A Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Method to Quantitate Organophosphate Flame Retardants, Isopropylated Phenyl Phosphate (IPP) and Triphenyl Phosphate (TPHP): Application to a Perinatal Dose-Range Finding Study

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Abstract

Organophosphate flame retardants such as IPP and TPHP are increasingly used in consumer products. The National Toxicology Program performed perinatal dose-range finding toxicity studies following exposure of Harlan Sprague Dawley (HSD) rats via feed to IPP and TPHP. The IPP test article used was an isomeric mixture of mostly mono-(36.9%), di- (21.9%), and tri-(8.5%) isopropyl substituted phenyl rings and TPHP (21.5 %). The objective of this work was to develop a method to quantitate TPHP and IPP isomers simultaneously in rodent matrices to assess maternal transfer and their potential to cross the blood brain barrier (BBB). Standards were prepared by spiking 100 µL of rat plasma with IPP or TPHP and $^{13}C_{18}$-TPHP (internal standard). Samples were extracted with acetonitrile, the supernatant was passed through a phospholipid removal filter, and analyzed by LC-MS/MS. The method was linear (r ≥ 0.99, 8 -150 ng/mL), and accurate (relative error ≤ ± 15%) with the limit of quantitation of 8 ng/mL for all analytes.

The method was applied to samples from dose-range finding studies following exposure of HSD rats to IPP or TPHP via feed at 0, 1000, and 10,000 ppm. In animals exposed to IPP, all isomers (IPP and TPHP) were quantifiable in GD 18 dam plasma with combined total isomer concentrations of 2120 (± 690) and 15,900 (± 1370) ng/mL for 1000 and 10,000 ppm, respectively. All isomers were also quantifiable in GD 18 fetal (450 (± 370) and 3340 (± 510)) ng/g 1000 and 10,000 ppm, respectively) and PND 4 pup homogenate (5230 (± 5190) ng/g for 1000 ppm)) demonstrating maternal transfer. Isomer pattern changed from that of the test article administered suggesting differences in disposition between isomers. Regardless of developmental stage, as the degree of substitution increased, the concentration increased with tri-IPP > di-IPP > mono-IPP > TPHP. Maternal transfer was also observed in animals exposed to TPHP via feed. All isomers were also quantifiable in the brains of pups on PND 28 with IPP isomer brain:plasma ratio decreasing from ~20:1 to ~ 1:1 as the number of substitutions increased. Our data shows that IPP and TPHP are transferred from the dam to pups during gestation and lactation and also cross the BBB.

Introduction

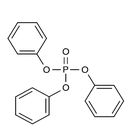
- IPP is a complex mixture used primarily as a flame retardant and is a component of Firemaster™550 and other commercial flame retardant mixtures. TPHP exists as a single isomer and in addition to its use as a flame retardant, is also used as a plasticizer in many consumer products, including nail polish[1].
- The major components of IPP are listed below.
  - Unsubstituted: triphenyl phosphate (TPHP, ~22%)
  - Mono-substituted isomers: m-, o-, or p-isopropylphenyl diphenyl phosphate (mono-IPP, ~37%)
  - Di-substituted isomers: m-, o-, or p-bis(isopropylphenyl) phenyl phosphate (di-IPP, ~22%)
  - Tri-substituted isomers: m-, o-, or p-tris(isopropylphenyl) phosphate (tri-IPP, ~8.5%)
- There is limited toxicological data available showing some evidence that IPP may specifically target reproductive or neurological endpoints[2]. The data available for TPHP is similarly scarce, but a few studies have demonstrated endocrine disrupting effects[3,4], and receptor-associated expression of mRNA[4] in zebrafish embryos. Acute oral toxicity for both compounds is relatively low in rats, with LD50 values of >5000 mg/kg and >3500 mg/kg for IPP and TPHP, respectively[5].
- Due to the potential for wide human exposure and the relative lack of toxicity data available for both chemicals, the National Toxicology Program (NTP) has begun a series of tiered developmental and developmental neurotoxicology studies of IPP and TPHP in Hsd:Sprague Dawley®SD (HSD) rats.
- Measurement of internal dose and potential maternal transfer, as well as the potential exposure to the brain, is important in understanding the outcomes of these studies.

