

A DEVICE FOR IMPLEMENTATION OF THE FORCED OSCILLATION METHOD ON STRIP PREPARATIONS OF BIOLOGICAL TISSUES IN VITRO IN PROLONGED EXPERIMENTS

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ABSTRACT: The viscoelasticity of blood vessels determines their biomechanical behavior. The present work aims to develop and validate a new device modification for the forced oscillation method and to elaborate an experimental protocol for investigating the direct effect of opioids on the arterial wall *in vitro* in a prolonged experiment. The construction and software of a previously constructed device were improved and upgraded. A new photo optic recording system and Analog-To-Discrete conversion and recording scheme were developed. The elaborated experimental protocol allows an accumulative treatment with opioids while keeping the preparation vital. The total error of the measurement modules is less than 4.45%. The device ensures sufficient repeatability and precision in recording the response oscillations and estimating the viscoelastic characteristics of biological tissues.

KEY WORDS: biomedical measurement, viscoelasticity, blood vessels, forced oscillations, data processing.

1 INTRODUCTION

The viscoelasticity of the blood vessel wall is highly important for the arterial behavior in norm and pathology and after physical or pharmacological influence on it. In 1972 Bergel [1] and Fung [2] formulated and theoretically described arterial viscoelasticity. As the arteries' elastic properties depend on the applied forces and deformations, the anisotropy of the arterial wall is widely investigated [2–7]. Weizsacker and Pinto [8] found that the arterial wall of rat arteries is isotropic in the physiological blood pressure range. Dobrin [9] has made a similar conclusion for dog arteries,

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and Mohan and Melvin [10] did not find a significant difference between both directions – longitudinal and circumferential, in human arteries. As was previously shown, data for the biomechanical characteristic of the arterial wall are pretty different and discrepant [11, 12]. The main reasons for that discrepancy are the differences in the experimental conditions (alive or post-mortem preparation, different ages [13, 14]), the arterial remodeling after some pathologies and influences [15, 16], the different types of loading – static, dynamic, or quasistatic.

The most informative remains the results from the dynamic testing. Most *in vivo* methods detect changes after treatment with vasoactive substances but do not give any results in unstimulated conditions [17]. Therefore, the dynamic *in vitro* methods are more promising. The investigations of the dynamic characteristics are conducted to obtain the compliance and distensibility of the arterial wall, as well as of the complex module of elasticity (formulated by Hardung in 1953 [18]). McDonald [19] represented a model of the pressure changes during the heart cycle that expands the pressure curve to a sum of mechanical sinusoidal loadings. Thanks to that work, applying just one simple harmonic loading facilitates the experiments, and the interpretation of the results is admissible. Apter and Marquez [20] and Hardung [21] applied such oscillating loading on rings of arteries stretched by two bars, and they registered resonance curves and estimated the natural frequency and the resonance frequency (both do not coincide for materials with nonlinear elasticity), and the complex modulus of elasticity.

The main advantage of the *in vitro* experiments on arteries is the characterization of the direct effect of any influence without the nervous or humoral regulation in the organism [22]. However, the preparation itself of a strip arterial segment damages its structure. Therefore, sufficient preparation size is required to develop a complete and adequate reaction on the applied mechanical loading.

The forced oscillation method affords additional opportunities to investigate the biomechanical characteristics of biological tissues. While it is a standard method for technical materials characterization, it still needs to be widely used in biomechanical studies. We cannot find in the literature any other authors to use that method in biomechanical experiments. This method might quantify the investigated specimen's natural frequency, dynamic modulus of elasticity, and coefficient of viscosity. Previously, we developed a device to implement the forced oscillation method in studying biological tissues. Previous publications [23–26] described some of the modifications of the device for experiments with strips or cylindrical segments of blood vessels. The main peculiarity of all device modifications is their ability to maintain the preparation vital during the entire experiment – as in the living organism. The particular modifications allow the method to be applied to various preparation geometry and any direct influence on the arterial wall. In the present work, a new modification is

represented for assessing opioids' influence on the arterial wall *in vitro*. In that case, a longer continuance of the experiment is needed, as will be described in the Method section. To this end, a new experimental protocol should be developed to allow an adequate extended duration of the experiment while keeping the vitality of the preparation. Besides, some refinements of the recording modules and the software will be represented here.

