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An improved high-performance liquid chromatography–tandem mass spectrometric method to measure atrazine and its metabolites in human urine☆

Parinya Panuwet[a], Paula A. Restrepo[b], Melina Magsumbol[a], Kyung Y. Jung[a], M. Angela Montesano[a], Larry L. Needham[a], Dana Boyd Barr[a,*]

[a] Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Highway NE, Mailstop F17, Atlanta, GA 30341, USA
[b] Battelle Memorial Institute, Atlanta, GA, USA

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ABSTRACT

We report an improved solid-phase extraction-high-performance liquid chromatography–tandem mass spectrometry method with isotope dilution quantification to measure seven atrazine metabolites in urine. The metabolites measured were hydroxyatrazine (HA), diaminochloroatrazine (DACT), desisopropylatrazine (DIA), desethylatrazine (DEA), desethylatrazine mercapturate (DEAM), atrazine mercapturate (ATZM), and atrazine (ATZ). Using offline mixed-mode reversed-phase/cation-exchange solid-phase extraction dramatically increased recovery and sensitivity by reducing the influence of matrix components during separation and analysis. DACT extraction recovery improved to greater than 80% while the other analytes had similar extraction efficiencies as previously observed. Limits of detection were lower than our previous method (0.05–0.19 ng/mL) with relative standard deviations less than 10%. The total runtime was shorter (18 min) than the previous on-line method, thus it is suitable for large-scale sample analyses. We increased the throughput of our method twofold by using the newer extraction technique.
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1. Introduction

Atrazine (6-chloro *N*-ethyl-*N*′-[1-methylethyl]-1,3,5-triazine-2,4-diamine; CAS Number: 1912-24-9; ATZ) is the second most abundantly used herbicide in the United States [1]. ATZ degradation occurs by both biotic and abiotic processes in the environment through N-dealkylation, dechlorination, and ring cleavage. ATZ also transforms to hydroxy atrazine (ATZ-OH) and other dealkylated hydroxyl compounds commonly found in the environment [2]. ATZ and its degradation products tend to migrate out of the soil into water systems, including surface runoff to streams, rivers, lakes, and eventually into deep ground water systems and aquifers, creating a high potential for human exposure [3].

In mammals, ATZ undergoes complex metabolic alterations. Typically, ATZ is N-dealkylated by the hepatic cytochrome P450 system; either the parent compound or the N-dealkylated metabolite then conjugates to glutathione, after which the terminal amino acids on glutathione cleave and the N-acetylation occurs to form a mercapturic acid conjugate. ATZ metabolites include its dealklylation products (diaminochloroatrazine [DACT], desethyl atrazine [DEA], and desisopropyl atrazine [DIA]); hydroxylated metabolites (hydroxy atrazine [ATZ-OH], hydroxy-DIA [DIA-OH], hydroxy-DEA [DEA-OH], and ammeline); and mercapturatic acid conjugates (diamino atrazine mercapturate [DAAM], DEA mercapturate [DEAM], DIA mercapturate [DIAM], and ATZ mercapturate [ATZM]) [4–12]. These metabolites are excreted in urine along with less than 2% of unchanged ATZ [10,11].

Measuring potential metabolites is necessary to accurately assess exposure to atrazine and its degradates. As exposure scenarios vary from occupational to environmental exposure to ATZ itself and/or one or more of the dealkylated or hydroxylated environmental degradates could occur [13]. However, multi-residue analysis of ATZ and its related degradates in biological samples is a challenge. The diverse chemical structures of ATZ metabolites with differing chemical properties (e.g., polarity, pK_a), require analyses using advanced analytical instruments with both great sensitivity and selectivity. Moreover, several preparatory steps are necessary to separate the analytes from interfering substances in the biological sample; urine samples require the most cleanup. Because of these limitations, several analytical methods reported in the literature can selectively quantify only a few of the ATZ metabo-

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

* Corresponding author. Present address: Rollins School of Public Health, Emory University, 1518 Clifton Road, Atlanta, GA 30322, USA. Tel.: +1 404 727 9605; fax: +1 404 727 8744.
E-mail address: dbbarr@emory.edu (D.B. Barr).

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lites in biological samples. None of these methods could analyze a broad spectrum of potential ATZ metabolites in a single analysis [4,8,9,11,12,14–19].