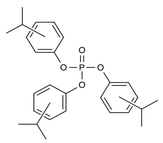
Objectives

- Present the chemical characterization results for the complex mixture, IPP.
- Develop and qualify an analytical method to determine concentrations of TPHP and multiple IPP isomers present in Hsd:Sprague Dawley®SD® (HSD) rat plasma, GD18 fetus and PND4 pup homogenate, and PND28 pup brain.
- Using samples from the NTP dose range-finding studies:
  - Determine maternal transfer.
  - Determine the potential for TPHP and isomers of IPP to cross the blood-brain barrier.

Materials

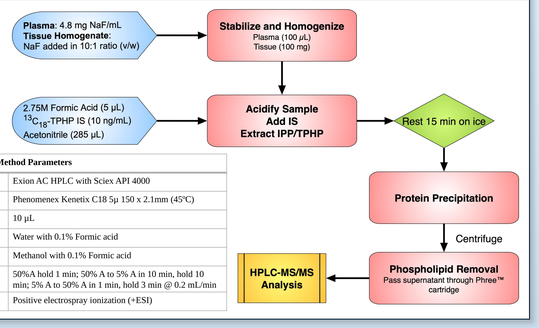
- **Triphenyl phosphate (TPHP)**
  - CASRN: 115-86-6
  - Molecular weight: 326.29 g/mol
- **Isopropylated phenyl phosphate (IPP)**
  - CASRN: 68937-41-7
  - Molecular weight: 452.53 g/mol





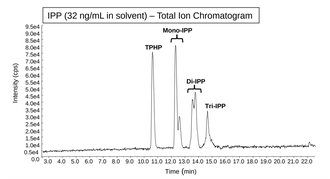
Major IPP Isomers by GC/MS	
Area %	Isomer
26.01	Triphenyl phosphate
32.50	Isopropylphenyl diphenyl phosphate
2.24	Bis(isopropylphenyl) phenyl phosphate
17.78	Bis(isopropylphenyl) phenyl phosphate
4.13	Tris(isopropylphenyl) phosphate
6.53	Tris(isopropylphenyl) phosphate
2.79	Tris(isopropylphenyl) phosphate
1.18	Tris(isopropylphenyl) phosphate

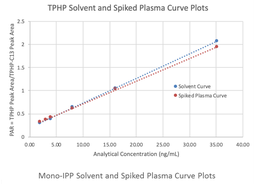
HPLC-MS/MS Method Summary

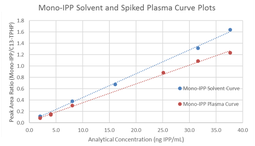


HPLC-MS/MS Method Parameters	
Instrument	Exion AC HPLC with Sciex API 4000
Column (Temp.)	Phenomenex Kenetix C18 5µ 150 x 2.1mm (45°C)
Injection Volume	10 µL
Mobile Phase A	Water with 0.1% Formic acid
Mobile Phase B	Methanol with 0.1% Formic acid
Gradient @ Flow Rate	50%A hold 1 min; 50% A to 5% A in 10 min, hold 10 min; 5% A to 50% A in 1 min, hold 3 min @ 0.2 mL/min
Ionization	Positive electrospray ionization (+ESI)

Quantitative Method Features – TPHP and IPP





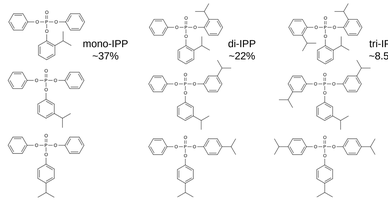


Isomer Group Category	Total Number Isopropyl Substituents	HPLC Retention Time (min)	Precursor Ion ((M+H)+); u)	Fragment Ions (u)^	Collision Energy (eV)
TPHP	0	10.5	327	**77** 152	61 51
Mono-IPP	1	12.0 – 12.6	369	**327** 152	29 61
Di-IPP	2	13.2 – 13.9	411	**327** 369	35 25
Tri-IPP	3	14.3 – 15.3	453	**327** 411	45 25

