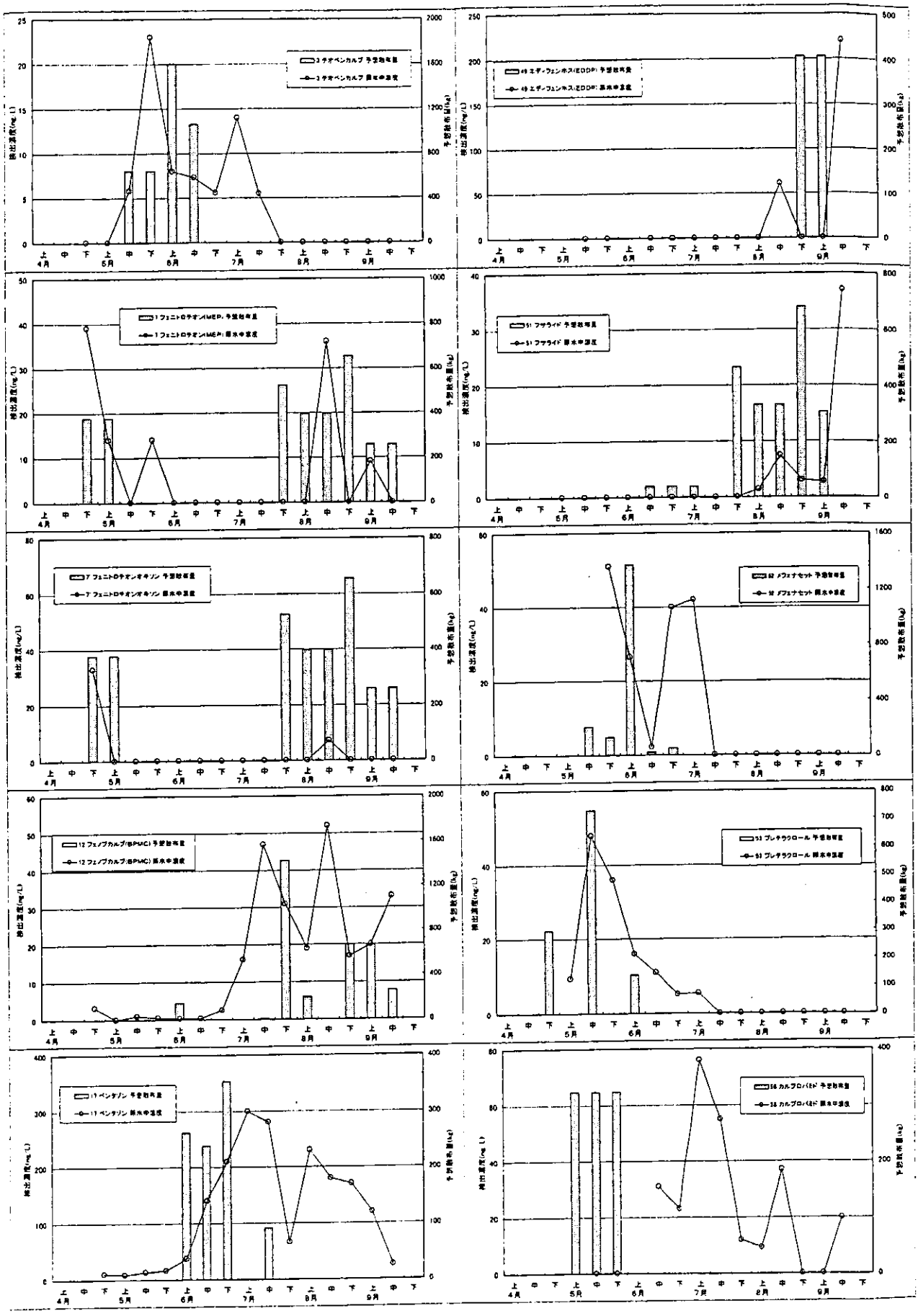
	農薬（新水道水質基準番号、農薬名、目標値（ng/Lで表示））		測定関連		測定数	高陽浄水場								緑井浄水場							
			測定方法*1	検出下限値の中央値*2		原水				浄水				原水				浄水			
						検出数	最大値	中央値	最小値	検出数	最大値	中央値	最小値	検出数	最大値	中央値	最小値	検出数	最大値	中央値	最小値
27	エトリジアゾール（エクロメゾール）	4000	SPE-GCMS	0.81	18	0				0				0				0			
28	オキシン銅	40000	SPE-HPLC	600	18	0				0				0				0			
29	キャプタン	300000	SPE-GCMS	8.7	18	0				0				0				0			
30	クロロネブ	50000	SPE-GCMS	0.35	18	1	17	17	17	1	19	19	19	1	19	19	19	1	24	24	24
31	トルクロホスメチル	200000	SPE-GCMS	1.5	18	0				0				0				0			
32	フルトラニル	200000	SPE-GCMS	0.62	18	9	38	7.4	0.79	7	36	4.5	0.19	9	9.9	4.6	0.45	6	7.5	6.0	2.0
33	ペンシクロン	40000	SPE-GCMS	200	18	0				0				0				0			
34	メタラキシル	50000	SPE-GCMS	6.3	18	0				0				0				0			
35	メプロニル	100000	SPE-GCMS	3.2	18	4	91	41	13	3	63	39	16	5	60	33	13	3	56	34	21
36	アシュラム	200000	SPE-HPLC	150	18	0				0				0				0			
37	ジチオピル	8000	SPE-GCMS	0.77	18	1	0.67	0.67	0.67	0				0				0			
38	テルブカルブ（MBPMC）	20000	SPE-GCMS	0.31	18	0				0				0				0			
39	ナプロパミド	30000	SPE-GCMS	1.8	18	0				0				0				0			
40	ピリブチカルブ	20000	SPE-GCMS	1.2	18	6	8.1	2.4	0.70	0				6	9.7	1.9	0.36	0			
41	ブタミホス	10000	SPE-GCMS	12	18	0				0				0				0			
42	ベンスリド（SAP）	100000	SPE-HPLC	30	18	0				0				0				0			
43	ベンフルラリン（ベスロジン）	80000	SPE-GCMS	0.47	18	0				0				1	1.3	1.3	1.3	0			
44	ペンディメタリン	100000	SPE-GCMS	20	18	1	17	17	17	1	23	23	23	1	47	47	47	0			
45	メコプロップ（MCPP）	5000	SPE-HPLC	150	18	0				0				0				0			
46	メチルダイムロン	30000	SPE-GCMS	1.1	18	1	3.4	3.4	3.4	0				0				0			
49	エディフェンホス（エジフェンホス、EDDP）	6000	SPE-GCMS	9.3	15	2	220	140	61	2	60	40	19	3	71	26	12	1	36	36	36
51	フサライド	100000	SPE-GCMS	0.73	18	5	37	3.0	1.4	2	9.4	6.9	4.4	3	3.9	3.5	2.7	3	10	6.6	4.0
52	メフェナセット	9000	SPE-GCMS	11	15	4	51	41	2.2	4	69	52	25	5	82	39	1.7	5	95	32	2.3
53	プレチラクロール	40000	SPE-GCMS	1.7	18	8	48	14	5.1	8	66	18	5.1	8	26	18	5.1	7	23	14	2.4
58	カルプロパミド	40000	SPE-GCMS	9.2	15	8	76	27	9.3	8	71	34	1.4	7	62	18	5.3	6	60	25	12
70	エトフェンプロックス	80000	SPE-GCMS	1.5	15	1	0.30	0.30	0.30	0				0				0			
83	エスプロカルブ	10000	SPE-GCMS	14	15	0				0				0				0			
87	トリシクラゾール	80000	SPE-GCMS	54	15	1	55	55	55	1	92	92	92	1	100	100	100	1	170	170	170

＊1　測定方法は旧水道水質基準の検査方法で実施。
SPE-GC：固相抽出-ガスクロマトグラフ法、SPE-GCMS：固相抽出-ガスクロマトグラフ質量分析法、SPEder-GCMS：固相抽出誘導体化-ガスクロマトグラフ質量分析法
HS-GCMS法：ヘッドスペース-ガスクロマトグラフ質量分析法、HPLC-GC法：高速液体クロマトグラフ-ポストカラム法

