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RESEARCH ARTICLE

Semiquantitative determination of meldonium and emoxypine in human urine by HPLC–MS/MS after receiving a single therapeutic dose of Brainmax® and milk from cows receiving a preventive course of Emidonol®

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Abstract

Objectives. Emidonol® is a veterinary drug used to treat pathological conditions associated with hypoxia in cattle. In addition to meldonium, which is included in the Prohibited List of the World Anti-Doping Agency, the biotransformation product of Emidonol® in animals is the antioxidant and antihypoxant emoxypine, which can act as a marker of contamination of food products with the above-mentioned widely known metabolic modulator. The study set out to semiquantitatively determine emoxypine and meldonium levels, as well as to compare the excretion profiles of these substances by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) in urine samples of volunteers after receiving a single oral administration of a therapeutic dose of Brainmax® and after consuming a large amount of milk from cows that had received a prophylactic course of Emidonol®.

Methods. Sample preparation of urine samples for the determination of meldonium was carried out using the "dilute and shoot" approach. Enzymatic hydrolysis with β-glucuronidase followed by purification by solid-phase extraction was used to determine emoxypine. Identification of meldonium and emoxypine was carried out by HPLC–MS/MS under conditions of electrospray ionization with registration of positively charged ions in the selective reaction monitoring (SRM) mode for the following transitions and collision energies: for meldonium, 147.1 > 147.1 (15), 147.1 > 132.1 (17), 147.1 > 58.1 (17), 147.1 > 59.1 (17), 147.1 > 42.1 (60); for emoxypine, 138.1 > 138.1 (7), 138.1 > 123.1 (15), 138.1 > 110.1 (20), 138.1 > 95.1 (20).

Results. The possibility of simultaneous identifying meldonium and emoxypine obtained after enzymatic hydrolysis with β-glucuronidase in urine samples of volunteers after oral intake of single dose of Brainmax® and consuming a large amount of Emidonol®-contaminated milk using the HPLC–MS/MS method with different numbers and variants of SRM transitions was demonstrated. Differences in the excretion profiles of these substances were found after ingestion of large amounts of contaminated milk and a single oral dose of Brainmax® 15–18 h later and further. After taking contaminated milk 12 h or more later, emoxypine is detected in concentrations 5 or more times higher than meldonium concentrations and is excreted for a longer period of time. Conversely, after taking a single dose of Brainmax®, which contains both substances, the content of meldonium in urine samples of volunteers 15–18 h after taking it is several times higher in relation to emoxypine. The constant ratio of estimated concentrations of meldonium and emoxypine in Emidonol® was found to be approximately 1 : 2.

Conclusions. Identification of meldonium in the presence of emoxypine in urine under certain conditions can be used to distinguish contamination of food products with a prohibited metabolic modulator from intentional ingestion of real doping.

Keywords

Emidonol®, emoxypine, meldonium, Brainmax®, high-performance liquid chromatography–tandem mass spectrometry

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НАУЧНАЯ СТАТЬЯ

Полуколичественное определение мельдония и эмоксипина в моче методом ВЭЖХ–МС/МС после приема однократной терапевтической дозы препарата Брейнмакс® и молока коров, получавших профилактический курс Эмидонола®

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Аннотация

Цели. Эмидонол® — лекарственный препарат ветеринарного назначения, применяемый для лечения у крупного рогатого скота патологических состояний, связанных с гипоксией. Продуктом биотрансформации Эмидонола® в организме животных помимо мельдония, входящего в Запрещенный список Всемирного антидопингового агентства, является антиоксидант и антигипоксант эмоксипин, который может выступать в качестве маркера контаминации продуктов питания вышеуказанным получившим широкую известность модулятором метаболизма. Цель исследования заключалась в полуколичественном определении эмоксипина и мельдония и сравнении профилей выведения этих веществ методом высокоэффективной жидкостной хроматографии–тандемной масс-спектрометрии (ВЭЖХ–МС/МС) в образцах мочи добровольцев после однократного перорального приема терапевтической дозы препарата Брейнмакс® и большого количества молока коров, получавших профилактический курс ветпрепаратом Эмидонол®.

Методы. Пробоподготовку образцов мочи для определения мельдония проводили посредством подхода «dilute and shoot», для определения эмоксипина использовали ферментативный гидролиз с β-глюкуронидазой и последующей очисткой методом твердофазной экстракции. Идентификация мельдония и эмоксипина осуществлялась методом ВЭЖХ–МС/МС в условиях электрораспылительной ионизации с регистрацией положительно-заряженных ионов в режиме мониторинга селективных (выбранных) реакций (SRM) по следующим переходам и энергиям соударения: 147.1 > 147.1 (15), 147.1 > 132.1 (17), 147.1 > 58.1 (17), 147.1 > 59.1 (17), 147.1 > 42.1 (60) для мельдония и 138.1 > 138.1 (7), 138.1 > 123.1 (15), 138.1 > 110.1 (20), 138.1 > 95.1 (20) для эмоксипина.

Результаты. Показана возможность одновременной идентификации мельдония, определенного прямым разбавлением, и эмоксипина, полученного после ферментативного гидролиза β-глюкуронидазой, в образцах мочи добровольцев после перорального приема однократной дозы препарата Брейнмакс® и употребления большого количества молока, загрязненного Эмидонолом®, методом ВЭЖХ–МС/МС с использованием различного количества и вариантов SRM-переходов. Установлены различия в профилях выведения данных веществ после приема больших количеств контаминированного молока и однократного перорального приема препарата Брейнмакс® спустя 15–18 ч и позже. После приема контаминированного молока спустя 12 ч и позже эмоксипин определяется в концентрациях в 5 и более раз превышающих концентрации мельдония и выводится более длительное время. При однократном приеме препарата Брейнмакс®, содержащего оба вещества, напротив, содержание мельдония в образцах мочи добровольцев спустя 15–18 ч и позднее после приема в несколько раз выше по отношению к эмоксипину. Также обнаружено, что постоянное соотношение оценочных концентраций мельдония и эмоксипина в чистом препарате Эмидонол® соответствует 1 : 2.

Выводы. Идентификация мельдония в присутствии эмоксипина в моче при определенных условиях может быть использована для отличия контаминации продуктов питания запрещенным модулятором метаболизма от намеренного приема реального допинга.

Ключевые слова
Эмидонол®, эмоксипин, мельдоний, Брейнмакс®, высокоэффективная жидкостная хроматография–тандемная масс-спектрометрия

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INTRODUCTION

The antihypoxic, antioxidant, and membrane-protective effects of veterinary drug Emidonol® (3-(2,2,2,2-trimethylhydrazinium) propionate-2-ethyl-6-methyl-3-hydroxy-pyridine disuccinate) are due to its components [1] (Fig. 1). Its use in cattle is authorized by the Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor) for various pathologies accompanied by hypoxia in the form of 5 and 10% solutions[1].

