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A Dilute-and-Shoot Flow-Injection Tandem Mass Spectrometry Method for Quantification of Phenobarbital in Urine

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A dilute-and-shoot flow-injection tandem mass spectrometry method for quantification of phenobarbital in urine

RATIONALE: Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is the gold standard of urine drug testing. However, current LC-based methods are time consuming, limiting the throughput of MS-based testing and increasing the cost. This is particularly problematic for quantification of drugs such as phenobarbital, which is often analyzed in a separate run because they must be negatively ionized.

METHODS: This study examined the feasibility of using a dilute-and-shoot flow-injection method without LC separation to quantify drugs with phenobarbital as a model system. Briefly, a urine sample containing phenobarbital was first diluted by 10 times, followed by flow injection of the diluted sample to mass spectrometer. Quantification and detection of phenobarbital were achieved by an electrospray negative ionization MS/MS system operated in the multiple reaction monitoring (MRM) mode with the stable-isotope-labeled drug as internal standard.

RESULTS: The dilute-and-shoot flow-injection method developed was linear with a dynamic range of 50–2000 ng/mL of phenobarbital and correlation coefficient > 0.9996. The coefficients of variation and relative errors for intra- and inter-assays at four quality control (QC) levels (50, 125, 445 and 1600 ng/mL) were 3.0% and 5.0%, respectively. The total run time to quantify one sample was 2 min, and the sensitivity and specificity of the method did not deteriorate even after 1200 consecutive injections.

CONCLUSIONS: Our method can accurately and robustly quantify phenobarbital in urine without LC separation. Because of its 2 min run time, the method can process 720 samples per day. This feasibility study shows that the dilute-and-shoot flow-injection method can be a general way for fast analysis of drugs in urine. Copyright © 2016 John Wiley & Sons, Ltd.

Pain management drugs are among the most commonly prescribed drugs and yet also the most abused drugs.[1–4] Therefore, it has become a general medical practice to monitor patients who are taking pain management drugs, for adherence to treatment as well as to detect presence of other illicit substances and unprescribed drugs. The urine drug test (UDT) is the current clinical procedure for such patient monitoring, and there are millions of UDTs performed each year.[5–8] Recently, liquid chromatography/tandem mass spectrometry (LC/MS/MS) has emerged as the gold standard UDT for quantification of pain management drugs in urine.

A routine UDT screening panel consists of more than 50 commonly prescribed pain management drugs along with other abused molecules, among which only ethyl glucuronide (EtG, a metabolite biomarker for alcohol abuse) and barbiturates (commonly abused drugs) must be negatively ionized,[9–15] while the rest of them are positively ionized. Moreover, although both of them are negatively ionized, they must be analyzed in different LC runs because EtG is highly polar, while barbiturates are hydrophobic. Therefore, three separate LC/MS/MS runs[16–19] are generally carried out to identify/quantify the whole panel of pain management drugs

and illicit drugs in a routine clinical operation. This means that a single LC/MS/MS run operated in the positive ionization mode simultaneously analyzes the overwhelming majority of the drugs, and two additional separate LC/MS/MS runs are needed just to analyze EtG and barbiturates. The equipment needed for LC/MS/MS is expensive, requiring HPLC separation along with the MS/MS detection of drug molecules. Compared to MS/MS, HPLC has a much longer run time to separate different molecules and reduce interferences, becoming the bottleneck of LC/MS/MS. Clearly, the general practice of running two separate LC/MS/MS runs just to analyze EtG and barbiturates greatly limits the throughput and increases the cost of a UDT. Hence, increasing the speed (throughput) of analyzing EtG and barbiturates is a major challenge faced by the clinical chemistry community.

