Journal of Chromatography B, 873 (2008) 180–186

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Cross validation and ruggedness testing of analytical methods used for the quantification of urinary phthalate metabolites

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ARTICLE INFO

Article history:
Received 16 June 2008
Accepted 25 August 2008
Available online 29 August 2008

Keywords:
Analytical method validation
Ruggedness resting
Phthalate metabolites
Cross method validation
HPLC–MS/MS

ABSTRACT

Since the publication of our first analytical method in 2000 to detect and quantify phthalate metabolites in human urine, we have modified the method several times to improve performance, reduce the volume of matrix and solvents used, and to increase the number of analytes in one analytical run. We performed cross method validation and ruggedness testing after each modification to ensure that the analytical method adopted is robust and produces accurate and reproducible data when compared to the previously used method. Here, we present the results from the evaluation of the ruggedness of our analytical approach under variable experimental conditions, using the current analytical method. Minor deviations of the standard experimental conditions, i.e., pH, incubation time, amount of deconjugation enzyme, and incubation temperature, had no effect on final analyte concentrations. Furthermore, we validated the method to ensure accuracy at concentrations beyond the highest calibration standard. The concentrations obtained by using a lower volume of urine agreed well with original levels, suggesting broad linear calibration range as well as complete hydrolysis of the glucuronide conjugates with the standard amount of β-glucuronidase used for deglucuronidation; also, the time of incubation (90 min) was adequate regardless of the amount of glucuronide present. We also summarize the precision of concentration data acquired by the five different analytical approaches we have used since 2000. The correlation plots of concentration data for each analyte obtained from split sample analysis, using three of these approaches, produced linear curves ($R^2 > 0.98$) with slopes and intercepts that were not statistically different ($p > 0.05$) from 1 and 0, respectively. These results suggest that the data are reproducible and accurate, regardless of the analytical method used. Furthermore, analysis of quality control urine samples made over the years confirmed the stability of the phthalate metabolites in urine at −70 °C for several years and the consistency of the analytical measurements obtained by using various methodological approaches over time.

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1. Introduction

Biomonitoring of environmental chemicals has become an increasingly used approach in exposure assessment. Epidemiological studies using biomonitoring may continue for years, thus emphasizing the need for accurate and consistent measurements throughout the study period [1–3]. Selective and sensitive analytical methods for the quantitative evaluation of the analytes of interest are critical for successful epidemiological studies [2,4–6]. Because of the ongoing advances in analytical chemistry technologies, analytical methods are updated over time; thus, validation and cross validation of methods become important for accurate interpretation of data collected over years [7–11].

Before the Centers for Disease Control and Prevention (CDC) developed a method to measure phthalate monoester metabolites in urine, the parent phthalate diesters were measured in exposure assessment studies except for a limited number of studies [12]. However, phthalate diesters are ubiquitous in the environment [13–15], and the accuracy of their concentration data may be affected by the potential for diester contamination [16–18]. Because phthalate metabolites are produced endogenously within the biological system [19–24], measuring metabolites in biological matrices are relevant to biomonitoring of exposure to phthalates [25].

At CDC, we began the exposure assessment of phthalates by analyzing human urine for phthalate metabolites in 1999 [26,27]. With the first method (method A, Fig. 1) [27], eight phthalate metabolites were manually extracted on a vacuum manifold, using solid-phase extraction (SPE), and the metabolites were quantified by isotope dilution-high-performance liquid chromatography, coupled with tandem mass spectrometry (ID-HPLC–MS/MS, Fig. 1). Over time, the method was modified several times to increase the number of

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1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2008.08.017

