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# Using the Waters Forensic Toxicology Screening Application Solution With UNIFI to Determine Diuretics in Urine

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## APPLICATION BENEFITS

Expanded Forensic Toxicology Screening Application Solution with UNIFI,® enabling the detection and quantitation of negative ionising diuretics in urine.

## WATERS SOLUTIONS

ACQUITY UPLC® I-Class System (FTN)

Xevo® G2-S QTof Mass Spectrometer

ACQUITY UPLC HSS Column

Forensic Toxicology Screening Application Solution with UNIFI

## KEY WORDS

Diuretics, World Anti-Doping Agency (WADA), sports doping, urine, UPLC-QTof-MS,$^{E}$ UNIFI

## INTRODUCTION

Diuretics are a class of pharmaceutical compounds whose primary aim is to promote urine production. As such they can be used to treat a number of medical conditions including congestive heart failure and hypertension. In sports, diuretics can be abused by athletes to generate rapid weight loss or to maintain low body weight. They can also be used to mask the presence of banned or illegal substances by facilitating dilution of the urine and aiding excretion. Consequently the use of diuretics is prohibited for athletes by the World Anti-Doping Agency (WADA). The list of banned compounds can be found in the WADA technical document[1] and includes at least 25 compounds with diuretic properties. To ensure consistency of measurement amongst doping control laboratories, WADA defines the minimum required performance level (MRPL), which is the concentration of a prohibited substance that laboratories are expected to detect; currently this is set at 200ng/mL.[2]

The Forensic Toxicology Screening Application Solution with UNIFI currently comprises acquisition of accurate mass data on an orthogonal acceleration time-of-flight mass spectrometer, operating in MS$^{E}$ mode using electrospray positive ionisation mode (ESI+), followed by comparison of the data with a comprehensive library containing more than 1000 toxicologically-relevant substances.[3-5] However, as a number of the diuretics only ionise in negative electrospray mode (ESI-), the aim of the recent work was to further extend the Forensic Toxicology Screening Application Solution with UNIFI to include compounds that ionise in negative mode and to use the method to determine the presence of diuretics in urine, particularly at concentrations below the WADA MRPL.

## EXPERIMENTAL

### ACQUITY UPLC conditions

| | |
|---|---|
| UPLC System: | ACQUITY UPLC I-Class (FTN) |
| Column: | ACQUITY UPLC HSS $C_{18}$, 100Å, 1.8 µm, 2.1 mm x 150 mm, (p/n 186003534) |
| Vials: | Maximum Recovery Vials, 12 x 32mm, screw neck (p/n 186000327c) |
| Column temp.: | 50 °C |
| Sample temp.: | 10 °C |
| Injection vol.: | 10 µL |
| Flow rate: | 0.4 mL/min |
| Mobile phase A: | Water containing 0.001% formic acid |
| Mobile phase B: | Acetonitrile containing 0.001% formic acid |
| Gradient: | Isocratic at 87% A for 0.5 min then to 5% A at 4.5 min, hold for 1 min before switching to 87% A |
| Run time: | 7.5 min |

### $MS^E$ conditions

| | |
|---|---|
| MS system: | Xevo G2-S QTof |
| Ionization mode: | ESI- |
| Source temp.: | 150 °C |
| Desolvation temp.: | 400 °C |
| Desolvation gas: | 800 L/h |
| Reference mass: | Leucine enkephalin $[M-H]^-$ *m/z* = 554.2620 |
| Acquisition range: | *m/z* 50-1000 |
| Scan time: | 0.1 s |
| Capillary voltage: | 1.5 KV |
| Cone voltage: | 20 V |
| Collision energy: | Function 1: 6 eV |
| | Function 2: ramped 10 to 40 eV |

### Materials

Ioxinyl, for use as internal standard (ISTD), was purchased from Sigma-Aldrich (Poole, UK). A stock solution was prepared at 1 mg/mL in methanol and stored at -20 °C. Prior to use, the stock was diluted to 100 ng/mL in 0.001% formic acid.

All other chemicals used were of the highest grade available and stored according to the supplier's instructions.

Bio-Rad normal control urine was obtained from Bio-Rad Laboratories (Hemel Hempstead, UK).

