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Concepts for a New Rapid and Simple HPLC Method for Simultaneous Determination of Metoprolol and Meldonium in Pharmaceutical Dosage Forms

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Abstract: Simultaneous determination of the tandem of drugs, like meldonium and metoprolol, with enormous polarity differences between them, requires thorough research and careful selection of chromatographic conditions. The three different CN-cyano groups with link-based particle columns, LiChrospher CN, Waters Spherisorb CNRP, Zorbax CN SB stationary phases, were tested, in an isocratic elution system, with a running mobile phase containing various concepts of composition contents. They were first with buffering salts which included acetonitrile and ammonium phosphate in one group, and then without buffering salts but with diluted acids, composed of acetonitrile and diluted acids as the second group. We can conclude that the most optimal concepts, in terms of expressiveness and environmental friendliness, were concepts using of column Zorbax CN SB (4.6 mm i.d. × 250 mm, 5 µm) and mobile phase ACN—0.15% $NH_4H_2PO_4$ (50:50 and 60:40, *v*/*v*). There are very poor available data about ideas and usable information about the development of methods for simultaneous determination of these two active substances with polarity differences between them. We suggest that our work offered detailed and successful solutions for the mentioned aim using less sophisticated equipment for quality control and a lab for routine manufacturing control.

Keywords: dosage form; HPLC; meldonium; metoprolol

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1. Introduction

Current standards for the treatment of patients with coronary heart diseases and hypertension include, as evidenced, neurohumoral modulation (β-adrenoceptor blockers, angiotensin-converting enzyme inhibitors), hemodynamic support (nitrates, calcium antagonists) and other means of improving patients' prognosis and quality of life (statins, acetylsalicylic acid). However, in real conditions where the use of basic drugs are not shown to be of sufficient clinical efficacy or there are clinical limitations for their use, other treatments can significantly help maintain the viability of the ischemic organ, and can provide metabolic agents that increase oxygen efficiency, switching the metabolism to be more economical in a way to protect the tissue from the effects of oxidative stress during reperfusion. In the arsenal of pharmacological agents that can affect the metabolic processes in the myocardium in ischemic conditions, a new class of antianginal drugs—partial fatty acid oxidation inhibitors (p-FOX). The mechanism of action of meldonium, which is a representative of partial inhibitors of fatty acid oxidation, is to reduce the rate of

biosynthesis of carnitine from its predecessor—gamma-butyrobetaine [1,2]. On the other hand, the accumulation of gamma-butyrobetaine stimulates the biosynthesis of nitric oxide (NO) [3], which leads to the normalization of the functional state of the endothelium and, consequently, to normalize the vascular tone (vasodilatory effect of meldonium), reduces platelet aggregation. However, it is impossible to exclude another mechanism of increasing the bioavailability of NO on the background of the use of meldonium, namely reducing the intensity of its free radical inactivation.

For the last few decades, beta-blockers have been one of the main drugs for the treatment of patients with cardiovascular disease. They affect the prognosis in patients with coronary heart disease before and after myocardial infarction, chronic heart failure. They provide effective blood pressure control in patients with hypertension, cardiac arrhythmias, and are used to prevent sudden coronary death, to treat hypertrophic cardiomyopathy, and mitral valve prolapse. Summarized data from clinical trials of thousands of patients show that long-term beta-blocker therapy reduces the risk of stroke, coronary heart disease and cardiovascular mortality [4]. In this study, metoprolol and meldonium were chosen as representative examples of a fixed dose combination which has the benefits of combined therapy for patients. The search of modern scientific publications for pharmaceutical development of combined dosage forms containing metoprolol and meldonium has not yielded any results. Our scientific group proposed using the ratio of (1/5) for metoprolol and meldonium, i.e., dosage forms containing 100 mg metoprolol and 500 mg meldonium. By surveying the literature review of these drugs, it was found that numerous methods have been reported for the detection of metoprolol alone in bulk or in its pharmaceutical formulation or in biological fluids, including spectrophotometric [5–10] and LC [11–20] methods. The European Pharmacopeia [21] has a monograph on metoprolol succinate and metoprolol tartrate. Meldonium was determined by different methods like spectrophotometric [22] and chromatographic [23–29] methods. In 2020, Berlato et al. [28] published an interesting reviewing article about the pharmacology, toxicology and analytical aspects of meldonium, which summarized in tabular form 15 analytical methods (13 HPLC, 1 HPTLC and 1 Capillary Electrophoresis methods), with operating conditions and remarks, but all of them are performed on biological samples. Meldonium dihydrate is described in the European Pharmacopoeia [21] but those methods of analysis are not suitable for dosage forms. There has been only one published analytical method for determining the two proposed drugs in human plasma, which was developed by our scientific group [29]. Hence, there was a need for the development of a simple, economic, fast, reliable and ecofriendly method for the simultaneous analysis of these two active substances with high robustness and flexibility [30,31]. Our aim of this work was to explore the possibilities and problems in the development of simultaneous HPLC concepts for the determination of metoprolol and meldoniumin solid pharmaceutical dosage forms, with less sophisticated equipment and budgets.

2. Materials and Methods

2.1. Chemicals

Metoprolol tartrate (≥98% (HPLC)) and meldonium dihydrate (≥98% (HPLC)) were purchased from MERCK, Sigma-Aldrich (Schaffhausen, Switzerland). All the used reagents: acetonitrile (ACN), ammonium dihydrogen phosphate ($NH_4H_2PO_4$) and trifluoroacetic acid(TFA) were of gradient chromatography quality and were purchased from Merck Darmstad, Germany [30].

The demineralized water produced from Merck Millipore UV-Simplicity system which generates 0.05 μS conductivity and is very important in low UV wavelength measuring signals.

2.2. Instrumentals

The following HPLC columns were tested: LiChrospher 100 CN with two dimensions (4 mm i.d. × 250 mm, 5 μm and 4 mm i.d. × 125 mm, 5 μm) (column A), Waters Spherisorb

CNRP (4.6 mm i.d. × 250 mm, 5 μm) (column B), ZORBAX StableBond CN (4.6 × 250 mm, 5 μm) (column C), and purchased from Merck Darmstadt, Germany and Agilent Technologies (Santa Clara, CA, USA).

The used chromatography equipment was a product of Shimadzu UPLC system LC-40 PDA, and Shimadzu Nexera-*i* LC-2040C 3D-Plus, controlled by software Lab Solution version 5.97, then ThermoDionex Ultimate 3000 UHPLCystem with 4-channels UV-Vis detector, controlled by software Chromeleon Version 6.80 (Thermo, Waltham, MA, USA) and Aglent HPLC 1260-II with PhotoDiodeArray Detector 1260 DAD HS G7117C with 1μL flow cell, controlled by OpenLab CDS Workstation Software (Agilent Technologies, CA, USA). Recommended wavelength for targeted sensitivity was selectable in the range UV = 190–205 nm, depending on the mobile phase concept, but in the case of using a concept with trifluoroacetic acid present in eluent, the recommended range for maximal sensitivity must be above 196 nm because of the higher UV absorbance cut-off value of TFA compared to a phosphate buffer. Acetonitrile was an organic solvent with the best UV absorption characteristics, lowest cut-off value of 190 nm, which made it a unique organic solvent in our experiments.

The main accent in our experiment, besides chromatographic column choice, was varying and selecting the best combinations of mobile phase constituents and concepts: combinations of acetonitrile (ACN) with ammonium phosphate ($NH_4H_2PO_4$), acetonitrile with trifluoroacetic acid (TFA). The best results are presented, compared and commented.

Other used instruments include: Analytical Balance Mettler Toledo AG285, pH–meter Metrohm 827 and IKA orbital shaker model KS4000i. The used nylon and regenerated cellulose RC 0.2 μm and 0.45 μm syringe filters were purchased from Agilent Technologies.

2.3. Sample Preparation

Preparation of combined standard solution: 100 mg metoprolol tartrate (standard sample) and 500 mg meldonium dihydrate (standard sample) were put in a 200 mL measuring flask and dissolved in 100 mL of demineralized water, treated using ultrasound for 3 min, mixed for 5 min on a rotational shaker, and filled to 200 mL with an adequate, certain mobile phase used for actual chromatographic analyses. The mobile phase was treated using ultrasound for 5 min and shaken for 10 min using a mechanical shaker. (Notation: The standards and samples can be prepared in 0.05% v/v 85% o-H_3PO_4 if $NH_4H_2PO_4$ is used in the mobile phase composition, or 0.07% v/v TFA if this acid is used in mobile phase for analysis. It is much better to always use mobile phase, since chromatogram baselines, recovery, precision, accuracy and sensitivity are achieved with dissolving them in freshly prepared mobile phase). After that, the measuring flask was filled up to mark with mobile phase and filtered through a 0.2 μm RC syringe filter before injection. Final targeted concentrations of 0.5 mg/mL of metoprolol and 2.5 mg/mL of meldonium were obtained, which should be done with standards too. After filtration, 2–5 μL were injected in the column, with preferential 2–3 μL to form minimal baseline disturbances and best flatness, especially when mobile phase constituents ACN, 10.15% $NH_4H_2PO_4$ or 0.07% TFA are separately pumped and mixed on-line or premixed and put in one bottle. The samples were kept at 8–10 °C in autosampler till injections for analyses. They were stable and refrigerated at 4–8 °C for a period of at least 24 h.

Twelve tablets (capsules) of each preparation were studied to obtain statistically significant results. The tablets (capsules) with declared contents of 100 mg metoprolol and 500 mg meldonium were purchased from local pharmacy. The tablets were put in a 200 mL measuring flask and dissolved in 100 mL of pure water, treated using ultrasound for 5 min and shaken for 10 min a using mechanical shaker. After that, the measuring flask was filled up to the mark of 200 mL with adequate, actual freshly prepared mobile phase for current chromatography analysis, and filtered through a 0.2 μm RC syringe filter before injection. Final concentrations of 0.5 mg/mL of metoprolol and 2.5 mg/mL of meldonium were obtained, as concepted targeting concentrations of analytes. After filtration, 1–5 μL

can be injected on the column, with preferring 2–3 µL as the optimum for minimal baseline disturbances. It is very important for mobile phase and samples to be prepared fresh daily.

In the case of chromatography analysis with mobile phases containing a higher percent of acetonitrile above 30%, the sample solvent was 100 mL mobile phase with an added 100 mL of pure water.

We have performed experiments with double the targeted concentrations of meldonium 5 mg/mL and 1 mg/mL metoprolol in samples, by preparing a 500 mg meldonium capsule and 100 mg metoprolol tablet in 100 mL of water acidified with 0.05% *v*/*v* o-H_3PO_4 or 0.05% *v*/*v* TFA, and these concentrations showed very good linearity and doubled sensitivity, but system suitability parameters peak symmetry tailing are worsening because of the high mass on column load. Working with this doubled concentration was unfavorable from the view of recovery and precision of the method, additional care should be taken in satisfying extraction in high concentrations sample solutions. Thereafter, we preferred and recommend working with the first stated concentrations of 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol, even method and analytical instruments and columns could stand with higher concentrations.

3. Results

In the beginning of the HPLC method development for simultaneous quantification of metoprolol and meldonium, main challenges were evoked. The first one was the difference in chemical structure and physico-chemical properties between metoprolol and meldonium. Metoprolol tartrate, bis[(2RS)-1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol] (2R,3R)-2,3-dihydroxybutanedioate, is a water soluble molecule (0.402 mg/mL) with log *p* = 1.8, pKa (strongest acidic) = 14.09, pKa (strongest basic) = 9.67 [30]. European Pharmacopeia (EP) proposed for the detection of related substances of metoprolol method of LC with the usage of stationary phase of end-capped octadecylsilyl silica gel for chromatography (5 µm) and mobile phase composing of 3.9 g of ammonium acetate R in 810 mL water R, 2.0 mL triethylamine R, 3.0 mL of phosphoric acid R, 10.0 mL of glacial acetic acid R and 146 mL of acetonitrile R, detection at 280 nm [21]. Meldonium dihydrate, 3-(2,2,2-trimethylhydrazin-2-ium-1-yl)propanoate dihydrate, is a water soluble molecule (20.2 mg/mL) with log *p* = −2.6, pKa (strongest acidic) = 4.14 [30,31]. Meldonium is an ammonium betaine that is beta-alaninate in which one of the amino hydrogens is replaced by a trimethylamino group. Meldonium is a very polar compound without any chromophores and cannot be readily retained and detected by conventional reserved-phase HPLC-UV methods. European Pharmacopeia proposes for detection of related substances of meldonium method of LC coupled with mass spectrometry (MS) with usage of stationary phase of end-capped octadecylsilyl silica gel for chromatography (5 µm) and gradient elution of mobile phase in the composing of mobile phase A (0.1% solution of heptafluorobutyric acid R in water for chromatography R) and mobile phase B (0.1% solution of heptafluorobutyric acid R in methanol R), detection—multiple reaction monitoring (MRM) mode, scanning mode 50–300 *m*/*z* [21]. This type of HPLC-MS method is very expensive, impractical and unsuitable for routine, high-throughput-based QC laboratory analyses of pharmaceutical dosage forms. Since the meldonium has extremely weak UV absorbing characteristics and quite inferior spectrum, usage of acetates and triethyalamine are quite inadequate for composing of mobile phase intended for use with UV-PDA measuring detector, since they have very high cut-off values which prohibits their use as eluent modifiers. This states, concerns especially for heptafluorobutyric acid as obligate component in mobile phase for chromatographic method for meldonium determination according to EP, as of essential importance in this method ion-pairing agent.

3.1. First Concept Based onLiChrospher®® 125 CN (5 µm) and LiChroSpher ®® 250 CN (5 µm) Columns

Immediately, we started our concept development using a denser bonded cyano particles filled column, with a carbon load of about 6.6% and a higher active surface of

about 350 m^2/g, pH Range 2–7.5, not end-capped and not base deactivated, but with a lower number of theoretical plats per meter (15,000–40,000) and per column LiChrospher®® 125 CN (5 µm) LiChroSpher®® 250 CN (5 µm). When these two-size length different columns LiChrospher CN 125 mm and 250 mm, we were performing scouting or shorter analyses with a shorter column of 125 mm with the aim to reduce the time and solvents. The chromatogram which we got with a 125 mm column LiChrospher CN are presented in Figure 1 and has been tested with other type of mobile phases, containing ACN and water acidified with 0.07% v/v TFA. Very interesting results have been achieved.

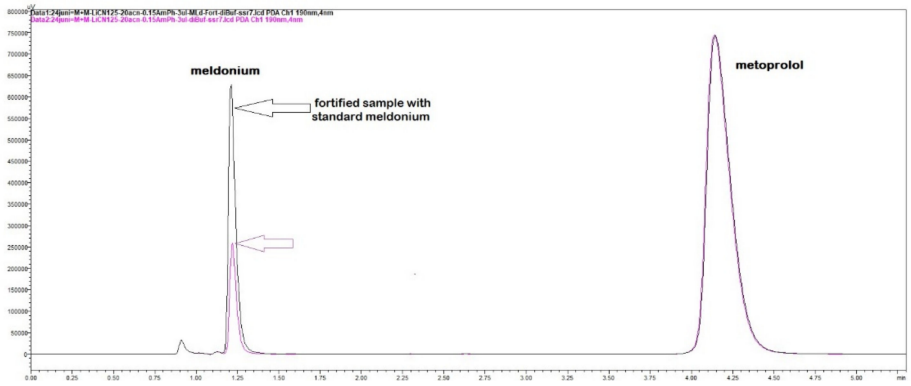


Figure 1. LiChrospher 100 CN column 125 × 4 mm, 2-wavelength comparison chromatogram, worked out with 20% ACN and 80% 12 mM (0.15% s/w) $NH_4H_2PO_4$ (Shimadzu LC-40).

The scouting experimenting chromatograms with 125 × 4 mm LiChrospher CN column are presented on Figures 1–6, all performed with 20% ACN and 80% 13 mM $NH_4H_2PO_4$. Unless otherwise stated, all the concentrations of standards and samples were adjusted to 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol. We have been surprised with the good peak symmetries of both peaks on the column which is type silia-A, non-end-capped and not-base deactivated (Figure 1). Figure 2 illustrates full 3-D chromatogram of analytes, with characteristic concave exponential UV-Abs spectrum of meldonium and two max Abs peak of metroprolol. This small 125 × 4 mm LiChrosher CN column, showed excellent mass balancing even at high quantities of concentrations, on-column mass injected analytes, especially meldonium, by creating perfect linearity curve presented on Figure 3.

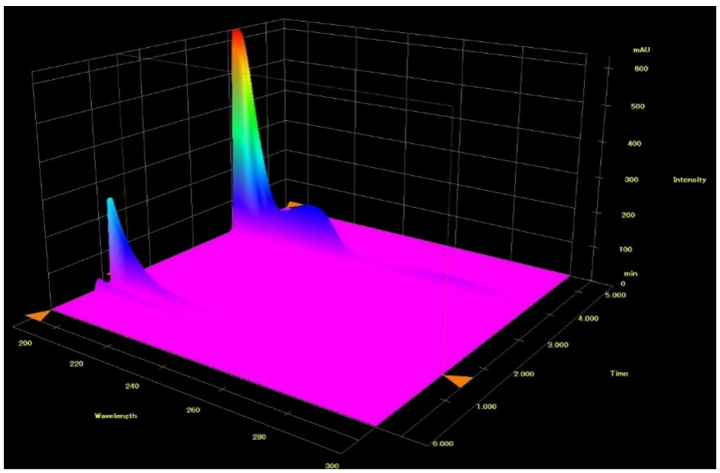


Figure 2. Full 3-D chromatogram of LiChrospher 125 × 4 mm column in acetonitrile and from the upper figure, got with 20% ACN and 80% 13 mM $NH_4H_2PO_4$. The first meldonium peak eluted at retention time about 1.25 min, which showed poor UV-abs spectrum exponentially falling in value as the wavelength increased from 190 nm to about 230–235 nm, while meldonium shows intense three max Abs peaks.

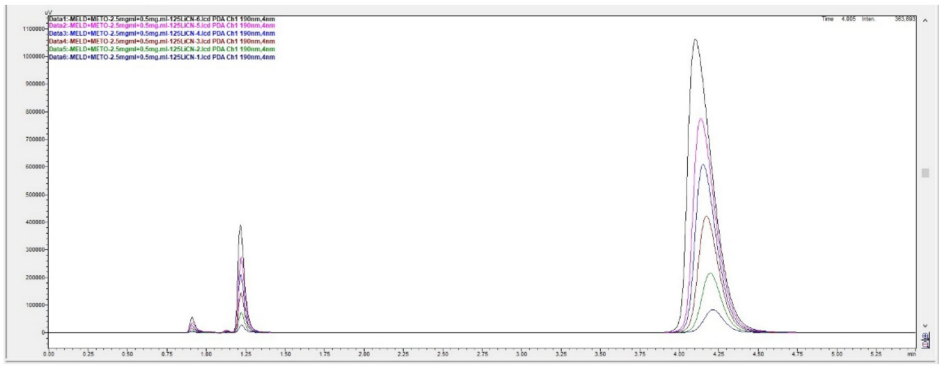


Figure 3. Overlaid chromatograms for linearity of peak responses to concentrations for meldonium and metoprolol with the short 125 × 4 mm LiChrospher column.