＊2　調査期間における検出下限値の中央値（SPE-GC法、SPE-GCMS法及びSPEder-GCMS法は測定毎に検出下限値を求めた）。



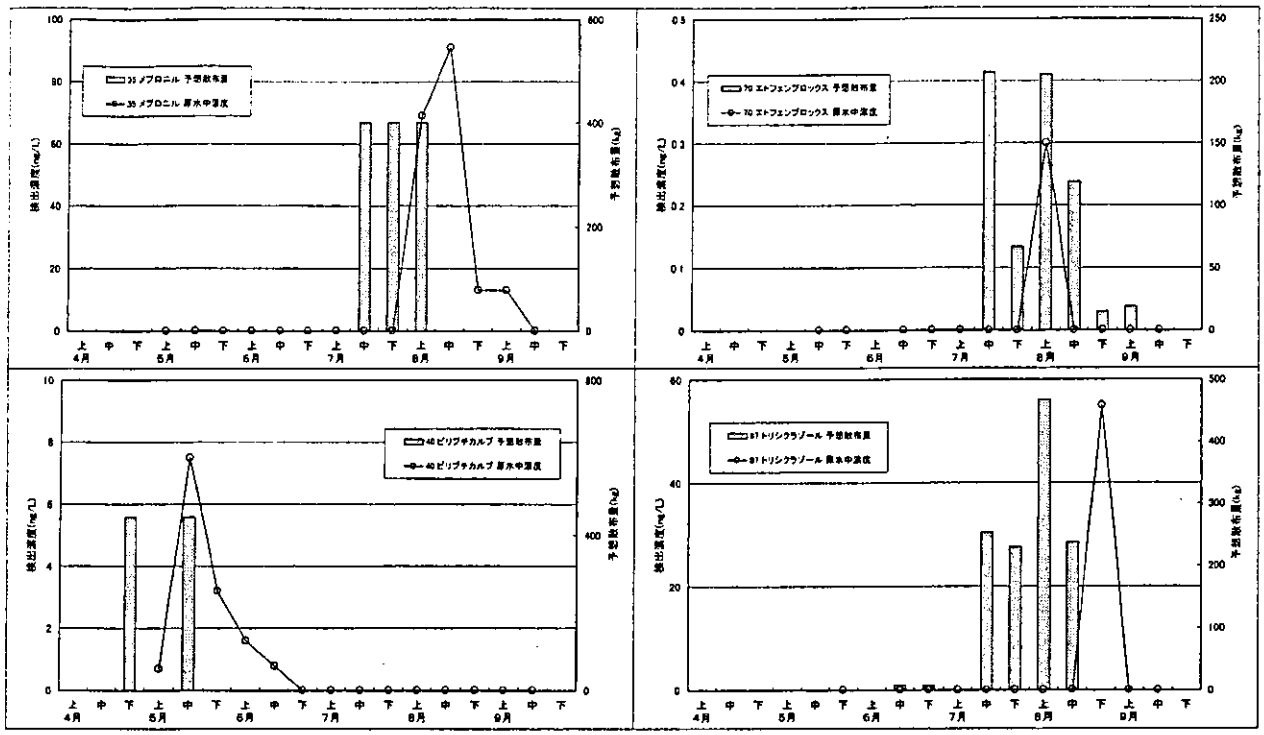


図-2　調査期間における農薬類の原水中濃度と予想散布量の推移（高陽浄水場）

(logKow) の関係を図-3に示す（カルプロパミドは logKow の値が不明だったので除外)。同じ散布量であれば、logKow が高い疎水性物質は土壌や河川中の有機物質に吸着しやすいので、logKow が低い物質に比べると水中濃度が低くなるために散布量と濃度の比は高くなると推測されるが、その通りの傾向が観察された。

3.2　浄水における農薬類

高陽浄水場の浄水（以下、高陽浄水)、緑井浄水場の浄水（以下、緑井浄水）ではそれぞれ延べ21物質、17物質が検出された。検出数が6以上の物質は、緑井浄水ではフェノブカルブ（BPMC)、

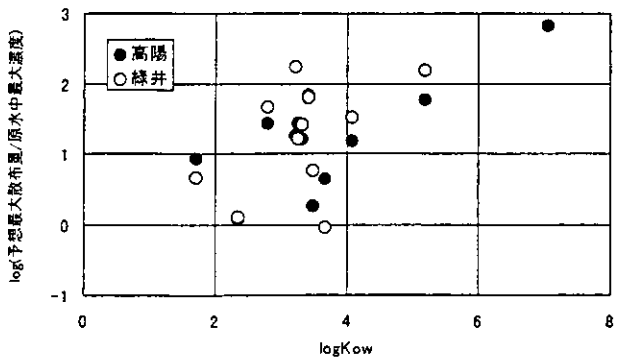


図-3　logKow と log（予想最大散布量/原水中最大濃度）の関係

フルトラニル、プレチラクロール、ベンタゾン、カルプロパミドで、高陽浄水ではこれらに加えてジクロルボス（DDVP）であった。

高陽あるいは緑井浄水場の原水において検出数が4以上の農薬で、原水より浄水での濃度値（最大値、中央値及び最小値)、検出数が減少したのはイプロベンホス（IBP)、シマジン、ダイアジノン、チオベンカルブ、ピリブチカルブ、フェニトロチオン（MEP)、フルトラニル、ベンタゾンであった。このうち IBP、MEP、ダイアジノン、チオベンカルブ、ピリブチカルブについては文献調査[9、10]、ビーカー試験の結果から塩素分解速度が大きいことを確認しているが、浄水工程における塩素による分解で減少したと推察される。

農薬類は新水道水質基準の水質管理目標設定項目となり、指定されている101農薬の検出濃度／目標値（以下、相対健康リスクという）の総和が1を超えないこととされている。そこで調査期間について農薬類の相対健康リスクを算出した。その総和の対数値の調査期間における推移を図-4に示す。なお、約20種類の浄水で検出された農薬のみ（オキソン体を除く）を計算対象とした。高陽及び緑井浄水におけるリスクの最大値はそれぞれ

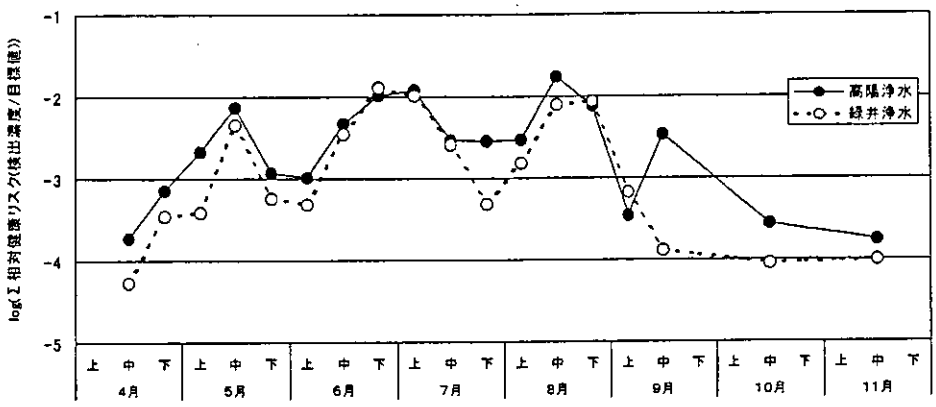


図-4　調査期間における農薬類の相対健康リスクの推移

0.017、0.013で、新水道水質基準の目標値1より2桁程度低かった。

4.　水環境における農薬類の濃度予測

4.1　予測方法

水環境における化学物質の濃度を予測するために環境動態モデル、統計モデル、物理モデルなど様々なモデルが構築されている[11~14]。福島ら[15]は5種類の物質特性、4種類の流域特性を説明変数とした統計モデルを作成した。本研究では、その中でも説明変数の入力が容易な重回帰分析モデルを用いて、広島太田川流域における農薬類の濃度予測への適用について検討することとした。福島らの予測式は以下の通りである。

log（農薬類濃度;mg/L）
＝−4.096−0.168×log(面積;km^2)
　+0.173×log（農地面積;km^2）
　+0.242×log(人口密度;人/km^2)
　+0.034×log（出荷額密度;万円/km^2）
　+0.010×log(ヘンリー定数；atm・m^3/mol 25℃)
　−0.236×logKow
　+1.574×(生分解速度定数；日$^{-1}$)
　−0.020×log(生産量；トン)
　+0.935×log（使用環境）

各説明変数であるが、

(1)　面積、農地面積、人口密度、出荷額密度は浄水場取水地点より上流に位置する市町村の平成12年度広島県統計データ[16]から算出した。

(2)　ヘンリー定数、logKow（オクタノール/水分配係数）はSyracuse Research Corporationのデータベース[17]から収集した。

(3)　生分解速度定数は福島らの方法と同様に収集あるいは予測した[18]。

(4)　生産量は国立環境研究所化学物質データベース[19]の平成12年度全国農薬出荷量データを用いた。

(5)　使用環境についてはすべての農薬類について開放的使用と考え、1.0とした。

なお、農薬類は上記の物質特性及び流域特性データがすべて収集できたものを解析対象とした。

4.2　予測結果

表-2に農薬類の重回帰分析モデルによる予測濃度と浄水場における原水中最大濃度を示す。このモデルによる予測濃度は原水中最大濃度とほぼ同程度であった。予測濃度の対数値と原水中最大濃度の対数値の差の中央値は、高陽浄水場で−0.040、緑井浄水場で0.15であり、重回帰分析モデルの予測値標準誤差が0.6オーダー程度であることを考慮すれば精度良く予測できるといえる。予測濃度と原水中最大濃度の差が1桁以上あったのは高陽浄水場、緑井浄水場ともに20物質のうち3物質であった。

次に検出下限値未満の農薬のデータを用いて検証を行った。本調査で検出されなかった農薬類24物質について重回帰分析モデルによる予測濃度を求め、検出下限値の中央値（HS-GCMS法及びSPE-HPLC法で測定した農薬類は検出下限値）と比較した（表-3）。予測濃度が検出下限値の中央値未満は8物質、検出下限値の中央値＋1桁以内は16物質とおよそ7割が1桁以内に入った。