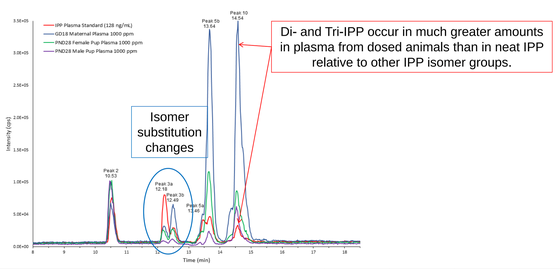
^ Transitions in bold were used for quantitation

- A quantitative LC-MS/MS method was developed and qualified for TPHP and 4 isomeric classes of IPP components in rat plasma, fetus and pup homogenate, and brain.
- The method was linear ((r ≥ 0.99, 8 -150 ng/mL) and accurate (relative error ≤ ± 15%) with the limit of quantitation of 8 ng/mL for all analytes.
- Peaks corresponding to mono-, di- and tri-IPP classes, were composed of multiple isomers.

Selected isomers present in neat IPP



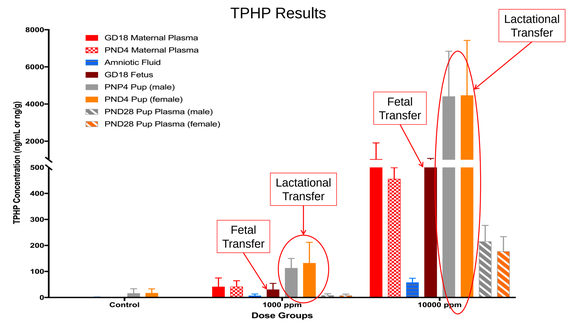
IPP Isomer Ratios: Neat IPP vs. Plasma IPP

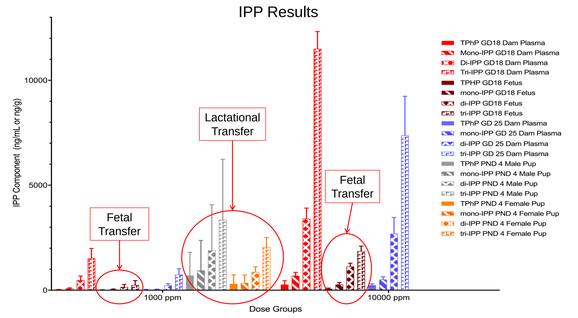


IPP Peak Ratio	Neat IPP	Dose (ppm)	GD18 Dam Plasma	GD18 Fetus	GD18 Amniotic Fluid	GD25 Dam Plasma	PND4 Pup	PND28 Pup Plasma
Peak 3:Peak 5 (mono:di)	1.56:1	1000	1:5.7	1:4.4	1:3.7	1:5.5	1:1.7 ♂ 1:1.4 ♀	1:2.0 ♂ 1:2.5 ♀
		10,000	1:4.9	1:4.2	1:4.0	1:2.0	--[a]	--[a]
Peak 3:Peak 10 (mono:tri)	3.60:1	1000	1:18.3	1:6.8	1:3.8	1:16.9	1:2.6 ♂ 1:2.8 ♀	1:3.6 ♂ 1:5.9 ♀
		10,000	1:16.6	1:6.8	1:5.7	1:14.7	--[a]	--[a]
Peak 5:Peak 10 (di:tri)	2.31:1	1000	1:3.2	1:1.6	1:1.0	1:3.1	1:1.5 ♂ 1:2.0 ♀	1:1.8 ♂ 1:2.4 ♀
		10,000	1:3.4	1:1.6	1:1.4	1:3.1	--[a]	--[a]

- Within an isomeric substitution group (e.g. mono-IPP) the proportion of each isomer present in plasma changes relative to neat IPP.
- More substituted IPP is present at higher concentrations in plasma than neat IPP.
- Plasma concentrations are in the order of: TPHP ≈ mono-IPP << di-IPP ≈ tri-IPP.