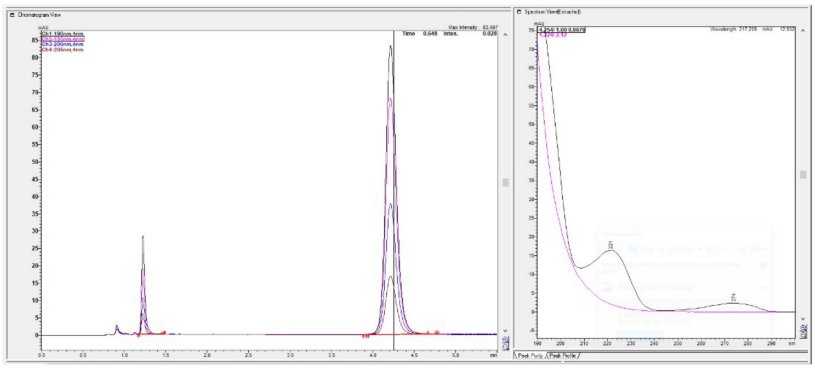


Figure 4. Chromatograms with first peak with 1.25 min of meldonium with its concave UV spectrum was overlaid with metoprolol spectrum, from the bigger peak eluted second at 4 min.

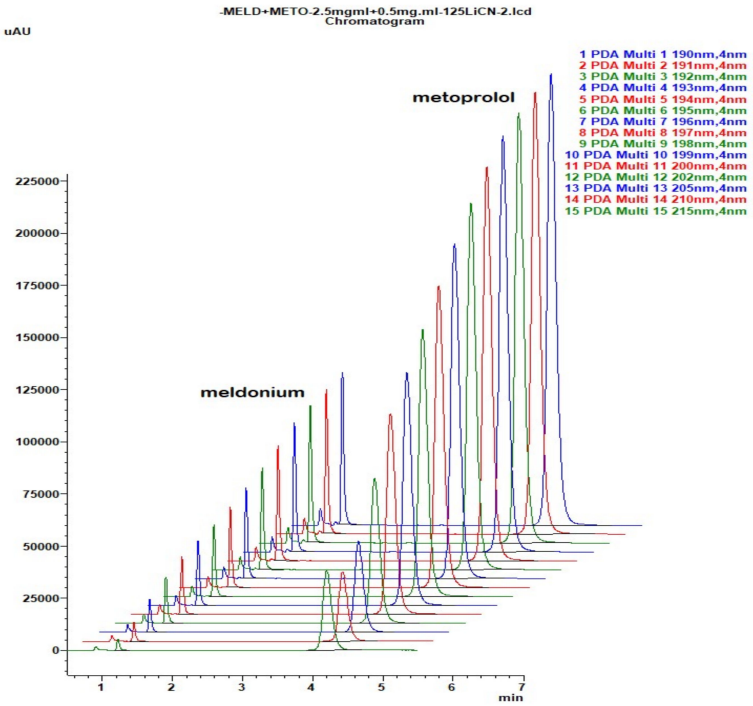


Figure 5. Chromatogram with LiChrospher CN 125 × 4 mm, mobile phase 20% ACN and 80% 13 mM $NH_4H_2PO_4$, monitored and overlaid from 15-channels overlaid, depending on peaks sizes with increments of 1 nm in UV-wavelength. Both analyte peaks were highly sensitive to the change of the wavelength, significantly, thus directly influencing the method of sensitivity for both API.

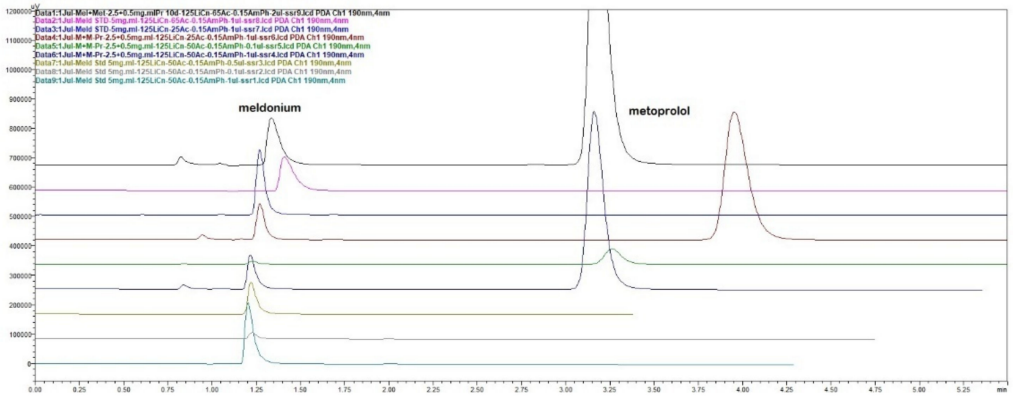


Figure 6. Retention time of meldonium and metoprolol mapping with variations of ACN percentage in composition of mobile phase with 13 mM $NH_4H_2PO_4$ with column LiChrospher CN 125 × 4 mm.

The dependence of peak size and shape of meldonium and metoprolol at 4-different wavelengths was illustrated overlaid at 4 wavelengths with isolated peak analytes UV spectrum, the "concave" shaped of meldonium and double-apex shaped of metoprolol, shown in Figure 4. Figure 5 illustrates 15-channels extraction, 15 wavelengths calculated changes of chromatogram with both analytes, with the aim to get the full conclusion about sensitivity power of the method for both peaks. Search for limits of variation of mobile phase composition with the shape of chromatogram and peaks, as retention time mapping and peak asymmetries were presented on overlaid set of chromatograms on Figure 6, with a 25%, 50% and 60% increase of ACN.

Figure 7 shows sample chromatogram of 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol at 45% ACN with 55% diluted TFA as eluent, with extracted peak UV-spectrum on right side. It is noticeable change of UV-spectrum of meldonium with disappearing of "concavity". The Figure 8 shows chromatograms baseline restrictions in visible noise, induced from high UV Cut-off value of TFA, about 205–210 nm, which complicates its use in this UV-region.

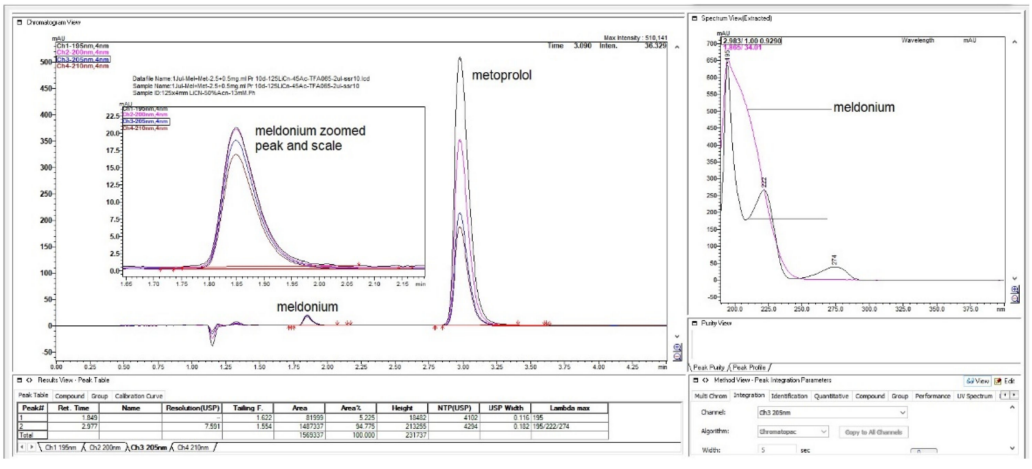


Figure 7. Chromatogram with LiChrospher CN125 × 4 mm with 45% ACN and 55% TFA 0.065 %, visible good retentions of meldonium, quite small peak and quite smaller peak height increasing with the decreasing wavelength in the lowest UV-range. While the baseline was remarkably different, changes in meldonium peak size were quite small, and very big to the peak of metoprolol. Other visible change was meldonium UV-abs spectrum shape, while the metoprolol UV-spectrum was unchanged.

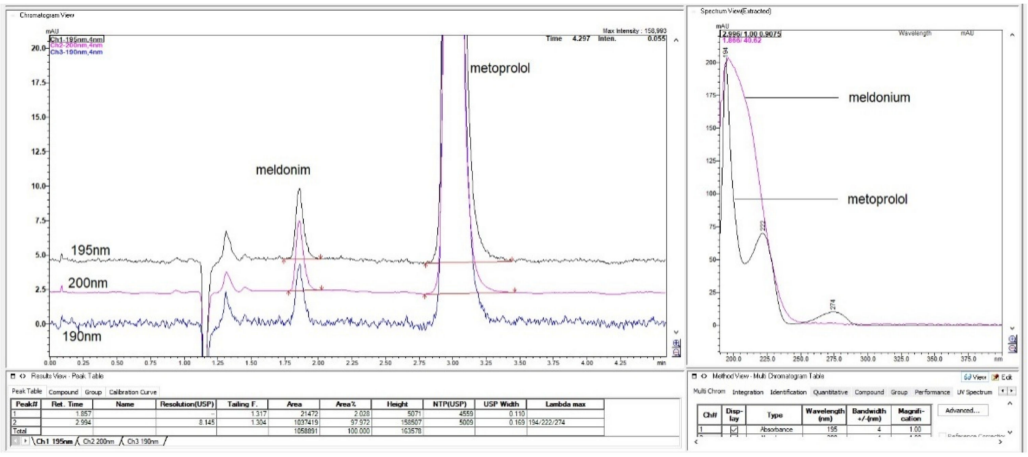


Figure 8. Column LiChrospher CN 125 × 4 mm baseline noise dependence of wavelength with mobile phase ingredient diluted TFA. While the baseline was remarkably different, changes in meldonium peak were quite small, and no more concave shaped as with ammonium phosphate used in mobile phases.

This column confirms the applicability of 125 mm column, by generating chromatograms with doubled run times of analytes. Figure 9 was 15-wavelenths channels monitored chromatogram of 250 × 4 mm LiChrospher CN column under identical operating HPLC parameters as previous 125 × 4 mm column. The extracted Abs-spectrum on right side are from meldonium (black) and first eluted adjacent unknown peak (pink) for confirmation of their distinction, recognition with the aim of the run time and spectral shapes.

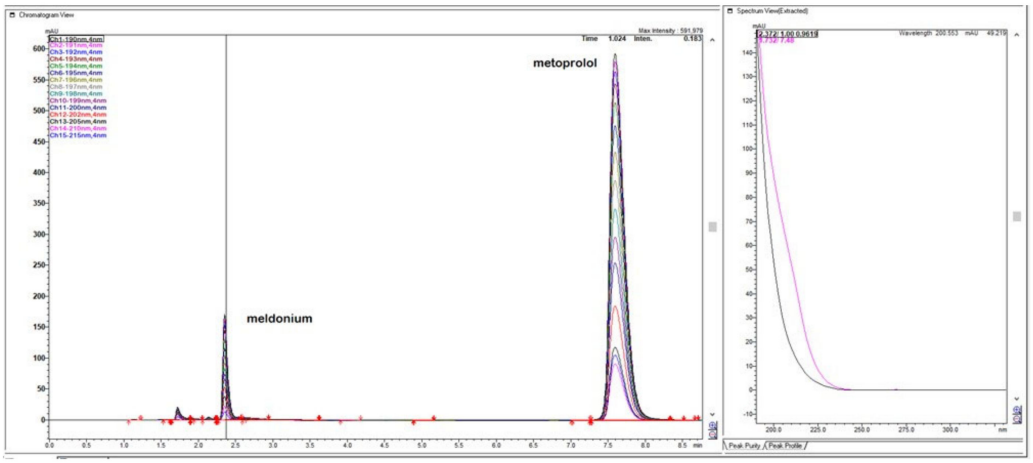


Figure 9. Multi 15-channel chromatogram 250 × 4 mm LiCnrospher 100 CN with 20% ACN -13 mM $NH_4H_2PO_4$. The two extracted peaks were from first eluted not-integrated small peak with tR about 1.7, with the characteristic meldonium concave Abs spectrum.

Figure 10 in acidic mobile phase, even at 40% presence of ACN in mobile phase, the chromatogram had a very different shape, and stronger interactions resulting in much higher retentions. Moreover, the peak shapes were especially highly distorted and skewed for the tailing peak of the meldonium, and much less for the metoprolol. The illustration shows chromatogram with analytes in full size for both, and overlaid the zoomed peak view of meldonium for better visibility, as a function of sequential overlaid increasing of recorded wavelengths 190–215 nm. Both peaks were tailing, severe asymmetry for meldonium. The right side of Figure 10 presents the UV-spectra of analytes.

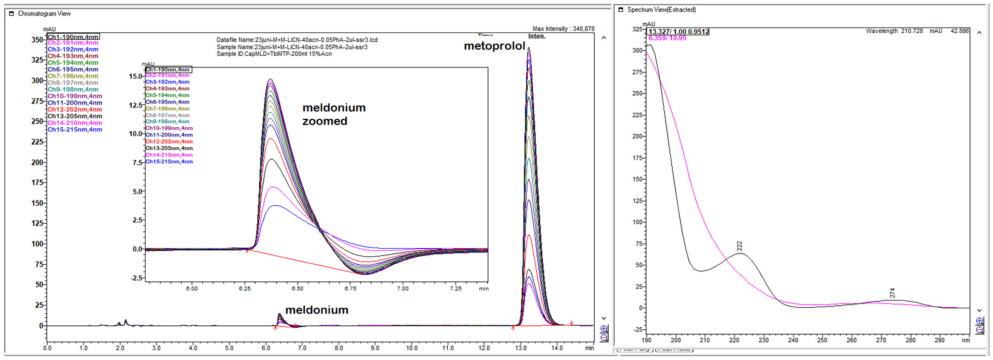


Figure 10. Chromatogram of 250 × 4 mm LiChrospher CN column with 40% ACN and 60% 0.05% $NH_4H_2PO_4$, with extracted spectrums of meldonium and metoprolol.

The chromatograms at Figure 11 confirm the selectivity of 250 × 4 mm LiChrospher CN column when 25% ACN with 75% diluted 0.065% v/v TFA was used at the mobile phase, simultaneously compared at 2 wavelengths 200 nm and 210 nm. Top chromatograms were with meldonium standard only, middle chromatograms with mixed standards of meldonium and metoprolol at 2.5 mg/mL and 0.5 mg/mL concentrations, and the bottom chromatograms were monitored solvents, which always contained full or diluted fresh mobile phases with water ×2. The chromatograms show two symmetric peaks of meldonium and metoprolol. Usage of TFA shows very good chromatograms, even at higher concentrations.

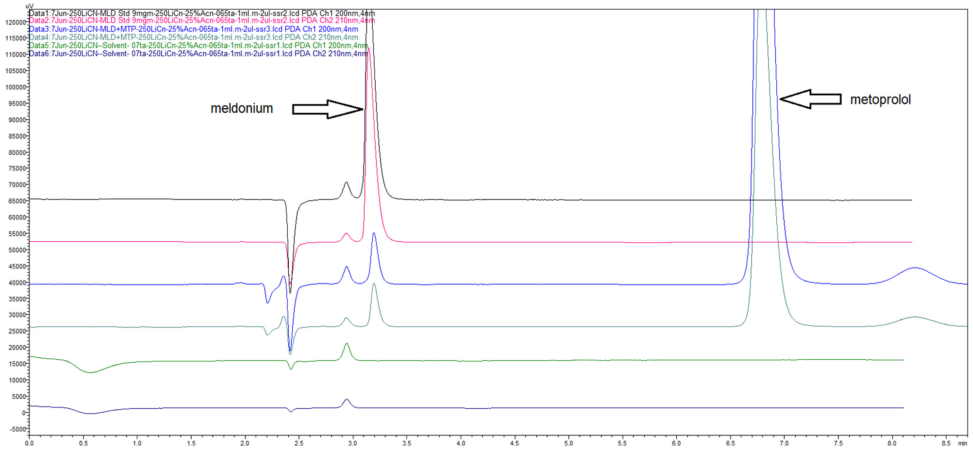


Figure 11. Chromatograms confirming the selectivity of 250 × 4 mm LiChrospher CN column when 25% ACN with 75% diluted 0.065% v/v TFA was used at mobile phase, simultaneously compared at 2 wavelengths 200 nm and 210 nm.

Figure 12 presents chromatogram of LiChrospher 250 × 4 mm, with 55% diluted TFA and 45% ACN at 2 comparison wavelengths: 195/210 nm. Operational and monitoring parameters were in the table on the right. The repositioning of the peaks of meldonium and methoprolol in a function of mobile phase variations of contents of ACN and diluted TFA is presented on Figure 13. These experiments are very useful for rough and fine tuning of analyte peaks according to the presence of other compounds, as peaks mapping as a function of ACN percentage in eluents, in the range of 25–70% ACN. Using diluted TFA in mobile phases generates significantly smaller peak size changes as in a function of wavelength decreasing changes for meldonium. The general conclusion is that a stronger acidic environment generates smaller increments in hyper-chromic effects on meldonium, compared with the presence of ammonium phosphate with a pH about 4.5, which increased by 0.2% with all 10% ACN presence. The peak of metoprolol showed increments with decreasing wavelength with different increments.

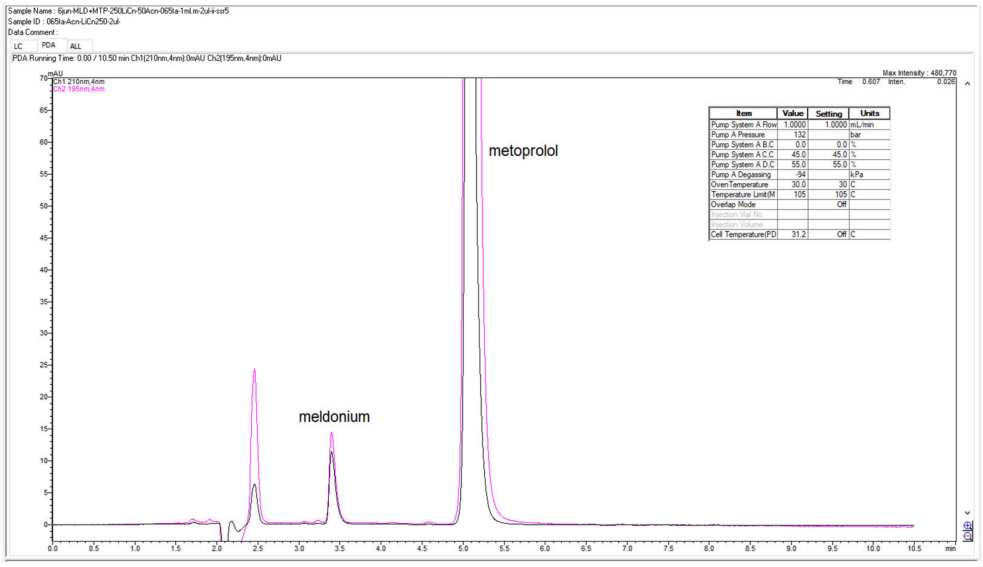


Figure 12. Chromatogram of LiChrospher 250 × 4 mm, with 55% diluted TFA and 45 %ACN at 2 comparison wavelengths, 195 nm and 210 nm. Operational and monitoring parameters presented on the table on the right.