5.　おわりに

広島市太田川流域に位置する浄水場の原水及び浄水について2002年4月～11月に57種類の農薬類

表-2　農薬類の重回帰分析モデルによる予測濃度と浄水場における原水中最大濃度

農薬（新水道水質基準番号、農薬名）		log（予測濃度）	高陽浄水場		緑井浄水場	
			log（原水中最大濃度）	差	log（原水中最大濃度）	差
2	シマジン（CAT）	1.81	1.48	0.34	1.30	0.51
3	チオベンカルブ	1.53	1.49	0.04	1.40	0.14
6	ダイアジノン	1.43	0.65	0.78	0.35	1.08
7	フェニトロチオン（MEP）	1.55	1.59	-0.04	1.40	0.15
8	イソプロチオラン（IPT）	1.68			1.00	0.68
11	ジクロルボス（DDVP）	1.99	0.89	1.10	0.83	1.16
12	フェノブカルブ（BPMC）	1.67	1.72	-0.04	1.48	0.20
15	イプロベンホス（IBP）	1.54	1.63	-0.09	1.45	0.10
17	ベンタゾン	1.77	2.48	-0.71	2.43	-0.66
20	トリクロピル	1.74	1.90	-0.17	1.61	0.12
30	クロロネブ	1.57	1.23	0.34	1.28	0.29
32	フルトラニル	1.45	1.58	-0.13	1.00	0.45
35	メプロニル	1.47	1.96	-0.49	1.78	-0.31
37	ジチオピル	1.25	-0.17	1.43		
43	ペンフルラリン（ペスロジン）	1.14			0.11	1.02
44	ペンディメタリン	1.12	1.23	-0.11	1.67	-0.55
49	エディフェンホス（エジフェンホス、EDDP）	1.50	2.34	-0.85	1.85	-0.35
51	フサライド	1.56	1.57	-0.01	0.59	0.97
52	メフェナセット	1.55	1.71	-0.16	1.91	-0.37
53	プレチラクロール	1.36	1.68	-0.32	1.41	-0.05
70	エトフェンプロックス	0.67	-0.52	1.19		
87	トリシクラゾール	1.90	1.74	0.16	2.00	-0.10

＊1　濃度の単位は ng/L である。
＊2　差とは log（予測濃度）と log（原水中最大濃度）の差を意味する。

の検出状況を調べた。調査期間には原水、浄水ともに延べ20物質程度の農薬類が検出され、検出率、検出濃度の平均的なものはそれぞれ20%程度、10ng/L オーダーであった。高陽浄水場及び緑井浄水場の浄水で検出された農薬類について相対健康リスク（検出濃度／目標値）の総和を算出すると0.017及び0.013と、新水道水質基準の目標値1より2桁程度低かった。

原水における検出濃度データを用いて、既往の簡易な統計予測モデルの適用について検討した。各農薬類の予測濃度と原水における検出最高濃度の差の中央値は0.1オーダー程度であり、精度良く予測できることを確認した。今後は、本研究で取り上げた重回帰分析モデルの継続的な検証、精度を高めるための物理モデルの構築を図り、多種多様な農薬類に対応可能な監視プライオリティーリストの作成を試みたい。

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表-3　農薬類の重回帰分析モデルによる予測濃度と検出下限値

	農　薬（新水道水質基準番号、農薬名）	a	b	a−b
1	チウラム	1.929	2.176	-0.247
4	1,3-ジクロロプロペン（D-D）	1.868	1.778	0.090
5	イソキサチオン	1.456	1.633	-0.177
9	クロロタロニル（TPN）	1.622	-0.102	1.724
10	プロピザミド	1.550	0.342	1.208
13	クロルニトロフェン（CNP）	1.254	0.740	0.513
16	EPN	1.221	0.845	0.376
18	カルボフラン	1.788	2.176	-0.388
19	2,4-ジクロロフェノキシ酢酸（2,4-D）	1.675	0.380	1.294
21	アセフェート	2.474	2.477	-0.003
23	クロルピリホス	1.186	0.431	0.754
25	ピリダフェンチオン	1.559	1.041	0.517
26	イプロジオン	1.619	2.079	-0.461
27	エトリジアゾール（エクロメゾール）	1.570	-0.092	1.662
29	キャプタン	1.656	0.940	0.716
31	トルクロホスメチル	1.296	0.176	1.120
33	ペンシクロン	1.160	2.301	-1.141
34	メタラキシル	1.944	0.799	1.145
38	テルブカルブ（MBPMC）	1.181	-0.509	1.690
39	ナプロパミド	1.542	0.255	1.287
41	ブタミホス	1.288	1.079	0.209
42	ベンスリド（SAP）	1.353	1.477	-0.124
45	メコプロップ（MCPP）	1.597	2.176	-0.579
83	エスプロカルブ	1.257	1.146	0.111

＊　a は log（予測濃度）、b は log（検出下限値の中央値）で、ともに濃度単位は ng/L。

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Short communication

Analysis of herbicides in water using temperature-responsive chromatography and an aqueous mobile phase

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Abstract

A simple and rapid method has been developed for herbicides in water using temperature-responsive liquid chromatography (LC) and a column packed with poly(*N*-isopropylacrylamide) (PNIPAAm), a polymer anchored on the stationary-phase surface of modified silica. PNIPAAm reversibly changes its hydrophilic/hydrophobic properties in water in response to temperature. The method was used to determine five sulfonylurea and three urea herbicides. Separation was achieved with a 10 mM ammonium acetate (pH 3.0) isocratic aqueous mobile phase, and by changing the column temperature. The analytes were extracted from water by off-line solid-phase extraction (SPE) with an *N*-vinyl-pyrrolidone polymer cartridge. The average recoveries of the eight herbicides from spiked pure water, tap water and river water were 70–130% with relative standard deviations (RSDs) of <10%. The limits of quantitation (LOQ) of the eight herbicides were between 1 and 4 $\mu g l^{-1}$.

Keywords: Poly(*N*-isopropylacrylamide); LC; Temperature-responsive chromatography; Sulfonylurea herbicides; Urea herbicides

1. Introduction

Herbicides are used in rice paddies, golf courses, and other types of fields. They are transported by aquifers in groundwater and are widely distributed in the environment. Sulfonylurea herbicides are labile, weakly acidic compounds. Sulfonylurea and urea herbicides are used at lower concentrations, and are more rapidly degraded in soil than older herbicides. Therefore, parts-per-billion concentrations of these herbicides are to be expected in the water supply. These herbicides have been analyzed in water by liquid chromatography (LC) with UV detection [1,2], capillary electrophoresis with UV [3], LC with mass spectrometry (MS) [4,5], immunoassay [6], bioassay [7] and radio immunoassay [1].

Recently, various polymers have been developed which change their structure in response to surrounding conditions, such as the pH, electric field, and temperature. Such polymers have been widely utilized in drug delivery systems [8], cell culture dishes [9], cell sheets [10] and bioconjugates [11]. Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of these; it exhibits a thermally reversible phase transition in response to temperature changes across a lower critical solution temperature (LCST) of 32 °C in aqueous solution [12]. In water, the polymer chains of PNIPAAm hydrate and expand below this LCST, while they dehydrate to form a compact conformation above it. We previously reported a considerable and reversible change in the hydrophilic/hydrophobic properties of PNIPAAm-grafted surfaces in response to a change in temperature. Taking advantage of this characteristic, we developed an LC column packed with PNIPAAm to selectively separate analytes by controlling the external column temperature [13–17].

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Temperature-responsive chromatography is a method with little load on the environment, because no organic solvent is used in the mobile phase. Urea herbicides in environmental water have been widely studied by Hogenboom and coworkers [2,18,19] and very rapid analyses were made by using a single short column for both SPE and analytical separation. However, there are fewer reports on sulfonylurea herbicides [5]. The aim of this study was to achieve the separation of both groups of herbicides by temperature-responsive LC with an aqueous mobile phase.

2. Experimental

2.1. Chemicals

Analytical-grade standards of bensulfuron-methyl (99.7%), imazosulfuron (99.7%), pyrazosulfuron-ethyl (99.9%), halosulfuron-methyl (100%), siduron (98.9%), daimuron (100.0%) and diuron (100.0%) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Analytical-grade flazasulfuron (99.9%) was purchased from Hayashi Pure Chemical Industries, Osaka, Japan. The structures of these herbicides are shown in Fig. 1. *N*-isopropylacrylamide (NIPAAm) was kindly provided by KOHJIN, Tokyo, Japan and was purified by recrystallization from *n*-hexane. 3-mercaptopropionic acid (MPA), 2,2′-azobisisobutyronitrile (AIBN), *N,N*-dimethylformamide (DMF), ethyl acetate, 1,4-dioxane, *N,N′*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide, HPLC-grade tetrahydrofran (THF) and ammonium acetate were purchased from Wako Pure Chemical Industries. Aminopropyl silica beads (average diameter, 5 μm; pore size, 120 Å) were purchased from Nishio Industries, Tokyo, Japan. The pure water used for sample preparation and the LC mobile phase was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The synthesis of PNIPAAm and a modification of aminopropyl silica with the NIPAAm polymer were carried out by radical polymerization, as previously reported [13,20].

2.2. Temperature-responsive LC

A PNIPAAm-grafted silica beads were packed into a stainless-steel column (150 mm × 4.6 mm i.d.). LC was carried out on an Agilent 1100 series (Agilent, Waldbronn, Germany) instrument equipped with a UV detector and a Rheodyne Model 7750 injector. The column oven was a product of Shodex AO-30C (Showa Denko, Tokyo, Japan). The mobile phase was 10 mM ammonium acetate (pH 3.0). The thermoresponsive elution behavior of the herbicides was monitored at 240 nm at a flow rate of 1.0 ml min^{-1} at various temperatures. The injection volume was 20 μl.

