OPTIMIZATION OF ANALYSIS OF POPULAR DOPING SUBSTANCES USING ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND GAS CHROMATOGRAPHY - MASS SPECTROMETRY (GC-MS) METHODS

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Background

Use of the prohibited and potentially dangerous substances in sports has become an actual problem for sports world. According to the list of sanctioned athletes, provided by Kazakhstan National Anti-Doping Centre. [1][3] Most frequently doped substances among them were: 1) anabolic androgenic steroids (AAS) 2) diuretics 3) metabolic modulators 4) beta-blockers. We will provide gas chromatographic analysis of these substances.

Anabolic androgenic steroids are synthetic or human-made variants of the male sex hormone testosterone. They provide several of physiological effects and the most predominant is an anabolic effect. The desire to gain muscles, lose body fat, and improve athletic performance is the most common motivation for anabolic steroid abuse. [2] AAS increases the number of satellite cells, which plays key role in muscle fiber growth by incorporation of them into preexisting fibers to maintain a constant nucleus to cytoplasm ratio. High abuse of AAS is observed among professional bodybuilders and non-professional athlete's cohort. [4]

Diuretics are misused by athletes for several reasons: 1) reduction of body weight to compete in the lower weight category, 2) to minimize fluid retention in the body caused by steroid usage, as well as the concentration of other prohibited drugs in urine, to avoid being tested positive. Loop diuretics are commonly used among our athlete's cohort. These is the type of diuretics binding to the chloride ions binding site in sodium+/potassium+/ chloride- transmembrane domain. It will reduce ability of kidney to concentrate urine and increases athletes' diuresis. [5]

Meldonium (MET-88), it is an active substance of medical product Mildronate. It is used to treat ischemic diseases of the brain and heart. Meldonium inhibits L-carnitine production and promotes its excretion. Athletes found that Meldonium increase endurance performance, improve rehabilitation process after exercise, and enhance activation of CNS function. According to a WADA study, 172 samples have tested positive for meldonium since it was declared a banned substance, spanning a variety of sports and countries. [6]

First line therapy of angina pectoris and hypertension are provided by using of the beta-blockers. By binding to beta-1, beta-2 receptors it provides negative inotropic and chronotropic effect. As a result, relaxation of muscle tissue and reduction of the heart rate will occur. [7] That is why, athletes participating in archery, shooting, gymnastics, golf, darts used it to improve their steadiness, equilibrium, and deftness. [8].

Instruments used in recent research publications on optimization of doping substances detection. (2020-21 y.) [9] (Table 1.)

Class	**Sub-group**	**GC-MS**	**LC-MS**
Anabolic agents	1. Anabolic androgenic steroids (AAS) 2. Other anabolic agents	+	+
Peptide hormones, growth factors, related substances, and mimetics	1. Erythropoietin-receptor agonists 2. Hypoxia-inducible factor activating agents 3. Growth hormone	+	+
Beta-2-Agonists	Beta-2-Agonists	+	-
Metabolic modulators	Metabolic modulators	+	+
Diuretics and masking agents	Diuretics and masking agents	-	+
Stimulants	Stimulants	+	+
Cannabinoids	Cannabinoids	+	+
Glucocorticoids	Glucocorticoids	-	+
Manipulations with blood and its components	-	+	+
Gene and cell doping	-	+	+

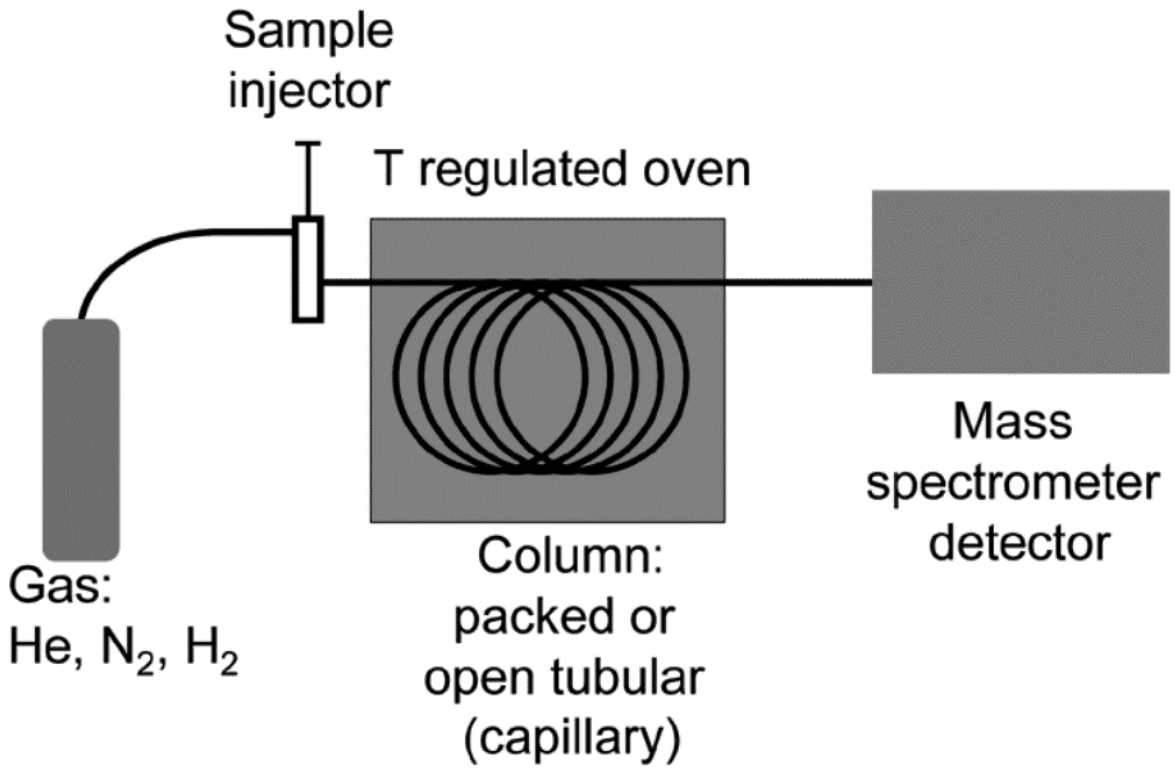
***Used instruments:** +, **not used instruments:** -

