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Determination of *ortho*-phenylphenol in human urine by gas chromatography–mass spectrometry

Michael J. Bartels[a,*], Kathy A. Brzak[a], Gary A. Bormett[b]

[a]*Health and Environmental Research Laboratories, The Dow Chemical Company, 1803 Building, Midland, MI 48674, USA*
[b]*Analytical Services Department, DowElanco, Indianapolis, IN, USA*

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Abstract

A sensitive gas chromatographic–mass spectrometric method was developed to quantitate total *o*-phenylphenol (OPP) (free plus conjugates) in human urine. Conjugates of OPP were acid-hydrolyzed to free OPP, derivatized to the pentafluorobenzoyl ester derivative and analyzed via negative-ion chemical ionization gas chromatography–mass spectrometry. Two stable isotope analogs of OPP were shown to be suitable as internal standards for this method (D_2-phenol ring, $^{13}C_6$-phenyl ring). A synthetic method is presented for the preparation of the D_2-OPP internal standard. The $^{13}C_6$-OPP analog was also shown to be useful as an alternate test material for laboratory-based exposure studies. The limit of quantitation for this method was 1 ng OPP/ml urine. Calibration curves were linear for the analyte over the concentration range of 0.5–1117 ng OPP/ml urine. Relative recovery of OPP from urine ranged from 97.0 to 104.7%. Low levels of OPP (mean=6±7 ng/ml; n=22) were found in control human urine samples. The method was validated with urine samples obtained from human volunteers undergoing a dermal exposure study with ^{12}C-/$^{13}C_6$-/^{14}C-OPP. This method was developed to aid in assessments of human exposure to OPP during a variety of uses of the compound. Published by Elsevier Science B.V.

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1. Introduction

The compound *o*-phenylphenol (OPP) is used as a fungicide for citrus fruits, a component of household and commercial disinfectant formulations as well as a preservative in a variety of industrial applications [1]. This compound has been shown to cause urinary bladder tumors in male Fisher 344 rats, following high, chronic administration [2]. No bladder toxicity/tumors have been seen in any other species tested chronically [3]. To assess human exposure during a variety of uses, a biomonitoring method is needed to quantitate the amount of OPP in human urine samples.

Numerous methods have been developed for the determination of similar phenols in biological media. Quantitation of chlorophenol isomers in human urine has been performed via high-performance liquid chromatography–mass spectrometry (HPLC–MS) as well as gas chromatography (GC) with either electron capture (ECD) or mass spectral (MS) detection [4–6]. Analysis of a variety of alkylphenols has also

*Corresponding author.

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been performed with HPLC and ultraviolet detection (HPLC–UV), GC of either organic extracts or direct headspace sampling, or GC–MS analysis [7–14]. These analyses generally employ chemical or enzymatic hydrolysis of any metabolic conjugates of the phenols to allow for the measurement of total analyte present in the urine samples.

Several methods have also been reported for the determination of OPP in biological matrices. Unconjugated OPP has been determined in urine as a dansyl derivative or underivatized in serum samples via HPLC with fluorescence detection (HPLC–FL) [15,16]. Detection limits for these methods were 20 ng/ml urine and 0.2 ng/ml serum. OPP has also been quantitated in serum samples (unconjugated) via GC–ECD with a detection limit of 20 ng/ml [17]. GC analysis with flame ionization detection (GC–FID) has also been utilized to measure total OPP in urine samples (free plus conjugates) with detection limits of 200 ng/ml and 780 ng/ml, respectively for the underivatized analyte [8,18].

In the current work, a sensitive and selective biomonitoring method is described for the quantitation of OPP in human urine samples. MS detection is employed to allow for the incorporation of stable-isotope labelled test materials and internal standards in this assay. Urine samples were also obtained from a recent dermal exposure study, in which 0.4 mg $^{13}C/^{14}C$-OPP was administered dermally to the forearm of six human volunteers for a period of 8 h [19]. OPP was well absorbed under these conditions (43%), with an absorption half-life of approximately 10 h. Independent validation data for this biomonitoring method, obtained from the analysis of selected urine samples from the recent human exposure study, are also presented.

