the manuscript.

The authors
Rat Cardiovascular Telemetry: Marginal Distribution Applied to Positive Control Drugs

M. V. Accardi\textsuperscript{1}, E. Troncy\textsuperscript{2}, S. Abtout\textsuperscript{1}, A. Ascah\textsuperscript{1}, S. Maghezzi\textsuperscript{1}, S. Authier\textsuperscript{1,2}

\textsuperscript{1}CiToxLAB North America, 445 Armand Frappier, Laval, QC, Canada, H7V 4B3
\textsuperscript{2}Faculty of Veterinary Medicine, University of Montreal, P.O. box 5000, St-Hyacinthe, QC, Canada, J2S 7C6

\textbf{Author of Correspondence:}

Simon Authier, DVM, MBA, PhD, DSP
CiToxLAB North America
445 Armand Frappier
Laval, Quebec, Canada
H7V 4B3
\textbf{E-mail:} authiers@ca.citoxlab.com
Abstract

Cardiovascular effects are considered frequent during drug safety testing. This investigation aimed to characterize the pharmacological response of the conscious telemetered rat in vivo model to known cardiovascular active agents. These effects were analyzed using statistical analysis and cloud representation with marginal distribution curves for the contractility index and heart rate as to assess the effect relationship between cardiac variables. Arterial blood pressure, left ventricular pressure, electrocardiogram and body temperature were monitored. The application of data cloud with marginal distribution curves to heart rate and contractility index provided an interesting tactic during the interpretation of drug-induced changes particularly during selective time resolution (i.e. marginal distribution curves restricted to $T_{\text{max}}$). Taken together, the present data suggests that marginal distribution curves can be a valuable interpretation strategy when using the rat cardiovascular telemetry model to detect drug-induced cardiovascular effects. Marginal distribution curves could also be considered during the interpretation of other inter-dependent parameters in safety pharmacology studies.
1. Introduction

Cardiovascular monitoring methods using telemetry have become an important component in preclinical safety assessments over the last decade, providing pharmacologists a simple but valuable tool to evaluate drug safety. Telemetry is widely employed to monitor a variety of cardiovascular parameters such as blood pressure, heart rate (HR), electrocardiogram (ECG), and body temperature (BT), among others, in awake, and freely moving laboratory animals (Kramer & Kinter, 2003). These systems are typically employed in regulatory safety pharmacology studies as detailed in the S7A guideline from the International Conference of Harmonisation (ICH) (FDA, 2001). The use of telemetry systems in safety assessments has since seen comprehensive implementation and validation in a variety of preclinical animal models (e.g., rodents, minipigs, dogs and nonhuman primates) (Authier et al., 2007; Authier et al. 2011; Deveney et al. 1998; Gauvin et al. 2006; Gelzer & Ball, 1997; Kramer & Kinter, 2003; Markert, et al., 2009; Segreti et al., 2016; Shiotani et al., 2007). While a broad range of species is available, the rat remains the most frequently used model for regulatory toxicology assessments (Gad, 2012) and a well-established preclinical cardiovascular safety testing model (Guth, 2007; Jacob, 2010). Apart from the obvious difference in size, the human and rat heart share similar morphological and physiological features including similar systolic, mean and diastolic pressures (Wessels & Sedmera, 2003). Moreover, the mRNA and expression profile of major cardiac ion channel proteins in both the atria and ventricle of rats and humans are also similar with the noted exception of the I Kr or human ether-à-go-go-related gene potassium channel (hERG)-like current which is absent in rats (Wymore, et al., 1997). The use of telemetry cardiovascular monitoring in rats has been employed and successfully validated (Brockway, Mills, & Kramer, 1998; Deveney, et al., 1998; Kramer, et al., 2001; Kramer & Kinter, 2003; Kramer & Remie, 2005) demonstrating expected cardiovascular and ECG modifications in responses to various cardioactive agents. However, the available scientific literature with rat telemetry presents limited consideration for agents inducing inotropic effects. Given this, we aimed to assess the pharmacodynamics response of telemetered rats to known pharmacological agents and document the corresponding electrocardiographic, hemodynamic, chronotropic and inotropic effects. In doing so, we provide a better understanding of this pre-clinical model as well as offer a
qualitative approach to beat-to-beat hemodynamic data presentation through the use of marginal
distribution curves.

