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SHORT COMMUNICATION

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Direct injection analysis of oxypurinol and metformin in wastewater by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry

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Abstract

The increasing global prevalence of gout and diabetes has led to a rise in the use of their respective medications, allopurinol and metformin. These are excreted via urine as oxypurinol and metformin and are discharged into wastewater and the environment. Current environmental monitoring of those two polar chemicals requires labour intensive and potentially inefficient sample pre-treatments, such as using solid-phase extraction or freeze-drying. This study validated a sensitive and simple method using direct-injection LC–MS/MS for the simultaneous measurement of oxypurinol and metformin in wastewater. The final method utilised a hydrophilic interaction liquid chromatography together with simple filtration through 0.2 μm regenerated cellulose filter followed by dilution in acetonitrile with a dilution factor of 10. The developed method was validated with the limit of quantifications (LOQ) of 0.11 and 0.34 μg/L for metformin and oxypurinol, respectively. The new method was applied to 42 influent wastewater samples and 6 effluent samples collected from 6 Australian wastewater treatment plants. Both compounds were detected well above the LOQ at concentrations 29–214 μg/L in influent and 2–53 μg/L in effluent for metformin, and 24–248 μg/L in influent and 4–81 μg/L in effluent for oxypurinol, demonstrating its high applicability.

KEYWORDS
direct injection, HILIC, metformin, oxypurinol, wastewater

1 | INTRODUCTION

Gout and type 2 diabetes are common chronic diseases and challenging public health problems worldwide. Due to the prevalence of these two diseases, high volumes of allopurinol and metformin, first-line medicines for their treatment, are prescribed and consumed globally.[1] Consequently, the residues of those drugs after human consumption, oxypurinol, and metformin excreted unchanged, have been detected ubiquitously in the urban water cycle, especially in wastewater.[2,3] These residues eventually released into the environment, raising concerns about their potential effects.[4] Therefore, cost effective and robust methods to routinely monitor these compounds in wastewater are

needed to assess their emission and environmental loading and to aid researchers in estimating their consumption in different populations.[5,6]

Oxypurinol and metformin in wastewater have been commonly analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS).[7,8] But they have very low LogP values of −1.8 and −0.86, respectively, suggesting that these compounds may not be retained on common reversed-phase HPLC columns, which results in co-eluting matrix compounds and poor reproducibility in LC–MS/MS analysis (Table S1).[2] Thus, alternative chromatographic columns such as C18 columns with polar-modified endcapping could be a solution to separate and retain extreme polar chemicals. Moreover, a few studies have achieved good retention of metformin and oxypurinol using hydrophilic interaction liquid chromatography (HILIC) columns (Tables S1 and S2).[2,3,9,10] Methods using HILIC columns typically utilise sample pre-treatments such as solid-phase extraction (SPE) or freeze-drying for solvent exchange from the water-base to high organic proportions to achieve appropriate peak symmetry.[2] However, these sample pre-treatments add extra labour, time costs and potentially have lower recoveries, which limits their application.[9]

This study aimed to develop a simple and rapid sample pre-treatment for the highly polar oxypurinol and metformin in wastewater matrix before separating them by direct injection LC–MS/MS for their simultaneous determination. Different LC columns (C8, C18 and HILIC) were assessed for optimal retention of the target compounds, and sample pre-treatment was further developed. The optimised method was then applied to influent and effluent samples collected from Australian wastewater treatment plants (WTTPs).

2 | MATERIAL AND METHODS

2.1 | Chemicals and reagents

The standards of metformin, oxypurinol and their labelled standards (metformin-D6 and oxypurinol-13C) were purchased from Toronto Research Chemicals (Canada). Parent stock solution for native and internal standards mixes were prepared in methanol at 1000 μg/L, respectively. LC grade methanol and acetonitrile, and LC–MS grade acetonitrile were purchased from Supelco (Bellefonte, USA). Formic acid and acetic acid were purchased from Fisher Scientific (USA). Deionized water was produced by an in-house Milli-Q system (Millipore, 0.22 μm filter, 18.2 MΩ·cm^{-1}).