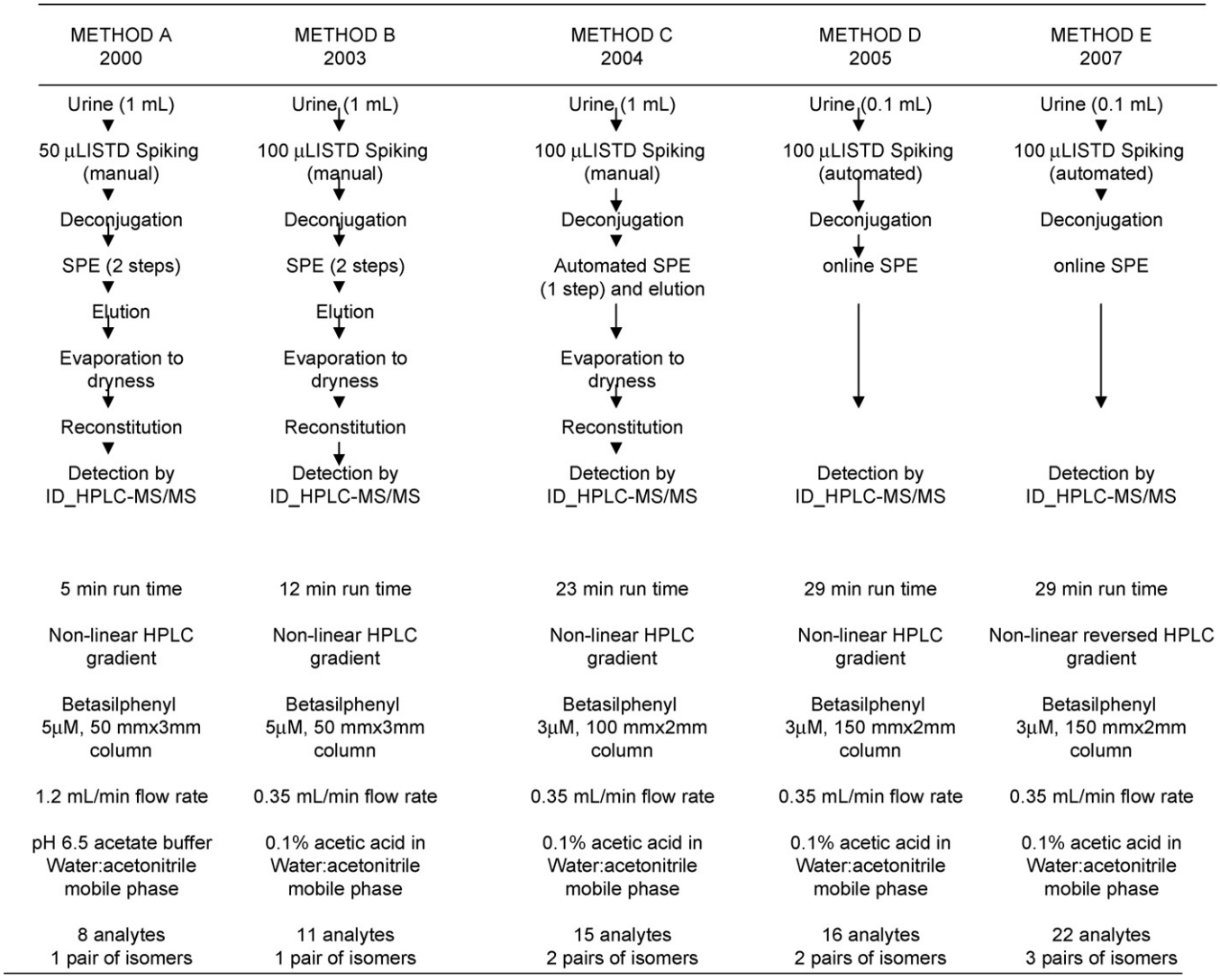

Fig. 1. Evolution time line of the method to measure phthalate metabolites in human urine at the Centers for Disease Control and Prevention.

metabolites measured from 8 to 22 and to reduce the urine volume required for analysis from 1 to 0.1 mL (Fig. 1) [28–31]. We also changed the extraction method from manual SPE (methods A and B) to automated SPE (method C) to on-line SPE (methods D and E). To accommodate the increasing number of analytes while maintaining sufficient resolution, we changed the dimensions of the HPLC analytical column and manipulated the solvent gradient (Fig. 1). With such changes, the limits of detection (LOD) were improved for most analytes, while allowing the selectivity needed to adequately resolve three pairs of structural isomers.

An integral component during method development involves the validation procedures to confirm the accuracy of data [7]. Here, we summarize the precision of concentration data acquired by the five different analytical approaches we have used since 2000 (Fig. 1) and also present the results from the evaluation of the ruggedness of the analytical method under variable experimental conditions, such as the amount of enzyme used, the pH, incubation time, and incubation temperature.

2. Subjects

Urine samples for method ruggedness evaluation were collected from both male and female donors with no documented exposure to phthalates. Samples were collected anonymously, and no personal or demographic data were available. A waiver of informed consent was requested under 45 CFR 46.116(d). The protocol for the collection of the samples was reviewed and approved by an Institutional Review Board of the Centers for Disease Control and Prevention. The urine samples were not first morning voids.