2. Materials and Methods

2.1. Statement on use and care of animals
During this investigation, care and use of animals were conducted in accordance with principles
outlined in the current Guide to the Care and Use of Experimental Animals published by the
Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals
published by the Institute of Laboratory Animal Resources. CiToxLAB’s facility is AAALAC
accredited and the procedures were reviewed and approved by the Institutional Animal Care and
Use Committee prior to conduct. All procedures were conducted as per Standard Operating
Procedures (SOPs) in place.

2.2. Animals and environment
All animals were housed under standard laboratory conditions with controlled temperature (21 ±
3 °C), humidity (30%–70%), 12 h light/dark cycle and 10–15 air changes per hour. Temperature
and relative humidity were monitored continuously. The animals were provided a standard
certified commercial chow (Harlan Teklad Certified Global Rodent Diet #2018C) and municipal
tap water (which has been exposed to ultraviolet light and purified by reverse osmosis) via water
bottles ad libitum. Male Sprague-Dawley rats (Charles River Laboratories, St-Constant, QC),
aged approximately 22 weeks old and weighing approximately 500 g at the beginning of the
study were used.

2.3. Surgical instrumentation

2.3.1. General anesthesia and surgical preparation
Penicillin G procaine (Procillin, 300 000IU/mL, Vetoquinol, Lavaltrie, Quebec, Canada) and
Buprenorphine (Temgesic™, 0.05 mg/kg, Schering-Plough, Welwyn Garden City, Hertfordshire,
UK), were administered by subcutaneous injection prophylactically. General anesthesia was
induced with 2–4% oxygen-isoflurane (AErrane™, Baxter Corporation, Mississauga, ON,
Canada) mixture and tracheal intubation was done with mechanical ventilation at a respiratory
rate of 60-85/minute and tidal volume of 2-2.5 ml. Anesthesia was maintained for the duration of the surgery and rats were closely monitored for the depth of anesthesia. Rats were placed on a heated surgical field in dorsal recumbency. The surgical sites were shaved and the skin was aseptically prepared and draped with sterile gauze.

2.3.2. Surgical procedure

All animals underwent surgery for telemetry transmitter implantation (Data Science International (DSI), Model DSI HD-S21) to monitor blood pressures, ECG, BT and locomotor activity. Surgeries were performed by a ventral midline incision on the linea alba using aseptic techniques. The telemetry transmitter was implanted in the left upper quadrant of abdominal cavity, parallel to the long axis of the body. The transmitter body was fixed to the abdominal wall using suture ribs (4-0 polypropylene). Local analgesics, bupivacaine (0.25%, 0.05 ml; Hospira, Montreal, Quebec, Canada) and lidocaine (20 mg/ml, 0.05 ml; Lurocaine, Vetoquinol, Lavaltrie, Quebec, Canada), were mixed and injected subcutaneously at the abdominal incision. The arterial blood pressure catheter was inserted into one of the iliac arteries and the electrocardiographic leads were implanted subcutaneously across the sternum in a Lead II configuration. For left ventricle pressure catheter placement, a 3-0 nylon suture was inserted through the xiphoid and retracted anteriorly to lift the thoracic cavity to allow maximum exposure of the diaphragm. A midline vertical incision was made in the diaphragm and a 5-0 propylene suture was passed through each side to retract the diaphragm, exposing the heart. The apex of the heart was located and the left ventricle was subsequently punctured with a 23 G needle and the tip of the telemetry pressure catheter was inserted into the ventricle up to the suture rib (approx. 8 mm). The catheter was fixed in position by tightening the purse string suture and secured. Once the catheters and lead placements were finalized, the diaphragm was sutured ensuring that the catheter exited the thoracic cavity through the diaphragm at the dorsal end of the incision. Air was aspirated from the thorax via a 25 G butterfly attached to 5 ml syringe inserted in the intercostals space. Negative pressure was re-established prior to removal of the syringe.