Previously, we developed an on-line solid-phase extraction (SPE) with isotope dilution high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) to determine seven ATZ metabolites including its parent compound in human urine [20]. Unfortunately, the method was extremely vulnerable to matrix effects and produced poor recoveries for the highly polar compounds. Because of these drawbacks, we have improved the method efficiency by using mixed-mode reversed-phase/strong cation-exchange SPE with isotope dilution HPLC–MS/MS. This current extraction method can capture all target compounds while reducing or eliminating most of matrix interferences found in urine, which increases the overall sensitivity. This method, combined with the selectivity of tandem mass spectrometry, provides better results than our previous method.

2. Materials and methods

2.1. Chemicals

We used analytical grade solvents. Methanol was purchased from Tedia Company Inc. (Fairfield, OH, USA). We purchased formic acid from Fisher Scientific (Phillipsburg, NJ, USA). We organically and biologically purified deionized water with a NANOpure® Infinity UF from Barnstead International (Dubuque, IA, USA). We purchased nitrogen and argon with a minimum purity of 99.999% from Airgas Inc. (Radnor, PA, USA).

We obtained the native standards of ATZ and DACT from Chem Services (West Chester, PA, USA). We purchased ATZ-OH, DIA, and DEA and its isotopically labeled standard including DACT from Dr. Ehrenstorfe GmbH (Augsburg, Germany). ATZM and DEAM and its isotopically labeled standards were custom synthesized by Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Preparation of standard solutions and quality control materials

We prepared nine standard spiking solutions containing every analyte by serial dilution of the initial stock solutions with methanol to cover the concentration ranges of 0.025–10.00 μg/mL for ATZ-OH, DACT, DIA, DEA, and DEAM and 0.010–4.00 μg/mL for ATZ and ATZM. We also prepared the isotope-labeled standard spiking solution in methanol, giving an approximate concentration of the individual labeled compounds of 0.625 μg/mL for DIA, DEA, DEAM, ATZ, and ATZM and 1.250 μg/mL for ATZ-OH and DACT.

We prepared three quality control (QC) spiking solutions containing all analytes by serial dilution of the initial stock solutions with methanol concordantly with standard spiking solutions. The designated concentrations of these spiking solutions were: low level (QCL), 0.125 μg/mL for ATZ-OH, DACT, DIA, DEA, and DEAM and 0.050 μg/mL for ATZM and ATZ; medium level (QCM), 0.625 μg/mL for ATZ-OH, DACT, DIA, DEA, and DEAM and 0.250 μg/mL for ATZM and ATZ; and high level (QCH), 1.50 μg/mL for ATZ-OH, DACT, DIA, DEA, and DEAM and 0.600 μg/mL for ATZM and ATZ.

All standard stock and spiking solutions were dispensed into amber vials and stored at −5 °C until used. To prepare a calibration set and QC materials, 20 μL of each standard solution and isotope-labeled standard solution was added to each 1 mL of blank urine. Based upon this procedure, the fortified concentration ranges of a calibration set were 0.5–200 ng/mL for ATZ-OH, DACT, DIA, DEA, and DEAM and 0.20–80.0 ng/mL for ATZ and ATZM; the fortified concentrations of QC materials were 2.50 ng/mL, 12.5 ng/mL, and 30.0 ng/mL for ATZ-OH, DACT, DIA, DEA, and DEAM and 1.00 ng/mL, 5.00 ng/mL, and 12.0 ng/mL for ATZM and ATZ.

2.3. Urine collection and storage

All of the urine used for calibration plots, blank samples, and QC materials was collected from multiple, anonymous donors and combined, mixed overnight at 20 °C, and then pressure filtered with a 0.45 μm filter capsule (Whatman Inc., Florham Park, NJ, USA). Various concentrations of urine samples were measured to ensure that the urine pool was free of endogenous chemicals before the urine was used. Aliquots of approximately 20 mL of urine were transferred into capped vials and stored in a freezer at −20 °C.