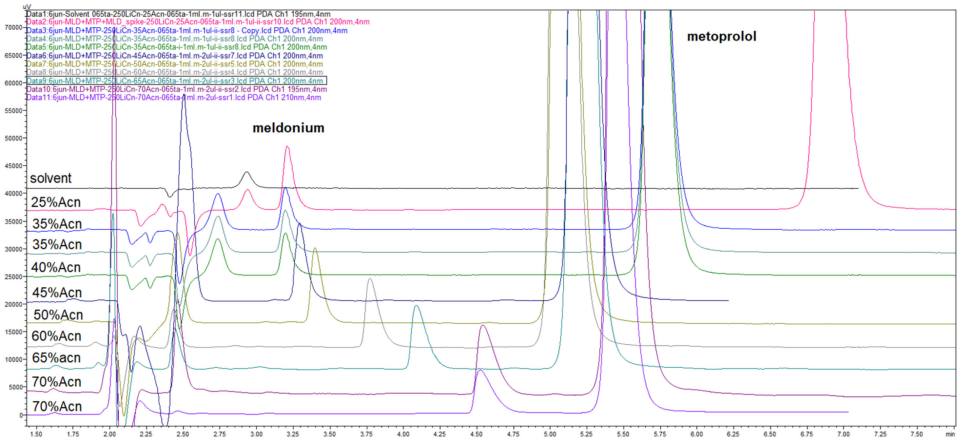


Figure 13. Overlaid chromatograms of LiChrospher CN 250 × 4 mm column experiments, that were useful for rough and fine tuning of analyte peaks according to presence of other compounds. Peaks mapping as a function of acetonitrile percentage in eluents, in the range of 25–70% ACN.

3.2. Second Concept Based on Waters Spherisob CNRP 250 × 4.6 mm 5 µm Column

Experiments with this column were performed, which resulted in different chromatograms, which was as expected due to different column characteristics from different vendors, as previously described. The first chromatogram which we have obtained using this column with 50% ACN and $NH_4H_2PO_4$ in a 50:50 ratio, is presented in Figure 14, which is recorded and presented as overlaid 5-channels extracted records This Figure presents a full-scale chromatogram with isolated UV-abs spectrums of meldonium and metoprolol on the right side. Figure 15 presents the same previous but with a expanded view of meldonium peaks with the system suitability parameters below in the table, monitored and recorded at 5 wavelengths.

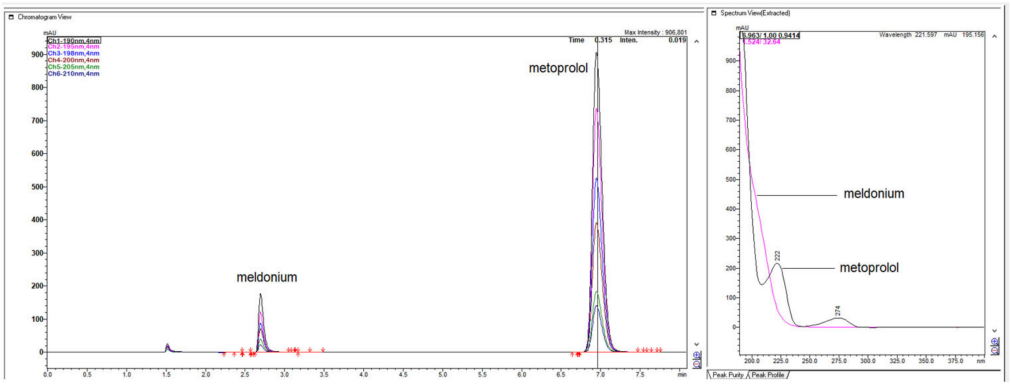


Figure 14. Chromatogram using Waters Spherisob CNRP 250 × 4.6 mm 5 µm column with 50% ACN and 0.15% $NH_4H_2PO_4$ in 50:50 ratio.

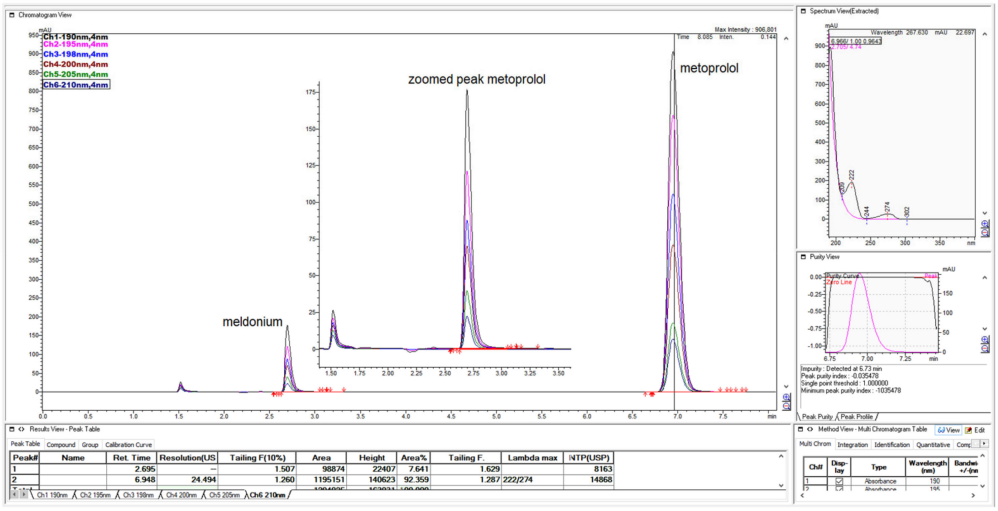


Figure 15. Chromatograms present expanded view of meldonium peaks with system suitability parameters below in the table, monitored and recorded at 5 wavelengths.

Perhaps the best visibility of the meldonium peak rising in the chromatogram depends on the wavelength, which is illustrated in Figure 16. It is mainly focused on the size of the meldonium peak increments, with visible peak increases in the height of the peak of metoprolol too. A full-scale view of chromatograms gained with 50% ACN and 50% $NH_4H_2PO_4$ is shown in the Figure 17, with direct comparisons of the peaks size changes as a function of wavelength absorbance. Further increasing percentage of ACN to 70% in the mobile phase with ammonium phosphate, results in the chromatograms illustrated in Figure 18, with visible increasing of run times of both analytes, with peaks of significant heights increasing with the decrease of the UV-wavelength.

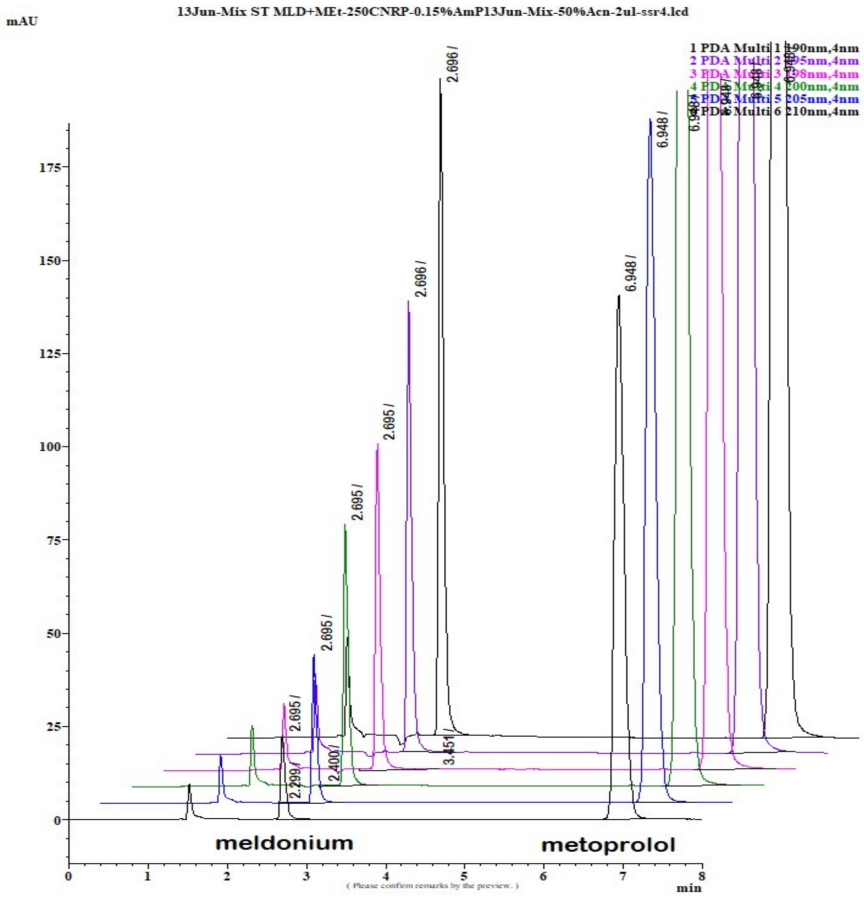


Figure 16. Chromatogram of sample 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol with Water Spherisorb CNRP-250 × 4.6 mm eluted 0.15% $NH_4H_2PO_4$ + 50% ACN meldonium expended view.

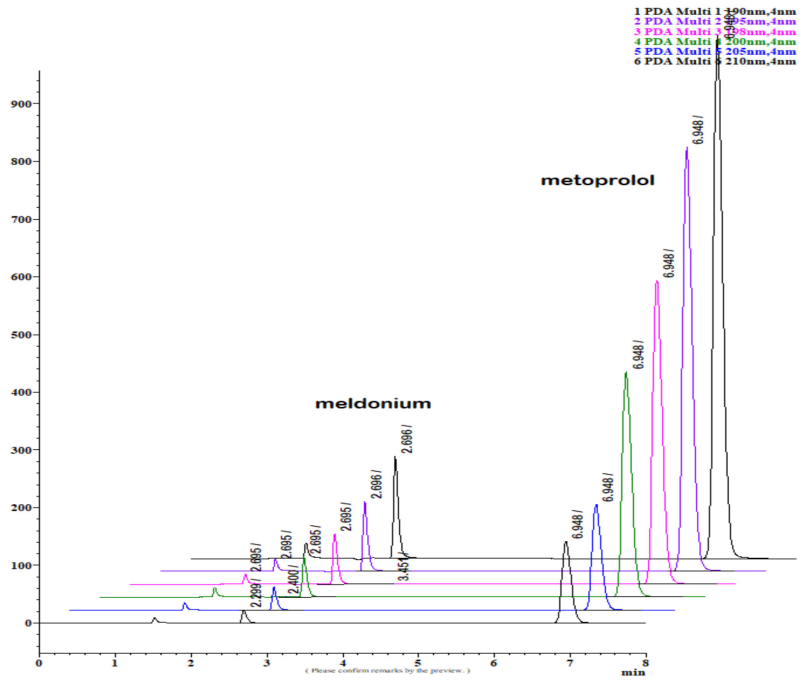


Figure 17. Full view-chromatogram of sample 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol with Waters Spherisorb CNRP-250 × 4.6 mm eluted 0.15% $NH_4H_2PO_4$ + 50% ACN full chromatogram view.

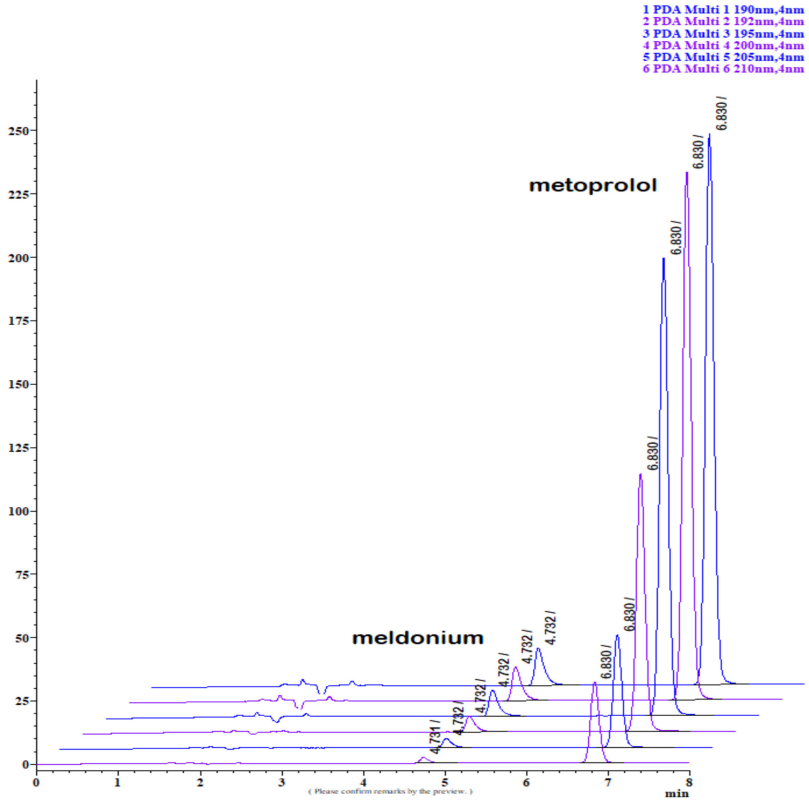
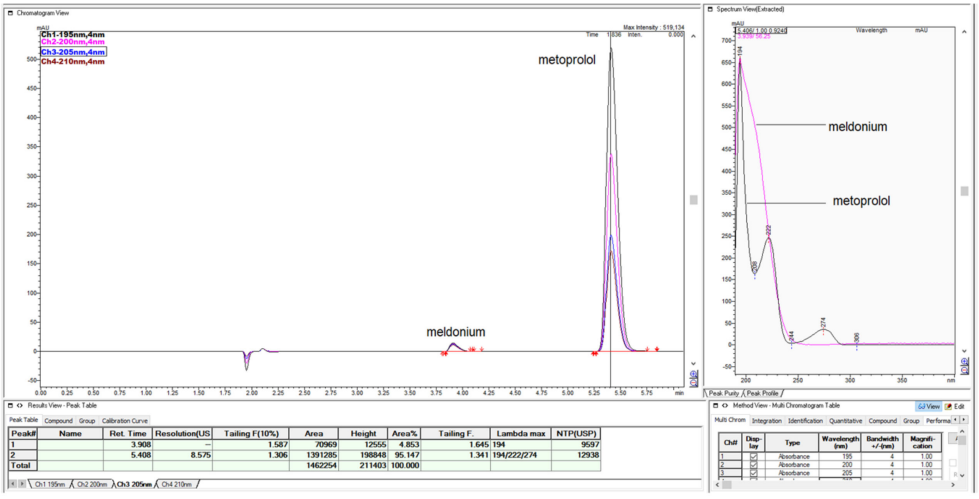


Figure 18. Chromatogram of sample 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol with Waters Spherisorb CNRP-250 × 4.6 mm eluted 0.15% $NH_4H_2PO_4$ + 70% ACN full view.

Figure 19 presents a chromatogram with 50% ACN and 50% diluted TFA, with full size chromatogram of meldonium peak monitored and overlaid at 4-channels with 4 different wavelengths. The closuring effects of the peaks of meldonium and metoprolol was very noticeable, with solid values for peak symmetries, the visible meldonium peak size resistance to change the height, size, compared to metoprolol. The right side of the figure contains extracted UV spectrums of meldonium and metoprolol. The system suitability of chromatogram could be seen in table below.



Peak#	Name	Ret. Time	Resolution(US)	Tailing F(10%)	Area	Height	Area%	Tailing F.	Lambda max	NTP(USP)
1		3.908	--	1.587	70969	12555	4.853	1.645	194	9597
2		5.408	8.575	1.306	1391285	198848	95.147	1.341	194/222/274	12938
Total					1462254	211403	100.000			

Figure 19. Chromatogram with 50% ACN and 50% diluted TFA with full size chromatogram below, monitored and overlaid at 4-channels with 4 different wavelengths.

The Figure 20 contains the same chromatogram with an enhanced, expanded, and zoomed view and scale of meldonium peak size changes for better comparison. The upper part of this Figure illustrates the baseline flatness depending on wavelength monitoring and measuring, with the worst case at the lowest of 195 nm. Figure 21 illustrates overlay in 3-D perspective of 4-channels with different wavelengths in full scale, with enhanced visibility of small increment peak height of meldonium peak, versus significant increases of the metoprolol peak. These experiments with Spherisorb CNRP column and diluted TFA were performed on UPLC Dionex with 4-channels UV-Vis detector, without PDA and spectra analysis possibility. We simultaneously monitored at 195 nm and 200 nm, with checking method transferability and precision. When samples and standards with adjusted targeted concentrations of 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol were tested, the following chromatograms were obtained as the results.