2.2 | Collection of wastewater samples

Wastewater samples (24 h composites) were collected from six Australian WWTPs. Specifically, seven consecutive daily wastewater influent samples were collected from each WWTP ($n = 42$) in April 2020, and one effluent sample was collected from each WWTP ($n = 6$) in August 2016. After collection, samples were immediately acidified using 2 M HCl and archived at −20°C until analysis.

2.3 | Instrumental method development

LC–MS/MS measurements were made on the Sciex 6500+ Qtrap coupled with a Shimadzu Nexera HPLC system (Kyoto, Japan), operated in ESI positive ionisation mode with scheduled MRM acquisitions. The method development and validation workflow is shown in Figure S1. For each analyte, two MRM transitions were monitored. Instrument and compound specific parameters were optimised for each compound (Table S3) All data were acquired and processed using Analyst 1.7.1 (Sciex) and MultiQuant 3.0.3 respectively.

After optimisation of the MS condition for each compound, chromatographic conditions were evaluated to obtain a narrow and symmetrical chromatographic peaks. All compounds were mixed at 10 μg/L in deionized water at pH 2, and the injection volume was set at 5 μl. The flow rate of the mobile phase was set at 0.4 ml/min, and the oven temperature was set at 40°C. Four different reversed phase C18 columns with polar modified endcapping (Luna Omega Polar C18; Luna Omega PS C18; Synergi™ Fusion-RP; Synergi™ Hydro-RP), 1 reversed phase C8 column (Luna® C8) and 2 HILIC columns (Luna® NH2; ACQUITY UPLC BEH Amide) were evaluated for chromatographic performance (Table S4).

For comparison purposes, all columns were tested with the same chromatographic parameters except the gradient of the mobile phase. This is due to the different mechanisms of separation between HILIC columns and reversed phase HPLC columns.[11,12] Gradient of mobile phase for C18 and C8 columns was described in Section S1. For HILIC columns, the gradient of mobile phase B was set as follows: 0–0.7 min: 100%B; 0.7–4.7 min: ramped down to 5%B; 4.7–8.0 min: 5%B; 8.0–8.5 min: ramped up to 100%B; 8.5–9.5 min: 100%B.

2.4 | Sample preparation optimisation

Three different sample pre-treatments (Procedure 1–3) were developed to match with different chromatographic conditions (Table S5). Based on the results from the chromatographic optimisation (Section 3.1), Procedure 3 worked best, showing narrow and symmetrical chromatographic peaks for both compounds and was thus selected for subsequent application. In detail, wastewater samples were defrosted and filtered through 0.2 μm regenerated cellulose syringe filters to remove suspended particles. Then 100 μl of the filtered wastewater samples were dissolved in 900 μl of acetonitrile and spiked with 10 μl (0.5 μg/ml) of labelled standard mixtures before instrument analysis.

2.5 | Method validation

The analytical method was validated for range and linearity, accuracy and precision, matrix effect, Limit of Detection (LOD) and Limit of Quantification (LOQ) by following the International Conference on Harmonisation (ICH) guidelines.[13] Detailed information for method validation process was described in Section S2.

TABLE 1 Retention time of metformin and oxypurinol in different types of columns

Mode	Column	Mobile phase	Estimated void time of the column[a] (min)	Metformin (RT, min)	Oxypurinol (RT, min)
Reversed phase	Luna® C8, 50 × 2 mm, 3 μm	A: 0.1% formic acid in deionized water B: 0.1% formic acid in methanol	0.25	0.33	0.72
	Luna Omega Polar C18, 50 × 2.1 mm, 1.6 μm		0.27	0.72	0.65
	Luna Omega PS C18, 50 × 2.1 mm, 1.6 μm		0.27	0.36	0.79
	Synergi™ Fusion-RP, 50 × 2.1 mm, 4 μm		0.25	0.43	0.81
	Synergi™ Hydro-RP, 150 × 3 mm, 4 μm		1.30	1.83	4.13
HILIC	Luna® NH2, 50 × 2 mm, 3 μm		0.25	0.37	-
	ACQUITY UPLC BEH Amide, 100 × 2.1 mm, 1.7 μm		0.22	3.18	1.06

[a]Calculated based on the provided column dead volume from the manufacturer and a flow rate of 0.4 ml/min.

