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Method Article

Simultaneous analysis of betrixaban and hexazinone using liquid chromatography/tandem mass spectrometry in aqueous solutions

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A B S T R A C T

A new analytical method has been developed, optimised, and validated for simultaneous detection and identification of betrixaban, an oral anticoagulant drug approved recently by food and drug administration (FDA), and hexazinone, a broad-spectrum triazine herbicide, in aqueous media by liquid chromatography/tandem mass spectrometry (LC–MS/MS) using multiple reaction monitoring (MRM). The method was validated by the limit of detection (LOD), the limit of quantification (LOQ), linearity, matrix effect, recovery, precision, and accuracy. The recovery experiments were carried out on raw wastewater samples, spiked with these two compounds, using solid phase extraction (SPE). It is the first time that betrixaban has been analysed for environmental samples. The developed analytical method can be applied for more in-depth studies on the fate and occurrence of these compounds in the engineered and natural aquatic environment. The key benefits of the method described here are:

- It is highly suitable for betrixaban and hexazinone detection and quantification in aqueous samples.
- It can be successfully applied for understanding the fate of betrixaban and hexazinone in real-world water samples.
- Method validation revealed that the method is repeatable, robust, and accurate and can be expanded to include detection of other pharmaceuticals and pesticides in aqueous media.

A R T I C L E I N F O

Method name: A modified method for simultaneous detection of betrixaban and hexazinone in aqueous solutions using liquid chromatography-tandem mass spectrometry

Keywords: Betrixaban, Hexazinone, Liquid chromatography/tandem mass spectrometry, Wastewater, SPE, MRM, PPCPs, Pesticides

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Specifications Table

Subject Area:	Environmental Science
More specific subject area:	Environmental Science and Engineering
Method name:	A modified method for simultaneous detection of betrixaban and hexazinone in aqueous solutions using liquid chromatography-tandem mass spectrometry
Name and reference of original method:	Simultaneous quantification of different anticoagulants using liquid chromatography-tandem mass spectrometry Foerster, K.I., et al., Simultaneous quantification of direct oral anticoagulants currently used in anticoagulation therapy. J Pharm Biomed Anal, 2018. 148: p. 238-244.
Resource availability:	If applicable, include links to resources necessary to reproduce the method (e.g. data, software, hardware, reagent)

Method details

The analytical method developed in this study is a modification of the method developed by Foerster et al. for betrixaban on patient plasma samples [1]. We successfully developed this method using LC–MS/MS for the analysis of betrixaban and hexazinone in aqueous solutions. This is the first method reported in the literature which can be applied for detection of betrixaban in environmental samples. The novelty for simultaneous hexazinone detection lies with the combination of the LC column and the eluents used which have not been used for the quantification of hexazinone before.

Materials

Betrixaban and hexazinone were purchased from Medkoo Biosciences Inc., U.S., and Sigma-Aldrich, New Zealand, respectively. Fig. 1 shows the chemical structures of betrixaban and hexazinone. Acetonitrile and methanol (Sigma-Aldrich, New Zealand) were of HPLC/MS grade (99.9% purity). Ammonium formate (Sigma-Aldrich, New Zealand; $\geq$99% purity) was used to prepare the aqueous UPLC-MS/MS eluent. Ultra-pure water was obtained from a Milli-Q water purification system. All other chemicals used in this study were of analytical grade and commercially available.

Sample preparation

Glassware was cleaned using a laboratory detergent, then rinsed with tap water and ultra-pure water, and finally baked at 300 °C for about 3 h. Betrixaban and hexazinone stock solutions were prepared by dissolving 22.6 and 12.6 mg betrixaban and hexazinone powder in 50 mL methanol, respectively, to give concentrations of 452 and 253 mg/L, representing 1 mM of each compound. The stock solutions were stored in amber glass bottles in the freezer (−20 °C) until use. The samples and calibration standards were diluted from the stocks in water and methanol, respectively.

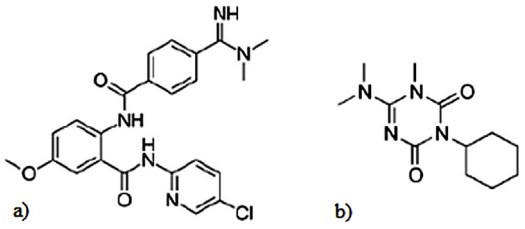

Fig. 1. Chemical structures of (a) betrixaban and (b) hexazinone.

Table 1
MRM parameters for betrixaban and hexazinone.