2.3.3. Post-surgical recovery

The abdominal site was flushed with warm saline and the incisions were closed with absorbable suture material (Novafil 4-0; Covidien, Saint-Laurent, QC) using simple continuous sutures. The
skin was closed with discontinuous buried sutures using absorbable suture material (Vicryl Polyglactin 4-0; Ethicon, Johnson & Johnson, Somerville, NJ, USA). Instrumented rats were single housed in their home cages and telemetry recordings commenced immediately. Penicillin G procaine and buprenorphine were subcutaneously injected BID for two days following the surgery. Objective end-points to body weight change, mobility were monitored cautiously for the first week post-surgery. If required, supplemental analgesia (buprenorphine) would be used.

2.4. Experimental design, data acquisition and cardiovascular monitoring
A range of pharmacological agents either negative (i.e. saline (IV/SC) or water (PO)), or positive (remifentanil, flecainide, dopamine, pimobendan, morphine, amrinone, atenolol, and itraconazole) control agents was selected to characterize the rat telemetry model (Table 1). All treatments were administered precisely at the same time of the day with at least 3 days of wash-out between doses. The staff was not allowed to enter the animal room during data acquisition. A negative control (saline or water treatment) using the same dose volume and administration route was administered 2 days prior to positive control administration for within-time comparison with all drugs. Positive chronotropic and inotropic drugs were selected to induce a battery of ECG and hemodynamic changes, and for several drugs, different doses were used for testing potential dose-effect (Table 1). Intravenous injections and infusions were performed using remote dosing from outside of the cage with a permanent catheter to avoid artifacts due to handling stress. Cardiovascular function including systemic arterial blood pressure (diastolic, mean and systolic SABP), left ventricular pressure (systolic, end diastolic LVP, dP/dt+ and contractility index) ECG (HR, intervals PR, QRS and QT), BT and locomotor activity were monitored using the radiotelemetry data acquisition program Dataquest ART (Version 4.39, DSI). Contractility index is defined as dP/dt+ divided by the pressure at that point. ECG analysis was conducted using semi-automated methods by a single reader to minimize variability (Authier et al., 2010). Cardiovascular parameters were recorded continuously for a period of at least 1 hour pre-dosing and for at least 24 hours post-dosing every 5 s for the duration of the recording. Data were analyzed and presented using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Marginal distribution curves using beat-to-beat contractility data were prepared using the Origin 2015 software (OriginLab, Northampton, MA, USA) for qualitative data interpretation. Within-
time statistical comparison was completed using an ANOVA for repeated measures (SAS 9.3, Cary, NC) with Dunnett’s post-hoc tests.
3. Results

3.1. Cardiovascular monitoring following drug exposure

3.1.1. Remifentanil

Following intravenous injection of remifentanil (n=4), there was an immediate decrease in BT of 4±0.5% (~1.5°C, p<0.05) that persisted for approximately 1.5 h. An immediate and severe bradycardia also developed shortly after remifentanil administration (slowing of up to ~71% baseline HR), showing a compensatory increase after 3 min (p<0.05) and slowly returning to control HR levels after only 30 min (Figure 1). This transient change in HR was also accompanied by a decrease in SABP (-15±5% decrease, p<0.05) and a prolongation of the QRS interval (up to 2 msec, p<0.05). It is noteworthy to mention that after saline injections, the HR (Figure 1) and SABP showed a slight increase when compared with the baseline (pre-dosing) values – an observation considered as a normal consequence after IV injections of room temperature saline. No other significant change was noted for ECG, LVP and locomotor activity.

