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Application of hydrophilic interaction chromatography for simultaneous separation of six impurities of mildronate substance

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ABSTRACT

The possibility of separating the impurities of mildronate, an antiischemic drug, by hydrophilic interaction chromatography (HILIC) was investigated on different polar stationary phases (silica, amino, cyano and zwitterionic sulfobetaine). The investigations have shown that HILIC is a useful alternative to reversed phase and ion-pair chromatography. The impact of HILIC separation conditions (acetonitrile content, buffer pH in mobile phase) on retention and selectivity has been systematically studied. Importance of these factors was found to be dependent on the structural properties of solutes. A HILIC method using a zwitterionic sulfobetaine stationary phase was developed and validated to determine six impurities in the drug substance. The method was validated in terms of specificity, limit of quantitation, limit of detection, linearity, accuracy and precision.

1. Introduction

3-(2,2,2-Trimethylhydrazinium)propionate dihydrate or mildronate (also known as THP, MET-88) is an antiischemic drug developed at the Latvian Institute of Organic synthesis more than 20 years ago. However, to the best of our knowledge, no HPLC method for simultaneous separation of mildronate (compound **4**) and its impurities (see Table 1), such as trimethylammonium bromide (compound **1**), 1,1,1-trimethylhydrazinium bromide (compound **2**), 3-hydroxy-1,1-dimethyl-4,5-dihydro-1H-pirazolium-1-betaine hydrate (compound **3**), 3-(2,2,2-trimethylhydrazinium)methylpropionate bromide (compound **5**), 3-(2,2,2-trimethylhydrazinium)ethylpropionate bromide (compound **6**) and 3-(2,2,2-trimethylhydrazinium)prop-2-yl propionate bromide (compound **7**) has been reported.

A good separation method for the studied compounds should combine sufficient retention, selectivity and sensitivity. Reversed-phase liquid chromatography (RPLC) is currently the most popular method in the field of HPLC. Although RPLC is a powerful separation mode, it has a major limitation: the lack of adequate retention of polar molecules. As mildronate and its related substances are polar solutes of small molecules, weak retention of these substances under reversed phases mode can be expected, and application of ion-pair chromatography seems more perspective in this case. However, there is another restriction, consisting in the lack of pronounced chromophores, which makes mass spectrometry the appropriate sensitive detection alternative. Unfortunately, ion-pair agents as well as high water content in the mobile phase usually reduce the sensitivity of MS detection [1,2].

Previously for determination of mildronate, HPLC method with evaporative light scattering detection (ELSD) was used [3,4]. It is known that stripping voltammetry [5], HPLC–MS–MS [6] or UV detection (with mildronate derivatization) [7] were used for mildronate detection in the plasma and urine. In all these methods, polar stationary phases (columns Silasorb 600 Silica, Inertsil NH_2, Inertsil CN-3) and mobile phases with water component content more than 60% are used. In our opinion, such chromatographic conditions suffer from insufficient sensitivity in HPLC–MS. None mentioned reference has studied impurities from Table 1.

Hydrophilic interaction chromatography (HILIC) is a HPLC mode based on the combination of hydrophilic stationary phases and hydrophobic, mostly organic, mobile phases. HILIC is an alternative approach to effectively separate low-molecular weight polar compounds [8–11]. HILIC mode was first discussed in detail in the

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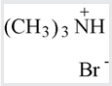
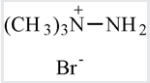
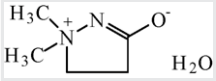
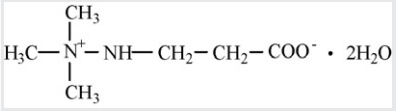
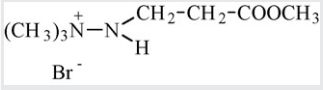
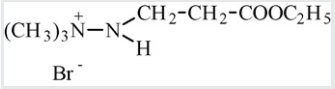
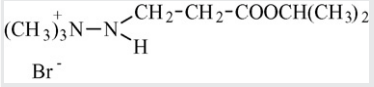
Table 1
Studied compounds

Formula	Compound number	Molecular weight	Cone voltage	Collision energy	MRM transition (*m*/*z*)
	**1**	60	20	18	60 → 45
	**2**	75	25	15	75 → 59
	**3**	115	35	19	115 → 72
	**4**	146	25	18	147 → 59
	**5**	161	20	23	161 → 59
	**6**	175	22	23	175 → 58
	**7**	189	25	22	189 → 58

works of Alpert [12]. The comprehensive review on the scope and limitations of HILIC approach for analysis of polar analyses was published recently by Hemstrom and Irgum [13]. This method has been used for pharmaceutical product analysis [14–18]. HILIC is more suitable to MS detection and improves the MS sensitivity [19–23].

