Cumulative plot of heart rate variability spectrum assesses kinetics of action of cholinergic drugs in rats

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Perlstein, I., and A. Hoffman. Cumulative plot of heart rate variability spectrum assesses kinetics of action of cholinergic drugs in rats. Am J Physiol Heart Circ Physiol 279: H110–H115, 2000.—A new approach to assess autonomic nervous system (ANS) activity and its response to drug action is presented. Our approach is based on the use of a cumulative plot of data obtained by power spectral analysis of heart rate variability, in defined frequency bands, during short time epochs (e.g., 2 min in rats). The substantial temporal variability in power evolving from the constant balancing nature of the ANS activity is minimized by this approach and produces a measurable index of ANS activity vs. time. The cumulative plot emphasizes the temporal response pattern of different components of the ANS and thereby facilitates the investigation of the kinetics of action of drugs affecting the ANS. We used this method to measure the activity of cholinergic drugs in freely moving Sabra rats. Bolus atropine doses between 0.5 and 2 mg/kg produced a similar magnitude of effect, reduction of the ascending slope by 0.003 power units/h, whereas the duration of this effect was dose dependent. A lower atropine dose (0.1 mg/kg) or 0.5 mg/kg scopolamine elevated the slope (0.074 and 0.054 power units/h for 206 and 216 min, respectively). The method was used similarly to assess the interaction between cholinergic drugs. Pretreatment with pyridostigmine produced temporal blockage of the anticholinergic activity of atropine.

Power spectral analysis; sum function; atropine; scopolamine; pyridostigmine

Power spectral analysis (PSA) of heart rate variability (HRV) has been demonstrated to be a noninvasive approach for the investigation of parameters of the autonomic nervous system (ANS). Data analysis typically produces three main spectral components: a high-frequency oscillation (HF) that represents the vagal respiratory activity mediated by the parasympathetic system, a low-frequency oscillation (LF), and a very-low-frequency oscillation (VLF) band. Unlike the HF band, these two frequency bands are affected by the sympathetic and parasympathetic nervous systems and other physiological mechanisms (3). Guidelines for uniform measurement methods of PSA of HRV and practical methods have been reviewed (11, 13).

Quantitative measurements of PSA of HRV are restricted by the inherent “noise” accompanying ANS activity. This noise is not a technical problem, but derives from the constant balancing activity of different autoregulatory systems, including sympathetic and parasympathetic activity. As these two systems continuously respond to various internal and external stimuli, analysis of consecutive short time epochs (e.g., 2 min in rat or 5 min in humans) shows huge variability between epochs (see Fig. 1). Thus a continuous plot of the power (of a specific frequency band) vs. time provides a highly fluctuating pattern (see Fig. 2A). This phenomenon imposes a rather subjective assessment of the spectral pattern of ANS activity, under defined conditions, that requires a considerable filtering process (4). Thus the assessment of the state of ANS activity from such inconsistent plots is rather complicated and hinders investigations of the kinetics of pharmacological and physiological processes (16).

In this paper, a practical approach is presented to minimize the noise problem, thereby improving the assessment of PSA of HRV measurements of ANS activity. It is based on cumulative data of the area under the power density curve (at a specific frequency band) obtained from short time epochs as a function of time. As shown (see Fig. 2B), the highly fluctuating graph (see Fig. 2A) is condensed into a single inclining slope (see Discussion). To demonstrate the applicability of this approach in measurement of kinetics of drug action on the ANS, the effects of two anticholinergic drugs (atropine and scopolamine) vs. time were assessed in preclinical (rat model) studies. In addition, the ability of this method to investigate the kinetics of interaction between pyridostigmine (cholinesterase inhibitor) and atropine was also determined.

Methods

Animals and Materials

Male Sabra rats weighing 300–400 g were housed separately in plastic cages and maintained in a 12:12-h light-dark cycle, with food and water available ad libitum. All experi-
ments of this investigation were performed in accordance with the guidelines of the National Research Council.

Commercial solutions of atropine sulfate (1 mg/ml) and scopolamine hydrobromide (0.5 mg/ml) were used (Teva Pharmaceutical Industries, Netanya, Israel). Pyridostigmine bromide (Rafa Laboratories, Jerusalem, Israel) was dissolved in double-distilled water just before the experiment to produce a 0.522-mg/ml solution (equivalent to 0.1 μM).