2.4. Sample preparation and injection

Prior to sample preparation, 1 mL of urine was spiked with 20 μL of internal standard (equivalent to 12.5 ng/mL or 25.0 ng/mL in urine depending on target analytes) and homogenized. A sample was then diluted with 2 mL of 2% formic acid, well mixed, and subsequently loaded onto a mixed-mode reversed-phase/strong cation-exchange SPE cartridge (Strata X-C, 60 mg/3 cm^3, Phenomenex, Torrance, CA, USA) that was previously conditioned with 2 mL of methanol followed by 2 mL of water. The cartridge was then washed twice with 1 mL of 2% formic acid in 20:80 water:methanol to reduce the interfering components. The cartridge was dried by vacuum prior to elution with 2 mL of 5% ammonium hydroxide in methanol. The eluate was concentrated to dryness using a TurboVap LV Evaporator (Zymark, Farmingham, MA, USA); the water temperature was set to 40 °C, and nitrogen (15 psi pressure) was used as an evaporating gas. The dried residue was kept at −70 °C. The residue was reconstituted with 100 μL of 0.1% formic acid before injection; the total injection volume was 10 μL.

2.5. Sample analysis

We separated samples by chromatography using an Agilent 1100 HPLC system (Agilent Tech., Waldbronn, Germany) consisting of one quaternary pump, two degassers, an auto sampler, and a temperature-stable column compartment. We used ChemStation B. 01.03 software (Agilent Tech., Waldbronn, Germany) to program and control all the HPLC modules. The analytical column was Gemini C6-Phenyl (100 mm × 4.6 mm, 3 μm particle size, 110 Å pore size, Phenomenex, Torrance, CA, USA). We applied a stepwise gradient elution using 0.1% formic acid (A) and 0.1% formic acid in methanol (B) for optimum separation, while achieving a shorter run time.

2.5.1. Mass spectrometry operating conditions

We used a TSQ Quantum Ultra triple quadrupole mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with a positive mode atmospheric pressure chemical ionization (APCI) interface to perform sample analysis with these parameters: 4.5 μA corona discharge current, 350 °C vaporizer temperature, 25 psi sheath gas (N_2), 5 arbitrary units auxiliary gas (Ar), 1 arbitrary unit ion sweep gas pressure, 260 °C capillary temperature, and 1.5 Torr collision pressure. The mass spectrometer was programmed and controlled using Xcalibur software (ThermoFisher, San Jose, CA, USA).

We identified four precursor/product ion pairs and confirmed that they were the same as those reported in previously published work; we chose other precursor/product ion pairs for the remainder of the analytes. Within a month, we selected both the quantification and confirmation ions by monitoring the intensity, peak shape, background level, and potential interferences in different urine samples. Table 1 shows the summary of selected precursor/product ion pairs for each analyte, including the internal standards.

Table 1
Characterized precursor/product ion pairs, the optimum collision offset energy (CE), and the retention time (RT) for the native compounds and corresponding isotopically labeled analogs.

Compound	Precursor ion (M + 1)	Product ions			CE (V) (Q, C1, C2)	RT (min)
		Q	C1	C2		
Native						
ATZ-OH	198	86	69	114	26, 32, 22	2.90
DACT	146	79	68	110	15, 26, 17	3.34
DEAM	315	185	144	102	12, 23, 37	7.54
DIA	174	79	104	68	20, 31, 31	7.60
DEA	188	104	79	110	18, 24, 22	9.98
ATZM	343	214	102	174	21, 39, 27	11.46
ATZ	216	174	104	68	18, 24, 52	14.21
Labeled						
ATZ-OH (d5)	203	161	NA	NA	19	2.89
DACT (d3)	149	113	NA	NA	20	3.30
DEAM (cysteine-13C3; 15N)	319	186	NA	NA	20	7.50
DIA (d5)	179	137	NA	NA	20	7.58
DEA (d6)	194	144	NA	NA	21	9.92
ATZM (ring-13C3)	346	217	NA	NA	19	11.46
ATZ (d5)	221	179	NA	NA	19	14.19

Q = quantitative ion; C = confirmative ion; RT = retention time; NA = not applicable.

We created and applied multiple segments containing specific MRM tables to increase the sensitivity during MS/MS acquisition of compounds that eluted from the analytical column at different times. The first and the last segments were set to detect only improbable "phantom" precursor/product ion transition (200 → 100 @ 26) because the divert valve was set to send the mobile phase to waste and the only thing that would be collected by the multiplier was electrical noise.