3.1.2. Flecainide

Following intravenous injection of flecainide (n=4), there was an immediate and severe decrease in BT of 4±0.5% (~1.4°C, p<0.05), which recovered to baseline levels at approximately 2 h. There were also noticeable increases in intervals PR (+181%±4, p<0.01), QRS (+94%±4 p<0.01), and QT (+121%±4, p<0.01) intervals, compared to baseline, the latter two of which occurred periodically for up to 8 and 16 h post-dosing, respectively (Figure 2). A mild increase in HR (+11±1%) was noted but did not reach statistical significance.

3.1.3. Dopamine

Single intravenous dosing of dopamine (n=4) had an immediate effect on HR leading to an elevation by 18±4% (p<0.05) from baseline (Figure 3A). Dopamine also largely increased systolic, diastolic and mean SABP (p<0.05), as well as pulse pressure by 27±4% (Figure 3A). Dopamine also presented positive inotropic effects on the contractility index (Figure 3B). Marginal distribution curves including the data at 0 – 0.25 h post-dosing was optimal to illustrate pharmacodynamics when compared to the entire 24 h period post-dosing.
3.1.4. Pimobendan

After oral administration of pimobendan (n=8), an immediate decrease in BT was observed which reached 35.8°C (-4±0.4% at 30 mg/kg, p<0.05) at 3 h post-dosing and progressively returned to baseline values at 14 h. A progressive decrease in systolic SABP at the high dose level (30 mg/kg) was also observed, when compared to the control, which started ~1 h post-dosing, reached nadir at 8 h post-dosing (-11±4%, p<0.05) and returning to basal levels thereafter with total recovery noted at 16 h post-dosing. This effect on systolic SABP was associated with an increase in HR (+37±3% at 30 mg/kg, p<0.05) during the same period. Mild effects were also observed on HR at both the mid (10 mg/kg) and low (3 mg/kg) doses, but these effects were not associated with any significant decrease in SABP. A clear dose-dependent increase in dP/dt max (+86±10% at 30 mg/kg, p<0.01) and contractility index (+85±7% at 30 mg/kg, p<0.01) was noted following administration of pimobendan (Figure 4A). Maximal effects are illustrated with marginal distribution curves restricted around T_{max} (Figure 4A, right) at 2 – 4 h post-dosing.

3.1.5. Morphine

Subcutaneous administration of morphine (n=9) was associated with an immediate and dose-dependent increase in BT lasting between 4-8 h reaching a maximum of 2.5±0.2°C (p<0.01), compared to the control. A trend (not statistically significant) towards dose dependent increases in systolic LVP, systolic, mean and diastolic SABP was also immediately noticeable after dosing, peaking at approximately 2 h and returning to basal levels at 4 h post-dosing. At the high dose (20 mg/kg) an increase in HR was observed immediately after dosing reaching a peak at 3.5 h (+51±5%, p<0.01) and returning to basal levels at approximately 10 h post-dosing. A similar, albeit less severe, elevation in HR was also observed at 2 and 6 mg/kg, both of which returned to basal levels at ~ 6 h post-dosing. The contractility index (+71±9%, at 20 mg/kg at 4 h post-Rx, p<0.01) and dP/dt max (+83±11%, at 20 mg/kg at 4 h post-Rx, p<0.01) parameters were increased in a dose dependent manner. The marginal distribution curve of morphine’s effects on HR and contractility index (Figure 4B) illustrates a trend towards dose dependent, positive chronotropic effect (considered secondary to central nervous system stress) with HR-mediated
positive inotropic effects – an effect further emphasized when analysis is restricted to $T_{\text{max}}$ (Figure 4A, right).