In HILIC mode, the mobile phase usually is an aqueous/organic mixture with the water component content less than 60%. Polar solutes usually are retained more strongly than non-polar ones. The retention mechanism for HILIC is a partitioning between the bulk eluent and a water-rich layer, partially immobilized on the stationary phase. But "pure HILIC" separation mode appears, however, quite rare [13]. Depending on the surface chemistry of the stationary phase, ion exchange in particular can contribute a lot to the retention of ionic solutes.

The aim of our study was to investigate the feasibility of HILIC method in the analysis of mildronate and related substances.

2. Experimental

2.1. Chemicals and solutions

HPLC grade acetonitrile (ACN) was obtained from Merck KGaA (Darmstadt, Germany), and HPLC grade water (18.2 MΩ cm) from a Millipore Milli-Q Gradient Purification System. Formic acid (FA, >99.0%), ammonium formate (purity 97%) were supplied by Acros Organics.

The parameters of compounds studied are given in Table 1. Mildronate (compound **4**) was manufactured at JSC "Grindeks" (Riga, Latvia), while the other six compounds were obtained from the Latvia Institute of Organic Synthesis (Riga, Latvia).

Solutions of 1 μg/ml concentrations were prepared from any of the seven compounds. For experiments a binary mixture acetonitrile–water (90/10, v/v) was used.

HILIC experiments were performed using binary mixtures of ACN and 0.1% FA in water (v/v) or ammonium formate buffers of

Table 2
Columns used and properties of stationary phases

Number	Column name	Phase type	Pore size (Å)	Particle size (μm)	Surface area (m^2/g)	Dimension (mm)
1	Discovery® cyano	Cyano	180	5	200	2.1 × 100
2	Hypersil APS-1	Amino	120	3	170	3.2 × 100
3	Atlantis HILIC silica	Silica	100	3	330	2.1 × 150
4	Alltima HP silica	Silica	100	3	450	2.1 × 150
5	Spherisorb® silica	Silica	80	3	220	2.1 × 100
6	ZIC®-HILIC	Sulfobetaine	200	5	135	2.1 × 100

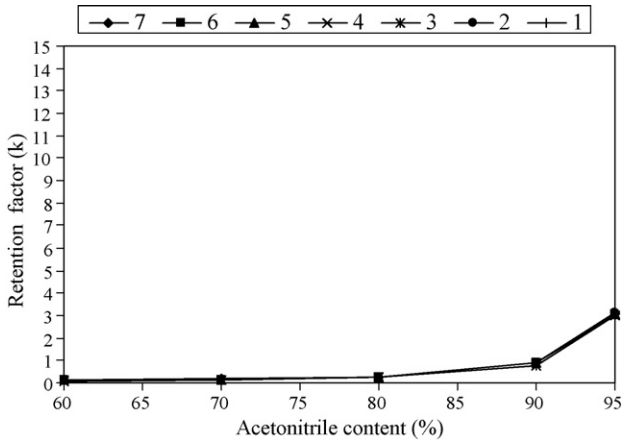


Fig. 1. Relationship between the retention factor (k) and the acetonitrile content. Column: Discovery Cyano, mobile phases: 0.1% FA and acetonitrile. For other chromatographic conditions see: Section 2.

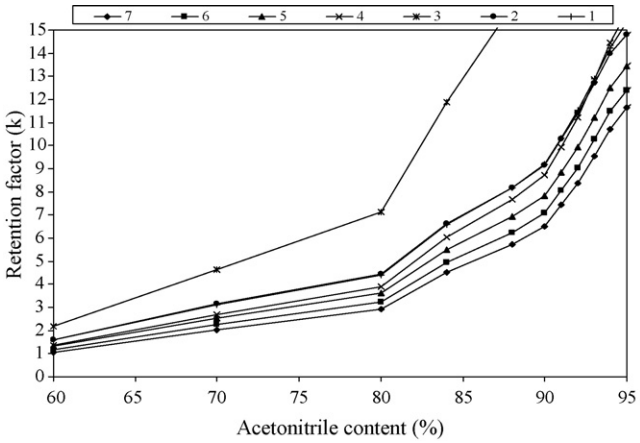


Fig. 3. Relationship between the retention factor (k) and the acetonitrile content. Column: Atlantis HILIC Silica, mobile phases: 0.1% FA and acetonitrile. For other chromatographic conditions see: Section 2.

various concentrations and pH values. Buffer pH was adjusted with 10% (v/v) formic acid in water (electrode accuracy ±0.01) before adding acetonitrile.

2.2. Equipment and methods

An Alliance 2695 liquid chromatograph (Waters, Milford, MA, USA) equipped with a 120-vial capacity sample management system was used in combination with a Quattro Micro API triple quadrupole-mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). Data acquisition was performed in the positive ion mode.

Interface parameters were set as follows:	
Capillary voltage	3.0 kV
Cone voltage	20–35 V
Extractor lens voltage	3 V
Source temperature	110 °C
Desolvation temperature	220 °C
RF lens	0.3 V
Dwell time	0.20 s
Interchannel delay	0.05 s
Desolvation gas (N_2, 99.5%)	500 L/h
Cone gas	20 L/h

The analytes were determined by using the multiple reaction monitoring mode (MRM) with specific transitions shown in Table 1.