3. Experimental

The analytical methods used have been published previously [27–31]. Briefly, the urine samples were spiked with $^{13}C_4$-labeled monoester internal standards, 4-methyl umbelliferone glucuronide (4MeUmb-glu), and its stable-isotope-labeled internal standard. The phthalate metabolites were extracted from the urine by either manual, automated off-line, or on-line SPE after addition of β-glucuronidase (*Escherichia coli* K12, Roche Biomedical, Mannheim, Germany) in pH 6.7 acetate buffer (0.1 M). The analytes were chromatographically resolved by Betasil phenyl columns (Thermo Scientific, San Jose, CA), using a non-linear water, acetonitrile solvent gradient in acetate buffer or in 0.1% acetic acid and detected by tandem mass spectrometry. 4-Methyl umbelliferone (4MeUmb) was used to monitor the completion of the enzymatic deglucuronidation reaction.

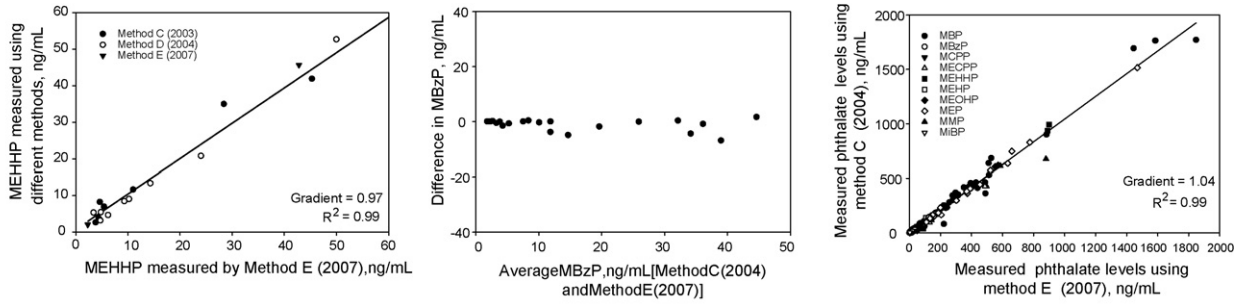

Fig. 2. Representative correlation (left) and Bland–Altman plots (middle) after repeat analysis of split urine samples using different analytical approaches. Correlation plot (right) constructed with 10 analytes using methods E and C.

With our most current method (method E, Fig. 1), the following urinary phthalate metabolites are measured in one analytical run: phthalic acid (PA), monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono-3-carboxypropyl phthalate (MCPP), mono-*n*-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP), monocyclohexyl phthalate (MCHP), monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-*n*-octyl phthalate (MOP), mono-isononyl phthalate (MNP), mono-isodecyl phthalate (MDP), mono-*n*-hexyl phthalate (MHxP), mono-*n*-heptyl phthalate (MHpP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-carboxy-*n*-heptyl phthalate (MCHpP), mono-carboxy-isooctyl phthalate (MCOP), mono-hydroxyisononyl phthalate (MHNP), mono-oxoisononyl phthalate (MONP), and mono-carboxyisononyl phthalate (MCNP).

3.1. Method validation and ruggedness testing

The most recent method (method E) was used as the reference method for method evaluation and ruggedness testing. However, because the experimental conditions are similar for all our analytical approaches, the results are applicable to all five analytical methods. Furthermore, with each analytical batch, water samples were processed and analyzed as reagent blanks (RBs). The analyte levels present in RB were subtracted from the unknown samples.

3.1.1. Repeat analysis of urine samples by different analytical approaches used over the years

Unknown samples and quality control (QC) materials (pooled urine samples collected from anonymous donors [27–31]) were analyzed by using the analytical approaches shown in Fig. 1. The results of these analyses are presented in a correlation plot and a difference plot with regression analysis (Fig. 2).

3.1.2. Assessment of long term stability of analytes in urine and the consistency of the measurements by analysing analyte concentrations in QC pools made over 8 years

The analyte concentrations in nine different QC pools prepared over 8 years (1999–2007) and stored at −70 °C since their preparation were re-measured using the current analytical method (method E) and compared to the original concentrations (Table 1).