2.6. Method validation

2.6.1. Limits of detection

We calculated the limits of detection (LODs) as three times the standard deviation of the noise at zero concentration (S_0) [21]. An estimate of the noise was based upon the variation in precision at concentrations close to the LODs. We estimated the LOD based upon standard injections then set our calibration standards to span this range. We used the three lowest calibration standards from 10 available validation and analytical runs. The inherent noise in the method at the low range would cause the imprecision that would allow us to estimate the precision at zero concentration.

2.6.2. Extraction efficiency

The extraction recovery of the method was determined at two concentrations for each analyte: 2.5 ng/mL and 30 ng/mL for all target analytes, except ATZ and ATZM, for which the concentrations were 1 ng/mL and 12 ng/mL. To begin this experiment, we spiked each of the five blank urine samples with the designated standard concentration, including the internal standard, and extracted according to the method (total $n = 10$). Meanwhile, we extracted 10 additional blank urine samples (spiked only with internal standard) concurrently. However, prior to the evaporation steps, each of the five additional extracts were spiked with the designated standard concentration to represent the 100% recovery. The samples were analyzed after they were evaporated and were reconstituted. We calculated the recovery by comparing the responses of the blank urine samples spiked before extraction to the responses of the blank urine samples spiked after the extraction.

2.6.3. Precision

We determined method precision by calculating the relative standard deviation (%RSD) of repeat measurements of samples from the QC materials at three different concentrations (2.50 ng/mL, 12.5 ng/mL, and 30.0 ng/mL for ATZ-OH, DACT, DEAM, DIA and DEA; 1.00 ng/mL, 5.00 ng/mL, and 12.0 ng/mL for ATZM and ATZ). We prepared and analyzed two samples from each of the QC materials daily during a 20-day period; the results were used to determine the inter-day precision ($n = 40$). We determined the intra-day precision by analyzing 10 samples from each level of the QC materials.

2.6.4. Accuracy and linearity

We determined the method accuracy by repeat measurements of spiked samples ($n = 20$) at two concentrations (1.00 ng/mL and 50.0 ng/mL for ATZ-OH, DACT, DEAM, DIA, and DEA; 0.40 ng/mL and 20.0 ng/mL for ATZM and ATZ) for 20 days. We calculated the deviation percent of observed mean concentration values against the nominal concentration values. We assessed the linearity of the calibration plot by determining an average r^2 value (20 analytical runs) of linear regression plots between nominal concentration values versus calculated concentration values of each target analyte across the entire range of calibration curve.

3. Results and discussion

In this study, we optimized the HPLC conditions, including the pH of mobile phases to achieve the best possible separation and retention for atrazine and its six metabolites in the same chromatographic system. Fig. 1 shows the elution profile of all target analytes in both a blank and spiked urine sample. We chose the quantification ion and the confirmation ions of each analyte based primarily on chromatographic behavior and intensity. We changed previously characterized precursor/product ion pairs of some compounds because our results indicated that those ions were heavily affected by the urinary matrix, primarily intermittent interferences. A summary of selected precursor/product ion pairs for each analyte, including its internal standard, is shown in Table 1.

In reversed-phase chromatography, retention and separation of compounds are strongly dependent upon the non-polar interactions with the column stationary phase and the composition of the mobile phase. Little information exists to determine whether pH plays an important role. Interestingly, we found that separation of particular atrazine metabolites from urinary matrix components, through multi-segment gradient conditions, was strongly influenced by the pH of the mobile phase. Fig. 2 shows the separation behavior of DACT, DEA, and ATZ at different pHs. When using non-acidic mobile phases, DEA showed the best separa-