Gas Chromatography – Mass Spectrometry (GC-MS) is a combinational method of analysis that uses gas chromatography and mass spectrometry features. It becomes the most accurate method for chemical substance detection when both analytical techniques are combined. With low polarity,

low bowling, or volatile after being derivatized, coupled GS-MS is one of the most suitable techniques. GC is mostly utilized for quantitative examination of chemicals, whereas MS is one of the most common detectors for qualitative analysis. Because it uses a 100% specific test, GS-MS is considered the “gold standard” in forensic material detection.

The capillary column used in the GC is dependent on the column diameters as well as the phase parameters. During the GC-MS analysis due to the differences between molecules chemical properties molecular separation process will occur. By splitting and ionization of each molecule MS will detect them using mass-to-charge ratio. (Fig 1.) [10]

Fig 1. [10]



***Structure of the GC-MS**

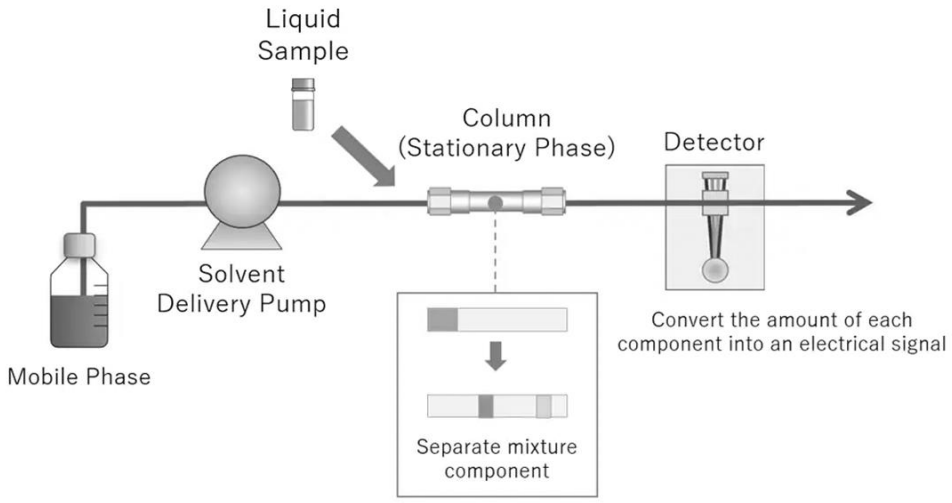
High Performance Liquid Chromatography (HPLC) is one of the widespread techniques used for splitting, detection, and scaling of the components of substances. While other instruments use gravity for passing the solvents through columns, HPLC pushes the substances using high pressure (400 bars) and the solvents can be separated to the several components constituents according to their comparative properties. [11]

HPLC uses pumps for the pressurizing of the dissolved substances, due to which samples will passes through column filled with the rigid absorptive material. According to the different flow rates, substance components will be divided into several particles.

With the mass exchange process, chromatography is also containing adsorption procedure. Most of the absorbent substances are granular rigid fragments varying in dimension from 2 to 50 micrometers (silica, polymers, etc.). Conforming to the various levels of the assertion with the retentive substances, separation of the sample particles occurred. Mixture of the substances (water, methanol, and acetonitrile) under the pressure is called “Mobile phase”. Under the influence of the mobile phase composition and temperature samples passed through absorbent material. [12]

There are two types of the HPLC is recognized: 1) classic "low weight" liquid chromatography using atmospheric pressure from 50 to 400 bars, 2) while during the partition chromatography it uses gravity for passing of the mobile phase through the segments. Columns used in HPLC are transversely from 2.1 mm to 4.6 mm and in length from 30 mm to 250 mm, according to the small number of substances separated during the HPLC run. Moreover, columns used for HPLC also have small sized absorbent material (from 2 to 50 mm). Due to the high sensitivity of the HPLC, it’s becoming one the most popular analytical instruments. [13] (Fig 2.) [14]

Fig 2. [14]



***Structure of the HPLC**

Limitations of the previous studies and premise of hypothesis:

1. Studies performed analysis of only 1 definite type of doping substances. While in our study we optimized methods for 3 groups of the drugs. (b-blockers, diuretics, and metabolic modulators) [2][5][6]
2. Some of the previous studies used obsolete equipment. Varian Inc. – CP-3800 Gas Chromatograph was introduced in 1997, while equipment used in our study Thermo Fisher

Scientific - UHPLC ultimate 3000 in 2014, and it is more sensitive and convenient for the detection of the doping substances. [18]

Impact

This study will give possibility to optimize the methods of detection of doping substances and with the help of this method, we will be able to identify any chemical substances that are used for doping in sport events. With the help of the HPLC instrument, detection of doping substances will be more specific and sensitive.