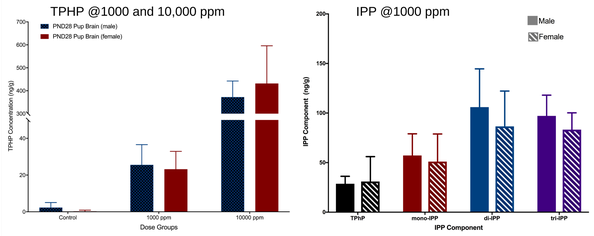
TPHP and IPP Lactational and Fetal Transfer





- Stability of the reported analytes in each matrix was not known at the time of the analysis, so values may be underestimated.
- Maternal transfer from HSD rat dams to fetuses and pups was demonstrated for TPHP and all 4 isomeric classes measured for IPP.
- The IPP isomer concentration in each matrix varied based on the degree of isopropyl substitution.
  - Unsubstituted IPP (TPHP) yielded the lowest matrix concentration followed by mono-IPP, di-IPP, and finally tri-IPP with the highest concentration.

TPHP and IPP Brain Results



	TPHP Mean (%RSD) ng/g	TPHP Mean (%RSD) ng/g	TPHP Mean (%RSD) ng/g	Mono-IPP Mean (%RSD) ng/g	Di-IPP Mean (%RSD) ng/g	Tri-IPP Mean (%RSD) ng/g
Study	TPHP DRF	TPHP DRF	IPP DRF	IPP DRF	IPP DRF	IPP DRF
Dose (ppm)	1000	10,000	1000	1000	1000	1000
PND28 ♂ Brain[1]	25.6 (42.9)	373 (18.8)	28.7 (26.2)	57.2 (38.2)	106 (36.4)	97.1 (21.6)
PND28 ♂ Plasma[1]	8.5 (69.2)	215.0 (29.9)	1.7 (1.8)	14.6 (8.2)	69.9 (45.0)	223 (122)
♂ Brain:Plasma[2]	3.01:1	1.72:1	17.2:1	3.9:1	1.5:1	0.44:1
PND28 ♀ Brain[1]	23.2 (41.9)	432 (32.0)	30.9 (81.7)	51.0 (54.8)	86.5 (41.2)	83.3 (20.3)
PND28 ♀ Plasma[1]	8.2 (59.4)	177.6 (31.6)	1.4 (2.1)	12.9 (10.8)	57.3 (41.9)	174 (95.4)
♀ Brain:Plasma[2]	2.82:1	2.43:1	22.6:1	4.0:1	1.5:1	0.48:1

[1] Stability of the reported analytes in each matrix was not known at the time of the analysis.

[2] Brains were not perfused. Experimentally determined blood:plasma ratio was found to be ~1:1 for TPHP and all IPP isomers, so plasma concentrations were used to calculate ratios.

- Detectable concentrations of TPHP and all 4 isomeric classes of IPP were measured in male and female PND28 HSD rat brain.
- No sex differences were observed.
- Brain to plasma ratios were generally >1 suggesting distribution of the isomers into brain.
- Differences in the uptake of IPP isomers was observed.
  - Higher substituted IPP isomers (di- and tri-IPP) were present in plasma and brain at higher concentrations than IPP-derived TPHP and mono-IPP.
  - Brain:Plasma ratios of the IPP isomers were reversed, with TPHP and mono-IPP ratios > di-IPP ~ tri-IPP.