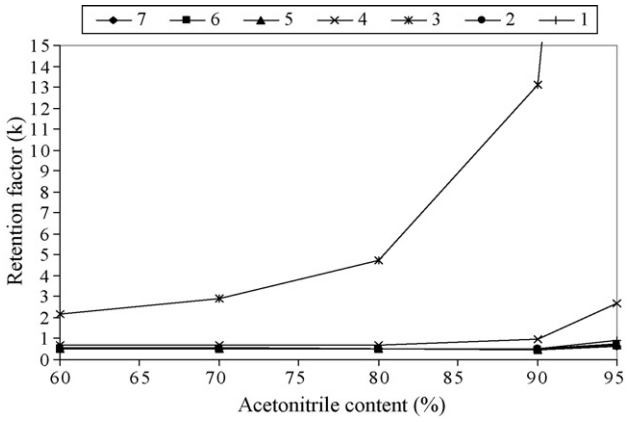


Fig. 2. Relationship between the retention factor (k) and the acetonitrile content. Column: Hypersil APS-1, mobile phases: 0.1% FA and acetonitrile. For other chromatographic conditions see: Section 2.

The collision gas was argon (99.99% purity). The MassLynx v. 4.0 Data System was applied for MS control and data processing.

pH meter INOLAB Level 1 (WTW, Germany) was used.

2.3. Columns

The columns used and properties of stationary phases are shown in Table 2. Atlantis HILIC Silica, Spherisorb® Silica columns were purchased from Waters (Milford, MA, USA). Hypersil APS-1 and Discovery® Cyano was purchased from Supelco (Bellefonte, PA, USA). ZIC®-HILIC column was purchased from SeQuant AB (Umea, Sweden). Alltima HP Silica was purchased from Alltech (Deerfield, IL, USA).

The column temperature 30 °C, flow rate 0.2 ml/min and injection volume 4 μl were used for all experiments. For the purpose of data uniformity in this paper it was assumed that t_0 corresponds to the first deflection point on the chromatogram [24].

3. Results and discussion

3.1. The effect of acetonitrile content on retention

From the point of view of typical structural moieties present, the studied substances (see Table 1) can be divided into three different

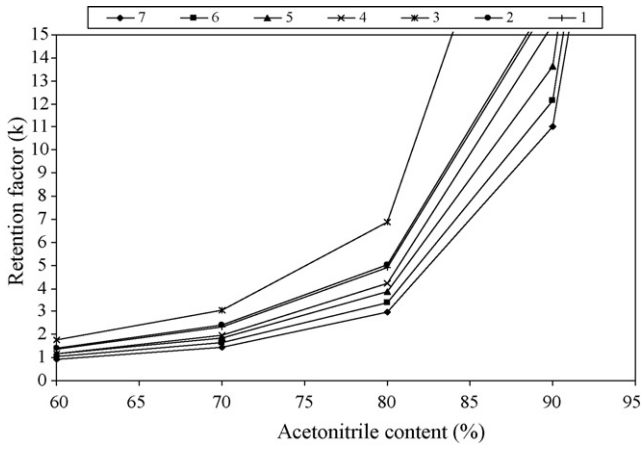


Fig. 4. Relationship between the retention factor (k) and the acetonitrile content. Column: Alltima HP Silica, mobile phases: 0.1% FA and acetonitrile. For other chromatographic conditions see: Section 2.

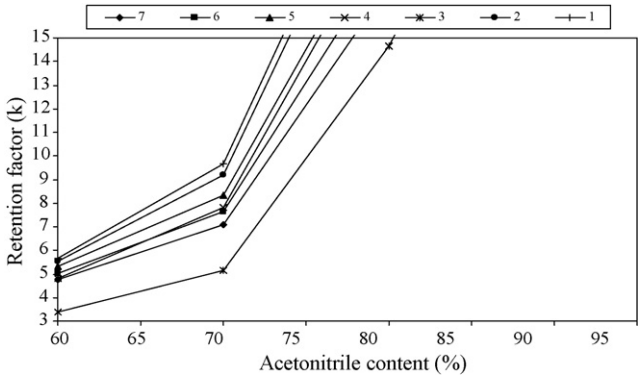


Fig. 5. Relationship between the retention factor (k) and the acetonitrile content. Column: Spherisorb Silica, mobile phases: 0.1% FA and acetonitrile. For other chromatographic conditions see: Section 2.