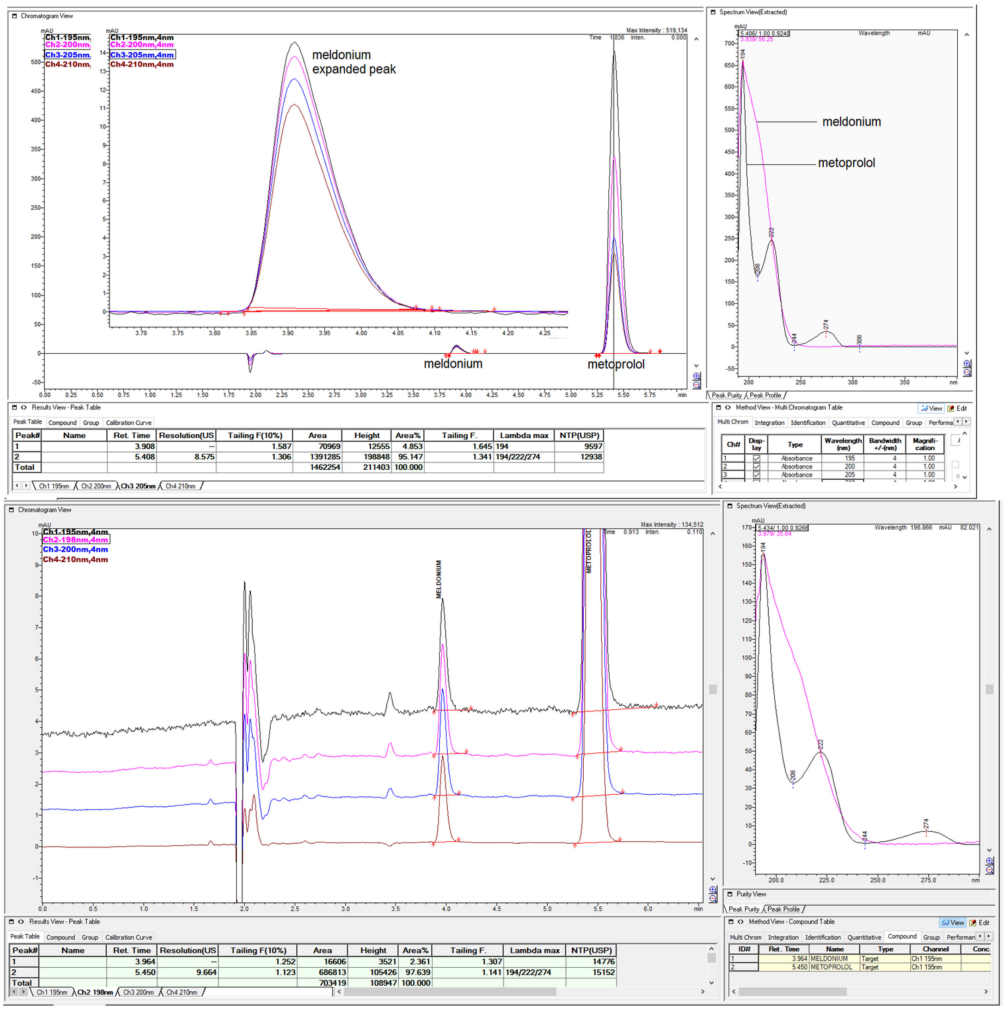


Figure 20. Chromatogram with 50% ACN and 50% diluted TFA, with full size chromatogram below and inserted expanded view of meldonium peak in zoomed scale above, monitored and overlaid at 4-channels with 4 different wavelengths. The right side of figure contains extracted UV spectrums of meldonium and metoprolol. The system suitability of chromatogram can be seen in the table below. The bottom figure illustrates mobile phase noises from upper chromatograms.

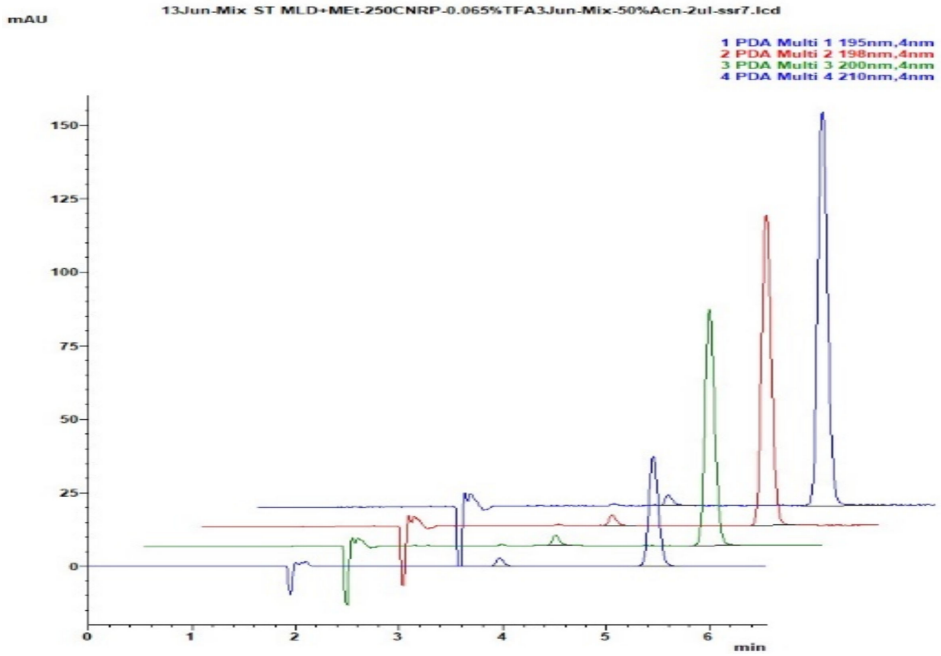


Figure 21. 4-channel multichannel view of full chromatograms with 50% ACN and 50% diluted TFA of samples with Waters Spherisorb CNRP-250 × 4.6 mm.

Figure 22 is a chromatogram performed with 50% ACN and 50% diluted TFA at 4 separated wavelengths channels, 195 nm and 200 nm. The figure illustrates the linearity check with the change of injection volumes 1–6 µm. It showed perfect linearity, but the peaks achieved with 6 µm injection manifested severe meldonium peak distortion with a shoulder, most probably as a result of mass overload. The Figure 23 presents the full figure on the left side and an expanded view of the same linearity overlaid chromatograms on the right side at the 195 nm monitoring, with system suitability parameters below on the table. It is easy to notify distorted peak with shoulder of meldonium when 6 µm was injected.

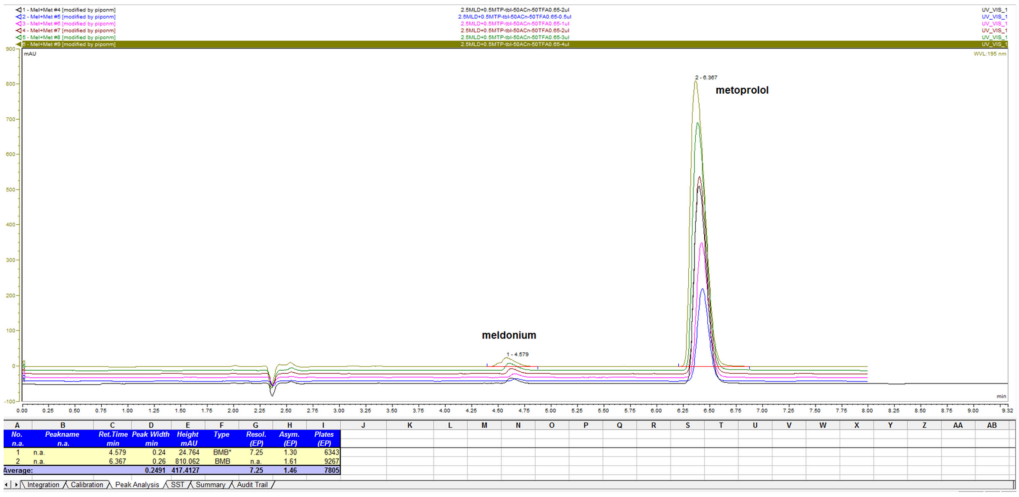
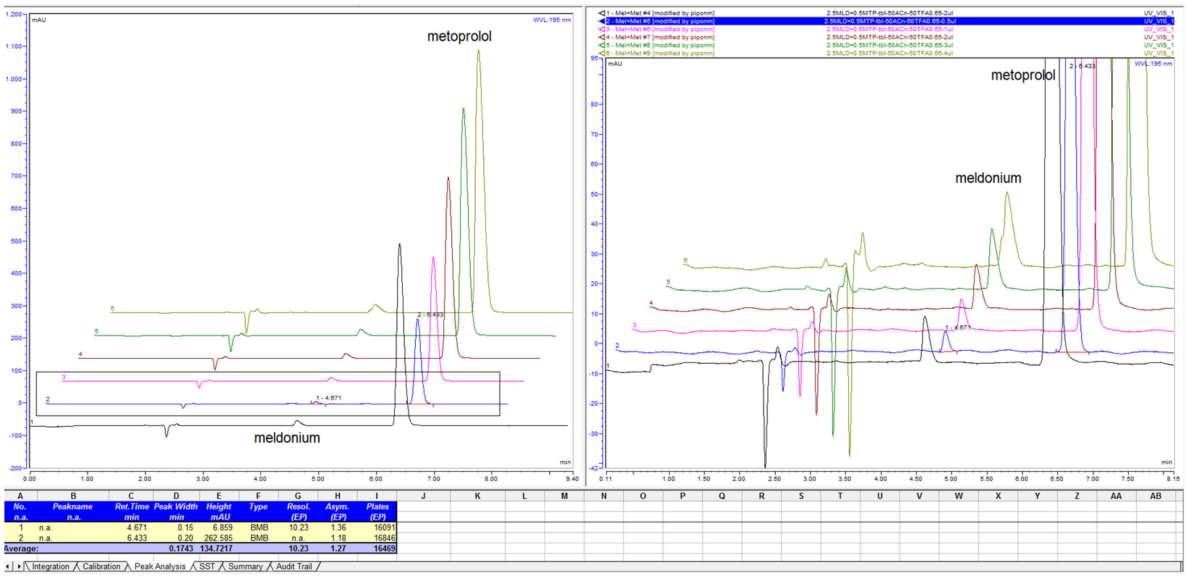


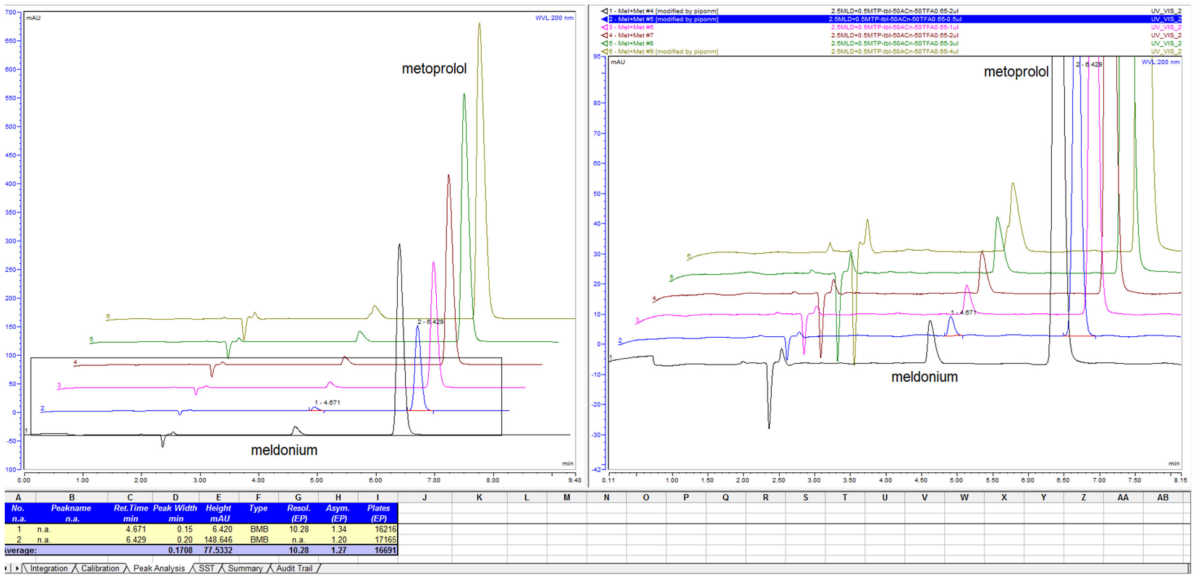
Figure 22. Dionex-Full scale overlaid chromatograms linearity of method with Waters Spherisorb CNRP-250 × 4.6 mm and 50% ACN and 50% diluted TFA at 195 nm, with system suitability parameters in table below.



No.	Peakname	Ret.Time min	Peak Width min	Height mAU	Type	Resol. (EP)	Asym. (EP)	Plates (EP)
1	n.a.	4.671	0.15	6.859	BMB	10.23	1.36	16091
2	n.a.	6.433	0.20	262.585	BMB	n.a.	1.18	16846
Average:			0.1743	134.7217		10.23	1.27	16469

Figure 23. Dionex-full scale overlaid and expanded zoomed Y-axis of overlaid chromatograms for linearity of method with Waters Spherisorb CNRP-250 × 4.6 mm and 50% ACN and 50% diluted TFA at 195 nm, with system suitability parameters in table below.

The Figure 24 illustrates the same sample linearity at 200 nm, in full scale on the left side and expanded scale on the right side of the linearity overlaid chromatograms. In summary, at the end of the experiments of testing the Waters Spherisob CNRP column, a final orientational overview of the behavior of these columns with different mobile phases, with same organic solvent ACN but with different modifiers, can be seen and analyzed at overlaid chromatograms in Figure 25.



No.	Peakname	Ret.Time min	Peak Width min	Height mAU	Type	Resol. (EP)	Asym. (EP)	Plates (EP)
1	n.a.	4.671	0.15	6.420	BMB	10.28	1.34	16216
2	n.a.	6.429	0.20	148.646	BMB	n.a.	1.20	17165
Average:			0.1708	77.5332		10.28	1.27	16691

Figure 24. Dionex full-scale overlaid and expanded zoomed Y-axis of overlaid chromatograms for the linearity of the method with Waters Spherisorb CNRP-250 × 4.6 mm and 50%ACN and 50% diluted TFA at 200 nm, with system suitability parameters in the table.

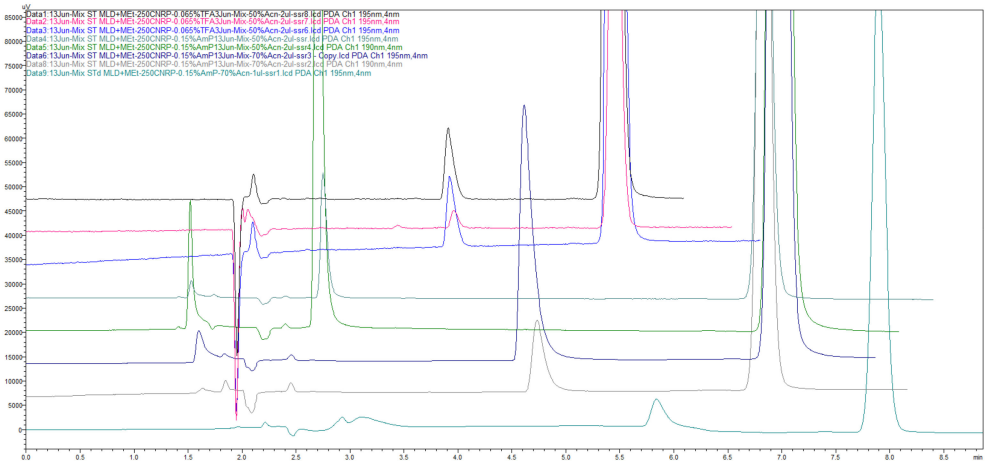


Figure 25. Overview of the retention map, testing Waters Spherisorb CNRP-250 × 4.6 mm, a final orientational overview of the behavior of these columns with different mobile phases, with the same organic solvent ACN but with different modifiers, can be seen and analyzed on the overlaid chromatograms.

3.3. Third Concept Based on Zorbax CN-SB 250 × 4.6 mm 5 µm Column

The most frequent successful combination of mobile phases in previous testing was 20–25% ACN with 13 mM (0.15% *w*/*v*) $NH_4H_2PO_4$, which was shown in the chromatogram results presented in Figure 26, and overlaid 15-channels of different UV-absorbing wavelengths on the left, and extracted UV-abs spectrum of recognized peaks on the right side. The bellow chromatograms are tables with integration and system suitability parameters for the injected sample with adjusted concentration of meldonium of 2.5 mg/mL and 0.5 mg/mL of metoprolol. This Figure illustrates that the column showed perfect symmetry for the meldonium peak (tailing F = 1.13) and very asymmetric tailing peak for metoprolol (tailing F = 2.1), for the first time compared to other previously tested column. For better comparison of meldonium peak height variation in a function of UV-wavelength, the same chromatogram from Figure 26 was inserted with zoomed peak and scaling for meldonium peak and illustrated in Figure 27.

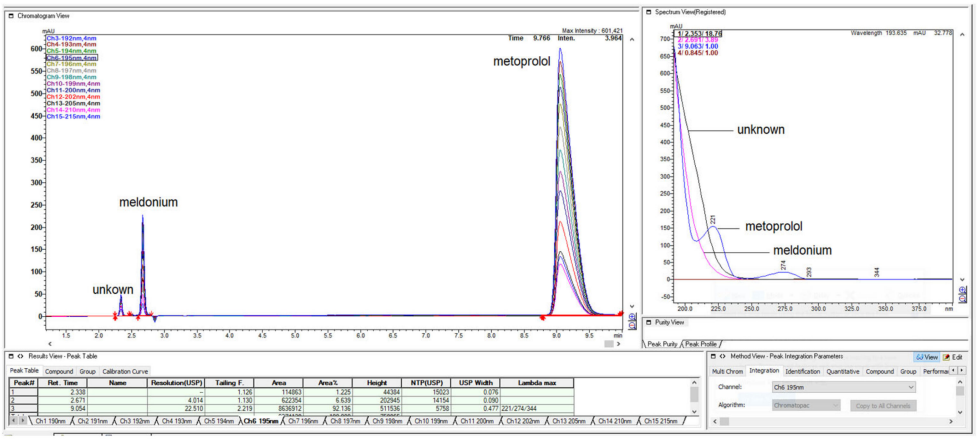


Figure 26. Chromatograms testing of column Zorbax CN-SB-250 × 4.6 mm with 20% ACN and 80% 13 mM (0.15% *w*/*v*) $NH_4H_2PO_4$ and overlaid 15-channels of different UV-absorbing wavelengths on the left, extracted spectrum on right.

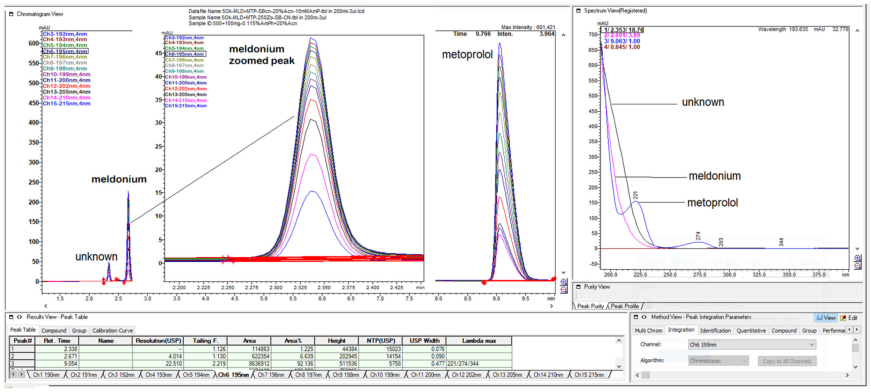


Figure 27. Chromatograms with column Zorbax CN-SB-250 × 4.6 mm with 20% ACN and 80% 13 mM (0.15% w/v) $NH_4H_2PO_4$ and overlaid 15-channels of different UV-absorbing wavelengths, with an expanded, zoomed peak of meldonium for better visibility in comparison with metoprolol.

Figure 28 presents Chromatograms with Zorbax CN-SB-250 × 4.6 mm with 50% ACN and 50% 13 mM (0.15% w/v) $NH_4H_2PO_4$ and overlaid 15-channels of different UV-absorbing wavelengths. Figure 29 with the same chromatogram as previous figure, but with expanded view of meldonium peaks, confirms our comment for quality and efficiency of the HPLC method with 50% ACN and 50% 13 mM $NH_4H_2PO_4$, monitored and presented for comparison at 15 different UV-wavelengths.

