

Analysis of Hypoxia-Inducible Factor Stabilizers by a Modified QuEChERS Extraction for Antidoping Analysis

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Abstract : An analytical method was developed for hypoxia-inducible factor (HIF) stabilizers based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) sample preparation and liquid chromatography–high resolution mass spectrometry analysis. HIF stabilizers potentially enhance the performance of athletes, and hence, they have been prohibited. However, the analysis of urinary HIF stabilizers is not easy owing to their unique structure and characteristics. Hence, we developed the QuEChERS preparation technique for a complementary method and optimized the pH, volume of extraction solvent, and number of extractions. We found that double extraction with 1% of formic acid in acetonitrile provided the highest recovery of HIF stabilizers. Moreover, the composition of the mobile phase was also optimized for better separation of molidustat and IOX4. The developed method was validated in terms of its precision, detection limit, matrix effect, and recovery for ISO accreditation. To the best of our knowledge, this is the first demonstration of the application of the QuEChERS method, which is suitable as a complementary analytical method, in antidoping.

Keywords : antidoping, hypoxia-inducible factor stabilizer, QuEChERS

Introduction

The World Anti-Doping Agency (WADA) annually announces a list of prohibited substances organized into various classes. Its list includes 12 classes and three forbidden methods of chemical and physical manipulation, gene doping, and blood and blood component manipulation.¹ A majority of newly added drugs have been included in established classes, but currently developed drugs are classified as specific groups owing to their unique medicinal effects or characteristics. Hypoxia-inducible factor (HIF) stabilizer is one of the new classes of prohibited drugs. Drugs of this class are believed to provide an alternative method for treating anemia and other ischemia-related diseases.² Additionally, HIF stabilizers are considered to activate genes related to hypoxic responses such as erythropoietin secretion.^{3–8} Hence, since 2011, they have been prohibited considering their potential performance-

enhancing effect of increasing oxygen transport capacity.⁹ HIF stabilizers are of great interest to WADA as the number of positive cases of HIF stabilizers has increased continuously since 2015, and the number of prohibited drugs has increased from 4 to 9. The number of prohibited HIF stabilizers have increased consistently, considering most of these drugs are still candidates for clinical use or are under development for clinical use.

Extensive research is being conducted on the analysis of excreted HIF stabilizers and their metabolites in urine.^{2,4,10–13} However, these drugs are known to be difficult to separate by using reverse-phase high performance liquid chromatography (HPLC) columns such as C18 columns. Although some successful analyses of HIF stabilizers have been reported, researchers have highlighted the problem of difficult of separation by using a C18 column.^{10,11} Against this background, the best strategy will be to realize an optimized column for HIF; however, this strategy will require an additional validated analytical method for only HIF stabilizers. In modern screening for antidoping analysis, hundreds of target compounds are monitored in a single liquid chromatography–mass spectrometry (LC–MS) run.^{13,14} Therefore, from the viewpoint of efficiency, it is desirable to include new compounds into an established method even if the separation efficiency or sensitivity is relatively low. For antidoping analysis, it is very important to establish a strategy to avoid false-negative or false-positive results, and the best way to achieve this goal may be to apply multiple analytical methods for cross-checking. Some

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substances can be analyzed by both gas chromatography-mass spectrometry (GC-MS) and LC-MS; however, establishing multiple instrumental analysis for hundreds of prohibited drugs and their metabolites will greatly inflate the cost. Therefore, adoption of complementary sample preparation methods with the same instrumental analysis may be a cost-efficient strategy. In this study, we selected a QuEChERS approach for cross-checking with solid phase extraction.