Premise of hypothesis

GC-MS and HPLC methods improve the specificity and selectivity of the doping substance analyses.

Rationale

With the help of this study, we can create sensitive and selective method for the analysis of doping substances. If our methodology confirmed as reliable, this method will be recommended to our local laboratories.

Specific aims

1. Get approval from the local Ethical Committee and recruit volunteers for sample collection
2. Obtain reagents and prepare samples for HPLC and GC/MS analysis
    - Reagents obtaining
    - Standard solutions preparation
    - Sample preparation
3. Chromatographic run and results analysis
    - GC-MS run
    - HPLC run
4. Results analysis and validation procedure

Experimental plan

1. Get approval from the local Ethical Committee

We got approval from NU-IREC (Nazarbayev University School of Medicine – Institutional Research Ethics Committee) on December 11th, 2021. (See appendix)

2. Reagents acquisition

Medications obtained from local pharmacies, vials, and cups for HPLC, and GC analysis obtained from «RIDDER» company (Karaganda).

3. Sample preparation for HPLC analysis

Standard solutions preparation:

For furosemide (4-chloro-N-furfuryl-5-sulfamoylanthranilic acid):

Preparation of standard stock solution of furosemide (400 μg/ml) was done by dissolving 40 mg furosemide in 100 ml alkali (mobile phase). Firstly, we powdered 1 tablet of furosemide (40 mg) and dissolved this powder in 100 ml of mobile phase (alkali). Mobile phase was prepared by dissolving of 1 granule of KOH in 100 ml of distilled water. After the dissolving of furosemide, we put this solution into the ultrasound bath for 20 minutes at 30 °C. After that we filtered this solution through a 30 mm filter. After that, we put standard stock solution into 50 ml volumetric flasks in a such way that concentration of furosemide was between 0.01 μg/ml – 5.0 μg/ml. [15]

For meldonium ((3-(2,2,2-trimethylhydraziniumyl) propionate):

Preparation of standard stock solution of meldonium (5000 μg/ml) was done by dissolving 500 mg of meldonium (capsule) in 100 ml of distilled water. Firstly, we open the capsule and took out a powder and dissolved this powder in 100 ml of distilled water. After the dissolving of meldonium, we put this solution into the ultrasound bath for 25 minutes at 30 °C. After that we filtered this solution through a 30 mm filter. After that, standard stock solution was taken into 50 ml volumetric flasks in a such way that concentration of meldonium was between 0.01 μg/ml – 5.0 μg/ml. [16]

For bisoprolol fumarate

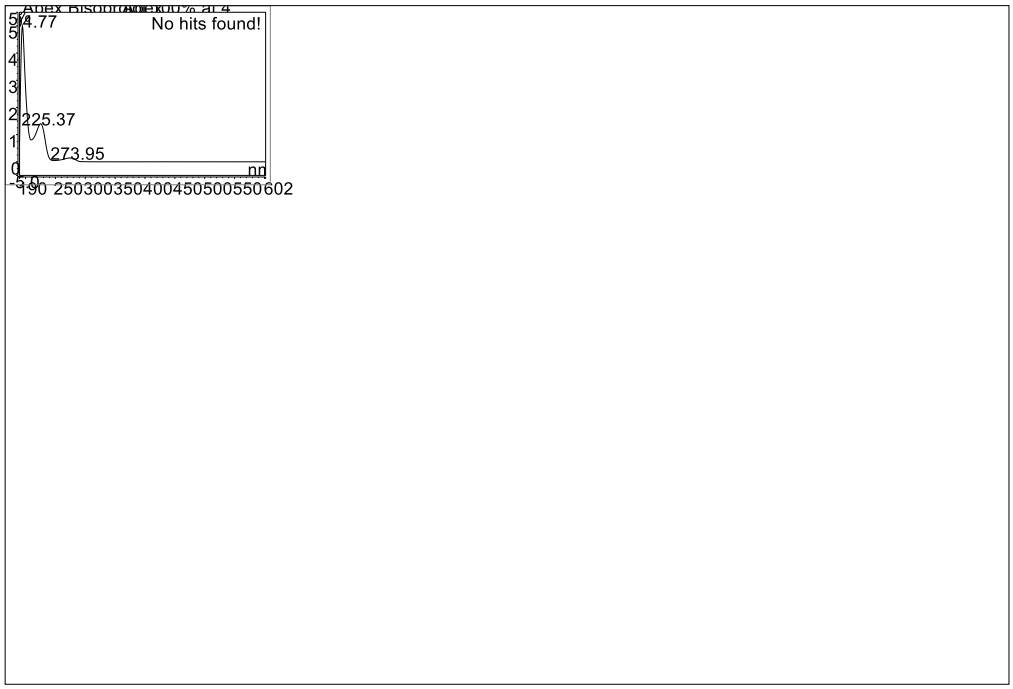
Preparation of standard stock solution of bisoprolol (250 μg/ml) was done by dissolving 25 mg of bisoprolol (10 tablets. 2.5 mg each) in 50 ml of methanol. Firstly, we powdered 10 tablets of bisoprolol (25 mg) and dissolved this powder in 50 ml of methanol solution. After the dissolving of bisoprolol, we put this solution into the ultrasound bath for 20 minutes at 30 °C. After that we filtered this solution through a 30 mm polypropylene filter. After that, standard stock solution was taken into 25 ml volumetric flasks in a such way that concentration of bisoprolol was between 0.01 μg/ml – 5.0 μg/ml. [17]