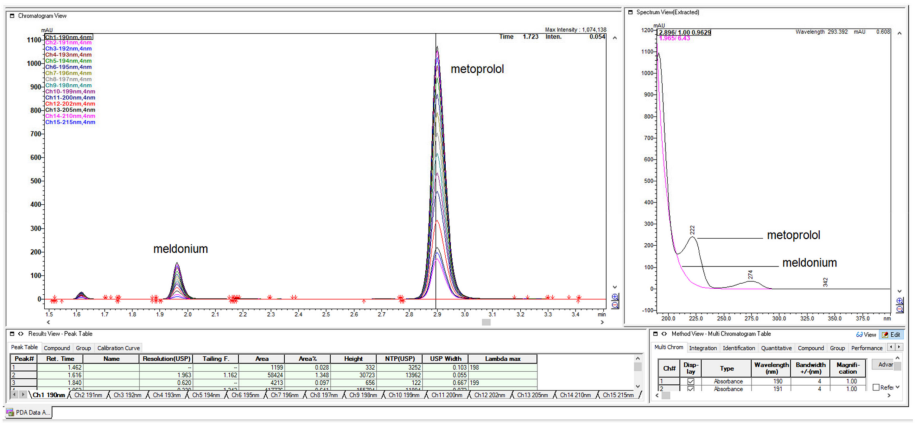


Figure 28. Chromatograms with Zorbax CN-SB-250 × 4.6 mm with 50% ACN and 50% 13 mM (0.15% w/v) $NH_4H_2PO_4$ and overlaid 15-channels of different UV-absorbing wavelengths.

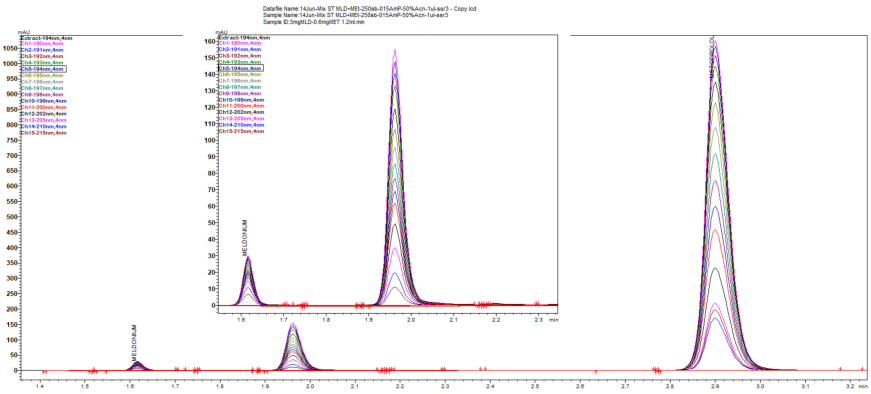


Figure 29. Chromatograms with Zorbax CN-SB-250 × 4.6 mm with 50% ACN and 50% 13 mM (0.15% w/v) $NH_4H_2PO_4$ and overlaid 15-channels of different UV-absorbing wavelengths, with expanded zoomed peak of meldonium inserted for better comparison with metoprolol peak.

Figure 30 also illustrates full scale of chromatogram and inserted expanded, zoomed peak and scaling of meldonium peak for better visibility and comparison at 4 different UV-wavelengths. All these chromatograms with Zorbax CN-SB column with mobile phase with 50% ACN and 50% 13 mM $NH_4H_2PO_4$ were performed with F = 1.2 mL/min.

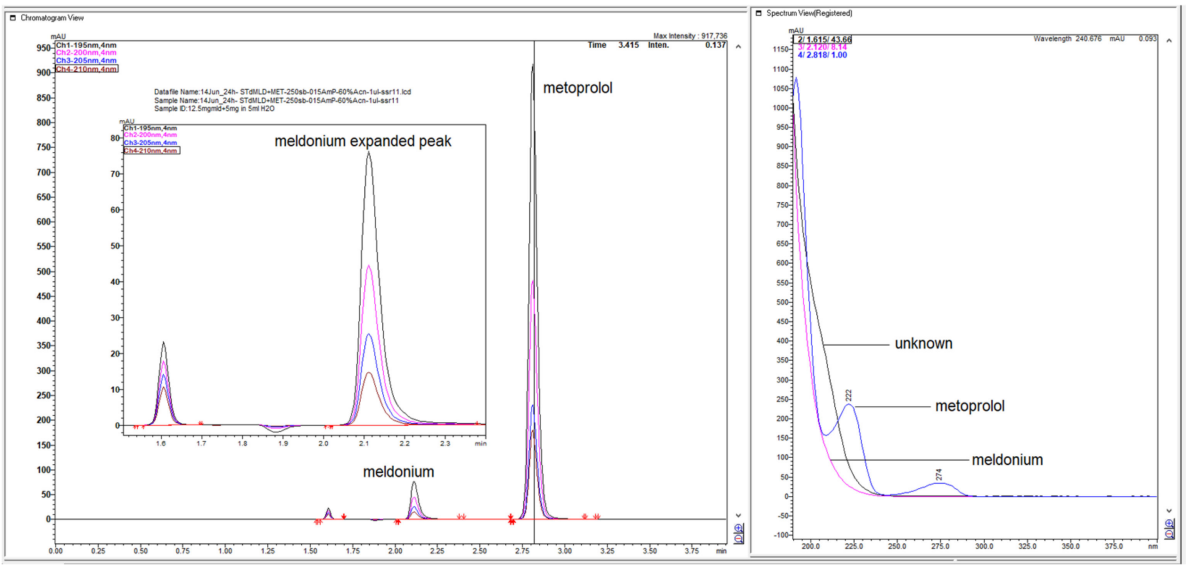


Figure 30. Chromatograms with Zorbax CN-SB-250 × 4.6 mm, gained with organic solvent ACN in mobile phase to 60% with 40% 13 mM $NH_4H_2PO_4$.

Figure 31 illustrates retention mapping of analytes as a function of eluate composition and pH. The overlay of mobile phase changes the experiments for chromatogram shaping results in trials with mobile phase composed of ACN and $NH_4H_2PO_4$. In the first experiments with the Agilent 1126-II-PDA detector, with reproducing the upper described experimental conditions with 50% ACN and 50% 13 mM $NH_4H_2PO_4$ with Zorbax CN-SB column and with a flow rate 1 mL/min, we have obtained the following chromatograms illustrated in Figure 32. The combined display contained extracted chromatogram at UV-197 nm below, and 3-D UV-spectrum of whole chromatogram as contour-map diagram on the left and extracted UV-spectrums of meldonium and metoprolol on the right.

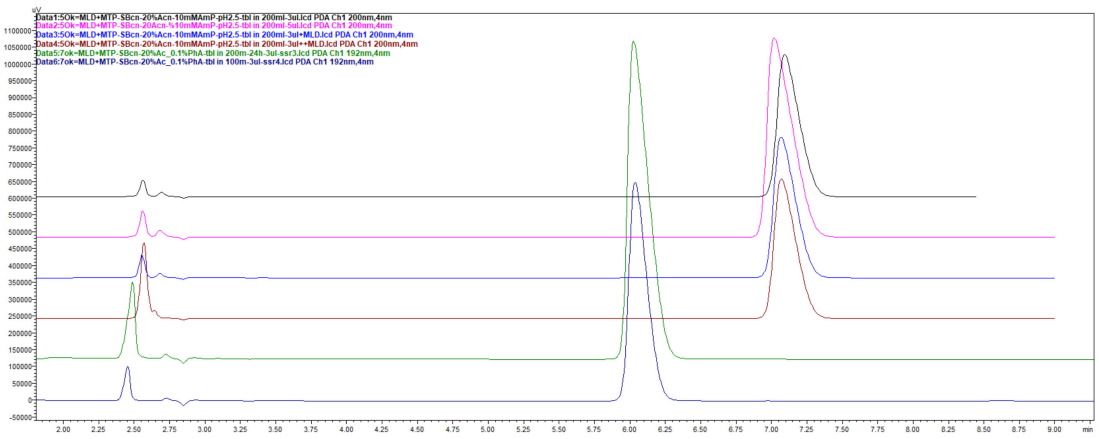


Figure 31. Chromatograms with Zorbax CN-SB-250 × 4.6 mm retention mapping of analytes as a function of eluate composition and pH, the overlay of mobile phase changes experiments for chromatogram shaping results, in trials with mobile phase composed of ACN,13 mM $NH_4H_2PO_4$, and 0.08% v/v o-H_3PO_4.

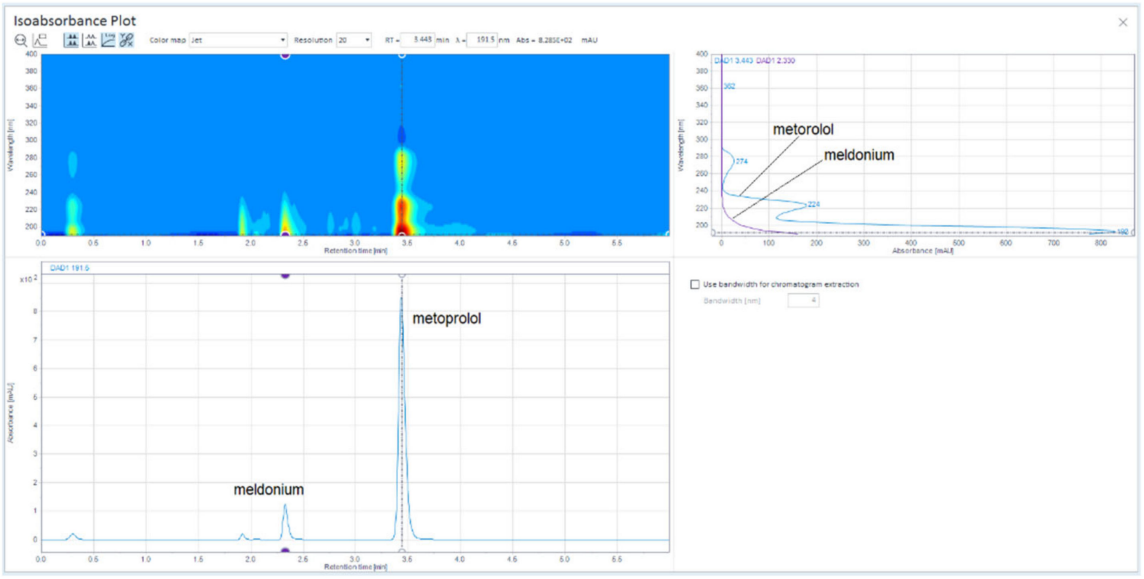


Figure 32. Full 3-D chromatogram of meldonium, extracted line at 197 nm and UV-spectrum of analytes with 50% ACN and 50% 13 mM $NH_4H_2PO_4$ using column Zorbax CN-SB-250 × 4.6 mm.

The peak identity for selectivity of meldonium has been checked again and confirmed, by comparison of meldonium standard only and sample meldonium with metoprolol with their chromatograms overlaid on Figure 33. Selectivity in overlaid chromatograms in Figure 34, are as a result of the injected meldonium standard of 5 mg/mL, meldonium standard with traces of spiked metoprolol in the middle and sample. The relatively small change of retention of meldonium in a wide range of acetonitrile partition changes in mobile phase effect is illustrated in Figure 35.

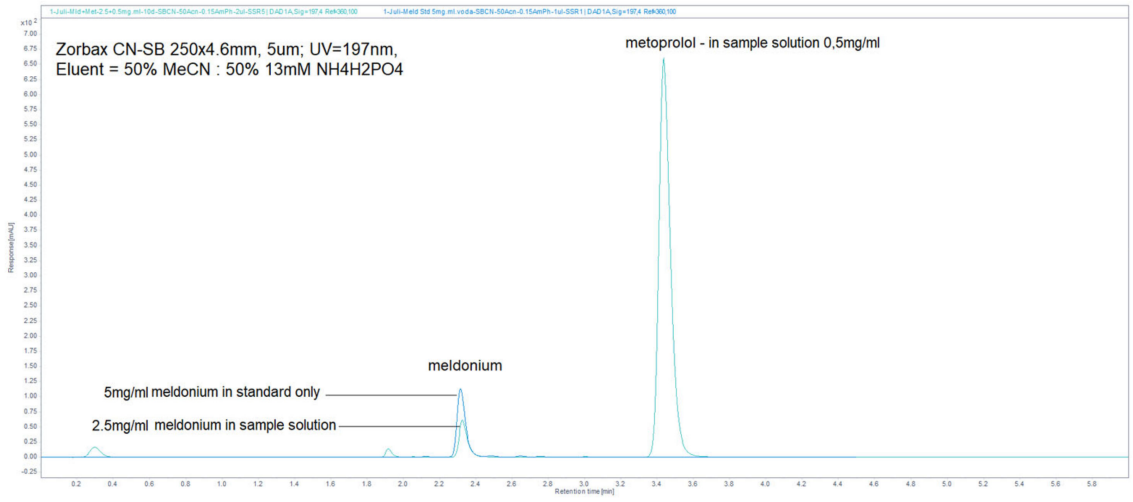


Figure 33. Chromatograms obtained with Zorbax CN-SB-250 × 4.6 mm in the term of confirmation of identities of meldonium and metoprolol with standard 50% ACN and 50% $NH_4H_2PO_4$.

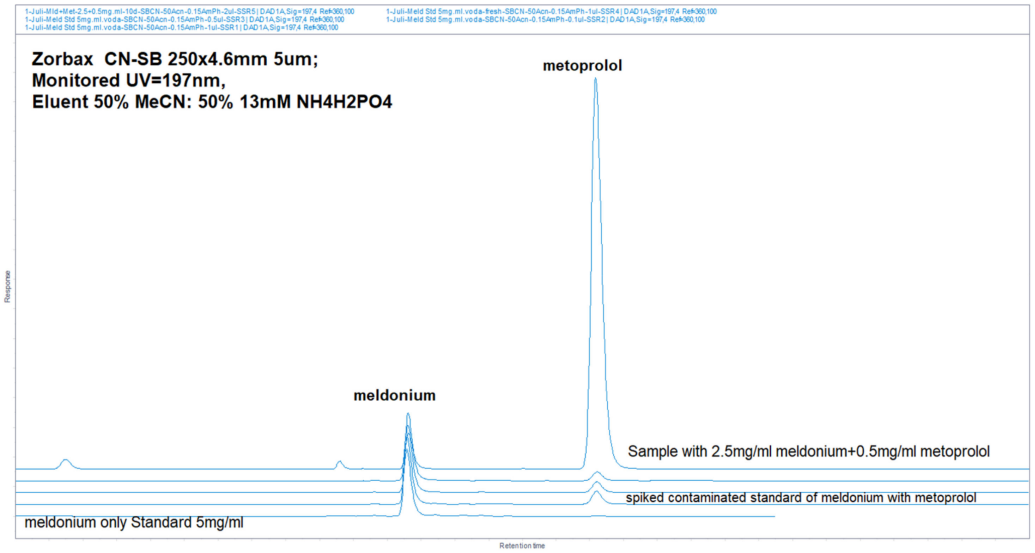


Figure 34. Overlaid chromatograms of meldonium standard pure, standard meldonium contaminated with trace of metoprolol and sample with 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol with ACN and 13 mM $NH_4H_2PO_4$ using column Zorbax CN-SB-250 × 4.6 mm.

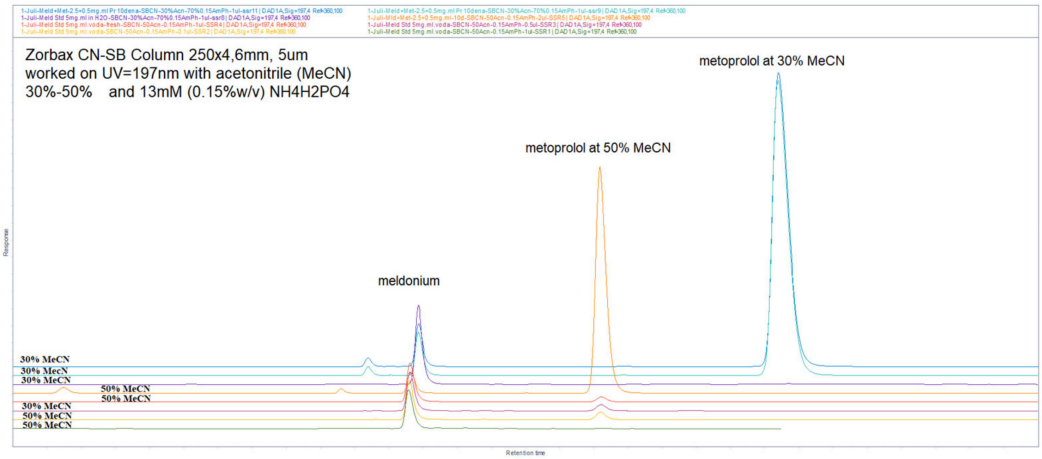


Figure 35. Chromatograms of retention time mapping with significant changes of organic percentage of ACN with 13 mM $NH_4H_2PO_4$ in mobile phase, comparison of overlaid retentions of analytes on chromatograms using column Zorbax CN-SB-250 × 4.6 mm.

The differences between Figures 36 and 37 were only a way of presenting the spectrum of peaks of chromatograms, in separated and common overlaid scale with normalization for better visibility and comparison. The only missing peak for UV-spectrum extraction was from the smallest fourth peak. With the aim to increase the resolution of the peaks, we have reduced the ACN content to 30% with 70% 13 mM $NH_4H_2PO_4$, and achieved better resolution function of increased retention, and overall run time of about 5 min, but significant peaks increased resolution, and were presented in Figure 38. The full-size comparison bellow overlay, and expanded view of overlaid chromatograms at top, were presenting 2 injections of freshly prepared samples with 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol, which did not have the second and the third peaks present, since they were the degrading products due to keeping samples for 3 days at room conditions. First peaks with increasing sizes were meldonium, and last one was metoprolol. This method was our favorite for reliable determination of meldonium and metoprolol in pharmaceutical dosage forms in presence of early degradation products.