3.1.6. Amrinone
Oral administration of amrinone (n=8) was associated with an increase in dP/dt max (+36±7% at 100 mg/kg, p<0.05) and contractility index (+31±8% at 100 mg/kg, p<0.05) with peak effect at 10 h post-dose. An increase in HR was noted at the high dose with peak effect at 3 h post-dose (+24±5%, p<0.05). No significant effect on SABP was noted. The marginal distribution curve of amrinone at $T_{\text{max}}$ (Figure 4C, right) showed a clear dose dependent positive inotropic effect.

3.1.7. Atenolol
When compared to control, oral administration of atenolol (n=9) at 10 and 100 mg/kg induced a progressive decrease in HR, diastolic SABP, dP/dt max, contractility index and systolic LVP (Fig. 5). At high dose (100 mg/kg), the peak effect on HR (-32±2%, p<0.01), diastolic SABP (-17±4%, p<0.01), dP/dt max (-36±3%, p<0.05) and contractility index (-17±4%, p<0.05) was noted around 16 h (960 min) post-dosing (Fig. 5). At 10 mg/kg, a trend towards lower diastolic SABP (-10±4%) was noted at 2 h post-dosing, while HR showed a mild but long lasting decrease from 2 to 13 h post-dose with a peak effect (-11±5%) at 9 h post-dosing (Fig. 5). No significant effect on HR and SABP was noted with atenolol at the lower doses (i.e. 1 and 3 mg/kg). When HR and contractility index were presented with marginal distribution curves (Figure 6A), atenolol’s negative chronotropic effects were evident at 100 mg/kg at 0 – 24 h (Figure 6A, left) and present for all doses at 2 – 4 h (Figure 6A, right) compared to saline.

3.1.8. Itraconazole
Unlike the other positive and negative control drugs used within this study, itraconazole (n=8) did not yield the expected negative inotropic effects at any dose level tested. Changes were considered to reflect inter-individual variations (Figure 6B). In this figure, neither time point (0 – 24 h nor 2 – 4 h) showed any noteworthy inotropic effects of itraconazole at any dose level.
4. Discussion

Marginal distribution curves, sometimes referred as border plots, are distribution curves that are added parallel to scatter plot axes to illustrate the data density across the range that is graphically presented. Marginal distribution curves are seldom used in safety pharmacology but were applied to various disciplines including statistical astronomy (Trumpler & Weaver, 1962) and computer science (Guo et al., 2015). In the current study, we explored the application of marginal distribution curves to safety pharmacology data focusing on cardiac contractility and heart rate relationships. Marginal distribution curves are typically applied to multivariate functional data with spatially heterogeneous shape characteristics (Staicu et al., 2012) which is commonly observed in safety pharmacology.

It is common knowledge that unlike other species used in cardiac safety testing (Nerbonne, 2000), rats possess little to no ventricular hERG-like current (Wymore, et al., 1997) making them unsuitable for QT interval assessments (Sanguinetti & Tristani-Firouzi, 2006). It remains that a range of other clinically relevant drug-induced cardiovascular effects can be identified with the rat model such as effects on arterial blood pressures, HR, ventricular pressures, PR and QRS intervals. As presented in the result section, expected drug effects were noted in this rat telemetry model with all positive drugs with the exception of itraconazole.

Flecainide induced an increase in the atrioventricular conduction (PR interval) and QRS interval duration (Salerno et al., 1986; Heath et al., 2011), two clinically relevant ECG parameters in drug safety. Chronotropic, inotropic and hemodynamic effects of dopamine were comparable to results observed in isolated rat hearts (Zausig et al, 2010). These effects were also comparable to results reported in the non-human primates (Authier et al. 2007) using telemetry.