2. Experimental

2.1. Chemicals

OPP was obtained from Bayer Chemical, Agricultural Division (Stilwell, KS, USA). The purity of OPP was determined to be 99.5% via HPLC. $^{13}C_6$-OPP (phenyl ring label) was obtained from Isotec (Miamisburg, OH, USA; purity=99.94% via GC). ^{14}C-OPP (uniformly labelled in the phenol ring; 48.37 mCi/mmol) was obtained from Wizard Laboratories (Sacramento, CA, USA; radiochemical purity=97.9% via HPLC). Pentafluorobenzoyl chloride, D_2O, CH_3OD and D_2SO_4 were obtained from Aldrich (Milwaukee, WI, USA). All other compounds and solvents were reagent grade or better.

D_2-OPP was synthesized as follows: unlabelled OPP (2.0 g; 12 mmol) was added to a solution of 20 ml D_2O (1107 mmol), 35 ml CH_3OD (860 mmol) and 20 ml concentrated D_2SO_4 (380 mmol). This mixture was heated to reflux for 4 h (OPP dissolved upon reaching reflux). The reaction mixture was then allowed to cool to room temperature, diluted with 150 ml distilled water and extracted with toluene (1×250 ml+1×150 ml). The first toluene extract was washed with 100 ml distilled water. The toluene extracts were then combined, taken to dryness (nitrogen stream, room temperature) and the residue recrystallized from hexane to afford 1.17 g (58%) of the desired product as an off-white crystalline solid, m.p. 58–59°C. Nuclear magnetic resonance (NMR) analysis showed incorporation of deuterium at the C_4 and C_6 positions of the phenol ring: ^{13}C NMR ($CDCl_3$) and 115.1 (t, 1, C_6) ($J_{C\text{-}D}$=2.5 Hz), 120.5 (t, 1, C_4) ($J_{C\text{-}D}$=2.5 Hz), 127.8 (s, 1, C_{10}), 128.2 (s, 1, C_2), 128.7 (s, 1, C_5), 129.0 (s, 1, C_9), 129.1 (s, 1, C_8), 130.0 (s, 1, C_3), 137.0 (s, 1, C_7), 152.3 (s, 1, C_1). High-resolution MS detection analysis (electron impact, 70 eV; 8000 resolving power) confirmed the elemental composition of the deuterated OPP: calculated=172.085719 u, measured=172.085439 u (0.3 ppm deviation).

2.2. Sample preparation

Weighed aliquots of urine from a controlled human exposure study [19] (1 ml) were transferred to 4-ml glass vials, acidified with 100 μl concentrated HCl and heated in a 90°C water bath for 15 h. The hydrolyzed samples were allowed to cool to room temperature, fortified with 20 μl of a 10 μg/ml solution of D_2-OPP in water and extracted with 1 ml toluene (vortexed 2 min, centrifuged at 1100 *g* 12–20 min). The toluene extract was transferred to clean 4-ml glass vials and derivatized by the addition of 0.5 ml 0.4 mol NaOH and 50 μl pentafluorobenzoyl chloride (vortexed 30 min at room temp; centrifuged

at 1100 g 5 min). The derivatized toluene layer was then transferred to a 2-ml glass GC autosampler vial for GC–MS analysis. Weighed aliquots of control human urine (1 ml) were fortified with 0.5–1000 ng OPP (0.08–81 μg/ml in water), hydrolyzed and derivatized as above for determination of relative and absolute recovery of OPP from urine. Selected samples were also prepared with $^{13}C_6$-OPP (25 μl of a 8.7 μg/ml solution) as an internal standard, instead of the deuterated OPP.

2.3. Instrumentation

Melting points were determined on an Electrothermal capillary melting point apparatus (UK) and are uncorrected. NMR spectra were recorded on a Bruker AC300 spectrometer (Billrica, MA, USA) using $CDCl_3$ as an internal standard. High-resolution mass spectra were obtained on a VG Autospec mass spectrometer (Manchester, UK) using a magnetic scan of 500–60 u, 10 s/decade. Acid hydrolysis efficiency experiments were performed with the radiolabelled human exposure study urine samples using the HPLC conditions with radiochemical detection, as described in that study [20].