As a part of our comprehensive effort to develop simple and rapid MS/MS-based methods for quantification of biomarkers in clinical specimens, we examined the feasibility of using the dilute-and-shoot flow-injection MS/MS (FI-MS/MS) method (without LC separation) for urine drug testing. In our method, a urine sample containing target drugs is spiked with internal standards, followed by enzymatic hydrolysis to cleave glucuronide conjugates.[20–22] The enzymatic treated sample is then diluted to minimize the matrix effect. Thereafter, the diluted sample is directly injected into the electrospray ionization (ESI) source of a tandem mass spectrometer operated in a MRM mode for identification and quantification. We hypotheses that this simple dilution along with selecting

good internal standards allows for fast, accurate and robust quantification of drugs in urine without pre-purification and LC separation.

In this study, we employed phenobarbital, the most prescribed barbiturate, as the model system to demonstrate the proof of principle. It was found that the dilute-shoot FI-MS/MS method developed was fast and robust for accurate quantification of phenobarbital in urine, where an autosampler directly introduces the sample along with the solvent into the mass spectrometer without a chromatographic column. The run time of our method is only 2 min long, enabling analysis of more than 720 urine samples per day, substantially increasing the throughput of MS-based UDT methods. To the best of our knowledge, this is the first report of accurate quantification of phenobarbital in urine by MS/MS without both pre-purification and LC separation. Reporting this study constitutes the focus of our communication.

EXPERIMENTAL

Materials and methods

Phenobarbital (analyte) and phenobarbital-D$_5$ (internal standard, IS) were purchased from Cerilliant Corporation (Round Rock, TX, USA). B-Glucurodinase, Type-1, was obtained from *Helix pomatia*, and ammonium acetate were purchased from Sigma-Aldrich (Allentown, PA, USA). HPLC grade methanol and acetonitrile were purchased from Pharmco-Apper (Philadelphia, PA, USA). Deionized water was obtained from a Barnstead Nano pure water purification system from Thermo Scientific (Waltham, MA, USA). Drug-free urine was donated by six different healthy volunteers and was verified to not contain drugs before analysis.

Sample preparation

The urine drug samples used in this study were prepared by spiking phenobarbital and phenobarbital-D$_5$ (IS) at the concentrations of 50, 100, 200, 500, 1000, and 2000 ng/mL and 500 ng/mL (IS) respectively, in human blank urine. Each of the samples (100 μL) was then subjected to enzymatic hydrolysis by adding 200 units of β-glucurodinase and 3 mM ammonium acetate buffer, vortexed, and then incubated at 55°C for 2 h. The hydrolyzed urine calibrators were diluted by ten times with deionized water and centrifuged at 13,000 *g* for 20 min. The supernatants were transferred into autosampler vials and analyzed using FI-MS/MS.

MS/MS instrumentation

Sample analysis was performed using 5500 QTRAP triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) with an electrospray ionization (ESI) source interfaced with a HPLC system containing two LC-30 AD pumps, a DUG-20A3R inline degasser, a SIL-30 AC autosampler, a CBM-20 A controller and a CTO-10AVP column oven (Shimadzu, Columbia, MD, USA).[23–25] It is noted that the HPLC system used herein was just for flow-injecting samples into the ESI source and has no chromatography column connected. Ammonium acetate (5 mM) in 70% acetonitrile was employed as a carrier solvent at a flow rate of 0.3 mL/min to inject the sample and the same solvent was used as a wash solvent, which was done only after injecting samples with the highest concentration (2000 ng/mL) in each batch. A volume of 10 μL of the sample was injected with a run time of 2 min per sample. All the MS and sample injection parameters were selected and controlled by Analyst software (version 1.5.2; AB Sciex, Toronto, Canada).

The ESI source and analyte-dependent MS parameters were optimized by a direct infusion of phenobarbital at 50 ng/mL in the negative ion mode. The parameters were selected based on a combination of high ion intensity, low noise background in urine matrix and reproducible analyte peak intensity. The MS parameters were as follows: (i) ESI source dependent parameters were optimized by direct infusion analysis: Curtain gas (35), nebulization gas (Gas I) (30), heating gas (Gas II) (30), ion spray voltage (−4000 eV), temperature (450°C). (ii) Analyte-dependent parameters were fine-tuned manually as follows: declustering potential (−110), entrance potential (−15), collision energy (−19), Cell exit potential (−15), dwell time for each multiple reaction monitoring (MRM) transition (150 ms).