Results and Discussion

Results (Bisoprolol)

For the detection of the bisoprolol Hypersil Gold^{TM} C18 (50 mm x 2.1 mm) column was used. During the process of the analysis of bisoprolol, we tried the number of the mobile phase and one of the most suitable was made up of water, methanol, and acetonitrile (50:25:25, v/v/v). Flow rate of the mobile phase was equal to 0.150 ml/min and the length of the UV wave for bisoprolol maximum absorption was equal to 194.77 nm (Fig 3.)

Figure 3. Chromatogram of Bisoprolol standard solution



Method of validation (Bisoprolol)

Validation process of our method was according to the guidelines approved by international committee. We validated our method for ensuing characteristics: linearity, limit of the detection, limit of the quantification, precision (injection repeatability). [19]

Linearity

For the examination of the linearity, we prepared six solutions with concentration from 0.01 to 5 μg/ml. Solutions prepared by the dissolving of the stock solution with the distilled water. Analysis of all solutions was provided at the identical state and the chromatographic area of the peak was identified. Regression equation for the area of the peak in the range from 0.01 to 5 μg/ml was equal to y = 0,6401x + 0,0039 and the concentration was equal to $R^2 = 0.9998$. On the figure 4 we can see graphical representation of the variety of the concentration values which was created according

to the regression equation. From these values, which was calculated in the range from 0.01 to 5 µg/ml, we can see that slope of the line equal to 2.0307, while intercept and coefficient of the regression (R^2) was 0.00596, 0.9959 respectively. From the figures 4, 5 we can see that theoretical concentration and concentration from equation showed interrelationship. We can see that slopes of this lines are close to the unification, and their intercepts are near to the 0.

Figure 4. HPLC calibration curve for bisoprolol detection

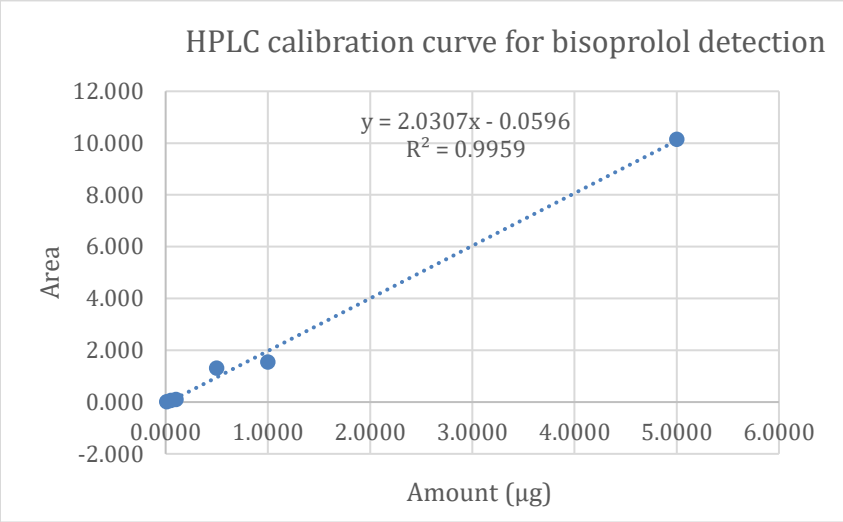
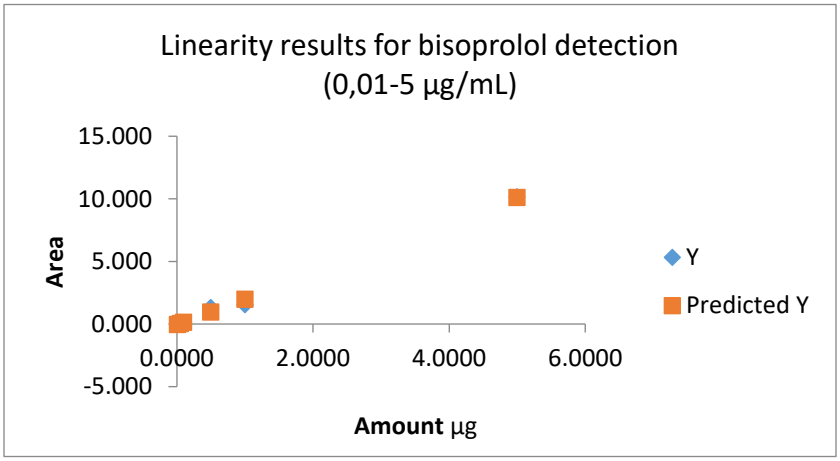