### Sample preparation

Acetonitrile (0.1 mL) was added to 0.2 mL urine and ISTD (0.7 mL). The sample was vortex-mixed, for 5 min at 1200 rpm, and then centrifuged at 8000 *g* for 10 min. Supernatant was transferred to a Maximum Recovery Vial (p/n 186000327c).

## RESULTS

Sixteen diuretics that ionise in ESI- were included in this analysis and are listed in Table 1, along with their exact neutral mass and UPLC® retention times. The list includes five compounds that solely ionise in negative mode (bendroflumethiazide, benzthiazide, furosemide, hydrochlorothiazide, and hydroflumethiazide).

The acceptance criteria for a positive identification of each analyte was as follows: retention time to be within 0.35 min of reference and the observed precursor mass to be within 5 ppm of expected. For additional confirmation, a minimum of one diagnostic fragment ion had to be found in the high energy function.

| Analyte | Neutral monoisotopic mass | Retention time (min) |
|---|---|---|
| Acetazolamide | 221.9881 | 1.7 |
| Chlorothiazide | 294.9488 | 1.8 |
| Hydrochlorothiazide | 296.9645 | 1.9 |
| Hydroflumethiazide | 330.9908 | 2.4 |
| Chlorthalidone | 338.0128 | 2.6 |
| Furosemide | 330.0077 | 3.1 |
| Metolazone | 365.0601 | 3.2 |
| Benzthiazide | 430.9835 | 3.4 |
| Indapamide | 365.0601 | 3.4 |
| Cyclothiazide | 389.0271 | 3.5 |
| Bendroflumethiazide | 421.0378 | 3.5 |
| Canrenoic acid | 358.2144 | 3.4 |
| Xipamide | 354.0441 | 3.7 |
| Bumetanide | 364.1093 | 3.8 |
| Probenecid | 285.1035 | 3.9 |
| Tolvaptan | 448.1554 | 4.1 |