Surgical Procedure

Transmitter implantation. After induction of anesthesia (50 mg/kg ketamine and 10 mg/kg xylazine ip injection), a two-lead electrocardiogram (ECG) telemetric transmitter was implanted in the peritoneal cavity. The leads were tunneled subcutaneously to their positions at the right acratchepzoideal muscle and the left gluteus muscle. After a 7-day recovery period, ECG was continuously monitored and the RR intervals (RRI) were recorded for baseline condition determination.

Catheter implantation. An indwelling polyethylene catheter was implanted in the right jugular vein under light ether anesthesia and filled with heparinized saline solution (20 IU/ml).

Experimental Protocol

To assess the effect of different doses of atropine and scopolamine on ANS activity, the rats received the following drug treatments: atropine sulfate 0.1, 0.5, 1.0, or 2.0 mg/kg; scopolamine hydrobromide 0.05 mg/kg; or normal saline solutions. Each treatment was given intravenously in a volume of 0.3 ml over 3 min. A washout period of at least 24 h was kept between each experiment. RRI data were collected for at least 24 h post-drug administration. The experimental design was block (crossover) randomization (n = 6).

To evaluate the protective effect of pyridostigmine against atropine activity, 0.261 mg/kg pyridostigmine bromide was administered to rats in a slow intravenous bolus (over 3 min). Twenty-five minutes later, a dose of atropine sulfate (1 mg/kg) was given in the same manner. One day later, the same rats received a saline solution (instead of the pyridostigmine solution) that was followed by atropine, according to the same protocol. RRI data were recorded for 24 h after drug administration for later off-line analysis (n = 6 for each treatment).

Signal Acquisition and Analysis

Data acquisition system. The data were acquired with the use of a telemetry system that contained an implantable radio frequency transmitter (TCA-F40, DSI) and a receiver (RCA-1020, DSI) located below each cage. The analog signals were transmitted to a computer (586 Pentium, 133 MHz) and digitized at a sampling rate of 600 Hz by an A/D converter (PCL 819 HG; ICPO). The RRI data were obtained online from the continuous ECG records by a threshold peak algorithm. RRI data, expressed in milliseconds, were recorded on continuous 1-h-length files.

Data acquisition and analysis. The RRI data were edited off-line, and RP-PR intervals were added in each case where there was a high P peak > R peak. The edited RRI data were divided into consecutive 2-min epochs. Data analysis was performed by software written by Sapoznikov et al. (14). A 301-point moving polynomial was subtracted from the original signal to reduce the effect of very slow nonperiodic variations of the parameters. PSA of each parameter was then obtained by an 11th-order autoregressive (AR) model by use of a Levinson algorithm (14, 15). More than 1% corrections of a single epoch was determined as the exclusion criterion. In general, approximately <3% of the data was omitted. Two distinct peaks were obtained by the HRV: a VLF peak (range 0.01–0.20 Hz) and a HF peak (range 1.35–2.65 Hz). The “cumulative plot” was constructed by summation of the power of the HF peak of successive (2 min) epochs and plotting of the cumulative data vs. time. The physiological intensity of the effect was defined by the magnitude of change in the slope (decrease/increase) and expressed in units of power per hour. The duration of the effect is derived directly from the curve and defined as the time between two vertex points. To combine the data sets obtained from different animals, the power units of each epoch were normalized by the mean baseline value. The cumulative plot yields a stable inclining slope. Baseline value was determined for each animal over 3 days between 1200 and 1800 (24-h time system) to avoid diurnal changes. The response to drug treatment was measured for each animal as the change in the inclining slope per minute, with respect to its own baseline value. To construct a uniform scale for data obtained for all the experiments, changes in the power values within each experiment were set to be in the scale of 0–1 (i.e., the maximal power value in each experiment was set to be 1).

Statistical Analysis

A stepwise linear regression analysis was used to determine the vertex points and, consequently, the slopes describing the data. The slope between each two vertex points was defined in units of power per hour, and goodness of fit was determined by the “least squares” method. Minimal error sum of squares (ESS) was used as the guideline measure for determination of vertex points (20). The stepwise linear regression program tested each cumulative plot for different number of vertex points (2–10 points). In general, increasing the number of vertex points reduces the value of the sum of ESSs. For the sake of simplicity, the selected value was the minimal number of points that could adequately fit the data, and an additional vertex point reduced the sum of ESSs by <15%. This simplification also facilitated the comparison among the results obtained from different animals and among different drug treatments.