2.3. Preparation of standard solutions

Stock solutions (1000 mg l^{-1}) of each analytical standard were prepared in THF. Next, working standard mixtures were prepared by diluting each herbicide stock solution with THF. These stock solutions were stored at 4 °C. Standard solutions

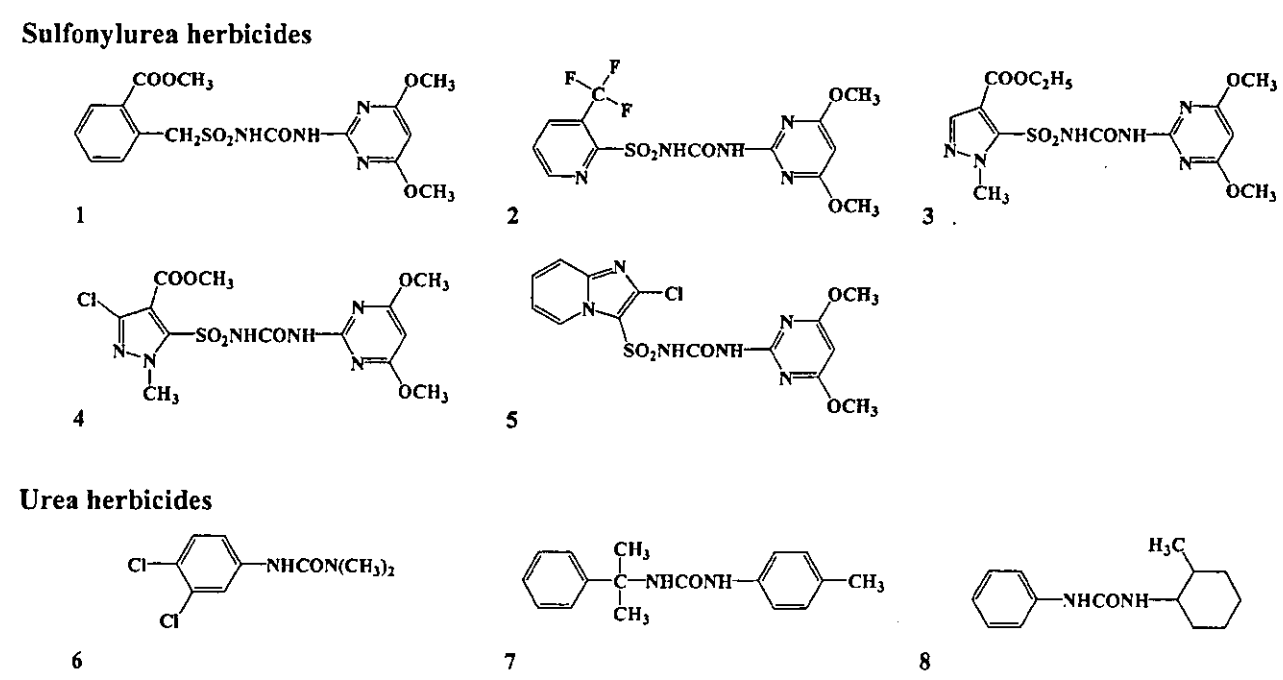

Fig. 1. Structures and common names of the eight herbicides. 1, bensulfuron-methyl; 2, flazasulfuron; 3, pyrazosulfuron-ethyl; 4, halosulfuron-methyl; 5, imazosulfuron; 6, diuron; 7, daimuron; and 8, siduron.

were prepared by diluting the stock solution with THF. The standard solutions were used for calibration plots and spiking of the water samples.

2.4. Water samples

Three types of water were analyzed: pure water, tap water and river water. The tap water was from a tap in the laboratory. L(+)-Ascorbic acid sodium salt (Wako Pure Chemical Industries) was added to the tap water at 0.005% (w/v), which eliminated chlorine that could react with and degrade some of the compounds of interest. The river water was collected from the Tama River near Tokyo; it was filtered through a glass-fiber filter before use.

2.5. Analytical methods

For recovery studies, three water samples (0.5 l each) were spiked with 1 ml of 2 mg l^{-1} (except for 0.5 mg l^{-1} diuron and daimuron) of the composite standard. Then, the spiked water samples were passed through a SPE cartridge to extract the analytes [5]. SPE was performed with cartridges prepacked with *N*-vinyl-pyrrolidone polymer resin (Oasis HLB Plus Extraction Cartridges) from Waters (Milford, MA, USA). The SPE cartridges were equilibrated with 5 ml of methanol and then 5 ml of pure water. The water samples were extracted at a 10 ml min^{-1} flow rate. Then, the cartridges were washed with 10 ml of pure water at a 5 ml min^{-1} flow rate and dried with air passed through the cartridge for 40 min. The herbicides were eluted from the cartridges with 3 ml of methanol at a speed of 1–2 drops s^{-1}. After evaporating the samples to near-dryness under a gentle nitrogen stream, the materials were dissolved to a final volume of 1.0 ml in THF.

3. Results and discussion

3.1. Sulfonylurea herbicides

Sulfonylurea herbicides were separated based on their temperature-controlled hydrophilic/hydrophobic properties by using an LC system connected to a column packed with PNIPAAm-modified silica beads. Fig. 2(a) shows van't Hoff plots for sulfonylurea herbicides separated using a PNIPAAm-modified column in 10 mM ammonium acetate (pH 3.0). The linearity in the van't Hoff plots is commonly observed for commercially available reversed-phase columns under standard chromatographic conditions. On the PNIPAAm-modified column, however, a deviation from linearity was found between ln *k* values and the reciprocal temperature (1/*T*). Interestingly, the slope of the van't Hoff plots of each analyte on the PNIPAAm-modified column changed markedly at the LCST boundary (Fig. 2 (a)). This corresponds to a phase transition of the polymer modified on the surface. Typical chromatograms for the standards of the five sulfonylurea herbicides using the PNIPAAm-modified column at 10 and 50 °C are shown in Fig. 3.

The log *P* values of these herbicides are given in Table 1. log *P* values were calculated by the CAChe system (Fujitsu, Japan). We reported in previous papers that the order of separation on a temperature-responsive-polymer-modified column depends on the hydrophobicities, corresponding to increasing log *P* values [13]. In this study, the retention time of the strongly hydrophobic imazosulfuron was remarkably increased, compared with four other sulfonylurea herbicides. When trying to separate the same herbicides on an ODS column using an aqueous/organic solvent, the three peaks of bensulfuron-methyl, flazasulfuron and imazosulfuron overlapped, and the two peaks of pyrazosulfuron-ethyl

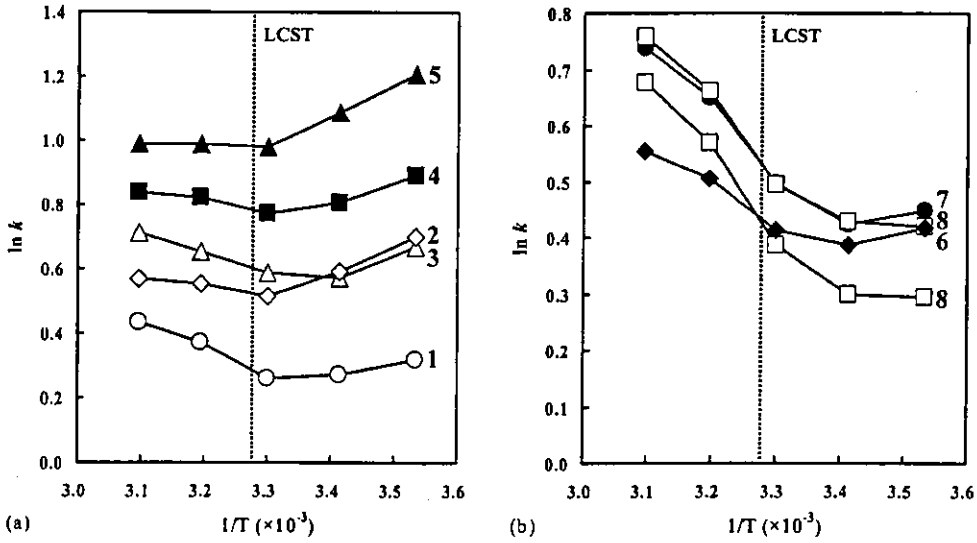

Fig. 2. van't Hoff plots of (a) sulfonylurea and (b) urea herbicides. For LC conditions, see Section 2. For peak numbers, see Fig. 1.

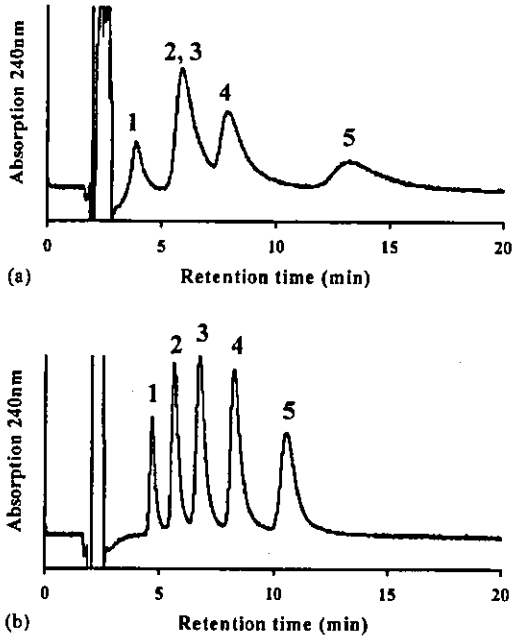

Fig. 3. LC–UV of standards using a PNIPAAm-modified silica column at (a) 10 °C and (b) 50 °C. For LC conditions, see Section 2. For peak numbers, see Fig. 1.