GC–MS analyses were performed on a Finnigan SSQ-710 mass spectrometer (Finnigan MAT, San Jose, CA, USA), equipped with a Hewlett-Packard 5890 gas chromatograph and a 7673A autosampler (Hewlett-Packard, Avondale, PA, USA). Separations were achieved using a J&W DB-17 fused-silica capillary column (J&W Scientific, Folsom, CA, USA) (30 m×0.25 mm I.D., 0.5 μm film); helium carrier gas (10 p.s.i.; 1 p.s.i. = 6894.76 Pa) at a flow-rate of approximately 0.5 ml/min; gas chromatograph oven temperature programmed from 180°C (1 min initial hold) to 280°C at 8°C/min; injector and capillary transfer line at 275°C; 2-μl autosampler injection (0.05 min splitless). The mass spectrometer conditions [negative-ion chemical ionization (NCI)] were: ion source temperature, 150°C; ionizing current, 0.4 mA; electron energy, 70 eV. Analysis of the pentaflurobenzoyl ester derivatives of OPP, D_2-OPP and $^{13}C_6$-OPP was achieved by either selected-ion monitoring (0.1 s/ion/scan) of the M^- ions m/z 364, 366 and 370, respectively or full-scan analysis (50–600 u, 0.5 s/scan).

2.4. Experimental calculations

The GC–MS peak areas for the derivatized OPP analogs were obtained by integration of the M^- parent ion peak areas using the data system of the mass spectrometer. Peak areas were corrected for isotope ''crossover'' according the method of Barbalas and Garland [21]. A linear least-squares standard curve was obtained from the OPP/internal standard corrected peak-area ratios of the solvent or matrix standard solutions. Concentrations of OPP in the samples and fortified control samples were then calculated using the experimentally derived standard curve.

3. Results and discussion

3.1. Internal standard synthesis

The deuterated analog of OPP was prepared in a simple one-step, acid-catalyzed proton exchange reaction. This aromatic electrophilic substitution reaction afforded a product with two deuterium atoms, at the *ortho* and *para* positions of the phenol ring. The deuterium label for this compound should be stable to a wide range of pH and chemical conditions, as evidenced by the harsh conditions required to incorporate the label. The deuterium label was stable to NCI mass spectral conditions (below).

3.2. Sample preparation and derivatization

A dermally absorbed dose of OPP has been shown to be metabolized primarily to the sulfate and glucuronic acid conjugates in human volunteers [20]. These conjugates, representing 73.0% of the absorbed dose, are rapidly excreted in the urine, with an estimated elimination $t_{1/2}$ of 0.8 h [19]. To permit quantitation of total OPP present in urine samples, acid-catalyzed hydrolysis of these conjugates is usually incorporated into this type of assay [8,18]. Initial hydrolysis efficiency experiments with the radiolabelled human exposure samples indicated that a minimum of 80°C was required for complete hydrolysis of both conjugates of OPP. A final hydrolysis temperature of 90°C was therefore utilized

to ensure complete conjugate cleavage within the 15 h hydrolysis interval.

Once hydrolyzed, the OPP was found to be well extracted from urine into toluene. The absolute extraction efficiency of OPP was determined to be 90.3±4.5% (n=4). Following extraction, the analyte and internal standard were converted to the corresponding pentaflurobenzoyl ester derivatives. As shown in Fig. 1, this derivative affords a simple NCI mass spectrum, consisting primarily of the M^- parent ion. This pentafluorobenzoyl ester derivative and the NCI mass spectral detection employed in this assay were chosen due to the high degree of selectivity and sensitivity that this derivative affords in combination with electron-capture negative-ion detection [22].

3.3. Chromatography

The NCI-GC–MS method employed in this assay was found to be quite selective and sensitive for the analysis of OPP in human urine. Analysis of a 0.5 ng/ml solvent standard of OPP afforded a GC–MS peak for the analyte at approximately 100×noise (Fig. 2A). The deuterated internal standard was quite isotopically pure, with contributions of only 0.11% and 0.03% to the analyte peak areas of m/z 364 and 370, respectively. The $^{13}C_6$-OPP, used as an analyte or an internal standard, was also relatively isotopically pure, contributing 4.37% and 0.25% of its parent ion peak area to m/z 364 and 366, respectively.

A representative set of selected ion chromatograms for a control human urine sample is shown in Fig. 2B. No interferences were observed for the D_2- or $^{13}C_6$-OPP in this sample (m/z 366 or 370, respectively). A minor GC–MS peak was found to have the same mass and retention time as the pentafluorobenzoyl derivative of OPP. This background OPP may be a result of exposure to this chemical or may be a metabolite of absorbed biphenyl [23–25]. This exogenous analyte was found in all control human urine samples analyzed, with a mean concentration of 6±7 ng/ml urine (1–36 ng/ml; n=22). The signal/noise for the background OPP shown in Fig. 2B (3 ng/ml) was approximately 200×noise. Based on this data, and the recovery data shown below, the limit of quantitation for this assay was set at 1 ng OPP/ml urine.