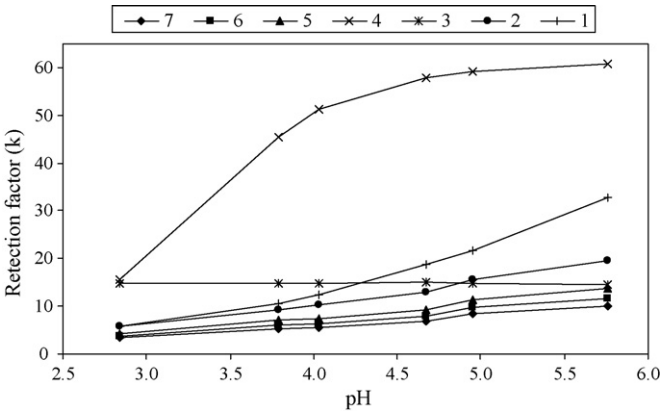


Fig. 7. Effect of the buffer pH on the retention factor (k). Column: Atlantis HILIC Silica, mobile phase: acetonitrile–5 mM ammonium formate (85/15, v/v). For other chromatographic conditions see: Section 2.

groups: compounds **1**–**2** (cations), compounds **3**–**4** (zwitterions), and compounds **5**–**7** (cationic esters).

The columns 1–6 (Table 2) were tested with respect to their ability of retaining the analytes 1–7 in typical HILIC conditions. The percentage of acetonitrile in the eluent was studied in the range between 60 and 95%, with deionized water and 0.1% FA.

It was established that the polar bonded phase Discovery Cyano appeared to be the least retentive (Fig. 1). At acetonitrile concentrations up to 90%, the retention was negligible. Even at 95% of acetonitrile, the retention factors were only about 3. This indicates that under the conditions used in these experiments the volume of dynamically generated water-rich stationary phase is small. Probably, the residual silanol groups are unable to stimulate accumulation of water because of surface shielding by organic ligands.

An interesting phenomenon was the almost complete absence of structural selectivity on this cyano column. All solutes irrespective of their molecular size, functionalities and ionized sites present elute within an extremely narrow k range. Obviously, such behavior is not attractive from purely analytical point of view. Still, its mechanism might be a subject of more thorough investigation in the future.

The amino phase (column Hypersil APS-1) showed a different behavior (Fig. 2). Cationic substances were retained very weakly in the entire range of acetonitrile concentrations studied (60–95%). On the other hand, zwitterions 3–4 were retained strongly what indicates electrostatic interaction between the amino groups of the packing (protonated at the mobile phase pH) and the negatively charged moieties of zwitterions.

Specialized HILIC silica (column Atlantis HILIC Silica) showed a very regular increase of retention with the increase in acetonitrile content in the mobile phase (Fig. 3). The cationic esters 5–7 differ by one carbon atom while having identical functionality. Retention behavior within such a structurally close groups of compounds is mainly dictated by balance of hydrophilic and hydrophobic parts. The linear relationships between the number of carbon atoms and retention is a common phenomenon in such cases, indicative of the role of partition mechanism between the stationary and mobile phases. The cationic compounds **1**–**2** cannot be separated on this column, even when retention is strong at 95% of acetonitrile. One could expect that compound **4** is retained much more strongly than cationic ester 5. In fact, our data show only slight difference between these two compounds. Most likely, in the acidic conditions used in these experiments, the carboxylic group does not influence partition values. The role of this group seems to be so insignificant that compounds **5**–**7** and 4 belonging to esters and a betaine behave like homologs (Fig. 3). Compound **3** is retained much stronger than 4–7 and falls out of the common line. This can be explained by a more pronounced contribution of ion exchange in the case of this cyclic structure.

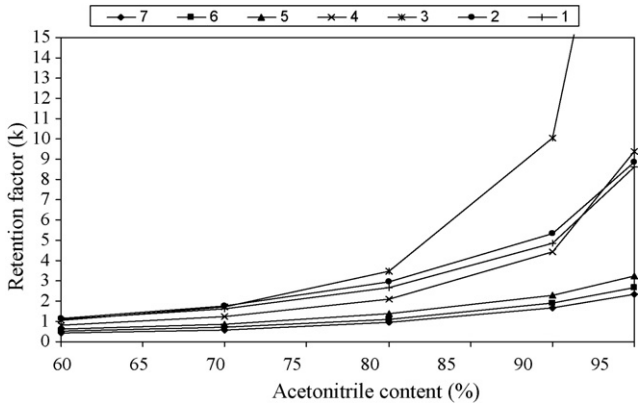


Fig. 6. Relationship between the retention factor (k) and the acetonitrile content. Column: ZIC-HILIC, mobile phases: 0.1% FA and acetonitrile. For other chromatographic conditions see: Section 2.

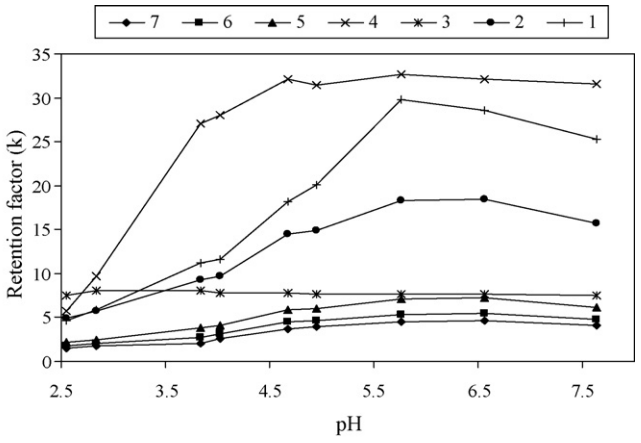


Fig. 8. Effect of buffer pH on the retention factor (k). Column: ZIC-HILIC, mobile phase: acetonitrile–5 mM ammonium formate (85/15, v/v). For other chromatographic conditions see: Section 2.