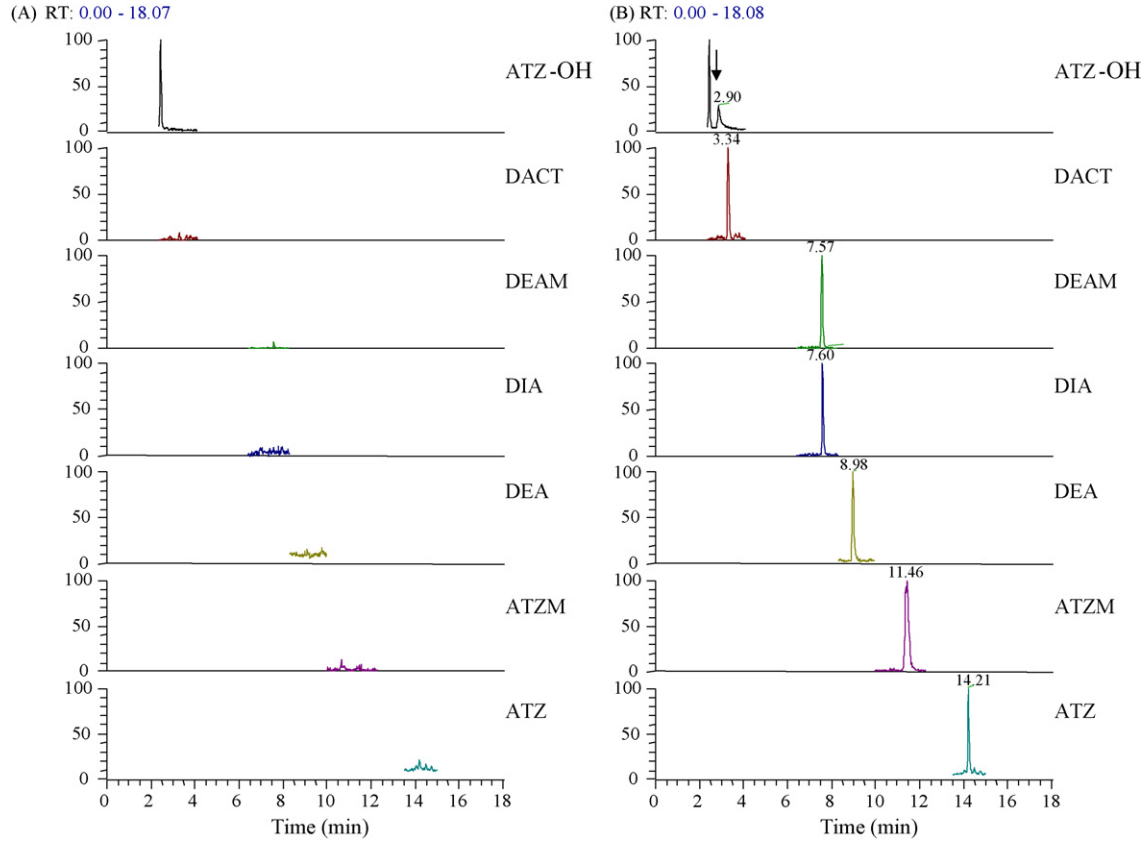

Fig. 1. Reconstructed ion chromatogram from a multiple reaction monitoring HPLC–MS/MS experiment showing the mass and chromatographic separation of the target analytes in a blank urine sample (A) and a urine sample spiked at 2.5 ng/mL for all analytes except for ATZM and ATZ which were 1 ng/mL.

tion from its interferents. However, this condition did not work well for other compounds, particularly ATZ, for which no separation occurred. Also, we observed an intensity reduction of mercapturate-conjugated compounds (DEAM and ATZM) because they are more completely ionized at acidic pH in the mass spectrometer source. Our results indicated that we obtained the best separation for all compounds using 0.1% formic acid in water and methanol at the mobile phase during multi-segment chromatographic separation.

We also found that separating target analytes becomes ineffective if the analytical column was overloaded with matrix components due to multiple injections of samples. We found that