The GC–MS data for one of the urine samples from a laboratory-based, human exposure study is shown in Fig. 2C. This urine sample was obtained from a volunteer given a dermal application of a mixture of OPP/^{14}C-OPP/$^{13}C_6$-OPP [19]. The radiotracer was employed in the study to obtain a mass balance of the administered dose in all of the samples collected from the experiment. The $^{13}C_6$-OPP was utilized to allow for the quantitation of low levels of applied dose regardless of the amount of

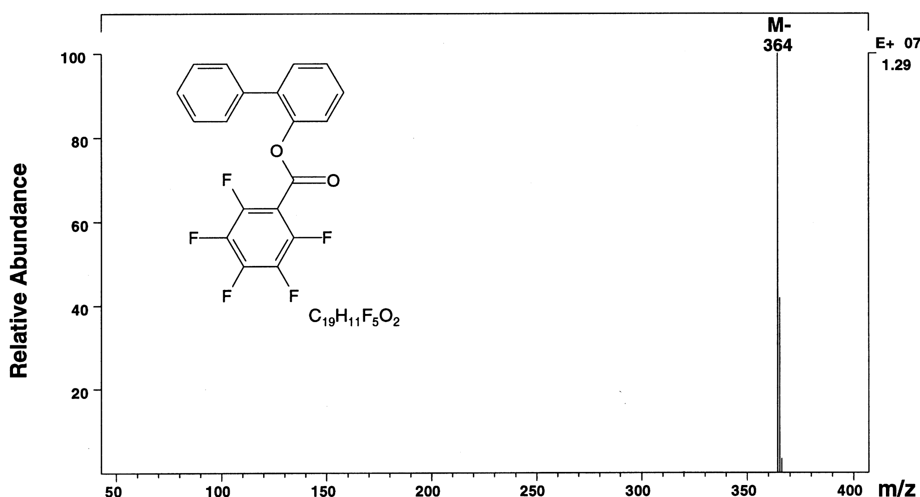

Fig. 1. Negative-ion chemical ionization mass spectrum of the pentafluorobenzoyl ester derivative of OPP (247 μg/ml).