Preparation of working solutions, calibrators, and quality control (QC) samples

Separate stock solutions of phenobarbital and phenobarbital-D$_5$ (IS) were prepared at 50 μg/mL and 10 μg/mL from 1 mg/mL and 100 μg/mL main stock solutions, respectively. The calibrators and QC samples were prepared from different sources of stock solutions. A set of phenobarbital working solutions of 50, 100, 200, 500, 1000, and 2000 ng/mL spiked with 500 ng/mL phenobarbital-D$_5$ (IS) were prepared by serial dilution from the stock solutions of phenobarbital and IS (in methanol), respectively. Similarly, the pooled urine calibrators (mixture of 6 lots of blank human urine) were prepared by spiking phenobarbital at 50–2000 ng/mL and 500 ng/mL IS followed by enzymatic hydrolysis and simple ten times (10×) dilution. Accuracy and precision measurements were assessed in QC samples at 50, 125, 445 and 1600 ng/mL, representing the lower limit of quantification (LLOQ), low QC (LQC), mid QC (MQC), and high QC (HQC) urine solutions, respectively. The working, QC and calibrators were then subjected to FI-MS/MS analysis. All the concentrations of phenobarbital reported are equivalent to the concentrations of phenobarbital in solvent without dilution.

RESULTS AND DISCUSSION

Method development

Optimization of flow injection mass spectrometric conditions for MRM quantitation

Because the dilute-and-shoot FI-MS/MS system has no LC column to separate the interfering background from the target drug and internal standard molecules, we first developed the conditions to minimize the effect of any potential interfering background on quantification. This was achieved by analyzing six different urine samples containing no phenobarbital under various conditions. Infusion experiments were performed to optimize MS/MS parameters, to select carrier solvent, buffers and fragment ions, and to produce strong signals of both the drug and IS. The negative ESI mode

was selected because phenobarbital is an acidic drug with a pH of 9–10, which carries a negative charge on the oxygen at position 4 of the barbiturate ring and thus can be more efficiently ionized in the negative ion mode. The internal standard (IS) used in this work was phenobarbital-D_5. The use of deuterated drug molecules as internal standards can improve the quantitative accuracy, which is essential to dilute-and-shoot methods.[26–28]

Selection of MRM transitions – phenobarbital and phenobarbital-D_5 (IS) fragmentation

Fragmentation of precursor ions by collision-induced dissociation (CID), with a collision energy of 20 eV and the dwell time at 300 ms, led to the identification of product ions, with the transitions set at m/z 231.1 → 42.1 (quantitative) and m/z 231.1 → 188.1 (qualitative) for phenobarbital, and m/z 234.2 → 85.1 for the IS (phenobarbital-D_5), respectively. The fragmentation patterns are shown in Fig. 1. Under the conditions, the signal to noise was high, while the interference from other molecules was significantly reduced.[29] It is noted that in this study, we have carefully evaluated the transitions m/z 231.1 → 188.1 and 231.1 → 42.1 in both the method development and validation phases. The transition m/z 231.1 → 42.1 was selected as the quantitative channel due to its high signal intensity and better signal-to-noise ratio compared to m/z 231.1 → 188.1. The transition m/z 231.1 → 188.1 was selected as a confirmation channel because

it is highly specific to phenobarbital. The fragment at m/z 42 (NCO^-) was derived via a retro-Diels–Alder reaction in CID, which is most commonly used for quantification of barbiturates.[30] The fragment at m/z 188.1 used as a confirmation channel was obtained by fragmentation at the N–C bond with a loss of a NCO- moiety. The fragment of m/z 85 ($ONONH^-$), which was used as the quantification channel for the IS, was due to the fragmentation at the negative charge at O^2 or O^4 of the phenobarbital-D_5 ring (IS). A quantitative channel is used to calculate the concentration based on the quantifier ion, while a qualitative channel is used to confirm the identification of the target analyte based on the ratio of the qualifier ion to quantifier ion.[27]