Data of magnitude and duration of effect are presented as means ± SD. The statistical significance of the differences between groups was assessed by one-way ANOVA.

RESULTS

Figures 1 and 2 demonstrate the way in which highly variable and fluctuating data can be more clearly presented with the use of the cumulative plot approach. The average baseline slope measured for the different animals was 0.048 ± 0.006 power units/h. The cumulative plot of the mean power values of rats that received the same drug treatment is presented in Fig. 3. Individual data of the magnitude and duration of response to each treatment are summarized in Table 1. Although a dose of 0.5 mg/kg caused a reduction of 0.026 from baseline for 64 min, the magnitude of effect was similar for both 1 and 2 mg/kg (−0.036 and −0.032, respectively), but the duration of action lasted for 140 and 145 min, respectively.

Increased parasympathetic activity was reflected by the cumulative PSA after a low dose of atropine (0.1 mg/kg) (+0.054 for 215 min). Similar activity was
found after a scopolamine bromide administration of 0.5 mg/kg (± 0.074 for 206 min).

The proposed methodology was utilized also to investigate interaction between atropine and the cholinesterase inhibitor pyridostigmine. The cumulative power plot demonstrated that 0.261 mg/kg of pyridostigmine bromide administered 20 min before 1 mg/kg of atropine sulfate partially prevented the parasympathetic effect of atropine for 120 min. Saline solution, administered 20 min before atropine sulfate a day later, served as a control group (atropine and pyridostigmine, 2 × 0.025 power units/h; atropine and saline solution, 2 × 0.032 power units/h for 120 min; n = 4).

**DISCUSSION**

Quantification of the impact of drugs on the ANS is important for both research and development of ANS acting drugs and for optimization of their clinical efficacy. The present work proposes a new data analysis approach that can be used as a practical tool for quantifying the activity of drug action on the ANS. Because Holter records of ECG for 24 h are widely available in the clinical setting, much work has been done on clinical assessment of PSA of HRV in various disease states (11). Much less work with this system has been done in preclinical studies. Apart from the ethical issues associated with human drug studies, preclinical investigations provide means to assess the impact of distinct variables without interference of multifactors usually associated in human studies. In the field of drug design and development, there is a clear advantage to recognizing the impact of different variables on the kinetics of drug action. Thus the overall goal of this work was to establish a preclinical model (in rats) to enable measurement of the kinetics of action of ANS drugs.

The currently available assessment methods of PSA of HRV data focus either on short-term records or on analysis of an entire 24-h period. Recently, wavelet transform (WT) methods have been adapted for HRV analysis, and by this approach the signals can be analyzed at different scales for HF, LF, and VLF components (1, 8, 19). However, these approaches do not provide consecutive information on the kinetics of changes of ANS activity. In general, to acquire continuous information on changes in ANS activity over time, the power at each frequency band obtained in the short-term records could be plotted vs. time. As shown in Fig. 2A, such a plot contains noisy information that is difficult to interpret in a quantitative manner. The solution to this problem, in our view, is to plot a cumulative graph of the power obtained (for a specific oscillation frequency range) in the short records (i.e., consecutive 2-min epochs in the rat studies) vs. time. As shown in Fig. 2B, the same information that is presented in Fig. 2A provides a relatively smooth line. As long as the ANS activity remains constant, the slope of this additive plot is steady. Changes in the overall ANS activity result in a corresponding shift of the slope. Thus the cumulative plot reveals the exact onset

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**Fig. 1.** Four consecutive 2-min epochs of power spectral analysis (PSA) of heart rate variability of a resting unrestrained rat. Focus is on high-frequency (HF) peak (1.35-2.65 Hz). Note spontaneous changes in power. F, frequency; PS, power spectrum.
and offset of drug action on ANS activity. A similar approach was suggested before for quantifying circadian blood pressure patterns (16).