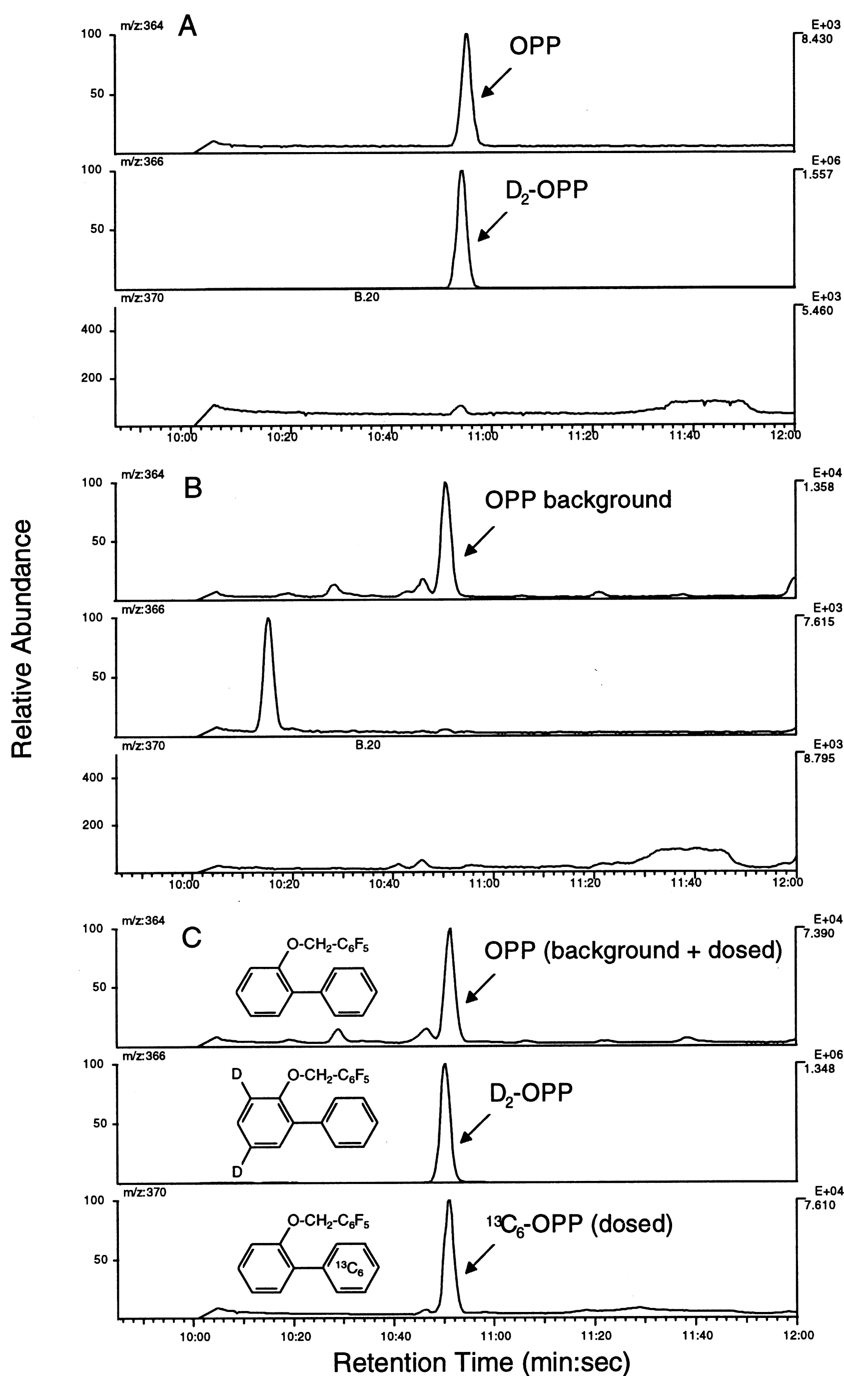

Fig. 2. Selected-ion chromatograms for OPP and stable isotope analogs as pentafluorobenzoyl ester derivatives (NCI). (A) Solvent standard containing 0.5 ng OPP and 200 ng D_2-OPP internal standard per ml; (B) extract of control urine sample containing 3 ng OPP per ml urine; (C) extract of exposure study urine sample containing 15 ng OPP (6 ng background + 9 ng dosed) per ml urine, 200 ng D_2-OPP internal standard per ml urine and 15 ng $^{13}C_6$-OPP (dosed) per ml urine.

background OPP present in the sample. From a standard curve of $^{13}C_6$-OPP/D_2-OPP, the concentration of $^{13}C_6$-OPP analyte in this sample was calculated to be 15 ng/ml. Analysis of the dose solution mixture afforded the ratio of the three analyte isotopomers, which could then be used to calculate the ratio of dosed/background unlabelled OPP in the sample (9 ng/ml dosed/6 ng/ml background for Fig. 2C). Utilization of the ^{13}C-OPP as a test material in this study was therefore extremely useful, in that it allowed for the quantitation of low levels of analyte from an exposure study, independent of background concentrations of the target analyte.

The mass spectral response for OPP was linear and reproducible over the concentration range of 0.5–1100 ng/ml. The regression coefficients for matrix standards containing OPP and the D_2-OPP internal standard were $m=0.0044\pm0.0004$ and $b=1.0001\pm0.0215$ for the standard curve $y=mx^b$, with a correlation coefficient of 0.999 ± 0.001 ($n=8$). Comparable results were obtained from solvent standards containing OPP and the deuterated internal standard ($m=0.0038$, $b=1.0090$, $r=0.999$; $n=1$) or matrix standards containing OPP and the $^{13}C_6$-OPP internal standard ($m=0.0074$, $b=0.9302$, $r=0.997$; $n=1$).

3.4. Quantitative analysis

Triplicate aliquots of control human urine were fortified with 1–466 ng OPP/ml urine and analyzed to measure intra-day accuracy of the method. The average relative recoveries for the analyte were found to range from 97.0–104.7% across this concentration range (Table 1). The precision of analysis at these concentrations was also quite good, with a mean standard deviation for the recoveries of 4.0%.