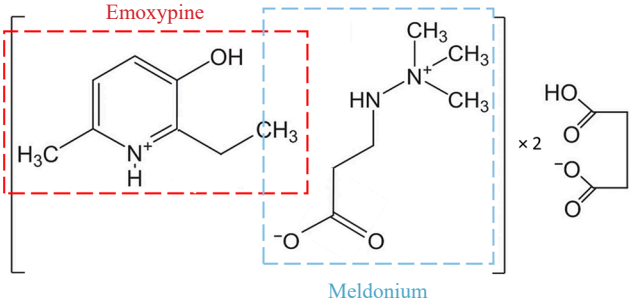


Fig. 1. Structural formula of Emidonol®

In animals, Emidonol® undergoes biotransformation to form trimethylhydrazinium propionate (meldonium) and emoxypine succinate, which is known in Russia as Mexidol®. The former is a metabolic modulator on the World Anti-Doping Agency (WADA) List of Prohibited Substances[2] [2]. The effect of the latter, which has been recently described by a number of authors as an antioxidant and antihypoxant, can also lead to an increase in performance, and is comparable to the effect of doping substances [2], but it has not yet been included in the WADA monitoring program[3] as a hypoxen [3].

The problem of contamination of foodstuffs with meldonium is extremely relevant for athletes due to the real risk of positive doping tests, given that it has been the most used doping substance for more than 15 years[4]. Earlier studies were published on the determination of the banned metabolic agent both in food [4] and in the urine of volunteers after consumption of milk from cows treated with the veterinary drug Emidonol® [5] or spiked with meldonium [6].

Our study proposes the use of emoxypine as an additional marker of Emidonol® degradation in urine biosamples simultaneously with the identification of meldonium. Of course, the simultaneous use of these two drugs to improve athletic performance is not excluded, given that emoxypine succinate (Mexidol®) is not prohibited and is often used to correct functional status in sports, but such a coincidence may be accidental and occur under conditions other than food contamination. Preparations containing meldonium and emoxypine succinate in one tablet (e.g., Brainmax®), which have recently appeared on the Russian pharmaceutical market, show a slightly different pattern of excretion with urine than contaminated milk consumption.

In this study, we conducted pilot studies comparing the excretion profiles of these two substances following a single oral administration of Brainmax® and ingestion of a large amount of milk from cows receiving a prophylactic course of Emidonol®. The proposed approaches for identifying biotransformation products of veterinary drugs meldonium and emoxypine using

[1] State Register of Medicines for Veterinary Use (list of medicines that have undergone state registration). URL: https://fsvps.gov.ru/files/gosudarstvennyj-reestr-lekarstvennyh-sredstv-dlja-veterinarnogo-primenenija-perechen-lekarstvennyh-preparatov-proshedshih-gosudarstvennuju-registraciju/. Accessed February 17, 2025.

[2] International Standard Prohibited List 2025. URL: https://www.wada-ama.org/sites/default/files/2024-09/2025list_en_final_clean_12_september_2024.pdf. Accessed March 10, 2025.

[3] WADA 2025 Monitoring Program. URL: https://www.wada-ama.org/sites/default/files/2024-09/2025_list_monitoring_program_en_final_clean_11_september_2024.pdf. Accessed December 15, 2024.

[4] RUSADA: the number of positive samples for meldonium in 2024 has decreased. URL: https://rsport.ria.ru/20240522/rusada-1947725070.html. Accessed December 17, 2024.

the method of high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) in the selective reaction monitoring (SRM) mode can be varied by using a different number of transitions. This approach can potentially be used to differentiate between the real ingestion of a prohibited metabolic modulator and contamination of foodstuffs by it when using a veterinary drug in farm animals.

MATERIALS AND METHODS

Analysis samples and reagents

The study was based on urine samples provided by volunteers (n = 3) before and after a single morning intake of three glasses (900 mL) of fresh milk of cows treated with the veterinary drug Emidonol® 10% (*AVZ Animal Health*, Russia) collected within 60 h. Dosages of Emidonol® were administered to animals for 15 days daily in the morning depending on animal weight according to the drug instructions. Milk samples were collected on the 15th (last) day of the course in plastic sterile bottles of 1 L.

Urine samples of other volunteers (n = 3) collected during 14–16 days were also used for experiments before and after a single intake of 1 tablet of the combination comparison drug Brainmax® containing 250 mg of meldonium and 250 mg of emoxypine succinate (Mexidol®), which is available in pharmacy chains without a prescription.

Volunteers (n = 3; age 35–52 years; body weight 60–92 kg; gender was not considered) had not previously taken any meldonium or mexidol preparations, nor had they consumed dietary supplements, milk or meat products 1–2 days before the urine specimen collection. Urine samples were collected in sterile 100 mL medical containers, which were labeled with the date and time of collection and stored at +4°C or frozen at −20°C before sample preparation.

The study follows the principles of the Declaration of Helsinki[5]. Written permission was obtained from the volunteers to use their biological material for research purposes.

A solution with a concentration of 1 mg/mL was prepared from deuterated meldonium (meldonium-d_3, certified standard) (*TLC PharmaChem Inc.*, Canada) used as an internal standard for the determination of meldonium. For preparation of positive urine control samples with meldonium content of 10, 100, and 1000 ng/mL, a stock solution of meldonium dihydrate reference standard (European Pharmacopoeia, Meldonium Dihydrate CRS, batch 1, European Directorate for the Quality of Medicines and HealthCare, France) having a concentration of 1 mg/mL was used.

A solution with a concentration of 1 mg/mL was prepared from Bupranolol (certified standard, *Clearsynth Labs*, 500 mg, India) used as an internal standard for the determination of emoxypine. To prepare positive control urine samples and solutions with concentrations of 10, 100, and 1000 ng/mL, a stock solution of the reference standard ethylmethylhydroxypyridine (emoxypine) succinate (MEZ-096, GEO 12209-2023, MCO 2931:2023 produced by the *Moscow Endocrine Plant*, Russia) having a concentration of 1 mg/mL was used. To estimate the concentrations of substances, calibration graphs were plotted for 3 solutions of standards in urine with the above concentrations.

The following substances were used for the study: methanol for chromatography (purity not less than 99.8%); acetonitrile; acetic acid (high-performance liquid chromatography (HPLC) grade, *JT Baker*, Netherlands); water (HPLC grade, *Thermo Scientific Chemical*, USA); sodium azide; ammonium acetate (purity not less than 99.9%, *Sigma-Aldrich*, USA); potassium carbonate; potassium hydrogen carbonate; potassium dihydrophosphate; sodium phosphate bivalent dihydrate (purity not less than 99%); β-glucuronidase from *E. Coli* K12 (*Roche*, Germany); compressed argon 5.0 with purity not less than 99.999%. To prepare buffer solutions, deionized water having resistivity of 18.2 mOsm·cm (*Millipore*, USA) was used.

Auxiliary equipment and materials

Crimper, decapper, 1.5 mL glass vials (*Macherey-Nagel GmbH & Co*, Düren, Germany); 0.3 mL polypropylene vials (*Macherey-Nagel GmbH & Co*, Düren, Germany); automatic variable volume pipettes 0.5–10 μL, 20–200 μL, 100–1000 μL, 500–5000 μL (*Eppendorf*, Germany) and their tips; liquid temperature thermostat (−30 ± 5°C), automatic orbital shaker, benchtop centrifuge with horizontal rotor for 16 × 125 mm tubes, incubator thermostat for glass tubes, Centrifuge 5430 benchtop centrifuge with rotor for 1.5–2 mL (*Eppendorf*, Germany); polypropylene test tubes 1.5 mL (*Eppendorf*, Germany); 15 mL and 50 mL Falcon tubes (*Greiner Bio-One*, Austria); Oasis HLB solid-phase extraction cartridges (60 mg, 3 mL) (*Waters*, USA); 16 × 125 mm glass tubes with screw cap; Ohaus Discovery DV215CD analytical scale (5-digit accuracy) (*OHAUS CORPORATION*, USA); Vortex liquid shaking apparatus.