The typical cardiovascular response to morphine in critically ill patients is a decrease in HR and arterial blood pressure (Rouby et al., 1981). Conversely, opioids were reported to increase HR and arterial pressures when administered to conscious cynomolgus (Authier et al., 2007) and humans (Glass et al., 1993). Morphine was reported to increase sympathetic nerve activity in healthy human volunteers (Carter et al., 2002). When studied in healthy volunteers, morphine was reported to induce a biphasic effect on venous tone with an initial contraction followed by venous relaxation with a reflex reduction in sympathetic alpha adrenergic tone (Zelis et al., 1974). In the current study, morphine induced an increase in HR, left ventricular
contractility with a trend for increasing SABP. Similar effects on systolic SABP had been reported following morphine administration in rats previously (Amagasa et al., 1996), but the effects on HR were not reported and left ventricular contractility was not monitored. The lack of effect on HR may be related to the lower morphine doses that had been used by Amagasa et al. (1996).

Positive inotropic effects of amrinone were identified in isolated rat hearts (Chevalier et al., 1987), in conscious rats (Derakhchan & Vargas, 2015), anesthetized dogs (Pagel et al., 1993) and conscious dogs (Guth et al., 2015). In the current study, dose-dependent positive chronotropic and inotropic effects of amrinone were identified as illustrated by marginal distribution curves (Fig. 4C). The chronotropic and inotropic effects of atenolol (3 mg/kg, PO) in conscious rats were absent when animals were maintained at a room temperature of 30°C (Derakhchan et al., 2015). Similarly, our results did not identify any significant effect of atenolol at low doses (i.e. 1 and 3 mg/kg, PO) on HR or contractility when animals were kept at a room temperature of 21°C. In contrast, we observed significant negative chronotropic and inotropic effects of atenolol at higher doses (i.e. 10 and mostly 100 mg/kg, PO) in the same environmental conditions (i.e. room temperature of 21°C).

The absence of an inotropic effect after itraconazole administration to conscious rats was previously reported (Qu et al., 2013a). The exact mechanism for the negative inotropic effects of itraconazole remains uncertain (Qu et al., 2013b) and the reason for the lack of effects noted in the rat model could not be fully explained. One hypothesis to explain the absence of effects is related to the limited bioavailability of itraconazole when administered orally and the importance of low gastric pH to ensure absorption (Miller et al., 2008).

It remains that the rat model enabled identification of inotropic effects with most drugs used. Some agents used in the current study induce direct positive chronotropic and inotropic effects such as pimobendan (Duncker et al., 1986) and amrinone, while others agents such as morphine were considered to have indirect (i.e. central nervous system mediated) positive chronotropic effects with HR dependent positive inotropic effects. Negative inotropic effects of atenolol were detected too.

Chronotropic and inotropic effects may result from changes in endogenous factors (e.g., epinephrine, norepinephrine, dopamine or cortisol). The circadian cycle modulates cardiovascular parameters in all species (Malpas & Purdie, 1990; Wang, et al., 1999). Within this
study, circadian influences were observed amongst all cardiovascular and ECG parameters as expected. The potentially confounding effects of the circadian cycle were best controlled by scheduling all activities precisely at the same time of the day throughout the experiment.