Compound	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
Betrixaban	7.8	452.20	324.20	−22
			279.2	−38
Hexazinone	7.6	253.15	171.05	−16
			71.10	−32

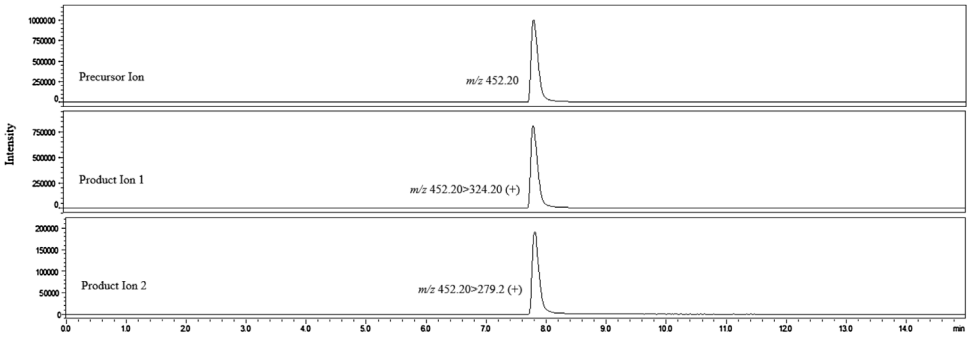

Fig. 2. Betrixaban chromatogram.

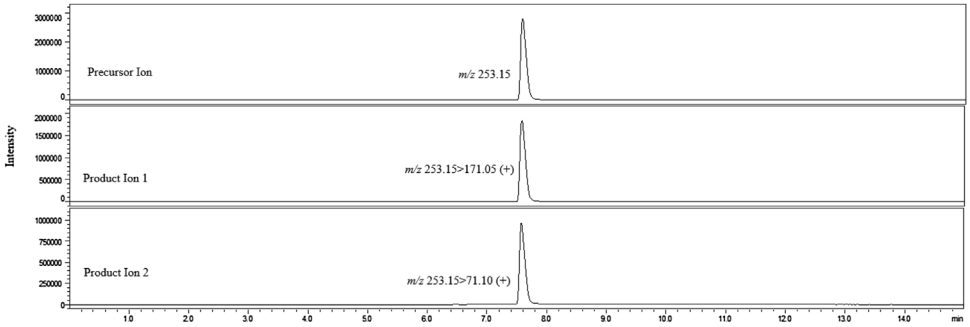

Fig. 3. Hexazinone chromatogram.

LC–MS/MS instrumentation

The quantification step was performed with a liquid chromatography-tandem mass spectrometer (LC–MS/MS) (Shimadzu Co., Japan) using multiple reaction monitoring (MRM). MRM parameters for betrixaban and hexazinone are shown in Table 1. The tandem mass spectrometer was operated in a positive electrospray ionization mode (+ESI) using external standards. Compounds were separated using a 2.1 × 100 mm, 3.5 μm, Eclipse Plus C18, Agilent column. The eluents consisted of 95% of 5 mM ammonium formate in water with 5% acetonitrile (mobile phase A) and 100% acetonitrile (mobile phase B). The injection volume was two μL, and retention times were 7.8 and 7.6 min for betrixaban and hexazinone, respectively. The LC–MS/MS chromatograms for betrixaban and hexazinone are shown in Figs. 2 and 3, respectively. Moreover, to show the clear separation of target compounds, the chromatogram of both compounds together is presented in Fig. 4. The mass spectra for betrixaban and hexazinone are also presented in Figs. 5 and 6, respectively.

The gradient program for identification of compounds was performed at a flow rate of 0.2 mL/min and started with 100% A and 0% B. Then the ratio was changed to 10% A and 90% B within 8 min. Within