The QuEChERS method is widely used, especially for the analysis of pesticides in food. In recent years, its applications have been studied for various matrix and target compounds, such as pollutants in blood^{15,16} or breast milk.¹⁷ QuEChERS has some advantages compared to classical liquid-liquid extraction (LLE) as is evident from its name—QuEChERS stands for “quick, easy, cheap, effective, rugged, and safe.” Most importantly, only a small

volume of organic solvent is needed for extraction, and hence, the costs are low, and the preparation time is small. However, adoption of the QuEChERS approach for antidoping analysis has not been reported, majority of HIF stabilizers analysis have been conducted with SPE^{4,10,11} or dilute-and-inject^{12,13} method. In this study, we demonstrated and evaluated a sample preparation method based on QuEChERS for the HIF stabilizers analysis of human urine and optimized the parameters for extraction efficiency. Moreover, the mobile phase composition for HPLC separation using a C18 column was optimized, and method validation was performed according to ISO 17025 guideline. This study is possibly the first report of application of the QuEChERS approach for antidoping analysis, and it may be helpful to establish a complementary analytical method for cross-checking with other classical methods.

Table 1. Information of target hypoxia-inducible factor (HIF) stabilizers, including parameters for LC-MS analysis.

Name	Formula	Polarity	m/z	RT (min.)	Manufacturer	Structure
Daprodustat	C ₁₉ H ₂₇ N ₃ O ₆	-	392.183	7.5	Chemscene	
Desidustat	C ₁₆ H ₁₆ N ₂ O ₆	+	333.108	5.6	MCE	
FG-2216	C ₁₂ H ₉ ClN ₂ O ₄	-	279.018	5.6	Selleckchem	
IOX2	C ₁₉ H ₁₆ N ₂ O ₅	+	353.113	6.0	MCE	
IOX4	C ₁₃ H ₁₆ N ₆ O ₃	+	329.136	5.8	MCE	
JNJ-42041935	C ₁₂ H ₆ ClF ₃ N ₄ O ₃	-	345.001	5.6	MCE	
Methaqualone (ISTD)	C ₁₆ H ₁₄ N ₂ O	+	251.118	5.3	Sigma	
Molidustat	C ₁₃ H ₁₄ N ₈ O ₂	+	315.131	3.9	Chemscene	
Roxadustat	C ₁₉ H ₁₆ N ₂ O ₅	+	353.113	6.3	MCE	
Vadadustat	C ₁₄ H ₁₁ ClN ₂ O ₄	+	307.048	5.8	MCE	

Experimental

Chemicals and Reagents

Water and acetonitrile (ACN) were purchased from J. T. Baker Chemicals (Phillipsburg, NJ, USA), and formic acid (FA) was obtained from Wako (Osaka, Japan). Anhydrous magnesium sulfate and sodium chloride used for salting out were supplied by Sigma (St Louis, MO, USA). Information of the target HIF stabilizers is shown in Table 1, and the internal standard (methaqualone) was purchased from Sigma (St Louis, MO, USA). Target substances were prepared to 1 mg/mL of solution in methanol or dimethyl sulfoxide for stock solution, and they were diluted to 1 µg/mL in ACN for spiking the mixture. The internal standard solution was prepared using 2 µg/mL of ACN solution separately.

Sample Preparation by Modified QuEChERS

For the sample preparation, 2 mL of pooled human urine was used as the blank matrix. The pooled urine, 20 µL of the mixture solution, and 10 µL of the internal standard solution was mixed in a 15 mL PP tube. Subsequently, 1 mL of ACN with 1% FA was added and vigorously mixed with a vortex mixer. Subsequently, 1 g of MgSO₄ and

250 mg of NaCl were added for salting out and phase separation and were vigorously mixed for 1 min. The water/ACN phase was separated via centrifugation for 5 min at 3,200 g. The organic phase layer (upper layer) was transferred into a new tube with 50 mg MgSO₄, and fresh 1 mL of ACN with 1% FA was added into the aqueous (bottom) layer and mixed for secondary extraction. After centrifugation, the secondary extraction solvent was mixed to the first extract and transferred into a new glass tube, and 2 mL of the extract was dried using a N₂ evaporator for 7 min at 40°C. The dried extract was reconstituted with 200 µL of 2% ACN + 0.2% FA in water for LC–MS analysis.