Figure 5. Linearity results for bisoprolol detection (0,01-5 µg/mL)



Limit of the detection and limit of the quantification

By using the regression slope and standard deviation values, we calculated the limit of detection = 0.4607 µg/ml) and the limit of quantification = 1.3962 µg/ml values. [20]

Precision: by providing the 10 consecutive HPLC runs of the lowest concentration of bisoprolol (Cx=0.01 µg/mL) we detect the system precision. Relative standard deviation was equal to 1.11 %. While maximum retention time was 4.137 minutes, and minimum retention time was 4.130 minutes**.** (Table 2.)

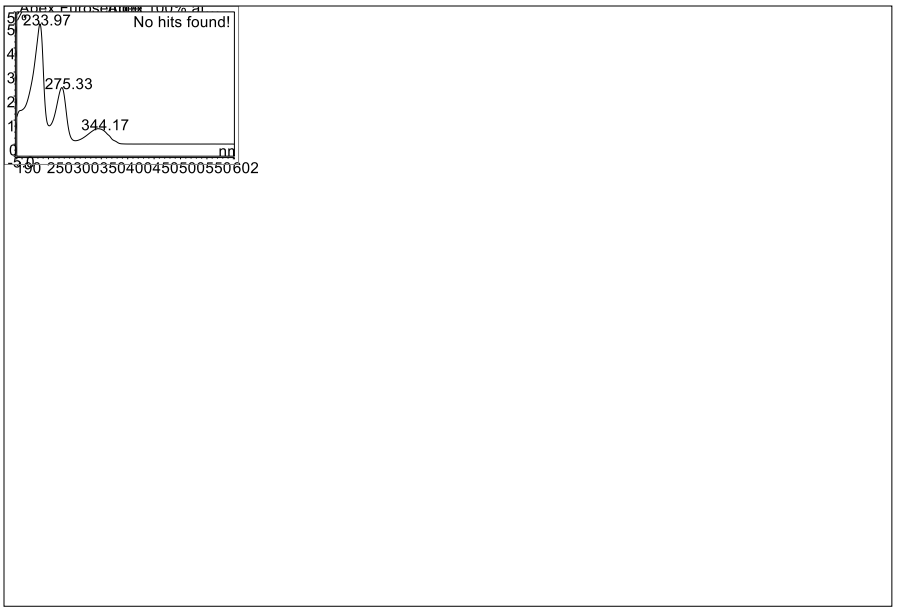
Table 2. Injection repeatability table (C_x=0.01 µg/ml)

Injection (No.)	Retention Time (min.)
1	4,133
2	4,133
3	4,135
4	4,13
5	4,133
6	4,135
7	4,135
8	4,137
9	4,137
10	4,137

Results (Furosemide)

For the detection of the furosemide Hypersil Gold™ C18 (50 mm x 2.1 mm) column was used. During the process of the analysis of the furosemide tablets, we tried the number of the mobile phase and one of the most suitable was made up of water, and acetonitrile (70:30, v/v). Flow rate of the mobile phase was equal to 0.200 ml/min and the length of the UV wave for furosemide maximum absorption was equal to 233.97 nm (Fig 6.)

Figure 6. Chromatogram of Furosemide standard solution



Method of validation (Furosemide)

Validation process of our method was according to the guidelines approved by international committee. We validated our method for ensuing characteristics: linearity, limit of the detection, limit of the quantification, precision (injection repeatability). [19]

Linearity

For the examination of the linearity, we prepared six solutions with concentration from 0.01 to 5 μg/ml. Solutions prepared by the dissolving of the stock solution with the distilled water. Analysis of all solutions was provided at the identical state and the chromatographic area of the peak was identified. Regression equation for the area of the peak in the range from 0.01 to 5 μg/ml was equal to y = 1,0416x + 0,0498 and the concentration was equal to R^2 =0.9996. On the figure 6 we can see graphical representation of the variety of the concentration values which was created according to the regression equation. From these values, which was calculated in the range from 0.01 to 5 μg/ml, we can see that slope of the line equal to 2.0307, while intercept and coefficient of the regression (R^2) was 0.00596, 0.9959 respectively. From the figures 6, 7 we can see that theoretical concentration and concentration from equation showed interrelationship. We can see that slopes of this lines are close to the unification, and their intercepts are near to the 0.