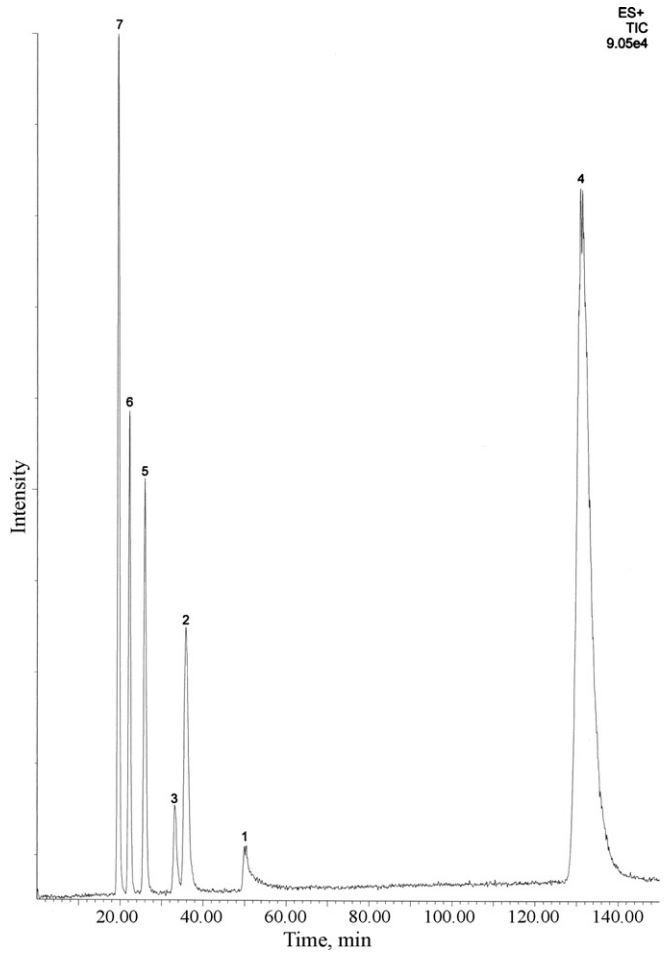


Fig. 9. Separation of test compounds **1**–**7** (see Table 1) under HILIC mode on Atlantis HILIC silica column, mobile phase: acetonitrile–5 mM ammonium formate pH 4.8 (85/15, v/v). For other chromatographic conditions see: Section 2.

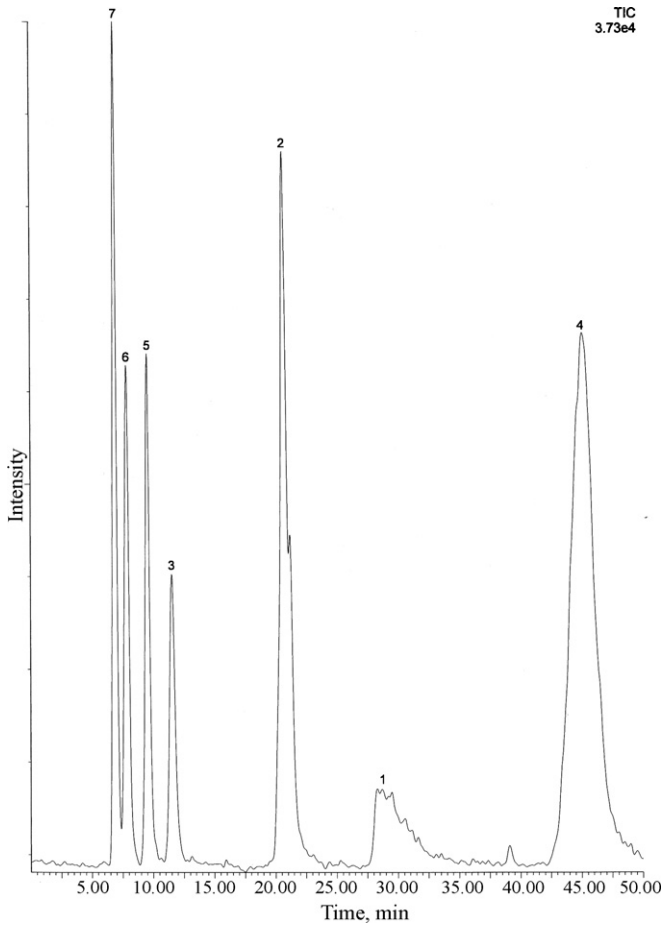


Fig. 10. Separation of test compounds **1**–**7** (see Table 1) under HILIC mode on ZIC-HILIC column, mobile phase: acetonitrile–5 mM ammonium formate pH 5.0 (85/15, v/v). For other chromatographic conditions see: Section 2.