The aim of the present work was to be developed and validated a new modification of the device for implementation of the forced oscillation method and to be elaborated a new experimental protocol for the investigation of the direct effect of opioids on the rat aorta *in vitro*.

2 MATERIALS AND METHODS

2.1 THEORETICAL GROUND

The theoretical ground of the method was previously described [23,27]. The method of forced oscillation is used here without changes. It applies sinusoidal oscillations with constant amplitude on the upper end of a vertically suspended preparation. The frequency of oscillations is made to sweep an interval in the physiologically normal range for arteries in both directions – increasing thereafter-decreasing frequency. The so-developed excitation oscillations excite response oscillations of the lower end of the preparation. The response oscillations frequency follows the excitation frequency with the same values and some time delay, but the response amplitudes are frequency dependent. When the excitation frequency increases, the response oscillations amplitude increases to resonance and diminishes. When the excitation frequency decreases, the response oscillation amplitude increases again to the resonance and diminishes. Because of the nonlinear elasticity, the resonance curve is not symmetric, and the resonance frequency is different by increasing/ decreasing frequency [28]. As mentioned in [14], the vital preparations possess a hardening type elasticity, while the no-vital preparations display a softening type elasticity. This fact is used for validation of the preparation vitality.

2.2 DEVICE

As mentioned above, it was essential to keep the vitality of the preparation during the entire experiment. A transparent box (a Perspex box) was used for this end in which the preparation was suspended, preserving the preparation against any draughts and sudden temperature changes. A nutrient solution was superfused on the suspended preparation (modified Tyrode solution), containing (mM): NaCl 136.9; KCl 3.2; MgSO₄ 0.6; NaH₂PO₄ 0.5; NaHCO₃ 11.9; CaCl₂ 2; glucose 11.5; pH 7.2-7.4, continuously gassed with 5% CO₂ in O₂. During the measurement, the superfusion was

stopped for a maximum of 14 seconds to avoid the influence of the additional mass of the solution on the response oscillations. It was proved that such a period without superfusion does not affect the vitality of the preparation.

2.2.1 EXPERIMENTAL SCHEME

The experimental scheme of the device is chosen in which the preparation is suspended vertically in a Perspex box, ensuring stable surrounding conditions and permanent superfusion with nutrient solution. Excitation oscillations were applied on the preparation's upper end, leading to response oscillations on the lower end. Electrical signals for the amplitude and the frequency of oscillations were taken by an ADC and processed by a personal computer.

In Fig. 1 is shown the scheme of the device.

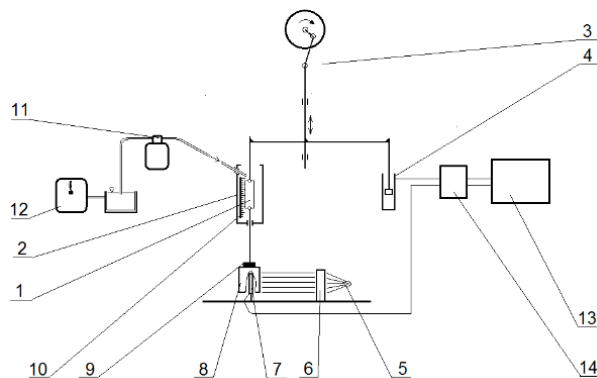


Fig. 1: Scheme of the device. Explanations in the text.

The driving and preparation modules are the same as in the previous device modification [26]. The preparation (1) is suspended vertically in the Perspex box (2). The upper end is connected to the elbow connecting rod mechanism (3) and a Hewlett Packard displacement transducer 7DCDT-250, Hewlett Packard, USA (4). The oscillation frequency is regulated by a driving mechanism, which ensures four frequency ranges adjustable for the specific object. For the present aim, the frequency can sweep the range of 3 to 30 Hz up and down. The photo optic module for measurement of the response oscillations is described in subsection 3.1. Experimental scheme (positions 5 to 9). A small plastic shield below the preparation preserves the nutrient medium to flow to the photo-optic module. The nutrient solution is drained from the Perspex box. A peristaltic pump Gilson Minipulce 3, Gilson, USA (11) ensures a 6 ml/min nutrient medium flow, heated to 37°C with a thermostat (12). Electric signals from the HP gauge and the photo element, respectively, for the excitation amplitude (Exci-

tation Amplitude Signal – EAS), and the response oscillations amplitudes (Response Amplitude Signal – RAS), are recorded in a PC (13) after conversion by Analogue to Digital Converter (ADC-14) described below in the subsection 3.3. Schematic implementation. Data are further developed.