[5] World Medical Association. Declaration of Helsinki. URL: https://asmu.ru/upload/iblock/067/Хельсинкская%20декларация.pdf. Accessed February 17, 2025.

Sample preparation

For the determination of meldonium, 100 μL of urine samples were collected from volunteers before (blank) and after milk intake, as well as before and after taking 1 tablet of Brainmax®. 100 μL of negative and positive (10, 100, and 1000 ng/mL) control urine samples were also taken into 1.5 mL Eppendorf tubes. 900 μL of diluent was added (to prepare the diluent, 22 μL of 0.1 mg/mL internal standard solution (meldonium-d_3) was added to a 100 mL volumetric flask and brought to the mark with methanol). After shaking, this was centrifuged for 10 min at 14000g. Then 800 μL of supernatant was collected into glass vials and covered with lids.

For the determination of emoxypine, 1 mL of blank urine, 1 mL of positive control urine samples with different emoxypine content (10, 100, and 1000 ng/mL) and 1 mL of the analyzed samples were collected into 16 mL tubes with screw cap. 1 mL of hydrolysis buffer mixture was added to each tube (the contents of 2 vials of β-glucuronidase (15 mL) were brought to 1000 mL in a measuring flask with freshly prepared phosphate buffer solution (54 g $Na_2HPO_4 \cdot 2H_2O$, 68 g K_2HPO_4, 0.5 g sodium azide was brought to 1000 mL with deionized water, pH 6.2–6.5), stirred and incubated at 55 ± 3°C for 60 ± 10 min. After incubation, 50 μL each of the internal standard (15 ng/mL bupranolol solution in methanol) was added to the tubes and mixed. 1 mL of the contents was then applied to Oasis HLB solid phase extraction cartridges (60 mg, 3 mL) preconditioned with 3 mL of methanol and then 3 mL of purified water. The cartridges were washed with 3 mL of 20% methanol solution in water and the contents eluted into clean tubes with 2 mL of 70% methanol solution in water. 800 μL of the eluate was placed in glass vials and sealed with lids.

For the determination of meldonium for the preparation of mobile phase A, 15 mL was removed from a 2.5 L water bottle and then 2.5 mL of concentrated acetic acid and 12.5 mL of 2 *M* ammonium acetate solution were added.

The mobile phase B was acetonitrile. For the determination of emoxypine, the mobile phase B consisted of acetonitrile/methanol mixture in the ratio 3 : 1.

The concentration of the determined substances was evaluated by selective SRM transitions: 147.1 > 58.1 for meldonium and 138.1 > 123.1 for emoxypine, respectively.

Parameters of instrumental analysis by HPLC–MS/MS method

For the determination of meldonium and emoxypine in urine samples of volunteers, HPLC–MS/MS analysis was performed using an Ultimate 3000 liquid chromatograph coupled to a TSQ Vantage model triple quadrupole mass spectrometer (*Thermo Fisher Scientific*, USA) with an electrospray ionization source with a heated spray gas stream in positive ion detection (ESI+) mode. Acquity UPLC® BEH HILIC 3.0 × 100 mm HPLC column with 1.7 μm particle size and Acquity UPLC® BEH HILIC 2.1 × 5 mm HPLC pre-column with 1.7 μm VanGuard™ particle size (*Waters*, USA) were used for analysis.

The following parameters were selected for the determination of meldonium: flow rate, 0.3 mL/min; sample injection volume, 10 μL; column temperature 40°C. The gradient elution program is shown in Table 1.

Table 1. Gradient elution program for the determination of meldonium

Time, min	Mobile phase A	Mobile phase B
0.0	5	95
0.5	5	95
4.0	95	5
5.5	95	5
5.51	5	95
9.0	5	95

The parameters for the determination of emoxypine are as follows: flow rate 0.5 mL/min; sample injection volume 10 μL; column temperature 40°C; gradient elution program is given in Table 2.

Table 2. Gradient elution program for the determination of emoxypine

Time, min	Mobile phase A	Mobile phase B
0.0	10	90
0.5	10	90
2.9	80	20
3.5	80	20
3.51	10	90
5.0	10	90

Positive ions were registered in SRM mode: MS run time, 9 min; collision gas pressure, 1.5 mTorr; full width at half maximum (FWHM) Q1 — 1.0, Q3 — 1.0; direct current voltage (DCV), 5 V; capillary temperature, 300°C;

evaporator temperature, 370°C; time of one complete cycle, 0.3 s; capillary voltage (ESI+), 4000 V; sheath gas pressure, 50.0; auxiliary atomizing gas flow, 20.0; ion sweep gas pressure, 0.0.

Evaluation of meldonium and emoxypine in the veterinary drug Emidonol®

In order to evaluate the content of the determined substances in the veterinary preparation Emidonol®, two solutions of the preparation in water (samples were diluted $5 \cdot 10^4$ times) were prepared. Solutions of meldonium and emoxypine in deionized water with concentrations of 10, 100, 250, and 1000 ng/mL were used as comparison solutions. To 100 μL of each of two samples of Emidonol® solution, in one of which meldonium was determined, while the other contained emoxypine, 900 μL of diluent containing meldonium-d_3 and bupranolol, respectively, were added. The assays were performed according to the programs for the determination of mexidol and meldonium described above.

RESULTS AND DISCUSSION

There are practically no data on the pharmacokinetics of Emidonol® in the literature. However, meldonium is known to be a biotransformation product of the veterinary drug in animals [4, 5]. Based on the structure of the drug, emoxypine in the form of succinate (mexidol) represents an additional metabolite. Meldonium, being an analog of gamma-butyrobetaine, contributes to the restoration of equilibrium of the processes of oxygen delivery and consumption in cells under conditions of ischemia, where glycolysis occurs without additional oxygen consumption to affect the key enzyme phosphofructokinase, which contributes to the reduction of mitochondrial damage and oxidative stress under hypoxia. In previously published studies on model experiments, we have shown the possibility of obtaining a positive doping test for meldonium after consumption of milk and meat of cows, which were administered Emidonol® veterinary drug for veterinary indications [4–6].

According to some authors, emoxypine succinate (the second component of Emidonol®) has similar biological effects to meldonium and trimetazidine included in the WADA Prohibited List and therefore should be considered as a candidate for inclusion in the monitoring program [2]. Jędrejko *et al.* state that combining emoxypine (a synthetic pyridoxine analog) with succinate in drugs increases their therapeutic efficacy. The authors claim that combinations of 3-oxypyridine derivatives with succinic acid salts can improve the performance of athletes due to their metabotropic and antihypoxic effects [2]. Lukyanova *et al.* have noted that hypoxia-induced expression of HIF-1α transcription factor is regulated by succinate and induced by succinate-containing drugs [7]. Succinate itself acts as a signaling molecule involved in the molecular adaptation of the organism to oxygen deficiency. In normoxia, succinate is rapidly eliminated with the formation of carbon dioxide and water; since its half-life is about 45 min, it is impossible to determine its content in the body.