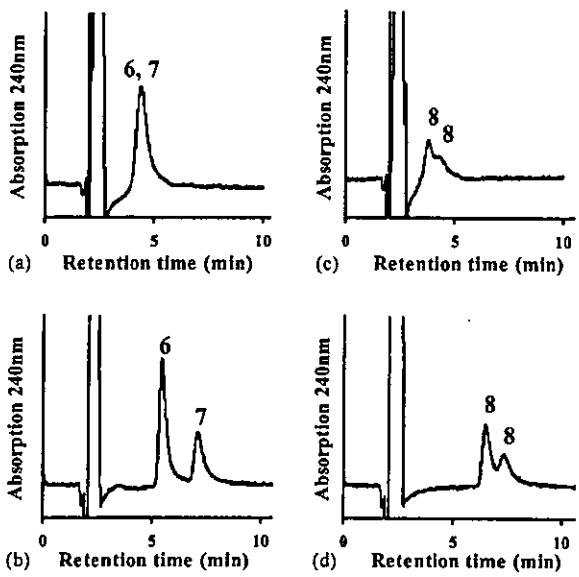

Fig. 4. LC–UV of standards using PNIPAAm-modified silica column at (a) and (c) 10 °C, and (b) and (d) 50 °C. For LC conditions, see Section 2. For peak numbers, see Fig. 1.

and halosulfuron-methyl also overlapped (data not shown). In contrast, upon raising the column temperature of the temperature-responsive system, these five sulfonylurea herbicides could be separated from each other with an aqueous mobile phase.

In this study, the mobile phase was adjusted to pH 3 which was lower than the pK_a values of these herbicides, bensulfuron-methyl (pK_a 5.2), flazasulfuron (pK_a 4.37) and imazosulfuron (pK_a 4.0), in order to suppress their ionization and effect their interaction with the surface of the stationary phase. With increasing temperature, the temperature-responsive surface of the stationary phase changed from hydrophilic to hydrophobic, the retention time increased as a result of hydrophobic interaction, and the separation of the five sulfonylurea herbicides markedly improved.

Table 1
Calibration, LOD and log P data for the eight herbicides

Compound	Calibration equation[a]	R^2	LOD (mg l^{-1})	log P
Bensulfuron-methyl	$y = 12.493x + 0.6557$	1.000	0.5	1.49
Flazasulfuron	$y = 9.8272x - 0.5951$	0.998	0.5	1.93
Pyrazosulfuron-ethyl	$y = 8.976x - 1.1398$	0.997	0.5	0.66
Halosulfuron-methyl	$y = 12.011x - 1.3876$	0.998	0.5	1.21
Imazosulfuron	$y = 16.043x - 0.951$	1.000	0.5	2.15
Diuron	$y = 20.209x + 0.6761$	0.996	0.5	2.15
Daimuron	$y = 11.74x - 0.2518$	0.995	0.2	3.61
Siduron	$y = 13.661x - 0.2925$	0.999	0.2	2.86

[a] y = area; x = concentration (mg l^{-1}).

3.2. Urea herbicides

The urea herbicides were separated using conditions similar to those for the sulfonyulurea herbicides. Fig. 2(b) shows van't Hoff plots for urea herbicides using a PNIPAAm-modified column. For urea herbicides, the ln k values increased markedly above the LCST (or lower $1/T$ values), indicating a hydrophobic interaction between the analyte molecules and the hydrophobized stationary phase surface of the column. The difference in retention behavior of the sulfonylurea and urea herbicides reflects differences in their physicochemical properties. Typical chromatograms for the standards of the two urea herbicides, and siduron using the PNIPAAm-modified column at 10 and 50 °C are shown in Fig. 4. Siduron gave two peaks corresponding to its cis/trans isomers. The retention times of urea herbicides also increased with the log P values. An increase in the retention times with increasing temperature was clearly observed.

3.3. Analytical performance

The calibration plots of all eight herbicides using temperature-responsive LC at 50 °C were linear. The concentrations range of the five sulfonylurea herbicides were 0.2–10 mg l^{-1} (six data points in triplicate), those of diuron and daimuron were 0.2–2.0 mg l^{-1} (four data points in triplicate), and those of siduron were 0.5–10.0 mg l^{-1} (five data points in triplicate). In all cases, the R^2 values were at least 0.995 (Table 1). Because siduron has two isomers, the area of the two isomer peaks was calculated and summed to give the total amount of siduron. The LODs of the eight herbicides were 0.2–0.5 mg l^{-1} (Table 1).

Table 2
Performance data for extracting five sulfonylureas and three ureas from pure water, tap water and river water

Compound	Pure water			Tap water			River water		
	Recovery[a] (%)	RSD (%)	LOQ ($\mu g l^{-1}$)	Recovery[a] (%)	RSD (%)	LOQ ($\mu g l^{-1}$)	Recovery[a] (%)	RSD (%)	LOQ ($\mu g l^{-1}$)
Bensulfuron-methyl	91	3.6	4	94	2.2	1	88	6.4	4
Flazasulfuron	90	1.9	1	86	1.7	1	72	9.7	4
Pyrazosulfuron-ethyl	93	1.6	1	98	2.5	1	100	5.0	4
Halosulfuron-methyl	90	2.7	1	98	1.1	1	97	4.5	4
Imazosulfuron	86	1.8	1	98	1.8	1	89	6.7	4
Diuron	91	4.5	1	84	6.8	1	97	4.5	1
Daimuron	127	2.8	1	100	5.3	1	94	6.0	1
Siduron	93	2.5	1	87	3.2	4	100	6.0	4

[a] Mean values from three individual samples.

3.4. Application

Water samples were prepared by adding 4 $\mu g l^{-1}$ (final concentration) of all herbicides, except for diuron and daimuron, which were added at a final concentration of 1 $\mu g l^{-1}$ to pure water, tap water, or river water. Then, 0.5 l of each sample was concentrated 500-fold by SPE. Using temperature-responsive chromatography, these eight herbicides were detected with acceptable recoveries and precisions (70–130% and relative standard deviation, RSD $\leq$10%, respectively) (Table 2).

4. Conclusions

Temperature-responsive LC with an aqueous solution without organic solvents as mobile phase can be used to determine sulfonylurea and urea herbicides. Combined with off-line SPE, trace levels of the herbicide can be quantified in real-life samples.

In temperature-responsive LC, analyte behavior is controlled merely by the temperature, without any changes in the mobile-phase composition.

Acknowledgement

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REVIEW ARTICLE

Principles of risk assessment for determining the safety of chemicals: Recent assessment of residual solvents in drugs and di(2-ethylhexyl) phthalate

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ABSTRACT **Risk assessment of chemicals is essential for the estimation of chemical safety, and animal toxicity data are typically used in the evaluation process, which consists of hazard identification, dose–response assessment, exposure assessment, and risk characterization. Hazard identification entails the collection of all available toxicity data and assessment of toxicity endpoints based on findings for repeated dose toxicity, carcinogenicity or genotoxicity and species-specificity. Once a review is compiled, the allowable lifetime exposure level of a chemical is estimated from a dose–response assessment based on several measures. For non-carcinogens and non-genotoxic carcinogens, the no-observed-adverse-effect-level (NOAEL) is divided by uncertainty factors (e.g. with environmental pollutants) or safety factors (e.g. with food additives) to derive a tolerable daily intake (TDI) or acceptable daily intake (ADI), respectively. These factors include interspecies and individual differences, duration of exposure, quality of data, and nature of toxicity such as carcinogenicity or neurotoxicity. For genotoxic carcinogens, low dose extrapolation is accomplished with mathematical modeling (e.g. linearized multistage model) from the point of departure to obtain exposure levels that will be associated with an excess lifetime cancer risk of a certain level. Data for levels of chemicals in food, water and air, are routinely used for exposure assessment. Finally, risk characterization is performed to ensure that the established 'safe' level of exposure exceeds the estimated level of actual exposure. These principles have led to the evaluation of several existing chemicals. To establish a guideline for residual solvents in medicine, the permitted daily exposure (PDE), equivalent to TDI, of N,N-dimethylformamide was derived on the basis of developmental toxicity (malformation) and of N-methylpyrrolidone on the basis of the developmental neurotoxicity. A TDI for di(2-ethylhexyl)phthalate was derived from assessment of testicular toxicity.**

Key Words: chemical risk assessment, DEHP, guideline for solvents in medicine, risk assessment

INTRODUCTION

Theophrastus Bombastus von Hohenheim (Philippus Aureolus, 1493–1541), better known as Paracelsius, wrote 'dosis sola facit venenum': all substances are poisons; there is none which is not a poison'. In other words, all chemicals can produce a toxic effect at some level and duration of exposure; however, the point at which toxicity may occur is unknown for the majority of chemicals that are utilized in society. The prediction of health effects of chemicals on the basis of available toxicity information is called risk assessment.