After optimization, we found that both the drug (phenobarbital) and the IS were well differentiated and that the background interference was minor, allowing for both the qualitative and quantitative determination of phenobarbital by MRM operated in the negative ionization mode. An isocratic flow of carrier solvent, 5 mM ammonium acetate in 70% acetonitrile at a flow rate of 0.3 mL/min, was used to inject samples, as this solvent led to stronger signals for both phenobarbital and the IS and more effectively reduced the interference, compared with other solvents and buffers studied. The run time of our FI-MS/MS method was set for 2 min for each sample with a 10 μL injection volume. Under our optimized conditions, the peak with the strongest phenobarbital and IS signals was at 0.7 min after injection.
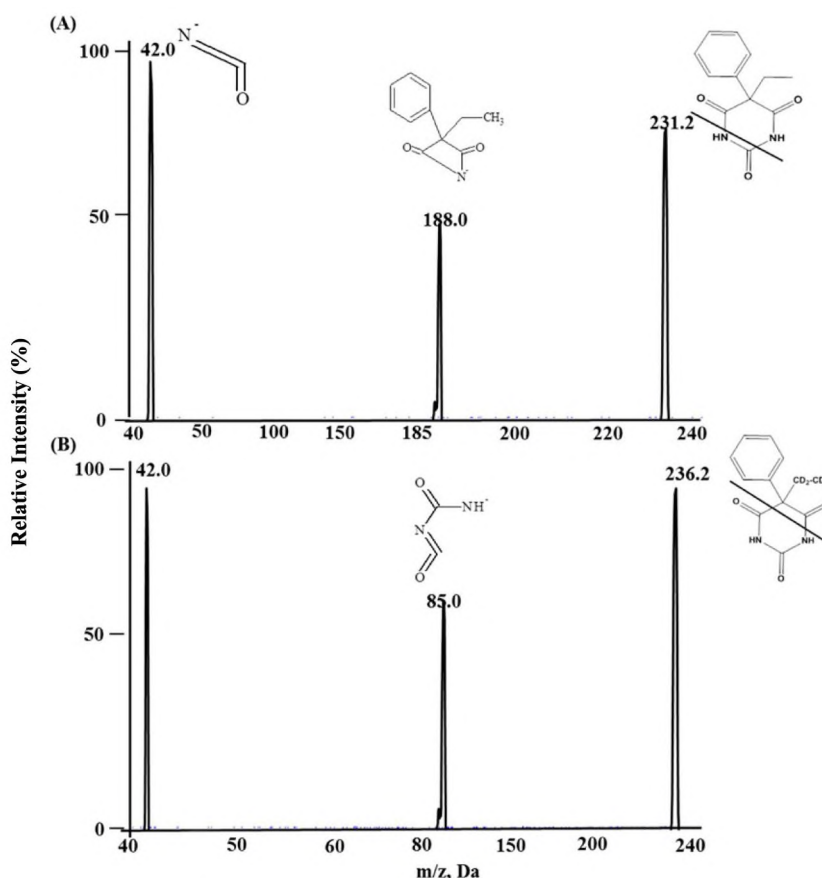
Figure 1. Precursor/product ion spectra and proposed fragmentation pathways for (A) phenobarbital and (B) internal standard phenobarbital-D_5.

## Phenobarbital cutoff concentration and calibration curve

In general, different predetermined cutoffs are used for different drugs, based on their distinct clinical significance. A too high cutoff can lead to false negatives, while false positives occur with a too low cutoff.[31,32] Based on clinical practice, a phenobarbital cutoff is generally set at 200 ng/mL (200 ng phenobarbital in 1 mL urine) by many reference laboratories.[27,33–35] With this in mind, we developed our calibration curve with the phenobarbital concentration ranging from 50 to 2000 ng/mL (Fig. 2), where the LLOQ (50 ng/mL) was set at 25% of the cutoff value (200 ng/mL phenobarbital). It is noted that the concentrations listed are the concentrations before 10× dilutions. Phenobarbital urine calibrators were prepared to obtain six urine calibrators: 50 ng/mL, 100 ng/mL, 200 ng/mL (cutoff), 500 ng/mL, 1000 ng/mL and 2000 ng/mL, where the cutoff concentration (200 ng/mL) in urine falls in the mid-point of our linear range, enabling our method to determine the concentrations both higher and lower than the cutoff value in urine samples.