*Table 1. Analyte retention times and neutral mass.*

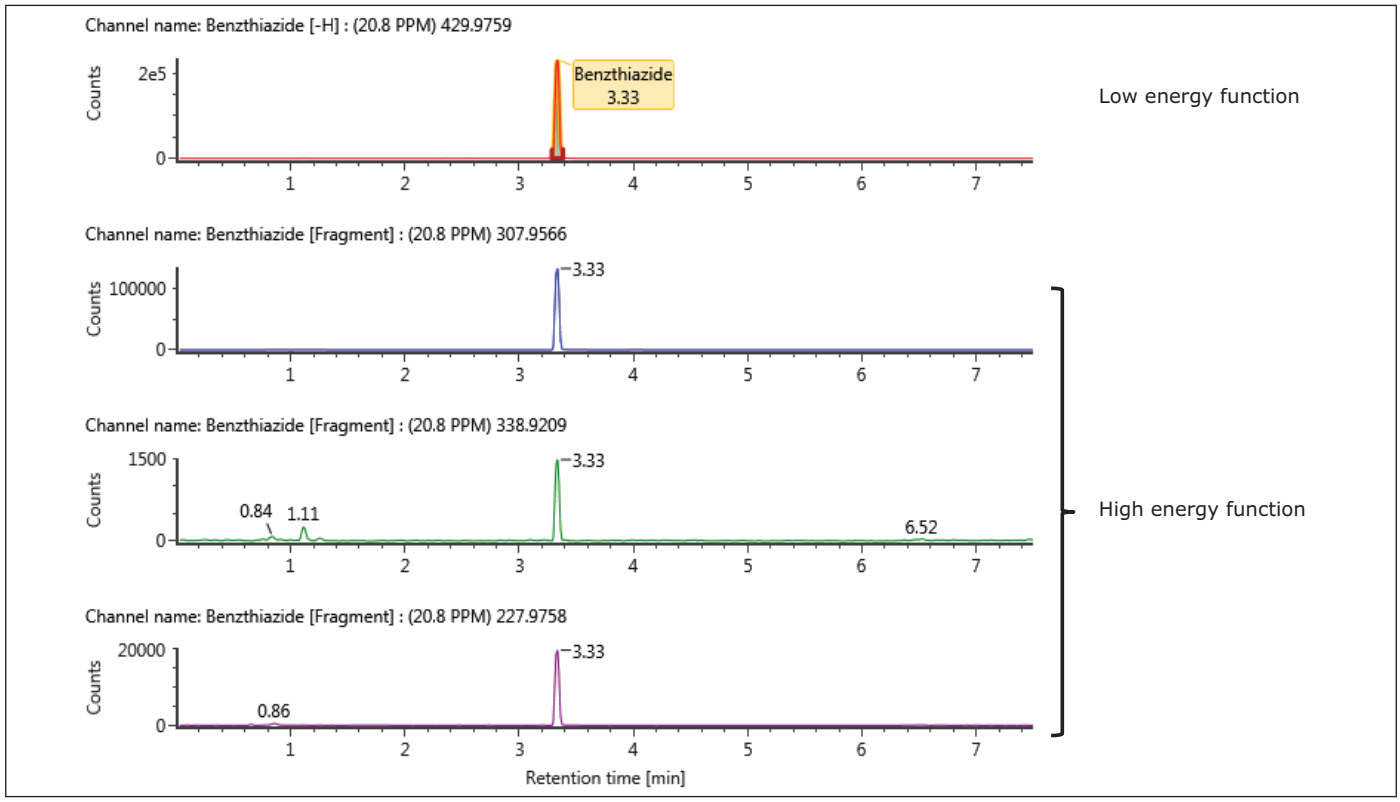


*Figure 1. Data for benzthiazide in a blank urine sample that has been spiked at the WADA MRPL (200 ng/mL); additional confirmation is achieved by the presence of three fragment ions in the high energy function.*

The utility of the $MS^E$ approach and associated fragment ion data generated under the higher energy condition is further demonstrated in Figure 2. The figure displays the high energy data for metolazone and indapamide which have identical elemental composition (C16H16ClN3O3S) and, under the chromatographic conditions employed here, are also closely-eluting i.e., within 0.2 min. Under these conditions it could be challenging to differentiate between the two diuretics; however the figure shows clear differentiatiation when the diagnostic fragment ions are taken into account.

To investigate linearity, control urine was spiked with the diuretics over a range from 0 to 2000 ng/mL and prepared, in duplicate, as described above. The response for each analyte trace was generated automatically during processing and referenced to the ISTD response. Semi-quantitative calibration curves were plotted using a 1/x weighting and a quadratic fit was applied to all the analytes. The correlation coefficient of determination was >0.99 for each analyte. A calibration curve from 0 to 2000 ng/mL for furosemide is shown in Figure 3. Urine spiked at the WADA MRPL was quantified against the relevant calibration curves; all analytes were positively identified at this level.

A comparison between the high energy fragments identified in an analytical standard and those identified in a blank urine sample spiked with furosemide is shown in Figure 4.