3.1.3. Assessment of potential variability in experimental conditions

Urine samples collected in 2008 from five individuals were spiked with the isotope-labeled internal standard solution, 4-MeUmb-glu solution, and β-glucuronidase solution in 0.1 M acetate buffer. We evaluated four experimental conditions, as follows: (A) the samples were incubated for 90 min at 25, 33, 37, 40, or 45 °C to assess the activity of the β-glucuronidase at different temperatures (Fig. 3A); (B) the samples were incubated at 37 °C for 0, 10, 20, 30, 60, 90, 120, and 240 min to assess the incubation time for complete deconjugation (Fig. 3B); (C) the samples were spiked with 0.25, 0.5, 1, 3, 5, 7, and 10 μL/mL urine of β-glucuronidase in 0.1 M pH 6.7 acetate buffer to assess the amount of enzyme needed for complete deglucuronidation (Fig. 3C), and the pH of the acetate buffer was changed from 4 to 8, to assess the effect of pH on deconjugation (Fig. 3D). In all cases, the analytes were extracted from urine (0.1 mL) using on-line SPE and detected by ID-HPLC–MS/MS by using method E. Except for the experimental conditions described in (C), for all other experiments 25 μL (5 μL of β-glucuronidase in 250 μL of acetate buffer) of enzyme solution was used to hydrolyze the glucuronides of the phthalate metabolites.

3.1.4. Assessment of 4-MeUmb-glu as an indicator for total deconjugation

Urine samples (0.1 mL) collected from five individuals were spiked with the internal standard solution, different concentrations of 4-MeUmb-glu (8, 26, 32, 80, 160, 1600, 6400, and 64,000 ng/mL) and β-glucuronidase in 0.1 M acetate buffer. The samples were incubated for 90 min at 37 °C. The analytes were extracted using on-line SPE and detected by MS–HPLC–MS/MS, using method E (Fig. 4) [31].

3.1.5. Variability in phthalate metabolite concentrations after adding the internal standard, manual vs. automated

Two pooled urine samples were spiked with all analytes of interest and were repeatedly analyzed ($N = 5$) after addition of the internal standard solution manually. The same procedure was repeated ($N = 5$) with the automated addition of the internal standard, using a Surveyor autosampler.

3.1.6. Proficiency testing (PT) and external quality assessment

Method accuracy was verified using pre-characterized PT pooled materials of three concentration ranges (i.e., low, medium, and high). At least two times per year, five PT samples were blind analyzed and the results were reported to an external QC officer for evaluation. Furthermore, we participate in the ongoing German External Quality Assessment Scheme (G-EQUAS) organized and managed by the Institute and Outpatient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (Erlangen, Germany). A minimum of once per year, we analyze two reference urine samples fortified with MBP, MEHHP, MEOHP, and MECPP (Table 2). Scheme, evaluation, and certification are based on the guidelines of the German Federal Medical Council (http://www.g-equas.de/).

Table 1
Re-quantification of selected phthalate metabolites in quality control (QC) pools made over several years using the current analytical method E

	Mean phthalate metabolite concentration[a] (ng/mL)								
	MCPP	MMP	MEP	MECPP	MBP	MiBP	MEOHP	MBzP	MEHP
Method A									
QCL[b]									
Expected	N/A	N/A	8.2	N/A	4.6	N/A	N/A	7.5	9.8
Observed	N/A	N/A	10.3	N/A	3.8	N/A	N/A	7.4	7.7
QCH[b]									
Expected	N/A	N/A	484.1	N/A	97.0	N/A	N/A	147.1	109.8
Observed	N/A	N/A	654.5	N/A	103.8	N/A	N/A	163.6	89.7
Method B[c]									
QCH									
Expected	N/A	213.5	486.3	N/A	110.3	N/A	N/A	128.9	33.4
Observed	N/A	160.9	658.3	N/A	118.7	N/A	N/A	144.8	27.2
Method C									
QCL									
Expected	37.9	56.6	154.7	N/A	55.3	28.2	26.4	63.7	30.3
Observed	39.9	58.0	171.9	N/A	71.5	32.7	26.4	64.8	22.0
QCH									
Expected	205.2	178.3	512.0	N/A	188.1	160.0	124.7	265.8	77.1
Observed	208.1	182.8	568.9	N/A	222.8	189.6	127.3	264.7	59.7
Method D									
QCL									
Expected	5.1	37.2	88.9	22.2	25.7	25.8	6.7	28.8	12.2
Observed	6.5	36.1	84.7	22.9	26.8	26.9	6.7	25.9	11.9
QCH									
Expected	14.9	216.4	393.8	88.4	142.9	135.1	45.5	212.3	70.2
Observed	18.0	200.3	376.1	90.6	140.0	133.0	45.4	183.2	67.4
Method E									
QCL									
Expected	4.1	5.5	62.1	12.9	14.0	13.3	12.9	8.4	13.9
Observed	5.0	5.7	56.0	13.5	14.5	13.8	13.1	8.3	15.5
QCH									
Expected	26.3	48.3	485.6	43.0	68.6	63.6	51.2	73.0	76.6
Observed	29.3	47.6	456.4	45.0	70.2	69.3	50.7	67.3	77.6