Pre-clinical models to assess ventricular contractility have received constant attention by the safety pharmacology community over the last decade (Adeyemi et al., 2009; Blasi et al., 2011; Cools et al., 2014; Ju et al., 2015; Markert et al., 2007; Markert et al., 2012; Mooney et al., 2012; Sarazan et al., 2012; Tontodonati et al., 2007; Wallis et al., 2015). The reason for this steady interest may be related to the high rate of cardiac toxicity reported to affect nearly one out of 3 drugs during development (Valentin et al., 2008). Recent trends in drug approval will likely maintain these sustained efforts to characterize pre-clinical contractility models. Oncology therapies represented approximately 16% of the total approved drugs and approximately 33% of the novel drug approvals by the FDA in 2015 up from approximately 6% and 22%, respectively, in 2014 (FDA, 2016a, 2016b). Of the oncology drugs approved in 2015, approximately 61% (11/18) exhibited cardiac dysfunction as an adverse effect, a number of which are known to alter cardiac contractility. Several anticancer therapies are well-known to cause cardiac dysfunction particularly amongst tyrosine kinase inhibitors and anthracycline treatments (Mellor et al., 2011; Pai & Nahata, 2000; Yeh & Bickford, 2009). Oncology drugs illustrate the broad range of molecules with cardiotoxicity liabilities. Small molecules such as doxorubicin (an anthracycline) are well known to induce a progressive cardiomyopathy (Chatterjee et al., 2010) and/or direct effects on calcium homeostasis regulation and function (Boucek et al., 1997; Olson, et al., 2005). Beyond chemotherapeutic drugs, biologics such as trastuzumab (Herceptin; a monoclonal antibody) have also been associated with cardiac dysfunction and decreased contractility (McNeil, 1998; Yeh & Bickford, 2009) but the relevance of the rat model to assess cardiac safety of biologics is often limited due to limited binding affinity.

Interpretation of cardiovascular safety pharmacology data relies on statistical analysis but with typical group sizes between n=4 and n=8, the experimental designs sharply contrast with the larger group sizes that are used in phase 1 clinical trials or thorough QT studies (e.g., n=18 to 56) (Malik et al., 2004; Dubois et al., 2015). In this context, qualitative interpretation of the cardiovascular safety pharmacology data remains an important part of the data analysis. An industry survey revealed that statistical analysis was not included in all safety pharmacology studies (Lindgren et al., 2008), although a trend towards generalization of the use of statistical
analysis was noted in recent years. Beat-to-beat monitoring using telemetry generates substantial amount of data which are typically averaged for interpretation purposes. Data bins (i.e., averages over a preset duration) can help reduce the random variability and the use of super-intervals (i.e. averages over longer data duration) was successfully applied to interpretation of cardiovascular safety pharmacology data (Sivarajah et al., 2010). The resulting average values translate into increased assay sensitivity by reducing random parameter variations. The major risk when using data bins is to overlook a drug effect shorter than the data bin duration. In such case, the drug effect is “diluted” with normal data included in the same data bin. To avoid such problem, the duration of each data bin needs to be carefully selected to separate altered cardiovascular data from normal/unaltered cardiovascular data. But the timing and duration of pharmacological effects are unknown when a safety pharmacology study is designed so selection of the bin interval can be challenging. Beat-to-beat graphical representation preserves data integrity and may be a sensitive qualitative data analysis strategy. Beat-to-beat data presentation has been applied to ECG parameters to assess the dynamic of QT-RR and QT-TQ interval relationships through quantification of heterogeneity, hysteresis and restitution (Fossa, 2008). Cloud analysis for beat to beat analysis was also recently applied to large animal telemetry data including left ventricular contractility parameters (Buchanan et al., 2015). While cloud data representation is an interesting approach to qualitative data evaluation, this approach can suffer from data overlap. In brief, cloud density may fail to present data density when the number of data points reaches a confluent pattern. To overcome this limitation of cloud data representation, marginal distribution curves may be an interesting tactic to preserve this dimension of the dataset. The data from the current study support the potential value of marginal distribution curves in the qualitative interpretation of rat ventricular contractility and HR data. Furthermore, marginal distribution curves may be considered for other parameters (cardiac or even respiratory) which exhibit rate dependent correlations.

Conflicts of Interest

None of the authors have any conflict of interest, other than their employment in a contract research organization.
References


Figure Legends

Figure 1. Remifentanil induced changes in HR. All data was obtained in conscious telemetered rats. Error bars represent SEM, n=4.

Figure 2. Flecainide induced changes in electrocardiographic parameters. A) Percent change from baseline of the PR interval after intravenous administration of either flecainide (16 mg/kg) or saline; B) Change in the QRS interval after intravenous administration of either flecainide (16 mg/kg) or saline. All data was obtained in conscious telemetered rats. Error bars represent SEM, n=4.