In a study by Voronina *et al.* [8], it was found that emoxypine succinate (Mexidol®) at single and sub-chronic intraperitoneal administration at doses of 50 and 100 mg/kg significantly increased the physical performance of mice in the swimming test with load. Mildronate®, being the comparison drug in this study, increased the physical performance of animals when administered at a dose of 100 mg/kg. The authors concluded that the effect of mexidol at doses of 50 and 100 mg/kg was comparable to that of Mildronate® at a dose of 100 mg/kg.

According to the data of P.A. Baranov's dissertation work[6] and experimental studies [9], emoxypine succinate (mexidol) is metabolized with the formation of two products of phase I (2,6-dimethyl-3-oxypyridine and 6-methyl-3-oxypyridine) and three conjugated products of metabolism phase II mainly comprised of glucuronoconjugate and small amounts of phosphate. It is noted that the unchanged substance can be detected in urine in insignificant amounts within 2 days following drug administration. Baranov also noted that 0.39 ± 0.02% of the unchanged drug, 20.71 ± 4.18% of the glucurono-conjugated derivative, 30.15 ± 4.27% of the sulfoconjugated forms, and 15.56 ± 1.54% of other forms of conjugated products are excreted with urine during the first 24 h after emoxypine succinate administration. Since 20% of emoxypine is excreted in the urine as glucurono-conjugate, its determination in urine samples was performed by enzymatic hydrolysis with β-glucuronidase followed by purification of the hydrolysate by solid-phase extraction, which minimized the matrix effect. Here, the degree of extraction of emoxypine was 97.5%. Determination of emoxypine in urine samples of volunteers without solid-phase extraction and hydrolysis by the "dilute and shoot" method was difficult due to interfering peaks of matrix components complicating identification.

In an earlier study [5], the possibility in principle to determine meldonium by HPLC–MS/MS in the SRM mode using five SRM transitions

[6] Baranov P.A. Investigation of glucuronoconjugation processes based on pharmacokinetic and biochemical studies: Cand. Sci. Thesis (Biol.). Moscow: 2009. 155 p.

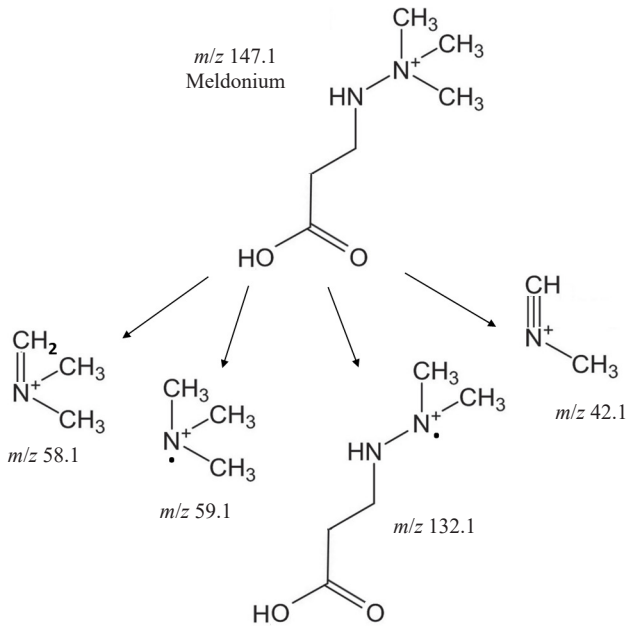


Fig. 2. Proposed fragmentation scheme of meldonium [6]

(147.1 > 147.1 (15), 147.1 > 132.1 (17), 147.1 > 59.1 (17), 147.1 > 58.1 (17), 147.1 > 42.1 (60)) after ingestion of large amounts of milk from cows (900 mL) having received a prophylactic course of the veterinary drug Emidonol® 4–12 h after consumption and 40–42 h later using the two longest-lived SRM transitions (147.1 > 59.1 (17), 147.1 > 58.1 (17)) was demonstrated. The fragmentation scheme of meldonium is shown in Fig. 2.

According to available data, meldonium is practically not metabolized and its significant part is excreted from the body in an unaltered form [10]; for this reason, the "dilute and shoot" approach was used for sample preparation. In this case, the maximum estimated concentrations of meldonium in analyzed urine samples of volunteers (taking into account 10-fold dilution according to the sample preparation method) of up to 400 ng/mL, which is higher than the minimum required performance level of WADA (Minimum Required Performance Level, MRPL[7], for meldonium—100 ng/mL), are reached after 5–10 h (when using morning milk samples) [5]. After 40–42 h, the concentrations of the metabolic modulator fell to 5 ng/mL (limit of detection) and below, and then its identification becomes difficult (Fig. 3) [5].

In the experiments performed, along with meldonium, emoxypine obtained during the hydrolysis of its glucurono-conjugated form is identified in the urine samples of all volunteers who consumed milk samples by HPLC–MS/MS using four SRM transitions (138.1 > 138.1 (7), 138.1 > 123.1 (15), 138.1 > 110.1 (20), 138.1 > 95.1 (20)). Emoxypine is identified at 32–34 h from hydrolysis of its glucurono-conjugated form; up to 50–52 h, it is identified using three SRM transitions (138.1 > 138.1 (7), 138.1 > 123.1 (15), 138.1 > 110.1 (20)) (see Appendix 1). Its maximum urinary concentrations in volunteers were 1250, 1400, and 1430 ng/mL (1360 ± 239.6 ng/mL, p = 0.95), reached 5–8 h after

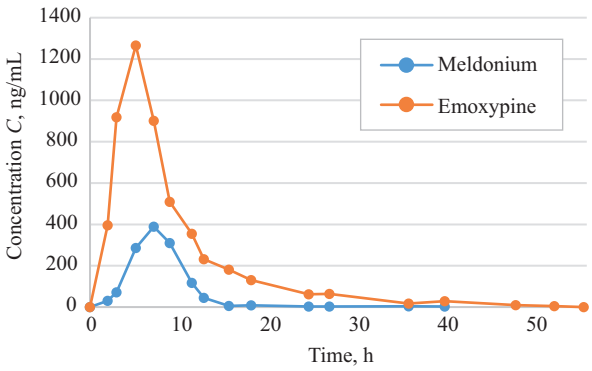


Fig. 3. Graph of meldonium and emoxypine excretion in urine for 54 h after a single consumption of a milk sample (900 mL) (volunteer sample). Estimated emoxypine concentrations are 5 or more times higher than meldonium concentrations 12 h and further after ingestion of contaminated milk

[7] WADA Technical Document – TD2022MRPL. URL: https://www.wada-ama.org/sites/default/files/2022-01/td2022mrpl_v1.1_eng_0.pdf. Accessed March 10, 2025.