[a] The observed measurements were made by repeat analysis of QC material using the current method (method E) for analysis of urinary phthalate metabolites. The expected measurements were made with the original method (methods A–D) [26,28–31].
[b] QCL, quality control low pool; QCH, quality control high pool.
[c] QCL was not available for measurement.

4. Results and discussion

Analytical chemistry is a dynamic research field in which analytical technologies evolve continuously. As a result, we modified our original method (method A, Fig. 1) [27] for the quantification of urinary phthalate metabolites four times from 2000 to 2007 (Fig. 1) [27–31]. These modifications resulted in higher sample throughput and an increased number of analytes measured in each sample, while reducing the solvent volume and the amount of matrix used. Furthermore, the analytical approach was gradually changed from manual SPE using vacuum manifolds (methods A and B) to off-line automated SPE (method C) and finally to automated sample preparation followed by on-line SPE (methods D and E), an approach that requires minimal human involvement. Upon each change, the updated method was validated against the previous method by the statistical evaluation of accuracy and precision data based on analysis of split urine and QC samples.

The agreement in analyte concentrations of repeat analysis of urine samples stored at −70 °C using different analytical approaches at different time periods was excellent (Fig. 2), suggesting that the agreement between methods is good. The correlation plots produced linear curves ($R^2 > 0.98$) in which the slopes and the intercepts were not statistically different ($p > 0.05$) from 1 and 0, respectively. These data indicate that the data are reproducible and accurate regardless of which method is used for analysis. The phthalate concentrations from repeat analysis of QC samples prepared over 8 years and stored at −70 °C since their preparation were comparable, suggesting method accuracy and consistency throughout this time period (Table 1). More importantly, these results further support the stability of the analytes over years at −70 °C and suggest that urine samples can be stored for a lengthy period without noticeable degradation that could affect the analytes' concentrations. The accuracy of the concentration measurements over years was further confirmed by blind analysis of PT samples (data not shown). Furthermore, the concentrations of MBP, MEOHP, MEHHP, and MECPP using our current method (method E) were within the reference levels established by G-EQUAS, an independent and objective monitor of the accuracy of the results submitted by different laboratories (Table 2), suggesting the reliability and compatibility of our results with those from other international laboratories.

The LOD for most analytes improved with each method (Table 3). However, for some analytes, such as MMP and MEHP, no improvement in the LOD was observed. This is likely related to the early

