Investigating the Urinary Concentrations and Distribution of Phthalate Metabolites in Cow Urine Distillate in India

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1. Optimization of HPLC-MS/MS Conditions and extraction procedure

Various columns, mobile phase compositions, and additive strength were tested to develop a sensitive, reproducible, and shorter analytical method. Zorbax Eclipse plus C18 (2.1 × 100 mm, 1.8 µm) (Agilent Technologies, Santa Clara, CA, USA) by maintaining a column temperature of 45 °C, column produced the most desirable peak shape and chromatographic run time of all the columns tested. In the next step, compositions of mobile phases were optimized, and acetonitrile and water with acetic acid were found to yield better peak shapes and good sensitivity for phthalates metabolites. As a result, acetonitrile and Milli-Q were used as organic and aqueous mobile phases in gradient mode, respectively, providing sharp and symmetrical phthalates metabolites and IS peak shapes. A negative mode of the AJS-ESI interface was found to be suitable for the detection of phthalates metabolites and IS. The optimized multiple reaction monitoring (MRM) transitions for phthalates metabolites are shown in **Table S1.**

A number of solvents, including acetonitrile and Milli-Q water, were tested for possible contamination with phthalates metabolites before use. Extracting phthalates from urine proved to be a challenging task. Different extraction techniques have been employed, like liquid-liquid extraction and solid-phase extraction, by using different cartridges. In this direction, solid-phase extraction was employed, which involved ABS Elut-NEXUS SPE cartridges (60 mg, 3mL, Agilent, Santa Clara, CA), preconditioning solvents, washing solvent, and extraction solvent for phthalates metabolites. Optimization efforts were made with respect to organic solvent types (methanol and acetonitrile) for preconditioning the cartridge and solvents used to elute the phthalate's metabolites. Out of the organic solvent tested for preconditioning and elution of the phthalate's metabolites, 1.0 mL of Acetonitrile and phosphate buffer (1.0 mL, pH 2) were used to precondition the cartridges. Next, 0.1 M formic acid (2 mL) and water (1.0 mL) were used to wash the cartridges. Targeted metabolites were eluted with Acetonitrile (900 µL) followed by ethyl acetate (900 µL to improve the recovery of the phthalate's metabolites. Combined eluents were evaporated to dryness under a vacuum concentrator and reconstituted with 180 µL of acetonitrile /water (1:9 v/v) and transferred into glass inserts. Further analysis was performed and conducted using LC-MS/MS.