The cumulative approach minimizes the temporal variations in ANS activity and causes the fluctuation in power values to be hidden in the line. This summation procedure lets us follow changes in the trend of the PSA of HRV of each component (i.e., HF, LF, and VLF) along the time axis. This way, the effect of a certain stimulus (e.g., drug activity) can be determined according to the slope of the cumulative line and its shift with time. The effect of drugs can be defined according to the curvature they impose on the baseline slope of the cumulative power line and the length of this change until it returns to baseline. It should be noted that in this approach, the baseline is always an ascending line. The magnitude of the pharmacological effect is defined by its angle (i.e., deviation from the baseline slope, which has to be determined for each individual subject according to the same x-y scale). To combine drug responses in several animals, the slope in each individual has to be normalized according to its baseline value. This approach enables the combination of several data sets, even when significant inter- and intra-variable differences in power scale occur. It should be noted that in this approach, single-power peaks (that are considerably higher than the average) have a disproportional impact on the overall slope. This feature is in accordance with the fact that the high-velocity “peaks” show high ANS activity. In addition, the cumulative plot emphasizes the pattern of small but sustained changes in the power vs. time data. It thereby facilitates the quantitative analysis of drug influence on HRV that is constricted within a relatively limited range, particularly in the (common) case of drugs that suppress HRV.

So far, several approaches using PSA data have been suggested to measure drug effect on the ANS. To over-
come the noise problem, other researchers have used different methods: 1) subjective selection of “representative data,” by choosing only typical stationary data; 2) filtering process, i.e., setting very tight exclusion criteria that leave out “atypical” results (17); 3) sequential analysis, i.e., 3-dimensional presentation of all frequency bands vs. time, which is an accurate data presentation but does not provide quantitative evaluation; 4) nonlinear/symbolic methods that present the data on a logarithmic scale (6, 11) and thereby reduce its sensitivity; and 5) utilization of averaged data of a large number of subjects at each time point. The WT approach, which provides considerably smaller fluctuations in consecutive power values, has not yet been utilized to assess the kinetics of drug action on the ANS.

In this work, we focused on the HF band that represents exclusively the parasympathetic system (2, 5, 9). The HF peak in the PSA of HRV is separated from all other frequency bands; it is easily detected (see Fig. 1), and its power can be directly determined, without the need for autoregressive analysis (12). To validate our cumulative approach as a useful measure to assess pharmacological activity, the effect of anticholinergic drugs on parasympathetic activity was examined. As shown in Fig. 3, the dose-response relationship of atropine could be assessed by the cumulative approach. Doses between 0.5 and 2 mg/kg produced (approximately) the same impacts on the slope of the line, whereas the duration of this effect was directly proportional to the dose. This data analysis demonstrates that the inhibitory effect of atropine on parasympathetic activity (as represented by the PSA of the HF band) appears to be all or none. This indicates that atropine effect on the parasympathetic activity reaches its maximal impact (i.e., maximal effect; $E_{\text{max}}$) at these doses. Thus, in this case, the dose-response relationship is demonstrated by the duration of action (7).

A low atropine dose (0.1 mg/kg) has an opposite effect on parasympathetic activity. It resulted in an elevation of the ascending cumulative slope that was similar to the effect of scopolamine (0.5 mg/kg) (see Fig. 3). This phenomenon, known as the paradoxical effect of atropine, is explained by differences in the functions of the two drugs on the autonomic nervous system.
of M₁ and M₂ (muscarinic) receptors (18) or by peripheral muscarinic receptor agonistic activity at low atropine and hyoscine concentrations (10).

The same data analysis approach enabled us to assess the kinetics of interaction between pyridostigmine and atropine. In this experiment, the protective activity of the cholinesterase inhibitor against the antagonsitic effect of atropine on the muscarinic receptors was determined. As was also found before in humans (8a), pyridostigmine did not elevate the power of the HF band, although it increases the amount of acetylcholine in the synapse. On the other hand, the elevated concentrations of the neurotransmitter competitively antagonized the influence of atropine, as reflected by our results. In contrast to the work in humans that assessed the effect of escalating doses at a single time point, the present work provides continuous assessment of the kinetics of activity of this drug combination on the respiratory peak.

In conclusion, the cumulative plot approach converts data obtained from frequency domain analysis into the time domain. It thereby enables one to follow the activity of certain components of the ANS activity vs. time. In our view, the present approach provides a relatively uncomplicated method to assess the effect of pharmacological agents on the ANS over time. It makes simple, rapid, and powerful assessments of changes in the slope of trends from continuously collected data and provides a more sensitive measure in the trends of the data than the data itself. Although the cumulative approach was used here with data obtained by the short-term Fourier transform, the same approach could be utilized also to convert the data obtained by other power analysis methods (e.g., the WT methods) from frequency domain into time domain, for similar purposes.

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