Analytical method validation

A full method validation was performed for precision, accuracy, selectivity, lower limit of quantification (LLOQ), matrix effect and sample stability. The entire method was validated according to the currently accepted FDA Bioanalytical Method guidelines.[36]

Linearity, selectivity, sensitivity and LLOQ

The calibration plots were established using six phenobarbital urine calibrators at the concentrations of 50, 100, 200, 500, 1000, and 2000 ng/mL, double blank and single blank (only IS). An excellent linearity was achieved with the mean correlation coefficient of $r = 0.9996$. Also, the calibration curves were evaluated by plotting standardized residual plots and checked for any outliers for each calibrator during the course of method validation for five batches on five separate days. The standard deviation (SD) for the residuals was found to be in the range of 0.83–2.02, which is acceptable ($< \pm 3$ SD). The LLOQ and
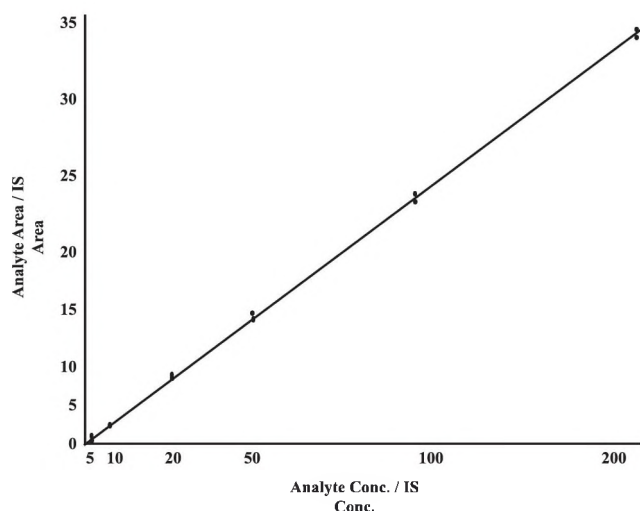
Figure 2. Calibration curve of phenobarbital in pooled blank human urine (mixture of 6 lots, 10× dilution). Linear regression (1/x weighting): y = 0.146x + −0.0278 (r = 0.9996).

selectivity of the method were assessed using the LLOQ (50 ng/mL phenobarbital) and double blank urine samples from six different urine sources. Phenobarbital (Fig. 3) and IS peaks (Fig. 4) at LLOQ were obtained at ~0.7 min and no significant interferences were detected in these time windows in blank urine samples, showing the high selectivity of our method. The S/N ratio of the quantitative transition (42.1) and the qualitative transition (188.1) was 20 and 10 at LLOQ, respectively (Figs. 3 and 4). The coefficient of variation (%CV) and accuracy for the LLOQ were 1.4% and 3.5%, respectively, meeting the requirement of FDA guidelines (Table 1).

Precision, accuracy and matrix effect

To evaluate intra-day (within the same day) and inter-day (5 different days) precision and accuracy, five replicates of three QC standards, LQC, MQC, and HQC of 125, 445 and 1600 ng/mL concentrations, along with the calibrators (50–2000 ng/mL) were analyzed within the same day and for 5 consecutive days, respectively. The values for intra- and inter-day precision and accuracies ranged from 2.0 to 2.5% and 1.0 to 3.0%, and 1.98 to 3.78% and 1.47% to 4.19%, respectively, indicating that the FI-MS/MS method developed is highly precise and accurate with negligible deviations (Table 2).