LC–MS Analysis

The liquid chromatography–mass spectrometry analysis was performed with a similar setup as in a previous study.¹⁸ The samples were separated by an ultrafast liquid chromatograph (UFLC) XR series HPLC system (Shimadzu, Japan) and a Synchronis C18 column (100 × 2.1 (I.D.) mm, 1.7-µm particle size; Phenomenex, Torrance, USA) was applied with a guard column (2.1 mm I.D.). The injection volume was 10 µL for each sample. Mobile phase A and B comprised 0.2% aqueous FA and

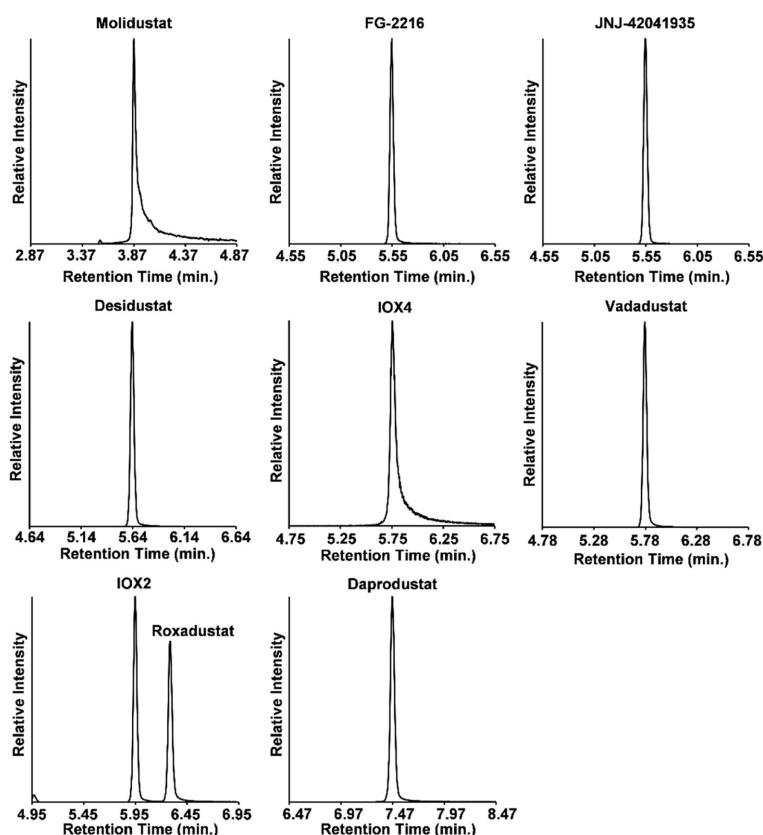


Figure 1. The representative chromatograms of HIF stabilizers. IOX2 and roxadustat were detected at equal *m/z*.

0.2% FA in ACN, respectively. Gradient elution was applied at a flow rate of 0.5 mL/min, and the 2% mobile phase B was held for 0.5 min, ramped to 95% B over 8.5 min, and then kept until 10.0 min. Subsequently, 2 min of re-equilibration for 2% B was applied. Therefore, the overall runtime was 12 min. For MS analysis, Q Exactive Plus tandem mass spectrometer from Thermo Scientific (San Jose, USA) was used. Both positive and negative ion modes were applied for each optimized ionization efficiency, and the capillary temperature was set at 300°C. The spray voltage was 4000 V (positive) and 3500 V (negative), and spectra acquisition were performed in the full scan mode. The *m/z* values and retention times of the target HIF stabilizers are listed in Table 1, and representative chromatograms were shown in Figure 1.