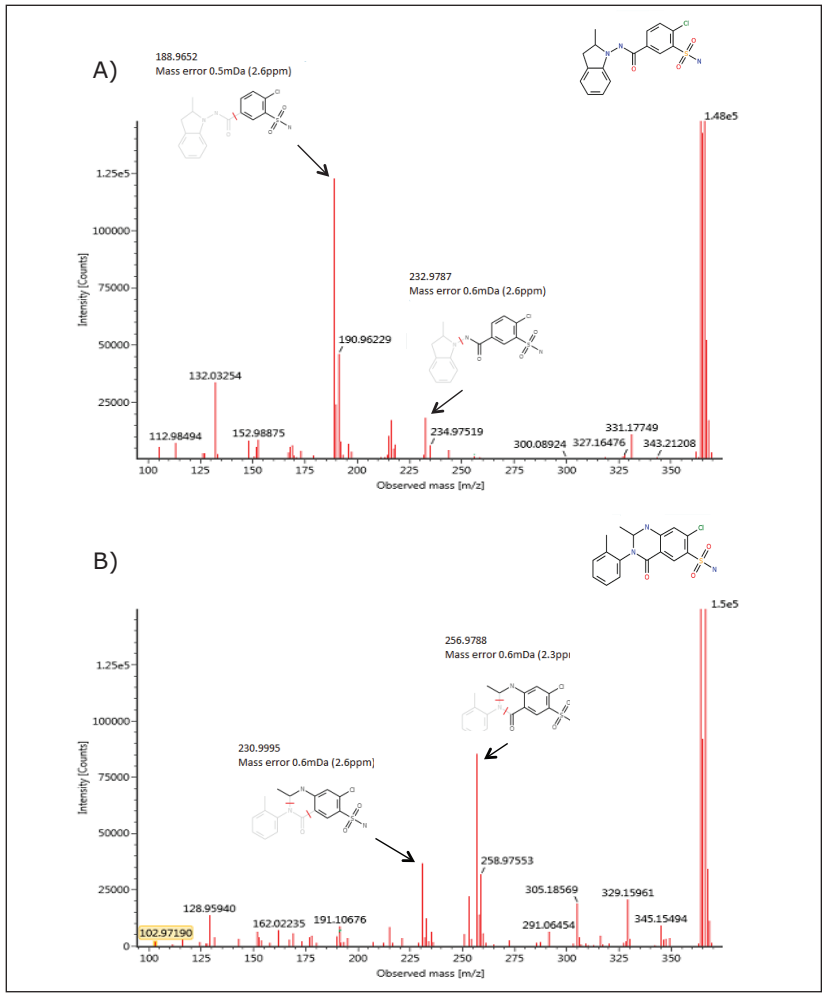


*Figure 2. Spectra showing the high energy data for indapamide (Figure 2A) and metolazone (Figure 2B) highlighting the differences in the high energy fragments detected.*

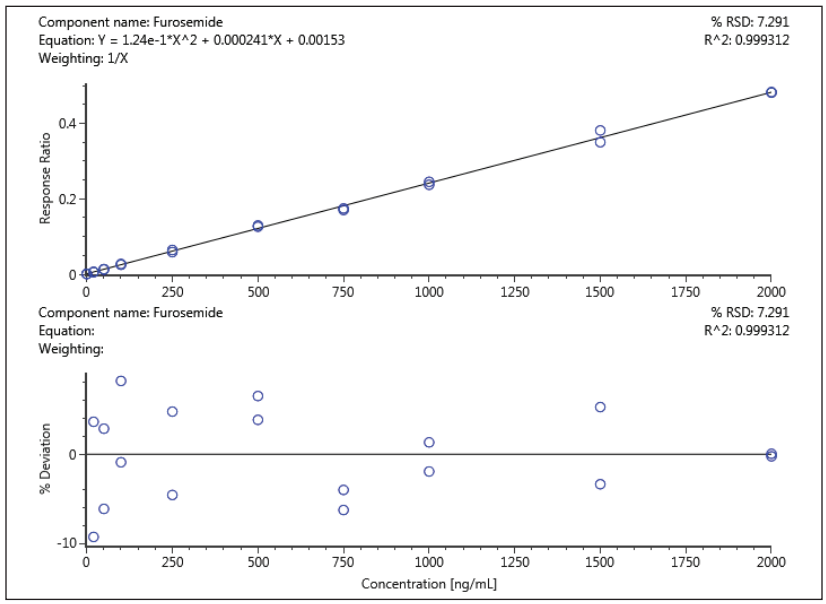


*Figure 3. A spiked urine calibration curve for furosemide over the range 0 to 2000 ng/mL using a quadratic fit with 1/x weighting applied.*

## Analysis of Authentic Urine Sample

An authentic urine sample was analysed following the sample preparation method described and was shown to contain the diuretic furosemide. Further investigation of the data using UNIFI's metabolite identification (Met ID) tools indicated the presence of both phase 1 and phase 2 metabolites. The retention times for the parent molecule and the most prevalent metabolite, a glucuronide conjugate, (2.69 min) are shown in Figure 5. The software highlights metabolic transformation with observed retention time along with observed *m/z* and the mass error in ppm.