In the case of pharmaceutical development, candidate medicines must be tested in healthy volunteers and patients, and adverse events (toxicity) as well as efficacy must be thoroughly evaluated before any approval is given. However, chemicals can not be tested in humans for toxicity evaluation. Therefore, human health effects of agents such as pesticides, food additives, drug excipients, environmental chemicals and industrial chemicals must be estimated on the bases of the results mostly from animal toxicity studies. In the article presented here, principles of chemical risk assessment are briefly described, and two examples of actual risk assessment (residual solvents in medicines and a major plasticizer) are introduced, focusing especially on malformations due to exposure.

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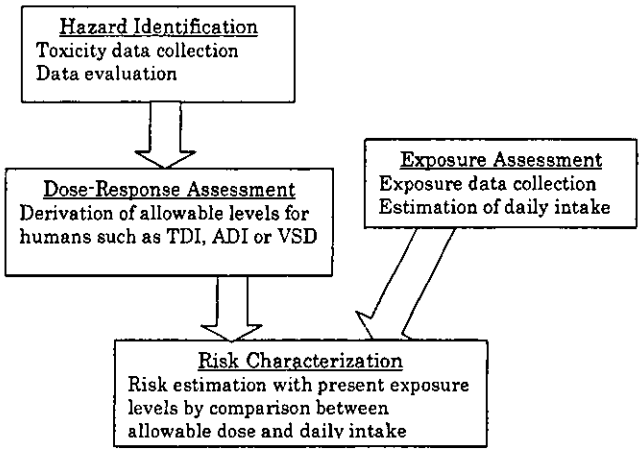

Fig. 1 Process of risk assessment. ADI, acceptable daily intake; TDI, tolerable daily intake; VSD, virtually safe dose.

Chemical risk assessment methodology

Risk assessment of chemicals is essential for the estimation of chemical safety for public health. The process consists of hazard identification, dose–response assessment, exposure assessment, and risk characterization (NRC 1983; Faustman & Omenn 2001). Toxicity assessment (hazard identification and dose–response assessment) and exposure assessment are generally conducted independently, and are merged at the final step, risk characterization (Fig 1). These practices include data collection, evaluation, and assessment, but are not necessarily the conduct of laboratory experiments. When the information is insufficient for risk assessment, additional research may be recommended.

Hazard identification

The first step entails the collection and evaluation of the available toxicity data. All information should be obtained from peer reviewed articles and if available, from pertinent reviews. Major toxicity endpoints such as short-term and long-term repeated dose toxicity, carcinogenicity, genotoxicity and reproductive/developmental toxicity are assessed. Other toxicity-related information, such as acute effects, irritation (in the eyes and skin), skin sensitization, toxicokinetics (absorption, distribution, metabolism and excretion), structure-activity relationships, and mode of action are also important to understand the toxicity profile of chemicals. Based on the available data, a no-observed-adverse-effect-level (NOAEL) or a lowest-observed-adverse-effect-level (LOAEL) for each endpoint is established, and judgment of genotoxicity and/or carcinogenicity is concluded. The following two important issues should be taken into account.

Evaluation of carcinogenicity in humans

Epidemiological information is the most important data source for assessment of human health, and is mainly derived from the following three kinds of studies:

1 Cross-sectional studies (relationship analysis between exposure and disease at a single time point in a specified population);
2 Cohort study (prospective examination of disease incidence in exposed and nonexposed populations); and
3 Case-control study (retrospective examination of exposure in disease-bearing and non-bearing populations).

Based on this information, in addition to animal carcinogenicity data, the IARC (International Agency for Research on Cancer) classifies chemicals into the following groups:

Group 1: Carcinogenic to humans;
Group 2A: Probably carcinogenic to humans;
Group 2B: Possibly carcinogenic to humans;
Group 3: Not classifiable as to its carcinogenicity to humans; and
Group 4: Probably not carcinogenic to humans.

The US EPA (Environmental Protection Agency) and the EC (European Commission) also have their own classifications into Groups A, B, C, D and E; and Categories 1, 2 and 3, respectively.

Such official conclusions regarding human carcinogenesis should be considered as key elements in hazard identification, in addition to separate full data analyzes of both epidemiological and animal carcinogenicity studies.

Animal toxicity cannot always be extrapolated to humans

Toxicity observed in some specific animal may not occur in humans. If the toxic mechanism was evidenced not to take place in humans, it could preclude the extrapolation to humans. Three typical examples are described below.

1 Rodents (rats and mice) are much more sensitive to peroxisome proliferators (fibrates, phthalates, etc.) than primates (cynomolgus monkeys and marmosets) and guinea pigs (IARC 1995). Peroxisome proliferation takes place via binding to a PPAR-α (peroxisome proliferator activated receptor-α) and no liver tumors have been induced by strong peroxisome proliferators in PPAR-α knockout mice (Peters *et al.* 1997; Ward *et al.* 1998). The m-RNA expression of PPAR-α in the livers of humans and guinea pigs is much lower than in the livers of rats and mice. Based on recent results from molecular biological studies the IARC re-classified di(2-ethylhexyl) phthalate (DEHP) from Group 2B to Group 3 in 2000 (IARC 2000).

2 α_{2U}-Globulin-related renal damages and tumors are male rat specific (Schnellman 2001). The protein is only produced in the male rat liver, appears in the blood and is excreted via the urine. When a chemical that can bind to α_{2U}-globulin is present in the blood, complexes are formed and reabsorbed to proximal epithelial cells in the kidneys after glomerular filtration. In epithelial cells, they become incorporated into lysosomes and accumulate over time due to

retarded degradation, leading to proximal tubular necrosis and finally tumors. Antibody-immunostaining can confirm this toxic mechanism. The most typical examples are unleaded gasoline, 2,2,4-trimethylpentane, d-limonene, lindane and 1,4-dichlorobenzene.

3 It is generally considered that thyroid hormone levels in humans are insensitive to chemical exposure, whereas in animals, especially male rats, they are extremely sensitive (Capen 2001). There are at least two major reasons. First, humans, monkeys and dogs have thyroxine-binding globulin in the blood, whereas rats, mice and chickens do not. Thus, rapid reduction of thyroid hormone levels in the blood can occur in the latter group, stimulating the release of thyroid hormone and leading to thyroid hypertrophy when hepatic metabolizing enzymes are induced. Second, thiourea and aniline derivatives inhibit thyroperoxidase in the thyroids of rats, mice and dogs, but not in humans, non-human primates and chickens.

Dose–response assessment

It is generally believed that there are two types of dose–response profiles. In one, toxic effects do not occur below a certain dose (i.e. threshold). In another, effects occur until the dose level reaches zero (i.e. non-threshold). Allowable lifetime exposure levels to a chemical at which no appreciable health risk would be expected over a lifetime are estimated via the different approaches for threshold and non-threshold cases.

For threshold cases, the NOAEL is divided by uncertainty factors (UF) or safety factors (SF) to derive a tolerable daily intake (TDI) or acceptable daily intake (ADI), respectively, as follows:

$$\frac{\text{NOAEL}}{\text{UF/SF}} = \text{TDI/ADI}$$

Usually, UF and TDI are used for undesirable chemicals, such as environmental pollutants and industrial chemicals, whereas SF and ADI are applied for permissible chemicals such as pesticides and food additives. However, UF and SF, TDI and ADI have basically the same meanings. UF/SF includes 5 variation components: interspecies differences; individual (intraspecies) differences; duration of exposure; quality of data; and nature of toxicity.

Interspecies differences

A factor of 10 or a body surface correction is used. Although it is generally difficult to compare toxicity levels between humans and experimental animals, information is available derived from cases in which anticancer drugs have been used for chemotherapy because they are administered up to dose levels, at which severe toxicity (the maximum tolerable) appears in patients. Data on 18 anticancer drugs in humans and from experiments with animals (rats, mice, hamsters, dogs and monkeys) showed that dose levels that induced the maximum tolerable effects were the same in humans and animals when the doses were expressed as mg/m^2 body surface area (Freireich *et al.* 1966). According to the following formula, the differences of body surface area/body weight between humans and animals are given as (human body weight)$^{1/3}$/(animal body weight)$^{1/3}$ (Freireich *et al.* 1966) as shown below:

$$\frac{\text{Body Surface}}{\text{Body Weight}} = \frac{\text{K}}{10^4 \times \text{W}^{1/3}} (\text{m}^2/\text{g})$$

$$\frac{\text{Animal (surface/weight)}}{\text{Human (surface/weight)}} \cong \frac{\text{Wh}^{1/3}}{\text{Wa}^{1/3}}$$

K is the correction factor (approximately the same in humans and animals); W is body weight (g); Wh is human body weight; and Wa is animal body weight.

For example, the human dose by body surface correction can be derived by dividing the animal dose (mg/kg body weight) by 11.4 ($60000^{1/3}/40^{1/3}$) for mice, 5.6 ($60000^{1/3}/350^{1/3}$) for rats, 2.7 ($60000^{1/3}/3000^{1/3}$) for monkeys, and 1.8 ($60000^{1/3}/10000^{1/3}$) for dogs, when the body weights are 60 kg for humans, 40 g for mice, 350 g for rats, 3 kg for monkeys, and 10 kg for dogs.

Individual differences

A factor of 10 has been commonly used from empiric findings.

Duration of exposure

Concerning the lifetime exposure, a 2-year period for rat and mouse studies for repeated dose toxicity is required. For shorter periods, factors of 2 for 1 year, 5 for 6 months and 10 for 3 months are applied.