Figure 7. HPLC calibration curve for furosemide detection

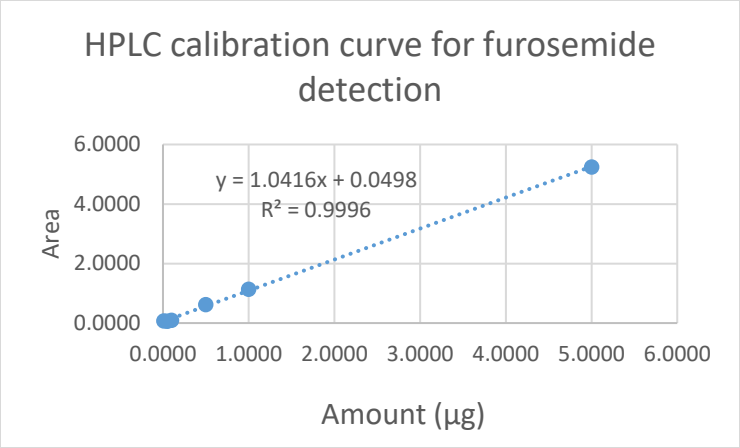
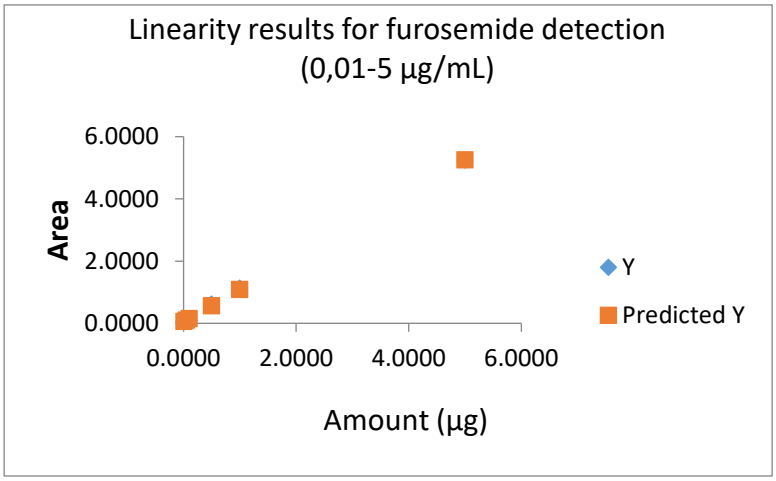


Figure 8. Linearity results for furosemide detection (0,01-5 µg/mL)



Limit of the detection and limit of the quantification

By using the regression slope and standard deviation values, we calculated the limit of detection = 0.1498 µg/ml) and the limit of quantification = 0.4540 µg/ml values. [20]

Precision: by providing the 10 consecutive HPLC runs of the lowest concentration of furosemide (Cx=0.01 µg/mL) we detect the system precision. Relative standard deviation was equal to 0.11 %. While maximum retention time was 10.758 minutes, and minimum retention time was 10.733 minutes**.** (Table 3.)

Table 3. Injection repeatability table (C_x=0.01 µg/ml)

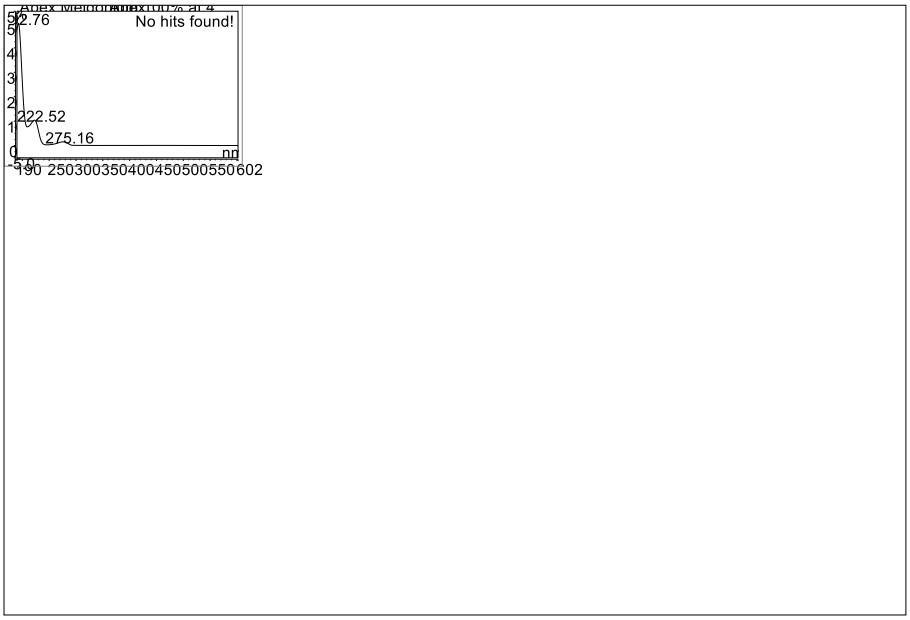
Injection (No.)	Retention Time
1	10,733
2	10,733
3	10,733
4	10,733
5	10,753
6	10,758
7	10,753
8	10,757
9	10,758
10	10,753

Results (Meldonium)

For the detection of the Meldonium Hypersil Gold™ C18 (50 mm x 2.1 mm) column was used. During the process of the analysis of the meldonium capsules, we tried the number of the mobile

phase and one of the most suitable was made up of water, and methanol (30:70, v/v). Flow rate of the mobile phase was equal to 0.300 ml/min and the length of the UV wave for the meldonium maximum absorption was equal to 192.76 nm (Fig 9.)