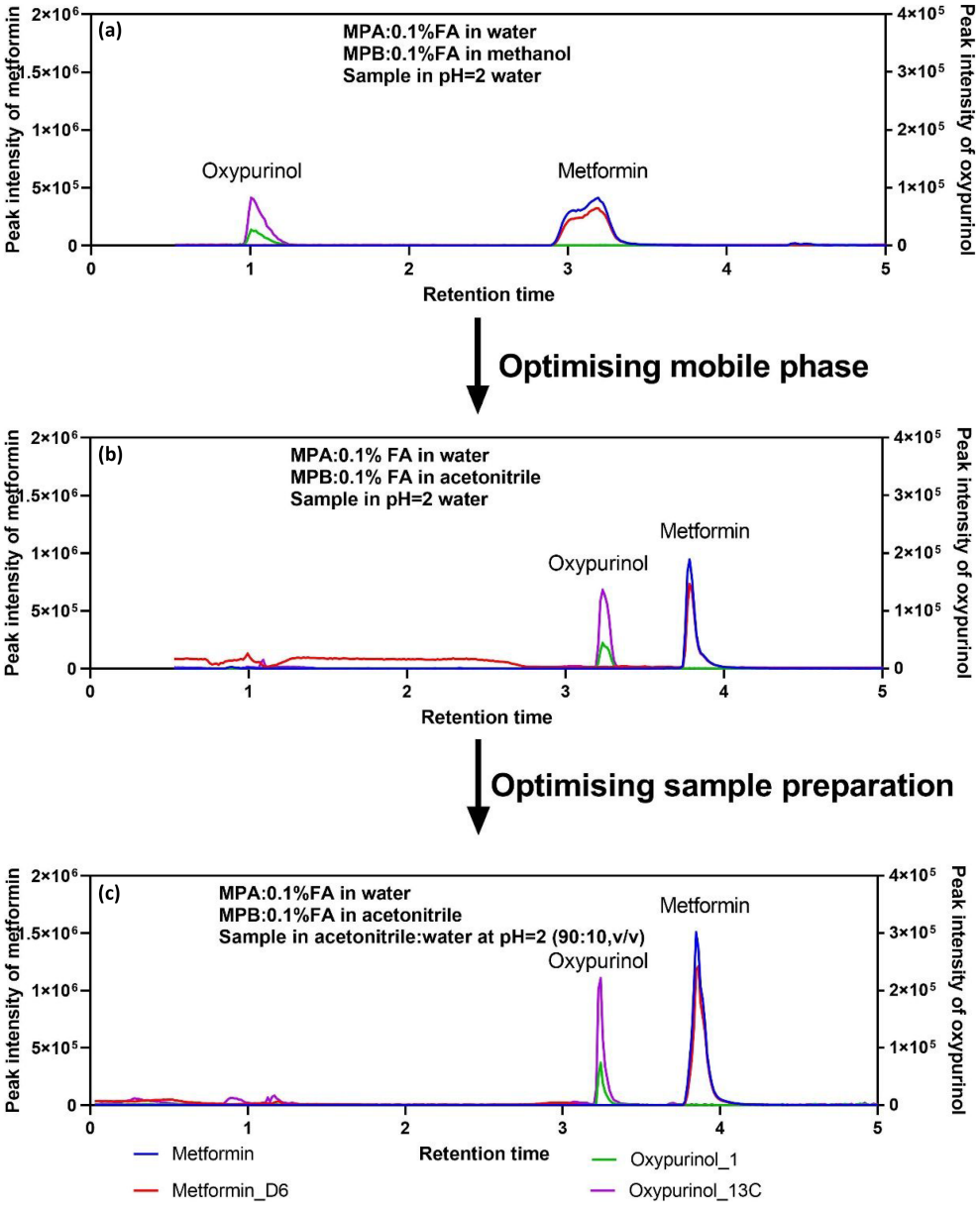

FIGURE 1 MRM chromatogram of metformin and oxypurinol (10 μg/L in deionized water at pH 2) in different mobile phases and sample diluent compositions using an ACQUITY UPLC BEH Amide column (MPA, mobile phase A; MPB, mobile phase B; FA, formic acid) [Colour figure can be viewed at wileyonlinelibrary.com]

2.6 | Quality assurance/quality control

The calibration curve, 2 quality assurance/quality control samples and 1 procedural blank were analysed at the beginning and end of the batch. A calibration point, a duplicate sample and a spiked sample were run every 10 samples. Detailed results of Quality Assurance/Quality Control are listed in Table S6.