It was established that the common silica column Alltima HP Silica when used with 70–80% acetonitrile mobile phase showed similar retention (Fig. 4) and similar elution order of the compounds under study to the specialized HILIC silica column Atlantis HILIC Silica. This suggests the retention mechanisms are similar on both silica packings. On the other hand, at higher (90–95%) acetonitrile concentrations, the retention on the common silica is much stronger than that on HILIC silica. A possible explanation of this phenomenon is the larger surface area of Alltima HP Silica (see Table 2). It can retain a larger volume of a water-rich layer acting as the stationary phase. Spherisorb silica column generally is about three times (Fig. 5) more retentive than Alltima HP silica if the mobile phase with 70% acetonitrile is used. At higher concentrations of ACN, the difference is even more pronounced. Retention regularities within homolog series 5–7 are similar to what was observed on Alltima HP and Atlantis HILIC Silica. At the same time, the profile of structural selectivity is different. For example, zwitterions are retained relatively weakly on this packing. Compound **3** is the least retained, and behavior of mildronate (compound **4**) is different to compare with esters (compounds **5**–**7**). A possible explanation lies in the properties of Spherisorb silica. It is one of the earliest types of HPLC silica (type A silica), most likely manufactured according to an old technology involving silicates and containing metal impurities, capable of secondary interactions with nitrogen containing compounds. The fact that considerable retention is observed even at 60% of acetonitrile (when the volume of the aqueous-rich stationary phase is relatively low) suggests a direct interaction with surface groups of the packing.

Retention of test solutes under HILIC conditions on ZIC-HILIC column is represented in Fig. 6. Average retention values on ZIC-HILIC are lower than those observed for silica columns, but the general character of plots is similar. On the other hand, the structural factors are more visible on ZIC-HILIC column. First, like it was on silica columns, the retention factors of cations 1 and 2 show very similar values. On the other hand, the retention of cationic esters 5–7 is much lower than that observed on silica. Zwitterions behave differently. At intermediate content of acetonitrile (70–80%), their behavior is similar to that of other solutes, but above 80% they show a more rapid increase in retention than other compounds do. A similar, still less pronounced effect was observed on HILIC silica.

Table 3
Linearity, limits of detection and quantitation

Compound	r^2	LOD (% of assay)	LOQ (% of assay)
1	0.991	0.003	0.01
2	0.997	0.0003	0.001
3	0.998	0.0005	0.002
5	0.992	0.0004	0.001
6	0.998	0.0001	0.0004
7	0.998	0.00006	0.0002

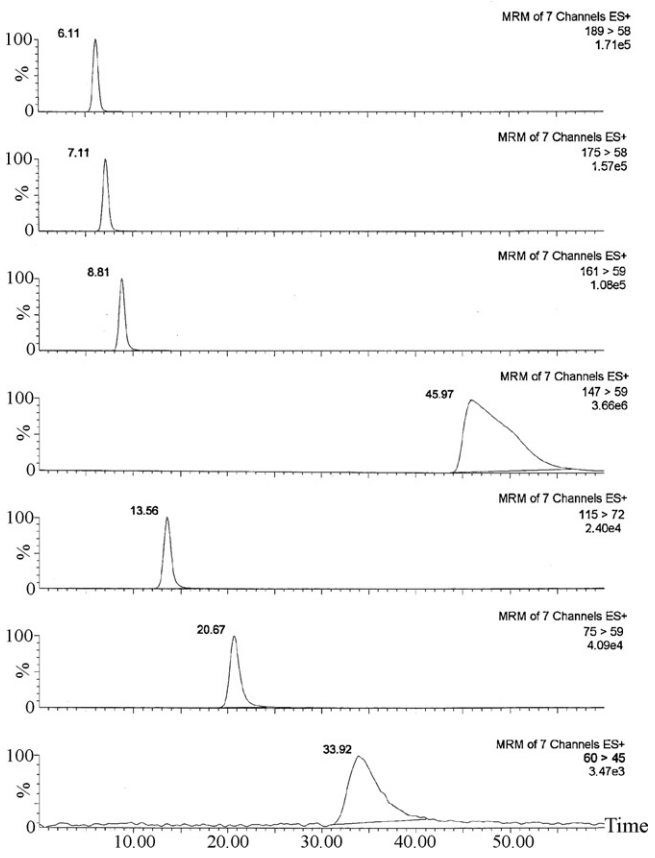


Fig. 11. MRM chromatogram of six standard impurities and main compound mixture. Column: ZIC-HILIC; concentration of mildronate 1 mg/ml, for other compounds: 1 μg/ml. Mobile phase: acetonitrile–5 mM ammonium formate pH 5.0 (85/15, v/v). Specific transition of compound **1**–**7** (from bottom to top). For other chromatographic conditions see: Section 2.