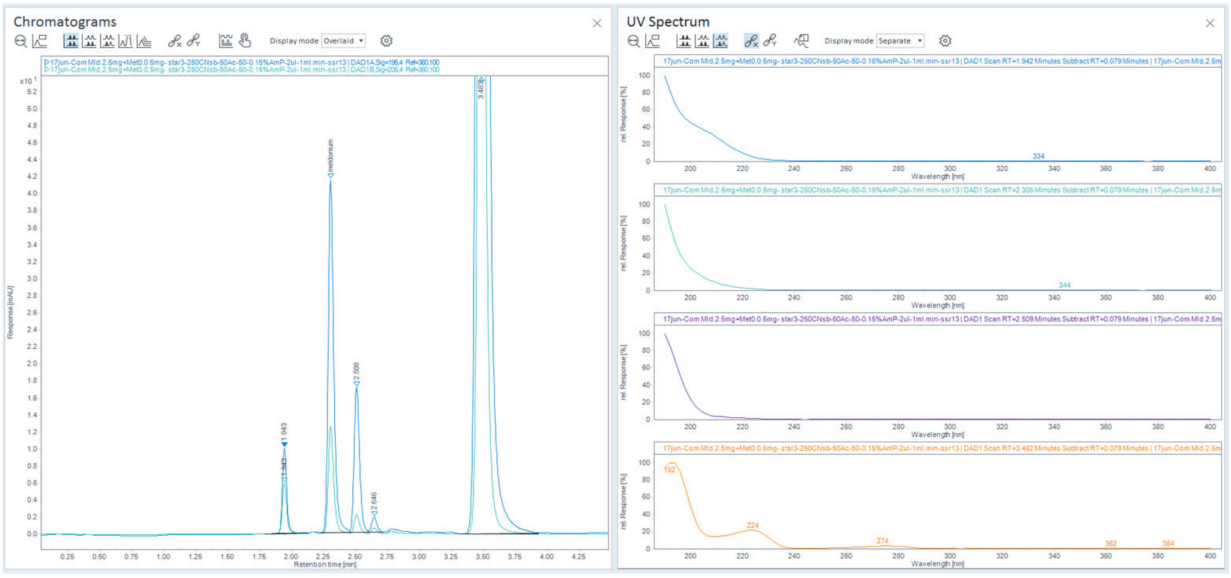


Figure 36. Chromatograms, eluted with 50% ACN and 50 % 13 mM $NH_4H_2PO_4$, were recorded and overlaid at 2-channels of 195 nm and 205 nm overlaid are on the left and their characteristic UV-spectrums on the right. The second peak is meldonium on chromatogram, and the last highest peak was metoprolol, third and fourth peaks were new appeared. From the 4 presented UV-Abs spectrums, the second from the top was the meldonium peak extracted and fourth at the bottom was of metoprolol.

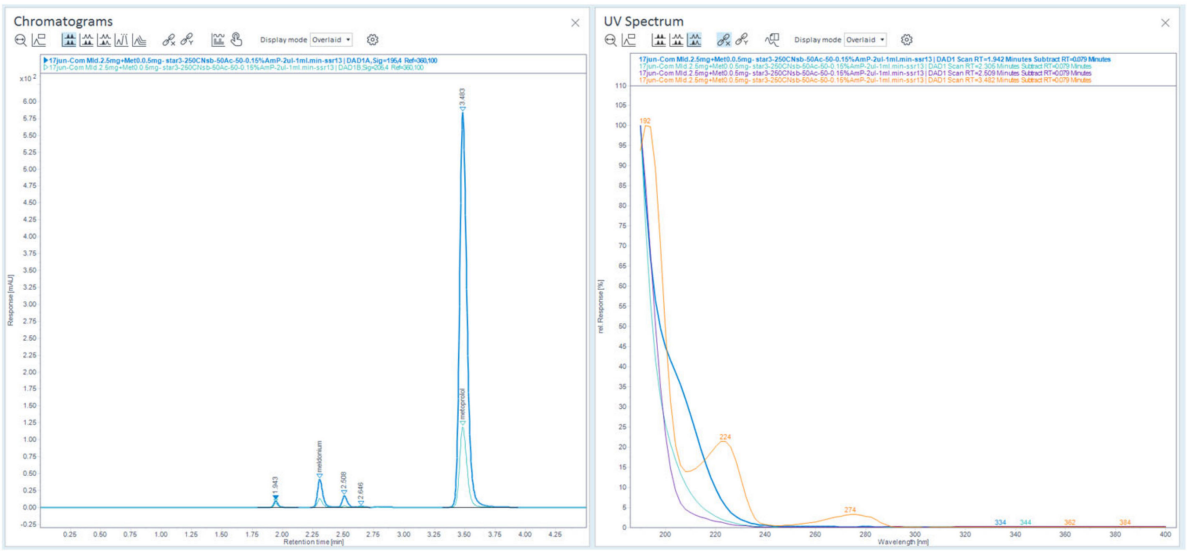


Figure 37. Chromatograms, obtained by usage mobile phase composed of 50% ACN and 50% 13 mM $NH_4H_2PO_4$, the most effective separation was achieved in less than a 4 min run time. The second peak was meldonium on chromatogram, and the last highest peak was metoprolol, the third and fourth peaks were new.

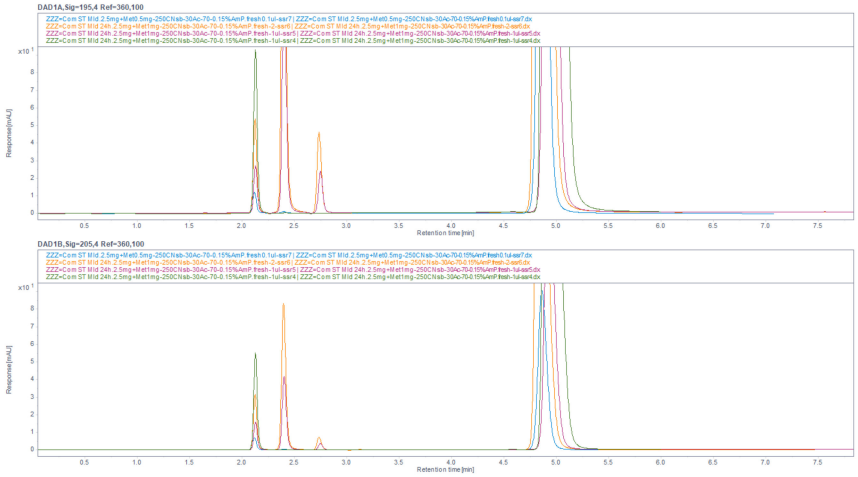


Figure 38. Chromatograms, obtained by usage mobile phase composed of 30% ACN and 70% 13 mM $NH_4H_2PO_4$, and achieved better resolutions function of increased retention, and overall run time of about 5 min, but significant peaks increased resolution. Overlaid 2 different situations of samples, freshly prepared and incubated for 3 days at room conditions. The first peak was meldonium, the second and third were degradation products, and the last one was metoprolol. The sizes of the peaks were different due to increasing injection volumes.

The Figure 39 presents direct comparisons of chromatograms gained with 30% and lower with 50% ACN, with their full and zoomed scalings, with the extracted UV-abs spectrum of four separated and integrated peaks, in overlay view on the right side. The first peak was meldonium and last peak was metoprolol, while the peaks in the middle were degradation products, present only in incubated samples for 3 days at room conditions. The influence on differently used phosphate salt on chromatogram was presented in Figure 40, in which the comparison was presented in mobile phase replacement of 70% 13 mM $NH_4H_2PO_4$ was replaced with 70%13 mM KH_2PO_4, combined with 30% ACN. The set of overlaid chromatograms contain fresh prepared sample of 2.5 mg/mL meldonium with 0.5 mg/mL metoprolol eluted with $NH_4H_2PO_4$. The 3 days stored samples prepared with $NH_4H_2PO_4$ and KH_2PO_4 were below, and only visible change was metoprolol peak spreading in a higher width, which confirms the advantage of using ammonium phosphate. Further experiments with Zorbax CN-SB columns were performed with diluted acids, without the use of phosphate salts.

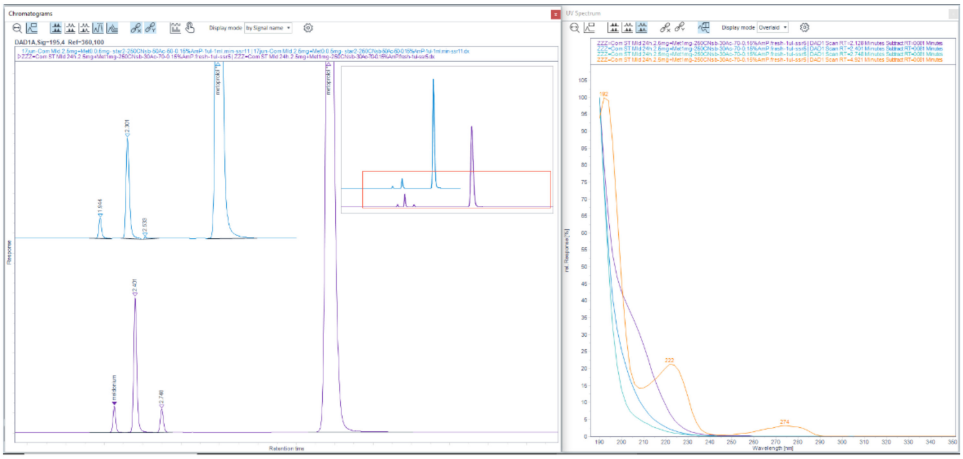


Figure 39. Chromatograms, obtained by usage ACN content to 50% (upper chromatogram) and with 30% ACN (bottom chromatogram) with of 13 mM $NH_4H_2PO_4$. Chromatograms were presented in full and expanded overlaid sizes, with UV-abs spectrum extracted at right.

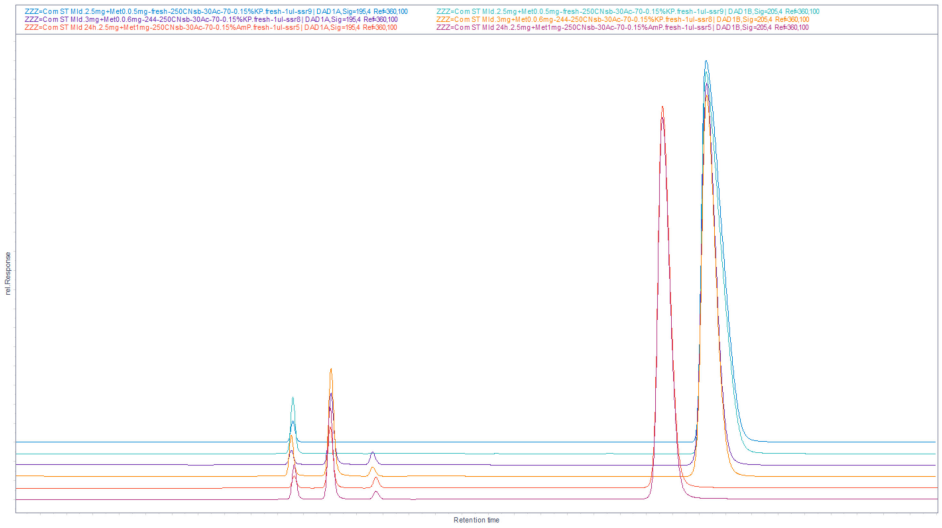


Figure 40. Chromatograms, obtained by usage of the ACN content to 30% with 70% 13 mM $NH_4H_2PO_4$ the last at bottom two chromatograms, and replaced with 13 mM KH_2PO_4 above them. The last top chromatograms were made from a fresh sample prepared with 13 mM KH_2PO_4 70% and 30% ACN, with noticeable width spread and small retention shift of metoprolol peak.

The first choice was 0.1% v/v o-H_3PO_4 80% and 20% ACN, illustrated on Figure 41, recorded and overlaid at 13-different UV-wavelengths. The meldonium peak was inserted as zoomed and rescaled for above full not scaled chromatogram for comparison of this peak changes to metoprolol peak more readily. The chromatograms gained with this mobile phase, generate high peaks asymmetry of both analytes, with meldonium peak leading distortion and metoprolol peak tailing, all recorded and presented in 13-channels with different wavelengths. It was notified that a significant increase of the meldonium peak as a function of the wavelength decrease, which was not the case previously. Metoprolol showed significant peak size changes, compared to meldonium. Due to meldonium peak leading, this area increment did not follow the height increments. When the o-H_3PO_4 was replaced with diluted 0.07% v/v TFA, quite interesting and substantial changes of the chromatogram view with peak shapes and retention appeared. Figure 42 shows both analytes peaks behavior in retention times and shapes, as a function of changes of percentage of ACN in 60–65–70–75–80% ACN, creating map of meldonium and metoprolol peak elution movement across the X-scale.

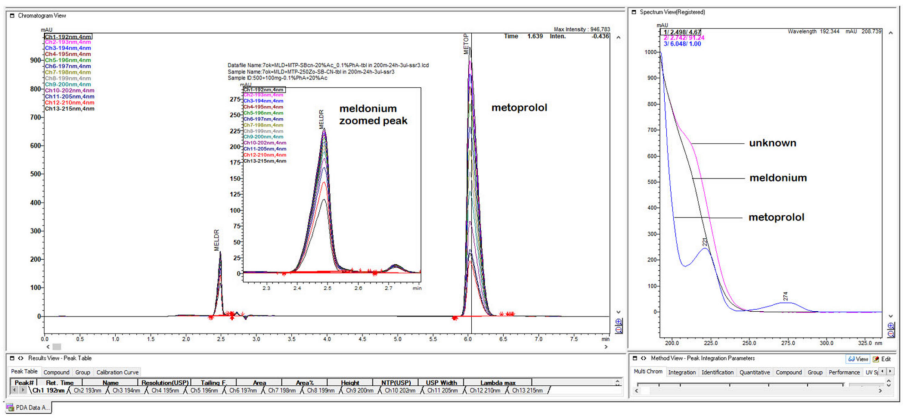


Figure 41. Chromatograms, performed with diluted acids, without use of phosphate salts. The first choice was 0.1% v/v o-H_3PO_4 80% and 20% ACN, recorded and overlaid at 13-different UV-wavelengths N- 20%ACN-80% 0.1% o-H_3PO_4. Distorted peaks with leading of meldonium and tailing of metoprolol were, for the first time, clearly visible.

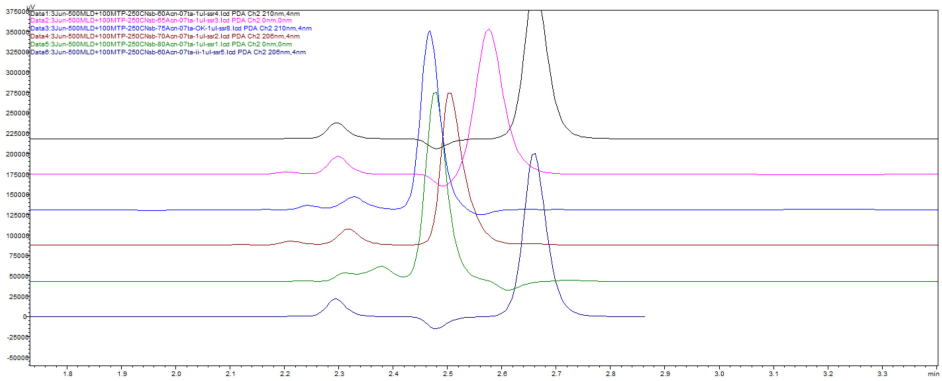


Figure 42. Chromatograms with TFA, a function of changes of percentage of ACN in 60–65–70–75–80% ACN, creating map of meldonium and metoprolol peak elution movement across the X-scale 60–80% increment of ACN.

The full-scale image of a very attractive chromatogram was presented in Figure 43, created with 70% ACN and 30% 0.07% v/v TFA, with an exceptionally short run time lasting less than 3 min, with all peaks perfectly shaped, but with no impressible resolution between them. The simplicity of method was impressive too, but the fine tuning of the peaks should be optimized further. Figure 44 presents the high-resolution PDA detector, full 3-D chromatogram confirms the identity of the peaks with implying a certain extent of confidence in peak recognition and measuring, with easily recognized characteristic UV-Abs spectrum of meldonium, annotated with a marking triangle located at the axis presenting the retention time. The simplest way to improve this situation with this mobile phase was purchasing and working with a column with a smaller particle diameter, from 5 µm to 3.5 µm. This will increase the resolutions by about 20%, which might be quite satisfying. Even with this 5 µm column, the method might be applicable for the determination of meldonium and metoprolol in high through put routine analyses of manufactured pharmaceutical dosage forms. This was an impressive chromatogram from the chromatographic point of view, the closest elution of meldonium to metoprolol, besides the enormous differences in polarities. Short retention times create short speeds and less diffused peaks with increasing the sensitivity parameters of the method.

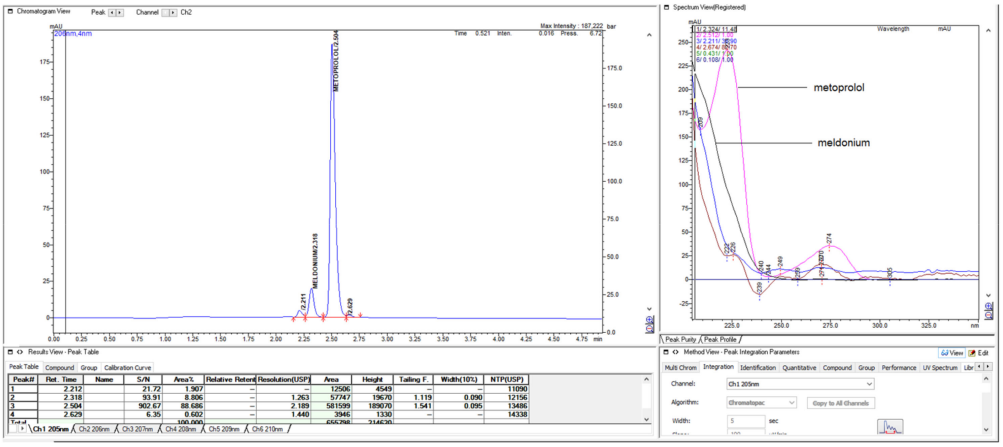


Figure 43. Chromatograms, obtained by usage mobile phase composed of 70% ACN and 30% 0.07% v/v TFA, with exceptionally short run times lasting less than 3 min, with perfect shaped all peaks, but with no impressible resolution between them. UV spectrum extracted peaks on the right.