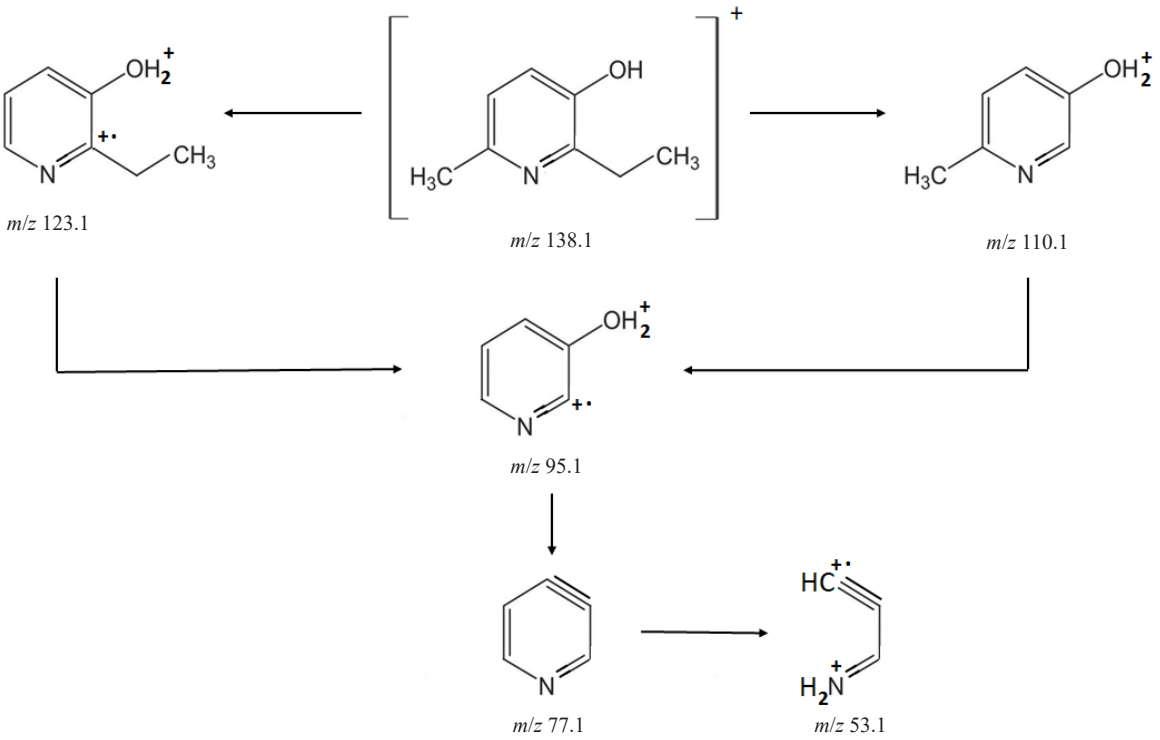


Fig. 4. Proposed fragmentation scheme of the protonated precursor ion of 2-ethyl-6-methyl-3-oxypyridine (emoxypine)[6]

administration, and fell to 5 ng/mL or lower by 52–54 h. The limit of detection of emoxypine was about 5 ng/mL. Figure 4 shows its putative fragmentation pattern.

Although the present study evaluated emoxypine released following enzymatic hydrolysis of its glucurono-conjugated form and unchanged during metabolism, the estimated concentrations in all volunteers are 3–4 times higher at the peak of excretion and 5–10 times higher at 12 h and later as compared with meldonium concentrations. This pattern of excretion is observed when analyzing urine samples from all three volunteers collected following ingestion of large doses of contaminated milk at up to 40–42 h when detection of meldonium becomes problematic due to the presence of extraneous interfering peaks of matrix components. Of course, urinary excretion depends on many factors, such as fluid intake, renal function, differences in glucuronoconjugation of emoxypine among volunteers, etc., and may vary slightly.

A different picture is observed with a single oral administration of the only combined drug Brainmax®, which contains both meldonium and mexidol and is included in the State Register of Medicinal Products of the Russian Federation. We would like to draw attention to the fact that the recommended daily dosage of meldonium is about 250–500 mg/day, while that of mexidol is 250–375 mg/day. The description of the drug states that its antioxidant, anti-ischemic, anxiolytic, nootropic, antihypoxic and membrane-protective effect exceeds the pharmacological effect of each of the components when used separately. It is also widely used for the treatment of post-coital asthenic syndrome in patients following viral infection, for complex therapy of acute and chronic cerebral circulatory disorders, as well as to improve cognitive functions and increase work capacity.

When analyzing urine samples of volunteers by HPLC–MS/MS in SRM mode, meldonium was reliably detected after a single oral administration of 1 tablet of Brainmax® using all five SRM transitions (147.1 > 147.1 (15), 147.1 > 132.1 (17), 147.1 > 59.1 (17), 147.1 > 58.1 (17), 147.1 > 42.1 (60)) for longer times, after 80–85 h; after 140–150 h using four SRM transitions (except 147.1 > 132.1 (17)); after 200–220 h using three SRM transitions (147.1 > 59.1 (17), 147.1 > 59.1 (17), 147.1 > 58.1 (17), 147.1 > 42.1 (60)) (Appendices 3d, 3e, 3f, respectively); up to 360–380 h using the two longest-lived SRM transitions (147.1 > 59.1 (17), 147.1 > 58.1 (17), data not shown). In this case, the maximum concentrations of meldonium in the urine samples of volunteers of 14070, 15280, and 15890 ng/mL (mean value 15080 ± 926.3 ng/mL) were reached 5–10 h after administration (Fig. 5). These figures take into account dilution according to the sample preparation technique. After 90–92 h, estimated metabolic modulator concentrations fell to 98, 105, and 110 ng/mL, respectively (mean 104.3 ± 15.0 ng/mL is at the MRPL level) (see Fig. 6); after 220 h, the respective concentrations fell to 18, 23,

and 20 ng/mL (20.3 ± 6.3 ng/mL). After 15–16 days (more than 360–380 h), the identification of meldonium from the above two SRM transitions became difficult (around 5 ng/mL and below) due to interfering peaks of matrix components; nevertheless, its trace amounts were still present in urine (data not shown).

Emoxypine after Brainmax® administration was significantly determined by HPLC–MS/MS using four SRM transitions (138.1 > 138.1 (7), 138. 1 > 123.1 (15), 138.1 > 110.1 (20), 138.1 > 95.1 (20)) over 50–55 h and using three SRM transitions (138.1 > 138.1 (7), 138.1 > 123.1 (15), 138.1 > 110.1 (20)) after 80–85 h (see Appendix 2). Its maximum urinary concentrations, which reached 59, 67, and 71 μg/mL (65.7 ± 15.2 μg/mL) 4–9 h after administration, were approximately 3–4 times higher than meldonium concentrations. However, 15–18 h later, concentrations fell to 690, 805, and 760 ng/mL (751.7 ± 144.0 ng/mL) and were then significantly lower than meldonium concentrations throughout the elimination time. After 90 h or more, emoxypine concentrations were below the established detection limit of 5 ng/mL; thereafter, the drug no longer detectable.