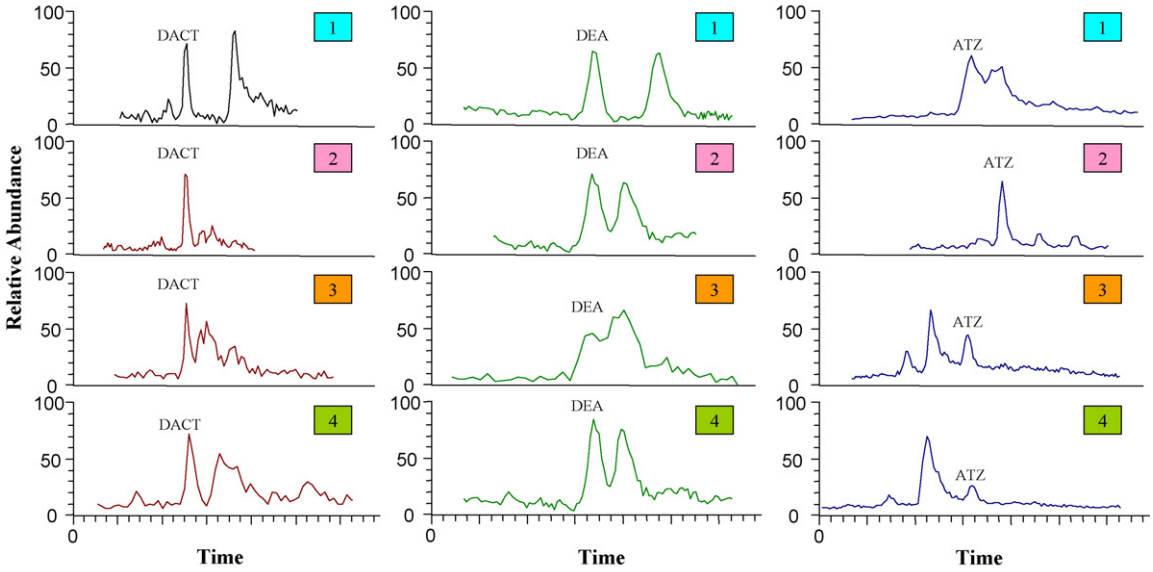

Fig. 2. The effect of pH of the mobile phase on the separation of DACT, DEA, and ATZ from interfering components (1 = water:MeOH; 2 = 0.1% formic acid in water:MeOH; 3 = 0.2% formic acid in water:MeOH; 4 = 0.5% formic acid in water:MeOH).

Table 2
Intra- and inter-day precision of the method.

Analyte	Precision (% relative standard deviation)					
	Intra-day (n = 10)			Inter-day (n = 40)		
	Low level	Medium level	High level	Low level	Medium level	High level
ATZ-OH	5.00	6.14	2.61	5.56	2.88	4.75
DACT	4.28	2.54	1.58	7.70	2.95	3.05
DEAM	4.13	2.37	2.86	5.33	5.31	3.27
DIA	3.47	2.77	1.82	4.58	1.98	5.92
DEA	2.15	3.23	2.32	9.02	2.93	5.06
ATZM	2.51	2.32	2.22	4.49	1.81	5.98
ATZ	2.87	2.93	3.13	3.97	2.83	3.59

Low level was 2.5 ng/mL for all analytes except ATZM and ATZ which were 1 ng/mL. Medium level was 12.5 for all analytes except for ATZM and ATZ which were 5 ng/mL. High level was 30 for all analytes except for ATZ and ATZ which were 12 ng/mL.

column cleaning and regeneration should be done periodically using 20:80 water and methanol solution to achieve optimal separation.

Apart from optimizing the HPLC separation, another major improvement of this method was better extraction recoveries, which led to elevated overall intensity and better LODs. Our previous method employed an on-line reversed-phase extraction, which limited the retention of highly polar compounds, especially DACT, and was vulnerable to matrix effects. We resolved this problem by applying mixed-mode SPE cartridges that allowed highly polar compounds and less polar compounds to be retained through strong cation-exchange mode and π–π interactions, respectively. The advantages of using a strong cation-exchange cartridge are that protonated compounds [H^+] are retained more strongly and non-polar matrix contaminants are eliminated completely when an acidified organic solvent wash is used. This is especially true for ATZ-OH; its matrix contaminant was significantly reduced when the cartridge was washed with 2% formic acid in 80:20 water:methanol. While removing unwanted interferences, we also observed a significant reduction in overall ion suppression from the matrix components. The overall extraction efficiencies of all target analytes are summarized in Fig. 3. DACT recovery was greatly improved compared with our previous method (68% to >86%). For other compounds, results indicated that the extraction recoveries were as good as the previous method.