Figure 9. Chromatogram of Meldonium standard solution



Method of validation (Meldonium)

Validation process of our method was according to the guidelines approved by international committee. We validated our method for ensuing characteristics: linearity, limit of the detection, limit of the quantification, precision (injection repeatability). [19]

Linearity

For the examination of the linearity, we prepared six solutions with concentration from 0.01 to 5 μg/ml. Solutions prepared by the dissolving of the stock solution with the distilled water. Analysis of all solutions was provided at the identical state and the chromatographic area of the peak was identified. Regression equation for the area of the peak in the range from 0.01 to 5 μg/ml was equal to y= 0,6401x + 0,0039 and the concentration was equal to $R^{2 =}$ 0.9998. On the figure 10 we can see graphical representation of the variety of the concentration values which was created according to the regression equation. From these values, which was calculated in the range from 0.01 to 5 μg/ml, we can see that slope of the line equal to 0.0045, while intercept and coefficient of the regression (R^2) was 0.0095, 0.9997 respectively. From the figures 10, 11 we can see that theoretical

concentration and concentration from equation showed interrelationship. We can see that slopes of this lines are close to the unification, and their intercepts are near to the 0.

Figure 10. HPLC calibration curve for meldonium detection

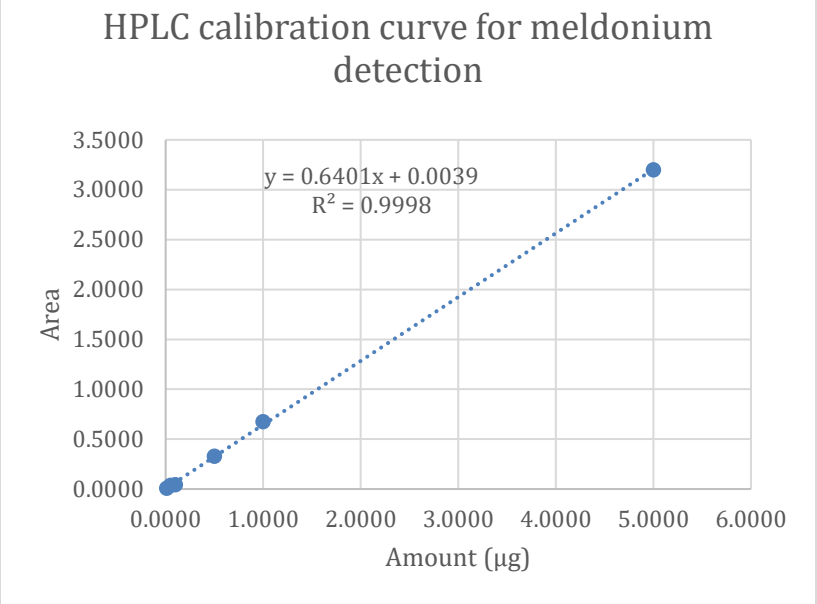
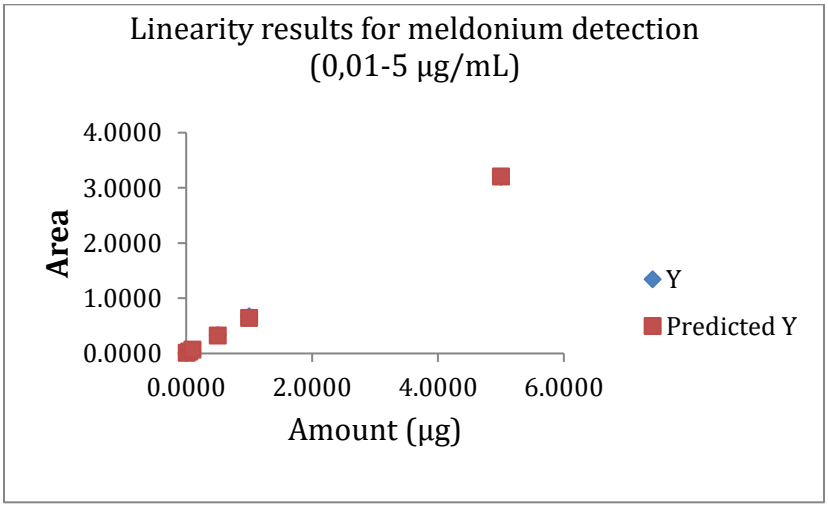


Figure 11. Linearity results for meldonium detection (0,01-5 µg/mL)



Limit of the detection and limit of the quantification

By using the regression slope and standard deviation values, we calculated the limit of detection = 0.1020 µg/ml) and the limit of quantification = 0.3091 µg/ml values. [20]

Precision: by providing the 10 consecutive HPLC runs of the lowest concentration of meldonium (Cx=0.01 µg/mL) we detect the system precision. Relative standard deviation was equal to 0.12

%. While maximum retention time was 9.013 minutes, and minimum retention time was 9.00 minutes**.** (Table 4.)