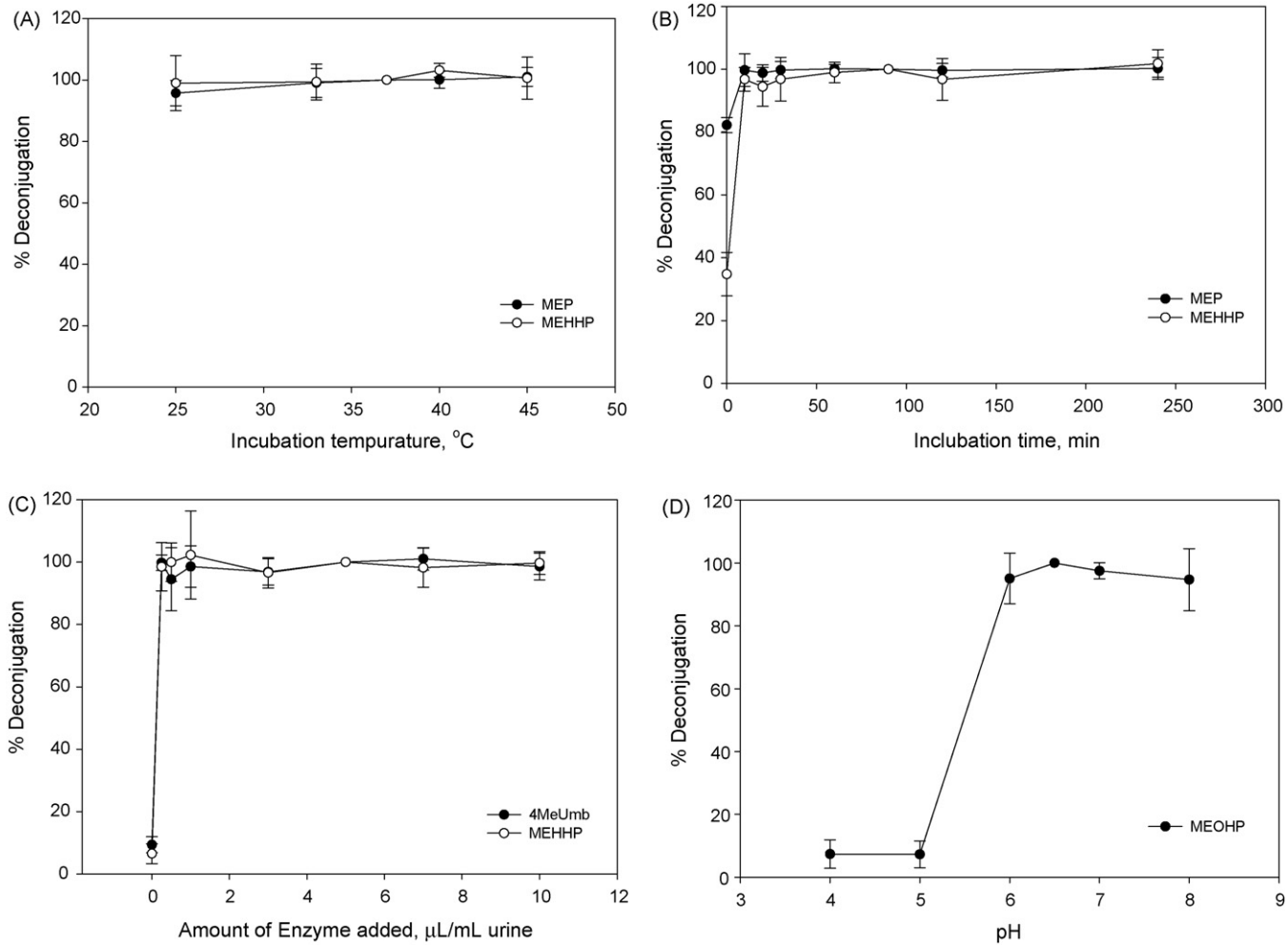

Fig. 3. The effect of incubation temperature (A), incubation time (B), amount of enzyme (C), and pH (D) on the deglucuronidation of selected phthalate metabolites.

Table 2
Results of the G-EQUAS 41 (http://www.g-equas.de/) conducted in the second half of 2007 for phthalate measurements in urine

Analyte	Sample	Measured concentration (ng/mL)	Reference value (ng/mL)	Tolerance range (ng/mL)
MBP	A	156.7	185.3	100.70–382.70
	B	243.9	265.1	147.50–382.70
MEHHP	A	113.4	109.84	83.47–136.21
	B	253.2	236.13	187.83–284.43
MEOHP	A	112.7	110.21	90.05–130.37
	B	267.6	265.92	225.12–306.72
MECPP	A	163.6	152.79	122.92–182.65
	B	384.0	359.93	310.13–409.73

elution of MMP with other hydrophilic urine contaminant components and to the presence of MEHP in reagents as a contaminant interfering with analysis at low trace levels.

Over 8 years, the number of analytes detected increased from 8 to 22 (Fig. 1); the HPLC column dimension and particle size changed, mobile phase pH changed and the analytical run time increased from 5 min (method A) [27] to 29 min (methods D and E) [28,31]. The flow rate was decreased from 1.5 to 0.35 mL/min. This increase in run time and the decrease in flow rate were necessary to move the most hydrophilic metabolites (e.g., MMP and MEP) away from the solvent front, adequately separate analytes, and to include MiBP, a structural isomer of MBP that uses the same mass spectrometric transitions for detection. We increased the number of analytes from 16 (method D) [28] to 22 (method E) [31], even with the addition of yet another isomeric pair (MCHpP/MECPP), by reversing the solvent gradient in the middle of the HPLC run. Furthermore, this novel approach adequately separated all 22 phthalate metabolites without increasing the analytical run time.