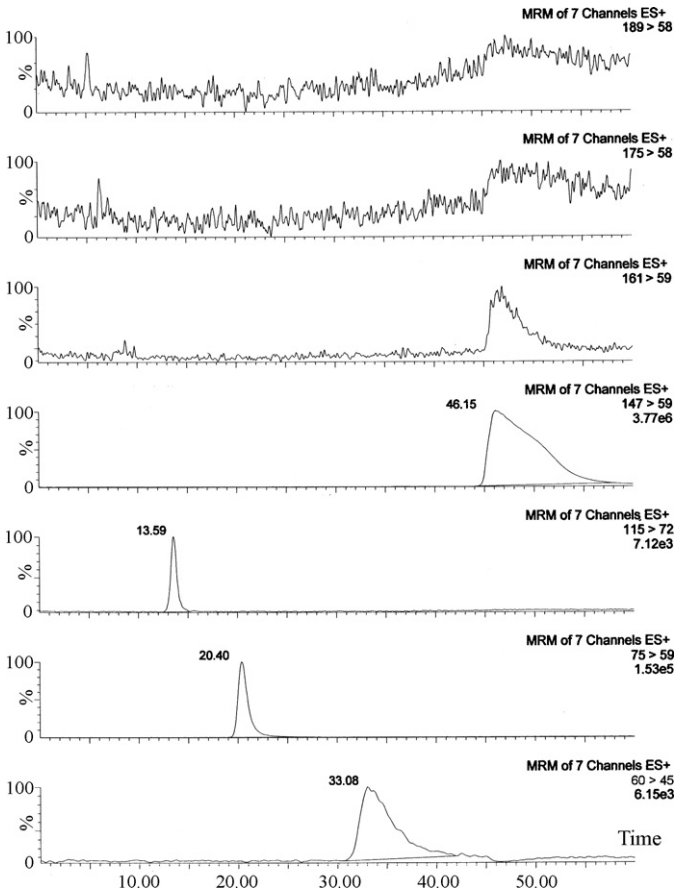


Fig. 12. MRM chromatogram of production batch 2374-06 of mildronate. Column: ZIC-HILIC; Concentration of mildronate 1 mg/ml. Mobile phase: acetonitrile–5 mM ammonium formate pH 5.0 (85/15, v/v). Specific transition of compound **1**–**7** (from bottom to top). For other chromatographic conditions see: Section 2.

Table 4
Results of accuracy

Compound	Level (%)	Recovery (%) for $n = 3$	%R.S.D.
1	50	104.7	1.8
	75	96.9	3.6
	100	103.2	10.1
	125	100.1	6.1
2	50	106.7	5.1
	75	110.6	1.1
	100	105.7	2.3
	125	114.0	1.9
3	50	113.4	2.9
	75	113.3	3.4
	100	111.2	7.9
	125	116.4	4.6
5	50	102.1	1.3
	75	112.8	1.4
	100	107.0	4.5
	125	114.4	1.1
6	50	106.2	1.1
	75	104.2	0.4
	100	105.0	2.3
	125	109.6	0.9
7	50	108.4	1.5
	75	105.8	2.6
	100	103.1	0.9
	125	107.9	1.8

A successful analytical method should avoid both too low retention (that usually leads to poor separation) and too high retention that makes the run longer and often is accompanied by unsatisfactory peak shape. Therefore two columns—ZIC-HILIC and Atlantis HILIC were chosen for further study.

3.2. The effect of buffer pH

Effect of pH on retention and selectivity of seven compounds was examined in pH range of 2.6–5.8 for Atlantis HILIC Silica column (Fig. 7), and in pH 2.5–7.6 for ZIC-HILIC column (Fig. 8).

The retention factor of cations 1 and 2 and cationic esters 5–7 on Atlantic HILIC Silica column increased steadily in the pH range between 2.8 and 5.8. The most probable reason of this is ion exchange. At higher pH, the degree of ionization of surface silanol group increases, thus creating more favorable conditions for such interactions. Mildronate 4 behaves in a similar way as cations 1