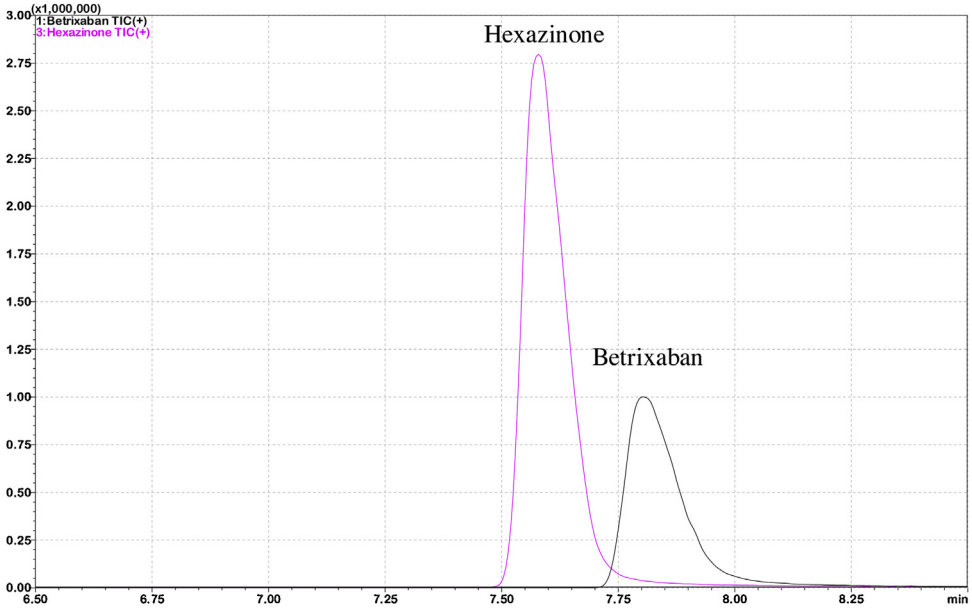

Fig. 4. Combined chromatogram of betrixaban and hexazinone.

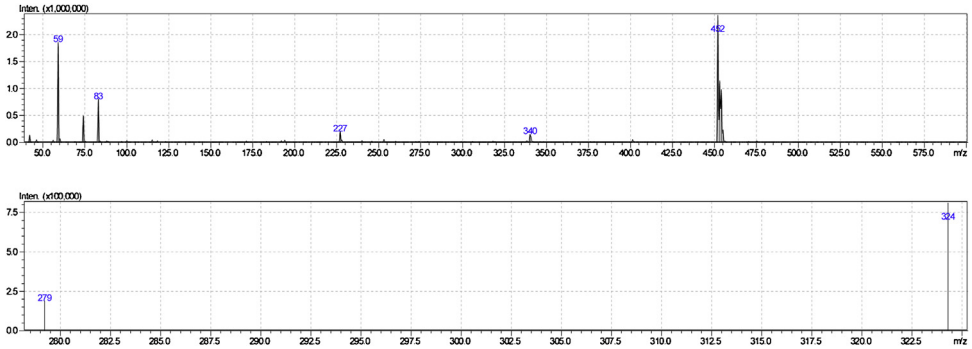

Fig. 5. Mass spectrum of betrixaban.

the next 2 min, the system returned to its initial conditions. The chromatographic gradient is shown in Table 2. Total run time was 15 min.

Method validation

The validation of the method was performed by the limit of detection (LOD), the limit of quantification (LOQ), the linearity of correlation coefficient (R^2), precision, accuracy, matrix effect, and recovery. The LODs and LOQs were calculated as the concentrations of analyte required to produce signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively [2]. The LODs and LOQs of spiked samples in a pure solvent (without any concentration factor) were estimated to be 0.3 and 0.9 μg/L for betrixaban and 0.2 and 0.8 μg/L for hexazinone, respectively. Furthermore, LODs and LOQs in spiked extracts of wastewater influent were estimated to be 1.6 and 4.9 μg/L for betrixaban and 0.5 and 1.4 μg/L for hexazinone, respectively.

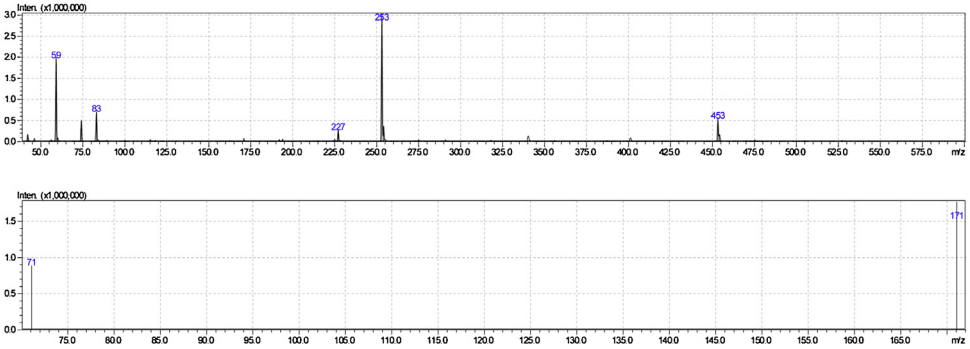

Fig. 6. Mass spectrum of hexazinone.

Table 2
Chromatographic gradient.

Time (min)	Solvent B (%)
0.01	0
1	0
8	90
10	0

Table 3
Calibration data, repeatability and inter-day precision of the target compounds analyzed by LC–MS/MS.