2.3 EXPERIMENTAL PROTOCOL

The experimental protocol for experiments with opioid analgesics as the pharmacological substance is specific. We intended to estimate the concentration dependence of opioids on the viscoelastic characteristics. The opioid application should be cumulative – each greater concentration should be applied after the previous (more minor) without washing away the previous. After that, the preparation should be washed down from the opioid solution for 30 minutes. A superfusion with naloxone (opioid receptor antagonist) in a higher concentration of 10^{-6} M/L should be applied (naloxone competitively bounds the opioid receptors). After that, the same concentrations of opioids must be applied in the presence of naloxone to assess the effect of opioids while the opioid receptors are occupied. All these actions: the control experiment (without any substance), the experiment for concentration dependence, and the experiment for concentration dependence in the presence of naloxone, extend the duration of the whole experiment. In addition, two preparations from one aorta might be taken, and they must be investigated immediately on the same day of the rat sacrifice. At this, the duration of the experiments with both preparations does not exceed four hours after taking the aorta, which ensures the vitality of both preparations during the entire experiment.

For the preliminary validation of the device, rat aorta preparations were used. The animal experiments were approved by Bulgarian Food Safety Agency permit No. 300/No5888–0183/10.05.2021, following EC Directive 2010/63/EU.

3 RESULTS

3.1 EXPERIMENTAL SCHEME

The experimental scheme of the device was described in general in the previous section (Materials and methods) – Fig. 1. Here only the new items are described.

The photo optic module comprises two infrared light diodes VTE3324LAH, 940 nm, Transfer Multisort Elektronik Sp. z o.o., Poland (5), and a semi-cylindrical lens (6) for making a parallel shaft of light to the photo element (7). The mask (8) is suspended to the lower end of the preparation and covers the photo element, varying the output voltage by oscillation. The mask is a 3D-printed cylinder with minimum mass. Outer, so-called concentrated masses (9) were suspended on the top of the mask, creating an elongation of the preparation equivalent to an intraluminal pressure in the physiological range of pressure values.

Four concentrated masses are chosen to create elongations of the preparations, corresponding to equivalent intraluminal pressures of 77.2, 111.8, 144.1, and 157.4 mmHg. The elongations made by each mass are measured by means of the scale (10) mounted in the Perspex box. A peculiarity of infrared light is that only platinum, aluminum, and copper shield it. That necessitates the mask to be covered by a thin copper foil and an aluminum box placed around the photo-optic system, preventing the exterior light from affecting the signal.

3.2 EXPERIMENTAL PROTOCOL

Because the most prolonged period to keep a preparation vital was measured at about 4 hours, the following experimental protocol was developed:

1. Initial adaptation consists of 30 min superfusion of the suspended preparation in the organ bath.
2. Control experiment:
 - (a) setting the concentrated mass,
 - (b) estimation of the static elongation of that step of the equivalent pressure,
 - (c) breaking the superfusion for 14 sec,
 - (d) recording of the oscillations at increasing and decreasing excitation frequency (sweeping the frequency interval between 3 and 25 Hz slowly),
 - (e) starting the superfusion again.
3. The control experiment is repeated for four concentrated masses, each repeating the procedures 2a) to 2e).
4. The experiment with the first (the lowest) concentration of the opioid is started immediately, repeating procedures 2) and 3).
5. The experiment continues with the second concentration and, generally, with four raising concentrations, repeating the same procedures 2) and 3). The nutrient solution with each opioid concentration replaces the previous; it is not added.
6. The opioid solution is washed down by 30 minutes of superfusion with a clear nutrient solution.
7. The procedures in points 2) and 3) are repeated.
8. The next is a 10 minutes superfusion with naloxone in the highest concentration (10^{-6} M/L) in the nutrient solution – which leads to bounding the opioid receptors with naloxone.
9. Procedures 2) and 3) are repeated to control the naloxone bounding.

10. Then, procedures 2) and 3) are repeated while each concentration is applied in the presence of naloxone (10^{-6} M/L).
11. End of the experiment with the first preparation.