Results and Discussion

Optimization of QuEChERS Extraction

QuEChERS extraction is similar to the classic LLE

except that phase separation between the aqueous solution and miscible organic solvents such as ACN is realized by salting out with excessive salt. Therefore, we performed optimization under an identical critical condition as that for the LLE with regard to pH, volume, and number of extractions. In normal QuEChERS, the addition of absorbents, such as C18 or primary secondary amines for the removal of matrix (e.g., lipids) is important. However, we did not observe any significant interference by the urine matrix, and the absorbent decreased the recovery of target compounds (data not shown). Therefore, the addition of the absorbent was excluded in this study. The optimization of pH was proceeded with the extraction solvent (ACN) with additives: no additives (neutral), 1% FA (weak acidic, pH 3.2), 5% FA (strong acidic, 2.2), and 1% NH₄OH (basic, 9.8). The extraction efficiencies are shown in Figure 2. For all target compounds, 1% FA in ACN had the highest extraction efficiency—in particular, for molidustat, desidustat, and FG-2216, the efficiency was significantly high. This result is ascribed to the fact that most of the HIF

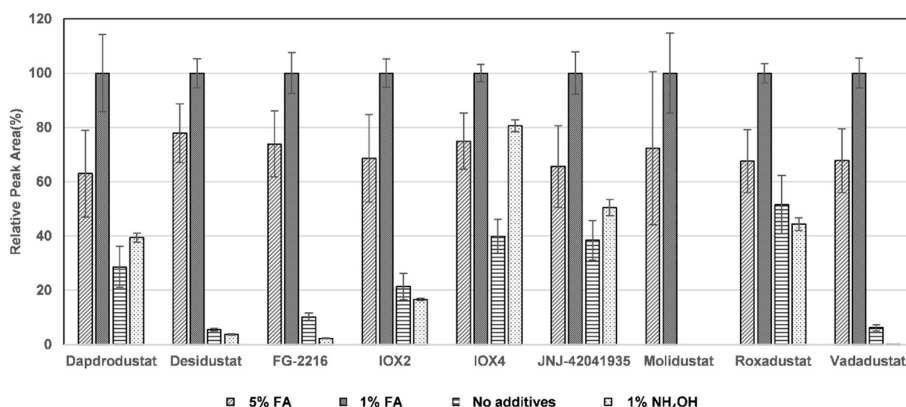


Figure 2. Comparison of the extraction efficiency by pH adjustment of the extraction solvent. The plot was normalized to the highest peak area for each compound.

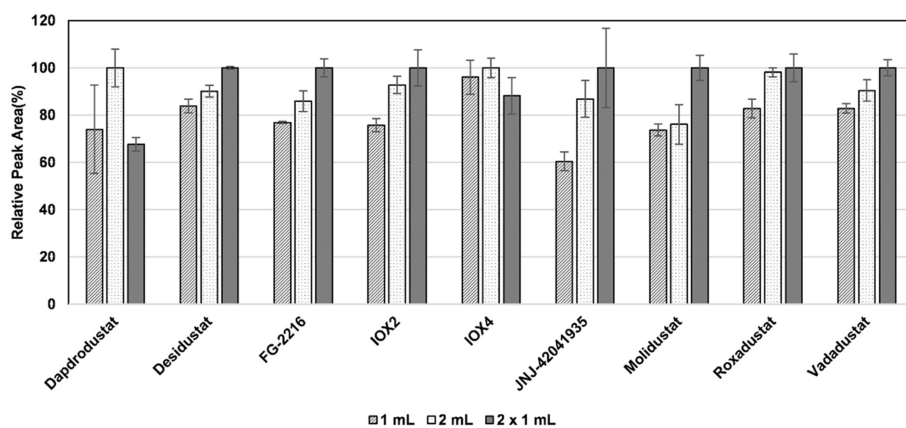


Figure 3. Comparison of the extraction efficiency by volume and number of extractions.

Table 2. Validation result for LOD, recovery, matrix effect, and precision.