Compound	Calibration data		Inter-day precision[a] (R.S.D., %)		Intra-day precision[a] (R.S.D., %)	
	Equation	R^2	t_R	Peak area	t_R	Peak area
Betrixaban	Y = 17836.8X	0.9986	1.25	11	0.087	5
Hexazinone	Y = 4526.35X	0.9951	0.8	10	0.072	7.8

[a] Relative standard deviation of retention time and peak area; Y = detector response; X = concentration (μg/L).

The results from the LODs and the LOQs showed that the proposed method is sensitive, accurate, and reproducible for the determination of these two organic compounds in aqueous samples. Furthermore, it has been used for the degradation studies of betrixaban and hexazinone in aqueous samples (manuscript under preparation) and has been found to be an effective analytical method.

The calibration curves for both compounds were obtained by injecting standards at concentrations of 3.9, 7.8, 15.63, 31.25, 62.5, 125, 250, 500, and 1000 μg/L. These were made from the stock solutions as described above. For each analysis, a new calibration curve was generated. Good linearity and correlation coefficients ($R^2 > 0.99$) were achieved for the nine-point calibrations.

The retention time and area precision of the method were tested by the intra- and inter-day precisions. For the intra-day precision, different concentrations of standards (n = 7) ranged from 0.01 to 0.7 mg/L were analysed on the same day with six replicates. The inter-day precision was determined at seven concentrations by preparing and analysing at four different days. The results are presented in Table 3.

The robustness of the method was examined during the method development by testing different columns, mobile phases, and injection volumes for the analysis of both target compounds in standards. The C18 (2.1 × 100 mm, 3.5 μm) and Ascentis RP-Amide (10 cm × 2.1 mm, 3 μm) columns were tested and finally, C18 column with better peak intensities and lower retention times (7.8 and 7.6 *vs* 11.25 and 8.11 for betrixaban and hexazinone, respectively) was chosen for further experimentation. Moreover, the removal of ammonium formate from mobile phase A did not influence significantly the

peaks of hexazinone while it negatively affected the intensities of the betrixaban peak. Therefore, the rest of the experiments were carried out in the mobile phase (A) containing ammonium formate. Lastly, since 1, 2, and 3 μL injection volumes did not change the performance of the method significantly, 2 μL was chosen as an injection volume for further experimentation.

The developed method was applied to raw wastewater samples collected from an urban wastewater treatment plant in the North Island of New Zealand for recovery experiments. We carried out the experiments on raw wastewater as it is the extreme environmental water matrix with a very high concentration of dissolved organics and represents maximum interference to test the validity of the method. These experiments were carried out at pH 2 as aqueous samples are almost always acidified before extraction of pharmaceuticals and pesticides [3,4].

Matrix effect and recovery in wastewater samples were calculated with the following formulas [5]:

$$\text{Matrix effect } (\%) = \frac{B}{A} \times 100$$

$$\text{Recovery } (\%) = \frac{C}{B} \times 100$$

Where A, B, and C are the average peak areas in the standard, the wastewater sample spiked after SPE, and the sample extract spiked before extraction, respectively.

We also carried out the recovery experiments on ultra-high pure water. The recovery in ultra-high pure water due to the lack of matrix effect was calculated with the following equation [5]:

$$\text{Recovery } (\%) = \frac{\text{The average peak area of analyte sample spiked before extraction}}{\text{The average peak area of analyte external standard in pure solvent}}$$

However, it should be noted that for a complete evaluation of matrix effects of betrixaban and hexazinone in aqueous solutions, matrix effect should be evaluated for different aqueous matrices, including drinking water, natural waters, surface waters, effluent, and influent wastewater. However, it was not within the scope of the current study.

Extraction experiments

Before SPE, the wastewater was vacuum filtered through a 0.7 μm glass microfiber filter of 47 mm diameter (Whatman, China) using a Bücher funnel and filter flask and vacuum pump. The filtered wastewater (200 mL) was transferred into amber glass bottles and spiked with 1 mL of 100, 500, and 1000 μg/L of betrixaban or hexazinone in respective bottles. The extraction experiments in ultra-high pure water were carried out in duplicates while for wastewater, those were carried out in triplicates. 200 mL of unspiked wastewater was used as control. For SPE in ultra-high pure water, the filtration step was not required.