In a study of the veterinary drug Emidonol®, the ratio of estimated concentrations of meldonium to mexidol was found to be about 1 : 2, i.e., there are two molecules of emoxypine per molecule of the prohibited metabolic modulator (see Appendix 4). In general, while the maximum concentrations of emoxypine in both cases (milk consumption/single tablet administration) exceed meldonium concentrations after approximately the same time 4–10 h and up to 42 h after milk consumption, meldonium concentrations significantly exceed emoxypine concentrations 15–18 h after Brainmax® administration to reveal the opposite pattern. At the same time, as noted above, the study evaluated the content of

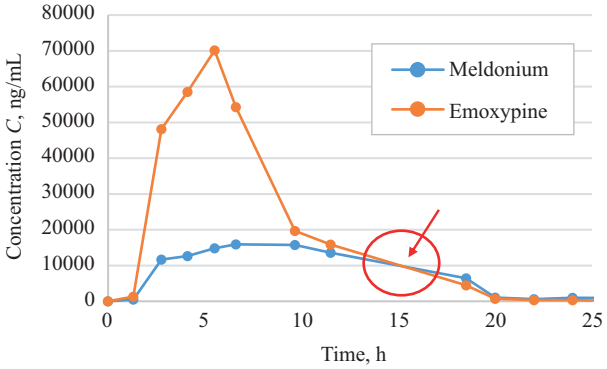


Fig. 5. Graph of meldonium and emoxypine excretion in urine during the first 25 h after oral administration of 1 tablet of Brainmax® (volunteer sample). Estimated emoxypine concentrations during the first 15 h exceed meldonium concentrations; however, the opposite scenario is observed after 15–18 h (see Fig. 6)

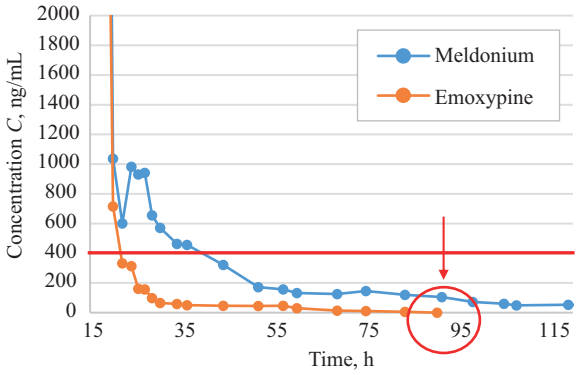


Fig. 6. Graph of meldonium and emoxypine excretion with urine during the following 15–120 h following oral administration of 1 tablet of Brainmax® (volunteer sample). After 18–20 h, the estimated concentrations of meldonium significantly exceed the concentrations of emoxypine. The red line highlights the maximum estimated concentration of meldonium in urine obtained after consumption of contaminated milk, while the arrow shows the impossibility of simultaneous determination of meldonium and emoxypine by the developed method after 90 h or more

emoxypine in urine samples released after enzymatic hydrolysis of its glucurono-conjugated form.

Similar results can be obtained after separate administration of Mildronate® and Mexidol®; however, emoxypine is evidently excreted faster, and the detection of higher concentrations of meldonium on the background of its presence in smaller amounts may indicate the same administration pattern as in the case of Brainmax®.

CONCLUSIONS

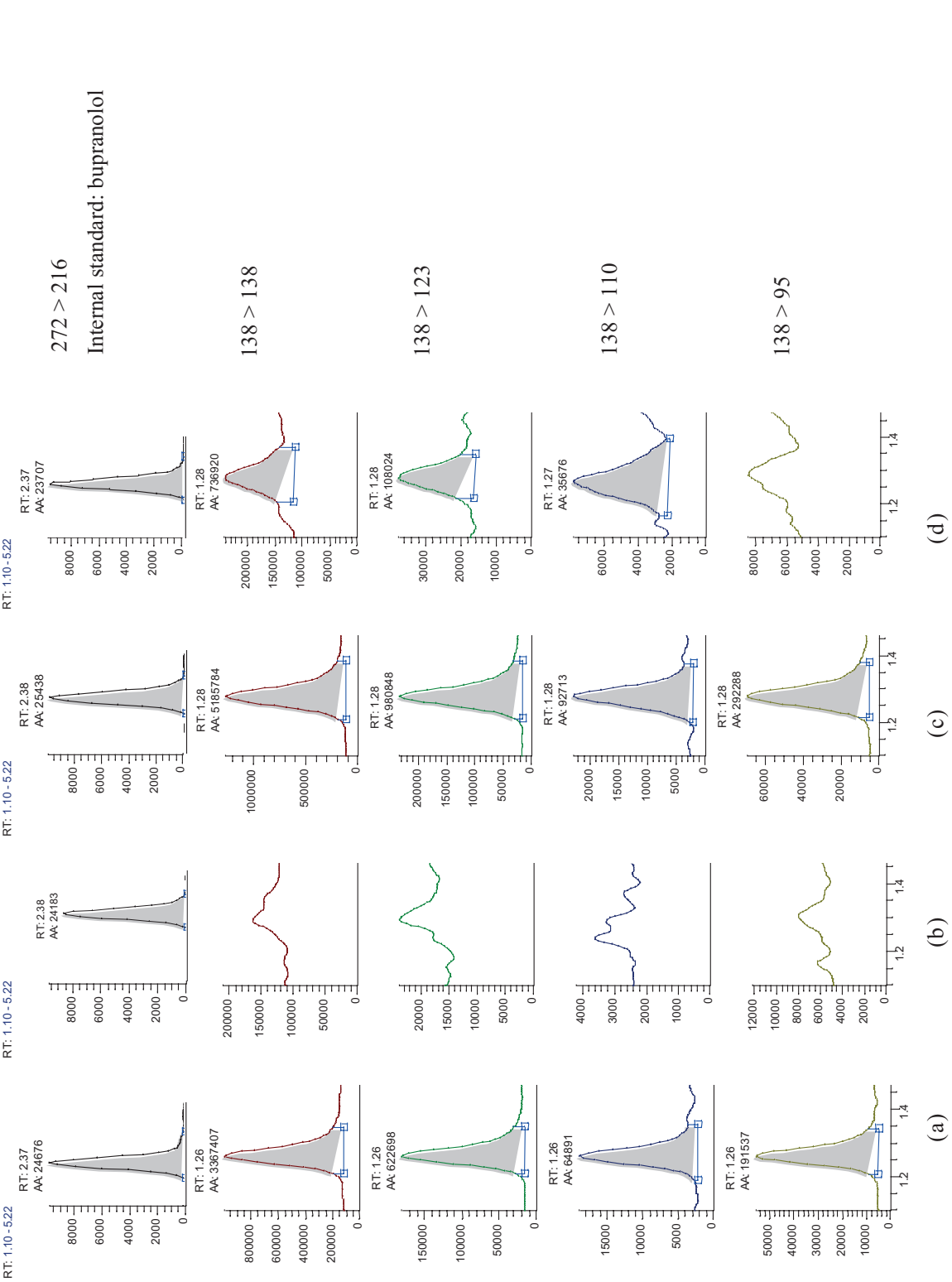
As a result of the undertaken pilot studies, it has been shown that the excretion profiles of meldonium and emoxypine are different when contaminated milk is consumed and 1 tablet of Brainmax® is taken orally. The possibility of identification of these substances in urine samples of volunteers by HPLC–MS/MS using different number and variants of SRM transitions was demonstrated.

In case of simultaneous detection of meldonium and emoxypine in a urine sample, attention should be paid to the ratio of their estimated concentrations. It appears that, in order to definitively resolve the problem of trace amounts of meldonium in urine, the two substances should be quantified: the content of emoxypine should be taken into account along with the different excretion patterns following consumption of contaminated milk and deliberate ingestion of illicit drugs. The veterinary drug Emidonol® has also been found to contain 2 times more emoxypine than meldonium, which may also be useful for identification purposes.