The inter-day variability of this method was also examined. As shown in Table 2, the average relative recovery of OPP from fortified urine samples ranged from 105.7% at the quantitation limit of 1 ng/ml to 92.4% for 186–323 ng/ml ($n=5$–7). The inter-day precision was also acceptable, ranging from 7.2–12.2%.

Comparable accuracy was also achieved when $^{13}C_6$-OPP was employed as an internal standard in this method. Relative recoveries of 99%, 125% and 102% were obtained at OPP concentrations of 1, 12 and 323 ng/ml in control human urine aliquots ($n=1$/concentration).

Both solvent standards and fortified urine matrix standards were found to be usable for the quantitation of OPP from human urine. The intra- and inter-day recovery data above was generated with matrix standards prepared by fortifying control human urine with OPP. While the use of matrix standards provides a more accurate correction for matrix effects on chromatographic or detector response, corrections must be made for the background

Table 2
Inter-day accuracy and precision of OPP quantitation in human urine

OPP concentration (ng/ml)	(%) Recovery	(%) Standard deviation	No. of days
1	105.7	12.2	6
10–30	93.2	7.2	7
186–322	92.4	10.3	5
Mean	97.1	9.9	

Table 1
Intra-day accuracy and precision of OPP quantitation in human urine

OPP concentration	(%) Recovery	(%) Standard deviation	*n*
1	101.7	5.5	3
14	103.0	2.0	3
24	100.6	2.9	3
186	97.0	11.3	3
404	100.0	1.8	3
466	104.7	0.6	3
Mean	101.2	4.0	

level of exogenous OPP present in the control human urine used for these standards. Alternately, the target analyte can be also be quantitated with the use of solvent standards, derivatized with pentafluorobenzoyl chloride. Analysis of ten separate control human urine samples, fortified with 25–30 ng OPP/ml urine, was performed with the use of solvent standards. The relative recovery for these standard addition samples was 93±5% ($n=10$). These results show that either solvent or matrix standard solutions can be used for the accurate determination of OPP in human urine.

Since biomonitoring urine samples are likely to be collected and stored frozen prior to analysis, the stability of total OPP was measured in fortified human urine samples. Pooled urine samples, fortified with 45–47 ng OPP/ml, were found to contain 86.2% of the initial concentration of the analyte after 79 days storage at −20°C ($n=2$). Repeated thawing and refreezing of these samples (three times; total thaw time approximately 8 h) did not contribute to degradation of the analyte (recovery=89.3%). In addition, the absolute recovery data (above), showing >90% recovery of OPP after 15 h acid hydrolysis at 90°C, also indicates excellent stability of the analyte at elevated temperatures.

As described above, urine samples were obtained from human volunteers administered a dermal application of OPP/^{14}C-OPP/$^{13}C_6$-OPP (8 h exposure, 0.4 mg/volunteer). These samples were analyzed via HPLC, with radiochemical detection (HPLC–RAD) and found to contain 73.0% of the absorbed dose, primarily as the sulfate and glucuronic acid conjugates of OPP with less than 1% of the absorbed dose present as free OPP [20]. The precision of analysis for the sulfate and glucuronide conjugates of OPP via this radiochemical method was 3% and 1%, respectively ($n=4$). The accuracy of the HPLC–RAD method, as measured by recovery of total radioactivity from the HPLC system, was 98±14%. To validate the accuracy of the GC–MS biomonitoring method, selected samples from the human exposure study were also analyzed by GC–MS and the results shown in Table 3. The quantitative results of the two methods were quite comparable, with an average of less than 15% difference between the two techniques. These results indicate that the urine biomonitoring method provides an accurate means of determining total OPP (free plus conjugates) present in the urine of humans exposed to this chemical.

4. Conclusions

A selective and sensitive method has been developed for the quantitation of total OPP (free plus conjugates) present in human urine samples. Conjugates of OPP are acid-hydrolyzed to free OPP, derivatized to the pentafluorobenzoyl ester derivative and analyzed via NCI-GC–MS. The limit of quantitation for this method was 1 ng OPP/ml urine. Two separate stable isotope-labelled internal standards were found to be suitable for this method (D_2-phenol ring, $^{13}C_6$-phenyl ring). The $^{13}C_6$-OPP was also shown to be useful as an alternate test material for laboratory-based exposure studies. This biomonitoring method should be extremely useful in a variety of studies to assess low-level dermal exposure of humans to this chemical.