Procedures 1) to 10) are repeated with the second preparation. In case of prolongation of the experiments longer than 4 hours, the bending of the resonance bone will show whether the preparation was devitalized. In such a case, the recorded data are discarded.

The excitation and response oscillation records are put into EXCEL tables and developed by built-in functions. The frequency is calculated; the excitation and response oscillations signals are filtered in the Origin 9.0 environment.

3.3 SCHEMATIC IMPLEMENTATION

Schematic implementation: For the reading of data from both sensors, a scheme was developed based on the universal plate Arduino Uno R3, IDII, Italy, and the Analog-to-Discrete-Converter ADC1015 from Texas Instrument, USA.

The ADC was connected to the universal plate Arduino Uno R3 via an I2C interface (Fig. 2). Having in mind the high output voltage of the HP sensor (the Excitation

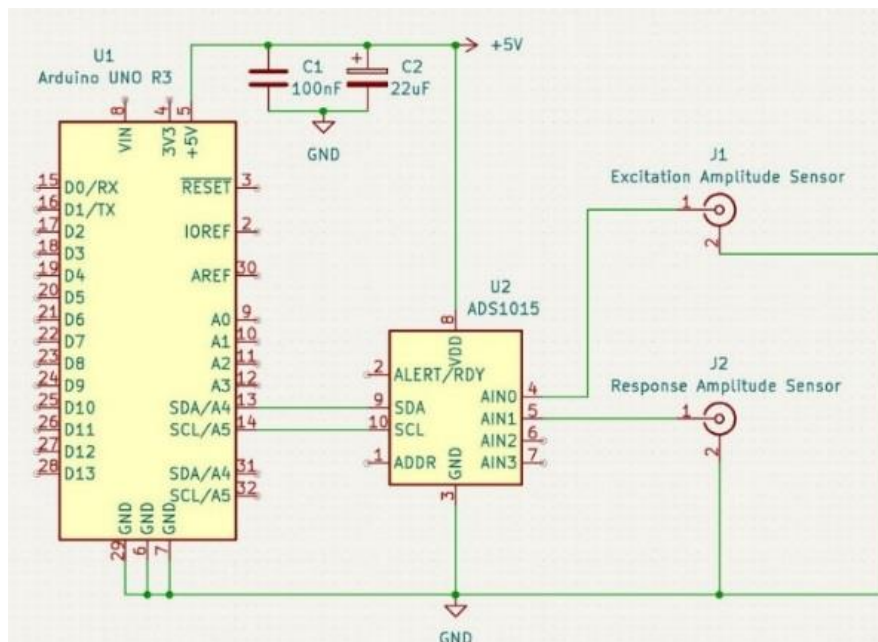


Fig. 2: Schematic implementation.

Amplitude Sensor – EAS), the amplification was chosen for a supply signal 0–5 V. They have used two analog channels – ADC0 and ADC1. The sensor was connected to channel ADC0, and the photosensor (the Response Amplitude Signal – RAS) – to channel ADC1. By the TIMER0 of the microcontroller ATMEGAA328P, built-in in the Arduino Uno R3, interruptions were set every ten milliseconds. At the development of the interruptions, the channels ADC0 and ADC1 were consecutively read. Data were transmitted to a personal computer via the USB port of the plate Arduino Uno R3.

Raw data were transmitted without any filtration or other development.

3.4 SOFTWARE ASSURANCE

A software package was developed, allowing data reading from the USB port and their visualization and storage (Fig. 3). A procedure for automatically scaling read data and automatically correcting the zero line was developed. That allowed a data visualization with a maximal dynamic range. Data recording was controlled by the operator, which allowed continuous monitoring on the one hand and the other – for recording chosen epoch with the needed beginning and duration.



Fig. 3: Software assurance.