3 | RESULT AND DISCUSSION

3.1 | Optimisation of chromatographic condition

3.1.1 | HPLC columns screening

Seven different HPLC columns were tested for the separation of metformin and oxypurinol following pre-treatment for Procedure 1 (Table 1, Figure S2). Either metformin or oxypurinol, or both, were poorly retained on the C8 column, NH2 column, and all C18 columns, which were evidenced by their retention times close to the void time of the columns, or poor peak shapes (Table 1 and Figure S2A–F). The use of a ACQUITY UPLC BEH Amide column for separation showed a good retention and separation for metformin and oxypurinol (Table 1). Oxypurinol was retained and separated at 1.06 min and metformin at 3.87 min (Figure 1a). However, the chromatographic peak of metformin was relatively wide. Such a distortion of peak shape was caused by the mismatch of the aqueous sample solvent and mobile phase (methanol) that impairs the interaction of metformin with the stationary phase. Hence, further chromatographic optimisation was required after the column screening.

3.1.2 | Optimisation of mobile phase and dissolution solvent

The composition of the mobile phases and sample diluents can strongly influence chromatographic peak shape when using HILIC columns.[14] To compare and better understand the effect of the mobile phase and dissolution solvent on the retention of metformin and oxypurinol in the ACQUITY UPLC BEH Amide column, different organic solvents (methanol, acetonitrile), a buffer (ammonium formate) during mobile phases and different proportions of aqueous and organic solutions in sample dilution were assessed (Aqueous; acetonitrile: deionized water, 90:10, v/v; methanol: deionized water, 90:10, v/v) (Table S7).

Best peak shape and ionization efficiency were achieved with 0.1% formic acid in deionized water and acetonitrile (Figure 1c). Correspondingly, sample preparation was optimised by following Procedure 3 as the pre-treated samples showed a more symmetrical peak shape (Figure 1c) than Procedures 1 or 2 (Figure 1b). Considering the high concentration of metformin and oxypurinol in wastewater such a dilution (10×) in sample preparation could still be sensitive enough to satisfy the detection limit of the method (Section 3.2).

TABLE 2 Method validation parameters using an ACQUITY UPLC BEH amide column for the determination of oxypurinol and metformin in wastewater

Compound name	Range (μg/L)	Linearity (R^2)	LOD (μg/L)	LOQ (μg/L)	Relative matrix effect (%)	Accuracy (%)			Intra-day precision (RSD,%)			Inter-day precision (RSD,%)		
						5 μg/L, $n = 3$	10 μg/L, $n = 3$	20 μg/L, $n = 3$	5 μg/L, $n = 3$	10 μg/L, $n = 3$	20 μg/L, $n = 3$	5 μg/L, $n = 3$	10 μg/L, $n = 3$	20 μg/L, $n = 3$
Metformin	0.1–50	0.997	0.04	0.11	−1.5	110	109	104	3.1	2.8	4.0	2.7	2.7	4.0
Oxypurinol	0.1–50	0.999	0.12	0.34	−0.3	104	102	99	2.4	3.5	4.5	3.0	3.0	4.5

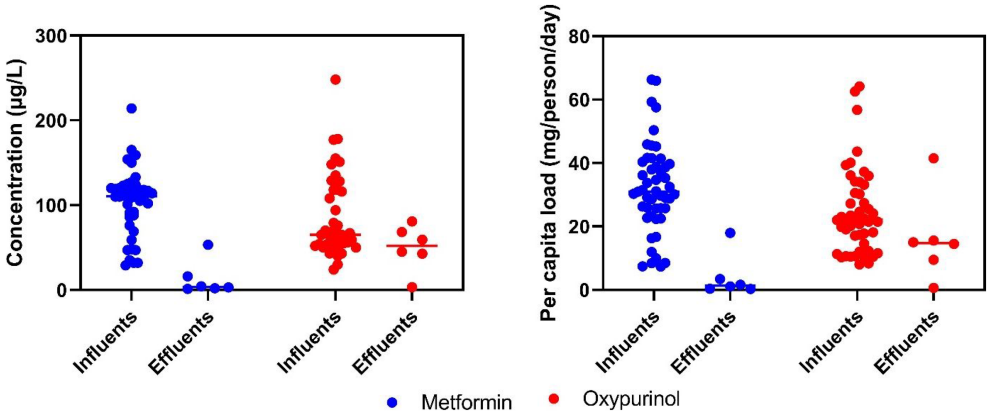

FIGURE 2 Concentrations and load of metformin and oxypurinol in influent and effluent wastewater samples in Australia [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Method validation