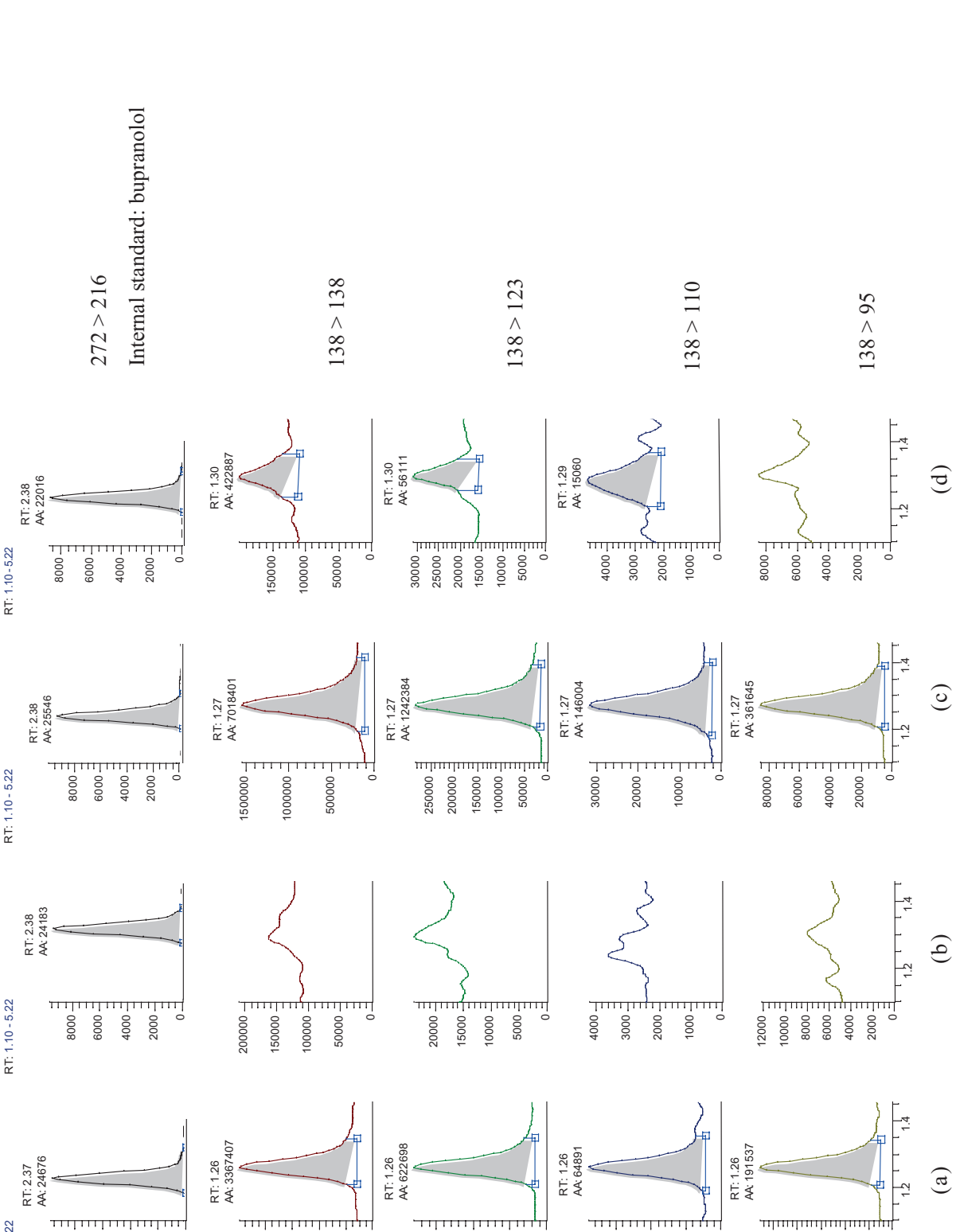
When its concentration exceeds several times in relation to meldonium, emoxypine can act as an additional marker of food contamination since also representing a biodegradation product of the veterinary drug Emidonol®. At the same time, even when large amounts of contaminated milk (900 mL) are consumed, meldonium concentrations do not exceed 320–400 ng/mL (within a few hours after consumption). Conversely, when taking 1 tablet of Brainmax®, meldonium is detected up to 80–85 h by all five SRM transitions, while emoxypine is almost undetectable. In our opinion, this approach can be used for differentiating between actual doping with products containing meldonium and the contamination of foodstuffs with meldonium due to the use of the veterinary drug Emidonol® in farm animals.

APPENDICES

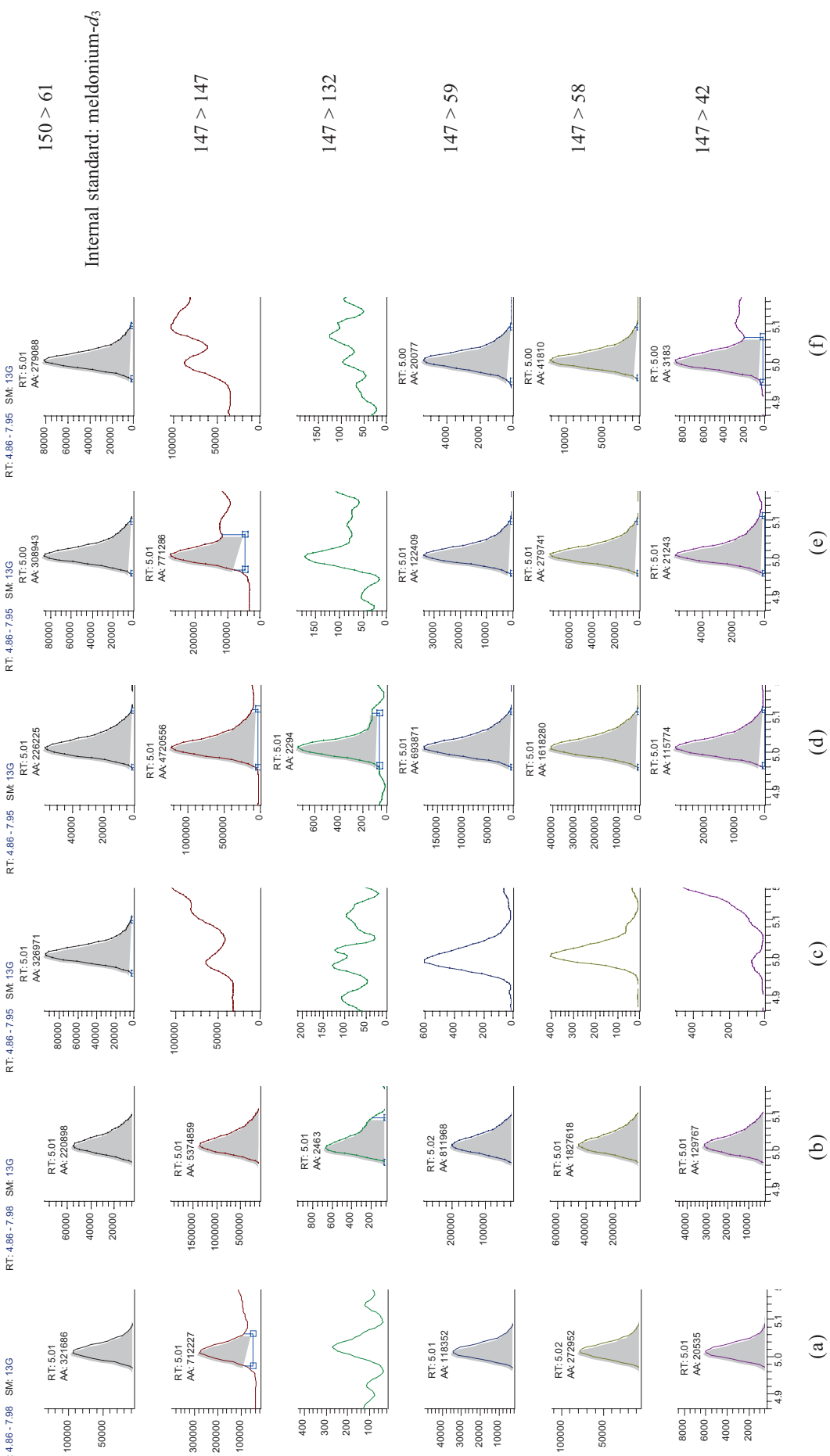
Appendix 1. Determination of emoxypine in a volunteer urine sample after consuming 900 mL of contaminated milk. (a) Positive control of urine containing 100 ng/mL of emoxypine; (b) negative control of urine; (c) volunteer urine sample in 18 h after drinking milk; (d) urine sample in 36 h after drinking milk