Absolute and relative matrix effects were investigated in triplicate at three QC concentrations (125, 445 and 1600 ng/mL) in both pooled blank urine (mixture of 6 lots) and six individual urine lots. The absolute matrix effect was calculated by comparing the peak areas of diluted blank urine samples (10× dilution, both pooled and individual blank urine) spiked with phenobarbital at three QC concentrations with those of corresponding standard solutions at equivalent concentrations. The relative matrix effect was calculated by comparing the peak area ratio of phenobarbital and IS (phenobarbital-D$_5$) spiked in the diluted blank urine samples at the same three QC concentrations with corresponding standard solutions at equivalent concentrations. The absolute matrix effect for each of the three QC pooled urine samples was 103.8%, 110.8%, and 112.8% and the relative matrix effect was found to be 98.7%, 108% and 110.5%, respectively (Table 3).

Similarly, absolute and relative matrix effect studies were performed in triplicate at three QC concentrations (125, 445 and 1600 ng/mL) with six individual urine lots to determine the extent of variation of ion suppression among each lot. The absolute matrix effect for each lot at three QC concentrations ranged from 102.6 to 113.2% and the relative matrix effect ranged from 92.1 to 112.4%, respectively. The variability of the matrix effect among different urine samples was expressed as percent coefficient variation (%CV), where acceptable values <12% were obtained. These studies clearly show that the dilute-and-shoot technique for sample preparation was effective and had minimal matrix effects.

Sample stability studies

The stability studies ($n = 3$) were performed using two different QC standards (125 and 1600 ng/mL), which were exposed to the following regimens: 6 h at room temperature (bench-top stability), 2 months at −20°C, and 3 freeze/thaw
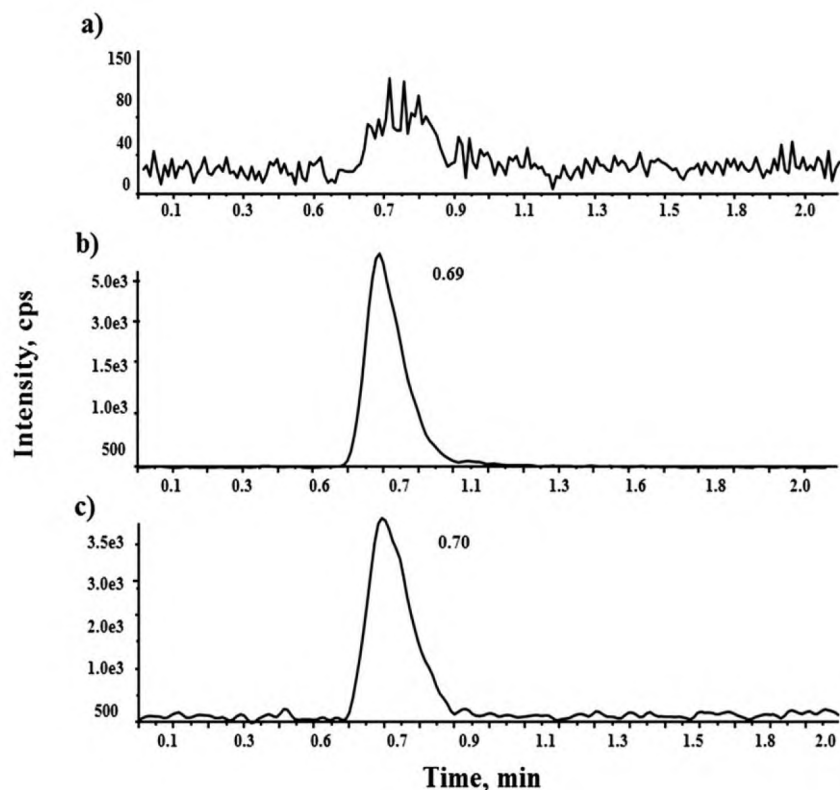
Figure 3. MRM extracted ion chromatograms (XICs) of (a) double blank pooled human urine (mixture of 6 lots, 10× dilution). (b) Blank pooled human urine (mixture of 6 lots, 10× dilution) spiked with phenobarbital at LLOQ level (50 ng/mL): Quantitative channel (42.0) and (c) Qualitative channel (188.0).
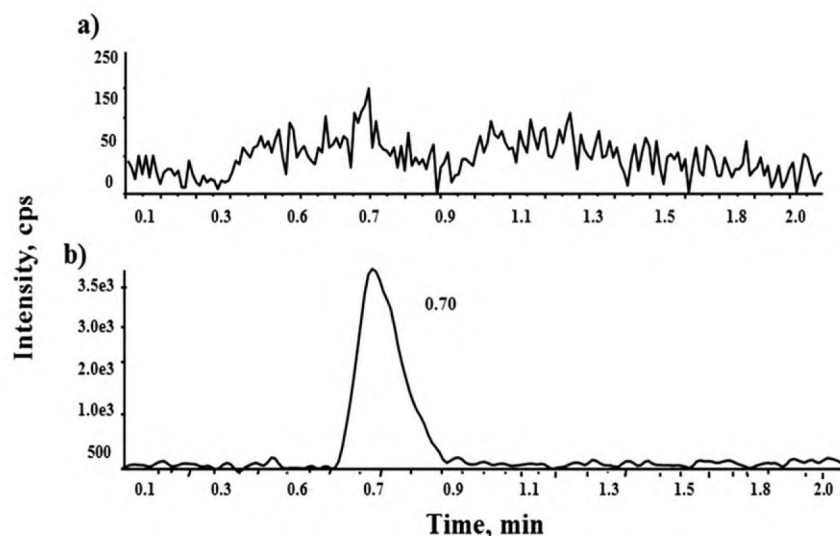
Figure 4. MRM extracted ion chromatograms (XICs) of (a) double blank pooled human urine (mixture of 6 lots, 10× dilution) and (b) single blank pooled human urine (mixture of 6 lots, 10× dilution) spiked with internal standard (phenobarbital-D$_5$) (500 ng/mL).