Method validation was carried out for the method selected above using the column ACQUITY UPLC BEH Amide, sample pre-treatment of Procedure 3 was followed. The calibration curve of both metformin and oxypurinol showed good linearity with correlation coefficients 0.997 and 0.999, respectively (Table 2). Regarding the method accuracy, both compounds showed accuracy ranging from 104% to 110% for metformin and 99% to 104% for oxypurinol at different spiking concentration levels in wastewater. The optimised analytical method showed less than 5% intra-day and inter-day variation in the analysis, indicating good reproducibility in the method. The matrix-matched calibration curves showed good correlation coefficients ($R^2 > 0.995$). LOD and LOQ were estimated as 0.04 and 0.11 µg/L for metformin, and 0.12 and 0.34 µg/L for oxypurinol, respectively (Table 2). Considering the low matrix effect of both compounds (<2%) and high concentration in wastewater samples, the LOQ in the current direct injection method was sufficiently sensitive for quantification of both oxypurinol and metformin in influent and effluent samples (Section 3.3).

3.3 | Application to wastewater samples

The validated method was applied to real influent and effluent wastewater samples collected from six Australian WWTPs. Oxypurinol and metformin were detected in all samples. Concentrations of influent samples ranged between 25 and 248 µg/L and 29 and 215 µg/L for oxypurinol and metformin, respectively. Their concentrations in effluent samples ranged from 4 to 80 µg/L and 2 to 53 µg/L, respectively (Figure 2).

The concentration of metformin in influent wastewater was similar to that reported in previous studies in Germany at 142 µg/L[15] and 109–250 µg/L,[9] lower than reported in Portugal at 325 µg/L,[16] higher than a recent study in China (0.16–50 µg/L).[5] The concentration of oxypurinol in the influent samples was within the same range as that reported for a recent study in Australia[6] but an order of magnitude greater than a previous study reported in Germany (2.8–26.6 µg/L). Metformin concentrations in the effluent samples in this study were higher than observed in Germany reported by Scheurer et al.[10] ranging from 2.2 to 21 µg/L and Oertel et al.[9] ranging from 0.50 to 1.37 µg/L.

The population normalised mass loads of oxypurinol and metformin were calculated from the concentrations, daily flow data and catchment population. The average per capita influent load of metformin and oxypurinol among WWTPs ranged from 32 to 48 and 13 to 43 mg/person/day, respectively. Per capita release of metformin and oxypurinol in effluent was estimated at 0.3 to 3.4 and 0.6 to 41 mg/person/day (Figure 2). Because the sampling date of influent samples is different from the effluent samples, we are unable to calculate the removal efficiency for the target compounds. In comparison with other studies, the estimated influent load of metformin is much higher than previously reported in the northeast of China.[17] The high influent mass load of metformin and oxypurinol observed in this study demonstrates high consumption of their parent medicines, metformin and allpurinol, in the Australian populations. Such observation fits well with the high level of prescription counts reported by the Australian Pharmaceutical Benefits Scheme and the prevalence of type 2 diabetes and gout in Australia.[18,19]

4 | CONCLUSION

A simple and sensitive direct injection LC–MS/MS method was developed and validated for the simultaneous determination of oxypurinol and metformin in wastewater samples. Among different HPLC columns, HILIC column was selected in combination with a specific gradient mobile phase and simple sample dilution with acetonitrile (1:10; v:v) to achieve appropriate separation and symmetrical peaks for these compounds. Our validation demonstrated the high selectivity, accuracy and precision of the method. The validated method was applied to Australian influent and effluent wastewater samples, with both compounds measured well above the detection limit. Compared with previous methods using SPE and freeze-drying, the new method can reduce labour and minimise inter-individual variation, allowing for high-throughput routine monitoring.

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SUPPORTING INFORMATION

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