Extraction procedure

1) The Oasis HLB (500 mg/6 mL) cartridges (Waters, Ireland) were placed into a 12-port vacuum manifold and were subjected to the vacuum of 5" Hg.
2) The cartridges were conditioned with 10 mL methanol and 10 mL of Milli-Q water.
3) The wastewater sample (200 mL) was loaded through the cartridges under vacuum at a flow rate of 1.2 mL/min
4) The cartridges were dried under vacuum for 30 min.
5) The pump was switched off, and centrifuge tubes were placed under the cartridges to collect the eluent.
6) 10 mL methanol was applied under vacuum.
7) The extracts were evaporated to 1 mL using a rotary evaporator concentrator (Thermo scientific, Savant RVT5105).
8) The samples were analysed using LC–MS/MS.

Table 4
Recoveries and matrix effects at pH 2.

Compound	Matrix effect (%) in wastewater influent	Recovery (%) in wastewater influent	Recovery (%) in ultra-high pure water
		Betrixaban	
100 (μg/L)	70 (±1)	91 (±3)	127 (±5)
500 (μg/L)	82 (±2)	96 (±38)	125 (±10)
1,000 (μg/L)	80 (±7)	114 (±46)	100 (±5)
		Hexazinone	
100 (μg/L)	45 (±16)	53 (±5)	124 (±24)
500 (μg/L)	55 (±1)	57 (±6)	113 (±18)
1,000 (μg/L)	60 (±11)	61 (±2)	98 (±9)

HLB cartridge used in this study showed the excellent capability of concentrating betrixaban and hexazinone. The recovery results from influent samples spiked at levels of 0.5, 2.5, and 5 μg/L for betrixaban and hexazinone are shown in Table 4. Moreover, these compounds were not detected in control samples (unspiked wastewater influent and ultra-high pure water samples). The recovery of hexazinone has been previously studied in different water matrices including natural waters, surface waters, and wastewater effluent samples and showed different recovery results (>70%) [6–8]. In the present study, spiked influent wastewater samples showed slightly lower recoveries (53%–61%). However, in ultra-high pure water samples, high recovery (>98%) was achieved for hexazinone. According to a study by Jordan et al. (2009), lower recovery in the real-world matrix, compared to the ultra-high pure water matrix, is due to the matrix interferences [9]. The use of other cartridges may improve the recovery of hexazinone in wastewater influent samples, but optimising SPE was not within the scope of this study.

In contrast, there is no SPE method in the literature that has been reported for betrixaban. However, similar to hexazinone, betrixaban extraction results showed higher recovery (>100%) for ultrapure water matrix than the recovery (>90%) obtained for influent wastewater samples. While the recovery in ultra-high pure water samples was very high at pH 2, recovery obtained at pH 7 was low (results not shown here). Therefore, pH is assumed to play an important role in recovery experiments by HLB cartridges for betrixaban. However, since acidification of wastewater samples is considered as the first step for analysis of PPCPs, recovery values at pH 2 are most relevant, and the discussion here is limited to pH 2 results.

Additional information

Pharmaceuticals and personal care products (PPCPs) and pesticides have been recognized as contaminants of emerging concern (CECs) due to their toxic, hazardous impacts on human and wildlife health [10]. In recent years, PPCPs have been detected in different aqueous environments such as surface water, groundwater, wastewater effluents, and even drinking water in trace or ultra-trace levels [3,4,11]. Pesticides, according to their targeted usage, are divided into subgroups, including herbicides, fungicides, insecticides, and bactericides [12]. Pesticides are widely used to control diseases, weeds, and insects in agriculture. They can migrate to different parts of the environment as a source of contamination through spillages, cleaning, leakages, and runoff [13]. The application of pesticides as an efficient solution for pests control is increasing globally, owing to intensive agricultural practices [14,15]. Therefore, the spillage of effluents containing high concentration levels of pesticides in water has been considered as one of the serious environmental problems. There is a need to reduce dependency on herbicides for forest management due to their detrimental effects on water quality [16].

Hence, the above-mentioned two classes of organic contaminants are a significant concern for the environment, and there is a need to improve their detection in environmental matrices. Betrixaban, recently approved by the FDA as an oral anticoagulant in 2017, is used on patients who are at risk of thromboembolic events [17]. The fact that betrixaban has the potential of being used in wide-scale

applications in the treatment, leading to the environmental presence, generates a need to understand its fate in the environment. Hexazinone, a broad-spectrum triazine herbicide, is the most common herbicide to control forest weeds, especially in New Zealand [15]. Therefore, it is important to develop a suitable method for their detection in environmental samples. Our study fills these research gaps through the development of a suitable method for the detection of both of these compounds simultaneously as described above.

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