Compound	LOD (ng/mL)	Recovery (%)	Matrix effect (%)	Intra-day precision (n = 7, %CV)			Inter-day precision (3 days, %CV)		
				low	mid	high	low	mid	high
Daprodustat	0.2	102.2	8.0	14.2	22.1	10.7	16.7	18.6	24.4
	0.2	93.2	48.4	7.9	7.6	6.8	11.3	14.3	7.3
	0.1	87.4	52.7	8.4	8.3	7.7	12.6	11.1	12.4
IOX2	0.5	94.2	21.8	11.7	12.4	8.0	11.3	15.1	15.8
IOX4	1.0	78.0	30.5	23.7	15.4	15.6	27.5	16.8	13.9
JNJ-42041935	0.5	91.5	18.1	10.8	12.3	9.6	17.0	20.4	15.6
Molidustat	1.0	73.2	15.1	28.1	23.5	12.6	32.0	25.5	12.4
Roxadustat	0.2	95.2	37.0	6.3	9.1	6.2	10.1	13.6	8.7
Vadadustat	0.1	95.3	42.0	7.6	7.2	6.5	9.5	9.1	7.4

stabilizers contain a carboxyl group, and hence, they can be protonated to an uncharged form under acidic condition. Therefore, uncharged HIF stabilizers were easily extracted by using an organic solvent, and the weak acid showed better efficiency than strong acid.

The volume of the extraction solvent and number of extractions were also tested. In this study, we tested both strategies with 1% FA in ACN. The results are shown in Figure 3. All substances except daprodustat and IOX4 followed the expected trend, and the efficiency of double extractions with low volume (2×1 mL) was better than that for single extraction with double volume (1×2 mL). When considering the differences between two extraction conditions, triple or more extractions did not result in better efficiency but resulted in longer time for drying the organic solvent or low reproducibility. Therefore, we set the extraction method to double extraction with 1 mL of the solvent for further analysis. By comparison with SPE method^{4,10,11} for HIF stabilizers, the sensitivity was similar, but required organic solvent volume was decreased to 20–65% and the preparation could be achieved with low cost.

Optimization of HPLC Separation

The major challenge in HIF analysis is the difficulty in separation using a reverse-phase HPLC column. However, separation by a reverse-phase column is essential for the simultaneous screening of numerous compounds by an established analytical method. Therefore, optimization was performed with a C18 column with various mobile phase compositions in this study. The base mobile phase was fixed to water/ACN for gradient elution based on previous studies.^{10,11} For the additives in mobile phases, different concentrations of formic acids or ammonium formate were tested from the perspectives of separation or ionization efficiency. Therefore, most substances showed satisfactory separation under our test conditions, but molidustat and IOX4 showed clear differences in the chromatogram with regard to the mobile phase composition. The chromatograms of both compounds under each condition are shown in Figure 4. In

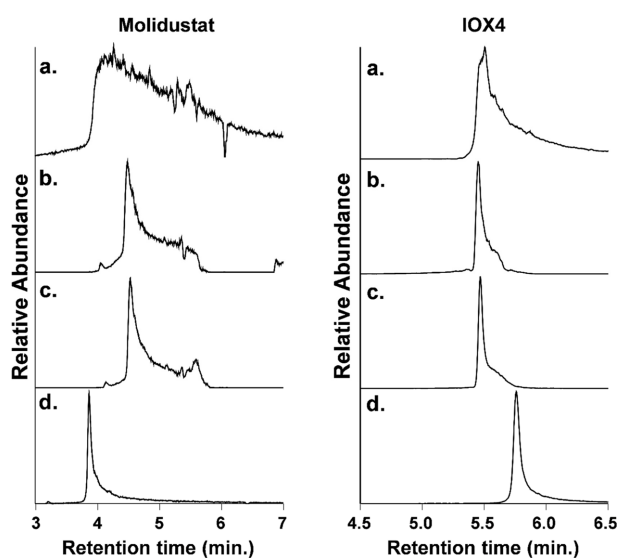


Figure 4. LC–MS chromatograms of molidustat and IOX4 in different mobile phase compositions. Each mobile phase composition as follows: a. 0.2% FA+10mM Ammonium formate in water / ACN b. 0.1% FA in water / ACN c. 0.1% FA in water / 0.1% FA in ACN d. 0.2% FA in water / 0.2% FA in ACN. Each chromatogram was normalized to relative abundance for easy comparison.