Quality of data

If there is insufficient data for a NOAEL to be established, a lack of sufficient information in the literature, or a small number of animals, a factor of up to 10 is applied on the judgment of toxicological experts.

Alternatively, a benchmark dose approach might be used if a NOAEL is not established (Crump 1984). Figure 2 illustrates the benchmark dose approach for a 10% response. First, a dose–response curve is obtained by the application of curve-fitting technology (mathematical dose–response model) to experimental data. Second, an estimated dose for a certain incidence of toxicity or a certain percentage change in a toxicity parameter, and the lower confidence limit dose with 90–99% confidence are obtained. The latter value is a benchmark dose. A 5% incidence is usually applied for developmental toxicity and a 10% change (increase or decrease) is applied for other toxicity parameters mostly with 95% confidence limits. Advantages of the benchmark dose

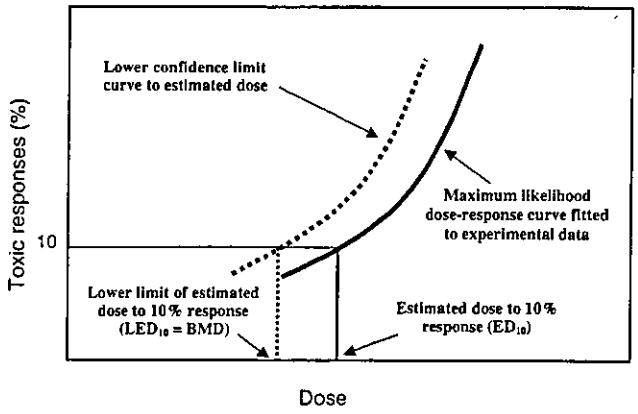

Fig. 2 Illustrated benchmark dose (BMD).

approach are: (i) its comparability to NOAEL (Farland & Dourson 1992; Allen *et al.* 1993); (ii) inclusion of the full dose–response curve and variability (number of animals and confidence limit); and (iii) generation of a realistic value because it is within the experimental range versus extrapolation from a high to a low dose. Regarding disadvantages, the benchmark dose approach is not generally applied to pathological changes because these may appear with various levels of severity and the diagnosis may change as the disease progress. The computational program compiled by US EPA can be downloaded from http://www.epa.gov/ncea/bmds.htm

Nature of toxicity

A factor of 10 is used for non-genotoxic carcinogenesis, neurotoxicity with pathological changes and malformations without maternal toxicity.

For non-threshold cases (genotoxic carcinogenesis), low dose extrapolation is accomplished with mathematical modeling (e.g. linearized multistage model) from the point of departure to obtain exposure levels that will be associated with an excess lifetime cancer risk of a certain level (generally 1 in 100 000). Usually, the lower confidence limit (95%) for the estimated dose level is called the virtually safe dose (VSD).

Exposure assessment

Measurement data for chemicals in food, water and air, are routinely used for exposure assessment. A 'market basket' methodology is often applied for estimating exposure through the food in the general population. Chemical analysis of outdoor and indoor air can be conducted, but it is very difficult to obtain constant and reliable values because winds can cause large fluctuations, and target sites and rooms can differ. Although chemical contents in drinking water can be determined in an easy and stable manner, the exposure allocation compared to other media is generally very low except with high contamination for a specific reason. Occupational exposure might provide the highest levels where sufficient protection is not in place, although the exposure data may not be generally available because they are not in the public domain.

Risk characterization

Finally, risk characterization is performed to ensure that the allowable lifetime exposure level exceeds the estimated level of exposure. Generally, practical safe levels of chemicals in food, air, water and household materials are established by regulatory authorities on the basis of TDI, ADI or VSD. If the estimated/measured levels exceed the safe level, regulatory action may be conducted case by case, concerning the excess and duration. This action is not a part of risk assessment but rather risk management.

Risk assessment is not simple because several complicating factors may be present and the political situation may interfere with the results. As recent international activities, Toxic Equivalent Factors (TEFs) and a TDI of dioxins have been established (van den Berg *et al.* 1998; WHO 1998; JECFA 2002) and revision of the WHO drinking water quality guideline is now in its final stages (WHO 2003). With these toxicity assessments, the above principles were used. New approaches such as subdivision of uncertainty factors into kinetics and dynamics (EHC 1994; Renwick & Lazarus 1998), application of benchmark dose for extrapolation of carcinogenesis assessment (US EPA, 2003) instead of mathematical models (such as a linearized multistage model), and margin of exposure or safety (Faustman & Omenn 2001), however, are also now being applied. As for Japanese risk assessment activities, we have contributed to the establishment of a Japanese drinking water standard (MHLW Japan 2003), with a proposal for risk assessment for drinking water contaminants (dichloroacetic acid, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone [MX], formaldehyde and methyl tertiary-butyl ether [MTBE]) (Hasegawa *et al.* 1999; Hirose *et al.* 1999, 2001, 2002), and establishment of a TDI for dioxins (Hirose *et al.* 1998) and phthalates (Koizumi *et al.* 2000, 2001b, 2002b). We have also conducted infant toxicity studies on 18 chemicals to compare toxicity levels and profiles between infants and young animals. We have already reported outcomes of detailed evaluation of data for four chemicals (Koizumi *et al.* 2001a, 2002a, 2003) and are now processing the other data that has been obtained. These analytical conclusions should provide valuable information on what UF/SF is sufficient or appropriate for dose–response assessment in view of child health.

Example 1: Derivation of permitted daily exposure (PDE) of residual solvents in drug materials and products

As one activity of ICH (International Conference on Harmonization of Technical Requirements for Registration of

Table 1 Key permitted daily exposure derivation and overall assessment for N,N-dimethylformamide

Teratogenicity study data (Hass *et al.*, 1994) and PDE derivation	
Method	Russian rabbits were given 46.4, 68.1 or 200 mL/kg during the organogenic period.
Results	There was no increase in uterine deaths but decreased fetal weight was noted at 200 mL/kg along with hydrocephalus at 68.1 and 200 mL/kg, as well as umbilical hernia at high dose. No maternal effects at 68.1 mL/kg
NOAEL	46.4 mL/kg
PDE calculation	46.4 mL/kg = 46.4 × 0.9445 = 43.8 mg/kg $\text{PDE} = \frac{43.8 \times 50}{2.5 \times 10 \times 1 \times 10 \times 1} = 8.76 \text{ mg/day}$ F1: 2.5 used for species differences from rabbits F4: 10 used for malformations without maternal toxicity
Overall assessment	
IARC	Not classifiable as to its carcinogenicity to humans (Group 3)
Genotoxicity	Negative results in *in vitro* studies (six reports)
Carcinogenicity (no tumors)	Rat PDE (oral) = 30 mg/day
Reproductive/developmental toxicity	Rabbit PDE (gavage) = 8.8 mg/day, rabbit PDE (skin) = 400 mg/day, rat PDE (skin) = 1200 mg/day
General toxicity	Rat PDE (diet) = 14.1 mg/day, rat PDE (ip) = 56.7 mg/day
Human data	No chronic data available
Conclusion	PDE = 8.8 mg/day based on generation of malformations

IARC, International Agency for Research on Cancer; NOAEL, no-observed-adverse-effect-level; PDE, permitted daily exposure.

Pharmaceuticals for Human Use), a guideline for residual solvents in drug materials and products was established on the basis of PDE derivation (Connelly *et al.* 1997) and is presently in the process of maintenance. To derive a PDE, a TDI approach in chemical risk assessment has been used because there is no risk assessment concept for medicines and genotoxic materials are not basically permitted for use in humans. The following equation is applied with a modifying factor (MF) instead of a UF.

$$\text{PDE} = \frac{\text{NOAEL} \times \text{Body Weight}}{\text{MF}} (\text{mg/day})$$

MF consists of F1 (interspecies differences), F2 (individual differences), F3 (duration of exposure), F4 (nature of toxicity) and F5 (quality of data). For F1, only a body surface correction is applied. For F4, a factor of 1 is applied to reproductive toxicity with maternal (general) toxicity, 5 to reproductive toxicity without maternal toxicity and malformations with maternal toxicity and 10 to malformations without maternal toxicity. The benchmark dose approach is not applied to F5. 50 kg is employed as the body weight for patients.

As one example of 52 PDEs established in 1997, Table 1 shows PDE derivation for *N,N-dimethylformamide* from the data of developmental toxicity study (Merkle & Zeller 1980). Because malformation (hydrocephalus) without maternal toxicity was observed in a rabbit study, factors of 2.5 and 10 were used for F1 and F4, respectively. From the overall assessment, the PDE was concluded to be 8.8 mg/day, as the lowest of all calculated values.