Method accuracy and precision are also as good as or better than previously reported values. The labeled internal standard for ATZ-OH is now used and significantly reduces %RSDs (<10%). Tables 2 and 3 summarize the precision and accuracy of this method, respectively. In general, accuracies (also called relative recoveries) ranged from 98% to 102% for all analytes with <2% variation. Our method precision was expressed as the %RSD values that ranged from 1.58% to 6.14% for intra-day variation and from 1.81% to 9.02% for inter-day variation. All reported RSD values were <15%, which is desired in bioanalytical analysis [22]. The results of linearity evaluations are summarized in Table 3. The correlation

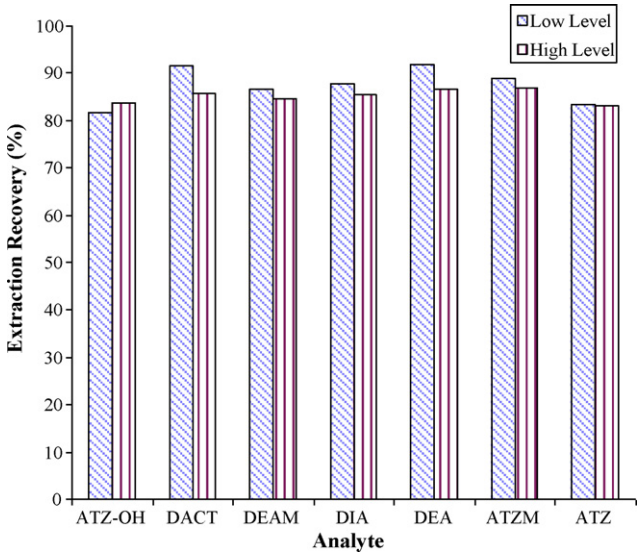

Fig. 3. Urinary extraction recoveries of all analytes at two different concentrations (1.00 ng/mL and 30.0 ng/mL for ATZ-OH, DACT, DEAM, DIA, and DEA; 0.40 ng/mL and 12.0 ng/mL for ATZM and ATZ).

coefficients of the calibration curves of all target compounds were >0.99 with errors about the slope of <3%, indicating the excellent quality of the calibration curves used for quantification.

Table 3 also summarizes the LODs of this method for individual analytes. All of our reported LODs are <1 ng/mL, which is sufficient for measuring concentrations in biological samples resulting from environmental exposures [23]. Compared to our previous method, better LODs are the result of an improved extraction method, better separation, and reduced matrix effects.

We achieved overall method efficiencies as well as improved method throughput. As a result of shorter run time, we can now

Table 3
Method accuracy, linearity, and limit of detection (LOD).

Analyte	Accuracy				r^2	Linear calibration range (ng/mL)	LOD (ng/mL)
	Low level (n = 20)		High level (n = 20)				
	Mean ± standard deviation (ng/mL)	Relative recovery (%)	Mean ± standard deviation (ng/mL)	Relative recovery (%)			
ATZ-OH	1.01 ± 0.08	101	49.8 ± 0.96	99.6	0.999	0.5–200	0.14
DACT	0.98 ± 0.14	98.0	50.0 ± 0.97	100	0.999	0.5–200	0.19
DEAM	1.01 ± 0.07	101	49.9 ± 1.26	99.8	0.999	0.5–200	0.09
DIA	0.98 ± 0.07	98.0	50.0 ± 1.45	100	0.999	0.5–200	0.12
DEA	1.01 ± 0.10	101	50.6 ± 1.03	101	0.999	0.5–200	0.10
ATZM	0.41 ± 0.03	102	20.2 ± 0.39	101	0.999	0.2–80	0.08
ATZ	0.40 ± 0.02	100	19.9 ± 0.46	99.5	1.000	0.2–80	0.05

measure ATZ and its related metabolites in 36 unknown urine samples, a standard calibration set, and QC materials in one analytical run. We have analyzed thousands of samples collected as a part of the National Health and Nutrition Examination Study (NHANES) [24] and would frequently detect many of the analytes. As is the policy of the NHANES study, the data will be released publicly this year, at which time, we can further examine the results. This indicates that our method is suitable for large epidemiologic studies that require analysis of numerous urine samples.

4. Conclusions

In summary, the significant improvements of this method compared to our previous one include better extraction recoveries, better accuracy and precision, and a shorter run time. Based upon the new LODs obtained, this method can be used to investigate environmental exposure to atrazine and its metabolites that result in low urine concentration (<1 ng/mL).

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