To evaluate the ruggedness of our analytical approach, we examined the influence of selected experimental conditions on the method performance. In particular, incomplete hydrolysis of phthalate glucuronides due to fluctuations in the incubation temperature, time, pH, and differences in activity of β-glucuronidase preparations may cause inaccurate quantification. We found that the β-glucuronidase was active if pH > 6 (Fig. 3). However, because we buffer each of the sample to pH 6.7; the pH at which the activ-

Table 3
Retention times (RTs) and the limits of detection (LOD) of phthalate metabolites quantified by using different analytical methods

Analyte	Method A [27]		Method B [29]		Method C [30]		Method D [28]		Method E [31]	
	LOD (ng/mL)	RT (min)	LOD (ng/mL)	RT (min)	LOD (ng/mL)	RT (min)	LOD (ng/mL)	RT (min)	LOD (ng/mL)	RT (min)
PA	–	–	–	–	1.6	3.1	1.0	4.5	0.9	4.9
MMP	–	–	0.7	3.1	1.2	5.4	1.2	6.8	1.1	6.7
MCPP	–	–	–	–	0.4	7.0	0.3	6.7	0.2	6.7
MEP	1.0	0.5	1.2	4.6	0.9	8.1	0.3	8.4	0.7	8.5
MEHHP	–	–	1.6	6.6	1.0	18.0	0.6	16.8	0.7	13.6
MiBP	–	–	–	–	1.0	17.1	0.3	16.4	0.3	14.1
MECPP	–	–	–	–	–	–	0.5	17.4	0.5	14.0
MBP	0.6	1.5	0.9	6.5	1.1	17.5	0.3	17.2	0.6	14.6
MCHpP	–	–	–	–	–	–	–	–	0.6	14.6
MEOHP	–	–	1.2	6.4	1.1	18.5	0.7	18.8	0.6	15.3
MHNP	–	–	–	–	–	–	–	–	0.9	16.6
MCOP	–	–	–	–	–	–	–	–	0.7	17.8
MBzP	0.8	2.0	0.5	7.4	0.3	19.5		21.9	0.3	19.7
MCHP	0.7	1.9	0.9	7.3	0.3	19.6	0.3	22.0	0.3	20.0
MONP	–	–	–	–	–	–	–	–	0.4	20.3
MCNP	–	–	–	–	–	–	–	–	0.5	23.0
MHxP	–	–	–	–	–	–	–	–	0.7	24.4
MHpP	–	–	–	–	–	–	–	–	0.5	25.4
MEHP	1.2	3.1	0.9	9.8	1.0	21.8	0.6	26.2	1.1	25.6
MOP	0.9	3.3	0.8	10.5	1.0	22.1	1.2	26.4	1.1	25.8
MNP	0.8	3.5	0.8	9.8	0.9	22.1	1.5	26.4	0.8	25.9
MDP	1.5	3.9	0.5	10.6	nd	22.7	nd	26.8	0.4	26.3

nd, not determined; –, not included in the method.

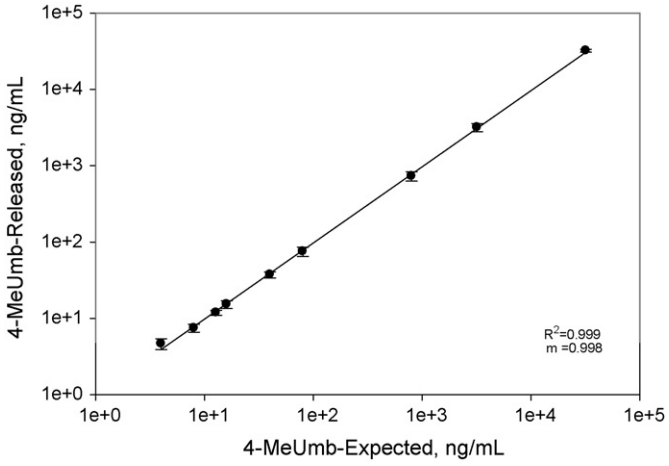

Fig. 4. Amount of 4-MeUmb released from 4-MeUmb-glucuronide after incubating the urine samples ($N = 5$) with 0.5 mL of β-glucuronidase enzyme in pH 6.5 acetate buffer for 90 min at 37 °C.

ity of the enzyme is optimal, large fluctuations in pH are unlikely. Our data also suggest adequate temperature tolerance (from 25 to 45 °C) and adequate activity of the β-glucuronidase to ensure the complete deglucuronidation of the urinary conjugates, even when the enzyme was used at concentrations 50 times less (Fig. 3) than the required amount established during method validation. Furthermore, the hydrolysis of glucuronides was rapid, resulting in the complete hydrolysis within 30 min of incubation (Fig. 3). Nevertheless, to ensure complete hydrolysis, we always incubated the samples for 90 min.