Table 5
Results of precision

Compound	Precision, R.S.D.% ($n = 6$)
1	9.4
2	3.3
3	7.7
5	2.7
6	2.1
7	2.7

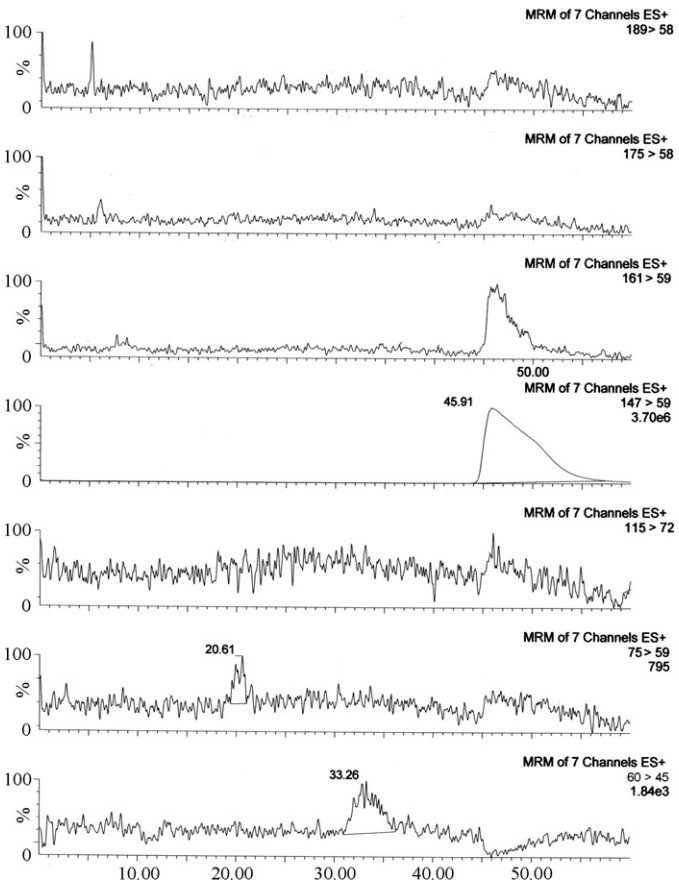


Fig. 13. MRM chromatogram of production batch 990208 of mildronate. Column: ZIC-HILIC; Concentration of mildronate 1 mg/ml. Mobile phase: acetonitrile–5 mM ammonium formate pH 5.0 (85/15, v/v). Specific transition of compounds **1**–**7** (from bottom to top). For other chromatographic conditions see: Section 2.

and 2. Another zwitterion 3 shows a completely different pattern: its retention is independent of pH.

While changes of retention on Atlantis HILIC are monotonous, similar series of experiments on ZIC-HILIC column, performed at a wider pH range, showed maximum at pH about 6. Again, the compound **3** is different, its retention steadily decreasing in the whole pH range studied.

Separation of the seven test compounds is visualized in Fig. 9 (pH 4.8; Atlantic HILIC Silica column) and Fig. 10 (pH 5.0; ZIC-HILIC column). ZIC-HILIC column shows the best selectivity for the seven tested compounds.

3.3. Method validation

The method was validated according to the ICH guidelines [25–28]. The validation parameters include specificity, accuracy, linearity, precision, limit of detection (LOD) and limit of quantitation (LOQ). The optimized conditions of the HILIC method are described in Fig. 11.

The method specificity has been demonstrated by separating all the known and potential degradation products. At start of studies, an assumption were made that any of impurities is allowable at concentrations not more that 0.1% of assay (preliminary quality specification (QS)). Concentration of mildronate during all validation was 1 mg/ml. Spiked samples were prepared (spike level: 50, 75, 100 and 125% of QS level, i.e., 0.0005–0.00125 mg/ml).

Linearity of method was established from 50 to 125% of the QS level (0.1% of the assay target concentration). Each compound was prepared and injected triplicate at each level. Correlation coefficients of linearity curve, LOD's and LOQ's results for six impurities are presented in Table 3.

Accuracy was established for the impurities at the level of 0.05, 0.075, 0.10 and 0.125% of the main compound's concentration. Each compound was prepared and injected triplicate at each level. Mean recovery and %R.S.D. for six impurities are presented in Table 4.

Precision is established at level 0.10% of the main compound's concentration. Six independent samples was prepared and injected triplicate. Precision results for six impurities are presented in Table 5.

Checking robustness of method, was found, that prepared solution of tested compounds (with 100% of QS spike) is stable (results show variations less than 15%) within at least 24 h.

Table 6
Quantitative results of analyses of technical and commercial mildronate, in percents

Compound	Technical, batch 2374-06	Commercial, batch 990208
1	0.20	0.01
2	0.35	Traces (~0.0003)
3	0.02	Not found
5	Not found	Not found
6	Not found	Not found
7	Not found	Not found

3.4. Application to real samples

The validated method was used to analyze technical batch (Fig. 12) and commercial batch (Fig. 13). Fig. 12 shows that technical product contains visible quantities of related substances, but Fig. 13 has just small traces of them. Comparative results of analysis are presented in Table 6. The developed method is suitable to control the quality of commercial mildronate API.

4. Conclusion

Mildronate and six of its related impurities have been separated in HILIC conditions.

Variation of polar stationary phase is used as a tool to change the selectivity of separation for the test solutes. It was established that the variation of acetonitrile concentration is a universal means of adjusting retention but does not significantly influence the selectivity.

It has been shown that HILIC separations of quaternary hydrazine derivatives are possible both on columns that are specifically positioned by the manufacturers as HILIC type and on common silica columns. Specialized columns show weaker retention and are more suitable for separations at high concentrations of acetonitrile. ZIC-HILIC column shows the best results.

ZIC-HILIC column was tested even more, the method validation having been performed for quantification of related substances. The validated method is suitable for purity analysis of the mildronate drug substance.

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