2. Method Validation

The developed LC-MS/MS method for determining phthalates metabolites in cow urine distillates product was validated with respect to linearity, accuracy, precision, recovery, and matrix effect. A representative MRM chromatogram from the analysis of a procedural blank sample and a matrix spiked with analytes at low levels is shown in **Figure S1**. The retention time of MMP, MCPP, MEP, MECPP, MEHHP, MiNP, MEOHP, MnBP, MiBP, MBzP, $^{13}C_2$ DEHP was 5.00, 5.00, 5.40, 5.94, 6.09, 6.17, 7.53, 6.24, 6.20, 6.30, 14.83 min, respectively. It was observed that no significant interferences occurred at the retention time of phthalates metabolites. Hence developed and validated LC-ESI-MS/MS method was specific for the quantification of phthalates metabolites. The calibration curve of phthalates metabolites was established using a weighing factor ($1/x$). A good linear relationship was observed between the peak area ratios of the phthalate metabolites to the IS and the phthalates metabolites concentration in the calibration curve. The calibration curves for all the phthalates metabolites were in the range of 1ng/mL to 200 ng/mL with a determinant coefficient of 0.995. The LOD for phthalates metabolites was in the range of 0.50 ng/mL to 1 ng/mL, whereas LOQ was in the range of 1 ng/mL to 5 ng/mL. In **Table S2**, the accuracy and precision of phthalates metabolites at all concentrations examined were well within acceptable limits at interday and intraday levels. Based on these results, the developed analytical method was accurate and precise for analysing phthalates metabolites in samples within the defined concentration range. The average percent recovery of phthalates metabolites at various concentration levels are >75%, suggesting that by using the proposed extraction procedure, an optimal analyte recovery could be obtained with no significant matrix interference from the samples.
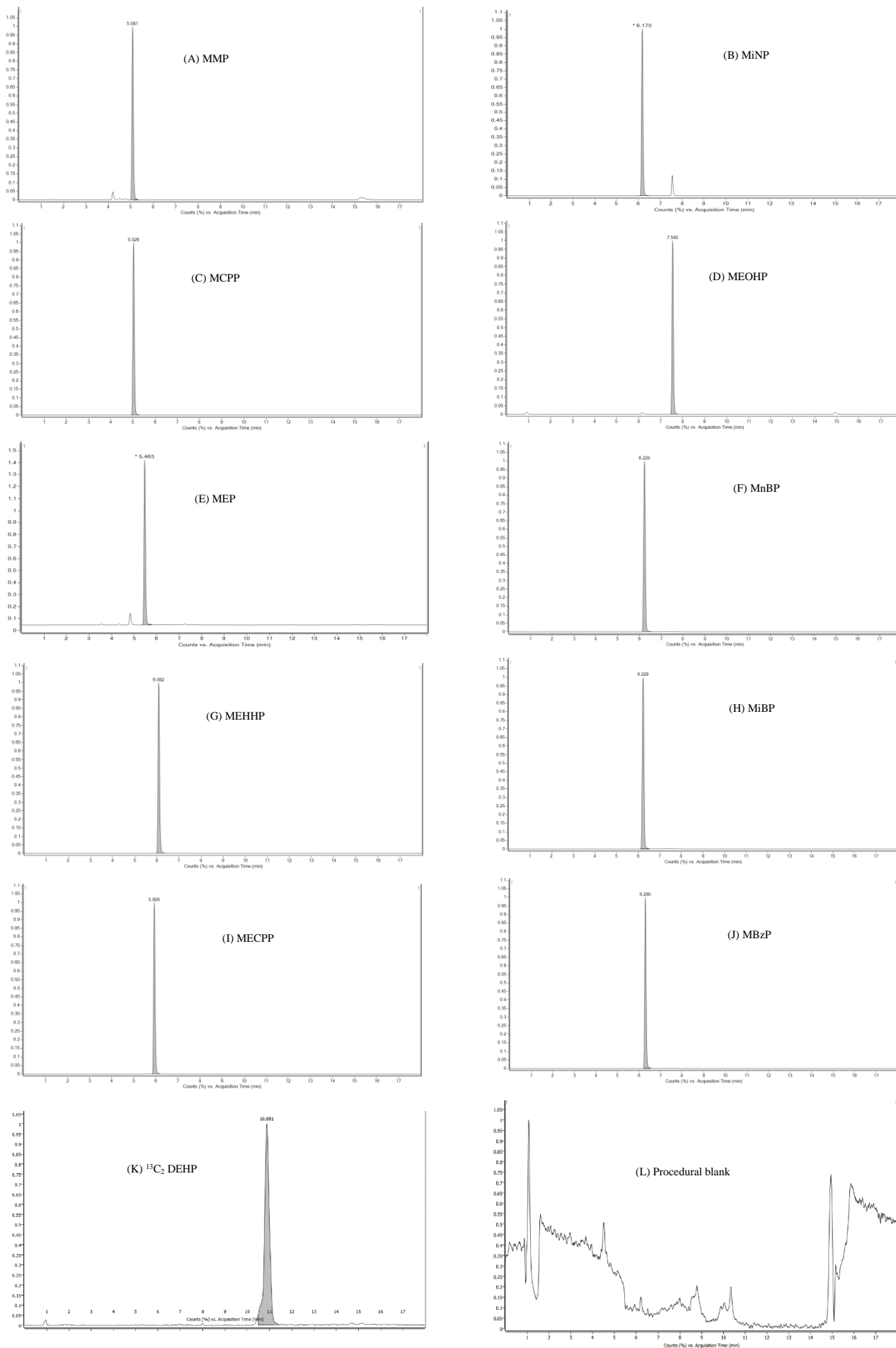
Figure S1. LC-MS/MS chromatograms of phthalate metabolites (A) MMP (B) MiNP (C) MCPP (D) MEOHP (E) MEP (F) MnBP (G) MEHHP (H) MiBP (I) MECPP (J) MBzP (K) $^{13}C_2$ DEHP (I.S) (L) Procedural Blank

Table S1. MRM transitions of phthalate metabolites and internal standard

Phthalate metabolites	Parent ion	Daughter ion	CE (eV)	RT (min)
MMP	179.00* 179.00	107.10 77.10	20.00	5.40
MCPP	251.10* 251.10	103.10 121.00	8.00	5.40
MEP	193.10* 193.10	77.10 148.30	16.00	5.40
MECPP	307.10* 307.10	159.10 113.10	8.00	5.94
MEHHP	293.10* 293.10	121.10 77.10	20.00	6.09
MiNP	291.20* 291.20	121.10 77.10	16.00	6.17
MEOHP	291.10* 291.10	141.10 121.10	20.00	7.53
MnBP	221.10* 221.10	77.10 71.10	16.00	6.24
MiBP	221.10* 221.10	77.10 134.10	16.00.	6.24
MBzP	255.10* 255.10	77.1 107.1	20.00	6.30
$^{13}C_2$ DEHP	393.00* 393.00	311.0 183.0	5.00	10.88