Table 4. Injection repeatability table (C_x=0.01 µg/ml)

Injection (No.)	Retention time
1	9,011
2	9,011
3	9,011
4	9,012
5	9,013
6	9,010
7	9,010
8	9,00
9	9,011
10	9,011

In comparison with other studies results, our study showed fastest retention time for Bisoprolol 4.13 minutes, and our study reveal acceptable sensitivity with LOD=0.4607 µg/ml, and LOQ=1.3962 µg/ml. While retention time of Furosemide was equal to 10.733 minutes, which was slower than in previous studies, but according to the LOD and LOQ our study showed satisfactory sensitivity with LOD equal to 0.1498 µg/ml and LOQ 0.454 µg/ml respectively. According to the Meldonium, our study provides adequate sensitivity with LOD=0.102 µg/ml, LOQ=1.3962 µg/ml and retention time was equal to 9 minutes. **(Table 5.)**

Table 5. Validation results comparison table

Name of the research work	Substance	Retention time (min)	LOD	LOQ
Our research	Furosemide	10,733	0,1498 µg/ml	0,454 µg/ml
Nagori, B. P., & Solanki, R. (2010). Indian journal of pharmaceutical sciences	Furosemide	3.038	-	-
Youm, I., & Youan, B. B. C. (2013). Journal of Analytical Methods in Chemistry	Furosemide	7.5	5.2 ng·mL−1	15.8 ng·mL−1
Sawant Ramesh, L., Bharat Anjali, V., Tanpure Kallyani, D., & Jadhav Kallyani, A. (2015). International Journal of Pharma Sciences and Research	Furosemide	4.58	-	-
Mohammed, S. B. D. A. K., & Alassaf, N. A. (2016). Chemistry and Materials Research	Furosemide	1.9	0.115 (µg.mL-1)	0.348 (µg.mL-1)
Our research	Bisoprolol	4,13	0,4607 µg/ml	1,3962 µg/ml
MAHU, S. C., SPAC, A. F., CIOBANU, C., HANCIANU, M., AGOROAEI, L., & BUTNARU, E.	Bisoprolol	-	1.3 g/ml	3.98 g/ml
Logoyda, L. I. L. I. Y. A., Kovalenko, S. E. R. G. I. Y., Abdel-Megied, A. M., Zhulkevych, I. G. O. R., Drapak, I. R. Y. N. A., Demchuk, I. N. N. A., & Netsyuk, O. L. E. H. (2019).	Bisoprolol	4.75	-	-
VU, B., Gaikwad, R. B., Chaudhari, F. M., & Kande, T. R. (2018).	Bisoprolol	5.7	0.27 µg/ml	0.83 µg/ml
Our research	Meldonium	9,00	0,102 µg/ml	0,309 µg/ml
Azaryan, A. A., Temerdashev, A. Z., & Dmitrieva, E. V. (2017). Journal of Analytical Chemistry	Meldonium	6,20	7.5 ng/ml	-

Limitations of the study

- **During 1st term we did not have possibility to work with GC/MS (was not working),** during the 2nd term, due to the time limitation we used only HPLC analysis
- **We did not receive reagents from Sigma-Aldrich company:** Due to the Covid-19 restrictions we had problems with obtaining of the reagents from foreign companies.
- **We did not have possibility to purchase Steroid drugs:** In our local pharmacies, we did not find suitable for HPLC analysis

Conclusion

1. The developed and validated HPLC method is simple, precise, and accurate, and was successfully applied to determine furosemide, bisoprolol in tablets, and meldonium in capsule forms

2. Our study showed fastest retention time for Bisoprolol 4.13 minutes, and our study reveal acceptable sensitivity with LOD=0.4607 μg/ml, and LOQ=1.3962 μg/ml. While retention time of Furosemide was equal to 10.733 minutes, which was slower than in previous studies, but according to the LOD and LOQ our study showed satisfactory sensitivity with LOD equal to 0.1498 μg/ml and LOQ 0.454 μg/ml respectively. According to the Meldonium, our study provides adequate sensitivity with LOD=0.102 μg/ml, LOQ=1.3962 μg/ml and retention time was equal to 9 minutes.

3. The method was validated in terms of linearity, precision, limit of detection and limit of quantification. The developed method was successfully applied for the estimation of furosemide, bisoprolol, meldonium in pharmaceutical dosage forms

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Thanks to co-supervisor: Yeltay Rakhmanov MD, PhD

Thanks to: Burkitkan Akbay, PhD

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Appendix

1. IREC approval letter

NAZARBAYEV UNIVERSITY
SCHOOL OF MEDICINE

Nur-Sultan, 11 December 2021

RE: Decision on the project "Optimization of analysis of popular doping substances using gas chromatography - Mass spectrometry method" *(OCT#02)*

The integrated student-driven project **"Optimization of analysis of popular doping substances using gas chromatography - mass spectrometry method"** having **Zhandos Bissarinov,** as Principal Investigator, and **Dr. Syed Ali** as Research Advisor, is part of the Master in Sport Medicine and Rehabilitation curriculum.

The above-mentioned student-driven project was evaluated by the NUSOM-IREC (Nazarbayev University School of Medicine – Institutional Research Ethics Committee) and is eligible for an **Exemption** from Research Ethics Review.

*This is to inform you that the aforementioned research has been **approved from the NUSOM- IREC as Application NUSOM-IREC-2021-OCT#02** on December 11th, 2021.*

Prof. Alessandro Salustri
Chair of NUSOM-IREC