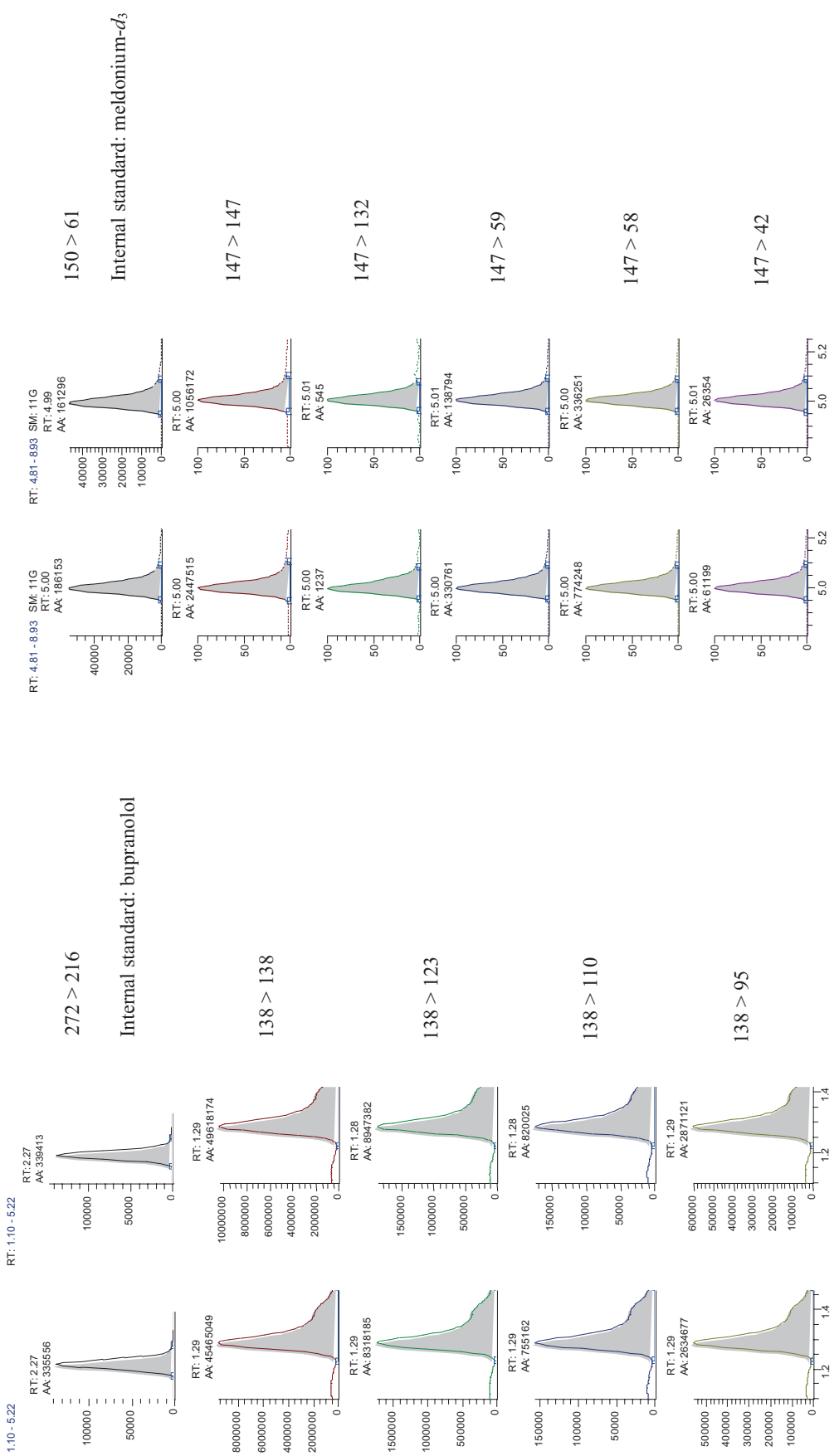


Appendix 2. Determination of emoxypine in a volunteer urine sample after oral administration of 1 tablet of Brainmax®. (a) Positive control of urine containing 100 ng/mL of emoxypine; (b) negative control of urine; (c) volunteer urine sample in 26 h after taking the drug; (d) urine sample in80 h after taking the drug



Appendix 3. Determination of meldonium in a urine sample of a volunteer after oral administration of 1 tablet of the drug Brainmax®. (a) Positive control of urine containing 100 ng/mL of meldonium; (b) positive control of urine containing 1000 ng/mL of meldonium; (c) negative control of urine; (d) volunteer urine sample in 24 h after taking the drug; (e) urine sample in 90–92 h after taking the drug; (f) urine sample in 200 h after taking the drug





Appendix 4. Determination of meldonium and emoxypine in the Emidonol® veterinary drug. (a) Positive control containing 1000 ng/mL of emoxypine (in water); (b) sample of the Emidonol® veterinary drug—solution in water (dilution by 50000 times); (c) positive control containing 1000 ng/mL of meldonium (in water); (d) sample of the Emidonol® veterinary drug—solution in water (dilution by 50000 times)

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Authors' contributions

P.V. Postnikov—writing the text of the article, formulation of aims and objectives, development of a plan for conducting experiments, conducting experimental research, discussion of experiments and results, editing the manuscript, editing the final version of the article, and preparing materials for publication.

A.D. Askretkov—conducting experimental research, discussion of experiments and results.

A.V. Polosin—conducting experimental research.

Yu.A. Efimova—editing the final version of the article and preparing materials for publication.

E.S. Mochalova—preparing materials for publication.

The authors declare no conflict of interest.

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