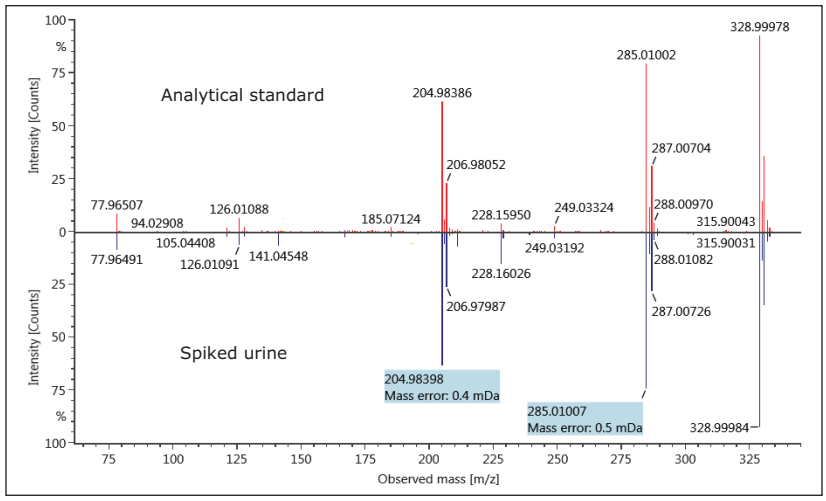


*Figure 4. Results of the binary comparison analysis for furosemide (the plot is scaled to the relative percentages of the identified fragments). The data have been generated using the binary compare tool in UNIFI and highlight the mass error between the standard (upper-trace) and the sample data (lower-trace).*

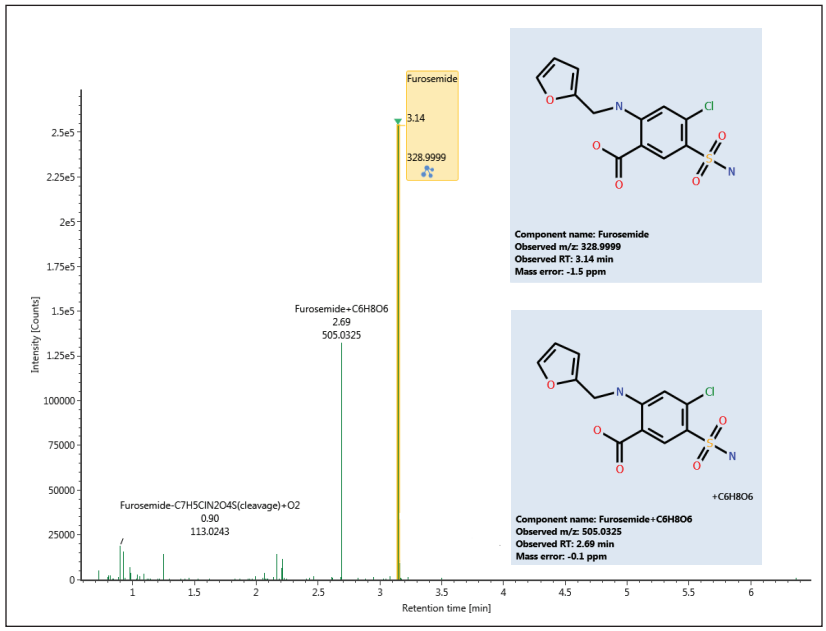


*Figure 5. A selection of UNIFI Met ID proposed metabolites (showing observed m/z, mass error in ppm, retention time, and transformation) detected in an authentic urine sample.*

## CONCLUSIONS

The rise of sports doping testing has highlighted the need for a quick, accurate, reliable, and robust method to initially screen large numbers of samples. Expanding the Forensic Toxicology Screening Application Solution with UNIFI to determine negative ionising compounds enables the determination of diuretics in diluted urine at levels which will allow this method to be applied to anti-doping labs that comply with the WADA guidelines.

The use of the binary compare and metabolite identification tools within UNIFI can increase the confidence in the data by highlighting high energy fragment matches and facilitates discovery of metabolites in the sample which are not present in the database. These metabolites can subsequently be added to the library.

## References


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3. Wood M. The Utility of $MS^E$ for Toxicological Screening. Waters technology brief. 2010. p/n 720005198en.
4. Forensic Toxicology Screening Application Solution. Waters Brochure. p/n 720004830EN.
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**A full validation by the user would be necessary prior to adoption in a laboratory.**

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