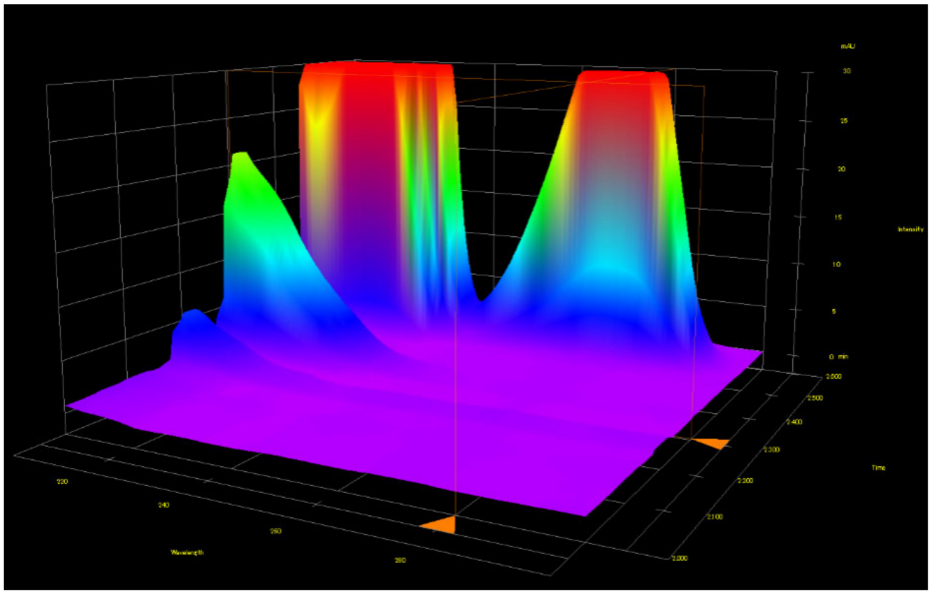


Figure 44. High resolution PDA detector full 3-D chromatogram confirms the identity of peaks with implying certain extent of confidence in peak recognition and measuring, with easily recognized characteristic UV-Abs spectrum of meldonium, annotated with marking triangle located at the axis presenting retention time.

Decreasing the percentage of ACN to 60% showed a very slight increase in retention for both analytes with very good shaped peaks, but with disappearing small peaks and appearing negative peak between meldonium and metoprolol, presented on the chromatogram in Figure 45. This situation might be with negative separating impact on smaller peaks, by hiding below the visible on the chromatogram. Further increasing the ACN to 80%, shown in Figure 46, guided the appearance of chromatogram with a slightly increased retention, appearing on new non separated peaks of unknown and meldonium, separated metoprolol peak, and with negative peak on baseline eluting after right behind metoprolol, jeopardizing this peak accuracy definition.

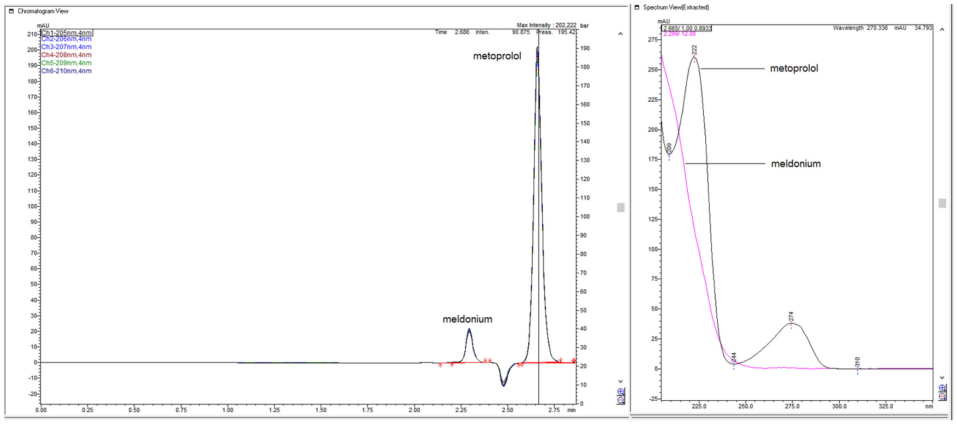


Figure 45. Chromatograms with application of percentage of ACN with 60%, showed a very slight increased retention for both analytes with very good shaped peaks, but with disappearing small peaks and appearing negative peaks between meldonium and metoprolol, with column Zorbax CN-SB 250 × 4.6 mm.

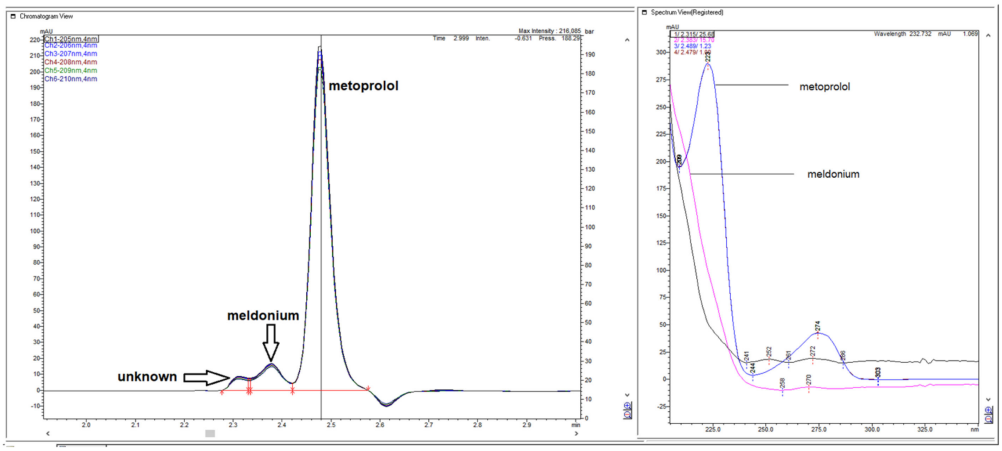


Figure 46. Chromatograms, obtained by increasing ACN to 80%, guided the appearance of chromatogram with a slightly increased retention, appearing on new non separated peaks of unknown and meldonium, separated metoprolol peak, and with a negative peak on the baseline eluting right behind metoprolol, jeopardizing this peak accuracy definition.

Two most illustrative chromatograms are changed due to the doubled percentage of ACN in mobile phases, are shown in Figure 47. Mobile phase with 80% ACN coelutes meldonium with the first peak and gap behind was jeopardizing integration of metoprolol. Decreasing ACN to 30% in mobile phases, increases separation of all peaks, with doubling the retention of metoprolol, but in this case the gap in the baseline jeopardize the accurate integration and determination of meldonium. This gap was a resolvable problem, by using the exact mobile phase with sample solvent, but increased presence of acetonitrile reduces dissolution of both analytes. Many experiments were performed for creating a global retention map of both analytes and checking of peaks symmetries at different percentage of acetonitrile in combination with diluted TFA, as illustrated on Supplementary Figure S1. When we wanted to check influences of inorganic mobile phase modifiers on chromatogram separation and peak shapes, with a constant part of 20% ACN and 80% of other inorganic constituent, we got the situation depicted in Figure 48, Zorbax CN-SB 250 × 4.6 mm chromatograms created with 20% ACN and 80% o-H_3PO_4 (the top 2 chromatograms), with acidified 13 mM $NH_4H_2PO_4$ to pH = 2.5 (medial 4 chromatograms) and with untreated 13.3 mM $NH_4H_2PO_4$.

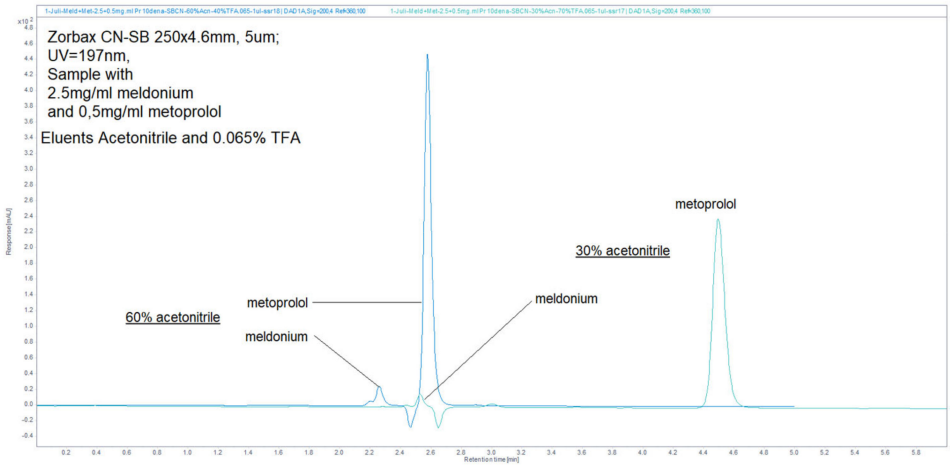


Figure 47. Two chromatograms with Zorbax CN-SB column of sample with doubled percentage of CAN in eluent. TFA enables enormous an approach of peak of metoprolol to meldonium, reducing their resolution to very low values approaching to 2.

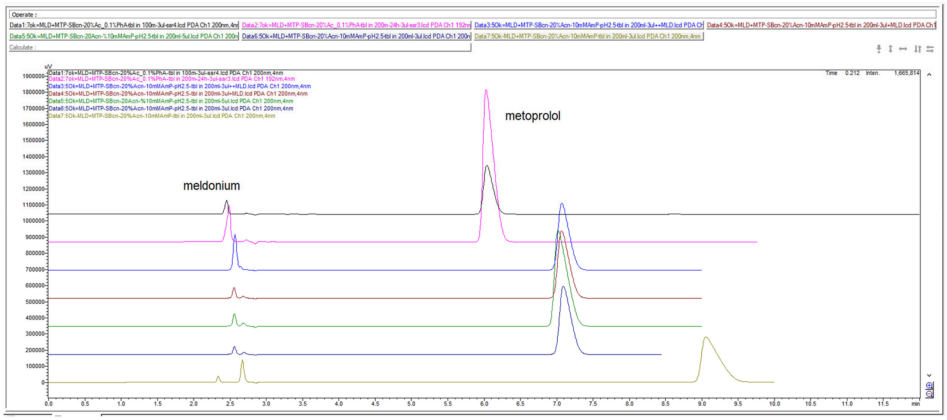


Figure 48. Chromatograms created with 20% ACN and 80% o-H_3PO_4 (the top 2 chromatograms), with acidified 13 mM $NH_4H_2PO_4$ to pH = 2.5 (medial 4 chromatograms) and with untreated 13.3 mM $NH_4H_2PO_4$. High acidity of eluents showed higher peaks asymmetries of some peak.

High acidity of eluents showed a higher probability for peak asymmetries of some peak. The short summary overview of created chromatograms of sample with 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol, with different percentages of ACN and different inorganic part composed of 0.065% TFA or 15 mM $NH_4H_2PO_4$ were presented for comparison in overlaid mode at Figure 49. This overview of retention peaks and shape mapping can help in the selection of the most adequate and proper situation for best mobile phase choice. Figure 50 presents summary peak mapping of meldonium and metoprolol due to different changing of percentages of ACN, and choice of inorganic modifier of mobile phase. Figure 50 confirms the necessity for use CN-cyano column, Zorbax CN-SB 250 mm × 4.6 mm for simultaneous determination of meldonium and metoprolol. Numerous combinations of ACN with 0.15% $NH_4H_2PO_4$ have been presented overlaid at 195 nm and 200 nm, compared with the last two chromatograms on the bottom, which were made of on 100 × 4 mm C18 Xterra column with 10% ACN and not eluting metoprolol even to 20 min.

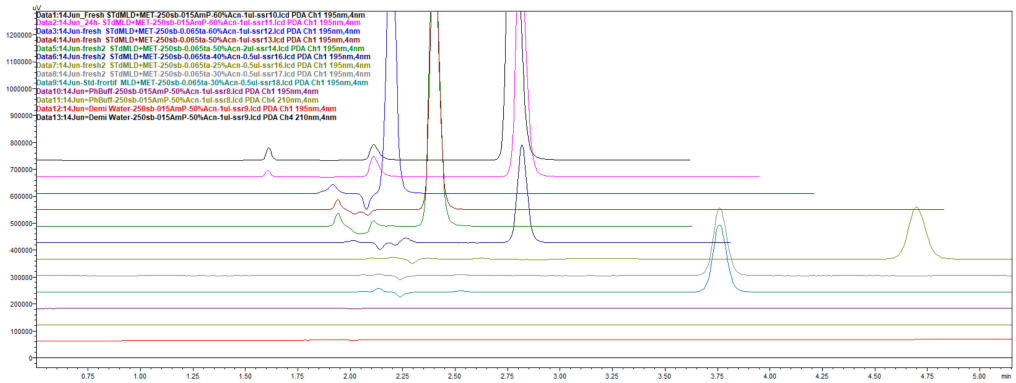


Figure 49. The short summary overview of created chromatograms of sample with 2.5 mg/mL meldonium and 0.5 mg/mL metoprolol, with different percentages of ACN and different inorganic part composed of 0.065% TFA or 15 mM $NH_4H_2PO_4$ were presented for comparison in overlaid mode, performed on Zorbax CN-SB 250 × 4.6 mm.

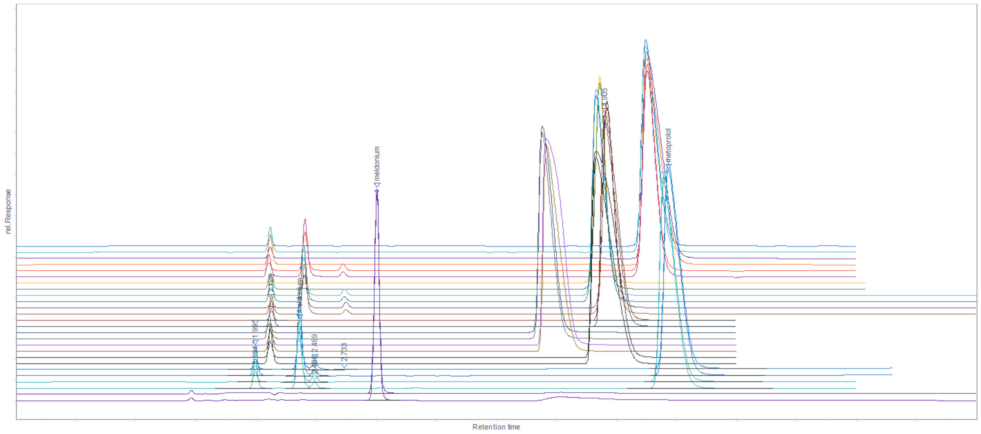


Figure 50. Chromatograms, obtained for use in CN-cyano column, Zorbax CN-SB 250 × 4.6 mm for simultaneous determination of meldonium with metoprolol. Numerous combinations of ACN with 0.15% $NH_4H_2PO_4$ have been presented overlaid at 195 nm and 200 nm, compared with the last two chromatograms on the bottom, which are made of on 100 × 4 mm C18 Xterra column with 10% ACN and not eluting metoprolol even to 20 min.

The last experimental performance was testing a method for applicability in cases of including other beta-blockers, like the one very often prescribed as bisoprolol, a chromatogram presented in Supplementary Figure S2.

4. Discussion

The main challenge in this work was to develop simple rapid UV-PDA-HPLC methods for simultaneous determination of metoprolol and meldonium with concepts and presentation solving problems in enormous polarity differences between these APIs. The physicochemical properties based on the chemical structures of the two analytes, meldonium and metoprolol, naturally manifested quite differently, significantly distinct interactions dictated from polarities interactions with octylsilane C-8 or octadecylsilabne C-18 alkyl-chains bonded to silica particles of most common used reversed phase chromatography column [30,31]. Herein, after elimination of the options for using classical reversed phase alkyl-chain of C-8 and C-18 columns, and phenyl particle based HPLC columns, because extremely polar zwiterionic structure meldonium does not poses aromatic-ring in structure, the next logical higher polarity mechanism was cyano or cyano-propyl bonded phase particles, in cyano or cyano-propyl CN columns.

First our concept was based on LiChrospher®® 125 CN (5 µm) and LiChroSpher®® 250 CN (5 µm) columns. It is well known that the cyanopropyl bonded column can function like reversed phase matrix obeying reversed-phase mechanism, but it can work as normal-phase column and obey in normal-phase mechanism when mobile phase exceeds 50% and higher. Depending on the structures, solubilities of analytes, the chromatograms can change dramatic. The description of results obtained with all columns tested, will follow our concept, beginning with mobile phases composed of acetonitrile and ammonium phosphate salt, after the experiments performed with mobile phases with diluted acids, TFA or o-H_3PO4, and without salts, buffer or else. It is clear visible separation of early, first eluting meldonium and late eluting peak of metoprolol, keeping in mind the fact that chromatograms in Figures 1–9 were performed on 125 × 4 mm 5 µm particles LiChrospher 100 CN, and Figure 6 presented chromatogram under identical conditions with used 250 × 4 mm 5 µm LiChrospher 100 CN-column, just double sized length, for checking influence of relationship Analytre Concentration-Peak Shapes. When the 13 mM ammonium phosphate was replaced with diluted TFA 0.065% v/v, a quite different situation has been achieved. Resolution of meldonium and metoprolol can be better regulated to decrease to minimum allowed, peak sizes of meldonium were significantly smaller, and very little dependance on the wavelength in lowest range 195–210 nm. The sensitivity of method

is lower with TFA in the mobile phase due to its high Cut-off UV abs value, increasing noise of the baseline. The applicability of shorter 125 mm LiChrospher CN method has been confirmed with doubled length LiChrospher CNcolumn 250 × 4 mm, with the aim to assure the "meldoniums peak vicinity area" for presence of contaminating, hiding peak monitored under PDA (DAD) detector. The increasing of both analytes peaks heights with reducing wavelength is visible, with higher impact on meldonium peak, which is logic when compared to UV-Abs spectra (Figure 9). The longer 250 mm LiChrospher CN column in further following experiments, was eluted with diluted 0.1% v/v o-H_3PO_4 was used instead of $NH_4H_2PO_4$ buffer. Chromatogram on Figure 10 confirms the possibility of CN column to retard and retain longer analytes if needed, under acidic pH. The common feature for use of phosphate salts and diluted o-H_3PO_4 was the possibility to measure and record under the lowest possible UV-wavelength down to 190 nm, without problems of baseline flatness. This was the result of high UV-transparency of mentioned o-H_3PO_4 and ACN. Main differences between $NH_4H_2PO_4$and o-H_3PO_4 presences, were meldonium severe peak distortion and its UV-spectrum without concave shape, higher UV-transparency and much higher retention of both analytes with diluted o-H_3PO_4. This eluent enables large retention time for both API on this column, but with high probability of meldonium peak skewing. Presence of $NH_4H_2PO_4$ with weak acidic pH value of about pH = 4.5, yields symmetric peak with shorter retentions for meldonium, and good baseline flatness, UV-transparence. Other significant difference is much higher peak differences of meldonium, depending on wavelength when worked with ammonium phosphate under mild acidic pH value, if compared to cases with high acidic pH when used o-H_3PO_4 in mobile phase. When the diluted 0.065–0.07% v/v TFA was used, a quite different situation has been achieved. This organic acid, TFA, is functioning like acidifier and shortest an ion-pairing molecule, but with higher cut-off values about 205–210 nm, which limits the wavelengths of detector monitoring. Monitoring with decreasing below 197 nm, rapidly increases short term and long-term noises of the baseline. As can be noticed from Figure 12, which presents chromatogram of LiChrospher 250 × 4 mm, with 55% diluted TFA and 45% ACN at 2 comparison wavelengths, 195/210 nm, besides very good peaks symmetry, under these conditions, increasing the percentage of mobile phase force closure elution of meldonium and metoprolol, which is very important for run time and sensitivity. Using diluted TFA in mobile phases (Figure 13) generates significantly smaller peak sizes changes as in a function of wavelength decreasing changes for meldonium. The general conclusion is that stronger acidic environment generates smaller increments in hyper-chromic effects on meldonium, compared with presence of ammonium phosphate with pH about 4.5, increased by 0.2% with all 10% ACN presence. The peak of metoprolol showed increment with decreasing wavelength with different increments.