Quantifier

Table S2. Accuracy and Precision of LC-MS/MS method

| Phthalate Metabolites | QC samples | Nominal Conc. (ng/mL) | Intra-Day | | | Inter-Day | | | % Recovery |
			Calculated Conc. (ng/mL)	Accuracy (%) ± SD	Precision (%RSD)	Calculated Conc. (ng/mL)	Accuracy (%) ± SD	Precision (%RSD)	
MCPP	LLOQ	1	0.89	89.54 ± 9.59	10.71	1.02	101.66 ± 11.27	11.09	95.86
	LQC	3	2.65	88.63 ± 4.73	6.53	2.61	87.02 ± 1.63	2.30	95.36
	MQC	100	100.16	100.16 ± 7.15	7.14	106.61	106.61 ± 2.29	2.15	96.95
	HQC	150	148.89	99.26 ± 0.79	0.79	150.83	100.55 ± 0.79	0.52	97.36
MMP	LLOQ	1	0.91	91.90 ± 5.57	6.06	0.81	80.80 ± 8.51	10.54	86.74
	LQC	3	2.81	93.79 ± 7.24	9.46	2.70	90.04 ± 3.45	4.69	82.66
	MQC	100	104.53	104.53 ± 5.73	6.72	107.00	107.00 ± 5.07	5.81	89.65
	HQC	150	148.56	99.04 ± 0.21	0.27	150.93	100.62 ± 1.24	1.51	94.59
MEP	LLOQ	5	5.75	111.07 ± 7.59	6.83	5.52	110.37 ± 3.91	3.54	95.64
	LQC	15	18.13	114.29 ± 1.10	0.96	17.02	113.45 ± 8.36	7.37	89.72
	MQC	100	97.75	97.75 ± 3.00	3.76	100.75	100.74 ± 3.14	3.82	81.45
	HQC	150	150.5	100.33 ± 0.56	0.69	153.20	102.13 ± 1.73	2.08	86.65
MiBP	LLOQ	1	0.97	97.085 ± 5.12	5.27	1.02	102.21 ± 6.00	5.88	84.95
	LQC	3	3.42	111.90 ± 6.22	5.56)	3.50	116.76 ± 1.97	1.69	89.49
	MQC	100	101.93	101.93 ± 3.45	4.14	108.08	108.07 ± 3.31	3.75	88.69
	HQC	150	156.04	104.02 ± 2.56	3.02	157.18	104.78 ± 1.29	1.52	90.65
MEOHP	LLOQ	1	1.09	107.77 ± 6.58	6.11	1.22	121.93 ± 6.11	5.01	85.14
	LQC	3	3.2	103.86 ±10.01	9.64	3.14	104.70 ± 6.17	5.90	86.69
	MQC	100	98.56	98.56 ± 4.98	6.19	102.26	102.26 ± 1.25	1.50	88.65
	HQC	150	149.22	99.48 ± 1.27	1.57	150.92	100.61 ± 0.94	1.15	85.65
MECPP	LLOQ	1	0.88	90.52 ± 11.79	13.02	1.01	100.62 ± 2.79	2.78	84.95
	LQC	3	2.49	86.44 ± 9.84	11.38	2.26	75.49 ± 4.11	5.45	89.90

	MQC	100	98.9	98.90 ± 5.76	7.13	104.78	104.77 ± 2.87	3.36	90.65
	HQC	150	146.15	97.43 ± 0.37	0.47	146.74	97.82 ± 1.21	1.53	88.65
MEHHP	LLOQ	1	0.86	86.42 ± 3.42	3.95	0.92	91.71 ± 8.74	1.14	86.14
	LQC	3	2.85	95.02 ± 11.45	12.05	2.73	91.15 ± 5.38	3.12	94.63
	MQC	100	97.79	97.79 ± 5.70	7.15	104.78	104.78 ± 2.67	5.91	80.77
	HQC	150	156.12	104.08 ± 2.74	3.23	152.51	101.67 ± 0.94	9.54	89.65
MBzP	LLOQ	1	1.06	106.68 ± 3.19	2.99	1.14	114.00 ± 10.25	9.00	94.08
	LQC	3	2.91	97.1 ± 7.43	7.65	3.04	101.20 ± 8.32	8.23	91.54
	MQC	100	98.42	98.42 ± 5.22	6.5	102.55	102.55 ± 1.13	1.36	88.56
	HQC	150	150.87	100.58 ± 0.77	0.94	154.46	102.97 ± 0.67	0.80	87.65
MiNP	LLOQ	1	0.85	85.79 ± 13.42	15.64	1.00	99.98 ± 2.48	2.49	85.65
	LQC	3	2.85	95.27 ± 9.71	10.19	2.83	94.31 ± 6.56	6.96	86.65
	MQC	100	97.41	97.41 ± 4.69	5.89	101.59	101.58 ± 1.96	2.37	88.65
	HQC	150	151.84	101.23 ± 0.75	0.91	158.10	105.39 ± 1.18	1.37	94.56