A maintenance process has been used since 1999 for unrecognized or new information to established PDE or new solvents. In late 1999, reproductive/developmental toxicity data for two solvents were submitted to and assessed by the ICH expert working group, and one PDE was revised (Connelly *et al.* 2003). Table 2 gives an assessment summary of newly submitted data for *N-methylpyrrolidone*. A rat developmental inhalation study showed a lowering of body weight in offspring up to 5 weeks after birth and impairment of higher cognitive functions at 150 p.p.m. (Hass *et al.* 1994). This study was conducted at only a single dose level but sufficient neurotoxicity examinations were performed. The toxicity is potentially serious because it is unclear if it is permanent or reversible. Furthermore, it is not determined

Table 2 Assessment of developmental neurotoxicity for N-methylpyrrolidone

Items	Developmental toxicity study data (Hass *et al.*, 1994) and permitted daily exposure derivation
Method	Wistar rats were exposed by inhalation to 150 p.p.m. for 6 h/day, daily from days 7–20 of gestation and were then allowed to litter.
Toxicity	No maternal toxicity was detected and litter size was unaffected by the treatment.
Abnormalities	No physical abnormalities were described. The offspring were reduced in body weight, the difference being statistically significant up to week 5 after birth.
Development	Pre-weaning development was impaired as was higher cognitive function related to solving of difficult tasks. Basal function of the central nervous system was normal and there were no effects on learning of low grade tasks.
NOAEL	Not established.
PDE calculation	$150\ \text{ppm} = \frac{150 \times 99.3}{24.45} = 608.16\ \text{mg/m}^3 = 0.608\ \text{mg/L}$
	$\text{For continuous dosing} = \frac{0.608 \times 6}{24} = 0.152\ \text{mg/L}$
	$\text{Daily dose} = \frac{0.152 \times 290}{0.33} = 133.58\ \text{mg/kg}$
	$\text{PDE} = \frac{133.58 \times 50}{5 \times 10 \times 1 \times 5 \times 5} = 5.3\ \text{mg/day}$
	F4: 5 used for impairment of only higher cognitive function of offspring
	F5: 5 used for NOAEL not being established

NOAEL, no-observed-adverse-effect-level, PDE, permitted daily exposure.

if the delayed development could be due to the lower body weight of the pups. However, the expert working group decided to be cautious in its interpretation and safety decision.

Ethylene glycol is a solvent that induces renal toxicity at low dose, and malformation and acute toxicity at high dose. Although a PDE of 6.2 mg/day on the basis of malformation was established in 1997, a higher value was proposed on metabolic consideration. The metabolic pathway described in Fig. 3 shows that the step from glycolic acid to glyoxylic acid catalyzed by lactate dehydrogenase is rate-limiting, meaning that high dose induce accumulation of glycolic acid, leading to skeletal malformation. In fact, glycolic acid may accumulate above 200 mg/kg BW for mice and 1000 mg/kg BW for rats, which were related with the increased incidences of skeletal malformation in mice and rats, respectively. (Neeper-Bradley *et al.* 1995; Frantz *et al.* 1996). However, there is a possibility of higher sensitivity of lactate dehydrogenase to glycolic acid in humans than mice and we were therefore not able to accept a proposal of a higher PDE. If sufficient evidence for an inhibitory profile in humans were provided, metabolic consideration could be taken into account for risk assessment. This is an example of risk assessment for malformations concerning metabolic characteristics.

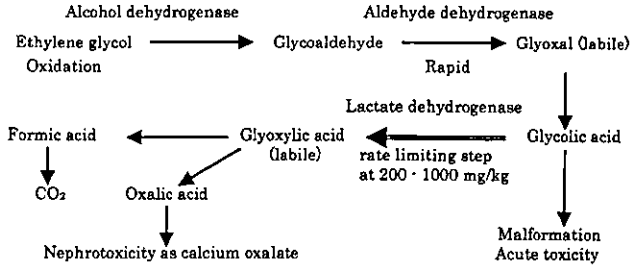

Fig. 3 Metabolic pathway of ethylene glycol.

Example 2: Establishment of a TDI for di(2-ethylhexyl) phthalate (DEHP) based on reproductive/developmental toxicity

Many kinds of phthalate esters have long been used as plasticizers. Among them, DEHP is the highest production volume chemical, found in various kinds of media. In 2001, the Division of Food Testing at our Institute reported extremely high amounts of phthalate esters, especially DEHP, to be present in certain cooked foods from convenience stores (Tsumura *et al.* 2001). Because the average amount of DEHP was 1700 μg in one Japanese style lunch package, the cause of contamination was examined. As the result, polyvinyl chloride gloves containing DEHP used in the final stage of food packing in factories were implicated.

It has been clearly shown that DEHP has two major toxicities, causing hepatic tumors and reproductive/developmental toxicity, at least in rodents. Hepatic tumors are excluded from the derivation of TDI, as the IARC (2000) concluded that hepatic tumor due to DEHP in rodents is not relevant to other animal species, including humans (Class 2B to 3), because of the association with peroxisome proliferation. Other toxic effects of DEHP can be divided into four regarding endpoints: (i) changes in male reproductive organs; (ii) alteration in female reproductive organs; (iii) reproduction toxicity; and (iv) developmental anomalies. As for male reproductive organs, the most critical toxicity data in rat testes give a NOAEL of 3.7 mg/kg/day in juveniles (Poon *et al.* 1997). It was reported that suppression of ovulation, disruption of estrus cycle and low plasma 17β-estradiol concentration occurred only at high dose level of DEHP in a single dose study (Davis *et al.* 1994). A NOAEL of 419 mg/kg/day for female reproductive organ toxicity was provided by Poon *et al.* 1997. Among a series of reproductive studies with continuous breeding in US NTP, a CD^{-1} mouse study showed the lowest NOAEL of 14 mg/kg/day (Lamb *et al.* 1987). Malformations such as hydrocephalus, cleft palate and skeletal malformation were found (Shiota *et al.* 1980) and a NOAEL of 44 mg/kg/day for developmental toxicity was apparent (Tyl *et al.* 1988).

The TDI was derived from dividing the NOAEL by a UF of 100 because the experimental conditions were sufficient for quality of data in all four cases, values for each endpoint being derived as follows:

1. Male reproductive organ toxicity
   NOAEL: 3.7 mg/kg/day → TDI: 40 μg/kg/day
2. Female reproductive organ toxicity
   NOAEL: 419 mg/kg/day → TDI: 4.2 mg/kg/day
3. Reproduction toxicity
   NOAEL: 14 mg/kg/day → TDI: 140 μg/kg/day
4. Effect on Development
   NOAEL: 44 mg/kg/day → TDI: 440 μg/kg/day

As to specific characteristics of DEHP, it is well known that there are strong species differences regarding testicular toxicity. DEHP caused severe seminiferous tubular atrophy of testes in rats and guinea pigs, and weak seminiferous tubular atrophy in mice, but none in hamsters (Gray *et al.* 1982). In two studies using marmosets and cynomolgus monkeys, no testicular toxicity was apparent (Kurata *et al.* 1998; Pugh *et al.* 2000). Considering these species differences, the Japanese government concluded a TDI of DEHP ranging from 40 to 140 μg/kg/day because it was unclear why the compound did not induce testicular toxicity in monkeys and whether this was relevant to humans (Koizumi *et al.* 2000, 2001b). As the exposure level of DEHP (118 μg/kg/day) in the worst case scenario exceeded the TDI, it was decided to ban the use of polyvinyl chloride gloves containing DEHP for food treatment.

At the same time, Gray *et al.* (2000) reported abnormalities of male reproductive organs to be observed in male offspring with exposure to several phthalate esters (500 or 750 mg/kg/day), but not to dimethyl or diethyl phthalates, from day 14 of pregnancy to lactation day 3 (Table 3). A NOAEL of 50 mg/kg/day for only di(n-butyl) phthalate was established from dose–response experiments (Mylchreest *et al.* 2000) before the establishment of any TDI for DEHP in Japan. Then, the 42nd Annual Meeting of the Society of Toxicology, Gray *et al.* (2003) described that slight antiandrogenic effects such as shortening of the anogenital distance and lowering of reproductive organ weights were observed at even 11 mg/kg/day of DEHP with maternal exposure from day 8 of pregnancy to post natal days 63–65. These seem more critical parameters than juvenile testicular toxicity. However, Gazouli *et al.* (2002) reported that DEHP can lower testosterone production *in vivo* via reduction of peripheral-type benzodiazepine receptor (PBR) expression

Table 3 Effects of phthalate esters on male offspring with exposure between late gestation to early lactation

Chemicals	Dose (mg/kg/day)	Effects on reproductive organs of male offspring	Reference
DMP	500 or 750	No effects	Gray *et al.* (2000)
DEP	500 or 750	No effects	Gray *et al.* (2000)
DBP	50	No effects	Mylchreest *et al.* (2000)
DBP	100~	Areola and nipple appearance	Mylchreest *et al.* (2000)
DBP	250~	Hypospadias, cryptorchidism and atrophy of accessory organs	Mylchreest *et al.* (2000)
DBP	500 or 750	Abnormal reproductive organs and cryptorchidism	Gray *et al.* (2000)
DEHP	500 or 750	Abnormal reproductive organs and cryptorchidism	Gray *et al.* (2000)
BBP	500 or 750	Abnormal reproductive organs and cryptorchidism	Gray *et al.* (2000)
DINP	750	Slightly abnormal reproductive organs	Gray *et al.* (2000)

BBP, n-butylbenzyl phthalate; DBP, di(n-butyl) phthalate; DEP, diethyl phthalate; DINP, di(iso-nonyl) phthalate; DMP, dimethyl phthalate.

in Leydig cells of the wild type mouse testis but it did not exert this effect in PPARα knockout mice. Therefore, there is a possibility that this antiandrogenic effect is rodent specific and would not take place in humans. The possible mechanisms underlying species differences should be clarified by further research.

As described above, species differences should need to be carefully considered for DEHP risk assessment, taking special account of appropriate mechanistic information.

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