The second concept was based on Waters Spherisob CNRP 250 × 4.6 mm 5 µm column. During the analyses of Figures 14 and 15, it can be seen that the first visible difference was a higher tailing of the meldonium peak, when compared with LiChrospher CN columns. The hyper-chromic effect of both analytes was almost proportional, meldonium and metoprolol, in the presence of $NH_4H_2PO_4$ was visible too, especially on a zoomed scale inserted view of meldonium peak above the standard view full chromatogram, but with almost twice the content of ACN. When $NH_4H_2PO_4$ in the mobile phase was replaced with diluted TFA, similar observations were noticed as with LiChrospher CN columns. The peaks of meldonium and metoprolol were getting closure as a significant increasing of retention of meldonium, followed with this peak tailing increment, but in a lesser extent compared with LiChrospher. Second, increasing of peak of meldonium was quite small compared to that of metoprolol in a function of decreasing wavelength, the same conclusion as for previously tested LiChropher columns.

Third concept was based on Zorbax CN-SB 250 × 4.6 mm 5 µm column. This column was most tested and exploited since its chemistry permits and prefer medium to highly acidic mobile phase, according to manufacturer specifications. Beside this feature, this column generates the highest number of theoretical plates, which means that the highest

efficiency should be created for separated peaks. The perfect symmetry of meldonium was in direct visible comparison highly superior to peak of metoprolol, which imputes, suggest change of mobile phase. When the organic solvent was increased to 50%, significant chromatogram shape difference appeared, illustrated on Figure 28. This mobile phase yielded perfect shaped symmetric peak of meldonium (tailing F = 1.31) and metoprolol (tailing F = 1.36), within an overall run time of less than 3.5 min. This concept, set of experimental chromatographic conditions of mobile phase and separating column, created the shortest run time effective method for the determination of meldonium and metoprolol in pharmaceutical dosage forms. Further increases in the percentage of organic solvent ACN in mobile phase to 60% with 40% 13 mM $NH_4H_2PO_4$, shown in Figure 30, does not significantly change the chromatogram with retention times of components, except some of worsening the peak symmetry of meldonium, with shortening of the run time to below 3 min. Acidification of mobile phase with $NH_4H_2PO_4$ to pH = 2.5 manifests some deformation, peak leading, skewing of peak of meldonium, compared with not the treated solution of the buffer salt in mobile phase. In a further research work, we tested the applicability and selectivity of so far the shortest HPLC method, with applying of samples which were kept on room temperatures 24–72 h, for checking of possibility for appearance and separation and determination of first degrading products. These experiments were performed on other HPLC system Agilent 1260-II with PDA detector with flow cell volume of 1 μL, and decrease flow rate to F = 1 mL/min, so run time increased to about 3.6 min. In varying from 30% to 50% ACN in eluent, retention of meldonium was changed by about 10%, while metoprolol retention was doubled in value. The percentages of ACN in mobile phases were labelled on the right on each chromatogram. A further figure illustrates the samples with constant concentrations of analytes adjusted to meldonium 2.5 mg/mL and metoprolol 0.5 mg/mL in mobile phase, and during testing and experimental work, samples were kept at room temperature for 3 days and injected. Monitored at two different overlaid UV-wavelengths, the chromatograms showed additional peaks which confirm the possibility of this method to register and measure new-born peaks as a degrading product. The following chromatograms performed on Zorbax CN-SB 250 × 4.6mm, recorded and overlaid at 2-channels of 195 nm and 205 nm overlaid are on left and their characteristic UV-spectrums on the right. With mobile phase composed of 50% ACN and 50% 13 mM $NH_4H_2PO_4$, the most effective separation was achieved in less than 4 min run time. The second peak was meldonium on chromatogram, and the last highest peak was metoprolol, third and fourth peak newly appeared. From the 4 presented UV-Abs spectrums, the second from top was of meldonium peak extracted and fourth at the bottom was of metoprolol. Figure 38 contains overlay of fresh sample of meldonium and metoprolol standards, and they lack the second and third peaks of meldonium degradation products, while other present samples were prepared and kept at room temperature for 3 days, they have second and third peak with UV-spectrums similar to meldonium, set of overlaid presentation at 2-different UV-wavelengths, 195 and 205 nm. The third early degradation product was easily noticeable with improved resolution and extended run time. The set was performed with intended increasing different injection volumes, for checking of influence of compound quantity on peak shapes. The first peak was meldonium, whilst the second and third which were absent in fresh standards of meldonium and last eluting highest peak of metoprolol.

These features suggested the inappropriateness of using reversed C-8 or even worse case C-18, since in one preliminary test with RP Select B 75 × 4 mm column (which is C-8 type silica B), chromatographic capacity factors for retaining this two molecules in a single isocratic run gained differences in retentivity more than a factor 20–30, which makes method invaluable, with increasing of run times followed by decreasing of peak height and area precision and accuracy. Quite long retention with capacity factors K' differing for more of factor 20–30×, enables unwanted peak widths expanding due to longitudinal diffusion of eluting zone of peaks. Increasing the percentage of organic solvents, in our case acetonitrile, decreases a decent retention time for metoprolol, but elutes the meldonium in void volume/time of column. In this way, every intention to

optimize mobile phase with using C-8 or C-18 based columns is quite impractical. Even looking for shortest RP column or looking for least retentive with lowest carbon load and bonded density ligands, cannot yield appropriate practical, high throughput method for simultaneous determination of meldonium and metoprolol. Using phenyl columns was out of choice, since the inappropriate meldonium structure again, no phenolic rings in molecule. Using ion-exchange columns was rejected due to known features, slow equilibration, wide peaks, low sensitivity and chromatographic system suitability parameters. The use of CN-cyano column enabled us with the possibility to govern analyte retentions and sensitivity, especially in cases where mobile phase constituents in composition is highly restricted for lowest measurable UV-region 190–205–210 nm. Testing many mobile phase and columns, enabled us even to govern the schedule of elution of analyte with reverting of their appearance in elution position, which is very interesting and important in situation of testing different pharmaceutical dosage forms with different excipients, or to resolve problems with coeluting in situations in biological samples.

Comparison between the proposed HPLC concepts presented in Table 1, Intra-and Inter-day accuracy and precision results—in Tables 2 and 3. Analyzing Tables 1–3, we can conclude that the most optimal in terms of expressiveness and environmental friendliness are concepts 9 and 10. It was achieved with the use of column Zorbax CN SB (4.6 mm i.d. $\times$ 250 mm, 5 μm) and mobile phase ACN—0.15% $NH_4H_2PO_4$ (50:50 and 60:40, v/v). In this point we would like to mention, that the greenness of the method in HPLC is limited for perfect green-values, due to limited solubilities of hydrophobic analytes. Decreasing the percentage of the organic solvent can directly influence the peak shape, which means worsening in accuracy and sensitivity. Using the HILIC column is helpless in some low hydro-soluble molecules and errors in determination are much more probable. In some logic, the best and greenest case would be to take shortest alkyl chain bonded stationary phase to elute faster the analytes with less organic consumables and better green coefficients, but it is always coupled with some obstacles, analytes solubility, satisfying retentions for appropriate run time, selectivity, and sensitivity.

Table 1. Comparison between the proposed HPLC concepts.

Concept	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Linearity range, mg/mL	0.2–1.0—metoprolol 1–5—meldonium											
Mobile phase	ACN—0.15% $NH_4H_2PO_4$ (20:80, *v*/*v*)	ACN—0.065% TFA (45:55, *v*/*v*)	ACN—0.15% $NH_4H_2PO_4$ (20:80, *v*/*v*)	ACN—0.065% TFA (25:75, *v*/*v*)	ACN—0.065% TFA (45:55, *v*/*v*)	ACN—0.15% $NH_4H_2PO_4$ (50:50, *v*/*v*)	ACN—0.065% TFA (50:50, *v*/*v*)	ACN—0.15% $NH_4H_2PO_4$ (20:80, *v*/*v*)	ACN—0.15% $NH_4H_2PO_4$ (50:50, *v*/*v*)	ACN—0.15% $NH_4H_2PO_4$ (60:40, *v*/*v*)	ACN—0.07% TFA (40:60, *v*/*v*)	ACN—0.07% TFA (30:70, *v*/*v*)
Stationary phase	LiChrospher®® 125 CN (5 µm) (4 mm i.d. × 125 mm, 5 µm)	LiChrospher®® 125 CN (5 µm) (4 mm i.d. × 125 mm, 5 µm)	LiChrospher®® 250 CN (5 µm) (4 mm i.d. × 250 mm, 5 µm)	LiChrospher®® 250 CN (5 µm) (4 mm i.d. × 250 mm, 5 µm)	LiChrospher®® 250 CN (5 µm) (4 mm i.d. × 250 mm, 5 µm)	Waters Spherisorb CNRP (4.6 mm i.d. × 250 mm, 5 µm)	Waters Spherisorb CNRP (4.6 mm i.d. × 250 mm, 5 µm)	Zorbax CN SB (4.6 mm i.d. × 250 mm, 5 µm)	Zorbax CN SB (4.6 mm i.d. × 250 mm, 5 µm)	Zorbax CN SB (4.6 mm i.d. × 250 mm, 5 µm)	Zorbax CN SB (4.6 mm i.d. × 250 mm, 5 µm)	Zorbax CN SB (4.6 mm i.d. × 250 mm, 5 µm)
Run time, min	5.5	4	9	8.5	7	8	6	10	3.5	3.5	5	3
Flow rate, mL min^{-1}	1	1	1	1	1	1.2	1.2	1	1	1	1	1
Wavelength, nm	200	200	200	200	200	200 and 210	200 and 210	200	200	210	210	210
LOD/LOQ, mg/mL	0.011/0.035—meldonium 0.00022/0.00075—metoprolol	0.032/0.011—meldonium 0.00041/0.00175—metoprolol	0.011/0.035—meldonium 0.00022/0.00075—metoprolol	0.043/0.133—meldonium 0.00038/0.00157—metoprolol	0.039/0.0.180—meldonium 0.00039/0.00143—metoprolol	0.015/0.047—meldonium 0.00022/0.0008—metoprolol	0.051/0.153—meldonium 0.00043/0.00164—metoprolol	0.017/0.056—meldonium 0.00016/0.00051—metoprolol	0.021/0.059—meldonium 0.00016/0.00052—metoprolol	0.017/0.056—meldonium 0.00019/0.00066—metoprolol	0.045/0.161—meldonium 0.0045/0.0015—metoprolol	0.052/0.173—meldonium 0.0053/0.0018—metoprolol
Linearity regression, R^2	y-16700× +9814.120.9998	y-15963× +5987.530.9999	y-13753× +5492.891.0000	y-19784× +8905.750.9999	y-17906× +7859.940.9999	y-18945× +7896.570.9997	y-17834× +7894.381.0000	y-18563× +9473.840.9997	y-15978x+ 6739.970.9999	y-17890× +6491.230.9997	y-18856× +7819.781.0000	y-17829× +6743.190.9998
Analytical GREEnness (AGREE)	0.76	0.69	0.74	0.67	0.68	0.75	0.68	0.74	0.77	0.77	0.68	0.69

Table 2. Intra-day accuracy and precision results.

Concept	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Analyte	**Mean, % (RSD, %)**											
Meldonium	99.86 (0.32)	99.67 (0.39)	100.48 (0.21)	100.87 (0.40)	99.56 (0.30)	100.09 (0.20)	100.36 (0.31)	100.03 (0.34)	100.15 (0.28)	100.09 (0.29)	99.89 (0.41)	100.14 (0.40)
	99.84 (0.38)	99.42 (0.42)	99.47 (0.46)	100.67 (0.31)	100.05 (0.24)	100.17 (0.41)	100.51 (0.27)	99.84 (0.44)	100.22 (0.34)	100.17 (0.21)	99.75 (0.23)	100.09 (0.35)
	100.12 (0.28)	99.53 (0.45)	100.32 (0.35)	100.76 (0.34)	100.18 (0.29)	100.15 (0.34)	100.60 (0.20)	100.01 (0.42)	100.11 (0.40)	99.81 (0.33)	99.69 (0.34)	100.29 (0.38)
Metoprolol	99.97 (0.22)	100.35 (0.27)	99.47 (0.25)	100.13 (0.29)	99.55 (0.35)	100.78 (0.45)	99.90 (0.18)	99.45 (0.44)	99.56 (0.31)	100.22 (0.37)	100.05 (0.22)	100.01 (0.27)
	99.84 (0.41)	99.95 (0.39)	99.67 (0.30)	100.35 (0.40)	99.78 (0.39)	100.66 (0.37)	100.17 (0.33)	99.48 (0.20)	99.77 (0.37)	100.45 (0.38)	100.01 (0.30)	100.18 (0.29)
	100.53 (0.36)	99.56 (0.27)	99.70 (0.27)	100.22 (0.28)	99.70 (0.23)	100.56 (0.29)	100.12 (0.28)	99.67 (0.31)	99.65 (0.40)	100.30 (0.31)	100.10 (0.27)	100.15 (0.35)

Table 3. Inter-day accuracy and precision results.

Concept	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Analyte	**Mean, % (RSD, %)**											
Meldonium	99.56 (0.21)	100.05 (0.30)	100.10 (0.26)	100.01 (0.22)	100.15 (0.20)	99.61 (0.48)	99.93 (0.28)	100.06 (0.32)	100.15 (0.16)	100.05 (0.18)	100.14 (0.30)	100.18 (0.26)
	99.51 (0.30)	100.02 (0.36)	99.93 (0.19)	99.64 (0.36)	100.01 (0.29)	99.82 (0.45)	99.84 (0.20)	100.27 (0.40)	100.22 (0.40)	100.22 (0.41)	99.95 (0.13)	100.12 (0.25)
	99.89 (0.34)	99.67 (0.24)	100.18 (0.26)	100.05 (0.42)	100.24 (0.36)	99.89 (0.38)	100.06 (0.25)	100.20 (0.23)	100.11 (0.35)	99.86 (0.20)	99.80 (0.23)	100.34 (0.44)
Metoprolol	100.18 (0.28)	100.06 (0.25)	99.83 (0.22)	99.91 (0.17)	99.70 (0.30)	100.03 (0.26)	99.95 (0.37)	99.97 (0.40)	99.56 (0.24)	100.12 (0.27)	100.17 (0.12)	99.69 (0.42)
	100.24 (0.22)	99.78 (0.28)	99.50 (0.29)	99.83 (0.32)	99.91 (0.26)	100.14 (0.17)	100.08 (0.31)	99.51 (0.44)	99.77 (0.31)	100.09 (0.18)	100.12 (0.28)	99.93 (0.15)
	100.13 (0.36)	99.85 (0.20)	99.89 (0.24)	99.63 (0.38)	99.95 (0.29)	100.25 (0.20)	100.17 (0.44)	99. (0.37)	99.65 (0.33)	100.01 (0.22)	100.03 (0.35)	99.74 (0.31)

In the very poor available data about getting idea and usable information about development of method for simultaneous determination of these two active substances with polarity differences between them, we can suggest our work with offered detailed and successful solutions for the mentioned aim with less sophisticated equipment for quality control lab for routine manufacturing control.

5. Conclusions

In solving the intention for creating, development and validating optimal HPLC method for simultaneous determination of the two analytes with extreme differences in polarities, we have tested 3 different cyano CN-bonded chromatographic column, with two main concepts of mobile phases composition, ACN with phosphate salts and ACN with diluted acids, inorganic o-H_3PO_4 and TFA. All concepts were performed with low and high percentages of ACN in mobile phases, and chromatograms were followed with full PhotoDiode Array detecting possibilities. All three columns are useful and powerful in the determination of meldonium and metoprolol under different conditions toward mobile phase composition. As an overall conclusion according to given results, some conclusion can be readily extracted. Using phosphate buffer enables stabile and very reproducible results, with high sensitivity for both analytes, which is enabled by the highest

UV-transparence of ACN and phosphate, enabling monitoring and measuring down to the shortest wavelength range 190–195–200 nm, and sensitivity with UV-PDA detector for meldonium is about 11–20 times better when compared to methods using diluted TFA, depending on selected comparative UV-wavelength. Acidifying of mobile phase induce worsening of peaks symmetry, especially meldonium. Increasing percentage of ACN with both types of inorganic parts of mobile phase, phosphate buffered and diluted TFA acid, howed approaching, closuring of peaks with reducing enormous excessive resolution, followed by spreading peak widths, which decreases method sensitivity, especially for meldonium. Usually, the presence of trifluoroacetic acid induces mild tailing of meldonium, while o-H_3PO_4 induces leading of meldonium peak. The peak size, area, height is far less sensitive to change in monitoring UV-wavelength in presence of TFA, compared to o-H_3PO_4, while presence of 13 mM $NH_4H_2PO_4$ induces enormous changes in both peaks size, meldonium and metoprolol. Using combination of ACN with diluted o-H_3PO_4 enables sensitive measuring to lowest 190 nm wavelength, same as with $NH_4H_2PO_4$ combinations. However, presence of even diluted 0.065–0.07% v/v TFA, does not permit sensitive measuring in ranges below 195 nm, because of high cut-off values of organic acid. In one sentence, in case of need using applicable method for LC-MS technique, combination of mobile phases with diluted TFA is highly recommended and achievable with all tested CN-cyanocolumns, with preferring Zorbax CN-SB, which is most stable in acidic pH. In cases for need method to use with UV-Vis, PDA, Fluorescent FLD, $NH_4H_2PO_4$ combination with ACN is superior and recommended option for method choice, enabling high-throughput analyses essential for QC laboratories. Both concepts of mobile phases are easily re-optimized for fine peak tuning elution positions and achievement of resolutions. Finally, since methods with $NH_4H_2PO_4$and o-H_3PO_4 with selected best choice of CN-column are far more sensitive for meldonium between 10–17 times, compared with methods with diluted TFA trifuoroacetic acid, the first are recommended form routine high-throughput analyses of meldonium and metoprolol in pharmaceutical dosage forms, while second group with TFA are ideal for biological samples analysis with LC-MS instant applicability, where MS detector will successfully compensate and upgrade the sensitivity of method for mentioned analytes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm90040065/s1. Figure S1: Testing the chromatograms elution positions and peak shapes of meldonium and metoprolol samples and meldonium standards only, in mobile phases with different procents of ACN and diluted TFA. Figure S2: Chromatogram gained with Zorbax CN-SB column with 3 clearly separated API molecules meldonium, metoprolol and bisoprolol, with their UV-absorption characteristic extracted spectrums on right side, which expand the applicability of method to perform determination of other beta-blocker drugs simultaneously.

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