Figure 3. Dopamine-induced effects on various cardiovascular parameters. A) Comparison of dopamine-induced changes on various cardiovascular parameters. Error bars represent SEM, n=4. HR, heart rate; sSABP, systolic systemic arterial blood pressure; mSABP, mean systemic arterial blood pressure; dSABP, diastolic systemic arterial blood pressure; Pulse, pulse pressure; B) Marginal distribution curve comparing dopamine-induced changes (green), with respect to saline (red), on the contractility index and heart rate at either 0 – 24 h post-dosing (left) or within a 0 – 0.25 h time window representing T_{max} (right). All data was obtained in conscious telemetered rats. * p < 0.05.

Figure 4. Marginal distribution curve for heart rate and contractility index following positive inotropic agent administration. Curves comparing pimobendan- (A), morphine- (B) and amrinone- (C) induced changes, with respect to saline, at various dosing levels at either 0 – 24 h post-dosing (left) or within a 2 – 4 h time window representing T_{max} (right). All data was obtained in conscious telemetered rats. Pimobendan and amrinone, n=8; morphine, n=9.

Figure 5. Effect of atenolol on Contractility Index and Heart rate. Curves comparing contractility index (top) and heart rate (bottom) after saline control or atenolol administration at 1, 3, 10 and 100 mg/kg (n=9). Error bars represent SEM.
Figure 6. Marginal distribution curve for heart rate and contractility index following atenolol and itraconazole administration. Curves comparing atenolol- (A) and itraconazole-(B) induced changes, with respect to saline, at various dosing levels at either 0 – 24 h post-dosing (left) or within a 2 – 4 h time window representing T_{max} (right). All data was obtained in conscious telemetered rats. Atenolol, n=9; itraconazole, n=8.
### Tables

Table 1. Cardiovascular positive and negative control drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Method of administration</th>
<th>Dose Level* (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline – Water</td>
<td>IV/SC – PO</td>
<td>0</td>
</tr>
<tr>
<td>Remifentanil</td>
<td>IV</td>
<td>0.03</td>
</tr>
<tr>
<td>Flecaainide</td>
<td>IV</td>
<td>16</td>
</tr>
<tr>
<td>Dopamine</td>
<td>IV</td>
<td>0.1</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>PO</td>
<td>3, 10, 30</td>
</tr>
<tr>
<td>Morphine</td>
<td>SC</td>
<td>2, 6, 20</td>
</tr>
<tr>
<td>Amrinone</td>
<td>PO</td>
<td>10, 30, 100</td>
</tr>
<tr>
<td>Atencolol</td>
<td>PO</td>
<td>1, 3, 10, 100</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>PO</td>
<td>10, 30, 100</td>
</tr>
</tbody>
</table>

Administration route: PO, oral; SC, subcutaneous; IV, intravenous (via tail vein).

* Dose selection was based on data previously obtained in conscious rats that were monitored using telemetry and the scientific literature available.
Figures

Figure 1

Heart Rate (% change from baseline)

Time (min)

-80%
-40%
0%
40%

- Remifentanil 0.03 mg/kg
- Saline
Figure 2

A

Change in PR

% change from baseline

Time (min)

B

Change in QRS

Duration (sec)

Time (min)

Flecainide 16 mg/kg
Saline
Figure 3
Figure 4
Accardi et al. 2016
*J Pharmacol Toxicol Meth*
3/25/2016

**Contractility Index (dP/dt+/P)**

| Control: Reverse Osmosis Water | Atenolol (1 mg/kg) | Atenolol (3 mg/kg) | Atenolol (10 mg/kg) |

**Heart Rate**

| Control: Reverse Osmosis Water | Atenolol (1 mg/kg) | Atenolol (3 mg/kg) | Atenolol (10 mg/kg) | Atenolol (100 mg/kg) |

Figure 5
Figure 6