TPHP Levels Following TPHP and IPP Exposure

Sample	Dose (ppm)	TPHP (ng/g)/(mg/kg)[a,c]	TPHP from IPP (ng/g)/(mg/kg)[a,c]
GD18 Dam Plasma	1000	0.042	0.132
	10,000	0.104	0.124
PND4 Dam Plasma	1000	0.0418	0.101
	10,000	0.046	0.112
Amniotic Fluid	1000	0.0074	0.051
	10,000	0.0058	0.013
GD18 Pooled Fetus	1000	0.031	0.061
	10,000	0.654	0.032
PND4 ♂ Pup	1000	0.113	3.26
	10,000	0.441	--[b]
PND4 ♀ Pup	1000	0.133	1.40
	10,000	0.447	--[b]
PND28 ♂ Pup Plasma	1000	0.0085	0.0077
	10,000	0.022	--[b]
PND28 ♀ Pup Plasma	1000	0.0082	0.0062
	10,000	0.018	--[b]
PND28 ♂ Pup Brain	1000	0.026	0.133
	10,000	0.037	--[b]
PND28 ♀ Pup Brain	1000	0.023	0.143
	10,000	0.043	--[b]

[a] Normalized data. TPHP dose normalization based on ppm dose and percent TPHP in IPP (21.5%). TPHP dose equivalent to 1000 ppm IPP dose = 215 ppm.

[b] No 10,000 ppm samples received.

[c] Stability of reported analytes in each matrix was not known at the time of the analysis.

- Dose-normalized internal TPHP exposure was higher in IPP-dosed animals than in TPHP dosed animals.
- Dose-normalized TPHP concentrations were similar between the 1000 or 10,000 ppm doses* indicating linear kinetics.

*Except GD18 fetus and PND28 male and female pup plasma.

Conclusions

- An HPLC-MS/MS method was developed and qualified for the analysis of TPHP and IPP component isomers in HSD rat plasma, fetus, pup homogenate and brain matrices.
- The analysis method was applied to samples from two dose-range finding studies of TPHP and IPP to determine whether maternal transfer occurred between dosed dams and their offspring during gestation or lactation and to see if TPHP or IPP component isomers crossed the blood brain barrier.
- Results of the analysis demonstrated maternal transfer during gestation and lactation for both TPHP and IPP components.
- Plasma and tissue concentrations of di-, and tri-substituted IPP isomers were higher than unsubstituted or mono-substituted IPP isomers, suggesting differences in the metabolism or distribution of the IPP isomers based upon substitution patterns.
- No sex differences were observed in any matrix.
- Brain:Plasma ratios from IPP-dosed animals were generally >1, suggesting distribution into brain.
- Di- and tri-substituted IPP isomers had lower Brain:Plasma ratios than mono-substituted IPP isomers or TPHP.
- Comparison of dose-normalized TPHP matrix concentrations from IPP- vs. TPHP-dosed animals showed generally higher TPHP matrix concentrations resulting from IPP dosing.
- Dose-normalized TPHP matrix concentrations were similar between the 1000 and 10,000 ppm dose groups suggesting linear TPHP kinetics.
- The analytical method is undergoing validation and stability of the analytes will be assessed in multiple matrices to support planned definitive toxicology studies.

References

1. Mendelsohn, E; Hagopian, A; Hoffman, K; Butt, CM; Lorenzo, A; Congleton, J; Webster, TF.; Stapleton, HM. (2016-01-01). "Nail polish as a source of exposure to triphenyl phosphate". Environment International. 86: 45–51. doi:10.1016/j.envint.2015.10.005.
2. ATSDR (2009)
3. Liu, C; Wang, Q; Liang K; Liu J; Zhou, B; Zhang, X; Liu, H; Giesy, JP; Yu, H. (2013). Aquatic Toxicology. 128-129: 147-147.
4. Zhang, Q; Ji, C; Yin, X; Yan, L; Lu, M; Zhao, M. (2016). Environmental Pollution. 210: 27-33.
5. USEPA Comptox Database. http://comptox.epa.gov (accessed 2/6/2019).

Acknowledgments

This work was funded through a contract with MRIGlobal from the Division of the National Toxicology Program within the U.S. Department of Health and Human Services, National Institutes of Health, National Institute of Environmental Health Sciences, Contract Award Number HHSN273201400020C.