the case of molidustat, no peak was observed under the no-additive condition, and increased baseline was only observed when ammonium formate was used. When 0.1% FA/ACN was applied, a peak was observed, but the S/N ratio was very poor, and extreme band broadening was observed. The largest peak was observed when a mobile phase composition of 0.1% FA in both water and ACN was used; however, excessive peak tailing persisted. This problem was significantly solved at 0.2% FA for both mobile phases. Under this condition, the peak width (full width at half maximum) also decreased to approximately 0.1 min, which is suitable for screening for antidoping.

IOX4 also showed a trend similar to that of molidustat, and the largest peak was observed in 0.1% FA in both mobile phases, and the peak shape was improved when 0.2% of FA was added. Unlike other HIF stabilizers, the poor separation efficiency of molidustat and IOX4 may be induced by their unique structures, which contain a triazole group substituted with carbonyl and pyrazole. The detailed mechanism of their behavior in HPLC should be further studied.

Method Validation

The developed method was validated according to ISO17025 guidelines for qualitative analysis. The validated characteristics were limit of detection (LOD), precision, matrix effect, and recovery. In order to determine LOD, seven replicates of the urine samples were prepared and analyzed which has different seven points of concentration in a range of 0.1~10 ng/mL ($n = 7$). LOD was defined as the lowest concentration at which all seven samples can be detected with a signal to noise ratio of ≥ 3 . As a result, the LODs of the HIFs ranged from 0.1~1.0 ng/mL, and all substances showed equal to less than a requirement of WADA guidelines (1 ng/mL) for screening. The matrix effect was assessed by comparing peak area of HIF-spiked urine samples and same concentration of HIFs mixture solution diluted in solvent for reconstitution. Matrix effect was calculated using the following equation, and the results that were obtained ranged from 8.0~52.7%

$$\text{Matrix effect (ME)} = \frac{\text{Peak area of the analyte in urine sample}}{\text{Peak area of analyte of sample in solvent}} \times 100 (\%)$$

The matrix effect was relatively larger than other studies, and it would be caused by ionization suppression, which was induced by the absence of absorbent during extraction.

The recovery was evaluated by analysis of three replicates of HIF-spiked samples (QC sample) and the recovery sample in which the HIFs were spiked after extracting the pooled urine (recovery sample). The peak areas were determined to acquire the recovery values. The peak area of the QC samples was divided into those of the recovery sample. As a result, the recovery values ranged from 73.2~102.2%. It is considered that QuEChERS approach could be a novel method for drug extraction in urine matrix.

The precision was determined by analysis seven replicates of urine samples that contains HIFs at three different concentration (1, 2, 10 ng/mL) in three days ($n = 7/7/7$ and $21/21/21$). Intra-day and inter-day precisions were evaluated with relative standard deviation (% CV) of the ratio of peak area against internal standard. The values for intra-day precision ranged from 6.2~28.1%, whereas those for the inter-day precision ranged from 7.3~32.0%.

Conclusions

In this study, a new QuEChERS method—a novel, simple, and low-cost method was developed for antidoping analysis. The method was optimized for HIF stabilizers in human urine by extraction methods such as pH adjustment. The method validation results indicated its suitability for screening in antidoping analysis. This approach can play the role of a novel complementary method to avoid false-positives and false-negatives. However, further evaluation for various prohibited drugs and detailed optimization are required.

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