Table 3
Comparison of total OPP determination in human exposure urine samples (8 h dermal application, 0.4 mg/volunteer) via HPLC–RAD and NCI-GC–MS

Exposure urine sample (h)	OPP concentration (ng/ml) via HPLC–RAD	OPP concentration (ng/ml) via NCI-GC–MS	% Difference (GC–MS vs. HPLC)
0–4	131	148	13.0
4–8	119	133	11.8
8–12	49	53	8.2
12–24	24	29	20.8
		Mean	13.4

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References

[1] W.B. Deichman and M.L. Keplinger, in G.D. Clayton and F.E. Clayton (Editors), Patty's Industrial Hygiene and Toxicology, Vol. 2A, Wiley, New York, 3rd ed., 1981, Ch. 36, p. 2616.
[2] K. Hiraga, T. Fujii, Food Cosmet. Toxicol. 22 (1984) 865–870.
[3] R.E. Allan, in G.D. Clayton and F.E. Clayton (Editors), Patty's Industrial Hygiene and Toxicology, Vol. 2B, Wiley, New York, 4th ed., 1994, Ch. 24, p. 1617.
[4] L.H. Wright, T.R. Edgerton, S.J. Arbes Jr., E.M. Lores, Biomed. Mass Spectrom. 8 (1981) 475–479.
[5] J.S. Holler, D.M. Fast, R.H. Hill Jr., F.L. Cardinali, G.D. Todd, J.M. McCraw, S.L. Bailey, L.L. Needham, J. Anal. Toxicol. 13 (1989) 152–157.
[6] M. Veningerová, V. Prachar, J. Uhnák, M. Lukáscová, T. Trnovec, J. Chromatogr. B 657 (1994) 103–110.
[7] A. Brega, P. Prandini, C. Amaglio, E. Pafumi, J. Chromatogr. 535 (1990) 311–316.
[8] M. Ikeda, T. Hirayama, T. Watanabe, I. Hara, Int. Arch. Occup. Environ. Health 41 (1978) 125–138.
[9] P.B. Van Roosmalen, J. Purdham, I. Drummond, Int. Arch. Occup. Environ. Health 48 (1981) 159–163.
[10] J.P. Buchet, Environmental Carcinogens: Methods of Analysis and Exposure Measurement, Vol. 10, WHO-International Agency for Research on Cancer, Lyon, France, 1988, pp. 281–286.
[11] M. Balíková, J. Kohlícek, J. Chromatogr. 497 (1989) 159–167.
[12] L. Weber, J. Chromatogr. 574 (1992) 349–351.
[13] W. Tashov, I. Benchev, N. Rizov, M. Kafedzhieva, A. Kolarsha, Chromatographia 32 (1991) 466–468.
[14] W.M. Pierce Jr., D.E. Nerland, J. Anal. Toxicol. 12 (1988) 344–347.
[15] R.M. Cassidy, D.S. LeGay, R.W. Frei, J. Chromatogr. Sci. 12 (1974) 85–89.
[16] L.L. Needham, R.H. Hill Jr., D.C. Paschal, S.L. Sirmans, Clin. Chim. Acta 107 (1980) 261–266.
[17] R.E. Cline, L.W. Yert, L.L. Needham, J. Chromatogr. 307 (1984) 420–425.
[18] H.-P. Harke, H. Klein, Int. J. Microbiol. Hyg. 174 (1981) 274–278.
[19] C. Timchalk, G.K. Songha and S. Selim, manuscript in preparation.
[20] M.J. Bartels, C. Timchalk, D.A. McNett, K.A. Brzak, A.L. Mendrala, G.K. Songha and W.R. Christenson, manuscript in preparation.
[21] M.P. Barbalas, W.A. Garland, J. Pharm. Sci. 80 (1991) 922–927.
[22] D.F. Hunt, F.W. Crow, Anal. Chem. 50 (1978) 1781–1784.
[23] T. Meyer, R.R. Scheline, Acta Pharmacol. Toxicol. 39 (1976) 419–432.
[24] T. Meyer, J.C. Larsen, E.V. Hansen, R.R. Scheline, Acta Pharmacol. Toxicol. 39 (1976) 433–441.
[25] T. Meyer, Acta Pharmacol. Toxicol. 40 (1977) 193–200.