Switching the method from the manual to the automated addition of the internal standards improved the sample throughput and potentially eliminated human errors and variability associated with the manipulation of samples by different analysts. Although increased variability is likely during manual preparation of samples, the repeat analysis of manually and automatically prepared spiked urine samples produced similar precision in the analyte concentrations (Table 4).

4-MeUmb-glu is used to measure the completion of the enzymatic hydrolysis of phthalate metabolites conjugates. Complete hydrolysis of 4-MeUmb-glu was observed to produce 4-MeUmb, even at concentrations up to 32,000 ng/mL (Fig. 4). Furthermore,

Table 4
Variability observed for selected phthalate metabolites when metabolite-enriched urine pools were repeatedly analyzed ($N = 10$) after manual and automated addition of the internal standard

Analyte	Low spike concentration ± STD (ng/mL)		High spike concentration ± STD (ng/mL)	
	Manual ISTD spiking[a]	Automated ISTD spiking[a]	Manual ISTD spiking	Automated ISTD spiking
PA	20.2 ± 0.3	22.6 ± 0.4	38.9 ± 0.9	43.0 ± 0.3
MCPP	16.9 ± 0.3	16.9 ± 0.6	32.1 ± 0.2	35.8 ± 0.8
MEP	168.3 ± 2.5	192.3 ± 2.1	372.4 ± 6.0	417.5 ± 4.1
MEHHP	28.1 ± 1.3	29.6 ± 0.8	51.7 ± 0.9	54.9 ± 1.6
MECPP	22.2 ± 0.5	23.4 ± 0.6	37.9 ± 1.1	42.0 ± 2.0
MIBP	29.7 ± 1.2	33.0 ± 1.1	52.3 ± 2.1	59.6 ± 3.8
MBP	33.7 ± 2.1	36.8 ± 1.8	63.2 ± 2.3	69.9 ± 2.6
MEOHP	24.7 ± 0.6	25.3 ± 0.8	42.4 ± 1.3	45.3 ± 0.7
MBzP	29.2 ± 0.9	31.8 ± 1.8	56.1 ± 2.0	59.7 ± 1.6
MEHP	45.7 ± 0.4	44.2 ± 3.7	76.5 ± 3.9	75.6 ± 2.7

[a] Measurements were made by using method E.

the hydrolysis of 4-MeUmb-glu followed a similar pattern to the hydrolysis of phthalate metabolites (Fig. 3C), suggesting that 4-MeUmb-glu is an excellent indicator of the hydrolysis of phthalate glucuronides. In *in vivo* systems, enzyme saturation may limit the extent of the enzymatic hydrolysis. However, with our method, because incubation is allowed to continue for 90 min, the catalytic activity of the β-glucuronidase was sustained until complete hydrolysis of the glucuronide was achieved, independent of the enzyme concentration.

MEP, MEHHP, MBP, MEOHP, and MECPP are the analytes that normally found at the highest median and upper concentration ranges. In particular, for some individuals, these analytes can be present at concentrations above the highest calibration standard. Nevertheless, the concentrations obtained by using a lesser amount of urine than the analytical method normally requires agreed well with that were obtained using the normal amount of urine, suggesting the adequacy of the enzyme activity and linearity of the calibration curve over a wide range of analyte concentrations, even beyond the highest calibration standard.

In conclusion, we confirmed that our method for detection and quantification of phthalate metabolites is accurate, rugged, and specific regardless of the analytical approach. Minor fluctuations in conditions during sample preparation or analysis have no noticeable effect on the final quantification of the analytes. Furthermore, the concentrations of phthalate metabolites in urine are stable over years at current storage conditions at −70 °C. These results, taken together, suggest that our analytical approach can produce consistent data throughout the years and is suitable for long-term epidemiological studies that require phthalate biomonitoring data.

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