cycles within 3 days. The stability of phenobarbital and IS in human urine was evaluated after each storage period and compared to the freshly prepared samples of equivalent concentrations. The drug molecules were found to be stable and no loss or degradation of the analyte in urine was observed in all the studies (Table 4).

Interference from other drugs

As described earlier, the current UDT typically detects two analytes in the negative ion mode. They are barbiturates and EtG. We have carried out a study to determine the effect of these molecules on the quantification of phenobarbital in

Table 1. Accuracy and precision of phenobarbital calibration standards in pooled blank human urine

Spiked conc. (ng/mL)	Determined conc. (ng/mL)	Accuracy (%RE)	Precision (%CV)
50	51.07 ± 0.07	3.50	1.4
100	101.02 ± 0.21	1.20	2.1
200	190.88 ± 0.48	4.50	2.5
500	497.3 ± 0.88	1.27	1.8
1000	1009.0 ± 1.11	0.98	1.1
2000	2000.0 ± 0.54	0.10	0.7

Mixture of 6 lots, 10× dilution (n = 5) over 50–2000 ng/mL

Table 3. Absolute (AME) and relative matrix effect (RME) of phenobarbital in pooled blank human urine

Phenobarbital conc. (ng/mL)	AME ± SD (%)	%CV	RME ± SD (%)	%CV
50	108.5 ± 0.33	7	95.3 ± 0.33	6.9
125	103.8 ± 0.22	2	98.7 ± 0.22	2.1
445	110.8 ± 1.07	2	108 ± 1.07	1.9
1600	112.8 ± 2.56	2	110.5 ± 2.56	2

Mixture of 6 lots, 10× Dilution (n = 3)

urine, where we spiked secobarbital, butalbital, pentobarbital and EtG into urine samples containing phenobarbital (LLOQ: 50 ng/mL, LQC: 125 ng/mL, MQC: 445 ng/mL, and HQC: 1600 ng/mL). This test was performed using both six separate lots of urine and one pooled lot (mixture of 6 individual lots). Our results showed that these molecules had no significant effects (either ion suppression or enhancement) on quantification of phenobarbital, suggesting that their presence is not a problem for quantifying phenobarbital for UDT.