The frequency values and oscillations amplitudes are estimated from the records, and the resonance curves are plotted. Each record represents one point of the dependency between the viscoelastic characteristics and the intraluminal (equivalent) pressure. The natural frequency (f_0), 3dB bandwidth (Δ), and octave length (Ω) are obtained from each resonance curve. The dynamic modulus of elasticity (E') and the coefficient of viscosity (β) are then calculated by the formulas having in mind the

geometry of the preparation (the mass – m , the initial length L_0 , the elongation ΔL , the current length $L_0 + \Delta L$, and the cross-sectional area S) [29]

$$(1) \quad E' = \frac{(2\pi f_0)^2 m (L_0 + \Delta L)}{S},$$

$$(2) \quad \beta = 2\pi f_0 m \left(2^{\Delta/\Omega} - 1 \right).$$

That way, one value of each characteristic at the respective equivalent pressure is estimated from each resonance curve. The dependency of the viscoelastic characteristics and the intraluminal (equivalent) pressure is then plotted for the natural frequency, dynamic modulus of elasticity, and coefficient of viscosity. In the present work, an additional characterization of the preparation could be applied – the static characteristic. It is derived from the readings of the elongation steps of the preparation and the corresponding stress. Static characteristic is also plotted as the dependency between the stress (σ) and strain (λ), calculated by formulas:

$$(3) \quad \lambda = \frac{L_0 + \Delta L}{L_0},$$

$$(4) \quad \sigma = E' \frac{\Delta L}{L_0}.$$

The relative error of the measurement is taken as the ratio sensitivity-to-signal percent. The sensitivity and the relative errors of the measurement modules are represented in Table 1. The total error is estimated as the sum of all relative errors.

Table 1: Assessment of the measurement errors

Sensor	Range			Sensitivity	Relative error, %
	Sensor input signal	ADC signal, bit	ADC signal, V		
HP7DCD	1.7 mm			0.02 mm	$\Delta_1 = 1.17 \%$
T-250 (EAS)		2300-3500	0 – 4 V	0.003 V	$\Delta_2 = 0.08 \%$
					$\Sigma \Delta_i = 1.25 \%$
Photo element (RAS)	± 6 mm			0.01 mm	$\Delta_1 = 0.16 \%$
		50-460	0 – 3 V	0.0073 V	$\Delta_2 = 0.24 \%$
					$\Sigma \Delta_i = 0.4 \%$
Elongation	3-10 mm			0.1 mm	$\Delta_{\max} = 3.3 \%$
Total error	Static experiments			$\Sigma \Delta = 3,3 + 0,4 = 3,7 \%$	
	Dynamic experiments			$\Sigma \Delta = 1,25 + 0,4 = 1,65 \%$	

4 DISCUSSION

The device's construction is proven suitable for the biomechanical investigation of blood vessels and other biological tissues *in vitro*, keeping their vitality during the entire experiment. Preliminary results [14] show that the resonance curves up to the fourth hour from the beginning of the experiment are of a hardening type of elasticity, i.e., the preparation is vital to the fourth hour. As noted, the basic construction of the device [26] is not changed vastly, but the changes facilitate the work and contribute to better measurement precision. The additional filtration of the signals eliminated the zero trend and the network interference, providing a better vision of the graphs. The optic system is improved with a higher photo element, which ensures a broader scope for developing and accurately measuring forced oscillations. The higher photo element needs a higher mask – in this case, the 3D-printed mask with only two layers of filaments is the best choice because of the minimal weight. The copper film (an opaque barrier against the infrared light) is also fine and does not add to the mask's weight. The infrared diodes are battery-supplied (with low consumption), so they do not cause electrical interference. An additional aluminum box is placed around the photo optic system to stop the external infrared light. The advantage of this system is its high sensitivity and low battery consumption.

The chosen ADC has the following advantages:

- Resolution – 12 bits, No Missing Code
- Speed of conversion – up to 3.3 kSPS
- Opportunity for the discretization of up to four analog channels (at that stage of work, only two channels are used).
- Programmable Gain Amplifier
- Built-in source of reference voltage with a minimal drift
- Built-in oscillator
- I2C interface
- Suitable supply voltage – 2.0–5.5 V
- Availability

The accuracy of all the recording modules is high enough – the relative errors vary from 0.4% to 3.3%, and the total error does not exceed 4% for static and 2% for dynamic experiments. The relative error of the new optic system is only 0.4%. It could be compared with one of the modifications with a similar optical system, which showed a 1,67% relative error. More records at the same conditions of one preparation take to the same resonance curve, evidencing good repeatability.

The experimental protocol is developed for a more extended experiment duration, maintaining the preparation vital.

The conclusion could be that the device ensures sufficient repeatability and precision in recording the response oscillations of strips to estimate the viscoelastic characteristics of the biological tissues.

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