Evaluation of analytical performance and robustness of our FI-MS/MS method

Because our method does not use LC, it has a run time of only 2 min. This means that with our method, the throughput of one MS/MS system can be as high as 720 samples per day, much higher than the throughout achieved by any LC/MS/MS method. Since the FI-MS/MS method developed is intended for clinical use, it must be reliable and robust. In other words, the performance of the method should not deteriorate after several hundreds of injections and results should be reproducible to avoid false positives and/or negatives, when implemented in clinical toxicology labs for routine UDTs. As demonstrated above, despite the presence of urine matrix, our FI-MS/MS method could still accurately quantify phenobarbital at 50 ng/mL (4 times lower than the cutoff currently used in clinical labs) and the signal intensity of both phenobarbital and IS was not compromised with the elimination of LC. The key to our FI-MS/MS method is the selection of proper fragmentation conditions and fragment channels so that the effect of the matrix background can be minimized. We have also tested the robustness of our

Table 4. Sample stability studies of phenobarbital in pooled blank human urine

		Spiked conc. (ng/mL)	Measured conc. (ng/mL) Mean ± SD	Stability (%Recovery)
Freeze/thaw (3 cycles)	Low	125	126.0 ± 0.6	100.7
	High	1600	1587.3 ± 4.3	98.5
Bench top (6 h) at room temp.	Low	125	125.2 ± 0.42	100.4
	High	1600	1590.7 ± 5.43	99.4
2 months at −20 °C	Low	125	127.3 ± 0.5	101.8
	High	1600	1597.2 ± 2.3	99.8

Mixture of 6 lots, 10× dilution (n = 3)

FI-MS/MS method with over 1200 consecutive injections during a 3-day period. It was found that the results were still reproducible even after 1200 injections, demonstrating that our method was robust enough for routine clinical operation.

CONCLUSIONS

We have successfully demonstrated the feasibility of using the dilute-an-shoot FI-MS/MS method for quantification of phenobarbital in urine. To the best of our knowledge, this is the first ESI-MS/MS study to quantify phenobarbital without LC separation. The method developed has the distinct advantage of being able to quantify and detect the presence of phenobarbital in urine even at 50 ng/mL. Importantly,

Table 2. Inter- and intra-assay accuracy and precision of phenobarbital in pooled blank human urine

	Spiked phenobarbital conc.(ng/mL)	Intra-assay			Inter-assay		
		Measured mean ± SD (ng/mL)	Precision (%RSD)	Accuracy (%RE)	Measured mean ± SD (ng/ml)	Precision (%RSD)	Accuracy (%RE)
Low QC	125	122.03 ± 0.22	2.5	2.13	122.32 ± 0.37	3.0	1.47
Mid QC	445	446.7 ± 1.07	2.0	3.78	446.88 ± 0.47	1.0	4.19
High QC	1600	1601.83 ± 2.56%	2.0	1.98	1598.83 ± 3.31	2.0	1.35

Mixture of 6 lots, 10× Dilution (n = 5)

the method is ultra-fast and robust with simple dilution for sample pretreatment, short run time of 2 min, excluding HPLC separation. The validation study has shown that this method is accurate and precise, meeting the requirements of FDA guidelines. We present a proof-of-concept that the application of this method represents a robust, high-throughput and turn-key analytical platform to address emerging concerns of analytical toxicological/clinical studies in urine. In spite of the single drug analysis in this study, the results are highly encouraging, and extending this approach to other drugs with similar ionization patterns will be highly beneficial. This method, an alternative approach to the current time-consuming LC/MS/MS method, meets the requirement of high-throughput UDT analysis with unparalleled speed. Its compelling analytical features and versatility offer a major improvement over existing methods. More importantly, the FI-MS/MS approach can be a general method for fast analysis of many other drug molecules present in clinical specimens. Finally, our method was developed as a model system only to test for the presence of phenobarbital in urine and the application of this method to quantify other drugs needs method validation for those drugs. Studies using this approach to quantify other drug molecules are in progress in our lab.

Acknowledgments

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